Kaohsiung Journal of Medical Sciences (2013) **29**, 422–429

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ORIGINAL ARTICLE

Effect of laminin 332 on motility and invasion in bladder cancer

Sung-Gu Kang [a], Young-Ran Ha [a], Young-Hwii Ko [a], Seok-Ho Kang [a], Kwan-Joong Joo [b], Hyun-Yee Cho [c], Hong-Seok Park [a], Chul-Hwan Kim [d], Soon-Young Kwon [e], Je-Jong Kim [a], Jun Cheon [a], Jeong-Gu Lee [a,*]

[a] *Department of Urology, Korea University School of Medicine, Seoul, South Korea*
[b] *Department of Urology, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, South Korea*
[c] *Department of Pathology, Gil Hospital, Gachon University College of Medicine, Incheon, South Korea*
[d] *Department of Pathology, Korea University School of Medicine, Seoul, South Korea*
[e] *Department of Otorhinolaryngology—Head and Neck Surgery, Korea University School of Medicine, Seoul, South Korea*

Received 28 November 2011; accepted 28 December 2011
Available online 14 February 2013

KEYWORDS
Invasion;
Laminin 332;
Prognosis;
Urothelial cell carcinoma

Abstract We examined the correlation between laminin 332 and malignancy in bladder cancer patients, and, using a strain of invasive bladder cancer cells, determined whether laminin 332 causes bladder cancer motility and invasion. To investigate the correlation between laminin 332 g2 distribution and patient outcome, we performed a semiquantitative immunohistochemical analysis of 35 paraffin-embedded samples using the antibody D4B5, which is specific for the laminin 5 γ2 chain. To evaluate the role of laminin 332 in NBT-II cell motility and invasion, we used a scratch assay and the Boyden chamber chemoinvasion system. Tumor stage and grade were significantly correlated with a loss of laminin 332 γ2 chain from the basement membrane ($p = 0.001$) and its retention in the cytoplasm ($p = 0.001$) (Kruskal–Wallis test). Kaplan–Meier survival curves revealed an association between the risk of progression and cytoplasmic retention of the laminin 332 γ2 chain. In addition, an *in vitro* scratch assay showed an increase in the migration of cells treated with laminin 332 from their cluster. The Boyden chamber assay showed that laminin 332 potentiated NBT-II cell invasion. Immunohistochemistry results showed that bladder cancer patients with a higher malignancy

* Corresponding author. Department of Urology, Korea University Hospital, 126-1, Anam-dong 5-ga, Sungbuk-gu, Seoul 136-705, South Korea.
E-mail address: kkangsung@naver.com (J.-G. Lee).

http://dx.doi.org/10.1016/j.kjms.2012.12.003

expressed more laminin 332. The *in vitro* scratch and invasion assay showed that laminin 332 stimulated the motility and invasion of bladder cancer cells. The invasion assay explains the correlation between laminin 332 expression and bladder cancer malignancy.

Introduction

To invade the surrounding tissue, tumor cells must dissolve cell—cell adhesions and transform into more migratory, invasive cells. These changes require various signaling pathways and molecular mechanisms. In particular, the interaction between cancer cells and the extracellular matrix is essential for invasion and metastasis [1]. Laminins are basement membrane (BM) proteins that influence cell proliferation, differentiation, adhesion, migration, and gene expression, while maintaining epithelial cell polarity and organization [2—5]. Specifically, laminin 332 is a component of the epithelial cell adhesion complex in hemidesmosomes, anchoring fibrils, and anchoring fibers [6].

Laminin 332, which consists of α3, β3, and γ2 chains, is a major component of the BM of transitional and stratified squamous epithelia, lung mucosa, and other epithelial glands [7,8]. In normal tissue, laminin 332 maintains tissue integrity, but in tumor cells, it promotes migration and dissemination [9]. Altered laminin 332 expression has been reported in squamous cell carcinoma, colorectal carcinoma, breast carcinoma, prostate carcinoma, pancreatic adenocarcinoma, and invasive cervical lesions [10—17]. Only a few articles, however, have studied laminin expression in patients with bladder cancer, and none of them have investigated whether laminin 332 expression affects bladder cancer migration [18,19]. Previously, Hindermann et al. [18] showed that the bladder cancer stage and differentiation clearly correlated with laminin γ2 expression. However, they could not determine whether laminin contributed to cancer invasion through abnormal hemidesmosomes or a reorganized extracellular matrix, and there is almost no other research on bladder cancer and laminin.

