Advances in Brief

Determination of High Mobility Group I(Y) Expression Level in Colorectal Neoplasias: A Potential Diagnostic Marker

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Abstract

High mobility group I(Y) [HMGI(Y)] proteins are architectural factors abundantly expressed during embryogenesis, and their overexpression is known to be closely associated with neoplastic transformation of cells. This study was performed to investigate whether determination of HMGI(Y) expression level could assist in (a) differential diagnosis between colorectal carcinoma, adenoma, and normal tissue and (b) determination of the prognosis of patients with colorectal cancer. To this end, HMGI(Y) expression was determined at both the protein and mRNA levels in 30 colorectal carcinomas, 26 adenomas, and 23 normal mucosa samples, and further correlations between the protein expression levels and various clinicopathological parameters, such as depth of tumor invasion, lymphatic and/or venous involvement, regional lymph node metastasis, and Dukes’ stage, were determined in 30 carcinoma cases. The expression of HMGI(Y) proteins was significantly increased in carcinoma and adenoma with severe atypia compared with that in adenoma with less atypia and normal colorectal mucosa. This increase in HMGI(Y) protein expression was found to be because of an increase in its mRNA expression by RNA in situ hybridization analysis. Clinicopathological analysis revealed that the level of HMGI(Y) protein expression was significantly correlated with parameters known to be indicative of a poor prognosis in colorectal cancer patients. These findings indicate that the determination of the HMGI(Y) protein expression level could be a potential marker for the diagnosis of colorectal neoplasias and can be of great value in predicting the prognosis of patients with colorectal cancer.

Introduction

HMGI is a family of low molecular mass, nonhistone nuclear DNA binding proteins (1). The HMGI family includes three proteins: HMGI, HMGY, and HMGI-C. The former two are derived from alternative processing of RNA transcripts from a single functional gene, whereas the latter is the product of a different gene (2, 3). Although the cellular functions of HMGI and HMGY [HMGI(Y)] proteins (which differ from each other by 11 amino acid; Ref. 4) remain to be determined, previous data indicate that they are involved in transcription of genes including IFN-β and interleukin 2 receptor α chain (5, 6), and their expression is known to be closely associated with neoplastic transformation of cells. This study was performed to investigate whether determination of HMGI(Y) expression level could assist in (a) differential diagnosis between colorectal carcinoma, adenoma, and normal tissue and (b) determination of the prognosis of patients with colorectal cancer. To this end, HMGI(Y) expression was determined both at the protein and mRNA levels in colorectal carcinoma, adenoma, and normal mucosa samples, and further correlations between the protein expression levels and various clinicopathological parameters, such as depth of tumor invasion, lymphatic and/or venous involvement, regional lymph node metastasis, and Dukes’ stage, were determined in 30 carcinoma cases.

Materials and Methods

Patients and Tissue Preparations. A total of 30 colorectal carcinomas, including 19 colon carcinomas and 11 rectal carcinomas, and 23 samples of adjacent normal colorectal mucosa were obtained from patients who underwent surgery for colorectal cancer during the period from October 1997 to February 1998 at the First Department of Surgery, Kyorin University Hospital (Tokyo, Japan). Of these patients, 17 were males and 13 were females. Their ages ranged from 34 to 85 years, with a median age of 63. Clinicopathological parameters, such as depth of tumor invasion, lymphatic and venous involvement, and regional lymph node metastasis, were evaluated according to the criteria of the Japanese Society of Cancer of the Colon and Rectum (24). Twenty-six adenoma samples were also obtained by colonoscopic removal or by surgery: 10 adenomas with histologically mild atypia, 9 adenomas with moderate atypia, and 7 adenomas with severe atypia (they were evaluated histologically according to the criteria of the Japanese Society of Cancer of the Colon and Rectum; Ref. 24). All patients gave their informed consent prior to inclusion in the study. All tissue samples were fixed immediately after surgical or colonoscopic removal in 4% paraformaldehyde in PBS at 4°C for 14 h, followed by cryoprotection in a graded concentration of sucrose in PBS. The
specimens were embedded in OCT compound and then frozen and stored at 
−80°C.

