Laminin-5 (γ2 chain) is a marker of invading cancer cells in human gallbladder carcinoma: Special emphasis on extension of carcinoma *in situ* along Rokitansky-Aschoff sinuses

TAKASHI EGUCHI[1,2], TAKAHIRO INOUE[2], KEI FUJII[2], HIROSHI YAMAGUCHI[2], KEN-ICHI NISHIYAMA[1], HIROTOSHI YONEMASU[4], TAKASHI YAO[2], MASAO TANAKA[3] and MASAZUMI TSUNEYOSHI[2]

[1]Kyushu Cancer Center, Departments of [2]Anatomic Pathology, [3]Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, [4]Ooita Red Cross Hospital; 3-1-1 Notame Minami-ku, Fukuoka 811-1395, Japan

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Abstract. Gallbladder carcinoma (GC) is a relatively uncommon malignancy and is often caused by diagnostic difficulties in distinguishing the extension of carcinoma *in situ* (CIS) from invasive carcinoma along Rokitansky-Aschoff sinuses (RAS). The laminin-5, a heterotrimer, is composed of α-3, ß-3 and γ-2 chains. The γ-2 chain is expressed in various invasive carcinoma cells. There are numerous reports that have described that laminin-5-γ-2 is associated with tissue invasion in many organs. However, few studies are found in gallbladder carcinoma. To clarify the relationship between laminin-5-γ-2 chain (LN-5) expression and the development of GCs, we performed an immunohistochemical study of 93 GCs. Cases were classified into three groups: CIS with/without the extension along RAS (Group A; n=17), carcinoma invading mucosa or muscular layer (Group B; n=5) and carcinoma invading beyond perimuscular connective tissue (Group C; n=71). The immunohistochemical intracytoplasmic expression was detected in the invasive fronts of the tumor. In the invasive components of Group B and C, LN-5 was expressed in 100 and 97% of cases, respectively, whereas in the CIS lesions of GCs, expression was not observed in Group A. LN-5 expression in the GCs tended to increase as tumors developed, which may be an indicator of the potential invasiveness of the tumor. Our results indicate that, in GCs, a strong intracytoplasmic expression of LN-5 was identified in the cancer cells whether *in situ* they extend along RAS or invading stroma.

Correspondence to: Dr Takashi Eguchi, Kyushu Cancer Center, 3-1-1 Notame Minami-ku, Fukuoka 811-1395, Japan
E-mail: eguchi.t@hamanomachi.jp

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Introduction

Most malignant epithelial tumors of the gallbladder are differentiated carcinomas, which are not difficult to recognize microscopically. However, when carcinomas of the gallbladder extend into epithelial invaginations known as Rokitansky-Aschoff sinuses (RAS), the surgical pathologists are faced with the dilemma of distinguishing between the extension into RAS from the stromal invasion. This distinction is not only one of academic interest but also has practical therapeutic and prognostic implications. Carcinoma *in situ* (CIS) of the gallbladder is cured by simple cholecystectomy with no recurrence, whereas invasive carcinomas are associated with a poor prognosis. It is important to judge the tumor invasion precisely.

Laminins are a family of basement membrane proteins that are associated with cell differentiation adhesion and migration, as well as being structural components. Laminin variants are composed of one heavy α-chain and lighter ß- and γ-chains, which are linked to each other through an α-helical coiled structure. These chains can form a variety of trimeric isoforms, though relatively little is known about their biological roles. The laminin-5-γ-2 chain (LN-5) is known to be expressed in cells of squamous cell carcinoma and various adenocarcinomas. According to some reports, the LN-5 is found in front-line invasive cancer cells at the epithelial-stromal interface and plays an important role in cancer cell invasion.

The purpose of this study is to analyze a relatively large series of gallbladder carcinomas (GCs) and to investigate the localization of the LN-5 protein in GCs in order to determine its usefulness in distinguishing the stromal invasion from the extension into RAS. The prognostic significance of this distinction is assessed by the clinical follow-up of the patients.

Materials and methods

Materials. Each of the 93 formalin-fixed, paraffin-embedded gallbladder carcinoma specimens registered at the Department

of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, between 1992 and 2002 were available for clinicopathological and immunohistochemical analyses. Of the 93 patients, 59 were women and 34 were men. The median age was 68 years (range 28 to 91 years). None of the patients received neoadjuvant therapy. Histologically, of the 93 tumors, 18 were papillary, 39 were well differentiated, 15 were moderately differentiated, 15 were poorly differentiated adenocarcinomas and 6 were adeno-squamous carcinomas (Table I).

