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

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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 process 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.

Keywords: Stromal tumor microenvironment; Stromal gene signature; CLL

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 nurse-like 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 tolerized 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.

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Methods

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 patients are provided in tables 1a-1c.

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. The 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 1d.

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

Table 1A: Peripheral Blood CLL patient samples information.

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Patients ID	Age	Gender	Time to treatment	Fluorescence in-situ Hybridization	Bulky disease	CD 38%	IgVH Mutation status
LN11	59	M	1	TRISOMY 12 (47%); 14q32 (25%), then 12% n 13% after that 17p del	Yes, diffuse abdominal, axilla, groin, neck LN	80%	Unmutated
LN34A	74	M	2	11q23 from 13qdel	BALA	63%	NA
LN34B	74	M	2	11q23 from 13qdel	BALA	63%	NA
LN40A	49	M	1	13q14 (68.5%),both11.5%,null 13.5%, then 17p13 in LN;	BALA	92%	Unmutated
LN40B	49	M	1	13q14 (68.5%),both11.5%,null 13.5%, then 17p13 in LN;	BALA	92%	Unmutated
LN59	62	F	108	NA	Lymphadenopathy		NA
LN64	72	F	48	17p13 *	Lymphadenopathy		NA
LN66A	61	F	11	11q22.3/ATM (78%), 13q14 (89%), 17p13.1 (3 copies in 46.3%)	BALA	84%	Unmutated
LN66B	61	F	11	11q22.3/ATM (78%), 13q14 (89%), 17p13.1 (3 copies in 46.3%)	BALA	84%	Unmutated
LN67	42	M	1	11q23, 13q14	BALA	21%	Unmutated
LN82	79	M	4	Trisomy 12 , 13q14 del	BALA	92%	Unmutated
LN83A	29	M	22	11q23 deletion (92%), 14q32 (64.5%)	BALA	98%	Unmutated
LN83B	29	M	23	11q23 deletion (92%), 14q32 (64.5%)	BALA	98%	Unmutated
LN93	50	M	1	14q-	BALA	52%	NA
LN149	41	M	6	17p- (11%), 13q- (33%), 14q- (12%)	LN (neck)	8%	NA

Table 1B: Lymph Nodes CLL patient samples information.

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

Table 1C: Bone marrow CLL patient samples information.

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

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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 and 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

Patients ID	Age	Gender	Time to treatment	Fluorescence in-situ Hybridization	Bulky disease	CD 38%	IgVH Mutation status
CLL 3	61	F	98	TRISOMY 12 (65.5%)	No lymphadenopathy	83%	Unmutated
CLL 11	59	M	1	TRISOMY 12 (47%); 14q32 (25%), then 12% n 13% after that 17p del	Yes, diffuse abdominal, axilla, groin, neck LN	80%	Unmutated
CLL 29	55	F	69	11q23- (91%);13q14 mono (95.5%),13q14(60%); 14q32 (65.5%)	BALA	17%	Unmutated
CLL 30	55	F	12	Trisomy 12 (70.5%)	Lymphadenopathy	55%	Unmutated
CLL 34	74	M	2	11q23 from 13qdel	BALA	63%	NA
CLL 40	49	M	1	13q14 (68.5%),both11.5%,null 13.5%, then 17p13 in LN;	BALA	92%	Unmutated
CLL 79	62	M	15	11q22.3 (78%), 13q14 (53.5%), null13q14 (34%)	No lymphadenopathy	23%	Unmutated
CLL 82	79	M	4	Trisomy 12 , 13q14 del	BALA	92%	Unmutated
CLL10	49	F	Never	Normal	No lymphadenopathy	6%	Mutated
CLL 13	83	M	1	Normal	No lymphadenopathy	NA	NA
CLL 19	56	F	Never	13q14-	No lymphadenopathy	NA	NA
CLL 75	34	M	96	Normal	No lymphadenopathy	3%	Mutated
CLL 100	70	F	Never	13q14	No lymphadenopathy	18%	NA
CLL 108	63	M	Never	13q14 and 14q32 rearrangement	No lymphadenopathy	9%	NA
CLL 164	64	F	12	Normal	No lymphadenopathy	NA	NA

Table 1D: Patients Clinical information used for Transcriptome.

