Expression of MUC1 Micins in the Subserosal Layer Correlates with Postsurgical Prognosis of Pathological Tumor Stage 2 Carcinoma of the Gallbladder

Toru Kawamoto, Junichi Shoda, Tatsumo Irimura, Naoki Miyahara, Masato Furukawa, Tetsuya Ueda, Toru Asano, Masahito Kano, Naoto Koike, Katashi Fukao, Naomi Tanaka, and Takeshi Todoroki

Departments of Gastrointestinal Surgery [T. K., N. K., K. F., T. T.] and Gastroenterology [J. S., T. A., M. K., N. T.], Institute of Clinical Medicine, University of Tsukuba, Ibaraki 305-8575; Department of Cancer Biology and Molecular Immunology, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033 [T. L., N. M.]; Second Department of Surgery, Nagasaki Central National Hospital, Nagasaki 856-0835 [M. F.]; Department of Pharmaceutical Research, Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., Tokyo 174-8555 [T. U.], Japan

ABSTRACT

The overall outcome of pT2 gallbladder carcinoma has not been favorable. Postsurgical recurrence at distant sites occurs in some cases, although the carcinoma was limited to the gallbladder wall. A high level expression of MUC1 micins with sialylated carbohydrates (sialylated MUC1 mucins) is correlated with poor survival in intrahepatic bile duct carcinoma. In the present study, immunohistochemical analysis was performed to determine the expression level of sialylated MUC1 mucins, detected by a monoclonal antibody, MY.1E12, in 31 cases of pT2 gallbladder carcinoma on which curative resections had been performed and to determine the correlation of the expression level of MY.1E12-reactive-MUC1 mucin with mode of recurrence and postsurgical survival. Immunostainings of the MUC1 mucin were recognized in different types of noncancerous pathological epithelia of the gallbladder except for intestinal metaplasia and cancerous epithelia. Immunohistochemical localization was classified into apical, cytoplasmic, and stromal types based on the predominant cellular distribution of MY.1E12-reactive-MUC1 mucin. In 31 cases of pT2 carcinoma, the localization was apical type in 64%, cytoplasmic type in 71%, and stromal type in 48% of the cases at the deepest invading sites in the subserosal layer. Distant recurrences, i.e., peritoneal dissemination in 8 patients and liver metastasis in 3 patients, were seen in 8 (53%) of 15 cases of pT2 carcinoma that had ≥10% of the cancerous epithelia showing stromal localization of the MUC1 mucin at the deepest invading sites and in 2 (12%) of 16 cases that had <10% of those showing the stromal localization. The postsurgical survival outcome was significantly poorer in the former than in the latter (P = 0.044). In pT2 gallbladder carcinoma, the presence of MY.1E12-reactive-MUC1 mucin in the stroma adjacent to the cancerous epithelia in the subserosal layer correlates with the aggressiveness of the disease, such as the tendency to form distant recurrences. This phenotype may serve as a unique biological feature associated with the malignant behavior of pT2 gallbladder carcinoma.

INTRODUCTION

Gallbladder carcinoma has always been associated with a dismal overall prognosis (1–5). The 5-year survival rate after surgery has been reported to be between 5 and 13% in recent studies (1–5). The clinical course of gallbladder carcinoma has been thought to depend on the depth of tumor invasion (6–8). The current study confirmed that only patients with pT1 carcinoma (a tumor confined to mucosa or muscle coat) have a real chance of being cured after simple cholecystectomy (9–11) and that the prognosis in cases of pT2 carcinoma (a tumor invading the perimuscular connective tissues but not extending beyond the serosa or into the liver) is worse (9–11). Despite a theoretical advantage for gallbladder carcinoma not involving the serosa, the prognosis of pT2 carcinoma is not necessarily favorable, and the 5-year postsurgical survival rate in cases of pT2 carcinoma, ~50–80%, is intermediate between that of pT1 carcinoma and those of pT3 and pT4 carcinomas (9–11). Some patients with pT2 carcinoma have no recurrence after simple cholecystectomy, whereas others have recurrence even after extended cholecystectomy. This is attributed to the variety of ways in which pT2 carcinoma progresses and to the fact that prognostic factors affecting the progression of less-advanced lesions, such as pT2 carcinoma, have not been fully elucidated.