We used human tissue to examine a correlation between laminin 332 and the clinicopathology of bladder cancer. We then attempted to demonstrate that laminin affects the motility or invasion of bladder cancer cells, as it does in other cancers. As far as we know, this is the first study that investigates the effects of laminin 332 on clinicopathologic parameters and *in vitro* invasion assays in bladder cancer.

In this study, we investigated the relationship between laminin 332 γ2, a marker of laminin 332, and tumor grade or stage, as well as patient outcome. Then, we investigated whether laminin 332 affected the motility and invasion of urothelial carcinoma cells *in vitro* to determine how laminin 332 contributes to bladder cancer invasion.

Material and methods

Patients and tumors

Thirty-five specimens of human urinary bladder were included. The specimens were surgically resected at the Department of Urology, School of Medicine, Korea University, from 2005 to 2009. The study was approved by the Institutional Review Board of Korea University Hospital, and informed consent was obtained from the participants. The surgical specimens included 13 muscle-invasive (pT2—pT4) and 22 nonmuscle-invasive (pTa—pT1) carcinomas. Nine patients had a recurrence, six had a progression, and three died of urothelial carcinoma during the follow-up period. Resected tissues were fixed in 10% formalin and embedded in paraffin. Histological type and grade, and disease stage were based on both the WHO classification criteria [20] and the TNM system. Clinical and pathological staging were done using 2002 TNM (tumor, lymph node, and metastasis) classification.

Immunohistochemistry

To detect laminin 332 γ2, tissue sections were deparaffinized with xylene, incubated with 0.3% hydrogen peroxide in methanol, and treated with protease XXIV (Sigma, St Louis, MO, USA) for 15 minutes at room temperature. Sections were then processed using a Cap-Plus Detection Kit (Invitrogen, Carlsbad, CA, USA), treated with a blocking solution for 20 minutes at room temperature, incubated with monoclonal laminin 332 γ2 antibody D4B5 (1:200) (Chemicon, Hampshire, UK) overnight at 4°C, and subsequently washed with Tris-buffered saline. Sections were incubated with secondary antibodies and streptavidin conjugated to horseradish peroxidase for 25 minutes, prior to incubating with 3,3-diaminobenzidine as a substrate/chromogen for 3 minutes and staining with hematoxylin as a nuclear counterstain for 2 minutes.

Evaluation of laminin 332 γ2 chain deposition

Immunoreactivity in tissues was independently judged by two pathologists (CHK and HYC) who were blind to the clinical data and other immunohistochemistry results. We evaluated laminin 332 γ2 in the BM, stromal deposition, and cytoplasmic retention. The BM staining by D4B5 was semiquantitatively scored using the following scale: 1, continuous linear staining (no BM loss); 2, loss of staining in less than 50% of the tumor—stroma interface (partial BM loss); and 3, loss of staining in more than 50% of the tumor—stroma interface (complete BM loss). Stromal deposition of laminin 332 γ2 was scored as follows: 1, no deposition; 2, focal deposition; and 3, diffuse deposition. Cellular retention of laminin 332 γ2 was scored as follows: 1, no retention; 2, retention in some tumor cells; and 3, retention in most tumor cells.

Cell lines and cell culture

A rat bladder carcinoma cell line, NBT-II, was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). NBT-II cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (complete medium) in a humidified atmosphere of 5% CO_2 at 37°C.

In vitro scratch assay

For the scratch assay, 1×10^5 NBT-II cells were seeded in 500 μL DMEM with 10% FBS in 24-well tissue culture plates. After 24 hours, confluent NBT-II monolayers were scratched with a p200 plastic pipette tip to create a cell-free zone. Scratched cells were washed with PBS, and new DMEM with 0.5% FBS was added to each well. To observe the effect of laminin 332 on motility, purified rat laminin 332 (Millipore Corporation, Billerica, MA, USA) 1 μg/mL was added to each well. After 24 hours, micrographs were taken (CKX31SF; Olympus, Tokyo, Japan).

