Molecular Pathogenesis of MALT Lymphoma

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Declarations

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

I also declare that this thesis is not substantially the same as any that I submitted for a degree or diploma or other qualification at any other University, and that no part has already been, or is currently being, submitted for any degree, diploma, or other qualification.

This dissertation comprises a total of 264 pages. Excluding tables, figures, references and appendices, the number of pages corresponding to text is 195.

Rifat Akram Hamoudi
September 2010
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Abbreviations

Some abbreviations used only once or a few times, particularly with gene names, may not be included in this list, but are explained in the main text. In general gene names and translocations are written in italics.

AP-1  Activator protein 1
CCR  Chemotactic cytokines receptor
cDNA  complementary DNA
CLL  Chronic lymphocytic leukaemia
cRNA  complementary RNA
DLBCL  Diffuse large B-cell lymphoma
DLR  Dual luciferase reporter assay
FL  Follicular lymphoma
gcRMA  GeneChip RMA
GO  Gene ontology
GSEA  Gene set enrichment analysis
H. pylori  Helicobactor pylori
IκB  Inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells
MALT  Mucosa associated lymphoid tissue
MAPK  Mitogen-activated protein kinases
MAS5  Affymetrix Microarray Suite 5
MCL  Mantle cell lymphoma
NF-κB  nuclear factor kappa-light-chain-enhancer of activated B cells
NHL  Non-Hodgkin lymphoma
PBS  Phosphate buffered saline
qPCR  Quantitative PCR
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<th>Abbreviation</th>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real time PCR</td>
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<tr>
<td>RMA</td>
<td>Robust Multiarray Averaging</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SMZL</td>
<td>Splenic marginal zone lymphoma</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>AP-1</td>
<td>Activator protein 1</td>
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<td>Follicular lymphoma</td>
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<td>MALT</td>
<td>Mucosa associated lymphoid tissue</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
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<tr>
<td>MAS5</td>
<td>Affymetrix Microarray Suite 5</td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
<td>MCL</td>
<td>Mantle cell lymphoma</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NHL</td>
<td>Non-Hodgkin lymphoma</td>
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<td>Quantitative real time PCR</td>
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<td>RMA</td>
<td>Robust Multiarray Averaging</td>
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Abstract

Molecular Pathogenesis of MALT Lymphoma

By Rifat Akram Hamoudi, Churchill College, University of Cambridge

Mucosa associated lymphoid tissue (MALT) lymphoma is characterized by \( t(11;18)(q21;q21)/API2-MALT1 \), \( t(1;14)(p22;q32)/BCL10-IGH \) and \( t(14;18)(q32;q21)/IGH-MALT1 \), which commonly activate the NF-\( \kappa \)B pathway. Gastric MALT lymphomas harbouring such translocation do not respond to \textit{Helicobacter pylori} eradication, while those without translocation can be cured by antibiotics.

To understand the molecular mechanism of MALT lymphoma with and without chromosome translocation, 24 cases (15 translocation-positive and 9 translocation-negative) of MALT lymphomas together with 7 follicular lymphomas and 7 mantle cell lymphomas were analysed by Affymetrix gene expression microarray platform. Unsupervised clustering showed that cases of MALT lymphoma were clustered as a single branch. However, within the MALT lymphoma group, translocation-positive cases were intermingled with translocation-negative cases. Gene set enrichment analysis (GSEA) of the NF-\( \kappa \)B target genes and 4394 additional gene sets covering various cellular pathways, biological processes and molecular functions showed that translocation-positive MALT lymphomas were characterized by an enhanced expression of NF-\( \kappa \)B target genes, particularly \( TLR6, CCR2, CD69 \) and \( BCL2 \), while translocation-negative cases were featured by active inflammatory and immune responses, such as \( IL8, CD86, CD28 \) and \( ICOS \). Separate analyses of the genes differentially expressed between translocation-positive and negative cases and measurement of gene ontology term in these differentially expressed genes by hypergeometric test reinforced the above findings by GSEA. The differential expression of these NF-\( \kappa \)B target genes between MALT lymphoma with and without translocation was confirmed by quantitative RT-PCR and immunohistochemistry or Western blot.
Expression of TLR6, in the presence of TLR2, enhanced both API2-MALT1 and BCL10 mediated NF-κB activation *in vitro*. In addition, there was cooperation between expression of BCL10, MALT1 or API2-MALT1, and stimulation of the antigen receptor or CD40 or TLR in NF-κB activation as shown by both reporter assay and IκBα degradation. Interestingly, expression of BCL10 but not API2-MALT1 and MALT1, in the presence of LPS stimulation, also triggered IκBβ degradation, suggesting activation of different NF-κB dimers between these oncogenic products.

Study by co-immunoprecipitation showed that BCL10 directly interacts with MALT1. Sub-cellular localisation experiments in BJAB B-cells, showed that BCL10 localisation was affected by MALT1. When BCL10 was over-expressed, the protein was predominantly expressed in the nuclei, but when MALT1 was over-expressed, BCL10 was mainly localised in the cytoplasm. When both BCL10 and MALT1 were over-expressed, BCL10 was expressed in the cytoplasm in the early hours when the protein level was low, but in both the cytoplasm and nuclei after 9 hours when the protein level was high. Over-expression of API2-MALT1 did not shown any apparent effect on BCL10 sub-cellular localisation *in vitro*.

Finally, comparison of MALT lymphoma expression microarray with other lymphomas showed *lactoferrin* to be highly expressed in MALT lymphoma. This was confirmed by qRT-PCR, showing *lactoferrin* to be significantly over-expressed in MALT lymphoma compared to FL and MCL. Thus *lactoferrin* may be a potential marker for MALT lymphoma.
Publications

Arising from this thesis:

   Differential expression of NF-kappaB target genes in MALT lymphoma with and
   without chromosome translocation: insights into molecular mechanism.
   Leukemia. 2010 Aug;24(8):1487-1497

   MALT lymphoma with t(14;18)(q32;q21)/IGH-MALT1 is characterized by strong
   cytoplasmic MALT1 and BCL10 expression.
   J Pathol. 2005 Feb;205(3):293-301.

Not arising from this thesis but related to MALT lymphoma:

   t(11;18)(q21;q21) of mucosa-associated lymphoid tissue lymphoma results from
   illegitimate non-homologous end joining following double strand breaks.

   T(11;18) is a marker for all stage gastric MALT lymphomas that will not respond to
   H. pylori eradication.
   Gastroenterology. 2002 May;122(5):1286-1294.

   T(11;18)(q21;q21) is associated with advanced mucosa-associated lymphoid tissue
   lymphoma that expresses nuclear BCL10.

   Resistance of t(11;18) positive gastric mucosa-associated lymphoid tissue lymphoma
   to Helicobacter pylori eradication therapy.
Acknowledgements

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Dr Hongxiang Liu and Dr. Jim Watkins for stimulating discussions.

Dr. Connie Parkinson, Dr. Tim Diss, Dr. Estelle Chanudet and Professor Dennis Wright for help in critically reading the thesis. Dr. Alex Freeman and Mr. Keith Miller for their support throughout the period of the thesis.

I would like to thank my wife Kamile for her patience and endless support for my work and putting up with the loneliness and emptiness in her life whilst I was working on this thesis. çok teşekkür ederim.

Finally, I would like to dedicate this thesis to all those who are about to give up on a difficult problem. This thesis is the proof that if you persevere long enough with a difficult problem, eventually a solution will be found.
CHAPTER 1 – General introduction

1.1 Brief overview of lymphomas

Lymphoma is defined as a neoplastic proliferation of lymphoid cells. 90% of all lymphoid malignancies are of B-cell lineage, whilst a minority are of T-cell (7%) or NK-cell lineage (<2%). In 1832 Thomas Hodgkin first described what became known as Hodgkin lymphoma. Hodgkin lymphoma (HL) accounts for approximately 30% of all lymphomas and comprises 2 distinct disease entities, the more frequent classical Hodgkin lymphoma (cHL) (95%) and the uncommon nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) (5%). In cHL, the neoplastic cells are usually a minority population whose survival appears to be dependent on the majority of reactive or inflammatory cells (Aldinucci et al., 2010). In the majority of cases the neoplastic (Reed-Sternberg (Reed, 1902)) cells appear to be of B-cell origin. NLPHL is characterised by lymphocytic and histocytic (L&H) or “popcorn” cells in that are distributed amidst abundant non-neoplastic inflammatory and accessory cells.

Approximately 70% of lymphomas do not have the clinical and pathological features of HL and have therefore been categorised historically as non-Hodgkin lymphomas (NHL). NHL are frequently disseminated and are all considered malignant or potentially malignant. Some are aggressive from the outset, while others are indolent for varying lengths of time, but may transform to more aggressive tumours. According to the World Health Organisation (WHO) 2008 classification (Swerdlow et al., 2008), these lymphoid neoplasms which together account for the majority of lymphomas are classified as distinct disease entities under the broader categories of precursor lymphoid neoplasms, mature B-cell neoplasms, mature T and NK-cell neoplasms, immunodeficiency-associated lymphoproliferative disorders and histocytic and dendritic cell neoplasms.
Most NHLs are mature B-cell neoplasms corresponding to a clonal proliferation of B cells at various stages of differentiation. They can be categorised into pre-germinal, germinal and post-germinal centre origin according to their differentiation stage using the germinal centre reaction as a reference. The most common type of NHL is diffuse large B-cell lymphoma (DLBCL) which represents 37% of all NHL and consists of a heterogeneous group of large B-cell tumours (Swerdlow et al., 2008), followed by follicular lymphoma (FL) which represents 29% of all NHL and is characterised by clonal expansion of neoplastic follicle centre-type cells. The third most frequent subtype of B-cell NHL is chronic lymphocytic leukaemia/small lymphocytic lymphoma (12%), followed closely by extranodal marginal zone lymphoma of mucosa associated lymphoid tissue (MALT lymphoma) which accounts for 9% of all B-cell lymphomas. Both MALT and follicular lymphomas may transform to a high grade lymphoma, most frequently diffuse large B-cell lymphoma. Nodal marginal zone (MGZ) lymphoma and splenic MGZ lymphomas are separate rare entities that involve lymph nodes (nodal MGZ lymphoma) and spleen usually with bone marrow and blood involvement (splenic MGZ lymphoma) respectively (Swerdlow et al., 2008).

MALT lymphoma can occur in a wide range of organs (Swerdlow et al., 2008). Amongst the various extranodal sites, gastric MALT lymphoma is the most common and thus the best characterised form of this disease (Du et al., 2002). Nodal MZL may be seen as an apparently primary disease as a result of either splenic MZL or extranodal MZL.
1.2 Overview of MALT lymphoma

MALT (Mucosa associated lymphoid tissue) lymphoma is an indolent neoplasm where tumours tend to stay localised at their site of origin until the late phase of the disease. It occurs at various extra-nodal sites. The most common site is the gastrointestinal (GI) tract, comprising 50% of all cases, and within the GI tract, the stomach is the most common location accounting for 85% of GI MALT lymphomas. The small intestine is typically involved in patients with immunoproliferative small intestinal disease (IPSID). Other frequent sites include salivary gland, lung, ocular adnexa, skin, thyroid and breast (Swerdlow et al., 2008). Apart from the small intestine, these anatomical sites are normally devoid of organised lymphoid tissues, thus MALT lymphoma appears to arise from acquired MALT, commonly due to chronic immunological stimulation, resulting either from pathogen infection or autoimmune disorders (Du, 2007). Patients generally present with MALT lymphoma at stages I or II. The average age of disease onset is 61 and the 5-year survival rate of patients has been as high as 93% in some studies. The median time before progression of the disease is approximately 5 years although it is significantly longer for cases with a gastrointestinal origin compared to those from other sites (Thieblemont et al., 1997). 2-20% of patients have bone marrow involvement and up to 10% have lymphoma in multiple extra-nodal sites (Isaacson et al., 1987; Wotherspoon et al., 1993), but they generally respond well to therapy and have a good overall prognosis (Fischbach et al., 2007).

1.2.1 Aetiology of MALT lymphoma

As mentioned in section 1.2, pathogen infection and autoimmune disorders play a major role in the development of MALT lymphoma. In this section, the aetiological role of pathogens such as Helicobacter pylori, Campylobacter jejuni, Borrelia burgdorferi and Chlamydia
psittaci and autoimmune disorders such as Sjögren’s syndrome and Hashimoto’s thyroiditis will be discussed.

1.2.1.1 Chronic infection

It has been shown that infection with certain microorganisms leads to the acquisition of lymphoid tissue at extra nodal sites that are normally devoid of any organised lymphoid tissues and hence plays a role in MALT lymphoma development (Banks, 2007).

*Helicobacter pylori*

In 1984 Marshall and Warren isolated a newly recognised bacterium, *Helicobacter pylori*, from patients with chronic gastritis and gastric ulcer (Marshall *et al.*, 1984). *H. pylori*, originally called *Campylobacter pylori*, is a unipolar, multiflagellate spiral shaped, microaerophilic, gram negative bacterium that lives in the luminal surface of the stomach and duodenum (Bolin *et al.*, 1995). *H. pylori* is widespread and has been implicated in several gastrointestinal diseases, such as chronic gastritis and peptic ulcer, (Howden *et al.*, 1998) gastric adenocarcinoma, (Asaka *et al.*, 1997) and MALT lymphoma (Hussell *et al.*, 1993b). In 1987, Smith *et al.* showed monoclonal rearrangements of the Ig-heavy chains in IPSID, including cases that responded to antibiotics (Smith *et al.*, 1987), and suggested the possible involvement of bacteria-driven antigen stimulation in the development of the lymphoma. Subsequent studies established that, in the stomach, MALT is acquired as a result of colonisation of the gastric mucosa by *H. pylori* (Stolte *et al.*, 2002; Wotherspoon *et al.*, 1991). In 1991, Wotherspoon *et al.* demonstrated the presence of *H. pylori* in 101 of 110 (92%) patients with gastric MALT lymphoma (Wotherspoon *et al.*, 1991) and suggested for the first time that gastric MALT lymphoma may develop from the MALT acquired in response to *H. pylori* infection.
At least 80-90% of patients with gastric MALT lymphoma are infected with *H. pylori*, which is much higher than the frequency of infection in the rest of the population (Wotherspoon *et al.*, 1991). The gastric mucosa is a hostile environment to most organisms due to its acidic environment. However, *H. pylori* secretes urease that raises the local pH so it is able to survive and colonise the gastric mucosa. The incidence of gastric MALT lymphoma was found to be high in North Eastern Italy where *H. pylori* infection is prevalent (Doglioni *et al.*, 1992).

*Campylobacter jejuni* and *Borrelia burgdorferi*

These were shown to be associated with a proportion of primary cutaneous MALT lymphoma and IPSID respectively (Cerroni *et al.*, 1997; Lecuit *et al.*, 2004).

*Chlamydia psittaci*

*Chlamydia psittaci* (*C. psittaci*) infection was recently shown to be associated with the development of ocular adnexal MALT lymphoma. In Italian studies, *Chlamydia psittaci* was detected in 80% of these lymphomas (Ferreri *et al.*, 2004). However, the association between *Chlamydia psittaci* and ocular adnexal MALT lymphoma was not reproduced by several other studies from the USA (Vargas *et al.*, 2006), Japan (Daibata *et al.*, 2006) and the Netherlands (Mulder *et al.*, 2006). Subsequently, Chanudet and co-workers confirmed geographical variation in the association between *Chlamydia psittaci* and ocular adnexal MALT lymphoma (Chanudet *et al.*, 2006).

Further investigations are needed to confirm or refute the causal association between the organisms described above and extranodal marginal zone lymphoma at various sites.
1.2.1.2 Autoimmune disease

In addition to microbial infections discussed in section 1.2.1.1, autoimmune disease plays a role in MALT lymphomagenesis. For example, thyroid MALT lymphoma is associated with Hashimoto’s thyroiditis and salivary gland MALT lymphoma is associated with lymphoepithelial sialadenitis (Kassan et al., 1978; Kato et al., 1985). Patients with Sjögren’s syndrome, and a number of other autoimmune disorders such as rheumatoid arthritis show an increased risk of lymphoma development (Smedby et al., 2006). These autoimmune diseases result in chronic immune responses and the formation of acquired MALT. Patients with these diseases are approximately 40 times more likely to develop lymphoma, and 85% of the lymphomas developed are MALT lymphoma (Kassan et al., 1978; Talal et al., 1967). In both Hashimoto thyroiditis and Sjögren’s syndrome, lymphomagenesis is thought to be mediated by sustained T-cell dependent antigenic stimulation, similarly to that in H. pylori-driven gastric MALT lymphoma (Yamamoto, 2003).
1.2.2 Histopathology of MALT lymphoma

Since the development of MALT lymphoma relies on the acquisition of organised MALT, it is best to describe MALT followed by MALT lymphoma.

1.2.2.1 Mucosa associated lymphoid tissue

The mucosa-associated lymphoid tissue is situated within mucosal tissues. The main function of MALT is to prevent foreign antigen invasion from the mucosal sites. In response to antigen specific T-cell activation, naïve B cells undergo clonal expansion, differentiation and become effector cells secreting immunoglobulins, thus conveying the mucosal immune response. Native MALT is found in the gastrointestinal tract where it is abundantly present in the Peyer’s patches of terminal ileum (Figure 1.1a). Typically, MALT in the Peyer’s patches consists of germinal centres where B cells encounter antigens and undergo a series of mutations to enhance their antigen specificity. Germinal centres contain activated B cells, named centroblasts, as well as their differentiated counterpart expressing immunoglobulins called centrocytes, macrophages and follicular dendritic cells essential to germinal centre reactions. The germinal centre is surrounded by a follicular mantle, formed by naïve B cells not yet exposed to antigen. Outer to the mantle zone is a marginal zone, where memory B cells reside. A distinct marginal zone, although a feature of Peyer’s patches is not present in human tonsillar tissue, and therefore not universally present in all MALT. However, there is evidence to show that intra-epithelial B-cells in tonsillar tissue could represent the equivalent of marginal zone B cells (Morente et al., 1992).
Figure 1.1 - Morphology of MALT and gastric MALT lymphoma from the gastrointestinal tract.

a. The Peyer’s patches are characterised histologically by the presence of a germinal centre (GC) surrounded by a follicular mantle (FM) and a marginal-zone (MGZ). Intraepithelial marginal-zone B cells (IEBC) are observed within the epithelium covering the Peyer’s patch, forming the lymphoepithelium characteristic of MALT.

b. The morphology of gastric MALT lymphoma. The germinal centre (GC), where B cells proliferate and mature following antigen stimulation, is surrounded by a follicular mantle (FM), which comprises naive B cells. The reactive B-cell follicle is surrounded by neoplastic marginal-zone (MGZ) B cells that infiltrate the neighbouring epithelium forming characteristic lymphoepithelial lesions (LEL).

Figure adapted from Isaacson et al. (2004). Nature Review Cancer, 4, 644-653.
Figure 1.2 - Cytological and histological features of MALT lymphoma.

a-c. Cytology: neoplastic cells can resemble small lymphocytes (a), or have the appearance of centrocytes but with more abundant cytoplasm (b), or rather have the features of monocytoid cells with abundant pale cytoplasm and well-defined borders (c). Scattered transformed centroblasts or immunoblast-like cells can also be observed (a). d-e. Histological features: MALT lymphoma can show prominent plasma cell differentiation (d). The neoplastic cells can infiltrate the surrounding epithelium, forming lymphoepithelial lesions (d), or invade B-cell reactive follicles (follicular colonisation) (e).

Figure adapted from Isaacson et al. (2005). Journal of Pathology, 205, 255-274.

1.2.2.2 Extranodal marginal zone B-cell lymphoma of MALT

Histologically, MALT lymphoma mimics the features of normal MALT (Figure 1.1b). The striking histological resemblance between some low grade B-cell lymphomas of the stomach, IPSID and the morphology of the Peyer’s patches which led to the proposition of the MALT lymphoma entity by Isaacson and Wright in 1983 (Isaacson et al., 1983). Similar to the structure of Peyer’s patches, MALT lymphoma consists of neoplastic B-cell infiltrates that are primarily located in the marginal zone (which lies outer to the mantle zone that surrounds reactive germinal centres) and can extend into the interfollicular region (Figure 1.1b). An important feature of MALT lymphoma is the presence of aggregates of neoplastic cells
infiltrating individual mucosal glands or other epithelial structures. Such aggregates, referred
to as lymphoepithelial lesions (Figure 1.1b, Figure 1.2d), resemble lymphoepithelium in
Peyer’s patches (Figure 1.1b) (Isaacson et al., 2004).
Cytologically, MALT lymphoma cells are variable in their appearance, resembling
centrocyte-like cells, sometimes with round nuclei and pale staining cytoplasm, monocytoid
B cells or small lymphocytes (Figure 1.2 a-c). Lymphoma cells may also show prominent
plasma cell differentiation particularly in the sub-epithelial lamina propria (Figure 1.2d). In
some cases the lymphoma cells may invade B-cell reactive follicles (Figure 1.2e). This so-
called follicular colonisation can lead to morphological resemblance to FL.

1.2.3 Immunophenotype
The typical immunophenotype of MALT lymphoma is detailed in Table 1.1. MALT
lymphoma cells mostly share the immunophenotype of non-neoplastic MGZ cells (Isaacson
et al., 1987). Currently there is no available marker specific to MALT lymphoma,
nevertheless immunophenotyping is helpful for the differential diagnosis, notably from other
small B-cell lymphomas (Table 1.1).
Table 1.1 - Typical immunophenotyping of MALT lymphoma cells.

<table>
<thead>
<tr>
<th>Cellular marker</th>
<th>MALT lymphoma</th>
<th>FL</th>
<th>MCL</th>
<th>SLL</th>
<th>DLBCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD20</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD79a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BCL2</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>CD43</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CD5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>CD10</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>CD23</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>BCL6</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>Ig heavy chain</td>
<td>M&gt;&gt;A&gt;G</td>
<td>G&gt;M+D</td>
<td>M+D</td>
<td>M</td>
<td>Variable</td>
</tr>
<tr>
<td>CD21 and FDC</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>meshwork</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: MALT, mucosa-associated lymphoid tissue; FL, follicular lymphoma; MCL, mantle-cell lymphoma; SLL, small lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; CD, cluster of differentiation; FDC, follicular dendritic cell; + positive; - negative.

The neoplastic cells of MALT lymphoma share the cytological features and immunophenotype (CD20\(^-\), IgM\(^+\), IgD\(^-\)) of marginal zone B cells (Spencer et al., 1985). MALT lymphoma cells are typically negative for CD10 and BCL6, which are characteristically positive in FL. Unlike mantle cell lymphoma (MCL) and small lymphocytic lymphoma, MALT lymphoma is only infrequently positive for CD5. It is negative for cyclin D1, a feature which helps to distinguish it from MCL (Table 1.1). The most important immunophenotypic feature favouring MALT lymphoma diagnosis is the presence of a diffuse infiltrate of CD20\(^-\), IgM\(^+\), IgD\(^-\) B cells outside the mantle zone of reactive follicles. Once the marginal zone phenotype is established, light chain restriction in this marginal zone population, or if present, within the plasma cells confirms the diagnosis. However, having a marker specific for MALT lymphoma would lead to more accurate MALT lymphoma diagnosis.
1.2.4 Pathogenesis of MALT lymphoma

The development of MALT lymphoma is a multistage process which is best understood in the gastric disease (Isaacson et al., 1994; Isaacson et al., 1995; Isaacson, 1995). Both gastric MALT lymphoma and pre-lymphomatous lesions are aetiologically related to *H. pylori* infection. Understanding the role of *H. pylori* has provided insights into the pathogenesis of MALT lymphoma.

1.2.4.1 *H. pylori* and its role in the development of gastric MALT lymphoma

There is now strong evidence that *H. pylori* is causally linked to gastric MALT lymphoma and this fulfils all criteria of Koch’s postulates set in 1884 (Koch, 1884); Firstly, *H. pylori* was found in the majority of gastric MALT lymphomas (Eidt et al., 1994). Secondly, *H. pylori* could be isolated from gastric MALT lymphoma, and grown in culture. Thirdly, infecting the stomach of pathogen-free mice with *H. pylori* could induce the development of MALT lymphoma (Enno et al., 1995).

Additionally, early functional and clinical studies provided evidence of the crucial role of *H. pylori* in the development of gastric MALT lymphoma. Hussell and colleagues demonstrated that lymphoma cell growth *in vitro* was dependent on *H. pylori* specific T cells (Hussell et al., 1996). Furthermore, Wotherspoon and colleagues first showed that *H. pylori* eradication by antibiotic treatment led to lymphoma regression in *H. pylori*–associated gastric MALT lymphomas (Wotherspoon et al., 1991). Taken together, these data established that *H. pylori* infection could cause gastric MALT lymphoma.

It is now known that the inflammatory process triggered by *H. pylori* infection is directly responsible not only for the acquisition of MALT in the gastric mucosa, but also for subsequent malignant transformation and the development of gastric MALT lymphoma.
As in other bacterial diseases, the development of \textit{H. pylori}-associated MALT lymphoma is hypothesised to be associated with the host response and bacterial status. However, the precise role of \textit{H. pylori} in MALT lymphomas is not clear.

1.2.4.2 Immunological stimulation

Several histological features of MALT lymphoma including the presence of plasma-cell differentiation, blasts, follicular colonisation and proliferation, suggest that MALT lymphoma cells preserve B-cell properties and that their growth may be partially driven by antigenic stimulation via antigen receptors (Isaacson \textit{et al.}, 2004) and T-cell and B-cell interaction. Recent studies indicate that both direct and indirect antigen stimulation mechanisms are involved.

1.2.4.2.1 Direct antigen stimulation

MALT lymphomas invariably express surface immunoglobulin. The anti-idiotype antibody has been shown to stimulate MALT lymphoma cell proliferation and synergise with mitogen stimulation (Hussell \textit{et al.}, 1993b). The data also showed that the tumour-derived immunoglobulin does not recognise \textit{H. pylori}, but recognises various autoantigens (Hussell \textit{et al.}, 1993a). Antibodies to gastric epithelial cells are commonly present in serum samples from patients with \textit{H. pylori} gastritis (Negrini \textit{et al.}, 1996). An anti-idiotype antibody to immunoglobulin of a gastric MALT lymphoma cross-reacts specifically with reactive B cells in \textit{H. pylori}-associated gastritis (Greiner \textit{et al.}, 1997). These findings suggest that gastric MALT lymphoma cells are transformed from autoreactive B cells, which are induced after \textit{H. pylori} infection.

Sequence analysis of the rearranged \textit{immunoglobulin} of MALT lymphoma also reveals evidence that MALT lymphoma cells respond to direct antigen stimulation, indicating the
tumour clone has undergone antigen selection (Bertoni et al., 1997; Du et al., 1996b). During the evolution of gastric MALT lymphoma, particularly in the early stage, the rearranged tumour immunoglobulin gene frequently showed further somatic mutations, commonly referred to as ongoing mutations (Bertoni et al., 1997; Du et al., 1996a). Since somatic mutations occurs in the rearranged immunoglobulin gene only during the germinal centre reaction, and depends on antigen and T cells, the finding of ongoing immunoglobulin mutations in MALT lymphoma suggests that tumour-cell growth is partially driven by direct antigen stimulation.

### 1.2.4.2.2 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 coculturing tumour cells with 13 clinical strains of heat-killed *H. pylori*, Hussell and co-workers demonstrated that *H. pylori* induced tumour cells to proliferate (Hussell et al., 1993b). The effect was strain-specific but was T-cell mediated and not due to specificity of lymphoma cells for *H. pylori* antigens. This effect was associated with expression of interleukin-2 (IL-2) receptors and secretion of immunoglobulin by tumour cells. Removal of tumour-infiltrating T cells before the experiment abolished all the effects of *H. pylori* on tumour cells. Furthermore, these authors confirmed that *H. pylori* did not directly stimulate tumour cells but did so via specifically activated tumour infiltrating T cells (Hussell et al., 1996). Furthermore, the stimulating effect of *H. pylori* on tumour B cells can be completely blocked by an antibody to CD40L. Thus, *H. pylori* stimulates lymphoma B cells through tumour-infiltrating T cells, involving CD40 and CD40L. It is possible that CD80/CD86 costimulatory molecules promote T-cell-mediated neoplastic B-cell growth.
The active role of tumour-infiltrating T cells in the growth of tumour B cells is further 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. These specific T-cell clones activated tumour B cells in a dose dependent manner (D'Elios *et al.*, 1997).

Additionally, complete regression of gastric MALT lymphoma following *H. pylori* eradication strongly indicates the role of *H. pylori* stimulation in the survival and growth of gastric MALT lymphoma (Begum *et al.*, 2000; Wotherspoon, 1996). Unlike low-grade tumour cells, high-grade tumour cells do not show any growth response to *H. pylori* mediated T-cell stimulation *in vitro* (Hussell *et al.*, 1993b). High grade lymphomas tend to be resistant to *H. pylori* eradication therapy (Bayerdorffer *et al.*, 1995; Thiede *et al.*, 1997), though recently published reports show a complete remission rate of 62.5% with a median follow-up of over 30 months in gastric DLBCLs (Chen *et al.*, 2001; Morgner *et al.*, 2001).

![Figure 1.3 - The role of T- and B- cell interaction in the development of MALT lymphoma.](image)

FH: Follicular Helper cells, TCR: T-cell receptor, MZ: marginal zone.

Figure adapted from Roulland *et al.* (2008). Trends in Immunology, 29, 25-33.
Therefore, it can be hypothesised that in MALT lymphomas, cells are autoreactive and can be stimulated at low levels by autoantigens, but additional stimuli are required for their enhanced proliferation and ultimate malignant transformation. In gastric MALT lymphoma, \textit{H. pylori} infection may cause two main events. Firstly, T-cell dependent immune responses in reactive components lead to the generation of a sustained pool of polyclonal \textit{H. pylori}-specific T cells. Secondly, these \textit{H. pylori} specific T cells in the marginal zone containing tumour cells provide non-cognate bystander T-cell help to the autoreactive MALT lymphoma cells (Figure 1.3). This working hypothesis explains how polyclonal \textit{H. pylori} specific T cells can be critical for the growth and survival of monoclonal neoplastic B cells, which recognise autoantigens rather than \textit{H. pylori}.

1.2.4.3 \textit{H. pylori} virulent factors and host genetics in the development of gastric MALT lymphoma

The fact that the majority of patients infected with \textit{H. pylori} are asymptomatic and only a small proportion of them develop gastric MALT lymphoma, indicates a role of bacterial virulence factors and host genetic susceptibility. The interaction between the bacterium and the host immune reaction may determine the risk of MALT lymphoma development.

1.2.4.3.1 Host factors

A number of gene polymorphisms are associated with susceptibility to autoimmune disorders (Lettre \textit{et al.}, 2008) and some have been shown to be associated with MALT lymphoma development. Polymorphisms in immune regulators such as cytokines are commonly involved in auto-reactive conditions (Hajeer \textit{et al.}, 2000). A recent large case control study on 1172 patients with NHL and 982 population-based controls demonstrated a significantly higher susceptibility to NHL among patients with an autoimmune disorder and \textit{TNFA}
(tumour necrosis factor A) G308A variant, or interleukin 10 (IL10) T3575A variant (Wang et al., 2007). TNFA G308A polymorphism associates with higher levels of TNFA expression which enhances chronic inflammatory responses (Bayley et al., 2004). Among NHLs, TNFA G308A polymorphism particularly increases the risk of several lymphoma subtypes, including MALT lymphoma and DLBCLs (Morton et al., 2008).

A number of other genetic polymorphisms have been investigated, particularly in genes encoding molecules involved in immune or inflammatory responses. For example, gene polymorphisms in toll-like receptors (TLR) have been shown to be associated with an increased risk of lymphoma, including MALT lymphoma (Nieters et al., 2006). The TLR4 Asp299Gly variant increases susceptibility to gram-negative bacteria infection, thus potentially representing one of the host factors affecting the risk of lymphoma among *H. pylori* infected individuals (Nieters et al., 2006). Likewise, polymorphisms in the T-cell receptor cytotoxic T-lymphocyte antigen 4 (CTLA4) gene, encoding a negative regulator of T-cell activation, increase the risk of gastric MALT lymphoma, especially in patients with *H. pylori* infection (Cheng et al., 2006).

1.2.4.3.2 Bacterial virulence factors

In addition to host genetic susceptibility, virulence factors associated with infectious agents also play a critical role in the outcome. The risk of developing gastric carcinoma has been shown to depend on the combined effect of pro-inflammatory cytokine gene polymorphisms in the host and virulence-associated genes in *H. pylori* (Figueiredo et al., 2002). Several *H. pylori* virulence factors are associated with an increased risk of gastric ulcer and gastric carcinoma (Maeda et al., 2007), especially the cytotoxin-associated antigen (CagA), vacuolating toxin (VacA) and outer membrane adhesion (BabA) virulence factors. CagA
antigens are known to induce a strong inflammatory response. CagA-positive strains contain a long pathogenicity island including over 30 genes (Prinz et al., 2003). Such pathogenesis-associated genes are usually absent in \textit{H. pylori} strains isolated from asymptomatic hosts (Baldwin \textit{et al.}, 2007). t(11;18)(q21;q21) associated gastric MALT lymphoma cases are significantly associated with infection by CagA strains of \textit{H. pylori} (Ye \textit{et al.}, 2003). CagA-positive strains of \textit{H. pylori} are also associated with a strong response mediated by neutrophils. This is thought to increase the release of ROS (reactive oxygen species) within the environment of inflammation, which may not only promote cell growth via activation of kinases (Cerutti \textit{et al.}, 1991), but may also contribute to the genomic instability already inherent in B cells. \textit{H. pylori} are strong inducers of interleukin-8, a potent chemokine for neutrophil activation. Activated neutrophils are known to release reactive oxygen species, which can cause a wide range of DNA damage, including double-strand breaks (Wiseman \textit{et al.}, 1996). \textit{H. pylori} VacA strains can alter intracellular vesicular trafficking, ultimately inducing the formation of large intracellular vacuoles and mediating mucosal damage (Smoot, 1997). BabA strains express the BabA adherence factor that can bind to Lewis blood sugar molecules present on the membrane of gastric epithelial cells. This results in enhanced adhesion and higher colonisation capacity. Nonetheless, no differences in the above \textit{H. pylori} virulent factors have yet been observed between patients with \textit{H. pylori}-associated MALT lymphoma and those with \textit{H. pylori}-induced gastritis (Lehours \textit{et al.}, 2004). Virulence factors associated with gastric MALT lymphoma remain largely unknown.

Similarly, \textit{C. jejuni} has been shown to produce a toxin that can directly cause DNA damage by inducing double-strand DNA breaks. This may be responsible for the truncated Ig observed in IPSID (Al Saleem \textit{et al.}, 2005).
1.2.4.4 Role of anti-microbial and other therapies in the treatment of MALT lymphoma

The role of *H. pylori* infection in the development of gastric MALT lymphomas has led to the successful use of antibiotic treatment in the cases localised to the stomach and had a profound implication in the clinical management of MALT lymphoma. Evidence indicates that eradication of *H. pylori* with antibiotics such as metronidazole, tetracycline, clarithromycin and amoxicillin can be effectively employed as the first line therapy of localised gastric MALT lymphoma. *H. pylori* eradication is the primary therapy for stage I gastric MALT lymphoma. Multiple trials carried out in independent reference centres on large cohorts of patients with long follow-up, have demonstrated that complete histological remission can be obtained in roughly 80% of early stage gastric MALT lymphomas following *H. pylori* eradication (Morgner *et al.*, 2007). Such a response is durable, as illustrated by the low incidence of lymphoma relapse (3%) at 75 months follow-up after *H. pylori* eradication (Wundisch *et al.*, 2005). When they occur, relapses are often due to *H. pylori* re-infection (Fischbach *et al.*, 2004; Montalban *et al.*, 2006). Presence of histological residual disease or clonal neoplastic cells is seen in gastric biopsies after *H. pylori* eradication. The cells are detected by PCR in a proportion of antibiotic-responsive cases, but are not associated with lymphoma progression, nor with transformation into DLBCL (Fischbach *et al.*, 2002; Fischbach *et al.*, 2007), supporting a “watch and wait” strategy with regular monitoring instead of a systematic second line treatment.

Overall, about 30% of gastric MALT lymphomas do not respond to *H. pylori* eradication, which suggests the acquisition of an antigen-independent autonomous growth. The majority of the non-responsive cases are at stage II or higher (Ruskone-Fourmestraux *et al.*, 2001), while a high proportion of stage I cases show complete remission following *H. pylori* eradication (Stolte *et al.*, 1989). Resistance to *H. pylori* treatment is mainly accompanied by
the presence of t(11;18)(q21;q21) (Liu et al., 2001a; Liu et al., 2002b) and infrequently to t(1;14)(p22;q32) (Ye et al., 2006). The role of *H. pylori* eradication in the treatment of transformed MALT lymphoma is controversial. Although gastric MALT lymphomas of stage II or higher are less likely to respond to *H. pylori* eradication (Ruskone-Fourmestraux et al., 2001; Sackmann et al., 1997), antibiotic treatment showed noticeable success in some *H. pylori*-positive cases, particularly those with limited dissemination. If the presence of the bacterium is confirmed, concurrent eradication of *H. pylori* is recommended, along with other treatment approaches, in order to limit the risk of lymphoma relapse (Morgner et al., 2007). This strategy also applies to transformed MALT lymphoma. A significant proportion of stage I *H. pylori* positive transformed MALT lymphomas achieve complete lymphoma regression after *H. pylori* eradication (Morgner et al., 2007).

MALT lymphoma that is non-responsive to antibiotic therapy can be treated with surgical resection alone or in combination with radiotherapy or chemotherapy. This treatment results in 90-100% 5-year survival for cases at stage I$_E$ and 82% survival at stage II$_E$ (Du et al., 2002; Schechter et al., 2000). Unfortunately, partial resection cannot remove all lymphoma cells, as they disseminate widely in the gastric mucosa. To completely remove the lymphoma, a total gastrectomy needs to be carried out which significantly reduces the patient’s quality of life (Zucca et al., 2000). Low-dose localised radiotherapy can be used alone to treat gastric MALT lymphoma. In a small study, patients with gastric MALT lymphoma with no evidence of *H. pylori* infection or with resistance to antibiotic therapy were treated with radiotherapy alone. 100% event-free survival was achieved by this treatment with a median follow-up time of 27 months (Parveen et al., 1993). Other treatments include the use of therapeutic monoclonal antibody drugs such as rituximab. The improvement of some autoimmune conditions following the administration of rituximab leads to new therapeutic strategies in the
treatment of autoimmune-related MALT lymphomas. A phase II clinical trial including patients with salivary gland MALT lymphoma associated with Sjögren’s syndrome showed that rituximab could lead to complete remission in 3 out of 7 patients (Pijpe et al., 2005). Further investigations are needed, especially as other B-cell targeting therapies are emerging. The therapeutic model of *H. pylori*-associated gastric MALT lymphoma may be extended to MALT lymphomas of other anatomical locations similarly associated with infectious agents. For example, eradication of *Borrelia burgdorferi* and *Campylobacter jejuni* infections has been shown to result in complete regression of some cases of cutaneous MALT lymphoma and IPSID respectively (Kutting et al., 1997; Lecuit et al., 2004). Ferreri and colleagues similarly demonstrated lymphoma regression in 13 out of 27 ocular adnexal MALT lymphomas (6 complete and 7 partial regressions) associated with *Chlamydia psittaci* infection, following eradicating antibiotic therapy with doxycycline (Ferreri et al., 2006).

### 1.3 Genetics of MALT lymphoma

#### 1.3.1 Chromosomal translocations

In addition to chronic immune stimulation, which plays an important role in the development of MALT lymphoma particularly at the early stages, the acquisition of genetic abnormalities contributes to both the genesis and the progression of the lymphoma. Four recurrent balanced chromosomal translocations have been described in MALT lymphoma (Du, 2007). Three of the four translocations involve *MALT1* or *BCL10* genes.
Figure 1.4. Structural domains of the oncogenic products resulting from MALT lymphoma associated chromosomal translocations.

BIR = baculovirus IAP repeats; UBA = ubiquitin associated domain; CARD = caspase associated recruitment domain; RING = really interesting new gene domain; Ig = immunoglobulin-like domain; ZF = zinc finger domain; LZ = leucine zipper domain. Arrows indicate the distribution of sites of fusion between API2 and MALT1 in individual MALT lymphomas.

API2 belongs to the inhibitor of apoptosis family and mediates apoptosis suppression by binding caspases via its caspase recruitment domain (CARD). API2 baculovirus inhibitor of apoptosis protein repeats (BIR) domains can mediate oligomerisation of BIR-domain containing proteins. MALT1 is a paracaspase. In addition to a caspase-like domain, MALT1 contains a death domain (DD) and 2 immunoglobulin-like domains (Ig). Although various breakpoints have been reported (Du, 2007), especially within the MALT1 gene, API2-MALT1 fusion transcript always contains the 3 intact BIR domains of API2 and the intact caspase-like domain of MALT1, suggesting a role for these domains in oncogenesis. BCL10 is an intracellular protein with a CARD domain and a serine/threonine-rich domain of unknown function. FOXP1 is a member of the FOX family of transcription factor containing both DNA-binding domains (zinc finger domains ZF; leucine zipper domain, LZ; winged helix) and protein-protein binding glutamine-rich domains.
1.3.1.1 t(11;18)(q21;q21)/API2-MALT1

t(11;18)(q21;q21) fuses in frame the amino-terminal sequence of the baculovirus inhibitor of apoptosis protein repeats (BIR) containing 3 (BIRC3, also known as API2) gene (11q21), to the carboxyl-terminal caspase-like domain of the MALT lymphoma translocation gene 1 (MALT1) (18q21) (Figure 1.4). API2 suppresses apoptosis by inhibiting specific caspases via its BIR domains (Roy et al., 1997). MALT1 is an essential component in the antigen-receptor-mediated activation of the master transcription factor nuclear factor κB (NF-κB) (Ho et al., 2005; Lin et al., 2004). t(11;18)(q21;q21) generates a functional fusion protein API2-MALT1 capable of activating NF-κB (Ho et al., 2005; Uren et al., 2000). An intact UBA (Ubiquitin Associated Domain) is present in roughly 98% of API2-MALT1 fusions and a functional UBA is required for efficient API2-MALT1 mediated NF-κB activation, although the precise molecular mechanism has yet to be established (Gyrd-Hansen et al., 2008).

Incidence. t(11;18)(q21;q21) is the most common and specific structural chromosomal abnormality in MALT lymphoma. It has not been described in other B-cell lymphoma subtypes, including nodal and splenic MGZ lymphoma, nor in MALT lymphoma associated inflammatory conditions such as H. pylori associated gastritis, lymphoepithelial sialadenitis or Hashimoto’s thyroiditis (Du, 2007). t(11;18)(q21;q21) occurs frequently in MALT lymphomas of the lung (40%), stomach (25%) and ocular adnexa (~10%), but not in those of the salivary gland and thyroid (Isaacson et al., 2004; Ye et al., 2003) (Figure 1.5).

Clinical impact. In gastric MALT lymphoma, t(11;18)(q21;q21) is associated with advanced stages, but not transformation into DLBCL (Huang et al., 2003; Liu et al., 2001a). t(11;18)(q21;q21) is associated with gastric MALT lymphomas that do not respond to H. pylori eradication, irrespective of the clinical stage of the lymphoma (Liu et al., 2001b; Liu et
al., 2002a), and that are resistant to oral alkylating agents (Levy et al., 2005). The detection of t(11;18)(q21;q21) is thus critical for the clinical management of gastric MALT lymphoma.

**Genetic correlations.** t(11;18)(q21;q21) is mutually exclusive from other MALT lymphoma-associated chromosomal translocations (Du, 2007; Liu et al., 2004a). t(11;18) positive cases rarely show aneuploidies frequently associated with translocation negative MALT lymphoma, such as trisomies 3, 12 and 18 (Remstein et al., 2002; Starostik et al., 2002).

**Function.** The biological importance of the API2–MALT1 fusion is indicated by the analysis of breakpoints at both the genomic and transcript levels. Although some genomic breakpoints were found to cause frameshifts, the API2–MALT1 fusion transcripts are always in frame, because of splicing out of the exon that causes the frameshift (Mestecky et al., 1999). The API2 gene contains three amino-terminal baculovirus IAP repeats, a central caspase recruitment domain (CARD) and a carboxy-terminal zinc-binding RING finger domain. The MALT1 protein comprises an N-terminal death domain, followed by two Ig-like domains and a caspase-like domain. Within the API2 gene, all breakpoints occur downstream of the third BIR domain, upstream of the C-terminal RING domain, with 91% occurring just before the CARD domain. In the *MALT1* gene, the breakpoints occur in four different introns upstream of the caspase-like domain (Liu et al., 2004a). Therefore, the resulting API2–MALT fusion transcripts always comprise the N-terminal region of API2 with three intact BIR domains and the C-terminal MALT1 region containing an intact caspase-like domain. The specific selection of these domains of the API2 and MALT1 gene to form a fusion product strongly indicates that they are required for the oncogenic activities of the fusion product. API2-MALT1 fusion product is capable of activating the NF-κB pathway in the absence of any surface receptor stimulation, although neither API2 nor MALT1 alone are able to activate NF-κB (Hu et al., 2006b).
1.3.1.2 \textit{t(1;14)(p22;q32)/BCL10-IGH}

t(1;14)(p22;q32) translocates the entire B-cell leukaemia/lymphoma 10 (\textit{BCL10}) gene on chromosome 1p22 under the control of the immunoglobulin heavy chain locus (\textit{IGH}) gene enhancer on chromosome 14q32 (Willis \textit{et al.}, 1999; Zhang \textit{et al.}, 1999). This translocation thus results in the over-expression of BCL10 which contains an N-terminal CARD, followed by a serine/threonine rich C-terminal.

The CARD motif of BCL10 can interact with the Ig-Like domains of MALT1 (Figure 1.4). \textit{In vivo} studies showed that BCL10 and MALT1 link antigen receptor signalling and NF-\kappaB activation pathway (Ruland \textit{et al.}, 2001; Xue \textit{et al.}, 2003).

