Clinical Significance of the *p53* and *c-myc* Proteins in Melanoma

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Abstract

Malignant melanoma continues to present significant epidemiological, prognostic and therapeutic problems that are unlikely to be resolved without greater understanding of its biology. Considerable interest centres on the molecular genetics of cancer, and in particular, the role of oncogenes known to be important in control of cellular growth and proliferation. In this thesis, dual parameter flow cytometry was used to study expression of the protein products of the tumour suppressor gene, p53, and the nuclear oncogene, *c-myc*, in patients with cutaneous melanoma. In addition, flow cytometry was also used to measure the proliferation kinetics of these tumours *in vivo* using bromodeoxyuridine. These parameters were related to each other and the clinico-pathological features of each tumour. p53 and *c-myc* protein expression were also investigated in benign and dysplastic naevi.

Expression of both oncoproteins was prevalent in melanoma. Eighty percent of ethanol-fixed melanomas were p53- immunopositive, but expression showed limited association with clinical parameters, other than ulceration and patient age. *c-myc* was detected in 79% of primary tumours and 89% of metastases and increased with thickness of the primary tumour (p = 0.008). Using univariate analysis, the level of cmyc expression was prognostically significant in patients with thick primary melanomas (>3mm) (p = 0.02) and stage 2 (p = 0.04) disease. Prevalence of both p53 and c-myc positivity in 94 paraffin-embedded melanomas were similar to those observed in ethanol-fixed tumours. Using multivariate analysis, the level of *c-myc* expression was associated with patient survival (p = 0.02) in patients with thick melanomas. The median tumour potential doubling time (Tpot) was shorter in thick primary melanomas (p = 0.03) and inversely associated with the level of *c*-myc oncoprotein expression (p = 0.02). Proliferation kinetics did not vary between primary and metastatic melanomas. BMN were immunonegative for p53, whilst 53% of DN were p53-positive. c-myc expression was prevalent in both types of naevi, but at significantly lower levels than observed in melanomas (p = 0.04).

In conclusion, the findings of this study suggest stabilisation of the p53 protein may represent an epiphenomenon and be of limited clinical significance. Proliferation kinetics may explain tumour behaviour in a further subgroup of patients. This study suggests *c-myc* oncoprotein has a more central role in melanoma biology than previously considered and may be of prognostic significance. Further investigation of this oncogene may provide greater understanding of melanoma behaviour and an objective platform upon which to design future therapy.

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Attribution

All the experimental work in this thesis was performed by myself except for the following; Christine Martindale calculated all the proliferation kinetics, Brigid Robinson performed all the immunohistochemistry.

Abbreviations

BrdUrd	5 -Bromo-2-deoxyuridine
DNA	Deoxyribonucleic acid
RNA	Ribonucleic Acid
mRNA	Messenger RNA
SSM	Superficial spreading melanoma
NM	Nodular melanoma
ALM	Acral Lentiginous melanoma
LM	Lentigo maligna melanoma
BMN	Benign melanocytic naevi
DN	Dysplastic naevi
PI	Propidium iodide
wt <i>p53</i>	Wild-type p53
FACS	Fluorescence activated cell sorter
HLA	Human leucocyte antigen
MHC	Major histocompatibility complex
PBS	Phosphate buffered saline
LI	Labelling index (%)
Ts	Duration of S phase (hrs)
Tpot	Potential doubling time (days)
UVr	Ultra-violet radiation
UVB	Ultra-violet-B radiation
IHC	Immunohistochemistry
VGP	Vertical growth phase
RGP	Radial growth phase
NMSC	Non-melanoma skin cancer
TGF-α	Transforming growth factor- α
NGF	Nerve growth factor
IL	Interleukin
TNF	Tumour necrosis factor
FGF	Fibroblast growth factor

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Chapter 1 General Introduction

Section 1 - Clinical aspects of melanoma

Section 2 - Cancer biology and melanoma

Section 3 - Flow cytometry and proliferation

Hypotheses

Chapter 1 Section 1

1.1 Clinical Aspects of Melanoma

1.1.1 Melanoma and the Need For Further Knowledge

Melanoma is a cancer of melanocytes, pigment-producing cells that arise from the embryological neural crest and migrate predominantly to the skin, but also the choroid of the eye, leptomeninges, gastro-intestinal and genito-urinary tract. Malignant transformation of these cells restores their early itinerant behaviour, to produce a cancer renowned for its metastatic capability and virulence.

The incidence of cutaneous melanoma has dramatically increased over the last five decades (Koh, 1991), changing a medical rarity to amongst the most common of cancers in certain parts of the world. In the United Kingdom this rise shows little sign of abating (Swerdlow & dos Santos Silva, 1993), resulting in an increasing number of deaths in people of all ages that has elevated the profile of this disease amongst both the lay and medico-scientific communities (Coggons & Inskip, 1994). Furthermore, this increase has occurred in the absence of effective curative therapy for the disseminated disease. Melanoma continues to present other significant clinical problems. Tumours may develop in association with benign pigmented naevi, but the magnitude of the malignant potential of these naevi remains controversial (Mooi and Krausz, 1992; Ackerman 1988). Once formed, thickness of the primary tumour remains the major prognostic determinant (Breslow & Macht, 1977; Balch *et al.*, 1978, 1992). However it may fail to predict outcome in a significant proportion of patients (Slingluff *et al*, 1988) and may provide little information about the behaviour of a particular tumour within a given individual or group of patients.

These problems are unlikely to be solved without greater understanding of the biology of this cancer and, in particular, the role of 'oncogenes' which appear to be responsible for control of cell growth and proliferation of both normal and transformed cells (Vile, 1992). Evidence from studies of these genes in other cancers suggests they may be of importance in regulating tumour development and metastatic behaviour (Fearon & Vogelstein, 1990; Bishop, 1991). Consequently, elucidation of their mechanism of action and effects of perturbation may yield valuable prognostic information and an objective platform upon which to develop effective therapy.

1.2. Melanoma - A Brief History of the Past and the Challenge to the Present

The first description of melanoma was made by Hippocrates in the 5th century. Relatively recent studies of pre-Columbian Inca mummies, approximately 2,400 years old, revealed widespread cutaneous and bony melanoma metastases but no evidence of a contemporary description of the disease (Urteaga and Pack, 1966). Ruphus of Ephesus gave a detailed account of skin cancers, including melanoma, in the 1st century AD. This was followed, centuries later, by a number of accounts of "fatal black tumours with metastases and black fluid in the body" between 1651 and 1757.

In 1812 René Laennec made the first published description of melanoma as a specific disease entity, using the term 'melanosis', from the Greek for black. Many of his descriptions concerned mediastinal lymph node metastases, as Laennec was principally interested in pulmonary disease and tuberculosis. In 1820 William Norris, a general practitioner from Stourbridge, provided the first report of disseminated melanoma in the English literature in which he described several key features of the disease (Norris, 1820). Norris presented a patient whose tumour arose from a longstanding mole, which over the course of time was accompanied by the arrival of several smaller nodules around it. Surgery was performed to remove the primary tumour. However it swiftly recurred to be accompanied by enlargement of nodes in the groin and eventually death from pulmonary complications. Norris performed the post-mortem himself, somewhat colourfully describing deposits of melanoma involving the liver, abdominal cavity and lungs, and also encrusting the heart and brain. Norris was clearly fascinated by this disease and committed himself to advancing knowledge of it. In 1857 he published a treatise discussing 'Eight Cases of Melanosis' (Norris, 1857) [Fig 1.1]. Through this handful of cases he was able to identify other aspects of the condition and suggested that people with moles were at increased risk. He observed a higher incidence in people of fair skin and that these few cases were more common in industrial rather than rural areas. Furthermore he postulated that melanoma may have a hereditary element and be associated with the presence of other cancers. His 40 years of observation also led him to comment on the treatment of melanoma. He advised a wide excision of tissue, to include both 'diseased and healthy parts', for narrow excision was followed by tumour recurrence. Finally he recognised that neither medicine nor surgery would be of benefit once the disease had spread.

Other 19th century doctors were also expressing an interest in this rarity. Robert Carswell (1838) coined the term 'melanoma' to replace melanosis and went on to describe four forms of melanoma in his '*Illustrations of the Elementary Forms of Disease'*. Later, in 1851, the first case of a node dissection for metastatic melanoma was reported in the Lancet (Fergusson, 1851). Melanoma was described in detail by both Sir James Paget (1853) and Oliver Pemberton (1858) in his '*Observations on the History, Pathology and Treatment of Cancerous Diseases'*. He reviewed 60 cases, reporting that melanoma was predominantly a disease of middle and old age, for which no effective therapy existed. At the same time Sir Jonathan Hutchinson, (1857) made the first formal descriptions of melanoma arising in the nailbed, known as sub-ungual melanoma and went on to describe melanomatous changes in large pigmented patches on the face, eponymously known as Hutchinson's melanotic freckle (Hutchinson, 1894).

The rapid increase in surgical expedition brought about by the introduction of anaesthesia, coupled with an increasing understanding of the natural history of malignant disease, led to a controversy that remains unresolved today, namely whether regional lymph nodes should be removed at the time of excising the primary tumour. In 1892, Snow noted in the Lancet;

"The utter futility of operative measures which are addressed to the primary lesion only ... We further see the paramount importance of securing, whenever possible, the perfect eradication of those lymph glands which will necessarily be first infected before enlargement takes place. Radical removal ... is a safe and easier measure", (Snow, 1892).

Frederic Eve was to echo these sentiments whilst discussing his experience of 45 cases in 1903;

"the treatment of melanoma of the skin can be given in a few words, (i.e. free excision or amputation), in accordance with the position and extent of the disease. The removal of the nearest chain of lymph glands, whether palpably enlarged or not, should never be omitted; for it may be taken as a matter of certainty that in the majority of cases they are infected" (Eve, 1903).

This passage reveals several features of contemporary understanding of melanoma, recognising it to have a great tendency to metastasise (probably reflecting the late

presentation of tumours at that time). It additionally illustrates the concept of metastasis involving a process of 'infection' with the cancer spreading at a microscopic level to vulnerable areas. These observations were crystallised in the work of William Sampson Handley, presented in his two Hunterian lectures of 1907, that were to dictate the surgical management of primary melanoma until almost the present day. (Handley, 1907). He advocated wide local excision of the tumour, regional lymph node dissection and amputation in selected cases.

EIGHT

CASES OF MELANOSIS,

WITH

PATHOLOGICAL AND THERAPEUTICAL REMARKS

ON THAT DISEASE.

BY WILLIAM NORRIS, M.D.,

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LONDON: LONGMAN, BROWN, GREEN, LONGMANS, AND ROBERTS. MANCHESTER: J. AND T. CORNISH. BIRMINGHAM: CORNISH BROTHERS, 37, NEW STREET.

Figure 1.1. Frontispiece to the first treatise on 'Melanosis' written by William Norris in 1857

1.3. Melanoma - The Clinical Entity

1.3.1. Epidemiology of melanoma

The characteristics of melanoma discussed in the previous Section have held true in almost every respect except that of epidemiology. Over the last 50 years the incidence of melanoma appears to have doubled approximately every decade and is continuing to grow at a faster rate than any other type of cancer (NCI, 1990; Glass, 1989; Coggons, 1994). This rise cannot entirely be explained by improvements in diagnostic accuracy or criteria (van der Esch *et al.*, 1991).

Epidemiological studies have identified a number of environmental and host factors known to be important in determining the risk of developing melanoma (Koh, 1991). These studies have confirmed melanoma to be predominantly a disease of fair-skinned peoples, being rare in native Africans and Asians (Grin-Jorgensen *et al.*, 1992). The concentration of cases in areas of high sun-exposure such as Australia, South Africa and the southern United States (Grin-Jorgensen *et al.*, 1992) has implicated excessive exposure to sunlight and particularly ultra-violet radiation (UVr) as a major aetiological agent.

Each year in the UK, melanoma affects approximately 6/100,000 of the population and results in the death of over 12,00 people (Swerdlow & dos Santos Silva, 1993). There is a small, though detectable, variation in the incidence of melanoma between varying parts of the country with the South-West being the most affected (Fig 1.2). A study of fourteen regions throughout the UK examined the relationship between melanoma incidence and sun-exposure (Swerdlow, 1979). A positive correlation was found between incidence and hours of sunshine and was also noted to be inversely related to latitude. Similar trends have been observed in the USA where each year 32,000 people are diagnosed with melanoma and from which almost 6,500 die (Koh, 1991). Again a marked variation in incidence is observed, ranging from 6/100,000 population on the eastern seaboard to almost 23/100,000 in caucasians in Hawaii (Muir *et al.*, 1987). In addition, the incidence of melanoma in caucasian Hawaiians has tripled over a seventeen year period (Muir *et al.*, 1987) and almost one in 90 of the US population have been predicted to develop melanoma by the turn of the century (Rigel *et al.*, 1987).

The highest incidence of melanoma is found in Queensland, Australia (Grin-Jorgensen *et al.*, 1992) and occurs predominantly in individuals with pale skin types poorly



Incidence of melanoma



Mean daily hours bright sunshine

Figure 1.2a & b. The distribution of melanoma in England and Wales suggests an association with the level of sun-exposure. The highest incidence is found in the south of England (Fig 12a, greatest shown in red), coinciding with the maximum mean daily hours of bright sunshine (Fig 12b, highest shown in black) (Swerdlow & dos Santos Silva, 1993). adapted to the sun (Bonett, 1986). The concentration of cases in areas such this has revealed other features to implicate the environment. Firstly, it is recognised that migrants to Queensland do not have the same risk of developing melanoma if they arrive after the age of ten (Armstrong *et al.*, 1982). Those arriving under this age acquire a risk similar to the host population, suggesting exposure to UVr may be more critical in childhood. Consequently, a major feature of current health policy in preventing melanoma has been to target and prevent excessive sun exposure in this vulnerable age group.

Although the median age of affected individuals is 53 years, data suggests melanoma is affecting and killing more people in younger and middle age. It has the highest incidence of any cancer in fair-skinned people between the ages of 25-29 and in males between 35-39 years of age (NCI, 1990). Incidence also varies between the sexes; In the UK the rise in incidence has particularly affected women, who are almost twice as likely to suffer from melanoma as men (Swerdlow *et al.*, 1979). In Ireland, females are three times more commonly affected than males (Gordon *et al.*, 1986). As in the UK, Northern European females are affected almost twice as commonly as males though in the United States and elsewhere variation is less evident and approximates to a 1:1 ratio (Grin-Jorgensen *et al.*, 1992). The sexes also vary as to site of the primary tumour. Females are more likely to develop melanomas on the lower limb whereas males have a higher incidence of tumours on the chest and back and this has been partially attributed to differences in clothing.

The incidence between populations also shows great variation, by a factor of up to 200-fold around the world. This ranges from as low as 0.2 per 100,000 in parts of Japan, to over 46 per 100,000 in Queensland (Muir *et al.*, 1987). In caucasian people, the incidence of melanoma increases with proximity to the equator (Grin-Jorgensen *et al.*, 1992). However the incidence of melanoma in northern Europe has also begun to increase and this has been attributed to changes in social behaviour. Melanoma is also more prevalent in people of higher socio-economic status (Lee *et al.*, 1980) and it has been speculated that social 'pressures' for a tan have led to holidays in the sun and an intense one or two week burst of exposure.

1.3.2. Clinical features of primary melanoma

Cutaneous melanoma presents in several different morphological forms that may be the consequence of varying tumour growth patterns.

Superficial spreading melanoma

Superficial spreading melanomas (SSM) account for 70 - 80% of melanomas in most studies (Mihm *et al.*, 1975; Fitzpatrick *et al.*, 1992) and may develop from a preexisting mole over the course of several months. Initially the lesion is flat, with irregular borders and variable pigmentation. Tumour cells grow in a horizontal plane above the basement membrane and (at this early stage) the lesion may be classified as melanoma-in-situ. This lateral spread of cells is termed the radial growth phase (RGP) (Clark *et al.*, 1984) (Fig 1.3 a & b). Over a period of months areas within the lesion may become nodular as the tumour begins to grow up into the more superficial layers of the skin and down into and through the basement membrane, to constitute the vertical growth phase (VGP) (Clark *et al.*, 1987). At this stage the tumour is invasive and liable to metastasise (Fig 1.4 a & b).

Nodular melanoma

Nodular melanomas (NM) comprise the second most-common type of lesion and contribute approximately 15-30% of cases (Fitzpatrick *et al.*, 1992). Nodular melanomas are more likely to arise de-novo in unaffected skin, but can develop in precursor naevi. They tend to have a shorter history and are more common in men (Fitzpatrick *et al.*, 1992). As their name implies, NMs are more protuberant, and symmetrical in shape than SSMs. Histologically they are characterised by a direct vertical growth phase which may account for their more aggressive behaviour and tendency to ulcerate (Fig 1.5a & b). Up to 5% of nodular melanomas may be devoid of pigmentation, (i.e. amelanotic).

Lentigo maligna melanoma

Lentigo maligna melanoma (LM) is a distinctive type of melanoma in both terms of site and morphology. It accounts for 4-10% of all melanomas and is usually found on the face of the elderly (Fitzpatrick *et al.*, 1992). It is the only type of melanoma to display an overt dose-response relationship with sun-exposure in that it arises on exposed regions over a period of many years. There is often a pre-malignant phase comprising a flat large pigmented patch, known as a lentigo maligna, which resembles a large freckle and is usually 1-3 cm in diameter. Over a period of years (as opposed to months) the lentigo may develop areas of increased pigmentation which represent patches of radial growth-phase tumour. In time this may progress to an invasive vertical growth phase, at which point the tumour will be of similar metastatic potential to a SSM or NM of similar thickness (Fig 1.6 a & b).

Acral lentiginous melanoma

Acral lentiginous melanomas (ALM) are rare amongst fair-skinned individuals, accounting for 1-5% of melanomas, but up to 90% of those seen in dark skinned or oriental people (Reintgen *et al.*, 1982). They usually arise on the palms of the hand or soles of the feet (Fig 1.7a), but even more rarely may affect the nail bed or ano-genital mucosa. Due to the rarity and difficulty of observing these lesions, they tend to present at a later, more advanced stage and are thus associated with a worse prognosis (Hiles 1990). Those arising under the nail bed are known as sub-ungual melanomas and may be sub-classified as a separate entity (Fig 1.7b). They account for 1% of melanomas in caucasians, but up to 35% of melanomas in Japan (Seiji *et al.*, 1982).

1.3.3. Dissemination of melanoma

In common with most cancers, melanoma disseminates through a number of routes including local invasion, lymphatic and vascular spread (reviewed by Fidler 1992). Local invasion and metastases result in the formation of smaller 'satellite' lesions (not to be confused with cutaneous metastases which result from widespread disease). Lymphatic spread is the major route of metastasis and accordingly the draining lymph nodes of patients are always examined as part of their routine follow-up in clinic. Spread to regional nodes may be heralded by 'in transit' metastases in the draining lymphatics. Melanomas arising on the leg tend to spread to the regional inguinal (groin) nodes whereas those on the upper limb drain to the ipsilateral axillary nodes. Tumours arising on the trunk can metastasise to either axilla or groin and are thus associated with a poorer prognosis than cutaneous melanomas arising elsewhere.

Metastases usually involve regional lymph nodes before moving onto more distant sites such as the skin, subcutaneous tissues and viscera (liver, lungs and brain etc.) via haematogenous spread. However direct haematogenous spread may occur in over 40% of cases (Fig 1.8).



Figure 1.3a. Superficial spreading melanoma. This long-standing naevus has begun to show evidence of malignant change. The lesion remains macular but has increased in size, along with darkening and variation in pigmentation.



Figure 1.3b. Schematic representation of malignant melanocytes in the radial growth phase (RGP) of a superficial spreading melanoma. Tumour cells migrate in a radial pattern, remaining above the papillary dermis. At this stage the tumour is not invasive and excision carries an excellent prognosis.



Figure 1.4a. Nodular regions have developed in this superficial spreading melanoma as tumour cells enter an invasive vertical growth phase (VGP). Cells penetrate the basal laminar and invade the dermis, increasing the potential for metastasis.



Figure 1.4b Schematic representation of vertical growth within a radial growth phase melanoma.



Figure 1.5a



Figure 1.5b

Figure 1.5 a and b. Nodular melanoma. The tumour shown in figure 1.5a shows a pure vertical growth phase melanoma. figure 1.5b shows a thick, ulcerated, nodular melanoma. Tumour ulceration has also been shown to indicate poor prognosis (see 1.4.2.).



Figure 1.6a. Superficial spreading melanoma arising within a lentigo maligna on the face of a 72 year-old male.



Figure 1.6b. Vertical growth phase, nodular melanoma developing within a lentigo maligna on the face of a 78 year-old female.


Figure 1.7a. An advanced acral lentiginous melanoma on the sole of a 55 year-old West Indian male. The tumour exhibits extensive radial growth, with several nodular regions indicating invasive tumour. The patient died of disseminated disease fourteen months after excision of the primary tumour.



Figure 1.7b. Sub-ungual melanoma. Note the characteristic pigmentation on the cuticle of the nail (Hutchinson's sign). The poor prognosis of this lesion is indicated by both its extent and ulceration.

1.4. Determination of Prognosis in Melanoma

1.4.1. Introduction

The estimate of prognosis has a fundamental role in determining how a clinician manages both the patient and their disease. The outcome of any malignant disease is dependent on a number of factors. In the first instance, as has been discussed, the site of the primary tumour is important as is the stage of disease at the time of presentation, (i.e. whether there is any evidence of spread). Melanomas on the hands and feet have a worse prognosis than those situated on the arms or legs (Day *et al.*, 1981b). Similarly lesions presenting on the scalp have been shown to metastasize more readily than melanomas arising on the ear, face or neck. The worsened prognosis of scalp tumours was found to be independent of patient sex and tumour thickness and may reflect the vascularity of this region (Urist *et al.*, 1984). Women have been observed to have a better prognosis than men in a number of studies (Drzewiecki *et al.*, 1982; Shaw *et al.*, 1987). This is partly explained by the greater proportion of lower limb tumours seen in females and the fact that patients with tumours at this site tend to present at an earlier stage.

Patients are clinically staged via physical examination and appropriate investigations to assess the extent of their disease and, as a consequence, determine appropriate therapy. A number of staging systems have been advocated for the classification of disease stage in melanoma. In this study the traditional three-stage classification (Ketcham *et al.*, 1992) has been used, as shown below.

Table 1.1. The three-stage classification system

Stage	1	Disease confined to primary site
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Stage 2 Metastases confined to local soft tissues or regional lymph nodes (loco-regional disease)

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Stage 3 Widely disseminated disease

1.4.2. Histological indicators of prognosis in primary melanoma

It has long been recognised that the microscopic, histological features of primary melanoma are associated with behaviour of the tumour and its progression. Allen and

Spitz (1953) suggested thicker primary tumours carried a worse prognosis and the same conclusion was reached by Peterson (1962). Mehnert and Heard (1965) proposed skin could be divided into four layers and that prognosis was related to the depth of invasion. Despite a sceptical reception to these proposals, others continued to seek a relationship between microscopic parameters and tumour progression. In 1969 Wallace Clark expanded upon Mehnert and Heard's levels and proposed the concepts of radial and vertical growth in the evolution of invasive melanomas, subsequently to be known as Clark's levels (Clark et al., 1969). Clark observed that outcome correlated with the depth of tumour invasion, associating prognosis with five levels of invasion, ranging from in-situ lesions confined to the epidermis (level 1), to melanoma invading the sub-cutaneous fat (level 5). Breslow simplified prognostic assessment further by utilising the vertical, micrographic height of the tumour alone (Breslow, 1970) [Fig 1.9]. This was shown by a number of studies, principally those of McGovern (1979) and Maize (1983), to be a better predictor of outcome than Clark's levels and confirmed that five-year survival rates are related to tumour thickness (Table 1.2).

Table 1.2	. Thickness of	of primar	y melanoma	and five y	year survival	(Koh,	1991)
						· · ·	

<u>Tumour Thickness (mm)</u>	<u>Survival Rate at 5 years (%)</u>		
≤ 0.75	96		
0.76-1.49	87		
1.50-2.49	75		
2.50-3.99	66		
≥ 4.00	47		

Other histological markers of poor prognosis have also been identified (reviewed by Mooi & Krausz, 1992a); ulceration has been shown to correlate with a worse prognosis in a number of studies (van der Esch *et al.*, 1981; Drzewiecki *et al.*, 1982), though this was largely attributed to the association of ulceration with thicker tumours. However it has been shown to be an independent variable in a small proportion of melanomas when tumour thickness is corrected (van der Esch *et al.*, 1981; Balch *et al.*, 1992b). Additionally, the width of ulceration is related to tumour thickness and can be used as a further variable in defining outcome (Balch *et al.*, 1980).

The number of mitotic figures per high power field has been shown to correlate with outcome, presumably as a marker of tumour cell turnover (van der Esch *et al.*, 1981). Various indices, made up of combinations of the preceding parameters, have also been shown to be of predictive value. The 'prognostic index', is calculated by multiplying tumour thickness by the number of mitotic figures per square millimetre and correlates with the risk of developing metastases (Schmoeckel *et al.*, 1983; Kopf *et al.*, 1987), as does tumour and nuclear volume (Sorenson *et al.*, 1989).

Lymphocytic infiltration has also been suggested as a favourable prognostic sign (Clark *et al.*, 1989). However, the significance of this observation disappears when adjustment for tumour thickness is made (Balch *et al.*, 1992b). Microscopic satellites in the reticular dermis and subcutaneous fat have also been shown to be of prognostic value (Day *et al.*, 1981a).

1.4.3. Limitations of histological parameters in determining prognosis

Whilst tumour thickness remains the major prognostic determinant in cutaneous melanoma, it may fail to predict disease progression in a significant proportion of tumours. Several studies (Slingluff *et al.*, 1988, Blessing *et al.*, 1990) have shown that up to 10% of tumours less than 1mm in thickness will metastasise and that many of these patients go on to die of their disease. Similarly, patients may present with thick lesions which do not spread. There are two possible explanations for this variance. Firstly, tumour thickness may be difficult to assess and secondly, thickness may not represent metastatic potential in certain tumours.

In addition there may be features of the biopsy or histological technique that compromise prognostic information. Incisional biopsies are to be discouraged in the diagnosis of primary melanoma as they only include a portion of the tumour and will compromise histological assessment of depth in 40% of cases (Lees *et al.*, 1991). Tumours will often show marked variation in their surface contour that require the pathologist to examine many sections in order to ascertain maximum thickness. Briggs and Rigby (1993) advised that at least four sections should be examined in small lesions and up to twenty in larger melanomas, in order to achieve adequate histological assessment of the tumour. This is particularly true of certain morphological variants, such as the verrucous melanoma, which has an undulating surface and is consequently more prone to observer error. Conversely, a proportion



Figure 1.8. Melanoma metastasises by both lymphatic and haematological routes. Limb tumours tend to metastasise to regional lymph nodes prior to wide dissemination that may involve viscera and soft tissues.



Figure 1.9. Schematic representation of the measurement of vertical tumour height using an optical micrometer. This currently represents the standard method of estimating prognosis.

of melanomas are characterised by epidermal hyperplasia where the bulk of tumour lies within the epidermis, (i.e. the tumour may be thick but only invade to Clark's level two or three). In this instance the Clark's level (of invasion) will carry more prognostic information than tumour thickness (Briggs & Rigby, 1993).

Histological assessment may be further compromised in melanomas that arise in preexisting naevi as it may be impracticable to measure the exact thickness. Distinguishing naevomelanocytes from malignant melanocytes may be impossible and only an 'at worst' measurement can be issued. The pathologist's task can be further hindered by the phenomenon of regression (Mooi & Krausz, 1992) where primary tumours may disappear partially or entirely, possibly as a result of a local immune response. In such cases the tumour may be macroscopically surrounded by a depigmented 'halo'. Microscopically the diagnosis may still be made but estimation of the original depth is impossible, and with it any prediction of tumour behaviour. Regression is indicated by several microscopic features, including presence of melanin-laden macrophages in the dermis and areas of fibrosis. Histological evidence of tumour regression has been suggested as a poor prognostic sign (Paladugu et al., 1983), implying the original tumour may have been thicker than present at the time of excision and examination. However, studies from the Sydney Melanoma Unit (McGovern et al., 1983) and University of Alabama (Trau et al., 1983) did not find regression to be an adverse prognostic indicator. Patients may present with nodal disease following an occult primary tumour and it is possible that in a proportion of these cases the primary had regressed completely at an early pre-diagnostic stage (Smith et al., 1965).

1.4.4. Prognostic factors in metastatic melanoma

Nodal metastases

Multivariate analysis of clinico-pathological features in 1,698 patients with nodal metastases revealed a number of factors associated with nodal spread (Balch *et al.*, 1981, Balch *et al.*, 1992a) which were assembled from the large co-operative study between the Sydney Melanoma Unit and the University of Alabama. Whilst no association was found between age and risk of nodal metastases, male patients above age 50 years were found to have a higher mortality rate than those below this age; this difference was not found in females. Tumours arising on the trunk, seen more commonly in males, were noted to show an increased tendency to spread to regional nodes, and this correlated with a worse survival.

Several studies have shown that prognosis for patients with stage two disease is associated with a number of independent factors. Adverse markers include features of the primary tumour discussed in Section 1.4.2. such as tumour thickness, tumour site and the presence of ulceration. Prognostic features of nodal disease itself include the number of affected nodes and presence of histologically-evident extra-nodal disease (Cascinelli *et al.*, 1980, Callery *et al.*, 1982). Overall, these studies revealed a 20% - 25% ten year survival, with up to 40% of patients surviving this period if the primary tumour was not ulcerated and disease was confined to four lymph nodes or less.

Distant metastases

The site(s) of distant widespread metastases are important when considering prognosis in metastatic melanoma. Melanoma may spread to both non-visceral and visceral sites (Balch *et al.*, 1992a). Non-visceral sites include lymph nodes and skin, whereas visceral sites most commonly comprise the lung, liver, brain and bones. At post mortem up to 75% of patients dying from melanoma will have lung metastases (de la Monte *et al.*, 1983) and almost 50% of patients will have intra-cranial disease (Patel *et al.*, 1978).

A number of studies have confirmed that mortality increases with involvement of multiple sites. A recent study of 635 patients with disseminated melanoma found poor prognosis to be indicated by being male, poor performance status (assessment of debility due to disease, [WHO 1979]) multiple disease-involved sites, hepatic involvement and a short (i.e. less than one year) interval between primary and secondary diagnosis (Ryan *et al.*, 1993). This study also noted favourable prognostic factors using a 'proportional hazards' model and identified several predictable symptomatic and clinical features including normal appetite, absence of nausea, vomiting or fever. The findings of this study confirm those of the earlier investigation by the South-Eastern Cancer Study Group (Presant *et al.*, 1982). Both studies found performance status and hepatic involvement to be the most important prognostic determinants in metastatic melanoma.

1.5. Aetiology of Melanoma

The aetiology of melanoma is unknown; however a number of host and environmental factors have been recognised to be important risk factors and of relevance in the causation of this condition.

1.5.1. Environmental factors

1.5.1.1. Biological effects of UV radiation

The putative carcinogen in sunlight is thought to be ultra-violet B radiation, (290 - 320 nm, UVB), which is known to produce a number of deleterious effects from both in vivo and in vitro studies. Non-melanoma skin cancers (NMSC) show a more direct dose-response relationship; they occur on sun-exposed areas of older people whose skin tans poorly and are particularly common in individuals with outdoor occupations (Armstrong & English, 1992; Grin-Jorgensen et al., 1992). In contrast melanoma has a variable and somewhat unpredictable distribution in that it occurs in younger individuals and often in sites normally kept protected from sunlight (i.e. the torso) (Crombie, 1981). Furthermore it is more likely to occur in professionals working indoors (Armstrong & English, 1992). The lack of a direct correlation between cumulative sun exposure and melanoma, as seen in basal and squamous carcinomas, suggests a complex relationship likely to involve other factors. There is no evidence for an unequivocal role for UVB in the aetiology of melanoma but a number of clinical and laboratory studies have illustrated various routes by which UVB may participate in the malignant process; UVB radiation can damage cellular control mechanisms by a number of routes. Ley et al., (1989) have shown that UVB radiation alone will induce melanocytic tumours, some of which are metastatic, in the South American Opossum. In mice, UVB acts as both a weak initiator and promotor of transformation, and greatly increases the carcinogenic action of croton oil and dimethyl benzanthracene to induce both melanocytic and NMSC's (Husain et al., 1991). In common with most epigenetic carcinogens, it is likely that the action of UVB is mediated by gene injury and mutation. However UVB is also capable of inducing a number of other effects which may contribute to transformation;

1) It produces DNA damage, cross linking the double helix, particularly at pyrimidine residues, to produce pyrimidine dimers. Patients with xeroderma pigmentosum have a genetic abnormality which limits repair of UV-induced DNA

injury, increasing their risk of developing both melanoma and non-melanoma skin cancers by almost 1000-fold (Elwood, 1992).

UVB inhibits the protective anti-oxidant system (Fuchs et al., 1990); free 2) radicals (O_2 ⁻ and OH·) are tissue-damaging agents known to be involved with the pathophysiology of several processes including inflammation, the effects of ionising radiation and carcinogenesis. These agents have a direct injuring action on cell membranes and DNA and are thought to contribute to transformation by inactivating certain growth control mechanisms and differentiation processes (Rabilloud et al., 1990). Cells combat the deleterious effects of these agents by the action of inactivating enzyme complexes, including superoxide dismutases, peroxidases and catalases. Deficiency of these protective enzymes has been observed in fibroblasts of patients with known radiation-sensitive skin disorders such as xeroderma pigmentosum and Bloom's syndrome (Vuillaume et al., 1987). Lowered catalase levels have also been shown to be associated with progression of cervical carcinoma and transformation of keratinocytes in vitro (Rabilloud et al., 1990). UVB has been demonstrated to reduce intracellular levels of the anti-oxidant system, which would allow intracellular free radical levels to rise, increasing cellular vulnerability to gene injury and the potential for transformation (Fuchs et al., 1990).

3) UVB modifies proto-oncogene and tumour suppressor gene activity (Ronai *et al.*, 1988). Mutated N-*ras* oncogenes in melanomas were found to be localised to tumours arising in sun-exposed sites (Van' T Veer *et al.*, 1989). Similarly UVB has been shown to induce mutations in the p53 tumour suppressor gene, which would further limit DNA repair (Brash *et al.*, 1991).

4) UVB acts as a growth factor and growth factor modulator. In vitro studies have shown UV radiation to induce the production of a number of growth factors in both melanocytes, primary melanoma cells and surrounding keratinocytes. These include production of TGF- α by melanocytes (Ellem *et al.*, 1988) and the synthesis of bFGF (Halaban 1988), IL-1 (Kupper *et al.*, 1987), IL-6 and TNF- α in keratinocytes (Kirnbauer *et al.*, 1991). The receptor for NGF is upregulated on melanocytes in response to UVB (Peacocke *et al.*, 1988), as are receptors for melanotropin on melanoma cells (Birchill *et al.*, 1991).

5) UVB suppresses local cell-mediated immune responses and in some respects this represents the most interesting mechanism. Melanoma cells injected into UV-irradiated areas on ears of C3H mice showed a greatly increased tendency to spread

when compared with inoculation into non-irradiated ears (Romerdahl *et al.*, 1989). This study, along with several others, has suggested UV radiation may also act by causing local suppression of the cell mediated response, allowing immune escape (Donawho *et al.*, 1991). Furthermore this effect is not specific for melanoma, as UVr enhanced the dissemination of other implanted tumour cells, such as small cell lung carcinoma (Donawho *et al.*, 1991). Epidemiological evidence from Australia and studies on animal models suggests UVr may have a similar effect in humans, and that melanoma may follow exposure to wavelengths other than UVB, (i.e. UVA and UVC) (Ley *et al.*, 1989, Setlow *et al.*, 1993). This has led to melanoma-prevention programmes advising against reliance on anti-UVB sun creams and greater advocacy of physical barriers such as shirts, hats and shade (Marks, 1994).

1.5.2. Host factors in the genesis of melanoma

1.5.2.1. Skin phototype and sunburn

A large number of epidemiological and clinical studies have indicated a strong aetiological role for sunlight in melanoma (reviewed by Armstrong *et al.*, 1992, Longstreth *et al.*, 1992). It is known the incidence of melanoma rises with proximity to the equator and is particularly high in those individuals whose skin is poorly adapted to solar exposure and burns as opposed to tans, a characteristic of Northern European, fair skin. Individuals can be classified on the basis of their tendency to tan or burn, as shown below in Table 1.3. A number of studies investigating sun exposure and melanoma have shown people with skin types one-to-three to have a two-to-three fold increase in risk of developing melanoma (Beitner *et al.*, 1990).

A pale complexion may be associated with red hair and blue eyes and both of these features have been shown to correlate with increased risk of melanoma, though the size of this risk is difficult to quantify. Freckles, or ephelides, are areas of hyperactive melanocytes within which the number of melanocytes remain unchanged from normal, but melanin production is greatly increased. This phenomenon is associated with a two to three fold elevated risk of melanoma, though one study estimated risk to be increased by as much as 20 fold (Dubin *et al.*, 1986). Some studies found freckling to be entirely independent risk factor (Mackie *et al.*, 1989), whereas others have noted a confounding factor to be the number of naevi (Elwood *et al.*, 1986).

White	1	yes	no	high
	2	yes	minimal	
	3	yes	yes	
	4	no	yes	
Brown	5	no	yes	
Black	6	no	yes	low
Brown Black	4 5 6	no no no	yes yes yes	low

Sunburn, particularly sustained in childhood, has been identified as an important predisposing factor for melanoma, though the relationship is complex (Hill et al., 1992). The last decade has seen over a dozen investigations concerning the link between sunburn and melanoma, but their variable design and complications of recall bias have produced questionable estimates of risk (Marks et al., 1994). Amongst these, six large population based, case-control studies from Europe (Osterlind et al., 1988; Zanetti et al., 1992), Australia (Green et al., 1985; Holman et al., 1986) and North America (Elwood et al., 1985; Weinstock et al., 1989) have shown frequent sunburn to be associated with a two-to-three fold increase in risk which was found, in three of the studies, to be independent of skin type (Osterlind *et al.*, 1988; Green *et al.*, 1985; Weinstock et al., 1989). In addition, a number of migration studies have confirmed these observations and gone on to show that childhood is a particularly vulnerable period for sun-induced injury (Holman et al., 1984; Osterlind et al., 1988). In Australia and elsewhere, great emphasis has been placed on the education of parents and children as to the risks of sun exposure in an effort to reduce the incidence of melanoma and non-melanoma skin cancers (see Section 1.3.1. on epidemiology of melanoma).

1.6. Precursor Lesions of Melanoma

1.6.1. Introduction

Six steps have been identified in the clinical evolution of melanomas (Clark *et al.*, 1984, Hart *et al.*, 1991). The development of the first of these, the common acquired melanocytic naevus, has been recognised as a host marker of increased susceptibility to melanoma (Mackie *et al.*, 1989). The second step consists of architecturally atypical naevi without cytological abnormalities. The third step involves naevi displaying both architectural and cytological atypia, often termed dysplastic naevi. In certain circumstances dysplastic naevi may be a histological precursor of melanoma, though this is not obligatory. Step four constitutes the radial growth phase of primary

melanoma, and step five the vertical, invasive growth phase melanoma. The final step is metastatic disease.

The first step, the benign melanocytic naevus, is important as it represents a benign melanocytic tumour and is common to every individual, though in varying numbers. The third stage, the dysplatic naevus, has become the source of considerable discussion and controversy regarding its diagnosis and putative role as a preneoplastic melanomatous lesion. They are both relevant to the formation of melanoma and will be briefly discussed.

1.6.2 Benign melanocytic naevi

Benign melanocytic naevi are defined on the basis of clinical and histological criteria which note the position of naevomelanocytes with respect to the dermis (reviewed by Barnhill *et al.*, 1992). Junctional naevi consist of nests of melanocytes along the dermo-epidermal border, usually at the crests of the rete pegs (Mooi & Krausz, 1992a). Compound naevi are composed of a similar array of melanocytes, but in addition, nests of melanocytes lie free within the dermis. Intradermal naevi, as their name implies, are composed of predominantly intradermal collections of naevus cells. Almost all melanomas associated with naevi arise in the junctional or intraepidermal components.

Depending on whether clinical or histological criteria are used, 18% to 72% of melanomas have been noted to develop in association with benign melanocytic naevi (BMN) (Elder *et al.*, 1981; Gruber *et al.*, 1989). Many patients present with tumours which appear to have arisen within naevi of many years standing and remnants of these can be histologically recognised within or immediately adjacent to 10%-20% of melanomas (Ackerman, 1988). However the actual relationship between multiple naevi and melanoma remains obscure, though it is evident that the chance of developing a melanoma in a naevus is several orders of magnitude greater than a corresponding region of normal skin. At the very least, this increased risk may be partially explained by the statistical likelihood of a tumour arising in the concentration of melanoma risk as opposed to actual precursor lesions. A number of case-controlled studies have repeatedly demonstrated an association between the risk of developing melanoma and the number of naevi (Evans *et al.*, 1988). An above average number of naevi has been associated with an increased risk of developing melanoma of between

melanoma and the number of naevi (Evans *et al.*, 1988). An above average number of naevi has been associated with an increased risk of developing melanoma of between three (Beral *et al.*, 1983, Bain *et al.*, 1988) and 64 times (Swerdlow *et al.*, 1986), though typically the factor is greater than ten.

BMN may be either congenital, or more often, acquired (become visible later) and are more common in fair skinned people. Congenital naevi are only found in 1-2% of the population, whilst the great majority appear in the first few decades of life. The Eastern Australia mole study (Rivers, 1993) examined prevalence of naevi in children of varying ages and at increasing distance from the equator. Children nearer the equator were observed to develop a greater number of moles, and more rapidly than those further away, but that by age 15 the mean naevus count was similar. Furthermore this study also confirmed the prevalence of naevi in the study group to be very much higher than those found in children from environments with less sun. The overall number of naevi correlated with other established risk factors, such as fair skin, red hair and eye colour. Not surprisingly the authors concluded that moves to limit development of naevi may be accompanied by reduction in the incidence of melanoma This argument was given added support by the study of Holman and Armstrong (1984), discussed above, which showed that migrants to Australia retained the melanoma risk of their birthplace if they emigrated after the age of 20.

1.6.3 Dysplastic naevi

In 1977 Frichot *et al.*, described an association between familial melanoma and large, clinically atypical moles. In a more extensive report, Clark *et al.*, (1978) observed a similar relationship in two families concluding that presence of these naevi were a very strong indicator for development of melanoma. This was confirmed in a prospective study of these atypical, or dysplastic, naevi by Greene (1985) in which 85% of melanomas were noted to have a pre-existent naevus at the margin of the tumour. In 70% of cases the histological features of these atypical naevi showed dysplasia, suggesting they were precursor lesions of melanomas.

Clinically, dysplastic naevi (DN) are larger than BMN, arising in both sun-exposed skin and unusual locations such as the breast, buttocks and scalp. They are usually at least 5mm in diameter, with irregular pigmentation and borders (Elder *et al.*, 1990). However the assessment of their relationship to melanoma is complicated by an absence of agreement on the clinical and histological criteria required to diagnose DN. These criteria are under continual review, though there is agreement on the necessity

of certain features noted above (Elder *et al.*, 1985). In spite of these classification problems, the presence of these naevi in large numbers, in the dysplastic naevus syndrome (or familial atypical multiple moles and malignant melanoma syndrome - FAMMM), are strongly associated with a high risk of melanoma and afflicted individuals require prolonged follow-up (Greene *et al.*, 1985).

Familial cases are uncommon and most attention has focused on the role of these lesions as precursors in sporadic melanoma. Several studies have shown that DN are not rare in the general population and may be found as solitary lesions in 2% to 9% of individuals without a family history of melanoma (Rhodes *et al.*, 1980, Cook *et al.*, 1989. Of particular note, Nordlund *et al.*, (1985) found that DN were present in 19% of those people with more than 30 BMN and also identified DN in 34% of people with a past history of melanoma, as opposed to 7% of the general population.

However considerable debate not only surrounds estimation of the pre-malignant potential of these lesions, but as to whether they actually exist as a separate histological entity. Ackerman (1985, 1988) has strongly argued against their existence, proposing they represent maturation of BMN. His argument is given some credence in that none of the histological features identified in DN are unique to these lesions. All can be found to a greater or lesser extent in BMN (Mooi & Krausz, 1992). Furthermore, estimations of risk have been compromised by the fact that diagnosis of these lesions has been reached in different studies using different criteria and histological consensus classifications. These and other limitations are discussed in more detail in both Chapter 6 and the extensive review by Mooi and Krausz (1992b).

1.7 Treatment of Melanoma

1.7.1 Management of primary melanoma

Surgery has remained the treatment of choice for primary melanoma since the observations of Norris (1857) on melanoma and its treatment. Sampson Handley (1907) advised that the tumour be removed with a wide cuff of normal tissue, extending down to and including the deep fascia (Fig 1.10). The skin excision was to be at least 2.5cm away from the tumour, extending a further 5cm subcutaneously in a circle. He also advised that the draining nodes be excised at the same time. This procedure was to become routine surgical practice for over 50 years, passing through

affected and altered by substances released from the primary tumour (Olsen, 1966). Similarly Wong (1970) had described atypical melanocytes up to 5cm away from the primary tumour.

However, such wide excisions presented a number of problems and patients were left with large, disfiguring wounds which invariably required skin-grafting (Fig 1.11). Such radical procedures required general anaesthesia and in-patient treatment for several days. Breslow and Macht (1977) suggested that the width of excision had no effect on the outcome of melanomas less than 0.76mm and that a 'narrow' (2cm) excision margin would be just as effective in providing local control (i.e. removing the tumour and minimising the risk of local recurrence). Yet a fundamental problem remained in that it was not known what effect excision margins had on the subsequent behaviour of the more common thicker lesions and their risk of recurrence.

Amidst considerable debate the last decade has seen a dramatic re-evaluation of the treatment of primary melanoma. A number of studies suggested that 'narrow' excision margins were safe for thin melanomas (less than 1mm) (reviewed by Seigler, 1990). However questions remained regarding the safety of such procedures, complicated by the small number of patients studied. In 1979 the World Health Organisation (WHO) Melanoma programme commenced a large prospective multinational trial, investigating excision margins for melanomas less than 2mm thick. The patient cohort was divided into two groups, one receiving a 1cm excision margin, and the second group receiving a 3cm margin (patients with melanomas arising on the face were excluded). The study entered 703 patients, of which 612 were evaluable and both groups were well matched for age, sex, tumour site and thickness. At the time of publication there had been five local recurrences in the narrow excision group, which occurred in those tumours greater than 1.2mm thick. There was only one local recurrence in the wide excision group. Width of excision did not affect the incidence of regional node metastases, which occurred in 4.6% of the narrow excision group and 6.4% of the wide excision group. Similarly width of excision did not affect formation of distant metastases. At 100 months median follow up survival was almost identical, with 91.2% of patients in the thin excision group alive at the end of this period and 90.7% in the wide excision group (Veronesi et al., 1988). This study concluded that an excision margin of 1cm was adequate for melanomas less than 2mm thick, however in view of the slightly higher incidence of local recurrence seen in lesions over 1.2mm thick, it was later advised that lesions between 1.2mm and 2mm required an excision margin of 2cm (Veronesi et al., 1991; Eva Singletary et al., 1992). As a result of these studies, excision of most melanomas is now carried out as

an outpatient procedure under local anaesthetic and skin grafting is much less common.

Currently two other trials are investigating excision margins and the treatment of primary melanoma. The Melanoma Intergroup is investigating whether 2cm or 4cm margins are appropriate for tumours 1mm to 4mm thick. The British Association of Surgical Oncologists, in conjunction with the British Association of Plastic Surgeons has initiated a trial to randomise patients with melanomas 2mm or thicker, to receive either 1cm or 3cm excision margins, to extend the study of Veronesi *et al.*, (1990) to thicker tumours. The reductionist movement in melanoma surgery has also influenced the treatment of very thin melanomas less than 0.76mm thick and with no evidence of invasion through the basal lamina; excision margins for these 'melanoma in situ' have now been reduced to 0.5cm (Eva Singletary *et al.*, 1992).

In all the studies reviewed above, the extent of surgical excision was based on the Breslow thickness. However, as has been discussed, tumour thickness may be a poor predictor of disease in the individual case.



Figure 1.10. Following a post mortem on a patient who had died of melanoma, Sampson Handley identified local lymphatic invasion as the major route of tumour spread. The dotted line in the figure shown above, taken from Sampson Handley's Hunterian Lecture of 1907, formed the basis of his recommendation to perfom wide excision of the primary tumour and include underlying lymphatics, fascia and muscle (Sampson Handley, 1907). This was to influence the surgical management of melanoma for over 70 years.



Figure 1.11. Tumour thickness dictates the width of excision. However, this may result in considerable disfigurement without improving patient survival.

1.7.2 Management of nodal disease

Removal of the regional lymph nodes does not routinely accompany excision of the primary tumour. This is primarily due to the fact that elective lymph node dissection (ELND) has not been shown to confer any survival advantage (McCarthy *et al.*, 1992). In the United Kingdom, current policy is to excise the primary tumour, followed by observation on a regular basis. Lymph node dissection (LND) is only performed when there is clinical suspicion of nodal metastases (therapeutic lymph node dissection, TLND).

