Effects of altered DNA repair genes on the tumorigenesis and progression of colorectal carcinomas with microsatellite instability

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Effects of altered DNA repair genes on the tumorigenesis and progression of colorectal carcinomas with microsatellite instability

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감사의 글

어느덧 꿈만 같았던 시간이 지나고, 미흡하지만 하나의 논문으로 결실을 맺었습니다. 오늘이 있기까지 저에게 많은 기회와 격려를 해주시며 더 큰 세계로 이끌어주신 모든 분들께 감사를 드립니다.

너무나 소중한 배움의 기회를 마련해 주신 지도교수 박찬일 교수님, 논문이 완성되기까지 학자로서의 열정으로 지도해주신 김호근 교수님께 깊은 감사의 말씀을 드립니다. 아울러 부족한 논문을 심사하여 주신 박전한 교수님, 김남규 교수님, 김원호 교수님께 깊은 감사를 드립니다. 항상 이 못난 제자를 걱정해 주시고 사랑해주신 손동식 교수님에게도 깊은 감사를 드립니다.

부족한 저에게 항상 힘이 되어주었던 김남균님, 김현기님, 따스한 마음으로 기쁨과 슬픔을 함께 해주었던 최연락님께 감사의 마음을 전합니다. 항상 열정을 가지고 실험을 하는 강현주님, 따스한 마음으로 많은 도움을 주었던 고귀혜님, 음으로 양으로 많은 도움을 주었던 백명진님, 오승연님, 김세경님, 김정진님, 그리고 이제 갓 대학원생이 된 유권태님, 양은기님께 깊은 감사를 드립니다. 그리고 제 연구에 많은 도움을 주셨던 김세훈 선생님, 강혜윤 선생님, 이환석 선생님, 신은하 선생님, 김혜령 선생님께 감사의 마음을 전합니다. 가까이에서 항상 격려를 해주었던 서정철형, 진정애 형수님, 이용, 김영호, 안연희, 양미자, 허성군, 현문호, 장규철, 김분희 친구들에게 고마움을 전합니다.

헌신적으로 뒷바라지 해주신 부모님과 누님, 매형, 동생, 그리고 항상 염려를 해주시는 모든 분들에게 감사와 사랑의 마음을 전해드립니다.

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ABSTRACT

Effects of altered DNA repair genes on the tumorigenesis and progression of colorectal carcinomas with microsatellite instability

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Tumors with microsatellite instability (MSI-H) are caused by defects in DNA mismatch repair genes and accumulated frameshift mutations in coding mononucleotide repeats (cMNR) of various tumor related genes in the tumorigenesis and progression of this type of tumors. This study elucidated the effects of altered DNA repair genes in the tumorigenesis of MSI-H colorectal carcinomas.

Frameshift mutations of 45 genes which have been reported as candidate target genes with mutation frequencies of more than 30%, and containing more than 10 mononucleotide repeats in its coding region searched by Unigene database, were included in this study. Expression of these genes was analyzed by using reverse transcriptase polymerase chain reaction (RT-PCR). Ten DNA repair genes were involved in this study. In these DNA repair genes, *hMSH3* (56%, 22/39) and *hRad50* (33%, 13/39) showed the highest frameshift mutation frequency. We could

not identify frameshift mutant transcripts of *hRad50* in RT-PCR analysis and the mutant transcripts reappeared when treated with puromycin, a kind of translation inhibitor, or treated with *hUpf1* siRNA or *hUpf2* siRNA which are known as key molecules in nonsense-mediated mRNA decay (NMD). To clarify the effects of altered DNA repair genes in the tumorigenesis and progression of MSI-H tumors, we analyzed genomic instabilities in MSI-H colorectal carcinomas. Analysis of relationship between chromosomal imbalances (CIs) and mutation status of DNA repair genes revealed that there is a significant relationship between *hRad50* mutation and CIs in MSI-H colorectal carcinomas. Expression of hRad50 was decreased in MSI-H colorectal carcinomas and mismatch repair (MMR)-deficient colorectal cancer cell lines. Expression of hMRE11, which form a complex with hRad50, was also decreased in MSI-H colorectal cancer cell lines. *hMRE11* showed frameshift mutation and alternative splicing in all of our 7 MMR-deficient cancer cell lines.

In conclusion, frameshift mutation of *hRad50* may play an important role in development of CIs in MSI-H colorectal carcinoma.

Key word: DNA repair, microsatellite instability, frameshift mutation, colorectal carcinoma

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I. INTRODUCTION

Human cancer can be viewed as a disease of underlying genetic instability. Most of human tumors display some form of genomic instability, including subtle DNA sequence alterations, gross chromosomal rearrangements, aneuploidy, and gene amplifications.[1] These alterations have the potential to affect the function of growth-regulating genes that are associated with the malignant transformation of cells. The basis of genomic instability is unfaithful transmission of genetic information from a cell to its daughters. This arises from failure of cellular functions that ensure the accuracy of DNA transactions such as DNA replication, DNA damage repair, or mitotic chromosome distribution. Specific functional defects can be associated with a characteristic pattern of genomic instability.[1]

It is widely accepted that the molecular genetics of human cancers can be used to categorize colorectal carcinomas into two major types of genomic instabilities, chromosomal instability (CIN) and microsatellite instability (MSI).[2] The majority of colorectal carcinomas are categorized into the CIN pathway, which is characterized by a high frequency of allelic losses, deletions and/or mutations of tumor suppressor genes like *APC* and *p53,* and abnormal tumor DNA.[3] Aneuploidy in CIN phenotype tumors had been demonstrated in colorectal cancer cell lines[4] and tumor tissues.[5] Although CIN is a common finding in colorectal carcinomas, the mechanism of CIN has not been clearly elucidated. Defects in DNA replication check point genes and many other genes increase the rate of genome rearrangement and it is suggested to be associated with CIN.[6-9]

The other pathway, namely the MSI pathway, begins with the inactivation of one of a group of genes responsible for DNA nucleotide mismatch repair, which leads to extensive mutations in both repetitive and non-repetitive DNA sequences with low frequencies of allelic losses and rare alterations of tumor DNA content.[10, 11] The mechanism of tumorigenesis in high microsatellite instability (MSI-H) tumors is thought to involve frameshift mutations of microsatellite repeats within coding regions of the affected target genes, and the inactivation of these target genes is believed to directly contribute to tumor development and progression. These target genes participate in a variety of essential cellular processes like signal transduction,[12] apoptosis,[13, 14] DNA repair,[15, 16] transcriptional regulation,[17, 18] protein translocation and modification,[19] or immune surveillance.[20]

Although these two distinct major genetic pathways of genetic instabilities are widely accepted, some tumors reveal different genetic pathways; i.e., some tumors show both types of genomic instabilities and some tumors do not show any of these two instabilities.[21-23] Tumors showing both type of genomic instabilities might be resulted from the genetic alterations evoking both types of genomic instabilities, the development of CIN in MSI-H tumors, or MSI in development of CIN tumors during tumor progression.

To clarify the responsible genes involving chromosome instabilities and microsatellite instabilities, we analyzed the type of genomic instabilities, mutation and expression status of DNA repair genes. Our findings suggest that alterations of DNA repair genes, *hRad50*, can be developed during the tumor progression of MSI-H tumors, and cause additional genomic instability.

II. MATERIALS AND METHODS

1. Patient selection

Three hundred and twenty-nine cases of colorectal carcinomas were included in this study. All cases were identified consecutively at the Gastrointestinal Tumor Working Group Tissue Bank at Yonsei University Medical Center (Seoul, Korea) between December 1996 and November 1999. Cases included in this study were sporadic tumors without relevant family history or clinical evidence of familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer.

2. Cell culture

Seven mismatch repair (MMR)-deficient colorectal cancer cell lines (LS174T, HCT8, SNU-C2A, SNU-C4, DLD-1, HCT116, LOVO) and 3 MMR-proficient colorectal cancer cell lines (NCI-H508, SW480, HT29) were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). All of the cell lines were cultured in RPMI 1640 (Life Technologies Inc., Grand Island, NY, USA) with 10% fetal bovine serum (Life technologie Inc.,) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Puromycin (Sigma, P 8833, St. Louis, MO, USA) was treated for 6 hours at the concentration of 30 μg/ml.

3. DNA, RNA extraction

DNAs were extracted from fresh frozen tissues. 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.[24] RNAs were extracted by using RNeasy mini Kit (Qiagen, Valencia, CA, USA).

4. Screening of Microsatellite Instability

DNAs from tumors and normal mucosae were PCR amplified at 6 microsatellite loci to evaluate the MSI. These markers included the recommended panel of five markers proposed at the National Cancer Institute Collaboratory Meeting and MSI in colorectal cancer [25] plus BAT40. 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 sample DNA; 1x PCR buffer; and 1.25 units of Taq polymerase (Life technologies, Inc.,). 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 55-60°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. MSI in three or more markers of which more than two mononucleotide repeat markers were included was classified as MSI-H; and those showing no instability were classified as MSS (Fig. 1). Methylation analysis of

hMLH1 and expression analysis of hMLH1 and hMSH2 were performed as described previously.[26, 27]

5. Detection of frameshift mutation

We chose the 45 target genes based on the reported frequency of frameshift mutations and their functions. Genes with mutation frequencies of more than 30% (24 genes), genes containing more than 10 mononucleotide repeats (16 genes), and genes which are reported to be involved in the suppression of genome instability (5 genes)[16, 28-32] were selected. The incidence of frameshift mutations had been previously reported in 38 out of the 45 genes in our 39 MSI-H colorectal carcinomas and 9 MSI-H colorectal cancer cell lines.[16, 28, 29] The MSI status of all cases and frameshift mutations of the 38 target genes have been previously reported.[16, 28, 29] The analyzed target genes were *ABCF1, ACVRII, AIM2, ATR, BAX, BLM, BRCA1, BRCA2, Caspase 5, CHD2, DKFZp564C2478, FLJ11186, FLJ11222, FLJ11383, FLJ11712, FLJ13615, FLJ20139, FLJ20333, GART, GRB-14, hMSH3, hMSH6, hRAD50, KIAA1052, KIAA1096, KIAA1268, KIAA1470, MAC30, MARCKS, MBD4, NADH-UOB, OGT, PRKDC, PRKWNK1, RFC3, SEC63, SLC23A1, SPINK5, SYCP1, TAF1B, TCF-4, TCF6L1, TGF-β RII, UVRAG,* and *WISP3.* All PCR products of these genes showed one band from the normal DNA, whereas monoallelic or biallelic mutations were present in some MSI-H tumors (Fig. 1). All PCR primers used in this study were listed in Table 1.

