Molecular Detection of Noninvasive and Invasive Bladder Tumor Tissues and Exfoliated Cells by Aberrant Promoter Methylation of Laminin-5 Encoding Genes

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ABSTRACT

Laminin-5 (LN5) anchors epithelial cells to the underlying basement membrane, and it is encoded by three distinct genes: LAMA3, LAMB3, and LAMC2. To metastasize and grow, cancer cells must invade and destroy the basement membrane. Our previous work has shown that epigenetic inactivation is a major mechanism of silencing LN5 genes in lung cancers. We extended our methylation studies to resected bladder tumors (n = 128) and exfoliated cell samples (bladder washes and voided urine; n = 71) and correlated the data with clinicopathologic findings. Nonmalignant urothelium had uniform expression of LN5 genes and lacked methylation. The methylation frequencies for LN5 genes in tumors were 21–45%, and there was excellent concordance between methylation in tumors and corresponding exfoliated cells. Methylation of LAMA3 and LAMB3 and the methylation index were correlated significantly with several parameters of poor prognosis (tumor grade, growth pattern, muscle invasion, tumor stage, and ploidy pattern), whereas methylation of LAMC2 and methylation index were associated with shortened patient survival. Of particular interest, methylation frequencies of LAMA3 helped to distinguish invasive (72%) from noninvasive (12%) tumors. These results suggest that methylation of LN5 genes has potential clinical applications in bladder cancers.

INTRODUCTION

Bladder carcinoma is the most common urothelial malignancy and occupies the 5th and the 10th ranks of neoplasms in men and women, respectively, in more developed countries (1). Histologically, ~90% of bladder carcinomas are urothelial carcinomas, characterized by malignant proliferation of the transitional epithelium [transitional cell carcinomas (TCCs)]. In ~25% of patients, bladder carcinoma is a multifocal disease. TCCs are subdivided into nonmalignant papillary and nonpapillary invasive carcinoma types. Papillary TCCs correspond to 70% of all the TCCs and usually are low grade, superficial, and noninvasive at the time of presentation (1–3). Papillary TCCs are characterized by high rates of recurrence, and ~10–30% will progress eventually to invasive disease (3–5). Invasive cancers may originate from papillary cancers or via a different pathogenesis involving dysplasia and carcinoma in situ. The distinction between these two forms of TCC is of major clinical, therapeutic, and prognostic importance. Conventional urine cytology has been the standard noninvasive method for cancer detection and disease monitoring. However, the sensitivity of this method is known to be low, especially for low-grade TCC (6). Noninvasive methods, such as molecular markers, to distinguish noninvasive cancers from invasive cancers would be of great benefit.

Laminin-5 (LN5), secreted by epithelial cells, is a large heterotrimeric glycoprotein consisting of α3, β3, and γ2 chains, which represent the products of three distinct genes (LAMA3, LAMB3, and LAMC2, respectively). LN5 is a core component of hemidesmosomes, which are the specialized attachment sites on the basement membrane (BM) for epithelial cell anchoring (7–9). Tumor invasion is one of the earliest steps in the multistep process of metastasis and is characterized by cancer cells invading and breaking the BM. It is widely known that human neoplasms, including bladder cancer, originate from the accumulation of multiple genetic events, leading to activation of proto-oncogenes or inactivation of tumor suppressor genes (10–13). Aberrant promoter methylation is emerging as one of the major mechanisms of inactivating tumor suppressor genes in many human cancers, and the number of methylated genes in individual cancers is estimated to be high (14–16). We have shown epigenetic inactivation is the major mechanism of silencing LN5 genes in lung cancers (17). We studied bladder tumors in resected specimens and exfoliated cells (bladder washes and voided urine) and correlated the data with clinicopathologic findings. Our results indicate the potential use of methylation of LN5-encoding genes as molecular markers to distinguish invasive from noninvasive bladder cancers.

MATERIALS AND METHODS

Cell Lines and Clinical Samples. RNA and DNA from five bladder cancer cell lines (UC2, UC3, UC9, UC11, and UC13) were used to validate the expression and methylation of LN5-encoding genes. RNA and DNA from eight tumors and corresponding nonmalignant urothelium and one urothelial cell culture were used to study gene expression and correlate it with methylation status. DNA from five samples of normal urothelium from subjects without bladder cancer was used to determine methylation status of normal urothelium.

