Epigenetic Inactivation of Laminin-5-encoding Genes in Lung Cancers

Ubaradka G. Sathyanarayana, Shinichi Toyooka, Asha Padar, Takashi Takahashi, Elizabeth Brambilla, John D. Minna, and Adi F. Gazdar

Hamon Center for Therapeutic Oncology Research [U. G. S., S. T., A. P., J. D. M., A. F. G.] and Departments of Pathology [U. G. S., A. F. G.] and Internal Medicine [J. D. M.], University of Texas Southwestern Medical Center, Dallas, Texas; Division of Molecular Oncology, Aichi Cancer Center, Nagoya; and Departments of Pathology [U. G. S., S. T., A. F. G.] and Internal Medicine [J. D. M.], University of Texas Southwestern Medical Center, Dallas, Texas 75390-8593; Division of Molecular Oncology, Aichi Cancer Center, Nagoya 464-8681, Japan [T. T.]; and Laboratoire de Pathologie Cellulaire, Centre Hospitalier Regional Universitaire, Grenoble 38043, France [E. B.]

ABSTRACT

Purpose: We investigated the loss of expression of three laminin-5 (LN5)-encoding genes in lung cancer cell lines and elucidated the mechanism of inactivation of the genes in lung cancer cell lines and tumors.

Experimental Design: We examined the expression of LN5-encoding genes by reverse transcription-PCR in 49 lung cancer cell lines. To elucidate the mechanism of gene silencing, we treated expression-negative cell lines (two for each gene) with a demethylating agent and examined the restoration of expression by reverse transcription-PCR. We dissected out the methylation patterns of CpG sites unique to the promoter regions of LN5-encoding genes by bisulfite genomic sequencing of expression-negative cell lines. We designed methylation-specific primers and validated the methylation status of the promoter regions in lung cancer cell lines using methylation-specific PCR. We further studied the methylation patterns of primary non-small cell lung cancer [NSCLC (n = 36)], small cell lung cancer [SCLC (n = 26)], and carcinoids (n = 24) tumors.

Results: We observed frequent losses of expression in NSCLC (20–60%) and SCLC (65–86%) cell lines. Expression of one or more genes was lost in 90% of SCLC cell lines and 65% of NSCLC cell lines. Treatment of expression-negative cell lines with demethylating agent restored expression in all of the cases. Methylation of LN5-encoding genes was present more frequently in SCLC cell lines (60–80%) than in NSCLC cell lines (15–60%), and at least one gene was methylated in 95% of SCLC and 60% of NSCLC cell lines. The concordances between loss of expression and methylation in 40 lung cancer cell lines for the three genes (90–95%) were statistically significant. Methylation was more frequent in SCLC tumors (58–77%) than in NSCLC tumors (22–42%) and carcinoids (13–33%), and at least one gene was methylated in 92% of SCLC tumors, 47% of NSCLC tumors, and 33% of carcinoids.

Conclusions: Our results demonstrate frequent epigenetic inactivation of LN5-encoding genes in lung cancers, and these findings are of biological interest and are potentially of clinical importance.

INTRODUCTION

BMs are characteristic of multicellular organisms, and they are the first ECM component produced during embryogenesis. The BM is a thin (20–200-nm) carpet-like ECM structure that regulates cell attachment, differentiation, and growth. It is a flat structure separating the epithelial cells from the underlying stromal tissues and forms an important barrier to invasion. Epithelial cells must be attached both to appropriate ECM components and to other similar epithelial cells to survive, a process termed anchorage dependence. ECM consists of several molecules; two of the most important are LN5 (secreted by the overlying epithelial cells) and collagen 4 (secreted by the stromal cells). Laminins, which are the major component of BM, play a major role in anchorage dependence. Whereas in situ carcinomas are intraepithelial by definition, invasion and destruction of the BM is the earliest morphological feature of invasive carcinomas.

The epithelium and underlying stroma function as a unit and constantly communicate. Two-way signaling occurs via extracellular proteins (laminins) and their transmembrane receptors, the integrins. In the BM, especially at specialized attachment units known as hemidesmosomes, the crucial integrin is α6β4, and its ligand is LN5. LN5 is a heterotrimetric protein member of the laminin family, and it consists of three polypeptide chains, A3, B3, and C2, which are the products of three different genes, LAMA3, LAMB3, and LAMC2 (3). The chains are assembled in a coiled cruciate-like structure, which is deposited in the BM. LN5 is specific to epithelium and is one of the pivotal hemidesmosomal proteins involved in the structural...
relationship between the epithelium and stroma. In addition to $\alpha_6\beta_4$, it can bind to $\alpha_5\beta_1$ integrins.

