Gene expression profiling reveals differences in microenvironment interaction between patients with chronic lymphocytic leukemia expressing high versus low ZAP70 mRNA

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ABSTRACT

Background

Zeta-associated protein 70 (ZAP70) is a widely recognized prognostic factor in chronic lymphocytic leukemia, but mechanisms by which its higher expression leads to a poor outcome must still be fully explained.

Design and Methods

In an attempt to unveil unfavorable cellular properties linked to high ZAP70 expression, we used gene expression profiling to identify genes associated with disparities in B cells from chronic lymphocytic leukemia patients expressing high versus low ZAP70 mRNA, measured by quantitative real-time PCR. Two groups of 7 patients were compared, selected on the basis of either high or low ZAP70 mRNA expression.

Results

Twenty-seven genes were differentially expressed with an FDR<10%, and several genes were significant predictors of treatment-free survival (TFS) and/or overall survival; PDE8A and FCRL family genes (down-regulated in ZAP70$^+$ patients) could predict TFS and overall survival; ITGA4 mRNA (up-regulated in ZAP70$^+$ patients) could significantly predict overall survival. Importantly, gene set enrichment analysis revealed overrepresentation of adhesion/migration genes. We therefore investigated *in vitro* adhesion/migration capacity of chronic lymphocytic leukemia cells into a stromal microenvironment or in response to conditioned medium. We showed that ZAP70$^+$ cells had better adhesion/migration capacities and only ZAP70$^+$ patient cells responded to microenvironment contact by CXCR4 downregulation.

Conclusions

We concluded that several prognostic factors are the reflection of microenvironment interactions and that the increased adhesion/migratory capacity of ZAP70$^+$ cells in their microenvironment can explain their better survival and thus the aggressiveness of the disease.

Key words: chronic lymphocytic leukemia, ZAP70, microarrays, microenvironment, prognosis.

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The online version of this article contains a supplementary appendix.

Introduction

Gene expression profile is a powerful tool to better understand the biology, the clinical outcome and molecular mechanisms implicated in chronic lymphocytic leukemia (CLL).[1,2] This disease, characterized by the accumulation of monoclonal CD5$^+$ B cells, displays an extremely variable clinical course with overall survival times ranging from months to decades. A plethora of prognostic factors classifying patients into poor or good predicted outcomes have been found in the last ten years: CD38 antigen expression,[3] microRNA expression,[4] cytogenetic aberrations,[5] mutational status of the immunoglobulin variable heavy chain region gene (IgVH)[6] and its surrogate markers ZAP70 (ζ-associated protein 70)[1] and LPL (lipoprotein lipase).[7]

ZAP70 expression in CLL correlates strongly with IgVH mutation status[8] and seems to be one of the most promising prognostic factors for future clinical use. In 2003, Wiestner *et al*. found a 93% correlation between mutational status and ZAP70:[1] patients with 20% of their B cells expressing ZAP70 by flow cytometry (FC) (i.e. at the same level as T cells) were generally unmutated; on the contrary, patients with less than 20% of their B cells expressing ZAP70 generally had mutated IgVH. ZAP70 status determination by FC, however, is often inaccurate at the positivity limit because of the low resolution between the positive (+) and negative (–) populations due to the gating procedure and the choice of antibody.[9] To offset these drawbacks, ZAP70 mRNA absolute quantification has been proposed. In a previous study, we showed that ZAP70 mRNA expression was highly correlated with IgVH mutational status, in addition to survival and treatment-free time.[10] Moreover, we and others suggested that ZAP70 could be an even better prognostic factor than mutational status.[10-12] We therefore used real-time RT-PCR quantification (qPCR) to select patients with high and low ZAP70 expression and compared their gene expression profiles in order to assess the biological significance of ZAP70 expression in CLL. We hypothesized that a gene expression profile comparison of these two CLL subsets' widely differing ZAP70 expression would reveal gene expression patterns associated not only with prognosis, but also with biology, particularly with regard to microenvironment interactions implicated in the survival of leukemic cells, a relevant aspect of the disease.

Design and Methods

Patients, sample collection, and RNA extraction

This study, approved by the Bordet Institute Ethics Committee, was based on peripheral blood samples obtained from CLL patients with informed consent and presenting a typical CD19$^+$CD5$^+$CD23$^+$ phenotype. Microarray and prognostication studies were based on CLL cells frozen at diagnosis before any treatment. For functional studies such as migration and adhesion assays, CLL cells of untreated patients or patients who had received no treatment for at least six months were used. The characteristics of patients included in gene expression comparison are summarized in Table 1. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation over Linfosep (Biomedics, Madrid, Spain). B cells were purified with a CD19$^+$ magnetic-bead system

Table 1. Characteristics of patients with low and high ZAP70 expression.

Patients					Mut. status[1,2]	VH homology (%)	VH gene	ZAP70 by qPCR		ZAP70 by FC		LPL qRT-PCR		CD38 by FC		Cytogenetic abnormalities[6]
Num	Group	Sex	Age	Stage				qPCR[3]	Status	% cell[4]	Status	qPCR[3]	Status	% cell[5]	Status	
1	High	F	69	A	UM	100.0	VH5-51	1507.7	+	66	+	142.9	+	58.0	+	del(11q)
2	High	M	68	C	UM	100.0	VH1-46	1475.8	+	41	+	3386.8	+	56.0	+	trisomy 12
3	High	F	57	B	UM	98.0	VH3-48	1179.2	+	57	+	1.8	–	50.0	+	trisomy 12
4	High	F	67	A	M	96.2	VH4-34	1137.0	+	39	+	0.1	–	68.0	+	del (6q)
5	High	M	61	C	UM	99.0	VH6-1	1074.7	+	22	+	247	+	14.0	+	del(6q)
6	High	F	74	C	UM	100.0	VH1-8	650.3	+	42	+	309.8	+	23.0	+	del (17p)
7	High	M	73	B	UM	100	VH1-69	643.9	+	77	+	447.8		38	+	del(13q) + complex karyotype
8	Low	F	67	A	M	94.2	VH3-74	61.8	–	2	–	0.1	–	94.0	+	normal
9	Low	F	68	A	M	95.2	VH4-39	50.4	–	2.5	–	0	–	0.0	–	del(13q)
10	Low	F	59	B	M	93.9	VH1-2	18.9	–	14	–	1.7	–	0.0	–	normal
11	Low	M	67	B	M	92.2	VH2-26	14.2	–	1	–	62.4	+	58.0	+	del(6q)
12	Low	M	72	B	M	95.2	VH4-34	9.5	–	3	–	1.9	–	0.0	–	del(6q)
13	Low	M	77	A	M	97.7	VH1-69	7.5	–	12	–	0.1	–	24.0	+	del(13q)
14	Low	M	69	A	M	94.8	VH3-9	2.7	–	10	–	0.3	–	1.0	–	normal

[1]Mutational status is based on a 98% cut-off value; [2]UM, unmutated; M, mutated; [3]The cut-off determined using ROC curve analysis is expressed in fold of target gene expression in the calibrator cell line; [4]The cut-off of 20% of CD19+ cells that express ZAP70 by flow cytometry, at the same level as T cell, was used; [5]The cut-off of 7% of CD19$^+$/CD38$^+$ cells was used as determined by ROC curve analysis; [6]Cytogenetic abnormalities were investigated by conventional karyotype analysis and CLL FISH panel detecting del(17p), del(11q), del(6q), del(13q) and trisomy 12.

