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Tumor Protein 53 Gene Mutations Without 17p13 Deletion Have No Significant Clinical Implications in Chronic Lymphocytic Leukemia. Detection of a New Mutation

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Abstract. *Background: The tumor protein p53 (TP53) gene may be inactivated through 17p13 deletion, somatic mutations, or both. In chronic lymphocytic leukemia (CLL) although 17p13 deletion is correlated with poor prognosis, the role of sole TP53 mutations remains controversial. Materials and Methods: We carried out a mutation analysis of TP53 gene in 72 patients with CLL. Results: Seventy-one (98.6%) patients carried the polymorphic site c.215C>G, p.Pro72Arg, but its presence was not correlated with overall survival (OS). Moreover, 19 (26.4%) patients carried a mutation of TP53. Among the eight detected mutations, to our knowledge, one (c.587G>A) has never been reported in the past. There was a correlation of the mutation burden with the stage of the disease (p=0.022), but not with OS. None of the detected mutations was individually correlated with OS. Conclusion. The clinical significance of TP53 mutations is still a matter of debate and larger studies and meta-analyses are required to reach an unequivocal conclusion.*

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A functional p53 protein is essential for the maintenance of the genetic integrity of a healthy cell and for the cytotoxic effect of DNA-damaging chemotherapy drugs on neoplastic cells. The clinical importance of abnormalities of its encoding gene, *TP53*, in chronic lymphocytic leukemia (CLL) has been extensively studied. These abnormalities consist of inactivation of *TP53* through 17p13 deletion, somatic mutations, or both. The clinical significance of 17p13 deletion detected by fluorescence *in situ* hybridization (FISH) is unequivocal and the poor prognosis marked by it is well documented in both the chemotherapy and immunochemotherapy era (1, 2). This chromosomal abnormality characterizes an advanced-stage, rapidly progressing disease requiring treatment. Deletion of 17p13 is detected in 4-8% of patients at diagnosis, 8-11% at the time the patient requires initial treatment for disease progression, ~30% in patients with purine-analog-refractory disease (3), and >40% in Richter syndrome (4). Almost 70% of patients with 17p13 deletion also harbor *TP53* mutations (5), while 17p13 deletion without TP53 mutation is much less frequent (~10%). On the other hand, 20-30% of patients with TP53 abnormalities carry only mutations and not 17p13 deletion at the time of diagnosis (6).

The clinical significance of sole *TP53* mutations without concomitant 17p13 deletion remains controversial. Several studies provide data about the prognostic significance of *TP53* somatic mutations, but the net result of the research so far is not conclusive.

In a series of 308 newly-diagnosed patients with CLL, multivariate analysis showed that *TP53* mutations carry a prognostic value that is independent of 17p13 deletion since

patients with *TP53* mutations without 17p13 deletion had a lower overall survival (OS) than patients without *TP53* mutations or 17p13 deletion ($p<0.001$) (7). Similarly, *TP53* mutations were evaluated as independent factors of a poor prognosis in 173 Chinese patients with CLL (8). Another study reported rapid disease progression in patients with CLL harboring isolated *TP53* mutations, but this study did not provide survival data for analysis (9).

Several other studies reported a poorer prognosis associated with TP53 mutations, but without testing for the confounding impact of a concomitant 17p13 deletion (5, 10-12).

On the other hand, there are several reports indicating that isolated *TP53* mutations do not affect prognosis. Thus, in a study where isolated *TP53* mutations were detected in 7.6% out of a total of 529 patients with CLL, there was no correlation with progression-free survival or OS (13). Moreover, in a study on 99 patients with fludarabine-refractory CLL, where 39% of the patients carried *TP53* mutations (18% without 17p13 deletion), there was no correlation of *TP53* mutation with survival (14).

Most *TP53* mutations occur in exons 4-9 and frequently disrupt the DNA-binding domain of the protein (15). Thus, the majority of the reports so far refer to studies on exons 4 to 9, with or without exons 2 and 3. Nevertheless, about 20% of the mutations occur in introns.

In order to contribute to this field, we present the results of a mutation analysis of the *TP53* gene in patients with CLL. The results of the analysis were correlated with the epidemiological and hematological characteristics of the patients, as well as their prognosis.

