# THE MOLECULAR BASIS OF HIGH-GRADE TRANSFORMATION OF B CELL LYMPHOMA OF MUCOSA ASSOCIATED LYMPHOID TISSUE (MALT)

**A Thesis Submitted for the Degree of Doctor of Philosophy in the Faculty of Clinical Sciences of the University of London**

By

**Huaizheng Peng**

**Supervised by Dr. Langxing Pan**

**Department of Histopathology**

**UCL Medical School**

**London**

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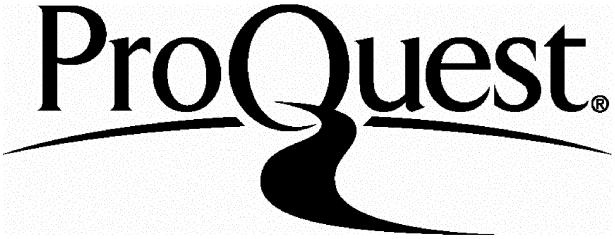

**Abstract**

Low-grade B cell MALT lymphomas are closely associated with chronic inflammation including autoimmune disorders, such as Hashimoto's disease in the thyroid and bacterial infection, such as Helicobacter pylori related gastritis in the stomach. They are clinically indolent, but can transform into high-grade tumours at a late stage. In this study, molecular biological methods have been used to investigate the molecular basis of this high-grade transformation.

To establish a clonal link between low and high-grade MALT lymphomas, clone-specific rearrangements of immunoglobulin heavy chain (***IgH***) gene were determined in coexisting low and high-grade lesions. Identical ***IgH*** rearrangements demonstrated in both lesions indicated that high-grade MALT lymphomas were directly derived from low-grade tumours.

*H pylori* strains containing the ***CagA*** gene are known to be more virulent than those without the gene. Using a sensitive PCR based assay, a higher frequency of infection by ***CagA***+ bacteria was observed in high-grade gastric lymphoma than in low-grade lymphoma and gastritis controls. This suggests that high-grade transformation may be more likely to occur following infection by ***CagA***+ *H pylori*.

More than half of the MALT lymphomas studied in this project were shown to possess an RER+ phenotype (an indicator of genetic instability), suggesting that genetic instability is a common feature of the tumour. Frequent somatic mutations in the ***c-myc*** regulatory regions were also observed, indicating that these mutations may be important in the development of MALT lymphoma. ***P53*** abnormalities (point mutation, loss of heterozygosity and over-expression) were found in a significant proportion of MALT lymphomas, with a higher incidence and more complex patterns in high-grade than low-grade tumours, indicating a possible role of these abnormalities in high-grade transformation. An interrelationship between ***p53*** abnormalities and RER+ phenotype was also demonstrated.

The results presented here show that, like other tumours, the development of MALT lymphomas is a multistage process. High incidence of genetic instability may be associated with chronic inflammation, which provides a basis for accumulation of genetic abnormalities leading to evolution of MALT lymphomas. Further alterations of vital genes, such as ***c-myc*** and ***p53*** may result in progression or high-grade transformation of the tumour.

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## Abbreviations

| | |
|---|---|
| ***BCL-1*** | B cell leukemia-lymphoma-1 |
| ***BCL-2*** | B cell leukemia-lymphoma-2 |
| ***BCL-6*** | B cell leukemia-lymphoma-6 |
| BL | Burkitt's lymphoma |
| Bp | Base pair |
| C | Constant region of ***Ig*** gene |
| ***CagA*** | Cytotoxic associated gene A of *H pylori* |
| CCL | Centrocyte-like cell |
| CDR II /III | Complementarity determining region 2 /3 |
| CLL | Chronic lymphocytic leukaemia |
| D | ***Ig*** gene diversity region |
| EBV | Epstein-Barr virus |
| FR 1/2/3 | ***Ig*** gene framework 1/2/3 |
| Gb | Genbank |
| GDB | Genetic data base |
| *H pylori* | Helicobacter pylori |
| HD | Hodgkin's disease |
| HE | Haematoxylin and eosin |
| IDL | Insertion-deletion loop type mismatched nucleotides |
| ***Ig*** | Immunoglobulin |
| ***IgH*** | Immunoglobulin heavy chain |
| ***IgL*** | Immunoglobulin light chain |
| IPSID | Immunoproliferative small intestinal disease |
| JH | ***IgH*** gene joining region |
| LEL | Lymphoepithelial lesion |
| LOH | Loss of heterozygosity |
| MALT | Mucosa-associated lymphoid tissue |
| MESA | Myoepithelial sialadenititis |
| MIF | Myc gene intron factor |
| NHL | Non-Hodgkin's lymphoma |
| NHPCC | Hereditary nonpolyposis colon cancer |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| RB | Retinoblastoma susceptibility gene |
| REAL | Revised European-American Lymphoma classification |
| RER | Replication error phenotype |
| SLL | Small lymphocytic lymphoma |
| SSCP | Single stranded conformation polymorphism |
| VH | ***IgH*** gene variable region |

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# Chapter 1 Introduction

## 1.1General Introduction

MALT lymphomas are a specific pathological entity, [1-3] which occur in a wide range of extranodal sites and morphologically mimic the appearance of acquired MALT. [1,4,5] These lymphomas form a major part of the extranodal lymphomas that account for 40% of non-Hodgkin's lymphomas (NHL). [1,6,7]

Typical MALT lymphomas are low-grade B cell lymphomas.[2,3,8-14] Their development is closely associated with chronic inflammation caused by autoimmune disorders and /or infections, [15,16] such as *H pylori* infection in gastric MALT lymphoma. [17] Largely because of its indolent clinical course, low-grade MALT lymphoma was wrongly considered as a benign lymphoid proliferation and was called pseudo-lymphoma before its recent characterisation. [13,18] Low-grade MALT lymphomas have been shown to be malignant by their monoclonal nature, [19,20] their invasive properties, [21-25] their ability to disseminate to both regional lymph nodes [26,27] and other mucosal organs, [28-31] and their cytogenetic and molecular biological aberrations. [32,33]

Although low-grade MALT lymphomas run an extremely indolent clinical course, transformation into high-grade disease may occur. [34] High-grade MALT lymphomas, like their nodal counterparts, exhibit aggressive clinical behaviour. Therefore understanding high-grade transformation of low-grade B cell lymphoma may not only help us to shed light on the development of the disease, but also indicate novel treatment protocols. Considerable progress has been made in defining the histological, [9-14] molecular [35] and biological [36,37] features of MALT lymphoma and distinguishing this tumour from its nodal counterpart, but little is known about the molecular basis of MALT lymphoma development, especially high-grade transformation.

The development of malignant tumours is thought to be driven by multiple specific genetic abnormalities, including tumour suppressor gene inactivation and oncogene activation. [38-41] These abnormalities are considered to result from genetic instability. [42,43] In addition, other non-genetic factors, such as autoimmune reaction, [15] chronic inflammation, [44,45] cytokine disturbance, [46,47] and viral infection [48-52] may also take part in this process. [38,40] Therefore, elucidation of the roles of these specific genetic abnormalities, along with genetic instability and other related non-genetic factors (such as bacterial infections in gastric MALT lymphoma) in both low and high-grade MALT lymphoma may lead to a better understanding of high-grade transformation. This speculation formed the working hypothesis of this thesis.

This chapter describes the clinical, pathological and genetic features of low and high-grade B cell MALT lymphoma. Aspects of tumour evolution, in particular, the development of gastric MALT lymphoma and its association with *H pylori* infection, and possible genetic abnormalities that may be involved in the development of the lymphoma are reviewed.

## 1.2 **Mucosa Associated Lymphoid Tissue (MALT)**

MALT represents a specialised part of the mucosal immune system, functioning as the primary immune defence against foreign antigens introduced through the mucosal surfaces of the body. [53]

Besides lamina propria lymphocytes, the intraepithelial lymphocytes and the mesenteric lymph nodes, the most prominent structural component of MALT is the organised nodular lymphoid tissue that occurs in the terminal ileum as Peyer's patches. [54] Peyer's patches are characterised by the lymphoid follicle, which is composed of a follicle centre, a thin mantle zone and a marginal

zone (see Figure 1-1). [1,55] On the lumenal side of the follicle, the marginal zone merges into a mixed cell infiltrate that covers the dome of the Peyer's patches. Histologically, the marginal zone is similar to the marginal zone of the splenic white pulp. It is composed predominantly of small B cells with cleaved nuclei (so called centrocyte-like cells). The mixed cell infiltrate of the dome region is composed of plasma cells, dendritic cells, macrophages, small lymphocytes and centrocyte-like cells. The centrocyte-like cells infiltrate the overlying epithelium. Animal models suggest that the function of Peyer's patches is associated with the generation of humoral immune response in the gut. [1,55-57]

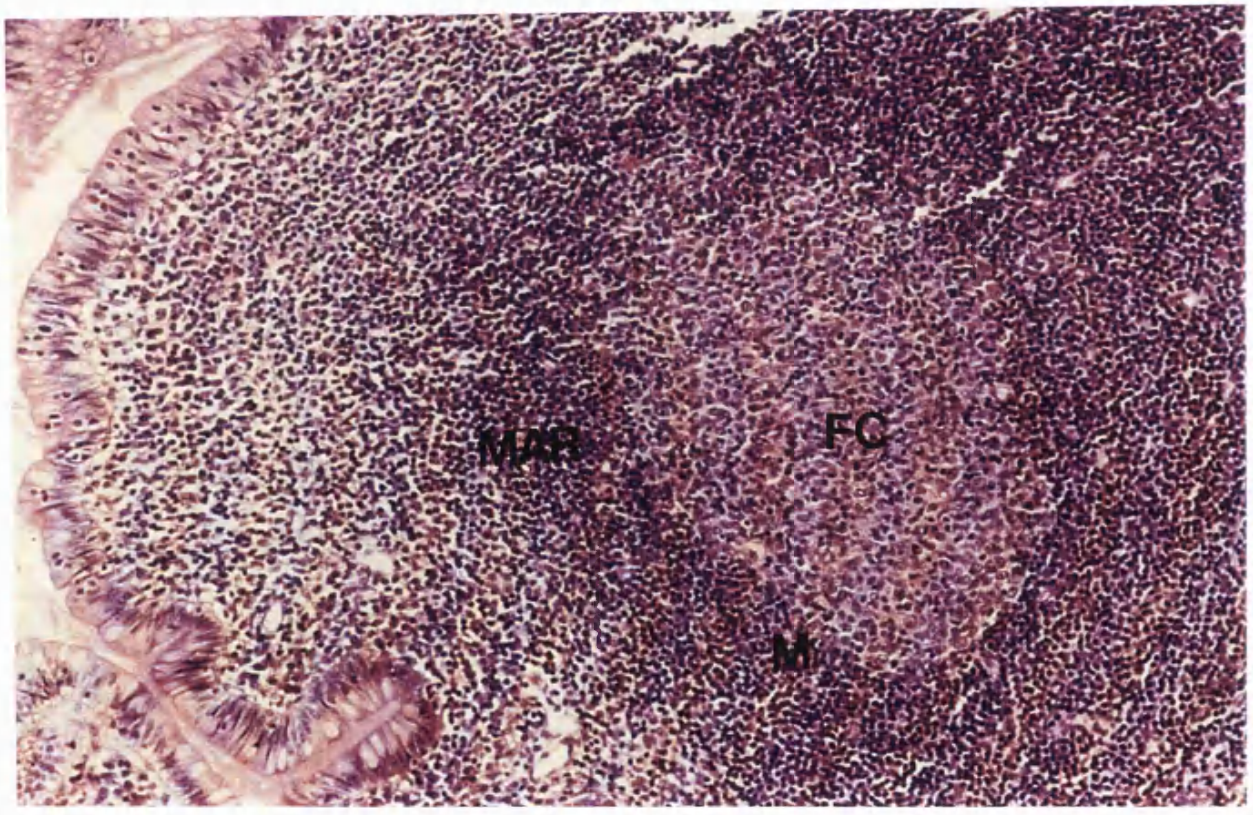


**Figure 1-1: Peyer's Patch,** showing follicle centre (FC), mantle zone (M) and marginal zone (MAR).

Unlike peripheral lymph nodes, which are structurally and functionally adapted to respond to antigen transported through afferent lymphatics, MALT has evolved to protect the mucosa from antigen in direct contact with the epithelial surface within the lumen. Antigens taken up by specialised mucosal epithelial cells (M cells) are transported to the mucosal organised lymphoid tissues where antigen specific B cells are stimulated to undergo switching from IgM to IgA producing B cells. [1,55] Following stimulation, the B cells leave the mucosa and enter the circulation via the mesenteric lymph nodes and the thoracic duct. These cells then home back to the mucosa.

## 1.3 MALT Lymphoma

### 1.3.1 Introduction

Lymphomas are neoplastic proliferations of B or T cells or their precursors.[58,59] They comprise about 5% of all malignancies diagnosed in adult males and about 6% in females. Three quarters of lymphomas are Non-Hodgkin's lymphomas (NHL). The majority of them are derived from B cells. Up to 20-40% occur in extranodal sites outside recognised lymphoid organs. [1]

In 1983, Isaacson and Wright noticed that, [9,10] just as follicular lymphomas mirrored the morphological features of lymphoid follicles, the extranodal lymphomas arising from the gastrointestinal tract closely resembled the morphology of MALT, as exemplified by Peyer's patches. They named these gastrointestinal lymphomas MALT lymphomas and considered them a specific group of NHL. [60,61] Later on, other extranodal lymphomas that arise from other mucosal organs, but share similar clinical and pathological features to gastric MALT lymphomas were also added to this group. [1,62]

Now, the concept of MALT lymphoma as a specific pathological entity is

accepted world-wide. The low-grade B cell MALT lymphoma has been incorporated into the recently proposed REAL (Revised European-American Lymphoma) Classification as a distinct lymphoma type, under the designation "extranodal marginal zone B cell lymphoma". [3,63,64] It is also a recognised entity in the new version (1997) of the World Health Organisation classification of hematolymphoid tumours. [65]

### 1.3.2 Clinical Features

B cell MALT lymphomas comprise the major proportion of the extranodal lymphomas. In Western countries, the stomach is the most common site accounting for 30-50% of cases, [7] followed by the small intestine and the colon. [1] In the Middle East, lymphomas of this type occur predominantly in the small intestine, presenting as a specific MALT lymphoma type, immunoproliferative small intestinal disease (IPSID). [66-68] They can also occur in a wide range of other mucosal organs, including salivary gland [11,69,70], thyroid [12,71], lung, [72-74], liver, [75] kidney [76] and breast, [77-79] and rarely, in orbit and conjunctiva,[14,80] bladder[81], thymus,[82] large intestine,[83], dura,[84] prostate, [85] testis, [86] larynx, [87-89] trachea, [90] and Waldeyer's ring. [91,92]

Low-grade MALT lymphoma often follows a previous history of chronic inflammation or autoimmune disease, [16] such as Hashimoto's thyroiditis in thyroid, [12] myoepithelial sialadenitis (MESA) in salivary glands, [11,82,93] follicular bronchiolitis in lung, [72] and chronic *H pylori* infection in stomach. [17]

The clinical presentation is usually more suggestive of a chronic inflammatory process than a lymphoma and naturally varies with the site of the disease. [1,4,93] For example, low-grade gastric MALT lymphoma occurs predominantly in individuals over age 50, with a peak in the 7th decade, although an increasing number of cases are being reported in patients in their 30s or

younger. The male-to-female ratio is approximately 1.5:1. The symptoms are usually those of non-specific inflammation. Severe abdominal pain and the presence of an abdominal mass are rare. The findings at endoscopy are usually those of non-specific gastritis and/or a peptic ulcer, the presence of a mass, again, being unusual.

One of the most striking clinical features of low-grade MALT lymphomas is that they have an indolent clinical nature compared with nodal lymphomas. [1,93] Some patients show no progression of disease for a number of years even without treatment. Although the involvement of bone marrow [21,22] and dissemination to other extranodal sites [28,30,31] may occur, the tumour is often localised to the primary site and/or regional lymph nodes for a long time. Therefore, the disease can potentially be cured by conservative therapy.

The clinical presentation of high-grade MALT lymphoma is similar to high-grade nodal lymphoma. [1,5] The mean age of patients is higher than that of patients with low-grade MALT lymphoma (64 v 55 years), but the sex distribution is the same. The duration of symptoms is shorter in patients with high-grade B cell lymphoma and the symptoms are similar to those seen in patients with gastric carcinoma and consist of pain, weight loss, and bleeding. Endoscopic examination usually shows an obvious tumour. The 5-year survival rate of patients with high-grade MALT lymphoma, although still better than that of comparable nodal lymphomas, is significantly worse than that of low-grade disease (75 versus 91%). [93]

### 1.3.3 Histopathology

Regardless of the sites of origin, all of the low-grade MALT lymphomas are closely similar to normal MALT, as seen in the Peyer's patches of the intestine (Figure 1-1). The histopathological features have been described by

Isaacson [1,2] and can be summarised as follows (Figure 1-2):

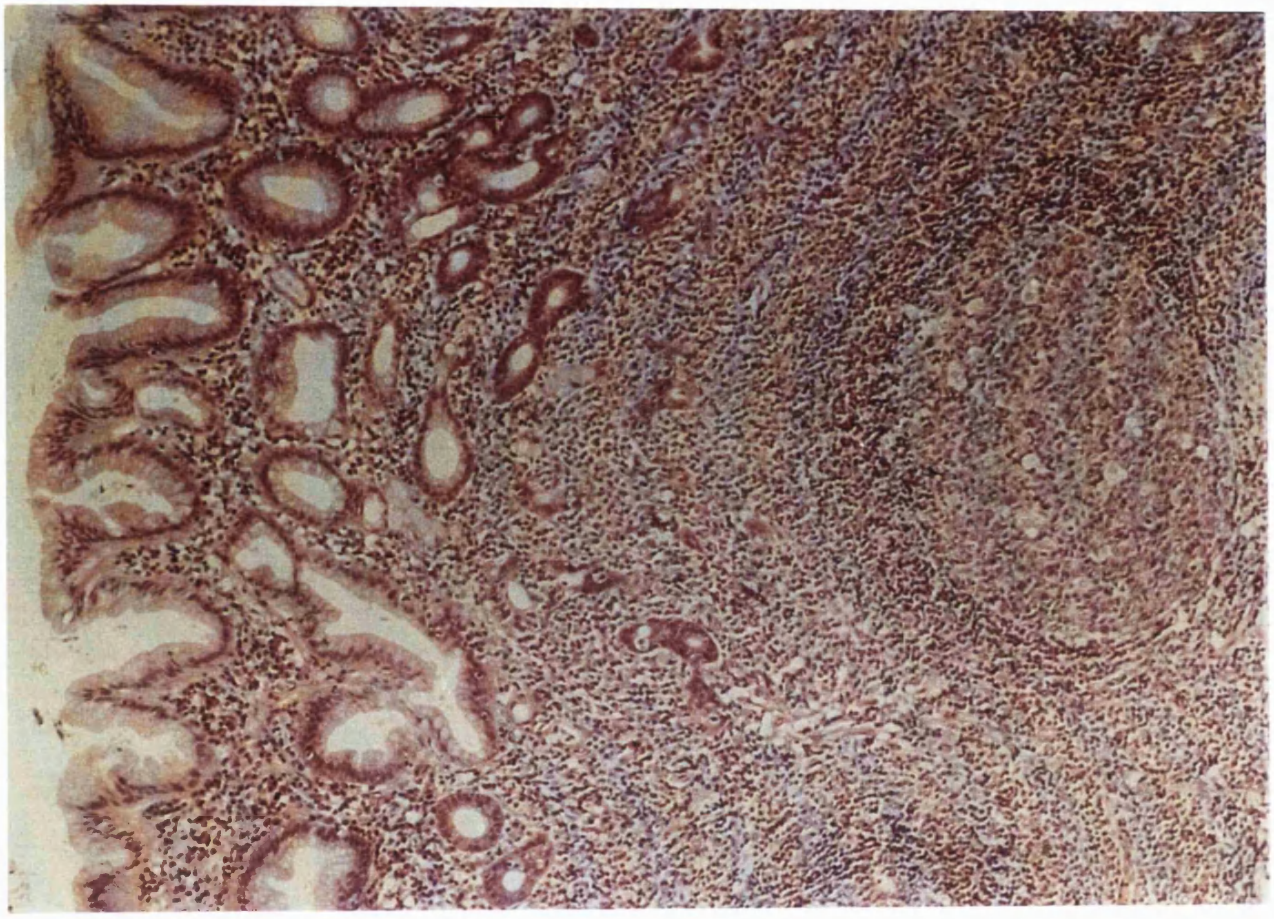

**Figure 1-2 A Low-grade B Cell MALT Lymphoma**

1. Reactive B cell follicles are an integral component.

2. Since the tumour cells bear a close resemblance to centrocytes, they are known as centrocyte-like (CCL) cells. In comparison with other nodal lymphomas, CCL cells have distinct phenotypes differing from mantle cell lymphoma with mantle cell origin and follicular lymphoma with follicular centre cell origin (see Table 1-1). However, they are morphologically and immunophenotypically almost identical to the marginal zone B cell

population (see Table 1-2), within which the lymphoma is thought to arise. [94-96]

3. CCL cells can not only infiltrate into pre-existing reactive B cell follicles and subsequently occupy the whole follicles (follicular colonisation), [71,97] but also selectively invade the epithelial lining of local glands, ducts or crypts, forming a central feature of low-grade MALT lymphoma, a lymphoepthelial lesion (LEL). [98]

**Table 1-1: The Phenotype of Tumour Cells from Low-grade B Cell MALT Lymphomas and Other Low-grade B Cell Lymphomas.**

| Lymphoma | CD5 | CD10 | CD23 | CD43 | Cyclin D1 | IgM | IgD |
|---|---|---|---|---|---|---|---|
| **Mantle cell** | + | -/+ | - | + | + | + | + |
| **Follicular** | - | +/- | +/- | - | - | + | +/- |
| **Marginal zone cell** | - | - | - | +/- | - | + | - |
| **MALT** | - | - | - | +/- | - | + | +/- |

**Table 1-2 Evidence Supporting the Hypothesis That Low-grade B Cell MALT Lymphoma Represents A Neoplasm of Marginal Zone B Cells**

| | |
|---|---|
| Morphologic | • Marginal zone growth pattern in the primary tumours<br>• Selective involvement of the marginal zones of lymphoid follicles in the regional lymph nodes and spleen when these sites are involved |
| Immunologic | • Lack of expression of CD5 (positive in small lymphocytic and mantle cell lymphoma)<br>• Lack of staining for cyclin D1 (mantle cell lymphoma)<br>• Lack of expression of CD10 (commonly expressed in follicular lymphoma)<br>• Immunophenotypic similarity to marginal zone B cells (predominantly CD5-, IgM+, IgD-) |
| Genotypic (See below) | • Lack of ***bcl-1***, ***bcl-2***, ***c-myc*** and ***bcl-6*** gene rearrangements<br>• Somatic hypermutation of ***Ig*** gene indicating an antigen selection process, consistent with a post-germinal centre stage of differentiation, and similar to the normal marginal zone B cells |

4. Plasma cell differentiation can be observed in approximately one third of MALT lymphomas. [1] Plasma cells may occur beneath the epithelium or

form the major part of the tumour in IPSID type MALT lymphoma. [66-68]

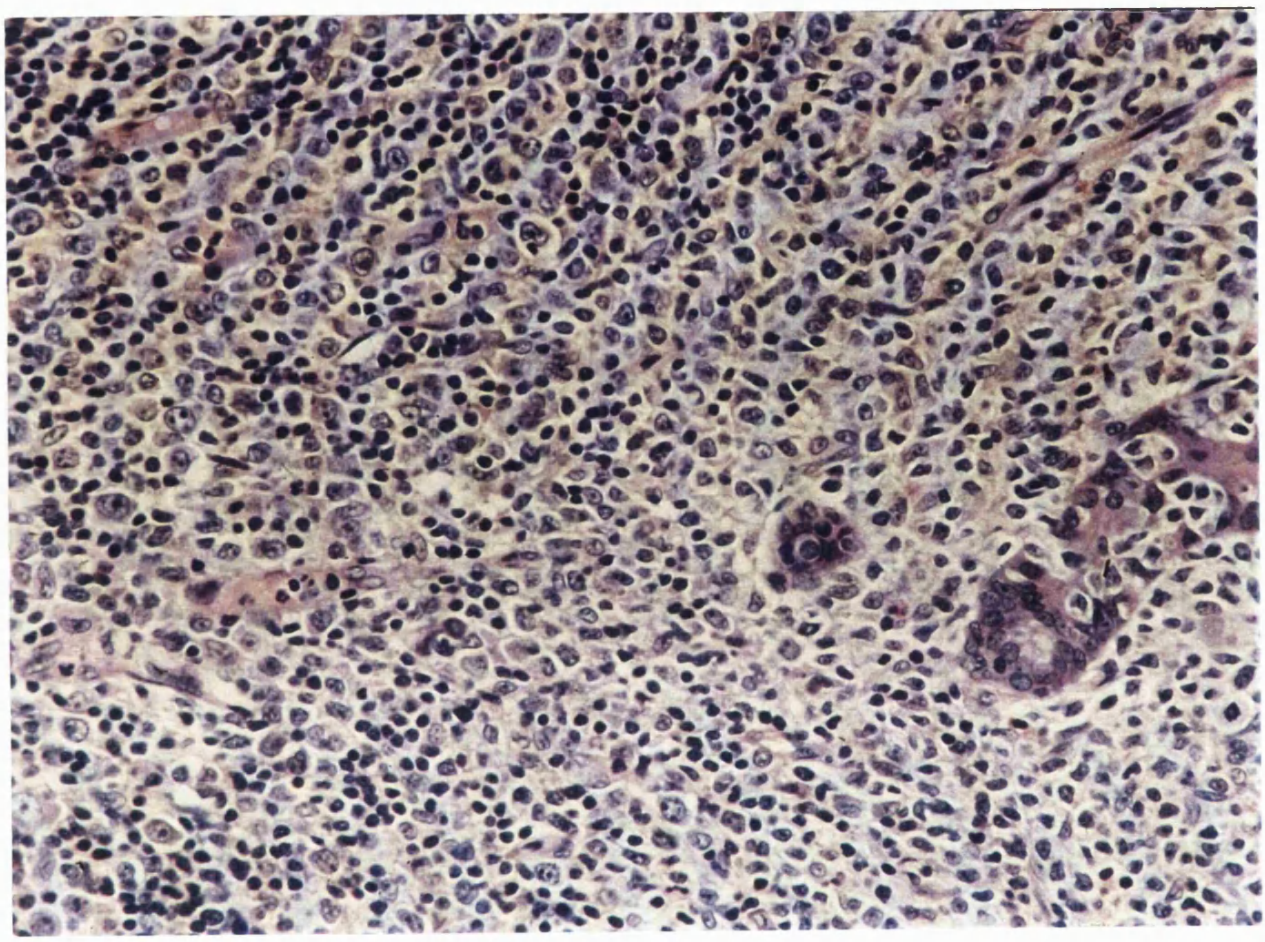

**Figure 1-3 A High-grade B cell MALT Lymphoma with Low-grade Components**

An indolent low-grade MALT lymphoma can eventually evolve into an aggressive high-grade tumour. [34] This may be particularly true for gastric MALT lymphoma since the stomach is the most common site for the tumour and most gastric MALT lymphomas are high-grade. [1,65] The pathological features of high-grade MALT lymphoma are not specific. [1,34] (Figure 1-3) The cells resemble centroblasts with abundant cytoplasm and large nuclei containing one to three nucleoli. The infiltrate is usually diffuse and the

glandular structures are destroyed in the centre of the lesion. At the periphery, the cells infiltrate around the glands but LELs may be identified. [99] Follicles may be present but are less common than in low-grade lymphoma and the follicular dendritic cell network is infrequently identifiable in the centre of the lesions.

The mechanism of high-grade transformation is not clear. Low-grade lymphoma often contains some small high-grade foci in colonised follicle centres; while high-grade lymphoma is characterised by a predominance of high-grade cells with small residual low-grade foci that can be difficult to find . [99] Immunocytochemistry demonstrates that coexisting low and high-grade lesions share the same ***IgL*** expression. These pathological and clinical observations have led to the hypothesis that most, if not all, high-grade MALT lymphomas directly develop from low-grade lesions. [34]

### 1.3.4 Molecular Genetics

#### 1.3.4.1 Clonal Features Demonstrated by Immunoglobulin Heavy Chain (*IgH*) Gene Rearrangement

It is widely accepted that the vast majority of cancers are of unicellular origin and therefore monoclonality is the hallmark of tumours. [100,101] In B cell lymphoma, monoclonality is commonly detected by demonstrating the specific ***IgH*** gene rearrangement of B cells using Southern blotting [19,102,103] or PCR.[20,104,105]

Human ***IgH*** gene is located on chromosome 14 at q32. It is composed of many non-identical gene segment repeats that must rearrange into a functional unit prior to transcription and production of the protein. The ***IgH*** gene consists of clusters of variable regions (V, >100), diversity regions (D,

about 30) and joining regions (J, 6) (Figure 1-4). Rearrangement of the gene involves excision of DNA between the D and J clusters resulting in D-J joining followed by a second excision between the D and V cluster resulting in V-DJ joining (Figure 1-4). The VDJ unit becomes the coding sequence for the variable part of the ***IgH*** protein, which is expressed in conjunction with constant (C) region exons. Variable numbers of untemplated nucleotides (N regions) are inserted at the junctions between the V, D and J segments. Diversity of ***IgH*** structure is achieved by unique combinations of different V, D and J regions (recombinatorial diversity), by variability in the precise joints between the segments (junctional diversity) and by somatic point hypermutation. Therefore, each B cell has a unique ***IgH*** gene rearrangement.[106-108] B cell populations originating from a single cell display a unique ***IgH*** rearrangement pattern, while the lymphocytes originating from polyclonal B cells display multiple rearrangements.

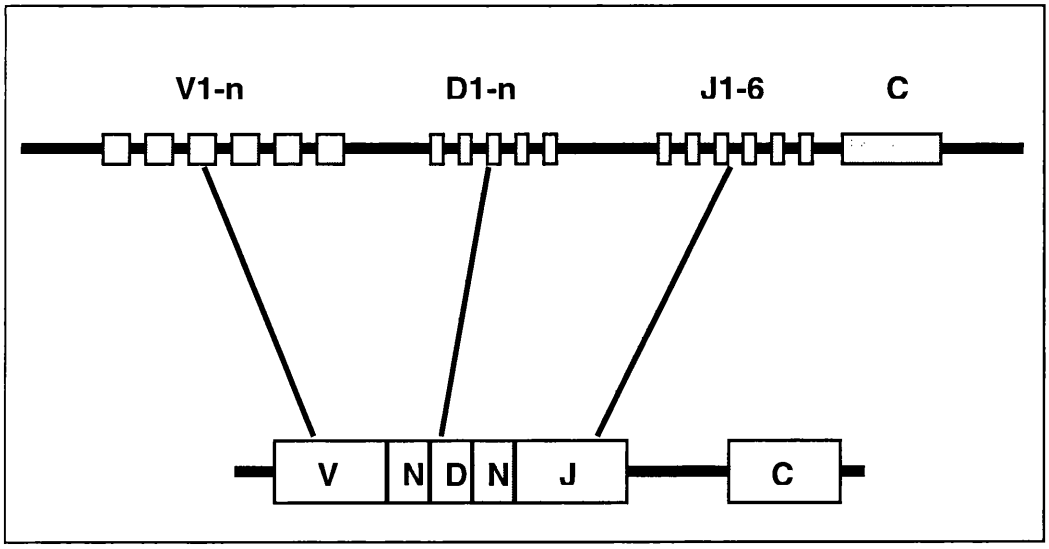


**Figure 1-4 *IgH* Gene Rearrangement.** The germline configuration of multiple segments (above) rearranges by D-J followed by V-DJ joining with insertion of N regions at each junction to form the coding sequence for the variable region (below). A singe constant region (C) is shown.

Detection of ***IgH*** gene rearrangement has been widely used in the clonality analysis of B cell lymphoma. [106,109] In early MALT lymphoma studies, ***IgH***

rearrangement detected by Southern blot hybridisation analysis has contributed significantly to the redefinition of lesions previously thought to be pseudo-lymphomas as malignant MALT lymphomas. [19] Recently, a strategy involving a semi-nested PCR protocol was developed. [20,109] The technique is based on amplification of the ***IgH*** gene variable regions, which are unique to each B cell, by primers directed to framework 3 (FR3) and JH regions. The principal of the strategy is illustrated in Figure 1-5. Using this method, more than 80% of low-grade and 35% of high-grade MALT lymphomas have been shown to contain a dominant ***IgH*** gene rearrangement, suggesting monoclonality. [20,109]

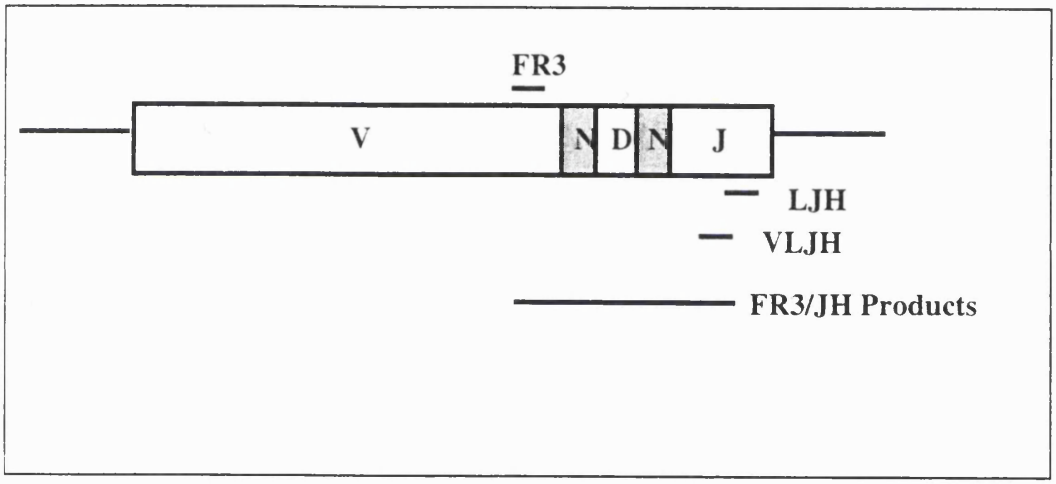


**Figure 1-5 Diagram to Show the Primer Sites on the Rearranged VDJ Regions of the *IgH* Gene.** The products from FR3/JH amplifications are shown below. The highly variable junctions between the V-D and D-J segments, which include the N regions and provide the high diversity of products sizes are shaded.

Rearranged ***IgH*** fragments can also be used as tumour specific clonal markers to trace tumour dissemination. By cloning and sequencing PCR products of rearranged ***IgH*** gene fragments, dissemination of a malignant clone from stomach to lip and then to bone marrow over a 13 year period in a patient with low-grade gastric B cell MALT lymphoma was traced. [30] The results provide important evidence for the tendency of MALT lymphomas to spread to other mucosal sites.

This technique may also be used to study the high-grade transformation of low-grade lymphomas. In a study of Richter's syndrome, [110] identical or different rearranged ***IgH*** genes were found in original chronic lymphocytic leukaemia (CLL)/small lymphocytic lymphoma (SLL) and transformed diffuse large cell lymphoma (DLL) of the same patients, suggesting DLL may be clonally-related to or clonally-distinct from the original CLL/SLL. Using this technique, the genetic link between coexisting low and high-grade lesions in MALT lymphomas has been studied (chapter 3).

### 1.3.4.2 Genetic Abnormalities

Low-grade B cell MALT lymphoma does not show the cytogenetic and molecular biological features typical of certain nodal lymphomas. [1,111] Thus, neither the t(8;14), t(14;18), t(11;14) nor the 3q27 abnormalities, which characterise Burkitt's lymphoma, [112,113] follicular lymphoma, [114-116] mantle cell lymphoma [113,117,118] and diffuse large cell lymphoma, [119] have been identified in cytogenetic studies of MALT lymphoma. [32] Similarly, low-grade MALT lymphomas do not show any gene rearrangements of ***c-myc***, [35] ***bcl-1***, [35,120] ***bcl-2***, [35] or ***bcl-6*** (unpublished data).

Abnormalities at 1p22 (translocation with chromosome 14 or inversion) have been found in four cases of B cell MALT lymphoma (1 unpublished data in the lab). [32,121] Histological examination has shown that the cases with these abnormalities have a wide extranodal dissemination, active proliferation and follicular colonisation. In-vitro experiments have shown that tumour cells from these cases can grow in ordinary culture without any of the external stimuli normally essential for in-vitro growth of MALT lymphoma cells. [122] These results suggest that deregulation of a gene at chromosome 1p22 is closely associated with the evolution and progression of these tumours. The cloning

of these breakpoints and identification of this potential oncogene, which is under way, should help our understanding of the pathogenesis of MALT lymphoma.

T(11;18)(q21;q21) has been found in more than 50% of low-grade MALT lymphomas, but not in high-grade MALT lymphomas by several individual groups in studies of a large series of cases. [123-127], suggesting that this translocation represents an early event in the genesis of MALT lymphoma. Genes located at the breakpoint sites of chromosome 11 and /or 18 may be also crucial to the pathogenesis of MALT lymphoma.

Another cytogenetic abnormality in low-grade MALT lymphoma is trisomy 3 observed by in-situ-hybridisation and cytogenetics, which has not been a consistent finding in other low-grade B cell lymphomas. [33,128] The role of trisomy 3 is unclear. It might be related to quantitative changes in the expression of oncogenes located on this chromosome, such as ***bcl-6***. Presumably, extra copies of the chromosome increase oncogene transcription.

The genetic abnormalities of high-grade MALT lymphoma are poorly understood. Available data show that, like low-grade MALT tumours, high-grade MALT lymphomas demonstrate an absence of ***bcl-1***, ***bcl-2*** and ***bcl-6*** rearrangement and the presence of trisomy 3. [35,128] However, in a single report with a small number of samples, [129] a high incidence of ***c-myc*** gene rearrangement was reported in high-grade MALT lymphomas, which suggested that ***c-myc*** gene alteration might play a role in high-grade transformation.

Limited karyotypic studies have shown deletion of chromosome 9p in several cases. [32] This finding is interesting, as the tumour suppressor genes encoding the cell cycle inhibitors, ***p15*** and ***p16***, are located on the short arm of

chromosome 9. [130-133] In a preliminary study in this lab, using Southern blot hybridisation, with a ***p16*** gene probe and an image analysis system, loss of the gene has been detected in 5 of 60 cases of MALT lymphoma. [134] More recently Neumeister et al examined 28 low-grade and 14 high-grade gastric MALT lymphomas and showed homozygous deletion of ***p16*** in 2 high-grade cases, suggesting a role for ***p16*** deletion in high-grade transformation in some cases.[135]

In addition, loss of heterozygosity (LOH) of other tumour suppressor genes including ***DCC, APC, RB1, WT1*** and ***NM23*** has also been examined in 12 cases in a single study by PCR-based analysis of appropriate microsatellite loci. [136] LOH of the ***DCC*** gene was seen in two cases during the transition from chronic gastritis to low-grade MALT lymphoma and in one case during the high-grade transformation of low-grade tumour. LOH of the ***APC*** gene was also noted in two high-grade tumours and in one case LOH occurred during high-grade transformation. Since the sample size was small, this finding needs to be confirmed.

