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ANALYSIS OF *LPL* GENE EXPRESSION IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Aim: **The *IGHV* mutational status is one of the most important markers for chronic lymphocytic leukemia (CLL) prognostication. Lipoprotein lipase (*LPL*) gene expression was found to correlate with *IGHV* status and was suggested as its surrogate marker. Recent data reported that *LPL* expression might be influenced by pivotal signalling pathways in CLL. This study aimed to assess *LPL* gene expression in relation to key immunogenetic and molecular markers of CLL, including *IGHV* mutational status, B-cell receptor (BCR) stereotypy, *TP53, NOTCH1,* and *SF3B1* gene mutations. *Materials and Methods:* Expression of LPL mRNA was measured in peripheral blood mononuclear cells of 73 CLL patients by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). *IGHV, NOTCH1, TP53,* and *SF3B1* gene mutation analysis was performed by PCR amplification and direct sequencing. *Results:* 44 of 73 (60%) CLL cases were categorized as *LPL*-positive based on the cut-off value established by ROC (receiver operating characteristic) curve analysis. *LPL* expression was significantly associated with *IGHV* mutation status ($r = 0.684$; $p < 0.0001$) and tended to correlate with presence of *NOTCH1* gene mutations ($p = 0.113$). BCR stereotyped cases showed higher *LPL* expression values in comparison to unstereotyped cases in the *LPL*-positive group of patients ($p = 0.041$). *LPL* expression was associated with a shorter overall survival in the entire CLL group (median 107 *vs* 143, $p = 0.048$) as well as in Binet A patients, albeit with borderline significance (median 139 *vs* not reached, $p = 0.086$). *Conclusion: LPL* expression was found to be closely correlated with *IGHV* gene mutational status and overall survival, proving *LPL* as prognostic marker in CLL. Our results also indicate a possible relationship between aberrant expression of *LPL* and BCR- and NOTCH1-dependent signalling pathways.**

Key Words: **CLL, *LPL, IGHV* mutational status, BCR stereotypy, *NOTCH1* mutations.**

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in adults, characterized by a highly variable clinical course. While one third of patients present with a stable asymptomatic disease, which does not require any treatment and is characterized by nearly normal life expectancy, others invariably progress and ultimately develop chemorefractoriness [1]. Such clinical heterogeneity is a reflection of biological heterogeneity. Several genetic and molecular features have been identified in CLL, which might affect significantly prognosis of the disease. These include the mutational status of the immunoglobulin heavy chain variable (*IGHV*) genes, certain recurrent genomic aberrations (deletions: 13q, 17p, 11q; trisomy 12), mutations in genes, which regulate apoptosis, DNA damage response or cell signalling (*TP53, ATM*, *NOTCH1, SF3B1, BIRC3*, *MYD88*, etc.), the expression of certain stereotyped B-cell receptors (BCRs) [2–5].

Currently, *IGHV* mutational status, which is associated with antigen binding capacity and functions of the BCR, is considered as one of the most important prognostic biomarkers in CLL independent of clinical stage [2, 3]. Patients carrying somatic hypermutations in their *IGHV* genes experience an indolent course compared to patients with unmutated (UM) *IGHV* genes and have better response to standard chemoimmunotherapy [6–8]. Gene expression profiling analysis of CLL revealed several genes correlating closely with the *IGHV* mutational status, including *COBLL1*, *ZAP70*, *ADAM29*, *TCL1A, CLLU1, LPL* [9–12].

Lipoprotein lipase (*LPL*) was identified as one of the most differentially expressed genes in *IGHV* UM *vs IGHV* mutated (M) CLL, with a significantly higher expression in the former group [9, 10]. High levels of LPL protein were detected also in the cytosol of CLL cells and on the cell surfaces [13, 14]. Additionally, several studies associated high LPL mRNA expression with short treatment-free (TFS) and decreased overall survival (OS) [13, 15–18]. *LPL* expression was also correlated with other prognostic markers: ZAP70, CD38, cytogenetic aberrations, and clinical stage [12, 13, 16, 19]. Thus, LPL is considered to be a reliable RNA-based marker for CLL prognosis. It is assumed, that it might also contribute to CLL cell survival and metabolism [20, 21].

