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Comparative Genomic in Situ Hybridization of Colon Carcinomas with Replication Error

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Abstract

The aim of the present study was to detect complex genetic alterations in colorectal carcinomas with and without microsatellite instability (MIN) by comparative genomic in situ hybridization. MIN due to replication errors is the hallmark of hereditary nonpolyposis colon cancer. None of 6 MIN-positive tumors showed amplifications, and only 2 tumors displayed deletions of one chromosomal segment each. In contrast, different gains and losses were observed in 11 of 12 MIN-negative carcinomas. The most frequent gains affected chromosomes 7, 13, and 20q, whereas deletions were observed on chromosomes 17, 18, and 9p. These results demonstrate different mechanisms of genetic instability in subgroups of colorectal carcinomas and may, therefore, support the hypothesis of different etiologies in tumors with and without MIN.

Introduction

MIN, i.e., expansion or reduction of the number of short tandem repeats) is the major characteristic of the mutator phenotype in tumors constituting HNPCCs (1–3). In these tumors, MIN is due to defective DNA repair enzymes. Mutations have been reported in the human mutS and mutL homologous genes hMSH2 and hMLH in patients suffering from HNPCC (4, 5). Moreover, it has been shown, by different studies, that not only hereditary but also sporadic colon carcinomas exhibiting the MIN phenotype are associated with several specific biological features (6, 7). They are mainly localized in the right colon and have a distinct histopathological pattern. Most colon carcinomas exhibiting the MIN phenotype are poorly differentiated G3 tumors with mucinous appearance. It has also been shown that patients suffering from tumors with MIN seem to have a more favorable clinical course (3). These data might indicate different etiologies of colon tumors with and without MIN mutator phenotype. Although characterized by genomic instability the great majority of tumors with MIN exhibit a diploid DNA status, whereas a high proportion of colon carcinomas without MIN is aneuploid (7). Thus far, no cytogenetic investigations have been published that address the question of chromosomal changes in MIN tumors. One limitation for cytogenetic investigation of colorectal tumors is the requirement of prior cell culture. CGH circumvents these problems; neither tissue culture nor specific probes are necessary (8, 9).

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The abbreviations used are: MIN, microsatellite instability; HNPCC, hereditary nonpolyposis colon cancer; CGH, comparative genomic in situ hybridization; LOH, loss of heterozygosity.

Materials and Methods

DNA Preparation and Labeling. Fresh tumor specimens of surgically removed colorectal carcinomas from 18 patients operated at the Department of Surgery, University of Regensburg, were snap frozen in liquid nitrogen immediately after resection and stored at −80°C until analysis. The mean age of the patients was 65 years (range, 40–84 years). Whole blood from the same patient was collected as a source of nonneoplastic control DNA. Preparation of genomic DNA was performed by standard methods. Control DNA from leukocytes of the same patient was applied in all experiments. For CGH, tumor and control DNA (1 μg each) were labeled by nick translation in the presence of either digoxigenin-11-dUTP or biotin-14-dATP. After determination of DNA concentration, a 10-μl mixture containing equal quantities of 200 ng of each DNA in hybridization solution (50% formamide-300 mM sodium chloride-30 mM sodium citrate-10% dextran sulfate) was prepared.

In Situ Hybridization. Metaphase chromosomes of normal blood lymphocytes (46,XY) were prepared according to standard protocols of Colcemid arrest, hypotonic treatment, and methanol:acetic acid (3:1, v/v) fixation. Chromosome spreads were aged for 70 min at 95°C on a heating block, followed by prehybridization with 10 μl of a mixture of 50% formamide-2× SSC-50 μg human Cot1 DNA under a 18×18-mm coverslip. After incubation at 37°C for 2 h, the coverslip was removed, and 10 μl of hybridization solution was applied to the same area and sealed with rubber cement under a coverslip (24×24 mm). After hybridization for 3 days at 37°C, the preparations were washed 3 times for 5 min in 0.05× SSC at 42°C, and immunodetection was performed as described (10).

