Co-expression of laminin β3 and γ2 chains and epigenetic inactivation of laminin α3 chain in gastric cancer

MASANORI II[1*], HIROYUKI YAMAMOTO[1*], HIROAKI TANIGUCHI[1], YASUSHI ADACHI[1], MAYUMI NAKAZAWA[1], HIROKAZU OHASHI[1], TOKUMA TANUMA[1], YASUTAKA SUKAWA[1], HIROMU SUZUKI[1], SHIGERU SASAKI[1], KOHZOH IMAI[2] and YASUHISA SHINOMURA[1]

[1]First Department of Internal Medicine, Sapporo Medical University, Sapporo, Hokkaido 060-8543; [2]The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

Received March 1, 2011; Accepted April 28, 2011

DOI: 10.3892/ijo.2011.1048

Abstract. Laminin-332 (LM-332, formerly termed laminin-5) is a heterotrimeric glycoprotein that regulates cell adhesion and migration. Molecular alterations of LM-332 are involved in cancer progression. The aim of this study was to clarify alterations of LM-332 in gastric carcinoma. The expression of LM-332 subunits in 10 gastric carcinoma cell lines was investigated by RT-PCR, Western blotting, and immunocytochemical/immunofluorescent analyses. The promoter methylation status of LM-332-encoding genes (LAMA3, LAMB3 and LAMC2) was analyzed by methylation-specific PCR (MSP). The relationship between cell migration and LM-332 expression was assessed by the scratch assay. The expression of LM-332 was analyzed immunohistochemically in 90 gastric cancer tissues. Co-expression of laminin β3 and γ2 chains was often observed in gastric carcinoma cell lines at mRNA and protein levels. In contrast, there was no expression of laminin α3 at either the mRNA or protein levels. Extracellular secretion of laminin β3 and γ2 chains was found in 2 of the 10 cell lines. The LAMA3 gene was transcriptionally silenced by methylation of the promoter CpG islands in all of the cell lines, while the LAMB3 and LAMC2 genes were silenced in several cell lines. Treatment with a demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC), restored expression of the LM-332-encoding genes. Methylation frequency of LAMA3 was higher than those of the LAMB2 and LAMC2 genes in gastric cancer tissues. Migration distances were significantly correlated with cytoplasmic laminin γ2 chain expression. Immunohistochemistry showed frequent co-expression of laminin β3 and γ2 chains in gastric carcinoma cells, which was significantly correlated with depth of invasion and advanced tumor stage. The results suggest that the laminin β3 and γ2 chains accumulate intracellularly and play a role in gastric cancer progression, while epigenetic silencing of the laminin α3 chain may lead to inability to synthesize the basement membrane and may affect cancer cell invasion. Cancer cell motility appears to be associated with the cytoplasmic laminin γ2 chain *in vitro*.

Correspondence to: Dr Hiroyuki Yamamoto, First Department of Internal Medicine, Sapporo Medical University, South-1, West-16, Chuo-ku, Sapporo 060-8543, Japan
E-mail: h-yama@sapmed.ac.jp

*Contributed equally

Abbreviations: 5-aza-dC, 5-aza-2'-deoxycytidine; BM, basement membrane; LM-332, laminin-332; MSP, methylation-specific PCR; RT-PCR, reverse transcription-polymerase chain reaction; TSA, trichostatin A

Key words: laminin-332, gastric cancer, methylation, laminin γ2, cell motility

Introduction

The basement membrane (BM) is a supporting structure underlying the epithelium or endothelium. BM provides a scaffold for cellular growth and differentiation, and breakdown of the BM is now regarded as an essential step for tumor invasion and metastasis. Laminin, one of the major components of the BM, constitutes a family of at least 15 distinct isoforms in humans (1,2). The laminin molecule is a cross-shaped heterotrimer assembled from α, β and γ glycoprotein subunits. Five α, three β, and three γ subunits are known at present. Each member of the laminin family is currently designated on the basis of a system in which their trimers can be identified by Arabic numerals for three α, β, and γ chain numbers.

Laminin-332 (LM-332, formerly termed laminin-5) is a heterotrimer of α3, β3 and γ2 subunits, which are encoded by three distinct genes (LAMA3, LAMB3 and LAMC2, respectively). LM-332 interacts with integrin α6β4 in a hemidesmosome and supports various cellular functions, i.e., adhesion to the BM, proliferation, migration, polarity, and apoptosis (2,3). Three subunits of LM-332 are synthesized separately, and subsequent formation of a heterotrimer is an essential step for the final composition of LM-332.

Neoplastic transformation essentially involves alterations of cell-cell and cell-matrix adhesion molecules between the epithelium and BM. The amount and distribution of LM-332 also undergo diverse changes during carcinogenesis. In colorectal carcinogenesis, the distribution of LM-332 is preserved

in the BM of adenomas but becomes discontinuous or reduced in carcinomas (4), resulting in loss of the BM. Several lines of evidence have demonstrated that the laminin γ2 monomer is overexpressed in invasive colorectal carcinoma, and this has been attributed to synergistic activation of the LAMC2 gene by TGF-β1 and HGF (5). We have reported that the laminin γ2 monomer is overexpressed in esophageal squamous cell carcinoma cells at the invasive front (6). Extracellular deposition or cytoplasmic accumulation of the laminin γ2 monomer has been reported in gastric adenocarcinoma (7,8). However, little is known about distribution patterns of LM-332 subunits in gastric carcinoma.

