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Stromal laminin-5γ2 chain expression is associated with the wall-invasion pattern of gallbladder adenocarcinoma

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ABSTRACT

Our previous study demonstrated that the pT2 and pT3-4 gallbladder carcinomas can be classified into two groups, *i.e.* infiltrative growth type (IG type) and destructive growth type (DG type) and that the DG type is associated with poor differentiation, aggressive infiltration, and decreased postoperative survival. The present study focused on the clinicopathologic significance of laminin-5γ2 chain expression as an indicator of local aggressiveness and Ki-67 labeling index (Ki-67 LI) as an indicator of the cell proliferation activity of gallbladder carcinoma. Ki-67 LI was higher in the DG type (26.3%) than in the IG type (21.4%), and the rate of high-grade cell proliferation cases (Ki-67 LI ≧ 30%) was high in the DG type ($P = 0.012$). Gallbladder carcinoma cases with high Ki-67 LI were significantly associated with poorly differentiation ($P = 0.089$) and distant lymph node metastasis ($P = 0.079$). Laminin-5γ2 expression patterns of gallbladder carcinoma were divided into two distinct types, extracellular staining and cytoplasmic staining. The extracellular staining was subclassified into two groups, basement membrane staining and stromal staining. In the basement membrane staining, laminin-5γ2 was present in the basement membranes surrounding neoplastic glandular structures. The basement membrane staining of laminin-5γ2 was more frequent in the IG type (40%) than in the DG type (12.9%) ($P = 0.025$). The stromal staining was more frequent in the DG type. Furthermore, the stroma-positive group was more closely associated with decreased overall survival than the stroma-negative group ($P = 0.028$). The cytoplasmic staining was not significantly correlated with invasion pattern in gallbladder carcinoma ($P = 0.545$). Univariate analysis demonstrated that laminin-5γ2 stromal staining is a predictor of lymphatic invasion, venous invasion, neural invasion, the mode of subserosal infiltration, and lymph nodal status. Multivariate analysis revealed the mode of subserosal infiltration is the strongest predictor of stromal invasion ($P = 0.068$). In conclusion, high-grade cell proliferation and stromal laminin-5γ2 staining were significantly correlated with a wall-invasion pattern of aggressive gallbladder carcinoma indicating destructive growth (DG type).

In a previous study, we subclassified the pT2 and pT3-4 gallbladder carcinomas into two groups, *i.e.* infiltrative growth type (IG type) and destructive growth type (DG type), and the DG type was significantly associated with poor differentiation, aggressive infiltration, vascular invasion, lymph node metastasis, and decreased postoperative survival (38). Therefore, subclassification of the IG/DG growth

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pattern is thought to be a useful indicator of the local aggressiveness of gallbladder carcinoma. Recently, immunohistochemical analyses of gallbladder carcinoma were performed for cell-cycle-related molecules (p53, retinoblastoma protein, cyclin D1, p27, Ki-67 *etc.*) (6, 12, 16, 26, 40, 46, 47, 49, 53–55) as they might reflect the invasion patterns of cancer cells. We continued this series of research, using immunohistochemical methods, involving the laminin-5γ2 chain and Ki-67, to clarify how gallbladder carcinoma develops its different invasion patterns and how the DG type obtains local aggressiveness when passing through the muscle layer.

Ki-67 is a nuclear protein that is expressed during the G1, S, G2, and M phases of continuously cycling cells, but not in G0 cells (6, 12, 16, 54). The genetic locus of Ki-67 is not well characterized, although it has been assigned to chromosome 10. Several studies have shown that cell proliferative activity, as defined by the Ki-67 labeling index (Ki-67 LI), correlates with cell growth (6, 12, 16, 47, 49, 54).

Laminins are a family of glycoproteins of the extracellular matrix that function in the development and maintenance of cellular organization on the basement membrane, and they regulate cell adhesion, migration, and differentiation (5, 10, 51). Structurally, the laminin molecule is a cross-shaped heterotrimer of polypeptide chains: one heavy α chain and two light β and γ chains (5, 51). These chains form a variety of laminin isoforms, which are tissue-specific and probably function differently (10). Recent studies have identified 11 laminin isoforms, formed by various combinations of laminin-chain variants (30). Laminin-5 is one of the isoforms of the laminin family and is composed of α3, β3, and 2 chains, one of which, the γ2 chain, is specific to laminin-5 (13, 18, 44, 45). Laminin-5 serves as an important adhesion protein for epithelial cells positioned on the basement membrane and plays an important role in cell migration (5, 10, 18, 30, 44, 51). For instance, it has been found in migrating keratinocytes in healing skin wounds (25, 41, 45). In addition, specific cleavage of laminin-5γ2 by matrix metalloproteinase-2 has been reported to be critical to cell migration during tumor invasion and tissue remodeling (14). Our present study focused on the clinicopathologic significance of laminin-5γ2 expression for local aggressiveness and Ki-67 LI for cell proliferating activity in gallbladder carcinoma.

