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Aberrant up-regulation of *LAMB3* and *LAMC2* by promoter demethylation in gastric cancer

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ABSTRACT

The *LAMB3* and *LAMC2* genes encode the laminin-5 β3 and γ2 chains, respectively, which are parts of laminin-5, one of the major components of the basement membrane zone. Here, we report the frequent up-regulation of *LAMB3* and *LAMC2* by promoter demethylation in gastric cancer. Gene expression data analysis showed that *LAMB3* and *LAMC2* were up-regulated in various tumor tissues. Combined analyses of DNA methylation and gene expression of both genes in gastric cancer cell lines and tissues showed that DNA hypomethylation was associated with the up-regulation of both genes. Treatment with a methylation inhibitor induced *LAMB3* and *LAMC2* expression in gastric cancer cell lines in which both genes were silenced. By chromatin immunoprecipitation assay, we showed the activation histone mark H3K4me3 was associated with the expression of both genes. The expression level of *LAMB3* affected multiple malignant phenotypes in gastric cancer cell lines. These results suggest that epigenetic activation of *LAMB3* and *LAMC2* may play an important role in gastric carcinogenesis.

1. Introduction

Gastric cancer is the second most common cause of cancer death in the world, and its incidence rate is highest in East Asia, Eastern Europe, and parts of Latin America [1]. However, the precise mechanisms underlying gastric carcinogenesis are not yet fully understood [2]. Promoter hypermethylation is involved in the repression of many tumor suppressor genes, and multiple approaches have identified many tumor suppressor genes silenced by promoter hypermethylation. On the contrary, promoter hypomethylation is associated with the expression of oncogenes including R-Ras in gastric cancer [3], c-Neu in transgenic mouse models [4], and Hox11 in leukemia [5]. These findings show that protooncogenes may be aberrantly re-expressed in cancers due to epigenetic changes such as DNA hypomethylation.

Laminins are large extracellular glycoproteins that are important components of all basement membrane zones (BMZs) and are involved in several important biological processes, including tissue development, wound healing, and tumorigenesis [6]. Three different polypeptide chains (α, β and γ) are components of laminins, and different combinations of these chains lead to the existence of 15 different laminin isoforms [7]. Laminin-5, a large molecule consisting of α3, β3, and γ2 chains, which are encoded by three distinct genes (*LAMA3*, *LAMB3*, and *LAMC2*, respectively) [8], is expressed in many epithelial tissues and in the tumor microenvironment of many carcinomas [6]. Laminin-5 promotes cell adhesion, migration, and scattering of various types of cultured cells more strongly than other known extracellular matrix proteins [9,10]. *LAMB3*, which is believed to be relatively resistant to proteolytic processing, is processed by both MT1-MMP [11] and matrilysin [12], and this cleavage increases carcinoma cell migration. Expression of *LAMC2* is associated with budding cancer cells located at the tip of invading malignant epithelium [13]. It has been reported recently that *LAMB3* and *LAMC2*, in conjunction with MMP7, play a key role in the progression of biliary tract cancer

Abbreviations: 5-Aza-dC, 5-aza-2′-deoxycytidine; ChIP, chromatin immunoprecipitation; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; shRNA, short hairpin RNA.

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[14], and coexpression of *LAMB3* and *LAMC2* has been reported in squamous cell carcinoma of the tongue, colorectal carcinoma, and basal cell carcinoma of the skin [14,15].

In this study, we showed that *LAMB3* and *LAMC2* were frequently overexpressed in gastric cancer tissues and promoter demethylation and histone modifications were associated with the overexpression of both genes in gastric cancer. We also showed that the increased expression of *LAMB3* was associated with increased proliferation, migration, and invasion of gastric cancer cells.

2. Materials and methods

2.1. Cell lines and tissue samples

Gastric cancer cell lines were cultured in complete RPMI 1640 medium. 293T and GP-293 packaging cell lines were maintained in complete DMEM media. All cell lines were obtained from the Korean Cell Line Bank (http://cellbank.snu.ac.kr/index.htm), and all complete media contained 10% fetal bovine serum (Hyclone), 100 U/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, and 0.5 mM HEPES. One hundred thirty frozen tumors were collected from Chungnam National University Hospital. All samples were obtained with informed consent, and their use was approved by the Internal Review Board at Chungnam National University Hospital.