LPL plays a central role in lipid metabolism and catalyzes the hydrolysis of very-low-density lipoproteins and chylomicrons, releasing free fatty acids (FFA). Additional noncatalytic binding functions lead to increased accumulation and cellular uptake of lipoproteins [22]. LPL is normally expressed in adipocytes and muscle cells. Lower levels of this protein are produced by macrophages, hormone-secreting cells in the adrenals and ovaries, certain neuronal cells, spleen, testes, lung and kidney [23]. LPL expression was not found in normal

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Abbreviations used: BCR – B-cell receptor; CLL – chronic lymphocytic leukemia; FFA – free fatty acids; *IGHV* – immunoglobulin heavy variable; LPL – lipoprotein lipase; M – mutated; OS – overall survival; PCR – polymerase chain reaction; TFS – treatment-free survival; UM – unmutated; VH CDR3 – variable heavy complementarity-determining region 3.

B-lymphocytes [13]. Despite aberrant *LPL* expression in CLL has been reported long ago, molecular mechanisms driving this expression as well as exact function of LPL in CLL cells and its effect on progressive disease development are not fully understood.

Recent data suggest that LPL might be involved in some key signalling pathways in CLL. It was demonstrated *in vitro* that in CLL cells *LPL* expression is increased by BCR cross-linking [24]. It was also shown, that *LPL* can be transcriptionally regulated by STAT3, a latent cytoplasmic transcription factor, which is constitutively serine-phosphorylated in CLL [25]. Additionally, Kristensen *et al*. [26] reported that LPL mRNA expression correlates closely with *NOTCH1* gene mutations in CLL patients and, based on the role of NOTCH1 signalling in adipose tissue, interference between the NOTCH1 pathway and *LPL* expression was suggested.

In our study we investigated *LPL* gene expression in relation to key immunogenetic and molecular markers, including *IGHV* mutational status, BCR stereotypy, *TP53, NOTCH1,* and *SF3B1* gene mutations. We also analysed the correlation of *LPL* expression status with clinical outcome.

MATERIALS AND METHODS

Patients and samples. The studied group consisted of 73 CLL patients (64 males and 9 females) referred to the State Institution "National Research Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine". The study was approved by the local Ethics Review Committee. All patients gave the informed consent. The diagnosis of CLL was based on clinical history, lymphocyte morphology and immunophenotypic criteria. The clinical stage of CLL was determined based on the Binet [27] and Rai [28] staging systems. Treatment requirement was estimated according to National Cancer Institute criteria [29].

The majority of patients (n = 64, 87.7%) were untreated at the time of blood collection and nine were treated with various therapeutic regimens using chlorambucil (5) and fludarabine (4). Previously treated patients were observed in relapse before the next course of therapy. At time of *LPL* expression analysis, mutational status of *IGHV* and *NOTCH1* genes was evaluated in all 73 patients, *TP53* — in 72, and *SF3B1* — in 69 patients.

Genomic DNA and total RNA were extracted from peripheral blood mononuclear cells (PBMCs) with the QIAamp Blood Mini Kit (Qiagen, Crawley, United Kingdom) and TRI Reagent® (Molecular Research Center Inc., Cincinnati, OH), respectively. The concentration and purity of RNA were assessed with NanoDrop Spectrophotometer (NanoDrop Technology). Purified RNA was stored in deep freeze until further use.

Determination of IGHV mutational status and DNA sequence analysis. The *IGHV* gene mutational status was assessed by polymerase chain reaction (PCR) amplification followed by direct sequencing as reported previously [30]. DNA sequence analysis and interpretation of *IGHV/IGHD/IGHJ* rearrangements were performed using the IMGT database (http://imgt.cines.fr, Montepellier, France) and the IMGT/V-QUEST tool [31, 32]. Clonotypic *IGHV* sequences with $\geqslant$ 98% homology to the closest germ-line gene were considered as UM, and cases with < 98% homology were considered as M [6, 7]. Assignment of cases to specific stereotyped subsets was performed following established guidelines and based on the following stringent criteria: the Ig sequences were connected if: 1) sharing at least 50% amino acid identity and 70% similarity within the variable heavy complementarity-determining region 3 (VH CDR3); 2) having identical VH CDR3 lengths and identical offsets of shared patterns between sequences; and 3) carrying *IGHV* genes of the same phylogenetic clan [33]. Stereotyped subsets were defined and named according to published criteria [33–35].

