Laminin-5–Encoding Genes in Breast Carcinoma

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ABSTRACT

Purpose: Down-regulation of Laminin-5 (LN5)–encoding genes (LAMA3, LAMB3, and LAMC2) has been reported in various human cancers. However, the mechanism of inactivation was not clearly understood until recently. In this study, we investigated the loss of expression of three LN5-encoding genes in breast cancer cell lines and elucidated the mechanism of silencing of the genes in breast cancer cell lines and tumors.

Experimental Design: We examined the expression of the three LN5-encoding genes by reverse transcription-PCR in breast cancer cell lines (n = 20). To elucidate the mechanism of silencing, we treated expression negative cell lines (n = 5) with a demethylating agent and examined restoration of expression by reverse transcription-PCR. By using methylation-specific primers designed by us, we validated the methylation status of the promoter regions in breast cancer cell lines using methylation-specific PCR. We additionally studied the methylation patterns in primary breast tumors (n = 74) and correlated the data with clinical parameters.

Results: We observed varied losses of expression (10–55%) of LN5-encoding genes in breast cancer cell lines. Expression of one or more genes was lost in 65% of breast cancer cell lines. Treatment of expression negative cell lines with demethylating agent restored expression in all cases. Methylation frequencies of LAMA3, LAMB3, and LAMC2 genes in 20 breast cancer cell lines were 40, 5, and 15%, respectively. The concordances between loss of expression and methylation in 20 breast cancer cell lines for the three genes (85–95%) were statistically significant. Nonmalignant breast tissues (n = 30) had very low frequencies of methylation (0–7%). In 74 breast tumors, methylation frequencies LAMA3, LAMB3, and LAMC2 were 44, 4, and 20%, respectively. The differences in methylation frequencies between cell lines and tumors were not statistically significant for all of the three genes. The methylation frequencies of LAMA3 and mean chain methylation index in cell lines and tumors were significantly different from methylation frequencies in nonmalignant tissues, and they were significantly higher in high stage and large size tumors as compared with low-stage and small size tumors. LAMA3 promoter methylation frequency in breast tumors was associated with increased tumor stage (P < 0.001) and tumor size (P < 0.001).

Conclusions: Our results demonstrate epigenetic inactivation of LN5-encoding genes in breast cancers and association of LAMA3 promoter methylation with increased tumor stage and tumor size. Our findings are of biological interest and potentially of clinical importance.

INTRODUCTION

Interactions between epithelial cells and extracellular matrix (ECM) are a prerequisite for the structural integrity and specialized function of breast epithelium (1–3). 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. Hemidesmosomes (HDs) are structures used by normal epithelia to adhere to basement membrane (BM). The major structural proteins of the HD are the integrin α6β4 and its ligand laminin 5 (LN5). In lobular and ductal structures of the breast, both myoepithelial and luminal cells bear HDs (4, 5). LN5, specific to epithelium, is a heterotrimERIC protein member of the laminin family and consists of three polypeptide chains α3, β3, and γ2, which are the products of three different genes LAMA3, LAMB3, and LAMC2. The chains are assembled in a coiled cruciate-like structure, which is deposited in the BM (6).

Although in situ carcinomas are intraepithelial by definition, invasion and destruction of the BM is the earliest morphological feature of invasive carcinomas. During progression from an in situ to an invasive phenotype, tumor cells break up their tight connections with neighboring cells, become motile, and subsequently force their path through surrounding obstacles such as BMs and dense interstitial mesenchyme (7). Alterations in BM structure and composition during malignant transformation of breast epithelium and in carcinoma progression have been described previously (2). Loss or down-regulation of LN5 and HDs have been reported in invasive lobular and ductal carcinomas (4, 8); however, the mechanism of inactivation of LN5–encoding genes has not been elucidated in breast cancers.