Phenotypic changes are involved in tumor progression, which in turn may modulate the biological behavior of carcinoma cells and allow the cells to disseminate, invade and survive at distant organ sites. The glycoproteins expressed on carcinoma cells representing a metastatic phenotype are qualitatively and quantitatively different from those of the nonmetastatic phenotype (12–15). Some altered glycoproteins presum-
ably affect the behavior of carcinoma cells during the process of metastasis (16). Detection of such altered glycoproteins in surgical specimens may provide information regarding the metastatic potential in the postsurgical period.

MUC1 mucin, a transmembrane glycoprotein with a large extracellular domain, is one of the glycoproteins expressed by the metastatic phenotype (17–19). Carcinoma-associated high-molecular-weight sialomucin MUC1 suppresses homotypic cellular aggregation (20) and cell-matrix adhesion, which in turn promotes invasion in Matrigel (21). These observations suggest that carcinoma cells with a high-level expression of MUC1 mucins with sialylated carbohydrates (sialylated MUC1 mucins) may be able to detach easily from the primary site and survive in circulation or in distant organs of metastasis by escaping from immune surveillance. High levels of expressions of sialylated MUC1 mucins, which was recognized by a novel mAb, MY.1E12-reactive mucins with sialylated carbohydrates (sialylated MUC1 mucins) promotes invasion in Matrigel (21). These observations suggest that carcinoma cells with a high-level expression of MUC1 mucin at the deepest invading sites in the subserosal layer of pT2 carcinoma, as a predictor for the invasive/metastatic potential, with the clinicopathological findings, mode of recurrence/metastasis-related substances at the deepest invading sites of gallbladder carcinoma may provide valuable information for understanding the mechanisms responsible for malignant behavior of carcinoma cells. Therefore, in 31 cases of pT2 carcinoma, correlations of the expression level of MY.1E12-reactive-MUC1 mucin at the deepest invading sites in the subserosal layer of pT2 carcinoma, as a predictor for the invasive/metastatic potential, with the clinicopathological findings, mode of recurrences, and postsurgical survival were investigated.

MATERIALS AND METHODS

Patients. Specimens from 45 patients (11 males and 34 females) with gallbladder carcinoma (8 with pT1, 31 with pT2, 5 with pT3, and 1 with pT4 carcinomas), all of which were curatively resected with a free surgical margin, were included in the present study. The mean age of the patients was 67 years (range, 51–89 years). The patients were diagnosed as having gallbladder carcinoma and underwent operations between October 1976 and September 1999 in the Hospital of the University of Tsukuba School of Medicine and in the Surgical Department of Nagasaki Central National Hospital. Gallbladder carcinoma was diagnosed on the basis of histological findings and was classified according to the tumor-node-metastasis (TNM) classification of the American Joint Committee on Cancer (Ref. 27): pT1, a tumor confined to mucosa or muscle coat; pT2, a tumor that has invaded the perimuscular connective tissues with no extension beyond the serosa or into the liver; pT3, a tumor that has perforated the serosa (visceral peritoneum) or has directly invaded one adjacent organ, or both (extension of 2 cm or less into the liver); and pT4, a tumor that has extended more than 2 cm into the liver and/or into two or more adjacent organs. The resection procedures are shown in Table 1. For pT1 carcinoma, simple cholecystectomy was performed on seven of the eight patients, and cholecystectomy combined with bile duct resection was performed on the other patient. For pT2 carcinoma, simple cholecystectomy was performed on 16 of the 31 patients, cholecystectomy combined with bile duct resection was performed on 9 patients, cholecystectomy with combined bile duct resection and hepatic resection was performed on 2 patients, cholecystectomy combined with bile duct resection and pancreatoduodenectomy was performed on 1 patient, cholecystectomy combined with bile duct resection and pancreatic resection was performed on 1 patient, and cholecystectomy combined with hepatic resection was performed on 2 patients. Histological examination revealed that all of the cases of pT2 carcinoma, in which the tumor had invaded perimuscular connective tissues with no extension beyond the serosa, had neither hepatic infiltration of carcinoma nor invasion into the hepatoduodenal ligament.

