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High Expression of *Lymphocyte-Activation Gene 3* (*LAG3*) in Chronic Lymphocytic Leukemia Cells Is Associated with Unmutated *Immunoglobulin Variable Heavy Chain Region* (*IGHV*) Gene and Reduced Treatment-Free Survival

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Chronic lymphocytic leukemia (CLL) is characterized by a monoclonal expansion of mature B-lymphocytes. Mutational status of the *immunoglobulin variable heavy chain region* (*IGHV*) gene stratifies CLL patients into two prognostic groups. We performed microarray analysis of CLL cells using the Agilent platform to detect the most important gene expression differences regarding *IGHV* status in CLL cells. We analyzed a cohort of 118 CLL patients with different *IGHV* mutational status and completely characterized all described prognostic markers using expression microarrays and quantitative real-time RT-PCR (reverse transcription PCR). We detected *lymphocyte-activation gene 3* (*LAG3*) as a novel prognostic marker: *LAG3* high expression in CLL cells correlates with unmutated *IGHV* ($P < 0.0001$) and reduced treatment-free survival ($P = 0.0087$). Furthermore, quantitative real-time RT-PCR analysis identified a gene-set (*LAG3*, *LPL*, *ZAP70*) whose overexpression is assigned to unmutated *IGHV* with 90% specificity ($P < 0.0001$). Moreover, high expression of tested gene-set and unmutated *IGHV* equally correlated with reduced treatment-free survival ($P = 7.7 * 10^{-11}$ vs. $P = 1.8 * 10^{-11}$). Our results suggest that IGHV status can be precisely assessed using the expression analysis of *LAG3*, *LPL*, and *ZAP70* genes. Expression data of tested markers provides a similar statistical concordance with treatment-free survival as that of the IGHV status itself. Our findings contribute to the elucidation of CLL pathogenesis and provide novel prognostic markers for possible application in routine diagnostics. *(J Mol Diagn 2010, 12:328–334; DOI: 10.2353/jmoldx.2010.090100)*

B-cell chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder with a highly variable clinical outcome, characterized by clonal expansion of mature B-cells expressing cell surface antigens CD5, CD23, and CD27, and low levels of surface Ig.[1,2] There are several independent prognostic factors used in prediction of clinical outcome of CLL disease. Apart from traditional Rai[3] and Binet[4] staging, lymphocyte doubling time, morphology, immunophenotype, β-2-microglobulin or lactate dehydrogenase, cytogenetics, and molecular markers assume an important place. There are four most common recurrent genomic alterations with prognostic significance detectable in CLL cases[5]: deletion 13q has a favorable clinical outcome compared with normal karyotype, deletions 17p, and 11q, and trisomy 12 are negative prognostic markers. The mutational status of the *immunoglobulin variable heavy chain region* (*IGHV*) gene is one of the most important molecular prognostic factors in CLL. The presence of unmutated *IGHV* gene identical with the germ line sequence by more than 98% is associated with a worse prognosis and shorter survival.[6,7] The reason for the close correlation of the *IGHV* mutational status and different clinical course remains a matter of intense debate. The presence of somatic hypermutation in *IGHV* in half of CLL cases led to the theory that CLL cases with unmutated *IGHV* emerged from naive B-cells meaning that these cells are not antigen-experienced. On the other hand, CLL with mutated *IGHV* were supposed to develop from post-germinal center (GC) B-cells. This hypothesis was consequently rejected with detailed immunophenotypic studies[8] and also with gene expression profiling using high-throughput approaches.[9–13] Moreover identification of a physiological analogue to CLL cells remains unsuccessful.