There has been much debate on the relative merits of ELND versus TLND (reviewed by McCarthy *et al.*, 1992). ELND is postulated to remove micrometastases present in regional nodes at the time of presentation of the primary. Protagonists of ELND argue that there are a number of specific situations in which it is appropriate and may confer a survival advantage, particularly when dealing with intermediate thickness lesions (between 1mm and 3mm thick) arising on the torso and head and neck. Evidence to support these views comes from a number of largely retrospective, single centre non-randomised trials comparing survival in patients between those who had and had not undergone LND (Balch *et al.*, 1982, Reintgen *et al.*, 1983). However, the two main randomised prospective trials of ELND performed to date have failed to confirm this hypothesis and argue against the elective use of LND (Veronesi *et al.*, 1977, Sim *et al.*, 1986). Furthermore, lymphadenectomy is a major procedure that involves a stay of five-to-seven days in hospital and can result in troubling complications such as prolonged discharge of lymph and infection. Late complications include limb oedema, which is both debilitating and unsightly.

Cochrane and Morton (1992) have proposed the method of 'intraoperative lymphatic mapping' that may clarify this issue and help to settle the debate (Morton *et al.*, 1992). The premise behind its application is to identify occult metastases in thicker melanomas and so influence the course of the disease. The method involves identifying the 'sentinel' node that drains the immediate region of skin within which the melanoma has arisen using pre-operative lymphoscintigraphy. Per-operatively, the actual node is identified following intra-dermal injection of patent-blue dye, which is excised and sent for frozen-section examination. If tumour cells are present the whole nodal basin is removed. If histology reveals no evidence of tumour then no dissection is performed and the patient is followed-up routinely. This method has been used for all melanomas at the Sydney Melanoma Unit for the last 20 months and at Morton's group in Santa Monica for several years, where it has been shown to improve patient survival (Morton *et al.*, 1991). These centres, along with several others in the USA and Australia, have combined to conduct a multicentre randomised trial to investigate whether SLNB affects patient survival. All patients with melanomas in excess of 1.2mm are randomised to receive either wide excision alone or undergo wide excision plus intraoperative lymph node mapping. The results of this study may help to answer questions as to whether ELND is of any benefit and for which group of patients. In patients with established nodal disease, TLND is the procedure of choice.

Non-surgical management of patients with stage two disease remains largely experimental and has involved a number of methods including use of adjuvent vaccines and chemotherapy. Melanoma has become an important model with which to study the development and application of tumour vaccines in solid tumours, as it is recognised to be particularly immunogenic (Donawho *et al.*, 1992). In particular, much emphasis has been placed on identifying melanoma surface antigens (Herlyn, 1993) in order to develop vaccines. In Australia, phase three trials are at an advanced stage, investigating the action of vaccinia-lysed allogeneic melanoma cell membranes (VMCL) in patients with stage two disease (Hersey, 1992). Over 450 patients have been recruited into this study to date, randomising patients with nodal disease to either undergo TLND alone or receive the VMCL vaccine course in addition to surgery. Early results suggest a significant survival advantage in the vaccine group, though a further follow-up is required.

Until recently there was little evidence to support the role of adjuvent therapy in the management of stage two disease. However the results of a randomised trial comparing the use of TLND and subcutaneous Interferon (three million units, three times a week for three months) compared to TLND alone has observed a definite survival advantage in the Interferon group at 19 months follow-up, and long term review is awaited with interest (Cascinelli *et al.*, 1994).

1.7.3 Management of disseminated melanoma

Once melanoma has spread outside regional lymph nodes, effective therapeutic options are few and the objective of management is palliation. Skin and subcutaneous metastases can be surgically excised or treated with a CO₂ laser and, in exceptional circumstances, may be managed in this way for several years. A number of studies have investigated the use of direct intra-lesional injections of agents such as bacille Calmette-Guérin (BCG) (Mastrangelo *et al.*, 1976), but patients must be able to mount a delayed-type hypersensitivity response. Disseminated disease confined to one limb

may be treated with intra-arterial limb perfusion of melphalan at 39°C. In specialist centres, complete remission (i.e. complete disappearance of local disease) is achieved in over 85% of patients, though this may be short-lived (Thompson, 1992).

Chemotherapy will only produce a response in 15-20% of patients. Dacarbazine is the agent most commonly used, and even combinations of dacarbazine and various other agents have proved disappointing. Some groups have reported encouraging response rates in excess of 50% using combinations of dacarbazine with cytokines, but with little increase in length of survival (Garbe *et al.*, 1993; Khayat *et al.*, 1993). The overall lack of efficacy has resulted in a large number of trials for combination chemotherapy, and for the present all new patients should be treated as part of such investigations. Biological agents have failed to live up to early expectations; both interferon- α and interleukin-2 (IL-2) have been shown to be effective in managing visceral metastases, but response rates are similar to those of dacarbazine (Creagan *et al.*, 1986).

Immunotherapy has been most extensively studied in the management of patients with stage three disease. A novel method involves isolation of tumour-infiltrating lymphocytes and expanding these *in vitro* prior to infusion back into the patient (Rosenberg, 1988, 1990). These studies have not progressed beyond phase two trials and have failed to fulfil the early expectations advanced by the authors. More recently, this group have presented the findings of studies in which the IL-2 gene has been transfected into LAK cells, though again this has not reached phase three clinical trials (Rosenberg *et al.*, 1990).

Section 2

1.8 Cancer Biology and Melanoma

1.8.1 Introduction: Cancer is a genetic disease

Tissue homeostasis is critically dependent on a balance between cell gain, through proliferation, and cell loss by a combination of several mechanisms including differentiation, cell migration and cell death. Generally, an inverse relationship exists between cellular proliferation and differentiation, each process being under the control of specific genes. Un-coupling of proliferation and differentiation upset this balance and represents a basic step towards tumour formation.

Proliferation and differentiation, like most cellular processes, are under the control of specific regulatory genes which in turn may be modulated by the action of other genes and extrinsic factors. It has been known for many years, following the work of Boveri on sea urchins in 1914, that chromosomal injury may result in cancer (Fischer, 1929). The appreciation that cancer has a genetic basis has allowed further understanding of the role of environmental factors in tumour aetiology. Almost all carcinogens have been shown to induce chromosomal injury and the capacity to damage DNA correlates well with the potential to induce tumours. Outwardly, free-growing cells have undergone a process of *transformation* in which they are able to grow in the absence of external growth factors and where they are refractory to other regulatory mechanisms, such as contact inhibition. The malignant phenotype becomes truly evident when, in addition to uncontrolled proliferation, cells are able to invade local tissues and metastasise to distant sites.

Cancer research is being increasingly directed towards elucidating mechanisms reponsible for regulation of cell growth and proliferation. In particular much interest centers on the role of certain *oncogenes* which represent altered normal regulatory genes, or *proto-oncogenes*. Oncogenes have been classified on the basis of their anatomical site of action (Fig 1.12) and also functionally on their mode of action. The term 'oncogene' more correctly refers to genes which act in a dominant manner, encoding proteins whose activity drives the cell towards transformation. A second class of gene has been recognised to be of great importance in cancer genesis, but these genes contribute to the malignant process by the absence of their protein product and are termed *tumour suppressor* genes.



Figure 1.12. The protein products of oncogenes and tumour suppressor genes (noted in italics) usually exert their effect at four main sites. (1) Within the cell nucleus; regulating the transcription of other genes. (2) The cytoplasm; (signal transduction) transmitting information that regulates cell proliferation and differentiation, either via (3) growth factors or (4) growth factor receptors within the cell membrane.

1.8.2 Oncogenes and transformation

Most proto-oncogenes encode proteins that are involved in proliferative pathways and their control. Extrinsic growth factors bind to extra-cellular receptors and these stimulatory signals are transduced across the cytoplasmic and nuclear membrane by proteins encoded by other proto-oncogenes. In the nucleus these signals induce transcriptional activation of proliferative genes and suppression of differentiation genes, to result in cell growth. Oncogenes are usually altered forms of these proto-oncogenes and function in such a way as to maintain continual growth stimulation (reviewed Vile *et al.*, 1993).

Malignant change does not occur in a single step nor by the action of one gene, but is postulated to require at least four to seven different genetic alterations before transformation occurs (Bishop, 1991, Vogelstein *et al.*, 1993). Transformation is the result of a cooperative process involving several genes, usually from different classes. As a broad generalisation nuclear-acting oncogenes will be activated in tandem with oncogenes encoding proteins that are active on the cell surface or membrane. The action of one gene alone may lead to immortilisation of cells in culture, but will lack tumourigenicity (the ability to produce tumours when implanted into nude mice) (Vile *et al.*, 1993). The growth-deranging effects of each oncogene combines to have an accumulative effect resulting in cell transformation. Several *in vitro* studies have shown that the *ras* oncogene will only transform cells in concert with other activated oncogenes such as mutated *p53* (Hinds *et al.*, 1989) or *c-myc* (Lu *et al.*, 1992). Similarly, greater transforming action is produced when cells are co-transfected with *ras*, mutated *p53* and *c-myc*, suggesting that *p53* mutation may augment the combined transforming action of *ras* and *myc* alone (Taylor *et al.*, 1992).

Appreciation of the central role that genetic derangements may play in the aetiology and pathology of cancer has led to investigation of the putative mechanisms by which these perturbations may act. These changes range from gross chromosomal abnormalities to deregulated oncogene expression. In investigating p53 and c-myc oncoprotein expression in melanoma, this study has examined the end-product of these deregulatory pathways. However, in order to assess the relative pathophysiological significance of these genetic changes in melanoma, it is necessary to consider other known gene alterations and a current overview is presented below in Section 1.9.

1.9 Cancer Genetics and Melanoma

The supposition that melanoma develops following a progressive accumulation of genetic defects is being increasingly confirmed at the molecular level (Fountain, 1990). However, cancer biologists have now to confront two formidable tasks: the first is to elucidate the precise nature and sequence of gene changes at each stage of tumour development. Secondly, it is necessary to define the biochemical and biological effect of these changes on those interdependent mechanisms which govern proliferation and differentiation.

Melanoma formation is likely to involve perturbation (induction, overexpression, mutation or deletion) of a number of different gene types; (i) genes altered by UV light, (i.e. oncogenes and tumour suppressor genes); (ii) genes regulating genomic stability (i.e. DNA repair genes); (iii) cell-cycle regulatory genes, (i.e. p53); (iv) genes encoding angiogenic and mitogenic factors; (v) genes involved in signal transduction (i.e. *ras*, tyrosine kinases and transcription factors); (vi) genes intrinsic to invasion and metastasis, (i.e. those that encode proteolytic enzymes, integrins and chemotactic factors); and finally, (vii) genes that either inactivate or confound immunological defence mechanisms.

Evidence from a range of studies conducted in vitro and in vivo have implicated proteins encoded by these genes in the aetiology and progression of melanoma (reviewed by Herlyn, 1990; Halaban, 1991, 1993). However convergent lines of evidence suggest that one of the fundamental defects may be deregulation, or disabling, of genes central to genomic stability and transcriptional regulation (Reviewed by Vile, 1992).

In order to identify the genetic and molecular correlates of progression from the melanocyte to the metastatic phenotype, the role of oncogenes in melanocyte transformation has been investigated by five approaches (Fountain, 1990).

- 1) Investigation of tumour chromosomes to detect any consistent abnormality suggesting the siting of an important gene or group of genes.
- 2) Examination of tumour DNA to detect any alteration in allele frequencies for a given gene in comparison to a control, normal gene.
- 3) Transfection assays of tumour DNA to identify activated oncogenes.

- 4) Transfection of activated oncogenes into melanocytes to determine their role in transformation.
- 5) Direct DNA sequencing of known oncogenes to discover whether any are mutated in melanoma.

1.9.1 Cytogenetic abnormalities associated with melanoma

Tumour progression is associated with increasing chromosomal abnormality, which in turn is inversely associated with prognosis (Balaban 1984, Trent *et al.*, 1990). Non-random karyotypic changes have been found in melanomas on chromosomes 1, 6, 7, 9 and 10.

Chromosome 1: Non-random karyotypic changes involving chromosome 1 were identified in 53 out of 58 advanced melanomas, most often consisting of deletion or translocation of the 1(p12-22) region (Parmiter *et al.*, 1988). Other studies have confirmed this observation and noted that loss of 1p is a late event in disease progression (Dracoploi *et al.*, 1989).

Chromosome 6: Trent has suggested that a melanoma-suppressor gene lies on the long arm of chromosome 6 and has described non-random deletions of this segment in a number of melanomas and metastatic melanoma cell lines. Introduction of normal 6q segments into these cell lines suppressed tumour formation (Trent *et al.*, 1989).

Chromosome 7: Overexpression of the Epidermal Growth Factor receptor on human melanoma cells has been associated with increased dosage (copies) of chromosome 7 (Koprowski *et al.*, 1985).

Chromosome 9: This chromosome has undergone increasing scrutiny as it may be the site of a melanoma-associated tumour suppressor gene. Cases of sporadic melanoma and the dysplastic naevus syndrome have been associated with abnormalities of this chromosome (Petty *et al.*, 1991); Fountain *et al.* (1991,1992) observed a region of 2-3 megabases that is deleted either heterozygously or homozygously in 85% of melanoma cell lines. Analysis of many melanomas has revealed linkage to 9p21, showing deletion early in tumour progression, which may be sufficient to induce transformation. Chromosome 10: In a study of 51 primary and metastatic melanomas, 18 tumours showed loss of either one or both copies of chromosome 10q. These deletions were more common in early lesions, suggesting that genes at this locus are involved in tumour development (Parmiter *et al.*, 1988).

Cytogenetic studies and linkage analysis remain largely descriptive and indicate little of the potential mechanisms by which melanocytes are transformed or how behaviour of melanoma cells is regulated. In order to elucidate these processes, understanding of the role of oncogenes is required.

1.9.2 Oncogene expression in melanoma

The methods outlined above have led to the identification and investigation of a number of different oncogenes in melanoma. The c-myc oncogene has been extensively studied in many cancers other than melanoma, as it is perceived to be central to the control of cellular proliferation, differentiation and apoptosis. The protein product of this gene is one of the main topics of investigation of this thesis and accordingly will be discussed in detail.

1.10 *c-myc* and Cancer

c-myc is a member of the *myc* family of proto-oncogenes that also includes *N-myc* and *L-myc*. Both *N-myc* and *L-myc* show considerable tissue and tumour specificity in their expression. However, *c-myc* is expressed in almost all normal tissues and deregulated in many human cancers. Consequently it has become the source of considerable attention (Littlewood *et al.*, 1990). The *c-myc* gene is located on chromosome 8(q24) and encodes two proteins of between 62 and 64kDa molecular weight, (see Section 1.10.2.). The protein is active within the nucleus and exhibits certain structural motifs to suggest it functions as a sequence-specific transcription factor. In cultured fibroblasts, the deregulated overexpressed *c-myc* oncogene will produce immortilisation and reduced growth factor requirements (Minks *et al.*, 1992). However, as noted above, *c-myc*-induced transformation usually requires co-transfection with other oncogenes, including *ras* and mutant *p53*.

1.10.1 The role of c-myc in human cancers

Several independent lines of evidence have suggested *c-myc* has a role in tumourigenesis of human cancers. The first came from analysis of the pathogenesis of avian bursal lymphoma and Burkitt's lymphoma in man (Hayward *et al.*, 1981). In the former, chronic infection by the avian leukosis virus results in insertion of a promotor/enhancer sequence near the *c-myc* gene. Excessive and inappropriate expression of this gene appeared to be associated with formation of lymphomas in birds. Subsequent investigations showed a similar mechanism involved in the formation of Burkitt's lymphoma in man. In this condition chromosomal translocation results in relocation of the *c-myc* gene from chromosome 8(q24) to a highly transcriptionally active region adjacent to the immunoglobulin heavy chain locus on chromosome 14(q32) and the κ and λ light chain loci on chromosomes 2(p12) and 22(q11) (Taub *et al.*, 1984). In such a location, the *c-myc* gene would be overexpressed both quantitatively and temporally.

c-myc overexpression has been observed in a wide range of haematopoeitic and solid cancers including those of the gastro-intestinal tract, urogenital tract, lung, breast and central nervous system (Prins & De Vries, 1993). However translocation and amplification only explain *c-myc* overexpression in a small proportion of cases, the remainder occurring by unknown mechanisms. Furthermore, in several of these cancers the level of expression may be of prognostic significance and this shall be expanded upon with respect to the findings of the present study in the general discussion (Chapter 8). It would appear, therefore, that loss of the regulatory mechanisms which control *c-myc* so tightly in normal cells may represent a frequent, if not essential, step in tumour evolution.

The role of c-myc in the pathogenesis of melanoma has been investigated to a very limited extent and is considered in more detail in the discussion to Chapter 4.

1.10.2 Structure and function of the c-myc oncogene and proteins

Like so many genes involved in control of cell growth and proliferation, the *c-myc* gene is highly conserved in evolutionary terms, particularly in certain regions or '*myc* homology boxes' thought to encode important functional domains of the *c-myc* oncoprotein (Littlewood *et al.*, 1990). The gene contains three exons, the first of

which is essentially non-coding along with four promotor regions, P0 to P3. Messenger RNA (mRNA) initiated at the P2 promotor, located in the first exon, provides 80% to 90% of *c-myc* mRNA in normal cells (Ross *et al.*, 1991).

Several studies have attempted to functionally map these regions; all three *myc* genes encode multiple transcript species which can be generated through the flexible usage of transcriptional start sites, polyadenylation species and post-transcriptional modification (Dang *et al.*, 1988). Deletion of three of these highly conserved areas in exon two showed that the amino acid sequences between aa44-65 and aa128-144 greatly reduced the transforming activity of the gene (Sarid *et al.*, 1987), though the significance of region aa44-65 was later questioned, highlighting the limitations of transformation assays in determining oncogenic activity (Stone *et al.*, 1987).

c-myc Binding to DNA and Max

DNA sequence-specific binding is mediated by a carboxyl terminal basic region helixloop-helix-leucine zipper domain (bHLH-LZ), common to all three *myc* proteins and many DNA binding transcription factors. This motif is essential for all *c-myc* functions (Evan *et al.*, 1992) in that it facilitates dimerisation to *Max*, another bHLH-LZ protein. Dimerisation is fundamental to *c-myc* function as this allows the heterodimer to bind to CACGTG, a core DNA sequence (Blackwood *et al.*, 1991) as a pre-requisite of transcriptional activity (*Max* is not thought to have any transcriptional activity (Amati *et al.*, 1992). Overexpression of *Max* results in formation of *Max-Max* homodimers which may play a part in regulating *c-myc* activity (Kretzner *et al.*, 1992). *Max* has a long half-life, and intra-cellular levels remain stable throughout proliferation. During proliferation, *c-myc* is upregulated and *Max* is likely to be present as part of the *c-myc-Max* heterodimer once more.

Specific DNA binding allows both activation and suppression of a number of genes including p53 and the HLA-B-gene, as well as those involved with growth and apoptosis (Blackwood *et al.*, 1992). The amino terminal plays the most important role in gene transactivation and neoplastic transformation (Bar-Ner *et al.*, 1992). Regulation of *c-myc* activity involves a number of mechanisms, including phosphorylation (Seth *et al.*, 1991). Gene expression may also be autoregulated, where increasing *c-myc* oncoprotein concentrations act by a putative negative feedback loop. However autoregulation of *c-myc* appears to be cell type-specifc and dependent on the *c-myc* concentration (Penn *et al.*, 1990). Of note, many transformed cells appear to have lost the ability to autoregulate *c-myc* expression and this may be in part due to the action of other oncogenes (Hirvonen *et al.*, 1991).

1.10.3 c-myc and the control of cellular proliferation and differentiation

The response of normally quiescent cells to growth factors is accompanied by the rapid induction of a number of genes including *c-myc*, *c-jun* and *c-fos* which appear to facilitate the early growth response. Several lines of evidence have shown *c-myc* to promote passage of quiescent cells from G0 to G1 of the cell cycle and to be expressed throughout proliferation. Moore (1987) measured the number of molecules of *c-myc* in individual, untransformed fibroblasts before and after mitogenic stimulation. At rest, each fibroblast contained approximately 300 molecules of *c-myc*, which increased rapidly to over 10,000 per cell in the early part of the G1 phase of the cell cycle. This concentration then fell to around 4,000 molecules per cell and persisted at this level throughout the remainder of the cell-cycle as long as the cell continued to divide. Similarly, *c-myc* mRNA increases 10-30 fold in fibroblasts when stimulated by external growth factors (DePinho *et al.*, 1991).

c-myc is able to stimulate proliferation of quiescent fibroblasts without the action of other immediate early response genes (Ellers et al., 1991). Antisense oligonucleotides to *c-myc* mRNA have been shown to inhibit progression throughout the cell cycle, interestingly not at the G0-G1 phase, but in G1 (Heikklia et al., 1987). Extrinsic cmyc relieves this inhibition, to allow continued progression through the cell cycle (Roussel et al., 1991). c-myc may also influence cell-cycle checkpoints at the S-G2 phase (Shibuya et al., 1992). Hence c-myc may regulate passage through the cell cycle at different stages in different cell types. Continued proliferation is dependent on maintainance of steady state *c-myc* expression and continued mitogenic stimulation. Withdrawal of mitogens produces a rapid down regulation of both c-myc mRNA and protein, consistent with a system of sensitive and rapid degradation. Contact inhibition of confluent cells in culture has also been shown to be accompanied by a fall in c-myc mRNA and protein levels (Moore et al., 1987). c-myc expression may involve control at several sites; at the DNA level, with modulation of transcriptioninitiation and elongation. Post-transcriptional mechanisms include mRNA turnover and translation (Marcu et al., 1992). The half-life of c-myc mRNA is also brief due to 3'-AU-rich untranslated regions which are found in a number of unstable mRNAs.

It is noteworthy that certain anti-proliferative cytokines, such as interferon- γ and transforming growth factor- β (TGF- β), also produce down regulation of *c*-*myc* mRNA, which may partly explain their mode of action (Pietenpol *et al.*, 1990). Two possible roles have been proposed for *c*-*myc* in control of proliferation. In the first,

continued c-myc expression is an essential requisite for proliferation, but control is limited to G1. The second model envisages c-myc to have no obvious role in cell cycle mechanisms, but determines whether cells proliferate or arrest in response to the modulating action of other genes and cytokines (Evan *et al.*, 1993).

Chemical induction of differentiation of cells *in vitro* produces a rapid fall in *c-myc* protein levels (Campisi *et al.*, 1984). However, if *c-myc* levels are artificially elevated, cells do not differentiate, presumably because they are under continual stimulation to proliferate and unable to exit the cell cycle. Under normal conditions *c-myc* is tightly regulated in response to external growth stimuli and continued expression of the gene is only seen in exponentially growing cells. Accordingly *c-myc* may be intimately involved in those processes which select cells to proliferate, differentiate or apoptose.

1.10.4 *c-myc* and apoptosis

Programmed cell death, or apoptosis, is a major process involved in the control of cell and tissue mass and differs from necrosis both morphologically and in being a genecontrolled process. It is central to embryogenesis and neuronal development and forms an integral part of those processes that regulate cell numbers in a given tissue. *c-myc* also plays a role in physiological apoptosis, as elevated *c-myc* protein levels accompany involution of thymocytes and breast tissue. Deregulation of apoptosis has come to be increasingly recognised as a potential mechanism for tumour formation (Wyllie 1987, 1993). Apoptosis is an active process that requires the induction of certain genes and their encoded proteins, such as endonucleolytic enzymes for digestion of DNA. Evan et al., (1992) showed that apoptosis in rat fibroblasts was cmyc dependent, though this only occurs in cells whose cell cycle has slowed. c-myc has been shown to induce apopotosis in serum-deprived fibroblasts or in cells where proliferation has been suppressed by anti-proliferative cytokines (Evan et al., 1993). Conversely, apoptosis may be inhibited by cytokines such as IL-3; withdrawal of IL-3 from cell medium results in quickened *c-myc*-induced apoptosis (Askew *et al.*, 1992). However *c-myc* sensitivity and apoptosis may be tissue specific as T-lymphocytes die in response to dexamethazone, which is accompanied by down regulation of *c-myc* (Alnemri et al., 1992).

Further evidence for the dual roles of c-myc have been advanced by study of the effects of the adenovirus E1A and cell proliferation. This virus shares the paradoxical

ability of c-myc in being able to stimulate proliferation, whilst retaining the capacity to initiate apoptosis (White *et al.*, 1991); it is clearly in the interest of the virus to suppress cell death. Thus it may not be possible to invoke proliferative mechanisms without priming the cell for apoptosis and this may represent a basic means of maintaining genomic stability during cell replication.

Recently other oncogenes have been discovered which regulate susceptibility to apoptosis. Fibroblasts transfected with both c-myc and bcl-2 show a significant reduction in their apoptotic potential compared to cells which express c-myc alone. Cells expressing both genes proliferate normally, suggesting that bcl-2 is only interacting with processes leading to death of the cell (Fanidi *et al.*, 1992). Consequently this represents a dynamic relationship where one gene prevents the lethal effects of another and in which both genes may be under the modulating influence of growth factors and cytokines (Fig 1.13). In uniting these findings to form a conceptual model of c-myc function in tumour formation, Evan and Littlewood (1993) suggest deregulated c-myc activity may not only stimulate inappropriate cell proliferation, but also allow survival of cells (with gene abnormalities) that would otherwise die.



Figure 1.13. Interaction between *c-myc*, *bcl-2* and other factors regulate apoptotic activity.

1.10.5 Other oncogenes studied in melanoma

To date the *ras* family of oncogenes have been studied more extensively in melanoma than any other gene. This is partly historical as this group of oncogenes were the first to be isolated from human tumours and subsequently shown to be expressed in a wide range of human malignancies (Weinberg *et al.*, 1984). The *ras* family consists of three genes; H-*ras*, Ki-*ras* and N-*ras*, each of which encodes a membrane-bound protein of 21 kD thought to be involved in signal transduction following stimulation of proliferation by growth factors.

In an important study, ras mutations were only found in melanomas that arose on sunexposed sites (Van' T Veer et al., 1989). Furthermore all mutations were found to occur at or near dypyrimidine sites, recognised as targets of UV irradiation. Bos found (1989) UV radiation to induce ras point mutations at codon 61. Both these lines of evidence implicate UV irradiation in the induction of ras mutations. However, Albino et al. (1989) found only 5-6% of human tumours contained mutated ras genes, whereas 25% of melanoma lines displayed the mutation. N-ras was most commonly affected, being mutated almost ten times more frequently than H-ras, whilst mutation of Ki-ras was not observed at all. Mutations were not detected in precursor lesions (i.e. dysplastic or benign melanocytic naevi) and this supported other studies which have proposed that ras mutation is a late event in melanoma formation. In addition, ras mutations did not correlate with pathological or clinical parameters. Thus ras mutations may only have an indirect role in the genesis of a subset of melanomas. The inconsistent relationship between ras and melanoma would suggest that mutation is largely the result of increased genetic instability associated with the malignant process rather than the cause of it (Bos 1989).

c-fos and *c-jun* are thought to form a heterodimer, AP-1, which is involved in recognising a DNA-sequence-specific region that mediates the transcriptional response to phorbol esters (there act as melanocyte growth factors). In a study of the mRNA of these oncogenes in benign and metastatic melanoma cell lines, a general increase in *jun-B* and *c-fos* mRNA was observed in metastatic lesions, with a fall in *c-jun* transcripts, suggesting a possible role in formation of metastases (Yamanishi *et al.*, 1991). Reduced expression of the *nm23* gene has been associated with tumour progression in a number of different tumour types and cell lines. In a study of 33 human metastatic melanomas, disease-free interval was found to correlate with *nm23* mRNA levels, supporting a proposed role for this gene in melanoma metastasis (Flørenes *et al.*, 1992).

Other isolated reports of oncogene activation in melanoma have been reported. However, again, the exact nature of their association and importance remains obscure. c-myb has been noted to be involved in chromosomal rearrangements but this does not appear to be related to tumour progression (Linnenbach *et al.*, 1988, Dasgupta *et al.*, 1989). c-kit encodes a transmembrane receptor protein kinase, the expression of which is reduced in melanomas compared to melanocytes (Lassam *et al.*, 1992). Suppression of c-kit is thought to confer a selective growth advantage on melanoma cells.

1.11 Tumour Suppressor Genes

1.11.1 Introduction

In contemplating the role of chromosomes in cancer 80 years ago, Boveri postulated the role of protective chromosome function;

The unlimited tendency to rapid proliferation in malignant tumour cells could result from a permanent predominance of the chromosomes that promote division ... Another possibility (to explain cancer) is the presence of definite chromosomes which inhibit division ... Cells of tumours with unlimited growth would arise if those 'inhibiting chromosomes' were eliminated ... since each kind of chromosome is represented twice in the normal cell, the depression of only one of these two might pass unnoticed ... (Fischer, 1929).

Early cytogenetic studies revealed that certain tumours exhibited specific chromosomal deletions and loss of genes from these sites implied they had a protective function. As a result of investigating such losses in familial retinoblastoma, Knudson (1971) postulated at least two independent mutations of a specific tumour-linked gene were required prior to tumour formation. In familial cases, the first mutation was present in the germ cell and thus present in every cell. Individuals inheriting this mutation would be more susceptible than the general population to developing retinoblastoma as they only had to suffer a single further mutation to become homozygous for loss of protection. In the much rarer sporadic cancer, two mutations would be required to occur in exactly the same cell and accordingly this was much more unlikely. Knudson's hypothesis has been increasingly supported by studies into other familial cancers which have revealed that these syndromes are linked to the inherited loss of a defined chromosomal locus at one allele. The retinoblastoma gene has gone on to be

extensively investigated as it is expressed in almost all cell types, suggesting a more widespread role in control of growth and proliferation (Cooper *et al.*, 1989). However, so far it has been strongly associated with only two cancers, retinoblastoma and osteosarcoma. The importance of this gene in itself is not to be underestimated but has been largely overshadowed by the discovery of another tumour suppressor gene, p53.

1.12 The Tumour Suppressor Gene *p53*

1.12.1 Introduction

The term 'cancer' comprises many different malignancies, arising from almost every cell type in the human body. One of the major aims of cancer research has been to identify the underlying mechanism in transformation and, better still, one that may unify these diseases in a common process; a common denominator that would reveal a route to better understanding, prevention and therapy. At present the criteria for a prevalent factor appear to be fulfilled by the tumour suppressor gene p53, which is mutated in the majority of human cancers (Nigro *et al.*, 1989, Hollstein *et al.*, 1991, Donehower *et al.*, 1993). The frequency of p53 gene mutations in human malignancies suggests it plays a central role in tumour biology (Vogelstein *et al.*, 1992). Recognition of the potential importance of p53 has made it the focus of more attention than any other oncogene and this exponential rise in interest has produced many publications. A concise review of current knowledge on p53 and its function is presented below.

1.12.2 The p53 gene and oncoprotein

p53 is so named for it denotes a nuclear phosophoprotein of 53 kilodaltons in molecular weight. The gene lies on the short arm of chromosome 17 (17p13.1) and encodes a protein of 393 amino acids. It was discovered whilst investigating the oncoprotein of the SV40 DNA virus (Lane *et al.*, 1979, Linzer *et al.*, 1979), which contains an oncogene encoding an oncoprotein known as large T antigen. Whilst attempting to isolate large T antigen, Lane and Crawford found a protein complexed to it, later identified as p53. It became apparent that large T antigen formed this complex to inactivate p53 and this was later established to be the route of action of other tumourigenic viruses, including the adenoviruses E1b and the papilloma virus E6. Interestingly these viruses bind to different sites on the p53 molecule (Lane, 1990).

Molecular genetic analysis of a diverse range of cancers has revealed alteration of both alleles of the gene, one by deletion and the other through point mutation, to support the contention that this is a tumour suppressor gene. The gene and its encoded protein are highly conserved, particularly in five specific domains (Soussi et al., 1987). There is 50% conservency between frog and human p53 and 80% between murine and human protein indicating the evolutionary importance of this gene. Somatic mutations of the p53 gene occur in four of these five conserved regions (or 'hotspots') in 50-80% of all sporadic cancers and are contained within exons 5 to 8; region A (codon 129-146), region B (codon 171-179), region C (codon 234-260) and region D (codon 270-287) (Hollstein et al., 1991). In an extensive study of p53 mutations in different tumour types, 98% of 280 point mutations were found to occur in a 600 base pair sequences from codon 110 to 307 (Hollstein et al., 1991). Mutations may occur outside these regions but they are rare. Germline mutations of p53 also occur in inherited cancers; for example, the Li-Fraumeni syndrome is a rare condition in which individuals inherit a missense or nonsense mutation of one allele of the gene in an autosomal, dominantly inherited syndrome (Malkin et al., 1990). Affected families are at increased risk of developing several malignancies by their early 30's, including breast cancer, ovarian carcinomas and possibly melanoma.

A number of studies have confirmed the tumour suppressive action of the wild-type (naturally occurring) protein; co-transfection assays with *ras* have shown *p53* to prevent transformation of mouse fibroblasts (Finlay *et al.*, 1989, Eliyahu *et al.*, 1989). This observation has been made in colorectal tumour lines (Baker *et al.*, 1990) and cultured prostatic tumour cells (Isaacs *et al.*, 1991).

In normal cells the protein is present in only minute quantities and is essentially undetectable by immunhistochemical techniques. Mutation of the gene may alter the tertiary structure of the protein to 'stabilise' it and so increase its half-life from 20 minutes to approximately 24 hours. This has the additional effect of resulting in relative intracellular overaccumulation (Iggo *et al.*, 1990). Secondly, the stable oncoprotein becomes detectable in the cytoplasm as well as its normal intra-nuclear localisation. The alteration in morphology between the wild-type and mutant protein makes it distinguishable by specific monoclonal antibodies. PAb240 is a 'mutant'-specific antibody that identifies an epitope in the central region of the protein which remains obscured in its wild-type conformation (see Section 2.4.1.). The latter can be identified using PAb246. However there is an emerging body of evidence to suggest

the p53 oncoprotein may be stabilised, and thus detectable, by processes other than p53 gene mutation. This is thought to occur by either the action of other oncogenes, such as *ras* and *c-myc* (Lu *et al.*, 1992), or post-transcriptional mechanisms (Han *et al.*, 1991).

1.12.3 Role of wild-type p53

The last few years have seen an expanding list of reported functions for wild-type p53, shown in Table 1.4. The protein encoded by the p53 gene is not essential for life, as mice lacking this gene develop normally. However they almost all go on to develop cancers in adulthood (Donehower *et al.*, 1992, Harvey *et al.*, 1993). A number of avenues of research have suggested that wild-type p53 acts to facilitate repair of injured DNA, primarily as a checkpoint for progression through the G1 phase of the cell cycle. DNA damage induced by γ or UV irradiation produces an elevation in intra-cellular *p53* levels which coincides with arrest at the G1 phase of the cell cycle (Kastan *et al.*, 1991, Zhan *et al.*, 1993). Growth arrest at this stage allows repair of the damaged genome before proceeding to the S phase of the cycle. Additional work has shown that overwhelming DNA injury leads to *p53*-dependent apoptosis (Clarke *et al.*, 1993, Lowe *et al.*, 1993). Lane (1992) has thus termed *p53* the 'guardian of the genome' as he envisages the function of *p53* to regulate the passage of replicating cells and minimise the transmission and accrual of potentially transforming gene mutations.

Biological Functions	Biochemical Functions
Induces growth arrest at G1	Transcriptional transactivation from
Induces apoptosis following	sequence-specific DNA sites
DNA damage	Regulates transcription of genes without
Inhibits malignant cell growth	p53 DNA binding sites
Maintains genomic stability	Promotes annealing of single-stranded
	DNA
	Inhibits DNA helicase activity
	Inhibits DNA replication

Table 1.4.	Functions	attributed	to	p53
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p53 is able to perform a number of different actions and this pleiotropy may, in part, be due to its ability to induce other genes, that each contain a specific DNA sequence in the upstream regulatory region (Vogelstein *et al.*, 1992). This sequence is found in a diverse range of genes including that for creatine kinase, GADD45 gene (growth arrest DNA damage inducible gene) and the *mdm*-2 (murine double minute) oncogene. Similarly, *p53* can suppress the expression of a number of genes which contain the TATA promoter sequence of nucleotides. Binding to this sequence represses transcription by obscuring an essential part of the transcription-initiation complex (Seto *et al.*, 1992, Mack *et al.*, 1993). Thus elevated intra-nuclear *p53* inhibits the expression of a number of key genes intimately involved with the regulation of cell proliferation and growth. These genes may be proto-oncogenes encoding transcription factors, or may even be other tumour suppressor genes (Shiio *et al.*, 1993).

Identifying proteins that bind to p53 modulating its activity has now become a central theme in p53 research, for elucidation of the genes under its control is likely to provide greater insight into the processes involved in cancer. Table 1.5. shows an expanding list of p53-associated proteins which includes an important recent addition, replication protein A (RPA). This multi-subunit complex is essential for viability in yeast and appears to be essential for DNA unwinding prior to replication. It has also been demonstrated to be necessary for DNA excision and repair in human cells *in vitro* (Coverley *et al.*, 1991). p53 binds to RPA, inhibiting its unwinding effect at replicative initiation origins, to possibly regulate onset of S phase. This role remains speculative as mutant p53 is also able to bind RPA but does not cause G1 arrest (Dutta *et al.*, 1993). It is unlikely that all the proteins listed in Table 1.5. bind to p53 simultaneously, as some may be cell-type or cell cycle specific.

The role of these proteins and p53 awaits considerable further clarification. However a major step forward was recently taken by the confluence of three outwardly different lines of research. Together this work suggests that p53 oncoprotein induces production of a second protein which inhibits enzymes central to progression of mitosis. Independent research by Harper *et al.* (1993) and El Deiry (1993) identified a gene that encodes a 21Kd protein which inhibits the action of cyclin dependent kinases (Cdk's) essential for passage between G1 and S phase of the cell cycle (Fig 1.14). Harper *et al.* termed the gene Cip1 (for Cdk-interacting protein 1), whilst El Deiry named the gene WAF1 (for wild-type p53-activated fragment 1). El Deiry (1993) demonstrated WAF1 acted identically to p53 in inhibiting cell growth and established a further important link in showing that WAF1 was induced by p53.

Table 1.5. Proteins associated with p53

Cellular proteins	Viral proteins			
· · · · · · · · · · · · · · · · · · ·				
CBF, CCAAT binding factor	Ad5 E1B, adenovirus type 5 E1B			
E6-AP, facilitates HPV E6 binding to	EBNA-5, Epstein-Barr nuclear antigen			
p53	5			
HSP70, heat-shock protein 70	HPV16/18 E6, Human papilloma			
mdm2, cellular oncoprotein	virus16/18- oncoprotein E6			
RPA, replication protein A	SV40 Tag, simian virus large T antigen			
SPI, transcription factor				
TBP, TATA binding protein				
WT1, Wilms tumour gene oncoprotein				

Efficient regulation of cell proliferation demands that p53 activity is also subject to control. In the first instance modulation occurs at the gene level but can also involve post-transcriptional modification via phosphorylation or interaction with other proteins (Hupp *et al.*, 1992). The encoded protein of a transcription factor, *mdm-2* (murine double minute) oncogene binds to p53 and prevents its transcriptional activity (Ullrich *et al.*, 1992, Momand *et al.*, 1992). Inhibition of p53 tumour suppressive activity by this means has been associated with development of soft-tissue sarcomas in almost 30% of cases (Leach *et al.*, 1993). It is also of interest that the first regulatory associate of p53 to be discovered is also another oncogene, binding to sites different from those of the DNA viral proteins, implying other regulatory proteins may do the same.

1.12.4 *p53 mutation and human cancer*

As noted above, mutations of *p53* have been mapped to largely one of four highly conserved regions on the *p53* gene (Nigro *et al.*, 1989), 80% of which are missense point mutations which involve substitution of one base pair for another (Bartek *et al.*, 1991). Of interest, mutations may be tumour-specific; G:C to T:A transversions are most commonly found in lung and liver cancers whereas point mutations at A:T pairs are seen more commonly in oesophageal carcinomas (Harris & Hollstein, 1993). Transitions also occur at CpG dinucleotide hotspots, particularly in colorectal and

reticuloendothelial cancers (Hollstein *et al.*, 1991). Tumour variation may indicate mechanisms of tumourigenesis (Greenblatt *et al.*, 1994); hepatoma arising in areas where hepatitis B and aflatoxin B1 are prevalent is characterised by p53 mutations at codon 249 (Harris & Hollstein, 1993). Susceptibility of G to T transversions at this site have been given added support by mutagenesis studies (Harris & Hollstein, 1993). Similarly in cutaneous squamous cell carcinoma, 20% of p53 mutations may be the result of UVr-induced gene injury, as indicated by tandem mutations in which two cytosine bases are replaced by thymine residues (Drobetsky *et al.*, 1987).

As might be predicted, variation in mutational site may result in several proteins with differing properties. In the first instance mutant proteins may be simply inactive, the mutation producing a 'loss of function'. More subtly the mutant protein forms heterooligomers with the wild-type protein, inhibiting it by a 'dominant-negative' effect (Oren et al., 1992). Mutant p53 may also bind 'heat shock' protein 70. Lastly, and of most interest, certain p53 mutations produce oncoproteins which act as dominant oncogenes, acquiring a 'gain of function' (Levine, 1993). Several studies (Kern et al., 1992, Scharer et al., 1992) have demonstrated this by placing the p53 binding consensus sequence upstream of a minimal promoter and reporter gene. They have then observed the effect of p53 on reporter expression and have found positive results in both yeast and human cells. p53 activity can also be inhibited by sequestration of the oncoprotein within the cytoplasm, preventing it entering and acting within the nucleus. Loss of the protective function of p53 could have three possible effects: one, the pool of proliferating cells would expand, passing on potentially mutated genes to increase the likelihood of transformation. Secondly, this pool may be less susceptable to apoptotic mechanisms, increasing the risk of developing a critical 'mutant gene mass'; and finally, the process of neoplastic transformation may be aided by the dominant oncogene activity of some p53 mutants (Lane et al., 1990).

A number of studies on different tumour types have demonstrated mutation of p53 to be a pre-invasive, even pre-malignant event and p53 immunopositivity has been found in mild dysplasias of the oesophagus, bronchus, breast and larynx (Harris & Hollstein, 1993). However it is far more common to identify gene mutations in established malignancies, particularly at a clinically advanced stage (Greenblatt *et al.*, 1994). Elegant studies have demonstrated the association of p53 mutations with progression of cervical carcinoma (Crook *et al.*, 1992) and advancement of astrocytomas to glioblastomas (Sidransky *et al.*, 1992). Furthermore, the interaction of p53 mutation with other genetic events has been dramatically illustrated in the model of colonic cancer evolution by Fearon and Vogelstein (1990). The association between p53 alteration and tumour progression has led to the proposal that p53 may be a putative prognostic marker in some cancers. Overexpression of p53 oncoprotein has been identified as an independent marker of poor prognosis in a number of cancers including lung (Quinlan *et al.*, 1992), breast (Thor *et al.*, 1992, Allred *et al.*, 1993) and gastric cancer (Martin *et al.*, 1992). In the case of breast cancer, several studies have shown p53 overexpression to be an important prognostic determinant in node-negative disease, possibly sub-selecting this group for more aggressive therapy in pre-menopausal women (Silvestrini *et al.*, 1993, Thor *et al.*, 1993).

The expanding interest in p53 has led to the potential application of this knowledge towards the early detection and therapy of cancer (Vile, 1993). Screening of urine to detect p53 from urothelial cancers is under evaluation as are methods to identify elevated p53 levels from early bowel cancers from stool specimens (Harris & Hollstein, 1993). New therapies may become available using gene therapy (Lemoine *et al.*, 1993) or pharmacological manipulation of the p53 oncoprotein and proteins that interact with it, such as *mdm-2* (Harris & Hollstein, 1993).

To date, nine papers have been published on p53 oncoprotein expression in cutaneous melanoma. All but one of these studies have utilised immunohistochemistry to detect the oncoprotein, and have made varying attempts to correlate the degree of expression with clinical behaviour of the tumour. The results and conclusions of these studies are reviewed in detail in context to the findings of the present investigation in the discussion sections of Chapters 4 and 5.

Section 3

1.13 Cell Proliferation and Human Tumours

1.13.1 Introduction

The impression that rapid tumour growth is associated with poor prognosis has been held for several decades (reviewed by Tubiana et al., 1989). Initial studies to elucidate this relationship were clinically based and simple, essentially recording the period over which a patients tumour had grown. From this information a crude and inaccurate estimation of growth rate could be made. Despite obvious limitations, clinical appraisal of growth-rate in breast cancers suggested rapidly growing tumours had a higher incidence of local recurrence and a worse prognosis than slower developing lesions (Richards et al., 1948, Rigby-Jones et al., 1963, Boyd et al., 1981). Subsequently, measurements of tumour size were made using sequential X-rays; however, this method depended on a considerable period of observation, clearly at odds with any objective of early treatment. In spite of this, several studies showed a long clinical tumour doubling time (Dt) to be associated with better prognosis (Joseph et al., 1971, Galante et al., 1986). As understanding of cellular biology grew, it became increasingly evident that greater insight into tumour behaviour may be derived from investigating proliferation kinetics at the cellular level. As discussed in the previous section, one of the repercussions of altered oncogene or tumour suppressor gene function may be uncontrolled proliferation. Consequently, an integral part of this thesis was to investigate the association between oncogene expression and tumour proliferation.

1.13.2 The cell cycle

Initial interest in tumour kinetics followed the description of the cell cycle in 1951 by Howard and Pelc (1951). Studying uptake of p^{32} into*Vicia Fabia* they noted mitosis to be preceeded and followed by periods of reduced uptake. This was later correlated with a number of steps in a replicative cell cycle which have now been well characterised. The G1 phase (Gap 1) represents the start of the cycle; once stimuli are received to commence replication, the cell proceeds along a biochemical pathway which includes an initial 'start' signal. Quiescent cells may move from a G0 phase into G1, though this distinction is essentially arbitrary. Once the cell passes the 'start' point it is committed to divide and proceed to the S phase (synthesis phase). During this period the necessary metabolites for DNA and chromosomal synthesis are amassed and the genome replicated. The S phase is followed by a second period of relative inactivity, denoted as the G2 (Gap 2) phase in which the cell contains a tetraploid number of chromosomes (i.e. four sets as opposed to a normal complement of two). The cell now undergoes mitosis, producing two daughter cells, to restore the normal diploid number of chromosomes. The progeny can either re-enter the cycle at the G0/G1 phase, proceed to differentiate, or undergo apoptosis (Fig 1.14).

CELL LOSS CELL PRODUCTION differentiation maturation Μ G2 resting (G0) nutrient deprivation G1 hypoxia exfoliation S cell death SHTDR BUDR SPF metastases

Figure 1.14. The cell cycle. All tissues are composed of cell populations that each reside within phases of a cycle. G0, G1 and G2 comprise 'gap' phases that contain cell cycle checkpoints. DNA is replicated in S phase and the cell divides into daughter cells in M (Mitosis).

Using tritiated thymidine, (3H-TdR), Taylor (1957) was able to estimate the number of cells actively involved in DNA synthesis in the S phase of the cell cycle and calculate a thymidine-labelling index (TLI) as a marker of proliferation. Measurement of the TLI led to a rapid growth of interest in measuring proliferation kinetics of both normal and malignant tissues. Clinicians began to utilise the data from these studies in an attempt to identify differences in the growth characteristics of malignant cells as opposed to normal tissues, with the findings of a number of these studies suggesting the following;

- TLI values show considerable variation, both within a group of similar tumours and between different histological types. The highest values were found in lymphomas (Malaise *et al.*, 1973) and the lowest in adenocarcinomas (Steele *et al.*, 1977).
- 2) An association exists between the doubling time of tumours of a given type and the TLI (Malaise *et al.*, 1973).
- 3) TLI is seen to be higher in more aggressive, less differentiated tumours (Tubiana *et al.*, 1989).
- 4) TLI may indicate the risk of disease progression, as first observed in breast carcinoma (Tubiana *et al.*, 1986).

However the impact of these studies on cancer management was disappointing due to a number of reasons. Firstly thymidine-labelling was lengthy, involving ten days of processing, precluding routine use. Secondly, it soon became evident that cell proliferation was not the only factor in determining tumour growth and analysis of it alone would be unlikely to predict failure of treatment. Nevertheless several groups persisted with this and other methods, frequently yielding data that showed measurement of the labelling index (LI) to be of prognostic importance (Tubiana *et al.*, 1989).

1.13.3 Use of Bromodeoxyuridine (BrdUrd) to measure cell-cycle kinetics

The thymidine analogue, 5-Bromo-2-deoxyuridine (BrdUrd), is taken up in place of this nucleotide during S phase and incorporated into newly synthesised DNA. Three important steps were taken in the early 1980's which were to greatly stimulate the resurgence of interest in tumour kinetics. Initially, a monoclonal antibody was raised to recognise BrdUrd (Gratzner *et al.*, 1982). Secondly, flow cytometry was used to simultaneously measure BrdUrd uptake as a function of DNA content (Dolbeare *et al.*, 1983). Finally, at the Gray Laboratory analyses were developed to facilitate flow cytometric measurement of the LI and duration of S phase (Ts), from a single observation (Begg *et al.*, 1985). These measurements would then allow calculation of the potential doubling time of the tumour, or Tpot (described in detail in Section 2.6.). Subsequently this technique was applied to measure tumour cell kinetics *in vivo* (Wilson *et al.*, 1985, Wilson 1991) and has remained the method of choice in a

number of centres and studies, particularly those considering the relationship between Tpot and radiotherapy dosage schedules (Bennett *et al.*, 1992). Together, these developments made rapid, accurate estimations of kinetic parameters feasable and with fewer limitations. Table 1.6 summarises the different techniques available for human tumour proliferation studies (Wilson, 1989).