Follow-up periods until September 1999 ranged from 2.2 to 156.3 months. Of the 31 patients, 18 were alive as of September 1999, 10 had died from distant metastasis (Table 2), and 3 had died from some other disease (cerebral infarction in 2 and pancreatic carcinoma in 1). The latter 3 patients were treated as lost cases. Survival curves were assessed by the Kaplan-Meier method. Statistical analysis were performed using Stat View and its survival tools (Abacus Concepts, Berkeley, CA) for Macintosh.

In addition, the intact gallbladder specimens and the specimens with chronic inflammatory changes were obtained at surgery from subjects who had undergone hepatectomy because of metastatic liver carcinoma and gallstone subjects who had undergone cholecystectomy, respectively.

A Cell Line and a mAb. Capan-1 pancreatic adenocarcinoma cell lines were obtained from Dr. M. L. Frazier (The University of Texas, M. D. Anderson Cancer Center, Houston, TX). Mz-ChA-1 gallbladder adenocarcinoma cell lines were obtained from Dr. A. Knuth (Johaness-Gutenberg University, Mainz, Germany). Mz-ChA-1 and Capan-1 cell lines were cultured in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 25 μg/ml gentamicin, and 2.5 μg/ml amphotericin B. Mz-ChA-1 cell line was obtained from Dr. A. Knuth (Johaness-Gutenberg University, Mainz, Germany). Mz-ChA-1 and Capan-1 cell lines were cultured in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 25 μg/ml gentamicin, and 2.5 μg/ml amphotericin B. Mz-ChA-1 cell line was obtained from Dr. A. Knuth (Johaness-Gutenberg University, Mainz, Germany).
Table 2  Postsurgical recurrent modes in pT2 gallbladder carcinoma

The 31 cases of pT2 carcinoma were divided into two groups based on the expression rate of stromal localization of MY.1E12-reactive-MUC1 mucin at the invading sites: one group of cases in which \( \geq 10\% \) of the cancerous epithelia showed stromal localization of the MUC1 mucin at the invading sites (group A) and the other group of cases in which \(< 10\% \) of the cancerous epithelia showed the stromal localization (group B).

<table>
<thead>
<tr>
<th>Peritoneal dissemination</th>
<th>Lymph nodes</th>
<th>Distant organs</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT2 carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (n = 15)</td>
<td>8</td>
<td>1</td>
<td>5*</td>
</tr>
<tr>
<td>B (n = 16)</td>
<td>2</td>
<td>1</td>
<td>10*</td>
</tr>
</tbody>
</table>

*P < 0.05, significantly different between A and B.

* Distant organs included the liver in 3 patients: the lung in 1, and the brain in 1 from group A and the liver in 1 patient from group B.

Mainz, Germany). The cells were maintained in a 1:1 mixture of DMEM and Ham’s F12 medium that contained 10% FCS in a humidified atmosphere with 5% carbon dioxide at 37°C. MAB MY.1E12 (IgG2a), specific for MUC1 mucin with sialylated O-linked oligosaccharides, was prepared as described previously (22). The epitope structure recognized by this mAb was described previously (22).

Immunoblot Analysis of mAb MY.1E12-reactive-MUC1 Mucin in Gallbladder Carcinoma. Immunoblot analysis was performed as described previously (22). Briefly, the frozen surgical specimens were thawed and homogenized with 0.5% NP40 in a desalting buffer consisting of 250 mM sucrose, 10 mM Tris-HCl, 50 mM calcium chloride, and 10 mM phenylmethylsulphonyl fluoride (pH 7.2). The Capan-1 cells and Mx-ChA-1 cells were also lysed and were used as positive controls. The supernatants of the lysates were mixed with 187.5 μg of Tris-HCl containing 5% SDS, 3% 2-mercaptoethanol, 30% glycerol, and 1.5 μg EDTA. The mixtures containing 200 μg of protein were heated for 5 min at 100°C and then electrophoretically separated on 4% polyacrylamide gels in the presence of 0.1% SDS-PAGE. They were transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Boston, MA). The membranes were incubated in 2% BSA in PBS at 4°C overnight and then mixed with mAb MY.1E12 (culture supernatant of hybrinoma cells) diluted in an equal volume of 2% BSA for 2 h. After washing with PBS containing Tween 20, they were incubated with rabbit antimouse IgG labeled with alkaline phosphatase (Zymed Laboratories Inc., San Francisco, CA) for 1 h. The membranes were washed with PBS containing 0.1% Tween 20 and then treated with ECL (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) to visualize the Abs that had bound.

