

## Detection of microsatellite instability but not truncating *APC* mutations in gastric adenocarcinomas in Brazilian patients

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### Abstract

A crucial role for the adenomatous polyposis colonic (*APC*) gene in colorectal carcinogenesis has been conclusively established, but, the role of *APC* in gastric tumors remains controversial. *APC* mutations have been detected at a relatively high frequency in gastric tumors of Japanese patients, yet such mutations have been reported to be extremely rare in British patients and patients from north-central-Italy. We here report the analysis of 40 primary sporadic gastric adenocarcinomas and 35 primary sporadic colon adenocarcinomas (from patients resident in São Paulo, Brazil), for mutations in the *APC* gene between codons 686 and 1693 using the protein truncation test. Although 19 truncating mutations were detected in 35 colon adenocarcinomas (54.2%) none were found in any of the gastric adenocarcinomas. As an internal control the tumor samples were also evaluated for microsatellite alterations, which are also common features of both tumor types. Microsatellite instability was present in 1 colon and 7 gastric tumor samples. This suggests that in relation to *APC* mutations gastric adenocarcinomas from Brazilian patients are similar to those that occur in Europe, and support a fundamental difference both between gastric carcinomas that occur in different geographical regions and between the molecular etiology of gastric and colorectal adenocarcinomas occurring in São Paulo, Brazil.

### INTRODUCTION

Gastric cancer is one of the most frequent cancers worldwide, especially in Japan, China, South America, Eastern Europe and north-central Italy. There are more than 20,000 new cancer cases per year in Brazil, with a 5-year survival rate < 10% (INCA, 1998). Although environmental agents such as tobacco, alcohol, highly salted food and *H. pylori* infection have been identified as risk factors in the development of gastric cancer (Fuchs and Mayer, 1995), the molecular mechanisms of gastric carcinogenesis remain relatively unexplored.

A number of genetic abnormalities have been identified in gastric cancers, including loss of heterozygosity (LOH) at various loci, mutations in protooncogenes and tumor suppressor genes, in addition to abnormal expression of growth factors, hormones and cytokines (Tahara, 1993). However, all these abnormalities individually exhibit frequencies of less than 50% in gastric tumors, and are very variable depending on the number and origin of the samples analyzed. The relevance and role of each of these changes in gastric tumorigenesis remain to be determined, but a more precise and detailed genetic model of tumor progression has been established for colorectal cancer (Vogelstein and Kinzler, 1993). Since gastric and colon tumors share some common genetic lesions (Uchino *et al.*, 1993) it is germane to inquire to what extent the two major tumor types of the gastrointestinal tract may be treated as molecularly related tumors, thus permitting a more rapid advance in our

understanding of the relatively less studied gastric tumors.

Frequent and fundamental lesions in colorectal tumors are mutations in the adenomatous polyposis coli gene (*APC*), which was first isolated in 1991 and demonstrated to be mutated both in the germline of patients with inherited familial adenomatous polyposis (FAP) as well as in approximately 50% of sporadic forms of colorectal tumors (Grodin *et al.*, 1991, Kinzler *et al.*, 1991; Miyoshi *et al.*, 1992a; Powel *et al.*, 1992; Uchino *et al.*, 1993). The majority of *APC* mutations identified to date are truncating in nature (Horii *et al.*, 1992; Miyoshi *et al.*, 1992a,b; Nakatsuru *et al.*, 1992). In FAP patients the mutations are distributed evenly along the 15 exons of the gene, while in the sporadic cases the mutations are highly concentrated between codons 1280 and 1500 encoded in exon 15 (Miyaki *et al.*, 1994).

Several somatic truncating and missense mutations of the *APC* gene have been described in gastric cancers and adenomas from Japanese patients (Horii *et al.*, 1992, Nakatsuru *et al.*, 1992, 1993; Tamura *et al.*, 1994), but other investigators have not been able to detect mutations in the *APC* gene in similar studies of gastric cancer in other geographical regions (Ogasawara *et al.*, 1994; Maesawa *et al.*, 1995). Thus, further studies of *APC* gene mutations in gastric cancer are required, especially in non-Japanese patients, in an attempt to determine their relevance to gastric tumor development. In the present study we report the results of the analysis of gastric tumors from Brazilian patients for truncating mutations in the *APC* gene.

Microsatellite instability (MSI) is a characteristic feature of tumors from patients affected with hereditary nonpolyposis colorectal carcinoma (Aaltonen *et al.*, 1993; Liu *et al.*, 1996). These alterations have also been found in a variety of sporadic tumors (Eshleman and Markowitz, 1995) including gastric carcinomas, where it has been reported at frequencies of between 15% (Strickler *et al.*, 1994) to 33% (Chong *et al.*, 1994) in tumors from diverse geographic areas. In addition, we have used MSI detection as a control of the quality of gastric tumor samples in terms of their capacity to permit detection of clonal molecular alterations.