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Several gene expression profiling studies have focused on comparison of cells with mutated and unmutated *IGHV*.[9,10,13] Surprisingly the differences between gene expression of CLL cells with mutated and unmutated *IGHV* were very low and the expression signatures did not correspond to any known physiological counterpart of B-cells in humans. However there were defined genes with slightly different gene expression between these two prognostic groups. Such studies also tried to establish a set of simply detectable markers that could help to improve stratification of newly diagnosed patients or even replace the analysis of *IGHV* mutational status. Using microarray approaches, *ZAP70* (*ζ-chain associated protein kinase 70 kDa*) was defined as a new prognostic marker,[9] and higher expression of this tyrosine kinase in CLL cells correlates to some extent with unmutated *IGHV* and worse prognosis. A lot of effort has been devoted to standardization of *ZAP70* routine quantification[14] and correlation between *ZAP70* expression and *IGHV* mutational status concluding that high expression of *ZAP70* is strongly associated with a worse prognosis independently of the *IGHV* mutational status.[15]

Vasconcelos et al[16] in their microarray study combined two independent prognostic factors, ie, *IGHV* mutational status and Binet staging. Using a comparison between the extreme ends of the disease spectrum—stable and *IGHV*-mutated versus progressive and *IGHV*-unmutated CLL—they confirmed a slight difference in the gene expression underlying these two entities. In a subsequent study,[17] the expression ratio of *LPL* (*lipoprotein lipase*) to *ADAM29* (*a disintegrin and metalloproteinase domain 29*) has been established as the best predictor for prognosis and a potential surrogate marker for *IGHV* status. However, a confirmatory study identified *LPL* expression itself as a better prognostic factor compared with the *LPL/ADAM29* ratio.[18] Furthermore it was reported that the *LPL* expression represents the best survival predictor, being as good as *IGHV* mutational status itself and better than *ZAP70* expression monitoring.[19,20] The fact that *LPL* is not expressed in any other blood cells but the IGHV unmutated CLL lymphocytes represents a major advantage.[21,22] Although *LPL* seems to be a superior surrogate marker for IGHV mutational status and a predictor of poor prognosis, some other genes have also been correlated with high-risk CLL and shorter survival. For example, high expression of *CLLU1* (*chronic lymphocytic leukemia up-regulated 1*),[23] *SEPT10* (*septin 10*),[22] *AICDA* (*activation-induced cytidine deaminase*),[24] and a low expression of *TCF7* (*transcription factor 7*)[16] were detectable in the CLL cells with unmutated *IGHV*.

In our study, we performed a microarray analysis using the Agilent expression arrays in contrast to majority of similar studies that used mainly Affymetrix platform. The Agilent microarrays used detected expression of approximately 22,000 human genes and due to the different probe design were able to provide additional information to already used Affymetrix arrays. We correlated gene expression with *IGHV* mutational status and identified *lymphocyte-activation gene 3* (*LAG3*, *CD223*) as a novel marker of CLL cases with unmutated *IGHV*. This gene has not been previously published in connection with CLL and *IGHV* mutational status. We tested expression level of *LAG3* in correlation with worse prognosis on a cohort of 118 CLL patients using quantitative real time RT-PCR. Moreover, we also validated a set of seven most frequently studied molecular markers alone and in different combinations (*LPL*, *ZAP70*, *AICDA*, *BCL2*, *CLLU1*, *SEPT10*, and *TCF7*) on the same cohort of CLL patients. Finally, we selected a set of three genes (*LAG3*, *LPL*, and *ZAP70*) with the best statistical prognostic value compared with *IGHV* mutational status itself.

Materials and Methods

Blood Samples

Heparinized peripheral blood samples were collected from 118 CLL patients with informed consent. Samples originated from previously untreated patients (70 males and 48 females) with characterized *IGHV* mutational status (59 unmutated vs. 59 mutated) and a median age of 63 years (range 42 to 82 years). Cytogenetic data of analyzed patients are summarized in Table 1.

Sample Preparation

CLL cells were separated from peripheral blood using RosetteSep B Cell Enrichment Kit (StemCell Technologies, Vancouver, Canada) according to manufacturer's instructions. Samples with CLL cells content higher than 95% were used for further analysis (assessed by flow-cytometry). Total RNA was isolated with RNeasy Mini Kit and digested with DNase I (Qiagen, Hilden, Germany) according to manufacturer's instructions.