Immunohistochemical Expression of mAb MY.1E12-reactive-MUC1 Mucin in Gallbladder Carcinoma. Immunostaining of sialylated MUC1 mucin was performed using mAb MY.1E12. Gallbladder carcinoma tissues, which had been preserved in 10% formalin and then embedded in paraffin, were serially sectioned at 2 mm in thickness, mounted on silane-coated slides, and later deparaffinized. The slides were immersed for 20 min in 0.3% hydrogen peroxide in methanol to deactivate endogenous peroxidase. After washing with PBS, the slides were incubated with a protein blocking agent for 5 min at room temperature in a humid chamber. The slides were then stained by the indirect immunoperoxidase method using mAB MY.1E12 (dilution, 1:1). Briefly, the slides were incubated with MY.1E12 at room temperature for 1 h and then washed with PBS three times. Then, a biotinylated secondary antibody was applied at room temperature for 10 min. After washing for 15 min, a streptavidin peroxidase reagent was applied and incubated at room temperature for 10 min. Finally, the reaction product was visualized using developing color by incubating the slides in a solution of 0.3% hydrogen peroxide, diazonobenidine tetrahydrochloride, and PBS. A negative control was made using BSA instead of MY.1E12. Counterstaining was done with hematoxylin.

Evaluation of sections was performed by a single pathologist who was blinded to clinical characteristics and pathological grade of response. The total number of cancerous epithelia in each section was evaluated. The immunohistochemical localization of MY.1E12-reactive-MUC1 mucin was classified into apical, cytoplasmic, and stromal types based on the predominant cellular distribution according to Hamada’s classification (28): apical type, MY.1E12-reactive-MUC1 mucin being restricted predominantly to the apical borders of the cancerous epithelia; cytoplasmic type, the MUC1 mucin being demonstrated not only on the apical surfaces but also on the basolateral surfaces and in the cytoplasm of the cancerous epithelia; and stromal type, the MUC1 mucin being found over the entire surfaces and cytoplasm of the cancerous epithelia, and being diffusely distributed in the surrounding stroma adjacent to the basolateral membrane of the epithelia. Fig. 1 shows the representative immunohistochemical localization of mAB MY.1E12-reactive-MUC1 mucin in the cancerous epithelia in the subserosal layer of pT2 carcinoma. The localization of MY.1E12-reactive MUC1 mucin was judged to be apical, cytoplasmic, or stromal type when more than 10% of the total number of cancerous epithelia in each section showed apical, cytoplasmic, or stromal cellular distribution, and was examined for both the mucosal or proper muscle layers (surface site) and the subserosal layer (invading site). Furthermore, the 31 cases of pT2 carcinoma were divided into two groups based on the expression rate of stromal localization of MY.1E12-reactive-MUC1 mucin at the deepest invading sites: one group of cases in which \( \geq 10\% \) of the cancerous epithelia showed stromal localization of the MUC1 mucin at the invading sites (group A) and the other group of cases in which \(< 10\% \) of the cancerous epithelia showed the stromal localization (group B). A comparison of groups A and B was made with special reference to clinicopathological findings, mode of recurrences, and postsurgical survival.

RNA Isolation and cDNA Synthesis. Total RNA was isolated from the gallbladder carcinoma specimens (from 6 with pT1, 14 with pT2, 4 with pT3, and 1 with pT4 carcinomas) and the gallbladder specimens (from 12 gallstone subjects) using Trizol reagent by the modified method described by Chomczynski and Sacchi (29). First-strand cDNAs were synthesized from total RNA with Moloney murine leukemia virus reverse transcriptase by the random primer method.

RT-PCR RT-PCR was performed as described previously (30) using a DNA Thermal Cycler (model PJ 2000; Applied Biosystems, Inc., Foster City, CA). PCR conditions were at 94°C for 1 min, at 55°C for 2 min, and at 72°C for 2 min...
for the following number of cycles: MUC1, 30; G3PDH, 25.

