Frameshift Mutations at Coding Mononucleotide Repeats of the hRAD50 Gene in Gastrointestinal Carcinomas with Microsatellite Instability

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Abstract

Microsatellite instability (MSI) and frameshift mutations in genes containing nucleotide repeats have been reported in a subset of colorectal and gastric carcinomas. This study describes the analysis of MSI-positive colorectal (39 cases) and gastric carcinomas (36 cases) for the presence of frameshift mutations of the six genes known to be involved in DNA repair and containing mononucleotide repeats in their coding region. Our mutational study of the 75 MSI-positive tumors revealed frequent mutations in hRAD50 (23 cases, 31%), BLM (16 cases, 21%), and hMSH6 (16 cases, 21%) rare mutations in BRCA1 (1 case, 1%) and ATM (3 cases, 4%); and no mutation in NBS1. In contrast, no frameshift mutation was found in 60 MSI-negative colorectal and gastric carcinomas. The mutation of hRAD50, a gene that is involved in the response to cellular DNA damage and forms a complex with hMRE11 and NBS1, has not been reported previously. Our results suggest that frameshift mutations of hRAD50, BLM, and hMSH6 are selected and play a role in the tumorigenesis of colorectal and gastric carcinomas with MSI. The MSI targeting of the hRAD50 and BLM genes represents an additional link between MSI and DNA repair because alteration of these genes could accelerate defective DNA repair.

Introduction

A subset of sporadic gastrointestinal carcinomas exhibits a molecular phenotype commonly referred to as MSI. MSI is detected as alterations in the size of microsatellite DNA sequences in DNA derived from tumor and matched normal tissue. MSI is a consequence of defects in the DNA mismatch repair genes, including hMSH2, hMLH1, hPMS1, hPMS2, hMSH3, and hMSH6. Recent studies indicate that hMLH1 promoter hypermethylation and lack of hMLH1 expression play a major pathogenic role in sporadic tumors with MSI. Defective DNA mismatch repair is thought to promote tumorigenesis by accelerating mutations in the oncogenes and tumor suppressor genes.

MSI has usually been demonstrated in the noncoding portions of the genes. However, in some cancer-related genes and in some of the mismatch repair genes, MSI has been identified in the protein coding regions. Known target genes affected by defective mismatch repair include TGF-βRII, IGFIIR, and BAX (5–8). Mutations targeting mononucleotide runs within the two mismatch repair genes hMSH3 and hMSH6 have also been identified in MSI-positive tumors (9). These findings suggest a multistep progression of MSI-positive tumors, a model in which the MSI mutator phenotype unfolds in gradual steps by successive actions of the different mutator genes. Therefore, the MSI-positive tumor cascade is composed of primary and secondary mutator genes, accelerating the level of genomic instability.

It was recently reported that the DNA mismatch repair proteins form a complex with other genes involved in DNA repair. DNA damage repair proteins (hMSH2, hMSH6, hMLH1, ATM, and BLM), hRAD50-hMRE11-NBS1 protein complex, and DNA replication factor C associate with BRCA1 to form a large complex named BASC that serves as a sensor for DNA damage (10). Interestingly, many genes in this BASC have mononucleotide repeats within the coding regions: (a) the (A)n tract in the hRAD50 and BLM genes; (b) the (A)n tract in the BRCA1 gene; (c) the (C)n tract in the hMSH6 gene; (d) the (A)n tract in the NBS1 gene; and (e) the (T)n tract of the ATM gene. Frameshift mutations of these genes, except for hRAD50, ATM, and NBS1 in the MSI-positive tumors, had been reported previously with variable incidences (8, 9, 11). Given that the mononucleotide repeats in hRAD50, ATM, and NBS1 might be another important target of tumors with MSI, we investigated the occurrence of frameshift mutations in these genes and compared these results with the occurrence of frameshift mutations in the other genes forming the BASC in primary colorectal and gastric carcinomas with MSI.

