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Telomere length, molecular cytogenetic findings, and immunophenotypic features in previously untreated patients with B-chronic lymphocytic leukemia

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Telomere length was evaluated by terminal repeat fragment method in 66 previously untreated patients with B-chronic lymphocytic leukemia (B-CLL) to ascertain whether telomere shortening was associated with genomic aberrations, immunoglobulin variable heavy chain (IgVH) mutational status, CD38 and ZAP-70 expression, and telomerase activity. Chromosomal aberrations were present in peripheral blood cells of 73% patients (48/66), no difference in telomere length between patients with good and intermediate prognosis according to cytogenetics was found. Association between telomere length and IgVH mutational status, ZAP-70 and CD38 expression was proved as significantly shorter telomeres in patients with unmutated IgVH status (p=0.01) and ZAP-70 positivity (p=0.01) and CD38 positivity (p=0.05) were detected. Telomerase activity was positive in 11 patients out of 21 examined, correlation between telomere length and telomerase activity was found (p=0.05). Telomere length and telomerase activity in combination with other prognostic parameters complete the risk profile of B-CLL patients and might serve for an easy decision on optimal treatment strategy.

Key words: B-chronic lymphocytic leukemia, telomere length, telomerase activity, chromosomal aberrations, prognosis

B-cell chronic lymphocytic leukemia is the most common leukemia in adults in the Western world, occurring predominantly in elderly people. It is a heterogeneous disorder characterized by a highly variable clinical course. Classical staging systems introduced by Rai et al. [1] and Binet et al. [2] help to predict survival in B-CLL, however they do not distinguish patients who will evolve more advanced, aggressive disease from those who will remain indolent. Therefore, other indicators related to the genetics and biology of B-CLL such as genomic aberrations, IgVH mutational status, CD38 and ZAP-70 expression, telomere length and telomerase activity, enable clinicians to identify patients with high risk B-CLL more precisely.

Interphase fluorescence in situ hybridization (I-FISH) allows the detection of specific chromosomal aberrations which would be missed by metaphase analysis or which are far beyond the resolution power of banding analysis. Using I-FISH, genomic aberrations are detected in approximately 80% of B-CLL cases [3], although recently, several immunostimulatory techniques yielding mitoses have been introduced thus, allowing more complex insight into the genome [4, 5]. The most common is 13q14 deletion and less frequently detected trisomy 12, ATM gene (11q22.3) and p53 gene (17p13) deletions. The prognostic impact of I-FISH is relevant in B-CLL: patients with deletion 13q14 as a sole aberration have good prognosis with survival better than those with a normal karyotype, deletions of ATM and /or p53 genes are associated with poor prognosis [6].

In 1999, Hamblin et al. [7] and Damle et al. [8] simultaneously and independently defined patients whose IgVH genes showed less than 98% homology with germline sequence as mutated CLL cases. Patients with unmutated IgVH genes had a significantly shorter median overall survival than patients with mutated IgVH genes. Since that time, the prognostic value of the IgVH mutational status has been confirmed by other groups (reviewed in [9]) and it is well established as one of the strongest prognostic features in B-CLL patients.

As the determination of IgVH was laborious and expensive, many efforts have been made to identify other biological

markers with prognostic value. Strong correlation between mutational status and CD38 expression was first reported by Damle et al. [8], however other studies proved that the association was not absolute and individual patients frequently show discordances between CD38 expression and IgVH mutations [10, 11]. The expression profiling studies revealed that the receptor kinase ZAP-70 is differentially expressed in IgVH mutated and IgVH unmutated B-CLL subtypes and expression level of this protein may predict IgVH mutational status and prognosis in 90% of B-CLL cases [12]. Recent research has focused on telomere length and telomerase activity that might be prognostically significant in patients with B-CLL. Telomere, a complex of guanine-rich repeat sequences and associated proteins, caps and protects the chromosomes from chromosomal fusion, recombination and terminal DNA degradation [13]. In humans, the telomeres have an average length of 5 kbp to 15 kbp. The gradual shortening of telomeres occurs at each cell division as a result of the incomplete replication of linear chromosomes. When telomere erosion reaches a critical point of 3-4 kbp, cells turn off the proliferation and undergo senescence or apoptosis. This physiological shortening is supposed to be enhanced during malignant transformation and results in an extensive DNA instability. Altered cells with a critical telomere length are rapidly eliminated, however, a small number of cells may survive and give rise to a clone with unlimited proliferative potential [14]. This is usually accompanied by activation of a ribonucleoprotein telomerase that synthesizes telomeric repeats. In normal somatic cells, telomerase activation is usually absent but for unlimited cell growth in tumorigenesis, telomerase activation plays the critical role.

