The predictive value of lipoprotein lipase for survival in chronic lymphocytic leukemia

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Background and Objectives. The mutational status of the immunoglobulin heavy chain variable region genes (*IGV*$_H$) is a strong indicator of prognosis in B-cell chronic lymphocytic leukaemia (CLL). Since the determination of the *IGV*$_H$ mutation status is very labor-intensive, alternative prognostically relevant markers would facilitate CLL diagnostics.

Design and Methods. Ten genes were selected from previously published gene expression profiling studies based on their differential expression in *IGV*$_H$ mutated versus unmutated cases of CLL, and tested with real-time quantitative polymerase chain reaction (RQ-PCR) in unpurified samples from 130 CLL patients. To ascertain potential contaminating effects by normal hematopoietic cells, the expression levels of the selected genes were determined in normal monocytes, B cells, T cells, NK cells and granulocytes.

Results. The selected genes, i.e., *ZAP70*, *LPL*, *SPG20*, *ADAM29, NRIP1, AKAP12*, *DMD*, *SEPT10, TPM2* and *CLECSF2*, showed prognostic significance. In multivariate logistic regression analysis expression levels of *LPL, ZAP70, ADAM29* and *SEPT10* were the most predictive for *IGV*$_H$ mutational status. In univariate analysis the expression of LPL was the best predictor. For survival, expression of *LPL* was the strongest prognostic factor. In combination with the three cytogenetic markers associated with a poor prognosis, i.e., deletions 17p13, 11q22 and trisomy 12, expression of *LPL* and *IGV*$_H$ mutational status performed equally well with regard to their predictive value for survival, both being more predictive than *ZAP70*.

Interpretations and Conclusion. This study demonstrates that *LPL* expression is a predictor for survival in CLL, and for this purpose is as good as *IGV*$_H$ mutational status and more reliable than *ZAP70* expression when tested in unpurified CLL samples.

Key words: CLL, mutational status, lipoprotein lipase, LPL, prognostic factor.

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Chronic lymphocytic leukemia (CLL) cells are characterized by the expression of CD19, CD5, CD23 and low levels of monoclonal surface bound IgM/IgD, a phenotype that is unknown in normal B-cell differentiation.[1-4] Normal CD5-positive peripheral blood B cells are characterized by rearranged immunoglobulin (IG) genes with unmutated variable region (V) gene segments.[5,6] In about 50% of CLL patients it appears that the *IGV* genes of malignant cells have undergone somatic hypermutation,[7-9] a process that in normal B-cell differentiation is seen in B cells that are subjected to a T-cell-dependent antigen response in the germinal center.[10] This finding suggests that, although all CLL cases share the same immunophenotype, the malignant cells from those CLL patients who have unmutated *IGV* genes are derived from cells responding to T-cell-independent antigens whereas the malignant cells from the other CLL cases showing mutated *IGV* genes have undergone a T-cell-dependent germinal center reaction.

Although the pathogenesis leading to these two distinct subgroups in CLL is still a matter of debate, in clinical practice the *IGV* mutational status, mostly measured in the heavy chain of the *IGV* gene (IGV$_H$), has shown to be an important single marker for prognosis.[8,9,11,12] CLL patients with unmutated *IGV*$_H$ genes have a significantly worse prognosis than those with hypermutated *IGV*$_H$ genes.

Gene expression profiling showed that both subtypes of CLL have homogeneous gene expression profiles, i.e., irrespective of their *IGV*$_H$ mutational status, pointing towards a common differentiation pathway or status.[13-16] In contrast, however, these studies also demonstrated that a few hundred genes are differentially expressed, which may reflect distinct biological differences in the two types of CLL. In fact,

these differentialy expressed genes may also be relevant prognostic markers. We selected ten genes that were commonly shown to be differentially expressed with regard to *IGV$_H$* mutational status (Table 1)[13–16] and studied their expression by reverse transcriptase polymerase chain reaction (RT-PCR) in 130 CLL patients.

The objective of this study was to evaluate the prognostic significance of the expression of each of these genes in relation to *IGV$_H$* mutational status, clinical stage at diagnosis and the presence of chromosomal abnormalities. We included *ZAP70*, which has already been shown to be a strong predictor of prognosis[17,18] but is also expressed on T lymphocytes and NK cells, which may, both in RT-PCR and in flow cytometric analyses, interfere with the interpretation of the results.[19,20] In multivariate analysis we aimed to establish the strength of the prognostic value of the selected genes.

