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Altersations of (CA)$_n$ DNA Repeats and Tumor Suppressor Genes in Human Gastric Cancer

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Abstract

We have examined whether alterations of simple (CA)$_n$ DNA repeats, as observed in human colon cancers, occur during human gastric carcinogenesis and whether such alterations reflect genomic instability that could lead to other genetic changes. A total of 22 gastric cancer samples were analyzed: 15 well or moderately differentiated adenocarcinomas, 6 signet-ring cell carcinomas, and 1 poorly differentiated adenocarcinoma. When (CA)$_n$ repeat sequences were examined at 10 loci, one adenocarcinoma showed a loss of repeat sequences at five loci, three adenocarcinomas gained a repeat at one locus, and one adenocarcinoma had new, repeated sequences at five loci. Three samples showed mutations in the p53 gene, two in exon 5 (both GC to AT transition at a CpG dinucleotide) and one in exon 7 (AT to GC transition). Only one sample with a p53 mutation also showed altered (CA)$_n$ repeats. A putative tumor suppressor gene, connexin 32, was not altered as assessed by single-strand conformation polymorphism analysis. These results suggest that genomic instability revealed by (CA)$_n$ repeat changes does not seem to contribute to induction of point mutations in p53 or connexin 32 genes but may participate in loss of heterozygosity at APC/MCC loci. The results are consistent with the hypothesis that different mechanisms are involved in the gain and loss of (CA)$_n$ repeats.

Introduction

Little is known concerning the sequence of genetic changes associated with the development of gastric cancer, although possible participation of oncogenes and tumor suppressor genes has recently been reviewed by Tahara (1). Because several specific mutations in the same cell are necessary in order for a tumor to develop, there must be a high mutation rate, probably based on a disturbance in the stability of the genome. Recently, it has been found that instability of simple di- and trinucleotide repeats might be associated with the function of a gene in chromosome 2 which determines predisposition to colorectal cancer (2-4). Such genomic instability, detectable by microsatellite sequences, could contribute to inactivation of tumor suppressor genes.

Among various tumor suppressor genes altered in human cancer, the most commonly implicated is the p53 gene, which has been found to be mutated in various types of tumors (5). LOH (6) on chromosome 5 (MCC/APC locus) is often associated with well-differentiated stomach adenocarcinoma (1). Mutations of the MCC and APC tumor suppressor genes were originally found in colorectal cancers, and germ-line mutation of the APC gene is associated with familial adenomatous polyposis coli (6).

During the process of carcinogenesis, gap junctional intercellular communication is often abnormal, and this could lead to loss of control of tumor cell proliferation. It has therefore been suggested that the connexin genes, which code for the gap junction proteins, could form a class of tumor suppressor genes (7). Various experimental results, including those on transfection of connexin genes into tumorigenic cells, have supported this hypothesis (8).

We have examined whether microsatellite alterations can be observed in stomach cancers and whether such alterations are due to genomic instability which is associated with other genetic alterations found in tumor suppressor genes, such as LOH at the APC/MCC locus and point mutations in p53 and connexin 32 (ex 32) genes.

Materials and Methods

Samples. Twenty-two pairs of primary gastric cancer tissue and corresponding normal mucosa were obtained from the Cancer Research Center, Moscow, Russia. The samples were frozen in liquid nitrogen immediately after surgical operations and stored at -80°C until use. Samples were histologically divided into three groups: adenocarcinomas (well or moderately differentiated), poorly differentiated adenocarcinomas, and signet ring cell carcinomas. DNA was extracted by a phenol-chloroform procedure.

Microsatellite Instability. Microsatellite instability was examined using ten separate (CA)$_n$ repeats localized at chromosomes 1q21–q23 (Mfd3), 5q15–q23 (DSS82), 12q22–q24.1 (Mfd1), 12 (Mfd44), 17p11–p12 (Mfd41), 18q (Mfd26), 18 (Mfd32), Xq13.3 (DXS441), and Xq21.1–q23 (Mfd72) (9-17). PCR was carried out in 25 µl with 1 µCi [c-35S]dATP (9). Samples were processed through 30 cycles comprising 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C; aliquots of the amplified DNA were electrophoresed on standard denaturing 8% polyacrylamide DNA sequencing gel. Gel size standards were products of dideoxy sequencing reaction using M13mpl8 as template.