We classified the GCs into three categories morphologically based on hematoxylin and eosin staining: CIS with/without the extention along RAS (Group A; n=17), carcinoma invading lamina propria or muscular layer (Group B; n=5) and carcinoma invading beyond perimuscular connective tissue (Group C; n=71). Morphological criteria for CIS with the extension along RAS is that the surface epithelium was replaced by columnar or cuboidal neoplastic cells similar to those of invasive carcinoma with no obvious stromal invasion. Overall survival was calculated from the date of surgery to the date of death or last follow-up. The median follow-up was 59 months (range 1-113 months).

Laminin-5-γ-2 chain immunohistochemistry. Mouse monoclonal antibody (Chemicon 1:100) was used for our immunohistochemical study. Formalin-fixed, paraffin-embedded sections were subjected to immunohistochemistry by the streptavidin-biotin peroxidase (SAB) method to characterize the tumor cells. Sections were deparaffinized with xylene, treated with 0.3% hydrogen peroxide in methanol, immersed in 10 mM citrate buffer (pH 6.0), heated to 99˚C in a microwave for 10 min and then allowed to cool at room temperature for 20 min. The sections were then preincubated in 2% normal porcine serum in phosphate-buffered saline, incubated with the purified monoclonal antibody at 4˚C overnight, washed with phosphate-buffered saline and incubated for 30 min with biotinylated rabbit anti-mouse immunoglobulin as the second antibody. They were then incubated for 30 min with avidin-biotinyl-peroxidase complex with a Vectastain ABC kit and subjected to the peroxidase reaction. This was followed by nuclear counterstaining with hematoxylin. Between each step, the slides were washed three times with phosphate-buffered saline. The positive control was a section of formalin-fixed, paraffin-embedded tissue of squamous cell carcinoma of the esophagus.

Immunoreactivity was evaluated as follows: Grade 0, no intracytoplasmic-positive cells; Grade 1, 1 to <30% intracytoplasmic-positive cells at the invasive front; and Grade 2, ≥30% intracytoplasmic-positive cells at the invasive front. The association between laminin-5-γ-2 chain expression and primary tumor depth is presented in Table II.

Statistical analyses. Correlations between clinical, immunohistochemical, histopathological variables and outcome during follow-up were analyzed by the Kaplan-Meier survival test and the differences were calculated with the log-rank test. For each variable evaluated, a series of cutoff values were tested. The cutoff that gave the best P-value is presented in the tables and used in the statistical analyses. In each of the calculations, $p<0.05$ was accepted as significant.

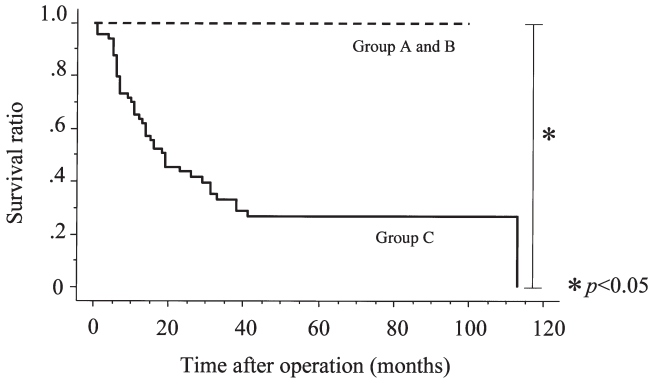

Figure 1. Survival according to primary tumor invasion.

Table I. The association between laminin-5-γ-2 chain expression and histological types.

Histological type	Staining grade 0	1	2	Total
Adenocarcinoma papillary	6	10	2	18
Well differentiated	13	22	4	39
Moderately differentiated	0	6	9	15
Poorly differentiated	0	3	12	15
Adenosquamous carcinoma	0	3	3	6
Total	19	44	30	93

Grade 0, no intracytoplasmic positive cells; Grade 1, 1 to <30% intracytoplasmic positive cells at the invasive front; Grade 2, ≥30% intracytoplasmic positive cells at the invasive front.

