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Prognostic value of *TP53* Pro47Ser and Arg72Pro single nucleotide polymorphisms and the susceptibility to gliomas in individuals from Southeast Brazil

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ABSTRACT. The *TP53* tumor suppressor gene codifies a protein responsible for preventing cells with genetic damage from growing and dividing by blocking cell growth or apoptosis pathways. A common single nucleotide polymorphism (SNP) in *TP53* codon 72 (Arg72Pro) induces a 15-fold decrease of apoptosis-inducing ability and has been associated with susceptibility to human cancers. Recently, another *TP53* SNP at codon 47 (Pro47Ser) was reported to have a low apoptosis-

inducing ability; however, there are no association studies between this SNP and cancer. Aiming to study the role of *TP53* Pro47Ser and Arg72Pro on glioma susceptibility and oncologic prognosis of patients, we investigated the genotype distribution of these SNPs in 94 gliomas (81 astrocytomas, 8 ependymomas and 5 oligodendrogliomas) and in 100 healthy subjects by the polymerase chain reaction-restriction fragment length polymorphism approach. Chi-square and Fisher exact test comparisons for genotype distributions and allele frequencies did not reveal any significant difference between patients and control groups. Overall and disease-free survivals were calculated by the Kaplan-Meier method, and the log-rank test was used for comparisons, but no significant statistical difference was observed between the two groups. Our data suggest that *TP53* Pro47Ser and Arg72Pro SNPs are not involved either in susceptibility to developing gliomas or in patient survival, at least in the Brazilian population.

Key words: Gliomas; Single nucleotide polymorphisms; *TP53*; Pro47Ser; Arg72Pro

INTRODUCTION

The *TP53* gene, located at 17p13.1, is responsible for the transcription of a site-specific DNA-binding protein and acts as a transcription factor of cell growth-regulator genes (Soussi and May, 1996). A frequency of *TP53* mutations of around 50% observed in human tumors points to complexity of antiproliferative pathways under the control of TP53 protein and demonstrates that it plays an important role in tumorigenesis (Bonafe et al., 2002). Wild-type TP53 protein prevents cells with genetic damage from growing and dividing by two distinct pathways associated with cell cycle block and apoptosis-inducing activity. This dichotomy leads to an appropriate biological response to the DNA damage: genomic integrity is reestablished by DNA repair and the transient cell cycle blockade is lifted, or the cell undergoes apoptosis if DNA damage persists (Lane, 1992; Wang et al., 1995).

A critical site in the TP53 protein for apoptosis-signaling is a proline-rich region located between codon 64 and 92. At codon 72, in exon 4, a frequent functional single nucleotide polymorphism (SNP) that leads to an arginine-proline amino acid change (Arg72Pro) has been reported. Dumont et al. (2003) reported that the Arg72 allele, if in homozygosis, has an apoptosis-inducing ability 15-fold higher than does the Pro72 allele. According to Leu et al. (2004), this high apoptosis-inducing ability of the Arg72 allele is in part due to its mitochondrial location which makes it possible for TP53 to have a direct interaction with pro-apoptotic BAK protein. Studies on this SNP function were the basis for testing its impact on the risk and progression of tumors, where the less apoptotic allele Pro72 was associated with increased risk for development of tumors (Granja et al., 2004; Hishida et al., 2004; Xi et al., 2004; Ignaszak-Szczepaniak et al., 2006; Perrone et al., 2007; Toyama et al., 2007).

Recently, another SNP that induces a proline-serine change in exon 4 at codon 47 of *TP53* gene (Pro47Ser) has also demonstrated a significant decrease in TP53 protein apoptosis-inducing ability (Li et al., 2005). A critical event in TP53-inducing apoptosis ability is phos-

phorylation of the serine residue at codon 46, where allele Pro47 acts as a substrate of proline-directed kinases, for example MAPK1 protein. Li et al. (2005) reported that allele Ser47, a poor substrate for MAPK1, has an apoptosis-inducing ability 5-fold lower than does the wild Pro47 allele. According to our present knowledge, there are no studies that show whether an association exists between *TP53* Pro47Ser SNP and tumoral processes.

