Exp Oncol 2020
42, 1, 60–65

Experimental ONCOLOGY

MYC COPY NUMBER AND mRNA EXPRESSION IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS EXPOSED TO IONIZING RADIATION DUE TO THE CHORNOBYL NPP ACCIDENT

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Some clinical and biological features indicating an unfavorable course of the disease were found in ionizing radiation (IR) — related chronic lymphocytic leukemia (CLL) patients. The *MYC* proto-oncogene is considered to contribute to CLL pathogenesis. Increased *MYC* copy number is associated with poor prognosis in CLL. *Aim:* To investigate the frequency of *MYC* gene copy number amplification in IR-exposed CLL patients and relate the findings to the *MYC* mRNA levels, the presence of unfavourable prognosis mutations (*TP53, SF3B1, NOTCH1*), and patient`s outcome. *Materials and Methods:* The analysis of *MYC* copy number was carried out by real-time quantitative polymerase chain reaction (PCR) in 70 IR-exposed CLL patients. The *MYC* mRNA expression was measured by real-time quantitative reverse transcription PCR. *Results:* Increased *MYC* gene copy number was present in 5.7% of cases. There was a statistically significant association between increased *MYC* copy number and increased *MYC* mRNA ($p < 0.014$). Additionally, somatic deletion in *MYC* locus was found in one patient. Most of patients (80%) with detected *MYC* aberrations were previously untreated, suggesting that these lesions might occur early in the course of the disease. The *MYC* aberrations were found mutually exclusive with high risk *TP53* and *SF3B1* mutations, while one case was identified, where *MYC* amplification and *NOTCH1* mutation coincided simultaneously. Regarding clinical outcome, the *MYC* aberrations were associated with a shorter time to first treatment (3 *vs* 25 months, $p = 0.008$) as well as reduced overall survival (60 *vs* 139 months). *Conclusion:* Our data suggest that *MYC* aberrations might be an early event in IR-related CLL and contribute to aggressive disease development in the absence of high risk *TP53* and *SF3B1* mutations.

Key Words: **chronic lymphocytic leukemia, *MYC* gene, 8q24.1, gene copy number.**

DOI: 10.32471/exp-oncology.2312-8852.vol-42-no-1.14214

Chronic lymphocytic leukemia (CLL) is the most common leukemia among the adults, which is characterized by a high clinical and biological variability [1]. Multiple genomic alterations and gene mutations have been identified as drivers of the disease, which might affect significantly clinical outcome. These alterations are associated with several key cellular signal pathways and processes: DNA damage response (*ATM*, *CHEK2, BRCC3*), apoptosis and cell cycle control (*TP53*), NOTCH1 signalling, RNA processing and export (*SF3B1, XPO4)*, B cell activity related pathways (*TRAF2, TRAF3),* MAPK signalling. Besides, dysregulation of MYC activity is considered as one of central cell pathways involved in CLL [2].

MYC gene, located at chromosome 8q24.21, is one of the proto-oncogenes, which is most frequently involved in human carcinogenesis [3]. *MYC* encodes for a transcription factor of the helix-loop-helix/leucine zipper protein family, which in form of dimers with the protein MAX binds to specific sequences — E-boxes (canonical sequence CACGTG) in regulatory regions of target genes. MYC/MAX dimers regulate 10–15% of all human genes and thus control a variety of cellular functions, including cell cycle progression, growth, survival, differentiation and biosynthesis [4–6]. Because of its central role in cell processes, *MYC* is tightly regulated at both the transcriptional and translational levels [7]. In normal cells *MYC* mRNA and protein have very short half-lives. Without appropriate positive regulatory signals MYC protein levels are low and insufficient to promote cellular proliferation [8–10]. In cancers the delicate balance of MYC regulation is often disturbed. Several mechanisms for *MYC* deregulation have been identified, including translocation, amplification, mutations, as well as mutations of cellular pathways involved in its regulation [3, 7].

