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Prognostic impact of *NOTCH1* and *MYD88* mutations in chronic lymphocytic leukemia patients

Ewelina Zakrzewska[1], Agnieszka Karczmarczyk[3], Joanna Purkot[3], Paulina Własiuk[2, 3], Krzysztof Giannopoulos[2, 3], Elżbieta Starosławska[1]

[1]Medical Diagnostic Laboratory, St John's Cancer Center, Lublin
[2]Department of Hematology, St John's Cancer Center, Lublin
[3]Department of Experimental Hematooncology, Medical University of Lublin

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Summary

Background. *Chronic lymphocytic leukemia (CLL) is one of the most common types of leukemia in adults with highly heterogeneous clinical course of the disease. Currently available prognostic factors are not fully efficient in predicting the course of CLL. New molecular mutations such as NOTCH1 and MYD88 could partly explain the CLL heterogeneity and help in identifying clinically relevant groups of patients.*

Material and methods. *NOTCH1 c.7544_7545delCT (n = 200) in PEST domain (exon 34) and MYD88 L265P (n = 60) mutations was investigated by amplification refractory mutation system (ARMS). Expression of MYD88 in CLL was assessed in peripheral blood (PB) (n = 60) and bone marrow (BM) (n = 92) of CLL patients and 25 healthy volunteers (HVs) using qRT-PCR.*

Results. *NOTCH1 mutation occurred in 18/200 (9.0%) CLL patients. Patients harboring NOTCH1 mutations prevalently belonged to aggressive cases, i.e. cases with an unmutated IGVH gene status, expression of CD38 and ZAP-70. MYD88 mutation occurred in 2/60 (3.3%) CLL patients. MYD88 mutations were strikingly enriched among patients expressing mutated IGVH genes. Our study demonstrated significantly higher PB MYD88 expression than in HVs and relevantly higher PB MYD88 expression in comparison with BM (respectively $p < 0.0001$ and $p = 0.0015$). There was no correlation between MYD88 expression in PB and BM and expression of ZAP-70, CD38 and IGVH mutational status.*

Conclusions. *NOTCH1 mutations are more frequently detected in cases with unfavorable biological markers and seem to be independent predictive markers for worse outcome in CLL patients. Further collaborative studies in CLL are obligate to study the prognostic and predictive relevance of MYD88 mutations and expression.*

Key words: chronic lymphocytic leukemia, NOTCH1, MYD88, prognostic factors, ARMS-PCR

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Correspondence address: Dr n. med. Ewelina Zakrzewska, Medical Diagnostic Laboratory, St John's Cancer Center, Lublin, Jaczewskiego 7, 20–090 Lublin, phone: 50 970 85 05, fax: 81 747 89 11; e-mail: evelin84@wp.pl

Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common types of leukemia in adults and is characterized by the accumulation of malignant B CD5+ lymphocytes in the peripheral blood (PB) and lymphoid organs [1]. The clinical course of the disease is highly heterogeneous, from patients with stable disease to rapid progression leading to death within a year of diagnosis or to transformation to an aggressive lymphoma, known as Richter's syndrome (RS) [2–5]. Clinical heterogeneity of CLL explains the need for identification of prognostic and predictive factors.

Currently available prognostic factors are not fully efficient in predicting the course of CLL, especially when the disease is diagnosed at an early stage. Therefore, we are looking for new prognostic and predictive factors, which allow the classification of patients into the groups of disease progression at the time of diagnosis. The currently used prognostic factors in the early clinical stages are based on biological parameters of B-cell leukemias such as the mutational status of *IGVH* (immunoglobulin heavy chain variable region) gene, gene aberrations, as well as expression of CD38 and ZAP-70 on B lymphocytes surface [6]. Moreover, Döhner et al. [7] used interphase fluorescence in situ hybridization (FISH) cytogenetic analysis to evaluate cytogenetic lesions in CLL, detecting chromosomal abnormalities in over 80% of patients. By correlating FISH changes with the course of the disease, a hierarchical model based on five risk categories was developed.