2.2. Genome-wide DNA methylation assay

From each sample, genomic DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA), and the converted DNA was amplified using the Infinium® Methylation Assay kit (Illumina, San Diego, CA, USA). Amplified DNA was hybridized to the HumanMethylation27 BeadChip (Illumina, San Diego, CA, USA), and the arrays were scanned using the BeadArray™ Reader (Illumina). Image processing and intensity data calculations were performed according to the manufacturer's instructions. Each methylation signal was used to compute a "Beta" value (β), which is a quantitative measure of DNA methylation ranging from 0 (no methylation) to 1 (complete methylation) [16].

2.3. Whole-genome gene expression assay

Total RNA was extracted from each gastric cancer cell line. We measured RNA concentration using the NanoDrop 1000 and RNA integrity and quality using the Bioanalyzer with Experion RNA Std-Sens analysis kit and accompanying software (Bio-rad, Montreal, Quebec, Canada). The cDNA was synthesized from 300 ng of total RNA using the GeneChip 3′ IVT Expression Kit and hybridized to the Human Genome U133 Plus 2.0 chip containing 47,000 transcripts and variants (Affymetrix Inc., Santa Clara, CA) according to the manufacturer's protocol. After washing and staining, arrays were scanned using the Affymetrix GeneChip Scanner 3000, and raw data were processed using the robust multichip average (RMA) method [17]. Both genome-wide DNA methylation and gene expression data were submitted to the NCBI Gene Expression Omnibus (GEO) database (Accession No. GSE25869).

2.4. Real-time reverse transcription-polymerase chain reaction and Western blot

Real-time RT-PCR and Western blot have been done as described previously (for details see Supplementary data) [18].

2.5. Methylated DNA immunoprecipitation sequencing and pyrosequencing

Methylated DNA immunoprecipitation sequencing and pyrosequencing were performed as described previously (for details see Supplementary data) [18].

2.6. Lentiviral packaging and transduction of LAMB3 small hairpin RNA (shRNA)

A non-targeting shRNA control vector (Catalog No. for shRNA: SHC002) and shRNA lentiviral vectors for targeting human *LAMB3* mRNA were purchased from Sigma–Aldrich (Catalog No. for shRNA: SHCLNG-NM 000228). For lentivirus production, the shRNA vector was cotransfected with lentiviral packaging mix (Sigma) into 293T cells. SNU-601 and MKN-1 cells were infected and selected with 1 mg/ml puromycin. *LAMB3* protein reduction was assessed by Western blotting.

2.7. Retroviral vectors and retroviral transduction of MKN-1 cells

The constructs presented herein were made using standard molecular biology techniques employing PCR and fragment replacement strategies (see Supplementary data). Retroviral vector was cotransfected with a pVSV-G plasmid (Clontech) into a pantropic packaging cell, GP-293. MKN-1 cells were transduced and selected with 1 μg/ml puromycin. The level of *LAMB3* protein was assessed by Western blotting.

2.8. Cell proliferation, anchorage-dependent colony formation, adhesion, and migration assay

These assays were performed as described previously (for details see Supplementary data) [18].

2.9. Statistical analysis

Statistical analyses of group differences were performed using a Student's *t*-test and ANOVA. A P value < 0.05 was considered significant. All statistical analyses were performed using the R package (Version 2.11.0). The following parameters were obtained from the medical records of the 130 patients studied: age, gender, Lauren's classification, and stage (Supplementary Tables 2 and 3).

3. Results

3.1. Negative correlation between DNA methylation and gene expression of LAMB3 and LAMC2 in gastric cancer cell lines

We observed the expression level of *LAMB3* and *LAMC2* in various cancers using the GENT database (available at http://medical-genome.kribb.re.kr/GENT/ or http://genome.kobic.re.kr/GENT/; submitted). *LAMB3* and *LAMC2* were overexpressed in many cancer types, including cervix, esophagus, head and neck, ovary, pancreas, and stomach (Supplementary Fig. 1A and B). To understand the mechanisms of overexpression of *LAMB3* and *LAMC2* in gastric cancer, we first examined the patterns of gene expression and DNA methylation of both genes in 10 gastric cancer cell lines. Interestingly, a strong negative correlation between DNA methylation and gene expression was observed in both genes (Fig. 1A and B). DNA methylation was bimodal, that is, either hypomethylated (less than 20%) or hypermethylated (more than 60%), and increased expression was correlated with hypomethylation in both genes (Fig. 1A and B). Methylated DNA immunoprecipitation sequencing also revealed the same result. SNU-668 cells, in which *LAMB3* and

