

# Aberrant expression of laminin gamma 2 chain and its prognostic significance in intrahepatic cholangiocarcinoma according to growth morphology

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Laminin gamma 2 chain is an extracellular matrix protein that plays an important role in cell migration and tumor invasion. We report altered expression and characteristic localization of this chain in a series of 105 cases of intrahepatic cholangiocarcinomas examined immunohistochemically. All tumors were grossly classified into the following three types: intraductal growth type ( $n=9$ ), periductal infiltrating type ( $n=8$ ) and mass-forming type ( $n=88$ ). The tumors exhibited three distinct staining types: basement membrane staining, cytoplasmic staining and stromal staining. The basement membranous staining of laminin gamma 2 chain was more frequent in biliary dysplasia, intraductal growth and periductal infiltrating type than in mass-forming type. The cytoplasmic staining of carcinoma cells was observed especially at the cancer–stromal interface or at the invasive front of tumors. Stromal staining of laminin gamma 2 chain was essentially localized in the stroma around cancer cells at the invasive area, and the expression was significantly correlated with tumor aggressive factors and a poor prognosis in patients with intrahepatic cholangiocarcinoma. We conclude that laminin gamma 2 chain exhibits aberrant expression in a stepwise manner through different aggressive stages of tumor progression.

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Laminin-5, which consists of alpha 3, beta 3 and gamma 2 chains, is a major component of the basement membrane of epithelial tissues.<sup>1–3</sup> It plays important roles in the static adhesion of epithelial cells to the basement membrane and in the promotion of epithelial cells.<sup>4,5</sup> The basement membrane has been considered to perform as a mechanical barrier against cancer cell invasion and, in contrast, the changes of the laminin distribution and composition have been associated with the malignant transformation of epithelia and cancer cell invasion.<sup>6</sup>

Laminin gamma 2 chain plays a key role in cell migration during tumor invasion. Overexpression of laminin gamma 2 chain has been particularly demonstrated at the invasive front of various carcinomas, including colorectal,<sup>7–11</sup> gastric,<sup>12</sup>

lung<sup>13,14</sup> and pancreatic<sup>15–17</sup> adenocarcinomas, and head and neck,<sup>18</sup> tongue<sup>19</sup> and esophageal<sup>20</sup> squamous cell carcinomas. The prognostic indicator of its expression has been reported in several tumors.<sup>7,13,19</sup> However, the regulatory mechanism for the laminin gamma 2 chain expression in tumor progression is currently unresolved.

Intrahepatic cholangiocarcinoma is the second most common primary liver cancer, and the incidence and mortality rate of intrahepatic cholangiocarcinoma have been increasing worldwide in recent years.<sup>21,22</sup> The prognosis of patients with cholangiocarcinoma is generally poor, although a minority subtype of intrahepatic cholangiocarcinoma showing an intraductal growth pattern had a more favorable prognosis when complete tumor resection was performed.<sup>23,24</sup> Intraductal growth type of intrahepatic cholangiocarcinoma frequently preserve basement membrane and are microscopically noninvasive or minimally invasive. It is important to evaluate the basement membrane molecule associated with tumor invasion among intrahepatic cholangiocarcinomas with various

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degrees of tumor extension. Up to now, the expression of laminin gamma 2 chain in intrahepatic cholangiocarcinomas and its premalignant lesion has not been elucidated. We currently investigated the expression and localization of laminin gamma 2 chain of dysplastic biliary epithelium, intraductal growth, periductal infiltrating and mass-forming types of intrahepatic cholangiocarcinoma. We then compared the results with the clinicopathological parameters and survival rates.

## Materials and methods

### Tissue Specimens

Tumor specimens were obtained from 105 patients who had undergone surgery for cholangiocarcinoma between 1987 and 2002. We also obtained 26 cases of surgically resected liver specimens for hepatolithiasis. All specimens were obtained from files at the Department of Anatomic Pathology of Kyushu University. We did not include patients with cholangiocarcinoma arising from the bile duct confluence or the extrahepatic bile duct. Patients who underwent incomplete resection were also excluded from this study. The cases of hepatolithiasis showed various degrees of dysplastic biliary epithelia with an increased nucleo-cytoplasmic ratio, loss of nuclear polarity and nuclear hyperchromasia. These dysplastic features were mild to severe epithelial atypia without definitive evidence of malignant cells. Tissues were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin for histological examination.