\textbf{Incidence.} \textit{t(1;14)(p22;q32)} is specifically associated with MALT lymphoma, though relatively infrequently. It is mostly seen in MALT lymphoma of the lung (7\%) and stomach (4\%) but not in those of other sites (Figure 1.5).

\textbf{Clinical impact.} Strong BCL10 nuclear localisation, characteristic of \textit{t(1;14)(p22;q32)}-positive MALT lymphoma, correlates with gastric MALT lymphomas of advanced stages and those not responsive to \textit{H. pylori} eradication (Ye \textit{et al.}, 2006). Also, nuclear BCL10 expression is associated with advanced MALT lymphoma (Liu \textit{et al.}, 2001b), and associated with shorter treatment failure-free survival in ocular adnexal MALT lymphomas (Franco \textit{et al.}, 2006).

\textbf{Genetic correlations.} \textit{t(1;14)(p22;q32)} is mutually exclusive from other MALT lymphoma-associated chromosomal translocations. Translocation positive cases commonly harbour trisomies 3, 12 and or 18 (Streubel \textit{et al.}, 2006).

\textbf{Function.} BCL10-knockout mice showed that BCL10 is essential for both the development and function of mature B and T cells, linking antigen-receptor signalling to the NF-\kappaB pathway (Ruland \textit{et al.}, 2001; Xue \textit{et al.}, 2003). Over-expression of BCL10 activates the IκB
kinase (IKK) complex through CARD domain-mediated oligomerisation, resulting in NF-κB activation (Hofmann et al., 1997). In line with these findings, a recent in vitro study indicated that BCL10 could prevent an immature B-cell line from antigen receptor induced apoptosis (Tian et al., 2005b).

1.3.1.3 t(14;18)(q32;q21)/IGH-MALT1

t(14;18)(q32;q21) brings the entire MALT1 gene (18q21) under the regulatory control of IGH (14q32), leading to the over-expression of MALT1 (Figure 1.4) (Streubel et al., 2003). The MALT1 gene is 5Mb away from the B-cell CLL/lymphoma 2 (BCL2) gene, targeted by t(14;18)(q32;q21) IGH/BCL2 characterising follicular lymphoma (Sagaert et al., 2007).

Incidence. t(14;18)(q32;q21)/IGH-MALT1 is a rare translocation, mainly described in MALT lymphomas of the lung (6%) and ocular adnexa (7%) (Remstein et al., 2006; Ye et al., 2005) (Figure 1.5). It is also seen in some DLBCLs (Du, 2007).

Clinical impact. As t(14;18)(q32;q21) occurs exclusively in extra-gastric MALT lymphoma, for which a specific therapy has not yet been established, the potential impact of this translocation in the clinical management of MALT lymphoma remains unknown.

Genetic correlations. t(14;18)(q32;q21) is mutually exclusive from other MALT lymphoma-associated chromosomal translocations. Translocation positive cases commonly harbour trisomies 3 and/or 18 (Streubel et al., 2003).

Function. Initial studies of MALT1 demonstrated that the wild-type protein has no independent ability to activate NF-κB and shows only modest enhancement of BCL10-mediated NF-κB activation, despite direct physical association with BCL10 (Uren et al., 2000). MALT1 over-expression led to increased constitutive NF-κB activity and enhanced IκB kinase (IKK) activation induced by CD40 stimulation (Ho et al., 2005).
1.3.1.4 **BCL10 and MALT1 expression patterns in MALT lymphoma with different translocation status.**

MALT1 and BCL10 proteins are essential components of NF-κB activation signalling pathway (Thome, 2004). In normal MGZ B cells, both MALT1 and BCL10 are weakly expressed, predominantly in the cytoplasm (Ye et al., 2005). In MALT lymphoma, tumour cells with different translocations show distinct BCL10 and MALT1 expression patterns (Ye et al., 2005). MALT lymphoma cells with t(11;18)(q21;q21)/API2-MALT1 display moderate BCL10 nuclear expression and weak MALT1 cytoplasmic expression (Figure 1.6). Tumour cells with t(1;14)(p22;q32)/BCL10-IGH show strong BCL10 nuclear expression and weak/negative cytoplasmic expression of MALT1. Tumour cells with t(14;18)(q32;q21)/IGH-MALT1 show strong homogeneous cytoplasmic expression of both BCL10 and MALT1 (Figure 1.6). Around 20% of MALT lymphoma cases without
translocation show BCL10 nuclear expression and around 6% show high BCL10 nuclear expression similar to that seen in t(1;14) positive MALT lymphomas (Liu et al., 2001b).

**Figure 1.6 - BCL10 and MALT1 staining patterns in MALT lymphoma translocations.**
BCL10 and MALT1 immunohistochemistry on FFPE translocation positive and negative MALT lymphoma tissue.

Figure adapted from Ye et al. (2005). Journal of Pathology, 205, 293-301.
Nakagawa and colleagues demonstrated that MALT1, but not its fusion counterpart API2-MALT1, was involved in BCL10 export from the nucleus to the cytoplasm, providing an explanation for the various BCL10 expression patterns observed in MALT lymphoma with different chromosomal translocations (Nakagawa et al., 2005). In cells with t(11;18)(q21;q21)/API2-MALT1, MALT1 expression is reduced by half due to the API2-MALT1 fusion, hence an expected reduced efficiency of BCL10 nuclear export. In cells with t(1;14)(p22;q32)/BCL10-IGH, MALT1 normal expression may not be sufficient for the export of the over-expressed BCL10, resulting in BCL10 nuclear retention. Finally, in cells with t(14;18)(q32;q21)/IGH-MALT1, the over-expression of MALT1 results in an increased export of BCL10 from the nucleus to the cytoplasm, where both proteins are thus strongly expressed in the cytoplasm (Ye et al., 2005). The temporal interplay between API2-MALT1, MALT1 and BCL10 in cellular localisation in vivo remains to be investigated.

1.3.1.5  t(3;14)(p13;q32)/FOXP1-IGH

T(3;14)(p13;q32) is a newly described MALT lymphoma-associated translocation involving IGH (Streubel et al., 2005). It deregulates the forkhead box protein P1 (FOXP1) gene (3p13), a member of the FOX transcription factor family, which includes numerous proteins involved in a variety of functions such as cellular differentiation and immune regulation. Foxp1 has been recently shown to be an essential regulator of early B-cell development (Hu et al., 2006a), but the molecular mechanism underlying its oncogenic activity in lymphoma remains to be investigated.

Incidence. Streubel et al. (Streubel et al., 2005) initially showed that FOXP1 was involved in t(3;14)(p13;q32) in MALT lymphoma which was present in 10% of MALT lymphomas, in those from the ocular adnexa (20%), thyroid (50%) and skin (10%), but not in those from the
salivary gland, stomach and lung (Streubel et al., 2005). However, the translocation was not found in 122 extranodal MALT lymphomas from various sites (9 cutaneous, 13 salivary gland, 50 pulmonary, 36 ocular adnexa, 8 thyroid gland and 6 gastric tumors) (Haralambieva et al., 2006b). The t(3;14)(p13;q32) was subsequently found in one case of MALT lymphoma of the stomach (Wlodarska et al., 2005), seven cases of diffuse large B-cell lymphoma of the stomach, thyroid and lymph nodes and also in two cases of B-cell non-Hodgkin lymphoma unclassified (Haralambieva et al., 2006b; Wlodarska et al., 2005). On a larger series of 321 MALT lymphomas, Goatly et al. showed that t(3;14)(p13;q32) is rare in MALT lymphoma, and primarily found in gastric cases (4%) (Goatly et al., 2008; Haralambieva et al., 2006a) (Figure 1.5).

**Clinical impact.** No correlation has yet been established between the presence of t(3;14)(p13;q32) and response to \textit{H. pylori} eradication in gastric MALT lymphoma. In DLBCL, strong expression of FOXP1 identifies cases with poor prognosis (Barrans et al., 2004). Similarly, FOXP1 over-expression is associated with MALT lymphomas with poor clinical outcome (Sagaert et al., 2006a). MALT lymphomas with concurrent over-expression of FOXP1 and trisomies 3 and 18 may be at risk of transformation into DLBCL (Sagaert et al., 2006a).

**Genetic correlations.** t(3;14)(p13;q32) is mutually exclusive from other MALT lymphoma-associated chromosomal translocations. Cases with t(3;14)(p13;q32) often harbour additional genetic abnormalities, especially trisomy 3 (Streubel et al., 2005).

**Function.** Activation of the NF-κB pathway by isoforms of FOXP1 has been investigated (unpublished data in Professor Ming Du’s laboratory). The full length FOXP1 protein and 2 short isoforms were able to activate NF-κB both alone and synergistically with cell surface stimulation of B cells by LPS and T cells by CD3 and CD28. The mechanism of NF-κB
activation is unknown but there is evidence that it is not through the classical or alternative pathways, in keeping with the nuclear localisation of FOXP1.

1.3.2 Other chromosomal translocations

In addition to the oncogenes mentioned in sections (1.3.1.1 – 1.3.1.4), novel translocations were identified using cytogenetics and long distance inverse PCR. Those include rearrangements of t(6;7)(q25;q11), t(1;14)(p22;q32)/CNN3-IGH, t(5;14)(q34;q32)/ODZ2-IGH and t(9;14)(p24;q32)/JMJD2C-IGH (Vinatzer et al., 2008), t(X;14)(p11;q32)/GPR34-IGH (Novak et al., 2008; Wlodarska et al., 2009) and t(3;13)(q24;p11) (Aamot et al., 2005).

It has been shown that over-expression of GPR34 activated NF-κB in vivo (Novak et al., 2008). However no functional characterisation for any of the translocations listed above has been performed. Nonetheless, the translocations above illustrate the heterogeneous nature of genetic alterations in MALT lymphoma.

1.3.3 Genetics of translocation negative MALT lymphoma

Trisomies are frequently associated with MALT lymphoma, particularly those without t(11;18)(q21;q21) (Brynes et al., 1996; Streubel et al., 2004). Trisomies 3 and 18 are found in about 35% and 25% of cases respectively. However, beyond those numerical aberrations, the molecular genetics of MALT lymphoma, especially those without chromosomal translocation, is poorly understood (Isaacson et al., 2004). The chromosomal gains and losses in translocation negative MALT lymphomas of the stomach and salivary glands were investigated using metaphase comparative genomic hybridisation (CGH) and recurrent chromosomal gains involving the whole or major part of chromosomes 3, 12 and 18, as well as recurrent discrete gains at 9q34 and 11q11-13 were found (Zhou et al., 2006; Zhou et al., 2007). Array comparative genomic hybridisation (aCGH) showed frequent microdeletions
involving 6p25.3 to be associated with outcome of *H. pylori* eradication in translocation negative gastric MALT lymphoma (Fukuhara *et al.*, 2007).

Recently, work in Professor Ming Du’s group showed that *A20* deletion was associated with copy number gain at the TNFA/B/C locus and occurred preferentially in translocation negative MALT lymphoma of the ocular adnexa and salivary glands but not in the stomach, lung and skin (Chanudet *et al.*, 2009). In addition to the *A20* gene deletions and mutations, promoter methylation was shown to be an alternative mechanism for *A20* inactivation in MALT lymphoma and *A20* complete inactivation was significantly associated with a shorter lymphoma-free survival in ocular adnexal MALT lymphoma (Chanudet *et al.*, 2010). The *A20* protein (also known as Tumour Necrosis Factor Alpha-Induced Protein 3 or TNFAIP3) is a key player in the negative feedback regulation of NF-κB signalling in response to multiple stimuli. For example, A20 regulates tumour necrosis factor (TNF)-induced NF-κB activation. Recent genetic studies demonstrated a clear association between several single nucleotide polymorphism (SNPs) in the *A20* locus and autoimmune disorders such as Crohn’s disease, Rheumatoid arthritis, systemic lupus erythematosus, psoriasis and type 1 diabetes (Vereecke *et al.*, 2009) further implicating its link with both autoimmune disease and lymphoma development.

More recently, inactivating mutations predicting truncated A20 products were identified in 6 (19%) of 32 marginal zone lymphomas, including 2 (18%) of 11 extranodal marginal zone lymphomas, 3 (33%) of 9 nodal marginal zone lymphomas, and 1 (8%) of 12 splenic marginal zone lymphomas (Novak *et al.*, 2009). Another study identified inactivating mutations in A20 in 21% MALT lymphoma patients (Kato *et al.*, 2009). Collectively, *A20* can be completely inactivated by homozygous deletion, hemizygous deletion plus mutation or promoter methylation and bi-allelic mutations. A20 inactivation abolishes the major negative
regulation of the NF-κB pathway, thus potentially contributing to the constitutive NF-κB activation in lymphoma subtypes such as DLBCL, particularly the (activated B-cell) ABC subtype, classic Hodgkin lymphoma and Burkitt’s lymphoma.

1.4 NF-κB pathway

As mentioned in section 1.3, MALT lymphoma associated oncogenes target the NF-κB pathway, thus in this section the NF-κB pathway will be discussed.

Nuclear Factor-κB (NF-κB) (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA. NF-κB was first discovered in the laboratory of Nobel Prize laureate David Baltimore via its interaction with an 11-base pair sequence in the immunoglobulin light-chain enhancer in B cells (Sen et al., 1986). NF-κB is a ubiquitous transcription factor found in almost all animal cell types and is involved in cellular responses to diverse stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized low density lipoproteins, and bacterial or viral antigens (Gilmore, 2006).

1.4.1 NF-κB family members

NF-κB is a family of 5 inducible transcription factors, sharing a highly conserved reticuloendotheliosis viral oncogene homology (REL) domain, REL homolog A (RelA, also known as p65), REL homolog B (RelB), NF-κB1 (also known as p50) and NF-κB2 (p52). All have a transactivation domain in their C-termini. 15 transcription factors can be formed from the homo- and heterodimerisation of the five NF-κB subunits (Gilmore, 2006). The NF-κB1 and NF-κB2 proteins are synthesised as large precursors, p105, and p100, which undergo processing to generate the mature NF-κB subunits, p50 and p52, respectively. The processing of p105 and p100 is mediated by the ubiquitin/proteasome pathway and involves selective
degradation of their C-terminal region containing ankyrin repeats. Whereas the generation of p52 from p100 is a tightly-regulated process, p50 is produced from constitutive processing of p105. The particular dimers present are dependent on the cell type, its stage of differentiation, and environmental signals (Hayden et al., 2004). As all dimers are able to bind to κB consensus sites in promoters and enhancers, they can all be termed as “NF-κB”. The components of each dimer affect its specificity for DNA binding, its interaction with other proteins, and the set of genes that it controls (Hayden et al., 2004). NF-κB dimers have the ability to activate or repress transcription of genes (Perkins, 2007). NF-κB dimers are sequestered in an inactive form in the cytoplasm by inhibitory proteins of the IκB family (also known as nuclear factor of κ light polypeptide gene enhancer in B-cell inhibitor, NFKBI). Diverse surface receptor signalling can activate the molecular pathways that allow NF-κB nuclear translocation and activate its target gene expression.

1.4.2 NF-κB activation pathways

There are two main pathways within a cell that result in NF-κB activation, namely canonical and non-canonical pathways (Figure 1.7). Recent data indicate the existence of a third NF-κB activation mechanism, referred to as linear ubiquitin chain assembly complex (LUBAC) pathway (Tokunaga et al., 2009) though this needs further experimental validation.
1.4.2.1 Canonical pathway

Receptor signalling

Whilst many different stimuli are able to activate NF-κB via surface membrane receptor signalling, the major ones can be broadly categorised into the following groups (Hayden et al., 2008):

1) Antigen receptors such as B-cell receptors (BCR) and T-cell receptors (TCR) which lead to NF-κB activation via antigenic stimulation

2) Toll-like receptors (TLRs) which respond to stimulations by bacterial or fungal products such as lipopolysaccharide (LPS), CagA and CpG, leading to NF-κB activation

3) Tumour necrosis factor receptor (TNFR) which lead to NF-κB activation by stimulation with TNF ligand and (TNF)-related apoptosis-inducing ligand (TRAIL)

Irrespective of the stimulus involved, the key event of canonical NF-κB activation is the phosphorylation of IκBα protein by an IκB kinase complex (IKK). This targets IκBα for ubiquitination and subsequent degradation, thus releasing NF-κB. NF-κB then translocates to the nucleus and binds the promoters of target genes to regulate their transcription (Figure 1.7a).

Signal transduction

As mentioned above, a number of surface receptor signals lead to activation of the canonical pathway in both B and T cells. In the context of this thesis, only the antigen receptor and TLR signalling will be outlined as this is most relevant to MALT lymphoma. Antigen receptor signalling triggers serial proximal signalling that causes the auto-phosphorylation of phosphoinositide-dependent kinase 1 (PDK1). In T cells, phosphorylated PDK1 binds Protein
Kinase Cθ (PKCθ), activating this kinase via phosphorylation of Thr538 (Lee et al., 2005; Park et al., 2009). Similarly, in B cells, antigen receptor signalling triggers phosphorylation and activation of PKCβ. Subsequently, PKCβ phosphorylates several residues in the “linker” region (also known as PKC receptor) of CARD11 (Matsumoto et al., 2005; Sommer et al., 2005). This phosphorylation event triggers a conformational change in CARD11 allowing its CARD domain to interact with the CARD of BCL10. Because BCL10 and MALT1 are constitutively associated in the cytoplasm (Su et al., 2005), the CARD11-BCL10 interaction also brings MALT1 into the complex, thereby forming the CARD11-BCL10-MALT1 (CBM) complex (Figure 1.8) (Matsumoto et al., 2005). CBM is further stabilised by a direct interaction between the coiled coil domain of CARD11 and the C-terminal portion of MALT1 (Che et al., 2004) (Figure 1.8).

Coincident with or downstream of formation of the CBM complex, a proportion of cellular BCL10 and MALT1 becomes oligomerised, as demonstrated by biochemical methodology (Sun et al., 2004) and FRET microscopy (Rossman et al., 2006), and it is these oligomeric species that are active in NF-κB signal transduction (Sun et al., 2004).

The active/oligomeric forms of BCL10 and MALT1 elicit the E3 ubiquitin ligase activity of the TRAF6 RING domain to synthesize a K63-linked polyubiquitin chain on TRAF6 itself (Stilo et al., 2004) (Thome, 2004; Thome, 2008) (Rossman et al., 2006; Sun et al., 2004) (Figure 1.8). Auto-ubiquitination increases the catalytic activity of TRAF6, enabling TRAF6-mediated K63-polyubiquitination of specific protein targets (Lamothe et al., 2007). Activated TRAF6 polyubiquitinates MALT1 on multiple C-terminal lysines and BCL10 on Lys31 and Lys63 (Oeckinghaus et al., 2007; Wu et al., 2008).

K63-polyubiquitination of BCL10 is enhanced by the presence of MALT1 (Wu et al., 2008), suggesting that MALT1-associated TRAF6 may contribute substantially to K63-
polyubiquitin modification of BCL10 in the CBM complex. Wu and colleagues showed that K63-polyubiquitination of BCL10 is essential for CBM association with the IKK complex and for consequent activation of NF-κB (Wu et al., 2008), while Duwel and colleagues showed an inability to detect K63-polyubiquitination of CBM-associated BCL10 (Duwel et al., 2009). Overall, data are consistent with a model proposing that NEMO is able to bind to K63-polyubiquitin chains on both BCL10 and MALT1, and that blockade of either of these interactions impairs NF-κB activation, by preventing efficient physical association between the CBM and IKK complexes. However, it is also possible that ubiquitin modification of BCL10 and/or MALT1 affects the function of one or both proteins in other as yet unidentified ways that are crucial for maximal activation of the IKK complex and do not contribute directly to physical association with the CBM.

CBM complex recruits the IKK complex through a recently identified ubiquitin binding domain (UBD) in the NEMO protein. This specialised UBD, called the UBAN (UBD in ABIN proteins and NEMO), facilitates association between the IKK complex and the K63-polyubiquitinated CBM complex (Wagner et al., 2008; Wu et al., 2006; Wu et al., 2008). The association of the CBM and IKK complexes then allows TRAF6 (in cooperation with Ubc13/Uev1A) to add K63-polyubiquitin chains to the C-terminal Zn-finger domain of NEMO at Lys399 and possibly additional lysines (Perkins, 2006).

Biochemical studies suggest that K63-polyubiquitination of NEMO enables physical association with the TAK1/TAB2/TAB3 kinase complex, via the UBD of TAB2. This association allows TAK1 to phosphorylate and activate the IKKβ catalytic subunit (Perkins, 2006). However, a more recent T-cell study has provided evidence that the phosphorylation of IKKβ is independent of CBM-mediated signalling and NEMO ubiquitination, suggesting that the mechanism of TAK1 association with the IKK complex remains to be defined.
(Shambharkar et al., 2007). Regardless of the details by which the IKK complex is modified, data from both groups strongly suggest that the combination of NEMO ubiquitination and IKKβ phosphorylation is required for activation of IKKβ kinase activity (Shambharkar et al., 2007; Shinohara et al., 2005). IKKβ then phosphorylates IκBα, triggering the terminal activation events of the canonical NF-κB activation cascade, as described above (Figure 1.7a).

**Toll-like receptor signalling pathway**

TLRs play a crucial role in the innate recognition of various molecular motifs in pathogens, termed pathogen associated molecular patterns (PAMPs) that are conserved in a large group of pathogens, including bacteria, viruses, fungi and parasites. At least 13 TLR family members in vertebrates and 10 TLRs in humans have been reported (Barton et al., 2002). All of these TLRs have extracellular leucine-rich repeats and an intracellular Toll/IL-1 receptor homology (TIR) domain (Takeda et al., 2003). These receptors transmit signals via several intracellular molecules, including myeloid differentiation factor-88 (MyD88), IL-1 receptor-associated kinases (IRAKs), TNF receptor-associated factor (TRAF) 6 and mitogen activated protein kinases (MAPKs). When associated with TLR, MyD88 recruits members of the IRAK family through (death domain-death domain) homophilic interactions. IRAK1 and IRAK4 are serine-threonine kinases involved in the phosphorylation and activation of TRAF6. After phosphorylation by IRAKs, TRAF6 forms a complex with Ubc13 and Uev1A, and activates a MAPK kinase kinase (MAPKKK) called transforming growth factor β–activated kinase (TAK-1) (Barton et al., 2003). Activated TAK-1, in turn, can phosphorylate MKK3 and MKK6, the kinases upstream of p38 MAPKs and JNK which in turn lead to the activation of AP-1 protein (Takeda et al., 2003). In addition, TAK-1 can activate the IKK
complex, which phosphorylates IκBα consequently leading to NF-κB activation as detailed above (Barton et al., 2003).

Different TLRs show variable recognition for microbial lipo-polysaccharides (LPS). For example *Escherichia coli* (E. coli) LPS is a ligand for TLR4. *H. pylori* associated LPS is recognized by the receptor complex containing TLR2–TLR1 or TLR2–TLR6 but not that containing TLR4 (Yokota et al., 2007).

**Chemokine receptor signalling pathway**

Chemokines are small peptides that are potent activators and chemoattractants for leukocyte subpopulations and some non-haemopoietic cells. Their actions are mediated by a family of 7-transmembrane G-protein–coupled receptors (Murphy et al., 2000). Chemokine receptors are divided into different families, CXC chemokine receptors, CC chemokine receptors, CX3C chemokine receptors and XC chemokine receptors that correspond to the 4 distinct subfamilies of chemokines they bind (Murphy et al., 2000). Some chemokine and cytokines activate central cellular pathways. For example, when CXCL8 (IL-8) binds to its specific receptors, CXCR1 or CXCR2, a rise in intracellular calcium activates the enzyme phospholipase D (PLD) that goes on to initiate an intracellular signalling cascade by activating the NF-κB and MAP kinase pathway. The initiated NF-κB and MAP kinase pathways activate specific cellular mechanisms involved in chemotaxis, degranulation, release of superoxide anions and changes in the avidity of cell adhesion molecules called integrins (Murdoch et al., 2000). In addition, the binding of chemokines to their respective leukocyte receptors initiates a series of cellular events including NF-κB and MAP kinase activation, all of which aim to eradicate the infiltrating inflammatory agents. These events include changes in cell shape leading to enhanced locomotion, secretion of lysosomal

enzymes, and production of superoxide anions. Once leukocytes reach the source of inflammation, a cytokine-rich milieu is generated that is sustained until the invading antigen is eliminated. In general, immune responses do not produce endothelial injury; however, on occasion acute or chronic inflammation may occur in which the endothelium and surrounding tissues become damaged (for example, by neutrophil generated products).

Several homeostatic chemokines have been shown to play an important role in mucosal immunology via germinal centre formation, homing mechanisms and local retention of activated mucosal B cells and dissemination of B cells to extra-intestinal secretory effector sites. Homing mechanisms play a role in B-cell recruitment to secondary lymphoid tissue. For example, recent mouse studies suggested that although the CCR7 ligand operating together with the CXCR4 ligand (CXCL12) are crucial for endothelial B-cell adhesion in lymph nodes, B-cell entry into Peyer’s patches depends on CXCR5 (Okada et al., 2002).

1.4.2.2 Non-canonical pathway

Receptor signalling

The non-canonical NF-κB pathway is activated largely through signalling via the following main receptors:

1) CD40 receptor (a TNFR family member) which leads to NF-κB activation via CD40 ligand stimulation

2) B-cell activating factor family (BAFF) receptor (BAFF-R)

3) lymphotixin α and β (LTβ also known as TNFC) receptor (LTβ-R)

Signal transduction

Receptor signalling activates NF-κB inducing kinase (NIK), which phosphorylates IKKα. Activated IKKα induces the partial proteolysis of p100 into p52. Subsequently, p52 binds to
RelB, forming an NF-κB dimer, which migrates to the nucleus and transactivates its target genes (Figure 1.7b) (Jost et al., 2007).

In contrast to the canonical signalling that relies upon NEMO-IKKβ mediated degradation of IκBa, -β, and -ε, the non-canonical signalling critically depends on NIK mediated processing of p100 into p52. Given their distinct modes of regulation, these two pathways were thought to be independent of each other. However, recent studies revealed that synthesis of the constituents of the non-canonical pathway, via RelB and p52, is controlled by the canonical IKKβ-IκB-RelA:p50 signalling (Basak et al., 2008). Moreover, generation of the canonical and non-canonical dimers, via RelA:p50 and RelB:p52, within the cellular milieu are also mechanistically interlinked (Basak et al., 2008). These data suggest that an integrated NF-κB system network underlies activation of both RelA and RelB containing dimer and that a malfunctioning canonical pathway will lead to an aberrant cellular response also through the non-canonical pathway. As with most cellular pathways, activation needs to be counterbalanced by inhibition or damping.

A variety of recent evidence, however, suggests that the control of the NF-κB pathway is more complex than simply IKK-mediated regulation of the IκB-NF-κB interaction. For example, RelA and p50 are regulated by ubiquitination, acetylation and prolyl isomerisation, and the transactivation activity of RelA and c-Rel can be affected by phosphorylation. In addition, as a consequence of the induction of NF-κB activity (at least by tumour necrosis factor), IKKα is also induced to enter the nucleus where it becomes associated with κB site promoters/enhancers to phosphorylate histone H3 which enhances the transcription of κB site-dependent genes (Chen et al., 2007).
Inactive NF-κB dimers are sequestered in the cytoplasm by inhibitory proteins of the IκB family, which includes IκBα, IκBβ, IκBγ, IκBδ, IκBε and BCL3, as well as p100 and p105 respective precursor of p52 and p50. In the canonical pathway, sequestered NF-κB mainly consists of heterodimer p50-RELA. The IKK complex is composed of two kinase subunits (IKKα and IKKβ) and a regulatory subunit, NEMO (also known as NF-κB essential modifier NEMO). The activation of the IKK complex by phosphorylation (P) of IKKβ leads to the phosphorylation of IκB. This targets IκB for ubiquitination (U) and subsequent degradation. In the non-canonical pathway, sequestered NF-κB mainly consists of heterodimer p100-RELB. The IKK complex is a dimer of IKKα. Activated IKKα phosphorylates p100 and triggers its proteolysis, producing p52. This allows the formation of NF-κB active complex p52-RELB. Both the canonical and the non-canonical pathways ultimately lead to the translocation of NF-κB dimers into the nucleus, where they transactivate target genes critical for cellular mechanisms such as proliferation and survival. Some target gene products negatively regulate the NF-κB pathway.
Figure 1.8 - B-cell receptor mediated NF-κB activation and constitutive NF-κB activation by MALT lymphoma associated translocations.

Following antigen recognition via PKCβ, CARD11 recruits BCL10 and MALT1 to the lipid raft at the activated receptor. Subsequently MALT1 binds TRAF6 activating its ubiquitin ligase. This results in TRAF6 and NEMO polyubiquitination and recruitment of kinase complex that phosphorylates IKKβ leading to IκBα phosphorylation and degradation. NF-κB is released and translocated into the nucleus, activating the transcription of target genes. The 3 MALT lymphoma associated translocations are believed to oligomerise and similarly recruit TRAF6 inducing constitutive NF-κB activation.
1.4.3 NF-κB negative regulators

As mentioned above, in unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs (Inhibitor of κB).

IκBs are related proteins in that they all have an N-terminal regulatory domain, followed by six or more ankyrin repeats and a PEST domain near their C terminus. Although the IκB family consists of IκBα, IκBβ, IκBγ and IκBε, and BCL3, the best-studied and major IκB protein is IκBα. Due to the presence of ankyrin repeats in their C-terminal halves, p105 and p100 also function as IκB proteins. The multiple copies of ankyrin repeats can mask the nuclear localisation signals (NLS) of NF-κB proteins sequestering them in an inactive state in the cytoplasm (Jacobs et al., 1998).

Of all the IκB members, IκBγ is unique in that it is synthesised from the NF-κB1 gene using an internal promoter, thereby resulting in a protein that is identical to the C-terminal half of p105 (Inoue et al., 1992). The C-terminal half of p100, that is often referred to as IκBδ, also functions as an inhibitor (Basak et al., 2007). IκBδ degradation in response to developmental stimuli, such as those transduced through LTβR, potentiate NF-κB dimer activation in of NIK dependent non-canonical pathway (Basak et al., 2007). Unlike other members of the IκB family, BCL3 was found to function as a transcriptional activator and enhance NF-κB activity, primarily via forming a complex with the NF-κB p50 heterodimer to block the latter’s mediated suppression of target gene expression (Li et al., 2006).

Activation of NF-κB is initiated by the signal-induced degradation of IκB proteins via their ubiquitination by the proteosome. With the degradation of the IκB inhibitor, the NF-κB complex is then free to enter the nucleus where it can activate the expression of specific genes that have DNA-binding sites for NF-κB nearby. The activation of these genes by NF-κB then leads to the given physiological response, for example, an inflammatory or immune
response, a cell survival response, or cellular proliferation. Interestingly, these IκBs are part of NF-κB target genes where the newly synthesised IκB repressors masks the NLS of NF-κB sequestering it in the cytoplasm thus, forming a negative feedback loop, which results in tighter control and hence oscillating levels of NF-κB activity (Nelson et al., 2004). This tight control is important as NF-κB regulates a multitude of target genes that play key roles in cell development and survival. In addition, NF-κB transactivates several other negative regulators such as A20 (Krikos et al., 1992) and its adaptor molecules such as ABIN-1 to 3 (Verstrepen et al., 2008).

1.4.4 NF-κB target genes and their biological implications

NF-κB activity is essential for development, activation, proliferation and survival of lymphocytes (Hoffmann et al., 2006). NF-κB has the ability to regulate the transcription of an extensive variety of genes with a κB site in their promoter. Promoters that respond to NF-κB also contain consensus-binding sites for other transcription factors and these may be clustered into enhancers. This indicates that NF-κB may not act alone in transactivating gene expression (Hoffmann et al., 2006).

Over 300 genes are regulated by NF-κB (http://people.bu.edu/gilmore/nf-kb/index.html) (Pahl, 1999), including NF-κB family members such as p50, p52 and RelB (Figure 1.9). A number of these NF-κB target genes encode NF-κB positive regulators, while several target genes encode negative regulators. According to their function, these NF-κB target genes can be categorised into the following groups:
Cytokines, chemokines and their modulators

NF-κB promotes the transcription of cytokines and chemokines (thus playing a critical role in inflammation) such as BAFF, TNF family members including TNFα, as well as numerous interleukins such as IL8 and IL2 and TRAIL (Apo2-Ligand) (Aggarwal, 2003).

Immunoreceptors

NF-κB target genes include those encoding immune system modulators such as Toll-like receptors (TLR) (TLR2 and TLR6), chemokine receptors (CCR5 and CCR2), CD40 (Hinz et al., 2001), CD86 (Zou et al., 2005), CD80 (Fong et al., 1996), CD69 (Lopez-Cabrera et al., 1995) and IRF4 (Grumont et al., 2000). In all cell types studied thus far, the expression of most of the genes induced by inflammatory stimuli are upregulated by NF-κB (Li et al., 2002) and interestingly the over-expression of surface immune receptor genes such as TLRs and CD40 can lead to positive feedback regulation of the NF-κB pathway.

Apoptosis regulators

NF-κB regulates genes involved in preventing apoptosis, such as GADD45β (Hoffmann et al., 2006) and BCL2 family members (Catz et al., 2001) as well as IAP, FLIP, TRAF1 and TRAF2 (Karin et al., 2002)

Cell cycle genes

NF-κB also regulates genes involved in cell cycle regulation cyclin D1 and MYC (Toualbi-Abed et al., 2008) (Hoffmann et al., 2006).

NF-κB negative regulators

NF-κB regulates the expression of its own inhibitors such as IκBα (Sun et al., 1993), and IκBe (Tian et al., 2005a) and NF-κB negative regulator genes such as A20 (Krikos et al., 1992) and ABIN (A20 binding and inhibitor of NF-B) gene family including ABIN-1 to 3 (Verstrepen et al., 2008) playing an important role in negative feedback regulation.
NF-κB regulates the transcription (shown by the blue arrows) of many target genes involved in cell survival and proliferation as well as immune response and homeostasis. These can be broadly classed as positive and negative regulators of NF-kB. Positive regulators of NF-kB, shown in blue arrows, include immune surface receptors (shown in dotted blue box) that are essential for cell signalling such as chemokine receptors, TLRs, CD40, TNF receptor, BAFF receptor and lymphotoxin receptors. They respond to many pro-inflammatory and environmental stimuli such as LPS, chemokines and cytokines and ligands and lead to positive feedback loop causing constitutive NF-κB activation promoting tumour cell survival. Negative regulators of NF-κB include A20 and IκB family. Interactions between the pro-apoptotic and pro-survival pathways, as well as feedback loops within each pathway contribute to a tightly regulated balance of cell death and survival. B- and T- cell receptors are not target genes but lead to NF-κB activation via CARD11-MALT1-BCL10 (CBM) complex.
1.5 MALT lymphoma associated translocations target common molecular pathways that cause NF-κB activation

BCL10 and MALT1 are central to the transduction of signals from cell surface immune receptors to the classical NF-κB activation pathway in both B and T cells (Farinha et al., 2005). The three common chromosomal translocations recurrently found in MALT lymphomas that involve the BCL10 and MALT1 genes are clearly implicated in the oncogenic process via deregulation of the NF-κB activation pathway. Mounting evidence indicates that the oncogenic activity of the three MALT-lymphoma associated chromosomal translocations is linked by roles of BCL10 and MALT1 in activating the NF-κB pathway in lymphocytes (Section 1.4) (Figure 1.8).

The API2-MALT1 fusion protein self oligomerises through a non-homotypic interaction mediated by the API2 region and is capable of activating NF-κB. However, neither MALT1 nor API2 alone is able to activate NF-κB in vitro (Du, 2007). Among the three BIR domains, only the first, BIR1, is essential for NF-κB activation by API2-MALT1 (Garrison et al., 2009; Zhou et al., 2005). BIR1 has been shown to contain an additional TRAF2/TRAF6 binding site, as well as a region that interacts with the C-terminal MALT1 responsible for a heterotypic oligomerisation that is critical for NF-κB activation (Garrison et al., 2009). It is likely that the constituting oligomerisation of the API2-MALT1 fusion protein contributes to its ability to interact with and oligomerise downstream ubiquitin ligase such as TRAF2/TRAF6. TRAF2 and TRAF6 (together with the ubiquitin conjugating enzyme complex, Ubc13/Uev1A) trigger the K63-polyubiquitination of the API2-MALT1 fusion product and the K63-autoubiquitination of the TRAFs, themselves. The polyubiquitinated API2-MALT1 complex then binds to NEMO (Wagner et al., 2008; Wu et al., 2006). This
molecular association allows the TRAFs associated with API2-MALT1 to K63-ubiquitinate NEMO and causing the terminal events in the canonical NF-κB activation cascade.

In addition to binding to TRAF2/TRAF6, the API2-MALT1 protein has further activities that can augment signals to NF-κB (Zhou et al., 2004). For example, some data suggest that API2-MALT1 itself possesses ubiquitin ligase activity that is capable of K63-polyubiquitinating NEMO and triggering NF-κB activation (Zhou et al., 2005). Also, recent data indicate that the API2-MALT1 fusion protein binds to K63-polyubiquitinated NEMO via the UBA domain of API2 (Gyrd-Hansen et al., 2008). It is possible that this interaction protects the NEMO protein from de-ubiquitination, thereby prolonging activation of the IKK complex. Another possibility is that the UBA-NEMO interaction stabilises the interaction between API2-MALT1 and the IKK complex, increasing the efficiency of NEMO K63-polyubiquitination by API2-MALT1-associated TRAF2 and TRAF6. There is also evidence that API2-MALT1, like MALT1, can mediate proteolytic cleavage of A20, a negative modulator of NF-κB signalling. This depends on the paracaspase activity of the caspase like domain (Coornaert et al., 2008). A point mutation that disrupts this proteolytic activity decreases API2-MALT1 mediated NF-κB activation by approximately 3-fold (Hu et al., 2006b). Thus, the proteolytic activity of API2-MALT1 appears to augment NF-κB activation by interfering with normal homeostatic mechanisms that serve to limit NF-κB activation. It remains to be investigated whether A20 is also cleaved when BCL10 or MALT1 is over-expressed.

BCL10 has been reported to interact with the API2-MALT1 fusion protein and synergistically enhance NF-κB activation in the absence of appropriate stimuli (Hu et al., 2006b). In this study, it was shown that BCL10 interacts with a sub-fragment of API2 (1-
which contains the three BIR domains and the UBA domain (Gyrd-Hansen et al., 2008) (Figure 1.4).

For the non-canonical (alternative) pathway, it has been shown that NF-κB signalling, once activated in a CD40-dependent immune response, is maintained and enhanced through deregulation of MALT1 or formation of an API2-MALT1 fusion (Ho et al., 2005).

In contrast to MALT1 and API2-MALT1, BCL10 deregulation was demonstrated to promote survival of antigen-simulated B lymphocytes, by showing that over-expression of BCL10 in primary B cells activated *ex vivo* promoted the survival of these cells after removal of activating stimuli (Tian et al., 2005b).

Although BCL10 and API2-MALT1 are potent activators of NF-κB, over-expression of these oncogenic products alone is not sufficient to induce malignant transformation. Transgenic mice expressing API2-MALT1 or BCL10 alone develop splenic marginal zone hyperplasia but not lymphoma (Baens et al., 2006; Macintyre et al., 2000; Li et al., 2009). However, when transgenic mice expressing the API2-MALT1 fusion protein are immunised with the Freund’s complete adjuvant, they develop splenic marginal zone lymphoma-like lymphoid hyperplasia (Sagaert et al., 2006a). In line with this, expression of either API2-MALT1 or MALT1 in BJAB B cells enhanced the activation of IKK and NF-κB by CD40/CD40L stimulation (Ho et al., 2005).

FOXP1-involved translocation occurs recurrently in MALT lymphoma and it is hypothesised that it may also confer oncogenesis through activation of NF-κB. Support for this hypothesis is provided by the observation that FOXP1 is highly expressed in the activated B-cell subtype of DLBCL in which NF-κB is constitutively active. However, these separate findings may be unrelated and offer no insight into the mechanism by which FOXP1 promotes oncogenesis. The full length and two short isoforms of FOXP1 were able to activate NF-κB alone and
synergistically with cell surface stimulation of B cells by LPS and T cells by CD3 and CD28. The mechanism of NF-κB activation is unknown but there is no evidence of activation of the canonical or non-canonical pathways in the cytoplasm, in keeping with the nuclear localisation of FOXP1 (unpublished data in Professor Ming Du’s laboratory). The extent of the oncogenic activities of these translocations is not yet fully understood as indicated by the aberrant pattern of BCL10 expression in some cases of MALT lymphoma. In normal B cells, including those of the marginal zone of B-cell follicles, BCL10 is expressed primarily in the cytoplasm (Ye et al., 2000). However, in MALT lymphoma with t(1;14)(p22;q32), BCL10 is strongly expressed in the nuclei (Ye et al., 2000). Moderate levels of nuclear BCL10 expression are also seen in up to 50% of t(1;14)(p22;q32)-negative MALT lymphomas, including almost all t(11;18)(q21;q21)-positive cases (Liu et al., 2001b; Maes et al., 2002; Ye et al., 2003). Furthermore, BCL10 was found to be expressed at high levels in the nuclei of splenic marginal-zone B cells in transgenic mice in which BCL10 expression is driven by Ig enhancers (Li et al., 2009). These observations indicate that aberrant nuclear BCL10 expression might have a role in MALT lymphoma development. However, the biological activity of nuclear BCL10 remains to be investigated.

1.6 Summary of current understanding of gastric MALT lymphoma

The sequential development of H. pylori-associated chronic gastritis, acquisition of MALT, low grade MALT lymphoma and transformation into DLBCL, clearly indicates a multistep process in the development of MALT lymphoma (Figure 1.10), primarily demonstrated by the histological presentation of these pathological conditions (Wotherspoon et al., 1993). Recent studies provided further understanding at both the cellular and molecular levels. MALT lymphomagenesis recapitulates many aspects of the normal immune response and
lymphocyte development. The patterns of spread of lymphomas reflect the homing patterns of normal lymphocytes, both microscopically, within lymph nodes, and macroscopically, at a clinical level.

Correlation of the genetic aberrations with clinical outcome will permit improved diagnostic, prognostic and therapeutic sub-classification of MALT lymphomas. Whilst NF-κB deregulation as a consequence of the MALT lymphoma translocations is clearly indicated, further investigation is necessary to clarify the role of these events in oncogenesis. The equivalent events in translocation negative lymphoma also require elucidation.

Figure 1.10 - Multistep development of gastric MALT lymphoma.
Figure adapted from Isaacson et al. (2004). Nature Review Cancer, 4, 644-653.
Infection by *H. pylori* first induces the formation of acquired mucosa-associated lymphoid tissue (MALT) in the gastric mucosa. The persistence of the bacterial infection results in chronic inflammation. Under sustained immunological stimulation, *H. pylori*-specific tumour-infiltrating T cells stimulate the proliferation of B cells. Genetic abnormalities such as chromosomal translocations (Figure 1.4) might be the result of oxidative stress associated with inflammation (Ye *et al.*, 2003). The acquisition of genetic abnormalities leads to malignant transformation. At this early stage, clonal expansion is *H. pylori*-dependent and the resulting MALT lymphoma can be effectively treated by *H. pylori* eradication with antibiotics. However, MALT lymphoma cells can gain autonomous growth ability, presumably through the acquisition of chromosomal translocations such as t(11;18) and t(1;14) or secondary genetic abnormalities such as A20 mutations, and thus, do not respond to *H. pylori* eradication.

The milieu of infection and chronic inflammation provide a common background for the development of MALT lymphomas. It is likely that NF-κB activation is involved in both translocation positive and negative MALT lymphoma. However despite the overlapping mechanisms there are important differences in the clinical and histological presentations between MALT lymphomas with and without chromosome translocation. Clinically, gastric MALT lymphomas with t(11;18) or t(1;14) are significantly associated with advanced clinical stages and resistance to *H. pylori* eradication (Liu *et al.*, 2002b; Ye *et al.*, 2006). Histologically, t(11;18) positive MALT lymphomas appear to be more monotonous, lacking apparent transformed blasts (Okabe *et al.*, 2003). These clinico-pathological characteristics may indicate the presence of significant differences in molecular mechanisms between MALT lymphomas with and without chromosome translocation. Thus, there are still many unanswered questions regarding MALT lymphomagenesis. Firstly, how chronic antigenic
stimulation in the presence of *H. pylori* or *C. psittaci* infection leads not only to chronic B-cell proliferation, but also to DNA damage which can result in the specific translocations that characterise most MALT lymphomas. Secondly, what are the molecular mechanisms that determine the response to *H. pylori* eradication. Thirdly, what is the molecular basis of NF-kB activation in MALT lymphoma. Fourthly, what are the molecular profiles and pathways targeted by MALT lymphoma with and without chromosomal translocations that can explain their differences in clinical and histological presentation. In order to answer some of these compelling questions, expression microarray investigations were applied in this thesis to MALT lymphoma cases with and without chromosomal translocation.

### 1.7 Gene expression microarray

Although all human somatic cells possess the same inherited genomic DNA, each cell transcribes different genes as mRNA according to the cell type. Further variation is provided by biological processes, both normal and abnormal conditions, amongst other parameters. The diversity in gene expression patterns has led to intensive research because of its biological and clinical relevance. Microarray technology allows the simultaneous profiling of the expression of tens of thousands of genes, thus painting a molecular portrait of the tissue or cell lines being studied. The technology, based on the micro-spotting of DNA, protein, tissue or small organic compounds, which can be probed with various labelled binding ligands (Howbrook *et al.*, 2003). Different microarray technologies are designed to address distinct biological questions. This thesis focuses on expression microarray technologies, which permit a broad overview of expression patterns.
1.7.1 Advantages of using gene expression microarray

It can be hypothesised within limits that changes in the phenotype of a cell or cell population should be reflected by concomitant transcriptional changes. Large-scale assessment of mRNA transcript abundance should thus provide a molecular signature of the state of gene activity of the biological system in question. Such assessment of gene expression has been considered a useful tool in the study of cellular biology in health and disease.