In this study, we investigated the expression patterns of both the mRNA and protein of LM-332 subunits and the relationship of migration activity with LM-322 expression in gastric cancer cell lines. Epigenetic alterations of the LAMA3, LAMB3, and LAMC2 genes were also analyzed. Finally, expression of LM-332 subunits was analyzed immunohistochemically in gastric cancer tissues.

Materials and methods

Cell culture and reagents. Ten human gastric cancer cell lines, NUGC3, SNU1, SNU638, AZ521, JRST, KATOIII, MKN7, MKN28, MKN45, and MKN74, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), Riken Cell Bank (Tokyo), or the American Type Culture Collection (Rockville, MD, USA). The cells were grown in RPMI-1640 medium (GIBCO BRL) containing 10% fetal bovine serum at 37˚C in a humidified atmosphere of 5% CO_2 in air.

Semiquantitative reverse transcription-polymerase chain reactions (RT-PCR). Expression of LAMA3, LAMB3, and LAMC2 was analyzed by semiquantitative RT-PCR. Total RNA was extracted from the cell lines. cDNA was synthesized from total RNA. Aliquots of cDNA (400 ng/μl) were subjected to PCR. cDNA generated from a human hepatoma cell line HuH-7 was used as a positive control for the LAMA3 gene, which was tested in our preliminary experiment (data not shown). GAPDH gene served as an internal control. Negative control reactions were run without a template cDNA. The sequences of PCR primers and thermal cycling conditions for amplifying each gene was determined as described previously (9,10). PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide under UV illumination. All PCR reactions were done at least twice to validate the results.

Preparation of cell lysates. Protein extracts of cells were prepared by scraping lysed cells in RIPA lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate (w/v), 0.1% SDS (w/v), and protease inhibitors in TBS). Samples were diluted and adjusted to the same protein concentration for quantitative Western blot analyses.

Preparation of conditioned media. To prepare conditioned media, cells were grown to semiconfluence in RPMI-1640 medium containing 10% FBS. The semiconfluent cultures were washed twice with PBS and incubated in serum-free RPMI-1640 medium for two days. MKN7 cells were incubated for about five days because this cell grew far slower than did other cell lines. The serum-free culture supernatants were collected and centrifuged at low speed to remove floating cells and debris. Proteins were concentrated 100-fold by a ultrafiltration method using a concentrator (Millipore). Samples were subjected to subsequent Western blot analysis.

Antibodies. Antibodies used for Western blot analysis, immunocytochemistry, and immunohistochemistry were rabbit polyclonal anti-laminin α3, goat polyclonal anti-laminin α3, rabbit polyclonal anti-laminin β3 (Santa Cruz Biotechnology), mouse monoclonal anti-laminin γ2 (Chemicon), and mouse monoclonal anti-actin (Thermo Fisher Scientific). All antibodies were applied at the concentration suggested by the manufacturer.

Western blot analyses for cell lysates and conditioned media. Samples of cell lysates and conditioned media were analyzed by conventional Western blot method. Proteins were resolved on 4-12% Bis-Tris gels (Invitrogen) under reducing condition. No protein controls were also electrophoresed, subsequently transferred to a PVDF membrane. Visualization was performed by chemiluminescence with specific antibodies.

Immunocytochemistry and immunofluorescence. Ten gastric cancer cell lines were cultured in Lab-Tek II CC2 Chamber Slide System (Thermo Fisher Scientific) in RPMI-1640 medium supplemented with 10% FBS. The media were removed and slides were washed with PBS. Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.3% Triton X-100 with PBS. After blocking with 3% FBS in PBS, endogenous peroxidase was quenched by 3% hydrogen peroxidase for 10 min. Slides were wiped again and incubated with a primary antibody against laminin β3 or laminin γ2 chain. Rabbit or mouse normal immunoglobulins were substituted for each primary antibody as negative controls. Detection of the laminin β3 chain was carried out by a biotinylated IgG and peroxidase-conjugated streptavidin kit (DakoCytomation), followed by development with 0.04% (w/v) diaminobenzidine solution with 0.01% hydrogen peroxidase for 5 min. Hematoxylin was used for counterstain. For an immunofluorescence study for the laminin γ2 chain, secondary antibody was conjugated to Alexa fluor 555 (Molecular Probes), and 4', 6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstain. Slides were mounted with aqueous mounting medium and sealed with coverslip. All staining procedures were carried out with a standard method to minimize variability.

Methylation-specific PCR (MSP). Methylation status of CpGs in the promoter regions of the LAMA3, LAMB3, and LAMC2 genes were determined by conventional MSP. Genomic DNA was extracted from the cell lines and 50 gastric cancer tissues. Informed consent was obtained from each patient. DNA was treated with sodium bisulfite using EZ DNA Methylation-Gold kit (ZYMO Research) and subjected to MSP. To detect methylation status of CpGs in the promoter regions of the LAMA3, LAMB3, and LAMC2 genes, two pairs of MSP primers specific for methylated or unmethylated alleles were used at appropriate temperature as described previously (11).