The aim of this study was to assess the telomere length in a cohort of previously untreated patients with B-CLL and to evaluate the association of telomere length with results of interphase FISH, telomerase activity, IgVH mutational status and ZAP-70 and CD38 expression analyses.

Table 1. Main clinical and biological features of B-CLL patients

Parameters			
Sex	32 male	34 female	
Median age	67 years	Range 44-89	
Rai staging			
0	38 patients	17 male	21 female
0-I	8 patients	5 male	3 female
I	14 patients	7 male	7 female
II	3 patients	1 male	2 female
unknown	3 patients	2 male	1 female
Mean TRF-L (kbp)	6.82	Range 3.65-15.50	
FISH[a]	27 good prognosis	35 intermediate	4 poor prognosis
IgVH status[b]	46 mutated	15 unmutated	5 not defined
ZAP-70 analysis[c]	43 negative	22 positive	
CD38 analysis[d]	52 negative	13 positive	
Telomerase activity[e]	10 negative	11 positive	

Abbreviations: CLL, chronic lymphocytic leukemia; FISH, fluorescence in situ hybridization

[a]For prognostic stratification patients were grouped as follows: 13q deletion alone (good prognosis), 17p and 11q deletion (poor prognosis), all the other (intermediate), data available in 66 patients

[b]Cut off for IgVH status: 2% (i.e. 98% of homology), data available in 61 patients

[c]Cut off for ZAP-70 expression: 20%, data available in 65 patients

[d]Cut off for CD38 expression: 30%, data available in 65 patients

[e]telomerase-positive: if the difference in absorbance is higher than the twofold background activity; data available in 21 patients

Patients and methods

Patients. Peripheral blood of 66 patients with B-CLL (32 male, 34 female, median age 67 years) was examined during the years 2007 – 2008. According to the Rai staging system, patients were classified to the low or intermediate risk group, not requiring the therapy. 19 patients were examined at the time of diagnosis and 47 patients during the course of the disease. Main clinical and biological features are summarized in Table 1. Informed consent was provided in all patients approving the use of their samples for research purposes.

Sample preparation for I-FISH. Peripheral blood smear preparations were prepared with conventional cytogenetic methods (hypotonic treatment of leukocytes and methanol-acetic acid 3:1 fixation). Cell suspensions were dropped onto microscopic slides and were directly used for FISH.

Analyses of genomic aberrations. I-FISH analyses were performed in all (66) patients on peripheral blood preparations using commercially available DNA probes:

1) Vysis CLL Probe Panel for regions 17p13.1 (gene p53), 11q22.3 (gene ATM), 13q14.3, 13q34 and 12p11.1-q11 (Abbott-Molecular, Downers Grove, IL);
2) Poseidon Repeat Free IGH (14q32) Break probe (Kreatech Diagnostics, Amsterdam, Netherlands) for detection of 14q32 aberrations.

Two hundred nuclei were analyzed for each probe mix. The cut off level for positive values were determined on samples obtained from 10 cytogenetically normal persons (5 male, 5 female) and were found to be 5% (mean ± 3SD).

Telomere length measurement. Mononuclear cells obtained from peripheral blood samples of 66 patients were separated by Ficoll-Paque density gradient centrifugation. High molecular weight genomic DNA was extracted by modification of the salting-out method according to the standard protocol recommended by manufacturers. Telomere lengths were assessed by Terminal Repeat Fragment (TRF) method performing non-radioactive chemiluminescent assay. This method is based on Southern blot hybridization with the oligonucleotides containing TTAGGG repeats labeled by digoxigenine after digestion of 3 μg high-molecular weight DNA with *Hin*fI and *Rsa*I restriction enzymes, followed by chemiluminescent detection (Telo*TAGGG* Telomere Length Assay, Roche Diagnostics GmbH, Germany). Telomere length in kilobases – TRF-L index, representing average data on telomeres from all chro-

