Mutaciones de *TP53* en pacientes con leucemia linfocítica crónica. Impacto clínico y consideraciones metodológicas

Mutations of *TP53* in chronic lymphocytic leukemia. Clinical impact and methodological considerations

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Resumen:

Las mutaciones del gen *TP53* son detectadas en ~5% de los pacientes con leucemia linfocítica crónica (LLC) al diagnóstico y se asocian con un resultado clínico desfavorable, independiente de la presencia de la deleción 17p. En este trabajo, describimos los resultados del análisis molecular de *TP53* en un grupo de pacientes con LLC de Argentina y Brasil. Las mutaciones de *TP53* se detectaron por secuenciación de Sanger en 22.56% de los casos; 8.7% en el diagnóstico y 39% en muestras pre-tratamiento / progresión (p <0.001; test exacto de Fisher). La frecuencia de mutación en 33 casos con del17p fue 42.4%. La técnica de NGS fue reproducible, todas las mutaciones, incluso en el diagnóstico, exhibieron VAF (frecuencia del alelo variante) >70%. Las mutaciones de *TP53* no se encontraban relacionadas con el estatus mutacional del gene de la cadena pesada de la inmunoglobulina (*IGVH*). Las mutaciones de *TP53* se asociaron a un menor tiempo para el primer tratamiento (p = 0.001; test log-rank). En los casos con necesidad de tratamiento entre 0-12 meses después del diagnóstico, la frecuencia de *TP53*-mutado fue 21.6%, comparada con 6% en pacientes tratados después de 12 meses y 0% en

los no tratados. Niveles de hemoglobina <10 g/dL, plaquetas menos de 100×10^9/L, linfadenomegalia, *IGVH* no mutado, *TP53* mutado y *NOTCH1* mutado se asociaron con una sobrevida global menor en el análisis univariado. En el multivariado, sólo trombocitopenia y las mutaciones de *TP53* mantuvieron el impacto independiente (HR 5.25; CI95% 2.13 – 12.94; *P*< 0.001). En conclusión, si bien el presente

estudio abarca un pequeño número de pacientes con tratamientos heterogéneos, nuestros datos son una contribución para el conocimiento de series de LLC en el mundo real, en regiones fuera de los grandes centros. Nuestros resultados también sugieren la utilidad del rastreo de mutaciones de *TP53* al momento del diagnóstico en la fracción de casos con tratamiento precoz.

Abstract

TP53 mutations are detected in ~5% of chronic lymphocytic leukemia (CLL) patients at diagnosis and are associated with unfavorable outcome, independent of the presence of 17p deletion. Most of knowledge on molecular and clinical characteristics of *TP53* mutation in CLL patients comes from developed countries. In this work, we aim to describe results of *TP5*3 molecular screening in a group of CLL patients from Brazil and Argentina. We studied a group of 133 CLL patients (110 from Rio de Janeiro, Brazil and 23 from Buenos Aires, Argentina). Detection of *TP53* mutations by Sanger sequencing disclosed a frequency of 22.56%; 8.7% at diagnosis, and 39% in pre-treatment/progression samples (p<0.001; Fisher's exact test). Mutation frequency in 33 cases with del17p was 42.4%. NGS analysis was reproducible, all mutations, even at diagnosis exhibited >70% VAF (variant allele frequency). *TP53* mutations were not significantly associated to the immunoglobulin heavy chain (IGVH) mutational status. Mutations were

associated to a shorter time to first treatment (p= 0.001; log-rank analysis). In cases needed to treat between 0-12 months after diagnosis, the frequency of *TP53* mutation was 21.6%, compared with 6% in patients needed to treat after 12 months and 0% in non-treated patients. Hemoglobin levels <10 g/dL, platelet count less than 100×10^9/L, lymphadenomegaly, an *IGVH* unmutated status, mutated *TP53* and mutated *NOTCH1* were associated with lower overall survival in univariate analysis. In multivariate analysis, only thrombocytopenia and *TP53* mutation status maintained an independent impact (HR 5.25; CI95% 2.13 – 12.94; p< 0.001). Although our study is limited by the small number of heterogeneously treated patients, we think that it is a contribution with sensible knowledge on a real-world CLL series outside the developed countries. Our results suggest that *TP53* mutation screening could be useful at diagnosis in the fraction of cases with early treatment intent.

