# MALT LYMPHOMA: t(11;18), t(1;14) AND t(14;18) AND THEIR ROLE IN PATHOGENESIS, DIAGNOSIS, PROGNOSIS AND TREATMENT

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


By

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**December 2004**

## Declaration

This thesis represents the work of the author unless indicated. Some of the data, which are contributed by my colleagues and are very much relevant to my own works, are included in the thesis in order to help interpretation of the overall results. The data contributed by my colleagues are indicated in the thesis where appropriate.

*Dedicated to*

*my wife Qian and my son Shen*

with love

## Abstract

MALT lymphoma is specifically associated with t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21). t(11;18)(q21;q21) fuses the N-terminal of the *API2* gene to the C-terminal of the *MALT1* gene and generates a functional API2-MALT1 fusion product. t(1;14)(p22;q32) and t(14;18)(q32;q21) bring the *BCL10* and *MALT1* gene respectively to the *IgH* locus and deregulate their expression. The oncogenic activity of the three independent chromosomal translocations is linked by the physiological role of BCL10 and MALT1 in antigen receptor mediated NFκB activation. In this thesis, immunohistochemical and molecular genetic methods have been developed and used to investigate the frequency of these translocations in MALT lymphomas of various sites and their role in the pathogenesis, diagnosis, prognosis and treatment of this malignancy.

t(11;18)(q21;q21) was detected by RT-PCR of the API2-MALT1 fusion transcript and was found most frequently in MALT lymphomas from the lung (38%) and stomach (24%), moderately in those from the ocular adnexae (16%), but rarely in those from the salivary gland (1%), thyroid, skin, and other rare sites. The translocation was not seen in the preceding diseases associated with the development of MALT lymphoma including *H. pylori* associated gastritis, lymphoepithelial sialadenitis and Hashimoto's thyroiditis, nor in other subtypes of non-Hodgkin's lymphomas. In gastric MALT lymphoma, t(11;18)(q21;q21) was significantly associated with advanced cases and was a reliable marker for those not responding to *H. pylori* eradication including cases at stage IE.

t(1;14)(p22;q32) positive MALT lymphoma was characterised by strong BCL10 nuclear expression in contrast to the cytoplasmic expression of the protein in normal B-cells. Moderate BCL10 nuclear expression was seen in t(1;14)(p22;q32) negative MALT lymphomas including all those with t(11;18)(q21;q21) and up to 20% of cases without t(11;18)(q21;q21). Based on BCL10 immunohistochemstry followed by interphase FISH, t(1;14)(p22;q32) was found in MALT lymphomas from the lung (12%) and stomach (5%), but not in those from the ocular adnexae, salivary gland, thyroid and skin. In gastric MALT lymphoma, t(1;14)(p22;q32) positive cases did not respond to *H. pylori* eradication.

t(14;18)(q32;q21) positive MALT lymphoma was characterised by strong homogeneous cytoplasmic expression of both MALT1 and BCL10. In MALT lymphomas without t(14;18)(q32;q21) including those with t(1;14)(p22;q32) or t(11;18)(q21;q21), MALT1 expression was generally weak or negative. Based on MALT1 and BCL10 immunohistochemistry followed by interphase FISH, t(14;18)(q32;q21) was found in MALT lymphomas of the lung (6%), ocular adnexae (7%) and liver (17%) but not in those from the stomach, salivary gland, thyroid and skin.

t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21) were variably involved in MALT lymphoma of different sites. Detection of these translocations has significant implications in diagnosis and prognosis of MALT lymphoma.

**Acknowledgements**

I would like to express my most sincere gratitude to Professor. Ming-Qing Du for his excellent supervision and incredible and endless support in all aspects of this study.

I would also like to thank Professor Ahmet Dogan for his supervision. I appreciate his continuous encouragement during the past 5 years.

I am grateful to Dr Hongxiang Liu for establishing the RT-PCR method for the translocation detecting of t(11;18) from both frozen and formalin-fixed and paraffin-embedded tissues, screening in a proportion of MALT lymphoma cases as well as his contribution to most of the sequencing work. I am also grateful to him for his encouragement and critical reading of the thesis.

I am grateful to Mr. Rifat A. Hamoudi for computing and some of sequencing analyses and Dr Liping Gong for performing all real-time RT-PCR.

I am grateful to Dr. Langxing Pan who provided me with the initial opportunity of working in Royal Free and University College Medical School.

I am also grateful to Dr Keith Miller and Phillipa Munson for their advice and technical support in immunohistochemistry.

I would like to thank all collaborators for providing the MALT lymphoma specimens.

I would like to thank Professor Peter G Isaacson for his supervision of Histopathology.

I would also like to thank all the staff of the Department of Histopathology at Royal Free and University College Medical School, University of London, chaired by Professor Peter G Isaacson, for supporting me during this PhD study.

I would, last but not least, like to thank my wife Qian and my son Shen for their infinite patience and constant support during the work.

The studies presented in this thesis were supported by the Cancer Research Campaign and the Leukaemia Research Fund, allowing me to work in the Department of Histopathology, Royal Free and University College Medical School as a Research Fellow and senior Research Fellow for the past 5 years.

**Publications Arising from This Thesis**

1. Ye H, Gong L, Liu H, Hamoudi RA, Shirali S, Ho L, Chott A, Streubel B, Siebert R, Gesk S, Martin-Subero JI, Radford JA, Banerjee S, Nicholson AG, Ranaldi R, Remstein ED, Gao ZF, Zheng J, Isaacson PG, Dogan A, Du MQ: MALT lymphoma with t(14;18)(q32;q21)/*IGH-MALT1* is characterised by strong cytoplasmic MALT1 and BCL10 expression. Journal of Pathology 2005;205:293-301.

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* contributed equally to this work.

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## Abbreviations

| | |
|---|---|
| ALPS I | Auto-immune lymphoproliferative syndrome type I |
| ATM | Ataxia-telangiectasia mutated |
| ATR | Ataxia-telangiectasia and rad3-related |
| *BabA* | Blood group antigen binding adhesin A |
| BCL2 | B cell leukemia-lymphoma-2 |
| BCL6 | B cell leukemia-lymphoma-2 |
| BCL10 | B cell leukemia-lymphoma-10 |
| BCR | B cell receptor |
| BIR | Baculoviral IAP repeat |
| Bp | Base pair |
| CagA | Cytotoxic associated gene A of *H. pylori* |
| CagE | Cytotoxic associated gene E of *H. pylori* |
| CARD | Caspase recruitment domain |
| CARMA1 | CARD, membrane-associated guanylate kinase, MAGUK, protein 1 |
| CCL | Centrocyte-like cell |
| Cdk | Cyclin-dependent-kinase |
| CDR | Complementarity determining region |
| CGH | Comprehensive geneomic hybridization |
| Chk1 | Checkpoint kinases-1 |
| Chk2 | Checkpoint kinases-2 |
| CLL | Chronic lymphocytic leukaemia |
| DAB | 3,3-Diaminobezidine tetrahydrochloride |
| DD | Death domain |
| DEME | Dulbecco's modified Eagle's medium |
| DLBCL | Diffuse large B-cell lymphoma |
| ECACC | European Collection of Animal Cell Cultures |
| ELISA | Enzyme linked immunosorbent assay |
| FCC | Follicle centre cell |
| FCS | Fetal calf serum |
| FDC | Follicular dendritic cell |
| G6PD | Glucose-6-phosphate dehydrogenase |
| GADD45 | Growth arrest and DNA damage 45 |
| GC | Germinal center |
| HAT | Hypoxanthine-aminopterin-thymidine |
| HBSS | Hank's balanced salts solution |
| HLs | Hodgkin's lymphomas |
| *hopQ* | Hypothetical protein |
| *H. pylori* | Helicobacter pylori |
| IAPs | Inhibitors of apoptosis proteins |
| IB | Immunoblotting |
| *IceA* | Induced by contact with epithelium A |
| IELSG | International Extranodal Lymphoma Study Group |
| *Ig* | Immunoglobulin |
| *IgH* | Immunoglobulin heavy chain gene |

| | |
|---|---|
| Ig-L | Ig-like C2 domain |
| IHC | Immunohistochemistry |
| IKKγ | IκB kinase-γ |
| IL-1 | Interleukin-1 |
| IL-2 | Interleukin-2 |
| IL-18 | Interleukin-1 |
| ILSG | International lymphoma study group |
| IPSID | Immunoproliferative small intestinal disease |
| LELs | Lymphoepithelial lesions |
| LOH | Loss of heterozygosity |
| MAdCAM-1 | Mucosa addressin cell adhesion molecule |
| MALT | Mucosa-associated lymphoid tissue |
| MESA | Myoepithelial sialadenititis |
| MSI | Microsatellite instability |
| MZ | Marginal zone |
| NEMO | NFκB essential modulator |
| NF-κB | Nuclear factor kappa B |
| NHLs | Non-Hodgkin's lymphomas |
| NSO | Mouse myeloma cells |
| *OipA* | Out inflammatory protein |
| PAGE | Polyacrylamide gel electrophoresis |
| PAI | Pathogenicity island |
| REAL | Revised European-American Lymphoma classification |
| ROS | Reactive oxygen species |
| RT-PCR | Revised transcript - polymerase chain reaction |
| SDS | Sodium dodecyl sulphate |
| TCR | T cell receptor |
| TNFRII | Tumour necrosis factor receptor II |
| TRAF6 | Tumour-necrosis factor receptor associated factor 6 |
| V | Volt |
| *VacA* | Vacuolating cytotoxin A |
| VDJ | Variable region (V), diversity region (D), and joining region (J) |
| VH | *IgH* gene variable region |

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# Chapter 1. General introduction

Lymphomas are neoplastic proliferations of B or T lymphocytes or their precursors, which account for 3-5% of all malignancies diagnosed[1]. Approximately 60% of lymphomas are Non-Hodgkin's lymphomas (NHLs), while the remaining 40% are Hodgkin's lymphomas (HLs). Unlike HLs that rarely arise outside lymph nodes, about 60% of NHLs arise in the lymph nodes and the remaining 40% arise from extranodal sites[1]. The most common site of extranodal lymphoma is the gastrointestinal tract followed by the skin, orbit, lung, thyroid, and salivary gland. These extranodal lymphomas, although broadly similar, behave somewhat differently from nodal lymphomas.

## 1.1 Histology of lymph nodes and mucosa-associated lymphoid tissue (MALT)

### 1.1.1 Lymph nodes

The anatomical distribution and structure of lymph nodes are adapted to deal with antigens carried to the node in the afferent lymphatics that drain sites at various distances from the node. The lymph nodes are situated at intervals along the lymphatics throughout the body, with well-recognised concentrations at certain sites, such as axilla, cervical region, groin, etc. Their position is in accordance with their function as a major part of the body's defence. In other words, lymph nodes are strategically placed to perform the function of immune surveillance.

There are three different cell groups in the lymph node. These are the B cells, T cells and accessory cells. Most of the lymph node B cells are present in the cortex, where they are concentrated in follicles. In unstimulated or resting lymph nodes, the follicles, known as primary follicles, are inconspicuous and consist of aggregates of small lymphocytes without a centre. In reactive nodes, however, the follicles, known as secondary follicles, have prominent structure consisting of a follicle centre and a zone of small lymphocytes, known as the mantle zone, partially surrounding the follicle centre. The follicle centre is populated principally with two types of B cells, the centroblast and centrocyte, and also contains macrophages and follicular dendritic cells (FDC). The lymphoid tissue between the B cell follicles and impinging on the medulla is known as the paracortex and it is here that the T cells are found[2].

### 1.1.2 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[3].

Besides lamina propria lymphocytes and the intra-epithelial lymphocytes, the most prominent structural component of MALT is the organised nodular lymphoid tissue that occurs in the terminal ileum as Peyer's patches[4]. Peyer's patches are characterised by the lymphoid follicle, which is composed of a follicle centre, a thin mantle zone and a marginal zone (Figure 1.1)[1,5]. The marginal zone represents one of the distinct compartments of the B cell area in lymphoid tissues. It is especially well developed in the white pulp of the spleen and in Peyer's patches of the gut, but not in lymph nodes, with the exception of the ones in the mesenterium[6,7]. The marginal zone comprises a

particular subset of lymphoid cells, which are characterised by abundant clear cytoplasm and a pale, irregular, centrally located nucleus[8]. Due to the resemblance to follicle centre cells, marginal zone B cells have been indicated as 'centrocyte-like (CCL) cells'. On the luminal side of the follicle, the marginal zone merges into a mixed cell infiltrate that covers the dome of the Peyer's patches. In the Peyer's patches, the mixed cell infiltrate of the dome region is composed of plasma cells, dendritic cells, macrophages, small lymphocytes and CCL cells. The CCL 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[1,5,9,10].

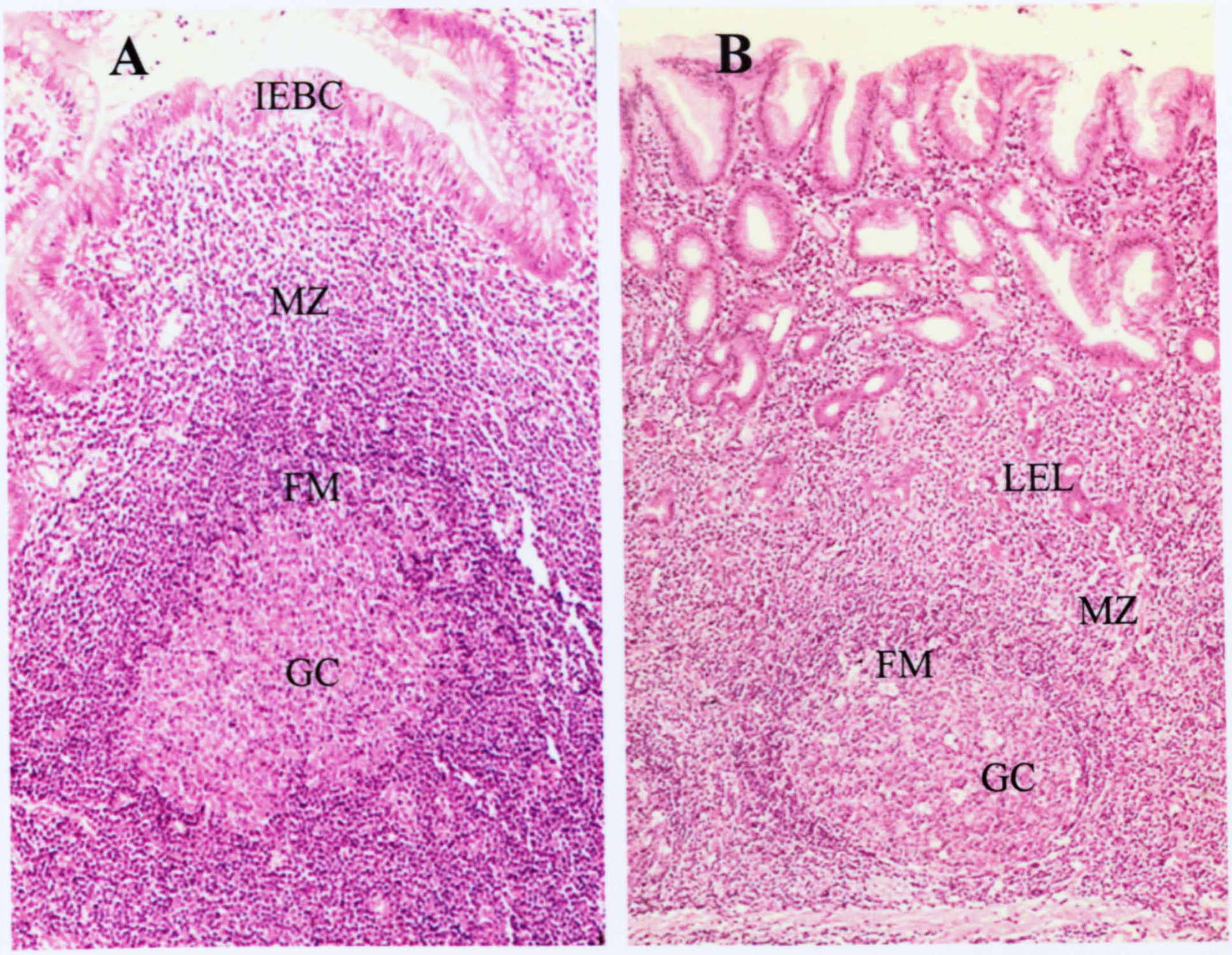



**Figure 1.1. MALT, Peyer's patches and MALT lymphoma A: Peyer's Patch**, a germinal centre (GC) is surrounded by an inner follicular mantle (FM), and an outer marginal zone (MZ). IEBC indicates intraepithelial B cells. **B: MALT lymphoma,** histological features are strikingly similar to those of the Peyer's patch, comprising a reactive B cell follicle surrounded by neoplastic marginal zone B cells that invade gastric gland epithelium to form lymphoepithelial lesions (LELs).

Unlike peripheral lymph nodes that 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 are transported to the mucosal organised lymphoid tissues where naïve B cells are stimulated to undergo germinal centre reaction and become memory B cells or plasma cells (detailed in the next section)[1,5]. These antigen-experienced B cells are programmed preferentially for mucosal immune defense. They may leave mucosa and enter circulation via the mesenteric lymph nodes and thoracic duct, then home back to the mucosa.

## 1.2 B cell development

Normal B cell differentiation begins with precursor B lymphoblasts (blast cells that are the precursors of the entire B cell lineage) that undergo immunoglobulin (Ig) variable region (*V*), diversity region (*D*) and joining region (*J*) (*VDJ*) gene rearrangement and differentiate into mature surface Ig (sIg) positive (IgM+ IgD+) naïve B cells that are often CD5+[11]. Naïve B cells are small resting lymphocytes that circulate in the blood and also occupy primary lymphoid follicles and follicular mantle zones[11,12]. On encountering antigen, naïve B cells migrate into the centre of a primary follicle and transform into blasts (centroblasts) and actively proliferate, forming a germinal centre[13,14]. The proliferating centroblasts occupy the dark zone and undergo somatic hypermutations in their rearranged *Ig* gene. This results in marked intraclonal diversity in a population of cells derived from only a few precursors, a characteristic feature of germinal centre B cells. The B cell progeny bearing mutated *Ig* gene move to the light zone, where they interact with antigen presented by FDC through their surface Ig. Those expressing surface Ig with high affinity to antigen are rescued by expression of BCL2 and selected to undergo Ig class switch from IgM to IgG or IgA. They exit the germinal centre as antibody secreting plasma cells or memory B cells, while those expressing surface Ig without high affinity to antigen die in situ by apoptosis. These apoptotic cells are cleared by macrophages, histologically characterised by tingible body macrophages.

The B cell maturation during the germinal centre reaction is a highly regulated complex process that is T cell dependent. Deregulation of the molecules that control the germinal centre reaction, such as BCL2, BCL6, plays an important role in lymphomagenesis.

## 1.3 MALT lymphoma

### 1.3.1 The MALT lymphoma concept

The concept that MALT lymphoma represents a distinct entity was proposed by Isaacson and Wright in 1983[15] based on clinico-pathological studies of low grade B cell lymphoma of the stomach and immunoproliferative small intestinal disease (IPSID). Clinically, both low grade B cell lymphoma of the stomach and IPSID are remarkably indolent and respond favorably to treatment. Histologically, these lymphomas recapitulate the morphology of Peyer's patches rather than lymph node[16]. Subsequently, the MALT lymphoma concept extends to include low grade B cell lymphomas derived from the lung[17], salivary gland[18], thyroid[19], ocular adnexae[20,21], and skin as they share similar clinical and histological features. Interestingly, these mucosal organs are normally devoid of any organised lymphoid tissue. However MALT can be acquired as a result of chronic inflammatory or autoimmune disorder. It is believed that MALT lymphoma originates from the acquired MALT[1].

The MALT lymphoma concept proposed entirely on the base of clinico-pathological observations is firmly supported by subsequent immunophenotypic and molecular studies. MALT lymphoma as a specific pathological entity was first included in the REAL classification in 1994[22]. In 2001, it is classified as extranodal marginal zone B cell lymphoma of MALT in the WHO Classification of Neoplastic Diseases of the Hematopoietic and Lymphoid Tissues[23].

### 1.3.2 MALT lymphoma and its preceding diseases

MALT lymphoma is the third most common NHLs, comprising about 40% of those arising from the extranodal sites[24]. The stomach is the most common site of MALT lymphoma, accounting for 30-50% of cases. Other common sites include lung (14%), salivary gland and thyroid (14%), ocular adnexae (12%), and skin (11%)[25].

MALT lymphoma is usually preceded by chronic inflammation or autoimmune disorder, such as *Helicobactor pylori* (*H. pylori)* associated chronic gastritis[26], Hashimoto's thyroiditis[19], Sjögren's syndrome / myoepithelial sialadenitis (MESA)[18], follicular bronchiolitis[27]. In addition, cutaneous marginal zone B cell lymphoma is linked to *Borrelia burgdoferi*infection[28,29], while IPSID and ocular adnexal MALT lymphoma has been recently shown to bo associated with *Campylobacter jejuni*[30] and *Chlamydia psittaci* infection in some cases[31].

Gastric MALT lymphoma occurs usually over the age of 50, with a peak in the 7th decade, although an increasing number of cases are being reported in patients at an earlier age. The male-to-female ratio is approximately 1.5:1. The clinical presentation is usually more suggestive of a chronic inflammatory process than a lymphoma and naturally varies with the site of the disease. The symptoms of gastric MALT lymphoma are usually those of chronic gastritis. Endoscopic findings are also usually not specific, and are those of gastritis and / or peptic ulcer.

### 1.3.3 Histopathology

All MALT lymphomas from different sites are similar, recapitulating the morphological features of the Peyer's patches of the terminal ileum. The histopathological features can be summarised as follows (Figure 1-1).

1. The MALT lymphoma cells are characterised by small to medium-sized, slightly irregular nuclei with moderately dispersed chromatin and inconspicuous nucleoli, resembling those of centrocytes, thus frequently referred as CCL cells. However, they are morphologically and immunophenotypically almost identical to the marginal zone B cells (Figure 1.1 and Table 1.1), from which MALT lymphoma is thought to arise[16].

2. CCL lymphoma cells infiltrate around reactive B cell follicles, external to a preserved follicular mantle, in a marginal zone distribution, and spread diffusely into the surrounding mucosa. They invade and destroy the epithelial lining of local glands, ducts or crypts, resulting in the so-called lymphoepithelial lesions (LELs), a characteristic feature of MALT lymphoma. LELs are aggregates of three or more neoplastic cells with distortion or destruction of the epithelium, often accompanied by eosinophilic degeneration of epithelial cells[32].

3. Plasma cell differentiation is present in approximately 30% of MALT lymphomas and tends to be maximal beneath the surface epithelium[1,25]. At the one extreme, there may be moderate numbers of plasma cells and demonstration of Ig light chain restriction by immuohistochemistry is necessary to determine whether they are a part of the

neoplastic clone. At the other extreme, plasma cell differentiation may be a striking feature, as seen in IPSID.

4. Residual reactive B cell follicles are a constant finding. They may either be naked or surrounded by a complete or partial lymphocytic corona, whereby the neoplastic proliferation forms a marginal zone pattern. Reactive germinal centres may be invaded or overrun by the neoplastic proliferation, known as 'follicular colonization'[33,34]. Residual germinal centres may become difficult to recognise and this can lead to a close resemblance to follicular lymphoma. There are three types of follicular colonization. Type I: reactive follicles are over-run and virtually replaced by CCL cells, resulting in confluent but poorly defined follicles in which fragmented residues of follicle centre cells (FCC) and small, darkly stained mantle zone cells are dispersed. Type II: follicle centres are selectively wholly or partialy replaced by CCL cells, resulting in expansion of the follicle centres massively, and loss of the characteristic zonal pattern of reactive follicle centres. Type III: the intrafollicular CCL cells show plasma cell differentiation.

5. Transformed centroblast- or immunoblast-like cells may be present in variable numbers in MALT lymphoma but when sheets of transformed cells are present the tumour should be diagnosed as diffuse large B cell lymphoma (DLBCL) and the presence of accompanying MALT lymphoma should be noted (section 1.4.3.5. of Chapter 1).

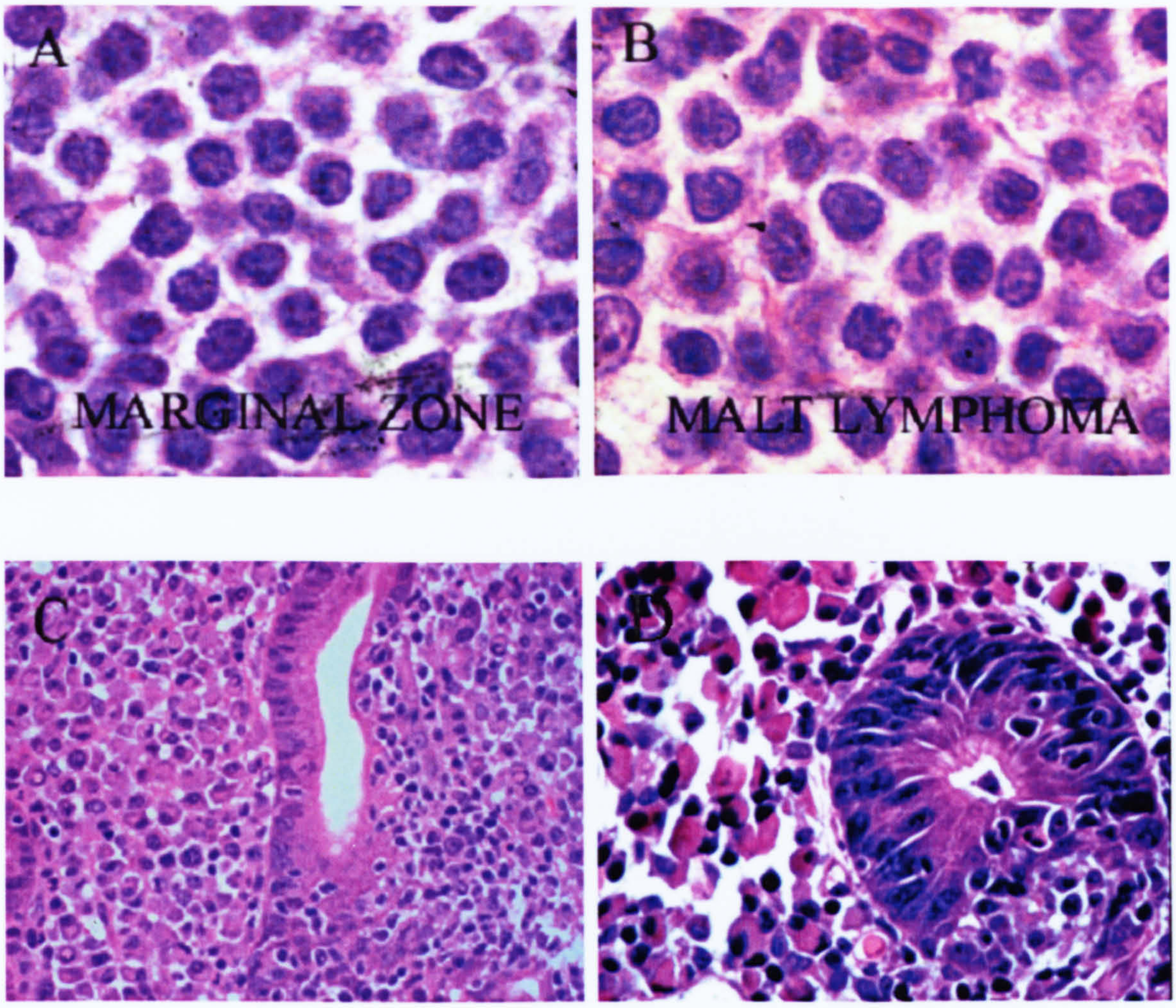



**Figure 1.2. Morphology of marginal zone B cells and MALT lymphoma A & B:** MALT lymphoma cells closely resemble marginal zone B cells. **C:** High magnification of a lymphoepithelial lesion showing infiltration and destruction of gastric gland epithelium by centrocyte-like cells. **D:** Gastric MALT lymphoma showing marked plasma cell differentiation. The plasma cells are distended with eosinophilic Ig.

### 1.3.3.1 Immunophenotypic features

The immunophenotype of MALT lymphoma is almost identical to that of normal marginal zone B cells[16]. Tumour cells typically express IgM, and less often IgA or IgG. The tumour cells of MALT lymphoma are CD20+, CD19+, CD79a+, CD5-, CD23-, CD10-, cyclin D1-, CD43+/-, CD11c+/-. BCL2 is positive in most cases. IgD expression may be variable[35](Table 1.1). There is no specific marker for MALT lymphoma at present. A recent study showed that IRTA1 (immunoglobulin superfamily

receptor translocation-associated 1) was selectively and consistently expressed by B-cells beneath and within the dome epithelium of the Peyer patches and tumour cells involved in LELs in MALT lymphoma[36]. However, whether IRTA1 can serve as a diagnostic marker for MALT lymphoma remains unknown.

**Table 1.1. Comparison of immunophenotypic and genotypic features of MALT lymphoma and normal marginal zone B cell**

| | Immunophenotype | | | | | | |
|---|---|---|---|---|---|---|---|
| | CD5 | CD10 | CD23 | CD43 | Cyclin D1 | IgD | IgM |
| MALT Lymphoma | - | - | - | +/- | - | +/- | + |
| Marginal Zone B Cells | - | - | - | - | - | - | + |

### 1.3.3.2 Genotypic features

Molecular investigations of MALT lymphoma have demonstrated that the lymphoma cells have clonally rearranged *Ig* heavy and light chain genes[37]. Their rearranged *Ig* genes show high levels of somatic mutations with features of antigen selection: high replacement mutation versus silent mutation in complementarity determining region (CDR) regions, consistent with their memory B cell phenotype.

### 1.3.3.3 Normal cell counterpart of MALT lymphoma

Several strands of evidence strongly indicate that MALT lymphoma originates from the marginal zone B cells of the acquired MALT. First, MALT lymphoma cells have a similar morphology to marginal zone B cells[1,16]. The lymphoma cells are distributed in marginal pattern. When they spread, they also preferentially occupy the marginal zone

of lymph node and spleen. Second, MALT lymphoma cells are immunophenotypically similar to marginal zone B cells (section 1.4.3.1). Finally, the rearranged *Ig* gene of MALT lymphoma shows similar frequencies and features of somatic mutations to those of the marginal zone B cells of the Peyer's patches and acquired MALT[38-40].

### 1.3.3.4 Multifocality and dissemination pattern

Histological examinations of surgical specimens of gastric MALT lymphoma showed multifocal microlymphomas with identical Ig light chain restriction scattered throughout the gastric mucosa[41]. A subsequent study by sequence analysis of the rearranged *Ig* heavy chain gene (*IGH*) confirmed the identity of different microlymphomatous lesions[42]. Further study by microdissection and clone-specific PCR demonstrated the presence of tumour cells in reactive lymphoid tissues without any histological evidence of the lymphoma[43]. A wide dissemination within gastric mucosa has also been well documented by Fend et al[44] and Hoshida et al[45].

MALT lymphomas generally remain localised for prolonged periods. However, regional lymph nodes may be involved by MALT lymphoma[1]. MALT lymphoma cells show a tendency to involve marginal zone when they disseminate to lymph nodes. Gastric MALT lymphoma has been shown preferentially to disseminate to the marginal zone of the spleen[46].

When remote spread occurs, gastric MALT lymphoma preferentially migrates to other mucosal sites such as the small intestine and salivary gland[46-48]. MALT lymphoma

rarely involves peripheral lymph nodes, but disseminates to the bone marrow in 5-10% of cases[49].

The propensity of gastric MALT lymphoma to disseminate to other parts of the gastrointestinal mucosa and splenic marginal zone is thought partially to reflect the homing properties of the tumour cells. Dogan et al[50] reported a case of gastric MALT lymphoma with secondary intestinal involvement and found that the mucosal homing receptor $\alpha4\beta7$ integrin was strongly expressed by the intestinal tumour but not by the gastric lesion. However, a high level of $\alpha4\beta7$ expression could be induced in gastric lymphoma cells following activation by a *H. pylori* generated T cell response. This suggests that $\alpha4\beta7$+ tumour cells could be similarly generated in gastric lymphoma *in vivo* and thus become 'programmed' to home to an appropriate microenvironment where the mucosal addressin cell adhesion molecule (MAdCAM-1), the ligand for $\alpha4\beta7$, is expressed.

### 1.3.3.5 High grade transformation

Transformed centroblasts or immunoblast-like cells are frequently seen in MALT lymphoma. These large cells may be scatter or form clusters or sheets, presenting as a DLBCL. The observation of the coexistence of low and high grade components in the same primary gastric lymphoma led to the assumption that high grade tumour evolves directly from low grade disease. However, the low and high grade lesions may not be always clonally related[51,52]. Chan et al examined a series of gastric MALT lymphomas and showed identical Ig light chain restriction in both low and high grade tumour components of the same case[53]. Peng et al and others further provided the genetic link

between low and high grade tumour components by microdissection and molecular analysis of the rearranged *Ig* genes[53-55]. It is believed that in cases with coexistence of MALT lymphoma and DLBCL, the DLBCL is most likely transformed from the low grade disease. However, in cases where only DLBCL is seen, it is a debate whether DLBCL originates from a MALT lymphoma or develops *de novo*. Given the fact that DLBCL frequently overruns the MALT lymphoma, it is believed that at least a proportion of DLBCL in the mocosal sites are transformed from an undetected MALT lymphoma.

De Jong et al[56] suggested dividing gastric MALT lymphoma into four categories. Category A refers to classical low grade MALT lymphoma in which transformed blasts comprise no more than 5% of cells and do not occur in clusters of more than ten cells. In category B, transformed cells may account for 10-20% of cells and occur in clusters of up to 20 cells. Category C is characterised by unequivocal high grade transformation with sheets of transformed cells that may leave only small foci of low grade lymphoma. In category D, no MALT lymphoma component is detectable and it is probably better classified as DLBCL. The classification of gastric MALT lymphoma is clinically relevant. Approximately, 90% of tumours of category A have a 10-year survival. The prognosis is slightly but significantly poorer in category B cases with a 10-year survival of approximately 78%. The survival for high grade cases is significantly worse but, interestingly, there was no difference in the clinical outcome between categories C and D where the 10-year survival for both was approximately 45%. Similarly, in a large series reported by Cogliatti et al, there was no significant difference in the survival between transformed MALT and *de novo* DLBCL[57].

### 1.3.3.6 Differential diagnosis

The differential diagnosis of MALT lymphoma includes *H. pylori* associated gastritis and other small B cell lymphomas such as follicular lymphoma, mantle cell lymphoma and lymphoplasmacytic lymphoma.

In severe *H. pylori*-associated chronic gastritis, sometimes known as follicular gastritis, the appearance can closely simulate lymphoma, making histological diagnosis difficult, especially based on small biopsies. Wotherspoon and Isaacson introduced a histological scoring system to help the differential diagnosis[26]. Follicular lymphoma, mantle cell lymphoma and small lymphocytic lymphoma very rarely occur as primary tumour of the stomach. Nonetheless, secondary gastric involvement by these lymphomas is relatively common. Distinction from these small B cell lymphomas is based on a combination of the characteristic morphologic, immunophenotypic and genotypic features as outlined in Table 1.2.

**Table 1.2. Comparison of immunophenotypic and genotypic features between MALT lymphoma and other small B cell lymphomas**

| | Immunophenotype | | | | | | | | | Genotype | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lymphoma subtypes** | **BCL2** | **BCL6** | **CD5** | **CD10** | **CD23** | **CD43** | **Cyclin-D1** | **IgD** | **IgM** | **Ig mutation** | **Translocation** |
| MALT Lymphoma | + | - | -/+ | - | - | +/- | - | -/+ | + | + | t(11;18); t(1;14); t(14;18)/MALT1 |
| Follicular Lymphoma | + | + | - | + | +/- | - | - | -/+ | + | + | t(14;18)/BCL2 |
| Mantle Cell Lymphoma | + | - | + | - | - | + | + | + | + | - | t(11;14) |
| Lymphoplasmacytic Lymphoma | + | - | - | - | - | +/- | - | - | + | + | t(9;14) |

## 1.4 The pathogenesis of MALT lymphoma

The development of MALT lymphoma is a multistage process. This is best understood in gastric MALT lymphoma since it is the most common and has been extensively studied among MALT lymphoma of different sites. The development of gastric MALT lymphoma involves not only acquisition of genetic abnormalities but also immunological stimulations mediated by *H. pylori* infection.

### 1.4.1 *H. pylori* infection and its role in development of gastric MALT lymphoma

*H. pylori* is an unipolar, multiflagellate spiral shaped, microaerophilic, Gram negative bacterium that lives in the luminal surface of the stomach[58,59]. The bacterium is implicated in several gastrointestinal diseases, such as peptic ulcer, gastric adenocarcinoma and MALT lymphoma[60].

In 1988, it was recognised that chronic *H. pylori* infection induced lymphoid infiltrate in the gastric mucosa[61,62], which gradually formed the acquired MALT. In 1991, Wotherspoon et al studied a large series of gastric MALT lymphoma and demonstrated *H. pylori* infection in the vast majority of cases[26]. This is confirmed by subsequent studies and further underlined by an epidemiological study in northeastern Italy, where a markedly high incidence of gastric MALT lymphoma is accompanied by a high prevalence of *H. pylori* infection[63]. Furthermore, in a case control study, Parsonnet et al showed a significant association between previous *H. pylori* infection and the development of gastric MALT lymphoma[64]. In addition to *H. pylori*, *H. heilmannii*

infection is also found to be associated with the development of gastric MALT lymphoma albeit its infection is infrequent[65].

The precise role of *H. pylori* in the development of gastric MALT lymphoma is not totally clear. Nonetheless, there is compelling evidence indicating that both the host responses to *H. pylori* infection and bacterial status play a critical role in the pathogenesis of gastric MALT lymphoma.

### 1.4.2 Immunological stimulation

Several histological features of MALT lymphoma, which include the presence of plasma-cell differentiation, blasts, follicular colonization and its association with active tumour cell proliferation[33,34], suggest that MALT lymphoma cells preserve B cell properties and that their growth may be partially driven by antigenic stimulation via surface receptors. Recent studies indicate that both direct and indirect antigen stimulation mechanisms are involved.