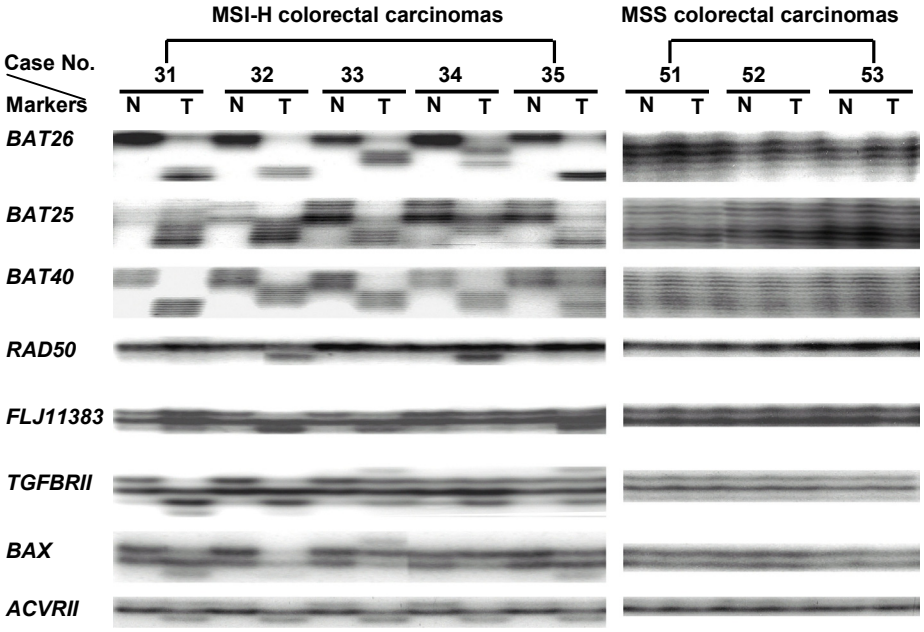


Figure 1. Frameshift mutations of target genes in MSI-H and MSS colorectal carcinomas. All of 5 MSI-H colorectal carcinomas showed shifted bands in all 3 mononucleotide repeat microsatellite markers (*BAT26, BAT25, and BAT40*). Fragment shifted bands of 5 target genes (*hRad50, FLJ11383, TGFβRII, BAX, and ACVRII*) showed in 5 MSI-H colorectal carcinomas, whereas no shifted bands showed in the 3 MSS colorectal carcinomas.

Table 1. Primers used for analysis of frameshift mutation of target genes

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product length
ABCF1	5'-CCTGGGCTTCATTTTCTCAC	CCTGCCTTTTCGGGTATCTC	73
ACVRII	GTTGCCATTTGAGGAGGAAA	GCATGTTTCTGCCAATAATCTC	111
AIM2	CCACTCATCGACTGCATCTC	TGGCTTGAATTGGTCCTTTT	102
ATR	GCTTCTGTCTGCAAGCCATT	TGAAAGCAAGTTTTACTGGACTAGG	70
BAX	ATCCAGGATCGAGCAGGGCG	ACTCGCTCAGCTTCTTGGTG	94
BLM	CTCTGCCACCAGGAAGAATC	ACAGCAGTGCTTGTGAGAAC	153
BRCA1	AGCCCACCTAATTGTACTG	CCATGAGTTGTAGGTTTCTG	115
BRCA2	TTGCTCACAGAAGGAGGACT	GCATTTGCTTCAAACTGGGCT	237
Caspase 5	CAGAGTTATGTCTTAGGTGAAGG	ACCATGAAGAACATCTTTGCCCAG	141
CHD2	CTATCCCTGTGGACCCTGAA	ACGGTACGACCATCTAAGCA	71
DKFZp564C2478	GGAGAGATGCCAAGGTGAAA	GCCTTGGGTTAGGATGACAG	143
FLJ11186	GCAAGAACAGCCATCAAGAA	GGAATGATTTGTTGTTTTCCTT	143
FLJ11222	GCTGCAGAAGACAAACGAAAC	GCAGCTGCTCATAAGCTTCC	106
FLJ11383	GGAAAATTATGAACAGCCACAA	GCAGCCAAATGCTTGTTATG	121
FLJ11712	GGCTAAAGTTGACAAGAGTGGAA	GTCAGGAAGGACAAACTGAAACA	147
FLJ13615	GTTGATTATTTTCTTGGCTGAAC	GCACTTCTTTTTCCTCTTTCTTGA	136
FLJ20139	GCCAACACAAAGTGTCTCCTC	GACTGTTGGATGGATGATGC	89
FLJ20333	GGCAAGGCAGCAAATTTAGA	GCATCTAAGGCACTATTCCAGA	124
GART	AGTGTTGAAGAATGGCTCCC	TGTTCCAGATATTAAGACAGCCAC	82
GRB-14	GGCATTTGCAGTTTTTCAGC	CCATAGTTAGTCGGTGCTCCA	130
hMSH3	GATGTCAATCCCCTAATCAAGC	ACTCCCACAATGCCAATAAAAA	150
hMSH6	GGGTGATGGTCCTATGTGTC	CGTAATGCAAGGATGGCGT	95
hRAD50	AACTGCGACTTGCTCCAGAT	CAAGTCCCAGCATTTCATCA	87
KIAA1052	GTCAACTTCTGGGGCCATTA	GAGGCATCCACTGACTCACC	104
KIAA1096	GGCAAATGAAGAATGGGAAA	GGAGGTAGAACATTCTCTCCAA	129
KIAA1268	GTTTCCTCTGTTTTGCAGGA	GACAGCCAGAGGCTACGAAC	90
KIAA1470	GCATTTGTTCTGGAAGCTCGT	GTGATGAGAAACCGGAGAGAA	143
MAC30	TGTTGCGGAGCCCCTAC	AACCACCCTGTAGGCATCTC	93
MARCKS	CCGCCTCCTCGACTTCTT	AGCCGCTCAGCTTGAAAGAC	120
MBD4	TGACCAGTGAAGAAACAGC	GTTGTGTTCTGAGTCTTTGG	138
NADH-UOB	TTTGCATCGCCAGCTTCTAT	AACATTTCACGGTCCCTCAC	102
OGT	TCACTTTTGGCTGGTCAGAG	GGGAGGGAAAGGAGGTAAAG	116
PRKDC	GACTCATGGATGAATTTAAAATTGG	TTTGAAAATAACATGTAAATGCATCTC	113
PRKWNK1	AGTTGGTACGGGAGGAGCA	CTGGGAAGCACTGGATTGTT	83
RFC3	TTTTCTTTGTCCACAGACTCCA	AAGGTGGTAGTTACTTGCAATGG	70
SEC63	AGTAAAGGACCCAAGAAAACTGC	TGCTTTTGTTTCTGTTGCTTTG	104
SLC23A1	GGTGACTACTACGCCTGTGC	TGGGACCAATCAGGAACTTG	102
SPINK5	TGAGGCGTTTGTTCACTTTG	TGCTCCTGTCTTCATCCTCTT	99
SYCP1	CCCCTTCATCTCTAACAACCC	CACTGATTCTCTGAAATTAAACAAATAAC	153
TAF1B	CCAAATAAAAGCCCTCAACC	TGTCCTGACATCATGAAGGTG	115
TCF4	GCCCTCTATTCACAGATAACTC	CTTCACCTTGTATGTAGCGAA	76
TCF6L1	TTGGAAAAAGAAATCATGGACA	AATCTCAATTCTTTACCATAAGAAAA	104
TGF-β RII	CTTTATTCTGGAAGATGCTGC	GAAGAAAGTCTCACCAGG	73
UVRAG	TTTATTTTTAAACATTGTGAGTATG	TTTTTAACTGCAGGCATTCAC	116
WISP3	CTTTTCTCCCTTTGTTTTAGCTT	CATGTTCTGGAGCAGGGAGT	99
hMRE11	GCTTTTGTCCCGTATGGAAA	TCGTCATGATTGCCATGAAT	123

6. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

First strand cDNA was synthesized from one μg of total RNA using random hexamer primers (QIAGEN) and M-MLV Reverse Transcriptase (Invitrogen, San Diego, CA, USA) following the protocols of the according to the manufacturer' s instructions. Twenty ng of cDNA from each sample was used in each reaction. RT-PCR reactions for MSI-H target genes 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; 1x PCR buffer; and 1.25 units of Taq polymerase. After denaturation at 95°C for 5 min, cDNA amplification was performed for 25-30 cycles consisting of denaturation at 95°C for 30 s, primer annealing at 55-60°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. Semi-quantitative RT-PCR was performed with primers for the specific genes (*hUpf1, hUpf2*) and for *β-actin*. All of the primers used in this study were listed in Table 2. The range of linear amplification for each gene and the *β-actin* gene was examined with serial PCR cycles, and the optimal PCR cycles were determined. The relative intensity of mRNA expression for each sample was then corrected for variable RNA recovery using the corresponding *β-actin* measurements as a surrogate for total mRNA.[33]

