TP53 Codon 72 and Intron 3 Polymorphisms and Mutational Status in Gastric Cancer: An Association with Tumor Onset and Prognosis

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Key Words
TP53 polymorphism • TP53 mutation • Gastric cancer

Abstract
Although TP53 alterations have been studied in human tumors, data considering the role of two common TP53 polymorphisms (Pro72Arg in codon 72 and Ins16bp in intron 3) and their associations with TP53 mutations in gastric cancer are very limited. Thus, we analyzed these parameters taking into consideration the clinicopathological data. DNA from 106 gastric tumor samples was available for TP53 Pro72Arg and TP53 Ins16bp polymorphism genotyping by PCR-RFLP and PCR, respectively. The mutational status of the TP53 exons 5–7 was assessed by the single-strand conformational polymorphism test. The TP53 72ArgArg genotype was statistically associated with patients aged ≥65 years (p = 0.039), and the intron 3 A2A2 genotype was correlated with late-stage tumors (III and IV; p = 0.043). Considering both polymorphisms, a negative correlation between the TP53 Pro-A1 haplotype and age >65 years (r = −0.211; p = 0.030) was found. Taking into account the TP53 mutations, the Pro/Pro genotype was positively correlated with the presence of exon 7 mutations (p = 0.049), and a correlation between this genotype and the number of mutations in TP53 was observed (p = 0.019). This study corroborates the understanding of TP53 polymorphisms in gastric carcinogenesis, especially regarding the genetic features in tumor onset and prognosis.

Introduction
Gastric adenocarcinoma is one of the most frequent malignancies worldwide and is globally the second leading cause of cancer-related deaths [1]. In this context, Brazil is one of the countries with a high incidence, mainly in the Southeast and Northeast regions [2]. As in other human cancers, gastric carcinogenesis is accompanied by the activation of oncogenes and the inactivation of tumor suppressor genes [3]. Among the tumor suppressor genes, the TP53 gene is one of the most frequent targets for genetic mutations [4]. The p53 pathway is crucial for effective prevention of genetically damaged cell propagation, either directly, by its participation in DNA repair mechanisms, or indirectly, by induction of apoptosis [5]. Thus, mutations in TP53 that compromise p53 function occur in >50% of human cancers, whereas alteration in p53 regulators occurs in many of the remainder [6].
Presently, several studies are focusing on genetic polymorphisms as an important contribution to cancer susceptibility and tumor behavior, such as those described in the TP53 gene [7]. One of the most studied TP53 polymorphism is Pro72Arg, located in codon 72 in exon 4, leading to a structural modification of the protein [8]. Another common polymorphism is the 16-bp insertion in intron 3 of the TP53 gene, which was suggested to promote alterations in messenger RNA processing. Interestingly, there is evidence that TP53 polymorphisms might be correlated with specific TP53 mutations depending on the tissue [9, 10], although the influence of these two TP53 polymorphisms on gastric carcinogenesis associated with the mutational profile of the TP53 gene has never been assessed.

Hence, the aim of this study was to evaluate the associations between the TP53 Pro72Arg and the TP53 Ins16bp polymorphisms and the presence of TP53 mutation in exon 5, 6 and 7 in gastric cancer, considering the clinicopathological data in this process.

Materials and Methods

Clinical Specimens

The present study was approved by the hospital ethics committee of the Federal University of Ceará, and all subjects signed the informed consent form before inclusion. Samples from 106 patients with gastric adenocarcinoma who had undergone gastrectomy were collected from the Walter Cantidio University Hospital and Santa Casa de Misericórdia Hospital, both located in Fortaleza, the Ceará state capital. Histological classification was done according to Lauren’s classification by our team of pathologists.

DNA Extraction

Genomic DNA was extracted from frozen tumor tissue using the cetyltrimethylammonium bromide technique, adapted from Foster and Twell [11]. DNA extraction was only performed in frozen tumor tissue that showed >80% of tumor cells determined by histopathological analysis. DNA quality was analyzed by 1% agarose gel electrophoresis and the amount was determined using the NanoDrop™ fluorospectrometer (Wilmington, Del., USA).

