Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

TP53 codon 72 polymorphism in adult soft tissue sarcomas

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Genet. Mol. Res. 7 (4): 1344-1352 (2008)
Received September 5, 2008
Accepted October 2, 2008
Published December 2, 2008

ABSTRACT. Soft tissue sarcomas (STS) are tumors of mesodermal origin, comprising about 1% of all adult neoplasms. Management of such tumors is an important medical challenge. TP53 codon 72 polymorphism results in either the arginine or proline form of the p53 protein; several studies have investigated whether codon 72 polymorphisms are risk and prognostic factors for cancer. We investigated p53 codon 72 polymorphism (Arg72Pro) frequencies with respect to the susceptibility and the clinical outcome of patients with STS. A series of 100 STS were genotyped for the p53 Arg72Pro polymorphism using polymerase chain reaction. Genotype frequencies were compared to a group of 85 healthy donors (controls). Possible associations between polymorphic genotypes, clinicopathological factors and survival of STS patients were also investigated. Genotypic frequencies obtained for STS patients did

not significantly differ from that obtained for controls. In the STS group, p53 codon 72 polymorphic variants were not significantly associated with gender, age, tumor size, clinical stage, tumor grade, histology, or nodal or distant metastasis. The five-year overall survival rate for the STS group was 48%; it was significantly affected by tumor grade, clinical stage, and nodal and distant metastasis. Soft tissue sarcoma patients with the Pro/Pro variant had a reduced survival rate (30%), when compared to the p53 Arg/Arg (45%) and the p53 Arg/Pro groups (55%). However, the differences between these groups were not significant (P = 0.44).

Key words: Soft tissue sarcomas; p53 Arg/Pro polymorphism; Prognostic factors; Single nucleotide polymorphism

INTRODUCTION

Soft tissue sarcomas (STS) are mesenchymal cancers with clinical significance disproportional to their frequency (Borden et al., 2003). They are responsible for about 1% of all adult malignant solid tumors (Clark et al., 2005), and their management is an important medical challenge (Manoel et al., 2008a,b). Five-year overall survival for STS patients is approximately 50% (Kotilingam et al., 2006; Skubitz and D'Adamo, 2007). Outcome for STS can be predicted according to prognostic factors, including tumor size, depth, grade, histological type, and the presence of node or distant metastasis (Manoel et al., 2008a,b). Genetic factors play a crucial role in the development of soft tissue sarcomas, including translocations and mutations in tumor suppressor genes and oncogenes (Antonescu, 2006; Kotilingam et al., 2006).

TP53 is the most common tumor suppressor gene studied in human cancers, since it controls both cellular development and cell cycle regulation (Levine et al., 2006). The protein encoded by TP53 is a 53-kDa nuclear phosphoprotein with 393 amino acids (Sutcliffe and Brehm, 2004; Levine et al., 2006). This protein plays a pivotal role in cellular response to several stress signals and is expressed during DNA damage (Sutcliffe and Brehm, 2004; Lima et al., 2006).

Single nucleotide polymorphisms (SNPs) are genetic variations in DNA sequence that can create or modify recognition sequences for restriction enzymes. The allele frequency for an SNP must be above 1% in a given population (Bojesen and Nordestgaard, 2008). A critical SNP has been extensively studied in the TP53 gene. It is located at exon 4, codon 72, and because it has an impact on the p53 protein sequence, its association with cancer susceptibility and prognosis has been widely investigated in human populations (Storey et al., 1998; Sourvinos et al., 2001; Bhattacharya et al., 2002; Cortezzi et al., 2004; Lattuada et al., 2004; Siddique et al., 2005; Nelson et al., 2005; Bond and Levine, 2007). In the TP53 gene, codon 72 can be CCC, which codes for p53Pro, and/or CGC, which codes for p53Arg. The two alleles can generate three different genotypes in human populations: two homozygotes for p53Arg/Arg and p53Pro/Pro, and one heterozygote for p53Arg/Pro.

TP53 codon 72 polymorphism is located in a proline-rich domain of p53 protein, which is necessary for the protein to efficiently induce apoptosis. A more efficient apoptotic potential was demonstrated for the p53Arg variant, resulting in a greater ability of this variant to enter the mitochondria, inducing cytochrome *c* release (Dumont et al., 2003). On the other hand, the p53Pro variant seems to be more competent in inducing cell cycle arrest and DNA-

repair (Dumont et al., 2003; Siddique and Sabapathy, 2006; Ørsted et al., 2007). Several studies have investigated the association of p53 codon 72 polymorphism with cancer susceptibility and prognosis, but, like a chain reaction, the results of one study represent a challenge for a new one (Sourvinos et al., 2001; Lattuada et al., 2004; Gallo et al., 2005). Based on a worldwide search of the literature, we believe this is the first study evaluating the potential role of TP53Arg/Pro codon 72 polymorphism in the susceptibility and prognosis of STS.