### Gross and Histologic Classification

Based on the criteria of The Liver Cancer Study Group of Japan,<sup>25</sup> intrahepatic cholangiocarcinomas were grossly divided into the following three types: intraductal growth type, periductal infiltrating type and mass-forming type. If more than one type was found, the predominant type was recorded in order of the degree of involvement. In this study, intra-

ductal growth type was histologically subclassified into intraductal growth without invasion into the basement membrane and intraductal growth with invasion to the liver parenchyma. Tumor size was recorded as the largest diameter in the fixed specimens. Histologic typing of tumors revealed eight papillary, 23 well-differentiated, 41 moderately differentiated and 33 poorly differentiated adenocarcinomas, according to their degree of papillary or tubular formation. The clinicopathologic parameters of the patients and tumors based on the gross features are summarized in Table 1.

### Immunohistochemistry

Sections (4  $\mu$ m thick) were cut and deparaffinized through xylene and ethanol. After the endogenous peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxidase for 30 min, the sections were treated with Protease XXIV (Sigma, St Louis, MO, USA) for 15 min at room temperature. The slides were exposed to 10% nonimmunized rabbit serum in phosphate-buffered saline (PBS) for 10 min, and then the sections were incubated overnight at 4°C with mouse monoclonal antibody against laminin gamma 2 chain (D4B5, Chemicon, Temecula, CA, USA) at the concentration of 10  $\mu$ g/ml. The antibody was developed against amino-acid residues 382–608 of human laminin gamma 2 chain, and it recognizes the 150- and 105-kDa chain proteins. The labeled antigen was detected by a HistoFine kit (Nichirei Pharmaceutical, Tokyo, Japan) and visualized by the 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen. No significant staining was observed in the negative controls, which were prepared using the mouse immunoglobulin at the same concentration.

### Immunohistochemical Evaluation

We evaluated the sections containing the maximum diameter of the tumor and the tumor–nontumorous border. In the nine cases of intraductal growth type,

**Table 1** Clinicopathologic features and follow-up according to the growth morphology of intrahepatic cholangiocarcinomas

Subtype	IG	PI	MF	Total
No. of cases	9	8	88	105
Age (years)	59.4	62.8	64.0	63.5
Gender (M/F)	4/5	5/3	53/35	62/43
Tumor size (cm)	4.5	2.1	4.8	4.5
<i>Histological differentiation</i>				
pap/well/mode/poor	6/3/0/0	1/5/2/0	1/15/39/33	8/23/41/33
Vascular invasion	4 (44%)	2 (25%)	56 (64%)	62 (59%)
Lymphatic invasion	2 (22%)	4 (50%)	52 (59%)	58 (55%)
Lymph node metastasis	2 (22%)	1 (13%)	30 (34%)	33 (31%)
No. of deaths	3 (33%)	3 (38%)	54 (61%)	60 (57%)
Due to tumor effects	2	2	50	54
Due to unrelated causes	1	1	4	6

IG, intraductal growth; PI, periductal infiltrating; MF, mass-forming.

we focused on the *in situ* components of the tumor without invasive cells. Carcinoma cells were often heterogeneous with respect to laminin gamma 2 chain staining within the same tumor. Localization of the laminin gamma 2 chain was divided into the staining of basement membrane, the staining of tumor cytoplasm and the staining of tumor stroma around the carcinoma cells. Expression of laminin gamma 2 chain was judged positive when more than 10% of clear membranous staining, cancer cell cytoplasmic staining or tumor stromal staining was present. All the hematoxylin and eosin-stained slides and the immunohistochemical slides were evaluated independently by three observers (SA, TT and MT), and the grading was evaluated without knowledge of the outcome of the patients.

