ANTICANCER RESEARCH *25*: 4069-4074 (2005)

Aneuploidy of Chromosome 8 and *C-MYC* Amplification in Individuals from Northern Brazil with Gastric Adenocarcinoma

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Abstract. *Background: Gastric cancer is the third most frequent type of neoplasia. In northern Brazil, the State of Para has a high incidence of this type of neoplasia. Limited data are available so far on the genetic events involved in this disease. Materials and Methods: Dual-color fluorescence in situ hybridization (FISH) for the C-MYC gene and chromosome 8 centromere was performed in 11 gastric adenocarcinomas. Results: All cases showed aneuploidy of chromosome 8 and C-MYC amplification, in both the diffuse and the intestinal histopathological types of Laurén. No correlation was found between polysomy 8 and the histopathological characteristics of the tumors. C-MYC amplification, like homogeneously-stained regions (HSRs) and double minutes (DMs), was observed only in the intestinal-type. Translocation of C-MYC was observed only in the diffuse-type. Conclusion: Chromosome 8 can be used as a marker in the diagnosis of gastric adenocarcinoma. The C-MYC oncogene requires further studies in order to verify if it is, when amplified, an etiological cause of transformation or a consequence of the proliferation process.*

Gastric cancer is the third most frequent type of cancer in the world (1). With regard to mortality, it represents the second most important cause of death in the world (2). In northern Brazil, the State of Para presents a high incidence of this type of neoplasia, and its capital, Belém, was 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 Para, especially the high consumption of salt-conserved food, the limited use of refrigerators and the low consumption of fresh fruit and vegetables (3).

Although gastric tumors are frequent neoplasias, papers on their cytogenetics are scarce in the literature (4). It is, therefore, necessary to conduct new studies aimed at identifying the peculiar genetic characteristics of a tumor, which might help in the diagnosis and prognosis of this disease, as well as to more accurately establish the therapeutic conduct.

Several chromosome alterations have been reported in gastric cancer, involving various chromosomes, such as chromosomes 3, 5, 6, 8, 12, 13 and 17 (5-7). Chromosome 8 abnormalities are frequent, not only in gastric neoplasias, but also in several types of hematopoietic proliferations and solid tumors (8-10).

Among the genes found on chromosome 8, *C-MYC*, located at 8q24, is the most studied one. The *C-MYC* gene is a regulator of the cell cycle and plays a major role in the control of cell growth, differentiation, apoptosis and neoplastic transformation (11). Overexpression of the *C-MYC* gene is a frequent alteration in human cancer and has been described in several types of the disease (12-14). An increased level of expression of the *C-MYC* gene has been found in gastric neoplasias (15-17). Nevertheless, there are few studies relating the ploidy of chromosome 8 with the number of alleles and/or level of amplification of the *C-MYC* gene in stomach cancer.

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Key Words: Fluorescence *in situ* hybridization, chromosome 8 aneuploidy, *C-MYC* amplification, gastric adenocarcinoma.