Table 2. Incidence of chromosomal aberrations in B-CLL patients

Aberration	No. of positive patients/ No. examined
del(13)(q14)	**42/66 (64%)**
del(13)(q14)	27
del(13)(q14), biallelic del(13)(q14)	9
biallelic del(13)(q14)	3
biallelic del(13)(q14), trisomy 12	1
del(13)(q14), del(11)(q22.3)	1
del(13)(q14), biallelic del(13)(q14), del(17)(p13.1)	1
trisomy 12	**5/66 (8%)**
trisomy 12	3
trisomy 12 biallelic del(13)(q14)	1
trisomy 12, del(17)(p13.1)	1
del(11)(q22.3)	**2/66 (3%)**
del(11)(q22.3)	1
del(11)(q22.3), del(13)(q14)	1
del(17)(p13.1)	**2/66 (3%)**
del(17)(p13.1), del(13)(q14), biallelic del(13)(q14)	1
del(17)(p13.1), trisomy 12	1
IgH rearrangement	**1/66 (2%)**

mosome ends, was estimated according to the manufacturer´s instructions. Normal and shortened telomeres lengths were determined with regard to the results of TRF-L measurement in 18 healthy age matched donors (median=61 years). We postulated cases with TRF-L shorter than 6.7 kbp as cases with reduced telomeres.

Detection of telomerase activity. Telomerase activity was detected in 21 patients by quantitative TRAP assay based on photometric enzyme immunoassay on solid phase from protein extract using *Telo*TAGGG Telomerase PCR ELISA-PLUS kit (Roche Diagnostics GmbH, Germany). Telomerase adds telomeric repeats (TTAGGG) to the 3´-end of the biotin labeled synthetic primer. The elongation products were amplified by PCR, split into two aliquots, denatured and hybridized separately to digoxigenin-labeled detection probes. The resulting products were immobilized via the biotin label to a streptavidin-coated microplate. Immobilized amplicons were then detected with an antibody against digoxigenin conjugated to horseradish peroxidase and the sensitive peroxidase substrate. When the difference in absorbance was higher than the twofold background activity, the samples were considered as telomerase-positive.

Analyses of IgVH mutational status. The assay was performed in 65 patients. Ficoll-Paq isolated mononuclears were lyzed and RNA isolated using TriZol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed to prepare cDNA using Superscript II (Invitrogen) which was subjected to PCR amplification with Ampli-Taq Gold polymerase (Applied Biosystems, Foster City, CA, USA) in six individual assays to detect clonal proliferation in 7 families of IgVH genes. The "touch down" methodology using degenerated primers is described elsewhere [15]. RT-PCR products were purified and sequenced using the Big Dye Terminator Kit v. 3 and ABIPrism 310Genetic Analyzer (both from Applied Biosystem). The BLAST program (www.ncbi.nlm.nih.gov/igblast) was used to compare the sequences of IgVH genes to their germ-line configuration. A cut off value of 2% (i. e. 98% of homology) was set to discriminate between mutated and unmutated IgVH sequences [16].

ZAP-70 expression analysis by flow cytometry. Cytoplasmatic ZAP-70 expression was measured in B-lymphocytes from peripheral blood, collected in K_3EDTA, using combination of three monoclonal antibodies: CD3-FITC (fluorescein isothiocyanate) (Immunotech/Beckman Coulter, Fullerton, CA, USA)/ZAP-70 – PE (phycoerythin) (Caltag/Invitrogen, Carlsbad, CA, USA)/CD-19 – PC-5 (phycoerythrincyanin-5) (Immunotech). The IntraPrem™ Kit (Immunotech) was used for the essential cell permeabilization, enabling the antibody penetration into cells. The samples were analyzed by flow cytometer FACSCalibur (Becton-Dickinson, La Jolla, CA, USA) and evaluated by CELLQuest™ software. The results represent the percentage share of ZAP-70 positive B cells in the total B-lymphocyte count. ZAP-70 expression in less than 20% of cells was considered ZAP-70 negative whereas the cases exhibiting ZAP-70 expression in ≥20% of cells were interpreted as ZAP-70 positive. Data were available in 65 patients.

CD38 antigen expression. Flow cytometric analyses were performed on Becton-Dickinson or Coulter Epics flow cytometry analyzers. CD38 antigen expression was analyzed in 65 patients and was deemed positive if ≥30% of cells stained positive [17].

