Research article

Interrelationship between *TP53* gene deletion, protein expression and chromosome 17 aneusomy in gastric adenocarcinoma

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Abstract

Background: This study evaluates the existence of numerical alterations of chromosome 17 and *TP53* gene deletion in gastric adenocarcinoma. The p53 protein expression was also evaluated, as well as, possible associations with clinicopathological characteristics.

Methods: Dual-color fluorescence *in situ* hybridization and immunostaining were performed in twenty gastric cancer samples of individuals from Northern Brazil.

Results: Deletion of *TP53* was found in all samples. *TP53* was inactivated mainly by single allelic deletion, varying to 7–39% of cells/case. Aneusomy of chromosome 17 was observed in 85% of cases. Chromosome 17 monosomy and gain were both observed in about half of cases. Cells with gain of chromosome 17 frequently presented *TP53* deletion. The frequency of cells with two chr17 and one *TP53* signals observed was higher in diffuse than in intestinal-type GC. Immunoreactivity of p53 was found only in intestinal-type samples. The frequency of cells with two chr17 and two *TP53* signals found was higher in samples with positive p53 expression than in negative cases in intestinal-type GC.

Conclusion: We suggest that *TP53* deletion and chromosome 17 aneusomy is a common event in GC and other *TP53* alterations, as mutation, may be implicated in the distinct carcinogenesis process of diffuse and intestinal types.

Background

Gastric cancer (GC) is the fourth most frequent malignancy and the second most common cause of cancer death in the World [1]. In the State of Pará (Northern Brazil), GC was the most common cause of cancer death in 2000. In Belém, State of Pará, the 5-year-survival rate is about 9–10% [2]. A better understanding of the biology of this neoplasia progression is crucial for the development of better tests to early neoplasia detection and also of new treatment strategies for GC.

Molecular events in the carcinogenesis of GC remain largely unknown [3]. A key feature in the pathogenesis of most GC, as in many other solid cancers, is chromosomal instability, resulting in gains and losses of parts or even whole chromosomes [4].

Gastrointestinal tract tumors are notorious for being difficult to be analyzed by standard cytogenetic techniques [5-9]. Fluorescence *in situ* hybridization (FISH) assay allows rapid detection of numerical genetics aberrations in interphase nuclei in tumor cells. FISH assay should be used to evaluate cell-to-cell heterogeneity in gene or loci copy number and detect small subpopulations of genetically aberrant cells [10]. FISH studies have shown numerical aberrations 1, 7, 8, 9, 17, 20, X and Y to be common in GC [[7,11-19], see also review [20]]. There are some studies in literature concerning *TP53*, located at chromosome 17p13.1, and chromosome 17 (chr17) copy number alterations by FISH assay in GC [[21-24], see also review [20]].

The *TP53* tumor suppressor plays a pivotal role in the coordination of the repair process or in the induction of apoptosis. *TP53* somatic alteration is described in approximately 50% of human cancers, including GC [25]. Deregulation of the *TP53* pathway has been shown to involve mutations, loss of heterozygosity (LOH), increased expression of the *TP53* inhibitor HDM2, or epigenetic silencing of the *TP53* promoter [26,27].

The aim of this study was to investigate chr17 and *TP53* numerical alterations in GC samples from Pará State by dual-color FISH technique. Immunostaining for p53 protein was also evaluated. These results were correlated with clinicopathological characteristics.

Methods

Samples

The study included 20 gastric adenocarcinoma samples. Samples of primary tumors submitted to surgical resection were obtained from João de Barros Barreto University Hospital (HUJBB). This study investigated cancer samples of patients from Pará State, where there is a mixed population composed of three main ethnic groups: Amerindian, African and European [28].

Patients' age, sex and tumor anatomical sites were obtained from tumor registries. The mean age of the twenty patients was 55 ± 14.67 years (range 24–77). The female/male ratio was 3:2. All samples were classified according to Laurén [29] and tumors were staged using standard criteria by TNM staging [30]. According to Laurén's classification, 6 were diffuse type (30%) and 14 were intestinal type (70%). Table 1 shows cases with their histopathological characteristics.

All patients had negative histories of exposure to either chemotherapy or radiotherapy prior to surgery; there was no other diagnosed cancer. An informed consent with approval of the ethics committee of HUJBB was obtained from the studied patients.