Signature	Overexpressed Genes			Under expressed Genes	
	High	Medium	Low		
Stromal 1	Apoe Clu Lum Mmp9 Mylk Robo1 Wnt2b Cxcl14 Pcolce Serpinf1 Cox7a1	Aebp1 Col4a4 Cyr61 Fbn1 Lama4 Parva Ptgds Evc	Adamdec1 Cald1 Chn1 Ctgf Ctsk Dzip1 Edg2 Itgb5 Ppa2b Rarres1 Sparc	Adra2a Angpl2 Cebpa Cspg2 Cyp27a1 Dlc1 Edil3 Gja1 Islr Maff	Pkd2 Ptprf Rab32 Rtn1 Ceecam Fap Itgb2
Stromal 2	Itga9 Saa1 Cav1 Cav2 Fabp4 Ced6 Ehd2	Col5a3 Lamb1 Prrg1 Sh3d5 Pa2ga Sparcl1 Aqp1		Sema4c Dlc1 Sdf-1 Procr Apm1 Kdr Itga6 Rbp4	

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.

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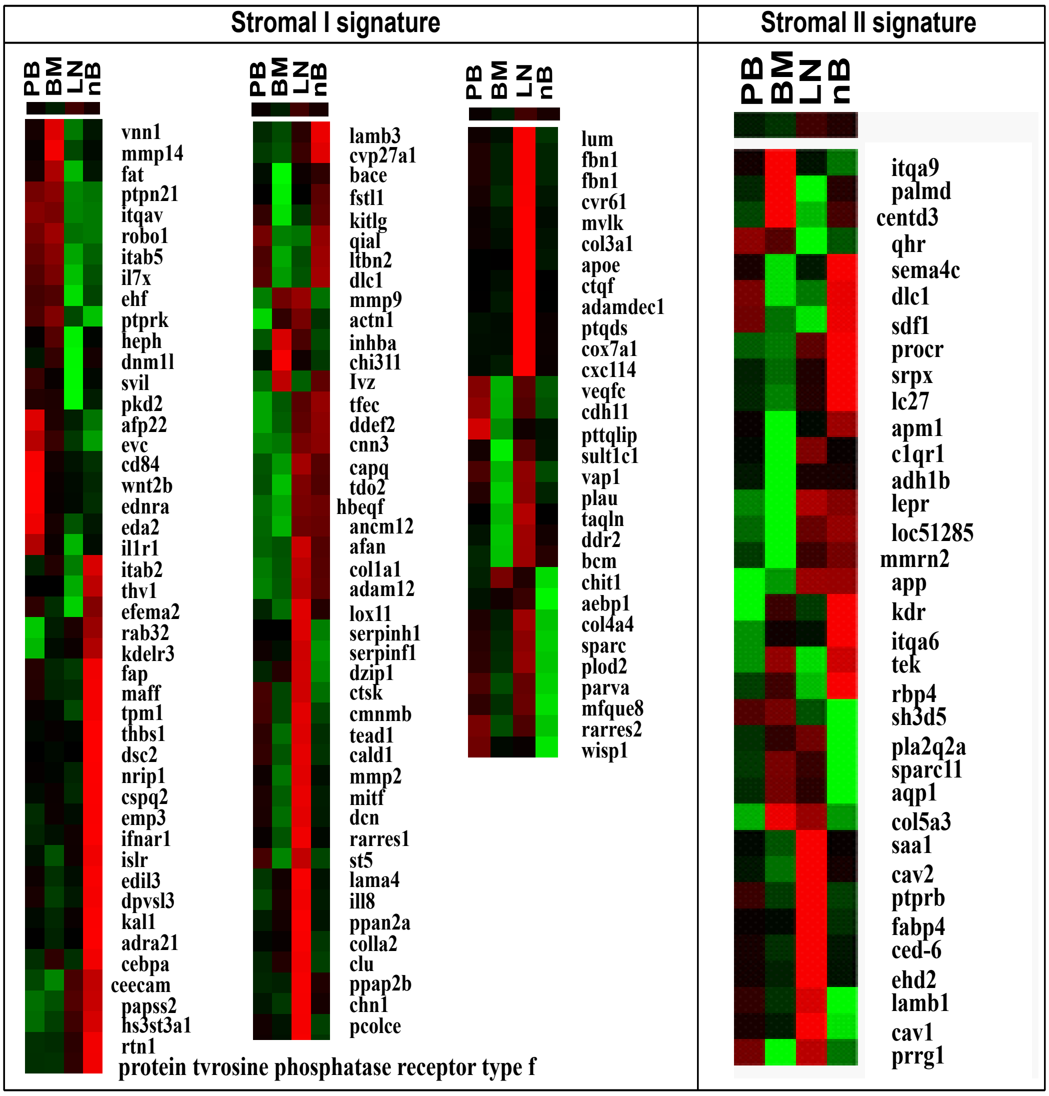