Statistical analyses. Statistical analyses were performed using SPSS statistical software (Chicago, IL). The correlation of TRF-L, age and gender were evaluated using Pearson´s test. Comparing of the means was done using Student´s t-test.

Results

Molecular cytogenetic analyses. A total of 66 patients were examined using I-FISH, 48 of them (73%) showed genetic abnormalities. Complete results are summarized in Table 2.

Deletion of 13q14 was proved in 42 (64%) patients, among these in 27 as a sole abnormality. In the remaining 15 cases, del(13)(q14) was combined with other chromosomal aberrations. Trisomy 12 was found in 5 patients (8%), as a sole aberration in 3 cases, in one case in combination with biallelic del(13)(q14), in one patient with del(17)(p13). Deletion of ATM gene – del(11)(q22.3) and deletion of p53 gene – del(17)(p13.1) were proved in 2 patients (3%) each. Rearrangement of IgH gene at 14q32 was proved in 1 patient (2%).

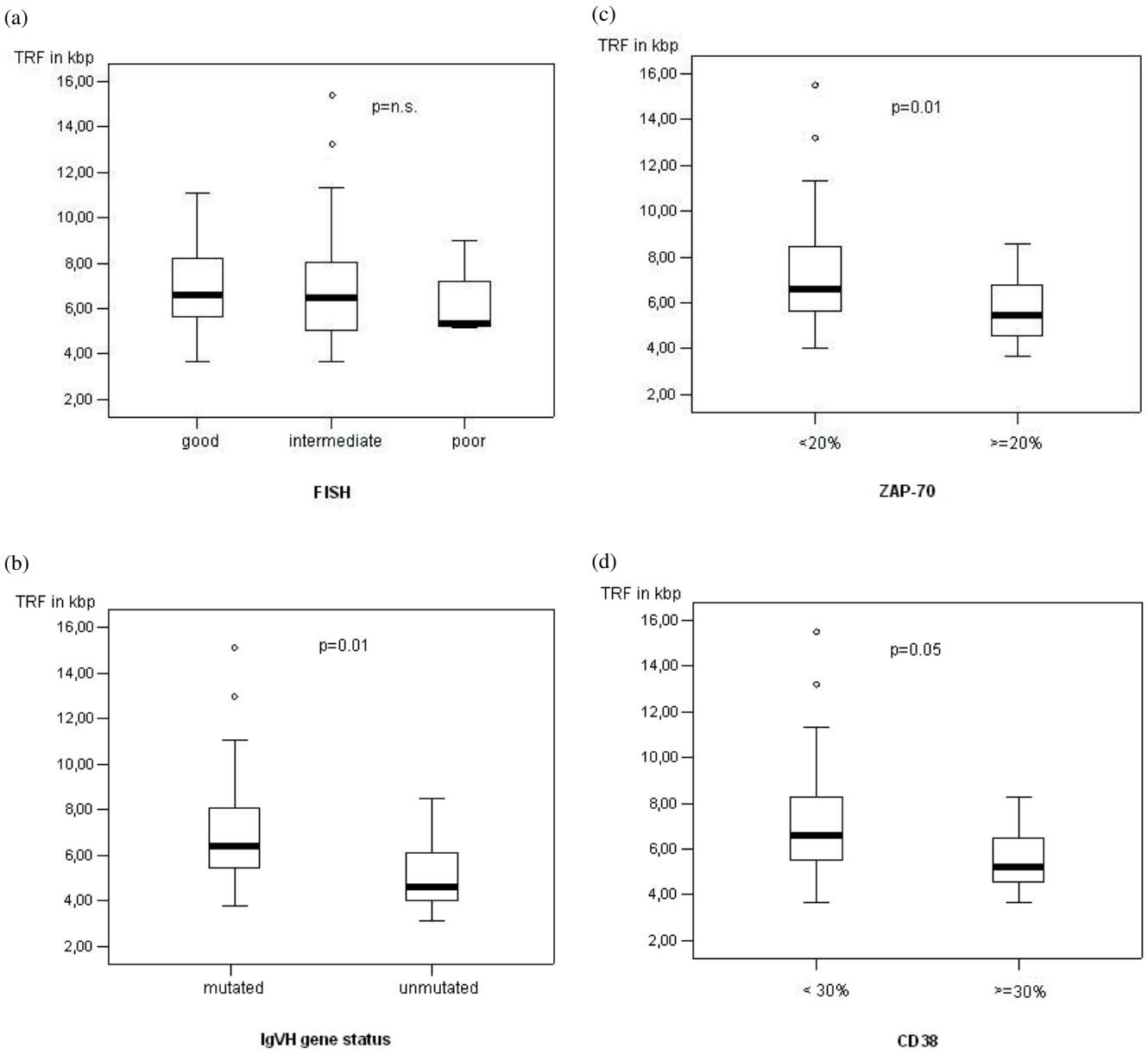

Figure 1. Telomere length distribution in relation to other prognostic factors: (a) telomere length versus prognosis according to I-FISH; (b) telomere length versus IgVH mutational status; (c) telomere length versus ZAP-70 expression; (d) telomere length versus CD38 expression; (e) telomere length versus age. The boxes shown present median value (bold line) and interquartile range and the whiskers minimum and maximal values, except for outliers (rings); n.s.= not significant

In addition, IgH gene variable segment partial deletion was found in 17 patients, however, this finding was classified as normal, for the genetic background of variable segment deletions is believed to be probably physiological.

Telomere length determination and association with other prognostic parameters. High heterogeneity in telomere length was observed with range 3.65 kbp – 15.50 kbp (mean 6.82 kbp), reduced telomeres were proved in 38 patients (58%). TRF-L did not correlate with sex or age. As for correlation of TRF-L with I-FISH, patients were grouped according to the results of molecular cytogenetic findings and thus according to the prognosis as follows: 27 patients in group 1 with good prognosis (13q deletion alone), 4 patients in group 3 with poor prognosis (17p and 11q deletion), 35 patients in intermediate prognostic group 2 (all other I-FISH findings). No significant difference in telomere length was observed between the three

