Title

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Relationship between the Immunohistochemical Expressions of Cathepsin B, Laminin and Tenascin and Clinicopathologic Features in Gallbladder Carcinomas

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In the present study, the expression of protease indicated by cathepsin B (CB) and the expression of extracellular matrix indicated by laminin (LN) and tenascin (TN) was immunohistochemically examined in 25 gallbladder carcinomas. The incidence of expression of CB and TN in normal epithelium was 1/25 (4%) and 0/25 (0%), respectively, and significantly increased in carcinomas (14/25 : 56% and 21/25 : 84%, respectively) (p<0.01). The LN expression was detected in the basement membrane of all normal epithelium, and the incidence of LN expression was significantly decreased in the carcinomas (7/25 : 28%) (p<0.01). The incidence of CB expression in poorly differentiated adenocarcinomas (1/8 : 13%) was significantly lower than that in papillary, well- and moderately differentiated adenocarcinomas (11/15 : 74%) (p<0.01). However, these responses were not significantly related with other histologic features or nuclear DNA ploidy pattern. The LN expression of the hepatic metastasis group (4/6 : 67%) was significantly greater than that in the non-metastatic group (3/19 : 16%) (p<0.05). The expressions of CB, LN and TN were not associated with the postoperative prognosis. In conclusion, the increased expressions of CB and TN, and the decreased expression of LN were cancer-associated alterations. The expression of CB was correlated with the histological grade of differentiation, and the expression of LN was correlated with the hepatic metastasis.

Key words: gallbladder carcinoma, cathepsin B, laminin, tenascin

Introduction

Gallbladder carcinoma is one of the frequent carcinomas in Japan. Despite improvements in the preoperative diagnosis and surgical procedure, the prognosis of gallbladder carcinoma is still poor among carcinomas of the digestive tract. In gallbladder carcinomas, the high frequency of direct invasion to surrounding organs, lymph node metastasis and liver metastasis through the lymphatic route are characteristic. Such characteristics in gall bladder cancer are influenced by tumor-related factors (e.g., the biological grade of the malignancy) and host-related factors (e.g., the anatomy and structure of the gallbladder wall).

In the present study, we have investigated tumor-related factors such as protease secreted from cancer cells and the extracellular matrix around the cancer. Cathepsin B (CB), a cysteine endopeptidase contained in the lysosome, is implicated in the invasion of cancer cells, and the expression of CB is closely associated with the staging or the prognosis of gastrointestinal and pulmonary cancers. Laminin (LN) is a glycoprotein and one of the major structural components of basement membrane. In gastric and colorectal carcinomas, LN was found to be closely linked to hepatic metastasis. Tenascin (TN) is also an extracellular matrix glycoprotein and is involved in epithelial proliferation, differentiation, cell adhesion, epithelial cell shedding from the surface, demarcation of tissue boundaries, hemagglutination and cell migration during embryonic development. An increased serum level of TN has been observed in various cancer stromas, increasing further with the advancement of the malignancy.

The aim of the present study was to clarify the biological behavior of gallbladder carcinomas by examining the relationship between the immunohistochemical expressions of CB, LN and TN, and the clinicopathologic features or the prognosis in resected gallbladder carcinomas.

Materials and Methods

Materials

Twenty-five specimens of gallbladder cancer including normal epithelium which had been surgically resected at the First Department of Surgery of the Nagasaki University School of Medicine between 1982 and 1993 were examined. Samples were fixed with 10% formalin and embedded in paraffin blocks.
Fig. 1. The immunohistochemical responses of cathepsin B, laminin and tenasin (×100). a. Cathepsin B expression was observed in the cytoplasm of cancer cells. b. Laminin was observed on the basement membrane of surrounding vessels but not on that of cancer cell nests. c. Laminin strongly expressed on the basement membrane of cancer cell nests in this case. d. Tenasin expression was observed in the stroma around the cancer cell nests.

**DNA flow cytometry**

The 50-μm sections of paraffin-embedded tissue were pretreated according to the method reported by Schutte et al.,* and the nuclear DNA content was measured by a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Ten thousand nuclei were examined in each specimen. The heterogeneity of DNA content was also evaluated and was determined when at least one different pattern of DNA content in five sections per specimen was detected.

**Immunohistochemical staining**

For immunohistochemical staining, 3-μm sections were cut. Sections were deparaffinized by xylene twice and rehydrated in an ethanol series (100%, 90%, 80%), followed by a rinse in distilled water.