#### 1.4.2.1 Direct antigen stimulation

MALT lymphoma cells invariably express surface Ig. Using anti-idiotype antibody to mimick antigen stimulation, Hussell et al showed that MALT lymphoma cells actively proliferated *in vitro*[66], suggesting that tumour cells may receive antigenic stimulations through surface antigen receptor. This notion is reinforced by subsequent studies of somatic mutation of the rearranged *Ig* gene. Most of MALT lymphomas show high levels of somatic mutation in the *Ig* gene, with features of antigen selection[38]. In

addition, the majority of cases show evidence of intraclonal sequence variations, i.e. ongoing mutation[38,48,67].

Since somatic mutation occurs in the rearranged *Ig* gene only during the germinal centre reaction, and depends on antigen and T cells, the finding of ongoing *Ig* somatic mutation in MALT lymphoma suggests that tumour-cell growth is partially driven by direct antigen stimulation. There are some preliminary data suggesting that the rate of ongoing mutations gradually declines during the evolution from an early to a late phase of low grade tumour and that the activity finally disappears in the high grade lesions[68,69]. Thus, it is likely that the role of direct antigen stimulation in the pathogenesis of this tumour progressively decreases during tumour evolution.

The antigens recognised by the surface Ig of MALT lymphoma cells are not fully characterised. The data collected so far suggest that the tumour-derived Ig does not recognise *H. pylori* but react with various autoantigens[70]. Antibodies to gastric epithelial cells are commonly present in serum samples from patients with *H. pylori* gastritis[71]. An anti-idiotype antibody to Ig of a gastric MALT lymphoma cross-reacts with reactive B cells in *H. pylori*-associated gastritis[72]. These findings suggest that gastric MALT lymphoma cells are transformed from autoreactive B cells, which are induced by *H. pylori* infection. In line with these findings, the $V_H$ germline used by MALT lymphoma cells is frequent those employed by auto-antibodies[38,48].

### 1.4.2.2 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-culturing tumour cells with 13 clinical strains of heat-killed *H. pylori*, Hussell et al demonstrated that *H. pylori* induced tumour cells to proliferate[73]. This effect was associated with expression of interleukin-2 (IL-2) receptors and secretion of Ig by tumour cells. The effect was strain-specific but not due to specificity of lymphoma cells for *H. pylori* antigens. Removal of tumour-infiltrating T cells before the experiment abolished all the effects of *H. pylori* on tumour cells. Furthermore, the stimulating effect of *H. pylori* on tumour B cells could be completely blocked by an antibody to CD40L[73]. Thus, *H. pylori* stimulated lymphoma B cells through tumour-infiltrating T cells, involving CD40 and CD40L costimulatory molecules.

The active role of tumour-infiltrating T cells in the growth of tumour B cells is supported by a study of T cell clones isolated from gastric MALT lymphoma. T cell clones responding to *H. pylori* stimulation were CD4-positive helper cells rather than CD8-positive cytotoxic cells[74]. These specific T cell clones activated tumour B cells in a dose dependent manner[74].

The critical role of *H. pylori* mediated immunological stimulation in the growth of gastric MALT lymphoma cells is further supported by the demonstration of complete tumour regression following *H. pylori* eradication. It is believed that eradication of *H. pylori* leads to disappearance of *H. pylori* specific tumour infiltrating T cells, consequently removing the critical growth support to the tumour B cells.

Unlike low grade tumour cells, transformed MALT lymphoma cells appeared to be unresponsive to *H. pylori* mediated T cell stimulation *in vitro*[66]. However, this observation is only based on study of a single case. More recent studies showed that some of transformed gastric MALT lymphomas were also responsive to *H. pylori* eradication[75-80], suggesting that *H. pylori* mediated immune response may also play a role in the growth of transformed MALT lymphomas.

### 1.4.3 *H. pylori* virulent factors and host responses to *H. pylori* infection in development of gastric MALT lymphoma

#### 1.4.3.1 *H. pylori* virulent factors

The incidence of *H. pylori* associated chronic gastritis is high, occurring in 50% - 90% of population depending on geographic variations[81,82]. Analysis of surgical specimens of gastric MALT lymphoma showed *H. pylori* associated chronic gastritis in 92% - 98.3%[26,83]. *H. pylori* virulent factors are highly associated with peptic ulcer and gastric cancer compared with gastritis[84,85].

There are a number of *H. pylori* virulent factors that are associated with enhanced pathogenicity of the bacterium. They include the *H. pylori* cytotoxin and *Cag* pathogenicity island (PAI) such as cytotoxin-associated gene A (*CagA*), *CagE*, vacuolating cytotoxin A *(vacA)*, induced by contact with epithelium A *(iceA)*, blood group antigen binding adhesin A *(babA)*, out inflammatory protein A *(oipA)*, and hypothetical protein Q *(hopQ)*[86].

These virulent factors have been extensively studied in peptic ulcers and gastric cancer. *Cag* PAI has been shown to be significantly associated with peptic ulcers and gastric cancer as compared with *H. pylori* associated chronic gastritis. However, it is unclear whether these virulent factors are associated with gastric MALT lymphoma. Several controversial results have been published. De Jong et al examined the primary *H. pylori* cultures using a PCR assay for the Cag*A* gene and showed that gastric MALT lymphoma was not associated with the *CagA* positive strain[87], while Eck et al observed a significant association using a serological test[88]. In a subsequent study, Peng et al examined the gastric biopsies using a PCR assay for the Cag*A* gene and demonstrated that the *CagA* positive strain of *H. pylori* was significantly associated with DLBCL but not MALT lymphomas of the stomach, as compared with *H. pylori* gastritis[89]. A recent study by Lehours et al investigated seven *H. pylori* virulence factors including *CagA*, *CagE*, *vacA*, *iceA*, *babA* and *hopQ* and two adhesins (*sabA* and *hopZ*) in 43 *H. pylori* strains isolated from patients with gastric MALT lymphoma and 39 strains from age-matched patients with gastritis alone. They found that only in patients infected with strains harbouring the *iceA1* allele, *sabA* functional status, and *hopZ* "off" status, the odds of developing a MALT lymphoma were 10 times higher. However, this triple association was only seen in 23% of gastric MALT lymphomas. The results remain to be validated in future studies. It also remains to be investigated whether there are other *H. pylori* virulence factors, which may be associated with MALT lymphoma development.

### 1.4.3.2 Host factors

*H. pylori* infection not only induces immune responses that stimulate the growth of infiltrating B and T cells, but also causes inflammatory responses that generate reactive oxygen species (ROS)[90]. ROS can cause a range of genetic damages, in particularly double strand break, which may lead to acquisition of genetic abnormalities critical to development of MALT lymphoma. The severity of DNA damage depends not only on the virulent factors of *H. pylori* but also the individual's ability to respond to *H. pylori* infection and detoxify ROS.

The immune response resulting from *H. pylori* infection is primarily via Th1, a proinflammatory response, mediated by a cascade of cytokines including interleukin 18 (IL-18), IL-2, IL-1, tumour necrosis factor-$\alpha$ and interferon-$\gamma$. These cytokines regulate their own levels of expression by autocrine effects, but they also influence downstream cytokine expression, thereby amplifying the inflammatory response. IL-1 is one of the principal proinflammatory cytokines that mediate the Th1 immune response following *H. pylori* infection, with IL-1$\beta$ production being up-regulated in the presence of *H. pylori*[91,92]. IL-1$\beta$ is also one of the most powerful inhibitors of gastric acid secretion, the hypochlorhydria associated with high-producer variants of IL-1 governing the extent of *H. pylori* infection and distribution of gastritis. As a consequence, polymorphic variants in IL-1 may affect not only the primary inflammatory response but also the level of response following amplification.

The data from two groups have shown an increased risk of gastric carcinoma associated with inheritance of the *IL-1B-31 (CC)/IL-1 RN 2/2* genotype[93-95]. The findings suggest

that the *IL-1B-31 C* allele in the context of *H. pylori* infection is associated with a proinflammatory phenotype and therefore an increased risk of DNA damage and ultimately contributing to gastric carcinoma development[93-95]. A recent study has demonstrated a significant association between gastric MALT lymphoma and inheritance of the *IL-1 RN 2/2* genotype[96], consistent with that previously finding in gastric carcinoma. However, they found no associated risk with *IL-1B-31* genotype. The study also showed that the glutathione S-transferase (GST) T1 null genotype was associated with risk of gastric MALT lymphoma. GST T1 has a strong antioxidant function in neutralising free radicals. These data support the hypothesis that the risk of developing gastric MALT lymphoma is influenced by interindividual variation in inflammatory response and antioxidative capacity. However, the study failed to include *H. pylori* associated gastritis as a control group and the significance of the finding remains to be investigated.

## 1.5 Genetic abnormalities

As discussed above, *H. pylori* infection induces acquisition of MALT in the gastric mucosa, a prerequisite for MALT lymphoma development, and its mediated immunological stimulation plays a direct role in the evolution of the transformed clone. On the other hand, *H. pylori* infection also promotes the acquisition of genetic abnormalities and malignant transformation of reactive B cells. *H. pylori* infection triggers inflammatory responses by attracting and activating neutrophils, which release ROS[90]. ROS is known to cause a range of genetic damage, particularly double strand break, thus may have a role in the acquisition of genetic abnormalities during MALT lymphoma development. There are a number of genetic abnormalities identified in

MALT lymphoma, including chromosomal translocations specifically associated with this lymphoma entity.

### 1.5.1 t(11;18)(q21;q21)/*API2-MALT1*

The t(11;18)(q21;q21) is the most frequent chromosomal translocation in MALT lymphomas[97-100]. Remarkably, the t(11;18)(q21;q21) is usually the sole chromosomal aberration. In contrast, t(11;18)(q21;q21) negative tumours show a wide range of abnormalities including trisomies 3, 12, 18. In 1999, Dierlamm et al[101], subsequently Akagi et al[102] and Morgan et al[103] showed that t(11;18)(q21;q21) causes a reciprocal fusion between *API2*, a known gene at 11q21and *MALT1*, a novel gene at 18q21. As the *API2-MALT1* fusion transcripts, but not the *MALT1-API2* fusion transcripts, were constantly expressed, the *API2-MALT1* fusion was thought to be most likely oncogenic.

Following identification of the genes involved in t(11;18)(q21;q21), RT-PCR and interphase FISH were developed for detection of the translocation[104,105]. These methods allow screening of large series of lymphoma cases. So far, studies from independent research groups showed that t(11;18)(q21;q21) occurred at variable frequencies in MALT lymphomas of various sites but not in closely related nodal or splenic marginal zone B cell lymphomas nor in other NHLs[104,106-111]. Nevertheless, it remains to be investigated: 1) whether the translocation occurs in the pre-malignant diseases associated with MALT lymphoma; 2) what the true incidence of the translocation in MALT lymphoma of different sites, particular those from sites other than stomach and lung is; 3) what the role of t(11;18)(q21;q21) in multistage development of gastric MALT

lymphoma, in particular with reference to staging and treatment responses to *H. pylori* eradication is.

### 1.5.1.1 The *API2* and *MALT1* genes: structure and function

The API2, together with API1, was originally identified as proteins recruited to the tumour necrosis factor receptor II (TNFRII) complex[112]. The *API2* gene, known as *c-IAP2*, *HIAP-1*, *MIHC*, belongs to a family of genes that encode inhibitors of apoptosis proteins (IAPs), which are characterised by a domain of about 70 amino acids termed BIR (baculoviral IAP repeat). It contains three BIR domains in its amino-terminus, a middle CARD (caspase recruitment domain) followed by a carboxy-terminal zinc binding RING finger domain (Figure 1.3)[113-115]. The BIR domains are important for inhibition of caspases and suppression of apoptosis[116] while the CARD has been shown to mediate protein-protein interactions[117]. The RING finger motif is involved in autoubiquitination and degradation of the API2 protein and has been shown to negatively regulate the function of the protein[113,118].

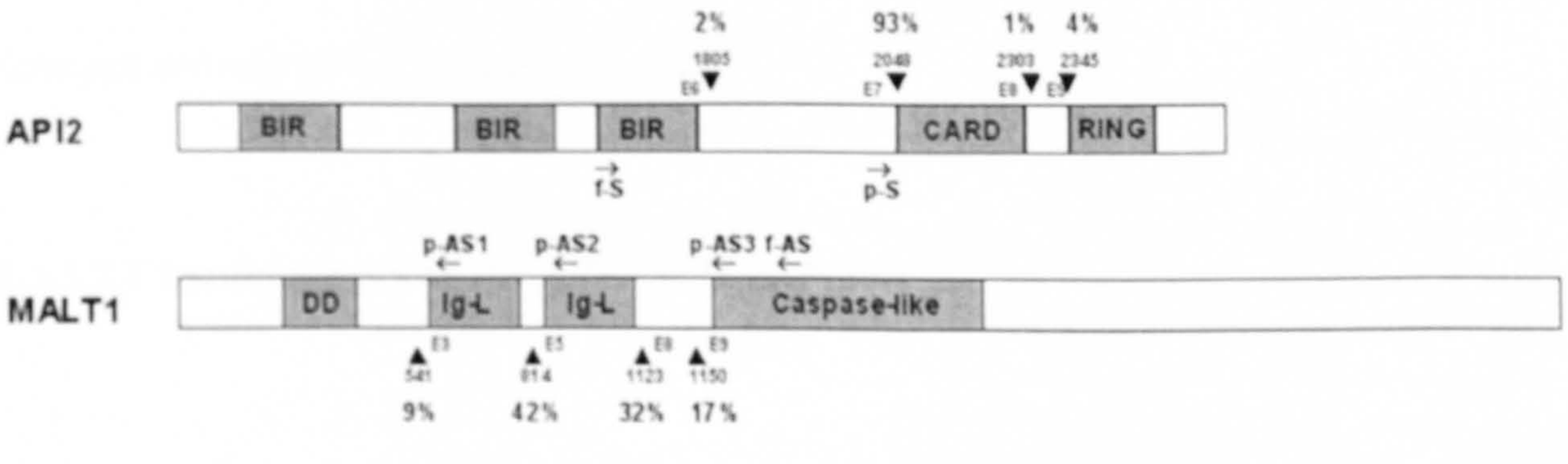



**Figure 1.3: Schematic presentation of *API2* and *MALT1* gene structure and primer positions.** Known breakpoints are indicated by *arrowheads*, and nucleic acids are numbered according to cDNA sequence of the *API2* (GenBank, NM_001165) and *MALT1* genes (AF130356). The frequency of individual known breakpoints was given[97,98,101-104,106-109,119]. *Arrows* indicate the position of primers used. f-S and f-AS, sense and anti-sense primer for PCR from frozen tissues; p-S and p-AS, sense and anti-sense primer for PCR from paraffin-embedded

tissues; BIR, baculovirus IAP repeat; CARD, caspase recruitment domain; DD, death domain; Ig-L, Ig-like C2 domain[104,110].

Wild type API2 is capable of inhibiting the biological activities of caspases 3, 7 and 9 in *in vitro* studies, and is thus believed to be an apoptosis inhibitor[114]. However, full length API2 did not protect cells from apoptosis induced by Bax over-expression and TNFα treatment[120]. Deletion of the C-terminal RING domain of API2 restores its antiapoptotic potential[120]. Engineered mutant IAPs that contain only BIR domains can bind and inhibit caspases, emphasising the importance of the BIR motifs in inhibiting apoptosis[121]. *API2* mRNA is highly expressed in lymphoid tissues (spleen and thymus)[122], indicating that it has a role in B and T cell function.

The *MALT1* gene, recently classed as a paracaspase, comprises an N-terminal death domain (DD), followed by two Ig-like C2 domains and a caspase-like domain (Figure 1.3)[123]. *MALT1* does not have classic activities of caspases[123]. *MALT1* knock out mice studies showed that MALT1 specifically links the antigen receptor signaling to NFκB activation pathway, and acts at the downstream of BCL10[124] However, MALT1 alone does not activate NFκB in *in vitro* assays[123,125].

### 1.5.1.2 Characteristics of the *API2-MALT1* fusion

The breakpoints of t(11;18)(q21;q21) are characteristic and most of the breakpoints for both the *API2* and *MALT1* genes occur in introns, appear to be in a random fashion[107,126]. In the *API2* gene, all the breakpoints occur downstream of the third BIR domain but upstream of the C-terminal RING, with 93% just before the CARD (Figure 1.1)[104,106-110]. In contrast, the breakpoints in the *MALT1* gene are more variable, occurring in four

different introns upstream of the caspase-like domain (Figure 1.1)[104,106-110]. Thus, the resulting *API2-MALT1* fusion transcripts always comprise the N-terminal *API2* with three intact BIR domains and C-terminal *MALT1* region containing an intact caspase-like C2 domain. Importantly, all *API2-MALT1* fusion transcripts are in frame, despite that some of breakpoints at the genomic level are in exons and potentially cause frame shift[126], indicating selection pressure for a functional fusion protein during lymphomagenesis.

The specific selection of certain functional domains of the *API2* and *MALT1* genes to form a fusion product strongly suggests the importance and synergy of these domains in oncogenic activities. The BIR domain of API2 has been shown to confer anti-apoptotic activity[120]. However, the anti-apoptotic activity of the API2 BIR domain was much weaker than that of the XIAP and has been shown to be suppressed by its C-terminal RING finger domain[120]. As a result, wild type API2 did not protect cells from apoptosis upon stimulation by death signals[120]. The negative effect of the RING finger on BIR function may be associated with its activity of promoting auto-ubiquitination and degradation[118,120]. Replacement of the C-terminal API2 with the C-terminal of MALT1 by the fusion product would release the intrinsic anti-apoptotic activity of the BIR domain and therefore make the new molecule anti-apoptotic. The incoming C-terminal MALT1 may further enhance the anti-apoptotic activity of the BIR domain. It has been recently shown that the API2-MALT1 fusion product, but not API2 or MALT1 alone was capable of activating NFκB[123,125,127]. API2-MALT1 fusion product with two Ig-like C2 domain is more potent in NFκB activation than those without Ig-like C2 domain.

The BIR domain is capable of mediating oligomerisation of BIR-containing protein[128]. It has been proposed that the API2-MALT1 fusion protein might be oligomerised through the BIR-BIR interaction, and through C-terminal MALT1 the fusion product can constitutively activate NFκB (Figure 1.1)[127]. Interestingly, the *API2* gene promoter is known to be NFκB responsive, so that expression of the *API2-MALT1* transcript is likely to be transactivated by NFκB. This could set a positive feedback loop resulting in unrelented NFκB activation. NFκB is a transcriptional factor and can transactivate genes, such as those encoding cytokines and growth factors, which are important for cellular activation, proliferation and survival. Thus, it is likely that the API2-MALT1 fusion product mediates its oncogenic activity through NFκB activation. It has been recently shown that the API2-MALT1 fusion products inhibit DNA damages induced p53 mediated apoptosis in an NFκB dependant manner[129].

In addition to its ability to activate NFκB, the API2-MALT1 fusion product has been shown to significantly suppress both UV- and etoposide-induced apoptosis in *in vitro*[130]. The anti-apoptotic activity of the API2-MALT1 fusion product appears to be mediated, at least in part, by direct interference of the proapoptotic activity of Smac[130], a mitochondrial protein that promotes cytochrome C-dependent caspase activation[131].

In line with the findings as discussed above, that the API2-MALT1 fusion products but not wild type API2 and MALT1 are potential oncogenic. It has been shown that the API2-MALT1 fusion protein product was stable, whereas both the full length API2 and MALT1 were unstable[132]. Thus, the API2-MALT1 fusion product gains properties potentially oncogenic.

### 1.5.2 t(1;14)(p22;q32)/*IGH-BCL10*

t(1;14)(p22;q32) is a rare but recurrent chromosomal translocation observed exclusively in MALT lymphoma. The lymphoma cells carrying t(1;14)(p22;q32) usually show other chromosomal aberrations such as trisomies 3, 12 and 18 and other chromosomal aberrations[133]. Early cell culture experiments showed that lymphoma cells carrying t(1;14)(p22;q32) were capable of growing for a few days in the absence of any mitogenic stimulations, in contrast those without t(1;14)(p22;q32) typically died by apoptosis under the same condition[66]. Interestingly, when stimulated with an anti-idiotype antibody, mimicking antigen receptor stimulation, tumour cells with t(1;14)(p22;q32) actively proliferated, 50 times higher than those without the translocation[66]. These findings suggest that t(1;14)(p22;q32) involves a critical gene, that may be closely associated with antigen receptor pathway.

In 1999, Willis et al[134] and Zhang et al[135] independently cloned the breakpoint of t(1;14)(p22;q32) and showed that the translocation brought the entire *BCL10* gene, a novel gene at 1p22, under the control of the Ig heavy chain gene enhancer and deregulated its expression. A subquent study also revealed translocation of the *BCL10* gene to the Igκ locus by t(1;2)(p22;p12)/*IGK-BCL10* in a case report[136]. BCL10 contains a CARD in its N-terminal region and is rich in serine and threonine in its C-terminal region. Subsequently, several research groups working on apoptosis also identified BCL10 through search of the EST databases for novel sequences containing CARD or yeast two-hybrid screening[137-141]. Independent studies from different laboratories consistently showed that wild type BCL10 was weakly proapoptotic despite that it also activated NFκB[134,135,137-141]. In line with this, wild type BCL10 was shown to

suppress *in vitro* transformation of rat embryonic fibroblasts by H-ras and mutant p53[134,135]. The pro-apoptotic activity of BCL10 requires both an intact CARD and the C-terminal domain[134]. The truncated mutant BCL10, identified from cDNA clones of MALT lymphoma with t(1;14)(p22;q32) lost the pro-apoptotic activity of the wild type but retained its ability to activate NFκB[134]. Moreover, truncated BCL10 mutants were shown to synergise with H-ras and mutant p53 in rat embryo fibroblast (REF) transformation assays[134]. Thus, mutation was initially thought to be important for BCL10 mediated oncogenesis.

### 1.5.2.1 *BCL10* gene mutation

The original mutation screening was based on sequencing of individual cDNA clones from MALT lymphomas with t(1;14)(p22;q32) and various tumour cell lines[134,135]. Complex mutations including insertions (particularly within a mononucleotide run of seven consecutive thymidines at nucleotides 493-499), deletions and point mutations were found in each case examined[134,135]. The majority of these mutations would result in truncated BCL10 products similar to those showing oncogenic activity in *in vitro* experiments.

To examine whether *BCL10* gene mutation plays a role in lymphomagenesis, Du et al screened 227 cases of lymphomas of various subtypes based on the genomic DNA and found *BCL10* gene mutation in 6.7% of cases. The majority of the mutations observed were in the C-terminus and would result in truncated BCL10 products, which were confirmed by Western blot analysis in some cases[142]. Similar frequencies of BCL10 mutation at the genomic level were subsequently reported by several independent

groups[143-146]. However, most intriguingly, BCL10 genomic mutation is not an essential feature of MALT lymphoma with t(1;14)(p22;q32), being found in only 1 of the 3 cases examined[142]. Taken together, BCL10 genomic mutation may play a role in the development of a small subset of lymphomas but is most likely not the only mechanism underlying the oncogenic activity of BCL10.

Comparison of BCL10 mutation at the genomic and transcript levels also revealed intriguingly that the mutations seen at the cDNA level were not observed at the genomic level[142]. Subsequent studies showed that the BCL10 mutations found in cDNA clones from t(1;14)(p22;q32) positive MALT lymphomas and various tumour cell lines were also present in cDNA clones from normal peripheral blood leukocytes[147,148]. Further studies indicated that the mutations observed from individual cDNA clones including those of recurrent mutations, such as the insertion in the mononuleotide run, were not detectable from uncloned cDNA samples either by PCR-single-strand conformation polymorphism (SSCP) or direct sequencing[149-154]. Extensive Western blot analysis of MALT lymphomas and various tumours cell lines also failed to detect truncated BCL10 products predicted from mutations in the cDNA clones (Du et al, unpublished data). It remains to be a debate whether mutations seen in cDNA clones were the results of "molecular misreading" during transcription or cloning artifacts.

### 1.5.2.2 BCL10 in B and T cell development

Studies of *BCL10* knockout mice[155,156] have shown that *BCL10*$^{-/-}$ cells retained normal susceptibility to various apoptotic stimuli. The *BCL10* deficiency mice showed profound defects in both B and T cell development. In the T cell lineage, the mice

showed an increased apoptosis of early immature thymocytes and a reduction of mature T cells, while in the B cell lineage the mice displayed an impaired development of both follicular and marginal zone B cells. *BCL10*$^{-/-}$ lymphocytes have profound functional defects characterised by impaired antigen receptor mediated activation and proliferation of both T and B cells. The *BCL10*$^{-/-}$ mice showed dramatically reduced basal levels of serum Igs and poor cellular and humoral responses to virus infection, including Ig class switch. These defects are primarily due to impairment of antigen receptor mediated NFκB activation. It has been shown that BCL10 specifically links the antigen receptor signaling to the NFκB pathway in both B and T cells. Thus, BCL10 acts as a positive regulator of B and T cell proliferation and activation. Interestingly, one third of *BCL10*$^{-/-}$ mice died at the embryonic stage due to neural tube closure defect. This finding suggests that BCL10 may confer additional function other than linking antigen receptor mediated NFκB activation in B and T cells. In line with the above findings, transgenic mice in which *BCL10* is linked to an Ig enhancer-containing construct to program expression of the protein in T and B cells showed a dramatic and specific expansion of the splenic marginal zone B cells, reminiscent of human marginal zone lymphoma[157]. Interestingly, t(1;14)(p22;q32) positive lymphoma cells showed active proliferation upon anti-idiotype antibody stimulation, 50 times higher than those without the translocation[66]. It is most likely that the over-expression of BCL10 in the cells with t(1;14)(p22;q32) sensitises the responses to the antigen receptor stimulation.

Recent studies have unraveled partially the role of BCL10 in antigen receptor mediated NFκB activation and some molecules both up and down stream of BCL10 have been identified. TCR with major histocompatibility complex (MHC)/antigen complex together with costimulation of CD28 initiates a tyrosine phosphorylation cascade that

leads to the activation of protein kinase C (PKC) $\theta$ and subsequent recruitment of the CARD domain-containing adaptor proteins CARMA1 (CARD, membrane-associated guanylate kinase, MAGUK, protein 1)[127,158,159]. CARMA1 subsequently recruits BCL10 via CARD-CARD interaction and induces BCL10 oligomerisation. This is followed by BCL10 mediated MALT1 oligomerisation, consequently triggering a cascade of events leading to NFκB activation. It is believed that CARMA1 links B cell receptor (BCR) to BCL10 and MALT1 in a similar way in B cells[160]. Oligomerised MALT1 binds to the tumour-necrosis factor receptor associated factor 6 (TRAF6) through its C-terminus and induces the oligomerisation of TRAF6, thereby activating its ubiquitin ligase activity. Activaed TRAF6 polyubiquitinates itself and NEMO at K63. Activated TRAF6 also activates TAK1, possibly by binding to TAB2 or TAB3 and promoting the autophosphorylation of TAK1. Poly-ubiquitination of NEMO may facilitate the recruitment of the IKK complex to the TAK1/TAB2 complex, allowing TAK1 to phosphorylate IKK$\beta$ in the activation loop, thereby activating IKK. In MALT lymphoma with t(1;14)(p22;q32), it is believed that over-expressed BCL10 forms oligomer through its CARD and activates down stream signaling leading to NFκB activation without the need of antigen receptor stimulation[137]. Nonetheless, the role of BCL10 in multistage of MALT lymphoma development and the incidence of t(1;14)(p22;q32) in MALT lymphoma of various sites remains to be investigated.

### 1.5.3 t(14;18)(q32;q21)/*IGH-MALT1*

t(14;18)(q32;q21) is characteristically seen in follicular lymphoma, in which it involves the *BCL2* gene at 18q21. The same chromosomal translocation, but not involving the *BCL2* gene, was described in MALT lymphoma in 1997[97]. However, it was not until

2003 that Streubel et al and Sanchez-Izquierdo et al demonstrated that the translocation brings the entire *MALT1* gene under the control of the Ig heavy chain enhancer[161,162]. Conventional cytogenetic and interphase cytogenetic studies showed that t(14;18)(q32;q21)/*IGH-MALT1* positive MALT lymphomas, similar to those with t(1;14)(p22;q32), frequently harbour other chromosomal aberrations such as trisomies 3, 12 and 18[161-163].

Following identification of the gene involved in t(14;18)(q32;q21) of MALT lymphoma, interphase FISH has been developed for detection of this translocation. So far, there are only three studies that examined the incidence of the translocation in MALT lymphoma[161-163]. This translocation appears to occur more often in MALT lymphomas from extra-gastrointestinal sites. It has been described in MALT lymphomas of the liver (4/4), skin (3/11), ocular adnexa (3/8), lung (3/27) and salivary gland (2/11) but not in those of the gastrointestinal tract (19), thyroid (4) and breast (2)[161,163]. In all cases reported, t(14;18)(q32;q21) is mutually exclusive from t(11;18)(q21;q21).

In addition to chromosomal translocation, *MALT1* gene appears to be targeted by gene amplification. Using array CGH and FISH, *MALT1* gene amplification was demonstrated in cell lines from 2 splenic marginal zone B cell lymphomas (SSK41 and Karpas 1718) and 1 Burkitt lymphoma (KHM10B)[162]. MALT1 over-expression in these cell lines was further confirmed by quantitative RT-PCR, suggesting that *MALT1* gene amplification may be involved in MALT lymphoma. However, the incidence of t(14;18)(q32;q21)/*IGH-MALT1* and *MALT1* gene amplification in MALT lymphoma of various sites is largely unknown. MALT1 protein expression, its subcellular localisation and correlation with BCL10 expression pattern are unclear.

### 1.5.3.1 MALT1 in B and T cell development

Most of our current understanding of MALT1 function are derived from studies of MALT1 knockout mice[124,164]. Similar to *BCL10*$^{-/-}$ mice, *MALT1*$^{-/-}$ mice showed profound defects in antigen receptor mediated activation and proliferation of both T and B cells. MALT1 acts downstream of BCL10 to induce NF$\kappa$B activation and is required for the normal development of both marginal zone B cells and peritoneal B1 cells. In contrast to BCL10 disruption, however, inactivation of MALT1 has only mild effects on B cell activation and does not cause defects during neurodevelopment. In essence, similar to BCL10, MALT1 is an essential regulator of adaptive immune responses.

The above findings are supported by biochemical characterisation of BCL10 and MALT1 induced NF$\kappa$B activation. It has been shown that BCL10 binds Ig-like C2 domain of MALT1 and induces MALT1 oligomerisation, consequently leading to NF$\kappa$B activation[123,127].

Interestingly, MALT1 does not have a structure domain that can mediate self-oligomerisation and over-expression of MALT1 alone does not activate NF$\kappa$B. However, artificial oligomerisation of the C-terminus of MALT1 containing the caspase-like domain is sufficient for NF$\kappa$B activation[127]. Co-expression of MALT1 and BCL10 shows synergistic activation of NF$\kappa$B. It is believed that MALT1 triggered NF$\kappa$B activation depends on its oligomerisation mediated by BCL10.

### 1.5.3.2 Molecular link among the three MALT lymphoma associated chromosomal translocations

As discussed above, MALT lymphoma is characterised by three specific chromosomal translocations involving different genes. Morphologically and immunophenotypically, MALT lymphomas with various chromosomal translocations do not show apparent differences, suggesting that these different chromosomal translocations may target the same or similar oncogenic pathways.

Mounting evidence indicates that the oncogenic activity of these three MALT lymphoma associated chromosomal translocations is linked by the physiological role of BCL10 and MALT1 in antigen receptor mediated activation. BCL10 forms a complex with MALT1 and the two molecules synergise in the activation of NF$\kappa$B[123,125]. BCL10 binds the Ig-like C2 domain of MALT1 through a short region (aminoacids 107-119) downstream of its CARD domain and induces conformational changes that allow MALT1 oligomerisation[125]. In addition to the regions responsible for the interaction between BCL10 and MALT1, the CARD domain of BCL10 and the caspase-like domain of MALT1 are crucial components for NF$\kappa$B activation. Using knockout mice, Ruland et al in 2001[155], subsequently Xue et al[156], showed that BCL10 specifically transduces antigen receptor signaling to activate the NF$\kappa$B pathway. Studies of MALT1-knockout mice by two different independent groups reinforced the above findings and showed that MALT1 operates downstream of BCL10[124,164]. Consistent with a role of BCL10 and MALT1 in activating NF$\kappa$B signaling, therefore regulating the proliferation and survival of B and T cells.

In 2001, three groups independently showed CARMA1 as the upstream activator of BCL10[127,165,166]. CARMA1 (also known as CARD11 or BIMP3) contains two potential protein-protein interaction domains: a CARD and a coiled-coil domain. It binds BCL10 through a CARD-CARD interaction and CARMA1, BCL10, and MALT1 form a ternary complex[127,165,166] leading to activation of IKK (section 1.5.2.2). Activation of the IKK complex leads to phosphorylation and subsequent ubiquitylation and proteolytic degradation of IκBα, and allows NFκB to translocate to the nucleus and activate genes, such as those encoding cytokines, growth factors that are important for lymphocyte activation and functions[167,168].

### 1.5.4 Other chromosomal aberrations

In addition to the chromosomal translocations discussed above, conventional cytogenetic studies revealed other chromosomal aberrations in MALT lymphomas. Among those chromosomal alterations, the most recurrent changes are whole or partial trisomies 3, 12, 18[133]. Using interphase FISH, Wotherspoon et al studied a large series of MALT lymphoma of various sites and showed trisomies 3 and 18 in 60% and 18% of cases respectively[133,169]. In contrast, both trisomies 3 and 18 were only seen in 16% of nodal low grade B cell lymphomas. Dierlamm et al subsequently confirmed these findings and further showed that similar frequency of trisomy 3 was also found in nodal and splenic marginal zone B cell lymphoma[99,170]. By analysis of allelic imbalance using microsatellite markers, Starostik et al showed frequent chromosomal gain or amplification at 3q26-27[171], where *BCL6*, an oncogene frequently deregulated in DLBCL by chromosomal translocation, is located. It remains to be examined whether *BCL6* is the target of chromosomal gains at 3q26-27 in MALT lymphoma. Nonetheless,

chromosomal translocation involving *BCL6* locus was reported in cases of MALT lymphoma[172,173].

C-myc involved chromosomal translocation has been reported in cases of transformed MALT lymphoma[174]. However, this translocation appears not to be associated with MALT lymphoma[174,175].

### 1.5.5 p53

The *p53* gene is located at chromosome 17p13 and encodes for a 393 amino acid nuclear phospho-protein. The protein contains five highly conserved regions: phosphorylation sites at both amino and carboxyl terminus, a central zinc-binding core domain, nuclear localisation and tetramerization domain at the C-terminus.

*P53* is a transcriptional factor and plays a critical role in maintenance of the genome integrity. This is mainly achieved by control of the G1/S and G2/M checkpoints.

The G1/S checkpoint prevents cells from entering the S phase in the presence of DNA damage by inhibiting the initiation of replication and therefore, monitors DNA repair, and negatively controls cell cycle. Maintenance of the G1/S arrest is achieved by *p53*, which is phosphorylated on Ser15 by ataxia-telangiectasia mutated (ATM) / ataxia-telangiectasia and rad3-related (ATR) and on Ser20 by activation of checkpoint kinases ChK1 or ChK2, respectively[176]. The phosphorylation of *p53* inhibits its nuclear export and degradation, thus resulting in increased levels of *p53*. *p53* activates its target genes, including $p21^{WAF\text{-}1/Cip1}$, which binds to and inhibits the S-phase-promoting cyclin-

dependent-kinase (CdK2)-CyclinE complex[177], thereby maintaining the G1/S arrest. *p21*$^{WAF\text{-}1/Cip1}$ also binds to the CdK4-CyclinD complex and prevents it from phosphorylating retinoblastoma (Rb)[178]. The phosphorylation of Rb results in its release of the E2F transcription factor, which is required for the transcription of S-phase genes in order for S phase to proceed[179].

The G2/M checkpoint prevents cells from undergoing mitosis in the presence of DNA damage. Part of the mechanisms by which *p53* blocks cells at the G2 checkpoint involves inhibition of Cdc2, the cyclin-dependent kinase required to enter mitosis[180]. The *p53* cell cycle arrest pathways involve *p21*(WAF1, Cip-1) and GADD45 (growth arrest and DNA damage). As mentioned above, *p21* is an inhibitor. It binds to a number of cyclin – cdk complexes and PCNA to block cell cycle progression in G1 and G2. GADD45 also binds to PCNA and arrests the cell cycle in G2. Binding of Cdc2 to Cyclin B1 is required for its activity, and repression of the cyclin B1 by *p53* also contributes to blocking entry into mitosis[179]. *p53* also represses Cdc3, to help ensure that cells do not escape the initial block[179].

Overwhelming evidence indicates that *p53* is a tumour suppressor. Inactivation of the *p53* tumour suppressor gene is the most frequent event in human cancer[181], and is commonly caused by mutation in one allele and loss of the other. This is exemplified by the findings in patients with familial Li – Fraumeni cancer syndrome: around 70% of cases carry a germline mutation and develop cancer following somatic loss of the remaining wild type allele of the *p53* gene. The affected individuals typically present sarcoma, breast cancer, brain tumours, leukemia and adrenal gland tumours, often at a young age[182,183].

Overall, *p53* mutations are found in about 50% of all human cancers[181] and majority of these mutations are clustered in the regions critical for its function, such as the region for DNA binding[179]. In general, the incidence of *p53* mutation in solid tumours is much higher than that in lymphoid malignancies. Various frequencies of *p53* mutation have been documented in lymphoid malignancies. In aggressive lymphoma such as DLBCL and Burkitt's lymphoma, *p53* mutation has been found in 26% cases[184]. Missense mutations of *p53* usually result in the stabilisation of the protein; therefore; increased levels of *p53* are typically detected in association with *p53* gene mutations. Over-expression of the p53 protein is relatively common in high grade NHL (*de novo* as well as those that transform from a low grade lymphoma) but not in low grade tumours[185].

Inactivation of the *p53* gene also plays a key role in the multistage development of MALT lymphoma and appears to be highly associated with its high grade transformation[68]. In MALT lymphoma, *p53* allelic loss and mutation have been found in 7% and 19% of cases, respectively, with concomitant allelic loss and mutation in only 1/11 affected cases[68]. However, in transformed MALT lymphomas, *p53* allele loss and mutation have been shown in 29% and 33%, respectively, with most affected cases (6/9) showing concomitant allelic loss and mutation[68]. These findings show that *p53* function is partly inactivated in low grade tumours, whereas complete inactivation of the *p53* gene is associated with high grade transformation.

