EXPRESSION OF $\alpha_1$-INTegrin, FIBronectin, Type IV Collagen, Tenascin and T CELL INFILTRATION IN GASTRIC CARCINOMA

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Abstract

To investigate the relationship between the progression of gastric carcinoma and cell adhesion molecules, extracellular matrices, and tumor infiltrating T cells, immunohistochemical examination for $\alpha_1$-integrin, fibronectin, type IV collagen, tenascin, and CD45RO was performed. Study materials consisted of 5 hyperplastic polyps, 9 adenomas, 55 early and 17 advanced gastric carcinomas.

The expression of $\alpha_1$-integrin, fibronectin, type IV collagen and tenascin was preserved in benign gastric lesions. However, their expression was decreased in gastric carcinoma according to the depth of invasion. $\alpha_1$-integrin was reserved in 32/38 (84.2%) of mucosal, 10/17 (58.8%) of submucosal, and 7/17 (41.2%) of advanced gastric carcinoma. Fibronectin was observed in 29/38 (76.3%), 15/17 (88.2%), 8/17 (47.1%), and type IV collagen expression was 25/38 (65.8%), 6/17 (35.3%), 2/17 (11.8%), of mucosal, submucosal, and advanced gastric carcinoma, respectively. Tenascin expression was observed in 31/38 (81.6%) of mucosal, 14/17 (82.4%) of submucosal, and 8/17 (47.1%) of advanced gastric carcinoma. In tenascin positive gastric carcinoma, a significantly large number of T cells was infiltrated in cancerous stroma ($21.0 \pm 14.0/0.16 \, \text{mm}^2$) than that of negative carcinoma ($9.6 \pm 11.6$), suggesting the activation of local cellular immune response against gastric carcinoma.

The decrease of the expression of $\alpha_1$-integrin, fibronectin, type IV collagen, and tenascin were associated with higher incidence of lymph vessel invasion, venous invasion and lymph node metastasis. These results suggest that the expression of $\alpha_1$-integrin, fibronectin, type IV collagen, and tenascin may play a possible role of biological barrier against gastric carcinoma.

Key Words:

Gastric carcinoma, $\alpha_1$-integrin, fibronectin, type IV collagen, tenascin

Introduction

Cancer cells are characterized by autonomous overgrowth, invasion into surrounding structures and formation of metastatic foci in distant organs. Most deaths from cancer after surgical removal of primary lesions are attributed to distant metastasis. Recent studies indicate that the cell adhesion molecules (CAM) and the extracellular matrix (ECM) play an essential role in the development of cancer invasion and metastasis\textsuperscript{1,2}. 

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Integrins constitute a family of transmembrane cell adhesion receptors which are composed of heterodimeric complexes of noncovalently linked $\alpha$ and $\beta$ chains. Integrins have specific receptors for fibronectin and function as signal transducers between cell-to-cell and cell-to-ECM interactions. Thus, they may play an important role in cancer invasion and metastasis. Fibronectin is one of the ECM components, and fibronectin-integrin interactions are involved in cancer cell migration, invasion, and metastasis. The multifunctional action of fibronectin is known, including the promotion of cell proliferation, chemotaxis and the induction and proliferation of cytotoxic T lymphocytes (CTLs) which produce various lymphokines. Type IV collagen and laminin are the major component of basement membrane, which delineate the boundary between the surface epithelium and the connective tissue. The basement membrane is regarded as the first biological barrier against cancer cell invasion. Tenascin is one of the ECM components, and it is expressed temporarily in fetal development, wound healing, inflammatory processes and neoplastic lesions. Tenascin is also induced by several growth factors and cytokines.

Gastric carcinoma constitutes the major cause of death from cancer in Japan. To investigate the relationship between the progression of gastric carcinoma and host microenvironmental CAMs and ECMs, immunohistochemical examination of $\alpha_1$-integrin, fibronectin, type IV collagen, and tenascin was performed using materials mainly composed of differentiated early gastric carcinomas from elderly patients. Furthermore, to clarify the mechanism in these expression, T cell infiltration was also evaluated.