MATERIAL AND METHODS

Samples

One hundred patients with histopathologic diagnosis of STS were included in this study. These patients were admitted to Hospital Araújo Jorge of the Association for the Combat of Cancer in Goiás, Goiânia, Brazil, from 1996 to 2000. Additionally, 85 healthy individuals were evaluated and constituted the control group. The tumor specimens were obtained from formaldehyde-fixed and paraffin-embedded tissue blocks stored in the Pathology Department of Hospital Araújo Jorge. The STS cases included patients with more than 18 years of age and with confirmed histopathological diagnosis. Clinicopathological information and archival tissue specimens were available for all patients. This study was approved by the Internal Review Board of the Association for the Combat of Cancer in Goiás. The samples consisted of 47 male and 53 female patients aged 19 to 83 years (mean age 48.3 years). The control group consisted of 54 males and 31 females. The histopathology was evaluated according to the criteria established by the World Health Organization, and staging was defined in accordance with the criteria established by the American Joint Committee on Cancer (AJCC) and the International Union against Cancer. The three most frequent sites of the primary tumor were lower limbs (35 cases), retroperitoneum (18 cases) and trunk (14 cases). Fibrosarcoma comprised 29 of the tumors, leiomyosarcoma 13 and liposarcoma 11. Most of the tumors were classified as high grade, larger than 5 cm and clinical stage III (data not shown).

DNA extraction

Genomic DNA from the control group was isolated from peripheral blood. Selected STS samples (0.5 to 2.0 mg tissue) were transferred to microtubes, and after paraffin removal with xylol baths at 65°C, they were rehydrated with three ethanol baths, at room temperature. DNA extraction, for both groups, was carried out by using the genomic DNA purifying kit Wizard® (Promega Corporation, USA), according to manufacturer instructions.

Polymerase chain reaction

TP53 codon 72 genotyping was carried out by polymerase chain reaction (PCR) amplification. Samples were submitted to PCR using two different primer sets for the amplification of the p53Pro and p53Arg alleles, according to Sourvinos et al. (2001). Glyceraldehyde-phosphate dehydrogenase gene amplification was used as internal control. Base sequence of primers was proposed by Storey et al. (1998). PCR products were analyzed on 8% silver stained polyacrylamide gels. A single band of approximately 177 bp indicated an individual homozygote for p53Pro; the presence of a band with 144 bp characterized an

individual homozygote for p53Arg. Heterozygous samples p53Arg/Pro showed fragments of both amplified fragments, one of 144 bp and another of 177 bp.

Statistical analysis

The chi-square test was used to determine the possible associations between clinicopathological features (i.e., tumor grade, size, stage, gender, age, and metastases) and TP53 variant frequencies. Significance was established at $P < 0.05$. The Kaplan-Meier method was used to estimate the 5-year survival curves. Differences in survival were evaluated using the log-rank test. Statistical analyses were performed using the SPSS software program (SPSS Inc.). Genotypic data were calculated using the GenePop® web version 3.4 software. This program allowed the analysis of allelic and genotypic frequencies and genetic and genotypic differentiation of cases and controls.

RESULTS

Allelic and genotypic frequencies were calculated for subjects and controls (Tables 1 and 2); however, no significant statistical difference was demonstrated between the two groups ($P > 0.05$). Possible associations between TP53 codon 72 polymorphic variants and different clinicopathological aspects such as gender, age, tumor size, grade, clinical stage, and the presence of nodal and distant metastasis were also investigated. No significant statistical association was demonstrated between such parameters (data not shown).

Table 1. Allele frequencies of case versus control populations.

Allele	Cases	Controls
p53Arg	159 (79.5%)	126 (74.1%)
p53Pro	41 (20.5%)	44 (25.9%)
Total	200 (100%)	170 (100%)

Allelic differentiation results from the GenePop® 3.4 web version analysis: $\chi^2 = 2.6$; d.f. = 2; 95%CI; $P = 0.267$.

Table 2. Genotypic frequencies of case versus control populations.