Cell invasion assay

To analyze cell invasion, a Bodyden chamber invasion assay was used. Cell culture inserts (BD Falcon, 8 μm pore, transparent polyethylene terephthalate (PET) track-etched membrane; BD Biosciences, Heidelberg, Germany) were coated with 50 μL growth factor-reduced Matrigel (BD Matrigel Basement Membrane Matrix; diluted 1:50 in DMEM) and incubated overnight at 37°C in 5% CO_2. NBT-II cells were suspended in culture medium at a concentration of 5×10^4 cells/mL in 24-well chambers. Laminin (0, 0.1, or 1 μg/mL) was added to the BD Falcon TC Companion plate as a chemoattractant and incubated in a humidified tissue culture incubator at 37°C in 5% CO_2. After 48 hours, non-invading cells were removed from the upper surface of the membrane. A cotton-tipped swab was inserted into the BD BioCoat Matrigel and moved gently and firmly over the membrane surface. Transwells were removed from the 24-well plates and stained with Hemacolor (Merck, Darmstadt, Germany) solution. The number of invasive cells was counted by light microscopy (CKX31SF; Olympus, Tokyo, Japan). Experiments were performed six times.

Statistical analysis

Invasion assay results were analyzed by the Kruskal–Wallis test with Bonferroni corrections for multiple tests. Associations between biomarkers and other clinicopathologic parameters were evaluated using the Mann–Whitney *U* and Kruskal–Wallis tests. Survival curves were constructed using the Kaplan–Meier method and compared using the log-rank test. The Cox proportional hazards regression model was used for a stepwise multivariate survival analysis (SPSS statistical software). A p value of <0.05 was considered statistically significant.

Results

Patient characteristics

A total of 35 patients, 32 males and three females, were studied. Their mean age was 68.6 years and the mean follow-up period was 24.5 months. Fifteen patients (42.9%) had low-grade cancer and 20 (57.1%) had high-grade cancer, with 11 patients in Ta (31.4%), 11 in T1 (31.4%), 10 in T2 (28.6%), and three in T3 (8.6%). Table 1 shows tumor stage and grade distributions. Of the 35 patients, nine (25.7%) developed a recurrence and six (17.1%) developed a progression to muscle-invasive disease. Three patients died during the 24-month follow-up period.

Relationship between the laminin 332 γ2 and pathologic characteristics

The semiquantitative results of evaluating laminin 332 γ2 distribution in patients with low- and high-grade cancer are provided in Table 2. Of 35 patients, 13 did not lose laminin 332 γ2 in the BM region (Fig. 1A), while the remaining 22 lost it in the BM region. Seventeen patients had a partial loss of laminin 332 γ2 (2 low-grade and 15 high-grade), and five had a complete loss (2 low-grade and 3 high-grade). Laminin 332 γ2 was retained in the cytoplasm of 21 tumors (3 low-grade and 18 high-grade) (Fig. 1B). Laminin 332 γ2 chain was deposited in the stroma of nine tumors, with focal deposition in seven tumors (2 low-grade and 5 high-grade) and diffuse deposition in two tumors (both high-grade) (Fig. 1C). Loss of laminin 332 γ2 from the BM and its cytoplasmic retention differed significantly between low- and high-grade tumors ($p = 0.002$ and 0.001, respectively), but stromal deposition did not ($p = 0.132$) (Table 3, Mann–Whitney test).

A semiquantitative evaluation of the laminin 332 γ2 distribution according to tumor stage is presented in Table 2. Seven patients with T1, nine with T2, and one with T3 had a partial loss of laminin 332 γ2 in the BM, while four with T1 and one with T3 had a complete loss. One patient with Ta, eight with T1, nine with T2, and three with T3 had cytoplasmic retention of laminin 332 γ2 in some tumors. Two patients with Ta, two with T1, and three with T2 had focal stromal deposition, while one with T2 and one with T3 had diffuse deposition. There were significant differences among patients with different tumor stages in loss from the BM ($p = 0.001$) and cytoplasmic retention of laminin 332 γ2 ($p = 0.001$), but not stromal deposition ($p = 0.536$) (Table 3, Kruskal–Wallis test).