In the present research, we conducted a case-control study and examined the genotype distribution of *TP53* Pro47Ser and Arg72Pro SNPs, using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach, to further evaluate its possible relevance in susceptibility to gliomas and in determining the oncologic prognosis of patients.

MATERIAL AND METHODS

Study population

A total of 94 gliomas were analyzed, which had been surgically resected from previously untreated patients under the care of the Neurosurgery Department of Fundação Pio XII, Cancer Hospital of Barretos (Barretos, SP, Brazil). Tumor types and stages were determined according to WHO criteria (Kleihues et al., 2002) by two experienced pathologists. The clinical outcome, including length of survival, was obtained from patient records and by contacting each patient's general practitioner. The mean follow-up period for all patients was 47.62 weeks (range = 0.12-118.14). Blood samples of 100 healthy individuals were collected for controls. Because of the highly heterogeneous ethnic composition of the Brazilian population, the individuals of the control group were selected from the general population of São Paulo State, with no family history of cancer in first-degree relatives. The control sample was matched for gender and mean age with the patient group. The mean age of both patient and control groups was 45 years old, and 69.14% of patients and 69% of controls were 40 years old or older. The collection and use of tumor and blood samples for this study were previously approved by the appropriate institutional Ethics Committee.

DNA extraction and primer construction

DNA extraction was performed using proteinase K and phenol-chloroform according to routine molecular biology protocols. Primers were constructed using the Gene Runner 3.05 program (Hasting Software, Inc.) from gene sequence of the *TP53* Pro47Ser and Arg72Pro polymorphisms, obtained in the dbSNP of NCBI (Accession numbers: rs1800371 and rs1042522, respectively). Table 1 shows the primers and PCR product sizes.

Table 1. Polymerase chain reaction (PCR) primers for the amplification of *TP53* Pro47Ser and Arg72Pro single nucleotide polymorphisms.

Gene	Primer	Sequence (5'- 3')	Length (bp)	PCR product (bp)
TP53	Pro47Ser-F	CTG GTA AGG ACA AGG GTT GG	20	201 or
	Pro47Ser-R	TCA TCT GGA CCT GGG TCT TC	20	185*
	Arg72Pro-F	GAA GAC CCA GGT CCA GAT GA	20	152
	Arg72Pro-R	CTG CCC TGG TAG GTT TTC TG	20	

*Size divergence due to a 16-bp in/del intronic polymorphism near the *TP53* Pro47Ser single nucleotide polymorphism.

Polymerase chain reaction-restriction fragment length polymorphism and sequencing

PCR was carried out in a final volume of 25 µL containing 50 ng genomic DNA template, 1X PCR buffer (Biotools) with 2 mM $MgCl_2$, 0.4 µM of each primer (Invitrogen), 50 µM dNTPs (Amersham Biosciences), and 0.5 U DNA polymerase (Biotools). For PCR amplification, the standard program was used as follows: one initial denaturation step at 94°C for 5 min, followed by 35 denaturation cycles of 30 s at 94°C, 30 s of annealing at 54°C, and 30 s of extension at 72°C, followed by a final elongation cycle at 72°C for 5 min.

For RFLP, the PCR products of *TP53* Pro47Ser and Arg72Pro SNPs were digested with *Msp*I (4 U at 37°C for 4 h) and *Bst*UI (2 U at 60°C for 4 h) (New England BioLabs), respectively. *Msp*I recognizes a restriction site at Pro47 allele (C▼CGG) and generates two fragments of different sizes (156 or 140 bp and 45 bp), while Ser47 allele generates only one fragment of 201 or 185 bp (size divergences due to a 16 bp in/del intronic polymorphism near the *TP53* SNP, as shown in Table 1). In the same way, *Bst*UI generates two fragments of different sizes (52 and 100 bp) by recognizing a restriction site at Arg72 allele (CG▼CG), while Pro72 allele generates only one (152 bp). DNA fragments were electrophoresed through a 10% acrylamide:bisacrylamide gel (19:1), and then stained with silver nitrate.