(MidiMACS, Miltenyi Biotec, Bergish Gladbash, Germany), according to the manufacturer's instructions. Mean B-cell purity was >98% as measured by FC. Total RNA was extracted from purified CD19+ cells in a single step using TriPure Isolation Reagent (Roche Applied Science, Vilvoorde, Belgium).

Gene expression profile

Microarray analysis was performed using 1.5 μg of RNA with Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array, which contained more than 54,000 probe sets for analysis of about 47,000 transcripts (Affymetrix, High Wycombe, United Kingdom). Amplification, hybridization, and scanning were done according to standard Affymetrix protocols (*www.affymetrix.com*) (*see also Online Supplementary Appendix*).

Bioinformatic analysis

A comparative gene expression profile was determined in 14 patients (7 ZAP70^{high} and 7 ZAP70^{low} patients). We identified significant differences between sample groups using BRB array tools (Biometric Research Branch, National Cancer Institute). Only genes defined as *present* by the Affymetrix algorithm in at least 30% of either of the two groups were considered for further analysis, and we calculated two-sample *t* tests (with a random variance model) of the two groups for each gene. We then addressed the multiple comparison problems by estimating the false discovery rate (FDR) in a simple manner as the ratio of expected number of false positives at this given *p* value threshold to the number of positives actually found. Using BRB gene set expression comparison tools, overrepresentation of gene ontology (GO) categories, Biocarta, KEGG, and Broad/MIT pathways were investigated by the Hotelling *T*-square test.

Flow cytometry analysis, IgVH gene mutational and cytogenetic abnormalities determination

Cytoplasmic ZAP70 protein by FC and IgVH mutational status were performed as previously described[10] (*see also Online Supplementary Appendix*). We evaluated the expression of CD38, CXCR4 (chemokine (C-X-C motif) receptor 4 or CD184)) and CD69 on the cell surface by FC in a CD19+ gate with a panel of fluorochrome-labeled monoclonal antibodies (phycoerythrin-conjugated, Immunosource). Classical cytogenetic abnormalities by standard karyotype analysis were investigated for the 14 patients included in gene expression profile analysis. Additional interphase FISH was performed to screen for most common aberrations using Chromoprobe Multiprobe® - CLL System (Cytocell, Amplitech, Compiegne, France).

Real-time PCR analysis

We used 25 ng of cDNA (produced by a standard reverse transcription) in a qPCR reaction with SYBR® Green PCR Master Mix (Applied Biosystems, Rotterdam, The Netherlands) and 0.32 μM of gene-specific forward and reverse primers (Invitrogen Life technologies, Merelbeke, Belgium). Primer sequences are listed in the *Online Supplementary Table S1*. Standard real-time PCR was performed as previously described[10] (*Online Supplementary Appendix*).

Co-culture of mesenchymal stromal cells and chronic lymphocytic leukemia cells

MSCs were generated from bone marrow (BM) aspirates of normal volunteers after obtaining informed consent, as previously described.[13] Passage one or two MSCs were used for the co-culture experiment. In order to study the influence of soluble factors produced by MSCs, conditioned serum-free medium (CM) from the culture of 1-day-old MSCs was prepared. Peripheral blood mononuclear cells from CLL ZAP70 + and - patients and also from healthy donors were resuspended in RPMI-1640 + 10% FBS (Biowhittaker, Verviers, Belgium) at a final concentration of 10^6/mL; 2 mL of cell suspension was placed in the wells of a 6-well plate or seeded on MSCs in identical plates. In parallel experiments, cells were seeded onto 24 mm Transwell diffusion chambers (0.4 μm microporous filter; Corning Incorporated, NY, USA) and placed into stroma-coated 6-well plates. ZAP70, CD69, and CXCR4 were all evaluated by FC in CLL cells cultured alone, with, and without contact with stromal cells. Moreover, in the case of contact with stromal cells, we separated cells in suspension in the medium from cells adhering to the stromal microenvironment in order to measure the above described markers. We also measured CXCR4 expression in fresh CLL blood samples from ZAP70 + and - patients.

Adhesion and migration assay

CLL cell suspensions were incubated in fibronectin (BDbioscience, Erembodegem, Belgium) precoated 24-well plates. After four hours of incubation, adherent cells were collected by trypsinization, concentrated, and counted by a Trypan blue exclusion assay (Invitrogen): 10^6 cells were also resuspended in 200 μL of medium and plated in the upper chamber of a 6.6 mm diameter Transwell culture insert in bare polycarbonate with a 5 μm pore size (Corning Incorporated, NY, USA). The lower chamber of each well contained 500 μL of medium alone, with SDF1α (stromal cell-derived factor 1 α also known as CXCL12 - chemokine (C-X-C motif) ligand 12) (200 ng/mL) (R&D Systems, Minneapolis, MN, USA) or MSC-CM. After four hours of incubation, cells were recovered from the lower chamber, concentrated and counted using a Trypan blue exclusion assay. Migration index was calculated as number of cells transmigrating in the presence of the chemoattractant per number of cells transmigrated in absence of the chemoattractant. In the cells found in the lower chamber, ZAP70 was measured by FC in order to compare its expression in migrating versus non-migrating cells in response to MSC-CM.

Statistical analysis

ROC curve analyses were performed to determine the "+" and "–" status cut-offs (for all studied variables) that best distinguished between + and – ZAP70 cases. Treatment-free survival (TFS) and overall survival (OS) distributions were plotted using Kaplan-Meier estimates and were compared using the log-rank test. Significant differences were evaluated using the Wilcoxon matched pairs or Mann Whitney test. All tests were two-sided. An effect was considered to be statistically significant at $p<0.05$, and all analyses were performed with Prism GraphPad 5.0 software.

