

Demethylation of Urokinase Promoter as a Prognostic Marker in Patients with Breast Carcinoma

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ABSTRACT

Purpose: Urokinase (uPA) is expressed in a number of highly invasive malignancies including breast cancer. Because production of uPA is associated with breast cancer progression and can serve as a useful prognostic marker, the purpose of this study was to examine the role of uPA promoter methylation as an indicator of uPA production in breast cancer patients.

Experimental Design: We examined the methylation status of the uPA promoter and the levels of uPA expression in normal human breast epithelial cells and several human breast cancer cells by bisulfite sequencing analysis and reverse transcription-PCR. We also analyzed the methylation status of the uPA promoter in surgical biopsy samples from patients with breast cancer of different grades, as determined by the Elston-Ellis histological grading system.

Results: Expression of uPA mRNA was only detected in the highly invasive estrogen receptor-negative breast cancer cell lines, where the promoter was completely demethylated. In normal and low invasive breast cancer cells, the uPA promoter was methylated, resulting in lack of uPA mRNA expression. Analysis of biopsy samples showed that demethylation of the uPA promoter is associated with malignant transformation. Reverse transcription-PCR analysis revealed that this demethylation of the uPA promoter is directly associated with induction of uPA mRNA expression, which is well known to be associated with poor prognosis in breast cancer patients.

Conclusions: This study indicated that uPA expression in breast cancer patients is under epigenetic control via methylation of its promoter. Determination of uPA promoter methylation can therefore serve as an early reliable indicator of uPA production in breast cancer patients.

INTRODUCTION

The leading cancer-associated cause of morbidity and mortality in patients with breast cancer is metastasis of tumor cells to different organs (1). Tumor metastasis is a multistep process involving local invasion, degradation of the extracellular matrix, angiogenesis, intravasation, survival of malignant cells in the circulation, extravasation, and, finally, establishment of a secondary growth in distant organs (2). One of the key mediators of this process is urokinase (uPA), a member of the serine protease family that catalyzes the conversion of inactive zymogen plasminogen to its active form, plasmin (3). When activated, plasmin degrades most components of the extracellular matrix, such as laminin, fibronectin, and collagen (3). In addition to its proteolytic activity, uPA is known to exert additional activities including stimulation of cellular proliferation, enhancement of cellular migration, alteration of cellular adhesive properties, and activation of specific growth factors such as vascular endothelial growth factor and hepatocyte growth factor that play an important role in angiogenesis (3, 4). Increased expression of uPA is directly related to higher tumor growth and metastasis due to the ability of uPA to induce these angiogenic factors (1). Recently, the tumor-promoting effects of uPA have been linked to the ability of uPA to prevent tumor cell apoptosis (5). Such functions of uPA have implicated this protease as a major player in promoting the process of tumor growth, invasion, metastasis, and angiogenesis of several malignancies, including breast cancer (6).

Over 14 years ago, it was proposed for the first time that breast cancer patients with high levels of uPA in their primary tumors have a lower overall survival rate than patients with low levels of uPA (7). Since then, a large number of studies by different groups have reported the prognostic value of uPA as a marker for breast cancer progression (8–10). It has been shown that determination of the levels of uPA production in breast cancer patients can serve as a reliable marker that is independent of and stronger than most traditional prognostic markers such as tumor size, tumor grade, patient age, axillary node status, and steroid receptor status (8–10). These studies have also identified uPA as an excellent therapeutic target for blocking tumor progression (10–12).

Cytosine methylation within the context of CpG dinucleotides in the genome is a molecular mechanism that causes epigenetic changes in the chromatin structure and leads to transcriptional silencing of genes in many human cancers (13). This epigenetic alteration is heritable but does not alter the nucleotide sequence, making the modification potentially reversible (14–16). DNA methylation markers, useful in both epidemiological and clinical studies, have been developed over the years using both targeted genes and genome-wide scanning techniques (17, 18). Hypermethylated CpG islands detected in serum, urine, bronchoalveolar lavage fluid, and lymph nodes derived from patients with various types of malignancies have

