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Association of *TP53* Polymorphisms on the Risk of Wilms Tumor

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Background. Molecular factors influencing Wilms tumor (WT) development remain largely unknown. *TP53* mutations seem to be restricted to the anaplastic WT subtype. However, *TP53* polymorphisms do not have a defined role in the disease. ***Procedure.*** To assess the impact of *TP53* mutations and polymorphisms (PIN2, PIN3, and PEX4) on risk of development, age at diagnosis, and survival in WT, we analyzed 46 blood DNA samples and 31 fresh tumor DNA samples from 52 patients with WT. Sequencing of *TP53* exons 2–11 was performed. ***Results.*** Tumor DNA analysis revealed *TP53* pathogenic missense mutations (p.V197M, p.R213Q, p. R248W, and p.R337C) in four samples (12.9%). Blood DNA samples revealed a novel intronic mutation, IVS2 + 37C > T, in one patient (2.2%). Bilaterality was associated with a twofold decrease in survival ($P = 0.00037$). Diffuse anaplasia also presented a lower survival probability compared to patients with non-anaplastic tumors, or with focal anaplasia ($P = 0.045$). Patients with a *TP53* somatic mutation showed survival probability of 37.5% versus 85.0% for patients with no somatic mutations, although the difference was not statistically significant ($P = 0.0706$). PIN3 duplicated allele was associated with a 20-month later mean age at diagnosis ($P = 0.0084$). *TP53* PEX4 C allele showed an increased risk for WT development ($P = 0.0379$). No relationship was found between survival and gender, age at diagnosis, or the less frequent alleles of PIN2, PIN3, and PEX4. ***Conclusions.*** Our results demonstrate an association between PIN3 and age at diagnosis, as well as an association of PEX4 and risk of development of WT. Pediatr Blood Cancer 2014;61:436–441.

Key words: PEX4; PIN2; PIN3; *TP53*; Wilms tumor

INTRODUCTION

Wilms tumor (WT) is the most common renal tumor of childhood, affecting 1:10,000 children [1]. In Brazil, there are approximately 10 new cases per 10 million children younger than 14 years old per year [2], figuring as one of the highest incidences in the world [3]. Genetics of WT is complex, and although mutations in *WT1*, *WTX*, and *CTNNB1* have shown to be common events related to the disease, they occur in only one third of the cases [4]. The tumor suppressor *TP53* remains the most frequently mutated gene in human malignancies. Somatic mutations are found in up to 50% of cancers [5], and germline mutations predispose to various early-onset cancers, condition known as the Li–Fraumeni syndrome (LFS) [6]. Also, *TP53* polymorphisms have shown to play a role in cancer. Three polymorphisms are of special interest in *TP53*: PIN2 (polymorphism in intron 2, or rs1642785), a G > C substitution; PIN3 (polymorphism in intron 3, or rs17878362), a 16 bp duplication; and PEX4 (polymorphism in exon 4, or rs1042522), a G > C transversion that leads to an arginine to proline substitution at codon 72. The three of them have been extensively studied and associated to risk of development of many cancers [7–12]. Although *TP53* mutations in patients with WT seem to be restricted to tumors of the anaplastic subtype [13–15], its polymorphisms do not have a defined role in the disease. Therefore, this study investigated the mutational spectrum of *TP53*, and assessed the frequency of PIN2, PIN3, and PEX4 polymorphisms, and their association with the risk of tumor development, age at diagnosis, and cumulative 60-month survival rate in patients with WT.

METHODS

Samples

Fifty-two patients with WT, diagnosed and treated at the Instituto Nacional de Câncer (INCA) between the years of 2000 and 2012, were included in this study. Parents were asked for the participation of their children during follow-up medical appointments. Because patients with bilateral and/or syndromic forms of WT are referred for genetic counseling clinic more often than patients with unilateral and/or sporadic disease, our cohort had a high proportion of bilateral and syndromic cases. Clinical files were reviewed for information regarding gender, age at diagnosis, histopathology, and laterality. Peripheral blood DNA samples were available for 46 of the 52 patients, while fresh primary tumor DNA samples from 31 patients were available. The study obtained institutional review board approval prior to its start, and informed consent was obtained from affected individuals' parents. All patients received preoperative chemotherapy, according to the SIOP 2001 protocol implemented in our institution. Part of the clinical data presented here was previously published by our group [16].

