NEW MOLECULAR MARKERS IN MANTLE CELL LYMPHOMA: STUDIES OF CANNABINOID RECEPTORS, 5-LIPOXYGENASE AND SOX11

Xiao Wang

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To my family with love

The journey of a thousand miles must begin with a single step

--Lao Tzu
ABSTRACT

Mantle cell lymphoma (MCL) is a malignant B-cell lymphoma that accounts for 5-10% of all lymphomas and tends to occur in older adults with a higher incidence in males. The genetic hallmark of this neoplasm is the t(11;14) translocation which results in overexpression of nuclear cyclin D1. The t(11;14) translocation is not sufficient for the development of MCL. Additional oncogenic events are required.

By global gene expression analysis of MCL, the cannabinoid receptor type 1 and 2 (CB1 and CB2), the key enzyme in leukotriene synthesis 5-lipoxygenase (5-LO) and transcription factor SOX11 were found to be highly express in MCL compared to reactive lymph nodes. In this thesis, the possible roles of these genes in MCL were investigated.

In paper I, the expression of CB receptors in the B-cell lymphomas was analyzed by quantitative real time PCR, Western Blot and immunohistochemistry. We found that the majority of B-cell lymphomas expressed CB1 and/or CB2 and that cannabinoids induced cell death in CB1 and CB2 expressing MCL and B-CLL cell lines. Moreover, a metabolically stable synthetic cannabinoid reduced tumor burden in mice xenografted with human MCL. From this study, we can conclude that CB receptors are broadly expressed in B-cell lymphomas and can be a potential target for therapy.

In paper II, the expression of 5-LO in the different subsets of normal B cells and corresponding B-cell lymphomas was investigated. Using reverse transcriptase PCR, Western Blot, and immunohistochemistry, we found that mantle zone, but not germinal centre B cells expressed high amounts of 5-LO. Similarly, primary MCL expressed high levels of 5-LO, while most of follicular lymphomas lacked 5-LO expression. Furthermore, MCL cell lines were capable of producing leukotrienes under certain conditions. Thus, our results strongly indicate that the expression of 5-LO in lymphomas can mimic the expression in the developmental stage of the B cells from which lymphomas arise.

In paper III, immunohistochemical analysis was used to assess a series of B-cell lymphomas. We found that nuclear SOX11 expression appears to detect most MCL, but not B-CLL or follicular lymphomas. SOX11 can therefore be considered as a new diagnostic marker. Importantly, a few MCL lacked nuclear SOX11 expression. Patients with SOX11 negative MCL had worse overall survival compared to those with nuclear expression.

In paper IV, the role of SOX11 in the pathogenesis of MCL was further analyzed. A siRNA knock down system in MCL cell line Granta 519 was used. After the reduction of more than 80% SOX11 mRNA and protein expression, we performed Affymetrix array to analyze the effect of SOX11 on global gene expression. A total of 26 genes were significantly downregulated in SOX11 siRNA treated cells compared to control cells. These genes were validated in gene expression data from two series of primary MCL. In these cohorts there was a strong correlation between SOX11 expression and the expression of DBN1, SETMAR and HIG2. Moreover, using ChiP, we found that SOX11 can directly target DBN1, SETMAR and HIG2 in MCL.

The conclusions from our SOX11 studies are that SOX11 is a new diagnostic marker for MCL and that SOX11 may be of prognostic importance in MCL. DBN1, SETMAR and HIG2 are directly targeted by SOX11 in MCL.
LIST OF PUBLICATIONS

I. Kristin Gustafsson, Xiao Wang, Denise Severa, Maeve Eriksson, Eva Kimby, Mats Merup, Birger Christensson, Jenny Flygare, Birgitta Sander
   Expression of Cannabinoid Receptors type 1 and type 2 in Non Hodgkin Lymphoma: Growth Inhibition by Receptor Activation.

II. Yilmaz Mahshid, Marcus-René Lisy, Xiao Wang, Rainer Spanbroek, Jenny Flygare, Birger Christensson, Magnus Björkholm, Birgitta Sander, Andreas JR Habenicht, Hans-Erik Claesson
    High Expression of 5-Lipoxygenase in Normal and Malignant Mantle Zone B Lymphocytes.
    BMC Immunol. 2009 Jan 9; 10:2

III. Xiao Wang, A.Charlotta Asplund, Anna Porwit, Jenny Flygare, C.I. Edvard Smith, Birger Christensson, Birgitta Sander
    The Subcellular Sox11 Distribution Pattern Identifies Subsets of Mantle Cell Lymphoma: Correlation to Overall Survival.

IV. Xiao Wang, Stefan Björklund, Agata M. Wasik, Alf Grandien, Patrik Andersson, Eva Kimby, Karin Dahlman-Wright, Chunyan Zhao, Birger Christensson, Birgitta Sander
    Gene Expression Profiling and Chromatin Immunoprecipitation Identify DBN1, SETMAR and HIG2 as Direct Transcriptional Targets of SOX11 in Mantle Cell Lymphoma.
    PLoS One, in press
LIST OF ABBREVIATIONS

2-AG  2-arachidonoylglycerol
5-LO  5-lipoxygenase
AEA   N-arachidonylethanolamine
B-CLL B-cell chronic lymphocytic leukaemia
BLT1  LTB4 receptor
BLT2  LTB4 receptor
CB1   Cannabinoid receptor 1
CB2   Cannabinoid receptor 2
DLBCL Diffuse large B-cell lymphoma
ERK   Extracellular-signal-regulated kinases
FDC   Follicular dendritic cell
FL    Follicular lymphoma
GC    Germinal centre
HMG   High-mobility group
JNK   c-Jun-N-terminal kinase
LT    Leukotriene
LTB4  Leukotriene B4
MCL   Mantle cell lymphoma
MZL   Marginal zone lymphoma
NHL   Non-Hodgkin lymphomas
NLS   Nuclear localization signal
PCR   Polymerase chain reaction
R(+)-MA (R+)-methanandamide
siRNA small interfering RNA
SOX11 SRY-related HMG-box11
TAD   Transactivation domain
TdT   Terminal deoxyribonucleotidyl transferase
TF    Transcription factor
THC   Δ9-tetrahydrocannabinol
TMA   Tissue microarray
UTR   Untranslated region
1 INTRODUCTION

By global gene expression analysis of mantle cell lymphoma (MCL), many genes were found overexpressed in MCL compared to reactive lymphoid tissues. This thesis will focus on some of them, the cannabinoid receptor type 1 and 2, the key enzyme in leukotriene synthesis 5-lipoxygenase and the transcription factor SOX11. The aim of this thesis is to investigate the role of these genes in MCL: their possible function, usefulness as diagnostic markers and as potential new targets for therapy.

1.1 B-CELL LYMPHOMA

1.1.1 B cell development and function

The lymphoid system acts as part of the defense mechanisms of the organism against pathogens. It consists of two parts: lymphoid tissue and recirculating lymphocytes. Two forms of lymphoid tissues have been distinguished: Primary (central) and secondary (peripheral) lymphoid tissue. The bone marrow and thymus constitute the primary lymphoid tissues involved in production and early selection of lymphocytes. B cells arise and differentiate to mature naïve B cells in the bone marrow. Mature B cells migrate to secondary lymphoid tissues, which include blood, spleen, lymph node and mucosa associated lymphoid tissue (MALT).

B cells were firstly discovered and characterized in the mid-1960s and early 1970s [1]. B-cell development in mice and human [2, 3] has since then been extensively studied. B-cells develop from hematopoietic stem cells (HSCs) located in the bone marrow. They first differentiate to hematopoietic multipotential progenitors (MPPs) and further to common lymphoid progenitors (CLPs). The CLP has the capacity to develop into T, B or natural killer (NK) cells, but not myeloid-lineage cells [4, 5]. The CLP can differentiate into the first precursor B-cell identified as the so called progenitor B cell or pro-B cell.

Normal B-cell differentiation begins with the first precursor B cell: pro-B cell which can initiate rearrangement of the immunoglobulin (Ig) heavy gene locus, which is the recombination of VDJ (variable, diversity and joining) genes in the heavy chain gene locus occurs. The next precursor stage is the pre-B cells express either the u heavy chain in the cytoplasm or later on express cell-surface μ heavy chain associated with surrogate light chain [6]. There are two specific markers expressed in the precursor B cell stages: CD34 and terminal deoxyribonucleotidyl transferase (TdT). The important function of TdT is that it inserts N-segments
between the V, D and J regions during the recombination of these genes. TdT switches off at the end of the recombination process [7].