Design and Methods

CLL patients

The clinical diagnosis of all patients was based on standard morphologic and immunophenotypic criteria.[1,2] Samples were taken after informed consent and the institutional ethic committee approved the study. Samples from 130 patients were included; 34 samples were collected at diagnosis and, forty-eight patients were known to have been previously treated with one or two regimens. The patients were equally distributed according to *IGV$_H$* mutated and unmutated status. Information on Binet stage at diagnosis was available for 98 patients. Mononuclear cells were isolated from peripheral blood by Ficoll-Hypaque (Nygaard, Oslo, Norway) density centrifugation and cryopreserved. After thawing the cells were processed for determination of the percentage of CLL cells by multiparametric flow cytometry (CD19, CD3 and CD45), DNA isolation and RNA extraction. All samples for analysis of mutational status, cytogenetics and gene expression were taken of the same date. Patients were untreated at the time of this examination (44%), or had received chlorambucil only (25%), or fludarabine as first (2%) or second-line treatment (14%); these treatment regimens may influence remission duration differently, but have no influence on overall survival. None of the patients received autologous or allogeneic stem cell transplantation, rituximab or alemtuzumab.

Isolation of normal hematopoietic subsets

Peripheral blood from an anonymous healthy donor was obtained from the blood bank. Buffy coat material was first separated by Ficoll-Hypaque density centrifugation into granulocyte and mononuclear cell fractions. The mononuclear cell fraction was further purified by cell sorting (FACS DiVa, Becton Dickinson, San Josè, CA, USA). Using CD3-FITC, CD16/CD56-PE, CD45-PerCP, and CD19-APC antibodies T lymphocytes, (CD3$^+$, CD16/56$^-$, CD19$^-$, CD45$^+$), B lymphocytes (CD19$^+$, CD3$^-$, CD16/56$^-$, CD45$^+$), NK cells (CD16/56$^+$, CD3$^-$, CD19-, CD45$^+$) and monocytes (CD3$^-$, CD16/56$^-$, CD19$^-$, CD45$^+$ and sideward scatter profile) were obtained to ~95% purity.

Analysis of IGV$_H$ somatic mutation status

Genomic DNA was prepared from cryopreserved cells and *IGH* gene rearrangements were amplified in duplicate by multiplex PCR using a mixture of V$_H$-FR1 (V$_H$1-V$_H$6) family primers in combination with a consensus JH primer, as designed by the BIOMED-2 Concerted Action.[21] Following amplification, heteroduplex analysis was performed to identify the presence of one or more clonal bands.[22] In the case of multiple clonal PCR products, bands were cut out from the polyacrylamide gel and eluted for sequencing; single monoclonal PCR products were directly sequenced using the ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Sequencing analysis was started using the consensus J$_H$ primer as the sequencing primer on PCR products from the two independent reactions. Following identification of the involved V$_H$ family member via the IMGT database (*http://imgt. cines.fr:8104*), the PCR products were sequenced with the appropriate V$_H$ FR1 family primer, for confirmation. A consensus sequence was made from the aligned sequences and compared to sequences in the IMGT database. Involved V$_H$, D$_H$ and J$_H$ gene segments and reading frame were determined, and the percentage homology to the closest germline V$_H$ gene segment was calculated. Percentage homology was defined as the number of identical nucleotides in the entire V$_H$ segment divided by the total number of nucleotides sequenced up to the last complete germline exon that could be recognized, excluding the primer sequence. The most discriminative cut-off point was considered, based on our own analysis (*data not shown*) and according to literature, to be 98% homology.[11,23]

Gene expression analysis

Total RNA was extracted using RNABee (Campro Scientific, Veenendaal, The Netherlands). cDNA was synthesized from 1 μg of RNA using random hexamer priming, essentially as described elsewhere.[24] cDNA prepared from 50 ng of RNA was used for all PCR amplifications, except for *ZAP70*, for which cDNA prepared from 100 ng of RNA was used. The expression levels of the following genes were determined by real-time quantitative PCR (RQ-PCR): *ZAP70, LPL, SPG20, ADAM29, NRIP1, AKAP12, DMD, SEPT10, TPM2* and *CLECSF2*. *RQ-PCR* amplification was performed with the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) using 50 μL mix containing 20 μM deoxyribonucleoside triphosphates (dNTP; Amersham Pharmacia Biotech, Roosendaal, The Netherlands); 15 pmol of both forward and reverse primers for the reference gene *PBGD*; 15 pmol of forward and 45 pmol reverse primer for the expression of *ZAP70* (Invitrogen,

Table 1. Identity, RQ-PCR primers, probes and calibrator cell lines of the candidate genes with prognostic value in CLL.

