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RAPID COMMUNICATION

Interrelationship between chromosome 8 aneuploidy, *C-MYC* amplification and increased expression in individuals from northern Brazil with gastric adenocarcinoma

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Abstract

AIM: To investigate chromosome 8 numerical aberrations, *C-MYC* oncogene alterations and its expression in gastric cancer and to correlate these findings with histopathological characteristics of gastric tumors.

METHODS: Specimens were collected surgically from seven patients with gastric adenocarcinomas. Immunostaining for C-MYC and dual-color fluorescence *in situ* hybridization (FISH) for *C-MYC* gene and chromosome 8 centromere were performed.

RESULTS: All the cases showed chromosome 8 aneuploidy and *C-MYC* amplification, in both the diffuse and intestinal histopathological types of Lauren. No significant difference ($P < 0.05$) was observed between the level of chromosome 8 ploidy and the site, stage or histological type of the adenocarcinomas. *C-MYC* high amplification, like homogeneously stained regions (HSRs) and double minutes (DMs), was observed only in the intestinal-type. Structural rearrangement of *C-MYC*, like translocation, was observed only in the diffuse type. Regarding *C-MYC* gene, a significant difference ($P < 0.05$) was observed between the two histological types. The C-MYC protein was expressed in all the studied cases. In the intestinal-type the C-MYC immunoreactivity was localized only in the nucleus and in the diffuse type in the nucleus and cytoplasm.

CONCLUSION: Distinct patterns of alterations between intestinal and diffuse types of gastric tumors support the hypothesis that these types follow different genetic pathways.

Key words: Chromosome 8 aneuploidy; *C-MYC* amplification; Immunostaining; Gastric adenocarcinoma

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INTRODUCTION

Gastric cancer is the third most frequent type of cancer in the world[1]. In Northern Brazil, the State of Pará presents a high incidence of this neoplasia type and its capital, Belém, is ranked eleventh in number of gastric cancers per inhabitant among all cities in the world with cancer records[2]. Food factors may be related to the high incidence of this neoplasia in Pará, specially high consumption of salt-conserved food, reduced use of refrigerators and little consumption of fresh fruit and

vegetables[3].

Gastrointestinal tract tumors are notorious for being difficult to analyze by standard cytogenetic techniques[4-8]. Fluorescence *in situ* hybridization (FISH) technique with specific DNA probes allows rapid detection of chromosome aberrations in tumor interphase nuclei. In FISH studies, numerical aberrations in chromosomes 1, 7, 8, 9, 17, 20, X and Y are common[9-12]. Chromosome 8 abnormalities are frequent, not only in gastric neoplasias, but also in several types of hematopoietic proliferations and solid tumors[13-15].

Among the genes found on chromosome 8, *C-MYC*, located at 8q24, has been the most studied. *C-MYC* gene is a regulator of cell cycle and plays a major role in control of cell growth, differentiation, apoptosis and neoplastic transformation[16]. *C-MYC* gene overexpression is a frequent alteration and has been described in several types of human cancer[17-19]. An increased *C-MYC* gene expression has been found in gastric neoplasias[20-23].

The aim of this study was to investigate chromosome 8 numerical aberrations, *C-MYC* oncogene alterations and its expression in gastric cancer samples from the State of Pará, using the FISH technique and immunohistochemistry. Possible correlations between these findings and histopathological characteristics were also evaluated.

MATERIALS AND METHODS

Cases studied

Seven samples of primary tumors submitted to surgical resection were obtained from male patients in Pará State João de Barros Barreto University Hospital (HUJBB). Patients' ages and tumors' anatomical sites were obtained from tumor registries (Table 1). The patients had never been submitted to chemotherapy or radiotherapy prior to surgery, nor had they any other diagnosed cancer. Genetic study of samples was approved by the Ethics Committee of HUJBB. A fraction of each sample was used for routine histopathological diagnosis according to Laurén's classification[24].

Immunostaining

For antigen retrieval, deparaffinized sections (5 μm) were pretreated by heating in a microwave oven in citrate buffer 10 mmol/L, pH 6.0 for 20 min. After cooling, sections were immersed in PBS containing 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then incubated in a humid chamber overnight at 4℃ with primary antibody C-MYC (clone 9E10.3; dilution 1:100). After rinsing with PBS, slides were incubated with secondary antibody followed by streptavidin-biotin-peroxidase complex, both for 30 min at room temperature with a PBS wash between each step. Slides were visualised with diaminobenzidine-H_2O_2 and counterstained with Harry's hematoxylin.