## MATERIAL AND METHODS

### Samples and DNA extraction

Forty primary gastric tumors and thirty-five primary colon tumors together with corresponding normal mucosa were obtained from the Hospital do Câncer, São Paulo. The samples were immediately frozen in liquid nitrogen after surgical resection and stored at -70°C until use. DNA was extracted by a phenol chloroform procedure. Hematoxylin- and eosin-stained sections were used to categorize the tumors according to the classifications of Lauren.

Gastric adenocarcinoma specimens were obtained from patients admitted to the Hospital do Câncer, São Paulo, Brazil. These tumors included all TNM stages, although 92.5% were classified as stage II or higher, and 7.5% classified as stages Ia and Ib. Most tumors had a distal location (body and antrum) but 13/40 (32.5%) were of the intestinal type and 27/40 (67.5%) of the diffuse type. The five-year survival rate of these patients is 32.5%. The colorectal tumors were all adenocarcinomas, also obtained from patients admitted to the Hospital do Câncer.

### Protein truncating test for the *APC* gene

Codons 686-1693 of the *APC* gene were analyzed for mutations using the *in vitro* synthesis protein (IVSP) assay exactly as previously described (Powell *et al.*, 1992; Van der Luijt *et al.*, 1994). In brief, two overlapping fragments of exon 15 of the *APC* gene, one encompassing codons 686 to 1217, and the other, codons 1099 to 1693, were amplified from the gastric and colorectal tumor samples using the polymerase chain reaction (PCR). The PCR reactions were undertaken in a 20- $\mu$ l reaction volume containing: 50 ng of genomic DNA, 20 pmol of each primer (for codons 686 to 1217: 5'-GCTAATACGACTCACTATAGGAACAG ACCACCATGGTGGCTGCAGCTTCA-3' and 5'-GCAGAGTGTTGTGCTACTAAGA-3', and for codons 1099 to 1693: 5'-GATCCTAATACGACTCACTATAGGGAGACCACC ATGGTTTCTCCATACAGGTCACGG-3' and 5'-GGAGGAT CCTGTAGGAATCCTATCTCG-3'), 200  $\mu$ M of each dNTP, PCR buffer containing 1.5 mM MgCl<sub>2</sub> and 1 U of taq polymerase. The amplification program consisted of 5 min at

95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 58°C and 4 min at 72°C, and a final extension for 7 min at 72°C. Successful amplification was demonstrated by electrophoretic analysis of the PCR products on 8% polyacrylamide gels and silver staining (Sanguinetti *et al.*, 1994). The PCR products were used directly as templates for the IVSP assay, which consisted of a coupled *in vitro* transcription and translation reaction capable of detecting translation-terminating mutations. The reaction was performed using the TnT kit (Promega) together with 40  $\mu$ Ci of [<sup>35</sup>S]-methionine (ICN Biochemicals). In this process synthesis is directed from the nucleotides included at the 5' end of each forward amplification primer which contains a T7-promoter and an eukaryotic translation initiation sequence. Samples were diluted in sample buffer and denatured before loading onto SDS-PAGE gels. Proteins were visualized by autoradiography.

### Microsatellite analysis

Microsatellite instability was detected using oligonucleotide primer pairs for microsatellite regions containing dinucleotide repeats (D2S119, D2S123, D2S134, D2S136, D2S147, D2S177, D10S89, D12S43, D13S71 and D17S250). PCR was carried out in the presence of 0.1  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]-dCTP. The amplification program consisted of 35 cycles comprising 1 min at 95°, 1 min at 56° and 1 min at 72°C. The PCR products were electrophoresed on denaturing 6% polyacrylamide DNA sequencing gels and visualized by autoradiography.

