CLL Patient Comparison According to ZAP70 mRNA Level : New Prognostic Factors, Differences In MicroRNA Expression And Distinct Interaction Capacities With The Microenvironment

B. STAMATOPOULOS*, B. HAIBE-KAINS, N. MEULEMAN, D. BRON, P. MARTIAT, L. LAGNEAUX. Experimental Hematology, Bordet Institute, Brussels, Belgium. * bstamato@ulb.ac.be

Background:

Gene expression profile is a powerful tool to better understand the biology, the clinical outcome and the molecular mechanism implicated in chronic lymphocytic leukemia (CLL). This disease presents an extremely variable clinical course with overall survival times ranging from months to decades. Therefore a plethora of prognostic factors which classified patients in poor or good behaviour have been investigated. Zeta-associated protein 70 (ZAP70) is one of the most promising prognostic factors to predict CLL evolution. Furthermore, we previously described a quantitative real-time PCR (qPCR) method to measure ZAP70 and demonstrated its prognostic power (Stamatopoulos et al, Clin. Chem., 2007).

PATIENTS					Mut. Status [a]	VH homology (%)	VH gene	Zap-70 by qPCR		Zap-70 by FC		LPL qRT-PCR		CD38 by FC		Follow-up or TFS Treatment		Death
#/um	Group	Sex	Age	Stage				qPCR [b]	Status	% cell [c]	Status	qPCR [b]	Status	% cell [c]	Status	months	Status	Status
1	high	F	69	A	UM	100.0	VH3-51	1907.7	+	66	+	1429	+	58.0	+	16.4	sys	sys
2	high	M	68	C	UM	100.0	VH1-46	1475.8	+	41	+	3388.8	+	56.0	+	0.5	sys	no
3	high	F	57	B	UM	98.0	VH3-48	1179.2	+	57	+	1.8	-	50.0	+	20.3	sys	sys
4	high	F	67	A	M	96.2	VH4-34	1137.0	+	38	+	0.1	-	Non defined		7.8	sys	sys
5	high	M	61	C	UM	99.0	VH6-1	1074.7	+	22	+	247	+	Non defined		18.7	sys	sys
6	high	F	74	C	UM	100.0	VH1-8	658.3	+	42	+	389.8	+	23.0	+	24.1	sys	sys
7	high	M	73	B	UM	100.0	VH1-69	643.9	+	77	+	447.8	+	20.0	+	2.4	sys	sys
8	low	F	67	A	M	94.2	VH3-74	61.8	-	2	-	0.1	-	94.0	+	111.9	no	no
9	low	F	68	A	M	95.2	VH4-39	50.4	-	2.5	-	0	-	0.0	-	35.4	no	no
10	low	F	59	B	M	93.9	VH1-2	18.9	-	14	-	1.7	-	0.0	-	44.6	no	no
11	low	M	67	B	M	92.2	VH2-26	14.2	-	1	-	62.4	+	50.0	+	208.6	no	no
12	low	M	72	B	M	95.2	VH4-34	9.5	-	3	-	1.9	-	0.0	-	172.3	sys	no
13	low	M	77	A	M	97.7	VH1-69	7.5	-	12	-	0.1	-	24.0	+	0.9	sys	no
14	low	M	69	A	M	94.8	VH3-9	2.7	-	10	-	0.3	-	1.0	-	88.1	sys	no

[a] Mutational status is based on a 98% cut-off value
[b] UM, unmutated; M, mutated; D, dead
[c] The cut-off determined using ROC curve analysis is expressed in fold of target gene expression in the calibrator cell line
[d] The cut-off of 20% of CD38+ cells that express Zap-70 by flow cytometry, at the same level than T cell, was used
[e] The cut-off of 7% of CD38+/CD38- cells was used as determined by ROC curves analysis

