MYC Insertions in Diffuse-type Gastric Adenocarcinoma

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Abstract. *Background: MYC is important in gastric carcinogenesis. A few studies reported MYC translocation or insertion associated with gastric cancer. Materials and Methods: MYC copy number and its insertion, as well as the chromosomes in which MYC was inserted, were evaluated by fluorescence in situ hybridization assay in interphase and metaphase cells of 12 diffuse-type gastric cancer samples. MYC protein expression was evaluated by immunohistochemistry. Results: The presence of 3 MYC signals was the most frequent alteration. All cases also presented 4 and 5 MYC signals. In all samples, we observed chromosome 8 trisomy with MYC copies and MYC insertion into the chromosomes 2, 7, 14, 17, 18 and 22. All samples presented nucleic and cytoplasmic immunoreactivity. Conclusion: MYC cytoplasmic immunoreactivity can be the result of MYC insertion with the breakpoints within or close to the regions that are able to target the nucleus. MYC insertion and cytoplasmatic immunoreactivity may be a common characteristic of diffuse-type gastric cancer.*

Gastric cancer (GC) is still a serious public health concern and is the second most prevalent cause of cancer death worldwide (1). In Pará state, Northern Brazil, the mortality rate of GC is higher than the national average rate (2).

Over 95% of gastric malignancies are adenocarcinomas (3). They are subdivided into two main histological types: well-differentiated or intestinal-type, and undifferentiated or diffuse-type (4). There are differences in the pathways of these two types of GC, including different genetic alterations, leading to two distinct entities of GC (5).

Molecular events in the carcinogenesis of GC remain largely unknown. Conventional cytogenetic studies have shown clonal chromosomal alterations in GC. However, conventional cytogenetics studies in this neoplasia are particularly problematic due to the difficulty in preparing metaphase spread of adequate quality and the complex nature of the chromosomal abnormalities (5). To our knowledge, only 130 gastric adenocarcinoma samples were analyzed by conventional cytogenetic (6).

Molecular cytogenetic assays have proven valuable in solving some of these problems. Our research group previously reported several aneusomies, such as of chromosomes 8, 9, 17 and X, in GC using GTG-banding, fluorescence *in situ* hybridization (FISH) with centromeric probes in interphase nuclei and comparative genome hybridization (CGH) techniques (7-12). Our studies have shown that chromosome 8 aneusomy is the most frequent cytogenetic alteration in GC samples, both intestinal- and diffuse-type, of individuals from Pará State (7, 8, 10, 12-14).

We also reported some differences between intestinal- and diffuse-type tumors, especially concerning the *MYC* oncogene. *MYC*, located at 8q24, is a transcription factor involved in cell cycle regulation, differentiation, apoptosis and neoplastic transformation (15). *MYC* amplification and its overexpression are observed in 15-30% of GC (16-20).

Our research group showed that the 8q24 region is highly amplified only in the intestinal-type GC using CGH assay (9). Moreover, we confirmed that intestinal-type tumors presented gain of *MYC* copy number, including high amplification as double minutes (DM) or homogeneous staining regions (HSR), using dual-color FISH technique for chromosome 8 alpha-satellite region (8q11) and for *MYC* gene region (8q24) in interphase nuclei. On the other hand, we also observed that all diffuse-type GC and no intestinal-type GC samples showed possible rearrangements between *MYC* gene and chromosome 8 centromere that were

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Table I. *Clinicopathological characteristics and MYC signal numbers (%) by interphase and metaphase nuclei.*