Currently, expression microarrays are much more reliable, optimised and permit higher throughput than any available proteome-assessing technology. Global and quantitative information on gene expression by measuring mRNA levels far outstrips the available proteomic technologies which are generally more technologically challenging and permit far lower throughput (Clarke et al., 2001). Indeed, expression microarray technology has matured impressively in the last 8 years and now has a resolution of approximately one transcript per known gene. Currently available expression microarray platforms can assess gene expression of over 300,000 distinct transcripts in any single experiment. Nonetheless, the complementation of expression with data from high throughput proteomic technologies such as high throughput crystallography and protein-based microarrays is eagerly awaited (Howbrook et al., 2003).

1.7.2 Limitations of gene expression microarray

The focus of expression microarray technology is the quantitative assessment of gene expression on a very large scale. Such an approach focusing on transcription is often questioned in relation to its ability to extrapolate findings at the protein level. This is one of the limitations of not only microarray-based, but all transcriptome-assessing technologies in
general. There is no doubt that results from expression technologies would be maximally
effective if accompanied by and integrated with studies assessing global protein expression
(Clarke et al., 2001). However, even this combined approach would not suffice to describe a
given cellular biological phenomenon in its entirety as an accurate description but would at
least also require extensive study of protein post-translational modification and localisation.
Microarray technology is sensitive to cellular heterogeneity. In this context where RNA
sample is obtained from complex tissues, expression results may relate to expression profiles
from a number of distinct cell types. Thus it is important to use highly homogenous, carefully
selected cell populations (for example lymphoma cells) as this can reduce the possibility of a
given expression result being affected by cell types of secondary interest. The use of
microdissected tissue may circumvent this problem by including mostly specific cell types in
a given expression profiling experiment. Finally, the requirement of often large quantities of
RNA (total or messenger) for these technologies may prove limiting especially for studies
focusing on rare clinical material. The development of RNA amplification protocols,
however, has addressed this issue successfully, significantly lowering the amount of RNA
needed per microarray experiment. More recently, the discovery of microRNAs (miRNAs)
(which are naturally occurring short non-coding RNA molecules that negative regulate
eukaryotic expression through binding to the complementary sequences in the 3’-UTR of
target mRNA) adds another limitation as some of the mRNA on the expression array may be
negatively regulated by some of those miRNAs. Also studies have shown that some miRNAs
such as miR-155, miR-210 and miR-21 are upregulated in the serum from DLBCL patients
(Lawrie et al., 2008a) whereas miR-150, miR-189, miR-223 and miR-768-3p are
downregulated in DLBCL and BL patients suggesting that dysfunctional expression of
miRNAs might be a feature in some haematological malignancies (Lawrie et al., 2008b).
1.7.3 Microarray platforms

There are several expression microarray platforms, including complementary DNA (cDNA) microarrays (Schena et al., 1995), oligonucleotide microarrays (Lockhart et al., 1996) and serial analysis of gene expression (SAGE) (Velculescu et al., 1995). The first two microarray technologies are the most commonly used platforms. The cDNA microarrays are based on standard microscopic glass slides on which cDNA fragments have been spotted. The oligonucleotide microarrays are constructed with oligonucleotides, 25- or 60-mer in length that are either synthesised in situ on a silicon wafer, or robotically spotted or injected on glass slides. The term commonly used to describe the DNA arrayed on a platform is “probe” and the cDNA or cRNA generated from a sample RNA, which represent the gene expression profile of the sample, are referred to as “target”. The types of probes and targets used in each microarray platform differ, but these differences are becoming less significant. The main difference between the two platforms is the method by which the mRNA levels are determined. In cDNA microarrays, the quantitation is made by comparing a selected sample to a ‘control’ sample, while in oligonucleotide microarrays, a well-defined arbitrary unit is produced without the need to compare with a different sample. Generally, it has been shown that oligonucleotide arrays give better results than cDNA microarrays (Woo et al., 2004). The most common oligonucleotide platform to study human genome expression is the Affymetrix HG-U133 GeneChip.

1.7.3.1 The Affymetrix HG-U133 GeneChip

The HG-U133 (Human Genome U133) GeneChip microarray set originally consisted of two GeneChips; HG-U133A and HG-U133B. Collectively, the HG-U133 set is capable of assessing a total of around 39,000 transcripts and variants, including more than 33,000 well-
substantiated human genes. The U133A chip alone assess 22,283 transcripts derived mostly from well-characterised genes and U133B can assess 22645 derived mostly from expressed sequence tags (ESTs). Sequences used in the design of the chip were selected from GenBank, dbEST and RefSeq. The sequence clusters were created from the UniGene database (Build 133, April 20, 2001) and then refined by analysis and comparison with a number of other publicly available databases. Subsequently around 2005, Affymetrix commercialised a new GeneChip that includes the whole human genome on one chip, named HG-U133 plus 2.0. This is capable of analysing the expression level of over 47,000 transcripts and variants, including 38,500 well-characterised human genes. It comprises more than 54,000 probe sets and 1,300,000 distinct oligonucleotide features. In addition, there are 9,921 new probe sets representing approximately 6,500 new genes. These gene sequences were also selected from GenBank, dbEST, and RefSeq. Sequence clusters were created from the UniGene database (Build 159, January 25, 2003) from manufacturer’s datasheet at


The unit of expression interrogation of the U133 chip is the probe set. Throughout this thesis, for the purpose of clarity, all genes mentioned will be associated to their probe set(s). The terms gene and probe set may be used interchangeably. It is stressed, however, that all observations refer to probe set signal intensity changes.

1.7.3.1.1 Probe versus Probe set

A major difference between spotted and GeneChip microarray technologies relates to probe design. Spotted microarray technologies employ probes that may either be synthetic oligonucleotides, long PCR products or cloned cDNAs. In all cases however, a single transcript is assessed by a single probe. For Affymetrix GeneChips, transcripts are
interrogated not by a single probe, but by a combination of probes collectively forming a probe set. Each probe in a probe set is designed to be complementary to a distinct region of the transcript queried. For example, the U133A Genechip used in this thesis, employs a total of 11 probes in the interrogation of each transcript assessed. These probes are referred to as perfect match (PM) as they are designed to be perfectly complementary to their respective transcript. In addition, for each probe set, a second set of mismatch probes (MM) with a mutation in the middle of the probe’s sequence is employed to control for hybridisation specificity and background.

The generation of probe set values from respective probe values can be addressed in a number of ways. These differ mainly in relation to the use of MM probes and the statistical approaches adopted for obtaining single probe set expression values from individual probe intensities. In the most simplistic scenario, probe set level data can be obtained by initially correcting for background by subtracting each MM probe from its respective PM one and then averaging corrected PM probe intensities.

1.7.4 Data analysis

Regardless of the microarray platform, each experiment produces a data set containing tens to hundreds of thousands of values of gene expression. This overwhelming abundance of data requires the use of powerful statistical and analytical tools. After normalisation and non-specific filtering, there are two basic approaches to analysing gene expression data set. The supervised approach is based on determining genes that fit a predetermined pattern, usually used to correlate between gene expression and clinical data. The two most common supervised techniques are: nearest neighbour analysis (Golub et al., 1999) and support vector machines (Brown et al., 2000). The unsupervised approach is based on characterising the
components of the data set without the *a priori* input or knowledge of a training signal. This approach is usually used to identify a distinct subgroup of tumours that share similar gene expression profiles. The four most common unsupervised techniques are: principle-component analysis (Raychaudhuri *et al.*, 2000), hierarchical clustering (Eisen *et al.*, 1998), self-organising maps (Tamayo *et al.*, 1999) and relevance networks (Butte *et al.*, 2000).

The first study utilising microarray technology demonstrated the power of this tool to classify and predict human acute leukaemias. These classifications and predictions were based solely on gene expression monitoring and were independent of previous biological knowledge (Golub *et al.*, 1999). Although histopathological evaluation, supplemented by cytogenetics and analysis of a few molecular markers, is still the gold standard in diagnosis and prognosis, gene expression profiling had proved capable of replacing these evaluations providing large numbers of patients are used in extracting the molecular signatures.

The rate-limiting step in functional genomics experiments is neither the handling of the biological samples nor the actual analysis, but instead the post-analytical work in determining what the results actually mean. This is largely restricted by the lack of biological knowledge of the gene studied, reliable bioinformatics methods in mining and interpretation of the massive data generated. Some advances have been achieved, such as the development of ONCOMINE, a cancer microarray database and web-based data-mining platform aimed at facilitating discovery from genome-wide expression analyses. To date, ONCOMINE contains 65 gene expression datasets comprising nearly 48 million gene expression measurements from over 4700 microarray experiments (Rhodes *et al.*, 2007) ([http://www.oncomine.org](http://www.oncomine.org)). Other pathway and data mining software such as Ingenuity Pathway Analysis ([http://www.ingenuity.com](http://www.ingenuity.com)) and GeneGO ([http://www.genego.com](http://www.genego.com)) help in the interpretation of microarray data. However, a precise method to map the genes that are differentially
expressed from the microarray studies to cellular pathways relevant to the cellular and molecular mechanisms of disease is required. Gene set enrichment analysis (GSEA) goes some way to achieve this. GSEA measures whether the individual genes in a signature are differentially expressed in a consistent fashion between two groups of samples (Mootha et al., 2003). This method was originally used to demonstrate that genes involved in oxidative phosphorylation are decreased in expression in diabetic muscle (Mootha et al., 2003). A recent enhancement of the GSEA method places added emphasis on those genes in a signature that are the most differentially expressed between two groups (Subramanian et al., 2005).

1.7.5 Advances in lymphoma by gene expression microarray investigations

Gene expression profiling using microarrays has made huge improvement in disease classification, disease sub-classification, biomarkers identification, and deciphering the molecular pathogenesis in a number of lymphoma subtypes. Highlighted below are examples of such advances:

**Disease sub-classification and characterisation**

The most prominent examples of gene expression microarray in lymphoma sub-classification are studies of DLBCL. These studies revealed new unexpected subgroups of DLBCL which cannot be classified by conventional histology and immunophenotype. While 40% of patients respond well to chemotherapy, the remaining 60% succumbs to the disease. Using unsupervised analysis of gene expression data, Alizadeh et al. (Alizadeh et al., 2000) identified two molecularly distinct forms of DLBCL, named germinal center B-cell-like (GCB) GCB-DLBCL and activated B-cell-like ABC-DLBCL. The different gene expression profiles apparently reflected the variation in tumour proliferation rate, host response and
differentiation state of the tumour. Importantly, this stratification proved to be clinically relevant in that activated B-cell (ABC) DLBCL is significantly associated with poor overall and event free survival. Subsequent studies showed that ABC-DLBCL is characterized by constitutive NF-κB activation and is more frequently associated with \textit{CARD11} and \textit{CD97B} inactivating mutations (Lenz \textit{et al.}, 2008; Davis \textit{et al.}, 2010).

\textbf{Disease classification}

Identification of molecular profiles that differentiate the lymphoma in question from other B-cell lymphomas can be seen in Burkitt’s lymphoma microarray studies. Burkitt’s lymphoma is a rare, aggressive B-cell lymphoma that accounts for 30\% to 50\% of lymphomas in children but only 1\% to 2\% of lymphomas in adults (NHL Lymphoma Classification Project, 1997; Swerdlow \textit{et al.}, 2008). The main diagnostic challenge in Burkitt’s lymphoma is to distinguish it from diffuse large B-cell lymphoma. The tumour cells of Burkitt’s lymphoma are characteristically medium-sized (smaller than the cells of most diffuse large-B-cell lymphomas) and very high fractions are proliferating. Dave \textit{et al.} (Dave \textit{et al.}, 2006) and Hummel \textit{et al.} (Hummel \textit{et al.}, 2006), used gene expression microarray technology to improve the accuracy of the diagnosis of Burkitt’s lymphoma. The two studies differ in many important ways, but both reach the same conclusion: the gene-expression profiling of cases classified as Burkitt’s lymphoma by expert pathologists identifies a characteristic genetic signature that clearly distinguishes this tumour from cases of diffuse large-B-cell lymphoma. Furthermore, the microarray method seems to outperform the expert pathologists: 17\% (Dave \textit{et al.}, 2006) and 34\% (Hummel \textit{et al.}, 2006) of cases with the gene-expression signature of Burkitt’s lymphoma had been called diffuse large-B-cell lymphoma or unclassifiable high-grade B-cell lymphoma; 0.4\% (Dave \textit{et al.}, 2006) and 4\% (Hummel \textit{et al.}, 2006) of cases
without the Burkitt’s signature had been called classic or atypical Burkitt’s lymphoma; and 3% (Dave et al., 2006) and 8% (Hummel et al., 2006) of cases diagnosed as diffuse large B-cell lymphoma or unclassifiable high-grade B-cell lymphoma had a Burkitt’s signature.

**Deciphering the molecular basis of lymphomagenesis**

Examples of the use of expression microarrays in understanding the molecular pathogenesis of lymphomas can be seen in mantle cell lymphoma (MCL) and follicular lymphoma (FL). The common cytogenetic alteration in MCL patients, t(11;14), leads to cyclin D1 overexpression. Nevertheless, overexpressing cyclin D1 in transgenic mice was not sufficient to induce lymphomas, and other oncogenic factors, were required (Bodrug et al., 1994). Thus, different mechanisms must be required for the development and progression of MCL. The first use of microarrays in studying MCL demonstrated altered apoptosis pathways, in addition to the known over-expression of cyclin D1 (Hofmann et al., 2001). A comparison of MCL with normal human B cells revealed a distinct gene expression signature affecting lymphocyte trafficking, differentiation and growth regulation (Ek et al., 2002). In follicular lymphoma, gene expression profiling showed that an interaction of tumour cells and the microenvironment determine the clinical behaviour (Glas et al., 2007) and that transformation of follicular lymphoma to diffuse large B-cell lymphoma is preceded by distinct oncogenic mechanisms (Davies et al., 2007).

**Biomarker identification**

Blenk et al. (Blenk et al., 2007) analysed a large data set on DLBCL gene-expression (248 patients, 12196 spots) and identified specific, activated B-cell-like (ABC) and germinal center B-cell-like (GCB) distinguishing genes. These include early (e.g. CDKN3) and late (e.g. CDKN2C) cell cycle genes. Independently from previous classification by marker genes
they confirmed a clear binary class distinction between the ABC and GCB subgroups. The biomarkers set distinguishing marked over-expression in ABC from that in GCB, is built by: ASB13, BCL2, BCL6, BCL7A, CCND2, COL3A1, CTGF, FN1, FOXP1, IGHM, IRF4, LMO2, LRMP, MAPK10, MME, MYBL1, NEIL1 and SH3BP5. It predicts and supports the aggressive behaviour of the ABC subgroup and help to understand target interactions, improve subgroup diagnosis, risk prognosis as well as therapy in the ABC and GCB DLBCL subgroups.

1.7.6 Gene expression microarray studies of MALT lymphomas

There are so far three gene expression microarray studies on MALT lymphomas. O'Rourke et al. (O'Rourke, 2008) investigated gastric mucosa of BALB/c mice infected with H. pylori and correlated transcriptional profile with histological changes during the progression from chronic inflammatory infiltrate to MALT lymphoma. Huynh et al. (Huynh et al., 2008) compared gene expression profiles of 21 gastric MALT lymphomas with corresponding H. pylori associated MALT B-cell follicles and aggregates. The study showed that gastric MALT lymphoma has a distinct gene expression profile, characterized by up-regulation of several surface receptor markers of haematopoietic cells such as CD1c, CD40, CD44, CD53, CD83 and CD86 and members of the HLA-D family, indicating antigen-dependent survival of lymphoma cells. Chng et al. (Chng et al., 2009) attempted to classify 35 cases of pulmonary MALT lymphoma (10 with t(11;18), 3 with t(14;18) translocations and 22 negative for all known MALT lymphoma translocations) with other B- and T- cell lymphomas. They showed that MALT lymphoma is a distinct entity with a prominent T-cell signature and a marginal zone/memory B-cell profile. Fifty genes were differentially expressed between MALT lymphoma and all the other samples, 13 of which showed over-
expression in MALT lymphoma. Only 4, MMP7, SIGLEC6, WSB1, and PRO1853, were specifically overexpressed in MALT lymphoma, and 2 of these, MMP7 and SIGLEC6 were validated using immunohistochemistry on MALT lymphoma tissue microarrays. Hierarchical clustering of pulmonary MALT lymphoma with and without translocations showed overlapping transcriptional profiles with over-expression of NF-κB and chemokine signalling pathways in MALT lymphomas with t(11;18). Additionally, spiked expression analysis showed high expression of MALT1 and RARA. Samples with plasmacytic differentiation had high FKBP11 expression, and samples with high RGS13 expression tended to have trisomy 3 and reactive follicles. However, the main criticism of this study is that only 7 out of the 33 cases used had 70% or higher tumour content and including cases that had tumour content as low as 15% (Chng et al., 2009). This can lead to false positives and under-powered study. In addition the study only focused on t(11;18) and t(14;18) in pulmonary MALT lymphomas only which can exclude other important genes implicated in MALT lymphoma such as BCL10.

In summary, none of the MALT lymphoma microarray studies thus far, convincingly showed the key molecular events explaining its lymphomagenesis.
1.8 Objectives of the thesis

1) To characterise the expression profile of MALT lymphoma with and without translocations with the aim to understand the common and unique molecular mechanisms underlying the disease;

2) To investigate the cooperation between the expression of MALT lymphoma oncogenes and immunological stimulation on NF-κB activation;

3) To identify novel phenotypic markers for MALT lymphoma by comparing expression microarray data of MALT lymphoma with other lymphomas such as FL, MCL, CLL and SMZL.
CHAPTER 2 – Materials and methods

2.1 Materials

2.1.1 Tissue materials and clinical data

2.1.1.1 Ethical considerations

The patient materials included in this study were archival fresh frozen and formalin-fixed paraffin-embedded tissues. Tissue material was retrieved from Professor Ming Du’s laboratory and his international collaborators. Local ethical guidelines were followed for the use of these archival tissues for research with the approval by the local ethics committees of the relevant institutions.

2.1.1.2 Tissue materials for expression microarray and phenotypic marker studies

A total of 26 well characterised MALT lymphomas, 14 SMZL, 7 nodal FL and 8 nodal MCL were used for the expression microarray study. The MALT lymphoma cases included 9 positive for t(11;18)(q21;q21)/API2-MALT1 (8 gastric and 1 pulmonary), 4 positive for t(1;14)(p22;q32)/BCL10-IGH or t(1;2)(p22;p11)/BCL10-IGk (3 gastric and 1 pulmonary), 2 positive for t(14;18)(q32;q21)/IGH-MALT1 (1 hepatic and 1 ocular adnexal), 1 positive for t(3;14)(p13;q32)/IGH-FOXP1 and 10 gastric cases negative for all known MALT lymphoma associated chromosome translocations (Table 2.1). In all cases, fresh frozen tissue from surgical resection specimens was available. Tumour content was checked based on histological examination of Haematoxylin and Eosin (H&E) slides and where necessary crude microdissection was performed to ensure at least 70% tumour cells were used for molecular investigations. The chromosomal translocation status in these cases was investigated in previous studies using conventional cytogenetics, interphase FISH and RT-PCR where appropriate (Table 2.1). Extensive immunophenotyping including BCL10,
MALT1 and FOXP1 immunohistochemical data was available from previous studies (Goatly et al., 2008; Liu et al., 2001b; Liu et al., 2001a; Liu et al., 2004a; Ye et al., 2000; Ye et al., 2006).

Table 2.1 - Summary of clinico-pathological, molecular and immunohistochemical data of MALT lymphoma cases used in gene expression microarray studies.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex</th>
<th>Age</th>
<th>Site</th>
<th>Diagnosis</th>
<th>Translocation status</th>
<th>Stage</th>
<th>Treatment</th>
<th>Follow-up</th>
<th>BCL10 immunohistochemistry</th>
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<td>1</td>
<td>M</td>
<td>41</td>
<td>Stomach</td>
<td>MALT lymphoma</td>
<td>t(11;18)(q21;q21)</td>
<td>IIIE</td>
<td></td>
<td></td>
<td>Moderate nuclear</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>72</td>
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<td>MALT lymphoma</td>
<td>t(11;18)(q21;q21)</td>
<td>IVE</td>
<td></td>
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</tr>
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<td>MALT lymphoma</td>
<td>t(11;18)(q21;q21)</td>
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<td>Moderate nuclear</td>
</tr>
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<td>F</td>
<td>52</td>
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<td>t(11;18)(q21;q21)</td>
<td>IIE</td>
<td></td>
<td></td>
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</tr>
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<td>Surgical resection,</td>
<td></td>
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<td>t(11;18)(q21;q21)</td>
<td>I</td>
<td>total gastrectomy</td>
<td>CR during 6 year follow up</td>
<td>Cytomplasmic</td>
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<td>7</td>
<td>M</td>
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<td>t(11;18)(q21;q21)</td>
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<td>t(11;18)(q21;q21)</td>
<td>III</td>
<td>total gastrectomy</td>
<td>CR during 10 year follow up</td>
<td>Cytomplasmic</td>
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<tr>
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<td>F</td>
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<td>MALT lymphoma</td>
<td>t(11;18)(q21;q21)</td>
<td>I</td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>71</td>
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<td>MALT lymphoma</td>
<td>t(1;14)(p22;q32)</td>
<td>IVE</td>
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</tr>
<tr>
<td>11</td>
<td>NA</td>
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<td>Lung</td>
<td>MALT lymphoma</td>
<td>t(1;14)(p22;q32)</td>
<td></td>
<td>Gastrectomy</td>
<td></td>
<td>Strong nuclear</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>55</td>
<td>Stomach</td>
<td>MALT lymphoma</td>
<td>t(1;14)(p22;q32)</td>
<td></td>
<td></td>
<td></td>
<td>Strong nuclear</td>
</tr>
<tr>
<td>13</td>
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<td>67</td>
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<td>MALT lymphoma</td>
<td>t(1;2)(p22;q12)</td>
<td>IIE</td>
<td>Total gastrectomy</td>
<td></td>
<td>Strong nuclear</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>62</td>
<td>Liver</td>
<td>MALT lymphoma</td>
<td>t(14;18)(q32;q21)</td>
<td>I</td>
<td>Surgical resection, no evidence of lymphoma during 6 years follow-up</td>
<td>Strong cytoplasmic</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>56</td>
<td>Ocular adnexa</td>
<td>MALT lymphoma</td>
<td>t(14;18)(q32;q21)</td>
<td>I</td>
<td>Treated by radiotherapy, no evidence of lymphoma during 10 years follow-up</td>
<td>Strong cytoplasmic</td>
<td></td>
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<tr>
<td>16</td>
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</tr>
<tr>
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<td>Stomach</td>
<td>MALT lymphoma</td>
<td>Negative</td>
<td></td>
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<td></td>
<td>Moderate nuclear</td>
</tr>
<tr>
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<td>Stomach</td>
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<td>Negative</td>
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<td>Moderate nuclear</td>
</tr>
<tr>
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<td>M</td>
<td>64</td>
<td>Stomach</td>
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<td>18</td>
<td>Stomach</td>
<td>MALT lymphoma</td>
<td>Negative</td>
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<td>22</td>
<td>M</td>
<td>57</td>
<td>Stomach</td>
<td>MALT lymphoma</td>
<td>Negative</td>
<td>III</td>
<td>Total gastrectomy</td>
<td>CR during 13 year follow up</td>
<td>Cytoplasmic</td>
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<tr>
<td>23</td>
<td>F</td>
<td>64</td>
<td>Stomach</td>
<td>MALT lymphoma</td>
<td>Negative</td>
<td>I</td>
<td>Total gastrectomy</td>
<td>CR during 6 year follow up</td>
<td>Cytoplasmic</td>
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<td>24</td>
<td>M</td>
<td>50</td>
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<td>MALT lymphoma</td>
<td>Negative</td>
<td>I</td>
<td>Total gastrectomy</td>
<td>CR during 2 year follow up</td>
<td>Cytoplasmic</td>
</tr>
<tr>
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<td>Stomach</td>
<td>MALT lymphoma</td>
<td>Negative</td>
<td></td>
<td></td>
<td></td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>26</td>
<td>NA</td>
<td>NA</td>
<td>Stomach</td>
<td>MALT lymphoma</td>
<td>t(3;14)(p14;q32)</td>
<td></td>
<td></td>
<td></td>
<td>Cytoplasmic</td>
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</tbody>
</table>

CR: complete remission
2.1.1.3 Tissue materials for qRT-PCR and immunohistochemistry

For qRT-PCR and immunohistochemical validation of the microarray study, an additional 73 cases of well-characterised MALT lymphoma were recruited. They included 18 cases positive for t(11;18), 8 cases positive for t(1;14) or variant, 9 cases positive for t(14;18), and 38 cases negative for these translocations.

2.1.2 Reagents

2.1.2.1 Reagents used in gene expression microarray and qRT-PCR

5x First Strand buffer for double stranded cDNA synthesis (Invitrogen)
250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM MgCl₂

5x Second Strand buffer for double stranded cDNA synthesis (Invitrogen)
100mM Tris-HCl (pH 6.9), 450mM KCl, 23mM MgCl₂, 0.75mM β-NAD⁺, 50mM (NH₄)₂SO₄

5x Fragmentation buffer
200mM Tris acetate pH 8.2, 500mM potassium acetate, 150mM magnesium acetate

1x Hybridisation buffer
100mM MES, 1M [Na⁺], 20mM EDTA, 0.01% Tween20

Hybridisation cocktail per chip
3nM Control Oligonucleotide B2, Eukaryotic Hybridisation Controls (bioB, bioC, bioD and cre, prepared in concentrations of 1.5, 5, 25 and 100pM respectively), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA and 1x Hybridisation buffer to a final volume of 275µl with ultrapure deionised water

MES (2-(N-Morpholino) ethanesulfonic acid sodium salt)
To make 12X MES Stock (1.22M MES, 0.89M [Na⁺]) in 1000 ml, add:

70.4g MES free acid monohydrate (Sigma)
193.3g MES Sodium Salt (Sigma)
800ml of Molecular Biology Grade water
Mix and adjust volume to 1000ml with DEPC-treated water
The pH should be between 6.5 and 6.7. Filter through a 0.2 µm filter.
Wash buffer
100mM MES, 0.1M [Na+], 0.01% Tween20

Streptavidin-Phycoerythrin (SAPE) solution per chip
10µg/ml SAPE stain, 1x MES stain buffer, 2mg/ml acetylated BSA to a final volume of 600µl with double distilled water

Biotinylated anti-streptavidin per chip
2mg/ml acetylated BSA, 0.1mg/ml normal goat IgG (Sigma), 0.5mg/ml biotinylated antibody (Vector Lab, Burlingane, California, USA), 1x MES stain buffer to a final volume of 600µl with double distilled water

2.1.2.2 Reagents used in immunohistochemistry

PBS-Tween
100µl of Tween 20 (Sigma-Aldrich) was added to 200ml of PBS to give a final concentration of 0.05%

Diaminobezidine tetrahydrochloride (DAB) substrate solution (DAKO)
20µl of Dako REAL DAB Chromogen (Dako Cytomation) was added to 1ml of Dako REAL Substrate Buffer (Dako Cytomation) to make the substrate working solution

Diaminobezidine tetrahydrochloride (DAB) substrate solution (Kem-En-Tec)
This solution was always prepared fresh just before use. One tablet of DAB (Kem-En-Tec, Denmark) was dissolved in 10ml of distilled water. 10µl of 30% hydrogen peroxide solution was added to the solution just before application

Haematoxylin
Mayers haematoxylin was filtered and two drops of Tween 20 (Sigma-Aldrich) were added per 500ml

Citrate Buffer pH 6.0
8.82g of sodium citrate tribasic dehydrate (Sigma-Aldrich) were dissolved in 3 litres of distilled water and the pH was adjusted to 6.0 with 1M HCl

Peroxidase block solution
This solution was prepared fresh before use and was composed of 200µl of 30% hydrogen peroxide (Sigma-Aldrich) in 12ml of methanol

Tris-buffered saline pH 7.6 (TBS)
6.05g of Tris (hydroxymethyl) aminomethane (Sigma-Aldrich) and 80g of NaCl were dissolved in 8 litres of distilled water, the pH was adjusted to 7.6 with 1M HCl and the volume brought up to 10 litres with distilled water

TBS-Tween
Tween 20 (Sigma-Aldrich) was added to TBS to give a final concentration of 0.05%
2.1.2.3 Reagents used in tissue culture

**RPMI 1640 10% fetal calf serum (FCS) medium & 10% P/S**  
50ml of FCS (Invitrogen) were added to 450ml of RPMI 1640 medium (Invitrogen). When culturing BJAB Tet-On and TRex cells, tetracycline (Tet) free FCS was used to prevent inadvertent induction of expression. Add penicillin/streptomycin (P/S) to a final concentration of 100U/ml penicillin and 100µg/ml streptomycin

**10x Phosphate buffered saline (PBS)**  
146.6g sodium chloride, 47.2g hydrogen phosphate and 26.4g sodium dihydrogen phosphate for 2 litres distilled water – pH 7.2

**Freezing medium**  
2ml of DMSO were added to 18ml of FCS

2.1.2.4 Reagents used in Western blotting and co-immunoprecipitation

**Triton Lysis Buffer**  
5ml of 50mM Tris pH 7.4, 30ml of 300mM NaCl, 10ml of 1% Triton X-100 (Sigma-Aldrich), and 400µl of 2mM EDTA were added to 100ml with sterile distilled water and mixed to make a stock solution. One tablet of Protease Inhibitor Cocktail (Roche, Penzberg, Germany) for inhibition of proteases was added to 10 ml of stock solution before use.

**Protein lysis buffer (2X)**  
Lysis buffer was composed of 100mM Tris-HCl (pH8.0), 300mM NaCl, 0.04% sodium azide, 0.2% sodium dodecyl sulphate (Sigma-Aldrich), 2% nonidet P-40 (BDH), 1% sodium deoxycholate (BDH), 2mM EDTA, 100mg/ml phenylmethylsulfonyl fluoride (Sigma-Aldrich), and 1mg/ml leupeptin (Sigma-Aldrich)

**Sample loading buffer**  
4µl of β-mercaptoethanol (BDH) was added to 96µl of 4X NuPAGE LDS Sample Loading Buffer (Invitrogen)

**Running Buffer**  
50ml of 20X NuPAGE Running Buffer (Invitrogen) were added to 950ml of deionised water

**Transfer Buffer**  
50ml of 20X NuPAGE Transfer Buffer (Invitrogen) and 100ml of methanol were added to 850ml of deionised water

**TBST**  
40ml of 1M Tris pH 7.4, 18g of NaCl and 2ml of Tween 20 (Sigma-Aldrich) were made up to 2 litres with deionised water and mixed
4% milk/TBST
4g of milk powder (Marvel, Premier Foods, UK) dissolved in 100ml of TBST

Stripping buffer
100ml of 10% SDS (10g in 100ml deionised water) were combined with 31.25ml of 1M Tris-HCl pH 6.8 and made up to 500ml with deionised water

2.1.2.5 Reagents used in cloning and DNA sequencing

LB medium
Autoclaved Luria-Bertani (LB) medium was provided in-house: 1% sodium chloride, 0.5% yeast extract and 1% tryptone (Sigma)

LB agar plates
15g/L bacteriological agar (Sigma) added to LB medium, autoclaved and poured into 100*15mm Petri dishes (Sigma)

1x Blue dextran mix
100mg/ml blue dextran (Sigma), 25mM EDTA

1x Tris-borate-EDTA electrophoresis buffer (TBE)
89mM Tris base (Sigma), 89mM boric acid (BDH), 2mM EDTA

Gel solution
To 900ml of Automatrix 4.5% 29:1 acrylamide:bisacrylamide ready made 6M urea gel solution (National Diagnostics, Hull) add 10ml of 1x TBE

2.1.3 Cell lines

BJAB cells: BJAB is a human Burkitt’s lymphoma cell line and is Epstein-Barr virus (EBV) negative (Steinitz et al., 1975). BJAB cells were a kind gift from Dr. Rolf Renne (University of Florida Shands Cancer Center, Florida, USA).
**BJAB-TetON cells:** BJAB-TetON cells were a kind gift from Dr. Rolf Renne (University of Florida Shands Cancer Center, Florida, USA) (An et al., 2005). Tetracycline free FCS was used when carrying out experiments involving inducible expression of the oncogene of interest.

**T-REx Jurkat cells:** Jurkat cells are a human T-cell line derived from a patient with acute lymphoblastic leukemia (Schneider et al., 1977). T-REx Jurkat cells (Invitrogen, Paisley, UK) stably express the tetracycline repressor protein. Tetracycline free FCS was used when carrying out experiments involving inducible expression of the oncogene of interest.

**BaF-3 cells:** An IL-3-independent clone of this murine pro-B cell line was established from the peripheral blood of a BALB/c mouse and was the kind gift of Dr Heike Laman, (University of Cambridge, UK).

**WEHI cells:** Immature murine pro-B cell line established from the peripheral blood of BALB/c mouse and was the kind gift of Dr Heike Laman, (University of Cambridge, UK).
2.2 Methods

2.2.1 Overview of the study plan

Figure 2.1 outlines the study plan in this thesis integrating the investigations using the primary lymphoma materials and *in vitro* cell lines.

![Diagram of study plan]

- **RNA extraction from fresh frozen cases**
- **Gene Expression Microarray**
- **Bioinformatics Analysis**
- **Candidate genes for further validation**
- **Validating candidate genes by qRT-PCR, IHC and Western blotting**
- **Preparation of BCL10, MALT1 and API2-MALT1 expression vector**
- **Generation of stably inducible BCL10, MALT1 and API2-MALT1 expressing cell lines**
- **Investigating their effect on NF-κB activation**
- **Study of their cellular localisation**
- **Validation of candidate genes by *in vitro* experiments**

**Figure 2.1 - Summary of the study plan.**

2.2.2 Crude microdissection

4μm sections of FFPE tissue were cut routinely and mounted on charged glass microscope slides. Sections were dried overnight at 56°C, deparaffinised in xylene and rehydrated using decreasing concentrations of ethanol (100%, 10 minutes; 95%, 5 minutes; 75%, 5 minutes) and immersed in distilled water for 5 minutes, and stained in Haematoxylin for 5 seconds.
The sections were covered in 50% ethanol and the unwanted tissue was scraped away using a needle (Figure 2.2). The selected cell populations were then scraped off the slide into sterile 1.5ml Eppendorf tubes. The microdissected cells were dried and used for RNA extraction.

![Crude microdissection of gastric MALT lymphoma.](image)

(a) Gastric MALT lymphoma with constitutive normal tissues
(b) Gastric MALT lymphoma after crude microdissection

**2.2.3 RNA preparation**

**2.2.3.1 RNA extraction from fresh frozen tissue**

RNA extraction was carried out using RNeasy™ Mini kit (Qiagen, East Sussex, UK) according to the manufacturer’s instructions. Briefly, crudely microdissected tumour cells from fresh frozen tissue sections (<5 µm) were lysed in 600 µl of RNeasy lysis buffer (Buffer RLT) containing 1% β-mercaptoethanol. The crude lysate was homogenised by spinning through a QIAshredder column (Qiagen, East Sussex, UK). After addition of absolute ethanol, the samples were applied onto an RNeasy spin column. The column was washed with buffers RW1 and RPE before elution with RNase-free water. To remove all traces of DNA from the samples, they were treated with Turbo DNase (Applied Biosystems,
Warrington, UK) for 30 minutes at 37°C. The DNAse was inactivated using the supplied Inactivation Reagent and the RNA yield was quantified.

2.2.3.2 RNA extraction from formalin fixed paraffin-embedded tissue

This was carried out on microdissected tissue using the Ambion RecoverAll Total Nucleic Acid Isolation kit (Applied Biosystems, Warrington, UK) essentially according to the manufacturer’s protocol with the exception that the tissue was digested in protease K at 50°C for 3.5 hours.

2.2.3.3 RNA extraction from cell lines

Around 7 million cells were centrifuged at 1200rpm for 5 minutes and the resulting pellet was resuspended in RLT buffer containing 1% β-mercaptoethanol before application to a QIAshredder column. RNA was extracted from cells and the contaminating DNA removed using the protocol described in section 2.2.3.1. The RNA quantity and quality were then measured (Section 2.2.3.5).

2.2.3.4 RNA linear amplification

For gene expression microarray using the HG-U133B GeneChips, there was insufficient RNA to carry out the hybridisation. Therefore a linear RNA amplification method was developed in house and tested with the RNA extracted from fresh frozen tissue. The in house method was based on data showing that reverse transcription using random pentadecamer primers increases yield and quality of resulting cDNA (Stangegaard et al., 2006) and the use of T7 and T3 RNA polymerase can be incorporated into the protocol to generate sense and anti-sense RNA (Marko et al., 2005; Xiang et al., 2003). The goal was to develop a strategy based upon the Eberwine method (Van Gelder et al., 1990) but with the ability to produce
sense RNA from small quantities of total or poly-(A)+ RNA extracted from both ideal samples (e.g. cell line RNA) and "real world" samples (e.g. tumours or tissues). This protocol avoids the need for PCR steps and requires two primers only. Additionally, the protocol is cost effective, efficient, and technically simple to perform. Finally, the method gives results consistent with similar amplification techniques when used with subsequent microarray analysis. The in house method used a T7 tagged oligo dT primer to generate a double stranded cDNA from the 3 prime end, followed by T3 tagged pentadecamer to generate a second cycle of double stranded cDNA. This avoided the problems of amplifying degraded RNA and allowed small amounts of RNA to be amplified faithfully for expression microarray studies. 1ng of total RNA was used to generate around 20µg of amplified anti-sense RNA ready to be used for the labelling step before hybridisation to microarray (Section 2.2.4).

2.2.3.5 RNA quantification and quality control

RNA concentration and quality were assessed spectrophotometrically using the GeneQuant Pro (Amersham Pharmacia Biotech, Uppsala, Sweden), and considered to be acceptable for further analysis at a concentration of greater than 500ng/µl RNA with an A\textsubscript{260}/A\textsubscript{280} ratio between 1.7 and 2.0. For gene expression microarray studies, the RNA quality was assessed further by running a 100-300ng aliquot on an Agilent 2100 Bioanalyzer (Agilent, Berkshire, UK) using Agilent RNA Nano Labchips. RNA extracted from all 26 cases of MALT lymphoma for microarray studies was of good quality varying from 1µg to 5µg total RNA with an A\textsubscript{260}/A\textsubscript{280} ratio between 1.7 and 2.0.
2.2.4 Expression microarray

2.2.4.1 Affymetrix HG-U133 GeneChips

Microarray experiments, during the early phase of the study, were carried out on MALT lymphoma cases using the HG-U133A and B, while in the latter phase of the study, FL and MCL cases were analysed using the updated HG-U133plus2 GeneChips.

2.2.4.2 Preparation of biotinylated cRNA target for Affymetrix GeneChips

Total RNA extracted was used to generate double stranded cDNA which acts as a template to generate biotin labelled cRNA that was fragmented using heat and high salt buffer and hybridised to the Affymetrix HG-U133 GeneChip as described below (Figure 2.3).

Figure 2.3 - Strategy for generating hybridisation target from total RNA.
2.2.4.2.1 Double stranded cDNA synthesis

First strand cDNA was synthesised from 5µg RNA using 100 pmol HPLC purified oligo(dT) primer conjugated to a T7 promoter (5’-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG (dT)_{24-3’} (Thermo Scientific, Surrey, UK), hybridised to the poly-A tail of the mRNA primer. Samples were incubated at 70°C for 10 minutes to denature the secondary RNA structures and placed on ice for the primer to anneal. First strand synthesis was performed in a reaction mix containing; 1× First Strand Buffer, 10mM DTT, 500µM each dNTP (Invitrogen, Paisley, UK) and 100U Superscript II reverse transcriptase (Invitrogen, Paisley, UK) at 42°C for 1 hour. The samples were incubated on ice for 2 minutes followed by brief centrifugation.

The second strand synthesis was carried out in a reaction mixture containing; 1× Second Strand Reaction Buffer, 200µM each dNTP, 10U E. coli DNA Ligase, 40U E. coli DNA Polymerase I, and 2U E. coli RNase H (Invitrogen, Paisley, UK) at 16°C for 2 hrs. T4 DNA Polymerase (Invitrogen, UK) was added at a final concentration of 10U followed by incubation at 16°C for a further 5 minutes. The reaction was terminated by addition of 10µl 0.5M EDTA, pH 8.0 and the double stranded cDNA immediately purified using the cDNA purification columns from the GeneChip sample cleanup module kit (Affymetrix, High Wycombe, UK). The purified cDNA was eluted with 12µl of ultrapure water.

2.2.4.2.2 cRNA labelling using IVT (In-Vitro Transcription)

The generation of cRNA target was carried out using the Enzo Bioarray HighYield RNA Transcript Labelling Kit (Affymetrix, High Wycombe, UK). The reaction was carried out in a 40µl mixture containing: 12µl template cDNA from second strand synthesis reaction, 4µl 10× HY Buffer, 4µl Biotin Labelled Ribonucleotides, 4µl DTT, 4µl RNase Inhibitor Mix,
2µl T7 RNA Polymerase, 10µl water at 37°C for 4.5 hours. The cRNA was purified using the cRNA purification columns from the GeneChip sample cleanup module kit (Affymetrix, High Wycombe, UK). 1µl of each cRNA sample was used to check for sample concentration and purity (Section 2.2.3.5) and the remainder stored at –20°C awaiting array hybridisation.

2.2.4.2.3 Fragmentation of cRNA

20µg biotinylated cRNA was fragmented by incubation in fragmentation buffer and RNase free water at 94°C for 40 minutes followed by 10 minutes incubation on ice. The fragmented cRNA was stored at –20°C until use for hybridisation.

2.2.4.3 Hybridisation to HG-U133 Affymetrix GeneChips and Data Acquisition

2.2.4.3.1 Hybridisation mix preparation

A hybridisation cocktail was prepared in a microfuge tube by adding reagents at the following concentrations; 3nM Control Oligonucleotide B2, Eukaryotic Hybridisation Controls (bioB, bioC, bioD and cre, prepared in concentrations of 1.5, 5, 25 and 100pM respectively), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA and 1× hybridisation buffer to a final volume of 275µl.

2.2.4.3.2 Hybridisation to HG-U133 GeneChips

The cRNA target samples were hybridised to HG-U133 chips. The chips were pre-equilibrated with 200µl 1× hybridisation buffer at 45°C for 10 minutes in a GeneChip 450 Hybridisation Oven (Affymetrix, High Wycombe, UK). The hybridisation cocktail containing the biotinylated cRNA was denatured at 99°C for 5 minutes, and transferred to a 45°C heat block for 5 minutes to pre-equilibrate before being added to the hybridisation chamber and incubated in a 45°C oven for 16 hours with rotation at 60rpm.
2.2.4.3 Hybridisation to Test3 GeneChip

A proportion of the biotinylated cRNA target samples was hybridised to Test chips (GeneChip Test3 Array) (Affymetrix, High Wycombe, UK) to ensure successful cRNA biotin labelling using IVT. This was similarly performed as above, with the exception that 80µl 1× hybridisation buffer was used for gene chip pre-equilibration, followed by 80µl hybridisation cocktail.

2.2.4.3.4 Staining of GeneChips

Hybridisation cocktail was removed and replaced with a non-stringent wash buffer (6× SSPE, 0.01% Tween 20). HG-U133A chips were placed in the corresponding slots of the Gene Chip Fluidics Station 450 (Affymetrix, High Wycombe, UK) and subjected to the washing and staining protocol EukGE WASH-WS2V4_450. The GeneChips were washed with a series of non-stringent and stringent wash buffers. Staining was carried out using the following procedure; 40 minutes incubation in Streptavidin-Phycoerythrin (SAPE) solution, then by 20 minutes incubation with biotinylated anti-streptavidin followed by a final 20 minutes incubation in SAPE solution.

2.2.4.4 HG-U133 GeneChip data acquisition and quality control

Chips were scanned at pixel value 2.5µm, wavelength 570 nm using argon laser Affymetrix GeneArray scanner 3000. Data were analysed using Affymetrix MAS and GCOS software. The array was inspected manually for image artefacts such as scratches, overall background, image intensity fluctuations and intensity of hybridisation controls. A grid was automatically placed over the image and correct alignment of grid to image was checked. The Microarray suite (MAS) software (version 5.0) (Affymetrix, High Wycombe, UK) was used to analyse
the scanned image and generate transcript expression data. To allow comparisons between samples, a global scaling technique was used to set the average intensity of each probe set within the array to a target intensity of 100.

The quality control of gene expression microarray varies between different studies and different platforms. Based on Brune et al. (Brune et al., 2008) and similar papers involving human tissue, the following quality control parameters for the HG-U133A and HG-U133plus2 GeneChips were used; overall background should be less than 130, scaling factor should be less than 10, the 5'3' ratio of the housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) should be less than 4, hybridisation control bioB is called ‘present’ by the software at least 50% of the time and bioC, bioD and cre called ‘present’ with increasing signal values representing their relative concentrations and percentage ‘present’ call of genes should be reproducible between tissues and higher than 20%.

In this study, the HG-U133B GeneChip samples were processed using RNA linear amplification protocol (Section 2.2.3.4) thus the quality control parameters according to (The Tumor Analysis Best Practices Working Group, 2004) and (Brune et al., 2008) were as follows; overall background should be less than 130, scaling factor should be less than 20, the 5'3' ratio of the housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) should be less than 13, hybridisation control bioB is called ‘present’ by the software at least 50% of the time and bioC, bioD and cre called ‘present’ with increasing signal values representing their relative concentrations and percentage ‘present’ call of genes should be reproducible between tissues and higher than 10%.
2.2.5 Bioinformatics and statistical analysis of gene expression microarray data

In general, bioinformatics analysis of gene expression microarray starts with normalisation followed by elimination of non-variant probes. The remaining variant probes are the ones that are changing across the samples, and are used for downstream analyses such as unsupervised clustering and differential expression analysis. Full bioinformatics comparison of methods and strategies are presented in Appendix I.