### Statistics

The correlation between laminin gamma 2 expression and clinicopathologic parameters was assessed by means of the  $\chi^2$  test and Mann–Whitney's *U*-test. Disease-specific survival was taken as the period of survival between surgery and the date of the last follow-up or death by disease. Patients who were alive or had died of a cause other than cholangiocarcinoma were censored for analysis of disease-specific survival. Survival curves were calculated by the Kaplan–Meier method, and the differences between the curves were analyzed by the log-rank test. Cox's proportional hazard model was used in the multivariate survival analysis. The results were considered significant if the *P*-value was <0.05.

## Results

### Biliary Dysplasia

No positive staining of laminin gamma 2 chain was detected in the basement membrane or cytoplasm of nondysplastic or nontumorous biliary epithelium (Figure 1a). In 17 (65%) of the 26 cases showing dysplastic biliary epithelium, laminin gamma 2 chain appeared to be located in the basement membrane of glandular epithelium. Basement membrane underlying the dysplastic epithelium is continuously stained (Figure 1b). In the 17 cases of positive basement membrane staining, two cases were positive in the cytoplasm of severely dysplastic epithelial cells.

### Intraductal Growth Type of Intrahepatic Cholangiocarcinoma

The nine cases of intraductal growth type were classified as four intraductal growth without invasion and five intraductal growth with invasion to the liver parenchyma. In six of the *in situ* components of the tumor, most parts of the basement membrane

surrounding neoplastic glands were intensely stained for laminin gamma 2 chain (Figure 1c). In four cases, the cytoplasm of neoplastic glands were focally positive (Figure 1d). This cytoplasmic staining pattern was observed only in cases of the intraductal growth type with invasion to the liver parenchyma (Table 2).