TP53 Analysis

TP53 exons 2–11, including splice-site junctions, and the entirety of introns 2, 3, 5, and 8, were amplified and then sequenced with Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA), according to manufacturer's

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protocol, using primer sequences described at the International Agency for Research on Cancer (IARC) *TP53* Database (http://www-p53.iarc.fr) [17] (Supplementary Material). Products were analyzed at an *ABI3130xl* platform (Applied Biosystems), and compared to the reference sequences obtained from NCBI (NG_017013.2; NP_000537.3).

Statistical Analysis

Molecular data concerning the polymorphisms were analyzed by chi-square and Fisher's exact test, using the website for statistical computation VassarStats (http://vassarstats.net/). Odds ratio was estimated to verify if the less frequent allele of each polymorphism was related to risk or protection in WT. Relation between the polymorphisms and laterality with age at diagnosis was assessed using the non-parametric Mann–Whitney test. To increase accuracy of this analysis, the two outliers observed in the sample were not considered (136 and 137 months). Clinical and molecular data were used to estimate the cumulative 60-month survival rates of the patients by the non-parametric Kaplan–Meier method. Survival curves were compared with the log-rank test using the *GraphPad Prism* software (www.graphpad.com/scientific-software/prism). Survival was estimated considering the time at hospital registration, corresponding to the beginning of the treatment, until last visit to the hospital. All statistical results were considered significant when $P < 0.05$.

RESULTS

Clinical Characterization

Clinical characteristics of the patients are summarized in Table I. The mean age at diagnosis for bilateral cases was 24 months (±19), while mean age for unilateral cases was 38 months (±20) ($P = 0.0563$). Syndromes or congenital anomalies presented in our cohort included Denys–Drash syndrome (n = 4), Beckwith–Wiedemann syndrome (n = 2), hemihypertrophy (n = 1), macrosomia (n = 2), WAGR syndrome (n = 1), Simpson–Golabi–Behmel syndrome (n = 1), and one patient who presented Moebius syndrome associated with hemihypertrophy. Three of the 12 patients with syndromes/anomalies (25%) developed bilateral WT (one with Beckwith–Wiedemann syndrome and two patients with Denys–Drash syndrome). Histological data of the tumors were available for 49 patients, and 8/49 tumors presented focal or diffuse anaplasia (16.3%): 4/49 (8.2%) with focal anaplasia, and 4/49 (8.2%) with diffuse anaplasia.

TABLE I. Epidemiological, Clinical, and Histopathological Characteristics of 52 Patients With WT

Characteristics	N (%)
Gender	
Female	24 (46.2)
Male	28 (53.8)
Laterality	
Unilateral	41 (78.8)
Bilateral	11 (21.2)
Age at diagnosis (months)	
0–24	16 (30.8)
25–48	21 (40.4)
49 or +	15 (28.8)
Mean	39 ± 28
Presence of tumor anaplasia[a]	
Focal anaplasia	4 (8.2)
Diffuse anaplasia	4 (8.2)
Associated syndromes/anomalies	
	12 (23.1)

[a]Histological data were available for only 49 patients.

TP53 Mutation Analysis

Five mutations were found in the analyzed samples (Table II). Among the 46 blood DNA samples, one constitutional mutation was present (2.2%). The novel intronic germline mutation IVS2 + 37 C > T (NG_017013.2: g.16067C > T) was found in homozygosity in patient P05 (Fig. 1). Among the 31 studied tumor DNA samples, 4 (12.9%) presented mutations, and 3 of these were tumors of the anaplastic subtype. Considering that seven of the eight anaplastic tumors were available for DNA analysis, a *TP53* mutation was observed in 3/7 (42.9%) of the studied anaplastic tumors. All tumor mutations were missense, and located between exons 6 and 10. Patients P16 and P17, in addition to carrying somatic missense mutations, also showed discordant genotypes between blood and tumor samples for polymorphisms PIN2 and PEX4, with loss of heterozygosity in tumor DNA. These results suggest the likely loss of one allele of *TP53* in tumor samples in these patients. According to the IARC *TP53* Database, the four observed missense mutations were previously described in both germline DNA from LFS patients, as well as in malignant tumors, being classified as pathogenic [17]. The germline mutation IVS2 + 37C > T was first identified in this study. None of the patients carrying mutations had clinical signs of congenital syndrome or anomaly, thereby being apparently sporadic WT cases.

