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GERMLINE TP53 MUTATIONS AND SINGLE NUCLEOTIDE POLYMORPHISMS IN CHILDREN

PAMELA VALVA[1], **PABLO D. BECKER**[2], **PATRICIA STREITEMBERGER**[3], **MERCEDES GARCIA LOMBARDI**[3], **GUADALUPE REY**[3], **CARLOS A. GUZMAN**[2], **MARIA VICTORIA PRECIADO**[1]

[1]*Laboratorio de Biología Molecular, Servicio de Anatomía Patológica;* [2]*Unidad de Oncología, Hospital de Niños Ricardo Gutiérrez, Buenos Aires;* [3]*Departament of Vaccinology and Applied Microbiology, Helmhotz Centre for Infection Research, Braunschweig*

Abstract Mutations in the gene TP53, which codifies the tumor suppressor protein p53, are found in about 50% of tumors. These mutations can occur not only at somatic level, but also in germline. Pediatric cancer patients, mostly with additional family history of malignancy, should be considered as potential TP53 germline mutation carriers. Germline TP53 mutations and polymorphisms have been widely studied to determine their relation with different tumors' pathogenesis. Our aim was to analyze the occurrence frequency of germline TP53 mutations and polymorphisms and to relate these to tumor development in a pediatric series. Peripheral blood mononuclear cell samples from 26 children with solid tumors [PST] and 21 pediatric healthy donors [HD] were analyzed for germline mutations and polymorphisms in TP53 gene spanning from exon 5 to 8 including introns 5 and 7. These PCR amplified fragments were sequenced to determine variations. A heterozygous mutation at codon 245 was found in 1/26 PST and 0/21 HD. Comparative polymorphisms distribution, at position 14181 and 14201(intron 7), between HD and PST revealed a trend of association (p= 0.07) with cancer risk. HD group disclosed a similar polymorphism distribution as published data for Caucasian and Central/South American populations. This is the first study about TP53 variant frequency and distribution in healthy individuals and cancer patients in Argentina.

Key words: TP53, germline, pediatric tumor, single nucleotide polymorphism, genotype frequency

Resumen ***Mutaciones y polimorfismos de un único nucleótido del gen TP53 en línea germinal en niños***. El gen que codifica para la proteína supresora de tumor p53 (TP53) se encuentra mutado en aproximadamente el 50% de los tumores. Estas mutaciones pueden presentarse como somáticas o en línea germinal. Los niños con tumores, sobre todo aquellos con historia familiar de enfermedad oncológica, deben considerarse potenciales portadores de mutaciones en línea germinal. Las mutaciones de TP53 y los polimorfismos son estudiados para determinar su relación con la patogénesis de diferentes tumores. El objetivo del trabajo fue analizar la frecuencia de mutaciones y polimorfismos en línea germinal de TP53 y relacionarlos con el desarrollo de tumor en un grupo de pacientes pediátricos. Se analizaron muestras de sangre periférica de 26 pacientes con tumores sólidos [PST] y 21 niños donantes sanos [HD] para determinar la presencia de mutaciones y polimorfismos de TP53 en línea germinal. Se analizó por PCR seguida de secuenciación, la región que comprende a los exones 5 a 8 (incluyendo intrones 5 y 7). En 1/26 PST se encontró una mutación heterocigótica en el codón 245. La distribución de los polimorfismos, en la posición 14181 y 14201 (intrón 7), entre HD y PST mostró una tendencia de asociación (p = 0.07) con el riesgo para desarrollar cáncer. La frecuencia de distribución de dichos polimorfismos en HD fue similar a la publicada para poblaciones caucásicas y de América Central/del Sur. Este estudio aporta información original sobre la frecuencia de distribución de las variantes TP53 en individuos sanos y con tumores en la Argentina.

Palabras clave: TP53, línea germinal, tumores pediátricos, polimorfismos de un único nucleótido, frecuencia de genotipo

TP53, a tumor suppressor gene, serves as a "guardian of the genome", preventing proliferation of a cell that has sustained genetic damage[1]. Tumor suppressor gene inactivation appears to be integral to the development of several types of human tumors[2] and mutated TP53 was found in a wide variety of them. Mutations of tumor suppressor genes can occur not only at the somatic level, but also in the germline, which has important clinical implications[3]. These last mutations were initially reported in patients with Li-Fraumeni syndrome (LFS)[4,5], with nearly 70% of them displaying heterozygous mutations[6]. Detection of germline mutations in TP53 should allow the iden-

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Postal address: Bioq. Pamela Valva, Laboratorio de Biología Molecular, Servicio de Anatomía Patológica, Hospital de Niños Ricardo Gutiérrez, Gallo 1330, 1425 Buenos Aires, Argentina
Fax: (54-11) 4963-4122 e-mail: valvapamela@yahoo.com

tification of subjects at high risk of developing cancer besides those affected by the LFS[3]. Seventy eight percent of the 376 mutations reported in germline are missense substitutions, most of them clustering within exons 5-8, the DNA binding domain of the protein[7].