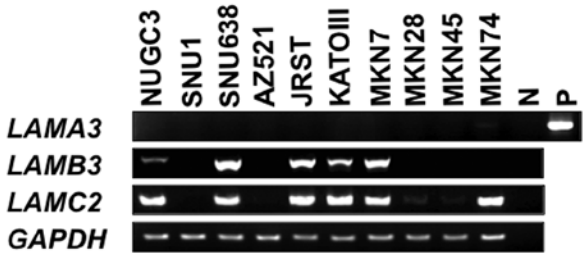
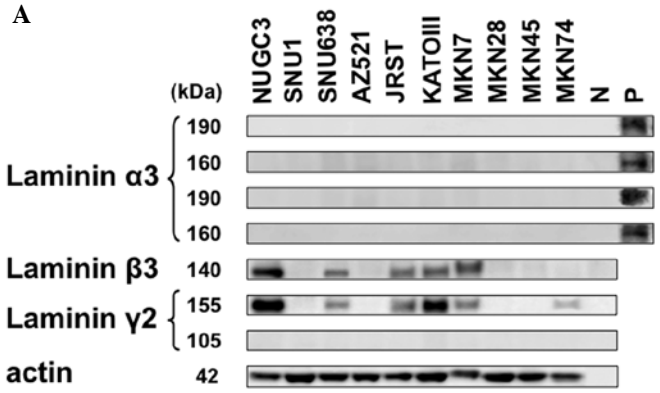


Figure 1. Expression of mRNA of the LM-332 encoding genes in gastric cancer cell lines analyzed by RT-PCR. HuH-7 cDNA served as a positive control for the LAMA3 gene. GAPDH was used as an internal control.



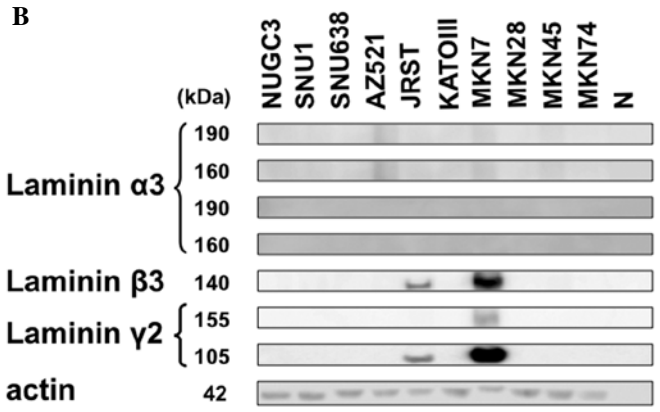


Figure 2. Protein expression of LM-332 in gastric cancer cell lines. (A) Western blot analyses for cell lysates. (B) Western blot analyses for conditioned media.

No template controls were used in the assays. MSP products were visualized on 2% TBE agarose gels containing 0.25 μg/ml ethidium bromide. Each sample was tested at least in duplicate.

5-aza-2'-deoxycytidine (5-aza-dC) and/or trichostatin A (TSA) treatment. To examine the role of CpG methylation and histone deacetylation in silencing of laminin-332-encoding genes, cancer cells were treated with 2 or 5 μM of 5-aza-dC (Sigma, St. Louis, MO, USA) for 72 h or with 100 nM of TSA for 24 h. Cells were also treated with 2 or 5 μM of 5-aza-dC for 72 h followed by 600 nM of TSA for an additional 24 h. The timing and sequencing of 5-aza-dC and/or TSA was based on similar preliminary studies as well as published studies (11,12). After the treatment, expression of laminin-332-encoding genes was analyzed by RT-PCR.

In vitro migration assay (scratch assay). Cells (1x10^6/well) of eight cell lines (except MKN7 and KATOIII) were seeded onto 6-well plates and cultured in RPMI-1640 medium with 10% FBS overnight at 37˚C with 5% CO_2. When the cells were semiconfluent, a straight scratch was gently made through the central axis of the plate using a pipette tip. The plates were rinsed with PBS and culture media were replaced. Initial widths of the scratches were immediately measured. After 24 h of incubation, the distance that remaining cells migrated across the scratch line was measured as described previously (13). Quantification was performed by photoshop. All scratch assays were done in triplicate. The results were expressed as the means ± SD.

Immunohistochemistry of gastric cancer tissues. Tissue microarray of gastric cancer tissues was purchased from SuperBioChips Laboratories (Seoul, Korea) and used for immunohistochemical analysis. Immunohistochemistry was carried out as described previously (3). Normal rabbit or mouse immunoglobulins were used as negative controls. Cytoplasmic expression was defined as positive when immunoreactivity was observed in >10% of carcinoma cells.

Statistical analysis. Statistical significance of associations between the migration distances and the relative quantitative levels of laminin γ2 protein in Western blot was determined by Pearson's correlation test. The correlation between immunohistochemical expression and clinicopathological characteristics was determined by the following statistical tests: Student's t-test for age, the Mann-Whitney test for lymph node metastasis and pTNM stage, and the χ^2 test or Fisher's exact test for the remaining parameter. A P-value of ≤0.05 was considered significant.