Recently, the use of next-generation sequencing (NGS) technologies has revealed previously unknown genomic alterations in this disease that are present at 5% to 15% (e.g. neurogenic locus notch homolog protein1 [*NOTCH1*], splicing factor 3B subunit 1 [*SF3B1*], ataxia-telangiectasia mutated [*ATM*]) and < 5% (e.g. myeloid differentiation primary response 88 [*MYD88*], baculoviral IAP repeat-containing protein 3 [*BIRC3*]) frequencies. These new mutations could partly explain the CLL heterogeneity and help in identifying clinically relevant groups of patients [8–11]. In the case of *NOTCH1*, mutations were detected more often among patients with progressive or high-risk CLL, while the role of *MYD88* has not yet been fully elucidated [12, 13]. Rossi et al. [14] believed that integration of these new mutational disorders with cytogenetic model results in more precise prediction of survival compared to the Döhner model alone. The 2016 revision of the World Health Organization classification suggested that these novel mutations have a potential clinical relevance and could be integrated into an updated cytogenetic risk profile [15]. However, a prognostic model created in the same year by the International Chronic Lymphocytic Leukemia–International Prognostic Index (CLL-IPI) Working Group included only IGVH and TP53 molecular mutations, recognizing the other mutations as showing no independent prognostic value. Therefore, there is a need to verify clinical significance of other novel prognostic factors [16].

The *NOTCH1* (neurogenic locus notch homolog protein 1) gene, encoded on chromosome 9q34.3, takes a fundamental biological role in hematopoiesis [8]. NOTCH1 receptors have been shown to have an essential role in the pathogenesis of some hematologic and solid malignancies [17, 18]. The extracellular domain of NOTCH1 (N-EC) consists of 36 epidermal growth factor-like repeats (EGFR), 3 cysteine-rich lin12/Notch repeats (LNR) and the (HD) heterodimerization domain. The intracellular part (N-IC) includes the RAM (R) domain, ankyrine repeats, transactivation domain (TAD) and the threonine-rich sequence (PEST) that marks N-IC for degradation by FBXW7 [19]. *NOTCH1* mutation in CLL patients most frequently concerns C-terminal PEST domain, which takes part in the proteasomal degradation of the activated form of *NOTCH1* [20]. In fact, mutated, short PEST domain results in stabilization of the active form of *NOTCH1*, the molecule impaired degradation, and thus upregulated *NOTCH1* signaling [21, 22]. *NOTCH1* mutations affect 5–10% newly diagnosed CLL and 15–20% in progressive CLL requiring first treatment and in relapsed cases [23, 24]. This mutation is associated with more aggressive clinical presentation of CLL such as chemorefractoriness and disease progression towards transformation into RS. *NOTCH1* mutations in RS occur in approximately 30% of cases and are the second, after *TP53*, most frequent genetic lesions [8, 23]. Studies on more numerous and specific subgroups of patients have revealed a particularly high frequency of *NOTCH1* mutations in CLL cases that harbor trisomy 12 (+12) as the sole cytogenetic abnormality (30%) [25]. Moreover, patients with *NOTCH1* mutations present a significantly shorter overall survival refining the intermediate prognosis of CLL patients with +12. Importantly, the high frequency of *NOTCH1* mutations in trisomy 12 CLL patients is associated with a characteristic gene-expression profile characterized by an overexpression of cell cycle-related

genes inserted on chromosome 12 [25]. *NOTCH1* mutations may represent potential new biomarker for the selection of poor-risk CLL patients. Patients with *NOTCH1* mutations are characterized by a significantly shorter OS (21–45% at 10 years) and present a more rapidly progressive disease compared to *NOTCH1* wild-type cases (56–66% at 10 years) [8, 23]. According to Mansouri et al., *NOTCH1* mutations similarly as *TP53* mutations seem to be strong, independent prognostic markers of poor prognosis [26].

