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Towards New Therapeutic Targets: Identification of Novel Tumor Markers in Chronic Lymphocytic Leukemia

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ÄNTLIGEN!!!!!
(Gert Fylking)

To my family

ABSTRACT

Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in the Western world and is caused by an abnormal accumulation of B lymphocytes in peripheral blood, bone marrow and lymphoid organs. It is a disease mainly of adults.

The clinical outcome of CLL may differ significantly. Some patients have an indolent leukemia with long survival, while others experience an aggressive disease, with an acute need for treatment. At present, no treatment regimen can be considered curative. Novel therapeutic approaches are required. Those aimed at directing the body's natural defense system against the tumor cells require well characterized targets that are exclusively expressed by the tumor cells.

In 2001, microarray studies revealed **FMOD**, a member of the Small Leucine Rich Proteoglycan family (SLRP) and **ROR1**, a member of the Receptor Tyrosine Kinase (RTK) family, as two genes being overexpressed in CLL compared to healthy controls. SLRPs are normally expressed and secreted into the extracellular matrix of collagen-rich tissues. They interact with collagen and participate in signaling regulation by the interaction with integrins, growth factors and their receptors. RTKs are transmembrane receptors for growth factors, cytokines and hormones and play important roles in cellular processes including proliferation, differentiation, migration, metabolism and survival.

The ectopic expression of FMOD and ROR1 in CLL was unexpected and was the prelude to this thesis. FMOD is located on chromosome 1 adjacent to two other members of the SLRP family; **PRELP** and **OPTC**.

In this project, we investigated FMOD, PRELP, OPTC and ROR1 at the gene as well as protein levels and their expression in CLL compared to healthy individuals and other hematological malignancies. We also investigated the effect of siRNA silencing of FMOD and ROR1 in CLL and normal control cells.

FMOD, PRELP, OPTC and ROR1 were expressed in all CLL patients but not in normal controls. ROR1 was detected on the surface of CLL cells, which corresponds to the natural ROR1 localization. PRELP and OPTC, on the other hand, seemed to be abnormally retained within the CLL cells, rather than secreted. The PRELP and OPTC proteins expressed in CLL were further investigated and found to be differentially glycosylated compared to their normal counterparts. The molecular weight of the detected PRELP and OPTC corresponded to completely unglycosylated core proteins. PRELP was detected in the cytosol of CLL cells, while CLL OPTC was found in the nucleus and endoplasmic reticulum.

Using siRNA technology, FMOD and ROR1 were efficiently downregulated which resulted in apoptosis of CLL cells but not of B cells from healthy donors. This suggests that FMOD and ROR1 may be important for the survival of CLL cells.

In conclusion, four genes, FMOD, PRELP, OPTC and ROR1, were found to be ectopically expressed by CLL cells. At least two of these genes, FMOD and ROR1, may be important for CLL cell survival. The reason for the aberrant expressions is not yet known, but once elucidated it may contribute to the understanding of the pathogenesis of CLL. Also, these novel markers might be suitable targets for future immunotherapy in CLL.

LIST OF PUBLICATIONS

The thesis is based on the following publications, which will be referred to in the text by their Roman numbers.

I. **Mikaelsson E**, Danesh-Manesh AH, Lüppert A, Jeddi-Tehrani M, Rezvany MR, Sharifian RA, Safaie R, Roohi A, Österborg A, Shokri F, Mellstedt H, and Rabbani H. Fibromodulin, an extracellular matrix protein: characterization of its unique gene and protein expression in B-cell chronic lymphocytic leukemia and mantle cell lymphoma. *Blood*. 2005 15;105(12):4828-35

II. **Mikaelsson E**, Jeddi-Tehrani M, Ostadkarampour M, Hadavi R, Gholamin M, Akhondi M, Österborg A, Shokri F, Mellstedt H, and Rabbani H. An Aberrant Unique Proline/Arginine-rich End Leucine-rich Repeat Protein (PRELP) is Ectopically Expressed in Chronic Lymphocytic Leukemia Cells. [manuscript]

III. **Mikaelsson E**, Tahmasebi Fard Z, Mahmoudi A, Mahmoudian J, Jeddi-Tehrani M, Akhondi M, Österborg A, Shokri F, Mellstedt H, and Rabbani H. The Small Leucine-rich Proteoglycan Opticin (OPTC) is Ectopically Expressed and Translocated to the Nucleus of Chronic Lymphocytic Leukemia Cells. [manuscript]

IV. DaneshManesh AH, **Mikaelsson E**, Jeddi-Tehrani M, Bayat AA, Ghods R, Ostadkarampour M, Akhondi M, Lagercrantz S, Larsson C, Österborg A, Shokri F, Mellstedt H, and Rabbani H. Ror1, a cell surface receptor tyrosine kinase is expressed in chronic lymphocytic leukemia and may serve as a putative target for therapy. *Int J Cancer*. 2008 1;123(5):1190-95

V. Choudhury A, Derkow K, Daneshmanesh AH, **Mikaelsson E**, Kiaii S, Kokhaei P, Österborg A, and Mellstedt H. Silencing of ROR1 and FMOD with siRNA results in apoptosis of CLL cells. *Br J Haematol*. 2010 Aug 31. [epub ahead of print]

LIST OF ABBREVIATIONS

aa	Amino Acid
ADCC	Antibody-Dependent Cellular Cytotoxicity
ALL	Acute Lymphocytic Leukemia
AML	Acute Myelogenous Leukemia
APC	Antigen Presenting Cell
APRIL	A Proliferation-Inducing Ligand
β2-M	β2-Microglobulin
BAFF	B cell Activation Factor of the TNF Family
BCR	B Cell Receptor
BM	Bone Marrow
CD	Cluster of Differentiation
CDC	Complement-Dependent Cytotoxicity
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeologenous Leukemia
CR	Complete Response
DC	Dendritic Cell
ECM	ExtraCellular Matrix
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
FC	Fludarabine/Cyclophosphamide
FCR	Fludarabine/Cyclophosphamide/Rituxumab
FDCs	Follicular Dendritic Cells
FGF	Fibroblast Growth Factor
FL	Follicular Lymphoma
FISH	Fluorescence In Situ Hybridization
FMOD	Fibromodulin
GAG	GlucosAminoGlycan
GM-CSF	Granulocyte-Macrophage-Colony-Stimulating-Factor
HCL	Hairy Cell Leukemia
HGF	Hepatocyte Growth Factor
ICAM-1	Inter-Cellular Adhesion Molecule-1
IFN	Interferon
Ig	Immunoglobulin
IgVH	Ig Variable region Heavy chain
IL	Interleukin
LDH	Lactate DeHydrogenase
LDT	Lymphocyte Doubling Time
LN	Lymph Node
LPL	LipoProtein Lipase

LRR	Leucine-Rich Repeat
mAb	Monoclonal AntiBody
MBL	Monoclonal B cell Lymphocytosis
MCL	Mantle Cell Lymphoma
MHC	Major Histocompatibility Complex
MM	Multiple Myeloma
NF-kB	Nuclear Factor Kappa B
NK cell	Natural Killer cell
NLC	Nurse-Like Cell
OPTC	Opticin
PB	Peripheral Blood
PBMC	Peripheral Blood Mononuclear Cell
PC	Proliferation Center
PDGF	Platelet Derived Growth Factor
PLL	Pro Lymphocytic Leukemia
PRELP	Proline/arginine-Rich End Leucine-rich repeat Protein
ROR1	Receptor tyrosine kinase-like Orphan Receptor 1
RTK	Receptor Tyrosine Kinase
SDF-1	Stromal cell Derived Factor-1
SLRP	Small Leucine-Rich Proteoglycan
TAA	Tumor Associated Antigen
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-β
TK	Thymidine Kinase
TKI	Tyrosine Kinase Inhibitors
TNF	Tumor Necrosis Factor
TSA	Tumor Specific Antigen
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
Zap-70	Tyrosine kinase zeta-Associated Protein-70

In this thesis, the gene and protein symbols for FMOD, PRELP, OPTC and ROR1 are all printed in non-italicized form for the sake of simplicity. For other genes and proteins, the designations are usually those used in the sited articles.

TABLE OF CONTENTS

1 CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

1.1 CLINICAL FEATURES OF CLL

CLL is the most common form of leukemia in the Western world, representing about 25-30% of all leukemias.[1] It is characterized by the abnormal accumulation of functionally incompetent B lymphocytes in peripheral blood (PB), bone marrow (BM), lymph nodes (LN) and spleen.[2] CLL is a disease of the elderly and the incidence increases with age. The median age at diagnosis is around 72 years.[3] The worldwide incidence is between <1 and 5.5 per 100.000 people and year.[4] The disease is more common in men than in women and in addition, the disease is more frequent in the European population compared to the African or Asian.[1, 5, 6] The etiology of CLL is still unknown but higher prevalence of the disease in relatives of CLL patients suggests a genetic role.[7-10] There is so far no association with either environmental factors or viral infections.[2, 11]

CLL is a heterogeneous disease with a highly variable clinical course, mainly falling into two subclasses; indolent or progressive. Indolent CLL may have a stable or a very slowly increasing peripheral lymphocyte count. It may not develop into a severe condition for many years, and patients can survive long periods without treatment. Progressive CLL, however, is characterized by a more aggressive disease with rapid doubling-time of the tumor cells, and can be fatal in a short period of time.[12, 13]

Although between 0,1% and 1,75% of CLL cells are actively dividing every day[14], the majority of cells are arrested in the G0 phase of the cell cycle.[15] Compared to normal B cells that have a life span of only a few days, circulating CLL cells can survive for several months.[13] The accumulation of CLL cells is due to an imbalance between birth and death rates such that the former exceeds the latter.[14]

With the help of improved diagnostic tools, 70–80% of the CLL cases are discovered in an early indolent phase.[16] There has also been a major progression in understanding the pathogenesis of the disease but despite new treatment regimens leading to improved overall survival, CLL is still considered an incurable disease.[17] There is a constant need for more knowledge and improved treatment strategies.

1.1.1 Diagnosis of CLL

CLL is often discovered by chance, when increased lymphocyte counts are found during routine check-ups.[18] However, some patients are diagnosed upon symptoms and classic first signs of CLL are fatigue, anemia and mild enlargement of LN, liver and spleen. As the disease progresses, the patients often experience fever, weight loss and night sweats. When the disease becomes more advanced, severe anemia, thrombocytopenia and neutropenia are seen due to BM infiltration by tumor cells.[11,12,18] As signs and symptoms develop gradually, it may be difficult to pinpoint the actual onset of the disease.

The diagnosis of CLL is based on the presence of more than 5.0×10^9/L B lymphocytes in blood. Immunophenotypic analysis is also required and CLL cells are $CD19^+$, $CD5^+$, $CD20^+$, $CD23^+$, $FMC7^-$ with weak expression of surface immunoglobulin

(sIg) and weak/neg expression of membrane CD79b. The leukemic cells are monoclonal, with light chain restriction (kappa or lambda Ig light chain).[12]

1.1.2 Prognosis of CLL

CLL is a disease with a highly variable course, with survival ranging from months to several years. Some patients, with very indolent disease, may not require therapy, but for patients with a more aggressive disease, the need for treatment may be urgent. Therefore, the prediction of disease course and selection of suitable treatment regimens are of great importance and the number of prognostic factors is steadily increasing.

1.1.2.1 Clinical prognostic factors

Rai (stage 0-IV)[19] and Binet (stage A-C)[20] staging systems (Table 1), both reflecting tumor burden, have been used for many years and are still the cornerstone in predicting the course of the disease.[3] The systems divide patients into prognostic groups predicting median survival. Patients with Rai stage 0 and Binet stage A disease belong to the low-risk group with a median survival of more than 10 years. Rai stage I + II and Binet stage B are classified as intermediate-risk with a median survival of 6-8 years. Rai stage III + IV and Binet stage C indicate high-risk patients with a median survival of less than 4 years. The drawback of these systems is that they fail to identify patients, who are diagnosed in an early stage and will face a rapid progression.[21]

Table 1. Rai classification and Binet staging systems for CLL (adapted from Gribben, 2010)[3]

System	Clinical features	Median survival, year
Rai stage (simplified 3-stage)		
0 (low risk)	Lymphocytosis in blood and bone marrow	>10
I and II (intermediate risk)	Lymphadenopathy, splenomegaly +/- hepatomegaly	7
III and IV (high risk)	Anemia, thrombocytopenia	0.75 – 4
Binet group		
A	Fewer than 3 areas of lymphadenopathy; no anemia or thrombocytopenia	12
B	More than 3 involved node areas; no anemia or thrombocytopenia	7
C	Hemoglobin <100 g/L, platelets <100 x10 g/L	2-4

Another traditional prognostic parameter is lymphocyte doubling time (LDT). The definition of LDT is “the number of months in which the lymphocytes double in number”.[22] A low LDT (<12 months) indicates an aggressive course and short survival.