PIN2, PIN3, and PEX4 Polymorphisms Analysis

Analysis of *TP53* polymorphisms PIN2, PIN3, and PEX4 was performed in available blood DNA samples from 46 patients with WT. Allele and genotype frequencies in patients were compared to a control group of 300 healthy adults from Brazil [18]. Intragroup genotype distributions were in Hardy-Weinberg equilibrium ($P > 0.05$), both in patients and controls. Chi-square test showed no statistically significant differences in intergroup genotype frequencies of PIN2, PIN3, and PEX4 between patients and controls ($P = 0.27$, $P = 0.91$, $P = 0.09$, respectively). For PIN2 and PEX4, the most prevalent genotype among patients was homozygous GG (Table III). For PIN3, the most common genotype was represented by two non-duplicated alleles (NN). For the odds ratio analysis, the most common genotype of each polymorphism in the control group was considered the reference genotype (i.e., GG for PIN2, NN for PIN3 and GG for PEX4). Likewise, the allele showing the higher frequency in each polymorphism in the control group was considered the reference allele. We found a significant risk associated with PEX4 CC genotype ($P = 0.0337$), and with PEX4 C allele in patients ($P = 0.0379$). The other analyses did not show statistical significance (Table III). Among the nine patients with PEX4 CC genotype, eight did not show tumor anaplasia, and from one patient the complete histopathological data were not available; this patient had bilateral WT and Denys–Drash syndrome and died before surgery. Also, no *TP53* mutation was observed among the nine PEX4 CC genotype patients.

TABLE II. *TP53* Mutational Spectrum Observed in 52 Patients With WT

Mutation	Patient	Age at diagnosis (months)	DNA		Tumor histology	Heterozygous	Location	Altered base/codon	Amino acid substitution	Mutation type
			Blood	Tumor						
IVS2 + 37C > T	P05	9	Yes	NA	Blastematous	No	Intron 2	C > T	—	Intronic
p.V197M	P49	16	NA	Yes	Focal anaplasia	No	Exon 6	GTG > ATG	Val → Met	Missense
p.R213Q	P16	29	No	Yes	Diffuse anaplasia	No	Exon 6	CGA > CAA	Arg → Gln	Missense
p.R248W	P29	10	NA	Yes	Epithelial	No	Exon 7	CGG > TGG	Arg → Trp	Missense
p.R337C	P17	50	No	Yes	Focal anaplasia	No	Exon 10	CGC > TGC	Arg → Cys	Missense

NA, non-available.

We assessed the influence of the less frequent allele of PIN2, PIN3, and PEX4 in age at diagnosis in our patients. For each polymorphism, we compared the mean age at diagnosis (in months) between the group of patients with the more frequent genotype (reference genotype), and the group of patients carrying at least one copy of the less frequent allele. For PIN2 and PEX4, there were no statistically significant differences between mean age at diagnosis of carriers and non-carriers of the less frequent allele C ($P = 0.2774$ and $P = 0.0858$, respectively). For PIN3, however, carriers of at least one duplicated allele were diagnosed, on average, 20 months later than non-carriers, a statistically significant difference ($P = 0.0084$). The mean age at diagnosis for the non-duplicated homozygous patients (NN) was 30 months (±20), while patients with heterozygous or homozygous duplication (ND or DD) had a mean age at diagnosis of 50 months (±17) (Table IV). Also, we sought to investigate a potential association between the less frequent alleles of PIN2, PIN3, and PEX4 and a higher frequency of bilateral tumor cases, what was not observed (Fisher's exact test, $P = 1.000$; $P = 0.700$; $P = 0.488$, respectively).

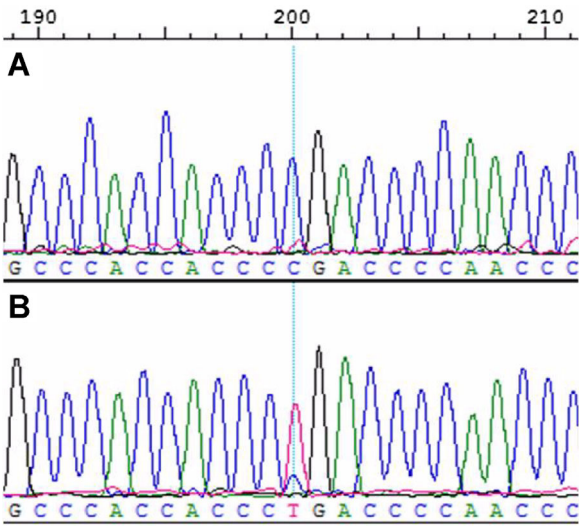

Fig. 1. Identification of the novel germline IVS2 + 37C > T mutation in intron 2 of the *TP53* gene. **A**: Patient with wild-type sequence; (**B**) germline homozygosity for IVS2 + 37C > T in patient P05.

