SPROUTY 2: A Novel Attenuator of B Cell Receptor and MAPK Signaling in Chronic Lymphocytic Leukemia

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SPROUTY 2: A NOVEL ATTENUATOR OF B CELL RECEPTOR AND MAPK-ERK SIGNALING IN CHRONIC LYMPHOCYTIC LEUKEMIA

By

Ashima Shukla

A DISSERTATION

Presented to the Faculty of the University of Nebraska Graduate College in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Genetics, Cell Biology & Anatomy Graduate Program

Under the Supervision of Professor Shantaram S. Joshi

University of Nebraska Medical Center Omaha, Nebraska

April, 2016

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SPROUTY 2: A NOVEL ATTENUATOR OF B CELL RECEPTOR AND MAPK SIGNALING IN CHRONIC LYMPHOCYTIC LEUKEMIA

Ashima Shukla, Ph.D.

University of Nebraska Medical Center, 2016

Supervisor: Shantaram S. Joshi, Ph.D.

Clinical heterogeneity is a major barrier to effective treatment of Chronic Lymphocytic Leukemia (CLL). Emerging evidence suggests that constitutive activation of various signaling pathways plays a role in the heterogeneous clinical outcome of CLL patients. MAPK-Erk signaling represents one such pathway with a demonstrated role in CLL pathogenesis. In this study, we have investigated the role of Sprouty2 (SPRY2) as a negative regulator of receptor and non-receptor tyrosine kinase signaling in the pathogenesis of CLL. We show that SPRY2 expression is significantly decreased in CLL cells, particularly from poor prognosis patients compared to those from good prognosis patients. Over-expression of SPRY2 in CLL cells from poor prognosis patients decreased their proliferation while increasing their apoptosis. Conversely, down-regulation of SPRY2 in CLL cells from good prognosis patients resulted in increased proliferation. Furthermore, CLL cells with low SPRY2 expression grew more rapidly in a xenograft model of CLL. Strikingly, B-cell specific transgenic over-expression of spry2 in mice led to a decrease in the frequency of B1 cells, the precursors of CLL cells in rodents. Mechanistically, we show that SPRY2 attenuates the BCR and MAPK-Erk signaling by binding to and antagonizing the activities of RAF1, BRAF and SYK in normal B cells and CLL cells. We identified that SPRY2 is targeted by miR-21 which in turn leads to increased activity of Syk and Erk in CLL cells. We also show that the
activation of miR-21 is mediated by IL-10 induced STAT3 signaling in CLL cells. Taken together, these results establish SPRY2 as a critical negative regulator of BCR-mediated MAPK-Erk signaling in CLL, thereby providing one of the molecular mechanisms to explain the clinical heterogeneity of CLL.
Chapter I

Introduction

“An Overview on Chronic Lymphocytic Leukemia”

Chronic Lymphocytic Leukemia (CLL) is the most prevalent form of adult leukemia, accounting for about 35% of adult leukemias diagnosed in the United States (1-3). There are around 15,000 newly diagnosed individual with CLL and approximately 4,500 deaths are caused by CLL each year (1). CLL is a clinically heterogeneous B-cell clonal lymphoproliferative disorder characterized by >5 × 10^9/L peripheral B-lymphocytes coexpressing CD5, CD19, and CD23 and a weak expression of CD20, CD79b, and surface immunoglobulin (sIg). The incidence of CLL significantly increases with age. With a median age lying in between 67 to 72 years CLL affects more male patients than female patients with a ratio of 2:1 (3,4).

Clinical heterogeneity makes CLL a challenging disease to provide effective treatment to patients. Along with the therapy of CLL, patients often requires an active management of a wide range of internal medicine problems related to chronic immune deficiency, infections, autoimmune complications and prevention of secondary cancers. The complexity of these related problems are mainly due to the clinical prognosis of CLL patients.

Clinical Heterogeneity and Prognosis in CLL:

CLL is clinically heterogeneous, with varying clinical outcomes and possibly distinct molecular pathogenesis (5). The overall median survival for patients with CLL is about 10 years however, the duration of survival differs substantially among individual patients. About one-third of CLL patients never need treatment. In another third, the
disease has an initial indolent phase, which subsequently leads to progression, whereas the remaining third manifest aggressive disease from the onset (3,4). Based on the Immunoglobulin variable heavy chain (IgVH) mutational status, chromosomal abnormalities and cell surface markers, CLL patients are categorized into subgroups with good or poor prognosis. Poor prognosis patients were defined by un-mutated immunoglobulin heavy chain variable (IgVH)-segments, high Zap70, 11q22 deletion, 17p deletion, trisomy 12 and/or high CD38 expression. Good prognosis patients were defined as having mutated IgVH, low Zap70, 13q14 deletions and/or low CD38 expression as shown in Figure 1.

Specific genomic alterations like deletion on the long arm of chromosome 13 (Del 13q14) leads to leukemic transformation (6). These abnormalities are caused by deletion of specific micro-RNA (miR) genes like miR-15a and miR16a which leads to impaired apoptosis in CLL cells (7). This aberration is found in around 55% of all CLL patients (1,7). Further, aberrations like deletion of the long arm of chromosome 11 (Del 11q), which is linked to deletion of ataxia-telangiectasia mutated (ATM) gene has much more aggressive disease onset (8). This aberration occurs in around 6% to 18% of CLL patients making it the second most common chromosomal aberration in CLL. Deletion of the short arm of chromosome 17 (Del 17p) which is associated with loss of p53 is found in about 7% of CLL patients and Trisomy 12 found in 12% to 16% patients whose role in pathophysiology of CLL is still unknown, seem to occur later in the course of the disease and predict a worse outcome (1,2).
Figure 1 Prognostic markers in chronic lymphocytic leukemia:

Definition of Criteria for Good vs Poor Prognosis in CLL

Schematic diagram showing the prognostic markers for classifying CLL patients into good and poor prognosis groups.
In general CLL can be categorized into two major groups: mutated and unmutated CLL (9). CLL derived from antigen inexperienced immature B cells (unmutated IgVH) determined by IgVH mutational status are predicted to have poorer clinical outcome when compared with antigen experienced post germinal center mature B cells (mutated IgVH). The molecular mechanism of unmutated IgVH leading to poor prognosis of CLL patients still remains ambiguous. However, gene expression profiling has shown that unmutated IgVh CLL and mutated IgVh CLL have distinct gene expression signature. The expression of Zeta-associated protein 70 (Zap70) is much higher in CLL cells isolated from unmutated IgVH CLL patients when compared with those from mutated IgVH CLL patients (10). Several reports have demonstrated that the high expression of Zap 70 can be used as a surrogate marker for unmutated IgVH CLL patients. It has been also demonstrated that the CLL cells expressing high levels of Zap70 also retains increased B cell receptor signaling.

Another molecule which is used as a prognostic indicator in CLL is CD38 molecule (11). CD38 is a single chain type II transmembrane glycoprotein which is present on variety of hematopoietic cells. The cellular function of CD38 includes a complex entoenzymatic activity, facilitating cell adhesion, signal transduction and calcium signaling (11). The expression of CD38 in normal B cells exhibit a discontinuous pattern during the process of B cell development. The CD38 molecule is detected in levels in B cells precursors, germinal center and plasma cells while circulating peripheral B cells and tonsillar B cells have markedly lower CD38 surface expression. The role of CD38 in CLL pathophysiology is presently unknown. However, it is tempting to speculate that differences in CD38 ligands interaction between CLL clones may influence their proliferative behavior (11).
Current Therapy used for CLL patients:

CLL is slow growing B cell neoplasm with highly variable clinical course. For more than a decade standard therapeutic approach for CLL patients has been “wait and watch”. For past few years CLL has undergone an exciting therapeutic transformation. Prior to this, patients with relapsed disease had generally unfavorable clinical outcome. Chemotherapy or single agent immunotherapy were the standard therapies. Stem cell transplantation is the only known curative therapy, which recuses about 50% of relapsed patients. However, transplanted related morbidity and mortality in older population still remain major concern. The introduction of two oral kinase inhibitor (Ibrutinib and Idelalisib) has significantly changed the standard care for relapsed patients (3).

Idelalisib is an oral reversible p110δ isoform specific phosphoinositide-3 kinase (PI3K) inhibitor. As δ isoform is specific to B lymphocytes therefore, this drug specifically targets CLL cells. It is currently approved along with rituximab for the treating relapsed and refractory CLL patients. Combination of idelalisib with rituximab led to overall response rate to 77% whereas rituximab alone was around 15%. On the other hand Ibrutinib is an oral, selective and reversible Brutons tyrosine kinase (BTK) inhibitor. Ibrutinib has been shown to inhibit the BCR signaling, prevention of CLL cells homing both in vitro and in patients. Currently, Ibrutinib is approved for relapsed or refractory CLL and CLL patients with Del 17p with an overall response rate of 91%. Along with these drug Venetoclax, BCL2 inhibitor is also a selective inhibitor however, it is not FDA approved yet (3,4). interestingly, in phase 1 study in combination with rituximab, the overall response rate was around 88%, at a median follow-up of 7 months (3).

As the target therapies has become more established in CLL, our understanding towards the molecular mechanism has also been evolved. Recent reports also indicate
emergence of resistance in a portion of CLL patients treated with targeted therapies. However, the mechanism through which CLL patients develop resistance to these drugs still remains ambiguous. Ibrutinib is the only targeted agent in CLL whose resistance mechanism is understood in certain percent of ibrutinib relapsed patients. Whole exome sequencing revealed the acquired mutations in BTK at the binding site of ibrutinib and in phospholipase Cy2 (PLCy2) following Ibrutinib therapy. The mutations in PLCy2 is gain of function mutation thus activates MAPK and Pi3K signaling in relapsed patients.

Although with the inclusion of oral selective drugs have improved the management of refractory and relapsed disease, still the major challenge is to manage the resistant disease. Consequently, CLL still remains incurable, however, these oral inhibitors have significantly improved the life span of CLL patients.

**Mutations and associated signaling pathways involved in CLL pathogenesis:**

Recent studies with whole genome sequencing and genome wide association study analysis has uncovered recurrent somatic gene mutations that occur in CLL cells in parallel to the above-mentioned structural genomic aberrations. Of these, mutations affecting the genes such as NOTCH1, MYD88, TP53, ATM, and SF3B1 seem to be more common and to have prognostic impact. However, these somatic mutations play central role in driving tumorigenesis, over course of therapy by deregulating several signaling pathways (12).

About 11% of those mutation are present in the pest domain of NOTCH1 which might be one the mechanism of prolonged activation of Notch signaling. Inactivating mutations found in TP53 and ATM genes, lead to reduction in DNA damage. Mutations in critical adaptor protein Myd88 leads constitutive activation of NFkβ signaling.
One such study with 58 CLL patient’s samples demonstrated 40% of mutation leads to activation of NOTCH signaling making it the most common deregulated signaling pathway (13). Furthermore, same study also demonstrated that 36% of CLL patients possess mutations associated with activation of MAPK signaling pathways (13). However, few other studies have shown mutations leading to activation MAPK signaling pathway are central to CLL driver events (12, 14). Recent studies have identified BRAF mutations in small cohort of CLL patients, however BRAF inhibitor has very little or no effect on CLL cells in vitro (13, 14). Highlighting some other mechanism leading to activation of MAPK signaling in CLL cells.

Although, historically CLL was considered as a malignancy of long lived matured B-cells, recently it was shown that there is a small actively proliferating population which resides in micro-anatomical sites known as proliferation centers in BM and LNs (15). CLL cells receive proliferation and survival signals in proliferation centers (16-18). These stimuli are primarily facilitated by B-cell receptors, chemokine receptors like CXCR4 receptor, BAFF receptors, CD40 and other CLL cell surface receptors (17). Once the ligand binds to its receptor they activate downstream signaling pathways including NF-kB, MAPK-Erk and Akt signaling known to promote survival and proliferation of CLL cells (18). In line with these findings, our recent studies on tumor microenvironment have demonstrated that constitutive activation of BAFF-APRIL, NF-kB, MAPK-Erk and BCR pathways are indeed important for proliferation and survival of CLL cells (18).

Our studies along with several other reports revealed a critical role for B-cell receptor (BCR) and MAPK-Erk signaling in the survival and proliferation of CLL cells (18-21). Similarly, BCR signaling is upregulated in CLL, providing a chronic stimulus for their proliferation (18-21). The role of BCR and MAPK signaling in CLL cells pathogenesis is now well-accepted.
A role for SPROUTY proteins in regulating CLL pathogenesis

Hyperactivation of MAPK-Erk and BCR signaling is hallmark of B cell malignancies including CLL (12-14). These pathways can be hyperactivated by above described genetic aberrations or by loss of function mutations or through deregulation of negative regulators like NF1, SPRED, or SPROUTY (SPRY) proteins (22-25). Therefore, to identify these deregulated genes which might be playing role in the pathogenesis of CLL we perform transcriptome analysis. We observed that the expression of SPRY2 was significantly downregulated in CLL cells isolated from poor prognosis patients when compared with those from good prognosis patients.

SPRY was first identified as a general inhibitor of RTK mediated signaling in Drosophila in a genetic screen for regulators of eye and tracheal development. SPRY2 is 1 of the 4 mammalian homologs of Drosophila sprouty gene, which encodes for 32- to 34 kDa protein (25, 24, 23). The vertebrate SPRY proteins are homologous to Drosophila protein at the cysteine rich C terminus whereas the N terminus differs dramatically. SPRY proteins specifically inhibits ligand induced RTK mediated signaling therefore might have a critical role in BCR signaling (25-30). Thus, typically these proteins negatively regulates the cellular proliferation, survival and migration. Alternatively, sprouty can increase cell proliferation by enhancing MAPK-ERK signaling in naive T cells after T-cell receptor (TCR) engagement or after EGF receptor (EGFR) activation in multiple nonlymphoid cell types (28,31).

Precise mechanism through which sprouty proteins modulate RTK signaling is still ambiguous, and several mechanisms for pathway inhibition or activation have been proposed, including antagonism of MAPK-ERK pathway signaling at the level of RAS activation, RAF activation, or upstream of RAS by sequestering the adaptor, GRB2 (26).
The interaction between SPRY2 and CBL sequesters CBL ability to ubiquinates of EGFR, leading to augmentation of epidermal growth factor (EGF)-dependent MAPK-ERK signaling pathway (27, 28).
Figure 2 Known interacting partners for SPROUTY2:

Schematic diagram generated using String database. The figure shows the known interacting partners for SPRY2 in several cellular systems.
The orange box shows the SH2-domain binding motif (amino acids 50-60) that binds to CBL, PP2A and GRB2; the key tyrosine residue Y55 is also indicated. The conserved cysteine-rich SPRY domain (amino acids 178-293) is crucial for its ability to interact with signaling molecules such as FRS2 and SHP2. The SPRY domain is also responsible for membrane translocation (MTD). There is an SH3-domain binding motif (amino acids 303-309) shown in blue at the C-terminal end of SPRY2 that also binds to GRB2. Proteins that interact with SPRY physically or functionally. Known functional consequences of the interactions are indicated in the boxed areas.
As critical attenuator of RTK signaling, SPRY proteins play an important role in attenuating ligand induced pro-growth signaling responses which can inhibit malignant transformation (32-36). Sprouty genes including Spry2 have been reported to be epigenetically repressed or targeted by miRNA-21 in several cancers of the prostate, lung, liver, lymphoma and breast (37-47). Where they function mainly as tumor suppressors by antagonizing the MAPK-ERK pathway (45-47). Consistent with this role, the expression of sprouty proteins is induced by negative feedback mechanism to reduce MAK-Erk signaling, by interacting with several proteins like RAF, BRAF, RAS, PTPN11, GRB2, EGFR, and SRC (48-51).

Role of miRNAs in CLL and their target genes

MicroRNAs (miRNAs) are small non-coding RNA molecules that modify the gene expression post-transcriptionally (37-39). MiRNA regulates the expression of multiple genes by inhibiting translation and/or stability of target messenger RNA (40,41). Over the past decade an essential role of miRNAs has emerged in cancer prognosis and pathogenesis. In CLL, miRNAs are important for disease prognosis and have been associated with the pathogenesis of the disease. Identifying the loss of miR-15a and miR-16-1 located at 13q14.3 (minimal deleted region) in CLL was the first study demonstrating the involvement of miRNAs in human disease (42). These miRNAs target several cell cycle regulators and anti-apoptotic genes like CDK6, CDK4, CHK1, BCL-2, etc. leading to decreased survival of CLL cells (43). Loss of 13q14.3 locus harboring miR15a/16-1; is the most common chromosomal aberration in CLL patients. Importantly, deletion of a region syntenic to human 13q14 or deletion of miR15a/16-1 alone leads to spontaneous CLL development at low penetrance in rodents (43). Interestingly, patients with 13q14 deletion have comparatively better prognosis (44).
Recently, several studies have shown that high miR-21 expression is associated with poor prognosis in CLL patients (10,11). MiR-21 is located at 17q.23.1 and is highly expressed in CLL patients with 17p locus deletion when compared to CLL patients with wild type 17p locus (11). Importantly, 17p deletion leads to loss of p53 and have been associated with poor patient outcomes (45). High miR-21 expression in CLL patients is linked to refractoriness to Fludarabine and associated to shorter overall survival (46). Also, Crespo et al reported that high Zap70 leads to induction of miR-21 expression in primary CLL cells (47). Together these lines of evidence demonstrate that miR-21 is overexpressed in CLL patients with poorer outcome.
Figure 4 MiR-21 targets SPRY2 at conserved 5'UTR region:

```
ANALYSIS OF MIR-21 TARGETS SPRY2 AT CONSERVED 5'UTR REGION

Figure 4 MiR-21 targets SPRY2 at conserved 5'UTR region:

This was generated using miR database, showing that Mir-21 targets SPRY2 at an evolutionary conserved region.
```
MiR-21 is an oncomir and is overexpressed in several other malignancies (48,49). Spry2, Spry1, PDCD4, TPM1 and PTEN are some of the known targets of miR-21 (12,49,50). However, it is uncertain whether Pten is a direct target of miR-21 or its destabilization occurs due to down regulation of Spry2 mediated by miR-21. Several groups have demonstrated that miR-21 directly targets and downregulates expression of Spry2 to activate MAPK-Erk signaling in human gliomas and many other malignancies (9,51). However, the role of miR-21 in the pathogenesis of CLL was not fully understood. Our studies have demonstrated that miR-21 targets SPRY2 to hyperactivate Syk mediated BCR and MAPK signaling.
Overall Significance:

Our recent studies revealed active MAPK and BCR signaling as a feature of CLL cells (18). Additionally, identification of mutations leading to constitutive activation of MAPK signaling in a significant proportion of CLL patients (36%) further entitle MAPK signaling to be crucial for CLL (12). Also genome wide association and whole exome sequencing studies have demonstrated that the activation of MAPK signaling plays a role as central driver event during the process of CLL disease progression (13,14). Therefore, it is imperative to study the role of Spry2 as a regulator of B cell receptor and MAPK signaling in CLL to better define the molecular pathogenesis of CLL. The insights gained from these studies can be used to identify new therapeutic targets for specific therapies against CLL. Thus, this research proposal is very significant because it advances the field and will identify the potential therapeutic targets to effectively treat the currently incurable, most common adult leukemia in the western world.

HYPOTHESIS:

Based on our preliminary results showing decreased expression of SPRY2 in CLL cells isolated from poor prognosis patients and activated MAPK-Erk and BCR signaling in CLL cells (18). I hypothesize that Spry2 negatively regulate the survival and proliferation of CLL cells in part by attenuating MAPK-Erk and B-cell Receptor signaling pathways.
APPROACHES:

Overview:

Our main objective is to investigate the role of Spry2 in pathogenesis of CLL and to define the molecular mechanism leading to inhibition of proliferation and survival CLL cells. We would also like to demonstrate the molecular mechanism for SPRY2 downregulation in CLL cells isolated from poor prognosis patients.

To address this we have used primary CLL cells isolated from the peripheral blood of good prognosis and poor prognosis patients. We have used Mec-1 cells a CLL cell line showing features of constitutively active BCR and MAPK-Erk signaling (52). Mec-1 cells are wide used human CLL cell line. Mouse models of CLL have contributed hugely to our present understanding of the disease. To address our specific aims we used three mouse models. 1. CD19-CRE mouse model which directs the expression of cre recombinase specifically in B-cells (53). We will use these mice to direct Spry2 expression only in B-cell. 2. Spry2 conditional transgenic mouse model, prior to CRE-mediated deletion, the transgene expresses LacZ, while after cre mediated deletion a bicistronic mRNA containing both mouse Spry2 and human placental alkaline phosphatase (PLAP) coding sequence is expressed (54). 3. We will use NOD-SCID immunodeficient mice for xenograft mouse models. Using these reagents we have addressed four specific aims:

AIM 1: To evaluate the role of Spry2 in CLL cell survival and proliferation:

In this aim we hypothesize that Spry2 negatively regulates the proliferation and survival of CLL cells. To test this hypothesis we will manipulate the levels of Spry2 expression in vitro and in vivo. Our working systems will help us in understanding the effect of Spry2 on the survival and proliferation of CLL cells. The transgenic mouse
model expressing Spry2 in B cells will provide valuable information for the role of Spry2 in normal B cell development. We will use the following sub-aims to achieve the goals of this Aim.

(a) To determine the effect of differential expression of Spry2 on primary CLL cells.

In this sub-aim we hypothesize that increased expression of Spry2 negatively regulates the survival and proliferation of primary human CLL cells. We used an IRB approved protocol to collect peripheral blood from good and poor prognosis CLL patients. We isolated CLL cells by negative selection using Miltenyi Biotech B-CLL cells isolation kit. We reconstituted the expression of Spry2 in primary CLL cells from poor prognosis patients using nucleofection as described previously (55). We used GFP coexpressing empty and Spry2-cDNA containing vector for transfection to primary patient CLL cells. Cells were analyzed by Annexin-V staining using Flow cytometry, 48 and 72 hours post transfection with respective vectors to determine the number of apoptotic cells. We also performed Spry2 knockdown in CLL cells using siRNAs from good prognosis patients and study their proliferation by Ki-67 staining.

(b) To examine the role of Spry2 in CLL initiation and progression.

In this sub-aim we hypothesize that increased Spry2 expression in B1 cells will delay CLL initiation and progression. B1 cells are the precursor cells of CLL in rodents. Based on our preliminary data and published literature, it appears that the ectopic expression of Spry2 decreases the viability of B cells and increases the apoptosis of CLL cells. In order to study the effect of Spry2 on...
CLL initiation and progression we crossed the CD19-Cre mouse to Spry2 transgenic mouse to generate Spry2 transgenic mouse expressing the transgene specifically in B cells (CD19cre-Spy2(tg)). We measured the absolute numbers of B1 cells and studied the proliferation of splenic B2 cells using CSFE labeling. We also used xenograft model of SPRY2 knockdown Mec-1 CLL cells to study SPRY2 role in CLL disease progression.