1.1.2.2 Serum markers

Lactate dehydrogenase

Lactate dehydrogenase (LDH) is an enzyme present in most organisms. Serum LDH level is considered a marker of cell turnover and increased LDH levels in CLL are associated with shorter survival.[21, 23] LDH may, however, also be increased due to autoimmune hemolytic anemia,[24] a not uncommon complication in CLL.

Thymidine kinase

Thymidine kinase (TK) is an enzyme found in most living cells. It plays an important role in DNA synthesis (and thereby in cell division), being part of the unique reaction chain to introduce deoxythymidine into the growing DNA strand. The predominant cytosolic form of TK is a useful marker of proliferation, since it is present in dividing cells but not in non-dividing cells. In CLL, high serum TK levels have been associated with progressive disease and bad prognosis.[12, 25]

β2-microglobulin

β2-microglobulin (β2-M) is a membrane protein associated with the α-chain of the MHC class I molecule. Normally, β2-M is present in small amounts in serum but the increased production or destruction of β2-M bearing cells causes β2-M levels in the blood to increase. In CLL, high serum levels of β2-M correlate with advanced stage and poor prognosis.[26]

Soluble CD23

CD23 is a membrane glycoprotein which may be cleaved into soluble fragments (sCD23). Functions of the soluble CD23 are among others (1) prevention of germinal center B cells from their entry into apoptosis[27] and (2) proliferation of myeloid precursor cells.[28] In CLL sera, sCD23 is elevated (3–500-fold) compared to control sera and provokes the CLL cells to enter the S phase of the cell cycle.[29] The levels of sCD23 in CLL correlate with disease activity as well as prognosis.[30-32]

1.1.2.3 Cytogenetic and molecular markers

Chromosomal aberrations

Several chromosomal abnormalities are reported in CLL and can be detected in up to 80% of the cases using fluorescence in situ hybridization (FISH). The most common alterations are 13q14 deletion, 11q22-q23 deletion, trisomy 12, 17p13 deletion and 6q21 deletion.[33] There is an important correlation between these aberrations and prognosis. Deletion of 13q14 is associated with a better prognosis, while 17p13 del and 11q22-q23 del are associated with worse prognosis.[33, 34] Cytogenetic changes at 17p13, which lead to loss of p53 function, is clinically the most important factor to consider, as it affects choice of therapy. Conventional therapy is not effective in cases with p53 mutations/ deletion.[35-37]

IgVH mutational status

In 1999, the correlation between IgVH (Ig variable region of the heavy chain) mutational status and prognosis was established and turned the diagnosis of CLL into a new path.[38, 39] During normal B cell development, B cells undergo a number of events to achieve binding specificity for the surface Igs. The initial steps (VDJ gene rearrangements, class switching and nucleotide ins/del) are followed by the introduction of point mutations (somatic hypermutations). 50-70% of the CLL patients have mutated IgVH genes (≥ 2% difference from the germline sequence)[40, 41] and show a more benign course with longer survival[38, 42] while the patients with unmutated IgVH chains genes (<2% difference from the germline sequence)[41] show a more aggressive disease with poorer outcome.[38, 42] However, according to the Swedish CLL guidelines, mutational status does not have influence on clinical decision-making in management of patients to the same extent as cytogenetics (http://www.swecll.org/Nationella-riktlinjer).

1.1.2.4 Surrogate markers

The sequencing of IgVH genes is costly and laborious and a number of surrogate markers have been introduced during the last decade.

Zap-70

Zap-70 is a protein tyrosine kinase, normally expressed by T cells and involved in T cell receptor (TCR) signaling. By gene array studies, it was shown to be expressed also by CLL cells[43-45], facilitating signaling through the B cell receptor (BCR).[46] ZAP-70 was reported to be one of the most differentially expressed genes between unmutated and mutated CLL.[44] High Zap-70 is detected in patients with unmutated IgVH and is associated with more aggressive disease and poor prognosis.[47-49] However, since Zap-70 is expressed also by T cells and NK (natural killer) cells, accurate measurements must be made on purified leukemic B cells, and there is not yet an international standardization on how to measure Zap-70 in CLL.

CD38

CD38 is a 45 kDa transmembrane glycoprotein found on the surface of many immune cells. The expression of CD38 is easily detected by flow cytometry but the reports correlating CD38 expression with mutation status are inconclusive. This may be due to the fact that 1) CD38 expression may vary over time, 2) different cut-off levels to discriminate between positive and negative cases have been used, and 3) CD38 expression show a bimodal profile in some CLL patients.[21]

The value of CD38 as a surrogate marker for IgVH mutation status has been debated but CD38 is still valuable as an additional predictor of prognosis; its expression correlates with poor prognosis.[50]

Lipoprotein lipase (LPL)

Gene array studies identified LPL as one of the most differentially expressed genes in mutated versus unmutated CLL.[43-45] LPL is an enzyme that hydrolyzes lipids in

lipoproteins and is normally expressed in adipose tissue, cardiac and skeletal muscles, and lactating mammary glands. The function of LPL in CLL is not understood, but high LPL values correlate with worse prognosis.[51] LPL is also judged in relation to disintegrin and metalloproteinase 29 (ADAM29), which is overexpressed in mutated CLL.[45] The ratio LPL/ADAM29 is a strong prognostic indicator in CLL (high ratio corresponding to unmutated IgVH and bad prognosis), especially in advanced stage disease.[52]

Telomere

A telomere is a region of hexameric repeats at the end of each chromosome that is essential for chromosome integrity and stability. In normal cells the telomeres become shorter during each cell division and finally the length reaches a critical point at which cell division stops. Thus, telomere length is a measure of a cell's proliferative history. The shortening of the telomeres may be counteracted by the enzyme telomerase, which is often upregulated in cancer.[53] In CLL, the cells from the subgroup with poor outcome (unmutated) have uniformly shorter telomeres and more telomerase activity than those from the subgroup with better outcome (mutated).[54]

Common prognostic parameters are summarized in Table 2.

Table 2. Prognostic factors in CLL (adapted from Herishanu, 2005)[55]

Prognostic factor	**Clinical risk**	
	Low	High
Patient gender	Female	Male
Clinical stage	Binet A Rai 0, I	Binet B or C Rai II, III, IV
Lymphocyte doubling time	>12 months	<12 months
IgVH gene status	Mutated	Unmutated
Genetic abnormalities	None Del 13q14	Del 11q22-23 Del 17p13
Expression of CD38	Low	High
Expression of Zap-70	Low	High
Serum levels of soluble CD23	Low	High
Serum levels of β2-microglobulin	Low	High
Serum levels of thymidine kinase	Low	High

1.1.3 Treatment of CLL

All treatment options for CLL may reduce disease progression and manage symptoms rather than provide curative therapy and the choice of first-line treatment is mostly dependent on the patient's age, risk factors and comorbidities. For patients with no adverse prognostic features and/or when the disease progresses very slowly, treatment is put on hold as long as the patient is asymptomatic. But when a patient becomes symptomatic or when the disease progresses, treatment is indicated. Young patients, patients with high stage disease and/or adverse prognostic factors may benefit from a

more aggressive combination of treatment regimens.[56, 57] Most often, the first treatment of choice is chemotherapy with or without monoclonal antibody (mAb) therapy.

1.1.3.1 Chemotherapy

For many years, alkylating agents such as chlorambucil were used as the front-line therapy for CLL because of their low toxicity, low cost and convenience. However, the complete response (CR) rates were very low.[1] Combining several different alkylating agents (e.g. CHOP; cyclophosphamide, hydroxydaunorubicin (doxorubicin), oncovin (vincristine), and prednisone/prednisolone) resulted in higher response rates but no survival benefits.[11]

Since mid 1980s, purine analogues like fludarabine, cladribine and pentostatin, are the most effective agents in the treatment of CLL. Purine analogues interfere with ribonucleotide reductase and DNA polymerase, thereby inhibiting DNA synthesis and promoting apoptosis. Fludarabine as a single agent has shown much higher response rates, including a prolonged survival at long-term follow up, than chlorambucil.[58, 59] These results, however, have not been confirmed by others.[60] Combining fludarabine with cyclophosphamide (FC regimen) has shown improved response rates, and progression-free survival compared to fludarabine alone.[61] Recently, a hybrid between an alkylator and a purine analogue, bentamustine, was shown to be effective as first line therapy.[62]

1.1.3.2 Monoclonal antibodies

The use of mAbs in CLL treatment started a new era in the mid 1990's. Alemtuzumab, a monoclonal human IgG antibody recognizing CD52, was the first antibody approved for CLL therapy. It is mainly used as a single agent for treatment of relapsed CLL, but also as first-line therapy in selected patients (e.g. 17p13 del).[63]

Rituximab, a chimeric mAb that targets CD20, has shown limited efficacy as single-agent treatment but the addition of rituximab to fludarabine and fludarabine plus cyclophosphamide (FCR regimen) has shown improved overall survival in previously untreated patients.[56, 57]

Ofatumumab, another human CD20 mAb, was recently approved for usage in multi-agent refractory CLL[64] and is currently being tested in combination with chemotherapy.

1.1.3.3 Stem cell transplantation

Allogeneic Stem Cell Transplantation (SCT) is the only CLL treatment capable of cure but is applicable only to a small number of patients. According to European Bone Marrow Transplant (EBMT) guidelines, reduced-dose allogeneic SCT is recommended for young high-risk CLL patients with poor prognostic features, in particular patients with tp53 abnormalities requiring therapy or patients that have relapse after autologous SCT.[65, 66]

1.1.3.4 Novel agents

Despite advances in first-line therapy, relapse rates are high and often accompanied by the development of resistance towards conventional chemotherapy. Therefore, new agents with novel mechanisms of action are needed, especially for relapsing CLL.[67] In addition to new mAbs and some new chemotherapeutic agents, such as bentamustine, there are several novel small interfering molecules, which are currently being evaluated in clinical trials. These molecules may act either by inhibiting protein synthesis or block the action of mature proteins participating in signaling cascades important for cell cycle progression or angiogenesis.

Oblimersen is an antisense oligonucleotide that targets the mRNA of the anti-apoptotic protein Bcl-2, preventing translation of the Bcl-2 protein, thereby preventing its anti-apoptotic effect. Oblimersen alone has shown modest activity but in combination with FC regimen, the overall survival was enhanced compared to FC alone.[68]

ABT-263 has an inhibitory effect on several members of the anti-apoptotic Bcl-2 family and thereby induces apoptosis. ABT-263 has shown significant tumor reduction in a few tested CLL patients.[68]

Obatoclax is a novel small molecule that inhibits not only Bcl-2, but also other anti-apoptotic proteins like BCL-XL and Mcl-1. It induces apoptosis in CLL cells but showed side effects like neurological toxicities, somnolence, euphoria and ataxia in a phase I trial.[69]

Flavopiridol inhibits the action of cyclin-dependent kinases important for cell cycle progression, thereby inducing cell cycle arrest. In CLL, it is believed that flavopiridol also induces apoptosis by down-regulation of the anti-apoptotic protein Mcl-1.[70] Flavopiridol has shown activity in CLL but also toxic side effects.[71]

Vascular endothelial growth factor (VEGF) is an important inducer of angiogenesis and CLL cells express VEGF as well as the receptor, resulting in autocrine stimulation.[72] The VEGF receptor may be inhibited by either tyrosine kinase inhibitors (TKIs) or VEGF-blocking antibodies, leading to inhibition of the VEGF-signaling pathway and induction of apoptosis in CLL.[73]

Several new mAbs are in various phases of clinical testing, targeting structures such as CD19, CD20, CD23, CD37, CD40, CD74 etc. Among these; TRU-016 targeting CD37 appears to be the most promising. CD37 is expressed on B cells and transformed mature B cell leukemias and lymphomas but not on T cells.[74]

1.2 PATHOPHYSIOLOGY OF CLL

1.2.1 Origin of the CLL cell

For a long time it was supposed that the CLL cells were small, non-dividing, immature, B cells that had not experienced antigens. It was also believed that defects in the apoptotic machinery were the reason behind the expansion of the leukemic clone. Today, CLL cells are considered as antigen-experienced, mature cells that escape apoptosis by the interaction with other cells, for example T cells and stromal cell.[75]

1.2.1.1 Cell proliferation and death

In normal tissue homeostasis, there is an ongoing regulation maintaining the balance between cell birth and cell death. However, in cancer this balance is disrupted, which can be due to increased proliferation, decreased cell death, or both, resulting in cell accumulation of the malignant clone.[14] The accumulation of CLL cells was long considered to be due to defective apoptosis in combination with minimal cell proliferation. This idea was re-evaluated when it was discovered that $1x10^9$ to $1x10^{12}$ CLL cells are produced every day. Even though this is a small fraction of the entire CLL clone (0,1-1,75%) CLL can be considered a disease of both cell proliferation and defective apoptosis, where there is a homeostatic balance in patients with stable lymphocyte counts and an imbalance in patients with increasing lymphocyte counts.[14]

1.2.1.2 Antigen stimulation / B cell receptor (BCR)

The BCR is a transmembrane protein composed of a membrane Ig (mIg) homodimer linked to a heterodimer Igα/Igβ (CD79a/CD79b).[76] (Figure 1).