Genotype	Cases	Controls
p53Arg/Arg	64 (64%)	51 (60%)
p53Arg/Pro	31 (31%)	24 (28.2%)
p53Pro/Pro	5 (5%)	10 (11.8%)
Total	100 (100%)	85 (100%)

Genotypic differentiation results from the GenePop® 3.4 web version analysis: $\chi^2 = 2.3$; d.f. = 2; 95%CI; $P = 0.308$.

The Kaplan-Meyer method was employed to estimate the 5-year survival rate for the STS patients. Overall survival was 48.1% (Figure 1A). Patients with low-grade tumors showed better survival compared to those with high-grade tumors (68.8 *vs* 32.6%; $P = 0.003$) (Figure 1B). Five-year survival was significantly different for patients with stage II (65.7%) compared to patients with stage III (46.2%) and stage IV (7.4%). Patients in stage I were censored because there were no deaths for this group during the period analyzed ($P < 0.001$) (Figure 1C). No significant difference was found with respect to tumor size, which is a well-established negative prognostic factor (data not shown). Node and distant metastasis were important prognostic factors. Patients who did not present

node metastasis had a 5-year survival of 50.3%, while patients who presented node metastasis had a survival of 20% (P = 0.029) (Figure 1D). The 5-year survival rate for patients with distant metastasis was significantly lower (0%) compared to those cases in which metastasis was not present (58.7%; P < 0.001) (Figure 1E). The 5-year survival rates were calculated according to the TP53 codon 72 polymorphic variant groups. The p53Pro/Pro homozygous group had a reduced survival (30%) compared to the 53Arg/Arg group (45%) and p53Arg/Pro heterozygous group (54.9%). However, differences between these three groups were not statistically significant (P = 0.444) (Figure 1F).