The rearrangement of the two Ig light chain genes also takes place in this stage resulting in an immature B cells with expression of a complete IgM on the cell surface. Even though the third B cell stage expresses IgM – the B-cell antigen receptor, it is unable to initiate an immune response to a foreign antigen. At this stage, the fate of cell is uncertain and the immature B cells may be eliminated or become anergic. A small part of the immature B cells (IgM⁺) are positively selected to leave the bone marrow to the spleen where they change to IgM⁺,IgD⁺ mature B cells. Those naïve mature B cells join the circulating pool of B cells [8]. In the peripheral lymphoid organs, they are ready to recognize and bind antigens.

Mature naïve B cells expressing CD5 circulate in the peripheral blood (PB) and the peripheral lymphoid organs where they occupy the primary B-cell follicles and the follicle mantle zone [9, 10]. The naïve B cells may be stimulated by two types of antigens, T-cell independent and T-cell dependent, leading to two types of B cell differentiation. In response to a T-cell independent antigen, mature naïve B cells undergo proliferation and become short-lived plasma cells that produce low affinity IgM. In response to a T-cell dependent antigen, naïve B cells are co-stimulated by T-cells. They move into the centre of a primary B-cell follicle, where they associated with follicular dendritic cell (FDC) proliferate and differentiate into centroblasts and centrocytes to form a germinal centre [11, 12]. In the germinal centre there is a “dark zone” consisting of centroblasts and a “light zone” composed mainly of centrocytes.

The germinal centre B cells express low level of surface Ig receptors. They also, in contrast to naïve B cells, memory B cells and plasma cells, express CD10 and the transcription factor BCL-6 [13]. Another important character of the germinal centre B cells is that they switch off expression of the anti-apoptosis protein Bcl-2, thus they are susceptible to death through apoptosis. Apoptosis of the germinal centre B cells can be prevented by survival and proliferation-promoting signals provided by FDCs and T cells [14].

In the germinal centre, B cells are involved in two different genetic processes: somatic mutation and isotype switching. Somatic mutation occurs mainly in the regions of the immunoglobulin heavy and light chain variable (IgV) genes that form the antigen binding site, aiming to increase the affinity of surface Ig receptors. Since this process is partially random, these mutations may result in decreased affinity of the Ig receptor. Such cells can not bind to the antigen retained on the FDC and therefore do not receive survival signals. Almost 90% of the germinal centre B cells die through apoptosis; only those B cells that have an increased affinity to the antigen presented by FDC will bind to the antigen and receive survival signals from FDC [14, 15]. The isotype switch process takes place on the Ig heavy chain and the switch goes from IgM to IgG, IgA or less commonly to IgE.
Importantly, somatic hypermutation may also occur in non-Ig genes. A fraction of normal GC and memory B cells carry BCL6 and CD95 (also called FAS) gene mutations [16, 17].

The centrocytes that survive in the germinal center mature into long-lived class switched plasma cells or memory cells [7]. Memory B cells, also called marginal zone B cells, comprise some of the cells in the follicular marginal zone of lymph nodes and spleen and also home to the bone marrow. Memory B cells express CD27 and strong IgM but little IgD [18-20]. Plasma cells produced in the germinal centre and these long-lived plasma cells enter the blood and home to the bone marrow. Plasma cells lack expression of B cell markers, CD19, CD20 and sIg, but express the adhesion molecule CD138 and CD38 [21].

Upon antigen stimulation naïve B cells become activated and can develop in three directions: clonal expansion and selection in GC, clonal differentiation extra-GC, or anergy. Eventually, B cells either die or differentiate to memory B cells or antibody-producing plasma cells.

**Figure 1. B-cell differentiation process.**

HSC: Hematopoietic stem cell; CLP: Common Lymphoid Progenitor; GC: germinal center
1.1.2 B-cell lymphoma

The incidence of malignant lymphoma in the western countries, is approximately 20 cases per 100,000 person [22]. According to the Swedish Cancer Registry, about 2000 new lymphoma cases are diagnosed annually in Sweden (http://www.socialstyrelsen.se). Traditionally, lymphomas are classified into Hodgkin and non-Hodgkin lymphomas (NHL). Most NHL are derived from B or T cells [23]. B-cell lymphomas comprise 95% of all lymphomas [24]. They are clonal tumors of immature or mature B cells at various stages of differentiation. More than 20 different types of mature B-cell lymphoma are identified according to the WHO classification [23]. The most common types of mature B-cell lymphomas are follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), B-cell chronic lymphocytic leukaemia/small lymphocytic lymphoma (B-CLL/SLL), mucosa-associated lymphoid tissue (MALT) lymphoma, mantle cell lymphoma (MCL), marginal zone lymphoma (MZL) and Burkitt lymphoma (BL).

Several risk factors for B-cell lymphomas have been established. One major risk factor for mature B-cell lymphoma is an impaired immune function, such as immunodeficiency and autoimmune disease. Underlying causes of immunodeficiency include infection with human immunodeficiency virus (HIV), iatrogenic immunosuppression to prevent allograft rejection or graft versus host disease. Patients infected with HIV have a significantly increased incidence of BL and DLBCL [25-27]. Certain lymphomas are related to viral infections, such as Epstein-Barr virus and hepatitis C virus [28-31], as well as bacterial infections, such as *Helicobacter pylori* [32].

1.1.2.1 Cellular origin of B-cell lymphomas

Assessed by a combination of microscopic appearance (morphology), gene expression profiling and immunophenotype, most B cell lymphomas mirror the characters of the different normal B-cell differentiation stages [24, 33] which thus is an important reference for classification of the B-cell lymphomas [34].

A common viewpoint is that the different types of mature B cell lymphomas are “trapped” in a specific B cell stage, with a specific gene expression profile and a characteristic immunoglobulin somatic hypermutation status. The mutation status of the immunoglobulin gene is characterized as either unmutated variable-region genes, ongoing somatic mutation or mutated variable-region genes [35]. When immunoglobulin genes are somatically mutated it indicates that the B cells are of GC or post-GC origin. FLs display ongoing somatic mutation and have a clear GC expression profile, and the tumor cells also express certain hallmarks of human
GC B cells, such as CD10 and BCL-6 which identify FL as a germinal centre B cell tumor [36, 37]. DLBLs are divided into two major groups, which resemble GC B cells or non-GC B cells, the latter resemble activated B cells (ABC). GC DLBCL subtype expresses genes that correspond well with the normal GC B cells and demonstrates ongoing somatic mutation, indicating its GC origin. The ABC DLBCL expresses genes normally induced during *in vitro* activation of peripheral blood B cells, suggesting its pre- or post-GC origin [37-39]. Among mature B cell lymphomas, only part of MCL and part of B-CLL express unmutated variable-region genes, and resemble pre-GC naïve B cells [40-42]. However, according to recent studies on gene expression profiling, B-CLL cells with unmutated Ig V-region genes were very similar to memory B cells that had undergone somatic hypermutation [43-45]. Moreover, a subset of MCL has been shown to have mutated Ig V region genes, indicating that some MCLs tumor cells have reached a GC stage of development [46-48].

![Cellular origin of human B-cell lymphomas. (Modified from [24])](image-url)
1.1.2.2 Transforming events

A hallmark of mature B-cell lymphomas is the presence of chromosomal translocations involving the Ig genes and a variety of partner genes. As a result of these translocations, oncogenes come under the control of Ig gene enhancers and become deregulated [33, 49]. Translocation occur at three stages in B cell development: during V(D)J recombination in the bone marrow, during somatic hypermutation and during class switch in the GC [24, 33]. Three types of breakpoints are distinguished in the Ig loci. Two paradigms of translocation involved in the V(D)J recombination stage are the t(11;14) (q13 ;q32 ) BCL1-IGH translocation in MCL and the (14;18) (q32 ;q21 ) BCL2-IGH translocation in FL [50, 51]. In MCL, the translocation results in overexpression of cyclin D1, a D-type cyclin involved in control of the cell cycle. Cyclin D1 is used as a diagnostic marker for MCL [52]. In FL, the translocation causes deregulated expression of the anti-apoptotic protein Bcl-2. Also Bcl-2 is used as a diagnostic marker since it can be used in distinguishing normal B cell follicles from the neoplastic follicles seen in FL.

In other translocations the breakpoints are found within or adjacent to rearranged V(D)J genes, and these V region are mostly somatically mutated [33, 53]. The MYC-IG translocations in BL and some translocations of the BCL6 gene in DLBCL show translocation breakpoints in somatically mutated V region genes [54, 55].