Apart from genetic alterations, Epstein-Barr Virus (EBV) infection has also been detected in both low and high-grade MALT lymphomas, [137-139] suggesting a pathogenic role of EBV in the development of MALT lymphoma. In situ hybridisation demonstrated that EBV infection was rare in primary low-grade tumour cells, but present in the local reactive lymphoid cells, indicating its possible involvement in autoimmune reactions in the tumours. However EBV infection was demonstrated in a portion of high-grade MALT lymphomas by in situ hybridisation (ISH), suggesting a role in high-grade transformation. [138] In addition, Hepatitis C virus (HCV) infection has been found to be associated with MALT lymphomas, [140,141] although its causal link with disease has not yet been established.

## 1.4 Development of Gastric MALT Lymphoma and Its Association with *H pylori*

### 1.4.1 Introduction

Although MALT lymphomas may arise at almost any extranodal site, most of our knowledge about their pathogenesis has been derived from the study of gastric lesions. [1,8,142-144] This is largely because it is the most common site of primary MALT lymphoma, but also because gastric MALT lymphomas are usually diagnosed prior to surgery, allowing arrangements for the collection of fresh tissue to be made.

Low-grade gastric MALT lymphoma arises from acquired organised MALT [17] and can eventually transform into a high-grade tumour. [34] Both low and high-grade gastric lymphomas, along with the putative pre-lymphomatous lesions (acquired organised MALT), are aetiologically related to *H pylori* infection.[17;142] Understanding the role of *H pylori* may provide insight into the pathogenesis of MALT lymphoma.

### 1.4.2 *H pylori* and Its Association with Gastric MALT Lymphoma

*H pylori*, originally named Campylobacter pylori, is a unipolar, multiflagellate spiral shaped, microaerophilic, Gram negative bacterium that lives in the luminal surface of the stomach and duodenum. [145,146]

*H pylori* infection is widely distributed in human populations (20-80%). [146,147] The prevalence of infection varies depending upon the age, the geographic location, and the socio-economic status of the subjects studied.[146] In most infected individuals, *H pylori* is well tolerated, with few or no symptoms for decades. However, infection with this organism is a significant risk factor for

development of gastritis, [148] peptic ulcer, [149] and gastric carcinoma. [150] There is ample evidence supporting an etiologic link between *H pylori* infection and the development of low-grade gastric B cell MALT lymphoma:

1. The organism has been detected in approximately 90% of gastric lymphomas, which is well over the prevalence of *H pylori* in most populations (Figure 1-6). [17,151,152]

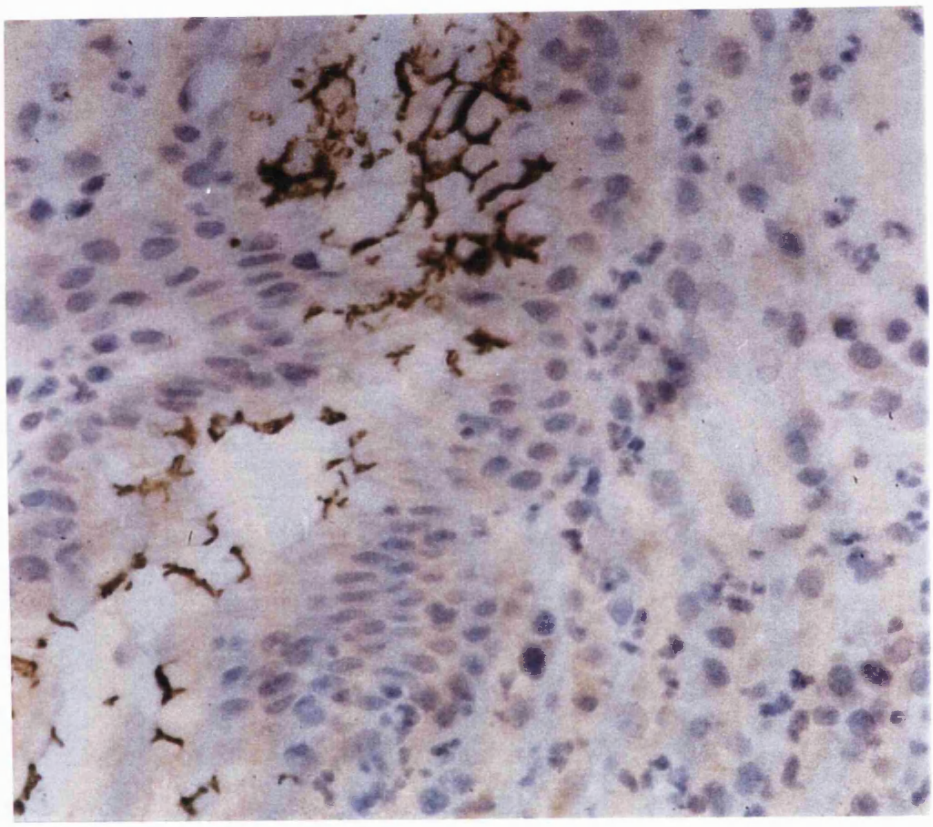

**Figure 1-6 *H pylori* Infection in a Low-grade B Cell MALT Lymphoma.**

2. There is a high incidence of gastric MALT lymphoma in north-eastern Italy, reflecting a high prevalence of *H pylori* infection in this area. [153] In contrast, there is a low prevalence of gastric lymphoma in a number of

British towns, which have a low prevalence of *H pylori*. [153]

3. A retrospective study of stored serum samples has shown that patients with gastric MALT lymphomas are more likely to have positive *H pylori* serology than matched controls. [154]

4. A significant proportion of gastric low-grade B cell MALT lymphomas have shown regression following anti-*H pylori* therapy. [155-158]

The precise role of *H pylori* in MALT lymphomas is not clear. As in other bacterial diseases, the development of *H pylori*-associated MALT lymphoma is hypothesised to be associated with the host response and bacterial status.[159] Since *H pylori* possesses a variety of cell surface and secreted proteins, it is a very immunogenic organism and therefore could be responsible for the generation of host humoral and cell mediated responses observed in infected individuals. [160] Thus, antigen stimulation by *H pylori* may play an important role in the development of MALT lymphomas. On the other hand, some *H pylori* strains contain certain genes that can produce cytotoxic materials and directly or indirectly promote tumour development. [161,162] One of these genes is cytotoxic associated gene A (***CagA***). [163] Bacteria expressing ***CagA*** have been closely associated with other serious *H pylori*-related diseases, such as gastric ulcer and gastric carcinoma, and, therefore, are considered more virulent. [161,162] There is a possibility that MALT lymphoma development is associated with the cytotoxic effects of these aggressive bacterial strains.

### 1.4.3 Antigen stimulation

As described previously, MALT lymphoma has a prolonged period of localised growth, although eventual systemic spread of the tumour cells is

frequent. [28-30,164] If the growth of gastric MALT lymphoma was influenced by local antigens, these features could be easily explained. This hypothesis was initially supported by two pieces of evidence:

1. Local antigen stimulation was first suggested by certain histological features, [4,5] including the presence of scattered transformed blasts, plasma cell differentiation and follicular colonisation. Plasma cell differentiation is usually most prevalent beneath the surface epithelium and suggests that the tumours respond to antigen either in the lumen of the stomach or in the gastric epithelium itself. The phenomenon of follicular colonisation is thought to be a recapitulation of the movement of marginal zone B cells into the follicle centre following antigen stimulation.

2. The second line of evidence has been obtained from observation of gastric MALT lymphoma development. Normal stomach is devoid of any organised lymphoid tissue. The majority of low-grade gastric MALT lymphomas arise within acquired MALT following *H pylori* infection in the gastric mucosa. [2,5,17,62,165] The eradication of *H pylori* with antibiotics may result in the histological and clinical regression of the lymphomas. [155-158] These observations suggest that the lymphocytes activated by *H pylori* within the acquired MALT are programmed to respond to this organism. The lymphoma cells that may develop within this acquired MALT retain the ability to respond to the immunological proliferative drive associated with the continued presence of the organism.

The nature of the antigen is not entirely clear. It appears that the lymphoma cells recognise autoantigen rather than antigens of *H pylori*. Hussell et al. have studied the tumour ***Ig*** specificity in 3 cases of gastric MALT lymphoma by immunohistochemistry and ELISA and clearly demonstrated distinct but specific immuno-reactivity to normal tissue components, such as follicular

dendritic cells, basement membrane and IgG, in these cases. [36] More recent studies on VH germline usage also provide indirect support for this hypothesis. In these studies, gastric MALT lymphomas were frequently shown to use the VH germline segments commonly involved in autoimmune diseases, [166-169] such as DP10 and DP54. [170-172] In view of the diversity of VH germline segments used by this tumour, different tumour precursor cells are most likely to recognise distinct autoantigens.

Physiologically, antigen exposure invokes the activation and proliferation of a polyclonal B cell population. In low-grade gastric MALT lymphoma, antigen stimulation may generate an actively proliferating B cell population and also directly trigger and sustain, at least in part, the expansion of the tumour clone. Recent studies indicate that both direct and indirect antigen stimulation mechanisms are involved.

1. **Direct antigen stimulation:** Several histological features of low-grade MALT lymphoma, such as the intimate interaction of tumour cells with reactive follicles (known as follicular colonisation) and its association with active tumour cell proliferation and blast transformation, [71,97] suggest that the tumour cells may receive antigen stimulation via antigen receptors. This notion is reinforced by more recent studies of somatic mutations of tumour derived ***Ig*** genes. Of the low-grade gastric MALT lymphomas examined, the majority of cases show evidence of intraclonal sequence variations i.e. ongoing mutations. [31,167,173] As somatic mutations occur at the ***Ig*** locus specifically in B cells engaged in the germinal centre reaction, an antigen driven immune response, [174] the finding of ongoing mutations strongly suggests that direct antigen stimulation plays a role in the clonal expansion of this tumour. This is supported by genetic evidence that evolution of the tumour clone is constantly under a positive or negative selection pressure. [31,167,175] Moreover, cross-linking surface ***Ig*** on tumour

cells in some low-grade MALT lymphomas has been shown to stimulate the tumour cells to proliferate. [122] There are some preliminary data suggesting that the rate of ongoing mutations gradually declines during the evolution from an early to a late stage of low-grade tumour and that finally the activity disappears in high-grade lesions. [31,167] Thus, it is likely that the role of direct antigen stimulation in the pathogenesis of this tumour progressively decreases during tumour evolution. Interestingly, gastric lymphoma cells recognise autoantigen rather than antigens of *H pylori*. [36]

2. **Indirect antigen stimulation:** The close association of *H pylori* infection with gastric MALT lymphoma development prompted research into the immunological responses of the tumour cells to *H pylori*. By co-culture of tumour cells with 13 clinical strains of heat-killed *H pylori*, Hussell et al. demonstrated that *H pylori* induced active proliferation of low-grade tumour cells. The effect was strain -specific but was T cell mediated and not due to specificity of lymphoma cells for *H pylori* antigens. [37] Subsequent study by these authors confirmed that *H pylori* did not directly stimulate the lymphoma cells but specifically activated tumour infiltrating T cells, [176] which then helped the malignant B cells to proliferate through direct T-B cell interaction via CD40-mediated signalling. [176,177] Despite the indirect role, the stimulatory effect of *H pylori* is of major importance since it has been shown in clinical studies that antibiotic eradication of the organism often leads to regression of low-grade MALT lymphoma. [155,178,179]

Unlike low-grade tumour cells, high-grade tumour cells do not show any growth response to *H pylori* mediated T cell stimulation in vitro. [37] Similarly, high-grade lymphomas do not respond to *H pylori* eradication therapy. [157,179] These findings suggest that the growth of high-grade tumour is no longer dependent on an *H pylori* mediated immune response.

### 1.4.4 CagA+ *H pylori* Strain Infection

*H pylori* is highly variable at the genetic level. Polymorphisms include point mutations, mosaicisms, non-conserved genes, size differences of particular genes, insertion sequences, chromosomal rearrangements and plasmids. [180] One feature that differentiates *H pylori* strains is the ***CagA*** locus. [181] *H pylori* strains may be classified as ***CagA***+ or ***CagA***- based on whether or not they possess this gene.

Depending on the geographic and ethnic distribution, as well as detection methods, the status of ***CagA***+ *H pylori* varies. [182,183] Serologic responses to ***CagA*** have been detected in *H pylori*-infected persons with peptic ulcers,[182,184] atrophic gastritis [185,186] and gastric carcinoma [187] more frequently than in infected individuals with gastritis alone. The incidence of ***CagA***+ strain bacterial infection in MALT lymphoma is not clear. There are two publications available from two groups on this issue. [188,189] DeJong et al [189] studied *H pylori* cultures isolated from 12 MALT lymphomas for ***CagA*** genes by PCR and did not observe a different frequency of ***CagA***+ strain infection between the gastritis controls and MALT lymphomas. However, because of the small sample size, it is hard to draw any firm conclusions. Eck et al [188] studied sera of 49 patients with *H pylori* infected gastric MALT lymphoma by immunoblotting and found that 95.5% of MALT lymphoma patients were seropositive for ***CagA***. However, since there is a discrepancy between local mucosal and systemic humoral responses to the ***CagA*** products, Eck's experiment needs to be reviewed by a more direct method.

It is still an open question whether the ***CagA*** gene represents a virulence factor itself or is only an aggression marker. [190] In-vitro and in-vivo studies showed that the strain containing the ***CagA*** gene could increase expression

levels of some mucosal cytokines, especially of IL-8, an important chemotactic and activation factor for neutrophils. [184,191,192] Continuous up-regulation of IL-8 and neutrophil activation could lead to increases of free radicals which have carcinogenic potential. [44,45] On the one hand, free radicals can cause transient disorders in the expression of some oncogenes, such as ***c-fos***, ***c-myc*** and ***c-jun.*** [45] On the other hand, they can induce a wide range of DNA damage, in the form of gene mutations, increasing sister chromatin exchanges, gene amplification, DNA strand breaks, deletions or translocations. [44,45,193] These genetic changes may directly promote the development and progression of tumours if they occur in tumour related genes. Furthermore, multiple genetic alterations occurring in the target cells may saturate the DNA damage repairing system and cause genetic instability, which may indirectly promote tumour formation.

## 1.5. The Molecular Basis of B Cell Lymphomagenesis

The molecular basis of MALT lymphoma progression remains unknown. The model of nodal B cell lymphomagenesis may provide some clues for the studies of MALT lymphoma.

### 1.5.1 Introduction

It is thought that B cell lymphomas generally arise by the clonal expansion of single B cells that have been arrested at a particular stage of maturation.[38,194,195] Despite the enormous variation in B cell lymphoma types, a common theme of B cell lymphomagenesis is that cell proliferation controls are lost at the level of either cell signalling, cell-cycle arrest, differentiation or apoptosis. [38,196,197] At the gene level, all of these cellular disturbances are caused by multiple specific genetic abnormalities, which are accumulated gradually during lymphoma development. These specific genetic

abnormalities can be broadly catalogued into two interrelated groups: activation of oncogenes and inactivation of tumour suppressor genes. [198,199] However since normal cells have a tightly controlled surveillance system to maintain genome integrity, these specific genetic abnormalities can only be accumulated when cells exhibit genetic instability (Figure 1-7).

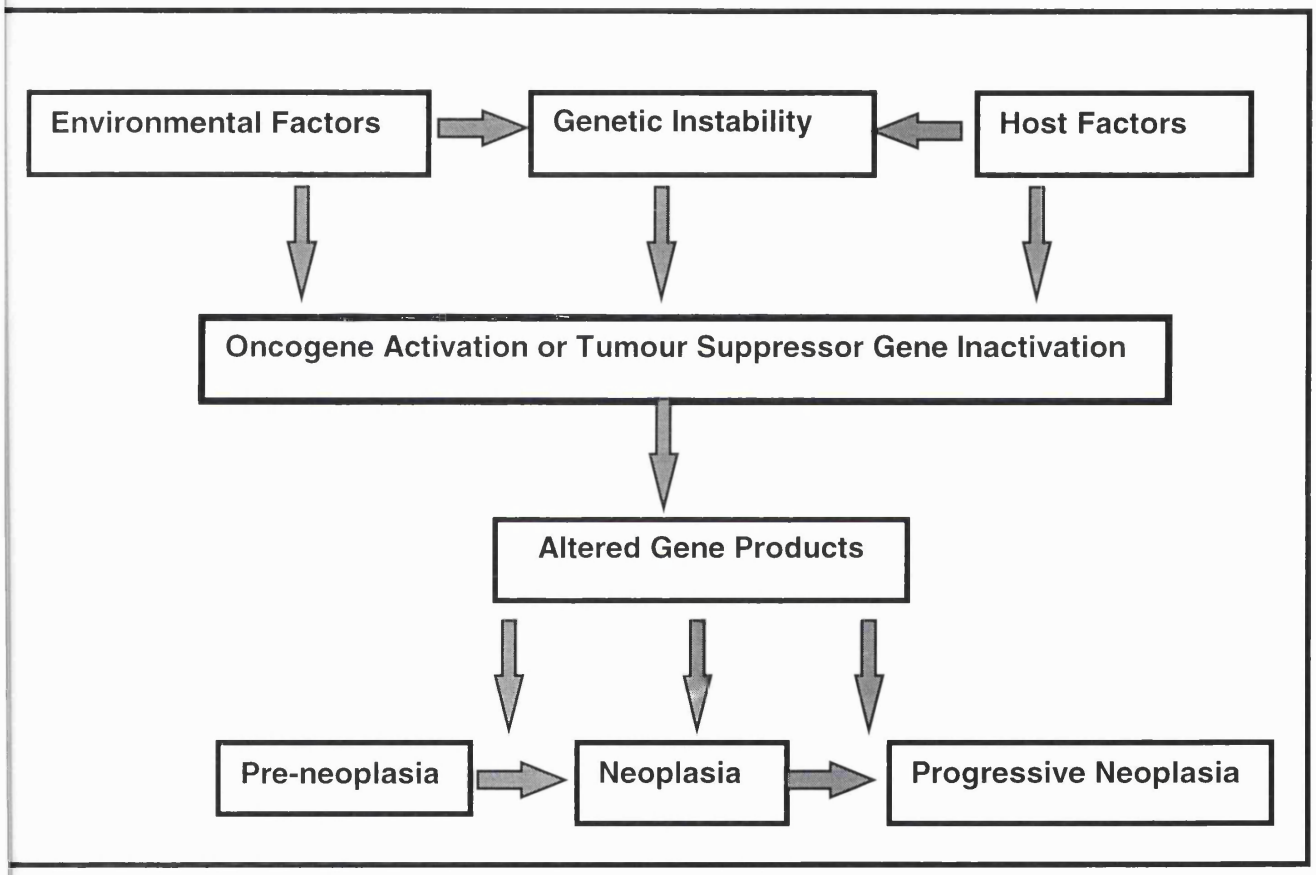


**Figure 1-7 Flowchart Depicting How an Interaction between Environmental and Genetic Factors May Give Rise to Neoplasia**. The interplay of these elements first induced genetic events, usually multiple, in somatic cells by such mechanisms as genetic instability, oncogene activation, tumour suppressor gene inactivation or all three. Such changes then lead to the synthesis of gene products that promote the formation of pre-neoplastic lesions as well as influence the progression from pre-neoplasia to neoplasia to clonally evolved, progressive neoplasia

### 1.5.2 Genetic Instability

### 1.5.2.1 Genetic Instability and Tumours

A malignant tumour is a disease caused by a combination of multiple specific genetic alterations, which are accumulated in a stepwise fashion from an enormous background of other non-specific genetic alterations. [38,41] However, in normal cells, the genetic integrity is finely controlled and guarded by many factors at different levels. [42,200] Alterations in the genome are prevented by the operation of an elaborate cellular apparatus that recognises and repairs genetic lesions. In addition, certain lesions that can not be readily repaired effectively trigger the cell to undergo apoptosis. In either case, the outgrowth of mutant cell clones is prevented. Thus, the spontaneous mutation rate in normal cells is very low, (estimated to be about $1.4 \times 10^{-10}$ mutation /base pair /cell division) [201] and inadequate to account for more than two genetic changes in a single cell. Therefore, it is hypothesised that in cancer cells, the normal protective mechanisms may be subverted at the genetic level and the mutation rate may somehow be substantially elevated. [42,200]

For example, individuals suffering from the disorders associated with defective DNA repair, such as Bloom's syndrome, [202] Fanconi's anaemia [203] and ataxia telangiectasia, [204] have a higher incidence of lymphomas and other tumours, along with a wide range of genetic abnormalities. [38] Furthermore, mutations of the ***p53*** gene, which is directly and /or indirectly involved in DNA repair and apoptosis in normal cells (see below), can result in the outgrowth of cells that may have sustained serious genomic damage. [205]

Recently a striking observation of genetic instability was made in hereditary nonpolyposis colon cancer (HNPCC) syndrome. The affected individuals demonstrated a high frequency of replication error (RER+) phenotype [206-211]

that has now become an indicator of genetic instability (see below). [212] The disease was linked with disorders of the DNA mismatch repair genes, such as ***hMSH2, hMLH1, hPMS1*** and ***hPMS2.*** [213-218]

Now, genetic instability, manifested as multiple genetic abnormalities, has been reported in a variety of human cancers, and is thought to be a hallmark of cancer cells. [42,43]

Little data are available about genetic instability in lymphomas. However, there are some lines of evidence suggesting that genetic instability may play a role in their pathogenesis. Firstly, multiple genetic alterations from the chromosome level to the gene level have been demonstrated. [219] Secondly, the incidence of most lymphomas exhibits an exponential rate of increase with age, suggesting a series of events each rendered more likely by the acquisition of a previous event. [38] Finally, a higher incidence of lymphomas occurs in the disorders associated with defective DNA repair, such as Bloom's syndrome, [202] Fanconi's anaemia [203] and ataxia telangiectasia, [204] although immunological deficiency is almost certainly a contributing factor in such individuals. [38]

### 1.5.2.2 The RER+ Phenotype- an Indicator of Genetic Instability

The RER+ phenotype was first described in HNPCC in 1993. [206,207,215] It was subsequently found in a wide range of other tumours with or without other known genetic abnormalities at early and /or late stages of the diseases. [220-230] Although it is not as common as in solid tumours, the RER+ phenotype has also been observed in lymphoid malignancies, such as chronic lymphocytic leukaemia, [231], Burkitt's lymphoma and HIV related lymphomas. [232]

The RER+ phenotype is defined by microsatellite abnormalities. [201,212,213]

Microsatellites are normal regions of the genome which are composed of one to six bases of repeated DNA sequences. [233] The most common repeat is (A)n, and the second most common is (CA)n, where n=10 to 60. They are highly polymorphic between individuals. The microsatellite pattern is genetically fixed for life in all of the tissues when the individual is born. However this germline pattern of microsatellites is unstable in some tumours and the instability is manifested as either an increase or a decrease in the number of the elements in the repeats. This results in an increase or decrease in the size of the microsatellite when measured by PCR and electrophoretic techniques. (Figure 1-8)

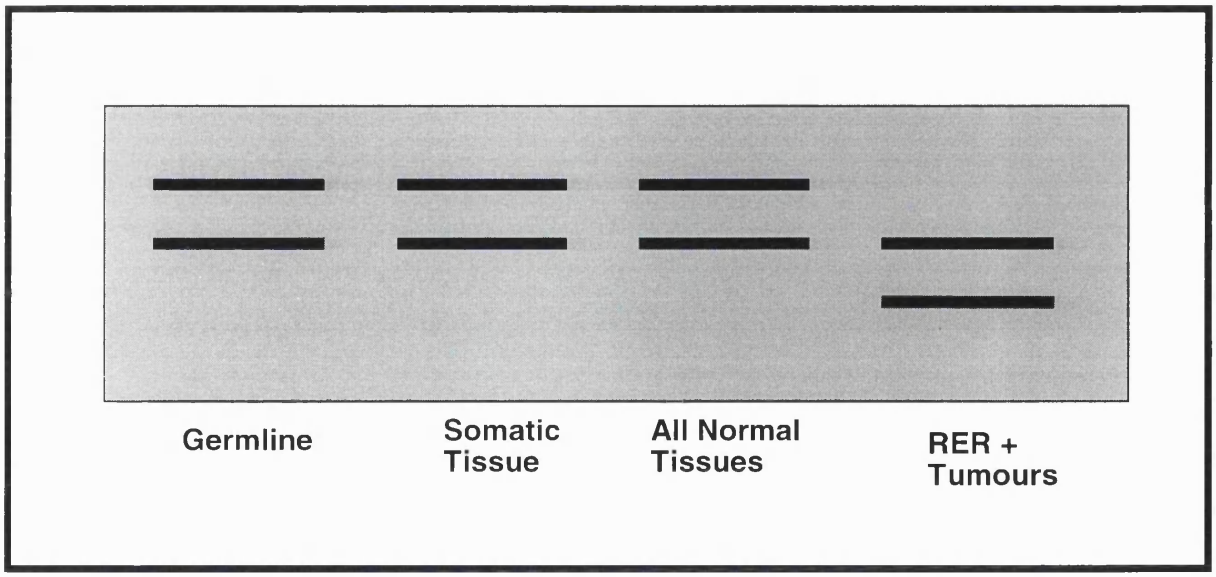


**Figure 1-8: PCR Amplification of Normal and Tumour Tissue at One Microsatellite Locus.** Because primers have been chose to span the microsatellite, the size of the band reflects the number of elements in the repeat. After PCR amplification of genomic DNA, the size of the microsatellite is measured by polyacrylamide gel electrophoresis. Two alleles present in each sample correspond to the maternally and paternally inherited copies. The individual shown here yield a germline pattern consisting of a (CA)n (top band) and (CA)m (bottom band). One would expect to see these same bands in all somatic tissues, including the individual's tumours. If the individual was susceptible to RER tumour, the size of the tumour's microsatellite could differ from the germline pattern.

The mechanisms underlying microsatellite abnormalities are not fully understood. One postulated model for microsatellite alteration involves DNA polymerase slippage during replication, resulting in insertion-deletion loop-type (IDL) mismatched nucleotides that contain integral numbers of the repeated sequences. [215] In normal conditions, the IDL can be recognised and repaired by post-replication-DNA-mismatch-repair-genes, such as ***hMSH2, hMLH1, hPMS1*** and ***hPMS2***. Therefore, the mutation rate in normal cells is very low, around $10^{-5}$-$10^{-6}$. In the absence of mismatch repair genes or other genes related to repair / replication pathways, these IDL mismatched nucleotides may be fixed into the genome in a subsequent round of replication, producing the microsatellite alterations.

The role of microsatellite alteration in tumour development remains unknown. Microsatellite sequences may play a role in chromatin structure maintenance and nucleosome placement. [233,234] The microsatellite alterations are assumed to result in transcriptional alterations. Some microsatellite sequences exist within tumour suppressor genes. One example is the alteration of a $G_8$ sequence in exon 3 of the ***bax*** gene observed in gastric carcinoma, [235] colon cancer [236] and some malignant lymphoid cell lines. [237-239] Increasing or decreasing the sequence length can produce truncated ***bax*** protein and is thought to promote cancer formation. However since the majority of microsatellites are located within non-coding sequences, [233,234] most of their alterations found in cancers are probably not directly involved in tumourigenesis. The genetic instability may predispose cells to the accumulation of other genetic alterations necessary to attain the transformed phenotype. This hypothesis has been confirmed by several reports. In an in-vitro study, a high rate of somatic mutations was observed in cells with the RER+ phenotype. [214] It has also been shown that this type of genetic instability is related to oncogene over-expression, [240] LOH, [227] ***TGF-β-RII*** gene

mutations, [225,241,242] ***bax*** gene mutations [235,236] and ***p53*** gene mutations. [223] Therefore, it is important to investigate the configuration of oncogenes and tumour suppressor genes and their association with genetic instability in order to understand carcinogenesis.

### 1.5.3 Oncogene Activation

#### 1.5.3.1 Introduction

Proto-oncogenes are tightly controlled normal cellular genes that promote cell growth by stimulating cell proliferation and /or inhibiting apoptosis. [41] Nowadays, more than 100 different oncogenes have been discovered. [41,196,243,244] Based on functional and biochemical properties, they can be classified into five groups: growth factors, growth factor receptors, signal transducers, transcription factors and apoptosis regulators. During the development of malignant tumours, proto-oncogenes can be activated to become oncogenic by prolonging cell life span and stimulating cell proliferation in an unimpeded fashion. This activation may be caused by gene rearrangement, mutation and / or amplification. [41]

In nodal lymphomas, activation of specific proto-oncogenes is usually associated with non-random chromosome translocations (including inversions) with ***IgH*** or ***IgL*** genes. [245,246] For example, activation of ***c-myc*** by t(8;14), t(8;22) or t(2;8),[112,247,248] ***bcl-1*** by t (11;14),[249] ***bcl-2*** by t(14;18) [250,251] and ***bcl-6*** by 3q27 abnormalities [252] characterise Burkitt's lymphoma, mantle cell lymphoma, follicular lymphoma and diffuse large cell lymphoma respectively.

As described before, no ***bcl-1***, ***bcl-2***, ***c-myc*** or ***bcl-6*** gene rearrangements have been found in low-grade MALT tumours. [1,35,120] However a high

incidence of ***c-myc*** gene rearrangement in high-grade MALT lymphoma is reported in a single paper, [129] suggesting that ***c-myc*** gene abnormalities may play a role in the high-grade transformation of MALT lymphoma. Therefore, the structure and function of the ***c-myc*** gene will be reviewed in detail in the next section.

### 1.5.3.2 The *C-myc* Gene

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#### 1.5.3.2.1 *C-myc* Gene Structure [253,254]

***C-myc*** was the first nuclear proto-oncogene discovered and has been the subject of intense investigation during the past 16 years. It is the major member of the myc family that contains at least seven closely related genes (***c-myc, n-myc, l-myc, p-myc, r-myc, s-myc*** and ***b-myc***). The gene is located on human chromosome 8q24 and consists of three exons (Figure 1-9). [255] It is highly conserved throughout vertebrate evolution. Gene expression is tightly controlled at several levels, encompassing transcription initiation and elongation, mRNA stability, and translation. Most of the regulatory elements are located at the boundary of exon I and intron I. Therefore, this region is considered as ***c-myc*** regulatory region.

RNA polymerase initiates ***c-myc*** transcription from distinct promoters that are relatively well conserved in evolution. In human ***c-myc*** gene, the major promoters P1 and P2 are separated by 161 bp and give rise to 95% of total ***c-myc*** mRNA. Anther promoter, P0, is located 550 bp upstream of P1 and yields less than 5% of total mRNA, while transcription from the P3 promoter found in the human, mouse and rat ***c-myc*** intron 1 is also detected, again yielding less than 5% of ***c-myc*** mRNA. However, in some translocations where the ***c-myc*** is deleted the promoters P0 to P2 are lost and all transcription occurs from P3.

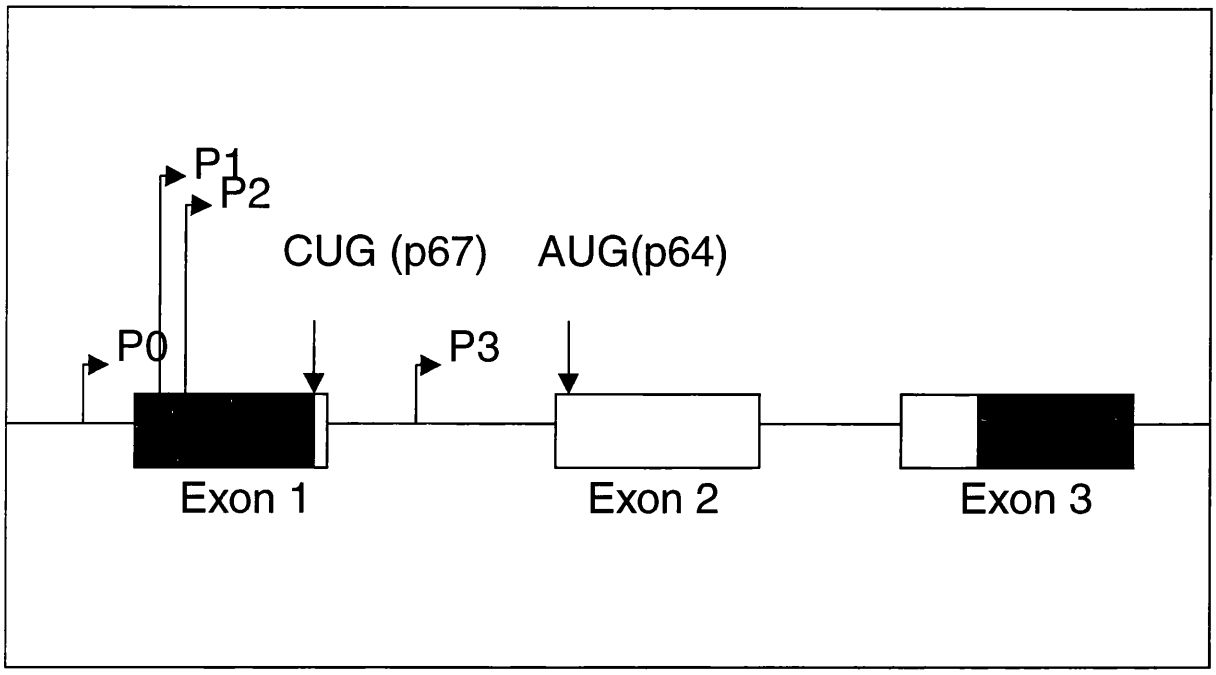


**Figure 1-9: *C-myc* Gene Organisation**. The ***c-myc*** gene consists of three exons, and the different promoters (P0, P1, P2 and P3), translation initiation codons (CUG for p67 and AUG for p64).

Despite the heterogeneous nature of the transcripts produced from the ***c-myc*** gene, one open-reading-frame (ORF) is observed in all mRNA species, spanning 439 amino acids in humans. It starts at an AUG initiation codon located at the 5' end of exon 2 and codes for the major ***c-myc*** protein observed in-vivo, of 64 kDa (p64). [256] A minor species of 67kDa is also observed, and is translated from a CUG codon located at the 3' end of exon 1 in frame with the previous ORF. The p67 protein therefore corresponds to an additional 14 amino acids at the N-terminus of the p64 sequences. [257] Both forms are relatively short-lived nuclear phosphor-proteins, and the conserved existence of these two forms in several species would suggest different functions for both proteins.

#### 1.5.3.2.2 Normal *C-myc* Gene Functions

The major ***c-myc*** protein is p64. It is expressed in a variety of adult tissues and at all stages of embryonic development. [253,258] The nuclear location of the protein suggests that ***c-myc*** might be a transcriptional regulator in the control of normal proliferation and differentiation, although its precise role remains unclear. In-vitro experiments showed that ***c-myc*** expression is rapidly activated by mitogenic stimuli and maintained throughout the cell cycle, [259] indicating that it is necessary for the exit from $G_0$ to $G_1$ and also for continuous proliferation. [254] Down-regulation of ***c-myc*** expression usually correlates with the onset of differentiation.

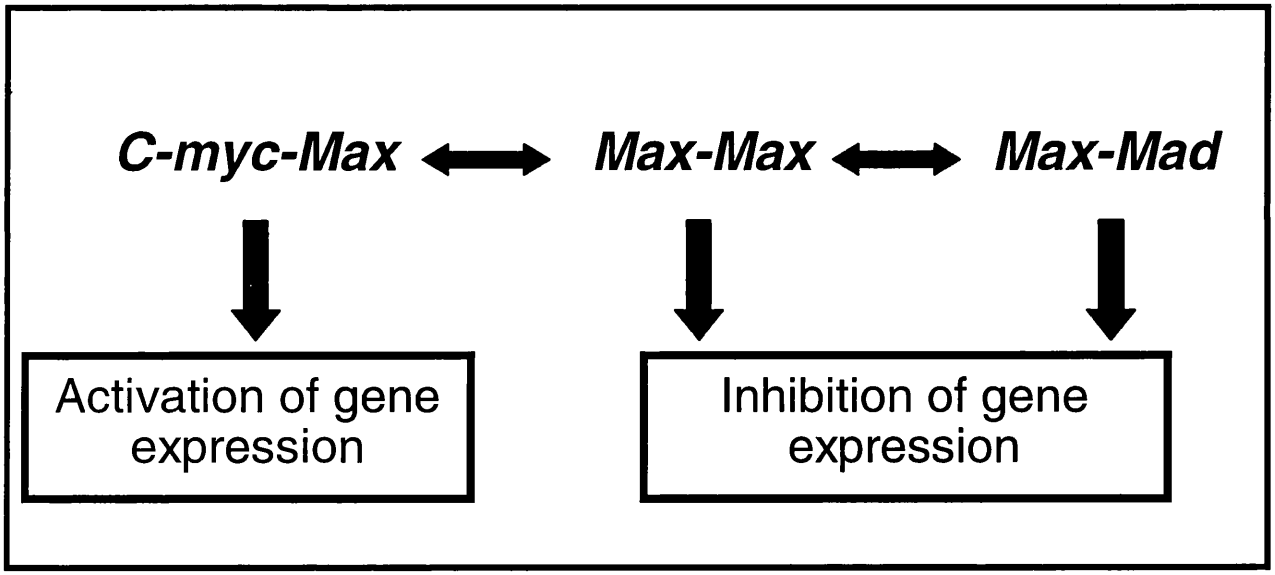


**Figure 1-10: the *C-myc* Gene Transcription Factor Activation Gene Expression When Dimerised with *Max* and Promotes Proliferation.** In contrast, when associated with itself or with ***mad, Max*** is a transcriptional repressor and is likely an inhibitor of cell cycles. Over-expression of ***c-myc*** disturbs this ***c-myc-max*** equilibrium by increasing the concentration of ***myc-Max*** and contributes to aberrant cell proliferation.