### Periductal Infiltrating Type of Intrahepatic Cholangiocarcinoma

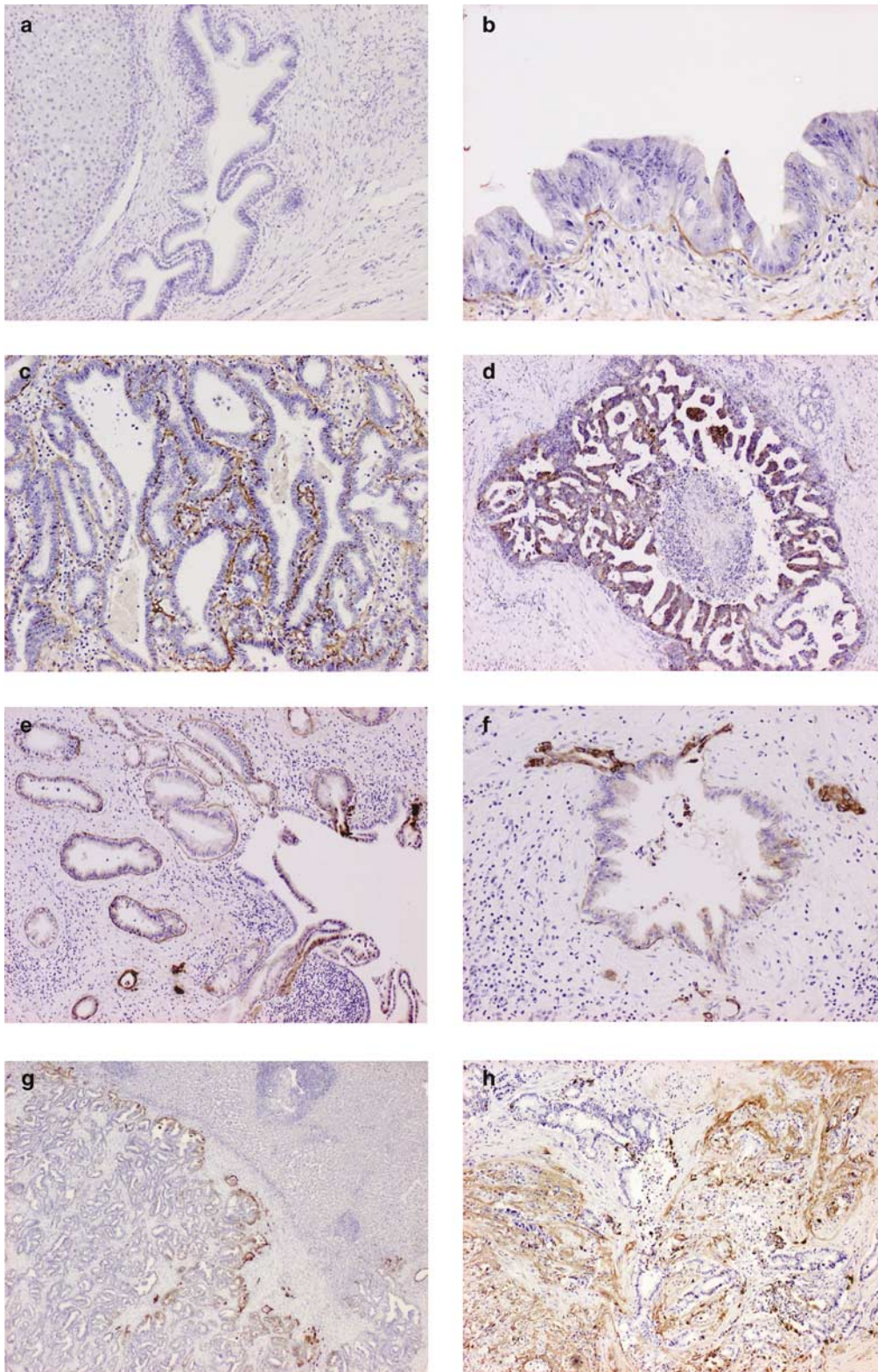
The tumor exhibited three distinct staining types: basement membrane staining, cytoplasmic staining and stromal staining. In six cases, varying degrees of positive staining for laminin gamma 2 chain were detected in the basement membrane surrounding neoplastic glands (Figure 1e). Cytoplasmic staining of carcinoma cells was observed in five cases, while stromal staining around carcinoma cells was observed in two cases (Table 2). The positive staining of carcinoma cell cytoplasm or the stromal component was clearly detected in the invasive areas (Figure 1f).

### Mass-forming Type of Intrahepatic Cholangiocarcinoma

The staining of laminin gamma 2 chain for mass-forming type was essentially identical to that observed in the cytoplasm of carcinoma cells at the cancer–stromal interface or at the invasive front of tumors in 53 of 88 cases (Figure 1g). We also found that tumors with microglandular structures or scattered individual cells showed more frequent laminin gamma 2 chain staining than tumors forming well-differentiated glandular structures. In 18 cases, focal or diffuse staining of the basement membrane surrounding neoplastic glands, preferentially well-differentiated tubular structures, was intensely stained, whereas in 21 cases, the stromal component around the carcinoma cells, especially in invasive areas, was positive for the laminin gamma 2 chain (Figure 1h). This staining pattern was observed in many cases of poorly differentiated carcinoma.

### Correlation between Laminin Gamma 2 Expression and Clinicopathologic Parameters

The results of the analysis of the correlation between laminin gamma 2 localization and the clinicopathologic parameters are presented in Table 3. The basement membrane expression of the laminin gamma 2 chain was significantly more frequent in well-differentiated carcinomas than in poorly differentiated carcinomas ( $P=0.0115$ ). In contrast, the stromal expression was more frequent in poorly differentiated carcinomas than in papillary and well-differentiated carcinomas ( $P=0.0303$ ). Other