groups (group 1 - mean TRF-L 6.86 kbp; group 2 - mean TRF-L 6.87 kbp; group 3 - mean TRF-L 6.21 kbp; see Figure 1a) but number of patients with poor prognosis was very low for statistical analysis.

IgVH mutational analysis was performed in 65 patients, in 46 mutated (71%), in 15 unmutated (23%) IgVH status was proved and in 4 patients (6%) the germline IgVH genes were not identified. Significantly reduced telomeres (p=0.01) were confirmed in patients with unmutated IgVH status (mean TRF-L 5.53 kbp) compared to IgVH mutated patients (mean TRF-L 7.30 kbp) and to the control group (mean TRF-L 6.70 kbp, p=0.05). However, IgVH mutated patients showing short telomeres and IgVH unmutated patients with long telomeres were also identified and defined as discordant (27 patients). TRF-L distribution in IgVH mutated and IgVH unmutated patients is shown in Figure 1b.

ZAP-70 positivity was detected in 22 patients of 65 examined (34%). Association between telomere length and ZAP-70 expression was found as significantly shorter telomeres in ZAP-70 positive patients (mean TRF-L 5.74 kbp; p=0.01) compared to ZAP-70 negative patients (mean TRF-L 7.35 kbp; see Figure 1c) and to the control group (p=0.05) were detected.

CD38 expression analysis was positive in 13 out of 65 examined patients (20%). We observed significant difference (p=0.05) in telomere length distribution between CD38 positive (mean TRF-L 5.58 kbp) and CD38 negative patients (mean TRF-L 7.14 kbp, see Figure 1d). Telomerase activity was determined in 21 patients, positive activity was detected in 11 patients. Correlation between telomerase positivity and short telomeres was confirmed (p=0.05).

The time from diagnosis to sampling varied in our cohort of patients. Since telomeres are shortering by time, we studied if the time span between diagnosis and sample collection could be of importance. No significant difference in telomere length was found between patients examined at time of diagnosis and during the disease without therapy (p=0.116).

Discussion

In this study, we analyzed telomere length, genomic aberrations, telomerase activity, IgVH mutational status, CD38 and ZAP-70 expression to evaluate the association between the telomere length and other prognostic factors in untreated patients with B-CLL. Determination of independent prognostic features in early and intermediate disease stage may contribute to consideration of appropriate therapeutic approach.

