Aberrant Promoter Methylation of Laminin-5-Encoding Genes in Prostate Cancers and Its Relationship to Clinicopathological Features

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ABSTRACT

Purpose: Laminin-5 (LN5) is an essential component of the basement membrane (BM) and is composed of three chains that are the products of three distinct genes (LAMA3, LAMB3, and LAMC2). Differential expression of LN5 genes has been reported in prostate and other cancers. Recently, in lung cancers, we developed methylation-specific PCR assays for each gene and demonstrated that the aberrant methylation as the mechanism of inactivation of genes. In this study, we investigated the aberrant promoter methylation of LN5 genes in prostate cancers and correlated the data with clinicopathological features.

Experimental Design: Promoter methylation of LN5-encoding genes was analyzed in 101 prostate cancer samples by methylation-specific PCR assay. In addition, we analyzed 32 nonmalignant prostate tissue samples. The methylation index (MI) was determined as the methylated fraction of the genes examined.

Results: The frequencies of loss of expression for the LAMA3, LAMB3, and LAMC2 genes in six prostate cancer cell lines were 83, 67, and 50%, respectively, whereas the methylation frequencies were 83, 67, and 33%, respectively. The concordances between loss of expression and methylation for the three genes were 100, 100, and 83%, respectively. The frequency of methylation of LN5-encoding genes in prostate cancers and nonmalignant prostate tissues, respectively, were: 44 and 12% for LAMA3 (P = 0.001); 18 and 6% for LAMB3; and 41 and 9% for LAMC2 (P = 0.001). In addition, methylation frequencies of any one or two genes, frequencies of at least one-gene methylation and mean chain MI were significantly higher in prostate cancers than in nonmalignant prostate tissues. For clinicopathological correlations, the high Gleason score (GS) group, high preoperative serum prostate-specific antigen (PSA) group, and high stage group had significantly higher methylation frequencies of LAMA3 than their corresponding low groups. Methylation frequency of at least one gene and mean chain MI was significantly higher in the high PSA group and high-stage group than their respective low groups. There was significant correlation between MI and PSA. The high GS group had higher frequencies of at least one gene methylation and mean chain MI than the low GS group.

Conclusions: Our results demonstrate frequent epigenetic silencing of LN5-encoding genes in prostate cancers and it correlates with clinicopathological features of poor prognosis. These findings are of biological interest and potentially of clinical importance.

INTRODUCTION

Prostate carcinoma is the commonest cancer and is the second leading cause of death in men. Human prostate cancer is a disease in which both latent and aggressive phenotypes exist. However, there is no conclusive way to distinguish between a microscopic cancer that will remain latent and one that will invade the surrounding matrix, spread to distant organs, and become life threatening. The molecular basis for these different phenotypes is currently unknown (1, 2).

Laminins are components of the extracellular matrix (ECM) that contribute to the architecture of the basal lamina surrounding the epithelial cells and mediate cell adhesion, growth, migration, proliferation, and differentiation. They are heterotrimeric glycoproteins composed of three different polypeptide chains (α, β, and γ) arranged in a cruciform structure. A separate gene encodes each polypeptide chain, and different combinations of these chains have led to the identification of 13 different laminin isoforms (2–4). Laminin-5 (LN5), secreted by epithelial cells, consists of α3, β3, and γ2 chains, which represent the products of three distinct genes (LAMA3, LAMB3, and LAMC2, respectively). LN5 is a primary component of hemidesmosomes, which are specialized attachment sites on the basement membrane (BM) for epithelial cell anchoring (5–7). Tumor invasion is one of the earliest steps in the multistep process of metastasis and is characterized by cancer cells crossing the BM. Association of loss of hemidesmosomes with invasive prostate carcinoma has been reported previously (2, 8). The hemidesmosomal instability or loss would suggest a less stable epithelial-stromal junction, increased invasion and...
migration of malignant cells, and disruption of normal integrin signaling pathways (2).

It is widely known that human neoplasms arise from the accumulation of multiple genetic events leading to activation of proto-oncogenes or inactivation of tumor suppressor genes (TSGs; Refs. 9, 10). Epigenetic phenomena inclusive of promoter methylation and histone deacetylation are emerging as the major mechanism of inactivating TSGs in many human cancers, and the number of methylated genes in individual cancers is estimated to be high (11–13). Differential expression of LN5 genes has been reported in prostate and other cancers (3, 14, 15). Recently, in lung cancers, we developed methylation-specific PCR (MSP) assays for each gene and demonstrated that the aberrant methylation as the mechanism of inactivation of genes (16). In this study, we investigated the aberrant promoter methylation of LN5 genes in prostate cancers and correlated the data with clinicopathological features.