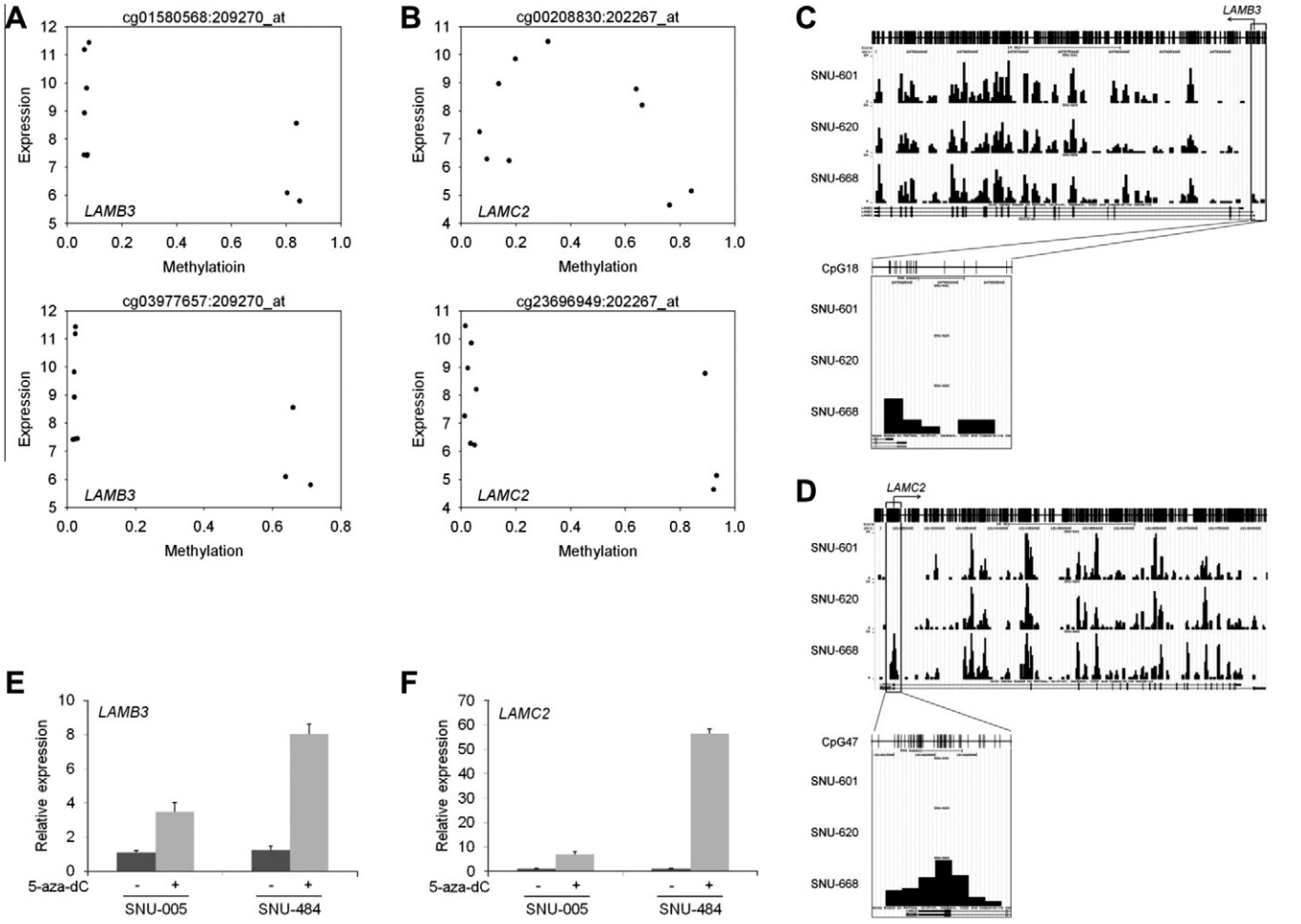

Fig. 1. Correlation between DNA methylation and gene expression of *LAMB3* and *LAMC2* in gastric cancer cell lines. **(A** and **B)** The relationship between expression level and methylation status of *LAMB3* and *LAMC2* in 10 gastric cancer cell lines. Expression and methylation values are expressed as the difference between the cell lines. **(C** and **D)** Methylated DNA immunoprecipitation sequencing analysis of cell lines SNU-601, -620 and -668. Methylated DNA immunoprecipitation sequencing data were visualized as a custom track in the University of California, Santa Cruz Genome Browser. CpG sites throughout *LAMB3* and *LAMC2* are shown. Black bars represent the methylation frequency of each 10 bp. **(E)** and **(F)** Cells were treated for 4 days with 2 μM of 5-Aza-dC, as described in Section 2. Quantitative real-time PCR was performed using specific primers for *LAMB3* and *LAMC2* gene. All expression levels are shown relative to 5-Aza-dC untreated cell line (set to a value of 1.0). Expression levels were normalized to β-*actin* control gene.