Introduction

The *TP53* gene plays a central role in maintaining genome integrity and responding to stress through cell cycle control, apoptosis induction or senescence[1].

Abnormalities in *TP53* occur in 10-15% of cases of CLL at diagnosis, in 10–12% of cases in first line treatment and in ~40% in cases refractory to treatment with fludarabine[2,3]. Importantly, *TP53* mutations are detected with a significant frequency in CLL patients at diagnosis (~ 5%) and are associated with short times of disease progression, poor responses to immuno-chemotherapeutic treatment

(fludarabine, cyclophosphamide and rituximab), regardless of the presence of 17p deletion[4,5]. This makes important to detect such mutations in the CLL patient prior to the clinical-therapeutic decision, which is usually performed by Sanger sequencing[6].

The advent of new molecular methods that can analyze the clonal architecture of the disease in depth using parallel mass sequencing methods (NGS; next generation sequencing) allowed to describe complex patterns of clonal evolution[7,8]. Regarding the *TP53* gene, it has been shown that patients with small mutated *TP53* subclones had

the same clinical phenotype and short survival as those patients with *TP53* clonal lesions. Longitudinal analysis showed that small *TP53* mutant subclones identified prior to treatment became the predominant population at the time of CLL relapse and anticipated the development of chemo-refractoriness[9-11]. Thus, quantification of clone size and detection of minor clones by NGS methods have become important in the understanding and clinical prediction of the disease.

In this work, we aim to present results on the study of *TP53* mutations in clinically defined group of CLL patients from Brazil and Argentina, as well as the efforts of optimization of NGS approaches for *TP53* mutation analysis.

Materials and methods
Patients
A group of 110 cases diagnosed with CLL at the Instituto Nacional de Câncer (INCA), Rio de Janeiro, Brazil (70 male and 40 female; median age: 59.7 years, range: 28 to 98 years; Rai stages 0: 18.75%, I: 31.25%, II: 13.75%, III: 17.5%, IV: 18.75%). Diagnosis was established following the International Workshop on Chronic Lymphocytic Leukemia Criteria[12]. Of the 110 patients, 69 were included at diagnosis while 41 were included prior to first or second line treatment, or during disease progression. Additionally, a selected group of 33 cases (23 from Argentina and 10 from Brazil) were studied, characterized for having del17p detected by FISH analysis. This project was approved by the Ethics Committee of INCA (CAAE 79155317.3.0000.5274) and the Ethics Committee of the National Academy of Medicine of Buenos Aires. All patients were included after signed informed consent. The study was conducted in compliance with the directives and norms regulating research involving human beings of the National Health Council (CNS, Brazil), Resolution nº 466/2012.

Detection of *TP53* mutations by Sanger sequencing
Mononuclear cells from EDTA-anticoagulated peripheral blood (PBMC) with average 60% lymphocytes were isolated by density centrifugation in Ficoll Hypaque. DNA was extracted from PBMC with commercial kits. PCR amplification of the entire coding region (exons 2-11) of the *TP53* gene and intron/exon boundaries (±2 intronic bp) was carried out according to standard protocols (IARC TP53 website http://p53.iarc.fr/ProtocolsAndTools.aspx) and followed the recommendations of the *European Research Initiative on CLL* (ERIC)[6]. Amplification products were isolated from 2% agarose gel and submitted to bi-directional sequence analysis in an ABI PRISM DNA Analyzer 3130xl. Positive results were validated by a separate PCR followed by Sanger sequencing. The analytical sensitivity of this assay was estimated in 20-25%.

