Gene expression profiling based on ZAP70 mRNA expression reveals differences in microenvironment interaction between patients with good and poor prognosis

Basile Stamatopoulos,[1] Benjamin Haibe-Kains,[2,3] Carole Equeter,[2] Nathalie Meuleman,[1] Anne Sorée,[1] Cécile De Bruyn,[1] Delphine Hanosset,[1] Dominique Bron,[1] Philippe Martiat[1] and Laurence Lagneaux[1]

[1]Laboratory of Experimental Hematology, Faculty of Medicine, Institut Jules Bordet, Université Libre de Bruxelles (ULB), Brussels; [2]Functional Genomics and Translational Research Unit, Faculty of Medicine, Institut Jules Bordet, Université Libre de Bruxelles (ULB), Brussels, and [3]Machine Learning Group, Faculty of Sciences, Université Libre de Bruxelles (ULB), Brussels, Belgium

Citation: Stamatopoulos B, Haibe-Kains B, Equeter C, Meuleman N, Sorée A, De Bruyn C, Hanosset D, Bron D, Martiat P, and Lagneaux L. Gene expression profiling based on ZAP70 mRNA expression reveals differences in microenvironment interaction between patients with good and poor prognosis. Haematologica 2009;doi:10.3324/haematol.2008.002626

Online Supplementary Appendix

Gene expression profiles

The quality of total RNA extracted from CLL CD19+ purified cells was assessed via the RNA profile generated by the Agilent bioanalyzer (Agilent Technology, Santa Clara, CA, USA). Samples with a total area under the 28S and 18S bands of less than 15% of the total RNA band area, as well as a 28S/18S ratio of less than 1.1, were considered to be degraded and were not further analyzed.

Amplification, hybridization, and scanning were done according to standard Affymetrix protocols. Briefly, double-stranded cDNA was synthesized using the One-Cycle cDNA synthesis Kit (Affymetrix). The cRNA was synthesized and biotinylated using the IVT Labeling kit according to the manufacturer's recommendations and was hybridized thereafter.

Image analysis and probe quantification were done with Affymetrix software, which produced raw probe intensity data in the Affymetrix CEL files. Normalization was done with the RMA method,[1] which processed a group of CEL files simultaneously.

ZAP70 flow cytometry analysis

We measured the expression of cytoplasmic ZAP70 protein by FC with the Fix and Perm Permeabilization KIT (ImTec Diagnostics), a ZAP70 phycoerythrin-conjugated antibody (clone 1E7.2, eBioscience), fluorescein isothiocyanate–conjugated CD3, and phycoerythrin-Cy5–conjugated CD19 (Immunotech). Because the choice of the threshold for ZAP70 positivity can critically affect the decision regarding ZAP70 status, we defined it on the basis of the lower limit of the region which included 99% of $ZAP70^+CD3^+$ cells. This threshold maximized the concordance between ZAP70 status and mutational status. After establishing the appropriate gating on $CD3^+$ cells, we fixed the cut-off for ZAP70 positivity and measured ZAP70 in $CD19^+$ cells.