Multiple mechanisms of gene silencing including loss of heterozygosity, point mutations, homozygous deletions, and aberrant promoter methylation have been reported in tumors (4). Aberrant methylation of CpG-rich sites (CpG islands) was identified as an epigenetic mechanism for the transcriptional silencing of tumor suppressor genes in many cancer types, and the number of methylated genes in individual cancers is estimated to be very high (5–7). Down-regulation of LN5 chains and hemidesmosomal component proteins has been reported in breast (8, 9), prostate (10), lung (11), colon (12), and other types of cancers. To investigate whether LN5-encoding genes are silenced by epigenetic phenomenon in lung cancers, we studied the methylation status of promoters of the three genes in lung cancer cell lines, tumors, and nonmalignant tissues. We report here that LAMA3, LAMB3, and LAMC2 genes are transcriptionally silenced by aberrant methylation of their respective promoters and that there is a high degree of concordance between loss of expression and methylation.

**MATERIALS AND METHODS**

**Cell Lines.** Human lung cancer cell lines (20 NSCLC lines and 29 SCLC lines) and B-lymphoblastoid cell lines (n = 2) were established by us (13, 14). Most NSCLC lines were established from primary tumors, and most SCLC lines were established from metastases. Cell cultures were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum and incubated in 5% CO$_2$ at 37°C.

**Clinical Samples.** Tumor samples from 36 primary NSCLCs (19 adenocarcinomas, 15 squamous cell carcinomas, 1 large cell carcinoma, and 1 adenosquamous carcinoma) and 21 corresponding nonmalignant lung tissues were obtained from surgical resections performed at the University of Texas Southwestern Medical Center and M. D. Anderson Cancer Center (Houston, TX). Tumor samples from 26 primary SCLCs and 24 bronchial carcinoids were obtained from surgical resections performed in the United States, France, and Japan. For gene expression studies, six nonmalignant tissue samples (four bronchial brushes, one airway epithelial cell culture, and two peripheral lung tissues) were obtained as far from the tumor tissue as possible. Epithelial cells from buccal swabs of 12 healthy non-smoking volunteers and peripheral blood lymphocytes from 14 healthy volunteers were also obtained. Appropriate institutional review board permission was obtained at participating centers, and written informed consent was obtained from all subjects. Tissues were stored at −80°C before testing.

**Expression of LAMA3, LAMB3, and LAMC2.** Expression of LN5-encoding genes was analyzed by the RT-PCR technique. Total RNA was extracted from 49 lung cancer cell lines, 4 bronchial brushes, 1 airway epithelial cell culture, and 2 peripheral lung tissues by using TRIzol reagent (Life Technologies, Inc.) following the manufacturer’s instructions. Two μg of total RNA treated with DNase I (1 unit; Life Technologies, Inc.) were reverse transcribed into cDNA using Superscript II First-Strand Synthesis System (Life Technologies, Inc.) at 42°C for 52 min using oligo(dT) primer according to the manufacturer’s instructions. The resulting cDNA was subjected to PCR using primers and temperature conditions as described in Fig. 2. For each primer set, the sense and antisense primer pairs were located on different exons to avoid amplification of contaminating genomic DNA. The housekeeping gene β-actin was used as an internal control to confirm the success of the RT-PCR (15). PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

**5-Aza-CdR Treatment.** Six tumor cell lines with loss of expression for one or more of the three genes were treated with the demethylating agent 5-aza-CdR (2 μg/ml) as described previously (16, 17).

**DNA Extraction.** Genomic DNA was extracted from cell lines, primary tumors, and nonmalignant cells by digestion with proteinase K (Life Technologies, Inc.) for 1 day at 50°C, followed by two extractions with phenol:chloroform (1:1; Ref. 18).

**Bisulfite Genomic Sequencing.** DNA was modified by sodium bisulfite as described previously (19). Modified DNA was stored at −80°C until use. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which is then converted to thymidine during subsequent PCR (19). To determine the methylation status of CpGs in the promoter regions of LAMA3, LAMB3, and LAMC2, primers specific to non-CpG regions were designed to perform PCR (see Fig. 1, A–C). The sequence information of cDNA (LAMA3, NM_000227; LAMB3, NM_000228; and LAMC2, NM_005562) and promoter region DNA was obtained from the University of California Santa Cruz genome browser. Using 2 μl of resuspended sodium bisulfite-treated DNA (from RT-PCR-positive and RT-PCR-negative cell lines; Fig. 1, A–C), PCR was performed in a 25-μl reaction using Hot star enzyme (Qiagen, Valencia, CA) as described in Fig. 1. PCR products were visualized on 2% agarose gels stained with ethidium bromide. The PCR products were gel purified, ethanol precipitated, and sequenced by the Applied Biosystems PRISM dye terminator cycle sequencing method from both ends by using the same primers used for amplification.