Determination of TP53 Polymorphism Genotypes

TP53 codon 72 genetic polymorphism was detected by PCR-RFLP, as previously described by Bonafè et al. [12]. PCR products of 155 bp were then digested with restriction endonuclease BsrUI. The detection of TP53 Ins16bp polymorphism was carried out by PCR, as described by Powell et al. [13], resulting in a product amplification of 135 bp (A2) or 119 bp (A1) depending on the presence or absence of the insertion. Both, the digestive and the PCR products were resolved by electrophoresis in 8% polyacrylamide gel with silver staining (fig. 1). Random samples were reanalyzed for laboratory procedure control.

Analysis of p53 Mutation

To detect the mutation in the TP53 gene, we used the single-strand conformational polymorphism (SSCP) test, under the premise that abnormal electrophoretic migration results from genetic mutation, as previously showed [4, 14]. Exons 5–7 of the p53 gene were amplified by PCR using three pairs of the primers described by Murakami et al. [14]. Each PCR reaction for a total of 25 µl final volume consisted of 0.2 mmol/l of the four deoxynucleotide triphosphate, 1.5 mmol/l MgCl₂, 0.4 µmol/l of each primer and 0.5 units of Taq DNA polymerase (Invitrogen®). The cycling temperature included an initial temperature at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 1 min for annealing temperature (55°C for exons 5 and 8/9, 63°C for exons 6 and 7), and extension at 72°C for 1 min. For SSCP analysis, 5 or 6 µl of each PCR product was mixed with an equal volume of stop solution (95% of formamide, 20 mmol/l EDTA, 0.05% of xylene cyanol and 0.05% of bromophenol blue), heated at 95°C for 5 min.
and immediately placed on ice and loaded onto a gel containing 12.5% acrylamide (GenePhor™, Amersham Pharmacia Biotech). Electrophoresis was performed using Electrophoresis Unit GenePhor (GE Healthcare) at 4°C for 3 h. The patterns of the bands were then visualized by a DNA Silver Staining Kit (Amersham Pharmacia Biotech).

**Statistical Analyses**
Analyses were carried out using the statistical program SPSS® version 15.0 (Chicago, Ill., USA). Statistically significant differences were evaluated by the χ² test. Correlations were analyzed by Spearman's rank correlation coefficient. The results were considered statistically significant when p values were <0.05.

**Results**

**Study Population**
Among the 106 analyzed cases, there was a higher percentage of males (68.9%, 73/106). The median age was 63.7 years, ranging from 23 to 92. The most frequent anatomic site was gastric non-cardia (74.5%, 79/106), and intestinal and diffuse subtypes were represented by 56.6 and 43.4%, respectively.

**TP53 Alterations in Gastric Cancer**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lauren’s histotype</th>
<th>Anatomic site</th>
<th>Age, years</th>
<th>Tumor stage</th>
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<tbody>
<tr>
<td></td>
<td>intestinal</td>
<td>cardia</td>
<td>non-cardia</td>
<td>&lt;65</td>
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<tr>
<td>Pro/Pro</td>
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<td>Pro/Arg</td>
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<td>Arg/Arg</td>
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<td>Pro allele</td>
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<td>Arg allele</td>
<td>45</td>
<td>37</td>
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<td>58</td>
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* p < 0.05.

**Frequencies of TP53 Genotypes and SSCP Analysis**
All 106 samples were available for TP53 polymorphism genotyping and SSCP analysis for exons 5, 6 and 7. At least one mutated exon was present in 38.7% of the cases. Exon 5 showed the most mutations (41.5%, 44/106), followed by exon 7 (33.0%, 35/106) and by exon 6 (31.1%, 33/106). Regarding the TP53 codon 72 polymorphism, all genotypes were well represented; the most frequent genotype was Arg/Arg (42.4%, 45/106), followed by Pro/Arg (35.0%, 37/106) and Pro/Pro (22.6%, 24/106). In contrast, for the TP53 Ins16bp (intron 3) polymorphism analysis, a high frequency of the A1A1 genotype (74.5%, 65/106) was observed, and A1A2 and A2A2 genotypes were present in 19.8 (21/106) and 5.7% (6/106) of the cases, respectively. All genotypes were in accordance with Hardy-Weinberg’s equilibrium.

**Associations between TP53 Polymorphisms and Clinicopathological Parameters**
No significant association was found between tumor localization (cardia and non-cardia), histological subtypes (intestinal and diffuse) and the TP53 genotypes for

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*Table 1.* Associations between TP53 codon 72 polymorphism and clinicopathological parameters

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<td>37</td>
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</table>

* p < 0.05.