**AIM 2: To elucidate the mechanism through which Spry2 regulates proliferation and survival in CLL cells.**

In this Aim we hypothesize that Spry2 negatively regulates the survival and proliferation of CLL cells by attenuating BCR and MAPK-Erk signaling pathways. To test this hypothesis we studied the effect of Spry2 on cells with constitutively active MAPK-Erk and BCR. We used calcium influx assay in SPRY2 modified normal human B cells and splenic B2 cells from CD19cre-Spy2(tg) mice to study BCR signaling.

**(a) To investigate the effect of Spry2 induced expression on constitutively active MAPK and BCR signaling in vitro.**

We hypothesize that Spry2 will inhibit constitutive activity of MAPK-Erk and BCR signaling in CLL cells. There are several studies reporting mutations leading to constitutively active MAPK-Erk signaling pathways in CLL patients. To demonstrate this we used doxycycline inducible system where we reconstituted the expression of SPRY2 in Mec-1 cells upon doxycycline treatment. Expression of pErk was measured in those cells. To study the effect on BCR signaling we measured the expression of pSyk in SPRY2 reconstituted Mec-1 cells using
immune-fluorescence. This helped us in determining that the effect of Spry2 on hyperactive and genetically altered MAPK and BCR signaling.

(b) To study the molecular mechanism through which SPRY2 downregulate BCR and MAPK-Erk signaling.

We hypothesize that Spry2 will attenuate MAPK and BCR signaling by interacting with SYK, BRAF and RAF in CLL cells. To demonstrate this we used BRAF and KRAS mutants co-transfected with SPRY2 constructs. We also used immunoprecipitation and immunofluorescence to study the co-localization and interaction of SPRY2 with SYK, RAF and BRAF in CLL cells and normal human B cells. In addition we used inhibitors to demonstrate the molecular mechanism through which SPRY2 downregulate survival and proliferation of CLL cells.

AIM 3: To demonstrate the role of STAT3 in activating miR-21 to target Spry2 in CLL cells.

In this aim we hypothesize that STAT3 induces the expression of miR-21 to inhibit Spry2 leading to activation of BCR mediated MAPK-Erk signaling in CLL cells. To demonstrate this we used stably pcDNA3.1-mir21 transfected Mec-1 cell line. For control we used stably transfected pcDNA3.1-Empty transfected and mec-1 cell line.

(a) To investigate the effect of miR-21 on the expression of Spry2 in CLL cells.

In this sub aim we hypothesize that high expression of miR-21 suppresses Spry2 expression in CLL cells. To study this stably transfected Mec-1 CLL cells with pcDNA-mir21 co-expressing GFP plasmid to upregulate mir-21
levels (54). After stable transfection, we measured the levels of miR-21 by performing qPCR. The protein levels of SPRY2, SYK and pErk were measured by using western blot.

(b) To demonstrate that STAT3 activates miR21 in CLL cells.

In this aim we hypothesize that STAT3 binds at miR21 promoter region to induce expression of miR-21 in CLL cells. To study this we stimulated STAT3 expression by stimulating Mec-1 CLL cells with IL-10 and IL-6. We performed chromatin immunoprecipitation of STAT3 and studied the enhancement of miR21 promoter region.

(c) To determine the effect of miR-21 inhibition on Spry2 in human CLL cells.

In this aim we hypothesize that miR-21 inhibition decreases the survival and proliferation by increasing Spry2 expression in primary human CLL cells. In this aim we will use primary CLL cells in vitro and Mec-1 CLL cells for testing anti-miR-21 and STAT3 inhibitors. To study the effect of miR-21 inhibition we used anti-miR-21 inhibitor, synthetic oligonucleotides designed to specifically bind and inhibit endogenous miRNAs from Ambion, Life Technologies (55). As miR-21 is overexpressed in CLL patients with poorer outcome we will use CLL cells from poor prognosis patients as described previously. We treated primary CLL cells from poor prognosis patients with STAT3 inhibitor, Stattic (CAS 19983-44-9) from SantaCruz biotechnology. We used three different concentrations of 2.5µM, 5µM and 10µM as previously described in other studies (ref). After 48 and 72 hours post Stattic treatment cell viability was measured using MTT assay. For negative control we will use DMSO vehicle only. As miR-21 is upregulated in relapsed and/or refractory CLL, we therefore, derived an Ibrutinib resistance CLL
cell line. To target MAPK signaling and miR-21 in these cells we treated Ibrutinib resistance cells with STAT3, MEK-1 and SYK inhibitors.
Chapter II

Material and Methods:

Isolation of CLL cells from patients and normal B-cells from healthy donors:

Peripheral blood from CLL patients/healthy donors was obtained using UNMC approved IRB protocols. Mononuclear cells were isolated using Lymphoprep (StemCell technologies, Vancouver, Canada) following manufacturers instruction. CLL/normal B-cells (n-B cells) were isolated by negative selection using B-CLL cells /B-cell isolation kit Milteny Biotech San Diego, CA. Purity of the isolated CLL/n-B cells were tested by flow cytometry using CD19+CD5+/only CD19+ cell surface markers. When the CLL cell number was more than 90% in the peripheral blood from patients, the cells purification step was not performed. CLL patients with High CD38, unmutated IgV, Del 17p and Del 11q were categorized as poor prognosis, whereas CLL patients with Low CD38, mutated IgV and only Del 13q14.3 were categorized into good prognosis.

Biological reagents and cell lines:

Mec-1 cell line was kindly provided by Dr. Runqing Lu, UNMC. Mec-1 cells were cultured in RMPI media with 10%FBS, 1% pen strep and 1% L-glutamine (RF-10). Primary CLL cells were co-cultured on S-17 stromal cells in RF-10 media for apoptosis and proliferation experiments. Normal B cells were co-cultured on OP-9 stromal cells in RF-10 media.

Antibodies:

Antibodies for Spry2, BRAF, RAF-1, Syk were purchased from Santa Cruz Biotechnologies and antibodies for Spry2, p-Syk, p-Erk, Erk, PI3K, BTK and B-actin were purchased from Cell Signaling Technology.
Flow cytometry:

Cells were washed with 1xPBS twice and resuspended in MACs buffer. Cells were stained with antibodies. Washed twice with MACs buffer and taken for flow analysis. For enumeration of apoptotic and proliferating cells Annexin-V (PE conjugated) apoptosis and Ki-67 proliferation assay (PE conjugated) kit was purchased from BD Biosciences, San Jose, CA. Cells were processed under manufacturer instructions. Flow cytometry was performed on FACSCalibur (BD Bioscience), using CellQuestPro software (BD Bioscience).

Immunoprecipitation:

Normal-B cells from healthy donor were stimulated by BCR crosslinking for 24 hours. Cells were washed two times with 1X PBS and then re-suspended in ice cold 500µl of NP40 lysis buffer with protease and phosphatase cocktail inhibitor. After 30 minutes incubation on ice, Primary antibody was added and incubated overnight at 4C on shaker. Agrose beads were added and incubated for 2 hours at 4C on shaker. Beads were washed twice with NP40 lysis buffer, beads were mix with 1x SDS loading buffer dye boiled at 95C for 10 mins. Beads were removed and protein lysate was loaded on 10%SDS gel for protein analyses.

Plasmids and Anti-sense:

Spry2 cDNA plasmids were kindly provided by Dr. Hamid Band, UNMC. Spry2 cDNA was cloned in migR1 and TET-pLKO construct by using Xhol and EcoRl restriction enzyme site. pcDNA3.1-mir-21 plasmid (56) was purchased from Addgene, Cambridge, MA. pLenti-Spry2, pLenti-Empty, pLenti-scramble, pLenti-s pry2siRNA(a) and pLenti-s pry2siRNA(b) was purchased from ABM Goods, Richmond, Canada. Spry2 siRNAs were purchased from Invitrogen Life technologies Carlsbad, CA.
BCR crosslinking:

Primary CLL cells and normal human B cells were stimulated with 5µg FCRII anti-IgM and anti-IgD antibodies (Sigma Aldrich, St. Lousi, MO) for different time points.

Flow cytometry:

Cells were washed with 1xPBS twice and resuspended in MACs buffer. Cells were stained with antibodies. Washed twice with MACs buffer and taken for flow analysis. For enumeration of apoptotic and proliferating cells Annexin-V (PE conjugated) apoptosis and Ki-67 proliferation assay (PE conjugated) kit was purchased from BD Biosciences, San Jose, CA. Cells were processed using manufacturer instructions. Flow cytometry was performed on FACSCalibur (BD Bioscience), using CellQuestPro software (BD Bioscience).

Calcium mobilization assay:

Human n-B cells from peripheral blood of healthy donor were isolated and murine B-cells were isolated from spleen by negative selection as previously described. Calcium influx assay was performed and analyzed as described by Ma et al. (57)

Animal studies:

CD19-rtTA-cre animals were kindly provided by Dr. Runqing Lu, UNMC. Spry2 conditional transgenic mouse (54) was purchased from MMRRC, UNC, Chapel Hill, NC. To generate CD19cre-spry2(tg) mice we crossed CD19-rtTA-cre and spry2(tg) flox/flox. Overexpression of spry2 in B-cells was tested by western blot. Expression of spry2 was tracked by GFP positive cells in flow cytometry experiments. NOD-SCID gamma (NSG) mice were purchased from Jackson
Laboratory, Bar Harbor, ME. All animal experiments were conducted under the guidelines of approved IACUC protocols.

**Nucleofection:**

Primary B cells/ CLL cells were nucleofected with Spry2 cDNA plasmid or Empty using primary B cells kit, Lonza, Basel program U-16. Mec-1 cells were nucleofected using lonza solution V kit program X-01 (55).

**Western blot analysis:**

As previously described by us, western blotting was performed to analyze protein expression (58). Antibodies for Spry2, BRAF, Raf-1, Syk were purchased from Santa Cruz Biotechnologies, Dallas, TX and whereas antibodies for Spry2, p-Syk, p-Erk, Erk, PI3K, BTK and B-actin were purchased from Cell Signaling Technology, Beverly, MA.

**Statistical Analysis:**

All data reported in graphs are expressed as SD+, p values were calculated by T-Test and were considered statistically significant when less than 0.05. All experiments were repeated at least 3 times unless specified.

**Ki-67 proliferation assay:**

Cells were harvested and fixed with 2% PFA for overnight. Add 1ml 70% - 80% ethanol/methanol dropwise into the cell pellet (1-5 x 10⁷ cells). Cells were incubated on ice for at least 1 hour. These fixed cells were then washed twice with 30-40 ml MACS buffer, centrifuge for 10 minutes at 200 x g. Cells were then resuspended to a concentration of 1 x 10⁷/ml. Transfer 100 µl (1 x 10⁶ cells) cell suspensions into each sample tube. Add 20 µl of properly diluted PE conjugated anti-Ki-67 antibody (BD Bioscience) according to the protocol into the tubes above.
Cells were mixed gently. Incubate the tubes at room temperature (RT) for 20-30 minutes in the dark. Cells were then washed with 2 ml of staining buffer at 200 x g for 5 minutes. Aspirate the supernatant. Wash with 2 times with MACS buffer. Add 0.5 ml of staining buffer to each tube. Proceed to flow cytometric analysis.

**DNA-Cell Cycle Analysis and Apoptosis Evaluation Telford Method**

DNA analysis was performed by staining the cells with propidium iodide, a fluorescent dye which intercalates between DNA base pairs. The fluorescent intensity of the dye within the nucleus is directly related to the amount of DNA present. Normal cells contain a diploid DNA content (2N) where N is equal to the number of chromosomes in a normal haploid gamete. Tumor cells often demonstrate abnormal DNA content. Aliquot 1 x 10^6 cells into a 12x75 mm test tube. Pellet the cells in the benchtop Immufuge for 1 min on “High”. Decant the supernatant and add 1 ml of 70% ethanol. Mix well and incubate at 4° C or on ice for 1 hour. Spin down for 1 min and decant ethanol. Wash 1X with 1 ml of PBS and spin down. Add 1 ml of Telford reagent (DNA stain for ethanol fixed cells). Incubate at 4° C overnight or for at least 30 min. Analyze by flow cytometry. Cells were incubated overnight at 4° C to allow better intercalation of the PI and diffusion of small DNA fragments to diffuse from the cells.

**Immunofluorescence staining of cells.**

Cells were harvested and cytospins were prepared. By adding 4% PFA for half an hour cells were fixed. Gently, perform two washes with 0.1M Glycine for 5 mins. Permeabilize the cells by adding Permeabilization Buffer drop wise for half an hour. After permeabilization cells were blocked by adding blocking Solution for half an hour. Incubate cells in primary antibody in 10% goat serum or 5%BSA overnight at 4C (or one hour for 37C). Wash cells three times with 1/10 permeabilization buffer in PBS for 10 mins. Incubate cells with secondary antibody in 10% Goat serum or 5%BSA for 40 mins to 1 hour at room temperature (or 37C).
Cells were washed three times with permeabilization buffer in PBS for 10 mins. Followed by one wash with 1/10 permeabilization buffer in PBS. Cells were then stained with DAPI for 5 minutes. Mount the cells with Mounting Media. These slides were then analyzed in immunofluorescence microscope and the expression of proteins were determined by the fluorescence intensity of specific protein antibodies.

**RNA isolation using Trizol**

Cells were harvested and washed twice with 1 x PBS. Add at least 1ml of TRI Reagent™ (Invitrogen, CA) per 10 million cells to resuspend. (If the cells are more or you see cloudy particles in tube split it into two and add more trizol). Mix well and incubate at room temperature for 5 mins. Add 300ul of chloroform and mix by inverting the tube for 15-20 secs. Set the tubes for 5 mins at room temperature. Centrifuge the sample at 12,000 rpm at 4C for 15 mins. Extract the clear upper supernatant (do not disturb the cellular layer) and put it in a fresh tube. Add 500ul isopropanol and 1ul of glycogen (RNA graded from invitrogen). You can stop at this step and store the samples at -80C. Keep the samples at room temperature for 5 mins. Spin for 10 mins @ 12000 rpm at 4C. Decant the supernatant and add 1ml 75% ethanol and mix and spin @ 7500rpm for 5 mins. Decant the supernatant and air dry for 5-10 mins. Re-dissolve in 20ul of ddH2O. Store RNA at -80C or process it for cDNA immediately.

**Quantitative Real Time PCR**

Total RNA from cells was extracted and purified using TRI Reagent™ as described above. Five micrograms of total RNA was then used for reverse transcription by using random hexamer primers and the superscript RT enzyme (Invitrogen, CA). The resulting cDNA was subjected to SYBR green real-time PCR to investigate the differential expression of genes. The cDNA was mixed with primers and SYBR green PCR master mix (Applied Biosystems, CA), and real-time
PCR was performed using the ABI Prism-7900HT real-time PCR detection system (Applied Biosystems, CA). The PCR reaction consisted of 36 cycles of 30 seconds at 95°C, then 30 seconds at 60°C, and finally 30 seconds at 72°C. Cycle threshold (Ct) values were used to determine fold changes in expression and compared among treatment groups after being normalized with GAPDH.
Chapter III

SPROUTY2: A novel regulator of B cell receptor and MAPK signaling pathways in CLL.

Introduction:

Precise regulation of cellular processes, such as those mediated by B-cells, requires homeostatic integration between intrinsic and extrinsic factors (59). Deregulation of such homeostatic processes in CLL cells could result in a state of constitutive activation of MAPK-Erk and BCR signaling. Constitutive activation of BCR and MAPK-Erk signaling promotes CLL cell survival and proliferation (12-14,18). However, the molecular mechanisms that lead to the constitutive activation of these pathways have not been fully explored. Identifying novel regulators of these pathways in CLL is crucial for understanding the disease biology and for the eventual development of targeted therapies.

To identify potential regulators of BCR and MAPK-Erk signaling in CLL, we performed a transcriptome analysis for genes that are differentially expressed in CLL patients with good vs poor prognosis. Of interest in relationship to MAPK-Erk signaling, we observed that expression of Sprouty-2 (SPRY2), a member of the sprouty protein family to be significantly down-regulated in CLL cells from poor prognosis patients compared to those from good prognosis patients. Sprouty proteins play key roles in maintaining cellular homeostasis by attenuating signaling, including MAPK-Erk pathway, downstream of several receptor tyrosine kinases (RTKs) (22-26). Notably, none of the other Sprouty family members (SPRY1, 3 and 4) showed significant differences in expression between the good and poor prognosis CLL cells. Therefore, we hypothesized that low levels of Sprouty 2 lead to a state of constitutive activation of BCR and MAPK-
Erk signaling in CLL patients with poorer clinical outcomes. Mechanistically, SPRY2 functions as an inhibitor of the ligand-induced RTK signaling. In addition, tyrosine phosphorylation of SPRY2 creates SH2 domain-binding motifs that are critical for its recruitment to signaling complexes at the plasma membrane and attenuation of signaling cascades triggered by membrane associated RTKs (22, 27). Therefore we reasoned that SPRY2 might act as a negative regulator of BCR signaling to inhibit the survival and proliferation of CLL cells. Consistent with such a possibility, a recent study demonstrated the induction of SPRY2, but not SPRY1, downstream of BCR signaling in mouse B-cells (32). This study also showed that SPRY2 levels negatively correlate with Erk signaling in mouse B cells, a finding similar to that described in other cellular systems (22-26, 32, 33). However, the molecular mechanism by which SPRY2 functions as a negative regulator of BCR signaling has not been deciphered. Moreover, the role of SPRY2 in B cell development and function are unknown. SPRY2 was previously shown to be down-regulated in Diffuse Large B cell Lymphoma but the functional significance of this down-regulation remains ambiguous (32).

In the present study, we identify SPRY2 down-regulation as a marker of poor prognosis in CLL and demonstrate that loss of SPRY2 provides a novel mechanism to constitutively activate BCR and MAPK-Erk signaling in CLL. Along with RAF and BRAF we have identified spleen tyrosine kinase (SYK) as a novel interacting partner of SPRY2 in normal B cells and CLL cells. We have also defined a novel role for SPRY2 as a negative regulator of BCR signaling. This result highlights a potentially complex role for sprouty proteins in cancer from distinct activities as either tumor suppressors. Finally, we show that SPRY2 is targeted by miR-21 in poor prognosis CLL that leads to constitutive activate state of BCR and MAPK-Erk signaling in CLL cells.
Results:

Spry2 expression is lower in CLL cells from poor prognosis patients compared to good prognosis patients:

CLL is clinically heterogeneous, with varying clinical outcomes and possibly distinct molecular pathogenesis. In order to identify molecular mechanisms associated with the poor prognosis form of CLL, we performed RNA-sequencing based transcriptome analysis of 15 CLL samples: 7 from good prognosis and 8 from poor prognosis patients. Poor prognosis patients were defined by un-mutated immunoglobulin heavy chain variable (IgVH)-segments, 11q22 deletion, 17p deletion, trisomy 12 and/or high CD38 expression. Good prognosis patients were defined as having mutated IgVH, 13q14 deletions and/or low CD38 expression (criteria used for Figure1-A, B and C). This method allowed us to simultaneously identify recurrent mutations and differential gene expression. We validated the differentially expressed genes using gene expression profiling data from a separate cohort of 38 CLL patients. Using these approaches, the expression levels of 146 genes were found to be significantly different between good versus poor prognosis CLL samples. We further selected genes based on their roles in B cell biology, or for their putative tumor suppressor or pro-oncogenic functions. Interestingly, spry2 was found to be significantly (3.4 log2-fold) down-regulated in the CLL cells from poor prognosis patients compared to those from good prognosis (Figure 1A). The differential expression was confirmed using western blotting and real time PCR of CLL cells from good vs. poor prognosis patients (Figure 1B & C). High CD38 positivity of CLL cells is associated with poor patient outcomes and constitutive activation of MAPK-Erk signaling (60). Therefore, we further compared spry2 expression in a larger cohort of 38 patients. Spry2 was significantly down-regulated (~2.8 folds) in CLL cells of patients with high CD38 expression compared to those with low CD38 expression (Figure 1D). We also observed low Spry2 mRNA expression in CLL
cells isolated from patients with unmutated IgVh and Del 17p when compared those from patients with mutated IgVh and Del 13q (Figure 1E & F). As CLL is clinical heterogeneous disease we also studied the expression of SPRY2 in a larger cohort of primary CLL cells in the study using Oncomine database and observed significant reduction in SPRY2 expression when compared with normal B cells. (Figure 1G).

As microenvironment plays a critical role in the survival of CLL cells. Indeed, the lymph node microenvironment is able to promote B-cell receptor signaling and tumor proliferation in CLL. In this respect, to study the effect of stromal co-culture of primary CLL samples on SPRY2 expression we co-cultured primary CLL cells on S-17 stromal cells and CLL cells alone. However there was no effect on SPRY2 levels in culturing CLL-cells alone vs CLL-cells co-cultured on S-17 cells (Figure 1H). Nevertheless, SPRY2 levels are much lower in CLL-cells isolated from bone marrow when compared with the CLL-cells isolated from peripheral blood from same patients as shown in Figure 1I. These results highlights that deregulation of Spry2 in CLL cells in CLL tumor microenvironment. Whereas examination of the expression of other sprouty family members (SPRY1, 3 and 4) in our data-set showed that, none of them were significantly differentially expressed between the good and poor prognosis CLL cells (data not shown). Thus our results show that the expression of SPRY2 is significantly down-regulated in CLL cells from patients with poor outcomes.

**SPRY2 is induced upon BCR cross-linking of normal B-cells and CLL cells, and functions as a negative regulator of BCR signaling:**

It is well established that BCR signaling is critical for survival and proliferation of CLL cells, however; no known recurrent mutations have so far been identified in this pathway among CLL patients. It has been established that SPRY2 functions as a negative
feedback regulator of RTKs (22-36). To investigate if SPRY2 is regulated by BCR signaling, we stimulated normal B cells from healthy donors and CLL cells from patients using BCR crosslinking with an anti-IgM/IgD antibody. Cells were collected at 0, 6, 12, 24 and 48 hours after stimulation and the expression of SPRY2 was determined by western blotting. We observed a gradual increase over time in SPRY2 expression in BCR stimulated normal B-cells (Figure 2A upper panel). On the other hand, primary CLL cells and Mec-1 CLL cell line exhibited a biphasic induction of SPRY2 (Figure 2A middle and lower panels). In CLL cells, SPRY2 expression peaked at 12 hours upon stimulation and declined at later time points (Figure 2A middle and lower panels). Thus, we conclude that SPRY2 expression induced by BCR crosslinking is sustained in normal B-cells but follows a cyclical expression pattern in CLL cells and is not sustained.