Materials and Methods

Patients and Tissue Samples. A total of 230 colorectal carcinomas and 414 gastric carcinomas were included in this study for the selection of tumors with MSI. All cases were identified consecutively for the Gastrointestinal Tumor Working Group Tissue Bank at Yonsei University Medical Center (Seoul, Korea) between December 1996 and November 1999. All cases were histologically confirmed as adenocarcinoma by two pathologists (H. K. and H. K.) without prior knowledge of the molecular data. Tumor specimens were microdissected on a cryostat and fractionated to enrich the tumor cell population. Genomic DNA was prepared by the SDS-proteinase K and phenol-chloroform extraction method.

Screening of MSI. DNAs from colorectal carcinomas (230) and gastric carcinomas (414) and matched normal DNAs were PCR amplified at five microsatellite loci (BAT26, BAT25, D2S123, D5S346, and D17S250) to evaluate MSI. PCR reactions were carried out in a mixture of 20 μl containing 1.5 mm MgCl₂; 20 pmol of primer; 0.2 mm each of dATP, dGTP, and dTTP; 5 μm dCTP; 1 μCi of [α-32P]dCTP (3000 Ci/mmol); DuPont New England Nuclear, Boston, MA); 50 ng of sample DNA; 1× PCR buffer; and 1.25 units of Taq DNA polymerase (Life Technologies, Inc., Grand Island, NY). After denaturation at 95°C for 5 min, DNA amplification was performed for 25–30 cycles consisting of denaturation at 95°C for 30 s, primer annealing at 55°C–60°C for 30 s, and elongation at 72°C for 15 s. PCR products were separated in 6% polyacrylamide gels containing 5.6 μM urea, followed by autoradiography. MSI was determined by the mobility shift of products from PCR. In tumors with MSI, additional bands were found in the normal allele regions. Based on the number of markers displaying instability per tumor, the tumors were initially divided into three groups: (a) those with two or more of the five markers showing...
instability (high MSI, MSI-H); (b) those with one of five markers showing instability (low MSI, MSI-L); and (c) those with no instability (MSI stable, MSS; Ref. 12). MSI-H tumors were classified as MSI positive, and MSI-L and MSS tumors were classified as MSI negative.

Detection of Frameshift Mutations. Frameshift mutation in the hRAD50 gene was detected using a PCR-based assay. Genomic DNA was amplified with primers RAD50F (5'-AATCTGGCTTGGTCCAGAT-3') and RAD50R (5'-CAAGTCCCAGATTTCATCA-3'), encompassing an 87-bp region of the hRAD50 segment [codon 704–733; (A)7 repeats are located between codon 719 and 722]. Frameshift mutations of the ATM and NBS1 genes were also analyzed using the same method. The primers for ATM were ATMF (5'-CATGCTGGTACCGAGATCTGC-3') and ATMR (5'-TCGCACTGAAAACTCCCGT-3'), encompassing an 88-bp region of the ATM segment [codon 192–221; (T)7 repeats are located between codon 213 and 215], and the primers for NBS1 were NBS1F (5'-ACGACCAACTTCCTCAGACGATA-3') and NBS1R (5'-CAGAGACATGAGAAGTTATC-3'), encompassing an 81-bp region of the NBS1 segment [codon 450–466; (A)9 repeats are located between codon 464 and 466].

Frameshift mutations in the coding nucleotide repeats of the other genes (BRCA1, BLM, and hMSH6) were also analyzed using a PCR-based assay by using previously described primers (8, 11). DNA denaturation, electrophoresis, and autoradiography were performed as described in the MSI analysis.

Sequencing Analysis of hRAD50 Frameshift Mutants. To confirm that the shifted band represents a frameshift mutation of the hRAD50 gene, genomic DNA fragments exhibiting bandshifts were excised and eluted from the polyacrylamide gel and subcloned to pT7Blue vector (Novagen, Madison, WI). Plasmids were sequenced using T7 sequencing kit (USB, Cleveland, OH) and autoradiography were performed as described in the MSI analysis.

Results

Frequency of MSI in Colorectal and Gastric Carcinomas. We defined a tumor as MSI positive when two or more of the five markers examined exhibited new microsatellite alleles in the tumor specimen compared with the corresponding nonneoplastic tissue. The frequency of MSI-positive tumors was higher in colorectal carcinomas than in gastric carcinomas: MSI was found in 39 of 230 (17%) colorectal carcinomas and in 36 of 414 (9%) gastric carcinomas (P = 0.002, χ2 test).