Materials and Methods

A consecutive series of 72 patients with CLL who were treated at our Center from June 2008 to June 2014 were included in the study. The patients were followed-up from diagnosis; six patients were lost to follow-up. All patients provided informed consent and the diagnosis of CLL was confirmed by flow cytometry in all cases. All patients had peripheral blood disease by flow cytometry at the time of sample collection.

We recorded the following variables at sample collection: (a) age, sex, date of diagnosis; (b) Binet stage at diagnosis and at sample collection; and (c) peripheral blood lymphocyte count, hemoglobin level, platelet count, lactate dehydrogenase (LDH) and β2-microglobulin levels. Date of last contact and survival status during last contact were also recorded.

FISH for 17p13 deletion was carried out according to the treating physician's decision (at diagnosis, disease progression, or treatment failure) and not at baseline in all cases. Thus, FISH for 17p13 deletion was carried out in 28 (38.9%) patients.

Peripheral blood samples were drawn in EDTA and DNA extraction was performed using standard procedures within 6 hours from collection. DNA was then stored at –20˚C. The *TP53* gene sequences from exons 4, 5-6, 7 and 8-9 were amplified by polymerase chain reaction (PCR) according to the instructions outlined in http://p53.iarc.fr/ProtocolsAndTools.aspx (accessed on March 19, 2017) and re-sequenced to identify the underlying sequence variations. Sequencing was performed twice per sample (two independent PCR products) in both forward and reverse orientations. Genomic DNA information was obtained from GenBank wild-type sequence [*TP53*: NG_017013.2, NM_000546.5]. Sequence variants/mutations were verified using the MegaBase 1000 DNA Sequencing System (Amersham Biosciences Corp, Piscataway, NJ, USA). The underlying variations in the cohort were detected by multiple alignment sequences using Chromas Lite 2.01 and Chromas Pro 1.5 software (Technelysium Pty Ltd, South Brisbane, Australia) and BLAST[National Center for Biotechnology Information (NCBI), Bethesda MD, USA].

SPSS statistics, v19.0 (IBM, Armonk, NY, USA), was used for the statistical analysis of the results. OS was calculated from date of diagnosis to date of last contact. Moreover, survival was also calculated from date of sample collection to date of last contact. Categorical variables were compared by chi-squared test and continuous variables were compared by Mann–Whitney test or Kruskal–Wallis test where appropriate. All *p*-values were double-sided. Survival analysis was performed using the Kaplan–Meier method and log-rank statistics to test for survival correlations.

Table I. *Patient characteristics*.

Parameter	Value
Number of patients, N (%)	72 (100)
Male to female ratio	1.48
Median age (range), years	70 (37-91)
Stage (Binet), N (%)	
At diagnosis	
A	34 (47.2)
B	20 (27.8)
C	16 (22.2)
At sample collection	
A	30 (41.7)
B	18 (25.0)
C	22 (30.6)
TP53 deletion, N (%)*	2 (7.1)
Median peripheral blood lymphocyte count (range), $\times 10^9$/l	18.2 (1.4-193.8)
Median hemoglobin (range), g/dl	13.3 (8.3-17.9)
Median platelet count (range), $\times 10^9$/l	168 (30-472)
Median LDH/ULN (range)	1.1 (0.6-7.5)
Median β2-microglobulin/ULN (range)†	2.6 (0.2-11.9)
TP53 polymorphism (p53Arg), N (%)	71 (98.6)
TP53 polymorphism homozygosity (Arg/Arg), N (%)	47 (56.2)
TP53 mutation, N (%)	19 (26.4.0)

LDH: Lactate dehydrogenase; ULN: upper limit of normal. *By fluorescence *in situ* hybridization in 28 patients. †Measured in 38 patients.

Results

Seventy-two patients with CLL were included in the study. There was a male predominance (male to female ratio: 1.48) and the median age was 70 (range=37-91) years. Sample

Table II. *Polymorphisms and mutations of TP53 detected.*

Codon change	Amino acid	Exon/intron position	dbSNP	Gene position	Result, N (%)
c.215C>G	P72R	Exon 4	rs1042522	16.397	71 (98.6)
c.375+109 T>C		Intron 4	-	17.666	1 (1.4)
c.459C>T	P153P	Exon 5	rs72661116	17.398	2 (2.8)
c.542G>A	R181H	Exon 5	rs 397514495	17.481	1 (1.4)
c.578 A>T	H193L	Exon 6	rs786201838	17.598	1 (1.4)
c.584T>C	I195T	Exon 6	rs760043106	17.604	1 (1.4)
c.587 G/A	R196Q	Exon 6	-	17.607	1 (1.4)
c.639 A>G	R213R	Exon 6	rs1800372	17.659	12 (14.6)
c.993+44 C>T		Intron 8	-	19.060	1 (1.4)

Reference sequences for TP53 NG_017013.2, mRNA: NM_000546.5, protein:NP_001119584.1.

