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# Mitotic Checkpoint Protein hsMAD2 as a Marker Predicting Liver Metastasis of Human Gastric Cancers

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**hsMAD2, the human homologue of mitotic arrest deficient 2 (MAD2), is a key component of the mitotic checkpoint system. Recently, mutations and decreased expression of mitotic checkpoint genes including hsMAD2 have been reported in cancer cell lines with defective mitotic checkpoint. However, the genetic alterations in the genomic *hsMAD2* gene have not been determined in gastric cancers. Moreover, the biological implications of the overexpressed hsMAD2 in primary cancers are unknown. In this study, we analyzed 32 primary gastric cancers with polymerase chain reaction (PCR) amplification of all exons, including flanking intronic sequences, of the genomic *hsMAD2* gene followed by direct DNA sequencing. We also measured the hsMAD2 protein levels in cancer and normal tissues by semi-quantitative immunoblotting. No mutations were found in the coding sequences, although three single nucleotide polymorphisms (SNPs) were identified in the noncoding sequences in 13 of 32 patients. These SNPs were not associated with either hsMAD2 expression or disease progression. The semi-quantitative western blot analysis showed hsMAD2 was significantly overexpressed in gastric cancer tissues compared with corresponding normal tissues ($P$<0.001). The calculated ratio of the hsMAD2 protein in cancer tissue (C) to that in corresponding normal tissue (N) (C/N ratio) was significantly higher in patients with well differentiated adenocarcinoma ($P$=0.0274) or with synchronous liver metastasis ($P$=0.0025). A C/N ratio greater than 3 was observed more frequently in patients with synchronous liver metastasis. Therefore, C/N ratio >3 may be clinically important as a predictive indicator for metachronous liver metastasis of gastric cancers.**

Key words: hsMAD2 — Human gastric cancer — Single nucleotide polymorphism — Liver metastasis — Predictive marker

The mitotic checkpoint is a surveillance system that ensures proper chromosome segregation during mitosis.[1] This system stops the cell cycle progression by inducing mitotic arrest when a single chromosome is unable to attach to spindle microtubules. Therefore, the mitotic checkpoint plays an important role in preventing the occurrence of cells with loss or gain of chromosomes.[2,3] In yeast, a loss of mitotic checkpoint frequently leads to abnormal chromosome numbers, resulting in aneuploidy or polyploidy. For instance, *MAD2* gene mutants showed a 15-fold increase in frequency of chromosome loss, compared with budding yeast harboring normal *MAD2* gene.[1]

Recently, mutations in *hBUB1* gene, one of the mitotic checkpoint genes, have been identified in 2 of 19 aneuploid colorectal cancer cell lines having defects in mitotic checkpoint function.[4] Like other mitotic checkpoint proteins, hsMAD2 protein, the product of the human homologue of *MAD2* gene, also plays a central role in activating the mitotic checkpoint during mitosis by sensing unattached kinetochores and sending a mitotic arrest signal in response to the improper spindle-kinetochore attachment.[5,6] Human breast cancer cell line T47D with a loss of mitotic checkpoint was previously reported to show a reduced protein level of hsMAD2 compared with HeLa cells and primary diploid fibroblast cells (F65) with normal mitotic checkpoint.[5]

These lines of evidence suggested that aneuploidy is in part a result of mitotic checkpoint gene mutations and that decreased mitotic checkpoint protein leads to a mitotic checkpoint defect in human cancer.[7–9] No mutations in *hsMAD2* gene have been reported,[10] although one silent polymorphism was found in the coding sequence.[11,12] Nei-

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The abbreviations used are: MAD2, mitotic arrest deficient 2; hsMAD2, human homologue of MAD2; PCR, polymerase chain reaction; SNPs, single nucleotide polymorphisms; hBUB1, human homologue of budding uninhibited by benomyl 1; OCT, optimal cutting temperature; 2ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene difluoride; RT, room temperature; GST, glutathione S-transferase; DAB, 3,3′-diaminobenzidine tetrahydrochloride; RT-PCR-SSCP, reverse transcriptase-polymerase chain reaction-single strand conformation polymorphism.

ther the genomic *hsMAD2* gene nor the hsMAD2 protein has been analyzed in primary gastric cancers.

In the present study, we carried out sequence analysis of the PCR products amplified from genomic DNAs of 32 primary gastric cancers. We also measured the hsMAD2 protein levels in cancer and normal tissues by semi-quantitative western blot analysis.