Table II. The association between laminin-5-γ-2 chain expression and primary tumor depth.

Group	Staining grade 0	1	2	Total
Group A	17	0	0	17
Group B	0	6	0	5
Group C	2	39	30	71
Total	19	44	30	93

Group A, carcinoma *in situ* with/without the extension along RAS; Group B, carcinoma invading lamina propria or muscular layer and Group C, carcinoma invading beyond perimuscular connective tissue.

Results

In this study, 93 primary GCs were characterized according to clinical, histopathological and immunohistochemical variables and the findings were then evaluated in relation to

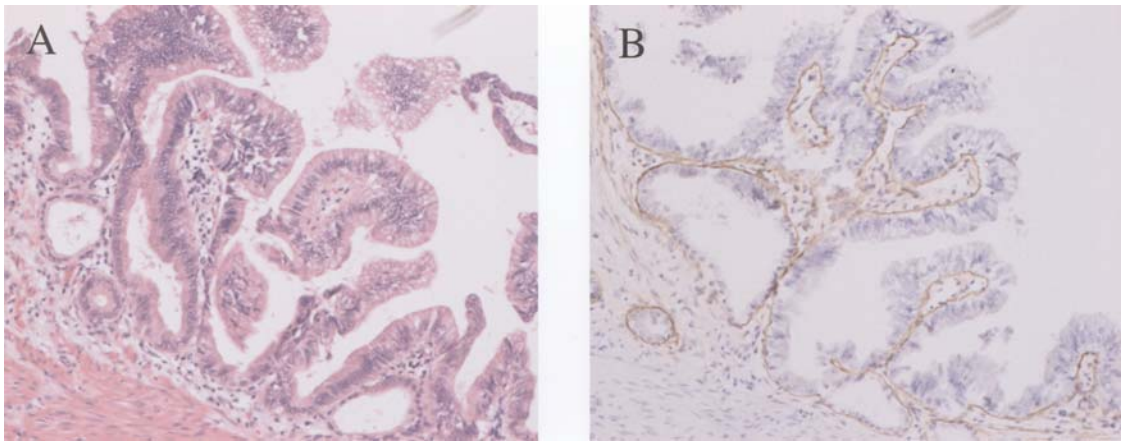

Figure 2. (A) Hematoxylin and eosin staining. (B) Immunohistochemical staining of the laminin-5-γ-2 chain. (A) High magnification of CIS extending along RAS. Papillary adenocarcinoma, the moderately to severe epithelial atypia are observed with no stromal invasion. (B) Grade 0 case. This case is also positive for linear basement membrane staining, though negative for intracytoplasmic expression (original magnification: A, x40; B, x100).

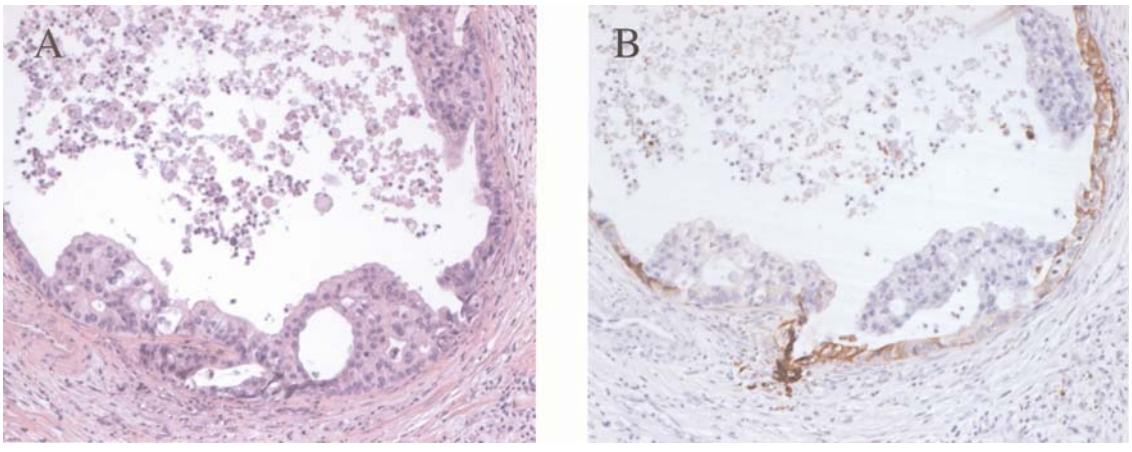

Figure 3. (A) Hematoxylin and eosin staining. (B) Immunohistochemical staining of the lamin-5-γ-2 chain. (A) Well differentiated adenocarcinoma invades the perimuscular connective tissue. (B) Grade 1 case. The hornlike invasive fronts of the tumor have stained positively (original magnification: A and B, x100).

the survival during follow-up. Tumors that invade beyond perimuscular connective tissue (Group C) were significantly correlated with an unfavorable outcome rather than CIS (Group A) and carcinomas invading the mucosa or muscular layer (Group B) (Fig. 1).