Multiple mechanisms of gene silencing, including loss of heterozygosity, point mutations, homozygous deletions and ab-
errant promoter methylation, have been reported in tumors (9). 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 (10–12). We have recently demonstrated that loss of LN5–encoding genes in lung cancers is because of aberrant methylation of their promoter regions (1). To investigate whether LN5–encoding genes are silenced by epigenetic phenomenon in breast cancers, we studied the methylation status of promoters of the three genes in breast cancer cell lines, tumors, and nonmalignant tissues and correlated methylation with clinicopathological parameters.

**MATERIALS AND METHODS**

**Cell Lines.** Human breast tumor cell lines (n = 20) were established by us (13). Cell cultures were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum and incubated in 5% CO2 at 37°C.

**Clinical Samples.** Surgically resected specimens from 74 breast tumors, 25 corresponding nonmalignant breast tissues, and 5 unpaired nonmalignant breast tissues from these patients were obtained from the Tumor and Tissue Repository of our center. The median age of the patients was 55 years (range, 31–90 years), and they consisted of whites (n = 24), blacks (n = 28), and Hispanics (n = 16), whereas the remaining six were of unknown race. By tumor-node-metastasis staging, the tumors consisted of tumor stage I (n = 13), II (n = 40), III (n = 11), and IV (n = 4). Tumor sizes were T1 (tumors ≤ 2 cm in diameter), n = 25; T2 (tumors between 2 and 5 cm in diameter), n = 25; T3 (tumors > 5 cm in diameter), n = 15; T4 (tumor of any size with distant metastases), n = 2; and the remaining (n = 7) were of unknown stage and size. Tumor stage and size categories were assigned using the criteria of the American Joint Committee on Cancer (14). The estrogen and progesterone receptor status was known for 50 (33 estrogen receptor positive and 17 estrogen receptor negative) and 49 (31 progesterone receptor status was known for 50 (33 estrogen receptor positive, 17 estrogen receptor negative, and 18 progesterone receptor negative) patients, respectively. The lobular/ductal status was known for 68 patients (11 lobular and 57 ductal), whereas Her 2/neu status was known for 42 patients (34 positive and 8 negative). For gene expression studies, RNA extracted from two nonmalignant breast tissue samples obtained as far from the tumor as possible and one nonmalignant breast epithelial cell culture were used. Epithelial cells from buccal swabs of 12 healthy nonsmoking volunteers and peripheral blood lymphocytes from 14 healthy volunteers were also obtained as controls for methylation assays (1). 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 reverse transcription-PCR technique. Total RNA was extracted from 20 lung cancer cell lines, 2 nonmalignant breast tissues, and the breast epithelial cell culture (n = 1) by using TRIzol (Life Technologies, Inc.) reagent 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)20 primer according to manufacturer’s instructions. The resulting cDNA was subjected to PCR using primers and temperature conditions as described elsewhere (1). The housekeeping gene 18S rRNA was used as an internal control to confirm the success of the reverse transcription reaction (15). PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

**5-Aza-CdR Treatment.** Five tumor cell lines with loss of expression for one or more of the three genes were treated with 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) (18).

**Methylation-Specific PCR (MSP).** The DNA was modified by sodium bisulfite treatment as described previously (19). Briefly, 1 μg of DNA was denatured by incubation with 0.2 m NaOH for 15 min at 37°C. Aliquots of 10 μl hydroquinone (30 μl; Sigma Chemical Co., St. Louis, MO) and 3 μl sodium bisulfite (pH 5.0, 520 μl; Sigma Chemical Co.) were added, and the solution was incubated at 52°C for 16 h. Treated DNA was purified by use of a Wizard DNA Purification System (Promega Corp., Madison, WI). Modified DNA was stored at −80°C until used. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which are then converted to thymidine during subsequent PCR (19). Thus, 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. The bisulfite-treated DNA was subjected to MSP using primers and temperature conditions as described elsewhere (1). Primers to detect the unmethylated form of P16 were used to confirm the integrity of tissue-extracted bisulfite-treated DNA (19). In this study, DNA from lymphocytes and nonmalignant breast tissues were used as negative controls for MSP assays. DNA from lymphocytes of healthy volunteers treated with 5-AzaCdr was then 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.