Table 1.6.	Summary	of methods for	or measurement	of cell	kinetics ir	human	tumours
(Wilson, 1	989)						

Method	Advantages	Disadvantages	
1. Mitotic Index		alow problems with	
a) Static method	applicable to all tumours	abnormal mitoses	
b) Stathmokinetic	measures mitotic rate	slow, requires spindle poison, mitotic collection curves never ideal	
2. Tritiated Thymidine			
a) LI	simple to perform on most tumours	slow, requires radioactive tracer to only yield static data	
b) PLM curves	data available from all parameters of cycle	multiple biopsies and <i>in-vivo</i> infusion of a radio-tracer	
3. Flow Cytometry			
a) S-phase fraction	fast, used in all tumours measures ploidy	static data, requires computer-aided analysis	
b) BrdUrd	rapid, assays doubling time and ploidy from single biopsy	requires IV injection	
c) Growth fraction	rapid, no DNA precursor, measures ploidy	static data, needs rapid fixation of fresh cell suspension	

1.14 Flow Cytometry

1.14.1 Introduction: what is flow cytometry?

Routine flow cytometry is less than 25 years old, but has already established itself as a powerful and versatile analytical tool. It allows the simple and rapid quantification of substances in minute quantities and has two main applications (reviewed by Carter *et al.*, 1994). Firstly, it can assay a wide range of intracellular parameters including individual proteins and nucleic acid content. Secondly, flow cytometry can sort cells, distinguishing one type from another. This has been clinically applied to haematological assays, including the red cell count and lymphocyte subgroup ratios in patients with Acquired Immune Deficiency Syndrome.

How does it work?

The principle of flow cytometry concerns the measurement of fluorescence derived from specifically labelled cells or proteins (Carter *et al.*, 1994). Flow cytometry is based on hydrodynamic principles which conduct a line of labelled particles, cells or nuclei, in a fluid system by virtue of 'hydrodynamic focusing'. Particles are delivered into a beam of laser light one at a time. Fluorochrome tags, specific to the cell or protein target, become excited by the laser and emit light of a specific wavelength and colour. Therefore, the amount of substance or number of cells are quantified and expressed as a function of their overall fluorescence. Of note, measurements are taken of each individual particle within the suspension and not just an average of the whole population.

This outwardly simple objective has three basic pre-requisites;

1) The sample is well prepared to minimise contamination. There is an axiom in flow cytometry; 'garbage in, garbage out'. Sample preparation is all important and this is particularly true when dealing with solid tissues where cells are separated using an enzyme 'cocktail'.

2) The target protein or cell requires a highly specific label, usually a monoclonal antibody or dye. This is, in turn, labelled with a fluorochrome tag, such as Fluorescein Isothiocyanate (FITC), or Phycoerythrin (PE). The last decade has seen a marked expansion in the range and type of monoclonal antibodies available, and with them the number of quantifiable factors. The choice of fluorochromes has also enabled two or three parameters to be simultaneously assayed and thus investigate any

apparent relationships. In multi-parameter analysis it is essential that emission spectra of the fluorochromes do not coincide to result in 'spectral overlap'.

3) Fluidic Systems: Particles are orientated within a flow chamber to intersect with the laser by hydrodynamic focusing, most commonly using laminar flow with viscous drag (Carter *et al.*, 1994); fluid at the centre of a moving channel flows more rapidly than that in contact with the walls of the chamber, which is slowed by viscous drag. The form of flow at the front thus assumes a parabola. As a result of this velocity gradient particles are drawn towards the centre by hydrodynamic focusing to form a stable parabola, prior to entry into the detection point.

Detection Systems

Fluorochromes emit light of specific wavlength in response to intense light stimulation (reviewed by Ormerod, 1994). Emitted light is scattered and focused prior to collection on photodetectors that convert light energy into electrical signals. In order to distinguish the output of several combined fluorochromes, light is optically filtered into separate bands of differing wavelengths. This is achieved with dichroic mirrors, interference phenomena and absorption filters, which selectively deflect or allow passage of various pulses of light, depending on their wavelength. Finally, separated signals are processed and digitally converted prior to graphic display on a screen. A number of highly powerful computer programmes facilitate simple analysis of this output (see Section 2.5.).

1.14.2 Attributes of flow cytometry

The principle attributes of FCM which make it an attractive technique for use in not only research but also clinical practice are as follows:

- Speed Tens of thousands of cells can be analysed in a few seconds. The technology dictates that most staining procedures and analysis can be carried out within a single day.
- Quantitation The use of computers to analyse the collected data ensure high quality statistics of measurement.
- 3) Simultaneous measurements This is the most important attribute as it facilitates multiparamater measurements on an individual cell basis.

1.15 Hypotheses

This project has two principle aims;

- 1) To use flow cytometry to investigate the range of expression of the *p53* and *c*-*myc* oncoproteins in primary cutaneous melanomas and their metastases.
- 2) To examine if any relationship existed between the level of expression of these oncoproteins and particular features of melanoma behaviour, including tumour cell kinetics and clinical parameters. To accomplish this, the following hypotheses were investigated:
- a) *p53* overexpression is a common event in melanoma and is associated with clinical outcome (Chapters 4 and 5).
- b) *c-myc* overexpression is a common event in melanoma and is associated with clinical outcome (Chapters 4 and 5).
- c) *p53* or *c-myc* oncoprotein overexpression occurs in pre-neoplastic lesions such as benign melanocytic naevi and dysplastic naevi (Chapter 6).
- d) Proliferation kinetics of malignant melanoma correlate with clinical outcome (Chapter 7).
- e) Abnormalities in *p53* and *c-myc* expression are related to proliferation kinetics (Chapter 7).

Chapter 2

Patient Material and Methods

2.1 Introduction

The prime objective of this study was to investigate expression of the p53 and c-myc proteins in cutaneous melanoma and, in so doing, further define their relevance to the clinical behaviour and pathology of this cancer. In an effort to study changes in oncoprotein expression with progression from benign to malignant tumours, a range of pigmented lesions were analysed principally by flow cytometry and, to a lesser extent, immunohistochemistry. Flow cytometry provides rapid, quantitative estimation of a given protein, whereas immunohistochemistry identifies the distribution of protein within cells and tissues. To date, little data has been published on the use of flow cytometry in the analysis of the p53 proteins and nothing on c-myc oncoprotein in melanoma. Consequently, it was desirable to further evaluate this method and assess the benefit of its application to the study of these proteins in melanoma. Pigmented lesions studied included:

- Benign melanocytic naevi (BMN)
- Dysplastic naevi (DN)
- Primary cutaneous melanomas
- Secondary melanomas, comprising nodal and cutaneous metastases.

2.2 Patient Material and Treatment of Melanoma

All material was derived from patients presenting to the outpatient department of the North-West Thames Regional Plastic Surgery Unit at Mount Vernon Hospital, Northwood, Middlesex.

Benign Melanocytic and Dysplastic Naevi

Clinically diagnosed BMN or DN were removed with a 1-2mm excision margin and sent for histological analysis. If the diagnosis was confirmed, no further clinical follow-up was carried out unless indicated.

Primary Melanoma

Primary melanomas were treated by surgical excision, usually under local anaesthetic as an out-patient procedure. If the diagnosis was uncertain the initial excision margin was confined to 2mm. Histological confirmation was then sought prior to a second, wider excision approximating to 1cm per mm thickness of tumour, up to a maximum of 3cm. Tumours arising on the face were excised with a maximum margin of 2cm. Tissue cover was provided using either skin grafts or local flaps. Sub-ungual melanomas were treated by amputation of either part or whole of the digit, depending on tumour thickness and extent.

Patients were routinely followed up in out-patient clinics. This occurred at three monthly intervals for the first two years, twice yearly for the following three years, and then once a year up until ten years post-diagnosis. Consultations consisted of a general symptomatic enquiry, followed by examination of the operation site and palpation of the regional lymph nodes and liver. In the case of axial tumours, this entailed examination of lymph nodes in both axillae and inguinal regions.

Metastatic Melanoma

Secondary disease clinically confined to regional lymph nodes (stage two) was treated by lymph node dissection. Localised cutaneous metastases were excised under local anaesthetic. Patients with evidence of widespread disease were referred to a medical oncologist for consideration of chemotherapy. Treatment was dependent on the patient's age and clinical status and consisted of dacarbazine alone, or in conjunction with other agents, including Interleukin-2 and Interferon α , as part of a trial. Two patients with multiple skin and sub-cutaneous metastases confined to the lower limb were treated with isolated limb perfusion, using melphalan at 39°C. Radiotherapy was given to a further two patients in this study, with the objective of obtaining control of locally recurrent disease unsuitable for surgical intervention.

2.2.1 Tissue fixation

Tissue samples were preserved by one of three methods:

1) Formalin-fixed tissue: Specimens were fixed in 10% formal saline prior to embedding in paraffin and sectioning. Sections for histological analysis were subsequently stained with haematoxylin and eosin (H & E).

2) Ethanol-fixed tissue: Fresh samples were directly fixed in 70% ethanol to allow prolonged storage at 4° C. Specimens were routinely preserved using this method for BrdUrd analysis and measurement of *p53* and *c-myc* oncoprotein levels.

3) Frozen tissue: In a limited number of tumour specimens, particularly nodal secondaries, sufficient material was available to additionally allow preservation of some tumour as fresh frozen tissue. The majority of samples were dissected into 3mm³ cubes and suspended in Hank's medium containing 10% dimethylsulfoxide (Sigma Chemical Co.) in Eppendorf tubes prior to storage in liquid nitrogen. In a few cases specimens were snap frozen by rapid immersion in liquid nitrogen and kept at -70°C. These samples were to form the basis of the validation studies.

2.2.2 Study design and aims

The study analysed both naevi and melanoma specimens in order to examine the hypotheses stated in Section 1.15. The aims were:

1) To use flow cytometry to establish the range of *p53* and *c-myc* oncoprotein levels in human melanoma samples and benign melanocytic lesions.

2) Assess the degree of heterogeneity and variation in oncoprotein levels between samples of the same tumour preserved by three different methods; fresh-frozen in liquid nitrogen, ethanol fixation and formalin fixed paraffin-embedded (archival) material.

3) Investigate whether any association existed between oncoprotein expression, pathological parameters and clinical behaviour.

4) Where possible, administer BrdUrd to patients to measure parameters of tumour proliferation. Again, with the intention of evaluating the clinical significance of cell kinetics and any putative associations between these parameters and tumour oncoprotein expression.

2.2.3 Clinical data

In order to evaluate the clinical significance of oncoprotein expression, the following clinical information was sought from each patient or their notes:

- 1) Age
- 2) Sex
- 3) Date of primary diagnosis
- 4) Presence of a pre-existing naevus
- 5) Relevant family history of melanoma
- 6) Site of primary tumour, coded to include:
 - Head and neck
 - Upper limb, front or back
 - Torso, chest or back
 - Lower limb, front or back
- 7) Morphological tumour type:
 - Superficial spreading melanoma
 - Nodular melanoma
 - Lentigo maligna melanoma
 - Acral lentiginous
 - Amelanotic variant of above types
- 8) Histological parameters
 - Maximal (Breslow) tumour thickness
 - Clark's level of invasion
 - +/- ulceration
 - +/- mitoses
 - +/- regression
 - +/- lymphocytic infiltrate
- 9) Site of first recurrence
 - wound/local skin
 - regional nodes
 - distant metastases
- 10) Time interval between primary and first recurrence (disease-free interval- months)
- 11) Time interval between primary diagnosis and death (months)
- 12) Duration of follow-up to January 1994 (months)
- 13) Clinical stage at January 1994:
 - Stage 1 Alive and well, clinically disease free
 - Stage 2 Loco-regional disease
 - Stage 3 Distant metastases present
 - Stage 4 Patient died.

2.3 Sample Preparation and Staining of Oncoproteins

In each of the methods discussed below, antibody concentrations were optimised using dilution studies performed on an ethanol-fixed melanoma metastasis (data not shown).

2.3.1 Ethanol-fixed melanomas

Fourteen primary and 36 secondary ethanol-fixed malignant melanomas were analysed for p53 and *c-myc* oncoprotein levels.

Method

1) Preparation of nuclei

A 3mm^3 sample of each specimen was minced and incubated in a 10ml pepsin solution (0.4mg/ml porcine pepsin in 0.1M hydrochloric acid, Sigma Chemical Co. Poole UK). The mixture was constantly agitated at 37° C for 45-55 min and then filtered through a 35μ m nylon mesh in a Swinnex holder. Nuclear concentration was estimated using a haemocytometer (Weber, UK) and adjusted to a concentration of less than 10^6 nuclei/mm³. The suspension was then aliquoted into four samples and washed twice in phosphate buffered saline (PBS), (centrifuged at 2000rpm, for 5 min between washes).

2) Primary antibodies and labelling of oncoproteins

One pair of samples were allocated for p53 analysis and a control, and the second pair for the *c-myc* assay and a control. In the case of test samples (as opposed to control samples), each nuclear pellet was incubated with the appropriate anti-oncoprotein antibody for 1hr at room temperature. Antibodies were added in 100µl of a 1:25 solution of PBS containing 0.5% normal goat serum (NGS) and a detergent, Tween 20 (Polyoxyethylenesorbitan monolaurate, Sigma Chemical Co. UK). p53oncoprotein was labelled using PAb1801, a mouse monoclonal antibody (Cambridge Research Biochemicals Ltd, Cheshire UK), which detects both wild-type and stabilised p53 oncoproteins. The isotypic control was provided by using mouse IgG at the same concentration as the antibody (Cambridge Research Biochemicals Ltd, Cheshire UK). *c-myc* was identified with a pan-*myc* polyclonal antibody (Cambridge Research Biochemicals Ltd, Cheshire UK). Antibody solution was not added to the control tubes.

3) Secondary antibodies

The suspension was then washed in PBS and incubated with the fluorochrome marker. For p53 this consisted of a goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (Sigma Immunochemicals, Poole UK). *c-myc* IgG was labelled

using a goat anti-rabbit IgG-FITC conjugate (Sigma Immunochemicals, Poole UK). Both fluorochromes were added in a 1:50 solution of NGS/Tween in PBS for 30 min at room temperature.

4) DNA staining

The mixture was then washed in PBS and resuspended in a 1ml solution of PBS containing 1mg/ml ribonuclease (Sigma Chemical Co., Poole UK), prior to addition of 20μ l of propidium iodide (Sigma Chemical Co., Poole UK). Ribonuclease was used to minimise labelling of extrachromosomal oligonucleotides and RNA which would otherwise interfere with measurement of DNA content. Flow cytometric analysis was usually performed on the day of processing and always carried out within 24 hours to minimise leakage and other potential artefacts.

2.3.2 Paraffin-embedded material

Nuclei were extracted from paraffin-embedded naevi and melanomas using an adaptation of the method of Hedley *et al.* (1983). Paraffin-embedded specimens analysed in this study comprised the following:

- 96 sections of both primary and metastatic melanoma excised from 96 patients who had received BrdUrd to measure cell kinetics.
- 30 BMN and 30 DN (diagnosed according to WHO criteria (Clemente *et al.*, 1991).

Method

1) De-waxing and preparation of nuclei

Four 35µm sections were cut from each paraffin-embedded block, applied to Sellotape and then onto a plain glass slide. Slides were stored at 4°C until re-hydrated. Attempts were made to limit analysis to tumour or naevus cells by excising the appropriate area from each slide, still retained by the Sellotape. Samples were dewaxed in conical glass tubes, and the Sellotape removed, using 5ml of xylene (BDH, Poole UK) for 10 min and the process repeated. Specimens were then rehydrated through successive increasingly hydrated alcohols, commencing with 100% ethanol (Hayman Ltd, UK), 90%, 70% and finally 50% solutions. In each case the sample was kept in 5ml of the alcohol for 10 min, prior to centrifugation at 1100rpm for 5 min. Samples could be stored in plastic universal containers with 50% ethanol indefinitely at 4°C.

2) Preparation of nuclei

Prior to staining, the sample was washed twice in 10ml of distilled water for 10 min and then minced with scissors. Pepsin enucleation was achieved using a greater concentration (4mg/ml) than employed for ethanol-fixed tumours, and the suspension incubated for a longer period of 50 to 60 min at 37°C. The sample was filtered, washed and aliquoted as before prior to incubation with the same concentrations of primary and secondary antibodies as used for ethanol-fixed samples. The yield of nuclei was less using this method, and thus the final labelled nuclear pellet was resuspended in only 0.5ml of PBS containing 1mg/ml RNAse and 20µg/ml propidium iodide. Again, FCM analysis was carried out the same day or within 24 hours.

2.3.3 Fresh-frozen tissue

Fresh-frozen melanomas were analysed as part of the validation studies presented in Chapter 3 to compare oncoprotein expression in tumours preserved by different methods.

Preparation of whole cells

Tumour samples were rapidly thawed and minced prior to incubation in a disaggregating solution of 0.2% type-II collagenase (Sigma Chemical Co.) and 0.02% DNAase type 1 (Sigma Chemical Co.) in Hank's buffered salt solution, pH 7.4 (Sigma Chemical Co.) for 35-50 min at 37°C. Following filtration through a 35μ m mesh in a Swinnex holder, the resulting suspension was centrifuged at 1000rpm for 5 min. The pellet was resuspended in 1ml PBS and then 9ml of cold 70% ethanol added whilst vortexing. Oncoproteins were labelled as outlined above in steps two to four of Section 2.3.1.

2.3.4 Staining of BrdUrd-labelled nuclei

Administration of BrdUrd

Administration of BrdUrd to patients in this study was commenced following Mount Vernon Hospital Ethical committee approval and only after gaining the patients informed, signed consent. Crystalline BrdUrd (CRC drug formulation unit, University of Strathclyde, Glasgow) was made up as a fresh solution (200mg in 20ml of 0.9% normal saline) immediately before each administration as it degrades within a short time in the aqueous form. The dose was administered as an intravenous bolus, four to six hours prior to surgery or biopsy. The dry, unfixed excision specimen was sectioned by the pathologist to ensure that the sample for BrdUrd analysis was not taken from an area that may hamper histological diagnosis or assessment of prognostic information. Consequently, for primary melanomas it was imperative that sampling did not occur in the thickest region. The sample allocated for analysis was then placed in 70% ethanol and stored at 4°C until processed.

Staining for BrdUrd

A 3mm³ segment of ethanol-fixed tumour was minced and enzymatically enucleated in 10ml of 0.4mg/ml pepsin in 0.1M HCl (Sigma Chemical Co.). The mixture was constantly agitated at 37°C for 45-55 min and then filtered through a 35µm nylon mesh. Nuclear concentration was estimated using an haemocytometer and adjusted to achieve a concentration of less than 10⁶ nuclei/mm³. The suspension was centrifuged at 2000rpm for 5 min and the nuclear pellet resuspended in 2.5 ml of 2M HCl for 12 min at room temperature (acidification partially unwinds the DNA to allow access of the anti-BrdUrd antibody to its epitope). The sample was then washed twice in PBS prior to addition of a 1:25 dilution of rat anti-BrdUrd monoclonal antibody (Hybridoma Unit, Institute of Cancer Research, Sutton, Surrey, UK) in 0.5ml of a mixture of PBS, 0.5% Tween-20 (Sigma Chemical Co.) and 0.5% normal goat serum (NGS) (Sigma Chemical Co.) and incubated at room temperature for one hour. After washing in PBS, the second antibody was added. This comprised a 1:25 solution of goat anti-rat IgG-FITC conjugate (Sigma Chemical Co.) in the PBS/Tween/NGS solution. The suspension was incubated at room temperature for a further 30 min, washed in PBS and resuspended in 2ml of PBS containing 20µl of 1mg/ml propidium iodide solution to label DNA, prior to flow cytometric analysis. RNAse was not necessary in this procedure as it is hydrolysed by the 2M HCl.

2.4 Antibodies

2.4.1 Detection of p53 oncoproteins

p53 antibodies

A number of antibodies are commercially available for the detection of the p53 gene product in both the wild-type and stable ('mutant') conformations; each antibody recognises different epitopes on the p53 molecule:

PAb 1801	Identifies both conformations
PAb 246	Wild-type specific
PAb 240	Stable ('mutant') conformation

PAb1801: (Cambridge Research Biochemicals Ltd.) is a mouse monoclonal antibody which recognises an epitope between amino acids 32 and 79 on the human p53 polypeptide (Banks *et al.*, 1986). It was used throughout this study as it is suitable for flow cytometric detection of p53 oncoprotein and can be used on pepsin-degraded material. The antibody is derived from murine ascites raised against a p53-beta-galactosidase fusion protein produced from a bacterial expression vector.

PAb240: (Cambridge Research Biochemicals Ltd.) was used in the validation studies to assess and compare p53 oncoprotein levels as measured using either PAb1801 (non-specific) or PAb240 ('mutant'-specific). It is derived from cell line supernatants, again raised against a p53-beta-galactosidase fusion protein in mice. It recognises an epitope between amino acid 156 and 214 of the stable ('mutant') conformation (Gannon *et al*, 1990).

PAb246: (Cambridge Research Biochemicals Ltd.) recognises the wild-type conformation of p53 and was used to measure wild-type p53 oncoprotein expression in the validation studies presented in Chapter 3. It is derived from murine cell line supernatants immunised against SV-40 transformed cell lines and recognises an epitope between amino acids 88 and 109 on the human wild-type protein.

The validation studies described in Chapter 3 demonstrate that in melanoma the great majority of detectable p53 oncoprotein was in the stable form and that PAb1801 reliably identified the stabilised conformation. This has also been shown in other studies which have compared PAb1801-p53 immunopositivity in sections of tumours with known p53 mutations (Lassam *et al.*, 1993). The use of PAb1801 was also

based on a second assumption which considered the wild-type protein to have a short half-life and, therefore, unlikely to be present in detectable amounts (Oren *et al.*, 1981; Finlay *et al.*, 1988).

2.4.2 Detection of c-myc oncoprotein

The *c-myc* protein was identified using a rabbit polyclonal antibody raised against *myc* oncoproteins (Cambridge Research Biochemicals Ltd.). Both N-*myc* and L-*myc* are highly tissue specific (Garte, 1993) and the oncoprotein products of these genes have not been described in melanoma. Furthermore, no evidence of either the mRNA or protein product of N-*myc* or L-*myc* were found in a study of 21 different melanoma cell lines of varying metastatic capability (Chevenix-Trench *et al.*, 1990). Consequently, it was assumed that neither of these genes would be expressed in benign or malignant melanocytes and that the polyclonal *myc* antibody would only label the *c-myc* oncoprotein. As with PAb1801, the *c-myc* oncoprotein using the *c-myc* monoclonal antibody, 6E10, has been described in several papers (Sikora *et al.*, 1987; Jack *et al.*, 1986). However, in the experience of the group at the Gray Laboratory and others (Lincoln & Bauer, 1989), 6E10 has not always proved reliable or stable using flow cytometry.

In an effort to ensure that we were indeed measuring c-myc, the validation studies compared both antibodies on sections of the same tumour, in both frozen and ethanol-preserved tumours.

c-myc antibodies

Rabbit polyclonal antibody to myc oncoproteins: (Cambridge Research Biochemical Ltd) is raised against a synthetic oligopeptide, Ala-Pro-Ser-Glu-Asp-Ile-Ttp-Lys-Lys-Phe-Glu-Leu-Cys, which is common to c-, L- and N-myc and between several species. The IgG is isolated directly from rabbit sera and affinity purified prior to stabilisation and lyophilisation.

6E10: (Cambridge Research Biochemical Ltd) is a mouse monoclonal antibody raised against a synthetic peptide sequence, which recognises an epitope between residues 171 and 188 on the human c-myc oncoprotein (Evan *et al.*, 1985). The immunoglobulin was obtained from a cell culture supernatant.

2.4.3 BrdUrd monoclonal antibody

This is derived from a rat hybridoma and isolated from cell line supernatant by the Hybridoma Unit, Institute of Cancer Research, Sutton, Surrey, UK.

2.4.4 Fluorochromes

These immunoglobulins bind to epitopes on the oncoprotein-labelling antibody to allow identification and quantification on the flow cytometer (see Section 2.5. on flow cytometry). The antiserum is raised against either mouse, rat or rabbit IgG; in the case of p53 this was a goat-anti mouse IgG, for *c-myc* it was a goat anti-rabbit IgG and for BrdUrd this was a goat anti-rat IgG. The antiserum is purified and then conjugated to FITC isomer 1 (Sigma Chemical Co.).

2.5 Flow Cytometry

The principles of flow cytometry are outlined in Section 1.14.

2.5.1 Description of the FACScan

The FACScan system is an automated cell analyser developed for both research and clinical applications. It consists of a bench top sensor module coupled with a computer module which controls both acquisition and analysis of data (Fig 2.1). Cells or nuclei enter the flow chamber one at a time and are irradiated by a 15mW, 488nm air-cooled argon-ion laser. Dichroic mirrors spectrally filter emitted light, separating and deflecting longer wavelengths whilst transmitting shorter wavelengths. Longer wavelengths are detected on two light scatter channels (front and side). These shorter wavelengths are further separated by other mirrors into the path of one of three photomultiplier detectors, FL1, FL2 and FL3, following which the signal is digitised and processed by the computer (Fig 2.2).

At 530nm the FL1 detector is optimised for FITC detection whilst the FL3 detector transmits wavelengths in excess of 650nm, suitable for detection of red light emitted by propidium iodide. The FL2 channel detects intermediate wavelengths (585nm), emitted by phycoerythrin in the red/orange band (this channel was not used in the present investigations). Dual parameter collection of data on FL1 and FL3 allows



Figure 2.1 The Becton Dickinson FACS scan. The sensor module is shown on the right of the computer module.





bivariate histograms to be formulated, recording expression of FITC-labelled data on the FL1 channel against DNA content on the FL3 channel. Examples of data are shown in Figures 2.3 and 2.4.

2.5.2 Examples of recorded data

1) Tumour Ploidy

Derangements in the chromosomal and DNA content of a cell are amongst the most common abnormalities found in tumour cells. The DNA histogram, produced by detection of DNA content using propidium iodide staining, shows whether tumours are diploid and contain the normal compliment of chromosomal DNA, or whether they are aneuploid, in which case there may be an excess or deficit of genetic material. Figures 2.3a &b show representative DNA histograms obtained from the FACScan.

Cells or nuclei may adhere to one another, producing 'doublets' with DNA echoes or peaks at regular points, giving the appearance of an aneuploid DNA histogram. This problem was addressed in two ways. Firstly, doublets were dissaggregated by gentle syringing of the sample through a 1ml syringe and a 25g needle. Secondly, utilising the doublet-discrimination mode on the flow cytometer prior to recording data, a region was set around the nuclear profile, using the FL3 area and width, to exclude any residual doublets, aggregates and cellular debris, to acquire a single nuclei suspension.

2) p53 and *c-myc* expression in diploid and aneuploid melanomas Bivariate histograms of oncoprotein content on the FL1 channel can be exhibited against DNA content on the FL3 channel. Figures 2.4a & b show expression of p53and *c-myc* in diploid and aneuploid melanomas respectively.

2.5.3 Data analysis and calculation of oncoprotein expression

Data was analysed using a computer acquisition/analysis programme, Lysys II (Becton Dickinson, San Jose Calif). Events are quantified by the imposition of computer generated windows (CGW) to define specific regions on the histogram or two-dimensional dot plot.

1) Use of Computer-Generated Windows

CGWs allow definition of specific populations of nuclei or cells and required phases of the cell cycle. Regions can be set around populations to omit extraneous interference from debris or from populations of cells whose data is not required. These regions are retained and superimposed on the control sample, to allow comparison of the number of nuclei and fluorescence in identical regions. In diploid specimens, seven regions were set around each sample, whilst eight were used in aneuploid melanomas. These are illustrated and explained in Figures 2.5a &b.

2) Calculation of Oncoprotein levels

Using Lysys II, oncoprotein positivity is calculated from comparison of the number of events within regions applied to the histograms of both the control and test sample (Fig 2.6). Initially a region is set around the control histogram to demarcate and differentiate labelled from unlabelled nuclei. However the secondary antibody (FITC) adheres non-specifically to a small proportion of nuclei and cellular debris, estimated to be less than 2% of the whole population of labelled material. To exclude this fluorescence, a region is set around those 2% of nuclei exhibiting the highest fluorescence values (R1). This region is automatically superimposed on the antibodylabelled histogram of the same specimen and subtraction of the event count of the antibody sample from the control gives the overall number of nuclei showing specific labelling due to oncoprotein overexpression. This is represented as a percentage of all nuclei, to give the % positivity (also referred to as % expression). Further regions can be set around populations of nuclei that lie within different phases of the cell-cycle, to allow analysis of oncoprotein expression within each phase. The mean fluorescence of the FITC labelled-nuclei gives an estimate of the relative amount of protein contained within each cell cycle phase and this can be represented numerically as a ratio of mean green fluorescence of positive cells compared to control (unlabelled) cells.



Figure 2.3a. DNA histogram of a diploid primary melanoma. The G1 peak represents fluorescence of PI-labelled nuclei in the Gap 1 phase of the cell cycle. The G2 peak is due to fluorescence in the Gap 2 phase, in which a tetraploid number of chromosomes are present prior to mitosis.



Figure 2.3b. DNA histogram of an aneuploid melanoma (nodal metastasis). Aneuploid nuclei contain an abnormal chromosomal content and are identifiable by their extra G1a (aneuploid) and G2a peaks.

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Figure 2.4 a. *p53* expression in a diploid primary melanoma. (Top) control sample, (Middle) Test sample, (Bottom) DNA histogram. Ordinate - fluorescense (FL1), abscissa - DNA content (FL3).



Figure 2.4 b. *c-myc* expression in an aneuploid primary melanoma. (Top) control sample, (Middle) Test sample, (Bottom) DNA histogram. Ordinate - fluorescence (FL1), abscissa - DNA content (FL3).



Figure 2.5a. The number of labelled nuclei in each phase of the cell cycle in diploid tumours are calculated by application of computer generated windows, or Regions, (described below) to the control and test histograms. The histogram above shows *c-myc* expression in a diploid primary melanoma;

- R1 Overall positive nuclei (specific and non-specific staining)
- R2 Labelled nuclei in G1
- R3 Labelled nuclei in S phase
- R4 Labelled nuclei in G2
- R5 Unlabelled nuclei in G1
- R6 Unlabelled nuclei in S phase
- R7 Unlabelled nuclei in G2



Figure 2.5b. Estimation of nuclear positivity in aneuploid tumours. Overlap of the G2d and G1a populations prevents an exact count of nuclei in each of these cell cycle phases. Top left - histogram of control sample, Top right - histogram of test sample, Bottom - DNA histogram.

- R1 Labelled nuclei in diploid G1
- R2 Labelled nuclei in aneuploid G1 (with diploid G2)
- R3 Labelled nuclei in aneuploid S phase
- R4 Labelled nuclei in aneploid G2
- R5 Unlabelled nuclei in diploid G1
- R6 Unlabelled nuclei in aneuplod G1 and diploid G2
- R7 Unlabelled nuclei in aneuploid S phase
- R8 Unlabelled nuclei in aneuploid G2

2.6 BrdUrd Labelling and Calculation of Potential Doubling Time (Tpot)

2.6.1 Use of BrdUrd to measure proliferation kinetics

Patient selection for BrdUrd

Whilst BrdUrd is free of acute toxicity, it carries a very small risk of mutagenicity, teratogenicity and may be cytotoxic in higher doses (Goz, 1978). Accordingly, it is only offered to patients over 20 years of age with a known, potentially life-threatening malignancy and who are not pregnant.

Time between injection and biopsy

BrdUrd is only taken up by cells in S phase, which then proceed through the cell cycle. The method depends on identifying BrdUrd-labelled cells as a function of their position in the cell cycle, which, in turn, can be determined from their DNA content. Therefore, the time between injection of BrdUrd and excision should be long enough to allow detectable cell movement, but insufficient for all those BrdUrd-labelled cells to have divided (Fig 2.7). An injection-biopsy interval of half the duration of S phase in hours (Ts) would be ideal to produce a profile of adequate distribution. However, a number of studies have shown considerable variation in the Ts within and between groups of tumours (reviewed by Wilson, 1993). The median Ts of oral cavity tumours is approximately 10hr, whilst that of bronchial carcinomas is about 15hr. In limited studies performed to date, the median Ts of melanomas is 10.7hr, with a range of 6.3hr to 20.5hr (Wilson, 1993). Therefore, an injection-biopsy interval of 4-6 hours has been used.

Processing of the tumour sample

In early studies whole cells were analysed, having been separated using an enzyme 'cocktail'. This had a number of drawbacks, including the need for immediate post-operative processing and the likelihood of a low cell yield. Schutte *et al.* (1987) modified this method, by fixing the biopsy sample in 70% ethanol, to allow storage at 4°C for an indefinite period and reveal opportunities for multi-centre studies.

Pepsin digestion confers a number of benefits and few disadvantages. It standardises procedures by excluding variation in enzyme 'cocktails'. Furthermore it produces a much larger yield of nuclei than other enzymes and thus reduces the amount of tissue required for analysis. This is of great importance in analysing melanoma samples,

where amounts of tumour may be very small. In analysing nuclei, the complications of non-specific staining due to cytoplasmic contaminants are excluded. However, other cytoplasmic markers of malignancy such as cytokeratin are also removed, and this may limit distinction between benign and malignant cells.

2.6.2 Data analysis

2.6.2.1 Principles underlying calculation of cell kinetic parameters

The labelling index

The labelling index (LI), is defined as the percentage of cells exhibiting significant BrdUrd uptake, making a simple correction for those cells which underwent division in the period between injection and biopsy (Wilson, 1991). The LI is calculated from analysis of the bivariate distribution of BrdUrd-labelled cells versus DNA content, as shown in Figure 2.7. In diploid tumours, calculation of the LI includes all cells, both malignant and non-malignant. In aneuploid tumours the LI can be calculated for the tumour population alone by setting regions according to DNA content.

The duration of S phase

Begg *et al.* (1985) proposed the principle of Relative Movement (RM) to calculate duration of S-phase. This is dependent on two assumptions. Firstly, that there is uniform distribution of labelling throughout S phase such that the mean DNA content of the population gives a value in mid-S. Begg coined the term 'relative movement' to describe the position of this population relative to G1 and G2. In order to measure RM, the mean DNA content of the G1 and G2 populations are calculated. The RM at time zero is estimated by subtracting the mean DNA content of G1 cells from that of the BrdUrd labelled population and dividing by the G1 DNA content subtracted from the G2.

 $RM = \frac{Fl(BrdUrd) - Fl(G1)}{Fl(G2) - Fl(G1)}$

Where Fl(BrdUrd), Fl(G1), and Fl(G2) denote the mean fluorescence values of these populations of cells. Thus the RM value at time 0 is 0.5. As labelled cells progress through S phase towards G2, the RM value will reach 1.0, at which point the only undivided BrdUrd-labelled cells will be found in G2.

Secondly, it is assumed that cells progress in a linear fashion through S-phase. Accordingly when the RM = 1.0, the labelled cells in G2 must represent cells that were in the early part of S phase at time 0. Therefore progression of cells with an RM value from 0.5 to 1.0 defines the Ts and allows calculation of this duration from a single observation:

$$Ts = \frac{1.0 - 0.5}{RM - 0.5}$$
 x t - where t is the time between injection and biopsy.

The potential doubling time (Tpot)

From these two parameters, the potential doubling time (Tpot) can be calculated, which represents the shortest possible time a cell population can double its number, taking into account the presence of dividing and non-dividing cells, but in the absence of cell loss. Tpot is calculated from the formula:

Tpot =
$$\lambda Ts$$

where λ is a correction factor for the age distribution of the tumour population (Steele 1977). λ varies between 0.693 and 1.38, but observations from experimental tumour systems has suggested a value of 0.8 (Wilson, 1992).

2.6.2.2 Derivation of LI and Ts from FCM profiles

Tumour samples were analysed on the flow cytometer to obtain a histogram showing the bivariate distribution of BrdUrd against DNA content. Figure 2.8a shows such a histogram, with the addition of regions to demarcate specific cell populations. The LI is the ratio of labelled to unlabelled nuclei, and is corrected to allow for passage of cells during the interval between injection of BrdUrd and biopsy. The corrected LI is determined by setting computer-generated regions around all the BrdUrd-labelled nuclei (R1) and around those labelled nuclei with G1 DNA content (R2). Having halved the number of events in the latter region, the resultant number is subtracted from the total number of BrdUrd labelled nuclei and the total number of nuclei. In order to calculate the relative movement of the BrdUrd-labelled cohort from G1 towards G2 and S phase, further regions are applied to the DNA histogram of total cells (2.8b). The DNA content of cells in the G1(R2), BrdUrd-labelled S (R3) and G2

(R4) phases are measured from the mean red fluorescence of nuclei within those regions.

From these values, cell kinetic indices can be calculated for a diploid tumour as follows:

Total Labelling Index (LI) = <u>Total BrdUrd cells</u> Total cells collected

Corrected LI = <u>Number of BrdUrd labelled cells - (1/2 G1 BrdUrd cells)</u> Total number of cells - (1/2 G1 BrdUrd cells)

In the case of an uploid melanomas, regions are set to measure the total number of an uploid cells and give a corrected an uploid LI:

Aneuploid LI = <u>No. of Aneuploid BrdUrd labelled cells - 1/2 Aneuploid G1 BrdUrd</u> Total Aneuploid population - 1/2 Aneuploid G1 BrdUrd

> $RM = \underline{Mean DNA \text{ content of } S - mean DNA \text{ content of } G1}$ Mean DNA content of G2 - mean DNA content of G1

 $Ts = \frac{0.5 \text{ x time interval (injection to biopsy in hours)}}{RM - 0.5}$

Tpot = $\underline{\text{Ts x } \lambda}$ (λ = 0.8 for human tumours) LI (corrected diploid/aneuploid)



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	Region S	stats						
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ste:	2/28/93	Gate G8=	R8					
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gn	Events	% Gated	% Total	Xmean	Ymean	P×,Py	resX,	rest
R1	7473	77.21	74.73	84.04	52.52	2, 1	1024,	1024
R2	Ø	0.00	0.00			0, 0	0,	6
R3	0	0.00	0.00			0, 0	0,	e
R4	0	0.00	0.00			0, 0	0,	e
	ile: ste: slect srame stal= gn R1 R2 R3 R3	Region S ile: DR30013 ste: 2/28/93 elected Prefer arameters: FL3 otal= 10000 gn Events R1 7473 R2 0 R3 0 R4 0	Region Stats ile: DR30013 Sample: H ste: 2/28/93 Gate G8= elected Preference: Ari arameters: FL3-A(LIN),F otal= 10000 Gated= gn Events % Gated 	Region Stats ile: DR30013 Sample: HUBU600, PS ste: 2/28/93 Gate G8= R8 elected Preference: Arithmetic/L: arameters: FL3-A(LIN),FL1-H(LIN) otal= 10000 Gated= 9679 gn Events % Gated % Total R1 7473 77.21 74.73 R2 0 0.00 0.00 R3 0 0.00 0.00 R4 0 0.00 0.00	Region Stats ile: DR30013 Sample: HUBU600, P53-F 013 ste: 2/28/93 Gate G8= R8 elected Preference: Arithmetic/Linear arameters: FL3-A(LIN),FL1-H(LIN) otal= 10000 Gated= 9679 gn Events % Gated % Total Xmean 	Region Stats ile: DR30013 Sample: HUBU600, P53-F 013 ste: 2/28/93 Gate G8= R8 elected Preference: Arithmetic/Linear arameters: FL3-A(LIN),FL1-H(LIN) otal= 10000 Gated= 9679 gn Events % Gated % Total Xmean Ymean 	Region Stats ile: DR30013 Sample: HUBU600, P53-F 013 ste: 2/28/93 Gate G8= R8 elected Preference: Arithmetic/Linear arameters: FL3-A(LIN),FL1-H(LIN) otal= 10000 Gated= 9679 gn Events % Gated % Total Xmean Ymean Px,Py 	Region Stats ile: DR30013 Sample: HUBU600, P53-F 013 ste: 2/28/93 Gate G8= R8 elected Preference: Arithmetic/Linear arameters: FL3-A(LIN),FL1-H(LIN) otal= 10000 Gated= 9679 gn Events % Gated % Total Xmean Ymean Px,Py resX,

Figure 2.6. Calculation of nuclear positivity. Application of R1 to the control histogram (left) defines the number of labelled nuclei due to non-specific staining. Application of this gate to the test histogram (right) estimates the number of both specific and non-specifically labelled nuclei (shown in red). Subtraction of the control R1 nuclei count from that of the test specimen, shown in the analysis tables below, gives the overall positivity for that tumour sample. In the histogram shown above, 77.5% of nuclei were p53 positive. Using the regions shown in figure 2.5, nuclear positivity can be calculated for each phase of the cell cycle.


Figure 2.7. Measurement of LI and Ts and calculation of Tpot from a single observation. The shaded area in the cell cycles indicate the position of a cohort of BrdUrd-labelled cells at the time of injection (only nuclei in S phase are labelled), the time of biopsy and the duration of S phase. The histograms below illustrate the predicted bivariate BrdUrd/DNA distribution at those times.



Figure 2.8a.Bivariate BrdUrd/DNA distribution from a diploid nodal metastasis.R1 - Total BrdUrd-labelled cells



Figure 2.8b. Application of additional regions to calculate DNA content of cells in G1(R2), S phase (R3) and G2 (R4).

- R2 Mean DNA content G1
- R3 Mean DNA content S
- R4 Mean DNA content G2

2.7 Histology and Immunohistochemistry

2.7.1 Introduction

Immunohistochemistry is the most frequently used technique for investigation of tumour oncoproteins and confers several advantages over other methods. Firstly, it is simple to perform and interpret. Secondly, it permits investigation of the architectural distribution of a given protein, to both identify its location within a cell as well as reveal the distribution of protein-positive cells within a tissue. In this respect it is a complimentary investigation to the quantitative facilities provided by flow cytometry. Immunohistochemistry has been the method of choice in investigating p53 oncoprotein expression in melanoma to date. Our departure from the exclusive use of immunohistochemistry was partly stimulated by the necessity of evaluating flow cytometry as an independent method of p53 and c-myc analysis, and also to allow further investigation of the potential benefits of combining both methods.

2.7.2 Conventional Histology

Four micron H & E sections of all tumours studied in this project were examined by a single observer, (Consultant Histopathologist, PR). Where possible, sections of tumours excised at hospitals outside Mount Vernon were also reviewed to obtain the required histological data. In the case of primary malignant melanomas, this consisted of the following:

Conformation of diagnosis Maximal tumour thickness (Breslow) Clark's level of invasion Presence of ulceration Comments on mitotic figures Presence of lymphocytic infiltrate Evidence of regression Presence of vascular and/or lymphatic invasion Completeness of excision

2.7.3 Immunohistochemistry and staining of oncoproteins

Staining for p53

Four micron sections of each specimen were applied to poly-L-lysine coated glass slides, dewaxed in xylene and rehydrated though increasingly aqueous alcohol solutions (100% to 70%). Slides were immersed in 10mM citrate buffer (adjusted to pH 6.0 using 2N NaOH) and heated for five periods of 5 min in a microwave oven (800W, Goldstar, Japan), ensuring that slides were always immersed in buffer. Microwaving of samples has been shown to lower the threshold of detection of a number of antigens (Shi *et al.*, 1991), including p53 and Ki-67, though the mechanism by which microwaves reveal increased amounts of antigen is not clear (Mckee *et al.*, 1993). Sections were left in buffer for a further 10 min at room temperature prior to washing with distilled water and then tris-buffered saline (TBS, 0.1M Tris [Boerhinger Mannheim], in 0.1M HCl and diluted to a 1:10 dilution with 0.9% normal saline, adjusted to pH 7.4 to 7.6). *p53* oncoprotein was identified with PAb1801, applied as a 1:50 dilution in TBS to the section and incubated overnight at 4° C.

Sections were then washed with TBS and the antibody visualised using an indirect technique. Firstly, biotinylated rabbit anti-mouse IgG was applied as a 1:300 dilution in TBS (Dako A/S, Denmark) and left for one hour at room temperature. Sections were then rinsed twice in TBS and streptavidin applied at a dilution of 1:800 in TBS, again for one hour at room temperature. Having washed the sections twice with TBS and tris buffer alone (0.1M Tris [Boerhinger Mannheim] in 0.1M HCl, pH adjusted to 7.4 - 7.6), diaminobenzidine (7.5mg/10ml plus 100 μ l of 1% H₂O₂) was placed over sections for ten minutes. Slides were then washed in tris buffer, followed by distilled water, prior to staining with Mayer's haematoxylin for one minute. Slides were finally washed under running water for five minutes, dehydrated through alcohols, cleared and mounted.

Staining for c-myc:

The procedure was identical to that for p53 except that 6E10, a *c-myc* monoclonal antibody was employed (Cambridge Biochemicals Ltd.).

Immunohistochemical assessment

All sections were reviewed by a single consultant histopathologist (PR) and graded for the degree of staining into one of four categories:

- No visible staining The specimen was negative for oncoprotein expression
- Focal staining Less than 10% of cells were immunopositive.
- Intermediate staining
 Positive nuclei were seen in between 10% and 50% of the specimen.
- Strong staining
 50% or more of the specimen was considered positively stained for oncoprotein.

2.8 Statistical Methods

All data was stored and manipulated on a Vax mainframe, using RS1 (BBN Software Products Corporation, Cambridge, MA. USA). All statistical analysis was also performed using this programme unless indicated. Data sets showing a normal distribution were compared using an unpaired Student's 't' test. The paired 't' test was used to compare oncoprotein expression within a given specimen (Chapter 3). Non-parametric data was evaluated using the Wilcoxon rank sum test (Mould, 1989).

In the validation experiments, described in Chapter 3, methods of preservation and different types of antibodies were compared according to the method proposed by Bland and Altman (1986) which allows assessment of the 'limits of agreement' between values as opposed to correlation. The correlation coefficient may be inappropriate in comparisons of methods as it gives no quantifiable estimate of agreement between measurements obtained using different methods, though correlation may be observed as they are assaying the same parameter. The 'limit of agreement' is derived from the mean difference between measurements obtained using two tests, followed by calculation of the standard deviation of the difference. The 95% normal range represents the mean +/- 2 x standard deviation.

Survival curves were produced using the method of Kaplan and Meier with significance being determined using the log-rank test (Kaplan & Meier, 1958; Peto, 1977). Survival data was analysed using the SUREAL package. Multivariate analysis was performed using Cox's proportional hazards model (Cox, 1972).

Chapter 3

Validation of Oncoprotein Detection Using Different Methods of Preservation and Antibodies

3.1 Introduction

The majority of clinical material in this study was obtained from ethanol-fixed and formalin-fixed paraffin-embedded material. Consequently it was important to perform validatory experiments to assess the effect of the method of preservation on oncoprotein expression. In addition it was essential to investigate the action of pepsin on both the epitopes and antibodies used in this study. Thus the aims of this first set of experiments were to:

- 1) Investigate what effect the method of preservation and preparation may have on detected oncoprotein levels.
- 2) Compare oncoprotein levels within a tumour using different antibodies.
- 3) Assess variation of oncoprotein levels within different regions of a tumour.
- 3.2 Investigation of the Effects of Method of Preservation on Oncoprotein Levels as Detected by Flow Cytometric Analysis

3.2.1. Introduction

Melanomas investigated in this study were mainly derived from ethanol-fixed and paraffin-embedded samples. Enucleation of cells from these samples requires the use of a proteolytic enzyme, pepsin. Therefore it was necessary to exclude the effects of this enzyme which may reveal or destroy epitopes on proteins and result in artefact.

One primary melanoma and ten metastatic melanomas were each divided into three samples and preserved by one of three different methods:- (1) ethanol-fixation, (2) formalin-fixation and paraffin-embedding and (3), freezing of fresh tumour at -70°C.

Ethanol-fixed and paraffin-embedded melanomas were prepared and stained for p53 and *c-myc* as described in Sections 2.3.1. and 2.3.2. p53 oncoprotein was labelled using PAb1801, whilst *c-myc* was detected using the pan-*myc* antibody. Fresh-frozen material was disaggregated as described in Section 2.3.3. and oncoprotein levels assayed in whole cells. The enzymes used in this process are free of proteolytic effects and analysis of these cells would be most likely to accurately reflect total intracellular protein levels. Data was collected and analysed using flow cytometry, as outlined in Section 2.5.

Assays of both p53 and *c-myc* oncoproteins were compared between the three methods of preservation in each tumour using a number of statistical analyses. Levels of expression were compared using the paired students 't' test, accompanied by the correlation coefficient (\mathbb{R}^2) and significance level (SL). In addition the effects of preservation technique were compared using the method proposed by Bland and Altman (1986), which measures the level of agreement between observations of a given parameter obtained using different methods (see Section 2.8.).

3.2.2. The effect of preservation method on p53 oncoprotein expression

Results

Table 3.1 shows p53 oncoprotein expression in one primary tumour and ten metastases preserved by different methods.

Oncoprotein levels were compared between all three methods of preservation using tests of similarity and correlation as discussed above. In comparing measurement of p53 levels between fresh-frozen and ethanol-fixed samples, shown in Tables 3.1 and 3.2, the null hypothesis examined in the 't' test proposed that sample measurements were different. The 'p' value of 0.24 clearly disputes this and indicates considerable similarity. The significance level (SL) and correlation coefficient (R²) show a highly significant correlation between these two methods of preservation. Similarly, p53 oncoprotein levels compared between ethanol-fixed and paraffin-embedded tumours were also found to be highly significantly correlated.

Table 3.1. p53 oncoprotein expression measured in melanomas preserved by different methods. Data shows the percentage of p53-positive cells/nuclei in each specimen, calculated as described in Section 2.5.3.