No intracytoplasmic positivity of LN-5 was seen in the non-neoplastic epithelial cells. Cases in Group A and B showed linear or focal basement membrane staining for LN-5 beneath the neoplastic epithelium (Figs. 2 and 3). In the hyperplastic and dysplastic epithelium, the linear basement membranes were also stained in certain cases.

In almost all cases of Group C, LN-5 staining was seen in the cytoplasms of the carcinoma cells and tended to be stronger in small tumor glands or tumor diffuse proliferation than in large glands/diffuse infiltration (Figs. 4 and 5). In the tumor nodules, staining was stronger in the carcinoma cells at the periphery than in the central area. The tips of the glandular structure stained strongly. In Group C, we could find only a few positive areas in the two cases.

Association between laminin-5-γ-2 chain expression and prognosis. Immunohistochemical stainings were performed for analyses of LN-5 expression levels. LN-5 expression was evident in 69 (74%) of the tumors and of these 30 (32%) were detected with Grade 2-positive cells.

High LN-5 expression was found to be significantly correlated with an unfavorable outcome (Fig. 6). However, in spite of cancer cells detected in the muscular layer or the perimuscular connective tissue, in six cases, LN-5 expression is negative and the patients are alive for over two years (Table III).

Tumors with positive staining for the LN-5 chain showed a cytoplasmic signal in the invasive front cells. The proportion of positive cells was scored at the 30% interval giving either negative (0% positive cells) or positive tumors (1 to <30% or ≥30% positive cells). When cases with positive and negative LN-5 staining were compared as a whole, a significant difference in outcome was revealed. Furthermore, cases with a high expression (≥30% positive cells) showed

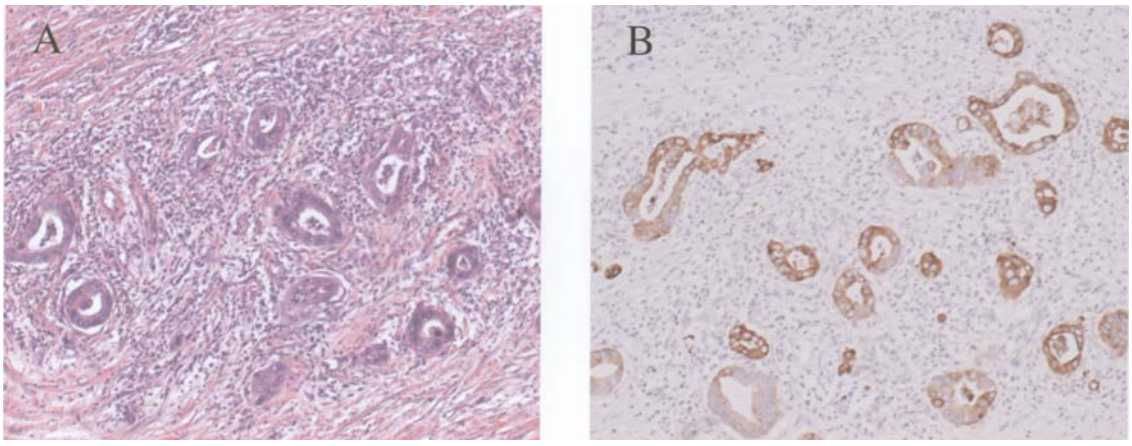

Figure 4. (A) Hematoxylin and eosin staining. (B) Immunohistochemical staining of the laminin-5-γ-2 chain. (A) Well differentiated adenocarcinoma invades the perimuscular connective tissue. (B) Grade 2 case. The small tumor glands of components of invasive carcinoma show strong intracytoplasmic positivity (original magnification: A and B, x100).