We next studied the effect of downregulating SPRY2 expression with siRNAs on BCR signaling in normal B cells obtained from healthy donors. We first tested the siRNAs in B cells. Compared to the scramble siRNA control, both SPRY2 siRNAs led to a decrease in the SPRY2 protein levels, but siRNA-B was more robust compared to siRNA-A (Figure 2B). Next, we transfected normal B-cells with the scrambled control or SPRY2 specific siRNAs and compared their anti-IgM-induced calcium influx with that of unperturbed control following with the Indo-1 dye. Compared to un-transfected and scrambled siRNA controls, B-cells with SPRY2 knockdown exhibited elevated calcium influx (Figure 2C). Even the basal levels of calcium were increased in B cells with SPRY2 knockdown. Taken together, these results establish that SPRY2 functions as a negative regulator of BCR signaling.
SPRY2 depletion in human CLL cells from Good Prognosis patients lead to increased proliferation.

As an approach to assess if the down-regulation of SPRY2 expression in poor vs good prognosis CLL cells is of functional consequence, we examined the impact of SPRY2 depletion in good prognosis CLL cells. For this purpose, we isolated CLL cells from newly-diagnosed patients with good prognosis CLL based on low CD38 expression, Del 13q and/or normal karyotype. We also used Mec-1 human CLL cell line, a widely-used cultured CLL cell model, for these studies. Mec-1 cells have an intermediate level of SPRY2 expression relative to primary good and poor prognosis CLL cells, as detected by western blotting (Figure 2A). As SPRY2 expression is induced on CLL cells upon BCR stimulation (Figure 2A), we assessed the impact of SPRY2 depletion on anti-IgM or anti-IgD induced Mec-1 cell proliferation, by analyzing changes in Ki-67+ fraction in FACS analyses. SPRY2 knockdown led to an increase in proliferation of Mec-1 cells relative to scrambled siRNA control under both anti-IgM and anti-IgD cross-linking condition (Figure 3A). Next, we transfected primary human CLL cells isolated from multiple good prognosis patients expressing higher levels of SPRY2 with siRNAs against SPRY2. 24 hours post transfection, the cells were subjected BCR cross-linking and level of proliferation was assessed 48 hours later. Knockdown of SPRY2 led to an increase in the number of proliferating cells compared to the scrambled siRNA transfected cells (Figure 3B, C & D). We confirmed this finding in CLL cells isolated from peripheral blood of 7 different CLL patients. We observed an increase in proliferation upon SPRY2 depletion compared to scrambled controls, with increased proliferation seen in 6 out of 7 with siRNA-B and in 5 out of 7 cases with siRNA-A mediated knockdown. The effect on proliferation were consistent with a more robust SPRY2 knockdown with siRNA-B with siRNA-A. We also measured the extent of apoptosis in these samples by Annexin-V staining, but we did not
observe significant differences between control and SPRY2 knockdown cells (data not shown). These findings indicate that SPRY2 functions as a key negative feedback regulator of BCR signaling in CLL cells and involved in limiting their BCR induced proliferation.

**SPRY2 expression induces apoptosis in human CLL cells from poor prognosis patients:**

To further elucidate the underlying basis for the down-regulation of SPRY2 expression in CLL cells from poor prognosis patients, we also examined the impact of ectopic SPRY2 expression on CLL cells with low SPRY2 expression. First, we examined the impact of increasing the expression of SPRY2 in Mec-1 cells, which express intermediate levels of SPRY2. We transfected Mec-1 cells with a SPRY2 expression construct that co-expresses a GFP as a reporter of transfection. At 48 and 72 hours post transfection, we observed an average of 58% and 18% of SPRY2 expressing (GFP+) Mec-1 cells to be Annexin V positive, respectively, compared to, 36% and 11% (Figure 4A & B). Next, we isolated CLL cells from five different poor prognosis patients, ectopically expressed Spry2 in these cells and measures the proportion of Annexin V staining cells after 48 hours (Figure 4C). An increase in apoptosis was observed in CLL cells from all five patients, SPRY2 as a negative regulator of CLL cell survival (Figure 4C & D). Moreover, we did not observe a significant impact on the proliferation of these cells compared to controls. Thus, our results demonstrate that low levels of SPRY2 in Poor prognosis patients CLL cells contribute to a survival advantage for cells.
B-cell-specific elevation of SPRY2 levels attenuates and suppresses B1 cells population:

To study the effect of SPRY2 on the development and function of B-cells in vivo we generated a B-cell-specific SPRY2 transgenic mouse model. For this purpose, we crossed the mice harboring a spry2 transgene preceded by a STOP cassette (Spry2(tg)) with mice carrying a CD19rtTa-cre transgene to obtain a mouse line (CD19-cre;Spry2(tg)) in which the expression of cre recombinase expression and hence SPRY2 expression are specifically induced in B-cells (Figure 5A). We observed that the generation of B-cells in these mice was apparently normal (Figure 5B). However, we observed a decrease in the number of a specialized B cell subset known as B1 cells in the CD19-cre;Spry2(tg) mice. This was particularly interesting because B1 cells are presumed to serve as precursors of CLL cells in mouse models of CLL. We found a decrease in the percentage of B1a (B220+ CD5+) cells in the peritoneal cavity of these mice (Figure 5C). Furthermore, we examined the effect of SPRY2 overexpression on BCR signaling in B-cells of CD19-cre;Spry2(tg) mice, compared to CD19-cre control mice by measuring their calcium influx over time in response to BCR cross-linking. The calcium influx in B-cells from CD19-cre;Spry2(tg) mice showed was significantly reduced compared to B cells isolated from CD19-cre control mice (Figure 5D). To study the effect of SPRY2 overexpression on proliferation, we isolated splenic B-cells from CD19-cre;Spry2(tg) mice labeled these with the CFSE dye and cultured the cells with and without anti-IgM antibodies. Strikingly, we detected very few or no cells undergoing division in CD19-cre;Spry2(tg) compared to CD19-cre control cells with or without BCR stimulation (Figure 5E). Taken together, these results show that Spry2 functions to limit the numbers of B1 cells (CLL precursors) in mice and negatively regulates BCR signaling and associated proliferation in mouse B-cells.
SPRY2 depletion in CLL cells results in more rapid lymphomagenesis in NSG mice:

In view of the effect of SPRY2 on the survival and proliferation in vitro, as well as its impact in regulating B1 pool of mouse B-cells, we modeled the low SPRY2 expression seen in human CLL disease in a mouse lymphomagenesis model. For this purpose, we used a xenograft model with Mec-1 CLL cells transplanted into NOD-SCID-Gamma chain deficient (NSG) mice. We derived SPRY2 knockdown version of Mec-1 cells by transducing them with pLenti-siRNA(A) or pLenti-siRNA(B), with pLenti-scramble siRNA (pLenti-scr) Mec-1 cell line as a control. Proliferating cell populations were measured by Ki-67 staining. We observed about 30% or 32% increase in proliferation of the pLenti-siRNA(A) and pLenti-siRNA(B) cell lines, respectively, when compared with the pLenti-scr cell line (Figure 6A). We also observed a significant decrease in the percentage of cell exhibiting apoptosis in pLenti-siRNA(A) and pLenti-siRNA(B) cell lines, compared to scrambled control (Figure 6B). Next, we injected 1.5x10^6 pLenti-siRNA(B) (higher degree of SPRY2 down-regulation) or pLenti-scr expressing Mec-1 cells subcutaneously into the left flank of sublethally irradiated NSG mice (5 mice per group). Tumors were palpable after 21 days of cell injections. Measurement of tumor volume using caliper demonstrated that pLenti-siRNA(B) Mec-1 cells gave rise to significantly larger tumors at the primary site of cell injection (Figure 6C&D). Dissemination of human CLL cells into other organs was measured by anti-human CD19 flow cytometry analysis. We observed significantly more human CD19 positive CLL cells in spleens of pLenti-siRNA(B) Mec-1 cell-injected mice (Figure 6E). Measured by H&E staining of spleen, liver and kidney also revealed more organ infiltration in pLenti-siRNA(B)-expressing CLL cells (Figure 6F). Western blot analysis of tumor isolated from NSG recipient mice confirmed the continuous down-regulation Spry2 in pLenti-siRNA(B) cell line-derived tumors compared to scrambled control cell line-derived tumors (Figure 6G). Furthermore, we also observed an increase
in pERK levels in tumors arising from SPRY2-depleted Mec-1 cells (Figure 6G). These results show that lower SPRY2 expression leads to the formation of more rapid and aggressive lymphomagensis in mice, indicating a role for SPRY2 down-regulation in CLL disease progression.

**Spry2 interacts with Raf-1, Braf and Syk to down regulate MAPK-Erk signaling in CLL cells.**

To elucidate the mechanism by which SPRY2 mediates a decrease in proliferation and survival of human CLL cells, we used a tet-on inducible system. We stably transfected Mec-1 cells with spry2 under the control of a tet-on promoter. Upon doxycycline treatment, we observed a dose-dependent increase in SPRY2 expression that was accompanied by a decrease in pERK levels (Figure 7A). Other groups have shown that SPRY2 interacts with Raf-1 and Braf to inhibit MAPK-Erk signaling in malignancies such as multiple myeloma (48-50). Interestingly, mutations in Raf-1 and Braf have also been identified in CLL patients (15). We first tested the conservation of these networks in human B-cells using immunoprecipitation of SPRY2 in BCR stimulated normal B-cells from healthy donors. Immunoprecipitation of SPRY2 in lysates of human B-cells led to co-immunoprecipitation of Raf-1 and Braf in suggesting that SPRY2 may attenuate MAPK-Erk signaling by inhibiting Raf-1 and Braf activities.

We next tested the effect of SPRY2 on MAPK-Erk signaling in the presence of Braf and Ras mutants. It has been demonstrated that Braf V600E mutant harboring a valine to glutamic acid substitution does not physically interact with SPRY2 and is resistant to SPRY2-mediated attenuation of MAPK-Erk signaling (50). Additionally, Ras mutants function upstream to Raf and can bypass SPRY2 mediated inhibition (51). Therefore, we
tested the effect of SPRY2 on these mutants in human CLL cells. Intriguingly, SPRY2 expression led to a decrease in p-Erk expression level even in the presence of BRAF V600 mutant however, while there was no impact of inducing SPRY2 expression on p-Erk level in cells transfected with the Kras V12 mutant (Figure 7B). These results indicate an additional and possibly parallel mechanism by which SPRY2 may attenuate MAPK-Erk signaling in B cells and CLL cells.

Interestingly, a recent study showed that in the presence of BRAF inhibitors, Syk undergoes upregulation to activate Erk signaling in CLL cells, an event mediated via Ras (61). Furthermore, inhibition of Syk reversed the Erk hyper-activation and led to a decrease in proliferation of CLL cells (61). Therefore, we speculated that SPRY2, in the presence of Raf inhibition may also inhibit Syk activity in B cells and CLL cells to attenuate MAPK-Erk signaling. To test this hypothesis we performed immuno-precipitation assays in stimulated normal B cells from healthy donors. Intriguingly, SPRY2 was found to physically interact with Syk but not with Bruton’s Tyrosine kinase (BTK) and PI3K in B cells as determined by reciprocal immuno-precipitation experiments (Figure 7C). Also, using immunofluorescence analysis, we observed a co-localization of SPRY2 with p-Syk in normal B cells and Mec-1 CLL cells (Figure 7D). We next tested the effect of SPRY2 overexpression on the activated form of Syk by measuring the levels of p-Syk in splenic B-cells from CD19-cre;Spry2(tg) mice and SPRY2 overexpressing Mec-1 cells by immunofluorescence. Notably, we observed a decrease in p-Syk expression in B-cells of CD19-cre;Spry2(tg) mice and Mec-1 cells overexpressing SPRY2 compared to B cells from CD19-cre mice and Empty vector Mec-1 cells, respectively (Figure 7E& F). We also observed a synergistic effect of SPRY2 with BRAF and Syk inhibitors in the presence of BRAF-V600 mutant, again highlighting the dual mechanism through with SPRY2 downregulates MAPK-Erk signaling in CLL cells (Figure 8). Thus, our results demonstrate
that Spry2 attenuates BCR mediated MAPK-Erk signaling by simultaneous inhibition of Raf and Syk activity in B cells and CLL cells.
Comparison of SPRY2 levels in CLL cells from Good and Poor Prognosis patients.

To compare the expression of SPRY2, CLL cells were isolated from PB of good and poor prognosis patients. (A) Relative mRNA level of spry2 from transcriptome analyses of 7 good prognosis and 8 poor prognosis CLL patients (n=15). RNA isolated from PB CLL cells was used for sequencing. The expression was normalized with GAPDH and genomic reference DNA was used as control for transcriptome analysis. (B) Real time-PCR measurement of relative expression of spry2 in CLL cells from good and poor prognosis patients, normalized with GAPH. (C) Levels of SPRY2 protein expression was measured in good prognosis patients (n=5) and poor prognosis patients (n=5). Displayed is a scanned western blot and densitometric measurements showing reduced protein levels of SPRY2 in poor prognosis CLL patients.
Mononuclear cells from healthy donor were used as positive control for antibody. 50µg of protein was loaded on 10%SDS gel. Beta actin is used as loading control. (D) Microarray data showing low relative expression of SPRY2 in patients with high CD38 expression. Patients with more than 30% of CD38 positive cells were considered CD38 High (n=15) and patients with less than 30% were considered Low CD38 (n=23) GAPDH was used to normalize the value, * p value=0.0045. (E) Shows SPRY2 mRNA levels in CLL cells from patients with IgVh unmutated vs mutated, n=18 p=0.0067. (F) Shows SPRY2 mRNA levels in CLL cells from patients with Del 17p vs Del 13q, n=20, p=0.0045.
(G) Shows the comparison of SPRY2 mRNA levels in primary CLL cells vs peripheral blood, this data was obtained from oncomine data set. Haferlach leukemia comparison of CLL (n=448) vs peripheral blood mononuclear cells (n=74) published in J Clin Oncol 2010/05/20.
(H) Shows the no change in the levels of SPRY2 when cultured alone vs cultured on S-17 stromal cells \textit{in vitro}. (I) Shows SPRY2 mRNA levels from in CLL cells isolated from paired Bone Marrow and Peripheral Blood from same patients $n=4$, $P=0.03$. 
Effect of SPRY2 on BCR signaling. To study the role of SPRY2 in CLL and normal B-cells, we isolated CLL and normal B cells from patients and healthy donors respectively. (A) Normal B-cells, primary human CLL cells and Mec-1 CLL cells were stimulated by BCR crosslinking for 0, 6, 12, 18, 24 and 48 hours. Cells were washed and protein lysate was prepared. Protein level of SPRY2 was determined by western blotting. First panel shows SPRY2 levels in normal B-cells, Second Panel shows SPRY2 levels in primary CLL cells from patient and third panel shows SPRY2 levels in Mec-1 CLL cells. (B) To test the efficacy of siRNAs against SPRY2 Normal B cells were transfected with siRNAa and siRNAb after 48 hours of transfection cells were washed and lysate was prepared.
Equal amount of protein was loaded in each well, Scramble siRNA and β-actin was used as control. Displayed is scanned western blot showing decrease in SPRY2 levels after siRNAs treatment. (C) Normal B-cells were isolated from healthy donors and nucleofected with scramble, siRNA A and siRNA B. After 48 hours of calcium influx assay was performed using Indo-1 dye dots represent 20 second time intervals. Displayed is mean graph of Indo-1 violet/Indo-1 Blue ratio, n=5 normal B cells samples from different healthy donors and p=0.0001.
Knockdown of SPRY2 increases proliferation of CLL cells from Good prognosis patients. To determine the effect of SPRY2 knockdown on human CLL cells we used Mec-1 cells and primary CLL cells from Good Prognosis CLL patients. (A) Displays mean bar graph of 3 repeats showing Ki-67 staining of Mec-1 cells after SPRY2 knockdown with anti-IgM and anti-IgD antibody stimulation. (B) Dot plot of patient’s sample CLL-1 showing increase in proliferation after SPRY2 knockdown using two different siRNA.
(C) CLL cells were isolated from peripheral blood of n=7 different good prognosis CLL patients. CLL cells were nucleofected with scramble, siRNA a and siRNA b and co culture on S-17 stromal layer. After 48 hours CLL cells were stained with Ki-67 stain and proliferation was measured. Displayed bar graph is fold in the rate of proliferation of CLL cells. (D) Displays the mean fold change of (C) showing significant p=0.01 increase in proliferation after SPRY2 knockdown with siRNA b.
Expression SPRY2 induces spontaneous apoptosis in CLL cells. (A) Mec-1 cells were transfected with Empty and spry2-cDNA GFP co-expressing vectors. (B) This figure shows Annexin-V staining done after 48 and 72 hours. Only live cells were gated for the analysis. The numbers represent the percentage of GFP positive cells also positive for Annexin-V.
(C) Primary CLL cells from n=5 different Poor Prognosis CLL patients were nucleofected with spry2-cDNA and Empty vector. Bar graph represents fold in percentage of apoptotic cells. (D) Mean Fold change of spry2-cDNA and Empty vector transfected cells from fig (C) p=0.009.
FIGURE 5.

(A) Displayed is a western blot showing overexpression of spry2 in splenic B-cells of CD19-cre;Spry2(tg). (B) Splenocytes from CD19creSpry2(wt) and CD19creSpry2(tg) mice were isolated and stained with anti-IgD and anti-IgM to study marginal zone and follicular B cells population. IgM<sup>int</sup>IgD<sup>nt</sup> shows marginal zone B cells and IgM<sup>nt</sup>IgD<sup>high</sup> demonstrate follicular B cells. (C) B1-cells were isolated from peritoneal cavity of CD19-cre;Spry2(wt) and CD19-cre;Spry2(tg) mice. B1-cells were stained with CD5 and B220 dye to determine the frequency of B1-cells using Flow cytometric analysis. Displayed is dot plot of showing B1a-cells frequency in CD19-cre;Spry2(wt) and CD19-cre;Spry2(tg) mice. Bar graph of absolute number of B1a-cells in peritoneal cavity of CD19-cre;Spry2(wt) and CD19-cre;Spry2(tg).
(D) Splenic B-cells were isolated by negative selection from rTTA positive CD19-cre;Spry2(wt) and CD19-cre;Spry2(tg) mice. GFP+ population displays CD19-cre expressed cells and displayed is calcium mobilization assay of splenic B-cells from above described mice n=3; p=0.0002. (E) Splenic B-cells were isolated from CD19-cre;Spry2(wt) and CD19-cre;Spry2(tg) stained with CFSE dye and cultured in vitro for six days with and without anti-IgM antibody. Displayed is the CFSE labeling of splenic B-cells of CD19-cre;Spry2(wt) and CD19-cre;Spry2(tg) mice.
SPRY2 knockdown leads to disease progression in NSG mice.

SPRY2 was stably knocked down in Mec-1 CLL cells using pLenti-siRNA A and pLenti-siRNA B, pLenti-scramble were used as control. (A) Displayed represents the mean bar graph of 3 repeats of propidium iodide to study the number of apoptotic cells in pLenti-scramble, pLenti-siRNA A and pLenti-siRNA B cell lines. (B) Displayed mean bar graph of 3 repeats of Ki-67 positive cells measured by flow cytometric analyses Mec-1 cells after pLenti-scramble, pLenti-siRNA A and pLenti-siRNA B stable transfection.
(C) Displayed average of tumor volume in n=5 mice in each group were transplanted with 1.5 million pLenti-scramble and pLenti-siRNA B Mec-1 CLL cells. Tumor volume was measured, using digital caliper we determine the length and width of the tumor. Tumor volume was calculated by \( V = \frac{L \times W \times W}{2} \) where \( V \) is the tumor volume, \( L \) is the tumor length and \( W \) is the width of the tumor. (D) Displays the tumor and spleen pictures from pLenti-scramble and pLenti-siRNA B cell line transplanted NSG mice. (E) Represents the frequency of Human CD19 positive cells in spleen of pLenti-scramble and pLenti-siRNA B CLL cells transplanted mice.
(F) Shows the hemotoxilin and eosin staining with tissue section of Kidney, Liver and Spleen to observe the number of CLL cells infiltration in pLenti-scramble and pLenti-siRNA B CLL cells transplanted mice. (G) Protein lysates were prepared from tumors; equal amount of protein was loaded in each lane of 10%SDS gel. Shown is scanned western blot to determine the protein levels of p-Erk and SPRY2 in tumors from pLenti-scramble and pLenti-siRNA B CLL-cells transplanted mice. Total Erk and β-actin were used as control. Densitometric measurements showing elevated of p-Erk normalized by total Erk in SPRY2 knockdown tumors.
FIGURE 7

SPRY2 downregulate MAPK-ERK signaling by interacting with RAF-1, Syk and BRAF. (A) Mec-1 cells were transfected with pLKO-TET-Empty and pLKO-TET-Spry2, Cells were treated with doxycycline for four days to induce the expression of SPRY2. Displayed is a scanned western blot of CLL cells transfected with Empty and spry2 cDNA to measure the protein levels of SPRY2 and p-Erk upon doxycycline treatment. β-actin and PAN Erk1/2 were used as controls. (B) KRAS-V12 and BRAF-V600 mutants were co-transfected with Empty and Spry2 vectors in Mec-1 cells. Scanned western blot showing the p-Erk and Erk levels in these cells.
Second panel shows scanned western blot of SPRY2 and BRAF protein levels in cells described in (B). β-actin was used as loading control. (C) Normal Human B cells were isolated from healthy donors, cells were stimulated by BCR crosslinking for 24 hours. Cells were lysed to prepare lysate for immunoprecipitation. Displayed is a scanned western blot of immunoprecipitation with SPRY2 and Syk showing pull down of Raf-1, Syk, BRAF and SPRY2. IgG, Pi3K and BTK were used as negative controls.
(D) Normal B cells were stimulated as described in (C) cytospin were prepared using these cells. Cells were stained with SPRY2, p-Syk and DAPI fluorescence antibody. Displayed are immunofluorescence images showing co-localization of SPRY2 and p-Syk in normal B cells and Mec-1 CLL cells. Unstimulated cell, Goat-IgG and Rabbit-IgG were used as control.
(E) Normal B cells were isolated from spleen of CD19-cre;Spry2(wt) and CD19-cre;Spry2(tg) mice and were stimulated for 30 mins with anti-IgM antibody. Displayed is scanned western blot of p-Syk and Syk and densitometric measurements showing normalized decreased levels of p-Syk.
(F) Mec-1 CLL cells were overexpressed with Spry2 cDNA and Empty vector co-expressing GFP, cytospins were prepared of these cells. Slides were stained with p-Syk (red) antibody. Displayed are immunofluorescence images and Densitometric measurements showing decreased levels of normalized p-Syk in SPRY2 co-expressing GFP positive cells.
(G) Normal B cells were isolated from healthy donor. Scanned western blot of 4G10 p-Tyrosine staining of nB-cells after treatment of siRNAs against SPRY2, scr siRNA was used as control.
Spry2 has synergistic effect with Syk and BRAF inhibitor in presence of BRAF V600 mutation. Mec-1 CLL cells were co-transfected of Empty and Spry2 vectors with BRAF V600 mutant and KRAS V12 mutant. (A) Displayed is bar graph of cell viability using MTT assay after treating BRAF-V600 mutated cells with BRAF inhibitor with 2.5µM, 5µM and 10µM (B) Displayed is bar graph of cell viability using MTT assay after treating KRAS V12 mutated cells with BRAF inhibitor with 2.5µM, 5µM and 10µM. (C) Displayed is bar graph using MTT assay after treating BRAF-V600 mutated cells with Syk inhibitor treatment with 2.5µM, 5µM and 10µM.
Table 1: Patient's information for samples used for western blotting and transcriptome analysis.

