

TP53 CODON 72 SINGLE NUCLEOTIDE POLYMORPHISM IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Defects in the tumor suppressor gene *TP53* are known to be important in chronic lymphocytic leukemia (CLL) and *TP53* inactivation is associated with a particularly aggressive form of the disease. The single nucleotide polymorphism in the *TP53* gene at codon 72 (rs1042522), results in amino acid substitution influencing apoptotic potential of *TP53* protein. *The aim* of the study was to evaluate the association of the *TP53* codon 72 polymorphism and incidence of *TP53* mutations in CLL patients. *Methods*: 261 CLL samples were analyzed by polymerase chain reaction and direct sequencing for *TP53* mutations and single nucleotide polymorphism. *Results*: The 72Pro/Pro genotype was associated with an increased incidence of *TP53* mutations in previously treated patients (OR = 2.503; 95% CI 1.142–5.487; $p = 0.001$). *Conclusion*: This study revealed that the *TP53* codon 72 polymorphism may be used as a risk factor for incidence of *TP53* mutations in CLL.

Key Words: chronic lymphocytic leukemia, *TP53* mutations, single nucleotide polymorphism.

Chronic lymphocytic leukemia (CLL) is characterized by a highly variable clinical course, ranging from indolent cases to cases with aggressive and rapidly progressing disease [1]. Several prognostic markers have been identified, including different genomic aberrations. Defects in the tumor suppressor gene *TP53* was found to be associated with particularly poor prognosis. The *TP53* protein plays a crucial role in cellular response to DNA damage by induction of apoptosis, cell cycle arrest and DNA repair, thus maintaining genomic integrity and mediating the action of DNA damaging chemotherapy, as well [2]. Inactivation of *TP53* due to deletion of *TP53* gene locus (17p13), which in the majority of cases (over 80%) is accompanied by mutation in the remaining *TP53* allele, or due to *TP53* mutation alone have been associated with short survival and chemotherapy resistance in a number of studies [3–7]. CLL patients with mutated *TP53* are considered a high-risk prognostic group, who should be offered alternative therapy strategies, acting independently of *TP53* pathway [8, 9].

The *TP53* gene aberrations are relatively infrequent at stage of CLL diagnosis or at time of initial therapy, ranging from 10% to 15% of cases. However, the incidence rises up to 40–50% in group of patients previously treated with chemotherapy and undergo refractory CLL [10, 11]. Some known markers of unfavorable CLL prognosis are reported as risk factors for *TP53* aberrations — an increased expression of CD38, ZAP-70, and unmutated status of immunoglobulin heavy chain variable region (*IGHV*) genes [12, 13]. Recently some preliminary data were presented indicating the association between *TP53* mutations and single nucleotide polymorphism

(SNP) within the *TP53* gene at codon 72 (rs1042522). This polymorphism encodes either arginine (72Arg, genotype CGC) or proline (72Pro, genotype CCC) and significantly affects *TP53* function. The 72Arg variant is more effective at inducing apoptosis than the 72Pro, while the 72Pro/Pro genotype was associated with an increased expression of cell cycle arrest genes [14]. Grossmann et al. and Dong et al. have found an increased incidence of *TP53* mutations and deletions in CLL patients with the 72Pro/Pro genotype [15, 16]. The same association was found in patients with non-melanoma skin cancer [17]. On the contrary, lung cancer patients who were the 72Pro allele carriers had significantly lower frequency of *TP53* mutations in comparison with 72Arg homozygotes [18].

To further substantiate the role of the *TP53* codon 72 polymorphism as a risk factor for *TP53* mutations, we have analyzed the *TP53* mutational status in CLL patients, carriers of different genotypes in relation to clinical characteristics and some molecular peculiarities of B-cell receptor of leukemic cells.

MATERIALS AND METHODS

The *TP53* codon 72 SNP was analyzed in 261 consecutive patients with CLL, 198 males (75.5%) and 63 females (24.5%), median age of 58 years at CLL diagnosis (range 29–86 years), referred to the State Institution “National Research Centre for Radiation Medicine, National Academy of Medical Sciences of Ukraine”, Kyiv, Ukraine. All patients were inhabitants of the central part of Ukraine. The study was approved by the local Ethics Review Committee, and all patients signed an informed consent form prior to participation in the study. CLL was diagnosed on the basis of clinical history, blood lymphocytosis lymphocyte morphology, and immunophenotypic criteria. The stage of the disease was assessed by Binet [19] and Rai classification [20]. The treatment was initiated in accordance with the National Cancer Institute of USA (NCI) criteria [21].