### 1.5.6 C-MYC

C-*MYC* 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 *MYC*-gene encodes for nuclear DNA binding proteins that are involved in transcriptional regulation. Myc proteins form homodimers or heterodimers with max, mad, and Mx11 through their C-terminal helix-loop-helix domains[186]. Max binds Myc to repress the transcriptional activities of Myc, whereas mad and Mx11 can bind Max and release Myc to function as a transcriptional activator[186]. Myc is implicated in the control of normal cell proliferation, transformation and differentiation[186]. Myc expression is essential for progression through the cell cycle, thus, cellular proliferation. In addition, C-*MYC* is implicated in apoptosis regulation. In absence of growth factors, Myc expression induces apoptosis[186].

The *C-MYC* gene is located on chromosome 8q24 and consists of 3 exons, of which the first exon is non-coding. The abnormalities of C-*MYC* gene found in human cancer include chromosomal translocation, gene amplification and mutation. *C-MYC* involved chromosomal translocations are commonly found in Burkitt's lymphoma[187,188] and DLBCL[189]. In 80% of cases, the translocation is between C-*MYC* and *IGH* on chromosome 14q32[190], while in the remaining cases, the translocation is between C-*MYC* and IG*K* or IG*L* sequences on chromosomes 2p12 and 22q11[191,192].

In Burkitt's lymphoma, a high frequency of mutations has been observed in the non-coding exon I and the exon I/intron I boundary region of the C-*MYC* gene[193,194]. Similar C-*MYC* mutations are subsequently observed in DLBCL including cases without the *c-*

*myc* involved translocation. The exon I and the exon I/intron I boundary region contains a transcriptional elongation block[195], a p67 protein initiation site, an intron splicing site[196], and 3 myc intron factor (MIF) binding sites[197,198]. Therefore, it is considered as the C-*MYC* regulatory region. Somatic alterations in this region have been shown to affect C-*MYC* expression based on *in vitro* study[194-198]. Thus, mutations in the regulatory region may alter myc transcription and expression in human tumour.

Although cytogenetic and Southern blot studies have shown no C-*MYC* translocation or rearrangement in MALT lymphoma[97,98], translocation involving the C-*MYC* locus has been reported in 3 of 24 transformed MALT lymphomas[98]. Sequence analysis of the regulatory regions of the C-*MYC* gene has shown somatic mutations in 16% of low grade tumours and 18% of transformed MALT lymphomas[54]. However, the importance of these mutations remains unclear.

### *1.5.7 p15/p16*

Both genes localise at chromosome 9q21 and are adjacent to each other. They inhibit the cyclin-dependent kinases Cdk4 and Cdk6, and are the major negative regulators of the cell cycle. Inactivation of these genes is commonly caused by hypermethylation in the promoter region or by homozygous deletion. In MALT lymphoma, hypermethylation has been found in both the *p15* (10%) and *p16* (44%) genes[199,200]. Deletion of the *p16* gene has been found only in transformed MALT lymphoma (14%)[201]. The inactivation of *p15* and *p16* might cause the loss of inhibition of the cyclin d-dependent kinase (Cdk4 and Cdk6) complexes, which promote the G1-S transition, thus leading to a deregulated cell cycling.

### 1.5.8 Fas

Fas (CD95; Apo-1) is a transmembrane receptor protein[202,203] and triggers apoptosis upon binding to its natural ligand, Fas ligand (FasL)[203-205]. The apoptosis signal is transmitted via an intracellular 'death domain' that interacts with a homologous motif in the adaptor protein Fas-associated death domain (FADD) which recruits pro-caspase 8[202,203]. Aggregation of pro-caspase 8 within the Fas signaling complex triggers its activation, which initiates a sequence of further downstream caspase activation, eventually causes apoptosis.

An alternative Fas mediated apoptosis pathway has been identified that involves mitochondria. The pathway employed depends on the cell type (type I and II cells) and is partially dependend on the BCL2 family member BID[206]. BID is cleaved by caspase-8 and its cleaved fragment *p15* translocates to the mitochondria membrane and perturbs the integrity of the outer mitochondrial membrane, which results in the release of cytochrome C[207]. Cytochrome C in turn causes apoptotic protease activating factor-1 (Apaf-1) to activate caspase-9, which results in downstream caspase activation[208].

Fas is widely expressed in different cell types and tissues. A physiologic role of Fas has been demonstrated in the immune system. The depletion of autoreactive T cells[209] and the elimination of activated lymphocytes at the end of an immune response depends on Fas-FasL interaction[204,205]. The importance of Fas and FasL in the immune system is illustrated by the MRL/Ipr-Ipr mouse (lympho-proliferation), which harbours a spontaneous mutation in the Fas gene and exhibits lymphoproliferation with massively

enlarged lymph nodes, splenomegaly, and various autoimmune disorders depending on the genetic background[210]. Similar clinical presentation is observed in patients with the auto-immune lymphoproliferative syndrome type I (ALPS I), also known as Canale-Smith syndrome, which is associated with mutations in the Fas gene[211,212]. The ALPS I is a rare inherited disease with manifestation in childhood. Affected patients show lymphoproliferation and autoimmunities with variable clinical manifestations (spleno- and hepatomegaly, lymph node enlargement – but rarely lymphomas)[211,212]. Lymphocytes from ALPS individuals are resistant or less sensitive to Fas mediated cell death[211,212].

Mutation of the *Fas* gene is also implicated in malignant diseases. Plasmacytomas harbour Fas mutations at a frequency of 10% (5/48)[213]. In a screen of different subtypes of B and T cell NHLs, *Fas* gene mutations were detected in 11% (16/150) of cases[214]. Interestingly, majority of the cases with *Fas* gene mutation were extranodal lymphoma accompanied by autoimmune diseases, such as hemolytic anemia and Sjögren's syndrome[214]. However, subsequent studies showed that *Fas* gene mutation rarely occurred in MALT lymphoma including those originated from a background of autoimmune disorder[215,216]. A recent study suggests that *Fas* gene mutation may be associated with high grade transformation of MALT lymphoma in a proportion of cases[216].

### 1.5.9 Microsatellite instability

Microsatellite instability (MSI) is a type of genomic instability. It is expressed as differences in the banding patterns of PCR amplified DNA from tumour cells versus

normal cells at various microsatellite loci. MSI was first reported as tumour specific in hereditary non-polyposis colorectal cancer[217,218], and sporadic colon cancer[217,219]. Subsequently, MSI was detected in many types of human solid tumours, albeit at different frequencies. MSI appears to be highly associated with defect in DNA mismatch repair genes[220,221].

In lymphoid malignancies with the exception of MALT lymphoma, MSI appears to be an infrequent or rare event. In MALT lymphoma, MSI has been reported in 53% of cases[222]. However, MSI is not associated with mutation of the DNA mismatch repair genes[223]. It is believed that MSI seen in gastric MALT lymphoma is most likely the result of genetic insults caused by *H. pylori* induced inflammatory responses.

## 1.6 Treatment of gastric MALT lymphoma

Before mid-1990's, MALT lymphoma was treated by various methods including surgery, radiotherapy and chemotherapy. The finding of the critical role of *H. pylori* infection in the development of gastric MALT lymphomas prompted the initial investigation by Wotherspoon et al in 1993[224], which examined the effect of *H. pylori* eradication on gastric MALT lymphoma. The study has led to not only novel therapy for gastric MALT lymphoma but also shed the light on development of potential therapeutic strategies for MALT lymphoma of other sites.

### 1.6.1 *H. pylori* eradication in gastric MALT lymphoma

In 1993, Wotherspoon et al treated 6 patients with *H. pylori* positive gastric MALT lymphoma using antibiotics alone and carefully followed up these patients with repeated endoscopic biopsies and both histological and molecular examinations. They showed complete regression of gastric MALT lymphomas in five out of the six patients 4-6 months after *H. pylori* eradication as judged by endoscopic and histological criteria. The histological remission was also accompanied by molecular evidence showing absence of tumour cells in the gastric biopsies[224]. With further follow-up, the patient whose tumour had initially failed to regress also showed histological and molecular remission 18 months after *H. pylori* eradication[225]. These six patients have been followed up for more than 6 years and they remain tumour free[225].

Above findings have now been confirmed by a number of independent studies worldwide, based on a total of more than 550 cases reported[226-240]. Overall, about 75% of patients show complete remission after eradication of *H. pylori*. In most cases, complete remission is achieved within 12 months of *H. pylori* eradication. However, a latent response of up to 45 months has occurred in some cases. On average, patients are followed up for more than 3 years and the tumour remission is stable. In 10% of cases, lymphoma relapse occurs and this often associated with *H. pylori* re-infection and can be controlled by antibiotics again. In the absence of *H. pylori* re-infection, relapse is frequently a transient self-limiting event. In 50% of the patients, tumour cells are detectable by PCR from post-remission biopsy samples, even though there is no histological evidence of a tumour lesion. Early observations suggest that this monoclonal tumour-cell population disapears during longer follow up[241,242].

The role of *H. pylori* eradication in the treatment of transformed MALT lymphoma is a debate. Several early studies indicated that transformed MALT lymphoma and MALT lymphomas with a high grade component did not respond to *H. pylori* eradication[235,239]. However, recent studies have shown complete remission of transformed MALT lymphoma in some cases after *H. pylori* eradication[78,80]. The value of *H. pylori* eradication as a treatment modality in transformed MALT lymphomas nonetheless remains to be further investigated.

### 1.6.2 Prediction of the response of gastric MALT lymphoma to *H. pylori* eradication

As discussed above, the time for a gastric MALT lymphoma to regress following *H. pylori* eradication can vary from a few weeks to 18 months. Prolonged follow-up with repeated endoscopy and gastric biopsy is essential to determine whether a lymphoma responds to *H. pylori* eradication or requires additional therapy. It is immensely beneficial, if the 25% of gastric MALT lymphoma that do not respond to *H. pylori* eradication can be identified at diagnosis. The prognostic value of clinical staging has been extensively examined by endoscopic ultrasonography, which allows assessment of the extent of tumour invasion into the gastric wall and regional lymph nodes[227,230,238,239]. In general, lymphomas of stage $II_E$ or above do not respond to *H. pylori* eradication[227,230,238,239]. In stage $I_E$ cases, the prognostic value of staging is limited although tumours that involve the muscularis propria or serosa (stage $I_{E2}$) show a higher failture rate than those restricted to the submucosa (stage $I_{E1}$)[227,230,238,239].

Attempts have been made to predict the response of gastric MALT lymphoma to *H. pylori* eradication using histological and immunophenotypical features. As discussed above, early studies showed that MALT lymphomas with high grade components tended to resist to *H. pylori* eradication. However, recent studies showed that early stage of transformed MALT lymphoma may respond to *H. pylori* eradication. Thus, histological features do not provide a reliable indication to predict the response of gastric MALT lymphoma to *H. pylori* eradication. In view of the critical role of *H. pylori* specific T cells in the growth of MALT lymphoma B cells, De Jong et al compared the expression of co-stimulatory molecules between gastric MALT lymphomas that responded to *H. pylori* eradication and those that resisted to the treatment. These authors found that CD86 expression was significantly associated with *H. pylori* eradication responsive cases[243]. Nonetheless, it remains to be evaluated whether CD86 expression level can be used as a reliable marker for identification of gastric MALT lymphoma that will respond to *H. pylori* eradication.

Majority of gastric MALT lymphomas are at stage $I_E$ at diagnosis. The response of these early stage gastric MALT lymphoma to *H. pylori* eradication can not be predicted neither by clinical staging nor by histological or immunophenotypic investigation. Clearly, other markers that can predict the treatment response of gastric MALT lymphoma to *H. pylori* eradication are highly desired. Identification of such treatment predictive markers may also help to unravel the molecular events that are responsible for MALT lymphoma cells to gain *H. pylori* independent growth capacity.

### 1.6.3 Treatment of *H. pylori* eradication non-responsive gastric MALT lymphoma

Surgery was widely and successfully used for treatment of gastric MALT lymphoma in the past[57]. It is highly curative for localised tumours, with a 5-year overall survival of 90-100% for those at stage $I_E$ and 82% for those at stage $II_E$[244,245]. However, lymphoma cells are unlikely to be totally removed by partial gastrectomy because they disseminate widely within the gastric mucosa[41]. This is also evident by the frequent observation of tumour relapse in the gastric stump[244,245]. Thus, to eradicate the lymphoma cells, a total gastrectomy is required, which seriously compromises the quality of patients' life. In view of this, and the similar survival rates achieved by various conservative therapies[244,246], surgical resection should be restricted to patients with complications such as gastric perforation and bleeding.

Gastric MALT lymphoma is sensitive to radiotherapy and the use of low-dose localised radiotherapy alone is becoming increasingly popular. The 5-year disease-free survival is about 80% and 5-year overall survival is greater than 90%[244,245]. Very encouraging results have been reported with low- to moderate-dose local radiotherapy in patients with stage I-II MALT lymphoma of the stomach, without evidence of *H. pylori* infection or with persistent lymphoma after antibiotics treatment, as well as in those with localised disease at non-gastric sites[247-251].

Chemotherapy has never been adequately evaluated in gastric MALT lymphomas because it was usually not administered, or given after surgery or radiotherapy. Only few compounds have been tested in MALT lymphomas. A non-randomised study reported that oral alkylating agents (either cyclophosphamide or chlorambucil, with a

median treatment duration of one year) can result in a high rate of disease control with projected 5-year event-free and overall survival of 50% and 75%, respectively[252]. A more recent phase II study demonstrated good anti-tumour activity of the purine analog cladribine (2-CDA) with a complete remission rate of 84%[253].

In the presence of disseminated disease, chemotherapy is an obvious choice. The role of the anti-CD20 monoclonal antibody rituximab in treatment of MALT lymphoma has also been explored in a phase II study, showing a response rate of about 70% (44% CR and 29% partial response)[254]. This may represent an additional option for the treatment of systemic disease, but the efficacy of its combination with chemotherapy remains to be further studied[255].

### 1.6.4 Treatment of MALT lymphoma of other sites

Most of the available information on the management of MALT lymphoma has been obtained from studies of gastric lymphoma. Non-gastric MALT lymphomas have been difficult to characterise because these tumours are distributed so widely throughout the body and it is difficult to assemble adequate series of any given site. Yet, few series have recently addressed the characteristics of non-gastric MALT lymphomas[246]. The International Extranodal Lymphoma Study Group (IELSG) published a retrospective survey of a large series of patients who were diagnosed as non-gastric MALT lymphoma. The IELSG study confirmed the indolent course of non-gastric MALT lymphomas despite the fact that one quarter of cases presented with stage IV disease and regardless of treatment type the 5-year survival was approximately 90%[256].

The optimal management of non-gastric MALT lymphomas has not yet been clearly established. Retrospective series included patients treated with surgery, radiotherapy and chemotherapy, alone or in combination. Whether different sites have a different natural history remains an open question[246,256]. Location can be an important factor because of organ-specific problems, which require different management strategies. Since optimal management of MALT lymphomas has not yet been clearly defined, the treatment choice should be patient-tailored, taking into account the site, the stage and the clinical characteristics of the individual patient.

Growing interest has been paid to explore the role of eradication of putative aetiological factors in treatment of non-gastric MALT lymphoma. Cutaneous marginal zone B cell lymphoma has been shown to be associated with *Borrelia burgdoferi* infection[28,29]. Kutting et al reported complete remission in 1 of 2 cases of cutaneous marginal zone B cell lymphoma after antibiotic therapy. Lecuit et al recently showed that *Campylobacter jejuni* was associated with IPSID[30]. As shown from this preliminary study, IPSID could be effectively treated by eradication of *Campylobacter jejuni* with antibiotics. This is accordance with the fact that IPSID has been known to respond to antibiotic therapy for many years[257,258]. A role for *Chlamydia psittaci* infection in the pathogenesis of ocular adnexal MALT lymphomas has been proposed by Ferreri et al[31]. They demonstrated that 2 of 4 patients respond antibiotic therapy for *Chlamydia psittaci*. In summary, these findings indicate that eradication of putative aetiological factors associated with non-gastric MALT lymphomas may have significant implication in treatment of these malignant diseases.

## 1.7 Pathogenesis of MALT lymphoma

Most of our current understanding of the pathogenesis of MALT lymphoma has been gained based on studies of those from the stomach. *H. pylori* infection stimulates the production of lymphoid infiltrates: B cells, T cells together with neutrophils, which lead to the formation of acquired MALT in the gastric mucosa. As a result of both direct and indirect immunological stimulation (by auto-antigens and *H. pylori* specific T cells, respectively), infiltrating B cells actively proliferate and occasionally undergo malignant transformation because of the acquisition of genetic abnormalities, such as t(11;18)(q21;q21), t(1;14)(p22;q32), trisomies of chromosomes 3, 12 and 18, allelic imbalance, partial *p53* inactivation. 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 MALT lymphoma. At this stage, the tumour is most frequently confined to the stomach and will regress following eradication of *H. pylori*. However, the lymphoma can progress, spread beyond the stomach and gain *H. pylori* independent growth capacity. By this stage, the lymphoma is no longer responsive to *H. pylori* eradication therapy. Nonetheless, the molecular events underlying the acquired *H. pylori* independent growth are not understood. Further genetic events such as complete inactivation of the *p53* and *p16* genes and other undetermined abnormalities can result in high grade transformation.

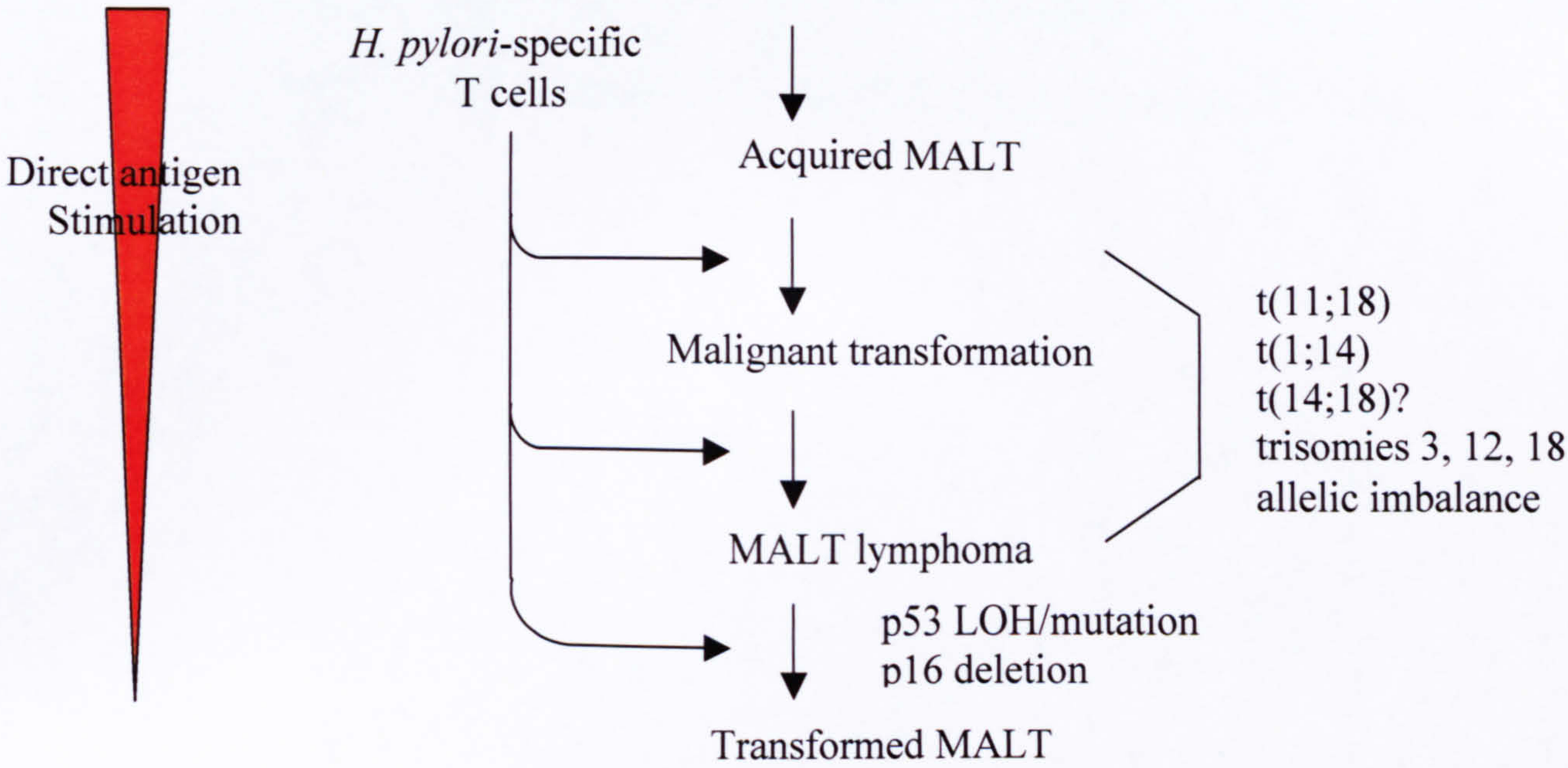



**Figure 1.4. Multistage development of gastric MALT lymphoma**

## 1.8 Aims

This study attempted to investigate the molecular events involved in pathogenesis of MALT lymphoma. The specific aims were as follows:

1. To investigate the frequencies of t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21) in MALT lymphoma of various sites and examine their roles during the multistage development of this tumour.

2. To correlate chromosomal translocations with clinical stages and treatment responses of gastric MALT lymphoma to *H. pylori* eradication

3. To study BCL10 and MALT1 expression pattern in normal and malignant lymphoid tissues, correlate their expression with different chromosomal translocations in MALT lymphoma and explore their value in detection of the translocations.

# Chapter 2. Materials and methods

## 2.1 Materials

### 2.1.1 Solutions

#### 2.1.1.1 Solutions used in immunohistochemistry and Enzyme-linked immunosorbent assay (ELISA)

**Tris buffered saline pH 7.6 (TBS):** Tris[hydroxymethyl] aminomethane (Sigma Chemical Co Ltd, Poole, Dorset, UK) of 6.05 g and NaCl (BDH Laboratory Supplies, Merck Ltd, Lutterworth, Leicestershire, UK) of 80 g were dissolved in 8 litres of distilled water, pH was adjusted to 7.6 with 1 M HCl (BDH) and the volume was brought to 10 litres with distilled water.

**TBS-Tween:** Tween 20 (Sigma) was added to TBS to give a final concentration of 0.05%.

**Phosphate buffered saline (PBS) pH 7.2:** One tablet of PBS (Sigma) was dissolved in 200 ml of distilled water, giving pH 7.2.

**Peroxidase block solution:** This solution was always prepared fresh just before use and composed of 200 $\mu$l of 30% $H_2O_2$ (hydrogen peroxide) (Sigma) in 12 ml of methanol (BDH).

**3,3-Diaminobezidine tetrahydrochloride (DAB) substrate solution:** This solution was always prepared fresh just before use. One tablet of DAB (Kem-En-Tec A/S, Denmark) was dissolved in 10 ml of distilled water. 10 $\mu$l of 30% $H_2O_2$ solution was added to the solution just before application.

**Alkaline phosphatase substrate solution:** 8 mg of Naphthol AS-MX phosphate (Sigma) was dissolved in 0.2 ml of dimethyl formamide (Sigma). This was added to 10 ml of 0.1 M tris solution. The pH was adjusted to 8.2 with 1 M HCl. Just before use, 2.5 mg of levamisole (Sigma) and 10 mg of Fast Blue (Sigma) or Fast Red (Sigma) were dissolved and the solution was filtered.

**Citrate buffer pH 6.0:** Sodium citrate tribasic dihydrate (Sigma) of 8.82 g was dissolved in 3 litres of distilled water and pH was adjusted to 6.0 with 1 M HCl.

**Tris-EDTA buffer pH 9.0:** 24 g of tris and 2 g of EDTA (BDH) were dissolved in 9 litres of distilled water, pH was adjusted to 9.0 with 1 M HCl (BDH) and the volume was brought to 10 litres with distilled water.

**Dako target retrieval solution pH 6.0:** 60 ml of Dako target retrieval solution pH 6.0 (DakoCytomation, UK) was mixed with 540 ml of distilled water.

**Dako target retrieval solution pH 9.9:** 60 ml of Dako target retrieval solution pH 9.9 (DakoCytomation, UK) was mixed with 540 ml of distilled water.

**Chymotrypsin (CT) solution:** CT (BDH) of 0.1 g and 0.1 g calcium chloride (BDH) were dissolved in 100 ml of distilled water and pH was adjusted to 7.8 with 0.1 N NaOH (BDH). The solution was warmed up to 37°C in a water bath before use.

**Protease solution:** Protease (Sigma) of 0.1 g was dissolved in 100 ml of distilled water and pH was adjusted to 7.8 with 0.1 N NaOH. The solution was warmed up to 37°C in a water bath before use.

**3% Paraformadehyde solution:** 3 g of paraformadehyde (Sigma) was dissolved in 100 ml of PBS.

**0.08% Triton 100 solution:** 800 $\mu$l of triton 100 was mixed with 1000 ml of PBS.

**Blocking buffer used in ELISA assays:** 3% of heat inactivated horse serum (Sigma) in TBS-Tween was used to block the non-specific binding sites in ELISA assays.

**O-Phenylenediamine substrate solution:** The solution was the mixture of 2.45 ml of 0.1 M citric acid (Sigma) and 2.55 ml of 0.2 M dibasic sodium phosphate (Sigma) with the volume adjusted to 10 ml with distilled water. Prior to use, 3.4 mg of o-phenylenediamine (Sigma) and 2 $\mu$l of 30% $H_2O_2$ solution were added.

### 2.1.1.2 Solutions used in tissue culture

**10% Sodium azide solution:** I g of sodium azide (Sigma) was dissolved in 10 ml of distilled water.

**Polyethylene glycol (PEG) solution:** 10 g of polyethylene glycol 1500 (BDH) was autoclaved and 10 ml of sterile serum free RPMI 1640 medium (Imperial Laboratories, Andover, Hampshire, UK) was added.

**Trypan blue solution:** Trypan blue was purchased from Sigma as a 0.4% solution for counting the viable cells.

**Calcium-magnesium Hanks's balanced salts solution (HBSS):** HBSS with 0.35 g/L sodium bicarbonate was purchased from Imperial Laboratories, Andover, Hampshire, UK, in sterile 500 ml bottles.

### 2.1.1.3 Solutions used in Western blotting

**Cell lysis buffer (2×):** Lysis buffer was composed of 100 mM tris-HCl (pH 8.0), 300 mM NaCl, 0.04% sodium azide, 0.2% sodium deoecyl sulfate (Sigma), 2% nonidet P-40 (BDH), 1% sodium deoxycholate (BDH), 2 mM EDTA, 100 mg/ml phenylmethylsulfonyl fluoride (Sigma), and 1 mg/ml leupetin (Sigma).

**Acrylamide/bis-acrylamide stock solution:** Ready mixed 40% acrylamide/bis-acrylamide stock solution was purchased from BDH and kept at 4°C until required.

**Stacking gel buffer stock (0.5 M Tris-HCl pH 6.8):** 6.0 g of tris was dissolved in 40 ml of distilled water, titrated to pH 6.8 with 1 M HCl and brought to 100 ml final volume with distilled water. The solution was stored at 4°C until required.

**Resolving gel buffer stock (1.5 M Tris-HCl pH 8.0):** 18.2 g of tris and 48.0 ml of 1 M HCl were mixed and brought to a final volume of 100 ml with distilled water. The solution was kept at 4°C.

**10% Sodium dodecyl sulphate (SDS) solution:** 10 g of SDS (BDH) was dissolved in 100 ml of distilled water and stored at room temperature.

**10% Ammonium persulphate solution:** 1 g of ammonium persulphate (Bio-Rad) was dissolved in 10 ml of distilled water. The solution was always prepared fresh just before use.

**Electrophoresis running buffer pH 8.3:** Electrophoresis running buffer was prepared as a 10 × concentrated stock solution and diluted to a working concentration in distilled water just before use. The 10 × concentrated running buffer was prepared by dissolving 30.3 g of tris, 150.14 g of glycine (Sigma), 10 g of SDS (BDH) and 3.7 g of EDTA in distilled water in a final volume of 1 litre. The solution was kept at room temperature.

**Blocking buffer:** Blocking buffer for Western blotting was composed of 5% skimmed milk (MARVEL) and 3% bovine albumin (Sigma) in TBS-Tween.

**Blotting buffer pH 9.2:** Blotting buffer pH 9.2 was made by dissolving 5.82 g of tris, 2.93 g of glycine and 0.375 g of SDS in distilled water followed by addition of 200 ml of methanol (BDH). The volume was adjusted to 1 litre with distilled water.

**Nitroblue tetrazolium chloride (NBT) – 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) substrate solution:** Substrate kit was purchased ready-made from Gibco. The substrate solution was prepared before application by mixing 44 $\mu$l of NBT solution and 33 $\mu$l of BCIP solution to 10.0 ml of 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$ (Sigma).

### 2.1.1.4 Solutions used in interphase fluorescence in situ hybridization (FISH)

**1 mM EDTA buffer (pH 8.0):** The solution was composed of 2 ml of 0.5 M EDTA (pH 8.0) (Merck) in 1 litre of distilled water.

**Pepsin solution:** 100 mg of pepsin was dissolved in 100 ml of distilled water and added 0.5 ml of 2 M HCl (Merck).

**1% paraformaldehyde solution:** 1 g of paraformaldehyde was dissolved in 100 ml of distilled water.

**0.4 × SSC/0.3% IGEPAL CA-630:** The solution was the mixture of 2 ml of 20 × SSC (Sigma) and 300 $\mu$l of IGEPAL CA-630 (Sigma) with the volume adjusted to 100 ml with distilled water.

**2 × SSC/0.1% IGEPAL CA-630:** The solution was the mixture of 10 ml of 20 × SSC and 100 $\mu$l of IGEPAL CA-630 with the volume adjusted to 100 ml with distilled water.

**2 × SSC:** 10 ml of 20 × SSC was mixed with 90 ml of distilled water.

### 2.1.2 Cell and culture media

**RPM1 1640:** Dutch modified RPMI medium 1640 with 20 mM HEPES was used as the standard medium in all cell culture experiments. In most cases it was supplemented with 10% heat inactivated fetal calf serum (FCS) (Imperial Laboratories), 2.05 mM L-glutamine (Sigma), 50 U/ml Penicillin G and 50$\mu$g/ml streptomycin (Sigma).

**Hypoxanthine-aminopterin-thymidine (HAT) medium:** 50 × HAT media supplement (Sigma) was reconstituted in 10 ml of RPMI 1640 and was added to 500 ml of RPMI 1640 plus 10% FCS to achieve final concentration of 100 $\mu$M hypoxanthine, 400 nM aminopterin and 16 $\mu$M thymidine.

**Dulbecco's Modified Eagle's Medium (DMEM):** Dulbecco's modified Eagle's medium with high glucose (Sigma) was used to culture HEK 293 cells and stable 293 transfected cell lines. It was supplemented with 10% heat inactivated FCS, 1 mg/ml G418 (Sigma), 100 g/ml of penicillin and 100 $\mu$g/ml of streptomycin.

**Freezing medium:** FCS containing 5% dimethyl sulphoxide (DMSO) (BDH) was used to freeze down cells for cryo-preservation in liquid nitrogen.

### 2.1.3 Antibodies

A wide range of murine monoclonal antibodies was used in the studies described in this thesis. The antibodies used and their specificities, dilutions, and sources are summarised

in Table 2.1. The secondary and third antibodies and reagents used to detect the reactivity of primary antibodies are shown in Table 2.2.

**Table 2.1. The characteristics of primary monoclonal antibodies used in immunohistochemistry (IHC), immunoblotting (IB)**

| SPCIFICITY | CLONE | DILUTION | PRETREATMENT | SOURCE/REFERENCE |
|---|---|---|---|---|
| BCL10 | 151 | 1/60 (IHC)<br>1/2000 (IB) | MW* pH 6.0 | Raised in House |
| CD 3 | UCHT-1 | 1/50 (IHC) | PC** 3 mins | DakoCytomation Ltd |
| CD20 | L26 | 1/400 (IHC) | MW 20 mins<br>Citrate buffer | DakoCytomation Ltd |
| C'-MALT1 | 25 | Neat (IHC)<br>1/100 (IB) | MW pH 9.9 | Raised in House |
| Neutrophil elastase | | 1/50 (IHC) | PC 3 mins | DakoCytomation Ltd |
| N'-MALT1 | | 1/50 (IHC)<br>1/2000 (IB) | PC 3 mins | Genetech, USA |

*MW: microwave oven **PC: pressure cooking

**Table 2.2. Characteristics of secondary and third reagents used to demonstrate the primary antibody immunoreactivity in IHC, ELISA and IB.**

| SECONDARY REAGENTS | DILUTION | SOURCE |
|---|---|---|
| Rabbit anti-mouse Ig, biotinylated | 1/300 (IHC)<br>1/4000 (IB) | DakoCytomation Ltd |
| Rabbit anti-mouse Ig, peroxidase | 1/500 (ELISA) | DakoCytomation Ltd |
| Extra-avidin, peroxidase | 1/200 (IHC) | Sigma |
| Extra-avidin, alkaline phosphatase | 1/1000 (IHC)<br>1/10000 (IB) | Sigma |

### 2.1.4 Cell lines

**NSO:** HAT sensitive, non-immunoglobulin-secreting mouse myeloma cell line NSO (European Collection of Animal Cell Cultures (ECACC), PHLS Centre for applied Microbiology and Research, Salisbury, Wilts, UK) was used as the fusion partner in all hybridoma works[259]. NSO cell line was grown in RPMI 1640 plus 10%FCS, maintained at a density of 3-9 × $10^5$ cells/ml and was used for fusion during exponential growth phase.

**BCL10 stable expression HEK 293 cell lines:** Full length BCL10, N and C terminal truncated BCL10 stable expression HEK293 cell lines and HEK 293 cells transfected with vector pcDNA3.1 were gift of Prof. Martin J.S. Dyer and colleagues. They were cultured in high-glucose Dulbecco's modified Eagle's medium containing 10% FCS and G418 antibiotics. These cell lines were used for Western blotting to test the specificity of mouse BCL10 monoclonal antibodies. In some experiments, these cells were pelleted, fixed in 10% of formalin and paraffin-embedded. Sections from these paraffin-embedded cell pellets were used for BCL10 immunohistochemistry.

**MALT1 or API2-MALT1 stable expression cell lines[260]:** Full-length MALT1 and API2-MALT1 stable expression Epstein-Barr virus (EBV)-negative BJAB human B cell lymphoma cell lines and BJAB transfected with vector pCDNA3.1 were gift of Dr L. Ho (Geneva, Switzerland). They were grown in RPMI-1640 with 10% FCS. These cell lines were used for Western blotting to test the specificity of mouse N-terminus of MALT1 and C-terminus of MALT1 monoclonal antibodies.

### 2.1.5 Tissues

**Frozen specimens:** All frozen tissue specimens were obtained from the tissue banks of the Department of Histopathology, Royal Free and University College Medical School, London, UK; Service de Gastro-enterologie/Service d'Anatomie Pathologique, Hotel-Dieu, Ap-Hp, Paris, France; Groupe d'Etude des Lymphomes Digestifs, Fondation Francaise de Cancérologie Digestive, France; the Department of Pathology/Gastroentorology, The Netherlands Cancer Institute, Amsterdam, The Netherlands; Servizi di Anatomia Patologica e Gatsroenterologia, Università degli Studi di Bologna, Italy; Labor Molekulare Diagnostik, Universitätsklinikum Carl Gustav Carus, Dresden, Germany; and Service d'Anatomie Pathologique, Hopital Lariboisiere, Paris, France.

**Paraffin embedded specimens:** All paraffin embedded tissue specimens were obtained from the surgical archives of Department of Histopathology, Royal Free and University College Medical School, London, UK; Department of Histopathology, The Royal Marsden NHS Trust , London; Department of Internal Medicine/Clinical Pathology, University of Vienna, Vienna, Austria; Service de Gastro-enterologie/Service d'Anatomie Pathologique, Hotel-Dieu, Paris, France; Department of Pathology/Gastroentorology, The Netherlands Cancer Institute, Amsterdam, The Netherlands; Servizi di Anatomia Patologica e Gastroenterologia, Università degli Studi di Bologna, Italy; Labor Molekulare Diagnostik, Universitätsklinikum Carl Gustav Carus, Dresden, Germany; Service d'Anatomie Pathologique, Hopital Lariboisiere, Paris, France; The Department of Haematology, Oncology and Immunology, Philipps University Marburg, Marburg, Germany; Institut fur Pathologie, Klinikum Bayreuth,

Germany; Department of Pathology, Institute of Pathological Anatomy and Histopathology, Ancona, Italy; Department of Pathology, Raigmore Hospital, Inverness, UK; Department of Haematology, University of Leicester, UK and Department of Pathology, Chi-Mei Medical Centre, Taiwan.

### 2.1.6 Animals

Six weeks old female Balb/c strain mice (Harlan, London, UK) were used for immunisation with recombinant BCL10 and MALT1 proteins, and production of mouse BCL10 and MALT1 monoclonal antibodies.

## 2.2 Methods

### 2.2.1 Immunohistochemistry

For immunohistochemical studies, microscopic slides (DakoCytomation) were used, and 6 $\mu$m frozen or 4 $\mu$m paraffin sections were cut from the tissue blocks. To stain non-adherent cells in culture, cytospins were prepared on slides. Monolayer cultures were grown on 1% gelatin-coated sterile coverslips in tissue culture dishes. At the end of the culture period, they were washed with TBS and air-dried.

### 2.2.1.1 Immunohistochemistry on formalin-fixed and paraffin-embedded tissue sections

Most of immunohistochemical studies were carried out on paraffin-embedded tissue sections using protocols described below unless specified.

Paraffin sections (4 $\mu$m) were deparaffinised in xylene (BDH), rehydrated using decreasing concentrations of ethanol (BDH), and incubated in peroxidase block solution for 10 minutes to block the endogenous peroxidase activity. Where indicated, antigen retrieval was carried out prior to immunostaining. To optimise a new primary antibody for immunohistochemistry, several antigen retrieval methods and a serial dilution of the primary antibody were systematically tested, and the conditions that gave the best staining were used in subsequent experiments. The antigen retrieval methods used for different antibodies are summarised in Table 2.1. Sections were then incubated with primary antibody at optimal dilution for 1 hour followed by biotinylated secondary antibody (1/200-1/300) and peroxidase conjugated ExtroAvidin (1/200) for 30 minutes, respectively. Finally, the staining was visualised with DAB in $H_2O_2$ and counter-stained with Mayer's haematoxylin. Throughout all immunohistochemistry procedures, the slides were washed in TBS-Tween, three times for 5 minutes each between all incubation steps.