MATERIALS AND METHODS

Gallbladder tissue specimens. All the tissue specimens were obtained by surgical resection of gallbladder adenocarcinomas at Tokai University Hospital. The stages of gallbladder carcinoma cases were based on the TNM classification (24). pT2 carcinomas invade subserosal connective tissue, and pT3-4 carcinomas extend to the visceral serosa. Sixty-six pT2/pT3-4 gallbladder carcinomas were examined. The age range of the patients (30 men and 36 women) from which the samples were taken was 40–93 (mean 64.1 ± 10.1) years. The median postoperative follow-up was 453.5 (228.0–1269.3) days.

Histological examination. The gallbladder tissue specimens were rapidly fixed in 10% buffered formalin for 24–48 h for histological and immunohistochemical analyses and were routinely embedded in paraffin. Tumor invasion was examined in 4 μm thick sections stained with hematoxylin and eosin. The degree of venous invasion was classified as: v0, no venous invasion; v1+, minimal venous invasion, *i.e.* one or two foci of venous invasion in one histological section; v2+, moderate venous invasion, *i.e.* three or four foci; or v3+, severe venous invasion with more than five foci. The degree of lymphatic invasion was classified as: ly0, no lymphatic invasion, ly1+, mild lymphatic invasion, ly2+, moderate lymphatic invasion, or ly3+, severe lymphatic invasion. The degree of perineural invasion was classified as: ne0, no perineural invasion, ne1+, mild perineural invasion, ne2+, moderate perineural invasion, or ne3+, severe perineural invasion. The modes of subserosal infiltration were classified into three groups according to the general rules for gastric cancer study of the Japanese Gastric Cancer Association (17), *i.e.* INFα, the tumor showing expanding growth and a distinct border from the surrounding tissue; INFβ, the tumor showing intermediate growth between INFβ and INFγ; and INFγ, the tumor showing scirrhous growth and an indistinct border from the surrounding tissue. The degree of lymph node metastasis was classified as: N0, no lymph node metastasis; N1, low-grade lymph node metastasis; or N2, high-grade lymph node metastasis based on TNM classification.

Immunohistochemical analysis. Deparaffinized and dehydrated sections were immersed in 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min to abolish endogenous peroxidase activity. Four-μm-thick paraffin sections were mounted on aminoacyl silane-

coated glass slides and used for immunohistochemical analyses of the Ki-67 and the laminin-5γ2. For Ki-67 antigen retrieval, the sections were penetrated with autoclave heating (ES-215, High-pressure steam sterilizer; TOMY, Japan) at 121°C for 4 min. Non-specific binding was abolished with diluted normal sheep serum (Cosmo Bio Co. Ltd., Tokyo, Japan). Next, the sections were overlayed with primary monoclonal antibodies diluted at 1 : 100 with 1% bovine serum albumin-containing phosphate-buffered saline (PBS) and were left overnight at 4°C in a moist chamber. After being washed with PBS, the secondary biotinylated anti-mouse $Ig(Fab)_2$ antibody at 1 : 100 (Amersham International plc., Buckinghamshire, UK) were applied for 60 min at room temperature. The sections were then treated with the streptavidin-conjugated horseradish peroxidase for 30 min at room temperature. The reaction products were visualized using diaminobentizine tetrahydrochloride (DAB) for 4 min in Tris buffer.

For laminin-5γ2 chain, mouse monoclonal antibody D4B5 was prepared using the human recombinant laminin-5γ2 chain (amino acid residues 382–608) as the antigen. All sections were treated with protease XXIV (Sigma, St Louis, MO) for 8 min at 37°C. The sections were then incubated with antibody at 4°C overnight. The labeled antigen was detected by a HistoFine kit (Nichirei Pharmaceutical, Tokyo, Japan) and visualized by the DAB reaction (1). The sections were counterstained with hematoxylin.