MATERIALS AND METHODS

Clinical Samples. The cell lines used in this study—MDAPCa2a, MDAPCa2b (17, 18), LAPC4 (17, 18), LuCaP, PC3, and Du 145 (17, 18)—were obtained from Dr. Jer-Tsong Hsieh at University of Texas Southwestern Medical Center at Dallas. The 101 tumor samples used in this study were collected from patients with prostate cancer. Adherent nonmalignant tissue was available from 7 patients. We also obtained nonmalignant tissues from 24 patients without cancer (benign prostatic hypertrophy or prostatitis from patients without cancer and prostate tissue adjacent to cancer). The patients underwent radical prostatectomy or transurethral resection at University of Texas Southwestern Medical Center-affiliated hospitals at Dallas, Texas, between 1994 and 2000, after Institutional Review Board approval and signed consent was obtained. The tissues were maintained frozen at −70°C until used. The histological grading was according to Gleason score (GS; Ref. 19), and the stage of the disease was by the clinical Tumor-Node-Metastasis classification of the American Joint Committee on Cancer (20). The clinicopathological features of cancer patients are described elsewhere (21). Briefly, the median age was 63 years (range, 43–81 years), and there were white (n = 69), black (n = 15), Hispanic (n = 5), and unknown (n = 12) races. Median ages (and the range in years) of patients from whom malignant and nonmalignant prostate tissue samples were collected are presented in Table 1. The Tumor-Node-Metastasis staging mainly consisted of three groups: I (n = 12), II (n = 14), and III (n = 25). The Tumor-Node-Metastasis staging of remainder samples was unknown. Peripheral blood lymphocytes from 13 healthy volunteers were also obtained.

Table 1 Frequency of promoter methylation of laminin-5–encoding genes in prostate cancers and control tissues

<table>
<thead>
<tr>
<th>Sample designation</th>
<th>Median age (yrs) (range in yrs)</th>
<th>Sample size (n)</th>
<th>Methylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate cancersa</td>
<td>63 (43–81)</td>
<td>101</td>
<td>45 (44)</td>
</tr>
<tr>
<td>Nonmalignant prostatic tissuesa</td>
<td>67 (49–86)</td>
<td>32</td>
<td>4 (12)</td>
</tr>
<tr>
<td>BPHb</td>
<td>70 (54–76)</td>
<td>7</td>
<td>1 (14)</td>
</tr>
<tr>
<td>Prostatiticc</td>
<td>65 (54–76)</td>
<td>1</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lymphocytesd</td>
<td>37 (32–65)</td>
<td>14</td>
<td>1 (7)</td>
</tr>
</tbody>
</table>

* The median age differences by Mann-Whitney U nonparametric statistical test was insignificant; for LAMA3, P = 0.39; for LAMB3, P = 0.68; and for LAMC2, P = 0.58. The Ps for the significant differences in methylation frequency between nonmalignant and malignant samples for all the three genes are presented in Fig. 1B.

a BPH, benign prostatic hypertrophy; NA, not available.
b From patients without cancer.
c From cancer patients.
d From healthy volunteers.

DNA Extraction. Genomic DNA was extracted from malignant and nonmalignant tissues by digestion with proteinase K (Life Technologies, Inc.) for 1 day at 50°C, followed by two extractions with phenol: chloroform (1:1) (22).

MSP. The DNA was modified by sodium bisulfite treatment as described previously (23). Briefly, 1 μg of DNA was denatured by incubation with 0.2 M NaOH for 15 min. at 37°C. Aliquots of 10 μM hydroquinone (30 μl; Sigma Chemical Co., St. Louis, MO) and 3 M 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 (23). 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 previously (16). P16-unmethylated primers were used to confirm the integrity of tissue-extracted bisulfite-treated DNA (23). DNA from lymphocytes of healthy volunteers and nonmalignant prostate tissues were used as negative controls for MSP assays. DNA from lymphocytes of healthy volunteers treated with SssI methyltransferase (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
RESULTS

Expression and Methylation Pattern of LAMA3, LAMB3, and LAMC2 in Prostate Cancer Cell Lines. The frequencies of loss of expression for the LAMA3, LAMB3, and LAMC2 genes in six prostate cancer cell lines were 83, 67, and 50%, respectively, whereas the methylation frequencies were 83, 67, and 33%, respectively. The frequency of loss of expression for at least one gene and the frequency of methylation for at least one gene were 83%. The concordances between loss of expression and methylation for the three genes were 100, 100, and 83%, respectively.