**DNA Sequencing of MSP Products.** The MSP products from 12 cell lines 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 methylation between two groups were compared using χ2 test and Fisher’s exact test with continuity correction. To compare the overall losses in expression and degree of methylation for all of the three genes examined, we calculated the chain loss index and methylation index (MI), respectively. The mean chain loss index is defined as mean number of chains 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 MI was determined. The mean chain MI is defined as the mean number of chains methylated/cell line. The methylation indices of different groups were compared using the Mann-Whitney U non-parametric test. Tests of trend for binary responses were carried out using the Cochran-Armitage test (for both tumor stage and tumor size). For continuous responses (trend in mean MI for increasing tumor stage and size), the Jonckheere-Terpstra Test was used. These analyses were carried out with either SAS or StatXact 3.0 software packages. For all of the tests, probability values $P < 0.05$ were considered as statistically significant. All of the statistical tests were two sided.

**RESULTS**

Expression of LN5-Encoding Genes: LAMA3, LAMB3, and LAMC2 in Breast Cancer Cell Lines and Nonmalignant Breast Tissues. Reverse transcription-PCR analysis revealed expression of LN5 genes in all control tissues (samples of nonmalignant breast tissues and breast epithelial cells). However, expression was lost in 11 (55%), 2 (10%), and 4 (20%) breast cancer cell lines for LAMA3, LAMB3, and LAMC2 genes, respectively (Fig. 1, A–C). Any one, two, three, and at least one gene frequencies of loss of expression were 40% (8 of 20), 25% (5 of 20), 0% (0 of 20), and 65% (13 of 20), respectively. The mean number of chains with lost expression/cell line (mean chain loss index) in breast cancer cell lines was 0.9 ± 0.17 (mean ± SE). All 5 cell lines tested with loss of gene expression had their expression restored after treatment with the demethylating agent 5-Aza-CdR (Fig. 2A).

Aberrant Promoter Methylation of LAMA3, LAMB3, and LAMC2 Genes in Breast Cancer Cell Lines, Tumors, and Control Tissues. To examine the methylation pattern of LN5-encoding genes in breast cancer samples and control tissues, we used the MSP assay as described in “Materials and Methods.” Results of aberrant methylation are detailed in Fig. 2B and representative examples are illustrated in Fig. 1 and Fig. 2C. Methylation frequencies of LAMA3, LAMB3, and LAMC2 in 20 breast cancer cell lines were 40, 5, and 15%, respectively, whereas in 74 breast tumors, methylation frequencies were 44, 4, and 20%, respectively. Both unmethylated and methylated bands were present in some cell lines at low frequencies (for LAMA3, 3 of 20, 15%; for LAMB3, 0 of 20, 0%; and for LAMC2, 0 of 20, 0%). The concordance between expression and methylation in 20 breast cancer cell lines for LAMA3, 17 of 20, 85%, $P = 0.001$; LAMB3, 19 of 20, 95%, $P = 0.002$; and LAMC2, 19 of 20, 95%, $P = 0.0002$ were statistically significant. Aberrant promoter methylation of the three genes was present at very low frequencies in peripheral blood lymphocytes (0–7%) and was completely absent in buccal swabs. We sequenced MSP products of methylated LAMA3, LAMB3, and LAMC2 amplicons from 12 cell lines. All of the CpGs in respective amplicons were 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. Nonmalignant breast tissues had very low frequency of methylation (for LAMA3, 2 of 30, 7%; for LAMB3, 0 of 30, 0%; and for LAMC2, 2 of 30, 7%). Differences in methylation between cell lines and tumors were not statistically significant (Fig. 2B). Methylation frequencies of any one gene, at least one gene and mean chain MI, were significantly higher in cell lines (for any one gene, 10 of 20, 50%, $P = 0.0007$; for at least one gene, 11 of 20, 55%, $P = 0.0008$ and mean ± SE = 0.6 ± 0.13, $P = 0.0009$) and tumors (for any one gene, 33 of 75, 44%, $P = 0.0003$; for at least one gene, 42 of 75, 56%, $P < 0.0001$ and mean ± SE = 0.7 ± 0.08, $P < 0.0001$) as compared with nonmalignant breast tissues (2 of 30, 6.7% and mean ± SE = 0.13 ± 0.08).