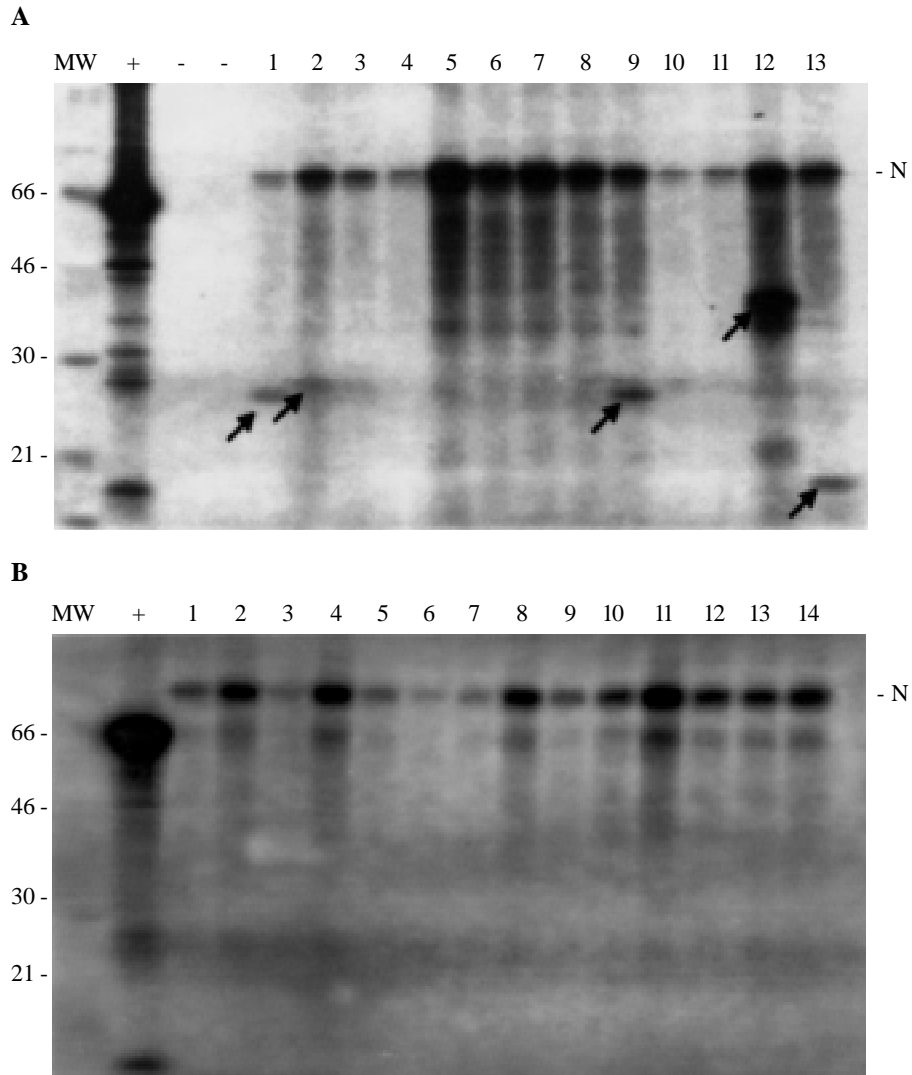
## RESULTS

### *APC* truncating mutations

We searched for truncating *APC* mutations between codons 686 and 1693 using the IVSP assay, which detects two-thirds of the *APC* mutations previously identified in colorectal tumors. In the samples tested truncating *APC* mutations were identified in 19 of the 35 colon tumors, examples of which are shown in Figure 1A, where a truncated peptide was detected in tumors 1, 2, 5, and 13, but truncated peptides were not detected in any of the gastric tumors (Figure 1B).

### Microsatellite instability

Microsatellite instability in at least one locus was identified in 10/40 (25.0%) of the gastric tumors. Of these ten, three exhibited only single locus alterations and were not considered to be MSI+. Seven gastric tumors showed alterations at two or more of the loci tested and were considered MSI+ 7/40 (17.5%) (Table I). Only one tumor, T13, showed LOH in D2S136 and D10S89 regions. These data clearly demonstrate that the lack of *APC* mutations is a meaningful negative finding.



**Figure 1** - Two overlapping segments of *APC*, encompassing codons 686 to 1217 and codons 1099 to 1693, were analyzed in 35 colon adenocarcinomas and 39 gastric adenocarcinomas. The figure shows the result of 13 and 14 representative samples of each tumor type obtained with the codon 1099 to 1693 fragment. **A**. Representative results obtained using DNA from colon tumors (1 to 13). Truncated protein products are indicated by the arrowheads. N indicates the position of the full length product. + and - are positive (luciferase) and negative (no DNA) controls, respectively. The numbers on the left are the size of the molecular weight (MW) markers. **B**. Representative results obtained using DNA from gastric tumors (1 to 14). N indicates the position of the full length product. + is the positive (luciferase) control. The numbers on the left are the size of the molecular weight markers (MW).

**Table I** - MSI in primary gastric tumors.

Patient	D2S119	D2S123	D2S134	D2S136	D2S147	D2S177	D10S89	D12S43	D13S71	D17S250
4	+	+	+	-	+	+	-	+	+	+
6	-	-	-	-	-	-	-	+	-	+
11	-	-	-	-	-	-	-	+	-	-
12	-	-	+	-	-	-	-	-	-	-
13	+	+	+	LOH	+	+	LOH	-	+	+
19	-	+	-	+	-	+	-	+	-	+
20	-	-	-	-	-	+	-	-	-	-
21	+	-	-	+	+	-	-	-	-	-
25	-	-	+	-	-	+	+	-	-	+
26	-	+	+	-	-	+	+	+	+	+

+, Altered alleles detected; -, altered alleles not detected; LOH, loss of heterozygosity detected.