## MATERIALS AND METHODS

**Patients and tumor specimens** Cancer tissues and corresponding normal tissues were obtained from 32 patients with primary gastric cancer who underwent surgery at Mie University Hospital from 1996 to 1999. Patients were 17 males and 15 females, ranging from 32 to 77 years old (mean age; 66 years). No patient received chemotherapy or radiation therapy prior to surgery. After surgical removal, cancer tissues and normal tissues for DNA extraction and protein extraction were collected into sterile tubes and were embedded in OCT compound (Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo). They were immediately frozen in liquid nitrogen and stored at −80°C until analyzed. Histopathological examination was done on the 10% formalin-fixed, paraffin-embedded specimens by a pathologist at the Pathology Division. Informed consent was obtained from all patients participating in this study.

**DNA extraction** Genomic DNA was extracted from 20-$\mu$m-thick sections of cancer tissues and corresponding normal tissues by proteinase K digestion and phenol-chloroform extraction as described.[13] To avoid contamination with normal cells as far as possible, areas rich in cancer cells were dissected from the normal cell components with reference to tissue sections stained with hematoxylin and eosin. The dissected tissue sections were used for DNA extraction from cancer tissues.

**PCR amplification** As shown in Table I, five sets of PCR primers were designed based upon the 9.8 kb genomic sequence (DDBJ: accession number AB056160) to amplify exons and flanking splice sites from 32 genomic DNA samples extracted from cancer tissues. The PCR amplification was carried out in a reaction mixture of 50 $\mu$l containing 0.1 $\mu$g of genomic DNA, 1× PCR buffer (10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin), 200 $\mu M$ of each deoxynucleoside triphosphate, 20 ng each of sense and antisense primers, and 0.5 units of Ampli Taq Gold DNA polymerase (PE Corporation Applied Biosystems, Foster City, CA). The PCR cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 60 s with a final extension of 10 min at 72°C. Aliquots of PCR products were electrophoresed on 2% agarose gels and were visualized by staining with ethidium bromide.

**Sequence analysis of *hsMAD2* gene** PCR products purified with a QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) were subjected to direct DNA sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Corporation Applied Biosystems) with forward primers for PCR amplification as sequencing primers on an ABI PRISM 377 DNA sequencer (PE Corporation Applied Biosystems). When base substitutions were found in DNA from cancer tissues, DNAs from corresponding normal tissues were used as templates for PCR amplification followed by direct DNA sequencing to see whether or not these changes were polymorphic.

**Protein extraction and western blot analysis** Both cancer and corresponding normal frozen tissues were minced and homogenized in lysis buffer (Tris-buffered saline, pH 7.5, containing 1% Triton X-100) for 5 min on ice. After spinning at 15 000 rpm for 15 min at 4°C, supernatants were collected and frozen at −20°C until use. The protein concentration was measured by the BCA protein assay (Pierce, Rockford, IL). Lysates containing 100 $\mu$g total protein were mixed with an equal volume of 2× Lammeli's loading buffer containing 2ME and heated at 100°C for 5 min. Samples, along with recombinant hsMAD2 protein, were electrophoretically separated on 7.5 to 15% gra-

Table I. Oligonucleotide Primers for PCR Amplification

| | Forward primer (5′-3′) | Reverse primer (5′-3′) | Expected size of PCR products (bp) |
|---|---|---|---|
| Exon 1 | AGACCACGACCAGAAGACACA | TGCAGCTCCACGTTAGCAA | 527 |
| Exon 2 | TGCTTTCAGCAAGATACACGTG | TTCAGCCCACTCACAATGTAGT | 473 |
| Exon 3 | TGGTAGGAATTTGCTGAGGATA | GCACAATTCTTTTCCATAGGTG | 385 |
| Exon 4 | CCTTTCTCAAATAAGCCCTGC | TGGTTTCAGGTATTCACTGTGG | 431 |
| Exon 5 | CCTTTTCCAGCAGTGAGTGGT | CCTCCTGGTTCCTTTTGAACA | 507 |