2.2.5.1 Normalisation

After testing many different strategies as outlined in Appendix I, the best strategy for generating variant probes that are differentially expressed between the groups was to normalise the array sets using gcRMA and MAS5 and cross reference the probes that passed through non-specific filtering based on coefficient of variation and absolute value thresholding. Raw gene expression data from Affymetrix CEL files were uploaded to bioconductor where MAS5 and gcRMA normalisation were performed separately for each Affymetrix platform. All MAS5 data were scaled to a target intensity of 100. The MAS5 normalised data was imported into Genespring 7.3.1 where they were log-transformed and median centred for further analysis. For comparison between microarray data obtained from HG-U133A&B and HG-U133 plus2 platforms, an additional median polishing normalisation step was applied. MAS5 normalised data were used for unsupervised clustering and fold change calculations; while gcRMA normalised data were used for gene set enrichment analysis (GSEA). Both MAS5 and gcRMA normalised data were subjected to non-specific filtering.
2.2.5.2 Non-specific filtering

To filter out non-variant genes, a combination of noise and variance filtering was applied. To filter out non-expressed genes, only probes with a value of 50 or higher in the MAS5 dataset in 2 or more samples were selected, since the minimum number of cases with a particular translocation, i.e. t(14;18)/IGH-MALT1, was two. To eliminate non-variant genes, only those with a coefficient of variation (CV) value of 10% or higher in the gcRMA dataset across all cases were considered to be variant and thus selected. CV was calculated as the mean / standard deviation of each gene across all cases. Finally, the genes that passed the above two filtering methods were intersected to obtain a common set of variant genes.

For comparison of microarray data between HG-U133A/B and HG-U133 plus2 platform where indicated, the non-specific filtering was similarly performed separately for each of these platforms as outlined above, then intersected to generate a final common set of variant genes. All the above analyses were carried out using scripts written in R programming language (Appendix II.I). The above procedure for analysis of expression microarray data from HG-U133A/B and HG-U133 plus2 platform was validated by a serial empirical testing using the published pulmonary MALT lymphoma expression microarray data from the HG-U133 plus2 platform as a reference (Gene Expression Omnibus: GSE13314) (Chng et al., 2009).

2.2.5.3 Clustering analysis

The microarray dataset after the above normalisation and filtering was used for unsupervised clustering and this was carried out using Pearson correlation coefficient and average linkage as the similarity measure and clustering algorithm respectively with Genespring GX 7.3.1.
Separate clustering was performed among all MALT lymphoma, FL and MCL cases and also within the MALT lymphoma cases.

**2.2.5.4 Gene Set Enrichment Analysis (GSEA)**

GSEA was used to identify gene sets differentially regulated between MALT lymphoma with and without chromosome translocation and this was performed essentially as previously described with minor modification (Subramanian et al., 2005). As the original GSEA only identifies the gene set showing either uniformly up or down regulation, for the gene sets displaying both up and down regulated genes, absolute GSEA was additionally performed as previously described (Saxena et al., 2006). A total of 4395 gene sets were analysed and they included:

1) NF-κB target genes, which were collated from online data base (http://www.nf-κb.org), published works (http://bioinfo.lifl.fr/NF-KB and http://people.bu.edu/gilmore/nf-κb/target/index.html) and systematic bioinformatics search


3) Gene sets from Molecular Signature database (http://www.broad.mit.edu/gsea/msigdb/index.jsp). The GSEA results were ranked according to the nominal P value and False Discovery Rate (FDR)
For the gene sets differentially regulated between MALT lymphoma with and without translocation, leading edge analysis was carried out to identify the biologically important gene subset (Subramanian et al., 2005). For absolute enrichment, a modification on the original GSEA was made to extract the leading edge set from either end of the list. When generating gene sets, for each sample, only the maximum expression value of the multiprobes for a given gene was used for GSEA as described previously (Subramanian et al., 2005), thus avoiding any potential biased representation due to multiple probes for the same gene.

2.2.5.5 Analysis of differential gene expression in MALT lymphomas with and without translocation

Differential gene expression between MALT lymphomas with and without chromosome translocation was investigated using one-way ANOVA with Cross-Gene Error Model in GeneSpring with P value of ≤ 0.05 considered to be significant. The MAS5 normalised and filtered dataset was used for this analysis and the genes differentially expressed between translocation positive and negative MALT lymphomas were obtained. For each of these significantly differentially expressed genes, fold change calculation was carried out and those showing more than 2 fold differences were selected for functional annotation using gene ontology.

2.2.5.6 Functional annotation using gene ontology (GO)

To further assess the biological implications of differential gene expression in MALT lymphomas with and without chromosome translocation, we measured the representation of gene ontology (GO) terms (association of gene products with regard to their associated biological processes, cellular components, and molecular functions) in the above
differentially expressed genes using Genespring and hypergeometric tests provided in the R package GOstats, version 2.8.0. This allowed us to examine whether any GO term was over or under-represented as compared to chance variations. Independent analyses of GO categories were performed for both over and under-expressed genes in translocation positive MALT lymphoma.

2.2.5.7 Phenotypic marker analysis

The 26 MALT lymphoma cases were compared to 14 SMZL, 7 FL, 8 MCL and 22 CLL. All expression microarrays were performed in house except for CLL which was from a previous study by Calin et al. (Calin et al., 2008) and the raw CEL files downloaded from ArrayExpress (http://www.ebi.ac.uk/microarray-ase/) using the query “E-MEXP-1482”. Normalisation and non-specific filtering was carried out as in sections 2.2.4.1 and 2.2.4.2. The 26 MALT lymphoma HG-U133A and HG-U133B and FL, MCL and SMZL HG-U133plus2 data were normalised separately using RMA algorithm (Irizarry et al., 2003) and combined using median polish step. Multivariate one-way ANOVA (Welch test) (using GeneSpring 7.3) and Bayesian statistical analysis (using in house R scripts) were carried out independently on all 77 lymphoma cases. The data from both algorithms were intersected to generate a list of common probes to both analyses. The common probes were subjected to SOM and Volcano plot analysis to further filter the gene set. Biological insight, literature search and pathway analysis were used to select the most meaningful phenotypic marker for the study.

2.2.5.8 Statistical analyses

Fisher’s exact test (“stats Package” in R version 2.8.0) is a non-parametric test used to assess the statistical significance of the association between two variables in a 2 by 2 contingency
The test is specifically adapted for small sample size, including unequally distributed data among the cells of the table, unlike a Chi-square test. Thus, it was more robust to compare categorical variables from the immunohistochemical staining of each antibody among the various MALT lymphomas with different translocation status.

Student’s t-test is a statistical hypothesis test in which the test statistic follows a Student's t distribution if the null hypothesis is supported. It is most commonly applied when the test statistic would follow a normal distribution if the value of a scaling term in the test statistic were known. It was used to calculate the statistics for comparing reporter assay experiments.

Mann–Whitney U is a non-parametric test for assessing whether two independent samples of observations have equally large values. It is one of the best known non-parametric significance tests. It is identical to performing an ordinary parametric two-sample t-test on the data after ranking over the combined samples. Thus it was used to compare the qRT-PCR data of each transcript between the MALT lymphomas with various translocation statuses.

In all the above statistical methods, the null hypothesis corresponded to the independence of the chosen variables. The null hypothesis was rejected if the probability value of the test of association was less than 0.05, meaning the variables were significantly associated.

### 2.2.6 Quantitative Real-time RT-PCR

#### 2.2.6.1 Primer design

Primers were designed for RT-PCR to validate the expression of candidate genes obtained from expression microarray analysis. All primers were designed using primer3 software (http://frodo.wi.mit.edu/primer3) initially and for difficult regions such as GC rich, Oligos software (Institute of Biotechnology, Finland) was used. Where possible, primers were designed to contain a GC clamp at the 3’ end. All primers were checked for any possible
primer dimer formation or self-complementarity and primer sequences were checked using BLAST tools (http://www.ncbi.nlm.nih.gov/BLAST) from National Centre for Biotechnology Information (NCBI). Details of the primers used for each candidate gene are shown in Table 2.2. Where possible, all gene-specific primer pairs were designed to span exons in order to make them suitable for degraded paraffin-embedded tissue nucleic acid where amplicons of less than 150 base pairs are desired (Liu et al., 2002a).

Table 2.2 - Primers used to investigate candidate genes by qRT-PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Exon targets</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Amplicon size</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>18SrRNA</strong></td>
<td>N/A</td>
<td>Sense</td>
<td>TGACTCAACACGGGAAACC</td>
<td>114bp</td>
<td>NR_003286</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>Anti-sense</td>
<td>TCGCTCCACCAACTAAGAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>N-MALT1</strong></td>
<td>Exon1</td>
<td>Sense</td>
<td>CTCCGCTCAGTTGCTAGA</td>
<td>104bp</td>
<td>NM_006785</td>
</tr>
<tr>
<td></td>
<td>Exon2</td>
<td>Anti-sense</td>
<td>CAACCTTTTTTCACCCATTAACTTCA</td>
<td>80bp</td>
<td>NM_003921</td>
</tr>
<tr>
<td><strong>BCL10</strong></td>
<td>Exon1</td>
<td>Sense</td>
<td>GAAGTGAAGAAGGACCCCTTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon2</td>
<td>Anti-sense</td>
<td>AGATGATCAAAAATGTCTCTCAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NR4A3</strong></td>
<td>Exon6</td>
<td>Sense</td>
<td>TTCCATCAAGTCAAACACTGC</td>
<td>84bp</td>
<td>NM_173198</td>
</tr>
<tr>
<td></td>
<td>Exon7</td>
<td>Anti-sense</td>
<td>AATCCACGAAGGCACTGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD86</strong></td>
<td>Exon1</td>
<td>Sense</td>
<td>GGAATGCTGCTGCTGCTATGC</td>
<td>121bp</td>
<td>NM_006889</td>
</tr>
<tr>
<td></td>
<td>Exon2/3</td>
<td>Anti-sense</td>
<td>AGCACCAGAGAGCAGGAGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD69</strong></td>
<td>Exon2</td>
<td>Sense</td>
<td>CCACACGTCCCCATTTCTCAA</td>
<td>125bp</td>
<td>NM_001781</td>
</tr>
<tr>
<td></td>
<td>Exon3</td>
<td>Anti-sense</td>
<td>TTGGCCACTGATAAGGCAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TLR6</strong></td>
<td>Only has one exon</td>
<td>Sense</td>
<td>ACAAAGTACCACAAAGCTGAAG</td>
<td>100bp</td>
<td>NM_006068</td>
</tr>
<tr>
<td><strong>CCR5</strong></td>
<td>Exon2</td>
<td>Sense</td>
<td>ATCCGTTCCCCTACAAAAGAACTC</td>
<td>100bp</td>
<td>NM_000579</td>
</tr>
<tr>
<td></td>
<td>Exon3</td>
<td>Anti-sense</td>
<td>GCAGGGCTCCGATGATAATAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CCR2A</strong></td>
<td>Exon3</td>
<td>Sense</td>
<td>GGTTTAAATCACTTGGAGGTGT</td>
<td>77bp</td>
<td>NM_001123041</td>
</tr>
<tr>
<td></td>
<td>Exon3</td>
<td>Anti-sense</td>
<td>CACTGGCAATATTAGGGAACAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BCL2</strong></td>
<td>Exon3</td>
<td>Sense</td>
<td>TTGCTTTACGTGGCTCTTTT</td>
<td>94bp</td>
<td>NM_000633</td>
</tr>
<tr>
<td></td>
<td>Exon3</td>
<td>Anti-sense</td>
<td>GAAGACCTGAAAGACAGCCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IRF4</strong></td>
<td>Exon7</td>
<td>Sense</td>
<td>GCCCAACAAACTGGAGAGAG</td>
<td>123bp</td>
<td>NM_002460</td>
</tr>
<tr>
<td></td>
<td>Exon8</td>
<td>Anti-sense</td>
<td>AAGCATAGAGTCACCTGGAAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lactoferrin</strong></td>
<td>Exon1</td>
<td>Sense</td>
<td>GCCACAAAAATGCTTCAATTGG</td>
<td>116bp</td>
<td>NM_002343</td>
</tr>
<tr>
<td></td>
<td>Exon2</td>
<td>Anti-sense</td>
<td>GCCCTGTTCACCAATGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.6.2 Complementary DNA (cDNA) synthesis

cDNA was generated using the Superscript III Reverse Transcriptase kit (Invitrogen, Paisley, UK) with gene specific primers. Typically, the reaction mixture contained: 1μM of each gene-specific anti-sense primer, 1μl of 10mM dNTP, and 200ng of RNA per reverse primer. After incubation at 65°C for 5 minutes the following were added: 2μl of 10 RT buffer, 4μl of 25mM MgCl₂, 2μl of 0.1M DTT, 1μl of RNAse inhibitor and 2U of Superscript III and incubated at 50°C for 50 minutes followed by incubation at 85°C for 15 minutes. RNAse H was added and the mixture incubated at 37°C for 20 minutes to remove the original RNA.

2.2.6.3 Quantitative PCR (qRT-PCR)

The expression of each target transcript was normalised against the level of expression of 18S rRNA in each case. Real-time PCR was performed using the iCycler IQ system (BioRad, Hertfordshire, UK) using SYBR Green I Supermix (BioRad, Hertfordshire, UK).

The conditions for real-time PCR were optimised prior to result collection. The specificity of the primers in producing PCR products was confirmed by melt-curve analysis. The efficiency of each primer was designed to be between 95% and 110% by generating standard curves for each candidate gene from serial dilutions of cDNA produced from tonsillar RNA. All qRT-PCR were carried out in triplicate and standard deviation of less than 0.5 was deemed to be acceptable. The average correlation coefficient value (R²) for each standard curve was above 0.99. The slope of the standard curves was used to determine the exponential amplification and efficiency of the qPCR reaction by the following equation (Tichopad et al., 2003):

\[
\text{Efficiency} = 10^{(-1/\text{slope})}
\]

cDNA was diluted 1/1000 for detection of 18S rRNA as it is highly expressed in most tissues (Ye et al., 2005). All samples were amplified by qRT-PCR on 96-well plates in triplicate using the following parameters: initial denaturation at 95°C for 3 minutes, followed by 45
cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. To ensure minimal intra-plate variation, a calibrator sample consisting of high and low C_t values was used in triplicate on each plate and was used to adjust the C_t. Only those samples that showed specific amplification by melt curve analysis were used for data analysis. The ΔC_t value for each sample was calculated by subtracting the C_t value for 18S from that of the target transcripts. The higher the ΔC_t value, the lower the expression of a transcript and vice versa. For all qRT-PCR, two negative controls were included; a negative control of water instead of RNA that goes through the cDNA synthesis and qPCR step and a negative control of RNA in the qPCR step to ensure that there is no genomic DNA contamination.

2.2.7 Immunohistochemistry

Immunohistochemistry was carried out with the help of Dr. Hongtao Ye, using the protocol described below. Paraffin sections (4μm) were deparaffinised in xylene (BDH, Leicestershire, UK), rehydrated using decreasing concentrations of ethanol (BDH Leicestershire, UK), and incubated in peroxidase blocking solution for 10 minutes to block the endogenous peroxidase activity. Antigen retrieval was carried out prior to immunostaining. Antigen retrieval conditions and primary antibody dilutions are detailed in Table 2.3. Sections were incubated with primary antibody at an 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 sites of antibody binding were visualised with DAB in H_2O_2 (Kem-En-Tec, North Carolina, USA) and counter-stained with Mayer’s haematoxylin. The slides were washed in TBS-Tween, three times for 5 minutes each between all incubation steps and mounted with cover slips for viewing.
Table 2.3 - Immunohistochemistry antibodies and conditions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary antibody</th>
<th>Source</th>
<th>Antigen retrieval method</th>
<th>Conditions for immunohistochemistry or Western blot</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL10 Mouse monoclonal antibody to human BCL10 (clone 151)</td>
<td>Mouse monoclonal antibody to human BCL10 (clone 151)</td>
<td>In house</td>
<td>Microwave in DAKO target retrieval solution pH 6.0 for 25-35 minutes</td>
<td>For immunohistochemistry:primary antibody (1/50), 1 hour at RT; biotinylated rabbit antimouse antibody, 30 mins at RT; peroxidase-conjugated extrAvidin, 30 mins at RT.</td>
</tr>
<tr>
<td>MALT1 Mouse monoclonal antibody to human C-MALT1</td>
<td>Mouse monoclonal antibody to human C-MALT1</td>
<td>In house</td>
<td>Microwave in DAKO target retrieval solution pH 9.9 for 25 minutes</td>
<td>For immunohistochemistry:primary antibody (1/50), 1 hour at RT; biotinylated rabbit antimouse antibody, 30 mins at RT; peroxidase-conjugated extrAvidin, 30 mins at RT.</td>
</tr>
<tr>
<td>BCL2 Mouse monoclonal antibody</td>
<td>Mouse monoclonal antibody</td>
<td>Novocastra</td>
<td>Pressure cooking with citrate buffer pH 6.0 for 3 minutes</td>
<td>For immunohistochemistry: primary antibody (1/120), 1 hr at RT; biotinylated rabbit antimouse antibody, 30 mins at RT; peroxidase-conjugated extrAvidin, 30 mins at RT.</td>
</tr>
<tr>
<td>CD69 Mouse monoclonal antibody</td>
<td>Mouse monoclonal antibody</td>
<td>NeoMarkers</td>
<td>Pressure cooking with 1mM EDTA for 3 minutes</td>
<td>For immunohistochemistry: primary antibody (1/20), 1 hr at RT; followed by polymer amplification system.</td>
</tr>
<tr>
<td>CD86 Sheep CD86 polyclonal antibody</td>
<td>Sheep CD86 polyclonal antibody</td>
<td>R &amp; D</td>
<td>Pressure cooking with citrate buffer pH 6.0 for 3 minutes</td>
<td>For immunohistochemistry: primary antibody (1/60), 1 hr at RT; biotinylated donkey anti-sheep antibody, 30 mins at RT; peroxidase-conjugated extrAvidin, 30 mins at RT.</td>
</tr>
<tr>
<td>IRF4 Mouse monoclonal antibody</td>
<td>Mouse monoclonal antibody</td>
<td>Dako Cytomation</td>
<td>Microwave in DAKO target retrieval solution pH 9.9 for 25 minutes</td>
<td>For immunohistochemistry: primary antibody (1/50), 1 hr at RT; biotinylated rabbit antimouse antibody, 30 mins at RT; peroxidase-conjugated extrAvidin, 30 mins at RT.</td>
</tr>
<tr>
<td>Lactoferrin Rabbit polyclonal antibody</td>
<td>Rabbit polyclonal antibody</td>
<td>Abcam</td>
<td>Pressure cooking with citrate buffer pH 6.0 for 3 minutes</td>
<td>For immunohistochemistry: primary antibody (1/50), 1 hr at RT; biotinylated rabbit antimouse antibody, 30 mins at RT; peroxidase-conjugated extrAvidin, 30 mins at RT.</td>
</tr>
</tbody>
</table>

2.2.8 Expression constructs preparation

2.2.8.1 Modification of pIRES vectors containing HA and FLAG tag sequences

pIRES contains internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one mRNA thus ensuring that the gene of interest is expressed together with a puromycin antibiotic resistance gene in the vector. This reduces the rate of false positives by ensuring that after selection with puromycin, nearly all surviving colonies will stably express the gene of interest (Jang *et al.*, 1988). A forward oligo was designed containing the second half of the *NheI* restriction site, an ATG translation start site and the sequence of the FLAG tag
(5’CTAGCATGGATTACAAGGATGACGACGATAAGG) or HA tag (5’CTAGCATGTACCCATACGATGTTCCAGATTACGCTG). A reverse oligo was designed containing the first half of the EcoRI restriction site, ATG translation start site and the sequence of the FLAG tag (5’AATTCCTTATCGTCGTCATCCTTGTAATCCTAG) or HA tag (5’AATTCAGCGTAATCTGGAACATCGTATGGGTACATG). The two oligos for each tag were annealed by mixing 1μg of each oligo with 100mM sodium chloride, 10mM Tris pH7.5 and 1mM EDTA and boiling for 90 seconds, before being allowed to cool at room temperature for 30 minutes. This generated a piece of double stranded sequence containing the NheI and EcoRI “sticky ends”. The pIRESpuro2 vector (Clontech Laboratories, UK) (Figure 2.4) was digested with NheI and EcoRI. The FLAG or HA tag sequence was ligated into the cut vector with T4 DNA ligase (NEB, UK) to generate FLAG and HA tagged pIRESpuro which were used to generate constructs to make the stable cell lines as described in section 2.2.9.
In order to generate cell lines that mimic the 3 main chromosome translocations; t(11;18), t(14;18) and t(1;14) three constructs were prepared, namely HA-tagged \textit{BCL10}, FLAG-tagged \textit{MALT1} and FLAG-tagged \textit{API2-MALT1} (Table 2.4).

\textbf{Figure 2.4 - pIRESpuro vector.}
### Table 2.4 - Primers used in construct preparation.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer Sequence (sense and antisense in 5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCL10</strong></td>
<td>Lead  <em>EcoRI</em>  TGAT GAATTC  ATGGAGCCCACCAGCCGCGGTC</td>
</tr>
<tr>
<td></td>
<td>Lead  <em>Nol</em>  TGAT GCGGCCGC  TCATTGTGTAACGAGTACG</td>
</tr>
<tr>
<td><strong>MALT1</strong></td>
<td>Lead  <em>BamHI</em>  TGAT GGATCC  ATGTCGCTGATGGGACCCGCTACAG</td>
</tr>
<tr>
<td></td>
<td>Lead  <em>Nol</em>  TGAT GCGGCCGC  TCATTGTGTAACGAGTACG</td>
</tr>
<tr>
<td><strong>API2-MALT1</strong></td>
<td>Lead  <em>EcoRI</em>  TGAT GAATTC  ATGAACATAGTAGAAAACAGCATATC</td>
</tr>
<tr>
<td></td>
<td>Lead  <em>Nol</em>  TGAT GCGGCCGC  TCATTGTGTAACGAGTACG</td>
</tr>
<tr>
<td><strong>pIRES</strong></td>
<td>ATCGATATCTGCGGCCTAGC</td>
</tr>
<tr>
<td></td>
<td>CCAGCAGACTTGTCATGTTATC</td>
</tr>
<tr>
<td><strong>pTRE2</strong></td>
<td>GGATCCTCTAGTGCTACGACG</td>
</tr>
<tr>
<td></td>
<td>TCTAGAGATATCGTCGACAAGC</td>
</tr>
</tbody>
</table>

### 2.2.8.2 Generation of tagged BCL10, MALT1 and API2-MALT1 expression constructs

A standard 25μl PCR reaction was carried out using 2mM MgCl₂, 0.2mM of each dNTP, 0.2μM of each primer and 1 unit of *Pfu* polymerase (Stratagene, Leicestershire, UK) on a Hybaid Px2 thermal cycler (Thermo Scientific, Surrey, UK). The PCR reaction was heated to 94°C for 2 minutes, followed by 30 cycles of denaturation for 45 seconds at 94°C, primer annealing for 45 seconds starting at 55°C and extension for 90 seconds at 72°C with a final extension time of 10 minutes at 72°C. PCR products were visualised by electrophoresis using 1.5% agarose gels.
2.2.8.2.1 Preparation of HA-tagged BCL10

The full length cDNA sequence of BCL10 was generated by RT-PCR of a t(1;14) MALT lymphoma and sub-cloned into TOPO-TA cloning vector (Invitrogen, Paisley, UK). The full length BCL10 cDNA sequence from the TOPO-TA cloning vector was then amplified by PCR using \textit{Pfu} polymerase with sense primer containing an \textit{EcoRI} site and anti-sense primer containing a \textit{NotI} site. The PCR products were cut with \textit{EcoRI} and \textit{NotI} to produce “sticky ends” and cleaned by gel extraction using the QIAQuick Gel Extraction Kit (Qiagen, East Sussex, UK). The HA-tag containing pIRESpuro2 vector was linearised by \textit{PvuI} restriction followed by \textit{EcoRI} and \textit{NotI} restriction and then ligated with the above BCL10 products. The vector sequences were checked for absence of mutations and to ensure that the gene of interest was in frame by sequencing in both directions.

2.2.8.2.2 Preparation of FLAG-tagged API2-MALT1 and FLAG- tagged MALT1

pcDNA3.1 vectors containing full length MALT1 and full length API2-MALT1 (joining nucleotides 1 to 2048 of API2 to nucleotides 814 to 2475 of MALT1 ) (Ho \textit{et al.}, 2005) were kindly provided by Dr. Liza Ho, Department of Clinical Pathology, Geneva, Switzerland. This API2-MALT1 product represents the most common breakpoint found in 93% of t(11;18) MALT lymphomas. Sense primer was designed with the addition of an \textit{EcoRI} restriction site targeting exon1 of the API2 gene while an anti-sense primer was designed with an addition of a \textit{NotI} site targeting exon 17 of the MALT1 gene. For the MALT1 construct, the sense primer has a \textit{BamHI} restriction site targeting exon1 of MALT1 and the anti-sense primer has a \textit{NotI} restriction site targeting exon 17. Both MALT1 and API2-MALT1 sequences were amplified by a standard PCR reaction using \textit{Pfu} polymerase. The PCR products were cut with \textit{EcoRI} (for API2-MALT1) or \textit{BamHI} (for MALT1) and \textit{NotI} to produce “sticky ends” and cleaned
by gel extraction using the QIAQuick Gel Extraction Kit (Qiagen). The FLAG-tag containing pIRESpuro2 vector was cut with EcoRI (API2-MALT1) or BamHI (MALT1) and NotI and the MALT1 or API2-MALT1 ligated with the above product using T4 DNA ligase. The vector sequences were checked for absence of mutations and to ensure that the gene of interest was in frame by sequencing in both directions.

2.2.8.2.3 Sub-cloning of oncogenes into pTRE2

pTRE2 vector (Clontech, Saint-Germain-en-Laye, France) is a response plasmid that can be used to express a gene of interest in Clontech’s Tet-On and Tet-Off cell lines. pTRE2 contains a multiple cloning site (MCS) immediately downstream of the Tet-responsive promoter (Figure 2.5).

![Figure 2.5 - pTRE vector.](image)
cDNA or genes inserted into the MCS will be responsive to the rTA and rtTA regulatory proteins in the Tet-Off and Tet-On systems respectively. \( P_{\text{hcmv}}-1 \) contains the Tet response element (TRE), which consists of seven copies of the 42-bp tet operator sequence (tetO). The TRE is just upstream of the minimal CMV promoter \( (P_{\text{min cmv}}) \), which lacks the enhancer that is part of the complete CMV promoter. Consequently, \( P_{\text{hcmv}}-1 \) is silent in the absence of binding of TetR or rTetR to the TetOs’ sequences. pTRE2 puro and pTRE hygro were used to generate inducible BCL10, MALT1 and API2-MALT1 expression cell lines.

In order to generate the cell lines, each oncogene construct and the pTRE vector was cut using \( NheI \) and \( NotI \) and the insert containing the oncogene in the correct reading frame was ligated into the pTRE vector with T4 DNA Ligase. Before transfection to the cell lines the vector containing the oncogene of interest was linearised by cutting with \( PvuI \) restriction enzyme to facilitate its integration into the host genome.

2.2.8.3 Toll-like receptors expression constructs

Toll-like receptor (TLR) 1, 2 and 6 in pFLAG-CMV-1 vector were a kind gift from Dr. Koichi Kuwano (Department of Bacteriology, Kurume University, Japan) (Shimizu et al., 2007). pFLAG-CMV-1 expression vector is used for expression and secretion of N-terminal FLAG fusion proteins in mammalian cells, its MCS has preprotrypsin which binds the expressed protein to the plasma membrane. All 3 vectors were verified by sequencing from both ends using pFLAG-CMV-1 vector primers.

2.2.8.4 DNA Sequencing

The sequencing reaction was carried out according to Hamoudi et al. (Hamoudi et al., 2002) using the dRhodamine Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). The sequencing reaction was carried out in a 10µl reaction mixture
containing 500ng of plasmid DNA, 4μl dRhodamine, 2μl of 5μM primer and 4μl ultrapure water in a thermal cycler (Hybaid Px2 thermal cycler, Thermo Scientific) using the following cycling protocol; 96°C for 30 seconds, 50°C for 15 seconds and 60°C for one minute, for 25 cycles. The products were precipitated with 3 volumes of ethanol and 0.5 volume of 3M sodium acetate at pH 5.2. DNA pellets were resuspended in 1μl of denaturing blue dye and denatured for 2 minutes at 90°C before loading on polyacrylamide gels (29:1 acrylamide:bisacrylamide ready made 6M urea) (National Diagnostics, Hull, UK), and electrophoresis was carried out using an ABI 377 DNA Sequencer (Applied Biosystems). Data was collected via the associated DNA Sequencer Collection software version 2.0 (Applied Biosystems) and the results were analysed using the BioEdit sequence alignment editor version 7 (Ibis Biosciences, California, USA), Sequence Navigator version 1.1 (Applied Biosystems) and online BLAST tools (http://www.ncbi.nlm.nih.gov/BLAST).

2.2.9 Cell culture

Cells were cultured in RPMI 1640 with 10% Fetal Calf Serum (FCS) and incubated at 37°C, 5% CO₂ in a humidified, automatically controlled incubator. All cells were checked for the presence of mycoplasma by PCR using VenorGeM kit (Minverva Biolabs, Germany).

2.2.9.1 Protocol for freezing cells

The cells to be frozen were centrifuged at 1200rpm for 10 minutes and the pellet was resuspended in Freezing Medium then transferred to cryotubes (Corning, Dorset, UK). The cell suspension was placed at –80°C overnight and then in liquid nitrogen for storage.
2.2.9.2 Protocol for thawing cells

The frozen cells were thawed at 37°C for 5 minutes with gentle shaking, briefly washed with 8ml of warm culture medium and transferred to a sterile tissue culture flask with appropriate culture medium and antibiotics.

2.2.9.3 Cell clot preparation

Around $5 \times 10^5$ cells were centrifuged at 1200rpm for 5 minutes and immediately placed on ice. Five drops of plasma were added to the cell pellet followed by gentle vortexing for 10 seconds. Three drops of thrombin (Diagnostic Reagents Ltd, Oxford, UK) were added and mixed using a plastic pipette in order to clot the cells. The clotted cells were transferred to Speci-wrap paper, which was folded and placed inside a formalin cassette. The cassette was placed inside a formalin jar and left for 4 hours. The samples were processed using a Shandon Excelsior machine to generate formalin fixed paraffin embedded blocks.

2.2.9.4 Transient transfection of cells

Baf-3 and WEHI cell lines were transiently transfected. The required number of cells were centrifuged to form a pellet and washed in PBS (Invitrogen, Paisley, UK). The clean cell pellet was resuspended in 100μl of appropriate Amaxa Cell Line Nucleofector Solution (Lonza, Berkshire, UK). The vector DNA was added at a ratio of around 1μg of DNA to 1 million cells and the cells were transfected using the appropriate programme on a Nucleofector I machine (Lonza, Berkshire, UK). The transfected cells were then routinely cultured. Nucleofector Solution T with programme T16 was used to transfect BJAB, Jurkat T-Rex and Baf-3 cells whereas solution V with programme T30 was used to transfect WEHI cells. These conditions were optimized by transfection of pmaxGFP and subsequent analysis.
of the transfected cells by Flow cytometry using FACSCalibur flow cytometer (Beckton Dickson, Oxford, UK).

2.2.9.5 Generation of stably inducible MALT lymphoma associated oncogenes cells

pTRE-BCL10, pTRE-MALT1, pTRE-API2-MALT1 and pTRE wild type were used to generate stable inducible cell lines, whilst pIRESpuro-BCL10 and pIRESpuro wild type were used to generate stable cell lines. 20µg of each different construct were linearised by digestion with PvuI, purified by phenol/chloroform extraction and resuspended in 20µl of sterile water to a final concentration of 1µg/µl. For each transfection, 2 ×10⁷ of BJAB, BJAB-TetON or Jurkat TetON (T-REx) cells were washed with PBS, resuspended in 700µl of PBS, mixed with 20µl of the linearised construct and transferred to a 4mm Gene Pulser cuvette. After 5-10 minutes on ice, electroporation was carried out using the Gene Pulser Apparatus (BioRad) at 250 volts and 950µFD (a time between 15 and 20 milliseconds indicates successful electroporation). The samples were incubated on ice for 10 minutes and transferred to a Falcon tube containing 20ml of RPMI 1640 medium with 10% FCS, 30% conditioned medium containing 1mM sodium pyruvate, 50µM α-thioglycerol and 20nM bathocuproindisulfonic acid disodium salt (reagents needed for cell growth and survival) (Brielmeier et al., 1998). Various concentrations of transfected cells (1.5 ×10⁵, 3.75 ×10³ and 1.25 ×10³ cells) were seeded in 96-well plates for 24 hours in medium containing 4µg/ml puromycin. Clones were checked after 10 to 14 days, and transferred to a 24-well culture plate, then to a small 10ml flask. The cloned cells were subjected to Western blot analysis to check their oncogene expression. Induction of the oncogene expression was carried out by adding 1µg/ml of doxycycline.
A total of 14 cell lines expressing MALT lymphoma associated oncogenes were generated and they included:

**Inducible expression cell lines**

1) BJAB Tet-ON pTREpuro wild type (control)  
2) BJAB Tet-ON pTREpuro FLAG-MALT1  
3) BJAB Tet-ON pTREpuro FLAG-API2-MALT1  
4) BJAB Tet-ON pTREhygro HA-BCL10  
5) BJAB Tet-ON pTREhygro HA-BCL10 and pTREpuro FLAG-MALT1  
6) BJAB Tet-ON pTREhygro HA-BCL10 and pTREpuro FLAG-API2-MALT1  
7) Jurkat Tet-ON (T-REx) pTREpuro wild type (control)  
8) Jurkat Tet-ON (T-REx) pTREpuro FLAG-MALT1  
9) Jurkat Tet-ON (T-REx) pTREpuro FLAG-API2-MALT1  
10) Jurkat Tet-ON (T-REx) pTREhygro HA-BCL10  
11) Jurkat Tet-ON (T-REx) pTREhygro HA-BCL10 and pTREpuro FLAG-MALT1  
12) Jurkat Tet-ON (T-REx) pTREhygro HA-BCL10 and pTREpuro FLAG-API2-MALT1

**Stable expression cell lines**

13) pIRESpuro wild type (control)  
14) pIRESpuro HA-BCL10

**2.2.10 Protein analysis**

**2.2.10.1 Protein extraction from whole cell lysate**

Cells were harvested by centrifugation and cell pellets were washed twice in 1×PBS then lysed in Triton Lysis Buffer, on ice for 30 minutes. The cell lysate was centrifuged at 7300rpm for 20 minutes at 4°C, and the supernatant was transferred to a new tube and quantified (2.2.10.4). Samples were stored at -80°C until required.

**2.2.10.2 Preparation of protein homogenate from frozen tissue**

Frozen tissue sections were homogenised in Triton lysis buffer by gentle pipetting at 4°C for 30 minutes. The lysate was centrifuged at 7300rpm for 20 minutes at 4°C and the supernatant
was transferred to a new tube and quantified as described in 2.2.10.4. Samples were stored at -80°C until required.

2.2.10.3 Protein extraction from nuclear and cytoplasmic fractions

Preparation of nuclear and cytoplasmic protein extracts from cells was carried using CelLytic NuCLEAR Extraction kit (Sigma, Dorset, UK) according to the manufacturer’s instructions. The procedure for nuclear protein extraction is to allow cells to swell with hypotonic buffer. The cells are then disrupted and cytoplasmic fraction is removed, and the nuclear proteins are released from the nuclei by a high salt buffer.

2.2.10.4 Protein quantitation

The concentrations of extracted proteins were measured using the Quant-iT Protein Assay kit (Invitrogen, Paisley, UK) according to the manufacturer’s instructions with the Victor 3 illuminometer (Perkin Elmer, Warrington, UK).

2.2.10.5 Antibodies used for functional and cellular work

Monoclonal and polyclonal antibodies used in the functional studies described in this thesis are shown in Table 2.5.
Table 2.5 - Primary and secondary antibodies used in co-immunoprecipitation and Western blotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source (Part No.)</th>
<th>Dilution</th>
<th>Application</th>
<th>Type</th>
<th>Host species and (primary/secondary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-MALT</td>
<td>In-house</td>
<td>1:500</td>
<td>Co-IP and Western blotting</td>
<td>Monoclonal</td>
<td>Mouse - primary</td>
</tr>
<tr>
<td>BCL10</td>
<td>In-house</td>
<td>1:10,000</td>
<td>Co-IP and Western blotting</td>
<td>Monoclonal</td>
<td>Mouse - primary</td>
</tr>
<tr>
<td>HA</td>
<td>Sigma-Aldrich</td>
<td>1:10,000</td>
<td>Western blotting</td>
<td>Monoclonal</td>
<td>Mouse - primary</td>
</tr>
<tr>
<td>FLAG</td>
<td>Sigma-Aldrich</td>
<td>1:10,000</td>
<td>Western blotting</td>
<td>Monoclonal</td>
<td>Mouse - primary</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sigma-Aldrich</td>
<td>1:1,000,000</td>
<td>Western blotting</td>
<td>Monoclonal</td>
<td>Mouse - primary</td>
</tr>
<tr>
<td>TLR6</td>
<td>Abcam (ab62569)</td>
<td>1:10,000</td>
<td>Western blotting</td>
<td>Polyclonal</td>
<td>Rabbit - primary</td>
</tr>
<tr>
<td>IκBβ</td>
<td>Cell Signaling Technology (9248)</td>
<td>1:20,000</td>
<td>Western blotting</td>
<td>Polyclonal</td>
<td>Rabbit - primary</td>
</tr>
<tr>
<td>IκBa</td>
<td>Santa Cruz Biotechnology (sc-203)</td>
<td>1:10,000</td>
<td>Western blotting</td>
<td>Polyclonal</td>
<td>Rabbit - primary</td>
</tr>
<tr>
<td>IκBe</td>
<td>Cell Signaling Technology (9249)</td>
<td>1:10,000</td>
<td>Western blotting</td>
<td>Polyclonal</td>
<td>Rabbit - primary</td>
</tr>
<tr>
<td>p65</td>
<td>Santa Cruz Biotechnology (sc-7151)</td>
<td>1:10,000</td>
<td>Western blotting</td>
<td>Polyclonal</td>
<td>Rabbit - primary</td>
</tr>
<tr>
<td>p50/p105</td>
<td>Santa Cruz Biotechnology (sc-7178)</td>
<td>1:10,000</td>
<td>Western blotting</td>
<td>Polyclonal</td>
<td>Rabbit - primary</td>
</tr>
<tr>
<td>p100/p52</td>
<td>Cell Signaling Technology (3017)</td>
<td>1:10,000</td>
<td>Western blotting</td>
<td>Polyclonal</td>
<td>Rabbit - primary</td>
</tr>
<tr>
<td>c-Rel</td>
<td>Cell Signaling Technology (4727)</td>
<td>1:10,000</td>
<td>Western blotting</td>
<td>Polyclonal</td>
<td>Rabbit - primary</td>
</tr>
<tr>
<td>RelB</td>
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<td>1:10,000</td>
<td>Western blotting</td>
<td>Polyclonal</td>
<td>Rabbit - primary</td>
</tr>
<tr>
<td>CD3</td>
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<td>Western blotting</td>
<td>Monoclonal</td>
<td>Mouse - primary</td>
</tr>
<tr>
<td>CD28</td>
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<td>Western blotting</td>
<td>Monoclonal</td>
<td>Mouse - primary</td>
</tr>
<tr>
<td>Donkey anti-rabbit HRP-conjugated</td>
<td>GE Healthcare (NA934)</td>
<td>1:20,000</td>
<td>Western blotting</td>
<td>Not applicable</td>
<td>Donkey - secondary</td>
</tr>
<tr>
<td>Sheep anti-mouse HRP-conjugated</td>
<td>GE Healthcare (NA931)</td>
<td>1:20,000</td>
<td>Western blotting</td>
<td>Not applicable</td>
<td>Sheep - secondary</td>
</tr>
</tbody>
</table>
2.2.10.6 Western blot

Protein lysates were mixed with 4X NuPAGE (Invitrogen, Paisley, UK) loading buffer containing 4% β-mercaptoethanol (BDH, Leicestershire, UK), heated to 99°C for 5 minutes and left on ice for 2 minutes then separated on NuPAGE gels (Invitrogen, Paisley, UK) by electrophoresis at 120V for 90 minutes using SeeBlue size standard (Invitrogen, Paisley, UK).

The proteins were transferred to an Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, USA) using XCell II Blot Module (Invitrogen, Paisley, UK) running at 30V for 90 minutes.

The membrane was first placed in TBST for 10 minutes followed by 4% milk/TBST for 1 hour whilst shaking to block non-specific binding then probed with primary antibody in 4% milk/TBST with 10% FCS overnight at 4°C. After washing in TBST, the membrane was incubated with an anti-species specific (according to Table 2.5) Horseradish Peroxidase (HRP)-conjugated secondary antibody in 4% milk/TBST for a minimum of 1 hour at room temperature. Finally, the protein was detected by the addition of an Immobilon HRP Substrate Peroxide Solution (Millipore Corporation, Watford, UK) and Immobilon HRP Substrate Luminol Reagent (Millipore Corporation, Watford, UK) for 5 minutes. The HRP chemiluminescent reaction resulted in the catalysed oxidation of luminol by peroxide. Oxidised luminol emits light as it decays. The chemiluminescence was detected by exposing an X-ray film to the membrane.
2.2.10.7 Stripping of Western blot

Bound antibody was removed from membranes by immersion in Stripping Buffer at 62°C for 2 hours, washed in TBST and placed in 4% milk/TBST for 1 hour, then probed and detected with the appropriate antibodies (Table 2.5) as described in section 2.2.10.6. The loading control antibody, β-actin was used on stripped blots. Also in section 5.4.5, IκBα were stripped and re-probed with IκBβ which was stripped and re-probed with β-actin.

2.2.10.8 Co-immunoprecipitation

Co-immunoprecipitation was carried out using the Anti-HA immunoprecipitation kit (Sigma, Dorset, UK) according to the manufacturer’s instructions. Some of the immunoprecipitated protein was then subjected to silver staining using the ProteoSilver Stain kit (Sigma, Dorset, UK) and dried for long term storage using the DryEase kit (Invitrogen, Paisley, UK) according to the manufacturer’s instructions.

2.2.11 Dual luciferase reporter assays

2.2.11.1 NF-κB luciferase reporter assay

NF-κB activity was measured in tissue culture cells using the Dual Luciferase Reporter Assay System (Promega, Southampton, UK). Cells were resuspended in Nucleofector Solution (Lonza, Berkshire, UK) and transfected with the pRL-TK (Promega, Southampton, UK) and pNF-κB-luc (Stratagene, Leicestershire, UK) reporter vectors, in combination with a pIRES vector containing the oncogene of interest or the pIRES control vector by electroporation or using an inducible stable cell using the Amaxa Nucleofector I machine (Lonza, Berkshire, UK). Luciferase is expressed from pNF-κB-luc after binding of NF-κB subunits to the vector. pRL-TK contains a cDNA encoding Renilla luciferase which was used as a control for
normalisation of the data. These cells were incubated at 37°C and 5% CO2 overnight and then stimulated as follows:

1) BaF3 cells with 10μg/ml LPS (Sigma, Dorset, UK) for 6 hours

2) Jurkat cells with 1μg/ml anti-CD3 (Sigma, Dorset, UK) and 1μg/ml anti-CD28 (Sigma, Dorset, UK) for 6 hours. For TLRs experiments, cells were stimulated with 10μg/ml LPS (Sigma, Dorset, UK) for 6 hours

3) WEHI cells with 10μg/ml LPS (Sigma, Dorset, UK) for 6 hours or 0.1μg/ml CD40 ligand (with 1μg/ml CD40 enhancer) or 10μg/ml anti-IgM

The cells were centrifuged to form a pellet and washed in 1X PBS before resuspension in 1X Passive Lysis Buffer (Promega, Southampton, UK). The cell suspension was rotated for 30 minutes to allow the cells to lyse completely. 15μl of whole cell extract was added to 45μl of Luciferase Assay Reagent 2 (LAR) (Promega, Southampton, UK) and the level of fluorescence produced by the firefly luciferase was detected with the Victor 3 luminometer (Perkin Elmer, Warrington, UK). The reaction was stopped with the addition of 45μl of Stop and Glo (Promega, Southampton, UK) and the fluorescence level produced by the Renilla luciferase was measured by the luminometer. The reading for firefly luciferase divided by the reading for Renilla luciferase gave the NF-κB activity.

2.2.11.2 AP-1 luciferase reporter assay

AP-1 activity was measured in transiently transfected BJAB cells using the Dual Luciferase Reporter Assay System (Promega) similar to that described for NF-κB luciferase reporter assay in section 2.2.11.1. The AP-1 vector used was the AP-1 cis-Reporting system (Stratagene, Leicestershire, UK). pRL-TK was used as the normalisation vector.
CHAPTER 3 – Characterisation of the gene expression profiles of MALT lymphoma with and without chromosome translocation

3.1 Introduction

As detailed in chapter 1, t(11;18)(q21;q21)/API2-MALT1, t(1;14)(p22;q32)/IGH-BCL10 and t(14;18)(q32;q21)/IGH-MALT1 are specifically associated with MALT lymphoma, occurring at variable frequencies in different anatomic sites. Although these translocations involve different oncogenes, their resultant oncogenic products commonly target the canonical NF-κB activation pathway. Expression of API2-MALT1, BCL10 and MALT1 (in the presence of BCL10) induces the activation of the NF-κB transcriptional factor, which transactivates a number of genes important for cellular proliferation and survival. The capacity of these oncogenic products to activate NF-κB is believed to be the crucial molecular mechanism underlying their oncogenic activity. Nonetheless, over-expression of these oncogenic products alone is insufficient for malignant transformation as both Eµ-API2-MALT1 and Eµ-BCL10 transgenic mice developed splenic marginal zone hyperplasia, but not lymphoma (Morris, 2001; Sagaert et al., 2006b; Li et al., 2009). In vitro assay showed that expression of both API2-MALT1 and MALT1 enhanced by the CD40 induced NF-κB activation in B cells (Ho et al., 2005). It is possible that such immunological stimulation is operational in MALT lymphoma. The extent and the nature of potential cooperation between MALT lymphoma associated oncogenic products and immune surface receptor signalling remain to be investigated.