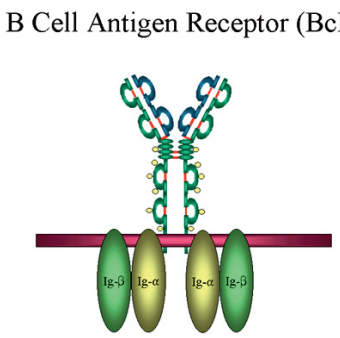

Figure 1. B cell receptor.
(http://pathmicro.med.sc.edu/mayer/stru-9.jpg)

The mIg subunit contains the antigen-binding site while the α/β subunits are responsible for the signal transduction into the cell interior. Ligand-binding to the BCR triggers a complex signaling cascade that involves a number of kinases, phosphatases and adaptor proteins that transmit signals resulting in cell differentiation, proliferation, survival or apoptosis. The BCR is considered a key molecule in CLL pathogenesis, since the clinical course of the disease is related to the IgVH mutational status, Zap-70 expression, the nature of the antigen and the strength and duration of the signal.

CLL cells generally express low levels of the BCR.[77] The synthesis of the BCR components is most likely normal but the assembly on the cell surface is defective due to folding and glycosylation defects of the μ and CD79a chains.[77, 78] However, the different CLL subsets exhibit differences in their capacity to signal through the BCR. CLL with unmutated VH genes appears to have more functional BCRs that may be stimulated by antigen binding and the result (proliferation or apoptosis) is determined by the balance of signals delivered by the activated BCR and survival signals from the microenvironment. CLL with mutated VH genes on the other hand, are less responsive with low capability of

triggering apoptosis, survival or proliferation. It is suggested that these cells may be either anergic, due to chronic antigen exposure, or incapable of responding to antigens because of BCR structure changes (e.g. posttranscriptional defects described above).[41, 79]

When a ligand binds to the BCR, the Ig-α and Ig-β units are phosphorylated by Lyn and other Src kinases. Syk is recruited, activated (phosphorylated) and may phosphorylate several signaling intermediates that in turn activate the downstream signaling molecules Akt, ERK, JNK, p38MAPK, NF-AT and NF-κB which transduce survival and antiapoptotic signals into the nucleus (Figure 2).[80] In CLL, Lyn is strongly overexpressed, leading to enhanced BCR signaling, favoring CLL cell survival.[81] Also, prolonged activation of MEK/ERK and PI3/Akt pathways has been noted in the anti-apoptotic signaling of the BCR in CLL.[82]

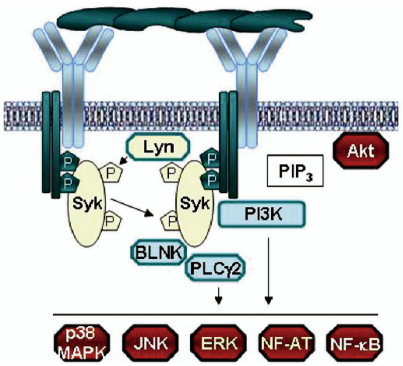

Figure 2. Signaling molecules activated upon BCR engagement.

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In contrast to what was previously known, several findings suggest that CLL founder cells are indeed antigen-experienced. There are a number of studies supporting this theory. The presence of somatic hypermutations indicates that at least the mutated cases are antigen-stimulated (since somatic mutations occur only after antigen stimulation). But also the unmutated cases might be antigen-experienced since they show an obvious skewing in IgVH gene usage, i.e. a high proportion of CLL patients express virtually identical IgVH genes, indicating that they have encountered the same antigen.[83-85] Also, unmutated and mutated CLL have very similar gene expression profiles sharing similarities with antigen-experienced memory B cells.[43, 44] The nature of the promoting antigens is unknown, but they could be of viral or bacterial origin, or may represent environmental antigens or self-antigens.[75, 86]

1.2.1.3 Genetic changes in CLL

Gene expression profiling has revealed a number of genes that are differentially expressed in CLL cells compared to normal B cells.[43] However, despite years of efforts, no common genetic aberration among all patients has been found.[87]

Nevertheless, some of the most common chromosomal alterations in CLL are affecting tumor suppressor genes; deletion 17p13 affects p53 and deletion of 11q23

affects ATM (ataxia telangiectasia). Both p53 and ATM play key roles in protection against DNA damage, cell cycle arrest and induction of apoptosis.[87]

Other genes (NPAT, CUL5 and PPP2R1B), affecting the regulation of cell cycle and apoptosis have been detected in the 11q22-23 segment and have been suggested a role in the pathogenesis of the disease.[88]

The most frequently deleted chromosomal region 13q14 holds 2 microRNA's (miR15a and miR16-1) that negatively regulate the anti-apoptotic protein Bcl-2. Bcl-2 repression by these microRNAs induces apoptosis and their down-regulation has been associated with the survival of CLL cells.[89]

The first disease specific gene that was identified, CLLU1, is located to chromosome 12q22 but there is no obvious difference in CLLU1 expression in patients with or without trisomy 12.[90]

Certain genetic changes found in CLL have been suggested to underlie the defects in apoptosis. Anti-apoptotic proteins like Bcl-2, BCL-XL, BAG1 and Mcl-1 are overexpressed while proapoptotic proteins like BAX and BCL-XS are downregulated.[15]

In addition to the defective apoptotic machinery of CLL, improper regulation of cell-cycle controlling genes may also contribute to the accumulation of CLL cells. Raised amounts of the negative cell cycle regulator CDKN1B are detected in many CLL patients and may explain the arrest of CLL cells in the G0/G1 phase.[91]

1.2.1.4 Epigenetic changes in CLL

The current definition of epigenetics is "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence".[92] These changes are essential for normal development and maintenance of tissue-specific gene expression patterns in mammals, and are transmitted to daughter cells during mitosis. However, disruption of epigenetic processes can lead to altered gene function and malignant cellular transformation.[93] The most common epigenetic alteration in CLL is DNA hypermethylation.

DNA methylation provides a stable gene silencing mechanism that plays an important part in regulating gene expression. Normally, a methylated gene is "turned off" while demethylation makes a gene "active" and available for transcription. Dysregulated hypo- or hypermethylation may cause upregulation of oncogenes and downregulation of tumor suppressor genes in various tumors.[94]

In CLL, the overexpression of Bcl-2 is due to hypomethylation of the Bcl-2 promotor.[95] Other genes affected by hypomethylation are the MYC oncogene[96], prosurvival gene Tcl-1A[97], oncogene Erb-A1[98] and human Ig light kappa constant genes.[93, 99]

Gain of methylation in GC-rich promoter regions, thereby silencing tumor suppressor genes are described for DAPK1[100], WIF1[101], ID4[102] and SFRP1.[103] Also the prognostic marker Zap-70 has been found to be methylated in CLL.[104]

One important post-translational modification is protein glycosylation, an enzyme-directed chemical reaction where glycan structures are added to proteins and lipids. Glycosylation takes place in the endoplasmic reticulum (ER) and in the Golgi apparatus

of the cell. Glycosylation within ER is important for the proteins' folding and stability while glycosylation in Golgi provides a direction "tag" to the proteins and tells them where to go. Glycosylation is also important for cell-cell communication and glycans are involved in several physiological processes such as host-pathogen interactions, cell differentiation, proliferation, migration.[105]

Embryonic development as well as cellular activation is typically accompanied by changes in cellular glycosylation profiles and it is becoming more and more evident that glycosylation changes also represent a universal feature of malignant transformation and tumor progression.[106] It is suggested that glycosylation might be epigenetically regulated, creating novel biological structures without introducing deleterious changes in the genome.[107]

Glycan changes in malignant cells can take a variety of forms. Examples have been found of loss of expression or excessive expression of certain structures, the persistence of incomplete or truncated structures, the accumulation of precursors and, less commonly, the appearance of novel structures.[106]

1.2.2 Microenvironment

CLL cells accumulate in vivo due to their resistance to undergo programmed cell death. Nevertheless, when cultured in vitro, CLL cells rapidly undergo apoptosis. This suggests a survival advantage provided by the microenvironment of the CLL cells. This microenvironment is divided in two compartments (Figure 3). As mentioned before, a small portion of the leukemic cells is proliferating and this takes places in the so called proliferation centers (PC) in the LN and BM where they interact with prolymphocytes and paraimmunoblasts. The majority of the CLL cell population, however, is in a resting phase and circulates and accumulates in the PB. This suggests a proliferating pool of cells in LN and BM that might feed the accumulating pool in the blood. In both PB and PC there are bystander cells like T cells, stromal cells, follicular dendritic cells (FDCs) and nurse-like cells (NLCs) that provide signals to support survival and growth. These cells may affect the leukemic cells by direct cell-cell interactions or by the secretion of various cytokines.[108, 109] T cells and stromal cells seem to be the main players and it is believed that activated T cells provide signals important for proliferation while stromal cells provide a long-term support favoring survival and accumulation of leukemic cells.[110]

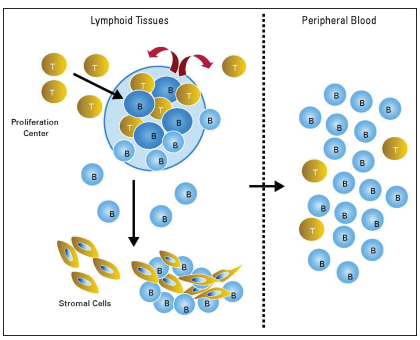

Figure 3. The 2 compartment model of CLL.

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Caligaris-Cappio F, Ghia P, The Journal of Clinical Oncology, vol 26, 2008, pp 4497-4503.

1.2.2.1 T cells

Despite the fact that CLL is a B cell malignancy, the number of T cells is also increased. This is mainly due to an increase in $CD8^+$ T cells but the number of $CD4^+$ T cells is also increased. There seems to be a small population of T cells (CD4 as well as CD8) with a natural and leukemia-specific response, in which they specifically recognize antigens expressed by tumor cells (e.g. survivin and telomerase). This population is seen more frequently in indolent patients. There is, however, increasing evidence that CLL T cells are immune-dysfunctional and this may contribute to the progress of the disease.[111, 112] The dysfunctional T cells show reduced CD25, CD28 and CD152 expression, proteins important for a normal immune response. Furthermore, the majority of CLL T cells not only lack anti-leukemic activity but are rather being part of the microenvironment, sustaining the growth of the malignant CLL clone.[113]

Individual T cell subsets show specific tissue distribution. In the PB, there is an increase in regulatory T cells (particularly in advanced disease) but whether they are contributing to the immune-dysfunction of the T cells is not clear.[112] In the PCs on the other hand, there is a marked presence of $CD4^+$ T cells, providing short-term proliferative support to the malignant cells.