Figure 1: Differential expression of stromal signature I and II associated genes in primary CLL cells: GEP showing differential expression of Stromal Signature I & II in cells isolated from peripheral blood (PB), bone marrow (BM) and lymph nodes (LN) from CLL patients in comparison with normal B cells 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.

suppressor [15], was under expressed in both stromal signatures I and 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 (Figures 2A-2D). 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-2G). 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 2I).

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

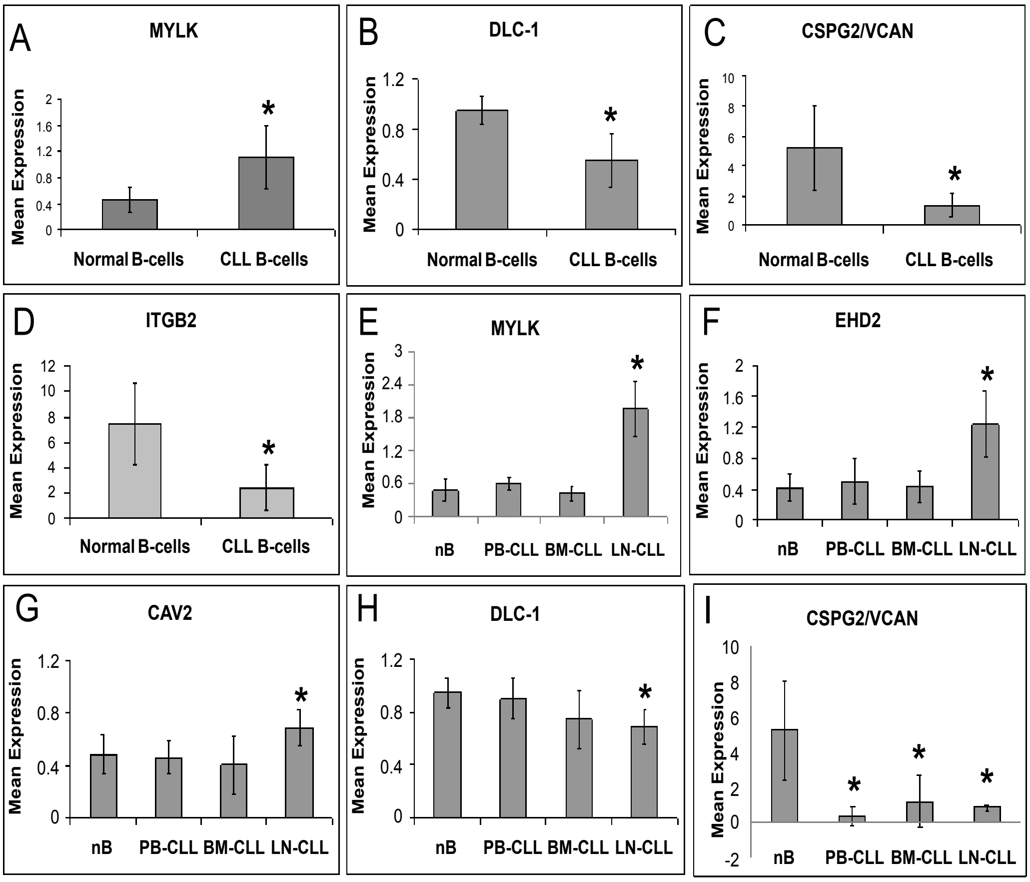

Figure 2: Expression levels of stromal genes in CLL cells from PB, BM and LN: Comparison of mRNA expression of (A) myosin light kinase (MYLK), (B) versican (VCAN), (C) deleted in liver cancer (DLC1), (D) Integrin, Beta 2 (ITGB2) in PB isolated B cells from 8 healthy donors and 20 CLL patients. mRNA expression 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.

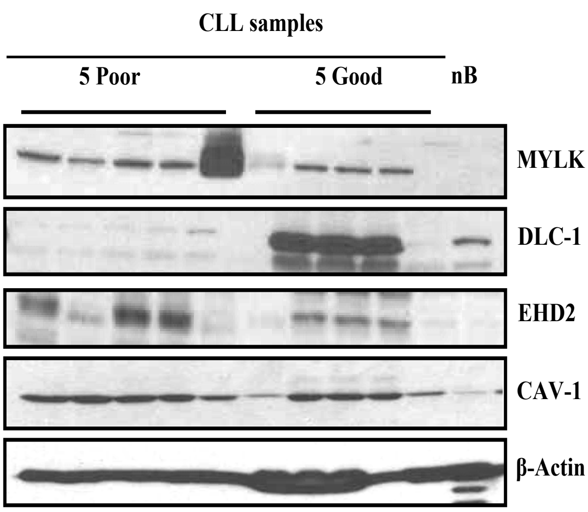

Figure 3: Analysis for protein expression: Expression of selected proteins in normal B and CLL cells from PB of 5 good prognosis and 5 poor prognosis patients using western blotting.

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) significantly correlated with poor clinical outcome. Recently Yamasaki et al., showed higher expression of CAV2 promotes cell proliferation,