Fresh bladder tumor tissues (n = 128) were obtained by transurethral resection or from cystectomy specimens at the M.D. Anderson Cancer Center (Houston, TX) or Affiliated Hospitals of the University of Texas Southwestern Medical Center (Dallas, TX). Appropriate institutional review board permission was obtained at both centers, and written informed consent was obtained from all of the subjects. The patients consisted of 81 men and 41 women. There were six patients whose sex is not known. The median age of the patients was 69 years (range, 40–96 years). The tumor classification, grading, pathologic staging, and other clinicopathologic features were determined as described elsewhere (18–22), and details are presented in Fig. 2, A–C and E and F. All of the tumors were TCCs except for two squamous cell carcinomas. For the resected tumors, there were patients with Tumor-Node-Metastasis stage 0 (n = 24), stage 1 (n = 7), stage 2 (n = 12), stage 3 (n = 33), and stage 4 (n = 4). By definition, muscle invasion was present in tumors stages T2–T4 and was absent in T0 and T1. Because full staging could not be determined for tumors resected transurethrally, there were 48 cases of unknown stage.

Exfoliated cells were available from 71 bladder cancer patients (bladder washes, n = 28; voided urine, n = 43). Of these cases, 24 corresponding tumor samples were available. These samples were subjected to clinical analysis for

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RESULTS

Expression and Methylation of LN5-Encoding Genes LAMA3, LAMB3, and LAMC2. Reverse transcription-PCR analysis revealed expression of all of the three LN5 genes in all of the samples of normal ureter urothelium, cultured normal urothelial cells, and nonmalignant urothelium adjacent to cancer. Expression of LAMA3, LAMB3, and LAMC2 genes was lost in four of five (80%), four of five (80%), and three of five (60%) bladder cancer cell lines, respectively. All of the five cell lines had lost expression of at least one gene. The methylation frequencies for LAMA3, LAMB3, and LAMC2 genes in bladder cancer cell lines were four of five (80%), three of five (60%), and three of five (60%), respectively. One or more genes were methylated in four of five (80%) cell lines. The overall concordance between loss of expression and methylation for the three genes in five bladder cancer cell lines was 93%.

Among tumors, expression of LAMA3, LAMB3, and LAMC2 genes was lost in six of eight (75%), six of eight (75%), and four of eight (50%) bladder tumors, respectively. All of the eight tumors had lost expression of at least one gene. Representative examples of expression patterns are illustrated in Fig. 1A. The overall concordance between loss of expression and methylation for the three genes in five tumors was 93%.

The methylation frequencies for LN5-encoding genes in 128 bladder tumors and 71 exfoliated cells, respectively, were as follows: LAMA3 (45% and 39%), LAMB3 (21% and 19%), and LAMC2 (23% and 15%). One or more genes were methylated in 55% (70 of 128) of tumors and 49% (35 of 71) of exfoliated cells. In tumors and exfoliated cells, methylation frequencies of any one gene were 30% (38 of 128) and 31% (22 of 71), respectively. The differences in methylation frequencies for the three LN5 genes and mean chain MI between tumors and exfoliated cells were not statistically significant. Representative examples of the methylation patterns of the LN5 genes in tumors and exfoliated cells are illustrated in Fig. 1B and C and summarized in Fig. 1D. There was no methylation in nonmalignant ureter samples and nonmalignant samples corresponding to tumor and bladder wash pairs. Among the 24 pairs of tumors and exfoliated cells, the concordances in methylation between tumor and corresponding exfoliated cells for LAMA3, LAMB3, and LAMC2 genes were 88% (P = 0.0006), 92% (P = 0.002), and 83% (P = 0.003), respectively.

Correlation of Methylation of LN5-Encoding Genes and Risk Factors. Fig. 2 illustrates the correlation of methylation frequencies of LN5 genes and the mean chain MI with five factors of increased risk, namely high tumor stage (Fig. 2A), nonpapillary growth pattern (Fig. 2B), aneuploidy (Fig. 2C; Refs. 26, 27), high tumor grade (Fig. 2E), and muscle invasion (Fig. 2F). In addition, we correlated the methylation frequencies of the three genes and MI with the number of risk factors (Fig. 2D). To get approximately equal numbers in each group, tumors were divided into a group with zero to two factors or a group with three to five factors. The MIs of tumors with any of these risk factors were significantly higher than those of tumors that were negative for the factors (Fig. 2, A–F). Tumors having one or more risk factors had significantly higher frequencies of methylation of LAMA3 and LAMB3 genes (Fig. 2, A–F). The methylation frequency of one or more genes was significantly higher in those tumors having one or more risk factors (other than aneuploidy; Fig. 2, A–F). Ploidy data were available from only a subset of tumors, and the aneuploid tumor group had significantly higher individual gene methylation frequencies for LAMA3 and LAMB3 and MI than the diploid tumor group (Fig. 2C). Although there were variable patterns in the number of genes methylated in the different risk factor categories, in the muscle invasion category, mean chain MI was significantly higher in invasive tumor samples than in noninvasive tumors (P < 0.0001; Fig. 2D). In tumors with three to five risk factors, mean chain MI (mean = 1.3) was significantly higher than in those with zero to two risk factors (mean = 0.43; P < 0.0001; Fig. 2D).