The third kind of translocation was detected in the chromosome breakpoints located in IGH switch regions. A lot of different translocations have been detected involving breakpoints in the IGH switch regions such as MYC in sporadic BL [56].

Although the chromosome translocations are important in B cell lymphomagenesis, it is not sufficient to induce lymphoma. Other genetic events are also necessary for lymphoma development and progression, such as mutations in tumor suppressor genes or deregulation of oncogenes such as MYC [24, 57, 58].
1.2 MCL

1.2.1 Epidemiology

Mantle cell lymphoma (MCL) is a mature B-cell neoplasm thought to be derived from the mantle zone B cells. This lymphoma was first described around 30 years ago, and then defined as “centrocytic lymphoma” by the Kiel classification [34], and finally accepted as a separate entity in the early 1990s [59]. MCL comprises approximately 4-10% of all cases of non-Hodgkin lymphoma (NHL) [60]. It occurs in the middle-aged to older individuals with a median age of approximately 60 years (range 29-85). This tumor has a marked male predominance with the ratio around 2-7:1 (male: female) [61]. MCL has one of the worst prognoses among lymphomas, with a median survival of approximately 3 years. MCL patients respond well to initial therapy but the remission time is short and there is no therapy that can be considered as standard [62, 63]. Recently, new treatment protocols have markedly improved the response duration and for patients less than 65 years of age, high dose chemotherapy and autologous stem cells transplantation may lead to cure [63]. However, there is still a need for new therapeutic strategies. Importantly, a subpopulation of MCL has a very indolent clinical course and these have to be identified in order to avoid overtreatment.

1.2.2 Tumor pathology

1.2.2.1 Morphology and site involvement of MCL

Most MCL are wide-spread at the time of diagnosis with involvement of lymph nodes, spleen and bone marrow. Leukemic form of MCL can occur early in the disease or at late stages. Involvement of extranodal sites, such as gastrointestinal tract and Waldeyer’s tonsillar ring [64, 65] is common.

The growth pattern in lymph nodes may be diffuse, nodular or involve the mantle zone, sparing the germinal centers. Most likely this represents different stages of tumor infiltration [61].

In the early stage growth pattern (mantle zone pattern) neoplastic cells surround residual reactive germinal centers and replace the normal follicle mantle [66]. In advanced stages MCL cells grow in nodules, replacing the germinal centers, or diffusely infiltrate the lymph node with effacement of the normal architecture [67]. Four cytologic variants of MCL can be recognized; a small cell variant, a marginal zone-like variant, a pleomorphic variant and a blastoid variant [67, 68]. The blastoid variant and pleomorphic variant are considered as aggressive variants and
are associated to a worse prognosis [68]. Recent findings suggest that the blastoid MCL may arise in patients with previously diagnosed classical MCL and therefore represents histological transformation of the original neoplastic clone rather than a de novo tumor [69].

1.2.2.2 Phenotype

The phenotype of MCL cells is similar to mature, naïve B cells expressing B-cell antigens (for example CD19+, CD20+, CD22+), and IgM and/or IgD surface immunoglobulins often associated with the lambda light chain [67]. MCL cells are usually CD5+ and CD43+, FMC-7+, but CD23-, BCL6 as well as CD10-. Some MCL tumors may be CD5- or weakly positive CD23 [70]. The phenotypic variants make the diagnosis difficult and some MCL may erroneously be diagnosed as CLL. The clinical and biological significance of these phenotypic variants is unclear.

1.2.3 Genetics

1.2.3.1 Antigen receptor genes

The immunoglobulin genes in MCL are rearranged. According to its origin from pre-germinal centre cells, the majority of MCL cases have no or very few somatic mutations in V-gene sequence of Ig (VH) genes. However, 15-40% of the MCL cases show somatic hypermutations in immunoglobulin genes, suggesting that those tumors may originate from a subset of B cells that was experienced in GC environment [47, 71, 72]. In contrast to CLL, the VH gene mutational status in MCL is not associated with patient outcome [47, 73, 74].

1.2.3.2 Cyclin D1 overexpression

The genetic hallmark of MCL is the t(11;14)(q13;q32) translocation. This translocation results in the positioning of the CCND1 gene under control of the immunoglobulin heavy-chain gene enhancer and leads to the overexpression of cyclin D1 mRNA and protein [75, 76]. The deregulated expression of cyclin D1 is present in almost all MCL and is considered the primary molecular event in the pathogenesis of MCL. Cyclin D1 is not detectable in normal B cells which instead may express cyclinD2 and/or D3 [61, 77].

Cyclin D1 cloning identified two major mRNA transcripts of approximately 4.5kb and 1.5kb that differ in the length of the 3’ untranslated region (UTR) but contain the whole coding region that generate a 36kDa polypeptide. Around 4-10% of MCLs lacks the long transcript but overexpress the shorter transcript. The short
transcript lacks the destabilizing AUUUA sequence in the 3’ UTR. The loss of this regulatory sequence is caused by the secondary rearrangement in the 3’ region of \textit{CCND1} gene [52, 78], or by genomic deletions and point mutations [79]. The short transcript has an increased half-life and is related to high level of cyclin D1 protein expression, increased proliferation and poor survival of the patients [76, 79-81].

Cyclin D1 binds to cyclin-dependent kinase 4 (CDK4) and CDK6 to form a cyclin D1-CDK4/CDK6 complex. These complexes promote phosphorylation of the tumor suppressor gene retinoblastoma protein (pRB). In normal cells, RB binds to E2F and then blocks its ability to activate the essential genes required for S phase entry and DNA replication. In MCL hyperphosphorylation of RB1 will lead to release of the E2F transcription factor which then leads to progression of the cell cycle into the S phase [82, 83].

There are two families of CDK inhibitors that can regulate the activity of CDK/cyclin complex. The INK4 family is specific to CDK4/CDK6 whereas the Cip/Kip family, including p27, whose action is broad and targets all the different CDK activities [84]. p27 together with the cyclinE-CDK2 complex may regulate the late G1 phase and the G1-S phase transition. Several ways of deregulating p27 function in MCL have been described. MCLs have normal p27 mRNA expression levels but increased p27 protein degradation activity by the proteasome pathway [85]. Furthermore, p27 is recruited by CDK4-cyclin D1 complexes which then prevent the p27-dependent inactivation of CDK2-cyclin E and G1 cell cycle arrest [86].

\textbf{1.2.3.3 Cyclin D1 negative MCL}

Some cases of MCL are negative for cyclin D1 and the t(11;14), but this particular subset of tumors share the same morphology, phenotype, global expression profile and clinical presentation as conventional MCL [81, 87, 88]. These cases have a high expression of cyclin D2 and cyclin D3. Deregulation of cyclin D2 is in some cases a result of t(2;12)(p12;p13) translocation fusing cyclin D2 to the kappa light chain locus [89]. It is difficult to recognize the cyclin D1 negative MCL since there are no specific diagnostic markers for this entity.

\textbf{1.2.3.4 Secondary genetic events}

The t(11;14) translocation is the important event for MCL initiation, but not sufficient for the development of MCL. Instead additional oncogenic events in the progression of MCL are needed [57, 58].
Such events could be deregulation of key genes involved in cell cycle control. 20-30% of blastoid variants have deletions of the CDKN2A locus on 9p21 [90] which encodes two key regulatory elements, the CDK4 inhibitor INK4a and the p53 regulator ARF. INK4a deletion together with cyclin D1 dysregulation increases the amount of active cyclin D1-CDK4 complexes, and then promotes G1/S phase transition in MCL. ARF stabilizes p53 protein by negatively regulating MDM2-mediated degradation. Mutations in the TP53 gene itself is associated with a poor prognosis in MCL [91].

Another important oncogenic alteration in the pathogenesis of MCL is in genes targeting the DNA damage response pathway. The most common secondary cytogenetic alterations in MCL are the deletions of the region on 11q22-23 including the ataxia-telangiectasia mutated (ATM) genes [92]. ATM plays an important role in the activation of p53 in response to DNA damage, such as double strand break lesions (DSBs) [93]. ATM deletion has been reported in 40-75% of MCL [94].

Deregulation of survival and apoptosis pathways may also contribute to the MCL oncogenesis. The most relevant apoptotic pathway is the BCL2 system. The overexpression of BCL2 protein in MCL can inhibit the cytochrome C1 system and thus prevent caspase 9-mediated cell death [95].