Gene			Primers and probes	Reference Sequences	Calibrator cell line
ZAP70	ζ-chain (TCR) associated protein kinase 70kDa	FW RV probe	5'-ACA CCC TCA ACT CAG ATG GAT ACA-3' 5'-TCG GCC GCG GTT TGT-3' 5'-CCC TGC GCC AGC ACG CAT AAC GT-3'	NM 001079	HPB
LPL	lipoprotein lipase	FW RV	5'-CCGCCGACCAAAGAAGAGAT-3' 5'-TTCCTGTTACCGTCCAGCCAT-3'	NM 000237	HL60
SPG20	spastic paraplegia 20, spartin	FW RV	5'-TAAGGGAGTGGGAGCTGGT-3' 5'-ATAGTAGTTCTTTGCTTCTTCC-3'	NM 015087	ME
ADAM29	disintegrin and metalloprotease (ADAM) domain 29	FW RV	5'-CAATACTCCTGTGATCGTATAA-3' 5'-CACTGCTCCTTTCCACTGC-3'	NM 014269	RAJI
NRIP1	nuclear receptor interacting protein 1	FW RV	5'-GCTTCTCGCCCACACAGC-3' 5'-AATCATGCTCTTATTTATACAGAT-3'	NM 003489	K562
AKAP12	kinase (PRKA) anchor protein (gravin) 12	FW RV	5'-GTCAGCTGTCCACCATCAAT-3' 5'-AGTCTCTCTGTCCAACCTCT-3'	NM 005100	TOM
DMD	dystrophin	FW RV	5'-GGATTCAACACAATGGCTGG-3' 5'-ACTGCTTGGTTTCTGTGATTTT-3'	NM 004023	K562
SEPT10	septin 10	FW RV	5'-GGCACCTGCTCTTTCAGTC-3' 5'-CCTTGCTGAATGGATCTGTT-3'	NM 144710	HL60
TPM2	tropomyosin 2 (β)	FW RV	5'-AGATGCAGCTGAAGGAGGC-3' 5'-TCCCCACATTTACTCTCGG-3'	NM 003289	HL60
CLECSF2	member of the C-type lectin/ C-type lectin-like domain superfamily	FW RV	5'-TTAAAAGAAGCACGGTATGATG-3' 5'-AATAATAGCATTTGTTTTGGAAAC-3'	NM 005127	RAJI
Reference gene *PBGD*	porphobilinogen deaminase	FW RV Probe	5'-GGCAATGCGGCTGCAA-3' 5'- GGGTACCCACGCGAATCAC -3' 5'-CATCTTTGGGCTGTTTTCTTCCGCC-3'	NM 000190	HPB

Breda, the Netherlands); 5 mM $MgCl_2$; 10 pmol of probe for *PBGD* and 7.5 pmol of probe for *ZAP70*, labeled at the 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein) and at the 3'end with the quencher dye molecule TAMRA (6-carboxy-tetramethylrhodamine) for both *ZAP70* and *PBGD* (Eurogentec, Maastricht, the Netherlands) (Table 1), 5 μL 10 × buffer A and 1.25 U AmpliTaq Gold with the *PBGD* and 2.5 U AmpliTaq Gold with the *ZAP70* amplification (Applied Biosystems). The thermal cycling conditions for *ZAP70* and *PBGD* were 10 minutes at 95°C followed by 45 cycles of denaturation for 20 seconds at 95°C, annealing at 58°C for 20 seconds and extension at 60°C for 30 seconds. All other genes were measured with 1× SYBR Green I dye (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). The cycling conditions for the SYBR Green I reactions were 10 minutes at 95°C followed by 45 cycles of denaturation for 15 seconds at 95°C, annealing at 60°C for 30 seconds, and extension at 62°C for 30 seconds; the annealing and extension temperature for *LPL* was 62°C. All RQ-PCR primers and probes (Table 1) were designed with Primer Express V2.0 software. To quantify the relative expression levels of the selected genes in CLL, the Ct values were normalized for the endogenous reference *PBGD* ($\Delta Ct = Ct_{target} - Ct_{PBGD}$) and compared with a calibrator that was chosen for its high expression level of the specific gene (Table 1), using the ΔΔ Ct method ($\Delta\Delta Ct = \Delta Ct_{CLL\ sample} - \Delta Ct_{Calibrator}$). We used the ΔΔCt value to calculate relative expression ($2^{-\Delta\Delta Ct}$).

