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Mutational Inactivation of Mitotic Checkpoint Genes, *hsMAD2* and *hBUB1*, Is Rare in Sporadic Digestive Tract Cancers

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Genetic instability is a key mechanism of tumorigenesis, and the instability exists at two distinct levels, the nucleotide and the chromosome levels. Disruption of the mitotic spindle checkpoint is one of the underlying mechanisms leading to aneuploidy and alterations of hsMAD2 and hBUB1, assumed to take part in the spindle checkpoint in human cells, have been found to be associated with chromosomal instability in some tumor cell lines. Therefore, we investigated the mutational status of the *hsMAD2* and *hBUB1* genes in 32 sporadic digestive tract cancers by reverse transcription-polymerase chain reaction-single strand conformation polymorphism analysis. The entire coding sequence of the *hsMAD2* gene, and conserved regions (codons 21–152 and codons 732–1043) presumed to be functionally important in the *hBUB1* gene were analyzed. Mutation of the *hsMAD2* gene was not observed at all and missense mutation of the *hBUB1* gene was noted in one rectal cancer case. Sequencing analysis revealed an AGT-to-GGT missense mutation, substituting glycine for serine, at codon 950, which is conserved between budding yeast and human. These results indicate that mutations of the *hsMAD2* and *hBUB1* genes are very rare and presumably play a very restricted role in tumor development of sporadic cancers.

Key words: *hsMAD2* — *hBUB1* — Mitotic checkpoint — Digestive tract cancers

Development of neoplastic cells is a multistep process and progressive stages of neoplasia are accompanied by accumulation of a half-dozen or more cellular gene mutations that directly or indirectly affect tumor cell proliferation.[1)] It has been postulated that generation of these multiple mutations is a result of genetic instability. One example is a subset of colorectal cancers exhibiting mismatch repair deficiency and consequently having various types of mutation at the nucleotide level, the most apparent of which occur in their microsatellite DNA stretches.[2, 3)] Tumors of this type usually have normal or quasi-normal chromosomal contents (euploidy).[4, 5)] However, most colorectal cancers do not show microsatellite instability (MSI), but reveal abnormal chromosomal number (aneuploidy) and loss of heterozygosity at many genetic loci.[1)] Thus, generation of aneuploidy, termed chromosomal instability (CIN), would be another underlying mechanism of tumor progression.

One of the mechanisms leading to aneuploidy is a disruption of the spindle assembly checkpoint machinery.[6)] The checkpoint control monitors proper assembly of mitotic spindles, which consist of microtubules and ensure stable attachment of chromosomes (at the kinetochore) to microtubules. By blocking the onset of anaphase, the mitotic checkpoint prevents improper allocation of the two chromatids of a chromosome to the two daughter cells. Unless the spindle assembly checkpoint works properly, cells exit mitosis prematurely and proceed rapidly to aneuploidy. By screening with drugs (taxol and/or nocodazol) that causes depolymerization of microtubules, three *MAD* (mitotic arrest deficient) genes and three *BUB* (budding uninhibited by benomyl) genes have been identified that are required for the execution of the mitotic checkpoint in *Saccharomyces cerevisiae*.[7, 8)] In mammalian cells, the MAD2 and BUB1 proteins have been found to make up the spindle checkpoint.[9, 10)] They bind to the kinetochore in the prophase of mitosis and are required for a normal mitotic delay in response to spindle destruction. In previous studies, reduced expression of *Homo sapiens* MAD2 (hsMAD2) was reported in one of two tumor cell lines with CIN, and mutational inactivation of the human *BUB1* (*hBUB1*) gene was noted in two of 19 colorectal cell lines with CIN.[9, 10)] However, to what extent alteration of these two genes might play a role in the development of cancers, especially in sporadic ones, has remained to be elucidated. In this context, we performed mutational analyses of these two genes in sporadic digestive tract cancers.