### 2.2.1.2 Immunohistochemistry on frozen tissue sections, cytospin preparations and monolayer cell cultures

In some cases, frozen tissue sections, cytospin preparations and monolayer cell cultures were used for immunohistochemistry. This was carried out essentially as described for paraffin-embedded tissue sections with following modifications.

Frozen sections (6 $\mu$m), cytospins and monolayer cultures on coverslips were air dried for 30 minutes and were either immediately stained or wrapped in cling film and kept at –20°C until required. Frozen sections were routinely fixed in acetone (BDH) for 10-20 minutes and air-dried. Cytospins and coverslips were fixed either in acetone for 10-20 minutes or in 3% paraformadehyde for 10 minutes followed by 0.5% Tirton-100 for 5 minutes. The staining process is similar to that for formalin-fixed and paraffin-embedded tissue sections except that they were washed in TBS instead of TBS-Tween. It is important not to use Tween or other surfactant on frozen sections, cytospins and coverslips as this will cause detachment of cells.

### 2.2.1.3 Double staining

The procedure of the first primary antibody was the same as that of immunohistochemistry on formalin-fixed and paraffin-embedded tissue sections. After developement with DAB, slides were incubated with the second primary antibody at an appropriate dilution for 1 hour followed by biotinylated secondary antibody and alkaline phosphatase anti-alkaline phosphatase for 30 minutes, respectively. Finally, the staining

was visualised with Fast Red or Fast blue substrate solution for 10-20 minutes and mounted with an aqueous mountant.

### 2.2.1.4 Quantification of neutrophil infiltration

Neutrophil infiltration in MALT lymphoma and its preceding diseases was quantified with the help of immunohistochemistry. Neutrophils were detected with a mouse monoclonal antibody to the neutrophil elastase. The extent of neutrophil infiltration was estimated in 10 randomly chosen fields using a Lennox eyepiece graticule (Graticules Ltd., Tonbrige, Kent, UK) under high power magnification and expressed as a mean number of neutrophils per high power field.

### 2.2.1.5 ELISA

ELISA was used to screen hybridoma clones to identify those expressing the Ig to the antigen of interested.

50 $\mu$g of protein used as antigen for mouse immunisation was coated on 96 well ELISA plates overnight at 4°C or one hour at room temperature. After incubation, non-specific reactivity was blocked by incubation in 3% heat inactivated horse serum in PBS for overnight at 4°C or 1 hour at room temperature. The hybridoma cells were allowed to grow to confluence in 96-well and 24-well culture plates. One hundred microlitres of culture supernatant of hybridoma clones as primary antibody against the antigen being investigated were applied to the plate coated with antigen for one hour at room temperature followed by peroxidase labelled rabbit antibody against mouse

immunoglobulins for one hour. Peroxidase reaction was visualised using o-phenylenediamine as substrate for 40 minutes and stopped by 50 $\mu$l of 0.5 M $H_2SO_4$. The reactivity was measured at 492 nm with Titertek® Uniscan II microplate reader (ICN). Final optical density was calculated as the difference between reactivity of the antibody against the antigen being investigated and the negative controls.

### 2.2.2 Microdissection[261]

Tissue sections were dewaxed and weak stained by Haematoxylin. A drop of 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. The 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 fresh new pipettes were used for each microdissection. The microdisseted cells were spinned down, dried and subjected to DNA and RNA extraction where indicated. .

### 2.2.3 Western blotting analysis

#### 2.2.3.1 Preparation of protein homogenate for Western blot

Frozen tissues and transfected HEK 293 cells were homogenised in lysis buffer. Transfected 293 cells were grown in a 15 ml small flask. The monolayer cells were

washed with HBSS and incubated in trypsin-EDTA solution at 37°C for 10-15 minutes. The loosened cells were then recovered, washed in tissue culture medium by centrifugation and resuspension.

The cells were transferred to 1.5 ml eppendof tubes, washed once with PBS, and 100 to 200 $\mu$l of cell lysis buffer (prechilled to 4°C) was added. The tubes were incubated on ice for 1 hour with occasional rocking. At the end of the incubation period, the samples were spun at 10,000 g for 10 minutes. The supernatant was transferred to a fresh tube and immediately used in immunoblotting studies as described below or kept at -70°C until required.

### 2.2.3.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The whole cell lysates as described above were analyzed by SDS-PAGE followed by immunoblotting. A vertical slab gel apparatus, mini-Protean II Dual Slab Cell® (Bio-Rad), was used. Separating (12%-5%) and stacking (4%) gels were prepared as shown in Table 2.3 and amonium persulphate solution and TEMED were added just before the mixture was poured to the gel apparatus.

Resolving gel solution was poured leaving 1 cm space below the teeth of the comb for the stacking gel. The resolving gel solution was overlaid with distilled water and left to polymerise for 45 minutes. When set, the overlay was rinsed off with distilled water. The stacking gel monomer was poured and the comb was placed in the gel sandwich. After polymerisation, the comb was removed and the wells were washed with running buffer; the gel apparatus was assembled and filled with running buffer. The cell lysates

were mixed with 6 × loading buffer and loaded to each well. In each run at least one well contained standard molecular weight markers (Amersham Life Science). The gel was run for approximately 45 minutes to 2 hours at a constant voltage of 150 volts (V). At the end of the run the gel was separated from the plates and blotted as described below.

**Table 2.3. Preparation of stacking (Total=5 ml) and 12%-5% resolving gels (Total=10 ml) for SDS-PAGE.**

| | Stacking Gel | 12% | 10% | 7.5% | 5% |
|---|---|---|---|---|---|
| 40% Acrylamide/ bisacrylamide | 0.5 ml | 3 ml | 2.5 ml | 1.875 ml | 1.25 ml |
| Stacking gel buffer stock | 1.25 ml | - | - | - | - |
| Resolving gel buffer stock | - | 2.5 ml | 2.5 ml | 2.5 ml | 2.5 ml |
| 10% SDS | 50 μl | 100 μl | 100 μl | 100 μl | 100 μl |
| Distilled Water | 3.17 ml | 4.5 ml | 4.85 ml | 5.475 ml | 6.095 ml |
| 10% APS | 25 ul | 50 ul | 50 ul | 50 ul | 50 ul |
| TEMED | 5 μl | 5 μl | 5 μl | 5 μl | 5 μl |

### 2.2.3.3 Western blotting

The proteins run on the SDS-PAGE as described above were transferred to nitrocellulose membranes (Bio-Rad) for immunostaining using a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad).

The gel was equilibrated in blotting buffer for 5 minutes. It was then put on a wet nitrocellulose membrane and placed in the blotting apparatus between layers of filter paper. The transfer was completed in 1 to 2 hours at a constant voltage of 10 V. The membrane was washed in TBS-Tween and non-specific binding was blocked by

incubation in a blocking solution (5% skimmed milk and 3% bovine albumin) overnight at 4°C or for 1 hour at room temperature with constant shaking. After blocking, the specific antibodies were applied for 1 hour at room temperature with constant shaking followed by biotinylated rabbit anti-mouse Ig for 30 minutes. Finally, alkaline phosphatase-conjugated avidin was added for 30 minutes, and NBT-BCIP solution was added for 5-10 minutes. At the end of the detection reaction, the blots were washed under running tap water and air-dried.

### 2.2.4 Tissue culture

#### 2.2.4.1 Cell culture

All tissue culture plastic-wares were purchased from Corning Inc., Corning, New York, USA. Unless otherwise stated, all tissue cultures were carried out at 37°C in 5% $CO_2$ using a humidified, automatically controlled incubator (Leec Ltd., Colwick, Nottingham, UK). Manipulation of the cultures was always handled in a class II laminar flow cabinet (ICN Biomedicals).

#### 2.2.4.2 Protocol for freezing cells

Cells to be frozen were spun down, the supernatant decanted and the pellet resuspended in cold medium up to a volume of 500 $\mu$l depending on the cell density. The cell suspension was transferred to a cryovial and appropriately labelled. Ideally, cells to be frozen were in a logarithmic growth and at a density of at least $10^6$ cells per vial. Cells were placed in a polystyrene container to control the rate of cooling and first frozen

down in a -70°C freezer. On the following day, vials were transferred into liquid nitrogen until required.

#### 2.2.4.3 Protocol for thawing cells

The vial to be thawed was retrieved from the liquid nitrogen Bank and rapidly warmed in a 37°C water bath. The cell suspension was transferred to a sterile container and media added slowly to the cells with gentle agitation. The cells were spun down to remove media and then were transferred to an appropriate culture flask in the required medium.

### 2.2.5 Generation of monoclonal antibody

#### 2.2.5.1 Recombinant BCL10 protein

The recombinant BCL10 protein was generated by Professor Ming-Qing Du. Briefly, the full length (amino acids 1-233) and amino terminus (amino acids 1-122) of BCL10 were PCR-amplified from a BCL10 cDNA clone using a forward primer containing NcoI site (5'ATCCATGGAGCCCACCGCACCGGTCC3') and a reverse primer containing NotI site 5'ATGCGGCCGCTTGTCGTGAAACAGTACGTGA3' for the full length and 5'ATGCGGCCGCACAACTGCTACATTTTAGTC3' for the amino terminus. The PCR products were cloned into the TA cloning vector pGEM®-T, subcloned into PUC119/Myc-His at the NcoI and NotI sites and then transformed into *E. coli* HB2151. Colonies were screened using PCR with vector primers (M13 forward and reverse) and positive clones were sequenced to check for correct sequence and reading frame. Ten

positive clones were induced to express BCL10 protein in a 5 ml culture with 1mM IPTG at 28°C for 10-16 hours and their BCL10 expression was assessed by Western blotting with 9E10 antibody (Sigma, Poole, U.K.), which recognises the c-Myc tag. The clone expressing the highest level was subjected to induction in a 2 litres culture under the same conditions. BCL10 was purified using Ni-NTA (QIAGEN, Crawley, U.K.) affinity chromatography under denaturing conditions with 8 M urea according to the manufacturer's instructions. Purified BCL10 was dialyzed against 30 mM Tris-HCl (pH 8.0) and concentrated using Centriplus concentrators (Amicon, Beverly, MA). The purity and yield were checked by SDS-PAGE.

### 2.2.5.2 Immunisation protocol

Balb/c strain mice were immunised by intraperitoneal injection of 50 $\mu$g of full-length recombinant BCL10 or C-terminal MALT1 protein (The gifts from Prof. Ming-Qing Du and Ms Sima Shirali) in complete Freund's adjuvant followed by two boost injections with 50 $\mu$g of the respective recombinant protein in incomplete Freund's adjuvant two weeks and 4 weeks after the priming. Three days after the second boost injection, the mice were sacrificed and the spleen was aseptically removed for production of hybridoma.

### 2.2.5.3 Preparation of feeder layer for growing hybridoma cells

To support growth of hybridoma cells, feeder cells were used. They were obtained by washing the peritoneal cavity of Balb/c mice with 10 ml of HAT medium. These feeder

cells were grown in 96 well plates for 1 day before they were used to support the growth of hybridoma cells.

### 2.2.5.4 Hybridoma fusion

Single cell suspension was prepared from the spleen of the immunised mice by cutting the splenic tissue with a steriled scalpel and washed in RPMI 1640 serum free medium for three times. NSO cultured under the standard condition were harvested and washed in RPMI 1640 serum free medium for three times. Mouse splenocytes (typically $1 \times 10^7$) were mixed with NSO cells in a ratio of 1.4:1 in 20 ml RPMI 1640, and 1 ml of pre-warmed (37°C) PEG 1500 solution was slowly added to the mixture with gentle shake. Immediately after this, 2 ml of HAT medium (pre-warmed to 37°C) was slowly added to the cell suspension followed by a further 18 ml of HAT medium within the next 2 minutes. The cells were spun down and resuspended in 10 ml of HAT medium and the number of live fusion cells was calculated by trypan blue exclusion method. The fusion cells were dispensed into 96 well tissue culture plates containing feeder cells at the final cell concentrations of $1 \times 10^5$ cells/well, $5 \times 10^4$ cells/well and $2.5 \times 10^4$ cells/well.

### 2.2.5.5 Hybridoma screening and single cell cloning

From 7 to 21 days after fusion, the 96-well plates were inspected daily and clones visible to naked eyes were subjected to ELISA screening. ELISA positive clones were transferred to a 24-well culture plate containing feeder cells. When they reached the exponential growth phase, supernatants from these wells were screened again by ELISA. Positive clones were then subjected to single cell cloning and the supernatant from these

clones were tested for immunohistochemistry of paraffin sections from tonsil and MALT lymphoma with relevant translocations. Positive clones after the second ELISA screening were cloned twice with limiting dilution. Briefly, hybridoma cells were plated into two 96-well plates at four concentrations so that 0.5, 1, 2 or 5 cells would be present in each well. The arising clones were screened using ELISA and immunohistochemistry as outlined above. Those with the desired reactivity were first transferred into a 24-well plate, and then grown in tissue culture flasks. Once a stable antibody secreting hybridoma was achieved, cells were transferred to standard medium RPMI 1640 plus 10% FCS. The supernatants from hybridoma cultures were used for immunohistochemistry and Western blotting and they were kept at -20°C until required. Once defrosted, sodium azide solution was added at a final concentration of 0.02% and the hybridoma supernatant was stored at 4°C.

#### 2.2.5.6 Determination of isotypes of monoclonal antibodies

The isotype of the mouse monoclonal antibodies generated was determined using the ImmunoType monoclonal antibody isotyping kit (Sigma) according to manufacturer's instructions.

### 2.2.6 DNA and RNA based molecular analysis

#### 2.2.6.1 DNA extraction

**DNA extraction from paraffin-embedded tissue sections:** Paraffin-embedded tissue sections were deparaffinised in xylene for 5 minutes at room temperature and washed

three times with 100% ethanol and air-dried at room temperature. Samples were then digested with proteinase K Qiagen solution (10 mM Tris pH9, 50 mM KCl, 0.1% Triton and 200 $\mu$g/ml proteinase K) at 37°C overnight or for three hours at 55°C. The proteinase K was inactivated at 95°C for 10 minutes.

### 2.2.6.2 RNA extraction

**RNA extraction from frozen tissues or fresh cells:** This was carried out using Qiagen RNeasy Mini kit (Qiagen, West Sussex, England) according to manufacturer's instructions. Briefly, fresh cells, or frozen tissue sections (<5 $\mu$m) were lysed in appropriate volume (600 $\mu$l) of RNeasy lysis buffer (Buffer RLT) containing $\beta$-mercaptoethanol (1%). Cells or tissues were homogenised by spinning the crude lysate through a QIAshredder column (Qiagen, UK). After addition of an appropriate amount of 100% ethanol, the samples were applied onto a RNeasy spin column. The column was washed with buffer RW1 and then Buffer RPE before elution with RNase-free water. RNA is quantified and stored at -70°C.

**RNA extraction from paraffin-embedded tissue sections:** This was carried out using Ambion RNA Isolation Kit (AMS Biotechnology, Oxon, England, United Kingdom). Paraffin embedded tissue sections were deparaffinzed in xylene for 20 minutes at room temperature and washed three times with 1 ml 100% ethanol and air-dried at room temperature. Samples were then digested with proteinase K (1 mg/ml) at 45°C for two hours and solubilised in a guanidium-based buffer. RNA was extracted with acid phenol/chloroform and precipitated in isopropanol with linear acrylamide as carrier.

RNA was washed with 75% ethanol, redissolved in 20 $\mu$l of RNA storage solution, quantified and stored at –70°C.

### 2.2.6.3 Quantification of RNA

2.5 $\mu$l of RNA extracted from frozen tissues or fresh cells or paraffin-embedded tissue sections was mixed with 77.5 $\mu$l of distilled water and quantified using GeneQuant pro (Amersham Pharmacia Biotech, Sweden) according to manufacturer's instructions.

### 2.2.6.4 Complementary DNA (cDNA) synthesis

**cDNA synthesis from RNA Samples prepared from frozen tissues and fresh cells:**

This was carried out with oligo(dT) primer using SuperScript Preamplification System (Invitrogen Ltd, Paisley, Scotland, United Kingdom). Briefly, 1 $\mu$l dNTP, 1 $\mu$l Oligo(dT), 2 $\mu$g RNA were mixed in 10 $\mu$l volume together and incubated at 65°C for 5 minutes, and placed on ice. If the amount of total RNA was below the measurable level, such as from biopsy tissue samples, a maximum volume of RNA preparation was used for cDNA syhthesis. After at least 1 minute, 2 $\mu$l 10 × RT buffer, 4 $\mu$l 25 mM $MgCl_2$, 2 $\mu$l 0.1 M DTT and 1 $\mu$l RNase inhibitor were added to the above RNA/primer mixture and incubated at 42°C for 2 minutes, and followed by additional of 1 $\mu$l of SuperSCRIP II RT enzyme and incubation at 42°C for 50 minutes. The reaction was terminated at 70°C for 15 minutes and 1 $\mu$l RNase H was applied and incubated for 20 minutes at 37°C.

**cDNA synthesis from RNA samples prepared from paraffin-embedded tissue sections:** For paraffin-embedded samples, cDNA was synthesised using SuperScript™ Preamplification System with the following modifications. In the case of detection of t(11;18), all three anti-sense primers (p-AS1, p-AS2 and p-AS3) to the *MALT1* gene together with anti-sense primer to the control gene glucose-6-phosphate dehydrogenase (G6PD) were included in the reverse-transcript (RT) reaction (Table 4.2). In addition, the temperature for primer annealing and cDNA synthesis was at 50°C rather than 42°C used for reverse transcription with oligo(dT) primer.

### 2.2.6.5 PCR and analysis of PCR products

**PCR optimisation:** Most PCR was carried out using standard routine procedures following optimisation. A typical PCR reaction was carried out in a 25 $\mu$l reaction volume containing 1 $\mu$l of cDNA extracted from fresh frozen tissues, 0.2 mM dNTP (Promega, Southampon, United Kingdom), 2 mM $MgCl_2$, 0.2 $\mu$M sense and anti-sense primers each and 1 unit Platinum® *Taq* DNA polymerase (Invitrogen Ltd) and amplified on a Phoenix thermal cycler (Helena BioSiences, U. K.). The PCR was conducted using a "hot-start touch-down" program, comprising hot start at 94°C for 4 min followed by denaturing at 94°C for 1 minute, annealing at 65-59°C (one degree down each cycle) for 1 minute and extension at 72°C for 1.5 minutes, and then 35 cycles of denaturing at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 1.5 minutes. A final extension step at 72°C for 10 minutes concluded the reaction.

Two sets of primers were used for detection of the *API2-MALT1* fusion transcript from frozen tissues (Table 2.4). The first set of primers, consisting of sense primer 5'-CTG

GTG TGA ATG ACA AGG TC from coding region 897-916 of *API2* (GeneBank, Accession No. NM_001165) and anti-sense primer 5'-TAG TCA ATT CGT ACA CAT CC from coding region 1124-1143 of *MALT1* (AF130356), were used for primary PCR covering all breakpoints of the fusion transcripts recorded in literature[97,98,101-104,106,108-110,262]. To improve specificity and sensitivity, the primary PCR products were further amplified using a nested set of primers consisting of sense primer 5'-ACA TTC TTT AAC TGG CCC TC from coding region 1505-1524 of API2 and anti-sense primer 5'-CAA AGG CTG GTC AGT TGT TT from coding region 1030-1049 of MALT1. All PCR reactions were performed in at least duplicate.

A 256 base pair fragment of *G6PD* gene was amplified in parallel as a control to verify RNA quality and RT-PCR efficiency for each sample, using sense primer 5'-GAG GCC GTG TAC ACC AAG ATG AT and anti-sense primer 5'-AAT ATA GGG GAT GGG CTT GG (Table 2.4). The primers were chosen to flank a region containing introns 10 (104bp) and 11(105bp) (GenBank X55448.1, M12996) so that amplification of contaminating DNA could be differentiated from that of cDNA by electrophoresis on agarose gels

For paraffin-embedded samples, in order to amplify the API2-MALT1 fusion product, primers were designed to flank a short segment of the fusion junction and hence were suitable for amplification of small fragments of cDNA typically prepared from RNA isolated from paraffin-embedded tissues. Three sets of PCR primers were designed: a common *API2* sense primer (p-S) that covered 93% of the known *API2* breakpoints, and three anti-sense primers that targeted all the four variable breakpoints on the *MALT1* gene (Table 2.4). A separate set of primers was designed for RT-PCR of the *G6PD* gene

(Table 2.4). The size [23,263] of fragments amplified with these primer pairs is shown in Table 2.4. PCR was performed separately with each primer pair using the same "hot-start touch-down" program as described above.

**Table 2.4. Primers for RT-PCR of the *API2-MALT1* fusion transcript and *G6PD***

| Tissue type | Gene target | Primer | Primer sequence | Expected major PCR products (base pairs) (bp)[a] |
|---|---|---|---|---|
| Frozen tissue | API2-MALT1[b] | Sense | 5'-ACA TTC TTT AAC TGG CCC TC | 669; 730; 1006; 1279 |
| | | Anti-sense | 5'-TAG TCA ATT CGT ACA CAT CC | |
| | G6PD | Sense | 5'-GAG GCC GTG TAC ACC AAG ATG AT | 258 |
| | | Anti-sense | 5'-AAT ATA GGG GAT GGG CTT GG | |
| Paraffin Tissue | API2-MALT1 | Sense | 5'-GGA AGA GGA GAG AGA AAG AGC A | 83 |
| | | Anti-sense 1 | 5'-CCA AGA CTG CCT TTG ACT CT | 67; 340 |
| | | Anti-sense 2 | 5'-GGA TTC AGA GAC GCC ATC AA | 73; 100; (133; 197; 230); 409 |
| | | Anti-sense 3 | 5'-CAA AGG CTG GTC AGT TGT TT | |
| | G6PD | Sense | 5'-ACG GCA ACA GAT ACA AGA AC | 87 |
| | | Anti-sense | 5'-CGA AGT GCA TCT GGC TCC | |

[a]Alternative splice variants are shown in parentheses

[b]Gene sequence used for primer design: *API2*, NM_001165; *MALT1*, AF130356; *G6PD*, X55448.1 and M12996.

Most of the primers used in this thesis were designed with Oligo 6.1 software (institute of Biotechnology, University of Helsinki, Finland) based on published sequences except the primer sets used for the amplification of the *H. pylori* urease A and the *CagA* genes of *H. pylori* which were adapted from published sequences[89,264]. All primers were synthesised by Oswel (Southampton, United Kingdom) or (Thermo Electron Corporation, Sedanstraβe, Germany).

Various laboratory procedures and precautions were strictly followed in order to prevent potential cross contamination. Briefly, RNA and DNA extraction and PCR experiment set up were carried out on a dedicated bench area in a "clean" laboratory, which is free

of PCR and cloning products. Once PCR experiments were set up, the PCR plates or tubes were transferred to a themo-cycler in a separate room, where the PCR amplification and PCR product analysis took place. In each PCR experiment, appropriate positive and negative controls were included.

**Analysis of PCR products:** RT-PCR products amplified from RNA of frozen tissues were analysed by electrophoresis on 0.9% agarose gels. Briefly, 3-10 $\mu$l of PCR product was mixed with loading buffer, loaded on a gel, electrophoresed at 100-150 V for 30 minutes and viewed under ultraviolet light after staining with ethidium bromide.

PCR or RT-PCR products amplified from RNA of paraffin embedded tissue sections were analysed by electrophoresis on 10% polyacrylamide gels. Briefly, 5-10 $\mu$l of PCR product was mixed with loading buffer, separated on polyacrylamide gel at 200 V for 1 hour and stained with ethidium bromide for 5 minutes and visualised under ultraviolet light.

### 2.2.6.6 PCR of *H. pylori* associated *urease* and *CagA* genes

For *H. pylori* associated *urease* gene, a pair of primers (sense primer 5'-CAT CTT GTT AGA GGG ATT GG-3'; anti-sense primer 5'-TAA CAA ACC GAT AAT GGC GC-3') were used, which yielded a 203bp product and was thus suitable for DNA samples prepared from formalin-fixed and paraffin-embedded tissues[264]. Similarly, the *CagA* gene was amplified with a primer set (sense primer 5'-TCAGAAATTTGGGGATCAG-3'; anti-sense primer 5'-TCATCARGGATAGGGGGTT-3') which gave rise to a 132bp product[89]. For both genes, PCR was carried out in a 25µl reaction on a thermocycler

(ThermaHybaid Px2) under the following conditions: 4 minutes at 94°C for initial denaturation, 40 cycles of 30 seconds at 94°C, 30 seconds at 53°C for and 45 seconds at 72°C, and finally 10 minutes at 72°C to conclude the reaction. PCR products were analysed on 10% polyacrylamide gels.

### 2.2.6.7 Purification of PCR products

PCR products were purified using a Concert Rapid PCR Purification System (Life Technologies) according to manufacturer's instructions. Briefly, the PCR products were dissolved in 400 $\mu$l of Binding Solution (H1) and loaded onto a spin cartridge and centrifuged in a microcentrifuge at $\geq$12,000g for 1 minute. The mixture was then washed with 700 $\mu$l of wash buffer (H2) by spinning at $\geq$12,000g for 1 minute twice and added 50 $\mu$l of warm TE Buffer and incubated at room temperature for 1 minute followed by centrifuging at 12,000g for 2 minutes.

### 2.2.6.8 Cloning

Some of PCR products from the alternative splicing variants of the *API2-MALT1* fusion transcript were cloned and sequenced. Briefly, PCR products were purified from 1.5% agrose gels using QIA Quick Gel Extraction Kit (Qiagen, UK) and ligated into the TOPO-TA cloning vector and transformed into One Shot competent Escherichia coli (Invitrogen, Chrlsbad, CA). The transformed cells were selected on Luria-Bertani (LB)-ampicillin agar plates and colonies were screened by PCR using vector primers T7 (5'-GTAATACGACTCACTATAGGGC-3') and T3 (5'-AATTAACCCTCACTAAAGGG-3'). The PCR products showing the expected insert size were sequenced in both

orientations with dRhodamine terminators (Perkin-Elmer, Foster City, CA) on an ABI 377 DNA sequencer (Perkin-Elmer, CA, USA).

### 2.2.6.9 Sequencing of PCR products

The RT-PCR products of the API2-MALT1 fusion transcript were directly sequenced from both orientations using ABI 377 DNA Sequencer. Briefly, 4 to 5 µl of PCR products (minimum of 800 ng in total) were mixed with 4 µl of dRhodamine Dye Terminator Mix and 5 µM (each) primers. After denaturation for 30 seconds at 96°C, each reaction mixture was amplified for 25 cycles as follows: 30 seconds at 96°C, 15 seconds of annealing at 50°C, and 1 minute of extension at 60°C. PCR products were cleaned by precipitaion with 100 µl of 80% ethanol and 6 µl of 3 M NaAc (pH 5.2) and analysed on an ABI 377 DNA sequencer.

Computer analysis of the DNA sequences obtained was carried out using BLAST online (http://www.ncbi.nlm.nih.gov/BLAST) and the Wisconsin GCG software (provided by the Human Genome Mapping Project, Cambridge, UK).

The RT-PCR method for detection of t(11;18) was established by Dr Hongxiang Liu. A proportion of MALT lymphoma cases were screened by Dr Hongxiang Liu. Most of the sequencing work was analysed by Dr Hongxiang Liu, some by Mr Rifat A. Hamoudi.

### 2.2.6.10 Interphase FISH

Interphase FISH was carried out using different probes. *BCL10* (BAC clones RP11-1080I1 and RP11-40K4 for telomeric as well as RP11-1077C10 and RP11-36L4 for centromeric) and *IGκ* (BAC RPCI-11 316G9 for telomeric as well as RPCI-11 102IF11 and RPCI-11 525L16 for centromeric) break-apart probes were the gifts of Dr Reiner Siebert (Institute of Human Genetics, University Hospital Schleswig-Holstein Campus Kiel, Germany) and *IGH/BCL10* (BAC 158A2 for *IGH* and BACs RP11-1080I1 and RP11-40K4 telomeric to *BCL10*) dual colour, dual fusion translocation probes were from Ellen D. Remstein (Divisions of Anatomic Pathology and Hematopathology, Mayo Clinic, Rochester, USA). The diagnostic reliability of the newly developed BCL10 break-apart assay was recently proven in a series of controls including four cases of cytogenetically proven t(1;14)(p22;q32) (Siebert et al., personal communication). *IGH* and *MALT1* break-apart probes, *IGH/MALT1* probes, and *API2/MALT1* probes were purchased from Abbott Dignostic (Downers Grove, IL, USA). Interphase FISH was performed on paraffin embedded tissue sections. Briefly, deparaffinised sections were pretreated by pressure cooking for 2 minutes and 40 seconds in 1 mM EDTA, pH 8.0 and subsequently incubated in pepsin solution for 20 minutes at 37°C to increase accessibility. Sections were then fixed in 1% paraformaldehyde for 1 minute, dehydrated and air-dried. Probe was applied to the tissue section, which was then covered with a 10 mm round coverslip. The probe and target DNA were thwn denatured at 80°C for 25-30 minutes and incubated for 3 days at 37°C. Sections were washed with 0.4 × SSC/0.3% IGEPAL CA-630 at 72°C for 2 minutes and 2 × SSC/0.1% IGEPAL CA-630 at room temperature for 1 minute, followed by 2 × SSC at room temperature for 2-5 minutes. Finally, the sections were counterstained with 4,6-Diamidino-2-phenylindole (DAPI) and mounted in Vectashield antifade mounting medium (Vector

Laboratories, Burlingame, CA). Sections were viewed under an Olympus Axioskop2 fluorescence microscope (Japan) using a 100 × oil immersion lens and appropriate filters and images were captured with ISIS imaging system (MetaSystems, Altlussheim, Germany).

#### 2.2.6.11 Real-time RT-PCR

Real-time RT-PCR was used to quantify *MALT1* and *BCL10* mRNA expression and 18S rRNA was used as an internal control. Total RNA was isolated from tumour cells microdissected from paraffin-embedded tissue sections[261]. cDNA was synthesised using random hexamer primers for *MALT1*/18S rRNA and gene specific primer for *BCL10*/18S rRNA. Real-time PCR was performed using an iCycler iQ system (BIO-RAD, UK) with SYBR Green I. One of each primer pair was designed to span an exon-exon junction to prevent any contaminated DNA from amplification (Table 5.2)[265]. The primers for the *MALT1* gene target its N-terminus, thus will only amplify wild type *MALT1* but not *API2-MALT1* transcripts.

**Table 2.5. Primers used for real-time quantitative PCR of *MALT1* and *BCL10* mRNA**

| Genes | | Primer sequence | Amplicon size (bp) |
|---|---|---|---|
| **MALT1*** | Sense | 5' ctc cgc ctc agt tgc cta ga | 104 |
| | anti-sense | 5' caa cct ttt tca ccc att aac ttc a | |
| **BCL10** | Sense | 5' gaa gtg aag aag gac gcc tta g | 80 |
| | anti-sense | 5' aga tga tca aaa tgt ctc tca gc | |
| **18SrRNA** | Sense | 5' tga ctc aac acg gga aac c | 114 |
| | anti-sense | 5' tcg ctc cac caa cta aga ac | |

* The primers for the *MALT1* gene were designed to target its N-terminus (nucleotides 363-466 according to its cDNA sequence AF130356), therefore will only amplify the wild type *MALT1* but not the *API2-MALT1* transcripts.

The conditions for real-time PCR were optimised prior to data collection. The specificity of the RT-PCR products for each primer set was confirmed by melt-curve analysis. The standard curves were generated by two-fold serial dilutions of 100 ng/µl *MALT1* cDNA and 1 ng/µl 18S rRNA cDNA prepared from fresh frozen tonsils, and 100 ng/µl *BCL10* cDNA and 1 ng/µl 18S rRNA cDNA prepared from t(1;14)(p22;q32) positive frozen tumor tissues. The average coefficient value ($R^2$) for each standard curve was above 0.99 and the relative efficiency of amplification of *MALT1* and *BCL10* was close to that of 18S rRNA since the absolute value of the slope of log-input amount of cDNA vs. $\Delta C_T$ was below 0.1.

Once the experimental conditions optimised, real-time PCR was performed in a 25 µl reaction-mixture containing 12.5 µl SYBR Green Super-Mix (BIO-RAD), 200 nM of each sense and anti-sense primer, and 100 ng (*MALT1* and *BCL10*) or 1 ng (18SrRNA) cDNA. All samples were amplified in triplicates using following parameters:

denaturation at 95°C for 3 minutes and annealing and extension at 60°C for 1 minute. Real-time PCR of 18S rRNA was run in parallel for each sample. Melt-curve analysis was performed immediately after the amplification protocol for each case and only samples that showed specific amplification were included in the data analysis. The $C_T$ numbers were obtained from each sample and $\Delta C_T$ value was calculated by subtracting the $C_T$ value of 18S rRNA from the $C_T$ value of *MALT1* or *BCL10*.

### 2.2.7 Statistical analysis

One-way Anova and student-test were used to examine the mean difference among different groups. Fisher exact, $\chi 2$, and pearson's correlation were used to analyze the correlation between different groups. Non-parametric Mann-Whitney U-Wilcoxon Rank SumW test was used to evaluate quantitative difference between groups.

# Chapter 3. t(11;18)(q21;q21)/*API2-MALT1*: incidence in MALT lymphoma of various sites and role in gastric MALT lymphoma development

## 3.1 Introduction

This study aimed to screen t(11;18)(q21;q21) in a large series of MALT lymphomas from different sites including the stomach, lung, salivary gland, thyroid, conjunctiva, orbit, skin and liver, as well as in IPSID and examine the incidence of the MALT lymphoma of different sites. In gastric MALT lymphoma, we also correlated the translocation with *CagA* strains of *H. pylori*, which are more virulent and pathogenic[266]. Based on gastric MALT lymphoma, we correlated the translocation with clinical staging and high grade transformation. We also correlated the translocation with the treatment response of gastric MALT lymphoma to *H. pylori* eradication and investigated its value as a molecular marker to identify those that do not respond to *H. pylori* eradication.

## 3.2 Case selection

Two large cohorts of MALT lymphoma cases were studied. The first cohort was consist of 417 cases randomly retrieved from collaborator's institutions (section 2.1.5 of Chapter 2) and used for investigation of the natural incidence of t(11;18)(q21;q21). They were composed of 173 cases from the gastrointestinal tract including 22 cases of IPSID, 47 from lung, 27 from conjunctiva, 28 from orbit, 72 from salivary gland, 18 from thyroid, 27 from skin, 6 from liver, and 19 from other rare sites (Table 3.1). Fresh frozen tissue was available in 72 cases, and the formalin-fixed and paraffin-embedded tissues were available in all cases. Clinical staging was available in 15 cases, whereas the extent of

tumour spread was determined in 26 gastric cases in which sufficient surgical materials were available for histological examination. In addition, frozen tissues were retrieved from 26 cases of mucosal DLBCL including 16 cases from stomach and 6 from lung. Paraffin-embedded tissues were retrieved from 22 cases of lymphoepithelial sialadenitis and 22 cases of Hashimoto's thyroiditis. Fresh-frozen gastric biopsies from 39 patients with gastritis were collected from Hospital of Ancona, Middle Italy. The diagnosis of gastritis was made on histological examination and lymphoid infiltration was mild in 25 cases and severe with aggregated follicles in 14 cases. Gastric ulcer was seen in 3 cases including 2 mild and 1 severe gastritis. *H. pylori* was identified in 33 of 39 gastric biopsies by Warthin Starry staining and histology.

The second cohort was composed of a series of 111 *H. pylori*-positive gastric MALT lymphoma patients, who were first treated with antibiotics alone, retrospectively recruited from Department of Histopathology, Royal Free and University College Medical School and our collaborators's institutions (section 2.1.5 of Chapter 2). They were used for investigation of the value of t(11;18)(q21;q21) in predication of the treatment response of gastric MALT lymphoma to *H. pylori* eradication. The selection of patients was biased towards those who showed no response to *H. pylori* eradication and the proportion *of H. pylori* eradication non-responsive cases from different participating centres was similar. Clinical staging was carried out according to the Ann Arbor system modified by Musshoff in each case prior to therapy[267]. In 64 cases, the extent of lymphoma invasion of the gastric wall and regional lymph nodes was determined by endoscopic ultrasonography, which allowed further division of stage $I_E$ tumours into $I_{E1}$ (restricted to the sub-mucosa) and $I_{E2}$ (extended to mucularis or serosa)[230]. *H. pylori* eradication was achieved by administration of patients with a 2

week course of amoxycillin (3 × 750 mg daily) and omeprazole (3 × 40 mg daily)[224,238,268]. One month after completing the antibiotic therapy, the first gastric endoscopy and biopsy were performed to detect *H. pylori* by histology, culture and PCR of the *H. pylori*-associated urease gene and tumour regression by histology and molecular analysis. These investigations were repeated every 3-4 months until lymphoma showed complete regression or was judged as non-responsive. After achieving complete regression, patients were examined further every 6 months. Lymphomas showing both complete endoscopic and histological regression were regarded as complete remission (CR). Those failed to show histological regression 12 months after successful eradication of *H. pylori* or progressed during follow-up were judged as non-responsive (NR).

Tissue specimens from diagnostic biopsies, including frozen tissues from 22 patients and formalin-fixed and paraffin-embedded tissues from 89 patients were retrieved for molecular analysis. Where indicated, follow-up biopsies were also analyzed.

In all cases, the diagnosis of MALT lymphoma was made according to the histological criteria described by Isaacson[1]. Where necessary, the histology and immunohistochemistry were reviewed by Professor P.G. Isaacson and Professor A. Dogan

## 3.3 Results

### 3.3.1 Establishment of RT-PCR for detection of t(11;18)(q21;q21)

RT-PCR of the *API2-MALT1* fusion transcript was first carried out on frozen tissues in 72 cases of gastric MALT lymphoma and 5 cases of tonsils as negative controls. RT-PCR was successful in all cases. No amplification was seen from all negative control cases. However, 25 of 72 cases of gastric MALT lymphoma were positive. Eleven cases showed a single band and the remaining 14 cases showed one dominant band with up to 4 faint smaller products (Figure 3.1). Direct sequencing or cloning and sequencing of the RT-PCR products confirmed the presence of *API2-MALT1* fusion transcript in all positive cases. The faint bands were alternative splice variants of the fusion transcript.