**Cathepsin B stain**: The avidin-biotin complex peroxidase method was applied. Using 1% hydrogen peroxide, the inhibition of endogenous peroxidase activity was performed. After incubation with normal rabbit serum (1:50; Vector Lab, Burlingame, CA), anti-cathepsin B antibody (1:100; sheep anti-sera purchased from the Binding Site Ltd., Birmingham, England) was applied at room temperature for 60 minutes. Each section was reacted with biotinylated rabbit anti-sheep gamma-globulin (1:200; Vector Lab.) and treated with avidin-biotin peroxydase complex solution. The peroxidase reaction was visualized with 0.05% of 3,3' diaminobenzidine (DAB) containing 0.01% of H₂O₂ in Tris-HCl buffered solution (pH = 7.6). The expression of CB was seen in the cytoplasm of the carcinoma cells (Fig. 1a).
Laminin stain: Initially, sections were incubated with 0.4% pepsin in 0.01N HCl for 90 minutes at 37°C. The avidin-biotin complex-alkaline phosphatase method was then performed for the detection of laminin. After treatment with normal goat serum (1:50; Nichirei, Tokyo), anti-laminin antibody (1:300; rabbit anti-sera purchased from Chemicon International, West Temecula, CA) was applied for 60 minutes at room temperature. Each section was reacted with biotinylated goat anti-rabbit gamma globulin (1:200; Nichirei) and treated with alkaline phosphatase-labelled streptavidin solution (Nichirei). The alkaline phosphatase reaction was detected by a solution containing phosphate ester of 6-bromo-2-hydroxy-3-naphthoic acid as buffered substrate solution, and diazotised 4'-amino-2',5'-diethoxy-benzanilide as phthalo-blue solution and activator solution (HistoMark Blue; Kirkegaard & Perry Lab., Gaithersburg, MD). Figures 1b and c show the negative and positive expression of LN in cancer tissues, respectively. The positive expressions of LN in cancer tissue was seen on the basement membrane of carcinoma cell nests (Fig. 1c).

Tenascin stain: First, sections were incubated with 0.4% pepsin in 0.01N HCl for 120 minutes at 37°C and treated with 1% hydrogen peroxide to inhibit endogeneous peroxidase activity. Anti-tenascin antibody (1:100; Life Technologies, Grand Island, NY) was applied for 24 hours at 4°C. The avidin-biotin complex peroxidase method was performed in the same manner as for the cathepsin B stain, and visualized with DAB. The expression of TN was seen in the stroma around the carcinoma cell nests (Fig. 1d).

Evaluation of stains

Each staining was determined in the superficial and the deepest layer of cancer tissues. The evaluation of CB was determined as the percentage of CB-positive cancer cells in the cancer tissue: stained less than 20% (−), 20-80% (+), more than 80% (++) were considered positive. When more than 20% of LN immuno-reactivity on the basement membrane of cancer nest was observed in a cancer tissue, the tissue was considered positive for LN. The evaluation of TN was determined as the percentage of TN positive around cancer tissues: stained less than 20% (−), 20-80% (+), more than 80% (++) were considered positive.

Statistics

The results of the immunohistochemical study were analyzed using the χ² test, and a p value less than 0.05 was considered significant. The survival rate was calculated by the Kaplan-Meier method and the differences were tested by the generalized-Wilcoxon test.

Results

Clinico-histological features

Of the 25 patients, 6 were male and 19 were female, and the age ranged from 46 to 78 years old (the mean age was 61.5 y. o.). Their gallbladder carcinomas consisted of 3 carcinomas invading the muscularis propria, 7 carcinomas invading the subserosa, 8 carcinomas that exposed the serosa, and 6 carcinomas infiltrating organs. Lymph node metastasis was detected in 8 cases (44%), and lymphatic involvement was detected in 18 cases (72%). Venous involvement was detected in 13 cases (52%), and perineural involvement was detected in 13 cases (52%). According to the General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract, 5 cases were in stage 1, 4 were in stage 2, 3 were in stage 3 and 14 were in stage 4. Histologically, 3 cases were papillary-, 5 were well-, 7 were moderately- and 8 were poorly-differentiated adenocarcinomas. Metachronous hepatic metastasis after operation was confirmed in 6 cases (24%). The mean survival time was 4.1 years, and both the three- and five-year survival rates were 34%.

DNA ploidy pattern

DNA diploidy and aneuploidy were detected in 2 cases (8.7%) and 21 cases (91.3%), respectively. Two of 22 specimens showed multiploidy. The heterogeneity of DNA ploidy was detected in 9 cases (39.1%). DNA ploidy and its heterogeneity were not associated with the histological features or prognosis of these carcinomas.

Expression in normal epithelium and gallbladder carcinomas (Table 1)

In the normal epithelium of the gallbladder, the expression of LN was detected in all specimens, and the expressions of CB and TN were hardly detected. In contrast, in the carcinomas, the incidence of LN expression was significantly decreased and those of CB and TN expression were significantly increased (p<0.01, respectively).