Table 2. Primers used for RT-PCR analysis

Gene name	Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product length
ABCF1	NM_001090	CAGCAGCAGCAACAGCAA	GCGGGTACTTCATCCTCCTC	158
ACVRII	NM_001616	GTTGCCATTTGAGGAGGAAA	CCAGCATGTTTCTGCCAATA	114
ATR	NM_001184	CTGAACACGGACATGTGGAC	TGAAAGCAAGTTTTACTGGACT	150
BAX	NM_004324	TGCTTCAGGGTTTCATCC	ACTCGCTCAGCTTCTTGGTG	108
FLJ11186	NM_018353	TGAAACCTTCACTCCTAACAGA	TGGACTGAGGCTCTTAGTGAAC	121
FLJ11222	NM_018365	ACGAAACAAAGCGAAAGCAC	TTTTTCCAACTGATCTTCTTCA	157
FLJ11383	NM_024938	GCCTGAAGCTCTTCTCTCCA	CCAGGACTTCAGGAAAATGA	152
FLJ20139	NM_017685	AGTGCCTGCTGCAATTTTCT	ATGGATGATGCAcTTGCTGA	125
GRB14	NM_004490	TTTTCAGCGAATTTGGCAAT	CTGCACAGAGCATTTTCAGG	138
hMSH3	NM_002439	CCAGCTATCTTCTGTGCATCTC	CAAACACAACCTCGCCTGT	114
hMSH6	NM_000179	ATCGCAGTGTTGGATGTTTT	CGTAATGCAAGGATGGCGT	141
hRad50	NM_005732	AACTGCGACTTGCTCCAGAT	CAATTATGCTTTGCCTCATGG	111
KIAA1052	NM_014956	GGAGCTTGGTGATCCAAGAG	CAAGGCCAGCGAACTTTT	122
MARCKS	NM_002356	CCGCCTCCTCGACTTCTT	AGCCGCTCAGCTTGAAAGAC	120
MBD4	NM_003925	TGAGACCCTCAGTGTGACCA	TTCTGAGTCTTTGGCTGAACAA	139
NADH-UOB	NM_004549	TACATCGGCTTCTTGGGCTA	CAGCATACAGGTAGTCTTCACG	157
OGT	NM_003605	TGGCTGGTCAGAGAAGGAAT	GAAGATGGGAGGGAAAGGAG	115
PRKWNK1	NM_018979	GAAATGGTAGAGTCTGGGTATG	GCTGTTTGAGACTGCTCTCTTC	160
PRO2198	NM_018621	ACTCCAGCCTGGGTGACA	CGTCCCACACCAGAGGTATAA	137
RFC3	NM_002915	TGGTGTTGGAGTGGAAAAAT	CTCGGTCACTATTTCCAGCA	137
RIZ	NM_012231	TTGGAAGCCTGAATAAACACG	CTTGCAGGTGATGAGTGTCC	112
SEC63	NM_007214	GGAGGATGGCAACAGAAGAG	CAGCTTCATTCCCAACGACT	151
TAF1B	NM_005680	GCACTTCTTGCCACAATGTT	CAATCCCAGCCTTTTTCAGT	130
TCF-4	NM_003199	GGGACAAGCAGCCGGGAG	CACCTTGTATGTAGCGAACGC	186
TCF6L1	NM_003201	TGGAAAAAGAAATCATGGACAA	AGCTTTTCCTGCGGTGAAT	160
TGFBRII	NM_003242	CAAGCTCCCCTACCATGACT	TGCTGGTGTTATATTCTTCTGA	158
hMRE11	NM_005590	GAGTGCATTTTCTGACATTTGAG TAC	CTGGCTAAAGCGAAGAACAC	1235
hRad50	NM_005732	TAATCAGACCAGGGACAGAC	GGTTGACTTGTTGGACAGTT	728
hNBS1	NM_002485	CAGGAGGAGAACCATACAGA	TTAATCCTGTACTGGGATGG	969
hUpf1	NM_002911	CATCATCACGCCCTACGAG	AGTTCAGCAGGTGGTTCCAG	299
hUpf2	NM_015542	GAAGCTGCTGGCAAATTCTC	CCAAATCATCGGGACTTGAT	335
β -actin	NM_001101	TGCTATCCCTGTACGCCTCT	GTACTTGCGCTCAGGAGGAG	596

7. Inactivation of nonsense-mediated mRNA decay (NMD) system by using siRNA

Most of the RNA products of frameshift mutant genes form premature translation termination codon (PTC), and might be decayed by NMD system.[34-36] To determine the RNA decay of the mutant transcript by NMD system, we tried to inactivate NMD system by using siRNA techniques. Twenty one nucleotide RNAs were chemically synthesized using Silencer™ siRNA Construction Kit (Ambion Inc., Austin, Tx, USA). Synthetic oligonucleotides were deprotected and gel-purified.[37] HCT116 and SNU C2A cells growing in 6 well dishes were transfected with 200 nM siRNA with oligofectamine (Invitrogen) according to the manufacturer's protocol. RNA was extracted after 72 h incubation for RT-PCR analysis. Targeted nucleotides, numbered relative to the start codon, were as follows: *rent1/hUpf1*, 1879-1901 (5'-AAGATGCAGTTCCGCTCCATTTT-3'); *rent2/hUpf2*, 1423-1445 (5'-AAGGCTTTTGTCCCAGCCATCTT-3').[38] *Luciferase GL2*, 153-173 (5'-AACACGTACGCGGAATACTTCGA-3').[39] The expressional inhibition of *hUpf1* and *hUpf2* of the siRNA targeting was evaluated by semi-quantitative RT-PCR.

8. Microarray Formulation

High-density spotted oligonucleotide microarrays were manufactured at the array core facility at Genome Institute of Singapore. The human Oligolibrary™ was

purchased from Compugen/Sigma-Genosys (Compugen USA Inc., Jamesburg, NJ, USA). It consisted of 18,861 oligonucleotides, representing 18,664 LEADS^{TM} clusters and 197 controls (GAPDH). Sixty mers of synthesized oligomers were robotically printed and processed. Seven cases of colorectal cancer cell lines were included in this study. Total RNA was extracted from 30 to 60 mg of microdissected frozen tissues using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Twenty μg of total RNA was used as input for cDNA target synthesis as previously described.[40] The targets and Universal Human Reference RNA (Stratagene, La Jolla, CA, USA) were hybridized to an oligonucleotide microarray containing 18,664 probe sets representing 18,664 unique (LEADS^{TM}) genes, and the array was scanned using GenePix scanners. Expression values for *hRad50, hMRE11, NBS1*, and other DNA repair genes were calculated by using the GenPix Pro 4.0 analysis software.

9. Western blotting

For Western blot analysis, tumor cell pellets were suspended in ice-cold lysis buffer(50 mM Tris (pH 7.4), 1% Triton X-100, 5 mM EDTA, 1 mM KCl, 140 mM NaCl, 2 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1% aprotinin, 1 uM leupeptin, and 1 mM sodium orthovanadate) for 45 min. After being centrifuged for 5 min at 4°C, the supernatant was collected and used for nuclear protein assay. Twenty μg of total protein lysates were loaded into each lane, size-fractionated by SDS-PAGE, and transferred to a polyvinylidene difluoride membranes that were blocked with Tris-buffered saline-Tween 20

containing 5% skim milk. Primary antibodies, hRad50 (C-19, polyclonal; Santa Cruz Biotech, Santa Cruz, CA, USA) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH; polyclonal; Trevigen, Gaithersburg, MD, USA) were diluted 1:5000 in blocking buffer and incubated for 1 h at room temperature. After washing, membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, UK), washed, and then developed with ECL-Plus (Amersham Biosciences UK Limited).

10. Comparative genomic hybridization and digital image analysis

Thirty-nine cases confirmed as MSI-H colorectal carcinomas, and 20 cases of right-sided MSS colorectal carcinomas were included in this study. In each case, grossly normal mucosa remote from the tumor was included as a control. Genomic DNA samples from tumors were labeled with Spectrum Green dUTP (Vysis Inc., Downers Grove, IL, USA), and normal reference genomic DNA was labeled with Spectrum Red dUTP (Vysis Inc.,) using the nick translation technique. Labeled tumor and reference DNA (200-400 ng), and 10 μg of unlabeled human Cot-1 DNA (Vysis Inc.,) were dissolved in 10 μl of hybridization buffer (50% formamide, 10% dextran sulfate, and 2 X SSC) and denatured at 72℃ for 2 min. Hybridization was performed at 37℃ on denatured normal metaphase spreads. After hybridization for 3 days, the slides were washed and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) in antifade solution. CGH

hybridizations were analyzed using an Olympus fluorescent microscope and the Cytovision image analysis system (Applied Imaging, Sunderland, Tyne & Wear, UK). Three digital images (DAPI, Spectrum Green, Spectrum Red) were acquired from 10-20 metaphases in each hybridization. Normal male DNA and DNA from tumor cell lines with known aberrations were used as DNA test controls. Green to red intensity ratio profiles were calculated for each chromosome and threshold values defining gains and losses were set at 1.25 and 0.75, respectively. Only the chromosomal losses and gains were analyzed, as reciprocal translocation can not be differentiated by CGH analysis. We regarded tumors as CIs when any chromosomal losses or gains were present in the tumors. The CIs of 5 chromosomal arms were validated by PCR-LOH analysis using 7 microsatellite markers (D9S195, D16S521, D17S578, D17S250, D18S58, D18S57, DCC).

11. Statistical Analysis

Clinicopathological variables and the presence of frameshift mutations of the target genes were cross-tabulated with the source (MSI-H colorectal carcinomas with CIs and those without CIs), and the significance of association was determined by using the Pearson's chi-square test or Fisher's exact test. The mean number and mean incidence of frameshift mutations of target genes in MSI-H colorectal carcinomas with and without CIs were determined using Student T-test. All calculations were performed using the SPSS 10.0 for Windows statistical software package.

III RESULTS

1. Identification of MSI-H colorectal carcinomas

Microsatellite instability screening revealed that 39 (11.9%) cases out of 329 colorectal carcinomas showed additional bands in the normal allele regions in 3 mononucleotide repeat markers and evaluated as MSI-H colorectal carcinomas. Summary of clinicopathological features of these 39 cases of MSI-H colorectal carcinomas is in the Table 3.

2. Alteration of Mismatch repair genes in MSI-H colorectal carcinomas

We analyzed the expression of hMLH1 and hMSH2, member of mismatch repair genes, in MSI-H colorectal carcinomas. Thirty one cases out of 39 (79%) MSI-H colorectal carcinomas did not express hMLH1 gene products by using tissue array immunostaining analysis, while all of 39 (100%) MSI-H colorectal carcinomas expressed hMSH2 gene products. *hMLH1* methylation analysis showed 34 cases out of 39 (87%) MSI-H colorectal carcinomas showed *hMLH1* gene promoter hypermethylation.