Data analysis and interpretation
Sequences were compared with the corresponding germline RefSeq sequence using BioEdit v7.2.5 and GLASS v0.2.9 softwares. Mutations were confirmed on both strands on independent PCR products and annotated by the International Agency for Research on Cancer (IARC) TP53 Mutation Database (http://www-p53.iarc.fr); functionality was inferred with the Seshat database (https://p53.fr/tp53-database/seshat). ClinVar and the Human Gene Mutation Database (HGMD) were also used. Mutation nomenclature was unified according to the HGVS guidelines (http://varnomen.hgvs.org/).

Detection of *TP53* mutations by NGS in an Ion Torrent PGM™platform
Twenty to 40 ng of DNA was amplified with the primers and reagents contained in the Ion AmpliSeq™ *TP53* Panel kit. After purification with magnetic beads, the product was digested and phosphorylated, followed by attachment of the adapters. Followed nick-translation and amplification steps a library dilution was submitted to enrichment of positive charged beads (ISPs) in OneTouch™ Ion apparatus. ISP-enriched libraries were sequenced on the Ion PGM™ Sequencer platform. Results were analyzed with the Torrent Suite software and annotation was performed with the Ion Reporter™ software. Analysis was performed with the Integrative Genomics Viewer (IGV) tool and the Genomic Analysis tool of Metacore™ software (Thomson Reuter). The results were validated when >90% amplicons showed at least 100 reads, ≥99% minimum coverage percentage were over 20x and a minimum of 20 reads supported the variant. Minimal limit of detection is 10% VAF (variant allele frequency). Development and optimization follow the recently published guidelines of (ERIC)[13].

Analysis of the mutational status of *IGHV* genes

Total RNA was extracted with Trizol reagent (Invitrogen) and retrotranscribed with random primers and Suprescript[TM] (Life Sciences). *IGHV* genes were PCR amplified using a set of primers for the leader region of the six VH families (VH1 to VH7), along with a consensus primer for the JH region[14]. Fragment analysis for evaluation of immunoglobulin clonality in selected cases[15], as well as bidirectional sequencing were performed in an ABI PRISM DNA Analyzer 3130xl (Applied Biosystems). The IGVH sequences were aligned with the germline sequence in the ImMunoGeneTics database (IMGT).

Statistical analysis

Categorical variables were compared with chi-square or Fisher's exact tests. Survival analyses were performed by the Kaplan-Meier method, differences were compared using the log-rank test, and risk rates were calculated by a Cox proportional hazards model. *P* values <.05 were considered significant. IBM SPSS v20.0 software was used for analysis.

