Expression of the \( \gamma_2 \) Chain of Laminin-5 at the Invasive Front Is Associated with Recurrence and Poor Prognosis in Human Esophageal Squamous Cell Carcinoma

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ABSTRACT

**Purpose:** Preferential expression of the \( \gamma_2 \) chain of laminin-5 in invading carcinoma cells has been implicated in tumor invasion. The aim of this study was to clarify the clinicopathological and prognostic significance of laminin \( \gamma_2 \) chain expression in esophageal squamous cell carcinoma (SCC).

**Experimental Design:** We analyzed the association between immunohistochemically detected laminin \( \gamma_2 \) chain expression in esophageal SCC and clinicopathological characteristics, and we investigated whether laminin \( \gamma_2 \) chain is a predictor of recurrence and/or survival.

**Results:** The cytoplasm of carcinoma cells was stained for laminin \( \gamma_2 \) at levels much stronger than those in normal esophageal basement membrane. The immunoreactivities at the invasive front were often more intense than those at the superficial layer. Sections with immunostaining signals in the invasive front were often more intense than those at the esophageal basement membrane. The immunoreactivities at the invasive front of tumor cells of the colon, pancreas, stomach (5, 15–19), and SCC of the cervix, vulva, and skin (15). It is notable that the laminin \( \gamma_2 \) chain has been predominantly detected at the invasive front of tumor cells of the colon, pancreas, and stomach (5, 15–19). It has been suggested that the laminin \( \gamma_2 \) chain is a marker for disease progression and malignancy (16).

**Conclusions:** Our results suggest that the laminin \( \gamma_2 \) chain plays a key role in the progression of esophageal carcinoma and that its detection is useful for the prediction of recurrence and poor prognosis.

INTRODUCTION

Laminin-5, also referred to as kalinin, nicein, epiligrin, and ladisin, consists of \( \alpha_3 \), \( \beta_3 \), and \( \gamma_2 \) chains. Laminin-5 is a laminin isoform that is present in the basement membranes of the skin and various epithelial tissues (1–5). Laminin-5 has been reported to be produced by normal keratinocytes, gastric carcinoma cell lines, and SCC cell lines (1–4, 6). In the skin, laminin-5 reportedly stabilizes the epidermal and dermal junction through binding with integrin \( \alpha_6\beta_4 \), forming a hemidesmosome structure (1, 5, 7). Laminin-5 has also been shown to promote the adhesion, migration, and scattering of a variety of cultured cells, mainly through integrin \( \alpha_6\beta_4 \), more strongly than other extracellular matrix proteins (2, 4, 8–11). Moreover, expression of laminin-5 has been shown to be stimulated by growth factors and a tumor promoter in vitro (6, 12). These properties of laminin-5 suggest its possible roles in tumor invasion and metastasis (5).

Expression of the three subunits of laminin-5 is regulated differentially in cancer cell lines and in normal and malignant tissues (6, 13, 14). Indeed, the laminin \( \gamma_2 \) chain has been shown to be secreted as a single subunit in gastric cancer (5). Several lines of evidence suggest that the tumor-derived laminin \( \gamma_2 \) chain contributes to invasion of tumor cells. Laminin \( \gamma_2 \) chain expression has been immunohistochemically detected in human colon adenocarcinoma, malignant melanoma, mammary ductal carcinoma, and SCC of the cervix, vulva, and skin (15). It is notable that the laminin \( \gamma_2 \) chain has been predominantly detected in the invasive front of tumor cells of the colon, pancreas, and stomach (5, 15–19). It has been suggested that laminin \( \gamma_2 \) chains are secreted by tumor cells to invasion of the cells by stimulating the cell motility activity of laminin-5 or by altering the extracellular location of laminin-5 (5). Preferential expression of laminin \( \gamma_2 \) chain-positive cells at the invasive front, where tumor cells with the most aggressive phenotype are localized (20), indicates the possibility that this molecule could be used as a marker for disease progression and malignancy (16).

Expression of the laminin \( \gamma_2 \) chain has been analyzed in a variety of cancers but not in esophageal SCC. Esophageal SCC is one of the most aggressive malignant tumors, and the prognosis of esophageal SCC is worse than that of other digestive tract cancers. Five-year overall survival after potentially curative surgical resection is still very low because of the high rate of recurrence and metastasis.

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[3] The abbreviations used are: SCC, squamous cell carcinoma; pTNM, pathological tumor-node-metastasis; MMP, matrix metalloproteinase.
Because the laminin γ2 chain has frequently been detected in SCC tissues of the cervix, vulva, and skin (16), it seems significant to examine laminin γ2 chain expression in esophageal SCC tissues. To clarify the possible involvement of the laminin γ2 chain in the progression of esophageal SCC, we immunohistochemically analyzed laminin γ2 chain expression in 100 primary esophageal SCC tissues.

