Frameshift Mutations at Mononucleotide Repeats in RAD50 Recombinational DNA Repair Gene in Colorectal Cancers with Microsatellite Instability

Tsuneo Ikenoue,1,2,4 Goichi Togo,1 Keiichi Nagai,3 Hideaki Iijichi,1 Jun Kato,1 Yutaka Yamaji,1 Makoto Okamoto,1 Naoya Kato,1 Takao Kawabe,1 Atsushi Tanaka,2 Masayuki Matsumura,2 Yasushi Shiratori1 and Masao Omata1

1Department of Gastroenterology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, 2Division of Gastroenterology, The Institute for Adult Diseases, Asahi Life Foundation, 1-9-14 Nishi-Shinjuku, Shinjuku-ku, Tokyo 160-0023 and 3Helix Research Institute, 1532-3 Yana, Kisarazu, Chiba 292-0812

To identify additional genes targeted for microsatellite instability (MSI), we search for human genes which contain mononucleotide repeats in their coding region, selected 7 genes (RAD50, DNA-PKcs, FLASH, Apaf-1, XPG, CtIP, and MLSN1), and analyzed frameshift mutations in them. Here we report that 60% (3 out of 5) of human colorectal cancer cell lines exhibiting a high frequency of MSI (MSI-H) and 46% (6 out of 13) of MSI-H primary colorectal tumors had mutations in the (A)9 repeat of RAD50 recombinational repair gene. In contrast, no frameshift mutations were found in any of the 5 MSI-negative colorectal cancer cell lines, 8 colorectal tumors exhibiting a low frequency of MSI (MSI-L), or 28 MSI-negative colorectal tumors. No mutations were found in the mononucleotide repeats of 6 other genes, even in MSI-H cancers. These results suggest that RAD50 frameshift mutations may play a role in the tumorigenesis of MSI-H colorectal cancers.

Key words: Microsatellite instability — Frameshift mutation — RAD50 — Recombinational DNA repair — Colorectal cancer

Microsatellites are repeating oligonucleotide tracts widely distributed throughout the human genome.1) DNA mismatch repair genes participate in the recognition and correction of base pairing anomalies and alterations of the microsatellite repeats during DNA replication.2) Mutations within these regions, consisting of the deletion or addition of repeated units, are referred to as microsatellite instability (MSI). The occurrence of MSI has been observed in patients with hereditary non-polyposis colorectal cancer (HNPCC) as well as in 10–15% of sporadic colorectal cancers.3, 4) Several genes have been shown to contain nucleotide repeats within their coding regions that are targeted by MSI. They include (A)10 of the transforming growth factor β type II receptor gene (TGFb-RII), (G)8 in the insulin-like growth factor II receptor gene (IGFIIIR), (A)8 and (C)8 in the DNA mismatch repair genes hMSH3 and hMSH6, (G)8 in the proapoptotic gene BAX, and (AGC)13 in the transcription factor E2F-4 gene.5–9)

To identify additional genes targeted for MSI, we searched for human genes which contain at least eight mononucleotide repeats in their coding region from the GenBank database. We identified more than 500 genes, including TGFb-RII, BAX, IGFIIIR, hMSH3, and hMSH6. Among these genes, we also found mononucleotide repeats in the coding region of the RAD50, DNA-PKcs, FLASH, Apaf-1, XPG (ERCC5), CtIP, and MLSN1 genes. RAD50 is one of the RAD52 epistasis group proteins that mediate DNA double-strand breaks (DSBs) repair. In response to human DNA damage, the RAD50 protein forms a complex with MRE11 and NBS1.10) Defect in this MRE11-RAD50-NBS1 protein complex has been implicated in the genomic instability-associated predisposition to malignancy. DNA-PK is a major component of the DNA double strand break repair apparatus, and cells deficient in one of its components are hypersensitive to ionizing radiation.11) DNA-PK is also required for lymphoid V(D)J recombination and its absence confers upon mice a severe combined immunodeficiency phenotype.12) Furthermore, inactivation of DNA-PKcs in mice reportedly leads to hyperplasia and dysplasia of the intestinal mucosa and production of aberrant crypt foci, suggesting a role of DNA-PKcs in tumor suppression, especially in the colon.13) FLASH was first reported to be a mammalian homolog of CED-4 involved in the activation of caspase-8 in Fas-mediated apoptosis.14) However, the function of this protein is now unclear.15) Apaf-1 is a mammalian homolog of CED-4 that regulates cell death by participating in a ternary complex with cytochrome c, and procaspase-9.16) XPG is one of the nucleotide-excision repair group proteins which act in mammalian cells to remove carcino-
genic DNA lesions caused by UV light and many other common mutagens. Defect of XPG causes xeroderma pigmentosum (XP) group G. Patients with XP are highly predisposed to develop sunlight-induced skin cancer. CtIP was recently identified as a protein that associates with BRCA1 and two other nuclear factors, CtBP1 and Rb1. CtIP is considered to potentially modulate the functions ascribed to BRCA1 in transcriptional regulation, DNA repair, and/or cell cycle checkpoint control. MLSN1 was reported to correlate inversely with potential for melanoma metastasis.