The genotypes of >10% of the samples were reassessed to confirm the results. Also, selected PCR products were purified and submitted to bidirectional sequencing to further confirm the authenticity of genotype analysis. PCR products were purified with ExoSAP (USB), followed by sequencing with DYEnamic ET Dye Terminator Kit (Amersham Biosciences), according to the manufacturer's specifications. Sequencing reactions were performed on MegaBACE 1000 (GE Healthcare).

Statistical analysis

The chi-square test was used to verify whether genotype distributions were in Hardy-Weinberg equilibrium. Observed frequencies of genotypes in gliomas were compared to controls using chi-square or Fisher exact tests when expected frequencies were small. Kaplan-Meier curves were constructed to assess overall survival and disease-free survival rates, and differences among groups were analyzed by the log-rank test. Statistical significance was set at $P < 0.05$. Statistical analyses were performed with S-Plus 2000 (Insightful, Inc.) and GraphPad Prism 4.0 (GraphPad Software, Inc.) softwares.

RESULTS

A total of 94 glioma patients and 100 control subjects were included in this study. The patients comprised 59 males and 35 females (M/F ratio = 1.69) and the control subjects consisted of 63 males and 37 females (M/F ratio = 1.7). Mean age in patient and control groups was 45 years (range = 1-75 and 18-72, respectively). No significant gender- or age-related differences were observed between the groups ($P > 0.05$). Eighty-one patients had astrocytomas (11 grade I, 23 grade II, 8 grade III, and 39 grade IV glioblastomas), 5 oligodendrogliomas (3 grade II and 2 grade III), and 8 ependymomas (5 grade II and 3 grade III). Forty-two tumors of 94 gliomas were classified as low grade (grades I and II) and 52 as high grade (grades III and IV). No significant difference in stratification by tumor grade was observed among the groups (*TP53* Pro47Ser and Arg72Pro, $P = 0.585$ and $P = 0.104$, respectively).

Genotype frequencies in controls and patients were in Hardy-Weinberg equilibrium. Allele and genotype frequencies of *TP53* Pro47Ser and Arg72Pro SNPs in controls and patients were not significantly different. As shown in Table 2, for *TP53* Pro47Ser SNP, Ser47 allele frequency in controls was 0.01, while in patients it was 0.02 (P = 1.000). The frequencies of Pro/Pro and Pro/Ser genotypes among controls were 98 and 2%, while in patients these were 96.8 and 3.2%, respectively (P = 0.675). For *TP53* Arg72Pro SNP, Pro72 allele frequency in controls was 0.31, while in patients it was 0.26 (P = 0.531). Frequencies of Arg/Arg, Arg/Pro, and Pro/Pro genotypes among controls were 48, 42 and 10%, while in patients these were 56.4, 36.2 and 7.4%, respectively (P = 0.488).

Table 2. Allele and genotype frequencies in controls and patients.

		Controls		Patients		P*
		N	%	N	%	
TP53 Pro47Ser	Pro/Pro	98	(98%)	91	(96.8%)	0.675
	Pro/Ser	2	(2%)	3	(3.2%)	
	Ser47 allele frequency		0.01		0.02	1.000
TP53 Arg72Pro	Arg/Arg	48	(48%)	53	(56.4%)	0.488
	Arg/Pro	42	(42%)	34	(36.2%)	
	Pro/Pro	10	(10%)	7	(7.4%)	
	Pro72 allele frequency		0.31		0.26	0.531

Data are reported as number with percent in parentheses. *P values were obtained using the Fisher exact test or the chi-square test.

The mean follow-up period for all patients was 47.62 weeks (range = 0.12-118.14). For 53 patients who survived the follow-up period (censored patients), mean follow-up was 62.07 weeks. For 41 patients who died during the follow-up period, mean follow-up time was 28.93 weeks. Survival curves for overall survival and disease-free survival in the patients were not significantly different for any of the *TP53* SNPs tested (Kaplan-Meier analysis; P > 0.05) (Figures 1-4).