dient polyacrylamide gels containing 0.1% SDS at 25 mA for 2 h followed by semi-dry transfer to an Immun-Blot PVDF membrane (Bio-Rad Lab., Hercules, CA) at 12 V for 2 h. The membranes were blocked for 1 h at RT using 5% skim milk in Tris-buffered saline, pH 7.5, supplemented with 0.1% Tween 20 (TBS-T). The blots were then incubated with mouse monoclonal anti-hsMAD2 antibody (Transduction Laboratories, Lexington, KY) at a 1:1000 dilution and mouse monoclonal anti-actin (clone C4) antibody (ICN Biomedicals, Inc., Aurora, OH) at a 1:3000 dilution in 5% skim milk in TBS-T overnight at 4°C. After having been washed three times in TBS-T, the blots were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Promega Corp., Madison, WI) at 1:10 000 dilution in 5% skim milk in TBS-T for 1 h at RT. Following treatment with an enhanced chemiluminescence detection solution, the blots were exposed to an X-ray film for autoradiographic visualization of the bands.

**Quantitation of hsMAD2 protein in primary gastric cancers** To estimate the content of hsMAD2, a semi-quantitative immunoblotting procedure was used. Various amounts of the purified hsMAD2-GST fusion protein were separated electrophoretically and western blot analysis was carried out as described above. The bands on the autoradiographs were scanned and analyzed densitometrically using Image Gauge, Version 3.2 (Fuji Photo Film Co., Ltd., Tokyo). Using a calibration curve obtained from the immunoreactive bands of the hsMAD2-GST fusion protein, hsMAD2 content in cancer tissues and their normal counterparts was quantitated.

**Immunohistochemistry** We first stained frozen tissues embedded in OCT compound to confirm that mouse monoclonal anti-hsMAD2 antibody was applicable to immunohistochemistry. After the immunoreactivity of this antibody with hsMAD2 protein was confirmed, immunostaining with anti-hsMAD2 antibody was carried out on sections prepared from formalin-fixed, paraffin-embedded tissues, using an antigen retrieval technique. In brief, formalin-fixed, paraffin-embedded tissues were cut to 5 to 6 $\mu$m in thickness and mounted on MAS coated slides (Matsunami Co., Ltd., Osaka). The sections were deparaffinized in xylene and rehydrated successively in graded concentrations of ethanol. For antigen retrieval, the slides immersed in 10 m*M* sodium citrate buffer, pH 6.0, were heated in a microwave oven for 10 min and cooled to RT. To inhibit endogenous peroxidase activity, air-dried slides were immersed in methanol containing 3% hydrogen peroxide and 15 m*M* sodium azide at RT for 30 min. Immunoreactivity was detected by an immunoperoxidase method (Vectastain *Elite* ABC, Vector Laboratories, Burlingame, CA). Nonspecific antibody-binding sites were blocked with normal horse serum. After having been washed three times with TBS (Tris-buffered saline, pH 7.6), sections were incubated overnight at 4°C with primary mouse monoclonal anti-hsMAD2 antibody at a dilution of 1:50 in TBS. The sections were then washed three times in TBS and incubated with secondary horse anti-mouse biotinylated antibody diluted at 1:200 in TBS for 30 min at RT. The sections were washed again and incubated with avidin conjugated to horseradish peroxidase (avidin-biotin complex reagent) for 30 min at RT. The color was developed with a Vector DAB substrate kit (Vector Laboratories) according to the instruction manual. Sections were lightly counterstained with Mayer's hematoxylin and mounted on slides with a crystal mounting solution after extensive washing in distilled water. As a negative control, sections were processed as described above, but without primary antibody.

**Statistical analysis** Statistical analysis of patients' data including various clinicopathological characteristics was performed using appropriate software (Stat View, version 5; Abacus Concepts, Inc., Berkeley, CA). Results are expressed as mean±SD. Unpaired Student's *t* test and Mann-Whitney *U* test were used for comparison among unpaired groups. Spearman rank correlations test was done to evaluate statistical correlations. *P* values less than 0.05 were considered statistically significant.

## RESULTS

**Sequence analysis of *hsMAD2* gene** Each exon along with flanking intronic sequences was amplified from 32 genomic DNA samples, followed by direct DNA sequencing as described in "Materials and Methods." Sequence analysis of the *hsMAD2* gene showed no base substitutions in the coding sequences of all 5 exons, although three base substitutions were found in the noncoding sequences in 13 (40.6%) of 32 samples. These three substitutions consisted of an A to C transversion and two G to A transitions, which reside at 227 bases upstream to the first ATG, at 8 bases upstream to exon 3, and at 60 bases downstream to the stop codon TGA, respectively. They all occurred simultaneously in the same DNA samples from cancer tissues. The same base changes were found in DNA samples from normal tissues in 13 patients whose cancer tissue DNA showed base changes. Therefore, these base substitutions were considered to be SNP. There was no significant association between the presence of SNPs and clinicopathological characteristics including tumor size, histological grade, lymphatic invasion, vessel invasion, lymph node metastasis, synchronous liver metastasis, peritoneal dissemination, and stage (data not shown).