<table>
<thead>
<tr>
<th>CLL Patient #</th>
<th>Source</th>
<th>Cytogenetics</th>
<th>IgV mutation status</th>
<th>CD38 positivity</th>
<th>Monotypic B-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL 10</td>
<td>PB</td>
<td>Normal</td>
<td>Mutated</td>
<td>Neg</td>
<td>82%</td>
</tr>
<tr>
<td>CLL 23</td>
<td>PB</td>
<td>Trizomy 12</td>
<td>Not done</td>
<td>Neg</td>
<td>67%</td>
</tr>
<tr>
<td>CLL 22</td>
<td>PB</td>
<td>Normal</td>
<td>Not done</td>
<td>Neg</td>
<td>75%</td>
</tr>
<tr>
<td>CLL 136</td>
<td>PB</td>
<td>13q14 del</td>
<td>Not done</td>
<td>Neg</td>
<td>74%</td>
</tr>
<tr>
<td>CLL 150</td>
<td>PB</td>
<td>14q</td>
<td>Not done</td>
<td>Neg</td>
<td>88%</td>
</tr>
<tr>
<td>CLL 3</td>
<td>PB</td>
<td>Trizomy 12</td>
<td>Unmutated</td>
<td>Pos</td>
<td>93%</td>
</tr>
<tr>
<td>CLL 34</td>
<td>PB</td>
<td>11q23</td>
<td>Not done</td>
<td>Pos</td>
<td>74%</td>
</tr>
<tr>
<td>CLL 40</td>
<td>PB</td>
<td>17p del, 13q</td>
<td>Unmutated</td>
<td>Pos</td>
<td>92%</td>
</tr>
<tr>
<td>CLL 80</td>
<td>PB</td>
<td>17p del, Trizomy 12</td>
<td>Unmutated</td>
<td>Pos</td>
<td>70%</td>
</tr>
<tr>
<td>CLL 70</td>
<td>PB</td>
<td>Trizomy 12, 13q</td>
<td>Unmutated</td>
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<td>84%</td>
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Table 2: CLL patient’s information used for proliferation and survival.

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<tr>
<th>LL Patient #</th>
<th>Source</th>
<th>Kappa</th>
<th>Lambda</th>
<th>CD38 positivity</th>
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</tr>
</thead>
<tbody>
<tr>
<td>CLL 1</td>
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<td>neg</td>
<td>pos</td>
<td>neg</td>
<td>81%</td>
</tr>
<tr>
<td>CLL 2</td>
<td>PB</td>
<td>neg</td>
<td>pos</td>
<td>neg</td>
<td>85%</td>
</tr>
<tr>
<td>CLL 4</td>
<td>PB</td>
<td>pos</td>
<td>neg</td>
<td>pos, dim, partial</td>
<td>82%</td>
</tr>
<tr>
<td>CLL 5</td>
<td>PB</td>
<td>neg</td>
<td>pos</td>
<td>neg</td>
<td>82%</td>
</tr>
<tr>
<td>CLL 8</td>
<td>PB</td>
<td>pos</td>
<td>neg</td>
<td>neg</td>
<td>81%</td>
</tr>
<tr>
<td>CLL 9</td>
<td>BM</td>
<td>pos</td>
<td>neg</td>
<td>neg</td>
<td>67%</td>
</tr>
<tr>
<td>CLL 10</td>
<td>BM</td>
<td>pos</td>
<td>neg</td>
<td>pos, dim, partial</td>
<td>87%</td>
</tr>
<tr>
<td>CLL 12</td>
<td>PB</td>
<td>pos</td>
<td>neg</td>
<td>pos, m</td>
<td>83%</td>
</tr>
<tr>
<td>CLL 13</td>
<td>PB</td>
<td>neg</td>
<td>pos</td>
<td>pos, m</td>
<td>72%</td>
</tr>
<tr>
<td>CLL 20</td>
<td>PB</td>
<td>neg</td>
<td>pos</td>
<td>pos, dim</td>
<td>82%</td>
</tr>
<tr>
<td>CLL 21</td>
<td>PB</td>
<td>pos</td>
<td>neg</td>
<td>pos</td>
<td>93%</td>
</tr>
<tr>
<td>CLL 145</td>
<td>PB</td>
<td>neg</td>
<td>pos</td>
<td>pos, dim</td>
<td>85%</td>
</tr>
</tbody>
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Discussion:

Clinical heterogeneity is a major problem in the management of CLL. The molecular basis of clinical heterogeneity is not well understood. The heterogeneous clinical outcome in patients appears to be the results of interactions between several molecules and cellular pathways. Consequently, in order to treat CLL effectively, a better understanding of the molecules and cellular pathways that that contribute to such heterogeneous clinical outcome is urgently needed. In the present study we have elucidated the molecular basis for BCR-mediated MAPK-Erk signaling where a negative regulator SPRY2 plays a major role in regulating the survival and proliferation of CLL cells. Moreover, SPRY2 may function as a molecule responsible for maintaining the clinical heterogeneity in CLL.

The BCR and its associated MAPK signaling are constitutively-activated in CLL cells and are critical for their survival, migration and proliferation. However, the precise molecular mechanisms leading to deregulation of these pathways in CLL cells still remain unclear. Our studies here have identified SPRY2 expression as a novel negative regulator of BCR-mediated MAPK-Erk signaling pathway in the pathogenesis of CLL. Spry2 expression is significantly lower specifically in patients with poor prognosis CLL (high CD38 expression) which possess features of constitutively-active BCR and MAPK-Erk signaling. The expression of other Sprouty family proteins, Spry1, 3 and 4 is particularly low across the CLL spectrum without any significant differential expression, thus, indicating that SPRY2 is the predominant member of Sprouty protein family of negative regulators of signaling whose expression is significantly altered in Good versus Poor prognosis CLL.
Notably, our findings have also underlined that SPRY2 expression is induced upon BCR stimulation and is critical for attenuating BCR-induced MAPK-Erk signaling. Moreover, SPRY2 may function as an attenuator of tonic BCR signaling in CLL cells and B cells as basal levels of signaling are elevated upon SPRY2 knockdown (Figure 2). While, SPRY2 over-expression in CD19-cre;Spry2(tg) mice led to impaired BCR signaling in B cells, we did not observe an apparent defect in the overall generation of B cells. Notably though, and directly relevant to role of SPRY2 down-regulation in CLL, we observed a decrease in the B1 subset of B-cells these mice. These results indicate a potential role for SPRY2 in the development of B1 cells and possibly in the initiation of CLL as B1 cells have been shown to function as precursors of CLL in mice in multiple model system (62). It will be of considerable interest to breed the CD19-cre;Spry2(tg) mice to established models of CLL such as Eµ-Tcl1 or IRF4−/−Vh11 mice to directly study the role of SPRY2 in development of CLL (62). Interestingly, SPRY2 levels are down-regulated in CLL cells isolated from Eµ-Tcl1 and IRF4−/−Vh11 mouse model (data not shown). Even though no other defects in B-cell development are apparent in CD19-cre;Spry2(tg) mice, it will be interesting to further study the induction of functional humoral responses in these mice given our findings that aberration of SPRY2 expression deregulate BCR signaling.

Functionally, our studies have shown that SPRY2 mediated regulation of BCR signaling is important for survival and proliferation of CLL cells. Moreover, SPRY2 plays a role in controlling the disease aggressiveness as knockdown of SPRY2 in Mec-1 CLL cells resulted in more aggressive disease in NSG mice. We show that SPRY2 expression in normal B cells and CLL cells leads to a decrease p-Syk levels. Mechanistically, BCR signaling in CLL cells constitutes a signaling axis whereby Syk can also function to regulate the activation of MAPK-Erk signaling (Figure 8E). Of significant interest in this regard, we observed that SPRY2 not only interacts with and antagonizes Raf/Braf
activities but that it also interacts and co-localizes with Syk near the plasma membrane to disrupt the MAPK-Erk signaling axis (Figure 8E). Therefore, we propose a model in which SPRY2 functions to regulate two different nodes of an overlapping signaling axis by attenuating Syk as well as Raf/Braf activity (Figure 8E). Importantly, our studies indicate that SPRY2 functions as a broad attenuator of BCR signaling via its regulation of Syk activity. This is evidenced by a decrease in calcium influx upon BCR stimulation, a process mediated by PLCγ2 signaling downstream of Syk activation.

SPRY2 has been shown to interact with RTKs and the associated adaptor molecules through its SH2 domain-binding motifs generated upon its phosphorylation (22). Syk is a non-receptor tyrosine kinase harboring multiple SH2 domains. Hence, it is tempting to speculate that SPRY2 might interact with Syk using one or more of its SH2 domain binding motifs. However, the precise domain(s) required for the interaction of SPRY2 with Syk is currently unknown. Additionally, how the functional inhibition of Syk is brought about by its interaction with Spry2 is an open question. Furthermore, SPRY2 overexpression in CLL cells induces an anti-survival effect that functions synergistically with Syk and BRAF inhibitors. Collectively, these studies highlight the presence of dual mechanisms through which SPRY2 regulates BCR-induced MAPK-Erk signaling in CLL and B cells as shown in Figure 8E. Thus, our findings provide a strong rationale for targeting of these pathways in the treatment of CLL patients, in particular those with MAPK pathway associated mutations. A recently study has identified a small subset of CLL patients that do not respond to the Btk inhibitor Ibrutinib (63). It will be of interest to evaluate the therapeutic potential of a combinatorial Syk and MAPK-Erk inhibition in such patients.

Our study shows that SPRY2 functions as a molecular rheostat important for fine-tuning the signaling cascades critical for survival and proliferation of CLL. By investigating
the mechanism of SPRY2 down-regulation and impact in human CLL cells and mouse models, our studies here identify and validate key molecular networks that can be therapeutically targeted in the treatment of CLL.
Chapter IV

**STAT3 regulates miRNA-21 to target SPRY2 in CLL patients with poor prognosis**

**Introduction**

Chronic Lymphocytic Leukemia (CLL), is the most prevalent adult leukemia in the western world (1-5). Several studies have identified molecular cues leading the pathogenesis of CLL (1,2). Despite this, the molecular aberrations leading to CLL onset are still under review. Specifically, understanding the role of oncogenes and tumor suppressor interplayed during the disease progression might unravels the mechanism leading to clinical heterogeneity. Emerging evidences have shown that microRNAs plays a critical role during the progression of CLL. MicroRNAs (miRNAs) are small non-coding RNAs which are expressed in tissue specific manner (64-66). MiRNAs are involved in several cellular processes to maintain the cellular homeostasis. Deregulation of miRNAs leads to alteration in expression levels of genes post-transcriptionally, which are involved during the development/progression of tumors. In CLL, along with the serving as a prognostic markers, miRNAs can function also oncogenes or tumor suppressors. MiRNA-21 (miR-21) is one such miRNA whose high levels are associated with the poor prognosis of CLL patients (41, 42). MiR-21 is located at 17q.23.1 and is highly expressed in CLL cells isolated from patients with 17p locus deletion (Del 17p) when compared those from CLL patients with wild type 17p locus (42). Notably, patients with chromosomal aberration Del 17p results in loss of p53 and has been associated with worst clinical outcomes (67). High expression of miR-21 is observed in patients who do not respond to Fludarabine and in patients who have shorter overall survival (68, 41, 42).

Recently, Crespo et al., reported that upon Zap70 expression leads to induction of miR-21 expression in B cell receptor (BCR) stimulated primary CLL cells, highlighting the
role of BCR signaling in CLL (69, 70). This might be one of the roles of activating MAPK and BCR signaling pathways to induce oncomirs like miR21 in CLL cells (70). These lines of evidence demonstrate that miR-21 is overexpressed in CLL patients with poor outcome. MiR-21 is overexpressed in several other malignancies and targets several tumor suppressors like Spry2, Spry1, PDCD4, TPM1 and PTEN (21, 39, 40, 71). However, it is uncertain whether Pten is a direct target of miR-21 or its destabilization occurs due to down regulation of Spry2 mediated by miR-21 (71). In CLL, miR-21 is often upregulated in patients with poor prognosis however, the molecular mechanism leading to its deregulation is not fully understood.

Tumor microenvironment plays a critical role in providing pro-survival signals to CLL cells. Several chemokines and cytokines secreted by stromal cells plays important role in activating these signaling pathways (72, 73). IL-10 is a classic anti-inflammatory cytokines, whose serum levels are often high in patients with CLL (74-76). Secreted IL-10 binds to IL-10R to activate Jak-STAT3 signaling pathway (75). Constitutive activation of signal transducer and activator of transcription (STAT) particularly STAT3, has been observed and is frequently associated with CLL disease progression. STAT proteins phosphorylates to form dimers to translocates to nucleus and activates transcription. Recent studies using STAT3-shRNAs have shown that STAT3 can alter the miRNA profiling in CLL cells (77). One of the main target of STAT3 is miR-21 (77). Several groups have demonstrated that miR-21 directly targets and downregulates expression of Spry2 to activate MAPK-Erk signaling in human gliomas and many other malignancies (37, 39, 40). Together, these studies suggests that targeting of SPRY2 by miR-21 might be one of the mechanism of SPRY2 deregulation. Therefore, in this study we have studied the SPRY2 as a target of miR-21 in CLL cells. We have also demonstrated that STAT3 binds at the promoter of miR-21 to induce its expression in CLL cells.
Results:

Mir-21 targets Spry2 in CLL cells to activate Syk and MAPK-Erk signaling.

Given the SPRY2 down-regulation in patients with poor prognosis CLL, and a cyclic pattern of SPRY2 expression in BCR cross-linked CLL cells (Chapter 4 Figure 2), we reasoned that an epigenetic mechanism may deregulate SPRY2 expression in CLL. While, spry2 gene is hyper-methylated in DLBCL, Plass et al did not observe any hyper-methylation of spry2 in patients with CLL (38). Therefore, we sought to identify other alternate mechanisms for spry2 down-regulation in poor prognosis CLL patients. Interestingly, SPRY2 has been shown to be a direct target of microRNA-21(miR-21) in other cell systems (37, 39, 40). Furthermore, mir-21 is an oncomir that has been reported to be highly overexpressed in CLL patients with poorer outcome in several studies (41, 42). Therefore, we overexpressed miR-21 in Mec-1 cells to study whether it can regulate Spry2 in CLL cells as shown Figure 1A. Notably, we observed a decrease in SPRY2 expression in Mec-1 cells expressing high levels of miR-21 compared to those with an empty vector control (Figure 1B). Next, we knock down miR-21 levels by using miR-21 inhibitors and observed significant upregulation of SPRY2 in human CLL cells (Figure 1C).

Intriguingly, concurrent with SPRY2 down-regulation in miR-21 overexpressing Mec-1 cells, we also observed an increase in the levels of p-Syk and p-Erk in the Mec-1 cells (Figure 2 A-C). In summary, our results here demonstrate that Spry2 is deregulated in CLL cells by the expression of miR-21. The results here also, provide a mechanism by which miR-21 promotes CLL progression via down-regulation of SPRY2.
STAT3 expression via IL-10 stimulation induces miR-21 expression in human CLL cells.

Emerging evidences have shown that miR-21 gets induce upon activation of STAT3 signaling pathway (77, 78). To study if STAT3 transcriptionally activate miR-21 in CLL cells we next perform chromatin Immunoprecipitation of STAT3. IL-10 mediated activation of JAK-STAT pathways is a well-established model used for studying these signaling pathways in vivo (75, 76). Therefore, we stimulated CLL cells in vitro with IL-10 for 12 hours and measured the levels of p-STAT3 and miR-21. Cells cultured without IL-10 were used as control. We observed that upon IL-10 stimulation there was robust induction of p-STAT3 expression when compared with Mec-1 cells alone (Figure 3 A). We next stimulated primary CLL cells with IL-10 and measured the levels of miR-21 in these CLL cells. After 12 hours incubation we observed a significant 2.7 fold increase in miR-21 expression (Figure 3 B). We next tested whether the increase in miR-21 levels is via STAT3 signaling. To test that after 12 hours post stimulation we performed chromatin immunoprecipitation of STAT3. We observed an enhanced amplification at the promoter region of miR-21 in CLL cells upon IL-10 stimulation measured by quantitation PCR (Figure 3 C). Hence, demonstrating that IL-10 induced STAT3 signaling indeed activate miR-21 transcription.

Inhibition of miR-21 via STAT3 inhibitor stabilizes SPRY2 expression in CLL cells.

Our previous studies have demonstrated that the SPRY2 possess an unstable biphasic cyclical expression in BCR stimulated CLL cells when compared it with normal B cells from healthy donors (Figure 2 Chapter III). First, to study if treatment of CLL cells with STAT3 inhibitor decreases the miR-21 expression we next measured the expression of miR-21 using q-PCR. We observed a significant reduction at the levels of miR-21 in CLL cells treated with STAT3 inhibitor when compared with those from cells only and
DMSO treated CLL cells (Figure 4 A). Therefore, we next studied if the inhibition of miR-21 in CLL cells can stabilize the expression of SPRY2 in CLL cells we used STAT3 inhibitors. We treated CLL cells shortly with STAT3 inhibitor for 12 hours. After 12 hours post incubation these treated CLL cells were washed and then stimulated with anti-IgM antibodies. We observed a stable expression of SPRY2 upon BCR stimulation in CLL cells treated with STAT3 inhibitor (Figure 4 B). Thus, demonstrating that STAT3 inhibition targets miR-21 and stabilizes SPRY2 expression in CLL cells.

**In vitro therapeutic efficacy of STAT3 inhibitor.**

To test the therapeutic efficacy of STAT3 inhibitor alone and in combination with other agents we used primary CLL cells and Mec-1 CLL cells. We first isolated primary CLL cells from the peripheral blood of CLL patients. We treated primary CLL cells isolated from 11 different patients with STAT3, SYK and BRAF inhibitor at 2.5 µM, 5 µM, 10 µM and 15 µM drug concentrations. After 24 and 48 hours post treatments CLL cells viability was measured using MTT assay. We observed statistically significant dose dependent response with STAT3 inhibitor treated primary CLL cells when compared those with SYK and BRAF inhibitors alone treated primary CLL cells (Figure 5 A-B). CLL cells treated with SYK and BRAF inhibitors showed significant decrease in cell viability at only higher dose of 10 µM. To study if targeting multiple pathways by combinational treatment of BRAF and SYK inhibitors with STAT3 inhibitor we used sub IC50 doses of each drug to obtain 2.5 µM, 5 µM, 10 µM and 15 µM combination drug concentrations (Figure 5 C).

As resistance towards Ibrutinib is major challenge in treating patients with CLL (3). Therefore, we have developed Ibrutinib resistant Mec-1 CLL cell line, by treating Mec-1 cells with standard dose of 10 µM for three months. Once the resistant cell line was developed, we treated those CLL cells with SYK, Mek-1 and STAT3 inhibitor. Mec-1 cells
alone and DMSO treated cells were used as control. We observed a significant reduction in cell viability measured by MTT assay of the Ibrutinib resistant CLL cells upon treatment with STAT3 inhibitor when compared with SYK and Mek-1 inhibitor at 24, 48 and 72 hours as shown in Figure 6 (A-C). Together these studies have demonstrated STAT3 inhibitor is more effective in treating CLL patients with Ibrutinib resistance and deregulated MAPK signaling.
MiR-21 targets SPRY2 in human CLL cells

Mec-1 cells were transfected with pCDNA-mir21 and pcDNA-Empty (A) Graph displaying the miR-21 expression measured by quantitative PCR in miR-21 overexpressing Mec-1 CLL cells. (B) Displayed is scanned western blot showing SPRY2 protein levels in miR-21 overexpressing CLL cells. β-actin was used as loading control. (C) Displayed is a scanned western blot of Mec-1 CLL cells after knockdown of miR-21 using miR-21 inhibitor.
MiR-21 activate BCR mediated MAPK-Erk signaling by targeting SPRY2 in CLL cells.

(A) Shows protein levels of p-Erk and Erk in miR-21 overexpressing cells. (B) Densitometric measurements showing elevated levels of p-Erk in miR-21 overexpressing CLL cells. (C) Displayed is scanned western blot showing the protein levels of p-Syk, Syk and SPRY2 in miR-21 overexpressing Mec-1 CLL cells.
Regulation of miR-21 and SPRY2 by IL-10 and STAT3 in human CLL cells.

CLL cells were isolated from the peripheral blood of patients and then incubated with IL-10 for 12 hours. (A) Displayed is the scanned western blot of pSTAT3 levels in IL-10 stimulated Mec-1 cells after 12 hours. Beta actin and Mec-1 cells stimulated for 0 hour were used as control. (B) MiRNA was isolated from these cells and quantitative PCR was performed using Taqman miR-21 assay. Displayed is quantitative PCR demonstrating the induction of miR-21 by IL-10 in primary human CLL cells. CLL only were used as control.
Figure 3 (C) Mec-1 cells were stimulated with IL-10 for overnight. Cells were washed and DNA was isolated and sonicated. Chromatin immunoprecipitation was performed using STAT3 antibody. Displayed is the ChIP analysis demonstrating the binding of STAT3 to miR-21 promoter region by enhancement measured by quantitative PCR.
FIGURE 4

Down regulation of STAT3 using STAT3 inhibitor in Mec-1 cells decreases miR-21 and stabilizes SPRY2 expression.

(A) Mec-1 CLL cells were treated with STAT3 inhibitor for 12 hours. After 12 hours cells were washed and miRNA was isolated. Displayed is the quantitative PCR graph measuring the level of miR-21 expression using taqman assay. (B) After treating Mec-1 cells with STAT3 inhibitor for 24 hours, cells were washed and stimulated with anti-IgM antibody. Cells were collected at 0, 6, 12, 24 and 48 hours. Displayed is the scanned western blot showing stabilization of SPRY2 expression after 24 hours treatment of Mec-1 CLL cells with STAT3 inhibitor. Mec-1 cell only was used as control.
FIGURE 5

Effects of STAT3, Syk and Braf inhibitors alone and in combination on survival of CLL cells.

(A) CLL cells were isolated from the peripheral blood of 11 different patients. CLL cells were treated with STAT3, SYK and BRAF inhibitors for (a) 24 hours and (b) 48 hours. Displayed is the measurement of the cell viability using MTT assay.
FIGURE 5 (B) Treatment of Mec-1 cells with STAT3 inhibitor (5µM) followed by SYK or BRAF inhibitor for 24 (a) and (b) 48 hours.
FIGURE 6

A

Therapeutic efficacy of STAT3 inhibitor over SYK and Mek-1 inhibitor in Ibrutinib resistant Mec-1 CLL cells.