Table I. *Number of signals by percentage of analyzed nuclei.*

						Percentage of nuclei Number of signals					
Case	Age	Origin	Histopathological type (HT)	UICC[1]	Probe	1	2	3	4	5	≥6
1	47	Anthrum	Diffuse	T3N0M0	*C-MYC*	0	17	31	40	2	10
					Chrom.8	2	61	31	2	2	2
2	59	Anthrum	Intestinal	T4N1M0	*C-MYC*	0	33	5	25	31	7.5
					Chrom.8	2	61	31	3	2	1
3	57	Anthrum	Diffuse	T3N2M0	*C-MYC*	0	24	7	43	6	20
					Chrom.8	0	58	36	5	1	0
4	71	Anthrum/body	Intestinal	T3N2M1	*C-MYC*	0	20	15	57	2	9.5
					Chrom.8	0	53	47	0	0	0
5	47	Anthrum	Intestinal	T3N2M0	*C-MYC*	0	45	39	10	4	4
					Chrom.8	1	63	35	1	0	0
6	51	Body	Intestinal	T3N2M0	*C-MYC*	0	45	8	32	15	0.5
					Chrom.8	2	63	29	4	2	0
7	55	Anthrum/body/fundus	Diffuse	T3N2M1	*C-MYC*	0	42	25	13	20	0
					Chrom.8	2	65	31.5	1.5	0	0
8	50	Cardia	Diffuse	T3N1M0	*C-MYC*	0	28	30	35	4	3
					Chrom.8	2	64	5	25	3	1
9	41	Anthrum	Intestinal	T3N2M1	*C-MYC*	0	36	15	31	15	5
					Chrom.8	4	61	30	4	1	0
10	62	Anthrum	Diffuse	T3N1M0	*C-MYC*	0	30	28	10	15	17
					Chrom.8	4	63	28	4	1	0
11	77	Anthrum	Intestinal	T2N1M0	*C-MYC*	2	55	27	6	6	5.5
					Chrom.8	2	57	27	5	5.5	3.5
Control	37	Lymphocytes	-	-	*C-MYC*	0.5	99.5	0	0	0	0
					Chrom.8	1	98.5	0.5	0	0	0

[1]Union Internationale Contre le Cancer

The fluorescence *in situ* hybridization (FISH) technique, a useful method in the analysis of numerical chromosome aberrations, also allows, when used with a probe labeled for specific genes, for the detection of genetic rearrangements and the increase of copy numbers, which can lead to the activation of oncogenes. This methodology, applied to cells in interphase, is very useful in the study of gastric cancer, as it does not require high-quality chromosome preparations which are difficult to obtain, and also avoids *in vitro* selection problems, thus revealing *in vivo* alterations.

The objective of this study was to investigate the existence of numerical alterations of chromosome 8 and of amplification and/or translocation of the oncogene *C-MYC* in gastric cancer samples from the State of Para, using the FISH technique. Possible correlations between these findings and histopathological characteristics, representing prognostic parameters of this type of malignancy, were also evaluated.

Materials and Methods

Eleven samples of primary tumors, submitted to surgical resection, were obtained from male African-Brazilian patients at the Para State Joao de Barros Barreto University Hospital (HUJBB) Brazil. The ethnicity, sex and age of the patients and the anatomical sites of the tumors were obtained from tumor registries. The patients had never been submitted to chemotherapy or radiotherapy prior to surgery, nor had they any other diagnosed cancer. The genetic study of the samples was approved by the Ethics Committee of HUJBB. A part of each sample was used for routine histopathological diagnosis according to Laurén's classification (18).

The tumor samples were processed for the cytogenetic study as described in other papers (19). The FISH method was performed on slides with cells fixed in methanol/acetic acid from all patients. A directly labeled dual-color Qbiogene probe was used for the alpha-satellite region of chromosome 8 (8q11) and for the region of the *C-MYC* gene (8q24). The slides were washed in 2x saline sodium citrate solution (SSC) and dehydrated in 70%, 80% and 95% ethanol. The samples were then denatured with 70% formamide/2x SSC (pH 7.0) at 70˚C for 2 min and transferred to an iced ethanol (–20˚C) series at 70%, 80% and 95%. The probe was denatured at 96˚C for 5 min. Then, 10μL were applied to the slide under a glass coverslip. *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. The molecular cytogenetic analysis was carried out under a ZEISS AXIOPHOT fluorescence microscope with a triple DAPI/FITC/TRICT filter and an ISIS capture and image analysis system. For each sample, 200 interphase nuclei were analyzed.