Fish

FISH was applied on cells fixed in methanol/acetic acid using recently made slides according to modified protocols [31]. The slides were washed in 2× saline sodium citrate (SSC)/0.5% NP-40 (pH 7.0) solution and dehydrated in 70%, 80% and 95% ethanol. To determine the chr17 and *TP53* copy numbers, cells were hybridized with 10 μL dual-color direct labeled probe (Qbiogene®, CA, USA) specific for chr17 α-satellite and *TP53* gene region, labeled with fluorescein and rhodamine respectively. The probe applied to the slide under a glass coverslip. The probe and sample were denatured at 75°C for 5 minutes and. *In situ* hybridization occurred at 37°C in a moist chamber overnight. Post-hybridization washings were done and the nuclei were counterstained with DAPI/antifade. Molecular cytogenetic analysis was carried out under an Olympus BX41 fluorescence microscope with triple DAPI/FITC/TRICT filter (Olympus, Japan) and the FISHView® of Applied Spectral Imaging® image analysis system (ASI Ldt., Israel). For each case, 200 interphase/metaphase nuclei were analyzed and were scored using the Hopman's criteria [32]. In our study, the cut-off level for interphase-FISH was 5%. To avoid misinterpretation due to technical error, gastric mucosal tissue (nonneoplastic) and normal lymphocyte nuclei were used as negative control.

Immunohistochemical staining

Deparaffinized tissue sections (4 μm) were incubated with primary monoclonal antibody p53 (DO-7, dilution 1:50, DakoCytomation, CA, USA) and secondary antibody followed by streptavidin-biotin-peroxidase complex (DakoCytomation, CA, USA) as previously described [18]. Slides were visualized with diaminobenzidine-H_2O_2 and counterstained with Harry's hematoxylin. The results were interpreted using the Ozturk's et al. criteria [33]. Positive p53 expression was defined as clear nuclear staining, whereas negative p53 immunostaining was considered when no positive cell was seen or rare cells were stained (less than 10% weakly stained tumor cells). A breast ade-

Table 1: Clinicopathological, immunohistochemistry and FISH results of GC samples.

							FISH chr17/*TP53* (%)												
Case	Age	Sex	Loc†	pTNM‡	LC§	IHC	2/2	2/1	1/1	3/2	3/1	3/3	4/2	4/1	1/2	2/3	4/4	4/3	5/4	2/4
1	77	M	An	T2N1Mx	Int	+	77.5	11.0	10.0	1.0	-	0.5	-	-	-	-	-	-	-	-
2	48	F	An	T4N0Mx	Int	-	52.2	16.0	4.1	1.2	2.4	0.6	8.2	-	-	-	13.5	1.8	-	-
3	58	M	Co/an	T1N1Mx	Int	+	68.5	14.5	4.5	-	3.0	-	3.5	2.5	1.5	0.5	-	1.5	-	-
4	48	F	Co/an	T3N0Mx	Int	+	62.2	15.6	10.0	3.2	0.5	-	6.5	1.0	-	0.5	0.5	-	-	-
5	24	F	Co/an	T3N3Mx	Int	-	50.7	39.0	9.6	-	-	-	-	-	0.7	-	-	-	-	-
6	71	F	An/py	T2N0Mx	Dif	-	65.0	20.0	12.0	1.0	-	2.0	-	-	-	-	-	-	-	-
7	41	F	Co/an/py	T4NxMx	Int	+	84.5	7.8	6.5	-	-	0.6	0.6	-	-	-	-	-	-	-
8	63	F	Co/an	T4N3M1	Dif	0	67.0	25.0	3.0	2.0	-	1.0	-	-	-	-	1.5	-	-	0.5
9	68	F	An	T1N1Mx	Int	+	70.0	11.5	4.5	3.0	2.0	-	4.0	1.5	1.0	0.5	2.0	-	-	-
10	76	M	An/py	T3N1M1	Int	-	68.0	14.0	2.0	2.0	6.0	-	5.0	0.5	2.5	-	-	-	-	-
11	41	F	All	T3N1M0	Dif	-	54.5	29.5	-	2.0	3.5	-	9.0	0.5	0.5	0.5	-	-	-	-
12	60	M	An	T3N2Mx	Int	0	88.0	7.0	5.0	-	-	-	-	-	-	-	-	-	-	-
13	65	M	Ca/fu	T3N1Mx	Int	0	69.0	16.0	10.5	-	0.5	2.0	1.0	0.5	-	-	-	-	0.5	-
14	52	M	Co/ca	T2N1Mx	Int	+	76.5	15.0	7.5	-	-	0.5	0.5	-	-	-	-	-	-	-
15	48	M	Co/an	T2N1Mx	Intl	+	54.0	24.0	1.5	5.0	5.5	2.5	6.0	1.5	-	-	-	-	-	-
16	52	F	An/py	T3N1Mx	Int	-	53.4	27.0	1.6	3.2	4.2	1.6	8.0	-	-	1.0	-	-	-	-
17	50	F	An/py	T4N1Mx	Int	+	63.0	19.0	4.0	2.0	0.5	1.0	7.5	-	2.0	1.0	-	-	-	-
18	47	F	An	T2N0Mx	Dif	-	65.0	17.5	9.5	1.0	2.5	1.5	1.5	1.0	-	-	0.5	-	-	-
19	74	F	Co/an/py	T4N1Mx	Dif	-	67.0	21.5	-	1.0	7.5	-	1.5	1.5	-	-	-	-	-	-
20	31	M	Co/an/py	T3N1Mx	Dif	-	62.7	24.3	2.7	5.5	1.6	-	2.7	0.5	-	-	-	-	-	-