Definition and histological identification of the invasion pattern. The following terminology is used to define and classify the two patterns of invasion through the muscle layer. a) Infiltrative growth (IG) type: cancer cells shows infiltrative growth into the muscle layer (through intermuscular space) without breaking it; and b) Destructive growth (DG) type: cancer cells shows massive growth with destruction of the muscle layer (38).

The cases which contain both DG and IG components were classified into the DG type because the DG type is thought to be an invasive and aggressive growth pattern.

Expression patterns of laminin-5γ2 chain. The immunohistochemical expression patterns of laminin-5γ2 were divided into four groups as follows (Figs. 1 and 2). a) Basement membrane type: the laminin-5γ2 was present in the basement membranes surrounding neoplastic glandular structures (laminin-5γ2 positivity in the basement membrane of more than 10%); b) Stromal type: the laminin-5γ2 was present in the stroma (laminin-5γ2 positivity in the stroma of more than 10%); c) Cytoplasmic type: the laminin-5γ2 was present in the cytoplasm (laminin-5γ2 positivity in the cytoplasm of more than 10%); and d) Negative type: all structural components were laminin-5γ2 negative.

Statistical analysis. Descriptive statistics were employed to examine the demographic characteristics of the study population. Data were expressed as

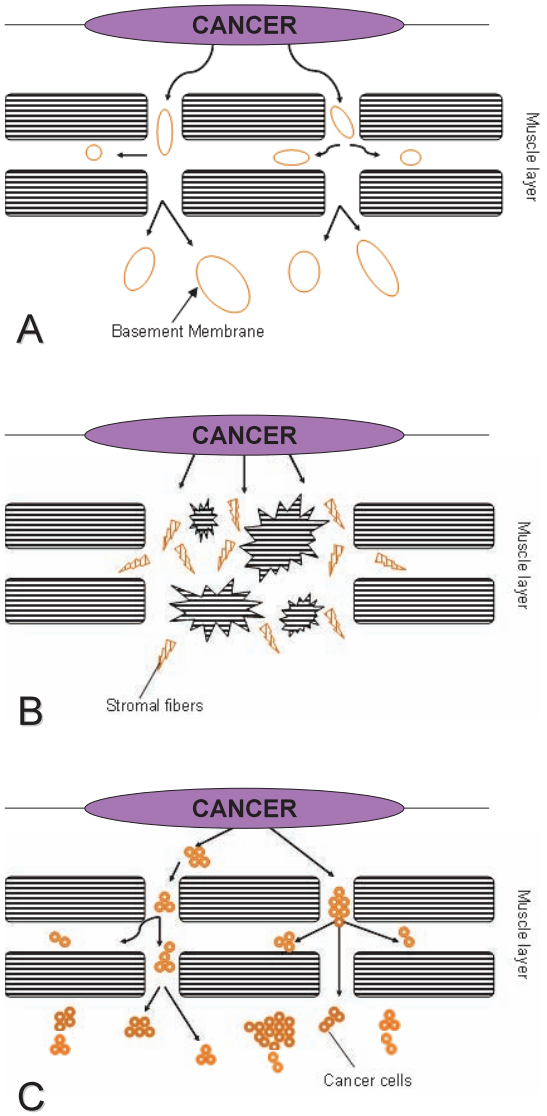

Fig. 1 A scheme of gallbladder carcinoma invasion and laminin-5γ2 staining patterns. A. Basement membrane type: laminin-5γ2 positivity in the basement membrane of more than 10%. B. Stromal type: laminin-5γ2 positivity in the stroma of more than 10%. C. Cytoplasmic type: laminin-5γ2 positivity in the cytoplasm of more than 10%.