Nine colorectal cancer cell lines, CaCO2, COLO320, HCT15, HCT116, HT29, LOVO, LS174T, SW48, and SW620, were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in MEM, DMEM, RPMI 1640, McCoy’s 5A, or Leivbovitz’s L-15 Medium (GIBCO/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO/BRL) at 37°C in a 5% CO2 atmosphere according to the supplier’s instructions. Tumor specimens from 49 patients with colorectal cancer (27 right-sided and 22 left-sided tumors) were obtained by surgical resections or autopsies at the Motojima General Hospital and the University of Tokyo Hospital. None of the patients included in this study had a known family history suggestive of HNPCC. All tumors were identified histopathologically as adenocarcinoma.

DNA extraction from tumors and adjacent nontumor tissue was performed as described previously. The MSI status of the 9 colorectal cancer cell lines has been previously described. Among 9 cell lines, 5 cell lines (HCT15, HCT116, LOVO, LS174T, and SW48) were MSI-H and the remaining 4 cell lines were non-MSI. The MSI status of the 49 colorectal cancer samples was analyzed in our previous report. Out of these 49 tumors, 13 were MSI-H, 8 were MSI-L, and the remaining 28 were non-MSI. All 13 MSI-H tumors were located in the right-side colon.

To detect mutations in the mononucleotide repeats of RAD50, DNA-PKcs, FLASH, Apaf-1, XPG (ERCC5), CtIP, and MLSN1 genes, we prepared PCR primers which amplify the fragments encompassing the mononucleotide repeats of each gene on the basis of the sequence obtained from GenBank database (Table I). The forward primer was fluorescently labeled with TET (Nihon Bioservice, Saitama). The 15-µl PCR mixture contained 0.05 µg of prepared DNA, 100 pmol of each primer, and 0.5 units of Pfu DNA polymerase (Stratagene, La Jolla, CA). After 30 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 30 s, PCR products were analyzed using the 373A DNA Sequencing System (Applied Biosystems, Foster City, CA). The appearance of one-base-shifted bands represented a mutation, and two bands with the same intensities represented heterozygous alleles.

Using the above-mentioned primer sets flanking the (A)9 repeat of the RAD50 gene, we could amplify the genomic DNA of all samples examined. Among the 5 MSI-H colorectal cancer cell lines, a 1-bp deletion was observed in the HCT116 and LOVO cell lines and a 1-bp

| Gene     | Type of repeat | GenBank accession no. | Primers (5′-3′)                           | Product length (bp) |
|----------|----------------|-----------------------|------------------------------------------|---------------------|
| RAD50    | (A)9           | U63139                | CTTAACTGGCACTTGCTCCA TCTTACCTCATGGGCAAG  | 107                 |
|          | (A)8           |                       | CAACATTGGAAAAGGTCCAG ACGTGGCTATGAACATAA   | 146                 |
| DNA-PKcs | (A)10          | U47077                | GACTTTTGAAGTTCAGAC CCTAAGTCTTATGCTAATAA   | 157                 |
|          | (A)8           |                       | TGGGAAATGTAGTAAAGGC ACCAAAGCTCATAACATTC   | 93                  |
| FLASH    | (A)9           | AF154415              | GTGACTACCTTACAGAAGAA TGGCCCTTCATCCTTCT    | 143                 |
| Apaf-1   | (A)8           | AF013263              | TGGGGAACATAACCATGTTA GACAATACAGGGAAAGAT   | 146                 |
| XPG      | (A)9           | X69978                | ATAAGACCTAATCCTCATGAA GATCCCTTGGATATCTC   | 128                 |
| CtIP     | (A)9           | U72066                | CCCCTTCTCTTACAGCCT GGCACTATCCTGATTTTG     | 110                 |
| MLSN1    | (A)14          | AF071787              | GAATGGAAGATGTAGAGCCCT GATACTGGAACCGACTCAG  | 111                 |

a) Sequences of complete cDNA.
b) Primers for RAD50 were designed on the basis of genomic sequence (GenBank accession no. AC004041).
c) Primers for Apaf-1 were designed on the basis of genomic sequence (GenBank accession no. AF098890 and AF098891).
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An insertion was observed in the LS174T cell line, whereas no alterations were observed in HCT15 and SW48 cell lines (Fig. 1). In 5 non-MSI cell lines, there were no observable alterations. In 13 MSI-H colorectal cancer samples, a 1-bp deletion was observed in 5 tumors and a 1-bp insertion was observed in one tumor (Fig. 2A). There were no observable alterations in the adjacent nontumor tissues of these 6 tumors (Fig. 2B). In the 8 MSI-L and 28 non-MSI tumors, no alterations were observed. All mutations detected were considered heterozygous since normal 107-bp PCR products were present in all cell lines and primary tumors.