Primary/	Fresh-frozen	Ethanol-fixed	Paraffin-emb
Metastases	(% +ve cells)	(% +ve nuclei)	(% +ve nuclei)
Primary	37.3	80.1	81.2
Met	74.5	87.1	75.2
Met	8.7	0.0	14.7
Met	16.8	18.7	20.7
Met	15.3	22.4	27.3
Met	63.3	96.3	71.7
Met	42.2	46.2	55.0
Met	44.5	51.0	70.6
Met	81.3	87.9	94.1
Met	64.7	72.6	85.3
Met	83.3	50.9	62.5

In contrast, a statistical difference was found when fresh-frozen and paraffinembedded melanomas were compared. p53 oncoprotein levels were higher in paraffin-embedded tumours than in fresh-frozen tumours (p = 0.04). However there was still a highly significant correlation between the two methods (SL = 0.0019, R² = 0.68). The highest degree of similarity and correlation was found between p53 levels derived from ethanol-fixed and paraffin-embedded tumours.

Examination of the 'agreement' of p53 protein levels, as opposed to the correlation, examined by the Bland-Altman method are shown in Figure 3.1. In all but one tumour the mean difference in p53 oncoprotein levels compared between the three methods of preservation were found to lie within boundaries of the 95% confidence limits and this suggests they provide results which do not statistically differ.





- a) Fresh vs ethanol-fixed melanomas
- b) Fresh vs paraffin-embedded melanomas
- c) Ethanol-fixed vs paraffin-embedded melanomas

Furthermore these graphs also show the mean difference between different methods does not exceed 10% in any of the three comparisons.

Statistical Test	Fresh vs Ethanol	Fresh vs Paraffin	Ethanol vs Paraffin
<i>'p</i> ' value paired <i>'t</i> ' test	0.24	0.04	0.31
Correlation Coefficient (R ²)	0.63	0.68	0.85
<i>'p'</i> value of correlation (SL)	0.0035	0.0019	0.00006

Table 3.2. Statistical comparison of p53 protein expression in melanomas preserved by different methods.

3.2.3. The effect of preservation method on c-myc expression

Results

c-myc oncoprotein levels were assayed in the same eleven tumours examined for p53, and results are presented in Tables 3.3 and 3.4. *c-myc* oncoprotein levels obtained from these three methods of preservation were statistically compared using the same tests as in comparison of methods of staining on p53 expression. Fresh and ethanol-fixed tumours were found to yield both statistically similar and correlated *c-myc* oncoprotein levels. As for measurements of p53, comparison of *c-myc* levels in both fresh and paraffin-embedded tumours showed the least similarity, but a high degree of correlation. Again, the greatest similarity and correlation in protein measurements were found between ethanol-fixed and paraffin-embedded tumours. Comparison of these methods on *c-myc* expression using the Bland-Altman method is shown in Figure 3.2. These graphs show a considerable degree of agreement in *c-myc* oncoprotein levels measured by all three methods of preservation, again with a low mean difference of under 10% in all three comparisons.



Figure 3.2. Comparison of *c-myc* protein positivity in melanomas preserved by different methods:

- a) Fresh vs ethanol-fixed melanomas
- b) Fresh vs paraffin-embedded melanomas
- c) Ethanol-fixed vs paraffin-embedded melanomas

Primary/ Metastasis	Fresh-frozen (%+ve cells)	Ethanol-fixed (%+ve nuclei)	Paraffin-emb (%+ve nuclei)
Primary	82.9	96.2	90.0
Met	54.4	96.2	92.4
Met	50.1	44.8	70.4
Met	22.1	16.1	29.4
Met	94.9	70.8	71.0
Met	45.6	91.7	54.7
Met	79.8	96.9	91.1
Met	90.1	83.0	91.2
Met	69.8	74.9	89.1
Met	57.6	63.4	80.2
Met	83.5	82.5	82.5

Table 3.3. *c-myc* oncoprotein positivity in tumours preserved by different methods

Table 3.4. Statistical analysis to compare effects of different methods of preservation on *c-myc* oncoprotein expression.

Statistical Test	Fresh vs Ethanol	Fresh vs Paraffin	Ethanol vs Paraffin
<i>'p</i> ' value paired 't' test	0.25	0.06	0.65
Correlation Coefficient (R ²)	0.38	0.52	0.56
<i>'p'</i> value of correlation (SL)	0.045	0.012	0.008

3.2.4 Discussion

Despite obvious differences between whole cells and nuclei, p53 and c-myc oncoprotein expression in fresh-frozen melanomas were similar to, and correlated with, protein levels in ethanol-fixed and paraffin-embedded melanomas. Furthermore there was satisfactory agreement between the methods of preservation according to the test proposed by Bland and Altman (1986). Lassam *et al.* (1993) compared the

intensity and distribution of immunostaining using fresh-frozen melanomas and paraffin-embedded material. They found no difference in either the frequency of staining or distribution of p53 within melanomas preserved by each method. The relatively high expression of both p53 and *c-myc* oncoproteins found in paraffin-embedded samples compared with fresh material has also been observed in bladder and bronchial carcinomas (Mørkve & Hostmark, 1991). Formalin-fixation followed by paraffin-embedding may either enhance antibody access to the epitope and/or result in further protein stabilisation. The authors of this paper also propose that lower oncoprotein levels may be obtained from fresh tumours due to a longer delay in freezing the sample, allowing degradation of the oncoprotein.

The concordance between ethanol-fixed and paraffin-embedded tumours may be due to the fact that oncoprotein analysis was performed on nuclei as opposed to whole cells. As both p53 and *c-myc* are intra-nuclear proteins, access of the antibody to epitopes may be improved in nuclear specimens. Access is potentially further enhanced in paraffin-embedded melanomas by the higher pepsin concentration used to enucleate cells as this may reveal more of the epitope. This study would suggest that pepsin does not affect detection of the p53 epitope recognised by PAb1801 and similar work on the effect of pepsin on p53 expression in the HSF4-T12 cell line has confirmed this (Mørkve & Hostmark, 1991).

As discussed in Section 1.12.3., one of the routes by which the p53 protein is inactivated involves retention and relocation within the cytoplasm. The findings of the present study suggest that cytoplasmic sequestration of p53 is not of such consequence as to cause a significant difference in oncoprotein levels assayed between whole cells and nuclei. A number of studies have used immunohistochemistry to examine p53 oncoprotein in melanoma and commented on its intra-cellular distribution. Stretch *et al.* (1991) found 82% of immunopositive melanomas to show combined nuclear and cytoplasmic staining and 17% to show intense nuclear staining alone. In the larger study of Lassam *et al.* (1993), referred to above, staining was observed to be exclusive to the nucleus. Despite these differences in qualitative assessment of distribution, quantitative measurements using flow cytometry would suggest that intra-cytoplasmic stores of p53 are not significant in malignant melanoma.

3.3. Comparison of *p53* and *c-myc* Oncoprotein Levels Identified Using Different Antibodies

3.3.1. Introduction

Several antibodies are commercially available for the detection of both the p53 and c-myc oncoproteins. The decision of which antibody to choose is dependent on several factors. Firstly, the choice of antibody may be determined by the method of preservation and whether material has been subjected to pepsin degradation. Secondly, antibodies vary with respect to their suitability for flow cytometry. As discussed in Section 2.4., neither PAb1801 nor the pan-myc antibody are entirely protein-specific. PAb1801 identifies both stabilised and wild-type p53 oncoproteins, consequently immunopositivity would not distinguish which type of protein was present in tumour specimens. Similarly the pan-myc antibody would also identify N-myc and L-myc oncoproteins if present. In order to investigate the effects of using different antibodies, samples from eleven ethanol-fixed melanomas (nuclei) and twelve fresh frozen tumours (whole cells) were analysed. Each tumour was divided into six aliquots and prepared and stained using the following panel of antibodies. The method is described in Sections 2.3. and 2.5.

p53

1)	PAb1801	- labels both wild-type and stabilised <i>p53</i> proteins
2)	PAb240	- identifies stabilised form of $p53$ ('mutant-specific') protein
3)	PAb246	- labels only wild-type p53 oncoprotein

c-myc 4) Pan-*myc* p62/64 5) 6E10

control

6) (IgG)

Antibodies were compared using the same statistical tests employed in comparing methods of preservation.

3.3.2. p53 oncoprotein expression compared in ethanol-fixed and fresh-frozen melanomas using different antibodies

Results

The results of p53 oncoprotein expression measured in eleven ethanol-fixed primary and metastatic melanomas using the panel of p53 antibodies noted above are shown in Tables 3.5 and 3.6. The same panel of antibodies were also used to assay p53 in twelve fresh-frozen melanomas and the findings from these studies are presented in Tables 3.7 and 3.8. The agreement between antibodies in these two groups of specimens, as measured by Bland-Altman analysis, is presented in Figures 3.3 and 3.4.

Table 3.5.	p53 positivity in ethanol-fixed melanomas stained using different
antibodies.	Figures represent the percentage of nuclei staining immunopositively for
p53 in each	tumour

Primary/	PAb1801	PAb240	PAb246
Metastasis	(% +ve)	(% +ve)	(% +ve)
pri	96.3	93.6	18.2
pri	14.3	12.9	0.2
met	96.9	93.4	9.4
met	62.6	76.1	2.5
met	50.9	24.1	1.6
met	91.9	93.3	29.7
met	42.9	41.5	8.1
met	94.1	90.1	7.3
met	22.4	15.5	2.7
met	80.9	54.2	5.2
met	96.3	95.0	5.1
Mean	68.3	62.7	8.2
SD	31.3	34.0	8.7

Statistical Tests	1801 vs 240	1801 vs 246	240 vs 246
<i>'p</i> ' value paired <i>'t</i> ' test	0.156	0.0001	0.00011
Correlation Coefficient (R ²)	0.88	0.31	0.35
<i>'p'</i> value of correlation (SL)	0.00002	0.08	0.55

Table 3.6. Statistical analysis to compare p53 oncoprotein levels measured by different p53 antibodies in ethanol-fixed tumours.

Table 3.7. Comparison of p53 oncoprotein levels in whole cells derived from twelve fresh-frozen melanomas. Figures represent percentage of p53-immunopositive cells.

Primary/	PAb1801	PAb240	PAb246
Metastasis	(% +ve)	(% +ve)	(% +ve)
pri	8.7	4.6	1.2
met	70.0	39.2	1.8
met	37.7	39.9	3.2
met	72.9	74.5	9.2
met	18.4	26.1	3.9
met	96.0	95.2	26.0
met	63.6	76.5	5.2
met	44.5	44.2	12.0
met	81.3	74.4	8.6
met	44.7	55.0	4.9
met	64.7	63.4	3.7
met	83.3	79.8	1.7
Mean	57.2	56.1	6.8
SD	26.7	26.1	6.9







- a) PAb1801 vs PAb240
- b) PAb1801 vs PAb246
- c) PAb240 vs PAb246

Statistical Test	1801 vs 240	1801 vs 246	240 vs 246
<i>'p</i> ' value paired <i>'t</i> -' test	0.74	0.0001	0.0001
Correlation Coefficient (R ²)	0.83	0.24	0.31
<i>'p'</i> value of correlation (SL)	0.00004	0.105	0.62

Table 3.8. Statistical analysis comparing p53 oncoprotein expression in fresh tumours labelled by various antibodies.

3.3.3. Discussion

Assays of p53 oncoprotein using PAb1801 and PAb240 in ethanol-fixed tumours showed considerable agreement. Column 1 of Table 3.6. confirms this, with no statistical evidence of a difference on the basis of the paired 't' test and an even more noteworthy level of correlation as illustrated by the high significance level and coefficient of correlation. These findings were confirmed in the comparison of antibodies in fresh melanoma, where the 't' test and estimates of correlation showed even greater concordance. Bland-Altman analysis, to compare p53 oncoprotein expression between PAb1801 and PAb240 in both ethanol-fixed and fresh-frozen tissues, revealed good agreement with values distributed equally around the mean difference. This similarity in p53 oncoprotein levels might be expected as both PAb1801 and PAb240 identify epitopes on the stabilised p53 protein conformation (see Section 2.4.1.) Other studies have also shown PAb1801 to reliably identify stabilised p53 protein when compared with PAb240 and other 'mutant'-specific p53antibodies in melanoma (Lassam *et al.*, 1993), non-small cell lung carcinoma (Mørkve *et al.*, 1992) and breast cancer (Walker *et al.*, 1991).

Although statistically insignificant, the slightly higher p53 oncoprotein levels observed using PAb1801 in ethanol-fixed tumours might be due to detection of wild-type p53oncoprotein present in the same tumour cells. Cells which possess both a normal p53allele, along with mutation of the other p53 gene, might be expected to produce small amounts of the wild-type protein. This would be labelled by PAb1801, but not PAb240, to produce a slightly higher overall p53 level. Oncoprotein levels detected by the p53 wild-type specific antibody, PAb246, were consistently lower than those measured by PAb1801 or PAb240 in both ethanol-fixed and fresh-frozen melanomas. The significant 'p' values confirmed the null hypothesis and the lack of correlation, as indicated by the SL and R². Bland-Altman analysis of these comparisons confirm the lack of agreement in measurements and show the mean difference between antibodies to increase with a rise in p53 positivity, as illustrated in Figures 3.3b & c and 3.4b & c. This evidence would suggest that PAb246 is identifying and labelling a different protein, and one in lesser amounts in melanomas, than that identified by either PAb1801 or PAb240.

3.3.4. Comparison of c-myc expression in ethanolfixed and fresh-frozen melanomas using two different antibodies

Using the methods described in Sections 2.3.1. and 2.3.3, *c-myc* immunopositivity was compared between the rabbit polyclonal pan-*myc* antibody and the mouse monoclonal 6E10 (antibodies are described in Section 2.4.2.). Assays were performed on the same eleven ethanol-fixed melanomas and twelve fresh-frozen melanomas used to investigate p53 expression. Results are shown in Tables 3.9. and 3.10. and Bland-Altman analysis in Figure 3.5.

Primary/ Metastasis	Pan- <i>myc</i> (%+ve)	6E10 (%+ve)	
pri	96.3	80.3	
pri	10.9	7.6	
met	97.3	61.2	
met	59.1	38.4	
met	82.5	83.7	
met	83.0	50.0	
met	31.1	30.3	
met	90.6	80.4	
met	70.8	61.1	
met	55.8	51.5	
met	91.7	89.1	

Table 3.9. *c-myc* positivity in ethanol-fixed tumours compared using the pan-*myc* antibody and a human *c-myc*-specific monoclonal, 6E10.





- a) Ethanol-fixed melanomas
- b) Fresh-frozen melanomas

Primary/ Motostosis	Pan-myc	6E10
wietastasis	(%+ve)	(%+ve)
pri	50.1	43.7
met	75.3	58.2
met	82.9	76.3
met	54.4	33.0
met	22.1	15.3
met	94.9	16.9
met	43.0	45.6
met	90.1	83.2
met	69.3	63.2
met	79.8	72.2
met	57.6	46.0
met	83.5	40.1

Table 3.10. *c-myc* oncoprotein expression in fresh-frozen melanomas. Whole cells were analysed and results expressed as the percentage of immunopositive cells.

3.3.5. Discussion

Comparison between the pan-*myc* antibody and 6E10 monoclonal antibody for detection of *c*-*myc* oncoprotein showed a consistent difference in that levels produced by the monoclonal antibody were generally lower. This was particularly true of fresh-frozen melanomas. Statistically this difference was confirmed, with 'p' values of 0.01 and 0.02 for ethanol-fixed and fresh-frozen specimens respectively. However ethanol-fixed tumours displayed a high degree of correlation, with a highly significant SL of 0.0002 and a R² of 0.79. In the comparative studies of *c*-*myc* staining performed on fresh tumours, correlation failed to reach statistical significance (SL = 0.12, R² = 0.23). However there was general agreement in values on the basis of the horizontal distribution of points using Bland-Altman analysis (Fig 3.5.).

The lower *c-myc* levels obtained by staining with 6E10, particularly in whole cells, may reflect the fact that this antibody is only identifying some of the available *c-myc* oncoprotein. *c-myc* oncoprotein is predominantly intra-nuclear and accordingly the 6E10 epitope may be more accessible in nuclei as opposed to whole cells. This may partially explain the greater agreement and correlation of detected values between the two antibodies in ethanol-fixed melanomas. As discussed in Section 1.10.2., the *c*-

myc gene encodes at least two proteins, of between 62kD and 64kD (Littlewood & Evan 1990). It is possible, therefore, that only part of the *c-myc* gene product is being identified by the monoclonal antibody.

An additional explanation for the lower level of expression seen with 6E10 may be due to the effects of tumour preparation for flow cytometry (Lincoln & Bauer 1989). Processing of tumours may result in loss of up to 50% of the available *c-myc* oncoprotein as detected by anti *c-myc* monoclonals, lowering observed protein levels. This may be an effect specific to the 6E10 epitope for in the same study detection of a second antigen, the proliferation-associated protein p105, was unaffected by pepsin enucleation.

It is highly unlikely that the higher oncoprotein levels produced by the pan-myc antibody are due to measurements of other myc proteins, such as N-myc and L-myc. Both these proteins are very tightly regulated in their embryological timing and pathological site of expression and have not been described in melanoma before. In an investigation of myc mRNA expression in 18 melanoma cell lines, N-myc and L-myc mRNA were not detected in any of the samples, whilst *c*-myc mRNA was present in 16 lines (Chevenix-Trench *et al.*, 1990). In a further study, amplification of the N-myc gene was reported in two surgically excised melanomas, but this was unaccompanied by elevation of the protein product (Bauer *et al.*, 1990). Multiple copies of genes would not be unusual in aneuploid tumours and thus this observation may merely represent an epiphenomenon.

3.4. Intra-tumour Variation of Oncoprotein Levels

3.4.1. Introduction

It was important to assess how representative measurements of oncoprotein levels derived from a single random biopsy were to a tumour specimen as a whole. In order to do this, oncoprotein levels were measured in several samples from the same tumour. Four biopsies were taken from the middle and periphery of seven surgically excised primary and metastatic melanomas and stained for p53 and c-myc as described in Section 2.3. Tumours assayed consisted of two primary melanomas and five metastases and were chosen as they had produced a range of oncoprotein levels on first analysis. Metastases comprised two skin secondaries and three nodal metastases. Data was collected and analysed as described in Section 2.5.

3.4.2. *Results*

Table 3.11. shows p53 oncoprotein levels measured in four biopsies, taken from seven primary and metastatic melanomas. Excluding one specimen in which p53 expression was essentially negative, the mean coefficient of variation in this group of melanomas was found to be 11.8%.

Table 3.11. p53 expression in multiple biopsies from seven primary and metastatic melanomas. Columns show the percentage of immunopositive cells observed in each sample.

Sample	1	2	3	4	Mean	CV
	(%+ve)	(%+ve)	(%+ve)	(%+ve)	(%)	(%)
Primary	0.0	2.2	1.7	0.8	1.2	83.3*
Primary	38.2	27.6	31.3	34.1	32.8	13.7
Skin	51.0	57.9	59.7	51.0	54.9	8.4
Skin	83.5	83.5	79.4	74.6	80.3	5.2
Node	81.0	73.3	86.2	83.7	81.1	6.9
Node	95.0	88.2	90.0	82.9	89.0	5.6
Node	14.7	17.8	25.7	13.3	17.9	30.7

Multiple biopsies were also analysed for c-myc oncoprotein levels in the same seven melanomas and the results are shown in Table 3.12. Calculation of the mean coefficient of variation in this group of melanomas, omitting the results of the negative tumour, yielded a value of 10%.

Table 3.12. *c-myc* oncoprotein expression from multiple biopsies of the same seven melanomas.

Sample	1	2	3	4	Mean	CV
	(%+ve)	(%+ve)	(%+ve)	(%+ve)	(%)	(%)
Primary	92.5	85.7	91.6	83.4	88.3	5.0
Primary	49.5	40.7	53.1	50.8	48.5	11.1
Skin	84.0	72.8	69.6	78.7	76.3	8.4
Skin	0.5	0.0	0.0	0.0	0.1	300*
Node	82.8	82.4	80.2	76.6	80.5	3.5
Node	75.2	67.0	47.1	61.1	62.6	18.8
Node	44.8	36.8	47.9	37.3	41.7	13.2

3.4.3. Discussion

Analysis of oncoprotein levels within different regions of the same tumour revealed considerable consistency between readings. Measurements of p53 oncoprotein levels showed similar intra-tumour values in all samples; the standard deviation was no more than +/-5.6% in all specimens studied, producing a mean coefficient of variation (CV) of 11.8%. Measurements of *c-myc* oncoprotein levels were equally consistent, with all but one of the specimens producing a maximum standard deviation of +/-6.4% and a mean CV of 10.0%. In the two samples where p53 and *c-myc* expression were almost zero or zero itself, (indicated by the * in Tables 3.11. and 3.12.) calculation of the CV was spurious. Any recorded expression above zero has the effect of raising the mean value, despite the fact that oncoprotein levels were effectively undetectable and classified as negative. This produces a misleading estimate of CV and, as a result, these two samples were not taken into account when calculating the mean CV.

Due to limitations in the number of primary tumours and their size, only two primary lesions were studied in this group. However results from primary lesions would be expected to be representative of the most active portion of the tumour as samples were obtained as close as possible to the thickest part taken for histological examination. None of the metastases studied were macroscopically necrotic. Analysis of such regions would be unlikely to yield any useful data with respect to protein levels across disparate, viable regions of a tumour.

Biological parameters such as proliferation kinetics show considerable heterogeneity within a specimen (Rew *et al.*, 1991). However the above data suggests that oncoprotein overexpression is more uniform throughout primary and metastatic melanomas. In part this may be due to the fact that primary and secondary melanomas are not usually as large, infected or as necrotic as larger tumours (i.e. colo-rectal malignancies) at the time of presentation. It is more likely, however, that oncoprotein levels are similar due to the monoclonality of tumours: every cell is affected due to the intrinsic genetic nature of the abnormality and its uniformity within a tumour. Biological parameters are affected by metabolic and physiological factors as well as genetically determined factors.

3.5. Summary to Chapter 3

These findings suggest the method of preservation does not adversely affect measurement of either p53 or c-myc oncoprotein expression in melanoma. The general agreement of results from each method implies that pepsin enucleation does not result in artefactual damage to either the target epitope or labelling antibodies.

PAb 1801 has been demonstrated to identify and label stabilised p53 oncoprotein with at least the efficacy of PAb240. The pan-myc antibody has been shown to produce greater immunopositivity than the *c*-myc monoclonal 6E10, but to be unaffected by the method of preservation. Consideration of other studies and the developmental and tissue specificity of N-myc and L-myc would suggest that the polyclonal antibody labels a greater proportion of *c*-myc protein than 6E10 when melanomas are preserved and prepared by the methods employed in this study.

Biopsies from disparate regions of non-necrotic primary and metastatic melanomas appear to yield oncoprotein levels which are representative of the whole specimen.

The following data presented in this thesis was acquired from a single specimen of ethanol or formalin-fixed material using pepsin digestion. Proteins were labelled using either PAb1801 or the pan-*myc* antibody.

Chapter 4

Investigation of *p53* and *c-myc* Oncoprotein Expression in Ethanol-Fixed Melanomas

4.1 Introduction

The validatory experiments discussed in Chapter 3 established that ethanol fixation did not adversely affect preservation or flow cytometric detection of p53 and c-myc oncoproteins in primary and metastatic melanomas. To date only one study has been published on the use of flow cytometry to investigate p53 oncoprotein expression in melanoma, and this was confined to paraffin-embedded archival material (Akslen & Mørkve, 1992). There have been no publications on the investigation of the c-myc oncoprotein in melanoma. To address this deficit, studies were performed on ethanolfixed melanomas, the aims of which were to:

- 1) Investigate *p53* and *c-myc* oncoprotein expression in cutaneous melanoma and establish the range of expression using flow cytometry.
- 2) Examine the relationship between oncoprotein expression of both oncogenes and clinico-pathological parameters.

The following chapter is divided into two main results sections. The first, Section 4.4, describes the findings of investigations into p53 oncoprotein expression in 50 ethanol-fixed primary and metastatic melanomas obtained from consecutive patients treated at the regional Plastic Surgery Unit at Mount Vernon Hospital. The second, Section 4.7, describes *c-myc* expression within the same tumours. Clinical and pathological details of the patient group studied in this series are reviewed in Section 4.3. All values are expressed as the median unless stated otherwise.

4.2 Materials and Methods

p53 and *c-myc* oncoprotein expression were studied in 50 primary and metastatic melanomas. Within 30 minutes of excision, a portion of the fresh, dry tumour was assigned to histological analysis and the remainder placed in 70% ethanol and stored at

4°C until processed. Tumours were processed and stained as described in Section 2.3.1.

Data was analysed on 10,000 events (nuclei) for each sample in two ways. Firstly, staining was assessed by measuring the number of labelled cells compared to the control, to provide the percentage p53 or *c-myc* positivity. DNA staining with propidium iodide also allows expression through the cell cycle to be measured. By plotting computer generated windows around and between cell cycle phases (described in Section 2.5.3.) total and phase-specific oncoprotein levels can be measured and compared to the control. DNA staining also displays tumour ploidy. Examination of these cell cycle associated changes allow two further calculations to be made. Firstly, the fluorescence level in each phase was estimated by taking the ratio of positive to negative fluorescence to obtain the expression ratio. Secondly, the p53 or *c-myc* positivity ratio was calculated by dividing the number of positive events by the total number of events in that phase. This was performed for the total population and for each cell cycle phase.

Information regarding the patient's history and clinical data were available for all the primary melanomas studied. Metastases made up the majority of melanomas investigated in this study and complete clinical and histological data of the primary tumour were available in all cases except for three patients. Two patients presented with secondary disease from an unknown primary and the third presented with her primary melanoma in 1953; no details other than the original diagnosis were available for review.

4.3 Results

4.3.1 Clinical and histopathological data

Tumour samples were obtained from 30 female and 20 male patients, with a median age of 54.4 years (range 20-93, mean 54.0 years). Tissue diagnosis was confirmed on formalin-fixed, paraffin-embedded sections stained with haematoxylin and eosin. Fourteen primary melanomas were investigated in this study, consisting of nine superficial spreading malignant melanomas and five nodular melanomas, with a median thickness of 3.8mm (range 1 to 10mm). Thirty six metastatic melanomas were analysed, comprising ten skin metastases and 26 nodal secondaries. The median follow-up was 39 months (range 12-120 months).

4.3.2 The relationship between oncoprotein immunopositivity and observed fluorescence

Flow cytometry not only displays the number of positive or negative events within a sample, but also expresses the overall amount of oncoprotein present as a function of fluorescence. In order to select the most appropriate parameter as a marker of tumour oncoprotein content, it was necessary to explore the relationship between the number of immunopositive nuclei within a sample and the resultant fluorescence.

In Figure 4.1a the percentage p53-positivity has been plotted against recorded fluorescence for nuclei in the G1/G0 phase of the cell cycle. This was an appropriate phase to select as the great majority of cells are usually resident within it at any one time. It is apparent from Figure 4.1a that as the number of p53-immunopositive nuclei increased, resultant fluorescence showed little change and the overall association is essentially flat. Whilst p53 positivity varied within this group from 0 to 95.3%, the p53 fluorescence (expression) ratio only ranges from 0 to 11, with a median value of 3.4 (mean 4.9 +/- 5.0). The upper range fluorescence value may be misleadingly high as it was only recorded in two samples (3.9%) and may not be indicative of a trend.

A similar relationship was observed between c-myc positivity and fluorescence ratio, as shown in Figure 4.1b. The median fluorescence ratio was 3.2, with a range of 0.0-44.8. Over this fluorescence range, c-myc oncoprotein expression ranged between 0.0% and 96.3%. As a result of the limited variation of the fluorescence ratio, percentage positivity has been used throughout as the index of tumour oncoprotein content.

4.4 *p53* Oncoprotein Expression in Ethanol-fixed Melanomas

4.4.1 p53 oncoprotein expression throughout the cell cycle.

p53 oncoprotein levels were measured with progression through phases of the cell cycle. As described in Section 4.2.1, p53 expression was represented by two parameters throughout each phase of the cell cycle, shown in Table 4.1 and Figures 4.2a and 4.2b.

Cell cycle phase	G1/G0	S	G2+M
<i>p53</i> positivity ratio	19.2	36.2	42.2
(+/- S.E.M) %	(4.9)	(5.1)	(5.0)
<i>p53</i> expression ratio	3.4	3.2	2.6
(+/- S.E.M) %	(0.7)	(0.7)	(0.8)

Table 4.1. Variation in p53 positivity ratio and expression ratio through phases of the cell cycle.

Figure 4.2a shows that as nuclei pass through the cell cycle the proportion of p53-positive nuclei in each phase increased as nuclei entered S phase before progressing onto G2+M. The relatively low positivity ratio seen for cells in G1/G0 reflects the fact that overall positivity is diluted by non-cycling cells also present within this phase, and that in diploid tumours normal cells will be included in this analysis window.

The variation in expression ratio, shown in Figure 4.2b, is effectively horizontal, suggesting that actual protein content per phase of the cycle remained uniform. This may be expected as intra-nuclear stabilised p53 protein content would be unlikely to increase as nuclei progress through the cell cycle.

4.4.2 p53 oncoprotein expression in primary and metastatic melanomas

p53 oncoprotein positivity was detected in 11 of the 14 primary melanomas studied (78.6%) and 29 out of the 36 metastases (80.5%), overall representing 40 out of 50 (80%) of all tumour specimens assayed. The proportion of p53-positive nuclei displayed considerable variation between melanomas (Fig 4.3), with the number of p53-immunopositive nuclei being distributed over a wide range from 0.1% to 95.3%. However, as the median percentage positivity was only 19.1%, it is clear that the majority of stained tumours contained relatively low numbers of immunopositive nuclei.



Figure 4.1a & b. p53 positivity and fluorescence in G0/G1. 4.1a - p53 expression, represented in relative fluorescence units, showed little change as the number of labelled nuclei, (p53 positivity) increased. A similar relationship was observed between *c-myc* positivity and fluorescence (4.1b).



Figure 4.2a. Variation in *p53* positivity ratio through the cell cycle.

Figure 4.2b. Variation in p53 expression ratio through the cell cycle.



Figure 4.3. Distribution of *p53* positivity in ethanol-fixed primary melanomas (filled symbols) and metastases (open symbols).

Dashed line - median Dotted line - mean Of the primary melanomas studied, 12.8% of nuclei (0 - 83.6) were p53 positive. In Table 4.2 primary melanomas have been divided on the basis of their thickness into two groups; those below 3mm in thickness and those greater than 3mm thick. Only five melanomas fell into the first group, of which four were p53-positive (80%). In this small sub-group, 6.3% (0.4 - 83.6) of nuclei stained positively for p53. Of the thicker primaries, eight out of nine tumours (88.8%) stained positively for p53, with 14.7%(5.7 - 86.0) of nuclei proving immunopositive. No statistical difference was found in the level of p53 staining between these two groups of primary melanomas (p = 0.68).

Following analysis of the metastatic melanomas, 22.5% of nuclei (range 0.1 - 95.3) were observed to be immunopositive. Metastases were sub-divided into nodal (n = 26) and skin (n = 10) secondaries. *p53* positivity was almost identical for each type of metastasis. Approximately 80% of each type proved *p53*-positive, with a median of 18.7% and 25.3% of nuclei staining positively in skin and nodal metastases respectively (p = 0.70). The increase in *p53* oncoprotein content observed in the progression from primary melanoma to metastases failed to reach statistical significance (p = 0.41).

Consideration of metachronous metastases in six patients revealed numbers of positive nuclei to increase in five out of six specimens from a median of 4.2% (range 0.0 - 80.5) in the first metastasis to 5.2% (0.7 - 93.2) in further metastases, but this was not statistically significant (p = 0.89). In one case both the primary tumour and metastasis was available; p53 positivity increased from 10% to 72% of nuclei in the primary and metastatic samples respectively.

4.4.3 DNA indices

Seven of 14 primary tumours (50%) and 17 of the 36 metastases (47.2%) were defined as an euploid on the basis of their DNA profile. p53 oncoprotein expression did not vary with tumour ploidy (p = 0.77); 23.0% (range 0.1 - 94.0) of nuclei were immunopositive in diploid melanomas. A similar level of expression was observed in an euploid tumours with 15.4% (range 0.7 - 95.3) of nuclei staining for p53. Consideration of metastatic site with tumour ploidy revealed a greater incidence of an euploid tumours in nodal metastases compared to skin metastases (p = 0.016).
4.4.4 Clinico-pathological parameters and prognosis

From the available clinico-pathological data, a number of parameters were evaluated for their role as potential prognostic indicators in melanoma. Univariate analysis (i.e. only taking that one factor into account and ignoring the possible influence of other factors) of the relationship between several of these parameters and survival suggested that primary melanoma thickness (p = 0.008, $\chi^2 = 7.1$), the patient's sex (p = 0.006, $\chi^2 = 7.62$) and the disease-free interval (p = 0.002, $\chi^2 = 9.96$) were of prognostic significance (Figs 4.4 a, b and c). However, consideration of these particular parameters together in a Cox's proportional hazards model found only Breslow thickness and patient sex to remain as independent prognostic indicators.

Table 4.2.	p53 oncoprotein	expression in	primary a	and metastatic	melanomas	measured
by flow cy	tometry using PA	b1801.				

Tumour Type	Number Analysed	<i>p53-</i> ve Tumours n (%)	p53+ve Tumours n (%)	% +ve nuclei (median)
1º Melanoma				
< 3.0mm	5	1 (20)	4 (80)	6.3
1º Melanoma				
> 3.0mm	9	1 (11.1)	8 (88.8)	14.7
2º Melanoma				
Skin Mets.	10	2 (20)	8 (80)	18.7
2º Melanoma				
Nodal Mets.	26	5 (19.2)	21 (80.8)	25.3

4.5 *p53* Oncoprotein Expression, Clinico-Pathological Parameters and Prognosis

The variables discussed in Sections 4.4.2 and 4.4.4 were further examined against p53 expression to see whether any correlation existed between clinical behaviour, histopathological parameters and tumour oncoprotein levels.

4.5.1 p53 expression, disease progression and patient survival

At a median follow-up of 39 months no survival difference was found in this small group of patients between primary melanomas on the basis of their p53 expression ($p = 0.82, X^2 = 0.05$) [Figure 4.5a]. However, in specimens analysed from patients with stage two disease, a trend was evident to suggest that tumours expressing low p53 oncoprotein content showed improved survival compared to tumours with high p53 oncoprotein positivity ($p = 0.08, X^2 = 3.06$) [Fig 4.5b]. Patients with elevated p53 (above median p53 level) expression were observed to have a shorter survival period (median 52 months) compared to those patients with low p53 expression (below median p53 expression, median survival 120 months). When all thick primary tumours (>3.0mm) were taken into account (i.e. including specimens from both primary and metastatic melanomas), a similar trend was observed between p53 expression and probability of survival (Fig 4.5c) but again, this was only of borderline significance ($p = 0.07, X^2 = 3.39$).

p53 expression did not vary significantly with the clinical tumour stage (shown in Figure 4.6). Melanomas from patients with no evidence of further spread (stage 1) had a median expression of 21.8% (0.0 - 93.4), whilst specimens from loco-regional metastases had a median expression of 18.7% (0.1 - 95.3). The median oncoprotein levels in primary and metastatic melanomas from patients who went on to die from their disease (stage four) were of a similar level, 21.0% (0.7 - 92.5). This is to be contrasted against the variation in *c-myc* oncoprotein levels with tumour stage, shown in Figure 4.6 (see Section 4.10.1).

4.5.2 Patient sex and variation in p53 expression

Sex of the patient was found to be of prognostic importance by both univariate and multivariate analysis. Female patients were observed to show prolonged survival when compared to males with melanoma. The median survival period was 115 months (12 - 150) for females and 28 months (13 - 56) for males. The median p53 expression in melanomas from male patients was 38.1% (0.4 - 91.9) compared to that in female patients of 17.4% (0.0 - 95.3). The span of p53-positivity values in both sexes precluded any statistically significant difference (p = 0.84, mean p53 expression in males = 34.5%, mean expression in females = 36.5%).



Figure 4.4a Survival and thickness of the primary melanoma. Upper curve - melanomas <2.5mm thick (n = 9), lower curve - melanomas >2.5mm thick (n = 30) [p = 0.008, $\chi 2 = 7.1$].

Figure 4.4b. Survival and sex of the patient. Upper curve - female (n = 25), lower curve - male (n = 16) [p = 0.006, $\chi 2 = 7.62$].

Figure 4.4c. Survival and disease-free interval. Upper curve - DFI >12 months, lower curve - DFI <12 months [p = 0.0016, $\chi 2 = 9.96$].

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Figure 4.5a. *p53* expression and survival in patients with Stage 1 melanoma. Upper curve - positivity <20% (n = 9), lower curve - positivity >20% (n = 5) [$p = 0.82, \chi 2 = 0.05$].

Figure 4.5b. *p53* expression and survival in patients with Stage 2 melanoma. Upper curve - positivity <20% (n = 13), lower curve - positivity >20% (n = 14) [p = 0.08, $\chi 2 = 3.07$].

Figure 4.5c. *p53* expression and survival in patients with thick primary melanomas. Upper curve - positivity <20% (n = 16), lower curve - positivity >20% (n = 13) [p = 0.07, $\chi 2 = 0.07$].

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4.5.3 Patient age and p53 expression

The bar-chart shown in Figure 4.7 displays incidence of p53-positive tumours against patient age. p53-positivity was uncommon in melanomas from patients under 60 years of age. Within this group of patients (n = 27) the median p53 expression was 6.6% per tumour (range 0.1 - 91.9). However, in melanomas from patients over 60 years of age (n=24) 57% of nuclei were p53-immunopositive (range 0.4 - 95.3). This difference was highly significant (p = 0.0001). A similar relationship was noted between c-myc expression and patient age (see Section 4.10.3) but the increase occurred a decade earlier; in patients over 50 years of age.

4.5.4 p53 expression and site of the primary or metastatic melanoma

In this group of patients a trend was evident suggesting a survival advantage for patients presenting with melanomas on the limbs, compared to the torso or head and neck, though the primary site was not found to be of prognostic significance by univariate analysis (p = 0.38, $\chi^2 = 1.03$). Patients presenting with melanomas on the limbs had a considerably longer median survival (115 months) compared to those presenting with tumours on the torso or head and neck (29 months). The distribution of primary melanoma according to site and sex is shown in Figure 4.8.

p53 expression did not vary significantly with tumour site; comparison of mean p53 expression between sites demonstrated a trend suggesting p53 expression in head and neck melanomas were higher than those arising on the torso (p = 0.08), but not in comparison to those originating on the limb (p = 0.24). Results are summarised in Table 4.3.



Figure 4.6. Variation in *p53* expression with disease stage.



Figure 4.7. *p53* expression and age of the patient at time of excision biopsy.

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Site of primary melanomas in females

Figure 4.8. Distribution of the site of presentation of primary melanomas in males and females.

	Head & Neck	Torso	Limb
Number of melanomas	8	10	28
Thickness (median)	4.6	3.8	3.6
<i>p53</i> positivity median (range %)	61.9 (5.7 - 94.0)	25.3 (0.4 - 66.0)	18.7 (0.1 - 95.3)

Table 4.3. Distribution of primary melanomas, median tumour thickness and p53 oncoprotein expression.

4.5.5 Histopathological parameters and p53 expression

In addition to tumour thickness, discussed in 4.5.1, the correlation between p53 expression and certain histological parameters were also examined. Ulceration was histologically evident in 14 (28%) of the primary melanomas of all 50 tumours investigated in this series. p53 oncoprotein expression was significantly higher in tumours derived from histologically ulcerated melanomas (p = 0.0001), with a median p53 expression of 64.1% (range 4.0 - 91.3), compared with 12.5% (range 0.1 - 95.3) in non-ulcerated melanomas.

Mitotic figures were present in 25 of the melanomas analysed (50%). A trend was observed in p53 oncoprotein levels between melanomas with or without mitotic figures. Melanomas with mitotic figures had a median p53 positivity of 32.9% (0.4 - 94.0). In comparison, those melanomas with no or few mitotic figures had a median expression of 16.1% (0.1 - 95.3), but this difference failed to reach statistical significance (p = 0.33).

Lymphocytic infiltration of the primary or metastatic tumour was seen in 32 tumours (64%). Presence of lymphocytic infiltration was found to correlate with p53 levels (p = 0.017). Melanomas with minimal histological evidence of infiltration or regression had a p53 positivity of 52.5% (0.1 - 95.3), whilst those melanomas without infiltration had a lower level of expression at 12.5% (0.7 - 91.3).

4.5.6 Disease-free interval and p53 expression

The disease-free interval (DFI) represents the period (in months) between diagnosis of the primary tumour and appearance of the first recurrence at any site. The median DFI in this series of patients was 14.3 months (range 0.0 - 150). By univariate analysis the DFI was found to act as a prognostic indicator (p = 0.0016, $\chi^2 = 9.96$) (Fig 4.4c), however this parameter was not significant on multivariate analysis. A statistically significant inverse relationship was found between p53 expression and the DFI (p = 0.04). p53 positivity in aggressive melanomas, with a below median DFI, was higher at 57% 0.1-93.9), compared to that found in melanomas with recurrences appearing after the median DFI; in these melanomas the median p53 positivity was 15.4 % (0.7 - 95.3).

4.6 Discussion

The clinical and pathological features of the small group of patients studied in this series appear to be reasonably representative of the distribution and behaviour of melanoma cases in the UK (Swerdlow, 1979); other than for the disproportionately high number of thick primary melanomas and metastases. As a regional unit, patients from outside the immediate catchment area of the hospital will usually have been referred following excision and diagnosis of the primary lesion, resulting in the relatively high proportion of metastatic melanomas seen in this group. In addition, limited amounts of tumour from thin primary melanomas were available for analysis following histological examination. The age and sex distribution of patients studied in this series are representative of individuals afflicted with melanoma as a whole and the median age at diagnosis of 54.4 years concurs well with the established estimate of 53 years (Koh, 1991). Similarly the sex distribution of cases is typical of that seen in the UK and elsewhere (Swerdlow, 1979; Grin-Jorgensen *et al.*, 1992).

The primary (Breslow) tumour thickness was found to be the major independent prognostic indicator in the series of patients studied in this group. Those of lesser importance, (i.e. patient sex and disease-free interval), were also identified in this group and are of recognised prognostic importance (Balch *et al.*, 1992a), (see Section 1.4). Tumour site was not identified as a prognostic indicator in this group, but a trend in survival advantage for those melanomas presenting on the limbs was clearly evident.

The findings of this study show elevated expression of p53 oncoprotein to be prevalent in both primary and metastatic melanomas, and at a level higher than found in almost any other tumour type (Bartek *et al.*, 1991). Bartek *et al.*, (1991), investigated p53 oncoprotein expression in 38 primary and secondary lesions using CM-1, a rabbit polyclonal antibody raised against human recombinant p53, and found 92% of tumour specimens stained positively for p53. In a subsequent study, Stretch *et al.* (1991) examined p53 oncoprotein expression in a range of melanomas from different stages of progression. They found 85% of all tumour specimens to be p53-positive, 70% of all primaries and 93% of metastases. The level of expression was significantly higher in metastatic tumours (p < 0.05). Over 80% of tumour-positive specimens showed combined nuclear and cytoplasmic staining, but the distribution of staining was not found to be associated with tumour stage. The findings of both these papers suggested two conclusions, firstly that p53 oncoprotein expression is more prevalent in melanoma than in other cancers assessed with similar methods, and secondly that overexpression may have a role in the development of the metastatic phenotype.

McGregor *et al.* (1993) and Lassam *at al.* (1993) both demonstrated a significant correlation between p53 immunoreactivity and poor prognosis in melanoma, using immunohistochemistry. McGregor *et al.* (1993) observed an association between p53 expression and primary tumour thickness; 77% of primary tumours greater than 1.5mm were p53 positive, as opposed to 66% of tumours less than 1.5mm thick. The highest levels were found in cutaneous metastases (6/7 specimens, 86%), but in contrast to all the other immunohistochemical studies, the prevalence of p53-positive nodal metastases were relatively low (40%). This may be explained by the fact that only five nodes were analysed and may be the result of sampling error.

The largest of the studies published to date used two different antibodies, DO-7 and PAb1801 (DO-7 is a mutant-specific antibody) to assess p53 oncoprotein expression in a range of benign and malignant melanocytic lesions (Lassam *et al.*, 1993). The lesions studied included 26 benign melanocytic naevi, 56 dysplastic naevi, 61 primary melanomas and 10 melanoma metastases. Their results confirmed the observation of high oncoprotein levels in secondary lesions and, in particular, nodal metastases made in earlier studies. However, only 5% of primary melanomas were found to stain positively for p53, with all staining noted to be intra-nuclear. This study concluded that p53 overexpression may be a late event in the progression of melanoma and of prognostic relevance, but is unlikely to act as a pre-neoplastic marker. In common with the above studies, staining was uniform and largely confined to the nucleus.

Cristolfolini *et al.* (1993) also observed an increase in the prevalence of staining in the progression from primary to metastatic melanoma. Almost 30% of primary melanomas exhibited sparse p53 staining, whilst six of seven metastases (86%) were found to be

immunopositive. The distribution of p53-positive cells was of interest in that relatively few cells were stained; in 90% of the primary melanomas studied less than 5% of nuclei were p53-positive. As in our study, although the prevalence of staining increased in metastatic melanomas, the overall nuclear positivity did not. This was less than that observed in the other studies noted above and would confirm the observations made in the present investigation to suggest that detection of the p53 protein is unlikely to be the result of a clonal mutation in the p53 gene. In early stages of melanoma development and radial growth-phase primary tumours, p53 mutation may be a focal event, whereas mutation of the gene itself may represent a late event in the development of the phenotype, but the clinical correlates of this change require further study. The diversity of findings (summarised in Table 4.4) between this and other studies reflect the variation in samples, methods of preservation and analysis used in each. These are considered in more detail in the following chapter (Section 5.11).

Ulceration of primary melanomas has been shown to be a dependent and independent prognostic variable in a number of studies (Balch *et al.*, 1992a, 1992b). Ulceration may result from local trauma but is more likely to be the result of dissociation between tumour proliferation and blood supply to the tumour. The significant association between elevated p53 oncoprotein levels and ulceration found in this group of tumours suggests that growth deregulation involving loss of p53 function may allow more rapid melanoma cell proliferation and ulceration as a secondary consequence. It is noteworthy that the groups of melanomas compared showed no statistical difference in thickness. However, this observation requires confirmation in a larger study that should also include more thin melanomas.

The results of this study failed to identify any difference in p53 protein expression between primary and metastatic melanomas. However a trend was observed between p53 expression and survival in those patients presenting with thick tumours(>3.0mm). p53 oncoprotein levels have been demonstrated to be independent prognostic markers in several different tumours including node-negative breast cancer (Thor *et al.*, 1993), lung cancer (Quinlan *et al.*, 1992), prostatic carcinoma (Visakorpi *et al.*, 1992), cervical cancer (Crook & Vousden, 1992) and gastric cancer (Martin *et al.*, 1992), reaffirming the impression that loss of cell-cycle control and tumour suppression by inactivation of p53 is a process central to tumourigenesis and progression of many cancers (Vile, 1993).

Study	Specimen	Number of	Number of
	Excn specimen/	primaries	metastases
	cell line	analysed	analysed
Bartek <i>et al.</i>	ES	8	30
(1991)		(not stated)	(35, 92%)
Volkenandt <i>et al.</i> (1991)	CL	-	9 (1, 11%)
Stretch <i>et al.</i>	ES	20	33
(1991)		(14, 70%)	(31, 93%)
Akslen & Mørkve	ES	30	13
(1992)		(29, 97%)	(13, 100%)
Lassam <i>et al</i> .	ES	61	10
(1993)		(3, 5%)	(7, 70%)
McGregor <i>et al.</i>	ES	24	12
(1993)		(15, 62.5%)	(8, 66.6%)
Critolfolini <i>et al.</i>	ES	40	7
(1993)		(11, 27.5%)	(6, 85.7%)
Weiss <i>et al.</i>	CL	3	10
(1993)		(2, 66%)	(10, 90%)
Akslen (1993)	ES	22 (19, 86.4%)	-
Flørenes <i>et al.</i>	ES	25	42
(1994)		(9, 36%)	(16, 38.1%)

Table 4.4. Review of publications investigating p53 immunoreactivity in melanoma (number and percentage of immunopositive specimens shown in parentheses).

The association between p53-overexpression and age is interesting and merits investigation in a larger series of patients. Initial findings suggest melanoma may arise in younger patients via mechanisms independent of p53 stabilisation. Tumours may arise as a result of mutations of other oncogenes or the action of specific 'melanoma genes' (Trent *et al.*, 1990). Recently a potential melanoma suppressor gene has been identified on chromosome 9p21 (Fountain *et al.*, 1992, Coleman *et al.*, 1994). Deletion or mutation of such a gene may be speculated to be of relatively greater importance in the development of melanomas in younger patients. This and other oncogenes may be more susceptible to actinic DNA injury, accounting for the increasing incidence of melanomas in younger people. The rise in incidence of p53 overexpression that accompanies age is consistent with the concept that cancer is a multi-step disorder in which the risk of achieving a critical, transforming gene mass increases with age.