Results

Coexpression of LAMB3 and LAMC2 mRNA in gastric cancer cell lines. Results of RT-PCR analyses are shown in Fig. 1. LAMB3 was intensely detected in 40% of the cell lines (SNU638, JRST, KATOIII and MKN7) and weakly detected in NUGC3 cells. LAMC2 was intensely detected in 60% of the cell lines (NUGC3, SNU638, JRST, KATOIII, MKN7 and MKN74) and very weakly detected in MKN28 and MKN45 cells. Notably, LAMA3 was not amplified after 35 PCR cycles in any of the tested cell lines, even in triplicate experiments. Expression of three LM-332-encoding genes was not associated with histological type (intestinal/diffuse type) or histological grade (poor/moderately/well-differentiated adenocarcinoma) of gastric cancer cell lines.

Intracellular coexpression of laminin β3 and γ2 proteins in gastric cancer cell lines. Cell lysate proteins were subjected to Western blot analyses for evaluation of intracellular synthesis of laminin-332 subunits (Fig. 2A). Laminin β3 protein was detected in 50% of cell lines (NUGC3, SNU638, JRST, KATOIII, and MKN7). Nonprocessed laminin γ2 protein

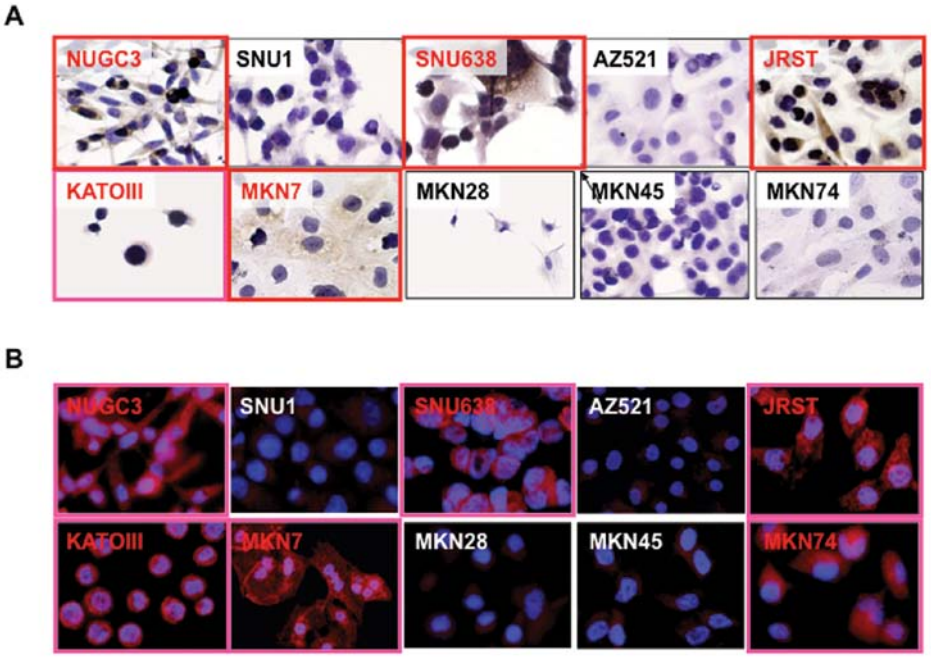


Figure 3. Immunocytochemistry for the laminin β3 chain and double immunofluorescent staining for the laminin γ2 chain in gastric cancer cell lines. (A) Immunocytochemistry for the laminin β3 chain. Positive staining is brown and nuclei counterstain is hematoxylin. Original magnification, x400. (B) Double immunofluorescent staining for the laminin γ2 chain. Nuclei were counterstained with DAPI (blue). Original magnification, x400.

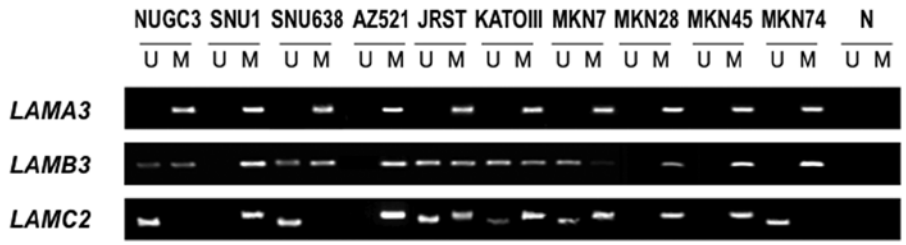


Figure 4. MSP analyses for the LM-332-encoding genes in gastric cancer cell lines. U, unmethylated PCR products; M, methylated PCR products.

(155 kDa) was detected in 60% of cell lines (NUGC3, SNU638, JRST, KATOIII, MKN7, and MKN74). Processed laminin γ2 protein (105 kDa) was not detected in any of the cell lines. Remarkably, laminin β3 and γ2 proteins were coexpressed in 50% of cell lines (NUGC3, SNU638, JRST, KATOIII and MKN7). The presence of laminin β3 and γ2 proteins was consistent with the presence of the LAMB3 and LAMC2 mRNA. Neither the non-processed (190 kDa) nor processed (160 kDa) form of laminin α3 protein was found in any of the cell lines despite the use of two kinds of antibodies.