Fluorescence in situ hybridization

Tumor samples from all patients were processed for cytogenetic study as described previously[25]. FISH assay was performed on slides with cells fixed in methanol/acetic acid. A directly labeled dual-color probe was used for chromosome 8 alpha-satellite region (8q11) and for *C-MYC* gene region (8q24). Slides were washed in 2 × saline sodium citrate solution (SSC) and dehydrated in 70%, 80% and 95% ethanol, respectively. Samples were then denatured with 70% formamide/2 × SSC (pH 7.0) at 70℃ for 2 min and transferred to an iced ethanol (-20℃) series at 70%, 80% and 95%. Probes were denatured at 96℃ for 5 min. Then, 10 μL was applied onto the slide under a glass coverslip. *In situ* hybridization occurred at 37℃ in a moist chamber overnight. Post-hybridization washings were done, and nuclei were counterstained with DAPI/antifade. Molecular cytogenetic analysis was carried out under an Olympus BX41 fluorescence microscope with triple DAPI/FITC/TRICT filter and an Applied Spectral Imaging image analysis. For each sample, 200 interphase nuclei were analyzed. To avoid misinterpretation due to technical error, normal lymphocyte nuclei and normal gastric tissue were used as a control.

Statistical analysis

For statistical evaluation, chi-square test was used. $P < 0.05$ was taken as significant.

RESULTS

All seven samples studied were histologically classified as gastric adenocarcinomas, 2 of them were diffuse type and 5 were intestinal type, according to Laurén's classification (Table 1).

C-MYC was expressed in all cases. C-MYC immunoreactivity was localized in nucleus and cytoplasm of diffuse type samples, but only in nucleus of intestinal type (Figure 1A and 1B).

In peripheral blood lymphocytes, two signals were observed in 98.5% of analyzed nuclei for chromosome 8 probe and 99.5% for *C-MYC* gene probe. Normal stomach tissue showed two signals in 96% of analyzed nuclei for chromosome 8 probe and 98% for *C-MYC* gene probe. All gastric adenocarcinoma cases showed numerical increase of chromosome 8 and *C-MYC* gene (Table 1).

Chromosome 8 trisomy was detected in all cases, varying from 19% (case 3) to 33% (case 1), and chromosome 8 tetrasomy (observed in all cases) varied from 1% (case 4) to 18% (case 3). Five signals for chromosome 8 were observed in 6 cases (85.7%) and the highest frequency was found in case 7 (4%). Six or more signals were observed in 1 case (case 7).

Presence of 6 or more signals for *C-MYC* gene was considered as an intermediary degree of amplification, whereas the cases which presented double minutes (DMs) and/or homogeneously stained regions (HSRs) were classified as presenting a high degree of gene amplification. Cells with more than six signals were found in 5 cases (71.42%). The frequency of cells with high amplification (Figure 1C) of this gene varied from 1% (case 4 and 5) to 6% (case 7) (Table 1). Thus, all intestinal type cases and none diffuse type presented intermediary and high amplification of *C-MYC*.

Table 1 Number of signals by percentage of analyzed nuclei

Case	Age (yr)	Origin	HT	UICC	Immunostaining	Probe	Percentage of nuclei: Number of signals 1	2	3	4	5	⩾ 6	HA
1	77	Antrum	Intestinal	T2N1M0	Nuclear	*C-MYC*	0	34	23	25	14	2	2
						Chrom.8	0	58	33	9	0	0	-
2	48	Antrum	Intestinal	T4N0M0	Nuclear	*C-MYC*	0	35	10	20	29	2	4
						Chrom.8	0	70	21	6	3	0	-
3	74	Antrum	Diffuse	T1N0M0	Nuclear/Cytoplasmatic	*C-MYC*	1	38	29	30	2	0	0
						Chrom.8	2	59	19	18	2	0	-
4	74	Antrum/body	Intestinal	T4N0M0	Nuclear	*C-MYC*	0	40	12	28	17	2	1
						Chrom.8	0	63	25	1	1	0	-
5	41	Antrum	Intestinal	T2N0M0	Nuclear	*C-MYC*	0	36	18	30	13	2	1
						Chrom.8	4	61	30	4	1	0	-
6	56	Antrum/body	Diffuse	T2N1M0	Nuclear/Cytoplasmatic	*C-MYC*	0	31	38	17	14	0	0
						Chrom.8	3	60	32	5	1	0	-
7	55	Antrum	Intestinal	T4N2M1	Nuclear	*C-MYC*	2	48	27	7	7	3	6
						Chrom.8	2	54	27	5	4	8	
Control	77	Stomach tissue	-	-	Without staining	*C-MYC*	2	98	0	0	0	0	0
						Chrom.8	1	96	3	0	0	0	-
Control	37	Lymphocytes	-	-	Without staining	*C-MYC*	0.5	99.5	0	0	0	0	0
						Chrom.8	1	98.5	0.5	0	0	0	-