Aliquots of the reaction mixture were electrophoresed on a 2% agarose gel. PCR primers were designed from cDNA sequences for human MUC1 (31), and then synthesized using an Applied Biosystems DNA synthesizer (model 392; Applied Biosystems., Inc.) as follows: G3PDH, sense 5′-GAA/GCG/GAA/GCT/CAC/TGG/CAT/GGC-3′ and antisense 5′-TGA/GGT/CCA/CCA/CCC/TGT/TGC/TG-3′; MUC1, sense 5′-CGT/CGT/GGA/CAT/TGA/TGG/TAC/C-3′ and antisense, 5′-GGT/ACC/TCC/TCT/ACC/CTC/CTC/CAA-3′.

In each experiment, RT-PCR was done in triplicate. In the quantitative assessment, the amounts of fluorescence intensity were measured using a FluorImager (Molecular Dynamics, Sunnyvale, CA). The data were expressed relative to the amount of G3PDH mRNA present in each specimen and then averaged.

Statistics. Values are given as means ± SE. A two-sided \( \chi^2 \) test was used for comparison of clinicopathological data between groups. The survival of patients was recorded every month, and patient survival was analyzed by the method of Kaplan-Meier. Differences in the survival of patients in subgroups were analyzed by the log-rank test. A \( P \) of <0.05 was defined as statistically significant.

RESULTS

Immunoblot Analysis of Gallbladder Carcinoma Lysates with mAb MY.1E12. MY.1E12-reactive-MUC1 mucin was included in the lysates of the gallbladder carcinoma tissues and Mz-ChA-1 and Capan-1 cell lines after SDS-PAGE (Fig. 2). The electrophoretic mobility of MUC1 mucin identified by mAb MY.1E12 in the carcinoma tissues and the cell lines was not identical. This may be dependent on polymorphism concerning the number of tandem repeats (17, 19, 31). A gallbladder carcinoma tissue from one patient, Mz-ChA-1 and Capan-1 cell lines apparently contained two distinct bands corresponding to two alleles of the MUC1 gene.
Immunohistochemical Localization of mAb MY.1E12-reactive-MUC1 Mucin in Gallbladder Carcinoma. The immunohistochemical localization of mAb MY.1E12-reactive-MUC1 mucin expressed in pT1-pT4 gallbladder carcinomas and in noncancerous pathological lesions of the gallbladder were studied (Table 3). The localization was heterogeneous in the gallbladder carcinomas with apical, cytoplasmic, or stromal type of the cellular distribution, in contrast to the localization in normal gallbladders showing solely apical type with prominent staining at the luminal epithelial surface (Fig. 3). The noncancerous pathological lesions in the gallbladder, i.e., hyperplasia, pseudopyloric gland metaplasia, and dysplasia, showed mostly apical type (Fig. 3), with some epithelia showing the mAb reactivity in their cytoplasm (cytoplasmic type). However, no stromal type of the cellular distribution was observed in either normal epithelial or the noncancerous pathological lesions of the gallbladder.

The localization of MY.1E12-reactive-MUC1 mucin appeared heterogeneous in carcinomas of different depths of invasion (Table 3), although it was observed that the immunohistochemical localization varied from pT1 carcinoma in the early stage to pT4 carcinomas in the advanced stage. In pT1 carcinoma, the localization was apical type in all of the cases except one, which showed both cytoplasmic and stromal types.

In pT2 carcinoma, the localization was apical type in 93%, cytoplasmic type in 58%, and stromal type in 10% of the cases at the noninvading surface site. At the deepest invading sites in the subserosal layer (Fig. 4), the localization was apical type in 64%, cytoplasmic type in 71%, and stromal type in 48% of the cases. In pT3 and pT4 carcinomas, the localization was apical type in 67%, cytoplasmic type in 83%, and stromal type in 17% of the cases at the noninvading surface site, and at the deepest invading sites, the localization was apical type in 17%, cytoplasmic type in 83%, and stromal type in 100% of the cases. Notably, the proportion of stromal localization of MY.1E12-reactive-MUC1 mucin at the deepest invading sites was significantly increased in pT2 carcinoma cases and tended to be increased in pT3 and pT4 carcinomas compared with the proportion in the noncancerous pathological lesions, pT1 carcinoma, and their corresponding surface sites of pT2-pT4 carcinomas. In contrast, the proportion of apical localization of the MUC1 mucin was significantly decreased at the deepest invading sites. It should be noted that the proportion of cancerous epithelia showing the stromal localization was increased at the deepest invasive sites in parallel with the depth of invasion.