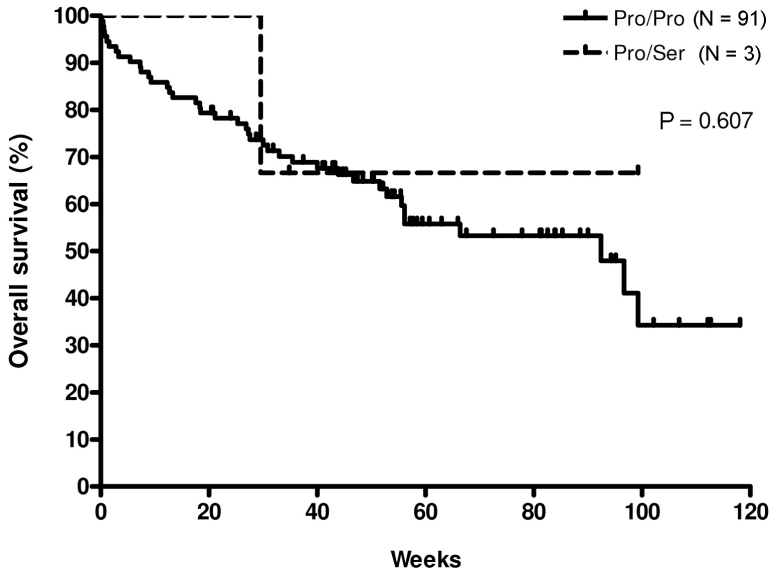

Figure 1. Overall survival in patients according to *TP53* Pro47Ser single nucleotide polymorphism.

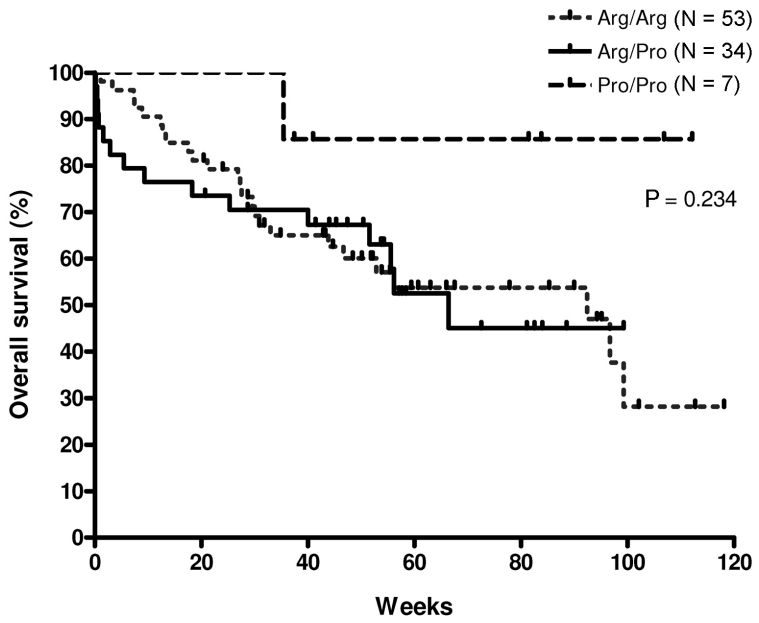

Figure 2. Overall survival in patients according to *TP53* Arg72Pro single nucleotide polymorphism.