Table 3. Clinicopathological features of 39 cases of MSI-H colorectal carcinomas

No.	BAT 26	BAT 25	BAT 40	Sex	Age	Location	TNM	Differentiation	Mucin Formation	Peritumoral Lymphoid reaction	Extramural vessel invasion
1	+	+	+	Male	62	right	II	Well	Absent	Present	Absent
2	+	+	+	Female	73	right	I	Moderate	Absent	Present	Absent
3	+	+	+	Female	53	right	III	Poor	Absent	Present	Absent
4	+	+	+	Male	52	right	IV	Poor	Present	Absent	Present
5	+	+	+	Female	66	right	III	Poor	Absent	Present	Absent
6	+	+	+	Male	62	left	I	Well	Absent	Absent	Absent
7	+	+	+	Male	40	right	III	Poor	Present	Present	Absent
8	+	+	+	Female	57	left	I	Moderate	Absent	Present	Absent
9	+	+	+	Male	45	right	I	Moderate	Present	Present	Absent
10	+	+	+	Male	34	right	III	Poor	Absent	Present	Absent
11	+	+	+	Male	39	right	II	Well	Present	Absent	Absent
12	+	+	+	Male	61	right	III	Poor	Absent	Present	Present
13	+	+	+	Male	37	left	II	Well	Present	Present	Absent
14	+	+	+	Male	45	left	II	Well	Present	Present	Absent
15	+	+	+	Male	57	left	II	Poor	Absent	Absent	Present
16	+	+	+	Male	66	right	II	Moderate	Present	Absent	Absent
17	+	+	+	Female	40	right	II	Moderate	Absent	Present	Absent
18	+	+	+	Female	68	right	II	Poor	Present	Present	Absent
19	+	+	+	Male	47	right	II	Moderate	Present	Absent	Absent
20	+	+	+	Female	67	right	III	Poor	Present	Absent	Present
21	+	+	+	Female	61	right	II	Poor	Absent	Present	Absent
22	+	+	+	Male	49	left	II	Poor	Absent	Present	Absent
23	+	+	+	Male	51	left	III	Moderate	Present	Present	Absent
24	+	+	+	Male	39	right	II	Poor	Present	Absent	Absent
25	+	+	+	Female	53	right	II	Well	Present	Present	Absent
26	+	+	+	Male	71	right	III	Moderate	Present	Present	Present
27	+	+	+	Female	68	right	II	Poor	Absent	Present	Absent
28	+	+	+	Male	46	right	II	Poor	Absent	Present	Absent
29	+	+	+	Female	51	right	I	Well	Present	Present	Absent
30	+	+	+	Male	53	right	II	Poor	Present	Present	Absent
31	+	+	+	Female	41	right	II	Poor	Present	Present	Absent
32	+	+	+	Female	57	right	II	Poor	Present	Present	Present
33	+	+	+	Female	65	right	II	Poor	Present	Present	Absent
34	+	+	+	Female	51	right	II	Moderate	Present	Absent	Absent
35	+	+	+	Male	70	right	II	Poor	Absent	Present	Absent
36	+	+	+	Female	67	right	III	Poor	Present	Present	Present
37	+	+	+	Female	42	right	II	Poor	Absent	Absent	Absent
38	+	+	+	Male	80	left	II	Moderate	Present	Present	Absent
39	+	+	+	Female	36	right	II	Poor	Present	Present	Absent

Table 4. Analysis of hMLH1 and hMSH2 gene expression and *hMLH1* promoter methylation in 39 MSI-H colorectal carcinomas

Case No.	hMLH1 expression	*hMLH1* methylation	hMSH2 expression
1	Unexpressed	Methylated	Expressed
2	Unexpressed	Methylated	Expressed
3	Unexpressed	Methylated	Expressed
4	Unexpressed	Methylated	Expressed
5	Unexpressed	Methylated	Expressed
6	Unexpressed	Methylated	Expressed
7	Unexpressed	Methylated	Expressed
8	Unexpressed	Methylated	Expressed
9	Unexpressed	Methylated	Expressed
10	Unexpressed	Methylated	Expressed
11	Unexpressed	Methylated	Expressed
12	Unexpressed	Methylated	Expressed
13	Unexpressed	Methylated	Expressed
14	Unexpressed	Methylated	Expressed
15	Unexpressed	Methylated	Expressed
16	Unexpressed	Methylated	Expressed
17	Unexpressed	Methylated	Expressed
18	Unexpressed	Methylated	Expressed
19	Expressed	Unmethylated	Expressed
20	Unexpressed	Methylated	Expressed
21	Unexpressed	Methylated	Expressed
22	Expressed	Unmethylated	Expressed
23	Unexpressed	Methylated	Expressed
24	Unexpressed	Methylated	Expressed
25	Unexpressed	Methylated	Expressed
26	Expressed	Methylated	Expressed
27	Unexpressed	Methylated	Expressed
28	Unexpressed	Methylated	Expressed
29	Expressed	Methylated	Expressed
30	Unexpressed	Methylated	Expressed
31	Expressed	Unmethylated	Expressed
32	Expressed	Unmethylated	Expressed
33	Unexpressed	Methylated	Expressed
34	Unexpressed	Methylated	Expressed
35	Unexpressed	Methylated	Expressed
36	Expressed	Methylated	Expressed
37	Unexpressed	Methylated	Expressed
38	Expressed	Unmethylated	Expressed
39	Unexpressed	Methylated	Expressed
Ratio	Unexpression: 31/39, 79%	Methylation: 34/39, 87%	Expression: 39/39, 100%

3. Identification of target genes in MSI-H colorectal carcinoma.

To identify target gene of MSI-H colorectal carcinomas, we analyzed 45 genes which have repeated mononucleotide sequence in its coding region. Frameshift mutation analysis revealed most of genes showed different frequency of mutation ratio (Fig. 2). Seven genes, *TGFβR II, TAF1B, MARCKS, ACVR II, FLJ11383, FLJ11186, and TCF-4* showed more than 50% of mutation frequency in MSI-H colorectal carcinomas and MMR-deficient colorectal cancer cell lines.

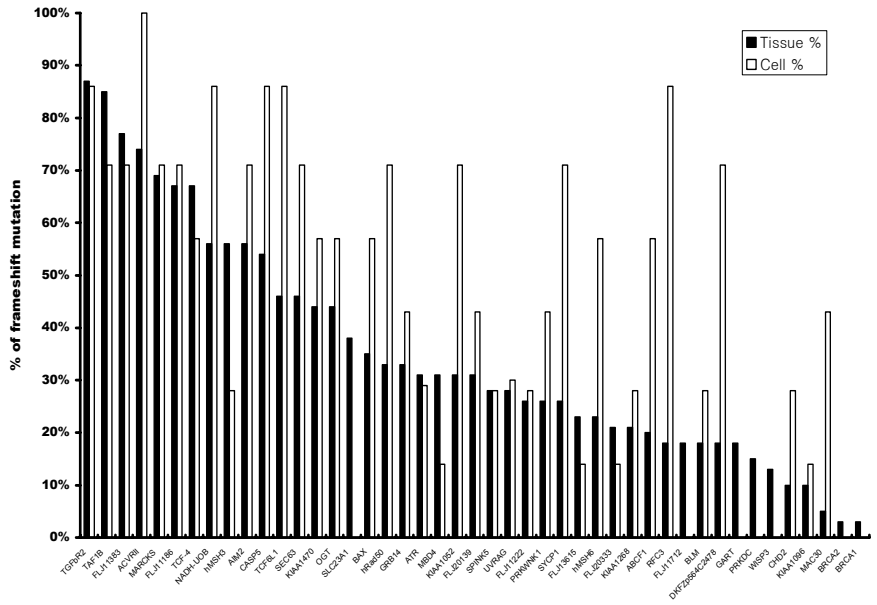


Figure 2. Frequency of frameshift mutations of 45 genes in 39 MSI-H colorectal carcinomas (dark bar) and 7 MMR-deficient colorectal cancer cell lines (white bar). Seven genes, *TGFβR II, TAF1B, MARCKS, ACVR II, FLJ11383, FLJ11186,* and *TCF-4* showed more than 50% of mutation frequency in MSI-H colorectal carcinomas and MMR-deficient colorectal cancer cell lines.

4. Expression of wild and mutant target gene products in the MMR-deficient colorectal cancer cell lines

We analyzed wild and frameshift mutant type mRNA expression of 25 target gene in 7 MMR-deficient colorectal cancer cell lines by using RT-PCR analysis. Three MMR-proficient colorectal cancer cell lines were used as control. We found that the expression of frameshift mutant gene products were different in the 25 target genes. Frameshift mutant transcripts were detected in 18 out of 25 target genes, while 7 target genes were not expressed mutant transcripts in our 7 MMR-deficient cancer cell lines (Table 5).