We also correlated the methylation frequencies of LN5-encoding genes in exfoliated cells collected from bladder cancer patients and mean chain MI with two factors of increased risk, namely high tumor grade (Fig. 2G) and muscle invasion (Fig. 2H). In exfoliated cells,
Fig. 1. Expression and methylation-specific PCR (MSP) analysis of laminin-5 (LN5) genes in bladder samples. A, agarose gel showing reverse transcription-PCR products for the three LN5 genes—LAMA3 (panel a), LAMB3 (panel b), LAMC2 (panel c)—and b-actin (panel d). Their respective amplicon sizes are given in parentheses. NU1 and NU2 are two normal urothelium samples; UA1 and T1 (P1), UA2 and T2 (P2), and UA3 and T3 (P3) are three pairs of bladder tumors and corresponding adjacent nonmalignant sites. UA, urothelium adjacent to malignant tissue; T, tumor; P, positive control or normal lymphocyte DNA treated with Sss I methyltransferase or known methylated DNA from lung cancer cell lines known to be methylated for the respective genes; N, negative control—or water blank or PCR mixture without template. B, agarose gel showing MSP products for P16 U control for DNA integrity (panel a) and three LN5 genes—LAMA3 (panel b), LAMB3 (panel c), and LAMC2 (panel d)—in normal urothelium and tumor samples. Their respective amplicon sizes are given in parentheses. NU1 and NU2 are two normal urothelium samples; 1–4 are noninvasive tumor samples (Non-INV), 5–8 are invasive tumor samples (INV); U, unmethylated form, M, methylated form; Cont, controls; P, positive control or normal lymphocyte DNA treated with Sss I methyltransferase or known methylated DNA from lung cancer cell lines known to be methylated for the respective genes; N, negative control or water blank or PCR mixture without template. C, agarose gel showing MSP products for P16 U (panel a) and three LN5 genes—LAMA3 (panel b), LAMB3 (panel c), and LAMC2 (panel d)—in exfoliated cells and tumors. Their respective amplicon sizes are given in parentheses. Non-INV, noninvasive samples; INV, invasive samples; Cont, controls. U1 to U6 are urine samples; T1 and BW1 and T2 and BW2 are two pairs of tumors and corresponding bladder washes; U, unmethylated form, M, methylated form; P, positive control or normal lymphocyte DNA treated with Sss I methyltransferase or known methylated DNA from lung cancer cell lines known to be methylated for the respective genes; N, negative control or water blank or PCR mixture without template. D, illustration of methylation frequencies of three LN5 genes—LAMA3, LAMB3, and LAMC2—at least one gene methylation (ANY), and mean chain methylation index in 114 bladder tumors and exfoliated cells (EF).

The frequency of methylation of LAMA3 was significantly higher in high-grade samples (16 of 25, 64%; P < 0.0001) than in low-grade samples (3 of 31, 10%). LAMA3 methylation frequency also was significantly higher in invasive samples (24 of 30, 80%; P < 0.0001) than in noninvasive samples (4 of 41, 10%). The methylation frequency of any one gene, at least one gene, and MI were significantly higher in exfoliated cells with one or more risk factors.

**Relationship between Methylation of LAMA3, LAMB3, and LAMC2 Genes and Prognosis.** By Kaplan-Meier analysis, neither the methylation status of individual genes nor the MI correlated with the recurrence-free intervals. However, in an analysis of overall survival by methylation status of these genes, the survival of patients with LAMC2-positive methylation in tumors was significantly shorter [P = 0.002, 5-year survival (%) of LAMC2-positive tumors (23.8) and LAMC2-negative tumors (70.4)] than of those patients with negative methylation in tumors as shown in Fig. 3A. There were no significant survival differences between methylation-positive and -negative tumors for LAMA3 and LAMB3. We divided the MI into two groups: low-MI group with zero or one gene methylated, and high-MI group with two or three genes methylated. The high-MI group had a significantly shorter survival than the low-MI group (Fig. 3B). In a multivariate analysis model that included tumor grade; growth pattern; muscle invasion; methylation status of LAMA3, LAMB3, and LAMC2 genes; and high- and low-MI groups, LAMC2 methylation-positive status was the only independent methylation-related prognostic factor as shown in Table 1. Tumor stage and ploidy pattern were excluded from this analysis because data were available from only a subset of tumors.