Results and discussion

In 133 cases with *TP53* mutation analysis, 42 mutations were detected, including synonymous and non-synonymous mutations (**Figure 1A**). Considering only the pathogenic mutations, i.e. missense mutations (17), nonsense (4) and insertions and deletions with frameshift (9), the frequency of *TP53* detection in the combined Argentinean-Brazilian group was 22.56%. The most frequent type of mutations was missense (40.55%), followed by indels (31.4%), as previously described for this gene[16]. Frequency and type of mutations varied according to the clinical setting. In diagnostic samples (N=69) 15 mutations were detected, but only 6 of them are considered pathogenic mutations (8.7%), while in pre-treatment or progression samples (N=41) 16/19 detected mutations were pathogenic (39%). This difference was statistically significant and highlights the selection of pathogenic variants during the course of the disease (**Figure 1B**).
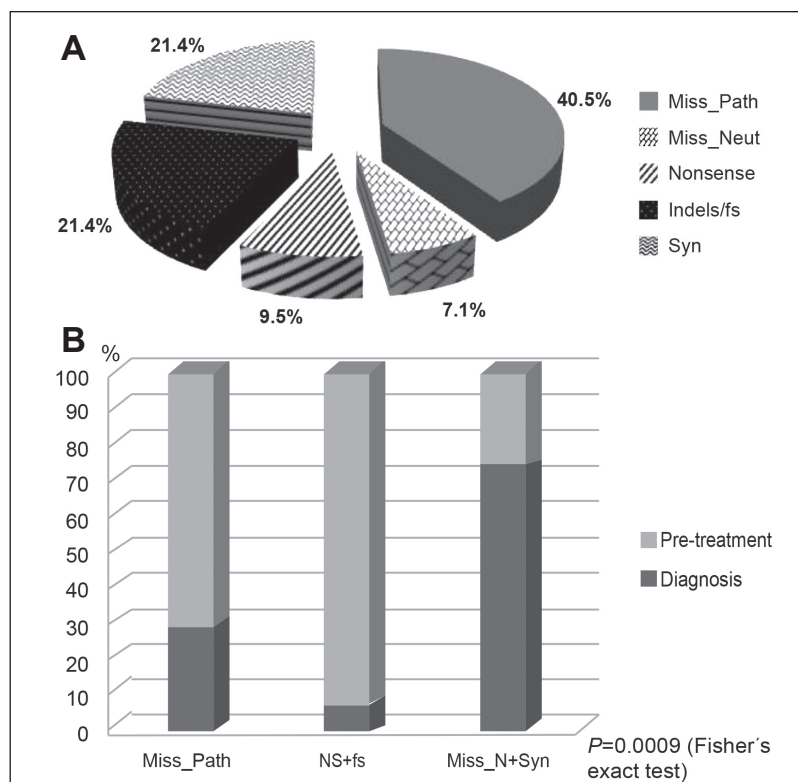

Figure 1. Frequency of TP53 mutations in Brazilian and Argentinean patients with chronic lymphocytic leukemia. (A) Mutations classified according to molecular subtype; (B) Frequency of mutations categorized according to functional effects at diagnosis and pre-treatment. Miss_Path: missense mutations with pathological functional effect; Miss_Neut, missense mutations with neutral functional effect; Syn, synonymous substitutions; Indels/fs: Insertions and deletions/frameshift

In the selected group of 33 cases (23 from Argentina and 10 from Brazil) harboring del17p or very aggressive disease, the frequency of mutation was 42.4% (14/33). All detected mutations belonged to the pathogenic type, as shown in **Table 1**.

Table 1. List of *TP53* mutations in selected chronic lymphocytic leukemia cases

Origin	Case	Sex/ Age	Exon/ Codon	Type	cDNA	Protein	IGHV	del17p13 (%)
ARG	1*	F/69	4/72	I-fs	c.214_214insC	p.V72fs	UM	35.1
ARG	2*#	M/73	5/156	Mi-TV	c.467G>C	p.R156P	UM	44.3
ARG	3	M/65	5/157	I-fs	c.469_472insTCCG	p.V157fs	UM	24.4
ARG	4*	M/?	5/187	Mi-TS	c.559G>A	p.G187C	-	88
ARG	5*#	M/51	6/209	del-fs	c.625_626delGA	p.R209fs	M	20.3
ARG	6*	F/74	8/272	Mi-TS	c.814G>A	p.V272M	M	60
ARG	7*	F/69	8/279	Mi-TS	c.386G>A	p.G279E	UM	25
ARG	8	M/60	7/248	Mi-TS	c.742C>T	p.R248W	UM	NN
BR	2	F/56	6/205	Mi-TV	c.803T>G	p.Y205D	UM	>20
BR	3	M/47	5/146	NS-TS	c.628G>A	p.W146*	UM	>20
BR	8	F/55	5/132	Mi-TS	c.585A>G	p.K132R	UM	>20
BR	11	F/72	5/154	Mi-TV	c.651G>T	p.G154V	UM	>20
BR	63	M/69	7/234	Mi-TV	c.890T>A	p.Y234N	UM	>20
BR	283	M/54	9/324	I-fs	c.1160_1161insCCAGCCAAAGAAGAAACCACTG	p.D324fs	UM	>20

ARG; Argentina; BR: Brazil; I: insertion; del: deletion; fs: frameshift; Mi: missense; NS: nonsense; TS: transition; TV: transversion; IGHV: immunoglobulin heavy chain variable gene, mutation status; UM: unmutated; M: mutated. Nomenclature according to HGVS (http://varnomen.hgvs.org/). * Abnormal karyotype; # Complex karyotype. NN: normal FISH.

