Increased Expression of S100A6 (Calcyclin), a Calcium-binding Protein of the S100 Family, in Human Colorectal Adenocarcinomas

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ABSTRACT

The expression of S100A6 (also known as Calcyclin/2A9/5B10/PRA) in surgically resected human colorectal adenocarcinomas was examined to investigate whether S100A6 plays a role in the malignancy of human tumor cells. Western blot analysis using the lysates from colorectal adenocarcinomas and adjacent normal mucosa from 10 patients revealed that the average S100A6 level of adenocarcinomas was significantly higher (about 2.4-fold) than that of normal mucosa. Immunohistochemical analysis using formalin-fixed paraffin-embedded surgical specimens and monoclonal anti-S100A6 antibody (mAbA6) demonstrated that 2 (5%) of 42 normal mucosa and 6 (46%) of 13 adenoma specimens were mAbA6-positive and showed granular staining localized at the supranuclear regions of epithelial cells, whereas 23 (55%) of 42 adenocarcinomas and 13 (100%) of 13 carcinoma cells that metastasized to the liver were mAbA6-positive and showed diffuse cytoplasmic staining. A significant correlation between S100A6 expression and Dukes' tumor stage or lymphatic permeation but not with other clinicopathological factors was shown. S100A6 was stained more intensely in peripheral portions than in central portions of adenocarcinomas, whereas Ki-67 (a growth marker) was stained equally in these two portions. These results suggest that S100A6 may be involved in the progression and invasive process of human colorectal adenocarcinomas.

INTRODUCTION

A number of S100-related low-molecular-weight calcium-binding proteins have been identified in mammalian cells (1) and are thought to mediate calcium signals in normal and transformed cells. One such protein, S100A6, is preferentially expressed in proliferating rather than quiescent cells (2, 3). The human S100A6 gene (2, 4), which is located on chromosome 1q21 (5), encodes an acidic 90-amino-acid protein (M, 10.5) containing two EF-hand motifs. The gene product has been implicated to be involved in growth of hair follicles (6), differentiation (7), regeneration (8, 9), secretion (10, 11), and metastasis (12, 13) in mammalian cells.

Colorectal cancer shows a clear step-wise progression from normal through premalignant and malignant stages to the metastatic state. There has been progress in molecular genetic analysis of colorectal tumorigenesis (14). In the present study, we investigated the expression of S100A6 in surgically resected normal human colonic mucosa, adenomatous polyps, adenocarcinomas, and metastatic nodules in the liver to clarify its biological relevance to the progression of human colorectal adenocarcinomas.

MATERIALS AND METHODS

Surgical Specimens. Fresh human tissues (primary colorectal adenomatous polyp, primary adenocarcinoma, and adjacent normal colorectal mucosa from specimens resected for carcinoma and liver metastases) were collected from patients undergoing surgical resection in our hospital. The primary tumors were staged according to Dukes’ classification system (15). Surgical specimens were immediately stored at −80°C for Western blotting (10 specimens) or fixed with 10% formalin in PBS for immunohistochemistry (42 specimens).

Preparation of Tissue Extracts. Frozen surgical specimens were thawed, minced with scissors, crushed in a solution consisting of 0.05 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 6% (v/v) β-mercaptoethanol, and 10% (w/v) glycerol using polytron, and centrifuged at 15,000 × g for 10 min. The supernatant was used immediately or stored frozen at −80°C for immunoblot analysis.

SDS-PAGE and Western Blot Analysis. SDS-PAGE was performed as described by Laemmli (16). Protein samples were electrophoresed on 15% polyacrylamide gel under reducing conditions. The resolved proteins were electrophoretically transferred to PVDF membrane (17). S100A6 and actin were detected using monoclonal antibodies against pig S100A6 (mAbA6; Sigma, St. Louis, Mo) and pan-actin (Anti-Actin,
Boehringer Mannheim, Mannheim, Germany), respectively. This mAbA6 cross-reacts with human, rabbit, and rat S100A6 and does not react with S100A2, S100a, and S100b. Polyclonal antimouse S100A4 antibody was kindly supplied by Dr. K. Takenaga. Antimouse IgG (H + L) AP conjugate or antirabbit IgG (Fc) AP conjugate (Promega, Madison, WI) and BCIP/NBT Color Substrate (Promega) were used for alkaline phosphatase detection. Both S100A6 and actin protein expression levels were quantitatively estimated by densitometric scanning performed with NIH Image 1.55f. S100A6 protein concentration was normalized to actin level and expressed as densitometric ratio. Protein concentration was determined by Protein Assay (Bio-Rad, Richmond, CA).