MATERIALS AND METHODS

Tumor specimens Tumor samples and their normal counterparts were surgically obtained from 32 patients with

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various digestive tract cancers at Tokyo Kousei Nenkin Hospital in 1998. All samples were snap-frozen in liquid nitrogen immediately after resection and were stored at –80°C until use. Tumors consisted of 23 colorectal cancers (termed C1T to C23T), 8 gastric cancers (G1T to G8T) and one esophageal cancer (E1T). The patients with colorectal cancers were 11 males and 12 females, ranging from 34 to 87 years old (mean, 65.0), those with gastric cancers were 6 males and 2 females, 58 to 84 years old (mean, 60.6) and the esophageal cancer patient was a 75-year-old female. The histological diagnoses were determined according to the criteria of Japanese Society for Cancer of the Colon and Rectum, Japanese Research Society for Gastric Cancer and Japanese Society for Esophageal Diseases using sections of the same samples as used for assays.[11–13]

RNA extraction Total RNA was prepared from frozen tissues by using an RNA/DNA Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To avoid DNA contamination in extracted total RNA, DNase I treatment was performed in a total volume of 30 μl containing 6 μg of total RNA, 0.6 μl of RNase-free DNase I (10 u/μl), 3 μl of 10× buffer (400 m*M* Tris-HCl (pH 7.5), 100 m*M* NaCl, 60 m*M* $MgCl_2$) at 37°C for 1 h. The reaction mixture was phenol/chloroform-extracted, the extract was ethanol-precipitated and the precipitate was resolved in 25 μl of RNase-free water.

Reverse transcription-polymerase chain reaction-single strand conformation polymorphism (RT-PCR-SSCP) analysis In this study, we investigated the entire coding sequence of the *hsMAD2* gene (open reading frame, 618 bases) and parts of the two highly conserved domains (CD) (CD1, codons 21–152, and CD2, codons 732–1043, respectively) of the *hBUB1* gene (open reading frame, 3258 bases) by using RT-PCR-SSCP analysis. In addition, codons 480–511 of the *hBUB1* gene, spanning the $(A)_7$ stretch (codons 504–506) and the formerly reported mutated codon 492, were also analyzed.[10] CD1 is assumed to direct kinetochore localization and binding to BUB3, another spindle checkpoint protein, and CD2 encodes the kinase domain.[14, 15] The entire coding sequence of the *hMAD2* gene was divided into five segments. CD1 of the *hBUB1* gene was divided into two (segments 1 and 2, covering codons 15–125), CD2 was divided into three (segments 4, 5 and 6, covering codons 815–968), and the region covering codons 480–511 was designated segment 3. The nucleotide sequences of the primer sets are listed in Table I. After heating total RNA at 65°C for 10 min, reverse transcription was performed in a total volume of 20 μl consisting of 3 μg of total RNA, 0.5 μl of 25 m*M* dNTP, 2 μl of random hexamer (1 nmol/μl), 1 μl of RNase inhibitor (2 u/μl), 4 μl of 5× single strand buffer, and 1 μl of Mu-MLV RTase (20 u/μl) at 37°C for 1 h. The reaction mixture was then heated at 80°C for 5 min, and cooled immediately on ice.

PCR was carried out in a total volume of 12.5 μl containing 1 μl of cDNA solution, 0.3 μl of [γ-^{32}P]dATP-labeled primers (10 μ*M*), 2 μl of dNTPs (2.5 m*M*), 1.25 μl of 10× reaction buffer, and 0.75 unit of *Taq* DNA polymerase. The reaction consisted of initial denaturing at 94°C for 5 min, followed by 40 cycles consisting of 94°C for 30 s, 55°C (for *hsMAD2* and segment 4 of *hBUB1*) or 52°C (for *hBUB1*) for 30 s, and 72°C for 30 s. After amplification, 5 μl of PCR product was mixed with 45 μl of SSCP-loading buffer and electrophoresed for 3–4 h at 20°C in a 5% non-denaturing polyacrylamide gel with or without 5% glycerol at 35 W.