Expression of laminin β3 and γ2 proteins released by gastric cancer cell lines. In Western blot analysis for conditioned media, neither non-processed (190 kDa) nor processed (160 kDa) laminin α3 proteins were found in any of the cell lines despite the use of two kinds of antibodies (Fig. 2B). The laminin β3 chain was detected in JRST and MKN7 cells. The non-processed laminin γ2 chain (155 kDa) was identified only in MKN7 cells. Noteworthy, the processed laminin γ2 chain (105 kDa) was also detected in JRST and MKN7 cells. The latter was not observed in Western blot analysis of the cell lysates. The other eight cell lines did not release any LM-332 subunits into the conditioned media.

Visualization of laminin β3 and γ2 proteins by immunocytochemical examination. Cultured cells on the chamber slides were subjected to immunocytochemical examination for evaluation of the expression of laminin β3 and γ2 chains (Fig. 3). There was no detectable immunoreactivity with a control antibody (data not shown). The laminin β3 chain was specifically immunostained in the cytoplasm of 50% of the cell lines (NUGC3, SNU638, JRST, KATOIII and MKN7). By means of intuitively comparing the saturation of fluorescent dye, the laminin γ2 chain was found in the cytoplasm of 60% of the cell lines (NUGC3, SNU638, JRST, KATOIII, MKN7, and MKN74). The results of both stainings were consistent with those of RT-PCR and Western blot analyses for cell lysates.

Methylation of the LAMA3 promoter correlates with lack of LAMA3 expression. MSP analysis showed PCR products for unmethylated and methylated promoter regions of the LAMA3, LAMB3 and LAMC2 genes (Fig. 4). Notably, the LAMA3 promoter was methylated in all of the 10 cell lines. The LAMB3 promoter was methylated in 50% of the cell lines (SNU1, AZ521, MKN28, MKN45 and MKN74) and partially unmethylated in the remaining cell lines. The

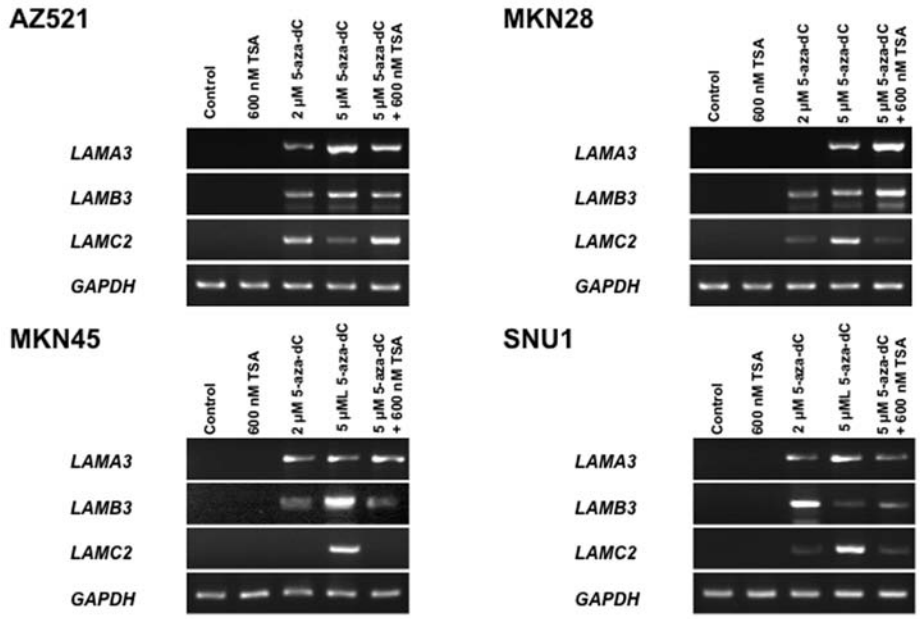


Figure 5. Re-activation of the LM-332 encoding genes by 5-aza-dC treatment in gastric cancer cell lines. As indicated, cells were incubated for 72 h with 2 μM or 5 μM 5-aza-dC and/or for 24 h with 600 nM TSA. After the treatment, expression of the LM-332 encoding genes was analyzed by RT-PCR.