TP53, NOTCH1 and SF3B1 mutation analysis. Mutation analysis was done by PCR amplification followed by direct sequencing. Screening for *TP53* gene mutations was performed for exons 3 to 10 as described previously [36]. *NOTCH1* mutations and *SF3B1* mutations were analyzed in the hotspot regions of these genes, where the vast majority of CLL-specific lesions were reported [37–39]. *NOTCH1* gene mutations were analyzed in c.7282_7680 region in exon 34, and in the 3′UTR region *(9:136,495553-136,495994)*, while *SF3B1* gene mutations were studied in exons 14, 15 and 16, as described before [40, 41].

Quantitative real-time PCR (RT-qPCR). For gene expression analysis, cDNA was synthesized from 5 μg RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), according to the supplier's instructions. RT-qPCR was performed with the Bio-Rad Real-time IQ Detector System (Bio-Rad) using the SYBR Green chemistry. Amplification was carried out with 2 μl of cDNA in a 25 μl reaction mixture containing 0.07 μM of each primer and Absolute Blue qPCR SYBR Green Fluorescein Mix (Thermo Scientific). PCR cycling conditions were: an initial denaturation step of 95 °C for 15 min, then 45 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. All reactions were done in duplicate. The expression levels of *LPL* were normalized to the expression of the housekeeping gene *ABL1* (v-abl Abelson murine leukemia viral oncogene homolog 1). The following primers were used: for *LPL* — 5′-CAGCAGCAAAACCTTCATGGT-3′ and 5′-AGTTTTGGCACCCAACTCTCA-3′ (reported by Van Bockstaele *et al.* [16]); for *ABL1* — 5′-TGGAGATAACACTCTAAGCATAACTAAAGGT-3′ and 5′-GATGTAGTTGCTTGGGACCCA-3′ (reported by Beillard *et al.* [42]). The relative *LPL* expression was determined using the comparative C_T ($2^{-\Delta\Delta Ct}$) method. Validation experiment was carried out preliminary following Van Bockstaele *et al*. [16], which confirmed the applicability of C_T ($2^{-\Delta\Delta Ct}$) method for evaluation of *LPL* expression. *LPL* expression in lymphocytes obtained from a healthy donor was used as a calibrator for relative quantification.

Statistical analysis. Receiver operating characteristic (ROC) curve analysis was performed to determine the *LPL* expression cut-off values that best distinguished between *IGHV* M and UM cases. Association between *LPL* and *IGHV* mutational status was described

using the Spearman correlation coefficient. Shapiro — Wilk's test was applied to assess normal distribution. The Mann — Whitney U test was used to compare median levels of *LPL* between groups. Associations between *LPL* expression status and other categorical clinico-biological variables were assessed with the chi-square test or the Fisher exact test, where appropriate. Kaplan — Meier method was used to analyse OS and TFS. The log-rank statistic was used to determine significant associations between individual markers and OS or TFS. p values < 0.05 were considered statistically significant. All analyses were performed with the SPSS software package, version 13.0 (SPSS).

RESULTS

Clinical and biological characteristics of CLL patients. The group of patients consisted of 64 men and 9 women, with a median age of 57 years (range: 39–76 years) at the time of diagnosis. Clinical and biological characteristics of patients are summarized in Table 1. Forty-eight patients (65.8%) had UM *IGHV* genes, whereas 25 (34.1%) patients had M *IGHV*. Seventeen patients (23.3%) were assigned to one of 12 previously reported BCR stereotyped subsets, based on VH CDR3 similarity [33–35]. Subset # 2 was the most frequently represented stereotyped variant (5 cases) in this CLL group followed by subset # 1 (2 cases). Other 10 identified subsets were represented by one case each (Table 2).

Table 1. Baseline clinical characteristics of observed CLL patients

Characteristics		Patients, n (%)
Total		73 (100)
Median age, years (range)		57 (39–76)
Gender	Male	64 (87.7)
	Female	9 (12.3)
Binet stage at diagnosis	A	38 (52.1)
	B	28 (38.3)
	C	7 (9.6)
Clinical phases of CLL	Did not require first treatment	34 (46.6)
	Required first treatment	30 (41.1)
	Relapsed	9 (12.3)
IGHV mutational status	M	25 (34.2)
	UM	48 (65.8)
BCR stereotypy	Stereotyped	17 (23.3)
	Unstereotyped	56 (76.7)
TP53	M	7 (9.7)
	UM	65 (90.3)
NOTCH1	M	11 (15.1)
	UM	62 (84.9)
SF3B1	M	8 (11.6)
	UM	61 (88.4)