The relatively high p53-positivity of head and neck melanomas compared to those situated elsewhere on the body may be due to increased sun-exposure at this site, and the result of actinic damage. In the first instance, elevation may be protective due to induction of wild-type p53, as opposed to a stabilised inactive form. However, the immunohistochemical studies on melanoma mentioned above show that elevation occurs principally in levels of the stabilised protein. In vivo experiments on the effect of UV irradiation (UVr) on normal human skin demonstrated induction of wild-type p53 in response to normal ambient levels of exposure (Hall et al., 1993). UVr-provoked genotoxicity has also been shown to increase intracellular p53 in vitro, with the level of oncoprotein correlating with the extent of DNA injury (Maltzman & Czyzyk, 1984; Kastan et al., 1991). However, in vitro elevation of intra-nuclear p53 in immortalised fibroblasts has been shown to follow accumulation of stabilised protein rather than increased p53 gene expression (Fritsche et al., 1993) and this is supported by studies in melanoma (Flørenes et al., 1994), (I shall return to this aspect in the General Discussion in Chapter 8). Thus one possible explanation for the differences in p53-positivity observed in the various studies on melanoma may be the site of origin of the primary tumour analysed. Tumourigenesis may be initiated by different routes and mechanisms to those in non-sun-exposed sites. This is partially supported by the natural history and development of invasive melanomas in lentigo maligna, which predominantly occur on the face in the elderly.

This study has shown flow cytometry to provide rapid, quantitative measurements of p53 oncoprotein levels in ethanol-fixed melanomas, allowing assessment of distribution in relation to the cell cycle. Immunohistochemistry has the advantage of allowing study of the anatomical distribution of a protein, but is not quantitative or rapid. Flow cytometry provides a rapid and simple method capable of overcoming these limitations and ideally both modalities should be used to assess protein localisation and quantification.

4.7 *c-myc* Oncoprotein Expression in Ethanol-fixed Melanomas

4.7.1 Materials and methods

c-myc oncoprotein was assayed in the same 50 melanomas used for p53 analysis (Section 4.3.1). The method for sample preparation and staining is described in Section 2.3.1 and flow cytometric analysis in Section 2.5. Similar parameters were calculated for *c-myc* expression, including percentage positivity, the fluorescence ratio and the cell cycle phase positivity ratio (Section 4.2).

4.8 Results

4.8.1 c-myc oncoprotein expression through the cell cycle

The variation in *c-myc* positivity ratio and expression ratio with cell cycle phase are shown in Figures 4.9a and b. A relative increase in intra-nuclear *c-myc* oncoprotein was observed as nuclei passed from G1/G0 to S phase, and then on to G2+M. As for p53, the relatively low positivity ratio seen in G1/G0 is partially explained by the diluting effect of non-cycling cells within this phase of the cell cycle. The median positivity ratio for nuclei in S and G2+M is higher than that for p53 at similar phases of the cycle (see Table 4.1). This is probably explained by the fact that as an acute growth response gene, intra-nuclear levels of *c-myc* are enhanced to a greater extent than those of p53 in passing from G0 to S.

The expression ratio is effectively horizontal, though it displays a slight increase in passing from G0/G1 to S (Fig 4.9b). This would imply only a slight increase in actual oncoprotein content between cell cycle phases and is at variance with what would be expected. Results are summarised in Table 4.5.

Phase of cell cycle	G1/G0	S	G2+M
<i>c-myc</i> positivity ratio	35.4	54.5	64.6
(+/- S.E.M) %	5.2	5.1	5.2
<i>c-myc</i> expression ratio	3.2	3.6	3.0
(+/- S.E.M)	1.0	1.2	1.6

Table 4.5. Variation in *c-myc* positivity ratio and expression ratio through phases of the cell cycle.

4.9 *c-myc* Oncoprotein Expression in Primary and Metastatic Melanomas

Eleven of the 14 primary melanomas (79%) and 32 out of 36 metastases (89%) were immunopositive for *c-myc*, representing 43 out of 50 (86%) of all melanomas assayed. The distribution of *c-myc* positivity, displayed in Figure 4.10 and summarised in Table 4.6, shows a wide span of values, ranging from 0.0% to 96.3%. The overall median positivity of 45.9% (mean = 49.4%) indicate this oncoprotein was prevalent in relatively high amounts in the melanomas examined in this series.

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Table / 6	C-muc	onconrotein	evpression 1	n nr	mary	and	metactatic	melanomae
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	~				~			

Tumour Type	Number Analysed	<i>myc-</i> ve Tumours	<i>myc</i> +ve Tumours	% +ve nuclei (Median)	
		(%)	(%)		
1º Melanoma					
< 3.0mm	5	3 (60)	2 (40)	7.7	
1º Melanoma					
> 3.0mm	9	0 (0.0)	9 (100)	49.5	
2º Melanoma					
Skin Mets.	10	2 (20)	8 (80)	70.7	
2º Melanoma					
Nodal Mets.	26	5 (19.2)	21 (80.8)	34.5	



Figure 4.9a. *c-myc* positivity ratio and cell cycle phase.

Figure 4.9b. *c-myc* expression ratio and cell cycle phase.



Figure 4.10. Distribution of *c-myc* positivity in ethanol-fixed primary melanomas (filled symbols) and metastases (open symbols).

Dashed line - median

Dotted line - mean

The *c-myc* positivity for all primary melanomas was 44.8% (0.0 - 93.0). However, of more interest, oncoprotein positivity was found to vary with tumour thickness (Fig 4.11); In the five primary melanomas less than 3.0mm thick, two tumours were immunopositive (40%), whereas nine of eleven (81.8%) thick melanomas (>3.0mm) stained positively for *c-myc*. Nuclear positivity also varied with tumour thickness; the median *c-myc* expression in thin melanomas (<3.0mm) was 7.7% (0.0 - 22.1), compared to 49.5% (20.9 - 93.0) in those tumours over 3.0mm. This difference was highly significant (p = 0.008).



Figure 4.11. Variation in *c-myc* expression with primary melanoma thickness.

Consideration of oncoprotein levels in all specimens on the basis of primary tumour thickness showed considerable variation in positivity. The median *c-myc* positivity for melanomas less than 1.5mm was 14.1% (0.0 - 94.8), whilst that for tumours over 3.0mm in thickness was 56.8%. The difference in *c-myc* expression between thick and thin melanomas was again statistically significant (p = 0.03).

Of the 36 metastases analysed, 32 (89%) were found to be *c-myc*-positive. The overall nuclear positivity was 47.8% (0.0 to 96.3). Nodal and skin metastases appeared to have a considerable difference in their median *c-myc* positivity, but this failed to reach statistical significance (p = 0.32). Nodal secondaries had a median positivity of 34.5% (0.0 - 96.3), whilst soft-tissue metastases had a median of 70.7% (8.4 - 94.9).

No statistical difference was seen in *c-myc* positivity between metachronous lesions (p = 0.84). Furthermore, the progression from primary melanoma to metastatic disease was not accompanied by a significant difference in oncoprotein levels (p = 0.32).

4.9.1 DNA indices

The higher median *c*-*myc* expression found in thick melanomas and soft tissue metastases was not significantly associated with tumour ploidy (p = 0.78). The median *c*-*myc* positivity in diploid melanomas was 47.7% (0.0 - 96.3); *c*-*myc* expression in aneuploid lesions was similar at 41.3% (0.0 - 95.8).

4.10 *c-myc* Oncoprotein Expression and Clinico-Pathological Parameters

In order to investigate the presence of any association between c-myc activity and clinical behaviour, a number of features from each patient and their tumour were examined in association with c-myc protein expression.

4.10.1 c-myc expression, disease progression and patient survival

On univariate analysis, survival in primary melanomas appeared to be worse in tumours with high *myc* expression (Fig 4.12a), though this was likely to reflect the association between *c*-*myc* expression and primary tumour thickness. However, of more interest, consideration of *c*-*myc* oncoprotein expression in patients with stage two disease also revealed the level of expression to be a significant prognostic indicator (p = 0.04, $\chi 2 = 4.21$, Fig 4.12b).

When c-myc expression was considered in thick tumours alone (>3.0mm and classified as of uniform risk using Breslow thickness), a marked difference was observed in the

survival of this group of patients depending on whether tumours were of high or low *c*-*myc* oncoprotein positivity (Fig 4.12c). Thick primary melanomas and their metastases, with low *c*-*myc* oncoprotein content, were found to have a significantly improved survival compared to those patients whose melanomas were high *myc* expressors (p = 0.02, $\chi 2 = 5.68$). At ten years follow-up, 16 of 18 of patients (89%) presenting with low *c*-*myc* positive thick melanomas were still alive. However by this time, all patients presenting with thick primary tumours that expressed elevated *c*-*myc* protein levels had died. Similarly, when all tumours were considered as a whole, elevated *c*-*myc* expression was again seen to act as a marker of poor prognosis (p = 0.01, $\chi 2 = 6.28$, Fig 4.12d).

Tumour oncoprotein content was observed to increase with disease progression and tumour stage (Fig 4.13). The median *c-myc* positivity of primary melanomas showing no evidence of progression at the time of follow up (stage one disease) was 22.4% (1.3 - 79.0), whereas that of samples from locoregional metastases (stage two) was 31.8% (0.0 - 96.3). This difference was not statistically significant (p = 0.42). However, *c-myc* levels measured in tumours from stage four patients, (i.e. those who had gone on to die from their disease), were significantly elevated (64.1%, range 0.0 - 94.9) compared to patients with stage one and two disease (p = 0.05). No such relationship was observed for *p53* expression and disease stage (Fig 4.6).

4.10.2 Patient sex and variation in c-myc expression

As discussed in Section 4.5.2, the sex of the patient was found to be of independent prognostic significance, with females showing improved survival. The median *c-myc* expression in females was 47.8% (0.0 - 95.8), whereas that in males was 35.2% (0.0 - 96.3). However, this difference was not statistically significant (p = 0.56).

4.10.3 Patient age and c-myc expression

Figure 4.14. shows the variation in *c-myc* expression with patient age. The association is similar to that seen for p53 in that expression was found to be greater in melanomas derived from patients above the median age (p = 0.002) and maximal







Figure 4.12a. *c-myc* expression and survival in patients with stage 1 disease. Upper curve - positivity <40% (n = 6), lower curve - positivity >40% (n = 7) [p = 0.08, $\chi 2 = 3.06$].

Figure 4.12b. *c-myc* expression and survival in patients with stage 2 disease. Upper curve - positivity <40% (n = 14), lower curve - positivity >40% (n = 13) [p = 0.04, $\chi 2 = 4.21$].





4.12d



Figure 4.12c. *c-myc* expression and survival in patients with primary melanomas >3mm thick. Upper curve - positivity <40% (n = 10), lower curve - positivity >40% (n = 18) [p = 0.02, $\chi 2 = 5.68$].

Figure 4.12d. *c-myc* expression and survival in all patients. Upper curve - positivity <40% (n = 20), lower curve - positivity >40% (n = 20) [p = 0.01, $\chi 2 = 6.28$].



Figure 4.13. *c-myc* expression and disease stage.



Figure 4.14. *c-myc* expression and age of the patient at time of excision biopsy.

in melanomas from patients in their sixties. However, the difference in oncoprotein levels between patients in their fifth and sixth decades is less obvious than that seen for p53. The median *c-myc* positivity in 13 melanomas from patients in their fifth decade was 47% (90.0 - 95.8), compared to 88.6% (22.6 - 94.3) in tumours from patients aged 60 to 70 years.

4.10.4 c-myc expression and site of the primary or metastatic melanoma

Primary melanomas and metastases arising on the head and neck were found to have the highest *c-myc* oncoprotein content, with 76.2% (range 20.9 - 96.3) of nuclei being immunopositive. This was significantly greater than those *c-myc* levels detected in melanomas arising on the torso (p = 0.001), where the median *c-myc* positivity was 22.7% (range 0.0 - 61.7). *c-myc* positivity in melanomas on the lower limb was 65.7% (range 0.0 - 95.8) which again was significantly greater than median oncoprotein levels found in melanomas on the torso (p = 0.01), but not from the head and neck (p = 0.29). Results are summarised in Table 4.7.

Table 4.7.	Variation in	c-myc	oncoprotein	expression	with	location	of	the	primary
melanoma									

	Head & Neck	Torso	Lower Limb
Number of melanomas	8	10	28
Thickness of primary (median)	4.6	3.8	3.6
<i>c-myc</i> positivity median (range %)	76.2 (20.8 - 96.3)	22.3 (0.0 - 61.7)	65.7 (0.0 - 95.8)

4.10.5 Histopathological parameters and c-myc expression

As for p53, a strong association was observed between elevated *c-myc* expression and the presence of ulceration in the primary melanoma (p = 0.0001). The median *c-myc* positivity in non-ulcerated melanomas and their metastases was 29.3% (0.1 - 95.3), whilst that in ulcerated lesions was 85.7% (30.0 - 94.9).

A trend was observed between *c-myc* expression and the presence of mitotic figures. The median *c-myc* positivity in melanomas with no or few figures was 33.1% (0.0 - 95.8). However, in tumours with several figures, *c-myc* positivity was elevated at 61.7% (90.0 - 94.9). This association failed to reach statistical significance (p = 0.10). Lymphocytic infiltration of the tumour was not found to be associated with *c-myc* levels (p = 0.5).

4.10.6 Disease-free interval and c-myc expression

The correlation between *c*-*myc* protein levels and survival were reflected in the association between this oncoprotein and the disease-free interval. The median *c*-*myc* positivity in patients with a low DFI was 72.5 % (range 0.0 - 96.3), compared to a median positivity of 32.7% (range 0.0 - 95.8) in patients with prolonged survival. This difference was highly significant (p = 0.004).

4.11 The Association Between *p53* and *c-myc* Expression

Figure 4.15 shows a plot of p53 positivity against *c-myc* positivity in all ethanol-fixed melanomas. Other than for one specimen in the lower right quadrant of the graph, an association is apparent which shows high p53 positivity (>80%) only occurred in those tumours where high levels of the *c-myc* oncoprotein were also present. The median *c-myc* expression in melanomas with p53 positivity above 80% was 79% (21.3 - 96.3). In melanomas with p53 positivity below 80%, median *c-myc* expression was 26% (0.0 - 93.0). This association proved highly significant (p = 0.0001). The relationship between *c-myc* positivity and p53 positivity also held true for comparisons in melanomas with p53 oncoprotein levels above and below the median (p = 0.0001).





Figure 4.15. Co-expression of c-myc and p53 proteins in ethanol-fixed primary melanomas (filled symbols) and metastases (open symbols). High p53 expression was significantly associated with overexpression of c-myc. This association was observed in oncoprotein assays of paraffin-embedded melanomas and dysplastic naevi, but not in benign melanocytic naevi.

4.12 Discussion

Using flow cytometry, this study found overexpression of the *c-myc* oncoprotein to be prevalent in both primary and metastatic melanomas. Overall, 86% of primary and metastatic melanomas stained positively for *c-myc* oncoprotein and this is consistent with the overall immunopositivity seen in other tumour types (Field & Spandidos, 1990). However, the major finding of this study suggests that elevated *c-myc* oncoprotein levels in melanoma may be associated with poor outcome and several clinical features of disease progression. These include ulceration of the primary tumour, presence of mitotic figures and a reduced disease-free interval.

In common with larger studies, primary tumour thickness was shown to be the major, independent prognostic indicator in this group of patients. The increased c-myc oncoprotein expression, noted in thick primary tumours as compared to thin lesions, was not the result of analysing a larger amount of tumour as the same number of nuclei were analysed in each sample. This provides corroborative evidence to support the argument that c-myc acts to potentiate the invasive behaviour of melanoma and is of prognostic significance. However, it will be necessary to examine this observation in a much larger group of primary melanomas, particularly thin lesions. The increase in c-myc observed in all melanomas, as a significantly higher median c-myc positivity was detected in melanomas of patients that went on to die compared to those tumours in patients whose disease remained confined to either the primary site or loco-regional metastases.

Using univariate analysis, the findings of this study suggest *c-myc* oncoprotein may act as a prognostic marker for patients when assayed in both thick primary melanomas (stage one) and locoregional metastases (stage two) disease. Furthermore, this survival difference could not be attributed to the site of the primary or the patients' sex. Presently, patients presenting with primary melanomas in excess of 3mm thick are classified as of uniform risk (poor prognosis) on the basis of this parameter alone (Balch *et al.*, 1992). The association between *c-myc* expression and primary tumour thickness may explain the difference in survival of patients, seen in Figure 4.12a, for as expected disease progression and death occurred in patients presenting with thick lesions. However, consideration of *c-myc* positivity in thick (>3mm) melanomas (Fig 4.12c) suggests that oncoprotein overexpression may further predict poor outcome in a patient group currently defined as of uniform risk of progression using Breslow thickness alone as a prognostic indicator. Confirmation of this observation in a larger series of patients may yield important prognostic information in addition to that obtained from the primary tumour thickness and may allow stratification of prognosis and further definition of outcome on the basis of *c-myc* oncoprotein content. It would be of additional benefit if it were shown *c-myc* also acted as a prognostic indicator in thin melanomas, as a proportion of these tumours (6 -10%) will metastasise in spite of the favourable prognosis predicted by their thickness (Park *et al.*, 1993).

The observation that *c-myc* expression may also predict tumour behaviour when assayed in patients with stage two disease may also be of potential value. Other than for consideration of the number of nodes and extra-capsular spread (Cascinelli *et al.*, 1984; Kissin *et al.*, 1987), no method is currently available to predict disease outcome in patients with locoregional metastases, despite the fact their disease course may be highly variable (Balch *et al.*, 1982, 1992). The association between *c-myc* protein levels and outcome apparent from this study would suggest that consideration of this marker may be of value in predicting outcome in patients with stage two disease and further understanding of melanoma biology. In addition, future consideration of biological markers, such as the *c-myc* oncoprotein, may act as a basis to determine patient management and stratify entrance into clinical studies.

Overexpression of *c-myc* has been observed in a wide range of haemopoeitic and solid cancers including colon, lung, breast and certain neurological tumours (reviewed by Alitalo & Schwab 1986, Garte 1993) and thought principally to be due to gene amplification (Seeger *et al.*, 1985). *c-myc* amplification has also been observed to accompany tumour progression and phenotypic changes consistent with malignant progression in small cell lung cancer cell lines (Johnson *et al.*, 1987; Garte, 1993). However, it would appear that translocation and amplification only explain overexpression of *c-myc* in a small proportion of cases, for in the majority *c-myc* overexpression is ubiquitous, and the level of expression is often related to disease outcome (Field *et al.*, 1989; Garte, 1993).

Overexpression of c-myc was shown by Bourhis *et al.* (1990) to be prognostically relevant in cervical carcinoma. This study used northern blotting to assay c-myc mRNA levels in 93 invasive cervical carcinomas and found overexpression in 33% of tumours. The level of overexpression and nodal status were found to provide a very accurate predictor of tumour recurrence (Bourhis *et al.*, 1990). Similarly, a number of studies have demonstrated an association between c-myc expression and progression of breast

cancer (Varley et al., 1987); (reviewed by Garte, 1993) and amplification of this gene has been shown to be an independent prognostic marker of poor outcome (Field & Spandidos, 1990). Other workers have found *c-myc* to be a superior prognostic indicator in breast cancer than other established oncogene markers such as *Her/neu* (Berns et al., 1992). Pertschuk and Feldman et al. (1993) found *c-myc* amplification to correlate with rapid disease progression and a reduced disease-free interval. A further study possibly explained these findings by observing that *c-myc* overamplification was associated with elevated cell proliferation in breast tumours as assessed by analysis of proliferation associated nuclear antigen, defined by Ki-S1 (Kreipe et al., 1993). However, Locker et al. (1989) failed to identify any prognostic role for *c-myc* in 141 breast cancers. Using ELISA, Field et al. (1989) found elevated *c-myc* oncoprotein levels to correlate with poor prognosis in head and neck squamous carcinomas.

In contrast, other studies have found low *c-myc* oncoprotein levels to be associated with poor prognosis. Invasive bladder carcinomas were observed to contain lower *c-myc* oncoprotein levels than well differentiated tumours (Masters *et al.*, 1988). This relationship has also been observed in testicular cancers (Watson *et al.*, 1986). Similar findings have been made in well-differentiated colonic carcinomas where increased levels of *c-myc* mRNA and oncoprotein, detected using immunohistochemistry, were found in comparison to poorly differentiated tumours (Sikora *et al.*, 1987). The authors of both papers proposed this paradoxical finding could be attributed to post-translational mechanisms or decreased protein stability in more aggressive tumours. It would appear, therefore, that the regulatory mechanisms which control *c-myc* so tightly in normal cells are lost, and that loss of *myc* homeostasis is a frequent, if not essential, step in tumour evolution.

Other members of the *myc* family have also been shown to be of prognostic significance. Overamplification of the N-*myc* gene in neuroblastoma is associated with disease progression and was found to be a strong indicator of poor outcome, independent of disease stage (Phillips *et al.*, 1991). The disease-free interval in neuroblastoma has also been shown to have an inverse relationship with the number of copies of the N-*myc* gene (Seeger *et al.*, 1985).

The relationship between tumour site and c-myc oncoprotein content suggests melanomas arising on sun-exposed sites may have higher c-myc oncoprotein expression. Tumours arising on the head and neck had similar oncoprotein positivities to those on the lower limb. Melanomas from both these sites had significantly higher cmyc expression in comparison to melanomas developing on the torso. This was observed in the study of p53 expression discussed in Section 4.5.4. (The association between p53 expression and *c-myc* is discussed in Chapter 8, the General Discussion). The underlying mechanism is likely to be similar, with UVr producing genetic changes that either result in gene amplification or, more likely, post-translational modification involving the deregulation and activation of other oncogenes. The association between the site of melanomas and oncogene mutation has been reported for *ras* mutations which were found to be confined to melanomas arising in sun-exposed areas (Van' T Veer *et al.*, 1989). Melanomas developing on peripheral sites tend to have a better prognosis than those on the torso and our finding of greater *c-myc* positivity in this group of tumours may be contradictory to that expected. However, the fact that *c-myc* expression acted as a prognostic indicator in all tumours, as well as all thick lesions, suggests that it predicts behaviour independently of tumour site; though clearly more patients need to be studied.

A trend was observed suggesting c-myc oncoprotein levels were much higher in skin and subcutaneous secondaries compared to lymph node metastases. This is in keeping with the proposed relationship between c-myc positivity and tumour aggression. Melanomas preferentially metastasise to regional lymph nodes, but invasion of extranodal sites (i.e. skin) may require the activation of additional metastatic mechanisms such as production of lytic enzymes and modulation of adhesion molecules. Therefore, it would seem consistent that in order for metastases to leave regional nodes, tumour cells would need to acquire more aggressive characteristics to overcome nodal immune defence mechanisms and disseminate more widely. This may follow further genetic degeneration or enhanced overexpression of already deregulated genes, as seen in this group of specimens. However, it is also evident that in the few metachronous lesions studied, oncoprotein levels did not vary significantly.

This study also found an association between *c-myc* positivity and a number of other markers of outcome, including tumour ulceration, the presence of mitotic figures and the disease-free interval. Ulceration of primary melanomas usually occurs in thicker lesions and has been attributed to several factors including trauma, and reduced blood supply to superficial, distant areas of the tumour. However, the results of this series would suggest that ulceration may also follow elevated *c-myc* expression producing secondary effects such as increased cellular proliferation. This could produce hyperproliferative regions in which cells are produced at a rate incompatible with nutritional supply and where other factors, such as loss of anchorage dependence may play a part. The relationship between elevated *c-myc* oncoprotein levels and decreased DFI has also been observed in head and neck squamous cell carcinomas (p < 0.02) (Field *et al.*, 1989) and in patients with breast cancer (p = 0.003) (Pertschuk *et al.*, 1993).

The role of *c-myc* in the pathogenesis of melanoma has been studied to a very limited extent. All publications to date have used northern blotting to assay *c-myc* mRNA in a limited sample of cell lines or tumour specimens. Husain *et al.* (1991) found *c-myc* mRNA to be elevated 9-14 times in two metastatic cell lines, compared with melanocytes, and this study also suggested that *myc* overexpression was associated with anchorage-independence and transfectability. A further study investigating expression of 21 oncogenes in metastatic melanoma cell lines found *c-myc* to be overexpressed in 16 of 18 cell lines (88.8%), but only in the order of 1.5 to 3 times (Chevenix-Trench *et al.*, 1990). Again no overexpression was found in melanocyte lines. Of interest, this study also found a correlation between *p53* and *c-myc* mRNA levels. In the final study, by Peris *et al.* (1991), *c-myc* mRNA levels were assayed in human excision specimens. Messenger RNA was found to be 1.4 times higher in naevus cells compared to melanomas, however this study only investigated four benign naevi and seven melanomas.

The widespread elevation of *c*-myc oncoprotein seen in this group of melanomas is consistent with the central role this gene is considered to have in growth control of both normal and malignant cells (Littlewood et al., 1990). As a transcription factor, binding of the oncoprotein to DNA-specific regions of the genome regulates expression and activity of at least three major groups of genes; those controlling proliferation, differentiation and apoptosis (Evan & Littlewood, 1993). As discussed in Section 1.10.3., a considerable body of *in vitro* work has shown that *c-myc* acts as a cell cycle control gene for cells as they pass from G0/G1 to S phase and also, in specific populations, from G2 to M. Inappropriate elevation of intra-nuclear oncoprotein levels would be expected to increase the number of cycling cells, resulting in a cell population that is predominantly proliferative and free of the retardive effects of differentiation. This growth promoting effect may confer a selective advantage on certain tumours accounting for their relatively aggressive behaviour. The mechanism by which *c*-myc protein participates in the malignant process awaits further clarification, as does the relationship between *c-myc* oncoprotein expression, *c-myc* mRNA and gene copy number (amplification).

4.13 Summary to Chapter 4

Both the p53 and *c-myc* oncoproteins were found to be overexpressed in the majority of melanomas examined in this study. A high positivity of both oncoproteins was associated with presentation above the median age and several histological parameters

including ulceration and presence of mitotic figures. A correlation was also observed between oncoprotein expression and site of the primary melanoma.

The major findings of this study were the association between high expression of the *c*-*myc* oncoprotein and increasing tumour thickness and survival in patients with both stage one and stage two melanoma. The observation that this was also true for melanomas over 3mm thick suggests measurement of this oncoprotein may yield prognostic information in addition to that provided by current prognostic markers. Verification of these observations are required in a greater number of patients.

Chapter 5

Investigation of *p53* and *c-myc* Oncoprotein Expression in Paraffin-Embedded Melanomas

5.1. Introduction

Investigation of the p53 and c-myc oncoproteins in ethanol-fixed melanomas suggested c-myc overexpression acts as a prognostic indicator in both primary and metastatic tumours. In order to verify this and other observations in a larger series of melanomas it was necessary to use paraffin-embedded specimens as an additional source of tumours. The aims of this set of experiments were to:

- 1) Establish the range of expression of both *p53* and *c-myc* oncoproteins in paraffin-embedded melanomas.
- Confirm whether the associations noted between expression of both oncoproteins and clinico-pathological parameters in ethanol-fixed melanomas were also displayed in this larger group of tumours.

5.2. Materials and Methods

Primary and metastatic melanomas were obtained from the Histopathology department at Mount Vernon Hospital. In all cases the diagnosis was confirmed and, where possible, original paraffin blocks were obtained for patients referred from other centres. p53 and *c-myc* oncoproteins were analysed in 96 primary and metastatic melanomas from 96 patients. Thick sections (35μ m) were cut from paraffin blocks and rehydrated using a modified technique described by Hedley *et al.* (1983). Melanomas were enucleated and stained as described in Section 2.3.2. This differs from the method used to enucleate ethanol-fixed melanomas as a higher concentration of pepsin solution is employed (4mg/ml for paraffin-embedded specimens versus 0.4mg/ml in ethanol-fixed tumours). Samples were analysed on the FACScan and the same indices calculated as for ethanol-fixed specimens (see Section 4.2.). Clinicopathological details were not made available until after flow cytometric data had been analysed.

5.3. Results

5.3.1. Clinical and histopathological data

Primary and metastatic melanomas were obtained from 96 patients, 37 males and 59 females, with a median age of 57.5 years (14 - 93). Of the 96 specimens analysed, 43 were primary melanomas, with a median thickness of 2.5mm (0.3 - 7.0). These comprised 6 lentigo maligna, 21 superficial spreading melanomas, 13 nodular melanomas, one acral lentiginous lesion and two subungual melanomas. Of the 53 metastases analysed, 28 were from regional nodes, 23 were skin or sub-cutaneous secondaries and two were from viscera. The median follow-up of this group was 32.5 months (10 - 237 months).

5.3.2. Oncoprotein positivity and the cell cycle

The pattern of p53 and c-myc positivity and expression ratios were very similar to those observed in ethanol-fixed melanomas. However, the actual ratios showed considerable variation between the two methods of preservation. The median p53 positivity ratio in G0/G1 (Fig 5.1a) was 9.2% (0.0 - 96.0) compared to 19.2% (0.0 - 99) in ethanol-fixed melanomas. Figure 5.1b shows the p53 expression ratios to be approximately 35% lower in paraffin-embedded tumours than those found in ethanol-fixed melanomas (see Fig 4.1). Similarly, the level of c-myc positivity was greater than that for ethanol-fixed melanomas (Fig 5.2a). In G0/G1, 61.3% of nuclei were immunopositive compared to 35.4% of ethanol-fixed melanomas in the same phase of the cell cycle.

5.3.3. Clinico-pathological parameters and prognosis

The clinical behaviour of this second group of patients were very similar to those studied in the ethanol-fixed tumour group. Univariate analysis showed the Breslow thickness (p = 0.0023, $\chi 2 = 9.3$) and disease-free interval (p = 0.001, $\chi 2 = 28.59$) to be of prognostic importance, but not the patient's sex (p = 0.11, $\chi 2 = 2.52$). Multivariate analysis confirmed Breslow thickness and the DFI to be independent factors in determining outcome and patient survival (Figs 5.3a to 5.3c).



Figure 5.1a. Variation in p53 positivity ratio with cell cycle. Positivity ratio increased with passage from G0/G1 to S phase, though phase positivity did not differ significantly from that in ethanol-fixed melanomas.

Figure 5.1b. Variation in p53 expression with the cell cycle. As in ethanolfixed melanomas, fluorescence did not significantly vary with cell cycle progression.



Figure 5.2a&b Variation in *c-myc* positivity ratio with the cell cycle. The phase -specific positivity ratios were higher than found in ethanol-fixed tumours. This was reflected in increased expression ratios at each phase (Figure 5.2b).
SURVIVAL BY BRESLOW THICKNESS





Figure 5.3a. Primary tumour thickness and patient survival. Survival was superior (p = 0.001, $\chi 2 = 10.72$) for melanomas below 3mm thick (upper curve, n = 42), compared to tumours >3mm in thickness (lower curve, n = 43).

Figure 5.3b. DFI and survival. A shortened DFI (less than 12 months, lower curve, n = 37) was associated with decreased survival compared to a DFI over 12 months (upper curve, n = 59) (p = <0.001, $\chi 2 = 28.59$).

Figure 5.3c. Patient sex and survival. Outcome was not influenced by sex ($p = 0.11, \chi 2 = 2.52$). Females (upper curve, n = 59), males (lower curve, n = 37).

5.4. *p53* Oncoprotein Expression in Paraffinembedded Melanomas

Of the 96 melanomas analysed, results were obtained in 86 specimens, 37 primary melanomas and 49 metastases. In ten specimens (10.4%) the flow cytometry profile was too contaminated with debris to yield interpretable data. Sixty-eight of the 86 specimens proved positive for p53, similar positivity was found between primary (30/37, 81.1%) and secondary disease (38/49, 77.6%).

The median percentage of p53-positive nuclei was lower, (11.5%; range 0.0 - 85.3) (Fig 5.4) compared to the median 19.2% positivity observed in ethanol-fixed tumours. However, distribution between the two methods of preservation did not significantly differ for either primary melanomas (p = 0.60) or metastases (p = 0.40). The median positivity for primary melanomas was 7.2% (0.0 - 80.2), whilst 13.2% (0.0 - 85.3) of nuclei in metastases were immunopositive. This trend did not prove statistically significant (p = 0.17).

The larger number of primary melanomas examined in this series allowed a more critical analysis of p53 expression and tumour thickness. Comparison of p53 positivity in 14 melanomas less than 1.5mm thick and 15 tumours in excess of 3.0mm, found no significant difference (p = 0.37). Tumour morphology was also not found to influence p53 positivity. The median p53 expression values of superficial spreading melanomas and nodular melanomas were very similar (7.5% and 5.3% respectively, p = 0.99). Both these sub-types produced similar p53 positivities to lentigo maligna melanomas (3.9%, p = 0.29).

Of the metastases studied, 28 were located in the regional nodes and 21 were either cutaneous or sub-cutaneous lesions. The median positivity in these two groups was very similar; 13.5% (0.0 - 85.3) in nodal metastases as compared with 13.2% (0.0 - 44.0) in skin secondaries (p = 0.47).

The incidence of an euploid DNA profiles in all tumours was 44.2% (38 tumours). The DNA profile showed 48 of all tumours studied to be diploid (55.8%). Within the primary melanomas, 15 (40.5%) were an euploid, whilst 23 of the metastases were aneuploid (47%). An euploid melanomas were noted to have a higher median p53positivity (16.9%, range 0.0 - 85.3) compared to diploid lesions (10.0%, range 0.0 -79.4), but this did not reach statistical significance (p = 0.16). Results are summarised in table 5.1.



Figure 5.4.Distribution of p53 positivity in all tumours.Dashed line - meanDotted line - median

Tumour Type	Number Analysed	<i>p53</i> +ve Tumours	% +ve nuclei (Median)
		n (%)	
1º Melanoma			
< 1.5mm	14	12 (86)	4.9
1º Melanoma			
1.5 - 3.0mm	8	6 (75)	7.4
1º Melanoma			
> 3.0mm	15	12 (86)	11.1
2º Melanoma			
Skin Mets.	21	16 (76)	13.2
2º Melanoma			
Nodal Mets.	28	22 (79)	13.5

Table 5.1. p53 oncoprotein expression in paraffin-embedded melanomas. Column two refers to the number of tumours yielding data.

5.5. *p53* Oncoprotein Expression and Clinicopathological Parameters

As for ethanol-fixed melanomas, p53 expression was examined in relation to a number of clinico-pathological parameters.

5.5.1. p53 expression, disease progression and patient survival

Patient survival was not influenced by the level of p53 protein expression in any of the groups analysed, including those with stage one disease (p = 0.39, $\chi 2 = 0.72$) [Fig 5.5a], thick (>3.0mm) melanomas alone (p = 0.96, $\chi 2 = 0.02$) [5.5b] and in patients with loco-regional metastases (p = 0.19, $\chi 2 = 1.71$)[Fig 5.5c]. However, the median p53 positivity of stage one melanomas was significantly lower than that of stage two tumours (p = 0.007), but not stage four melanomas (p = 0.19), though the trend was still apparent as illustrated in Figure 5.6.



Figure 5.5a p53 expression and survival in stage 1 melanoma. ($p = 0.39, \chi 2 = 0.72$). Upper curve - above median p53 positivity, (n = 24). Lower curve - below median (n = 56).

Figure 5.5b. *p53* expression and survival in thick (>3mm) melanomas. ($p = 0.96, \chi 2 = 0.02$). Upper curve - above median *p53* positivity, (n = 11). Lower curve - below median (n = 25).

Figure 5.5c p53 expression and survival in stage 2 melanoma. ($p = 0.19, \chi 2 = 1.71$). Upper curve - above median p53 positivity, (n = 13). Lower curve - below median (n = 30).

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5.5.2. Patient sex and variation in p53 expression

A trend was observed in this group of patients which showed that females had a better prognosis than males with melanoma, but this failed to reach statistical significance ($p = 0.11, \chi 2 = 2.52$). However, as for ethanol-fixed lesions, no correlation was found between p53 positivity and patient sex (p = 0.68).

5.5.3. Patient age and p53 expression

The study on ethanol-fixed melanomas showed a distinct relationship between age and p53 expression. However, Figure 5.7 shows that whilst there is an observable trend of increasing p53 oncoprotein expression with age, it was less obvious in paraffinembedded tumours (p = 0.34). Patients below the median age had a median p53 positivity of 8.8% (0.0 - 85.3), whilst those above it had a positivity of 9.7% (0.0 - 35.7).

5.5.4. p53 expression and site of the primary or metastatic melanoma

Melanomas arising on the limbs displayed a statistically insignificant trend showing better survival than those on the head and neck, $(p = 0.3, \chi 2 = 2.4)$ as seen in ethanol-fixed tumours. However, no difference in p53 positivity was found between melanomas arising at different sites (p = 0.9).

5.5.5. Histological parameters and p53 expression

As opposed to those findings seen in ethanol-fixed lesions, no correlation was observed between p53 oncoprotein levels and presence of ulceration in the primary melanoma (p = 0.77). No relationship was found between p53 positivity and frequent mitotic figures (p = 0.31), or with the presence of a lymphocytic infiltrate (p = 0.35).



Figure 5.6 . Variation in *p53* positivity with disease stage



Figure 5.7. *p53* expression and patient age

5.5.6. Disease-free interval and p53 expression

The DFI (median 14 months, range 0.0 - 156) was found to be an independent marker of prognosis using multivariate analysis (Fig 5.4b). In contrast to ethanol-fixed melanomas, no association was found between *p53* positivity and the DFI (*p* = 0.69). The median *p53* positivity in tumours with a short DFI (less than the median DFI) was 14 .0% (0.0 - 85.3), whilst that in tumours with a longer DFI (> median DFI) was 12.7% (0.0 - 81.2).

5.6. Discussion

Overexpression of the p53 protein was found to occur in the majority of both primary and metastatic paraffin-embedded melanomas. The proportion of positive samples was very similar to that observed in ethanol-fixed melanomas, confirming the observations of other studies that perturbation of the oncoprotein is prevalent in melanoma and is amongst the highest found in any tumour type (Bartek *et al.*, 1991; Stretch *et al.*, 1991). Flow cytometric analysis of melanomas preserved by both these methods have shown that approximately 80% of both primary and metastatic melanomas are immunopositive for the p53 oncoprotein.

However, the actual number of immunopositive nuclei in most ethanol-fixed and paraffin-embedded melanomas were low. This would suggest that p53 immunopositivity is not the result of a clonal population containing a missense mutation of the gene, but that immunodetection follows overexpression secondary to other processes. p53 overexpression may be the consequence of a gene damage response, or reflect polyclonality in the nature of p53 lesions within the tumour; immunonegativity may result from deletion or truncation of both alleles of the p53 gene (Greenblatt *et al.*, 1994), though this has not been reported in melanoma (see Section 8.1.). Although a trend was seen in both groups to suggest that the metastatic phenotype is accompanied by a greater number of immunopositive nuclei, compared to primary melanomas this was not statistically significant and would suggest that p53 stabilisation (or mutation) is not a prerequisite for metastasis in melanoma.

Studies in a number of other tumour types have confirmed this finding; immunohistochemical and ELISA analysis of p53 oncoprotein expression in 136 matched primary and metastatic breast carcinomas found 'no gain of positivity' in metastases as compared to primary cancers (Bartkova *et al.*, 1993). Scrutiny of the p53 protein itself confirmed that the amino acid sequence and conformation remained unchanged in progression from the primary to secondary tumour. In several tumours, metastases that had developed from p53 immunopositive primary tumours were found to be p53-negative. This may be explained by the loss of the second p53 allele in progressing from the primary to the secondary phenotype. DNA sequencing in matched pairs of tumours has also been performed in a limited number of other studies on breast malignancies (Davidoff *et al.*, 1991) and small cell lung carcinomas (Sameshima *et al.*, 1992). The present study did not sequence the p53 gene in any of the melanomas analysed and therefore activity of the gene can only be interpreted from expression of the oncoprotein. However, the findings are consistent with the concept that p53 stabilisation is required in the early phase of tumourigenesis and perpetuation of the malignant phenotype, but that the actual progression from primary to metastatic phenotype requires the action of other genes.

To date only one study has been published on the use of flow cytometric analysis of p53 oncoprotein in paraffin-embedded melanomas and this was confined to measurements in nodular melanomas (Akslen & Mørkve, 1992). Akslen and Mørkve (1992), found 29 of 30 tumours (97%) to be p53 immunopostive using PAb1801. As in the present study, DNA histograms were found to be less consistent in paraffin embedded material. Furthermore, no association was found between the level of nuclear positivity and outcome. However, Akslen and Mørkve (1992) observed significantly lower p53 levels in metastatic melanomas compared to primary tumours and again speculated that this may be due to loss of the normal p53 allele in progressing from primary to metastatic disease.

Overall, the population of paraffin-embedded melanomas analysed in the present study were found to contain an identical proportion of aneuploid melanomas to those found in ethanol-fixed tumours (47%). The incidence of aneuploidy in primary tumours alone (40.5%) was similar to that observed by Akslen and Mørkve (1992) of 29%. However, in contradiction to the findings of their study, a trend was observed in this group of paraffin-embedded melanomas that suggested aneuploid melanomas overexpress the p53 oncoprotein to a greater extent than diploid lesions. This may be due to amplification of the number of copies of the p53 gene, but equally, aneuploid lesions may be more prone to stabilising p53 oncoprotein by post-translational mechanisms.

At 7.2%, the overall median level of p53 positivity in primary melanomas was lower than that in ethanol-fixed tumours (12.8%), though this difference was far from statistically significant (p = 0.60). However, expression was sufficiently reduced to exclude or obscure any of the associations observed between p53 oncoprotein expression and characteristics of the primary melanoma found in the group of ethanolfixed melanomas; including ulceration and presence of a lymphocytic infiltrate. Other clinical associations that were absent in paraffin-embedded melanomas included parameters such as patient age, the DFI and primary site. This may partially be explained by the fact that a separate group of patients and specimens were analysed. In addition, the effects of different methods of preservation and preparation may also have to be taken into account.

The validation experiments presented in Chapter 3 found the method of preservation and preparation had no significant influence on oncoprotein levels. However, this set of experiments were unable to account for the effect of time and possible degradation of the target oncoprotein over many months or years whilst stored as archival material, as all the validation samples were less than twelve months old. Prileau & Schnitt (1995) studied p53 immunoreactivity in newly prepared breast carcinoma sections and compared results with slides from the same tumours stored for two months at room temperature. Immunoreactivity was significantly decreased in all the stored specimens, suggesting degradation of the p53 protein with time may result in potential errors in interpreting data. Consequently, consideration of the effects of time and storage conditions on p53 positivity may provide, in part, a plausible explanation for the observed differences between in ethanol-fixed and paraffin-embedded melanomas.

These results confirm the findings of the observations made in ethanol fixed melanomas that overexpression does not appear to be of prognostic significance and that overexpression is not related to patient survival. Paraffin-embedded melanomas did not show an association between p53 oncoprotein expression and the DFI, though stage two tumours were found to exhibit greater oncoprotein levels than primary melanomas. This last finding is of interest in that it concurs with the findings of Stretch et al. (1991), who made similar observations using immunohistochemistry. Both Stretch et al. (1991) and McGregor et al. (1993) noted that nuclear staining was particularly strong in metastatic lesions. Other studies have not noted a variation in *p53* immunopositivity between primary and metastatic melanoma (Lassam *et al.*, 1993; Akslen & Mørkve, 1992). Accordingly, consideration of the variation between findings in the two groups of patients studied in this study, and those noted in other studies, would suggest that stabilisation of the p53 oncoprotein is a common event in melanoma, but that it may not play a central role in the genesis and progression of melanoma, and indeed may be an epiphenomenon. The putative role of p53 in melanoma biology is further contemplated in the General Discussion (Chapter 8).

5.7. *c-myc* Oncoprotein Expression in Primary and Metastatic Melanomas

Of the 86 melanomas in which complete data was achieved, 33 of 37 primary melanomas stained positively for *c-myc* (89.2%) and 42 of 49 metastases (85.7%); producing an overall positivity of 87.2%. The distribution of *c-myc* positivity ranged between 0.0% and 96.5% of nuclei per tumour, producing an overall median positivity of 68.9% (Fig 5.8).

The median *c-myc* positivity of primary melanomas was 58.3% (0.0 - 96.5%), as opposed to 44.8% in ethanol-fixed primaries, but the difference between the two groups of samples was not significant (p = 0.23). Primary melanomas less than 1.5mm had a median positivity of 44.3% (0.9 - 96.5), whilst thick melanomas (>3.0mm) had a median positivity of 58.8% (13.0 - 94.3). However, again, this trend was not statistically significant (p = 0.45).

The absence of an association between *c-myc* positivity and tumour thickness was also reflected in the observed relationship between oncoprotein expression and primary tumour morphology. No significant difference was found between oncoprotein positivity in any of the four morphological sub-types, though a trend was evident to suggest ALMs had a higher *c-myc* positivity than LMs (p = 0.18). Oncoprotein positivity is summarised for each morphological type in Table 5.3. *c-myc* oncoprotein expression did not vary between ALMs and NMs (p = 0.71), or ALMs and SSMs (p = 0.36).

The median *c-myc* positivity of all metastases in this group was 75.3% (0.0 - 96.1), the difference between primary paraffin-embedded melanomas and metastases being highly significant (p = 0.006). This did not differ significantly from the median *c-myc* expression in ethanol-fixed melanomas (p = 0.37). No difference was observed in the median *c-myc* positivity between nodal and cutaneous metastases (77.6% versus 77.8% respectively, p = 0.92). Diploid melanomas had a median *c-myc* positivity of 62.0% (0.0 - 96.5), whilst that of an euploid tumours was 73.8 (0.0 - 85.3). Again, this difference failed to reach statistical significance (p = 0.16).



Figure 5.8.Distribution of *c-myc* expression in all melanomas.Dashed line - meanDotted line - median

Tumour Type	Number Analysed	<i>myc</i> +ve Tumours	% +ve nuclei (Median)
- 5 P -	1111119504	n (%)	(())(())())
1º Melanoma			
< 1.5mm	14	13 (93)	44.3
1º Melanoma			
1.5 - 3.0mm	8	7 (88)	58.3
1º Melanoma			
> 3.0mm	15	13 (87)	58.8
2º Melanoma			
Skin Mets.	21	18 (86)	77.8
2º Melanoma			
Nodal Mets.	28	24 (86)	77.6

Table 5.2. *c-myc* oncoprotein expression in paraffin-embedded primary and metastatic melanomas.

Table 5.3. c-myc positivity in morphological subtypes of primary melanoma.

Morphological subtype of melanoma	<i>c-myc</i> positivity median % (range)
Lentigo maligna	44.3 (0.9 - 87.1)
Superficial spreading	53.3 (28.2 - 85.1)
Nodular	58.8 (4.7 - 96.5)
Acral lentiginous	84.0 (43.1 - 88.7)

5.8. *c-myc* Oncoprotein Expression, Clinicopathological Parameters and Prognosis

The aims of this section, as for p53, were to investigate the association of *c-myc* expression to the range of clinico-pathological parameters analysed in ethanol-fixed melanomas. However, one of the major objectives was to further investigate whether

c-myc oncoprotein overexpression acted as a marker of poor prognosis in this larger group of patients.

5.8.1. c-myc oncoprotein, disease progression and patient survival

Consideration of *c-myc* expression and outcome confirmed the findings in ethanolfixed melanoma; high *c-myc* oncoprotein content was strongly associated with poor prognosis. In these paraffin-embedded melanomas, Cox's proportional hazards model revealed *c-myc* to be an independent prognostic indicator in patients presenting with thick primary melanomas (p = 0.02, $\chi 2 = 5.77$) [Fig 5.9a]. Though trends were evident, it was not found to be a significant prognostic indicator in patients with stage two disease (p = 0.2, $\chi 2 = 1.64$)[Fig 5.9b] or when all tumours were considered as a whole (p = 0.23, $\chi 2 = 1.44$).

The variation in median *c-myc* positivity with disease stage is shown in Figure 5.10. Patients with stage one disease had a median *c-myc* positivity of 55.5% (0.9 - 85.1) at a median 32.5 months follow-up. However, patients with stage two and four disease had median *c-myc* positivities of 81.4% and 73.4% respectively. Comparison of *c-myc* positivity between stage one and two/four disease is highly significant at this period of follow-up (p = 0.003).

5.8.2. Patient sex and variation in c-myc expression

c-myc positivity was very similar in melanomas derived from both male and female patients (p = 0.94). The median positivity in males was 66.2% (0.9 - 96.5), whilst that in females was 72.3% (0.0 - 96.1).

5.8.3. Patient age and c-myc expression

Figure 5.11. shows the variation of *c-myc* positivity with the age of the patient. In this group of specimens, a trend was seen indicating median *c-myc* positivity was greater in melanomas from patients presenting above the median age, with a median positivity of 71.5% (6.7 - 96.1). Melanomas from patients below the median age had a positivity of 62.0% (6.7 - 96.1), but no trend was statistically evident (p = 0.84).



Figure 5.9a. *c-myc* expression and survival in melanomas >3mm thickness. Elevated oncoprotein expression was associated with poor survival in thick melanomas (p = 0.02, $\chi 2 = 5.77$). Upper curve - below median expression (n = 10), lower curve - above median expression (n = 27).

Figure 5.9b. *c-myc* expression and survival in patients with loco-regional metastases (stage 2 disease). Survival was not significantly improved for melanomas with low *c-myc* expression ($p = 0.2, \chi 2 = 1.64$), though any survival advantage may have been obscured due to the limited number of tumours exhibiting low *c-myc* expression (upper curve, n = 6). Lower curve, high *c-myc* positivity (n = 38).



Figure 5.10. Variation in *c-myc* positivity with disease stage



Figure 5.11. *c-myc* expression and patient age. The association observed in ethanol-fixed melanomas was not found in these paraffin-embedded specimens.

5.8.4. c-myc expression and site of the primary or metastatic melanoma

The site-specific *c-myc* expression observed in ethanol-fixed melanomas was not seen in paraffin-embedded tumours. Median *c-myc* positivity did not vary between melanomas arising on the head and neck or torso (p = 0.9), or head and neck and limbs (p = 0.9).