Interphase fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) was performed on thawed or fresh peripheral mononuclear blood cells. Cytospins were made and cells were fixed with methanol. The following commercially available DNA probes (Vysis, Downers Grove, IL, USA) were used: LSI D13RB-1, LSI D13S319 and LSI D13S25 including the retinoblastoma gene and the markers S319 and S25 on chromosome 13q14, LSI p53 for the *p53* gene on chromosome 17p13, LSI ATM for the ataxia telangiectasia gene (*ATM*) on chromosome 11q22 as well as a centromeric probe for chromosome 12 (CEP12) (Vysis). To exclude patients with leukemic mantle cell lymphoma all blood samples were studied for t(11;14)(q13;q32) using the LSI IgH/CCND1 dual fusion translocation probe which can detect the juxtaposition of the *IGH* locus and the cycin D1 gene (*CCND1*).[25] FISH analysis was performed according to the manufacturer's instructions. In most cases 300, but in all case at least

Table 2. Clinical characteristics and IGV_H mutational status of the CLL patient cohort.

	n	*IGV_H Mutated*	*IGV_H Unmutated*
Number	130	80 (62%)	50 (38%)
Male	81 (62%)	46 (57%)	35 (43%)
Age median (range), years	66 (33-92)	65 (39-92)	66 (33-86)
VH1-69	14	4 (28%)	10 (72%)
VH3-21	16	11(69%)	5 (31%)
VH4-34	12	12 (100%)	0 (0%)
Binet A	59	45 (76%)	14 (24%)
Binet B	24	11 (46%)	13 (56%)
Binet C	15	4 (27%)	11 (73%)
Binet unknown	32	20 (63%)	12 (37%)

100, interphase nuclei were evaluated per probe per patient. Clonality was accepted to be present when the percentage of cells with an abnormal number of FISH spots exceeded the normal cut-off value.

Statistical analysis

For the evaluation of the predictive value of gene expression towards *IGV_H* mutational status, multivariate logistic regression was used. Cut-off points for expression values were determined by optimizing the prognostic value of the expression value towards *IGV_H* mutational status using isotonic regression analysis. As it is not known whether the expression of the genes under study was stable during the course of the disease and testing was retrospective for a number of patients, we defined overall survival as the time from sample collection to death. Patients alive at the date of last contact were censored at that time for this endpoint. Univariate Cox regression analysis was applied to evaluate the prognostic value of the continuous expression values, cytogenetic variables and *IGV_H* mutational status towards overall survival. Differences with p values < 0.05 were regarded as statistically significant. Kaplan-Meier estimates of survival were made, dividing patients according to low and high levels of expression of each gene.

Results

Clinical data

Samples from 130 patients with CLL that were send to our referral laboratory between December 1999 and July 2004 were selected on the basis of availability of material, taken at diagnosis or during the course of the disease, and the possibility of determining the *IGV_H* mutational status. Clinical data are shown in Table 2. The median follow-up from inclusion date of the 108 patients alive was 17 months (range 0 – 57). The median proportion of B cells within this cohort was 90.8% (range 31.3 – 98.1) and that of T cells was 4.0% (range 0.9 – 52.0).

Analysis of IGV_H mutational status

Clonal *IGV_H* gene rearrangements could be amplified using the V_H-FR1 BIOMED-2 multiplex PCR in 130 patients. These patients' samples were included for further analysis of gene expression levels and chromosome aberrations. More detailed analysis of *IGV_H* gene segment usage in these 130 patients showed preferential use of V_H1-69 in non-mutated B-CLL (10/14; 71%) and V_H4-34 in mutated CLL (12/12; 100%), whereas V_H3-21 was also frequently found in mutated cases, although to a much lesser extent (10/16; 63%). Similar evaluation of D_H and J_H gene segment usage did not reveal any dominant associations in CLL with non-mutated or mutated *IGV_H* genes.

Table 3. Gene expression in relation to the known prognostic factors in CLL (univariate analysis).