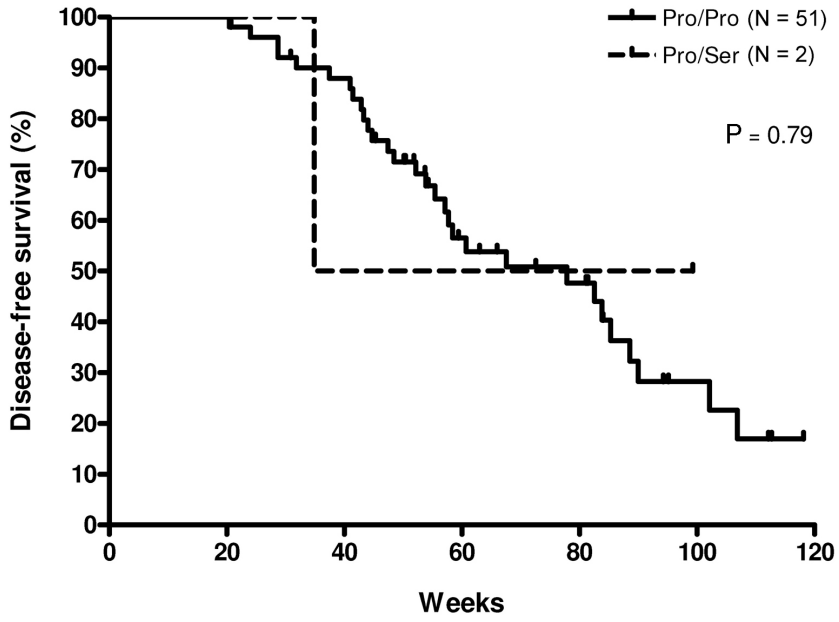

Figure 3. Disease-free survival in patients according to *TP53* Pro47Ser single nucleotide polymorphism.

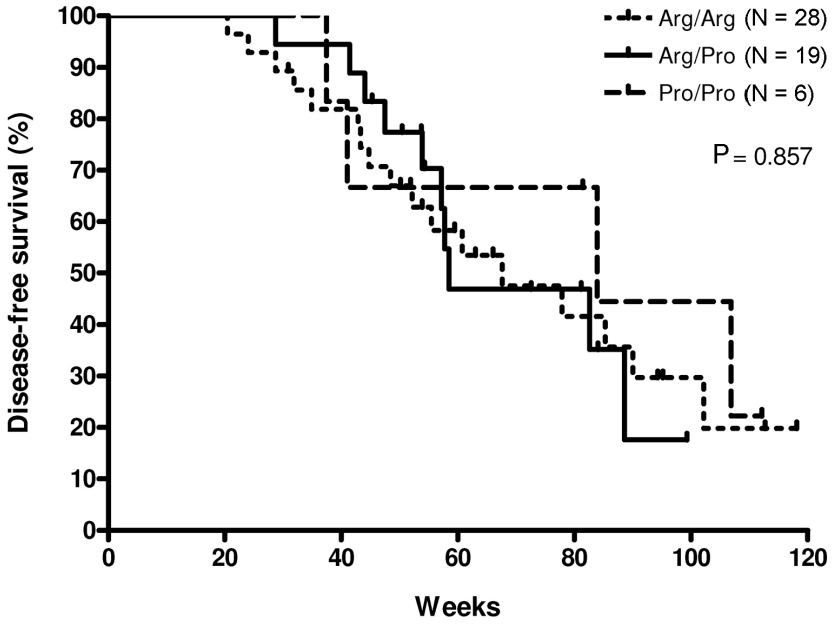

Figure 4. Disease-free survival in patients according to *TP53* Arg72Pro single nucleotide polymorphism.

DISCUSSION

SNPs are the most abundant variations in the human genome and have become increasingly popular in the genetic study of several diseases, due to their quick, inexpensive and accurate analysis. The identification of SNPs as risk factors for different cancer types can be important for prevention, diagnosis and prognosis (Kirk et al., 2002). For this reason, the major aim of this case-control study was to investigate the relationship between *TP53* Pro47Ser and Arg72Pro SNPs and susceptibility to glioma and patient survival.

Published research substantially lacks information on *TP53* Pro47Ser SNP. Felley-Bosco et al. (1993) were the first to report about the Ser47 allele prevalence in Afro-American patients, showing a frequency less than 0.05. However, they did not find this allele in any of the 69 Caucasians from their cohort. Our results show a frequency of 0.01 for the Ser47 allele in the control group and 0.02 in patients, similar to a recent survey in 200 Afro-American individuals who showed a Ser47 allele frequency of 0.01 (Li et al., 2005).

As far we know, this is the first study that investigates the association between *TP53* Pro47Ser SNP and cancer susceptibility and oncologic prognosis of patients, more specifically with regard to glioma. Our data showed that neither glioma susceptibility ($P = 0.675$) nor patient survival (overall and disease-free survival, $P = 0.607$ and 0.79, respectively) was associated with variant alleles.