Table 1 Tumor stage and grade of the 35 urothelial carcinomas used in this study.

	Stage				Total
	Ta	T1	T2	T3	
Grade					
Low	11	3	1	0	15 (42.9%)
High	0	8	9	3	20 (57.1%)
Total	11 (31.4%)	11 (31.4%)	10 (28.6%)	3 (8.6%)	35 (100%)

Table 2 Semiquantitative evaluation of the laminin 332 γ2 chain distribution in BM, stromal deposition, and cytoplasmic retention according to tumor grade and stage.

	Loss of laminin from the BM			Cytoplasmic retention		Stromal deposition			Total
	No	Partial	Complete	No	In some tumors	No	Focal	Diffuse	
Stage									
Ta	11	0	0	10	1	9	2	0	11
T1	0	7	4	3	8	9	2	0	11
T2	1	9	0	1	9	6	3	1	10
T3	1	1	1	0	3	2	0	1	3
Grade									
Low	11	2	2	12	3	13	2	0	15
High	2	15	3	2	18	13	5	2	20
Total	13	17	5	14	21	26	7	2	35

BM = basement membrane.

Association of laminin 332 γ2 chain distribution with disease progression and recurrence

During the follow-up period (mean 24.5 months), nine of 35 patients (25.7%) developed a recurrence and six (17.1%) had a progression to muscle-invasive disease. The mean interval to progression was 15.4 months. Overall, there were significant differences in focal loss and cytoplasmic retention of laminin 332 γ2 between low- and high-grade tumors, as well as between tumor stages (Mann–Whitney and Kruskal–Wallis tests). Kaplan–Meier survival curves and a univariate analysis using the log-rank test showed an increased risk of progression associated with increased cytoplasmic retention of laminin 332 γ2 (Fig. 2). Although a multivariate analysis using the Cox proportional hazard regression model showed no independent prognostic parameters, cytoplasmic retention of laminin 332 γ2 was the most important parameter for predicting prognosis in our study ($p = 0.18$).

In vitro scratch and Boyden chamber invasion assay

In the *in vitro* scratch assay, cells had migrated from their cluster of origin after 24 hours. Fig. 3 shows few migrating