LAMC2 are expressed at a low level, had extensive DNA methylation near the transcription start site, while SNU-601 and SNU-620 cells, in which both genes are expressed at a high level (Fig. 1C and D), had no DNA methylation near the transcription start site.

We then treated two cell lines (SNU-005 and SNU-484), which did not express both genes, with the demethylating agent 5-Aza-dCs and found that the loss of expression of both genes in SNU-005 and SNU-484 cells could be restored by 5-Aza-dC treatment, suggesting that DNA methylation is one of the mechanisms responsible for the expression of both genes (Fig. 1E and F).

3.2. Negative correlation between methylation and gene expression of LAMB3 and LAMC2 in gastric cancer tissues

We examined the pattern of gene expression and DNA methylation of *LAMB3* and *LAMC2* in 130 paired gastric cancer tissues. We first performed real-time RT-PCR to find any difference between normal and cancer tissues in mRNA expression level. A majority of patients (77 of 130 for *LAMB3*, 92 of 130 for *LAMC2*) showed higher expression levels of *LAMB3* and *LAMC2* mRNA in tumor tissues than in normal tissues. The mean expression level of *LAMB3* and *LAMC2* mRNA in tumor tissues was 1.648 ± 1.263 and 2.264 ± 1.983 (mean ± SD), respectively, which were higher than values from the corresponding normal tissues (1.361 ± 0.843 for LAMB3, $P = 0.005$; 1.167 ± 0.569 for *LAMC2*, $P = 2.8E\text{-}0.9$) (Fig. 2A). We then performed pyrosequencing to evaluate the methylation status of promoters of *LAMB3* and *LAMC2* in the same tissues. The mean of *LAMB3* CpG sites methylation level in the tumor tissues was 35.4 ± 11.2%, but it was 39.2 ± 8.6% in normal tissues. Of the 130 tumors, 80 were hypomethylated at the two *LAMB3* CpG sites ($P = 0.0003$). The mean of *LAMC2* CpG sites methylation level in tumor tissues was 31.8 ± 8.4%, but it was 36.5 ± 5.2% in normal tissues (Fig. 2B). Of the 130 tumors, 93 showed hypomethylation in the three *LAMC2* CpG sites ($P = 4.4E\text{-}09$).

We assessed whether methylation of the *LAMB3* and *LAMC2* CpG sites was associated with expression of both genes. A significant correlation between expression and methylation ($R = -0.206$, $P = 0.0008$ for *LAMB3*, $R = -0.162$, $P = 0.0089$ for *LAMC2*) was observed, indicating that an increase in *LAMB3* and *LAMC2* expression was associated with DNA hypomethylation (Fig. 2C and D). Interestingly, the methylation of *LAMB3* was highly correlated with *LAMC2* methylation in gastric cancer tissues ($R = 0.655$, $P = 0.0001$) (Fig. 2E). We performed chromatin immunoprecipitation (ChIP) assay to determine whether the expression of *LAMB3* and *LAMC2* is associated with histone modification. We found that