**Association of Aberrant Promoter Methylation of LAMA3, LAMB3, and LAMC2 Genes in Breast Tumors with Clinicopathological Features.** Frequencies of methylation of LN5-encoding genes in 74 tumors were correlated with patient age, race, estrogen receptor and progesterone receptor status, nodal status, lobular/ductal status, Her2/neu status, tumor stage, and tumor size. The promoter methylation frequency of LAMA3, at least one gene methylation frequency and mean chain MI, were significantly higher in large size ($T_1$ plus $T_2$) tumors (14 of 17, 82%, $P = 0.0002$; 15 of 17, 88%, $P = 0.002$ and mean ± SE = 1.1 ± 0.15, $P = 0.001$), respectively, as compared with small size ($T_1$ plus $T_2$) tumors (15 of 50, 30%; 22 of 50, 44%...
and mean ± SE = 0.5 ± 0.1, respectively); also, they were significantly higher in high-stage (III plus IV) tumors (12 of 14, 86%; \( P = 0.0003 \); 12 of 14, 86%; \( P = 0.01 \) and mean ± SE = 1.1 ± 0.165, \( P = 0.01 \), respectively) as compared with low-stage (I plus II) tumors (17 of 53, 32%; 25 of 53, 47% and mean ± SE = 0.6 ± 0.1, respectively). The LAMA3 promoter methylation (not LAMB3 and LAMC2) frequency in breast tumors was associated with increased tumor stage (\( P < 0.001 \)) and tumor size (\( P < 0.001 \); Fig. 3A). There was a statistically significant increasing trend in mean MI for both tumor stage (stage I, 0.18; stage II, 0.19; stage III, 0.36; stage IV, 0.33; \( P = 0.03 \)) and size (size I, 0.13; size II, 0.21; size III, 0.35; stage IV, 0.5; \( P = 0.0008 \); Fig. 3B). The differences in promoter methylation frequencies of LAMA3 between tumor stage and size were statistically insignificant. Methylation frequency of LAMC2 was higher in large size (4 of 17, 24%) and high-stage tumors (3 of 14, 21%) as compared with small size (9 of 50, 18%) and low-stage tumors (10 of 53, 19%), whereas methylation frequency of LAMB3 was higher in large size tumors (1 of 17, 6%) only as compared with small size (2 of 50, 4%) tumors. These differences were not statistically significant. There was no association of methylation frequencies of LAMA3, LAMB3, and LAMC2 with the other patient and tumor characteristics in breast tumors.

**DISCUSSION**

Apart from providing structural integrity and scaffolding of a tissue, the ECM encodes a large variety of specific signals, which directly influence growth, migration, and differentiation of cells. Epithelial cells attach to BM through adhesive contacts between the basal cells of the epithelium and proteins of the ECM. During invasion and metastasis, dissemination of cells begins with the escape of cells from their local environment. The first step in an invasive, mobile tumor cell is to break all stable physical contacts with neighboring cells. Subsequently, the tumor cells move through the surrounding BM followed by micro- or macrometastasis. Tumor cell-secreted proteases and matrix-proteinas, the expression of which is influenced by ECM constituents, are one of the well-known causatives in the degradation of BMs around epithelial cells. They are also involved in the penetration of other physical barriers such as dense mesenchyme (7). HDs are small multiprotein complexes that mediate adhesion of epithelial cells to the underlying BM and connect elements of cytoskeleton to the ECM (20). The core of HD is formed by the crucial integrin \( \alpha_5\beta_1 \) and its ligand LN5 (21). Both of these proteins are elaborated by epithelial cells. It has been reported that malignant transformation of breast epithelium with expression of an invasive phenotype was associated with loss of HDs and reduction in immunostaining of LN5 (4, 5). We have shown previously that methylation is a major mechanism of silencing of LN5–encoding genes in lung cancers (1).