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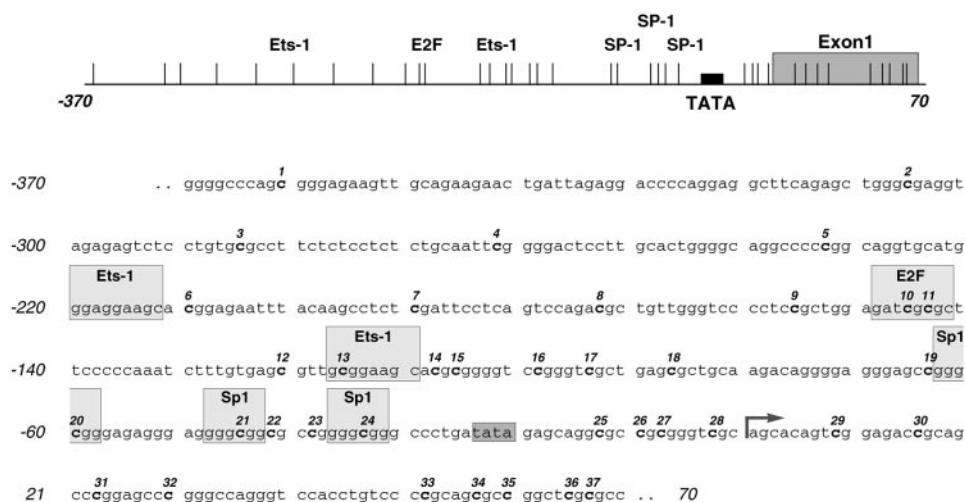


Fig. 1 Map of urokinase promoter (uPA). A map of a 440-bp-long region of the uPA promoter in which the location of exon 1, TATA box, and several transcription factor binding sites is outlined. Clusters of CpG dinucleotides are shown as vertical bars (top panel). The potential methylation sites within the uPA promoter sequence (-370 to +70) are shown in bold and numbered from 1 to 37. The TATA box and the location of the Sp1, Ets-1, and E2F transcription factor binding sites are indicated. The arrow represents the transcription initiation site (bottom panel).

been proposed to be a potential early molecular marker for detection of cancer (19, 20). Moreover, gene-specific methylation has been suggested as a useful tool for prediction of prognosis or treatment response in certain cancers (21, 22).

In recent studies, we have demonstrated that silencing of the uPA gene in early-stage prostate cancer cells is due to hypermethylation of its promoter, resulting in decreased tumor cell invasion *in vitro* and tumor volume *in vivo* (23). In this study, to determine a correlation between DNA methylation of the uPA promoter and uPA gene expression, we carried out a detailed methylation analysis of the uPA promoter in different human breast cancer cell lines and in surgical biopsy samples of different grades isolated from patients with breast cancer. We

propose that methylation status of the uPA promoter is possibly the earliest reliable method for predicting induction of uPA, which has already been shown to be an excellent prognostic marker in patients with breast cancer.

MATERIALS AND METHODS

Cell Lines and Reagents. Human mammary epithelial HMEC cells were obtained from Clonetics (San Diego, CA), and the human breast cancer cell lines MCF-7, ZR-75, T47D, BT474, HS578T, BT549, and MDA-231 were obtained from American Type Culture Collection (Manassas, VA). All cells were maintained as recommended by the manufacturer.