### Table 3 Immunohistochemical localization of MY.1E12- Reactive-MUC1 Mucin in noncancerous lesions and carcinomas of the gallbladder

<table>
<thead>
<tr>
<th>Localization type</th>
<th>Noncancerous lesions</th>
<th>Gallbladder carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal (n = 7)</td>
<td>pT1 (n = 8)</td>
</tr>
<tr>
<td></td>
<td>Hyperplasia (n = 24)</td>
<td>pT1 (n = 31)</td>
</tr>
<tr>
<td></td>
<td>P.P.G. (n = 16)</td>
<td>Surface site 4 (67)</td>
</tr>
<tr>
<td></td>
<td>I.M. (n = 5)</td>
<td>Invading site 64 (20)</td>
</tr>
<tr>
<td></td>
<td>Dysplasia (n = 13)</td>
<td>pT1-pT4 (n = 6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Surface site 4 (67)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Invading site 1 (17)</td>
</tr>
</tbody>
</table>

**Note:** Values in parentheses represent percentages.

- P < 0.05, significantly different from normal epithelia.
- P < 0.01, significantly different from normal epithelia, hyperplasia, and P.P.G.
- P < 0.05, significantly different from normal epithelia, hyperplasia, and P.P.G.
- P < 0.01, significantly different from normal epithelia, hyperplasia, and P.P.G.
- P < 0.01, significantly different from normal epithelia, hyperplasia, and P.P.G.
- P < 0.01, significantly different from normal epithelia, hyperplasia, and P.P.G.
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- P < 0.01, significantly different from normal epithelia, hyperplasia, and P.P.G.
- P < 0.01, significantly different from normal epithelia, hyperplasia, and P.P.G.
- P < 0.01, significantly different from normal epithelia, hyperplasia, and P.P.G.

### mRNA Level of Human MUC1 MUCs in Gallbladder Carcinoma.
To determine whether the high-level expression of stromal localization of MY.1E12-reactive-MUC1 mucin in pT2-pT4 gallbladder carcinomas was caused by an increased level of MUC1 MUCs, the steady-state mRNA level of the MUC1 gene in carcinomas of different depths of invasion (6 with pT1, 14 with pT2, 4 with pT3, and 1 with pT4 carcinomas) was measured by RT-PCR (Fig. 5). The mRNA level of MUC1 MUCs did not differ significantly either among the pT1-pT4 carcinomas [pT1; 159 ± 15% (mean ± SE) of G3PDH mRNA; pT2; 149 ± 5; pT3; and pT4; 150 ± 12] or between the carcinomas and the gallbladders with gallstones (12 gallstone subjects, 167 ± 12). These results clearly indicated that the stromal localization of MY.1E12-reactive-MUC1 mucin in the deepest invading sites in pT2-pT4 carcinomas were not caused by an increased level of MUC1 MUCs.

### Relationship between Pathological Malignancies and Expression Rate of Stromal Localization of mAb MY.1E12-reactive-MUC1 Mucin in Patients with pT2 Carcinoma.
The 31 patients with pT2 carcinoma were divided into two groups based on the expression rate of stromal localization of MY.1E12-reactive-MUC1 mucin in pT2-pT4 gallbladder carcinomas and the postsurgical recurrent modes in patients with pT2 carcinoma (Fig. 4, group A) and the other group of cases in which less than 10% of the cancerous epithelia showed stromal localization of the MUC1 mucin at the deepest invading sites (Fig. 4, group B). A comparison of groups A and B was made with special reference to pathological malignancies, i.e., histological grade, lymphatic permeation, venous permeation, and lymph node metastasis. The results revealed no significant difference in the parameters of pathological malignancies between groups A and B (Table 4).

### Relationship between Mode of Recurrence in Patients with pT2 Carcinoma and Expression Rate of Stromal Localization of mAb MY.1E12-reactive-MUC1 Mucin in the Specimens.
The postsurgical recurrent modes in patients with pT2 carcinoma in groups A and B were compared. Of the 15 patients in group A, 8 had peritoneal dissemination, 5 had metastasis in
distant organs, and 1 had lymph node metastasis (Table 2). Notably, based on the histopathological records, no lymph node metastasis was seen at the time of surgery in four of the eight cases with postsurgical peritoneal dissemination. On the other hand, of the 16 patients in group B, 2 had peritoneal dissemination, 1 had metastasis in distant organs, and 1 had lymph node metastasis (Table 2). Interestingly, it should be noted that in pT2 carcinoma, the postsurgical peritoneal dissemination was found to be more frequent in the patients in group A (53%) than in those in group B (12%). The difference was statistically significant ($P < 0.05$). The expression rate of stromal localization of MY.1E12-reactive-MUC1 mucin at the deepest invading sites may, therefore, be an important predictor of postsurgical peritoneal dissemination in pT2 carcinoma.