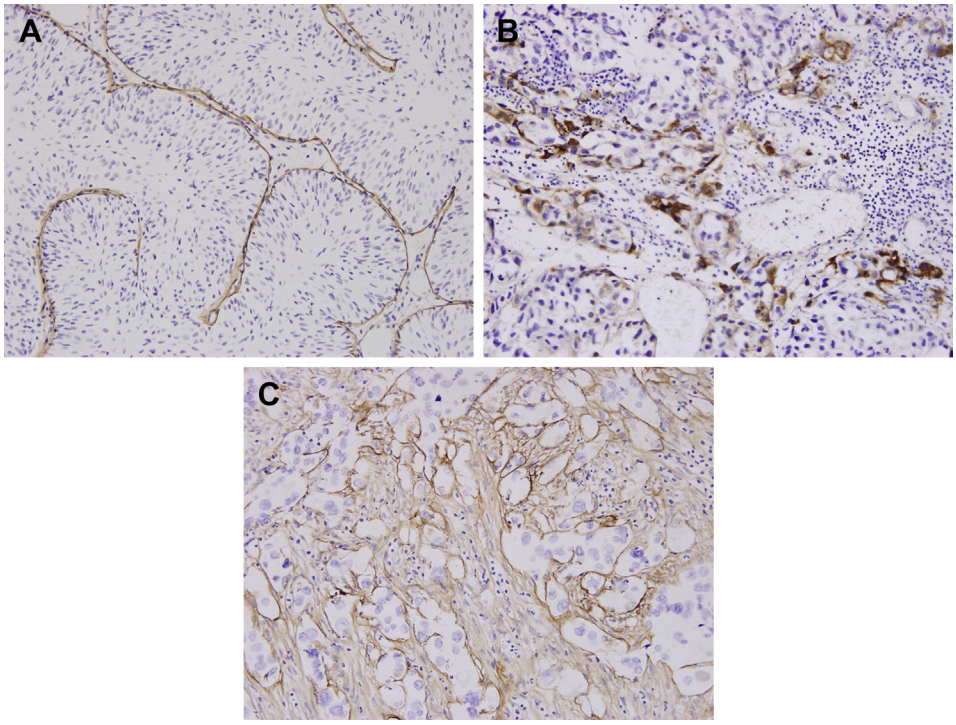

Figure 1. (A) Papillary low-grade carcinoma with no BM loss, no cytoplasmic retention, and no stromal deposition (laminin γ2 chain immunostained, 200×). (B) Invasive high-grade carcinoma with partial BM loss, cytoplasmic retention in some tumors, and no stromal deposition (laminin γ2 chain immunostained, 200×). (C) Invasive, high-grade urothelial carcinoma with partial BM loss, no cytoplasmic retention, and diffuse stromal deposition (laminin γ2 chain immunostained, 200×). BM = basement membrane.

Table 3 Statistical relationship of the semiquantitative evaluation of the laminin 332 $\gamma 2$ chain distribution according to grade and stage (p values).

Laminin	Mann—Whitney test	Kruskal—Wallis test	Log-rank test
	Grade	Stage	
Loss from the basement membrane	0.002	0.001	0.108
Cytoplasmic retention	0.001	0.001	0.022
Stromal deposition	0.132	0.536	0.884

cells in the control group, and significantly more cells migrating from the cluster in the laminin 332-treated group (Fig. 3).

Using a Boyden chamber invasion assay, we investigated whether laminin 332 promoted NBT-II cell invasion. Laminin 332 added to the lower compartment of the Boyden chambers increased the number of cells that migrated into the lower compartment in a concentration-dependent manner. This was repeated six times, and the mean number of invading cells, from six independent experiments, significantly depended on the laminin concentration (Kruskal—Wallis test, $p < 0.001$) (Fig. 4). Fig. 5 shows laminin-untreated and laminin-treated NBT-II cells invading the Matrigel (Fig. 5A and B).

Discussion

Laminin 332 has two opposing activities, maintaining tissue integrity and promoting cell motility, in the extracellular matrix [9]. Laminin expression depends on the tissue, but in general many cancers upregulate laminin 332 and it is found at the migrating edge of tumor cells [21]. Although studies are available on laminin 332 in a variety of tumors, such as squamous cell carcinoma, breast cancer, melanoma, and colorectal cancer, few reports have examined laminin 332 in bladder cancer. Although many techniques have been used to assess laminin 332 in cancer specimens, immunohistochemistry is one of the most common techniques [22]. By immunohistochemistry, normal urothelial expression of laminin 332 $\gamma 2$ is restricted to the BM region in a continuous pattern. However, there are three tumor-specific laminin 332 $\gamma 2$ staining patterns: laminin 332 $\gamma 2$ is lost from the BM, deposited in the stroma, and retained in the cytoplasm. These patterns have also been described in other carcinomas [7]. To our knowledge, only two studies have reported on the relationship between the laminin 332 $\gamma 2$ and the clinicopathologic parameters of urinary bladder cancer [18,19]. In our study, we showed that cytoplasmic expression of laminin 332 $\gamma 2$ was related to tumor invasiveness, by Kruskal—Wallis tests, and to increased risk of progression, by Kaplan—Meier curves.