Immunohistochemical Analysis. Immunohistochemical examinations were performed with the avidin-biotin complex immunoperoxidase technique using the Avidin-Biotinylated Enzyme Complex Kit (Vector Laboratories). The primary antibody used in this study, against an HMGI(Y)-specific synthetic peptide corresponding to the NH2-terminal region of the molecule (19), was derived from rabbit serum. This antibody specifically recognizes HMGI(Y) antigen in colorectal carcinoma cells (22).

Frozen sections (5 μm) were cut, transferred onto poly-L-lysine-coated slides, air-dried, and then washed in PBS, followed by quenching of endogenous peroxidase activity by 0.3% hydrogen peroxide in methanol. After further rinsing with PBS, the sections were incubated with normal goat serum for 20 min at room temperature to block nonspecific binding and then incubated with primary anti-HMGI(Y) antibody at a dilution of 1:75 for 14 h at 4°C. After washes in 0.2% Triton X-100 in PBS, the sections were further incubated with biotinylated anti-rabbit IgG for 30 min at room temperature and washed in 0.2% Triton X-100 in PBS. After addition of streptavidin-biotin-conjugated peroxidase and incubation for 30 min at room temperature, the sections were washed in 0.2% Triton X-100 in PBS, and the location of the HMGI(Y) proteins was visualized by incubating the sections with 3,3’-diaminobenzidine.

Negative controls were made by replacing the primary antibody with normal rabbit serum, under the same experimental conditions.

The slides were counterstained with Mayer’s hematoxylin (B–E).
was scored by counting ~1000 epithelial cells in three randomly selected fields. For clinicopathological study, slides of carcinomas were assigned one of three staining scores: 3+, >60% of cancer cells with positive nuclear staining; 2+, 20–60% of cancer cells with positive nuclear staining; or 1+, <20% of cancer cells with positive nuclear staining.

**RNA in Situ Hybridization.** Frozen sections from normal mucosae, adenomas, and carcinomas were processed for RNA in situ hybridization, using a nonradioactive system, to investigate the expression of HMGI(Y) mRNA. The probe for HMGI(Y) was generated from a 309-bp cDNA subcloned in pBluescript II (KS+) (Stratagene, Germany), corresponding to exons 5–8 of the human HMGI(Y) gene. This probe specifically recognizes HMGI(Y) transcripts on Northern blot analysis (data not shown). Antisense and sense HMGI(Y) riboprobes were generated by *in vitro* transcription of 1 μg of the linearized plasmid digested with *Bam*HI (antisense) and *Hind*III (sense), using T7 and T3 RNA polymerases, respectively, in the presence of digoxigenin 11-UTP (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions. For hybridization experiments, 8-μm frozen sections were incubated in 0.3% Triton X-100 for 10 min, followed by digestion in 1 μg/ml proteinase K at 37°C for 12 min. After this treatment, the sections were incubated in 0.2% glycine in PBS for 10 min to inhibit proteinase K activity, followed by postfixation with 4% paraformaldehyde in PBS for 5 min. Thereafter, the sections were acetylated in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine for 15 min and then finally washed with distilled water prior to hybridization. The sections were hybridized by incubation in a moist chamber for 16 h at 50°C with a hybridization mixture containing 50% formamide, 3× SSC, 2× Denhardt’s solution, 10 mM EDTA (pH 8.0), 10% dextran sulfate, 300 μg/ml yeast tRNA, 300 μg/ml boiled salmon sperm DNA, and 6 μg/ml riboprobe labeled with digoxigenin. After hybridization, the sections were washed in 2× SSC with 50% formamide at 46°C for 30 min. The sections were incubated in 20 μg/ml RNase-A for 30 min at 37°C to remove unhybridized excess labeled RNA and then washed in 1× SSC with 50% formamide at 46°C for 30 min and 0.5× SSC with 50% formamide at the same temperature for 30 min. After incubation in 10% heat-inactivated FCS in 25 mM Tris-HCl (pH 7.5) for 30 min at room temperature, the sections were incubated in 0.2% alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim) in 1% heat-inactivated FCS in 25 mM Tris-HCl for 30 min at 37°C and then washed in 25 mM Tris-HCl (pH 7.5) twice to remove unbound antidigoxigenin antibody. Immobilized digoxigenin was revealed by alkaline phosphatase staining with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate cytochemical stain. Finally, the sections were counterstained with methyl green and mounted in gelatin.