Results

Patient selection

In our previous study, ZAP70 was measured by qPCR in a 108 patient cohort.[10] Based on these data, two groups of 7 patients were chosen from the top-20 patients expressing the highest or lowest levels of ZAP70 mRNA, after checking the yield and quality of RNA. The median TFS of the ZAP70^{high} group was 12.1 months, while this value reached 172.3 months in the ZAP70^{low} group [p=0.0133; $\chi^2(1)$=6.132]. Moreover, the median OS was also significantly different [p=0.0018; $\chi^2(1)$=9.701]. ZAP70 average expression assessed by qPCR was 23.57±8.71 in the low group and 1096±131.50 in the high group (p<0.0001). These results were confirmed in a patient cohort of 85 patients with a median follow-up of 74 months (range 8-299 months) (Figure 1A and B). This cohort was derived from the 108 cohort patient cohort previously published[10] for which enough RNA was available. The characteristics of these patients are stated in the *Online Supplementary Table S2*. ZAP70 expression was expressed as fold change of ZAP70 in the Namalwa cell line and normalized with cyclophilin expression. Patients expressing ZAP70 with a fold change above 114 were deemed "+".

ZAP70-associated genes and validation of single targets

Gene expression profile revealed 937 probe sets differentially expressed between the two prementioned groups (p<0.05) with a fold change of 1.5-fold (increase or decrease), and a p<0.001 allowed us to identify a list of 135 probe sets that were differentially expressed (*Online Supplementary Table S3*).

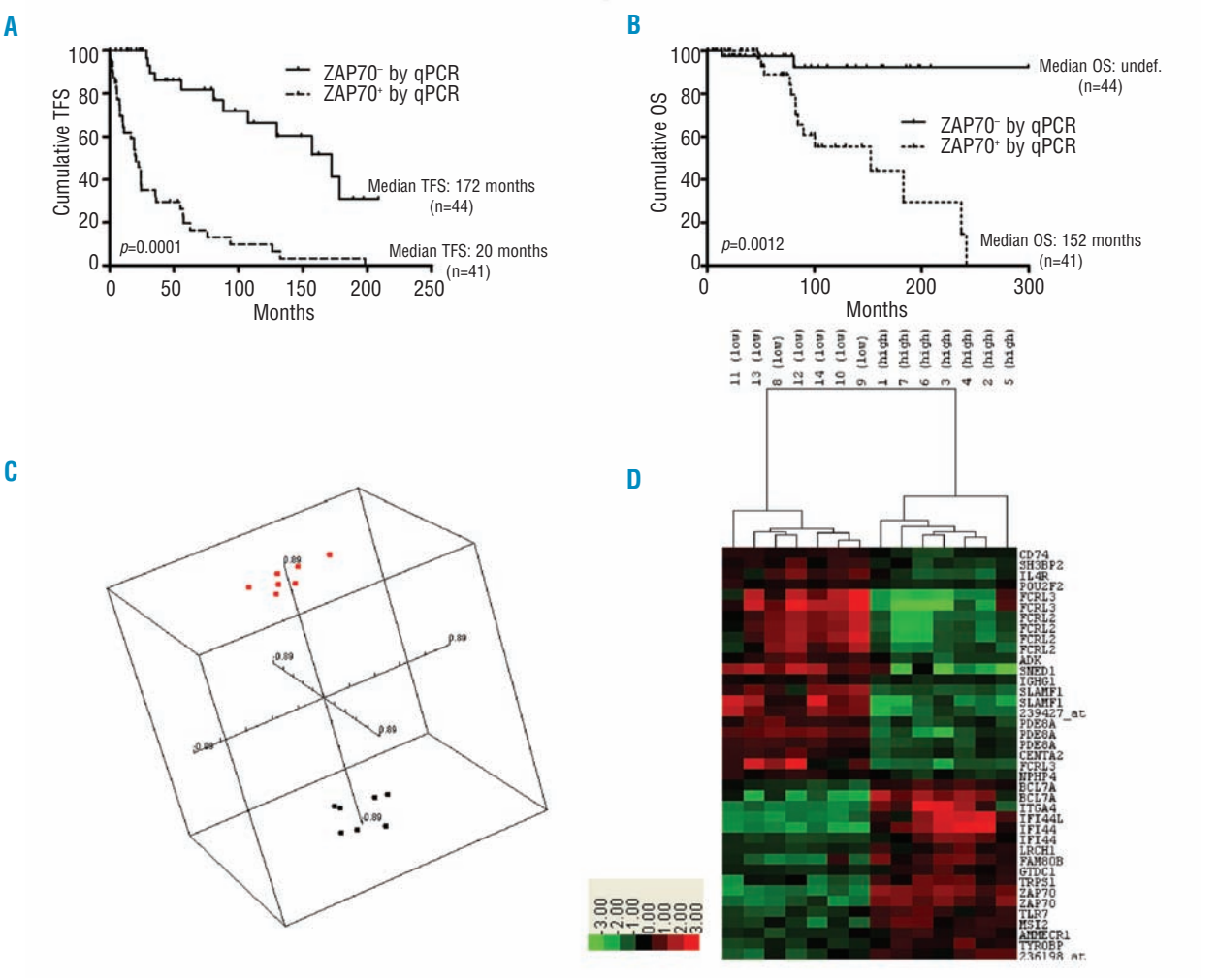


Figure 1. Disease progression and underlying gene expression in chronic lymphocytic leukemia patients with high versus low ZAP70 expression. ZAP70 expression was expressed as fold change of ZAP70 in the Namalwa cell line and normalized with cyclophilin expression. Patients expressing ZAP70 more than 114 fold were deemed "+" (n=41) while patients expressing ZAP70 with less than 114-fold were deemed "–" (n=44). (A) Treatment-free survival as a function of ZAP70 measured by qPCR. (B) Overall Survival as a function of ZAP70 measured by qPCR plotted using Kaplan-Meier analysis and compared using the log-rank test. (C) Multidimensional scaling of samples using the ZAP70 gene expression signature (n=39, FDR<10%). Red samples represent ZAP70^{high} and black samples represent ZAP70^{low} patients. (D) Hierarchical clustering of 39 probe sets discriminated between ZAP70^{high} and ZAP70^{low} chronic lymphocytic leukemia samples. Color scale indicates units of standard deviation from the mean expression of each gene.

After applying an FDR, we found only 39 probe sets representing 27 different genes with an FDR ≤10% (Figure 1D). Of these, 13 genes were up-regulated and 14 were down-regulated in the ZAP70^{high} group. The two ZAP70 probe sets were, not surprisingly, at the top of this list with an FDR<10^{-7}. As expected, a multidimensional scaling of samples as well as a hierarchical clustering using this ZAP70-specific gene signature exhibited a clear separation of ZAP70^{high} and ZAP70^{low} patients (Figure 1C and D).