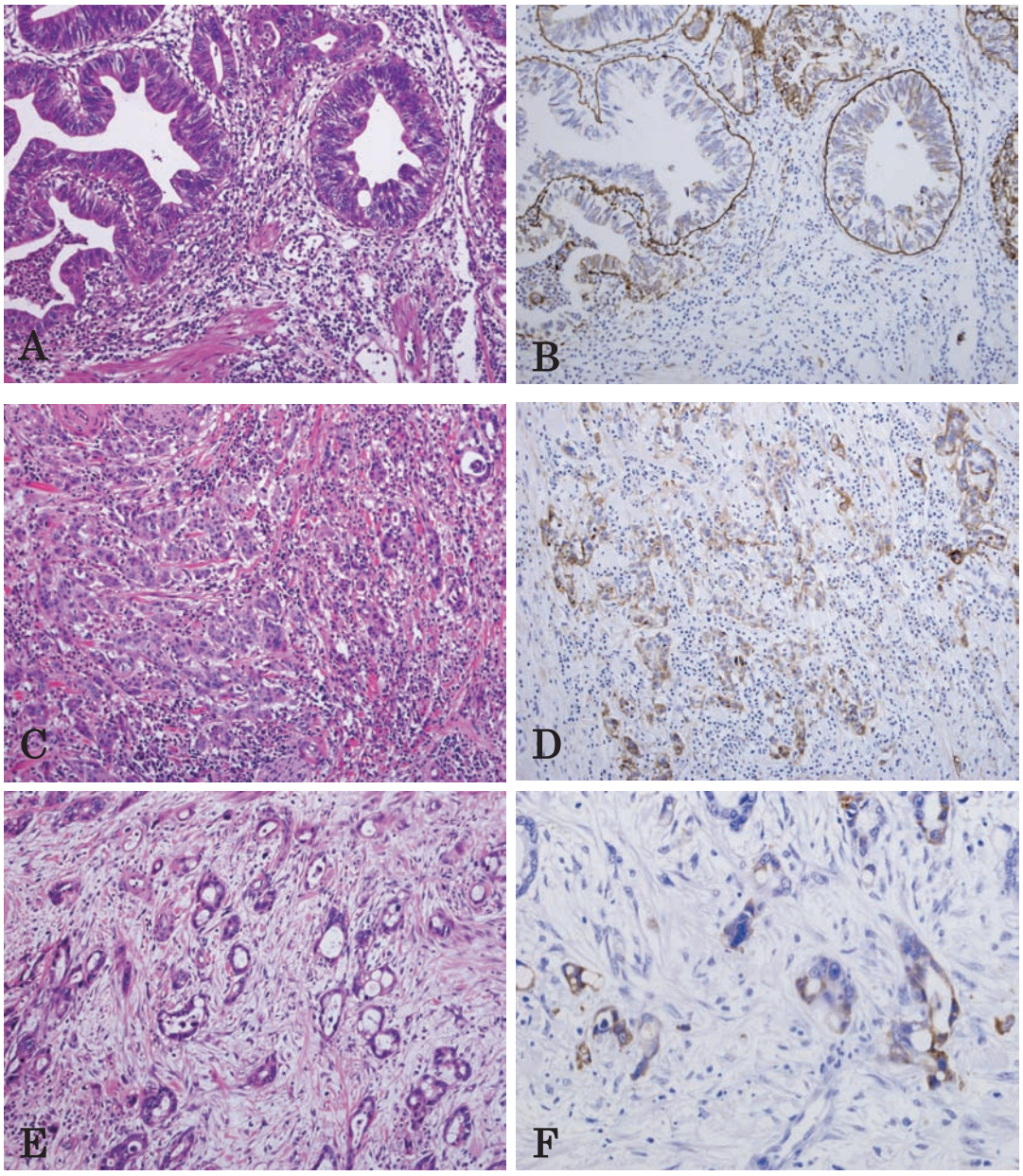

Fig. 2 Hematoxylin-eosin and immunohistochemical findings of gallbladder carcinoma invasion and laminin-5γ2 staining patterns. In the basement membrane type (A, B), basement membranes surrounding neoplastic glandular structures showed intense and continuous immunoreactivity for laminin-5γ2. In the stromal type (C, D), the immunoreactivity for laminin-5γ2 was irregular and fibrous around tumor cells scattered in the stroma. In the cytoplasmic type (E, F), the immunoreactivity for laminin-5γ2 was positive in the intracellular matrix.

mean ± SD and median (25–75, percentile). Univariate analyses (Chi-square test) were primarily used for selection of variables, based on a P-value < 0.05. The significant variables and clinically effective factors were entered into a forward logistic regression analysis to determine the net effect of each predictor while controlling for the others. Odds ratios (OR) and their 95% confidence intervals (CI) were used to assess the independent contributions of significant factors. A value of $P < 0.05$ was considered to indicate statistical significance. After statistical analysis concerning the laminin-5γ2 staining pattern, negative cases were excluded because it was impossible to identify their immunostaining patterns. Survival curves were traced with the Kaplan-Meier method. All the analyses were performed using the standard

statistical software package SPSS II (version 11.0; SPSS, Tokyo, Japan).