Table 1: Patient characteristics

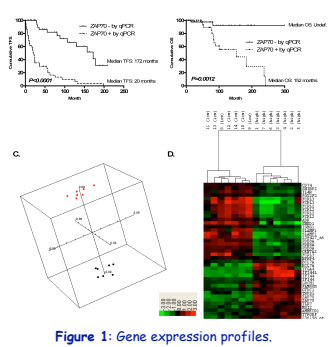

Figure 1: Gene expression profiles.
A. TFS according to ZAP70 (qPCR); B. OS according to ZAP70 (qPCR); C. Multidimentional scaling; D. microarray analysis

Aims:

In this study, we compared gene expression profile of patients expressing high versus low ZAP70 mRNA level in order to find genes not only associated with prognosis but also with cell biology. We also confirmed some microRNA differentially expressed between these two groups and linked them to treatment-free survival (TFS) and overall survival (OS).

Methods:

ZAP70 was evaluated by qPCR in a cohort of 108 patients ; two groups of 7 patients were chosen in the top-20 of patients expressing high and low level of ZAP70 mRNA and their gene expression profiles were compared using Affymetrix technology (Table 1). Selected genes were verified by qPCR in an extended patient cohort (n=85) with a median follow-up of 72 months.

Adhesion/migratory capacities into a stromal microenvironment (SM) or in response to conditioned medium were also evaluated. Finally, we investigated some microRNA differential expression by qPCR in a cohort of 61 patients with a median follow-up of 74 months.

Results:

43 probe sets were differentially expressed with a FDR<10% (Figure 1), 135 with a P<0.001 and 932 with a P<0.05. Several of these genes were TFS and/or OS significant predictors: PDE8A and FCRL family genes were downregulated in ZAP70+ patients and can predict TFS and OS; ITGA4 mRNA was upregulated in ZAP70+ patients and can significantly predict OS (Figure 2 and Table 2).

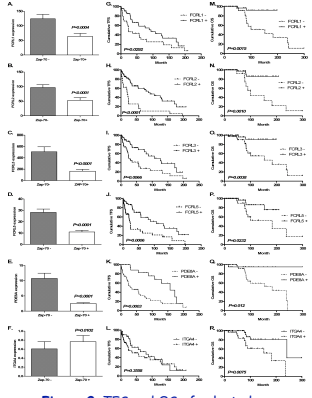

Figure 2: TFS and OS of selected genes

Symbol	Gene description	Differences between ZAP70+ (n=41) and ZAP70- (n=44) cases			Target gene status [a]	n	Number of ZAP70+ patients	Treatment-free Survival (TFS)		Overall Survival	(OS)
		P	Mann Whitney U	Fold Change [c]				P	Median TFS	P	Median OS [c]
PDE8A	phosphodiesterase 8A	P<0.0001	422.5	-4.3	-	62	39	0.0003	35	0.012	183
					+	23	2		157		UD
FCRL1	Fc receptor-like 1	0.0004	483	-1.9	-	39	27	0.0292	24.13	0.0015	152.5
					+	46	14		93.67		UD
FCRL2	Fc receptor-like 2	P<0.0001	450	-1.8	-	22	21	P<0.0001	20.27	0.001	106.5
					+	63	20		93.67		UD
FCRL3	Fc receptor-like 3	P<0.0001	422	-3.1	-	43	10	0.0006	29.2	0.0038	152.5
					+	42	31		107.2		UD
FCRL5	Fc receptor-like 5	P<0.0001	304	-2.6	-	49	30	0.0006	22.3	0.0232	183
					+	36	11		107.2		UD
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	0.0102	605.5	1.3	-	54	7	0.3558	88.67	0.0075	241.9
					+	31	34		57		183
TLR7	toll-like receptor 7	0.0002	472	2.0	-	22	21	0.0263	88.07	0.0992	UD
					+	63	20		30.07		152.5
LPL	lipoprotein lipase	P<0.0001	381	11.8	-	42	11	0.0031	126	0.1593	241.9
					+	43	30		28.1		237.1
CLEC2B	C-type lectin domain family 2, member B	P<0.0001	386.5	5.5	-	34	14	0.0449	80.47	0.1838	UD
					+	51	27		29.33		237.1
PCDH9	protocadherin 9	0.0044	577.5	2.6	-	47	11	0.0266	126	0.0194	UD
					+	38	30		29.33		183
BCL7A	B-cell CLL/lymphoma 7A	0.0004	497.5	5.6	-	32	17	0.0754	88.07	0.0576	251.9
					+	53	24		24.13		237.1
CTLA4	cytotoxic T-lymphocyte-associated protein 4	0.0308	707	-2.3	-	61	26	0.0087	35.5	0.0157	183
					+	24	5		88.07		UD
MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	P<0.0001	354	-6.6	-	55	35	0.0005	29.9	0.0154	183
					+	30	6		172.27		UD