Table II. *Number of cells with amplification alterations and/or a structural rearrangement for oncogene C-MYC.*

		Number of amplified and/or translocated *C-MYC* probe signals					
Case/HT	Alteration	1	2	3	4	5	≥6
1 (D)	Rearrangement	-	3 cells	1 cell	1 cell	-	-
	HSR	-	-	-	-	-	-
	DM	-	-	-	-	-	-
2 (I)	Rearrangement	-	-	-	-	-	-
	HSR	-	-	-	-	-	1 cell
	DM	-	-	-	-	-	4 cells
3 (D)	Rearrangement	-	-	3 cells	1 cell	-	-
	HSR	-	-	-	-	-	-
	DM	-	-	-	-	-	-
4 (I)	Rearrangement	-	-	-	-	-	-
	HSR	-	-	-	-	-	1 cell
	DM	-	-	-	-	-	6 cells
5 (I)	Rearrangement	-	-	-	-	-	-
	HSR	-	-	-	-	-	2 cells
	DM	-	-	-	-	-	2 cells
6 (I)	Rearrangement	-	-	-	-	-	-
	HSR	-	-	-	-	-	1 cell
	DM	-	-	-	-	-	-
7 (D)	Rearrangement	-	4 cells	-	1 cell	1 cell	-
	HSR	-	-	-	-	-	-
	DM	-	-	-	-	-	-
8 (D)	Rearrangement	-	-	2 cells	1 cell	-	-
	HSR	-	-	-	-	-	-
	DM	-	-	-	-	-	-
9 (I)	Rearrangement	-	-	-	-	-	-
	HSR	-	-	-	-	-	2 cells
	DM	-	-	-	-	-	2 cells
10 (D)	Rearrangement	-	-	2 cells	-	-	-
	HSR	-	-	-	-	-	-
	DM	-	-	-	-	-	-
11 (I)	Rearrangement	-	-	-	-	-	-
	HSR	-	-	-	-	-	2 cells
	DM	-	-	-	-	-	1 cell

HT=histological type; I=intestinal-type; D=diffuse type; DM=double minute; HSR=homogeneously-stained region.

To avoid misinterpretation due to technical error, normal lymphocyte nuclei were used as a control. For statistical evaluation, the Chi-square test was used.

Results

All 11 samples studied were histologically classified as gastric adenocarcinomas, 5 of them of the diffuse type and 6 of the intestinal type, according to Laurén (Table I).

In peripheral blood lymphocytes, 98.5% of the analyzed nuclei had two signals for the chromosome 8 probe, and 99.5% had two signals for the *C-MYC* gene. All cases of gastric adenocarcinoma studied showed chromosome 8 aneuploidies and amplification of *C-MYC* (Table I).

Most of the chromosome 8 alterations involved a numerical increase of this chromosome. The presence of chromosome 8 trisomy was detected in all cases, varying from 5% (case #8) to 47% (case #3), and the presence of chromosome 8 tetrasomy (observed in 90.9% of the cases) varied from 0% (case #4) to 25% (case #8). The presence of five signals for chromosome 8 was observed in 8 cases (72.7%), the highest frequency having been found in case #11 (5.5%). The presence of 6 or more signals was observed in 4 cases, with a frequency of up to 3.5% in case #11.

Analysis of the numerical difference between the total number of signals for the *C-MYC* gene and the number of signals for the centromere of chromosome 8, revealed that, in all cases studied, the number of signals for the gene was greater than the number of signals for chromosome 8 ploidy.

Cells with more than 6 alleles of the *C-MYC* gene were found in 10 cases (90.9%). Cells with amplification of the