Case	Age (years)	Location	pTNM	IHC	*MYC* signals, no. (%) by nuclei						
					1	2	3	4	5	≥6	HA
1	47	Antrum	T3N0M0	+	3 (1.4)	85 (41.1)	58 (28)	53 (25.6)	8 (3.9)	0	0
2	57	Antrum	T3N2M0	+	1 (0.5)	80 (39)	66 (32.2)	36 (17.6)	22 (10.7)	0	0
3	55	Antrum/body	T3N2M1	+	6 (2.9)	92 (44.8)	75 (36.4)	20 (9.7)	5 (2.4)	4 (1.9)	4 (1.9)
4	50	Cardia	T3N1M0	+	2 (1)	75 (36.6)	82 (40)	41 (20)	5 (2.4)	0	0
5	62	Antrum	T3N1M0	+	3 (1.5)	83 (40.5)	78 (38)	39 (19)	2 (1)	0	0
6	54	Antrum	T3N0M0	+	7 (3.4)	84 (40.2)	82 (39.2)	21 (10)	15 (7.2)	0	0
7	45	Antrum	T3N2M0	+	1 (0.5)	84 (40.4)	84 (40.4)	31 (14.9)	8 (3.8)	0	0
8	50	Antrum/body/fundus	T3N2M1	+	5 (2.5)	91 (43.3)	66 (31.4)	32 (15.2)	12 (5.7)	4 (1.9)	0
9	46	Antrum	T3N1M0	+	7 (3.3)	99 (48.3)	65 (31.6)	15 (7.1)	12 (5.7)	4 (2)	4 (2)
10	60	Antrum/body	T3N2M0	+	1 (0.5)	99 (48.3)	67 (32.7)	29 (14.1)	5 (2.4)	2 (1)	2 (1)
11	61	Antrum/body	T3N1M1	+	8 (3.9)	114 (55.6)	59 (28.8)	23 (11.2)	1 (0.5)	0	0
12	65	Antrum	T4N2M1	+	10 (4.8)	90 (43.5)	65 (31.4)	30 (14.5)	10 (4.8)	2 (1)	10 (4.8)
Control	77	Stomach tissue		–	4 (2)	194 (97)	2 (1)	0	0	0	0
Control	37	Lymphocytes		–	8 (4)	192 (92)	0	0	0	0	0

IHC, Immunohistochemistry; HA, High amplification.

discriminated by the evident separation of the two signals (7). In the same population, our research group observed that all GC samples, including early tumors, presented nuclear immunoreactivity for MYC protein (10, 12). However, advanced diffuse-type GC (2 samples) also showed cytoplasmic MYC immunoreactivity.

In the present study, we evaluated the *MYC* copy number and its insertion as well as its protein expression, and we identified the chromosomes in which *MYC* was inserted.

Materials and Methods

Specimens. The study included 12 diffuse-type GC samples according to Laurén's classification (21). Tumors were staged using standard criteria by TNM staging (22). All diffuse-type GC samples were of advanced stage (Table I). 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 the population is composed of interethnic crosses among three main origin groups: European (mainly represented by Portuguese), Africans and Amerindians (23). All patients were males, with a mean of 54.3±6.8 years (range 45-65). All patients had negative histories of exposure to either chemotherapy or radiotherapy before surgery and there was no case of other concurrent diagnosed cancer. Informed consent with approval of the Ethics Committee of HUJBB was obtained.

Fluorescence *in situ* hybridization. Cancer samples were processed by direct chromosomal analysis technique as described by Xiao *et al*. (24). FISH was applied to cells fixed in methanol/acetic acid using recently made slides according to modified protocols (14, 25). To determine *MYC* gene copy number, cells were hybridized with digoxigenin-labeled probe for the *MYC* gene region (TU801-SP O. 8q24.1-q24.2, Technogenetics, France). Nuclei were counterstained with DAPI/antifade. Molecular cytogenetic analysis was carried out under an Olympus BX41 fluorescence microscope with double FITC/TRICT filter (Olympus, Japan) and an Applied Spectral Imaging image analysis system (ASI Ldt., Israel). For each case, 200 interphase nuclei and 5-10 metaphases were analyzed using criteria of Hopman *et al*. (26). To evaluate the chromosome in which *MYC* was inserted, biotin-labeled α-satellite centromeric probes (Cytocell, UK) were co-hybridized with *MYC* probe in metaphase cells. *MYC* insertion was only considered as a clonal aberration when it was present in more than two metaphases (27).

To avoid misinterpretation due to technical error, gastric mucosal tissue (nonneoplastic) and lymphocyte nuclei and metaphases were used as negative control.