There are important differences in the clinical and histological presentations between MALT lymphomas with and without chromosome translocation. Clinically, gastric MALT
lymphomas with t(11;18) or t(1;14) are significantly associated with advanced clinical stages (Liu et al., 2001b; Ye et al., 2006) and resistance to *H. pylori* eradication (Isaacson et al., 2004; Liu et al., 2001a; Liu et al., 2002b; Ye et al., 2006). Histologically, t(11;18) positive MALT lymphomas appear to be more monotonous, lacking apparent transformed blasts (Okabe et al., 2003). These distinct clinico-pathological characteristics indicate the presence of significant differences in molecular mechanisms between MALT lymphomas with and without chromosome translocation. In order to investigate this and understand further the molecular mechanism of MALT lymphomagenesis, the transcriptional profiles of a well characterised series of MALT lymphomas with different chromosome translocation status were determined.

### 3.2 Aims of the study

1) To characterise gene expression profiling of MALT lymphoma with and without chromosome translocation

2) To identify the molecular mechanisms involved in translocation positive MALT lymphoma

3) To identify the molecular mechanisms involved in translocation negative MALT lymphoma
3.3 Experimental design

3.3.1 Case selection

Fresh frozen tissues from 24 well-characterised MALT lymphomas (case numbers 1 to 24 in Table 2.1), 7 nodal follicular lymphomas (FL) and 8 nodal mantle cell lymphomas (MCL) were used for this part of the gene expression microarray analysis. The MALT lymphoma cases included 9 positive for t(11;18)(q21;q21)/API2-MALT1 (8 from stomach and 1 from lung), 4 positive for t(1;14)(p22;q32)/BCL10-IGH or t(1;2)(p22;p11)/BCL10-IGκ (3 from stomach and 1 from lung), 2 positive for t(14;18)(q32;q21)/IGH-MALT1 (1 from liver and 1 from ocular adnexa) and 9 cases negative for all known MALT lymphoma associated chromosome translocations (all from stomach with two having nuclear BCL10 staining). MALT lymphomas case number 25 was excluded from the analysis due to the fact that it was a mixture of MALT and diffuse large B-cell lymphoma and case number 26 was excluded due to the fact that it is the only fresh frozen case of t(3;14(p14;q32)/IGH-FOXP1 and a minimum of two cases are needed to obtain useful mechanistic information. However both cases were included in the phenotypic marker analysis carried out as described in chapter 6.

The percentage of tumour cells are estimated on haematoxylin & eosin stained sections and where necessary, crude microdissection was carried out to enrich tumour cells and ensure that a sample containing at least 70% tumour cells was used for expression microarray analysis.

3.3.2 RNA quality check using Agilent nanochip

Total RNA, complementary RNA and fragmented RNA were subjected to quality control assessment using the Agilent nanochip. The criteria for passing the quality control were as follows: for total RNA both the 18S and 28S bands should be around 2000 and 4000bp
respectively, for cRNA the mean of the size distribution should be around 1000bp and for fragmented RNA, the mean of the size distribution should be around 60 to 100bp.

3.3.3 Validation of the in house linear RNA amplification protocol
To validate the fidelity of the linear amplification protocol developed in house, RNA from a fresh frozen tissue specimen of a t(11;18) positive MALT lymphoma was amplified, then labelled and hybridised to HG-U133A chip according to the method described in section 2.2.5. Pearson correlation comparing the values of all 22283 probes from the amplified RNA and total RNA of HG-U133A GeneChip from the same case was carried out.

3.3.4 Unsupervised clustering analysis
After normalisation and non-specific filtering, the 24 MALT lymphoma cases derived from HG-U133A and HG-U133B, 7 FL and 8 MCL on the HG-U133plus2 platform were subjected to unsupervised clustering using Pearson correlation coefficient and average linkage as the similarity measure and clustering algorithm respectively within Genespring GX 7.3.1. Unsupervised clustering was also carried out within the MALT lymphoma group to investigate whether there are distinct subsets.

3.3.5 Characterisation of gene expression features of MALT lymphoma with and without chromosome translocation
Normalisation followed by non-specific filtering was used to derive set of variant probes which were collapsed and used in interrogating 4395 pathways using GSEA (Section 2.2.5.4). The same set of probes was subjected to one-way ANOVA multivariate analysis to derive a set of differentially expressed genes between MALT lymphoma with and without chromosomal translocation (Section 2.2.5.5). The differentially expressed genes were subjected to Gene Ontology (GO) analysis using hypergeometric testing (Section 2.2.5.6).
Results from both GSEA and GO analysis were compared to characterise the features of MALT lymphoma with and without translocation.

### 3.4 Results

Details of the bioinformatics analysis including choice of algorithms and software are given in Appendix I. In this section, results of the analysis of gene expression microarray of MALT lymphoma are presented.

#### 3.4.1 RNA quality control

In each case the total RNA, complimentary RNA and fragmented RNA were of good quality as shown by electrophoresis on RNA nanochip (Figure 3.1).

![Figure 3.1 - Typical good quality data of total, complementary and fragment RNA on RNA nanochip.](image)
3.4.2 Comparison of total RNA with amplified RNA

Total RNA and linearly amplified RNA from the same t(11;18) positive MALT lymphoma fresh frozen specimen were hybridised on a HG-U133A GeneChip. The Pearson correlation coefficient ($R^2$) between the two RNA preparations was 97.2% with $p < 0.001$ (Figure 3.2), indicating that linearly amplified RNA faithfully amplifies the same distribution of the starting total RNA.

![Figure 3.2 - Correlation between total and amplified RNA from the same fresh frozen specimen of t(11;18) positive MALT lymphoma. Red line is the line of best fit through the points with a correlation coefficient of 97.2%.

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3.4.3 Validation of microarray data using biological controls

As expected, the hybridisation signal of the N-terminal MALT1 probe was the highest in the two cases of t(14;18) positive MALT lymphoma, whereas the signals of two C-terminal MALT1 probes were much higher in the cases with t(11;18) than those with t(1;14) or without translocation (Figure 3.3). Cyclin D1 was highly expressed in MCL, while CD10 and BCL6 showed the highest expression in FL. The CD10 expression in MALT lymphoma was low, suggesting the presence of minimal reactive B cells components in the fresh frozen materials used for expression microarray. Despite the fact that \( BCL2 \) t(14;18)(q32;q21)/\( IgH-BCL2 \) translocation is the genetic hallmark of follicular lymphoma, it was not in the hallmark genes probably because \( BCL2 \) was shown to be highly expressed in translocation positive MALT lymphomas (Section 3.4.6.1). Overall, the expected results from the biological controls indicated that the labelling, hybridisation and basic bioinformatics analysis were successfully carried out.

![Figure 3.3 - Expression of hallmark genes in MALT lymphoma, FL and MCL.](image)

Correlation of known expression levels of genes with different lymphoma types. N-MALT1 probe (210017_at) detects only wild type MALT1 transcripts, while C-MALT (210018_x_at & 208309_s_at) probes detect both MALT1 and API2-MALT1 transcripts. The chromosome translocation status of MALT lymphoma is indicated by colour. On the heatmap, red represents up-regulated genes and green down-regulated genes, with the scale showed at the bottom of the figure.
3.4.4 Unsupervised clustering between MALT, MCL and FL defines MALT lymphoma as a distinct entity

The methodology used to combine MALT lymphoma HG-U133A with FL and MCL HG-U133plus2 is summarised (Figure 3.4).

Figure 3.4 - Summary of bioinformatics strategy to combine FL and MCL with MALT microarray GeneChips.
Normalisation is followed by non-specific filtering and exclusion of housekeeping probes for both U133AB and U133plus2. The probes from the two platforms are combined using median polish transformation and the probes used for unsupervised clustering.

Analysis of HG-U133A and B and plus2 were done separately then a median polish step was used to normalise the data from the two different platforms giving rise to 6822 probes. These were used for unsupervised clustering of the 24 MALT lymphomas, 7 follicular lymphoma (FL) and 8 mantle cell lymphoma (MCL).
Figure 3.5 - MALT lymphoma shows distinct gene expression profiles from follicular lymphoma and mantle cell lymphoma using unsupervised hierarchical clustering. After normalisation and filtering across both U133A&B and U133plus2 sets, a set of 6822 probes were obtained and used for unsupervised hierarchical clustering analysis. FL: follicular lymphoma; MCL: mantle cell lymphoma; NEG: translocation negative MALT lymphoma. The chromosome translocation status of MALT lymphoma, FL and MCL are indicated by colour. On the heatmap, red represents up-regulated genes and green down-regulated genes, with the scale shown at the bottom of the figure.
MALT lymphomas were clustered as a single branch, irrespective of their origin from different anatomic sites or chromosome translocation. Within the MALT lymphoma group, the chromosome translocation status appeared to have little impact on the hierarchical clustering as translocation positive MALT lymphomas were intermingled with translocation negative cases (Figure 3.5). The two translocation negative cases with BCL10 nuclear staining clustered together (Figure 3.5).

In order to derive the functional groups in Figure 3.5, the gene tree was ordered according to biological processes defined by gene ontology, and the gene clusters enriched for a particular biological process in a lymphoma subtype as shown by hypergeometric testing as indicated. The gene sets for cell cycle (GO:7049) and regulation of progression through cell cycle (GO:74) were highly enriched in MCL, while those for immune response (GO:6955) and immune response to biotic stimulus (GO:9607) were enriched in FL. The gene sets for immune response to external biotic stimulus such as pest, pathogen or parasite (GO:9613) and cellular defence response (GO:6968) were highly enriched in MALT lymphoma.
3.4.5 Unsupervised clustering confirms overlapping features in gene expression profiling between MALT lymphoma with and without chromosome translocation

The strategy used to compare MALT lymphoma with and without translocation is summarised in Figure 3.6.

**Figure 3.6 - Summary of bioinformatics strategy used to compare MALT lymphoma with and without chromosome translocations.**

Normalisation is followed by non-specific filtering and exclusion of housekeeping probes. The probe sets were used to determine differentially expressed genes using multivariate analysis, this is followed by pathway analysis using GSEAs and GO and in house datamining. Candidates from this analysis were validated further by qRT-PCR and immunohistochemistry (IHC).
Figure 3.7 - Unsupervised hierarchical clustering of MALT lymphoma with different translocation status.

After normalisation and filtering, a set of 8107 probes were obtained and used for unsupervised hierarchical clustering analysis. MALT lymphomas with and without chromosome translocation are intermingled together. The chromosome translocation status of MALT lymphoma is indicated by different colour scheme. On the heatmap, red represents up-regulated genes and green down-regulated genes, with the scale shown at the bottom of the figure.
The result of unsupervised clustering of MALT lymphoma cases again showed that the chromosome translocation status had little impact on the hierarchical clustering as translocation positive MALT lymphoma cases were intermingled with translocation negative cases (Figure 3.7). The results suggest an overlap in gene expression profile and hence molecular mechanisms between MALT lymphoma with and without chromosome translocation. The clustering showed that the two translocation negative cases with BCL10 nuclear staining clustered together with a group consisting of predominantly translocation positive MALT lymphomas (Figure 3.7). In order to investigate this further, supervised clustering with 733 probes was carried out on the four t(1;14) and nine translocation negative cases including two with nuclear BCL10 staining. The 733 probes were derived by applying one-way ANOVA multivariate analysis across MALT lymphoma with and without translocation groups to the 8107 probes obtained after normalisation and non-specific filtering. The two translocation negative MALT lymphomas with nuclear BCL10 expression still clustered with the four cases with t(1;14) (Figure 3.8).
Figure 3.8. Supervised hierarchical clustering of MALT lymphoma with different translocation status.
After normalisation and filtering, a set of 733 probes were obtained and used for supervised hierarchical clustering analysis. MALT lymphoma cases without chromosome translocation but with nuclear BCL10 staining are clustering with t(1;14) positive cases. The chromosome translocation status of MALT lymphoma is indicated by different colour scheme. On the heatmap, red represents up-regulated genes and green down-regulated genes, with the scale shown at the bottom of the figure.
3.4.6 Characterisation of the gene expression profiles of MALT lymphoma with and without chromosome translocation

To gain further insights into the potential difference in molecular mechanisms between MALT lymphomas with and without chromosome translocation, GSEA and absolute GSEA were performed on 4395 gene sets covering various cellular pathways, biological processes or molecular functions derived from in house analysis and molecular signature database (Subramanian et al., 2005) as described in Appendix I.III. Results of GSEA was cross validated using GO hypergeometric testing as described in section 2.2.5.6. Summary of the methodology used for GSEA (Figure 3.9).

Figure 3.9 - Summary of bioinformatics strategy used for GSEA on MALT lymphoma with and without chromosome translocation.
Normalisation is followed by non-specific filtering and exclusion of housekeeping probes. The probes were then combined and mapped to the RMA normalised data. The probes are collapsed and used as an expression dataset in GSEA together with the relevant geneset and phenotype.
GSEA identified a total of 51 gene sets (not including those with very general terms) with a statistical significance (P<0.05 and FDR<0.05) and were thus differentially over-represented between MALT lymphomas with and without translocation. Remarkably, the NF-κB target gene signature was the most differentially enriched gene set (p < 0.0001 and FDR < 0.0001) (Table 3.1).
Table 3.1 - Gene sets differentially over-represented between MALT lymphoma with and
without chromosome translocation.
Gene Set

Norm Nominal P
FWER P
FDR q value
Tag % Gene %
ES
value
value

Size

Source

ES

NF-κB target genes

167

Appendix III.I for details

0.531

1.826

0.0001

0.0001

0.0000

0.455

0.249

positive regulation of IKK and NF-κB cascade

62

GO_0043123

0.466

1.541

0.0088

0.0446

0.1235

0.274

0.157

Inflammation

73

Immunome database

0.590

1.865

0.0000

0.0065

0.0076

0.548

0.257

Cellular cation homeostasis

56

GO:0030003

0.519

1.842

0.0000

0.0116

0.0710

0.429

0.264

Inflammatory response

166

GO_0006954

0.530

1.775

0.0022

0.0116

0.0128

0.482

0.267

Response to other organism

47

GO:0051707

0.575

1.829

0.0000

0.0124

0.0840

0.404

0.183

Locomotory behaviour

54

GO:0007626

0.601

1.800

0.0000

0.0153

0.1210

0.556

0.257

Chemokine

128

Immunome database

0.510

1.725

0.0022

0.0163

0.0293

0.367

0.175

Immune responses

119

GO_0006955

0.517

1.648

0.0067

0.0185

0.0266

0.429

0.259

Defence response

135

GO:0006952

0.511

1.757

0.0000

0.0210

0.1870

0.467

0.274

B-cell activation

26

GO_0042113

0.623

1.779

0.0000

0.0222

0.0117

0.423

0.152

Innate immune response

52

GO_0045087

0.506

1.595

0.0065

0.0311

0.0724

0.519

0.301

Cell adhesion molecules

86

HSA04514

0.456

1.812

0.0000

0.0321

0.0480

0.326

0.209

Lymphocyte activation

77

GO_0046649

0.525

1.647

0.0292

0.0322

0.0501

0.338

0.152

Response to biotic stimulus

71

GO:0009607

0.507

1.687

0.0000

0.0378

0.3500

0.423

0.262

Cellular homeostasis

74

GO:0019725

0.448

1.692

0.0000

0.0380

0.3310

0.351

0.264

HumoralImmunity

65

Immunome database

0.545

1.526

0.0550

0.0395

0.1312

0.477

0.257

Inflammatory response

66

GO:0006954

0.534

1.662

0.0065

0.0420

0.4020

0.455

0.274

TLR sigalling pathway from GeneGo

33

GeneGO database

0.520

1.616

0.0088

0.0421

0.0674

0.606

0.366

Cellular immunity

47

Immunome database

0.542

1.537

0.0214

0.0456

0.1269

0.489

0.262

Chemokine

29

GO_0042379

0.638

1.519

0.0338

0.0463

0.1438

0.655

0.257

Cell adhesion

50

GO:0016337

0.483

1.634

0.0058

0.0482

0.4860

0.42

0.259

Rceptor signalling protein activity

54

GO:0005057

0.529

2.061

0.0000

0.0015

0.0010

0.574

0.355

Regulation of MAP kinase activity

41

GO:0043405

0.493

1.979

0.0000

0.0026

0.0140

0.537

0.292

Regulation of protein kinase activity

95

GO:0045859

0.455

1.894

0.0000

0.0066

0.0340

0.453

0.316

Regulation of kinase activity

96

GO:0043549

0.450

1.875

0.0000

0.0079

0.0420

0.448

0.316

Activation of MAPK activity

22

GO:0000187

0.549

1.845

0.0020

0.0118

0.0690

0.591

0.287

MAPKKK cascade

61

GO:0000165

0.443

1.847

0.0000

0.0121

0.0660

0.623

0.44

Transmembrane receptor protein tyrosine kinase signalling pathway

48

GO:0007169

0.465

1.832

0.0000

0.0125

0.0820

0.354

0.213

JNK cascade

28

GO:0007254

0.444

1.727

0.0000

0.0282

0.2550

0.5

0.343

MAPK signalling pathway

152

HSA04010

0.442

1.826

0.0000

0.0383

0.0380

0.467

0.343

G protein coupled receptor protein signalling pathway

125

GO:0007186

0.424

1.670

0.0061

0.0412

0.3870

0.32

0.256

Protein kinase cascade

184

GO:0007243

0.421

1.672

0.0021

0.0414

0.3830

0.413

0.317

JAK STAT cascade

20

GO:0007259

0.559

1.661

0.0151

0.0419

0.4070

0.55

0.3

Peptide GPCRS

25

C2 genmapp

0.609

1.829

0.0020

0.0457

0.0200

0.32

0.107

G protein signalling coupled to cyclic nucleotide second messenger

36

GO:0007187

0.502

1.644

0.0083

0.0475

0.4560

0.278

0.154

Regulation of cell growth

29

GO:0001558

0.632

2.014

0.0000

0.0013

0.0060

0.448

0.205

Negative regulation of growth

24

GO:0045926

0.681

2.019

0.0000

0.0016

0.0060

0.458

0.183

Positive regulation of cell proliferation

87

GO:0008284

0.500

1.958

0.0000

0.0027

0.0140

0.483

0.308

Regulation of cell proliferation

179

GO:0042127

0.465

1.813

0.0000

0.0136

0.1030

0.43

0.308

Negative regulation of cell cycle

49

GO:0045786

0.548

1.690

0.0000

0.0370

0.3380

0.51

0.282

Cell cycle arrest

36

GO:0007050

0.564

1.640

0.0022

0.0480

0.4680

0.472

0.223

Cation homeostasis

57

GO:0055080

0.509

1.820

0.0000

0.0131

0.0970

0.421

0.264

Reproductive process

59

GO:0022414

0.516

1.803

0.0000

0.0150

0.1150

0.424

0.257

Ion homeostasis

63

GO:0050801.

0.481

1.748

0.0021

0.0218

0.2010

0.54

0.377

mitochondrion organization and biogenesis

30

GO:0007005

0.551

1.690

0.0000

0.0376

0.3360

0.4

0.23

Positive regulation of transcription

80

GO:0045941

0.414

1.681

0.0000

0.0379

0.3620

0.325

0.282

Angiogenesis

22

GO:0001525

0.563

1.648

0.0206

0.0474

0.4400

0.364

0.137

Cyclic nucleotide mediated signalling

36

GO:0019935

0.502

1.644

0.0083

0.0475

0.4560

0.278

0.154

Protein processing

26

GO:0016485

0.511

1.642

0.0019

0.0475

0.4600

0.385

0.26

Carbohydrate biosynthetic process

20

GO:0016051

0.542

1.636

0.0200

0.0484

0.4810

0.45

0.294

NF-κB related

Inflammation and immune responses

MAPK pathway realted

Cell cycle related

Others

ES: Enrichment score; Norm ES: Normalised ES; FDR: False discovery rate; FWER: Family wise-error rate; Tag%: the percentage of gene tags before (for
positive ES) or after (for negative ES) the peak in the running enrichment score; Gene %: the percentage of genes in the gene list before (for positive ES) or after
(for negative ES) the peak in the running enrichment score.

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3.4.6.1 NF-κB target genes are significantly differentially expressed between MALT lymphoma with and without chromosome translocation

GSEA showed that the expression of the NF-κB target genes was enhanced in both translocation positive and negative cases but with a different signature for each group. A subset of the NF-κB target genes was over-represented in translocation positive MALT lymphomas, while another subset was enriched in translocation negative MALT lymphomas. Leading edge analysis showed that 20 core genes accounted for the significant enrichment in translocation positive MALT lymphomas including \textit{CCR2A}, \textit{BCL2}, \textit{TFEC}, \textit{CD69}, \textit{BCL10}, \textit{TLR6}, \textit{REL}, \textit{LTB}, \textit{IRF4}, \textit{CCR7}, \textit{CCR5} and \textit{MAP4K1} (Figure 3.10A). Similarly, 70 core genes underscored the significant enrichment in translocation negative MALT lymphomas and \textit{CXCL5}, \textit{PTGIS2}, \textit{NR4A3}, \textit{CCL11}, \textit{PTGIS}, \textit{IL8}, \textit{MMP3}, \textit{CXCL2}, \textit{CXCL1} and \textit{CD86} were the top 10 of this biologically significant gene subset (Figure 3.10A). The differential expression of these genes was clearly seen in the heatmap illustration of Figure 3.10A.
Figure 3.10 - Gene set enrichment analysis (GSEA) of NF-κB target genes in MALT lymphomas with and without chromosome translocation.

(A) NF-κB target genes and (B) Immune response set.

Left panel shows the distribution of NF-κB target genes according to their rank position. Right panel shows heatmap illustration of their expression between MALT lymphoma with and without chromosome translocation. The top 20 leading edge core genes are shown. trans -ve: translocation negative MALT lymphoma; trans +ve: translocation positive MALT lymphoma.

3.4.6.2 Other gene sets differentially enriched between MALT lymphoma with and without chromosome translocation

As there was considerable overlap among some of the gene sets that were associated with the related cellular pathways, biological processes or molecular functions, they were grouped according to their involvement in the NF-κB activation pathway, inflammation/immune responses, MAPK pathways, cell cycle and others as shown in Table 3.1. Leading edge analysis was carried out to identify the core subset genes that underscored the significant
enrichment and were thus most likely to be biologically important. The NF-κB target genes were frequently represented in each of these core subset genes, often on top end of the list particularly in the gene sets related to inflammation/immune responses (Figure 3.11).

![Graph showing the proportion of NF-κB target genes in the leading edge core set.](image)

**Figure 3.11 - Presence of high proportion of NF-κB target genes in the leading edge core set of various gene sets related to inflammation and immune responses.**

Leading edge analysis of the positive regulation NF-κB cascade group (GO:43123) shows CD40 as one of the leading edge genes enriched in translocation positive MALT lymphoma and leading edge analysis of immune response genes (GO:6955) (Figure 3.10B) shows CD1D enriched in the translocation positive MALT lymphoma group.
3.4.6.3 Gene Ontology annotations of genes differentially expressed between MALT lymphoma with and without chromosome translocation confirms findings by GSEA

The strategy for analysis of differentially expressed genes between MALT lymphoma with and without chromosome translocation by Gene Ontology (GO) annotation is shown in Figure 3.12.

Figure 3.12 - Summary of bioinformatics strategy used for Gene Ontology analysis on MALT lymphoma with and without chromosome translocation.
After normalisation and non-specific filtering, the data is mapped to MAS5 normalised values and a one-way ANOVA was applied to it and reduced further by calculated fold change. The final probes are entered into GO to obtain any relevant pathways.

To gain further insights into the molecular pathways affected by the oncogenic products of MALT lymphoma associated chromosome translocations, genes that were significantly over or under-expressed in translocation positive MALT lymphoma in comparison with translocation negative cases were identified using the one-way ANOVA test. These
differentially expressed genes were further filtered using 2-fold change as a threshold. Ninety six genes were over-expressed in translocation positive MALT lymphoma, while 174 genes were under-expressed in these cases, i.e. over-expressed in translocation negative cases. TLR6, CCR2, BCL2, CD1D and CD69 identified by GSEA were most highly expressed in translocation positive MALT lymphoma, and conversely IL8, NR4A3 and CXCL5 were on the most highly expressed in translocation negative MALT lymphoma as shown in Table (Appendix III.II).

To further assess the biological implications of the differential gene expression in MALT lymphoma with and without chromosome translocation, the representation of gene ontology (GO) terms in the above gene sets that were over or under-represented in translocation positive MALT lymphoma were measured using hypergeometric tests (Falcon et al., 2007). Among the genes over-expressed in translocation positive MALT lymphoma, the GO terms relating to NF-κB pathway activation, defense/immune responses and CCR signalling were significantly over-represented (Table 3.2). While among the genes under-expressed in translocation positive MALT lymphoma, i.e. over-expressed in translocation negative cases, the GO terms relating to chemotaxis, inflammatory response and CCR signalling were significantly over-represented (Table 3.2). These findings from the analysis of differentially expressed genes between MALT lymphomas with and without chromosome translocation reinforce the GSEA results described in sections 3.2.6.1 and 3.2.6.2.
### Table 3.2 - Representation of gene ontology terms in over-expressed genes in MALT lymphoma with and without chromosome translocation.

<table>
<thead>
<tr>
<th>Gene ontology term</th>
<th>GO category</th>
<th>Genes in Category</th>
<th>% of Genes in Category</th>
<th>Genes in List in Category</th>
<th>% of Genes in List in Category</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene ontology term over-represented in translocation positive MALT lymphoma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:6952: defense response</td>
<td>Biological process</td>
<td>1306</td>
<td>8.029</td>
<td>17</td>
<td>23.61</td>
<td>4.18E-05</td>
</tr>
<tr>
<td>GO:9607: response to biotic stimulus</td>
<td>Biological process</td>
<td>1361</td>
<td>8.367</td>
<td>17</td>
<td>23.61</td>
<td>7.02E-05</td>
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<td>GO:7243: protein kinase cascade</td>
<td>Biological process</td>
<td>474</td>
<td>2.914</td>
<td>8</td>
<td>11.11</td>
<td>0.00114</td>
</tr>
<tr>
<td>GO:7249: I-kappaB kinase/NF-kappaB cascade</td>
<td>Biological process</td>
<td>881</td>
<td>5.416</td>
<td>11</td>
<td>15.28</td>
<td>0.00161</td>
</tr>
<tr>
<td>GO:48522: positive regulation of cellular process</td>
<td>Biological process</td>
<td>1028</td>
<td>6.32</td>
<td>12</td>
<td>16.67</td>
<td>0.00173</td>
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<tr>
<td>GO:48518: positive regulation of biological process</td>
<td>Biological process</td>
<td>1187</td>
<td>7.297</td>
<td>13</td>
<td>18.06</td>
<td>0.00193</td>
</tr>
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<td>GO:42981: regulation of apoptosis</td>
<td>Biological process</td>
<td>525</td>
<td>3.227</td>
<td>8</td>
<td>11.11</td>
<td>0.00218</td>
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<td>GO:43067: regulation of programmed cell death</td>
<td>Biological process</td>
<td>531</td>
<td>3.264</td>
<td>8</td>
<td>11.11</td>
<td>0.00234</td>
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<td>GO:7250: activation of NF-kappaB-inducing kinase</td>
<td>Biological process</td>
<td>17</td>
<td>0.105</td>
<td>2</td>
<td>2.778</td>
<td>0.00252</td>
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<tr>
<td>GO:43122: regulation of I-kappaB kinase/NF-kappaB cascade</td>
<td>Biological process</td>
<td>133</td>
<td>0.818</td>
<td>4</td>
<td>5.556</td>
<td>0.00286</td>
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<tr>
<td>GO:43123: positive regulation of I-kappaB kinase/NF-kappaB cascade</td>
<td>Biological process</td>
<td>141</td>
<td>0.867</td>
<td>4</td>
<td>5.556</td>
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<td>GO:16493: C-C chemokine receptor activity</td>
<td>Molecular function</td>
<td>22</td>
<td>0.128</td>
<td>2</td>
<td>2.857</td>
<td>3.58E-03</td>
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<tr>
<td>GO:19957: C-C chemokine binding</td>
<td>Molecular function</td>
<td>155</td>
<td>0.901</td>
<td>4</td>
<td>5.714</td>
<td>3.67E-03</td>
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<td><strong>Gene ontology term over-represented in translocation negative MALT lymphoma</strong></td>
<td></td>
<td></td>
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<td>GO:46870: cadmium ion binding</td>
<td>Molecular function</td>
<td>10</td>
<td>0.0582</td>
<td>5</td>
<td>3.378</td>
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<td>GO:1664: G-protein-coupled receptor binding</td>
<td>Molecular function</td>
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<td>0.39</td>
<td>7</td>
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<td>1.71E-06</td>
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<tr>
<td>GO:5507: copper ion binding</td>
<td>Molecular function</td>
<td>79</td>
<td>0.459</td>
<td>7</td>
<td>4.73</td>
<td>5.24E-06</td>
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<tr>
<td>GO:42379: chemokine receptor binding</td>
<td>Molecular function</td>
<td>58</td>
<td>0.337</td>
<td>6</td>
<td>4.054</td>
<td>1.03E-05</td>
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<td>GO:8009: chemokine activity</td>
<td>Molecular function</td>
<td>58</td>
<td>0.337</td>
<td>6</td>
<td>4.054</td>
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<td>GO:5102: receptor binding</td>
<td>Molecular function</td>
<td>902</td>
<td>5.246</td>
<td>21</td>
<td>14.19</td>
<td>3.05E-05</td>
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<td>Biological process</td>
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<td>0.984</td>
<td>8</td>
<td>5.882</td>
<td>5.96E-05</td>
</tr>
<tr>
<td>GO:6935: chemotaxis</td>
<td>Biological process</td>
<td>160</td>
<td>0.984</td>
<td>8</td>
<td>5.882</td>
<td>5.96E-05</td>
</tr>
<tr>
<td>GO:7155: cell adhesion</td>
<td>Biological process</td>
<td>978</td>
<td>5.012</td>
<td>21</td>
<td>15.44</td>
<td>6.11E-05</td>
</tr>
<tr>
<td>GO:6954: inflammatory response</td>
<td>Biological process</td>
<td>263</td>
<td>1.617</td>
<td>10</td>
<td>7.335</td>
<td>7.33E-05</td>
</tr>
<tr>
<td>GO:6817: phosphate transport</td>
<td>Biological process</td>
<td>124</td>
<td>0.762</td>
<td>7</td>
<td>5.147</td>
<td>8.17E-05</td>
</tr>
<tr>
<td>GO:9611: response to wounding</td>
<td>Biological process</td>
<td>568</td>
<td>3.492</td>
<td>15</td>
<td>11.03</td>
<td>8.17E-05</td>
</tr>
<tr>
<td>GO:7610: behaviour</td>
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<td>465</td>
<td>2.859</td>
<td>13</td>
<td>9.559</td>
<td>1.43E-04</td>
</tr>
<tr>
<td>GO:4295: trypsin activity</td>
<td>Molecular function</td>
<td>14</td>
<td>0.0814</td>
<td>3</td>
<td>2.027</td>
<td>2.12E-04</td>
</tr>
<tr>
<td>GO:5125: cytokine activity</td>
<td>Molecular function</td>
<td>295</td>
<td>1.716</td>
<td>10</td>
<td>6.757</td>
<td>2.41E-04</td>
</tr>
</tbody>
</table>

**Category:** the name of the category within the ontology; **Genes in Category:** the total number of genes in the genome that have been assigned to the category; **% of Genes in Category:** the percentage of genes in this category assigned to this GO term; **Genes in List in Category:** the total number of genes that are present both in the selected gene list and in the category; **% of Genes in List in Category:** the percentage of genes of this category in the selected gene list that are assigned to this GO term; **P-value** (hypergeometric p-value): this is a measure of the statistical significance of the overlap, i.e. the likelihood that it is a coincidence that this many genes were in both the gene list and the category. Only the top 15 are shown.
3.4.7 Pathway analysis of molecules identified by gene expression microarray and potentially important in MALT lymphoma pathogenesis

Basic pathway analysis was carried out to investigate the pathways linked to the molecules derived from the microarray analysis.

For translocation positive MALT lymphoma, immune receptor molecules were TLR6, CD69 and CD1D, chemokine receptors were CCR2, CXCR4, CCR5, CCR6 and CCR7 and the apoptosis inhibitor BCL2. For translocation negative MALT lymphoma, pro-inflammatory cytokines were IL8 and IL1B and co-stimulatory molecules were CD86, CD28 and ICOS. MALT lymphoma with and without translocation were analysed separately using Ingenuity Pathway Analysis.
Figure 3.13 - Pathway analysis of molecules derived from MALT lymphoma microarray analysis.
Pathway analysis of molecules involved in (A) translocation negative MALT lymphoma and (B) translocation positive MALT lymphoma. The molecules highlighted with grey colour are derived from expression microarray results. A solid line indicates a direct interaction while a dashed line indicates an indirect interaction.

Pathway analysis is highly dependent on existing literature knowledge and experimental data. There is not much understood about the mechanism of translocation positive MALT lymphoma, interestingly the pathway analysis showed a possible link to the JAK3 and STAT5 pathway most likely via NF-κB inducible genes (Figure 3.13B). However, translocation negative MALT lymphoma molecules also show involvement of the NF-κB pathway especially via IL8, IL1B and CD86 as shown in (Figure 3.13A).
3.5 Discussion

By investigating the transcriptional profile, the present study demonstrated that MALT lymphoma is characterised by a distinct expression profile in comparison with FL and MCL, in line with the recent study by Chng et al. (Chng et al., 2009). Although there was considerable overlap in the gene expression profiles between MALT lymphomas with and without chromosome translocation as demonstrated by unsupervised clustering analyses, there were important differences in the expression of NF-κB target genes between these subgroups. Systematic GSEA of various molecular pathways and biological processes, showed that the NF-κB target genes are the most differentially over-represented gene set between MALT lymphomas with and without chromosome translocation, followed by those related to inflammation, cellular homeostasis and immune responses. Importantly, several of these molecular pathways or biological processes also lead to NF-κB activation. These findings were confirmed by independent analyses of differentially expressed genes between MALT lymphomas with and without chromosome translocation using hypergeometric tests of Gene Ontology groups. Our observations provide several novel insights into the molecular mechanisms of both translocation positive and negative MALT lymphomas that potentially explain their different clinical and histological presentations.

3.5.1 Molecular mechanism of translocation positive MALT lymphoma

In comparison with translocation negative MALT lymphoma, GSEA and leading edge analysis revealed a common core subset genes that were enriched and over-expressed in translocation positive MALT lymphoma and a high proportion of these genes are NF-κB target genes involving multiple related biological processes or molecular pathways. The most
significant ones included immune receptors such as TLR6, TLR7, CD40, CD83, CD1D and CD69, chemokine receptors such as CCR2, CXCR4, CCR6 and CCR7, the apoptosis inhibitor BCL2, positive regulators of the NF-κB pathway such as REL (a component of NF-κB transcription factor), LTβ (a powerful proinflammatory cytokine) and molecules involved in MAPK pathways (Figure 3.10). All these molecules are involved in promoting tumour cell survival and proliferation either directly or indirectly. Among these, the over-expression of the above immune surface receptors is particularly interesting as this enhances the interaction between tumour cells and their microenvironment, which is known to be critical for MALT lymphoma development.

For example, TLRs are innate immune receptors critical for recognising the conserved microbial structures known as pathogen associated molecular patterns (PAMP), and are capable of activating the NF-κB pathway (Shimizu et al., 2007). CD40, CD83 and CD69 are activation markers and co-stimulating molecules which are also likely to play a role in NF-κB activation. BCL2 is a classic apoptosis inhibitor, and may account for the prolonged survival of tumour cells.

The expression of these molecules was thus further investigated in independent cohorts of MALT lymphomas and discussed in the next chapter.

3.5.2 Molecular mechanism of translocation negative MALT lymphoma

In contrast to translocation positive MALT lymphoma, translocation negative cases were characterised by expression of a strong inflammatory gene signature. GSEA and leading edge analysis also revealed common core subset genes involving several related biological processes or molecular pathways, which were enriched and over-expressed in translocation negative MALT lymphoma. These included pro-inflammatory cytokines such IL8, IL1β and
LTA, molecules involved in B- and T- cell interaction such as CD86, CD28 and ICOS, several chemokine and chemokine receptors, TLR2 and NR4A3 (also known as MINOR, or NOR1) (Figure 3.10).

IL8, IL1β and LTA are the hallmark of a pro-inflammatory cytokine profile in response to *H. pylori* infection. IL8 is critical for neutrophil infiltration and activation, while IL1β and LTA induce gastrin release, inhibit acid secretion and promotes apoptosis of epithelial cells, thus affecting *H. pylori* colonisation (McNamara *et al.*, 2008). The finding of up-regulation of these pro-inflammatory cytokines in translocation negative MALT lymphomas, all from the stomach, indicates the presence of active *H. pylori* infection. In line with this, the expression of a number of chemokines and chemokine receptors was enriched in translocation negative gastric MALT lymphoma. This may reflect the trafficking and retention of various immune cells in response to an active *H. pylori* infection. In keeping with this, translocation negative gastric MALT lymphomas show more frequently an increased number of blast cells than translocation positive cases (Okabe *et al.*, 2003).

Most importantly, GSEA showed that the expression of the surface molecules involved in B- and T- cell interaction, namely CD86, CD28 and ICOS was enriched in translocation negative gastric MALT lymphoma, and this was accompanied by unregulated *IL10* expression, a well known outcome of ICOS stimulation (van Berkel *et al.*, 2006).

### 3.5.3 Nuclear BCL10

Both supervised and unsupervised clustering analysis showed that translocation negative MALT lymphoma cases with nuclear BCL10 staining clustered together with cases harbouring t(1;14)/BCL10-IGH (Figure 3.8). It was recently demonstrated that nuclear expression of BCL10 is significantly associated with resistance to gastric MALT lymphoma
to *H. pylori* eradication (Kuo *et al.*, 2004). In ocular adnexal MALT lymphoma, nuclear BCL10 seemed to correlate with a shorter treatment failure-free survival (Franco *et al.*, 2006), although this could not be confirmed by other groups (Vejabhuti *et al.*, 2005). Gallardo *et al.* (Gallardo *et al.*, 2006) found a significant association between nuclear BCL10 expression and a higher risk of extra-cutaneous involvement in primary cutaneous marginal zone B-cell lymphoma, whereas Li *et al.* (Li *et al.*, 2003) reported that nuclear BCL10 was associated with the development of a locally aggressive course. Based on these observations, a role for nuclear BCL10 in lymphomagenesis is suggested, although the precise pathological significance remains unclear. Based on its interaction with transcription factor IIB and its ability to activate transcription as a fusion protein linked to the Gal4-DNA-binding domain in HeLa cells, Liu *et al.* (Liu *et al.*, 2004d) suggested a role for BCL10 as a transcriptional activator, whereas Yeh *et al.* (Yeh *et al.*, 2006) found nuclear BCL10 to be involved in the transcriptional activity of NF-κB following TNF-α signalling in MCF7 cells. The current microarray analysis further enforces a role for nuclear BCL10 in the development of MALT lymphoma. However, further functional studies on the role of BCL10 in the nucleus are needed to determine which pathways and interacting molecules are involved.

### 3.5.4 Summary and conclusion

In summary, gene expression microarray studies showed that MALT lymphoma is a distinct entity, but with overlapping gene expression signatures between MALT lymphoma with and without chromosomal translocation. In both supervised and unsupervised clustering analysis, translocation negative MALT lymphoma with nuclear BCL10 expression clustered with translocation positive MALT lymphoma cases suggesting a role of nuclear BCL10 in MALT lymphomagenesis. Gene set enrichment analysis and Gene Ontology hypergeometric
analysis, showed differences in the molecular pathways involved in MALT lymphoma with and without chromosome translocation. Many of these differences were attributed to different involvement of the of NF-κB target genes. Leading edge analysis identified a number of important molecules that are potentially important for the pathogenesis of MALT lymphoma with or without chromosome translocation which will be investigated and discussed in the next chapter.
CHAPTER 4 – Validation of the genes identified by expression microarray of MALT lymphoma with and without chromosome translocation using qRT-PCR and immunohistochemistry

4.1 Introduction

Gene expression microarray study of MALT lymphoma with and without chromosome translocation identified a number of genes preferentially over-expressed in either subset (Chapter 3). Translocation positive MALT lymphomas were highly enriched in the expression of $\text{CD69}$, $\text{CCR2A}$, $\text{CCR5}$, $\text{TLR6}$, $\text{BCL2}$ and $\text{IRF4}$ (also known as $\text{MUM1}$) in addition to the already characterised genes $\text{BCL10}$, specific to t(1;14), and $\text{MALT1}$ specific to t(11;18) and t(14;18). In contrast, translocation negative MALT lymphomas were highly enriched in the expression of $\text{CD86}$ and $\text{NR4A3}$. The differential expression of these molecules was thought to play an important role in the pathogenesis of the respective MALT lymphoma subgroups. Thus, it was necessary to validate further their differential expression in MALT lymphomas with and without chromosome translocation in independent cohorts by qRT-PCR and immunohistochemistry and Western blotting.

4.2 Aims of the study

To validate the molecules derived from gene expression microarray studies in a large cohort of MALT lymphoma specimens using qRT-PCR and immunohistochemistry or Western blotting and to correlate the transcript and protein expression with the chromosomal translocation status.
4.3 Experimental design

4.3.1 Case selection

A total of 73 cases of MALT lymphoma with known translocation status were investigated. These included 8 cases with t(1;14), 18 cases with t(11;18), 9 cases with t(14;18), 38 cases without MALT lymphoma associated chromosomal translocations including 10 with nuclear and 28 with cytoplasmic BCL10 expression. These MALT lymphomas originated from the stomach (48), liver (2), ocular adnexa (4), small intestine (4) and lung (15).

4.3.2 Validation of gene expression by qRT-PCR

The mRNA expression level of the genes differentially expressed between MALT lymphomas with and without chromosome translocation (CD69, CCR2A, CCR5, TLR6, BCL2, IRF4, CD86, NR4A3, BCL10 and N-terminal part of MALT1) was investigated by qRT-PCR. RNA was extracted from microdissected tumour cells of FFPE MALT lymphoma specimens and treated with Turbo DNase to remove genomic DNA (Section 2.2.3.1). Where possible, primer pairs were designed to span exons to prevent amplification of any residual genomic DNA and to target up to 150bp (Table 2.2) (Section 2.2.6.1), and were thus suitable for FFPE tissues. This was the case for all genes except TLR6 which had only one coding exon and CCR2A which shared the sequence with CCR2B except for a small region. For those two primer pairs within exons, the negative control of the RNA that has undergone Turbo DNAase was checked to confirm the amplification was that of the cDNA rather than genomic DNA. All primers were checked on tonsillar RNA to ensure that they gave specific PCR products and ran on gels to ensure they amplified the expected size. The results are presented as ΔCT values calculated as (ΔCT = T_{Reference Gene} − T_{Test Gene}), so the higher the value, the lower the transcript expression and vice versa. Unsupervised clustering on subset
of the genes across all samples using Ward similarity measure and average linkage was carried out to determine the discriminatory power of those genes on the MALT lymphoma cases. Both BCL2 and IRF4 were excluded from clustering as their qRT-PCR was carried out on a smaller cohort of MALT lymphomas (25 and 24 cases respectively) due to lack of sufficient tissue in the remaining MALT lymphomas. The results of the qRT-PCR were correlated with the known chromosomal translocation established by conventional cytogenetics, interphase FISH and RT-PCR, as described in Chapter 3. The Mann-Whitney U Test determined whether there were statistically significant differences in the expression of particular transcripts between groups of cases with different chromosome translocation status.

4.3.3 BCL2, CD69, IRF4, MALT1, BCL10 and CD86 immunohistochemistry

Immunohistochemistry was carried out by Dr. Hongtao Ye on all cases using the BCL2, CD69, IRF4 and CD86 antibodies. Data on BCL10 and MALT1 expression from same cases of MALT lymphoma subgroups was obtained from a previous immunohistochemistry based study (Ye et al., 2005) (Figure 1.6). The immunohistochemical conditions, including antigen retrieval, antibody dilution and incubation times, were systematically optimised as described in Chapter 2 (Table 2.3). The immunostaining was evaluated independently by two assessors (Professor Ming Du and Dr. Hongtao Ye) and scored according to the percentage of positive cells in a section (<30%, 30-70%, >70%) and the intensity of staining (weak, moderate, strong). Mr. Rifat Hamoudi helped in section cutting, slide preparation, scoring of immunohistochemistry under the guidance of Dr. Hongtao Ye, collation of the immunohistochemical data and analysis. Cases were considered positive if 30% or more tumour cells were stained. Cases with scoring discrepancies between the assessors were
reviewed. The results of the immunostaining were then correlated with the known chromosome translocation status as determined by qRT-PCR and interphase FISH (detailed in Chapter 3). Differences in the proportion of cases staining positive for each of the six proteins across the different chromosome translocation groups were assessed by the Fisher’s exact test.

4.3.4 Validation of TLR6 using Western blotting

Imunohistochemistry of TLR6 antibody did not yield satisfactory results thus, western blotting was carried out using the TLR6 antibody on 5 fresh frozen t(11;18) positive and 9 translocation negative MALT lymphoma cases with one FL case and one Jurkat cell line used as control. Protein extraction and Western blotting were carried out as detailed in Chapter 2. The filter was stripped and re-probed with β-actin used as a loading control, followed by stripping and re-probing with MALT1 (97 kD) as an integrity control to ensure all archival samples used were adequate for investigation using TLR6 (92 kD). TLR6 and β-actin bands were quantified using AIDA (Advanced Image Data Analyzer, version 4.18) (Raytest, Straubenhardt, Germany), and normalised TLR6 expression was calculated as the ratio of TLR6 / β-actin. Differences in the cases with different TLR6 expression across the various chromosomal translocation groups were assessed by Fisher’s exact test.
4.4 Results

4.4.1 Correlation of mRNA expression of CD69, CCR2A, CCR5, TLR6, BCL2, IRF4, CD86, NR4A3, BCL10 and MALT1 genes in MALT lymphoma with and without chromosomal translocation

BCL10 transcript expression was highest in the t(1;14) group (p = 0.001) whereas 5' end of MALT1 (encoding the N-terminus of MALT1) was highest in the t(14;18) group (p = 0.02) reflecting the pattern seen in the gene expression microarray data and confirming the validity of the qRT-PCR methodology (Figure 4.1). In keeping with the results of the gene expression microarray study, CCR5 was highly expressed in t(1;14) and TLR6, CCR2A, BCL2, CD69 and IRF4 were significantly highly expressed in t(1;14) and t(11;18) positive cases in comparison with translocation negative cases (Figure 4.1), whereas CD86 and NR4A3 were significantly highly expressed in translocation negative cases in comparison with translocation positive cases (Figure 4.1).
Figure 4.1 - Validation of gene expression in MALT lymphoma with and without chromosome translocation by real-time quantitative RT-PCR.