Gene	*Expression value relative to calibrator cell line*	*Total n=130 (%)*	*$IGHV_H$ >98% n=50 (%)*	*$IGHV_H$ ≤98% n=80 (%)*	*Binet stage A* n=59 (%)*	*del 13q14 n=86 (%)*	*del 17p13 n=11 (%)*	*del 11q22 n=18 (%)*	*+12 n=14 (%)*
ZAP70	≤4.6	75	54	88	78	78	67	61	57
	> 4.6	25	46	12	22	22	33	39	43
LPL	≤0.07	70	32	94	76	69	67	39	86
	>0.07	30	68	6	24	31	33	61	14
ADAM29	≤3.25	21	36	11	25	19	17	33	21
	> 3.25	79	64	89	75	81	83	67	79
SPG20	≤0.04	75	58	86	83	79	67	50	71
	> 0.04	25	42	14	17	21	33	50	29
NRIP1	≤11	83	100	73	71	84	92	100	86
	>11	17	0	27	29	16	8	0	14
AKAP12	≤0.94	76	62	90	83	73	91	47	86
	> 0.94	24	38	10	17	27	9	53	14
DMD	≤ 4.63	63	38	84	78	70	64	29	50
	> 4.63	37	62	16	22	30	36	71	50
CLECSF2	≤0.31	56	41	69	65	63	42	44	36
	> 0.31	44	59	31	35	37	58	56	64
TPM2	≤4.9	50	23	70	56	57	55	24	57
	> 4.9	50	77	30	44	43	45	76	43
SEPT10	≤75	73	46	95	81	76	73	41	79
	> 75	27	54	5	19	24	27	59	21

**Binet stage was known in 98 of 130 patients.*

Gene expression in relation to IGV_H mutational status

The *IGV_H* mutational status did not correlate with either age or gender (Table 2). The relation between gene expression and *IGV_H* mutational status is tabulated in Table 3. The relation of all expression values together and *IGV_H* mutational status were evaluated with a multivariate logistic regression analysis (Table 4). Stepwise exclusion of factors with the least significant value until p<0.05 left the expression of *ZAP70*, *LPL*, *ADAM29* and *SEPT10* as the most predictive genes for *IGV_H* mutational status. The optimal predictive model based on these expressions yielded an area under the receiver operating characteristic (ROC) curve of 0.90. When *LPL*, *ADAM29* and *SEPT10* were used as variables, the area under the ROC curve was 0.88.

Table 4. Ranking of gene expression with respect to their predictive value for *IGV$_H$* mutation status as determined in multivariate logistic regression analysis.

	full model			final model		
	odds ratio	(95% CI)*	p-value	odds ratio	(95% CI)	p value
LPL	0.21	(0.09–0.48)	< 0.001	0.22	(0.62–0.94)	0.010
ZAP70	0.76	(0.62–0.94)	0.013	0.764	(0.12–0.46)	0.010
ADAM29	2.21	(0.97–5.03)	0.059	2.301	(1.16–4.58)	0.018
SEPT10	0.52	(0.26–1.06)	0.073	0.486	(0.26–0.90)	0.022
NRIP1	2.07	(0.80–5.33)	0.132		-	
DMD	0.84	(0.48–1.46)	0.530		-	
AKAP12	1.27	(0.57–2.84)	0.559		-	
CLECSF2	0.77	(0.27–2.14)	0.612		-	
SPG20	0.97	(0.45–2.10)	0.944		-	
TPM2	1.01	(0.41–2.50)	0.986			

** For all expression values except for ZAP70 the logarithm (base 10) is taken because of the skewed distribution of the variables. The odds ratio corresponds to an increment of 1-log.*

Table 5. Gene expression in relation to *IGV$_H$* gene segment usage.

	V$_H$1-69 n=14 (%)	V$_H$3-21 n=16 (%)	V$_H$4-34 n=12 (%)
ZAP70 ≤4.6	64	47	100
LPL ≤0.07	29	80	91
ADAM29 ≤3.25	36	7	18
SEPT10 ≤75	36	67	91

Table 6. Levels of expression of the individual genes in normal hematopoietic subsets.

Gene	Cut -off point expression value relative to calibrator cell line	Expression value relative to calibrator cell line				
		monocytes	B cells	T cells	NK cells	granulocytes
ZAP70	4.6	0.1	0.1	9.5	31.8	3.66
LPL	0.07	0.05	0.07	0.01	0.09	0.13
ADAM29	3.25	8.60	34.75	16.63	209.70	3196.43
SPG20	0.04	4.62	9.73	4.93	10.57	145.71
NRIP1	11	349	444	77	1406	107
AKAP12	0.94	0.08	0.24	0.18	0.17	2.43
DMD	4.63	0.06	0.08	0.10	0.40	1.16
CLECSF2	0.31	0.84	10.75	3.68	48.17	295.29
TPM2	4.9	221.7	28.4	305.7	561.0	706.3
SEPT10	75	297	15	86	22	1178.86

When *ZAP70*, *ADAM29* and *SEPT10* were used in the logistic regression analysis the area under the ROC curve was 0.84. As a single marker *LPL* was the best predictor for *IGV$_H$* mutation (p<0.001) with an area under the ROC curve of 0.84. The correlations of the expression of *ZAP70, LPL, ADAM29* and *SEPT10* with V$_H$1-69, V$_H$3-21 and V$_H$4-34 are given in Table 5. For the other genes, a low expression of *SPG20*, *AKAP12*, and *DMD* was associated with V$_H$4-34, and a low expression of *NRIP1* with V$_H$1-69 (*data not shown*).