† Tumor location: An = antrum; Ca = cardia; Co = corpus; Fu = fundus; Py = pylorus | ‡ TNM pathological staging | §Laurén's classification: Int = Intestinal; Dif = Diffuse | Immunostaining: 0 = not done; (-) without immunoreactivity; (+) with immunoreactivity.

nocarcinoma sample with known p53 immunoreactivity was used as positive control and a normal gastric mucosa as negative control. Two pathologists evaluated the immunostaining results independently.

Statistical analyses

Statistical analyses were performed using Fisher's exact test and Mann-Whitney test. P value < 0.05 was considered to be statistically significant.

Results

FISH

Lymphocyte nucleus and normal gastric mucosa showed two signals to chr17 and *TP53* in 97.5% and 96.5% of analyzed cells respectively. All cancer samples presented numerical alterations of chr17 and *TP53* gene. Normal nuclei were observed in 50.7–88% of cells/case (Table 1).

The main *TP53* alteration observed was the single allelic deletion. This alteration was present in all cases, varying to 7–39% of cells/case (Figure 1A). Chr17 monosomy observed in 45% of samples, ranging 5–12% of cells/case. Chr17 gain was also detected in 45% of cases. Chr17 trisomy was observed in 20% of the cases in a frequency up to 7.5% cells/case (case 19), in which 40% and 60% of these cases showed two and one single *TP53* copy number respectively. Chr17 tetrasomy with two *TP53* signals was detected in 35% of cases in a frequency up to 9% cells/

case. Four signals to chr17 and *TP53* was observed in one case (case 2) in 13.5% of cells (Table 1).

The frequency of cells with two chr17 and one *TP53* signals observed was higher in diffuse than in intestinal-type GC (p = 0.026). Chromosome alterations were not associated with other clinicopathological characteristics.

p53 protein expression

In the present study, breast adenocarcinoma (positive control) showed p53 overexpression and the normal gastric mucosa (negative control) showed lack of p53 immunoreactivity.

Seventeen GC samples were analyzed by immunostaining. Immunoreactivity of p53 (positive) was found in 8 cases (47%) (Figure 1B). All of these cases were intestinal-type (8 of 12 samples). Statistical analysis showed an association between intestinal-type GC and p53 expression (p = 0.0294). In our sample, p53 immunoreactivity was not associated with age, gender, location and TNM status (p > 0.05) (Table 1).