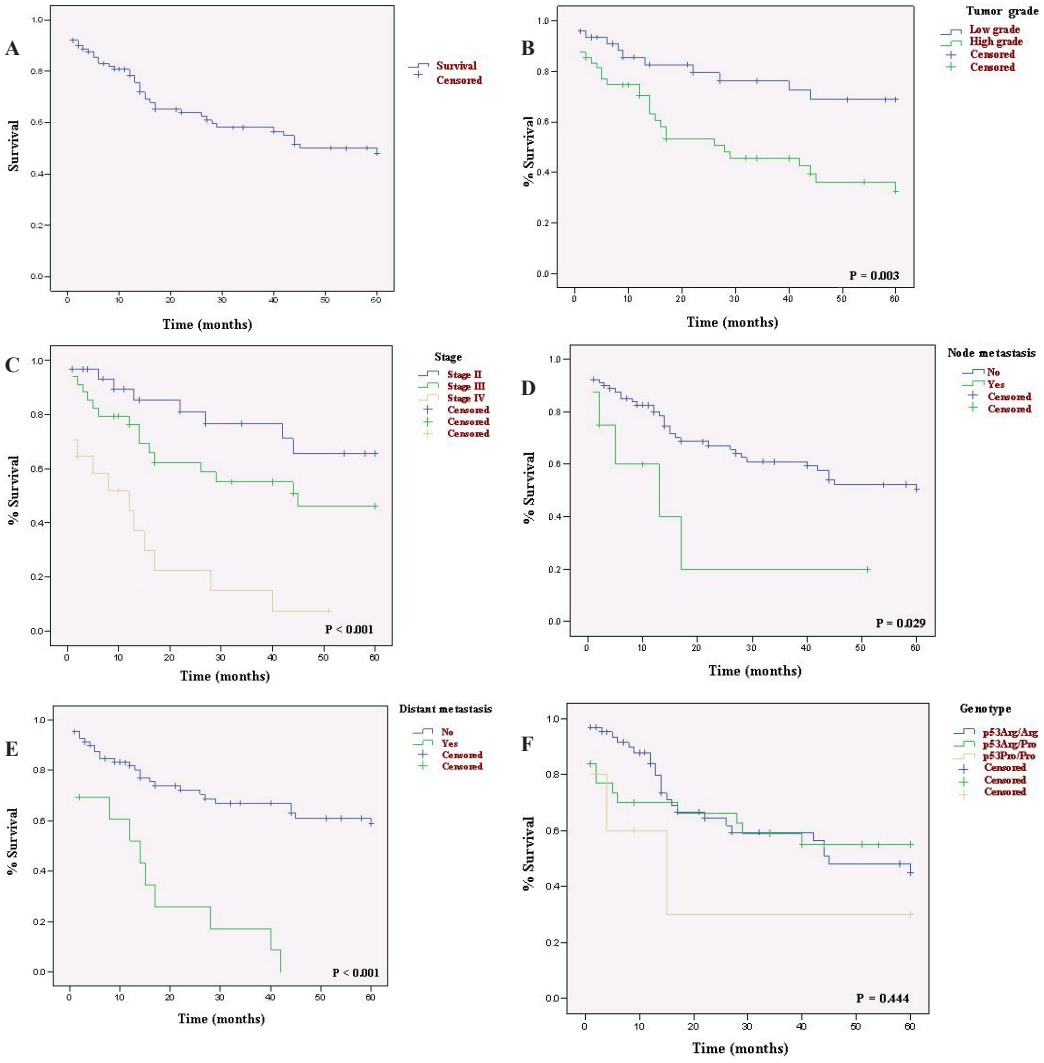

Figure 1. Five-year survival rates for soft tissue sarcoma patients. **A.** Five-year overall survival for soft tissue sarcoma patients. **B.** Five-year survival according to histopathological grade in soft tissue sarcoma patients. **C.** Five-year survival according to stage in soft tissue sarcoma patients. **D.** Five-year survival according to node metastasis in soft tissue sarcoma patients. **E.** Five-year survival according to distant metastasis in soft tissue sarcoma patients. **F.** Five-year survival according to genotype of soft tissue sarcoma patients.

DISCUSSION

Several studies have associated TP53 gene mutations with a high risk for STS development, tumor aggressiveness, poor prognosis, and diminished survival (Komuro et al., 1993; Hieken and Das Gupta, 1996; Taubert et al., 1996; Dirix and van Oosterom, 1999; Schneider-Stock et al., 1999; de Alava et al., 2000; Taylor et al., 2000; Savage et al., 2006; Das et al., 2007; Sabah et al., 2007; Muret et al., 2008). There is also evidence showing that STS expressing mutated p53 protein has a worse prognosis (Bond and Levine, 2007; Das et al., 2007; Muret et al., 2008). The TP53 codon 72 polymorphism is widely studied because it affects the gene coding sequence, generating polymorphic variants with different biochemical and biological activities. It has been associated with higher risk for several cancer types, but no published study on the TP53 codon 72 polymorphism and adult STS was found in the literature. In our study, allelic frequencies calculated for subjects and controls were 79.5 and 74.1% for p53Arg and 20.5 and 25.9% for p53Pro, respectively. Genotypic frequencies calculated for subjects and controls were 64 and 60% for homozygotes p53Arg/Arg, 31 and 28.2% for heterozygotes p53Arg/Pro, and 5 and 11.8% for homozygotes p53Pro/Pro, respectively. Although, no significant difference was demonstrated for the genetic and genotypic frequencies in the two groups, a higher frequency was observed for the p53Arg allele in both groups. Similar frequency patterns have been observed for different tumors (Bonafé et al., 2003; Brenna et al., 2004; Cortezzi et al., 2004; Lima et al., 2006). Our study supports the evidence that p53Arg is the most common allele in Latin American populations (Gallo et al., 2005). However, worldwide literature data on genetic and genotypic frequencies are conflicting, due to ethnic differences among the populations studied (Brenna et al., 2004; Siddique et al., 2005). Different confounding factors should be considered as well, including sample size, the source of DNA, the detection methods, and the inter-laboratory variations in the protocols affecting the ability to detect TP53 polymorphisms (Brenna et al., 2004).

According to our results, no statistically significant association was demonstrated between TP53 codon 72 polymorphism and clinicopathological aspects, such as gender, age, tumor localization, histology, tumor size, stage, grade, and node and distant metastasis. The STS cases enrolled in this study showed clinicopathological characteristics very similar to those described in different studies (Hieken and Das Gupta, 1996; Taubert et al., 1996; Clark et al., 2005; Kotilingam et al., 2006; Sabah et al., 2007; Muret et al., 2008). The overall 5-year survival rate was 48.1%. Patients with low-grade tumors had a statistically significant better survival than those with high-grade tumors (68.8 *vs* 32.6%; $P = 0.003$). Five-year survival was significantly different for patients with stage II (65.7%) compared to stage III (46.2%) and stage IV patients (0.07%; $P < 0.001$). These data agree with those in the literature (Kotilingam et al., 2006; Skubitz and D'Adamo, 2007). Node and distant metastases were good prognostic factors. Patients who did not present node and/or distant metastasis had a better survival. Patients showing the homozygote p53Pro/Pro genotype had a worse survival (30%) when compared to those showing the homozygote p53Arg/Arg (45%) and heterozygote p53Arg/Pro (54.9%) genotypes. However, these differences were not statistically different ($P = 0.444$). Considering this, we were not able to associate the p53Pro/Pro homozygote genotype with a worse prognosis in STS patients. In the analysis, the p53Pro/Pro genotype became a confounding factor, since the number of subjects with such genotype was too small (only 5 subjects). If the sample size could be increased, the analysis would certainly reach statistical significance.