5.8.5. Histological parameters and c-myc expression

Ulcerated primary melanomas had a median *c-myc* positivity of 77.3%, as opposed to 65.5% (7.6 - 96.5) in non-ulcerated lesions. This trend was not statistically significant (p = 0.18). *c-myc* positivity was also greater in tumours with increased mitotic figures; 73.7% (0.9 - 96.5) of nuclei were immunopositive in these tumours compared to 58.6% (6.7 - 88.7) in primary melanomas without mitoses. This trend just failed to reach statistical significance (p = 0.08). The presence of a lymphocytic infiltrate also showed a borderline association with *c-myc* positivity (p = 0.07). Primary melanomas with a marked lymphocytic infiltrate had a median positivity of 69.2% (0.9 - 96.5) compared to 53.3% (0.0 - 93.4) in tumours with no infiltrate.

5.8.6. Disease-free interval and c-myc expression

DFI was found to act as an independent prognostic marker in this group of specimens $(p = 0.001, \chi 2 = 28.59)$. However, the significant inverse association seen between *c*-*myc* positivity and DFI in ethanol-fixed tumours was not found in paraffinembedded melanomas, though a clear trend was present (p = 0.13). Median *c*-*myc* positivity in patients with a short DFI was 88.5% (13.0 - 96.5), whilst that in patients with prolonged survival was 70.6% (0 - 94.4).

5.9. Comparison between Ethanol-fixed and Paraffin-embedded Methods of Preservation on p53 and c-myc Parameters

The following tables summarise the association between oncoprotein positivities and various clinical parameters, as measured in the two groups of melanomas.

Oncoprotein positivity (%)	<i>p53</i> Ethanol	<i>p53</i> Paraffin	<i>c-myc</i> Ethanol	<i>c-myc</i> Paraffin
primaries	12.8	7.2	44.8	58.3
metastases	22.5	13.2	47.8	75.3
ʻp' 1° vs 2°	0.41	0.17	0.32	0.06

Table 5.4. Median oncoprotein positivity in ethanol-fixed and paraffin-embedded melanomas. 'p' value, Wilcoxon rank sum test.

Table 5.5. This table summarises the statistical association between p53 and c-myc oncoprotein expression and clinico-pathological parameters. 'p' values show comparisons between oncoprotein expression above and below median parameters in ethanol-fixed and paraffin-embedded melanomas ('t' test).

	<i>'p'</i> value			
Parameter	p53	p53	c-myc	c-myc
	Ethanol	Paraffin	Ethanol	Paraffin
Survival -				
All tumours	0.13	0.50	0.01	0.29
Thick (> 3mm)	0.07	0.96	0.02	0.02
Patient sex	0.84	0.68	0.56	0.94
Age	0.0001	0.34	0.002	0.84
Tumour site				
(h&n vs torso)	0.085	0. 9	0.001	0.9
Ulceration	0.0001	0.77	0.0001	0.18
Mitoses	0.33	0.31	0.10	0.08
Lymphocytic infiltrate	0.017	0.35	0.50	0.07
DFI	0.04	0.69	0.004	0.13



Figure 5.12. *p53* versus *c-myc* expression in paraffin-embedded primary melanomas (filled symbols) and metastases (empty symbols).

5.10. The Association Between *p53* and *c-myc* Expression

Figure 5.12 shows the relationship between p53 oncoprotein and *c-myc* oncoprotein expression. As in the case of ethanol-fixed melanomas, a clear trend was seen indicating that p53 overexpression only occurred in the presence of high levels of *c-myc* positivity. The median *c-myc* positivity in the ten melanomas with the highest p53 oncoprotein values was 82.5% (54.3 - 96.5). In comparison, melanomas with below median p53 expression had a significantly lower median *c-myc* positivity 65.6% (0.0 - 94.8; p = 0.008). Of importance, this relationship was unilateral; for taking all melanomas into account, high *c-myc* positivity did not correlate with elevated p53 expression (p = 0.39). Furthermore, *c-myc* positivity did not significantly vary above and below the median p53 positivity in both primary melanomas (p = 0.42) and metastases (p = 0.41).

5.11. Discussion

Overexpression of the *c-myc* oncoprotein was observed to be as prevalent in this group of melanomas as detected in the ethanol-fixed specimens. Almost 87% of all paraffin embedded melanomas studied in this series were immunopositive for *c-myc*, a similar proportion to that found in ethanol-fixed tumours. Overall, *c-myc* positivity was similar in primary melanomas for both ethanol-fixed and paraffin-embedded melanomas.

However, a number of significant differences were found between characteristics of c-myc expression in primary melanomas between the two groups. Although a trend was present no significant association was found between tumour thickness and c-myc expression in paraffin-embedded melanomas as seen in ethanol-fixed tumours. c-myc expression was significantly higher in paraffin-embedded melanomas less than 3mm thick, compared to the values measured in thin ethanol-fixed tumours. In the latter group, only five specimens were analysed, whilst 22 paraffin-embedded melanomas were available for study. Thus the low values seen in ethanol-fixed tumours may have either been the result of selection bias or an effect of the method of preservation and preparation not evident from the validation experiments. Despite the absence of an obvious association between c-myc positivity and tumour thickness, there was a trend to suggest that LMs had a lower oncoprotein content than ALMs. As they arise on the

face, patients with LMs tend to present at an earlier stage and behave less aggressively than ALMs, which tend to be thicker at the time of diagnosis. The median thickness of LMs studied in this series was 1.0mm (0.4 - 2.8), whilst that of the ALMs was 3.0mm (2.6 - 3.4). However, only six LMs and four ALMs were studied in this series, and caution should be exercised in over-interpreting this data. Nevertheless, the results of this small sample suggest that thinner, more slowly progressing LMs had lower oncoprotein positivity than thicker, more aggressive NMs and ALMs.

Ethanol fixation may lower p53 oncoprotein fluorescence values, as observed in comparison of flow cytometric analysis of ethanol-fixed and paraffin-embedded bronchial and bladder carcinomas (Mørkve & Hostmark, 1991). However, the relatively high *c-myc* values found in thin paraffin-embedded primary melanomas may also have been the effect of the method of preservation and enucleation. Formalin fixation crosslinks protein end groups, to stabilise epitopes, and possibly produce enhanced fluorescence when compared to fresh and ethanol-fixed tumours (Bauer, 1988). Furthermore this has been demonstrated in melanoma, where formalin fixation alone has been shown to make cells fluorescent (Jacobsen *et al.*, 1988). These effects may be compounded in thin melanomas, and further modified by the action of pepsin.

Due to the limited amount of available tumour, thin melanomas were exposed to relatively higher concentrations of pepsin than other tumour specimens and this may have either revealed greater amounts of epitope or produced artefactual results. The action of these relatively high pepsin levels on a sub-group of thin melanomas were not examined in the validation studies, due to the paucity of available specimens. However from these studies it was observed that paraffin-embedded samples tended to produce higher estimates of oncoprotein content (although these differences were not significant). Pepsin enucleation has also been shown to produce relatively elevated oncoprotein levels in other studies (Mørkve & Hostmark, 1991).

Overexpression of c-myc was strongly associated with tumour progression in this group of patients, confirming the prognostic role suggested by observations on ethanol-fixed melanomas discussed in Section 4.10.1. Tumours analysed from patients with stage two disease were found to have significantly higher c-myc oncoprotein levels than primary melanomas and this was further reflected in the close association between the level of oncoprotein overexpression and the DFI.

The most significant observation of this set of experiments was the finding that the level of c-myc expression acted as an independent prognostic indicator in patients with primary melanomas in excess of 3mm in thickness. Using current prognostic

indicators, (i.e. the Breslow thickness), all these patients would have been categorised as having been at uniform risk of disease progression and poor outcome. However, the survival analysis shown in Figure 5.9a shows that patients with thick tumours but low *c-myc* oncoprotein expression had a significantly better outcome than patients with high *c-myc* oncoprotein content. This improved survival was not the result of patients receiving additional treatment (i.e. chemotherapy or adjuvant therapy). Consequently, assay of *c-myc* positivity may allow stratification of prognosis within groups previously defined by tumour thickness and carry considerable clinical implications. Analysis of *c-myc* content in the primary tumour, and to a lesser extent loco-regional metastases, may yield important prognostic information that is unavailable using current methods.

Consideration of the effect of c-myc expression in all tumours also revealed a trend indicating a survival difference between tumours with high and low c-myc positivity. However, the lack of statistical significance is most likely to have been the result of the unexpectedly high c-myc positivity observed in thin melanomas. On the basis of Breslow thickness these lesions would be expected to carry a good prognosis, even though the c-myc expression may indicate otherwise. Figure 5.9b illustrates a further limitation; deaths that occurred in the relatively small group of tumours with low cmyc positivity may have resulted in potentially artefactual approximation of survival curves and the concealment of any difference in outcome.

The association, of borderline significance, between c-myc positivity and the presence of mitoses in primary melanomas is consistent with the role of c-myc, established in cell culture, as an early growth response gene (Littlewood & Evan, 1990). This association suggests that c-myc overexpression within the tumour may be histologically manifested by increased numbers of mitotic figures and an overall rise in cell proliferation, and this is further analysed in Chapter 7. Similarly, ulceration of the primary tumour also exhibited a clear trend to suggest that this morphological sub-type may be, in part, the result of increased expression, mediated either by increased cell turnover, or as a result of increased hypoxic sensitivity.

The absence of any association between *c-myc* expression and primary site in paraffinembedded melanomas may partly be due to sample bias of the specimens investigated in the ethanol-fixed specimens. However, the relatively high expression in thin melanomas discussed above, may have obscured this and other associations noted in ethanol-fixed tumours, such as the variation with tumour thickness and patient age. From the available evidence, the findings of this study would suggest that ethanolfixed melanomas may provide a more reliable source of data. Enucleation from ethanol-fixed material involves a shorter and potentially less destructive process than that required for isolating nuclei from paraffin-embedded melanomas. The increased pepsin concentration and number of steps required to process paraffin-embedded melanomas may have contributed to artefact and loss of nuclei. As noted in Section 5.6, longterm storage of paraffin-embedded tumour samples may affect oncoprotein preservation and immunoreactivity as detected by immunohistochemistry (Prileau & Schnitt, 1995). The effect of time on *c-myc* protein contained within paraffinembedded and ethanol-fixed melanomas was not examined in this study and may have also modified protein conformation or immunoreactivity. To resolve these issues, a greater number of paraffin-embedded and ethanol-fixed specimens derived from the same, thin, melanomas need to be analysed. In addition, the effect of storage time on *c-myc* protein positivity will also require investigation.

The prognostic role of c-myc in melanoma will need further evaluation in a greater number of patients and as such continues to form an integral part of the next phase of the RAFT melanoma project. In addition, further analysis needs to be performed in patients presenting with thin melanomas. Where possible, assays should be carried out on both ethanol-fixed and paraffin-embedded samples derived from the same tumour.

5.12. Summary to Chapter 5

Similar proportions of melanomas were found to be immunopositive for both p53 and c-myc as observed in ethanol-fixed tumours, confirming the prevalence of overexpression of both these proteins in cutaneous melanoma. p53 overexpression did not appear to be significantly associated with any of the parameters examined in paraffin-embedded specimens, suggesting that stabilisation of this oncoprotein may accompany other genetic changes intrinsic to melanoma genesis and progression, and that modification of the protein or mutation of the p53 gene itself may not represent an essential step. Indeed, as will be discussed in Chapter 8, the association between overexpression of p53 and c-myc suggests that in some tumours, p53 stabilisation may be an epiphenomenon.

The method of preservation may affect the quality and results of dual parameter flow cytometry and this effect may be more obvious in thin melanomas. Larger studies are

required to evaluate the optimum application of this assay in melanomas preserved using different methods.

In the paraffin-embedded melanomas studied in this group of patients, c-myc overexpression was found to act as an independent prognostic indicator in thick melanomas, providing information not available from current parameters. Elucidation of the mechanism of the association between c-myc overexpression and tumour spread may yield information to confirm a central role for perturbation of c-myc in the genesis and progression of melanoma. The relationship between c-myc and tumour morphology, Breslow thickness and primary site awaits clarification in a greater number of tumours and patients.

Chapter 6

p53 and *c-myc* Oncoprotein Expression in Benign Melanocytic Naevi and Dysplastic Naevi

6.1. Introduction

Patients commonly give a history describing a pre-existing naevus at the site of a melanoma and histological examination will reveal remnants of a benign melanocytic naevus (BMN) adjacent to 10-20% of melanomas in caucasians (Crucioli & Stilwell, 1982; Ackerman & Mihara, 1985). The diversity in morphology and type of naevi has posed the question as to whether a specific form of naevus is particularly associated with malignant transformation, one that is definable both histologically and clinically. The dysplastic naevus has been advanced as the most likely candidate following descriptions by Frichot (Frichot *et al.*, 1977) and Clark (Clark *et al.*, 1978) of clinically atypical naevi, later termed dysplastic naevi (DN), in association with familial melanoma. These naevi have been well characterised clinically but much debate surrounds their histological definition, which has only been reached by consensus (Table 6.1 and illustrated in Fig 6.1). Furthermore, there is argument as to the extent of their pre-malignant potential, particularly in sporadic cases of melanoma. Assessing the nature of their relationship to melanoma has been complicated by a number of factors discussed in Section 1.6.3.

This relationship would be clarified if it were found that clinical atypia was accompanied by specific genetic changes, which in themselves were insufficient to cause melanocyte transformation, but which indicated preliminary change. This would aid reclassification of naevi and allow accurate identification of pre-neoplastic naevi. Having established that overexpression of both the p53 and c-myc oncoproteins are a prevalent event in cutaneous melanoma, it was therefore necessary to investigate the expression of these oncoproteins in potentially pre-neoplastic lesions, particularly DN. This would provide information as to whether early oncoprotein perturbation was evident in a putative, intermediate stage of melanoma development.

Therefore the aim of this set of experiments were to:

- 1) Investigate expression of both the p53 and c-myc oncoproteins in clinically and histologically diagnosed BMN and DN, and subsequently determine the range of expression.
- 2) Establish any correlation between oncoprotein expression and histological atypia.
- 3) Compare *p53* oncoprotein levels detected by flow cytometry and immunohistochemistry.

Table 6.1.	Clinical and histological features of dysplastic naevi.
Histological	criteria as defined by Clemente et al. (1991)

Clinical Features	Histological Features	
Greater than 5mm in diameter	Architectural	
Irregular margin	Melanocyte proliferation along dermo-	
Macular, with occasional papular centre	epidermal junction	
Variable pigmentation	Proliferation in a lentiginous pattern	
Peripheral inflammatory hue	Bridging of rete ridges	
Atypical locations i.e. breast, buttocks	Lamellar/ eosinophilic fibrosis	
	neovascularisation/capillary proliferation	
	Lymphocytic infiltrate	
	Cytological	
	Nuclear atypia; colour, size	
	Prominent nucleoli	



Figure 6.1a. Dysplastic naevi, on the back of a 24 year old male. Note the lesion diameter, the irregular borders and variable pigmentation (compare to BMN in Fig 6.1b below).



Fig 6.1b. Benign melanocytic naevus. These naevi are usually less than 5mm in diameter, with regular borders and uniform pigmentation.

6.2. Materials and Method

Thirty surgically excised paraffin-embedded BMN and 30 DN were analysed for p53 and *c-myc* oncoproteins as described in Section 2.3.3. All 30 DN were clinically diagnosed sporadic cases with histological features of atypia (see Table 6.1). None of these patients had a personal or family history of melanoma. Thick sections were rehydrated using a modified method of Hedley *et al.* (1983) prior to oncoprotein staining and flow cytometric analysis.

In addition, thin $(4\mu m)$ sections of all 30 DN and 10 BMN were stained for p53 using immunohistochemistry, as described in Section 2.7.3., to compare the two methods of staining and identify architectural distribution of the oncoprotein.

6.3. *p53* Oncoprotein Expression in Benign and Dysplastic Naevi

6.3.1. p53 oncoprotein expression in benign melanocytic naevi

All 30 BMN investigated using flow cytometry were effectively immunonegative for p53 oncoproteins. In 19 specimens no positive nuclei were detectable at all. In the remainder, very few nuclei stained for p53, producing an overall median positivity of 0.5% (0.0 - 3.9). This was significantly less than oncoprotein levels detected in paraffin-embedded primary melanomas (p = 0.0007) and ethanol-fixed primary tumours (p = 0.001) (Fig 6.2).

None of the ten BMN examined using immunohistochemistry were p53-positive.

6.3.2. p53 oncoprotein expression in dysplastic naevi

Of the DN analysed 16 out of 30 (53.3%) stained positively for p53, compared to 80.5% of all melanomas (Fig 6.2). The median nuclear positivity of 7.2% (0.0 - 90.2) was similar to that found in paraffin-embedded primary melanomas (p = 0.99) (Fig 6.3), but considerably greater than levels present in BMN (p = 0.002). Nineteen of the 30 sections (63.3%) analysed using immunohistochemistry stained



Figure 6.2. p53 protein expression in BMN, DN and melanomas, analysed using flow cytometry. Cross-hatched bars represent the median nuclear positivity (%). Open bars show the proportion of immunopositive specimens for each type of lesion (%).



Figure 6.3. Distribution of *p53* in dysplastic naevi, as measured by flow cytometry.Dashed line - meanDotted line - median

positively for p53. There was considerable correlation between immunopositive histological sections and flow cytometric analysis as summarised in Table 6.2.

Table 6.2. Correlation between flow cytometric analysis and immunohistochemical detection of p53 oncoprotein in dysplastic naevi.

Histological staining pattern	Flow cytometric <i>p53</i> positivity (%, range)	Correlation Coeff. (R ²)
Negative	0.6 (0.0 - 28.0)	0.59
Focal	13.0 (0 - 68.1)	0.47
Widespread	14.2 (0 - 90.2)	0.52

Samples with low p53 oncoprotein content, measured by flow cytometry, were also either negative or weakly stained using immunohistochemistry (p53 oncoprotein positivity in negative versus widespread staining, p = 0.015). Similarly focal staining also correlated well with flow cytometry analysis (focal versus widespread, p =0.024) as did widespread histological staining (Fig 6.4). However, no difference in p53 levels were detected between focal and widespread staining using flow cytometry (p = 0.27). p53 staining in histological sections was variable, but usually dominated by staining of nuclei within the basal layer of the epidermis and keratinocytes. Deeply stained melanocytes were few and sporadic, with staining predominantly confined to the nucleus, though some sections displayed occasional cytoplasmic positivity.

6.3.3. p53 oncoprotein expression and histological atypia

p53 oncoprotein levels were correlated with a number of architectural features considered to be important in the histological diagnosis of dysplastic naevi, as shown in Table 6.1. Nuclear morphology was examined in addition to these criteria, that according to the WHO classification (Clemente *et al.*, 1991) have been divided into major and minor.



Figure 6.4. Immunohistochemical section of a DN stained for p53; widespread strong immunopositivity is associated with several features of architectural and cytological atypia. Melanocyte proliferation is accompanied by nest formation and bridging of the rete ridges. Nuclear hyperchromasia is also present (x 20).

Major criteria

1)

Proliferation of melanocytes at the epidermo-dermal junction in a lentiginous pattern with or without nests

This was observed in 19 of the 30 sections examined (63.3%). The median p53 positivity in specimens with melanocyte proliferation was 14.2% (0.0 - 90.2) compared to 4.2% (0.0 - 69.0) in naevi without evidence of proliferation. However this trend was not statistically significant (p = 0.15) (Figs 6.4 & 6.5).

2) Junctional component extending more than three rete ridges beyond margins of the dermal component (shoulder phenomenon)

Similar oncoprotein levels were detected in DN with and without this feature, which was identified in nine DN (30%). A median 8.0% (0.0 - 90.2) of nuclei was immunopositive in those sections without extension of the junctional component, compared to 4.5% (0.0 - 68.1) of lesions with shouldering (p = 0.68).

Minor criteria

1) Lamellar or eosinophilic fibrosis

Lamellar fibrosis was observed in almost all the DN examined. In 16 DN the distribution of fibrosis was focal, whereas in 12 specimens fibroplasia was more widespread. The median p53 positivity in lesions with focal fibrosis was 4.3% (0.0 - 90.2), whilst that in DN with widespread fibrosis was 14.5% (0.0 - 69.2). This trend was not statistically significant in this group of specimens (p = 0.26).

2) Capillary proliferation

Capillary proliferation within the superficial, papillary, dermis was observed in seven sections of DN (23.3%). No difference in p53 positivity was found between naevi with neovascularisation (9.4%) and without (6.5%) (p = 0.88).

3) *Lymphocytic infiltrate*

No infiltrate was seen in nine sections (30%), a mild infiltrate was present in 11 specimens (36.6%) and a relatively intense inflammatory response observed in 10 sections (33.3%). The median p53 positivity in each of these three subgroups was 8.0%, 7.0% and 0.6% respectively. The trend suggesting an increased infiltrate is accompanied by a reduction in p53 oncoprotein content was not significant (p = 0.56).

4) Bridging of rete ridges

Fusion of adjacent rete ridges were present in focal locations in seven sections (23.3%), and more widespread in 15 specimens (50%). There was a distinct trend indicating p53 oncoprotein expression was higher in lesions with bridging compared

to those without (p = 0.09). However, of interest, p53 positivity in naevi with widespread bridging was significantly greater than in lesions with only sporadic, focal fusion of the rete ridges (p = 0.005). The median p53 positivity in lesions without bridging was 7.3% (0.0 - 69.2), as opposed to a median 18.7% in lesions with widespread fusion.



Figure 6.5. Melanocyte proliferation and *p53* expression in dysplastic naevi.

Absence of the rete tip was seen in 8 sections (26.7%) but was not associated with a difference in p53 positivity (p = 0.53). Similarly irregular shape and orientation of rete ridges were also not found to correlate with p53 positivity (p = 0.48).

5) Nuclear atypia

No association was found between p53 positivity and nuclear hyperchromasia (p = 0.44), the presence of prominent nucleoli (p = 0.97) and nuclear or melanocytic enlargement (p = 0.5). Results are summarised in Table 6.3.

6.4. Discussion

None of the benign naevi and just over half (53.3%) of all dysplastic naevi examined in this series using flow cytometry were found to be immunopositive for p53. Similarly none of the BMN examined with immunohistochemistry proved positive for p53, whilst 19 of the 30 sections (63%) of DN showed positive staining. Good correlation was found between oncoprotein levels measured by flow cytometry and intensity of staining on histological sections. However, flow cytometry did not detect differences in oncoprotein levels between naevi displaying histologically focal and widespread immunopositivity. These observations show that whilst flow cytometry gives rapid measurement of tissue oncoprotein content it is unable to distinguish potentially important variations in architectural distribution.

The observations of this study regarding the absence of p53 positivity in BMN are consistent with a number of other studies, all of which used immunohistochemistry to assess p53 positivity in paraffin-embedded naevi and these are summarised in Table 6.4. In all these studies, BMN and DN were examined as part of investigations of p53 expression in cutaneous melanoma. The study by Stretch *et al.* (1991) mentions analysis of 10 BMN but does not state how they were preserved. Neither of the studies by Lassam (1993) or McGregor (1993) detected p53 positivity in a total of 53 BMN examined. However, conflicting observations were made by Cristolfolini *et al.* (1993) and Akslen (1993). In the former study, 12 out of 75 BMN were observed to be immunopositive for p53, though the authors state that in these samples, less than 1% of cells of each section were immunopositive, with staining usually confined to the nucleus (Cristolfolini *et al.* 1993). These levels of positivity are similar to p53 levels in lesions observed in the present study, which were classified as immunonegative (median 0.5% of nuclei) using flow cytometry. At these low levels, isolated cellular p53 positivity is likely to represent physiological activity of wild-type p53, (i.e. a

damage-response) (Lane, 1992), or as part of events leading to differentiation (Kastan et al., 1991b).

Table 6.3. The association between p53 oncoprotein positivity and histological atypia. Figures represent % p53 positivity, parentheses show the number of sections exhibiting a given feature.

Histological	Negative	Focal	Widespread	'p' value
Feature	% (n)	% (n)	% (n)	(<i>'t'</i> test)
Melanocytic	4.2 (11)	_	14.2 (19)	0.15
proliferation				
Junctional	8.0 (21)		4.5 (9)	0.68
extension				
Fibrosis	Γ_	4.3 (16)	14.5 (12)	0.26
Capillary	6.5 (23)	_	9.4 (7)	0.88
proliferation				
Lymphocytic	8.0 (9)	7.0 (11)	0.6 (10)	0.56
infiltrate				
Bridging of	7.3 (8)	3.3 (7)	18.7 (15)	0.09
rete ridges				
Nuclear				
Atypia	6.5 (17)	6.8 (4)	14.7 (9)	0.44
Hyperchromasia				
	5.5 (8)	13.0 (6)	4.2 (16)	0.97
Prom. nucleoli				
	4.5 (9)	13.6 (10)	3.2 (11)	0.50
Nuclear				
enlargement				

In this study over half (53.3%), of DN examined using flow cytometry were immunopositive for p53 and 63% of sections examined using immunohistochemistry. The median p53 positivity varied significantly between BMN and DN (p = 0.002), but not between DN and paraffin-embedded primary melanomas. However, a considerably greater proportion of melanomas were immunopositive for p53 (78.4%)

compared with DN (53.3%). Taken alone, this observation would suggest that DN exhibit stabilisation of p53 at levels that may indicate pre-neoplastic change. However, this is a simplistic conclusion which might be misleading, as clinically very few (if any) lesions are observed to undergo malignant change (Mooi & Krausz, 1992b). Table 6.4 summarises the findings of several other studies which have examined p53 positivity in DN. Stretch *et al.* (1991) only examined three lesions and did not comment on the criteria used to diagnose naevi as dysplastic. Furthermore with so few specimens analysed, their conclusions are of limited significance and open to obvious sample error. McGregor et al. (1993) found 25% of the 12 DN they examined to be immunopositive, but again they failed to state the criteria of diagnosis. As in our study, this paper examined a reasonable number of BMN (27) and noted no positive sections, concluding that p53 immunopositivity was highly correlated with malignancy in melanocytic lesions. Lassam et al. (1993) were more thorough in examining 60 DN; 47 from Kraemer types A - C (patients with sporadic DN and nonfamilial melanomas) and 14 DN from high risk familial DN kindreds. They did not observe any immunopositive lesions in either group using both PAb1801 or DO-7, both of which recognise the wild-type and stabilised oncoprotein. However, the lack of p53 immunopositivity may be explained by the fact that sections were not microwaved for antigen retrieval.

Taken in the context of these other studies our findings raise two contentious issues. Firstly, they question the validity of p53 positivity as a marker of neoplastic change, as advocated by Hall *et al.* (1991). Secondly, they question whether the above studies were in fact comparing similar clinical and histological entities.

In the study by Akslen (1993), both paraffin-embedded BMN and DN were analysed for p53 oncoprotein expression using PAb1801 and PAb240. He reported 100% immunopositive staining in BMN and 82% of DN. This observation has been made to an equally limited extent in other benign tumour types, including those of the breast (Heyderman & Dagg, 1991) and other soft tissues (Chang *et al.*, 1991; Dei Toss *et al.*, 1993). Clearly these findings imply that whilst p53 immunopositivity may be of use as a marker of malignancy or its potential development in other tumour types, its application in this context to melanoma is premature and possibly incorrect. In some cases, as for BMN, the presence of detectable oncoprotein may be due to a physiological response and this is given support by the lack of correlation between the presence of these lesions and development of sporadic melanomas (Mooi & Krausz, 1992b). The positive p53 staining observed in DN may be attributed to degradation of the formalin-embedded epitope with time (Kawasaki *et al.*, 1992), though this seems unlikely as paraffin-embedded BMN should have been affected in the same manner.
Although it is speculative, the lack of clinical progression of these lesions in sporadic cases of DN may imply that p53 is overexpressed as part of other processes, including naevus differentiation and maturation, extensively described by Clark *et al.* (1984). The recognition that types and distribution of naevi vary considerably with age and development would suggest that several differentiation pathways and changes are involved, some of which may require specific p53-controlled regulation. Rotter (1993) has most elegantly shown that during spermatogenesis, p53 expression is primarily confined to populations of cells undergoing differentiation.

Dysplastic naevi were diagnosed in Lassam's (1993) study using the criteria of Clark and Elder (1984), which includes consideration of the two major criteria advised by the WHO consensus paper (Clemente et al., 1991). The importance of uniformity of diagnostic criteria becomes evident as it is possible that different studies diagnosed atypia and dysplasia in different ways and that different populations were grouped together or compared, to result in spurious data. Furthermore, this may have resulted in true BMN being classified as DN, producing a lower observed proportion of immunopositive lesions. Similarly, early melanomas may be grouped with atypical naevi. The Yale melanoma study (Titus-Ernstoff et al., 1988) examined the presence of DN in association with multiple primary melanomas, using very different criteria to those of Clark et al. (1984) or Clemente et al. (1991). These required the presence of two of the following features; nuclear enlargement, pleiomorphic nuclei, hyperchromatic nuclei and pale or dusty cytoplasm. The potential for variation in histological diagnostic criteria to result in confusion is further highlighted as the authors of this paper admitted that two of the lesions diagnosed as DN turned out to be early melanomas. However, even application of specific criteria for the diagnosis of these lesions does not ensure consistency and may result in inter-observer differences in diagnosis; a recent study has shown that a group of experienced pathologists within the same department found it difficult to agree on features of cytological atypia (Hastrup et al., 1994).

None of the above studies commented upon the relationship between the intensity or distribution of p53 staining and features of histological atypia. The small number of DN examined in this series showed distinctive trends that suggested a statistically significant relationship may have been identified in a larger group of specimens. In particular, bridging of rete ridges and the presence of melanocytic proliferation were particularly associated with increased p53 expression. Examination of histological sections displaying this property showed some variation in the p53 pattern; staining was usually confined to the basal layer of the epidermis, but in a few sections extended into the dermis in a non-specific manner. It would have been beneficial to

not only examine a greater number of these atypical naevi, but to also investigate p53 expression with a stable-protein specific antibody, such as PAb240, to elucidate the nature of the underlying process (Akslen [1993] found seven of eleven DN to be immunopositive using PAb240). The observation that a lymphocytic infiltrate is accompanied by a reduction in detected p53 levels may be explained by the fact that lymphocytes would be expected to be p53 immunonegative. This would have a diluting effect on naevomelanocytes assayed through the flow cytometer, which (without appropriate markers) is unable to distinguish cell types.

The limited discussion of particular features of atypia in the studies referred to above reflect the underlying fact that many of the features described as being important in the diagnosis of DN are, in fact, common to BMN. The distinction between BMN and melanoma is a well recognised area of difficulty in histopathology (Mooi & Krausz, 1992b) and the development or identification of a marker of malignancy would be of great benefit. Certain histological differences between BMN, DN and melanomas may be quantitative rather than qualitative. The association of atypical naevi with familial melanoma has demarcated these lesions as of potential neoplastic potential, but preemptive excision has precluded any reliable comment on their natural history (Mooi & Krausz, 1992b). Furthermore there is an increasing tide of opinion which is questioning any relevance of these naevi to both sporadic and familial melanoma, this view being most strongly promulgated and extensively reviewed by Ackerman (1985, 1988). Ackerman and Mihara (1985) proposed that dysplasia was an inappropriate term and that these naevi were very common in the general population (indeed, Ackerman wishes to rename them as 'common naevi'). Ackerman believes that cytological atypia is unnecessary in the diagnosis of clinically atypical lesions. His view has received both support and criticism; Murphy and Halpern (1990) concluded that "using architectural criteria alone, many acquired naevi will be erroneously judged to be dysplastic". Equally, others are just as fervent in their support of the concept of DN and the importance of cytological atypia (Elder 1985, Steijlen et al., 1988). Nevertheless there is sufficient corroborative data to support an argument for the existence of a specific sub-group of naevi which display clinical, architectural and cytological atypia and which, as in this series, have shown distinctive p53 expression when compared to BMN. The spectrum of histological appearance of atypical naevi increase the argument for greater study of these naevi at the cellular and molecular genetic level, for this may provide definitive answers to the neoplastic potential of these lesions and further definition of their histological characteristics.

Table 6.4. Summary of the findings from other studies which have investigated p53 oncoprotein expression in BMN and DN using immunohistochemistry. P-E; Paraffinembedded specimen

Study	Frozen/ Paraffin-emb	<i>p53</i> Antibody	No. Naevi examined	No. +ve sections (%)
BMN				
Stretch				
(1991)	?	PAb 240	10	0 (0)
McGregor				
(1993)	PE	CM-I	27	0 (0)
Cristolfolini		PAb1801,		
(1993)	PE	DO7, CM-1	75	12 (16)
Lassam		PAb1801,		
(1993)	PE	DO7	26	0 (0)
Akslen		PAb1801		
(1993)	PE	PAb240	10	10 (100)
DN				
Stretch				
(1991)	?	PAb240	3	0 (0)
McGregor				
(1993)	PE	CM-1	12	3 (25)
Lassam		PAb1801	47 (low risk)	0 (0)
(1993)	PE	D07	13 (high risk)	0 (0)
Akslen		PAb1801	11	9 (82)
(1993)	PE	PAb240		8 (73)

6.5. *c-myc* Oncoprotein Expression in Benign and Dysplastic Naevi

6.5.1. c-myc oncoprotein expression in benign naevi

Using flow cytometry, *c-myc* oncoprotein was detected in 21 of the 30 BMN analysed (70%), with a median protein positivity of 26.1% (0.0 - 94.7) (Fig 6.6). This was significantly less than the median oncoprotein level observed in paraffin-embedded primary melanomas (p = 0.002), but not from the overall *c-myc* positivity detected in ethanol-fixed tumours (p = 0.12).

6.5.2. c-myc oncoprotein expression in dysplastic naevi

Twenty of the 30 DN specimens analysed (66.6%) were immunopositive for the *c*-*myc* oncoprotein. The median positivity per naevus was 24.0%, with a range of 0.0 to 81.7% (Fig 6.7). This was significantly lower than that for paraffin-embedded primary melanomas (p = 0.008) and ethanol-fixed tumours (p = 0.039). However, it was similar to the level of oncoprotein positivity found in BMN (p = 0.6).

Attempts to examine the architectural distribution of *c-myc* oncoprotein using immunohistochemistry on paraffin-embedded sections were unsuccessful and impossible to assess. 'Immunopositive' sections displayed widespread, low-intensity background staining, devoid of cellular or tissue specificity.

6.5.3. c-myc expression and histological atypia

c-myc expression was considered in relation to the same set of major and minor histological criteria as investigated for p53.

Major criteria

1) Proliferation of melanocytes at the dermo-epidermal junction in a lentiginous pattern with or without nests

The relationship between histologically evident melanocyte proliferation and c-myc oncoprotein positivity is illustrated in Figure 6.8. A trend of borderline significance was observed suggesting widespread melanocyte proliferation was accompanied by

increased *c-myc* oncoprotein expression (p = 0.08). Median *c-myc* positivity in specimens with melanocyte proliferation was 30.9% (0.0 - 81.7) compared to 8.6% (0.0 - 61.2) in naevi without evidence of proliferation.

2) Junctional component extending more than three rete ridges beyond margins of the dermal component (shoulder phenomenon)

No association was found between *c-myc* positivity and the presence or absence of junctional extension of melanocytes (p = 0.75). The median positivity in specimens with and without shouldering were 23.8% and 24.2% respectively.

Minor criteria

1) Lamellar or eosinophilic fibrosis

As for p53, no difference was observed in *c*-myc content between DN with and without fibroplasia (p = 0.26). The median positivity in lesions with focal fibrosis was 15.3% (0.0 - 80.6) compared to 40.5% (4.3 - 81.7) in sections with diffuse fibrosis.

2) Capillary proliferation

No difference was found in *c-myc* positivity in the seven sections of DN with neovascularisation. DN with capillary proliferation had a median *c-myc* positivity of 22.0% (0.0 - 80.6) as opposed to 24.2% (0.0 - 81.7) in naevi where this feature was absent.

3) *Lymphocytic infiltrate*

In specimens without an inflammatory response, the median *c-myc* positivity was 23.8% (0.0 - 80.6), whilst that in lesions with a marked lymphocytic infiltrate was 30.9% (0.0 - 81.7). These values were very similar (p = 0.9).

4) Bridging of rete ridges

Bridging of the rete ridges was associated with a trend suggesting *c-myc* oncoprotein levels were elevated in naevi displaying this feature. *c-myc* positivity increased from 23.0% (0.0 - 78.6) in naevi without bridging to 49.2% (0.0 - 81.7%) in lesions with prominent rete fusion; but this failed to reach statistical significance (p = 0.17). However, as for *p53*, a significant difference was observed between *c-myc* oncoprotein levels in lesions with focal bridging compared to a more widespread distribution (p = 0.007).

Irregular shape and orientation of rete ridges was not found to correlate with *c-myc* positivity, though a definite trend was evident (p = 0.15). Similarly, absence of rete tips was not associated with variation in *c-myc* positivity (p = 0.41).

5) Nuclear atypia

No association was found between *c-myc* positivity and nuclear hyperchromasia (p = 0.95) or the presence of prominent nucleoli (p = 0.54). Nuclear and melanocytic enlargement were also independent of *c-myc* oncoprotein content (p = 0.97). Results are summarised in Table 6.5.

Table 6.5. Median *c-myc* positivity (%) in dysplastic naevi showing histological atypia. Parentheses show number of sections with a given feature.

Histological	Negative	Focal	Widespread	<i>'p'</i> value
Feature	% (n)	% (n)	% (n)	(' <i>t</i> ' test)
Melanocytic	8.6 (11)	-	30.9 (19)	0.08
proliferation				
Junctional	24.2 (21)	_	23.8 (9)	0.75
extension				
Fibrosis	_(2)	15.3 (16)	40.5 (12)	0.26
Capillary	24.2 (23)	-	22.0 (7)	0.51
proliferation				
Lymphocytic	23.8 (9)	22.3 (11)	30.9 (10)	0.90
infiltrate				
Bridging of	23.0 (8)	4.9 (7)	49.2 (15)	0.17
rete ridges				
Nuclear				
Atypia	22.6 (17)	14.5 (4)	29.6 (9)	0.95
Hyperchromasia				
	16.2 (8)	22.0 (6)	29.6 (16)	0.54
Prom. nucleoli				
	21.7 (9)	23.2 (10)	29.6 (11)	0.97
Nuclear				
enlargement				



Figure 6.6. *c-myc* oncoprotein expression in BMN, DN and primary melanomas, analysed using flow cytometry. Cross-hatched bars; median nuclear positivity (%). Open bars; proportion of immunopositive specimens for each type of lesion (%).



Figure 6.7.Flow cytometric distribution of *c-myc* positivity in DN.Dashed line - meanDotted line - median



Figure 6.8. Melanocyte proliferation and *c-myc* expression in dysplastic naevi.



Figure 6.9a & b. p53 vs *c-myc* expression in BMN (6.9a, upper graph) and DN (6.9b, lower graph). As observed in melanomas, high p53 expression was associated with *c-myc* overexpression.

6.6. Co-expression of *p53* and *c-myc* in Naevi

Benign melanocytic naevi

The association between p53 and c-myc expression in BMN is shown in Figure 6.9a. From this, no obvious relationship is apparent between p53 and c-myc expression, as observed in melanomas. In the few BMN expressing any p53 oncoprotein, the range of c-myc recorded is wide and non-specific.

Dysplastic naevi

Elevated p53 oncoprotein expression was only observed in DN where *c-myc* levels were also highly overexpressed (Fig 6.9b). Only five of the p53-positive DN exhibited positivity values in excess of 60%. In these five naevi, *c-myc* expression was considerably greater than the median value of 24% at 72.2% (21.8 - 80.8) compared with a median positivity of 22.0% (0.0 - 81.7) in the remaining naevi with p53 expression below this level. This difference was highly statistically significant (p= 0.002). The three DN with p53 expressed in over 80% of their nuclei all had particularly high *c-myc* positivities. However, as this figure also demonstrates, overexpression of *c-myc* occurred in the absence of a concomitant rise in p53.

6.7. Discussion

Using flow cytometry, expression of c-myc oncoprotein was found to be prevalent in the BMN and DN examined in this study. Almost 70% of each type of naevus was found to be immunopositive for the c-myc oncoprotein. The levels of oncoprotein found in each group were also similar to each other, but not to primary or metastatic melanomas in which both the proportion of immunopositive tumours and numbers of positive nuclei were higher. Taken in isolation, this observation would suggest that observed differences in clinical and histological atypia between BMN and DN are independent of this oncoprotein, but that a particular level of overexpression may be associated with transformation and melanoma progression.

Unlike the detection of stabilised p53 in non-malignant melanocytic lesions, the presence of *c-myc* oncoprotein is not contradictory to its putative role. *c-myc* is expressed in a wide range of both normal and malignant tissues (Littlewood & Evan, 1990) emphasising its importance in the control of cellular growth and proliferation (Evan & Littlewood, 1993) (see Section 1.10.). Overexpression of *c-myc* mRNA has been associated with the development of dysplastic changes in other tissues, including

prostate (Pylkkanen *et al.*, 1993) and colonic adenomata (Sharrard *et al.*, 1992), suggesting that increased cellular proliferation and deregulated differentiation may initiate or accompany architectural and cytological atypia (Urso *et al.*, 1992).

Consideration of the association between histological atypia and *c-myc* oncoprotein levels found a relationship of borderline significance between elevated oncoprotein expression and melanocyte proliferation (p = 0.09), which is consistent with the role of *c-myc* as an early growth response gene that acts as a cell cycle checkpoint (Evan & Littlewood, 1993). Oligonucleotides to c-myc mRNA inhibit proliferation of cells in culture and potentiate differentiation pathways (Bacon & Wickstrom, 1991). Elevated levels of the *c-myc* oncoprotein would be expected to be present in cells which were proliferating, but presumably not to the abnormally elevated levels seen in malignant melanomas. The similar median oncoprotein expression of *c-myc* positivity observed in both BMN and DN may suggest that excessive proliferation is not a feature of DN, though widespread melanocyte proliferation in DN was associated with elevated *c-myc* protein expression. Furthermore, measurements of LI in both these lesion types suggests DN proliferate at a level intermediate between BMN and melanomas (Urso et al., 1992) (see Chapter 7). In addition, the co-operative action of other growth response genes, such as *c-fos*, which have also been shown to be expressed in BMN must also be considered (Peris et al., 1991; Ogiso et al., 1988). It would be both interesting and worthwhile to assess intrinsic proliferation markers in these naevi, e.g. Ki67 or PCNA, to investigate whether elevated oncoprotein levels were indeed accompanied by increased melanocyte turnover (though proliferation cannot be considered in isolation and does not take cell death into account). Morphologically, BMN are well defined naevi, whereas DN show considerable variation in their pigmentation and border; the accompanying architectural disorder seen in DN may, in part, be due to excessive proliferation and variation in melanocyte differentiation. Equally, sporadic apoptosis of melanocytes may also partly account for the irregular appearance of these naevi, and merits further study. With the ubiquitous distribution of the *c*-myc gene, a significant proportion of assayed oncoprotein may also be derived from keratinocyte progenitor cells in the basal layer of the epidermis, which is proliferating much of the time.

No other significant correlation was found between *c-myc* oncoprotein expression and histological atypia, though a trend was observed associating bridging of rete ridges and elevated oncoprotein levels (p = 0.15). This was also associated with increased p53 levels and accordingly may have been the secondary effect of *c-myc-* induced stabilisation of p53; it is noteworthy that a similar relationship to that seen between p53 expression and *c-myc* positivity in melanoma was also observed in DN. High

p53 positivity was only seen to occur in lesions where *c-myc* expression was also relatively high. However, elevated *c-myc* oncoprotein expression was also observed in naevi where p53 levels were low, suggesting this was not simply a direct, interdependent artefact. Other than for BMN, an association has been observed between *c-myc* overexpression and elevated p53 oncoprotein levels in all the types of melanocytic lesions studied in this thesis and this is explored in more detail in the General Discussion (Chapter 8).

A very limited number of studies have investigated *c-myc* activity in BMN, though none appear to have examined this gene or its products in DN. Husain et al., (1990) observed c-myc mRNA levels to be very low in cultured melanocytes, but considerably higher (1-14x) in melanoma cell lines. Chevenix-Trench et al. (1990) reported *c-myc* mRNA to be undetectable in melanocyte cell lines, but present in melanoma lines. However, the authors of this paper comment that absence of *c-myc* mRNA in BMN was likely to be the result of inadequate RNA on the gel. Both these papers provide evidence to support the findings of this present study in that c-mvc production is overexpressed in melanoma compared to untransformed melanocytes. Peris et al. (1991) found up to three times higher levels of c-myc mRNA in the four BMN they examined compared to those in seven melanomas they analysed with northern blotting. c-myc mRNA was detected in all specimens, including normal skin, but levels were higher in naevi. The authors proposed that expression of *c-myc* was central to the growth and development of these naevi, with the implication that additional perturbation of other genes may result in transformation. These differing findings are not readily explainable other than to comment on the fact that the studies by Husain (1990) and Chevenix-Trench (1990) were performed on melanocyte cell lines, whilst Peris reported on mRNA levels in only four human BMN.

This present study did not explore changes in *c-myc* mRNA and accordingly it is impossible to comment on the relationship between mRNA levels and oncoprotein expression. Due to the complexities of post-translational modification and intracellular regulatory mechanisms there is little evidence to support a directly proportional relationship between gene expression, mRNA levels and the resultant oncoprotein produced, as was seen for p53 (Flørenes *et al.*, 1994). None of the studies discussed here reported on oncoprotein expression, and the association between activation of the *c-myc* gene and protein production requires further study.

There have been no reports of oncogene expression in cultured DN cells for they are difficult to identify, isolate and grow (Herlyn, 1993). Furthermore, they may only

represent a small portion of cells in a naevus, and are easily overgrown with common naevus cells.

As discussed, *c-myc* is able to transform cells in co-operation with *ras*, the *p-21* oncoprotein of which has been identified in both naevi and melanomas (Yasuda *et al.*, 1989). In the study by Yasuda *et al.* (1989), dermal, but not junctional naevi, were observed to stain immunopositively for *p-21 ras*. Furthermore, the intensity of staining was seen to increase with progressively thick primary melanomas. The potential for co-transformation between *ras*, *p53* and *c-myc* (Lu *et al.*, 1992) underlines the importance of investigating the relationship between these, and other, key oncogenes in an attempt to identify and classify truly pre-neoplastic naevi.

6.8. Summary to Chapter 6

This study has shown p53 oncoprotein expression occurs in over 50% of clinically atypical naevi but not in clinically or histologically benign naevi. p53 oncoprotein detected in DN was expressed at a similar level to that found in primary melanomas, but in a considerably smaller proportion of specimens. This confirms the findings of several other studies which have questioned the view that p53 immunopositivity is a marker of malignancy. *c-myc* oncoprotein was detected in the majority of both benign and dysplastic naevi, but at significantly lower levels than found in melanomas and in a smaller proportion of lesions. This would suggest that marked overexpression of cmyc is associated with development of the malignant phenotype, which requires the co-operative action of other oncogenes such as ras. Clarification of the relationship between naevi and melanoma will require further investigation of the cytogenetic and molecular genetic changes associated with the development of these lesions. Furthermore, these studies will need to be carried out on an adequate number of naevi that have been classified according to uniform clinical and histological criteria. It may then be possible to elucidate the interaction of certain key oncogenes in normal melanocytes and the ensuing changes that accompany progression from naevus to melanoma. This would have the additional benefit of providing further objective evidence to clinically and histologically distinguish types of naevi and classify those that may be potentially pre-neoplastic.

Chapter 7

Tumour Proliferation in Melanoma

7.1 Introduction

Proliferation has been extensively investigated in human cancer to increase understanding of tumour biology and in an effort to identify distinguishing features between normal and tumour cells that might be exploited therapeutically. Various methods have been developed to assay certain parameters of proliferation (outlined in Section 1.13.3), however, most of these yield static 'snapshot' measurements of cells in a particular cell cycle phase or at a particular time. In the past, the most widely used method involved the uptake of tritiated thymidine into cells, followed by detection using autoradiography. However, this method is laborious and the radioactive nature of the DNA precursor generally precludes it from use *in vivo*. The capability of flow cytometry to rapidly measure two parameters independently and simultaneously has made it a valuable tool with which to quantitate numbers of cells in particular phases of the cell cycle. The use of the thymidine analogue, 5-bromo-2-deoxyuridine (BrdUrd), to label cells in S phase, along with the development of a BrdUrd-specific antibody, resulted in a method capable of measuring *in vivo* tumour cell kinetics.

From analysis of the distribution of cell populations in given phases of the cell cycle, key proliferation indices may be calculated, as explained in Section 2.6. The labelling index (LI) represents the percentage of cells incorporating a DNA precursor, such as BrdUrd, which will be taken up by DNA-synthesising cells in S phase, at the time of injection. The duration of S phase (Ts) can be calculated from analysis of the bivariate distribution of BrdUrd uptake (measured on the y axis) against DNA content on the x axis at time intervals after injection of the DNA precursor. It represents the time taken to complete the DNA replication phase. From these parameters a further calculation can be made to estimate the potential doubling time (Tpot). Tpot represents the time taken by a cell population to double its number, assuming cell loss did not occur (see Section 2.6) but taking into account that some cells will be out of cycle.