Immunohistochemical staining. Deparaffinized tissue sections were incubated with primary antibody to MYC (clone 9E10.3; dilution 1:100; Labvision®, USA) and secondary antibody followed by streptavidin-biotin-peroxidase complex (DakoCytomation, USA) as described elsewhere (10). Slides were visualized with diamino-benzidine-H_2O_2 and counterstained with Harry's hematoxylin. Normal gastric mucosa was used as negative control. Two pathologists evaluated the immunostaining results independently. Positive MYC expression was defined as clear staining.

Results

Lymphocyte nuclei and normal gastric mucosa showed two signals to *MYC* in 96% and 97% of analyzed cells respectively. All cancer samples presented numerical alterations of the *MYC* gene in both interphase and metaphase cells (Table I). The presence of 3 copies of the *MYC* gene per cell was the most frequent alteration observed. This alteration was present in all cases, varying from 28% (case #1) to 40.4% (case #7).

The presence of 4 (range 9.7-25.6% of cells) and 5 (range 0.5-10.7% cells) *MYC* signals were also observed in all cases

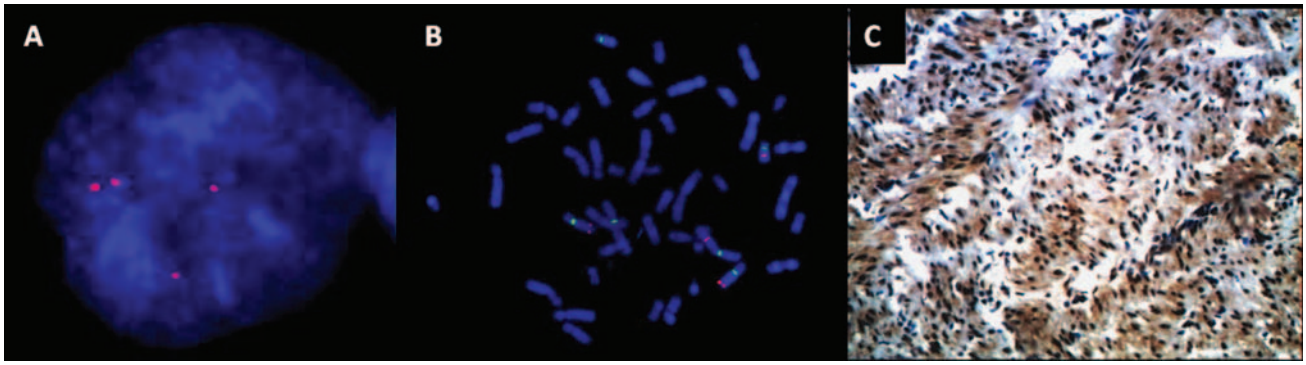

Figure 1. *Cells submitted to immunohistochemistry and FISH techniques. A, Interphase nuclei presenting 4 MYC signals; B, metaphase cell presenting MYC gene in one homolog of each chromosome 2 and 18 pairs, and in both chromosomes 8; C, diffuse-type GC with nucleic and cytoplasmatic MYC location, ×400.*

(Figure 1A). In all samples, the frequency of these alterations by case was lower than the frequency of 3 *MYC* copy numbers. The presence of 6 or more *MYC* copies and its high amplification as DM and HSR were observed in only 1-2% of cell of 5 (41.7%) and 3 (25%) cancer samples respectively.

Clonal *MYC* insertion was observed in all cases, especially into chromosome 2 (41.7% of cases), 7 (33.3%), 14 (41.7%), 17 (25%), 18 (33.3%) and 22 (25%), in which only one homolog of each chromosome pair presented this alteration (Table II) (Figure 1B). The frequency of *MYC* insertion ranged from 29% (case #12) to 100% (case #9) of metaphase cells.

In metaphase cells, chromosome 8 trisomy was observed in all cancer samples. Chromosome 8 tetrasomy was also observed in three samples (25%). The frequency of chromosome 8 aneusomy varied from 33.3% to 62.5%. All chromosome 8 presented one *MYC* gene copy (Figure 1B).

In this study, the normal gastric mucosa tissues were shown to have a lack of MYC immunoreactivity. MYC protein expression, with nucleic and cytoplasmic location, was observed in all cancer samples by immunohistochemistry (Figure 1C).