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE12734(*http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12734*). In order to validate this pattern, a panel of six genes in the list of 27 (with FDR<10%), five genes among the list of 135 probe sets (with p<0.001) and two genes in the list of 937 probe sets (p<0.05) were selected for qPCR validation on CD19^{+} purified samples in an extended cohort of 85 patients with a median follow-up of 74 months (range 8-299 months). These 13 randomly chosen genes were all confirmed as differentially expressed (Table 2, *Online Supplementary Figure S1*).

Genes associated with clinical outcome

qPCR data were analyzed for correlation with TFS and OS by Kaplan-Meier analysis, using a cut-off determined by ROC analysis, and optimizing the concordance with ZAP70 status. We calculated gene expression as a fold change of the target gene in the Namalwa cell line and normalized with cyclophilin expression. All cut-offs for each investigated gene are shown in Table 2. Some of the analyzed genes could significantly predict TFS (*TLR7, LPL*), OS (*ITGA4*) or both (FCRL family, *PDE8A, PCDH9, CTLA4, MYBL1*) (Table 2, *Online Supplementary Figure S1*).

Gene set expression comparison reveals interaction differences with the microenvironment

Using BRB array tools, we performed gene set enrichment analysis investigating gene GO categories, in addition to Biocarta, KEGG, and Broad/MIT pathways that had a higher than expected number of genes differentially

Table 2. Real-time PCR validation of selected genes differentially expressed between ZAP70 + and - patients.

Symbol	Gene description	Differences between ZAP70^{+} (n=41) and ZAP70- (n=44) cases			Cut-off[2]	Target gene status[3]	n	Number of ZAP70+ patients	Treatment-free survival (TFS)		Overall survival (OS)	
		p	Mann Witney U	Fold Change[1]					p	Median TFS	p	Median OS[4]
PDE8A	Phosphodiesterase 8A	p<0.0001	422.5	-4.3	6.815	– +	62 23	39 2	0.0003	35 157	0.012	183 UD
FCRL1	Fc receptor-like 1	0.0004	483	-1.9	67.330	– +	39 46	27 14	0.0292	24.13 93.67	0.0015	152.5 UD
FCRL2	Fc receptor-like 2	p<0.0001	450	-1.8	26.650	– +	22 63	21 20	p<0.0001	20.27 93.67	0.001	100.5 UD
FCRL3	Fc receptor-like 3	p<0.0001	422	-3.1	186.2	– +	43 42	10 31	0.0066	24.2 107.2	0.0038	152.5 UD
FCRL5	Fc receptor-like 5	p<0.0001	304	-2.6	13.230	– +	49 36	30 11	0.0006	22.3 107.2	0.0232	183 UD
ITGA4	Integrin, α 4 (antigen CD49D, α4 subunit of VLA-4 receptor)	0.0102	605.5	1.3	0.085	– +	54 31	7 34	0.3558	80.47 57	0.0075	241.9 183
TLR7	Toll-like receptor 7	0.0002	472	2.0	6.610	– +	22 63	21 20	0.0263	88.07 30.07	0.0992	UD 152.5
LPL	Lipoprotein lipase	p<0.0001	381	11.8	6.000	– +	42 43	11 30	0.0031	126 24.1	0.1593	241.9 237.1
CLEC2B	C-type lectin domain family 2, member B	p<0.0001	386.5	5.5	0.530	– +	34 51	14 27	0.0849	80.47 29.33	0.1838	UD 237.1
PCDH9	Protocadherin 9	0.0044	577.5	2.6	10.00	– +	47 38	11 30	0.0266	126 29.33	0.0194	UD 183
BCL7A	B-cell CLL/lymphoma 7A	0.0004	497.5	5.6	0.300	– +	32 53	17 24	0.0754	88.07 24.13	0.0576	251.9 237.1
CTLA4	Cytotoxic T-lymphocyte -associated protein 4	0.0308	707	-2.3	419187	– +	61 24	36 5	0.0087	35.5 157	0.0157	183 UD
MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	p<0.0001	354	-6.6	1.240	– +	55 30	35 6	0.0005	29.9 172.27	0.0154	183 UD

[1]Positive values represent an upregulation and negative values a downregulation in ZAP70+ patients; [2]Cut-offs were calculated using ROC curve analysis maximising the concordance with ZAP70 status and are expressed as a fold change of the target gene in the Namalwa cell line and normalized with cyclophilin expression; [3]Optimal cut-offs were determined using ROC curve analysis in order to maximize the concordance with ZAP70 status; [4]UD, undefined.

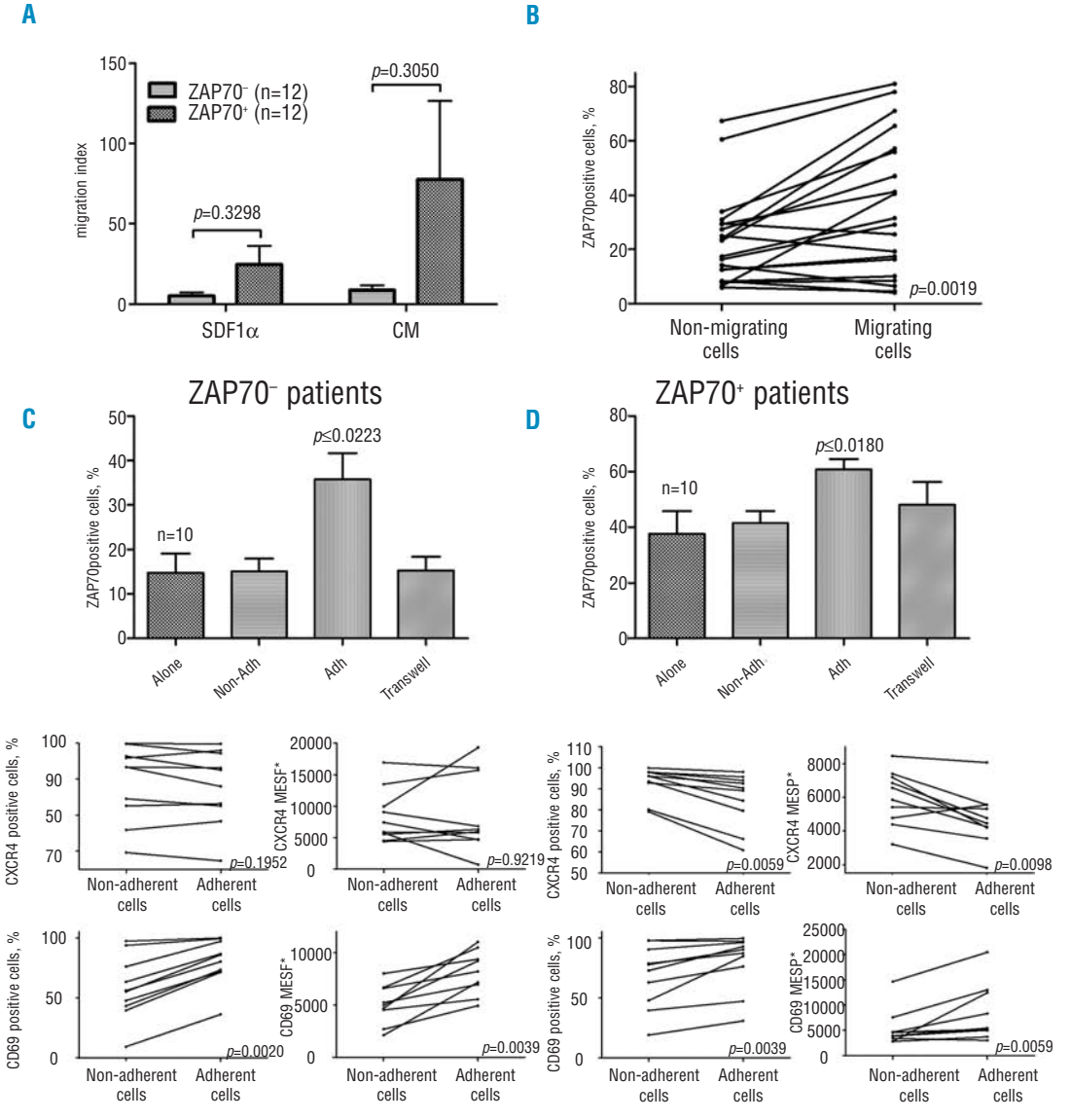
expressed between our two classes of samples using Hotelling *T*-square test. In other words, among differentially expressed genes we examined whether an enrichment/abnormal rate of genes involved in a same pathway/function is overrepresented in comparison with what is expected by hazard.