Cumulative 60-Month Survival

To evaluate the impact of clinical, epidemiological and molecular data, we calculated the 60-month survival probability of patients according to the variables: laterality, gender, age at diagnosis, presence of tumor anaplasia, presence of *TP53* somatic mutation, and PIN2, PIN3, and PEX4 genotypes. Overall survival (n = 52) was 80.8% (95% CI = 0.701 to 0.931). Bilaterality represented the highest difference in survival (n = 52; $P = 0.00037$) (Fig. 2A). Patients with unilateral disease showed a survival rate of 92.4% (95% CI = 0.845 to 1), while patients with bilateral tumors had an approximately twofold decrease in survival, 45.5% (95% CI = 0.238 to 0.868). When considering only patients with available tumor samples (n = 31), we observed that patients with no pathogenic somatic mutations in *TP53* showed a survival probability of 85.0% (95% CI = 0.725 to 0.997), while patients with a *TP53* somatic mutation showed survival probability of only 37.5% (95% CI = 0.084 to 1), a difference that did not show a statistical significance ($P = 0.0706$) (Fig. 2B). The presence of diffuse anaplasia was associated with an impact on survival probability, showing rates that tended to zero, versus the 86.7% and 100% survival probabilities for patients with non-anaplastic tumors and patients with tumors with focal anaplasia (n = 49; $P = 0.045$) (Fig. 2C). No association was observed between survival and gender (n = 52; $P = 0.15$), age at diagnosis (n = 52; $P = 0.818$), or the less frequent allele of PIN2, PIN3, and PEX4 (n = 46; $P = 0.342$; $P = 0.975$; and $P = 0.812$, respectively). Patients without any syndromes/anomalies showed a survival rate of 85.4% (95% CI = 0.742 to 0.982), while patients presenting such clinical signs showed a survival rate of 65.6% (95% CI = 0.432 to 0.997), a difference that tended towards statistical significance (n = 52; $P = 0.0551$) (Fig. 2D).

DISCUSSION

We investigated germline and somatic mutations, as well as polymorphisms in *TP53* in 52 patients with WT, and the influence of genotype and clinical variables in their survival rates. One novel germline mutation was found in one patient, corresponding to 2.2% (1/46) of the available blood DNA samples. It is known that germline mutations in *TP53* are more frequently related to soft tissue sarcomas, osteosarcomas, premenopausal breast cancer,

TABLE III. Distribution of Allele and Genotype Frequencies, and Odds Ratio Determined for Each *TP53* Polymorphism in Patients With WT and Controls

Polymorphism	Patients (%), N = 46	Controls (%), N = 300[a]	OR (95% CI)	*P* (Fisher's exact test)
PIN2				
GG	27 (58.7)	166 (55.3)	1.00 (Reference)	—
GC	13 (28.3)	112 (37.3)	0.71 (0.35–1.44)	0.3901
CC	6 (13.0)	22 (7.3)	1.68 (0.62–4.51)	0.3915
Allele frequency G	67 (72.8)	444 (74.0)	1.00 (Reference)	—
Allele frequency C	25 (27.2)	156 (26.0)	1.06 (0.65–1.74)	0.8987
PIN3				
NN	33 (71.7)	222 (74.0)	1.00 (Reference)	—
ND	12 (26.1)	70 (23.3)	1.15 (0.57–2.35)	0.7103
DD	1 (2.2)	8 (2.7)	0.84 (0.10–6.94)	1.0000
Allele frequency N	78 (84.8)	514 (85.7)	1.00 (Reference)	—
Allele frequency D	14 (15.2)	86 (14.3)	1.07 (0.58–1.98)	0.8734
PEX4				
GG	19 (41.3)	158 (52.7)	1.00 (Reference)	—
GC	18 (39.1)	114 (38.0)	1.31 (0.66–2.61)	0.4809
CC	9 (19.6)	28 (9.3)	2.67 (1.10–6.50)	0.0337
Allele frequency G	56 (60.9)	430 (71.7)	1.00 (Reference)	—
Allele frequency C	36 (39.1)	170 (28.3)	1.63 (1.03–2.56)	0.0379

N, non-duplicated allele; D, duplicated allele. [a]Control group genotyped by Marcel et al. [18].