**Expression of the hsMAD2 protein in gastric cancers and its relationship with clinicopathological characteristics** The hsMAD2 protein levels in cancer tissues and corresponding normal tissues were determined as described in "Materials and Methods" (Figs. 1 and 2). The hsMAD2 protein (ng/mg protein) in cancer tissues was

19.09±10.20 (mean±SD) ranging from 3.36 to 44.36, whereas in corresponding normal tissues it was 8.62±5.06 (mean±SD) ranging from 2.42 to 20.63. The hsMAD2 protein in cancer tissues was significantly overexpressed compared with that in corresponding normal tissues ($P$<0.001) (Fig. 2). The calculated ratio of the hsMAD2 protein in cancer tissue (C) to that in corresponding normal tissue (N) (hereafter, C/N ratio) was 2.54±1.39 (mean±SD), ranging from 0.86 to 5.54, indicating that hsMAD2 expression was approximately 2.5-fold higher in cancer tissues than in corresponding normal tissues. No significant association was found between the presence of SNPs and the content of hsMAD2 protein in either cancer or normal tissue (data not shown). Furthermore, we analyzed the association between the C/N ratio of hsMAD2 expression and clinicopathological parameters including tumor size, histopathological grade, lymphatic invasion, vessel invasion, lymph node metastasis, synchronous liver metastasis, peritoneal dissemination, and stage. The results are summarized in Table II. The C/N ratio in well differentiated adenocarcinomas was significantly higher than that in poorly differentiated adenocarcinomas ($P$=0.0274). The C/N ratio in 6 patients with synchronous liver metastasis was significantly higher than that in 26 patients without synchronous liver metastasis ($P$=0.0025). Three of 6 patients with synchronous liver metastasis had the histological type of well differentiated adenocarcinoma. There was no statistically significant association between the C/N ratio and other clinicopathological parameters.

**Immunohistochemical staining of gastric adenocarcinomas** Immunohistochemical staining was done to determine the expression of hsMAD2 on a single cell basis. The immunoreactive hsMAD2 protein was mainly detected in the cytoplasm of cancer cells, while adjacent normal gastric epithelial cells were stained negatively or very weakly. These findings were consistent with the results obtained from western blot analysis (shown in Fig. 1). Since the C/N ratio in patients with synchronous liver metastasis was higher than that in patients without synchronous liver metastasis, the immunoreactivity of hsMAD2 was determined to see whether or not the immunostaining intensity correlated with synchronous liver metastasis. As shown in Fig. 3, strong immunoreactivity of hsMAD2 was observed in patients with synchronous

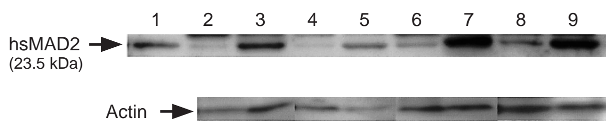


Fig. 1. Representative western blot analysis of hsMAD2 protein in primary gastric cancers. Tissue lysates extracted from cancer tissues and corresponding normal tissues were analyzed along with Jurkat cell extract as a positive control as described in "Materials and Methods." An arrow indicates a 23.5-kDa hsMAD2 protein, and human actin protein is shown as an internal control to verify equal loading. Lanes were: 1, Jurkat cell extract; 2, 4, 6, and 8, normal tissues; 3, 5, 7, and 9, cancer tissues. Lanes 2 and 3, 4 and 5, 6 and 7, and 8 and 9 were pairs of normal and cancer tissues from the same patients.

