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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