IgVH gene mutational analysis

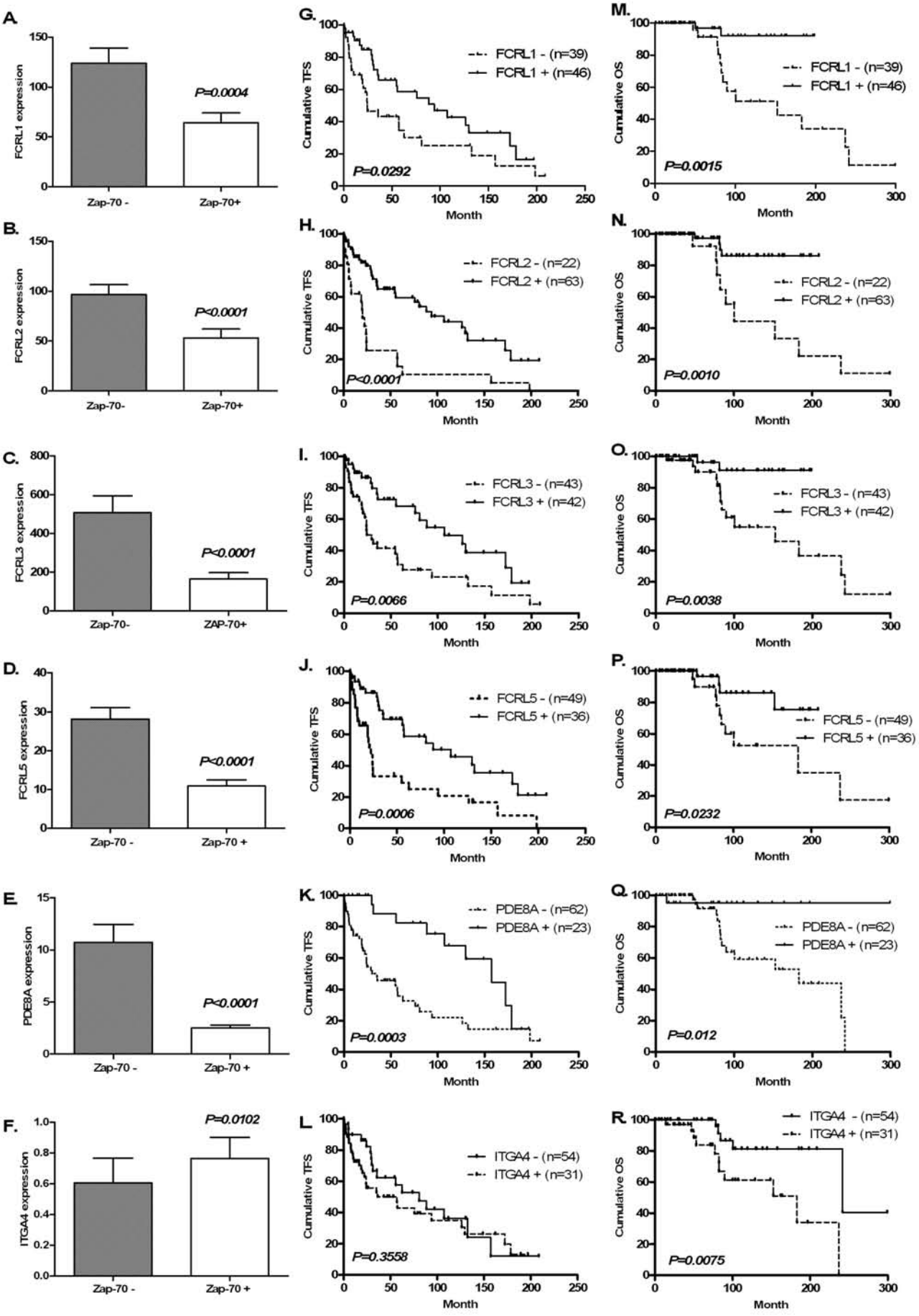
IgVH gene mutational analysis was performed as previously described[2] and aligned sequences with those in the international ImMunoGeneTics information system database (http://imgt.cines.fr). Sequences with <2% deviation from any germ line IgVH sequence were considered unmutated.[3]

Real-time PCR analysis

Standard real-time PCR was performed as previously described[4] on an ABI Prism 7900 HT (Applied Biosystems). Five housekeeping genes [*LMNB1*, lamin B1; *EIF1AX*, eukaryotic translation initiation factor 1A, X-linked; CASC3 (also known as *MLN51*), cancer susceptibility candidate 3; *PPIA*; and *PGK1*, phosphoglycerate kinase 1] were tested as endogenous controls (*data not shown*). Finally, we standardized all results using *PPIA* gene expression, which was the most stable. A calibrator sample (cDNA from the Namalwa cell line, a human B-lymphoid leukemia cell line; ATCC) was included as a control in each experiment and all data were normalized using cyclophilin A (PPI) gene expression. In all cases, we created dissociation curves to confirm PCR specificity. Data were analyzed with the comparative DDCt method.

References

1. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003;19:185-93.
2. Campbell MJ, Zelenetz AD, Levy S, Levy R. Use of family specific leader region primers for PCR amplification of the human heavy chain variable region gene repertoire. Mol Immunol 1992; 29:193-203.
3. 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.
4. Stamatopoulos B, Meuleman N, Haibe-Kains B, Duvillier H, Massy M, Martiat P, Bron D, Lagneaux L. 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.



Online Supplementary Figure S1. Confirmation and prognostic power of differentially expressed genes between ZAP70 + and - cases. Figures A to F represent the expression distribution between ZAP70 + (n=41) and - (n=44) patients measured by real-time PCR; G to L, the treatment-free survival (TFS) (n=85); M to R, the overall survival (OS) (n=85), respectively for FCRL1, FCRL2, FCRL3, FCRL5, PDE8A and ITGA4. Expression of these genes is given in terms of fold change of the target gene over that in the calibrator Namalwa cell line and normalized using cyclophilin expression. Optimal cut-offs to determine + and - cases were calculated by ROC curve analysis in order to maximize the concordance with ZAP70 expression. Significant differences were calculated using the Mann Whitney test, and differences between curves in TFS and OS analysis were evaluated using the log-rank test.