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Abbreviations used: CLL – chronic lymphocytic leukemia; *IGHV* – immunoglobulin heavy chain variable region; SNP – single nucleotide polymorphism.

Genomic DNA was extracted from peripheral blood mononuclear cells with the QIAamp Blood Mini Kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's protocol. At the time of blood samples collection, 172 (65.9%) patients were either at diagnosis or before the initial treatment (median of observation was 41 months) and remained 89 of 261 (34.1%) patients received therapy (median of observation was 75 months). The *IGHV* gene mutational status was assessed by PCR amplification followed by direct sequencing according to the BIOMED-2 consortium rules [22], as described above [23]. Sequences were analyzed using the IgBlast and IMGT databases. The *IGHV* gene was considered unmutated when the identity to the corresponding germline gene was more than or equal 98%.

TP53 gene mutation analysis was performed for exons 3–10 in the majority of cases (245/261), and for exons 5–9 (region, where the vast majority of mutations are located), in 16 cases. Study was done by PCR amplification followed by direct sequencing with Big-Dye Term v3.1 cycle sequencing chemistry (Applied Biosystems Foster City, CA). Data were compared with reference *TP53* gene sequence NC_000017.10, and mutations were validated using the International Agency for Research on Cancer (IARC) *TP53* Mutation Database (www.p53.iarc.fr) [24]. For exons 3–10 analysis five fragments were amplified, spanning 3–4, 5–6, 7, 8–9 and 10 exons. Primers were kindly designed by Dr. V. Saenko (Atomic Bomb Disease Institute, Nagasaki University), all primer sequences are available on demand. PCR was performed using 10 ng of DNA in a 25 µL reaction mixture containing 0.3 µM of each primer, 1.5 mM MgCl₂, 0.8 mM dNTPs, and 0.125 U of AmpliTaqGold DNA polymerase (Applied Biosystems, Foster City, CA, USA), or 0.25 U of KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan). PCR conditions were optimized for each primer pair. In cases, where exons 5–9 only were analyzed, PCR primers and reaction conditions according to IARC protocol, 2010 were used (www.p53.iarc.fr). Primers and length of amplified fragments are listed in Table 1. PCR was performed with 50 ng of DNA in a total volume of 25 µL reaction mixture consisting of 0.4 µM of each primer, 1.5 mM MgCl₂, 0.8 mM dNTPs, 0.125 U of AmpliTaqGold DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: initial denaturation step of 3 min at 95 °C, 20 cycles of 30 s at 95 °C, 45 s at 63 °C (reducing at 0.5 °C every 3 cycles) and 45 s at 72 °C, with following 30 cycles of 30 s at 95 °C, 45 s at 60 °C and 45 s at 72 °C.

Table 1. Primer sequences used for the *TP53* gene mutation analysis (exons 5–9)

Region amplified	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplification size
Exons 5–6	TGTTCACTTGTGCCCTGACT	TTAACCCTCCTCCAGAGA	467 bp
Exon 7	AGGCGCACTGGCCATCTT	TGTGCAGGTGGCAAGTGGC	177 bp
Exons 8–9	TTGGGAGTAGATGGAGCCT	AGTGTTAGACTGGAACTTT	445 bp

The *TP53* codon 72 SNP was assessed by analysis of exon 4 fragment sequences (Fig. 1), or by PCR-restriction fragment length polymorphism (RFLP) method

according to Hirata et al. [25] in that cases, where exons 5–9 only were analyzed. For genotyping by RFLP method PCR was carried out using 50 ng of DNA in a total volume of 20 µL consisting of 0.5 µM each forward primer (5'-TCTGGGAAGGGACAGAAGATGAC-3') and reverse primer (5'-TTGCCGTCCCAAGCAATGGATGA-3'), 1.5 mM MgCl₂, 0.8 mM dNTPs, 1.25 unit *Taq* DNA polymerase (Thermo Scientific). The PCR conditions were as follows: initial denaturation step of 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s of annealing at 57 °C, and 30 s of extension at 72 °C. The amplified 199-bp fragments were digested with *Bst*UI (Thermo Scientific) by the manufacturers' protocols. The digested PCR products were resolved on 3% agarose gel containing ethidium bromide and visualized under UV light. The presence of the *TP53* 72G allele resulted in the digestion of the amplicon to 113 bp and 86 bp products, the presence of 72C allele displayed as non-digested 199-bp product (Fig. 2).

