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

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Cancer Res 2001;61:36-38.

[CANCER RESEARCH 61, 36–38, January 1, 2001]

Advances in Brief

Frameshift Mutations at Coding Mononucleotide Repeats of the *hRAD50* Gene in Gastrointestinal Carcinomas with Microsatellite Instability[1]

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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.[3] 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* (1). Recent studies indicate that *hMLH1* promoter hypermethylation and lack of hMLH1 expression play a major pathogenic role in sporadic tumors with MSI (2, 3). Defective DNA mismatch repair is thought to promote tumorigenesis by accelerating mutations in the oncogenes and tumor suppressor genes (4).

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 monotonic 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 $(A)_9$ tract in the *hRAD50* and *BLM* genes; (*b*) the $(A)_8$ tract in the *BRCA1* gene; (*c*) the $(C)_8$ tract in the *hMSH6* gene; (*d*) the $(A)_7$ tract in the *NBS1* gene; and (*e*) the $(T)_7$ 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. Ka. and H. Ki.) 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 $MgCl_2$; 20 pmol of primer; 0.2 mM each of dATP, dGTP, and dTTP; 5 μM dCTP; 1 μCi of [α-^{32}P]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 M 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

Received 7/27/00; accepted 11/13/00.

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[1] Supported in part by grants from the Korean Science and Engineering Foundation through the Cancer Metastasis Research Center Yonsei University College of Medicine and 1999 Good Health R&D Project Grant HMP-99-M-03-0001 from the Ministry of Health and Welfare, Republic of Korea.

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[3] The abbreviations used are: MSI, microsatellite instability; BASC, BRCA1-associated genome surveillance complex.

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′-AACTGCGACTTGCTCCAGAT-3′) and RAD50R (5′-CAAGTCCCAGCATTTCATCA-3′) encompassing an 87-bp region of the *hRAD50* segment [codon 704–733; $(A)_9$ 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′-CATGCTGTTAC-CAAAGGATGC-3′) and ATMR (5′-TCGCACACTGAATAGCCTTG-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′-AGCAGACCAACTCCATCAGA-3′) and NBS1R (5′-CAGAGACAT-GAGAGAAGTTATC-3′), encompassing an 81-bp region of the *NBS1* segment [codon 450–466; $(A)_7$ 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 separated in 8% denaturing polyacrylamide gels.

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 ($P = 0.002$, χ^2 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 BASC complex by PCR amplification of the regions comprising the $(A)_9$ tract in the *hRAD50* and *BLM* gene, the $(C)_8$ tract in the *hMSH6* gene, the $(A)_8$ 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 tract 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.

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

Gene	Type of nucleotide repeat	Incidence of frameshift mutation			
		Colon [$N = 39$]		Stomach [$N = 36$]	
		No.	(%)	No.	(%)
hRAD50	$(A)_9$	13	(33)	10	(28)
BLM	$(A)_9$	7	(18)	9	(25)
hMSH6	$(C)_8$	9	(23)	7	(19)
BRCA1	$(A)_8$	1	(3)	0	
ATM	$(T)_7$	1	(3)	2	(6)
NBS1	$(A)_7$	0		0	

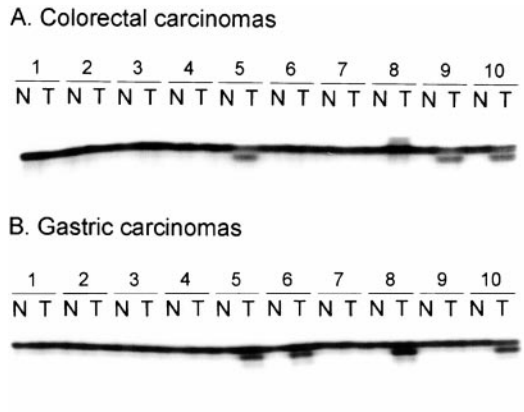

Fig. 1. Alteration of the coding polydeoxyadenosine mononucleotide repeat numbers of the *hRAD50* gene in the MSI-positive colorectal (*A*) and gastric carcinomas (*B*). *N*, DNA from normal tissue; *T*, DNA from carcinoma tissue.

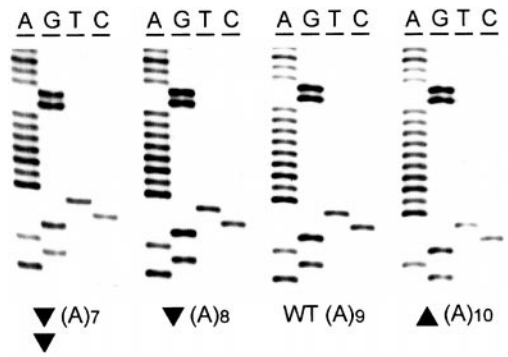

Fig. 2. Nucleotide sequence analysis of the representative clones of *hRAD50* from MSI-positive gastric carcinomas. *Triangles pointing up* or *down* indicate insertion or deletion of one nucleotide in the polydeoxyadenosine mononucleotide repeats, respectively.

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-

entiate 30 homozygous mutations of 58 frameshift mutations of the $(A)_{10}$ repeats of the *TGF-βRII* 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 the 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_r 83,000 in size, as opposed to M_r 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_2-terminal and the 752 amino acids located upstream from the $(A)_9$ 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-hMRE11-NBS1 protein complex will increase the genomic instability and is likely to contribute to tumor formation and progression. The defective formation of hRAD50-hMRE1-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.

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