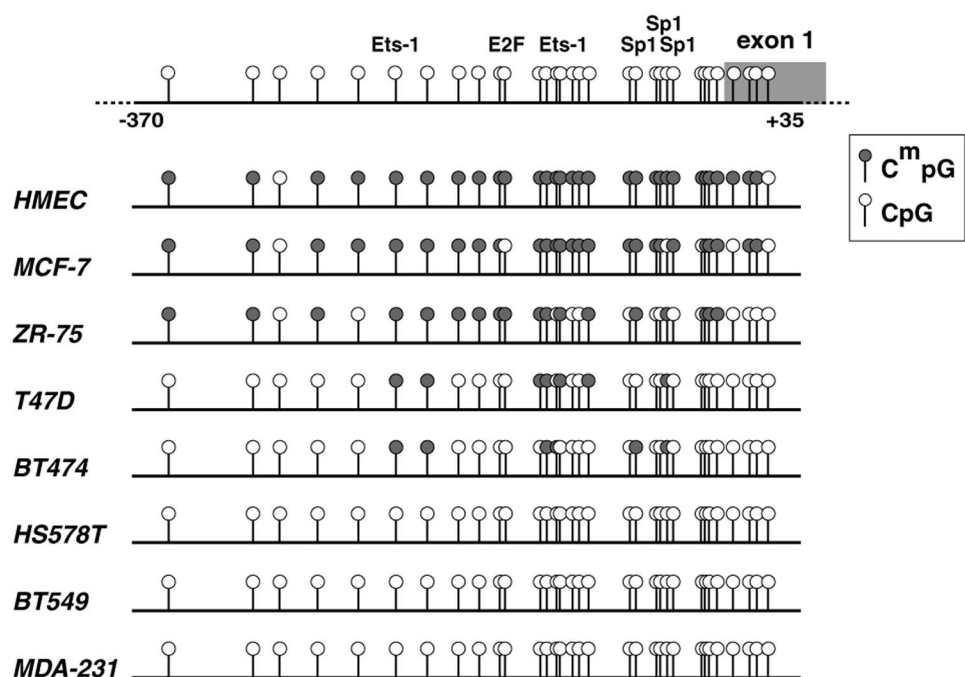


Fig. 2 Analysis of the methylation status of the urokinase promoter in human breast cancer cell lines by sodium bisulfite sequencing. Genomic DNA isolated from different human breast cancer cell lines was analyzed for methylation of the urokinase promoter by bisulfite sequencing. The amplified products were subcloned into TOPO pCR2.1 vector and sequenced by automated sequencer. Methylated cytosines are distinguished from unmethylated cytosines because they are resistant to bisulfite treatment and remain unchanged. Open and closed circles represent unmethylated and methylated CpG dinucleotides, respectively. The results are representative sequencing of at least five different clones for each cell line.

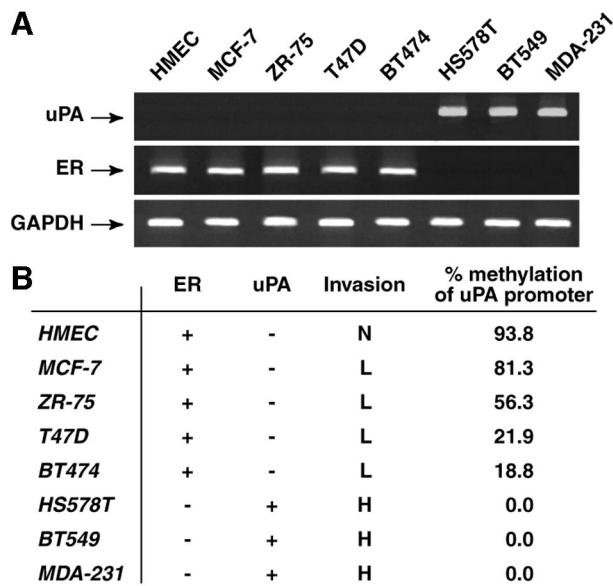


Fig. 3 Characterization of human breast cancer cell lines. **A**, reverse transcription-PCR analysis of total cellular RNA isolated from human breast cancer cell lines to determine the levels of urokinase (uPA) and estrogen receptor mRNA expression in these cells. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were determined as control. **B**, the presence and absence of uPA and estrogen receptor expression in these cell lines are indicated as + and -. The invasive capacity of these cells was determined by Boyden chamber Matrigel invasion assay. The noninvasive, low invasive, and high invasive capacity of the cells is indicated as *N*, *L*, and *H*, respectively. The ratio of total methylated sites to all sites within the amplified region of the promoter was determined and recorded as the percentage of methylation of the uPA promoter; a value of 100% represents total methylation of the CpG sites within this region. All results are representative of three independent experiments.

Genomic DNA and cellular RNA were extracted from cell pellets using DNazol and TRIZOL (Life Technologies Inc.) following the manufacturer's instructions.

Boyden Chamber Invasion Assay. Using two-compartment Boyden chambers (Transwell; Costar, Cambridge, MA) and basement membrane Matrigel (Becton Dickinson Labware, Bedford, MA), the invasive capacities of human breast cancer cell lines were determined as described previously (24). The 8- μ m-pore polycarbonate filters were coated with basement membrane Matrigel (50 μ g/filter) and used to analyze 5×10^4 cells in each chamber as described previously (24). The filters were then fixed for 30 min in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature, washed with PBS, and, finally, stained with 1.5% toluidine blue and mounted onto glass slides. Cells were examined under a light microscope. Under $\times 400$ magnification, 10 randomly selected fields were examined, and the average number of cells invaded was calculated.