Gene expression in normal hematopoietic subsets

To investigate the potential risk of contamination by normal hematopoietic subsets, we determined the expression levels of the ten selected genes in normal granulocytes and FACS-sorted monocytes, B cells, T cells and NK cells (Table 6). *ZAP70* expression was, as expected, high in purified T- and NK-cells. In fact, the relative expression levels of *ZAP70, ADAM29, SPG20, NRIP1, CLECSF2, TPM2* and *SEPT10* were, in at least one of the normal subsets, well above the respective cut-off points. Contamination by these normal subsets would affect the prediction of the *IGV$_H$* mutational status on the basis of mRNA expression in unpurified CLL samples. The levels of expression of *LPL, AKAP12* and *DMD* are relatively low in normal hematopoietic subsets. The relatively high expression levels of all the selected genes in normal granulocytes are mainly due to the relative low expression of the reference gene in these granulocytes. Since all CLL samples were Ficoll-Hypaque separated, the CLL samples were generally devoid of granulocytes.

Gene expression in relation to other clinical prognostic factors

Based on the expression cut-off points (Table 3) the correlation of the expression of each single gene with respect to age, gender and Binet stage at diagnosis was calculated (Table 2). No correlation was found for age or gender (*data not shown*). The percentages of patients with Binet stage A disease and low gene expression levels are given in Table 3. These percentages are calculated for the 98 patients for whom Binet known stage at diagnosis was known. Prognostically favorable gene expression levels of *SPG20*, *AKAP12* and *SEPT10* showed the highest predictive value for this favorable clinical stage, each including about 80% of the patients. About 75% of the patients with Binet stage A had a low expression of *ZAP70* or *LPL*.

Gene expression and chromosomal aberrations

Interphase cytogenetics was performed on samples from all 130 patients. Abnormalities were seen in 86 patients (66%). Deletion 17p13 (*p53* gene) was found in 11 (8%) patients, trisomy 12 in 14 (11%), deletion 11q22 (*ATM* gene) in 18 (14%) and hemizygous or homozygous deletions of 13q14 as a single aberration were seen in 63 (48%) samples. In 28 (22%) patients none of these aberrations was observed. Translocation (11;14)(q13;q32), a characteristic occurrence in mantle cell lymphoma, was not found. The correlation of the expression of the different genes and the presence of the different chromosomal aberrations is shown in Table 3. Loss of 13q14, which as a single abnormality is associated with a favorable prognosis,[23] was associated with a low expression of all the genes under study, except for *ADAM29* which was highly expressed. Deletion 17p13, associated with short treatment-free intervals, was more frequent in *ADAM29* positive cases and in *NRIP1* and *AKAP12* negative cases. Deletion 11q22, which is also accepted as a indicator of unfavorable disease[23] was only seen in *NRIP1* negative cases, but did not show any association with the expression of the other genes. Trisomy 12, associ-

Table 7. Survival in relation to *IGVH* mutational status or level of expression of the selected genes.

	n	survival at 24 months (%)	p value
IGVH mutation			
no	50	62	<0.001
yes	80	92	
ZAP 70			
no	97	82	0.22
yes	33	76	
LPL			
no	91	89	0.007
yes	39	63	
ADAM29			
no	27	74	0.45
yes	103	82	
SPG20			
no	98	86	0.017
yes	32	62	
NRIP1			
no	108	79	0.17
yes	22	86	
AKAP12			
no	99	82	0.15
yes	26	75	
DMD			
no	82	89	0.008
yes	42	64	
CLECSF2			
no	73	80	0.37
yes	53	81	
SEPT10			
no	95	85	0.005
yes	30	66	
TPM2			
no	65	89	0.020
yes	60	72	

Cut-off points as in Table 3.