## DISCUSSION

Little is known about which genetic alterations are significant in gastric cancer and, in contrast to colorectal cancer, no clear sequence of genetic changes has been elucidated. The only gene consistently demonstrated to be frequently altered in gastric carcinomas is *p53* (Uchino *et al.*, 1993; Strickler *et al.*, 1994). Other genetic changes that have been reported include LOH on chromosomes 1q, 5q, 17p (Sano *et al.*, 1991), 7p (Kuniyasu *et al.*, 1994) and 18q (Uchino *et al.*, 1992), amplification of the *erbB-2* oncogene (Park *et al.*, 1989), alterations of *E-cadherin*, microsatellite instability and abnormalities of growth factor and growth factor receptors and TRP-MET rearrangement (Tahara, 1993). Our data, added to those of Ogasawara *et al.*, 1994 and Maesawa *et al.*, 1995, show that the possible role of *APC* mutations in gastric cancer needs to be carefully evaluated. Significant allelic loss on chromosome 5q loci at or near the *APC* gene has been reported in gastric adenocarcinomas, varying from 20 to 86% depending on sample preparation and methods of analysis (Sano *et al.*, 1991; Tamura *et al.*, 1993; Rhyu *et al.*, 1994a). In addition, several somatic truncating and missense mutations of *APC* have been recently noted on partial single stranded conformational polymorphism or ribonuclease protection screening analysis of gastric cancers and adenomas from Japanese patients (Horii *et al.*, 1992; Nakatsuru *et al.*, 1992; Tamura *et al.*, 1994), but these data have not been reproduced in patients from Brazil, Great Britain or Italy. This suggests that there may be an alternative tumor suppressor gene close to *APC* involved in gastric cancer (Powell *et al.*, 1996; Sud *et al.*, 1996).

We cannot exclude the possibility that our samples could have mutations outside the domain explored or missense mutations, which would not be identified by our assay. This would imply, however, a different mechanism of *APC* involvement in gastric and colorectal tumorigenesis (given the clear preponderance of truncating mutations in exon 15 in the latter) supporting the notion of dichotomy between the two tumor types. The data obtained with the colorectal tumors agree with the findings of others, thus serving as a good positive control for the assay (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992).

Although we have used microsatellite instability here principally as a control the findings are worthy of comment. We found microsatellite instability in 7 of 40 human gastric cancers (17.5%). Although our results are similar to the studies of Mironov *et al.*, 1994, Chong *et al.*, 1994 and Rhyu *et al.*, 1994b, we did not find an association between MSI and clinicopathological features, such as age, sex, size, histological differentiation, depth of invasion, lymph node metastasis or survival rate. Nevertheless, our data show that MSI at a high frequency is a constant feature of gastric tumors irrespective of geographic (and hence environmental) considerations.

In summary, although we detected 7 of 40% (17.5%)

gastric samples with microsatellite instability, our results indicate that genetic alterations of the *APC* gene commonly present in colorectal tumors are infrequent in sporadic gastric adenocarcinomas from Brazilian patients, adding to the evidence that colon and gastric cancers do not share the same carcinogenic pathway, at least in British, north Italian and Brazilian patients.

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## RESUMO

Um papel crucial para o gene da polipose cólica adenomatosa (*APC*) na carcinogênese colo-retal já está estabelecido, mas sua participação nos tumores gástricos permanece controversa. Mutações no *APC* foram detectadas com uma frequência relativamente alta em tumores gástricos em pacientes japoneses, mas tais mutações foram relatadas como sendo muito raras em britânicos e em pacientes da região norte-central da Itália. Nós relatamos aqui a análise de 40 adenocarcinomas gástricos esporádicos primários e 35 adenocarcinomas cólicos esporádicos primários (de pacientes residentes em São Paulo, Brasil), quanto a mutações no gene *APC* entre os códons 686 e 1693, usando o teste da proteína truncada (PTT). Embora 19 mutações que levem a formação de uma proteína truncada tenham sido detectadas em 35 adenocarcinomas do colo (54,2%), não se encontrou nenhuma nos adenocarcinomas gástricos. Como um controle interno, as amostras tumorais foram também avaliadas quanto à presença de alterações de microssatélites, que também são características comuns a ambos os tipos de tumores. Encontrou-se instabilidade de microssatélites em uma amostra de tumor cólico e em 7 de tumores gástricos. Este fato sugere que, no que diz respeito a mutações do *APC*, os adenocarcinomas gástricos de pacientes brasileiros são semelhantes aos que ocorrem na Europa, e confirmam a diferença fundamental que existe tanto entre os carcinomas gástricos que ocorrem em diferentes regiões geográficas como entre a etiologia molecular de adenocarcinomas gástricos e colo-retais que ocorrem em São Paulo, Brasil.

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