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 (8). To understand the role of LN5–encoding genes in breast cancers, we examined the expression of LAMA3, LAMB3, and LAMC2 genes. All of the three genes were expressed in nonmalignant breast epithelial cells and breast tissues while breast 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 reverse transcription cell lines, indicating methylation as a mechanism of transcriptional silencing of three LN5-encoding genes. Using the same set of primers previously published from our lab (1), we analyzed the methylation status of LN5-encoding genes in breast cancer cell lines, tumors, and control tissues. The excellent overall concordance between loss of expression and methylation in breast cancer cell lines provides powerful evidence that methylation results in gene silencing. There was uniform methylation of all of the Cpgs in the amplicons of promoter regions of the LN5-encoding genes in the cell lines studied. Methylation frequencies of LAMA3 promoter, any one gene, at least one gene and mean chain MI, were significantly higher in cell lines and tumors as compared with control tissues. These findings indicate that the epigenetic inactivation of LN5-encoding genes occurs specifically during tumorigenesis and the potential use of these markers in distinguishing malignant breast tissues from nonmalignant samples. There were no significant differences in the methylation frequencies between cell lines and tumors, indicating that cell lines are suitable models for studying promoter methylation of LN5 genes. Only occasional methylation was present in nonmalignant tissues adjacent to cancers. With the assay conditions used, almost all control tissues from healthy volunteers were negative for LAMA3, LAMB3, and LAMC2 promoter methylation, and all of the three genes were expressed in nonmalignant breast tissues.

We also correlated the methylation data of LN5-encoding genes with clinicopathological features of poor prognosis specifically high tumor stage and high tumor size. The promoter methylation frequencies of LAMA3, at least one gene methylation frequency and mean chain MI, were significantly higher in high stage and large size tumors as compared with low-stage and small size tumors. LAMA3 promoter methylation in breast tumors was associated with increased tumor stage and tumor size. Thus, methylation of LAMA3 is a potential molecular marker for breast cancer prognosis.

The major receptor for ligand LN5 is integrin α6β4. It is probable that loss of any of the five component chains of laminin-integrin complex (LN5 and α6β4) could disrupt the HD and basement membrane and lead to invasion (4, 22–24). We and others (21, 24) have found losses of expression of integrin β4 in lung cancers. We also found that integrin genes are not inactivated by promoter methylation (unpublished data). Thus, we limited our methylation studies to the LN5-encoding genes in lung and breast cancers. Individual components of LN5 can be down- or up-regulated depending on specific microenvironmental features, although its absence could favor disassembly or reduction in the number of HDs with a consequent failure of cell anchoring leading to an invasive and metastatic phenotype. Thus, although loss of any chain results in loss of the functional molecule, unopposed expression of one or more chains (especially C2) may aid invasion (6). Of the three LN5-encoding genes, LAMA3 was the most frequently methylated in breast and lung cancers. The frequency of methylation of LAMA3 was similar in breast (44%) and non-small cell lung cancer (42%) but was more frequent in the highly metastatic small cell lung cancer (65%). LAMC2 methylation frequency was the least among three genes in lung cancers, whereas LAMB3 methylation frequency was the least among three genes in breast cancers. The mean chain MI was highest in small cell lung cancer tumors followed by non-small cell lung cancer tumors, breast cancers, and carcinoids. Our data satisfy the criteria required for the demonstration of biological significance of methylation (17, 25): (a) aberrant methylation is frequent in the tumor type studied; (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 LN5-encoding genes, especially of LAMA3 gene by methylation, plays an important role in pathogenesis of breast cancers and may play a role in tumor progression.

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Laminin-5 Methylation in Breast Cancer

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