Myeloid differentiation primary response 88 (MYD88) is a protein that plays a key role in the innate and adaptive immune response and is encoded by the *MYD88* gene locating on the short arm of chromosome 3 (p) at position 22 (3p22) [27]. MYD88 acts as a signaling adaptor protein that activates the NF-kB pathway after stimulation of toll-like receptors (TLRs) and receptors for IL-1 and IL-18 on independent and dependent signaling pathways [28]. Moreover, MYD88 co-ordinates the gathering of a multi-subunit signaling complex consisting of various members of the IRAK family of serine-threonine kinases [29]. The most common mutation is a single-nucleotide change (c.794T.C) that leads to switching of leucine to proline at codon 265 (p.L265P) [30]. That dominant mutation triggers constitutive NF-kB stimulation, thus conferring a proliferation and survival advantage to the mutant cells. Ngo et al. described mutations in *MYD88* in 39% of patients of (ABC-DLBCL) activated B cell type diffuse large B cell lymphoma, with a single L265P substitution accounting for 75% of the mutations [31]. The L265P mutation occurs in 2–10% of cases of CLL [8, 30] and almost 100% of cases of Waldenström's macroglobulinemia [32]. *MYD88* mutations in CLL patients are strikingly enriched among (M-CLL) patients expressing mutated *IGHV* genes [33]. Baliakas et al. [34] in a collaborative multicenter series of 1039 well-annotated CLL cases identified *MYD88* mutations in 24/1080 (2.2%) CLL patients and 92% cases implemented the hotspot p.L265P substitution. Xia et al. [33] in a study on Chinese population with CLL, found *MYD88* mutations (exons 3-5) in 23 of 295 (8%) analyzed cases. These mutations were more common in patients with mutated *IGVH* (2/115 vs. 21/172; $p = 0.001$). In another study, Jeromin et al. [35] investigated a large cohort of 1160 untreated CLL patients for novel prognostic markers considering *MYD88*. This mutation was detected in 15 of 969 cases (1.5%) and it was associated with mutated *IGVH* status.

Material and methods

Characteristics of patients

The research material was peripheral blood and bone marrow of CLL patients. The study included 200 newly diagnosed and previously untreated patients with CLL. The *NOTCH1* (n = 200) cohort included 88 women and 112 men at a median age of 66 years (range, 42–90 years). The *MYD88* (n = 60) cohort included 38 women and 22 men at a median age of 67 years (range, 50–84 years). Distribution of disease stages according to the Rai classification in *NOTCH1* group was as follows: stage 0, 63 patients; stage I, 33 patients; stage II, 48 patients; stage III, 12 patients; and stage IV, 20 patients. Distribution of disease stages according to the Rai classification in *MYD88* group was as follows: stage 0, 18 patients; stage I, 10 patients; stage II, 14 patients; stage III, 4 patients; and stage IV, 8 patients.

Additionally, expression of *MYD88* in CLL was assessed in peripheral blood (PB) (n = 60) and bone marrow (BM) (n = 92) of CLL patients. The detailed clinical characteristics of patients are presented in Table 1.

Ethical approval was granted by local review committees and informed consent was collected according to the principles laid by the Declaration of Helsinki. The approval number of the Ethical Commission is KE-0254/231/2015.

Peripheral blood mononuclear cells and DNA isolation

Peripheral blood mononuclear cells (PBMCs) from CLL patients were isolated by Ficoll density gradient centrifugation (Biochrom, Berlin, Germany). For DNA preparation form PBMCs, QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. DNA was quantified by BioSpec-nano (Shimadzu, Kyoto, Japan).

Amplification refractory mutation system for *NOTCH1* and *MYD88* mutations

MYD88 L265P (n = 60) and *NOTCH1* c.7544_7545delCT (n = 200) in the PEST domain (exon 34) mutations were investigated by (ARMS-PCR) amplification refractory mutation system polymerase chain reaction. Reactions for *NOTCH1* were performed in a 20 μl reaction volume containing the following: primer for the wild-type gene sequence, primer for the mutated gene sequence, reverse primer and multiplex. Reactions for *MYD88* were performed in 2 samples, a 20 μl

Table 1. Clinical characteristic of CLL patients

Characteristic	*NOTCH1* mutation	*MYD88* mutation
Number of patients	200	60
Sex		
Females	88	38
Males	112	22
Age median (range)	66 (42–90)	67 (50–84)
Rai stage		
0	63	18
1	33	10
2	48	14
3	12	4
4	20	8
Not available	24	6
ZAP-70 (cut-off 20%)		
Positive	53	12
Negative	102	40
Not available	45	8
CD38 (cutt-off 30%)		
Positive	53	11
Negative	111	42
Not available	36	7
IGVH mutation status		
Mutated	76	20
Unmutated	67	31
Not available	57	9

reaction volume each; the first sample contained multiplex, forward primer, and reverse primer for the wild-type gene; the second sample contained multiplex, forward primer, and reverse primer for the mutated gene. ARMS-PCR products were separated by gel electrophoresis.