*Table 2.* Associations between TP53 Ins16bp polymorphism and clinicopathological parameters

<table>
<thead>
<tr>
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<td>non-cardia</td>
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<tr>
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<td>36</td>
<td>19</td>
<td>60</td>
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<tr>
<td>A1A2</td>
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<td>8</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>A2A2</td>
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<td>2</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>A1 allele</td>
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<td>44</td>
<td>26</td>
<td>74</td>
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<tr>
<td>A2 allele</td>
<td>17</td>
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* p < 0.05.
both studied polymorphisms (table 1, 2). However, a positive correlation was found between the Arg/Arg genotype and age ≥65 years (r = 0.200; p = 0.040). In fact, the Arg/Arg genotype was statistically more often associated with higher ages at a cutoff of 65 years when compared to the Pro/Arg genotype (p = 0.016) or to the Pro allele carriers (Pro/Pro + Pro/Arg; p = 0.039).

When we analyzed the TP53 genotype distribution according to tumor stage, we observed that patients carrying the A2A2 genotype significantly correlated with late-stage tumors (III and IV; r = 0.198; p = 0.041). In addition, considering stage III and IV tumors, the A2A2 genotype was statistically more frequent than the A1A2 genotype (p = 0.020), and also than the A1 carriers (A1A1 + A1A2; p = 0.043). However, when we analyzed the lymph node status, the invasion and the presence of metastasis, no significant association was observed with any TP53 genotype.

Analyzing the associations between both TP53 polymorphisms, the Pro/Pro genotype was positively correlated with the A2A2 genotype (r = 0.258; p = 0.008). However, there was no significant correlation between the A2 and the Pro carriers (r = 0.327; p = 0.001). Interestingly, the heterozygous genotypes (Pro/Arg and A1A2) were also positively correlated with each other (r = 0.282; p = 0.003). When we analyzed the associations between the TP53 haplotypes and the clinicopathological data, we found a negative correlation between the TP53 Pro-A1 haplotype and age <65 years (r = –0.211; p = 0.030).

Associations between TP53 Polymorphisms and TP53 Mutations

In this study, the Pro/Pro genotype was positively correlated with the presence of exon 7 mutations (r = 0.192; p = 0.049). Additionally, a correlation between this genotype and the number of mutations in TP53 was observed (r = 0.229; p = 0.019). No correlation was observed between the TP53 Ins16bp polymorphism and the presence of TP53 mutation.

Among the non-mutated cases, the frequencies of the ProArg and the ArgArg genotypes were similar [45.4% (15/33) and 42.5% (14/33), respectively], and the ProPro genotype was present in 12.1% (4/33) of the cases. For the TP53 intron 3 polymorphism, the genotypic frequencies were as follows: 78.8% (26/33) for the A1A1, 18.2% (6/33) for the A1A2 and 3.0% (1/33) for the A1A1 genotype. No significant association was found among these genotypes and any of the clinicopathological parameters.

**Discussion**

Although the risk of gastric cancer has been widely studied regarding the TP53 codon 72 polymorphism, few studies focused on the associations between the TP53 Ins16bp polymorphism (intron 3) and the clinicopathological features [15, 16]. Also, the TP53 mutational status has not been taken into account when these polymorphisms were assessed in gastric cancer series [15, 16]. Given that both polymorphisms and the presence of mutation might modify or drive the protein function into a preferred direction, it is important that these alterations are also analyzed in association, in an attempt to better characterize some aspects of gastric cancer pathogenesis. Our study aimed to analyze these parameters in association with the clinicopathological data.

The distribution of the TP53 codon 72 polymorphism in our study was regular, with no clear preponderance of any specific genotype. However, this polymorphism has been demonstrated to vary pronouncedly depending on the studied population, as the Pro allele, considered to be the ancestral allele, presents a frequency of approximately 95% in Africans and the Arg allele a frequency as high as about 85% in northern Europeans [17, 18]. Our findings may represent the ethnical mixture seen in our population. On the other hand, the TP53 intron 3 polymorphism has not been associated with ethnical variability [19]. This study showed a marked prevalence of the wild genotype, in accordance with the study of De Feo et al. [20], which also showed no significant genotypic difference between gastric cancer patients and healthy controls.