HT: Histological type; UICC: Union Internationale Contre le Cancer; HA: High amplification.

Rearrangements between *C-MYC* gene and chromosome 8 centromere were discriminated by evident separation of the two signals. In tumor samples, translocation was observed between *C-MYC* gene and another chromosome in case 3 (2%) and case 6 (4%), both of them of diffuse type (Figure 1D).

Analyzing the total number of signals for *C-MYC* gene and for chromosome 8 centromere, it was found that the number of signals for the gene was greater than the number of signals for chromosome 8 ploidy in all cases studied.

No significant difference ($P < 0.05$) was observed between chromosome 8 ploidy level and adenocarcinoma site, stage or histological type. Regarding *C-MYC* gene, a significant difference was observed between the two histological types. This difference was due to the presence of HSRs and/or DMs in Laurén's intestinal type, where multiple (uncountable) signals were found per cell.

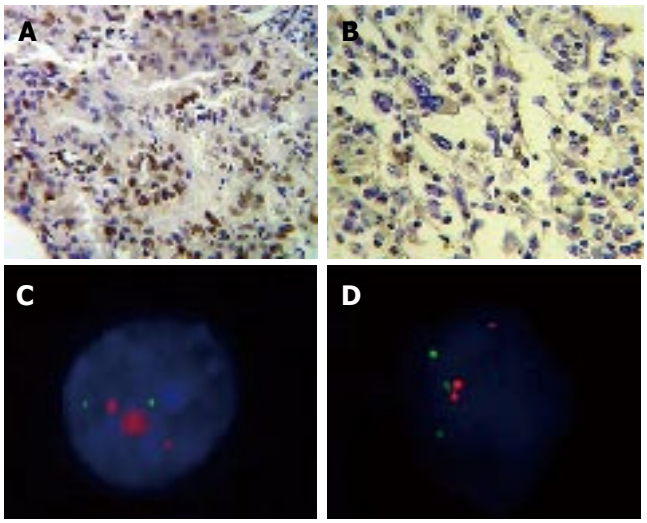

Figure 1 Cells submitted to immunohistochemistry and FISH techniques. **A**: Infiltrating gastric adenocarcinoma of intestinal type shows intense nuclear marcation for C-MYC, × 400; **B**: Gastric adenocarcinoma of diffuse type nuclear marcation and cytoplasmatic light marcation for C-MYC, × 400; **C**: Interphase nuclei presenting *C-MYC* high amplification (red) and chromosome 8 (green); **D**: Interphase nuclei presenting chromosome 8/*C-MYC* rearrangement.

DISCUSSION

Gastric cancer is one of the most common neoplasias and both environmental and genetic factors contribute to its occurrence[26]. The present study used interphase dual-color FISH with direct fluorescent labeling for the chromosome 8 centromere/*C-MYC* gene and compared the copy number observed in 7 gastric adenocarcinoma samples with C-MYC expression by immunohistochemistry.

C-MYC amplification has been reported in a small percent of gastric carcinomas[21,27,28]. *C-MYC* expression was shown to be more frequent in diffuse than in intestinal type gastric cancer cells, and more frequent in gastric adenocarcinoma than in adenoma[29,30]. In the present study, C-MYC was expressed in all cases. C-MYC immunoreactivity was localized in the nucleus in intestinal-type and in cytoplasm and nucleus in diffuse type. More samples need to be investigated, in order to clarify if this immunostaining differences exist.

Our laboratory has previously observed the presence of chromosome 8 trisomy in all 16 cases studied by direct chromosome analysis and centromeric FISH[31] and in 60% of analyzed cells of ACP01 gastric adenocarcinoma cell line[32]. The gain of signals for the two probes analyzed in all samples in the present study corroborates with these data.