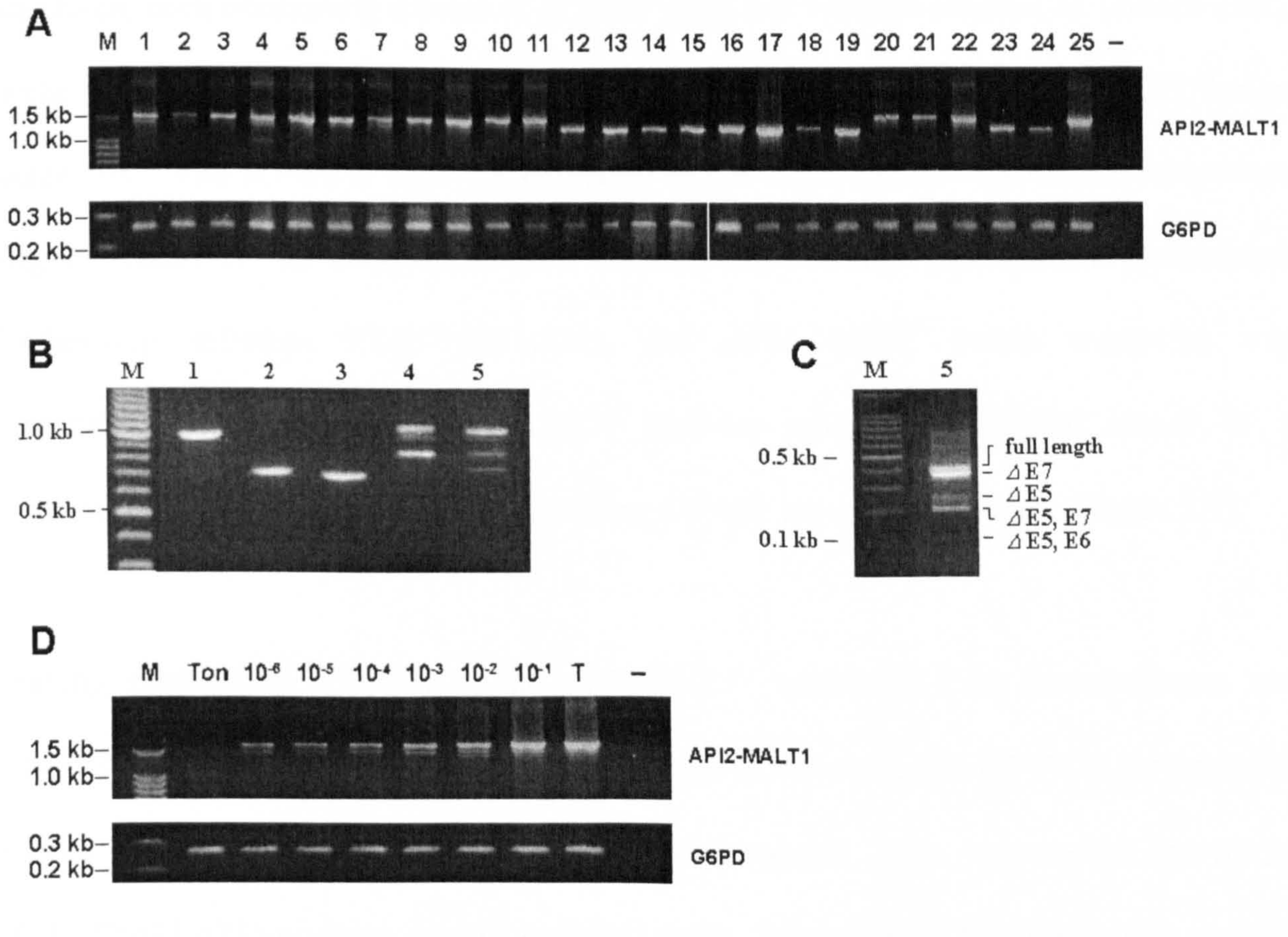



**Figure 3.1. Detection of *API2-MALT1* fusion transcript by RT-PCR from frozen tissue samples**. **A:** 25 t(11;18)(q21;q21) positive MALT lymphoma cases. -, negative control. **B:** Examples of RT-PCR using primer f-S and f-AS. Cases 1, 2 and 3 show a single *API2-MALT1* fusion band and cases 4 and 5 harbour an *API2-MALT1* fusion with breakpoint immediately upstream of the exon 5 of the *MALT1* gene and show alternative splice variants of the fusion transcript. M: molecular weight marker. **C:** RT-PCR with primer p-S and f-AS in case 5 shows alternative splice variants of the *API2-MALT1* fusion. Deleted exons are indicated. **D:**

Sensitivity of RT-PCR for detection of the *API2-MALT1* fusion transcript. Tumour cells harbouring t(11;18)(q21;q21) were serially diluted with tonsillar cells and were then subjected to RNA extraction and RT-PCR. Using the first set of PCR primers (f-S and f-AS), the *API2-MALT1* fusion transcript was detectable when the t(11;18)(q21;q21) positive cells were diluted down to a concentration of 1 in $10^6$ tonsillar cells. M, molecular weight marker; Ton, tonsillar cell; $10^{-6}$to $10^{-1}$, various tumour cell concentration; T, undiluted tumour cells -, negative control

To assess the sensitivity of the RT-PCR for detection of the *API2-MALT1* fusion transcript from fresh tissues, cell suspensions of two gastric MALT lymphomas with different t(11;18)(q21;q21) were serially diluted with tonsillar lymphocytes. The cell mixtures, each containing a total of 2 x $10^6$ cells but variable amount of tumour cells, were subjected to RNA isolation and RT-PCR for the *G6PD* and *API2-MALT1* fusion transcript. The RT-PCR used for detection of the *API2-MALT1* fusion transcript was highly sensitive. In each of the two dilution experiments with MALT lymphomas harbouring different t(11;18)(q21;q21), the *API2-MALT1* fusion transcript was detectable when the t(11;18)(q21;q21) positive cells were diluted down to a concentration of 1 in $10^6$ tonsillar cells using a single set of PCR primers (Figure 3.1).

Having established RT-PCR of the *API2-MALT1* transcript from frozen tissues and identified a number of cases with t(11;18)(q21;q21), we evaluated RT-PCR for detection of the fusion transcript from paraffin-embedded tissues. This was carried out on 20 t(11;18)(q21;q21)-positive and 10 negative cases. In each of t(11;18)(q21;q21) positive cases, RT-PCR based on paraffin-embedded tissues showed specific products from one or two primer sets (Figure 3.2). Some of the PCR products showed one dominant band with up to 3 faint smaller products. Sequencing analysis confirmed that the dominant band was *API2-MALT1* fusion product. The faint bands were alternative splicing

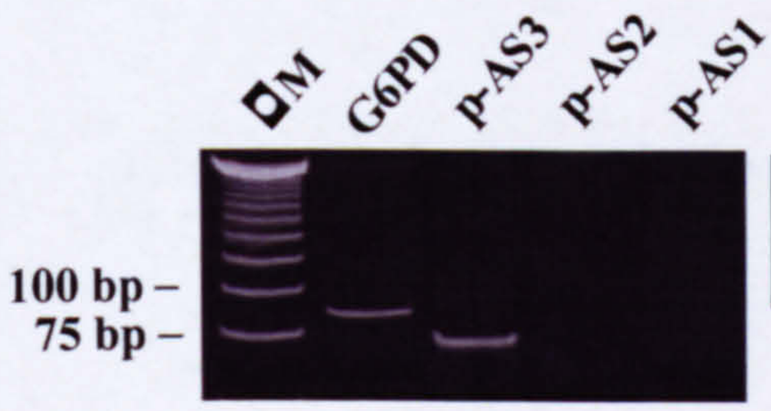
variants. None of the t(11;18)(q21;q21) negative cases showed an amplified product. Given that specific sized products are anticipated from each primer set, analysis of PCR products based on polyacrylamide gels can provide reliable evidence of presence or absence of the translocation in majority cases without the need of sequencing of PCR products. To further determine whether the system can be applied to tissues from small biopsies, we carried out RT-PCR on microdissected cells (100 ~ 200 cells) from 3 t(11;18)(q21;q21) positive cases and the translocation was detected in each occasion. Having established the reliability of the system, we screened unknown cases for t(11;18)(q21;q21). The molecular detection of t(11;18)(q21;q21) was carried out without the knowledge of the clinico-pathological data.


(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAAATGATT
TATTATTAATCCGGAAGAATAGAATGGCACTTTTTCAACATTTGACTTGTGTAATTC
CAATCCTGGATAGTCTACTAACTGCCGGAATTATTAATGAACAAGAACATGATGTT
ATTAAACAGAAGACACAGACGTCTTTACAAGCAAGAGAACTGATTGATACGATTTT
AGTAAAAGGAAATATTGCAGCCACTGTATTCAGAAACTCTCTGCAAGAAGCTGAAG
CTGTGTTATATGAGCATTTATTTGTGCAACAGGACATAAAATATATTCCCACAGAA
GATGTTTCAGAGAAGTGTTGATGGCGTCTCTGAATCC(MALT1-AS2-814)


(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAGAAGTG
TTGATGGCGTCTCTGAATCC(MALT1-AS2-814)
(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAGAATAA
AGATTACTGTAAACCCAGAGTCAAAGGCAGTCTTGG(MALT1-AS1-541)


(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAATAATAAAGAGC
AAACAACTGACCAGCCTTTG(MALT1-AS3-1150)
(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAATGAATTAAATA
ATCTTGGTCATCCTGATAATAAAGAGCAAACAACTGACCAGCCTTTG(MALT1-AS3-1123)
(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAGAAGAACAGATG
AGGCAGTGGAGTGCACTGAAGATGAATTAAATAATCTTGGTCATCCTGATAATAAAGAGCAA
ACAACTGACCAGCCTTTGG(MALT1-AS3-1090)
(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAGATCGAGACAGT
CAAGATAGCAAGAAGGTAGAAATCATCATAGGAAGAACAGATGAGGCAGTGGAGTGCACTG
AAGATGAATTAAATAATCTTGGTCATCCTGATAATAAAGAGCAAACAACTGACCAGCCTTTG
G(MALT1-AS3-993)
(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAGTGCCTTATGTGG
ATTTGGAACACCAAGGAACCTACTGGTGTCATGTATATAATGATCGAGACAGTCAAGATAGC
AAGAAGGTAGAAATCATCATAGGAAGAACAGATGAGGCAGTGGAGTGCACTGAAGATGAAT
TAAATAATCTTGGTCATCCTGATAATAAAGAGCAAACAACTGACCAGCCTTTGG(MALT1-
AS3-894)
(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAGAAGTGTTGATG
GCGTCTCTGAATCCAAGTTGCAAATCTGTGTTGAACCAACTTCCCAAAAGCTGATGCCAGGC
AGCACATTGGTTTTACAGTGTGTTGCTGTTGGAAGCCCTATTCCTCACTACCAGTGGTTCAAA
AATGAATTACCATTAACACATGAGACCAAAAAGCTATACATGGTGCCTTATGTGGATTTGGA
ACACCAAGGAACCTACTGGTGTCATGTATATAATGATCGAGACAGTCAAGATAGCAAGAAGG
TAGAAATCATCATAGGAAGAACAGATGAGGCAGTGGAGTGCACTGAAGATGAATTAAATAA
TCTTGGTCATCCTGATAATAAAGAGCAAACAACTGACCAGCCTTTGG(MALT1-AS3-814)


(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAATGAA
TTAAATAATCTTGGTCATCCTGATAATAAAGAGCAAACAACTGACCAGCCTTTG
(MALT1-AS3-1123)




(API2-sense)GGAAGAGGAGAGAGAAAGAGCAACTGAGGAAAAAGAATCAATAAT
AAAGAGCAAACAACTGACCAGCCTTTG(MALT1-AS3-1150)

**Figure 3.2. Detection of the *API2-MALT1* fusion transcript from paraffin-embedded tissues by RT-PCR**. PCR products derived from primers p-AS2 or p-AS3 show splice variants, which are indicated by white arrowheads. The sequences of API2-MALT1 fusion products are shown on the right side. Letters in blue are from the *API2* gene, while letters in red are from the *MALT1* gene. The letters underlined are primer sequences. M: molecular weight marker.

### 3.3.2 Frequencies of t(11;18)(q21;q21) in MALT lymphoma of different sites

All the cases included in Table 3.1 were successful for RT-PCR of the reference gene *G6PD*. RT-PCR products of the *API2-MALT1* fusion transcript from frozen tissues were further confirmed by sequencing, while those from paraffin-embedded tissues were characteristic for each breakpoint on polyacrylamide gels, allowing confident detection of t(11;18)(q21;q21) without the need of sequencing confirmation in 95% cases as discussed above.

**Table 3.1. Frequency of t(11;18)(q21;q21) in MALT lymphoma of different sites**

| Site of MALT lymphoma | No. of cases | t(11;18)(q21;q21) (%) |
|---|---|---|
| Gastrointestinal tract | | |
| Stomach | 138 | 33 (23.9) |
| Small intestine | 8 | 5 (62.5) |
| IPSID | 22 | 0 (0) |
| Large intestine | 5 | 1 (20) |
| Lung | 47 | 18 (38.3) |
| Conjunctiva | 27 | 5 (18.5) |
| Orbit | 28 | 4 (14.3) |
| Salivary gland | 72 | 1 (1.4) |
| Thyroid | 18 | 0 (0) |
| Skin | 27 | 0 (0) |
| Liver | 6 | 0 (0) |
| Other* | 19 | 0 (0) |
| Total | 417 | 67 (16.2) |

*Including brain (4), bladder (4), tonsil (2), thymus (2), lacrimal (1), eye lid (2), breast (1), gall bladder (1), lip (1), and ovary (1).

The cases included for the purpose of this investigation were selected sequentially from the contributor's archive and thus the frequency of t(11;18)(q21;q21) found in MALT lymphoma of different sites reflected their natural incidences (Table 3.1). Of the 8 major sites from which MALT lymphoma commonly arises, t(11;18)(q21;q21) was found at the highest frequencies in those derived from the lung (38.3%) and stomach (23.9%), and at a moderate frequencies in those from the conjunctiva (18.5%) and orbit (14.3%). The translocation was seen in only 1 case of salivary gland MALT lymphoma (1.4%) but was absent in those from the thyroid, skin, liver and other rare sites, as well as in IPSID.

t(11;18)(q21;q21) was not found in 39 cases of *H. pylori* associated gastritis, 22 cases of lymphoepithelial sialadenitis and 22 cases of Hashimoto's thyroiditis, the preceding disease associated with gastric, salivary gland and thyroid MALT lymphomas respectively.

### 3.3.3 t(11;18)(q21;q21) positive gastric MALT lymphoma is significantly associated with *CagA* stains of *H. pylori*

Because *CagA* positive strains of *H. pylori* are more virulent and pathogenic, we correlated t(11;18)(q21;q21) with *CagA* status in gastric MALT lymphomas. DNA samples from all gastric MALT lymphomas were first screened for the presence of *H. pylori* by amplification of the urease gene and the positive samples were subsequently subjected to PCR of the *CagA* gene. Overall, *CagA* positive strains of *H. pylori* were found in 28 of the 42 cases (67%) of gastric MALT lymphoma in which PCR of the *H. pylori urease* gene was positive. *CagA* positive strains of *H. pylori* were significantly

higher in t(11;18)(q21;q21) positive gastric MALT lymphoma (14/15=93.3%) than the translocation negative cases (14/27=51.9%) ($p<0.01$).

### 3.3.4 Neutrophil infiltration in MALT lymphoma preceding diseases and its implication in occurrence of t(11;18)(q21;q21)

In view of the finding that t(11;18)(q21q;21) occurred at markedly variable frequencies in MALT lymphomas of different sites, it is highly likely that the occurrence of the translocation is influenced by the preceding disease associated with MALT lymphomas. In gastric MALT lymphoma, t(11;18)(q21;q21) is significantly associated with *CagA* positive strains of *H. pylori* that are strong inducers of interleukin-8, a potent chemokine for neutrophil activation[266]. Activated neutrophils are known to release reactive oxygen species, which can cause a wide range of DNA damage including double strand breaks[90]. We therefore examined the extent of neutrophil infiltration in *H. pylori* associated gastritis, lymphoepithelial sialadenitis and Hashimoto's thyroiditis to further understand whether there is any difference in the exposure of potential genetic insults among these pre-malignant diseases. As expected, neutrophil infiltration was significantly higher in *H. pylori* associated gastritis (8.19±0.92 neutrophils/high power filed) than in lymphoepithelial sialadenitis (0.09±0.03 neutrophils/high power field) and Hashimoto's thyroiditis (0.38±0.04 neutrophils/high power filed) ($p<0.01$ for both) (Figure 3.3).

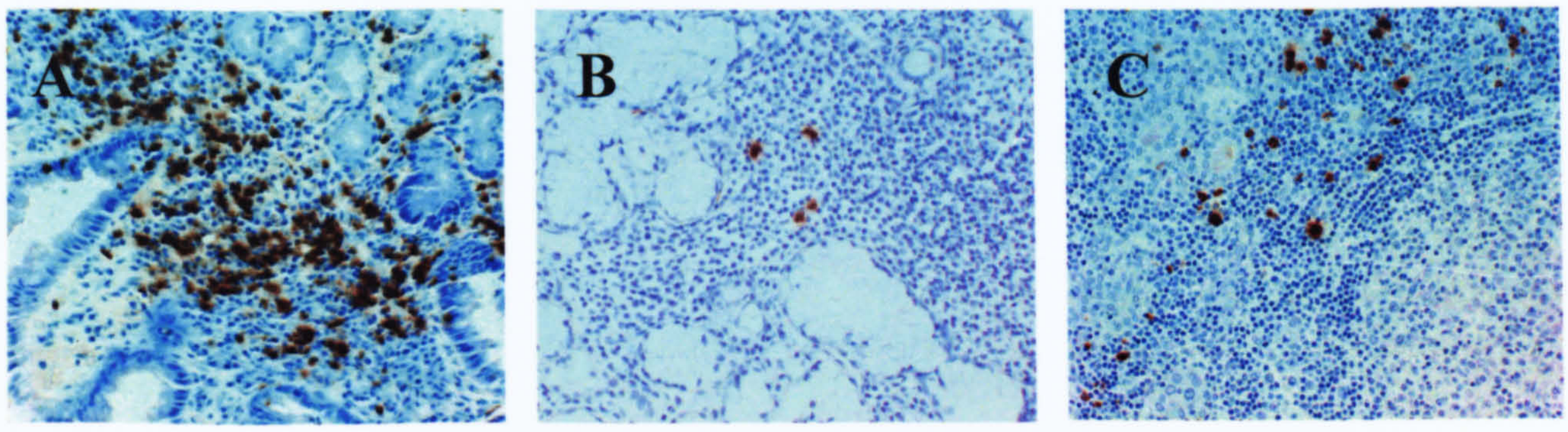



**Figure 3.3. Neutrophil infiltration in MALT lymphoma preceding diseases.** Neutrophil infiltration in *H. pylori* associated gastritis (A), lymphoepithelial sialadenitis (B) and Hashimoto's thyroiditis (C).

### 3.3.5 t(11;18)(q21;q21) is associated with gastric MALT lymphoma at advanced stage

Among various groups of MALT lymphoma examined for t(11;18)(q21;q21), those from the stomach constituted the largest group. In total, 249 cases of gastric MALT lymphoma were studied and the extent of tumour spread could be assessed by clinical staging or histological examination of surgical resected specimens in 158 cases. We corrected t(11;18)(q21;q21) with clinical stage of gastric MALT lymphoma in order to understand its role in the multistage development of this tumour.

Of the 158 cases with clinical staging, the translocation was found in 32/45 (71%) cases at stage $II_E$ or above, but only in 29/113 (26%) cases at stage $I_E$ (Figure 3.4). Statistical analyses showed that t(11;18)(q21;q21) was significantly associated with cases at advanced stages than those at early stage ($P<0.0001$, $\chi^2$ test). Despite that t(11;18)(q21;q21) is significantly associated with MALT lymphoma at advanced stage, the translocation was not detected in 26 DLBCL including 16 and 6 cases from the

stomach and lung, respectively, where the translocation was most frequently seen in MALT lymphoma.

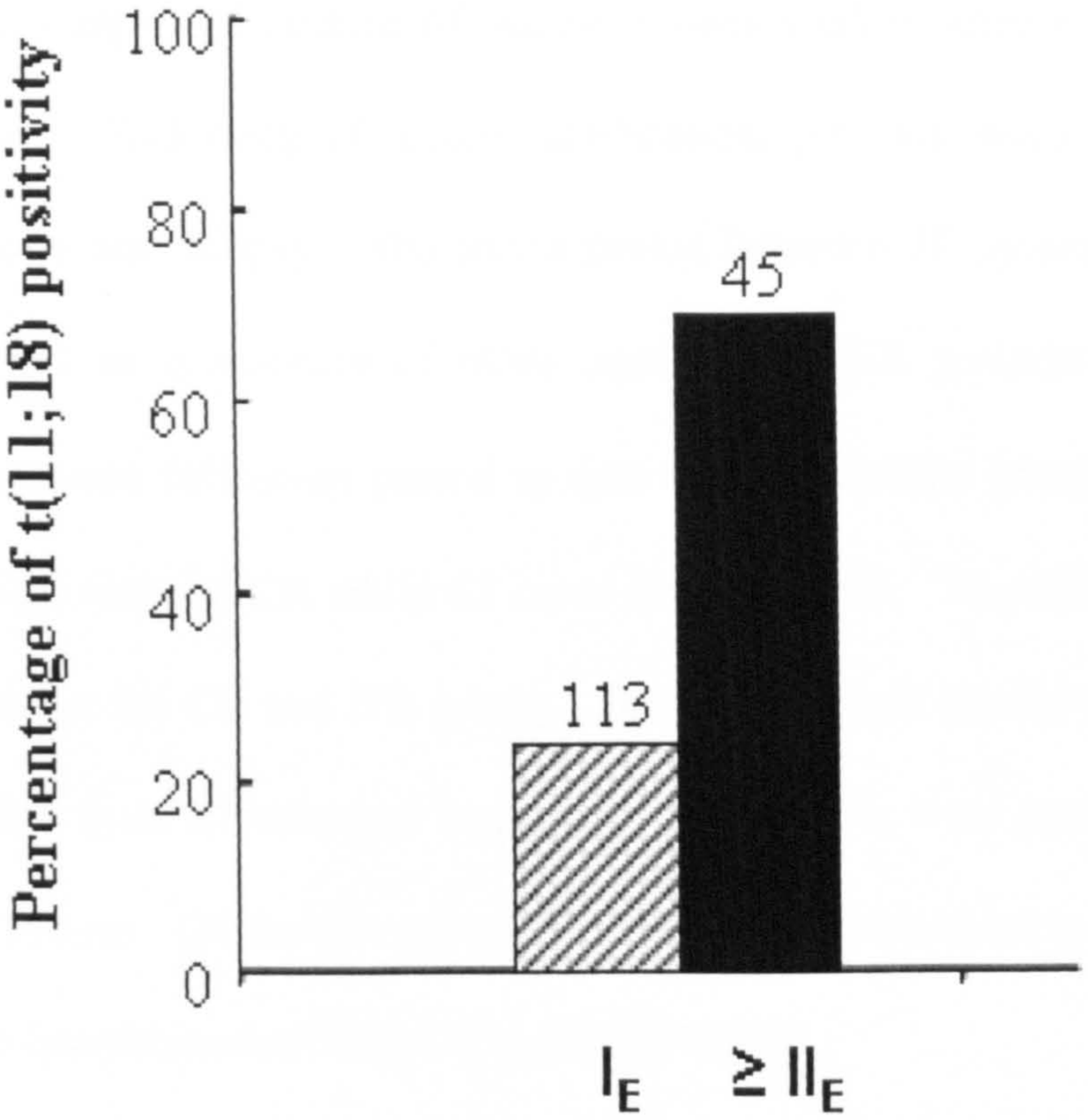



**Figure 3.4. Correlation between clinical staging of gastric MALT lymphoma and t(11;18)(q21;q21).** The number of cases in individual subgroups is indicated. $p<0.005$.

### 3.3.6 t(11;18)(q21;q21) is a marker for gastric MALT lymphomas that do not respond to *H. pylori* eradication

This investigation was carried out on the second cohort of gastric MALT lymphomas, for which *H. pylori* eradication was used as the first line treatment and clinical follow up data were available.

To examine whether t(11;18)(q21;q21) bears any value in predication of the treatment response of gastric MALT lymphoma to *H. pylori* eradication, we investigated the translocation in 111 cases of *H. pylori* positive gastric MALT lymphoma treated with

antibiotics. The clinical and histological features of these cases with stage $I_E$ are summarised in Table 3.2. *H. pylori* infection was successfully cured in all cases as confirmed by histology and culture of gastric biopsies taken after completion of the antibiotic therapy. Following *H. pylori* eradication, patients were followed up by repeated endoscopy and biopsy. The mean period between *H. pylori* eradication and achievement of CR or commence of other treatment in NR patients was 12 months (range 1-75) and mean follow-up period to date was 35 months (range 9-85). During follow-up, 48 cases showed CR while 63 cases displayed NR. There is no difference in age and sex between the CR and NR group. Both groups had similar length of follow-up. Histologically, focal transformed high grade components were seen in 3 NR but not in any of the CR cases. Of the CR group, 2 of the 48 cases showed tumour relapse and in both cases the lymphoma harboured t(11;18)(q21;q21).

**Table 3.2. Clinical and histopathological features of stage $I_E$ gastric MALT lymphomas and their responses to *H. pylori* eradication therapy.**

| | Complete regression | No regression |
|---|---|---|
| Number of patients | 47 | 43 |
| Age (years) | | |
| Mean | 60 | 57 |
| Range | 25-85 | 30-88 |
| Sex | | |
| M | 29 | 21 |
| F | 18 | 22 |
| Histology with high grade component | 0 | 3 |
| Stages by endoscopic ultrasonography | | |
| $I_{E1}$ | 29 | 30 |
| $I_{E2}$ | 3 | 2 |
| Follow-up period (months) | | |
| Intervals* | | |
| Mean | 8.2 | 15 |
| Range | 1-26 | 5-75 |
| Follow-up to date | | |
| Mean | 38 | 30 |
| Range | 10-82 | 9-85 |

*The time between *H. pylori* eradication and complete regression or commence of other treatment in non-responsive cases.

Among the 48 CR cases, 47 were at stage $I_E$ with only the remaining one being at stage $II_E$. The stage $II_E$ CR case is one of the two that showed lymphoma relapse. Of the 63 NR cases, 20 were at stage $II_E$ or above and the remaining 43 cases were at stage $I_E$. Despite that the vast majority of lymphomas at stage $II_E$ or above (20/21=95%) did not respond to *H. pylori* eradication ($P<0.001$), there were almost half of stage $I_E$ tumours

which also did not respond to *H. pylori* eradication (43/90=48%, $P>0.05$). Therefore, the staging failed to predict the response of stage $I_E$ gastric MALT lymphoma to *H. pylori* eradication. Among cases with stage $I_E$ lymphoma, there was no difference in age, sex and follow-up periods between CR group and NR groups ($P>0.05$) (Table 3.2). The extent of lymphoma invasion within the gastric wall was assessed by endoscopic ultrasonography in 64 cases with stage $I_E$ lymphoma. There was no difference in the response of gastric MALT lymphoma to *H. pylori* eradication between cases showed stage $I_{E1}$ and stage $I_{E2}$ disease ($P>0.05$) (Table 3.2).

Detection of t(11;18)(q21;q21) was carried out by RT-PCR of the *API2-MALT1* fusion transcript from frozen tissues in 22 cases and from paraffin-embedded tissues in 89 cases. All 111cases showed successful RT-PCR of the reference gene *G6PD*. In majority of cases, the characteristic PCR product pattern on polyacrylamide gels allowed confident detection of t(11;18)(q21;q21). However, in 13 cases, PCR bands were weak and sequencing confirmation was carried out. Overall, t(11;18)(q21;q21) was positive in 40% (44 of 111) of cases detected.

Of the 48 CR cases, 2 were t(11;18)(q21;q21) positive (Figure 3.5). One of these 2 cases, a stage $I_E$ tumour, achieved CR 25 months after *H. pylori* eradication. The remission lasted for 56 months but tumour reoccurred later in the absence of *H. pylori* re-infection. PCR of the rearranged *Ig* gene confirmed the clonal lineage between the original lymphoma and the recurrence. t(11;18)(q21;q21) was detected only in follow-up biopsies showing the tumour relapse but not in those that displayed complete remission. The other t(11;18)(q21;q21)-positive case was a stage $II_E$ tumour, in which CR was achieved 9 months after *H. pylori* eradication and the remission was maintained so far

for 32 months. However, t(11;18)(q21;q21) was detected in the last follow-up biopsy. A review of histology of the biopsy revealed a small crushed fragment of lymphoid tissue suspicious of tumour relapse. *H. pylori* was not seen.

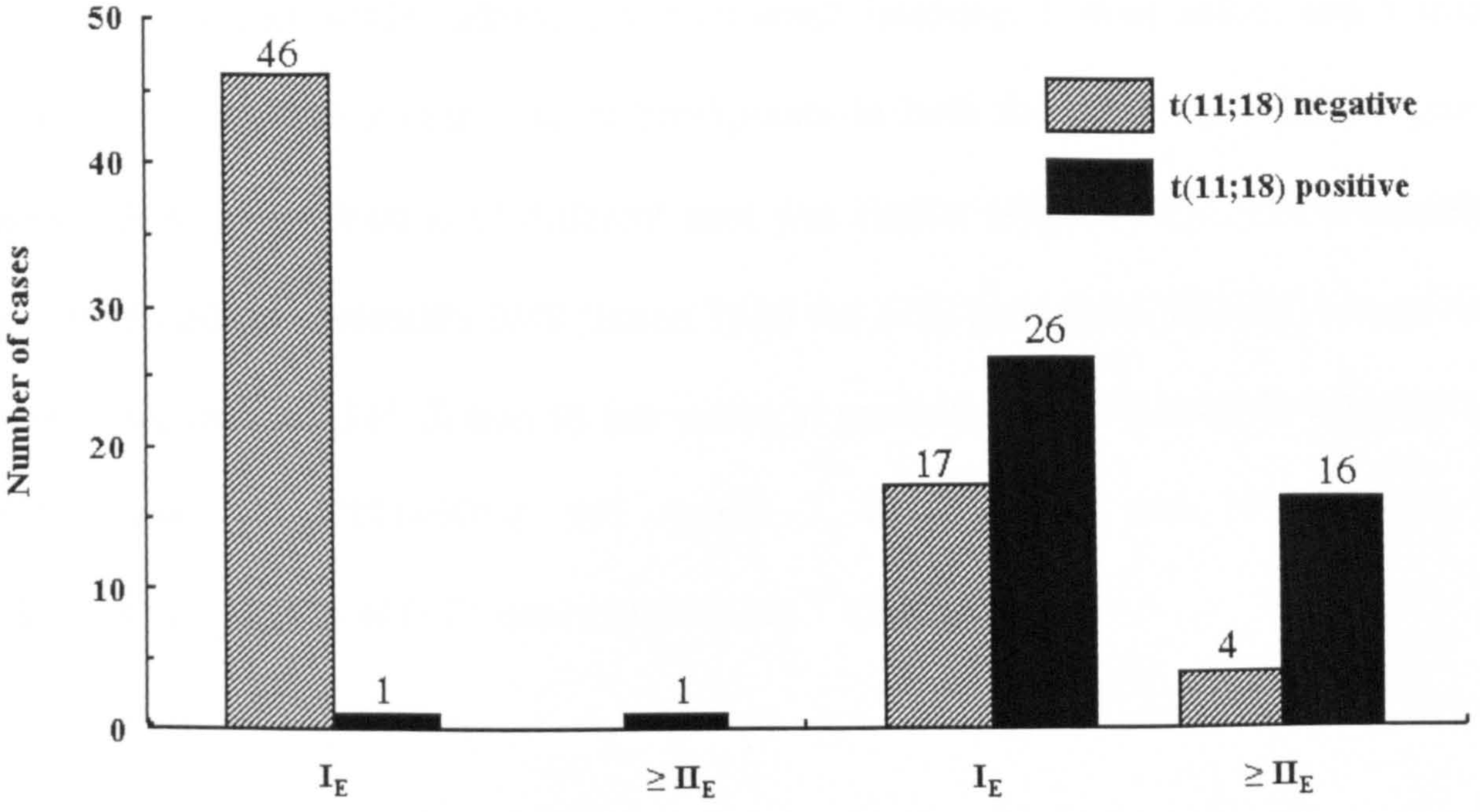



**Figure 3.5. Correlation between response of gastric MALT lymphoma to *H. pylori* eradication therapy and clinical staging and presence of t(11;18)(q21;q21).** Clinical staging has little value in predication of the response of stage $I_E$ gastric MALT lymphoma to *H. pylori* eradication therapy. In contrast, the translocation can predict 60% of *H. pylori* therapy non-responsive cases at stage $I_E$.

In contrast to the CR group, 42 of the 63 (67%) NR cases were positive for the translocation, including 26 of the 43 (60%) stage $I_E$ tumours (Figure 3.5). Thus, t(11;18)(q21;q21) could predict the response of the majority of early gastric MALT lymphomas to *H. pylori* eradication ($P<0.001$). As expected, the frequency of t(11;18)(q21;q21) was much higher in lymphomas at stage $II_E$ or above (16/20=80%) than those at stage $I_E$ ($P<0.001$) (Figure 3.5).

### 3.3.7 Characteristics of t(11;18)(q21;q21) breakpoint

In total, there were 111 t(11;18)(q21;q21) positive cases including 77 from stomach, 18 from lung, 9 from ocular adnexae, 5 from small intestine, 1 from colon, and 1 from salivary gland. The distribution of breakpoints in both the *API2* and *MALT1* genes among MALT lymphomas of different sites was similar (Figure 3.2). The breakpoint was invariable at nucleotide 2048 (intron 7) on the *API2* gene (NM_001165) except one case at nucleotide 2345 (intron 9) but varied at nucleotides 413 (intron 2, 2/111=2%), 715 (intron 4, 67/111=60%), 991 (intron 7, 23/111=21%) and 1018 (intron 8, 18/111=16%) on the *MALT1* gene (AB026118)[107] (Figure 3.6).

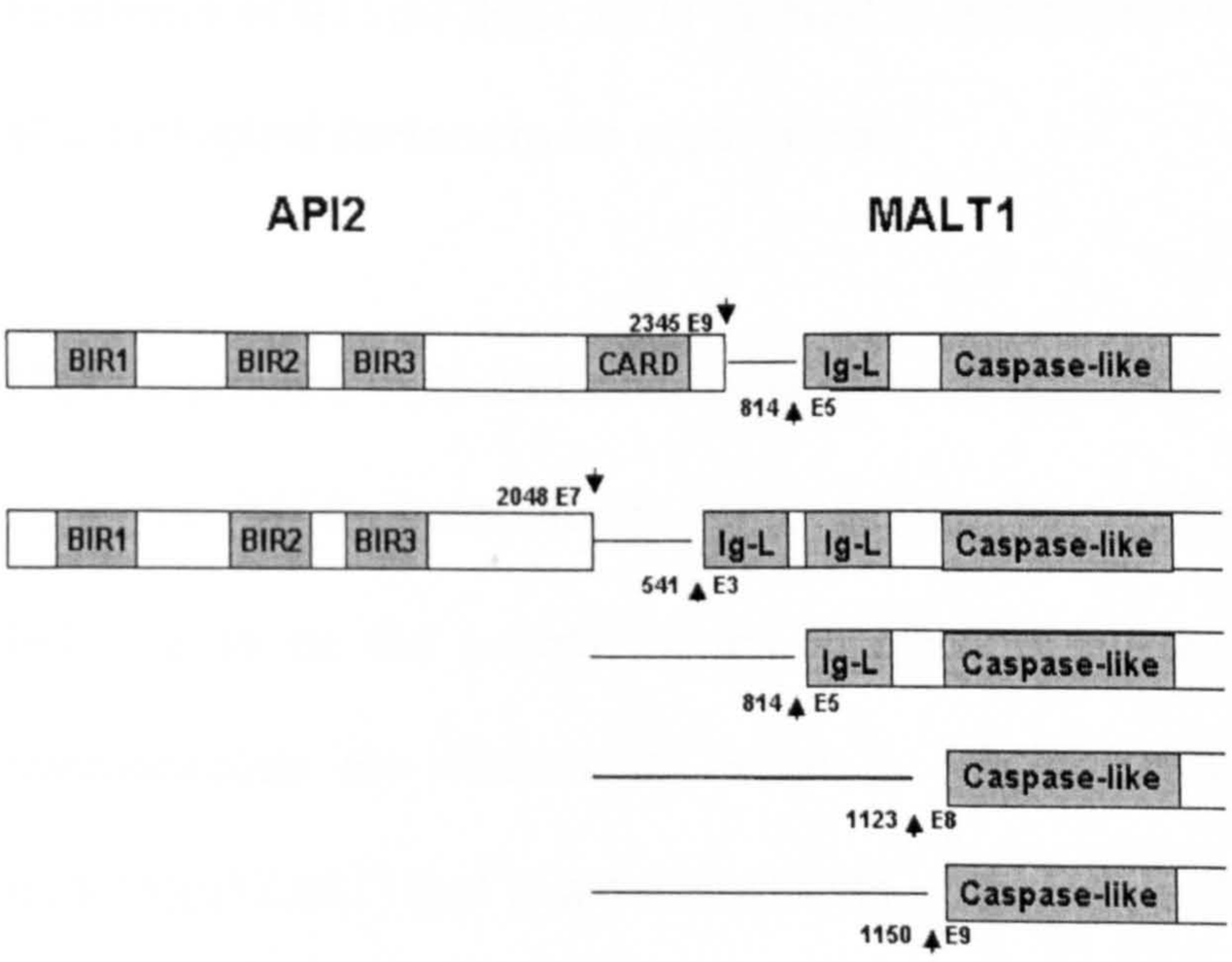



**Figure 3.6. Characteristics of t(11;18)(q21;q21) breakpoints.** Representative *API2-MALT1* fusion products detected by RT-PCR from paraffin-embedded tissues are illustrated schematically and their breakpoint and frequency of occurrence are shown.