Many reports suggest that CD40/CD40L is the most important interaction between T cells and CLL cells. The CD40 receptor is a 47-50 kDa glycoprotein, a member of the tumor necrosis factor (TNF) receptor family. It is expressed by B cells, monocytes and dendritic cells (DCs). CD40L is a member of the TNF family and is expressed by activated $CD4^+$ T cells, preferably in LN and BM. The CD40-CD40L interaction rescues CLL cells from apoptosis and induces proliferation. This is mediated by upregulation of cell surface molecules (CD80 and CD95), cytokine production (IL-4, TNF-α, GM-CSF), and chemokine production (CCL-22 and CCL-17). In addition, a subset of the leukemic CLL cells express both CD40 and CD40L enabling an autocrine stimulation where the CLL cells promote survival signals to themselves.[114]

CD95/Fas

CD95/Fas is a 40- to 50 kDa glycoprotein that belongs to the TNF receptor family and is expressed by T cells, B cells and NK cells. The Fas ligand (FasL) is a member of the TNF family and it is expressed by activated T cells. Normal Fas-FasL interaction induces apoptosis. However, CLL cells express little or no Fas and are thereby relatively resistant to Fas-mediated apoptosis. CD40 stimulation induces expression of Fas on CLL cells but paradoxically also provides a strong NF-κB mediated survival signal to the CLL cells.[115]

CCL-22/CCL-17

CCL22 and CCL17 are two chemokines expressed by CLL B cells from LN and BM (not PB). The receptor for these two chemokines, CCR4, is expressed on $CD4^+$/$CD40L^+$ T cells that upon interaction induce a strong chemokine production by the leukemic clone, attracting new T cells, resulting in a vicious circle, leading to CLL cell survival.[116]

1.2.2.2 Stromal cells

Stromal cells are a heterogeneous population of cells, located in the BM, that are important in normal B cell development. These cells produce several cytokines; IL-6, IL-7, IL-10, TGF-β, stemcellfactor B and colony-stimulating factor. However, it seems that the direct cell-cell contact is the most crucial event protecting the CLL cells from apoptosis. Several adhesion molecules are involved in the interactions between CLL cells and stromal cells.[117]

A small population of these stromal cells expresses stromal cell derived factor-1 (SDF-1), a CXC chemokine that plays an important role in B cell development. SDF-1 binds to CXCR4, a chemokine receptor that is consistently overexpressed by CLL cells. The interaction between SDF-1 and CXCR4 protects CLL cells from apoptosis and allows their spontaneous migration beneath BM stromal cells. The latter suggests a mechanism for BM infiltration.[118, 119]

Stromal cells also express vascular cell adhesion molecule-1 (VCAM-1) and inter-cellular adhesion molecule-1 (ICAM-1). They bind to integrin receptors that are frequently expressed by CLL cells. VCAM-1 binds VLA-4 (α4β1 integrin) and ICAM-1 binds LFA-1 (αLβ2 integrin). The interaction between stroma cells and CLL cells via β1 and β2 integrins protects the CLL cells from apoptosis, probably correlating with Bcl-2 expression.[120, 121]

CD100/Plexin-B1 is another ligand-receptor pair that seems to influence the survival of CLL cells. CD100 is a transmembrane protein, expressed on CLL cells. Plexin-B1 is expressed on BM stromal cells (as well as FDCs and activated T cells) and its binding to CD100 sustains the proliferation and survival of the CLL cells.[122]

1.2.2.3 Nurse-like cells (NCLs)

NLCs derive from $CD14^+$ cells but have an expression profile distinct from monocytes, macrophages and blood-derived DCs.[123] In vitro, these cells can attract and protect CLL cells from apoptosis and also promote migration. It is believed that they may promote both CLL cell survival and BM infiltration also in vivo.[124]

NCLs express several factors that promote interactions with the CLL cells; SDF-1, CD31, B cell-activating factor of the tumor necrosis factor family (BAFF) and a proliferation-inducing ligand (APRIL).[124, 125]

As stromal cells, NLCs express SDF-1 and its binding to the CLL receptor CXCR4 protects CLL cells from apoptosis and enhances BM infiltration.[2, 118, 125]

By CD31, NLCs may bind CD38 expressed on CLL cells and this interaction increases cell proliferation and survival.[126] Also, it is suggested that CLL cells may express both CD31 and CD38, which could result in an autocrine stimulation of the leukemic cells.

BAFF and APRIL are two members of the TNF family. BAFF binds to three known receptors, transmembrane activator and CAML interactor (TACI), B cell maturation antigen (BCMA) and BAFF receptor (BAFF-R or BR3). APRIL also binds to TACI and BCMA, but not BAFF-R. CLL cells express all three receptors. This interaction results in activation of NF-kB and enhanced expression of the anti-apoptotic protein Mcl-1.[124]

1.2.2.4 Follicular dendritic cells (FDCs)

FDCs are accessory cells normally found in peripheral lymphoid organs but are absent in normal BM.[127] In CLL however, FDCs have been detected in LN as well as in BM of CLL patients with early BM involvement.[128] It has been suggested that FDCs provide proliferation and survival signals in B cell lymphoma and CLL cells cultured together with FDCs were rescued from apoptosis. These signals may be mediated by CD44 interaction associated with up-regulation of the anti-apoptotic gene Mcl-1.[129]

As stromal cells, FDCs express Plexin-B1 and the interaction with CD100 on CLL cells results in sustained proliferation and survival of the CLL cells.[122]

1.2.2.5 CLL cells

The CLL cells themselves are active players, shaping the microenvironment according to their needs. They secrete chemokines that attract stromal cells as well as activated T cells, which in turn provide signals leading to cell growth and survival.[108] The CLL cells may also co-express cytokines and their receptors, leading to autocrine cell growth signaling. Some examples are IL-2, IL-4, IL-8, TNF-α, IFN- α, IFN-γ, GM-CSF, VEGF and their receptors.[130]

BAFF and APRIL are a recently described survival factors for CLL cells. In addition to being expressed by NLCs, they are also expressed by the CLL cells that thereby provide survival signals to themselves.[131]

CXCR4, CCL22 and CCL17 are chemokines expressed by CLL B cells and their interaction with SDF-1 on stromal cells and CCR4 on $CD4^+$/$CD40L^+$ T cells have been discussed above.

Survivin belongs to the protein family inhibitors of apoptosis proteins (IAPs). Upon CD40 stimulation, survivin is expressed by CLL cells. The survivin positive cells are localized in the PC of LN and BM, interspersed with T cells. Survivin blocks apoptosis by inhibition of caspase-3 and caspase-7, terminal effectors in the apoptosis protease cascade. Survivin also participates in cell cycle progression and the low proliferation activity in CLL cells might be associated with survivin expression.[132]

LPA (Lysophosphatidic acid) 1 is a naturally occurring soluble phospholipid that is expressed at higher levels in primary CLL cells as compared with normal B cells. In normal B cells, LPA acts as a growth factor increasing cell proliferation, intracellular calcium, and Ig formation. In CLL, LPA1 is suggested to be a survival factor, protecting the leukemic cells from apoptosis using the PI3K/Act signaling pathway.[133]

2 SMALL LEUCINE-RICH PROTEOGLYCANS

2.1 INTRODUCTION

The small leucine-rich proteoglycans (SLRPs) are a family of structurally and functionally related proteins that are normally present in the extracellular matrix (ECM) of collagen-rich tissues. The family has expanded in the last years and includes 18 genes divided into 5 classes; the traditionally defined classes I-III and the non-canonical classes IV-V (Figure 4).[134] The classification is based on number of exons encoding the gene, number of leucine-rich repeats (LRR), cysteine-rich regions, conservation and homology at the protein and genomic level, and chromosomal organization.[135]

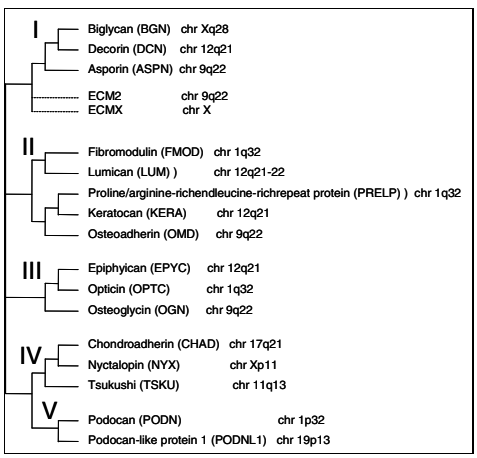

Figure 4. Small leucine-rich proteoglycan family; classification and chromosomal location.
(Adapted from Schaefer and Iozzo, 2008)[136]

2.1.1 STRUCTURE AND FUNCTION

SLRPs are small proteins (protein cores are 40-50 kDa) composed of a central domain containing LRRs, small cysteine clusters, ear repeats (classes I-III) and attached polysaccharides.[137] Some of the SLRPs may undergo proteolytic cleavage, and some may be tyrosine sulfated.[138, 139] The structure of the proteotype member, decorin, is shown in Figure 5.

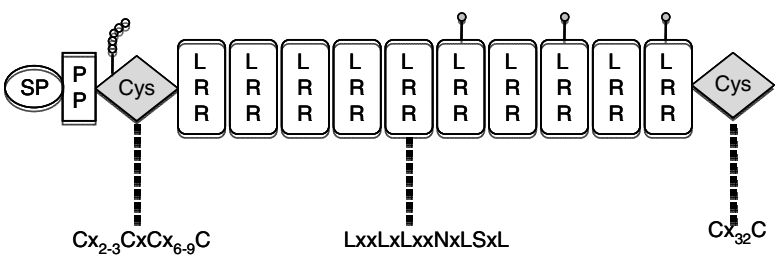

Figure 5. Schematic representation of the structural features of decorin. SP, signal peptide; PP, propeptide; Cys, cysteine-rich region; LRR, leucine-rich repeats. The glucosaminoglycan side chain and potential N-linked oligosaccharides are indicated with small circles. (Adapted from Iozzo, 1998)[138]

The LRR region is formed by 7-20 tandem repeats of 20-29 amino acids (aa) with leucine (L) and asparagines (N) residues in conserved positions.[136] It can be found in most organisms, from bacteria to man.[140] There are more than 100 identified proteins with LRR domains, all are involved in protein-protein interactions.[140] The LRR region forms a horse-shoe, or banana-formed structure and it is thought that the ligand-binding site is located on the concave surface. Several SLRPs may form dimers and this is done by the concave surface of the molecules (Figure 6).[135, 141, 142]

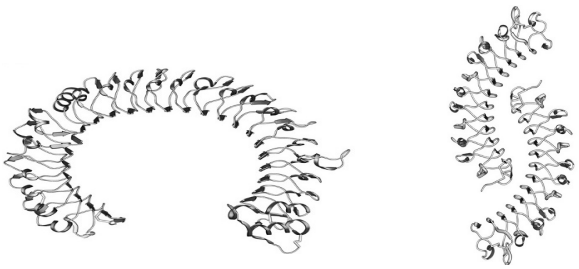

Figure 6. The horseshoe shape of a LRR and the dimeric core protein of decorin.
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The LRR region is flanked by small cysteine clusters; in most cases four cysteine residues in the N-terminal end and two cysteine residues in the C-terminal end, forming disulfide bonds.

Most SLRPs are proteoglycans carrying one or more glucosaminoglycan (GAG) chains (chondroitin-, dermatan- or keratin sulfate). Others are synthesized as glycoproteins carrying O-linked or N-linked oligosaccharides. Some SLRPs have been seen with varying glycosylation suggesting different roles in different organs or species.[143-145]

The ear repeat is a recently discovered distinctive feature of the SLRPs (classes I – III). The second last LRR is longer than the rest, typically 30 aa (classes I and III) or 31 aa (class II) and contains a Cys residue that forms a disulfide bond with another Cys residue in the final LRR. The first 18–19 residues of the ear repeat seem to follow a conserved pattern similar to that in other LRRs. However, the latter residues are not highly conserved, suggesting that this part could be of functional importance, for example in ligand binding.[141]

The majority of the SLRP family members map to only a few chromosomes. The genes encoding decorin, lumican, keratocan and epiphycan are all located on chromosome 12q21-23, while fibromodulin (FMOD), proline/arginine-rich end leucine-rich repeat protein (PRELP) and opticin (OPTC) are located adjacent to each other on chromosome 1q32. The genes for asporin, ECM2, osteoadherin and osteoglycin are all found on chromosome 9q22. The clustered organization on different chromosomes suggests that the SLRP family has arisen from several duplication events.[146]

The biological role of the SLRPs is diverse. Mostly, these proteins are secreted and bind to membrane receptors or ECM proteins. Many SLRPs bind collagen and other ECM proteins, thereby regulating the structure and hydration of the ECM. Several SLRPs bind growth factors and their receptors, including integrins, thereby affecting cell proliferation and cell migration.[147]

In addition to being secreted proteins, it is becoming evident that SLRPs may also be located intracellularly. An intracellular role has been proposed for decorin in binding the cytoskeletal protein filamin.[148] FMOD is located intracytoplasmically in basal and stratified keratinocytes.[149] Glypican and biglycan have been found in the nuclei of neurons and glioma cells.[150] For lumican, an intracellular as well as a secreted form was detected in lung cancer cells.[151]

2.2 SLRPs IN CANCER

The expression of SLRPs in cancer is becoming more and more frequently reported. However, the expression pattern is diverse. Decorin is upregulated in pancreatic cancer but downregulated in nonsmall cell lung cancer, adenocarcinoma and squamous cell carcinoma.[152-154] Lumican is overexpressed in a variety of malignancies, sometimes expressed by the tumor cells (in colorectal, cervical squamous cell carcinoma, pancreatic adenocarcinoma) and sometimes by fibroblasts in the ECM (breast cancer).[155-158]

As mentioned above, changes in glycosylation are a universal feature of malignant transformation and tumor progression[106] and there are indeed reports about SLRPs being differentially glycosylated in cancer. Decorin is expressed both glycanated and non-glycanated in laryngeal squamous cell carcinoma.[159] Lumican on the other hand is expressed as a nonsulphated glycoprotein in colorectal and pancreatic tumor cells while expressed in the proteoglycan form (with keratin sulphates) in melanoma cancer cells.[156, 158, 160]

Also, as mentioned above, lumican is expressed both intracellularly as well as secreted in lung cancer and the molecular weight of the cytoplasmic lumican differed from that in the culture medium owing to glycosylation of the protein.[151]

Much more is to be learned regarding the function of SLRPs in cancer but most reports suggest that they may play an anti-tumor role. Decorin inhibits tumor growth[161], prevents metastatic spreading[162] and suppresses angiogenesis.[163] Lumican inhibits melanoma growth and invasion.[164]

2.3 FIBROMODULIN, PRELP AND OPTICIN

In 2001, a gene array study showed the enhanced expression of the SLRP FMOD in CLL.[43] As mentioned, FMOD is located on chromosome 1q32, adjacent to two other SLRP members; PRELP and OPTC.