The frequency of cells with two chr17 and one *TP53* signals observed was higher in samples with immunoreactivity of p53 negative than in cases with positive p53 expression (p = 0.016). In intestinal-type GC, the frequency of cells with two chr17 and two *TP53* signals
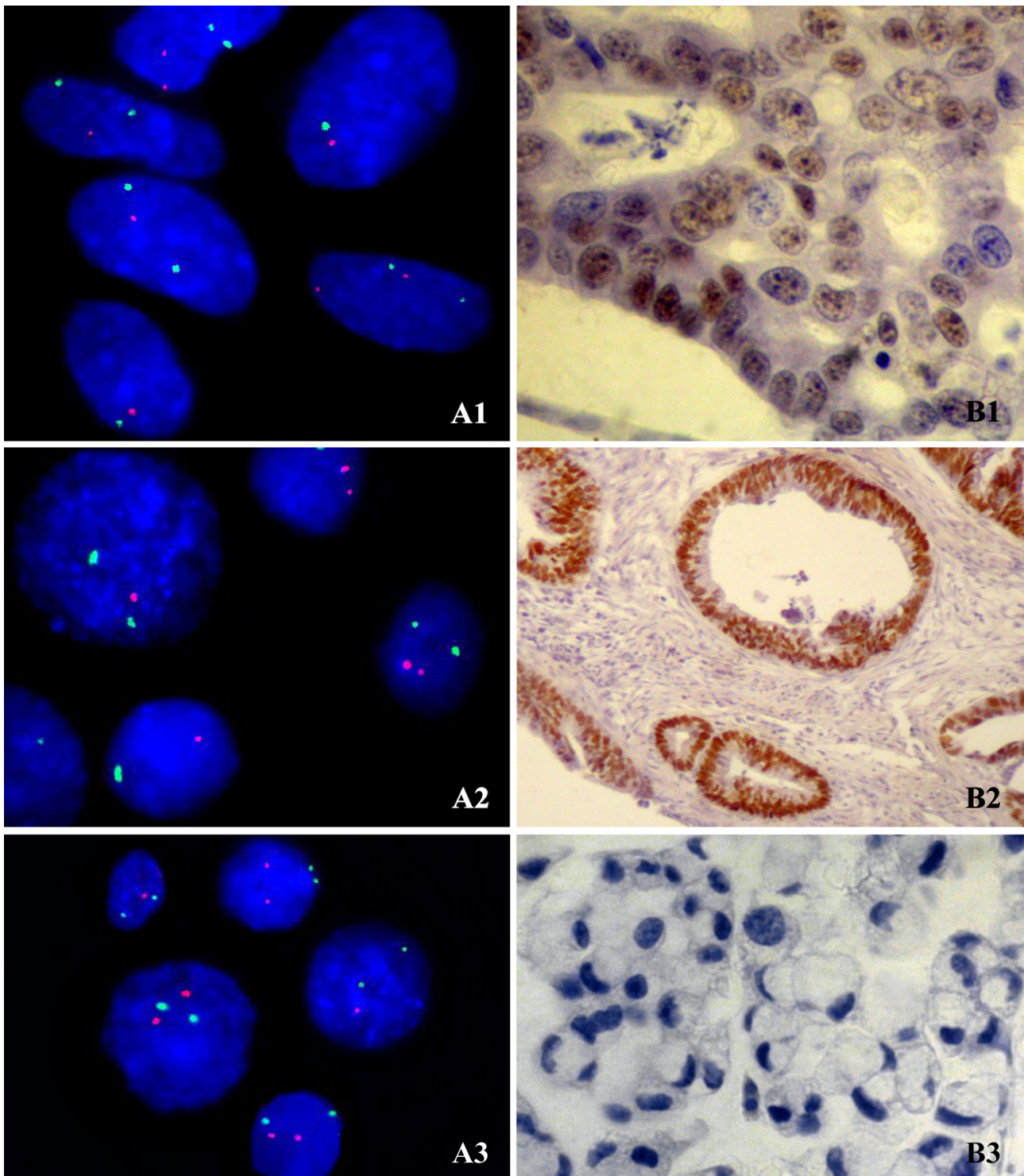
Figure 1
Cells submitted to FISH (A) and immunohistochemistry (B) assays. A1 (case 14), A2 (case 5) and A3 (case 6): interphase nuclei presenting chr17 monosomy (green signal) with one copy of *TP53* (red signal), and nuclei presenting chr17 disomy with one or two copies of *TP53* – 1000× magnification; B1 (case 14) and B2 (case 17): tissue with nuclear p53 immunoreactivity (brown stain) – 400× and 100× magnification, respectively; B3 (case 6): tissue without p53 immunoreactivity – 400× magnification.

found was higher in samples with positive p53 expression than in negative cases (p = 0.027).

Discussion

Aneusomy is one of the most common findings in human cancer. Chromosome copy number changes encompass a continuum ranging from sporadic events to a change of chromosome numbers with each cell division. Although aneusomy can be detected at early stages of transformation and even in certain premalignant lesions, the degree of numerical chromosomal aberrations generally increases with tumor progression, and tumors with aggressive clinical behavior are more likely to be aneusomy than less malignant tumors. Aneusomy has also been found to be associated with poor treatment outcome in cancer patients [34].

Regarding chr17 and *TP53* copy number, we observed normal nuclei in 50.7–88% of cells/case. This result corroborates our previous conventional and molecular cytogenetic studies, which demonstrated that chr17 aneusomy is not a frequent in GC samples of individual from Northern Brazil [7,35,36].

There are few studies in literature concerning chr17 and *TP53* copy number alterations. Our findings corroborated Kobayashi et al. [21] that found deletion of *TP53* in 39% of 67 tumors and all of these samples showed chr17 polysomy. Takahashi et al. [22] also observed that p53 signal count was lower than the chromosome 17 signal count in 1 of 3 intestinal-type GC.