Reverse Transcription-PCR (RT-PCR). Total RNA (2 μ g) isolated from cells or tumors was used for reverse transcription and amplification. The primers used for RT-PCR were designed so that there was an intron between the amplified regions to recognize DNA contamination. The uPA (5'-ACAT-TCACTGGTGCACACTGC-3' and 5'-CAAGCGTGTCAGCGCT-

GTAG-3'), estrogen receptor [ER (5'-CTGAACCGTCCGC-AGCTCAAG-3' and 5'-CCAGGTAGTAGGGCACCTGCT-3')], and glyceraldehyde-3-phosphate dehydrogenase (5'-CCCTTCAT-TGACCTCAACTACATGGT-3' and 5'-GAGGGGCCATCC-ACAGTCTTCTG-3') primers were used for each RNA sample, and the reverse transcription reactions were carried out using standard protocols. The DNA was amplified under the following conditions: 95°C for 3 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. The PCR products were then analyzed on a 1% agarose gel.

Sodium Bisulfite Sequencing. Sodium bisulfite treatment of the genomic DNA was performed as described previously (23, 25). Primers were designed to amplify the modified DNA fragment within the uPA promoter located at -423 (5'-TTTATAGTTTTATTAGTTG-3') or at -320 (5'-TAGGTGTATGGGAGGAAGTA-3') and 109 (5'-CAGGAGGTTT-TAGAGTTGGG-3') of the uPA promoter sequence. The amplification reaction was performed under the following conditions: 95°C for 3 min; 10 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 45 s; 20 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 5 min. The PCR products were purified and subcloned into TOPO-pCR2.1 cloning vectors according to the manufacturer's instruction (Clontec) or sent directly for DNA sequencing analysis (Bio S&T, Montreal, Canada).

Surgical Biopsy Samples. Forty-three breast cancer specimens were randomly selected to have an equal number of each grade from the Cancer Research Center Tumor Bank at L'Hotel-Dieu de Québec. All specimens were snap frozen within 1 h of resection and kept at -75°C. The quality of the tissue and the presence of cancer were confirmed by histological examination of a section stained with H&E.

Statistical Analysis. Results are expressed as the mean \pm SE of at least triplicate determinations, and statistical comparisons are based on the nonparametric Kruskal-Wallis test and ANOVA. A probability value of <0.05 was considered to be significant.

RESULTS

We have shown previously that methylation of the CpG dinucleotides concentrated within the 5' regulatory region of uPA is the mechanism that silences uPA promoter activity (23, 24). Analysis of the uPA promoter region (-370 to +70 bp) showed the presence of at least 37 CpG dinucleotides as potential methylation sites. This GC-rich region of the uPA promoter was chosen because of its close proximity to the transcription initiation site and the presence of several important transcription factor binding sites such as Sp-1, Ets-1, and E2F (26). The Transcription Element Search System (TESS) DNA analysis program was used to identify the location of these transcription factor binding sites (Fig. 1). We have shown previously that methylation of this region can block uPA promoter activity *in vitro*, the mechanism that was found to be responsible for regulation of uPA gene transcription in both MCF-7 breast and LNCaP prostate cancer cell lines (23, 24).

Methylation Analysis of uPA Promoter in Different Human Breast Cancer Cell Lines. To examine methylation status of the uPA promoter in different human breast cancer

cells, sodium bisulfite sequencing analysis of the isolated genomic DNA was used to characterize methylation of the CpG dinucleotides within the uPA promoter region in normal HMEC cells and in different human breast cancer cell lines. Bisulfite sequencing analysis showed that the uPA promoter was highly methylated in HMEC cells and the MCF-7, ZR-75, T47D, and BT474 human breast cancer cell lines. In contrast, the uPA promoter was found to be completely unmethylated in the HS578T, BT549, and MDA-231 cell lines (Fig. 2).

Characterization of Human Breast Cancer Cell Lines.