2.3.1 Fibromodulin (FMOD)

FMOD belongs to class II of the SLRP family. As the other members of class II, the FMOD gene comprises three exons. The protein core (43 kDa) consists of 376 aa supplemented with four keratin sulfate chains giving rise to a 59 kDa mature protein where the signal peptide of 18 aa has been removed. Twelve LRR have been defined[140] and in the N-terminal region, 9 tyrosine sulfation sites have been detected.[139] FMOD is normally expressed in cartilage, tendons and ligaments.[165]

FMOD participates in the assembly of the ECM as it interacts with type I and type II collagen fibrils and inhibits fibrillogenesis in vitro.[166] It may also regulate TGF-β activities by sequestering TGF-β into the ECM.[167, 168]) FMOD may also participate in inflammation by activating complement by directly binding complement protein C1q.[169] FMOD expression has been detected by gene expression profiling in lung, breast, glioblastomas, prostate carcinomas and benign uterine tumors as leiomyomas.[170-174]

2.3.2 Proline/arginine-rich end leucine-rich repeat protein (PRELP)

PRELP is also a member of class II SLRPs. The PRELP gene consists of three exons. The primary sequence corresponds to 382 aa residues, including a signal peptide of 20 aa. Twelve LRR have been identified[140] as well as 4 and 2 cysteine residues in the N- and C-terminal parts, respectively. The N-terminal part is unusual in that it is basic and rich in arginine and proline residues.[175] The protein core has a molecular weight of 42 kDa and contains 4 potential N-linked glycosylation sites. The mature PRELP has a molecular weight of 55 kDa. The existence of PRELP as a proteoglycan (with GAG substitution) has been debated[175] but at least in human cornea and sclera, PRELP was detected with keratin sulfate substitution. In addition, PRELP may also bind O-linked oligosaccharides.[176] PRELP is normally expressed in the ECM of connective tissues, mainly in cartilage, lung, kidney, skin, and tendon.[175] The function of PRELP is unclear, but the interactions between PRELP and collagen type I and II as well as heparin and heparan sulphate[177] suggest that PRELP may be a molecule anchoring basement membranes to connective tissue.[178] PRELP has rarely been associated with diseases. In Hutchinson-Gilford progeria, lack of binding of collagen in basement membranes and cartilage suggests PRELP involvement.[179] Similar to FMOD, PRELP also interacts with complement proteins and may be involved in inflammation processes.[180] Also, a PRELP-derived peptide was shown to bind bacterial membranes and had antibacterial activity.[181] To our knowledge, there are no reports about PRELP in cancer.

2.3.3 Opticin (OPTC)

Belonging to SLRP class III, the genome of human OPTC comprises eight exons. The core protein (37 kDa) consists of 332 aa, the first 19 representing the signal sequence. Seven LRR have been identified.[140] OPTC was first described as a 45 kDa glycoprotein of the bovine eye[182] but has later also been detected in non-ocular tissues like cartilage, brain, ligament, liver, testis, muscle and skin.[182-184]

The OPTC protein varies in size between species; human 42–48 kDa, bovine 45 kDa and rat 37 kDa, probably due to differences in glycosylation and/or other post-translational modifications. OPTC is not substituted with GAGs. Instead it is a glycoprotein holding a cluster of sialylated O-linked oligosaccharides in the N-terminal region.[182] OPTC interacts with collagen, heparin sulphate and growth hormone and may play a role in anchorage and growth factor reservoir.[182, 185, 186] However, the precise function of OPTC is still to be determined.

Little is reported about OPTC in disease but it has been suggested a role in human proliferative retinal disease[187] and its downregulation is reported in neoplastic lesions of ciliary body epithelium.[188] It is suggested that OPTC may have anti-angiogenic properties by inhibiting fibroblast growth factor (FGF).[189]

3 RECEPTOR TYROSINE KINASES

3.1 INTRODUCTION

The receptor tyrosine kinases (RTKs) is a family of high-affinity membrane spanning cell surface receptors involved in signal transduction in all eukaryotes.[190] This family has 58 members grouped into 20 classes based on conserved primary sequence and domain structure. The RTK genes are found on 19 of the 24 human chromosomes. Examples of well-defined RTKs are the receptors for FGF, VEGF, platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), insulin, epidermal growth factor (EGF). For many of the RTKs; the ligands are known while for others, the ligands are still to be discovered.[191]

The RTKs have a tyrosine kinase domain that attaches phosphate groups to tyrosine residues, either on themselves or on other proteins. This process called phosphorylation converts the target protein into an active state. Many enzymes and receptors are switched "on" or "off" by phosphorylation and dephosphorylation and thus, phosphorylation of proteins plays a significant role in a wide range of cellular processes.[192]

3.1.1 Structure and function

RTKs are receptors that bind growth factors, cytokines, and hormones and play important roles in cellular processes including proliferation, differentiation, migration, metabolism and survival.[190]

Each receptor consists of an extracellular part binding the ligand, a single transmembrane domain and an intracellular part with a tyrosine kinase domain. The precise mechanism of activation and signaling differs among the receptor families[193] but may be generally described in a simplified version. Upon ligand-binding; the receptors undergo dimerization or oligomerization, bringing the intracellular tyrosine kinase domains in close proximity. This leads to autophosphorylation of catalytic as well as noncatalytic domains within the tyrosine kinase region (Figure 7).

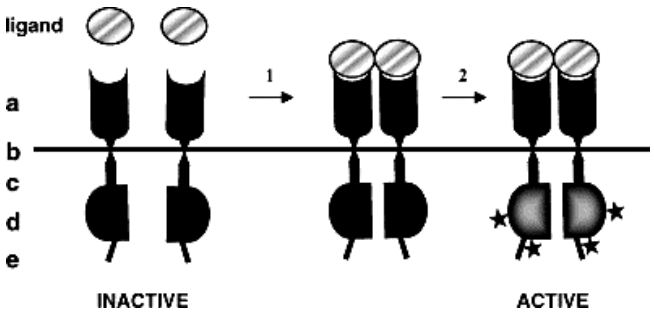

Figure 7. Schematic structure of receptor tyrosine kinases and their mechanism of action.
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Phosphorylation of the catalytic domain enhances the enzymatic activity of the receptor while phosphorylation of noncatalytic domains creates docking sites for downstream signaling proteins which are recruited and activated. The result is a signaling cascade and altered transcription of genes involved in proliferation, differentiation and migration (Figure 8). The most common signaling pathways for the RTKs are ras/raf/MAPK and PI3/Akt pathways.[193-195]

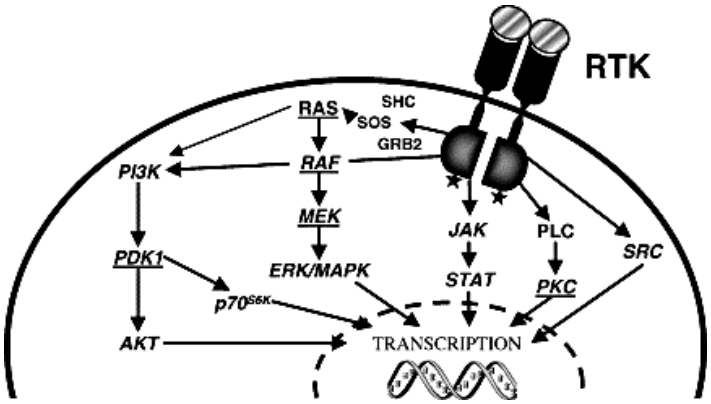

Figure 8. Schematic view of RTK signaling.
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3.2 RTKs IN CANCER

Many RTKs are involved in the signaling controlling cell cycle progression, cell proliferation, apoptosis and angiogenesis. Signaling via the receptors for EGF- and IGF (insulin-like growth factor) promotes cell growth and cell survival, respectively, while angiogenesis is initiated by signaling through VEGF, PDGF and FGF receptors.[194] In cancer, the receptors may be overexpressed or structurally altered due to mutations in the RTK genes. When the receptors are high in number, they might come in such a close contact that dimerization, phosphorylation and activation occurs, even without binding of ligand.

Also, cancer cells may be abnormally stimulated by autocrine growth factor loops, in which RTKs play an important role. This leads to increased receptor signaling and dysregulated cell growth.[195]

The approach to disturb the RTK signaling is a fairly novel way of disturbing growth advantages gained by tumor cells. Small-molecule TKIs like Gleevec (imatinib mesylate) as well as mAbs like cetuximab, trastuzumab, and bevacizumab are in clinical use to treat patients with breast cancer, colorectal cancer, chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors.[195, 196]

3.3 RECEPTOR TYROSINE KINASE-LIKE ORPHAN RECEPTORS

Receptor tyrosine kinase-like orphan receptors (RORs) belong to the RTK family and are involved in skeletal and neuronal development, cell movement and cell polarity. Vertebrates have two RORs; ROR1 and ROR2[197], which are highly homologous but are transcribed from different genes.[198] In addition to humans, ROR genes have been found in fruit flies, round worms, sea slugs, zebrafish, chicken, frogs and mice.[197] In 2001, gene array studies showed the elevated expression of ROR1 in CLL.[43, 44]

3.3.1 Receptor tyrosine kinase-like orphan receptor 1 (ROR1)

The ROR1 gene is localized at chromosome 1p31.3 (http://www.ensembl.org). The human ROR1 protein is 105 kDa and is composed of an extracellular region including one Ig domain, one cysteine-rich domain ("frizzled domain"), one kringle domain, followed by a transmembrane region and an intracellular region with a tyrosine kinase domain, a proline-rich domain straddled by two Serine/Threonine –rich domains (Figure 9).[197, 199]

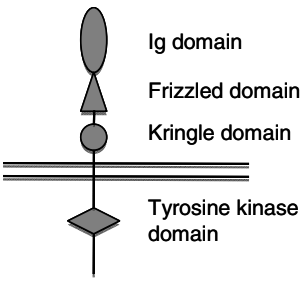

Figure 9. Domain structure of the ROR1 protein. (Adapted from Baskar, 2008).[199]

The ROR1 protein is highly conserved between species; mouse, human, drosophila and C.elegans, which verifies its importance during evolution. Ligands for ROR1 remain unknown but secreted proteins of the Wnt family have been proposed, since they signal through other cell surface receptors containing frizzled domains.[197]

ROR1 is expressed during development in heart, lung, kidney, and to a lesser extent in pancreas and skeletal muscle[198], is attenuated after embryonic development, and has not been detected in human adult brain, kidney, heart, skeletal muscle, prostate, testis or ovary tissue.[199-201] ROR1 is also expressed during early stage of normal B cell development.[201] ROR1 has been implicated in cancer development. In human cervical cancer cell line HeLa, 650 known and putative kinases were silenced by siRNA technology, identifying 73 kinases as survival kinases (inhibiting apoptosis), of which ROR1 was one of the most potent.[202] ROR1 was also over-expressed in mature subsets of B cell acute lymphoblastic leukemia (ALL) cells.[203, 204]

The precise ligand and signaling pathway for ROR1 is still to be determined, and also the function of ROR1 in CLL is unclear. It is suggested that the expression of ROR1 in CLL is activated by the transcription factor STAT3.[205] Another report describes the

interaction between ROR1 and Wnt5a, leading to activation of NF-kB and enhanced CLL cell survival in vitro.[200]

ROR1 is expressed in undifferentiated embryonic stem cells and has characteristics of an oncofetal protein (i.e. a protein typically present only during fetal development but found in adults with certain kinds of cancer http://en.wikipedia.org). It is expressed on the surface of CLL cells, is recognized and lysed by T cells, and may thereby be a suitable candidate for therapy.[201]

4 CANCER IMMUNOTHERAPY

4.1 INTRODUCTION

The definition of "immunotherapy" is "treatment of disease by inducing, enhancing, or suppressing an immune response" (www.dictionary.com). Immunotherapies designed to elicit or amplify an immune response, e.g. in cancer, are classified as *activation immunotherapies* whereas immunotherapies designed to reduce or suppress an existing immune response, as in cases of autoimmunity or allergy, are classified as *suppression immunotherapies*.