Mutational Analysis of hRAD50, BLM, hMSH6, BRCA1, ATM, and NBS1 in Colorectal and Gastric Carcinomas with MSI. We analyzed the frameshift mutations of the mononucleotide repeat sequences in the genes of the BASIC complex by PCR amplification of the regions comprising the (A)9 tract in the hRAD50 and BLM gene, the (C)9 tract in the hMSH6 gene, the (A)9 tract in the BRCA1 gene, the (T)7 tract in the ATM gene, and the (A)7 tract in the NBS1 gene (Table 1).

Alterations of hRAD50 were found in 13 colorectal carcinomas (33%) and 10 gastric carcinomas (28%). The alterations of the hRAD50 included either 1- or 2-bp deletions or 1-bp insertions in the (A)9 repeats of the coding region of the hRAD50 gene (Fig. 1). Sequencing analysis confirmed that the deletion and insertion of nucleotides in the polydeoxyadenosine tracts from the hRAD50 gene accounted for the observed bandshift (Fig. 2). Frameshift mutations in the BLM gene were found in seven colorectal carcinomas (18%) and nine gastric carcinomas (25%). All of the alterations of BLM were 1-bp deletions and were confirmed by sequencing analysis. The hMSH6 gene microsatellite variants were found in nine colorectal carcinomas (23%) and seven gastric carcinomas (19%). Frameshift mutation analysis in the mononucleotide repeat sequences of the BRCA1, ATM, and NBS1 genes revealed no mutation or rare mutation of these genes. The BRCA1 frameshift mutation was found only in one colorectal carcinoma (3%). The sequencing analysis of BRCA1 confirmed a 1-bp deletion in the tumor DNA, whereas DNA from the matched normal mucosa showed the normal sequence, indicating that this mutation is a somatic mutation rather than a germ-line mutation. Similarly, ATM frameshift mutations were found in one colorectal carcinoma (3%) and two gastric carcinomas (6%). No frameshift mutation of the NBS1 gene was observed in MSI-positive colorectal and gastric carcinomas. As controls, none of the 60 MSI-negative carcinomas had mutations in any of the genes.

The overall mutational profiles of the six evaluated genes revealed diverse combinations. Among the 75 MSI-positive carcinomas, 44 (59%) had mutations in more than 1 gene, 15 had mutations in 2 genes, and 29 had mutation in 1 gene. The mutations of hRAD50 and BLM were not mutually exclusive. Of the 23 cases with hRAD50 mutation and the 16 cases with the BLM mutation, 5 revealed concomitant mutations within the same tumor.

We evaluated the homozygous and heterozygous status of the mutations by comparing the intensity of the normal and abnormal (shifted) band. The percentage of tumor cells, determined on the histological slides for the tumors with mutations, was about 50–90%. Taking the percentage of tumor cells into account, we could differ-

Table 1 Frequency of frameshift mutations of the six genes involved in DNA repair in 75 MSI-positive gastrointestinal carcinomas

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type of nucleotide repeat</th>
<th>Colon [N = 39]</th>
<th>Stomach [N = 36]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td>hRAD50</td>
<td>(A)9</td>
<td>13 (33)</td>
<td>10 (28)</td>
</tr>
<tr>
<td>BLM</td>
<td>(A)9</td>
<td>7 (18)</td>
<td>9 (25)</td>
</tr>
<tr>
<td>hMSH6</td>
<td>(C)9</td>
<td>9 (23)</td>
<td>7 (19)</td>
</tr>
<tr>
<td>BRCA1</td>
<td>(A)9</td>
<td>1 (3)</td>
<td>0</td>
</tr>
<tr>
<td>ATM</td>
<td>(T)7</td>
<td>1 (3)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>NBS1</td>
<td>(A)7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
entiate 30 homozygous mutations of 58 frameshift mutations of the (A)\textsubscript{10} repeats of the TGF-BRII gene from our 75 MSI-positive tumors (data not shown). Among the frameshift mutations of the six genes in the BASC, homozygous mutations were rare; 2 of 16 hMSH6 mutations were homozygous, whereas all of the mutations of the other five genes were heterozygous.