On the other hand, several TP53 polymorphisms have been also identified in humans. Their pattern may show wide differences, not only among tumor types but also among different populations depending on the genetic background, environmental factors and socio-economic status[7, 8]. In the present study, a pediatric cohort from Buenos Aires was analyzed for the presence of germline TP53 mutations and polymorphisms to relate them to tumor development.

Material and Methods

Forty-seven peripheral blood mononuclear cell (PBMC) samples corresponding to 26 children with solid tumors (PST) (Table 1) and 21 pediatric healthy donors (HD), attending at Ricardo Gutiérrez Children Hospital, Buenos Aires, were screened for the presence of germline alterations in exons 5-8 and the corresponding introns of TP53. PST median age was 5 years (range 3 mo-16 yrs) and the male:female ratio was 0.733. HD median age was 11 years (range 1-15 years) and the male:female ratio was 0.75. Hospital's ethic committee reviewed and approved this study and written informed consent was obtained from all subjects' parents or guardians. Collected family data disclosed that 11/26 patients (42.31%) had additional family records of malignancy; however, none of them presented history consistent with LFS.

PBMC from EDTA-collected blood were obtained by osmotic lysis of red blood cells with 2M Tris-ClH, 1M $MgCl_2$, pH 7.5. DNA was isolated by SDS-Proteinase K lysis and phenolchloroform extraction.

The TP53 analyzed region was amplified using the following primers and conditions:

A) exon 5-6S: 5'TTCCTCTTCCTGCAGTACTC3', (13040-13059) and exon 5-6 AS:5'AGTTGCAAACCAGACCTCAG3' (13447-13428) (44 cycles of 30s at 95 °C, 1 min at 55 °C and 1 min at 72 °C) to amplify exon 5, intron 5 and exon 6 full length.

B) exon 7-8S 5'GTGTTGTCTCCTAGGTTGGC3' (13986-14006), exon 7-8AS 5'AAGTGAATCTGAGGCATAAC3' (14653-14631) (44 cycles of 30s at 95 °C, 1 min at 56 °C and 1 min at 72 °C) to amplify exon 7, intron 7 and exon 8 full length.

Primers positions refer to TP53 complete genome Genbank X54156.

In a separate reaction tube, a set of primers for the beta-globin gene was incubated with the template DNA and served as a control to monitor the amplification ability of a single copy gene. PCR amplified DNA was subjected to electrophoresis on a 2% agarose gel and purified with QIAEX^R II extraction kit (Valencia, USA). DNA sequencing was performed using big dye terminator cycle sequencing kit version 1.1 and the 3100 genetic analyzer (Applied Biosystems). Analysis of mutation spectra was carried out with the CLUSTAL W software using sequence X54156 (GenBank accession number) as reference.

Genotype frequencies were calculated from observed genotypes and then compared HD vs. PST, as well as HD vs. other populations (Central/South America, African, Caucasian, East Asia; data available on http://www.ncbi.nlm.nih.gov/SNP). Expected genotype frequencies were calculated from the Hardy-Weinberg equilibrium.

Fisher's exact test was used to assess the association between categorical variables; $p < 0.05$ values were considered significant.

TABLE 1.– *Cancer type and polymorphism at positions 14181 and 14201 in PST*

Cancer type	*n*	Homozygous C/C (14181) T/T (14201) *n*	Homozygous T/T (14181) G/G (14201) *n*	Heterozygous C/T (14181) T/G (14201) *n*
Rhabdomyosarcoma *	6	4	0	2
Osteosarcoma	2	0	0	2
Synovial sarcoma	1	0	0	1
Ewing's sarcoma	3	3	0	0
Neuroblastoma	1	1	0	0
Teratoma	1	0	0	1
Schwannoma	1	1	0	0
Retinoblastoma	4	3	0	1
Wilms´ tumor	1	0	0	1
Ependymoma	2	0	0	2
Glioma	1	1	0	0
Ovarian tumor	1	0	0	1
Medulloblastoma	1	1	0	0
Hepatoblastoma	1	1	0	0

** One homozygous case presents an extra point mutation G→A at position 14060.*
Nucleotide sequence data reported are available in the GenBank databases under the accession number: EF445551-EF445626.