1.2.4 Prognosis and predictive factors

The most important prognostic marker in MCL patients is a high mitotic index or high frequency of cells expressing the proliferation-associated antigen ki67 [96, 97]. Gene expression analysis identified a series of genes which predict the survival in MCL patients [81]. This set of genes is called the proliferation signature and consists of 20 genes. Recent studies reported that a five-gene model that can predict survival in MCL using quantitative RT-PCR. The five-gene signature consists of RNA, MYC, TNFRSF10B, POLE2, and SLC29A2 [98].
Figure 3. Molecular pathways involved in the pathogenesis of MCL. Proteins overexpressed are shown in red. (Modified from [99])
1.3 THE ENDOCANNABINOID SYSTEM

The endocannabinoid system consists of the endocannabinoids, the cannabinoid receptors, CB1 and CB2 and the enzymes involved in the endocannabinoid synthesis (N-acyl phosphatidylethanolamine phospholipase D, NAPE-PLD) and degradation (fatty acid amid hydrolase, FAAH) [100-102].

1.3.1 Cannabinoids

There are three types of cannabinoids, including the natural plant derived cannabinoid, such as cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC), endogenous cannabinoids, endocannabinoids which are endogenous agonists to CB1 and CB2 and synthetic cannabinoids which are developed in the laboratory. The endocannabinoids N-arachidonylethanolamine (anandamide, AEA) [103] and 2-arachidonoyl glycerol (2-AG) are best studied [104]. Commonly used synthetic cannabinoids are WIN 55,212-2, (R)-methanandamide [105, 106].

The first selective antagonist to the CB1 receptor, SR141716A (Rimonabant or Acomplia) [107] was introduced in the mid-1990, SR141716A showed high binding affinity to CB1, but low to CB2 [108]. Rimonabant can significantly reduce the food intake and body weight gain in obese animals [109, 110].SR144528 is the first selective and active antagonist to CB2 receptor [111].

1.3.2 Cannabinoid receptors and their functions

1.3.2.1 Cannabinoid receptors

In the early 1990s, the two seven-transmembrane G-proteins coupled (GPCR) receptors, CB1 and CB2, were discovered [100, 101, 112]. CB1 was denoted the central cannabinoid receptor based on its predominant expression in the CNS, while CB2 was denoted the peripheral cannabinoid receptor based on its expression in the peripheral immune system. Recent evidence show that CB1 can also be expressed in non-neuronal tissues, such as immune cells and reproductive tissues [113, 114], and CB2 is expressed also in CNS [115]. The CB1 receptor is highly conserved across species, while the CB2 receptor is less conserved. The two human receptors have only 44% identity in the total protein sequences [101], but they have a conserved ligand binding region [116]. Two splice variants of CB1 have been described, CB1a and CB1b. They are expressed at low levels in a variety of tissues and display increased
binding affinity for WIN 55,212-2 compared to the full length CB1 receptor [117].

In addition to these two receptors, other receptors such as transient receptor potential vanilloid type-1 (TRPV1) receptor can also bind cannabinoids including AEA [118]. The G protein coupled receptor GPR55 expressed in the brain and some peripheral organs and sharing little identity with CB1 and CB2, was recently discovered to bind several cannabinoids [119].

1.3.2.2 The functions of CB receptors

The major function of CB1 is inhibition of neurotransmitters release, while CB2 regulates cell migration and cytokine release in the immune system [120]. In addition to their roles in neuromodulation and immune function, the cannabinoid signaling pathway is also involved in the regulation of cell fate, including activation of mitogen-activated protein (MAP) kinase cascades: extracellular-signal-regulated kinases (ERK), c-Jun-N-terminal kinase (JNK) and p38, and ceramide generation [120, 121].

1.3.3 Cannabinoids and cannabinoid receptors in cancer

The natural cannabinoid, THC, was firstly used as an anti-cancer agent for treatment of Lewis lung carcinoma in 1975 [122]. However, there were no further studies in this field carried out until in the 1990s. Since then, great effort has been put into the investigation of the anti-cancer effect of cannabinoids in different types of cancer [121].

Increased expression of the CB1 and CB2 receptors has been shown in several cancers, such as lymphoma, acute myeloid leukemia (AML), breast cancer, hepatocellular carcinoma, skin cancer and prostate cancer cells in comparison to their normal counterparts [123-127]. Interestingly, high expression of CB1 receptor in the prostate, pancreatic cancers patients is associated with worse outcome compared to those with less expression [128, 129]. However, in hepatocellular carcinoma, overexpression of CB1 and CB2 receptors correlates with improved prognosis of patients [130].

Cannabinoids have been found to induce tumor cell apoptosis, cell cycle arrest, anti-angiogenic activity and reduce migration of tumor cells [120, 121]. The effects of cannabinoids on several cancers have been reported. In colon cancer, glioma and leukemia, THC binding to CB1 receptor can induce apoptosis through dephosphorylation of ERK and AKT and activation of BAD [131-133]. In prostate cancer, AEA induced a decreased EFGFR expression by
acting on CB1 receptor, which in turn inhibits the EGF-stimulated growth of tumor cells [134].

*In vivo*, anti-tumor effects of cannabinoids have mainly been studied in xenograft models. If tumors express CB receptors, the tumor cells are targeted by cannabinoids with in turn inhibiting tumor growth [122, 135, 136]. However, tumor cells lacking expression of CB receptors may not been affected by cannabinoids [137]. In human study, Salazar *et al.* showed that targeting CB1 receptor with THC in human glioma cells caused cell death via ceramide accumulation, ER stress, autophagy and ultimately apoptosis [138].

Thus, cannabinoids in combination with other conventional therapy could be used in the treatment of cancers.

### 1.4 5-LIPOXYGENASE

#### 1.4.1 Regulation of 5-LO expression and activity

The human 5-LO is a 674 aa, 78 kDa enzymatic protein. The enzyme contains a non-heme iron involved in catalysis which is active in its ferric (Fe$^{3+}$) form [139]. 5-LO is expressed mainly in leukocytes, in line with the function of LTs as mediators of immune reactions. Granulocytes, mast cells, dendritic cells, monocytes/macrophages and B lymphocytes express 5-LO [140].

A few factors can regulate the 5-LO expression and activity. The regulation of 5-LO expression at transcriptional level is complex and is related to the methylation of 5-LO promoter [141]. Also the localization of the enzyme affects its function. Phosphorylation of 5-LO can modulate its subcellular localization as well as its activation [142].

In resting cells, 5-LO is localized in either the cytosol or inside the nucleus. Upon activation, 5-LO moves to the nuclear membrane, where interacts with cytosolic phospholipase of class A2 (cPLA2) and 5-LO activating protein (FLAP) for LT biosynthesis. The regulation of 5-LO activity is influenced by many factors. Calcium ions in combination with phosphatidylcholine (PC) or coactosin-like protein (CLP) can stimulate 5-LO translocation to the nuclear membrane and induce 5-LO activity [140].

#### 1.4.2 Biosynthesis of leukotrienes

5-LO has a central role of biosynthesis of leukotrienes (LTs). LTs are lipid messengers involved in autocrine and paracrine cellular signaling. They are
synthesized from arachidonic acid (AA). In the first step 5-LO in concert with FLAP converts AA to 5-HPETE and 5-HPETE can be further converted to an intermediate leukotriene A4 (LTA4). LTA4 can be hydrolyzed by the enzyme LTA4 hydrolase to leukotriene B4 (LTB4) or conjugated with glutathione by LTC4 synthase to produce leukotriene C4 (LTC4) [143, 144]. LTC4 can be future converted to leukotriene D4 (LTD4) and leukotriene E4 (LTE4). LTC4, LTD4 and LTE4 are also called cysteiny1 leukotrienes (cys-LTs).

**Figure 4.** 5-lipoxygenase (LO) activation in the cell.

cPLA2: cytosolic phospholipase A2.
FLAP: 5-LO activating protein. LTs: leukotrienes (modified from[140])
1.4.3 The role of 5-LO and LTB4 on B cells

Biosynthesis of LTs from AA has for a long time been thought to be restricted to myeloid cells. Not until in the 1990s, the expression of 5-LO and the ability of human B lymphocytes to convert AA to LTB4 was reported [145, 146]. The activation of LT synthesis in B cells is quite different from myeloid cells. Myeloid cells produce LTs upon stimulation with calcium. However, the intact B cell can synthesize LTB4 after stimulated by glutathione depleting agents (Diamide) plus AA and calcium. It seems that the cellular oxidative status is important for inducing biosynthesis of LT in intact B cells, although the mechanism of activation of leukotriene biosynthesis is unclear [147, 148].