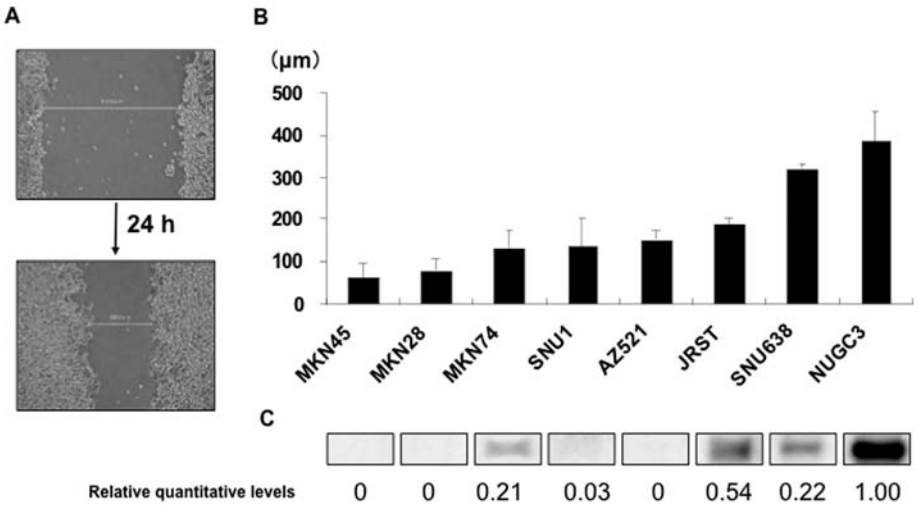


Figure 6. A correlation between migration analyzed by scratch assay and laminin γ2 expression. (A) Representative images of straight scratch made in the migration assay. Bar, 612.9 and 286 μm. (B) Distance that cells migrated across the scratch line. C, Cytoplasmic laminin γ2 expression analyzed by Western blotting is also shown side by side. Relative quantitative levels are shown below band images. The migration distances were significantly correlated with the quantitative levels of laminin γ2 proteins ($p<0.05$).

LAMC2 promoter was methylated in 40% of the cell lines (SNU1, AZ521, MKN28, and MKN45) and partially or completely unmethylated in the remaining cell lines. The methylation status of LAMB3 and LAMC promoters was consistent with the lack of gene expression determined by RT-PCR.

Re-activation of the laminin-332-encoding genes expression by 5-aza-dC/TSA treatment. To further examine the role of CpG methylation and histone deacetylation in silencing of the laminin-332-encoding genes, cancer cell lines were treated with 5-aza-dC and/or TSA. 5-aza-dC restored expression of the laminin-332-encoding genes in cancer cell lines (Fig. 5). Treatment with TSA alone had no effect. Combined treatment with 5-aza-dC and TSA restored expression of the laminin-332-encoding genes synergistically in several cancer cell lines.

Migration activity is associated with laminin γ2 protein expression. In the scratch assay, NUGC3, SNU638 and JRST were more motile than other cell lines (Fig. 6A and B). The migration distances were significantly correlated with quantitative levels of cytoplasmic laminin γ2 proteins determined by Western blot analysis ($p<0.05$; Fig. 6C).

Methylation of laminin-332-encoding genes in gastric cancer tissues. Methylation status of laminin-332-encoding genes was analyzed by MSP in gastric cancer tissues. Methylation of

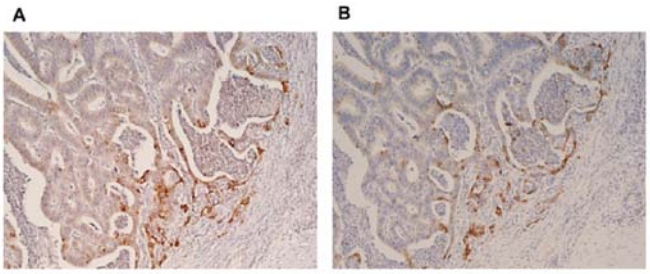


Figure 7. Representative results of immunohistochemistry for laminin β3 (A) and γ2 (B) in gastric cancer tissues. Original magnification, x400.

LAMA3, LAMB2 and LAMC2 genes was detected in 70, 24 and 20% of the 50 gastric cancer tissues, respectively. Methylation frequency of LAMA3 was significantly higher than methylation frequencies of LAMB2 and LAMC2 in gastric cancer tissues ($p<0.001$). Methylation status of laminin-332-encoding genes was not siginificantly correlated with clinicopathological features (data not shown).

Association of the expression of laminin β3 and γ2 chains with clinicopathological characteristics. Fig. 7 shows representative results of immunohistochemistry for laminin β3 and γ2 chains. Expression of laminin α3 was detected in normal BMs but not in carcinoma cells (data not shown). There was no detectable immunoreactivity with negative controls (data not shown). Positive staining for laminin β3 and γ2 chains in the cytoplasm of carcinoma cells was observed in 50 (56%) and 53 (59%) of the gastric cancer tissues. Notably, laminin β3 and γ2 chains were significantly coexpressed ($p< 0.05$). For both laminin β3 and γ2 chains, positivity was significantly correlated with depth of invasion and advanced tumor stage ($p<0.05$).

Discussion

In the present study, analysis of expression profiles of laminin-332-encoding genes by RT-PCR showed that LAMB3 mRNA and LAMC2 mRNA were often coexpressed in gastric cancer cell lines. These results suggest that LAMB3 and LAMC2 genes are regulated by a similar mechanism or undergo gene transcription dependently. In contrast, LAMA3 mRNA expression was not detected in any of the cell lines, suggesting that transcription of the LAMA3 gene is regulated by a mechanism different from that for LAMB3 and LAMC2 genes.

Coexpression of laminin β3 and γ2 chains in gastric cancer cell lines was further supported by results of Western blot analyses of cultured cell lysates and immunostaining. These results suggest that cultured gastric cancer cells generate laminin β3 and γ2 chains during autonomous growth *in vitro*.