Table 8. Survival according to the combination of *IGVH* mutational status or *LPL* expression with cytogenetics in multivariate Cox regression model.

	n	*IGVH* mutation model HR	(CI)	p value	LPL model HR	(CI)	p value
LPL 10 log	130				1.821	(1.15- 2.88)	0.011
IGVH mutation no	50	1					
yes	80	0.28	(0.10–0.73)	0.010			
17p12 no	119	1			1		
yes	11	9.21	(3.37–25.13)	< 0.001	10.59	(3.93–28.51)	<0.001
11q22 no	112	1			1		
yes	18	2.13	(0.76–5.95)	0.149	2.32	(0.83 – 6.46)	0.109
tr12 no	116	1			1		
yes	14	0.54	(0.12 – 2.52)	0.433	0.65	(0.14 – 3.07)	0.582

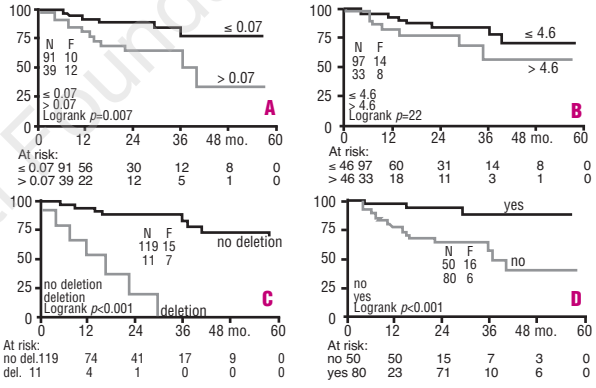

Figure 1. CLL survival analyses with single prognostic factors. Kaplan-Meier estimates of overall survival with regard to LPL expression (A), ZAP70 expression (B), 17p13 deletion (C) and IGVH mutational status (D). x axis: cumulative percentage.

ated with an intermediate prognosis, showed no obvious correlation with the expression of any of the genes investigated.

Gene expression and survival

The Kaplan-Meier estimates of overall survival at two years were calculated with regard to *IGVH* mutation status and the expression of the genes studied (Table 7). Among the ten genes studied, five were significantly associated with survival ($p<0.05$). All genes were included in a multivariate Cox model and a stepwise exclusion of factors with the least significant value until $p<0.05$ was performed. Only *LPL* remained in the model, with a hazard ratio of 2.00 (1.29–3.07). The multivariate prognostic model with *IGVH* mutation and *LPL* separately included are presented in Table 8. When Binet stage was included, a similar coefficient was observed but with a lower strength because of the substantial number of missing values (33 patients) (*data not shown*). Because of the high correlation of *LPL* expression and *IGVH* mutational status, their simultaneous inclusion in the model would weaken the model due to co-linearity. Both *LPL* expression and *IGVH* mutational status had a similar additional prognostic value to cytogenetic factors with respect to survival (p value 0.011 and 0.010, respectively). Survival curves in relation to *LPL* and *ZAP70* expression, 17p13 deletion and IGVH mutational status as a single factor are shown in Figure 1.

Discussion

In this study we confirm that *IGVH* mutational status is one of the most important prognostic factors in CLL. In our series of 130 patients, which we analyzed using the BIOMED-2 FR1 multiplex PCR approach, we found 98% homology to the nearest germline segment to be the most discriminatory value for survival, as was previously published .[11,23] Detailed analysis of gene

segment usage in non-mutated versus mutated CLL in our series of 130 patients showed preferential use of V_H1-69 in non-mutated CLL, whereas V_H3-21 and especially V_H4-34 were predominantly used in mutated CLL cases. These patterns are in accordance with earlier published studies,[26-29] thereby confirming that our series is representative of CLL with two distinct biological types.

Since mutational status is a good predictor of survival, genes that are differentially expressed by gene expression profiling in mutated versus unmutated CLL cases will also be prognostically significant. We selected ten genes consistently differentially expressed in published CLL gene expression profiling studies[13,14], and tested these genes with RQ-PCR in a panel of 130 well-characterized CLL patients, showing that the expression of these genes had prognostic significance. The strength of the predictive value was calculated by univariate analysis and in a multivariate model. Taken as a single factor, *LPL*, encoding lipoprotein lipase, showed the highest predictive value. *ZAP70,* also included in our selection and known to be associated with survival,[17,19,20] performed less well (both with respect to the association with mutational status and survival). This may be explained by the fact that we performed the investigations in cell suspensions that were isolated by density gradient centrifugation, but not purified for CLL cells by positive or negative selection. Thus, T lymphocytes and NK cells may falsely contribute to the *ZAP70* signal in *IGV$_H$* mutated cases (Table 6). Another explanation may be that *ZAP70* expression really does perform less well as a predictor for survival, as was recently reported by Opezzo *et al.*[30] who compared *LPL* and *ZAP70* in both unpurified and purified CLL suspensions. There is also a technical aspect which makes *ZAP70* less suitable for routine diagnostics, since in multiparametic flow cytrometry the marker setting for the discrimination between positive and negative *ZAP70* cases is subjective, as the brightness of expression between positive and negative cases is continuous.[19,20] Interestingly, we found that *LPL* is not expressed or only very weakly expressed in normal peripheral blood cells (Table 6) and, therefore, determination of its expression does not require extensive purification of the sample. Our data are in agreement with those of a very recent study by Heintel *et al.*,[31] who described *LPL* as a poor prognostic marker with strong correlation to mutational status, when tested in unsorted blood samples. None of the genes showed a bi-modal expression pattern. Cut-off points for positive or negative expression are, therefore, artificial and depend on technical aspects such as the calibrator cell line used. We used isotonic regression analysis for the determination of these cut-off points for each of the genes in order to find the best association with the unmutated status. These values were used throughout the study for the comparison with clinical prognostic factors, cytogenetic aberrations and survival.