**Clinicopathological Analysis.** To assess the clinical significance of HMGI(Y) expression, we analyzed the relationship between HMGI(Y) protein expression level (as measured by immunohistochemical staining) in 30 carcinomas cases and several clinicopathological parameters: depth of tumor invasion, lymphatic and/or venous involvement, lymph node metastasis, and Dukes’ stage.
Fig. 4. HMGI(Y) mRNA expression in colon carcinoma and normal colonic mucosa by RNA in situ hybridization. A, in a section including carcinoma and normal epithelial cells (CA, carcinoma lesion; NM, normal mucosa), no specific reactivity was found in both regions with the control HMGI(Y) sense riboprobe. B, in a section equivalent to that shown in A, the HMGI(Y) antisense riboprobe hybridized strongly with carcinoma cells (purple staining) and also with epithelial cells in adjacent normal colonic mucosa. C and D, parts of sections in A and B observed under higher magnification. C, the HMGI(Y) antisense riboprobe hybridized strongly with carcinoma cells. D, the HMGI(Y) antisense riboprobe also hybridized with epithelial cells in the adjacent normal colonic mucosa, although the signals were much weaker than those found for carcinoma cells. Objective magnifications, ×100 (A and B) and ×400 (D and E). Sections were counterstained with methyl green.

Results and Discussion

Expression of HMGI(Y) Proteins. Immunohistochemical analysis using a specific antibody revealed HMGI(Y) immunoreactivity in carcinoma cells but not in epithelial cells in adjacent normal colorectal mucosa (Fig. 1, A and B) when observed at lower magnification, as reported previously (22). In carcinomas, although HMGI(Y) immunoreactivity was localized mainly in the nuclei, faint staining was also observed within the cytoplasm (Fig. 1D). Negative control sections did not show any corresponding staining (Fig. 1C). Observation at higher magnification revealed HMGI(Y) immunoreactivity in a small proportion of epithelial cells in normal colorectal mucosa, although the intensity was much lower than that in carcinoma cells (Fig. 1E). The percentages of HMGI(Y)-positive cells were then calculated. The proportion of HMGI(Y)-positive cells in the carcinoma tissue (62.5 ± 23.1%, mean ± SD) was significantly higher than that in normal colorectal mucosa (4.7 ± 2.9%; P = 0.0001, Mann-Whitney test; Fig. 2), indicating that an increased expression of HMGI(Y) proteins is closely associated with the malignant phenotype in colorectal tissue.