To establish a correlation between hormone responsiveness, tumor cell invasiveness, uPA gene expression, and methylation status of the uPA promoter, the levels of uPA and ER mRNA expression in normal and malignant human breast cancer cell lines were examined by RT-PCR analysis (Fig. 3A). High levels of ER are expressed in normal HMEC cells and in MCF-7 cells, where the uPA gene is silenced, and the promoter is >80% methylated. ER mRNA is also expressed in ZR-75, T47D, and BT474, where the uPA promoter is 18–60% methylated, resulting in silencing of uPA gene expression. In contrast, no detectable levels of ER mRNA were expressed in the uPA-expressing HS578T, BT549, and MDA-231 cells, where the uPA promoter is completely unmethylated. Demethylation of the uPA pro-

motor results in the induction of uPA mRNA expression in these ER-negative breast cancer cell lines. Boyden chamber Matrigel invasion assay was used to determine the invasive capacity of these cells (Fig. 3B). HMEC cells were completely noninvasive, whereas MCF-7, ZR-75, T47D, and BT-474 cells exhibited low invasive capacity. On the other hand, the ER-negative HS578T, BT549, and MDA-231 cells that express high levels of uPA exhibited high levels of tumor cell invasive capacity.

These results showed that there is in fact a clear correlation among methylation of the uPA promoter, ER mRNA expression, and low invasive capacity of these cells. All low invasive, ER-positive cell lines failed to express any detectable levels of uPA mRNA, whereas the highly invasive, ER-negative tumor cells expressed high levels of uPA. These results suggest that complete demethylation of the uPA promoter is required for uPA mRNA expression and the subsequent high invasive capacity of these cells.

Methylation of the uPA Promoter in Surgical Biopsy Samples from Patients with Breast Cancer.

Methylation status of the uPA promoter was analyzed in surgical biopsy samples from patients with breast cancer of different grades, as determined by the Elston-Ellis histological grading system, which classifies tumors based on tubular architecture, nuclear