brain tumors, adrenocortical carcinoma and acute leukemias, the core tumors of the LFS [19]. Although WT has been reported as more frequent in families carrying *TP53* germline mutations [20], it is considered a rare component of this syndrome [21,22]. The novel intronic mutation IVS2 + 37C > T is located one nucleotide upstream of PIN2. According to the Human Splice Finder software (http://www.umd.be/HSF) [23], this substitution creates a new exonic splicing regulatory sequence, although *in vitro* studies would be necessary to characterize its impact on the protein. It is noteworthy, though, that the patient carrying the mutation died 28 months after diagnosis. Family history of cancer was negative. Parents were unavailable for molecular analysis. Pathogenic somatic mutations were found in four tumor DNA samples, and three of them were obtained from anaplastic areas. There is a clear relationship between *TP53* mutations and anaplastic WT, the histologic subtype with poorer prognosis. This indicates these mutations are related to tumor progression, and associated with a more aggressive type of the disease [13–15]. Our patients were treated with preoperative chemotherapy according to the SIOP 2001 protocol. However, we could not find evidence that somatic or germline mutations within *TP53* gene could be influenced by this treatment modality. Interestingly, one study used comparative genomic hybridization to search for chromosomal rearrangements in tumor samples of 41 patients with WT, of whom 19 had received preoperative chemotherapy, and observed fewer chromosomal rearrangements in the group that received this modality of therapy [24].

TABLE IV. Distribution of Mean Age at Diagnosis According to *TP53* Polymorphisms Status

Polymorphism	Mean age in months (SD)	*P*
PIN2		
GG	34 (±23)	0.2774
GC + CC	39 (±18)	
PIN3		
NN	30 (±20)	0.0084
ND + DD	50 (±17)	
PEX4		
GG	30 (±20)	0.0858
GC + CC	41 (±21)	

N, non-duplicated allele; D, duplicated allele.

Besides pathogenic mutations, *TP53* polymorphisms may also be sufficient to alter the expression or activity of the protein [5,25], emphasizing the importance of investigating *TP53* polymorphisms as possible risk factors, or phenotype modifiers in cancer. PIN3 duplicated allele was a modifier of age at diagnosis in our cohort, since age of tumor presentation in patients with this allele was, on average, 20 months later than in non-carriers. A similar effect has already been observed in patients with LFS, in which patients with the duplicated allele developed their first tumor, on average, 19 years later than patients without the duplicated allele. However, this effect was restricted to patients with germline *TP53* mutations [18]. The PIN3 16 bp sequence bends in a tertiary structure of pre-RNA called G-quadruplex. G-quadruplexes operate in the regulation of pre-RNA processing, modifying synthesis rates of the different p53 isoforms [26]. One of these isoforms, Δ40p53, is produced with retention of intron 2 by alternative splicing, and lacks the N-terminal domain and part of the transactivation domain. The truncated protein Δ40p53 lacks functional activity, and acts as a negative regulator of functional p53 [27]. G-quadruplex structures in the stable RNA increase efficiency of intron 2 excision, thus reducing the production of mRNA encoding Δ40p53, and increasing the production of mRNA encoding functional p53. Thus, PIN3 duplicated allele may contribute to this regulation, having a potential regulatory role on the expression of p53, but its mechanisms of action are not well determined [26]. Interestingly, most studies involving PIN3 report an association between the