In 1992, Amati and co-workers showed that when ectopically expressed in yeast, ***c-myc*** is able to activate transcription of a reporter gene containing the CACGTG ***myc***-binding site in its promoter. [260] Similar results were also

reported in mammalian cells. [261] Sequence-specific binding by ***c-myc*** was strictly dependent on dimer formation with ***max*** that behaves as a ***c-myc*** functional partner. [262,263] Several studies led to the conclusive demonstration that both ***c-myc*** and ***max*** together but not ***c-myc*** alone can directly regulate gene transcription. [254] The ***c-myc*** protein binds ***max*** to form a heterodimer that is a sequence specific transcriptional activator. ***Max*** forms homodimers with itself or heterodimers with a related molecule, the ***mad*** protein, both of which are transcriptionally inactive. The relative ratio of ***c-myc-max*** and ***max-max*** complexes modulates expression of target genes (Figure 1-10). Tumour-associated ***c-myc*** alteration may be related to unbalance in the ***c-myc-max*** system by sequestration of most of the available ***max*** with excess ***c-myc*** into ***c-myc-max*** heterodimers, which in turn would up-regulate genetic programs associated with cell proliferation. [263]

Unexpectedly, in addition to stimulating cell division, a newly discovered biological function of ***c-myc*** is its role in controlling apoptosis. [264-266] ***C-myc*** related apoptosis might be involved with many mechanisms, such as the ***fas/fas*** ligand pathway [267], mediation of ***p53*** [264], co-operation with ***bcl-2***, [265] and inhibition of specific cytokines (e.g., IGF and PDGF). [268]

An attractive model to explain how cellular fate is chosen after the simultaneous induction of the apoptosis and proliferation pathways by ***c-myc*** has been proposed by Evan and Littlewood (Figure 1-11). [254] Achievement of one or the other cellular programmes is dependent on the second action of survival factors such as ***bcl-2*** or cytostatic factors such as transforming growth factor-β and interferon. The requirement for dimerisation with ***max*** in both these processes suggests that, once established, regulation is dependent upon downstream sets of target genes. Such ***myc-max*** activated genes might be common to the two processes or totally independent. It is also possible that ***myc***-induced regulation of these two programmes might be achieved by as yet

unidentified non-transcriptional mechanisms. The model further suggested how such a system might safeguard the development of an organism. Since all cells undergoing proliferation would also be primed for apoptosis, only those receiving the appropriate survival signals would continue to grow. Cells undergoing aberrant proliferation due to a single genetic lesion would not receive these signals, activating the apoptosis pathways and resulting in their elimination. For example, cells proliferating due to deregulated ***c-myc*** expression would normally undergo apoptosis, unless a survival factor such as ***bcl-2*** was activated due to a specific developmental programme, or in the case of carcinogenesis, by secondary genetic alterations.

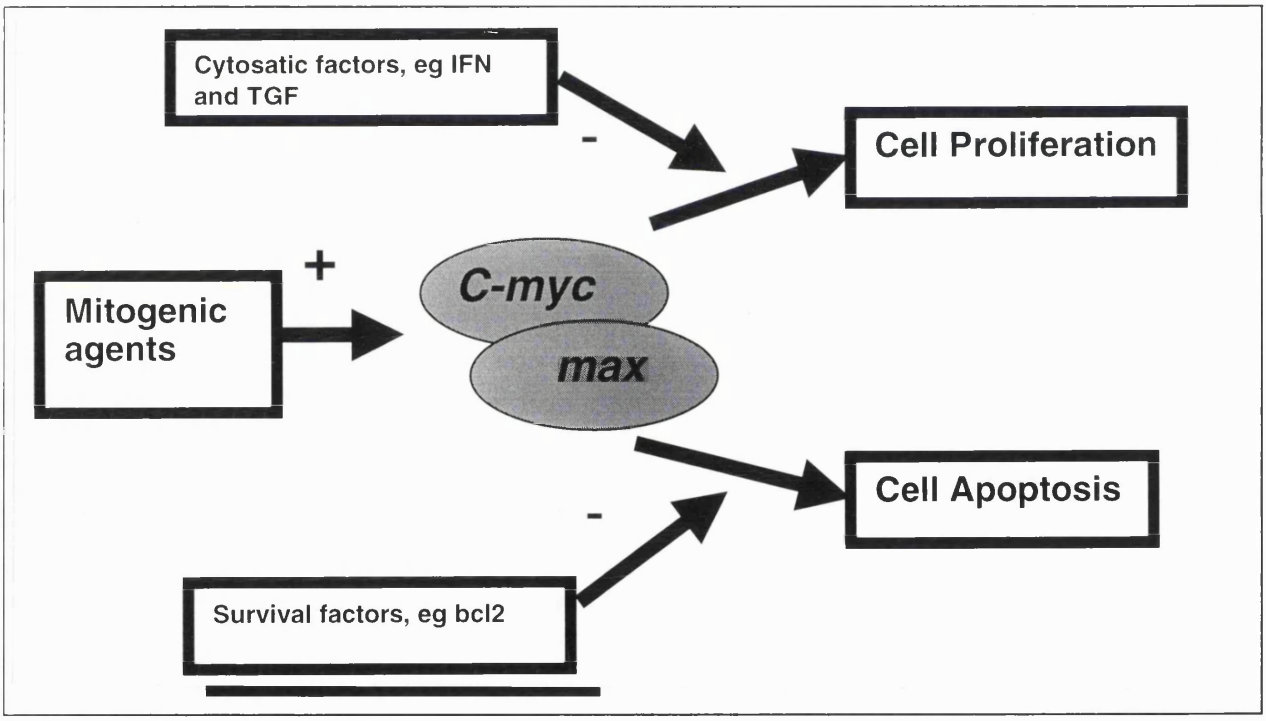


**Figure 1-11: Model for the Induction of Apoptosis and Proliferation Mediated by *C-myc*.** Induction of ***c-myc*** expression by mitogenic agents seems to be responsible for the simultaneous induction of both the apoptosis and proliferation pathways. Achievement of one or other of these cellular programmers is depended upon the secondary action of survival factors or cytostatic factors, which block the apoptosis or proliferation pathways, respectively.

The function of minor species of ***c-myc*** protein, p67, is not clear. It might be a growth-inhibitor that negatively controls p64. This view was initially supported by two pieces of evidence:

(1) In Burkitt's lymphoma p64 ***c-myc*** is the only one synthesised, since initiation at the CUG codon is suppressed by a rearrangement; [256]

(2) There is a 5- to 10-fold increase in p67 synthesis as cells approach quiescence. [257]

The distinct cellular roles of p64 and p67, if any, were initially hypothesised to be associated with the heterodimer-formation of both proteins. [257] Further studies showed that functional differences between them might be related to the difference in their abilities to induce transcription. Both p64 and p67 bind ***max*** with equal affinities and activate transcription from reporter genes containing the CACGTG motif in their promoters, [269,270] However ***myc/max*** dimers can also bind noncanonical sequences. At one such site, the EFII, enhancer element from Rous sarcoma virus long terminal repeat, the p67 and p64 ***c-myc*** proteins have different effects. In this case, p64 is unable to activate transcription when this sequence is linked to a reporter gene, in contrast to p67, which efficiently stimulates transcription. [270]

#### 1.5.3.2.3 *C-myc* Gene Abnormalities in Lymphoma

***C-myc*** gene abnormalities are found in Burkitt's lymphomas, [271] diffuse large cell lymphomas, [272] follicular lymphomas with high-grade transformation, [273] as well as carcinomas, such as breast cancer, [274,275] and small cell lung cancer. [276] The abnormalities are mainly in the form of chromosome translocations, gene mutations and gene amplification. At least three mechanisms are involved in ***c-myc*** activation.

#### 1.5.3.2.3.1 Immunoglobulin Gene Enhancers

The analysis of translocations involving ***c-myc*** in Burkitt's lymphoma [255,271] and mouse plasmacytomas [277] provided the first molecular evidence for activation of proto-oncogenes by chromosome translocation. The translocation involving the ***c-myc*** gene on chromosome 8 and one of the ***Ig*** loci are of three types. About 80% of cases involve translocation between ***c-myc*** and ***IgH*** on chromosome 14q. [278] The remaining cases involve translocation between ***c-myc*** and ***IgL*** sequences on chromosomes 2p and 22q. [279,280]

Molecular characterisation of the structure and expression of the rearranged ***c-myc*** gene has led to the consensus that the translocation results in a loss of normal gene regulation, leading to constitutive ***c-myc*** expression. In plasmacytomas, the translocation usually occurs within the first intron of ***c-myc*** (in mice, like in humans, the first exon is non-coding). In Burkitt's lymphoma, the translocations are more variable, sometimes occurring in either 5' or 3' flanking sequences rather than within the ***c-myc*** gene itself. The mechanisms by which these different rearrangements affect ***c-myc*** gene expression are not entirely understood. However, it is hypothesised that the translocation can activate ***c-myc*** in two ways. Firstly, the translocation can relocate ***c-myc*** into the vicinity of the strong transcription enhancers of the ***Ig*** gene that will aberrantly control expression of ***c-myc***. In the most frequent example (Figure 1-12), the transcription enhancer of the ***IgH*** gene is embedded within that of ***c-myc*** and becomes the controlling factor for the expression of ***c-myc***. The trancriptional enhancers of the ***Ig*** gene may include $E_{\mu}$ in the JH-CH intron, [278] an enhancer in the 3' end of the rat ***IgH*** loci, [281] and others. [282] In any event, the consequence of these rearrangements is

abnormal constitutive expression of a ***c-myc*** coding sequence that is generally unaltered compared with its normal allele.

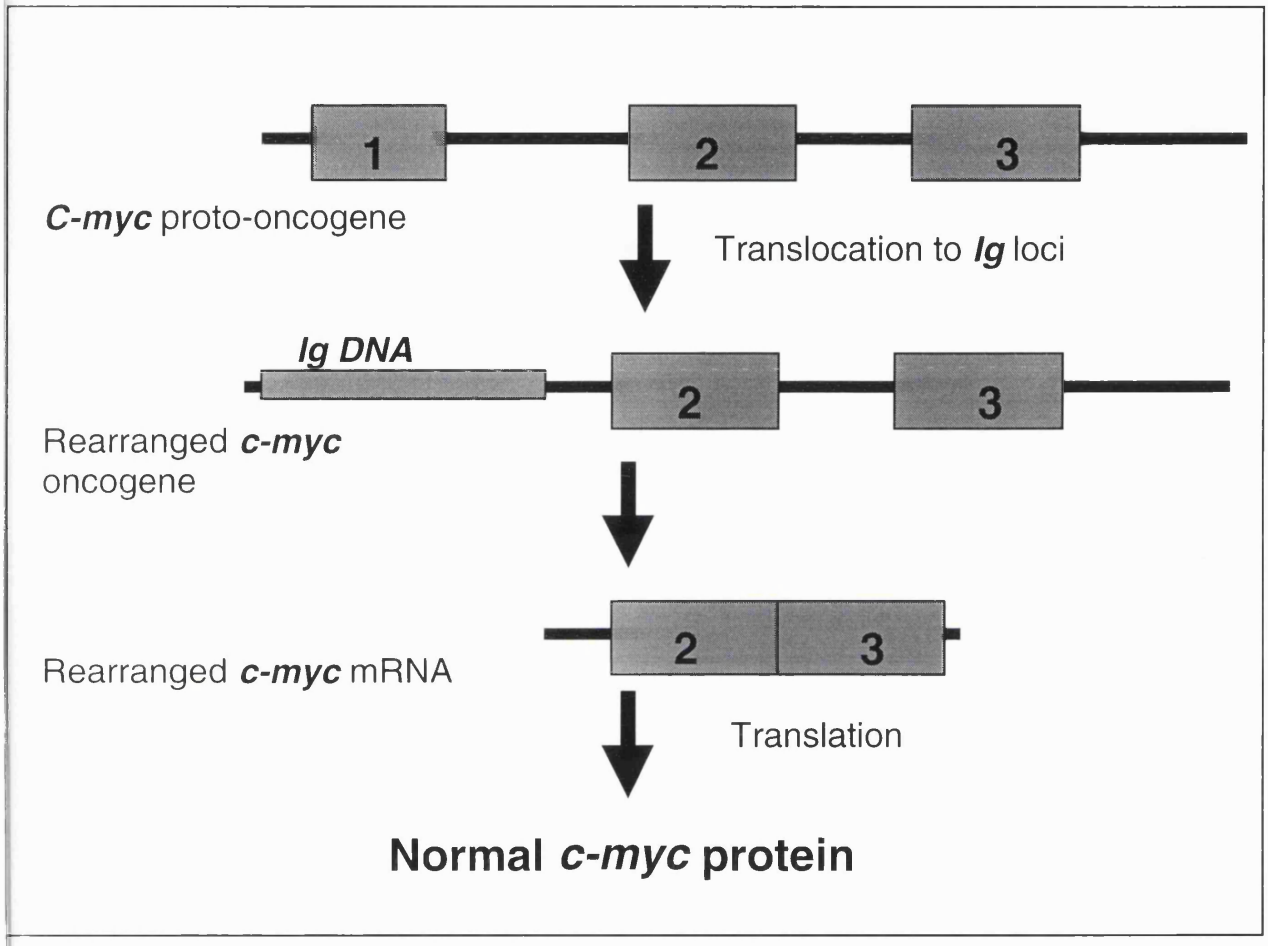


**Figure 1-12: Activation of *C-myc* by Translocation**. Translocation of ***c-myc*** to the ***Ig*** locus most frequently results in deletion of non-coding exon 1 and 5' flanking sequences. Transcription of the rearranged gene is initiated from a cryptic promoter in the first intron, leading to constitutive expression of the normal ***c-myc*** protein.

#### 1.5.3.2.3.2 Mutations in *C-myc* Gene

In Burkitt's lymphoma, a high frequency of mutations has been observed in the non-coding ***c-myc*** gene exon I and at the exon I/intron I boundary with or without ***c-myc*** gene translocation. [283,284] This region includes a transcriptional

elongation block, [285] a p67 protein initiation site, an intron splicing site, [257] and 3 myc intron factor (MIF) binding sites [286,287] and is responsible for mRNA stability. [288,289] Therefore, it is considered as the ***c-myc*** regulatory region. Deregulation of ***c-myc*** expression has been found to be associated with somatic alterations in this region. [257,284-287] These alterations may alter ***myc*** transcription and expression by removing a normal block to transcriptional elongation, increasing the stability of ***c-myc*** mRNA.

In addition, ***c-myc*** gene mutation, including point mutation, small deletion and duplications, can also occur in some coding regions, such as the 3' ***c-myc*** transactivation domain, which may also have some pathological effects. [284,290-292]

The mechanisms by which these mutations are caused are unknown. Since the mutations often accompany with the ***c-myc*** translocation into the an ***Ig*** gene and the frequency and type of mutation are similar to those seen in ***Ig*** gene somatic hypermutations, these mutations may be ongoing somatic mutations associated with antigen stimulation. [284,293] However other mechanisms, such as genetic instability [42,43] or non-***Ig*** gene hypermutaion in lymphocytes, [294] can not be ruled out since mutation can also be observed before translocation. [290]

#### 1.5.3.2.3.3 *C-myc* Amplification

In some non-lymphoid malignancies, such as small cell lung cancer, [295] ovarian carcinoma [296] and breast cancer, [274] deregulation of ***c-myc*** is associated with its amplification. Amplifications of ***c-myc*** are rare in lymphoid cells, but have been reported in at least one T-NHL cell line [297] and a B-NHL cell line. [298] A few cases have also been documented in which a translocated ***c-myc*** allele has been duplicated. [299]

### 1.5.4 Inactivation of Tumour Suppressor Genes

#### 1.5.4.1 Introduction

Tumour suppressor genes may be defined as growth inhibitory genes. [300,301] Examples include the retinoblastoma susceptibility gene (***RB1***), the adenomatous polyposis gene (***APC***), the ***p53*** gene and the Wilms' tumour gene (***WT-1***). Mutations or deletions in both alleles can result in the uncontrolled cell growth encountered in cancer.

In nodal lymphomas, the inactivation of tumour suppressor genes appears to be rare as a primary mechanism of tumour development, but may be associated with tumour progression. They may act as the targets for second hits in aggressive lymphomas. Examples include ***p53*** mutations in Burkitt's[302,303] and other high-grade lymphomas. [304] ***RB*** gene abnormalities have been recorded in high-grade lymphomas, [305] and two uncharacterised genes in 6q25-27 and 6q21-23 in low and high-grade B cell lymphomas. [306] However, more recently, a high frequency of ***p53*** mutation has also been reported in low-grade splenic marginal zone B cell lymphoma. [307] Since this tumour is analogous to MALT lymphoma in its origin, immunophenotype, histology and genetic features, [1,65] inactivation of the ***p53*** gene may also be important in the tumourigenesis of MALT lymphoma. In this thesis ***p53*** gene structure and function has been reviewed and studied in MALT lymphoma.

#### 1.5.4.2 *P53*

##### 1.5.4.2.1 *P53* Gene Structure [308,309]

The ***p53*** gene consists of 11 exons within a 16-20 kb region of DNA located

on the short arm of human chromosome 17 at position 17p13. (Figure 1-13) It encodes for a 393-amino-acid nuclear phosphor-protein, which is highly conserved in diverse organisms, suggesting that the encoded protein plays a central and critical role.

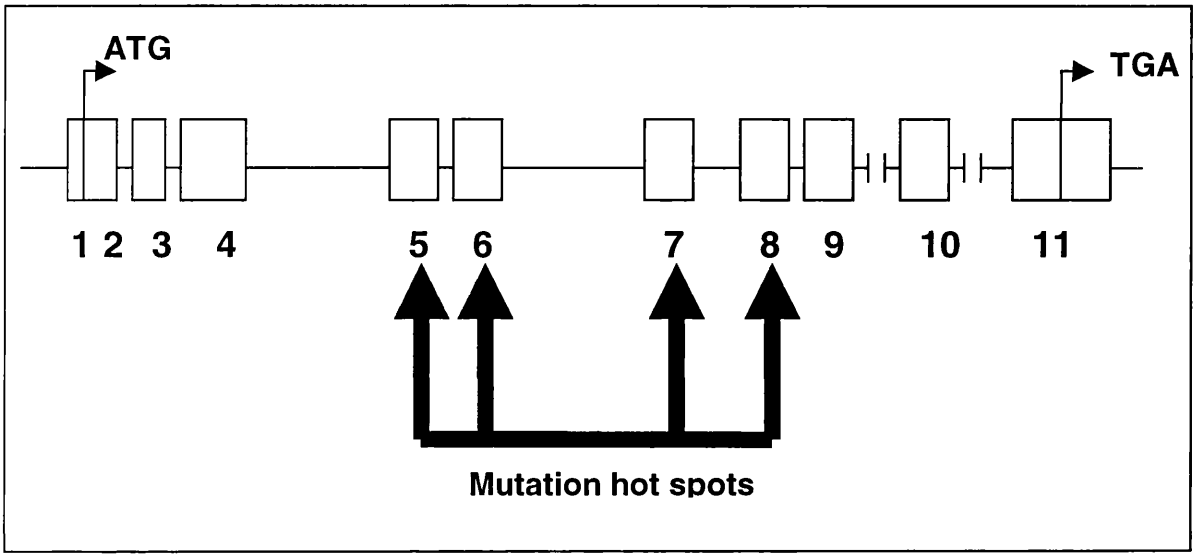


**Figure 1-13, The Gene Structure of P53 Gene.** Numbered open boxes represent coding exons. The hot spots for gene mutation are indicated by arrow.

#### 1.5.4.2.2 *P53* Function

Numerous in-vitro and animal experiments show that ***p53*** is one of the major factors controlling cell proliferation. It acts as "guardian of the genome" [310] by preventing cells bearing damaged DNA from proliferating. This is achieved, either temporarily, by arresting the cell division cycle and directly or indirectly provoking the DNA damage repairing system to repair DNA damage, [311] or permanently, by pushing the damaged cell down an irreversible apoptosis pathway. [312]

***P53*** functions are associated with its transcriptional control effect. ***P53***

protein accumulates quickly after DNA damage. It specifically binds to DNA sequences in upstream promoter regions of a range of target genes, and transcriptionally activates them. These genes include the ***p53*** mediated cyclin-kinase inhibitor, ***p21$^{cip/WAF1/Sdi1}$***, [311] the growth arrest and DNA damage protein ***GADD45***, [313] the apoptosis-inducing factor, ***bax***, [238] and a putative cell cycle associated protein, ***cyclin G***. [314]

#### 1.5.4.2.3 *P53* Abnormalities

Two lines of evidence clearly demonstrate that ***p53*** is a tumour suppressor gene:

1. ***P53*** abnormalities have been found in more than half of all human tumours. [308] The vast majority of ***p53*** abnormalities occur at the gene level and manifest as gene mutation with or without deletion, [315] or gene rearrangement. [316,317] Most of the mutations are missense mutations. Gene mutation can lead to a stabilised protein product as a result of conformational alterations, which cause failure by protease to recognise and break down the mutant ***p53*** protein in the cells. [318] Therefore the mutant protein can be detected by immunohistochemical methods. [319] In addition, the abnormalities may occur at the protein level, by binding to either tumour associated virus proteins (such as the SV40 large T antigen,[320,321] the adenovirus E1B protein [322] or papillomavirus E6 protein[323,324] ), or specific cellular gene products (such as murine double minute-2 (***MDM2***) [325,326] ).
2. Mice that contain mutations of both ***p53*** alleles that eliminate ***p53*** functional proteins are normal at birth, but 100% of these mice develop cancer in 6-9 months. [327] These null mutations prove that the loss of ***p53*** function results in a predisposition to cancer.

While analysing the spectrum of ***p53*** gene mutation patterns, [315] it is found that most mutations, the majority of which are located between codons 120-298 (exons 5-8), are missense mutations, resulting in a faulty or altered protein in cells. This contrasts with other tumour suppressor genes (***RB, APC***) which have much higher frequencies of chain termination, deletion, exon-skipping and frame-shift. [315,328] Clearly these mutations are selected during tumour development, suggesting that the nature of ***p53*** mutations is more complex than a simple loss of function. Indeed, missense mutant ***p53*** protein does exhibit several oncogenic functions. The ***p53*** gene can co-operate with an activated ras oncogene and transform primary rat embryo fibroblasts in cell culture. [329,330] Similarly, missense ***p53*** mutant alleles enhance colony-formation ability or plating efficiency of primary rat cells in culture.[331] In these cases, it is thought that the faulty ***p53*** protein forms a tetrameric protein complex with the wild-type ***p53*** in the cell, which inactivates the function of the wild-type ***p53*** proteins. [332] This is a dominant loss of function phenotype which can explain how mutant ***p53*** proteins actively contribute to cell transformation and in this way, act like oncogenes. [331] The mutant missense ***p53*** proteins also posses a gain of function phenotype. [333] If mutant ***p53*** missense alleles are introduced into a cell that has no ***p53*** genes or protein, these cells show an enhanced ability to induce tumours in nude mice. Since these cells contain no endogenous wild-type ***p53*** protein, these data indicate that the altered ***p53*** protein itself can contribute a new function to these cells.

These observations indicate that ***p53*** missense mutants have lost some functions, as expected for a tumour suppressor gene, but also gained new activities [333,334] and therefore this gene is more like a hermaphrodite in the scheme of oncogenes and tumour suppressor genes. This may account for the fact that mutations at the ***p53*** locus are the single most common genetic alteration so far observed in human cancers.

### 1.5.4.2.4 *P53* Abnormalities in Lymphoma [302,335]

**Table 1-3 *P53* Alterations in B Cell Lymphoma**

| Lymphoma | Frequency of abnormalities |
|---|---|
| Low-grade B cell lymphoma | Rare |
| Common acute lymphocytic leukaemia | 3% |
| Chronic lymphocytic leukaemia | 15% |
| High-grade B cell lymphoma | 30% |
| Richter's syndrome | 40% |
| Burkitt's lymphoma | 40% |
| Burkitt's type acute lymphocytic leukaemia | 50% |

***P53*** gene abnormalities may play a role in the initiation of some lymphomas, as seen in the lymphomas of Li-Fraumeni syndrome families. [336] However, they are more commonly associated with progression of haematopoietic malignancies (tables 1-3 and 1-4). For example, low-grade NHLs rarely have ***p53*** alterations (although a high incidence of ***p53*** gene mutations was observed in low-grade splenic marginal zone B cell lymphoma), but their progression to high-grade lymphoma may be associated with development of ***p53*** mutations. [304] In a recent study, serial biopsies of patients with follicular NHL who underwent histologic transformation to high grade showed that 1/3 of the transformed samples acquired a ***p53*** mutation that was not detected in the follicular stage of the disease. [304] Another study found that 4 of 5 cases with transformation of follicular to diffuse large cell NHL were associated with ***p53*** mutations. [337] In one case which had scattered regions of high-grade NHL mixed with low-grade disease, only high-grade regions contained a mutant ***p53*** gene. ***P53*** positive staining cells were detected before transformation to high-grade lymphoma, but they represented a minority of the lymphomatous cells. [304] Therefore, individuals with low-grade follicular NHL that have ***p53*** staining cells may be at increased risk for transformation to an aggressive lymphoma.

**Table 1-4 *P53* Mutations Associated with Disease Progression**

| |
|---|
| 1. Evolution from chronic phase to myeloid blast crisis of chronic myeloid leukaemia |
| 2. Evolution from myelodysplastic syndrome to acute myelogenous leukaemia |
| 3. Evolution from follicular to high-grade lymphoma |
| 4. Evolution from chronic lymphoid leukaemia to high-grade Richter's syndrome |
| 5. Progression to a refractory phase of multiple myeloma |
| 6. Development of relapsed B or T cell acute lymphoid leukaemia |

### 1.5.5 The Multistage Process of Carcinogenesis

#### 1.5.5.1 Introduction

Tumour cells must acquire a number of distinct genetic aberrant traits to proliferate. Reflecting this requirement is the fact that the genomes of certain well-studied tumours carry several different independently altered genes, including activated oncogenes and inactivated tumour suppressor genes. Each of these genetic changes appears to be responsible for imparting some of the traits that, in total, represent the full neoplastic phenotype. [198,338] A combination of several genetic alterations must occur for the initiation and progression of most types of human cancer. This can be clearly seen in studies of colon cancer in which the clinical course of the disease has been correlated with the alteration of specific genes. [339,340] This model can also be extended to lymphomagenesis.

#### 1.5.5.2 The Multistage Process of Lymphoma Initiation

The genetic abnormalities of Burkitt's lymphoma have been well studied. [303] Burkitt's lymphoma is a human B cell lymphoma in which the ***c-myc*** gene is activated by translocation to an ***Ig*** gene. [248] This disease occurs with high incidence in some areas of Africa, where it is strongly associated with infection by EBV. [50,341] EBV can inhibit the apoptosis process [342-345] and

immortalise B cells to proliferate continuously in culture.[346-348] In-vitro experiments showed that EBV infection or ***c-myc*** activation alone can not fully transform normal B cells. However, introduction of an activated ***c-myc*** oncogene into EBV immortalised B cells converts them into the full tumourigenic phenotype. [349,350] The results suggest that both EBV infection and ***c-myc*** translocation figure in the pathogenesis of this neoplasm, most likely representing two distinct events in lymphomagenesis.

However, ***c-myc*** activation and EBV infection may not be sufficient to maintain the malignant phenotype, [351] suggesting that some other gene abnormalities are involved. It has been observed that a major portion of Burkitt's lymphomas carry ***p53*** mutations. [352,353] More than 50% of these mutations are clustered in a small stretch of 33 amino acids (codon 213 to 248), with codons 213 and 248 being the most frequently mutated sites. [354] In-vitro experiments showed that introducing wild type ***p53*** into Burkitt's lymphoma cell lines could reduce tumourigenesis. [302] This result suggests that the ***p53*** abnormalities may also play a critical role in the development of Burkitt's lymphoma. [303]

### 1.5.5.3 Lymphoma Progression

Tumour progression is also a multiple-gene process. It has been observed that, over time, all types of tumour may become clinically more aggressive and less responsive to chemotherapy as a result of the accumulation of multiple genetic alterations. Clinically advanced cancers generally have more chromosomal aberrations than early stages of malignant disease. However, in some tumours, specific genetic alterations have been associated with progression.

More than 80% of low-grade follicular lymphomas harbour the

t(14;18)(q32;q21). Deregulation of oncogene ***bcl-2*** enhances its apoptosis inhibition function. [250,355] After acquiring a further translocation of the ***c-myc*** gene, the relatively indolent low-grade tumour can transform to a high-grade diffuse large cell lymphoma. [265] This observation has also been confirmed by transgenic mice experiments. [356,357]

However, ***c-myc*** deregulation only accounts for a small proportion of high-grade transformed follicular lymphomas. [358] In the remaining cases, other mechanisms may contribute to tumour progression. Levine and colleagues analysed sequential karyotypes in NHL and reported several secondary abnormalities including gene rearrangements of chromosome 1, 2 and 14, and loss of 17p. [359] Whang-Peng et al noted that progression of follicular lymphoma in four cases was associated with acquisition of der(18), +7 and / or +12, all abnormalities which are associated with high-grade disease or short survival. [360] Others found frequent losses of 9p sequences in subtypes of high-grade lymphomas but it was not shown whether these were acquired as the disease progressed from low-grade lymphoma. [361,362] Many groups have reported a high frequency of ***p53*** abnormalities in high-grade NHL and an association of ***p53*** mutation with progression of follicular lymphoma in 25-30% of cases. [273,304]

### 1.6 Aims

Taking advantage of the availability of a large number of MALT lymphoma samples in the Department of Histopathology at UCL, this study attempted to investigate the molecular mechanisms involved in high-grade transformation of MALT lymphoma. The specific aims were as follows:

1. To examine rearrangements of ***IgH*** genes derived from coexisting low and high-grade lesions in MALT lymphoma patients by microdissection, PCR

and sequencing in order to establish the genetic link between the two lesions.

2. To investigate the incidence of ***CagA+*** *H pylori* infection in low and high-grade gastric MALT lymphoma in order to determine whether specific bacterial strains are involved in the development of low and high-grade tumours.

3. To investigate the incidence of genetic instability indicated by the RER+ phenotype in order to study its role in MALT lymphomagenesis.

4. To investigate ***c-myc*** gene configuration, including gene rearrangement and mutation of regulatory regions in order to study its role in high-grade transformation of MALT lymphoma.

5. To investigate ***p53*** gene abnormalities including LOH, mutations and gene expression abnormalities in high-grade transformation of MALT lymphoma.

It is hoped that examination of the interplay between these genetic abnormalities, together with the data accumulating in the literature, will help in understanding the high-grade transformation of MALT lymphoma at the molecular level.

## Chapter 2 Materials and Methods

### 2.1 Materials

#### 2.1.1 Chemicals and Solutions

The routine chemical reagents used in the molecular biology laboratory were provided by Sigma, UK. The common buffers are listed in Table 2-1. The reagents or solutions used for individual methods are described in the text of this chapter.

**Table 2-1: Commonly Used Solutions and Buffers**

| Solution | Working solution |
|---|---|
| **Sequencing stop buffer** | 98% formamide, 10 mM NaOH, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF |
| **10 X PCR buffer** | 100mM Tris pH 9, 500mM KCl, 1% Triton X-100. |
| **20 X SSC** | 876.5g NaCl, 441g $C_6H_5Na_3O_7$, distilled water to 5 litres. |
| **DAB buffer** | 5mg DAB, 10ml Tris-HCl buffer (0.05M Tris, 0.05M HCl, pH 7.6), 0.1 ml $H_2O_2$ . |
| **Electrophoresis loading buffer** | 0.25% bromophenol blue, 0.25% xylene cyanol, 6 X TBE, 40% sucrose. |
| **TBS (0.005M)** | 80g NaCl, 6.05g Tris, 44ml of 1M HCl, 10 litres $dH_2O$. pH adjusted to 7.6 with 1M HCl or 0.2M Tris. (0.05% Tween added). |
| **Tris-borate (TBE, 10X)** | 108g Tris, 55g boric acid, 9.3g EDTA, distilled water to 1 litre. |

#### 2.1.2 Tissue Samples

The majority of fresh tissue and paraffin blocks of low and high-grade MALT

lymphomas used in this thesis, along with their corresponding normal tissues when available, were obtained from the archives of the Department of Histopathology, University College London Medical School. Additional cases were collected by other institutes, as indicated in the material and methods sections of the relevant chapters. The tumours originated from stomach, salivary gland, small intestine, conjunctiva and lung. The tissue samples were routinely examined by pathologists and the diagnosis of MALT lymphoma was established by the characteristic histological appearances together with immuno-phenotyping [1,2,8,143] and clonal analysis of the ***IgH*** gene. [20]

In addition, various positive and negative control tissues were used as indicated in individual chapters.

### 2.1.3 Tissue Storage and Handling

#### 2.1.3.1 Fresh Tissue

Fresh, unfixed samples were snap frozen in liquid nitrogen and stored in cryotubes at -70°C before use.

#### 2.1.3.2 Fixed Tissue: Paraffin Blocks and Sections

Tissue blocks processed at UCL Medical School were fixed in unbuffered formal saline and paraffin wax embedded using a standard protocol. Tissue samples referred from other laboratories were fixed and processed by unknown methods. In some cases without fresh tissue or paraffin blocks, unstained or stained sections were used as a source of DNA.

One to five (depending on the size of the tissue) 5 μm sections were cut from tissue blocks and placed into clean Eppendorf tubes. Unstained or stained

cut sections were scraped from slides, after removal of cover slips and mountant using xylene if necessary, and placed into Eppendorf tubes.

### 2.1.3.3 Microdissection*[363]

Areas of interest on tissue sections were selected and marked with ink on top of the cover slips under the microscope. The marked areas were recorded diagrammatically. Slides were then soaked in xylene for 3 to 5 days to remove the cover slips. The slides were washed and stored separately in 50% ethanol.

The recorded areas were relocated under the microscope and sections were left to dry. A drop of 20-50% ethanol was subsequently applied to cover the defined area using a drawn out glass pipette (cotton plugged) controlled by suction through a rubber tube. The selected cell populations were scraped gently under the microscope and transferred to Eppendorf tubes using the same glass pipette device. The 20-50% ethanol solution was essential for floating the dissected cells and preventing them from adhering to the slide.

To avoid cross-contamination, sections were rinsed with ethanol and the glass pipettes were washed thoroughly (2X with water and 2X with ethanol) after each manipulation. The pipettes were replaced for each new section. Samples from washing solutions were taken for PCR amplification controls.

An example of microdissection from a low-grade MALT lymphoma is illustrated in Figure 2-1.

*The microdissection was mainly carried out by Dr Langxing Pan.

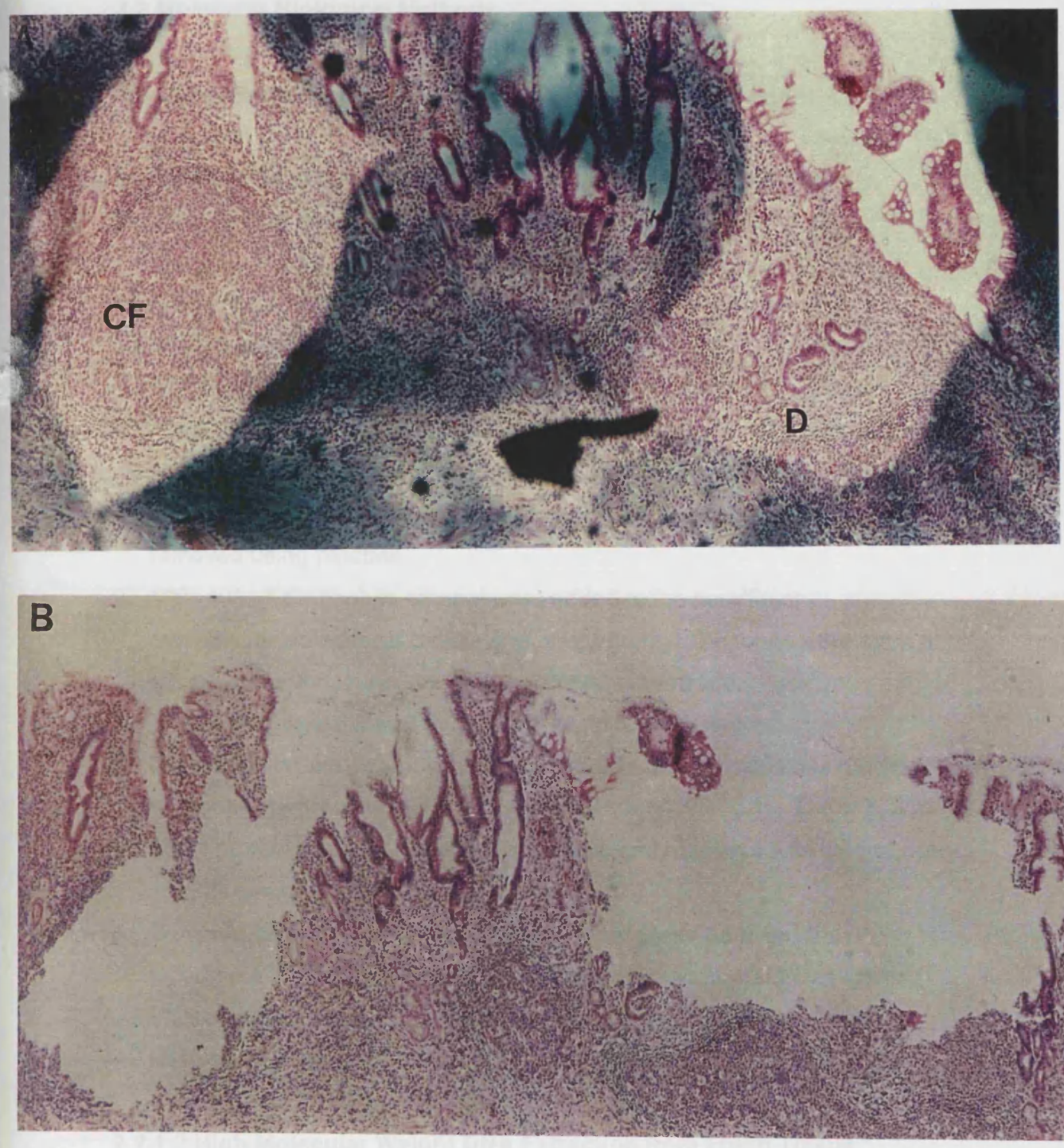


**Figure 2-1 Microdissection of Colonised (CF) and Diffused Follicle (D) in a Low-grade MALT Lymphoma. A: before, B: after.**

## 2.2 Molecular Biological Methods

### 2.2.1 DNA Preparation

#### 2.2.1.1 DNA Extraction from Paraffin Embedded Tissues and Microdissected Materials [20,363]

DNA was extracted from paraffin embedded tissue and microdissected tissues by proteolysis using proteinase K buffer:

1. For paraffin sections (but not microdissected materials), 400 μl of xylene were added to an Eppendorf tube and mixed for 1-2 minutes and spun at full speed on a microcentrifuge for 5 minutes. The supernatants were removed using pipettes.
2. 100- 400 μl of absolute ethanol were added to the paraffin or microdissected material pellets and mixed briefly. The tubes were spun at full speed for 5 minutes and the supernatants removed.
3. The pellets were allowed to dry completely at room temperature
4. Depending on the size of sections or microdissected materials, 10-200 μl of proteinase K solution (10mM Tris pH 9, 50mM KCl, 0.1% Triton X, 200 μg/ml proteinase k) were added to the tube and digestion was carried out at 37°C overnight, or for three hours at 55 °C.
5. Samples were then centrifuged to the bottom of the tube and the proteinase K inactivated at 95°C for 10 minutes in a Hybaid Omnigene PCR machine (Hybaid Ltd, 111-113 Waldegrave road, Teddington, Middlesex, England).

#### 2.2.1.2 High Molecular Weight DNA Extraction from Fresh Tissue *[364]

High molecular weight DNA was extracted from fresh tissues using standard

proteinase K digestion and phenol/ chloroform purification.