IGHV *Mutational Status Determination*

IGHV mutational status was determined according to ERIC (European Research Initiative on CLL) recommendations.[25] Briefly, total RNA was amplified by reverse transcription

Table 1. Summary of Cytogenetic Data

IGHV Number of patients	Cytogenetics Number of patients												
		13q−					11q−						
	No aberration	Alone	Plus 11q−	Plus 12+	Plus 17p−	Plus 11q− and 12+	Alone	Plus 12+	Plus 17p−	Plus 12+ and 17p−	12+ Alone	17p− Alone	All aberrations
59 mutated	17	33	1	2	1	1	1	–	–	–	3	–	–
59 unmutated	10	13	9	1	2	–	8	1	1	1	9	2	2

polymerase chain reaction (RT-PCR) using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) with heavy chain variable region (VH) family specific forward primers and common reverse heavy chain joining region (JH) primer. Clonal product was consequently sequenced. The nucleotide sequences were aligned with IMGT/V-QUEST. An unmutated status was assigned in case of ≤ 2% deviation from the germ line *IGHV* sequences.

Microarray Analysis

Ten samples with mutated and 10 samples with unmutated *IGHV* were randomly chosen from the studied cohort of 118 CLL patients for microarray analysis. Linear amplification of 1.2 μg total RNA (Low RNA Input Linear Amplification Kit, Agilent, Palo Alto, CA, modified protocol) including incorporation of aminoallyl-UTPs (Epicentre, Madison, WI) was performed and cRNA was consequently fluorescently labeled (Dy547-NHS-ester, Dy647-NHS-ester, Dyomics, Jena, Germany) and cohybridized with differently labeled reference RNA (Universal Human Reference RNA, Stratagene, Cedar Creek, TX) on Human 1A Arrays (Agilent). All patient samples were hybridized with the same reference. This reference was labeled with both dyes; part of the samples labeled with green and the other part with red fluorescent dye. Random dye assignment to the reference and patient samples controlled dye related bias. Maximizing the number of samples to control an interindividual variability was preferred over technical replication that would only help to estimate nonbiological sources of variation. With a certain number of arrays, the approach omitting dye-swap (technical replication) is beneficial in contrast to a dye-swap design.[26,27] Microarray image analysis was performed using QuantArray software (PerkinElmer, Waltham, MA). Raw data were normalized in R and R Bioconductor[28] and analyzed using MEV software[29] and Significance Analysis of Microarrays [30] supervised algorithm. The microarray data has been deposited in ArrayExpress (*http://www.ebi.ac.uk/microarray-as/ae/*; accession no: E-TABM-696).

Quantitative Real Time RT-PCR of Selected Genes

cDNA synthesis from 500 ng total RNA was performed using SuperScript III Reverse Transcriptase with oligo$(dT)_{12-18}$ primer (Invitrogen). Each sample was analyzed in triplicate using the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions (for list of used TaqMan Gene Expression Assays see Supplemental Table S1 at *http://jmd.amjpathol.org*). DNA amplification was detected using the 7300 Real Time PCR System (Applied Biosystems). Data were analyzed using the Sequence Detection System software version 1.3.1 (Applied Biosystems). Relative gene expression was normalized to the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) expression. *GAPDH* was selected as a gene with the lowest variability among tested housekeeping genes on our set of samples.

Statistical Analyses

Statistical significance of differential expression between *IGHV* mutated and unmutated cases analyzed with quantitative real time (RT-PCR) was determined using the Wilcoxon rank sum test. A training set of 40 randomly chosen samples (20 with mutated and 20 with unmutated *IGHV*) was examined with series of threshold analysis to maximize the confidence of correct discrimination between cases with mutated and unmutated *IGHV*. Every possible combination of selected genes was tested to improve the discriminating potency of the gene expression analysis and determine the gene combination with the best predictive value. Every possible combination of threshold ΔCt values in the range 2 to 12 in increments of 0.1 was tested. The combination giving the best sensitivity and specificity values (Youden's index, for combinations with the same Youden's indexes, the one with highest sensitivity was preferred) was applied to the test set. A verifying set of 78 samples was consequently analyzed; sensitivity, specificity, positive prognostic value (PPV), negative prognostic value (NPV) was determined (Table 2). To further verify the performance of selected gene-set linear discriminant analysis was used to construct classifier. For linear discriminant analysis, the data set was divided into training group of 70 samples and testing group of 48 samples.

Treatment-free survival (TFS) was evaluated as from the time of diagnosis to the beginning of CLL-related therapy using the Kaplan-Meier estimator, and statistical significance was calculated using the log-rank test.