Both p53 and *c-myc* are known to be intimately involved with the control of growth and proliferation in tumour cells. The association between *c-myc* positivity and disease outcome observed in this study may be explained, in part, by increased

tumour cell turnover at the expense of retardive differentiation processes. Therefore the aims of this section were to;

- Measure *in vivo* tumour proliferation, using BrdUrd, in both primary and metastatic melanomas, to establish and compare the range of kinetic parameters.
- 2) Investigate the association between clinical behaviour of melanoma and proliferation indices.
- 3) Determine if an association exists between *c-myc* and *p53* overexpression and proliferation. In particular, to investigate whether the mechanism of *c-myc* induced melanoma progression involves increased tumour cell turnover.

7.2 Materials and Methods

Chapter 5 described the investigation and findings of a study into *p53* and *c-myc* oncoprotein expression in paraffin-embedded primary and metastatic melanomas derived from 96 patients. The present investigation into proliferation was performed on the same patient group and tumours. Following informed and signed consent, BrdUrd was administered as an intravenous bolus of 200mg in saline to pulse-label all cells actively synthesising DNA in S phase. Tumours were excised 4 to 6 hours post-injection and a sample allocated for analysis by the pathologist. The remainder was preserved in 70% ethanol and stored at 4°C until processed. Tumour nuclei were labelled for BrdUrd, and proliferation indices, including Tpot, calculated as described in Section 2.6. Oncoproteins were assayed as described in Section 2.3.

7.3 Proliferation in Primary and Metastatic Melanoma

In this first section, the range of proliferation indices are presented and then examined in relation to the clinical behaviour of both primary and metastatic melanomas. Clinical details on the patient population studied in this series are described in Section 5.3.1. BrdUrd was administered to 96 patients, providing interpretable results in 79 (82.3%). Thirty of these were obtained from patients presenting with primary melanoma and 49 from patients with metastatic disease. In the remaining 17 specimens, analysis was prevented due to either excessive debris in the sample or inadequate tumour material.

7.3.1 Proliferation kinetics in primary melanoma

The median LI of primary melanomas yielding data in this series was 4% (1.3-18.0). The median Ts was 13.0 hours (4.4 - 25.7), with the median Tpot showing a greater range of distribution at 9.4 days (2.5 - 50.6). Cell kinetics varied considerably between thick and thin melanomas. Tumours were arbitrarily divided into those below 3.0mm thick (n =16, 'thin') and thick lesions in excess of 3.0mm (n = 14). The median LI of 2.9% (1.3 - 9.0) in thin lesions increased to 4.6% (1.4 - 18.0) in thick lesions but this trend was not statistically significant (p = 0.19). However, an association was found between tumour thickness and Ts and Tpot. The duration of Ts was significantly lower in thick lesions, at 10.2 hrs (4.4 - 24.8), compared to 16.4 hrs (7.2 - 25.7) in thin melanomas (p = 0.015). Similarly, Tpot was found to be significantly lower in thick melanomas compared to thin tumours (p = 0.027). The median Tpot in thin lesions was 12.5 days (6.8 - 50.6), decreasing to 6.9 days (2.5 - 27.0) in thick melanomas. These finding are summarised in Table 7.1.

Table 7.1. Tumour kinetics in primary melanoma and variation with tumour thickness. Values represent the median.

Primary	LI (%)	Ts (hrs)	Tpot (days)
Melanoma			
All primaries	4.0	13.0	9.4
Thin melanomas			
(< 3.0mm)	2.9	16.4	12.5
Thick melanomas			
(> 3.0mm)	4.6	10.2	6.9

Variation in proliferation with site of the primary melanoma is shown in Table 7.2. Measurements of the LI suggested that melanomas arising on the head and neck had a lower number of labelled cycling cells than those elsewhere on the body, but the difference was not statistically significant. However, the duration of S phase varied

considerably between tumour sites. Head and neck melanomas had a median Ts of 7.8 hrs (4.4 - 16.0), which was significantly shorter when compared to axial tumours (p = 0.046) and those arising on the lower limb (p = 0.008). An obvious trend was also present to suggest Tpot differed between sites, though this was not significant. Tpot in head and neck melanomas, at 8.2 days (2.9 - 24.9), was less than that observed in either those on the torso (p = 0.26) or limbs (p = 0.1). As can be seen from Table 7.2, proliferation indices were very similar in limb and axial melanomas.

Tumour Site	LI (%)	Ts (hrs)	Tpot (days)
Head and Neck	3.0	7.8	8.2
Torso	4.1	13.9	11.3
Limbs	4.3	14.3	9.0

Table 7.2. Variation in proliferation with site of the primary melanoma. Values represent the median.

7.3.2 Proliferation kinetics in metastatic melanoma

Data was obtained from 49 of the 53 metastatic melanomas analysed (92.5%). None of the calculated indices varied from those observed in primary melanomas. The overall median LI was 4.4% (0.6 - 16.0), which did not differ significantly from that observed in primary tumours (p = 0.43). The median Ts in secondary melanomas at 12.3 hrs (6.1 - 46.0) was also similar to that found in primary tumours (p = 0.61). The median Tpot of 9.6 days (2.3 - 13.9) was almost identical to that detected in primary tumours (p = 0.95).

Comparison of kinetics between skin/sub-cutaneous metastases and nodal secondaries implied that a larger population of cells were cycling in skin metastases and dividing more rapidly than those in nodal disease. The median LI of skin metastases was 5.4% (0.6 - 16.0), compared to 3.4% (0.9 - 15.1) in nodal secondaries (p = 0.22). Likewise, the median Tpot showed a trend suggesting Tpot was shorter in skin metastases. The median Tpot in skin metastases was 7.1 days (2.5 - 139.0),

compared to 13.1 days (2.3 - 84.2) in nodal metastases, but again this trend was not significant (p = 0.15). Ts did not vary significantly between sites of secondary disease (p = 0.42). Results are summarised in Table 7.3.

None of the kinetic parameters investigated in this study showed significant change in the progression from primary to metastatic disease. Comparison of the LI in all primary melanomas versus metastases showed little difference (p = 0.43), as was the case for the Ts (p = 0.61) and Tpot (p = 0.95) [Fig 7.1].

Site of	LI (%)	Ts (hrs)	Tpot (days)
Metastatic Disease			
All metastases			
	4.4	12.3	9.6
Nodal Mets.			
	3.4	12.9	13.1
Skin/Sub-cutaneous.			
Mets.	5.4	11.2	7.2

Table 7.3. Proliferation in metastatic melanoma. Values represent the median.

7.4 Tumour Proliferation and Clinical Behaviour

Study of cell kinetics in other tumour types has led to the concept that faster proliferating tumours may behave more aggressively. In order to investigate the relationship between proliferation and tumour behaviour, melanoma cell kinetics were examined in relation to a number of clinical parameters. Results for this section are summarised in Table 7.6.

7.4.1 Ploidy, proliferation and patient survival

Within this group of patients, a borderline, univariate, association was found between tumour ploidy and survival (Fig 7.2a), with diploid melanomas showing an improved prognosis compared to aneuploid tumours (p = 0.06, $\chi^2 = 3.5$). The LI (p = 0.0027, $\chi^2 = 8.98$) and Tpot (p = 0.01, $\chi^2 = 5.97$) were found to be markers of poor prognosis by univariate analysis alone (Fig 7.2b & c). No association was found

between Ts and survival. However, neither the LI nor Tpot were found to be independent prognostic variables using a multivariate model because both correlated strongly with Breslow thickness, and in particular, with each other. This might be expected as the LI is used to calculate Tpot.

7.4.2 Melanoma kinetics and the disease-free interval

The association between LI, Tpot and survival were reflected in their relationship with the disease-free interval (DFI). As noted in Section 5.5, the DFI was observed to be an independent prognostic variable in this group of patients. The median LI in patients with a shortened DFI was significantly elevated, at 8.4% (5.3 - 9.6), compared to 3.0% (2.5 - 4.6) in patients with a DFI below the median of 14 months (p = 0.004). There was also a borderline association between a reduced Tpot and a shortened DFI (p = 0.07). The Tpot in these melanomas was 5.9 days (2.5 - 9.0) as opposed to 12.5 days (6.6 - 18.3) in tumours from patients showing prolonged survival.

7.4.3 Proliferation and patient age

Age was not associated with any of the indices measured. The LI was similar in patients above and below the median age (p = 0.47), as was the duration of S phase (p = 0.78). Tpot was very similar in both groups (p = 0.92).

7.4.4 Proliferation and patient sex

The absence of any difference in survival between male and female patients in this group was reflected in the similarity of cell kinetics in tumours derived from each sex. The median LI in male patients was 4.1% (1.6 - 18.0), as opposed to 3.1% (1.3 - 9.0) in female patients (p = 0.55). Equally, both the Ts (p = 0.62) and Tpot (p = 0.44) were observed to be similar in male and female patients.



Figure 7.1.Proliferation kinetics in primary and metastatic melanoma.Primary (open bar), metastases (cross-hatch). Error bars, s.e.m.





Figure 7.2a. Tumour ploidy and patient survival. Upper curve - diploid melanomas (n = 44), lower curve - aneuploid (n = 35) [p = 0.06, $\chi 2 = 3.51$].

Figure 7.2b. LI and patient survival. Upper curve - below median LI (n = 46), lower curve - above median LI (n = 33) [p = 0.0027, $\chi 2 = 8.98$].

Figure 7.2c. Tpot and patient survival. Upper curve - below median Tpot (n = 37), lower curve - above median Tpot (n = 42) [p = 0.01, $\chi 2 = 5.97$]. Note, Tpot was not associated with tumour ploidy (see Section 7.4.6).

7.4.5 Tumour kinetics and clinical features of the primary melanoma

As noted in Section 7.4.1, proliferation indices suggested that cells from thick melanomas proliferated more rapidly than those in thin melanomas. A trend was evident to suggest that lentigo maligna melanomas proliferated more slowly than any of the other types of melanoma examined and values are shown in Table 7.4. The median Tpot of lentigo maligna melanomas (LM) was significantly longer than that measured in acral lentiginous melanomas (ALM) (p = 0.03) but only a trend in the LI was present between this melanoma (NM) (p = 0.1). Acral lentiginous melanomas (NM) (p = 0.16) and nodular melanomas (NM) (p = 0.12). Acral lentiginous melanomas were observed to be the most rapidly proliferating morphological subtype, possessing a significantly higher median LI than both LMs (p = 0.02) and SSMs (p = 0.03), but not NMs (p = 0.42). In keeping with the elevated LI's in this type of melanoma, ALMs also had the shortest median Tpot. However, of note, only four specimens of this type were analysed (three sub-ungual melanomas, one plantar tumour).

Morphology of	LI (%)	Ts (hrs)	Tpot (days)
primary			
melanoma			
Lentigo Maligna	2.2	12.1	18.7
n = 6			
Sup. Spreading	3.0	13.8	11.3
n = 20			
Nodular	5.2	10.2	7.9
n = 13			
Acral Lentiginous	6.3	13.1	6.6
n = 4			

Table 7.4. Proliferation and morphology of the primary melanoma

A shortened Ts and Tpot both correlated with increased mitotic figures, though the LI did not. The median Ts in melanomas with increased mitotic figures was considerably shorter, at 10.2 hrs (4.4 - 16.5), compared to tumours with sparse mitoses where the Ts was 16.8 hrs (7.2 - 25.7) (p = 0.05). Similarly the median Tpot was significantly faster (p = 0.05) at 9.0 days (2.9 - 25.7) in melanomas with increased mitotic figures,

compared to 11.1 days (4.6 - 50.6) in tumours with few mitoses. Increased mitotic figures were not associated with an elevated LI (p = 0.62).

There was an association of borderline significance between ulceration of the primary tumour and an increased LI, compared with tumours in which ulceration was absent (p = 0.08). The LI in ulcerated lesions was 4.8% (2.5 - 18) compared with 3.0% (1.3 - 9.0) in non-ulcerated tumours. However no such trend was observed between Ts and ulceration, in which estimations of Ts were very similar (p = 0.8). The Ts in non-ulcerated melanomas was 10.5 hrs (4.4 - 13.9), compared with 10.2 hrs (7.3 - 24.8) in ulcerated primaries. Ulcerated melanomas had a median Tpot of 8.4 days (4.2 - 11.3) as opposed to 10.4 days (2.9 - 50.6) measured in non-ulcerated melanomas, but again this was not of statistical significance (p = 0.13).

To exclude the effect of tumour thickness on cell kinetics, indices were compared in thick primary lesions alone. Again, no statistically significant relationships were obvious, though the trend suggesting that ulcerated melanomas had an elevated LI was maintained. Results are shown in Table 7.5.

Table 7.5. Cell kinetics in thick (> 3mm) ulcerated and non-ulcerated primary melanomas. Values represent the median.

Tumour Ulcertion	LI (%)	Ts (hrs)	Tpot (days)
Non-ulcerated melanomas	3.9	10.5	6.9
Ulcerated melanomas	5.3	10.2	7.9
<i>'p'</i> value (<i>'t'</i> test)	0.17	0.28	0.35

7.4.6 Proliferation and tumour ploidy

A significantly greater number of cells were observed to be cycling in an euploid melanomas compared to diploid tumours. The median LI in an euploid melanomas was 6.3% (0.9 - 18.0) compared with 3.1% (0.6 - 14.0) in diploid tumours (p = 0.017). Ts was also associated with tumour ploidy (p = 0.036), with an euploid tumours exhibiting a longer S phase duration than diploid melanomas. However, the potential doubling time was not associated with tumour ploidy (p = 0.38). Proliferation indices and clinical parameters are summarised in Table 7.6.

Table 7.6. Summary of median proliferation values and their association with clinicopathological parameters. 'p' values; 't' test.

Clinical Parameter	LI (%)	' <i>p</i> ' value	Ts (hrs)	<i>ʻp'</i> value	Tpot (days)	<i>ʻp'</i> value
Patient Age						
Below median	3.0		12.9		10.4	
Above median	4.6	0.47	13.1	0.78	8.6	0.92
Sex						
Male	4.1		13.8		8.6	
female	3.1	0.55	12.9	0.62	10.4	0.44
DFI						
Below median	8.4		12.3		5.9	
Above median	3.0	0.004	11.5	0.76	12.5	0.07
Ploidy						
Diploid	3.1		11.1		10.9	
Aneuploid	6.3	0.17	14.1	0.036	7.3	0.38
Ulceration						
+ve	3.0		13.8		10.4	
-ve	4.8	0.08	12.1	0.80	8.4	0.13
Mitoses						
+ve	3.2		16.8		11.1	
-ve	4.1	0.62	10.2	0.05	9.0	0.05

7.5 Melanoma Proliferation and Expression of *p53* Oncoprotein

The association between p53 oncoprotein expression and proliferation indices is shown in Figure 7.3. Although Figure 7.3a suggests an inverse relationship between p53 positivity and the LI, oncoprotein levels were similar in melanomas with a labelling index above and below the median value (p = 0.55). Melanomas with an LI below the median of 4.1% (0.6 - 18.0) had a median p53 oncoprotein positivity of 10.8% (0.0 - 85.3), whilst tumours with an LI above the median had a p53 positivity of 14.1% (0 - 85.3). Equally, although a trend suggested an association between elevated p53 expression and the duration of S phase, this did not prove significant (p = 0.15) [Fig 7.3b]. A borderline association was found between p53 expression and Tpot (p = 0.06). Figure 7.3c illustrates a trend in which the Tpot reduced with increasing p53 expression. The median p53 oncoprotein positivity of melanomas with a shorter Tpot (below median) was 15.6% (4.6 - 80.2) compared with 10.6% (0.0 - 60.3) in tumours with a prolonged doubling time.

7.6 Melanoma Proliferation and Expression of *c-myc* Oncoprotein

Overexpression of *c-myc* was found to be associated with rapid proliferation as measured by all three indices. Figure 7.4a illustrates a trend indicating that proliferation rate increased with *c-myc* overexpression, though this was more evident in metastatic melanomas. The LI was observed to show a borderline association with *c-myc* positivity (p = 0.07). The median *c-myc* expression in melanomas with an elevated LI was 77.0% (0.0 - 96.5), whilst tumours exhibiting a lower LI had a median *c-myc* oncoprotein positivity of 66.5% (0.9 - 94.8). Equally, elevated *c-myc* expression was found to accompany a shortened Ts (p = 0.06), showing an inverse relationship between *c-myc* oncoprotein expression and the duration of S phase (Fig 7.4b). Melanomas with a short Ts (below the median value) had a *c-myc* positivity of 78.3% (8.4 - 96.5) compared with 64.2% (0.0 - 96.1) in tumours with a longer S phase.

Tpot was significantly associated with *c-myc* expression (p = 0.002). As can be seen from Figure 7.4c, high *c-myc* expression was associated with a shortened Tpot, and consequently a more rapid cell turnover. The *c-myc* positivity in melanomas with a low Tpot was significantly higher at 72.3% (7.6 - 96.5), than that found in tumours with a prolonged doubling time in which the median oncoprotein positivity was only 43.3% (0.9 - 88.7). The association between *p53* and *c-myc* oncoprotein expression, and cell kinetics are summarised in Table 7.7.



Figure 7.3a, b & c.

Proliferation Kinetics and expression of *p53* protein.
(a) LI, (b) Ts, (c) Tpot - versus *p53* positivity.
Primary melanoma (squares), metastases (circles).



Figure 7.4a, b & c.Proliferation Kinetics and expression of c-myc protein.(a) LI, (b) Ts, (c) Tpot - versus c-myc positivity.Primary melanoma (squares), metastases (circles).

Kinetic Parameter	p53 positivity (%)	' <i>p</i> ' value	c-myc positivity (%)	'p' value
LI				
Below median	10.8		77.0	
Above median	14.1	0.55	66.5	0.07
Ts				
Below median	13.0		78.3	
Above median	16.1	0.15	64.2	0.06
Tpot				
Below median	15.6		72.3	
Above median	10.6	0.06	43.3	0.002

Table 7.7. Oncoprotein expression and cell kinetic parameters in primary and metastatic melanoma. 'p' value; 't' test.

7.7 Discussion

Considerable efforts are being made to investigate the processes central to the cellular proliferation of human tumours and their regulation, for it is becoming increasingly evident that perturbation of these pathways may lie at the core of tumour genesis. Furthermore, the information provided from these studies may have both therapeutic and prognostic relevance. Tumour growth is dependent on three main variables:- (1) the proportion of cells cycling at any one moment (the growth fraction), (2) the duration of the cell cycle, and (3) the proportion of cells which take no further part in division (the cell loss factor). The potential doubling time, Tpot, was proposed by Steel (1977) as a theoretical measure of proliferation in the absence of cell loss. Prior to the application of flow cytometry, and the introduction of BrdUrd to the study of cell kinetics, measurement of these parameters was both difficult and tedious, requiring the use of radioactive precursors and a number of pre-requisite biopsies to construct curves for pulse label mitosis analysis (Newburger & Weinstein, 1980). Furthermore, it took a number of weeks before results became available.

Our understanding of tumour cell kinetics has been substantially increased by the use of flow cytometry and BrdUrd incorporation (Begg *et al.*, 1985; Wilson *et al.*, 1985; Wilson, 1991). Assessment of LI and the duration of the S phase can be made from a single observation using a method that is both acceptable to the patient and rapid to analyse and calculate. From these two parameters the Tpot can be calculated and provides a superior and more complete index of proliferative activity than either the LI or Ts (Wilson, 1993). The objective of the experiments presented in this chapter were to measure these proliferative parameters *in vivo* in patients with melanoma and establish whether any association existed between them and clinical behaviour. In this respect, several observations of note were made, all of which suggest that aggressive tumour behaviour in melanoma is associated with increased cellular proliferation.

Thicker melanomas were observed to contain cell populations with a more rapid turnover, as reflected by the shorter Ts and Tpot compared to thin melanomas. Similar findings have been suggested from the few studies of cell kinetics in melanoma (Muggia & DeVita, 1972; Costa et al., 1987). These studies have used tritiated thymidine/autoradiography or immunohistochemistry to measure proliferation by either calculation of the LI, or a proliferative index using intrinsic markers such as Ki-67 (Kaudewitz et al., 1989). The estimates of the LI made in this present study of primary melanomas using BrdUrd are consistent with estimates made in several other studies, all of which have used tritiated thymidine labelling. In these studies, estimates of the LI have varied from between 5.1% and 23% (Newburger & Weinstein, 1980). This variation is entirely within the limits of biological variability of this parameter and discrepancies may be further compounded by methodological differences. Shirakawa et al. (1970) and Muggia & Devita (1972) both made similar estimates of the LI in melanoma, of 5.1% and 8% respectively. Costa et al. (1987) investigated the role of cell kinetics as a prognostic tool in melanoma, using tritiated thymidine to measure the LI. The median LI in the few primary tumours analysed gave a median value of 4.2%, very similar to that estimated in our set of patients. They noted a two-fold increase in the LI of thick melanomas compared to primaries, however as in our study, the wide range of values obscured any statistical significance. Kaudewitz et al. (1989) measured the growth fraction of 72 primary melanomas by assessing immunohistochemical staining of the nuclear proliferationassociated antigen, Ki-67. They found a positive correlation between the growth fraction and both tumour thickness and mitotic figures.

Costa *et al.* (1987) found nodular melanomas to have a greater LI than superficial spreading melanomas. In our study, acral lentiginous melanomas had the highest median LI and the shortest Tpot. Nodular melanomas were also observed to have a

considerably higher LI and Tpot than superficial spreading melanomas, though the broad range of values precluded statistical significance. Nevertheless, a clear trend was evident to show that the LI and Tpot all varied with tumour thickness and morphology. Lentigo maligna melanomas were observed to have a considerably longer natural history than other morphological types. The Tpot for this type of lesion was considerably longer than the more aggressive nodular and acral lentiginous tumours. The slow prelude in development of LMs prior to initiation of a more rapid vertical growth phase gives support to the suggestion that invasive tumour behaviour may follow further cytogenetic instability, producing tumour cells capable of increased proliferation and progressive independence from external inhibitory growth factors (Weinert & Lydall, 1993). As a group, tumours arising on the head and neck region had proliferation indices that were not dissimilar from melanomas elsewhere. This may partly be explained by the fact that this group included a mixture of both relatively slow proliferating LMs and thicker primary tumours and metastases. The difference in clinical behaviour of melanomas with similar proliferation indices is noteworthy for it emphasises that calculation of kinetic parameters cannot account for cell loss. Thus tumours with a low Tpot, indicating rapid proliferation, may clinically still grow slowly as a result of cell necrosis or apoptosis.

Acral lentiginous melanomas are usually associated with a worse prognosis than other morphological types of melanoma due to their relatively late presentation at a thicker stage. Sub-ungual melanomas are particularly associated with poor outcome, even when matched for thickness with cutaneous melanomas (Hiles, 1990). This has been partly attributed to the vascularity of the nail bed in which this form of melanoma arises, though it may also reflect specific biological characteristics of the tumour type. However, due to the limited numbers of acral lentiginous lesions analysed in this study it would be speculative to interpret any more from these observations.

Ulcerated primary melanomas were discovered to have an increased LI and a reduced Tpot compared to non-ulcerated melanomas. This may partly be explained by the fact that in the group of melanomas studied, ulcerated melanomas tended to be thicker than non-ulcerated lesions. However, when tumours were matched for thickness, no statistical difference was found between any of the kinetics parameters. Ulceration of a primary melanoma may be expected to be the result of its biological properties; proliferating melanoma cells may either outgrow their nutrient blood supply to produce superficial necrosis or, because of cell turnover, be particularly sensitive to hypoxia. Equally, rapid proliferation could result in the accumulation of genetically compromised cells with reduced survival capabilities.

Both the Ts and Tpot were associated with increased mitotic figures, though the LI was not. Assessment of the numbers of mitotic figures is open to considerable observer variation (Larson *et al.*, 1980) and can be complicated by the presence of abnormal mitoses due to genetic abnormalities. This would interfere with the relationship that should exist between LI and mitotic index (MI). Furthermore, and of significance, it has been observed that up to 15% of metastasising thin melanomas (< 1.5mm) do not have any histological evidence of mitoses, limiting the assessment of mitotic figures in exactly the group of melanomas that additional prognostic information may be most of benefit (Kuenhnl-Petzoldt *et al.*, 1984). This might arise as a result of rapid cell turnover, as mitotic cells, which are found in the shortest cell cycle phase (typically 30 minutes to one hour) might be difficult to identify because of their small numbers.

This highlights the problem of static cell cycle measurements, such as LI or MI. It is entirely conceivable that low LIs or MIs may not necessarily mean that the tumour population is slowly proliferating. Without the knowledge of the Ts or duration of mitosis (Tm), it is possible that the cell turnover is rapid because of a shorter cell cycle phase duration. Indeed, from basic cell kinetic principles the number of cells present in any cell cycle phase is dependent on the length of that phase relative to the cell cycle time and, of course, the growth fraction. Therefore, a short Ts or Tm would be associated with a low LI or MI. However, the population doubling time depends on both the number of cells in a particular cell cycle phase and the duration of that phase. This means that a tumour with a low LI or MI could have the same Tpot as a tumour with a high LI or MI because it's Ts or Tm is faster.

The present study did not find any correlation between melanoma cell kinetics and clinical parameters, such as the age of the patient or their sex, and is in agreement with the findings of the other studies referred to above. None of the indices studied were found to act as independent prognostic variables on multivariate analysis, though the univariate association between Tpot and survival and LI and survival could be explained by their correlation with each other and the Breslow thickness. Both the LI and Tpot showed significant associations with the DFI, though this may be partially explained by the relationship between these variables and tumour thickness.

Cell kinetics in metastatic melanomas were very similar to those observed in primary tumours. This similarity is not unexpected as presumably other processes would be invoked and of greater importance in order to facilitate metastasis, such as modulation of surface immunogens and adhesion molecules (Hart & Easty, 1991). Costa *et al.* (1987) did not observe such a trend, though in their study, the overall LI of

metastases was significantly greater than in primary melanomas. A distinct trend suggested that skin secondaries, as reflected by the LI and Tpot, proliferated more rapidly than nodal deposits, though this was not statistically significant.

Despite differences in the LI and Ts, aneuploid tumours did not proliferate more rapidly than diploid melanomas (Tpot, p = 0.38). This was because aneuploid tumours had a longer Ts than diploid tumours, which would counteract the differences in LI on the overall calculation of Tpot. The findings of a similar study, performed on squamous cell cancers of the head and neck, suggested that no systematic difference in cell kinetics were detectable between diploid and aneuploid tumours (Bennett *et al.*, 1992). Other studies at the Gray laboratory have found diploid lung and oesophageal carcinomas to also proliferate as rapidly as their aneuploid counterparts (Bennett *et al.*, 1992). Furthermore, there is little direct evidence in the literature to support a relationship between tumour ploidy and proliferative activity. Indirect evidence has come from correlating proliferation indices with clinical parameters, but tumour behaviour cannot be attributed to this variable alone.

The major limitation of flow cytometry is that in preparing a nuclear or single cell suspension, tissue architecture is lost and with it any information on the spatial distribution of the cells of interest. This problem may be compounded by the potential for cell selection, which would yield inaccurate estimations of the DNA index as well as assays of proliferation. Using flow cytometry to calculate the DNA index, 45% of the primary melanomas studied in this group were found to be aneuploid. A similar study of DNA indices in 53 paraffin-embedded melanomas found a similar proportion (41%), observing the incidence to increase with tumour thickness (Von Roenn *et al.*, 1986). In addition, aneuploid melanomas showed an increased recurrence rate at two years follow-up. Similar findings were made in a larger study of 177 stage one melanomas, though in addition this study also noted an association between tumour ploidy and ulceration (Kheir *et al.*, 1987).

Following intravenous infusion, uptake of BrdUrd into tumour cells is dependent on a number of physical and pharmacokinetic factors, including tissue vascularity and diffusion. Alone or in tandem, these processes will affect labelling of cycling cells and overall estimates of kinetic indices. In view of this the significance of cell kinetics as a prognostic assay has been questioned due to observed intra-tumour heterogeneity, evident in multiple biopsies of colorectal and breast carcinomas, (Rew *et al.*, 1991; 1992). This has additionally been attributed to the size of these tumours at the time of excision and areas of necrosis. Due to the limited amount of tumour material available, particularly in the case of primary melanomas, multiple biopsies were only

performed in three tumours. In these, little variation was noted in indices. In contrast to colorectal and breast cancers, this may reflect the relatively small size of the lesions. However, kinetics in larger necrotic metastases might be expected to be less consistent in their values.

Study of the association between expression of the p53 and c-myc oncoproteins and cell kinetics in melanoma revealed a number of features that indicate the potential mode of action of these proteins and the effects of their perturbation in melanoma. Although no association was found between the p53 positivity and the LI and Ts, a borderline correlation was observed between oncoprotein overexpression and a shortened Tpot. As mentioned in the introduction, wild-type (wt) p53 is known to act as a cell cycle checkpoint at the G1-S phase transition, usually in response to DNA damage. wt p53 can also induce cell cycle arrest in response to depleted nucleotide precursors prior to replication. γ -irradiation of mammalian bone marrow cell lines produces DNA damage which results in G1 arrest. However, arrest does not occur in cells that are p53 null (Kastan *et al.*, 1991), but can be restored following transfection of the gene (Kuerbitz *et al.*, 1992). The loss of this protective function is being increasingly appreciated as a key step in the loss of genomic stability, resulting in accumulation and accruing of further gene mutations (Lane, 1992).

The actions of p53 noted above are mediated by its role as a transcriptional transactivator, suppressing proliferation by the induction of other specific regulatory genes. A basic hypothesis might therefore propose that the wt p53 oncoprotein limits cell proliferation by inhibiting expression of genes that promote cell cycle progression. Loss of p53-regulated cell cycle arrest may simplistically be envisaged to result in tumour cell populations proliferating more rapidly. However, presence of a stabilised, ineffective, oncoprotein may contribute to increased proliferation by other routes. p53 has been shown to participate in regulating differentiation in certain cell types, including pre-B cell lines and in spermatogenesis (Shaulsky *et al.*, 1991; Rotter, 1993). Cells committed to differentiation have, as a rule, down-regulated activity of proliferative genes; similarly proliferation is usually accompanied by down regulation of differentiation pathways (Hart & Easty, 1991). Consequently, inactivation of normal p53 function may also indirectly promote proliferation by incapacitation pathways.

Experiments with temperature-sensitive p53 mutant M1 myeloid leukaemia cell lines have suggested an additional route by which p53 may promote proliferation (Yonish-Rouach *et al.*, 1991). In this cell line wtp53 induces IL-6 dependent apoptosis which implies that, in certain cell populations, loss of this control may allow increased numbers of deregulated cells to undergo division. This and other work has suggested that in certain cell types, *p53* and other regulatory genes may act by controlling cell viability by altering their sensitivity to specific survival factors (Yonish-Rouach *et al.*, 1993).

A further potential mechanism by which abrogation of p53 function may contribute towards proliferation is suggested by the central role p53 plays in maintaining genomic conservation and karyotypic structure. Transformed cells often show aneuploid karyotypes with amplification of certain genes. The loss of wt p53 from cells *in vitro* has been observed to result in cells with an increased aptitude to develop karyotypic abnormalities, including gene amplification (Livingstone *et al.*, 1992; Yin *et al.*, 1992). This has been observed in fibroblasts from Li-Fraumeni patients with two mutant p53 alleles in which certain marker genes, such as CAD (a three enzyme complex, carbamoyl phosphotase synthetase, aspartate transcarbamylase and dihydroortase) underwent amplification (Yin *et al.*, 1992) restitution of wt p53function in these cells prevents further amplification. The actual mechanism of gene amplification is poorly understood, but recombination of chromosomal segments may produce arrays of amplified genes (Smith *et al.*, 1990).

In the appropriate conditions (i.e. with the additional loss of p53-mediated G1 arrest or apoptosis), it is conceivable that amplification of specific oncogenes intrinsic to cell proliferation, such as *c-myc*, may upset regulatory control processes and have an excessively stimulant action on the cell cycle. p53 may prevent such translocations and inhibit division of cells that have undergone genomic alterations. However, these two functions are not interdependent, for amplification does not automatically follow loss of G1 arrest. Therefore, it would appear that inactivation of the wtp53oncoprotein may result in a number of processes, each of which co-operate to facilitate transformation and increase cell proliferation.

This study has detected the stabilised, and therefore partially or wholly inactive, p53 oncoprotein to be present in the great majority of both primary and metastatic melanomas analysed. This raises the simple question as to why should p53 be such a frequent target in transformed cells? Part of the answer must lie in the importance of its function to normal cells and specifically the central role the wtp53 oncoprotein occupies in regulation of the G1-S checkpoint. However, deregulation may also involve mechanisms within the cell cycle. As a result, elucidation of the exact machinery of the cell cycle is becoming of increasing importance in order to understand proliferation control and tumourigenesis.

The cell cycle is run by specific proteins known as cyclins, eight of which have been identified in mammalian cells to date. These proteins form the interface between external regulatory factor, such as p53, and enzymes which drive the cell cycle itself, known as cell-dependent kinases (Cdks). Recently a direct connection has been made between p53 and certain cyclins which may explain its restraining action on the cell cycle. Cdk-2 is an enzyme which binds to G1-cyclins to activate cells through the 'start' phase of G1, following which cells are committed to divide. Independent studies simultaneously identified a gene that encoded a 21kd protein which binds to Cdk-2 to block its action. This gene was named by its various discoverers as either WAF1 (wild-type p53-activated fragment 1) [El-Deiry et al., 1993] or Cip1 (Cdkinteracting protein 1) [Harper et al., 1993]. Transfection of the WAF1/Cip1 gene alone into transformed cells suppressed cell growth in a similar fashion to p53, emphasising its potential role as an intermediary in p53 activity. Significantly this protein has been found to be absent in several cancer cell lines (Marx, 1993), raising the possibility that cells may transform in the presence of a normal wt p53 gene compliment. Furthermore, unpublished work from the Memorial Sloane-Kettering has identified such a scenario in melanoma cells (A. Albino, personal communication).

Work such as this indicates that inactivation of p53 may produce a block midway through processes that regulate the cell cycle. The even more intriguing corollary of this is that abnormalities may occur in steps downwind of oncogene-related control, to also precipitate deregulated proliferation. In support of this, cyclin D1, one of the eight Cdk-regulatory proteins, has been shown to be overexpressed in both benign parathyroid tumours and malignancies (Marx, 1994). Further work found the gene encoding cyclin D1 to be the oncogene *bcl1*. This gene is overexpressed in approximately one third of oesophageal cancers and 15% of breast carcinomas (Hinds, 1994). This connection between the multi-gene steps involved in tumour aetiology and the complexities of proliferation control have been further highlighted by the discovery that the cyclin D gene can co-transform cells *in vitro* with the *ras* oncogene. Other cyclins (D2 and D3) have been shown to limit differentiation, an additional requisite of malignant change (Sherr, 1993).

Increasing efforts are underway to illuminate the action of a number of other cyclins, such as cyclin E, which is overexpressed in breast cancer (Keyomarsi, 1994) and abnormalities of the Cdks as well. This has raised the further question: what are the substrate targets of the Cdks? The retinoblastoma gene (Rb) was the first gene discovered to have a tumour-suppressive effect, but significantly its protein product has also been identified as a target for Cdks. The Rb gene acts by binding to and

inhibiting a transcription factor that induces other gene-directed cell growth. The inhibitory action of the Rb oncoprotein can be prevented by activation of Cdks. Cyclins D and E, and their associated Cdks, have also been shown to be capable of undermining the growth suppressive action of the Rb gene (Marx, 1994) and it would seem reasonable to assume that other suppressor genes, such as p53, may be overridden by similar mechanisms.

Increased *c-myc* oncoprotein levels were shown to act as an independent prognostic variable in this group of patients and be closely associated with a number of important clinical and pathological parameters associated with poor outcome, including thickness of the primary tumour and the DFI. *c-myc* has been recognised to be intimately involved in regulation of the cell cycle and other processes which may modulate tissue homeostasis. The deregulation of this oncogene, principally by gene amplification, has been recognised to play a potentially important role in the genesis and progression of a number of human cancers (Section 4.10). As outlined in Section 1.10, this may stimulate cell proliferative processes, a reduction in differentiation pathways and induction of apoptosis.

Co-analysis of c-myc oncoprotein and cell kinetics in the group of patients with melanoma presented in the present study represented a novel attempt at elucidating the putative relationship between c-myc and one of these pathways. Overexpression of the c-myc oncoprotein was found to be closely associated with all three proliferation indices, and in turn each of these showed close association with the two major clinical prognostic indicators, Breslow thickness and the DFI. The relationship between clinical parameters and melanoma cell kinetics would suggest that increased cell proliferation may be one of the routes by which c-myc potentiates malignant behaviour in this tumour. This would also possibly explain the relationship observed between c-myc overexpression and tumour thickness and that between Tpot and tumour thickness. As noted above, several other studies have observed an association between primary tumour thickness and the LI.

c-myc overexpression was noted to be more prevalent in nodular melanomas than lentigo maligna melanomas. Similarly the latter were observed to have a considerably shorter median Tpot than nodular lesions. In the first instance this may be partly an effect of tumour thickness. However, the relatively prolonged Tpot observed in lentigo maligna melanomas is consistent with their natural history, and also with the relatively low *c-myc* oncoprotein expression measured within them.
Ulceration and the presence of increased mitotic figures in primary melanomas were both found to be associated with *c-myc* overexpression and displayed trends in their kinetics to indicate that these tumours were also proliferating more rapidly. The LI showed a borderline association with ulceration, whilst both Ts and the Tpot were significantly associated with increased mitotic figures. The lack of correlation with the LI is difficult to explain in the light of these findings, though it underlines the fact that mitotic figures may not be entirely representative of cell proliferation and their assessment may be subject to observer bias and abnormalities in the cell division process.

These observations are consistent with the proposed role of c-myc in regulation of cell turnover, described in Section 1.10.4. One of the effects of c-myc induction is to increase the proportion of cells in the G1 population by recruitment from the resting, G0 population. Expression of c-myc stimulates quiescent fibroblasts to proliferate (Eilers *et al.*, 1991). Crucially, antisense oligonucleotides to c-myc mRNA do not prevent transition from G0 to G1, but do so in G1 (Heikkila *et al.*, 1987). Extrinsic c-myc oncoprotein will relieve this inhibition, allowing continued progression through the cell cycle. One of the key features of malignant cells in culture is a lack of dependence on external growth factors. These agents stimulate proliferation by induction of early growth medium of normal cells results in a rapid down-regulation of c-myc expression and cessation of the cell cycle in G1. Overexpression of c-myc oncoprotein in melanoma may be envisaged to occur without the influence of extrinsic growth factors and can provoke cell proliferation by both increasing the proliferating population of cells and maintaining cell cycle checkpoints in an open mode.

In addition, as discussed in Section 1.10.4, *c-myc* plays an important role in selecting cells for apoptosis. Cells undergoing transformation will show a survival advantage if proliferative mechanisms remain in the 'on' position and de-selective pathways, such as apoptosis, are inactivated. This might have the cumulative effect of selecting clones of tumourigenic cells that are proliferating rapidly in an uncontrolled fashion, and which may behave more aggressively.

These mechanisms may explain the close association observed between the LI and *c*-*myc* positivity, for increased oncoprotein activity would be expected to result in an increased number of cells taking up BrdUrd in S phase. As discussed in Section 1.10.4. of the introduction, *c-myc* acts as an early growth-response gene, regulating passage of cells at both the G1 and S-G2 transition (Shibuya *et al.*, 1992), raising the possibility that *c-myc* may be both cell and tissue-specific with regard to the type of

cell cycle control involved. Overexpression may also reduce differentiation processes, further increasing the number of cycling cells and the LI. The accompanying deregulation of cell cycle checkpoints would explain the association between overexpression of the *c-myc* oncoprotein and a reduction in the duration of S phase (Ts). Both these effects would contribute to the close correlation found between Tpot and *c-myc* overexpression. In the General Discussion (Chapter 8), it is proposed that the mode of action of the *c-myc* oncoprotein may be mediated by a number of other routes, including stabilisation of the *p53* protein.

One limitation to the understanding of a central role of *c-myc* in cell proliferation in general has been the observation that once the cell commences division, gene expression remains at a steady state level. This implies that regulation does not involve modulation of the oncoprotein level, though little is known of the other genes induced by *c-myc* as part of its role as a transcriptional transactivator. It would be of considerable interest and value to be able to identify what other genes are regulated by *c-myc* and how their protein products interact with the cell cycle control checkpoints.

7.8 Summary to Chapter 7

Analysis of cell kinetics in primary and metastatic melanomas suggests that clinicopathological behaviour may be associated with variation in the rate of cell proliferation. Thick primary melanomas, known to have a worse prognosis, were observed to possess a shorter Tpot than thin melanomas, and this association was further reflected in the DFI. Similarly, consideration of the relationship between tumour kinetics and primary morphology also confirmed that certain types, such as ALMs proliferated more rapidly than SSMs or LMs, even when corrections were made for tumour thickness.

There would appear to be many potential routes by which deregulation of the cell cycle may be brought about and cell proliferation increased. Mechanisms may involve several factors extrinsic to the cell cycle machinery, including the p53 and c-myc oncogenes, or intrinsic proteins such as the cyclins and Cdks. The results of the investigation into melanoma kinetics and p53 expression confirm the complexity of this picture; deregulation of p53 function may explain part of the increase in proliferation seen in melanoma, but the extent of its influence in this regard may vary from cell to cell as proliferation remains a biological variable susceptible to a number of extra and intra-cellular factors. Overexpression of the c-myc oncoprotein appears

to be intimately associated with increased proliferation in both primary and metastatic melanoma. Furthermore, this relationship may partially explain the mechanism by which c-myc influences the clinical and pathological behaviour of these tumours.

Chapter 8

General Discussion

8.1 *p53*: Cause or Epiphenomenon?

8.1.1 Introduction

The p53 gene has become the focus of cancer research world-wide due to the increasing awareness that mutation of this gene represents the most common gene abnormality identified in human cancers to date (Harris *et al.*, 1993). This study attempted to evaluate the role that this gene may play in melanoma biology by assaying the expression of its protein product. As outlined in Section 1.12., mutation of the gene alters the conformation of its encoded oncoprotein to not only modify or inhibit normal function, but, as a consequence, to make the oncoprotein detectable using appropriate antibodies. As part of this study, surgical excision specimens were assayed for p53 oncoprotein using flow cytometry to provide a rapid and objective method of detecting immunopositive nuclei. In the two studies described in this thesis, presented in Chapters 4 and 5, p53 oncoprotein was detected in approximately 80% of melanomas analysed, but benign naevi were immunonegative. Initially this observation confirmed those of several other studies (Bartek *et al.*, 1991; Stretch *et al.*, 1993) supporting the impression that p53 overexpression was so prevalent as to infer a key role in melanoma biology.

The validation studies, described in Chapter 3, found the great majority of detectable p53 oncoprotein to be present in a stable 'mutant' conformation, as evidenced by the close correlation of staining between PAb1801 and PAb240. In addition, the low proportion of wtp53 oncoprotein identified by PAb246 would suggest that only a limited proportion of the PAb1801-detected protein was produced as a protective response. Detection of p53 protein using 'mutant' specific antibodies, such as PAb240, implied that the conformational change was secondary to gene mutation. Immunodetection of p53 oncoprotein in tumours using PAb240 has been employed as a basic screening mechanism prior to DNA sequencing in a number of reports (reviewed by Greenblatt *et al.*, 1994). Therefore, by association, the prevalence of p53 oncoprotein positivity implied that p53 gene mutation must represent a key step in the development and dissemination of melanoma. However, recently there has been a growing awareness that immunopositivity may not always be the result of gene

mutation (Volkenandt *et al.*, 1991; Castresana *et al.*, 1993) and this has considerable implications as to how the role of the p53 gene is perceived in this and other cancers, and how the gene and its products may be exploited for diagnostic and therapeutic purposes. The separation of gene mutation and protein stabilisation has raised three important questions that will need to be addressed in order to assess the role of p53 in melanoma.

- 1) Is immunopositivity always associated with *p53* gene mutation?
- 2) Is mutation common in cutaneous melanoma and important to the development and clinical behaviour of this tumour?
- 3) What other processes may produce *p53* oncoprotein overexpression in melanoma?

8.1.2 *p53 immunopositivity and p53 gene mutation*

The modest size of the p53 gene (11 exons, 393 amino acids) has made sequencing of the total encoding region possible. In a recent review, Greenblatt *et al.* (1994) analysed the incidence of mutations in the p53 gene from over 300 reports in which sequencing had been performed on human tumours or cell lines derived from human cancers. In total, 2,567 mutations were described and a selection of these are summarised in Table 8.1. In the more common cancers, such as colon and lung, 50% or more of tumours studied were found to contain p53 mutations. However, review of sequence analysis performed on 70 melanomas (tumours and cell lines) found a gene mutation prevalence of only 9%.

These findings were at variance with the observed incidence of p53 oncoprotein positively reported in our, and several other studies, in which up to 98% of melanomas were found to be immunopositive (Akslen & Mørkve, 1992; Cristolfolini *et al.*, 1993). Furthermore, the study by Stretch *et al.* (1991) used PAb240 to detect 'mutant' p53 in clinical samples of melanoma and found 93% of metastases to be immunopositive, concluding that almost all these tumours contained p53 gene mutations. The present study did not sequence the p53 gene, though the estimates of prevalence of p53 stabilisation are consistent with those of the other studies referred to above.

Tumour type	Number of tumours analysed	Prevalence of mutations (%)
Lung	897	56
Colon	50	50
Oesophagus	45	45
Non-melanoma		
skin cancers	44	44
Breast	1536	22
Melanoma	70	9
Testis	40	0

Table 8.1. Prevalence of gene mutations in human cancers. Adapted from Greenblatt *et al.* (1994).

The belief that immunopositive p53 protein is the product of a mutated p53 gene has come under increasing scrutiny since a number of studies have failed to discover p53gene mutations in association with the detected oncoprotein (Dunn et al., 1993; Weiss et al., 1993; Flørenes et al., 1994). In the first instance, an association between p53 gene mutations and the high prevalence of stabilised p53 in melanoma may be unexpected considering that chromosome 17 aberrations are detected in only 22% of melanomas (Fountain et al., 1990) (though point mutations may not be detectable as gross chromosomal defects). To date, four studies have reported the relationship between p53 oncoprotein overexpression and mutation of the p53 gene. Volkenandt et al. (1991) investigated p53 expression in nine melanoma cell lines using PAb1801. Only one melanoma line stained positively for p53 (11%) and this occurred in the only tumour found to have a detectable gene mutation. This consisted of a C to T transversion at codon 248, resulting in substitution of a tryptophan residue for arginine. The low prevalence of immunoreactivity was partly explained by the possibility that p53 mutation only occurs in a subset of melanomas, and/or that it was merely a secondary event to other gene abnormalities and had no primary role in the biology of this tumour. In comparison to the findings of the present studies

performed on clinical specimens, it would appear the limited selection of cell lines investigated in the study by Volkenandt *et al.* (1991) underestimated the prevalence of immunopositivity. In addition the senior author of this paper has subsequently expressed his doubts as to the purity and efficacy of the antibody used in that study (A. Albino, personal communication).

Weiss *et al.* (1993) examined p53 oncoprotein expression in 13 melanoma cell lines, using both PAb1801 and PAb240, followed by sequencing of exons 5 to 8 of the gene. Eleven of these tumours were found to show nuclear positivity (84.6%). PAb240 produced strong nuclear immunopositivity in four of these lines, that was subsequently found to coincide with point mutations, including C-T transversions in three tumours; representing 30.7% of the cell lines studied. However, again, this was a small study carried out on cell lines and may be unrepresentative of p53 expression in clinical specimens.

Attempts to further resolve this issue have been made in two extensive studies investigating p53 allele loss and p53 gene mutations in surgical excision samples of melanoma. Castresana et al. (1993) examined 46 primary and metastatic melanomas for loss of heterozygosity (LOH) using a combination of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. In a further 51 paraffin-embedded melanomas, DNA was extracted to sequence exons 5 to 8, using PCR and single strand conformation polymorphism (SSCP). Allelic loss was not detected in any of the samples analysed, nor were any mutations discovered in the exons analysed. More recently, Flørenes et al. (1994) studied 30 fresh and 12 paraffin-embedded human melanoma specimens in order to investigate the association between p53 gene mutation, p53 mRNA levels and oncoprotein expression. p53 gene aberrations were detected in seven of the 30 of melanomas (23%), including six tumours with LOH and one point mutation. Intriguingly, elevated mRNA levels were only present in one of these tumours, but detected in six other specimens with apparently normal p53 gene sequences. Immunohistochemical detection of the p53protein proved equally interesting in that 33% of specimens were found to be immunopositive, with detection occurring in 40% of tumours with apparently normal levels of p53 mRNA. Only two of the seven tumours with gene abnormalities were p53 immunopositive. In 25 cases both primary and metastatic tissue was available, but no significant difference in immunoreactivity was found between the two groups. However, a significant correlation was found between p53 immunoreactivity and the disease-free interval in patients presenting with superficial spreading melanomas. These studies suggested several conclusions of import; firstly, p53 gene mutation was not a significant factor in elevating p53 mRNA or overexpression of immunopositive

p53. Secondly, mutation of the p53 gene was not a prerequisite for the genesis of malignant melanoma or its subsequent progression.

A potential explanation for the observed absence of p53 gene mutations in these studies may be the fact that analysis was confined to exons 5 to 8, and that mutations may have been missed which occur outside this region. The recognition that the majority of mutations occur within this region (Hollstein et al., 1991) has produced a bias in mutation analysis of this gene, resulting in over 95% of mutations being identified within this location. However, in their survey Greenblatt et al., (1994) suggested 14% of mutations may lie in exons 4 and 10 of the p53 gene, (i.e. regions not normally screened). Furthermore, the distribution of mutations appears to be tumour specific, with 28% of mutations in bladder carcinomas residing in these exons as opposed to only 2% in head and neck malignancies. The present study did not sequence the p53 gene in any of the melanomas studied, though the findings of the investigations discussed above would suggest that mutation is not as prevalent as stabilisation or overexpression of the oncoprotein. Neither the study by Weiss (1993), nor Flørenes (1994) reported an association between clinical behaviour and tumours in which mutations were found. It would be of value to sequence exons 4 to 10 of the p53 gene in the specimens analysed in our study and further examine the relationship between gene mutation and oncoprotein expression, and also investigate if an association existed between gene perturbation and clinical behaviour. However, this would represent a formidable exercise, both in terms of time and cost.