<table>
<thead>
<tr>
<th>Immunohistochemical response</th>
<th>cathepsin B</th>
<th>laminin</th>
<th>tenascin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal epithelium</td>
<td>1/25</td>
<td>25/25</td>
<td>0/25</td>
</tr>
<tr>
<td>(4%)</td>
<td>(100%)</td>
<td>(0%)</td>
<td></td>
</tr>
<tr>
<td>Carcinomas</td>
<td>14/25</td>
<td>7/25</td>
<td>21/25</td>
</tr>
<tr>
<td>(56%)</td>
<td>(28%)</td>
<td>(84%)</td>
<td></td>
</tr>
</tbody>
</table>

**: p<0.01 χ² test
Inter-layer difference of expression in carcinomas (Table 2)

There was no significant difference of these immunohistochemical expressions between the superficial and the deeper layer of carcinomas.

**Relationship with histological features and DNA content (Table 3)**

<table>
<thead>
<tr>
<th>Table 2. Inter-layer difference of expression in carcinomas</th>
<th>Table 4. Relationship with the hepatic metastasis after operation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunohistochemical response</td>
<td>Immunohistochemical response</td>
</tr>
<tr>
<td>cathepsin B</td>
<td>laminin</td>
</tr>
<tr>
<td>Superficial layer</td>
<td>10/25 (40%)</td>
</tr>
<tr>
<td>Deepest layer</td>
<td>14/25 (56%)</td>
</tr>
</tbody>
</table>

# : p<0.08 $\chi^2$ test

The positive expression of CB was significantly lower in poorly differentiated adenocarcinomas compared with papillary-, well- and moderately-differentiated adenocarcinomas ($p<0.01$). However, the expressions of CB, LN and TN were not associated with other parameters at all.

**Relationship with the hepatic metastasis after operation (Table 4)**

<table>
<thead>
<tr>
<th>Table 3. Relationship with histologic features and DNA ploidy pattern</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>cathepsin B</td>
<td>laminin</td>
</tr>
<tr>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>depth</td>
<td>m, pm, ss</td>
</tr>
<tr>
<td></td>
<td>se, si</td>
</tr>
<tr>
<td>lymphnode</td>
<td>meta (−)</td>
</tr>
<tr>
<td></td>
<td>meta (+)</td>
</tr>
<tr>
<td>lymphatic</td>
<td>inv. (−)</td>
</tr>
<tr>
<td></td>
<td>inv. (+)</td>
</tr>
<tr>
<td>venous</td>
<td>inv. (−)</td>
</tr>
<tr>
<td></td>
<td>inv. (+)</td>
</tr>
<tr>
<td>perineural</td>
<td>inv. (−)</td>
</tr>
<tr>
<td></td>
<td>inv. (+)</td>
</tr>
<tr>
<td>stage</td>
<td>1, 2</td>
</tr>
<tr>
<td>3, 4, 5</td>
<td>6</td>
</tr>
<tr>
<td>differentiation</td>
<td>pap, tub 1, tub2</td>
</tr>
<tr>
<td></td>
<td>poor</td>
</tr>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>DNA ploidy</td>
<td>diploidy</td>
</tr>
<tr>
<td></td>
<td>aneuploidy</td>
</tr>
<tr>
<td>DNA heterogeneity</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>positive</td>
</tr>
</tbody>
</table>

**: p<0.01 $\chi^2$ test
Although the incidences of CB and TN expression were not different between the liver metastasis and non-liver metastasis group, that of LN expression was significantly greater in the liver metastasis group (4 of 6 cases; 67%) compared with the non-liver metastasis group (three of 19 cases; 16%) (p<0.05).

Relationship with post-operative survival
The expressions of CB, LN and TN were not associated with postoperative prognosis at all.

Discussion
The aggressive invasiveness and poor prognosis of gallbladder carcinoma may be caused by the characteristic anatomy and wall structure of the gall bladder (thin wall, Rokitansky-Aschoff sinus and lack of muscularis mucosa). The cellular biology of this carcinoma may also be associated with these aspects of gallbladder carcinoma. The biological grade of the malignancy in carcinomas has recently been the focus of attention. However, no studies of the biological grade of malignancy and the biological characteristics of gall bladder carcinomas have been done to date, to our knowledge.

Proliferative activity is closely associated with the biological grade of malignancy. The count, area and ratio of Argyrophil Nucleolar Organizer Region associated proteins (AgNORs) were observed to be increased in conjunction with deeper invasion, lymph node metastasis, distant metastasis, low grade of histological differentiation, lymphatic invasion and perineural invasion, and the AgNORs count was shown to be an independent prognostic factor for survival in patients of gallbladder cancers. A positive correlation between the nuclear DNA content in gallbladder carcinomas and prognostic values was reported, whereas another study found a negative correlation. In a previous study in our laboratory, the DNA ploidy pattern was not associated with the clinicopathological features or the prognosis of gallbladder carcinoma. Moreover, the correlations between these parameters, and the pathological features or the prognosis of oncogenes (such as c-myc and c-fos) in cholangiocarcinomas were not clarified.