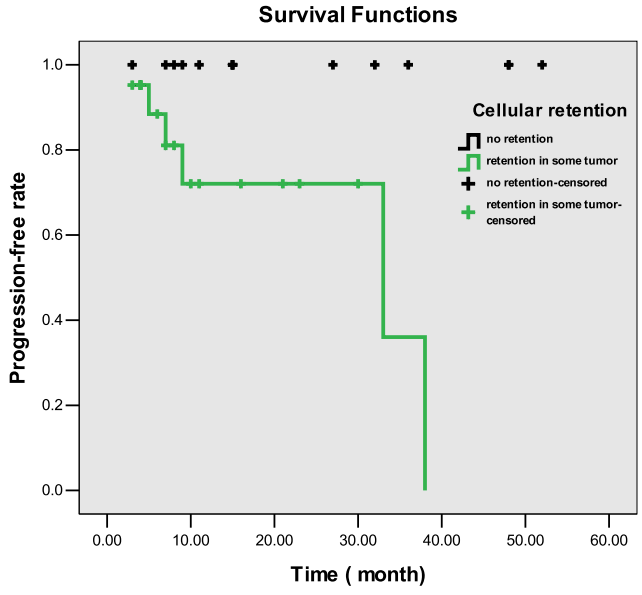

Figure 2. Kaplan—Meier survival curves and univariate analysis using the log-rank test showed an increased risk of progression associated with increased cellular retention of laminin 332 $\gamma 2$ ($p = 0.004$).

This study confirmed a meaningful association between laminin 332 $\gamma 2$ and tumor staging or differentiation. Although results were univariate, there was an association between cytoplasmic retention and time to progression. A previous study found that laminin 332 expression appeared to be associated with the time to recurrence, but could not clearly explain this result [19]. One possible explanation may be that laminin 332 subunits have differential functions, but there is no evidence to support this conclusion. In another study, a univariate analysis showed that laminin 332 expression correlated with survival, but multivariate analysis did not produce any statistically significant results [18]. Although a Cox proportional analysis did not provide any meaningful conclusions, intracytoplasmic retention was indicated as the most important factor. The study found *de novo* synthesis and accumulation of laminin 332 in aggressive tumors by western blot, but could not provide a mechanistic link between laminin 332 and tumor aggressiveness. The two possible explanations provided were that laminin 332 caused abnormal hemidesmosomes and that it contributed to tumor invasion through reorganization of the extracellular matrix. However, these were not supported by experimental evidence. There are no obvious conclusions regarding bladder cancer and laminin 332, but this and previous studies have consistently shown a clear correlation between tumor stage, differentiation, and laminin expression. Also, a correlation between laminin 332 and time to progression seems more reasonable than that between laminin 332 and recurrence, since laminin 332 causes invasion, which leads to progression. Much to our regret, this relationship was not statistically significant. Future research involving more patients may generate statistically meaningful outcomes.

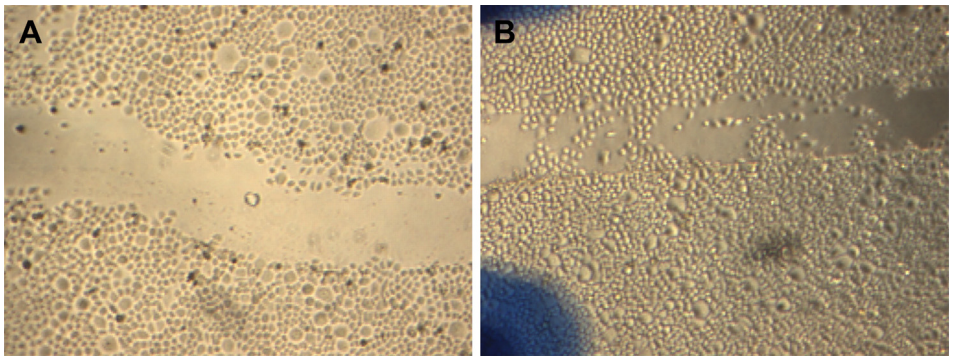

Figure 3. *In vitro* scratch assay. (A) Control group after 24 hours and (B) laminin 332-treated group after 24 hours.