mRNA preparation, reverse transcription and quantitative reverse transcriptase-polymerase chain reaction for *MYD88* expression

For total RNA isolation from PBMCs QIAamp RNA Blood Mini Kit (Qiagen, Venlo, Netherlands) was used according to the manufacturer's instructions. From each sample, 1 μg of total RNA was reverse transcribed to 20 μl of cDNA using QuantiTect Reverse Transcription Kit (Qiagen). For quantitative RT-PCR reactions, 1 μl of cDNA of each sample was used.

Expression of *MYD88* in CLL was assessed in PB (n = 60) and BM (n = 92) of CLL patients and 25 healthy volunteers (HVs) using (qRT-PCR) quantitative reverse transcriptase-polymerase chain reaction. qRT-PCR was performed using TaqMan Gene Expression Assays methodology according to the manufacturer protocol (Applied Biosystems, Foster City). As a constitutively expressed housekeeping gene, (*GAPDH*) glyceraldehyde-3-phosphate dehydrogenase was used. Thermocycling program was set for 40 cycles of 15 sec at 95°C, 1 min at 60°C on the ABI Prism 7300 Sequence Detector (Applied Biosystems). Expression levels were calculated as an inverse ratio of the difference in cycle threshold (ΔCt), where ΔCt is the Ct value of the target receptors minus Ct value of *GAPDH*.

Flow cytometry analysis for ZAP-70 and CD38

The expression of ZAP-70 and CD-38 was assessed by flow cytometry. Based on a ZAP-70 cut-off value of 20%, patients with CLL were categorized into a ZAP-70-positive ($\geq$ 20%) or a ZAP-70-negative subgroup ($<$ 20%). For CD-38, the cut-off value was defined as 30% and patients were categorized into a CD-38-positive ($\geq$ 30%) or a CD-38-negative subgroup ($<$ 30%).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (La Jolla, California, United States). The Mann-Whitney and Kruskal-Wallis tests were used to evaluate the differences between the subgroups. The correlations of variables were computed with the Spearman rank correlation coefficient.

Results

NOTCH1 mutations

NOTCH1 c.7544_7545delCT mutation occurred in 18 of 200 patients (9%) with CLL. Patients harboring the *NOTCH1* mutation prevalently belonged to aggressive cases, i.e. were significantly more often characterized by the unmutated *IGVH* gene status (n = 17 of 18, 94%) than patients with the wild-type *NOTCH1* gene (n = 50 of 112, 44%); (p = 0.0001). The mutation was significantly more often associated with high level of CD38 expression. It accounted for 10 of 18 CD38-positive cases (55%) among individuals with the *NOTCH1* mutation in comparison with 43 of 141 cases (30%) with the wild-type gene (p = 0.02). The expression of ZAP-70 was also significantly higher in individuals with mutated *NOTCH1* gene (n = 12 of 18, 66%) than in those with wild-type *NOTCH1* gene (n = 41 of 136, 30%) (p = 0.0008). Representative results of the *NOTCH1* ARMS assay are shown in Figure 1. The