Interestingly, several GO categories/pathways involved in migration, motility, adhesion, cytoskeleton, actin modification, and microenvironment interaction (via the CXCR4 pathway) were significantly over-represented (Table 3). Additional details on involved genes are available in the *Online Supplementary Table S4.*

B cells from ZAP70⁺ patients adhere better to fibronectin and ZAP70⁺ cells show higher migration capacity in response to stromal cell conditioned medium

After subtraction of spontaneous plastic adhesion, we demonstrated that cells isolated from ZAP70⁺ patients (n=12) adhered to fibronectin 2.7 fold more than cells from ZAP70⁻ patients (n=12) (p=0.0110).

Migration in response to SDF1α was very low in comparison to MSC-CM (p<0.0001). However, no significant difference in terms of migration was found between ZAP70⁺ (n=12) and - (n=12) patients (Figure 2A) but there was a clear trend towards greater migration in cells from ZAP70⁺ patients. We also measured and compared ZAP70



*MESF: Molecules of Equivalent Soluble Fluorochrome

Figure 2. Migration of chronic lymphocytic leukemia cells in response to SDF1α and MSC conditioned medium and modulation of ZAP70, CXCR4 and CD69 in response to the stromal microenvironment. (A) Results are expressed as the mean of migration index in response to SDF1α or MSC conditioned medium (CM). (B) ZAP70 measured by FC in the upper and lower migration chamber. Chronic lymphocytic leukemia cells from ZAP70– (C) and + (D) patients were co-plated either with stromal cells, with stromal cells separated by a 0.45 μm transwell not allowing contact between the two cell types, or alone. After 4 h of incubation, ZAP70, CXCR4 and CD69 were measured by FC on adherent and non-adherent cells. Furthermore, ZAP70 was measured on chronic lymphocytic leukemia cells cultured alone and in transwell conditions.

expression using FC in cells from the upper and lower chamber: the number of ZAP70$^+$ cells was significantly increased in the migrating cell population (n=20; p=0.0019) (Figure 2B). We verified that ZAP70 was not induced by CM (*data not shown*), indicating that this difference was linked to migrating cells.

ZAP70, CXCR4 and CD69 are modulated by stromal cell contact

FC analyses revealed that MSC-adherent cells were significantly enriched with ZAP70$^+$ cells, indicating that ZAP70$^+$ cells had better adhesion/migration capacity. This remained true in ZAP70 - (n=10, p≤0.0223) and + (n=10, p≤0.0180) patient cells but also in normal B cells isolated from healthy donors (n=10, p≤0.0020; *Online Supplementary Figure S2*). Moreover, there was no statistical difference between ZAP70 expression among non-adherent cells, cells separated by a transwell, or cells cultured in medium (Figure 2C and D, *Online Supplementary Figure S2*). Although CXCR4 mRNA was not differentially expressed between our two groups, CXCR4 signaling pathway genes were over-represented. Therefore, we also measured CXCR4, the receptor for SDF1α, on adherent B cells. Very interestingly, CXCR4 cell surface expression between adherent and non-adherent cells was only significantly down-regulated in ZAP70$^+$ patients (% of cells: p=0.0059; Molecules of Equivalent Soluble Fluorochrome (MESF): p=0.0098), but not in ZAP70$^-$ patients (% of cells: p=0.1952; MESF: p=0.9219) (Figure 2C and D). Furthermore, when CXCR4 was measured in 54 fresh CLL blood samples (29 ZAP70$^-$ and 25 ZAP70$^+$), ZAP70$^+$ patients expressed significantly less CXCR4 molecules per cell (p=0.0183). The characteristics of these patients are stated in the *Online Supplementary Table* S5. We also

Table 3. Over-represented gene list between ZAP70^{high} and ZAP70^{low} patients.