### Materials and Methods

#### Materials

The study materials consisted of 5 hyperplastic polyps, 9 adenomas, 55 early (tumor invasion limited to the mucosa and submucosa) and 17 advanced (tumor invasion beyond the muscularis propria) gastric carcinomas that had been surgically resected from 56 elderly patients, 34 men and 22 women, with a mean age of 73.1 ± 6.5 (mean ± SD) years. There were 23 patients who had multiple gastric lesions. None of the patients received chemotherapy or radiation therapy prior to operation. Histopathological diagnosis was made according to the Japanese Classification of Gastric Carcinoma. Gastric carcinoma was histologically classified into two types as the differentiated type (papillary or tubular adenocarcinoma) and the undifferentiated type (poorly differentiated adenocarcinoma and signet-ring cell carcinoma).

**Preparation of the tissue specimens**

The resected surgical specimens were fixed in 20% formaldehyde solution and processed in the usual manner for histopathological examination. Paraffin-embedded tissue blocks including the center of gastric lesions and adjacent gastric mucosa were selected from each specimen, and the serial tissue sections, 4 μm thickness, were sliced for immunohistochemical stainings.

**Immunohistochemical Staining**

As the primary antibodies, rabbit polyclonal anti-human fibronectin (A0245), mouse monoclonal anti-human-collagen IV (Clone CIV22), and mouse monoclonal anti-human T cell (CD45RO, UCHL1) were purchased from DAKO(Copenhagen, Denmark). Mouse monoclonal anti-human-$\alpha_1$-integrin (Clone DF7) and mouse monoclonal anti-human-tenascin (Clone DB7) were purchased from Biohit(Kajaani, Finland). Specificities and working dilutions of each antibody were listed in Table 1.

**Immunohistochemical staining** was performed according to the avidin-biotin-peroxidase complex method, using Vectastain ABC kit according to manufacturers' instruction (Vector Laboratories, Burlingame, USA).
Negative controls were obtained by equivalently diluted mouse or rabbit serum instead of the primary antibodies. Normal gastric mucosa included in tissue specimens was used as 'build-in' positive control of the procedure. In fibronectin staining, pretreatment by microwave oven heating in 10 mM citrate buffer (sodium citrate-citric acid), pH 6.0 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), at 95°C for 10 minutes was performed for retrieval of antigen. Enzymatic digestion by incubation in 0.01 M HCl containing 1 mg/ml pepsin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), pH 2.0, at 37°C for 60 minutes were performed in the stainings of Integrin, tenascin, and type IV collagen. Color reaction was developed with 0.05 M Tris-HCl pH 7.6 (SIGMA CHEMICAL CO., St.Louis, USA), containing 0.15 % diaminobenzidine tetrahydrochloride (Funakoshi CO., Ltd., Tokyo, Japan). Nuclear counterstaining was performed with Meyer's hematoxylin (MUTO PURE CHEMICAL, LTD., Tokyo, Japan).

Grading of staining intensity

The tissue sections were examined with a conventional light microscope (OLYMPUS OPTICAL CO., Ltd., Tokyo, Japan). The immunoreactivity and the localization of Integrin, fibronectin, type IV collagen, and tenascin were observed in gastric mucosa and gastric lesions. Gastric lesions were regarded as normally preserved for Integrin, fibronectin, and type IV collagen when staining of the cellular membrane or the matrix component was observed throughout the gastric lesions in the tissue sections. Gastric lesions exhibiting aberrant or decreased expression were regarded as reduced expression. Gastric lesions were regarded as positive for tenascin when tenascin immunoreactivity was observed in the stroma around gastric lesions. Gastric lesions lacking tenascin staining were regarded as negative.

Enumeration of T cells in cancerous stroma.