Table III. *Statistical correlations.*

Parameter	Mutated *TP53*	Wild-type *TP53*	*p*-Value*
Median age (range), years	68 (37-76)	72 (49-91)	0.335
Gender (male/female)	3.5	1.16	0.071
Stage (at diagnosis), N (%)			0.022
A	5	29	
B	6	16	
C	8	8	
Stage (at sample collection), N (%)			0.090
A	5	25	
B	4	15	
C	10	13	
Median peripheral blood lymphocyte count (range), $\times 10^9$/l	19.2 (3.0-193.8)	15.2 (1.4-131.0)	0.765
Median hemoglobin (range), g/dl	13.2 (9.0-16.1)	13.3 (8.3-17.9)	0.816
Median platelet count (range), $\times 10^9$/l	150 (30-257)	171 (52-472)	0.400
Median LDH/ULN (range)	1.2 (0.7-3.1)	1.1 (0.6-7.5)	0.422
Median β2-microglobulin/ULN (range)†	2.4 (0.2-3.1)	2.7 (0.8-11.9)	0.276

LDH: Lactate dehydrogenase; ULN: upper limit of normal. *Two-sided.

collection was performed at diagnosis in 25 (34.7%) cases and during the disease course in the rest (65.3%). Forty-six (63.9%) patients were treatment-naïve. The patients' epidemiological characteristics, stage of disease at diagnosis and at sample collection and blood count values are given in detail in Table I. FISH analysis for the detection of 17p13 deletion had been performed in 28 (39.9%) patients, according to common clinical practice. Only two (7.1%) of the tested patients had 17p13 deletion. Because of the very low rate of 17p13 deletion, no statistical significant correlations with prognosis were found. One of the patients with 17p13 deletion also carried a *TP53* mutation (c.639A>G, rs1800372). This was a 65-year-old male with stage C disease that progressed to Richter's syndrome and who had an OS of 117 months. The second patient with 17p13 deletion was a 77-year-old man with stage A disease who did not carry any *TP53* mutation and showed no progression over a period of 98 months of follow-up.

Almost all (n=71, 98.6%) patients carried the polymorphic site encoding for arginine at the residue 72 of exon 4, c.215C>G (p.Pro72Arg, rs1042522). Forty-seven of them (47/72, 66.2%) carried the polymorphic site in homozygosity (Arg/Arg) and 24 (33.8%) in heterozygosity (Pro/Arg). The presence of the polymorphism in homozygosity was not correlated to any of the epidemiological and hematological characteristics of the patients, nor to OS. No correlation was found between this polymorphism and the incidence of *TP53* mutation (p=0.961). Moreover, the survival of patients carrying a *TP53* mutation was not affected by the concomitant presence of this polymorphism (p=0.145).

Mutation analysis revealed that 19 (26.4%) patients carried a mutation of *TP53*. The detected mutations were as follows: c.639A>G [rs1800372] in 12 (16.7%) patients, c.459C>T [rs72661116] and c.542G>A [rs397514495] in exon 5 in two (2.8%) and one (1.4%) patient, respectively, and c.584T>C

[rs760043106] and c.578A>T [rs786201838] in exon 6 in one (1.4%) patient each. One new mutation was detected in exon 6, c. 587G>A. Moreover, another two mutations in introns were detected: c.375+109 T>C in intron 4, and c.993+44 C>T in intron 8, in one (1.4%) patient each (Table II).

One patient, in addition to the presence of the homozygote polymorphic site c.215C>G, carried another two mutations in exon 6: c.639A>G [rs1800372] in homozygosity and c.584T>C [rs760043106] in heterozygosity. Moreover, two other patients were heterozygous for mutations in introns 4 and 8 in addition to the polymorphic site for arginine (p53Arg) in exon 4.