Laminin 332 forms a rod-like structure with truncated short arms and lacks some of the domains present in other laminins [2,5]. Laminin 332 heterotrimers comprise $\alpha 3$, $\beta 3$, and $\gamma 2$ chains, with the $\gamma 2$ chain being unique to laminin 332 [22]. Therefore, the $\gamma 2$ chain is frequently used as a marker. We used the $\gamma 2$ chain to examine the correlation between laminin 332 and cancer malignancy. The $\gamma 2$ chain exists as monomers, $\gamma 2/\beta 3$ as heterodimers, and laminin 332 as heterotrimers. Differentiated gastric carcinoma cells that form glandular structures express laminin $\alpha 3$, $\beta 3$, and $\gamma 2$. However, the $\gamma 2$ chain typically accumulated in the cytoplasm of invading or budding tumor cells, without laminin $\alpha 3$ or $\beta 3$ [23]; thus, the $\gamma 2$ chain may be important for tumor invasiveness. A recent study of the $\gamma 2$ monomeric form also found that the $\gamma 2$ chain itself may affect cancer motility or invasion. Of the laminin 332 subunits, $\gamma 2$ appears to play the largest role in tumor motility and invasion [24]. Still, there are contrasting results and this conclusion requires additional study. Laminin 332 $\gamma 2$ is thought to affect tumor motility in a heterotrimer or a monomer. No matter what the contribution of the $\gamma 2$ chain, there is a clear correlation between $\gamma 2$ immunofluorescence and tumor aggressiveness. As seen in this experiment, aggressive cancers with a higher stage or worse differentiation had clearly increased laminin 332 $\gamma 2$ expression.

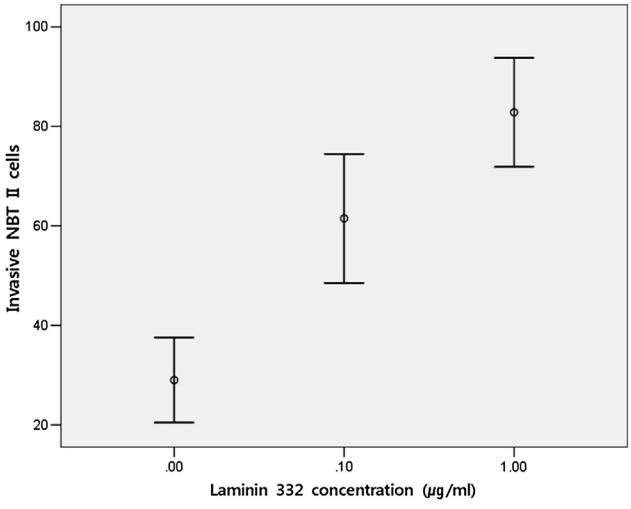

Figure 4. Matrigel invasion assay. NBT-II cell invasion in the presence of varying laminin 332 concentrations (Kruskal–Wallis test, $p < 0.001$).

In this study, using *in vitro* scratch and Matrigel invasion assays, we demonstrated that laminin 332 enhanced the motility and invasion of NBT-II urothelial carcinoma cells. *In vivo* NBT-II vesicle rat carcinoma cells secrete a laminin 332-related protein that causes scattering [25]. Also, there is an increase in laminin $\gamma 2$ expression and *de novo* synthesis of laminin in bladder cancer [18]. How the increased laminin synthesis contributes to cancer invasion, however, is not clear. We used NBT-II cells, which secrete laminin 332, in order to show that laminin 332 increases the motility and invasion of this cell strain. Research on the influence of laminin 332 and its proteolytic fragments on motility and invasion, although somewhat controversial, has shown that laminin 332 enhances motility by affecting cell receptors and their associated signaling pathways. The enhanced invasiveness of pancreatic carcinoma, colorectal adenocarcinoma, head and neck squamous cell carcinoma, and melanoma cells results from enhanced laminin 332 $\alpha 3\beta 1$ integrin signaling [26]. Laminin 332 $\alpha 4\beta 6$ integrin can also affect cancer cell motility [27]. Laminin 332 activity in epithelial cell scattering and cytoskeletal reorganization depends on the activation of the small guanosine triphosphate (GTP)-binding protein, Rho, and is probably independent of integrin receptors [25]. In our study, laminin-treated NBT-II cells had lamellipodia, demonstrating cytoskeletal reorganization; however, more study is needed to elucidate the exact signaling pathway.