Microscopic Evaluation. Preparations were embedded in VectaShield anti-bleach medium (Vector Laboratories, Inc., Burlingame, CA) and examined in a Zeiss Axioskop fluorescence microscope (Zeiss, Munich, Germany) equipped with a double band pass filter for simultaneous excitation of green and red fluorescence. Pictures were recorded on Kodak 400 color slide films and subsequently stored as PCD files on CD-ROM. For evaluation of chromosomal copy number, pictures were evaluated on a PC monitor, and individual chromosomes were identified by diaminophenylindole banding. Copy number changes of chromosomal regions were apparent by a shift of the chromosomal hybridization image (yellow) toward red (loss) or green (gain). Using this method, the particular chromosomal regions displaying gains and losses could be unequivocally identified. In addition, quantitative analysis of the red:green fluorescence ratio along individual chromosomes was performed using the analySIS (SIS, Muenster, Germany) software package. CGH analysis was performed independently by two investigators (J. S. and G. S.) without knowledge of which samples were MIN positive and MIN negative.

PCR Analysis. PCR analysis of matched samples of DNA from tumor specimens and blood of the same patient was performed as described previously (11). The microsatellite loci were localized on chromosomes 5q (ACP), 9 (D9S171), 10 (D10S89, Mfd28), 17p (P53), and 18q (D18S34, Mfd26). Oligonucleotides used as primers for the PCR were AFM186x3 for D9S171, ACP, Mfd28, P53, and Mfd26; primer sequences have been published previously (11).
CGH OF COLON CARCINOMAS

Results

Microsatellite PCR. Six of 18 colorectal carcinomas investigated in the present study showed MIN consisting of expansion and contraction of (CA)_n repeats. Four of these 6 tumors showed MIN at 3 or more loci, whereas 2 tumors exhibited MIN at 1 (CA)_n repeat. In addition, we also detected LOH of chromosomes 5q (APC), 9p (D9S171), 17p (p53) and 18q (DCC) in some tumors using this approach. One tumor exhibited MIN at one locus (p53) and LOH at another site (APC). Of the 12 tumors without MIN, 8 exhibited LOH, 2 showed LOH at 5q, 1 showed LOH at 9p, 5 showed LOH at 17p, and 3 showed LOH at 18q. Three tumors showed LOH at 2 loci. Results regarding MIN and LOH are summarized in Table 1.

CGH Analysis. By CGH, using tumor DNA of 18 human colorectal carcinomas, we found a striking difference between tumors with and without MIN phenotype. Tumors exhibiting MIN rarely demonstrated numerical genetic alterations (Fig. 1A). None of 6 MIN tumors showed gene amplifications by increased signal intensities. We observed deletions of chromosomal segments in only 2 MIN tumors. The two tumors showed deletions of chromosome 13 and the short arm of chromosome 1, respectively. In contrast, we found gross chromosomal aberrations in a high proportion of colorectal carcinomas without MIN phenotype. Eight of 12 MIN-negative tumors exhibited gains of different chromosomal segments, 5 of these at more than 1 locus. Gain of chromosomal DNA was frequently observed on chromosomes 7 (4 tumors), 13 (5 tumors), and 20q (3 tumors) (Fig. 1B). Two carcinomas showed polysomy of chromosome 3, and one tumor exhibited amplification on 8q21. In addition, 9 MIN-negative tumors showed recurrent deletions on chromosomes 9p (4 tumors), 17 (5 tumors), and 18 (3 tumors). Six MIN-negative tumors exhibited deletions on other chromosomes (Table 1). There was a close correlation between LOH results obtained by microsatellite PCR and CGH. By CGH, 4 of 5 carcinomas with LOH at chromosome 17 also showed deletions of 17p. In 1 of 2 tumors with LOH at chromosome 5 and all 3 tumors with LOH at chromosome 18, CGH revealed deletions of chromosomes 5 and 18, respectively. However, none of the carcinomas with 9p deletions exhibited LOH at D9S171, whereas one tumor with LOH at chromosome 9p showed no alteration on chromosome 9 by CGH.