Because the *API2-MALT1* fusion products with intact Ig-like C2 domains are more potent activators of NFκB than those without Ig-like C2 domains[123] and therefore may be more oncogenic[125], we correlated the type of *API2-MALT1* fusion with clinical staging in gastric MALT lymphoma. Of the 44 t(11;18)(q21;q21) positive cases for which clinical staging was available, 33 fusion transcripts had one or two intact Ig-like C2 domains, while 11 did not contain Ig-like C2 domains (Figure 3.3). Tumours bearing the fusion product with intact Ig-like C2 domains (22/43=51%) were more often at stage $II_E$ or above than those harbouring the fusion product without Ig-like C2 domains (5/18=28%) although statistical analysis did not reveal any significant difference ($P$>0.05).

## 3.4 Discussion

### Incidence of t(11;18)(q21;q21) in MALT lymphoma of various sites and implication of aetiological factors in its occurrence

Chromosomal translocations associated with B cell lymphomas commonly involve the Ig locus, which most likely occur during the VDJ recombination process and are believed to be the primary event in lymphomagenesis[269]. At least some of these translocations are known to occur in pre-lymphomatous lesions. For example, t(14;18)(q32;q21) that is associated with up to 90% follicular lymphomas has also been found in about 50% lymphoid hyperplasias and peripheral blood lymphocytes from normal individuals[270-272]. To examine whether t(11;18)(q21;q21), a frequent translocation in MALT lymphoma[104,107,108,119], is present in pre-lymphomatous lesions, we examined *H. pylori*-associated gastritis from Italy where the frequency of *H. pylori*

associated gastric MALT lymphoma is high[63] and lymphoepithelial sialadenitis and Hashimoto's thyroiditis, the preceding disease associated with salivary gland and thyroid MALT lymphomas respectively. Our results indicate that t(11;18)(q21;q21) is absent or at least not a frequent event in *H. pylori* associated gastritis, lymphoepithelial sialadenitis and Hashimoto's thyroiditis.

Previous studies showed t(11;18)(q21;q21) in 35-50% of gastric MALT lymphomas and 55-75% of pulmonary MALT lymphomas, but the incidence of t(11;18)(q21;q21) in MALT lymphoma of other sites is largely unknown as only limited cases have been examined[106-109,119]. By screening 417 cases of MALT lymphoma of 9 major sites for t(11;18)(q21;q21), and we showed that the translocation occurred at the highest frequencies in those from the lung (38.3%) and stomach (23.9%) and at moderate frequencies in those from the conjunctiva (18.5%) and orbit (14.3%). The translocation was present in only a single example of salivary gland MALT lymphoma but was absent in those from the thyroid, skin, liver and other rare sites, and in IPSID.

The incidence of t(11;18)(q21;q21) in both pulmonary and gastric MALT lymphoma in the present study is much lower than reported previously even taking into account the fact that our RT-PCR approach for paraffin-embedded tissues would miss approximately 7% of rare breakpoints on the *API2* gene. The number of cases studied in the previous reports was relatively small and the investigations were commonly based on those treated by surgery and were therefore biased towards the advanced cases. At least in gastric MALT lymphoma, t(11;18)(q21;q21) has been shown to be significantly associated with cases of more advanced stage. Thus, the previous studies may have over-estimated the incidence of t(11;18)(q21;q21) in pulmonary and gastric MALT

lymphomas. In the present study, the cases included were randomly selected and a large cohort were examined and thus the frequency of t(11;18)(q21;q21) reported here should be much closer to its natural incidence. In line with this, the frequencies of t(11;18)(q21;q21) in MALT lymphoma of various sites as shown in this study are very similar to those reported by a recent study in which 252 cases of MALT lymphoma randomly selected were studied[111]. The t(11;18)(q21;q21) was found at the highest frequencies in MALT lymphomas derived from the lung (53%) and stomach (24%), at a moderate frequencies in those from the intestine (13%), and rarely in those from the salivary gland (2%), liver and other rare sites[111].

The higher incidence of the translocation in pulmonary MALT lymphoma may be in part the result of the disease being diagnosed at relatively more advanced stages. Approximately 50% of patients with pulmonary MALT lymphoma are asymptomatic and 25-47% of cases are at stage III or above at the time of diagnosis[273,274]. In contrast, patients with gastric MALT lymphoma commonly present upper stomach discomfort and only 13% of cases are at stage III or above at diagnosis[273].

Unlike the majority of chromosomal translocations associated with lymphoma, t(11;18)(q21;q21) does not involve the *Ig* locus and its occurrence is most likely not associated with the VDJ recombination event. The finding of dramatically variable incidences of t(11;18)(q21;q21) in MALT lymphoma of various sites indicates that the occurrence of the translocation is influenced by different preceding diseases associated with MALT lymphoma. *H. pylori* associated gastritis is strongly related to the development of gastric MALT lymphoma, while lymphoepithelial sialadenitis and Hashimoto's thyroiditis are closely associated with the genesis of salivary gland and

thyroid MALT lymphoma, respectively. The mechanisms underlying the pathogenesis of these diseases are different. Lymphoepithelial sialadenitis and Hashimoto's thyroiditis are principally due to generation of auto-reactive B cells, while *H. pylori* infection causes damage of gastric mucosa through bacterial toxins and host responses. *CagA* positive strains of *H. pylori* are known to be more virulent and pathogenic and are significantly associated with increased risk of development of gastric cancer and peptic ulcer.

The role of infection of *CagA* strains of *H. pylori* in the development of gastric MALT lymphoma remains unclear since controversial results have been reported[87-89]. To examine whether the occurrence of t(11;18)(q21;q21) is related to aetiological factors, we first correlated the translocation with *CagA* status in gastric MALT lymphoma. Our results showed that *CagA* positive strains of *H. pylori* were significantly associated with t(11;18)(q21q;21), suggesting that *CagA* positive strains of *H. pylori* may be highly potent in promoting the occurrence of t(11;18)(q21;q21). *H. pylori* strains harbouring the *CagA* island may cause strong inflammatory responses, of which the key element is induction of interleukin-8, a potent chemokine for neutrophil activation[266]. Activated neutrophils release reactive oxygen species, which can cause a wide range of DNA damage including double strand breaks[90]. It is possible that the occurrence of t(11;18)(q21;q21) is related to oxidative damage induced by *H. pylori* infection. In line with this hypothesis, the genomic breakpoints of t(11;18)(q21;q21) on both derivative chromosomes were random and showed no association with sequence motifs known to be associated with chromosomal recombination[126,262]. Furthermore, deletions ranging from a few to several kilo-base pair is a common finding at the breakpoint for both the *API2* and *MALT1* loci[126,262].

To further examine whether there is any difference in the potential exposure of genetic insults among different pre-malignant diseases associated with MALT lymphoma, we examined the extent of neutrophil infiltration in *H. pylori* associated gastritis, lymphoepithelial sialadenitis and Hashimoto's thyroiditis. As expected, neutrophil infiltration was prominent in *H. pylori* associated gastritis but not in lymphoepithelial sialadenitis and Hashimoto's thyroiditis. The difference in neutrophil infiltration among these pre-malignant diseases correlated well with the incidence of t(11;18)(q21;q21) detected in prospective MALT lymphoma. Thus, it is possible that the presence or absence of genotoxic factors such as activated neutrophils in the pre-malignant disease may influence the incidence of t(11;18)(q21;q21) in prospective MALT lymphoma.

The above hypothesis may also explain the finding of a high incidence of t(11;18)(q21;q21) in pulmonary MALT lymphoma. Acquired MALT in the lung is seen in the inflammatory disease known as follicular bronchiolitis[275]. Although the etiology of follicular bronchiolitis is unknown, in at least 50% of cases, histological examination shows suppurative exudates in bronchiolar lumina and neutrophils in adjacent alveoli[275], suggesting that genotoxic factors could well be present.

**t(11;18)(q21;q21) is associated with gastric MALT lymphoma at advanced stage and those failed to respond to *H. pylori* eradication**

To understand the role of t(11;18)(q21;q21) in multistage development of MALT lymphoma, we correlated the translocation with clinical stage of gastric MALT lymphomas and their treatment response to *H. pylori* eradication. In line with the

expected oncogenic role of t(11;18)(q21;q21), we showed that the translocation was significantly associated with gastric MALT lymphoma at advanced stage and those failed to respond to *H. pylori* eradication.

*H. pylori* eradication leads to complete regression of gastric MALT lymphoma in 75% of cases and is widely accepted as the first line treatment for this tumour[224,228-230,238,239,268]. One of the major dilemmas in clinical management of patients with this disease is the identification of those that will not respond to *H. pylori* eradication and require chemo- or radiotherapy. At present, this requires prolonged follow-up with repeated endoscopy and gastric biopsy. Clinical staging is helpful in predicting the response since lymphomas at stage $II_E$ or above rarely respond to *H. pylori* eradication. However, the predictive value of clinical staging for stage $I_E$ tumours is limited[224,228-230,238,239,268] and better prognostic markers are needed. We have shown that t(11;18)(q21;q21) is a marker for non-responsive gastric MALT lymphomas including those at stage $I_E$. In the stage $I_E$ cases, the translocation allows this prediction in 60% of non-responsive cases. None of the CR cases were positive for t(11;18)(q21;q21) with the exception of the 2 equivocal cases described.

Our findings indicate that t(11;18)(q21;q21)-positive gastric MALT lymphomas do not undergo regression following *H. pylori* eradication and require other conventional therapies up front. However, Fischbach reported that 4 cases of gastric MALT lymphomas bearing t(11;18)(q21;q21) responded to *H. pylori* eradication with durable remission[276]. Nevertheless, *H. pylori* should be eradicated in all cases as this not only eliminates reactive lymphoid infiltrates but most likely has an adjuvant effect since *in vitro* experiments have shown that *H. pylori* also stimulates t(11;18)(q21;q21)-positive

lymphoma cells to proliferate via T cell help[73,263]. Moreover, eradication of *H. pylori* and reactive lymphoid infiltrates may reduce the risk of developing secondary tumours in the stomach.

Among the NR cases, 33% failed to show t(11;18)(q21;q21) by RT-PCR. Our RT-PCR strategy for frozen tissues would theoretically detect 100% of known breakpoints in both the *API2* and *MALT1* genes. However, the RT-PCR methodology for paraffin-embedded tissues would miss three minor *API2* breakpoints, which account for 7% of the total API2-MALT1 fusions[101-104,106-109,119]. Thus, our current results may slightly underestimate the true frequency of t(11;18)(q21;q21) in *H. pylori* eradication non-responsive gastric MALT lymphoma. For prospective clinical screening, PCR with primers for these minor breakpoints should be included and multiplex amplification in a single tube may offer a practical approach[277]. Alternatively, the translocation can be detected by interphase FISH.

In about 25% of cases, resistance of gastric MALT lymphoma to *H. pylori* eradication appears to be due to other factors. MALT lymphomas with chromosomal translocation involving the *BCL10* locus, such as t(1;14)(p22;q32)[134,135] and t(1;2)(p22;p12)[136], are typically those at advanced stages and are unlikely to respond to *H. pylori* eradication[142] (Chapter 4). *H. pylori* associated gastric MALT lymphoma in patients with autoimmune disease has been shown to be resistant to antibiotic treatment[278]. The *fas* gene is frequently mutated in MALT lymphoma in patients with autoimmunity[214] and *fas* gene mutations may confer resistance of gastric MALT lymphoma to *H. pylori* eradication.

In view of the significant role of t(11;18)(q21;q21) during MALT lymphoma progression, it is intriguing that the translocation is only rarely found in transformed MALT lymphoma [104,107-110,279]. Although mucosal DLBCL may arise *de novo*, at least a proportion of mucosal DLBCL are transformed from low grade MALT lymphomas[53,54]. It is unlikely that such an important translocation is lost during high grade transformation. Alternatively, t(11;18)(q21;q21) positive MALT lymphoma is a distinct subgroup of this lymphoma entity that does not or rarely undergoes high grade transformation. There are clear differences in both gross chromosomal and microsatellite alterations between t(11;18)(q21;q21) positive and negative MALT lymphomas: the former do not usually show any chromosomal aberrations other than t(11;18)(q21;q21) and allelic imbalances, whereas the latter including those with t(1;14)(p22;q32) display various abnormalities including both recurrent and rare aberrations and frequently allelic imbalances[97,98,133,171]. It remains to be determined whether further differences in histology, immunophenotype and genetic abnormalities such as genetic instability exist between the two groups.

**Characteristics of t(11;18)(q21;q21) breakpoint and their implication in the oncogenetic activity of the API2-MALT1 fusion product**

All the breakpoints in the *API2* gene occur between the third BIR domain and the C-terminal RING, with 91% being just upstream of the CARD[104,107-109,119]. In contrast, the breakpoints in the *MALT1* gene are more variable but are always upstream the carboxyl caspase-like domain[104,107-109,119]. Thus, the resulting *API2-MALT1* fusion transcripts always comprise the amino terminal *API2* with three intact BIR domains and the carboxyl terminal *MALT1* region containing an intact caspase-like domain. The specific

selection of these domains of the *API2* and *MALT1* genes to form a fusion product strongly suggests their importance and synergy in oncogenesis. The BIR domain of *API2* has been shown to be anti-apoptotic[120]. However, the anti-apoptotic activity of the *API2* BIR domain was weak and has been shown to be suppressed by its C-terminal RING finger domain[120]. As a result, wild type API2 did not protect cells from apoptosis upon stimulation by death signals[120]. The negative effect of the RING finger on BIR function may be associated with its ability to promote auto-ubiquitination and degradation[118,120]. Replacement of the C-terminal of API2 with the C-terminal of MALT1 by the fusion product would release the intrinsic anti-apoptotic activity of the BIR domain and therefore make the new molecule anti-apoptotic. Indeed, the API2-MALT1 fusion product, but not API2 or MALT1 alone, has been shown to activate NFκB and the caspase-like domain is required for this function[123]. Moreover, the fusion products with intact Ig-like C2 domains are more potent activators of NFκB than those without Ig-like C2 domains[123,125]. In keeping with this, we found that tumours bearing the fusion product with one or two intact Ig-like C2 domains were more often at stage $II_E$ or above than those harbouring the fusion without the Ig-like C2 domain.

## 3.5 Conclusion

In summary, our results demonstrate that t(11;18)(q21;q21) occurs at markedly variable frequencies in MALT lymphoma of different sites, suggesting that the occurrence of the translocation is influenced by the nature of pre-malignant diseases associated with MALT lymphoma. Oxidative damage might play a role in development of t(11;18)(q21;q21). In gastric MALT lymphoma, t(11;18)(q21;q21) occurs more frequently in those at stage $II_E$ or above than cases at stage $I_E$. Irrespective of its

association with staging, the translocation is a reliable marker for all stage gastric MALT lymphomas that do not respond to *H. pylori* eradication.

# Chapter 4. t(1;14)(p22;q32)/*BCL10-IGH* and BCL10 deregulation in MALT lymphoma

## 4.1 Introduction

This study attempted to study BCL10 protein expression pattern in normal and malignant lymphoid tissues, particularly those with t(1;14)(p22;q32) and investigate the incidence of t(1;14)(p22;q32) in MALT lymphoma of various sites and its role in multistage development of gastric MALT lymphoma.

## 4.2 Case selection

Formalin-fixed and paraffin-embedded tissue specimens from 463 cases of lymphomas comprising of 331 MALT lymphomas (Table 4.1), 20 mucosal DLBCLs (14 of them with low grade MALT lymphoma component), 21 follicular lymphomas, 17 mantle cell lymphomas, 18 nodal DLBCLs. 31 normal lymphoid tissues including 8 fetal thymuses at 16-40 weeks of gestation, 4 appendices, 8 tonsils, 6 lymph nodes and 5 spleens, and 74 normal non-lymphoid tissues of 21 different types were retrieved from the surgical files of Department of Histopathology, Royal Free and University College Medical School and our collaborators's institutions (section 2.1.5 of Chapter 2). Among MALT lymphoma cases, 4 cases were known positive for t(1;14)(p22;q32)[133,280] and 1 case positive for t(1;2)(p22;p12)[136] by conventional cytogenetic investigation. The histology of all lymphoma cases was reviewed by specialised haematopathologists: Professor P.G. Isaacson and Prof. Ahmet Dogan.

111 cases of *H. pylori*-positive gastric MALT lymphomas initially treated with antibiotics alone were retrospectively retrieved from the surgical files of Department of Histopathology, Royal Free and University College Medical School and our collaborators's institutions (section 2.1.5 of Chapter 2).

**Table 4.1. Frequency of BCL10 nuclear expression in MALT lymphoma of various sites**

| Site of MALT Lymphoma | No. of cases | BCL10 nuclear expression | | |
|---|---|---|---|---|
| | | Strong like in t(1;14) (%) | Moderate with API2-MALT1 Fusion (%) | Moderate but lacking API2-MALT1 fusion (%) |
| Gastrointestinal tract | | | | |
| Stomach | 123 | 6 (4.9) | 30 of 30 (100) | 26 of 87 (30) |
| Small intestine | 4 | 0 | 1 of 1 (100) | 0 of 3 (0) |
| IPSID | 5 | 0 | - | 1 of 5 (20) |
| Large intestine | 3 | 0 | 1 of 1 (100) | 0 of 2 (0) |
| Lung | 41 | 5 (12) | 17 of 17 (100) | 5 of 19 (26) |
| Conjunctiva | 19 | 0 | 3 of 3 (100) | 8 of 16 (50) |
| Orbit | 19 | 0 | 3 of 3 (100) | 1 of 15 (6.7) |
| Salivary gland | 70 | 0 | 1 of 1 (100) | 15 of 69 (21.7) |
| Thyroid | 14 | 0 | – | 0 of 14 (0) |
| Skin | 13 | 1 (7.7) | – | 0 of 12 (0) |
| Liver | 4 | 0 | – | 1 of 4 (25) |
| Other* | 16 | 0 | – | 3 of 16 (18.7) |
| Total | 331 | 12 (3.6) | 56 of 56 (100) | 60 of 263 (23) |

*Includes brain (4), bladder (4), tonsil (2), thymus (2), lacrimal (1), eye lid (2), breast (1), gall bladder (1), lip (1), and ovary (1).

– Not applicable

## 4.3 Results

### 4.3.1 Characterisation of BCL10 antibodies

The 8 positive single clones were examined for their specificity by Western blotting analysis with the full length recombinant BCL10 protein. Seven clones showed specific recognition of the BCL10 protein. To map the amino acid residues recognised by these monoclonal antibodies, Western blotting analysis of the HEK 293 cells transfected with various BCL10 deletion constructs as well as the recombinant amino terminal BCL10 product was carried out. Three clones including clone 151 recognised the full length (amino acids 1-233), the truncated (1-168) and the carboxyl terminal (amino acids 101-233) BCL10 products expressed in HEK 293 cells, but did not react with the recombinant amino terminal BCL10 product (1-122) (Figure 4.1), indicating that these clones recognised amino acids between 123-168. The remaining 4 clones recognised the full length and the carboxyl terminal BCL10 product but only weakly reacted with or did not recognise the truncated BCL10 product, suggesting that these antibodies recognised amino acid residues further toward the carboxyl terminus than those recognised by clone 151. No clones recognised epitopes within the amino-terminal CARD. All 7 monoclonal antibodies were further tested by immunohistochemistry of formalin-fixed paraffin-embedded tissue sections from MALT lymphomas with t(1;14)(p22;q32) and all 7 showed characteristic staining. The staining was specific as no staining was seen if hybridoma culture supernatant was omitted or immunoabsorbed with the recombinant BCL10 protein prior to immunohistochemistry. Of the 7 monoclonal antibodies, clone 151 gave the best immunostaining on paraffin embedded tissue sections and was used for all subsequent experiments. Western blotting analysis of frozen tissues from tonsil,

lymph node and spleen showed that BCL10 was present as a predominant 32kD with a weaker 37kD band (Figure 4.1).

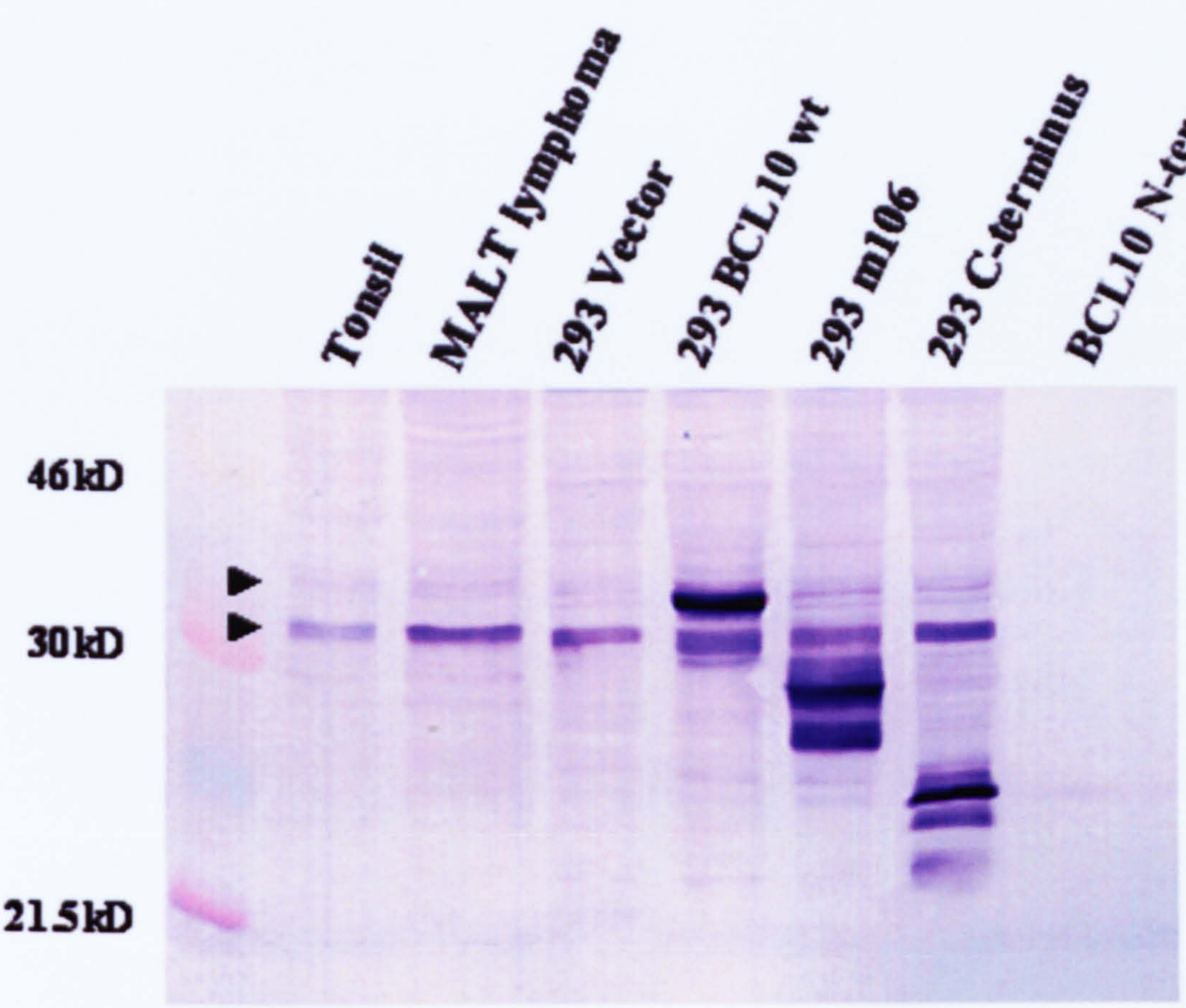



**Figure 4.1. Western blotting analysis**. Mouse BCL10 monoclonal antibody clone 151 recognises the expressed BCL10 products in 293 cells by constructs containing the full length wild type (293 BCL10 wt, amino acids 1-233), the truncated (293 m106, amino acids 1-168) and the carboxyl terminal BCL10 sequence (293 C-terminus, amino acids 101-233), and does not react with the recombinant BCL10 amino terminal product (BCL10 N-terminus, amino acids 1-122). Tonsil, MALT lymphoma and the 293 cells transfected with control vector show two forms of BCL10: a predominant 32 kD and a weaker 37 kD band, indicated by arrow heads.

### 4.3.2 BCL10 expression in normal tissues

BCL10 protein was expressed in normal spleen, reactive tonsil, lymph node and MALT of the appendix. In B cell follicles, the protein was expressed abundantly in the germinal

centre B cells, moderately in the marginal zone but only weakly in 40-60% of the mantle zone B cells (Figure 4.2 A-D). Within the germinal centre, dark zone centroblasts expressed more BCL10 than light zone centrocytes (Figure 4.2 A). Both the germinal centre and marginal zone B cells expressed BCL10 protein in the cytoplasm (Figure 4.2 B and D). The subcellular localisation of BCL10 in the mantle zone B cells could not be confidently determined due to their low expression level and scanty cytoplasm.

BCL10 protein was also expressed in foetal thymus. At early stages of gestation (16-25 weeks), BCL10 was expressed in the medulla and occasionally in the cortex (Figure 4.2 E), whereas at late stages of gestation (>28 weeks), the protein was found exclusively in the medulla. Double immunostaining for BCL10 and CD20 or CD3 (Dako, U.K.) revealed that BCL10 positive cells are both B and T cell lineages, with the majority of positive cells being T cells (Figure 4.2 G and H). In both cases, BCL10 was expressed only in the cytoplasm (Figure 4.2 F).

Of 21 types of normal solid tissues examined, including tongue, oesophagus, duodenum, rectum, liver, gall bladder, pancreas, bronchus, heart, lung, thyroid, breast, adrenal gland, kidney, bladder, uterus, cervix, ovary and tube, placenta and cord, testis and skin, only breast showed BCL10 protein expression. BCL10 expression appeared to be restricted to the cytoplasm of the luminal epithelial cells of breast.

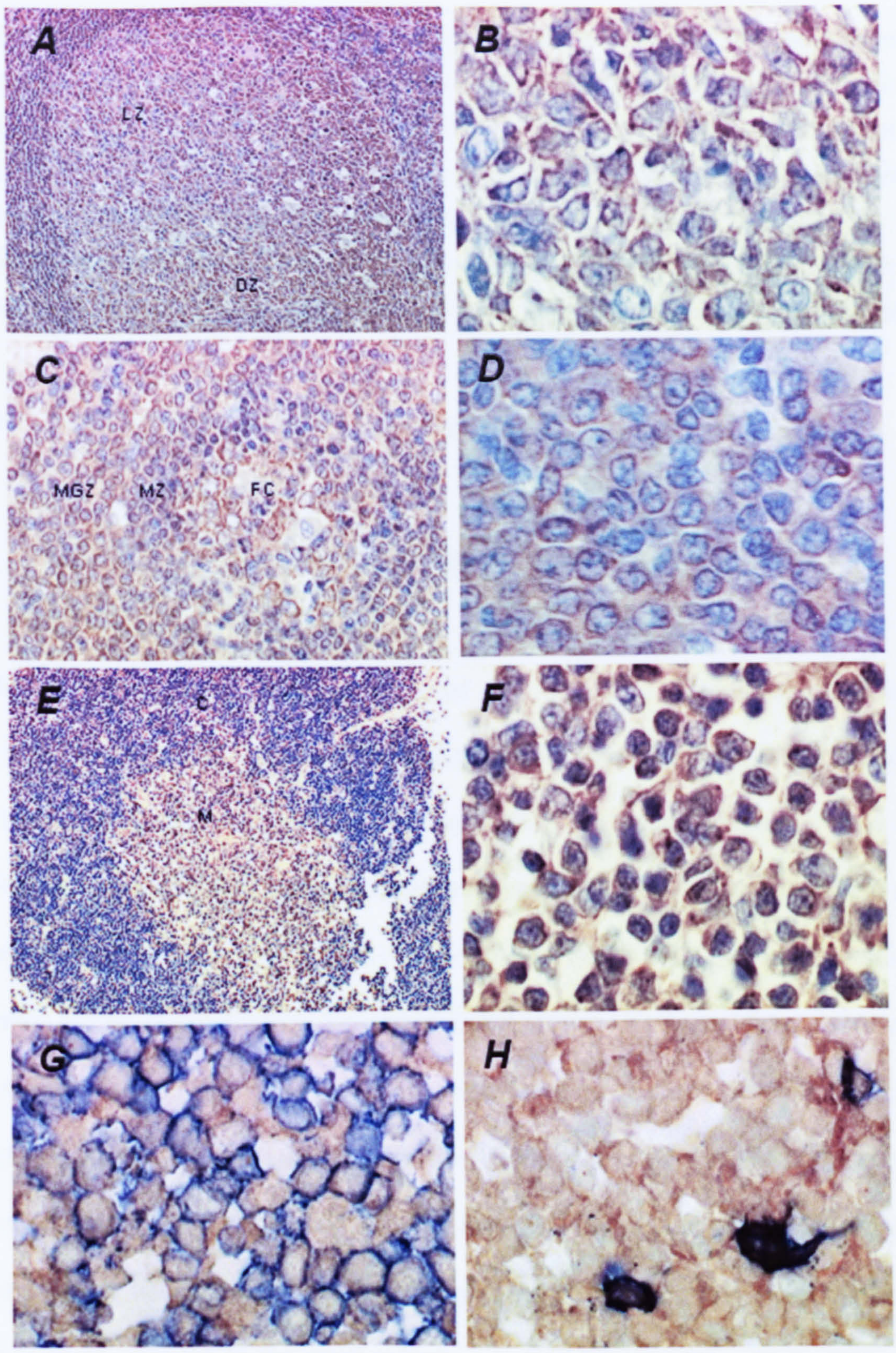



**Figure 4.2. BCL10 protein expression in normal lymphoid tissues.A:** A low magnification (x150); of tonsil shows the level of BCL10 expression is much higher in centroblasts of the dark zone (DZ) than in centrocytes of the light zone (LZ); **B:** Germinal centre B cells show BCL10 expression in the cytoplasm (magnification x480); **C:** A low magnification (x250) of spleen shows differential BCL10 expression in marginal zone (MGZ), mantle zone (MZ), and follicle centre (FC) B cells; **D:** Splenic marginal zone B cells show BCL10 expression in the cytoplasm

(magnification x480); **E:** Thymus (16 weeks of gestation) shows BCL10 expression mainly in the medulla (M) and occasionally in the cortex (C) (magnification x80); **F:** Cytoplasmic BCL10 expression in medullar lymphocytes (magnification x480); **G:** Double immunostaining with BCL10 and CD3 antibodies (magnification x480); BCL10 is in brown, showing parenuclear staining, while CD3 is in blue, displaying a membrane staining pattern; **H:** Double immunostaining with BCL10 and CD20 antibodies (magnification x480). BCL10 is in brown, showing paranuclear staining, while CD20 is in blue, displaying a membrane-staining pattern.

### 4.3.3 BCL10 expression in MALT lymphoma

#### (1) BCL10 expression in MALT lymphoma with t(1;14)(p22;q32)

Unlike marginal zone B cells, each of the 4 MALT lymphomas known with t(1;14)(p22;q32) showed strong BCL10 expression in both the nucleus and cytoplasm in all tumour cells (Figure 4.3 A-D). Strong BCL10 nuclear expression in these tumour cells distinguished them from non-lymphomatous reactive lymphocytes, which showed either weak or no cytoplasmic BCL10 expression. The BCL10 expressing tumour cells invaded gastric glands forming lymphoepithelial lesions (Figure 4.3 C) and disseminated to other parts of the gastric mucosa intermingling with reactive lymphocytic infiltrates (Figure 4.3 E). Discrete tumour cells identified by strong BCL10 nuclear expression were also found in the marginal zone of the spleen in one gastric MALT lymphoma where splenic tissue was available. Strong nuclear BCL10 expression in tumour cells was in sharp contrast to the weak cytoplasmic BCL10 expression in normal marginal zone B cells (Figure 4.3 F). Presence of the tumour cells in the marginal zone has been confirmed in a previous study by microdissection and PCR of the rearranged *Ig* gene[43]. The gastric MALT lymphoma with t(1;2)(p22;p12) showed BCL10 staining pattern identical to that of t(1;14)(p22;q32) positive tumour cells.

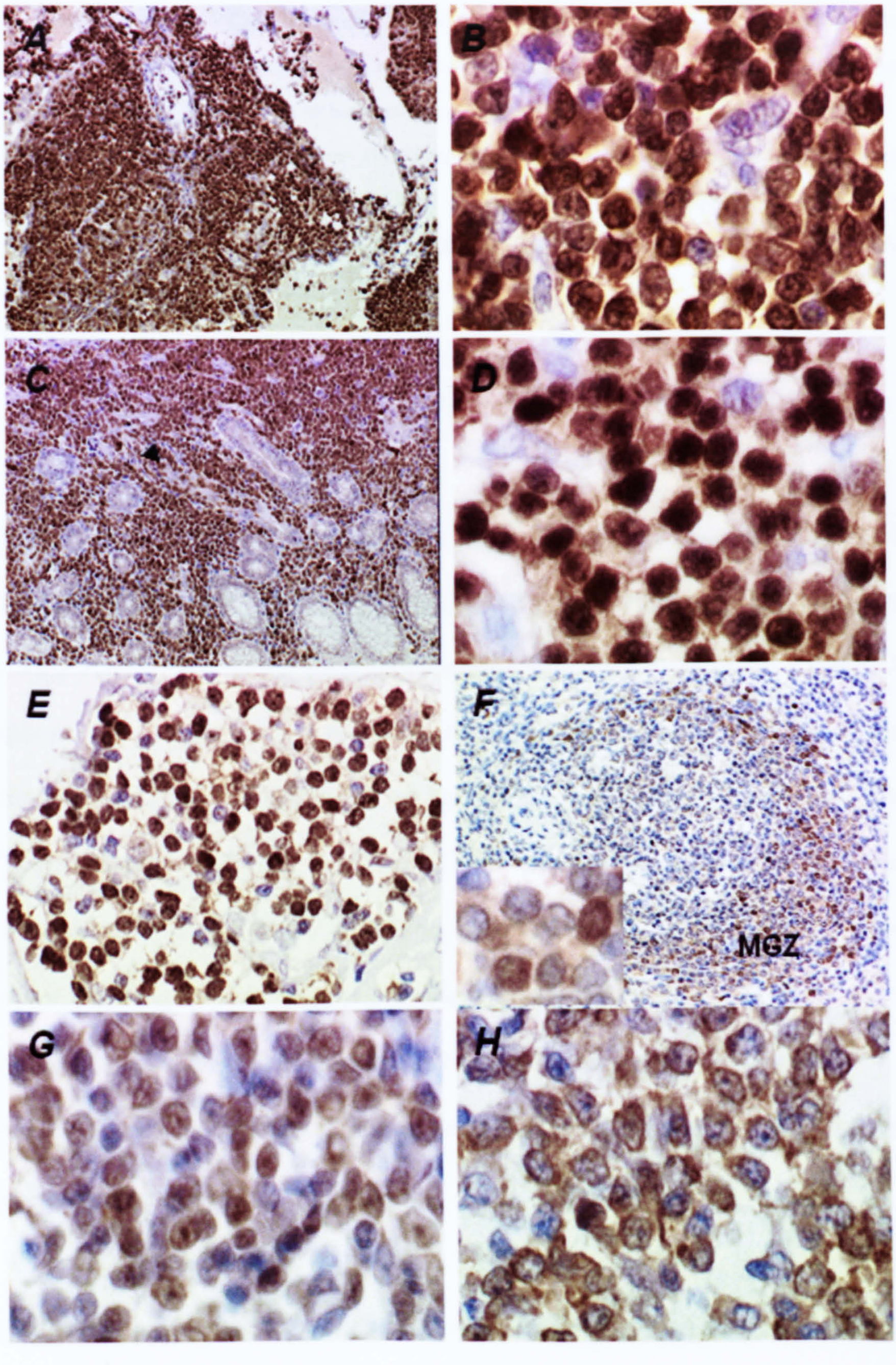



**Figure 4.3. BCL10 protein expression in malignant B cell lymphoma**. **A& B:** A pulmonary MALT lymphoma with t(1;14)(p22;q32) (A: magnification x150; B: magnification x480); **C & D:** A gastric MALT lymphoma with t(1;14)(p22;q32). Arrow-head indicates lymphoepithelial lesion (C: magnification x150; D: magnification x480); **E:** BCL10 expressing tumour cells

disseminate to other part of the gastric mucosa intermingling with reactive lymphocytes (magnification x250). **F:** Discrete tumour cells are also found in the marginal zone of the spleen, where the strong nuclear BCL10 expression in tumour cells is in sharp contrast to the weak cytoplasmic BCL10 expression in normal marginal zone B cells (magnification x150); **G:** A gastric MALT lymphoma without t(11;18)(q21;q21) shows moderate BCL10 expression predominantly in the nucleus (magnification x480); **H:** A gastric MALT lymphoma without t(1;14)(p22;q32) and t(11;18)(q21;q21) shows BCL10 expression in the cytoplasm (magnification x480).

### (2) Incidence of t(1;14)(p22;q32) in MALT lymphoma of various sites

In order to understand whether strong BCL10 nuclear staging is characteristic of MALT lymphoma cells with t(1;14)(p22;q32) and to determine the incidence of t(1;14)(p22;q32), BCL10 immunohistochemistry was carried out in 331 cases of MALT lymphoma of 8 sites (Table 4.1). In all the cases, BCL10 staining was performed on either surgical resected specimens, or large tissue biopsies to confidently interprete BCL10 staining. Strong nuclear BCL10 expression similar to that seen in t(1;14)(p22;q32) or t(1;2)(p22;p12) was observed in 12 cases and they were from stomach (6), lung (5) and skin (1) (Table 4.1 and Figure 4.4). All these cases were negative for t(11;18)(q21;q21) as described in section 3.3.2 of Chapter 3. Of the remaining cases, 116 showed moderate BCL10 nuclear staining and the rest displayed cytoplasmic staining.