The major activities of LTB4 include inflammatory cell recruitment, activation of inflammatory cell effector functions, and prolongation of inflammatory cell survival [149]. LTB4 can activate and stimulate effector functions of lymphocytes by affecting the proliferation and differentiation of B cells and the expression of CD23 [150].

Meanwhile, some studies focused on the biological effects of LTB4 on B cells. LTB4 in synergy with interleukin-2 (IL-2) and interleukin-4 (IL-4) played a role in B cell differentiation, which was measured by the increased CD23 expression, DNA synthesis, cell numbers and the increased secretion of IgG and IgM [150, 151].

1.4.4 The role of 5-LO and leukotrienes in cancer

1.4.4.1 The role of 5-LO in cancer

Over expression of 5-LO has been found in several cancers, such as prostate, lung, pancreas, bladder, breast, colon, testicle, esophagus and B-CLL [152-159]. The high expression of 5-LO in tumor tissues make it possible to target the cancer cells by inhibiting 5-LO activity.

Imbalance between cellular proliferation and cell death is the hallmark of cancer. In colon cancer, inhibition of 5-LO significantly inhibited the activation of NFkB and subsequent cell proliferation [160]. In prostate cancer cells, EGF and neurotensin-induced cell growth via the MAPK pathway is highly dependent on the 5-LO activity [161].
In vivo study, it was found that 5-LO inhibition by zileuton significantly inhibited esophageal carcinogenesis in rats [162].

The expression and activity of 5-LO in malignant B cells was first investigated in B-CLL in 1995 [147]. Subsequent studies showed that B-CLL cells produced LTs, and specific LT biosynthesis inhibitors counteracted CD40-dependent activation of B-CLLs. The effects were measured by the inhibition of CD40-induced DNA synthesis and CD40-induced expression of CD23, CD54 and CD150 [159].

1.4.4.2 The role of leukotrienes in cancer

LTB4 can prevent apoptosis of pancreatic carcinoma both in vitro and in vivo. Treatment with LY293111, a LTB4 receptor antagonist in pancreatic carcinoma xenografted mice induced tumor cell apoptosis and resulted in the inhibition of tumor growth [153, 163]. Similar result was found in another study with anaplastic large-cell lymphoma (ALCL). LY293111 treatment has been shown to inhibit the proliferation of an ALCL cell line and induce G1-S cell cycle arrest [164].

Cys-LTs are produced by mast cells, macrophages, basophils and eosinophils[165]. These substances are potent constrictors of human bronchial smooth muscle cells both in vitro and in vivo [166]. Cys-LTs also increase vascular permeability in postcapillary venules and recruit inflammatory cells [167]. It is well known that chronic inflammation can increase the risk of cancer. It was reported that in inflammatory bowel disease, LTD4 can activate β-catenin signaling, resulting in up-regulation of Bcl-2 which could increase tumor cell survival [168].

It seems that the 5-LO may be involved in cancer biology, and treatments aimed at inhibiting its activity should improve the efficacy of conventional treatments. However, before my study started, the expression, the activity and the function of 5-LO in MCL was still unknown. It is challenging to investigate the possible role of 5-LO in MCL.

1.5 THE SOX TRANSCRIPTION FACTORS

SOX (SRY-related HMG-box) proteins are a family of transcription factors (TFs) which comprises 20 members in most vertebrates [169]. SOX proteins were named due to their high similarity to the sex-determining region on the chromosome Y (Sry)–related high-mobility (HMG) group box proteins. Thus,
members of SOX gene family were first identified by their homology to the HMG domain of the testis-determining factor in the 1990s [170].

1.5.1 The molecular features of SOX proteins

1.5.1.1 HMG domain

SOX proteins bind to DNA sequence-specifically via a HMG domain with a consensus motif: 5’-(A/T) (A/T) CAA (A/T) G-3’, which allow them to function as TFs. This HMG domain is highly conserved in the SOX family [171, 172]. Unlike most other DNA binding TFs which mainly target the major groove, SOX proteins contacts the minor groove of DNA [173], thus leaving space for other TFs to bind the major groove. SOX proteins may therefore bring together several regulatory elements and promote the formation of protein complexes [174].

The SOX proteins have been subdivided into eight groups (A, B1, B2, and C-H) based on the degree of homology within and outside the HMG domain. SOX proteins belonging to the same group usually have more than 80% sequence identity in their DNA binding HMG-domain and share other well conserved regions [175-177].

1.5.1.2 DNA interaction

The HMG box domain performs the functions of DNA binding, DNA bending, protein interactions, and nuclear import and export. SOX proteins have their own unique preference for the DNA binding hexametric core sequence, 5’-(A/T) (A/T) CAA (A/T) G-3’. For instance, SOX9 and SOX17 prefer 5’AG, but SOX9 prefers 3’GG, and SOX17 prefers 3’G nucleotide [176, 178]. After binding to DNA, SOX proteins also have the ability to bend DNA which may be an essential SOX function. SRY and SOX2 mutations which directly interfere with DNA bending lead to a disease phenotype [179, 180].

SOX proteins are capable to bind DNA, but the binding affinity is lower than other TFs. How to improve the binding efficiency? Several mechanisms that could improve the binding have been identified which will be discussed below. Also, each SOX protein may be expressed in more than one cell type. Furthermore several SOX proteins may use similar DNA binding motifs. How, then, can SOX proteins recognize their appropriate target genes? To answer these questions, another important property of SOX proteins, protein interactions, must be mentioned.
1.5.1.3  Protein partner and co-factors of SOX proteins

SOX protein can interact with three classes of proteins to activate or repress the transcription of the targeted genes. These proteins include (1) other TFs; (2) importins, required for the nuclear import of SOX proteins; and (3) adaptor proteins, which link SOX factors to other proteins.

**SOX interactions with other TFs**

Several TF proteins from different families have been reported to partner with SOX proteins, with the function either to activate or repress transcription from target promoters. The first described example is SOX2 interacting with Oct3 to regulate the expression of fibroblast growth factor 4 gene (Fgf4) in embryonic stem cells [181]. SOX2 can also pair off with other partners in other cell lineages. Kamachi et al reported that Sox2 and TF Pax6 act cooperatively to activate transcription from the enhancer of the lens-specific gene DC5 both *in vivo* and *in vitro* [182].

SOX proteins can also recruit transcriptional repressors binding to promoter sequences. SOX6 and CtBP2 interact to repress Fgf3 transcription through the PS4A promoter [183]. SOX proteins may be expressed in multiple cell types and their target genes in a particular tissue are determined by the presence of specific protein partners.

In all these cases, the SOX proteins and their partners interact through their DNA binding domain.

**Nuclear import proteins**

In addition to binding to DNA and mediating interactions with TFs, the HMG domain also contains signals for nuclear import. This process is mediated by a group of transporter proteins called importins, which recognize a nuclear localization signal (NLS) in the protein. Two NLS sequences localized at N and C termini of the HMG domain have been identified in some SOX proteins (SRY and SOX9) [184]. Nuclear import of SRY is mediated by importin β, which requires the HMG domain C-terminal NLS of SRY. Mutation in this NLS results in cytoplasmic accumulation of SRY and sex reversal [185, 186].

**Adaptor proteins**

Apart from HMG domain, other parts of the SOX proteins also play a role in the regulation of gene transcription. The protein PSD-95, Discs large, zO-1 (PDZ)
class can interact with SOX proteins on other domain. PDZ proteins are a group of adaptor proteins which can assemble multi-protein complexes in appropriate regions of the cells. They usually bind to C-terminal residues of other proteins [187]. During B cell development, both SOX4 and IL-5 have important functions. By using yeast two-hybrid system, interleukin 5 (IL-5) mediated transcriptional regulations via an interleukin 5 receptor α subunit (IL-5Rα) interacting protein, syntenin, was identified [188]. When IL-5 regulate SOX4 activation through its receptor IL-5Rα, syntenin acts as an adaptor to connect SOX4 with IL-5Rα [188].

Figure 5. Three different types of SOX protein-protein interaction. Modified from [189].
1.5.1.4  Transactivation and transrepression

Transactivation domain (TAD) has been identified in many SOX proteins including SOXB1, SOXC, SOXE, and SOXF groups. TADs are well conserved between the different members of the same SOX group, but show only little similarity among different groups [176]. In the SOXC group, the TAD is localized in the C-terminal of the protein and corresponds to the last 33 amino acids [190]. In contrast, the SOXE and SOXF proteins have two separate TADs [191].