Mutations in *NOTCH1* gene were the most frequent genetic abnormality in the present group, being detected in 11 of 73 (15.1%) of patients. They were mostly represented by a typical two base pair deletion c.7544_7545delCT (P2514fs*4) localized in exon 34 (10 (13.7%)) cases. In one case (1.4%) missense substitutions g.139390152 A>G in 3`UTR region of *NOTCH1* gene was revealed. Mutations in *TP53* gene were found in seven of 72 (9.7%) cases and were represented mainly by missense substitutions (5 cases, 71%). *SF3B1* mutations were detected in eight of 69 (11.6%) cases and were represented by missense substitutions in all detected cases, which is consistent with previous reports [37, 38].

LPL expression and correlation with IGHV mutational status. Relative *LPL* expression levels in CLL samples ranged from 0 to 1663.5. UM *IGHV* cases showed a significantly higher *LPL* expression (median = 84.5, range 0.3–1663.5) in comparison with M cases (median = 1.1, range 0–27.9), $p < 0.0001$ (Fig. 1). A strong correlation between individual *LPL* expression levels and *IGHV* mutational status was found (r = 0.684; $p < 0.0001$). We applied ROC curve analysis to calculate cut-off value for the *LPL* expression to distinguish *IGHV* M and UM cases. The cut-off value of 17 showed the best degree of discrimination. With this cut-off value 65 of 73 patients (89%) were classified correctly into the *IGHV* M and UM group, and this value was used for further analysis of associations of *LPL* expression with clinico-biological variables and clinical outcome.

Prognostic value of LPL expression. Based on the cut-off value of 17, 44 (60.3%) CLL cases were classified as *LPL*-positive and 29 (39.7%) cases as *LPL*-negative. Follow-up information was available for all patients, 29 (39.7%) of whom died during the period of observation due to CLL-related causes. The estimated median OS was 106 months (95% CI 68.2–143.8 months). Log-rank tests for the Kaplan — Meier curves demonstrated significantly reduced OS for *LPL*-positive CLL patients in comparison with *LPL*-negative patients (107 *vs* 143 months), $p = 0.048$. Similar results were obtained, when the analysis was restricted to patients with stage A disease. The median

Table 2. Immunogenetic characteristics of CLL cases assigned to BCR stereotyped subsets

Subset	*IGHV* mutational status	*IGHV* gene	*IGHD* gene	*IGHJ* gene	VH CDR3 pattern	VH CDR3 length	Clinical outcome*
# 1	UM	IGHV1-69	IGHD6-19	IGHJ4	ARDQWLPITSFDY	13	Aggressive
# 1	UM	IGHV1-2	IGHD6-19	IGHJ4	ARDQWLVLPNFDY	13	Aggressive
# 2	M	IGHV3-21	ND	IGHJ6	ARDANGMDV	9	Aggressive
# 2	M	IGHV3-21	ND	IGHJ6	ARDANGMDV	9	Aggressive
# 2	M	IGHV3-21	ND	IGHJ6	AREQNAMDV	9	Aggressive
# 2	M	IGHV3-21	ND	IGHJ6	ASDRNGMDV	9	Aggressive
# 2	M	IGHV3-21	ND	IGHJ6	VRDANGMDV	9	Aggressive
# 3	UM	IGHV1-69	IGHD2-2	IGHJ6	AREAPDIVVVPAAIRRVYGMDV	22	Aggressive
# 5	UM	IGHV1-69	IGHD3-3	IGHJ6	ARVQVFGVVNTYYYYYYMDV	20	Aggressive
# 7B	UM	IGHV1-69	IGHD3-3	IGHJ6	ARGRNYDFWSGPTWGYYYYYMDV	23	Aggressive
# 16	M	IGHV4-34	IGHD2-15	IGHJ6	AGRFYCSGATCHAAQFYYYYGLDA	24	Very indolent
# 26	UM	IGHV3-21	IGHD6-13	IGHJ6	ARDRGVSSSWYLSYYYYMDV	20	*ND*
# 28A	UM	IGHV1-2	IGHD1-26	IGHJ6	ARPYSGSYPWYYYGMDV	17	*ND*
# 77	M	IGHV4-59	IGHD6-19	IGHJ4	GRGPNESGWLGLDS	14	Indolent
# 98	UM	IGHV3-30	IGHD3-3	IGHJ6	ARAVATYYDFWSGLTPHWYYYGMDV	25	*ND*
# 109	UM	IGHV3-11	IGHD3-10	IGHJ5	ARDNVLYYGSGSYFNWFDP	19	*ND*
UA1	UM	IGHV3-33	IGHD3-22	IGHJ4	ARDTTYYYDSSGYYRGWGHAFDI	23	*ND*