Aberrant Promoter Methylation of LAMA3, LAMB3, and LAMC2 Genes in Prostatic Tissues. Results of aberrant methylation in malignant prostatic and control tissues are detailed in Table 1 and Fig. 1B, and representative examples of methylation patterns in tumors and control tissues are illustrated in Fig. 1A. The frequency of methylation of LN5-encoding genes in prostate cancers and nonmalignant prostate tissues, respectively, were: 44 and 12% for LAMA3 (P = 0.001); 18 and 6% for LAMB3; and 41 and 9% for LAMC2 (P = 0.001). The unmethylated form of P16, run as a control for DNA integrity, was present in all samples. Methylation frequencies of any one gene (39 of 101, 39%; P < 0.002), two genes (22 of 101, 22%; P = 0.004), at least one gene (68 of 101, 67%; P < 0.0001) and mean chain MI (P < 0.0001) were significantly higher in prostate cancers than in nonmalignant prostate tissues (for one gene, 3 of 32, 9%; for two genes, 0 of 32, 0%; and for at least one gene, 5 of 32, 16%). Of interest, we also correlated the methylation status of LAMA3 with the methylation status of other known TSGs studied on the same set of samples (previously published from our lab; Ref. 21). The concordance between LAMA3 and RASSF1A (65%, P = 0.002), LAMA3, and CDH1 (62%, P = 0.04) was statistically significant.

Correlation between Methylation of LAMA3, LAMB3, and LAMC2 Genes and Clinicopathological Features. We compared the methylation frequencies of LN5-encoding genes with the GS, the preoperative serum prostate-specific antigen (PSA), and the tumor stage. It was reported that prostatic tumors with GS values of 5–6 have a significantly better clinical course than those with values of ≥7 (24). Hence, we divided our tumors into those with values of 6 or lower (low GS group) and those with values ≥7 (high GS group). The median value of preoperative serum PSA in our cancer patients was 7.5 ng/ml. To obtain approximately equal number of patients in each category, we divided our patients into a low PSA group (≤8 ng/ml) and a high PSA group (≥8 ng/ml). The median values of PSA in the low and high PSA groups were 5.6 and 14.2 ng/ml,
respectively. Tumor staging information was available for 60 of the patients. Because of the relatively small numbers in each of the four grades, we pooled patients into low-stage (stages I and II) or high-stage (stages III and IV) groups.

The correlation of methylation frequencies and the mean chain MI with different clinicopathological features are shown in Fig. 2. The high GS group (Fig. 2A), high preoperative serum PSA group (Fig. 2B), and high stage group (Fig. 2C) had significantly higher methylation frequencies of LAMA3 (36 of 65, 55%, $P = 0.003$; 40 of 45, 89%, $P < 0.0001$; and 21 of 34, 62%, $P = 0.02$, respectively) than their corresponding low groups. At least one gene methylation frequency and mean chain MI were significantly higher in high PSA group ($P = 0.0001$) and high-stage group ($P = 0.02$ for at least one gene and $P = 0.008$ for MI) than their respective low groups. According to the Spearman rank-correlation test, there was significant correlation between MI and PSA (coefficient $= 0.48$, $n = 101$, $P < 0.0001$). The high GS group had higher frequency of at least one gene methylation (69%) and mean chain MI (mean $\pm$ SE = 0.4 $\pm$ 0.04) than low GS group (64%; mean $\pm$ SE = 0.3 $\pm$ 0.05).

Patients from whom the nonmalignant prostate tissues were collected had a slightly higher median age than those from whom the malignant tissues were collected (Table 1). Mann-Whitney $U$ nonparametric statistical test for the median age differences between malignant and nonmalignant prostate samples revealed that the differences are not statistically significant (for LAMA3, $P = 0.39$; for LAMB3, $P = 0.68$; and for LAMC2, $P = 0.58$). The frequencies of methylation of the three genes in nonmalignant tissues were low. Survival data were available for 44 prostate cancer patients with a median follow-up period of 27 months. Although methylation status of LN5 genes did not correlate with survival, no deaths were noted until 48 months after surgery. Thus, a lengthy follow-up period will be required to determine whether methylation is a prognostic factor for survival.

DISCUSSION

The mechanism of initiation and progression of prostate cancer is not well understood, but the progression is highly variable and is associated with alterations in the composition of the prostatic ECM (1, 2, 8). The variable nature of the disease constitutes a major problem in clinical management of the individual patient. A more thorough understanding of the composition of the ECM, attachment mechanisms, and signaling factors that contribute to the degradation of the ECM and migration of primary metastatic cells would advance the ability to predict the clinical progression rate and prognostic outcome of the disease (1).