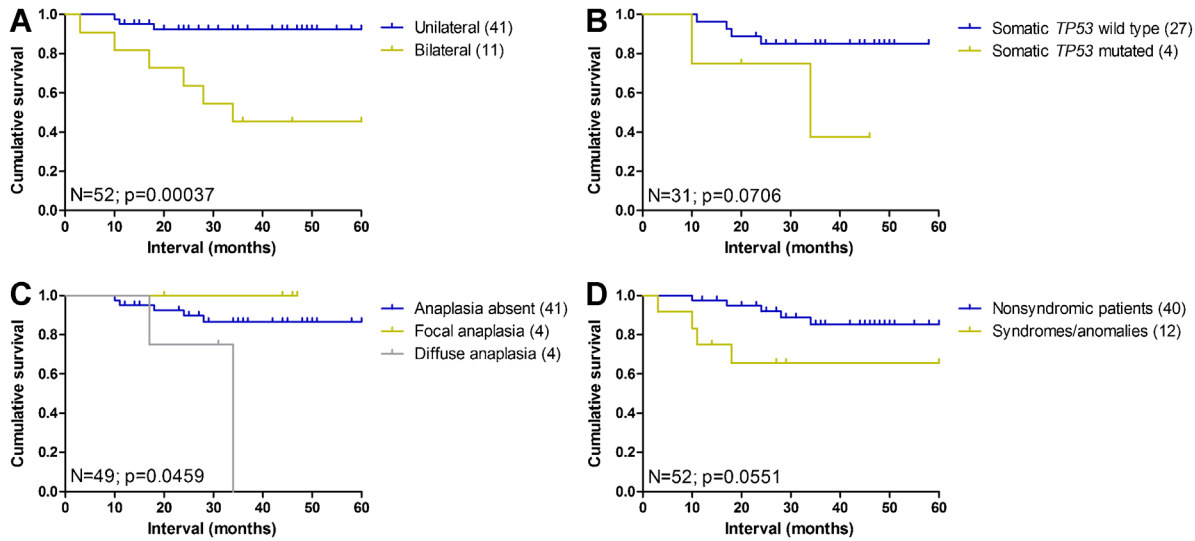

Fig. 2. Kaplan–Meier survival estimates for Wilms tumor patients. **A**: Survival curves of the patients according to tumor laterality; (**B**) shows only patients who had tumor DNA samples available stratified by the absence or presence of a *TP53* somatic mutation; (**C**) patients who had accessible histological data are divided in groups with non-anaplastic tumors, focal anaplasia tumors, and diffuse anaplasia tumors; (**D**) survival curves of patients according to the presence of congenital syndromes/anomalies.

duplicated allele and the risk of developing several cancers, including ovarian, colorectal, esophageal, and gastric cancer [28–30]. Wu et al. [31] reported lower rates of apoptosis and DNA repair in lymphoblastoid cells that had a duplicated allele of PIN3 in specific haplotype combinations with PEX4 and rs1625895 *TP53* SNPs.

Concerning the odds ratio for PIN2, PIN3, and PEX4 polymorphisms in our cohort, PIN2 and PIN3 showed no association with risk for developing the disease ($P > 0.05$), although several studies have linked them to other types of cancer [7,8,28–30]. PEX4 C allele showed a higher risk for development of WT than G allele. There is no consensus regarding the impact of PEX4 polymorphism in p53 protein function. While some studies demonstrate that the protein carrying the Arg72 residue (G allele) has a higher ability to induce apoptosis [32,33], other studies showed that the protein carrying Pro72 residue (Allele C) has greater efficacy in activating DNA repair genes [34] and in suppressing cell cycle at G1 phase [35]. Thus, it is expected that studies investigating the influence of PEX4 in development of various cancers do not show a consensus, reporting both an increased risk for the G allele [36,37], and for the C allele [9,38,39]. A recent study with 23 favorable histology WT samples showed PEX4 had no influence on age of onset of tumors, staging, or tumor recurrence [40]. The same authors, however, found an over-representation of the G allele in their samples, compared to reported frequencies in general population. Such association was not observed in our cohort, when comparing to Brazilian control data. It is necessary to enlarge our cohort to verify if the effect of PIN3 and PEX4 in age at diagnosis and risk of development remains the same.

The overall 60-month survival rate in our cohort was 80.8%. Age at diagnosis had no influence on patients' survival. Although patients diagnosed after 48 months usually have a higher frequency of tumors in stage III and IV [41], advances in treatment may be related to the high survival rates even in cases diagnosed later in childhood. In relation to the presence of anaplasia, our study is in accordance to postulated data from the National Wilms Tumor Study Group, reporting that only the diffuse subtype of anaplasia can be considered unfavorable histology, being related to a poorer prognosis [42]. Bilaterality, on the other hand, showed a 60-month survival probability rate about two times lower than survival of patients with unilateral disease, corresponding to the most important prognostic factor. Bilateral disease remains a challenge for the need to preserve both renal parenchyma, avoiding renal failure and reaching a better survival rate. The high frequency of bilateral disease and/or syndromic patients in our cohort may be due to the fact that both characteristics are commonly related to genetic predisposition, and thus the patients tend to be directed to genetic counseling and molecular studies more often than unilateral cases and children with no syndrome/anomaly. It is noteworthy, however, that all mutations observed in our cohort were present in non-syndromic patients. The presence of the less frequent alleles and genotypes of PIN2, PIN3, and PEX4 showed no influence on patients' survival, although an association of PEX4 and risk of tumor development was apparent in our sample, as well as the impact of PIN3, associated with a difference of 20 months in the mean age at diagnosis. The exact mechanisms of action concerning *TP53* polymorphisms in WT remain to be elucidated.

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