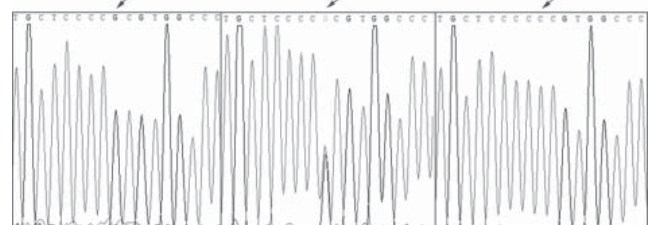


Fig. 1. Sequencing data of observed CLL patients' samples for *TP53* exon 4 analysis (region of *TP53* codon 72 SNP location): GG (Arg/Arg), CG (Arg/Pro), CC (Pro/Pro) genotypes

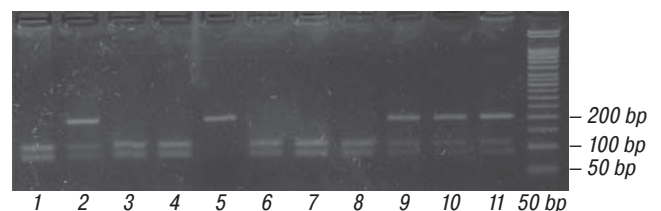


Fig. 2. Results of *TP53* codon 72 polymorphism detection by polymerase chain reaction and digestion with *Bst*UI in observed CLL patients: Line 1, 3, 4, 6, 7 and 8 — Arg/Arg genotype (86 bp and 113 bp bands); line 2, 9, 10 and 11 — Arg/Pro genotype (86 bp, 113 bp and 199 bp bands); line 5 — Pro/Pro genotype (199 bp band); 50 bp DNA size markers

Statistics were performed using the SPSS 16.0 software package (SPSS, Chicago, IL). *TP53* rs1042522 polymorphism was tested for deviation from the Hardy — Weinberg equilibrium by comparing the observed and expected genotype frequencies using the χ^2 test with one degree of freedom. Data shown are the means plus or minus standard deviations, and medians. The χ^2 test for categorical variables was used to compare characteristics between different subgroups of patients. The relative significance of different factors on *TP53* mutation occurrence was evaluated by univariate and multivariate analysis. All tests were two-sided and considered to be statistically significant with a *p*-value of < 0.05.

RESULTS AND DISCUSSION

Analysis of the *TP53* codon 72 polymorphism in 261 CLL patients resulted in frequencies of 0.68 and 0.32 for the G and C allele, respectively (Arg/Arg — 124 cases, 47.5%; Arg/Pro — 109 cases, 41.8%; Pro/Pro — 28 cases,

10.7%). Genotype frequencies did not differ significantly from those predicted by Hardy — Weinberg equilibrium ($\chi^2 = 1.29$; $p = 0.273$). These results are comparable with data from CLL group presented by Sturm et al. [26] (Arg/Arg — 57.3%; Arg/Pro — 36.1%; Pro/Pro — 6.6%).

TP53 mutations were found in 21 of 261 patients (8%) in this study. Most of mutations were detected in the DNA-binding domain (exons 5–8), namely, in exon 5 (4 patients), exon 6 (5 patients), exon 7 (4 patients), and exon 8 (3 patients). In three patients *TP53* mutations were detected in exon 9 (C-terminal region) and in two patients — in exon 4 (proline rich region). Of the 28 cases homozygous for the 72Pro allele, 7 cases (25%) carried mutation in the *TP53* gene. In contrast, of the 124 cases which were homozygous for the 72Arg allele, 6 cases only (4.8%) had the *TP53* mutation (Table 2). Thus, *TP53* mutations were significantly more often found in CLL patients carriers of Pro/Pro genotype ($p = 0.002$).

Table 2. *TP53* mutations in relation to the *TP53* codon 72 genotypes and previous therapy

CLL patients	The frequency of <i>TP53</i> mutations, cases (%)			<i>p</i> value
	Arg/Arg	Arg/Pro	Pro/Pro	
All patients, n=261	6 of 124 (4.8)	8 of 109 (7.3)	7 of 28 (25.0)	0.002
Untreated, n=172	5 of 85 (5.9)	2 of 70 (2.9)	0 of 17 (0.0)	0.427
Treated, n=89	1 of 39 (2.6)	6 of 39 (15.4)	7 of 11 (63.6)	0.0001