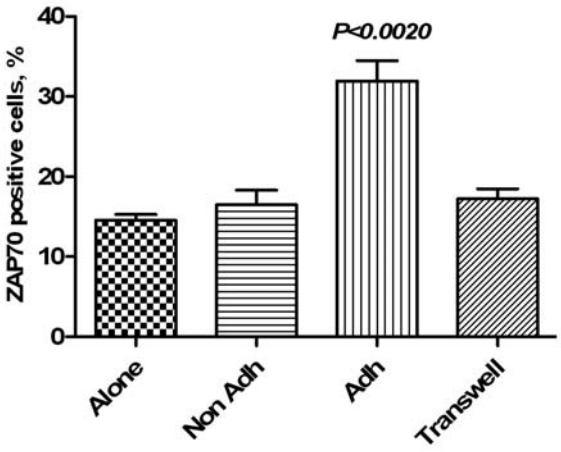
Online Supplementary Table S1. Real-time PCR primer sequences.

Symbol	Gene description	Foward primer	Reverse primer
PDE8A	phosphodiesterase 8A	AGGCGCCCATCACCAA	TGTCACAGGCATGGGACTACTT
FCRL1	Fc receptor-like 1	TATTTTGCTACGGCCTCAAAAGA	GGGAAGGCTCCTGAGTGGAT
FCRL2	Fc receptor-like 2	CAACGGCCATGTGCCTATC	CAGGGCGAGACACTGGAATT
FCRL3	Fc receptor-like 3	TGACTCCTGGAAGAGAACAATCAG	TGTGGACCATGGAGGATTGA
FCRL5	Fc receptor-like 5	CCCAGCGCAGTGAGACAGT	GCAAAAGGGCCACTTCTGTTC
ITGA4	integrin, α 4 (antigen CD49D, α 4 subunit of VLA-4 receptor)	AGGTGTCCAGCAGAGAAGCTAACT	GGATGTCCCGCACATCTTTC
TLR7	toll-like receptor 7	ATATAGGATCACTCCATGCCATCA	GGTGCCAAGATCAGCTTGAGA
LPL	lipoprotein lipase	CCGCCGACCAAAGAAGAGAT	TTCCTGTTACCGTCCAGCCAT
CLEC2B	C-type lectin domain family 2, member B	AGATGGAGCTACATTTACCAAATCG	TCATCGCTGAGGTAGGCACAT
PCDH9	protocadherin 9	TCAGAGTGCAGTTCCCAAGGA	GCTGCGACTGTCTGGTGTGT
BCL7A	B-cell CLL/lymphoma 7A	GGGAGCCCATTGGGTTGT	AGGCCTCCAGTTTCATCTTTTTAG
CTLA4	cytotoxic T-lymphocyte-associated protein 4	TGC AGC AGT TAG TTC GGG GTT GTT	CTG GCT CTG TTG GGG GCA TTT TC
MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	AACAACATGGAACTGATGATTGGA	GGCACTGAAAATCAGAGCGATT
ZAP70	zeta associated protein 70	GTT GAC TCA TCC TCA GAG ACG AAT	AGG TTA TCG CGC TTC AGG AA
PPI	Cyclophylin A	GCTCGTGCCGTTTTGCA	GCAAACAGCTCAAAGGAGACG

Online Supplementary Table S2. Characteristics of the 85 patient cohort.

		n	%
Patients		85	100
	Male	46	54
	Female	39	6
Binet		85	100
	Stage A	56	66
	Stage B	19	22
	Stage C	10	12
Mutational status[1]		83	100
	IgVH - Unmutated	40	48
	IgVH - Mutated	43	52
	Non Defined	2	
ZAP-70[2]		85	100
	>114 (positive)	41	48
	<114 (negative)	44	52
LPL[2]		85	100
	>6 (positive)	40	47
	<6 (negative)	45	53
CD38[2]		80	100
	>7% (positive)	37	46
	<7% (negative)	43	54
	Non Defined	5	
Treatment status		85	100
	Patients not requiring treatment	32	38
	Patients requiring treatment	53	62
Death status		85	100
	Patients still alive	66	78
	Patients died during the study	19	22

This cohort is a part of the 108 patient cohort previously published (Stamatopoulos et al., Clin Chem, 2007) for which enough RNA was available. The median age at diagnosis was 65 years (range, 45-89 years). The median TFS of this cohort was 61 months (range, 2-226 months), while the median OS was 237 months (range, 2-299 months). The median follow-up duration was 74 months (range, 2-299 months). [1]Mutational status is based on a 98% cut-off value. [2]The cut-off determined using ROC curve analysis maximizing the concordance with the IgVH status.



Online Supplementary Figure S2. Modulation of ZAP70 in response to the stromal microenvironment in normal B cells. Normal B cells isolated from healthy donors (n=10) 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 was measured by FC in adherent and non-adherent cells. Furthermore, ZAP70 was measured on cells cultured alone and in transwell conditions.