Having shown that an increased expression of HMGI(Y) proteins is a characteristic feature of colorectal carcinoma, we then examined their expression in colorectal adenoma. HMGI(Y) immunoreactivity in adenoma with mild (Fig. 3A) or moderate (Fig. 3B) atypia remained as weak as that in normal colorectal mucosa (Fig. 1E), whereas adenomas with severe atypia (Fig. 3C) showed strong immunoreactivity equivalent to that observed in carcinoma cells (Fig. 1D). The percentages of HMGI(Y)-positive cells were then calculated. The proportion of HMGI(Y)-positive cells in all adenoma samples (15.8 ± 16.8%) was significantly lower than that in carcinoma samples (P = 0.0001, Mann-Whitney test) and significantly higher than that in normal mucosa samples (P = 0.002). When the HMGI(Y) protein expression levels and histological cellular atypia grading in adenomas were compared, the proportion of HMGI(Y)-positive cells in adenoma with severe atypia (39.5 ± 12.4%) was significantly higher than that in adenomas with mild atypia (7.1 ± 3.9%; P = 0.0001) or moderate atypia (6.9 ± 4.8%; P = 0.0001) or in normal colorectal mucosa (4.7 ± 2.9%; P = 0.0001; Fig. 2). There was also no significant difference in the percentages of HMGI(Y)-positive cells between adenomas with mild atypia and those with moderate atypia. However, the proportion of HMGI(Y)-positive cells in adenomas with severe atypia was significantly lower than that in carcinomas (P = 0.007). In other words, a significant elevation of the HMGI(Y) protein expression level appeared in adenomas with severe atypia, whereas it remained normal in adenoma with less atypia. Considering that HMGI(Y) is overexpressed in colorectal carcinoma cells and that HMGI(Y) overexpression has been shown to be closely associated with neoplastic transformation of cells and malignant phenotypes in experimental tumor cell lines (25, 26), these findings may support the view that colorectal adenoma with severe atypia is a precancerous lesion. It may be possible that some event (or events) involving alteration in the expression of HMGI(Y) proteins takes place in severely dysplastic adenomas, presumably preceding the morphological changes associated with carcinogenic transformation in colorectal tissue. It remains to be determined, however, whether or how such alteration in the expression of HMGI(Y) proteins is related to colorectal carcinogenesis per se. Such alteration of HMGI(Y) expression in cellular atypia, however, appears not to be a feature unique to colorectal adenomas. Increased HMGI(Y) protein expression was observed in intraepithelial cellular dysplasia of the uterine cervix (23). In these lesions, HMGI(Y) expression is not only elevated but also positively correlated with cellular dysplasia grading.

Expression of HMGI(Y) mRNA. To determine whether the altered HMGI(Y) protein expression observed in colorectal carcinoma is associated with an alteration of mRNA expression levels, we analyzed the expression of HMGI(Y) mRNA by RNA in situ hybridization. Under conditions in which no specific cellular reactivity was
found with the control HMGI(Y) sense riboprobe (Fig. 4A), the HMGI(Y) antisense riboprobe hybridized strongly with carcinoma cells (Fig. 4, B and C). The HMGI(Y) antisense riboprobe also hybridized with epithelial cells in the adjacent normal mucosa (Fig. 4D), although the signals were much weaker than those found for carcinoma cells. In adenomas, the signal intensity again correlated well with the cellular atypia grading, similar to the analysis at the protein level. That is, signals observed in adenomas with histologically mild or moderate atypia remained as weak as those in normal mucosa, whereas adenomas with severe atypia showed strong signals almost equivalent to those observed in carcinoma cells (Fig. 3, D–F). These results indicate that an increased expression of HMGI(Y) proteins in colorectal carcinoma cells occurs as a result of an increase in its mRNA expression.

Relationship between HMGI(Y) Protein Expression Level and Clinicopathological Parameters. Of the 30 carcinoma cases investigated in the study, 18 (60%) showed high-level HMGI(Y) expression (3+), 9 (30%) moderate-level expression (2+), and 3 (10%) showed low-level expression (1+), when categorized based on the criteria mentioned in “Materials and Methods” (Fig. 5). There was a significant difference in the HMGI(Y) protein expression level between cases with lymphatic and/or venous involvement and those without it. In fact, 14 of 17 (82.4%) invasion-positive cases showed high levels of HMGI(Y) protein expression, whereas 8 of 11 (72.7%) invasion-negative cases showed low to moderate levels of HMGI(Y) protein expression (Table 1). A significant correlation was detected between high-level HMGI(Y) protein expression level and the presence of lymph node metastasis and advanced clinical stage according to Dukes’ classification. Factors found to be associated with the elevated expression of HMGI(Y) proteins, including lymphatic and/or venous involvement, lymph node metastasis, and advanced stage according to Dukes’ classification, are known to be indicative of poor prognosis of patients with colorectal cancer (27, 28). Thus, this study revealed a striking correlation between the level of HMGI(Y) protein