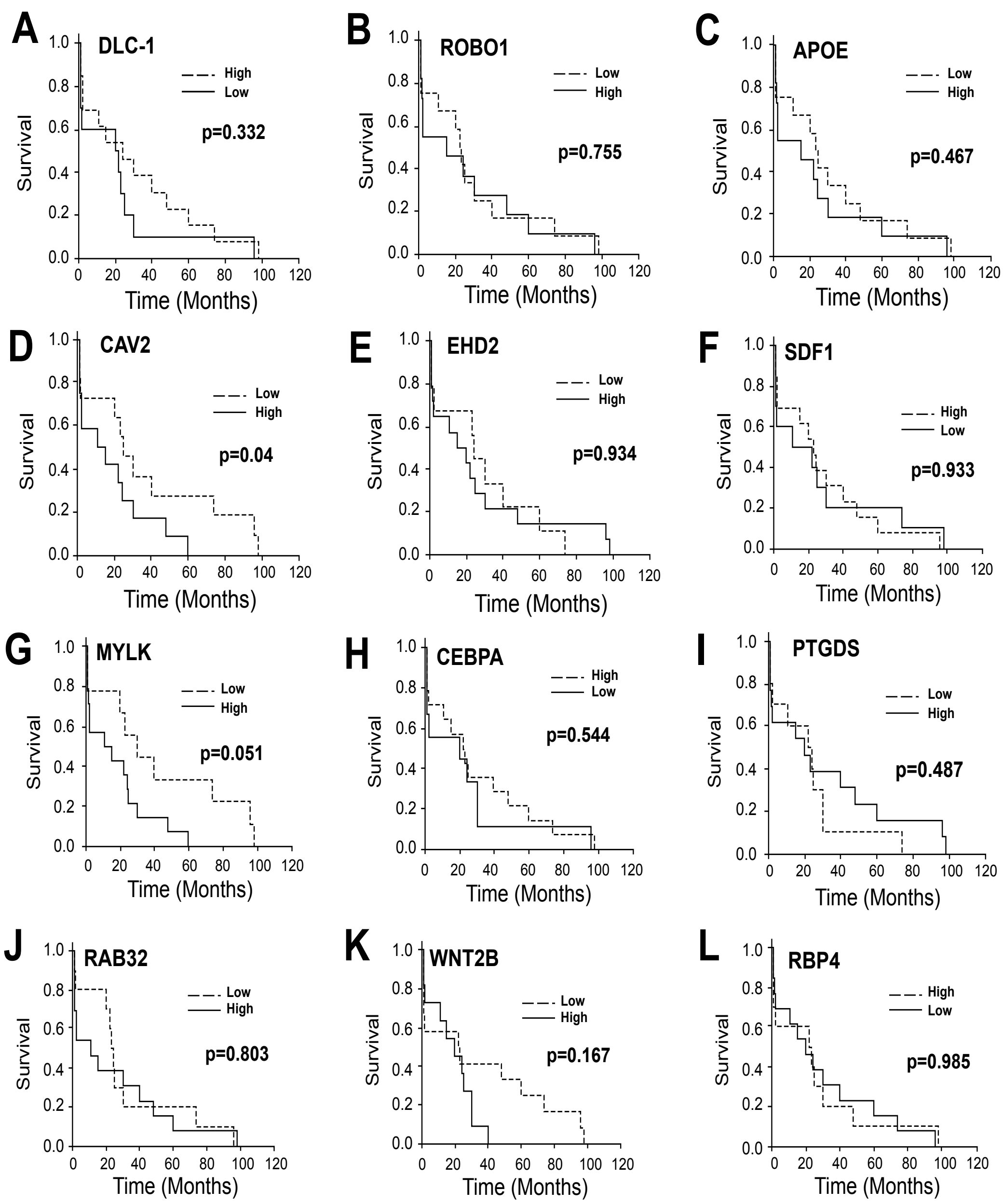

Figure 4: Clinical significance of stromal genes: Clinical correlation of expression levels of stromal signature I associated genes (CEBPA, MYLK, APOE, RAB32, PTGDS and WNT2B) and stromal signature II associated genes (DLC1, CAV2, EHD2, SDF1, RBP4 and ROBO1) in CLL patients using Kaplan Meier analyses 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.

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). 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 (Table 2) 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.

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 (Table 3) of stromal signature I and II highlighted cytoskeleton associated genes like MYLK, CAV1, CAV2 and EHD2 which were significantly upregulated in CLL

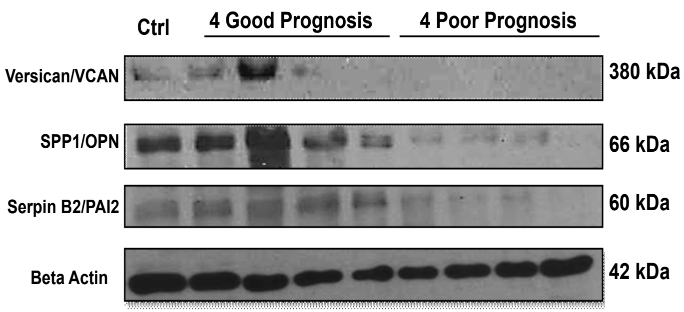

Figure 5: Validation of differentially expressed extracellular matrix associated genes in good and poor prognosis CLL samples using analyses for protein expression: Expression of selected extracellular matrix associated proteins in CLL cells from PB of 4 good prognosis and 4 poor prognosis patients using western blotting method.

Gene Symbol	Gene Name	Upregulated in Good Prognosis (Relative Fold Change)	q value
VCAN	Versican Core Protein isoform 3 precursor	50	0.00453231
SPP1	Osteopontin isoform a precursor	955	0.0463267
IL8	Interleukin-8 precursor	40	0.00027589
CD93	Complement component C1q receptor precursor	20	0.0081508
CXCL1	Growth-regulated alpha protein precursor	77	0.00492674
CXCL3	C-X-C motif chemokine 3	111	0.00090857
SERPINB2	Plasminogen activator inhibitor 2 precursor	1271	1.2298E-09

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.

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.

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