Online Supplementary Table S3. Differentially expressed probe sets between ZAP70^{high} and ZAP70^{low} patients. (*please refer to the corresponding_.PDF*)

Online Supplementary Table S4. Gene set expression comparison.

GO categories	Description	Total number of involved items in significant pathways		Number of deregulated items involved in significant pathways		*p* value
		probe sets*	genes	probe sets*	genes	
GO0015629	Actin cytoskeleton	253	88	20	17	0.0021
GO0030036	Actin cytoskeleton organization and biogenesis	130	38	9	8	0.0027
GO0030029	Actin filament-based process	215	57	12	10	0.0014
GO0007155	Cell adhesion	879	357	53	31	8.10^{-7}
GO0016337	Cell-cell adhesion	80	21	13	7	0.0053
GO0007160	Cell-matrix adhesion	92	26	10	4	0.0003
GO0006935	Chemotaxis	57	31	6	5	0.0023
GO0005856	Cytoskeleton	990	351	78	57	0.0002
GO0007010	Cytoskeleton organization and biogenesis	104	43	34	29	0.0009
GO0005874	Microtubule	381	149	16	13	0.0003
GO0007018	Microtubule based movement	187	86	8	8	0.0108
Kegg Pathway						
hsa04514	Cell adhesion molecules (CAMs)	223	72	5	5	0.0020
hsa04530	Tight junction	503	185	20	14	$2.6.10^{-7}$
hsa04520	Adherens junction	769	267	20	12	$2.5.\ 10^{-6}$
hsa04540	Gap junction	690	254	22	13	$3.9\ .\ 10^{-5}$
hsa04670	Leukocyte transendothelial migration	420	142	13	11	$<10^{-7}$
hsa04810	Regulation of actin cytoskeleton	478	168	22	14	$<10^{-7}$
hsa04510	Focal adhesion	638	226	26	18	$<10^{-7}$
Broad Pathway						
SIG_CHEMOTAXIS_h	Signaling Alliance	157	45	11	6	0.0002
cell_adhesion_receptor_activity_h	Combining with cell adhesion molecules to initiate a change in cell activity.	107	38	6	3	0.0121
SIG_Regulation_of_the_actin_cytoskeleton_by_Rho_GTPases_h	Signaling Alliance	102	35	6	5	0.0069
cell_adhesion_molecule_activity_h	Mediates the adhesion of the cell to other cells or to the extracellular matrix.	277	120	10	7	0.0011
cell_motility_h	Any process involved in the controlled movement of a cell.	272	118	7	5	0.0005
cell_adhesion_h	The attachment of a cell, either to another cell or to the extracellular matrix, via cell adhesion molecules.	444	201	20	11	0.0004
h_ST_Integrin_Signaling_Pathway	Signaling Transduction KE	295	82	15	7	$1.9\ .\ 10^{-5}$
Biocarta pathways						
h_lympathway	Adhesion and diapedis of lymphocytes	40	18	6	3	0.0012
h_integrinPathway	Integrin Signaling Pathway	130	37	6	5	0.0015
h_lymphocytePathway	Adhesion Molecules on Lymphocyte	31	10	6	3	0.0012
h_cxcr4Pathway	CXCR4 Signaling Pathway	78	23	8	7	0.0006

**In Affymetrix technology, several probe sets (targeting different gene sequences) can be associated to one single gene.*

Online Supplementary Table S5. Characteristics of the 54 patient cohort.

		n	%
Patients		54	100
	Male	32	59
	Female	22	41
Binet		54	100
	Stage A	36	67
	Stage B	13	24
	Stage C	5	9
Mutational status[1]		54	100
	IgVH-unmutated	25	46
	IgVH-mutated	29	54
ZAP-70[2]		54	100
	>114 (positive)	24	44
	<114 (negative)	30	56
LPL[2]	54	100	
	>6 (positive)	25	46
	<6 (negative)	29	54
CD38[2]	54	100	
	>7% (positive)	32	59
	<7% (negative)	22	41
Treatment status		54	100
	Patients not requiring treatment	20	37
	Patients requiring treatment	34	63
Death status		54	100
	Patients still alive	48	89
	Patients died during the study	6	11

This cohort is a part of the 108 cohort previously published (Stamatopoulos et al., Clin. Chem, 2007) for which cells were available. The median age at diagnosis was 65 years (range, 46-86 years). The median TFS of this cohort was 57 months (range, 2-226 months), while the median OS was not reached (range, 2-299 months). The median follow-up duration was 91 months (range, 2-299 months). [1]Mutational status is based on a 98% cut-off value; [2]The cut-off determined using ROC curve analysis maximizing the concordance with the IgVH status.