This was performed in triplicate using RNA samples extracted from tumour cells microdissected from paraffin-embedded tissue sections. Asterisk indicates statistically significant differences between various translocation positive groups and translocation negative group by Mann-Whitney non-parametric statistical test. The medians are indicated by horizontal bars in the rectangular boxes. Error bars show the standard deviation of the results in each group. Neg: translocation negative MALT lymphoma. High ΔCt values reflect low transcript expression and vice versa.
Figure 4.2 - Unsupervised hierarchical clustering of qRT-PCR data on MALT lymphoma cases with various translocation status.

(A) Unsupervised clustering heatmap of the qRT-PCR genes that are differentially expressed between translocation positive and negative MALT lymphoma. Red colour indicates high expression and green low expression. (B) dendrogram of the MALT lymphoma cases shown in the heatmap of part A.
The unsupervised clustering analysis did not show clear segregation between MALT lymphoma with and without translocation. Nevertheless, the dendrogram (Figure 4.2 B) showed 3 main groups; the far right had mainly translocation negative MALT lymphomas (16 negative and 3 t(11;18) positive), the middle group contained a mixture of translocation positive and negative MALT lymphomas but with nuclear BCL10 expression (12 positive, 5 negative with nuclear and 1 cytoplasmic BCL10 expression), and the far left contained mainly translocation positive MALT lymphomas (9 positive and 2 negative one with nuclear and the other with cytoplasmic BCL10 expression) (Figure 4.2B). Both the heatmap and dendrogram, revealed on the left, one MALT lymphoma negative case with cytoplasmic BCL10 expression labelled “NEG_Cyto_12” had high expression for all 8 genes but low expression for TLR6 (Figure 4.2 A and B).
4.4.2 Comparison of protein expression of CD69, BCL2, CD86 and IRF4, MALT1 and BCL10 in MALT lymphoma with and without chromosomal translocation using immunohistochemistry

A

B

Figure 4.3 - Immunohistochemistry of BCL2, CD69, CD86 and IRF4.
(A) BCL2, CD69 and CD86 immunohistochemistry in MALT lymphomas with and without chromosome translocation.
(B) Summaries of immunohistochemistry showing that BCL2 and CD69 are more strongly and homogeneously expressed in translocation positive than translocation negative MALT lymphoma (p < 6.9 × 10^-5, p < 2.2 × 10^-4 respectively by Fisher’s exact test), while CD86 is more strongly expressed in translocation negative than translocation positive MALT lymphoma (p < 6.4 × 10^-7 by Fisher’s exact test). IRF4 is typically expressed in activated B cells and cells showing plasma cell differentiation.

FC: follicle centre; Trans-ve: translocation negative.
Table 4.1 - Correlation of BCL2, CD69 and CD86 expression in MALT lymphoma with and without chromosome translocation.

<table>
<thead>
<tr>
<th>Marker</th>
<th>MALT lymphoma translocation</th>
<th>No. of cases</th>
<th>Expression in &gt;70% cells</th>
<th>Expression in 30-70% cells</th>
<th>Expression in &lt;30% cells</th>
<th>Negative or expression in &lt;30%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strong or moderate staining</td>
<td>Weak staining</td>
<td>Strong or moderate staining</td>
<td>Weak staining</td>
</tr>
<tr>
<td>BCL2</td>
<td>Positive</td>
<td>28</td>
<td>27 (96%)</td>
<td>1 (4%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>26</td>
<td>3 (12%)</td>
<td>2 (8%)</td>
<td>6 (23%)</td>
<td>5 (19%)</td>
</tr>
<tr>
<td>CD69</td>
<td>Positive</td>
<td>29</td>
<td>28 (97%)</td>
<td>-</td>
<td>1 (3%)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>41</td>
<td>12 (29%)</td>
<td>-</td>
<td>12 (29%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>CD86</td>
<td>Positive</td>
<td>33</td>
<td>-</td>
<td>-</td>
<td>1 (3%)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>26</td>
<td>3 (12%)</td>
<td>2 (8%)</td>
<td>6 (23%)</td>
<td>5 (19%)</td>
</tr>
</tbody>
</table>

BCL10 and MALT1 staining were carried out as part of the study by Ye et al. (Ye et al., 2005) and discussed in section 1.3.1.4. CD69, BCL2, CD86 and IRF4 protein expressions were further investigated by immunohistochemistry in MALT lymphomas (Table 4.1). IRF4, known to be expressed in activated B cells and plasma cells, was highly expressed in the neoplastic plasma cells (confirmed by immunoglobulin light chain staining) of t(1;14) positive MALT lymphoma, which were prominent in the lamina propria. Since BL10, MALT1 and IRF4 gave staining patterns similar to that reported in the literature, both BCL2 and CD69 showed homogeneous and strong expression in 97% (28/29) of both t(11;18) and t(1;14) translocation positive MALT lymphoma cases, whereas their expression was heterogeneous and much weaker expression in 29% (12/41) of translocation negative cases (Figure 4.3 and Table 4.1). For example, BCL2 was strongly expressed in at least 70% tumour cells in 96% (27/28) of translocation positive cases but only in 12% (3/26) translocation negative cases. In contrast, CD86 showed heterogeneous and strong expression in translocation negative MALT lymphomas (Figure 4.2) but weak or negative staining in
most (97% or 32/33) translocation positive MALT lymphomas. Statistical analysis using the Fisher’s exact test showed that BCL2, CD69 and CD86 were significantly different between translocation positive and negative MALT lymphoma.

4.4.3 Comparison of protein expression of TLR6 in MALT lymphoma with and without chromosome translocation using Western blotting

All MALT lymphoma cases expressed a MALT1 protein of the expected molecular weight (approximately 90 kDa) indicating that the archival protein extracts are adequate for Western blot analysis (Figure 4.4A). TLR6 was highly expressed in translocation positive MALT lymphoma, but at low levels in translocation negative cases (Figure 4.4). Interestingly, case M6 which is a translocation negative MALT lymphoma with nuclear BCL10 showed relatively high expression of TLR6 (ratio of TLR6/actin = 0.67) comparable to t(11;18) positive cases (Figure 4.4B).
Figure 4.4 - TLR6 expression in MALT lymphoma with and without chromosomal translocation using Western blot analysis.

(A) Western blot showing that TLR6 is highly expressed in translocation positive MALT lymphoma, but at low levels in translocation negative cases. M: MALT lymphoma; M6 and M7 are translocation negative MALT lymphoma with nuclear BCL10 staining; FL: follicular lymphoma. Jurkat cell line used as control.

(B) Graphical representation of the ratio of TLR6 expression across MALT lymphoma cases with and without chromosomal translocation.
4.5 Discussion

*MALT1* was highly expressed in cases with t(11;18) and t(14;18) translocation. *TLR6, CCR2A, CD69, BCL2* and *IRF4* were highly expressed in cases with t(11;18) translocation. *BCL10* and *CCR5* were highly expressed in cases with t(1;14) translocation. *CD86* and *NR4A3* were highly expressed in translocation negative MALT lymphomas. This was the case for 20 MALT lymphoma cases that were subjected to microarray analysis, qRT-PCR and immunohistochemistry for MALT1, BCL10, BCL2, CD69 and CD86. Thus this shows that mRNA correlates with protein expression for the above proteins and validates both methodologies as well as providing further evidence of the reliability of the gene expression microarray results in this thesis.

Translocation negative MALT lymphomas were characterised by high expression of CD86 and N4RA3. CD86 is a surface molecule involved in B- and T- cell interaction and thus might play a role in response to *H. pylori* eradication. In line with this finding, a previous study showed significantly higher CD86 expression in gastric MALT lymphomas that responded to *H. pylori* eradication when compared with those resistant to the therapy (66% VS 10%) (de Jong *et al.*, 2001). Though the chromosome translocation status in these cases is not available, it is most likely that the cases that responded to *H. pylori* were translocation-negative (Liu *et al.*, 2002b). Although residual reactive follicles may be present and contribute to the high CD86 expression in translocation negative cases, the over-expression of CD86 in tumour cells was clearly demonstrated by microdissection, qRT-PCR and immunohistochemistry. Taken together, these findings suggest that there is an active immune response to *H. pylori* infection in translocation-negative gastric MALT lymphoma, and this most likely underscores the tumour cell survival and expansion and thus determines their response to *H. pylori* eradication.
NR4A3 is another molecule significantly enriched and over-expressed in translocation negative MALT lymphoma. NR4A3 is a member of the nerve growth factor-1B (NGF1B, or NR4A1 or Nur77) subfamily of nuclear orphan receptors. In T cells, NGF1B and NR4A3 are involved in TCR mediated cell death and thymocyte negative selection (He, 2002). These nuclear orphan receptors are also involved in the apoptotic process of other cell types in response to external signals (Hashida et al., 2007). The function of NR4A3 in B cells is currently unclear. Nonetheless, NR4A3 is one of the top over-expressed genes in curable, as opposed to fatal/refractory, DLBCL (Shipp et al., 2002). It is possible that over-expression of NR4A3 in lymphoma cells might sensitise their response to pro-apoptotic signals following \textit{H. pylori} eradication and elimination of the microbial mediated immune stimulations.

The over-expression of IRF4, BCL2, CD69, CCR2A, CCR5 and TLR6 in translocation positive cases was further confirmed in a separate cohort of MALT lymphomas by qRT-PCR and immunohistochemistry or Western blot analysis.

IRF4 encodes a transcriptional factor and is expressed in activated B cells and cells showing plasma cell differentiation (Pernis, 2002). In line with its known expression pattern, IRF4 is highly expressed in the plasma cell component of MALT lymphoma. Among different subgroups of MALT lymphoma, plasma cell differentiation of neoplastic B cells is most prominent in those with t(1;14) translocation. IRF4 is transcriptionally activated by t(6;14)(p25;q32) in multiple myeloma and strong IRF4 expression has also been found in several lymphoma subtypes including lymphoplasmacytic lymphoma and 75% of diffuse large B-cell lymphoma and primary effusion lymphoma, which are not associated with t(6;14) (Falini et al., 2002). IRF4 was one of the molecules found in the activated DLBCL signature profile using cDNA microarrays (Alizadeh et al., 2000). The oncogenic activity of IRF4 is thought to be related to its transcriptional repression of interferon (IFN) inducible
genes and thus suppression of the anti-proliferative effects of IFN (Hrdlickova et al., 2001; Pernis, 2002). Interestingly, NF-κB transactivates IFN and a simultaneous up-regulation of IRF4 may block the effect of NF-κB on IFN activation.

BCL2 is an apoptosis inhibitor protein; its over-expression in lymphocytes alone was shown to be insufficient for malignant transformation, but simultaneous over-expression of BCL2 and the proto-oncogene MYC may produce aggressive B-cell malignancies (Otake et al., 2007). Hence in MALT lymphomas, over-expression of BCL2 may lead to oncogenesis by reducing cell death. Currently, the exact anti-apoptotic pathways through which BCL2 exerts its role are only partially understood, involving decreased mitochondrial release of cytochrome C, which in turn is required for the activation of pro-caspase-9 and the subsequent initiation of the apoptotic cascade (Adams et al., 1998).

CD69, a type II transmembrane glycoprotein with an extracellular C-type lectin binding domain, is another potential co-stimulatory receptor and may also have an immunoregulatory role (Sancho et al., 2005). Although the precise function of CD69 in B cells is largely unknown, it is a well described activation marker in a number of cell types. CD69 is frequently expressed in low grade B-cell non-Hodgkin lymphomas and in follicular lymphoma, its expression on tumour cells is associated with poor treatment outcome (de Jong et al., 2009; Erlanson et al., 1998). The finding of enriched expression of CD69 in translocation positive MALT lymphoma in the present study further implicates its role in lymphoma pathogenesis.

Other types of molecule enriched in translocation positive MALT lymphomas are chemokine receptors such as CCR2 and CCR5. These are G protein coupled receptors (GPR) whose major function is to regulate leukocyte trafficking and mediate immune cell migration and their retention in inflammatory sites (Murphy et al., 2000). Other functions include
angiogenic activity, apoptosis, T-cell differentiation and phagocyte activation. Following interaction with their specific chemokine ligands, chemokine receptors trigger a flux of intracellular calcium (Ca^{2+}) ions (calcium signalling). This causes cellular responses, including the onset of a process known as chemotaxis that triggers cells to migrate to specific anatomical sites. Several homeostatic chemokines have been shown to play an important role in mucosal immunology including germinal centre formation, homing mechanisms, migration and retention of lymphocytes to the sites of inflammation. Recently a study showed that CCR2 is expressed in several haematopoetic cell lineages and is critical for migration of haematopoetic stem and progenitor cells to sites of inflammation (Si et al., 2010). Although the specific role of CCR2 in B-cell trafficking and homing is unclear, it forms a heterodimer with CXCR4 that is critical for B-cell homing to the Peyer’s patches and splenic marginal zone (Springael et al., 2005), thus potentially playing a role in mature B-cell homing processes. CCR5 is a receptor for a number of inflammatory CC-chemokines including MIP-1-alpha, MIP-1-beta and RANTES, which may prove to be important in translocation positive MALT lymphoma.

Another interesting molecule highly expressed in translocation positive MALT lymphoma is Toll-like receptor 6 (TLR6). Toll-like receptors play a role in innate immunity by recognising conserved microbial structures known as pathogen associated molecular patterns (PAMPs). TLR6 is activated by bacterial lipopolysaccharide (LPS) and signals through MyD88 to activate NFκB; the signalling pathway of TLR6 is similar to that of the IL-1 receptor upon activation by the cytokine IL-1. In a mouse model, it has been shown that TLR signalling promotes marginal zone B-cell activation and migration (Rubitsov et al., 2008). TLR6 typically forms heterodimers with TLR2 on the cell surface to recognize bacterial antigens (Gomariz et al., 2007). TLR2/TLR6 signalling activates not only IKK complex that leads to
activation of the NF-κB transcriptional factor, but also the MAP kinase p38 and Jun amino-terminal kinase (JNK) that leads to activation of the AP-1 transcriptional factor (Akira et al., 2004). Hence, over-expression of TLR6 in translocation-positive MALT lymphoma could potentially augment the NF-κB activity mediated by MALT lymphoma associated oncogenic products and also activate the MAP kinase pathways. In order to test the former hypothesis, functional studies involving the expression of TLR6, in the presence of TLR2, were carried out to investigate whether they could enhance both BCL10 and API2-MALT1 mediated NF-κB activation in vitro and whether this effect was particularly significant upon LPS stimulation.

In summary, all of the key NF-κB target genes, found to be over-expressed by expression microarrays, were confirmed by qRT-PCR and immunohistochemistry. Those genes are involved in promoting tumour cell survival and proliferation either directly or indirectly and their over-expression may enhance the interaction between tumour cells and their microenvironment, which is known to be critical for MALT lymphoma development. Thus further investigation of the role of some of those genes in MALT lymphogenesis is warranted and the effect of some of the identified genes (such as TLR6) on NF-κB activation, will be investigated using in vitro model as described in the next chapter.
CHAPTER 5 – Cooperation between MALT lymphoma oncogenes and immunological stimulation in activating the NF-κB pathway

5.1 Introduction

MALT lymphoma commonly occurs in sites that are normally devoid of organised lymphoid tissues where the lymphoma is preceded by the accumulation of reactive lymphoid tissue, suggesting that the tumours arise during a chronic immune response. Several previous studies suggest that surface receptor stimulation may play an important role in MALT lymphoma pathogenesis. MALT lymphomas invariably express surface Ig. Stimulation by the antidiotypic antibody has been shown to enhance MALT lymphoma cell proliferation and this synergises with mitogenic stimulation in vitro (Hussell et al., 1993b). In line with this, expression of either API2-MALT1 or MALT1 in BJAB B-cells enhances the activation of IKK and NF-κB by CD40/CD40L stimulation (Ho et al., 2005). Both API2-MALT1 and BCL10 transgenic mice acquired expansion of the white pulp of the spleen but not lymphoma (Baens et al., 2006). However, treatment of these mice with the Freund’s complete adjuvant led to the development of marginal zone hyperplasia reminiscent of human MALT lymphoma (Baens et al., 2006).

Thus antigen stimulation may play a critical role in the clonal expansion and survival of MALT lymphoma cells. However, the surface receptors involved and the molecular mechanisms underlying the proliferation and survival of MALT lymphoma cells remain to be investigated. Also, TLR signalling activates the IKK complex that leads to activation of the NF-κB transcriptional factor, thus the effect of TLR6/2 on MALT lymphoma associated oncogenes mediated NF-κB activation was investigated. In addition, the potential cooperation
between MALT lymphoma associated oncogenes and immune receptor signalling such as those from TLR, B- and T-cell antigen receptors and CD40 was investigated. The possible molecular mechanisms underlying such cooperation were explored.

In view of the finding of enriched TLR6 expression in translocation positive MALT lymphoma as described in chapter 4, cooperation between TLR6 and MALT lymphoma associated oncogenes in NF-κB activation in vivo was first investigated.

5.2 Aims of the study

1) To test the hypothesis that there is cooperation between BCL10, MALT1 and API2-MALT1 and TLR6 on NF-κB activation;

2) To investigate whether there is a synergistic effect between expression of MALT1, BCL10 and API2-MALT1 and antigen receptor stimulation on NF-κB activation;

3) To explore the effect of expression of MALT1, BCL10 and API2-MALT1 on key regulators of canonical NF-κB activation pathway in B- and T- cell lines;

4) To determine BCL10 and MALT1 sub-cellular localisation and the effect of MALT1 on BCL10 sub-cellular localisation.
5.3 Experimental design

5.3.1 Cell lines

Murine BaF3 and WEHI cell lines were selected to investigate the effect of expression of \textit{BCL10, MALT1} and \textit{API2-MALT1} on NF-κB with and without stimulation by LPS, anti-IgM and CD40L, as they are responsive to such stimulation. The BaF3 clone used was IL-3 independent. Jurkat human T-cell line was selected to investigate the effect of expression of \textit{BCL10, MALT1} and \textit{API2-MALT1} on NF-κB with and without stimulation using anti-CD3 and anti-CD28. BJAB human B-cell line was used to investigate the effect of expression of \textit{BCL10, MALT1} and \textit{API2-MALT1} on their sub-cellular localisation. It was not possible to use the BJAB B-cell line for NF-κB luciferase reporter assays as no increased activity has been observed in response to known NF-κB activators such as LPS, anti-IgM and CD40L.

5.3.2 Investigation of the cooperation between \textit{BCL10, MALT1} and \textit{API2-MALT1} and TLR6 on NF-κB activation with and without LPS stimulation

\textit{BCL10, MALT1} and \textit{API2-MALT1} together with \textit{TLR2} and \textit{TLR6} or \textit{TLR1} and \textit{TLR6} expression constructs along with a control vector \textit{pIRES} were transiently transfected into human Jurkat T cells, which are known to be non-responsive to LPS stimulation and thus are a good model to investigate LPS mediated NF-κB activation. The transfected cells were seeded in multi-well plates, cultured for 18 hours and then treated with LPS or vehicle alone for 6 hours. Twenty four hours after transfection, the cells were harvested and measured for NF-κB activity using a luciferase assay (Section 2.2.11.1). NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. The experimental work in this section was carried out with the help of Dr. Alex Appert.
5.3.3 Investigation of the effect of BCL10, MALT1 and API2-MALT1 expression and antigen receptor stimulation on NF-κB activation

Murine B-cell lines BaF3, WEHI and Jurkat human T cells were transfected with BCL10, MALT1 and API2-MALT1 vectors and a control vector independently (Section 2.2.11.1). The transfected cells were seeded in multi-well plates, cultured for 18 hours. Prior to harvest for NF-κB luciferase assay, BaF3 cells were stimulated for 6 hours with LPS, WEHI cells were stimulated for 6 hours with LPS, anti-IgM or CD40L and Jurkat cells were stimulated for 6 hours with anti-CD3 and anti-CD28. Each of these experiments was performed three times independently. NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. The experimental work in this section was carried out with the help of Dr. Alex Appert.

5.3.4 Investigation of the effect of MALT lymphoma associated oncogenes expression on canonical NF-κB pathway activation

This was carried out using BCL10, MALT1, API2-MALT1 and BCL10/MALT1 double inducible BJAB cells and BCL10 stably expressed BJAB cells. BJAB cells transfected with pIRES vector were used as control (Section 2.2.9.5).

The following proteins were investigated by Western blotting: 3 NF-κB negative regulators, IκBα, IκBβ and IκBε; 3 central molecules, IKKα, IKKβ and NEMO; and 5 NF-κB subunits including c-Rel, RelB, p65, p50/p105 and p52/p100. IκBα and IκBβ are phosphorylated and degraded during activation of the classical NF-κB pathway. p105 is cleaved into p50 and p100 is cleaved into p52 during activation of the canonical and non-canonical NF-κB pathway respectively. β-actin was used as loading control. Western blotting results were quantified (Section 4.3.4) and ratio of each molecule over β-actin was calculated.
5.3.5 Investigation of the effect of \textit{BCL10} and \textit{MALT1} expression on their sub-cellular localisation

This was carried out using \textit{BCL10}, \textit{MALT1}, \textit{API2-MALT1} and \textit{BCL10/MALT1} double inducible BJAB cells and \textit{BCL10} stably expressed BJAB cells. BJAB cells transfected with \textit{pIRES vector} were used as control. The expression of each of the oncogenes mentioned above in the BJAB cells was investigated at 9, 16 and 24 hours following induction by the addition of doxycycline to the culture medium. Cell clots were prepared (section 2.2.9.3) and the expression of these oncogenes was investigated by immunocytochemistry using the BCL10 and C-MALT1 antibodies. In addition, co-immunoprecipitation was carried out on BCL10 and MALT1-expressing BJAB cells (section 2.2.10.8). Western blots were probed with BCL10 and MALT1 antibodies. The immunostaining with BCL10 and C-MALT1 was carried out by Dr. Hongtao Ye.
5.4 Results

5.4.1 TLR6 (in the presence of TLR2), enhances BCL10 and API2-MALT1 mediated NF-κB activation in Jurkat T cells

A

![Graph showing the fold increase of NF-κB activity with no stimulation and LPS stimulation for different conditions.](image-url)
Figure 5.1 - TLR6 enhances BCL10 and API2-MALT1 mediated NF-κB activation, in the presence of TLR2 but not TLR1, in Jurkat T cells.

Jurkat T cells were co-transfected with vector (pIRESpuro2) or plasmids containing FLAG-tagged BCL10, API2-MALT1 (AM), MALT1, TLR6, TLR2 and TLR1 as indicated, together with NF-κB luciferase reporter gene. (A) NF-κB luciferase reporter assay data for each oncogene alone and in combination with TLR6 or TLR2 with and without LPS stimulation. (B) NF-κB luciferase reporter assay data for each oncogene alone and in combination with either TLR6/2 or TLR6/1 with and without LPS stimulation. The transfected cells were seeded in multi-well plate, cultured for 18 hours and then treated with 10μg/ml LPS or vehicle alone for 6 hours. NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. Western blots in (B) show the appropriate expression of the various constructs. *P<0.01 by Student’s t-test. Arrows show that API2-MALT1, MALT1 and TLRs expression were detected using the anti-FLAG antibody with MALT1 and TLRs having approximately 90 kDa (which is the expected molecular weight) and API2-MALT1 have slightly higher molecular weight of approximately 125 kDa (which is the expected molecular weight).

Expression of TLR1, TLR2, TLR6 alone was insufficient to induce NF-κB reporter activity (Figure 5.1 A). TLR6 alone and TLR2 alone, both failed to enhance BCL10 and API2-MALT1 mediated NF-κB activation in Jurkat cells even in the presence of LPS stimulation (Figure 5.1 A). However, the expression of both TLR6 and TLR2 significantly enhanced BCL10 (4.9 fold increase) and API2-MALT1 (19.6 fold increase), but not MALT1, mediated
NF-κB activation, and this effect was much potent in the presence of LPS stimulation. Co-expression of TLR6/2 and BCL10 or API2-MALT1 appeared to be synergistic in NF-κB activation as shown by the reporter assay (Figure 5.1 B), with API2-MALT1 showing the highest synergistic effect (Figure 5.1 B). In contrast, there was no co-operation between co-expression of TLR6/1 and MALT lymphoma associated oncogenes. These results are consistent with the previous finding that TLR6 typically forms a heterodimer with TLR2 in response to stimulation by bacterial antigens (Shimizu et al., 2007; Takeuchi et al., 2001).
5.4.2 Cooperation of BCL10, MALT1, API2-MALT1 and immune receptor signalling in NF-κB activation in B-cells

To further understand the potential co-operation between MALT lymphoma associated oncogenes and immunological stimulation in NF-κB activation, NF-κB reporter assays in B- and T- cell lines were performed in the presence of stimulation to TLR, antigen receptors and CD40.

Figure 5.2. BCL10, MALT1 and API2-MALT1 mediated NF-κB activation, with and without LPS stimulation in BaF3 murine B-cells.

BaF3 B-cells were co-transfected with vector (pIRESpuro2) or plasmids containing HA-tagged BCL10, FLAG-tagged MALT1 and API2-MALT1 (AM) as indicated, together with NF-κB luciferase reporter gene. The transfected cells were seeded in multi-well plate, cultured for 18 hours and then treated with 10μg/ml LPS or vehicle alone for 6 hours. NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. Western blot in the lower panel shows appropriate expression of the various constructs.
In BaF3 B-cells, expression of each of the three MALT lymphoma associated oncogenes yielded moderate increase of NF-κB activity with API2-MALT1 showing the greatest NF-κB activity in comparison with vector control (Figure 5.2). LPS stimulation of BaF3 B-cells transfected with the pIRES control vector produced a mean of 19.7 fold increase of NF-κB activity over un-stimulated cells (Figure 5.2). The expression of the three oncogenes in BaF3 cells was comparable as shown in the Western blots (Figure 5.2). Thus, it can be concluded that the expression of each of the three oncogenes enhanced the LPS mediated activation of NF-κB in BaF3 B-cells in a synergistic manner with fold increase of 29.1, 34.5, and 33.3 for BCL10, MALT1 and API2-MALT1 respectively (Figure 5.2). Similar results were also seen in WEHI cells (Figure 5.3).
Figure 5.3 - BCL10, MALT1 and API2-MALT1 mediated NF-κB activation, with and without 6 hours of LPS, anti-IgM and CD40-L stimulation in WEHI murine B-cells.

WEHI B-cells were co-transfected with vector (pIRESpuro2) or plasmids containing HA-tagged BCL10, FLAG-tagged MALT1 and API2-MALT1 (AM) as indicated, together with NF-κB luciferase reporter gene. The transfected cells were seeded in multi-well plates, cultured for 18 hours and then treated with 10μg/ml LPS, 0.1μg/ml CD40 ligand (with 1μg/ml CD40 enhancer), 10μg/ml anti-IgM or vehicle alone for 6 hours where indicated. NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. Western blot in the lower panel shows appropriate expression of the various constructs.

The expression of the three oncogenes in WEHI cells was comparable as shown in the Western blots (Figure 5.3). However, unlike LPS stimulation, antigen receptor stimulation by anti-IgM showed variable co-operation with the expression of MALT lymphoma associated oncogenes in NF-κB activation in WEHI cells (Figure 5.3). BCL10 expression slightly enhanced anti-IgM mediated NF-κB activation in an additive manner (Figure 5.3). However
both MALT1 and API2-MALT1 expression failed to enhance anti-IgM mediated NF-κB activation. Similarly, stimulation using CD40L enhanced NF-κB activation mediated by the expression of BCL10 (but not MALT1 or API2-MALT1) in an additive manner (Figure 5.3).

5.4.3 Cooperation between BCL10, MALT1, API2-MALT1 immune receptor signalling on NF-κB activation in Jurkat T cells

![Graph showing NF-κB activation](image)

**Figure 5.4** - BCL10, MALT1 and API2-MALT1 mediated NF-κB activation, with and without 6 hours of CD3/CD28 stimulation in Jurkat T cells. Jurkat T cells were co-transfected with vector (pIRESpuro2) or plasmids containing HA-tagged BCL10, FLAG-tagged MALT1 and API2-MALT1 (AM) as indicated, together with NF-κB luciferase reporter gene. The transfected cells were seeded in multi-well plates, cultured for 18 hours and then treated with 1μg/ml anti-CD3 and 1μg/ml anti-CD28 or vehicle alone for 6 hours where indicated. NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. Western blot in the lower panel shows appropriate expression of the various constructs.
The expression of the three oncogenes in Jurkat T cells was comparable as shown in the Western blots (Figure 5.4). Thus it can be concluded that the expression of \textit{BCL10}, \textit{MALT1} and \textit{API2-MALT1} alone showed 5.0, 2.7 and 19.2 fold increase of NF-κB activity respectively in Jurkat T cells (Figure 5.4). Co-stimulation of CD3 and CD28 produced a mean of 2.7 fold increase of NF-κB activity over un-stimulated cells (Figure 5.4). In comparison with the control, CD3/CD28 co-stimulation clearly enhanced BCL10 but not MALT1 or API2-MALT1 mediated NF-κB activation (Figure 5.4).
5.4.4 Investigation of the effect of BCL10 and MALT1 expression on their sub-cellular localisation

 BJAB B-cell lines that express various inducible MALT lymphoma associated oncogenes were generated and used for investigation of their subcellular localisation. The expression of MALT lymphoma associated oncogenes were induced by doxycycline (Dox) and cells were harvested at 9, 16 and 24 hours followed by preparation of cell clots and immunocytochemistry with antibodies against BCL10 or C-terminal MALT1 where indicated. For each cell line, a control of cells without doxycycline treatment was used as reference.

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**Figure 5.5 - Immunohistochemistry on cell clots expressing various MALT lymphoma associated oncogenes.**

BJAB B-cell lines that express various inducible MALT lymphoma associated oncogenes were generated and used for investigation of their subcellular localisation. The expression of MALT lymphoma associated oncogenes were induced by doxycycline (Dox) and cells were harvested at 9, 16 and 24 hours followed by preparation of cell clots and immunocytochemistry with antibodies against BCL10 or C-terminal MALT1 where indicated. For each cell line, a control of cells without doxycycline treatment was used as reference.
BJAB cells transfected with vector only showed weak cytoplasmic BCL10 and MALT1 expression (Figure 5.5 A). In contrast, BJAB cells transfected with inducible BCL10 expression construct showed both strong nuclear and cytoplasmic expression in a proportion (10 - 15%) of cells after 9 hours induction by doxycycline, which increased to around 60% of cells at 24 hours induction (Figure 5.5 B). Interestingly, in BJAB with inducible MALT1 expression, immunostaining showed strong BCL10 in the cytoplasm, suggesting that MALT1 resulted in BCL10 accumulation and retainment in the cytoplasm (Figure 5.5 C). In BJAB cells with both BCL10 and MALT1 inducible expression, BCL10 was largely in the cytoplasm at 9 and 16 hours, but was predominantly in the nuclei at 24 hours after doxycycline induction (Figure 5.5 G and D). In contrast, co-expression of API2-MALT1 did not have any apparent effect on BCL10 sub-cellular localisation (Figure 5.5 E, F and H).

Based on the co-localisation of BCL10 and MALT1 upon doxycycline induction, it can be hypothesised that these two molecules may interact. To investigate this further, co-immunoprecipitation investigating the interaction of BCL10 with MALT1 was carried out next.
It is known that BCL10 interacts with MALT1 and mediates MALT1 oligomerisation in response to upstream signalling. In line with this, co-immunoprecipitation experiments showed that BCL10 was capable of interacting with both endogenous and exogenous MALT1 (Figure 5.6 A and B). It is most likely that through such direct interaction, MALT1 affects the subcellular localization of BCL10 especially in the cytoplasm where over-expression of BCL10 leads to strong nuclear and cytoplasmic accumulation of BCL10 initially (Figure 5.5 B). In addition, subcellular localization data (Figure 5.5 D) and co-IP data (Figure 5.6 B)
showed more BCL10 in the presence of MALT1 indicating the possibility that increased MALT1-BCL10 interaction might lead to more BCL10 expression.

5.4.5 Effect of BCL10, MALT1 and API2-MALT1 expression on canonical NF-κB pathway activation

Figure 5.7 - Activation of canonical NF-κB pathway by BCL10, MALT1 and API2-MALT1 and association of IκBβ degradation with BCL10 expression.

Various BJAB B-cells and Jurkat T cells expressing inducible BCL10, MALT1, API2-MALT1 were subjected to doxycycline (dox) treatment for 18 hours, followed by stimulation with 10μg/ml LPS for BJAB and 1μg/ml anti-CD3/anti-CD28 for Jurkat for 30 minutes, 2, 6 hours where indicated. Protein extracts from each experiment were subjected to Western blotting with IκBα and IκBβ and β-actin as the loading control. The bands were quantified and the ratio of each over β-actin is calculated in the tables accompanying each figure.

The effect of over-expression of BCL10, MALT1 and API2-MALT1 on canonical NF-κB pathway was investigated using various BJAB B-cells and Jurkat T-cell lines that inducibly
or stably expressed these proteins. As expected, LPS stimulation, induced prominent IκBα degradation in both B- and T- cells (Figure 5.7 A-E). Very interestingly, expression of BCL10 together with LPS stimulation, also caused IκBβ degradation in BJAB B-cells (Figure 5.7 A-C). IκBβ degradation was strongest with stable BJAB BCL10 cell lines (Figure 5.7 B), possibly due to the fact that the inducible cell lines have the Tet element which may slightly affect the expression of the transgene.

However, neither LPS stimulation alone nor its combination with MALT1 or API2-MALT1 expression induced IκBβ degradation (Figure 5.7 D and E). In Jurkat T cells, expression of BCL10, together with CD3/CD28 stimulation, also induced IκBβ degradation (Figure 5.7 F). All other regulators of the canonical NF-κB pathway including the NF-κB negative regulators, IκBε; 3 central molecules, IKKα, IKKβ and NEMO; and 5 NF-κB subunits including c-Rel, RelB, p65, p50/p105 and p52/p100 did not show any significant difference among the three MALT lymphoma associated oncogenes when they were over-expressed (data not shown).

Similar experiments were carried out with anti-IgM stimulation in BJAB cells but no IκBβ degradation was seen in presence or absence of expression of the above MALT lymphoma associated oncogenes (data not shown).

Taken together, these results suggest that under these conditions, expression of BCL10 may trigger the release of NF-κB dimer inactivated by IκBβ. In this context, BCL10 may play a more significant role than MALT1 or API2-MALT1.
5.5 Discussion

In this chapter, the potential cooperation between MALT lymphoma associated oncogenes and immune receptors stimulation in NF-κB activation was investigated and the common and distinct features of MALT lymphoma associated oncogenes in the activation of the canonical NF-κB pathway were explored.

5.5.1 Cooperation between the expression of MALT lymphoma associated oncogenes and TLR stimulation

Reporter assay results in Jurkat T cells showed a synergy between MALT lymphoma associated oncogenes, in particular BCL10 and API2-MALT1, and TLR6/2 expression on NF-κB activation upon LPS stimulation. Similarly, in BaF3 and WEHI B-cells, co-expression of TLR6/2 enhanced BCL10 and API2-MALT1 mediated NF-κB activation in the presence of LPS stimulation.

Based on the results of in vitro assays and expression microarray data, it is pertinent to speculate that TLR signalling may play a role in the pathogenesis of translocation positive MALT lymphoma. This is further supported by the following observations: 1) *H. pylori* activates NF-κB via both the classical and alternative pathway in B lymphocytes and this effect is dependent on LPS but not the Cag pathogenecity island (Ohmae et al., 2005); and 2) *H. pylori* associated LPS induced NF-κB activation requires TLR2/TLR6 or TLR2/TLR1 complex but not those containing TLR4 (Yokota et al., 2007), which typically recognises LPS from other Gram-negative bacteria (Akira et al., 2004).
5.5.2 Effect of antigen receptor stimulation on MALT lymphomagenesis

The three recurrent translocations identified in MALT lymphoma all induce lymphomagenesis through constitutive activation of the NF-κB pathway (Zhou et al., 2005). Functional analysis presented here showed that expression of BCL10, MALT1 and API2-MALT1 alone was capable of activating NF-κB in both B- and T- cells which are in line with the literature findings. In addition, the *in vitro* experiments showed that the expression of BCL10 enhanced the antigen receptor CD40 mediated NF-κB activation in B-cells. This finding is important in the context of MALT lymphoma as the lymphoma cells express functional BCRs and remain responsive to antigen, at least in the earlier stages of the disease (Hussell et al., 1993b). For example, in an early study by Hussell et al., gastric MALT lymphoma cells are stimulated to proliferate through CD40-CD40L interactions with T cells that recognise *H. pylori* antigens (Hussell et al., 1993b). If these gastric MALT lymphoma cells already over-express BCL10, API2-MALT1 or MALT1, then the activation of NF-κB by CD40 stimulation will be most likely enhanced. In addition, the only case that responded to anti-idiotype antibody stimulation and showed enhanced MALT lymphoma cell proliferation was a t(1;14) positive case (Hussell et al., 1993b) suggesting that a combination of high BCL10 protein expression and surface immune receptor stimulation may play an important role in MALT lymphoma development.

Recent study showed that CD40 signalling can enhance both API2-MALT1 and MALT1 mediated NF-κB activation in B-cells *in vitro* (Ho et al., 2005), however the results in this thesis showed that out of the three MALT lymphoma oncogenes, only BCL10 over-expression moderately enhanced CD40 mediated NF-κB activation. This discrepancy might be due to the fact that the study by Ho et al. used a different strain of the BJAB cell line to
the one used in this thesis which had high constitutive NF-κB activity. Another possible explanation might be that the NF-κB activation was assessed indirectly by measuring the degradation of IκBα (which is transiently degraded) (Ho et al., 2005) rather than by measuring the level of NF-κB transcriptional activity as done in the present study.

Taken together, it is tempting to speculate that the microbe mediated immune responses including help from T cells may also play a role in the pathogenesis of translocation positive MALT lymphoma. The potential involvement of TLR, CD40 and IgM signalling in translocation positive MALT lymphoma may explain the finding that rare cases of translocation positive gastric MALT lymphoma responded to H. pylori eradication (Liu et al., 2002b; Wundisch et al., 2005). The finding that API2-MALT1 and BCL10 mediated the strongest NF-κB activity is interesting considering the fact that both are highly expressed in translocation positive MALT lymphoma cases. Thus, the constitutive NF-κB activation seen in translocation positive MALT lymphoma may be, at least in part, due to preferential expression of the API2-MALT1 or BCL10 oncogenic products.

5.5.3 BCL10 expression associated with IκBβ degradation

All cell lines showed an intact IκBα mechanism, however unlike MALT1 and API2-MALT1, the expression of BCL10 also induced IκBβ degradation in the presence of CD3/CD28 and LPS stimulation in both Jurkat T- and BJAB B-cells respectively. IκBα and IκBβ interact with various NF-κB/Rel dimers with similar affinity, but recent studies demonstrated that they have slightly different mechanisms and operate through distinct properties. IκBα can shuttle NF-κB/Rel complexes in and out of the nucleus, whereas IκBβ is more efficient at sequestering NF-κB/Rel complexes in the cytoplasm. IκBβ causes cytoplasmic retention of NF-κB due to the masking of two NLSs on the NF-κB dimers. Interaction between the NF-
κB/IκBβ complex and the small guanosine triphosphatases κB-Ras-1, -2 also contributes to the NF-κB activation. When binding to κB-Ras, IκBβ cannot be phosphorylated by IKK, thus blocking the NF-κB activation signal from IKK (Fenwick et al., 2000). The differential control between IκBα and IκBβ may lead to biphasic activation of NF-κB. IκBα is promptly upregulated upon NF-κB activation and thus controls the fast transient activation of NF-κB, whereas IκBβ controls the persistent activation of NF-κB (Ladner et al., 2003). It can be speculated that BCL10 may affect the regulation of this biphasic activation of NF-κB, and thus lead to further NF-κB activation and perhaps the expression of a different set of NF-κB target genes.

5.5.4 BCL10 sub-cellular localisation and its relationship with MALT1 expression

The temporal interplay between API2-MALT1, MALT1 and BCL10 in cellular localisation in vivo showed that over-expression of BCL10 leads to the movement of excess BCL10 to the nucleus. This confirms the observation that tumour cells with t(1;14)(p22;q32)/BCL10-IGH show strong BCL10 nuclear expression (Liu et al., 2001b), but interestingly the results also showed that over-expression of BCL10 led to the presence of BCL10 in both the cytoplasmic and nuclear compartments. Over-expression of MALT1 resulted in strong homogeneous cytoplasmic localisation of both BCL10 and MALT1. But over-expression of API2-MALT1 had no effect on BCL10 localisation probably because MALT1 expression is reduced by half due to the API2-MALT1 fusion, hence the expected reduced efficiency of the nuclear export of BCL10. In cells with t(1;14)(p22;q32)/BCL10-IGH, MALT1 endogenous expression may not be sufficient for the export of over-expressed BCL10, resulting in BCL10 nuclear retention. Finally, in cells with t(14;18)(q32;q21)/IGH-MALT1, the over expression of MALT1 results in an increased retention of BCL10 in the cytoplasm, mimicking the
phenomenon seen in t(14;18) MALT lymphomas, where both proteins are thus strongly expressed in the cytoplasm (Ye et al., 2005). In addition, subcellular localization and co-IP data showed more BCL10 in the presence of MALT1 reflecting the possibility that increased MALT1-BCL10 interaction might lead to increase in BCL10 expression.

The phenomenon of nuclear BCL10 warrants further investigation, as it may have an unidentified role in the deregulation of NF-κB or other cellular pathways that may be linked to MALT lymphomagenesis. Also, BCL10 does not contain NLS, so the mechanism by which it moves to the nucleus remains to be investigated. Co-immunoprecipitation results showed that over-expression of BCL10 protein was able to interact with both endogenous and exogenous MALT1.

Taken together, it can be suggested that MALT1 and BCL10 may play an important role in the generation of the CBM complex, facilitating the initial surface receptors’ signal transduction to NF-κB. Following stimulation of surface receptors such as LPS, IgM or CD40 in B- cells or CD3/CD28 in T- cells, over-expression of BCL10 also leads to the degradation of IκBβ, facilitating the activation of NF-κB pathway. Thus, BCL10 probably plays a dual role in the NF-κB pathway by transducing the signals from B- and T- cells to the NF-κB subunits as well as facilitating NF-κB activation partly via IκBβ degradation.

In addition to the mechanistic details of how MALT lymphoma associated oncogenes affects NF-κB activation, the data also showed the heterogeneity of MALT lymphoma in that translocation positive MALT lymphoma has four recurrent translocations, each having different mechanisms of NF-κB activation. Also, other translocation positive MALT lymphoma rearrangements were recently found such as t(1;14)(p22;q32)/CNN3-IGH, t(5;14)(q34;q32)/ODZ2-IGH and t(9;14)(p24;q32)/JMJD2C-IGH (Vinatzer et al., 2008) which so far do not seem to be directly affecting the NF-κB pathway. Some of the
translocation negative MALT lymphomas have recently been shown to be associated with negative regulators of NF-κB such as A20 deletion (Chanudet et al., 2010; Kato et al., 2009). Taken together, it can be concluded that at the molecular level, different molecules and pathways play a role in the pathogenesis of MALT lymphoma. Thus it would be useful to use the expression microarray data to identify putative phenotypic markers that are specifically highly expressed in both translocation positive and negative MALT lymphoma. This will aid in the diagnosis and prognosis of MALT lymphoma and may provide further insights into the molecular pathogenesis of MALT lymphoma.
CHAPTER 6 – Identification of MALT lymphoma specific phenotypic markers using gene expression microarray

6.1 Introduction

Currently, there is no MALT lymphoma specific phenotypic marker. The differential diagnosis of MALT lymphoma includes the reactive inflammatory processes that typically precede the lymphoma including \textit{H. pylori} gastritis, lymphoepithelial sialadenitis, Hashimoto thyroiditis and other small B-cell lymphomas such as follicular lymphoma, mantle cell lymphoma and small lymphocytic lymphoma. Distinction from reactive processes is based mainly on the presence of destructive infiltrates of extra-follicular B cells, typically with the morphology of marginal zone cells (Wotherspoon \textit{et al.}, 1993). Immunophenotyping and molecular genetics analysis is used in borderline cases to assess B-cell clonality to help establish or exclude a diagnosis of MALT lymphoma, however molecular analysis may also demonstrate clonal B cells in some non-neoplastic MALT proliferations or persistant clonal population in gastric MALT lymphomas even after histologic complete remissions (Wundisch \textit{et al.}, 2003). Distinction from other small B-cell lymphomas is based on a combination of the characteristic morphologic and immunophenotypic features such as the presence of a diffuse infiltrate of CD20\(^+\), IgM\(^+\), IgD\(^-\) B cells beyond the reactive follicles outside the mantle zone. Once the marginal zone phenotype is established, light chain restriction in this marginal zone population or if present, within the plasma cells, would define the diagnosis. Thus, MALT lymphoma diagnosis is rather difficult as well as prone to errors (Pongpruttipan \textit{et al.}, 2007) and having a marker specific for MALT lymphoma would
lead to more accurate MALT lymphoma diagnosis and would help both haemtopathologists and non-haemtopathologists to make a correct diagnosis.

6.2 Aims of the study

1) To identify genes highly and specifically expressed in MALT lymphoma by comparing the gene expression microarray data of MALT lymphoma with those of FL, MCL, SMZL and CLL;

2) To validate the putative phenotypic markers identified by the gene expression microarray study in a large cohort of FFPE MALT lymphomas, FL, MCL and SMZL specimens using qRT-PCR and immunohistochemistry.