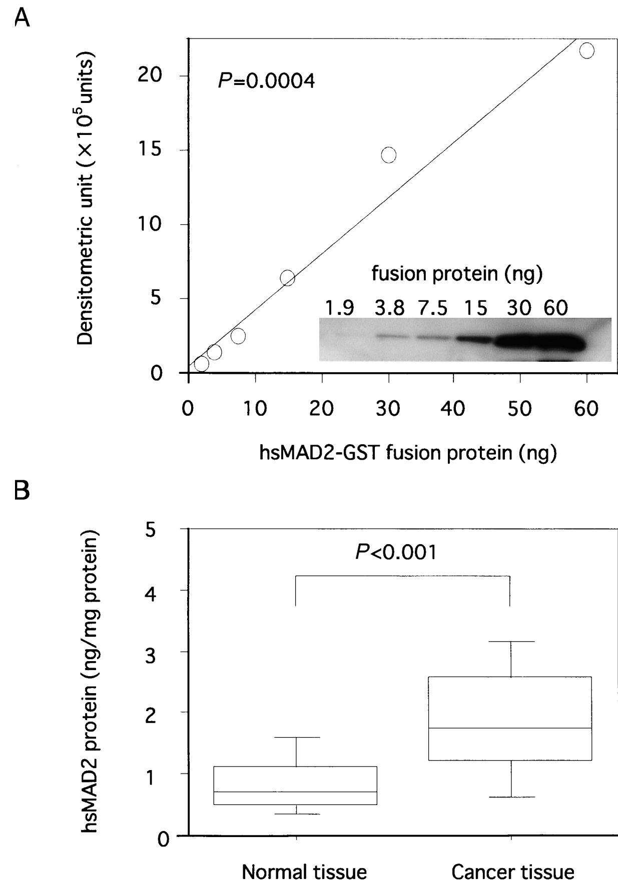


Fig. 2. A, A calibration curve for quantitation of hsMAD2 protein in primary gastric cancers. In this experiment, purified hsMAD2-GST fusion protein in the indicated amounts was separated electrophoretically, transferred to a membrane, and probed with anti-hsMAD2 antibody. Following the densitometric determination of the band intensities on the autoradiographs, a calibration curve was prepared. Linear regression analysis indicated a significant linear correlation ($P$=0.0004) between the densitometric units and the amounts of the fusion protein. B, Comparison of the hsMAD2 content in gastric cancer tissues with that in matched normal tissues. The hsMAD2 protein levels in tissues were determined by semi-quantitative western blot analysis as shown in Fig. 1. The line in the box indicates the median value, while the top and the bottom of the box indicate the 25th and 75th percentiles, respectively. The ends of the vertical line represent the 10th and 90th percentiles, respectively. The hsMAD2 protein levels in cancer tissues were significantly higher than those in matched normal tissues ($P$<0.001, Mann-Whitney $U$ test).

Table II. Association between the C/N Ratio of hsMAD2 Expression and Clinicopathological Parameters

| Variables | | No. of cases | C/N ratio[a] | *P* value |
|---|---|---|---|---|
| Tumor size | 55 mm > | 16 | 2.32±1.38[b] | NS[c] |
| | 55 mm ≤ | 16 | 2.75±1.40 | |
| Histological type (differentiation) | | | | |
| Well differentiated adenocarcinoma | | 9 | 3.29±1.61 | 0.0274 |
| Moderately differentiated adenocarcinoma | | 7 | 2.77±1.31 | |
| Poorly differentiated adenocarcinoma | | 16 | 2.01±1.11 | |
| Lymphatic invasion | (+) | 31 | 2.57±1.40 | NS |
| | (−) | 1 | 1.661 | |
| Vessel invasion | (+) | 18 | 2.47±1.34 | NS |
| | (−) | 14 | 2.63±1.48 | |
| Lymph node metastasis | (+) | 22 | 2.33±1.19 | NS |
| | (−) | 10 | 2.99±1.72 | |
| Synchronous liver metastasis | (+) | 6 | 4.00±1.25 | 0.0025 |
| | (−) | 26 | 2.20±1.20 | |
| Peritoneal dissemination | (+) | 7 | 1.80±0.75 | NS |
| | (−) | 25 | 2.74±1.46 | |
| Stage (TNM) | 0 | 0 | — | NS |
| | IA | 0 | — | |
| | IB | 4 | 3.42±1.69 | |
| | II | 7 | 2.17±1.18 | |
| | IIIA | 4 | 1.45±0.96 | |
| | IIIB | 3 | 2.01±0.25 | |
| | IV | 14 | 2.89±1.48 | |

*a*) The ratio of hsMAD2 protein content in cancer tissues (C) to that in corresponding normal tissues (N).
*b*) Mean±SD.
*c*) Not significant.

liver metastasis compared to patients without synchronous liver metastasis.