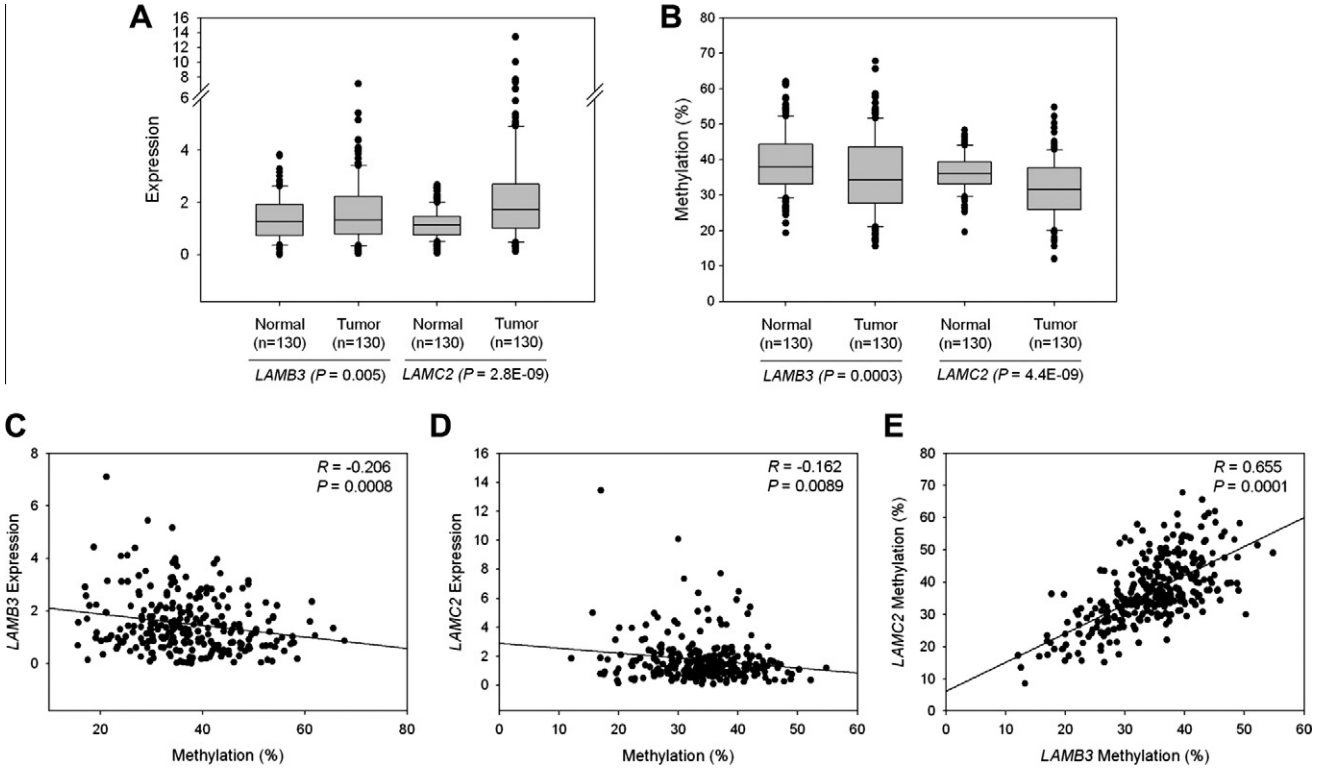

Fig. 2. Correlation between expression level and methylation status of *LAMB3* and *LAMC2* in gastric cancer tissues. **(A)** Expression level of the mRNA for *LAMB3* and *LAMC2* in 130 paired human tumor and adjacent normal tissues. mRNA values were statistically different between the samples from normal and tumor tissues. **(B)** *LAMB3* and *LAMC2* methylation for 130 paired gastric normal and tumor tissues. Pyrosequencing analysis was performed at the two CpG sites of *LAMB3* and three CpG sites of *LAMC2* promoter. The boxes are bounded above and below by the 25th and 75th percentiles. The lines in the boxes indicate the median values. **(C)** and **(D)** Correlation between methylation and expression of *LAMB3* and *LAMC2*. **(E)** Correlation between the methylation levels of both genes.

the active H3K4me3 mark was involved in up-regulation of both genes in gastric cancer cell lines (Supplementary Fig. 2B and C). These results showed that the expression of *LAMB3* and *LAMC2* was regulated by histone methylation as well as DNA methylation.

3.3. Targeting LAMB3 expression alleviated malignant phenotypes in SNU-601 cells

We first confirmed that *LAMB3* shRNAs reduced levels of *LAMB3* protein in SNU-601 cells (Fig. 3A). We then assessed the malignant properties of SNU-601 cells transduced with *LAMB3* shRNA (shβ3-A or shβ3-B) compared with the control cells transduced with non-targeting shRNA (shCTL). SNU-601 cells transduced with *LAMB3* shRNA grew more slowly than control shRNA-treated cells. However, in MKN-1 cells whose *LAMB3* expression was undetectable, there was no difference between *LAMB3* and control shRNA treatment (Fig. 3B). Similar results were obtained from a anchorage-dependent colony-forming assay. Whereas *LAMB3* shRNA treatment reduced the size and number of colonies in SNU-601 cells, the same treatment did not have an effect on colony formation in MNN-1 cells (Fig. 3C). In cell culture, we found that *LAMB3* shRNA-treated cells were more readily detached than control cells. This feature suggested that decreased *LAMB3* expression might cause an interruption of cell-extracellular matrix (ECM) adhesions. We performed a cell detachment assay to address this question. *LAMB3* shRNA-treated SNU-601 cells were sensitive to trypsin-induced detachment compared with control shRNA-treated cells (Fig. 3D). In a short-term attachment assay using fibronectin, which is one of the important parts of the basement membrane, cell adhesion was decreased in *LAMB3* shRNA-treated SNU-601 cells (Fig. 3E). We attempted to perform a migration assay for SNU-601 cells, but the assay was unsuccessful because SNU-601 cells moved randomly as reported previously [19]. Taken together, these results showed that knockdown of *LAMB3* reduced the malignant phenotypes of SU-601 cells.