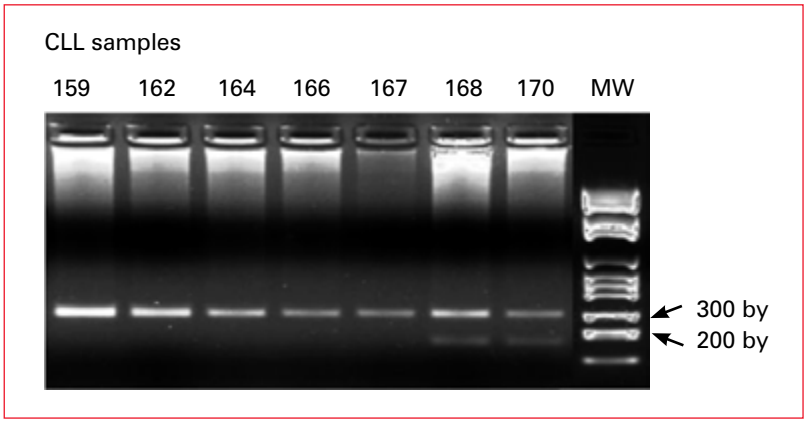
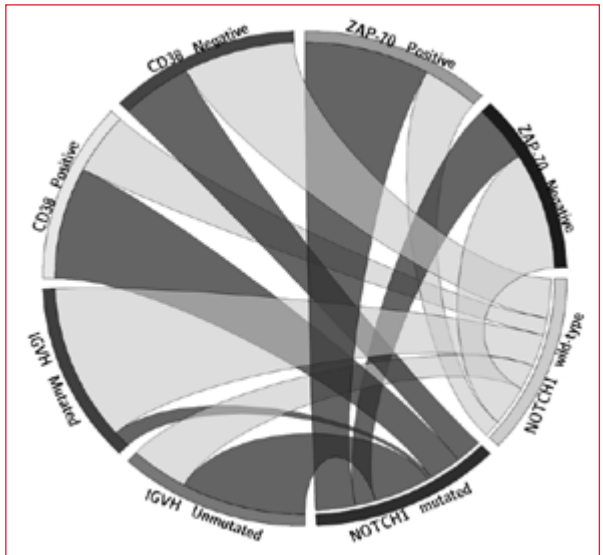


Figure 1. ARMS to detect *NOTCH1* c.7544_7545delCT mutation. Representative results of the ARMS assay showing 2 CLL samples that scored positive for the c.7544_7545delCT mutation (codes 168, 170) and 5 CLL samples that scored negative for the c.7544_7545delCT mutation (codes 159, 162, 164, 166, 167). Negative samples show a normal band of 284 bp. Positive samples show an additional mutant band of 183 bp. Molecular Weight (MW) is the 100-bp DNA ladder

complete analysis of the association between the *NOTCH1* mutation and prognostic markers in CLL is presented in Figure 2.

MYD88 mutations

The *MYD88* L265P (exon 5) mutation occurred in 2 of 60 patients (3.3%) with CLL. *MYD88* mutations were strikingly enriched among patients expressing mutated *IGVH* genes. The median expression of *MYD88* in PB (1/ΔCt) was 0.178, in the HVs we observed lower expression of *MYD88* (1/ΔCt of 0.128). The median expression of *MYD88* in PB (1/ΔCt) was 0.178, in the BM we observed lower expression of *MYD88* (1/ΔCt of 0.160). These differences reached statistically significance (respectively $p < 0.0001$ and $p = 0.0015$). The median expression of *MYD88* (1/ΔCt) in BM was significantly higher in CLL patients with stage Rai I (0.172) than in patients with stage II (0.152) and III (0.155) (respectively $p = 0.03$ and $p = 0.04$). There was no correlation between *MYD88* expression in PB and BM and expression of ZAP-70, CD38 and *IGVH* mutational status. Representative results of the *MYD88* ARMS assay are shown in Figure 3. Characterization of *MYD88* mRNA expression in CLL patients is presented in Figure 4A, 4B and 4C.



Clinical characteristic	Percentage of CLL patients		
	NOTCH1-mut	NOTCH1-wt	P-value
IGVH-UM/ /IGVH-M	94/6	44/56	0.0001
ZAP-70$_{positive}$/ /ZAP-70$_{negative}$	66/34	30/70	0.0008
CD38$_{positive}$/ /CD38$_{negative}$	55/45	30/70	0.02

Figure 2. Association between *NOTCH1* gene mutation status and clinical characteristic of CLL patients (created using Circos) [36]

Discussion

Thanks to the development of modern methods of molecular biology, the prognostic value of previously unknown factors has been identified and the usefulness of subsequent ones is verified in numerous scientific studies. Because CLL is still an incurable disease, there is a need for research in which the prognostic value of the new factors will be assessed. Since 2000, CLL patients' stratification is workable thanks to the cytogenetic model demonstrated by Döhner et al.