1. A frozen section from the tissue sample was cut and Hematoxylin and esion stained to confirm that the tissue contained the appropriate cell populations for the study.
2. A block or a discrete part of a block containing the desired cell population was minced finely with a sterile scalpel blade in a clean petri dish and collected into a 15 ml tube.
3. An appropriate volume (2-5 ml) of proteinase K solution (0.5 X SSC, 0.5% SDS, 5mM Tris, 0.5mM EDTA, 100mg/ml proteinase K) was added to the tube. The mixture was mixed well and incubated at 37°C overnight or 3 hours at 55°C. If digestion was not complete, additional proteinase K was added and incubated for another 1-3 hours.
4. An equal volume of water-saturated phenol was added to the tube, which was rotated end over end for 10 minutes at room temperature and spun at 2000 rpm for 10 minutes.
5. The upper aqueous layer was collected into a fresh tube using a blunt end pipette and re-extracted with phenol/chloroform (49% phenol, 49% chloroform, 2% isoamyl alcohol) until a clean interface was achieved (usually two or three repeats).
6. The upper aqueous layer was extracted once with an equal volume of chloroform in the same way.
7. 1/10 volume of 3 M NaAc was added, followed by 2.5 volumes of 100% ethanol, and incubated overnight at –20 °C, or for 3 hours at -70°C, or for 5 minutes on dry ice.
8. The DNA pellet was transferred into 10ml of clean 70% ethanol at -20 °C using a sterile pipette tip. After gentle inversion for 1-2 minute the pellet was transferred to a 1.5ml Eppendorf tube, allowed to dry and 0.2-1 ml of sterile TE buffer added, the volume being determined by the size of the pellet.

9. The DNA sample was allowed to dissolve overnight at 4°C (or for up to three days) and a 5 μl aliquot removed for estimation of DNA concentration. The optical density was measured at 260 and 280nm using a spectrophotometer. Samples with a 260/280 ratio of 1.6-2 were considered suitable for further analysis and those with ratios outside this range were re-extracted. The DNA concentration was calculated by the equation: DNA concentration (mg/ml) = $OD_{260}$ X 50.
10. The DNA concentration was adjusted to 0.5 μg/μl and checked on an agrose mini-gel
11. DNA samples were stored at -20 °C.

*Most of the high molecular weight DNA was extracted by Dr. T Diss.

### 2.2.2 Southern Blotting and Hybridisation

Southern blotting and hybridisation were used to detect ***c-myc*** gene rearrangement. Two cDNA probes were used: one covered exons 2 and 3 of the ***c-myc*** gene, and the other covered the full length of the ***c-myc*** gene coding sequences. The method was adapted from well-established protocols.[19,35,74,120]

#### 2.2.2.1 Probe-labelling

1. Purified recombinant plasmids containing ***c-myc*** cDNA were digested by EcoRI and Hind III. The digested mixtures were electrophoresed on 0.5% low-melting-point agarose gels (Sigma, UK). The 1.5 kb or 1.2 kb fragment was collected into an Eppendorf tube using a sterile scalpel blade. 3 volumes of water were added to the tube which was then boiled for 5 minutes. The probe was stored at -20°C before use.
2. 9 μl (25-50ng) probe DNA was placed in an Eppendorf tube and denatured at

95° C in a pre-set hot block for 5 minutes, and chilled on ice immediately.

3. The following reagents were immediately added to the Eppendorf tube in order and mixed well: 5 μl of 5x oligo-labelling-buffer, 1 μl bovine serum albumin; 0.5 μl Klenow enzyme (Promega, UK) and 2.5 μl (25 μCi) of $\alpha$-$^{32}$P-dCTP (Amersham, UK).
4. The mixture was kept at room temperature overnight or at 37° C for 1.5 hours.
5. The probe was purified by Nick column (Pharmacia, UK) using a protocol recommended by the manufacturer:
    a) The storage solution was tipped off from the column.
    b) The column was flushed through with 1XTE (1mM Tris-HCl pH 7.5, 0.1mM EDTA), 3 times.
    c) The sample was added into the column.
    d) 400 μl 1XTE was added onto the column and the solution was collected in an Eppendorf tube.
    e) 400 μl 1XTE was added onto the column and the second solution (containing labelled probe) was collected into another Eppendorf tube.

#### 2.2.2.2 Southern Blotting

1. 10 μg of high molecular weight DNA were digested using 40 units of Eco R I, Hind III or Pst I in a volume of 30 μl, using the buffers supplied by the manufacturer, at 37°C for 7-16 hours.
2. After completion of digestion, 6 μl of electrophoresis loading buffer were added to each sample before loading on to 0.8% agarose gels. Electrophoresis was carried out at 50V for approximately 16 hours.
3. The gels were viewed under UV light and photographed using a Polaroid C5 camera, to provide a record of the DNA load and the success of restriction digestion in each sample.
4. The gels were transferred to plastic trays containing 0.25M hydrochloric acid and agitated gently for 15-20 minutes to reduce the average DNA

fragment size.

5. Gels were rinsed in ultra pure water, transferred to 0.4M NaOH and agitated gently for 30min to denature the DNA.
6. After denaturation, gels were inverted and placed on a filter paper wick (Whatman No 1) suspended in a reservoir of 0.4M NaOH. Gels were covered with a layer of cling film, a window the size of the area to be blotted cut and folded back to allow positioning of a sheet of Hybond-$N^+$ nylon membrane, cut precisely to size. Four sheets of filter paper cut to the size of the membrane and soaked in 0.4M NaOH were placed on top of the membrane and a 4cm thick pile of green paper tissues placed above the filter papers followed by a plastic plate and a 0.5kg weight. Blotting was allowed to proceed for 16 hours.
7. After blotting, the membranes were retrieved by removal of filter and tissue papers, the gel inverted with the membrane in place and well positions marked with a pencil. The gels were discarded and membranes rinsed in 2 X SSC for 1 minute, dried briefly on filter paper, wrapped in cling film and stored at 4°C prior to hybridisation.

#### 2.2.2.3 Hybridisation

Hybridisation was carried out in a hybridisation oven (Hybaid, UK), using the protocol recommended by the manufacturer.

1. Membranes were separated by sheets of nylon mesh, rolled up, placed in glass bottles containing 10-20 ml of hybridisation buffer (6 X SSC, 1% SDS, 10% dextran sulphate, 50 μg/ml sonicated and denatured salmon sperm DNA), and rotated in a hybridisation oven at 65°C for at least 1 hour.
2. Buffer was decanted and replaced with 10-20ml of fresh buffer containing 25ng of denatured DNA probe oligo-labelled with 25 μCi $^{32}$P-dCTP. Tubes were replaced in the oven and rotated for 16 hours.

3. Membranes were removed from the tubes and washed in two changes of 500 ml of 2 X SSC/0.1% SDS at 65°C for 5 minutes followed by 500ml of 0.1 X SSC/0.1% SDS at 65°C for 10 minutes.
4. Membranes were monitored using a Geiger counter, calibrated for use with $^{32}P$. If background counts were significantly over 10 counts per second (cps) at 1cm the membranes were replaced in buffer for further periods of washing until counts were reduced to appropriate levels.
5. Membranes were wrapped in cling film and exposed to x-ray film in lightproof cassettes with intensifying screens for 3-7 days before development using an automatic machine.

### 2.2.3 PCR and Analysis

#### 2.2.3.1 PCR Protocols

Most PCR protocols used were standard one-round methods involving 30-40 cycles of denaturing, annealing and extension, using one or more pairs of primers (Table 2-2). In order to increase the amplification sensitivity and specificity for the ***IgH*** gene CDR III regions, a semi-nested PCR was used.

##### 2.2.3.1.1 Standard One-Round PCR Protocol

All PCRs were carried out under optimised conditions:

Most of the primers used in this thesis were adapted from published sequences except the primer set used for the amplification of the ***CagA*** gene of *H pylori* which was designed by the PRIMER programme (HGMP, Cambridge, UK) based on published sequences. All primers were synthesised by Oswel (Southampton, UK) without purification. Some were labelled with a fluorescent dye (Hex or FAM) by the manufacturer (for

automated sequencer), or with $\gamma$-$^{32}$P-dATP using T4 kinase end-labelling by the protocol recommended by the manufacturer (Promega, UK) immediately before use (for LOH studies).

The PCR reaction mixture consisted of 1 μl DNA extracted from fresh, paraffin-embedded or microdissected tissue, 1 X PCR buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP (Promega, Southampton, UK), 0.2 μM of each primer, and 0.25-0.5 units of Taq polymerase (Promega, Southampton, UK) in a total volume of 12.5 (multiple fluorescent PCR and LOH study) or 25 μl. The reaction mixture was covered with one drop of mineral oil.

Forty cycles of PCR were carried out on a Hybaid Omnigene PCR machine (Hybaid Ltd). The PCR profiles were 95°C for 30 seconds, 45-58°C (depending on the primer sequences, normally about (4(C+G)+2(A+T)-2)°C) for 30 seconds, and 72°C for 45 seconds. A denaturation at 95°C for 5 minute before adding Taq polymerase was used before cycling and an extended period at 72°C for 5-10 minutes concluded the reaction.

#### 2.2.3.1.2 Semi-nested PCR Protocol [20,363]

Amplification of ***IgH*** genes was achieved using primers directed to conserved sequences in the framework (FR) 3 and joining regions of ***IgH***. A semi-nested PCR protocol was employed using 2 rounds of amplification. The first round involved the FR3 primer (ACACGGCTGTGTATTACTGT) to the LJH primer (outer J region) sequence (TGAGGAGACGGTGACC). The second round used the same FR3 primer and an inner J primer (VLJH) (GTGACCAGGGTACCTTGGCCCCAG).

PCR reactions were performed in a volume of 50 μl using 1 μl of DNA sample (first round) or 1 μl of the product of round 1 reaction (second round), 3mM

(first round) or 2 mM (second round) of $MgCl_2$, 0.25 unit of Taq polymerase (Promega, Southampton, UK), 10 μM each dNTP (Promega, Southampton, UK) and 100ng of each primer, 1 X Taq polymerase buffer provided with the Taq polymerase. The reaction mixture was overlaid with one drop of mineral oil. Prior to the addition of enzyme the PCR mixture was heated to 95°C for 7 minutes and cooled to the annealing temperature (hot-start).

PCR profiles used in the first and second rounds were as follows: 93°C, 45 seconds; 50°C, 45 seconds; 72°C, 120 seconds, for 30 (round 1) or 20 cycles (round 2), followed by an elongation step of 72°C, 5-10 minutes.

### 2.2.3.3 Analysis of PCR Products

### 2.2.3.1 Preparation of Gels

Different gel types were used for the analysis of PCR products according to the product size and required resolution.

#### 2.2.3.1.1 Agarose Gels [364]

A horizontal midi-gel apparatus (Bio-Rad, CA, USA) was used for agarose gel electrophoresis of PCR products of all the standard PCRs:

1. 1-3g of agarose was dissolved in 100ml of 1 X TBE buffer (containing 0.1mg/ml ethidium bromide) by boiling in a microwave.
2. The agarose was cooled to 50°C at room temperature.
3. 75 ml of agarose was poured into the apparatus and the comb added.
4. When the gel was completely set (about 20-30 minutes), the comb was carefully removed and 1 X TBE buffer (containing 0.1mg/ml ethidium Bromide) added to cover the gel to a depth of about 1 mm.

5. 3-10μl of PCR product was mixed with 1-2.5μl of loading buffer (5X) and together with molecular weight markers (ϕX174 Hinf I, Promega, UK) applied to the wells
6. The gels were run at 100-150V for 30 minutes.
7. The gels were removed from the midi-gel apparatus and viewed under ultra-violet light. Polaroid photographs were taken to provide a permanent record of the results

#### 2.2.2.3.2 Polyacrylamide Gels [364]

10% polyacrylamide gels in a vertical mini-gel apparatus (Bio-Rad, USA) were used to analyse the products of semi-nested PCR of ***IgH*** CDR III regions:

1. The gel tank was set up as recommended by manufacturer.
2. 10 ml pre-mixed 10% polyacrylamide gel (BDH) was added to the gel-apparatus after adding 100 μl of 10% APS and 10 μl of tetramethylethylenediamine (TEMED, Sigma, UK), and well-forming combs inserted.
3. Once set, the combs were removed and the wells washed 3 times with 1xTBE buffer.
4. 1X TBE running buffer was added to the system. 10μl of PCR product was mixed with 2.5μl of loading buffer and together with molecular weight markers (ϕX174 Hinf I, Promega, UK) applied to the wells.
5. The gel was run at 125V for 1.5 hours.
6. The gel was then removed and stained with ethidium bromide for 5 minutes and visualised under ultra-violet light.

#### 2.2.2.3.3 Composite Gels [365,366]

60 ml of composite gels containing 5% polyacrylamide and 0.25% agarose for a 200mm X 240mm X 0.75mm gel (Bio-Rad, USA) were prepared as follows:

1. 30 ml of 10% polyacrylamide (polyacrylamide:bis-acrylamide = 99:1, National Diagnostics, England) with 20% glycerol was prepared and warmed to 30 °C in a water bath.
2. 30 ml of 0.5% agarose (Bio-Rad, USA) in 2 X TBE buffer was separately boiled and cooled down to 30 °C.
3. The polyacrylamide and agarose solutions were then mixed. After addition of 200 μl 10% ammonium persulfate and 20 μl TEMED, the mixture was immediately poured into a vertical gel apparatus (Protean II, Bio-Rad, CA, USA) and the gel allowed to set for one hour at room temperature.

**2.2.2.3.4 Denaturing Gels** [364]

6% denaturing gels were used for the LOH, automated sequencing and direct sequencing analysis.

1. 0.5 ml 10% ammonium persulphate and 50 μl TEMED were added to the gel mix (49 ml diluent, 7 ml 10X buffer, 14ml polyacrylamide) (National Diagnostics, USA), which was poured into the gel assembly and the comb inserted with the flat side down. After setting, combs were removed from the gels, the tops washed with buffer to remove excess urea and combs replaced teeth down.
2. 4 μl of samples was loaded into each well and gels run at 60W for 2-3 hours.
3. After electrophoresis gels were transferred to filter paper, dried and exposed to X-ray film for 2-5 days.

**2.2.3.3 SSCP Analysis Using a Composite Gel and A Background-Free**

**Silver Staining Method** [365,366]

### 2.2.3.3.1 SSCP Electrophoresis

PCR products (***p53***) or digested PCR products (5 μl of PCR products of ***c-myc*** exon I and intron I region digested by 1.5μl (Pharmacia, 1000u/μl) of Taq I restriction enzyme at 37°C for 0.5-1 hour) were mixed with 3 volumes of sequencing stop buffer. The mixtures were denatured at 98°C for 5 minutes and loaded onto composite gels. Electrophoresis on composites gels was carried out at 6-8 W in 1XTBE running buffer for 6-15 hours at 4°C maintained by a thermostatically controlled refrigerated circulator (LKB, Sweden).

### 2.2.3.3.2 Silver Staining

1. Gels were fixed in a solution containing 12% acetic acid, 50% methanol and 0.02% formaldehyde for 2-16 hours with gentle agitation.
2. Gels were washed in 50% ethanol for 20 minutes twice.
3. Gels were pre-treated in freshly prepared 0.02% sodium thiosulphate for 1 minute and rinsed in distilled water three times.
4. Gels were then impregnated with 0.2% $AgNO_3$ and 0.03% formaldehyde for 20-30 minutes, and rinsed in distilled water twice.
5. PCR products were then visualised in a solution containing 6% $Na_2CO_3$, 0.02% formaldehyde and 0.0005% sodium thiosulphate for 3-5 minutes and the reaction was stopped by transferring gels into a solution containing 50% methanol and 16% acetic acid.
6. Gels were dried onto cellophane or filter paper.

### 2.3.2.2. Electrophoresis on ABI Automated Sequencer and Data Analysis

Following multiplex fluorescent PCR amplification, 0.5 μl of PCR product was mixed with 0.6 μl of internal lane size standard (GeneScan 2500-ROX) and 4 μl blue formamide loading buffer (ABI, Foster city, CA, USA). Samples were denatured at 94°C for 3 minutes and loaded onto 6% denaturing polyacrylamide gels on a model 373A sequencer (ABI, Foster city, CA, USA) and electrophoresed for 4 hours.

The fluorescent gel data were automatically collected and analysed using GeneScan™ 672 (version 1.2) software following electrophoresis as described in the manufacturer's manual. The PCR products were distinguished from each other by different sizes and dyes.

### 2.2.3.4 Direct Sequencing of PCR Products

Direct sequencing of PCR products was used to analyse the ***IgH*** CDR III regions and identify mutations of the ***c-myc*** and ***p53*** genes. The method was adapted from the protocol recommended by the manufacturer (UBS, Amersham, UK), with some modifications:

#### 2.2.3.4.1 Sequencing Reaction

1. 5 μl of PCR product were added to 1 μl of exonuclease 1, incubated at 37 °C for 15 minuets and heated to 85 °C for 5 minutes to inactivate the enzyme.
2. 1 μl of shrimp alkaline phosphatase was added and mixtures incubated at 37 °C for 15 minutes followed by 5 minutes at 85 °C.
3. 3 μl primer (30pmol) which had been used in the PCR amplification was added and the tubes heated to 99 °C for 5 minutes, placed on ice for 5 minutes and spun.
4. A total of 7.5 μl of sequencing reagent mixtures (2 μl reaction buffer, 1 μl

0.1M DTT, 0.2 μl deaza-dGTP, 0.4 μl Sequenase enzyme, 1.6 μl enzyme buffer, 0.5 μl $^{35}$S-dATP and 1.8 μl $dH_2O$) were added to each DNA preparation. Tubes were spun and left at room temperature for 5 minutes. 3.5 μl of each preparation was added to 2.5 μl each of ddATP, ddCTP, ddGTP, ddTTP in separate tubes and incubated at 37 °C for 5 minutes.

5. 4 μl of stop solution were added to each tube and samples were heated to 95 °C for 2 minutes prior to loading onto denaturing gels from ice.

To rule out artefacts, the sequencing was conducted from both directions or repeated at least twice.

#### 2.2.3.4.2 Sequence Analysis

Computer analysis of the DNA sequences obtained was carried out using a BLAST WWW online server in the National Centre for Biotechnology Information at the National Library of Medicine, USA, (http://www.ncbi.nlm.nih.gov/Blast) and Wisconsin GCG software (provided by the Human Genome Mapping Project, Cambridge, UK).

### 2.3 Immunostaining of *p53* [367]

1. 3-5 μm sections were cut from paraffin blocks on to Vectabond coated microscope slides. Sections were dried at 56°C overnight.
2. Sections were de-waxed using two changes of xylene and taken to absolute alcohol.
3. Slides were incubated in 0.5% hydrogen peroxide in methanol for 10 minutes (to block endogenous peroxidase activity), then rinsed thoroughly in tap water.
4. Paraffin sections were treated in 0.01 M sodium citrate buffer (pH 6.0) in a pressure cooker for two minutes in order to retrieve the antigen.
5. ***P53*** protein was routinely immunostained using the CM1 polyclonal

antibody (1/5000 dilution) for 16 hours at room temperature, followed by biotinylated swine anti-rabbit immunoglobulin (1/250 dilution) for 40 minutes and StreptABComplex/HRP (1/100 dilution) for 40 minutes.

6. Sections were visualised by incubation with diaminobenzidine (DAB) and $H_2O_2$.

*Most of the immunohistochemistry was carried out by Miss Nalini Singh.

**Table 2-2 PCR Primers Used in Single-Round PCR**

| Chapter | Target genes | Size (bp) | Analysis | Primer Sequences |
|---|---|---|---|---|
| 4 | ***CagA*** gene (new) | 133 | Agarose | TCAGAAATTTGGGGA(C)TCAG ;TCATCAA(G)GGA(G)TAGGGGTTG |
| | ***CagA*** gene (old) | 350 | Agarose | GATAACAGGCAAGCTTTTGAGG; CTGCAAAAGATTGTTTGCGAGA |
| | ***Urease A*** gene | 140 | Agarose | GCCAATGGTAAATTAGTT; CTCCTTAATTGTTTTTAC |
| 5[a] | D3A1262 (3q) [368] | 112-126 | | FAM*CGGCCCTAGGATATTTTCAA; CCAGTTTTTATGGACGGGGT |
| | D3s1265 (3q27-qTER) [368] | 126-150 | Automated | HEX*TTCTATAAGGGCAGGGACAC; GCTCGCAATTTCTCCTTAAT |
| | D3S11 (3p24.2-p22) [368] | 135-147 | Sequencer | FAM*CAAACTTTCCACAGTATCGTTC; GTTTCCTTGAGAAGAATGGAGC |
| | D3S1261(3p14-p12) [368] | 84-116 | | HEX*GAGGTGATGTGAGAGTGGAT; CTCAGCACCACAGTATGTGT |
| | D6S262 (6p23.1-21.3) [368] | 167-183 | | HEX*ATTCTTACTGCTGGAAAACCAT; GGAGCATAGTTACCCTTAAAATC |
| 6 | ***c-myc*** exon I-intron I | 650 | SSCP | GCACTGGAACTTACAACACC; CTGGCTCACACAGGCGAT |
| 7 | ***P53*** Exon 5[369] | 245 | | TTCCTCTTCCTGCAGTACTC; ACCCTGGGCAACCAGCCCTGT |
| | ***P53*** Exon 6[369] | 163 | SSCP | AGTTGCAAACCAGACCTCAG; ACAGGGCTGGTTGCCCAGGGT |
| | ***P53*** Exon 7[369] | 138 | | GTGTTGCCTCCTAGGTTGGC; CAAGTGGCTCCTGACCTGGA |
| | ***P53*** Exon 8[369] | 165 | | CCTATCCTGAGTAGTGGTAA; GTCCTGCTTGCTTACCTCGC |
| | ***P53*** CA repeat [b 370] | 103-135 | LOH | AGGGATACTATTCAGCCCGAGGTG; ACTGCCACTCCTTGCCCCATTC |

[a]: multiplex fluorescent PCR.

*: FAM or HEX fluorescent-labelled primer

[b]: One of the primers was end-labelled using $\gamma$-$^{32}$P-dATP and T4 polynucleotide kinase.

# Chapter 3 Genetic Evidence for a Clonal Link between Low and High-grade MALT Lymphoma

## 3.1 Introduction

Low-grade MALT lymphomas usually run an indolent clinical course, but may be followed by aggressive high-grade tumours. [2,34,371] Many high-grade tumours contain low-grade components. [34] These clinical and pathological features have led to the hypothesis that high-grade MALT lymphomas evolve directly from low-grade disease. This may be particularly true of gastric MALT lymphoma since the stomach is the most common site for the tumour, and most gastric MALT lymphomas are high-grade. [4] Immunophenotypic studies have shown the same immunoglobulin (***Ig***) light chain restriction in coexisting low and high-grade lesions of gastric MALT lymphomas. [34]

However, the relationship between the coexisting low and high-grade lesions is difficult to establish with morphologic examination alone. Immunophenotypic studies are not entirely reliable determinants of clonal identity of two cell populations. [372,373] In addition, because patients with lymphoid malignancies have a greater tendency than the normal population to have secondary lymphoma, [374,375] there is also a possibility that the high-grade lesions represent a secondary unrelated malignancy. An example is Richter's syndrome (chronic lymphocytic leukaemia (CLL) or small lymphocytic lymphoma (SLL) and subsequent diffuse large cell lymphoma (DLL)). It has been suggested, based on immunohistological, ***Ig*** isotype, anti-idiotype, ***IgH*** gene rearrangement and cytogenetic studies, that the DLL develops as a clonal evolution from CLL/SLL, or alternatively, that the two neoplasms are distinct, unrelated clonal proliferations. [110] Therefore it is necessary to establish a genetic link between both low and high-grade lesions of MALT lymphoma.

In order to investigate the clonal link between low and high-grade lesions, the ***IgH*** CDR III regions of four gastric MALT lymphomas were analysed using

PCR and direct sequencing. As high and low-grade tumour populations were present within each tissue section and were accompanied by substantial reactive lymphoid infiltrates, microdissection was used to enrich target cell populations.

## 3.2. Materials and Methods

### 3.2.1. Case Selection

All of the high-grade lymphomas in the tissue bank were reviewed histologically. The only criteria for case selection was the availability of clear-cut coexisting low and high-grade lesions and feasibility of PCR amplification of ***IgH*** from both lesions. Four cases met these criteria and were suitable for this study.

### 3.2.2 Microdissection and DNA Extraction

The low and high-grade lesions of each case were identified and separated by microdissection and DNA was extracted (see 2.1.3.3 and 2.2.1.1 ). [363]

### 3.2.3 PCR of *IgH* FR3/JH

All DNA samples from both lesions of each case were amplified in parallel using PCR primers directed to framework (FR) 3 and the joining (JH) region of ***IgH***. (See 2.2.3.1.2) [20] Test samples were run in parallel with a monoclonal control (follicular lymphoma) and a negative control (no template DNA). Extractions from each specimen were carried out at different times and duplicated to minimise the risk of cross-contamination. Products were analysed on 10% polyacrylamide gels, stained with ethidium bromide and viewed under UV radiation. (See 2.2.2.3.2)

### 3.2.4 Direct Sequencing

PCR products from each case were directly sequenced in both directions with JH or FR3 primers (see 2.2.3.4).

### 3.2.5 Sequence Analysis

The sequence analysis was carried out by WWW-based BLAST software and GCG package. The D and J regions were determined from published germline sequences. [376,377]

## 3.3. Results

Using FR3/JH primers, PCR amplification of all extracts from microdissected low and high-grade lesions of each lymphoma produced a discrete fragment within the expected size range (70-120 bp), with or without a background smear, representing monoclonal rearrangement of the ***IgH*** gene. Both lesions yielded bands of identical size in each case (Figure 3-1). No products were seen in negative control reactions and the positive control yielded the expected dominant band. Amplification of duplicate samples produced identical products.

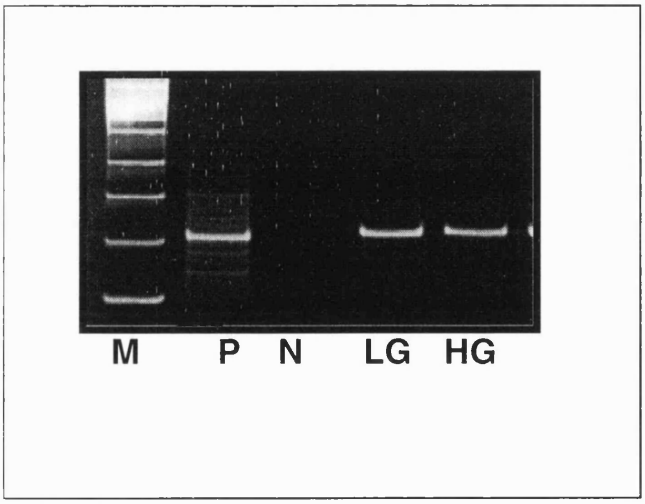


**Figure 3-1. Ethidium Bromide Stained Polyacrylamide Gel Showing PCR Products from Low and High-grade Lesions of A MALT Lymphoma**. Lanes: M - molecular weight markers; P – positive control; N - negative control (no DNA template); LG - low-grade lesion; HG high-grade lesion

Following direct sequencing, paired PCR products from low and high-grade lesions in cases 1 and 2 showed identical sequence (Figure 3-2). In cases 3 and 4, the sequences were identical except for differences at 2 and 3

nucleotide positions respectively. Computer analysis confirmed that each sequence was a rearranged ***IgH*** gene containing combinations of V, D, and J regions and containing a variable number of nucleotides at the V-D (N1) and D-J (N2) junctions (Table 3-1). The differences between the sequences derived from the low and high-grade lesions were located within the D or N regions.

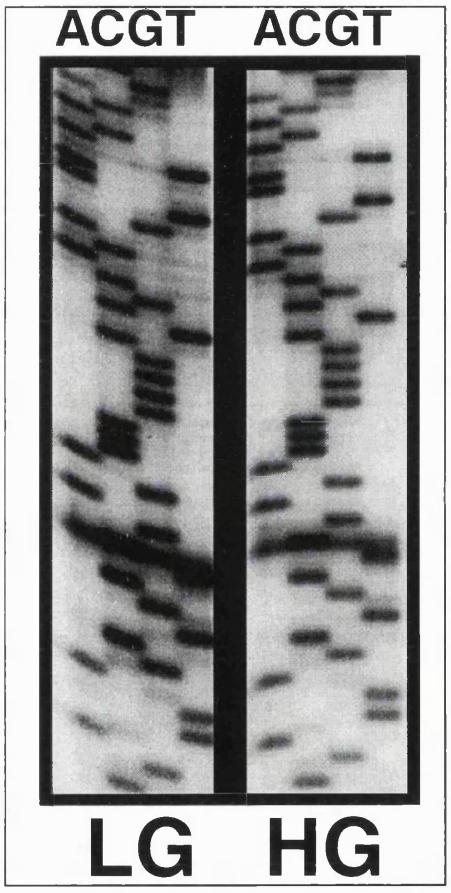


**Figure 3-2: IgH CDR III Sequences Derived from Low (LG) and High-grade (HG) Lesions of a High-grade B Cell MALT Lymphoma.**

### 3.4. Discussion

In this study, PCR and direct sequence analysis were used to examine the rearranged ***IgH*** genes of low and high-grade lesions microdissected from four MALT lymphomas. The amplification of dominant ***IgH*** PCR products of identical size from both lesions in each case implies the presence of the same neoplastic clone in both lesions in each patient. This has been further confirmed by direct sequencing that revealed common clones in each case.

**Table 3-1 The IgH CDR III Sequences Derived From Low and High-grade Lesions**

| Cases | | N1 | D | N2 | J |
|---|---|---|---|---|---|
| | | | *DXP-3* | | *J5* |
| 1 | LG | GCCAG | TTACTATTATGGAAGTCGTGA | ACGCCGGAG | CTGGTTCGACCCC |
| | HG | ----- | -------------------- | --------- | ------------- |
| | | | *DN1* | | *J4* |
| 2 | LG | GCGAGCCCCGGGTCTCG | AGCAGCT | AATCG | TATCACT |
| | HG | ----------------- | ------- | ----- | ------- |
| | | | *DLR-1* | | *J4* |
| 3 | LG | GCGAAATCCGAC | GACGATAAT | | CACTTTGACTAC |
| | HG | -----T------ | ---A----- | | ------------ |
| | | | *D23-7* | | *J4* |
| 4 | LG | GCGCG | TGGAGAG | GACCGATTTTGCC | GACTA |
| | HG | --C-- | --C---- | -----T------- | ----- |

Identical nucleotides between low and high-grade lesions are represented by dashes, and sequence variations are shown by appropriate nucleotides.

The result provides solid evidence supporting a genetic link between the low and high-grade lesions of MALT lymphoma. This finding is in agreement with the observations of shared microsatellite alterations in low and high-grade lesions in chapter 5. [378] It is therefore likely that high-grade MALT lymphomas arise by further transformation of low-grade lesions.

Transformation from preceding low-grade disease may be a general feature of high-grade MALT lymphomas, including the cases without visible low-grade lesions. This is supported by the shared features of low and high-grade tumours, such as association with preceding *H pylori* infections, [17] the frequent presence of replication error (RER+) phenotype (chapter 5) [378], ***p53*** alterations (see chapter 7) [379] and trisomy of chromosome 3. [128] Low-grade lesions may not be identified in some high-grade tumours due to sampling bias [380] or to overgrowth by high-grade cells. [99] However, it can not be excluded that some high-grade tumours may arise *de novo*.

The mechanisms involved in the high-grade transformation of low-grade MALT lymphoma have yet to be identified. In this study, some nucleotide differences between the ***IgH*** CDR III sequences derived from low and high-grade lesions were observed. These may represent ongoing mutations that resulted from antigen stimulation or aberrant function of the somatic hypermutation mechanism. However, the relationship of this process to high-grade transformation is unclear. In-vitro studies have shown that the proliferation of high-grade tumour cells, unlike low-grade cells, does not depend on T cell mediated specific antigen stimulation. [37] Therefore, it is possible that during a prolonged antigen driven life course some low-grade tumour cells escape from immune dependency by transformation into high-grade lesions through acquisition of additional genetic changes, such as ***p53*** alteration (chapter 7) [379] and ***c-myc*** activation (chapter 6). [381]

### 3.5 Conclusion

High-grade B cell MALT lymphomas generally evolve from low-grade clones.

## Chapter 4 High Frequency of *CagA+ H pylori* Infection in High-grade Gastric B Cell MALT Lymphomas

### 4.1 Introduction

A high incidence of *H pylori* infection has been observed in patients with gastric B cell MALT lymphoma. [17,142] Although in vitro experiments have shown that the bacterium can provoke an immune response in low-grade B cell MALT lymphoma through T cell mediation, [37,176,382] the role of *H pylori* in the pathogenesis of the tumour is not fully understood. Recent studies have indicated that, in addition to immune stimulation, [160] the bacterium has other direct oncogenic effects which may be associated with certain strains. [383] Investigation of *H pylori* subtypes may help to further understand the pathogenic role of the bacterium in MALT lymphoma.

*H pylori* has numerous genetically distinct strains. [180] These can be classified into two types: ***CagA***+ and ***CagA***- based on the presence of the ***CagA*** gene.[163] ***CagA***+ *H pylori* are found in the vast majority of gastric pre-cancerous lesions (atrophic gastritis and peptic ulcer) [149,186] and gastric carcinoma, [187,384] and considered to be particularly virulent. [385] The role of the ***CagA*** gene in tumourigenesis is still not clear. It has been observed that the ***CagA***+ *H pylori* harbours a 40 kb fragment known as the "pathogenicity island" which contains genes with direct virulent effects. [386] In vitro and in vivo studies have shown that ***CagA***+ bacterial infection can increase secretion of IL-8, which is an important chemotactic and activation factor for neutrophils. [191,192,387] Continuous up-regulation of IL-8 and neutrophil activation could lead to increased free radical formation and directly or indirectly promote tumour development and progression. [45,383]

***CagA***+ *H pylori* infection in MALT lymphoma has been investigated

previously by two groups, [188,189] with discordant results. One found a high incidence of ***CagA***+ *H pylori* infection in gastric MALT lymphoma, [188] while the other observed no significant association of this strain with the tumour. [189] This difference may be due to limited numbers of cases analysed and different methods used. In the present study, an effective and sensitive PCR assay was specifically developed for archival tissue samples. This assay allowed examination of the ***CagA*** genotype in a larger number of *H pylori* associated low and high-grade gastric MALT lymphomas.

## 4.2 Materials and Methods

### 4.2.1 Materials

123 fresh frozen (13) and paraffin-embedded (110) *H pylori* infected gastric biopsies were collected from Italy (Institute of Pathological Anatomy and Histopathology, Nuovo Ospedale Regionale, Ancona, Italy). These included 56 cases of gastritis, and 37 low-grade and 30 high-grade MALT lymphomas. The age distribution of all cases was in the range of 50-70. The histological diagnoses had been made using histological and immunohistological criteria. [2,8] DNA was extracted as described previously. [20] The presence of *H pylori* was confirmed by PCR amplification of the ***urease A*** gene. [155] To verify the specificity of our PCR protocols, DNA samples from 10 *H pylori* strain cultures with known ***CagA*** status (4 ***CagA*** negative and 6 ***CagA*** positive), [388] obtained from Dr JC Atherton (Nottingham University Hospital) and Dr A Basarab (Southampton General Hospital) as a gift, were used as controls.

### 4.2.2 Primers and PCR

All available ***CagA*** gene sequences listed in the GenBank database [389] were retrieved and analysed by the GCG pileup program (HGMP, MRC, Cambridge, UK) for conserved regions. These regions were further analysed

by PRIMER program (HGMP, MRC, Cambridge, UK) for primer design.

The amplification efficiency of the newly designed primer set was compared with the previously published ***CagA*** primers (covering 350bp, Table 2.2) [189,390] on DNA extracts from both fresh and paraffin embedded tissue samples of 10 cases of *H pylori* positive MALT lymphoma. The specificity of the adapted and newly designed primer set was examined on known ***CagA***+/- bacterial culture controls. The PCR products generated with the new primer set on the positive controls were directly sequenced and the sequences were compared with published data by WWW-based BLAST analysis.

PCR was carried out as described in section 2.2.3.

In order to rule out false negatives the ***urease A*** gene of *H pylori* [155] was amplified in parallel with amplification of the ***CagA*** gene.

### 4.2.3 Statistical Analysis

Differences in the incidence of ***CagA*** positivity among low and high-grade lymphomas and gastritis controls were evaluated by the $\chi^2$ test.

## 4.3 Results

Using the PileUp programme in the GCG software package, a conserved region was found in the ***CagA*** region (Figure 4-1). Based on the sequence of this region, a pair of primers was selected using the PRIMER programme (Table 5-1) with an expected PCR fragment of 133 bp in size.