SSCP Analysis of p53 and Connexin 32 Genes. PCR reactions with p53 genes were performed as described by Hollstein et al. (18). For analysis of the cx 32 gene, eight pairs of primers were synthesized to produce overlapping fragments that cover the coding part of the gene sequence. Pairs of primers used were (5'-3'): (a) AACTGCCAGGTGTGGTACAC and AGATGAGGAGGATTTCTCA; (b) CCGAGTATGGCTCTCGGTCA and TFCTCTATGTGTIGCTGGTGG; (c) TCTTCTCCATCCTCATTAG and TTCCATCCTCCTCCAGGTGTAG; (d) ATGCTACGGCTTGAGGGCCA and GCATAGGCCAGGTAGGACGAG; (e) TCATACGGCTTGTTTCCCG and CAGA-TGCGCAAGGAGCGTAG; (f) TGGCTCCGCCCCCTGGGAGCAAA and TTGC-GCGGAAAGTGTTAGTTGGA; (g) CTCGATCATCCCACTCAAGTG and TATGC-TCTTTCAGGGAGCACAT; (h) GAGATGGGAGGGGGTCTGTTGG and CAAAGTGCTTCCTTGGAGACTGA. The 30 cycles of PCR were programmed as 30 s at 94°C, 1 min at 60°C, and 30 s at 72°C.

For SSCP, two µl of PCR products were dried in Speed Vac and dissolved in 2 µl of a sample buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue). The samples were denaturated for 4 min at 94°C and applied to 8% polyacrylamide DNA sequencing gel. Gel electrophoresis was carried out in the same buffer. After electrophoresis, the gel was stained using a Silver Stain Plus kit (Bio-Rad).

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2 To whom requests for reprints should be addressed, at International Agency for Research on Cancer, 150, cours Albert Thomas, 69372 Lyon cedex 08, France.
3 The abbreviations used are: LOH, loss of heterozygosity; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.
LOH at the APC/MCC Locus. LOH at the APC/MCC loci was investigated by three approaches: Rsal polymorphism within exon 11 of the APC gene detected by SSCP, polymorphism within exon 10 of the MCC gene detected by electrophoresis in agarose (19), and polymorphism at the D5S82 locus proximal to the APC gene detected by electrophoresis in denaturing polyacrylamide gel as a loss of the bands corresponding to one allele (12).

Sequencing. The PCR fragments of exon 7 of the p53 gene were sequenced directly (18). For exon 5, the PCR product was cloned in pBluescript SK(+) (Stratagene). Several subclones were sequenced for each tumor by Sequenase Version 2.0 kit (United States Biochemical Corp.).

Results

By amplifying DNA fragments at 10 microsatellite sites, we detected differences between tumor and normal DNA in 5 adenocarcinomas of 15 studied (Fig. 1; Table 1). Among these five tumors, patient 1 had loss of one allele present in normal mucosa at chromosomes 5q15–q23, 12q22–q24.1, 13, 17p11.1–p12, and Xq13.3. In contrast, four other patients showed a gain of additional microsatellite sequences: patient 4 at chromosome 18q; patient 5 at chromosome 18; patient 15 at chromosome 17p11.1–p12; and patient 19 at multiple loci, i.e., 1q21–q23, 5q15–q23, 12q22–q24.1, 13, and Xq21.1–q23. The size of gained repeats could be longer or shorter than the original repeats, and at some loci both longer and shorter size bands appeared in the same tumor (Fig. 1; patient 19).

LOH at APC/MCC loci, studied by three approaches, is summarized in Table 1. Patients 10 and 19 were homozygous (uninformative) at all three loci studied. Only one sample, the adenocarcinoma from patient no. 1, showed LOH at one D5S82 locus of the three studied, which was heterozygous in this sample (Table 1). This sample also showed a loss of microsatellite repeat sequences at four other loci (Table 1).

Among twenty-two tumor samples screened for p53 gene mutations within exons 5–8, three tumor samples (patients 1, 20, and 22) had a detectable shift of the bands on SSCP gel. No band shift was detectable in the adjacent mucosa samples. Sequence analysis of these samples revealed point mutations in codon 234 (TAC --- TGC, Tyr --- Cys), codon 154 (GGC --- AGC, Gly --- Ser) and codon 175 (CGC --- CAC, Arg --- His) for patients 1, 20, and 22, respectively (Fig. 2; Table 1). All three tumors with mutated p53 genes were adenocarcinomas, and two of the tumors with mutated genes had metastases.

Possible alterations in the whole coding region of the cx 32 gene were analyzed by the PCR/SSCP method, but no evidence of any alteration was found in the 22 stomach tumors studied (data not shown).