To further ascertain whether the cases showing a strong BCL10 nuclear staining harbour a *BCL10*-involved chromosomal translocation, interphase FISH was performed to detect chromosomal breakpoint affecting the *BCL10* locus, like t(1;14)(p22;q32) or variants. The reliability of the *BCL10* break-apart assay for detection of the BCL10 involved chromosomal translocation was first validated in 4 t(1;14)(p22;q32)/*IGH-BCL10* and 1

t(1;2)(p22;p12)/*IGκ-BCL10* positive MALT lymphomas proven by conventional cytogenetics, and 5 negative controls. *BCL10* break-apart assay showed BCL10 involved translocation in 5/5 positive cases but not in any of the negative controls. The *BCL10* break-apart assay was then performed on 4 pulmonary and 3 gastric cases showing strong BCL10 nuclear expression with which sufficient tissue materials were available. Five cases showed a signal constellation, indicating a chromosomal translocation affecting the *BCL10* locus (Figure 4.4). The remaining 2 cases lacked evidence for a breakpoint involving the *BCL10* gene, and one case contained a heavy reactive component that may have compromised the FISH analysis. None of these cases showed *BCL10* gene amplification.

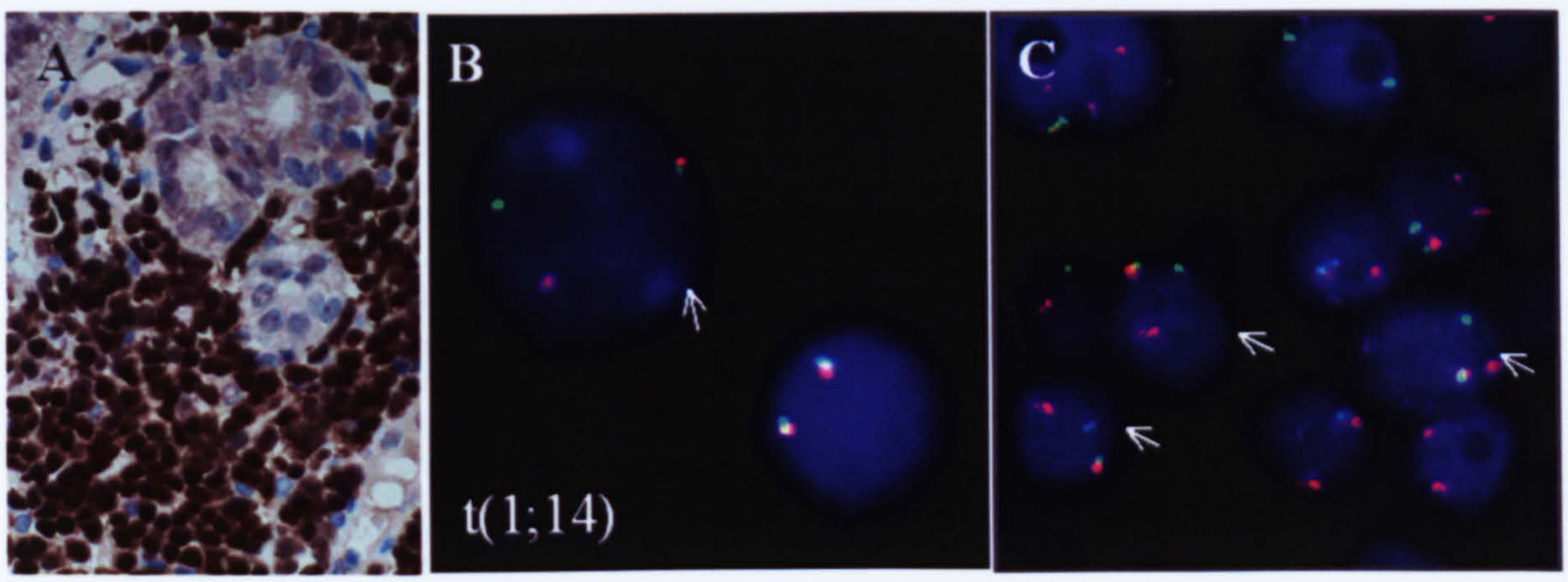



**Figure 4.4. Strong BCL10 nuclear expression and Break-apart double colour interphase FISH for the detection of breakpoints in the BCL10 locus.** **A:** A gastric MALT lymphoma shows strong BCL10 nuclear expression similar to that found in those with t(1;14)(p22;q32). **B:** A case with cytogenetically proven t(1;14)(p22;q32). The arrow points to a tumour cell in which the dissociation of the red and green signals indicates the presence of a chromosomal breakpoint in the *BCL10* locus. The nucleus on the right displays 2 colocalised signals pointing to 2 intact copies of the *BCL10* locus. **C:** The gastric MALT lymphoma with strong BCL10 nuclear expression as shown in A. Interphase nuclei show that several cells (arrows) display a split of the red and green signals, suggestive of t(1;14)(p22;q32) or variants.

To further investigate that t(1;14)(p22;q32) or variants was truly negative in MALT lymphoma lacking strong nuclear expression of BCL10, we performed interphase FISH using *BCL10* break-apart dual colour probes on 12 and 18 cases showing moderate nuclear BCL10 staining and cytoplamic staining pattern, respectively. All cases were negative for both BCL10 involved chromosomal translocation and gene amplification. These observations confirmed that strong BCL10 nuclear expression pattern is characteristic of MALT lymphoma with t(1;14)(p22;q32) or variants.

Based on BCL10 staining of 331 cases of MALT lymphoma from 8 major sites, the overall incidence of t(1;14)(p22;q32) was around 4%, with the highest frequency (12%) in pulmonary MALT lymphoma.

**(3) BCL10 expression in MALT lymphoma without t(1;14)(p22;q32) or variants**

As mentioned above, of the 331 cases examined for BCL10 expression, 319 cases did not show strong BCL10 nuclear expression. However, 116 cases showed a moderate nuclear BCL10 expression in 20 – 90% tumour cell population (Figure 4.3 G) and the rest displayed cytoplasmic BCL10 expression (Figure 4.3 H). In all cases, t(11;18)(q21;q21) was detected by RT-PCR for the *API2-MALT1* fusion transcripts from formalin-fixed and paraffin-embedded tissues, where possible, frozen tissues were preferentially used for RT-PCR. We correlated BCL10 expression pattern with presence or absence of t(11;18)(q21;q21). It was found that all 56 cases of t(11;18)(q21;q21)-positive lymphoma showed moderate BCL10 expression in the nuclei of most tumour cells (Figure 4.3 G and Table 4.1). BCL10 nuclear expression, similar to that seen in t(11;18)(q21;q21)-positive cases, was also found in 60 (23%) of the 263 cases that were

negative for the translocation by RT-PCR. The remaining 203 cases displayed only BCL10 cytoplasmic expression (Figure 4.3 H).

In view of the significant association between t(11;18)(q21;q21) and nuclear BCL10 expression, we re-examined the 7 cases showing nuclear BCL10 expression but not t(11;18)(q21;q21) by RT-PCR to ascertain whether the absence of the translocation in these cases was due to failed detection by the primer set used. A new MALT1 primer (MALT1-3: 5'-TTT TTC AGA AAT TCT GAG CCT G-3), which targets the 3' end of its coding region, together with the API2 primer was used for PCR (Table 2.4 in Chapter 2). All the seven cases consistently showed absence of the API2-MALT1 fusion. In addition, a lack of t(11;18)(q21;q21) in these cases was further confirmed by interphase FISH with MALT1 break-apart probes (Chapter 5).

**(4) BCL10 nuclear expression is associated with MALT lymphoma at advanced stage**

The findings of BCL10 nuclear expression in MALT lymphoma with t(1;14)(p22;q32) and a subset of MALT lymphoma without evidence of t(1;14)(p22;q32) suggest that nuclear BCL10 expression may be associated with its oncogenic activity. To further explore the role of BCL10 nuclear expression in multistage development of MALT lymphoma, we correlated BCL10 expression pattern with clinical staging in gastric MALT lymphoma. Of 41 gastric MALT lymphomas in which the lymphoma staging was available, nuclear BCL10 expression was found in 37% (7 of 19) tumours confined to the mucosa or sub-mucosa, 43% (3 of 7) cases in which tumour invaded to muscular

layer or serosa, and in 93% (14 of 15) cases in which tumour disseminated beyond the stomach ($P$<0.005, Chi-square test) (Figure 4.5).

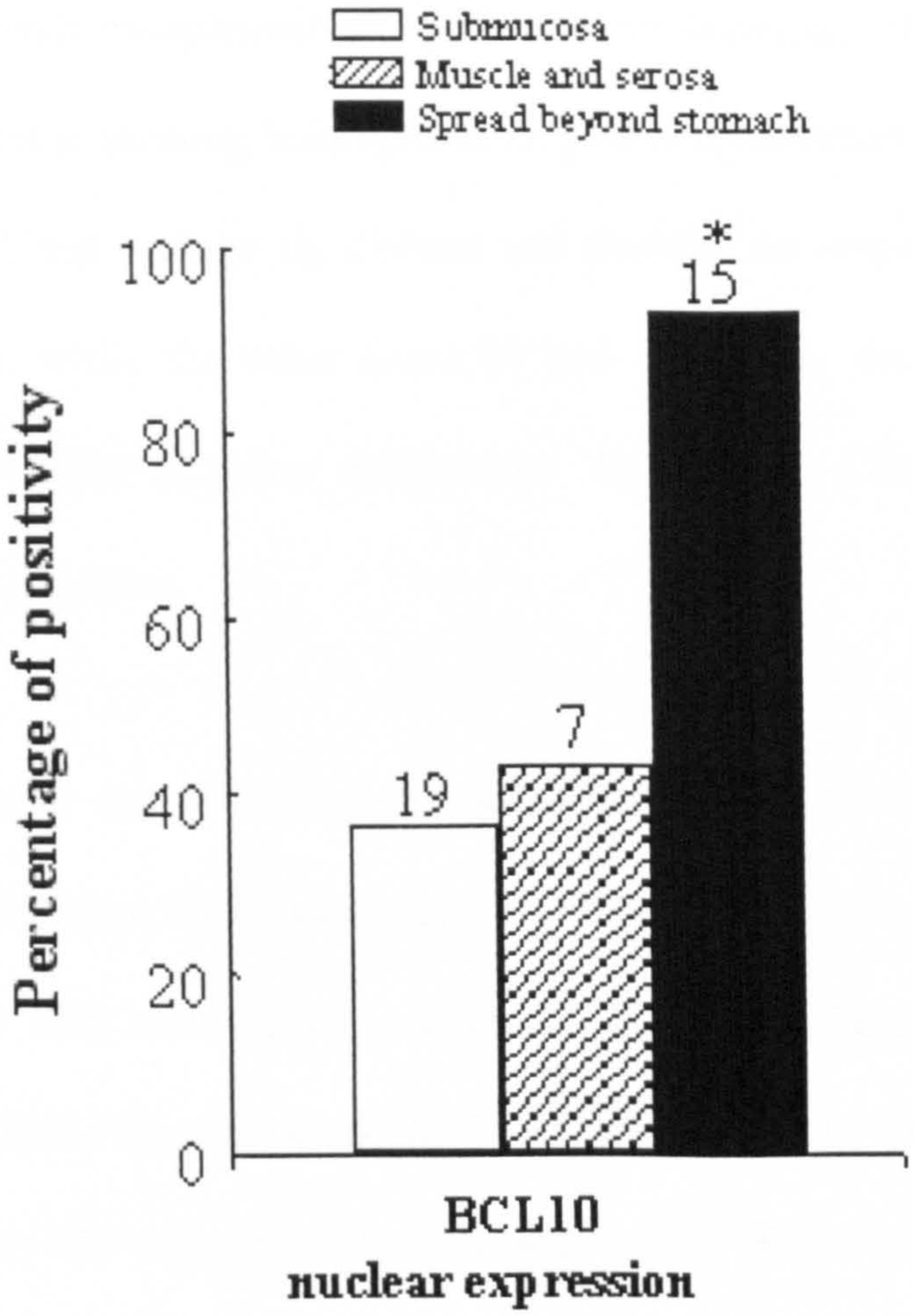



**Figure 4.5. Correlation between clinical staging of gastric MALT lymphoma and BCL10 nuclear expression**. The number of cases in individual subgroups is indicated on the top of the corresponding histogram. *$p$<0.005.

## (5) Gastric MALT lymphoma with strong BCL10 nuclear expression failed to respond to *H. pylori* eradication

The cohort of gastric MALT lymphoma, which treated by *H. pylori* eradication and investigated for t(11;18)(q21;q21) as detailed in Chapter 3, were also studied for BCL10 expression pattern. Of the 111 cases of gastric MALT lymphoma examined, 35 from the complete regression group and 40 from the non-response group had tissue sections containing an adequate tumour cell population for evaluation of BCL10 staining. Two

cases showed strong BCL10 nuclear staining in virtually all tumour cells (Figure 4.4 A), similar to that seen in t(1;14)(p22;q32) positive cells, while the remaining cases displayed either weak cytoplasmic or weak nuclear staining. Both cases that showed strong BCL10 nuclear staining belonged to *H. pylori* eradication non-responsive group: one cases (case 1) had a stage $II_E$ disease and showed no response 8 months after *H. pylori* eradication, while the other (case 2) had a stage $I_E$ disease and displayed no response 12 months after *H. pylori* eradication. As shown in Chapter 3, both cases were t(11;18)(q21;q21) negative.

To ascertain whether the two cases that showed strong BCL10 nuclear staining were positive for t(1;14)(p22;q32) or variants. Interphase FISH with break-apart dual colour probes for *BCL10*, *IGH* and *IGκ* was performed. Both cases failed to show evidence of a breakpoint at the *BCL10* locus nor *BCL10* gene amplification. However, case 2 showed a breakpoint at the *IGH* locus in 10% of tumour cells. Given the strong BCL10 nuclear staining and evidence of an IGH breakpoint in one case, a false negative result of interphase FISH with *BCL10* break-apart probes cannot be excluded. To further investigate whether these cases harboured a *BCL10* involved chromosome translocation, we carried out real time quantitative RT-PCR of *BCL10* mRNA expression. Unfortunately, adequate tissue materials were only available in case 2. The level ($\Delta$Ct=3.4) of *BCL10* mRNA expression in this case was compatible to MALT lymphoma with t(1;14)(p22;q32) (mean ± standard deviation = 1.60 ± 2.37), well above those without the chromosomal translocation (mean ± standard deviation = 6.94 ± 1.72) (section 5.3.3 of Chapter 5).

To further assess the value of t(1;14)(p22;q32) in prediction of the response of gastric MALT lymphoma to *H. pylori* eradication, we retrospectively reviewed the clinico-pathological features of gastric MALT lymphoma with t(1;14)(p22;q32) or variants, 4/5 cases were at stage $II_E$ or above at the time of diagnosis.

### (6) BCL10 expression in other NHLs

Similar to the BCL10 expression in normal germinal centre B cells, 19/21 follicular lymphomas expressed the protein in the cytoplasm and the remaining 2 expressed the protein in both the nucleus and cytoplasm in 30% and 80% of tumour cells, respectively. Mantle cell lymphomas showed either weak (14/17=82%) or no (3/17=18%) BCL10 expression. As the expression level is low, the sub-cellular localisation of BCL10 protein in this lymphoma subtype could not be confidently determined. All DLBCL of mucosal sites showed weak cytoplasmic BCL10 expression. Of nodal DLBCL, 14 of the 18 cases expressed BCL10 protein in the cytoplasm and the remaining 4 cases expressed the protein in both the nucleus and cytoplasm in 10-20% of tumour cells.

## 4.4 Discussion

### BCL10 expression in normal lymphoid tissues

BCL10 mRNA, as previously shown by *in-situ* hybridization, was highly expressed by germinal centre B cells, moderately by marginal zone but weakly by mantle zone B cells[134,135]. BCL10 protein, as shown in the present study, was also differentially expressed among various B cell subsets of the B cell follicle, from abundant expression

in antigen activated highly proliferating germinal centre centroblasts to low or no expression in non-dividing naïve mantle zone B cells. The BCL10 protein was also differentially expressed at various stages of T cell maturation in thymus. At an early stage, the T cells, which reside in the cortex and undergo T cell receptor rearrangements and positive selection, lacked BCL10 expression; while at a late stage, T cells, which move to the medulla and undergo negative selection to delete those highly reactive to self-major histocompatibility complex (MHC) and self peptides[281], expressed BCL10 in the cytoplasm. Our findings of different BCL10 expression during both B and T cell maturation are in line with the physiological role of BCL10 in antigen receptor mediated NFκB activation in both B and T cells[155,156].

Among other normal tissues, BCL10 protein was found by immunohistochemistry only in breast but not in 20 other types of tissues examined. The lack of BCL10 protein expression in these normal tissues is intriguing. It is possible that BCL10 protein is expressed in these normal tissues but at a low level below the detection limit of the current immunohistochemical system. Alternatively, BCL10 protein expression may be tissue and cell type specific.

**BCL10 deregulation in MALT lymphoma**

As mentioned above, BCL10 is found primarily in the cytoplasm of normal B cells, including the marginal zone B cells which are the normal cell counterpart of MALT lymphoma. By contrast, BCL10 is predominantly expressed in the nuclei of MALT lymphoma cells with t(1;14)(p22;q32) or variants. Moreover, BCL10 nuclear expression is associated with the presence of t(11;18)(q21;q21) and up to 20% of MALT

lymphomas without the translocation, predominantly in the nucleus, at a moderate level. BCL10 nuclear expression is found in a higher proportion of lymphomas with dissemination to local lymph nodes or distal sites (14 of 15 cases, 93%) than those confined to the gastric wall (10 of 26 cases, 38%). t(1;14)(p22;q32) and t(1;2)(p22;p12) are typically those at advanced stages. Furthermore, BCL10 has been found to be expressed at high levels in the nuclei of splenic marginal zone B cells in the transgenic mice in which *BCL10* expression is driven by an Ig enhancer (M-Q. Du and S. Morris, unpublished observations). These findings suggest that the nuclear expression may relate to its oncogenic activity. However, the molecular mechanisms underlying the deregulated BCL10 nuclear expression in malignant B cells are unclear. Overall, BCL10 genomic mutation is an infrequent event, occurring in about 5% of MALT lymphoma[142,143]. Intriguingly, BCL10 genomic mutation is not a common feature of MALT lymphoma with t(1;14)(p22;q32) and has been found in only one of three cases examined[142]. The findings that the frequency of nuclear BCL10 expression is 10 times as high as that of *BCL10* gene mutation in MALT lymphoma suggest that events other than genomic mutations are responsible for the nuclear localisation of the protein[142,143]. BCL10 nuclear expression is independent of both t(1;14)(p22;q32) and the level of protein expression, as well as *BCL10* gene amplification. BCL10 does not contain any known nuclear localisation signals[282]. The translocation of BCL10 from cytoplasm to nucleus is mostly likely mediated by its interaction proteins. The presence of nuclear BCL10 suggests that the protein may have functions other than those identified so far.

The studies of *BCL10*-knockout mice have shown that BCL10 is essential for both the development and function of mature B and T cells, linking antigen receptor signaling to the NF$\kappa$B activation[124,125]. NF$\kappa$B activation in lymphocytes leads primarily to cellular

activation, proliferation, survival and induction of effector function. In line with these observations, the findings of an association between BCL10 nuclear expression and nuclear localisation of NF$\kappa$B in nasal NK/T cell lymphomas and DLBCLs of the stomach suggest that nuclear BCL10 expression may be related to NF$\kappa$B transcription activation[283,284].

Unlike MALT lymphoma, both mantle cell and follicular lymphomas generally showed BCL10 expression patterns comparable to those seen in their normal cell counterparts, suggesting that BCL10 is unlikely to be involved in the development of these tumours.

**Incidence of t(1;14)(p22;q32) in MALT lymphoma of various sites**

In the present study, we showed that BCL10 was expressed strongly in both the nuclei and cytoplasm of tumour cells with t(1;14)(p22;q32). Based on BCL10 staining of 331 cases of MALT lymphoma from 8 major sites followed by interphase FISH, the overall incidence of t(1;14)(p22;q32) is approximately 4% of MALT lymphomas, with the highest frequency in those from the lung (12%) followed by stomach (5%), but absent in those from the salivary gland, thyroid, conjunctive and orbit. Like translocation-negative MALT lymphomas, t(1;14)(p22;q32)-positive cases usually contain other chromosomal aberrations such as trisomies 3, 12 and 18[280]. In view of the finding of t(1;14)(p22;q32) in DLBCL, MALT lymphomas with this translocation may undergo high grade transformation (The Cancer Genome Anatomy Project by National Cancer Institute).

**Gastric MALT lymphomas with strong BCL10 nuclear expression do not respond to *H. pylori* eradication**

*H. pylori* eradication can cure 75% of patients with gastric MALT lymphoma and is now the first line therapy for this disease. Because the assessment of the treatment response requires a prolonged follow-up with repeated endoscopy and gastric biopsies, it would be immensely beneficial if the *H. pylori* eradication non-responsive cases can be identified at diagnosis. In Chapter 3, t(11;18)(q21;q21) was shown to be nearly always associated with *H. pylori* eradication non-responsive gastric MALT lymphomas and detection of this translocation could identify 75% of such cases. In the present study, it was further shown that detection of t(1;14)(p22;q32) could identify a further 5% of gastric MALT lymphomas that did not respond to *H. pylori* eradication.

The finding that t(1;14)(p22;q32) is associated with *H. pylori* eradication non-responsive gastric MALT lymphoma is, to a certain extent, expected and is in line with the molecular and cellular properties of tumour cells carrying the translocation and their clinical presentation. The translocation deregulated the expression of the *BCL10* gene, which, like the API2-MALT1 fusion product of t(11;18)(q21;q21), is a potent activator of the transcriptional factor NF$\kappa$B. NF$\kappa$B activation in lymphocytes leads to expression of genes important for cellular activation and proliferation. A previous *in vitro* study showed that tumour cells with t(1;14)(p22;q32) could survive longer than those without this translocation in absence of any mitogenic stimulations[66]. Clinically, t(1;14)(p22;q32) positive gastric MALT lymphomas are typically those that show dissemination to regional lymph nodes or remote sites as shown by both previous and current studies[134].

t(1;14)(p22;q32) is a relatively infrequent chromosomal translocation in gastric MALT lymphoma, occurring in approximately 4% of cases. In view of that MALT lymphoma cells carrying t(1;14)(p22;q32) or variants show characteristic strong BCL10 nuclear expression, this translocation may be screened by BCL10 immunohistochemistry followed by interphase FISH analysis. The interpretation of BCL10 immunostaining particularly on small tissue biopsies can be difficult, however, those with t(1;14)(p22;q32) or variants are easily recognisable as they show strong homogeneous BCL10 nuclear staining in tumour cells but weak or no staining in the accompanied reactive T cells. For MALT lymphoma with t(11;18)(q21;q21), BCL10 immunohistochemistry does not provide a strong indication for the presence of the translocation as up to 20% of t(11;18)(q21;q21) negative cases also show a moderate BCL10 nuclear expression. Detection of this translocation is best carried out by RT-PCR of the *API2-MALT1* fusion transcripts or interphase FISH, both of which can be applied to formalin-fixed and paraffin-embedded tissue biopsies as described in section 2.2 of Chapter 2. Detection of both chromosomal translocations can identify 80% of gastric MALT lymphomas that will not respond to *H. pylori* eradication.

The molecular mechanisms underlying the remaining 20% of *H. pylori* eradication non-responsive gastric MALT lymphomas are not clear. It has been shown that *H. pylori* associated gastric MALT lymphoma in patients with autoimmune disease was resistant to antibiotic treatment[278] and *fas* gene was mutated in MALT lymphoma of patients with autoimmunity[214]. Additional novel chromosomal translocations may also be present in MALT lymphoma. Study of these genetic targets may allow identification of further molecular markers for *H. pylori* eradication non-responsive gastric MALT lymphoma.

Recently, two novel chromosomal translocations have been described in MALT lymphomas. One is t(14;18)(q32;q21)/*IGH-MALT1* involving the *MALT1* gene[161,162], the other is t(3;14)(p14;q32) involving *FOXP1* gene[285]. These translocations appear to occur more often in non-gastrointestinal sites, particularly those of the lung, liver, thyroid and ocular adnexa. However, their incidence in MALT lymphoma of various sites and their clinical relevance remain to be investigated.

## 4.5 Conclusion

In summary, the present study showed that BCL10 was expressed differentially among different B cell populations of the B cell follicle, in line with its role in NF$\kappa$B activation and B cell activation. The sub-cellular localisation of BCL10 is frequently altered in MALT lymphoma in comparison with its normal cell counterparts, suggesting that this may be important in lymphomagenesis. Strong nuclear BCL10 expression is seen in MALT lymphoma with t(1;14)(p22;q32) or variants, while moderate nuclear BCL10 expression is found in t(11;18)(q21;q21) positive MALT lymphoma and 20% of cases without evidence of both t(1;14)(p22;q32) and t(11;18)(q21;q21). Although the mechanisms underlying the BCL10 nuclear expression are unclear, nuclear BCL10 expression appears to be significantly associated with cases at advanced stages. In gastric MALT lymphoma, those expressing strong nuclear BCL10 do not respond to *H. pylori* eradication.

# Chapter 5. t(14;18)(q32;q21)/*IGH-MALT1* and MALT1 deregulation in MALT lymphoma

## 5.1 Introduction

This study aimed to investigate the incidence of t(14;18)(q32;q21)/*IGH-MALT1* in MALT lymphoma of various sites and examine MALT1 expression in both normal and malignant lymphoid tissues and correlated its expression pattern with that of BCL10 as well as the presence of the three chromosomal translocations in MALT lymphomas.

## 5.2 Case selection

These included 30 cases of formalin-fixed and paraffin-embedded normal lymphoid tissues, 490 cases of B cell lymphomas including 423 cases of MALT lymphomas of eight sites (Table 5.1), 22 cases of follicular lymphoma, 18 cases of mantle cell lymphoma, and 27 cases of DLBCL. Of the MALT lymphoma cases, 6 cases were known to be t(14;18)(q32;q21)/*IGH-MALT1* positive as shown in previous studies[161,286]. In addition, 86 normal non-lymphoid tissues of 21 different types were studied.

**Table 5.1. MALT1 expression pattern in MALT lymphomas with different chromosomal translocations**

| Site of MALT lymphoma | Number of cases | Translocation status* | Number of cases | Intensity of MALT1 expression | | |
|---|---|---|---|---|---|---|
| | | | | Strong | Moderate | Weak/negative |
| Stomach | 185 | t(11;18) +ve | 40 | - | 2 | 38 |
| | | t(1;14) +ve | 8 | - | - | 8 |
| | | t(14;18) +ve | - | - | - | - |
| | | translocation -ve | 137 | - | 7 | 130 |
| Lung | 47 | t(11;18) +ve | 18 | - | - | 18 |
| | | t(1;14) +ve | 4 | - | - | 4 |
| | | t(14;18) +ve | 3 | 3 | - | - |
| | | translocation -ve | 22 | - | - | 22 |
| Ocular adnexae | 73 | t(11;18) +ve | 7 | - | 1 | 6 |
| | | t(1;14) +ve | - | - | - | - |
| | | t(14;18) +ve | 5 | 5 | - | - |
| | | translocation -ve | 61 | - | 7 | 54 |
| Salivary gland | 59 | t(11;18) +ve | 1 | - | - | 1 |
| | | t(1;14) +ve | - | - | - | - |
| | | t(14;18) +ve | - | - | - | - |
| | | translocation -ve | 58 | - | 8 | 50 |
| Thyroid | 12 | t(11;18) +ve | - | - | - | - |
| | | t(1;14) +ve | - | - | - | - |
| | | t(14;18) +ve | - | - | - | - |
| | | translocation -ve | 12 | - | 2 | 10 |
| Skin | 37 | t(11;18) +ve | - | - | - | - |
| | | t(1;14) +ve | - | - | - | - |
| | | t(14;18) +ve | - | - | - | - |
| | | translocation -ve | 37 | - | 9 | 28 |
| Liver | 6 | t(11;18) +ve | - | - | - | - |
| | | t(1;14) +ve | - | - | - | - |
| | | t(14;18) +ve | 1 | 1 | - | - |
| | | translocation -ve | 5 | - | - | 5 |
| Intestine | 4 | t(11;18) +ve | 1 | - | - | 1 |
| | | t(1;14) +ve | - | - | - | - |
| | | t(14;18) +ve | - | - | - | - |
| | | translocation -ve | 3 | - | - | 3 |
| Total | 423 | | 9 (2.1%) | 36 | | 378 |

* t(14;18)(q32;q21)/*IGH-MALT1* and *BCL10* break/t(1;14)(p22;q32) were primarily detected by MALT1 and BCL10 immunohistochemistry followed by interphase FISH with appropriate probes. t(11;18)(q21;q21)/*API2-MALT1* positive cases was detected by RT-PCR of the *API2-*

*MALT1* fusion transcript with exception of one pulmonary case that was initially identified by interphase FISH with *MALT1* break-apart probes.

## 5.3 Results

### 5.3.1 Characterisation of MALT1 antibodies

The N-terminal MALT1 antibody recognises wild type MALT1 but not the API2-MALT1 fusion product, while the C-terminal MALT1 antibody reacts with both MALT1 and API2-MALT1 fusion product (Figure 5.1).

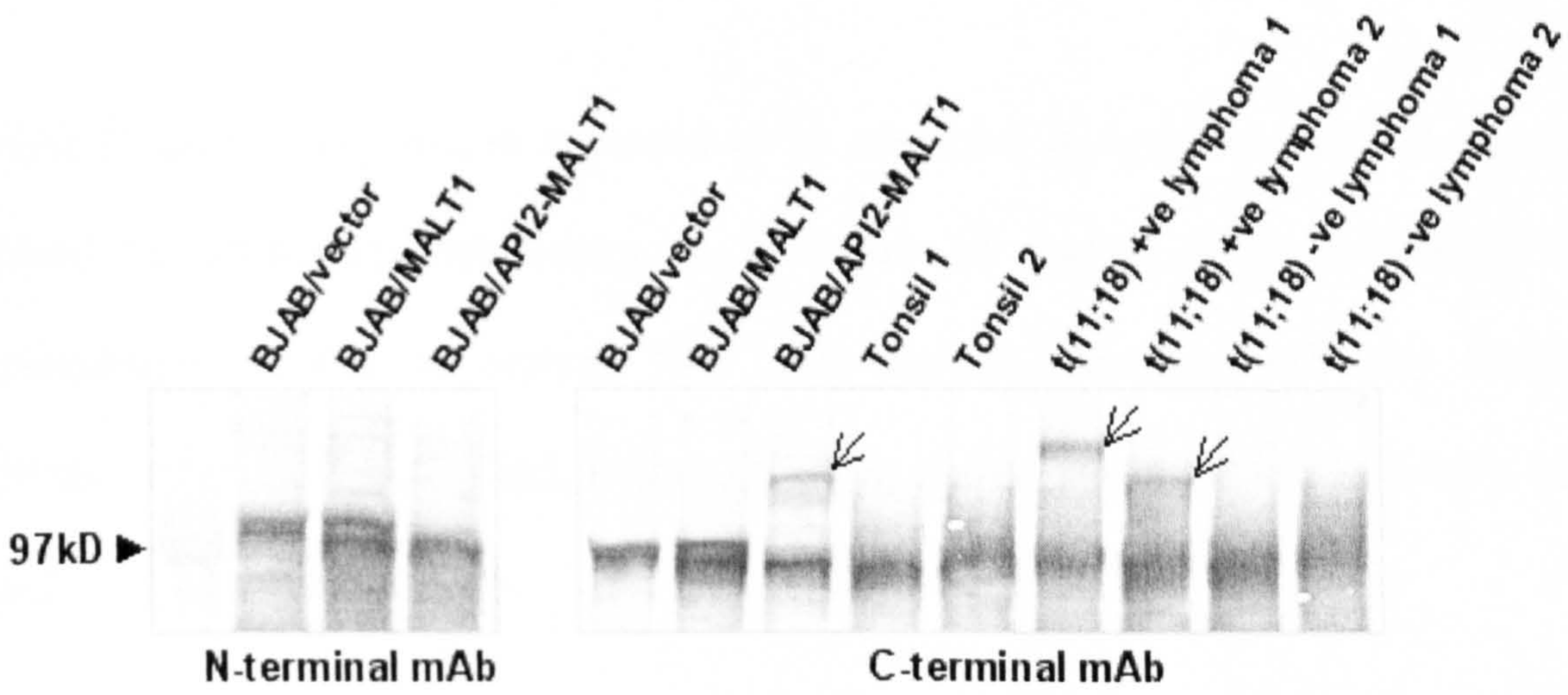



**Figure 5.1. Western blot analysis of the human B cell lymphoma cells (BJAB) transfected with MALT1 or API2-MALT1 expression construct[260], and MALT lymphoma with and without t(11;18)(q21;q21).** The mouse monoclonal antibody (mAb) to the N-terminus of the MALT1 recognises full length MALT1, but not the API2-MALT1 fusion product, while the mouse monoclonal antibody to the C-terminus of MALT1 reacts with full length MALT1 as well as the API2-MALT1 fusion product (indicated by arrows).

### 5.3.2 MALT1 expression in normal tissues

Immunohistochemistry with both N-terminal and C-terminal MALT1 mouse monoclonal antibodies showed that the protein expression pattern was identical to that of BCL10 in both B cell follicles and thymus (Figure 5.2 and section 4.3.2 of Chapter 4). In B cell follicles of tonsil, lymph node and spleen, both MALT1 and BCL10 are differentially expressed in various germinal centre B cells, strong in centroblasts, moderate in centrocytes, and weak/negative in mantle zone B cells (Figure 5.2). In thymus, MALT1 was weakly, while BCL10 was moderately expressed in medullar T cells. Irrespective of different cell types, both MALT1 and BCL10 are predominantly expressed in the cytoplasm.

MALT1 protein expression appeared to be restricted to lymphoid tissues. It was not found by immunohistochemistry in 21 types of normal tissue, including tongue, oesophagus, duodenum, rectum, liver, gall bladder, pancreas, bronchus, heart, lung, thyroid, breast, adrenal gland, kidney, bladder, uterus, cervix, ovary and tube, placenta and cord, testis and skin.

### 5.3.3 MALT1 expression in MALT lymphoma

MALT1 expression in MALT lymphoma was investigated with N-terminal MALT1 antibody, which recognises the wild type MALT1 but not the API2-MALT1 fusion product (Figure 5.1). As reactive lymphoid follicles are commonly seen in MALT lymphoma, MALT1 and BCL10 expression in B cells of reactive germinal centres provides an excellent internal control. The level of MALT1 and BCL10 expression in

lymphoma cells was therefore recorded with reference to that in centroblasts (strong), centrocytes (moderate), and mantle zone B cells (weak) of reactive lymphoid follicles.

The expression of these proteins was studied in 423 cases of MALT lymphoma and their expression was correlated with the presence of chromosomal translocations. For all cases, data for BCL10 expression, *BCL10* rearrangement indicating t(1;14)(p22;q32) or variants, and t(11;18)(q21;q21) were from previous studies (Chapter 3 and Chapter 4).

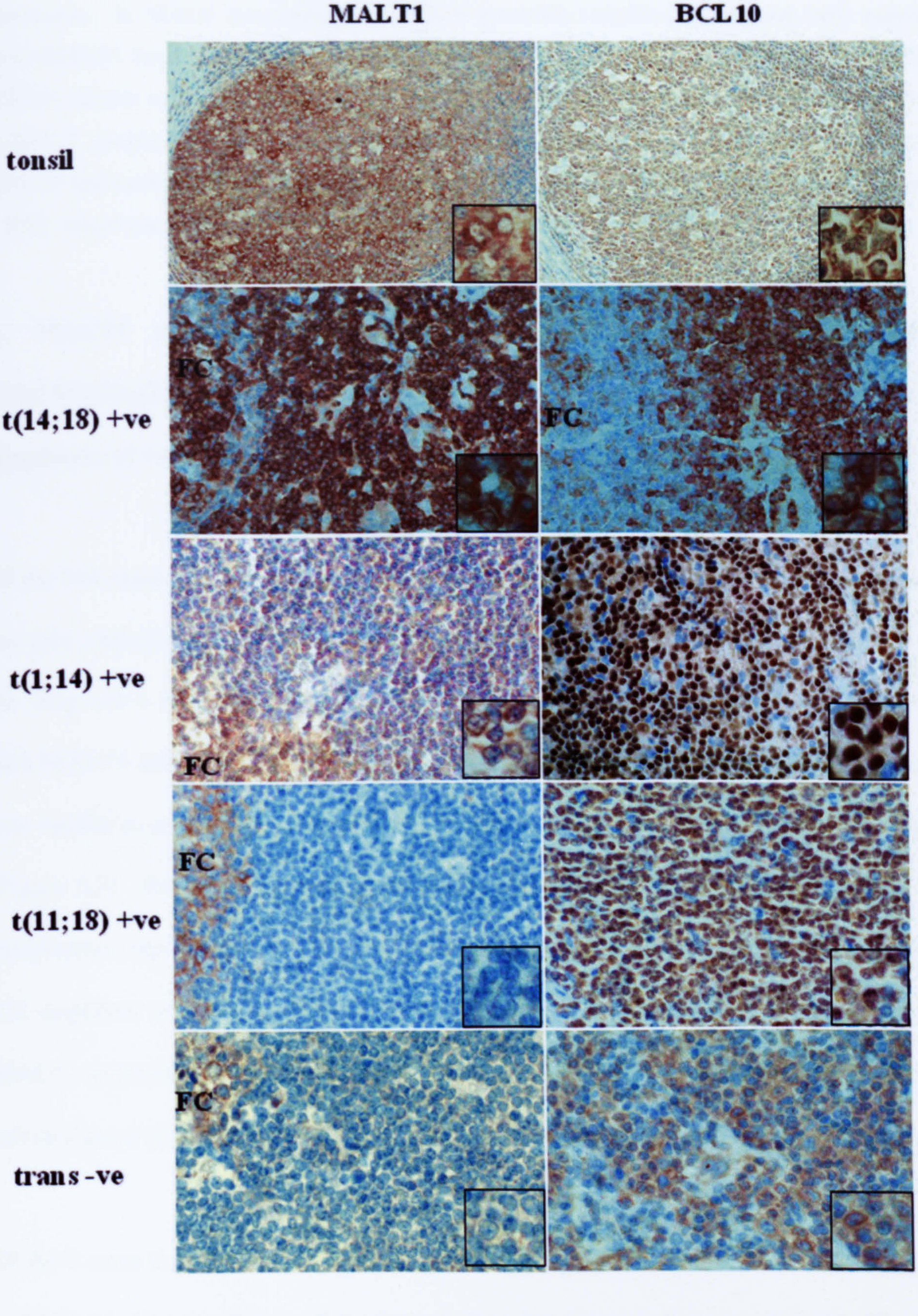



**Figure 5.2. MALT1 and BCL10 expression in reactive tonsil and MALT lymphomas with and without chromosomal translocations**. Both MALT1 and BCL10 are similarly expressed in the cytoplasm of various B cells in reactive tonsil, high in centroblasts, moderate in centrocytes and weak/negative in the mantle zone B cells. In MALT lymphoma with t(14;18)(q32;q21)/*IGH-MALT1*, all tumour cells show strong MALT1 and BCL10 cytoplasmic

expression. In MALT lymphoma with *BCL10* break/t(1;14)(p22;q32), tumour cells express weak MALT1 but strong nuclear BCL10. In MALT lymphoma with t(11;18)(q21;q21)/*API2-MALT1*, tumour cells generally show lack of MALT1 expression but moderate nuclear BCL10. In MALT lymphoma without the above chromosomal translocations, tumour cells show weak MALT1 and moderate BCL10 expression in the cytoplasm. FC: follicle centre. (magnification x 200); Insert (magnification x 400).