We could also amplify the genomic DNA of all samples examined using the above-mentioned primer sets flanking the (A)8 repeat of the RAD50, the (A)10 and (A)8 repeats of the DNA-PKcs, the (A)9 repeat of the FLASH, the (A)8 repeat of the Apaf-1, the (A)9 repeat of the CtIP, and the (A)14 repeat of the MLSN1. However, no alterations were observed in 9 cell lines and 49 primary tumors. These 13 MSI-H tumors were also analyzed for mononucleotide repeats in TGFb-RII (12/13, 92%), BAX (5/13, 38%), IGFIIIR (2/12, 17%), hMSH3 (4/13, 31%), and hMSH6 (3/13, 23%) genes in our previous study.23

In this study, we found frequent mutations in primary human colorectal cancers within the recombinational DNA repair gene, RAD50. Alterations of the (A)9 repeat within the RAD50 coding region were observed in 3 of 5 (60%) and 6 of 13 (46%) MSI-H cell lines and primary tumors, respectively. In contrast, such alterations were observed in none of the 4 non-MSI cell lines, 8 MSI-L, or 28 non-MSI primary tumors (P=0.0001, Fisher’s exact test). These results suggest that the RAD50 gene may be one of the frequent targets in MSI-H colorectal tumors.

The RAD50 protein forms a complex with Mre11 and NBS1 that has a pivotal role in diverse aspects of the cellular response to DSBs, including detection of DNA damage, activation of cell cycle checkpoints, and DSB repair by DNA recombination. Two recent observations link the complex to DSB recognition and cell cycle-checkpoint functions: (i) the MRE11-RAD50-NBS1 complex localizes to DSBs after ionizing radiation exposure, and remains associated until repair is complete,24 and (ii) NBS1 mutations cause Nijmegen breakage syndrome, a chromosomal instability disorder associated with cell cycle checkpoint defects.25, 26

Since chromosomal instability represented by chromosomal rearrangements or chromosome number changes and escape from cell cycle control are common features of malignant cells, deficiency in this recombinational DNA repair protein complex has been suggested to play an important role in neoplasia. Of 3 MSI-H cell lines in which RAD50 mutations were detected in this study, the LOVO cell line has been characterized as having both microsatellite and chromosomal instability.27 The results of the present study suggest that some MSI-H tumors, genetically unstable due to a deficiency of mismatch repair genes, also acquire the feature of chromosomal instability due to mutations in the RAD50 recombinational DNA repair gene.

The human RAD50 protein is a 153 kDa protein containing an N-terminal nucleotide-binding domain and two long heptad repeat regions. The C-terminal heptad repeat is required for interaction with MRE11.28 Both poly(A)8 and poly(A)10 RAD50 mutants code for truncated proteins of 734 and 726 amino acids, respectively, in comparison...
with the normal length of 1313 amino acids. Thus, both frameshift mutants lose the ability to bind to MRE11 due to a lack of the C-terminal heptad repeat. Furthermore, it is noteworthy that mutations were heterozygous in all the examined cell lines and primary tumors, with no sign of homozygous mutations. Since homozygous disruption of the RAD50 gene causes embryonic stem cell lethality in mice, the RAD50 protein is supposed to mediate functions that are essential for cell viability. Thus, cancer cells with homozygous mutations of the RAD50 gene may not be capable of surviving, while those with heterozygous mutations may. It is to be expected that normal RAD50 gene may not be capable of surviving, while those with heterozygous mutations may. It is to be expected that normal RAD50 protein is still present in these heterozygous mutant tumors, although in lower amounts.

In conclusion, the RAD50 recombinational DNA repair gene is frequently mutated in human microsatellite-unstable colorectal cancers, although mutations were heterozygous in all the examined cell lines and primary tumors of this study. These mutations may represent an additional mechanism that allows tumor cells to acquire the genomic instability associated with neoplastic transformation through the inability to produce normal RAD50 products, leading to insufficiencies in the MRE11-RAD50-NBS1 repair complex.

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