Results

One out of 26 samples analyzed from PST was found to have a heterozygous missense mutation (Gly to Ser) in germline TP53 exon 7, at codon 245, that was G:C→A:T transition at the CpG site. This patient had been diagnosed with rhabdomyosarcoma (RMS) at the age of 22 months. None of the other PST displayed mutations in the studied exons.

Concerning introns, the previously reported polymorphisms C→T at position 14181 and T→G at position 14201, were analyzed in PST and HD samples (Tables 1 and 2). They showed complete linkage between C at 14181 and T at 14201 as well as between T at 14181 and G at 14201, as has also been reported by Bergreen et al[9]. Comparative polymorphism distribution between HD and PST revealed a trend of association for cancer susceptibility ($p = 0.07$).

TP53 intron polymorphism frequencies have also revealed significant differences among populations. The present HD group showed no significant difference in genotype frequencies with Caucasian and Central/South America populations, however, it displayed a statistically different distribution from Africa and East Asia populations, considering the data published in IARC database[7] for these positions (Table 2). The patient who carried the germline mutation at codon 245 was homozygous (C/C, T/T) for these polymorphisms. Interestingly, all HD and PST samples presented a T at position 14168, a TT to CC transition at positions 14234-14235, a C to T transition at position 14271, an insertion of a G at position 14243, and a complex change from CACCT to ACCTA at position 14316 to 14320 of intron 7, as was previously observed in other GenBank reported sequences and described by Eicheler and Baumann[10].

Discussion

Germline mutations, besides those occurring in LFS, have been observed mostly in patients with an unusual history of cancer, i.e. multiple malignancies or a family history of cancer; however the frequency of mutations is lower than in LFS[3]. In particular, TP53 germline mutation carriers had a remarkable risk for developing multiple cancers[11, 12, 13]. In the present study, none of the studied patients had multiple tumors whereas 11/26 had a family history of cancer. About 30% of the mutations are located at five "hotspot" codons (175, 245, 248, 273 and 282) and have been found in almost every type of cancer[8]. The mutation reported in this series was localized at codon 245, one of the hotspot codons mentioned above. Interestingly, this codon is at a CpG island, which has been previously described as a highly mutable dinucleotide where several TP53 missense mutations were found[14]. This germline mutation corresponded to a RMS patient, the most frequent type of sarcoma in children[15], with family history of cancer (1/11). The frequency for germline mutations in pediatric RMS (3/33) reported by Diller et al was comparable to the one assessed for RMS in this study (1/6), although few cases were included[16]. As previously reported for other series of PST outside LFS, the overall

TABLE 2.– *Allele and genotype frequencies of TP53 intron 7*

		Allele frequencies		Genotypes frequencies						
				14181			14201			p^b
Population	*n*	14181	14201	C/C	T/T	C/T	T/T	G/G	G/T	
Healthy donors	21	C 0.678	0.678	0.62	0.14	0.24	0.62	0.14	0.24	
		T 0.322	G 0.322							
Pediatric patients with	26	C 0.678	T 0.678	0.58	0	0.42	0.58	0	0.42	0.07
solid tumors		T 0.322	G 0.322							
Central/South America[a]	23	C 0.913	T 0.913	0.870	0.043	0.087	0.870	0.043	0.087	0.21
		T 0.087	G 0.087							
Caucasian[a]	31	C 0.863	T 0.863	0.745	0.020	0.235	0.745	0.020	0.235	0.38
		T 0.137	G 0.137							
African[a]	48	C 0.854	T 0.854	0.708	0	0.292	0.708	0	0.292	0.05
		T 0.146	G 0.146							
East Asia[a]	48	C 0.688	T 0.688	0.417	0.041	0.542	0.417	0.041	0.542	0.03
		T 0.312	G 0.312							

[a] *Data available on http://www.ncbi.nlm.nih.gov/SNP*
[b] p *value was calculated using HD as reference group.*

frequency of TP53 germline mutation detected was low (1/26, 3.8%)[11, 17, 18] and it was associated with a family history of cancer[19]. This observation suggests that *de novo* germline p53 mutations are rare; they are therefore probably most often inherited.

Finally, most of the reported mutations in germline TP53 are in hot spots clustering within exons 5-8, but that certain mutations may occur outside them[7]. Moreover, some patients may carry wild-type TP53 alleles, but inherit a predisposition for the development of cancer through a different gene or through a mechanism that functionally inactivates wild-type P53 protein[16].