3.4. Increased colony formation, migration, and adhesion in MKN-1 cells stably expressing LAMB3

To further investigate the oncogenic capabilities of *LAMB3*, MKN-1 cells were transduced with empty vector (Vector) or *LAMB3*-expressing retroviral vector (β3) in cell-free retroviral supernatants. The expression of *LAMB3* in mixed clones after selection was estimated by Western blot analysis (Fig. 4A). We first examined whether *LAMB3* overexpression affected cell growth in MKN-1 cells but found no significant difference in growth between *LAMB3*-overexpressing and control cells at 24, 48, 72, 96, 120 and 144 h after plating (Fig. 4B). However, in the anchorage-dependent colony forming assay, *LAMB3* overexpressing cells formed more and larger colonies than control cells (Fig. 4C).

We also tested whether *LAMB3* overexpression affects migration and adhesion of MKN-1 cells. *LAMB3* overexpression significantly increased migration after 18 h (Fig. 4D), and *LAMB3* overexpression increased cell adhesion of MKN-1 cells in a short-term attachment assay using fibronectin (Fig. 4E).

4. Discussion

Laminin-5 is one of the major factors that stimulate the invasion and metastasis of several types of tumors [13,20]. The role of laminin-5 in tumorigenesis has been well established at the biological and signaling levels. However, the molecular mechanisms