**DISCUSSION**

Invasion and metastasis are biological hallmarks of malignant tumors, and metastases are the major cause of cancer deaths. Disruption of organization or integrity of the BM is a key histologic marker of a tumor’s transition to an invasive carcinoma, and invasion and destruction of BM are the earliest morphologic features of invasive tumors. Carcinoma in situ is a flat, superficial lesion and is the most common precursor to invasive bladder cancer (13). The most important fundamental question is what causes in situ cancers to become invasive even though cancer cells at the preinvasive and invasive stages are morphologically similar. One of the well-established mechanisms of invading and destroying BM is by matrix metalloproteinases, which are up-regulated during invasion and metastasis (28). We selected for molecular markers that mark the transition of in situ to invasive cancers because they might predict for those at highest risk or for those with early invasive cancers. Such markers normally should be expressed in epithelial cells, which are tethered to BM, and provide...
defense against invasion of preinvasive cancers. Epithelial cells attach to the BM through adhesive contacts between the basal cells of the epithelium and the proteins of the extracellular matrix. The hemidesmosome is a specialized cell-extracellular matrix contact that mediates the attachment of the epithelial cell basal cell surface to the extracellular matrix. The core of the hemidesmosome is formed by the crucial integrin α6β4 and its ligand LN5 (9). We have shown previously that epigenetic inactivation is the major mechanism of silencing of LN5 genes in lung cancers (17).

In this report, we analyzed the methylation status of LN5-encoding...
genes in bladder tumors and exfoliated cells and correlated the data with clinicopathologic features of poor prognosis. Tumors with one or more risk factors had significantly higher frequencies of methylation of LAMA3 and LAMB3 genes. The methylation frequencies of one or more genes were significantly higher in those tumors with one or more risk factors (other than aneuploidy). Of particular interest, in the muscle invasion category, methylation frequencies for any one, any two, or all of the three genes were significantly higher in invasive tumor samples than in noninvasive tumors. Of particular interest, methylation frequencies of LAMA3 helped to distinguish invasive (72%) from noninvasive (12%) tumors.

Of the three LN5-encoding genes, LAMA3 was methylated more frequently in bladder cancers than the other two genes. The three chains of LN5 form a cruciate-like protein structure with LAMA3 located centrally and having globular domains at its N- and COOH-terminal that interact with transmembrane receptor integrins. LAM3 and LAMC2 are intertwined around LAMA3. Thus, the loss of the central LAMA3 chain leads to breakdown of the entire cruciate structure. From our data, LAMA3 is methylated and lost most frequently, and it is linked most strongly to clinical parameters. Loss of LN5 structure may affect the formation of hemidesmosomes and hence aid in invasion. Our findings are consistent with our previous reports (17, 29, 30) and those of others (31) on other tumors.

When the methylation frequencies of the three genes were compared in invasive and noninvasive tumor tissues and exfoliated cells, there were no significant differences. The concordances in methylation were similar in invasive and noninvasive tissue. However, the concordances in methylation between tumor and corresponding exfoliated cells for the three genes were significantly higher in invasive tumors than in noninvasive tumors. This suggests that methylation of LN5-encoding genes in urine may be useful to distinguish invasive from noninvasive cancers (72%). Kaplan-Meier analyses demonstrated that methylation of LAMC2 and high MI were associated with shortened survival. In a multivariate analysis, LAMC2 was the only independent methylation-related prognostic factor. Thus, LAMC2 methylation may be used as a marker for survival. Our findings suggest that LAMA3 methylation is associated with tumor invasion, whereas LAMC2 methylation is associated with improved patient survival. What are the explanations for these seemingly contradictory findings? Loss of any hemidesmosome components (LN5 and its receptor α6β4) presumably disrupts the hemidesmosome and leads to invasion (7, 31–33). 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 extracellular matrix, 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 (8).

The staging of bladder cancer is critical to therapy and outcome (34–37). Historically, the parameters used to help define therapy were the degree of bladder wall penetration and histologic grade. Because the treatment approaches to noninvasive superficial cancers are different from those required for invasive cancers, the distinction between these cancers is critical. Although such a distinction may appear straightforward, growing clinical evidence has demonstrated that understaging often occurs, resulting in the administration of inefficient therapy. In addition, it is important to identify those superficial cancers that are at increased risk to eventually become invasive. Molecular markers that help identify such tumors offer the promise of considerable clinical utility. Our findings suggest that detection of methylation of LN5-encoding genes in urine may be useful to distinguish invasive from noninvasive cancers (LAMA3) and to predict survival (LAMC2).

**REFERENCES**


LAMININ-5 METHYLATION IN BLADDER CANCER

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