**Figure 1** Immunohistochemical results of laminin gamma 2 chain. No positive reaction was detected in the basement membrane or cytoplasm of normal epithelium (a). Basement membrane underlying the dysplastic epithelium is continuously stained (b). Intraductal growth type without invasion represents intense staining of basement membrane surrounding neoplastic glands (c). Intraductal growth type with invasion shows focally positive staining of the cytoplasm of neoplastic glands (d). Periductal infiltrating type shows mainly positive staining of basement membrane surrounding neoplastic glands (e), and partly positive staining of carcinoma cell cytoplasm at the invasive areas (f). In mass-forming type, the cytoplasmic staining of carcinoma cells is observed at the cancer-stromal interface or at the invasive front of a tumor (g), but the stromal component around the carcinoma cells is positively stained, especially in invasive areas (h).

pathologic parameters, lymphatic permeation ( $P=0.0002$ ) and lymph node metastasis ( $P=0.0014$ ) were significantly correlated with stromal expression of laminin gamma 2 chain. In

addition, only the lymph node metastasis was significantly correlated with cytoplasmic expression of laminin gamma 2 chain ( $P=0.027$ ).

**Table 2** Laminin gamma 2 expression according to the growth morphology of intrahepatic cholangiocarcinoma

Subtype	Dysplasia	IG		PI	MF
		inv (-)	inv (+) <sup>a</sup>		
No. of cases	26	4	5	8	88
BM	17 (65%)	3 (75%)	3 (60%)	6 (75%)	18 (20%)
CYT	2 (8%)	0 (0%)	4 (80%)	5 (63%)	53 (60%)
ST	0 (0%)	0 (0%)	0 (0%)	2 (25%)	21 (24%)

IG, intraductal growth; PI, periductal infiltrating; MF, mass-forming; BM, basement membrane staining; CYT, cytoplasmic staining; ST, stromal staining.

<sup>a</sup>Inv (-), intraductal growth without invasion to the basement membrane; inv (+), intraductal growth with liver invasion.

**Univariate and Multivariate Analysis of Prognostic Parameters**

The results of the univariate analysis of the conventional prognostic factors for disease-specific survival are shown in Table 4. We found that tumor size ( $P<0.0001$ ), growth morphology ( $P=0.0167$ ), histologic differentiation ( $P<0.0001$ ), vascular invasion ( $P=0.0001$ ), lymphatic permeation ( $P<0.0001$ ) and lymph node metastasis ( $P<0.0001$ ) exhibited prognostic implications. Stromal expression of laminin gamma 2 chain was also significantly correlated with a poor prognosis ( $P=0.0006$ ), although cytoplasmic expression of laminin gamma 2 chain was not (Figure 2). The prognostic parameters and laminin gamma 2 chain expression were analyzed

**Table 3** Correlation between laminin gamma 2 chain localization and clinicopathologic parameters

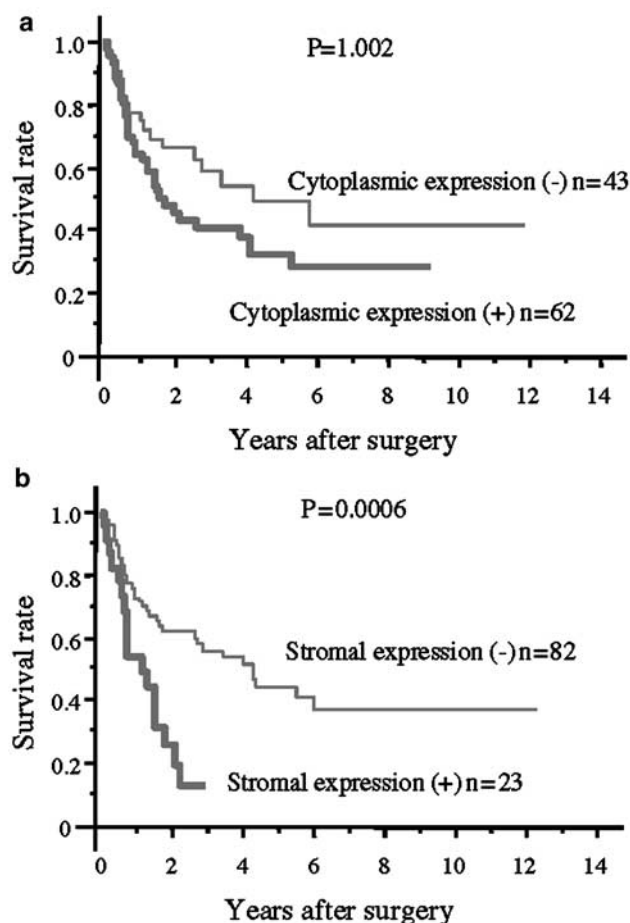
Parameters	BM			CYT			STR		
	(+)	(-)	P-value	(+)	(-)	P-value	(+)	(-)	P-value
No. of cases	30	75		64	41		23	82	
<i>Histological differentiation</i>			0.0115*			0.1699			0.0303*
Papillary/well	15	16		14	17		2	29	
Moderately	7	34		27	14		10	31	
Poorly	8	25		21	12		11	22	
Lymphatic invasion (+)	20	38	0.2033	39	19	0.0897	21	37	0.0002
Lymph node metastasis (+)	12	21	0.3351	27	6	0.0027*	14	19	0.0014*