## DISCUSSION

Previous studies have indicated that mutations of mitotic checkpoint gene or lower expression of mitotic checkpoint protein might lead to the failure of mitotic checkpoint function, which might in turn lead to tumorigenesis, especially aneuploid cancers. Li and Benezra have reported that one breast cancer cell line with a mitotic checkpoint defect expressed hsMAD2 at a level one-third less than that of diploid normal cell lines.[5] Cahill *et al.* have also demonstrated that aneuploid colorectal cancer cells consistently exhibit loss of mitotic checkpoint function and they found mutations in the *hBUB1* gene in 2 of 19 aneuploid colorectal cancer cell lines exhibiting defective mitotic checkpoint function. Furthermore, transfection of one mutant allele of *hBUB1* gene dominantly led to the disruption of a mitotic checkpoint in near-diploid colorectal cancer cells with a normal checkpoint.[4] Recently, Takahashi *et al.* have reported impaired mitotic checkpoint function in 4 (44%) of 9 human lung cancer cell lines.[11] The previous reports on the *hsMAD2* gene indicated that a silent polymorphism was present at codon 143 (CCA to CCG) of the *hsMAD2* gene in cell lines derived from lung and colorectal cancer.[11, 12] On the other hand, Imai *et al.* found no mutation of *hsMAD2* gene in esophageal, gastric, and colorectal cancers by RT-PCR-SSCP analysis.[10] The results of our present study revealed no mutations in the entire coding sequence of *hsMAD2* gene in 32 primary gastric cancers. However, three SNPs in the noncoding region of the *hsMAD2* gene were found in 13 (40.6%) of 32 patients. The SNPs reported here were not associated