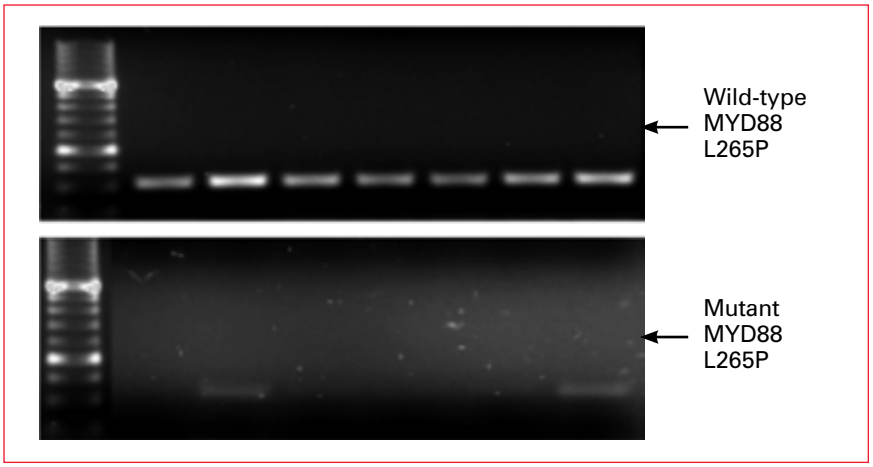


Figure 3. AS-PCR assay for detection of *MYD88* L265P. Representative results of the AS-PCR assay showing 2 CLL samples that scored positive for the L265P mutation and 5 CLL samples with *MYD88* wild-type sequence

[7]. It concentrates on chromosomal abnormalities, which are detectable in more than 80% of patients with CLL. Nevertheless, it occurs that molecular lesions provide additional information that also may have prognostic impact. Revision of the classification of lymphoid tumors of the World Health Organization in 2016 showed the potential clinical significance of molecular changes, such as *TP53*, *NOTCH1*, *SF3B1*, *ATM* and *BIRC3* [15]. Some of these changes have been proposed to be included in the updated cytogenetic risk profile, which also contains well-known recurrent chromosomal abnormalities typically identified with fluorescence in situ hybridization studies [34]. In 2016, the International CLL-IPI Working Group created a prognostic model combining biochemical, genetic, and clinical parameters. It includes five independent factors: age, β2-microglobulin concentration, clinical stage, *IGVH* mutation status, and *TP53* status. Other than *TP53* disorders, recurrent genetic abnormalities, such as *NOTCH1* and *SF3B1*, did not show independent prognostic information [16].

The prognostic impact of the molecular factors such as *NOTCH1* and *MYD88* has been confirmed by several research groups, but still has not been used in clinical practice [11–14, 34]. These mutations are thought to be part of the mechanisms explaining the heterogeneity of the disease and may point to new potential therapeutic targets in CLL [11].

To our knowledge, we were the first to assess the occurrence of the analyzed mutations in the cohort of Polish patients. In our study the frequency of the *NOTCH1* mutation in newly diagnosed patients with CLL reached 9.0% and was in agreement with the findings of other investigators [11, 24]. Really, it has been shown that the frequency of mutations can increase as the disease progresses, even up to 20% during relapse [9, 30]. The *NOTCH1* mutation is associated with especially poor prognosis. The comparison with the presently available prognostic factors revealed the accumulation of negative markers in *NOTCH1*-mutated individuals. A significant correlation was found with the unmutated *IGVH* status, confirming the results of previous studies [11, 30, 37–39]. Additionally, the *NOTCH1* mutation was significantly more frequently associated with positive expression of CD38 and ZAP-70, which is associated with poor prognosis. Studies revealed that the *NOTCH1* mutation was more common among patients in advanced clinical stage, mainly enrolled or treated in clinical trials [34]. *NOTCH1* mutation has also a strong positive association with trisomy +12 and negative association with isolated del(13q). What's more, it has been associated with decreased overall survival in Rossi et. al and Oscier et al. studies [11, 24], but was not an independent marker of survival [5].