Lymphocytes expressing membranous staining with CD45RO were considered as CD45RO positive cells. The mean number of T cells infiltrating in cancerous stroma was calculated by counting CD45RO positive cells in three consecutive areas with a 10 μm calibrated grid in the ocular at 200 magnification (cells/0.16 mm²).

Statistical analysis

Student's t-test and the χ² test with Yates correction were used for statistical analysis, and the level of significance was designated as p < 0.05.

Results

The study materials consisted of 5 hyperplastic polyps, 9 adenomas, 38 mucosal, 17 submucosal, and 17
advanced gastric carcinomas. Because the materials were obtained from elderly patients, mucosal atrophy and focal or severe intestinal metaplasia was observed in almost all of the sections, and 66 of 72 gastric carcinomas were classified histologically as the differentiated type.

Expression on \( \alpha_1 \)-integrin in gastric carcinoma.

In normal gastric mucosa, uniform membranous staining of \( \alpha_1 \)-integrin was observed throughout the cellular surface of the gastric gland epithelium. Muscular structures, blood vessels, and some stromal cells in the gastric wall were also stained (Figure 1a, b).

In benign gastric lesions, the immunoreactivity for \( \alpha_1 \)-integrin was preserved in 5/5 (100 %) of hyperplastic polyps and 7/9 (77.8 %) of gastric adenomas (Table 2). In contrast, gastric carcinomas exhibited aberrant or reduced expression of \( \alpha_1 \)-integrin (Figure 1c, d). The expression of \( \alpha_1 \)-integrin was significantly decreased in advanced gastric carcinomas: 32/38 (84.2 %) of mucosal, 10/17 (58.8 %) of submucosal, and 7/17 (41.2 %) of advanced gastric carcinoma \((p < 0.01, \text{Table } 3)\).

There was no statistical significance between \( \alpha_1 \)-integrin expression and differentiation grade, venous invasion and lymph node metastasis. However, lymph vessel invasion was not identified in the \( \alpha_1 \)-integrin preserved gastric carcinoma group, while 39/52 (75.0 %) and 13/52 (25.0 %) in \( \alpha_1 \)-integrin reduced carcinoma \((p < 0.05, \text{Table } 3)\).

Expression on fibronectin in gastric carcinoma.

Fibronectin was observed in the stroma around the gastric glands in normal gastric mucosa. Irregular staining in the lamina propria was also identified. Muscular structures, blood vessels, and some stromal cells in the

Table 2. Expression of \( \alpha_1 \)-integrin, fibronectin, type IV collagen, and tenascin in gastric lesions.

<table>
<thead>
<tr>
<th>Gastric Lesion</th>
<th>( \alpha_1 )-integrin</th>
<th>Fibronectin</th>
<th>Type IV Collagen</th>
<th>Tenascin</th>
<th>Differentiation Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa</td>
<td>Uniform</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Hyperplastic polyp</td>
<td>Preserved</td>
<td></td>
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<tr>
<td>Gastric adenoma</td>
<td>Preserved</td>
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<tr>
<td>Gastric carcinoma</td>
<td>Reduced</td>
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</table>

Table 2. Expression of \( \alpha_1 \)-integrin, fibronectin, type IV collagen, tenascin and clinicopathological features of gastric carcinoma.

<table>
<thead>
<tr>
<th>Clinicopathological Features</th>
<th>( \alpha_1 )-integrin</th>
<th>Fibronectin</th>
<th>Type IV Collagen</th>
<th>Tenascin</th>
<th>Lymph Node Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal</td>
<td>32/38 (84.2 %)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Submucosal</td>
<td>10/17 (58.8 %)</td>
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<tr>
<td>Advanced</td>
<td>7/17 (41.2 %)</td>
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</table>

There was no statistical significance between \( \alpha_1 \)-integrin expression and differentiation grade, venous invasion and lymph node metastasis. However, lymph vessel invasion was not identified in the \( \alpha_1 \)-integrin preserved gastric carcinoma group, while 39/52 (75.0 %) and 13/52 (25.0 %) in \( \alpha_1 \)-integrin reduced carcinoma \((p < 0.05, \text{Table } 3)\).
Expression of CAM, ECM in gastric carcinoma

Gastric wall were also stained (Figure 2a, b).