PCR products of expected sizes were successfully amplified from each of the 6 ***CagA*** positive bacterial strains, using either the newly designed (133bp products ) or previously published (350bp products) ***CagA*** primer sets. The

sequencing confirmed that the 133 bp PCR products from all 6 positive controls were fragments within the ***CagA*** gene of *H pylori* (Figure 4-1). None of the 4 ***CagA*** negative strains showed any PCR amplification with either of the two primer sets.

```
           1                GA                                          50
           TCAGAAATTT GGGACTCAG Sense primer
AB003397   TCAGAAATTT GGGACTCAGC GTTACCGAAT TTTCACAAGT TGGGTGTCCC
AF001357   TCAGAAATTT GGGACTCAGC GTTACCGAAT TTTCACAAGT TGGGTGTCCC
HPU60176   TCAGAAATTT GGGGATCAGC GTTACCGAAT TTTCACAAGT TGGGTGTCCC
  X70039   TCAGAAATTT GGGGATCAGC GTTACCGAAT TTTCACAAGT TGGGTGTCCC
AE000569   TCAGAAATTT GGGGATCAGC GTTACCAAAT TTTCACAAGT TGGGTGTCCC
  L11714   TCAGAAATTT GGGGATCAGC GTTACCGAAT TTTCACAAGT TGGGTGTCCC
       4a  TCAGAAATTT GGGGATCAGC GTTACCAAAT TTTTATGAAT TGGGTGTCCC
       7a  TCAGAAATTT GGGGATCAGC GTTACCAAAT TTTTATGAAT TGGGTGTCCC
       5a  TCANAAATTT GGGGATCAGC GTTACCAAAT TTTTATGAAT TGGGTGTCCC
  U80066   TAAGAAATTT GGGGATCAGC GTTACCAAAT TTTTACGAGT TGGGTGTCCC
  U80065   TCGGAAATTT GGGGATCAGC GTTACCAAAT TTTTACGAGT TGGGTGTCCC

           51                                                       100
AB003397   ATCAAAACGA TCCGTCTAAA ATCAACACCC GATCGATCCG AAATTTTATG
AF001357   ATCAAAACGA TCCGTCTAAA ATCAACACCC GATCGATCCG AAATTTTATG
HPU60176   ATCAAAACGA TCCGTCTAAA ATCAACACCC GATCGATCCG AAATTTTATG
  X70039   ATCAAAACGA TCCGTCTAAA ATCAACACCC GATCGATCCG AAATTTTATG
AE000569   ATCAAAAAGA TCCGTCTAAA ATCAACACCC GATCGATCCG AAATTTTATG
  L11714   ATCAAAACGA TCCGTCTAAA ATCAACACCC GATGCATCCG AAATTTTATG
       4a  ATCAAAAAGA TCCGTCTCAA ATCAACACCC AAAAAATCCG AGGTTTTATG
       7a  ATCAAAAAGA TCCGTCTCAA ATCAACACCC AAAAAATCCG AGGTTTTATG
       5a  ATCNAAAAGA TCCGTCTCAA ATCAACACCC ANNAAATCCG AGGTTTTATG
  U80066   TTCAAAAAGA TCCGTCTGAA ATCAACACCC AACAAATCCG AAATTTTATG
  U80065   TTCAAAAAGA TCCGTCTAAA ATCAACACCC AACAAATCCA AAATTTTATG

           101                                  133
AB003397   GAAAATATCA TACAACCCCC TACCCCTGAT GA
AF001357   GAAAATATCA TACAACCCCC TACCCCTGAT GA
HPU60176   GAAAATATCA TACAACCCCC TATCCTTGAT GA
  X70039   GAAAATATCA TACAACCCCC TATCCTTGAT GA
AE000569   GAAAATATCA TACAACCCCC TATCCCTGAT GA
  L11714   GAACATACCA TACAACCCCC TATCCCTGAT GA
       4a  GAAAATATCA TACAACCCCC TATCTCTGAT GA
       7a  GAAAATATCA TACAACCCCC TATCTCTGAT GA
       5a  GAAAATNNCA TGCAACCCCC TATCTCTGAT GA
  U80066   GAAAATATCA TACAACCCCC TATCTCTGAT GA
  U80065   GAAAATATCA TACAACCCCC TATCTCTGAT GA
                     GTTGGGGG ATAGAGACTA CT Anti-sense Primer
                              C GA
```

**Figure 4-1: PileUp Result of *CagA* gene**: AB003397, AF001357,HPU60176, X70039, AE000569, L11714, U80066 and U80065 are the published sequences retrieved from the GenBank database.[389] 4a, 5a and 7a are the sequences of the PCR products amplified from 3 known ***CagA***+ *H pylori* strains. The primer positions are bold and underlined, and the degenerate nucleotides are indicated.

Using DNA extracted from fresh tissues, one sharp band of the expected size was demonstrated in 5 of the 10 selected cases with both primer sets. When these primers were tested on the extracts from paraffin embedded tissues of the same cases, the ***CagA*** gene in the five positive cases was also amplified using the new primer set. However, only one of the five cases was amplified using the previously published primer set (Figure 4-2).

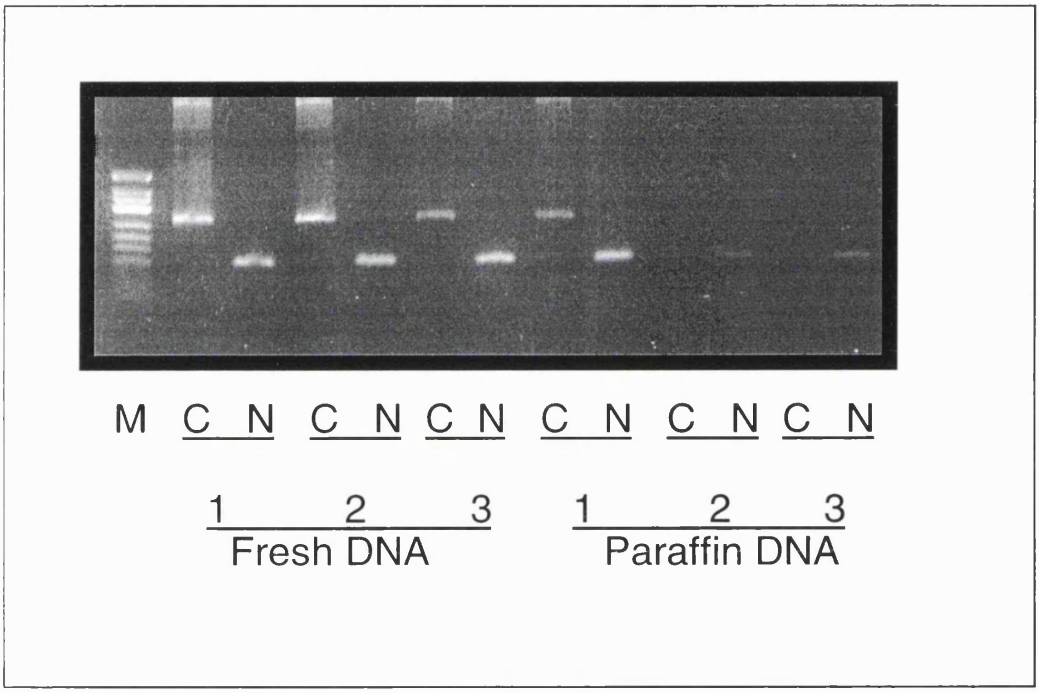


**Figure 4-2: Efficiency of PCR Amplification of the *H pylori CagA* Gene Using Novel (N) and Conventional (C) Primer Sets.** Successful amplification was achieved from fresh tissue extracts (DNA 1-3) by both primer sets. From paraffin embedded tissue extracts of the same cases, the conventional primer set was successful in only one case (case 1), whereas the novel primer set was successful in all three cases (cases 1-3)

All of the 123 cases tested showed a distinct band of PCR products for the *H pylori* ***urease A*** gene. With the new ***CagA*** gene primers, the gene was identified in 37 of 67 (55.2%) gastric MALT lymphomas with an incidence of 37.8% (14/37) in low-grade and 76.8% (23/30) in high-grade tumours (Table

4-1 and Figure 4-3). The ***CagA*** gene was also detected in 17/56 (30.3%) of the gastritis controls. Statistical analysis showed that the incidence of ***CagA*** *H pylori* infection in high-grade MALT lymphoma was significantly higher than that in low-grade MALT lymphomas or gastritis ($p<0.05$). There was no significant difference between low-grade MALT lymphoma and gastritis ($p>0.05$).

**Table 4-1 *CagA* Gene in the MALT Lymphomas**

| | Gastric MALT lymphoma | | Gastritis |
|---|---|---|---|
| | **Low-grade** | **High-grade** | |
| **Cases** | 37 | 30 | 56 |
| **Positive** | 14(37.8%) | 23 (76.8%) | 17(30.3%) |
| **Total** | 37/67=55.2% | | 17/56=30.3% |

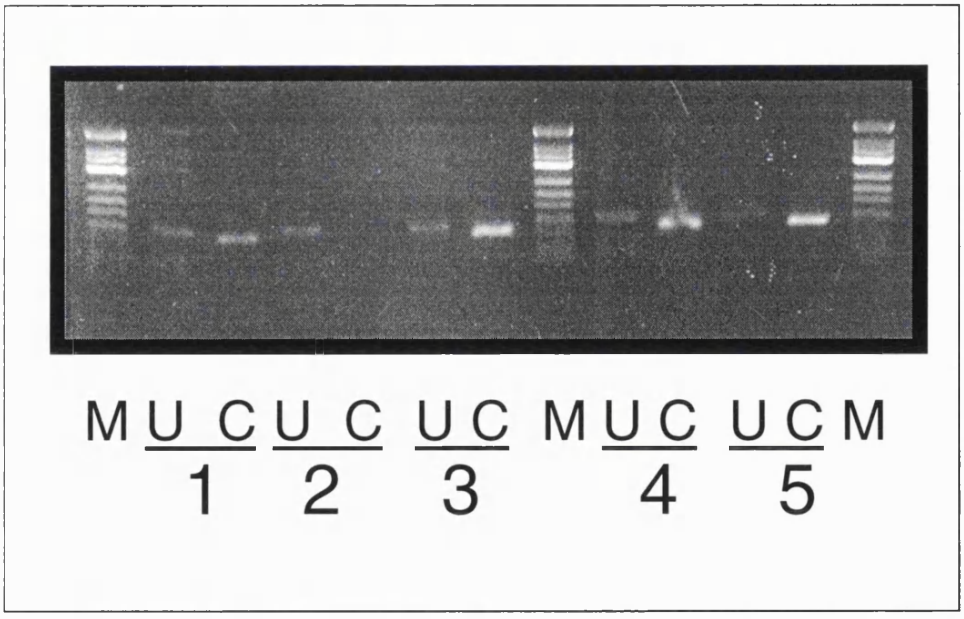


**Figure 4-3: PCR Amplification of *H pylori CagA* (C) and *Urease A* (U) Genes from Low-grade MALT Lymphomas (cases 1-2), High-grade MALT Lymphomas (cases 3-4) and Gastritis (case 5).**

## 4.4 Discussion

Based on an extensive search of the GenBank database [389] and GCG PileUp analysis, conserved sequences for the ***CagA*** gene in *H pylori* were identified. Using these sequences, a set of primers with the potential to target the ***CagA*** gene in any ***CagA***+ *H pylori* strain and suitable for amplification of DNA from archival paraffin-embedded tissues was designed. The specificity of the primer set was validated by amplification of the expected PCR products using the positive control bacterial DNAs and sequence analysis of the PCR products. When compared with the primer set used in previous studies in a trial experiment, this primer set demonstrated a much higher sensitivity and efficiency in amplification of archival tissues and should provide an improved means of ***CagA*** genotyping.

The incidence of ***CagA***+ *H pylori* infection in low-grade gastric MALT lymphoma was similar to that in gastritis. Therefore, the development of low-grade B cell gastric MALT lymphoma is unlikely to be associated with the ***CagA*** status of *H pylori*. This finding is not surprising, since low-grade gastric MALT lymphomas commonly arise from acquired MALT lymphoid follicles[2,5,6,62], that can be induced by any strain of *H pylori*. [165] The role of *H pylori* infection in the pathogenesis of low-grade MALT lymphoma may, thus, be generally related to immune response rather than the ***CagA*** associated cytotoxic effects of the bacterium.

The incidence of ***CagA***+ *H pylori* infection in high-grade gastric MALT lymphoma was found to be significantly higher than in low-grade gastric MALT lymphoma or gastritis. This finding indicates that high-grade transformation may be more likely to occur in gastric MALT lymphomas associated with ***CagA***+ *H pylori*. In-vitro studies have shown that stimulation of *H pylori* that provokes an immune response in low-grade MALT lymphoma has no effect on high-grade MALT lymphoma. [37] It is possible that the ***CagA*** associated cytotoxic effect has an association with the pathogenesis of high-

grade MALT lymphomas.

***CagA+*** *H pylori* infection can enhance inflammation through up-regulation of IL8, the neutrophil chemotactic and activating peptide. [160,184,191,192] The enhanced inflammatory process can increase the production of free radicals[45] which are extremely genotoxic and can cause a wide range of genetic abnormalities, [42,43,391] favouring the accumulation of specific genetic abnormalities in cells. It is not clear whether these factors are important in high-grade MALT lymphomas.

### 4.5 Conclusion

High-grade transformation of gastric B cell MALT lymphoma may be more likely to occur following infection of ***CagA+*** H pylori.

# Chapter 5 Genetic Instability Indicated by the RER+ Phenotype in MALT Lymphomas

## 5.1. Introduction

Low-grade MALT lymphomas arise from a background of inflammatory lesions and can transform into high-grade lymphomas at a later stage. [1,34] Since chronic inflammation may be associated with genetic instability and the latter can serve as a basis for inactivation of tumour suppressor genes and activation of oncogenes, [42,43] both of which directly contribute to tumour transformation, [198,199] it is possible that genetic instability may play a role in the evolution and progression of this type of lymphoma.

In this study, a newly defined manifestation of genetic instability, the replication error (RER+) phenotype, [201] has been investigated in various lesions microdissected from 40 MALT lymphomas by multiplex fluorescent PCR. The aim of this study was to investigate the incidence of the RER+ phenotype and its role in the development of MALT lymphoma.

## 5.2. Materials and Methods

### 1.2.1 Materials

Forty lymphomas (16 high-grade and 24 low-grade) were collected. Tumour cell populations (high and/or low-grade lesions), along with normal tissues (mucosa, spleen, liver, muscle, and lymphoid infiltrates distant from tumours when available), from each MALT lymphoma were microdissected from either paraffin-embedded (10 cases) or frozen sections (30 cases). In five high-grade lymphomas with low-grade lesions, both components were microdissected separately. In addition, tumour-adjacent reactive lymphoid infiltrates from 7 lymphomas (five low-grade and two high-grade) were also

microdissected. The nature of the microdissected lesions was confirmed by PCR clonality analysis for ***IgH*** gene rearrangement using FR3/JH primers.

### 5.2.2 Multiplex Fluorescent PCR and GeneScan™ analysis

Five pairs of primers (listed in Table 2-2) were chosen for microsatellite fluorescent PCR from the Genetic Database (GDB) at Genethon. [368] The markers were selected to enable amplification of all 5 loci in a single PCR reaction. One of each primer pair was fluorescently labelled with either FAM (blue) or HEX (yellow).

Paired normal and tumour DNA samples from each patient were amplified simultaneously.

The paired DNA samples were subjected to multiplex fluorescent PCR of five chromosome loci (the primer sequences and their labelled dyes are listed in Table 2-2). The PCR products were analysed on a model 373A sequencer and the data were analysed using GeneScan™ 672 (version 1.2) software.

## 5.3. Results

### 5.3.1 Interpretation of Microsatellite Alteration and RER+ Phenotype

A major peak surrounded by one or two minor peaks represents a normal allele. A microsatellite alteration was defined as a locus that gained one or more new peaks, or showed peak shifts when compared with the patterns from the corresponding normal tissue. If a sample contained 2 or more microsatellite alterations among 5 loci examined, it was classified as RER+ as proposed previously. [392]

### 5.3.2 Microsatellite Alteration and RER+ Phenotype in Chronic

**Inflammatory Lesions.**

Normal tissues from different sites of the same patients showed identical microsatellite migration patterns. No microsatellite alterations were observed in reactive lymphoid infiltrates distant from the lymphomas. However, among tumour-adjacent lymphoid infiltrates isolated from seven cases of lymphoma, five showed one microsatellite alteration, which in each case was identical or different to that of the corresponding tumours.

### 5.3.3 Microsatellite Alteration and RER+ Phenotype in MALT Lymphomas

Of 40 MALT lymphomas, 33 showed microsatellite alterations. The microsatellite alterations at 5 loci, D3S1262, D3S11, D3S1261, D3S1265, and D6S262 were found in 16 (40%), 8 (20%), 17 (42.5%), 19 (47.5%) and 12 (30%) of 40 tumour cases respectively (see Figure 5-1). The comparable percentage at different loci suggests that genetic instability is widespread, involving multiple genetic sites. The frequency at which specific numbers of microsatellite alterations were seen in high and low-grade lymphoma is illustrated in Figure 5-2. There were a consistently higher percentage of alterations in high-grade lymphoma.

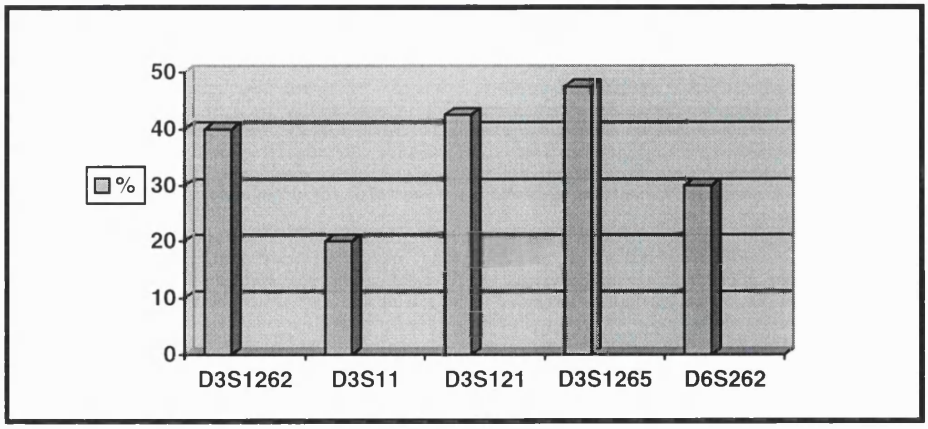


**Figure 5-1 Microsatellite Alteration Frequency at 5 Loci in MALT Lymphoma**

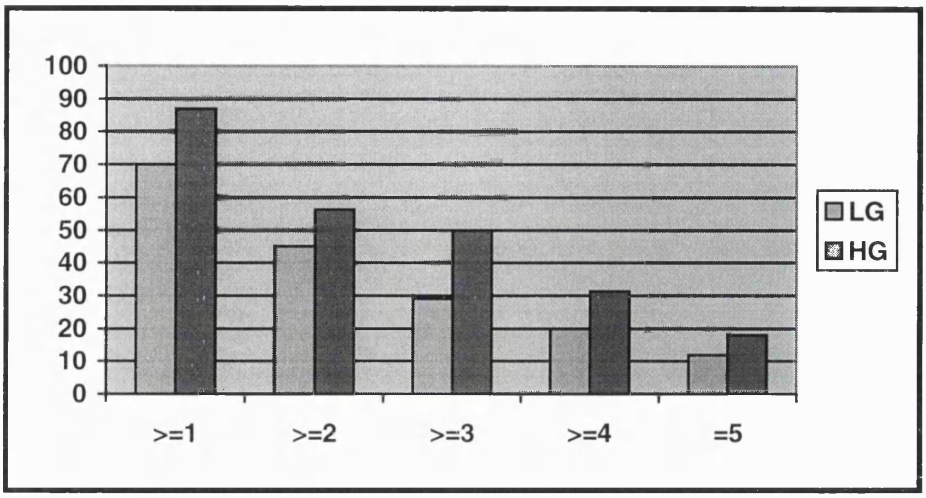


**Figure 5-2. Comparison of Microsatellite Alterations between Low and High-grade Lymphomas**

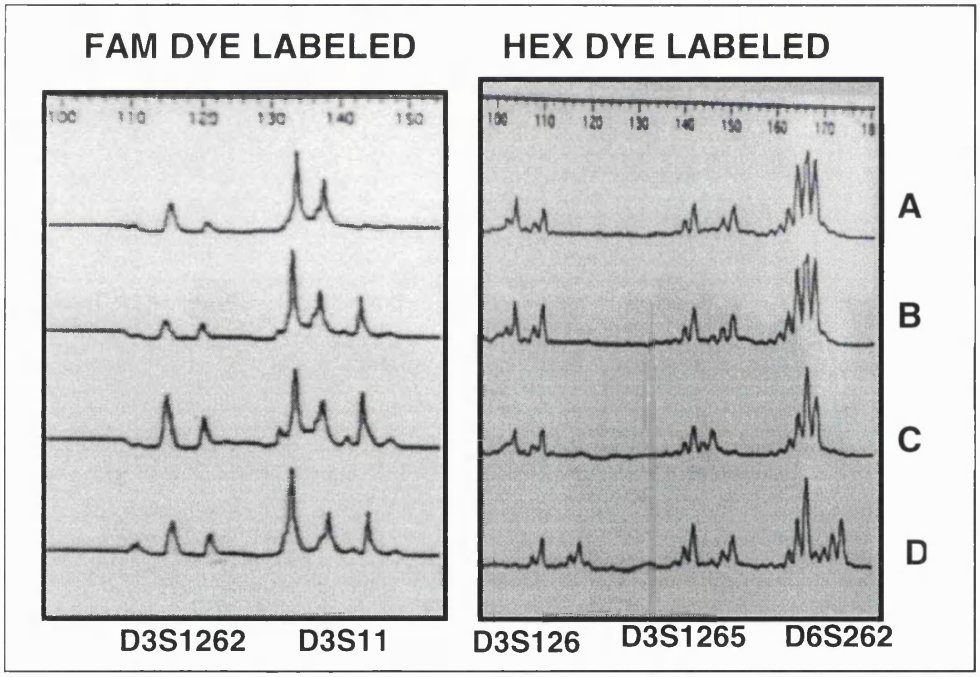


**Figure 5-3. The Microsatellite Alterations in a High-grade MALT Lymphoma with Adjacent Low-grade Components and Reactive Lymphoid Infiltrates**. A: normal tissues. B: adjacent reactive lymphoid infiltrates. C: low-grade components and D: high-grade components.

Only 21 (52.5%) lymphomas were RER+. The frequency of the RER+ phenotype was slightly higher in high-grade (9/16, 56.2%) than in low-grade (12/24, 50%) MALT lymphomas, without a statistically significant difference ($p>0.05$).

Four of the 5 microsatellite alterations observed in tumour-adjacent lymphoid infiltrates were also identified in the corresponding lymphoma lesions (Figure 5-3). In the 5 high-grade lymphomas with low-grade components, 2 or more microsatellite alterations were observed in both tumour lesions from each case. Some of these alterations appeared to be heterogeneous, but at least one was identical in both low and high-grade components (Figure 5-3).

## 5.4. Discussion

In this study, 52.5% of MALT lymphomas were shown to have the RER+ phenotype. There are limited data available about the RER+ phenotype in the lymphomas. The frequency presented here is similar to that reported in HIV-related NHL, [232] but is much higher than that of other nodal lymphomas where reported frequencies vary from 0% to 20%. [232,393,394] The results further demonstrate that MALT lymphomas are a distinct entity, differing from nodal lymphomas at the genetic level.

Genetic instability may occur at different stages in malignant development, depending on the type of tumour. For example, the RER+ phenotype has been found in the early stage of NHPCC [395] and gastric lymphoma [222,396] and in the late stage of non-NHPCC. [395] The results here have shown that genetic instability persists throughout the development of MALT lymphoma and may play a role at early as well as late stages of tumour development.

Reactive lymphoid infiltrates adjacent to the lymphoma, in contrast to those distant from the tumour, demonstrated microsatellite alterations which were

also identified in the corresponding low or high-grade lesions. This is the first direct evidence of a relationship between reactive lymphoid infiltrates and the adjacent MALT lymphomas. It also suggests that microsatellite alterations occur before the histological appearance of a tumour. This evidence supports the hypothesis that lymphoid infiltrates induced by chronic inflammatory stimulation are precursors of MALT lymphomas. The role of chronic inflammation in tumour promotion remains unclear, through it is considered to be related to oxidant mediated genetic instability. [44,45,193]

The identical and different microsatellite alterations observed between the low and high-grade lymphomas suggest tumour evolution. However, the homogeneous microsatellite alterations observed between low and high-grade lesions of the same patients indicates a common genetic lineage between these two components. This is , in agreement with the finding in chapter 3. [397] The heterogeneous alterations may reflect the genetic diversity of the lymphoma. This genetic diversity may lead to aberrations which drive the tumours from indolent low-grade to aggressive high-grade lymphomas.

## 5.5 Conclusion

The genetic instability indicated by the RER+ phenotype is one of the common genetic abnormalities in the development of MALT lymphoma. It may occur and persist throughout the whole spectrum of the development of the disease.

## Chapter 6 Abnormalities of the *C-myc* Gene in MALT Lymphomas

### 6.1 Introduction

The ***c-myc*** gene is one of the most frequent targets for abnormalities in B cell lymphomagenesis. The gene is located on chromosome 8. [253] It contains 3 exons, of which exons II and III encode a dominant function protein (p64) (see Figure 6-1). [253] Translation of ***c-myc*** mRNA can also be initiated from exon I, resulting in a large polypeptide (p67). ***C-myc*** protein, p64 is widely considered as a transcription factor, which modulates two overlapping sets of genes, one involved in cell proliferation, and the other in cell death. [254] The function of p67 is unknown, though it has been suggested that the peptide may act as a negative regulator to p64 activity. [257,284] Studies have indicated that over-expression of p64 plays an important role in the development and progression of many tumours, while loss of p67 confers a selective growth advantage. [398,399] At the genetic level, deregulation of ***c-myc*** expression has been found to be associated with somatic alterations, mainly resulting from chromosomal translocation within or close to regulatory sequences in exon I and intron I including a transcriptional elongation block, [285] a p67 protein initiation site and an intron splicing site, [257] and three myc intron factor (MIF) binding sites. [286,287]

The configuration of the ***c-myc*** gene in MALT lymphomas is largely unknown, although in a single study, [129] a high incidence of ***c-myc*** gene rearrangements was detected by Southern blot hybridisation in a small series of high-grade gastric lymphomas. In order to understand the role of ***c-myc*** gene abnormalities in the pathogenesis of MALT lymphomas, ***c-myc*** gene rearrangements were examined in 54 MALT lymphomas, along with 36 nodal lymphomas, using the same techniques and probes. In addition somatic mutations in the exon I/intron I regulatory region were also investigated by

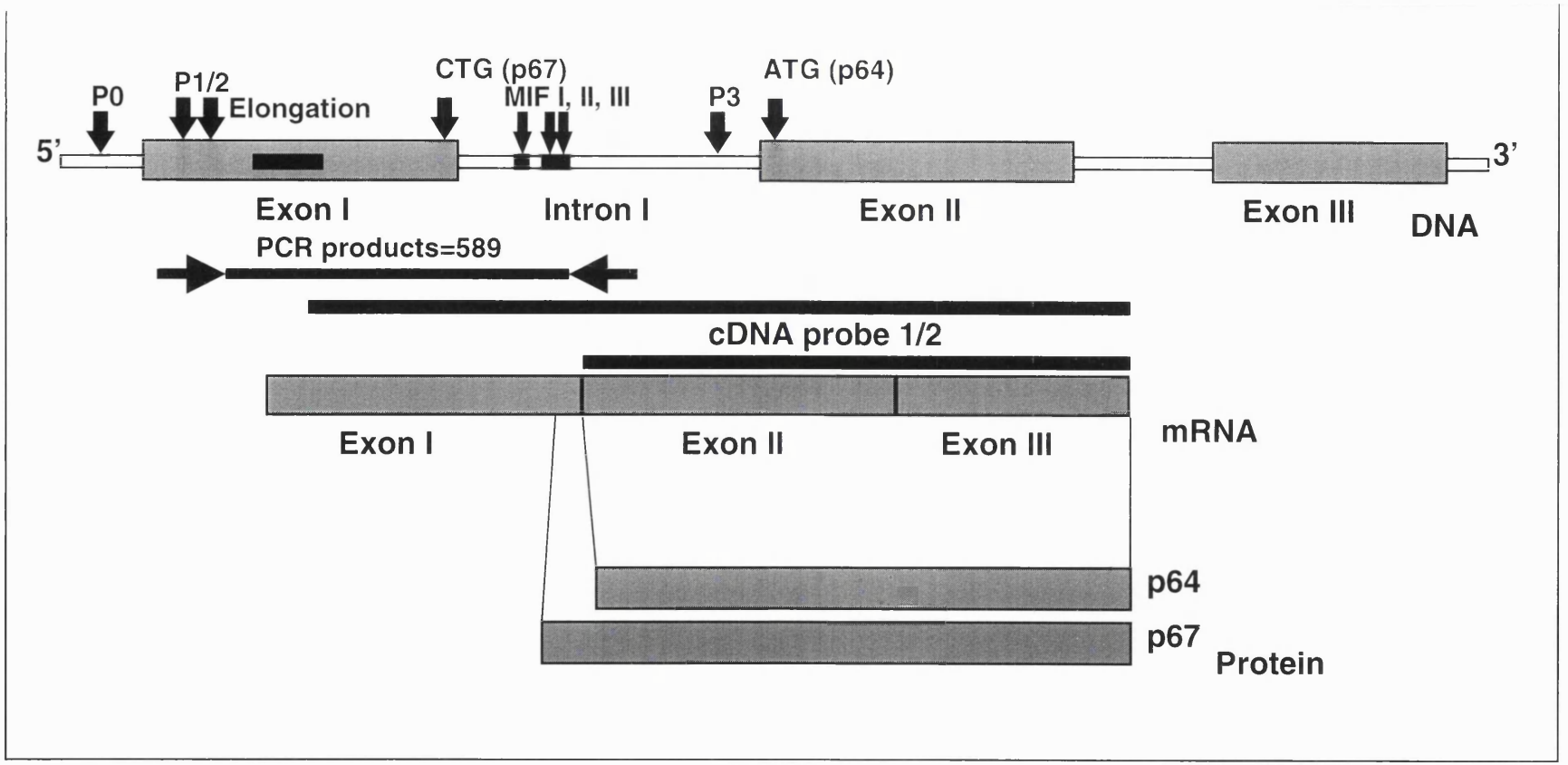


**Figure 6-1. The Structure and Regulatory Elements of the Human *C-myc* Gene**. The solid boxes indicate exons and the open boxes represent introns. P1 and P2 are major and P0 and P3 are minor promoters. The small bar inside exon I is the site of the conditional block to transcriptional elongation. Myc intron factor binding sites (MIF I-III) are indicated as solid bars inside intron I. Synthesis of the dominant ***c-myc*** protein, p64 is initiated from the AUG codon at the 5' end of exon II (AUG, p64), while synthesis of the larger protein, p67 starts from the CUG codon at the 3' end of exon 1 (CUG, p67). In addtion the PCR and Southern blotting hybridisation strategy are illustrated.

PCR-SSCP/direct sequencing in the same cases. Since MALT lymphomas, unlike their nodal counterparts, commonly contain a heavy inflammatory infiltrate, to obtain representative results, tumour populations were isolated by microdissection for PCR mutation analysis.

## 6.2 Material and Methods

### 6.2.1 Materials

DNA was extracted from frozen tissue blocks of 54 MALT lymphomas (43 low and 11 high-grade) and 36 cases of nodal lymphoma (27 low and 9 high-grade). DNA extracted from tonsil and placenta was used as a control.

### 6.2.2 Southern Blotting

Southern blot hybridisation was carried out as described in 2.2.2.

### 6.2.3. Microdissection

The percentage of malignant cells was estimated either by histopathological examination, or by comparison of the intensity of rearranged ***IgH*** bands with the germline ***IgH*** bands in Southern blot hybridisation with a JH probe. All of the nodal lymphomas analysed in this study contained at least 60% tumour cells. Therefore, they could be analysed directly from the whole tissue samples. However, the percentages of malignant cells in MALT lymphomas were smaller (10% to 50%), necessitating microdissection of the tumour cells in these cases. Microdissection and subsequent DNA extraction were performed as described in chapter 2. [363]

### 6.2.4. PCR-SSCP and Direct Sequencing

PCR-SSCP and direct sequencing was carried out as described in 2.2.3.3 and 2.2.3.4. In order to understand whether the mutations observed were homogeneous within the same tumours or represented an ongoing process (ongoing mutations) as the tumour clones expanded, multiple tumour lesions were analysed in 4 of the cases with ***c-myc*** mutations.

## 6.3. Results

### 6.3.1. Southern Blot Hybridisation

Control DNA from placenta and tonsil digested with the three restriction enzymes, showed germline patterns of the ***c-myc*** gene. The same germline patterns without rearranged bands were also detected in all the MALT lymphomas. Of the 36 nodal lymphomas, 2 (1 low and 1 high-grade lymphoma) showed one or more rearranged ***c-myc*** gene segments when hybridised with each of the two ***c-myc*** probes in all the digests (see Figure6-2).

### 6.3.2. PCR-SSCP

The results are summarised in Table 5-1 and an example of an SSCP gel is shown in Figure 6-3. Abnormal migration patterns of digested PCR products were observed in 9 out of 54 MALT lymphomas (16.6%), including 2 in high-grade and 7 in low-grade tumours. The incidence in high-grade (2/9=18%) lymphomas was similar to that in low-grade (7/43=16%). Only the two (2/36=5.6%) nodal lymphomas which had been shown to have ***c-myc*** rearrangements by Southern blotting exhibited abnormal SSCP patterns.

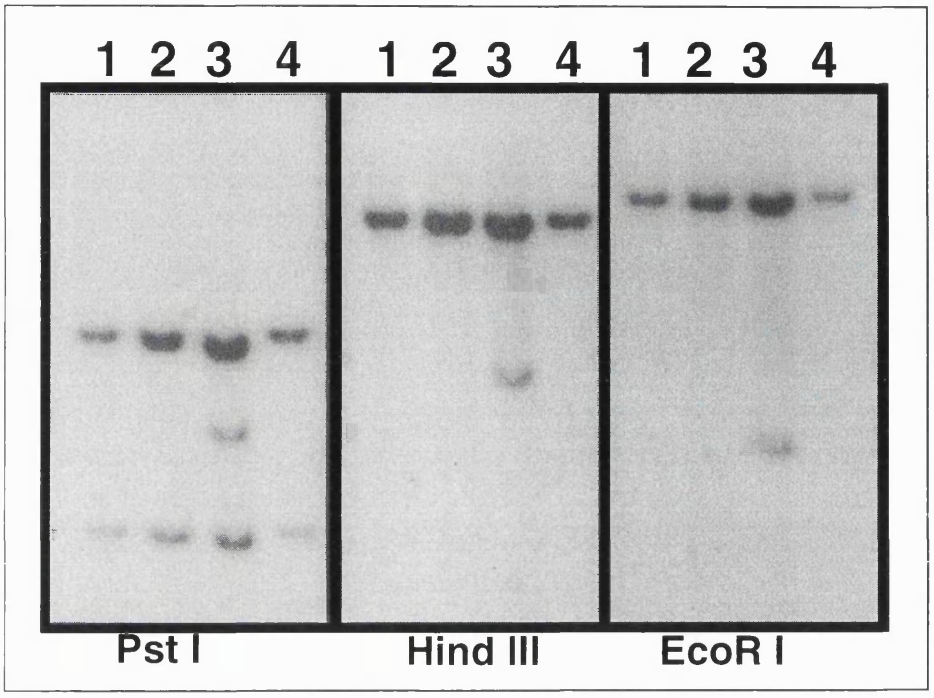


**Figure 6-2. Southern Blot Analysis of the *C-myc* Gene.** Lane 1 (placenta) and lanes 2 and 4 (MALT lymphomas) showing germline bands of the ***c-myc*** gene. Lane 3 (a nodal lymphoma) showing rearrangement of the gene in all 3 restriction enzyme digests.

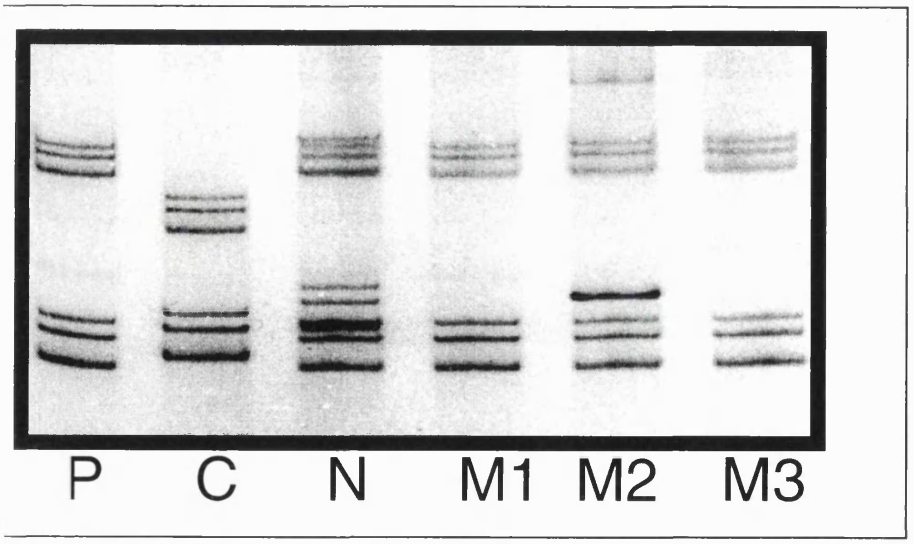


**Figure 6-3. Multiplex SSCP of *c-myc* Regulatory Regions. P** - placenta; **C** - mutated cell line; **N** - nodal lymphoma with abnormal SSCP pattern; **M** - MALT lymphoma, M2 with abnormal SSCP.

**Table 6-1, *C-myc* gene abnormalities in MALT lymphomas and nodal lymphomas**

| Abnormalities | MALT lymphomas | | Nodal Lymphomas | |
|---|---|---|---|---|
| | Low-grade | High-grade | Low-grade | High-grade |
| **Rearrangement #** | 0 | 0 | 1/27=3.7% | 1/9=11.1% |
| **Mutation @** | 7/43=16.2% | 2/11=18.1% | 1/27=3.7%* | 1/9=11.1%* |
| **Total** | 9/54=16.7% | | 2/36=5.6% | |

Note: #: as determined by Southern blots ; @: As determined by SSCP / sequencing *: Same case as with Southern blotting

### 6.3.3. Direct Sequencing Analysis

To characterise the type and distribution of mutations, the 11 lymphoma samples with abnormal PCR-SSCP patterns were analysed by direct sequencing. Mutations were confirmed in each case. The distribution of mutations is shown in Figure 6-4 and examples of the direct sequencing results are shown in Figure 6-5 (MALT lymphoma) and Figure 6-6 (nodal lymphoma).

Eight MALT lymphomas with ***c-myc*** mutations showed a single base change. One case had 2 point mutations. The 10 mutations in these 9 cases were concentrated in 2 regions. Seven mutations from 6 cases were located near the border of exon I and intron I, within 95 bp up-or down stream of the Pvu II site in exon I. They included 1 near and 2 within the Pvu II site, 1 immediately next to the p67 initiation site, and 2 close to and 1 within the intron/exon splicing site. The 3 point mutations in the remaining 3 cases were located within the three MIF (I-III) binding sites respectively. Analysis of multiple tumour lesions in 4 of the cases with mutations showed no variation between different lesions in the same cases. The 2 nodal lymphomas exhibited multiple single base changes, one possessing 4 and the other 5 alterations. These mutations were scattered or clustered within the 589 bp DNA fragment analysed.