**Relationship between Postsurgical Survival of Patients with pT2 Carcinoma and Expression Rate of Stromal Localization of mAb MY.1E12-reactive-MUC1 Mucin in the Specimens.** The overall postsurgical survival rate in 31 patients with pT2 gallbladder carcinoma was poor in contrast to the quite favorable prognosis of patients with pT1 carcinoma (Table 4). Similarly, a comparison of groups A and B with special reference to postsurgical survival showed that the survival rate of patients in group A was significantly lower than that of patients in group B ($P = 0.043$; Fig. 6). To exclude the influence of lymph node metastasis on postsurgical survival, a comparison was made between the 11 patients in group A without metastasis and the corresponding 11 patients in group B. The analysis revealed that the survival rate of the patients in group A was
significantly lower than that of the patients in group B, but the difference was not statistically significant (Fig. 6). Furthermore, a comparison was made between the four patients in group A with metastasis and the corresponding five patients in group B. The analysis also revealed that the survival rate of the patients in group A was lower than that of the patients in group B, but the difference was not statistically significant (Fig. 6).

DISCUSSION

Among the patients who had undergone surgery with curative intent for less-advanced pT2 gallbladder carcinoma, those with a high expression rate of stromal localization of MY.1E12-reactive-MUC1 mucin at the deepest invading sites in the subserosal layer had a poorer postsurgical survival rate, attributable to peritoneal dissemination or metastasis at distant organs (Table 2), than those with a low expression rate ($P = 0.044$). The results indicate that gallbladder carcinoma cells in the deepest invading sites showing a high expression rate of stromal localization of the MUC1 mucin have a strong potential for metastasis and that micrometastasis may already have occurred at the time of surgery if the expression rate was high at the deepest invading sites. Notably, in relation to the depth of invasion, there were significant differences in cellular localization of the MUC1 mucin at the deepest invading sites. The depth of invasion may affect the expression of the MUC1 mucin in gallbladder carcinomas. The results are consistent with those of a recent study on colorectal carcinomas showing that primary colon carcinomas at advanced stages have a high-level expression of matured MUC1 mucin (18, 32).

It has been reported that the clinical outcome of patients
with gallbladder carcinomas depends on the depth of invasion (10). However, as mentioned above, despite a theoretical advantage for gallbladder carcinoma not involving the serosa, the prognosis of pT1 carcinoma is not necessarily favorable, and the 5-year postsurgical survival rate in cases of pT2 carcinoma, ~50–80%, is intermediate between that of pT1 carcinoma and those of pT3 and pT4 carcinomas (9–11). This may be because approximately one-half of the patients had malignant infiltration into the lymphatic, venous, and perineural spaces and that the frequency of lymph node metastasis was 50% (9, 10). In the present study, the expression rate of stromal localization of MY.1E12-reactive-MUC1 mucin at the deepest invading sites may be considered to be an independent prognostic marker for pT2 carcinoma, and mAb MY.1E12 should, therefore, be useful in identifying a unique subgroup of MUC1 mucins.

It is also possible that the alteration in cellular localization of MY1E.12-reactive-MUC1 mucin is simply associated with the status of tumor differentiation and may not influence the carcinoma cells’ malignant behavior. This is related to the abnormality in the cell surface expression of the MUC1 mucin caused by the lower differentiation, that is, the failure to establish or maintain polar expression of normal epithelial surface glycoprotein. The localization of the MUC1 mucin, therefore, may be determined by the degree of tumor differentiation. However, in cases of pT2 gallbladder carcinoma, there was no significant correlation between the degree of tumor differentiation and the expression rate of stromal localization of the MUC1 mucin at the deepest invading sites.