Note: *ND* – not defined. *According to Baliakas *et al.* [44].

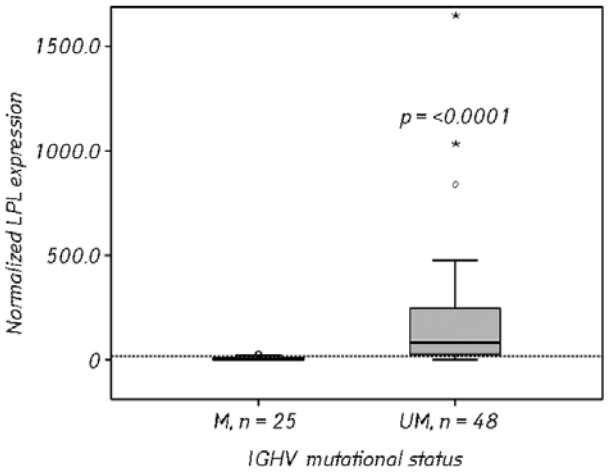

Fig. 1. *LPL* expression in relation to *IGHV* mutation status. Boxplots show relative expression of *LPL* in CLL patients with M and UM *IGHV* genes. Median, 25 and 75 percentile values, non-outlier ranges, outliers and extremes are indicated. The *p* value was calculated using the Mann — Whitney U test. Optimal cut-off value for *LPL* expression was determined by ROC curve analysis and is indicated by a dot line

OS in *LPL*-positive patients was 139 months, while the median OS was not reached in the *LPL*-negative group, although this difference did not reach statistical significance ($p = 0.086$) (Fig. 2). For the entire CLL group, significant association was also found between OS and Binet staging ($p = 0.002$), *IGHV* mutational status ($p = 0.014$), *TP53* and *SF3B1* mutations ($p = 0.001$) (Table 3). The median OS of the *IGHV* UM group was similar to that of the *LPL*-positive group (101 and 107 months respectively). For Binet A patients, *IGHV* mutation status and *TP53* status remained significant predictors of OS ($p = 0.041$ and 0.038, respectively) (Table 4).

Regarding TFS, although *LPL*-positive patients had shorter TFS in comparison with *LPL*-negative patients (medians 24 months vs 42 months), the difference was not statistically significant ($p = 0.101$). Meanwhile, significant difference in TFS was found for patients differing in *IGHV* mutation status ($p = 0.002$).

LPL expression in relation to other prognostic markers. High *LPL* expression correlated with Binet stage, while no significant differences in the

Table 3. OS and time to treatment in CLL patients according to clinical and molecular characteristics

Characteristics	N	OS, median, months	*p*	Time to treatment, median, months	*p*
Binet stage at diagnosis			0.002		< 0.0001
A	38	142		60	
B	28	75		6	
C	7	68		4	
IGHV mutational status			0.014		0.002
UM	48	101		12	
M	25	*NR*		88	
BCR stereotypy			0.123		0.839
Stereotyped*	15	101		59	
Unstereotyped	56	114		25	
TP53			0.001		0.103
M	7	52		6	
UM	65	133		38	
NOTCH1			0.179		0.133
M	11	80		6	
UM	62	117		34	
SF3B1			0.001		0.116
M	8	52		6	
UM	61	139		38	
LPL expression			0.048		0.101
Positive	44	107		24	
Negative	29	143		42	

Note: *NR* – not reached. *Stereotyped cases excluding # 16 and # 77 subsets, associated with indolent clinical course.