Previously treated patients showed a higher frequency of *TP53* mutations in comparison with untreated patients ($p = 0.001$). Time to treatment ($p = 0.446$), duration of therapy ($p = 0.997$) did not differ significantly in carriers of different *TP53* codon 72 genotypes. The incidence of *TP53* mutations among previously treated patients was also higher in carriers of the Pro/Pro genotype comparing to carriers of the other genotypes ($p = 0.0001$), and in carriers of the Pro allele comparing to carriers of the Arg/Arg genotype (13 of 50 patients, 26.0% and 1 of 39 patients, 2.6%, correspondingly; $p = 0.003$). At the same time, among untreated patients the distribution of *TP53* mutations did not differ in carriers of different genotypes ($p = 0.427$) and in carriers of the Pro allele in comparison with carriers of the Arg/Arg genotype (2 of 87 patients, 2.3% and 5 of 85 patients, 5.9%, correspondingly; $p = 0.234$). The risk of *TP53* mutations occurrence among pretreated patients was increased in carriers of the Pro allele (Odds ratio, OR = 1.317; 95% confidence interval, CI 1.109–1.564) and in carriers of the Pro/Pro genotype (OR = 2.503; 95% CI 1.142–5.487).

To estimate the impact of some clinical and biological factors on the incidence of *TP53* mutations we used univariate Cox regression analysis. Clinical data at diagnosis were available for the most of CLL cases. Several factors were included: age of patients (cut-off point at 65 years), gender, Binet stage at diagnosis, initial WBC count (cut-off point at $100 \cdot 10^9/L$), *IGHV* mutational status, previous treatment, *TP53* codon 72 genotype. As shown in Table 3, the 72Pro/Pro genotype, previous treatment and Binet stage B only correlated significantly with the occurrence of *TP53* mutations.

Multivariate Cox regression analysis confirmed an independent significance of abovementioned factors for *TP53* mutations occurrence.

Table 3. Significance of some clinical and biological factors for occurrence of *TP53* mutations under univariate and multivariate Cox regression analysis

Factors	N	Univariate analysis		Multivariate analysis
		<i>TP53</i> mutations, n (%)	<i>p</i> value	<i>p</i> value
Age (years)				
> 65	62	9.7 (6)	0.629	0.344
< 65	194	7.7 (15)		
Sex			0.102	0.741
Male	195	9.6 (19)		
Female	63	3.2 (2)		
Binet stage at diagnosis			0.076	–
A	133	4.5 (6)		
B	97	13.4 (13)		
C	31	6.5 (2)		
Binet stage at diagnosis			0.011	0.023
B	97	13.4 (13)		
A + C	164	4.9 (8)		
Initial WBC counts, $\cdot 10^9/L$			0.703	0.509
> 100	30	10.0 (3)		
< 100	226	8.0 (18)		
<i>IGHV</i> genes			0.346	0.396
M	92	5.4 (5)		
UM	161	8.7 (14)		
<i>TP53</i> codon 72 genotypes			0.001	0.001
Pro/Pro	28	25.0 (7)		
Arg/Arg+Arg/Pro	231	6.1 (14)		
Treatment			0.001	0.001
Untreated	172	4.1 (7)		
Treated	89	15.7 (14)		

Thus, our data are in agreement with results of Grossmann et al. [15] and Dong et al. [16], who have found an increased incidence of *TP53* mutations in CLL patients, carriers of the 72Pro/Pro genotype. However, we found that the frequency of *TP53* mutations in 72Pro homozygotes was increased only after previous treatment.

The *TP53* codon 72 alleles encode an arginine amino acid with a positive-charged basic side chain and a proline residue with a nonpolar-aliphatic side chain, which have different biochemical properties and different binding to components of the transcriptional machinery [27]. On the other hand, it is known that purine analogues and alkylating agents induce a *TP53*-dependent gene expression response [28, 29], and the occurrence of *TP53* gene alterations in CLL patients is associated with previous chemotherapy by these drugs [30]. It is possible, that some conformational features of TP53 protein and TP53-containing complexes in carriers of the Pro/Pro genotype contribute to the development of *TP53* mutations under drug-induced high expression of this gene. Another possible explanation of our findings may be related to different functional properties of TP53 polymorphic variants. The 72Arg is more effective at inducing apoptosis than the 72Pro variant, while the Pro/Pro genotype is associated with an increased expression of cell cycle arrest genes [14]. Preferential cell cycle arrest and slowing apoptosis may contribute to an inadequate repair of DNA damages under action of genotoxic stimulus and accumulation of cells with *TP53* mutations.

In summary, our preliminary data suggested that CLL patients with the Pro/Pro genotype are a risk group for development of *TP53* mutations under modern treatment.

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