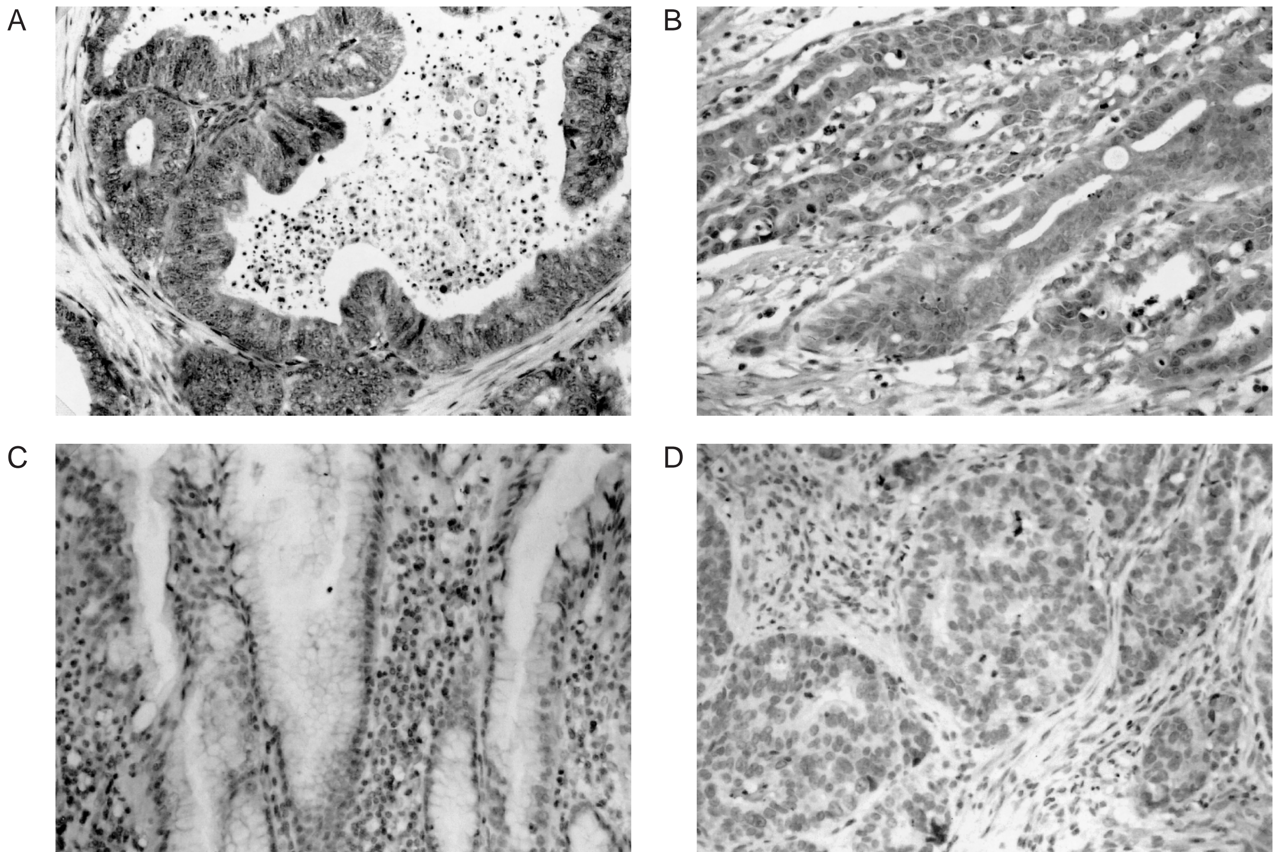


Fig. 3. Immunohistochemical staining of hsMAD2 in gastric cancers. Immunohistochemical staining was performed as described in "Materials and Methods." Gastric cancer cells were strongly positive for hsMAD2 in gastric cancer tissues from patients with liver metastasis (A), whereas they were moderately positive in gastric cancer tissues from patients without liver metastasis (B). Adjacent normal gastric epithelial cells were stained negatively or very weakly (C). As a negative control, sections were processed without primary antibody (D). (Original magnification ×200)

with either expression or disease progression in human gastric cancers. In our preliminary experiments, the hsMAD2 protein levels in 9 near-diploid and aneuploid colorectal cancer cell lines were found to have no apparent relationship with the DNA ploidy (data not shown).

Taken together, these findings suggest that the hsMAD2 expression status has novel biological implications in the genesis or progression of human gastric cancer. Therefore, we determined the expression status of hsMAD2 in both cancer and normal tissues by a semi-quantitative immunoblotting procedure. Our results on the expression of hsMAD2 were not consistent with the finding of reduced hsMAD2 expression in a breast cancer cell line reported by Li and Benezra.[5] The hsMAD2 expression in gastric adenocarcinomas was markedly increased compared with corresponding normal mucosa in 30 (93.8%) of 32 patients. In budding yeast, the amount of MAD2 protein appeared to be constant during the cell cycle, although the level of Clb2p, a mitotic cyclin, changes periodically in response to cell cycle progression.[14] Similarly, a certain amount of hsMAD2 protein is thought to be required to maintain the normal checkpoint system in human normal cells. However, hsMAD2 was significantly overexpressed in gastric cancer tissues comapared with corresponding normal tissues ($P<0.001$).

Very recently, Poelzl *et al.* reported that the estrogen receptor β, which may be involved in the regulation of cellular proliferation, interacts with hsMAD2 directly and specifically.[15] The hsMAD2 protein also interacts with the cytoplasmic domain of the insulin receptor, which is thought to be a regulator of cellular growth.[16, 17] The hsMAD2 protein overexpressed in cancer tissues was exclusively present in the cytoplasm of cancer cells (Fig. 3). Taking these findings together, we speculate that cytoplasmic hsMAD2 protein enhances the positive regulatory action of the estrogen receptor β and the insulin receptor

on cellular proliferation. Further investigations will be needed to prove this hypothesis.

The C/N ratio of hsMAD2 protein was found to be associated with histological differentiation and synchronous liver metastasis of gastric cancers. A C/N ratio greater than 3 was observed more frequently in patients with synchronous liver metastasis. Therefore, a C/N ratio >3 may be clinically important as a predictive indicator for metachronous liver metastasis of gastric cancers. As shown in Fig. 3, immunohistochemical detection of hsMAD2 protein in the tissue sections will be of help to identify gastric cancers prone to metastasize to the liver. It is necessary to study the relationship between immunoreactive hsMAD2 expression and metachronous liver metastasis retrospectively. However, it remains to be answered how hsMAD2 is overexpressed in cancer tissues compared with normal tissues in gastric cancers and how the overexpressed hsMAD2 protein accelerates metastasis to the liver in gastric cancers.

In conclusion, although three SNPs were detected in the noncoding region, these changes did not affect the expression of *hsMAD2* gene. The protein level was higher in cancer tissues than in normal tissues. The higher the protein level in cancer tissues was, the more frequently gastric cancers had liver metastasis. Since the difference in the protein level between cancer cells and normal mucosa was easily detected by immunohistochemistry, the hsMAD2 protein will be a good marker predicting liver metastasis of gastric cancers.

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