Fig. 5. Typical HMGI(Y) immunostaining representing 3+ (A), 2+ (B), and 1+ (C). Immunostained slides were evaluated microscopically, and the percentage of HMGI(Y)-positive cells was scored by counting ~1000 epithelial cells in three randomly selected fields. A, a carcinoma sample assigned as 3+ (≥60% of carcinoma cells with positive nuclear staining); B, a carcinoma sample assigned as 2+ (20–60% of carcinoma cells with positive nuclear staining); C, a carcinoma sample assigned as 1+ (<20% of carcinoma cells with positive nuclear staining). Objective magnification, ×400. Sections were counterstained with Mayer’s hematoxylin.

Table 1 Relationship between HMGI(Y) protein expression level and clinicopathological parameters

<table>
<thead>
<tr>
<th>Parameter (n)</th>
<th>HMGI(Y) protein expression level&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
<th>P</th>
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<tbody>
<tr>
<td>Serosal/adventitia invasion</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Negative (22)</td>
<td></td>
<td>3/22 (13.6%)</td>
<td>7/22 (31.8%)</td>
<td>12/22 (54.6%)</td>
<td>0.107</td>
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<tr>
<td>Positive (8)</td>
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<td>1/8 (12.5%)</td>
<td>7/8 (87.5%)</td>
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<td></td>
</tr>
<tr>
<td>Lymphatic and/or venous involvement</td>
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<td>3/11 (27.3%)</td>
<td>5/11 (45.4%)</td>
<td>3/11 (27.3%)</td>
<td>0.005</td>
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<tr>
<td>Negative (11)</td>
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<td>4/19 (21.0%)</td>
<td>14/17 (82.4%)</td>
<td></td>
<td></td>
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<tr>
<td>Positive (19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nodal metastasis</td>
<td></td>
<td>3/16 (18.8%)</td>
<td>7/16 (43.7%)</td>
<td>6/16 (37.5%)</td>
<td>0.009</td>
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<tr>
<td>Negative (16)</td>
<td></td>
<td>2/14 (14.3%)</td>
<td>12/14 (85.7%)</td>
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<td></td>
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<tr>
<td>Positive (14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dukes’ classification&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>3/16 (18.8%)</td>
<td>7/16 (43.7%)</td>
<td>6/16 (37.5%)</td>
<td>0.009</td>
</tr>
<tr>
<td>A and B (16)</td>
<td></td>
<td>2/14 (14.3%)</td>
<td>12/14 (85.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C and D (14)</td>
<td></td>
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<sup>a</sup> 1+, <20% of carcinoma cells with positive nuclear staining; 2+, 20–60% of carcinoma cells with positive nuclear staining; 3+, >60% of carcinoma cells with positive nuclear staining.

<sup>b</sup> A, invasion limited to tunica muscularis propria and no lymph node metastasis; B, invasion beyond tunica muscularis propria and no lymph node metastasis; C, metastasis to lymph nodes; D, metastasis to distant organs.
expression and factors closely associated with a poor prognosis of patients with colorectal cancer. One possible interpretation would be that the more HMGI(Y)-positive cells or biologically aggressive cells a tumor contains, the more it proliferates or acquires metastatic capability, resulting in fulminant clinical progression. These results for colorectal cancer corroborate a previous report on prostate cancer demonstrating a correlation between the HMGI(Y) mRNA level and clinicopathological parameters such as clinical stage, Gleason grade, and recurrence rate (21). Thus, determination of the level of HMGI(Y) protein expression could be a potential tumor progression marker, not only in colorectal cancer but also in other types of cancer. Studies on larger numbers of patients, including complete follow-up studies, will further elucidate the clinical significance of HMGI(Y) protein expression in colorectal cancer.

In conclusion, these results indicate that the determination of the HMGI(Y) protein expression level could be a potential marker for the diagnosis of colorectal neoplasias and can be of great value in predicting the prognosis of patients with colorectal cancer.

References
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