Most SOX proteins predominantly function as transcriptional activators. The SOXB group is of particular interest in that. It includes a B1 subgroup of three transcriptional activators: SOX1, SOX2, and SOX3, and a B2 subgroup of two transcriptional repressors (SOX14 and SOX21). Since these five genes have very similar HMG boxes and overlapping expression patterns, the target genes must be regulated by the counterbalance of activating and repressing SOX proteins [176, 192]. SOX13, another interesting SOX protein, can directly bind T-cell factor (TCF1) and modify its activity, and thereby regulate the developmental fate of two lineages of T lymphocytes [193]. From the evidences described above, it seems that the transcriptional actions of the SOX proteins are not only related to the cellular context, but also dependent on the context of the target gene.

1.5.1.5  Post-translational modifications of SOX proteins

Post-translational modification (PTM) regulates the activity, stability and partner protein interactions of TFs [194]. The most common PTMs include modification of SOX protein by phosphorylation, acetylation, and sumoylation [195].

Phosphorylation of SOX proteins has been best studied in SOX9. SOX9 is phosphorylated by the cyclic AMP-dependent protein kinase (PKA) at two residues resulting in increased DNA binding efficacy and transcriptional activity of SOX9 [196]. Furthermore, phosphorylation of SOX9 can modify the nuclear translocation of SOX9 protein in the testis [197].

Acetylation regulates the function of SRY in sex determination by influencing its subcellular localization [198]. Lysine residues are important for another PTM, sumoylation. For examples, SOX2, SOX4 and SOXE proteins can be sumoylated or ubiquitinated in vitro, with the variable consequences for DNA binding efficiency and transcriptional activity [199-201].

These findings will be of critical importance for our understanding the precise mechanisms of SOX function.
1.5.2 The roles of SOX proteins *in vivo*

The SOX proteins have broad functions, and many members contain the ability to regulate several different aspects of development. Furthermore several SOX protein may be involved in one developmental process. The following table shows the most common roles of SOX proteins *in vivo*.

Table1. Roles of SOX proteins *in vivo*

<table>
<thead>
<tr>
<th>Group</th>
<th>Sox members</th>
<th>Majors roles</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>SRY</td>
<td>Sex determination [202]</td>
</tr>
<tr>
<td>B1</td>
<td>SOX1,SOX2,SOX3</td>
<td>Neurogenesis and eye development [203, 204]</td>
</tr>
<tr>
<td>B2</td>
<td>SOX14, SOX21</td>
<td>Neurogenesis: counteraction of SOX1-3 to promote neuronal differentiation [205]</td>
</tr>
<tr>
<td>C</td>
<td>SOX4, SOX11, SOX12</td>
<td>Lymphopoiesis [206]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neurogenesis [207] Organ development [208]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>unknown</td>
</tr>
<tr>
<td>D</td>
<td>SOX5, SOX6, SOX13</td>
<td>Gliogenesis [209]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erythropoiesis [210]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lymphopoiesis [193]</td>
</tr>
<tr>
<td>E</td>
<td>SOX8, SOX10, SOX9, SOX15</td>
<td>Sex determination [211]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neural crest development [176]</td>
</tr>
<tr>
<td>F</td>
<td>SOX7, SOX17, SOX18</td>
<td>Cardiogenesis [212]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hair follicle development [213]</td>
</tr>
<tr>
<td>G</td>
<td>SOX15</td>
<td>Skeletal muscle regeneration [214]</td>
</tr>
<tr>
<td>H</td>
<td>SOX30</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
1.5.3 SOX11

SOX11 together with SOX4 and SOX12 constitute the SOXC group. These one-exon genes are highly conserved among vertebrates [175]. All SOXC members contain two functional domains, the HMG box domain, located in the N-terminus, and the TAD, located at the C-terminus [190]. They share 84% identity in the HMG box and 67% identity in TAD. They are co-expressed in many tissues and might functionally interact in many processes. This thesis will focus on the role of SOX11 in MCL.

1.5.3.1 The human SOX11 molecular features

The human SOX11 gene was first cloned and mapped in 1995 [215]. It was mapped to chromosome 2p25. The full length cDNA is 8743bp possessing a long 3’ UTR. The human SOX11 protein has 441 amino acids (aa) with molecular weight of 46.7 kDa [216, 217]. In SOX11, the HMG box is in the N-terminal third (47-122) and the TAD domain at the C-terminal (408-441) [190, 216].

![Figure 6. The structure of human SOXC proteins, highlighting the two functional domains in each protein.](image)

HMG: High-mobility group box DNA binding domain;
TAD: Transactivation domain.
1.5.3.2 The expression of SOX11

SOX11 is widely expressed during organogenesis. It is also highly expressed in human and mouse fetal tissues, such as CNS and PNS, lung, kidneys, gastrointestinal tract, pancreas, spleen, gonads, bronchial arches and mesenchyme [208, 215, 218]. The SOX11 expression is restricted after birth, and only low transcript levels of SOX11 were detected in adult colon, small intestine, heart and brain [219]. High level of SOX11 is however present in normal human adult prostate tissue and mouse brain [220, 221]. SOX11 expression was not detected in any other adult human or mouse tissues [215].

1.5.3.3 The functions of SOX11

The expression pattern of SOX11 can give some insight into the biological role of this protein. Several pieces of evidence suggested that SOX11 may be essential for embryonic development in both CNS and PNS, tissue remodeling including heart arterial outflow tract formation, skeletal development, and the developing anterior eye segment [208, 218, 222]. SOX11, together with SOX4, can regulate the differentiation of neuronal progenitors [207]. Both SOX4 and SOX11 are required during development of the sympathetic nervous system, but they perform different functions in different stages [223]. SOX11 is involved in the transcriptional regulation of specific gene expression programs in adult neurogenesis, and it functions at the stage of the immature neuron by regulating early steps in neuronal differentiation, migration, and maturation [221]. SOX11 is able to control morphological maturation, such as neurite growth and modulate the peripheral nerve regeneration following the nerve injury [224, 225]. Several studies have shown that the SOX11 protein is able to cooperate with protein partners. SOX11 may synergize with Brn-1/Brn-2 and exhibit cooperate effects in glial cells [226, 227].

In the mouse, two genes which are direct targets of SOX11 protein have been identified, Tubb3 and Tead2. Tubb3 is the class-III β-tubulin gene. Tead2 is TEA domain family member 2, a transcription factor involved in mediating the intrinsic organ growth function of the Hippo signaling pathway [228].

In summary, SOX11 has critical roles in embryonic neurogenesis and tissue remodeling. It also required for neuron survival and neurite growth. However, to date, little is known about the role of SOX11 in hematopoiesis. SOX4 is so far the only gene in SOXC group has been found to be important in lymphocytes development. In SOX4-deficient mice, B cell development can be blocked at the pro-B stage [229, 230].
1.5.4 SOX11 in cancer

High expression of SOX11 and SOX4 has been found in most medulloblastomas. The different expression profiles of the two SOX genes in medulloblastomas may reflect their maturation-dependent expression characteristics during normal tissue development, and they may supply cell specific markers for tumor classifications [231].

Increased SOX11 expression was also reported in gliomas [219]. However, recent evidence showed that human glioma-initiating cell (GIC) from malignant gliomas lacked SOX11 expression. Overexpression of SOX11 prevented tumorigenesis by inducing neuronal differentiation. Clinical studies also showed that downregulation of SOX11 mRNA correlated with a significant decrease in survival [232]. These studies suggest that lack of SOX11 expression was correlated to a less differentiated and more aggressive tumor subtype. Also in ovarian carcinoma, lack of SOX11 is associated to poor prognosis [220]. On the other hand, glioma-directed cytotoxic T lymphocytes (CTLs) were identified to target SOX11 positive gliomas [233].

Thus, it is possible that SOX11 have different effects on tumor cells based on different primary transformation mechanisms. Our understanding of these functions is just at its beginning. More studies are needed to clarify the complicated roles of SOX11 in regulating tumor proliferation and survival in multiple pathological processes.
2 AIMS OF THE PRESENT STUDY

The general aim of the study was to broaden our understanding on the molecular pathogenesis of MCL and to identify possible new therapeutic targets for this disease.