6.3 Experimental design

6.3.1 Case selection

A total of 77 cases from five different lymphoma subtypes with expression microarray data were included in this study. These were composed of 26 cases of MALT lymphomas (21 from stomach, 3 from lung, 1 from ocular adnexa and 1 from liver) with Affymetrix HG-U133A/B GeneChips; 7 cases of nodal FL; 8 cases of nodal MCL; 14 cases of SMZL and 22 cases of CLL with Affymetrix HG-U133plus2 GeneChip. The CLL expression microarrays were from a previous study carried out by Calin et al. (Calin et al., 2008), while all the other expression microarray data were obtained as part of this study.
6.3.2 Identification of genes highly and specifically expressed in MALT lymphoma by gene expression microarray analysis

After normalisation using RMA, the MALT lymphoma, SMZL, CLL, FL and MCL gene expression microarrays were subjected to the following two different analyses: Firstly, One-way ANOVA with Bonferroni multiple testing correction using Genespring GX 7.3.1 and secondly, Baysian statistics using in house programs written in R (version 2.8.0). Genes that had p < 0.05 were considered significant. Probes that were common in the two analyses were further filtered using SOM and Volcano plot and finally narrowed down using fold change calculation on the raw expression data as well as literature search of the genes preliminarily identified. Supervised clustering was carried out using Pearson correlation and average linkage as the similarity measure and clustering algorithm respectively within Genespring GX 7.3.1 (Figure 6.1).

6.3.3 Validation of genes highly expressed in MALT lymphoma by qRT-PCR and immunohistochemistry

The genes that were found to be specifically expressed in MALT lymphoma were validated by qRT-PCR and immunohistochemistry. mRNA expression was measured using qRT-PCR on a total of 79 cases of lymphoma including 44 MALT lymphomas (38 from stomach, 4 from lung, 1 from ocular adnexa and 1 from liver); 13 nodal FL; 11 nodal MCL and 11 SMZL. No CLL samples were available thus no CLL cases were included in the downstream validation. 18s rRNA was used as the housekeeping gene. RNA was extracted from microdissected tumour cells of FFPE tissue specimens of MALT lymphoma cases and qRT-PCR was carried out as described in Chapter 2. Where possible, primer pairs were designed to span exons to prevent any amplification of genomic DNA and to target up to 150bp (Table 3 in section 2.2.6), and were thus suitable for FFPE tissues. The results were presented as ∆Ct
values, so the higher the value, the lower the transcript expression, and vice versa. The Mann-Whitney U Test was used to determine whether there were statistically significant differences in the expression of a particular transcript between the various lymphoma groups. Immunohistochemistry using Lactoferrin antibody was carried out by Dr. Hongtao Ye on 5 gastric MALT lymphoma, 2 FL and 2 MCL specimens using 1:200 dilution of rabbit polyclonal antibody (product no. ab15811, Abcam) as based on the information given, seemed suited for immunohistochemistry on FFPE tissue.
6.4 Results

6.4.1 Identification of genes highly and specifically expressed in MALT lymphoma

The strategy used to combine HG-U133A&B microarray data of MALT lymphoma with HG-U133plus2 microarray data of FL, MCL, SMZL and CLL is summarised in Figure 6.1.

Figure 6.1 - Summary of bioinformatics strategy to combine MALT lymphoma, FL, MCL, SMZL and CLL microarray data and identify the genes highly and specifically expressed in MALT lymphoma.

Both the U133AB and U133plus2 sets were normalised using RMA and combined using median polish. The normalised set was then subjected to multivariate ANOVA and Bayesian statistics (using eBayes) analysis. Common probes from both analyses were reduced by subjecting them to self organising maps (SOM) and volcano plot analyses. The remaining probes were further reduced using biological knowledge and studies form the literature.
Multivariate ANOVA with Bonferroni’s stringent multiple testing criterion yielded 4907 probes whereas Baysian statistical analysis (which tends to shrink the data and eliminate non-variant probes using model fitting) gave rise to 390 probes. The common set between the two analyses was 234 probes. SOM and Volcano plot analysis further filtered the set to 178 probes. The 178 probes were ranked according to their fold change between MALT lymphoma and the other lymphomas. The top 20 probes were selected and known genes that are expressed in non-neoplastic cells were excluded.

Using this approach, 5 probes were selected; 2 probes mapped to Dermatopontin, one to Decorin, one to Tetraspanin 8 and one to Lactoferrin. Fold change, minimum and maximum raw expression signals and coefficient of variation were calculated for the 5 probes across the 26 MALT lymphoma and the 51 other lymphoma cases separately (Table 6.1). The descriptive statistics showed that Lactoferrin had the lowest CV (67%) and the highest average (1027.05) in the MALT lymphoma group indicating that Lactoferrin is expressed uniformly at higher level in the MALT lymphoma group (Table 6.1). Plot of the raw expression microarray Lactoferrin data confirmed that it is most highly expressed in MALT lymphoma group compared to the other 3 genes (Figure 6.1). This in addition to the fact that a literature search showed Lactoferrin to inhibit the immunostimulatory effect on human B cells (Britigan et al., 2001) and might play a role in lymphocyte migration in lymphoid malignancy (de Sousa et al., 1978) making it the most likely phenotypic marker and was used for downstream validation.
Figure 6.2 - (A) Supervised hierarchical clustering of MALT lymphoma, FL, MCL, SMZL and CLL (B) Volcano plot of MALT v other lymphomas with an arrow showing *Lactoferrin* position near the top.
Figure 6.3 - Variation of the raw gene expression microarray data of *Lactoferrin* across the 5 lymphoma groups.
Five probes came in the final analysis two of them localised to the Dermatopontin gene. The groups used are; 26 MALT lymphomas, 14 SMZLs, 22 CLLs, 7 FLs and 8 MCLs.

Table 6.1 - Descriptive statistical properties of the 5 potential phenotypic marker probes calculated for MALT and other lymphomas separately.

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>MALT Average</th>
<th>MALT SD</th>
<th>MALT CV</th>
<th>MALT Max</th>
<th>MALT Min</th>
<th>Lymphomas Average</th>
<th>Lymphomas SD</th>
<th>Lymphomas CV</th>
</tr>
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<tr>
<td>202018_s_at</td>
<td>Lactoferrin</td>
<td>9.61</td>
<td>1027.05</td>
<td>683.09</td>
<td>0.67</td>
<td>3081.00</td>
<td>274.80</td>
<td>106.92</td>
<td>133.93</td>
<td>1.25</td>
</tr>
<tr>
<td>203824_at</td>
<td>Tetraspanin 8</td>
<td>23.59</td>
<td>266.72</td>
<td>468.33</td>
<td>1.76</td>
<td>1887.00</td>
<td>10.85</td>
<td>11.30</td>
<td>7.02</td>
<td>0.62</td>
</tr>
<tr>
<td>211896_s_at</td>
<td>Decorin</td>
<td>8.29</td>
<td>457.07</td>
<td>519.97</td>
<td>1.14</td>
<td>1829.00</td>
<td>34.28</td>
<td>35.13</td>
<td>105.75</td>
<td>1.92</td>
</tr>
<tr>
<td>213008_at</td>
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<td>173.85</td>
<td>167.19</td>
<td>0.96</td>
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<td>17.90</td>
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<td>34.23</td>
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<tr>
<td>213071_at</td>
<td>Dermatopontin</td>
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<td>77.23</td>
<td>16.94</td>
<td>17.33</td>
<td>1.02</td>
</tr>
</tbody>
</table>
6.4.2 Validation of Lactoferrin expression in MALT lymphoma

The expression of Lactoferrin in MALT lymphoma and other lymphoma subtypes was first compared using qRT-PCR on microdissected tumour cells from FFPE tissues. All the expression values of Lactoferrin in SMZL were negative, most likely due to tissue degradation and thus the SMZL data were not used in the analysis. Lactoferrin mRNA expression was the highest in the 44 MALT lymphomas group as compared to 11 MCL and 13 FL.

Although the range of Lactoferrin expression in MALT lymphoma was rather large, there was only small overlap between MALT lymphoma and FL or MCL. Not surprisingly, the Mann-Whitney U test showed a significant difference in the gene expression between MALT lymphoma and FL or MCL (Figure 6.4). Thus Lactoferrin could be a potential marker for MALT lymphoma.

To further validate this, immunohistochemistry was performed with Lactoferrin antibody (ab15811) on 5 gastric MALT lymphoma, 2 FL and 2 MCL. The preliminary immunohistochemical results showed high and scattered staining in MALT lymphoma compared to tonsils (Figure 6.5). The intensity and distribution of staining was similar between five cases of MALT lymphoma, FL and MCL (data not shown), probably because this anti-Lactoferrin antibody seem to detect inflammatory infiltrating tissue which can occur as a result of the lymphoma so it was a poor choice to use for this study. Thus, immunohistochemistry need to be repeated with perhaps a different anti-Lactoferrin antibody such as the polyclonal rabbit anti-human (product no. A0061, DakoCytomation, Cambridge, UK) or the monoclonal mouse anti-human (Clone 1A1, product no. H86024M, Meridian Life Science, Maine, USA) where both were successfully used on clear cell carcinomas FFPE tissue (Giuffre et al., 2007). Another antibody is the polyclonal rabbit anti-human anti-
Lactoferrin (product no. 07-685, Upstate (Millipore), Massachusetts, USA) which was successfully used on nasopharyngeal carcinoma tissue microarray (Zhou et al., 2008). However, it has also been shown that Lactoferrin binding in B-lymphocytes may increase during certain stages of cell maturation (Butler et al., 1990) and Lactoferrin is expressed in cells from the upper gastrointestinal tract (possibly related to mucosal defence mechanisms) but has low background staining (Mason et al., 1978), thus immunostaining with either of the two antibodies mentioned above need to be interpreted with caution.
Figure 6.4 - Validation of Lactoferrin expression in 44 MALT, 13 FL and 11 MCL lymphomas by real-time quantitative RT-PCR.
This was performed in triplicate using RNA samples extracted from tumour cells microdissected from paraffin-embedded tissue sections. Asterisk indicates statistical significant differences between FL, MCL and combined FL with MCL and MALT lymphoma group by Mann-Whitney non-parametric statistical test. The medians are indicated by horizontal bars in the rectangular boxes. Error bars show the standard deviation of the results in each group. High values reflect low transcript expression and vice versa.

Figure 6.5 - Validation of Lactoferrin expression in MALT lymphomas by immunohistochemistry.
Summary of Lactoferrin immunohistochemistry showing high and scattered staining in MALT lymphoma compared to tonsils. The pattern of staining between MALT lymphoma, FL and MCL was similar.
6.5 Discussion

By analysis of gene expression profiles of MALT lymphoma, FL, MCL, SMZL and CLL, several genes including Lactoferrin were found to be highly expressed in MALT lymphoma whereas the other genes were not found to be highly expressed in MALT lymphoma (Figure 6.3). High expression of Lactoferrin in MALT lymphoma was also confirmed by qRT-PCR and awaiting further validation by immunohistochemistry. Lactoferrin belongs to a family of iron-binding proteins that modulate iron metabolism, haematopoiesis and immunologic reactions. Lactoferrin is an iron binding glycoprotein with approximate weight of 78 kDa. It is present in secretary fluids of mammals and contained in secondary granules of neutrophils (Boxer et al., 1982) that defends against microbial pathogens in innate immunity. It has been shown to be bacteriostatic and bactericidic against various infectious agents including H. pylori (Ellison, III, 1994). The specific receptor for Lactoferrin has been reported on several cell types including mitogen-stimulated human peripheral blood lymphocytes (Maneva et al., 1983), macrophages (Maneva et al., 1983), platelets and epithelial cells of human mammary gland (Bennett et al., 1983). It is thought that receptor binding is the first step in cell functions related to Lactoferrin. For instance, interaction of Lactoferrin with cells of the immune system induces the release of cytokines (Van Snick et al., 1976) and can protect mice against a lethal dose of E. coli in experimental infection (Van Snick et al., 1976). Lactoferrin is also capable of promoting the proliferation of phytohaemagglutinin-stimulated human peripheral blood lymphocytes (Maneva et al., 1983), as well as both human B- and T- cell lines (Dagg et al., 1981).

Unmethylated CpG dinucleotide motifs in bacterial DNA, as well as oligodeoxynucleotide (ODN) containing these motifs, are potent stimuli for many host immunological responses. Lactoferrin has been shown to inhibit CpG (ODN) stimulation of CD86 expression in the
human Ramos B-cell line leading to a decrease in the cellular uptake of ODN, a process required for CpG bioactivity (Britigan et al., 2001). Lactoferrin binding of CpG-containing ODN may serve to modulate and terminate host response to immunostimulatory molecules such as Heparin and LPS at mucosal surfaces and sites of bacterial infection (Britigan et al., 2001).

Lactoferrin has also been shown to play a role in the control of lymphoid cell migration (de Sousa et al., 1978) perhaps via the control of chemokines and cytokine release. Thus, Lactoferrin plays an important role in B-cell physiology.

Mounting evidence from the literature suggest that there is a link between increase in Lactoferrin expression and H. pylori infection and gastric inflammation. Microarray analysis of antral biopsies from patients with and without H. pylori infection showed Lactoferrin expression to be 4.2 folds higher in patients with H. pylori infection versus normal controls (Mannick et al., 2004). Microarray and qRT-PCR analysis showed Lactoferrin expression was upregulated in gastric biopsies with H. pylori infection with 19.4 fold change in H. pylori positive compared to negative biopsies by qRT-PCR (Wen et al., 2004). In a similar study, Lactoferrin expression had 14.6 and 16.8 fold induction in H. pylori positive compared to negative biopsies using microarray and qRT-PCR respectively (Wen et al., 2007). Molecular analysis of H. pylori associated gastric inflammation in chronically infected and immune mice, showed Lactoferrin to be expressed 38 fold higher in H. pylori infected mice compared to naïve animals (Rahn, 2003). In addition, a study by Zou et al showed that supplementation with Lactoferrin could be effective in increasing eradication rates of anti-H. pylori therapy, and could be helpful for patients with H. pylori eradication failure (Zou et al., 2009) and Hirata et al. showed that faecal Lactoferrin levels were elevated and this was
found to be useful in the detection of colorectal diseases including MALT lymphoma (Hirata et al., 2007).

Analyses of B-cell malignancies comprising cells representing different maturational stages showed variable Lactoferrin expression (Butler et al., 1990). Acute lymphoblastic leukaemia (ALL) derived from progenitor B cells, and hairy cell leukaemia (HCL) derived from late activated memory B cells do not express surface Lactoferrin. In addition, EBV-transformed B-cell lines, representing activated B cells, were virtually negative for Lactoferrin. The same study showed that CLL had the highest percentage of surface Lactoferrin positivity (Butler et al., 1990). Microarray analysis in this thesis showed that mRNA expression of Lactoferrin in CLL is minimal. However the CLL microarray data were obtained from a study by Calin et al. (Calin et al., 2008) in public domain and no CLL tissue was available, thus it was not possible to confirm this finding by qRT-PCR as was the case with FL and MCL. If the qRT-PCR results confirm the microarray data, it can be hypothesised that perhaps the binding (but not the expression) of Lactoferrin to B lymphocytes might increase during certain stages of cell maturation. This might slightly reduce the use of Lactoferrin as a potential phenotypic marker for MALT lymphoma, however the diagnosis of MALT lymphoma is not usually confused with CLL/SLL, as the latter typically is CD5+ and CD23+. Furthermore, the disease usually manifests as CLL (rather than SLL) with an associated with lymphocytosis at presentation, a feature not associated with MALT lymphoma. Attempts of Lactoferrin immunohistochemistry with commercial antibody failed to yield any conclusive results and validation of Lactoferrin expression in MALT lymphoma remains to be investigated.
CHAPTER 7 – GENERAL DISCUSSION

7.1 MALT lymphoma is a distinct entity within the lymphomas but with heterogeneity between MALT lymphoma with and without chromosome translocation as indicated by gene expression profiling

Gene expression microarray results showed that MALT lymphoma is an entity distinct from other lymphoma subtypes, FL (thought to originate from germinal centre B cells) and MCL (thought to originate from the mantle zone B cells) (Kuppers, 2005). Significantly, MALT lymphomas expressed at high levels, many of the genes related to response to external biotic stimulus. On the other hand, in FL, immune response related genes were highly expressed, and in MCL, cell cycle related genes were highly expressed.

Unsupervised clustering between translocation positive and negative MALT lymphomas showed no clear separation between these cases indicating overlap in the molecular mechanism between the two groups. In all unsupervised clustering analyses, translocation negative MALT lymphomas with BCL10 nuclear staining tended to cluster with translocation positive cases suggesting that such cases at the molecular level resemble more to those with chromosome translocation.

7.2 Overview of the molecular mechanisms underlying the pathogenesis of MALT lymphoma

Although unsupervised clustering analyses showed considerable overlap in the gene expression profiles between MALT lymphomas with and without chromosome translocation, there was a significant difference in the expression of NF-κB target genes between the two subgroups. Exhaustive and systematic GSEA of various molecular pathways and biological processes, showed that gene sets related to inflammation, immune responses, chemokine and
GPR signalling, were differentially over-represented between these different subgroups. Importantly, several of these molecular pathways or biological processes also lead to NF-κB activation. These findings were also reinforced by independent analyses of differentially expressed genes between MALT lymphomas with and without translocation using hypergeometric tests. These observations provide several novel insights into the molecular mechanisms of both translocation-positive and negative MALT lymphomas which potentially explain their different clinical and histological presentations.

7.2.1 Aberrant molecular mechanisms of translocation positive MALT lymphoma

GSEA and leading edge analyses revealed a common core subset of genes that were overexpressed in translocation positive cases and a high proportion of them were NF-κB target genes involving multiple related biological processes or molecular pathways.

Potentially critical immune receptors highly expressed in translocation positive MALT lymphoma were TLR6, CD40, CD83, CD1D and CD69 as shown by GSEA of gene expression microarray study in this thesis.

TLR6 typically forms heterodimers with TLR2 on the cell surface to recognize bacterial antigens (Gomariz et al., 2007). TLR2/TLR6 signalling activates not only the IKK complex that leads to activation of the NF-κB transcriptional factor, but also the MAP kinase p38 and Jun amino-terminal kinase (JNK) that lead to activation of the AP-1 transcriptional factor (Akira et al., 2004). Hence, over-expression of TLR6 in translocation-positive MALT lymphoma could potentially augment the NF-κB activity mediated by MALT lymphoma associated oncogenic products and also activate the MAP kinase pathways. Data from this thesis in Baf3 B cells and Jurkat T cells showed that expression of TLR6, in the presence of
TLR2, enhanced both BCL10 and API2-MALT1 mediated NF-κB activation. This effect was particularly significant upon LPS stimulation suggesting a potential biological cooperation between MALT lymphoma associated translocations and TLR signalling in lymphomagenesis. In addition, data from this thesis demonstrated that TLR6 with TLR2 enhanced API2-MALT1, BCL10 and MALT1 mediated AP-1 activation in BJAB B cells regardless of LPS stimulation.

The growth of gastric MALT lymphoma cells is critically dependent on their cognate interaction with \textit{H. pylori} specific tumour infiltrating T cells, involving co-stimulatory molecules such as CD40/CD40L (D'Elios \textit{et al.}, 2003; Hussell \textit{et al.}, 1996). A recent study showed that CD40 signalling could enhance both API2-MALT1 and MALT1 mediated NF-κB activation in B cells (Ho \textit{et al.}, 2005). In addition, previous studies have also indicated a role of B-cell receptor signalling in \textit{H. pylori} induced MALT lymphomagensis (Craig \textit{et al.}, 2010). In this thesis, the potential cooperation between expression of MALT lymphoma associated oncogenes and BCR and CD40 signalling were investigated. As expected, functional studies in WEHI B cells showed additive effect between CD40L stimulation and BCL10 in NF-κB activation. GSEA of the expression microarray data demonstrated that the expression of co-stimulating molecules CD40 and CD83 was enriched in translocation positive MALT lymphoma, further supporting their role in lymphomagenesis. In line with this hypothesis, the GSEA also revealed up-regulation of CD1D in translocation positive MALT lymphoma, which is involved in the presentation of microbial lipid and lipopeptide antigens to T cells (Brigl \textit{et al.}, 2004). Thus, it is tempting to speculate that the microbe mediated immune responses including T cells may also play a role in the pathogenesis of translocation positive MALT lymphoma. The potential involvement of TLR, CD40 and CD83 signalling in translocation positive MALT lymphoma may explain the finding that rare
cases of translocation positive gastric MALT lymphoma respond to *H. pylori* eradication (Liu *et al.*, 2002b; Wundisch *et al.*, 2005).

CD69 is an early cell activation antigen expressed on the surface of activated immune cells by small subset of T and B cells in peripheral lymphoid organs (Hara *et al.*, 1986). Although the precise function of CD69 in B cells is largely unknown, it is a well-described activation marker in several cell types, and its expression is up-regulated in marginal zone B cells upon TLR stimulation (Rubtsov *et al.*, 2008). CD69 is frequently expressed in low-grade B-cell lymphomas, and in FL, its expression is associated with poor treatment outcome (de Jong *et al.*, 2009; Erlanson *et al.*, 1998). Also, it has been shown that CD69 functions downstream of IFNα and IFNβ, and possibly other activating stimuli, to promote lymphocyte retention in lymphoid organs (Shiow *et al.*, 2006). The finding here of enriched expression of CD69 in translocation-positive MALT lymphoma further implicates its role in lymphomagenesis.

Among the genes up-regulated in translocation positive cases, IRF4 showed a significant increase in cases with t(1;14) chromosomal translocation. IRF4 encodes a transcriptional factor and is expressed in activated B cells and cells showing plasma cell differentiation (Pernis, 2002). It is required during an immune response for lymphocyte activation and the generation of immunoglobulin-secreting plasma cells. In line with its known expression pattern, IRF4 is highly expressed in the plasma cell component of MALT lymphoma. Furthermore, plasma cell differentiation of neoplastic B cells is most prominent in MALT lymphoma with t(1;14) translocation. IRF4 is transcriptionally activated by t(6;14)(p25;q32) in multiple myeloma where gene expression profiling and genome wide chromatin immunoprecipitation analysis uncovered an extensive network of IRF4 target genes and showed that although IRF4 is not genetically altered in most myelomas, they are nonetheless
addicted to an aberrant IRF4 regulatory network that fuses the gene expression programmes of normal plasma cells and activated B cells (Shaffer et al., 2008). Strong IRF4 expression has also been found in several lymphoma subtypes including lymphoplasmacytic lymphoma, 75% of diffuse large B-cell lymphoma and primary effusion lymphoma, which are not associated with t(6;14) (Falini et al., 2002). IRF4 is one of the molecules found in the ABC-DLBCL signature (Alizadeh et al., 2000). The oncogenic activity of IRF4 is thought to be related to its transcriptional repression of IFN-inducible genes, and thus the suppression of the anti-proliferative effects of IFN (Hrdlickova et al., 2001; Pernis, 2002). NFκB transactivates IFN, and IFNα and IFNβ were moderately up-regulated in t(11;18) or t(1;14) positive MALT lymphoma. A simultaneous up-regulation of IRF4 may block the side-effects of NF-κB on IFN activation.

In addition to immune receptors discussed above, several potentially critical chemokine receptors were highly expressed in translocation positive MALT lymphoma and they included CCR2, CXCR4, CCR5 and CCR7 as shown by GSEA of gene expression microarray study in this thesis.

Several homeostatic chemokines have been shown to play an important role in mucosal immunology including germinal centre formation, homing and trafficking of activated mucosal B cells. Germinal centre formation involves the trafficking and positioning of lymphocytes in the organized lymphoid tissue (Moser et al., 2001) and homing mechanisms play a role in B-cell recruitment to the secondary lymphoid tissue. CCR7 expression is up-regulated in activated B cells allowing them to acquire the capacity to migrate into the T-cell zone and to follicles in Peyer’s patches, where the CCR7 ligands, CCL19 and CCL21 are highly expressed (Okada et al., 2002; Reif et al., 2002). CCR7 is shown to play a central role
in regulation of normal mucosal lymphocyte re-circulation and homeostasis, particularly in
the stomach (Hopken et al., 2007). CXCR4 is expressed in B cells at multiple stages of their
development. It is required for retention of B-cell precursors in the bone marrow. CXCR4-
deficient B-cell precursors that migrated prematurely became localised in splenic follicles
despite their unresponsiveness to CXCL13. CXCR4 is also critical for B-cell homing to the
Peyer’s patches and splenic marginal zone (Nie et al., 2004). In both low grade B-cell NHL
and classic Hodgkin lymphomas, CCR7 and CXCR4 over-expression were associated with a
wide lymph node spread, supporting their role in lymphoma pathogenesis (Lopez-Giral et al.,
2004; Hopken et al., 2002; Trentin et al., 2004).

B cells express CCR5 on their cell surfaces, and RANTES, one of four chemokine ligands of
CCR5, is mitogenic for B cells (Rabkin et al., 1999). Thus, it is tempting to speculate that
RANTES and hence CCR5 may play a role in lymphoma expansion by avoiding immune
surveillance.

CCR2 (CC chemokine receptor 2) is a receptor for MCP-1 which attracts monocytes and T
cells to sites of injury as part of the inflammatory response. Two isoforms of CCR2, namely
CCR2A and CCR2B have been cloned (Charo et al., 1994; Charo, 1999). It has been
suggested that these two isoforms of the receptor might be splice variants of a single gene
(Charo et al., 1994). CCR2B is the major form of the receptor readily detected in monocytes,
whereas CCR2A is less abundant (Charo, 1999). The physiological role of CCR2A is not
fully understood (Charo, 1999). Data in this thesis has shown that CCR2A and CCR2B were
differentially expressed between translocation positive and negative MALT lymphoma cases
with greater variability in CCR2A expression. Flaishon et al. described a novel role for CCR2
and its ligand CCL2/JE in inhibiting the chemotactic response of immature B cells to the
chemokine CXCL12/stromal cell-derived factor 1 (SDF-1), suggesting that CCR2 and its
ligand act as negative regulators of the homing of immature B cells (Flaishon et al., 2004). They also showed that CCR2 is transcribed in immature B cells, while its mRNA is dramatically down regulated at the mature B cells stage. CCR2-deficient mice showed massive accumulation of immature B cells in the lymph nodes in comparison with wild type mice (Flaishon et al., 2004). Beside its expression in immature B cells, CCR2 is found to be expressed in mature B-cell neoplasms such as marginal zone B-cell lymphoma (Trentin et al., 2004). It has been shown that CCR2 is up-regulated in response to the formation of superoxide free radical molecules and that IL-2 induced the expression of CCR2 in T lymphocytes, which correlated with the response of these cells to MCP-1 in chemotaxis assays (Loetscher et al., 1996). Also, IL-10 selectively up-regulated the expression of CCR2 in monocytes by prolonging the mRNA half-life (Sozzani et al., 1998). More recently, CCR2 was shown to mediate hematopoietic stem and progenitor cell trafficking to sites of inflammation (Si et al., 2010). However, it is still unclear how the up-regulation of CCR2 expression contributes to MALT lymphoma development or whether it is induced by the presence of oxygen free radicals which would occur during H. pylori mediated inflammatory responses. It may be that infection with H. pylori leads to chronic inflammation which may later cause the B cells to initiate the expression of CCR2 amongst other chemokines leading to their trafficking to inflammation sites. Once the inflammation subsides, CCR2 expression becomes reduced, however some of the cells within the inflammatory site may acquire an oncogenic even such as t(11;18)/API2-MALT1 leading to the constant expression of CCR2 via aberrant NF-κB and MAPK pathways activation. Over-expression of CCR2A and B, CCR5 and CXCR4 has been shown to activate NF-κB, Jak/STAT and MAPK pathways (Okada et al., 2002). This, together with the TCR, BCR and CD40 signalling may form a
positive feedback autoregulatory loop in API2-MALT1 or BCL10 mediated NF-κB activation, thus leading to constitutive NF-κB activation.

7.2.2 Aberrant molecular mechanisms of translocation negative MALT lymphoma

In contrast to translocation-positive MALT lymphoma, translocation-negative cases were characterised by expression of a strong inflammatory gene signature. GSEA and leading edge analysis also revealed common core subset genes involving several related biological processes or molecular pathways, which were enriched in translocation-negative MALT lymphoma. The top examples included proinflammatory cytokines IL8 and IL1β, molecules involved in B- and T- cell interaction such as CD86, CD28 and ICOS, several chemokine and chemokine receptors, NR4A3 (also known as MINOR) and TLR2 (Figure 3.10).

IL8 and IL1β are the hallmark of a proinflammatory cytokine profile in response to *H. pylori* infection. IL8 is critical for neutrophil infiltration and activation, while IL1β induces gastrin release, inhibits acid secretion and promotes apoptosis of epithelial cells (McNamara *et al.*, 2008). The finding of over-expression of these proinflammatory cytokines in translocation-negative gastric MALT lymphomas, indicates the presence of active *H. pylori* infection. In keeping with this, translocation-negative gastric MALT lymphomas show a higher number of blast cells than translocation-positive cases (Okabe *et al.*, 2003). In addition, a number of chemokines and chemokine receptors was highly expressed in the translocation-negative cases. This may reflect the trafficking and retention of various immune cells in response to an active *H. pylori* infection.

Most importantly, GSEA showed up-regulated expression of the surface molecules involved in B- and T- cell interaction namely CD86, CD28 and ICOS in translocation-negative gastric
MALT lymphoma. Although residual reactive follicles may be present and contribute to the high CD86, CD28 and ICOS expression in translocation negative cases, the germinal centre markers CD10 and BCL6 were expressed in much lower levels in MALT lymphoma (Figure 3.3). More importantly, over-expression of CD86 in tumour cells was clearly demonstrated by qRT-PCR on microdissected samples and immunohistochemistry. In line with these findings, a previous study showed significantly higher CD86 expression in gastric MALT lymphomas that responded to *H. pylori* eradication than those resistant to the therapy (66% VS 10%) (de Jong *et al.*, 2001). Although the chromosome translocation status in these cases is not available, it is most likely that the cases responded to *H. pylori* were translocation-negative (Liu *et al.*, 2002b). Taken together, these findings suggest that there is an active immune response to *H. pylori* infection in translocation-negative gastric MALT lymphoma, and this most likely underscores the tumour cell survival and expansion, and thus determines its response to *H. pylori* eradication.

NR4A3 is another molecule significantly enriched and over-expressed in translocation negative MALT lymphoma. NR4A3 is a member of the nerve growth factor-1B (NGF1B, or NR4A1 or Nur77) subfamily of nuclear orphan receptors. In T cells, NGF1B and NR4A3 are involved in TCR mediated cell death and thymocyte negative selection (He, 2002). These nuclear orphan receptors are also involved in the apoptotic process of other cell types in response to external signals (Hashida *et al.*, 2007). The function of NR4A3 in B cells is currently unclear. Nonetheless, NR4A3 is one of the top over-expressed genes in cured, as opposed to fatal/refractory, DLBCL (Shipp *et al.*, 2002). It is possible that over-expression of NR4A3 in lymphoma cells may predispose them to apoptosis following *H. pylori* eradication and elimination of the microbial mediated immune stimulation.
7.2.3 Molecular mechanisms of BCL10, MALT1 and API2-MALT1 mediated NF-κB activation

Cytospin and immunohistochemistry data from this thesis showed that over-expression of BCL10 led to its subcellular localisation in the nucleus. Co-immunoprecipitation data showed that over-expression of BCL10 led to its interaction with MALT1 in BJAB B cells. Functional data showed that only BCL10 over-expression with LPS stimulation led to IκBβ degradation. In addition, subcellular localization and co-IP data showed more BCL10 in the presence of MALT1 reflecting the possibility that increased MALT1-BCL10 interaction might lead to increase in BCL10 expression. However, BCL10 was shown to interact with transcription factor IIB which plays an important role in the assembly of transcription activators that make up the RNA polymerase II pre-initiation complex (Liu et al., 2004c) suggesting a possible role as a transcriptional activator. Taken together, it can be hypothesised that excess BCL10 interacts with MALT1 either by affecting its stabilisation or its expression. Either way, the cytoplasmic level of BCL10 and MALT1 needs to be maintained to allow the formation of the CBM signalosome upstream of the NF-κB subunits. Closer to the NF-κB subunits, BCL10 over-expression together with stimulation by LPS in B cells or CD3/CD28 in T cells stimulation led to constitutive NF-κB activation via IκBβ degradation. It has been shown that TCR/CD28 co-stimulation induces IκBα, IκBβ and IκBε degradation (Li et al., 2005). IκBα and IκBβ use slightly different mechanisms of NF-κB activation. One hypothesis proposes that IκBα masks the NLS of p65 in addition to the fact that it contains nuclear export sequencing that enables newly synthesized IκBα to shuttle nuclear NF-κB/Rel dimers into the cytoplasm (Phelps et al., 2000). In contrast, IκBβ does not contain nuclear export sequence and is able to mask the NLS domains of both p65 and p50. While detailed control of IκB nuclear import and export has yet to be defined, these unique
properties of IκBα and IκBβ are thought to provide the fine tuned regulation of NF-κB/Rel proteins, whereby IκBα controls transient NF-κB/Rel activation and IκBβ regulates sustained NF-κB/Rel activity. Studies using embryonic fibroblasts derived from various IκB knockout mice (Hoffmann et al., 2002), showed that IκBα resulted in high oscillatory NF-κB nuclear activity, whereas IκBβ displayed a constant steady increase in nuclear NF-κB activity that plateaued without subsequent decline, allowing sustainable NF-κB activation in the case of prolonged stimulation (Hoffmann et al., 2002). Also, studies have shown that when IκBβ is degraded, NF-κB activation becomes persistent; even though newly synthesized IκBα accumulates to high levels in unstimulated cells. This is partly because the newly synthesized IκBβ is an unphosphorylated protein that binds to a portion of newly made NF-κB and sequesters it from IκBα. The unphosphorylated IκBβ, however, fails to mask the NLS and DNA binding domain on NF-κB, resulting in the nuclear uptake of the unphosphorylated NF-κB/IκBβ complex (Suyang et al., 1996). Thus, data from this thesis showed that over-expression of BCL10 leads to IκBβ degradation causing constitutive NF-κB activation. This partly explains the biochemical mechanisms behind the NF-κB reporter assay observations showing that BCL10 expression together with surface receptor stimulation led to increased NF-κB activation. In addition, phosphorylated BCL10 was shown to form a complex with another NF-κB inhibitor, BCL3, to enter the nucleus (Yeh et al., 2006), thus it might be that BCL10 may move to the nucleus with BCL3. However, details of BCL10 nuclear import and export as well as the function of nuclear BCL10 remain to be determined. Collectively, it can be hypothesised that BCL10 may indirectly (e.g. via interaction with the IKK complex or some unknown molecule) degrades IκBβ, since BCL10 is not part of the transcription factor complex that binds the NF-κB promoter, nor does it affect the ability of NF-κB to bind the DNA. This degradation of IκBβ may cause different NF-κB to transactivate slightly different
set of NF-κB target genes thus explaining the slightly different gene expression profiles of the NF-κB target genes between MALT lymphoma with and without chromosome translocation. A hypothesis on the molecular mechanisms underlying MALT lymphoma with and without chromosomal translocation can be generated from the data described above. In translocation positive MALT lymphoma, over-expression of API2-MALT1, BCL10 or MALT1 activates the canonical NF-κB pathway. Canonical NF-κB activation is augmented by B-cell receptor signalling, TLR signalling and potentially CCR2 signalling. The non-canonical NF-κB pathway may be activated by CD40 and LTβ receptor signalling. Activation of the canonical and non-canonical NF-κB pathways leads to enhanced expression of the NF-κB target genes, particularly TLR6, CCR2A, CCR2B CD69, IRF4 and BCL2. Over-expression of these immune receptors may provide a further positive feedback to the activation of the NF-κB pathways. In addition, expression of TLR6 and CCR2 may trigger activation of the MAPK pathway. Over-expression of BCL2 is expected to promote tumour cell survival. In essence, the above chromosome translocations cause constitutive NF-κB activation with expression of their target genes forming a potential positive feedback loop, and the relentless NF-κB activation, which in the case of gastric MALT lymphoma confers its resistance to _H. pylori_ eradication (Figure 7.1).

In translocation negative MALT lymphoma, the ongoing inflammatory and immune responses maintain active cognate B- and T- cell interaction via co-stimulating molecules CD86/CD28, B7RP1/ICOS, which are the major determinants of tumour cell survival and thus explain, in the cases of gastric MALT lymphoma, their responses to _H. pylori_ eradication (Figure 7.1).
Figure 7.1 - Summary and hypothesis on molecular mechanisms of MALT lymphoma with and without chromosomal translocation.

In translocation positive MALT lymphoma, over-expression of API2-MALT1, BCL10 and MALT1 activates the canonical NF-κB pathway (e.g. in the case of BCL10 via degradation of IκBβ), leading to enhanced expression of the NF-κB target genes, particularly TLR6, CCR2, CD69 and BCL2. Over-expression of TLR6 may provide a further positive feedback to the activation of the NF-κB pathway. Similar positive feedback may also be expected from the CCR2 signalling, and in addition both TLR6 and CCR2 may trigger activation of the MAPK pathway. The pathogenic implication of enhanced CD69 expression is currently unknown. Over-expression of BCL2 is expected to promote the tumor cell survival. In essence, the above chromosome translocations cause constitutive NF-κB activation with the expression of their target genes forming a potential positive feedback loop, and the relentless NF-κB activation, which in the case of gastric MALT lymphoma, confers its resistance to H. pylori eradication.

In translocation negative MALT lymphoma, the ongoing inflammatory and immune responses maintain active cognate B- and T- cell interaction via the co-stimulating molecules; CD86/CD28, B7RP1/ICOS, which are the major determinants of tumor cell survival and thus explain, in the cases of gastric MALT lymphoma, their responses to H. pylori eradication.
7.3 MALT lymphoma specific phenotypic marker identification

Comparison of MALT expression microarray data with other lymphomas demonstrated Lactoferrin to be highly expressed in MALT lymphoma but not in other lymphoma subtypes. This was confirmed by qRT-PCR showing Lactoferrin to be significantly over expressed in all MALT lymphoma cases compared to FL or MCL. Currently, the only reported marker for marginal zone lymphomas is myeloid cell nuclear differentiation antigen (MNDA), a nuclear protein expressed by myeloid cells and a subset of B cells (Kanellis et al., 2009). It was shown to be expressed in normal tissue by a subset of the marginal zone B cells as well as subgroups of CLL, MCL and DLBCL, but it is highly expressed by MALT, SMZL and nodal marginal zone lymphoma (NMZL) and rarely expressed in FL making it potentially a useful marker for distinguishing between NMZL and FL (Kanellis et al., 2009). Both microarray and qRT-PCR results from this thesis showed that Lactoferrin mRNA is more highly expressed in MALT compared to other lymphomas. It can be hypothesised that if the Lactoferrin protein expression follows a similar pattern as its mRNA expression, then Lactoferrin may prove to be a better phenotypic marker for MALT lymphoma than MNDA.

Lactoferrin belongs to a family of iron-binding proteins that modulate iron metabolism, haematopoiesis and immunologic reactions. It has been shown that Lactoferrin which is present at mucosal surfaces and neutrophil specific granules (Bacciolini et al., 1970) (Raphael et al., 1989), readily binds CpG-containing DNA which binds to B cells via interaction with DNA on the cell surface (Bennett et al., 1983), mediated through the highly charged N-terminal sequence of Lactoferrin (Kawasaki et al., 2000). Lactoferrin inhibited CpG ODN stimulation of CD86 expression in the human Ramos B cell line and decreased cellular uptake of ODN, a process required for CpG bioactivity. Lactoferrin binding of CpG-containing ODN may serve to modulate and terminate host response to immunostimulatory
molecules such as CD86 and CD80 at mucosal surfaces and sites of bacterial infection. (Britigan et al., 2001). In addition, various studies show that there is an increase in Lactoferrin expression and H. pylori infection and gastric inflammation (Mannick et al) (Wen and Wen). Also, clinical studies showed that supplementation with Lactoferrin could be effective in increasing eradication rates of anti-H. pylori therapy, which could be helpful for patients with H. pylori eradication failure (Zou et al.) and that fecal Lactoferrin levels were elevated and this was found to be useful in the detection of colorectal diseases including MALT lymphoma (Hirata et al.).

Since the starting point of MALT lymphoma is thought to be infection with a pathogen and most of the samples analysed were H. pylori positive gastric MALT lymphoma, this may explain why Lactoferrin seem to be more highly expressed in MALT compared to other lymphomas. Besides its inhibitory effect on CpG-containing ODN in human B cells, Lactoferrin has been shown to affect phenotypic changes in immature B-cell populations and has an effect on the antigen presenting function of these cells (Zimecki et al., 1995). Lactoferrin also plays a role in the maturation of cells of the immune system and, together with the demonstration of the involvement of Lactoferrin in the maturation of T cells (Zimecki et al., 1991), this provides evidence that Lactoferrin can enhance the induction phase of the immune response.

In conclusion, Lactoferrin is a likely phenotypic marker for MALT lymphoma but further confirmation by immunohistochemistry is needed.
7.4 Conclusions

The results detailed in this study allow the following conclusions to be drawn:

1) Unsupervised clustering of MALT lymphoma with FL and MCL shows that MALT lymphoma is a distinct entity. Nonetheless, there is an overlap in the gene expression profiles between translocation positive and negative MALT lymphomas as both activate NF-κB pathway but leading to the expression of different sets of NF-κB target genes.

2) Translocation positive MALT lymphoma was characterised by an enhanced expression of NF-κB target genes, particularly CCR2, TLR6, CD69, IRF4 and BCL2.

3) Translocation negative MALT lymphoma was featured by active inflammatory and immune responses to *H. pylori* infection. Tumour cell interaction with infiltrating T cells through co-stimulatory molecules (especially CD86/CD28) may have an important role in their survival and clonal expansion.

4) *In vitro* assays show cooperation between the expression of MALT lymphoma associated oncogenes and signalling via surface receptors including BCR, TLR and TCR. Such cooperation may be operational *in vivo*.

5) BCL10 expression with surface receptor stimulation leads to IκBβ degradation. Over-expression of BCL10 together with LPS stimulation in BJAB B cells may also activate NF-κB inactivated by IκBβ.

6) Comparison of MALT lymphoma expression microarrays with other lymphomas showed Lactoferrin to be a putative MALT lymphoma specific marker.
7.5 Future perspectives

This thesis has identified novel mechanisms involved in MALT lymphoma pathogenesis, however many questions remain to be addressed.

7.5.1 CCR2 involvement in the molecular mechanism of MALT lymphomagenesis

Genes highly expressed in translocation positive MALT lymphoma include CCR2A and B isoforms. It would be useful to determine the role of each of these in MALT lymphomagenesis. This can be done in a similar way to the functional experiments carried out on TLR6 by identifying which pathways (e.g. NF-κB and MAPK) they affect and whether there is a synergy between them and MALT lymphoma associated oncogenes in the activation of those pathways. Once this is established, it would be helpful to construct inducible stable cellular models with each of the above genes together with MALT lymphoma associated oncogenes to confirm any synergy between each of the above genes and MALT lymphoma associated oncogenes. Migration assays can be carried out on the cellular model to study the effect of CCR2 expression on B-cell migration. Expression microarray experiments can be carried out on the cellular models by expression of CCR2 alone and together with MALT lymphoma associated oncogenes. GSEA and GO analysis will then lead to the identification of the specific pathways affected by the above genes that may be involved in MALT lymphomagenesis. Validation of genes involved in those pathways can be carried out on MALT lymphoma patient samples.
7.5.2 Nuclear BCL10 function

Identification of BCL10 binding partners would help to determine the functions of nuclear BCL10 which may help to explain its role in the regulation of the NF-κB pathway and its interaction with MALT1. Co-immunoprecipitation of nuclear BCL10 can be carried out by isolating the nuclear BCL10 fraction from BCL10 BJAB cells. BCL10 has been shown to form a complex with BCL3 which is a transcriptional co-activator of NF-κB (Yeh et al., 2006), thus co-IP products of nuclear BCL10 can be investigated for the potential presence of NF-κB subunits as potential binding partners by Western blotting. Co-IP extracts can also be investigated for the presence of unknown binding partners. Extracts can be separated by PAGE and the proteins visualised by silver staining. The proteins within bands unique to extracts from cells expressing nuclear BCL10 protein can be identified by mass spectrometry. Confirmation of the identity of any putative nuclear BCL10 binding partners can then be achieved by Western blotting of co-IP products for the presence of these targets. Site-specific mutagenesis of BCL10 expression constructs could be carried out to determine the exact region of the BCL10 protein required for binding to these partners.

BCL10 has been shown to bind to transcriptional activator TBII (Liu et al., 2004b). The role of BCL10 as a transcriptional activator could be investigated by Chromatin immunoprecipitation (ChIP) assay on cell lines with over-expressed BCL10 and compared to those with normal BCL10 expression. This will answer the question whether BCL10 may play a role as a transcriptional activator and if so whether it affects MALT1 expression amongst other genes which might partly explain the mechanism by which over-expression of BCL10 leads to MALT1 interaction.
7.5.3 Lactoferrin expression in MALT lymphoma by immunohistochemistry

A practically useful specific marker of MALT lymphoma needs to be developed. Lactoferrin seems to be the most promising candidate as confirmed by qRT-PCR. However, the initial Lactoferrin antibody used to validate the microarray and qRT-PCR produced inconclusive immunohistochemical data and the immunohistochemistry need to be repeated with a different Lactoferrin antibody. Two antibodies; the polyclonal rabbit anti-human (product no. A0061, DakoCytomation, Cambridge, UK) and the monoclonal mouse anti-human (Clone 1A1, product no. H86024M, Meridian Life Science, Maine, USA) were successfully used on FFPE tissue of clear cell carcinoma (Giuffre et al., 2007). Another antibody is the polyclonal rabbit anti-human anti-Lactoferrin (product no. 07-685, Upstate (Millipore), Massachusetts, USA) which was successfully used on nasopharyngeal carcinoma tissue microarray (Zhou et al., 2008). MNDA was shown to be a useful marker for distinguishing between NMZL and FL. Thus comparison between MNDA and the above two Lactoferrin marker on the same series of NHL B-cell lymphomas including CLL/SLL is warranted.
References


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Appendix I – Details of bioinformatics analysis

I.1 Preprocessing algorithm for Affymetrix expression arrays

Preprocessing Affymetrix expression arrays usually involves three steps: background adjustment, normalisation and summarisation. The bioconductor software (http://www.bioconductor.org) which use the R programming platform (http://www.r-project.org/), implements a wide range of methods for each of these steps. Self-contained routines for background correction and normalisation usually take an AffyBatch as input and return a process AffyBatch. Routines for summarisation produce exprSet objects containing expression summary values.