GO categories	Description	*p* value
GO0015629	Actin cytoskeleton	0.0021
GO0030036	Actin cytoskeleton organization and biogenesis	0.0027
GO0030029	Actin filament-based process	0.0014
GO0008154	Actin polymerization and/or depolimerization	0.0010
GO0007155	Cell adhesion	8.10^{-7}
GO0016337	Cell-cell adhesion	0.0053
GO0007160	Cell-matrix adhesion	0.0003
GO0006935	Chemotaxis	0.0023
GO0005856	Cytoskeleton	0.0002
GO0007010	Cytoskeleton organization and biogenesis	0.0009
GO0040011	Locomotion	0.0060
GO0005874	Microtubule	0.0003
GO0007018	Microtubule based movement	0.0108
Kegg Pathway		
hsa04514	Cell adhesion molecules (CAMs)	0.0020
hsa04530	Tight junction	$2.6.10^{-7}$
hsa04520	Adherens junction	$2.5.10^{-6}$
hsa04540	Gap junction	$3.9.10^{-5}$
hsa04670	Leukocyte transendothelial migration	$<10^{-7}$
hsa04810	Regulation of actin cytoskeleton	$<10^{-7}$
hsa04510	Focal adhesion	$<10^{-7}$
Broad Pathway		
SIG_CHEMOTAXIS_	SignalingAlliance	0.0002
cell_adhesion_receptor_activity_h	Combining with cell adhesion molecules to initiate a change in cell activity	0.0121
SIG_Regulation_of_the_actin _cytoskeleton_by_Rho_GTPases_h	SignalingAlliance Mediates the adhesion of the cell to other cells or to the extracellular matrix.	0.0069
cell_adhesion_molecule_activity_h	Any process involved in the controlled movement of a cell.	0.0011
cell_motility_h	The attachment of a cell, either to another cell or to the extracellular matrix,	0.000
cell_adhesion_h	Via cell adhesion molecules.	0.0004
h_ST_Integrin_Signaling_Pathway	Signaling Transduction KE	$1.9 . 10^{-5}$
Biocarta Pathways		
h_lympathway	Adhesion and diapedis of lymphocytes	0.0012
h_integrinPathway	Integrin Signaling Pathway	0.0015
h_lymphocytePathway	Adhesion Molecules on Lymphocyte	0.0012
h_cxcr4Pathway	CXCR4 Signaling Pathway	0.0006

observed that CD69 mRNA was significantly increased in ZAP70+ patients (*Online Supplementary Table S2*) and that CD69 protein was up-regulated after stromal cell contact (Figure 2C and 2D).

Discussion

ZAP70 is a powerful prognostic factor confirmed by several studies.[8,10,11,14,15] However, little is known about the underlying molecular mechanisms in which ZAP70 is involved or its prognostic value. Therefore, we investigated the transcriptome of CLL cells presenting high or low ZAP70 expression, employing Affymetrix technology in order to identify a molecular signature explaining the different clinical outcomes of these groups. Preview studies already investigated gene expression profile comparison of CLL subsets with different prognosis. Firstly, different signatures have been proposed according to IgVH mutational status and obtained independently of ZAP70 expression.[16,17] Our signature shared some overlapping genes with them[17,18] but all these signatures are globally different from ours. Indeed, our patient selection considerably increased the number of ZAP70-linked genes and thus ZAP70-linked pathways. It is also notable that previous signatures did not describe a difference in migration/adhesion pathways. Secondly, Schroers *et al*. in 2005, and Hüttmann *et al.* in 2006, compared transcriptome of CLL subgroups based on the combination of ZAP70 and CD38 expression (both measured by flow cytometry).[19,20] Schroers *et al*. found interferon-stimulated genes as differentially expressed between $ZAP70^{+}CD38^{+}$ (poor prognosis) and $ZAP70^{-}CD38^{-}$ (good prognosis) subgroups indicating that T cells constituting the normal microenvironment could also influence CLL cell survival. Based on the same subset comparison, microarray study of Hüttmann *et al*. underlined genes implicated in BCR pathway (over-expressed in poor prognosis patient). These results are in line with the better responsiveness of $ZAP70^{+}$ cells to external microenvironment stimuli that we observed. Unlike these previously published microarray studies, the present study employed a patient selection approach based on ZAP70 quantification using qPCR in a cohort of 108 patients. Here, we show for the first time that $ZAP70^{high}$ and $ZAP70^{low}$ patients display a distinct gene expression profile, composed of 27 genes differentially expressed with an FDR<10%. This signature can clearly separate $ZAP70^{high}$ and $ZAP70^{low}$ patients as illustrated by a multidimensional scaling analysis. Several of these genes were confirmed by qPCR in an extended cohort of 85 patients and were subsequently investigated for their TFS and OS prognostic power. Several of them could predict TFS, OS or both and were independently confirmed by other studies (LPL,[7] FCRL2,[21] CTLA4,[22] ITGA4).[23] Furthermore, other genes found in our study (which we did not confirm by qPCR) have also been proposed as prognostic markers in the literature (CD38,[3] CD69).[24]

More interestingly, in addition to their prognostic value, the expression of these factors is linked to microenvironment crosstalk. LPL expression could be induced by B-cell receptor (BCR) stimulation in CLL cells but not in normal B cells.[25] Several studies have shown significantly higher LPL expression in IgVH-unmutated patients[7,10,26] which is associated with autoreactive BCR activity.[27] Taken together, our data strongly suggest an important role of continual stimulation by the tumoral microenvironment in patients with a poor prognosis. Fc receptor-like (FCRL) genes (also known as FcRH, IRTA, IFGP, SPAP) were up-regulated in ZAP70- patients. These genes belong to a large family of lymphocyte receptors with immunoreceptor tyrosine–based inhibition motifs (ITIM).[28,29] These ITIMs can be phosphorylated following external stimuli and can subsequently recruit phosphatases. They can therefore have an inhibitory role in BCR[30] or in MAPK signaling.[31] Stimuli given by the microenvironment could confer growth advantage and extended survival to leukemic cells.[32] Moreover, BCR stimulation is linked to cell survival, activation, and G1 progression.[33] All these finding are in line with higher expression of FCRL genes in ZAP70- patients, with potential ITIM inhibitory pathways explaining their lower response to external survival stimuli, which would be associated with a good prognosis. Finally, we also showed that CD69 (recently proposed as a strong predictor of CLL prognosis)[24] could be up-regulated while CXCR4 could be down-regulated by microenvironment contact. Similarly, Ocana *et al*. demonstrated that low CXCR3 was associated with Rai disease stages III and IV[34] and recently Ding *et al*. showed that CD38 expression (in CLL cells with evidence for CD38 expression) was up-regulated after two weeks in contact with MSCs. In contrast, CD38 expression remained unchanged in cells with minimal CD38 expression after co-culture with MSC.[35] Considering all these data, we can tentatively conclude that expression of markers such as *ZAP70, LPL, CD38, CD69, FCRL, CXCR4*, and *CXCR3* are probably linked to the microenvironment, and classification of patients into poor or good prognosis groups with regard to these factors seems to be a reflection of microenvironment interactions.