**MSP.** The MSP reaction uses an initial bisulfite reaction to modify the DNA (19) as described above. After bisulfite treatment, alleles that were originally methylated have DNA sequences different from those of their corresponding unmethylated alleles, and these differences can be used to design PCR primers that are specific for methylated or unmethylated alleles. Based on the bisulfite genomic sequencing information on methylation status of CpGs in the promoter regions of LAMA3, LAMB3, and LAMC2 (Fig. 1, A–C), we designed two sets of methylating-specific (i.e., unmodified by bisulfite treatment) and unmethylation-specific primers (i.e., modified by bisulfite to UpG) to amplify each region of interest. The primer sequences and temperature conditions are described in Fig. 2. p16 unmethylated primer was used as control in MSP to check the integrity of tissue-extracted bisulfite-treated DNA (19). DNA from peripheral blood lymphocytes (n = 14) and buccal swabs (n = 12) from healthy nonsmoking subjects was used as negative control for MSP assays. DNA from lymphocytes of healthy
volunteers treated with 5-aza-2′-deoxycytidine (New England Biolabs, Beverly, MA) and subjected to bisulfite treatment was used as a positive control for methylated alleles. Water blanks and PCR mixtures (without template) were used as negative controls in each assay. PCR products were visualized on 2% agarose gels stained with ethidium bromide. Results were confirmed by repeating bisulfite treatment and MSP assays for all samples.

**DNA Sequencing of MSP Products.** The MSP products of 12 cell lines (four for each gene) with promoter methylation were isolated from the gels and purified. After amplification with the same primers used for MSP, 20 ng of PCR products were sequenced by ABI automated sequencer.

**Data Analysis.** The frequencies of loss of expression and methylation between two groups were compared using \( \chi^2 \) test and Fisher’s exact test with continuity correction. To compare the overall losses in expression and degree of methylation for all three of the genes examined, we calculated the chain LI and MI, respectively. The chain LI is defined as the number of chains with lost expression in each cell line. The MI is defined as the total number of genes methylated divided by the total number of genes analyzed. Then the mean values of expression loss and MI were determined. The methylation indices of different groups were compared using the Mann-Whitney nonparametric \( U \) test. For all of the tests, probability values of \( P < 0.05 \) were considered statistically significant. All of the statistical tests were two-sided.

**RESULTS**

**Expression of LN5-encoding Genes LAMA3, LAMB3, and LAMC2 in Lung Cancer Cell Lines and Nonmalignant Tissues.** RT-PCR analysis revealed expression of LN5 genes in all control tissues (samples of bronchial brushes, airway epithelial cells, and peripheral lung tissues). However, expression was lost in 12 of 20 (60%), 4 of 20 (20%), and 5 of 20 (25%) NSCLC cell lines and in 25 of 29 (86%), 20 of 29 (69%), and 19 of 29 (65%) SCLC cell lines for LAMA3, LAMB3, and LAMC2 genes, respectively (Fig. 2, A–C). The differences in loss of expression between NSCLC and SCLC cell lines for all three of the genes were compared using \( \chi^2 \) test, and they were statistically significant (\( P = 0.034 \) for LAMA3, \( P = 0.001 \) for LAMB3, and \( P = 0.002 \) for LAMC2). In NSCLC cell lines, 13 of 20 (65%) lost at least one of the three chains, whereas in SCLC cell lines, 26 of 29 (90%) lost at least one of the three chains (Fig. 3A). The above differences in chain loss between NSCLC and SCLC cell lines were statistically significant (\( P = 0.04 \)). Mean chain loss in SCLC cell lines (mean ± SE, 2.24 ± 0.190) was statistically significant (\( P = 0.004 \)) from mean chain loss in NSCLC cell lines (mean ± SE, 1.05 ± 0.223). All six cell lines tested (two for each gene) with loss of gene expression had expression restored after treatment with the demethylating agent 5-Aza-CdR (Fig. 3B).

