***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 ob-

served ($p = 0.019$). This study corroborates the understanding of *TP53* polymorphisms in gastric carcinogenesis, especially regarding the genetic features in tumor onset and prognosis.

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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].

Fig. 1. Representative electrophoresis of the *TP53* codon 72 polymorphism in 8% polyacrylamide gel with silver staining.

Fig. 2. Representative electrophoresis of the *TP53* intron 3 polymorphism in 8% polyacrylamide gel with silver staining.

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 Cantídio 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™ 3300 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 *Bst*UI. 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 $MgCl_2$, 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

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

Genotype	Lauren's histotype		Anatomic site		Age, years		Tumor stage	
	intestinal	diffuse	cardia	non-cardia	<65	≥65	I and II	III and IV
Pro/Pro	15	9	3	21	11	13	8	16
Pro/Arg	17	20	10	27	23	14	15	22
Arg/Arg	28	17	14	31	16	29*	19	26
Pro allele	32	29	13	48	34	27	23	38
Arg allele	45	37	24	58	39	43	34	48

* p < 0.05.

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

Genotype	Lauren's histotype		Anatomic site		Age, years		Tumor stage	
	intestinal	diffuse	cardia	non-cardia	<65	≥65	I and II	III and IV
A1A1	43	36	19	60	35	44	30	49
A1A2	13	8	7	14	13	8	12	9
A2A2	4	2	1	5	2	4	0	6*
A1 allele	56	44	26	74	48	52	42	58
A2 allele	17	10	8	19	15	12	12	15

* p < 0.05.

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 χ^2 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.

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

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 ($r = 0.258$; $p = 0.008$). In fact, there was a 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 carriers 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 carrier patients with lung cancer. 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 marker of earlier age of cancer onset. Also, the more advanced tumors (stages III and IV) point to being a feature of *TP53* intron 3 A2A2 genotype 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.

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