Table 5. Expression of frameshift mutant mRNAs in 7 MMR-deficient colorectal cancer cell lines

Gene name	Type of frameshift mutation	Type of repeat	Position of mononucleotide repeat	Position of PTC	Distance from last exon-exon junction	Detection of mRNA (Detected No. of mutant mRNA/ No. of frameshift mutation)
ABCF1	-1	A10	312	497	1853	- (0/3)
	-2			359	1990	- (0/1)
hMSH6	-1	C8	3341	3352	735	- (0/2)
	+1			3361	728	- (0/2) *
hRad50	-1	A9	2544	2590	1548	- (0/4)
	+1			2566	1574	- (0/1)
PRKWNK1	-1	A10	1739	1777	5053	- (0/3)
RFC3	-1	A10	317	398	562	- (0/6)
SEC63	-1	A10	1776	1864	454	- (0/4)
	-2			1852	465	- (0/1)
FLJ11186	-1	A11	711	788	2756	- (0/3)
	-2			740	2804	- (0/1)
BAX	-1	G8	183	244	193	+ (4/4)
	+1			286	153	+ (1/1)
hMSH3	-1	A8	1130	1229	2061	+ (2/2)
TAF1B	-1	A11	285	369	1293	+ (1/1)
	-2			300	1361	+ (4/4)
TFAM	-1	A10	564	577	148	+ (5/5)
	-2			616	108	+ (1/1)
TGFβRII	-1	A10	709	819	1039	+ (6/6)
	-2			723	1134	+ (1/1)
ACVRII	-1	A8	1466	1484	25	+ (7/7)
	-2			1526	-18	+ (1/1)
MARCKS	-1	A10	823	865	Last exon	+ (4/4)
	-2			913	Last exon	+ (1/1)
TCF-4	-1	A9	1684	1757	Last exon	+ (4/4)
KIAA1052	+1	A11	484	757	3677	+ (1/1)
	-1			556	3876	+ (2/2)
	-2			754	3677	+ (3/3)
FLJ20139	-1	A10	433	470	Last exon	+ (3/3)
FLJ11222	-1	A10	761	793	766	+ (2/2)
OGT	-1	T10	340	404	2448	+ (4/4)
ATR	-1	A10	2416	2437	5428	+ (2/2)
MBD4	-1	A10	1106	1125	715	+ (1/1)
GRB-14	-1	A9	1423	1495	520	+ (3/3)
NDUFC2	-1	T9	340	364	86	+ (6/6)
RIZ	-1	A9	5315	5543	492	+ (4/4)

5. Identification of frameshift mutant target gene product after inhibition of NMD system

The frameshift mutant transcrips of 7 genes, which were not found in the MMR-deficient colorectal cancer cell lines, can be identified when treated cell lines with puromycin, a kind of translation inhibitor (Fig. 3). These findings indicate that the frameshift mutant transcripts of 7 genes are actively degraded after transcription. Most of the frameshift mutant transcripts show premature stop codon, and it is well known that the transcripts with premature stop codon are actively degraded by nonsense-mediated mRNA decay (NMD).[34-36] We therefore assumed that the aberrant transcripts from frameshift mutant products are degraded by NMD system. To clarify this issue, we applied gene knowndown system with siRNA in two genes, *hUpf1* and *hUpf2*, of the NMD system. Synthetic short-interfering RNA (siRNA) duplexes were used to inhibit expression of *hUpf1* and *hUpf2* which are reported as key players in NMD system.[41, 42] RT-PCR analysis revealed siRNA repressed more than 50% loss of expression of both targeted transcripts (Fig. 4). We found the frameshift mutant transcripts of 7 genes in the MMR-deficient colorectal cancer cell lines when the expression of *hUpf1* and *hUpf2* were inhibited by the siRNA treatment (Fig. 5).

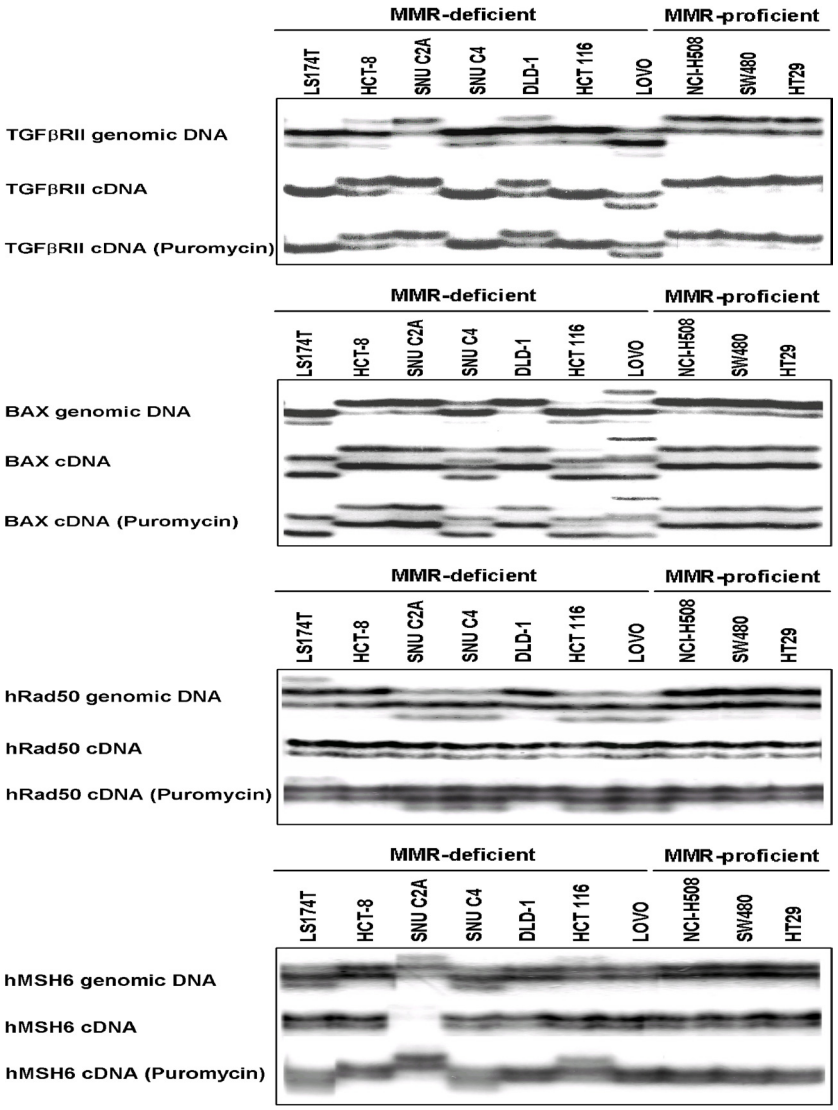


Figure 3. Analysis of frameshift mutations and expression profiles of *TGFβR II, BAX, hRad50, and hMSH6* in 7 MMR-deficient and 3 MMR-proficient colorectal cancer cell lines. RT-PCR analysis showed that disappeared hRad50 and hMSH6 mutant transcripts reappeared after puromycin treatment, whereas TGFβR II and BAX mutant transcripts were not changed.

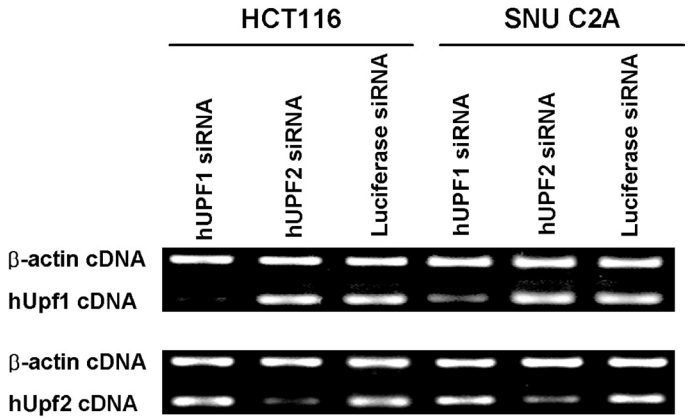


Figure 4. Sequence-specific inhibition of gene expression with RNAi in mammalian cells. HCT116 and SNU C2A cells were transfected with siRNA duplexes directed against *hUpf1, hUpf2*, and firefly luciferase (a negative control). Seventy-two hours after transfection, cells were harvested and RNAs were extracted. Expression of hUpf1 and hUpf2 were analyzed by RT-PCR. *β-actin* was used as a control for nonspecific effects of RNAi treatment.

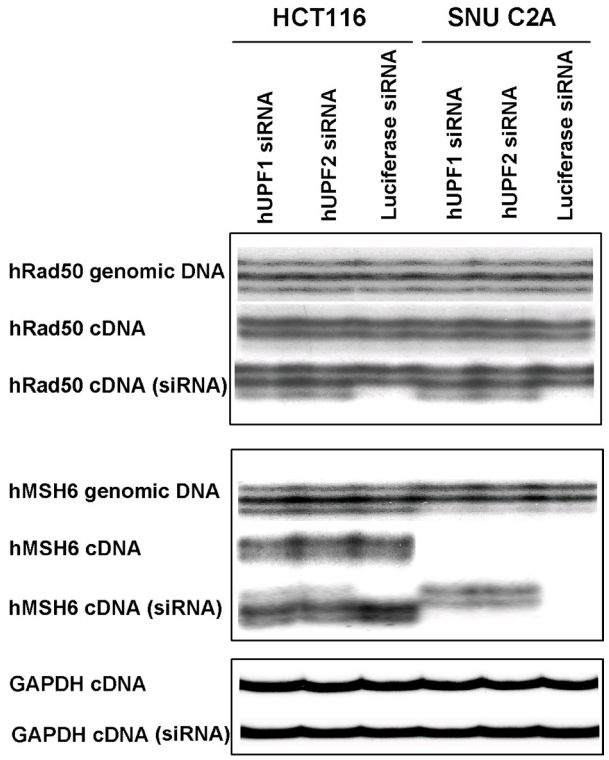


Figure 5. RT-PCR analysis of *hRad50* and *hMSH6* gene expression in HCT116 and SNU C2A cell lines after treated with *hUpf1* siRNA and *hUpf2* siRNA. Disappeared hRad50 and hMSH6 mutant transcripts were reappeared after *hUpf1* siRNA and *hUpf2* siRNA treatment.

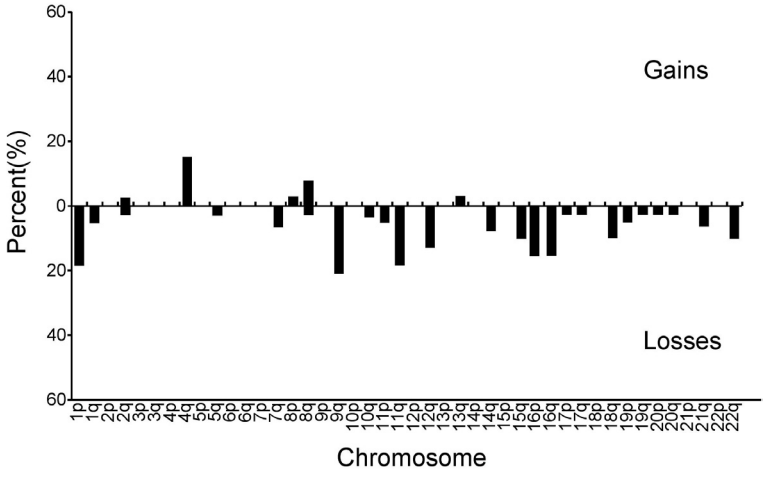
6. CIs in MSI-H colorectal carcinomas

In order to clarify the relationship between CIN and MSI, we analyzed CIN in our 39 MSI-H colorectal carcinomas and compared the results with changes in 20 MSS carcinomas. We found CIs in 12 (31%) of 39 MSI-H colorectal carcinomas by CGH analysis and the average number of CIs in MSI-H carcinomas was 2.1. CIs

showed a rather random distribution over the chromosomes, although there were more losses than gains (ratio of losses to gains; 5.9:1). Chromosomal arm imbalances did not exceed 25% in MSI-H tumors. Most frequent CIs in MSI-H tumors were gains of 4q (15%) and losses of 9q (21%), 1p (18%) and 11q (18%).