The frequent silent mutation (c.639A>G, R213R) was correlated with advanced disease stage (p=0.024), and was far more common in males (10 men, two women, p=0.06), but no correlation with OS was found.

No correlations were found between the presence of *TP53* mutations and the epidemiological and hematological characteristics of the patients. There was a statistically significant correlation with the stage at diagnosis (higher stage was accompanied by higher mutation rate, p=0.022), but not with the stage at sample collection (p=0.09). Detailed results are shown in Table III.

OS seemed to be unaffected by the mutation status of the patients both that from diagnosis (p=0.079) and that from sample collection (p=0.336). None of the individual mutations detected was correlated with OS.

Discussion

The common single nucleotide polymorphism (SNP) located within the proline-rich domain of p53, at codon 72 of exon 4 (c.215C>G), results in either a proline or an arginine residue (p.Pro72Arg). The polymorphism varies with latitude and race and is maintained at different allelic frequencies across populations (16). It has been shown that the two alleles may produce functionally-distinct proteins and that the p53Arg variant induces apoptosis markedly better than the p53Pro variant (17), while the p53Pro variant appears to induce a higher level of G_1 phase arrest than the p53Arg variant (18). It has been speculated that these differences may affect response to treatment and survival in CLL. Such a speculation has not been proven (19), but it has been reported that the presence of the polymorphism is associated with an increased incidence of *TP53* mutation (20). In our cohort of patients, the detection of the polymorphic site encoding arginine in homozygosity or heterozygosity was very high (only one patient was Pro/Pro, 24 were Arg/Pro and 47 were Arg/Arg), higher than any other reported rate to date. However, we did not find any correlations of the polymorphism with the epidemiological and hematological characteristics of the patients, the stage or OS. Moreover, the presence of the polymorphism was not correlated with an increased incidence of *TP53* mutations.

All detected mutations except for c.587G>A are well-characterized by previous researchers and the relevant information was obtained from the NCBI Search database.

The most frequently detected mutation was c.639A>G [rs1800372]. This is a silent mutation at exon 6, detected in Li-Fraumeni syndrome and hereditary cancer-predisposing syndrome, and is considered of benign clinical significance. This mutation was detected in 12 (16.7%) patients and was correlated with an advanced disease stage, but not with OS, a result that is in accordance with the benign nature of the mutation as given in the NCBI database.

None of the other mutations detected were correlated to the stage or the prognosis of the disease. In detail, the pathogenic c.542G>A mutation was found in a male patient with stage A disease that had not progressed during the follow-up period (26 months). The c.375G>T mutation of uncertain significance was detected in a 73-year-old male patient with stage B disease and no progression for 77 months. The pathogenic c.584T>C mutation was detected in a 72-year-old male patient with stage C disease and an OS of 92 months (27 months from the detection of the mutation). The same patient also carried c.639A>G. The deemed benign c.459C>T mutation was detected in a 62-year-old male patient with stage A disease and no progression during a follow-up period of 41 months, as well as in an 81-year-old male patient with stage C disease and an OS of 10 months. The likely pathogenic c.578A>T mutation was detected in a 65-year-old female patient with stage B disease and no progression from diagnosis for a total of 86 months. c.993C>T, a mutation of uncertain significance was detected in a 68-year-old female patient with stage B disease and no progression for 106 months. Finally, a 42-year-old man carried the new mutation c.587G>A, which is probably pathogenic since another change at the same site (c. 587G>C, rs483352697) has been correlated to hereditary cancer-predisposing syndrome according to the lats from the NCBI database. Although the presence of a mutation was correlated with a higher disease stage, this correlation was not accompanied by a survival disadvantage.

In conclusion, our results lean towards the absence of clinical significance of *TP53* mutations when not accompanied by 17p13 deletion and this conclusion is in accordance with several research studies in the field. Therefore, the clinical significance of *TP53* mutations is still a matter of debate, and we need larger studies, or a meta-analysis of the existing ones, in order to reach an unequivocal conclusion. The clinical implication of the results of the studies so far is that in order to assess for *TP53* abnormalities, the use of both FISH to detect 17p13 deletion and gene sequencing is recommended in order for the complete range of *TP53* abnormalities to be covered (21).

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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