In CLL, mutations in multiple genes were identified, although most of them with low frequency, which might influence MYC activity — *NOTCH1, MGA, FBXW7*, *PTPN11, FUBP1* [2]. *MYC* amplification and translocation were reported rare in general CLL cohorts (up to 4%) [11], however, their frequency was found increased in CLL subsets with aggressive disease, and especially in Richter transformation (up to 40%) [12, 13]. Recently Edelmann *et al.* [14] showed that *MYC* amplification was highly enriched in relapsed/refractory *TP53*-deficient CLL group (17%). Besides, both *MYC* amplification and high *MYC* expression were reported to be associated with poor prognosis in CLL patients [11, 15].

We previously found some clinical and biological features of CLL patients exposed to ionizing radiation (IR) due to Chornobyl NPP accident indicating unfavorable course of disease, such as high frequency

Submitted: November 07, 2019.
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Abbreviations used: CLL – chronic lymphocytic leukemia; CN – copy number; IR – ionizing radiation; OS – overall survival; PCR – polymerase chain reaction; TTFT – time to first treatment.

of secondary solid tumors and Richter transformation, mainly unmutated status of heavy chain variable region (*IGHV*) genes with increased usage of *IGHV1-69* and *IGHV3-21* [16]. Thus, we were tempted to study the frequency of *MYC* copy number (CN) amplification in IR-related CLL patients to estimate the influence on disease development. In this study we investigated *MYC* CN amplification in relation to the *MYC* mRNA levels, molecular markers (mutations in *TP53, NOTCH1,* and *SF3B1* genes), and patient`s outcome.

MATERIALS AND METHODS

Patients and samples. The studied cohort included 70 IR-exposed CLL patients (61 males and 9 females), referred to the State Institution "National Research Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine". Fifty-four of 70 CLL patients were clean-up workers, 10 — inhabitants of radionuclide contaminated areas, and 6 patients — evacuees. The study was approved by the local Ethics Review Committee, and all patients gave informed consent prior to participation in it. 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 [17] and Rai [18] staging systems, and treatment requirement was according to National Cancer Institute criteria [19].

Most patients (n = 61, 87.1%) were untreated at the time of sample collection, and 9 patients 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. Molecular characteristics such as mutational status of *TP53, NOTCH1* genes were known for all 70 patients, *SF3B1* — for 68 patients, and mutational status of *IGHV* genes — for 69 patients, as a result of previous studies [16, 20].

Genomic DNA and total RNA were extracted from peripheral blood mononuclear cells with the QIAamp Blood Mini Kit (Qiagen, Crawley, United Kingdom) and TRI Reagent® (Molecular Research Center Inc., Cincinnati, OH) respectively, and were stored in deep freeze until further use.

Quantitative real-time polymerase chain reaction (PCR). For *MYC* CN analysis quantitative real-time PCR (qPCR) was performed with the Real-time IQ Detector System (Bio-Rad) using SYBR Green chemistry. Amplification was carried out with 50 ng DNA in a 25 µl reaction mixture containing 80 nM of each primer and Maxima SYBR Green qPCR Master Mix (Thermo Scientific). The *BRG1* gene served as an internal control. The following primers were used: for *MYC* — 5′-TGGATCACCTTCTGCTGGA-3′ and 5′-TCTGACACTGTCCAACTTGACC-3′ (reported by Chen H *et al.* [21]), for *BRG1* — 5′-AAGAAGACTGAGCCCCGACATTC-3′ and 5′-CCGTTACTGCTAAGGCCTATGC-3′ (reported by Buscarlet *et al.* [22]). qPCR cycling conditions were as follows: an initial denaturation step of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. All reactions were done in triplicate, and all qPCR runs included 2 calibrator samples (DNA of healthy donors). The relative CN of *MYC* gene was determined using the comparative ΔΔCt method and was calculated as $CN = 2^{-\Delta\Delta Ct}$ ($2 \times 2^{-\Delta\Delta Ct}$ — per diploid genome) [23]. To estimate boundary values for 2 copies of *MYC* gene, qPCR amplification was performed with DNA samples of 8 healthy donors following Kindich *et al.* [24]. In this study, the *MYC* gene CN was considered increased in sample when CN was > 2.4.