All analyses were performed in R statistical environment.

Table 2. Summary of quantitative real time RT-PCR Results

Gene	Sensitivity %	Specificity %	PPV %	NPV %	Wilcoxon rank sum test
Set of three genes: *LAG3, LPL,* and *ZAP70*	86	90	89	87	$<2.2 * 10^{-16}$
Set of two genes: *LAG3* and *ZAP70*	79	80	83	78	$<2.2 * 10^{-16}$
Set of two genes: *LAG3* and *LPL*	85	80	80	85	$<2.2 * 10^{-16}$
Set of two genes: *LPL* and *ZAP70*	85	89	89	85	$<2.2 * 10^{-16}$
LAG3 (lymphocyte activation gene 3)	93	32	58	83	$1.186 * 10^{-13}$
LPL (lipoprotein lipase)	88	80	81	87	$<2.2 * 10^{-16}$
ZAP70 (ζ-chain associated protein kinase 70 kd)	93	60	70	90	$1.063 * 10^{-08}$
AICDA (activation-induced cytidine deaminase)	69	56	66	68	$2.735 * 10^{-07}$
BCL2 (B-cell-leukemia/lymphoma 2)	74	62	67	69	$<2.2 * 10^{-16}$
CLLU1 (chronic lymphocytic leukemia up-regulated 1)	63	60	61	62	$<2.2 * 10^{-16}$
TCF7 (transcription factor 7)	84	80	82	83	$1.544 * 10^{-11}$
SEPT10 (septin 10)	72	67	70	69	$1.471 * 10^{-11}$

Expression of selected genes in CLL cells with different IGHV mutational status. Parameters calculated in relation to unmutated IGHV for tested genes. PPV, positive predictive value; NPV, negative predictive value.

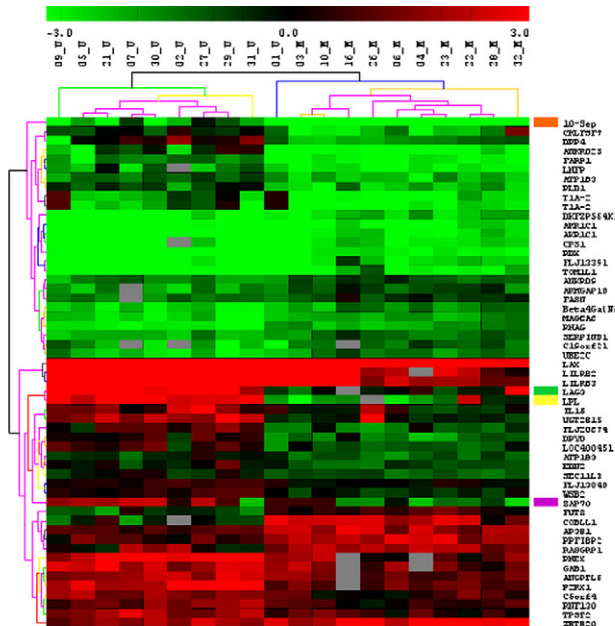

Figure 1. Significance Analysis of Microarrays supervised analysis of differently expressed genes. Expression profiles obtained using microarray analysis were divided into groups according to *IGHV* mutational status (M, mutated *IGHV*; $n = 10$ and U, unmutated *IGHV*; $n = 10$). Significant genes were selected with Significance Analysis of Microarrays and grouped into clusters. Gene coloring is based on normalized patient-to-reference RNA log2 ratios as shown at the top of the figure. Genes selected for further validation using quantitative real time RT-PCR are marked in colors on the side.

Results

Our microarray study detected a set of ~ 50 genes with different expression levels in CLL cells with different *IGHV* mutational status. Figure 1 shows the expression patterns of 20 microarray-tested CLL patients and a differentially expressed gene set, with analysis settings of median false discovery rate < 1%. Correlation of the reference RNA expression in between the individual arrays was characterized by Pearson's Correlation Coefficient $r > 0.97$.

We detected *lipoprotein lipase* (*LPL*) as the gene with the best differentiating potential. Identification of *LPL* in our experimental set of patients was in line with recently reported data.[17,21] In concordance with previously published studies, we also confirmed the overexpression of *ZAP70*[9] and *SEPT10*[22] in CLL cells with unmutated *IGHV*. Moreover, we detected the *lymphocyte-activation gene 3* (*LAG3*), also known as CD223, as a novel independent marker of CLL cells harboring the unmutated *IGHV* gene.