In the 20 MSS colorectal carcinomas, we found CIs in 14 (70%) cases of 20 MSS colorectal carcinomas (Fig. 6A). In addition to more frequent CIs in the MSS tumors (31% versus 70%, p=0.004), the average number of CIs was significantly higher in the MSS carcinomas (2.1 versus 4.4, p=0.04). Moreover, the MSS carcinomas showed distinct and different CIs from the MSI-H carcinomas. Frequent changes in MSS carcinomas were gains of 8q (50%), 13q (35%), and 20q (25%), and losses of 18q (55%), 15q (35%), and 17p (30%) (Fig. 6B). None of these changes were included in the chromosomal arms showing frequent changes in our 39 MSI-H colorectal carcinomas. CIs in all of the 5 chromosomal arms were validated by 7 microsatellite markers. The PCR-LOH results were matched with all 20 MSS tumors, however it could not be evaluated in most of the MSI-H tumors due to frequent microsatellite instability in these 7 markers (Fig.7).

A. MSI-H colorectal carcinomas



B. MSS colorectal carcinomas

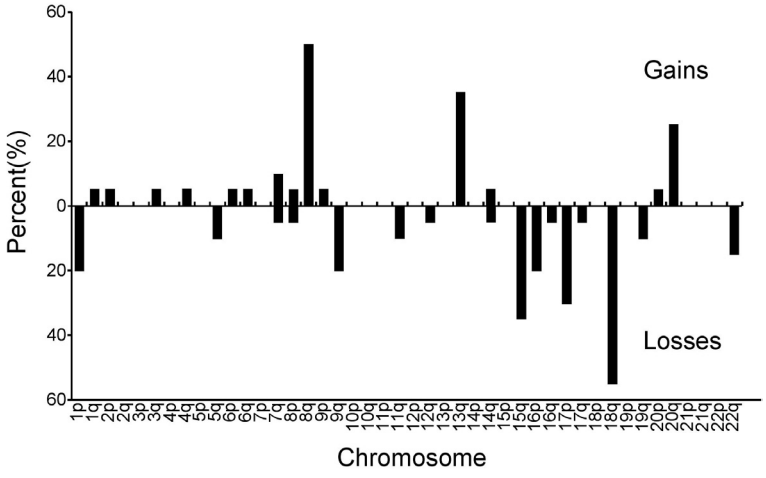


Figure 6. The rate of chromosomal losses and gains observed in 39 designated non-acrocentric chromosomal arms of MSI-H colorectal carcinomas (**panel A**) and 20 MSS colorectal carcinomas (**panel B**). Each bar represents the percentage loss (**lower**) or gain (**upper**) of a chromosomal arm.

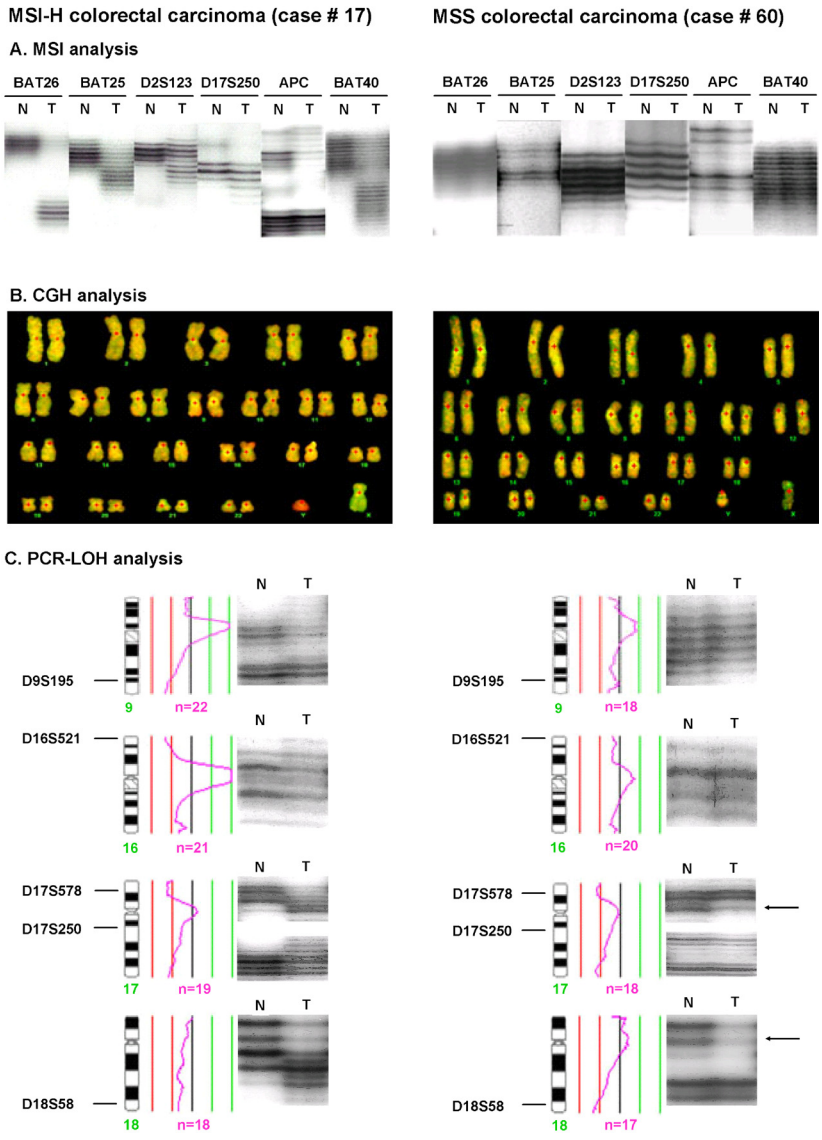


Figure 7. Examples of MSI, CGH and PCR-LOH analysis in MSI-H (case No. 17) and MSS (case No. 60) colorectal carcinoma with CIs. Panel A: MSI analysis. Additional alleles were found at 6 microsatellite markers in a MSI-H colorectal carcinoma and no changes were found in a MSS colorectal carcinoma. Panel B: CGH analysis. CIs were found in 9q, 16p, 17p, and 17q in MSI-H colorectal carcinoma (left) and 8q, 16p, 17p, 17q, 18q in MSS colorectal carcinoma (right).

Panel C: Comparison of CGH analysis and PCR-LOH. PCR-LOH analysis showed same allelic imbalance (arrow) in 17p and 18q in MSS colorectal carcinoma (right), whereas comparison was not possible in MSI-H carcinoma due to shifted bands in 9q, 16p, 17p, 17q, and 18q (left).

7. Relationship between CIs and target gene mutations in MSI-H colorectal carcinomas

We analyzed the relationship between the incidence of CIs and the incidence of frameshift mutations of the 45 target genes we analyzed previously.[16, 28, 29] The mean number of frameshift mutations of the 45 target genes was 18.3 in the MSI-H carcinomas with CIs and 15.4 in the MSI-H carcinomas without CIs (p=0.20). The mean incidence of frameshift mutations of the 45 target genes in MSI-H colorectal carcinomas with CIs was 41%, and in MSI-H colorectal carcinomas without CIs was 34% (p=0.20). There was no difference in the incidence of frameshift mutations of the 45 target genes in MSI-H carcinomas with and without CIs, except for the *hRAD50* and *FLJ11383* genes. Mutation of *hRAD50*, a member of the DNA repair genes, was significantly related to MSI-H colorectal carcinomas with CIs (p=0.01). Mutation of *FLJ11383*, the function of which is unknown, was also frequent in MSI-H tumors with CIs (p=0.02). There was also a tendency towards more frequent frameshift mutations of *ATR* and *MBD4*, both of which are DNA repair genes, in MSI-H colorectal carcinomas with CIs (Table 6).

Table 6. Comparison of frameshift mutations of target genes in MSI-H colorectal carcinomas with and without CIs

	% of frameshift mutations in MSI-H colorectal carcinomas							
	With CIs (no.)		Without CIs (no.)					
Genes	Total mutation *	Biallelic mutation **	Total mutation *	Biallelic mutation **	P value *	P value **	Type of repeat	Reported function of wild type gene product
hRAD50	67(8)	0(0)	19(5)	0(0)	0.01		A(9)	DNA double strand breaks repair
FLJ11383	100(12)	58(7)	63(17)	29(5)	0.02	0.12	A(10)	Unknown
ATR	50(6)	0(0)	22(6)	0(0)	0.13		A(10)	Checkpoint kinase
MBD4	50(6)	33(2)	22(6)	0(0)	0.13	0.46	A(10)	DNA glycosylase, Methyl-CpG binding protein
RFC3	33(4)	0(0)	15(4)	0(0)	0.22		A(10)	DNA replication factor
FLJ13615	42(5)	0(0)	19(5)	0(0)	0.23		A(11)	Unknown
TGFβRII	100(12)	83(10)	81(22)	68(15)	0.30	0.68	A(10)	Tumor suppressor
BRCA2	8(1)	0(0)	0(0)	0(0)	0.32		A(8)	Tumor suppressor
FLJ11712	8(1)	8(1)	22(6)	0(0)	0.4	0.31	A(10)	Unknown
PRKDC	8(1)	0(0)	22(6)	0(0)	0.4		A(10)	DNA-dependent protein kinase
ABCF1	33(4)	0(0)	19(5)	0(0)	0.42		A(10)	Unknown
MARCKS	83(10)	20(2)	67(18)	28(5)	0.45	0.68	A(11)	Cell proliferation and differentiation
FLJ11186	75(9)	0(0)	59(16)	4(1)	0.48	1	A(11)	Unknown
GRB-14	42(5)	0(0)	30(8)	0(0)	0.49		A(9)	Growth factor bound protein
NADH-UOB	67(8)	0(0)	52(14)	0(0)	0.49		T(9)	NADH ubiquinone oxydoreductase
Caspase 5	42(5)	17(1)	59(16)	40(6)	0.50	0.61	A(10)	Apoptosis
MAC30	8(1)	0(0)	4(1)	0(0)	0.53		A(10)	Unknown
BLM	25(3)	0(0)	15(4)	0(0)	0.65		A(9)	DNA helicase
DKFZp564C2478	25(3)	8(1)	15(4)	4(1)	0.65	0.53	A(10)	Unknown
TAF1B	92(11)	0(0)	81(22)	11(3)	0.65	0.54	A(11)	Transcription factor
TCF6L1	58(7)	0(0)	44(12)	0(0)	0.65		A(10)	Transcription factor
GART	25(3)	0(0)	19(5)	0(0)	0.68		A(10)	Unknown
ACVR II	83(10)	50(5)	70(19)	47(9)	0.69	1	A(8)	Growth factor receptor
KIAA1052	25(3)	0(0)	37(10)	0(0)	0.71		A(11)	Unknown
SPINK5	25(3)	0(0)	37(10)	0(0)	0.71		A(10)	Serine protease inhibitor
TCF4	75(9)	33(3)	63(17)	24(4)	0.71	0.66	A(9)	Transcription factor
BAX	42(5)	20(1)	33(9)	22(2)	0.72	1	G(8)	Apoptosis
UVRAG	42(5)	0(0)	33(9)	0(0)	0.72		A(10)	Unknown