For *MYC* expression analysis, cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the supplier's instructions. qPCR was performed with the Real-time IQ Detector System (Bio-Rad) using Absolute Blue qPCR SYBR Green Master Mix (Thermo Scientific) as reported previously [25]. The expression levels of *MYC* were normalized to the expression of the housekeeping gene *ABL1.* The following primers were used: for *MYC* — 5′-TCGGATTCTCTGCTCTCCTC-3′ and 5′- GAGCCTGCCTCTTTTCCAC-3′ (reported by Caraballo *et al.* [12]); for *ABL1* — 5′-TGG AGATAACACTCTAAGCATAACTAAAGGT-3′ and 5′-GATGTAGTTGCTTGGGACCCA-3′ (reported by Beillard *et al.* [26]). The relative expression levels were calculated using the ΔΔCt method. The *MYC* expression of peripheral blood sample of healthy donor was used as a calibrator for relative quantification. Additionally, qPCR products we analysed by electrophoresis in 1.5% agarose gel and visualization under UV light after ethidium bromide staining.

Statistical analysis. Associations with categorical clinico-biological variables were assessed with the chi-square test or the Fisher exact test, where appropriate. The Mann-Whitney U test was used to compare median levels of *MYC* between groups. Kaplan — Meier method was used to analyse overall survival (OS) and time to first treatment (TTFT), the log-rank statistic was used to determine significant associations between individual markers and OS or TTFT. *P* values <0.05 were considered statistically significant. All analyses were performed with the SPSS software package, version 13.0 (SPSS).

RESULTS

CLL group characteristics. The studied CLL group consisted of 61 men and 9 women, with a median age of 57.7 years (range: 39–76 years) at the time of diagnosis. Clinical and biological characteristics of patients are provided in Table 1. Sixty-one (87.1%) of patients were untreated at the time of sample collection, while 9 (12.9%) were previously treated. In the subsequent period, 48 (68.6%) of patients received therapy. Overall, 57 (81.4%) patients were treated, with the median time from diagnosis to treatment of 33 months (1–156 months).

Forty-eight (69.6%) patients had unmutated *IGHV* genes, whereas 21 (30.4%) patients had mutated *IGHV*. Mutation in at least one of the three common mutated genes (*TP53, SF3B1* or *NOTCH1*) was detected in 18 (25.7%) cases. Eight (11.4%) of patients

Table 1. Baseline characteristics of observed CLL patients

Characteristics		Patients, n = 70
Median age, years (range)		57.7 (39–76)
Gender, n (%)	Male	61 (87.1)
	Female	9 (12.9)
Binet stage at diagnosis, n (%)	A	39 (55.7)
	B	26 (37.1)
	C	5 (7.2)
Clinical phases of CLL, n (%)	Not requiring first treatment	31 (44.3)
	Requiring first treatment	30 (42.9)
	Relapsed	9 (12.9)
IGHV mutational status*, n (%)	Unmutated	48 (69.6)
	Mutated	21 (30.4)
TP53 mutated, n (%)		7 (10)
NOTCH1 mutated, n (%)		8 (11.4)
SF3B1 mutated**, n (%)		7 (10.3)

Note: **IGHV* mutation status data were available for 69 patients. ***SF3B1* status data were available for 68 patients.

harbored *NOTCH1* mutation, in 4 (5.7%) of cases both *TP53* and *SF3B1* genes were found mutated, isolated *TP53* and *SF3B1* mutations were found in 3 cases each (3.3%). Unmutated *IGHV* status and presence of *TP53* or *SF3B1* mutations were highly predictive factors of reduced TTFT and OS in this CLL group (Table 2).

Frequency of MYC aberrations. Increased *MYC* gene CN (CN > 2.4) was found in 4 cases (5.7% patients). The *MYC* CN values ranged from 2.8 to 3.4 copies/cell. Three of these patients did not receive previous treatment and one patient was previously treated. We did not reveal a decrease in *MYC* CN in any case of studied CLL group.