For cases with del17p, it is expected a high frequency of mutation detection, up to 90% [2,3] which is higher than the frequency detected in our cases. The discrepancy can be due to sensitivity issues, as well as the small size of our group.

As previously described for *TP53*, mutations have a preferred topological distribution along the gene[16-18], with missense mutations accumulating in the DNA-binding domain (exon 5–8). In the central domain we found 73% of mutations, mostly represented by missense mutations (71%). Nonsense and frameshift mutations, which are described to accumulate outside the central core domain (exons 2–4 and exons 9–11) were also detected mostly in the central core (4/6 frameshift and 3/4 nonsense mutations) (**Figure 2**). This may be due to the small sample size, or to biological differences that need further research.
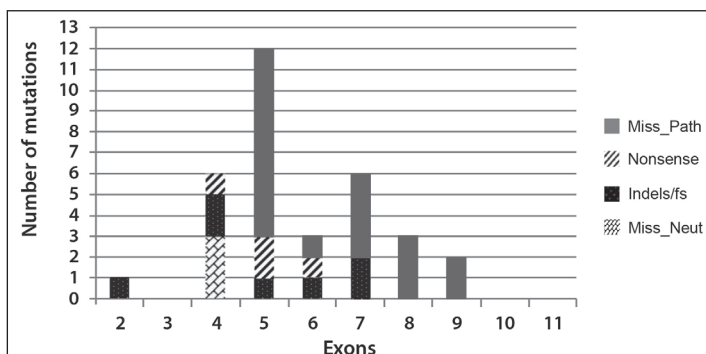

Figure 2. *Topological distribution of non-synonymous TP53 mutations according to molecular subtype in Brazilian and Argentinean patients with chronic lymphocytic leukemia. Miss_Path, missense mutations with pathological functional effect; Miss_Neut, missense mutations with neutral functional effect; Indels: insertions and deletions with frameshift effect.*

In respect of *TP53* mutation detection by NGS, a group of 30 cases were tested in parallel by Sanger and NGS sequencing. All missense and nonsense mutations were readily detected by both methods. Insertion and deletion were more difficult to call and annotate by NGS sequencing, as already described[10,13]. All mutations, even when detected in diagnostic samples had a variant allele frequency (VAF) >70%. In the group studied here, no minor *TP53* clones were detected. In two cases with available samples, the *TP53* clone detected at disease progression could not be tracked down to diagnostic samples, suggesting that clonal evolution occurred during the disease progression. The increase of samples studied at diagnosis and during clonal evolution will help to determine whether our cases behave alike the international reported series [10,11].

Regarding the clinical aspects of our patient group, this was a very high risk series, with 80% of cases requiring treatment. Median time of overall survival was 38.5 months (range 1-215 months). The detection of a pathogenic *TP53* mutation was associated with shorter time to first treatment and overall survival (**Figure 3**).