**Aberrant Promoter Methylation of LAMA3, LAMB3, and LAMC2 Genes in Lung Cancer Cell Lines, Lymphocytes, and Buccal Swabs.** To examine the methylation patterns of the 5′-region of LNS5 genes, we designed methylation-independent primers (to non-CpG sites) for each gene separately and did bisulfite genomic sequencing of DNA from RT-PCR.
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Expression and MSP analysis of LN5-encoding genes in representative lung cancer cell lines. *Lanes 1–10*, lung cancer cell lines NCI-H522 and NCI-H1703 (NSCLC); NCI-H2141 and NCI-H1963 (SCLC); and NCI-H1770, NCI-HCCS15, NCI-HCC78, NCI-H2009, NCI-H2595, and NCI-H1395 (NSCLC), respectively. E, expression by RT-PCR; U, unmethylated form; M, methylated form. Positive control (Lane *P*), RNA from bronchial brushes or airway epithelial cells or /H11032/H11001, sense (/, antisense (/H11001/H11032), M primers: 5'-TATAGGAATTATAGAGTGGTGT-3' specific (/H11032/H11002), antisense (M) primers: 5'-TATAGGAATTATAGAGTGGTGT-3'.

Reactions were hot started at 95 °C for 30 s, and 72 °C for 1 min; followed by 1 cycle of 72 °C for 5 min. Temperature conditions for PCR for U primer were as follows: 35 cycles of 95 °C for 30 s, and 72 °C for 1 min; followed by 1 cycle of 72 °C for 5 min. Temperature conditions for PCR for M primer were as follows: 7 cycles of 95 °C for 12 min. Temperature conditions for PCR for U primer were as follows: 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; followed by 1 cycle of 72 °C for 5 min. Temperature conditions for PCR for U primer were as follows: 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; followed by 1 cycle of 72 °C for 5 min. White arrow indicates both unmethylated and methylated form in H1770 cell line. B, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line. C, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line. D, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line. E, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line. F, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line. G, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line. H, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line. I, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line. J, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line. K, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line. L, agarose gel showing RT-PCR products (491 bp) and MSP products (unmethylated form, 271 bp; methylated form, 269 bp) showing RT-PCR products (491 bp) and MSP products (unmethylated and methylated form in H1770 cell line.
Examples are illustrated in Fig. 4A. The differences in methylation and MI between NSCLC and SCLC tumors for LAMC2 ($P = 0.0043$), LAMB3 ($P < 0.0001$), and MI ($P = 0.0002$) were statistically significant, as were those between NSCLC and neuroendocrine tumors for LAMB3 ($P = 0.004$) and those between SCLC tumors and carcinoids for LAMA3 ($P = 0.0005$), LAMB3 ($P = 0.002$), LAMC2 ($P < 0.001$), and MI ($P < 0.0001$). The differences in methylation between nonmalignant and malignant samples were statistically significant ($P < 0.0001$). The presence of unmethylated p16 promoter sequences in all of the tissues analyzed confirmed the integrity of the DNA in these samples (Fig. 4A). Differences in methylation between respective tumors and their cell lines were not significant. Aberrant methylation was present at a low frequency in nonmalignant tissues from NSCLC resections (Table 1). The corresponding tumor samples were also methylated in the two cases where the nonmalignant tissues were methylated. In tumor samples, which consist of mixtures of tumor cells and nonmalignant cells, either the unmethylated band only or both the methylated and unmethylated bands were present (data not shown). The differences in methylation frequencies between NSCLC primary tumors of adenocarcinoma and squamous cell carcinoma for all three of the genes of LN5 (Table 1) were not statistically significant. In NSCLC tumors, 17 of 36 (47%) had at least one of the three chains methylated, whereas in SCLC tumors, 24 of 26 (92%) had at least one of the three chains methylated. In carcinoids, 8 of 24 (33%) had at least one of the three chains methylated (Fig. 4B). The above-mentioned differences in the number of chains methylated between NSCLC and SCLC tumors ($P = 0.0003$) and between SCLC tumors and carcinoids ($P < 0.0001$) were statistically significant. Differences in mean chain MI between NSCLC tumors (mean ± SE, 0.889 ± 0.186) and SCLC tumors (mean ± SE, 2.00 ± 0.175; $P = 0.0002$) and between SCLC tumors (mean ± SE, 2.00 ± 0.175) and carcinoids (mean ± SE, 0.625 ± 0.207; $P < 0.0001$) were statistically significant.