Table 4. OS and time to treatment in Binet A patients according to clinical and molecular characteristics

Characteristics	N	OS, median, months	*p*	Time to treatment, median, months	*p*
IGHV mutational status			0.041		0.037
UM	20	117		45	
M	18	*NR*		96	
BCR stereotypy			0.195		0.976
Stereotyped*	6	117		60	
Unstereotyped	30	146		60	
TP53			0.038		0.001
M	3	42		14	
UM	35	143		60	
NOTCH1			0.716		0.740
M	4	139		53	
UM	34	142		60	
SF3B1			0.595		0.751
M	2	133		59	
UM	35	143		60	
LPL expression			0.086		0.235
High	20	139		59	
Low	18	*NR*		96	

Note: *NR* – not reached. *Stereotyped cases excluding # 16 and # 77 subsets, associated with indolent clinical course.

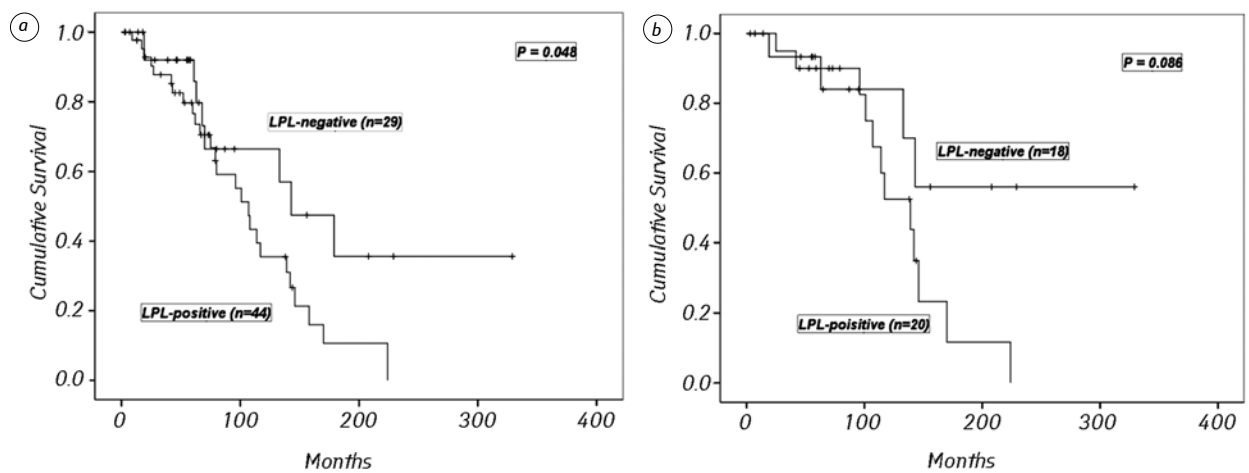

Fig. 2. Survival analysis: *a* — OS in CLL patients with positive and negative *LPL* status; *b* — OS in stage A patients regarding *LPL* expression status

expression were found concerning patient gender, age at diagnosis and previous treatment. There was no correlation between *LPL* values and the presence of *TP53* or *SF3B1* mutations. The vast majority of *NOTCH1* mutated cases (9 of 11; 81.8%) demonstrated *LPL*-positive status, although the difference between groups did not reach significance, $p = 0.113$ (Table 5).

LPL expression in relation to BCR stereotyped subsets. It was reported before, that *LPL* is differently expressed in some prognostically distinct stereotyped subsets [14, 43]. Twelve of 17 BCR stereotyped cases identified in our CLL group were assigned to seven major stereotyped subsets with defined clinical outcome [44], namely poor-prognostic subsets (# 1, # 2, # 3, # 5, # 7B), and favourable-prognostic subsets (# 16 and # 77) (Table 2). Comparison of these two groups regarding to *LPL* expression status showed correlation of *LPL*-positivity with poor prognostic subsets ($p = 0.048$), when subset # 2 cases were excluded. Stereotyped subset # 2 is characterized by restricted usage of *IGHV3-21* gene, which is often M and a typical short VH CDR3 pattern [33]. In our CLL group, all 5 subset # 2 cases were *IGHV3-21*/M and were classified as *LPL*-negative using cut-off value defined by ROC analysis. This is consistent with data by Mansouri *et al.* [14], where M/stereotyped *IGHV3-21* cases showed low *LPL* mRNA expression and did not differ in this regard from other M CLL cases. In line with this, a strong correlation was found between *LPL* expression and *IGHV* mutational status in the entire group of patients with stereotyped BCR — all 10 stereotyped/UM cases were categorised as *LPL*-positive, while all 7 stereotyped/M cases — as *LPL*-negative ($p = 0.0001$). Thus, differential *LPL* expression in certain stereotyped subsets might simply reflect correlation between *LPL* expression and *IGHV* mutational status. Additionally, we compared the *LPL* expression levels between BCR stereotyped and unstereotyped cases in *LPL*-positive CLL group (all cases were *IGHV* UM). Stereotyped cases showed higher *LPL* mRNA expression in comparison to unstereotyped cases (median, 251 *vs* 82), the differences were statistically significant, $p = 0.041$ (Fig. 3).