**PATIENTS AND METHODS**

**Patients and Tissue Samples.** Paraffin-embedded tumor specimens from 100 Japanese patients with esophageal SCC were used for immunohistochemical analysis. All patients had undergone curative surgical resection at Keiyukai Sapporo Hospital. Patients who died of causes other than carcinoma were not included in the study. The clinicopathological characteristics...
were evaluated according to the guidelines of the Union Internationale Contre le Cancer. Informed consent in writing was obtained from each patient, and the experiments were approved by the institutional review committee.

Immunohistochemistry. Sections of 5 μm in thickness were dewaxed in xylene and rehydrated in alcohol and then heated to 105°C in an autoclave for 10 min. The endogenous peroxidase activity was suppressed by a solution of 3% hydrogen peroxide in methanol for 5 min. After being rinsed twice in PBS, the sections were treated for 18 h with an antihuman laminin γ2 monoclonal antibody (Chemicon, Temecula, CA) at the concentration of 10 μg/ml. The characteristics of this antibody have been previously described (22). An anti-idiotypic monoclonal antibody AI-206 was used as a negative control. After washing three times in PBS, the sections were treated with biotinylated goat antimouse immunoglobulin (DAKO, Glostrup, Denmark) for 10 min and then by horseradish peroxidase-avidin complex, diluted as recommended by the manufacturer, for 10 min. The slides were then washed in PBS and developed in 0.05 M Tris-HCl (pH 7.5) containing 0.6 mg/ml 3,3′-diaminobenzidine at room temperature. The sections were counterstained in Mayer’s hematoxylin and mounted. Immunostaining signals at the invasive front were scored in two sections each by two independent observers. The scores were calculated as the number of stained cells divided by the total number of carcinoma cells according to the previous report (23). Sections with immunostaining signals in >30% of carcinoma cells at the invasive front were judged to be positive for laminin γ2 chain.

Statistical Analysis. Laminin γ2 chain expression was assessed for associations with clinicopathological parameters using the following statistical tests: Student’s t test for age, the Mann-Whitney test for the depth of invasion and pTNM stage, and the χ² two-tailed test or Fisher’s exact test for the remaining parameters. Probability distributions of disease-free and overall survival were calculated using the Kaplan-Meier method and compared by the log-rank test. Cox model was used for univariate and multivariate analyses. P < 0.05 was judged to be statistically significant.

RESULTS

In the normal esophageal tissues, immunoreactivity to laminin γ2 was observed in basement membrane (22). There was no detectable immunoreactivity with the control AI-206 antibody (data not shown). In the carcinoma tissues, the cytoplasm of carcinoma cells was stained for laminin γ2 at levels much stronger than those in normal basement membranes. In addition, extracellular staining in a basement membrane-like pattern surrounding cancer cells was observed in some specimens. The cytoplasmic immunoreactivities at the invasive front were often more intense than those at the superficial layer (Fig. 1A–C). Cancer cells budding or dissociating from the tumor nests showed intense cytoplasmic staining (Fig. 1E). Sections with immunostaining signals in >30% of carcinoma cells at the invasive front, which were observed in 44 (44%) cases, were judged to be positive for laminin γ2 chain expression. The relationship between laminin γ2 chain positivity and clinicopathological characteristics is summarized in Table 1. Laminin γ2 chain positivity was significantly correlated with depth of invasion (P = 0.0022), lymph node metastasis (P = 0.0090), distant metastasis (P = 0.0163), advanced pTNM stage (P = 0.0022), recurrence (P < 0.00001), and recurrence within the first postoperative year (P = 0.0014). On the other hand, there were no significant relationships between laminin γ2 chain

<table>
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<th>Association of laminin γ2 chain expression with clinicopathological characteristics</th>
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<td>Distant metastasis (pM)</td>
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<td>pTNM stage</td>
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positivity and age, gender, or tumor differentiation. (Table 1 and data not shown).

Patients whose tumors were laminin γ2 chain positive in immunohistochemistry had a significantly shorter disease-free and overall survival time than did those with laminin γ2 chain-negative tumors ($P = 0.0001$ and $P < 0.0001$, respectively; Fig. 2). In univariate analysis, significant prognostic variables for predicting both disease-free and overall survival were laminin γ2 chain expression, depth of invasion, lymph node metastasis, distant metastasis, and pTNM stage (Table 2). In multivariate analysis of these variables, only the laminin γ2 chain retained its significant predictive value for disease-free and overall survival (Table 2).