Gene set enrichment analysis revealed several pathways and GO categories linked to migration, motility, adhesion, cytoskeleton, actin modification, and microenvironment interaction (via the CXCR4 pathway). These results are in line with findings by Deaglio *et al*. who reported a genetic signature based simply on migration index and independent of molecular factors.[36] This signature is composed of genes involved in the control of cell motility, adhesion, cell-cell contact and cytoskeletal organization and also included *ZAP70* indicating that *ZAP70* is linked to migration, thus reinforcing our hypothesis. The microenvironment is considered to be crucial in determining expansion[37] and survival[38] of CLL cells. Therefore, we investigated the effects of microenvironment in a BM-MSC model, which we previously established.[38] We showed that $ZAP70^{+}$ cells whether from $ZAP70^{+}$ or ZAP70- patients or normal B cells isolated from healthy donors adhered significantly more to MSCs, indicating that ZAP70 played an important role in the chemotaxis and/or the adhesion process, even in normal B cells. Moreover, ITGA4 is significantly over-expressed on cells from $ZAP70^{+}$ patients. Interestingly, this gene has been implicated in migration into lymph nodes[39] and is modulated by SDF1α.[40] In CLL, the interaction of ITGA4 with

fibronectin enhances BCL2 expression[41] and influences chemosusceptibility.[42] Furthermore, our qPCR data indicated that ITGA could significantly predict OS (p=0.0075).

Surprisingly, we and others[36] did not find significant differences in cell migration in response to SDF1α between cells from ZAP70 + and - patients. However, we observed that ZAP70$^+$ cells migrated significantly more than ZAP70$^-$ cells when we compared the upper and lower chamber cells in our migration assay. Although there were no significant migratory differences between ZAP70 + and - patients, there was a clear trend towards increased migration of cells from ZAP70$^+$ patients in response to MSC-CM. The better responsiveness of ZAP70$^+$ cells to their microenvironment that we observed is in line with the observation of Richardson *et al*. who showed that ZAP70 identifies cells with an increased propensity to migrate to the lymph node and an increased ability to respond to survival signals.[43] Moreover, we showed that only ZAP70$^+$ patients respond to microenvironment contact by a downmodulation of CXCR4 (probably by protein internalization, as previously described)[44] indicating once more that only ZAP70$^+$ patients could respond to microenvironment stimuli and suggesting an association between aggressiveness of the disease and chemotaxis towards the microenvironment via the SDF1α/CXCR4 pathway. Furthermore, the role of ZAP70 in migration has already been demonstrated in T cells.[45,46] Indeed, expression of wild type ZAP70 in ZAP70-deficient cells (P116 neo) strongly enhanced its migratory capability and this remains true even in absence of CD3 complex surface expression and thus of functional TCRζ subunits as is the case in B cells.[46] Tacchini *et al*. also showed that SDF1α induces a rapid and transient tyrosine phosphorylation of ZAP70, suggesting that this chemokine can directly activate ZAP70. We could thus speculate that, in CLL cells, microenvironment stimuli could transitorily activate ZAP70 resulting in downstream cascade phosphorylation. Stimulation by the *SDF1α/CXCR4* pathway could activate ZAP70-dependent genes (such as Vav1[46] which contributes to signal amplification and diversification events)[47] leading to actin cytoskeleton changes and thus chemotaxis.

In conclusion, our data demonstrated that in ZAP70$^+$ patients, leukemic cells can better interact and cross-talk with their protective microenvironment, explaining their increased survival and the aggressiveness of the disease.

Authorship and Disclosures

BS performed research and statistical analysis, analyzed data, made figures and tables, and wrote manuscript. BH-K performed and revised statistical analysis. CE contributed to microarray experiments. AS performed real-time PCR confirmations. CDB contributed to flow cytometry experiments. DH. initiated project and performed research. NM, DB and PM contributed to patient samples and data. LL supervised and designed research, and revised manuscript.

The authors declare no competing financial interests.

References

1. Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, Henrickson SE et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 2003;101:4944-51.
2. Bilban M, Heintel D, Scharl T, Woelfel T, Auer MM, Porpaczy E, et al. Deregulated expression of fat and muscle genes in B-cell chronic lymphocytic leukemia with high lipoprotein lipase expression. Leukemia 2006;20:1080-8.
3. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999;94:1840-7.
4. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 2005;353:1793-801.
5. Dohner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med 2000;343:1910-6.
6. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999;94:1848-54.
7. Van Bockstaele F, Pede V, Janssens A, Callewaert F, Offner F, Verhasselt B, et al. Lipoprotein lipase mRNA expression in whole blood is a prognostic marker in B cell chronic lymphocytic leukemia. Clin Chem 2007;53:204-12.
8. Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 2003;348:1764-75.
9. Catherwood MA, Matthews C, Niblock R, Dobbin E, Morris TC, Alexander HD. ZAP-70 mRNA quantification in B-cell chronic lymphocytic leukaemia. Eur J Haematol 2006;76:294-8.
10. Stamatopoulos B, Meuleman N, Haibe-Kains B, Duvillier H, Massy M, Martiat P, et al. Quantification of ZAP70 mRNA in B cells by real-time PCR is a powerful prognostic factor in chronic lymphocytic leukemia. Clin Chem 2007;53:1757-66.
11. Del Principe MI, Del Poeta G, Buccisano F, Maurillo L, Venditti A, Zucchetto A, et al. Clinical significance of ZAP-70 protein expression in B-cell chronic lymphocytic leukemia. Blood 2006;108:853-61.
12. Rassenti LZ, Huynh L, Toy TL, Chen L, Keating MJ, Gribben JG, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. N Engl J Med 2004; 351:893-901.
13. Tondreau T, Meuleman N, Delforge A, Dejeneffe M, Leroy R, Massy M, et al. Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. Stem Cells 2005;23:1105-12.
14. Orchard JA, Ibbotson RE, Davis Z, Wiestner A, Rosenwald A, Thomas PW, et al. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. Lancet 2004;363:105-11.
15. Durig J, Nuckel H, Cremer M, Fuhrer A, Halfmeyer K, Fandrey J, et al. ZAP-70 expression is a prognostic factor in chronic lymphocytic leukemia. Leukemia 2003;17:2426-34.
16. Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H, et al. Gene expression profiling of B cell chronic lymphocytic leukemia