Expression of fibronectin was preserved in all benign gastric lesions (Table 2). Fibronectin staining was also observed in gastric carcinoma. However, its expression was irregular or reduced, especially in advanced gastric carcinoma (Figure 2c, d). Expression of fibronectin was decreased according to the depth of invasion: 29/38 (76.3 %) of mucosal, 15/17 (88.2 %) of submucosal, and 8/17 (47.1 %) of advanced gastric carcinoma ($p < 0.05$, Table 3).

The expression of fibronectin was 50/66 (75.8 %) in differentiated and 2/6 (33.3 %) in undifferentiated gastric carcinoma, and a statistically significant decrease was identified in undifferentiated gastric carcinoma ($p < 0.05$). Its expression was associated with a lower incidence of lymphatic involvement: 41/52 (78.8 %) of lymphatic invasion negative and 11/20 (55.0 %) of positive gastric

Figure 2. Expression of fibronectin in normal gastric mucosa and gastric carcinoma.

Fibronectin was observed in the stroma around the gastric glands. Irregular staining in the lamina propria was also identified (a; HE, b; fibronectin immunostain. $\times$ 40). In gastric carcinoma, aberrant or reduced stromal staining was observed. Reduced at the upper part and aberrantly expressed at the lower part (c; HE, d; fibronectin immunostain. $\times$ 100).

Figure 3. Expression of type IV collagen in normal gastric mucosa and gastric carcinoma.

The basement membrane surrounding the gastric glands was lineally stained with type IV collagen (a; HE, b; type IV collagen immunostain. $\times$ 40). Irregular or reduced staining of type IV collagen was observed in gastric carcinoma. Negative at the upper part and partially positive at the lower part (c; HE, d; type IV collagen immunostain. $\times$ 100).
carcinomas ($p < 0.05$). However, there was no significant difference between the fibronectin expression and venous invasion or lymph node metastasis (Table 3).

**Expression on type IV collagen in gastric carcinoma.**

In normal gastric mucosa, type IV collagen was observed lineally along the basement membrane surrounding the gastric glands. Blood vessels and muscular structures were also immunoreactive to the antibody (Figure 3a, b). All hyperplastic polyps and gastric adenomas reserved type IV collagen immunoreactivity (Table 2).

In gastric carcinomas, type IV collagen was also observed like the basement membrane surrounding the cancer nests. However, aberrant or reduced expression was observed especially in advanced gastric carcinoma (Figure 3c, d). Strong correlation was observed between type IV collagen immunoreactivity and the depth of invasion; type IV collagen was preserved in 25/38 (65.8 %) of mucosal, 6/17 (35.3 %) of submucosal and 2/17 (11.8 %) of advanced gastric carcinoma ($p < 0.01$, Table 3). The expression of type IV collagen was observed in 33/66 (50 %) of differentiated gastric carcinoma, but not observed at all in undifferentiated carcinoma ($p < 0.05$). Reduced expression of type IV collagen was associated with a statistically significant high incidence of lymph vessel invasion and lymph node metastasis: 22/52 (42.3 %) of lymph vessel invasion negative and 17/20 (85.0 %) of positive carcinomas ($p < 0.01$); and 30/62 (48.4 %) of lymph node metastasis negative and 9/10 (90.0 %) of positive carcinomas ($p < 0.05$, Table 3).

In mucosal gastric carcinoma, no immunoreactivity was observed in undifferentiated mucosal carcinomas. In contrast, 25 of 36 differentiated mucosal carcinomas preserved type IV collagen immunoreactivity (data not shown).