Additionally, analysis of PCR products of c.1066-1226 *MYC* region amplification by electrophoresis showed shortened band in one patient (Fig. 1). This patient was previously untreated. The shortened amplicon differed by more than 10 bp compared to intact PCR product (160 bp) indicating the deletion in c.1066-1226 region of *MYC* (protein position: 235–287 aa). We did not find similar size deletion among COSMIC-listed *MYC* mutations [COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/]. However, that MYC region contains known phosphorylation sites, where it was demonstrated in Burkitt lymphoma, somatic mutations clustered significantly [27]. Since phosphorylation is required for ubiquitination and subsequent degradation of the MYC protein by the proteasome, abrogation of phosphorylation could lead to a decrease of protein degradation and hence, increase the stability of the MYC protein [28]. Thus, protein stabilization due to mutations in close to phosphorylation regions might be one of mechanisms to deregulate MYC in CLL.

Table 2. OS and TTFT in CLL patients according to clinical and molecular characteristics

Characteristics	N	OS Median, months	*p*	TTFT Median, months	*p*
Binet stage at diagnosis			0.001		0.0001
A	39	146		45	
B	26	75		6	
C	5	68		1	
IGHV mutational status			0.004		0.005
Unmutated	48	107		13	
Mutated	21	*NR*		88	
TP53			0.0001		0.015
Mutated	7	52		6	
Unmutated	63	139		36	
NOTCH1			0.641		0.263
Mutated	8	80		3	
Unmutated	62	114		24	
SF3B1			0.0001		0.012
Mutated	7	57		6	
Unmutated	61	139		34	
MYC aberrations			0.001		0.008
Detected	5	60		3	
Not detected	65	139		25	
MYC expression			0.319		0.403
Increased	19	114		22	
Low	49	158		36	

Note: *NR* – not reached.

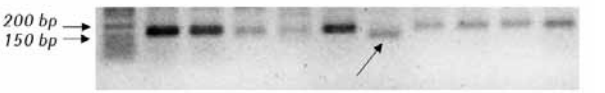

Fig. 1. Results of PCR amplification of c.1066-1226 *MYC* region (160 bp) in CLL patients. The shortened band of about 150 bp indicates somatic deletion (marked by arrow)

MYC aberrations are almost mutual exclusive with common CLL mutations. *MYC* aberrations (increased *MYC* CN and *MYC* deletion) were found mutually exclusive with mutations in the *TP53* gene as well as *SF3B1* mutations (Table 3). At that, more than half (4 of 7; 57.1%) of *TP53* mutated cases were represented simultaneously with *SF3B1* mutation. This frequent representation of both *TP53* and *SF3B1* mutations was described by us previously as distinctive feature of IR-related CLL group [20]. Concerning *NOTCH1* gene, one case was identified, where *MYC* amplification and *NOTCH1* mutation coincided simultaneously.

MYC aberrations are associated with unfavorable clinical outcome. Follow-up information was available for all patients, 32 (45.7%) of whom died during the period of observation due to CLL-related causes. The estimated median OS was 117 months (95% CI = 77.5–156.5 months). Log-rank tests for the Kaplan — Meier curves demonstrated significantly reduced TTFT in CLL patients with *MYC* aberrations in comparison with the rest of cohort — 3 *vs* 25 months, $p = 0.008$. These patients experienced also shorter overall survival — 60 *vs* 139 months, $p = 0.001$ (Fig. 2).

MYC expression. Overall *MYC* expression was low in most CLL cases studied. The average relative *MYC* expression level was 5.7 (range, 0–48.5). The cases with revealed *MYC* CN amplification (n = 4) showed increased *MYC* expression (median = 8.5, range 6.2–19.5) in comparison with the rest of the group (median = 2.7, range 0–48.5), $p < 0.014$ (Fig. 3). In the case with *MYC* deletion, low *MYC* level was detected.