[a] Positive values represent an upregulation and negative value a downregulation in ZAP70+ patients.
[c] Optimal cut-offs were determined using ROC curve analysis in order to maximize the concordance with ZAP70 status
[c] UD, undefined.

Table 2: TFS and OS of selected genes

Among the 4 microRNAs tested, we confirmed the differential expression of miR-29c and miR-223. We showed for the first time that miR-29c and miR-223 had a TFS and OS individual prognostic power (Figure 5).

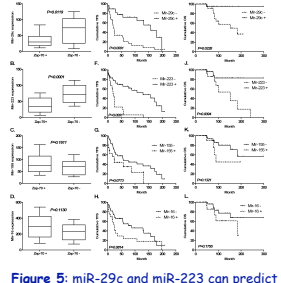

Figure 5: miR-29c and miR-223 can predict TFS and OS

Moreover pathway analysis reveals an overrepresentation of adhesion / migration genes (Table 3). We plated CLL cells in presence of a SM (with or without contact). We found that significantly more ZAP70+ cells adhere to this SM. We also observed a downregulation of CXCR4 in stromal-adherent cells only in ZAP70+ patients indicating that only these patient cells can respond to SM stimulus. CD69, recently described as a poor prognosis factor, was also up-regulated in adherent cells (Figure 3). Furthermore, ZAP70+ patient cells can significantly better adhere to fibronectin and have better migration capacities (Figure 4).

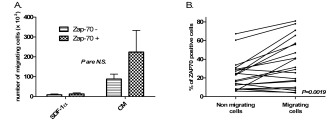

Figure 4: ZAP70+ cells have better migration capacities

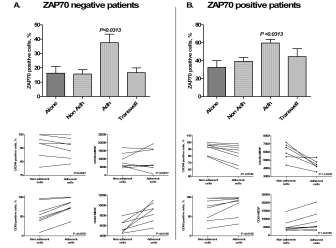

Figure 3: ZAP70+ cells adhere better to the SM

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	actine polymerization and/or depolimerization	0.0010
GO0007155	cell adhesion	8.10^{-7}
GO0016387	cell-cell adhesion	0.0053
GO0007160	cell-matrix adhesion	0.0003
GO0006935	chemotaxis	0.0023
GO0005856	cytoskeleton	0.0002
GO0007010	cytoskeleton organisation 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	$2.6.10^{-2}$
hsa04520	adherens junction	$2.5.10^{-6}$
hsa04540	gap junction	$3.9.10^{-3}$
hsa04670	transendothelial leukocyte migration	$<10^{-7}$
hsa04810	regulation of actin cytoskeleton	$<10^{-7}$
hsa04510	Focal adhesion	$<10^{-7}$
Broad Pathway		
SIG_CHEMOTAXIS_h	SignallingAlliance	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	SignallingAlliance 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, via cell adhesion molecules.	0.0005
cell_adhesion_h		0.0004
h_ST_Integrin_Signaling_Pathway	Signaling Transduction KE	$1.9.10^{-4}$
Biocarta pathways		
h_lymphPathway	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

Table 3: Gene set enrichment analysis

Conclusions:

Considering all these data, we can tentatively conclude that markers such as ZAP70, LPL, CD38, CD69 or CXCR4 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. Moreover, this study identifies new prognostic factors (genes and microRNA) and shows the better adhesion/migratory capacities of ZAP70+ cells in their microenvironment explaining their better survival and the aggressiveness of the disease.