**Expression on tenascin in gastric carcinoma.**

In normal gastric mucosa, tenascin immunoreactivity was observed in the subepithelial layer of the surface epithelium. Muscular structures and blood vessels were also stained with the antibody (Figure 4a, b). All benign lesions were associated with the expression of tenascin (Table 2). The expression of tenascin was observed in the stroma of gastric carcinoma (Figure 4c, d). Tenascin expression was decreased according to the progression of gastric carcinoma; 31/38 (81.6 %) of mucosal, 14/17 (82.4 %) of submucosal and, 8/17 (47.1 %) of advanced gastric carcinoma ($p < 0.05$, Table 3). Although no correlation was found between the expression of tenascin and the differentiation grade, lymphatic and venous invasion, tenascin positive gastric carcinoma showed a statistically significant low incidence of lymph node metastasis; 49/62 (79.0 %) of lymph node metastasis negative and 4/10 (40.0 %) of positive gastric carcinomas ($p < 0.05$, Table 3).
T cell infiltration in gastric carcinoma.

T cells were observed as membranous staining. Three consecutive areas were counted for CD45RO positive cells, and the mean number of T cells infiltrating the cancerous stroma was derived (Figure 5a, b). The mean number of T cells in 1-integrin preserved and reduced gastric carcinoma was 17.2 ± 19.8 and 19.8 ± 17.1/0.16 mm² (mean ± SD) and it was not statistically significant. The mean number of T cells in fibronectin preserved and reduced carcinomas was 17.5 ± 15.0 and 19.3 ± 12.3, and 15.5 ± 12.2 and 20.1 ± 15.6 in type IV collagen preserved and reduced carcinomas, respectively, and no statistical significance was found. However, a significantly large number of stromal T cells was infiltrated in gastric carcinoma expressing tenascin; 21.0 ± 14.0 in tenascin positive and 9.6 ± 11.6 in negative carcinomas (p < 0.01, Table 3).

Discussion

Recent advances in tumor biology revealed that the complex interactions between cancer cells and host CAMs, ECMs, and tumor infiltrating lymphocytes play a crucial role in the process of cancer cell proliferation, invasion, and metastasis. Integrins are a family of CAMs and constitute transmembrane receptor proteins. 4/1 and 5/1 integrins are known to act as specific fibronectin receptors and function as a signal transducer between cell and ECMs. Fibronectin is known as a multifunctional ECM glycoprotein and integrin-fibronectin interactions are important in the process of cancer cell migration, invasion, and metastasis. The proliferation of CTLs, the production of lymphokines such as interleukin-2, interferon-α, and granulocyte macrophage colony stimulating factor by fibronectin stimulation is reported, indicating a close relationship between fibronectin and the host immune mechanism against cancer. In this study, the expressions of 1-integrin and fibronectin were almost preserved in benign gastric lesions. However, a significant decrease of 1-integrin and fibronectin was observed according to the progression of gastric carcinoma. Loss of 1-integrin and fibronectin in advanced gastric carcinoma may suggest the acquisition of anchorage independent growth of deeply invasive cancer cells, which are considered as more malignant phenotype. Loss of cell-to-cell adhesions by integrin-fibronectin interactions and substantial alterations of the integrin subunits in highly metastatic phenotypes were also demonstrated in in vitro experiments.

Type IV collagen is a major component of the basement membrane, which separates the surface epithelium from lamina propria mucosae of the gastric mucosa. Gastric carcinomas arising in the epithelium must traverse the basement membrane during its invasive process. Thus, the basement membrane is regarded as the first biological barrier against cancer cell invasion. The acquisition of matrix metalloproteinase (MMP) and proteolysis of ECM components including type IV collagen is reported in invading and metastasizing tumors. In this study, type IV collagen was not observed at all around gastric carcinomas of the undifferentiated type, even in mucosal carcinomas. Loss of type IV collagen may indicate the invasive nature of undifferentiated gastric carcinoma. The restricted expression of type IV collagen in differentiated gastric carcinoma has also been reported in gastric cancers of foreign countries. In contrast, 25 of the 36 differentiated mucosal carcinomas maintained type IV collagen expression around cancer, suggesting the
existence of non-invasive intraepithelial or in situ gastric carcinomas [17]. Basement membrane staining, including type IV collagen is regarded as an essential diagnostic tool to differentiate intraepithelial carcinoma from carcinoma invading lamina propria mucosae. A significantly low incidence of lymphatic invasion and lymph node metastasis in type IV collagen positive gastric carcinomas, may indicates the possible role of type IV collagen as a biological barrier against cancer invasion and metastasis.