With respect to the clinical factors, clinical stage was weakly associated with the expression of the genes, which could be expected since the association of both clinical stage and gene expression with mutational status is known.[11,23] The expression of *TPM2* showed the weakest association with both clinical stage and mutational status. Cytogenetic aberrations were found in a relatively small number of cases. Hence, no strong conclusions can be drawn from their association with mutational status, although deletion 13q14, associated with a favorable prognosis,[11,23] was seen more often in cases with favorable gene expression values.

The samples taken for this retrospective study were obtained at diagnosis or during the course of the disease. There is no change in mutational status during the disease, whereas some markers, e.g. deletion 17p13, may be acquired during the disease. It is not known whether the expression of the genes under study varies over time. It does, however, seem unlikely that *LPL* expression changes, for two reasons: the correlation between *LPL* expression and mutational status is high, irrespective of the time point at which blood is taken during the course of the disease and preliminary data in our laboratory showed no changes during longitudinal disease evolution. Nevertheless, because these data are circumstantial, we took as a measure of survival the duration from the day of sample collection until death from any cause or last contact. This is different from most studies that use the date of diagnosis as the first time point or time from diagnosis to first treatment as a surrogate for prognosis. Another reason for our choice was the probability of selecting in favor of long survivors in this retrospective analysis, if the date of diagnosis had been used. This might obscure possible unexpected expression values in patients who died before the date of analysis. A consequence of this choice is that the follow-up time in our study is rather short. Nevertheless, using this definition a clear separation with respect to survival is seen between mutated and unmutated cases. *LPL*, as a single gene, was the best predictor for survival in the univariate regression analysis. Using a multivariate analysis with the three cytogenetic abnormalities associated with a poor prognosis, deletions 17p13 and 11q22 and trisomy 12, we found *LPL* and *IGV$_H$* mutational status equal in their predictive value for survival. When clinical stage was included in the model, similar figures were obtained for mutational status and *LPL* expression values, but the model was weaker, due to missing data.

In summary, we have demonstrated that the expression of the ten genes that we selected from published gene expression profiling studies, is associated with either IGV$_H$ mutated or unmutated CLL cases when studied by RQ-PCR. The strongest association with mutation status was found for *LPL* mRNA levels, as very recently reported by Opezzo *et al.*[30] and Heintel *et al.*[31] The latter study found the same for LPL protein expression. *LPL* expression was also the strongest predictor for prognosis and appeared to have a complementary value to chromosomal abnormalities as a prognostic factor. LPL is a key enzyme in the hydrolysis of triglyceride-rich lipoproteins.[32] It may play a modulating role in the intensity and duration of activation by cell surface bound receptors, possibly by

inhibiting transcription factor NFκB and by abrogating IκB-α.[32] The function of this gene in connection with B-cell differentiation or antigen responses is not, as yet, known.[33] Nevertheless, its strength as a prognostic factor in CLL, is comparable to that of *IGV$_H$* mutational status and *ZAP70*. These three factors are closely linked and add no mutual advantage, in contrary to cytogenetic factors and probably clinical stage. However, in clinical practice the use of *LPL* as a prognostic factor has advantages over the other two. For instance, *LPL* expression is much less laborious to investigate than is *IGV$_H$* mutational status and the results are not affected by the presence of other blood cells in the sample, as is the case with *ZAP70*.

MBvV, AWL, KvL and PJMV: research design, performed research, analyzed data, wrote manuscript; AMB, BV and CSG: performed research, analyzed data; : research design, performed research, analyzed data; WJG: performed research, analyzed data, wrote manuscript.

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