Mec-1 CLL cells were treated with 10 µM per milliliter for about 3 months. After spontaneous acquired Ibrutinib resistance cells were treated with Syk, STAT3 and Mek-1 inhibitor for 24 hours (A).
(B) Treatment for 48 hours with SYK, STAT3 and Mek-1 inhibitor.
(C) Treatment for 72 hours with SYK, STAT3 and Mek-1 inhibitors. Cells viability was measured by MTT assay for different doses A- 2.5 µM, B- 5 µM and 10 µM. Mec-1 cells only and DMSO treated were used as control.
Discussion

Our previous studies identified SPRY2 as one of the molecules which might be playing critical role in clinical heterogeneity of patients with CLL. However, the mechanism leading to SPRY2 differential expression in between CLL patients with good and poor clinical outcome was unclear. In this study we have identified the molecular mechanism through which SPRY2 is deregulated in CLL patients. We demonstrated that high levels of miR-21 leads to suppression of SPRY2 in CLL cells. Upon knockdown of miR-21 we observed the upregulation of SPRY2. We further demonstrated the role of STAT3 signaling in CLL progression by upregulating miR-21 levels in poor prognosis CLL. Using STAT3 inhibitor we identified STAT3 as potential therapeutic target in CLL patients with poor prognosis and also in Ibrutinib resistant CLL patients.

Spry2 is either epigenetically silenced or repressed by miR-21 in several cancers including breast, prostrate, lungs, liver and lymphoma (37-40). In human CLL, Chen et al, have demonstrated that the promoter region of spry2 was only hypermethylated in a small fraction of the 55 CLL patients that were profiled, signifying alternate mechanisms that lead to Spry2 down-regulation in CLL (38). Interestingly, we observed that SPRY2 is a direct target of miR-21 in human CLL cells. There are several reports that have correlated high miR-21 expression in CLL patients with poorer outcome (41, 42). High MiR-21 expression has been shown to activate MAPK-Erk signaling in several malignancies by suppressing SPRY2 levels (41, 42). However, in this report we have shown the molecular mechanism through which miR-21 leads to disease advancement. We observed elevated p-Syk and p-Erk levels and low levels of SPRY2 in high miR-21 expressing CLL cells. Together, these findings suggest that miR-21 targets SPRY2 to activate Syk-mediated BCR and MAPK-Erk signaling in poor prognosis CLL.
Persistent activation of STAT3 signaling via IL-10 is critical for oncogenic transformation in several cellular systems (79). In CLL patients, several reports have shown the elevated serum levels of IL-10 cytokine (76). However, the role of IL-10 signaling in CLL was controversial. Our studies revealed the critical molecular mechanism played by IL-10 mediated STAT3 signaling in CLL cells. We have shown that upon IL-10 stimulation, miR-21 levels were increased via STAT3 transcription factor in human CLL cells. Hence, highlighting STAT3 as a potential therapeutic target in CLL cells.

Using primary CLL cells, Ibrutinib resistant Mec-1 CLL cell line in this study we demonstrated the higher therapeutic efficacy of STAT3 inhibitor over SYK, BRAF and Mek-1 inhibitors. Upon treating CLL cells with STAT3 inhibitor we observed a significant reduction in the levels miR-21 and stable expression of SPRY2 in BCR stimulated CLL cells. Resistance to Ibrutinib acquired by patients is major challenge in treating CLL with current therapy (3-6). Elevation of MAPK-signaling and miR-21 is seen in such relapsed patients. Intriguingly, we observed more effective therapeutic efficacy of STAT3 inhibitor over Mek-1, SYK and BRAF inhibitors. Highlighting the different mechanisms leading to activation of these pathways in Ibrutinib resistant CLL cells. These studies warrants further in depth analysis of these signaling pathways in CLL patients with acquired resistance to Ibrutinib. Together our studies have demonstrated the molecular mechanism through which SPRY2 is deregulated in CLL patients and by targeting STAT3 we can inhibit the effects of miR-21 in CLL cells.
Chapter V

Conclusion, Caveats and Future Directions:

Conclusion and Caveats

Sprouty is significantly reduced in CLL cells isolated from poor prognosis patients when compared those from good prognosis.

Chronic Lymphocytic Leukemia (CLL) is the most prevalent adult leukemia where clinical heterogeneity and therapy resistance are the major challenges. Several studies have highlighted the critical role played by MAPK signaling in CLL cells proliferation and survival (80). About 36% of mutations found in CLL cells isolated from the patients leads to constitutive activation of MAPK signaling. Constitutive activation of MAPK signaling is one of the driver event for clonal evolution during the CLL disease progression. However, the molecular mechanism leading such deregulation is not fully understood. In an attempt to unravel these signaling pathways cues we performed transcriptome analysis which highlighted that Sprouty (SPRY)2 is significantly downregulated in CLL cells isolated from poor prognosis patients when compared with those from good prognosis patients.

SPRY2 belongs to SPROUTY protein family. These protein plays crucial role in providing negative feedback for signaling cascade activated via receptor tyrosine kinase (RTK). Interestingly, none of the other SPROUTY proteins (SPRY1, SPRY3 and SPRY4) showed significant difference in expression between good and poor prognosis CLL. First, to provide in vitro evidence we tested the differential expression level of SPRY2 in CLL cells isolated from good and poor prognosis CLL patients. We independently tested the expression level of SPRY2 in CLL cells isolated from unmutated-IgVh vs mutated-IgVh CLL patients, High CD38 vs Low CD38 CLL patients and Del 17p vs Del 13q CLL patients.
We observed significant reduction in SPRY2 expression in CLL cells isolated from all poor prognosis patients categorized by different prognostic markers. To further validate this, we also measured the protein level by western blotting and mRNA levels by RT-PCR of SPRY2 in CLL cells isolated from good and poor prognosis patients. These results conclude that SPRY2 is significantly down regulated in CLL cells isolated from poor prognosis when compared with those from good prognosis patients.

**SPRY2 is a novel attenuator of BCR signaling.**

Next we studied the role of SPRY2 as a negative regulator in B cell receptor (BCR) signaling. To demonstrate this we first stimulated normal B cells isolated from healthy donor with anti-IgM antibodies and collected cells at 0, 6, 12, 18, 24 and 48 hours. We observed a gradual increase in SPRY2 expression over period of time in normal B cells. Whereas, primary CLL cells as well as Mec-1 CLL cells displayed a biphasic cyclical expression of SPRY2 suggesting the instability of SPRY2 in CLL cells. Interestingly, we observed two bands of SPRY2 upon stimulation. The upper band is due the post-translational modification of SPRY2 by palmitoylation. Upon addition of palmitoylation SPRY2 gets recruited to lipid rafts on the plasma membrane. Using siRNAs mediated knockdown of SPRY2 in normal B cells we demonstrated prolonged BCR activation in B cells with low levels of SPRY2 shown by the calcium influx assay. Based on results of these studies we concluded that SPRY2 is a negative regulator of BCR signaling.

**Caveats:**

The differences are slight in calcium influx but these results are highly repeatable and statistically significant. Therefore, more samples must be analyzed.

**SPRY2 negatively regulates the proliferation and survival of CLL cells.**
To elucidate the role of SPRY2 in CLL cell proliferation and survival, we first used CLL cells isolated from good prognosis patients and Mec-1 CLL cells. As good prognosis CLL cells possess higher levels of SPRY2 when compared with those from poor prognosis patients, we knockdown SPRY2 expression using siRNAs. We observed increase in proliferation in BCR stimulated primary CLL cells upon SPRY2 knockdown. To study the effect of reconstitution of SPRY2 expression in CLL cells we used CLL cells isolated from poor prognosis patients, as they possess very low levels of SPRY2 levels. We expressed SPRY2 by transfecting these cells with SPRY2 cDNA followed by a reported GFP tag constructs and measured the rate of apoptosis in these cells. Upon reconstitution of SPRY2 expression in CLL cells from poor prognosis patients the increase in the rate of apoptosis when compared with Empty vector construct transfected cells. These results helped us to conclude that SPRY2 negatively regulates the proliferation and survival of human CLL cells.

To determine if reconstitution of SPRY2 expression in B cells have any effect on B cell signaling and B cell development we created a B cell specific Spry2 transgenic mouse model (CD19cre-Spry2(tg)). Although, we did not observe any disparity in B cell development we did observe that the number of special B cell population, B1 cells was significantly reduced. This was particularly interesting as B1 cells were initially presumed and lately shown as precursor of CLL cells in mice. The percentage and absolute number of B1 cells was specifically reduced in peritoneal cavity of CD19cre-Spry2(tg) when compared with those from CD19cre mice. Intriguingly, splenic B cells (B2 cells) showed significant reduction in the BCR activation measured by calcium influx in response to BCR crosslinking. Next, using this mouse model we have also established the role of SPRY2 in B cell proliferation. We demonstrated that splenic B2 cells showed no or few cells proliferating measured by CSFE labeling in response to anti-IgM and LPS when compared
with those from CD19cre mice. Together, these results concluded that SPRY2 negatively regulates the survival of B1 cells and attenuates the proliferation of splenic B2 cells in response to external stimuli.

**Caveats:**

In our study for survival and proliferation we did not demonstrated the opposite effects of SPRY2 knockdown and overexpression. Although we did observe decrease in survival upon SPRY2 knockdown in primary CLL-cells but was not statistically significant. The subtle changes on survival due to SPRY2 knockdown in primary CLL-cells might have been overshadowed by anti-IgM antibody effects on CLL-cells survival in vitro. In an overexpression system with SPRY2-cDNA expressing plasmid, we did not stimulate those CLL-cells as BCR stimulation would further increase the levels of SPRY2 in those cells. Therefore we did not observe much difference in rate of proliferation. However, further in depth analysis are needed with many more primary CLL samples to confirm and conclude the opposite effects.

**Low levels of SPRY2 leads to CLL disease advancement.**

To study the role of SPRY2 during CLL disease progression, we modeled the low SPRY2 expression seen in human CLL disease in a mouse lymphomagenesis model. For this purpose, we established a xenograft model with low SPRY2 expressing Mec-1 CLL cells transplanted into NSG mice. We observed mice transplanted with low SPRY2 expressing Mec-1 CLL developed more rapid and significantly larger tumors when compared with those transplanted with control Mec-1 cells. Interestingly, CLL cells isolated from these tumors retained low SPRY2 levels and increased levels of p-Erk. Using Tet-on inducible system we also observed a dose dependent decrease in p-Erk levels on SPRY2
induction. Together, these results concluded that low levels of SPRY2 promotes CLL disease progression in xenograft mouse model by activating MAPK-Erk signaling.

**SPRY2 interacts with SYK and RAF-1/BRAF to attenuates BCR mediated MAPK signaling.**

Next to study the molecular mechanism by which SPRY2 negatively regulates BCR and MAPK signaling in CLL we used stimulated normal B cell as a model to induce endogenous SPRY2. As, it was well established that SPRY2 interacts with Ras and/or Raf proteins to attenuates MAPK-Erk signaling. However, SPRY2 interaction with these proteins varies with cell types. Therefore, we first tested the conservation of these networks and showed that SPRY2 interacts with RAF-1 and BRAF in normal B cells upon stimulation.

As, KRAS and BRAF mutations are very commonly found in patients with CLL, we next tested if SPRY2 can attenuate MAPK-Erk signaling in the presence of KRAS and BRAF mutations in CLL cells. We observed that SPRY2 was still able to attenuate MAPK-Erk signaling even in the presence of BRAF mutant but not in the presence of KRAS mutations. This is was particularly interesting as lately it was shown that upon treating a Multiple Myeloma patients with preexisting Monoclonal B lymphocytosis (MBL) with BRAF inhibitor led to CLL transformation. The CLL cells were found to activate MAPK-Erk signaling via SYK. Also, as SPRY2 possess SH2-domain binding motifs and gets recruited in lipid rafts of plasma membrane, we speculated that SPRY2 might be interacting with SYK. By performing immunoprecipitation of SPRY2 and SYK in stimulated normal B cells we have shown that SPRY2 interacts with SYK upon B cells stimulation. Using immunofluorescence we also demonstrated that SPRY2 co-localizes with p-Syk upon BCR stimulation.
To test if SPRY2 attenuates Syk mediated BCR signaling, we used SPRY2 expressing Mec-1 cells and splenic B2 cells from CD19cre-Spry2(tg). We observed low levels of p-Syk in immunofluorescence of SPRY2 expressing Mec-1 cells and western blot of splenic B cells from CD19cre-Spry2(tg) mice when compared with respective controls. These results concluded that SPRY2 attenuates BCR and MAPK-Erk signaling through a parallel mechanism by interacting with Syk and RAF-1/BRAF in normal B and CLL cells.

**Caveats:**

We demonstrates that SPRY2 interacts with Syk however, it is unclear if SPRY2 and Syk interacts directly or they are part of same complex. Therefore, identification of domains critical for SPRY2 and SYK interaction is essential to understand the process at the molecular level.

**MiRNA-21 targets SPRY2 to activate BCR and MAPK-Erk signaling in CLL cells from poor prognosis patients**

SPRY2 is either epigenetically silenced or targeted with miR-21 in several malignancies. It was previously demonstrated that SPRY2 is not epigenetically repressed in CLL and miR-21 is highly expressed in CLL cells from patients, we therefore speculated that miR-21 targets SPRY2 in CLL. We observed decreased SPRY2 levels in miR-21 expressing CLL cells and upon knockdown of miR-21, SPRY2 levels were increased in these cells. This was particularly interesting as the levels p-Syk and p-Erk increased in high miR-21 expressing CLL cells.

**Caveats:**

To establish if SPRY2 is the major target of miR-21 in CLL, rescue experiments are needed. Reconstitution of SPRY2 expression in high miR-21 expressing CLL cells will demonstrate if SPRY2 is the main target of miR-21 during the pathogenesis of CLL. This
will establish if SPRY2 expression can inhibits the phenotype of high miR-21 expressing CLL cells.

**IL-10 stimulation activates STAT3 signaling pathways to increases miR-21 levels in CLL cells.**

IL-10 is classical example of inflammatory cytokine whose levels are elevated in CLL patients. STAT3 signaling is activated by IL-10 and/or IL-6 stimulation, however the role of IL-10 signaling in CLL is still unclear. As it is very well established that STAT3 activation affects the levels of miR-21 in CLL. We showed that upon IL-10 stimulation STAT3 binds at the promoter region of miR-21 to increase the levels of miR-21. We also demonstrated that upon treating CLL cells with STAT3 inhibitor, the levels of miR-21 goes down and SPRY2 expression is stabilized. Also, STAT3 is a better therapeutic target in CLL cells with mutations associated with MAPK signaling pathways and in Ibrutinib refractory CLL. Together, we conclude that STAT3 signaling is one of the molecular mechanism for the high miR-21 expression in CLL cells.

**Caveats:**

The role of IL-10 on CLL cells survival and proliferation is not known. Therefore, IL-10 stimulated CLL cells must be evaluated for their survival and proliferation.
We conclude that SPRY2 is a target of miR21 in CLL cells from poor prognosis patients. MiR21 targets SPRY2 to activate Syk mediated BCR and MAPK signaling in CLL patients. IL-10 induces miR-21 expression in CLL cells via activation of STAT3 signaling pathway. Also, STAT3 can be used as therapeutic target for poor prognosis CLL patients with high miR21 expression.
Future Directions:

1. **To identify the role of SPRY2 in CLL disease initiation.** Although, we demonstrated that constitutive expression of SPRY2 *in vivo* decreases the number of B1 cells the role of SPRY2 in CLL initiation is not get understood. To study this CD19cre-Spry2(tg) can be crossed with a well-established CLL mouse model. As SPRY2 levels are low in Vh11IRF4 deficient mouse model therefore, reconstitution of SPRY2 expression in these mice will provide a demonstration of SPRY2 role in CLL initiation. **Expected Results:** We expect to see delay in the development of CLL in these mice.

2. **To identify the domains critical for SPRY2 and SYK interaction.** SPRY2 possess SH2-domain binding motifs, it will be interesting to study if SPRY2 binds with SYK at SH-2 domains. To test this we will use SYK and SPRY2 mutants lacking these critical domains and perform immunoprecipitation of SPRY2 and SYK. Using this approach we will demonstrate the domains important for Syk and SPRY2 interaction. **Expected Results:** We expect that SPRY2 interacts with SH-2 domains of Syk.

3. **To identify the molecular mechanism for biphasic expression of SPRY2 in CLL cells.** We have shown that SPRY2 possess a biphasic cyclical expression upon BCR stimulation in CLL cells. Therefore, to elucidate the molecular mechanism we will evaluate the expression of miR-21 and SPRY2 together in BCR stimulated CLL cells. We will treat CLL cells with Mir-21 inhibitor and study if the expression of SPRY2 expression is stabilized. **Expected Results:** We expect that the levels of miR-21 inversely correlates with SPRY2 levels in stimulated CLL cells.

4. **To elucidate the role of miR-21 in CLL initiation and disease progression.** We have shown that miR-21 targets SPRY2 to activate BCR mediated MAPK-erk
signaling. As miR-21 is specifically highly expressed and deregulated in relapsed and refractory CLL patients. Therefore, it will be interesting to study the role of miR-21 in CLL disease progression by crossing miR-21 knockout mouse model with Eμ-TCL1 mouse model. We will also perform gene expression profiling of CLL cells isolated from patients with high miR-21 expression and low mir-21 expression. This will allow us to study the targets of miR-21 important in CLL disease progression. **Expected Results:** We expect SPRY2 is the major target of miR-21 in CLL disease progression.
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Appendix 1

Stromal Tumor Microenvironment in Chronic Lymphocytic Leukemia: Regulation of Leukemic Progression


Abstract

Chronic Lymphocytic Leukemia (CLL), the most prevalent adult leukemia in western countries, which is highly heterogeneous with a very variable clinical outcome. Emerging evidence indicates that the stromal tumor microenvironment (STME) and stromal associated genes (SAG) play important roles in the pathogenesis and progression of CLL. However, the precise mechanisms by which STME and SAG are involved in this processes remain unknown. In an attempt to explore the role of STME in this process, we examined the expression levels of stromal associated genes using gene expression profiling (GEP) of CLL cells from lymph nodes (LN) (n=15), bone marrow (BM) (n=18), and peripheral blood (PB) (n=20). Interestingly, LUM, MMP9, MYLK, ITGA9, CAV1, CAV2, FBN1, PARVA, CALD1, ITGB5 and EHD2 were found to be overexpressed while ITGB2, DLC1 and ITGA6 were under expressed in LN-CLL compared to BM-CLL and PB-CLL. This is suggestive of a role for LN-mediated TME in CLL cell survival/progression. Among these genes, expression of MYLK, CAV1 and CAV2 correlated with clinical outcome as determined by time to first treatment. Together, our
studies show that members of the stromal signature, particularly in the CLL cells from lymph nodes, regulate CLL cell survival and proliferation and thus leukemic progression.
Introduction:

Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous, incurable B cell malignancy affecting elderly population in the western world. Emerging evidence suggest that CLL cells depend on complex communications with their microenvironment for survival. Due to an overt dependence of CLL cells on these interactions, their survival is greatly reduced when cultured in vitro by themselves. We and others [1,2] have shown that the tumor microenvironment (TME) in lymph nodes (LN) provide pro-survival/proliferation signals to CLL cells and induces host immune suppression [3]. Furthermore, prolonged survival of CLL cells in the proliferation centers in bone marrow (BM) and in LNs is mediated by several stromal micro-environmental (STME) cues; however, the precise nature of these interactions remains ambiguous. LN microenvironment is comprised of stromal and other cells along with the associated extra cellular matrix. Extra cellular matrix is comprised of proteoglycans, integrins, hyaluronic acid and reticular network. On the other hand, stromal and other cells in the lymph nodes represented by lymphatic endothelial cells, mesenchymal cells, T cells, follicular dendritic cells and monocytes-derived nurselike cells have been shown to enhance CLL cell survival [4]. The STME helps them to escape from therapy resulting in increased relapse rate in CLL patients [4]. Once the tumor cells colonize in LNs, they shape their microenvironment to support their own survival and growth. This partly involves the activation of immune tolerance genes in CLL cells [3]. Recently Garcia-Munoz et al. have suggested that immunoglobulin gene mutated CLL cells in the LN, acquire self-reactivity for auto antigens while being tolerated with receptor editing [5]. As a consequence, the normal function and proliferation of nonmalignant B cells in the TME are also affected [5]. Lenz et al. have eloquently demonstrated that survival of diffuse large B cell lymphoma following treatment is influenced by the differences in immune
cells, fibrosis and angiogenesis in the TME [6]. This conclusion was based on the analyses of stromal gene signatures in large B cell lymphomas.

Orimo et al. have reported that stromal fibroblasts present in invasive human breast cancer promote tumor growth and angiogenesis through elevated SDF/CXCL12 secretion [7]. Together, these reports advocate the importance of complex interactions between CLL and other tumor cells with their microenvironment for increased proliferation leading to disease progression. In B cells, such interactions involve cytoskeletal changes possibly mediated by stromal microenvironment leading to enhanced B cell activation by BCR clustering [8,9]. Similar mechanisms may impose altered cytoskeletal changes in CLL cells leading to better survival and proliferation. Therefore, identification of molecular network involved in modification of LN microenvironment by CLL cells will lead to a better understanding of the disease. In an attempt to understand the molecular basis of stromal associated regulation of CLL progression and its prognostic implication we performed a gene expression profiling (GEP) of CLL cells from PB, BM and LN. We also performed transcriptome analysis of PB-CLL cells from patients with good versus poor prognosis to identify stromal gene signatures associated with disease aggressiveness. We identified two genes, MYLK and CAV2, whose transcript and protein expression is upregulated in patients with poor prognosis than good prognosis and significantly associated with patient’s outcome.

**CLL patient information:**

Using an Institutional Review Board approved-protocol and informed consent, CLL samples were obtained from patients. PB (n=20), BM (n=18) and LN (n=15) samples from 53 CLL samples were collected from 37 different patients. In addition, to validate the results, additional peripheral blood CLL samples from 20 patients and another 15 patients with good prognosis (n=7) and poor prognosis (n=8) patient
peripheral blood samples were also used. In this study, we included CLL patients who are untreated and who did not receive any treatment for six months prior to sample collection. For control, B cells from age matched normal donors were obtained. The clinical information on these 72 patient are provided in Table 1 a-c.

**CLL cells isolation and characterization:**

All CLL samples for this study were obtained using an UNMC Institutional Review Board approved protocol. CLL cells were isolated from PB and BM using density gradient centrifugation with lymphocyte separation medium, LymphoPrep, followed by negative selection using magnetic bead separation method as needed [10, 11]. Frozen LN samples were obtained from the UNMC tissue bank. CLL cells were localized on frozen LN samples using immunohistochemistry by staining CD19 and CD5 positive cells. Fifteen to twenty sections of 8-10 micron thickness were made at 4°C and fixed immediately by ice-cold acetone and stained with cresyl violet (LCM Staining Kit, Ambion, TX, USA) for 30 seconds. These cells were micro dissected for RNA isolation [2]. For CLL cells isolated from PB and BM, the purity was tested by flow cytometry using the combination of CD3-FITC, CD19-PE, CD5-PE, CD38-PE and CD19-FITC (BD Biosciences, San Jose, CA, USA).