For many years, chemotherapeutic agents and ionizing radiation have been the major treatment modalities in primary cancer treatments with the goal to eliminate tumor cells. These therapies have offered substantial benefit and some cures, but in addition to sometimes severe side effects, development of drug resistance leading to tumor relapses is a significant problem. Therefore, additional therapeutic approaches to eliminate these resistant tumor cells must be established.

The concept of immunotherapy is based on the body's natural defense system and the main strategies for cancer immunotherapy is to provide antitumor effectors to patients (passive immunotherapy) and/or to stimulate the patient's own antitumor immune responses (active immunotherapy).[206]

Antigens specifically expressed by the tumors may be recognized by immune cells. These cells and many released signaling molecules are involved in the intriguing network to concur tumor cells (Figure 10). However, in many cases it seems that although the immune system prevents or delays tumor growth, it is eventually overwhelmed or evaded, and the tumor growth progresses. This is partly due to the tumors' capacity to escape immune surveillance. Tumor cells develop a number of different strategies to avoid detection. They often lose the expression of tumor antigens or downregulate the MHC molecules in order to avoid detection by T cells. They may also secrete cytokines (e.g. VEGF, IL-10 and TGF-β) that inhibit immune responses. In addition, immune tolerance may develop against tumor antigens, whereby the immune system no longer attacks the tumor cells.[207] Therefore, cancer vaccines must stimulate specific immune responses against the correct target. And secondly, the immune responses must be powerful enough to overcome the barriers that cancer cells use to protect themselves from attack by B cells and killer T cells.[208]

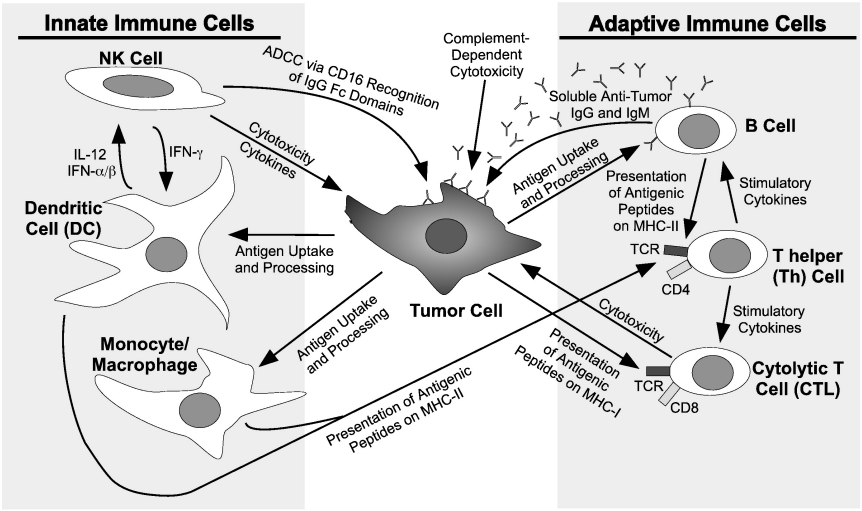

Figure 10. Major cells of the innate and adaptive immune systems and their functions in response to a tumor cell. Reprinted from European Journal of Pharmacology, vol 625, Borghaei H, Smith MR, Campbell KS, Immunotherapy of cancer, pp 41-54. Copyright (2009), with permission from Elsevier.

Historically, successful immunotherapy to treat cancer was used already in the 1890's and involved the use of toxins from Streptococcus erysipelatis and Bacillus prodigious.[209, 210] Later, cytokine therapy using IFNγ or IL-2, became the first type of immunotherapy to be applied in patients with various types of cancer.[206] In more recent years, the use of mAbs has become standard treatment in many cancers. There are many ongoing clinical trials with attempt to achieve effective cancer vaccines but still, no therapeutic vaccine to fight cancer has yet been approved by US and European regulatory agencies.[211] In the last years, however, vaccines have been used in cancer prevention against tumor-causing hepatitis B virus[212] and papilloma virus.[213] With the increased knowledge of basic immune mechanisms, a wide array of immune pathways has been identified as attractive targets to promote anti-tumor responses in cancer patients.

4.2 IMMUNOTHERAPY STRATEGIES

4.2.1 Tumor antigens

The definition of a tumor antigen is "a substance produced in tumor cells that triggers an immune response in the host" (http://en.wikipedia.org). Tumor antigens may broadly be divided into two categories: Tumor-Specific Antigens (TSAs) and Tumor-Associated Antigens (TAAs). TSAs comprise gene products that are uniquely expressed in tumors while TAAs may also be expressed by other cells. However, this classification is somewhat risky since some antigens thought to be tumor-specific may be found to be expressed on some normal cells as well. Tumor antigens are targets of both mAbs and T cell based vaccination.

The ideal tumor antigen would be expressed exclusively be the tumor cells and not by the normal tissue (to decrease the risk of autoimmune reaction). It should also be detectable in all tumor cells and in the majority of patients. To give the acquired effect, it also needs to be essential for the survival of the tumor cells. Most tumor antigens do not possess these ideal characteristics and are therefore limited in their use.[214]

4.2.2 Antibody-based immunotherapy (passive immunization)

The earliest strategies for tumor immunotherapy relied on passive immunization, in which immune effectors are injected into cancer patients. MAbs can be used as carriers to target radioisotopes, drugs or toxins to directly kill tumor cells, but they are also used to stimulate components of the immune system or to interfere with signaling pathways within the tumor cell.[215]

When antibodies bind to TSAs on cancer cells, a process called ADCC (antibody-dependent cellular cytotoxicity) is activated.[216] NK cells express Fc receptors that may bind to the Fc portion of the bound antibody and this interaction leads to activation of the NK cells that release cytotoxic granules which contain proteins (perforin and granzymes) that kill the target cell.[217]

Most mAbs that mediate ADCC also induce complement-dependent cytotoxicity (CDC). The complement system consists of a number of proteins found in blood that upon stimulation trigger activation of other complement proteins. The end-point of this cascade is the formation of pores on the tumor cell surface by proteolytic enzymes, leading to tumor cell lysis. Antigen-presenting cells (APCs) may then present tumor-derived peptides on MHC class II molecules, leading to $CD4^+$ T cell activation.[217] In addition, complement activation also leads to the recruitment and activation of immune effector cells such as macrophages, neutrophils, basophils, mast cells and eosinophils, that help eliminate the tumor cells.[218]

Antibodies can also be used to interfere with intracellular signaling pathways. Common targets are growth factor receptors and by blocking ligand binding and/or signaling through the receptor, the regulation of cell growth, apoptosis and angiogenesis is disturbed.[217]

As mentioned before, tumor cells often receive survival support from the microenvironment and antibodies may also be used to target ECM structures, inhibiting processes such as angiogenesis.[217]

4.2.3 Vaccination (active immunization)

The purpose of tumor vaccines is to either induce immune recognition against tumor cells or to enhance an existing anti-tumor immune response. Successful vaccination is dependent upon three factors; expression and presentation of tumor antigens, host cells being capable of recognizing the tumor antigens, and generation of an effective immune response.[219] The main advantage of vaccination is the possibility to specifically target cancer cells without damaging normal tissues or causing debilitating side effects.

Single antigens or a mixture of antigens may be used as specific targets. When these are presented to the host, an immune response is triggered, where both T cells and B cells become activated and battle against the tumor cells by means of antibody production, cytokine production and cytotoxicity.[220]

Numerous efforts have been made to find the most suitable way of delivering the vaccine to the host and to activate the most efficient immune response. Whole-tumor cell vaccines utilize either tumor cells from a patient, or tumor cells from established tumor cell lines. In general, the major advantage of whole-tumor cell vaccines is that a broad range of TAAs are presented to the T cells, including antigens that are not yet known. However, whole cell vaccines might share fragments with normal cellular proteins and therefore involve an enhanced risk of autoimmunity compared to defined purified TAA's.[221]

DCs are considered to be the most potent APCs and, therefore, one of the most powerful tools for immunization. The antigen loading techniques are numerous and include: (1) DCs transfected with tumor-derived RNA or DNA (2) DCs loaded with whole tumor lysates or apoptotic tumor cells (3) DCs pulsed with tumor-derived peptides or whole proteins (4) DC electrofused with CLL cell (tumor-cell/DC hybrids). Other ways to distribute the vaccine into the host include vector-based vaccines using viral, bacterial or yeast gene transfer, or plasmid DNA.[222]

Most antigens do not induce an immune response strong enough to make effective cancer treatment vaccines. Therefore, the vaccine is usually accompanied by an adjuvant; a substance that will enhance the immune response that has been set in motion by exposure to the antigen. There are various types of adjuvants[221] utilizing various mechanisms of action:

1) Incomplete Freund's adjuvant (IFA) helps in keeping the antigen at the injection site so that infiltrating APCs and effector cells can initiate a stronger immune response. This type of action is relevant for peptides or proteinbased vaccines that tend to rapidly diffuse from the site of injection.
2) The use of aluminum-based salts has a similar function as the antigen is clumped with the aluminum salt.
3) Bacterial products, such as Bacillus Calmette-Guérin (BCG) or lipopolysaccharide (LPS), induce strong immunostimulatory activity.
4) Cytokines like GM-CSF, INF–α and IL-2, are commonly used as adjuvants, as they enhance immune responses by promoting the differentiation, activation, or recruitment of APCs, therefore, enhancing antigen presentation and activation of antigen-specific T cells.

4.2.4 Adoptive T cell therapy

In cancer patients, the number of naturally occurring tumor-specific T cells is usually very low. Adoptive T cell therapy is a method to drastically change the balance between cancer cells and immune cells. In this method, antigen-specific T cells are isolated from a cancer patient, activated and expanded in vitro, and re-infused into the patient. The goal

is a more potent anti-tumor attack, resulting in elimination of the tumor and preventing its recurrence.[223]

5 IMMUNOTHERAPY IN CLL

5.1 GENERAL ASPECTS

Immunotherapy of CLL includes passive antibody treatment, active immunization and adoptive T cell transfer. Since CLL patients suffer from impaired immune functions, the task of generating immune responses against the leukemic cells may be challenging, but even though T cell function as well as antigen presentation by DCs are impaired in CLL[111, 224], cytotoxic T cell responses against a number of CLL TAAs have been generated.[225, 226]

5.2 IMMUNOTHERAPY STRATEGIES IN CLL

5.2.1 Tumor antigens in CLL

CLL cells express a number of TAAs with the potential to induce a specific anti-tumor response.[227] Idiotype Ig[228], Adipophilin[229], CD23[225], CD229[230], FMNL-1[231], MDM2[226], OFAiLRP[232], SLLP1[233], RHAMM[234], hTERT[235], survivin[236], FMOD[237] and ROR1[200] are some examples. The two latter were investigated in papers I and IV.

5.2.2 Antibody therapy in CLL

As discussed earlier (§ 1.1.3.2), the monoclonal antibodies rituximab (targeting CD20) and alemtuzumab (targeting CD52) have remarkably changed the treatment of CLL patients[238] by improving both CR rates as well as progression free survival. As most mAbs, rituximab and alemtuzumab act by ADCC and/or CDC.[239]

5.2.3 Vaccination in CLL

Theoretically, the CLL cells would be a suitable target for T cell mediated responses for several reasons. First, CLL arises from B cells that can act as APCs, express TAAs and can be targeted by allogeneic T cells. Second, since CLL is a slow-growing malignancy, it allows time for an immune response against the tumor cells to be generated. Third, the tumor cells are easily isolated as they circulate in large numbers in PB. Nevertheless, it is difficult to induce immune responses in CLL, mainly due to the CLL cells being inefficient APCs and in addition, as mentioned earlier, both CLL T cells as well as DCs are immune deficient.

The numbers of vaccination clinical trials that have been performed in CLL are limited and due to the difficulties in finding suitable TAAs in CLL, the majority is performed using whole-cell vaccines.