The p53Pro/Pro genotype has been associated with poorer disease-free survival in breast cancer patients (Toyama et al., 2007). In contrast, a different study (Bonafé et al., 2003), which also evaluated TP53 codon 72 polymorphism in breast cancer subjects, concluded that p53Arg variant was associated with a significantly reduced overall survival. Another study (Ørsted et al., 2007) associated the p53Pro/Pro genotype with a better prognosis in different cancer types. It is possible that in our study, the prognostic value of the TP53 variants could have been underestimated, because mutations in these alleles can also occur (Nelson et al., 2005; Petitjean et al., 2007) and, in this context, they may affect the response pattern to therapy in the STS patients.

A possible association between TP53 codon 72 polymorphism and STS prognosis would be of special interest, since it could provide a potential genetic marker related to a better response to treatment. Studies have demonstrated that p53Arg variant has a better apoptotic performance (Thomas et al., 1999; Dumont et al., 2003), and this feature could be advantageous to the patient. There is evidence that resistance to apoptosis is one of the most important characteristic of malignant tumors (Grivicich et al., 2007). Since apoptosis is an important anticancer defense mechanism, and several chemotherapeutic agents exert their effects through cell death induction, the influence of p53 codon 72 polymorphism on the apoptotic response induced by chemotherapy in cancer cells must be better understood. Codon 72 polymorphism has been shown to modulate TP53-dependent apoptosis, and therefore, it can modify sensitivity to chemotherapeutic agents (Toyama et al., 2007). This idea is supported by clinical data, which show that p53Arg patients without TP53 mutations respond much better to chemotherapy and survive longer compared to p53Pro patients (Sullivan et al., 2004; Siddique and Sabapathy, 2006). Nonetheless, it has been reported that p53Pro is more efficient in the DNA-repair function, as well as in the induction of cell-cycle arrest (Thomas et al., 1999; Dumont et al., 2003; Siddique and Sabapathy, 2006; Ørsted et al., 2007; Bojesen and Nordestgaard, 2008). A better repair mechanism prevents cellular genetic instabilities making cancer development more difficult. However, some studies associating the p53Pro allele with a worse prognosis in some cancer types, such as breast cancer (Toyama et al., 2007), hepatocellular carcinoma (Zhu et al., 2005), lung cancer (Nelson et al., 2005), and gastric cancer (Yi and Lee, 2006) have been published.

It is important to note that both variants are able to carry out both apoptosis and DNA-repair functions, but with different efficiencies. Thus, the polymorphism at codon 72 may serve as a genetic modifier, thereby allowing fine-tuning of these biological processes, perhaps as a consequence of ecological adaptation (Siddique and Sabapathy, 2006).

Another interesting aspect of our analysis is that the 5-year survival rate was higher for the heterozygote p53Arg/Pro patients. Since these subjects had both p53 variants, we could foresee that apoptosis and repair mechanisms for this group are equally efficient. However, a differential expression of these variants may influence prognosis. The first study indicating a selective expression of the TP53 codon 72 polymorphic alleles was carried out by Siddique and his group (2005). They suggested that healthy heterozygous Asians seem to preferentially express the p53Pro allele and that healthy Caucasians express the p53Arg variant. When allelic expression was examined in Chinese breast cancer heterozygotes, they found that this population preferentially expressed the p53Arg allele in the tumor tissue, which was in contrast to the data obtained in healthy subjects. These heterozygote p53Arg expressers did not show any traces of the expression of the p53Pro allele, suggesting that the p53Arg allele was selectively activated and the p53Pro allele was silenced in the heterozygote cancers. This

raises the possibility that the p53Arg allele may not be functionally involved, but its expression may simply relate to tumorigenesis. Together, these data indicate that the p53Arg allele is associated with breast cancer susceptibility. Our results about genetic frequencies are in contrast with this latter study because the p53Arg allele was the most frequent variant found in the population studied. The histological types and ethnic characteristics of the individuals evaluated in our study are different from those evaluated by Siddique, and this may explain the differences between the results.

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