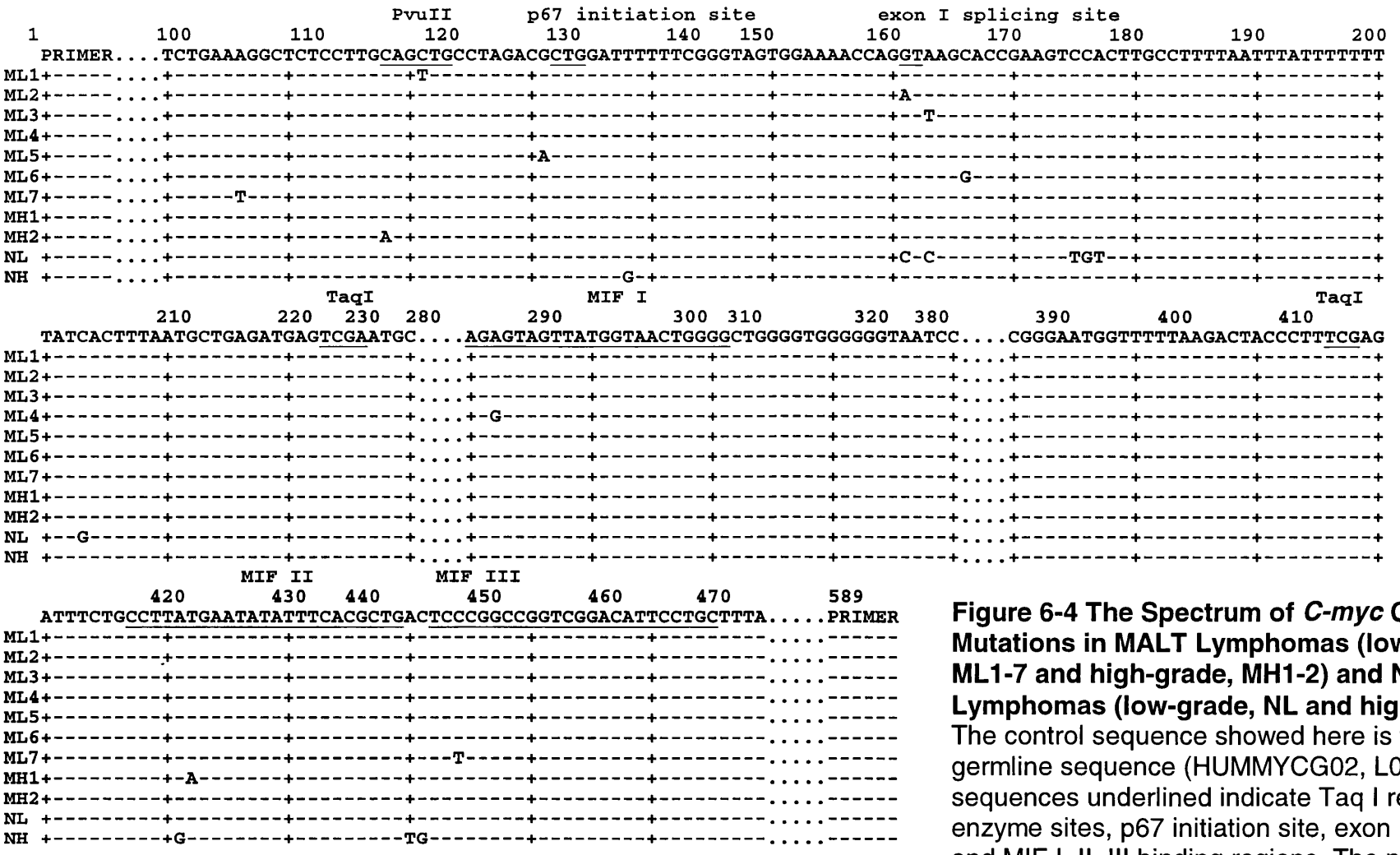


**Figure 6-4 The Spectrum of *C-myc* Gene Mutations in MALT Lymphomas (low-grade, ML1-7 and high-grade, MH1-2) and Nodal Lymphomas (low-grade, NL and high-grade, NH).** The control sequence showed here is the ***c-myc*** germline sequence (HUMMYCG02, L00057). The sequences underlined indicate Taq I restriction enzyme sites, p67 initiation site, exon I splicing site and MIF I, II, III binding regions. The positions of the mutated bases in each case are indicated.

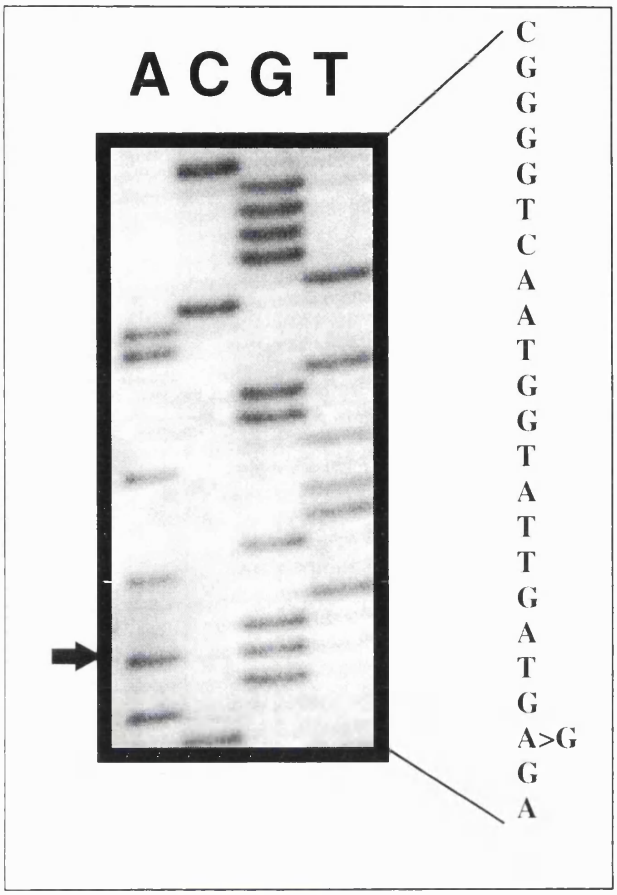


**Figure 6-5. Direct Sequencing of the PCR Product from an SSCP Positive MALT Lymphoma,** showing an A to G mutation in the MIF I region

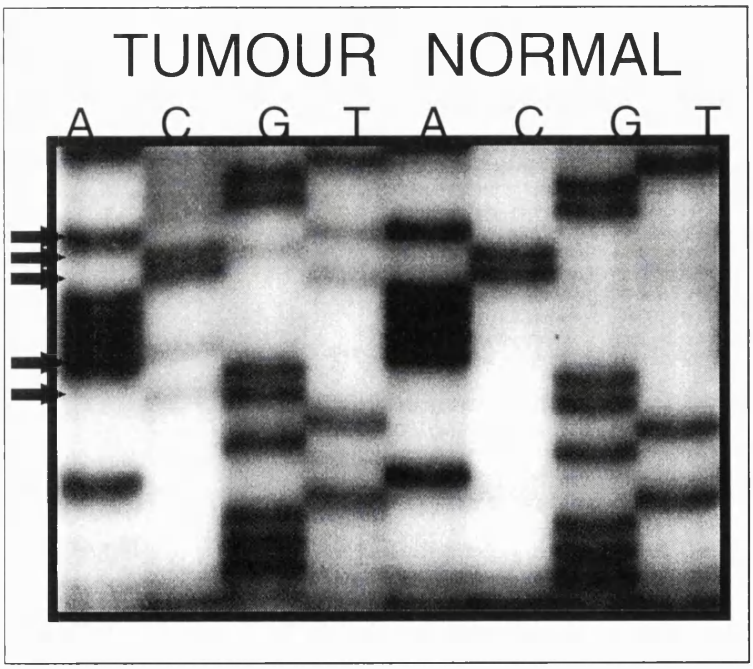


**Figure 6-6 Direct Sequencing of the PCR Product from an SSCP Positive Nodal Lymphoma,** showing multiple mutations.

## 6.4. Discussion

One report of a high incidence of ***c-myc*** gene rearrangement in high-grade gastric "MALT" lymphomas [129] and the long speculated role of the gene in lymphomagenesis prompted this study of ***c-myc*** abnormalities in MALT lymphoma. ***C-myc*** rearrangement could not be identified in any of the 54 cases of MALT lymphomas examined, but was seen in two of the 36 cases of nodal lymphomas. However, a series of point mutations in the ***c-myc*** exon I and intron I regulatory region were observed in a significant proportion of these MALT lymphomas. These results, together with the documented cytogenetic studies [32,74] showing no evidence of chromosome 8 translocations in MALT lymphomas, indicate that it is unlikely that the lymphomas are characterised by ***c-myc*** gene rearrangements as previously suggested. [129]

As shown in Figure 6-2, all mutations in the 9 cases of MALT lymphoma were clustered in two regions, i.e. exon I / intron I border and MIF sites which are known to be essential for the regulation of ***c-myc*** expression. As implicated in many studies, [257,284,398,399] somatic alterations in p67 mRNA splicing and translation initiation sites near the exon I/intron I border can down-regulate or abolish expression of p67 protein which is believed to act as a negative regulator to the activity of p64, the functional ***c-myc*** protein. In addition, mutations within the border (95 bp up and down stream of the Pvu II site) may remove a block on transcription elongation, [285] or increase p64 ***c-myc*** mRNA stability. [288,289] Mutations in the MIF sites may affect the binding of the ***c-myc*** intron factor, a 138 kDa phosphor-protein which functions as a negative regulator to ***c-myc*** transcription. [286] Therefore, all of the mutations identified have the potential to cause over-expression or increased activity of the ***c-myc*** protein. This has been shown in previous studies to be a critical step in lymphomagenesis, [253,284,400,401] and therefore may contribute to the development of at least some MALT lymphomas. However, further studies are

needed to verify this possibility.

In other types of lymphomas, somatic mutations in the ***c-myc*** exon I / intron I region are almost exclusively accompanied by chromosome translocation and probably acquired as a result of the hypermutation capability of translocated ***Ig*** genes. [284] Although these alterations have rarely been described in low-grade nodal lymphomas, they have been reported as "second hits" during high-grade transformation of some low-grade nodal lymphomas [358] or as essential initiating genetic factors in aggressive lymphomas, such as Burkitt's lymphoma, [284] AIDS-related lymphomas [292] and anaplastic large cell lymphoma. [402] Since none of the 54 cases of MALT lymphoma showed ***c-myc*** gene rearrangement, the mutations observed in the tumours are likely to be chromosome translocation independent. There is no significant difference in the mutation frequency of the ***c-myc*** gene between low and high-grade MALT lymphomas, suggesting that the mutations may be acquired at an early stage of development and kept throughout tumour progression.

The mechanisms leading to ***c-myc*** gene mutation in MALT lymphomas are not clear. Since the tumours are always preceded or accompanied by chronic inflammation, it is possible that the mutations are related to inflammation. There is accumulating evidence that chronic inflammation can induce instability in the cellular genome, leading to DNA damage, in the forms of gene mutations, gene amplification, DNA strand breaks, deletions or translocations in the affected cells. [42,193] In chapter 5, over 50% of MALT lymphomas have been shown to exhibit genetic instability, as indicated by an RER+ phenotype. [378] Since genetic instability may cause widespread gene mutation in the genome [200] and has been related to the activation of oncogenes and inactivation of tumour suppressor genes, [223,225,235,236,240-242] the results presented here were compared with data of chapter 5. Four out of six cases with ***c-myc*** mutations also showed genetic instability, as indicated by an RER+ phenotype. Although the sample number is small, it is possible that

***c-myc*** gene mutation in MALT lymphomas is related to genetic instability.

## 6.5 Conclusion

Abnormalities of the ***c-myc*** gene are associated with some MALT lymphomas, but the involvement of ***c-myc*** in these cases differs from its involvement in lymphomas of other types. It is postulated that mutation of ***c-myc*** in these lymphomas is an early event that may be associated with an RER+ phenotype. Further detailed study is required to confirm the relationship between genetic instability and ***c-myc*** abnormalities.

## Chapter 7 The Accumulation of *P53* Abnormalities Is Associated with Progression of MALT Lymphoma

### 7.1. Introduction

As described in Chapter 1, ***p53*** acts as a cell cycle check point protein which induces cell cycle arrest in the late G1 phase or apoptosis following DNA damage. [310,311] Inactivation of ***p53*** tumour suppressor activity during tumour development is a process of accumulation of genetic abnormalities within the gene. [309] Abnormalities of ***p53*** including point mutation, allele loss and abnormal protein accumulation have been found in a wide range of tumours. [308] In lymphoma, ***p53*** abnormalities are thought to be associated with the high-grade transformation of follicular lymphoma. [273,403] Recently, a high frequency of ***p53*** mutation has been reported in marginal zone splenic B cell lymphoma, [307] which is analogous to MALT lymphoma in its origin of cell lineage, immuno-phenotype, histology and genetic features. [1,65] Thus, inactivation of the ***p53*** gene may also be important in the pathogenesis of MALT lymphoma.

In the present study, mutation of the ***p53*** gene was investigated by PCR-SSCP and direct sequencing in 75 cases of MALT lymphoma. ***P53*** allele loss and protein accumulation were examined in the same cases by PCR-LOH analysis and immunocytochemistry. The aim of this study was to define the incidence of ***p53*** abnormality and its role in the evolution and high-grade transformation of MALT lymphomas.

### 7.2 Materials and Methods

#### 7.2.1 Materials

Seventy-five cases (paraffin embedded tissues available in 63, frozen tissues

available in 24) of B cell MALT lymphoma were retrieved and reviewed. They comprised 48 low-grade and 27 high-grade tumours.

### 7.2.2 Microdissection

The tumour and non-tumour cell populations were separately microdissected and DNA extracted from each case. [363]

### 7.2.3 PCR-SSCP of *P53* Exons 5, 6, 7 and 8 and Direct Sequencing

***P53*** exons 5, 6, 7 and 8 were amplified separately and SSCP analysis of the PCR products was carried out.[369] The samples with abnormal SSCP patterns were further analysed by direct sequencing.

### 7.2.4 LOH

LOH of the ***p53*** gene was detected by PCR-based analysis of the ***p53*** dinucleotide repeat (CA25) polymorphism. [370] PCR was carried out using primers flanking this polymorphism. One of the primers was end-labelled with $^{32}$P-dATP using T4 kinase. The PCR products were electrophoresed on a denaturing gel, followed by autoradiography.

### 7.2.5 Immunostaining of *P53*

Immunostaining for the ***p53*** protein was carried out using pressure cooker pre-treatment [367] in cases with paraffin embedded tissues, which were composed of 37 low-grade and 26 high-grade tumours.

For each experiment, positive (a breast carcinoma known to express mutant ***p53*** protein) and negative controls (without the primary antibody) were included.

## 7.3 Results

### 7.3.1 Mutation of the *P53* Gene in MALT Lymphoma.

In comparison with the placental control, 21 of 75 cases showed abnormal SSCP patterns. They included 7 with abnormalities in exon 5, 8 in exon 6, 4 in exon 7 and 2 in exon 8 (Figure 7-1).

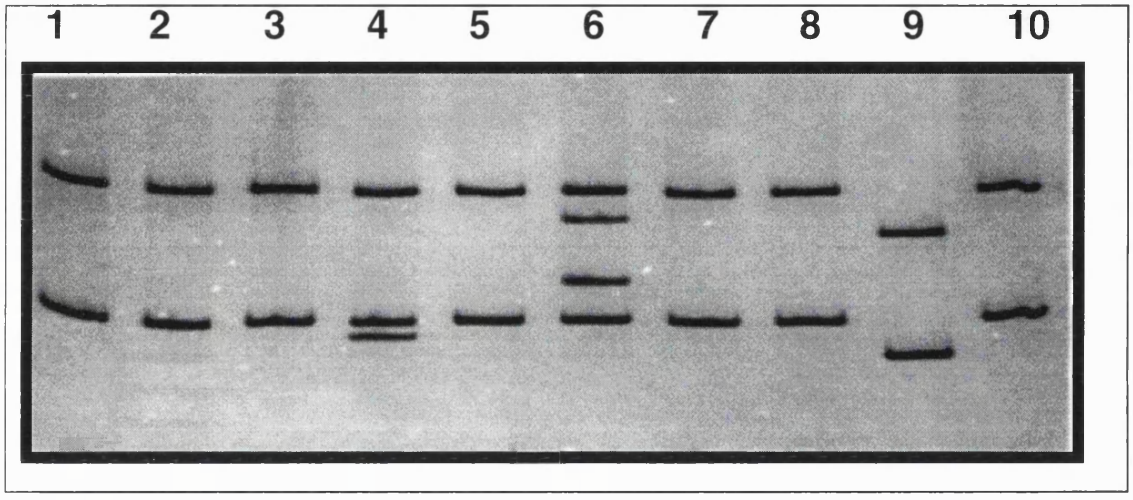


**Figure 7-1: Detection of *P53* Mutation in MALT Lymphomas by PCR-SSCP Analysis.** PCR products of ***p53*** exon 6 were denatured, separated on a polyacrylamide-agaose gel and revealed by silver staining. Lanes 1 and 10, placental DNA as negative controls; Lanes 2-8, individual MALT lymphoma samples; lane 9, A3/kawa cell line containing a missense mutation in exon 6 used as a positive control. Lanes 4 (case 1) and 6 (case 17) showed fragments migrating differently from the normal control.

All 21 SSCP pattern alterations were confirmed to be point mutations or point deletions by direct sequencing. The results are presented in Table 7-1 and an example is shown in Figure 7-2. The mutations identified showed a wide spectrum with a similar frequency of transition (10/20) and transversion (10/20) mutations. Four out of 20 mutations including 2 C>T, 1 G>A transition and 1 G>T transversion occurred at CpG dinucleotides. However, no predominant type of mutation was observed.

**Table 7-1. Summary of MALT Lymphomas Showing *P53* Abnormalities.**

| Case | L/H | Staining | LOH | SSCP | Codon | Mutation | Substitution |
|---|---|---|---|---|---|---|---|
| 1 | L | - | - | Exon 6 + | 193 | CAT → CAC | His → His |
| 2 | L | ND‡ | - | Exon 6 + | 197 | GTG → GTA | Val → Val |
| 3 | L | - | + | - | | | |
| 4 | L | ND | + | - | | | |
| 5 | L | - | - | Exon 6 + | 196 | CGA → TGA | Arg → End |
| 6 | L | - | - | Exon 5 + | 178 | CAC → *AC | Frameshift |
| 7 | L | - | - | Exon 5 + | 160 | ATG → CTG | Met → Leu |
| 8 | L | - | - | Exon 6 + | 197 | GTG → ATG | Val →Met |
| 9 | L | - | - | Exon 5 + | 165 | CAG → CTG | Gln → Leu |
| 10 | L | - | - | Exon 8 + | 273 | CGT → TGT | Arg → Cys |
| 11 | L | - | - | Exon 5 + | 165 | CAG → CCG | Gln → Pro |
| 12 | L | - | - | Exon 5 + | 165 | CAG → CCG | Gln → Pro |
| 13 | L | - | + | Exon 7 + | 243 | ATG → GTG | Met → Val |
| 14 | H | - | - | Exon 7 + | 253 | ACC → ACT | Thr → Thr |
| 15 | H | - | - | Exon 6 + | 206 | TTG → T*G | Frameshift |
| 16 | H | - | - | Exon 6 + | 194 | CTT → CAT | Leu → His |
| 17 | H | + | - | Exon 6 + | 212 | TTT → TTA | Phe → Leu |
| 18 | H | - | + | Exon 5+ | 158 | CGC → CTC | Arg → Leu |
| 19 | H | + | + | Exon 8 + | 274 | GTT → GAT | Val → Asp |
| 20 | H | + | + | Exon 5 + | 151 | CCC → CAC | Pro → His |
| 21 | H | + | + | Exon 6 + | 192 | CAG → CGG | Gln → Arg |
| 22 | H | + | + | Exon 7 + | 248 | CGG → CAG | Arg → Gln |
| 23 | H | + | + | Exon 7 + | 254 | ATC → GAC | Ile → Asp |

note: L/H= low or high grade lymphoma, LOH= lose of heterozygosity, SSCP= single strand polymorphism; * = deletion

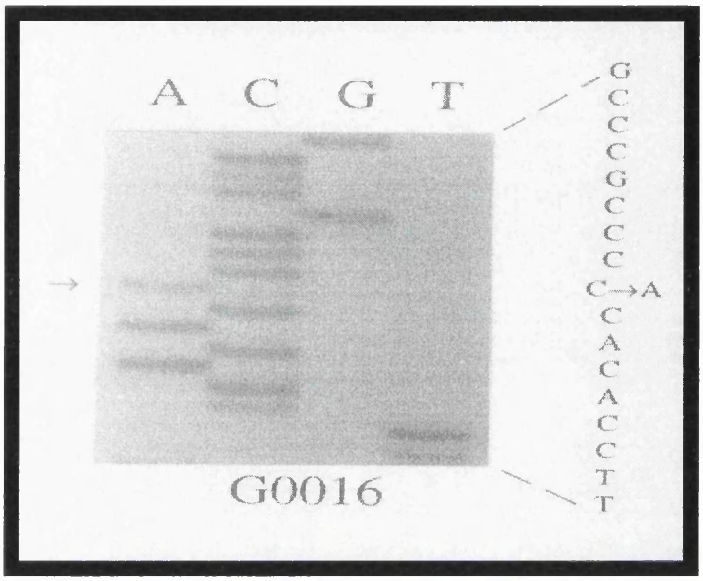


**Figure 7-2: Direct sequencing of the PCR products from the SSCP positive case**. Case 20 showed a missense mutation (CCC → CAC) in codon 151

Among these 21 mutations, 18 (18/75 =24%) led to alterations in amino acid sequence, including 15 amino acid substitutions by missense mutations and 3 truncated amino acid sequences by frame shift mutations which produced stop codons. These mutations were more frequent in high-grade (9/27, 33.3%) than low-grade (9/48, 18.8%) tumours. Mutations in the remaining 3 cases with alterations were silent and did not alter the amino acid sequence.

### 7.3.2 LOH of the *P53* Gene in MALT Lymphoma

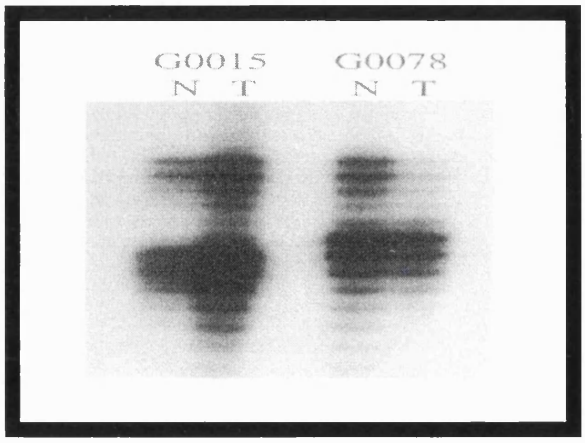


**Figure 7-3: Detection of LOH of the *P53* Gene in MALT Lymphomas**.. N and T indicate non-tumour and tumour samples from the same patient. Case G0078 showing loss of one ***p53*** allele in the tumour sample.

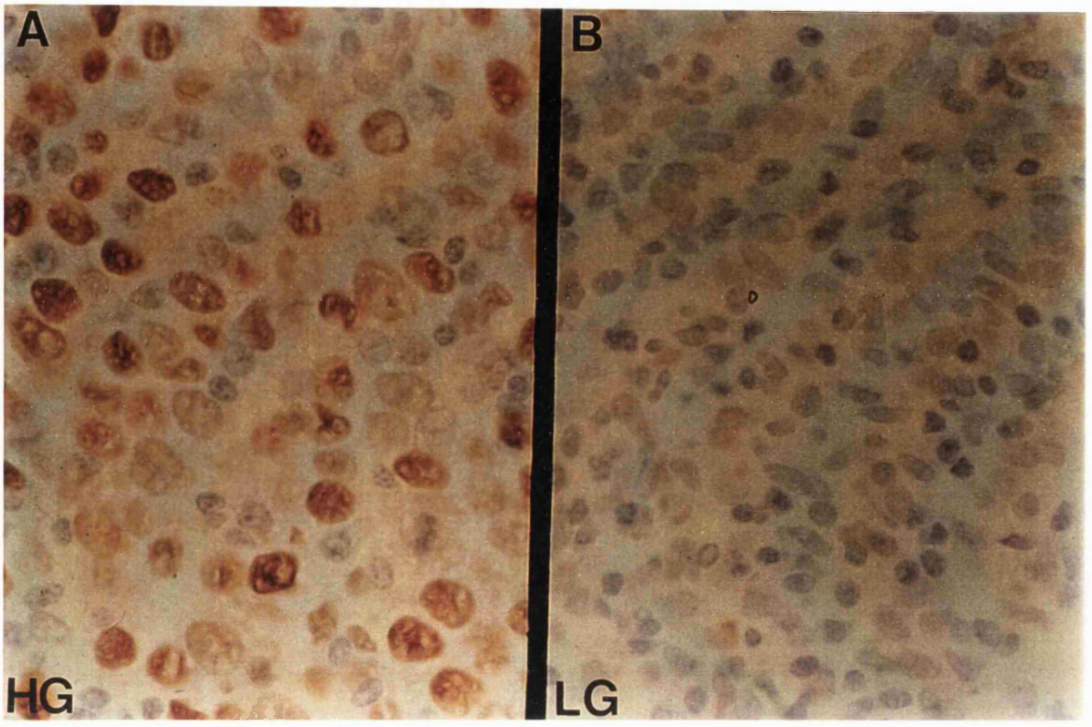


**Figure 7-4 : Accumulation of the *P53* Protein in MALT Lymphomas.** ***P53*** was immunostained with CM1 antibody. Nuclear staining in the majority of the high-grade (HG) cell but also in a few low-grade (LG) tumour cells. The high and low-grade foci are from the same histological section.

Of the 75 MALT lymphomas studied, non-tumour DNA preparations were available from 74 cases. Among these cases, 65 (87.8%) were heterozygous for the ***p53*** CA repeat and thus were informative for LOH analysis. As the analysis was carried out from DNA samples prepared from microdissected cell populations, this largely eliminated the interference from non-tumour cells and LOH was easily determined by a direct comparison of the allelic density between tumour and non-tumour samples of each patient (Figure 7-3). In total, LOH of the ***p53*** gene was found in 9 (13.8%) cases, with a much higher frequency in high-grade (6/21, 28.6%) than low-grade (3/44, 6.8%) tumours.

### 7.3.3. Accumulation of *P53* Protein in MALT Lymphoma.

Of the 63 cases in which ***p53*** staining was carried out, positive staining was found in 6 cases. All positive cases (6/26, 23%) were high-grade tumours and none of the 37 low-grade tumours studied showed any staining for ***p53***. Nuclear staining was found in the majority of tumour cells in the positive cases. Strong nuclear staining was observed in 5 cases, while moderate nuclear staining was seen in the remaining case. Of the positive cases, 1 showed the presence of abundant low and high-grade tumour components together with follicles colonised by high-grade tumour cells. ***P53*** staining in this case was observed mainly in high-grade blasts, but also in a few low-grade tumour cells (Figure. 7-4).

### 7.3.4 Interrelationships between *P53* Mutation and *P53* Staining or *P53* Allele Loss in MALT Lymphoma.

Cases showing ***p53*** staining, LOH and mutation are summarised in Table 1. All tumours showing ***p53*** staining contained missense mutations in the ***p53*** gene. However, 14 cases harbouring ***p53*** mutations including 9 cases with missense mutations, 2 cases with silent mutations, 2 cases with frame shift mutations and 1 case with a nonsense mutation did not show any staining for ***p53*** in at least two separate staining experiments using more concentrated CM1 antibody.

In addition to the difference in the frequency of ***p53*** allele loss and mutation between low and high-grade tumours, differences in the extent of ***p53*** abnormalities were also observed between the two groups. Of the 11 low-grade cases with ***p53*** abnormalities, only 1 case showed both ***p53*** mutation and LOH, whereas in the high-grade tumours, 6/9 involved cases displayed both ***p53*** mutation and LOH, suggesting an association between the accumulation of ***p53*** abnormalities and disease progression.

## 7.4 Discussion

The results show that frequent ***p53*** mutation and allelic loss are associated with both low (LOH 6.8%, mutation 18.8%) and high (LOH 28.6%, mutation 33.3%) grade MALT lymphomas, particularly in the high-grade tumours. The data also indicate that there is a significant difference in the extent of ***p53*** abnormalities between low and high-grade MALT lymphomas. In low-grade tumours, most ***p53*** defective cases (10/11) showed either one allele mutation or one allele loss, suggesting only partial loss of ***p53*** function. Whereas, in high-grade tumours, the majority (6/9) of affected cases exhibited both ***p53*** mutation and allele loss, implying complete loss of ***p53*** function. Thus, the extent of ***p53*** inactivation is closely associated with the progression of MALT lymphoma. A partial inactivation of ***p53*** function may be involved in the development of some low-grade MALT lymphomas, whereas a complete loss of ***p53*** function may be important for high-grade transformation. In this aspect, the pathological role of ***p53*** inactivation in MALT lymphoma is different from that in follicular lymphoma in which ***p53*** mutation has been suggested to be mainly associated with high-grade transformation, [304] or that in splenic marginal zone B cell lymphoma in which ***p53*** inactivation appears to be involved with the development of low-grade tumours. [307]

The observation of distinct ***p53*** gene abnormalities at different stages of MALT lymphoma shows the multistage nature of tumourigenesis and highlights the fact that the loss of normal ***p53*** function is a process of accumulation of genetic abnormalities within the genome. Distinct ***p53*** gene abnormalities (allele loss or mutation or both) may cause different degrees of ***p53*** inactivation, and exert different tumourigenic effects, thus playing different roles during tumour evolution.

Mutant ***p53*** protein frequently accumulates in cell nuclei due to its increased half-life. [318] Therefore, it can be detected by immunocytochemical staining. ***P53*** staining frequently indicates mutation of the gene. In the present study,

all cases showing ***p53*** staining contained missense mutations in the gene. However, 14 cases harbouring ***p53*** mutation did not show any ***p53*** staining with CM1 antibody. Staining was not expected for 5 cases, including 2 cases in which the mutation was silent and another 3 cases in which mutations resulted in truncated protein products. The mechanism underlying the absence of ***p53*** staining in the remaining 9 cases, in which missense mutations were found, is unclear. Lack of staining in tumours harbouring ***p53*** missense mutation has been reported previously. [404] There are two possibilities: (1) the accumulation of mutant ***p53*** may not reach the level detectable by immunochemistry. This could be due to insufficient stabilisation of some mutations or lack of sufficient protein expression; (2) the antibody used may not recognise all mutant ***p53***. However, this possibility is small in this study since ***p53*** staining was carried out using CM1 and an antigen retrieval method, which has been shown to be a highly sensitive method. [405] In addition, the CM1 antibody is polyclonal and, theoretically, should recognise all ***p53*** protein. Nevertheless, the use of immunostaining with CM1 antibody alone as a marker for ***p53*** mutation in MALT lymphomas is limited.

Gradually increasing expression of mutant ***p53*** during tumour progression has been observed previously. [404] This suggests that additional mechanisms other than gene mutation may affect protein expression [319] and the expression of mutant ***p53*** protein may be functionally important. Increasing evidence suggests that mutant ***p53*** has a dominant negative effect on the wild type.[333,406] Thus, the lack of mutant ***p53*** expression in tumours harbouring ***p53*** mutation may indicate the absence of such dominant negative effects and represent a relatively weak model of mutation mediated ***p53*** inactivation. That the majority of the low-grade cases tested in this study did not show protein accumulation and loss of the other allele, but contained mutation is in agreement with this hypothesis. Further studies correlating ***p53*** expression and disease progression in these cases are necessary to understand the significance of mutant ***p53*** expression.

Analysis of the spectrum of ***p53*** mutation in a tumour may reveal the aetiology involved in its carcinogenesis. [308,309] For example, the high frequency of G>T transversion in codon 249 in human hepatocellular carcinoma is related to alfatoxin B1 mediated mutagenesis, [309] whereas the predominant transition mutation in the CpG island observed in colon cancer and lymphomas suggests endogenous mutagenesis. [309] No predominant type of mutation in the ***p53*** gene was found in MALT lymphoma and the mechanism underlying the diverse ***p53*** mutations in this tumour remains unclear. Oxygen-reactive species, which are actively produced during inflammatory disease [193] and cause various types of mutations in DNA, [44,45,193] may be important in the carcinogenesis of MALT lymphoma because this tumour is frequently derived from a background of inflammatory disease.

## 7.5 Conclusion

Partial inactivation of ***p53*** may play an important role in the development of low-grade MALT lymphomas, whereas complete inactivation may be associated with high-grade transformation.

## Chapter 8 The Relationship between the RER+ Phenotype and *P53* Mutations

### 8.1 Introduction

As discussed in chapter 5, using multiplex fluorescent PCR, a high incidence of the RER+ phenotype was found in both low and high-grade MALT lymphomas, suggesting that genetic instability is a common genetic feature of MALT lymphoma. Frequent mutations of the ***p53*** gene have also been observed in this lymphoma (chapter 7), indicating its role in tumour development. Since genetic instability is caused by the disturbance of the system that maintains genetic integrity, [42,200] of which the normal ***p53*** gene is one of the essential factors, [308] ***p53*** mutation may contribute to the genetic instability in MALT lymphomas. However, because different cases have been examined in these two studies, the relationship between the RER+ phenotype and ***p53*** mutation is unknown. In this study, using PCR-SSCP and direct sequencing, the same cases tested for RER+ phenotype (chapter 5) were re-examined for ***p53*** mutations.

### 8.2 Materials and Methods

All of the 40 low and high-grade lymphomas, along with their normal and reactive tissues examined in chapter 5 were used for this study. Among them, 21 (21/40=52.5%) cases were RER+ in tumour samples, including 9 in high (9/16=56.2%) and 12 in low (12/24=50%) grade lymphoma. The same DNA extracts from microdissected populations used in chapter 5 were analysed for mutations of ***p53*** exons 5-8 by PCR-SSCP. PCR products showing abnormal SSCP patterns were directly sequenced. These methods are described in chapters 2 and 7.

The $\chi^2$ test was used to assess the association between RER+ phenotype

and ***p53*** mutations.

### 8.3 Results

No mutations were observed in normal tissues or reactive lymphoid infiltrates by PCR-SSCP analysis. Among 40 tumour samples screened for ***p53*** gene mutations within exons 5-8, abnormal electrophoresis mobility shifts on SSCP gels were detected in 14 cases (Figure 8-1). All of the mutations were confirmed by direct sequencing (Table 8-1). These included 9 replacement mutations resulting in amino acid substitution, 2 deletion mutations resulting in frame shifts and 3 silent mutations. Therefore, a total of 11/40 (27.5%) MALT lymphomas showed ***p53*** gene mutations that resulted in alteration of ***p53*** amino acid sequences. A higher mutation frequency was observed in high-grade (6/16, 37.5%) than in low-grade (5/24, 20.8%) MALT lymphomas.

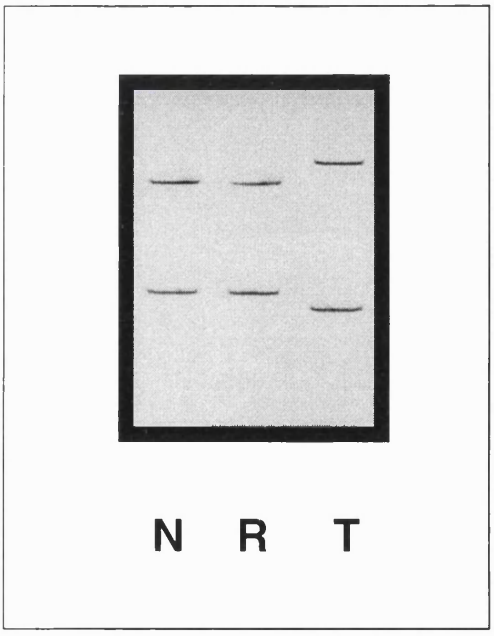


**Figure 8-1. An SSCP Analysis of *P53* Gene Exon 5 for Microdisected Tissues from a High-grade MALT Lymphoma.** N: normal control, R: reactive lesion adjacent to lymphoma. T: high-grade tumour lesion.

**Table 8-1. *P53* Mutations in MALT Lymphomas**

| CASE | L/H | RER | CODON | MUTATION | SUBSTITUTION |
|---|---|---|---|---|---|
| 1 | H | + | 212 | TTT>TTA | PHE>LEU |
| 2 | H | + | 194 | CTT>CAT | LEU>HIS |
| 3 | H | - | 158 | CGC>CTC | ARG>LEU |
| 4 | H | + | 206 | TTG>T*G | FRAMESHIFT |
| 5 | H | + | 253 | ACC>ACT | SILENT |
| 6 | H | + | 274 | GTT>GAT | VAL>ASP |
| 7 | H | + | 254 | ATC>GAC | ILE>ASP |
| 8 | L | + | 193 | CAT>CAC | SILENT |
| 9 | L | + | 160 | ATG>CTG | MET>LEU |
| 10 | L | + | 178 | CAA>*AA | FRAMESHIFT |
| 11 | L | - | 273 | CAT>TAT | HIS>TRY |
| 12 | L | + | 234 | TAC>CAC | TRY>HIS |
| 13 | L | - | 179 | CAT>TAT | HIS>TRY |
| 14 | L | - | 197 | CTG>GTA | SILENT |

The association between the RER+ phenotype and ***p53*** gene mutation is shown in Table 8-2. The $\chi 2$ test shows that there is a significant association between the RER+ phenotype and ***p53*** mutations ($p<0.05$). ***P53*** mutations ccurred more frequently in RER+ cases (8/21, 38%) than in RER- cases (3/19, 15%). In addition, the RER+ phenotype is more often seen in tumours with ***p53*** mutation (8/11, 72%) than tumours without ***p53*** mutations (13/29, 44.8%).

**Table 8-2. The Relationship between *P53* Mutation and RER+ Phenotype**

| | RER+ | RER- |
|---|---|---|
| ***P53* mutation +** | 8 | 3 |
| ***P53* mutation -** | 13 | 16 |
| ***P<0.05 by $\chi^2$ test** | | |

## 8.4 Discussion

In this study, frequent ***p53*** gene mutations were observed in B cell MALT lymphomas, with a higher incidence in high-grade than that in low-grade

disease. This result, in agreement with the data obtained from the study in chapter 7, [379] suggests that ***p53*** abnormalities may play an important role in the development of MALT lymphomas, particularly in high-grade transformation.

It was also observed that ***p53*** mutation frequencies were higher in the tumours with the RER+ phenotype and vice versa. This result suggests that ***p53*** gene mutations and genetic instability may affect each other. Genetic instability may promote ***p53*** mutations that cause failure of maintenance of genetic integrity and lead to further genetic instability. Thus, both genetic instability and ***p53*** mutation allow accumulation of further genetic alterations, which promote tumour formation and development.

The mechanisms by which ***p53*** affects genetic stability remain largely unknown. It is thought to be most likely linked to its function as a control protein in the cell cycle. [318,407] Normal cells arrest in $G_1$ before entering S phase in response to DNA damage. This approach temporarily halts the cell cycle and allows the cell time for DNA to be repaired before being copied. Although it is not known how ***p53*** senses DNA damage, it is observed that cells harbouring DNA damage accumulate ***p53*** protein immediately. ***P53*** can induce the expression of the ***p21*** gene that has been shown to inhibit the cell cycle in $G_1$ phase through direct interaction with a cyclin-dependent kinase.[311] In addition, a recent study showed that the ***p53*** gene may also take part in the DNA repair system indirectly by stimulating ***GADD45*** and/ or directly by interacting with ***RECC3,*** a DNA repair molecule (Figure 8-2). [408] In some cases, ***p53*** may also cause cells to undergo apoptosis before their damaged genome can be handed down to progeny cells. [312] Cells without wild-type ***p53*** protein lack sensitivity to DNA damage [409] and do not show $G_1$ arrest. This may lead to accumulation of unrepaired genetic lesions, which are subsequently copied to progeny cells and to increasing mutation frequencies.

## 8.5 Conclusion

There is an interplay between genetic instability as defined by the RER+ phenotype and ***p53*** gene mutation in MALT lymphoma.