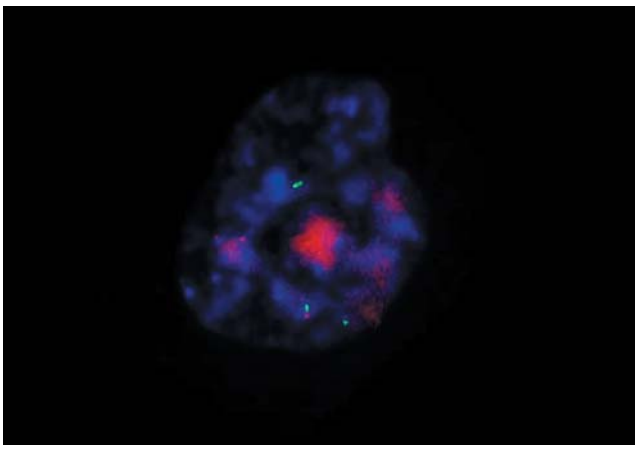

Figure 1. *Interphase nuclei presenting DMs and chromosome 8 trisomy.*

gene in the form of double minutes (DMs) and of homogeneously-stained regions (HSRs) were included in this group (Table II). Up to 6 cells with DMs (case #4) and up to 2 cells with HSRs (cases #5, 9, and 11) were observed. An amplification of *C-MYC* as HSR and DMs was seen only in the histological intestinal-type (Figure 1).

Rearrangements between the *C-MYC* gene and the chromosome 8 centromere were discriminated by the evident separation of the 2 signals. In the tumor samples studied, the occurrence of a translocation between the *C-MYC* gene and another chromosome was observed in 45.5% of the cases, all of them of the histological diffuse-type.

No statistically significant difference ($p<0.05$) was observed between the level of chromosome 8 ploidy and the site, stage or histological type of the adenocarcinomas studied ($p=0.35$). Regarding the *C-MYC* gene, a statistically significant difference was observed between the two histological types. This difference was due to the presence of HSRs and DMs in Laurén's intestinal-type, where multiple (uncountable) signals were found per cell.

Discussion

In solid tumors, the detection of clonal genetic alterations by conventional cytogenetics was limited by the complexity of these alterations and by the difficulties in preparing adequate metaphases. Molecular cytogenetic techniques have proven useful in solving some of these problems (20).

The present study used interphase dual-color FISH with direct fluorescent labeling for the chromosome 8 centromere and for the *C-MYC* gene and compared the number of copies observed in 11 gastric adenocarcinoma samples. In all samples studied, a gain of signals, both for chromosome 8 and for the *C-MYC* gene, was observed.

Chromosome aberrations involving chromosome 8 have been described in several types of solid tumors, mainly in gastric cancer (6, 7, 21-31). The findings of the present study regarding the presence of chromosome 8 trisomy as a chromosome alteration that is present in all samples corroborates previous data obtained by our research team in gastric adenocarcinoma samples, where this trisomy was found in all 10 cases studied by direct chromosome analysis (32). In another study conducted by our team (33), chromosome 8 trisomy was found in 60% of the analyzed cells of an ACP01 gastric adenocarcinoma cell line.

In a more recent study, Panani *et al*. (20) analyzed 33 gastric tumor samples by FISH, using a chromosome 8 alpha-satellite probe. Numerical aberrations involving chromosome 8 were observed in 62.16% of the studied samples, where trisomy was detected in 43.24%, tetrasomy in 10.81% and monosomy in 8.10%. Our results confirm that trisomy of chromosome 8 is a common biological phenomenon in adenocarcinoma of the stomach and can be used as a gastric mucosa malignancy marker. In our study, 100% of the samples presented a gain of chromosome 8 as a clonal alteration. In case #8, tetrasomy was more frequent than trisomy of this chromosome, and this was the only case where the tumor was located in the cardia region of the stomach. A larger sample number appears to be necessary to verify this percentage, but, if this finding is confirmed, it may represent either a regional characteristic or a cytogenetic subgroup of this neoplasia.

Numerical abnormalities of chromosome 8, on which *C-MYC* is located, are suggested to be an important mechanism in the increase of the *C-MYC* copy number. Xia *et al*. (7) suggested, after comparing their results with those of the literature, that trisomy of chromosome 8, associated or not to other chromosomal aberrations, could occur in early stages of the disease, possibly prior to the occurrence of metastases.