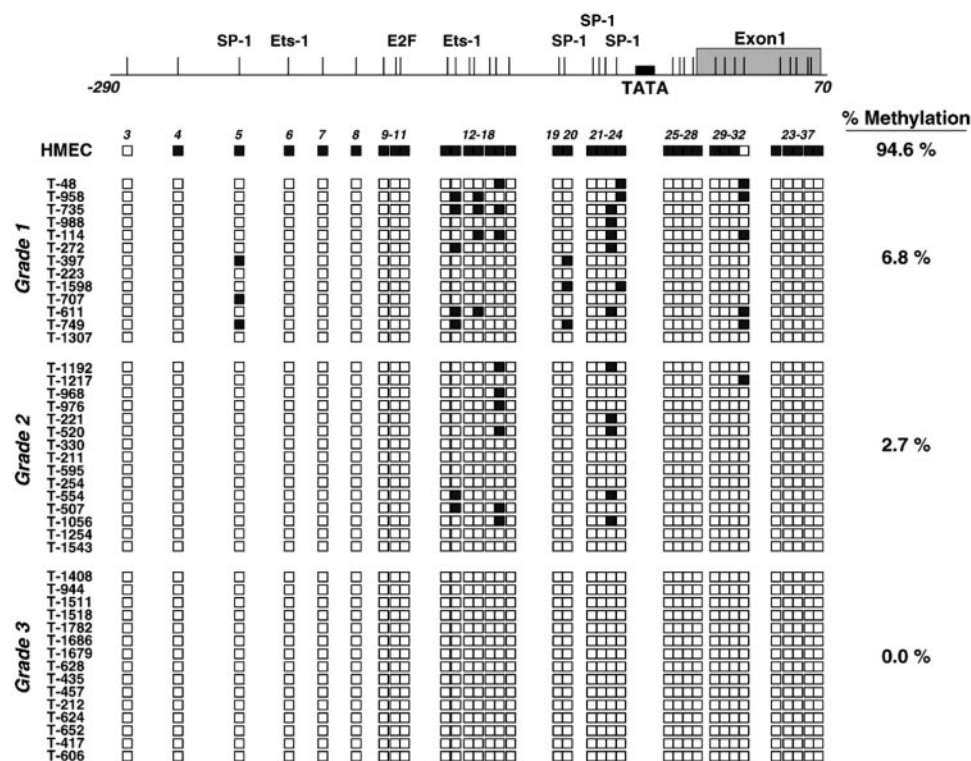


Fig. 4 Sodium bisulfite sequencing analysis of urokinase (uPA) promoter in surgical biopsy samples from patients with breast cancer. Genomic DNA was isolated from HMEC cells and surgical biopsy samples of grades 1, 2, and 3, as determined by Elston-Ellis histological grading system. The DNA was treated with sodium bisulfite and amplified using a set of uPA primers specific for the modified DNA. The amplified products were subcloned into TOPO pCR2.1 vector and sequenced by automated sequencer. The methylated cytosines were distinguished from unmethylated cytosines because they are resistant to bisulfite treatment and remain unchanged. Open and closed boxes represent unmethylated and methylated CpG dinucleotides, respectively. All results are representative sequencing of five different amplified samples repeated three times. The ratio of total methylated sites to all sites was determined and recorded as the percentage of methylation of the uPA promoter; a value of 100% represents total methylation of the potential methylation sites analyzed in this region.

		ER	uPA
	HMEC	+	-
Grade 1	T-48	+	-
	T-958	+	-
	T-735	+	-
	T-988	+	-
	T-114	+	-
	T-272	+	-
	T-397	-	-
	T-223	-	-
	T-1598	+	-
	T-707	-	-
	T-611	+	-
T-749	+	-	
T-1307	-	+	
Grade 2	T-1192	+	-
	T-1217	-	-
	T-968	+	-
	T-976	+	-
	T-221	-	-
	T-520	-	-
	T-330	-	+
	T-211	-	-
	T-595	-	+
	T-254	-	+
	T-554	-	-
	T-507	+	-
	T-1056	+	-
	T-1254	-	+
T-1543	-	+	
Grade 3	T-1408	-	+
	T-944	+	+
	T-1511	-	+
	T-1518	-	+
	T-1782	-	+
	T-1686	-	+
	T-1679	+	-
	T-628	-	+
	T-435	-	+
	T-457	-	+
	T-212	-	+
	T-624	-	+
	T-652	+	+
	T-417	+	-
	T-606	+	+

Fig. 5 Analysis of urokinase and estrogen receptor mRNA levels in biopsy samples. Total RNA was isolated from the gross surgical biopsy samples and subjected to reverse transcription-PCR analysis to determine the levels of urokinase and estrogen receptor mRNA expression in these tumors. The presence and absence of mRNA expression are indicated by + and -, respectively. Results are representative of at least three independent analyses.

pleomorphism, and mitotic counts (27). Methylation sites within the uPA promoter (-290 to +70) were mapped by sodium bisulfite sequencing of the genomic DNA isolated from these tumors. Results showed that 6.8% of the CpG dinucleotides within the analyzed region of the uPA promoter were methylated in grade 1 patients. Partial methylation of the uPA promoter was detected in 11 of 13 patients in this group. In grade 2 patients, partial methylation of the uPA promoter was detected in 9 of 15 patients, and only 2.7% of the potential sites were methylated. In contrast, no methylated sites were found in the 15 grade 3 patients analyzed. Results are representative of sequencing analysis of five different clones for each sample, repeated three times. Collectively, the bisulfite sequencing results

showed that unlike HMEC cells, where the uPA promoter is >94% methylated within this region, the percentage of methylation of the uPA promoter has been found to be significantly reduced after malignant transformation in patients with breast cancer (Fig. 4). This demethylation of the uPA gene was directly associated with induction of uPA mRNA expression as determined by RT-PCR analysis of the total RNA isolated from these tumors (Fig. 5). Levels of ER mRNA expression were also analyzed by RT-PCR, and results showed that 9 of 13 grade 1 patients expressed detectable levels of ER mRNA. In the grade 2 and grade 3 patient groups, only 5 of 15 patients in each group expressed detectable ER levels (Fig. 5).

The percentage of methylation of the uPA promoter and the percentage of uPA and ER mRNA expression in these samples were summarized and graphed, and the results showed a direct correlation between demethylation of the uPA promoter and induction of uPA mRNA expression in these patients (Fig. 6). Thus, demethylation of the uPA promoter has a direct impact on uPA mRNA expression. Additionally, whereas ER expression decreases from grade 1 to grade 3 patients, changes in uPA mRNA expression are not completely dependent on the ER status of these breast cancer patients (Fig. 6).