Sequencing RT-PCR products recognized as aberrant bands were eluted from the isolated gel in 30 μl of distilled water at 95°C for 10 min. They were used as templates for PCR with the same primers and the products were directly sequenced. When an aberrant band was very close to wild ones, RT-PCR products prior to SSCP were directly sequenced. Sequence primers were the same as the RT-PCR primers.

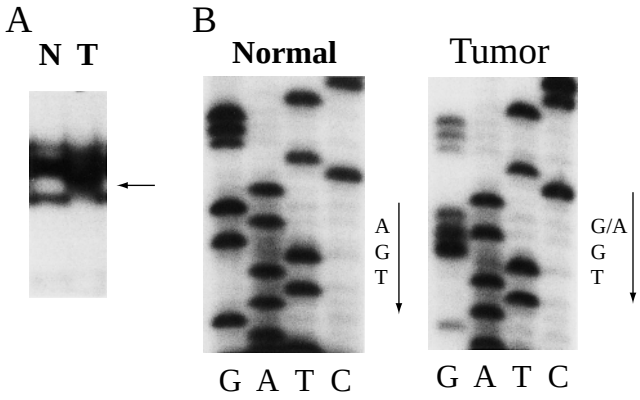
Table I. Primers for PCR Amplification

Gene	Region	Sense	Antisense
hsMAD2	Seg. 1	5′-TGGAAGCGCGTGCTTTTGTTTGTGT-3′	5′-CAAGCAAGGTGAGTCCGTATTTCTG-3′
	Seg. 2	5′-GGCATATATCCATCTGAAACCTTTA-3′	5′-TGCCATCTTTCCAGGACCTCACCAC-3′
	Seg. 3	5′-AGTTCAGAAACTGGTTGTAGTTATC-3′	5′-TTCCAACAGTGGCAGAAATGTCACC-3′
	Seg. 4	5′-TGAAATCCGTTCAGTGATCAGACAG-3′	5′-GGATTGTAGTAGTAAATGAACGAAG-3′
	Seg. 5	5′-GAGTCGGGACCACAGTTTATTACCA-3′	5′-AGGAAAACCACATTTCAAAATTACA-3′
hBUB1	Seg. 1	5′-CAGAGCTACAAGGGCAATGA-3′	5′-ATTGATGGAGGTCACTGTTG-3′
	Seg. 2	5′-CCCAAGATTCATCAGTTATT-3′	5′-TCTGGGTTCAGCCTGGTTTT-3′
	Seg. 3	5′-GTTTCAGGCTCCTACACTTC-3′	5′-TATTGACTCCCCAAGCCCCA-3′
	Seg. 4	5′-GAGATCTGAATGATGCTAAAAATAAA-3′	5′-GCATTTAATAATGTTCCATAGCTGTA-3′
	Seg. 5	5′-GGCAGTGTATTAGTAGGAGA-3′	5′-AAATCCGTTTCCAAGTATGA-3′
	Seg. 6	5′-TCATTCATGGAGACATTAAAC-3′	5′-CTGAGCATCTCAACACACTG-3′

RESULTS

Histopathological findings All of the tumors were in the advanced stage, infiltrating proper muscle or further. As for colorectal cancers, 15 cases were rectosigmoid colon cancers, 3 cases were cecal cancers and the remaining 5 cases were others. Grossly, 3 cases were Borrmann type 1 and 20 cases were Borrmann type 2. Histologically, 6 cases were well differentiated papillotubular adenocarcinoma and the remaining 17 were moderately differentiated papillotubular adenocarcinoma. With regard to gastric cancers, 2 cases were Borrmann type 1 and 6 cases were Borrmann type 2. Histologically, 4 cases were moderately differentiated tubular adenocarcinoma and the remaining 4 were poorly differentiated adenocarcinoma with a solid growth pattern. The esophageal cancer was macroscopically Borrmann type 3 and histologically moderately differentiated squamous cell carcinoma.