The specific aims were:

1) To investigate the expression and functional roles of cannabinoid receptors in MCL as well as other malignant B-cell lymphomas. To study the possible of the anti-tumor effects of cannabinoids in an animal model for MCL.

2) To study the expression and function of 5-lipoxygenase on different subsets of B cells and MCL.

3) To determine if SOX11 can be used as a diagnostic and prognostic marker in MCL.

4) To further investigate the role of SOX11 in the pathogenesis of MCL.
3 COMMENTS ON METHODOLOGY

The methods used in this thesis are described in the individual papers. Some methodological considerations are discussed below.

3.1 MATERIALS

3.1.1 Cell lines and patient samples

In this thesis, four MCL cell lines Rec-1, Granta 519, JeKo-1, and JVM2 were used. All these MCL cell lines carry the t (11; 14) translocation. Two B-CLL cell lines (MEC1 and MEC2), two BL cell lines (Raji and Namalwa), and a plasma cell line (SKMM-2) were also used.

Different types of B-cell lymphomas diagnosed in the Department of Pathology at Karolinska University Hospital were used. (Paper I, II, III, IV)

3.1.2 Animal model

Ten week old female immunodeficient NOD/SCID mice lacking B cells and T cells were subcutaneously injected with the MCL cell line JeKo. Tumors were visible in 3 weeks. (Paper I)

3.2 METHODS

3.2.1 Quantitative real time PCR

In molecular biology, real-time PCR is a widely used method based on the PCR. It is used to amplify and quantify a gene product. Two common methods for detection of products in real-time PCR: non-specific fluorescent dyes which intercalate with any double-strand DNA, and sequence-specific DNA probes consisting of oligonucleotides which are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its cDNA target. We used SYBR green binding to amplified cDNA and the ΔΔCT method to calculate real-time PCR results. B-actin was used as the endogenous control. (Paper I, IV)
3.2.2 Western Blot (WB)

WB separate and identify proteins based on the molecular size. Proteins are separated by gel electrophoresis. The proteins are then transferred to a PVDF membrane where they are exposed to a primary antibody specific to the target protein. Thereafter a horseradish peroxidase (HRP)-linked secondary antibody is applied. The HPR cleave a chemiluminescent substance, and the reaction product produces luminescence which can be detected by photographic film or image instrument.

Using WB, we detected the protein expression of CB1, 5-LO and SOX11. (Paper I, II, III, IV)

3.2.3 Immunohistochemistry (IHC)

IHC is the most commonly applied immunostaining technique. It is widely used in the basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in the different tissues. Labeled antibodies bind proteins in tissue sections, which then are visualized by a marker such as enzyme. The most common enzyme is HRP which is reacted with 3, 3’-Diaminobenzidine (DAB) to produce a brown staining. The distribution of the stain can be examined by light microscopy.

In our studies, we used peroxidase-DAB system to detect CB1, 5-LO and SOX11 in lymphomas. (Paper I, II, III)

3.2.4 Cell death ELISA

In our paper I, we used cell death ELISA to examine R (+)-MA induced cell death via CB1 and CB2 receptors in lymphoma cell lines. The cell death ELISA is a quantitative sandwich ELISA that detects histone and intranucleosomal DNA fragmentation by binding two different monoclonal antibodies. It allows specific determination of mononucleosomes and oligonucleosomes from the cell lysates. (Paper I)

3.2.5 RNA interference (RNAi)

Small interfering RNAs (siRNA), also known as short interfering RNAs, is a class of 20-25 nucleotides double-strand RNA (ds RNA). siRNA induces effective posttranscriptional silencing of specific target genes in a variety of organisms. RNA interference (RNAi) is the process by which dsRNA induces specific inhibition of gene expression through degradation of complementary messenger RNA (mRNA). In vivo, long dsRNA is processed
by an enzyme called Dicer to form 21-25 nucleotide dsRNA-siRNA. siRNA is incorporated into a large protein complex named RNAi-induced silencing complex (RISC), then the antisense strand of the siRNA is used by RISC to guide mRNA cleavage, so promoting mRNA degradation. siRNA can also be exogenously introduced into cells by different methods to specifically knockdown a gene of interest.

Using RNAi technology, we successfully knocked down the SOX11 gene in MCL cell lines. (Paper IV)

3.2.6 Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) is a widely used method to explore interactions of proteins and DNA in vivo. ChIP can be used to map DNA target sites for TFs and other chromosome-associated proteins. It used to identify TFs in a given promoter. A brief summary of the method, formaldehyde cross links proteins and DNA molecules, the DNA-protein complexes are then fragmented by sonication into uniform fragments of 200-1000 base pair (bp), followed by immunoprecipitation with an antibody to the protein (for example TF) of interest, then the DNA fragments are purified and sequence are determined.

Using ChIP assay, we demonstrated that SOX11 can directly target DBN1, SETMAR, and HIG2 in MCL. (Paper IV)
4 RESULTS AND DISCUSSION

4.1 PAPER I

Expression of cannabinoid receptors type of 1 and 2 in non Hodgkin lymphoma: Growth inhibition by receptor activation.

The expression and function of the cannabinoid receptors in MCL has been described in the previous studies in our group [105, 106, 127]. The aim of this paper is to extend our knowledge about the cannabinoid receptors in lymphomas.

In the present study, we investigated the expression of cannabinoid receptors in a series of B-cell lymphomas (n=62) including B-CLL/SLL, FL, DLBCL, MCL, BL, MZL, and pre-B-ALL. The mRNAs level of CB1 and CB2 was measured by quantitative real-time PCR. The majority of lymphomas expressed high levels of CB1 and/or CB2 mRNA compared to reactive lymphoid tissues. In MCL, uniform overexpression of the CB receptors was found, while the expression was highly variable in other lymphomas.

Two splice variants of CB1 was recently identified in human brain [117], CB1a and CB1b. CB1a and CB1b showed different ligand binding affinity compared to the full length receptor. We investigated the expression of these two splice variants in the lymphoma tissues by PCR, and CB1a could be detected in 27 of the 62 samples (44%), while CB1b was not expressed.

Next, we performed Western Blot and immunohistochemistry in patient samples from different entities expressing various CB1 mRNA levels. From the staining, we found that CB1 expression was not only detected in tumor cells but also in non malignant cells such as endothelium. In some lymphomas, CB1 expression was highly variable in the tumor population. The higher variable expression of CB receptors was also reported in other malignancies, such as glioma and breast cancer [126, 135].

Our previous results showed that treatment with cannabinoid receptor ligands can induce growth suppression and apoptosis in MCL [105, 106]. What could be the functional role of CB receptors in other lymphomas? We investigated the levels of CB receptors on SKMM-2 derived from plasma cell leukemia, Raji and Namalwa (Burkitt lymphoma), MEC1 and MEC2 (chronic lymphocytic leukemia) and Rec1 (MCL), and the cells were treated with endocannabinoid analog R(+)-MA. To confirm the role of the receptors we applied nM doses of SR141716 (SR1) or SR144528 (SR2), specific antagonist
to CB1 and CB2 respectively. The studies showed R(+)-MA can induce caspase-3 activity and cell death in cell lines from MCL and CLL which express both CB1 and CB2 receptors, but not in BL cell lines expressing low levels of CB2 or in the plasma cell line expressing low levels of CB1. In MCL and CLL cell lines, the R(+)-MA induced cell death was abrogated by pretreatment with either SR1 or SR2, which suggested that ligation of both receptors is needed to induce cell death.

To determine the therapeutic potential of cannabinoids in malignant lymphoma in vivo, we xenografted mice with human MCL cells JeKo expressing both CB1 and CB2 receptors. The mice were treated with R(+)-MA twice per day by injection at the same site as the tumor cells injection. We found that the tumors in R(+)-MA treated mice grew slower and were significantly smaller compared with the control group. There was a 25% reduction in mitotic index in the tumors treated with R(+)-MA.

These data suggest that targeting of the endocannabinoid system could be part of future therapies for certain of malignant lymphomas.

4.2 PAPER II

High expression of 5-lipoxygenase in normal and malignant mantle zone B lymphocytes

Our gene expression data showed that 5-LO expression is much higher in MCL than in reactive lymphoid tissues [127].

It was therefore of interest to investigate the expression and the activity of 5-LO in MCL, as well as the expression of 5-LO in different subsets of normal B cells. In normal tonsillar B cells, higher expression of 5-LO was found in mantle zone B cells than GC B cells at both mRNA and protein level.