**Clinical Characterization of CLL patient:**

Patients with high CD38 positive, bulky lymphadenopathy, chromosome 11q deletion, 17p deletion and trisomy 12, unmutated IgVH and shorter time to first treatment were considered as a poor prognosis patient, whereas patients with only 13q deletion or normal karyotype, mutated IgVH and longer time to treatment were considered as a good prognosis patient [11, 12].
**Microarray analysis:**

RNA from CLL cells was extracted using TRIzol™ (Invitrogen, Grand Island, NY, USA) as per the manufacturer's instruction. Gene expression profiling was performed using a DNA microarray chip (MWG Biotech, Germany, Human 30K oligo set B) consisting of a 50-mer oligonucleotide representing 10,000 different genes. cDNAs were obtained from RNA using Stratagene manufacturer’s instructions. The hybridized slides were scanned and images were collected by an Axon 4000B scanner (Axon Instruments, Grand Terrace, CA, USA). Differentially expressed stromal signature genes were identified using significance analyses of microarray (SAM).

**Transcriptome analysis:**

RNA from peripheral blood B cells of seven good prognosis CLL patients and eight poor prognosis CLL patients was isolated as mentioned above. mRNA levels of stromal related genes with significant p value were compared between good and poor prognosis patients. The RNA sequencing was done at the UNMC Next Generation Sequencing Core facility using Illumina HiSeq 2000 sequence analyzer. RNA sequence alignment was done by TopHat alignment software; this was followed by Picard processing for removal of duplicates. To identify differential gene expression levels among the good versus poor prognosis, Cuffdiff method was used. To determine the relative fold change the values were normalized with GAPDH. Clinical information of these 15 patients is provided in Table 1 (d).

**Clinical correlation of the gene expression levels:**

The Kaplan Meier analysis log-rank test was used to analyze clinical correlation of gene expression levels or clinical parameters with time to first treatment. Time to
treatment is the interval in months between diagnosis and initiation of the first treatment in months among the CLL patients. In some cases the CLL cells used in the study were classified based on cytogenetic chromosomal abnormality, where CLL cells 13q deletion and normal karyotype were considered as good prognosis and CLL cells with 11qdel, trisomy 12 and 17p deletion as poor prognosis group. Also CD38 low (less than 30% positive) or immunoglobulin gene mutation and CD38 high (more than 30%) or unmutated immunoglobulin gene were also grouped as good and poor prognosis, respectively.

**Western blotting:**

The expression of MYLK, EHD2, DLC1 and CAV1 in B cells from a normal healthy donor, five good prognosis CLL patients and five poor prognosis CLL patients were determined using western blot analyses. 50 µg of protein was loaded on 10% SDS-PAGE gel, which was separated by electrophoresis, and blotted on PVDF membrane. In brief, the membrane was incubated with primary antibodies of MYLK and DLC1 (Santa Cruz Biotechnologies, Dallas, TX, USA), CAV1 (Abcam, Cambridge, MA, USA), EHD2 (homemade antibody was kindly provided by Dr. Hamid Band, UNMC) and β-actin (Sigma Aldrich, St. Louis, MO, USA). This was followed by incubation of membranes with appropriate secondary antibodies and blot was developed using Enhanced Chemiluminescence, Pierce ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA).

**Statistical Analysis:**

For the identification of differentially expressed genes, a significant analysis of microarray (SAM) was used. To identify the tissue specific gene signatures, analysis was performed using random variance t-test with p-value of 0.01 and false discovery
rate (FDR) of 0.08. The most of analyses were performed at p<0.05 and FDR<0.25, unless specified otherwise. The Kaplan-Meier method using log-rank test was used to study the association of gene expression or clinical parameter with the clinical outcome as done previously [13].

Results

**Supervised cluster analyses of differential expression of stromal signature I and II associated genes in primary CLL cells:**

Figure 1 shows a supervised cluster analyses of the differential expression of stromal signature I & II genes in CLL cells isolated from peripheral blood (PB), bone marrow (BM) and lymph nodes (LN) from patients in comparison to each other and with normal B cells from healthy donors (nB). In the case of stromal signature I, 119 genes were analyzed. There were in total 47 genes that are differentially expressed. Among these 47 genes, 30 genes were overexpressed and 17 genes were under expressed compared to each other. Among the 30 genes overexpressed, based on the transcript levels, we divided them into the categories of high (11 genes), medium (8 genes) and low (11 genes) expressing genes based on the fold change in their expression compared to reference as shown in Table 1. In this Stromal I gene signature, these differentially expressed genes are associated with extracellular matrix, cytoskeleton maintenance, cell migration, and biosynthesis of collagen. We have recently shown that the CLL cells in the LNs induce immune tolerance against themselves to facilitate their uninhibited growth [2]. Interestingly, many of the genes including SERPINH1, SERPINF1, FBN1, APOE, PTGDS, LUM, CALD1 and MYLK were significantly overexpressed in CLL cells in the LNs compared to BM, PB and nB.
In the case of stromal II genes, there were a total of 35 genes; of these, 22 genes were differentially expressed genes. Among these 22 genes, 14 genes were overexpressed and 8 genes were under expressed in CLL cells compared to normal B cells. Among the overexpressed genes, 7 genes had high expression levels and 7 genes had medium expression levels and there were no genes in which expression was relatively low compared to other genes in the category. Stromal signature II shows the mRNA level of genes which are associated with intracellular compartment: EHD2, SDF1, PTPRB, CAV2 and CAV1 [14] were overexpressed, whereas DLC1, which is a known tumor suppressor [15], was under expressed in both stromal signatures I & II.

Validation of differential expression using transcript levels of selected stromal genes in CLL cells from PB, BM and LN:

We confirmed the differential expression of selected genes by evaluating the mRNA levels of each gene. In these analyses, we either compared the gene expression to the expression levels in normal B cells, and/or compared to normal B cells as well as to CLL cells from PB, BM and LNs. Figure 2 shows the results of these analyses. There was an increased expression of MYLK and decreased expression of DLC1, CSPG2/VCAN and ITGB2 in CLL B cells (Figure 2A, B, C and D). Further comparison of these three and additional genes with CLL cells from PB, BM and LN and normal B cells showed there was a significantly increased expression (p <0.05) of MYLK, EHD2, CAV2 in CLL cells from the LNs compared other cells in these analyses group (Figure 2E, F and G). In contrast, the expression of DLC-1 and CSPG2/VCAN were significantly decreased (p< 0.05) in the CLL cells from the lymph nodes compared to rest of the cells in this analyses group (Figure 2H and I).
Validation of differential expression of selected genes at protein levels using western blotting:

As further validation for higher expression of MYLK, CAV1 and EHD2 and lower expression of DLC1 seen in the transcript analyses, we performed comparison of protein expression on the basis of disease progression. The evaluation of protein levels of these genes was done using PB CLL cells from five patients with good prognosis and CLL cells from five poor prognosis and normal donor B cells. Figure 3 shows the results of these analyses. There was an increased expression of MYLK1, EHD2 and CAV2 in all five CLL patients with poor prognosis compared to CLL cells from five good prognosis patients, whereas the expression levels of DLC1 was significantly higher in good prognosis patients compared to CLL cells from poor prognosis patients. In these analyses, β-Actin was used as housekeeping gene control. Together these analyses confirmed the differential expression these selected genes.

Clinical significance of stromal genes:

Next, in order to understand the clinical significance of the differentially expressed genes, we compared the expression levels of expression of stromal signature I associated genes whose transcript analysis were statistically significant, namely CEBPA, MYLK, APOE, RAB32, PTGDS and WNT2B and levels of expression of stromal signature II associated genes, namely DLC1, CAV2, EHD2, SDF1, RBP4 and ROBO1, with the time to first treatment in patients. Figure 4 shows the results of these analyses using Kaplan Meier analyses with log-rank test. We have previously shown that high expression CAV1 correlated with clinical outcomes, also knockdown of CAV1 impaired CLL cells to migrate and formation of immune synapse [2]. Among the 12 genes we evaluated, higher expression of MYLK and CAV2 (Figure 4 D and G) significantly
correlated with poor clinical outcome. Recently Yamasaki T et al, showed higher expression of CAV2 promotes cell proliferation, migration and invasion in renal cell carcinoma [16]. DLC1 is a known tumor suppressor and the western blot analysis confirmed the down regulation at the protein level which made it interesting to see the clinical outcome. However, although not statistically significant, higher expression of WNT2B and EHD2 and lower expression of DLC1 showed clinical correlation (Figure 4 A, E and K). The rest of genes did not show clinical correlation with time to first treatment in patients.

**Gene expression levels determined by RNA sequencing:**

In order to identify additional genes which are associated with extracellular matrix we did comparative analysis of differential gene expression in Good Prognosis CLL and Poor prognosis CLL using RNA transcriptome analysis. The mRNA level of genes encoding for cell surface proteoglycan and glycoprotein those also associated with stromal signature I & II as mentioned above like VCAN and CD93 were 50 and 20 fold up-regulated respectively; inflammatory related cytokines associated genes like IL8, CXCL1 and CXCL3 were 40, 77 and 111 fold up-regulated respectively in good prognosis; and SPP1 and SERPINB2 were 955 and 1271 fold higher than poor prognosis respectively. Among these differentially expressed genes identified in our transcriptome analyses, expression levels of proteins of selected extracellular matrix genes were evaluated using Western blot analyses. There was a significantly increased protein level of VCAN, SPP1 and SERPINB2 in CLL cells from good prognosis compared to CLL cells from poor prognosis patients (Figure 5). Thus these results validated the differential expression of certain extracellular matrix associated genes as identified in transcriptome analyses.
Figures and Tables:

**Figure 1**

### Stromal I signature

- **PB**: vank1, fraf, nton41, ittn, rob1, itud3, trc, eki, stoxk, henh, dnn11, avl1, rbd2, oit3, cve, cdl4, vbm6, edm5, edr2, lld1, itdb1, thv1, eoz1, ocm2, rih3, kde1, kfa, nhs1, lbu1, thb1, dcb2, arc1, cmgr2, cew3, idnr1, rnr, cdl1, dbe13, hcl1, odc5a, ceh1a, cec2, cecam, cecam2, hmg3, bta, stox1, rital, mtl, ntrk1, ttbd, mame3, ppas2, ntrk, neuropilin receptor type 1, tki.

### Stromal II signature

- **PB**: lmb3, cvef1a1, bace, fslt1, hlf, stl, li, myk, cki1, cvx, cki2, cki3, cmx, cno, tdo2, vhec, amstl2, atn, col1a1, adml2, loki1, semn, sem1, sem2, stl, ctk, mno, tmd, cald1, mm2, mitf, ccm, crrcs1, s17, lama4, vnc1, cck2, cki2, clu, vnc2, cki1, cki1a, cki1b, cki1c, cki1d, cki1e, cki1f, cki1g, cki1h, cki1i, cki1j, cki1k, cki1l, cki1m, cki1n, cki1o, cki1p, cki1q, cki1r, cki1s, cki1t, cki1u, cki1v, cki1w, cki1x, cki1y, cki1z, cki1aa, cki1ab, cki1ac, cki1ad, cki1ae, cki1af, cki1ag, cki1ah, cki1ai, cki1aj, cki1ak, cki1al, cki1am, cki1an, cki1ao, cki1ap, cki1aq, cki1ar, cki1as, cki1at, cki1au, cki1av, cki1aw, cki1ax, cki1ay, cki1az, cki1ba, cki1bb, cki1bc, cki1bd, cki1be, cki1bf, cki1bg, cki1bh, cki1bi, cki1bj, cki1bk, cki1bl, cki1bm, cki1bn, cki1bo, cki1bp, cki1bq, cki1br, cki1bs, cki1bt, cki1bu, cki1bv, cki1bw, cki1bx, cki1by, cki1bz, cki1ca, cki1cb, cki1cc, cki1cd, cki1ce, cki1cf, cki1cg, cki1ch, cki1ci, cki1cj, cki1ck, cki1cl, cki1cm, cki1cn, cki1co, cki1cp, cki1cq, cki1cr, cki1cs, cki1ct, cki1cu, cki1cv, cki1cw, cki1cx, cki1cy, cki1cz, cki1da, cki1db, cki1dc, cki1dd, cki1de, cki1df, cki1dg, cki1dh, cki1di, cki1dj, cki1dk, cki1dl, cki1dm, cki1dn, cki1do, cki1dp, cki1dq, cki1dr, cki1ds, cki1dt, cki1du, cki1dv, cki1dw, cki1dx, cki1dy, cki1dz, cki1ea, cki1eb, cki1ec, cki1ed, cki1ee, cki1ef, cki1eg, cki1eh, cki1ei, cki1ej, cki1ek, cki1el, cki1em, cki1en, cki1eo, cki1ep, cki1eq, cki1er, cki1es, cki1et, cki1eu, cki1ev, cki1ew, cki1ex, cki1ey, cki1ez, cki1fa, cki1fb, cki1fc, cki1fd, cki1fe, cki1ff, cki1fg, cki1fh, cki1fi, cki1fj, cki1fk, cki1fl, cki1fm, cki1fn, cki1fo, cki1fp, cki1fq, cki1fr, cki1fs, cki1ft, cki1fu, cki1fv, cki1fw, cki1fx, cki1fy, cki1fz, cki1ga, cki1gb, cki1gc, cki1gd, cki1ge, cki1gf, cki1gg, cki1gh, cki1gi, cki1gj, cki1gk, cki1gl, cki1gm, cki1gn, cki1go, cki1gp, cki1gq, cki1gr, cki1gs, cki1gt, cki1gu, cki1gv, cki1gw, cki1gx, cki1gy, cki1gz, cki1ha, cki1hb, cki1hc, cki1hd, cki1he, cki1hf, cki1hg, cki1hi, cki1hj, cki1hk, cki1hl, cki1hm, cki1hn, cki1ho, cki1hp, cki1hq, cki1hr, cki1hs, cki1ht, cki1hu, cki1hv, cki1hw, cki1hx, cki1hy, cki1hz, cki1ia, cki1ib, cki1ic, cki1id, cki1ie, cki1if, cki1ig, cki1ih, cki1ij, cki1ik, cki1il, cki1im, cki1in, cki1io, cki1ip, cki1iq, cki1ir, cki1is, cki1it, cki1iu, cki1iv, cki1iw, cki1ix, cki1iy, cki1iz, cki1ja, cki1jb, cki1jc, cki1jd, cki1je, cki1jf, cki1jg, cki1jh, cki1ji, cki1jj, cki1jk, cki1jl, cki1jm, cki1jn, cki1jo, cki1jp, cki1jq, cki1jr, cki1js, cki1jt, cki1ju, cki1jv, cki1jw, cki1jx, cki1jy, cki1jz.

**Differential expression of stromal signature I and II associated genes in primary CLL cells:** GEP showing differential expression of Stromal Signature I & II in cells isolated from peripheral blood (PB), bone marrow (BM) and lymph nodes (LN) from CLL patients in comparison with normal B cells from healthy donors (nB). Stromal Signature I and II showing the differential gene expression in extra cellular matrix compartment and cellular components of CLL patients and healthy donors. mRNA level of some cytoskeleton related genes like MYLK, APOE and PTGDS from stroma I, EHD2, CAV2 and CAV1 from stroma II were high, whereas tumor suppressor genes like DLC1 was down regulated in both stroma I & II.
Expression levels of stromal genes in CLL cells from PB, BM and LN: Comparison of mRNA expression of (A) myosin light kinase (MYLK), (B) versican (VCAN), (C) deleted in liver cancer (DLC1), (D) Integrin, Beta 2 (ITGB2) in PB isolated B cells from 8 healthy donors and 20 CLL patients. mRNA expression comparison of (E) myosin light kinase (MYLK), (F) EH domain containing 2 (EHD2), (G) caveolin 2 (CAV2), (H) deleted in liver cancer (DLC1) and (I) versican (VCAN) in B cells from PB of 8 healthy donors and B cells from PB, BM and LN samples from 37 CLL patients samples. Student t-TEST was applied to determine the statistically significance among the normal healthy donors and CLL patients. "*" determine p value of ≤ 0.05. The relative expression was normalized by GAPDH.
Figure 3: Analysis for protein expression: Expression of selected proteins in normal B and CLL cells from PB of 5 good prognosis and 5 poor prognosis patients using western blotting.

<table>
<thead>
<tr>
<th>Protein</th>
<th>5 Poor</th>
<th>5 Good</th>
<th>nB</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYLK</td>
<td><img src="MYLK.png" alt="Image" /></td>
<td><img src="MYLK.png" alt="Image" /></td>
<td><img src="MYLK.png" alt="Image" /></td>
</tr>
<tr>
<td>DLC-1</td>
<td><img src="DLC-1.png" alt="Image" /></td>
<td><img src="DLC-1.png" alt="Image" /></td>
<td><img src="DLC-1.png" alt="Image" /></td>
</tr>
<tr>
<td>EHD2</td>
<td><img src="EHD2.png" alt="Image" /></td>
<td><img src="EHD2.png" alt="Image" /></td>
<td><img src="EHD2.png" alt="Image" /></td>
</tr>
<tr>
<td>CAV-1</td>
<td><img src="CAV-1.png" alt="Image" /></td>
<td><img src="CAV-1.png" alt="Image" /></td>
<td><img src="CAV-1.png" alt="Image" /></td>
</tr>
<tr>
<td>β-Actin</td>
<td><img src="%CE%B2-Actin.png" alt="Image" /></td>
<td><img src="%CE%B2-Actin.png" alt="Image" /></td>
<td><img src="%CE%B2-Actin.png" alt="Image" /></td>
</tr>
</tbody>
</table>
**Clinical significance of stromal genes:** Clinical correlation of expression levels of stromal signature I associated genes (CEBPA, MYLK, APOE, RAB32, PTGDS and WNT2B) and stromal signature II associated genes (DLC1, CAV2, EHD2, SDF1, RBP4 and ROBO1) in CLL patients using Kaplan Meier analyses with log-rank test. Among these genes, expression of MYLK, WNT2B, DLC1, EHD2 and CAV2 in stromal signature I and II correlated with clinical outcome as determined by time to treatment.
Validation of differentially expressed extracellular matrix associated genes in good and poor prognosis CLL samples using analyses for protein expression:

Expression of selected extracellular matrix associated proteins in CLL cells from PB of 4 good prognosis and 4 poor prognosis patients using western blotting method.
Table 1: (a) Peripheral Blood CLL patient samples information:

<table>
<thead>
<tr>
<th>Patients ID</th>
<th>Age</th>
<th>Gender</th>
<th>Time to treatment</th>
<th>Fluorescence in-situ Hybridization</th>
<th>Bulky disease</th>
<th>CD 38%</th>
<th>IgVH Mutation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB12</td>
<td>69</td>
<td>M</td>
<td>1</td>
<td>NA</td>
<td>No lymphadenopathy</td>
<td>45%</td>
<td>NA</td>
</tr>
<tr>
<td>PB16</td>
<td>51</td>
<td>M</td>
<td>1</td>
<td>Trisomy 12; 17p-; 13q14-</td>
<td>Bulky adenopathy</td>
<td>89%</td>
<td>NA</td>
</tr>
<tr>
<td>PB22</td>
<td>66</td>
<td>F</td>
<td>Never</td>
<td>NORMAL</td>
<td>No lymphadenopathy</td>
<td>12%</td>
<td>NA</td>
</tr>
<tr>
<td>PB37</td>
<td>53</td>
<td>M</td>
<td>NA</td>
<td>NORMAL</td>
<td>No lymphadenopathy</td>
<td>4%</td>
<td>NA</td>
</tr>
<tr>
<td>PB82</td>
<td>79</td>
<td>M</td>
<td>4</td>
<td>Trisomy 12, 13q14 del</td>
<td>BALA</td>
<td>92%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>PB89</td>
<td>39</td>
<td>M</td>
<td>10</td>
<td>Trisomy 12 (23% - 56%)</td>
<td>Mild lymphadenopathy</td>
<td>3%</td>
<td>NA</td>
</tr>
<tr>
<td>PB90</td>
<td>66</td>
<td>F</td>
<td>Never</td>
<td>13q14 del</td>
<td>No lymphadenopathy</td>
<td>20%</td>
<td>NA</td>
</tr>
<tr>
<td>PB96</td>
<td>85</td>
<td>F</td>
<td>1</td>
<td>NORMAL</td>
<td>Mild lymphadenopathy</td>
<td>9%</td>
<td>NA</td>
</tr>
<tr>
<td>PB97</td>
<td>54</td>
<td>F</td>
<td>2</td>
<td>NORMAL</td>
<td>Lymphadenopathy</td>
<td>51%</td>
<td>NA</td>
</tr>
<tr>
<td>PB106</td>
<td>65</td>
<td>M</td>
<td>Never</td>
<td>13q null</td>
<td>No lymphadenopathy</td>
<td>27%</td>
<td>NA</td>
</tr>
<tr>
<td>PB107</td>
<td>58</td>
<td>M</td>
<td>2</td>
<td>11q22.3/ATM (96%); 13q14 deletion (96.5%)</td>
<td>LN (axilla, supraclavicular, mediastinal)</td>
<td>1.50%</td>
<td>NA</td>
</tr>
<tr>
<td>PB109</td>
<td>44</td>
<td>M</td>
<td>0.25</td>
<td>11q22.3/ATM (96%), 13q14 (12%), (14q32 del, 77.5%)</td>
<td>LN (neck and axilla)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PB117</td>
<td>80</td>
<td>M</td>
<td>48</td>
<td>13q14 (87.5% - 96%) IgH (12.5%)</td>
<td>BALA (axillary)</td>
<td>32.45%</td>
<td>NA</td>
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<tr>
<td>PB121</td>
<td>47</td>
<td>M</td>
<td>Never</td>
<td>13q14 (72%)</td>
<td>No lymphadenopathy</td>
<td>7%</td>
<td>NA</td>
</tr>
<tr>
<td>PB124</td>
<td>53</td>
<td>F</td>
<td>Never</td>
<td>Blood14q32 (35.5%)</td>
<td>No lymphadenopathy</td>
<td>11%</td>
<td>NA</td>
</tr>
<tr>
<td>PB134</td>
<td>57</td>
<td>M</td>
<td>82</td>
<td>13q nullosomy</td>
<td>No lymphadenopathy</td>
<td>23%</td>
<td>NA</td>
</tr>
<tr>
<td>PB141</td>
<td>71</td>
<td>M</td>
<td>Never</td>
<td>13q14 (38.5%)</td>
<td>No lymphadenopathy</td>
<td>24%</td>
<td>NA</td>
</tr>
<tr>
<td>PB143</td>
<td>69</td>
<td>F</td>
<td>Never</td>
<td>13q14 (53.5%), cyto-N; 13q14 (83%)</td>
<td>No lymphadenopathy</td>
<td>12%</td>
<td>NA</td>
</tr>
<tr>
<td>PB146</td>
<td>76</td>
<td>F</td>
<td>17</td>
<td>11q23 (84.5%), 13q14 (15.5%)</td>
<td>No lymphadenopathy</td>
<td>43%</td>
<td>NA</td>
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<tr>
<td>PB149</td>
<td>41</td>
<td>M</td>
<td>6</td>
<td>17p- (11%), 13q- (33%), 14q- (12%)</td>
<td>LN (neck)</td>
<td>8%</td>
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</table>
### Lymph Nodes CLL patient samples information:

<table>
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<tr>
<th>Patients ID</th>
<th>Age</th>
<th>Gender</th>
<th>Time to treatment</th>
<th>Fluorescence in-situ Hybridization</th>
<th>Bulky disease</th>
<th>CD 38%</th>
<th>IgVH Mutation status</th>
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<tbody>
<tr>
<td>LN11</td>
<td>59</td>
<td>M</td>
<td>1</td>
<td>TRISOMY 12 (47%); 14q32 (25%); yes, diffuse abdominal, axilla, then 12% n 13% after that 17p del groin, neck LN</td>
<td>80%</td>
<td>Unmutated</td>
<td></td>
</tr>
<tr>
<td>LN34A</td>
<td>74</td>
<td>M</td>
<td>2</td>
<td>11q23 from 13qdel</td>
<td>BALA</td>
<td>63%</td>
<td>NA</td>
</tr>
<tr>
<td>LN34B</td>
<td>74</td>
<td>M</td>
<td>2</td>
<td>11q23 from 13qdel</td>
<td>BALA</td>
<td>63%</td>
<td>NA</td>
</tr>
<tr>
<td>LN40A</td>
<td>49</td>
<td>M</td>
<td>1</td>
<td>13q14 (68.5%), both11.5%,null 13.5%, then 17p13 in LN;</td>
<td>BALA</td>
<td>92%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>LN40B</td>
<td>49</td>
<td>M</td>
<td>1</td>
<td>13q14 (68.5%), both11.5%,null 13.5%, then 17p13 in LN;</td>
<td>BALA</td>
<td>92%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>LN59</td>
<td>62</td>
<td>F</td>
<td>108</td>
<td>NA</td>
<td>Lymphadenopathy</td>
<td>NA</td>
<td></td>
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<tr>
<td>LN64</td>
<td>72</td>
<td>F</td>
<td>48</td>
<td>17p13 *</td>
<td>Lymphadenopathy</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>LN66A</td>
<td>61</td>
<td>F</td>
<td>11</td>
<td>11q22.3/ATM (78%), 13q14 (89%), 17p13.1 (3 copies in 46.3%)</td>
<td>BALA</td>
<td>84%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>LN66B</td>
<td>61</td>
<td>F</td>
<td>11</td>
<td>11q22.3/ATM (78%), 13q14 (89%), 17p13.1 (3 copies in 46.3%)</td>
<td>BALA</td>
<td>84%</td>
<td>Unmutated</td>
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<tr>
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<td>M</td>
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<td>11q23, 13q14</td>
<td>BALA</td>
<td>21%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>LN82</td>
<td>79</td>
<td>M</td>
<td>4</td>
<td>Trisomy 12, 13q14 del</td>
<td>BALA</td>
<td>92%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>LN83A</td>
<td>29</td>
<td>M</td>
<td>22</td>
<td>11q23 deletion (92%), 14q32 (64.5%)</td>
<td>BALA</td>
<td>98%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>LN83B</td>
<td>29</td>
<td>M</td>
<td>23</td>
<td>11q23 deletion (92%), 14q32 (64.5%)</td>
<td>BALA</td>
<td>98%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>LN93</td>
<td>50</td>
<td>M</td>
<td>1</td>
<td>14q-</td>
<td>BALA</td>
<td>52%</td>
<td>NA</td>
</tr>
<tr>
<td>LN149</td>
<td>41</td>
<td>M</td>
<td>6</td>
<td>17p- (11%), 13q- (33%), 14q- (12%)</td>
<td>Lymph (neck)</td>
<td>8%</td>
<td>NA</td>
</tr>
</tbody>
</table>
(c) Bone marrow CLL patient samples information:

<table>
<thead>
<tr>
<th>Patients ID</th>
<th>Age</th>
<th>Gender</th>
<th>Time to treatment</th>
<th>Karyotype</th>
<th>Bulky disease</th>
<th>CD 38%</th>
<th>IgVH Mutation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM12</td>
<td>69</td>
<td>M</td>
<td>1</td>
<td>NA</td>
<td>No lymphadenopathy</td>
<td>45%</td>
<td>NA</td>
</tr>
<tr>
<td>BM16</td>
<td>51</td>
<td>M</td>
<td>1</td>
<td>Trisomy 12; 17p-; 13q14-</td>
<td>Bulky adenopathy</td>
<td>89%</td>
<td>NA</td>
</tr>
<tr>
<td>BM32</td>
<td>65</td>
<td>F</td>
<td>NA</td>
<td>13q14</td>
<td>Lymphadenopathy</td>
<td>34%</td>
<td>NA</td>
</tr>
<tr>
<td>BM34</td>
<td>74</td>
<td>M</td>
<td>2</td>
<td>11q23 from 13qdel</td>
<td>Lymphadenopathy</td>
<td>63%</td>
<td>NA</td>
</tr>
<tr>
<td>BM37</td>
<td>53</td>
<td>M</td>
<td>NA</td>
<td>NORMAL</td>
<td>No lymphadenopathy</td>
<td>4%</td>
<td>NA</td>
</tr>
<tr>
<td>BM67</td>
<td>42</td>
<td>M</td>
<td>1</td>
<td>11q23, 13q14</td>
<td>BALA</td>
<td>21%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>BM74</td>
<td>55</td>
<td>M</td>
<td>Never</td>
<td>13q13 deletion</td>
<td>No lymphadenopathy</td>
<td>5%</td>
<td>Mutated</td>
</tr>
<tr>
<td>BM83</td>
<td>29</td>
<td>M</td>
<td>22</td>
<td>11q23 deletion (92%), 14q32 (64.5%)</td>
<td>BALA</td>
<td>98%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>BM89</td>
<td>39</td>
<td>M</td>
<td>10</td>
<td>Trisomy 12 (23% - 56%)</td>
<td>Mild lymphadenopathy</td>
<td>3%</td>
<td>NA</td>
</tr>
<tr>
<td>BM106</td>
<td>65</td>
<td>M</td>
<td>Never</td>
<td>13q null</td>
<td>No lymphadenopathy</td>
<td>27%</td>
<td>NA</td>
</tr>
<tr>
<td>BM117</td>
<td>80</td>
<td>M</td>
<td>48</td>
<td>13q14 (87.5% - 96%) IgH (12.5%)</td>
<td>BALA (axillary)</td>
<td>32.45%</td>
<td>NA</td>
</tr>
<tr>
<td>BM120</td>
<td>65</td>
<td>M</td>
<td>3</td>
<td>14q-</td>
<td>LN (neck)</td>
<td>34%</td>
<td>NA</td>
</tr>
<tr>
<td>BM122</td>
<td>50</td>
<td>M</td>
<td>17</td>
<td>various del, 17p del, 20q-, 7p-</td>
<td>No lymphadenopathy</td>
<td>33.50%</td>
<td>NA</td>
</tr>
<tr>
<td>BM124</td>
<td>53</td>
<td>F</td>
<td>Never</td>
<td>blood- normal in BM-14q32 (35.5%)</td>
<td>No lymphadenopathy</td>
<td>11%</td>
<td>NA</td>
</tr>
<tr>
<td>BM152</td>
<td>60</td>
<td>M</td>
<td>Never</td>
<td>13q- (48%)</td>
<td>No lymphadenopathy</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>BM163</td>
<td>70</td>
<td>M</td>
<td>Never</td>
<td>13q null (24%), 13q- (46%)</td>
<td>No lymphadenopathy</td>
<td>23%</td>
<td>NA</td>
</tr>
<tr>
<td>BM166</td>
<td>52</td>
<td>M</td>
<td>Never</td>
<td>-normal</td>
<td>LN(axillary)</td>
<td>28%</td>
<td>NA</td>
</tr>
<tr>
<td>BM168</td>
<td>56</td>
<td>F</td>
<td>Never</td>
<td>13q null (11%), 13q del (66%)</td>
<td>mild LA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
(d) Patients Clinical information used for Transcriptome:

<table>
<thead>
<tr>
<th>Patients ID</th>
<th>Age</th>
<th>Gender</th>
<th>Time to treatment</th>
<th>Fluorescence in-situ Hybridization</th>
<th>Bulky disease</th>
<th>CD 38%</th>
<th>IgVH Mutation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL 3</td>
<td>61</td>
<td>F</td>
<td>98</td>
<td>TRISOMY 12 (65.5%)</td>
<td>No lymphadenopathy</td>
<td>83%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>CLL 11</td>
<td>59</td>
<td>M</td>
<td>1</td>
<td>TRISOMY 12 (47%); 14q32 (25%); then Yes, diffuse abdominal, 12% n 13% after that 17p del axilla, groin, neck LN</td>
<td>No lymphadenopathy</td>
<td>80%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>CLL 29</td>
<td>55</td>
<td>F</td>
<td>1</td>
<td>11q23- (91%); 13q14 mono (95.5%),13q14(60%); 14q32 (65.5%)</td>
<td>BALA</td>
<td>17%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>CLL 30</td>
<td>55</td>
<td>F</td>
<td>11q23 from 13qdel</td>
<td>Trisomy 12 (70.5%)</td>
<td>Lymphadenopathy</td>
<td>55%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>CLL 34</td>
<td>74</td>
<td>M</td>
<td>2</td>
<td>BALA</td>
<td>Lymphadenopathy</td>
<td>63%</td>
<td>NA</td>
</tr>
<tr>
<td>CLL 40</td>
<td>49</td>
<td>M</td>
<td>1</td>
<td>13q14 (68.5%), both 11.5%, null 13.5%, then 17p13 in LN;</td>
<td>BALA</td>
<td>92%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>CLL 79</td>
<td>62</td>
<td>M</td>
<td>15</td>
<td>11q22.3 (78%), 13q14 (53.5%), null13q14 (34%)</td>
<td>No lymphadenopathy</td>
<td>23%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>CLL 82</td>
<td>79</td>
<td>M</td>
<td>4</td>
<td>Trisomy 12; 13q14 del</td>
<td>BALA</td>
<td>92%</td>
<td>Unmutated</td>
</tr>
<tr>
<td>CLL 10</td>
<td>49</td>
<td>F</td>
<td>Never</td>
<td>Normal</td>
<td>No lymphadenopathy</td>
<td>6%</td>
<td>Mutated</td>
</tr>
<tr>
<td>CLL 13</td>
<td>83</td>
<td>M</td>
<td>1</td>
<td>Normal</td>
<td>No lymphadenopathy</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CLL 19</td>
<td>56</td>
<td>F</td>
<td>Never</td>
<td>13q14-</td>
<td>No lymphadenopathy</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CLL 75</td>
<td>34</td>
<td>M</td>
<td>96</td>
<td>Normal</td>
<td>No lymphadenopathy</td>
<td>3%</td>
<td>Mutated</td>
</tr>
<tr>
<td>CLL 100</td>
<td>70</td>
<td>F</td>
<td>Never</td>
<td>13q14</td>
<td>No lymphadenopathy</td>
<td>18%</td>
<td>NA</td>
</tr>
<tr>
<td>CLL 108</td>
<td>63</td>
<td>M</td>
<td>Never</td>
<td>13q14 and 14q32 rearrangement</td>
<td>No lymphadenopathy</td>
<td>9%</td>
<td>NA</td>
</tr>
<tr>
<td>CLL 164</td>
<td>64</td>
<td>F</td>
<td>12</td>
<td>Normal</td>
<td>No lymphadenopathy</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
Table 2: Expression of the stromal signature 1 and 2 genes in LN-CLL compared to PB-CLL, BM-CLL and normal B-cells in gene microarray analysis. The Overexpressed genes were categorized into high (>2 fold), medium (>1.5 fold) and low (~1.5 fold) higher expressed genes compared to PB-, BM-CLL and normal B-cells.

<table>
<thead>
<tr>
<th>Signature</th>
<th>Overexpressed Genes</th>
<th>Underexpressed Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Medium</td>
</tr>
<tr>
<td>Stromal 1</td>
<td>Apoe</td>
<td>Clu</td>
</tr>
<tr>
<td></td>
<td>Mmp9</td>
<td>Mylk</td>
</tr>
<tr>
<td></td>
<td>Wnt2b</td>
<td>Cxcl14</td>
</tr>
<tr>
<td></td>
<td>Serpinf1 Cox7a1</td>
<td>Parva</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromal 2</td>
<td>Itga9</td>
<td>Saa1</td>
</tr>
<tr>
<td></td>
<td>Cav2</td>
<td>Fabp4</td>
</tr>
<tr>
<td></td>
<td>Ehd2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adra2a Angpl2 Cebpa Cspg2 Cyp27a1 Dlc1 Edil3 Gja1 Islr Maff Pkd2 Ptprf Rab32 Rtn1 Ceecam Fap Itgb2
**Table 3:** Comparative analysis of differential gene expression of genes encoding extra cellular matrix components in Good Prognosis CLL and Poor prognosis CLL using RNA transcriptome analysis. The result shows relative fold change in the mRNA expression of particular genes states the upregulated genes in good prognosis.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Upregulated in Good Prognosis (Relative Fold Change)</th>
<th>q value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAN</td>
<td>Versican Core Protein isoform 3 precursor</td>
<td>50</td>
<td>0.00453231</td>
</tr>
<tr>
<td>SPP1</td>
<td>Osteopontin isoform a precursor</td>
<td>955</td>
<td>0.0463267</td>
</tr>
<tr>
<td>IL8</td>
<td>Interleukin-8 precursor</td>
<td>40</td>
<td>0.00027589</td>
</tr>
<tr>
<td>CD93</td>
<td>Complement component C1q receptor precursor</td>
<td>20</td>
<td>0.0081508</td>
</tr>
<tr>
<td>CXCL1</td>
<td>Growth-regulated alpha protein precursor</td>
<td>77</td>
<td>0.00492674</td>
</tr>
<tr>
<td>CXCL3</td>
<td>C-X-C motif chemokine 3</td>
<td>111</td>
<td>0.00090857</td>
</tr>
<tr>
<td>SERPINE2</td>
<td>Plasminogen activator inhibitor 2 precursor</td>
<td>1271</td>
<td>1.2298E-09</td>
</tr>
</tbody>
</table>
Discussion

In this report, we have studied the nature of expression of stromal signature genes in CLL cells from PB, BM and LNs. CLL cells, particularly in the patient’s body, proliferate and survive for a long time; however, they do not survive long once they are removed from the body, suggesting the role of in vivo microenvironment. Evidence from literature indicates that CLL cell’s inability to survive in vitro is due to lack of complex interactions between CLL cells and the surrounding microenvironment. We and others [1, 2] have shown that the tumor microenvironment (TME) in the LN provides pro-survival/proliferation signals to CLL cells leading to the formation of proliferation centers (PCs) with varied sizes from small to extensively large. It is previously demonstrated that CLL cells at the tissue sites such as LNs induce host immune suppression via differential expression of tolerogenic genes reported earlier in T cell malignancy [17]. Based on our previous studies, we believe that the CLL lymph node induced host immunosuppression significantly contributes to the leukemic progression in CLL patients [2]. However, the precise mechanism of the process and particularly the role played by stromal genes in CLL in the tissue microenvironment is not known. Therefore, the current study was undertaken.

Lenz et al [5] have reported that in the case of diffuse large B cell lymphoma, stromal signature predicted the clinical outcome. In the present study, differentially expressed genes from both stromal I and II signatures were involved in the poor prognosis CLL with LN involvement. For example in the case of one of the overexpressed genes MYLK, a member of the stromal signature I, higher expression of this gene was correlated with poor prognosis. Similarly, CAV1 [2] and CAV2 members of the stromal II signature are overexpressed in CLL cells from LN and correlated with poor clinical outcome in patients. Furthermore, in the current study, the higher expression of
CAV2 is correlated with poor prognosis. In addition, Myosin Light Chain Kinases (MLCKs) are a group of proteins found in smooth muscle and phosphorylates myosin II regulatory light chains at Ser19, allowing myosin cross bridges to bind to actin filaments and initiate contraction [18, 19]. Interestingly, we have recently reported [2] that over expression of CAV1 in CLL cells in the lymph nodes might be involved in inhibiting immune synapse formation via regulating the actin polymerization. This leads us to speculate that MYLK might be involved in the CAV1 mediated inhibition of immune synapse formation of CLL cells in the LNs. Further, in-depth analyses are needed to confirm the role for MYLK in the CAV1 mediated immunosuppression specifically inhibition of immune synapse formation. In this regard, we also see the over expression of CAV2, associated with poor prognosis in CLL, might also be involved in interacting directly with G-protein alpha subunits and thus functionally regulate their activity through phosphorylation of Ser-36 to modulate mitosis in CLL cells. This might be through being a positive regulator of cellular mitogenesis of the MAPK signaling pathway.

Our transcriptome analyses of extracellular matrix associated genes revealed an over expression of VCAN, SPP1 and SERPINB2 in CLL cells from good prognosis patients. Although we do not know the significance of the elevated expression of these genes in CLL biology it is possible that these genes interact with CAV1 and CAV2 in regulating immune response towards CLL, this need to be addressed to understand the role of these overexpression genes.

In summary, differential gene expression of stromal signature I and II highlighted cytoskeleton associated genes like MYLK, CAV1, CAV2 and EHD2 which were significantly upregulated in CLL cells from patient’s LNs. Expression levels of stromal associated genes MYLK, DLC1, WNT2B, EHD2 and CAV2 correlated with clinical
outcome. Thus, our results suggest that STME provides survival signals to CLL cells and facilitates the resistance to therapy which might be leading to leukemic progression.

In addition our transcriptome analyses showed several genes associated with extracellular matrix significantly upregulated in good prognosis. These results lay the foundation for in-depth analyses of these genes to elucidate the functional significance at the mechanistic levels of the differential expression of the relevant genes.

Acknowledgement

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References


Appendix II

Absence of caveolin-1 leads to delayed development of chronic lymphocytic leukemia in Em-TCL1 mouse model


Abstract

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the United States. The tissue microenvironment, specifically the lymph nodes, influences the biological and clinical behavior of CLL cells. Gene expression profiling of CLL cells from peripheral blood, bone marrow, and lymph nodes revealed Cav-1 as one of the genes that might be involved in the pathogenesis of CLL. We have previously reported that the knockdown of Cav-1 in primary CLL cells exhibits a significant decrease in cell migration and immune synapse formation. However, the precise role of Cav-1 in CLL initiation and progression in vivo is not known. Therefore, we decreased the expression of Cav-1 in vivo by breeding Em-TCL1 with cav-1 knockout mice. We observed a significant decrease in the number of CLL cells and rate of proliferation of CLL cells in spleen, liver, and bone marrow from Em-TCL1- Cav1/+ and Em-TCL1-Cav1/ mice as compared with Em-TCL1 mice. In addition, there was a significant increase in survival of Em-TCL1-Cav1/+ and Em-TCL1-Cav1/ compared with Em-TCL1 mice. Mechanistically, we observed a decrease in MAPK-Erk signaling measured by p-Erk levels in Em-TCL1-Cav1/+ mice when compared with Em-TCL1- Cavwt/wt. Together these results indicate that decreased Cav-1 in Em-TCL1 mice significantly delays the onset of CLL and
decreases leukemic progression by inhibiting MAPK-Erk signaling, suggesting a role for Cav-1 in the proliferation and progression of CLL.
INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is a clinically heterogeneous, currently incurable and the most common adult leukemia in the Western world (1,2). Despite advancement in treatment strategies, the overall survival of CLL patients is still about 7 years. Therefore, in order to develop effective therapy we need to understand the molecular basis of clinical heterogeneity. Emerging evidence has convincingly demonstrated that the tumor microenvironment (TME) plays an important role on clinical and biological behavior of CLL (3-8). We have earlier reported that CLL cells in the lymph node (LN) TME have aggressive clinical outcome and express B cell associated proliferation and survival genes including a tolerogenic gene signature (host immune tolerance associated genes) (7). Among the tolerogenic gene signature, Caveolin-1 (Cav-1) is the most highly overexpressed gene in CLL cells from LN compared to peripheral blood (PB) and bone marrow (BM) (3,6).

Earlier we have shown that Cav-1 is indeed associated with the regulation of human CLL cell behavior in vitro (7). Besides being a scaffolding molecule involved in vesicular transport, Cav-1 is also shown to be a multifunctional protein with tumor associated functions such as cellular transformation, tumor growth and progression, multi-drug resistance, angiogenesis, cell migration and metastasis. Cav-1 also functions as an oncogene or tumor suppressor gene depending on the tissue microenvironment (9,10). In a clinical study Burgermeister et al have shown that Cav-1 upregulation is associated with tumor progression and metastasis in different human cancers (11). We have shown earlier that Cav-1 is also involved in the regulation of immunological synapse formation of CLL cells (7). In addition, it has been shown that Cav-1 regulates actin polymerization; these studies indicate a role for Cav-1 in the CLL leukemogenesis and/or progression (12).
Therefore, in this study we crossed *cav-1* null mice with the Eµ-"TCL1" transgenic mice, a murine model for aggressive CLL. The offspring mice were examined for the onset and dissemination of CLL cells in the bone marrow, spleen, lymph node, liver and peripheral blood using antibodies to CD5/CD19/B220 molecules and flow cytometry. Our results have shown that there was a significant decrease in CLL in the Eµ-"TCL1-Cav1"/+ and Eµ-"TCL1-Cav1"/- mice as demonstrated by flow cytometry and a significant decrease in rate of proliferation of CLL cells in spleen as demonstrated by the Ki-67 immunohistochemical analyses. Together these results demonstrate that *cav-1* is involved in the leukemogenesis and progression of CLL.

**METHODS**

**CLL cell isolation from mouse:**

Spleen, bone marrow and lymph nodes were isolated from animals and minced into single cell suspension. Splenocytes and bone marrow cells were incubated in red blood cell (RBC) lysis buffer for 5 minutes at room temperature. After incubation, cells were washed twice with phosphate buffered saline (PBS) to remove lysed RBCs. Following RBC lysis, mouse CLL cells were isolated using negative selection with a mouse B cell isolation kit by Miltenyi Biotech. Purified cells were processed for either RNA isolation or flow cytometric analysis see supplementary figure 1.