There are currently 4 main algorithms that can be used for preprocessing the samples; MAS5, RMA, gcRMA and dChip. Literature studies comparing the above algorithms showed that dChip does not perform in a consistent manner, however none of the comparison studies showed the best algorithm to use to preprocess data from Affymetrix expression arrays. Some studies preferred MAS5 while others suggested that either RMA or gcRMA is best. Therefore, for this thesis a novel strategy was used to preprocess the raw CEL files using both gcRMA and MAS5. This was followed by non-specific filtering of the probes to eliminate non-variant probes.
The algorithm for MAS5 non-specific filtering was as follows:

```
FOR (each probe)
{
    IF ((probe raw value > 50) in > x MALT lymphoma samples)) THEN
        { Label that probe as variant and keep }
    ELSE
        { Discard that probe and move to the next probe }
}
x = is the group containing the least samples

So x in this thesis = 2 because the group containing the least samples is the t(14;18) and this has 2 cases
```

The algorithm for gcRMA non-specific filtering was as follows:

```
FOR (each probe)
{
    Calculate the CV for the probe across all samples
    IF (probe CV > 10% across all MALT lymphoma samples) THEN
        { Label that probe as variant and keep }
    ELSE
        { Discard that probe and move to the next probe }
}
```

Once this is done, a new set of probes is constructed from common probes that passed both gcRMA and MAS5 preprocessing.
The advantage of using this strategy is that MAS5 is used to filter the probes on their absolute values, thus it will keep the low copy variant probes as well as the obvious highly variant probes. gcRMA is used to eliminate any non-variant probes across all samples, which is good where some probes might be high due to the tissue specificity and not because their contribution to the tumour part of the sample.

This strategy is totally unsupervised and proved to be the best for normalising and filtering probes from difficult studies such as in this thesis where; firstly, there are no controls as such, thus the comparison is made between groups of the same entity i.e. MALT lymphoma. Secondly, the number of cases in some of the groups is very small to be statistically feasible e.g. only two cases of t(14;18) and 4 cases of t(1;14) (3 t(1;14) and 1 t(1;2)) were available.

Systematic testing of individual preprocessing algorithms and combination of (RMA and MAS5) and (RMA and gcRMA) followed by false discovery rate (FDR) multiple testing corrections showed the consistent loss of probes mapping to BCL10, TLR6 and CD69.

However gcRMA and MAS5 gave the best trade-off with MAS5 giving high number of probes with some false positive but gcRMA giving lower number of probes with few false positive. This strategy was used as the initial step for analysis of all the array data in this thesis.
I.II Analysis of differentially expressed genes

Many microarray studies are designed to detect genes associated with different groups (phenotypes), for example in this thesis, the comparison of MALT lymphoma with and without chromosome translocation cases and comparison of MALT lymphoma against other lymphomas. The distribution of gene expression data is generally parametric thus the array data was log transformed in order to make the distribution of the replicated measurements per gene roughly symmetric and close to normal. A variance stabilizing transformation derived from an error model for microarray measurements was employed to make the variance of the measured intensities independent of their expected value. This can be advantageous for gene-wise statistical tests that rely on variance homogeneity, because it will diminish differences in variance between experimental conditions that are due to differences in the intensity level, but differences in variance between conditions may also have gene-specific biological reasons, and these will remain untouched.

Generally, for the comparison of MALT lymphoma with and without chromosomal translocation in chapter 3, t-test (ANOVA on two groups) was applied with the error model to obtain set of genes that are differentially expressed between the two groups. However, for the phenotypic marker study in chapter 6, multiple group ANOVA and eBayes which is part of the limma package in bioconductor, were applied separately to the same set of probes and the common probes from each analysis were combined to create a new set.

eBayes fits the probe data to a linear model and works best when the variability of the log-ratios is as homogenous as possible across the probes, whereas multiple group ANOVA can cope with slight heterogeneity in the data. eBayes is more stringent because it shrinks the data and results in few genes across the groups whereas multiple group ANOVA is less stringent and give larger set of genes with more false positives even after the stringent Bonferroni multiple testing correction. Thus a combination of both for the phenotypic marker study proved the best strategy. Finally in order to manage the vast amount of testing between the groups, a relational database management software was constructed as described in Appendix II.
I.III Construction and annotation of gene sets for GSEA

Creation and annotation of human immune gene sets

The Gene Set Enrichment Analysis (GSEA) is a powerful technique for elucidating various groups of genes that may be important from gene expression data. However, one drawback of the current implementation of GSEA is that the gene sets are only as good as the annotation and the immunology gene set annotations from GO are poor and do not follow a certain pathway. Thus in addition to running the 4395 pathways as mentioned in section 2.2.5.4, the challenge was to see which of the immune system pathways are significantly enriched in MALT lymphomas with and without chromosome translocations. For this thesis, the genes and proteins of the essential human immunome were identified and collected by literature search, reviewing the existing databases such as Immunome at: http://bioinf.uta.fi/Immunome/, ImmTree at: http://bioinf.uta.fi/ImmTree/, immune pathways in GeneGo (http://www.genego.com/) and immune pathways in Ingenuity (http://www.ingenuity.com/). It is difficult to strictly define immunome genes. In this thesis, a pragmatic approach was taken, where the gene products have to be essential for immunity, but not be widely expressed in many cells and tissues. Using this strategy and in house software tools such as relational database management systems, human immune gene sets were created that broadly fall into the following categories:

1) Antigen Presentation
2) CD genes
3) Cellular Immunity
4) Chemokine
5) Complement System
6) Humoral Immunity
7) Inflammation
8) Innate Immunity
9) Phagocytosis
10) Transcription Factor
11) B-cell receptor signalling
12) T-cell receptor signalling
13) TLR signalling pathway
14) Chemotaxis of leukocytes
15) Immune response to bacteria
Creation and annotation of NF-κB target genes set

NF-κB target gene is defined in broad terms as a gene that has a κB site in its promoter. To date there is no comprehensive list of NF-κB target genes, thus a comprehensive list of NF-κB target genes was collated by bioinformatics, literature search and Internet search of NF-κB target genes list at: http://people.bu.edu/gilmore/nf-kb/target/index.html and http://bioinfo.lifl.fr/NF-KB/

Bioinformatics strategy

Bioinformatics search algorithm is summarised as follows:

Find promoter of gene of interest

Using TRANSFAC
Does the promoter have NF-κB site?

NO

YES

Preliminary candidate for NF-κB inducible gene

Check using Ensembl, SwissProt and other software if the human NF-κB site is conserved in mouse or Drosophila genome

YES

The gene of interest is NF-κB inducible
This strategy identified the following genes:

<table>
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<tr>
<th>Gene symbol</th>
<th>Gene full name</th>
</tr>
</thead>
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<td>interleukin 32</td>
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<td>RCP9</td>
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<td>PR domain containing 2, with ZNF domain</td>
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<td>beta-site APP-cleaving enzyme 2</td>
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</tr>
<tr>
<td>LAMC2</td>
<td>laminin, gamma 2</td>
</tr>
<tr>
<td>BCL2L10</td>
<td>BCL-2-like 10 (apoptosis facilitator)</td>
</tr>
<tr>
<td>TNFRSF6</td>
<td>tumor necrosis factor superfamily, member 6</td>
</tr>
<tr>
<td>CD105</td>
<td>homodimeric transmembrane protein which is a major gl</td>
</tr>
<tr>
<td>TNFRSF6</td>
<td>tumor necrosis factor receptor superfamily, member 6, d</td>
</tr>
<tr>
<td>TNFSF5</td>
<td>tumor necrosis factor superfamily, member 5</td>
</tr>
<tr>
<td>BM2</td>
<td>influenza B virus BM2</td>
</tr>
<tr>
<td>HC3</td>
<td>proteasome subunit HC3</td>
</tr>
<tr>
<td>SIAT8A</td>
<td>ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransfer</td>
</tr>
<tr>
<td>TBR</td>
<td>tuberin</td>
</tr>
<tr>
<td>TNFRSF5</td>
<td>tumor necrosis factor receptor superfamily, member 5, d</td>
</tr>
<tr>
<td>RBCK1</td>
<td>RanBP-type and C3HC4-type zinc finger containing 1</td>
</tr>
<tr>
<td>CCR2A</td>
<td>chemokine (C-C motif) receptor 2 isoform A</td>
</tr>
<tr>
<td>CCR2B</td>
<td>chemokine (C-C motif) receptor 2 isoform B</td>
</tr>
</tbody>
</table>

Those genes are not found on the websites mentioned above or literature search.
Literature search

Literature search identified the following genes:

BIRC2, ICAM1, CX3CL1, NR4A3 and BCL10

Internet search

Internet search of NF-κB target genes list was carried out at:


Summary of the categories and genes found in each is presented in the following table:

<table>
<thead>
<tr>
<th>Category</th>
<th>Number of Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines/Chemokines and their modulators</td>
<td>29</td>
</tr>
<tr>
<td>Immunoreceptors</td>
<td>20</td>
</tr>
<tr>
<td>Acute phase proteins</td>
<td>1</td>
</tr>
<tr>
<td>Stress response genes</td>
<td>5</td>
</tr>
<tr>
<td>Growth factors, ligands and their modulators</td>
<td>15</td>
</tr>
<tr>
<td>Early response genes</td>
<td>3</td>
</tr>
<tr>
<td>Proteins involved in antigen</td>
<td>1</td>
</tr>
<tr>
<td>Cell adhesion molecules</td>
<td>6</td>
</tr>
<tr>
<td>Cell surface receptors</td>
<td>10</td>
</tr>
<tr>
<td>Regulators of apoptosis</td>
<td>9</td>
</tr>
<tr>
<td>Transcription factors</td>
<td>20</td>
</tr>
<tr>
<td>Viruses</td>
<td>4</td>
</tr>
<tr>
<td>Enzymes</td>
<td>33</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>41</td>
</tr>
<tr>
<td><strong>Total no.</strong></td>
<td><strong>200</strong></td>
</tr>
</tbody>
</table>

Using all 3 strategies but removing duplication a total of 271 NF-κB target genes were identified. A full list is provided in the complementary DVD attached to the back cover of this thesis.
Overall, a total of 4395 gene sets were identified including 56 custom sets which was constructed as mentioned in this appendix.

Gene set details are summarised in the following table:

<table>
<thead>
<tr>
<th>Name of Gene set</th>
<th>No. of gene set</th>
<th>No. of genes</th>
<th>Access</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5: GO biological process gene sets</td>
<td>825</td>
<td></td>
<td></td>
<td>GO biological process gene sets</td>
</tr>
<tr>
<td>C2: BioCarta gene sets</td>
<td>249</td>
<td></td>
<td></td>
<td>BioCarta gene sets</td>
</tr>
<tr>
<td>C2: KEGG gene sets</td>
<td>200</td>
<td></td>
<td></td>
<td>KEGG gene sets</td>
</tr>
<tr>
<td>Gene Sets from Immunome Database</td>
<td>131</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-kB target genes</td>
<td>271</td>
<td></td>
<td></td>
<td>Genes contain kB binding sites in their promoters and are transactivated by NF-kB</td>
</tr>
<tr>
<td>Innate immunity</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antigen processing and presentation</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Innate immunity</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene Sets from Molecular Signature Database</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular signature pathways that are derived ...</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR signalling</td>
<td>1</td>
<td>33</td>
<td><a href="http://www.ingenuity.com">http://www.ingenuity.com</a></td>
<td>Genes to do with TLR signalling derived from the gene ontology part of the Ingenuity pathway analysis software</td>
</tr>
<tr>
<td>T cell receptor signalling</td>
<td>1</td>
<td>57</td>
<td><a href="http://www.ingenuity.com">http://www.ingenuity.com</a></td>
<td>Genes to do with T cell receptor signalling derived from the gene ontology part of theIngenuity pathway analysis software</td>
</tr>
<tr>
<td>Chemokine signalling</td>
<td>1</td>
<td>36</td>
<td><a href="http://www.ingenuity.com">http://www.ingenuity.com</a></td>
<td>Genes to do with chemokine signalling derived from the gene ontology part of the Ingenuity pathway analysis software</td>
</tr>
<tr>
<td>Antigen Receptor GO1</td>
<td>1</td>
<td>17</td>
<td><a href="http://www.ingenuity.com">http://www.ingenuity.com</a></td>
<td>Genes to do with antigen receptor signalling derived from the gene ontology part of the Ingenuity pathway analysis software</td>
</tr>
<tr>
<td>Gene set derived from Pathways annotated by Ingenuity Systems</td>
<td>75</td>
<td></td>
<td><a href="http://www.genego.com/">http://www.genego.com/</a></td>
<td>Genes related to B cell receptor signalling pathway, derived from the GeneGo Metacore software</td>
</tr>
<tr>
<td>BCR Pathway</td>
<td>1</td>
<td>75</td>
<td><a href="http://www.genego.com/">http://www.genego.com/</a></td>
<td>Genes related to B cell receptor signalling pathway, derived from the GeneGo Metacore software</td>
</tr>
<tr>
<td>ICOS</td>
<td>1</td>
<td>93</td>
<td><a href="http://www.genego.com/">http://www.genego.com/</a></td>
<td>Genes related to ICOS pathway, derived from the GeneGo Metacore software</td>
</tr>
<tr>
<td>B Cell Lymphoma</td>
<td>1</td>
<td>50</td>
<td><a href="http://www.genego.com/">http://www.genego.com/</a></td>
<td>Genes involved in B cell lymphoma derived from the GeneGo Metacore software</td>
</tr>
<tr>
<td>Lymphocyte activation (GO:0046649)</td>
<td>1</td>
<td>137</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Genes involved in lymphocyte activation derived from gene ontology root category of the term GO:0046649</td>
</tr>
<tr>
<td>Activation of NF kappaB inducing kinase (GO:0007250)</td>
<td>1</td>
<td>13</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Genes involved in the activation of NF kappaB inducing kinase derived from gene ontology root category of the term GO:0007250</td>
</tr>
<tr>
<td>Anti apoptosis (GO:0006916)</td>
<td>1</td>
<td>165</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Genes involved in anti-apoptosis derived from gene ontology root category of the term GO:0006916</td>
</tr>
<tr>
<td>T cell mediated immunity (GO:0002456)</td>
<td>1</td>
<td>11</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Genes involved in t cell mediated immunity derived from gene ontology root category of the term GO:0002456</td>
</tr>
<tr>
<td>Lymphocyte mediated immunity (GO:0002449)</td>
<td>1</td>
<td>38</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Genes involved in lymphocyte mediated immunity derived from gene ontology root category of the term GO:0002449</td>
</tr>
<tr>
<td>Positive regulation of immune response (GO:0050778)</td>
<td>1</td>
<td>60</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Genes involved with positive regulation of immune response derived from gene ontology root category of the term GO:0050778</td>
</tr>
<tr>
<td>Negative regulation of immune response (GO:0050777)</td>
<td>1</td>
<td>9</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Genes involved with negative regulation of immune response derived from gene ontology root category of the term GO:0050777</td>
</tr>
<tr>
<td>Regulation of immune response (GO:0050776)</td>
<td>1</td>
<td>77</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Genes involved in regulation of immune response derived from gene ontology root category of the term GO:0050776</td>
</tr>
<tr>
<td>Regulation of adaptive immune response (GO:0002819)</td>
<td>1</td>
<td>11</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Genes from regulation of adaptive immune response derived from gene ontology root category of the term GO:0002819</td>
</tr>
<tr>
<td>Immune response (GO:0006955)</td>
<td>1</td>
<td>596</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Immune response genes derived from gene ontology root category of the term GO:0006955</td>
</tr>
<tr>
<td>B and T cell receptor signalling pathway</td>
<td>1</td>
<td>113</td>
<td><a href="http://www.geneontology.org/">http://www.geneontology.org/</a></td>
<td>Contains 69 and 44 genes for B and T cell receptor signalling respectively</td>
</tr>
<tr>
<td>Finally, in order to manage and query the vast amount of data gathered from microarrays and gene set enrichment analysis, a relational database management software was constructed and included in the complementary DVD attached to the back cover of this thesis.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix II – In house software for gene expression microarray analysis

Two main programming languages were used to write custom made software for analysis of microarray data generated from this thesis; R programming platform with use of some of the bioconductor libraries and Visual Basic programming platform embedded within Microsoft Access in order to write the relational database management software.

II.I R software

R custom software were used to preprocess and analyse the 24 CEL files from MALT lymphoma and 15 CEL files from FL and MCL microarray data, as well as to help post process some of the GSEA data and carry out unsupervised clustering and statistical analysis e.g. for qRT-PCR and identification of differentially expressed genes using Bayesian analysis such as eBayes.

A list of all the custom software is included in the complementary DVD attached to the back cover of this thesis.

The main software R code for pre-processing and analysing microarray data from both the U133A&B and U133plus2 platforms is as follows:
```r
# Analysis of U133A&B MALT lymphoma and U133plus2 FL and MCL
# Software written by Rifat Hamoudi, 2010

library(affy)
library(gcrma)
library(genefilter)
library(gplots)
library(annotate)
library(hgu133a.db)
library(hgu133b.db)
library(hgu133aprobe)
library(hgu133bprobe)
library(gsubfn)

##########################################################################
# Read the U133plus chips of FL & MCL
###########################################################################

setwd("/media/disk/RawArrayData/Cel Files/U133/FL_MCL")

MCL_FL<-ReadAffy()  # read affy files after changedir. Read into
                   # AffyBatch object

sampleNames(MCL_FL)

sampleNames(MCL_FL)[1]<-"FL14"
sampleNames(MCL_FL)[2]<-"FL16"
sampleNames(MCL_FL)[3]<-"FL17"
sampleNames(MCL_FL)[4]<-"FL18"
sampleNames(MCL_FL)[5]<-"FL19"
sampleNames(MCL_FL)[6]<-"FL20"
sampleNames(MCL_FL)[7]<-"FL21"
sampleNames(MCL_FL)[8]<-"MCL22"
sampleNames(MCL_FL)[9]<-"MCL23"
sampleNames(MCL_FL)[10]<-"MCL24"
sampleNames(MCL_FL)[11]<-"MCL25"
sampleNames(MCL_FL)[12]<-"MCL26"
sampleNames(MCL_FL)[13]<-"MCL27"
sampleNames(MCL_FL)[14]<-"MCL28"
sampleNames(MCL_FL)[15]<-"MCL29"

gcrma_mcl_fl<-gcrma(MCL_FL)  # normalization via gcrma
mas_mcl_fl<-mas5(MCL_FL, sc=100)  # normalization via MAS5

# MCL FL groups definition

flnum <- seq(1:7)
mclnum <- c(8,9,10,11,12,13,14,15)
```

allnum <- c(flnum, mclnum)

# GCRMA groups implementation
flgc <- gcrma_mcl_fl[,flnum]
mclgc <- gcrma_mcl_fl[,mclnum]
allflmclgc <- gcrma_mcl_fl[,allnum]

# MAS5 groups implementation
flmas <- mas_mcl_fl[,flnum]
mclmas <- mas_mcl_fl[,mclnum]
allflmclmas <- mas_mcl_fl[,allnum]

# filter stuff on MAS5 abs values
f1<-kOverA(7, 50) # if a gene is 50 or more raw value in more than 7 samples then pass it
ff <-filterfun(f1)
masselect_flmcl <- genefilter(mas_mcl_fl, ff)
sum(masselect_flmcl)
esetmasflmcl <- mas_mcl_fl[masselect_flmcl,]

# filtering gcRMA on CV
cvfun <- cv(0.1, 1.0)
ffun <- filterfun(cvfun)
gcselect_flmcl <- genefilter(gcrma_mcl_fl, ffun)
sum(gcselect_flmcl)
esetgcrmaflmcl <- gcrma_mcl_fl[gcselect_flmcl,]

# Extract the correct set on gcRMA and MAS5
selectgenes_flmcl <- intersect(featureNames(esetmasflmcl),
featureNames(esetgcrmaflmcl))
selectgenes_flmcl
length(selectgenes_flmcl)
esetgcmoodleflmcl <- gcrma_mcl_fl[selectgenes_flmcl,] # gcRMA
esetgcmoodleflmcl

esetmasgoodflmcl <- mas_mcl_fl[selectgenes_flmcl,] # MAS5
esetmasgoodflmcl

# Extract the correct set on gcRMA and MAS5
selectgenes_flmcl <- intersect(featureNames(esetmasflmcl),
featureNames(esetgcrmaflmcl))
selectgenes_flmcl
length(selectgenes_flmcl)
esetgcmoodleflmcl <- gcrma_mcl_fl[selectgenes_flmcl,] # gcRMA
esetgcmoodleflmcl

esetmasgoodflmcl <- mas_mcl_fl[selectgenes_flmcl,] # MAS5
esetmasgoodflmcl

gn <- featureNames(MCL_FL)
ps <- probeset(MCL_FL, gn[1:2])
probeNames(MCL_FL)[1:5]

gcrmaflmclexp <- exprs(gcrma_mcl_f1)
# eliminate AFFX and _x_
idsflmcl <- featureNames(esetmasgoodflmcl)
ids.affx <- grep("^AFFX", idsflmcl)
#noX <- grep("_x_", ids)
ids.noaffx_flmcl <- setdiff(c(1:length(idsflmcl)), ids.affx)
#ids.noaffx <- setdiff(c(1:length(idsflmcl)), noX)
esetgcfinalflmcl <- esetgcgoodflmcl[ids.noaffx_flmcl,]
esetgcfinalflmcl
esetmasfinalflmcl <- esetmasgoodflmcl[ids.noaffx_flmcl,]
esetmasfinalflmcl

###########################################################
#  Read U133A MALT lymphomas
###########################################################
setwd("/media/disk/RawArrayData/Cel Files/U133/MALT/HG133_A")
MALT_A<-ReadAffy()  # read affy files after changedir. Read into
                   # AffyBatch object
sampleNames(MALT_A)
sampleNames(MALT_A)[1] <- "11_18_G0015_A"
sampleNames(MALT_A)[2] <- "11_18_G5125_A"
sampleNames(MALT_A)[3] <- "11_18_G5661_A"
sampleNames(MALT_A)[4] <- "11_18_G6071_A"
sampleNames(MALT_A)[5] <- "11_18_86_14635_Samp11V_A"
sampleNames(MALT_A)[6] <- "11_18_95_10509_Samp1P_A"
sampleNames(MALT_A)[7] <- "11_18_92_10232_Samp2F_A"
sampleNames(MALT_A)[8] <- "11_18_96_8361_Samp3F_A"
sampleNames(MALT_A)[9] <- "11_18_97_107717_Samp7V_A"
sampleNames(MALT_A)[10] <- "1_14_Bel_A"
sampleNames(MALT_A)[11] <- "1_14_G0186_A"
sampleNames(MALT_A)[12] <- "1_14_G0262_A"
sampleNames(MALT_A)[13] <- "1_2_G6389_A"
sampleNames(MALT_A)[14] <- "14_18_02_101211_Samp8V_A"
sampleNames(MALT_A)[15] <- "14_18_97_21350_Samp16V_A"
sampleNames(MALT_A)[16] <- "3_14_G0046_A"
sampleNames(MALT_A)[17] <- "NEG_G0019_A"
sampleNames(MALT_A)[18] <- "NEG_G0055_Nuc_A"
sampleNames(MALT_A)[19] <- "NEG_G0078_Nuc_A"
sampleNames(MALT_A)[20] <- "NEG_G5018_A"
sampleNames(MALT_A)[21] <- "NEG_G6352_Nuc_A"
sampleNames(MALT_A)[22] <- "NEG_88_20237_Samp12V_A"
sampleNames(MALT_A)[23] <- "NEG_92_8149_Samp4F_A"
sampleNames(MALT_A)[24] <- "NEG_91_6360_Samp5F_A"
sampleNames(MALT_A)[25] <- "NEG_96_9991_Samp6F_A"
sampleNames(MALT_A)[26] <- "NEG_89_01810_Samp13V_A"
gcrmamalt_a<-gcrma(MALT_A)  # normalization via gcrma
masmalt_a <- mas5(MALT_A, sc=100)  # normalization via MAS5

# groups definition

neg_A <- c(17,18,19,20,21,22,23,24,25,26)
t11_18_A <- c(1,2,3,4,5,6,7,8,9)
t11_14_A <- c(10,11,12,13)
t14_18_A <- c(14,15)

t11_18set_A <- c(tr11_18_A, neg_A)
t1_14set_A <- c(tr1_14_A, neg_A)
t14_18set_A <- c(tr14_18_A, neg_A)

# GCRMA groups implementation

posneggcrma_A <- c(tr1_14_A, tr11_18_A, tr14_18_A, neg_A)
posneggcrma_A <- gcrmamalt_a[,posneggcrma_A]
pData(posneggcrma_A)$sample <- c(1:25)

t11_18gc_A <- gcrmamalt_a[,t11_18set_A]
t1_14gc_A <- gcrmamalt_a[,t1_14set_A]
t14_18gc_A <- gcrmamalt_a[,t14_18set_A]

# MAS5 groups implementation

posnegv_A <- c(tr1_14_A, tr11_18_A, tr14_18_A, neg_A)
posnegmas_A <- masmalt_a[,posnegv_A]
pData(posnegmas_A)$sample <- c(1:25)

t11_18mas_A <- masmalt_a[,t11_18set_A]
t1_14mas_A <- masmalt_a[,t1_14set_A]
t14_18mas_A <- masmalt_a[,t14_18set_A]

# Read U133B MALT lymphomas

setwd("/media/disk/RawArrayData/Cel Files/U133/MALT/HG133_B")
MALT_B<-ReadAffy()  # read affy files after changedir. Read into
                   # AffyBatch object

sampleNames(MALT_B)

sampleNames(MALT_B)[1] <- "11_18_G0015_B"
sampleNames(MALT_B)[2] <- "11_18_G5125_B"
sampleNames(MALT_B)[3] <- "11_18_G5661_B"
sampleNames(MALT_B)[4] <- "11_18_G6071_B"
sampleNames(MALT_B)[5] <- "11_18_86_14635_Samp11V_B"
sampleNames(MALT_B)[6] <- "11_18_95_10509_Samp1F_B"
sampleNames(MALT_B)[7] <- "11_18_92_10232_Samp2F_B"
sampleNames(MALT_B)[8] <- "11_18_96_8361_Samp3F_B"
sampleNames(MALT_B)[9] <- "11_18_97_107717_Samp7V_B"
sampleNames(MALT_B)[10] <- "1_14_Bel_B"
sampleNames(MALT_B)[11] <- "1_14_G0186_B"
sampleNames(MALT_B)[12] <- "1_14_G0262_B"
sampleNames(MALT_B)[13] <- "1_2_G6389_B"
sampleNames(MALT_B)[14] <- "14_18_02_101211_Samp16V_B"
sampleNames(MALT_B)[15] <- "14_18_97_21350_Samp8V_B"
sampleNames(MALT_B)[16] <- "3_14_G0046_B"
sampleNames(MALT_B)[17] <- "NEG_G0019_B"
sampleNames(MALT_B)[18] <- "NEG_G0055_Nuc_B"
sampleNames(MALT_B)[19] <- "NEG_G0078_Nuc_B"
sampleNames(MALT_B)[20] <- "NEG_G5018_B"
sampleNames(MALT_B)[21] <- "NEG_G6352_Nuc_B"
sampleNames(MALT_B)[22] <- "NEG_88_20237_Samp12V_B"
sampleNames(MALT_B)[23] <- "NEG_92_8149_Samp4F_B"
sampleNames(MALT_B)[24] <- "NEG_91_6360_Samp5F_B"
sampleNames(MALT_B)[25] <- "NEG_96_9991_Samp6F_B"
sampleNames(MALT_B)[26] <- "NEG_89_01810_Samp13V_B"

gcrmamalt_b <- gcrma(MALT_B)  # normalization via gcrma
masmalt_b <- mas5(MALT_B, sc=100)  # normalization via MAS5

# groups definition Bchips
neg_B <- c(17,18,19,20,21,22,23,24,25,26)
t11_18_B <- c(1,2,3,4,5,6,7,8,9)
t1_14_B <- c(10,11,12,13)
t14_18_B <- c(14,15)

t11_18set_B <- c(tr11_18_B, neg_B)
t1_14set_B <- c(tr1_14_B, neg_B)
t14_18set_B <- c(tr14_18_B, neg_B)

# GCRMA groups implementation
posneggcrmav_B <- c(tr1_14_B, tr11_18_B, tr14_18_B, neg_B)
posneggcrma_B <- gcrmamalt_b[,posneggcrmav_B]
pData(posneggcrma_B)$sample <- c(1:25)
t11_18gc_B <- gcrmamalt_b[,t11_18set_B]
t1_14gc_B <- gcrmamalt_b[,t1_14set_B]
t14_18gc_B <- gcrmamalt_b[,t14_18set_B]

# MAS5 groups implementation
posnegv_B <- c(tr1_14_B, tr11_18_B, tr14_18_B, neg_B)

- 255 -
posnegmas_B <- masmalt_b[,posnegv_B]
pData(posnegmas_B)$sample <- c(1:25)

t11_18mas_B <- masmalt_b[,t11_18set_B]
t1_14mas_B <- masmalt_b[,t1_14set_B]
t14_18mas_B <- masmalt_b[,t14_18set_B]

# MALT_A nonspecific filtering
# # filter stuff on MAS5 abs values A chip
f1<-kOverA(2, 50)# if a gene is 50 or more raw value in more than 2 samples then pass it
ff <-filterfun(f1)
maselect_malt_a <- genefilter(masmalt_a, ff)
sum(maselect_malt_a)
esetmasposneg_a <- posnegmas_A[maselect_malt_a,]

# filtering gcRMA on CV
cvfun <- cv(0.1, 1.0)
ffun <- filterfun(cvfun)
gcselect_malt_a <- genefilter(gcrmamalt_a, ffun)
sum(gcselect_malt_a)
esetgcrmaposneg_a <- posneggcrma_A[gcselect_malt_a,]

# Extract the correct set on gcRMA and MAS5
selectgenes_malt_a <- intersect(featureNames(esetmasposneg_a),
featureNames(esetgcrmaposneg_a))
length(selectgenes_malt_a)
esetgcmcgoodposneg_a <- posneggcrma_A[selectgenes_malt_a,] # gcRMA
esetgcmgoodposneg_a

esetmasgoodposneg_a <- posnegmas_A[selectgenes_malt_a,] # MAS5
esetmasgoodposneg_a

gn_a <- featureNames(MALT_A)
ps_a <- probeset(MALT_A, gn_a[1:2])
probeNames(MALT_A)[1:5]
gcrmamaltexp_a <- exprs(gcrmamalt_a)

# eliminate AFFX and _x_
ids_a <- featureNames(esetgcmgoodposneg_a)
ids.affx_a <- grep("^AFFX", ids_a)
ids.noaffx_malt_a <- setdiff(c(1:length(ids_a)), ids.affx_a)
#noX <- grep("_x_", ids)
#ids.noaffx <- setdiff(c(1:length(ids)), noX)

esetgcfinalposneg_a <- esetgcgoodposneg_a[ids.noaffx_malt_a,]
esetmasfinalposneg_a <- esetmasgoodposneg_a[ids.noaffx_malt_a,]

###########################################################
# MALT_B nonspecific filtering
###########################################################

# filter stuff on MAS5 abs values B chip
f1<-kOverA(2, 50) # if a gene is 50 or more raw value in more than 2 
samples then pass it
ff <-filterfun(f1)
masselect_malt_b <- genefilter(masmalt_b, ff)
sum(masselect_malt_b)
esetmasposneg_b <- posnegmas_B[masselect_malt_b,]

# filtering gcRMA on CV
cvfun <- cv(0.1, 1.0)
ffun <- filterfun(cvfun)
gcselect_malt_b <- genefilter(gcrmamalt_b, ffun)
sum(gcselect_malt_b)
esetgcrmaposneg_b <- posneggcrma_B[gcselect_malt_b,]

# Extract the correct set on gcRMA and MAS5
selectgenes_malt_b <- intersect(featureNames(esetmasposneg_b),
featureNames(esetgcrmaposneg_b))
selectgenes_malt_b
length(selectgenes_malt_b)
esetgcgoodposneg_b <- posneggcrma_B[selectgenes_malt_b,] # gcRMA
esetgcgoodposneg_b
esetmasgoodposneg_b <- posnegmas_B[selectgenes_malt_b,] # MAS5
esetmasgoodposneg_b

gn_b <- featureNames(MALT_B)
ps_b <- probeset(MALT_B, gn_b[1:2])
probeNames(MALT_B)[1:5]

gcrmamaltexp_b <- exprs(gcrmamalt_b)

# eliminate AFFX and _x_
ids_b <- featureNames(esetgcgoodposneg_b)
ids.affx_b <- grep("^AFFX", ids_b)
ids.noaffx_malt_b <- setdiff(c(1:length(ids_b)), ids.affx_b)
#noX <- grep("_x_", ids)
#ids.noaffx <- setdiff(c(1:length(ids)), noX)


esetgcfinalposneg_b <- esetgcgoodposneg_b[ids.noaffx_malt_b,]
esetfinalflmclmas_a <- mas_mcl_fl[featureNames(esetmasfinalposneg_a),]

# Map all the MALT_A probes on the FLMCL
#
maltprobes_a <- featureNames(esetmasfinalposneg_a)
esetfinalflmclgc_a <- gcrma_mcl_fl[maltprobes_a,]
esetfinalflmclmas_a <- mas_mcl_fl[maltprobes_a,]

# Map all the MALT_B probes on the FLMCL
#
maltprobes_b <- featureNames(esetmasfinalposneg_b)
esetfinalflmclgc_b <- gcrma_mcl_fl[maltprobes_b,]
esetfinalflmclmas_b <- mas_mcl_fl[maltprobes_b,]

# Find common probes between MALT_A and MALT_B and FLMCL
#
flmclmaltprobes_a <- intersect(featureNames(esetmasfinalflmcl),
featureNames(esetmasfinalposneg_a))
intersectflmclmaltgc_a <- esetgcgoodflmcl[flmclmaltprobes_a,]
intersectflmclmaltmas_a <- esetmasgoodflmcl[flmclmaltprobes_a,]

intersectmaltgc_a <- esetgcgoodposneg_a[flmclmaltprobes_a,]
intersectmaltmas_a <- esetmasgoodposneg_a[flmclmaltprobes_a,]

# MALT_B

flmclmaltprobes_b <- intersect(featureNames(esetmasfinalflmcl),
                              featureNames(esetmasfinalposneg_b))
intersectflmclmaltgc_b <- esetgcgoodflmcl[flmclmaltprobes_b,]
intersectflmclmaltmas_b <- esetmasgoodflmcl[flmclmaltprobes_b,]

intersectmaltgc_b <- esetgcgoodposneg_b[flmclmaltprobes_b,]
intersectmaltmas_b <- esetmasgoodposneg_b[flmclmaltprobes_b,]

setwd("/media/Linux/malt_flmcl_res")

# MALT_A writeout

write.table(exprs(posnegmas_A), file="MALT MAS5 Achip 22283.xls",
append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

write.table(exprs(posneggcrma_A), file="MALT GCRMA Achip 22283.xls",
append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

write.table(exprs(esetgcfinalposneg_a), file="MALT GCRMA Filtered Achip
norm 8181.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

write.table(exprs(esetmasfinalposneg_a), file="MALT MAS5 Filtered Achip
raw 8181.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

# MALT_B writeout

write.table(exprs(posnegmas_B), file="MALT MAS5 Bchip 22645.xls",
append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

write.table(exprs(posneggcrma_B), file="MALT GCRMA Bchip 22645.xls",
append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

write.table(exprs(esetgcfinalposneg_b), file="MALT GCRMA Filtered Bchip
norm 8622.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))
write.table(exprs(esetmasfinalposneg_b), file="MALT MAS5 Filtered Bchip raw 8622.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

# FLMCL writeout
write.table(exprs(mas_mcl_f1), file="FLMCL MAS5 U133plus2 54675.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))
write.table(exprs(gcrma_mcl_f1), file="FLMCL GCRMA U133plus2 54675.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))
write.table(exprs(esetgcfinalflmcl), file="FLMCL GCRMA Filtered U133plus2 norm 10652.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))
write.table(exprs(esetmasfinalflmcl), file="FLMCL MAS5 Filtered U133plus2 raw 10652.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

# FLMCL on MALT writeout
write.table(exprs(esetfinalflmclgc_a), file="FLMCL_MALT_GC Achip 8181.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))
write.table(exprs(esetfinalflmclmas_a), file="FLMCL_MALT_MAS Achip 8181.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))
write.table(exprs(esetfinalflmclgc_b), file="FLMCL_MALT_GC Bchip 8622.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))
write.table(exprs(esetfinalflmclmas_b), file="FLMCL_MALT_MAS Bchip 8622.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

# FLMCL on MALT intersect writeout
write.table(exprs(intersectflmclmaltgc_a), file="FLMCL_MALT_A_GC FLMCL intersect 3871.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))
write.table(exprs(intersectflmclmaltmas_a), file="FLMCL_MALT_A_MAS FLMCL intersect 3871.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))
write.table(exprs(intersectmaltgc_a), file="FLMCL_MALT_GC MALT_A intersect 3871.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(intersectmaltmas_a), file="FLMCL_MALT_MAS MALT_A intersect 3871.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(intersectflmclmaltgc_b), file="FLMCL_MALT_B_GC FLMCL intersect 3034.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(intersectflmclmaltmas_b), file="FLMCL_MALT_B_MAS FLMCL intersect 3034.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

II.II Visual Basic software for relational database management software

Two relational database management software (RDBMS) were written for this thesis. Firstly, an RDBMS entitled “ArrayExplorer RDBMS” which allowed the query and data management generated from the microarray experiments. Secondly an RDBMS entitled “GSEA RDBMS” which contained query and data management functionalities that helped in collating the information generated from GSEA.

Both of these software are included in the complementary DVD attached to the back cover of this thesis.
III. NF-κB target gene set

A list of 271 NF-κB target genes was compiled as described in appendix I.III. 223 genes were obtained from the website:

However the remaining 48 genes were obtained through bioinformatics and literature search as described in appendix I.III. The 48 genes are listed below:

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene full name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIP10</td>
<td>thyroid hormone receptor interactor 10</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>IL32</td>
<td>interleukin 32</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>RCP9</td>
<td>calcitonin gene-related peptide-receptor component pre</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>ANKR1D1</td>
<td>ankyrin repeat domain 1 (cardiac muscle)</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TNFRSF10B</td>
<td>tumor necrosis factor receptor superfamily, member 10</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>AKR1C2</td>
<td>aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase)</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>LDHB</td>
<td>lactate dehydrogenase B</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TEAD1</td>
<td>TEA domain family member 1</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>PRDM2</td>
<td>PR domain containing 2, with ZNF domain</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>BACE2</td>
<td>beta-site APP-cleaving enzyme 2</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>SUZ39H1</td>
<td>suppressor of variegation 3-9 homolog 1 (Drosophila)</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>IL1F9</td>
<td>interleukin 1 family, member 9</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>ALOX12B</td>
<td>arachidonate 12-lipoxygenase, 12R type</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>CARD15</td>
<td>caspase recruitment domain family, member 15</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>CD74</td>
<td>CD74 antigen (invariant polypeptide of major histocompatibility complex class I)</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>CXCL2</td>
<td>chemokine (C-X-C motif) ligand 2</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>DFFB4</td>
<td>defensin, beta 4</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>IL15RA</td>
<td>interleukin 15 receptor, alpha</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TPMT</td>
<td>thiorpurine S-methyltransferase</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TL1R6</td>
<td>toll-like receptor 6</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TL1R4</td>
<td>toll-like receptor 4</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>SH3GLR1L3</td>
<td>SH3 domain binding glutamic acid-rich protein like 3</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>PLA2G2E</td>
<td>phospholipase A2, group IIE</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>ADAMTS12</td>
<td>ADAM metalloprotease with thrombospondin type 1 motif</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>CF2R2A</td>
<td>colony stimulating factor 2 receptor, alpha, low-affinity</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>MMP8</td>
<td>matrix metalloproteinase 8 (neutrophil collagenase)</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>CCL7</td>
<td>chemokine (C-C motif) ligand 7</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TNFRSF21</td>
<td>tumor necrosis factor receptor superfamily, member 21</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>PLA2G4A</td>
<td>phospholipase A2, group IVA (cytosolic, calcium-dependent)</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>LAMC2</td>
<td>laminin, gamma 2</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>BCL2L10</td>
<td>BCL2-like 10 (apoptosis facilitator)</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TNFRSF6</td>
<td>tumor necrosis factor superfamily, member 6</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>CD105</td>
<td>homodimeric transmembrane protein which is a major glycoprotein of the monocyte and macrophage cell surface</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TNFRSF6</td>
<td>tumor necrosis factor receptor superfamily, member 6</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TNFRSF5</td>
<td>tumor necrosis factor superfamily, member 5</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>BM2</td>
<td>influenza B virus BM2</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>HC3</td>
<td>proteasome subunit HC3</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>SIAT8A</td>
<td>ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TBR</td>
<td>tuberin</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>TNFRSF5</td>
<td>tumor necrosis factor receptor superfamily, member 5</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>RIKC1</td>
<td>RanBP-type and C3HC4-type zinc finger containing 1</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>CCR2A</td>
<td>chemokine (C-C motif) receptor 2 isoform A</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>CCR2B</td>
<td>chemokine (C-C motif) receptor 2 isoform B</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>BIRC2</td>
<td>baculoviral IAP repeat-containing 2</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>ICAM1</td>
<td>intercellular adhesion molecule 1 (CD54), human rhinovirus-1a receptor</td>
<td>Blood, 15th of August, 2005 ; 106(4) : 1392 - 1399</td>
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<tr>
<td>CXCL1</td>
<td>chemokine (C-X-C motif) ligand 1</td>
<td>Blood, 15th of August, 2005 ; 106(4) : 1392 - 1399</td>
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<tr>
<td>NR4A3</td>
<td>nuclear receptor subfamily 4, group A, member 3</td>
<td>Journal of Biological Chemistry, 12th of August, 2005 ; 280(32) : 29256-29262</td>
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<tr>
<td>BCL10</td>
<td>B-cell CLL/lymphoma 10</td>
<td>Journal of Biological Chemistry, 6th of January, 2006 ; 281(1) : 167 - 175</td>
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</tbody>
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### III.II Leading edge core set of NF-κB target genes enriched in MALT lymphoma with and without chromosome translocation

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene</th>
<th>Description</th>
<th>Chromosome Band</th>
<th>Entrez ID</th>
<th>Signal to noise</th>
<th>Enrichment Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CXCL5</td>
<td>chemokine (C-X-C motif) ligand 5</td>
<td>4q12-q13</td>
<td>4634</td>
<td>0.530</td>
<td>0.020</td>
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<tr>
<td>2</td>
<td>PTGS2</td>
<td>prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase 2)</td>
<td>2q23-2q25.3</td>
<td>5743</td>
<td>0.519</td>
<td>0.039</td>
</tr>
<tr>
<td>3</td>
<td>NFKB1</td>
<td>nuclear receptor subfamily 4, group A, member 3</td>
<td>1q32</td>
<td>4032</td>
<td>0.485</td>
<td>0.057</td>
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<tr>
<td>4</td>
<td>CCL11</td>
<td>chemokine (C-C motif) ligand 11</td>
<td>17q21.1-q21.2</td>
<td>6356</td>
<td>0.455</td>
<td>0.073</td>
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<tr>
<td>5</td>
<td>PTGDS</td>
<td>prostaglandin D2 synthase</td>
<td>20q11.23</td>
<td>4315</td>
<td>0.388</td>
<td>0.114</td>
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<tr>
<td>6</td>
<td>ABCD1</td>
<td>matrix metalloproteinase 3 (stromelysin 1, progelatinase)</td>
<td>17q12.1-q12.2</td>
<td>4212</td>
<td>0.372</td>
<td>0.128</td>
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<tr>
<td>7</td>
<td>CXCL12</td>
<td>chemokine (C-X-C motif) ligand 2</td>
<td>4q21.3</td>
<td>2920</td>
<td>0.372</td>
<td>0.128</td>
</tr>
<tr>
<td>8</td>
<td>CXCL1</td>
<td>chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)</td>
<td>4q21.3</td>
<td>2919</td>
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<td>0.140</td>
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<td>9</td>
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<tr>
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<td>17q11.2-q11.3</td>
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<tr>
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<td>CD86</td>
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<tr>
<td>12</td>
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<td>20q13.13</td>
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<td>0.161</td>
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<tr>
<td>13</td>
<td>PTGS1</td>
<td>prostaglandin endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase 1)</td>
<td>1q25.2-q25.3</td>
<td>5743</td>
<td>0.519</td>
<td>0.039</td>
</tr>
<tr>
<td>14</td>
<td>NR4A3</td>
<td>nuclear receptor subfamily 4, group A, member 3</td>
<td>4q21</td>
<td>4032</td>
<td>0.485</td>
<td>0.057</td>
</tr>
<tr>
<td>15</td>
<td>CCL7</td>
<td>chemokine (C-C motif) ligand 7</td>
<td>17q21.1-q21.2</td>
<td>6356</td>
<td>0.455</td>
<td>0.073</td>
</tr>
</tbody>
</table>

*Expression of genes enriched in translocation negative MALT lymphoma*
<table>
<thead>
<tr>
<th>Expression of genes enriched in translocation positive MALT lymphoma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes</strong></td>
</tr>
<tr>
<td>KLK3</td>
</tr>
<tr>
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