Several studies have investigated association between *TP53* Arg72Pro SNP and an increased risk for developing tumors and results have shown that this SNP is a risk factor for adrenocortical, colorectal, breast, lung, head and neck, and cervical-uterine cancers, among

many others (Papadakis et al., 2002; Shen et al., 2002; Buyru et al., 2003; Sifuentes and Reyes, 2003; Ignaszak-Szczepaniak et al., 2006; Zhu et al., 2007). However, none of the investigations have shown strong evidence, and this association remains unclear (Drummond et al., 2002; Oren, 2003; Khadang et al., 2007). With regard to Arg72Pro alleles, Arg72 in homozygosis has been reported as a risk factor for cervical cancer (Qie et al., 2002) while the Pro72 allele in homozygosis has been reported as a high risk factor for pulmonary, hepatic and nasopharyngeal cancers (Wang et al., 1995; Yu et al., 1999; Tsai et al., 2002). Genotype Arg/Pro has been associated with an increased susceptibility to tobacco-induced lung adenocarcinoma (Fan et al., 2000). In our study, we did not observe an association between *TP53* Arg72Pro SNP and susceptibility to glioma (P = 0.488).

The heterogeneity of association of *TP53* Arg72Pro SNP is at least in part due to its large ethnic and geographical variation (Zehbe et al., 1998). The Pro72 allele, for example, has shown a north-south frequency gradient of 0.17 in Sweden to 0.63 in Nigeria (Beckman et al., 1994). In Western Europe (France, Switzerland, and Norway), the United States, Central and South America (Mexico, Costa Rica and Peru), and Japan, Arg72 allele is the most frequent, ranging from 0.60 to 0.83. However, frequencies higher than 0.40 for Pro72 allele were reported in Afro-Americans and Chinese (Weston et al., 1992; Jin et al., 1995; Ngan et al., 1999; Peixoto et al., 2001). In our study, we identified a frequency of 0.69 for Arg72 allele in the control group and 0.74 in patients, results similar to other studies conducted in Brazil. Granja et al. (2004), besides demonstrating an association between the Pro/Pro genotype and thyroid cancer, also reported a frequency of 0.65 for Arg72 allele in a control group from southeast Brazil. Khayat et al. (2005), in a case-control study of gastric adenocarcinoma in the State of Pará, northern Brazil, described a frequency of 0.69 for Arg72 allele in their control group.

The *TP53* Arg72Pro SNP role in cancer patient prognosis is still more conflicting. While several studies have revealed that *TP53* Arg72Pro SNP has no significant effect on oncologic prognosis for patients with pancreatic, testicular and prostate tumors (Wu et al., 1995; Dong et al., 2003), other studies have confirmed this association for patients with breast and lung tumors (Wang et al., 1995; Toyama et al., 2007). In our study, we were not able to demonstrate an association between *TP53* Arg72Pro SNP and glioma patient survival (overall and disease-free survival, P = 0.234 and 0.857, respectively).

Jones et al. (2004) reported that homozygote individuals for Pro72 allele from non-polyposis colorectal cancer-predisposed families have disease manifestation 13 years earlier than homozygote individuals for Arg72 allele. However, unlike Jones et al. (2004), we did not observe an association between *TP53* Arg72Pro alleles and age of glioma patients (P > 0.05).

There are few studies on the association between *TP53* Arg72Pro SNP and gliomas. Biros et al. (2002) and Uno et al. (2006) did not show any association of this SNP in astrocytoma samples. Furthermore, Parhar et al. (2005) have suggested a possible association between *TP53* Arg72Pro SNP and susceptibility to brain tumors, particularly high-grade astrocytomas. Our results corroborate only the studies of Biros et al. (2002) and Uno et al. (2006), since we were not able to demonstrate any association between *TP53* Arg72Pro SNP and tumor grade (P = 0.104), as demonstrated by Parhar et al. (2005).

We conclude that there is no association between *TP53* Pro47Ser and Arg72Pro genotypes and glioma susceptibility or patient survival, at least in the Brazilian population. Nevertheless, future studies on larger populations from other parts of the world are essential for a definitive conclusion about these results.

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