Two studies using 1) allogenetic DCs pulsed with tumor lysates or apoptotic bodies[240] and 2) autologous DCs pulsed with tumor lysates[241] reported minor clinical effects but without significant clinical responses.

Oxidized autologous tumor vaccines were evaluated in a phase I/II trial.[242] Partial response was noted but the response duration was short.

A phase I study evaluating safety and immunogenicity of a vaccine consisting of irradiated apoptotic autologous leukemic cells, showed some immunological and minor clinical responses.[243]

One recent phase I trial evaluated vaccination with a specific TAA-derived epitope. Apart from being "safe" the RHAMM-derived epitope R3 could to some extent elicit specific T cell responses against the tumor antigen RHAMM.[244]

6 AIMS OF THE THESIS

Within the scope of a long-term research programme on CLL and its treatment, the studies underlying this thesis aimed at finding new tumor markers that might be used as novel targets for immunotherapy. The specific objectives were:

Study 1
To investigate the gene and protein expression of FMOD in CLL, other hematological malignancies and healthy controls.

Study 2
To investigate the gene and protein expression of PRELP in CLL, other hematological malignancies and healthy controls.
To characterize the glycosylation pattern of PRELP in CLL cells.

Study 3
To investigate the protein expression of OPTC in CLL patients and healthy controls.
To characterize the glycosylation pattern of OPTC in CLL cells.
To investigate the cellular location of OPTC in CLL cells.

Study 4
To investigate the gene and protein expression of ROR1 in CLL and healthy controls.

Study 5
To investigate the result of downregulation (siRNA) of FMOD and ROR1 in CLL cells.

7 RESULTS

7.1 PAPER I

Fibromodulin, an extracellular matrix protein: characterization of its unique gene and protein expression in B-cell chronic lymphocytic leukemia and mantle cell lymphoma
(Blood. 2005;105(12):4828-35)

FMOD belongs to the family of SLRPs and is expressed in the ECM of collagen-rich tissues. It interacts with type I and type II collagen fibrils and inhibits fibrillogenesis in vitro, and may thereby participate in the assembly of the ECM. FMOD may also regulate TGF-β activities by sequestering TGF-β into the extracellular matrix.

In 2001, gene expression profiling revealed FMOD as one of many genes overexpressed in CLL. The aim of the present study was to further investigate FMOD expression in CLL patients, patients with other hematological malignancies and healthy controls.

FMOD was expressed at the mRNA level (RT-PCR) in all CLL patients tested (n=75) and in most patients with MCL (5 of 7). FMOD was not expressed in tumor cells of patients with T-CLL (n=5), B-PLL (n=1), T-PLL (n=1), HCL (n=10), FL (n=2), MM (n=4), AML (n=5), ALL (n=14) or CML (n=15). Neither was FMOD expressed in fresh PBMC (n=70), isolated B cells (n=6), isolated T cells (n=4), or isolated blood granulocytes of healthy donors (n=10). The FMOD protein was detected in the cytoplasm of the CLL cells (flow cytometry) and in the supernatant after in vitro cultivation (Western blot). Mutation analysis did not reveal any mutations in the FMOD gene of CLL patients (n=10). Stimulation (phorbol 12-myristate 13-acetate [PMA]/ionomycin) of normal B- and T lymphocytes induced weak FMOD expression, but not to the extent seen in freshly isolated CLL cells.

To summarize, this study showed that FMOD is exclusively expressed in CLL and MCL. The unique ectopic expression in all CLL patients tested suggested that FMOD may play a role in the pathogenesis of the disease. Thus, this finding gave an impetus to further studies of the SLRP family.

7.2 PAPER II

An Aberrant Unique Proline/Arginine-rich End Leucine-rich Repeat Protein (PRELP) is Ectopically Expressed in Chronic Lymphocytic Leukemia Cells
(manuscript)

PRELP is also a member of the SLRP family and is normally expressed in the ECM of connective tissue, mainly in cartilage, kidney, skin, and tendon. The function of PRELP is unclear, but the interactions between PRELP and collagen type I and II as well as heparin and heparan sulphate suggest that PRELP may be a molecule anchoring basement membranes to connective tissue. The PRELP gene is located in close proximity to the FMOD gene on chromosome 1 (1q32.1). The unexpected finding of an aberrantly expressed ECM protein (FMOD) raised the question whether also other SLRP family members might be expressed in CLL. The aim of the present study was to analyze PRELP in CLL cells as cluster upregulation of genes is occasionally noted in malignancies.

PRELP was expressed at gene level (RT-PCR) in all CLL patients examined (n=30) irrespective of clinical phase (non-progressive / progressive). PRELP was also expressed in tumor cells of MCL patients (3/5) but not in AML (0/5), FL (0/2), T- or B-PLL (0/5), HCL (0/2), MM (0/6), CML (0/5), and ALL (0/10). PRELP was not expressed in fresh PBMC of healthy donors (0/10), enriched normal blood B cells (0/6), T cells (0/4), or granulocytes (0/5). PRELP was expressed in four CLL cell lines (EHEB, I83-E95, 232-B4, WAC3-CD5) but not in cell lines derived from MM (0/1), T cell leukemia (0/1), ALL (0/4), AML (0/1), CML (0/1), and NK cell lymphoma (0/1). Sequencing of cDNA from 10 CLL patients revealed no major mutations in the PRELP gene.

Stimulation of normal B cells, normal T cells and CLL cells (CD40L-transfected fibroblasts, α-CD3 mAb and PMA/ionomycin) did not induce/enhance the PRELP expression.

PBMC from CLL patients were tested for PRELP protein expression in Western blot. A PRELP protein was detected in all CLL samples tested (n=30) but not in leukocytes of healthy donors (n=10). The molecular weight of the normal glycosylated PRELP protein was in the range 50-58 kDa. However, the PRELP expressed by CLL cells was 38 kDa. Molecular analysis suggests that the 38 kDa PRELP is a unique unglycosylated core protein, with an intact signal peptide, localized in the cytosol of the CLL cells. This protein was not detected in serum which, in combination with the uncleaved signal peptide, suggests cellular retention. An additional 76 kDa PRELP (possibly a dimer) was detected in a lysate fraction containing membrane and intracellular organelles. All four CLL lines also expressed the 38 kDa PRELP as well as the 76 kDa dimer.

We also analyzed serum from 8 CLL patients and 8 healthy control donors by western blot. All serum samples showed two bands, 50 and 58 kDa, representing mature glycosylated PRELP, probably produced by fibroblasts in the surroundings. The 38 kDa and 76 kDa CLL PRELP proteins were not detected in serum from either patients or normal donors.

This is the first report connecting PRELP to cancer. An unglycosylated form of PRELP is expressed in CLL- and MCL-cells but not in normal white blood cells. Unglycosylated PRELP may be a good target for immunotherapy in that it is distinguishable from normal PRELP in other tissues.

7.3 PAPER III

The Small Leucine-rich Proteoglycan Opticin (OPTC) is Ectopically Expressed and Translocated to the Nucleus of Chronic Lymphocytic Leukemia Cells
(manuscript)

As FMOD and PRELP, OPTC is also a member of the SLRP family, first identified in the extracellular matrix of the eye and subsequently in cartilage, brain, ligament, liver, testis, muscle and skin. OPTC interacts with collagen, growth hormone and heparin sulphate and may potentially play a role in cell anchorage, growth factor sequestration and regulation of angiogenesis. OPTC is located next to FMOD and PRELP on chromosome 1 (1q32.1). Cluster upregulation of genes is not an uncommon phenomenon in malignancies and the present study examining the potential expression of OPTC by CLL cells was performed as a logical extension to previous studies on FMOD and PRELP.

This study focused on the protein expression of OPTC. Chemical deglycosylation was performed to determine molecular weight and glycosylation pattern while four cell fractionation methods were used to determine the cellular location of CLL OPTC.

The molecular weight of the normal glycosylated OPTC protein varies between 45 and 50 kDa. A 50 kDa OPTC protein band was detected (Western blot) in the cytosol from CLL cells (n=30), as well as normal leukocytes (n=10). However, the CLL cells, irrespective of clinical phase (non-progressive/progressive) also demonstrated a 37 kDa OPTC band that was not detected in healthy donors. This 37 kDa OPTC protein was localized in the cell nucleus and ER of the CLL cells. It was recognized both by a C-terminal and an N-terminal antibody, indicating that it is probably not a degradation or cleavage product. The performed glycosylation experiments suggest that the unique 37 kDa OPTC is an unglycosylated core protein.

We also analyzed serum of CLL patients and healthy control donors by Western blot. All serum samples showed a 50 kDa band representing the mature glycosylated OPTC. The 37 kDa OPTC protein was not detected in sera from either group of donors.

In addition to FMOD and PRELP, a third SLRP, OPTC, was shown for the first time to be expressed by CLL cells. This finding suggests that a cluster of genes are upregulated on chromosome 1. The unexpected localization of OPTC in the nucleus of CLL cells might be a useful lead in the future search for information about OPTC's role in CLL.

7.4 PAPER IV

Ror1, a cell surface receptor tyrosine kinase is expressed in chronic lymphocytic leukemia and may serve as a putative target for therapy
(Int J Cancer. 2008;123(5):1190-95)

ROR1 belongs to the family of RTKs; transmembrane receptors involved in cellular processes including differentiation, proliferation, migration, angiogenesis and survival. ROR1 is highly conserved between species, e.g. human, mouse, drosophila and C.elegans and is normally expressed during development and to a lower extent in adults.

In 2001, gene expression profiling revealed ROR1 as one of many genes overexpressed in CLL. The aim of the present study was to analyze gene and protein expression of ROR1 in CLL cells and normal blood leukocytes.

ROR1 was detected at the mRNA level (RT-PCR) in PBMC of all CLL patients tested (n=100) but not in healthy donor PBMC (n=10), isolated normal B cells (n=6), normal T cells (n=3), or enriched blood granulocytes (n=10). Mutation analysis of cloned extracellular and cytoplasmic kinase domains of the ROR1 gene was performed in 10 CLL patients and showed no major genomic aberrations.

Protein expression was analyzed by Western blot as well as flow cytometry. ROR1 was detected on the cell surface (PBMCs) from all CLL patients tested (n=18) but not on PBMC from healthy donors (n=10). The CLL cells demonstrated two different variants of the Ror 1 protein, 105 kDa and 130 kDa, neither of which was detected in healthy donors.

Stimulation using PMA/ionomycin induced ROR1 expression in normal B and T lymphocytes, but did not enhance the fundamental ROR1 expression in CLL cells.

FISH analysis was performed of PBMC from 3 CLL patients and showed no rearrangement in the chromosome 1p region.

To summarize, this fourth study of potential targets for immunotherapy demonstrated that also ROR1, belonging to the RTK family, is expressed by CLL cells but not by normal leukocytes. The surface expression of ROR1 in all CLL patients makes it a promising target for future therapy.

7.5 PAPER V

Silencing of ROR1 and FMOD with siRNA results in apoptosis of CLL cells.
(Br J Haematol. 2010 Aug 31. [epub ahead of print])

As shown in papers I and IV, FMOD and ROR1 are two genes upregulated in CLL cells in contrast to normal B cells. However, the function of ROR1 and FMOD in CLL is not known. siRNA is today an established technique by which essentially any gene of interest may be knocked down, and functional effects thereof can be investigated.

In this study, we have used siRNAs to specifically silence ROR1 and FMOD gene expression in CLL cells, healthy B cells and human fibroblast cell lines.

siRNA treatment induced a significant decrease in FMOD and ROR1 both at mRNA (RT-PCR and realtime PCR) and protein (Western blot) levels.

Silencing of FMOD and ROR1 resulted in statistically significant apoptosis of CLL cells but not of B cells from normal donors. The latter was somewhat expected since normal B cells do not express neither FMOD nor ROR1. However, human fibroblast cell lines expressing FMOD and ROR1, did not undergo apoptosis when treated with FMOD and ROR1 siRNA.

This is the first report demonstrating that ROR1 and FMOD may be involved in the survival of CLL cells. FMOD may be relevant as a target for active immunotherapy but its expression in connective tissues necessitates caution due to the risk of autoimmune reactions. ROR1 on the other hand, is not expressed in normal tissue and may be a good target for therapy in CLL.

8 DISCUSSION

8.1 GENERAL CONSIDERATIONS

During the past decade, considerable progress has been achieved in the diagnosis and treatment of CLL. Among the new interventions various forms of immunotherapy, often in combination with existing therapy, are being explored in the effort to find a curative treatment for CLL. Passive immunization, using mAbs, has been used with favorable results in some cases, while the progress with active immunization has been limited. The main reason for the difficulties is probably the impaired immune response, inherent in the disease. Another problem is the adverse effects caused by autoimmune reactions.