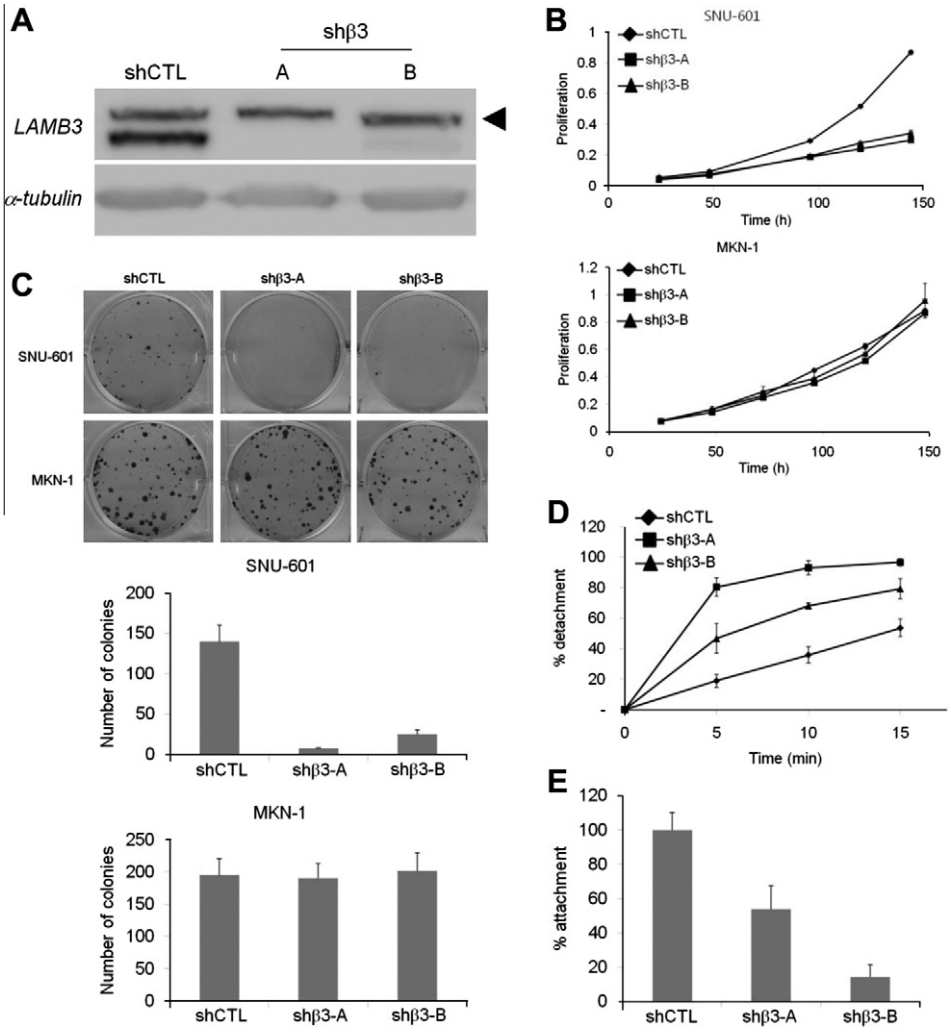

Fig. 3. Effect of *LAMB3* knockdown on malignant phenotypes of SNU-601 gastric cancer cells. **(A)** Western blot analysis of *LAMB3* expression in SNU-601. The expression of *LAMB3* was suppressed by lentiviral-mediated transduction of *LAMB3* shRNA. Arrowhead indicates nonspecific band. **(B)** Proliferation was measured at 24, 48, 72, 96, and 120 h. Columns, results of triplicate experiments; error bars, SD. **(C)** Anchorage-dependent colony formation assay in monolayer culture. Stable SNU-601 and MKN-1 cells transduced with control shRNA (shCTL) or *LAMB3* shRNA (shβ3-A or shβ3-B) were plated in 6-well plates. After 2 weeks of incubation, the cells were stained with crystal violet. The graph shows the number of colonies. Columns, results of triplicate experiments; error bars, SD. **(D)** *LAMB3* knockdown decreases resistance to trypsin dissociation. The indicated cells were subjected to diluted trypsin at the indicated intervals and percentage of cells dissociated was quantified. Columns, results of triplicate experiments; error bars, SD. **(E)** The short-term attachment assay was performed by seeding equal amounts of the indicated cells in 96-well tissue culture plates coated with fibronectin. Columns, results of triplicate experiments; error bars, SD.

regulating *LAMB3* and *LAMC2* expression in gastric cancer are not well known. We showed that the expression levels of *LAMB3* and *LAMC2* were regulated by both DNA methylation and histone modification, and the expression level of *LAMB3* affected multiple malignant phenotypes in gastric cancer cell lines.

DNA methylation is an important mechanism for regulating gene expression during carcinogenesis. Many tumor suppressor genes are repressed in cancers by promoter hypermethylation [21]. Interestingly, cancer-associated DNA hypomethylation is as prevalent as cancer-linked hypermethylation, and it is often associated with increased expression of oncogenes. *c-Myc* is one of the widely reported hypomethylated genes in some cancers [22,23]. We showed that the expression of *LAMB3* and *LAMC2* was increased in many tumor tissues but decreased in some tumors (Supplementary Fig. 1). Interestingly, promoter hypermethylation is involved in the silencing of laminin-5-encoding genes in cancers such as breast and prostate cancer [24,25]. We showed that promoter demethylation was one mechanism for the increased expression of *LAMB3* and *LAMC2* in gastric cancer cell lines and tumor tissues (Figs. 1 and 2). Thus, DNA methylation seems to be important in the regulation of laminin-5 encoding genes, but its effect on gene expression differs across different tissue types.

Another important epigenetic mechanism that regulates gene expression is histone modification. Here, we showed that histone modification was involved in the regulation of both *LAMB3* and *LAMC2* expression. The activating histone mark H3K4me3 was enriched in the *LAMB3* and *LAMC2* overexpressing SNU-601 cells but not in SNU-005 cells in which both genes are silenced (Supplementary Fig. 2). However, few differences were observed in the pattern of the repressive histone mark H3K27me3 between SNU-601 and SNU-005 cells, suggesting that another repressive mark might be involved in silencing these genes [26]. Taken together, our data indicates that the expression of *LAMB3* and *LAMC2* are regulated in gastric cancer by epigenetic mechanisms including DNA methylation and histone modification.

Autocrine laminin-5 mediates anchorage-independent survival in breast tumors through ligation of α6β4 integrin [27]. Laminin-5 also stimulates HCC cell proliferation via a different function of integrins α6β4 and α3β1 [28]. We showed that shRNA-mediated knockdown of *LAMB3* inhibited proliferation and monolayer colony