Table II. *MYC copy number by chromosome.*

Case	Chromosome							CN/MN
	2	7	8	14	17	18	22	
1	3	-	17	-	-	2	-	22/7
2	-	-	12	2	2	-	-	16/5
3	2	2	14	-	-	-	2	20/6
4	2	-	12	2	-	2	-	18/5
5	-	-	12	2	2	-	-	18/5
6	-	2	24	-	-	-	2	28/9
7	2	-	24	2	-	2	-	30/8
8	-	-	27	2	-	-	2	31/10
9	-	4	12	-	-	2	-	18/5
10	2	-	12	-	-	-	-	14/5
11	-	-	12	-	2	-	-	14/5
12	-	2	17	-	-	-	-	19/7

CN, Total *MYC* copy number; MN, analyzed metaphase number.

Discussion

Oncogenic alterations of the *MYC* gene are commonly induced by events such as point mutations, gene amplification, chromosomal translocation, viral insertion at *MYC* locus (28). In the present study, we observed *MYC* amplification in interphase and metaphase cells, corroborating our previous findings (7, 10, 12). However, for the first time, we observed high amplification of *MYC* in diffuse-type CG, but at a lower frequency than that in the intestinal-type.

In this study, we found chromosome 8 trisomy in all cases. The *MYC* copy number was higher than the chromosome 8 copy number in metaphase cells. We found 4 or more *MYC* signals in all GC samples, in up to 25.6% of nuclei (case #1), which may have been due to chromosome 8 tetrasomy or *MYC* insertion (or translocation).

MYC translocation is frequently described in Burkitt's lymphoma. A few studies have also found insertion or translocation of the *MYC* locus associated with gastric carcinogenesis. Stamouli *et al*. (29) observed that structural abnormalities predominate and normal homologs were mostly absent in a sample of poorly differentiated diffuse GC analyzed by multicolor-FISH. Yamashita *et al*. (30) reported that structural rearrangements in GC cell lines and cancerous ascitic fluids most frequently involved 8q24 breakpoint by spectral karyotyping analysis (SKY). The authors demonstrated that the *MYC* locus was involved in several types of chromosomal rearrangements including translocation, HSRs and frequent insertion, resulting in gain and amplification of

the gene in two poorly differentiated GC cell line by dual-color FISH. Takahashi *et al*. (31) also performed dual-color FISH for chromosome 8 and the *MYC* locus in metaphase cells of one poorly differentiated GC. The authors observed that 2 *MYC* signals were present on chromosome 8 and another 3 signals were detectable on other chromosomes.

Our findings confirm that *MYC* gene can be inserted into other chromosomes besides chromosome 8 in diffuse-type GC. All diffuse-type GC samples presented *MYC* insertion in up to 100% of metaphase cells. In our GC samples, we detected clonal *MYC* insertion only into chromosomes 2, 7, 14, 17, 18 and 22. *MYC* locus insertion into any of the three immunoglobulin gene loci on chromosomes 14q32, 2p11 or 22q11 is common in Burkitt's lymphoma (32). In the present study, it was not possible to analyze the breakpoints involved in *MYC* insertion.

We also observed that MYC immunoreactivity was localized in nucleus and cytoplasm of all diffuse-type GC. MYC cytoplasmatic immunoreactivity can be the result of *MYC* insertion. *MYC* insertion breakpoints can be within or close to the regions that are able to target the nucleus (M1 or M2) (33). Dang and Fee (33) demonstrated that the deletion of the M1 peptide from the *MYC* sequence results in a protein that is mostly, but not completely, cytoplasmic in distribution.

FISH assay in metaphase cells, as well as other molecular cytogenetic techniques, can help understand structural rearrangements in GC. In the present study, we reported *MYC* copy number alteration and chromosome 8 trisomy in all diffuse-type GC of individuals from Northern Brazil. We also confirm the presence of *MYC* in other chromosomes besides chromosome 8 and its cytoplasmic protein immunoreactivity as common findings in diffuse-type GC that may be a specific characteristic of this neoplasia.

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