Data acquired from microarray analysis was further validated using quantitative real time RT-PCR on 118 patients (including 20 patients initially microarray-tested). We analyzed the expression levels of *LAG3*, *LPL*, *ZAP70*, and *SEPT10* that resulted from our microarray analysis, together with four other candidate genes selected from similar studies (*AICDA*, *BCL2*, *CLLU1*, and *TCF7*) to evaluate their prognostic relevance on our set of samples. Threshold values were set for each gene: 8.9 (ΔCt) for *LAG3*, 8.8 (ΔCt) for *LPL*, 5.4 (ΔCt) for *ZAP70*, 14 (ΔCt) for *CLLU1*, 14 (ΔCt) for *AICDA*, 12 (ΔCt) for *SEPT10*, 6 (ΔCt) for *TCF7*, and 2.2 (ΔCt) for *BCL2*. The values in the Table 2 present results obtained on the test set. Statistical significance of the quantitative real time RT-PCR data were analyzed using the Wilcoxon rank sum test and confirmed a strong association of high expression of *LAG3*, *LPL*, *ZAP70*, *AICDA*, *BCL2*, *CLLU1*, *SEPT10*, and low expression of *TCF7* with unmutated IGHV ($P < 0.0001$ in all tested genes) summarized in Table 2. High expression of *LAG3* alone correlates with the absence of IGHV mutations with 93% sensitivity. Relative gene expression levels of tested genes in groups with mutated and unmutated IGHV are presented as box plots (see Supplemental Figure S1 at *http://jmd.amjpathol.org*). We also tested the best gene combination to obtain a gene-set the expression of which has the highest correlation with IGHV mutational status. Our results showed that combined assessment of *LPL*, *ZAP70*, and *LAG3* expression may correctly assign IGHV status with 90% specificity ($P < 0.0001$) and 86% sensitivity ($P < 0.0001$) (Table 2) with 89% positive predictive value. Linear discriminant analysis using the expression values of three best genes and *IGHV* mutational status trained on 70 samples correctly classified 84% of samples. The combination of three markers (*LAG3* + *LPL* + *ZAP70*) provides higher sensitivity and specificity than any combination of two markers (ie, *LAG3* + *ZAP70*, *LAG3* + *LPL*, and *LPL* and *ZAP70*), as indicated in Table 2.

In addition to this, we correlated the expression of tested genes with TFS using the Kaplan-Meier estimator (Figure 2, A–F). Patients manifesting a high expression of *LAG3*, *LPL*, or *ZAP70* required therapy significantly earlier than patients with a low expression of the tested genes. TFS median in patients with low *LAG3* expression was not reached compared with a median of 50 months in cases with high expression ($P < 0.0089$) (Figure 2C). TFS median in patients with low *LPL* expression was 157 vs. 17 months in patients with high *LPL* expression ($P < 0.001$) (Figure 2B). TFS median in patients with low *ZAP70* expression was not reached compared with 33 months in those with high *ZAP70* expression ($P < 0.001$) (Figure 2D). The *IGHV* mutational status had TFS median of 13 months in unmutated cases and median not reached in mutated ($P = 1.8 * 10^{-11}$) (Figure 2A), our tested gene-set (*LPL* and *LAG3* together with *ZAP70*) showed TFS median of 157 months in cases with low vs. 15 months with high expression ($P = 7.71 * 10^{-11}$) (Figure 2E). Furthermore, we compared the differences in TFS with respect to the number of overexpressed genes (Figure 2F). TFS median was not reached in arms with all three genes down-regulated or only one up-regulated (both $P < 0.001$). TFS median in cases with two genes up-regulated was 157 months ($P < 0.001$) in comparison with TFS median 15 months, in cases with all three genes up-regulated.