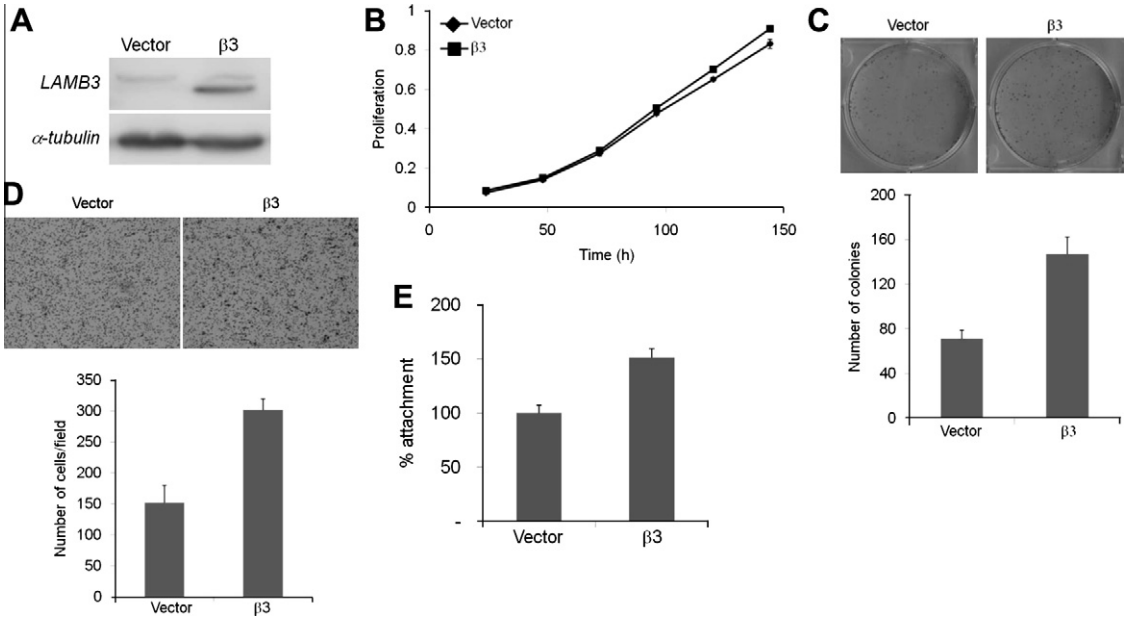

Fig. 4. Effect of *LAMB3* overexpression on malignant phenotypes of MKN-1 gastric cancer cells. **(A)** Western analysis of *LAMB3* expression in MKN-1. MKN-1 cells were transduced with pLPCX *LAMB3* or control pLPCX (Vector) retroviral supernatants. **(B)** Proliferation was measured at 24, 48, 72, 96, and 120 h. Columns, results of triplicate experiments; error bars, SD. **(C)** Anchorage-dependent colony formation assay in monolayer culture. Stable MKN-1 cells were plated in 6-well plates. After 2 weeks of incubation, the cells were stained with crystal violet. The graph shows the number of colonies. Columns, results of triplicate experiments; error bars, SD. **(D)** Photographs of MKN-1 cells from the underside of Boyden chamber membrane. Number of migrated cells observed using microscope and representative fields (200× magnification) are shown. The graph displays average number of cells migrated. Columns, results of triplicate experiments; error bars, SD. **(E)** The short-term attachment assay was performed by seeding equal amounts of the indicated cells in 96-well tissue culture plates coated with fibronectin. Columns, results of triplicate experiments; error bars, SD.

growth of SNU-601 cells. In contrast, the forced expression of *LAMB3* increased monolayer colony growth of MKN-1 cells, although *LAMB3* overexpression did not affect short-term proliferation. Cell growth in monolayer culture was measured for 2 weeks, whereas the cell proliferation assay was performed for 5 days. We estimate that a weaker effect of overexpression relative to knockdown may have caused the lack of effect on short-term proliferation.

To invade the surrounding tissues, cancer cells need to penetrate through the basement membrane zone, which is composed of a number of extracellular matrix proteins. Laminn-5-ligated β4 integrin supports epithelial migration and invasion [29]. The heterodimer of the *LAMB3* and *LAMC2* is accumulated in the cytoplasm of dissociating tumor cells from neoplastic tubules or colon carcinomas [30]. *LAMB3* and *LAMC2* are frequently coexpressed at the cancer stromal interface and at the invasive front of tumors [15]. Another study reported that gastric tumor tissues expressed *LAMA3*, *LAMB3,* and *LAMC2* in the underlying basement membrane [13]. We showed that migration of MKN-1 cells was increased by ectopic expression of *LAMB3*. Adhesion signaling plays an important role in the tumor microenvironment, contributing to cancer progression, invasion, and metastasis. Laminin-5 enhances adhesion ability of glioma cells through interaction with integrin [31]. We revealed that shRNA-mediated knockdown caused morphological changes of cells and resulted in a decrease in cell adhesion. We showed that knockdown or enhancement of *LAMB3* expression resulted in significant changes in malignant phenotype of gastric cancer cell lines. These results suggest that *LAMB3* may play an important role in the progression of gastric cancer.

In conclusion, we showed that the expression of *LAMB3* and *LAMC2* genes was up-regulated in various cancers including gastric cancer and was regulated by epigenetic mechanisms in gastric cancer tissues and cell lines. We also showed that the malignant phenotypes of gastric cancer cell lines were dependent on the amount of *LAMB3* expression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.082.

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