Gomyo et al. [23] demonstrated 3 or 4 signals for chr17 in 46% of 13 intestinal-type GC samples and 77% of these cases showed *TP53* deletion by FISH assay. In our sample, 45% of all cases presented 3 or 4 signals for chr17 and *TP53* deletion was detected in all cases.

Suzuki et al. [24] observed an increased of chr17 polysomy frequency and the incidence of *TP53* deletion ranged from 55% to 90% in ten GC samples. They also described that *TP53* deletion was significantly higher in intestinal than in diffuse-type cancers. However, in our sample we found *TP53* deletion in up to 49% cells/case and the frequency of cells with two chr17 and one *TP53* signals observed was higher in diffuse than in intestinal-type GC. Inconsistencies regarding the frequency of *TP53* deletion in GC between our study and Suzuki et al. [24] may be suggestive of distinct gastric carcinogenesis pathways in different ethnic composition or differences in stage when the analysis was done. It is widely reported that differences between carcinogenesis processes can be the result of distinct environmental and genetic factors.

Suzuki et al. [24] also observed that chr17 monosomy was present in 70% of 10 cases and the most frequent pattern in these cases was the combination of one copy of chr17 and one of *TP53*. On the other hand, in our sample we observed chr17 monosomy in 45% of 20 cases (cut-off level of 5%) and the more frequent pattern was the combination of two copies of chr17 with one *TP53* copy by cell.

In the present study, chr17 tetrasomy with two *TP53* signals was frequently observed. We also could observe that chr17 gain tended to be more frequently found in tumors with higher extension (T3 or T4 stages). This finding suggests that tetrasomy event is a subsequent step after gene deletion, which could justify the higher frequency of cells with two copies of chr17 and one *TP53* copy and also the tendency of increased level of chr17 gain in tumors with higher extension. Galipeau et al. [37] suggested that increased polysomy level is associated with inactivation of the *TP53* in Barrett's esophagus in vivo, supporting our hypothesis.

Williams et al. [38] described that *TP53* deletion was the most common aberration in gastritis, intestinal metaplasia, dysplasia e GC by FISH assay. The author suggested that this abnormality may exist in the initiation and progression to gastric cancer.

TP53 deletion, as well as chromosome 17 aneusomy, was observed in all analyzed samples, despite Laurén's histopathologic types. However, differential p53 expression was detected between these groups.

Increased immunostaining of p53 can depend on either increased synthesis of wild-type protein or accumulation of mutated protein in the cell, since the antibody recognizes both types of the protein [39]. In the present study, we observed an increased frequency of immunostained nuclei and the greater staining intensity in 47% of GC samples, as compared to normal gastric mucosa. We suggest that the p53 overexpression may be related to the mutated type of this protein. The frequency of p53 overexpression in GC has been described varying from 19% to 57.5% of cases [23,40-44] and some studies also described *TP53* mutations related with its protein overexpression [23,41].

In the present study, only intestinal-type GC presented p53 immunoreactivity. Our research suggests that, beside *TP53* loss by allelic deletion or chr17 aneusomy, a mutation in the remaining *TP53* allele may exist in intestinal-type GC samples, which would explain the protein immunoreactivity. On the other hand, two possibilities might be considered to the absence of immunoreactivity in diffuse-type GC: this absence was not due to mutations in

TP53 gene or an eventual mutation in this gene would not interfere in the protein accumulation. In both situations the immunoreactivity cannot be detected.

The p53 expression was also associated with a higher frequency of cells with two chr17 and two TP53 signals in intestinal-type GC. We hypothesize that these cells may present TP53 with mutations and this event could be occurring earlier than allelic deletion in intestinal-type gastric carcinogenesis. Further investigations concerning *TP53* mutations and expression should be done in larger samples, also including early GC specimens.

Conclusion
Our findings showed that *TP53* deletion and chromosome 17 aneusomy are common events in GC. Our results also suggest that LOH is an important *TP53* alteration in GC. However, other *TP53* alterations than allelic deletion may be implicated in the carcinogenesis process.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
RRB and MACS designed the study. ASK, ACG, DQC, ADS, EML, MFL were involved in data collection, literature searches, genetic and statistical analysis. MHGF, SHBR, SD were involved in pathological analysis. PPA recruited patients and was responsible by samples collection. ASK wrote the first draft of the manuscript. All authors listed have contributed to all subsequent drafts, and have approved the final manuscript.

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