### DNA Sequencing of MSP Products

We sequenced MSP products of methylated LAMA3 [in four cell lines (HCC2157, HCC1500, NCI-H1155, and NCI-H1703)], LAMB3 [in four cell lines (NCI-H1994, NCI-H2196, NCI-H1963, and NCI-H2029)], and LAMC2 [in four cell lines (HCC1569, HCC1954, NCI-H249, and NCI-H522)] amplicons. There were 9 (in LAMA3), 5 (in LAMB3), and 14 (in LAMC2) CpGs in respective amplicons, and they were all methylated, confirming the uniform methylation of CpGs in the amplicons of promoter regions of respective genes in those cell lines from which we sequenced the MSP products.

### DISCUSSION

Metastases are the major cause of cancer deaths. To metastasize and grow, neoplastic cells must invade and migrate into surrounding tissues. The ability to block these processes offers a new approach to treating and perhaps preventing invasive cancers. The roles of the components of BM in tumorigenesis are complex (21). The reported role of LN5 in tumorigenesis shows apparent dichotomy, in part because immunostaining techniques do not examine all three gene products. In breast cancer, a down-regulation of LN5 mRNA (for all three genes) has been observed, and a role for LN5 in controlling tumor growth has therefore been proposed (9).

To understand the role of LN5-encoding genes in lung cancers, we examined the expression of LAMA3, LAMB3, and LAMC2. All of the three genes were expressed in bronchial cells, cultured airway epithelial cells, and lung tissues, whereas lung cancer cell lines had varying frequencies of losses of all
three genes, especially of LAMA3. Treatment with 5-Aza-CdR restored the expression of all of the three genes in RT-PCR-negative cell lines, indicating methylation as a mechanism of transcriptional silencing of the LAMA3, LAMB3, and LAMC2 genes. Of interest, as reported previously (22) in a cDNA microarray analysis to screen for genes that are epigenetically silenced, LAMA3 was up-regulated by demethylation and histone deacetylation inhibition in a human colorectal cancer cell line. The methylation frequencies of both NSCLC and SCLC tumors were not significantly different from their respective cell lines, indicating that cell lines are suitable models for studying promoter methylation of LN5 genes in SCLC tumors, whereas NSCLC tumors showed predominantly inactivation of any one gene. Theoretically, loss of any of the five component chains of this laminin-integrin complex could disrupt the hemidesmosome and lead to invasion (27-30). LN5 can be down- or up-regulated, depending on specific microenvironmental features, whereas its absence could favor disassembly or reduction in the number of hemidesmosomes with a consequent failure of cell anchoring leading to an invasive and metastatic phenotype. Thus, whereas loss of any chain results in loss of the functional molecule, unopposed expression of one or more chains (especially C2) may aid invasion (3). The loss of LN5 may cause perturbations of the ECM and integrin signaling, affecting growth factors and cell cycle regulators and apoptosis. As postulated, these gene expression changes may cause potential genetic instability in lung carcinoma due to loss of an ECM protein (31). Because LN5 is a component of BM, which is a major barrier for invasion of cancer, we presume that methylation of LN5 genes may help distinguish invasive from noninvasive cancers. We have found that methylation of LN5-encoding genes distinguishes invasive from noninvasive bladder cancers.5

Our data satisfy the criteria required for the demonstration of biological significance of methylation (24, 32): (a) aberrant methylation is frequent in tumors; (b) methylation is a rare event in nonmalignant and control tissues; (c) loss of expression is frequent in tumors; (d) aberrant methylation and expression are concordant; and (e) gene expression is restored after exposure to a demethylating agent. Our results strongly suggest that silencing of LAMA3, LAMB3, and LAMC2 genes by...
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logical and potential clinical importance. has been reported in SCLC (34, 35). Our findings are of bio-
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confirm and extend these findings and demonstrate the mecha-
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of lung cancers.
methylation plays an important role in pathogenesis of all types
of lung cancers.

Higher frequencies of loss of LN5 chains have been ob-
erved in SCLC, as compared with NSCLC (11). Our results
confirm and extend these findings and demonstrate the mecha-
ism of loss of the LN5 chains. Whereas promoter methylation
of LN5-encoding genes appears to be an important factor in the
pathogenesis of invasive lung cancers, alternative mechanisms
for disruption of the BM may exist. These include inactivation
of LN5-encoding genes by mechanisms other than methylation,
loss of \( \alpha_5\beta_4 \)-encoding genes, or overexpression of matrix met-
alloproteinase 9 (33). Of interest, selective loss of integrin \( \beta_4 \)
has been reported in SCLC (34, 35). Our findings are of bi-
ological and potential clinical importance.

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