Discussion

Expression analysis of different subgroups of CLL patients identified *LAG3* as an important prognostic marker with possible relationship to CLL pathogenesis. *LAG3* (*CD223*) was identified in 1990 by authors Triebel et al.[31]

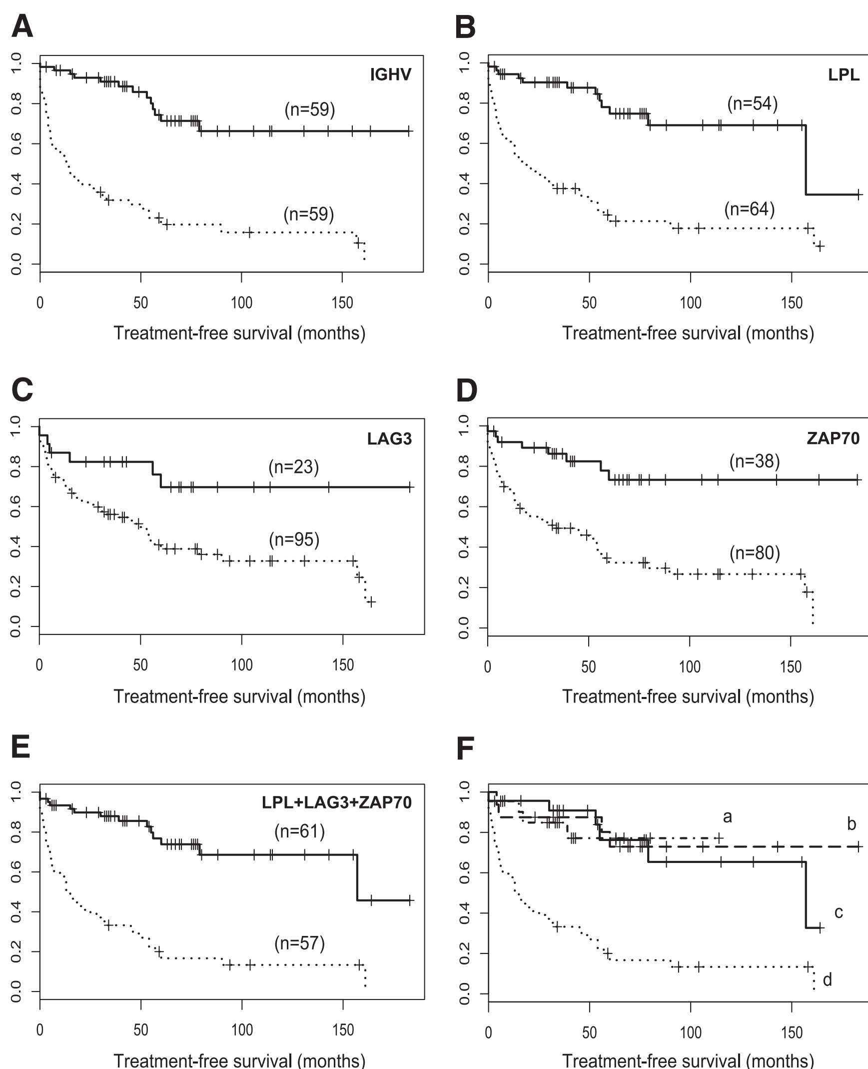

Figure 2. Kaplan-Meier curves for treatment-free survival (TFS) from diagnosis to onset of CLL-related therapy according to *IGHV* mutational status (**A**) and expression of tested genes (**B, C, D, E,** and **F**). Statistical significance was calculated using a log-rank test. **A:** Patients were grouped according to *IGHV* mutational status; the TFS median was not reached vs. 13 months in mutated and in unmutated arms, respectively ($P < 0.001$). **Solid line** corresponds to mutated *IGHV*; **dotted line** corresponds to unmutated IGHV. **B:** Patients were grouped according to *LPL* gene expression; the TFS median was 157 vs. 17 months in low and high expression arms, respectively ($P < 0.001$). **Solid line** corresponds to low expression; **dotted line** corresponds to high expression of *LPL*. **C:** Patients were grouped according to *LAG3* gene expression; the TFS median was not reached vs. 50 months in low and high expression arms, respectively ($P < 0.0089$). **Solid line** corresponds to low expression; **dotted line** corresponds to high expression of *LAG3*. **D:** Patients were grouped according to *ZAP70* gene expression; the TFS median was not reached vs. 33 months in low and high expression arms, respectively ($P < 0.001$). **Solid line** corresponds to low expression; **dotted line** corresponds to high expression of *ZAP70*. **E:** Patients were grouped according to combined 3-gene set expression (*LPL* + *LAG3* + *ZAP70*); the TFS median was 157 vs. 15 months in low and high expression arms, respectively ($P < 0.001$). **Solid line** corresponds to low expression and **dotted line** corresponds to high expression of gene set. **F:** Patients were grouped according to combined 3-gene set expression (*LPL* + *LAG3* + *ZAP70*) considering number of up-regulated genes; the TFS median was: (**a**) not reached in arm with all 3 genes down-regulated ($n = 16$), **dash-and-dot line**, $P < 0.001$, as compared with (**d**); (**b**) not reached in arm with 1 gene up-regulated ($n = 23$), **dashed line**, $P < 0.001$, as compared with (**d**); (**c**) 157 months in arm with 2 genes up-regulated ($n = 22$), **solid line**, $P < 0.001$, as compared with (**d**); and (**d**) 15 months in arm with all three genes up-regulated ($n = 57$), **dotted line**.