For the *MYC* expression stratification, the overall mean *MYC* value (5.7) was used as the cut-off to designate increased *vs* low expression, following Stasik *et al.* [29]. Thus, in the study 19 (27.9%) of cases were classified as cases with increased *MYC,* while 49 (72.1%) — as low *MYC* expressed cases. Analysis of associations of *MYC* expression with clinical and biological variables, and clinical outcome was done using this stratification (see Table 3).

Table 3. *MYC* aberrations and *MYC* expression in relation to clinical and molecular characteristics

Characteristics		*MYC* aberrations, n = 70		*p*	*MYC* expression, n = 68		*p*
		Detected, n (%)	Not detected, n (%)		Increased, n (%)	Low, n (%)	
Age at diagnosis				0.643			0.594
	< 57.7 years	2 (5.7)	33 (94.3)		11 (31.4)	24 (68.6)	
	57.7 years and more	3 (8.6)	32 (91.4)		8 (24.2)	25 (75.8)	
Gender, n (%)				0.492			0.216
	Male	5 (8.2)	56 (91.8)		18 (30.5)	41 (69.5)	
	Female	0	9 (100)		1 (11.1)	8 (88.9)	
Binet stage at diagnosis				0.198			0.814
	A	1 (2.6)	38 (97.4)		10 (22.3)	28 (73.7)	
	B	3 (11.5)	23 (88.5)		7 (28)	18 (72)	
	C	1 (20)	4 (80)		2 (40)	3 (60)	
Clinical phases of CLL				0.557			0.236
Not requiring first treatment		3 (9.7)	28 (90.3)		11 (35.5)	20 (64.5)	
Requiring first treatment		1 (3.3)	29 (96.7)		5 (17.2)	24 (82.8)	
Relapsed		1 (11.1)	8 (88.9)		3 (37.5)	5 (62.5)	
IGHV				0.599			0.538
	Unmutated	4 (8.3)	44 (91.7)		13 (27.7)	34 (72.5)	
	Mutated	1(4.8)	20 (95.2)		5 (25)	15 (75)	
TP53				0.439			0.175
	Mutated	0	7 (100)		0	6 (100)	
	Unmutated	5 (7.9)	58 (92.1)		19 (30.6)	43 (69.4)	
NOTCH1				0.465			0.427
	Mutated	1 (12.5)	7 (87.5)		1 (16.7)	7 (83.3)	
	Unmutated	4 (6.5)	58 (93.5)		18 (12.5)	42 (87.5)	
SF3B1				0.432			0.632
	Mutated	0	7 (100)		1 (16.7)	5 (83.3)	
	Unmutated	5 (8.2)	56 (91.8)		18 (30)	42 (70)	