**Immunohistochemical Staining.** Four-μm sections from formalin-fixed, paraffin-embedded tissues were mounted on poly-L-lysine-coated slides. They were then air-dried and deparaffinized. Endogenous peroxidase activity was blocked with 0.35% hydrogen peroxide in 50% methanol for 15 min at room temperature. The sections were rehydrated and washed with PBS. After blocking nonspecific binding sites with 2% normal horse serum in PBS for 30 min at room temperature, the sections were incubated with mAbA6 or monoclonal anti-Ki-67 antibody (MIB-1, Immunotech, Westbrook, ME) in PBS containing 0.1% BSA overnight at 4°C. After rinsing with PBS, the sections were incubated with biotinylated horse antimouse IgG (Vector, Burlingame, CA) for 30 min at room temperature followed by washing with PBS. Immunoreactivity was detected with an avidin-biotin system (Vector) using 0.025% 3,3’-diaminobenzidine tetrahydrochloride as a chromogen for 2.5 min. The sections were lightly counterstained with Mayer’s hematoxylin.

**Evaluation of Degree of Antibody Reactivity.** The degree of monoclonal anti-S100A6 or anti-Ki-67 reactivity with each tissue section was scored by the percentage of stained normal or neoplastic epithelial cells in the section. In this study, normal and neoplastic epithelial tissues were divided into three categories: negative, reduced, and positive. As shown in Table 1, 30% of the sections were determined to be negative, 36% were defined as “positive” and others (34%) as “reduced.” Three persons (K. K., A. A., and H. N.) independently judged the stained cells.

**Statistical Analysis.** Correlations between positive expression and clinicopathological factors were tested by the χ² test except for age parameter, which was assessed by Student’s t test.

**RESULTS**

**Western Blotting of S100A6 in Human Colorectal Adenocarcinomas.** We first subjected recombinant rabbit S100A6, recombinant mouse S100A4, and human colorectal adenocarcinoma lysate to SDS-PAGE to examine whether the mAbA6 used in this study cross-reacts with human S100A4 or not. As shown in Fig. 1, A and B, S100A6 ran faster than S100A4 in SDS-PAGE, and mAbA6 reacted with human S100A6, but it did not cross-react with human S100A4.

Fig. 2A shows S100A6 and actin protein expression of the matched colorectal adenocarcinomas (T) and adjacent normal colorectal mucosa (N) from 10 patients. In 9 of 10 adenocarcinomas, S100A6 levels were higher than those of normal mucosa. Average S100A6 level of adenocarcinomas was significantly higher (about 2.4-fold; P = 0.001) than that of normal mucosa (Fig. 2B).

**Immunohistochemical Analysis of S100A6 Expression in Human Colorectal Adenocarcinomas.** In order to examine the expression of S100A6 at the histological level, we performed immunohistochemical analysis. The staining was abolished when an adjacent serial section was incubated with mAbA6 that had been previously absorbed with excess recombinant S100A6 protein and further abolished by incubating with normal mouse IgG (data not shown).

Two (5%) of 42 normal mucosa and 6 (46%) of 13 adenoma specimens showed mAbA6-positive and granular staining localized at the supranuclear regions of epithelial cells (Table 1; Fig. 3, A and B). In adenocarcinomas, 23 (55%) of 42 cases were mAbA6-positive and diffusely stained in whole cytoplasts (Table 1; Fig. 3C). There was a significant correlation (P < 0.01) between S100A6 level and Dukes’ tumor stage or lymphatic permeation but no other clinicopathological factors (Table 2). The carcinoma cells that invaded into lymphatic vessels were immunopositive (Fig. 3 E). All of the carcinoma cells that metastasized to the liver [13 (100%) of 13 cases] were mAbA6-positive (Table 1; Fig. 3D). In normal colorectal tissues, smooth muscle of blood vessels (in most but not in all cases) and nerve bundle were strongly stained (Fig. 3F).

**Comparison of the Staining Pattern between S100A6 and Ki-67 in Human Colorectal Adenocarcinomas.** Fig. 4 shows S100A6 and Ki-67 staining in a serial section of colorectal adenocarcinoma. Ki-67 is a growth marker which is present in the nuclei (especially nucleoli) of growing normal and tumor cells, although its function is still obscure (18). S100A6 staining was more intense in peripheral portion than in central portion of the carcinoma (Fig. 4A), whereas Ki-67 staining pattern did not show such a tendency (Fig. 4B). Thirty-four (89%) of 38 colorectal adenocarcinoma specimens were stained as mAbA6-positive in the peripheral portions, whereas 11 (29%) of 38 specimens were stained as mAbA6-positive in the central portions of the carcinomas (Table 3). This staining pattern was statistically significant (P < 0.0001). On the other hand, the
staining pattern of Ki-67 in peripheral portions of adenocarcinoma specimens was similar to that in central portions (Table 3).