Mutation of the *hsMAD2* and *hBUB1* genes RT-PCR-SSCP analysis was performed by using total RNA without DNase I digestion as a template. Analysis of the *hsMAD2* gene revealed mobility-shifted bands in segments 1 and 4 in some cases, and subsequent sequencing revealed nucleotide changes at codons 21 (GCC to GAC), 42 (ACC to ATC), 165 (GAA to GGA, Glu to Gly) and silent mutations at codons 14, 16, 37, 138, and 156 (data not shown). These changes were on the same polynucleotide molecule, as demonstrated by TA-cloning and sequencing. After DNase I digestion of total RNA, however, the aberrant bands disappeared, suggesting that the phenomenon was caused by contamination with genomic DNA. Therefore, mutation of the *hsMAD2* gene was not observed in any of the tumors. With regard to the *hBUB1* gene, an aberrant band of segment 6 was observed in RT-PCR-SSCP analysis and a missense mutation at codon 950 (AGT to GGT, substituting glycine for serine) was identified in one colorectal tumor case (C1T) (Fig. 1). The mutation was heterozygous, and analysis of the corresponding normal tissue confirmed the somatic nature of the mutation. The case with *hBUB1* gene mutation (C1T) was that of a rectal cancer which was grossly Borrmann type 2, 3.2×3 cm in size, and histologically moderately differentiated papillotubular adenocarcinoma in a 61-year-old male patient. The case, however, did not show any peculiar clinicopathological features.



ig. 1. RT-PCR-SSCP and direct sequencing analysis of *hBUB1* egment 6 in case C1T. A, RT-PCR-SSCP analysis. B, Direct equencing of the RT-PCR product that showed an altered mobilty in SSCP analysis revealed an AGT-to-GGT (Ser-to-Gly) misense mutation at codon 950 of the *hBUB1* gene. Results for DNA from both tumor and corresponding normal tissue are hown.

DISCUSSION

This is the first study to investigate the mutational status of the *hsMAD2* and *hBUB1* genes in human sporadic cancers. The hsMAD2 and hBUB1 proteins have emerged as molecules closely associated with the mitotic spindle checkpoint. In previous studies, decreased expression of the hsMAD2 protein in one of two tumor cell lines and mutation of the *hBUB1* gene in two of 19 cell lines sensitive to nocodazole and/or taxol were reported.[9, 10] Because aneuploidy is postulated to be responsible for development of a large proportion of sporadic cancers and some of the molecular mechanisms of aneuploidy are supposed to be disruption of the mitotic spindle checkpoint, we reasoned that mutational inactivation of the *hsMAD2* and *hBUB1* genes, if present, might be a feature of the carcinogenetic mechanisms in a subset of sporadic cancers. When the *hsMAD2* gene was analyzed using total RNA prior to DNase I digestion, a polymorphic *hsMAD2* sequence was obtained in some cases. Because it disappeared after treating total RNA with DNase I, the aberrant bands were considered to be a result of genomic DNA contamination. Very recently, Cahill *et al*. reported the *hsMAD2* pseudogene, located at chromosome 14q21-23, which is identical in nucleotide sequence with the polymorphic *hsMAD2* gene we observed.[16] In summary, mutational inactivation of the *hsMAD2* gene was not observed at all and mutation of the *hBUB1* gene was noted in only one sporadic case. The mutation was a missense mutation (AGT to GGT) at codon 950, conserved between budding yeast and human. Although the mutation was heterozygous, mutant hBUB1 has been demonstrated to operate in a dominant-negative manner, thereby completely abolishing its function in the cell.[10] Therefore, the present missense mutation might also have influenced the activity of hBUB1. However, the mutation frequencies of the two genes were very low as compared to a larger proportion of cancers with aneuploidy. Because only parts of the *hBUB1* gene were analyzed in this study, the mutation frequency of the gene might have been underestimated. Another explanation

would be that the putative inactivating mechanism might work at the transcriptional or translational levels, as has been demonstrated in the hsMAD2 protein.[9)]

In conclusion, mutational inactivation of the *hsMAD2* and *hBUB1* genes is very rare and probably plays a very restricted role in tumor development of sporadic digestive tract cancers.

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