With regard to TP53 polymorphisms, most of them have been identified in introns as single nucleotide polymorphisms (SNPs) outside the consensus splicing sites. Some of these natural variants have also been related to an increased risk of cancer development, although there is no apparent indication that such variants alter p53 function; it remains feasible that these findings are the result of linkage to other functionally significant TP53 alterations[20]. If the association of the polymorphism with cancer risk is true, genotype frequency in cancer patients will differ from that in healthy populations. Many groups explored the association between several distinct polymorphisms and cancer susceptibility. Concerning the polymorphism at position 14181 and 14201, Li et al proposed that it could be associated with genetic susceptibility for oral neoplasm[21]. However, Berggren et al suggested that there is no relation between urinary bladder cancer pathogenesis and this polymorphism[22]. Although our analysis of genotype frequency differences between the PST and HD groups at those positions did not show a significant relationship with cancer risk, a trend for genetic susceptibility for cancer (p= 0.07) was evidenced. A larger number of samples may confirm this tendency.

As it is well known, non-coding region polymorphism frequencies show significant differences among populations and they have been also correlated to ethnical variations. Remarkably, genotypes C/C, T/T and C/T at position 14181, as well as T/T, G/G and T/G at position 14201 do not occur at the same frequency within different populations[22]. According to IARC database for TP53 polymorphisms[7], Caucasian and Central/South American populations do not disclose any significant difference in genotype distribution (p = 0.39). However, it was statistically different if Africa and East Asia populations (p = 0.006) were compared. Our HD series genotype comparative analysis disclosed a distribution similar to Caucasian and Central/South American populations, but significantly different from Africa and East Asia (Table 2). Since the ethnical background of the studied series included children who are the product of mating of Southamerican Indians with European people, these results were foreseeable[23].

In conclusion, this is the first study that sheds light on the Argentine situation about TP53 germline mutations in normal and cancer patients. Further analysis aimed at integrating our results with other Latin American studies should provide a comprehensive overview to improve our understanding of cancer pathogenesis, as well as to evaluate the impact of TP53 variants in different ethnic groups.

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Conflict of interest: Nothing to report.

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Ce ne sont pas les médicaments d'origine chimique mais plutôt les préparations d'origine biologique qui dominent au début du XXe siècle… Surtout l'opothérapie, la sérothérapie et la vaccinothérapie… la vaccin contre la rage (1884) et le sérum antidiphtérique (1892). […] Les années 1930 correspondent du point de vue de l'innovation thérapeutique,… à l'émancipation de la synthèse en chimie organique, la multiplication de médicaments d'origine biologique et enfin, par l'établissement progressif de pratiques de screening systématique et d'études cliniques contrôlées […], la mise au point des vitamines (A, B, C, D) des hormones sexuelles (oestrogène et testostérone), des antibiotiques et des corticostéroïdes. Les premiers antipaludéens de synthèse (mepacrine), les sulfamides et les antihistaminiques. Entre 1950 et le milieu des années 1960 c'est la mise au point des premiers anti-hypertenseurs (diurétiques et beta-bloquants), …de la psychopharmacologie…, des antibiotiques semi-synthetiques (pheneticilline), des anti-inflammatoires non stéroïdiens (ibuprofen) et au début des années 1960 des premiers contraceptifs oestrogestatifs oraux.

No son los medicamentos de origen químico, sino más bien las preparaciones de origen biológico que dominan a principios del siglo XX… Sobre todo la opoterapia, seroterapia y vacunoterapia… la vacuna antirrábica (1884) y el suero antidiftérico (1892). […] Los años 30 corresponden desde el punto de vista de la innovación terapéutica a la emancipación de la síntesis química orgánica, la multiplicación de medicamentos de origen biológico y en fin, al establecimiento progresivo de prácticas de *screening* sistemático y de estudios clínicos controlados […], la puesta a punto de vitaminas (A, B, C, D), de hormonas sexuales (estrógeno y testosterona), antibióticos y corticosteroides. Los primeros antipalúdicos de síntesis (*mepacrine*), las sulfamidas y los antihistamínicos. Entre 1950 y la mitad de la década de los 60 es la puesta a punto de los primeros anti-hipertensores (diuréticos y beta-bloqueantes), de la psicofarmacología, …de los antibióticos semisintéticos (*feneticilina*), de los anti-inflamatorios no esteroides (*ibuprofeno*), y a principios de los 60, de los primeros contraceptivos estrogénicos orales.

Ch. Bonah, A. Rasmussen

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