Of note, laminin β3 and γ2 chains were also assumed to be secreted into culture media by JRST and MKN7 cell lines. This notion is supported by previous data showing that tumor cell lines expressing laminin β3 and γ2 chains, but not the α3 chain, could secrete γ2 monomer or β3γ2 heterodimer (14,15). Simultaneous detection of processed (105 kDa) and nonprocessed (155 kDa) laminin γ2 chain in the conditioned medium of MKN7, which was not observed in the cell lysate of MKN7, is consistent with the results of a previous study showing that a γ2 subunit is digested into a mature form after extracellular secretion (16).

By using MSP, we demonstrated that silencing of the LM-332-encoding genes was correlated with hypermethylation of the promoter in gastric cancer cell lines. To confirm the role of epigenetic alterations in transcriptional repression of the LM-332-encoding genes, we treated gastric cancer cell lines, in which the LM-332-encoding genes was methylated, with 5-aza-dC alone or in combination with TSA. Treatment with 5-aza-dC restored the LM-332-encoding genes expression in cancer cell lines. Moreover, combined treatment with 5-aza-dC and TSA restored expression synergistically, indicating CpG methylation and histone deacetylation have an important role in silencing the LM-332-encoding genes.

Epigenetic inactivation of LM-332-encoding genes through aberrant promoter methylation has been reported in lung, bladder, breast, and prostate cancer cell lines and cancer tissues (11,17-19). Frequencies of LAMA3 methylation have generally been higher than frequencies of methylation of LAMB3 and LAMC. Similar results were obtained in the present study, suggesting that epigenetic regulation of LM-332-encoding genes, especially LAMA3 silencing, plays a role in the progression of gastric cancer. The LM-332 molecule has been demonstrated to be synthesized initially as three monomers of each subunit, which are then glycosylated (16). Subsequently, β3 and γ2 chains assemble into a β3γ2 heterodimer, followed by incorporation of the α3 subunit to form an α3β3γ2 heterotrimer. Therefore, frequent laminin α3 chain suppression due to DNA methylation may play a critical role in the cytoplasmic accumulation of laminin β3 and γ2 chains in gastric cancer cells.

The laminin γ2 monomer is frequently expressed in invasive carcinomas and has recently been recognized as an excellent invasion marker for epithelial tumors (3,6-8). In this regard, it is interesting that cytoplasmic laminin γ2 chain expression was significantly associated with gastric cancer cell motility *in vitro* as shown by the migration assay. LM-332 is a constituent of the BM that provides static adhesion and hemidesmosome formation, but the monomeric laminin γ2 chain does not act as a morphogenesis protein. The results suggest that the cytoplasmic laminin γ2 chain plays a positive role in gastric cancer cell motility.

The results of *in vitro* expression studies were further supported by results for primary tumor tissues. Laminin β3 and γ2 chains were often coexpressed in cancer cells of gastric cancer tissues, and both of them were associated with depth of invasion and advanced tumor stage. The results suggest that coexpression of laminin β3 and γ2 chains is involved in the aggressive phenotype of gastric cancer cells, resulting in the progression of disease. Previous immunohistochemical studies have shown significant coexpression of laminin β3 and γ2 chains in the cytoplasm of invading or budding tumor cells in colorectal carcinoma, lingual squamous cell carcinoma, hepatocellular carcinoma, and basal cell carcinoma of the skin (4,9,20,21). We have also reported overexpression of laminin β3 and γ2 chains in biliary cancer (3). Our results further support the notion that laminin β3 and γ2 chains play an important role in cancer progression.

Mechanisms underlying cytoplasmic accumulation of laminin β3 and γ2 chains in cancer cells *in vivo* are not

known. Firstly, interactions of cancer cells with stromal cells may promote an accumulation of laminin β3 and γ2 chains (3). Secondly, it is thought that suppression of α3 chain expression may hinder LM-332 trimer secretion, leading to abnormal accumulation of the β3γ2 heterodimer (4). Our results support the latter possibility, but further investigation is necessary.

Loss of any of the five component chains of the laminin-integrin complex (LM-332 and α6β4) could disrupt the hemidesmosome and BM, leading to cancer cell invasion (11,18). Loss of LM-332 could favor disassembly or reduction in the number of hemidesmosomes with a consequent failure of cell anchoring, leading to an invasive and metastatic phenotype (11). While loss of any chains results in loss of the functional molecule, expression of one or more chains (especially β3 and γ2) may promote cancer cell invasion (2). Since LM-332 is a component of the BM, which is a major barrier for invasion of cancer, detection of methylation of LM-332-encoding genes may be useful for distinguishing aggressive gastric cancers from non-aggressive ones (11).

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (H.Y., K.I. and Y.S.) and supported in part by Program for developing the supporting system for upgrading the education and research from the Ministry of Education, Culture, Sports, Science, and Technology (Y.S.).