hMSH3	50(6)	17(1)	59(16)	13(2)	0.85	1	A(8)	DNA mismatch repair
KIAA1470	50(6)	8(1)	41(11)	11(3)	0.85	1	A(10)	Unknown
SEC63	58(7)	17(2)	56(15)	15(4)	0.85	1	A(10)	ER membrane protein
AIM2	67(8)	8(1)	67(18)	19(5)	1	0.65	A(10)	IFN inducible
BRCA1	0(0)	0(0)	4(1)	0(0)	1		A(8)	Tumor sppresor
CHD2	8(1)	8(1)	15(4)	0(0)	1	0.31	A(10)	Sequence-selective DNA binding protein
FLJ11222	25(3)	0(0)	26(7)	0(0)	1		A(100	Unknown
FLJ20139	25(3)	8(1)	30(8)	19(5)	1	0.65	A(10)	Unknown
FLJ20333	17(2)	0(0)	22(6)	0(0)	1		A(10)	Unknown
hMSH6	17(2)	0(0)	26(7)	29(2)	1	1	C(8)	DNA mismatch repair
KIAA1096	8(1)	0(0)	15(4)	0(0)	1		A(10)	Unknown
KIAA1268	25(3)	8(1)	22(6)	0(0)	1	0.31	A(10)	Unknown
OGT	42(5)	8(1)	41(11)	11(3)	1	1	T(10)	O-linked GlcNAc transferase
PRKWNK1	25(3)	0(0)	26(7)	4(1)	1	1	A(10)	Unknown
SLC23A1	42(5)	0(0)	37(10)	0(0)	1		C(9)	Nucleobase transporter
SYCP1	25(3)	0(0)	22(6)	0(0)	1		A(10)	Synaptonemal complex
WISP3	8(1)	0(0)	15(4)	25(1)	1	1	A(9)	Growth factor (Wnt pathway)

* p value of total allelic mutations with and without CIs
** p value of biallelic mutations with and without CIs

8. hRad50 complex expression in MSI-H colorectal carcinomas

We evaluated the expression of hRad50 because mutation of *hRad50* was frequent in MSI-H tumors, and mutation of *hRad50* is related to the CIs in the MSI-H tumors (p=0.01). hRad50 gene product forms a complex with hMRE11 and hNBS1, and involve both in the homologous recombination (HR) and nonhomologous end joining (NHEJ). We therefore analyzed the expression of hRad50, hMRE11, and hNBS1 transcripts in MMR-deficient colorectal cancer cell lines. Microarry analysis showed transcription expression value of hRad50 and hMRE11 gene were decreased, while hNBS1 was not changed (Fig. 8). Semi-

quantitative RT-PCR analysis showed transcript expression of hMRE11 was decreased and alternative spliced in MMR-deficient colorectal cancer cell lines (Fig. 9). Mutation analysis revealed that repeated (T11) is located in intron 4 of *hMRE11* gene and showed frameshift mutations in MMR-deficient colorectal cancer cell lines (confirmed by automatic sequencing). hRad50 protein expression was decreased in frameshift mutant MMR-deficient colorectal carcinoma cell lines (Fig. 10).

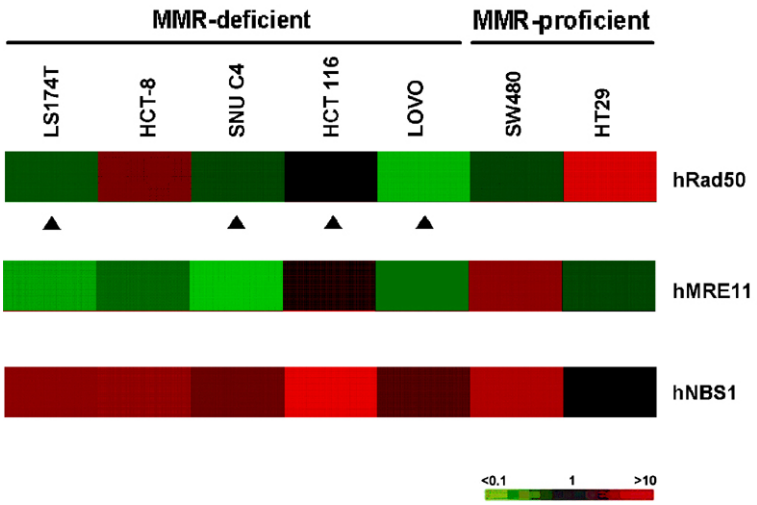


Figure. 8. Identification of hRad50, hMRE11, and hNBS1 transcript expression in MMR-deficient and MMR-proficient colorectal cancer cell lines. hRad50 transcript was decreased in frameshift mutant MMR-deficient colorectal cancer cell lines and hMRE11 transcript was also decreased in MMR-deficient colorectal cancer cell lines, whereas hNBS1 was not changed.

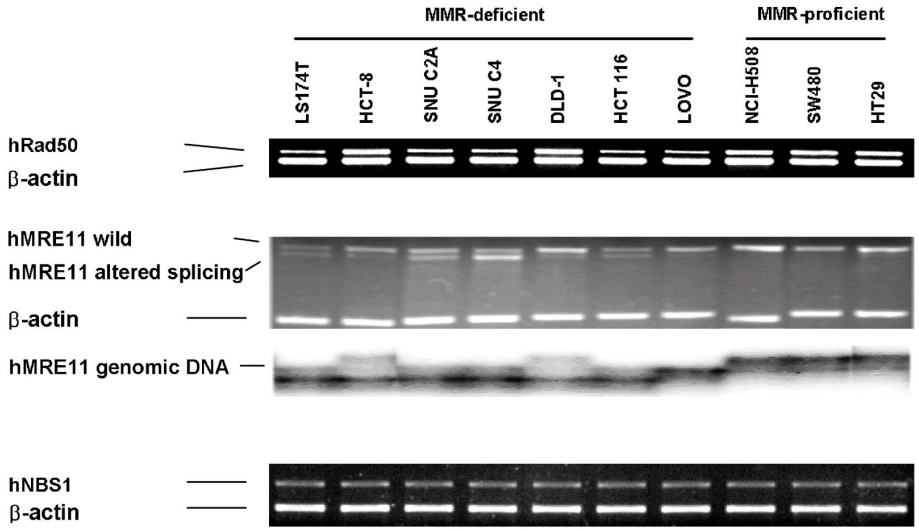


Figure. 9. Identification of hRad50, hMRE11, and hNBS1 gene expression by using RT-PCR analysis. hRad50 transcripts were decreased in frameshift mutant colorectal cancer cell lines and hMRE11 showed frameshift mutation and altered splicing in all of MMR-deficient colorectal cancer cell lines, whereas hNBS1 transcripts were not changed.

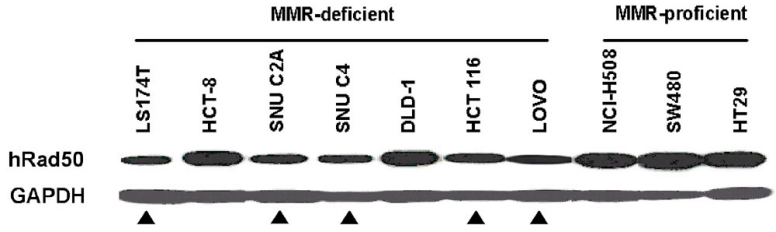


Figure. 10. Western blotting analysis of hRad50 in MMR-deficient and MMR-proficient colorectal cancer cell lines. Protein expression of hRad50 was decreased in 5 frameshift mutant colorectal cancer cell lines. (▲: frameshift mutation of *hRad50*)