The absence of a clear relationship between p53 oncoprotein immunopositivity and gene mutation in melanoma questions the importance of deregulation at the gene level for tumour development and progression; abrogation of p53 may represent only one of several pathways that leads to transformation. A lack of correlation between p53immunopositivity and gene mutation has been described in breast carcinoma (Dunn et al., 1993), whilst in testicular cancer cell lines (Peng et al., 1993) no p53 mutations were discovered at all. Furthermore, there is considerable evidence to suggest that the association between p53 overexpression is tumour specific; studies in head and neck squamous cell carcinomas have found a close correlation between gene mutation and protein stabilisation (Burns et al., 1993). Equally, in certain tumour types the acquisition of a p53 gene mutation is recognised as a crucial step in advancement to a more aggressive phenotype; the progression from low grade gliomas to high grade invasive tumours has been shown to be accompanied by accrual of p53 gene mutations in five of six cases studied (Sidransky et al., 1992). In the study on ethanol-fixed melanomas presented in Chapter 4, a trend was observed correlating the level of p53expression with survival in patients with thick (> 3mm) melanomas. It would be of interest and value to sequence the p53 gene in these lesions and establish whether mutations are more commonly seen in this subgroup of tumours, and whether they account for the increase in tumour progression and invasion, possibly as a result of co-transformation with other genes such as *c-myc* and *ras* (see below).

The study carried out by Akslen and Mørkve (1992) concentrated on paraffinembedded nodular melanomas and observed a high level of p53 protein in 97% of tumours. It is conceivable that p53 mutations may be accrued in vertical growth phase melanomas to modify behaviour, leading to invasion and metastasis. Equally, the relatively low prevalence of p53 mutations in melanoma may be explained by postulating that they occur more frequently in nodular melanomas, which only comprise 15%-20% of primary tumours. Mutations in this sub-group of melanomas may facilitate the action of other genes that control invasive processes, and these hypotheses clearly warrant further study. The relationship between ulceration and p53expression seen in the present study also suggests that gene mutations should be sought in this sub-group of primary tumours as well. Mutation or overexpression of inactivated p53 in melanoma cells may partially account for the development of tumours that are comprised of rapidly proliferating cells which are genetically unstable and prone to hypoxia and necrosis.

The absence of an obvious association with gene mutation may explain the range of conclusions regarding the timing of p53 mutation and stabilisation. The present study, along with that of Stretch *et al.* (1991), Akslen & Mørkve (1992) and McGregor *et al.* (1993) support the view that protein stabilisation occurs at an early stage of melanoma development, and that a trend exists to suggest intra-cellular p53 increases with progression. Others, such as Lassam *et al.* (1993) regard stabilisation, and therefore possibly mutation, of p53 to be a late event in melanoma progression. The prevalence of p53 overexpression in the series of melanomas examined in our study may be attributed to one, or a combination of, several possibilities:-

1) The detected oncoprotein was a wild-type conformation, induced in response to genomic injury and the presence of other mutated genes. The response to DNA injury and other mitogenic agents, such as UVr has been well documented. (Fritsche *et al.*, 1993; Zhan *et al.*, 1993). However, as noted above this is unlikely in view of the proportion of wtp53 oncoprotein observed in the validation experiments presented in Chapter 6.

2) The PAb1801 antibody is detecting a stabilised, inactivated oncoprotein which has lost the potential to induce G1 arrest or bring about apoptosis. In so doing, the

transformational effects of other mutated oncogenes are either left unchecked or potentiated.

3) The oncoprotein consisted of denatured wild-type protein which also crossreacts with mutant specific antibodies (Gannon *et al.*, 1990).

4) wtp53 may be accumulated and stabilised by binding to intracellular or viral proteins (Greenblatt *et al.*, 1994).

5) wtp53 is induced as part of a failed negative feedback loop in response to inactivation of downstream effector genes that interact with Cdks, (i.e. WAF1/Cip1). This would be a plausible scenario in tumours such as melanoma where immunopositivity is prevalent, but rarely accompanied by p53 gene mutations.

6) The overexpression of p53 follows post-translational modification, to result in accumulation of a conformationally stable and inactivated protein. p53 inactivation further facilitates the oncogenic behaviour of other regulatory genes and may confer a selective survival advantage on particular clones of cells.

Whilst any or all of the above may explain the prevalence of p53 immunopositivity in the melanomas investigated in this study, the last of these theories is given weight by the observed association between p53 and c-myc expression and merits further enquiry; p53 overexpression was consistently seen to only occur in the presence of elevated c-myc oncoprotein levels, and this is further contemplated in the following section (Section 8.2). The studies on p53 and melanoma reviewed above and in the discussion sections to both Chapters 4 and 5 have revealed a diverse range of findings and proposals. Attempts to assemble these disparate conclusions into a coherent model for the role of p53 in melanoma are limited by the fact that these studies analysed different specimens, preserved and prepared by different methods. In addition, there were inconsistencies in the type of antibody used and the method of detection employed.

In order to finally establish the role of p53 overexpression and gene mutation in the biology of melanoma, a large series of patient material will need to be studied using uniform methods of tumour preservation and processing, along with consistent techniques to assay the oncoprotein and sequence the gene, including regions outside exons 5 to 8. The findings of this study indicate that overexpression of p53 oncoprotein is common in melanoma and may be associated with the architectural and cytological atypia found in dysplastic naevi. The absence of p53 positivity in benign

naevi would suggest that alterations in the p53 oncoprotein capable of facilitating flow cytometric immunodetection are associated with development of architectural and cytological atypia and the malignant phenotype, but that the exact timing and mode of overexpression remain unclear. Ideally, FCM should be performed in tandem with IHC, to provide both quantitative estimates of protein content and data on the architectural distribution of the protein.

The findings of this study are consistent with the impression of a tumour in which p53 protein stabilisation is a common event, but where gene mutation is not essential, and this may explain the lack of correlation between the level of p53 expression and clinical outcome. However, the trend observed between p53 expression and survival in patients with thick primary melanomas is noteworthy and may prove an exception. An association between tumour grade and p53 mutations have been shown for several other tumour types, and the role of p53 oncoprotein as a prognostic marker has already been discussed in Section 1.12.4.

8.2 *p53* and *c-myc*: Do They Interact?

The possibility that p53 expression and function in melanoma may be influenced by the level of *c-myc* expression was suggested by the relationship observed between the co-expression of these two oncoproteins in all the tumours and atypical naevi examined in this study. In all these lesions an association was observed between p53and *c-myc* oncoprotein positivity that intimates overexpression of p53 was sustained by elevated *c-myc* positivity. The converse of this relationship did not apply as *c-myc* overexpression occurred in the absence of elevated p53 levels.

The association between p53 and c-myc expression has also been observed in studies of melanoma cell lines. Chevenix-Trench *et al.* (1990) found a strong correlation between levels of p53 mRNA and c-myc mRNA isolated from 18 metastatic melanoma cell lines ($\mathbf{r} = 0.55$, p < 0.05), which were seen in each of three different northern blots of specimens. The authors of this paper suggested the action of a common regulatory factor that controls the expression of both genes in a co-ordinated manner, such as UVr. Mørkve *et al.* (1992) investigated co-expression of p53 and c-myc oncoproteins in 15 non-small cell lung carcinomas using flow cytometry. p53positivity was observed in all 15 specimens, whilst c-myc was detected in ten (67%) tumours. As in our study, c-myc-positive tumours were found to have a significantly higher p53 expression than tumours which were immunonegative for c-myc. Furthermore, in keeping with the findings of our studies, no significant association was demonstrated between expression of either oncoprotein and tumour ploidy. A similar relationship between *c-myc* and p53 has also been described in one other study of p53 expression in lung carcinomas (Volm *et al.*, 1992).

Our findings concur with the qualitative nature of the relationships observed in the above studies, but propose a more significant role for c-myc in the regulation of p53oncoprotein levels than has been previously reported. The level of expression of cmyc was observed to be closely associated with a number of features of melanoma behaviour, including survival, suggesting a more active role in the clinico-pathological characteristics of melanoma and these are explored more closely in Section 8.3. It is possible that *c*-myc overexpression initially induces and then inactivates wtp53 at the post-translational level, to produce a stabilised, detectable but inactive oncoprotein, and this may be particularly relevant in tumours in which mutation of p53 does not occur. The inactivation of p53 by *c*-myc activity may confer a tumourigenic and survival advantage on tumour clones expressing this gene combination, selecting cells with a limited ability to undergo protective cell cycle arrest or apoptosis. The absence of an association between p53 expression and *c*-myc levels in benign naevi raises a number of possibilities. Firstly, it suggests that additional changes may accompany transformation, within either the p53 or c-myc gene, to facilitate the actions of c-myc on p53 expression in atypical naevi and, in particular, melanomas. Secondly, in keeping with the multi-step concept of tumourigenesis, perturbation of other genes (i.e. ras), (Taylor et al., 1992); nm23 (Flørenes et al., 1992) may be required in order to perform a 'priming' role on the p53 gene or its products as a prelude to modification by other factors, including *c-myc*.

Indirect evidence for these proposals comes from several sources. Firstly, *c-myc* alone is unable to transform cells in culture and requires the co-transfection and activity of other oncogenes (Littlewood & Evan, 1990; Garte, 1993; Prins & De Vries, 1993). Secondly, studies have shown that *c-myc* and mutated p53 act synergistically to induce cell transformation and metastasis, particularly in combination with *ras* (Taylor *et al.*, 1992). Finally, and of most interest, it has been demonstrated that co-transfection of *ras* and *c-myc* may transform cells in a rat prostate model in the presence of wtp53, bypassing it's anti-proliferative effects (Lu *et al.*, 1992). Heikkila *et al.*, (1987) demonstrated *c-myc* anti-sense oligonucleotides inhibit passage of cells at the G1/S phase checkpoint, but not from G0 to G1. Of importance, *p53* exerts it's anti-proliferative effect at the same G1/S phase checkpoint (Lane, 1992) and *c-myc* overexpression may override proliferation controls at this site. Lu *et al.* (1992) proposed that cells may become neoplastic via several routes. One route may involve inactivation of *p53* through mutation and the production of ineffective tumour

suppressor proteins. The second envisages tumour clones which are refractory to the effects of the wtp53 protein (possibly due to deregulation of downstream mechanisms, i.e. WAF1/Cip1) in which growth deregulation continues unabated. This model is consistent with the relationship observed between p53 and c-myc in this study and the relatively low prevalence of p53 gene mutations noted in melanomas from other studies.

The relationship between *c-myc* activity and p53 expression requires further study, particularly at the gene and post-translational level, in order to evaluate the effect of *c-myc* on both p53 transcription and protein conformation.

8.3 *c-myc*: A Central Role in Melanoma Biology?

The association between c-myc and clinico-pathological prognostic determinants in melanoma proved to be the most striking finding of this study. In particular, overexpression of the c-myc oncoprotein was found, on univariate analysis, to be a marker of poor prognosis in all ethanol-fixed tumours, including thick primary melanomas and patients with stage two disease. In the larger series of paraffinembedded melanomas, multivariate analysis identified c-myc overexpression as an independent prognostic marker in patients with thick primary melanomas. These observations pose the question as to whether c-myc may play a more central role in melanoma biology than has been previously contemplated. Evidence can be summarised from both the findings of this study and others, discussed below, to support such a position in this cancer:-

1. Prevalence of overexpression

c-myc was detected in almost 80% of ethanol-fixed primary and metastatic melanomas and 87% of paraffin-embedded tumours studied. *c-myc* is expressed in a wide range of both normal and malignant tissues (Littlewood & Evan, 1990), though overexpression was more prevalent in melanomas than found in benign and dysplastic naevi.

2. Level of c-myc expression

The level of c-myc expression was found to be associated with a number of recognised clinical prognostic determinants; positivity increased with tumour thickness and was also higher in ulcerated primary melanomas. Furthermore, the level of expression assayed in locoregional metastases of patients that went on to die were significantly higher than those with stage one or two disease at the time of follow-up.

3. c-myc and proliferation

This study observed clear associations between all three kinetic indices and the level of intra-nuclear *c-myc* positivity. Both LI and Ts displayed correlations of borderline significance, whilst that between *c-myc* expression and Tpot was highly significant. These *in vivo* observations are consistent with those made *in vitro*, demonstrating *c-myc* to both increase the number of cycling cells in a given population (reflected in the LI), by transfer of cells from G0 to G1, and the rate of proliferation (as indicated by Tpot and Ts) (reviewed by Littlewood & Evan, 1990). In addition, *in vitro* work has also shown *c-myc* to potentiate proliferative activity by suppression of differentiating pathways (Prins & De Vries, 1993).

4. The association between p53 and c-myc

The relationship between the expression of these two proteins in melanoma, also described in other tumour types (see Section 8.2), suggest that pathologically elevated c-myc levels may modify p53 to either inactivate or bypass the tumour-suppressor effect of its protein. The loss of gene injury-associated G1 arrest would allow the passage and accrual of further gene mutations prior to acquisition of a 'critical mass', adequate to initiate transformation (Bishop, 1991; Vogelstein *et al.*, 1993). Furthermore, the loss of ancillary protective mechanisms such as apoptotic removal of potentially dangerous cell clones, would further ensure inheritance of an increasingly mutated genetic legacy by progeny cells.

These observations provide compelling evidence to support the argument that c-myc occupies a central role in melanoma biology, as opposed to that of an epiphenomenon. In addition, evidence from other allied studies, discussed below, exists to both further substantiate this role and allow proposal of a model as to how c-myc may contribute to melanoma growth and progression. The most significant of these putative routes concerns the established association between melanoma and the immune system. Elucidation of the role of c-myc in this relationship may carry significant implications for the development and design of future therapeutic strategies.

8.3.1 Melanoma, c-myc and the immune system

An association has been both clinically and experimentally established between certain cancers and the immune system for many years (reviewed by Scott, 1991) and this has been particularly true for melanoma (Ruiter *et al.*, 1984; van Duinen, 1988). Indeed, melanoma has been the subject of a disproportionate amount of research on tumour immunology (Donawho & Kripke, 1992), largely the consequence of the following clinical observations:-

- 1. Depigmentation can occur around naevi ('halo'), associated with a profuse mononuclear infiltrate (Barnhill *et al.*, 1992).
- 2. Rare reports of partial or complete spontaneous clinical tumour regression, associated with histological removal of tumour by an inflammatory infiltrate (Smith & Stehlin, 1965).
- 3. The unpredictable and often long latency period between primary melanoma excision and appearance of metastases.
- 4. An increased incidence of skin cancers, including melanoma, in immunosuppressed patients (Boyle *et al.*, 1984; King *et al.*, 1995).

Further important evidence for the relationship between melanoma and the immune system has come from experimental investigations of melanoma immunology (Ruiter *et al.*, 1991), and in particular the identification of melanoma-specific antigens (Van den Eynde *et al.*, 1989; Gaugler *et al.*, 1994). This work has revealed that melanomas exhibit at least three different classes of cell surface antigen (Fig 8.1):

- 1. Tumour antigens unique to a particular patient and their melanoma.
- 2. Melanoma-specific antigens, found only on melanomas.
- 3. Common, major histocompatibility (MHC) antigens.



Figure 8.1. Melanomas may express and shed antigens derived from many sources. Pigmentation-related antigens, extracellular matrix proteins, cytoskeletal proteins and intermediate filaments are not expressed on the cell surface, though all other antigens illustrated above are detectable as surface markers, even if only transiently.

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It has been well established that melanoma cells exhibit decreased expression of MHC class one molecules with disease progression (Ruiter et al., 1984). Radial growth phase melanomas display high HLA class one expression on their surface, but the density of these antigens decrease with vertical growth characteristics (Brocker et al., 1985). This has been substantiated in a number of other studies of HLA class one expression in melanoma (reviewed by Ruiter et al., 1991) and subsequent studies that have gone on to show melanoma progression is accompanied by downregulation of specific HLA class one sub-groups of antigens, including HLA-B, HLA-A2 (Pandolfi et al., 1991) and adhesion molecules (Natali et al., 1990). Cancer cells can only be recognised by cytotoxic T lymphocytes if tumour antigens are presented in conjunction with MHC class one molecules and a co-stimulatory signal, involving the CD28-B7 complex (Nossal 1993) [Fig 8.2], inferring that metastasis incorporates processes to evade host immune mechanisms in an otherwise immunocompetent host. In the study by Pandolfi et al. (1991), gene-transfected restoration of the expression of HLA-A2 to the surface of melanoma cell lines was accompanied by regaining of immune recognition by cytotoxic lymphocytes. The obvious therapeutic implications of these studies have led to intensive efforts to both characterise and manipulate these antigens with a view to the development of both passive and adjuvant tumour vaccines (reviewed by Hellström et al., 1992). The identification and cloning of tumourinfiltrating lymphocytes (TIL), followed by IL-2 activation, has also produced novel treatment in the form of lymphokine activated killer cells (LAK) (Rosenberg et al., 1990), discussed in Section 1.7.3.

The role of the immune system in dissemination of melanoma has also been indicated by several other clinical and experimental observations. The presence of TIL in both primary and metastatic melanomas has been correlated with improved survival (Clark et al., 1989); HLA antigens may be required for the recruitment and proliferation of CD8⁺ (cytotoxic) lymphocytes which is lost following downregulation of HLA class one antigens. The other observation of note is that in vitro expression of HLA class one antigens on melanoma cells can be modulated by incubation with the interferons, γ and α , which act to upregulate surface expression of these antigens (Basham *et al.*, 1982; Anichini et al., 1986). This is of particular interest as the tumouricidal effect of this, and other lymphokines, have been documented in melanoma, particularly in combination with other cytotoxic agents such as dacarbazine (Falkson et al., 1991) or vindesine (Vorobiof et al., 1993) and alteration of these tumour immune markers may partially explain their mode of action. Recently, regular administration of interferon γ has been shown to confer a survival advantage on patients with stage two melanoma compared to a control population to whom interferon was not given (Cascinelli et al., 1994).



Figure 8.2. Overexpression of *c-myc* may result in downregulation of immune markers on the surface of melanomas, including MHC antigens, that may facilitate evasion of host defence mechanisms.

8.3.2 c-myc, early growth response genes and regulation of melanoma surface antigens

A potential route by which *c-myc* overexpression may interact in the relationship between melanoma progression and the immune system was initially suggested by the observation of an inverse correlation between levels of *c-myc* mRNA and HLA class one antigens in melanoma cell lines (Versteeg *et al.*, 1988). Transfection of *c-myc*, to produce gene amplification and overexpression of *c-myc* mRNA, was found to provoke downregulation of HLA antigens in cell lines with otherwise normal compliments of HLA class one and β 2-microglobulin expression. Furthermore, this study found that down-regulation of these antigens was reversed by exposure to interferon γ , which appeared to act by suppressing overexpression of the *c-myc* gene just prior to reappearance of surface antigens. Thus a central mechanism of the association between *c-myc* and melanoma progression may involve oncogene induced suppression of MHC class one antigens and induction of immune-escape processes (Fig 8.2.)

This fascinating aspect of the multi-functional role of c-myc has been further substantiated by observations that other members of the myc family are known to induce similar suppression of HLA antigens in other tumour types, including small cell lung carcinoma and neuroblastoma (L- and N-myc respectively) (Nau et al., 1985). Recently, overexpression of other types of early growth response genes have also been shown to affect MHC class one expression (Yamit-Hezi et al., 1994). This finding is of particular interest as this group of genes are also known to exert multiple effects on different biological processes, including signal transduction and control of cellular proliferation, similar to the myc family (reviewed by Diamond et al., 1990). In addition, they have been shown to have variable effects on HLA expression that were found to be tumour-specific; c-fos levels were found to be overexpressed in immunogenic tumours with low metastatic capabilities. Interferons have also been shown to induce *c*-fos expression in highly metastatic cell lines which is followed by upregulation of MHC class one antigens (Kushtai et al., 1990), similar to their effect on HLA antigens noted in the study by Versteeg et al. (1988). Investigation of the expression of MHC class one antigens in B16 melanoma cell lines confirmed the correlation between *c-fos* expression and HLA expression, but found that *jun*B expression acted similarly to *c-myc* in downregulating MHC transcription. Cotransfection with both *c-fos* and *c-jun* into B16 melanoma cells reduced their tumourigenicity and this was accompanied by an elevation in MHC class one expression (Yamit-Hezi et al., 1994).

These findings imply that control of the immunogenicity of both normal and tumour cells is regulated by several genes, that are part of the same functional group (i.e. early growth response genes). The relative importance of each gene in this process may vary with tumour type and individual. In melanoma, *c-myc* expression may play an central role in the regulation of a number of processes intrinsic to melanoma growth and progression, summarised in Figure 8.3. The relative importance of each aspect of *c-myc* activity may vary from tumour to tumour. Thus progression may be more dependent on proliferation, as opposed to immune escape, in one individual compared to another. However, other early growth response genes may exhibit similar associations to some or all of these processes and further research into the activity of these genes in melanoma is awaited with interest.



Figure 8.3. Putative routes by which *c-myc* protein overexpression may promote growth and metastasis of melanoma.

Despite the fact that over 100 oncogenes have been identified to date, of which over 30 are known to be active in man, little is known about how they actually participate in the process of transformation or metastasis. To elucidate how given groups of genes

actually orchestrate these processes and in what time sequence will require complex studies that may reveal mechanisms unique to a tumour or individual.

The findings of this study suggest that *c-myc* plays a central role in the biology of cutaneous melanoma, but much more research will need to be performed in order to identify what genes are most commonly mutated in transformation and metastasis, and the sequence and timing of their expression or inactivation. Furthermore, the nature of the perturbation and the level of the lesion (i.e. gene, mRNA, or protein) and how these genes interact, will also need considerable clarification. Finally, much more needs to be understood about how genes actually regulate other genes and proteins. Bit by bit, pieces of a jigsaw are becoming available, but assembly into a coherent picture will only be possible when an image is available to model it upon and all the components are known.

8.4 Is Studying Melanoma Proliferation Worthwhile?

8.4.1 Introduction

The limitations of the clinical staging of melanoma to yield accurate, predictive data on disease outcome are not unexpected. In the first instance, histological primary tumour thickness has been assumed to reflect the invasive potential of the tumour, in spite of the fact that measurement of this parameter follows fixation and considerable manipulation, the limitations of which were outlined in Section 1.4.3. However, of most significance, histology can only be expected to provide limited information on the biological characteristics of the tumour, yet it is upon this that the behaviour, and prognosis, of a given cancer will largely depend. One of the major aims of this study has been to evaluate biological parameters that may increase understanding of melanoma biology and to evaluate their prognostic significance. The study of proliferation in melanoma may prove of considerable importance and value in three main areas. Firstly, it is an important biological variable that may allow further insight into the significance of cell proliferation to tumour growth and metastasis, and this may be of particular value when studied in conjunction with other putative genetic or biological regulatory factors of cell turnover. A further, equally valuable, benefit is the prospect that it may yield information of prognostic significance in addition to that already available from histological criteria. However, the most potentially significant benefit of measuring tumour kinetics may be the possibility of being able to identify and predict response to therapy in an individual or given tumour type.

8.4.2 Proliferation as a prognostic indicator in stage 1 melanoma

The investigation into melanoma proliferation reported in this study identified a number of associations between proliferation indices, in particular Tpot, and clinical parameters, including primary tumour thickness. Thick primary melanomas were found to have a significantly shorter Tpot and Ts than thin tumours. This, in part, may be due to perturbation of growth regulatory mechanisms secondary to *c-myc* overexpression. The relationship observed between *c-myc* expression and Tpot and *c*myc and tumour thickness implies an association between these two variables in melanoma, substantiated by studies of the effects of *c-myc* on cell proliferation in vitro (Dang, 1991; Marcu et al., 1992) (i.e. that increased c-myc activity stimulates proliferation and tumour aggression, to produce the association observed between patient survival and both these variables). This premise is almost certainly over simplistic, as it fails to take into account the action of other gene-directed processes or the effect of growth modulating agents such as those of hormones or local growth factors. However, investigation of these and other biological parameters may provide meaningful information to increase understanding of disease pathophysiology and how other regulatory factors, (i.e. oncogenes, influence tumour proliferation, progression and ultimately, clinical behaviour).

8.4.3 Proliferation as a prognostic indicator in stage 2 melanoma

The additional, major, limitation of current histological prognostic criteria is the inability to predict tumour behaviour in patients with stage two melanoma (Koh *et al.*, 1986; Cascinelli *et al.*, 1984). Following regional lymph node dissection, the patient's clinical course is unpredictable (Kissin *et al.*, 1987) and nodal status may act only as a prognostic indicator and not a determinant of outcome (Cady, 1984). The present study found clear associations between the level of *c-myc* expression and disease progression in patients with stage two disease, emphasising the importance of evaluating this and other biological parameters at junctures beyond stage one disease. In a follow-up to their earlier study, Costa *et al.* (1990) identified measurements of the LI, established using tritiated-labelled thymidine([³H] dt LI), to be an independent prognostic marker in patients with stage two disease, even taking the number of affected nodes into account. The risk of recurrence and eventual death from melanoma was found to be almost two-fold higher in patients with tumours showing a high LI

compared with more slowly proliferating metastases. In the present study, on univariate analysis *c-myc* expression was found to act as a prognostic marker in the 36 patients whose ethanol-fixed lymph node metastases were investigated and described in Chapter 4. Similarly, both Tpot and LI were found, on univariate analysis, to act as prognostic indicators in all tumours considered as a whole. These findings would indicate that using BrdUrd, measurement of proliferation indices can be made to provide prognostic information not available following routine clinical or histological examination of the primary or secondary lesion alone. These findings merit the further study of *in vivo* cell proliferation in a larger series of patients, in conjunction with investigation of *c-myc* oncoprotein expression.

8.4.4 Proliferation kinetics as a predictive assay in melanoma

A further major benefit of studying melanoma proliferation may lie in the potential to predict response to therapy in an individual patient. As observed in other tumours (Chauval et al., 1984; Wilson, 1991), the present study noted considerable variation in tumour kinetics from one patient to another, which may have implications for the chosen type and schedule of adjuvent therapy (Wilson, 1993). This is of importance as it would be of considerable value to investigate whether the outcome of such treatment is affected by dosage schedules that are tailored to an individual patient's tumour proliferation profile. The influence of cell kinetics on tumour therapy is the subject of several multicentre phase three trials investigating the role of CHART (Continuous Hyperfractionated Accelerated Radiotherapy) schedules (Dische & Saunders, 1989) versus conventional radiotherapy protocols in the treatment of squamous cell carcinomas of the head and neck (Wilson, 1993). Only interim reports are available at present, but these suggest that rapidly proliferating tumours do less well if treated with conventional dosage schedules (Begg et al., 1990; Saunders et al., 1991) which may allow rapidly proliferating tumours to repopulate between treatments.

Consequently, whilst *c-myc* expression and tumour kinetics may prove to be of value as predictive assays of tumour behaviour in patients with primary melanomas of similar thickness, their major value may lie in predicting which treatment modality is most suitable for a given patient with stage two and three disease. One potential factor to limit efficacy of chemotherapy may be the timing and dosage of drugs used and this may be of particular relevance in combination protocols (Wilson, 1993). Future studies of adjuvant therapies in association with measurement of genetic and biological variables may identify sub-groups of patients that are more responsive to a particular regimen, or in whom the scheduling of dosage is important. Whilst trials have compared the frequency of administration of chemotherapy, either as single agents or in combination, in the management of disseminated melanoma (Creagan *et al.*, 1988; Allen, 1992; Kirkwood, 1992) none have utilised BrdUrd to identify whether variation in timing and frequency of the doses, as directed by the patient's tumour kinetics, has any affect on treatment response and outcome.

Despite the intrinsic chemoresistance of many solid tumours, including melanoma, efforts have continued to identify a relationship between cell kinetics and specific tumour types in order to investigate their relevance to chemotherapy dosage schedules (Silvestrini, 1989). However, an association is rarely obvious; testicular and colonic cancers exhibit amongst the most rapidly reported LIs, but represent amongst the most and least chemosensitive tumours respectively (Silvestrini, 1994). This observation alone suggests that chemoresistance is tumour-specific and independent of proliferation in certain tumours. However, in other tumour types, the rate of proliferation has been shown to predict response to therapy (Molinari et al., 1991; Remvikos et al., 1989). Patients with rapidly proliferating intra-oral carcinomas (as determined by estimation of [³H] dTR LI), showed improved long-term survival when treated with a combination of intra-arterial vincristine, bleomycin and methotrexate compared to vincristine and bleomycin alone (Molinari et al., 1991). However, in slowly proliferating tumours, the addition of methotrexate was not found to confer any additional survival advantage. Similar findings have been made from a retrospective study investigating the relevance of proliferation kinetics to the response following either single or combination chemotherapy for ovarian carcinoma (Silvestrini et al., 1992). In this study, rapidly proliferating tumours were found to be more sensitive to polychemotherapy, whereas slowly proliferating cancers were more responsive to single agent therapy. Similar observations that correlate a therapeutic response for breast cancer with cell kinetics have also been made in studies of chemotherapy (Remvikos et al., 1989) and hormonal treatment (Nicholson et al., 1991).

Combined analysis of different biological variables, although independent, may be useful in improving prognostic definition both overall and within specific disease stages (Silvestrini 1994). Therefore, the working hypothesis must be that a greater prognostic profile is established when information from several parameters are considered in concert, and that these may also be utilised to predict response to therapy. An example of this is the relationship between response to chemotherapy and oestrogen-receptor positivity in breast cancers (Osborne, 1991). The value of *c-myc*

and cell kinetic parameters as predictive assays will only be ascertained by measurement of these variables in a large series of patients as part of controlled, randomised trials with adequate follow-up. The application of BrdUrd and flow cytometry to provide a rapid and simple method of estimating *in vivo* tumour proliferation presents a unique opportunity to study the prognostic relevance of cell kinetics as both a prognostic marker and predictive assay of response to therapy. In addition it may be used to monitor the effect of adjuvant therapies on the biological behaviour of the tumour (Silvestrini 1994). However, the present study has shown a more fundamental application of this knowledge, to understand more of the biology of cutaneous melanoma and how cell kinetics may be associated with tumour behaviour. In addition, the investigation of these parameters in conjunction with oncoprotein analysis has afforded insight into how key genes may manifest their regulatory control and participate in the malignant process.

8.5 Implications for the Clinical Management of Melanoma

The dramatic advances in tumour biology and cancer genetics have begun to identify specific gene lesions that may underlie progression from benign precursors through to the metastatic phenotype, as seen in colo-rectal cancer (Fearon & Vogelstein, 1990). The pinpointing of such lesions, along with the resurgence of interest in melanoma immunology, suggests a number of areas in which the results of this study may find potential application in the future management of melanoma, and provide a model upon which to consider the contribution of both these oncoproteins, and other biological markers, to future clinical practice.

1. Primary health care and epidemiology of cancer

Data on the association between distribution of disease and gene mutations has led to the recognition that molecular epidemiology may make an increasingly significant contribution to understanding cancer aetiology and the role of the environment (Greenblatt *et al.*, 1994). UVr is associated with recognisable p53 mutations in nonmelanoma cancers (Harris *et al.*, 1993), whilst other distinct p53 mutations have been identified in the liver and oesophagus (Greenblatt *et al.*, 1994). Molecular epidemiology of p53 and other genes in melanoma would aid clarification of the role of sun exposure in melanoma aetiology, and identify future areas of laboratory and clinical research. This may also have implications in the design of preventative health measures for a particular individual, family or population.

2. Identification of those at risk

As for the single gene disorders, considerable efforts are being directed towards the identification of cytogenetic and oncogenic lesions as markers of risk for several sporadic and familial types of cancer. In melanoma, attention has focused on both chromosome six (Fountain *et al.*, 1990; Trent *et al.*, 1990) and more recently, 9p21, as putative sites of melanoma susceptibility genes and melanoma suppressor genes respectively (Fountain *et al.*, 1992; Kefford, 1992). Identification of specific loci would allow counselling of individuals with a familial history and, ultimately, the general population as to their risk of melanoma. Once a gene or panel of marker genes are isolated, their encoded proteins can be identified, to both uncover a further step in the pathogenesis pathway and afford other potential clinical applications.

3. Detection of residual disease

The role of oncoproteins and tumour suppressor genes as tumour markers have been investigated in certain cancers. Overexpression of p53 oncoprotein has been shown to correlate with tumour grade and stage in bladder cancer (Esrig *et al.*, 1993) and analysis of urine samples for mutant p53 in tumour cells has been investigated as a screening method for recurrent transitional bladder carcinoma (Sidransky *et al.*, 1991). Studies have also examined serum levels of p53 oncoproteins as a tumour marker in breast cancer (Crawford *et al.*, 1982) and ovarian cancer (Labreque *et al.*, 1993), thought to follow either tumour necrosis or as a result of immunogenicity against a complex formed between p53 protein and heat shock 70 protein (Davidoff *et al.*, 1992). p53 antibodies have also been detected in patients with lung cancer (Winter *et al.*, 1992) and lymphoma (Carol de Fromentel *et al.*, 1987). In melanoma, elevated serum levels of soluble ICAM-1, an adhesion molecule, have been shown to closely correlate with both presence of primary melanoma and progression to disseminated disease (Altomonte *et al.*, 1992).

Tumour markers may be utilised to detect sub-clinical signs of recurrent disease and monitor both the course of disease and response to therapy, as for β -HCG in testicular carcinoma. However, tumours identified to display specific immune or surface protein changes may be susceptible as therapeutic targets to antibodies to which either a radiation source or chemotoxic agent is attached (Connett *et al.*, 1991). A number of other markers have been shown to vary with progression from the primary to metastatic phenotype, summarised in Table 8.2. Some of these, including the gangliosides and HLA antigens, have been incorporated into novel therapies (see on).

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Considerable work is required to fully evaluate the therapeutic potential of these and the remaining factors.

4. Advances in histopathology

The histopathological diagnosis of melanoma is often very difficult to make, particularly when attempting to distinguish melanocytic lesions that may exhibit features common to both benign naevi and melanomas (Mooi & Krausz, 1992b). A significant contribution would be made if an oncoprotein tumour marker was identified that was specific to malignant melanocytes. *c-myc* was found to be overexpressed in all types of melanocytic lesions studied in this investigation and is therefore unsuitable. The discovery of p53 expression in over 50% of atypical naevi would also suggest, contrary to other studies (Lassam et al., 1993; McGregor et al., 1993), that p53 overexpression is not specific to malignant melanoma either. Caution has been expressed with respect to the interpretation of p53 immunopositivity as a marker of malignancy in other tumours using immunocytochemistry (Wynford-Thomas, 1992), though the presence of mutant p53 may be a reliable malignant marker in breast cancer (Hall et al., 1991), further illustrating the tumour-specific heterogeneity of p53 mutations and their clinical significance (Harris et al., 1993). High p53 expression on immunohistochemical sections may indicate particular behavioural characteristics, including invasive potential in prostatic carcinomas (Visakorpi et al., 1992), whilst other studies have shown that p53 immunodetection may aid differential diagnosis of cytological aspirates when considered in conjunction with other proteins (Betta et al., 1993). In the case of p53, analysis of the pattern and distribution of oncoprotein positivity may also indicate the nature of the underlying gene mutation (Harris et al., 1993).

The study of oncogenes and their oncoproteins may also further define tumour types and their classification, in a similar manner to oestrogen receptor status in breast cancers. In addition to clinical characteristics, tumours may display histologically distinct morphological features that correlate with oncogene mutation or oncoprotein overexpression, as described in bladder cancer (Esrig *et al.*, 1993). Ramon Y Cajal *et al.* (1991) investigated the effect of oncogene transfection on tumour morphology in melanoma cell lines that were transfected with one of several oncogenes, including Hras, avian MC29 gag-myc, neu and E1a. Transfected lines were transplanted into syngeneic mice, followed by histological examination of the resultant tumour morphology. H-ras transfected lines tended to produce tumours with epithelioid cells, neu transfection resulted in a predominant spindle cell population and myc produced an anaplastic appearance. The ability of viral myc genes to alter tissue conformation has also been observed in myelocytic cells, in which the differentiation phenotype could be modified by altering the level of gene expression (Symonds *et al.*, 1989). Consequently, these findings would suggest that certain features of tumour morphology may be the result of specific oncogenes. The functional activity of several oncogenes in a population of tumour cells may explain the histological tumour heterogeneity frequently observed in melanomas (Mooi & Krausz, 1992b), but this proposal awaits further study.

5. Oncoprotein expression as a prognostic indicator

The observations of an association between c-myc expression and prognosis for patients with both stage one and, in particular, stage two melanoma have been discussed in Section 8.2. A prognostic role of c-myc has also been noted in other cancers, including those of the cervix (Bourhis *et al.*, 1990), colon and breast (Alitalo & Schwab, 1986) (see Section 4.12). In the patients investigated in this series, p53 expression did not act as a prognostic marker. However, in other tumour types this is not the case; p53 oncoprotein expression has been shown to be of prognostic significance in node-negative breast cancer (Thor & Yandell, 1993) and lung cancer (Quinlan *et al.*, 1992) (see Section 1.12.4). The selection of reliable, repeatable, oncoprotein (and other biological) markers raises the possibility of constructing a prognostic profile for each tumour type and patient, as a part of each patient's routine clinical and pathological assessment.

6. Development and design of novel therapies

The correlation between melanoma behaviour and *c-myc* expression requires considerable work to elucidate the exact mechanism of how oncoprotein overexpression may interact with other systems, in particular those regulating cell surface antigen expression, proliferation and differentiation. The major implication lies in the recognition that identifiable biological lesions exist within melanoma that may be susceptible to manipulation by pharmaceutical means or otherwise. However, in order to achieve this, the mode of action of both the *c-myc* gene and oncoprotein will need considerable elucidation. Clarification of these pathways may be exploited by several novel treatment strategies:-

a. Anti-sense oligonucleotides against c-myc

The inverse relationship observed between c-myc expression and downregulation of HLA class one antigens in melanoma cell lines (Versteeg *et al.*, 1988) raises the

question as to whether the tumour-progressive effect of the *c-myc* gene or oncoprotein can be inhibited, either with the use of anti-sense oligonucleotides, or pharmacological antagonists (reviewed by Carter & Lemoine, 1993). Anti-sense oligonucleotides have been shown to inhibit part or all of its proliferative effect in culture (Heikkila *et al.*, 1987; Wickstrom *et al.*, 1988) and have been shown to act against other transforming oncogenes *in vitro* (Helene, 1991). Of particular interest, oligonucleotide inhibition of N-*myc* expression in neuroblastoma cell lines was accompanied by a reduction in proliferation and phenotypic changes consistent with increased cellular differentiation (Negroni *et al.*, 1991). Three major obstacles remain prior to introduction of antisense therapy. Firstly, considerably more needs to be known of the potential effects of downregulating gene activity that is common to many cell types, both normal and neoplastic. Secondly, anti-sense therapy needs to be shown to be effective *in vivo*. Thirdly, a suitable delivery system will need to be developed to locate oligonucleotides to the required site of action in order to minimise their metabolism and potential sideeffects.

b. Melanoma immunotherapy

A resurgence of interest in methods of manipulating the immune system as an adjuvant treatment in stage two and three melanoma has led to a number of trials of melanoma vaccines (Hersey, 1992). Immunotherapy against the melanoma antigens, such as GD3 has been shown to limit growth of metastases in humans (Houghton et al., 1985). However, the greatest interest has concentrated on the use of either autologous or allogeneic melanoma tumour vaccines (reviewed by Hellström et al., 1992), with or without addition of an adjuvant agent (Hersey & Coates, 1994) as outlined in Sections 1.7.2 and 1.7.3. Considerable efforts are being made to modify and increase the immunogenicity of vaccines to incite a greater host-immune response. Studies with a polyvalent melanoma vaccine derived from three melanoma cell lines (Hoon et al., 1990) have been shown to confer a survival advantage in patients with disseminated disease compared to historical controls (Tai et al., 1985; Morton et al., 1990; 1992b). Efforts continue to devise methods of increasing vaccine immunogenicity. Hayashi et al. (1993) have shown that expression of HLA-A (HLA-A2 in particular) antigens, made allogeneic melanoma vaccine cells as effective as autologous melanoma cells in activating cytotoxic lymphocyte killing of autologous melanoma cells. In view of the proposed relationship between *c*-myc and HLA class one expression, it would be of interest to investigate whether HLA antigens could be upregulated to increase immunogenecity of vaccine cells using *c-myc* anti-sense oligonucleotides, or by pharmacological inhibition of the oncoprotein.

c. Gene therapy

Ultimately, the main objective of identifying aetiological lesions at the gene level must be the hope that techniques may become available to correct such deficits *in vivo*, using gene therapy (Lemoine & Sikora, 1993; Cournoyer & Caskey, 1990). However, this proposed form of therapy is based on several assumptions: firstly it is supposed that single, identifiable lesions may be identified and that replacement of the defective gene or oncoprotein may restore cell and tissue homeostasis. Secondly, it is assumed that the identifiable gene lesion is still responsible for the phenotypic behaviour of the tumour cell at the time of diagnosis. Thirdly, that vectors are available to deliver genes to the correct location. Consequently, in melanomas or other cancers where a mutation has been detected, the aim would be to transfect copies of the wild type gene into tumour cells in order to correct or compensate the deficit. Thus potentially, normal copies of c-myc, or other genes known to downregulate its activity, such as the interferon genes, could be transfected into melanoma cells to limit its tumourigenic activity.

Virally directed enzyme pro-drug therapy delivers vectors to cells that contain specific promoter sequences (Huber *et al.*, 1991) (i.e. tyrosinase gene promoter in melanomas and melanocytes). The promoter is coupled to a non-mammalian enzyme to activate metabolism of a pro-drug to a cytotoxic agent. Melanoma cells have provided a chosen model for these studies (Vile 1993). Vile and Hart (1993) delivered constructs containing β -galactosidase to cells in tumours by direct injection, distributing the enzyme DNA into surrounding cells by a 'bystander' effect. Similar methods could be used to deliver copies of immunomodulatory genes including cytokine genes, or those encoding HLA class one antigens, to modify tumour cell immunogenicity. This approach has been used with some success in a mouse colonic cancer model (Fearon & Vogelstein, 1990) and has been performed on tumour-infiltrating lymphocytes isolated from patients undergoing treatment for metastatic melanoma (Rosenberg *et al.*, 1990).

Cancer is a multi-gene disorder, and is likely to be less responsive to transfection of one gene, than the single gene disorders, such as cystic fibrosis (Davies *et al.*, 1993). However, much interest centres on the transfection of p53 and restoration of tumour suppression in growth deregulated cells. Trials to introduce wild type p53 into bronchial carcinomas, using a retroviral vector (Fujiwara *et al.*, 1993) or liposomes (Davies *et al.*, 1993), are presently under consideration.

Advances in medicine and the basic sciences have resulted in stratification of the methods available to assess cancers and an individual patient's prognosis. At the

foundation, the patient's history and clinical examination assess the type, differential diagnosis and extent of disease. The next level has followed developments, particularly in basic medical sciences and technology, to improve imaging and analyse biological markers such as proliferation parameters, steroid receptors and hormonal markers. Investigations directed at aspects of melanoma biology, other than those discussed in this study, have identified factors that vary with specific stages of melanoma growth and metastasis, summarised in Table 8.2 (Herlyn 1993b). Several of these, such as the adhesion molecules (Altomonte *et al.*, 1992) and gangliosides (Morton *et al.*, 1990), have been investigated for their prognostic and therapeutic potential. The third level has exploited recent technological advances to investigate the observations first made by Rous over 80 years ago, (i.e. the study of oncogenes in human cancer and their interaction with epigenetic factors).

At present, an enormous challenge lies ahead in being able to ascend to the final level, in which the actual mode of action of each oncogene is explained and orientated in context to other genes and epigenetic factors. Once the processes that lead to deregulated growth control and invasion are elucidated, specifically designed and effective therapy may become available, with the realistic aim of curing the patient and involving minimal risk and morbidity.

8.6 Conclusions: Hypotheses Answered?

This thesis was researched to investigate the hypotheses stated in Section 1.15. From the observations of this work the following conclusions may be drawn:-

1. Expression of the p53 oncoprotein is prevalent in both primary and metastatic melanoma and is found in approximately 80% of tumours. The level of expression does not appear to be associated with outcome or other parameters of clinical behaviour, but perturbation may be related to patient age and ulceration of the primary tumour.

2. Overexpression of the c-myc oncoprotein was detected in approximately 80% of ethanol-fixed melanomas and 87% of paraffin-embedded tumours. The level of expression appears to show an inverse relationship with patient survival and tumour cell proliferation, as measured using an *in vivo* technique. *c*-myc positivity correlates with primary tumour thickness and ulceration and an association was found between c-myc positivity and the number of mitotic figures. In addition, the level of expression was observed to stratify survival of patients with thick primary

melanomas, yielding prognostic data in addition to that provided by routine histological parameters. Of equal significance, *c-myc* expression in ethanol-fixed melanomas may act as a prognostic marker when measured in patients with stage 2 disease.

3. BrdUrd can be used to analyse *in vivo* tumour cell kinetics in melanoma. Tpot was observed to decrease with increasing primary tumour thickness, with the proliferation rate of metastases being similar to that found in primaries. Tpot and LI were both found to be of prognostic significance by univariate analysis alone.

4. Rapid melanoma cell proliferation, indicated by a lowered Ts and Tpot, were both found to be significantly associated with c-myc overexpression. p53 immunopositivity did not correlate with tumour cell kinetics.

5. p53 was not detected in benign melanocytic naevi using either immunohistochemistry or flow cytometry. Approximately 50% of histologically diagnosed sporadic dysplastic naevi were found to be p53 immunopositive, using both methods of detection, indicating that detection of this protein in melanocytic naevi does not act as a marker of malignancy. The level of p53 oncoprotein may be associated with certain features of architectural atypia, but further studies are required in a larger number of specimens to clarify the significance of this relationship.

8.7 Future Studies

I would hope that the work presented in this thesis may stimulate further research in selected related areas. In the first instance, measurement of c-myc oncoprotein and cell kinetics is continuing in an ongoing study to confirm the observations made in the present study in a larger series of patients. It would be of great interest to investigate the mechanism of c-myc overexpression in the melanomas analysed in this series, by examining changes at the gene and mRNA level. The immunohistochemical features of c-myc oncoprotein expression in fresh naevi and melanomas, at different stages of development also merit investigation. However, it is the putative relationship between c-myc expression and melanoma HLA antigens that is of most scientific and therapeutic interest. This should be further studied in excision tumour specimens, along with the expression of other co-stimulatory molecules, such as B7 (Nossal, 1993). The continued evaluation of the association between c-myc expression and melanoma behaviour provides a model upon which to explore potential novel

therapies, particularly in the study of anti-sense therapy and the design of tumour vaccines.

The relevance of the prevalent p53 oncoprotein stabilisation in melanoma requires further evaluation; although p53 mutations in melanoma would appear to be rare, it would be very worthwhile to investigate the clinico-pathological features of the subgroup of melanomas that do possess detectable mutations.

The ultimate aims of this thesis were to both acquire laboratory skills applicable to cancer research, and further insight into the biology of melanoma. An appreciation of the method and principles of investigating tumour biology are essential skills for any clinician specialising in the care of patients with cancer. This broadening of vision is particularly important for a surgeon such as myself, as past principles of cancer surgery based on a Virchowian model, of 'the wider the excision, the greater the chance of cure' stratagem, are no longer applicable. Our concepts of tumourigenesis and metastasis have undergone profound changes over this last quarter century, bringing with them the hopes of novel, effective therapies. The surgical management of solid tumours must adopt and adapt these new concepts, methods and philosophies in order to optimise patient management most effectively as part of a multi-disciplinary approach. It is likely that in the near future, cancer surgery will require application of additional diagnostic and therapeutic methods that work at a subtle, molecular level not amenable to the knife. The appreciation that surgery may be limited in its application and ambition is not new, and in closing I would ask the reader to contemplate the words of John Hunter, the father of modern surgery, who wrote in 1786:

"This last part of surgery, namely operations, is a reflection on the healing art; it is a tacit acknowledgement of the insufficiency of surgery. It is like an armed savage who attempts to get that by force which a civilised man would get by stratagem". (Palmer, 1835)

"Surgeons tend to forget that they are not masters but only servants. They can assist the natural powers within all living flesh but cannot replace them". (Palmer, 1835)

Upregulated	Downregulated
Adhesion molecules	
$\alpha_v\beta_3$	α6β1
ICAM-1	
MUC18 (gp113)	
ECM proteins	
Tenascin	collagen
Growth factor receptors	
EGF receptor	
Transferrin receptor	c-kit
MHC antigens	
HLA class 2	HLA Class -1
	Thy-1
Gangliosides	
GD2	GM3
GD3/GM3 ratio > 1.0	
Proliferation markers	
Ki-67	
LI	
Tpot	
Angiogenesis	
Laminin receptor	
Enzymes	
	Dipeptydy peptidase
	(CD26 or ADA-binding protein)
Oncogenes	
ras	
с-тус	
c-fos	
c-jun	
nm23	
Tumour Suppressor genes	
<i>p53</i>	

Table 8.2. Progression factors associated with growth and metastasis of melanoma

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