A common viewpoint is that the different types of mature B cell lymphomas are “trapped” in a specific B cell stage. The cell of origin in most MCL is mantle zone B cells and FL originates from GC B cells. Then, we investigated if the 5-LO expression in MCL and FL resembled 5-LO expression observed in normal B subtype B cells. We found that most MCL samples showed high expression of 5-LO, while most of FL samples did not express 5-LO. Thus we can draw the conclusion that the 5-LO expression in MCL and FL corresponds to the subset of normal B cells from which they origin.
We further investigated the activity of 5-LO in MCL. MCL primary tumor cells and the MCL cell lines Granta 519, Rec-1 and JeKo-1 all expressed 5-LO protein. When stimulated with Diamide plus AA and calcium ionophore, they produced similar amounts of LTB4 as human neutrophils.

Put together, this study demonstrated that the expression of 5-LO in MCL can mimic their origin from mantle zone B cells. The activity of 5-LO in MCL is similar to previous findings in other reports using malignant B cells [159, 234]. However, it is still unclear how 5-LO can be activated in B cells \textit{in vivo}.

4.3 \textbf{PAPER III}

The subcellular SOX11 distribution pattern identifies subsets of MCL: correlation to overall survival

In this study, we investigated the expression of SOX11 in MCL cell lines and tumor tissues.

First, we performed reverse transcriptase PCR (RT-PCR) to investigate the expression of SOX11 in lymphomas using RNA from human and mouse brain as positive controls. The primers had been published in a study of brain tumors [231]. We found that SOX11 was strongly expressed in all the primary MCL samples (5/5), whereas only one of four reactive controls had weak expression. The expression of SOX11 mRNA was variable in 5/6 follicular lymphomas (FL). Among the cell lines, the MCL cell lines Granta 519 and Rec1 (cyclin D1+) were strongly positive as well as the breast cancer cell line MCF-7 (cyclin D1+). T cell lines Jurkat and Molt-4 (cyclin D1-) weakly expressed SOX11 and plasma cell leukemia cell line SKMM-2 (cyclin D1+) had no expression. Thus, SOX11 mRNA was expressed in both lymphomas and reactive lymphoid tissues, and the expression seemed to be cyclin D1 independent.

Subsequently, we performed Western Blot to investigate the protein level of SOX11 in MCL cell lines, Granta 519, Rec-1 and JeKo. The results showed that there were obvious bands in Granta 519, Rec-1 and JeKo, but weak bands in Jurkat and Molt-4, no band in SKMM-2.

Then, it is interesting to identify the distribution and localization of SOX11 in MCL tissues. We constructed a series of tissue microarrays (TMA). In the first TMA we compared 5 MCL, 7 FL, 5 SLL tumors and reactive lymphoid tissues. In MCL, SOX11 was expressed in the nucleus. We found that in reactive lymphoid tissues and FL, SOX11 was weakly expressed in some
biopsies and localized to the cytoplasm of the cells in germinal centre and in neoplastic follicles, respectively. The same pattern was found in few cells in SLL. These results were consistent with a recently published article analyzing more cases of SLL and FL [235]. However, when we analyzed the expression of SOX11 in the second TMA, constructed from a larger cohort of MCL, we found that while most MCL (48/53) had nuclear SOX11 staining, a few cases (5/53) had only cytoplasmic expression. Nuclear SOX11 expression therefore appears to detect most MCL tumors and can be considered as a new diagnostic marker in MCL.

Tumor proliferation is the strongest prognostic marker in MCL. We therefore investigated if SOX11 expression was related to tumor cell proliferation. Our results showed that SOX11 negative MCL had moderate proliferation, but the overall survival of these patients was worse compared with those with nuclear expression.

The present study demonstrates that SOX11 may act not only as a new diagnostic marker but also may carry information of clinical and biologic behavior of MCL.

4.4 PAPER IV

Gene expression profiling and chromatin immunoprecipitation identify DBN1, SETMAR and HIG2 as direct transcriptional targets of SOX11 in mantle cell lymphoma

From our previous study on SOX11 and recent publications from other groups, we know that expression of SOX11 can be a diagnostic marker independent of cyclin D1 in MCL [235-239]. SOX11 can also detect the rare cyclin D1 negative MCL. Certain other subtypes of B-cell lymphomas may be SOX11 positive. However, the role of SOX11 in hematopoiesis is still unclear. In this study, we intended to determine the possible role of SOX11 in pathogenesis of MCL.

In order to investigate the SOX11 target genes, we used siRNA to knock down SOX11 in the MCL cell line Granta 519, which expresses high SOX11 protein but low SOX4 and SOX12 mRNA compared to other MCL cell lines. We evaluated the transfection efficiency of SOX11 siRNA by quantitative real time PCR and WB. The results showed that SOX11 was downregulated both at the transcriptional and translational level. We then tested the effect of SOX on the expression of the class-III β-tubulin gene (TUBB3), which is up-regulated by SOX11 in neural cells [190]. Our results showed that TUBB3
expression was reduced by SOX11 downregulation in MCL cell line Granta 519.

Subsequently, we used the Affymetrix platform to analyze the effect of SOX11 downregulation on global gene expression in Granta 519 cells. 26 genes were significantly downregulated upon SOX11 siRNA treatment compared to cells treated with control siRNA.

To further investigate the interaction between SOX11 and these 26 genes, we analyzed the correlation coefficients between SOX11 and these genes in two sets of gene expression data publically available from studies of primary MCL. We found a strong correlation between SOX11 expression level and the expression of DBN1, SETMAR and HIG2.

To validate the results of the gene expression analysis, we performed quantitative RT-PCR in available primary MCL cells together with three MCL cell lines. We found a significant correlation between the expression of SOX11 and DBN1.

We further investigated whether DBN1, SETMAR and HIG2 are the direct targets of SOX11 by ChIP assay, and it was shown that SOX11 was significantly recruited to DBN1, SETMAR, and HIG2 promoter regions close to transcription start site. DBN1 encodes for the actin binding protein Drebrin 1 which plays a role in the morphogenesis in neurons. SETMAR encodes Metnase could influence cell division and response to cytostatic treatment [240].

In summary, our study showed that SOX11 can directly target DBN1, SETMAR and HIG2. SOX11 may involve in cell migration and cell deviation.
5 CONCLUSIONS

This thesis has focused on the expression and the functional role of four genes overexpressed in MCL, the cannabinoid receptor type 1 and 2, the key enzyme in leukotriene synthesis 5-lipoxygenase and the transcription factor SOX11. The major findings of this thesis are:

Not only in MCL, but also in other mature B cell malignancies cases, the expressions of CB receptors were identified. Treatment of cannabinoid was found to induce apoptosis \textit{in vitro} and to inhibit tumor growth \textit{in vivo}. Therefore, targeting of the cannabinoid system may be of therapeutic use in lymphomas in the future.

High 5-LO expression was found in MCL as well as in normal mantle zone B cells. 5-LO could be activated in MCL cell lines \textit{in vitro}. However, the functional role of 5-LO in MCL is still unknown.

SOX11 was expressed in most MCL and may serve as a new diagnostic marker. The SOX11 transcription factor can directly target DBN1, SETMAR and HIG2 and possibly regulates their expression in MCL.
6 FUTURE PERSPECTIVES

We have identified that SOX11 can be a diagnostic marker in MCL, and it also can directly target DBN1, SETMAR and HIG2 through direct binding to DNA. Therefore, it is interesting to map SOX11 binding sites on a genome-wide scale. ChIP-on-ChIP is a good technique for this project.

SOX11 protein can interact with other proteins to activate or repress the transcription of the targeted genes. However, to date, little is known about the role of SOX11 in hematopoiesis. Thus, a future perspective study would be interesting to investigate if SOX11 needs other partners to regulate genes and if so, what kinds of proteins are involved and in which ways to interact? Two-hybrid screening is considered to a good assay for discovering protein-protein interactions.
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REFERENCES


34. Lennert, K., ed. Histopathology of Non-Hodgkin's Lymphomas: Based on the Kiel Classification. . 1981, Springer-Verlag: Berlin, Germany,


Tsujimoto, Y., et al., *The reciprocal partners of both the t(14; 18) and the t(11; 14) translocations involved in B-cell neoplasms are rearranged by the same mechanism*. Oncogene, 1988. 2(4): p. 347-51.


239. Chen, Y.H., et al., Nuclear expression of sox11 is highly associated with mantle cell lymphoma but is independent of t(11;14)(q13;q32) in non-mantle cell B-cell neoplasms. Mod Pathol. 23(1): p. 105-12.