In addition, individual pictures were quantitatively analyzed using the analySIS software package. Using this computer program, the red:green fluorescence ratio along individual chromosomes was calculated. We found no differences between visual judgment and the computer profiles (i.e., we were not able to identify gains or losses in MIN-positive tumors by image analysis). In tumors without MIN, no additional alterations were detected by quantitative analysis.

Discussion

Genetic instability is a constant feature of malignant tumors. The concept of a step-wise carcinogenesis involving genetic changes of dominant acting genes ("oncogenes") and recessive "tumor suppressor genes" has been evolved in colorectal cancer (12). The end point is a highly malignant tumor exhibiting aneuploidy with multiple chromosomal aberrations. Assessment of the ploidy status of tumor genomes has, therefore, been introduced as a prognostic method in tumor pathology. Recently, another mechanism leading to genetic instability has been demonstrated in a subgroup of colorectal carcinomas. MIN is the hallmark of HNPCC, which is the most common familial cancer syndrome (13). Not only hereditary colon carcinomas but also sporadic MIN tumors exhibit a distinct phenotype including a predominance of localization in the proximal colon, histological subtype, and diploid DNA distribution (3, 6, 7). These data might indicate a unique etiology of carcinomas with MIN phenotype.

In the present study, we used CGH to record complex genetic alterations in colorectal carcinomas with and without MIN. CGH is a novel technique that permits a molecular cytogenetic analysis of tumor genomes (8, 9). Use of tumor DNA as a probe also allows identification and chromosomal mapping of de novo amplified, previously unknown DNA sequences (14). It has been shown by several authors (8, 9) that the data obtained by CGH are reproducible and comparable to the results of classical cytogenetic investigations. CGH is therefore of potential interest for the cytogenetic analysis of colon carcinomas, which hardly can be cultured in vitro. However, cell culture is the prerequisite of classic cytogenetic analysis. In accordance with previously published flow cytometric (7) and molecular genetic data (3), we found statistically significant more gross chromosomal aberrations in MIN-negative colorectal carcinomas using CGH. Main alterations consisted of gains of different chromosomal segments most often detected on chromosomes 7, 13, and 20q, whereas chromosomes 9p, 17, and 18 frequently showed deletions. These results corroborate data from molecular and cytogenetic investigations demonstrating polysomy of chromosomes 7, 13, and 20, as well as deletions and LOH on chromosomes 17 and 18 (15). In

<table>
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<tr>
<th>Tumor</th>
<th>Site</th>
<th>MIN</th>
<th>LOH</th>
<th>Frequent gains</th>
<th>Frequent losses</th>
<th>Rare chromosomal alterations</th>
</tr>
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<tbody>
<tr>
<td>MIN+</td>
<td>1 R</td>
<td>5</td>
<td></td>
<td>7 13 20q 9p 17 18</td>
<td>LOH</td>
<td>1p--13</td>
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<td>2 R</td>
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| MIN-  | 7 L  | 17  |     | 20p-14       |     |                             |
| 8 L   |     |     | +   | +           |     |                             |
| 9 L   |     | +   |     | +           |     |                             |
| 10 R  |     | +   |     | +           |     |                             |
| 11 L  |     | 17  |     | +           |     |                             |
| 12 L  |     | 9,17|     | +           |     |                             |
| 13 L  |     |     | +   | +           |     |                             |
| 14 R  |     | 5,17|     | 8q21+,--3,--13|    |                             |
| 15 R  |     |     |     | 5q--,--14,--22|    |                             |
| 16 R  |     |     |     | 2q+,--10q-  |     |                             |
| 17 L  |     |     | +   | +           |     |                             |
| 18 L  |     | 17,18| + | +           |     |                             |

MIN, number of loci exhibiting MIN (total investigated = 5).
LOH, chromosomal loci demonstrating LOH.