This surface protein is related to CD4 molecule and physiologically expressed on T-cells and natural killer-cells after cell activation and not on resting peripheral blood lymphocytes.[31] Surface *LAG3* expression on activated human T-cells is up-regulated by interleukin IL-2, IL-7, and IL-12,[32] and is detectable on all human T-cells 2 to 3 days after activation.[33] Therefore *LAG3* probably does not take part in induction phase of immune response but may play essential role during activation. Foa et al[34] reported that IL-2 released by B-CLL T-lymphocytes may be used by the neoplastic B-cell clone expressing the IL-2 receptor. Decreased availability of IL-2 could therefore play part in some of the T-cell defects in B-CLL. Moreover, Huard et al[35] reported that *LAG3* down-regulates $CD4^+$ antigen-specific T-cell proliferation through interaction with MHC II molecules and Workman and Vingali[36] suggest that *LAG3* functions as a negative regulator of T-cell homeostasis. These findings correlate with facts that total amount of T-cells in B-CLL is often increased and the ratio of helper CD4 to suppressor CD8 T-cells is in many cases reversed.[37]

During normal B-cell maturation naive B-cells enter the lymph node, are activated with antigen, form GCs inside the lymph node follicles and start to proliferate with the contribution of $CD4^+$ helper T-cells. In this respect Kisielow et al[38] reported an interesting finding that *LAG3* can be also expressed on activated murine B-lymphocytes, but only on B-cells activated with T-cells. They suggested that *LAG3* can serve as a marker of T-cell induced B-cell activation while the strength of B-cell activation could influence the formation of GCs.[39] Activated extrafollicular B-cells form short-lived plasmablasts, enter GC reaction and differentiate into memory cells. Insufficient specific T-cell help together with strong B-cell activation lead to transient GC formation and extrafollicular differentiation of activated B-cells. Aberrant expression of activating markers on CLL cells like *ZAP70* and *LAG3* together with detectable expression of *AICDA* outside the germinal centers could therefore indicate improperly finished or prematurely terminated process of B-lymphocyte affinity maturation of CLL cells with unmutated *IGHV*.

Analysis of the *IGHV* mutational status as a prognostic marker in CLL has been widely used; however this process is time consuming and includes several steps. Proposed gene-set (*LPL*, *ZAP70*, and *LAG3*) detection

enables fast testing of a large set of samples in only two-steps analysis. In addition, many laboratories introduce *ZAP70* analysis as a part of routine CLL diagnostics and prognosis assessment. *ZAP70* expression can be detected on mRNA level using quantitative real time RT-PCR or on protein level using flow-cytometry, but proper standardization of flow-cytometric analysis still remains controversial. *ZAP70* is physiologically expressed in T-cells, depletion of these cells is crucial for correct quantitative real time RT-PCR analysis. Therefore the enlargement of a set of analyzed markers could increase the confidence of the prognosis assessment. We proved that combined expression analysis of tested surrogate markers (*LPL*, *LAG3*, and *ZAP70*) provides a similar statistical concordance with TFS as that of the *IGHV* status itself and could be potentially used in diagnostics.

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