DISCUSSION

Altered expression of S100A6 has been reported in several human neoplastic cells (19–23). No functional implications of S100A6 in tumor development, however, have been established, although biochemical studies have shown to specifically interact with annexins, tropomyosin, caldesmon, and other proteins (24–29). Another link between S100 family members and tumorigenicity comes from the location of the S100 gene cluster, because the chromosome region 1q21 is frequently rearranged in various tumors, especially in human breast carcinomas (30).

In the present study, S100A6 levels in human colorectal adenocarcinoma and matched normal mucosa were quantitatively measured by Western blotting (Fig. 2A). The expression level was about 2.4-fold higher in adenocarcinomas than in normal mucosa (Fig. 2B). Because S100A6 has been reported to be expressed in a variety of cell types [such as fibroblasts and epithelial cells (31), nerve bundles, and blood vessel endothelial cells (20)], it is premature to conclude that the higher expression of S100A6 protein in adenocarcinoma specimens indeed reflects the expression in carcinoma cells themselves. To evaluate this point and to examine the expression of S100A6 in adenocarcinoma cells more closely, we performed immunohistochemical analyses using mAbA6.

Table 1  mAbA6 reactivity in human colorectal normal and neoplastic epithelial cells examined immunohistochemically

<table>
<thead>
<tr>
<th></th>
<th>Positive/Total</th>
<th>%</th>
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<tbody>
<tr>
<td>Normal mucosa</td>
<td>2/42</td>
<td>5</td>
</tr>
<tr>
<td>Adenoma</td>
<td>6/13</td>
<td>46</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>23/42</td>
<td>55</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>13/13</td>
<td>100</td>
</tr>
</tbody>
</table>

Normal colorectal mucosa and adenoma cells showed granular staining localized at the supranuclear regions (Fig. 3, A and B). Furthermore, we also observed such a staining pattern in normal small intestinal mucosa (data not shown). A role for S100A6 in the process of mucus secretion in the epithelia that lines the gastrointestinal, respiratory, and urinary tracts and in the process of insulin release from the pancreatic β cells was suggested (10, 11). Therefore, S100A6, which we observed in normal colorectal mucosa, may play a role in mucus secretion.

In adenocarcinoma cells, S100A6 was stained more intensely and diffusely in the cytoplasm (Fig. 3C). Such a staining pattern was also observed in the tumor cells permeated in lymphatic vessels and metastasized to the liver (Fig. 3, D and E), whereas normal liver cells were not stained at all. As our results indicate in relation to clinicopathological factors, S100A6 expression in colorectal adenocarcinomas was significantly associated with Dukes’ tumor status (i.e., nodal status) and lymphatic permeation (Table 2). These results suggest that S100A6 expression may be linked to the progression of colorectal neoplasms.

When normal and neoplastic epithelial tissues containing more than 50% stained cells were defined as mAbA6 positive, there was not so much difference in immunoreactivity between adenoma (46%) and adenocarcinoma (55%) cells (Table 1). However, when more than 10% stained cells were defined as positive, immunoreactivity was as follows: (a) normal mucosa 4 (10%) of 42; (b) adenoma 6 (46%) of 13; (c) adenocarcinoma 39

Fig. 2  Expression of S100A6 in human colorectal adenocarcinomas and adjacent normal mucosa. A, Western blot analysis of the S100A6 protein expression. Tissue extracts (1 μg) were run on SDS-PAGE under reducing conditions and blotted onto PVDF membrane. S100A6 immunoblot in normal mucosa (N) and adenocarcinoma (T) in matched samples from 10 patients (1—10) is shown (upper row). Actin immunoblot is also shown (lower row). B, comparison of average S100A6 expression levels in human normal colorectal mucosa and adenocarcinoma. “Normal” and “Tumor” indicate the means of densitometric ratio (S100A6:actin) of N and T samples, respectively, from 10 patients shown in A.
(93%) of 42; (d) liver metastasis 13 (100%) of 13. Thus, the immunoreactivity in adenoma (46%) was less than in adenocarcinoma (93%) and more than in normal mucosa (10%). In colorectal tissues, several types of cells were stained with mAbA6; smooth muscle cells of blood vessels and nerve bundles invariably showed strong staining (Fig. 3F). The variation of S100A6 contents in normal mucosa specimens by Western blotting may be derived from these immunopositive cells.