In our cohort of patients, TRF-L did not correlate with sex or age. This finding is consistent with studies indicating that demographic factors impacting TRF-L of normal cells have little or no effect on cancer cell telomeres [18, 19]. The frequencies of chromosomal aberrations examined by I-FISH are distinct from our preliminary reported data [17, 20]. The discrepancy in number of trisomy 12 and del(11)(q22.3)/del(17)(p13) can be explained by smaller cohort size and by majority of Rai 0 – I patients included in presented study as we focused on patients with low or intermediate risk not requiring the therapy. We have statistically evaluated correlation of TRF-L with individual I-FISH prognostic groups (good, intermediate and poor) but no association was found. In hematological malignancies, an increased frequency of genetic alterations has been connected with short telomeres [21, 22, 23], but only a few studies of this correlation in CLL were published. Ricca et al. [19] have reported a nonsignificant trend toward an association between short telomere length and high risk cytogenetics. Roos et al. [24] proved in a study of 152 patients with CLL strong association of telomere length with specific cytogenetic abnormalities, namely short telomeres with 17p- or 11q- abnormalities whereas patients with a sole 13q- were characterized by long telomeres, respectively. In the present study, the cohort of patients with poor prognostic cytogenetic features was too small for statistical evaluation, however, in three out of four patients in this cohort the telomeres were short. This finding contributes to the possible association of short telomeres with adverse prognostic cytogenetic features but it is necessary to perform further studies with larger cohorts of patients.

One of the possibilities how to reveal other cytogenetic aberrations in patients with CLL is the evaluation of immunostimulated metaphases [4, 5]. This technique allows a more comprehensive cytogenetic analysis providing information on the whole genome and could lead to the detection of additional aberrations compared to I-FISH. Evaluation of TRF-L in patients examined by this technique may bring new evidence in relationship of telomere length and genomic aberrations.

Telomerase activity was examined in 21 patients in our CLL cohort, positive activity was proved in 11 patients, in 9 of them (82%) significantly shorter telomeres were found and the correlation between telomere length and telomerase activity was proved (p=0.05). Evidence, that almost all human tumors have increased telomerase activity [25], supports key role of telomerase in the neoplastic process, however, its prognostic role is still unclear. Some studies proved the relationship between telomerase activity and clinical outcome, while some authors did not confirm telomerase activity as a prognostic factor in B-CLL [26, 27, 28, 29]. Even thought we found the association of short telomeres and higher telomerase activity in our cohort, number of patients is low and for confirmation of this association as a prognostic factor it will be necessary to analyze larger cohorts of patients.

The mutational status of the IgVH gene is one of the strongest prognostic indicators of survival in patients with B-CLL, however, some patients with unmutated IgVH status do not have progressive disease but others with mutated IgVH genes have a highly aggressive clinical course [30]. To identify the groups of patients with different clinical outcome, the evaluation of more prognostic features is needed. It seems that telomere length in combination with IgVH mutational status can be helpful to refine prognostication of CLL patients. In our study, we proved significantly shorter telomeres in patients with unmutated IgVH status, however

IgVH mutated patients showing shorter telomers and IgVH unmutated patients with long telomers were identified as well. Our results are consistent with data of Ricca et al. [19], who defined new subgroups according to the correlation between telomere length and IgVH mutational status - 41% of IgVH unmutated showed long and 5% of IgVH mutated short telomeres - as discordant. IgVH unmutated discordant patients had a better clinical outcome than IgVH unmutated concordant. Similarly, worse overall survival in the IgVH mutated group with short telomeres compared to mutated patients with long telomeres was reported by Grabowski et al. [18]. Detection of such groups of patients seems to be very important from the clinical point of view and needs further characterization.

Telomere length in relation to ZAP-70 and CD38 expression seems to be of prognostical relevance in CLL patients but the data differ in various studies. In our cohort, we proved significantly shorter telomeres in patients with ZAP-70 and CD38 positivity compared to patients ZAP-70/CD38 negative, however discordant patients were also revealed. Similarly Ross et al. [24] confirmed that telomere length correlates strongly with IgVH mutational status, CD38 and ZAP-70 expression. On the contrary, Ricca et al. [19] did not find any association between telomere length and ZAP-70/CD38 expression in 201 B-CLL patients.

Determination of independent prognostic features in early stages of the disease is a subject of intensive research in patients with B-CLL. We conclude that telomere length and telomerase activity in combination with other prognostic parameters like genetic aberrations, IgVH mutational status, ZAP-70 and CD38 expression may complete the risk profile of the patients without therapy. Further studies with larger cohort of patients are needed to determine these associations.

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