IV. *DISCUSSION*

The development of genomic instability is an important event in the multistep progression of colorectal carcinogenesis. Molecular genetics of human cancers can be used to categorize colorectal carcinomas into two major types of genomic instabilities, chromosomal instability (CIN) and microsatellite instability (MSI).[2] CIs are common phenomena in human cancers, but their causes and consequences are not well defined. Of the two main forms of genomic instability, CIN and MSI, CIN is known to be closely associated with CIs, and the CIN phenotype is characterized by gross rearrangement of chromosomes. Common CIs include the loss or gain of whole chromosomes or chromosomal fragments, and amplification of chromosome.[43] Chromosomal loss can lead to the inactivation of tumor suppressor genes, whereas chromosomal gains can lead to the activation of proto-oncogenes. The other frequent CIs involves translocation, which can lead to the deregulation of gene expression or aberrant gene expression related to tumorigenesis by causing two genes to fuse.[44] In contrast, the MSI pathway begins with the inactivation of one of a group of genes responsible for DNA nucleotide mismatch repair, which leads to extensive point mutations and frameshfit mutations in repetitive DNA sequences with low frequencies of allelic losses and rare alterations of tumor DNA content.[45] Frequent somatic frameshift mutations in the genes containing nucleotide repeats in their coding sequences have been reported,[15, 16, 28, 46, 47] and many of these genes are regarded as target genes in MSI-H tumors. Recent studies have found that most of the sporadic MSI-H carcinomas result in the

inactivation of the hMLH1 gene, principally by transcriptional silencing.[48, 49] In this study, we evaluated 39 (11.9%) cases out of 329 colorectal carcinomas as MSI-H colorectal carcinomas. In these MSI_H colorectal carcinomas, 31 (79.5%) cases out of 39 showed absent or decreased expression of hMLH1 by using tissue array immunostaining analysis, while all of MSI-H colorectal carcinomas showed positive expression of hMSH2 gene product. Thirty-four out of 39 (87.2%) MSI-H colorectal carcinomas showed *hMLH1* gene promoter hypermethylation. This result indicate that most of the sporadic MSI-H carcinomas result from the inactivation of the hMLH1 gene, principally by transcriptional silencing. The reported target genes of MSI-H tumors can be functionally categorized as tumor suppressors, and genes involved in apoptosis and DNA repair. [14, 16, 28, 50, 51] Many genes involved in DNA damage signaling and DNA repair pathways play critical roles in the suppression of genome instabilities. Inactivating mutations of *ATM, BRCA1, BRCA2, NBS1*, and *BLM* genes cause defects in DNA damage signaling and DNA repair, give rise to some form of CIN, and contribute to carcinogenesis.[1] We and others have reported that several genes involved in DNA repair have mononucleotide repeats in their coding sequences and are frequently mutated in MSI-H colorectal carcinomas.[16, 28, 47, 52] Among the 10 DNA repair genes involved in this study, *hMSH3* and *hRad50* showed more than 30% mutation frequency in MSI-H colorectal carcinomas and MMR-deficient colorectal cancer cell lines.

An important consequence of the frameshift mutations of target genes is the formation of premature translation termination codon (PTC). In the mammalian cells it is well known that the degradation of mRNAs containing PTC either by

nonsense mutations or by frameshift mutations are actively degraded by nonsense mediated mRNA decay (NMD), thus avoiding the production of potentially deleterious truncated proteins.[42, 53] We analyzed PTC in MSI-H target genes and found that most of the MSI-H target genes form PTC result in frameshift mutation. We assumed that these target genes may be degraded by NMD. mRNA expression of frameshift mutant genes were different in the 25 genes. mRNA derived from frameshift mutant genes can be detected in 18 out of 25 genes, while 7 target genes such as *hRad50, hMSH6, SEC63, ABCF1, PRKWNK1, FLJ11186*, and *RFC3* showed no aberrant mRNAs in our 7 MMR-deficient colorectal cancer cell lines. These 7 genes with frameshift mutations and no mRNAs for the frameshift mutant, the mutant transcripts were detected after NMD inhibition.

The tumor development stage and the genetic events that initiate CIs in MSI-H colorectal carcinomas are important for the understanding of the molecular pathogenesis of colorectal carcinomas. We found CIs in a subset of MSI-H colorectal carcinomas. Although it is widely accepted that CIs are rare in MSI-H tumors, a few studies on CIs in MSI-H carcinomas are available. The majority of previous studies about CIs in MSI-H tumors have included a small number of cases, and the results obtained have been conflicting.[21, 22, 54-56] The coexistence of two kinds of genomic instability in several colorectal cancer cell lines had been reported.[23] In this study, we examined CIs in a relatively large number of cases (39), and found CIs in 12 (31%) cases. These findings indicate that some colorectal carcinomas have both types of genomic instability, and therefore, it might show biologic behaviors that differ from those showing only one of the two typical

genetic instabilities. We previously proposed a role for frameshift mutations during malignant transformation. We found that the incidence and type of inactivation patterns of the MMR genes were the same in MSI-H gastric adenomas and carcinomas, but that frameshift mutations were much more frequent in MSI-H gastric carcinomas.[57] This suggests that the accumulated frameshift mutations of the target genes related to the manlignant transformation of MSI-H tumors. The incidence of frameshift mutations of the target genes in our MSI-H tumors with CIs was slightly higher than that of the MSI-H tumors without CIs, suggesting that the progression period of the MSI pathway might be longer in MSI-H tumors with CIs. We also found that chromosomal changes in MSI-H colon carcinomas are much less frequent than changes of MSS carcinomas and that the types of chromosomes involved are quite different. These findings suggest that some tumors following the MSI pathway can have mutations in the genes responsible for CIN and these defects might be selected[23] during tumor progression. However, the genetic pathways responsible for inducing CIN might differ from that of MSS tumors.

We analyzed the relationship between target gene mutation and CIs in MSI-h tumors, and found that frameshift mutation of *hRad50* is significantly related to CIs in MSI-H tumors. hRad50 forms a comples with hMRE11 and hNBS1. Gene product expression of hRad50, hMRE11, and hNBS1 revealed that hRad50 transcript and protein were decreased and hMRE11 transcript was also decreased and alternative spliced in MMR-deficient colorectal cancer cell lines, while hNBS1 was not changed. Frameshift mutation analysis showed that (T11) was located in intron 4 in *hMRE11* genes and frameshift mutated in 7 MMR-deficient colorectal

cancer cell lines. The role of this hererotrimer in DNA damage signaling and chromosome instability had been reported.[58-60] Based on the reported functional characteristics of the hRad50/hMRE11/hNBS1 complex, our findings indicate that CIN in MSI-H colorectal carcinomas might be induced from the functional alterations of hRad50/hMRE11/hNBS1 complex.

We cannot explain the significance of the high frequency of mutations of *FLJ11383* in MSI-H tumors with CIs, because the functionality of *FLJ11383* is unknown. In addition to *hRad50* and *FLJ11383*, mutations of the other genes involved in DNA repair, *ATR* and *MBD4*, also showed trends toward more frequent mutations in MSI-H tumors with CIs. Future analysis of a larger series of cases and the determination of the functional mechanism of the CIN of these genes should clarify the associations between mutations of these genes and genetic progression via the CIN pathway in MSI-H carcinomas.

V. CONCLUSION

To clarify the effects of altered DNA repair genes in the tumorigenesis and progression of colorectal carcinomas with microsatellite instability, we analyzed type of genetic instabilities, frameshift mutation and expression status of DNA repair genes.

1. Most of the sporadic MSI-H colorectal carcinomas induced by the inactivation of *hMLH1* promoter hypermethylation.
2. Frameshift mutation of target genes result in the appearance of premature translation termination codon (PTC) from the generation of alternative reading frame. In 25 target genes, mutant transcripts of 7 target genes were decayed by NMD, whereas mutant transcripts of 18 target genes immune to NMD.
3. Frameshift mutation of *hRad50* induce down expression of wild type mRNA and protein.
4. Down-regulation of hRad50 is related to the chromosome instability in MSI-H colorectal carcinomas.

The result obtained in our study suggest that unique subset of CIs existed in MSI-H colorectal carcinomas and alterations of DNA repair gene, *hRad50*, can be developed during the tumor progression of MSI-H tumors, and cause additional genomic instability.

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<ABSTRACT IN KOREA>

DNA 복구 유전자들의 이상이 현미부수체 불안정형 대장암의 발생 및 진행에 미치는 영향

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고빈도 현미부수체 불안정형(microsatellite instability, MSI-H) 종양은 DNA 복제오류 교정 유전자의 결합으로 발생하며, 이로 인해 종양연관 유전자의 단백질 지령부위에 존재하는 현미부수체 (coding mononucleotide repeats, cMNR) 에 체이동 돌연변이가 발생하여 결과적으로 해당유전자의 기능적 이상이 초래되어 암 발생 및 진행이 일어나는 종양이다. 본 연구에서는 MSI-H 종양에서 표적이 되는 종양연관 유전자중 DNA 수선에 관련된 유전자의 변이가 암발생에 어떤 영향을 주는가를 밝히고자 하였다.

MSI-H 종양의 표적유전자로 이전에 보고 된 표적유전자와 unigene 데이터베이스 탐색을 통해 10 개 이상의 cMNR 을 포함하는 45 개를 대상으로 체이동 돌연변이를 조사하였으며, 이들 돌연변이형 전사체 발현을 역전사 중합효소 연쇄반응 (reverse transcriptase polymerase chain reaction, RT-PCR)로 조사하였다. 검색된 45개의 표적유전자중 DNA 수선에 관여하는 유전자는 10 개 이었으며 이중 *hMSH3*, *hRad50* 유전자의 돌연변이 율이 56% (22/39), 33% (13/39) 로 가장 높았다. *hRad50* 유전자의 돌연변이형 전사체는 발현되지 않았으며, 단백질 합성 억제제인 puromycin 을 처리하거나 nonsense mediated decay (NMD) system 구성 유전자인 *hUpf1* 또는 *hUpf2* 를 처리하였을 때 발현되었다. 표적유전자의 돌연변이와 chromosomal imbalances (CIs) 의 관계를 조사한 결과 *hRad50* 유전자의 이상은 CIs 와 관계가 있음을 밝혀 *hRad50* 의 돌연변이에 의해 MSI-H 종양에서 염색체변형이 촉진됨을 시사하였다. MSI-H 종양 및 세포에서 hRad50의 발현은 *hRad50* 유전자의 돌연변이에 의해 감소되었으며 *hRad50* 과 복합체를 이루는 *hMRE11*은 MSI-H 종양 모두에서 감소되어 있었다. 이와 함께 MSI-H 종양에서는 *hMRE11*의 splicing variant 가 모두 관찰되었다. 따라서 MSI-H 종양에서 *hRad50*의 돌연변이로 인해 정상 hRad50 유전자의 발현양이 저하되고 결국은 DNA 수선에 작용하는 복합체의 양이 줄어듬을 알수 있었다.

이러한 결과는 MSI-H 종양에서 일어난 *hRad50* 유전자의 체이동 돌연변이가 CIs 를 일으키는 직접적인 원인이 될수 있음을 시사한다.

핵심되는 말: DNA 복제오류 교정, 현미부수체 불안정형, 체이동 돌연변이, 대장암