Our study demonstrated significantly higher PB *MYD88* expression than in HVs and relevantly higher PB *MYD88* expression in comparison with BM (respectively $p < 0.0001$ and $p = 0.0015$). There was no correlation between *MYD88* expression in PB and BM and expression of ZAP-70, CD38 and *IGVH* mutational status. Apart from *MYD88* expression in current study we also investigated *MYD88* mutations. These mutations occurred in 3.3% CLL patients and were strikingly enriched among patients expressing mutated *IGVH* genes. Our data argue with previous reports by Xia et al. that demonstrated more common occurrence *MYD88* mutations in patients with mutated *IGVH*

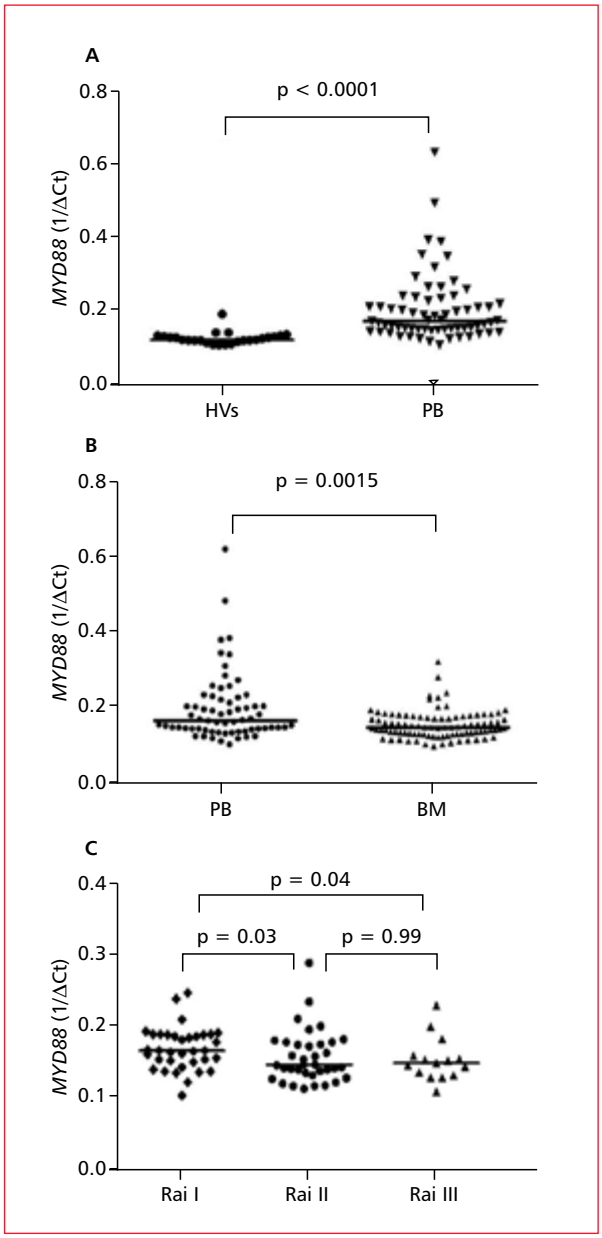


Figure 4. Characterization of *MYD88* mRNA expression in chronic lymphocytic leukemia (CLL) patients. **A.** Expression of *MYD88* in peripheral blood of healthy volunteers (HVs) and CLL patients; **B.** Expression of *MYD88* in peripheral blood (PB) and bone marrow (BM) of CLL patients; **C.** Expression of *MYD88* in BM of CLL patients according to the stage of the disease

[33]. The study of Jeromin et al. on the large cohort of CLL patients revealed *MYD88* mutations in 1.5% cases and was also associated with mutated *IGVH* status [35]. Therefore, the relevance of the *MYD88* mutation as a prognostic factor remains unclear and larger collaborative studies are required.

Conclusions

In conclusion, *NOTCH1* mutations are associated with certain, unfavorable biological markers and seem to be independent predictive markers for worse outcome in CLL patients. Definitely, these mutations may help the identification of patients with high-risk CLL, and in combination with established negative prognostic factors of the CLL-IPI, they might provide additional information on disease prognosis. Further collaborative studies in CLL are obligate to study the prognostic and predictive relevance of *MYD88* mutations and expression.

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