Panani *et al*[9] analyzed 33 gastric tumor samples by FISH, using a chromosome 8 alpha-satellite probe. Numerical aberrations in this chromosome were observed in 62.16% of studied samples, in which trisomy was detected in 43.24%, tetrasomy in 10.81% and monosomy

in 8.10%. Our results confirmed that chromosome 8 trisomy is a common biological phenomenon in adenocarcinoma of stomach and can be used as a gastric mucosa malignancy marker. In our study, 100% of samples presented a gain of chromosome 8 as a clonal alteration.

Chromosome 8 numerical abnormalities, in which *C-MYC* is located, are suggested to be an important mechanism in *C-MYC* copy number increase. Xia *et al*[5] suggested that chromosome 8 trisomy, associated or not with other chromosomal aberrations, could occur even in less advanced stages of the disease, possibly prior to the occurrence of metastases.

Kitayama *et al*[12] analyzed interphase nuclei of 51 gastric cancer cases from pathology archives using 18 centromeric probes, including chromosome 8 probe, and a probe for *C-MYC* gene. They observed chromosome 8 numerical abnormalities in 56.9% of samples, which placed them among the most frequent alterations; in 12 cases, a *C-MYC* gain was observed and all of them presented chromosome 8 gain. Amplification of this oncogene was also reported in other FISH studies in gastric neoplasias[33-35].

C-MYC oncogene seems to be fundamental in the oncogenesis process. Thus, increased *C-MYC* allele number is directly related to the degree of tumor aggressiveness, considering that the greater the gene copy number, the higher its level of expression. This gene was amplified in all samples studied, but without numerically accompanying chromosome 8 ploidy; that is, in all cases there were more *C-MYC* gene alleles than this chromosome copies.

In another study conducted in our laboratory[36], 90.9% of cases presented intermediary amplification, 60% of which presented DMs and HSRs. It seems that, as our sample presented chromosome 8 gain as a clonal characteristic, *C-MYC* amplification is a later step, a consequence of carcinogenesis clonal expansion.

We have previously demonstrated, by comparative genomic hybridization, gains at region 8q24.1 (38.1%), which were found exclusively, in all intestinal type adenocarcinomas with systemic metastasis (M1). Results from this work also showed the highest amplification level in gastric cancer of intestinal type. *C-MYC* locus amplification may be an aggressiveness predictor in intestinal type gastric cancer, playing an important role in its development and progression. These results should provide useful information for developing more effective strategies in management of gastric cancer[37].

Stamouli *et al*[25] studied, by multicolor FISH, two primary gastric adenocarcinoma cases: a well-differentiated intestinal-type and a poorly differentiated diffuse type. The intestinal-type exhibited few structural abnormalities, in contrast to the diffuse type. In our analysis, all diffuse type cases presented at least four cells with translocation. It seems that this histological type is more susceptible to chromosomal rearrangements than intestinal type.

Correa[38] suggested that the intestinal type fits the multiple-step process. Thus, it is plausible that the intestinal type presents a greater number of DMs and/or HSRs than the diffuse type. Our findings support that these two histological types follow different genetic tumorigenesis mechanisms[39]. Moreover, it could be observed that translocations were restricted to diffuse type. This result can be explained by the fact that gene amplification is not necessarily associated or required for its overexpression. Leukemias, lymphomas and sarcomas commonly elicit specific balanced translocations which mediate proto-oncogenes activation, by their juxtaposition with promoter sequences or generating gene fusion[40].

Enhanced C-MYC protein expression contributes to almost every aspect of tumor cell biology. Although the ability of C-MYC to drive unrestricted cell proliferation and to inhibit cell differentiation had been well recognized, a recent work showed that deregulated C-MYC expression can drive cell growth and vasculogenesis, reduce cell adhesion, and promote metastasis and genomic instability. On the other hand, C-MYC loss not only inhibits cell proliferation and cell growth, but can also accelerate differentiation, increase cell adhesion and lead to an excessive response to DNA damage. Studies in animal models suggest that C-MYC may be a target for human cancer treatment, but it is still unknown whether such drugs will be useful[41].

The alterations found in this study have been described in the literature, even though their frequency was higher in our sample. Considering that external factors, such as eating habits and other environmental agents, have a direct influence on the development of this neoplasia, many genetic alterations may be regional characteristics of a given population.

Based on our findings we could also affirm that gastric adenocarcinomas of differing histopathological features are associated with distinct patterns of genetic alterations, suggesting that they evolve through different genetic pathways.

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