**DISCUSSION**

In the current study, laminin γ2 chain positivity in carcinoma cells at the invasive front was immunohistochemically seen in 44% of patients with esophageal SCC, being associated with depth of invasion, lymph node metastasis, distant metastasis, and advanced pTNM stage. These results suggest that laminin γ2 chain expression in carcinoma cells at the invasive front contributes to the more aggressive phenotype of carcinoma cells, resulting in the progression of esophageal SCC.

Preferential expression of the laminin γ2 chain in carcinoma cells at the invasive front and its correlation with tumor progression suggest that this molecule plays a role in the acquisition of a migrating and invading epithelial cell phenotype that is a prerequisite for malignancy (16). Predominant expression of the laminin γ2 chain at the invasive front has been reported in carcinomas of the colon, pancreas, and stomach (5, 15–19). Our results extend these observations to esophageal SCC. The mechanism underlying the preferential distribution of laminin γ2 chains at the invasive front in cancer is not known. It is known that activation of cancer-related genes in carcinoma cells affects their associated stromal cells (16). Certain stromal cell populations lying close to carcinoma cells may be induced to assist the invasion process by signals sent out by the cancer cells, stimulating the synthesis of gene products that facilitate cancer cell invasion and migration (24). Interactions of carcinoma cells with stromal cells or with the surrounding extracellular matrix at the invasive front may result in an accumulation of laminin γ2 chains at the invasive front, where they may play a direct role in tumor invasion processes (25).

The implication of laminin γ2 chain positivity at the invasive front was further substantiated by its correlation with disease recurrence and shorter disease-free and overall survival time. Moreover, only the laminin γ2 chain retained its significant predictive value for disease-free and overall survival in multivariate analysis that included conventional clinicopathological factors. These results suggest that laminin γ2 chain expression is a powerful predictor of recurrence and poor prognosis with a significance equaling or surpassing that of other conventional clinicopathological factors.

Identification of the laminin γ2 chain as a molecular marker that is correlated with disease recurrence and poor prognosis would provide new insights into disease management by making it possible to define a high risk of recurrence, thus providing a more accurate estimation of the prognosis of patients with esophageal SCC. Consequently, early postoperative screening and/or intense postoperative therapy should be performed on patients with laminin γ2 chain-positive carcinoma. Immunohistochemical analysis is a technique that is available in daily clinical practice and, therefore, analysis of laminin γ2 chain expression could be an important routine part of the management of patients with esophageal SCC. The diagnostic strategy shown in this study and advances in therapeutic approaches should improve the prognosis of patients with esophageal SCC.

Cytoplasmic accumulation of the laminin γ2 chains in invading cancer cells has been reported in a variety of human malignancies, including adenocarcinomas of colon and stomach and SCCs of the cervix, vulva, and skin (5, 15–19). It is known that laminin-5 in the basement membranes functions through binding with integrins such as α6β4 and α6β4. In contrast, roles of cytoplasmic laminin γ2 chain in tumor invasion are unclear and several possibilities can be considered (5). First, the cytoplasmic accumulation of the laminin γ2 chain may disturb the formation of other laminin species, resulting in the enhancement of tumor invasion. It has been suggested that in the absence of the α4 and β4 chains, the laminin γ2 chain is accumulated intracellularly in cancer cells, but a part of the overexpressed γ2 chain is secreted. Secreted laminin γ2 monomer or its proteolytic fragments may exhibit biological activities that promote tumor cell invasion. Because the amino-terminal region of the laminin γ2 chain contains binding sites with fibulin-2 and type VII collagen, the secreted laminin γ2 chain monomer may alter the extracellular location and interaction of laminin-5 with target cells. Further work is needed to clarify the mechanism and to provide evidence of a causative role of laminin γ2 chain in invasive potential of cancer cells. With the attention to the
reactivity of antibodies and sensitivity of immunodetection, additional immunohistochemical analyses using a panel of antibodies against the three chains of laminin-5 are also warranted. It has been suggested that the controlled up-regulation of gene products is one of the characteristics of invading cancer cells and that these gene products have functions crucial for the invasive phenotype of cancer cells (16). We have recently reported that expression of the MMP matrixin at the invasive front is associated with recurrence and poor prognosis in esophageal SCC (26). It is notable that limited proteolysis of the laminin γ3 chain by MMP-2 increases the cell motility activity of laminin-5 (25). However, expression of MMP-2 has hardly been detected in esophageal SCCs (data not shown; Ref. 27). In this regard, it would be intriguing to analyze whether the laminin γ3 chain is cleaved by matrixin.

REFERENCES

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