Table 5. *LPL* expression in relation to clinical and molecular characteristics

Characteristics		*LPL*, n (%) positive	*LPL*, n (%) negative	*p*
Age at diagnosis	< 57 years	24 (63.2)	14 (36.8)	0.6
	57 years and more	20 (57.1)	15 (42.9)	
Gender	Male	39 (60.9)	25 (39.1)	0.985
	Female	5 (55.6)	4 (44.4)	
Binet stage at diagnosis	A	20 (52.6)	18 (47.4)	0.02
	B	22 (78.6)	6 (21.4)	
	C	2 (28.6)	5 (71.4)	
TP53	UM	39 (60)	26 (40)	0.556
	M	5 (71.4)	2 (28.6)	
NOTCH1	UM	35 (56.5)	25 (43.5)	0.113
	M	9 (81.8)	2 (18.2)	
SF3B1	UM	36 (59)	25 (41)	0.850
	M	5 (62.5)	3 (37.5)	
BCR stereotypy	Stereotyped	10 (58.8)	7 (41.2)	0.889
	Unstereotyped	34 (60.7)	22 (39.3)	
BCR stereotypy/*IGHV* status	Stereotyped/UM	10 (100)	0	0.000
	Stereotyped/M	0	7 (100)	

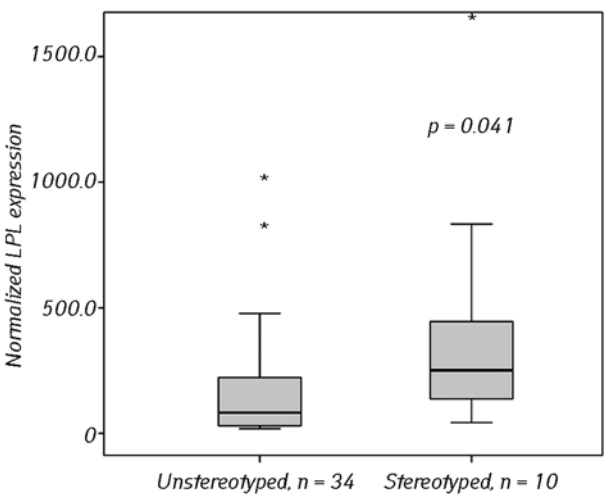

Fig. 3. The expression levels of *LPL* in relation to BCR stereotypy. Boxplots represent BCR stereotyped and unstereotyped CLL cases. The *p* value was calculated using the Mann — Whitney U test

DISCUSSION

Nowadays, the *IGHV* mutational status is one of the most important prognostic biomarkers in CLL. Besides, it is also regarded as a powerful predictive marker for estimation of response duration after standard therapy with FCR (fludarabine, cyclophosphamide and rituximab). CLL patients harbouring M *IGHV* genes, in particular, when combined with favorable cytogenetics, were shown to have a high chance of achieving a durable remission after FCR [8, 45, 46]. *IGHV* mutational status assessment is recommended by the current guidelines in CLL patients requiring therapy [47]. Along with that, the suggestion was made to include *IGHV* evaluation as standard clinical tests for all patients with newly diagnosed CLL since a greater understanding of the risk of disease progression at the time of diagnosis is of importance in the context of individual risk-adapted clinical management [48].