BM, basement membrane staining; CYT, cytoplasmic staining; STR, stromal staining.

\* $P<0.05$ .

**Table 4** Univariate analysis of disease-specific survival for 105 patients with intrahepatic cholangiocarcinoma

Variables	No. of cases	Death with disease	5-year survival (%)	P-value
<i>Tumor size (cm)</i>				
4.5 >	59	20	56	<0.0001
4.5 ≤	46	34	16	
<i>Growth morphology</i>				
Intraductal growth	9	2	65	0.0167
Periductal infiltrating	8	2	75	
Mass-forming	88	50	81	
<i>Histological differentiation</i>				
Papillary/Well	31	7	67	<0.0001
Moderately	41	21	38	
Poorly	33	26	8	
<i>Vascular invasion</i>				
Negative	43	14	63	0.0001
Positive	62	40	18	
<i>Lymphatic permeation</i>				
Negative	47	12	56	<0.0001
Positive	58	42	22	
<i>Lymph node metastasis</i>				
Negative	72	25	54	<0.0001
Positive	33	29	0	
<i>Stromal expression of laminin gamma 2 chain</i>				
Positive	23	17	0	0.0006
Negative	82	37	44	



**Figure 2** Survival curves of patients with intrahepatic cholangiocarcinoma. (a), Disease-specific survival rate of patients is not correlated with cytoplasmic expression of laminin gamma 2 chain. (b), Disease-specific survival rate of patients with positive stromal expression of laminin gamma 2 chain is significantly higher than that of patients with negative stromal expression.

**Table 5** Multivariate analysis of disease-specific survival

Variable	RR	95% CI	P-value
Tumor size 4 cm $\leq$	2.347	1.265–4.347	0.0067
Histological differentiation <sup>a</sup>	2.3584	1.290–4.310	0.0053
Lymphatic permeation	4.184	1.992–8.771	0.0002
Lymph node metastasis	3.096	1.589–6.024	0.0009

RR: risk ratio; 95% CI: 95% confidence interval.

<sup>a</sup>Papillary, well and moderately vs poorly.

using multivariate analysis. Tumor size ( $P=0.0067$ ), histological differentiation ( $P=0.0053$ ), lymphatic permeation ( $P=0.0002$ ) and lymph node metastasis ( $P=0.0009$ ) were independent prognostic parameters (Table 5). Stromal expression of laminin gamma 2 chain is not an independent prognostic marker.