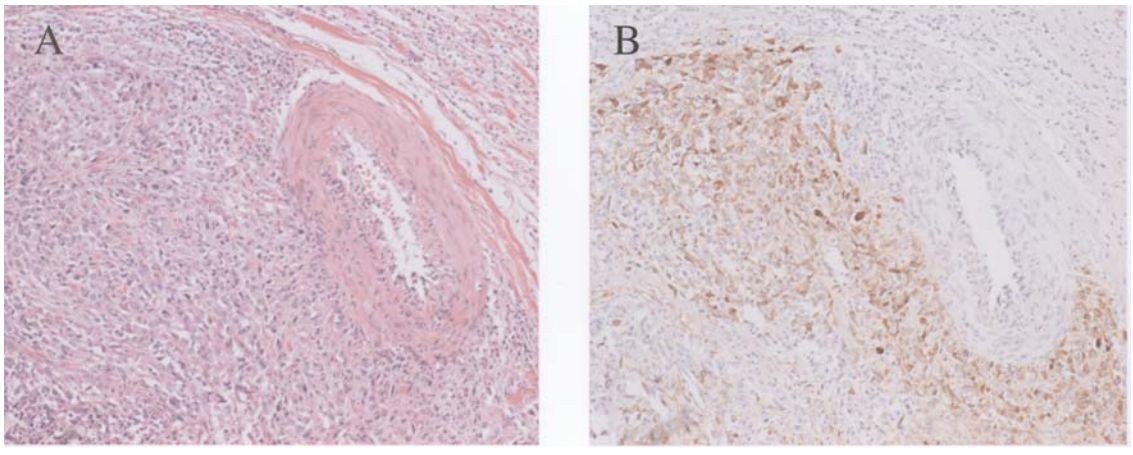

Figure 5. (A) Hematoxylin and eosin staining. (B) Immunohistochemical staining of the laminin-5-γ-2 chain. (A) Poorly differentiated adenocarcinoma invades the perimuscular connective tissue. (B) Grade 2 case. Positive staining is visible, predominantly in the periphery of the tumor nests (original magnification: A and B, x100).

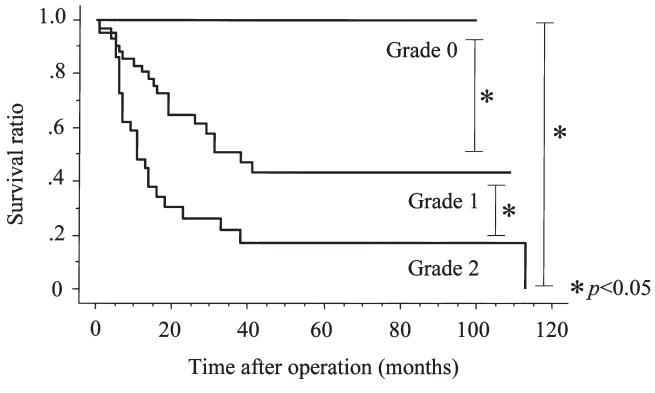

Figure 6. Survival according to laminin-5 expression.

significantly worse survival compared to cases with no or lower expression levels (<30%) of positive cells (Fig. 6).

Association between laminin-5-γ-2 chain expression and histological factors. The histopathological and immunohistochemical variables evaluated in this study were all pairwise compared to the correlation analyses. This revealed that lymphatic permeation, venous invasion, perineural invasion and lymph node metastasis are positively correlated to LN-5 expression levels (Tables IV and V).

Discussion

Gallbladder carcinoma (GC) is a relatively uncommon malignancy and not enough is known about its pathogenesis (1-9). In CIS or invasive carcinoma of gallbladder, the tumor extends replacing the pre-existing normal epithelium. This phenomenon simulates a stromal invasion in the carcinoma and has been described in many organs (10,11). However, it is practically important to separate the above two tumor growth patterns in order to perform an adequate therapy.

Albores-Saavedra *et al* reported that they could separate RAS with CIS from tubular neoplastic invasive glands just

Table III. The negative staining cases for LN-5 in spite of cancer cells in the muscular layer or perimuscular connective tissue.