RESULTS

Thirty-five (53.0%) cases displayed the IG type and thirty-one (47.0%) cases displayed the DG type. Fifty cases (75.8%) were well to moderate differentiated adenocarcinomas, while the other sixteen cases (24.2%) were poorly differentiated adenocarcinomas or other histological types such as signet-ring cell carcinoma, adenosquamous cell carcinoma, mucinous carcinoma, small cell carcinoma, and undifferentiated carcinoma. Cell proliferation as evaluated by Ki-67 LI was higher in the DG type (26.3%) than in the IG type (21.4%) ($P = 0.038$), and the rate of high-grade cell proliferation cases (Ki-67 LI ≧ 30%) was high in the DG type (P=0.012, Table 1). Poorly differentiated adenocarcinomas, INFγ (scirrhous growth) and N2 (high-grade lymph node metastasis) showed higher Ki-67 LI values than well to moderate differentiated adenocarcinomas ($P = 0.089$), INFα, β ($P = 0.085$) and N0, 1 ($P = 0.079$), respectively, otherwise no correlation was present between Ki-67 LI and local aggressiveness in this study (Table 2).

Laminin-5γ2 expression patterns were divided into three distinct types, and results of the staining are summarized in Table 3. Laminin-5γ2 basement membrane type was found in 40% of IG type and 12.9% of DG type of gallbladder carcinomas. Laminin-5γ2 stromal type was detected in 25.7% of

Table 1 *The relationship between the invasion pattern and the Ki-67 labeling index (MIB-1)*

Invasion pattern	Mann-Whitney U test Ki-67 LI (mean ± SD, %)	P Value	Ki-67 LI		χ^2 test P Value
			< 30%(n)	≧ 30%(n)	
IG	21.4 ± 7.8		30	5	
		0.038			0.012
DG	26.3 ± 13.1		18	13	

LI: labeling Index
n: number of cases
IG: infiltrative growth pattern
DG: destructive growth pattern

Table 2 *Univariate analysis: Predictors of proliferation in gallbladder cancer patients*

Factor	Ki-67 LI		χ^2 test P-Value	Odds Ratio	95% Confidence Interval
	< 30% (n = 48)	≧ 30% (n = 18)			
Age (y.o) mean ± SD	65.01 ± 10.1	62.2 ± 10.2	0.303	0.972	0.919–1.027
Histological differentiation Well, Mod. (n)	38	11	0.089	2.758	0.836–9.092
Lymphatic invasion ly0, 1 (n)	24	10	0.688	0.800	0.269–2.375
Venous invasion v0, 1 (n)	25	10	0.392	1.607	0.540–4.783
Neural invasion ne0, 1(n)	24	12	0.512	0.692	0.229–2.086
Spread pattern INFα, β (n)	30	7	0.085	2.619	0.860–7.974
Lymph nodal status N0, 1 (n)	32	11	0.079	0.305	0.078–1.199

Well, Mod.: well or moderately differentiated adenocarcinoma
ly0, 1: no lymphatic invasion or mild lymphatic invasion
v0, 1: no venous invasion or mild venous invasion
ne0, 1: no neural invasion or mild neural invasion
INFα, β: expansive or intermediate growth of subserosal infiltration
N: lymph node status based on the TNM classification
n: number of cases
LI: labeling index

Table 3 *A summary of immunohistochemical analyses of human gallbladder carcinomas for the laminin-5γ2 chain*

Invasion pattern	Total no. of cases	Positive cases of laminin expression			Negative cases
		Extracellular staining		Cytoplasmic staining	
		BM	ST	CP	–
IG	35	14 (40.0%)	9 (25.7%)	25 (71.4%)	0
DG	31	4 (12.9%)	21 (67.7%)	18 (58.1%)	3
χ^2 test *P* Value		0.025*	0.001*	0.545	n.e.