Although no association between the TP53 genotypes or mutational status and tumor location or histological subtype was found, a correlation between older patients (age ≥65 years) and the Arg/Arg genotype was observed. This age-associated change regarding the TP53 codon 72 genotypes in gastric cancer is considered in only few studies, with contradictory results. Mojtahedi et al. [21] and Kim et al. [16] found no differences between genotype or allele frequencies by age, while Zhang et al. [22] observed a strong correlation between the TP53 72Arg/Arg genotype and aging in gastric cancer patients. Likewise, Wang et al. [23], analyzing lung cancer, suggested a relationship between the TP53 72Arg/Arg genotype and patients ≥60 years old. Our data add further evidence to the association of the presence of the TP53 72Arg/Arg genotype in older patients with cancer. It is suggested that the TP53 72Arg allele is more efficient in triggering the apoptotic process [24–26] because it has a greater binding
activity with MDM2 [27]. Therefore, it may act as a survival factor in gastric cancer patients when compared to those TP53 72Pro carriers which justify the data from the studies that pointed to TP53 with 72Arg, conferring a later cancer onset.

When we analyzed the tumor stage and the TP53 polymorphisms, we found an association between the TP53 intron 3 A2A2 genotype and the late gastric tumor stages (III and IV). Although a relationship between the TP53 Ins16bp polymorphic allele and the genotype with advanced tumor stages has been highlighted in many studies involving breast cancer [7, 8], this is the first evidence given in gastric tumors. The biological effect of this polymorphism is unclear; however, it has been pointed out that this polymorphism could affect messenger RNA splicing, altering the coding regions and thus being implicated in the regulation of gene expression and DNA-protein interactions, resulting in an impaired protein that could lead to a more aggressive type of cancer [28].

Concerning the associations between the studied polymorphisms, we found a statistical association between the presence of both TP53 72Pro and TP53 intron 3 A2 alleles. Thus, we speculated if this could be an important factor affecting tumor clinicopathological features. In the combined allele analyses, a significant correlation between the TP53 Pro-A1 alleles and patient age <65 years was found. No association with this TP53 haplotype has been described in gastric cancer. Nevertheless, Osorio et al. [29], studying breast cancer, found that the TP53 Pro-A1 haplotype was also associated with an earlier age at cancer onset in BRCA2 mutation carriers, corroborating our findings.

In our study, the TP53 polymorphisms were also analyzed focusing on their association with the TP53 mutational status. We found that the number of mutations tends to increase in the presence of the TP53 72ProPro genotype, being consistent with the study of Mechanic et al. [30] that suggested an increased frequency of somatic TP53 mutation in TP53 72Pro carriers. Additionally, we found a statistically significant correlation between the TP53 72Pro/Pro genotype and mutation in TP53 exon 7. Studies involving an association between the TP53 codon 72 polymorphism and TP53 somatic mutations show conflicting results. Studying gastric cancer, Kim et al. [31] found no correlation between any TP53 Pro72Arg allele and TP53 mutation; however, in their study, the TP53 mutational status was predicted by p53 protein immunopositivity, which might provide several false-negative cases [32, 33]. On the other hand, El Hallani et al. [34], studying glioblastomas, pointed to a relationship between the 72Pro allele and a TP53 somatic mutation. Indeed, this association seems to be dependent on the tumor tissue. A study carried out in Norway [10] involving breast and colorectal cancer found differences in the relationship between TP53 codon 72 polymorphism alleles and the TP53 mutational status according to the tumor tissue, where no association was found in the colorectal cancer, while the 72Arg allele was related to the occurrence of TP53 somatic mutation in breast cancer.

In conclusion, the TP53 72ArgArg seems to be a survival factor in gastric cancer, as these patients’ tumor onset apparently occurs at a later age, while the TP53 Pro-A1 haplotype seems to be a maker of earlier age of cancer onset. Also, the more advanced tumors (stages III and IV) point to being a feature of TP53 72ProPro carriers. Additionally, the TP53 72ProPro is potentially associated with the accumulation of TP53 somatic mutations, especially in exon 7. This study corroborates the understanding of the TP53 polymorphisms in cancer pathogenesis, especially filling a gap in gastric cancer. Studies involving patient follow-up could extend our understanding of these TP53 alterations and their relationship with gastric cancer development.

References


TP53 Alterations in Gastric Cancer

Pathobiology 2012;79:323–328 327