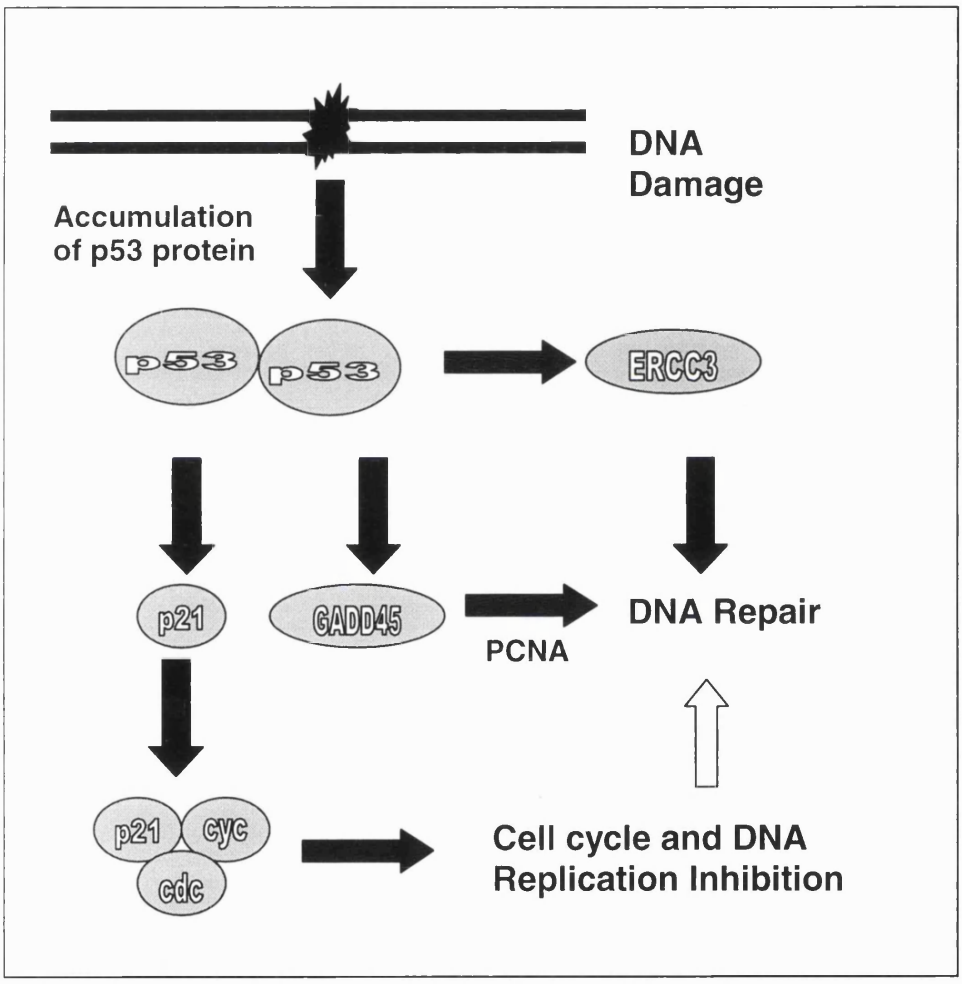


**Figure 8-2. The Links between *P53* and DNA Repair**

## Chapter 9 General Discussion and Future Directions

### 9.1 Introduction

MALT lymphomas constitute a distinct clinicopathological entity and differ significantly from their nodal counterparts. Low-grade tumours arise within and expand from a background of chronic inflammation caused by infection, autoimmune disease or both. [1,12,16,17,72] At a later stage, low-grade cells can disseminate to other mucosal sites [21,22,28-30] and transform into a high-grade tumour. [34] Both low and high-grade gastric MALT lymphomas are associated with ***H pylori*** infection. [17,153] In-vitro cell culture experiments have shown that their development is dependent on constant bacterial stimulation via T cell help. [37,122,382] At the molecular level, low-grade MALT lymphomas do not show any rearrangement of ***bcl-1***, [35] ***bcl-2*** [35,120] and ***c-myc***, [35] or ***bcl-6*** (unpublished data), which are typical features of certain types of nodal lymphoma. However MALT lymphomas do have unique genetic abnormalities such as trisomy 3 [33,128] and 1p22 abnormalities. [32] Although many efforts have been made, little is known about the genetic basis of MALT lymphoma development.

It is believed that MALT lymphoma development, like all other malignancies, [38,194,195] is a multistage clonal evolution process associated with the accumulation of genetic abnormalities which cause oncogene activation and tumour suppressor gene inactivation as a result of genetic instability. This formed the working hypothesis of this thesis.

### 9.2 The Genetic Link Between Low and High-grade MALT Lymphomas

Clinical follow-up suggests that low-grade MALT lymphoma may directly transform into a high-grade disease at a late stage. [34] Pathological studies also showed that high-grade MALT lymphomas often contain low-grade

tumour components; both cell populations have been shown to express the same ***IgL*** previously. [34] To investigate the clonal link between these low and high-grade components, both lesions were microdissected from tissue sections of four high-grade gastric MALT lymphomas (chapter 3). PCR and sequence analyses were performed to identify clone specific rearranged ***IgH*** sequences. In each of these cases, the PCR products from the two components were identical in size by electrophoresis. Direct sequencing revealed common clone-specific ***IgH*** gene rearrangements in both lesions of each case, providing genetic evidence for a clonal link. These results support the proposal that high-grade MALT lymphomas generally evolve from low-grade clones.

## 9.3 The Role of *H pylori* in the Development of Gastric MALT Lymphomas

Beside the role of antigen stimulation in the proliferation of low-grade (but not in high-grade) gastric MALT lymphoma, [37,122,382] *H pylori* has cytotoxic effects,[163] which may be directly involved in the oncogenesis of gastric carcinoma. [187,384] The bacterial strains possessing the ***CagA*** gene have been linked to severe gastritis, [186] duodenal ulceration [149] and gastric carcinoma. [187,384] In chapter 4, the ***CagA*** status was examined in 127 *H pylori*-related gastric biopsies collected from Italy, including 56 cases of chronic gastritis, 47 low-grade and 24 high-grade MALT lymphomas. A novel PCR-based assay was designed for the amplification of the bacterial ***CagA*** gene from DNA extracted from paraffin sections. 25% of gastritis cases (14/56) and 31.9% of low-grade MALT lymphoma (15/47) showed ***CagA*** gene positivity. The incidence was statistically significantly higher in high-grade lymphomas (14/24=58.3%). The results suggest that *H pylori* may play different roles in low and high-grade MALT lymphomas. High-grade transformation may be more likely to result following an infection by ***CagA*** expressing strains of *H pylori.*

## 9.4 The RER+ Phenotype Is a Common Phenomenon in Low and High-grade MALT Lymphoma

The RER+ phenotype is a newly defined manifestation of genetic instability. It is characterised by alterations of microsatellite sequences (microsatellite instability) scattered throughout the human genome. The RER+ phenotype has now also been observed in many other types of tumour. [221-228,232,410-413] In chapter 5, microsatellite instability was examined in 40 cases of MALT lymphoma, and 21 (52.2%) were identified to be RER+. [378] This RER+ frequency is significantly higher than that reported in nodal lymphomas (0 to 20%). [232,393] The results suggest the genetic instability indicated by the RER+ phenotype is a common genetic abnormality in MALT lymphomas. Since high-grade MALT lymphomas always harboured more microsatellite alterations than low-grade tumours [378] and showed more heterogeneous microsatellite alterations than low-grade lesions, [378,414] these findings strongly suggest that microsatellite alterations are an ongoing event in MALT lymphoma, other than a reflection of the heterogeneous nature of the normal cell populations. [415] This genetic instability may contribute to the accumulation of genetic abnormalities during the evolution of gastric MALT lymphoma and also explain the increased incidence of additional solid tumours observed in patients with this malignancy in southern Switzerland and northern Italy [374] and in other gastric lymphoma series. [141,416] The genetic instability may occur and persist throughout the whole spectrum of tumour development.

It is very interesting to note that, in 4 cases where tissues were available, both homogeneous and heterogeneous microsatellite alterations were found to accumulate during expansion from chronic inflammatory through low-grade to high-grade lesions. This suggests that genetic instability may occur in the pre-lymphomatous MALT lesions. The relationship between genetic instability and chronic inflammation remains largely unclear. It is speculated that the

inflammatory cells can produce a large amount of free radicals, which are genotoxic and can cause a wide range of genetic damage. This damage, in the form of gene mutations, gene amplification, DNA strand breaks, deletions or translocations, may cause loss of genome integrity (genetic instability), which may favour tumour formation, and development. [212,214] In *H pylori* associated gastric diseases, the production of free radicals is linked to the expression of a bacterial inflammatory enhancer gene, ***CagA***. [160,184,191,192] However, in chapter 4, whist high-grade MALT lymphoma showed a high incidence of ***CagA***+ *H pylori* infection, this was not seen in gastritis and low-grade MALT lymphoma. Although an exact relationship between ***CagA***+ *H pylori* infection and genetic instability is still not clear due to different samples examined in these two studies, this result indirectly suggests that ***CagA*** associated genetic instability, if any, may be related only to the high-grade tumours. Study of the relationship of RER+ phenotypes, the status of free radicals and ***CagA***+ *H pylori* infection in gastric MALT lymphoma may help us to evaluate this proposal. In addition, other mechanisms may also take part in this process, such as non-***Ig*** gene hypermutation induced by antigen stimulation. [294]

### 9.5 Genetic Alterations Involved in MALT Lymphomagenesis

Like all malignant tumours, the evolution of MALT lymphoma is likely to be a multistage process involving different genetic abnormalities. In this thesis, the abnormalities of the ***c-myc*** and ***p53*** gene have been studied systematically.

#### 9.5.1 *C-myc* Gene Abnormalities

A large body of evidence supports the hypothesis that the deregulation of ***c-myc*** expression by genetic abnormalities plays a pivotal role in the pathogenesis of some types of lymphoma. [253] These abnormalities include gene rearrangement and somatic mutation in regulatory regions between

exon I and intron I. In a single study, a high incidence of ***c-myc*** gene rearrangement has been detected by Southern blot hybridisation in high-grade gastric lymphomas. [129] In chapter 6, ***c-myc*** gene abnormalities (gene rearrangements and mutations of the regulatory region) have been studied in 54 MALT lymphomas (43 low and 11 high-grade) and 36 nodal lymphomas (27 low and 9 high-grade). Based on Southern blot hybridisation analysis, none of the 54 MALT lymphomas, but 2 of 36 nodal lymphomas had ***c-myc*** gene rearrangements by two different ***c-myc*** cDNA probes. Defined tumour cell populations from all MALT lymphoma cases were isolated by microdissection from frozen tissues and analysed by PCR-SSCP and direct sequencing for somatic mutations in the exon I / intron I region of the gene. Point mutations in the region were identified in 9 MALT lymphomas (7/43=16.2% of low-grade; 2/11=18.1% of high-grade). These mutations were located at either the exon I/intron I border or myc intron factor (MIF) binding sites which are critical in the negative regulation of ***c-myc*** expression. Of the nodal lymphomas, only the 2 cases (5.6%) with ***c-myc*** gene rearrangements showed scattered or clustered mutations. The results suggest that ***c-myc*** mutations in MALT lymphomas are unlikely to be associated with chromosome translocation, which is the main cause of somatic mutations of the gene, observed in other types of lymphoma. However this association can not be completely ruled out since translocations undetectable by the probes used in this study may exist. The mutations involving the ***c-myc*** regulatory regions may play a pathogenetic role in at least a proportion of MALT lymphomas. However further studies are needed to determine the effects of mutations on ***c-myc*** expression and its relationship with the RER+ phenotype.

### 9.5.2 *P53* Gene Abnormalities

***P53*** gene abnormalities have been found in splenic marginal zone B cell lymphomas, [307] which are analogous to B cell MALT lymphoma. [1,65] In chapter

7, to investigate the role of the ***p53*** gene in the pathogenesis of MALT lymphoma, a comprehensive examination of ***p53*** gene mutation, allele loss and expression in a series of 75 cases was conducted. [379] More than 20% of cases showed ***p53*** gene abnormalities. The nature of the abnormalities was found to be closely associated with tumour progression. Low-grade cases showed only partial inactivation of the gene, while high-grade tumours predominantly exhibited complete inactivation. The accumulation of ***p53*** abnormalities during the progression of the tumours studied also provides genetic evidence for a multistage process in MALT lymphomagenesis.

***P53*** gene abnormalities are the most common genetic abnormalities in human tumours. [310,315] However ***p53*** abnormalities in B cell lymphoma do not appear to be as common as in solid tumours. This suggests that other genetic abnormalities related to ***p53*** function may play a role in lymphomas. It has been shown that over 50% of leukaemias [417] and low-grade NHL [418] had increased expression of ***MDM2***, which can bind with ***p53*** protein and inhibit its transcriptional activity. [325,326] It was also observed that a portion of Burkitt's lymphoma cell lines demonstrated frequent mutations of ***WAF-1***. [419] ***WAF-1*** is a cyclin-dependent kinase inhibitor, which is regulated by ***p53*** and takes part in the ***p53*** mediated G1 arrest. [420] Therefore, over-expression of ***MDM2*** and defects of ***WAF-1*** by gene mutation may also play a role in lymphomagenesis. Whether this is true for the development of B cell MALT lymphoma remains unknown.

### 9.5.3 Other Genetic Abnormalities

Since lymphomagenesis is a multiple-gene process, many other genetic alterations, beside the abnormalities of ***p53*** and ***c-myc*** genes are likely to be involved in MALT lymphoma development. These genetic abnormalities may include abnormal expression or mutation of the ***bax*** gene, [237] ***bcl-2*** amplification, [421,422] ***bcl-6*** gene rearrangement or mutations, [39,423,424] ***bcl-8***

gene rearrangement or abnormal expression, [425] ***p15/p16*** deletion, mutation or hypermethylation, [132,133,426] all of which have recently been associated with nodal lymphoma progression. Studying their role in MALT lymphoma may improve our knowledge of the molecular basis of MALT lymphoma development.

## 9.6. The Relationship between the RER+ Phenotype and Genetic Abnormalities

Genetic instability is caused by abnormalities of the genes which maintain genetic integrity, and is characterised by the presence of a wide range of other specific and non-specific gene abnormalities. [42,43] In chapter 7, ***p53*** mutation and genetic instability indicated by the RER+ phenotype were studied. It was observed that the ***p53*** mutation frequency was higher in tumours with the RER+ phenotype than those with the RER- phenotype and vice versa, which probably reflects an interaction between these two types of genetic abnormalities. It is possible that during the development of MALT lymphoma genetic instability induced by chronic inflammation may promote ***p53*** mutations, which, in turn, cause failure of maintenance of genetic integrity and lead to further genetic instability. More studies are needed to confirm this hypothesis.

However, it is clear that ***p53*** abnormality is not the only factor, which contributes to genetic instability since the RER+ phenotype was also observed in a portion of the lymphomas without ***p53*** gene mutation. In hereditary nonpolyposis colorectal cancer (HNPCC), the RER+ phenotype results from germline mutations in the four DNA mismatch repair genes so far identified - ***hMSH2, hMLH1, hPMS1*** and ***hPMS2***. [206,207,213,216-218,427] In a recent study, mutation and loss of expression of ***hMLH1*** has been observed in leukaemia and lymphoma cell lines. [428] Studying the configuration of these genes may help us to further understand the role of the RER+ phenotype in

MALT lymphomas.

## 9.7 Summary

In summary, the data presented here along with previous studies suggest that the evolution of MALT lymphoma is a multistage process, which comprises sequential development of chronic inflammation caused by infection and /or autoimmune reaction, low-grade and high-grade tumour. In general, tumour cells gradually accumulate genetic abnormalities and gain the ability for autonomous growth while gradually losing dependence on immunological stimulation during tumour evolution.

Taking gastric MALT lymphoma as an example, a tentative scheme for the pathogenesis of the tumour is illustrated in Figure 9-1. As a result of *H pylori* infection, B and T cells, together with neutrophils (the primary effector cells in the host defence), are recruited to the gastric mucosa and form MALT. The host immune response to *H pylori* induces and sustains an actively proliferating B cell population, which is constantly subject to genotoxic insult by activation of neutrophils. During the course of this chronic inflammatory disease, the genetic instability may develop and genetic abnormalities such as t (11;18), trisomy 3 and partial ***p53*** inactivation may accumulate in B cells, particularly in those which recognise autoantigen and already possess a growth advantage, and consequently lead to a partially transformed B cell clone in rare cases. In the presence of growth help from *H pylori* specific T cells, this abnormal B cell clone may undergo clonal expansion and gradually form a frank low-grade MALT lymphoma. At this stage, the tumour is most frequently confined to the stomach and will regress following eradication of *H pylori.* Some low-grade MALT lymphomas, perhaps related to ***CagA***+ *H pylori* strain infection, may acquire further genetic instability, which promoter additional genetic abnormalities, such as the t (1;14) translocation and others uncharacterised, and completely transform this abnormal B cell clone and

result in escape from T cell dependency. Tumour may now present beyond the stomach and no longer be responsive to *H pylori* eradication therapy. Finally, further genetic events such as complete inactivation of the tumour suppressor genes ***p53*** and ***p16***, possibly activation of ***c-myc*** oncogene by translocation and other undetermined abnormalities can result in high-grade transformation.

Although some genetic events, such as t (1;14) translocation and inactivation of tumour suppressor genes, ***p53*** and ***p16,*** are thought to be responsible for the escape from *H pylori* mediated T cell dependent growth and high-grade transformation of low-grade MALT lymphoma, these abnormalities are found only in a minority cases. Clearly, alternative genetic mechanisms exist and will be the focus for future investigation.

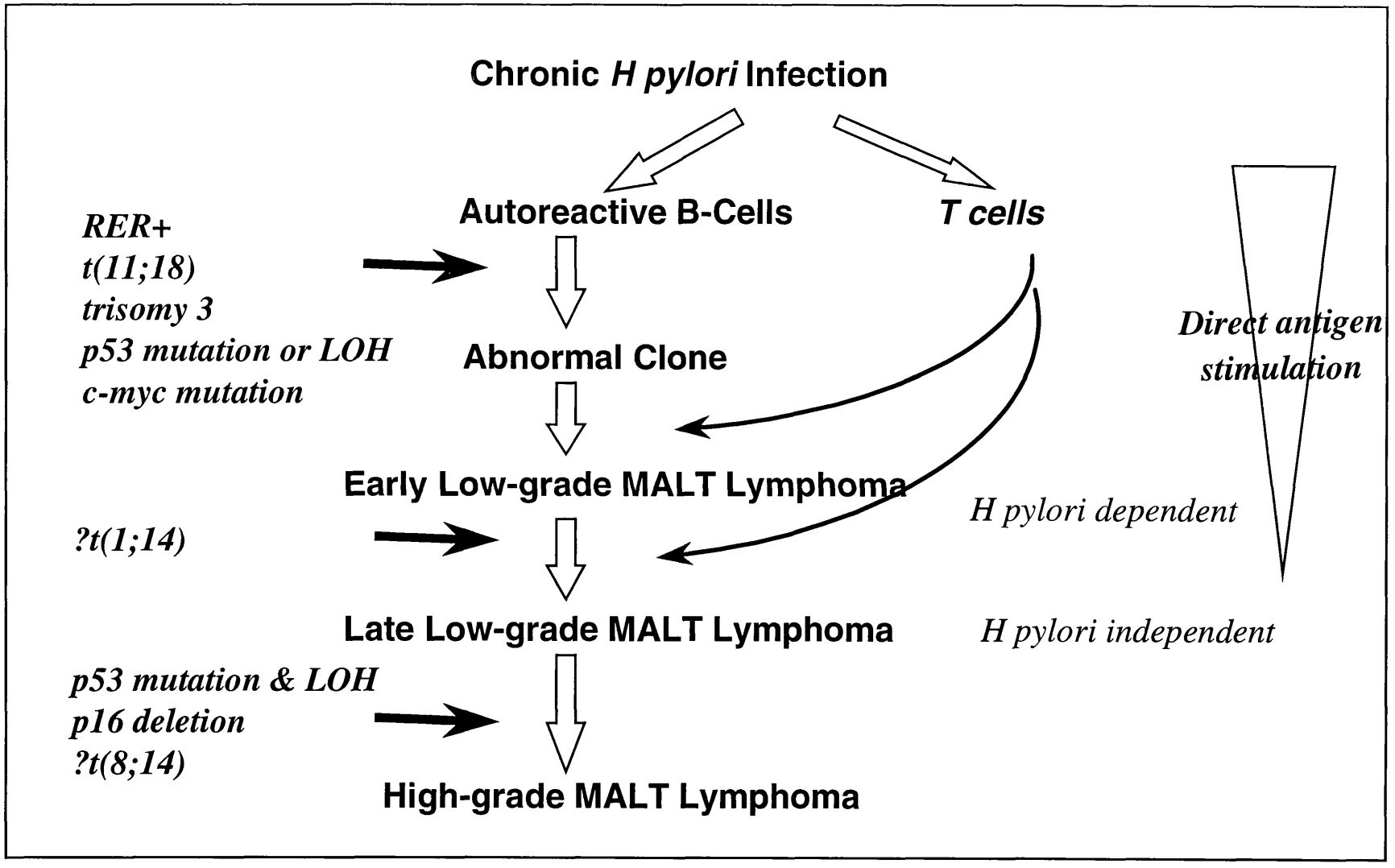


**Figure 9-1 A Tentative Scheme for the Pathogenesis of Gastric MALT Lymphoma.**

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2. Peng HZ et al: Infrequent ***bax*** gene mutation in B cell lymphomas, Journal of Pathology, 1998, In press.

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# Genetic evidence for a clonal link between low and high-grade components in gastric MALT B-cell lymphoma

H.PENG, M.DU, T.C.DISS, P.G.ISAACSON & L.PAN
*Department of Histopathology, University College London Medical School, London, UK*

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**Genetic evidence for a clonal link between low and high-grade components in gastric MALT B-cell lymphoma**

High-grade MALT lymphomas often contain low-grade tumour components; both cell populations have been shown to express the same immunoglobulin light chain previously. However, the clonal link between the low and high-grade components has not been established at the genetic level. To investigate this link, we have examined low- and high-grade components microdissected from tissue sections of four high-grade gastric MALT lymphomas. PCR and sequence analyses were performed to identify clone-specific rearranged immunoglobulin heavy chain gene sequences. In each of these cases, the PCR products from the two components were identical in size by electrophoresis. Direct sequencing revealed common clone-specific immunoglobulin heavy chain gene rearrangements in both lesions of each case, providing genetic evidence for a clonal link. These results support the proposal that high-grade MALT lymphomas generally evolve from low-grade clones.

Keywords: clonality, high grade transformation, MALT lymphoma

## Introduction

Low-grade MALT lymphomas usually run an indolent clinical course, but may be followed by aggressive high-grade tumours[1–3]. Many high-grade MALT lymphomas contain low-grade tumour components[4–9]. These clinical and pathological features support the hypothesis that high-grade MALT lymphomas directly evolve from low-grade disease. Immunophenotypic studies have shown expression of the same immunoglobulin (Ig) light chain in coexisting low and high-grade lesions of gastric MALT lymphomas[5]. Rearranged Ig heavy chain genes of identical size have been PCR-amplified from a low-grade and subsequent high-grade gastric B-cell MALT lymphoma[10]. These findings suggest, but do not prove, a clonal link between low- and high-grade components.

The rearrangement of Ig heavy chain (IgH) genes creats unique sequences in the junctions between variable (V), diversity (D) and joining (J) regions which form clone-specific complementarity determining region III (CDR III)[11–13]. These sequences can be amplified by PCR and used as genetic markers of clonal identity[14–16]. By analysing sequential samples of histologically transformed small lymphocytic lymphoma for these unique sequences[17], it has been shown that the majority of high-grade tumours represent the same clone as preceding low-grade lymphomas, suggesting progression of a single clone.

In order to investigate the clonal link between low- and high-grade lesions, we have analysed the Ig CDR III regions of four gastric MALT lymphomas using PCR and direct sequencing. As high- and low-grade tumour populations were present within each tissue section and were accompanied by substantial reactive lymphoid infiltrates, microdissection was used to enrich target cell populations.

## Materials and methods

Four cases of high-grade gastric MALT lymphoma with coexisting low-grade lesions were selected from the tissue bank of the Histopathology Department at the

Address for correspondence: Dr L.Pan, Department of Histopathology, University College London Medical School, University Street, London WC1E 6JJ, UK.

University College London Medical School, London, UK. The diagnoses of high-grade MALT lymphoma were established using histological and immunophenotypical criteria as previously described[2,3]. In each case, the rearranged immunoglobulin heavy chain (IgH) genes of both high- and low-grade lesions could be PCR amplified (as described below).

Well defined low- and high-grade lesions and reactive lymphoid lesions of each case were identified histologically (see Figure 1) and separated by microdissection from stained tissues sections prior to DNA extraction, as described previously[18].

The DNA extracts from each lesion in each case were amplified in parallel, along with a monoclonal control (follicular lymphoma) and a negative control (no template DNA), using PCR primers directed to framework (FR) 3 and the joining (JH) region of IgH. A previously described semi-nested PCR protocol[14] was employed. A first round of 25 cycles (93°C for 45 s, 50°C for 45 s and 72°C for 110 s) of amplification of the DNA extracts with primers FR3 (ACACGGC[C/T][G/C]TGTATTACTGT) and LJH (TGAGGAGACGGTGACC) was followed by 20 cycles of amplification with an aliquot of 0.5 $\mu$l from the first round, using primers FR3 and VLJH (GTGACCAGCGTNCCTTGGCCCCAG). In each round, an initial denaturation at 95°C for 5 min preceded the addition of Taq polymerase and the final extension period was increased to 7 min. Each reaction contained 10 mM tris (pH 8.3), 50 mM KCl, 200 $\mu$M each dNTP, 250 ng each primer, 0.1% Triton X-100, 9 mM (first round) or 3.5 mM (second round) magnesium chloride and 0.25 units of Taq polymerase (Promega UK). Products were analysed on 10% polyacrylamide gels, stained with ethidium bromide and viewed under UV radiation. Microdissection, extraction and PCR amplification of each specimen were duplicated to control for cross-contamination.

PCR products from each case were directly sequenced in both directions using the USB sequenase II sequencing kit (Amersham, UK) according to the manufacturer's protocol. Computer analysis of the DNA sequences obtained was carried out using a BLAST

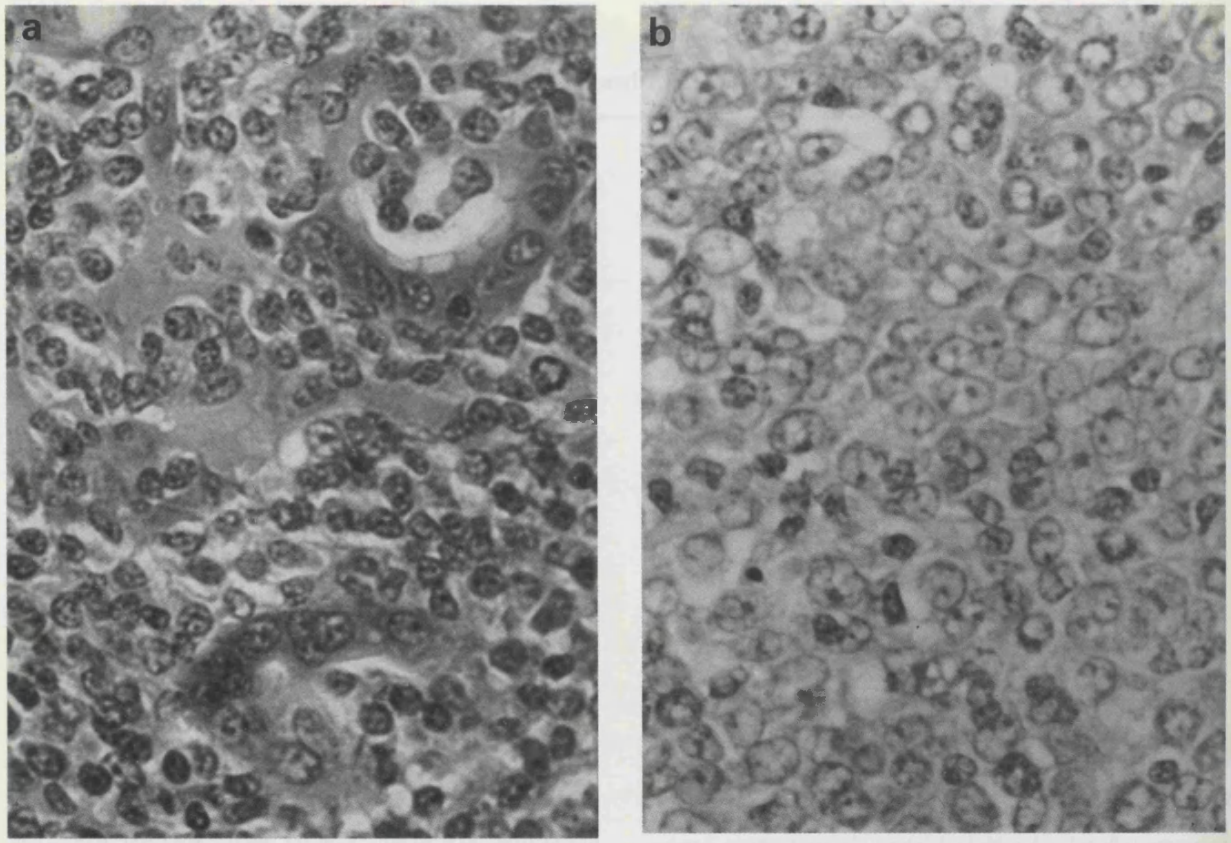


**Figure 1.** H & E stained tissue section showing **a** low-grade and **b** high-grade components of case 1.

WWW online server in the National Centre for Biotechnology Information at the National Library of Medicine, USA (http://www.ncbi.nlm.nih.gov/Blast) and Wisconsin GCG software (provided by the Human Genome Mapping Project, Cambridge, UK). The D and J regions were determined from published germline sequences[19,20].

## Results

PCR amplification of IgH gene of all extracts from microdissected low- and high-grade lesions of each lymphoma produced a discrete fragment within the 70–150 bp size range, representing monoclonal rearrangement of the IgH gene. Both lesions yielded bands of identical size in each case (Figure 2). No dominant bands were amplified from the reactive lymphoid lesions. The positive control yielded the expected dominant band and the negative control showed no products. Amplification of duplicated extracts and reactions produced identical products.

Following direct sequencing, paired PCR products from low- and high-grade lesions in cases 1 and 2 showed identical sequence (Figure 3). In cases 3 and 4, the sequences were identical except for differences at 2 and 3 nucleotide positions, respectively. Computer analysis confirmed that each sequence was a rearranged IgH gene containing combinations of V, D and J regions and contained a variable number of nucleotides at the V-D (N1) and D-J (N2) junctions (Table 1). The differences between the sequences derived from the low and high-grade lesions were located within the D or N regions.

## Discussion

In this study, PCR and direct sequence analysis were used to examine the rearranged IgH genes of low- and high-grade lesions microdissected from four MALT lymphomas. The amplification of dominant IgH PCR products of identical size from both lesions in each case implies the presence of the same neoplastic clone in both lesions in each patient. This has been further confirmed by direct sequencing which revealed common clones in each case. The result provides solid evidence supporting a genetic link between the low- and high-grade lesions of MALT lymphoma. This finding is in agreement with previous observations of shared microsatellite alterations in low- and high-grade lesions[21]. It is therefore likely that high-grade MALT

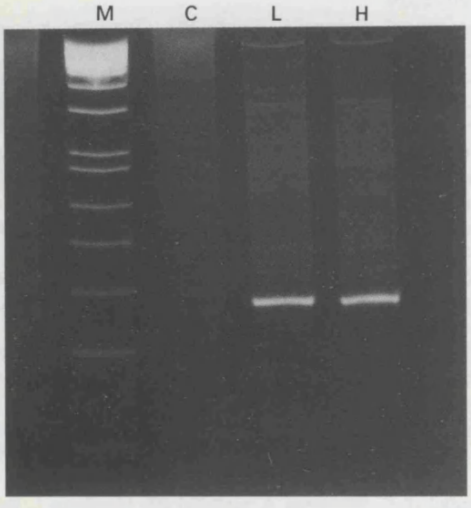


**Figure 2.** Ethidium bromide stained polyacrylamide gel showing PCR products from low and high grade lesions of a MALT lymphoma. Lanes: M – molecular weight markers; C – negative control (no DNA template); L – low-grade lesion; H – high-grade lesion.

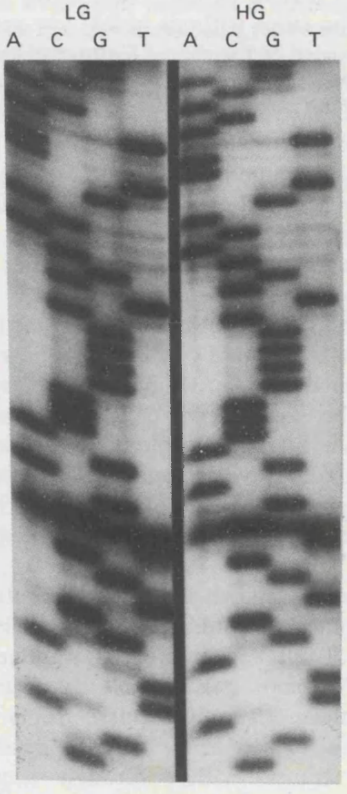


**Figure 3.** IgH CDR III sequences derived from low (LG) and high-(HG) grade lesions of a MALT lymphoma.

**Table 1.** The IgH CDR III sequences derived from low- and high-grade lesions of four cases

| Cases | | N1 | D | N2 | J |
|---|---|---|---|---|---|
| | | | *DXP-3* | | *J5* |
| 1 | LG | GCCAG | TTACTATTATGGAAGTCGTGA | ACGCCGGAG | CTGGTTCGACCCC |
| | HG | - - - - - | - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - | - - - - - - - - - - - - |
| | | | *DN1* | | *J4* |
| 2 | LG | GCGAGCCCCGGGTCTCG | AGCAGCT | AATCG | TATCACT |
| | HG | - - - - - - - - - - - - - - - - - | - - - - - - - | - - - - - | - - - - - - - |
| | | | *DLR-1* | | *J4* |
| 3 | LG | GCGAAATCCGAC | GACGATAAT | | CACTTTGACTAC |
| | HG | - - - - - T - - - - - - | - - - A - - - - - | | - - - - - - - - - - - - |
| | | | *D23-7* | | *J4* |
| 4 | LG | GCGCG | TGGAGAG | GACCGATTTTGCC | GACTA |
| | HG | - - C - - | - - C - - - - | - - - - - T - - - - - - - | - - - - - |

Note. Identical nucleotides between low- and high-grade lesions are represented by dashes, and sequence variations are shown by appropriate nucleotides.

lymphomas arise by further transformation of low-grade cells.

Transformation of preceding low-grade disease may be a general feature of high grade MALT lymphomas, including the cases without visible low-grade lesions. This is supported by the shared features of low and high-grade tumours, such as association with preceding *Helicobacter pylori* infection[22–24], the frequent presence of replication error (RER+) phenotype[21], p53 alterations[25] and chromosome trisomy 3[26]. Low-grade lesions may not be identified in some high-grade tumours due to sampling bias[27] or to overgrowth by high-grade cells[28]. However, it cannot be excluded that some high-grade tumours may arise *de novo*.

The mechanisms involved in high-grade transformation of low-grade MALT lymphoma have yet to be identified. In this study, we observed some nucleotide differences between the IgH CDR III sequences derived from low- and high-grade lesions. These may represent ongoing mutations which resulted from antigen stimulation or aberrant function of the somatic hypermutation mechanism. However, the relationship of this process to high-grade transformation is unclear. *In vitro* studies have shown that the proliferation of high-grade tumour cells, unlike low-grade cells, does not depend on T-cell mediated specific antigen stimulation[29,30]. Therefore, it is possible that during a prolonged antigen-driven life-course some low-grade tumour cells escape from immune dependency by high-grade transformation through acquisition of additional genetic changes, such as p53 alteration[25] and c-*myc* activation[31].

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# c-*myc* GENE ABNORMALITIES IN MUCOSA-ASSOCIATED LYMPHOID TISSUE (MALT) LYMPHOMAS

HUAIZHENG PENG, TIM DISS, PETER G. ISAACSON AND LANGXING PAN*

*Department of Histopathology, University College London Medical School, University Street, London WC1E 6JJ, U.K.*

## SUMMARY

**c-*myc* gene abnormalities associated with lymphomagenesis, including rearrangements and mutations in the regulatory region between exon I and intron I, have been studied in 54 MALT lymphomas (43 low and 11 high grade) and 36 nodal lymphomas (27 low and 9 high grade). By Southern blot analysis, none of the 54 MALT lymphomas but 2 of 36 nodal lymphomas had c-*myc* gene rearrangements. Defined tumour cell populations from all MALT lymphoma cases were isolated by microdissection from frozen tissue sections and analysed by polymerase chain reaction–single-strand conformational polymorphism (PCR–SSCP) and direct sequencing for somatic mutations in the exon I/intron I region of the gene. Point mutations in this region were identified in nine cases of MALT lymphoma (7/43=16·2 per cent of low grade; 2/11=18·1 per cent of high grade). These mutations were located at either the exon I/intron I border of *myc* intron factor (MIF) binding sites, which are critical in the negative regulation of c-*myc* expression. Of the nodal lymphomas, only the two cases (5·6 per cent) with c-*myc* gene rearrangement showed scattered or clustered mutations. These results suggest that c-*myc* mutations in MALT lymphomas are unlikely to be associated with chromosome translocation, which is the main cause of somatic mutations observed in other types of lymphoma. The mutations involving the c-*myc* regulatory regions may play a pathogenetic role in at least a proportion of MALT lymphomas.** © **1997 by John Wiley & Sons, Ltd.**

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KEY WORDS—**MALT lymphoma; c-*myc*; translocation; mutation; Southern blotting; PCR–SSCP; direct sequencing**

## INTRODUCTION

Primary B-cell lymphomas of mucosa-associated lymphoid tissue (MALT) constitute a distinct clinicopathological entity which commonly arises in a background of chronic inflammation occurring in mucosal organs, such as the gastrointestinal tract, lung, thyroid, and salivary gland. These tumours are clinically indolent, but can transform into aggressive high-grade lymphomas at a later stage.[1] The molecular basis for the development and progression of the tumours is still unclear. However, in a search for genetic abnormalities in MALT lymphomas, we recently identified a high incidence of replication error (RER)-related genetic instability indicating a mutator phenotype of the tumours,[2] which might be associated with chronic inflammation. We also noted that a significant proportion of RER-positive MALT lymphomas showed p53 mutations which together with p53 allele loss or overexpression were found to be associated with high-grade transformation.[3] It is likely that other vital genes may also be involved in the pathogenesis of MALT lymphomas.