reveals a homogeneous phenotype related to memory B cells. J Exp Med 2001;194:1625-38.
17. Haslinger C, Schweifer N, Stilgenbauer S, Dohner H, Lichter P, Kraut N, et al. Microarray gene expression profiling of B-cell chronic lymphocytic leukemia subgroups defined by genomic aberrations and VH mutation status. J Clin Oncol 2004;22:3937-49.
18. Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X, et al. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med 2001;194:1639-47.
19. Schroers R, Griesinger F, Trumper L, Haase D, Kulle B, Klein-Hitpass L, et al. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. Leukemia 2005;19:750-8.
20. Huttmann A, Klein-Hitpass L, Thomale J, Deenen R, Carpinteiro A, Nuckel H, et al. Gene expression signatures separate B-cell chronic lymphocytic leukaemia prognostic subgroups defined by ZAP-70 and CD38 expression status. Leukemia 2006;20: 1774-82.
21. Li FJ, Ding S, Pan J, Shakhmatov MA, Kashentseva E, Wu J, et al. FCRL2 expression predicts IGHV mutation status and clinical progression in chronic lymphocytic leukemia. Blood 2008;112:179-87.
22. Joshi AD, Hegde GV, Dickinson JD, Mittal AK, Lynch JC, Eudy JD, et al. ATM, CTLA4, MNDA, and HEM1 in high versus low CD38 expressing B-cell chronic lymphocytic leukemia. Clin Cancer Res 2007;13:5295-304.
23. Gattei V, Bulian P, Del Principe MI, Zucchetto A, Maurillo L, Buccisano F, et al. Relevance of CD49d protein expression as overall survival and progressive disease prognosticator in chronic lymphocytic leukemia. Blood 2008;111:865-73.
24. Del Poeta G, Del Principe MI, Luciano F, Maurillo L, Buccisano F, Catalano G, et al. High CD69 protein expression predicts a poor prognosis in B-cell chronic lymphocytic leukemia (B-CLL). 49th American Society of Hematology (ASH) meeting, Altanta, Georgie, USA. 2007. [Abstract].
25. Pallasch CP, Schwamb J, Konigs S, Schulz A, Debey S, Kofler D, et al. Targeting lipid metabolism by the lipoprotein lipase inhibitor orlistat results in apoptosis of B-cell chronic lymphocytic leukemia cells. Leukemia 2008;22:585-92.
26. Heintel D, Kienle D, Shehata M, Krober A, Kroemer E, Schwarzinger I, et al. High expression of lipoprotein lipase in poor risk B-cell chronic lymphocytic leukemia. Leukemia 2005;19:1216-23.
27. Herve M, Xu K, Ng YS, Wardemann H, Albesiano E, Messmer BT, et al. Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. J Clin Invest 2005;115:1636-43.
28. Ehrhardt GR, Leu CM, Zhang S, Aksu G, Jackson T, Haga C, et al. Fc receptor-like proteins (FCRL): immunomodulators of B cell function. Adv Exp Med Biol 2007;596: 155-62.
29. Miller I, Hatzivassiliou G, Cattoretti G, Mendelsohn C, Dalla-Favera R. IRTAs: a new family of immunoglobulinlike receptors differentially expressed in B cells. Blood 2002;99: 2662-9.
30. Xu MJ, Zhao R, Zhao ZJ. Molecular cloning and characterization of SPAP1, an inhibitory receptor. Biochem Biophys Res Commun 2001;280:768-75.
31. Rodriguez A, Villuendas R, Yanez L, Gomez ME, Diaz R, Pollan M, et al. Molecular heterogeneity in chronic lymphocytic leukemia is dependent on BCR signaling: clinical correlation. Leukemia 2007;21:1984-91.
32. Ghia P, Circosta P, Scielzo C, Vallario A, Camporeale A, Granziero L, et al. Differential effects on CLL cell survival exerted by different microenvironmental elements. Curr Top Microbiol Immunol 2005;294:135-45.
33. Deglesne PA, Chevallier N, Letestu R, Baran-Marszak F, Beitar T, Salanoubat C, et al. Survival response to B-cell receptor ligation is restricted to progressive chronic lymphocytic leukemia cells irrespective of Zap70 expression. Cancer Res 2006;66:7158-66.
34. Ocana E, Delgado-Perez L, Campos-Caro A, Munoz J, Paz A, Franco R, et al. The prognostic role of CXCR3 expression by chronic lymphocytic leukemia B cells. Haematologica 2007;92:349-56.
35. Ding W, Nowakowski G, Abrahamzon JL, Wellik LE, Ghosh AK, Secreto C, et al. Crosstalk between Chronic Lymphocytic Leukemia (CLL) B-Cells and Marrow Stromal Cells: Implication for CLL B-Cell Activation and Survival. 49th American Society of Hematology (ASH) meeting, Altanta, Georgie, USA. [Abstract].
36. Deaglio S, Vaisitti T, Aydin S, Bergui L, D'Arena G, Bonello L, et al. CD38 and ZAP-70 are functionally linked and mark CLL cells with high migratory potential. Blood 2007;110:4012-21.
37. Ghia P, Granziero L, Chilosi M, Caligaris-Cappio F. Chronic B cell malignancies and bone marrow microenvironment. Semin Cancer Biol 2002;12:149-55.
38. Lagneaux L, Delforge A, Bron D, De Bruyn C, Stryckmans P. Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. Blood 1998;91:2387-96.
39. Till KJ, Lin K, Zuzel M, Cawley JC. The chemokine receptor CCR7 and α4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes. Blood 2002;99:2977-84.
40. Sanz-Rodriguez F, Hidalgo A, Teixido J. Chemokine stromal cell-derived factor-1α modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. Blood 2001;97:346-51.
41. Matsunaga T, Takemoto N, Sato T, Takimoto R, Tanaka I, Fujimi A, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nat Med 2003;9:1158-65.
42. de La Fuente MT, Casanova B, Moyano JV, Garcia-Gila M, Sanz L, Garcia-Marco J, et al. Engagement of α4β1 integrin by fibronectin induces in vitro resistance of B chronic lymphocytic leukemia cells to fludarabine. J Leukoc Biol 2002;71:495-502.
43. Richardson SJ, Matthews C, Catherwood MA, Alexander HD, Carey BS, Farrugia J, et al. ZAP-70 expression is associated with enhanced ability to respond to migratory and survival signals in B-cell chronic lymphocytic leukemia (B-CLL). Blood 2006;107:3584-92.
44. Minina S, Reichman-Fried M, Raz E. Control of receptor internalization, signaling level, and precise arrival at the target in guided cell migration. Curr Biol 2007;17:1164-72.
45. Ottoson NC, Pribila JT, Chan AS, Shimizu Y. Cutting edge: T cell migration regulated by CXCR4 chemokine receptor signaling to ZAP-70 tyrosine kinase. J Immunol 2001;167:1857-61.
46. Ticchioni M, Charvet C, Noraz N, Lamy L, Steinberg M, Bernard A, et al. Signaling through ZAP-70 is required for CXCL12-mediated T-cell transendothelial migration. Blood 2002;99:3111-8.
47. Caloca MJ, Zugaza JL, Bustelo XR. Mechanistic Analysis of the Amplification and Diversification Events Induced by Vav Proteins in B-lymphocytes. J Biol Chem 2008;283: 36454-64.