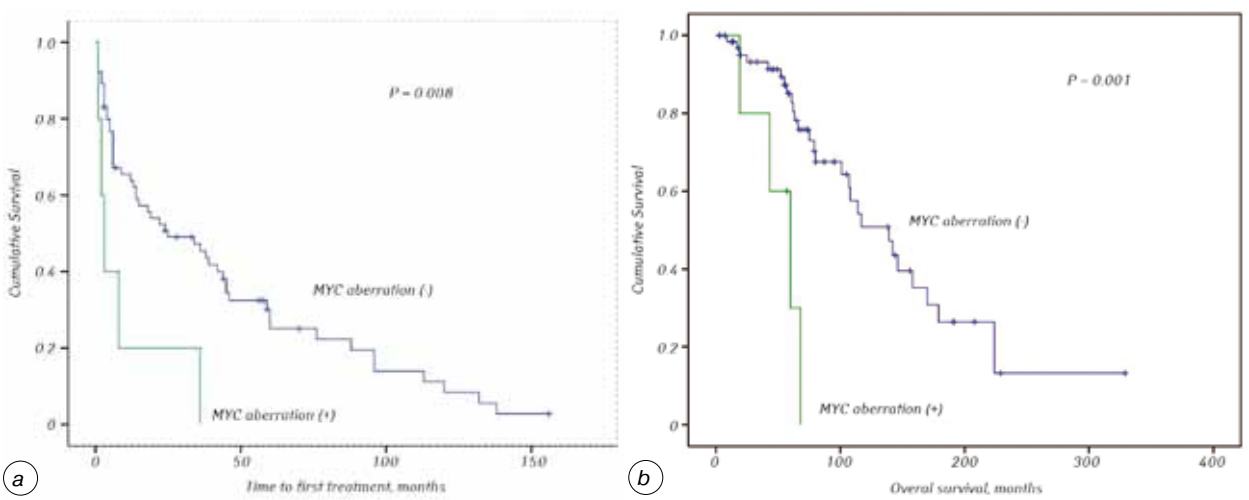

Fig. 2. Survival analysis. TTFT (a) and OS (b) in CLL patients regarding presence of *MYC* aberrations

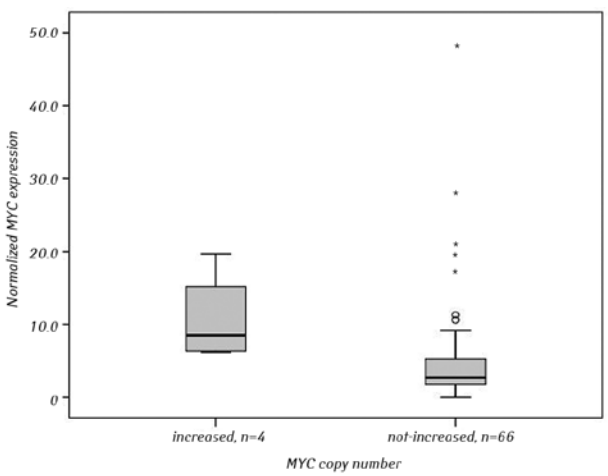

Fig. 3. Expression levels of *MYC* in relation to *MYC* copy number. Boxplots show relative expression of *MYC* in CLL patients with increased *MYC* copy number and not-increased *MYC* copy number. Median, 25 and 75 percentile values, non-outlier ranges, outliers and extremes are indicated. The *p* value was calculated using the Mann — Whitney U test

We failed to find a significant correlation between *MYC* values and clinical variables. We did not detect also any correlation between *MYC* expression and any of the molecular markers analysed (such as unmutated *IGHV*, mutated *TP53, SF3B1* and *NOTCH1* genes). Besides, there was no significant difference in TTFT and OS between groups with increased *MYC* and low *MYC* expression. That is in line with report by Caraballo *et al.* [12], where also no associations were found between MYC expression and several clinical and biological variables, including *NOTCH1* and *TP53* mutations. Besides, authors reported absence of close correlation between *MYC* mRNA and protein level. Contradictory results were also reported, where high expression *MYC* levels have been described [15, 30]. Further study is necessary to clarify this issue taking in view that *MYC* is tightly regulated at both the transcriptional and translational levels [7].

DISCUSSION

The *MYC* gene amplification and mutations are, in addition to mutations in MYC-related pathways,

mechanisms of MYC protein deregulation in CLL. *MYC* aberrations were detected in 5 (7.1%) cases in our study: CN amplification was present in 4 (5.7%) cases, and in one case somatic deletion was detected in *MYC* locus. *MYC* amplification was found to be associated with increased *MYC* mRNA expression that agrees with previous reports [11, 14]. Most of patients with *MYC* aberrations (80%) were previously untreated indicating that these lesions might occur early in the course of the disease. Whole-genome sequencing studies analysing clonal heterogeneity also suggested gains at 8q24 (where *MYC* maps) as early events during CLL development [2].