A number of genes have been studied extensively for their roles in lymphomagenesis in humans. Among them, the c-*myc* gene is one of the most frequent targets. The gene is located at chromosome band 8q24. It contains three exons, of which exons II and III encode a dominant functional protein (p64). Translation of c-*myc* mRNA can also be initiated from exon I, resulting in a larger polypeptide (p67) (Fig. 1).[4] c-*myc* p64 is a transcription factor which modulates two overlapping sets of genes, one involved in cell proliferation, the other in cell death.[5] The function of p67 is unknown. It has been suggested that the peptide may act as a negative regulator of p64 activity.[6,7] Studies have indicated that overexpression of p64 plays an important role in the development and progression of many tumours, while loss of p67 confers a selective growth advantage.[8,9] c-*myc* expression is controlled at multiple levels, from transcription to post-translation stages. Most of the regulatory elements, such as a transcription elongation block,[10] a p67 protein initiation site, an intron splicing site,[6] and three *myc* intron factor (MIF) binding sites,[11,12] are located within a short region between the 3′ end of exon I and the 5′ end of intron I. Deregulation of c-*myc* expression in lymphoid tumours has been found to be associated with somatic alterations within or close to this region, commonly resulting from chromosomal translocation with immunoglobulin (Ig) genes. These alterations may cause aberrant control of c-*myc* by Ig regulatory sequences, promoter shifts, alternative p64/p67 expression ratios, and the loss of the block to transcription elongation.[4–12]

The configuration of the c-*myc* gene in MALT lymphomas is largely unknown, although in a single study,[13] a high incidence of c-*myc* gene rearrangements was

*Correspondence to: Dr Langxing Pan, PhD, MB, Department of Histopathology, University College London Medical School, University Street, London WC1E 6JJ, U.K. email: l.pan@ucl.ac.uk

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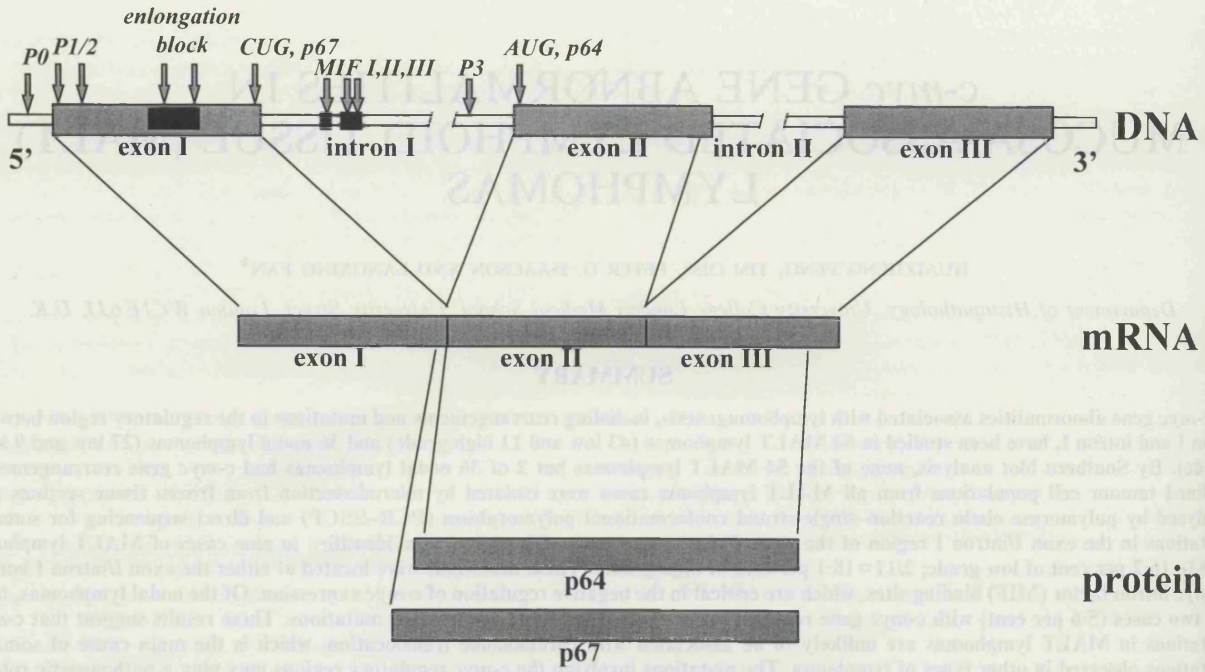


Fig. 1—The structure and regulatory elements of human c-*myc*. The solid boxes indicate exons and the open boxes represent introns. P1 and P2 are major promoters and P0 and P3 are minor promoters. The small bar inside exon I is the site of the conditional block to transcriptional elongation. *myc* intron factor, a negative regulator of c-*myc* transcription, binding sites (MIF I–III) are indicated as solid bars inside intron I. Synthesis of the dominant c-*myc* protein, p64, is initiated from the AUG codon at the 5′ end of exon II (AUG, p64), while synthesis of the larger protein, p67, starts from the CUG codon at the 3′ end of exon I (CUG, p67)

detected by Southern blot hybridization in a small series of high-grade gastric lymphomas. In the present study, using the same technique and probes, we sought c-*myc* gene rearrangements in 54 cases of MALT lymphoma. We also examined somatic mutations in the exon I/intron I regulatory region by PCR–SSCP/direct sequencing in the same cases. Since MALT lymphomas, unlike their nodal counterparts, commonly contain a heavy inflammatory infiltrate, tumour populations were isolated by microdissection prior to PCR mutation analysis in order to obtain representative results. Thirty-six cases of nodal lymphomas were analysed in parallel. We expected that the results generated would help us to understand the role of the c-*myc* gene in the pathogenesis of MALT lymphomas.

## MATERIALS AND METHODS

### *Materials*

Frozen tissue blocks of 54 cases of MALT lymphoma (43 low and 11 high grade) and 36 cases of nodal lymphoma (27 low and 9 high grade) were obtained from the tissue bank of the Histopathology Department at the University College London Medical School, London, U.K. All cases were shown to be monoclonal by Ig light chain immunophenotyping or immunoglobulin heavy chain gene rearrangement analysis.[14] Standard procedures were used to extract DNA from snap-frozen tumour biopsy specimens.[15] DNA extracts from normal tissues (placenta and tonsil) and the Raji cell line were used as negative and positive controls.

### *Microdissection*

The samples were reviewed for malignant cell purity in order to check whether a reliable PCR–SSCP result could be obtained. The percentage of malignant cells was estimated either by histopathological examination or by comparison of the intensity of rearranged $IgV_H$ bands with the germline $IgV_H$ bands in Southern blots hybridized with a $J_H$ probe. All of the nodal lymphomas examined contained at least 60 per cent tumour cells and were therefore analysed directly from whole tissue samples. However, the percentages of malignant cells in the MALT lymphomas ranged from 10 to 50 per cent, necessitating microdissection of the tumour cells in these cases. Microdissection and subsequent DNA extraction were performed as described previously.[16]

### *Southern blotting*

DNA extracts from frozen tissues were digested separately with restriction enzymes Eco RI, Hind III, and Bam HI or Pst I. DNA digests were fractionated according to size in 0·8 per cent agarose gels and transferred to nylon membranes by Southern blotting. A 1·4 kb Eco RI DNA fragment spanning from c-*myc* intron 1 to exon 3[15] and a 2·0 kB Eco RI c-*myc* cDNA fragment containing exons 2–3 (kindly provided by HGMP probe bank, Cambridge, U.K.) were used as hybridization probes. The probes were separately radio-labelled with $^{32}$P-dCTP by the random hexamer method and hybridized to the membranes using the conditions described previously.[15] The membranes were washed at the

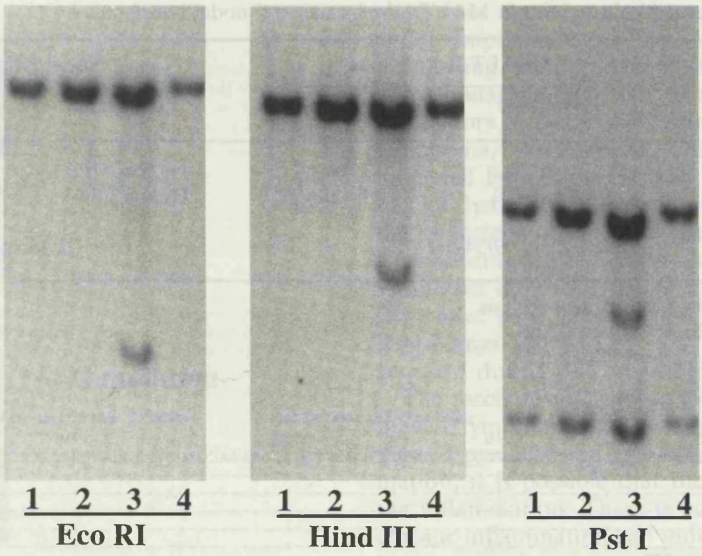


Fig. 2—Southern blot analysis of c-*myc* gene. Lane 1 (placenta) and lanes 2 and 4 (MALT lymphomas) show germline bands of the c-*myc* gene. Lane 3 (a nodal lymphoma) shows rearrangement of the gene in all three different restriction enzyme digests

appropriate stringency and exposed to X-ray films at −70°C for 2–7 days. Before rehybridization, the radiolabelled probe was removed from the blots by boiling in 0·5 per cent SDS.

### *PCR–SSCP and direct sequencing*

Primers designed to amplify the c-*myc* exon I–intron I regulatory region contained the sequences 5′-GCA CTG GAA CTT ACA ACA CC-3′ and 5′-CTG GCT CAC ACA GGC GAT-3′. The PCR products were 589 bp in size and included 162 bp in exon I and 427 bp in intron I. The PCR reaction mix consisted of 1 $\mu$l of DNA extract, 10 mM Tris (pH 8·3) 50 mM KCl, 1·5 mM $MgCl_2$, 0·1 per cent Triton X-100, 200 $\mu$M of each dNTP (Promega, Southampton, U.K.), 15 pmol of each primer, 0·001 per cent gelatin, and 0·25 units of Taq polymerase (Promega, Southampton, U.K.) in a total volume of 25 $\mu$l. Forty cycles of PCR were carried out on a thermal cycler (Hybaid, U.K.), consisting of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 45 s. An initial denaturation step at 95°C for 5 min preceded the addition of enzyme and an extension step at 72°C for 5 min concluded the reaction. Three microlitres of PCR products was checked for yield size on a 1 per cent agarose gel.

A total of 5 $\mu$l of PCR products was digested by Taq I to generate one 223 bp and two 183 bp fragments. SSCP analysis of these digested PCR products was carried out using a recently published polyacrylamide/agarose gel and a background free silver staining system.[17] PCR products showing abnormal SSCP patterns were directly sequenced using the USB PCR sequencing kit (Amersham, U.K.) according to the manufacturer's protocol, with minor modification. The sequencing reactions were either repeated at least twice or performed from both directions with independent template preparations to eliminate PCR artefacts.

In order to understand whether the mutations observed were homogeneous in each tumour or represented an ongoing process (ongoing mutations) during the tumour expansion, multiple tumour lesions were analysed in four of the cases with c-*myc* mutations.

## RESULTS

### *Southern blot hybridization*

DNA samples from all of the MALT lymphomas and controls from placenta and tonsil DNA yielded germline patterns of the c-*myc* gene, when digested with the three restriction enzymes. Of the 36 nodal lymphomas, two (one low- and one high-grade lymphoma) showed one or more rearranged bands in all digests when hybridized with each of the two c-*myc* probes (Fig. 2).

### *SSCP*

The results are summarized in Table I. Abnormal migration patterns of digested PCR products were observed in 9 of 54 MALT lymphomas (16·6 per cent), including two high- and seven low-grade tumours. The incidence in high-grade (2/9=18 per cent) lymphomas was similar to that in low-grade (7/43=16 per cent). Only the two (2/36=5·6 per cent) nodal lymphomas which had been shown to have c-*myc* rearrangements by Southern blotting exhibited abnormal SSCP patterns.

### *Direct sequencing analysis*

To characterize the type and distribution of mutations, the 11 lymphoma samples with abnormal PCR–SSCP patterns were analysed by direct sequencing.

Table I—c-*myc* gene abnormalities in MALT lymphomas and nodal lymphomas

| Abnormalities | MALT lymphomas | | Nodal lymphomas | |
|---|---|---|---|---|
| | Low grade | High grade | Low grade | High grade |
| Rearrangement* | 0 | 0 | 1/27=3·7% | 1/9=11·1% |
| Mutation† | 7/43=16·2% | 2/11=18·1% | 1‡/27=3·7% | 1‡/9=11·1% |
| Total | 9/54=16·7% | | 2/36=5·6% | |

*As determined by Southern blot analysis.
†As determined by SSCP/sequencing.
‡Same case with c-*myc* rearrangement.

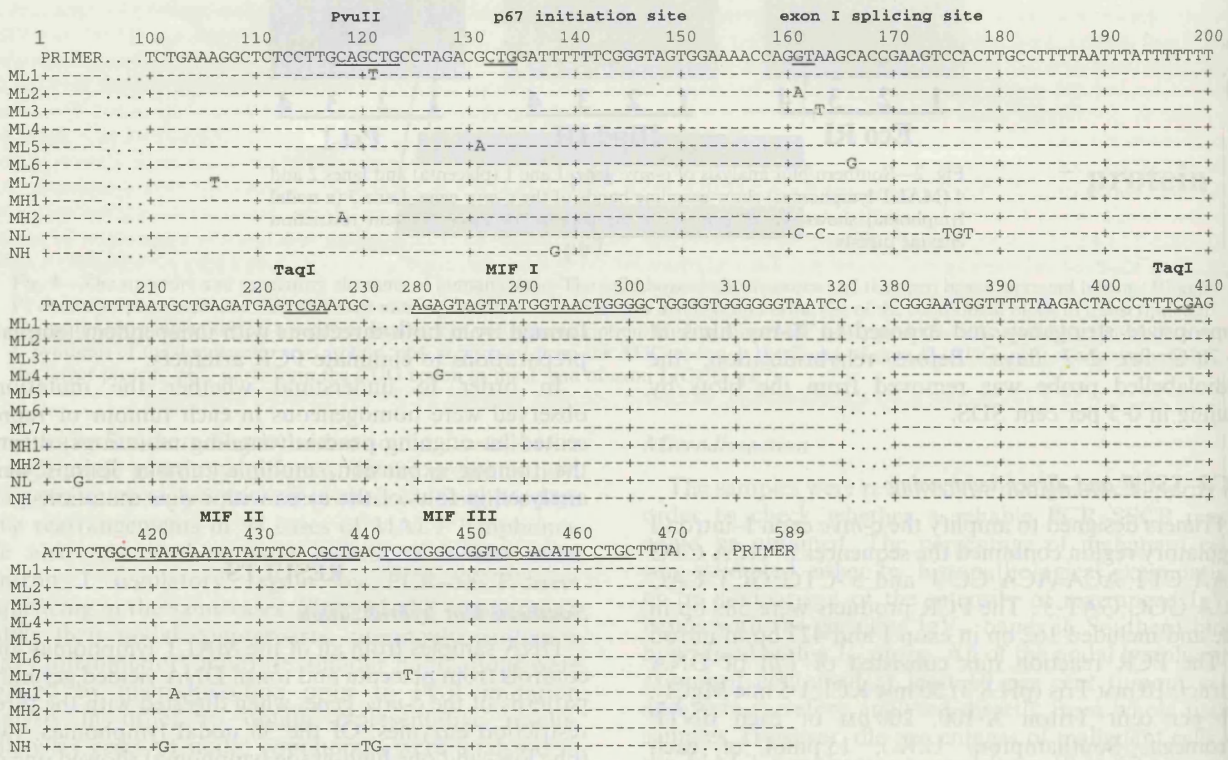


Fig. 3—The spectrum of c-*myc* gene mutations in MALT lymphomas (low grade, ML1–7 and high grade, MH1–2) and nodal lymphomas (low grade, NL and high grade, NH). The control sequence shown here is the c-*myc* germline sequence (HUMMYCG02, L00057). The sequences underlined indicate Taq I restriction enzyme sites, p67 initiation site, exon I splicing site, and MIF I, II, III binding regions. The positions of the mutated bases in each case are indicated

Mutations in all these cases were confirmed. The distribution of the mutations is shown in Fig. 3 and an example of the direct sequencing results is shown in Fig. 4.

The nine MALT lymphomas with c-*myc* mutations showed a single base change, except for one case which had two point mutations. All ten mutations in these nine cases were concentrated in two regions. Seven mutations from six cases were located near the border of exon I and intron I, within 95 bp up- or down-stream of the Pvu II site in exon I. They included one near and two within the Pvu II site, one immediately next to the p67 initiation site, and two close to and one within the intron/exon splicing site. The three point mutations in the remaining three cases were located within the MIF (I–III) binding sites. Analysis of multiple tumour lesions in four of the cases with mutations showed no variation between different lesions in the same cases. The two nodal lymphomas exhibited multiple single base changes, one possessing four and the other five alterations. These mutations within the 589 bp long DNA fragment analysed were widely scattered in one case and clustered in the other.

## DISCUSSION

The single report of a high incidence of c-*myc* gene rearrangement in high-grade gastric 'MALT' lymphomas[13] and the proposed role of the gene in

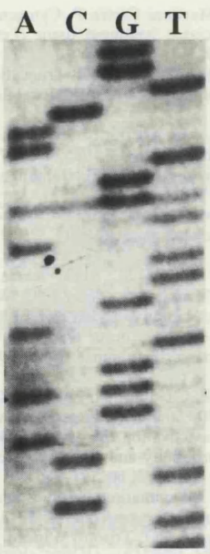


Fig. 4—Direct sequencing of the PCR product from an SSCP-positive MALT lymphoma, showing an A to G mutation in the MIF I region.

lymphomagenesis prompted us to undertake this study. We could not identify c-*myc* rearrangement in any of the 54 MALT lymphomas examined, although we detected rearranged c-*myc* gene in two of the 36 nodal lymphomas. We observed a series of point mutations in the c-*myc* exon I and intron I regulatory region in a significant proportion of MALT lymphomas. Our results, together with documented cytogenetic studies[18–21] showing no evidence of chromosome 8 translocations in MALT lymphomas, indicate that it is unlikely that these lymphomas are characterized by c-*myc* gene rearrangements as previously suggested.[13]

As shown in Fig. 3, all the mutations in the nine MALT lymphomas are concentrated in exon I/intron I border and MIF sites which are known to be essential for regulation of c-*myc* expression. As described in several studies,[6–9] somatic alterations in p67 mRNA splicing and translation initiation sites near the exon I/intron I border can down-regulate or abolish expression of p67 protein, which is believed to act as a negative regulator of p64, the functional c-*myc* protein. In addition, mutations within the 95 bp either side of the Pvu II site may remove a block to transcription elongation,[10] or increase p64 c-*myc* mRNA stability.[22,23] Mutations in the MIF sites may affect the binding of the c-*myc* intron factor, a 138 kD phosphoprotein which functions as a negative regulator of c-*myc* transcription.[11] Therefore, all of the mutations identified have the potential to cause overexpression or increased activity of c-*myc* protein, a critical step in lymphomagenesis.[4,7,24,25] This may contribute to the development of at least a proportion of MALT lymphomas. However, further studies are needed to validate this proposal.

In other types of lymphoma, somatic mutations in the c-*myc* exon I/intron I region are almost exclusively accompanied by chromosome translocation and are probably acquired as a result of the hypermutation capability of translocated Ig genes.[7] Although these alterations have rarely been described in low-grade nodal lymphomas, they have been reported as a 'second hit' during high-grade transformation of some low-grade nodal lymphomas[26] or as essential initiating genetic factors in aggressive lymphomas, such as Burkitt's lymphoma,[7] AIDS-related lymphoma,[27] and anaplastic large cell lymphoma.[28] Since none of our 54 cases of MALT lymphoma showed c-*myc* gene rearrangement, the mutations observed in the tumours are likely to be chromosome translocation-independent. There was no significant difference in the mutation frequency within the c-*myc* gene between low- and high-grade MALT lymphomas, suggesting that the mutations may be acquired during early development of the tumours.

The mechanisms leading to c-*myc* gene mutations in MALT lymphomas are not clear. Since the tumours are always preceded or accompanied by chronic inflammation, it is possible that the mutations are related to the inflammation. There is accumulating evidence that chronic inflammation can induce instability in the cellular genome, leading to DNA damage, in the form of gene mutations, gene amplification, DNA strand breaks, deletions or translocations in the affected cells.[29,30] In a recent study, over 50 per cent of MALT lymphomas have been shown to exhibit genetic instability, as indicated by an RER-positive phenotype.[2] These RER-positive tumours are accompanied by a high frequency of p53 gene mutations. Therefore, the inflammation-associated genetic instability and the resulting somatic mutations and other genetic abnormalities, such as trisomy of chromosome 3[31] or t(1;14),[19] may form the molecular basis for evolution and progression of MALT lymphomas.

## ACKNOWLEDGEMENTS

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# Replication Error Phenotype and *p53* Gene Mutation in Lymphomas of Mucosa-Associated Lymphoid Tissue

Huaizheng Peng,* Gong Chen,† Mingqing Du,* Nalini Singh,* Peter G. Isaacson,* and Langxing Pan*

*From the Department of Histopathology,* University College London Medical School, and The William Harvey Research Institute,† St. Bartholomew's Hospital Medical College, London, United Kingdom*

***Low grade mucosa-associated lymphoid tissue (MALT) lymphomas commonly arise from a background of chronic inflammatory lesions and can transform into high grade tumors at a late stage. Because chronic inflammation is closely associated with genetic instability, which is one of the mechanisms leading to activation of oncogenes and inactivation of tumor suppressor genes, it is possible that genetic instability plays an important role in MALT lymphomagenesis. In this study, we have examined the frequency of replication error (RER$^+$) phenotype, a newly defined manifestation of genetic instability, and its relationship to p53 mutations in 40 MALT lymphomas (16 high grade and 24 low grade). RER$^+$ phenotype was detected in 21/40 (52.5%) MALT lymphomas (12/24, 50% in low grade; 9/16, 56.2% in high grade). Five of seven reactive lymphoid infiltrates adjacent to tumors also showed one microsatellite alteration, four of which were identified in the corresponding lymphoma lesions in the same patient. In five RER$^+$ high grade lymphomas with low grade lesions, homogeneous and heterogeneous microsatellite alterations were observed between the two components. The same 40 cases were investigated for p53 gene mutations at exons 5 to 8 by PCR-SSCP and direct sequencing. p53 point mutations were found in 11 (27.5%) of the 40 cases. These mutations were statistically related to RER$^+$ phenotype ($P < 0.05$). Our results demonstrate that the RER$^+$ phenotype is a common genetic feature of MALT lymphomas. Genetic instability occurs throughout the spectrum of the lymphoma development and may be related to the accumulation of genetic aberrations such as p53 mutations. The observation of identical microsatellite alterations between the adjacent lymphoid infiltrates and their corresponding lymphomas provides genetic evidence for evolutionary link of the two lesions. The homogeneous and heterogeneous microsatellite alterations observed between low and high grade components indicate their clonal lineage and genetic diversity. (Am J Pathol 1996, 148:643–648)***

Low grade B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) is characterized by a favorable clinical behavior.[1] It usually arises from lymphoid tissue acquired as a result of chronic inflammation, and can transform to high grade lymphoma at a late stage.[1] Little is known about the molecular pathogenesis of MALT lymphomas, although the clinical, pathological, and immunophenotypic features have been well documented.[1] Because chronic inflammatory stimuli can result in genetic instability,[2] which is a necessary step to accumulate tumor-related genetic aberrations,[3] it is possible that genetic instability is related to the development of MALT lymphoma.

Replication error (RER$^+$) phenotype, a recently defined manifestation of genetic instability, has been observed in a wide range of tumors.[4–7] It is caused by a deficiency of DNA mismatch repair genes.[8,9] The cells with the RER$^+$ phenotype demonstrate markedly increased mutation rates in non-coding regions (eg, microsatellite sequences) as well as in coding regions[10] of the genome. Mutations in micro-

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Address reprint requests to Dr. Langxing Pan, Ph. D., M. B., Department of Histopathology, University College London Medical School, University Street, London WC1E 6JJ, UK.

Table 1. *Primers for Multiplex Fluorescent PCR*

| Locus | Heterozygosity rate | Location | Size | Dye | Primer sequences |
|---|---|---|---|---|---|
| D3S1262 | 81% | 3q | 112–126 | FAM | *CGGCCCTAGGATATTTTCAA and CCAGTTTTTATGGACGGGGT |
| D3S1265 | 84% | 3q27–qTER | 126–150 | HEX | *TTCTATAAGGGCAGGGACAC and GCTCGCAATTTCTCCTTAAT |
| D3S11 | 93% | 3p24.2–p22 | 135–147 | FAM | *CAAACTTTCCACAGTATCGTTC and GTTTCCTTGAGAAGAATGGAGC |
| D3S1261 | 85% | 3p14–p12 | 84–116 | HEX | *GAGGTGATGTGAGAGTGGAT and CTCAGCACCACAGTATGTGT |
| D6S262 | 84% | 6p23.1–21.3 | 167–183 | HEX | *ATTCTTACTGCTGGAAAACCAT and GGAGCATAGTTACCCTTAAAATC |

*Fluorescent-labeled primer.

satellites (microsatellite alterations) provide a marker for this phenotype. The mutations in coding regions may lead to oncogene activation and tumor suppressor gene inactivation and therefore contribute to tumor development.

*p53* gene mutation is one of the most common genetic alterations in human malignancy, contributing to tumor development and progression.[11] Normal *p53* gene is thought to play both a direct and indirect role in maintaining genetic integrity,[12] and therefore *p53* mutation may contribute to genetic instability. However, the relationship between the $RER^+$ phenotype and *p53* mutation is still not clear, although some inconclusive reports are available.[13–15]

We have examined the frequency of $RER^+$ phenotype and *p53* mutations in 40 MALT lymphomas by fluorescent multiplex polymerase chain reaction (PCR) and PCR-SSCP. Our purpose was to understand their roles and association in the development of this lymphoma.

## Materials and Methods

### Tissue Samples

Frozen (30 cases) or formalin-fixed paraffin-embedded tissue samples (10 cases) of MALT lymphomas, including 16 high grade and 24 low grade, were obtained from the Department of Histopathology at the University College London Medical School. The diagnosis of these lymphomas was established by histological findings together with immunophenotyping and clonal analysis of rearranged immunoglobulin heavy chain genes.

### Microdissection and DNA Preparation

The sections were carefully reviewed histologically. Tumor cell populations (high and/or low grade lesions) from each MALT lymphoma were microdissected from either paraffin-embedded or frozen sections. In five high grade lymphomas with low grade lesions, both components were microdissected separately. In addition, tumor-adjacent reactive lymphoid infiltrates from seven lymphomas (five low grade and two high grade) were microdissected. Normal tissues including mucosa, spleen, liver, and/or muscle, and lymphoid infiltrates distant from tumors when available, were used as controls in each case. The methods of microdissection and DNA extraction were as described previously.[16] The nature of the microdissected lymphoid tissues was further confirmed by PCR clonality analysis for immunoglobulin *VH* gene rearrangement using Fr3-Jh primers.[16]

### Multiplex Fluorescent PCR of Microsatellite Repeats

Five pairs of primers were chosen for microsatellite fluorescent PCR from the Genetic Database GDB in Genethon and are listed in Table 1. The markers were selected to enable amplification of all five loci in a single PCR reaction. One of each primer pair was fluorescently labeled with either FAM (blue) or HEX (yellow) dye (Oswell DNA Service, Edinburgh, UK).

Paired normal and tumor DNA samples from each patient were amplified simultaneously. The PCR reaction mixture consisted of 1 μl DNA extract, 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 200 μmol/L each deoxynucleoside triphosphate (Promega, Southampton, UK), 1 pmol of each of the 10 primers, 0.001% gelatin, and 0.25 units of *Taq* polymerase (Promega) in a total volume of 10 μl. Thirty cycles of PCR were carried out on a thermal cycler (Hybaid, UK). The PCR profile consisted of a denaturation at 94°C for 30 seconds, an annealing at 55°C for 30 seconds and an extension at 72°C for 45 seconds. An initial denaturation step at 95°C for 5 minutes preceded the addition of enzyme, and a primer extension step at 72°C for 5 minutes concluded the reaction. Before electrophoresis on an automated sequencer, 3 μl PCR products were routinely checked for yield and size on a 2% agarose gel.

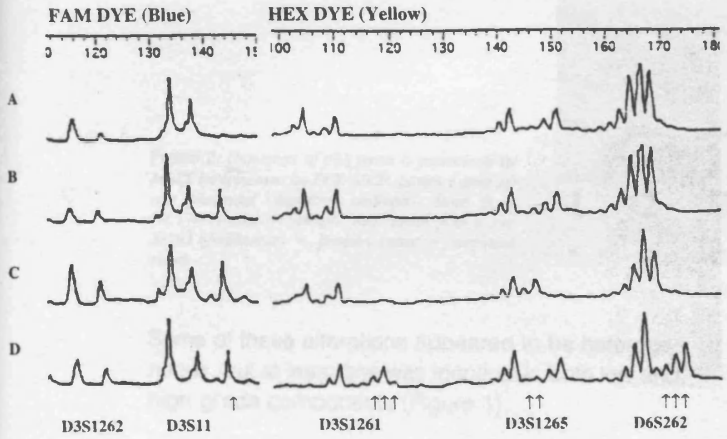


Figure 1. *The microsatellite alterations (arrows) in a high grade MALT lymphoma with adjacent reactive lymphoid infiltrates and low grade component. (A) Normal tissue. (B) Adjacent reactive lymphoid infiltrates. (C) Low grade component. (D) High grade component.*

To exclude technical artifacts or specimen contamination, PCR was repeated in duplicate or triplicate.

### *Electrophoresis and Data Analysis*

After amplification, 0.5 μl of PCR product was mixed with 0.6 μl of internal lane size standard (GENSCAN 2500-ROX) and 4 μl blue formamide loading buffer (ABI, Foster City, CA). Samples were denatured at 94°C for 3 minutes and loaded onto 6% denaturing polyacrylamide gels (Severn Biotech Ltd., Worcestershire, UK) on a model 373A sequencer (ABI) and electrophoresed for 4 hours. The fluorescent gel data were automatically collected and analyzed using GENESCAN 672 (version 1.2) software after electrophoresis as described in the manufacturer's manual. The PCR products were distinguished from each other by different sizes and dyes.

### *PCR-SSCP Analysis and Direct Sequencing of* p53 *Genes*

*p53* exons 5 to 8 were separately amplified by PCR as described previously.[17] SSCP analysis of these PCR products was carried out using our recently published protocol,[18] which utilized a polyacrylamide/agarose gel and a background-free silver staining method. PCR products showing abnormal SSCP patterns were directly sequenced using the Fmol sequencing kit (Promega) according to the manufacturer's protocol. The sequencing reactions were either repeated at least twice or performed in both directions from independent template preparations to ensure that mutations were not the result of PCR artifacts.

### *Statistical Method*

The $\chi^2$ test was used to assess the association between RER+ phenotype and *p53* mutations.

## *Results*

### *Microsatellite Alterations and RER+ Phenotype in MALT Lymphomas*

A microsatellite alteration was defined as a locus that gained one or more new peaks, or showed peak shifts when compared with the patterns from the corresponding normal tissue. If a sample contained two or more microsatellite alterations among five loci examined, it was classified as RER+ as proposed previously.[19]

Normal tissues from different sites of the same patients showed identical microsatellite migration patterns. No microsatellite alteration was observed in reactive lymphoid infiltrate distant from the lymphomas. However, among tumor-adjacent lymphoid infiltrates isolated from seven cases of lymphoma, five showed one microsatellite alteration.

In 40 MALT lymphomas, 33 showed microsatellite alterations. But only 21 (52.5%) contained two or more microsatellite alterations and therefore were RER+. The frequency of the RER+ phenotype was slightly higher in high grade (9/16, 56.2%) than in low grade (12/24, 50%) MALT lymphomas.

Four of the five microsatellite alterations observed in tumor-adjacent lymphoid infiltrates were also identified in the corresponding lymphoma lesions (Figure 1). In the five high grade lymphomas with low grade components, two or more microsatellite alterations were observed in both tumor lesions from each case.

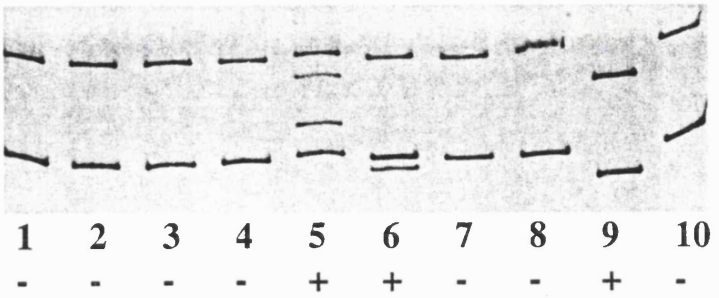


Figure 2. *Detection of* p53 *exon 6 mutations in MALT lymphomas by PCR-SSCP. Lanes 1 and 10 are placenta (negative controls), lane 9 is HUT78 (positive control), and lanes 3 to 8 are MALT lymphomas. +, positive cases; −, negative cases*

Some of these alterations appeared to be heterogeneous, but at least one was identical in both low and high grade components (Figure 1).

## p53 *Mutations in MALT Lymphomas*

No mutations were observed in normal tissues or reactive lymphoid infiltrates by SSCP analysis. Among 40 tumor samples screened for *p53* gene mutations within exons 5 to 8, abnormal electrophoresis mobility shifts on SSCP gels were detected in 14 cases (Figure 2). All the mutations were confirmed by direct sequencing (Figure 3 and Table 2). These included nine replacement mutations resulting in amino acid substitution, two deletion mutations resulting in frame shifts, and three silent mutations. Therefore, a total of 11/40 (27.5%) MALT lymphomas showed *p53* gene mutations that resulted in alterations of *p53* amino acid sequences. A higher mutation frequency was observed in high grade (6/16, 37.5%) than in low grade (5/24, 20.8%) MALT lymphomas.

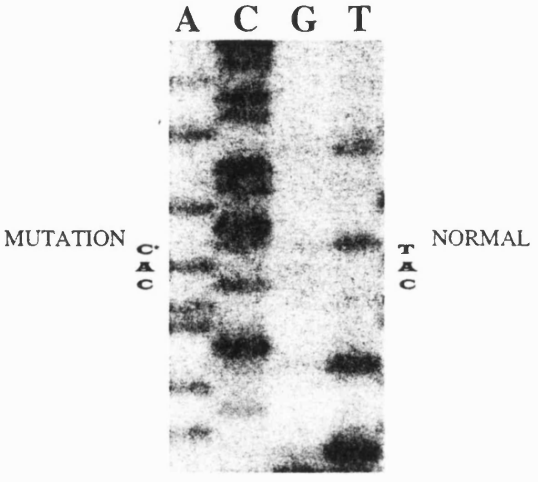


Figure 3. *Direct sequencing of the PCR product from an SSCP-positive case, showing a missence mutation (TAC → CAC) in codon 234, p53, exon 6.*

## *Association between the RER⁺ Phenotype and* p53 *Mutations*

The $\chi^2$ test shows that there is a significant association between the RER⁺ phenotype and *p53* mutation ($P < 0.05$). *p53* mutations occurred more frequently in RER⁺ cases (8/21, 38%) than in RER⁻ cases (3/19, 15%). In addition, the RER⁺ phenotype is more often seen in tumors with *p53* mutation (8/11, 72%) than tumors without *p53* mutation (13/29, 44.8%).

## *Discussion*

To achieve sensitive and reliable results, particular attention was paid to methodology. 1) Defined normal, reactive, and tumor populations for microdissection were subject to careful histological examination. To confirm the nature of the microdissected lymphoid tissues, PCR clonality analysis based on *IgVH* gene rearrangements was performed. This is extremely important for MALT lymphoma, as the tumor is always accompanied by a substantial reactive lymphoid component.[1] 2) Multiplex PCR was used for all microsatellite amplifications. These enabled us not only to speed up the analysis, but also to reduce the risk of sample contamination. 3) A highly sensitive automated sequencer was used to detect fluorescent PCR products, and GeneScan software to analyze the data. The advantages of this technique are accurate sizing of PCR products, rapid generation of results, high sample throughput, a requirement for very little DNA, and the non-radioactive nature of the assay.

The RER⁺ phenotype is common in MALT lymphomas (52.5%) as shown in the present study. This suggests that genetic instability may cause the functional genetic alterations required for the manifestation of the malignant phenotype. There are limited data available about RER⁺ phenotype in lymphomas. The frequency presented here is similar to that reported in human immune deficiency virus-related

Table 2. *p53* Mutations in MALT Lymphomas

| Case | Grade | RER | Codon | Mutation | Substitution |
|---|---|---|---|---|---|
| 1 | H | + | 212 | TTT → TTA | Phe → Leu |
| 2 | H | + | 194 | CTT → CAT | Leu → His |
| 3 | H | − | 158 | CGC → CTC | Arg → Leu |
| 4 | H | + | 206 | TTG → T*G | Frameshift |
| 5 | H | + | 253 | ACC → ACT | Silent |
| 6 | H | + | 274 | GTT → GAT | Val → Asp |
| 7 | H | + | 254 | ATC → GAC | Ile → Asp |
| 8 | L | + | 193 | CAT → CAC | Silent |
| 9 | L | + | 160 | ATG → CTG | Met → Leu |
| 10 | L | + | 178 | CAA → *AA | Frameshift |
| 11 | L | − | 273 | CAT → TAT | His → x Tyr |
| 12 | L | + | 234 | TAC → CAC | Tyr → His |
| 13 | L | − | 179 | CAT → TAT | His → Tyr |
| 14 | L | − | 197 | CTG → GTA | Silent |

non-Hodgkin's lymphoma,[20] but is much higher than that of other nodal lymphomas where reported frequencies vary from 0 to 20%.[20,21] Our results further demonstrate that MALT lymphomas are a distinct entity differing from nodal lymphomas at the genetic level.

Our results have shown that genetic instability indicated by microsatellite alterations persists throughout the development of MALT lymphoma. Reactive lymphoid infiltrates adjacent to the lymphoma, in contrast to those distant from the tumor, demonstrate microsatellite alterations that were also identified in corresponding low or high grade lesions. This is the first direct evidence of a relationship between reactive lymphoid infiltrates and the adjacent MALT lymphomas. It also suggests that microsatellite alterations occur before histological tumor appearance. This evidence supports the hypothesis that lymphoid infiltrates induced by chronic inflammatory stimulation are precursors of MALT lymphomas. The homogeneous microsatellite alterations observed between low and high grade lesions of the same patients may indicate a genetic lineage between these two components, while the heterogeneous alterations may reflect genetic diversity of the lesions.

A high incidence of *p53* gene mutation has been found in MALT lymphomas with a higher frequency in high grade lymphomas. This, together with other data from our group,[22] suggests that the *p53* gene plays an important role in the development of MALT lymphomas, particularly in high grade transformation. The association between *p53* abnormality and RER+ phenotype suggests that they may affect each other. Both genetic instability and *p53* mutations allow accumulation of further genetic alterations that promote tumor development and progression.

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