Interestingly, S100A6 was more intensely stained in peripheral portions with structural atypia or with deeply invaded portions than in central portions with differentiated structure of colorectal adenocarcinomas (Table 3; Fig. 4A). On the other hand, the Ki-67 staining pattern was similar in these two portions (Table 3 and Fig. 4B). These results were unexpected because expression of S100A6 has been thought to be involved in cell growth. However, a dissociation between S100A6 ex-
pression and cell growth was reported by Gong et al. in human endometrial carcinoma cell lines (32). In these cell lines, phorbol esters inhibit cellular proliferation but enhance S100A6 expression, which may result from the activation of protein kinase C. We also observed that the deeply invaded adenocarcinoma cells consisting of single cells were intensely stained with mAbA6 but not with anti-Ki-67 antibody at all. The infiltrating neutrophils and macrophages in the stroma were also mAbA6-positive (data not shown). Furthermore, Guo et al. found elevated S100A6 expression in metastatic H-ras-transformed NIH 3T3 cells as compared with nonmetastatic ones (12). Weterman et al. showed that S100A6 expression is elevated in highly metastatic human melanoma cell lines as compared with low metastatic ones (13). They also reported that a stronger S100A6 staining in a higher percentage of positive cells is observed in the more advanced vertical growth phase of human primary melanoma as compared with the early growth phase of the melanoma (20). Our present observation showed that all of the carcinoma cells in metastatic nodules in the liver were mAbA6-positive (100%) when compared with primary adenocarcinomas (55%) (Table 1). These results suggest the involvement of S100A6 in the progression and invasive process of human colorectal adenocarcinoma cells.

The immunohistochemical staining pattern of S100A6 in colorectal adenocarcinomas was similar to that of S100A4 reported by Takenaga et al. (33). They reported that the incidence of S100A4 immunopositive cells increases according to the depth of invasion. However, in our study, S100A6 showed a granular staining pattern localized at the supranuclear regions of epithelial cells in normal colorectal mucosa and adenomas, whereas Takenaga et al. reported that these epithelial cells are not stained at all with anti-S100A4 antibody. Mandinova et al. also showed a distinct subcellular localization of S100A6 and S100A4 in human vascular smooth muscle cells (34). These observations suggest that S100A6 and S100A4 play different roles in the physiology of normal colorectal epithelial cells. Although the staining pattern of these 2 proteins seemed to be similar in adenocarcinomas, their functional roles in the tumor remain to be elucidated. Further work is required to provide evidence for a causative role of S100A6 in invasive and meta-

Table 2  Relationship between S100A6 expression and clinicopathological factors

<table>
<thead>
<tr>
<th>S100A6 level*</th>
<th>Reduced</th>
<th>Positive</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD, years)</td>
<td>62.8 ± 8.3</td>
<td>60.1 ± 10.3</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>16/3</td>
<td>16/7</td>
<td>NS</td>
</tr>
<tr>
<td>Dukes' classification</td>
<td>17</td>
<td>9</td>
<td>0.0025</td>
</tr>
<tr>
<td>A, B C</td>
<td>2</td>
<td>14</td>
<td>0.0022</td>
</tr>
<tr>
<td>Lymphatic permeation</td>
<td>Absent</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Present</td>
<td>11</td>
<td>23</td>
<td>0.0022</td>
</tr>
<tr>
<td>Vascular permeation</td>
<td>Absent</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Present</td>
<td>13</td>
<td>19</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor size (mm)</td>
<td>&lt;40</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>≥40</td>
<td>10</td>
<td>11</td>
<td>NS</td>
</tr>
<tr>
<td>Histological differentiation</td>
<td>Well</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Moderate, poor</td>
<td>9</td>
<td>13</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Colorectal adenocarcinoma tissues with more than 50% stained cells were defined as mAbA6 positive and others as reduced.

Table 3  Relationship between S100A6 and Ki-67 staining in central and peripheral portions of human colorectal adenocarcinomas*

<table>
<thead>
<tr>
<th>Portion of tumor</th>
<th>S100A6</th>
<th>Ki-67</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>27</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>Peripheral</td>
<td>4</td>
<td>34</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Colorectal adenocarcinoma tissues with more than 50% stained cells were defined as positive and others as reduced.

b NS, not significant.
static potential, especially lymphatic permeation and nodal metastasis of colorectal adenocarcinoma cells. In addition, whether S100A6 expression could be used as a marker for prognosis in colorectal carcinoma patients should be evaluated.

ACKNOWLEDGMENTS

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