Tenascin, also called cytoactin or neuronectin, is a hexameric multidomain protein transiently expressed during embryonic and fetal development. However, it is reexpressed in the stroma of neoplastic lesions and considered one of the oncofetal proteins [18]. The expression of tenasin associated with favorable long-term prognosis has been reported in breast cancer [19,20]. Moreover, diploid DNA ploidy patterns predominating in tenasin positive colon cancer, in contrast to aneuploidy in negative cancers has been reported, suggesting more malignant nature of tenasin negative colon cancers [20]. In gastric carcinoma, the expression of tenasin has also been reported in cancerous stroma, but there was no convincing evidence suggesting an antimetastatic effect or prognostic determinant of tenasin [22,23]. The induction of tenasin by interleukin-1, interleukin-4, fibroblast growth factors, transforming growth factor-β, and tumor necrosis factor-α is also reported, suggesting complex interactions between cancer and host microenvironmental cells [7]. In this study, expression of tenasin was relatively preserved in early gastric carcinomas, but significant decrease was observed in advanced gastric carcinomas. A significantly large number of T cells and lower incidence of lymph node involvement was identified in tenasin positive gastric carcinomas. These results may indicate the local cellular immunity against gastric carcinoma may be activated in tenasin positive gastric carcinoma [12].

Further investigation is necessary to clarify the genetic regulation of the expression or disappearance of CAMs and ECMs and the relationship to MMPs, as well as their biological role in progression and metastatic process of gastric carcinoma.

In conclusion, the expressions of 1-integrin, fibronectin, type IV collagen, and tenasin decreased according to the progression of gastric carcinoma, associated with a high incidence of vessel invasion and lymph node metastasis.

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References

9) Shi SR, Key ME and Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues; An


胃癌におけるⅠ-インテグリン、フィブロネクチン、Ⅳ型コラーゲン、
テネーシンおよび腫瘍内浸潤T細胞に関する免疫組織学的研究

抄録

胃癌の進展と細胞間接着分子および細胞外マトリックスの関係を検討する目的で、Ⅰ-インテグリン、フィブロネクチン、Ⅳ型コラーゲン、テネーシンおよび腫瘍内浸潤T細胞に関する免疫組織学的検討を行った。対象は過形成性ポリープ5病変、胃腺腫9病変、早期胃癌55病変、進行胃癌17病変の計86病変である。バラフィン包埋ブロックより、病変の中心と周辺の胃粘膜を含む連続切片を作製し、酵素抗体ABC法により染色した。良性病変におけるこれらの発現は正常胃粘膜同様の染色性を示した。胃癌における発現率は、m癌、sm癌、進行癌の順にⅠ-インテグリンでは84.2％、58.8％、41.2％、フィブロネクチンでは76.3％、88.2％、47.1％、Ⅳ型コラーゲンでは65.8％、35.3％、11.8％、テネーシンでは81.6％、82.4％、47.1％と、いずれも癌の深達度に従い低下した。テネーシン発現胃癌においては、腫瘍内浸潤T細胞数が陰性胃癌に比べ有意に多く、宿主の細胞性免疫応答機構との関わりが示唆された。リンパ節転移、静脈侵襲、リンパ管浸潤が認められた群では、Ⅰ-インテグリン、フィブロネクチン、Ⅳ型コラーゲン、テ

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