Table 1 Chromosomal abnormalities in colorectal carcinomas detected by PCR and CGH

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Fig. 1. Colorectal carcinomas with (A) and without (B) MIN. A, no cytogenetic alterations are detectable by CGH. B, CGH illustrates different cytogenetic alterations, including gains of chromosomes 7, 13, and 20q, as well as loss of 9p (arrows) and of the short arm of chromosome 20.
addition, we observed deletions of chromosome 9p, which have been demonstrated in a broad variety of human malignant tumors. Previously, the MTS1 (multiple tumor suppressor) gene has been mapped to the deleted region. It has been shown that this gene is identical to the CDKN2/CDK4/INK4 gene, which encodes the cyclin-dependent kinase 4 inhibitor protein p16 (16). Another homologous gene encoding the cell cycle inhibitory protein p15 is located in the same region. The CDKN2 gene is homozygously deleted in several tumor entities, with highest mutation/deletion rates in melanomas and astrocytic brain tumors. However, in a first investigation, colorectal cancer cell lines showed no alterations of this locus (16). In the present study, we found deletions on the short arm of chromosome 9; however, none of these tumors showed LOH at the D9S171 locus, which is closely linked to the p15/p16 gene cluster. Moreover, chromosomal deletions at 9p in colorectal carcinomas seemed to be more telomeric, involving bands 9p22–ter. These results might indicate the existence of one or more as yet unidentified genes on chromosome 9p, which are involved in the tumor progression of colorectal carcinomas.

The characteristic genetic features of MIN tumors are subtle expansions and deletions in repetitive motifs throughout the genome. However, it is not clear in which way small alterations at multiple (CA)n repeats could contribute to the development of malignant tumors. Thus far, there are only few experimental results indicating higher mutation rates affecting genes without microsatellite sequences in MIN tumor cell lines. Bhattacharyya et al. (17) found a 200–600-fold increase of mutation rates at the HPRT locus in Oua-bain- or 6-thioguanine-treated MIN cells, compared with normal fibroblasts. Recently, Lazar et al. (18) showed frequent mutations at the APC and TP53 loci in some members of HNPCC families. These data indicate a general genomic instability in MIN tumors involving identical genes, compared with MIN-negative colon cancer. Therefore, one would expect a similar course of tumor progression resulting in aneuploid tumors. This, however, seems not to be the case. Although no cytogenetic analyses have been performed thus far, MIN tumors are rarely aneuploid by flow cytometry (7). These data are substantiated by the results of the present study. We found gross chromosomal alterations in two MIN tumors; each exhibited only one deletion by CGH. Consequently, colorectal carcinomas showing the MIN phenotype are mainly devoid of numerical chromosomal aberrations.

It has been shown that the MIN phenotype is due to defects in DNA repair enzymes. Mutations of the hMSH2 and hMLH genes have been demonstrated in 80–90% of carcinomas from HNPCC patients (4, 5). However, only 10% of sporadic carcinomas exhibited aberrations of these genes. MIN in nonhereditary cases seems to be due to other genetic changes; one of these may involve polymerase δ (19). DNA mismatch repair enzymes recognize small mutations of up to 6 bases in DNA mismatches repair enzymes recognize small mutations of up to 6 bases. This could explain the lack of gross genetic alterations in the initial steps of tumorigenesis. However, during malignant transformation, other genes may be affected by mutations due to incorrect mismatch repair. These genetic alterations seem not to proceed with aneuploidy. Possibly, MIN tumors with defects in mismatch repair are more sensitive to cell damage. Tumor cells with gross mutations could be more easily eliminated by cell death. This mechanism may involve p53-inducible apoptosis, because the p53 gene is not mutated or stabilized at the protein level in MIN tumors (7). In that case, tumor cells with small but efficient mutations would have a selective advantage. The identification of these genetic alterations could elucidate the mechanism of carcinogenesis in colorectal carcinomas with MIN. In conclusion, the results of the present study demonstrate different mechanisms of genetic instability in subgroups of colorectal carcinomas and may, therefore, support the hypothesis of different etiologies in tumors with and without MIN.

References
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