Finding new specific TAAs will substantially improve the possibility to target tumor cells without harming normal cells. The data presented in this thesis identify four new proteins, FMOD, PRELP, OPTC and ROR1, all expressed by CLL cells but not by normal white blood cells. In view of the high degree of specificity, these proteins might be suitable future targets for immunotherapy.

The reason for the unexpected expression of FMOD, PRELP, OPTC and ROR1 in CLL is not known. Abnormalities of chromosome 1, where the four genes are located, are rarely seen in patients with CLL, and no mutations for either of the genes were found.

The fact that the neighboring genes encoding FMOD, PRELP and OPTC are all aberrantly expressed in CLL is intriguing and might imply that this cluster of genes has a common gene control mechanism. Such upregulation of gene clusters has been reported in other malignant diseases. The possible connection in this case between gene regulation and pathogenesis in CLL is a topic beyond the scope of the present thesis, but further studies are certainly warranted.

FMOD, PRELP and OPTC all belong to the SLRP family. There are several reports about other SLRPs being differentially expressed in cancer, with abnormal function as well as altered location compared to normal [156, 157, 159, 245] but this is the first time that FMOD, PRELP and OPTC have been associated with cancer.

The results in this thesis show that FMOD, PRELP and OPTC (papers I-III) are all expressed in CLL with altered glycosylation (PRELP and OPTC) and abnormal location (OPTC). These novel findings may be of great importance in 1) understanding the function of these proteins in CLL and 2) the search for future treatment targets.

ROR1 belongs to the RTK family and members of this family are occasionally upregulated in cancer.[246] The ROR1 protein was shown to be expressed on the surface of CLL cells in all patients tested but not in healthy individuals (paper IV). In contrast to FMOD, PRELP and OPTC, ROR1 is expressed in several other hematological malignancies such as MCL, marginal zone lymphoma, diffuse large B cell lymphoma and FL.[247]

8.2 FUNCTION

The function of FMOD, PRELP, OPTC and ROR1 in CLL is still to be discovered, but eventually the knowledge may provide useful information in understanding the disease. ROR1 is normally expressed during development and is detected in undifferentiated embryonic stem cells.[201] Whether FMOD, PRELP and OPTC have the same characteristics as ROR1 is not yet known, but there are reports about proteoglycans being expressed during B cell development, interestingly with modulations in glycosylation during distinct phases of B cell differentiation.[248, 249] Perhaps FMOD, PRELP and OPTC are expressed during B cell development and are left in an "on-state" after tumorigenesis, exerting their specific, yet unknown, functions. A similar scenario could be hypothesized for the proteoglycan Syndecan-1 which is expressed in normal pre-B cells and in plasma cells as well as in CLL cells.[250]

Unglycosylated forms of PRELP and OPTC were detected in CLL whereas ROR1 was expressed in two forms, of which one showed higher molecular weight, possibly due to enhanced glycosylation. This novel finding of altered glycosylation of PRELP, OPTC and ROR1 in CLL needs to be further investigated. Aberrant glycosylation has been reported in colorectal cancer[251] thyroid cancer[252], breast cancer[253], and recently also in CLL.[254] There are, however, few reports about proteins being expressed in completely non-glycosylated forms. In Esophagus cancer, a glycoprotein called clusterin was reported to be expressed uncleaved and nonglycosylated in the nucleus of Esophagus cancer cells.[255] Another protein, Mucin 1, is overexpressed in a largely deglycosylated form in advanced tumors of non–small-cell lung cancer.[256] In patients with Ehlers-Danlos syndrome, decorin is partly secreted lacking its single glycosaminoglycan side chain.[257] A study about proteoglycan expression in aging skin reported decorin to be reduced in size in mature skin compared to fetal. The small decorin was regarded as non-glycosylated but was speculated to be a catabolic fragment of normal decorin.[258]

Regardless of glycosylation, the core protein of several proteoglycans may act as the binding part responsible for interactions with other proteins. The core protein of decorin interacts with both collagen and fibronectin[259, 260] and may also bind TGF-β and TNF-α as has been shown for biglycan.[167, 261] Whether the core proteins of PRELP and OPTC have similar capabilities needs to be further studied. However, in vitro, ectopic expression of decorin protein core resulted in retarded growth and induced apoptosis of a variety of tumor cells. This was mediated via interaction of the decorin protein core with EGF receptors.[262]

Also, the localization of OPTC in the nucleus of CLL cells needs to be further investigated. Interestingly, the closely related SLRP decorin was reported aberrantly expressed and localized to the nucleus of dysplastic oral epithelial cells. It was speculated by the authors that these cells had lost their ability to inhibit TGF-α signaling and activation of the EGF receptor signaling pathways because of such aberrant nuclear localization.[245]

Most of what is known about the normal function of FMOD, PRELP and OPTC concerns their interactions with ECM proteins. The microenvironment is important for CLL cell survival[263] and direct contact between leukemic and BM stromal cells are

essential to tumor cell survival.[121, 264] It is not unlikely that FMOD, PRELP and OPTC are involved in the communication between CLL cells and their surroundings. Thus, it is somewhat contradictory that PRELP and OPTC seem to be retained within the CLL cells. However, as mentioned previously, SLRPs may also have intracellular functions. The core protein of the closely related decorin has been found both in a secreted form binding to fibronectin[259] as well as an intracellular form binding the cytoskeletal protein filamin.[148] An intracellular location has even been proposed for FMOD in basal and stratified keratinocytes, though without any suggestion being made regarding its function.[149]

In addition to their interactions with ECM proteins, FMOD, PRELP and OPTC also seem to interact with TGF-β[167] NF-KB[265] and retinal growth hormone[186], respectively. The closely related SLRP decorin interacts with EGF-R, TGF-β and p21, (a potent inhibitor of cell cycle progression), thereby showing cell proliferation- as well as apoptosis- regulatory functions.[266] The possible non-ECM interactions of FMOD, PRELP and OPTC need to be investigated.

8.3 DIAGNOSIS AND PROGNOSIS

The diagnosis of CLL is well established and is based on >5.0x10^9/L lymphocytes in peripheral blood with a characteristic phenotype; $CD19^+$, $CD5^+$, $CD20^+$, $CD23^+$, $FMC7^-$, sIG^{weak}, $CD79b^{weak/neg}$. However, even though flow cytometry methods are becoming more and more sophisticated using multi-color systems, CLL is sometimes misdiagnosed as MCL, marginal zone lymphoma, B cell PLL (prolymphocytic leukemia) or lymphoplasmacytic lymphoma. FMOD, PRELP, and OPTC are not expressed in other hematological malignancies than CLL and MCL and could be a good complement to the markers used today.

Our unpublished data show that the expression of ROR1 is elevated in progressive patients compared to indolent, suggesting ROR1 as a future prognostic marker of CLL. Neither FMOD, PRELP or OPTC have yet shown any potential in being a surrogate marker. However, more sensitive methods (e.g. real-time PCR) might detect differences in mRNA levels. Also, there might be differences in the degree of glycosylation that has not yet been discovered. More profound analyses need to be performed.

It is known today that CLL is preceded by a condition called monoclonal B cell lymphocytosis (MBL). MBL is detected in a subset of the healthy population, with B cells immunophenotypically identical to CLL cells being present in PB.[267] Several studies have investigated the prevalence of MBL in the general population and the reported numbers varies between 0,6 and 12% depending on the detection method used.[268] There has been a considerable interest in the biology of MBL to better understand the mechanisms of CLL tumorigenesis. Although the majority of CLL cases appear to be preceded by MBL[269] the majority of individuals with MBL will not develop a hematologic malignancy. Whether or not MBL cells express FMOD, PRELP, OPTC and/or ROR1 needs to be investigated, but if so, the four genes might be useful tools in defining the MBLs that will progress into CLL.

8.4 TREATMENT

Our results demonstrate that FMOD is expressed in all CLL patients but not in normal control PBMC, purified B or T cells (paper I). FMOD has also been shown to be naturally processed and presented as a TAA in primary CLL cells, enabling the expansion of autologous tumor-specific T cells.[237] Also, in a vaccination study where autologous dendritic cells were loaded with CLL cell lysate, the frequency of FMOD-reactive T cells was enhanced.[241] These findings suggest that FMOD could be a suitable target for clinical vaccination trials or adoptive T cell transfer.[227, 237] However, though not expressed by normal blood cells, FMOD is expressed in connective tissues and the risk of autoimmune reactions needs to be considered.

PRELP and OPTC are also expressed in normal connective tissue. Immunotherapy directed against these targets could, as for FMOD, involve a risk of autoimmune reactions. However, the finding of altered glycosylation of PRELP and OPTC in CLL (papers II and III) enhances the favorable aspects of these two proteins as possible vaccine targets since the glycosylation pattern distinguishes the CLL cells from normal cells. One possible approach would be to trigger T cells against a specific PRELP- or OPTC derived peptide that is unglycosylated in CLL but glycosylated in normal cells.

ROR1 is the fourth gene found to be overexpressed in CLL (paper IV). ROR1 belongs to the RTK family and the role of these receptors in tumor development has been well documented.[194, 246] Mutations in RTK genes, receptor overexpression or abnormal autocrine growth factor loops lead to constitutive RTK signaling, resulting in dysregulated cell growth and cancer.

RTKs are multifunctional transmembrane proteins, expressed at the cell surface. These characteristics make them potential multisite targets that can be inhibited by antibodies, ligand antagonists or TKIs. The anti-ErbB2 antibody Herceptin and the TKI Gleevec (PDGF-R) and Iressa (EGFR) were among the first anti-RTK drugs used in the clinic for the therapy of patients with advanced breast cancer, CML and lung cancer, respectively.[195] Many have followed their paths and there are numerous ongoing clinical trials using mAbs or inhibitory molecules targeting RTKs both in solid tumors (as non-small cell lung cancer, renal cell carcinoma, breast cancer, pancreatic cancer, head- and neck cancer, liver carcinoma) and in hematological malignancies (MM and AML) (http://clinicaltrials.gov).

T cells genetically modified to express a chimeric antigen receptor specific for ROR1 lysed primary CLL cells but not normal mature B cells in vitro.[201] T cell therapies targeting ROR1 may therefore be effective in CLL. However, ROR1 might also be a suitable target for mAb therapy as well as small molecule inhibitors. Today, mAb therapy in CLL includes rituxumab, targeting CD20 and alemtuzumab, targeting CD52. Although these mAbs have fewer side effects than standard chemotherapy, they also target normal cells expressing CD20 and CD52. ROR1 is not expressed by normal cells and may therefore give fewer side effects. In addition, ROR1 is detected at very low levels in sera from CLL patients[199] (which is not the case for CD52) and is therefore less likely to interfere with the administered mAbs.

FMOD, PRELP and ROR1[201] are all expressed in CLL as well as MCL (OPTC not yet analyzed in MCL). Both diseases are presumed to derive from mature antigen-experienced B cells but the mechanism, timing, and role of the ectopic expression is not known.[270] ROR1 and FMOD may be important for the survival of CLL and MCL cells, as siRNA silencing of FMOD and ROR1 individually led to enhanced apoptosis of CLL cells (paper V). The hypothesis of ROR1 as a survival kinase was strengthened by a study where shRNA technology silencing of ROR1 led to induced apoptosis of Hela cervical carcinoma cells.[202]

However, most reports about SLRPs in cancer describe an anti-tumor effect, reducing tumor growth as well as inducing apoptosis. If this would be the case for FMOD, PRELP and OPTC, one approach in treating CLL would be to stimulate their expression. Our results, however, where siRNA silencing of FMOD induced apoptosis in CLL cells, suggest a role for FMOD, PRELP and OPTC that would rather favor the survival of CLL cells.

The traditional approach in the treatment of CLL has been to delay treatment until onset of symptoms. Most patients with early-stage disease continue to live for decades and never require treatment. However, some patients progress rapidly and will, despite treatment, die of the disease within 2-3 years.[68] Historical trials, which form the basis for the wait-and-watch approach, used single alkylating agents and yielded a CR in only <10% of the patients. Recent years combination treatments have achieved CR rates of up to 70%.[271] Patients with indolent disease show better immune responses and would theoretically be the ones that benefit from early immunotherapeutic intervention. The four novel genes investigated in this thesis are expressed in all CLL patients, irrespective of clinical phase (indolent/progressive). Ror expression seems to increase upon progression (outside the scope of this thesis). At least FMOD and ROR1, seem to be important for the CLL cell survival. Obviously further studies are needed, but the present findings imply that at least some of the newly detected tumor antigens might be suitable candidate targets for future immunotherapy.

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