Kitayama *et al*. (34) analyzed interphase nuclei of 51 cases of gastric cancer from pathology archives using 18 centromeric probes – including the chromosome 8 probe – and a probe for the *C-MYC* gene. They observed numerical abnormalities of chromosome 8 in 56.9% of the samples, which places them among the most frequent alterations; in 12 cases, a gain of *C-MYC* was observed, and all of them presented gain of chromosome 8. Amplification of this oncogene was also reported in other FISH studies in gastric neoplasias (35-37).

The *C-MYC* oncogene seems to be fundamental in the carcinogenesis process. Thus, the increase of the number of alleles of the proto-oncogene *C-MYC* is directly related to the degree of aggressiveness of the tumor, considering that the more copies of it there are, the higher its level of expression. This gene was amplified in all the samples studied by us, but without numerically accompanying the ploidy of chromosome 8 to which it maps, that is, in all cases there were more alleles of the *C-MYC* gene than copies of this chromosome.

In this study, the presence of 6 or more alleles of the *C-MYC* gene was considered as an intermediary degree of amplification, whereas the cases which presented DMs and HSRs were classified as presenting a high degree of gene amplification. Thus, 10 cases (90.9%) presented intermediary amplification, 6 of which (60%) presented DMs and HSRs (high degree of amplification). To our knowledge, there are no reports in the literature regarding this evidence in primary tumors of the stomach, and only one describing such a finding in a gastric carcinoma transplanted to a mouse line (38). It seems likely that, as our sample presented the chromosome 8 gain as a clonal characteristic, the amplification of the *C-MYC* gene is a late step, a consequence of the clonal expansion of carcinogenesis in general.

According to the criteria of *Union Internationale Contre le Cancer* (39), the tumors studied are classified as advanced. Our findings reinforce the hypothesis brought forward by Han *et al*. (40), who suggested that there is a relationship between *C-MYC* expression and the aggressive phenotype, based on which patients with a high *C-MYC* expression would have a worse prognosis.

It is plausible that the intestinal-type presents a greater number of DMs and HSRs than the diffuse-type, because the intestinal-type better fits Correa's multiple-step process (41), and these aberrations are the result of clonal expansion. However, it should be taken into account that this sequential model has been questioned lately. Our findings reinforce the idea that the two histological types follow different genetic tumorigenesis mechanisms. Moreover, it could be observed that translocations, even if not very frequent, were restricted to the histological diffuse-type.

Stamouli *et al*. (19) studied two primary gastric adenocarcinoma cases, one of which a well-differentiated intestinal-type and the other a poorly-differentiated diffuse-type, using the multicolor FISH (M-FISH) technique. The intestinal-type exhibited few structural abnormalities, in contrast to the diffuse type. In our analysis, all diffuse-type cases were found to present at least two cells with a translocation. It seems likely that this histological type is more susceptible to chromosomal rearrangements than the intestinal-type, and this finding supports the hypothesis that the intestinal- and the diffuse-types follow different genetic pathways (42).

The alterations found in this study have been previously described in the literature, but the frequencies were higher in our sample. Given the fact 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.

Thus, chromosome 8 can be used as a chromosomal marker of the establishment of gastric adenocarcinoma. However, the *C-MYC* oncogene needs to be better investigated in other stages of gastric neoplasias, in order to clarify if it truly is, when amplified in a greater number of copies of chromosome 8, an etiologic cause of malignant transformation or a consequence of the proliferation process.

Acknowledgements

This work was supported by Financiadora de Estudos e Projetos (FINEP CT-INFRA/FADESP) grant No. 1017-01 and Fundaçao de Amparo à Pesquisa do Estado de Sao Paulo (FAPESP) grant 2003/06540-5; RRB had a post-doctorate fellowship (number 151127/2002-6) granted by Conselho Nacional de Desenvolvimento Cientıfico e Tecnologico (CNPq), Brazil.

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Received May 23, 2005
Accepted September 2, 2005