IG: infiltrative growth pattern
DG: destructive growth pattern
BM: basement menbrane pattern
ST: stromal pattern
CP: cytoplasmic pattern
n.e.: not evaluated

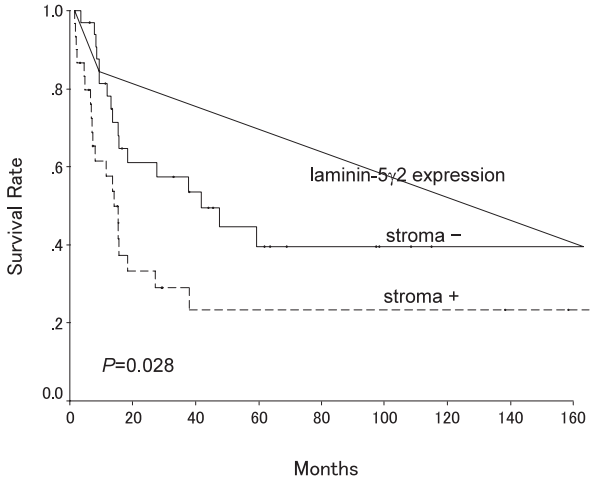

Fig. 3 Stromal laminin-5γ2 expression and overall survival of patients with gallbladder carcinomas. The patients with pT2/pT3-4 carcinomas were divided into the two groups, according to stromal laminin-5γ2 expression. Patients in the stroma-positive group (---, n = 30) had a significantly poorer prognosis compared to that of patients in the stroma-negative group (—, n = 33; *P* = 0.028 log-rank test).

IG type and 67.7% of DG type. The cytoplasmic type was found in 71.4% of IG type and 58.1% of DG type. Laminin-5γ2 basement membrane type was more frequent in the IG type than DG type of gallbladder carcinomas ($P = 0.025$, Table 3). On the other hand, the most typical staining in DG type was stromal type, as characterized by the laminin-5γ2 positive staining in the stroma adjacent to the small carcinoma cell nests ($P = 0.001$, Table 3). Furthermore, patients in the stroma-positive group had a significantly poorer prognosis compared to that of patients in the stroma-negative group ($P = 0.028$, log-rank test) (Fig. 3). In our study, there was no correlation between invasion pattern and cytoplasmic staining ($P = 0.545$, Table 3). We examined univariate analysis for determining predictive clinicopathological factors of the local aggressiveness of gallbladder carcinomas (Table 4). Univariate analysis identified that laminin-5γ2 stromal type was correlated with five factors, ly, v, ne, INF, and lymph nodal status. In addition, multivariate logistic regression analysis demonstrated that laminin-5γ2 stromal type was correlated with the mode of subserosal infiltration (INF) (Table 5).

DISCUSSION

The number of surgically resected cases of gallbladder carcinoma has recently increased because of advances in imaging diagnosis and operative procedures. In this study, we reviewed 66 surgically resected cases of gallbladder carcinoma to clarify cancer aggressiveness by evaluating cell proliferation activity, as well as cancer invasiveness by classifying laminin-5γ2 staining patterns. High-grade cell proliferation cases (Ki-67 LI $\geqq$ 30%) were common in the DG type. Laminin-5γ2 basement membrane type was more frequent in the IG type than in the DG type. The most common staining pattern in the DG type was the stromal type. To our knowledge, this is the first report that describes the relationship between wall-invasion pattern and the aggressiveness/invasiveness of gallbladder carcinomas.

The layers of the gallbladder wall include the surface epithelium, lamina propria, smooth muscle, subserosal connective tissue, and serosa, but lack a muscularis mucosae and submucosa (2). The smooth muscle layer is approximately 400–500 μm thick and consists of loosely arranged bundles of muscle fibers. Therefore, gallbladder carcinomas can easily invade into the subserosal layer through the smooth muscle layer, and show frequent vascular permeation and perineural invasion, which means high malignancy histologically and clinically (8, 19, 20–

Table 4 *Univariate analysis: Laminin-5γ2 chain stromal staining in gallbladder cancer*

Factor	Laminin-5γ2 stromal staining		*P* Value	Odds Ratio	95% Confidence Interval
	negative (n = 33)	positive (n = 30)			
Age (y.o) mean ± SD	64.9 ± 11.2	63.6 ± 9.1	0.632	0.988	0.941– 1.038
Histological differentiation Well, Mod. (n)	29	21	0.08	3.107	0.843–11.456
Lymphatic invasion ly0, 1 (n)	24	9	0.001	6.222	2.084–18.579
Venous invasion v0, 1 (n)	22	11	0.017	3.455	1.225– 9.744
Neural invasion ne0, 1(n)	24	10	0.002	5.333	1.814–15.681
Spread pattern INFα, β (n)	26	10	< 0.001	7.429	2.404–22.954
Lymph nodal status N0, 1 (n)	26	14	0.008	4.245	1.412–12.758