Overall, the frequency of *MYC* amplification in studied group is slightly higher in comparison with CLL cohorts reported (up to 4%) [11, 14]. At that, *MYC* amplification might be underestimated in our studied group since we have assessed *MYC* locus only. Other *MYC*-related CN aberrations were identified in CLL using high resolution array-based technologies recently. In addition to *MYC* locus amplification, focal amplifications in 8q24.21 affecting a super-enhancer region approximately 360 kb centromeric to *MYC* locus were detected [11, 14]. The 8q24.21 "gene desert" regulatory region contains multiple single nucleotide polymorphisms that have been associated with susceptibility to various cancers including CLL [31, 32]. According to Edelman *et al.* [14], minimally gained region in 8q24.21 in CLL encompassed three long non-coding RNAs — *CASC19*, *CCAT1*, and *CASC21*. *CCAT1* (colon cancer associated 1) was associated with adverse risk in several solid cancers, its transcript was shown to stabilize a chromosome loop between *MYC* and the enhancer region [33]. Besides, the 8q24.21 super-enhancer region was shown to contain binding sites for the NICD1 (NOTCH1 intracellular domain) transcription factor [34]. Edelman *et al.* [14] reported focal amplifications in 8q24.21 in 1.3% of cases and *MYC* locus amplification — in 2.2% of cases in standard risk CLL subset.

In one case in our cohort, somatic deletion was detected in *MYC* region c.1066-1226 (protein position: 235-287 aa), which included partially the second and the third exons. We did not find similar deletion among COSMIC-listed *MYC* mutations. This *MYC* region contains known phosphorylation sites, in proximity to which somatic mutations clustered significantly in Burkitt lymphoma [27]. Phosphorylation is required for ubiquitination and degradation of the MYC protein by the proteasome, and abolition of phosphorylation could lead to a decrease of protein degradation, increase the stability of the MYC protein, and thus MYC activity [28]. Broun *et al.* [11] reported one CLL case with missense substitution Thr58Ala related to regulatory phosphorylation site in the first exon of *MYC*. That substitution was previously identified in Burkitt lymphoma and was shown to impair FBXW7-mediated proteasomal degradation of MYC that resulted in its activation [35]. Thus, protein stabilization due to mutations close to phosphorylation regions might be one of mechanisms, although infrequent, to deregulate MYC in CLL.

MYC aberrations were found to be represented almost mutually exclusive with *TP53, SF3B1* and *NOTCH1* mutations in studied cohort. Regarding *NOTCH1* mutations, this agrees with the finding that *MYC* is one of main target gene of NOTCH1. Tight relation was demonstrated between aberrantly strong NOTCH1 signaling, caused particularly by activating mutations in *NOTCH1* gene, and increased MYC activity in CLL [34]. *SF3B1* mutations are supposed to constitute another frequent mechanism to strengthen the NOTCH1-MYC signaling axis [14]. It was shown that the strength of NOTCH1 signaling depends on DVL2 (multi-domain protein Dishevelled 2), which inhibits transcriptional activation by NOTCH1 [36]. Mutations in *SF3B1* lead to alternative splicing of *DVL2* and the resulting splice variant was shown to lack its ability to modulate NOTCH1 signaling [37]. In line with these, *NOTCH1* and *SF3B1* mutations are often mutually exclusive in CLL [14].

Possible involvement of *SF3B1* mutations in NOTCH1-MYC signaling axis might explain our results indicating mutual exclusivity of *MYC* aberrations and *TP53* mutations in studied cohort. This contradicts the study, where the frequency of *MYC* amplification was found significantly increased in *TP53*-deficient CLL subset [14]. However, in more than half (57.1%) of *TP53* mutated cases in our cohort *SF3B1* mutation was presented simultaneously. The frequent coincidence of *TP53* and *SF3B1* mutations was described by us previously as distinctive feature of IR-related CLL subset [20].

MYC aberrations were associated with reduced TTFT and OS in studied CLL cohort. The association with unfavorable outcome was observed in the absence of high risk *TP53* and *SF3B1* mutations. Broun et al. [11] previously reported that association of *MYC* amplification with short TTFT was independent of co-occurrence of high risk deletions 11q and 17p.

In conclusion, the results of our study suggest that *MYC* aberrations might be early events in IR-related CLL and contribute to aggressive disease development in the absence of high risk *TP53* and *SF3B1* mutations. Further studies are needed to precise the incidence and value of *MYC* lesions in IR-related CLL, including analysis of the 8q24 risk region near *MYC* for focal amplifications and somatic mutations of the entire *MYC* locus.

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