BMs are characteristic of multicellular organisms and they are the first ECM component produced during embryogenesis (25). 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 (26). ECM consists of several molecules, two of the most important being LN5 (of epithelial origin) and collagen 4 (secreted by the stromal cells). The epithelium and underlying stroma function as a unit and constantly communicate. Although in situ carcinomas are intraepithelial by definition, invasion and destruction of the BM are the earliest morphological feature of invasive carcinomas.
Multiple mechanisms of inactivating TSGs such as loss of heterozygosity, point mutations, homozygous deletions, and aberrant promoter methylation have been reported in various types of cancers (27). Inactivation of multiple TSGs by epigenetic phenomenon has been reported in prostate cancers (21). To develop molecular markers for prostate cancer risk assessment, we investigated the epigenetic inactivation of LN5-encoding genes in prostate cancer cell lines, malignant, and control prostate samples. There was high degree of concordance between loss of expression and methylation in the prostate cancer cell lines studied. Methylation frequencies of LAMA3 and LAMC2 genes, at least one gene methylation frequency and mean chain MI, were significantly higher in prostate cancers than in nonmalignant tissues. These findings indicate the epigenetic inactivation of LN5 genes is tumor specific. Also, lymphocytes from healthy volunteers had a very low level of methylation frequency further supporting the observed methylation frequency for LN5 genes as tumor specific. This indicates the potential use of these markers in distinguishing malignant from nonmalignant prostate tissues. We did not have access to cases of prostatic intraepithelial tumors in specimens free of invasive cancers. Thus, we do not know if methylation of LN5 genes can distinguish between invasive and noninvasive cancers. Methylation of LAMA3 was significantly higher in tumors of patients with increased risk of death, namely high GS, high preoperative PSA, and high stage. At least one gene methylation frequency and mean chain MI were significantly higher in tumors of patients with high PSA group and high-stage group than in the corresponding low groups. Other studies have demonstrated that the preoperative serum PSA reflects both tumor grade (28) and tumor volume (29).

Progressive age-related increase in methylation has been previously reported for genes such as estrogen receptor in colorectal mucosa (30). To address whether the observed methylation frequency for LN5-encoding genes in malignant prostate samples was age dependent, we compared the median age (and range) of patients from whom malignant and nonmalignant prostate tissues were collected. We found that those patients from whom the nonmalignant prostate tissues were collected had slightly higher median age (and range) than those from whom the malignant tissues were collected although these differences were not significant. Thus, we can exclude the possibility of age-dependent methylation of LN5 genes in prostate cancer patients.

In prostate carcinoma, loss of expression of LN5 or variable pattern of expression of different chains of LN5 (2, 3, 31), loss of basal lamina or change in components of basal lamina during tumorigenesis (1, 32), and loss of hemidesmosome (8) have been reported. The original data have shown that LAMC2 and LAMB3 are major chains showing down-regulation, while expression of LAMA3 remains unchanged (1, 2), were inconsistent because nonspecific rabbit polyclonal antibodies were used for immunostaining against LAMA3 (31). In the more recent study, authors (31) show using a monoclonal antibody loss of expression of all three chains in prostate carcinoma, in particular, LAMA3 and LAMC2. These findings are consistent with our data in both cell lines and tumor samples. It has been reported that there was progressive loss of basal lamina with increasing GS (33). Our data are consistent with these observations and extend these findings by demonstrating the mechanism of loss of the LN5 chains. At least one LN5 chain was inactivated by methylation in ~70% of invasive prostate cancers. As the BM must be disrupted in all invasive cancers, alternative mechanisms of disruption must exist, including loss of integrin αβ3 encoding genes or overexpression of matrix metalloproteinase 9 (34–36). Loss of any of hemidesmosome components (LN5 and its receptor α5β1) presumably disrupts the hemidesmosome and leads to invasion (6, 32, 34, 37). 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 epithelial cell anchoring to ECM, leading to an invasive and metastatic phenotype. LN5 chain loss was not random, with losses LAMA3 and LAMC2 more frequent than LAMB3. This pattern is consistent with the previously published data for methylation of LN5 genes in non-small cell lung carcinoma samples (16). Thus, although loss of any chain results in loss of the functional molecule, unopposed expression of one or more chains (especially LAMC2) may aid invasion (5).

Our results demonstrate epigenetic inactivation of LN5-encoding genes in prostate cancers and also the potential use of methylation of LAMA3 as a potential molecular marker in distinguishing the different stages in the progression of prostate carcinoma. We also found significant concordance between the methylation status of LAMA3 and other known TSGs—RASSF1A and CDH1, which further indicates that association of several molecular markers on the same set of tumor samples potentiates the use of these markers in prostate cancer risk assessment and follow-up procedures. Our findings may be of clinical importance and may help to identify cancer patients at increased risk of tumor progression.

REFERENCES

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