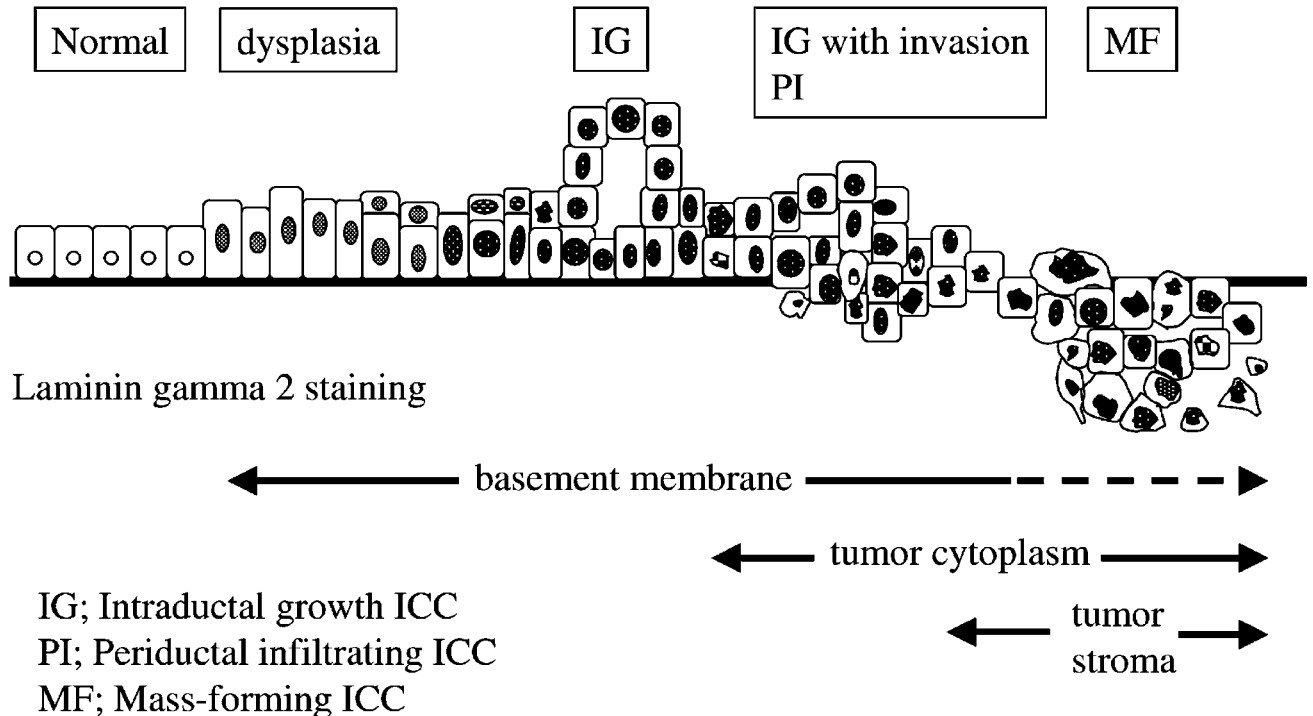
## Discussion

In our study, the *in situ* component of intraductal growth type with stromal invasion showed focal cytoplasmic expression of carcinoma cells, whereas noninvasive intraductal growth types revealed no cytoplasmic expression of carcinoma cells. It has been shown that laminin gamma 2 chain expression in the *in situ* component of intraductal papillary-mucinous tumors (IPMTs) of the pancreas tends to increase with tumor development.<sup>15</sup> The expression of intraductal components in both intraductal growth type of intrahepatic cholangiocarcinoma and IPMT had a similar pattern, which suggests that the cytoplasmic expression of carcinoma cells for laminin gamma 2 chain plays an important role in the progression from noninvasive intraductal tumor to an invasive tumor.

Tumor invasion is the basic indicator for progression from premalignant to malignant tumors. In this study, we considered the biliary dysplasia of hepatolithiasis to be a premalignant lesion. Previous reports noted that human colonic adenomas and various normal epithelial tissues show the basement membranous expression of laminin gamma 2, not cytoplasmic expression of this chain.<sup>2,10</sup> Our findings of dominant expression of laminin gamma 2 chain in basement membrane of biliary dysplastic epithelium and noninvasive intraductal growth type of intrahepatic cholangiocarcinoma suggest that the basement membranous type of laminin gamma 2 chain is associated with the anchorage of epithelial cells to the underlying basement membrane. However, very little has been reported about the laminin gamma 2 expression in premalignant lesions, such as colorectal adenoma and dysplasia, so further investigation into the role of laminin gamma 2 chain in the basement membrane of premalignant lesions is needed. Our data and previous studies also demonstrated that a well-differentiated adenocarcinoma component showed more frequent basement membrane staining than does a poorly differentiated adenocarcinoma.<sup>12,17</sup> Laminin gamma 2 chain in basement membrane may maintain the differentiated phenotype of adenocarcinoma.