The three parameters of CB, LN and TN were observed to be closely associated with the biological grade of malignancy in carcinomas of other digestive organs. To the best of our knowledge, no study of the expressions of CB, LN and TN in gallbladder carcinomas has been published in the English literature, although the expression of TN and LN in intrahepatic bile duct carcinomas was reported. In the present study, the expression of CB and TN were hardly detected in normal epithelial glands and were increased in the gallbladder carcinomas. These expressions may thus be cancer-associated responses. However, cholecystitis, dysplasia and adenomas in the gallbladder were not examined in this study. In colorectal neoplasms, CB expression was detected in 22% of adenomas, although the incidence was lower than that of carcinomas (67%). TN expression was detected in gastric ulcers, local inflammatory response and wound healing except regulation during the embryonic stage, and TN may be also an important stromal component in inflammatory lesions undergoing active repair and remodeling. Therefore, TN expression may be also detected in cholecystitis.

We observed that LN was continually stained in the basement membrane of all normal epithelial glands whereas the incidence of LN expression in the carcinomas was remarkably decreased. We noticed that the LN stain in the basement membrane of the carcinomas was thicker and more remarkable compared to that of the normal epithelium, and sometimes intermittent. In studies of gastrointestinal carcinomas and pulmonary carcinomas, the incidence of LN expression was also decreased following deeper invasion. A similar result was obtained in the present study of gallbladder carcinomas. Perhaps the disappearance of the structural components of basement membrane including LN or type IV collagen is important for the development of carcinomas.

It was reported that the expressions of CB, LN and TN were related to the depth of invasion in gastrointestinal carcinomas. In the present study, we also examined the heterogeneity of CB, LN and TN expression in the superficial and the deeper layer of gallbladder carcinomas. However, there was no interlayer difference in carcinomas and the incidence of these expressions was not correlated with the depth of invasion. Overall, no significant relationship with the invasion of carcinoma was observed in gallbladder carcinomas.

In the present study, the expression of CB, LN and TN were not correlated with the malignant behavior or prognosis of gallbladder carcinomas, such as lymph node metastasis, lymphatic-, venous- and perineural- involvement, and staging. Only CB expression was significantly correlated with the histologic grade of differentiation. Carcinomas with the low grade of differentiation might have produced such proteases. In colorectal carcinomas, the CB expression was also associated with the histologic grade of differentiation.

As described above, the LN expression decreased following the invasion of carcinoma. However, it is interesting that the LN expression of advanced carcinoma is closely associated with hepatic metastasis of gastric and colorectal carcinomas. The LN expression on the basement membrane of a carcinoma nest may be different from that of normal epithelial glands, since the nest is thicker and multiple layers compared with that of normal glands. Similar findings were observed in this study. Such
LN expression of carcinomas may be the product of carcinoma cells derived themselves from LN in the basement membrane of normal epithelium. LN produced by cancers may play a role in the adhesion of migrating carcinoma cells to vessels in primary and metastatic organs. In the present study, 4 (67%) of the 6 cases revealing hepatic metastasis after operation showed the LN expression in primary sites. This incidence was greater than that of the non-liver metastasis group, but the number of liver metastasis patients was rather small. A larger number of metastatic patients must be examined.

It was reported that the expressions of CB, LN and TN were closely associated with postoperative survivals in various cancers.\textsuperscript{6,11,25} Mizumoto and Ogura reported that TN expression was significantly associated with poor prognosis after resection, and that the expression of LN and fibronectin were not associated with the bile duct carcinomas.\textsuperscript{30} However, in our present study, the expression of all parameters including TN were not associated with the postoperative prognosis of gallbladder carcinoma. The biological characteristics of gallbladder carcinoma and those of bile duct carcinoma may be different. After all, the expressions of CB, LN and TN were not prognostic markers of gallbladder carcinomas. We also examined the survival periods in our patients; there was no difference of survival rates among the three parameters.

In conclusion, the increased immunohistochemical expression of CB in cancer cells and that of TN around cancer nests, and the lack of LN on the basement membrane of carcinomas were observed in cancer tissues of the gallbladder. The increased expression of CB was significantly greater in papillary, well- and moderately differentiated adenocarcinomas. The expression of LN in carcinoma tissue was significantly associated with hepatic metastasis. These parameters were not associated with the postoperative prognosis.

Acknowledgment

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References