## A. MALT1 and BCL10 expression pattern in MALT lymphoma with t(14;18)(q32;q21)/*IGH-MALT1* and incidence of the translocation in MALT lymphoma of various sites

Of the 344 cases lacking evidence of t(1;14)(p22;q32) by BCL10 immunohistochemistry and t(11;18)(q21;q21) by RT-PCR, 9 cases including 5 from the ocular adnexae, 3 from the lung and 1 from the liver showed strong homogeneous cytoplasmic expression of both MALT1 and BCL10 in virtually all tumour cells. The intensity of MALT1 staining was similar to or stronger than that seen in centroblasts of the reactive germinal centre (Figure 5.2). Remarkably, these tumour cells also showed strong homogenous BCL10 cytoplasmic expression (Figure 5.2). Of the remaining cases, 33 showed moderate and 302 displayed weak/negative MALT1 cytoplasmic staining. Cases showing moderate MALT1 expression were from the stomach (7/185=3.8%), ocular adnexae (7/73=9.6%), salivary gland (8/59=13.6%) thyroid (2/12=16.7%), and skin (9/37=24.3%) (Table 5.1).

Of the 9 cases that showed strong homogeneous cytoplasmic expression of both MALT1 and BCL10, 6 were subjects of previous studies and shown to harbour t(14;18)(q32;q21). For the remaining 3 cases without previous information on t(14;18)(q32;q21) status, we performed interphase FISH using IGH/MALT1 dual colour, dual fusion translocation

probes. In each case, signal patterns indicating presence of the chromosomal translocation were detected (Figure 5.3).

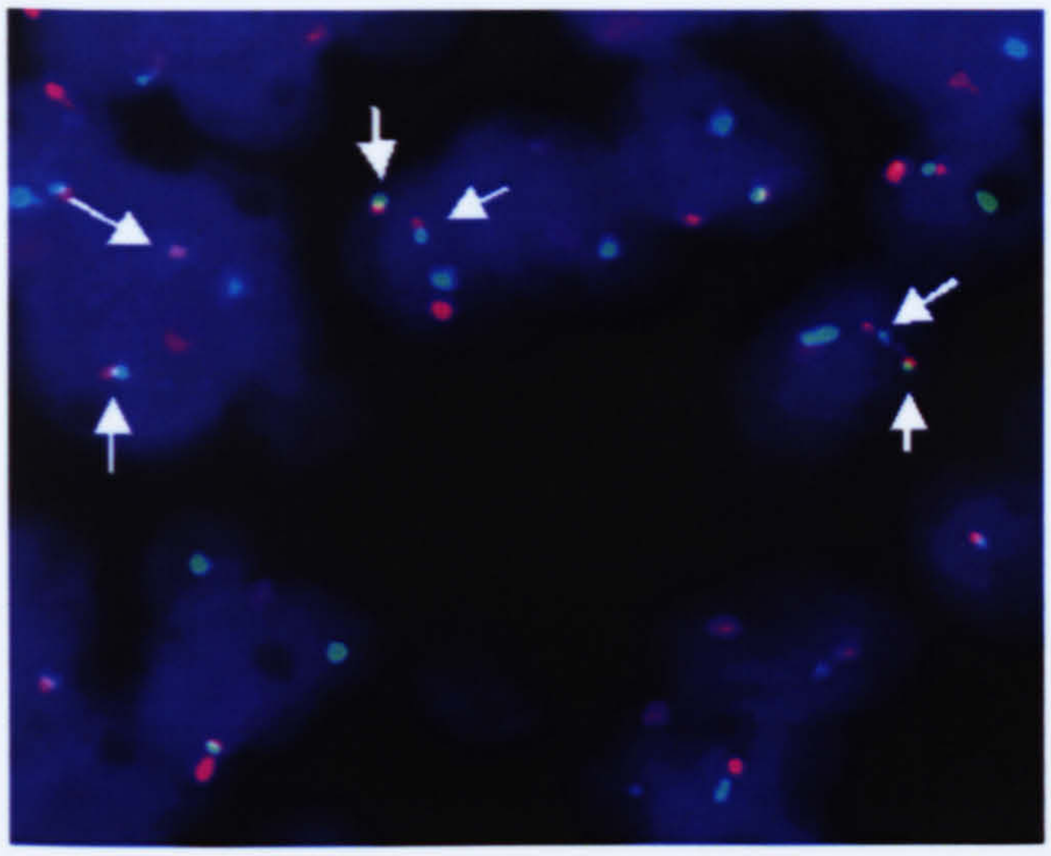


**Figure 5.3. Detection of t(14;18)(q32;q21)/*IGH-MALT1* by interphase FISH with dual colour, dual fusion translocation probes**. An ocular MALT lymphoma with strong cytoplasmic expression of both MALT1 and BCL10 shows co-localisation of green and red signals in interphase nuclei, suggestive of t(14;18)(q32;q21)/*IGH-MALT1*.

To investigate further that t(14;18)(q32;q21)/*IGH-MALT1* was truly negative in MALT lymphoma lacking high cytoplasmic expression of MALT1 and BCL10, we performed interphase FISH using *MALT1* break-apart dual colour probes. The reliability of the *MALT1* break-apart assay for detection of chromosomal translocation involving *MALT1* was first validated in 34 cases of t(11;18)(q21;q21) positive MALT lymphoma, 5 cases of t(14;18)(q32;q21)/*IGH-MALT1* positive MALT lymphoma and 5 negative controls. *MALT1* break-apart assay showed *MALT1* involved chromosomal translocation in 39/39 positive cases but not in any of the negative controls.

The *MALT1* break-apart assay was performed in 174 cases of MALT lymphoma that showed no evidence of t(11;18)(q21;q21) by RT-PCR and t(1;14)(p22;q32) or variants

by BCL10 immunohistochemistry. They included 48 from salivary gland, 45 from ocular adnexae, 23 from lung, 31 from stomach, 12 from thyroid, 12 from skin, 2 from liver and 1 from small intestine. With the exception of one case, none of the remaining cases, including 26 cases that showed a moderate MALT1 expression, displayed evidence of a chromosomal breakpoint affecting the *MALT1* gene. The only case showed a breakpoint at the *MALT1* locus was from lung and subsequently confirmed to be t(11;18)(q21;q21) by interphase FISH with *API2/MALT1* dual colour, dual fusion translocation probes. This case was not detected by RT-PCR of the *API2-MALT1* fusion transcript on RNA samples prepared from the formalin-fixed and paraffin-embedded tissues. It is well documented from our previous studies that the RT-PCR protocol used misses 7% of rare breakpoints on the *API2* gene (Chapter 3). Nonetheless, 28 cases showed three-copies of the *MALT1* gene in more than 80% of tumour cells, but only 3 of them displayed a moderate MALT1 expression, with the remaining cases exhibiting weak MALT1 expression. There was no evidence of *MALT1* gene amplification in all the cases examined by interphase FISH. Figure 5.4 summarises the frequencies of t(14;18)(q32;q21)/*IGH-MALT1*, t(11;18)(q21;q21)/*API2-MALT1* and *BCL10* break/t(1;14)(p22;q32) in MALT lymphomas of various sites.

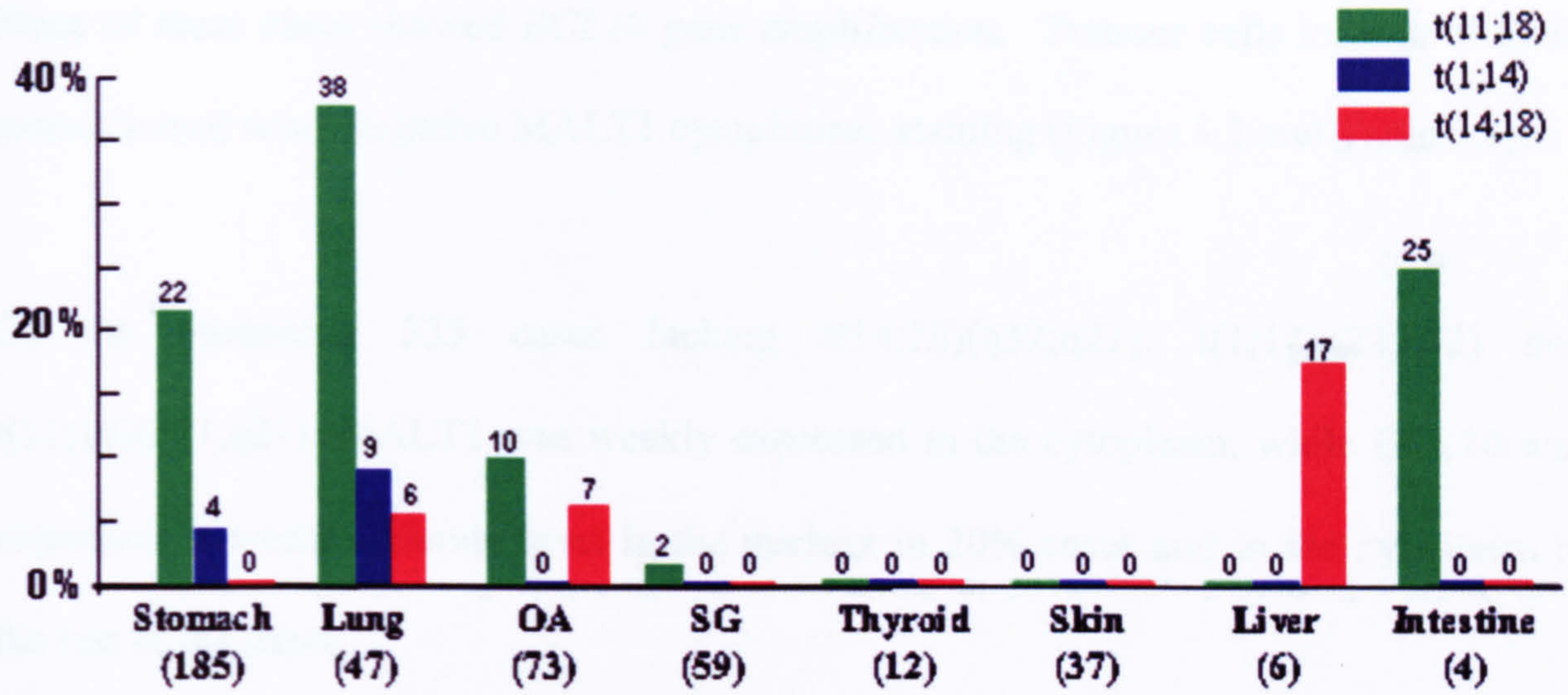



**Figure 5.4. Frequency of t(14;18)(q32;q21)/*IGH-MALT1*, *BCL10* break/t(1;14)(p22;q32) and t(11;18)(q21;q21)/*API2-MALT1* in MALT lymphomas of various sites.** OA: Ocular adnexae; SG: salivary gland. Number in bracket indicates the number of cases studied. t(14;18)(q32;q21)/*IGH-MALT1* and *BCL10* break/t(1;14)(p22;q32) were detected by MALT1 and BCL10 immunohistochemistry followed by interphase FISH. t(11;18)(q21;q21)/*API2-MALT1* was detected by RT-PCR of the *API2-MALT1* fusion transcript with exception of one pulmonary case that was identified by interphase FISH with *MALT1* break-apart probes.

### B. MALT1 and BCL10 expression pattern in MALT lymphoma without t(14;18)(q32;q21)/*IGH-MALT1*

Sixty seven cases were t(11;18)(q21;q21) positive as proven by RT-PCR of the *API2-MALT1* fusion transcript or interphase FISH. As detailed in previous Chapter (Chapter 3), tumour cells in these cases showed moderate or weak BCL10 nuclear staining (Figure 5.2). Among t(11;18)(q21;q21) positive cases, 3 showed moderate and the remaining cases displayed weak/negative MALT1 cytoplasmic staining (Table 5.1).

In total, 12 cases showed strong BCL10 nuclear staining, and among them 10 showed evidence of t(1;14)(p22;q32) by conventional cytogenetics or *BCL10* interphase FISH.

None of these cases showed *BCL10* gene amplification. Tumour cells in each of these cases showed weak/negative MALT1 cytoplasmic staining (Figure 5.2 and Table 5.1).

Of the remaining 335 cases lacking t(14;18)(q32;q21), t(1;14)p22;q32) and t(11;18)(q21;q21), MALT1 was weakly expressed in the cytoplasm, while BCL10 was expressed at weak/moderate level in the nucleus in 20% cases and in the cytoplasm in the rest of the cases.

### 5.3.4 Correlation of MALT1 and BCL10 protein expression with their mRNA expression

In keeping with MALT1 protein expression, *MALT1* mRNA expression was the highest in MALT lymphomas with t(14;18)(q32;q21)/*IGH-MALT1*, significantly higher than those with t(11;18)(q21;q21) ($p<0.01$) and those without any of the chromosomal translocations studied ($p<0.05$) (Figure 5.5). Interestingly, *MALT1* mRNA expression was also significantly higher in MALT lymphomas without any of the chromosomal translocations than those with t(11;18)(q21;q21) ($P<0.01$) (Figure 5.5).

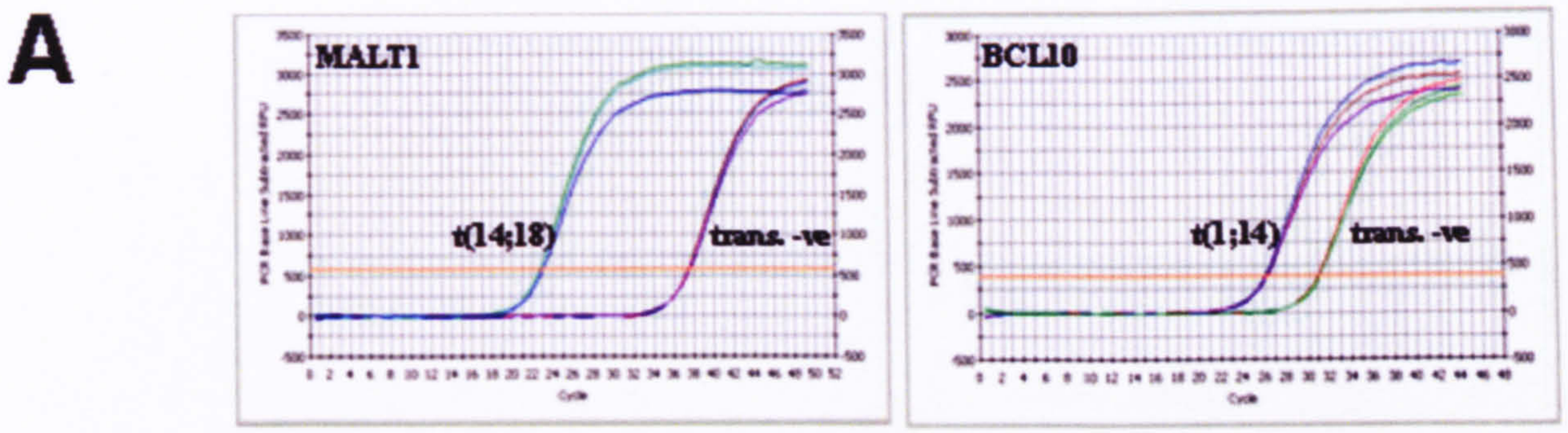



**Figure 5.5.** ***MALT1*** **and** ***BCL10*** **mRNA expression in MALT lymphoma with different chromosomal translocations.** **A:** Examples of real-time quantitative RT-PCR with SYBR Green I using an iCycler iQ system. This was carried out in triplicates using RNA samples extracted from the tumour cells microdissected from paraffin embedded tissue sections. For simplicity, reference control 18S rRNA is not shown in the figure. Y-axis: relative fluorescent units; X-axis: number of PCR cycles; trans. –ve: translocation negative case. **B:** Comparison of *MALT1* and *BCL10* mRNA expression in MALT lymphomas with different chromosomal

translocation status. *indicates statistical significant difference. The medians are indicated by the horisontal bars in the rectangular boxes.

Similarly, *BCL10* mRNA expression was the highest in MALT lymphomas with t(1;14)(p22;q32), significantly higher than those with t(14;18)(q32;q21)/*IGH-MALT1*, or t(11;18)(q21;q21) or without any of these translocations (all $p<0.01$) (Figure 5.5). There was no correlation between *MALT1* and *BCL10* mRNA expression in individual groups with different chromosomal translocation status or in all groups combined together.

### 5.3.5 MALT1 expression in other NHLs

We also examined MALT1 expression in follicular lymphoma, mantle cell lymphoma and diffuse large B cell lymphoma. Of the 22 cases of follicular lymphoma examined, 17 cases showed a moderate to strong cytoplasmic MALT1 staining, and the remaining 5 cases displayed a weak MALT1 staining. In mantle cell lymphoma, MALT1 expression was weak in 14 cases and moderate in 4 cases. Among the 27 cases of diffuse large B cell lymphoma studied, 20 cases showed moderate to strong MALT1 expression and the remaining 7 cases displayed weak MALT1 expression.

## 5.4 Discussion

Mounting evidence indicates that BCL10 and MALT1 specifically transduce antigen receptor signaling to activate NFκB and play a critical role in the biology of both B and T cells[160]. This is best demonstrated by studies of BCL10 and MALT1 deficient mice which are characterised by impaired B cell development and function, showing a reduced number of marginal zone B cells and poor humoral responses to both T cell

dependent and independent stimulations[124,155,156,164]. It is believed that in normal B and T cells, upon antigen receptor stimulation, Carma1 is activated to recruit BCL10 via CARD-CARD interaction and induces BCL10 oligomerisation. BCL10 then binds the Ig-like domain of MALT1 through a short region downstream of its CARD and induces MALT1 oligomerisation, subsequently leading to NFκB activation[123,125]. The finding of identical expression pattern of MALT1 and BCL10 in B cell follicle in the present study is in line with their roles in B cell activation and maturation.

One of the remarkable findings of the present study is the observation of characteristic expression pattern of MALT1 and BCL10 in MALT lymphoma with different chromosomal translocations. In those with t(14;18)(q32;q21)/*IGH-MALT1*, the tumour cells are characterised by strong cytoplasmic expression of both MALT1 and BCL10, while MALT lymphoma cells with *BCL10* break/t(1;14)(p22;q32) or t(11;18)(q21;q21) show strong or moderate BCL10 nuclear expression respectively, but generally a weak MALT1 cytoplasmic expression. Such differential MALT1 and BCL10 expression patterns in MALT lymphoma with various chromosomal translocations may reflect not only the consequence of these chromosomal translocations but also the molecular mechanisms involved.

In MALT lymphoma with t(14;18)(q32;q21)/*IGH-MALT1*, strong cytoplasmic MALT1 expression is expected given the strong transcriptional activity of the *IGH* enhancer. The strong cytoplasmic BCL10 expression is, to some extent initially, a surprising finding, but this could well be explained by the molecular mechanism of MALT1 mediated NFκB activation. MALT1 does not have a structural domain that is capable of mediating its self-oligomerisation and over-expression of MALT1 alone in fibroblasts *in*

*vitro* does not activate NFκB[123,125,127]. However, MALT1 is synergistic with BCL10 in NFκB activation and it is believed that the oligomerisation and activation of MALT1 depend on BCL10[127]. Thus, it is likely that in MALT lymphoma cells with t(14;18)(q32;q21)/*IGH-MALT1*, MALT1 interacts with BCL10 and stabilises it in the cytoplasm, consequently leading to its accumulation. In line with this notion, there was no alteration in *BCL10* mRNA expression in MALT lymphoma with t(14;18)(q32;q21)/*IGH-MALT1* as compared with other MALT lymphomas lacking the *BCL10* associated chromosomal translocation.

Similarly, in view of the direct interaction between BCL10 and MALT1 in their mediated NFκB activation as demonstrated in *in vitro* studies, one may expect to see MALT1 protein accumulation in MALT lymphoma cells with *BCL10* break/t(1;14)(p22;q32). Intriguingly, this is not the case: MALT1 is only weakly expressed in the cytoplasm of *BCL10* break/t(1;14)(p22;q32) positive cells, in contrast to strong BCL10 expression in the nuclei. Such differential expression of the two proteins in terms of both level and sub-cellular localisation suggests that MALT1 may not be required for BCL10 function. This is supported by knockout mice studies. While BCL10 is essential for antigen receptor mediated NFκB activation in both B and T cells, deficiency of MALT1 expression does not critically affect BCL10 mediated NFκB activation in B cells as it does in T cells[124,155]. It is believed that there is an alternative pathway in BCL10 mediated NFκB activation in B cells, which is MALT1 independent. In addition, BCL10 plays an extra role during neurodevelopment, indicating that BCL10 has additional biological activity compared with MALT1[155].

Similar to *BCL10* break/t(1;14)(p22;q32) positive MALT lymphoma, tumour cells with t(11;18)(q21;q21) generally show a weak/negative MALT1 cytoplasmic expression but moderate BCL10 nuclear expression. Given that the oligomerisation of the API2-MALT1 fusion product is most likely mediated by the N-terminal BIR domains of the API2 molecule, the API2-MALT1 mediated NFκB activation unlikely requires BCL10 or MALT1. In addition, only one allele of the intact *MALT1* gene remains in MALT lymphoma with t(11;18)(q21;q21) and the level of *MALT1* mRNA is much lower in these tumours than those without this translocation. Hence, a weak MALT1 cytoplasmic expression in MALT lymphoma with t(11;18)(q21;q21) is expected, however, moderate nuclear BCL10 expression is a surprising finding. The mechanism underlying BCL10 nuclear expression is unclear. Nonetheless this is unlikely related to the level of *BCL10* mRNA expression since there was no significant difference in its transcript expression between cases expressing nuclear BCL10 (excluding those with BCL10 involved chromosomal translocation) or those expressing cytoplasmic BCL10 (M-Q Du, unpublished data, 2004).

As shown in Chapter 4, strong nuclear BCL10 staining is highly indicative of presence of t(1;14)(p22;q32) or variants. In the present study, we further showed that high levels of cytoplasmic expression of both MALT1 and BCL10 are characteristic of MALT lymphoma with t(14;18)(q32;q21)/*IGH-MALT1*. Such characteristic MALT1 and BCL10 expression pattern was seen in 9/9 MALT lymphomas with t(14;18)(q32;q21)/*IGH-MALT1*. The absence of t(14;18)(q32;q21)/*IGH-MALT1* in MALT lymphoma lacking strong MALT1 and BCL10 cytoplasmic expression was further confirmed by interphase FISH analysis of 174 cases of t(11;18)(q21;q21) and t(1;14)(p22;q32) negative cases. Based on MALT1 and BCL10 immunohistochemistry

followed by interphase FISH analysis, we have shown t(14;18)(q32;q21)/*IGH-MALT1* in MALT lymphoma of the lung (6%), ocular adnexae (7%) and liver (17%) but not in those of the stomach, salivary gland, thyroid and skin. Our current findings on the occurrence of t(14;18)(q32;q21)/*IGH-MALT1* in MALT lymphoma of various sites are in line with those reported from the previous studies[161,286-288]: the translocation is mutually exclusive from t(11;18)(q21;q21) and *BCL10* break/t(1;14)(p22;q32), and occurs biased to extra-gastrointestinal sites.

In view of the characteristic pattern of BCL10 and MALT1 expression in MALT lymphoma with different chromosomal translocations, BCL10 and MALT1 immunohistochemistry may be used as a screening method pointing to the presence of these chromosomal translocations. Since both BCL10 and MALT1 expression patterns in MALT lymphomas with *BCL10* break/t(1;14)(p22;q32) or t(14;18)(q32;q21)/*IGH-MALT1* are characteristic, much easy to recognise and the incidence of both chromosomal translocations are relatively infrequent in MALT lymphoma, it would be rational to screen these translocations first by BCL10 and MALT1 immunohistochemistry, followed by confirmation with interphase FISH. For MALT lymphoma with t(11;18)(q21;q21), BCL10 and MALT1 immunohistochemistry does not provide a strong indication for the presence of the translocation as up to 20% of t(11;18)(q21;q21) negative MALT lymphomas also show a moderate BCL10 nuclear expression. Detection of this translocation is best carried out by RT-PCR of the *API2-MALT1* fusion transcript or interphase FISH.

## 5.5 Conclusion

In summary, we have shown that MALT1 expression pattern is identical to that of BCL10 in normal lymphoid tissues but varies in MALT lymphomas, with high levels of cytoplasmic expression of both MALT1 and BCL10 characterising those with t(14;18)(q32;q21)/*IGH-MALT1*.

## Chapter 6. General discussion

The MALT lymphoma concept, proposed by Isaacson and Wright in 1983, was entirely based on clinical and histological investigations[289]. The concept was reinforced by subsequent immunophentypic studies and analysis of the rearranged *Ig* genes. This is now further supported by recent findings of molecular studies that MALT lymphoma is characterised by three unique chromosomal translocations: t(11;18)(q21;q21)/*API2-MALT1*, t(1;14)(p22;q32)/*IGH-BCL10* and t(14;18)(q32;q21)/*IGH-MALT1*[49]. These chromosomal translocations are specifically associated with MALT lymphoma entity, and are not seen in the chronic inflammatory diseases associated with this lymphoma such as *H. pylori*-associated chronic gastritis, Hashimoto's thyroiditis and lymphoepithelial sialadenitis nor in other NHLs including nodal and splenic marginal zone B cell lymphomas[104,106,290]. Interestingly, although these chromosomal translocations are specifically associated with MALT lymphoma entity, they occur at markedly variable incidences at different sites as shown in this thesis and other studies[106,111,290]. For example, t(11;18)(q21;q21) occurs most frequently in MALT lymphomas of the lung and stomach, but rarely or not in those of the salivary gland, thyroid and skin. These findings suggest that the occurrence of the translocation may be influenced by different aetiological factors associated with MALT lymphoma of different sites. Notably, *H. pylori*-associated chronic gastritis is strongly related to the development of gastric MALT lymphoma. *H. pylori* infection triggers inflammatory responses by attracting and activating neutrophils, which release ROS[90]. These ROS can cause a wide range of genetic damages, particularly double strand break, thus may have a role in the acquisition of genetic abnormalities seen in MALT lymphoma. It is possible that the occurrence of t(11;18)(q21;q21) is related to oxidative damage induced

by *H. pylori* infection. In line with this hypothesis, the genomic breakpoint of t(11;18)(q21;q21) on both derivative chromosomes was random and showed no association with sequence motifs known to be associated with chromosomal recombination[126]. Furthermore, deletions and duplications ranging from a few to several kilobase pair are a common finding at the breakpoint for both *API2* and *MALT1* loci, indicating t(11;18)(q21;q21) may result from illegitimate non-homologous end joining following double strand breaks[126].

However, it is surprising to find that the incidence of t(1;14)(p22;q32) and t(14;18)(q32;q21) is also dramatically variable in MALT lymphoma of different sites[133,161,290,291]. t(1;14)(p22;q32) occurs frequently in MALT lymphomas of the lung and stomach, but not in those of the other mucosal sites, while t(14;18)(q32;q21) is seen frequently in MALT lymphomas of the lung and ocular adenxae, but not in those of the stomach. Like most chromosomal translocations associated with lymphoma, t(1;14)(p22;q32) and t(14;18)(q32;q21) involve the *Ig* locus, and their occurrence is most likely associated with the VDJ recombination event. It remains to be investigated whether their occurrence is also influenced by different aetiological factors associated with MALT lymphoma.

In view of the specific association of t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21) with MALT lymphoma, detection of these chromosomal translocations may be valuable in MALT lymphoma diagnosis and differential diagnosis. For example, by detecting these chromosomal translocations, we have successfully diagnosed MALT lymphoma in a case with Waldenstrom's Macroglobulinemia, for

which it was impossible to distinguish lymphoplasmacytic lymphoma based on histological presentations alone[292].

In addition, detection of these translocations plays a significant role in management of gastric MALT lymphoma. Around 75% of patients with gastric MALT lymphoma can be cured by *H. pylori* eradication. To assess the treatment response, a prolonged follow-up with repeated endoscopy and gastric biopsies is essential. Clinically, it would be immensely beneficial if the *H. pylori* eradication non-responsive cases can be identified at diagnosis. In the present studies, we have shown that t(11;18)(q21;q21) and t(1;14)(p22;q32) or variants were nearly exclusively associated with *H. pylori* eradication non-responsive gastric MALT lymphomas and detection of these translocations could identify 75% and 5% of such cases, respectively. Detection of these chromosomal translocations can be readily carried out on routine diagnostic biopsies. Given that BCL10 expression in MALT lymphomas with t(1;14)(p22;q32) is characteristic and the incidence of this translocations is relatively infrequent, it would be rational to screen this translocation first by BCL10 immunohistochemistry, followed by confirmation with interphase FISH. For MALT lymphoma with t(11;18)(q21;q21), BCL10 immunohistochemistry does not provide a strong indication for the presence of the translocation as up to 20% of t(11;18)(q21;q21) negative cases also show a moderate BCL10 nuclear expression. Detection of this translocation is best carried out by RT-PCR or interphase FISH.

The molecular mechanisms underlying the remaining 20% of *H. pylori* eradication non-responsive gastric MALT lymphomas are not clear. In approximately 5% of gastric MALT lymphomas, there is no evidence of *H. pylori* infection, and these cases do not

respond to *H. pylori* eradication[238,293]. We have previously studied 17 cases. In each case, the lack of *H. pylori* infection was confirmed by serology, histology/immunohistochemistry and none of them responded to antibiotic treatment[293]. Additional chromosomal translocations have been reported in MALT lymphoma and they may account for the remaining cases that do not respond to *H. pylori* eradication. The *BCL6* proto-oncogene involved chromosomal translocation has been reported in a few cases of MALT lymphomas[172,173,294]. In a recent study, we have screened 305 cases of MALT lymphoma for BCL6 involved translocation by interphase FISH and found the translocation in 2/133 cases of gastric MALT lymphoma[295]. Immunohistochemistry also revealed aberrant *BCL6* expression in these cases. It remains to be examined whether *BCL6* translocation positive gastric MALT lymphoma responds to *H. pylori* eradication.

Another chromosomal translocation recently reported in MALT lymphomas is t(3;14)(p14;q32)/*IGH-FOXP1*[285,296]. Although the study by Streubel et al[285] failed to detect t(3;14)(p14;q32) in gastric MALT lymphoma, the translocation was seen in a case of gastric MALT lymphoma as well as two cases of DLBCL in the report by Wlodarska et al[296]. t(3;14)(p14;q32)-positive MALT lymphomas, like those with t(1;14)(p22;q32) and t(14;18)(q32;q21), frequently harbour other chromosomal aberrations such as trisomy 3[285]. However, the clinical behaviour of t(3;14)(p14;q32)-positive gastric MALT lymphoma is unknown. By screening 366 cases of MALT lymphoma of different sites with interphase FISH using *IGH* break-apart probes, we have identified two cases of gastric MALT lymphoma harbouring a breakpoint in the *IGH* locus (M.Q. Du, unpublished data). One case was confirmed carrying t(3;14)(p14;q32)/*IGH-FOXP1* by interphase FISH. Interestingly, this case did not respond to *H. pylori* eradication despite being at stage $I_E$. It remains to be validated in a large cohort whether

t(3;14)(p14;q32)-positive gastric MALT lymphoma resists to *H. pylori* eradication. The other case, while lacks evidence of common lymphoma associated chromosomal translocations, is currently under investigation.

In addition to gastric MALT lymphoma, detection of chromosomal translocations may also help to predict the treatment response of MALT lymphoma of other sites. A recent study by Ferreri et al showed that *Chlamydia psittaci* infection was associated with MALT lymphoma of the ocular adnexae and 2 of the 4 patients studied responded to antibiotic therapy for *Chlamydia psittaci*[31]. Given both t(11;18)(q21;q21) and t(14;18)(q32;q21) frequently occur in ocular MALT lymphoma, it would be pertinent to examine the impact of these chromosomal translocations on antibiotic therapy of this tumour. Nonetheless, the association between ocular MALT lymphoma *Chlamydia psittaci* infection and the lymphoma respond to antibiotic therapy remain to be validated in future investigations.

In general, different lymphoma subtypes are characterised by distinct chromosomal translocations. In a given lymphoma subtype, the same oncogene is frequently deregulated by chromosomal translocations involving different partners. For example, c-myc is deregulated by chromosomal translocations involving various *Ig* loci in Burkitt's lymphoma[297]. Similarly, the *BCL6* involved translocations, frequently seen in DLBCL, may involve several loci including both *Ig* and non-*Ig* gene loci[297]. In MALT lymphoma, notably, different oncogenes are involved by different chromosomal translocations, with t(11;18)(q21;q21) generating a chimeric API2-MALT1 fusion product.

There are no apparent differences in histology and immunophenotype among MALT lymphomas with different chromosomal translocation status although t(11;18)(q21;q21)-positive cases tend to be more homogeneous, lacking transformed blasts[298]. Such histological homogeneity may be explained by the recent findings that the three major MALT lymphoma associated chromosomal translocations essentially target the same molecular pathway and they are linked by the physiological role of BCL10 and MALT1 in antigen receptor mediated NFκB activation. BCL10 specifically transduces antigen receptor signalling to activate the NFκB pathway, operating upstream of MALT1. In response to the upstream signal, BCL10 binds MALT1 and induces conformational changes of MALT1, which allow MALT1 oligomerisation and trigger the downstream events leading to NFκB activation[123,125]. In MALT lymphoma with t(1;14)(p22;q32), BCL10 is over-expressed and is thought to form oligomers through its CARD domain without the need of upstream signals, leading to constitutive NFκB activation. In those with t(14;18)(q32;q21), the oligomerisation of the over-expressed MALT1 is thought to be dependent on BCL10. In MALT lymphoma with t(11;18)(q21;q21), the API2-MALT1 fusion product is believed to self-oligomerise through the BIR domain of the API2 molecule, therefore leading to constitutive NFκB activation.

In view of the above findings and hypothesis, it is interesting to investigate whether there is any molecular link between the BCL10/MALT1-NFκB activation pathway and BCL6 as well as FOXP1.

BCL6 is a transcriptional repressor and is believed to exert its oncogenic activity by repression of a group of genes involved in B cell activation and terminal differentiation (such as B-lymphocyte-induced maturation protein 1 (Blimp1) and cell cycle control

(cyclin D2, p27), thus contributing to lymphomagenesis[297]. BCL6 is preferentially expressed by B and T cells in germinal centre and is critical for germinal centre development[297]. In view of that BCL10 is highly expressed in germinal centre B cells and is critical for B cell activation and maturation, there might be molecular link between the physiological role of BCL10 and BCL6.

FOXP1 is a member of the FOXP subfamily of transcription factors. Although its function in B cell development and maturation is unclear, the protein is expressed in activated B cells including those in the germinal centre. Interestingly, high FOXP1 expression is strongly associated with non-germinal centre type of DLBCL, which is characterised by high NF$\kappa$B activity[299]. Nonetheless, it remains to be examined whether FOXP1 is functionally connected to the NF$\kappa$B activation pathway.

One of the most intriguing findings in this thesis is the altered subcellular localisation of BCL10 protein in MALT lymphoma. In line with the physiologic role of BCL10, BCL10 is expressed primarily in the cytoplasm of normal lymphocytes. However, the protein is strongly expressed in the nuclei of MALT lymphoma cells with t(1;14)(p22;q32). Moderate levels of nuclear BCL10 expression are also seen in all t(11;18)(q21;q21)-positive cases and up to 20% of MALT lymphomas lacking these chromosomal translocations. BCL10 nuclear expression is not related to BCL10 mutation or BCL10 gene amplification[142,291]. In BCL10 transgeneic mice, in which *BCL10* gene expression is linked to Ig enhancers, BCL10 is also highly preferentially expressed in the nucleus of splenic marginal zone B cells (M.Q. Du and S. Morris, unpublished observations). These data strongly indicate that aberrant nuclear BCL10

expression might confer oncogenic activity. It remains a mystery how BCL10 is translocated into nuclei, and what is the function of nuclear BCL10.

BCL10 does not harbour nuclear localisation signals and its nuclear translocation must be mediated by its interacting proteins. Several BCL10 interacting proteins including CARMA1, MALT1 and TRAF6 have been identified[160]. However, none of them containing a nuclear localisation signal. It is interesting to note that three NF$\kappa$B interacting molecules including I$\kappa$B$\alpha$, NF$\kappa$B-inducing kinase (NIK) and I$\kappa$B kinase 1 (IKK1) are capable of shuttling between cytoplasm and nucleus[300]. When these proteins together with NF$\kappa$B are translocated to the nucleus, they continue to modulate NF$\kappa$B transcriptional activity[300-302]. It is possible that BCL10 is a member of such protein complex and is translocated to nucleus as a part of the NF$\kappa$B/regulating partner complex. Nonetheless, the hypothesis remains to be tested.

In summary, the thesis developed the methodology/strategy for screening MALT lymphoma associated chromosomal translocations, i.e. t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21), and examined their frequencies in MALT lymphoma of different sites. Detection of these chromosomal translocations plays an important role in clinical management of gastric MALT lymphoma although such role in extra-gastric MALT lymphomas remains to be investigated. In view the findings of this thesis, it is pertinent to suggest the following approach for management of gastric MALT lymphoma. t(11;18)(q21;q21) and t(1;14)(p22;q32) should be routinely screened using methods discussed as above. Those positive for the chromosomal translocations should be given *H. pylori* eradication, followed by chemotherapy or radiotherapy, while those negative for these chromosomal translocations having clinical or histological

evidence of *H. pylori* infection, patients should be treated first with *H. pylori* eradication, periodically followed up with endoscopic and histological investigation. Patients, not responding to *H. pylori* eradication by 12 to 18 months, should be treated additionally with chemotherapy or radiotherapy.

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