Age	Sex	Histological type	1	v	p	n	Depth of extension	Prognosis (month)
91	F	Well differentiated	Absent	Absent	Absent	Absent	ml	Alive (36)
78	F	Well differentiated	Absent	Absent	Absent	Absent	ml	Alive (57)
80	M	Papillary	Absent	Absent	Absent	Absent	pct	Alive (27)
70	F	Well differentiated	Absent	Absent	Absent	Absent	pct	Alive (42)
59	M	Well differentiated	Absent	Absent	Absent	Absent	pct	Alive (59)
68	M	Well differentiated	Absent	Absent	Absent	Absent	pct	Alive (100)

1, Lymphatic permeation; v, venous invasion; p, perineural invasion; n, lymph node metastasis; ml, muscular layer pct and perimuscular connective tissue.

Table IV. Association between laminin-5γ-2 chain expression and histological factors.

Laminin-5	n	Histological factors			
		Lymphatic permeation		Venous invasion	
		Absent	Present	Absent	Present
Grade					
0	20	20	0	20	0
1	43	12	31	16	27
2	30	6	24	8	22
Total	93	38	55	44	49

[a]P<0.05; [b]NS, not significant.

Table V. Association between laminin-5-γ-2 chain expression and histological factors.

Laminin-5	n	Histological factors			
		Perineural invasion		Lymph node metastasis	
		Absent	Present	Absent	Present
Grade					
0	20	20	0	8	0
1	43	12	31	14	15
2	30	6	24	4	17
Total	93	38	55	26	32

[a]P<0.05. [b]NS, not significant.

by a morphological feature (12). CIS spreading along RAS consisted of long tubular and often dilated structures extending through the intermuscular connective tissue, whereas neoplastic glands were usually small or of medium size that

invaded smooth muscle bundles or intermuscular connective tissue. However, this would not apply to every surgical pathologist.

In this study, once CIS was recognized on the surface epithelium, we tried to determine whether the long duct-like structures in the tumor represented CIS in RAS or whether these structures were invasive neoplastic glands, based on morphological findings and immunohistochemical studies by LN-5 expression. The latter was considered as useful clues in solving the diagnostic dilemma.

Laminins are a family of basement membrane proteins, which are associated with cell differentiation, adhesion and migration, as well as being structural components (13-15). The LN-5 is known to be expressed in tumor cells of squamous cell carcinoma and various adenocarcinomas, and in organs such as the esophagus, cervix, breast, colon and pancreas (16-23). According to certain studies, the LN-5 is found in the invasive front at the epithelial-stromal interface and plays an important role in cancer cell invasion (24).

In this study, most of the 71 GCs, which invaded beyond the perimuscular connective tissue, contained LN-5-positive cells on immunohistochemistry. Soini *et al* reported that intracytoplasmic positivity for LN-5 was seen in 98% of the pancreatic adenocarcinomas (25). They also found that the tumors forming solid nests or large glandular structures showed less LN-5 positivity than that of the tumors with small invasive glandular structures of scattered individual invasive cells. The expression pattern of invasive carcinoma cells in our study was in accordance with current results and other reports (19,25) and these results suggest that the expression of LN-5 is associated with the interaction between the epithelium and stroma.

High LN-5 expression in GCs, was associated with a poor prognosis as shown by lymphatic permeation, venous invasion, perineural invasion and lymph node metastasis. On the contrary, all cases of Group A, which were diagnosed with CIS, including 7 cases with the extension along RAS, were negative for LN-5 intracytoplasmic staining. These findings indicate non-invasive features in these tumors, which are confirmed by immunohistochemical studies on LN-5. However, two cases of Group C had negative immunoreactivity for LN-5. We determined these tumors as with the invasive growth pattern, since, these neoplastic glands were small or medium sized and lined by columnar cells with considerable nuclear atypia and located in perimuscular connective tissue. It should be emphasized that these atypical glands had no desmoplastic reaction in the surrounding stroma and no obvious tumor budding or sprouting. Furthermore, these patients were alive at 55 and 75 months respectively, so we evaluated these cases as CIS with the extension along RAS, retrospectively.

In the current study, a high LN-5 expression in GCs, was significantly associated with poor prognosis, which was indicated by lymphatic permeation, venous invasion, perineural invasion and lymph node metastasis, whereas, CIS including the extension along RAS, was negative for LN-5 immunoreactivity. In human GCs, we suggest that an expression of LN-5 identifies the cancer cells as *in situ* extending along RAS or invading stroma.

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