Well, Mod.: well or moderately differentiated adenocarcinoma
ly0, 1: no lymphatic invasion or mild lymphatic invasion
v0, 1: no venous invasion or mild venous invasion
ne0, 1: no neural invasion or mild neural invasion
INFα, β: expansive or intermediate growth of subserosal infiltration
N: lymph node status based on the TNM classification
n: number of cases

Table 5 *Multivariate analysis: Laminin-5γ2 chain stromal staining in gallbladder cancer*

Factor	*P* Value	Odds Ratio	95% Confidence Interval
Age < 65	0.389	0.972	0.911–1.037
Lymphatic invasion ly0, 1 (n)	0.643	1.452	0.300–7.026
Venous invasion v0, 1 (n)	0.463	1.695	0.414–6.931
Neural invasion ne0, 1 (n)	0.179	2.568	0.649–10.173
Nodal Status N0, 1 (n)	0.215	2.507	0.586–10.737
Spread pattern INFα, β (n)	0.068	3.689	0.909–14.978

Well, Mod.: well or moderately differentiated adenocarcinoma
ly0, 1: no lymphatic invasion or mild lymphatic invasion
v0, 1: no venous invasion or mild venous invasion
ne0, 1: no neural invasion or mild neural invasion
INFα, β: expansive or intermediate growth of subserosal infiltration
N: lymph node status based on the TNM classification
n: number of cases

23, 34, 37, 43). We previously reported that the wall-invasion pattern is correlated with histological aggressiveness and the survival rate of patients with gallbladder carcinoma (38). In this study we demonstrated invasion patterns and their ways of obtaining local aggressiveness when invading through the muscle layer. We used the Ki-67 LI to evaluate the cell proliferation of gallbladder carcinomas and indicate cancer aggressiveness. Ki-67 is a nuclear protein that is expressed during the G1, S, G2, and M phases of continuously cycling cells, but not in G0 cells (6, 12, 16, 54). Ki-67 expression was significantly higher in the DG type than in the IG type, *i.e.* high-grade cell proliferation might be correlated with muscle destruction. Laminins are now known to play a central role in organizing and establishing the basement membrane (5, 10, 51). Laminin-5 is a recently identified laminin isoform, which acts as a functional adhesion component for epithelial cells (44). Laminin-5 contains unique laminin variant chains, one of which, the γ2 chain, has recently been cloned and sequenced. Some investigations have indicated that laminin-5 serves as an important adhesion protein for epithelial cells positioned on the basement membrane. Some studies have reported that laminin-5γ2 was intensely expressed in the invasive front of cancers in some digestive organs, while others demonstrated that loss of laminin-5 in the epithelium-stroma interface is an immunohisto-

chemical marker of malignancy in epithelial lesions (4, 11, 15, 24, 27–29, 31, 33, 39, 42, 48, 50).

In previous studies, the laminin-5γ2 staining pattern was classified into three patterns, *i.e.* basement membrane staining, diffuse staining around tumor cells, and cytoplasmic staining (24). We introduced different laminin-5γ2 expression patterns, which are described above. In the present study, cytoplasmic staining of laminin-5γ2 was not correlated with wall-invasion pattern. However, basement membrane staining of laminin-5γ2 was correlated with IG type and stromal staining of laminin-5γ2 was correlated with DG type. The basement membrane is composed of major structural proteins such as collagen type IV, laminin, heparan sulfate proteoglycan, nidogen (entactin), and BM-40 (osteonectin, SPARC) (51). The formation of the basement membrane plays a barrier role against cancer invasion. Advanced gallbladder carcinomas show vertical invasive growth accompanied by fibrosis (desmoplasia) (2, 3, 8, 17, 19–22, 34, 37), which is produced by the interaction between the cancer and the stroma in the absence of basement membrane. A recent paper demonstrated a complete loss or only fragmentary remnants of laminin-5γ2 immunostaining in the carcinoma-stroma interface (15). Our study demonstrated that stromal staining of laminin-5γ2 reflected the invasiveness of cancer nests indicating an interaction between cancer cells and stromal tissue.

In conclusion, high-grade cell proliferation as measured by the Ki-67 LI and invasiveness with stromal laminin-5γ2 staining were significantly correlated with a wall-invasion pattern of aggressive gallbladder carcinoma indicating destructive growth (DG type).

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