In cases needed to treat between 0-12 months after diagnosis, the frequency of *TP53* mutation was 21.6%, compared with 6% in patients needed to treat after 12 months and 0% in non-treated patients. Therefore, even when mutation frequency was lower at diagnosis, patients bearing *TP53* mutations at diagnosis needed to treat between 15 days and 6 months from being admitted to the hospital.
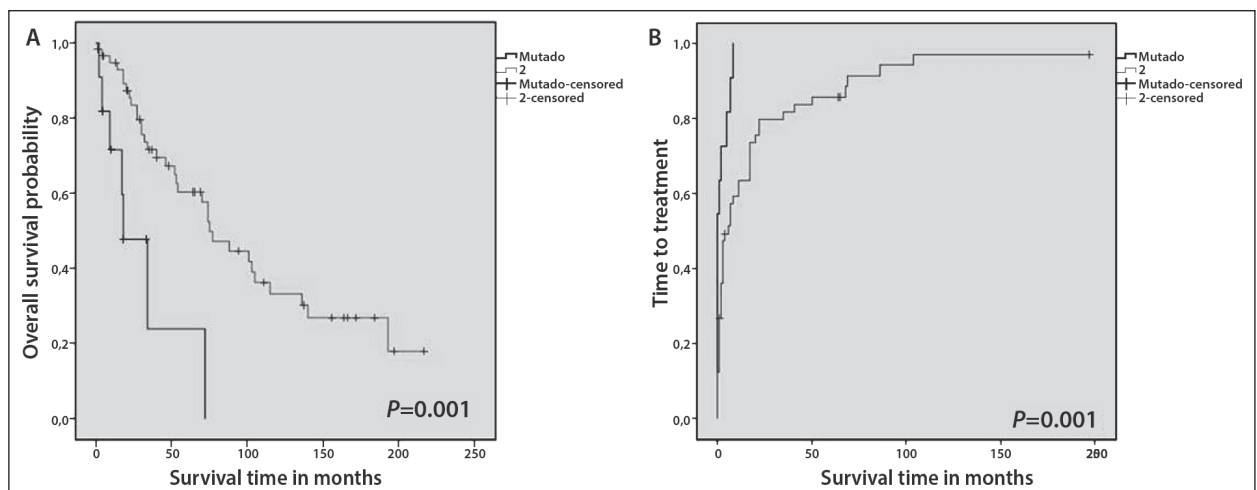

Figure 3. *Survival analysis according to the mutational status of TP53. (A) Kaplan–Meier curves for overall survival; (B) Time to first treatment. p < 0. 05 calculated by log-rank test.*

The presence of *TP53* mutations was not significantly associated to *IGHV* mutational status, with 71% of cases with mutated *TP53* being *IGHV* unmutated, compared to 59.5% of cases with wild-type *TP53* (*P*= 0.445).

The presence of hemoglobin levels <10 g/dL, platelet count less than 100×10^9/L, lymphadenomegaly, an *IGHV* unmutated status, mutated *TP53* and mutated *NOTCH1* were associated with lower overall survival in univariate analysis. In multivariate analysis, only thrombocytopenia and *TP53* mutation status maintained an independent impact (HR 5.25; CI95% 2.13 – 12.94; *P*< 0.001).

In this study we described the *TP53* mutation profile in CLL patients from two South American countries, and reinforce the need for uniformization of protocols and approaches in order to make it comparable results from different countries and foster collaborations. While detection of *TP53* mutations by Sanger sequencing is a mature and reproducible approach, NGS can contribute with important knowledge on the size of *TP53* mutated clones, but it is still a technique in need of optimization and uniformization.

We also present clinical data from a patient series treated in a tertiary public cancer hospital serving a low resource population. Although our study is limited by the small number of heterogeneously treated patients, we think that it is a contribution with sensible knowledge on a real-world CLL series outside the developed countries.

TP53 mutation screening is advised before any therapeutic decision, especially if FC protocols are being

considered. It is known that ~5% of untreated CLL patients have a *TP53* mutation in the absence of 17p deletion (~9% in our series) and that the presence of a mutated clone, either a major or a minor one is associated with poor response to standard treatment approaches[4,5]. This open an opportunity for including *TP53* molecular testing at diagnosis at least in the fraction of cases with early treatment intent.

Declaration of conflicts of interest:
The authors declare no conflicts of interest.

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