References

1. Colognato H and Yurchenco PD: Form and function: the laminin family of heterotrimers. Dev Dyn 218: 213-234, 2000.
2. Giannelli G and Antonaci S: Biological and clinical relevance of laminin-5 in cancer. Clin Exp Metastasis 18: 439-443, 2000.
3. Oka T, Yamamoto H, Sasaki S, Ii M, Hizaki K, Taniguchi H, Adachi Y, Imai K and Shinomura Y: Overexpression of β3/γ2 chains of laminin-5 and MMP7 in biliary cancer. World J Gastroenterol 15: 3865-3873, 2009.
4. Sordat I, Bosman FT, Dorta G, Rousselle P, Aberdam D, Blum AL and Sordat B: Differential expression of laminin-5 subunits and integrin receptors in human colorectal neoplasia. J Pathol 185: 44-52, 1998.
5. Olsen J, Kirkeby LT, Brorsson MM, Dabelsteen S, Troelsen JT, Bordoy R, Fenger K, Larsson L-I and Simon-Assmann P: Converging signals synergistically activate the LAMC2 promoter and lead to accumulation of the laminin gamma 2 chain in human colon carcinoma cells. Biochem J 371: 211-221, 2003.
6. Yamamoto H, Itoh F, Iku S, Hosokawa M and Imai K: Expression of the γ2 chain of laminin-5 at the invasive front is associated with recurrence and poor prognosis in human esophageal squamous cell carcinoma. Clin Cancer Res 7: 896-900, 2001.
7. Koshikawa N, Moriyama K, Takamura H, Mizushima H, Nagashima Y, Yanoma S and Miyazaki K: Overexpression of laminin γ2 chain monomer in invading gastric carcinoma cells. Cancer Res 59: 5596-5601, 1999.
8. Yamamoto H, Kitadai Y, Yamamoto H, Oue N, Ohdan H, Yasui W and Kikuchi A: Laminin gamma2 mediates Wnt5a-induced invasion of gastric cancer cells. Gastroenterology 137: 242-252, 2009.
9. Giannelli G, Fransvea E, Bergamini C, Marinosci F and Antonaci S: Laminin-5 chains are expressed differentially in metastatic and nonmetastatic hepatocellular carcinoma. Clin Cancer Res 9: 3684-3691, 2003.
10. Giannelli G, Bergamini C, Fransvea E, Sgarra C and Antonaci S: Laminin-5 with transforming growth factor-beta 1 induces epithelial to mesenchymal transition in hepatocellular carcinoma. Gastroenterology 129: 1375-1383, 2005.
11. Sathyanarayana UG, Toyooka S, Padar A, Takahashi T, Brambilla E, Minna JD and Gazdar AF: Epigenetic inactivation of laminin-5-encoding genes in lung cancers. Clin Cancer Res 9: 2665-2672, 2003.
12. Cameron EE, Bachman KE, Myohanen S, Myöhänen S, Herman JG and Baylin SB: Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21: 103-107, 1999.
13. Zhou Y, Zhang J, Liu Q, Bell R, Muruve DA, Forsyth P, Arcellana-Panlilio M, Robbins S and Yong VW: The chemokine GRO-α (CXCL1) confers increased tumorigenicity to glioma cells. Carcinogenesis 26: 2058-2068, 2005.
14. Niessen CM, Hogervorst F, Jaspars LH, de Melker AA, Delwel GO, Hulsman EH, Kuikman I and Sonnenberg A: The alpha 6 beta 4 integrin is a receptor for both laminin and kalinin. Exp Cell Res 211: 360-367, 1994.
15. Mizushima H, Miyagi Y, Kikkawa Y, Yamanaka N, Yasumitsu H, Misugi K and Miyazaki K: Differential expression of laminin-5/ladsin subunits in human tissues and cancer cell lines and their induction by tumor promoter and growth factors. J Biochem 120: 1196-1202, 1996.
16. Matsui C, Wang CK, Nelson CF, Bauer EA and Hoeffler WK: The assembly of laminin-5 subunits. J Biol Chem 270: 23496-23503, 1995.
17. Sathyanarayana UG, Maruyama R, Padar A, Suzuki M, Bondaruk J, Sagalowsky A, Minna JD, Frenkel EP, Grossman HB, Czerniak B and Gazdar AF: Molecular detection of noninvasive and invasive bladder tumor tissues and exfoliated cells by aberrant promoter methylation of laminin-5 encoding genes. Cancer Res 64: 1425-1430, 2004.
18. Sathyanarayana UG, Padar A, Huang CX, Suzuki M, Shigematsu H, Bekele BN and Gazdar AF: Aberrant promoter methylation and silencing of laminin-5-encoding genes in breast carcinoma. Clin Cancer Res 9: 6389-6394, 2003.
19. Sathyanarayana UG, Padar A, Suzuki M, Maruyama R, Shigematsu H, Hsieh J-T, Frenkel EP and Gazdar AF: Aberrant promoter methylation of laminin-5-encoding genes in prostate cancers and its relationship to clinicopathological features. Clin Cancer Res 9: 6395-6400, 2003.
20. Akimoto S, Nakanishi Y, Sakamoto M, Kanai Y and Hirohashi S: Laminin 5 beta3 and gamma2 chains are frequently coexpressed in cancer cells. Pathol Int 54: 688-692, 2004.
21. Svensson Mansson S, Reis-Filho J and Landberg G: Transcriptional upregulation and unmethylation of the promoter region of p16 in invasive basal cell carcinoma cells and partial co-localization with the gamma 2 chain of laminin-332. J Pathol 212: 102-111, 2007.