Discussion

In the present study, we found microsatellite DNA alterations in 5 of 22 primary human gastric cancers (22.7%). While the number of samples examined is small, our results indicate that microsatellite instability is rather common in gastric cancer. Microsatellite alterations have so far been reported only in human colon cancers, both hereditary and sporadic cases (3, 4). Our finding that human gastric cancers contain such alterations suggests that the somatic instability represented by alterations of DNA repeats may be common in many types of cancer.

The human genome contains approximately 50,000–100,000 simple DNA repeat microsatellites (9). Only ten of these loci were examined in the present study. One tumor showed loss of repeated sequences at five loci and another a gain of repeats also at five loci. These results imply that DNA in these tumors has alterations at thousands of loci. Even an alteration at 1 of 10 loci, as seen in 3 tumors, may represent changes at up to 10,000 loci in the whole genome. It has been proposed that such changes are results of genomic instability which causes numerous nonspecific genetic alterations; involvement of DNA replication errors had been suggested (3, 4). This has very recently been supported by the study by Strand et al. (20) who found that simple, repetitive DNA destabilization in yeast is associated with a reduction in heteroduplex repair efficiency which leaves replication errors uncorrected. It is likely that similar mechanisms are involved in microsatellite instability of the mammalian genome, although this has not yet been demonstrated.

There were two distinct patterns of (CA)n repeat alterations in the stomach tumors we studied: (a) loss of sequences which contain (CA)n repeats; and (b) gain of new (shorter or longer) repeats. The former type is essentially LOH, and only one tumor sample (patient no. 1) showed such a loss of repeats, although it was seen at five different loci. Moreover, this tumor sample was the only one that exhibited LOH at D5S82 proximal to the APC/MCC locus. It may be
that this tumor has the trait for the production of LOH at various loci. The second type of alteration, “gain” of (CA)$_n$ repeats, was observed in 4 of 22 stomach tumors (18.2%), 3 at only 1 locus and 1 at 5 of the 10 loci examined. In the case of hereditary nonpolyposis colorectal cancer, chromosome 2 has been identified as a locus responsible for the generation of new, single DNA repeats (2, 3). Because the tumor from our patient 1 had LOH at 5 (CA)$_n$ repeat loci but no gain at any of the 10 loci examined and since the tumor from patient 19 had a gain of new (CA)$_n$ repeats at 5 different loci but no loss in any locus, it appears that the mechanisms for the loss and gain of (CA)$_n$ repeats are distinct.

Microsatellite alterations were found either at only 1 locus or 5 loci of the 10 examined; there were no tumors which showed alterations at 2, 3, or 4 loci. Since we do not know the spontaneous somatic mutation frequency of microsatellites, it is possible that only those tumors with multiple microsatellite alterations represent true genomic instability, while tumors with only single microsatellite alterations just demonstrate fragility of these sites. The spontaneous mutation frequency of these microsatellites needs to be established.

Our results show that genomic instability revealed by (CA)$_n$ repeat alterations does not confer an ability to produce abundant point mutations in tumor suppressor gene p53. Three tumors showed mutations in this gene and none in the $cx$.32 gene, and only one tumor with a p53 gene mutation also had (CA)$_n$ repeat alterations (a loss). Similarly, in hereditary nonpolyposis colorectal cancers in which abundant microsatellite alterations were found, the prevalence of mutations in K-ras, p53, and APC genes was similar to that in sporadic cases (3). Thus, although microsatellite instability can partially explain how multiple genetic alterations can occur in the same cell during carcinogenesis, other types of genomic instability are also likely to be involved in multistage carcinogenesis.

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**References**


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**Table 1 Summary of gastric carcinoma samples**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Histology</th>
<th>TNM stage$^a$</th>
<th>Microsatellites (altered per 10 used)</th>
<th>LOH$^b$ at APC/MCC loci</th>
<th>Mutations of p53</th>
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<td>1</td>
<td>69</td>
<td>F</td>
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<td>T2N3M0</td>
<td>5$^c$</td>
<td>LOH H H</td>
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<td>H H H</td>
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<td>1 H</td>
<td>H H H</td>
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<td>1 H</td>
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<td>H H H</td>
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</tbody>
</table>

$^a$ TNM, tumor, node, metastases.

$^b$ H, homozygous (uninformative); -, heterozygous sample without LOH.

$^c$ Includes result at DSS82 locus.

$^d$ New, repeated sequence appeared in tumor at this locus.

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![Fig. 2: Mutations of p53 gene in gastric tumors. Transition mutations were seen in exon 7 (TAC → TGG) and exon 5 (GGC → CAC and GGC → AGC).](image-url)
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