**Discussion**

In this study, we identified the hRAD50 gene as an important target in tumors with MSI. Frameshift mutations of hRAD50 were present in both MSI-positive colorectal and gastric carcinomas, and the frequency of this mutation was higher compared with other genes of the DNA repair system.

Alteration of the mismatch repair genes is the cause of MSI, and the genetic progression of MSI-positive tumors has been proposed to be based on the occurrence of accelerating mutations in tumor-related genes, such as oncogenes, tumor suppressor genes, apoptosis-related genes, and genes involved in DNA repair (5–9, 13–15). Several genes involved in DNA repair contain mononucleotide repeats in their coding region, and frameshift mutations of these regions have been reported (8, 9). We found frequent frameshift mutations in BLM and hMSH6 and rare BRCA1 mutations in our MSI-positive tumors. We also examined the incidence of frameshift mutations in three additional DNA repair genes containing mononucleotide repeats in their coding region, which have not been examined previously. We could not find frequent frameshift mutations in the ATM and NBS1 genes; however, frequent frameshift mutations of hRAD50 were found in MSI-positive tumors, suggesting that hRAD50 might be another target gene in the tumors with MSI.

All of the hRAD50 frameshift mutations found in this study were expected to result in truncated proteins of approximately M, 83,000 in size, as opposed to M, 154,000 for the normal hRAD50 protein. The expression of truncated hRAD50 protein and its direct implications in tumorigenesis should be elucidated in the future, along with those of the other truncated proteins in the MSI-positive tumors. Until now, functional and structural analysis of hRAD50 protein was not completely characterized. hRAD50 is a coiled-coiled structural maintenance of chromosome-like protein with ATP-dependent DNA binding activity (16). hRAD50 has a binding domain of BRCA1 that is known to be located between the NH\textsubscript{2}-terminal and the 752 amino acids located upstream from the (A)\textsubscript{10} repeat (17). hRAD50 forms a complex with hMRE11 and NBS1 and functions in homologous recombination, activation of cell cycle checkpoint, nonhomologous end joining, meiotic recombination, and telomere maintenance (18, 19). DNA double-strand breaks are repaired by homologous recombination and nonhomologous end joining, and the hRAD50-MRE11-NBS1 complex is involved in both pathways (18). Based on the aforementioned structural and functional characteristics, the truncated proteins from the mutated hRAD50 are expected to cause functional interference in protein complex formation with the other proteins as well as accelerate defective DNA repair. The defective DNA repair that arose from the abnormalities of the hRAD50-MRE11-NBS1 protein complex will increase the genomic instability and is likely to contribute to tumor formation and progression. The defective formation of hRAD50-MRE11-NBS1 protein is shown to be a cause of Nijmegen breakage syndrome, an autosomal recessive disorder characterized by chromosomal instability, ionizing radiation sensitivity, and increased cancer incidence (20).

In addition to the frequent mutations of the hRAD50 gene, mutations of the BLM and hMSH6 gene were also frequent in our MSI-positive tumors. The hMSH6 gene, a DNA mismatch repair gene, is known to be a secondary mutator gene. The mutations of hMSH6 in MSI-positive tumors are proposed to accelerate genomic instability associated with MSI (9). The BLM gene is a member of the RecQ gene family of helicases, and its germ-line mutation is responsible for Bloom syndrome. The possible mechanisms of altered BLM in tumorigenesis, which include disturbing DNA replication, repair, and recombination or chromosomal segregation, had been proposed previously (11). Moreover, Bloom syndrome is a cancer-prone disease and is well known for its chromosomal instability. Therefore, the mutations of hRAD50 and BLM, either single or putative, may selectively have effects on the genomic instability associated with chromosomal instability, thus contributing to cancer formation and/or progression.

In conclusion, we have identified frequent frameshift mutations of hRAD50, BLM, and hMSH6 in tumors with MSI. Based on their proposed function, the hRAD50 and BLM genes could represent an additional link between MSI and DNA repair because MSI targeting of these genes could accelerate defective repair of the damaged DNA.

**References**


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Cancer Res 2001;61:36-38.

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