**Reverse transcription-PCR:**

Total RNA was extracted and purified from CLL cells using the TRIZol™ method (Invitrogen, Carlsbad, CA). Template cDNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA). To determine the expression levels of selected tolerogenic genes in CLL cells from mice, we performed real-time PCR using a cocktail of 10µl
Power SYBR green master mix (Applied Biosystems, Foster City, CA), a final concentration of 10pm of primer, 2μl of cDNA, and brought to a final volume of 20μl per reaction with water. We assessed the integrity of isolated RNA by mass spectrometry and 0.8% agarose gel electrophoresis. Results were compared to a housekeeping gene, Gapdh. List of primers of all the genes is presented in Table 1 in supplementary data.

**Generation of Eμ-TCL1-Cav1−/− and Eμ-TCL1-Cav1−/+ mice:**

Eμ-TCL1 mice were obtained from Dr. Carlo Croce (Ohio State University, Columbus, OH) and were bred with C57BL/6J (Jackson Laboratories, Bar Harbor, Maine) to establish a colony. Mice were housed at Creighton University Medical Center or the University of Nebraska Medical Center, Omaha, NE. Mice had access to food and water ad libitum in microisolater cages. Eμ-TCL1 mice and wild-type (WT) age-matched controls were sacrificed once transgenic mice appeared to be moribund. Additional mice were euthanized at 6, 12, 24, 36, and 36+ weeks of age to perform time-course analyses of disease progression. Cav-1 knock out (STOCK Cavtm1Mls/J) mouse model was purchased from Jackson Laboratory and bred with Eμ-TCL1 and C57BL/6 to establish a colony. Genotyping was performed to obtain Eμ-TCL1-Cav1−/− and Eμ-TCL1-Cav1−/+.

**Immunophenotyping:**

Cells from Eμ-TCL1, Eμ-TCL1-Cav1−/− and Eμ-TCL1-Cav1−/+ mice from lymph nodes, spleen and bone marrow. Cell suspensions from spleen, BM, and LNs were prepared from Eμ-TCL1 and littermate controls. Cells were blocked with serum containing medium (PBS with 5% fetal bovine serum). Splenocytes were flow sorted for CD5+B220+CD19+ cells. Both CD5+CD19+ and CD5-CD19+ cells were collected for downstream analyses. Cells from spleen, bone marrow and lymph nodes were stained with the following antibodies: CD5 (eBioscience, San Diego, CA), B220 (BD Bioscience,
San Diego, CA) and CD19 (BD Bioscience). All data generated by flow cytometry were analyzed using FlowJo Flow Cytometry Analysis Software version 9.2 (Ashland, OR).

**Immunohistochemistry and H&E staining:**

Liver, spleen, and lymph nodes were collected from each mouse, fixed in 10% buffered formalin for 72 hours, and embedded the next day. Five-micron sections of each tissue were stained with H&E to determine gross histology. Additionally, serial sections were used for immunohistochemistry of these tissues. Briefly, paraffin-embedded samples were incubated in two changes of xylene for 5 minutes each. Next, slides were transferred to 100% alcohol for 2 changes, 3 minutes each, followed by incubation in 90%, 80%, and 70% alcohol for 3 minutes each. Endogenous peroxidase activity was blocked by incubating sections in 3% hydrogen peroxide, then in methanol for 10 minutes and then was rinsed for two changes at 5 minutes each in PBS. Antigen retrieval was performed by boiling the sections in 10mM sodium citrate buffer, pH 6.0, and then slides were allowed to cool for 10-20 minutes. Slides were washed with PBS for two changes at 5 minutes each. Tissues were then incubated with blocking buffer (10% fetal bovine serum in PBS) for 1 hour in a humidified chamber. Appropriate primary antibody (*Ki-67* 1:200 (BD biosciences, San Jose, CA), *Cav-1* 1:100 (Abcam, Cambridge, MA), *p-Erk* 1:200 (Cell signaling technologies, Danvers, MA), CD5 1:150, and B220 1:100) was added and samples were incubated overnight in a humidified chamber at 4°C. Slides were then washed two times with PBS and secondary antibody (goat anti-mouse HRP conjugated, Santa Cruz Biologicals, Santa Cruz, CA) was added and tissues were incubated at room temperature for 4 hours. Tissues were then washed twice more and DAB was added for developing.

**Survival Analyses:**
The survival of these offspring mice was monitored and compared using the Kaplan–Meier method. One event represents death of one mouse; mice were sacrificed upon getting sick. Survival analyzed in the log rank test using sigma plot software (13).

**Statistical Analyses:**

To determine the significance of our results, statistical analysis was performed using student t-test with the p>0.05 was considered significant unless specified. For the in vivo studies, we analyzed at least three mice of each unless specified. There was difficulty in obtaining Eµ-TCL1-Cav1−/− therefore, we have used Eµ-TCL1-Cav1+/− more in our analysis.

**RESULTS:**

The goal of this study was to determine the role of cav-1 in CLL disease progression in vivo by crossing cav1−/− mice with Eµ-TCL1 transgenic mice. These studies yielded the following results.

*Cav-1 expression is significantly increased in mice with CLL.*

We have previously shown the importance of Cav-1 in immune synapse formation, survival and migration in human CLL in vitro (7). However, the role of Cav-1 in tumor microenvironment was not precisely studied. Therefore, to understand the role of tolerogenic signature in tumor microenvironment of mouse CLL, we selected few significantly deregulated genes in human CLL naming Ido1, Tgfbr1, Il22, Cav1, Il2Ra, Zwint, Tgfb, Il10ra and Foxp3 from the gene expression profiling we completed previously. We next looked for their expression in primary CLL cells from lymph nodes of
CLL bearing Eµ-TCL1 mouse model. By performing RT-PCR we found all of the genes except Tgfβ and Tgfβr1 were also significantly overexpressed in CLL cells from Eµ- TCL1 mice (Figure-1). These results demonstrated that the Eµ-TCL1 mouse model possesses a tolerogenic signature similar to human CLL. Therefore, we selected this model to study the role of cav-1 in CLL disease progression.

The expression level of cav-1 increases with disease progression in Eµ-TCL1-Cav1wt/wt mouse model.

CLL is a heterogeneous disease whose clinical outcome varies. In our previous studies we have shown that Cav-1 is significantly overexpressed in CLL cells from poor prognosis CLL patients. The expression of Cav-1 was specifically high in CLL cells from lymph node proliferation centers. Therefore, we wanted to study whether the expression of Cav-1 and two associated genes varies along with the disease progression. To examine that we isolated CLL cells from LNs of Eµ-TCL1 mice at the age of 12, 24 and 36 weeks. For control we used C-57B6 wild type mice. Interestingly, the expression of cav-1 increased 4 fold with the disease progression whereas gene like Prkcb1 shows initial decreased and then increase expression with the disease progression (Figure-2). On the other hand, Ptpn6 shows a decrease in expression along with the disease progression. This establishes the importance of cav-1 in CLL disease progression in Eµ-TCL1 mice. Therefore, the increase in cav-1 expression supported our previous finding showing the importance of cav-1 in human CLL.

Delayed and Decrease in CLL development in Eµ-TCL1-Cav1+/− and Eµ-TCL1-Cav1+/+ mice.

It has already been reported by our group that cav-1 knock down induces spontaneous apoptosis in primary CLL cells (7). Based on this, we wanted to study the
effect of loss of cav-1 in CLL disease progression. The Eµ-TCL1 mouse model starts showing expansion of CLL cells at the age of 24 weeks. As shown previously by Nganga et al., CLL cells in Eµ-TCL1 mouse model are CD19 positive and B220(low)CD5 positive as shown in Figure 3 (A) (14). For control we used aged matched C57B6 mice. In Eµ-TCL1 mice, splenomegaly starts due to CLL cells infiltration and proliferation around at the age of 24 weeks as measured by spleen size, weight and confirmed by flow cytometry (15). To study if the loss of cav-1 in Eµ-TCL1 will delay or decrease the CLL development/progression in Eµ-TCL1 mice we used a cav-1 germ line knockout mouse model. We used the germ line knock out model considering cav-1 is a part of the cytoskeleton as well as stromal signature which might be providing a tumor microenvironment to CLL cells (8). To address this, we crossed cav-1 knockout mice from Jackson’s laboratory with Eµ-TCL1-Cav1<sup>wt/wt</sup> mice kindly provided by Dr. Rene Opavsky at UNMC. Resulting offspring were Eµ-TCL1-Cav1<sup>/+</sup> and Eµ-TCL1-Cav1<sup>−/−</sup> mice. Decrease in Cav-1 expression was measured by IHC of spleen sections from Eµ-TCL1-Cav1<sup>−/−</sup> and Eµ-TCL1-Cav1<sup>wt/wt</sup> animals from 9 month of age Figure 4 A.

In Eµ-TCL1-Cav1<sup>wt/wt</sup> we first observed the expansion of B220 and CD5 positive CLL cells in spleen at 24 weeks of age and at the age of 36 weeks, these mice have fully developed CLL as shown in Figure 3 A&B. At the age of 36 weeks (9 months), Eµ-TCL1-Cav1<sup>wt/wt</sup> gets aggressive CLL with about 75-90% CLL cells in the spleen Figure 3 C. We therefore analyzed all the mice at the age of 36 weeks (age of 9 months). We next examined Eµ-TCL1-Cav1<sup>/+</sup> and Eµ-TCL1-Cav1<sup>−/−</sup> for frequency of CLL cells in the spleen and bone marrow. In Eµ-TCL1-Cav1<sup>/+</sup> and Eµ-TCL1-Cav1<sup>−/−</sup>, there was significant decrease in B220/CD5 positive B cells in Bone Marrow and Spleen see Figure-4 (A). In spleen there were 12% B220+CD5+ cells in Eµ-TCL1-Cav1<sup>−/−</sup> whereas in Eµ-TCL1-Cav1<sup>−/−</sup> there were 23% CLL cells. In bone marrow we observed 6% and 11% CLL cells in Eµ-
TCL1-Cav1\(^{+/−}\) and Eµ-TCL1-Cav1\(^{+/−}\) respectively. Also the spleen size was significantly reduced as shown in Figure 4 B. With regard to spleen size and weight, in the case of Eµ-TCL1-Cav1\(^{−/−}\) mice, we observed varying size and weight ranging from small, medium and larger medium with corresponding frequency of CLL cells as shown in Figure 4 B. To this end we analyzed five Eµ-TCL1, ten Eµ-TCL1-Cav1\(^{−/−}\) and one Eµ-TCL1-Cav1\(^{+/−}\). There was significant reduction in the average number of CLL cells in spleen of Eµ-TCL1-Cav1\(^{−/−}\) and Eµ-TCL1-Cav1\(^{+/−}\) as shown in Figure 4 C. Hence, we concluded that Cav-1 is important for CLL disease progression. In addition we monitored the survival of the offspring mice Eµ-TCL1-Cav1\(^{wt/wt}\) and Eµ-TCL1-Cav1\(^{+/−}\). There was a significant increase in the survival of Eµ-TCL1-Cav1\(^{−/−}\) mice compared to Eµ-TCL1-Cav1\(^{wt/wt}\) mice (p= 0.002) as shown in Figure 4D.

**Decreased CLL cells infiltration and proliferation in cav-1 deleted Eµ-TCL1 mice.**

We have previously shown that Cav-1 is important for CLL cells migration in vitro. Therefore, to study its effect in vivo we analyzed the infiltration of CLL cells in the spleen, lymph nodes, liver, kidney and lungs in these mice. In Eµ-TCL1-Cav1\(^{wt/wt}\) mice, the CLL cell infiltration is in all the organs, specifically liver and spleen therefore, hepatosplenomagly was observed. In contrast, the size of spleen and liver and infiltration of CLL cells was significantly reduced in Eµ-TCL1-Cav1\(^{−/−}\) and Eµ-TCL1-Cav1\(^{+/−}\) mice as shown in Figure 5 A. We also performed immunohistochemistry of continuous spleen sections with B220 and CD5 to ensure presence of CLL cells in these organs Figure 5 B. As discussed earlier, Cav-1 plays an important role in tumor progression therefore we next wanted to study its effect on CLL cells proliferation in splenic CLL cells of these mice. We performed immunohistochemistry of spleen section with Ki-67 stain to measure proliferation of CLL cells in Eµ-TCL1-Cav1\(^{−/−}\) and Eµ-TCL1-Cav1\(^{wt/wt}\) animals from 9 month of age. We observed a significant reduction in proliferating CLL cells in
spleen of Eµ-TCL1-Cav1/− when compared with spleen of Eµ-TCL1-Cav1wt/wt as shown in Figure-5 C. Cav-1 is known to regulate MAPK-Erk signaling by increasing p-Erk levels in several cancers including prostate cancer (16). Therefore, to elucidate the molecular mechanism leading to decrease in CLL cell proliferation we examined the levels of p-Erk in these tissues. We observed significant reduction in the p-Erk levels in spleen of Eµ-TCL1-Cav1/− when compared with spleen of Eµ-TCL1-Cav1wt/wt as shown in Figure-5 D. Therefore, we concluded that deletion of cav-1 in Eµ-TCL1 lead to a decrease in CLL cells proliferation and infiltration with delayed onset of leukemia.
Cav-1 expression is significantly increased in mice with CLL.

Expression of selected genes of the Tolerogenic Signature was measured in CLL cells from lymph nodes of Eµ-TCL1 mice using quantitative real time PCR. The expression levels are expressed as fold change in relative gene expression of Ido-1, Tgfbr1, IL22, Cav-1, IL2Ra, Zwint, Tgfb, IL10ra and Foxp3 in CLL cells from Eµ-TCL1 and in comparison to normal B cells from B57BL-6 mice. GAPDH, a house keeping gene was used for normalizing the expression. N=3 mice, 36 weeks of age; *p≤0.01, #p≤0.05.
The expression level of Cav-1 increases with leukemic progression in Eμ-TCL1-Cav1wt/wt mouse model.

Expression of Cav-1, Prkcb1 and Ptpn6 was measured in CLL cells from Eμ-TCL1 mice during CLL progression using quantitative real time PCR. CLL cells were isolated from LNs of Eμ-TCL1 and C57BL-6 mice at 12, 24 and 36 weeks of age. Expression levels were measured and expressed as fold change in relative expression comparison to normal B cells. GAPDH, a house keeping gene was used for normalizing the expression.
**Figure 3:**

**A.**

Development of CLL in Eµ-TCL1 mice:

To determine the development of CLL, the frequencies of B220\(^{low}\) CD19 CD5 positive CLL cells in spleen and bone marrow of Eµ-TCL1 mice at 12 and 36 weeks of age in comparison with C57BL-6 mice was measured using flow cytometry (Figure 3A). Figure 3 B shows the gain in spleen weight n=3 in each group. Spleen weight of wild type mice
36+ weeks was not done. Figure 3 C shows CD19+CD5+ CLL cells in spleen and bone marrow cells from the 36+ week old E\textsubscript{\mu}-TCL1 mice.
Significantly reduced CLL progression in Eμ-TCL1-Cav1+/mice.

Eμ-TCL1-Cav1wt/wt, Eμ-TCL1-Cav1+/− and Eμ-TCL1-Cav1+/+ mice were euthanized at 9 month of age. Figure 4 A shows the decreased expression of Cav1 in Eμ-TCL1-Cav1+/− and no expression in Cav1−/− mice when compared with Eμ-TCL1-Cav1+/+ mice. CLL cells from spleen and bone marrow were stained with B220 (APC) and CD5 (PE). Figure 4 B shows B220 and CD5 positive cells in spleen and bone marrow in Eμ-TCL1-Cav1+/− and Eμ-TCL1-Cav1+/− comparing with Eμ-TCL1-Cav1wt/wt. Figure 4 C shows the decrease in size of spleen from Eμ-TCL1-Cav1−/+, Eμ-TCL1-Cav1+/− and Eμ-TCL1-Cav1wt/wt 9 months of age mice. Figure 4 D shows the average percentage of CLL cells in spleen of Eμ-TCL1-Cav1wt/wt (n=3), Eμ-TCL1-Cav1+/− (n=10) and Eμ-TCL1-Cav1+/− (n=1). The difference between Eμ-TCL1-Cav1wt/wt and Eμ-TCL1-Cav1+/− is statistically significant with p=0.01. Figure 4 E shows a significant increase in survival time of the Eμ-TCL1-Cav1−/− compared to Eμ-TCL1-Cav1 wt/wt mice as determined by Kaplan Meier curve method and analyzed by the log rank test.
Figure 5:

**Decreased CLL cells infiltration and proliferation in Cav-1 deleted Eµ-TCL1 mice.**

A. Sections of organs of Eµ-TCL1-Cav1<sup>−/−</sup>, Eµ-TCL1-Cav1<sup>−/+</sup> and Eµ-TCL1-Cav1<sup>−/−</sup> animals from 9 month of age. Mice were euthanized at 9 months of age and a part of spleen, and liver were fixed in formaldehyde and later sectioned for staining with hematoxylin and eosin. Photographs were taken at 2.5X, 10X and 20X. Tumor-bearing animals had visually larger lymphoid organs with diffuse lymphocyte infiltration. Additionally, aggressive disease was ensured as we could detect lymphocytes near vessels in the mouse liver and the livers of transgenic animals enlarged significantly.

Figure 5 B shows immunohistochemistry of Eµ-TCL1-Cav1<sup>−/−</sup> and Eµ-TCL1-Cav1<sup>−/−</sup> mice spleen sections showing less CLL cells. Spleen sections of Eµ-TCL1-Cav1<sup>−/−</sup> and Eµ-TCL1-Cav1<sup>−/−</sup> animals from 9 month of age. Mice were euthanized at 9 months of age and a part of spleen were fixed in formaldehyde and later sectioned for staining with
CD5 and B220 antibodies. Photographs were taken at 20X and 40X. A reduced number of B220 and CD5 positive cells were observed in Eμ-TCL1-Cav1/+ when compared with Eμ-TCL1-Cav1/wt/wt mice. Figure 5 C Shows immunohistochemistry staining of Ki-67 stain to measure the proliferating cells in Eμ-TCL1-Cav1/+ and Eμ-TCL1-Cav1/wt/wt animals from 9 month of age.

![Image of immunohistochemistry staining](image)

Figure 5 D shows the immunohistochemistry staining of p-Erk in CLL cells in spleen of Eμ-TCL1-Cav1/+ and Eμ-TCL1-Cav1/wt/wt animals from 9 month of age.
SUPPLEMENTAL DATA

Supplemental Figure 1: Showing the percentage of the CLL cells in lymph nodes after purification used for analyses in Figure 1 and Figure 2.
**Supplemental Table I.** The following table describes the primers used for analysis of tolerogenic genes for this study.

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<tr>
<th>Primer/ Gene Name</th>
<th>Sequence 5’ to 3’ (F/R)</th>
<th>Expected Product</th>
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<td>AKT</td>
<td>CCAACACCTTCATCATCCGC/CACCTCCATCTC TTCAGCCC</td>
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<tr>
<td>BCL2</td>
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<td>129bp</td>
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<tr>
<td>c-MYC</td>
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Discussion:

*Cav-1* regulates a variety of cellular processes in normal and pathologic conditions such as cancer. In this report, we have demonstrated that decreased expression of *cav-1* results in significantly delayed onset, increased survival and reduced the number of CLL cells in tissues such as spleen, bone marrow and liver. Like in other cancers, over expression of *Cav-1* is associated with leukemia progression in CLL as we have seen in Figure 2 that a parallel increase in *cav-1* expression along with CLL progresses in *Eμ-TCL1* murine model for CLL (17-19). In this regard our study opens up avenues to explore and elucidate the precise role(s) played by *cav-1* in the pathogenesis of CLL.

Multifunctional *Cav-1*, a well-studied molecule for its scaffolding function, is also involved in the regulation of initiation and progression of cancers including CLL. Some of these *Cav-1* functions are mediated via its binding partners such as *Prkcb1* and *Ptpn6* as there is an inverse relationship between the expression of *cav-1* and *Ptpn6/Prkcb1* expression in CLL (Figure 2).

Our data represents the first report on the definitive role for *cav-1* in the leukemogenesis and progression of CLL using appropriate murine models. Interestingly, decreased *Cav-1* expression in these mice produced different results in *Eμ-TCL1-Cav1*−/− versus *Eμ-TCL1-Cav1*+/− mice. Varying degree of CLL infiltrations created varying spleen size/weight, which was observed in the *Eμ-TCL1-Cav1*+/− mice. The reason for this we do not know at this time. However, additional studies at the molecular levels will be required to address this question.

We have earlier reported that the knocking down of *Cav-1* using siRNA in primary CLL cells resulted in the significant decrease in immune synapse formation by the CLL cells (7). At this time, we have not observed any gross alteration in the morphology of the
lymphoid tissues in these offspring resulted from crossing $E\mu\cdot TCI1$ and $cav1^{-/}$ mice. Although alterations in the immune functions in the $cav-1^{-/}$ mice have been reported (20-23), in this study we did not perform analyses of immune function in these mice. Cav-1 has been shown to regulate the cell migration; possibly via regulating the actin polymerization (24-28). The decreased infiltration of CLL in the offspring might be due to altered migration of CLL cells or it could be due to general decrease in leukemic progression. Mechanistically, we observed that decrease in $cav-1$ leads to decrease in p-Erk expression in CLL cells from spleen. As MAPK-Erk signaling is critical for CLL cell proliferation and migration, we reasoned MAPK-Erk signaling to be the major target of $cav-1$ in these mice. Due to decrease in Erk signaling we observed reduction in CLL cell proliferation and delayed in disease progression in these mice leading to better survival. However, the precise mechanism through which $cav-1$ regulates p-Erk levels still remains unclear.

In this study we have not evaluated the normal B cell development particularly the B1 cells, the precursors of CLL in mouse. However, Bai et al (2014) have reported that frequencies of B cells was significantly decreased in $cav-1$ deficient mice as determined by the hematopoietic reconstitution method using $cav-1$ deficient bone marrow transplantation into their wild type counterpart animals (29). In this regard in our study we have observed a decrease in CLL cell frequency and delayed onset of leukemia in $E\mu\cdot TCI1\cdot Cav1^{-/-}$ mice. This means pan decrease in B cells might not be the main reason for the reduced severity and delayed onset of CLL in the offspring mice.

In CLL cells from lymph nodes, the Cav-1 expression is significantly high compared to CLL cells from the bone marrow and peripheral blood (6, 7). We also have shown that CLL cells in the lymph nodes have more aggressive phenotype compared to CLL cells from the other tissue site. The molecular basis of lymph node associated
aggressiveness of CLL and increased expression of Cav-1 in lymph node CLL cells is not known at this time (6).

In order to understand the precise role of Cav-1 in the pathogenesis of CLL, our results warrant further studies exploring the additional proteins/molecules that interact with Cav-1 in regulating the CLL biology. In this regard, it would be interesting to study the activity of the Pkrcb1 or Pten in these mice offspring.

Overall, our study showed that Cav-1 play a key role in the initiation and progression of CLL and decrease expression of Cav-1 in Eµ-TCL1 mice leads to delayed CLL development by decreasing p-Erk signaling.

**Acknowledgements:** We want to thank the department of Genetics, Cell Biology and Anatomy for the pilot project grant. University of Nebraska, College of Medicine, Dean's Research and Development funds.

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