Using reconstituted BM Matrigel, we demonstrated that laminin 332 enhances invasion. Laminin 332 promotes the production of matrix metallopeptidase 9 (MMP-9) in A375 melanomas [26]. Type IV collagenase (MMP-9) is thought to be essential for invasive cells to degrade BM and is involved in tumor cell invasion [28,29]. Laminin 332 has been localized at the invasion front in colon adenocarcinomas and likely plays a role in tumor invasion [30]. In this study, invasion was investigated by adding laminin 332 to NBT-II cells and examining its penetration into reconstituted BM. According to the immunohistochemistry and invasion assay results, synthesis and accumulation of laminin 332 increase in aggressive tumors with higher tumor stage and grade. This synthesis increases cytoplasmic retention, which causes tumor migration and invasion through autocrine signaling. The correlation between BM loss and tumor aggressiveness could also be explained as follows. MMP-9,

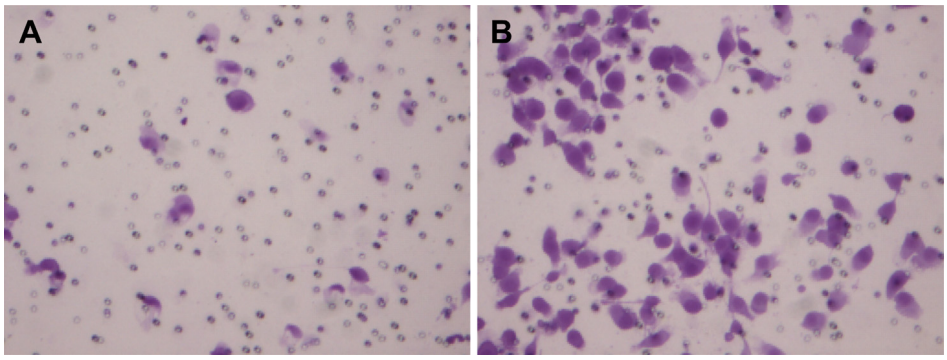

Figure 5. NBT-II cells invading Matrigel. Invading NBT-II cells in the laminin-treated group had lamellipodia and there were significantly more cells compared with the control group. (A) Invading NBT-II cells in the control group. (B) Invading NBT-II cells in the laminin-treated group.

which is induced as laminin 332, degraded BM, facilitating tumor invasion. In addition, invasive cells treated with laminin 332 appeared to have a more mesenchymal-like morphology. Therefore, laminin could also affect the epithelial-to-mesenchymal transition of tumor cells.

Generally, treatment strategies for bladder cancer are divided into radical cystectomy or intravesical treatment, depending on the results from the initial transurethral resection. The risk of progression to muscle-invasive disease is an important decision standard in bladder cancer treatment [31]. Therefore, trials have focused on determining prognostic markers to predict progression; however, until now, potential markers have had limited ability to predict tumor recurrence, progression, development of metastases, response to therapy, or patient survival. Since cell migration and invasion are essential to cancer progression, these may be important for predicting progression. We demonstrated that loss of laminin 332 γ2 from the BM and cytoplasmic retention indicated urothelial carcinoma invasiveness. As shown by both the Kaplan—Meier survival curves and the Kruskal—Wallis test, cytoplasmic expression of laminin 332 γ2 was associated with invasive behavior and tumor progression of urothelial carcinoma cells. Cellular retention of laminin 332 was the most important prognostic parameter in the multivariate analysis, although the results were not significant ($p = 0.180$).

The potential limitations of our findings are that a standardized scoring protocol and criteria would have reduced subjectivity in the immunohistochemistry studies. Additionally, the study population was small and the follow-up period was short. Future large-scale studies using the Cox proportional hazard regression model may provide significant information. Despite its limitations, it is the first study of the combined effects of laminin 332 on clinicopathologic parameters and *in vitro* invasion assays in bladder cancer.

Conclusions

The present study demonstrates that expression of laminin 332 γ2 is significantly correlated with the clinicopathologic parameters of bladder cancer, and that laminin 332 can promote NBT-II cell motility and invasion. Considering the significance of invasiveness and progression in treatment planning and prognosis for bladder cancer patients, investigating laminin 332 and its proteolytic subunits may lead to the development of new targeted therapies that can inhibit the ability of laminin 332 to drive tumor growth, dissemination, or both. However, prior to employing this promising prognostic marker in bladder cancer treatment, a better understanding of laminin 332 signaling and a large-scale, long-term population study are needed.

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