Determination of the *IGHV* mutation status is quite laborious and not readily available in most routine diagnostic laboratories. This induced the search for surrogate markers with the same prognostic value as *IGHV.* Gene expression studies have suggested a number of possible alternatives with *LPL* expression being one of the better markers for distinguishing *IGHV* M and UM cases [12–16]. *LPL* was identified as one of the most differentially expressed genes between M and UM CLL cases in the initial gene expression profiling studies of Rosenwald *et al*. [9] and Klein *et al*. [10]. Considerable differences in the *LPL* expression were confirmed by other researches. In particular, Heintel *et al*. [13] reported 34-fold higher, and Kaderi *et al*. [12] — 73-fold higher median *LPL* expression in UM *vs* M CLL cases, making a clear distinction between the two groups of patients. Additionally, several studies showed that *LPL* mRNA expression is associated with short TFS and decreased OS in CLL [12, 13, 16–19, 26].

Our results, being in accordance with previous studies, proved *IGHV* mutation status as a reliable prognostic marker for OS ($p = 0.014$) as well as TFS ($p = 0.002$). It also remained significant predictor when only Binet A patients were considered ($p = 0.041$ and $p = 0.037$ for OS and TFS, respectively). It is of importance, since the majority of CLL patients nowadays are diagnosed at the early disease stages.

We also revealed a strong correlation of *LPL* expression with *IGHV* mutational status in our CLL group. The discriminating cut-off value defined by ROC curve analysis allowed classification into the *IGHV* M or UM subgroups in 89% of patients. *LPL* expression showed a positive predictive value of 95% (42 of 44 *LPL*-positive cases were *IGHV* UM), and a negative predictive value of 79% (23 of 29 *LPL*-negative cases were *IGHV* M). This is comparable with previous reports on different CLL cohorts [13, 15, 16]. When analysed with regard to clinical outcome, *LPL* expression was associated with OS ($p = 0.048$), but the differences concerning TFS did not reach statistical significance ($p = 0.101$).

Thus, our data in line with previous reports [12–19] suggest that *LPL* expression could be a useful surrogate marker for *IGHV* mutational status in CLL. The important advantage of *LPL* as a marker is that it might be measured in unselected peripheral blood mononuclear cells, since its expression in normal hematopoietic subsets are found at very low levels [13, 17].

Besides aberrant *LPL* gene expression, it was demonstrated that CLL cells are able to synthesize LPL protein and capture it on the cell surface [13]. The functional role of LPL as well as its influence on progressive disease phenotype development, if any, are still not fully understood. It was suggested that LPL might play an important role in fatty acid metabolism and energy supply to B-CLL cells and could help the leukemic clone to increase survival and proliferative signalling, leading to disease progression [20, 21].

Molecular mechanisms, which drive aberrant expression of *LPL* gene in CLL are also still unknown. It is assumed that *LPL* expression might be influenced by BCR-dependent signaling pathways. Pallasch *et al*. [24] demonstrated that in CLL cells *LPL* expression was increased by BCR cross-linking. Rozovski *et al*. [25] showed that *LPL* expression can be transcriptionally regulated by signal transduction and activator of transcription 3 (STAT3) phosphorylation, and nuclear translocation where it can bind *LPL* promoter. Later, it was demonstrated that in CLL cells, the STAT3 phosphorylation is induced under activation of BCR [49]. Recently it was shown also that the BCR inhibitor ibrutinib reduced *LPL* mRNA and protein levels and inhibited FFA metabolism in CLL cells *in vitro*. Likewise, in CLL cells from ibrutinib-treated patients, FFA metabolism was reduced and eventually stopped [50]. In our CLL cohort we observed higher *LPL* mRNA expression in BCR stereotyped compared to unstereotyped cases ($p = 0.042$). These also may be in favor of relationship between *LPL* expression and BCR-driven signaling, since stereotyped BCRs were shown to exhibit higher BCR reactivity [51, 52].

Other possible molecular mechanisms regulating *LPL* expression in CLL are also reported. Kristensen *et al*. [26] recently showed that LPL mRNA expression correlates closely with *NOTCH1* gene mutations in CLL patients and, based on the role of NOTCH1 signalling in adipose tissue, interference between the NOTCH1-dependent pathway and *LPL* expression was suggested. In line with this we found, that most of *NOTCH1* mutated cases in observed CLL group were *LPL*-positive (9 of 11 cases, 81.8%), although the differences did not reach statistical significance ($p = 0.113$).

In summary, the results of our study show close correlation of *LPL* expression with *IGHV* gene mutational status and OS, proving *LPL* as a reliable RNA-based prognostic marker in CLL. Relationships between *LPL* aberrant expression and BCR- and NOTCH1-dependent signalling pathways in CLL needs to be studied further.

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