In normal epithelia, MUC1 mucins are expressed only on the apical (luminal) side of the cells. In most carcinoma cells, this polarization is lost and overexpression of MUC1 mucins on the whole cell membrane destabilizes the cell-to-cell adhesion and permits carcinoma cells to migrate and metastasize (17). This tendency to lose homotypic cell-to-cell adhesion was experimentally observed under the influence of E-cadherin (17), which is a 120,000 glycoprotein present in epithelial cells and acts as intercellular adhesion molecules (41), modulating gap junctional intercellular communication and cell surface polarity. The frequency of expression of E-cadherin was observed to be high at the invading sites of pT2–pT4 gallbladder carcinomas (current data not shown). If MY1E.12-reactive-MUC1 mucin has an antagonizing effect on E-cadherin, a decrease in E-cadherin function may favor the detachment of carcinoma cells, resulting in a more aggressive tumor (42, 43). The antagonizing effect of the MUC1 mucin on E-cadherin at the deepest invading sites may be enhanced if the presence of the MUC1 mucin in the surrounding stroma adjacent to the cancerous epithelia is due to a direct leakage from living carcinoma cells producing and containing abundant MUC1 mucin.

The mechanistic basis for the alterations in stromal localization of MY1E.12-reactive-MUC1 mucin at the deepest invasive sites of gallbladder carcinoma and for the high expression rate in pT2 carcinoma with poor prognosis remains to be further

### Table 4 Histopathological findings of gallbladder carcinoma by depth of invasion

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>Lymphatic permeation</th>
<th>Venous permeation</th>
<th>Lymph node metastasis</th>
<th>Involved surgical margin</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1, G2-4</td>
<td>n</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1-year</td>
</tr>
<tr>
<td>pT1a</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>100 ± 0*</td>
</tr>
<tr>
<td>pT1b</td>
<td>31</td>
<td>25</td>
<td>6</td>
<td>11</td>
<td>83 ± 7</td>
</tr>
<tr>
<td>pT2</td>
<td>15</td>
<td>12</td>
<td>3</td>
<td>4</td>
<td>73 ± 11</td>
</tr>
<tr>
<td>pT3, pT4</td>
<td>16</td>
<td>13</td>
<td>6</td>
<td>2</td>
<td>93 ± 6</td>
</tr>
</tbody>
</table>

* Values represent means ± SE.

P < 0.05, significantly different between the two groups.
MUC1 MUCs is not likely to be the cause as shown in Fig. 5. Host microenvironmental factors may affect the production and cellular distribution of the MUC1 mucin. In gallbladder carcinomas with excessive desmoplasia, the cancerous epithelia are separated by fibrous stroma (44). At the deepest invasive sites of gallbladder carcinomas, invasion/metastasis-related substances may be produced in the proliferated stromal cells (fibroblasts) surrounding cancerous epithelia, possibly by a communication with the cancerous epithelia. A soluble factor, produced by connective tissue of the colon, reportedly increases the expression of MUC1 mucins by colon carcinoma cells in vitro (39, 45, 46). A similar protein that increases MUC1 mucin production and underlies a high level expression of stromal localization of MY1E12-reactive-MUC1 mucin may be produced in the stroma of gallbladder carcinoma tissues.

REFERENCES

Fig. 6 Relationship between postsurgical prognosis and expression rate of stromal localization of mAb MY.1E12-reactive-MUC1 mucin at the deepest invading sites in surgical specimens of pT2 carcinoma by Kaplan-Meier’s survival curves. I. the 31 cases of pT2 carcinoma were divided into two groups based on the expression rate of stromal localization of the MUC1 mucin at the deepest invading sites: one group of cases in which $\geq 10\%$ of the cancerous epithelia showed the stromal localization (group A); and the other group of cases in which $< 10\%$ of the cancerous epithelia showed the stromal localization (group B; $P = 0.043$ according to the log-rank test). II. the 22 cases of pT2 carcinoma without lymph node metastasis were divided into groups A and B according to the same criteria. III. the nine cases of pT2 carcinoma with lymph node metastasis were divided into groups A and B according to the same criteria.


Expression of MUC1 Mucins in the Subserosal Layer Correlates with Postsurgical Prognosis of Pathological Tumor Stage 2 Carcinoma of the Gallbladder

Toru Kawamoto, Junichi Shoda, Tatsuro Irimura, et al.

*Clin Cancer Res* 2001;7:1333-1342.

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