Laminin gamma 2 positive expression was observed in the basement membrane of normal gastric epithelia with the anti-laminin gamma 2 antibody, D4B5.<sup>12</sup> In our study, using the same antibody, basement membrane underlying normal biliary epithelia is not stained. Other studies showed that the non-neoplastic tissue, including pancreatic duct, colonic mucosa and mammary duct, fails to demonstrate laminin gamma 2 by immunohistochemical approach and *in situ* hybridization.<sup>8,15,16</sup> Soini *et al*<sup>16</sup> suggested that the concentration of laminin gamma 2 in non-neoplastic duct is so low that it cannot be detected by immunohistochemistry.

In our study, laminin gamma 2 chain appeared to be expressed in the stromal component around the carcinoma cells within tumors. Koshikawa *et al*<sup>12</sup>



**Figure 3** Progression model of intrahepatic cholangiocarcinoma according to the growth morphology and localization of laminin gamma 2 chain. The basement membranous staining continues from premalignant lesions to malignant tumors and decreases in the advanced stage. The cytoplasmic staining of carcinoma cells is correlated with the step of carcinoma invasion to the stroma and the formation of an invasive tumor. The stromal staining appeared in the more advanced stages of tumor.

reported that most laminin gamma 2 chain was accumulated in the cytoplasm of the tumor cells; however, diffuse extracellular staining for the gamma 2 chain was detected in only limited poorly differentiated gastric adenocarcinomas. The authors indicated that a part of the laminin gamma 2 chain may be secreted into the tumor stroma from the carcinoma cells. The hypothesis is explained by other studies showing that extracellular stromal staining of laminin gamma 2 chain was detected by immunohistochemical analysis, but the signal for laminin gamma 2 chain mRNA was not observed in the non-neoplastic stromal cells.<sup>8</sup> Furthermore, matrix metalloproteinase-2 (MMP2) and membrane-type 1 matrix metalloproteinase (MT1-MMP) are considered to be candidates for laminin gamma 2 processing protease.<sup>26,27</sup> Processing of the 155-kD gamma 2 chain involves a cleavage within domain III leading to a 105-kD polypeptide. It appears that the resultant degraded laminin gamma 2 chain is an active form that enhances epithelial cell migration.<sup>28</sup> We used commercially available antibody against laminin gamma 2 chain, which recognizes the 150- and 105-kDa chain proteins. Therefore, the antibody is considered to react to the proteolytically processed form of laminin gamma 2 chain. Katayama *et al*<sup>29</sup> reported that degraded laminin gamma 2

chain after receiving proteolytic processing by MMPs may be released into circulation. According to these results, stromal accumulation of laminin gamma 2 chain in intrahepatic cholangiocarcinomas may result from a secreted and active form of laminin gamma 2 chain, which may be processed proteolytically. Our interesting results that stromal expression of laminin gamma 2 chain is correlated with a poorly differentiated tumor feature, invasive potential such as lymph node metastasis and lymphatic permeation suggest that stromal expression of laminin gamma 2 chain is a potential marker of aggressive behavior of intrahepatic cholangiocarcinomas.

We propose a progression model of intrahepatic cholangiocarcinoma according to the growth morphology and localization of laminin gamma 2 chain (Figure 3). The basement membranous staining continues from premalignant lesions to malignant tumors and decreases in the advanced tumor stage. The cytoplasmic staining of carcinoma cells is correlated with the step of carcinoma invasion to the stroma and the formation of an invasive tumor. The stromal staining appeared in the more advanced stages of tumor. Laminin gamma 2 chain appears to exhibit aberrant expression in a stepwise manner through different aggressive stages of tumor progression.

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