DISCUSSION

Substantial evidence has demonstrated in different studies over the years the importance of epigenetic mechanisms in the transcriptional regulation of genes that play critical roles in the process of cancer progression. Regional hypermethylation of a number of tumor suppressor genes (Rb, p16, and VHL) has been characterized and shown to be responsible for silencing of these genes in tumor cells (28, 29). In contrast, little attention has been paid to the cancer-specific global hypomethylation of the genome, which is known to be responsible for the activation of several tumor-promoting genes such as uPA, insulin-like growth factor, and ras oncogene (30, 31). Data presented in this study confirm that demethylation of the uPA promoter is the key mechanism responsible for induction of uPA gene expression in aggressive stages of breast cancer. We analyzed normal human mammary epithelial HMEC cells and seven different human breast cancer cell lines, and we showed that uPA expression clearly correlates with lack of ER expression and high invasive capacity of tumor cells. Methylation analysis of the uPA promoter in these cells provided a detailed methylation pattern of the uPA promoter and showed that even partial methylation of the uPA promoter was accompanied by ER expression, low invasive capacity, and silencing of uPA gene expression. These results suggest that in late stages of breast cancer, when the disease is characterized by the loss of functional ER expression, and the cancer cells become refractory to hormonal treatments, the uPA promoter becomes unmethylated, leading to high expression of uPA and increased invasive capacity of the cancer cells. Moreover, we have previously shown that *in vitro* methylation of the uPA promoter results in inactivation of uPA promoter activity (23, 24). All these findings therefore suggest that demethylation of the uPA promoter can be the mechanism responsible for initiation of the process of enhanced tumor invasion and metastasis in breast cancer patients.

We analyzed the methylation status of the uPA promoter in

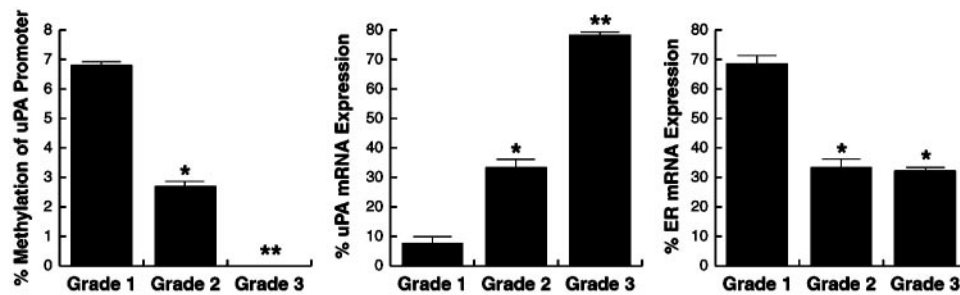


Fig. 6 Reverse correlation between percentage of methylation of the urokinase promoter (uPA) and uPA mRNA expression in biopsy samples. Percentage of methylation of the uPA promoter and the uPA and estrogen receptor mRNA expression in the biopsy samples analyzed are summarized and graphed. Results are the mean \pm SE of at least three independent analyses of 13 grade 1 samples, 15 grade 2 samples, and 15 grade 3 samples. Significant difference from grade 1 is shown by an *asterisk*, and significant difference from both grade 1 and 2 is shown by *two asterisks* ($P < 0.05$).

43 surgical biopsy samples isolated from patients with breast cancer. These biopsy samples were grades 1 (13 samples), 2 (15 samples), and 3 (15 samples), as determined by the Elston-Ellis histological grading system. The genomic DNA of the gross tumor was isolated from each sample, and the methylation status of uPA was determined and compared with the normal mammary epithelial HMEC cells because we were not able to obtain normal breast tissues at this point. Results showed complete or significant demethylation of the uPA promoter associated with malignant transformation. No methylation sites were found in grade 3 tumors, where high levels of uPA mRNA were expressed as determined by RT-PCR analysis. Due to the limited amount of specimens available, we were unable to determine the production of uPA protein in all these patients. However, it has been reported previously that in representative samples of grade 1, 2, and 3 breast cancer patients, the production of uPA determined by ELISA was associated with poorer overall survival (8, 32). Results obtained from both the cell lines and the clinical samples suggest that even partial methylation of the uPA promoter can result in silencing of uPA expression. Determination of whether it is methylation of specific sites within the promoter or partial methylation anywhere within this region that is actually responsible for silencing of promoter activity awaits further analysis of a much larger number of clinical samples.

Methylation-specific PCR and bisulfite sequencing-based assays have been developed and used for rapidly and accurately detecting hypermethylation of promoter regions of cancer-associated genes as markers (20, 33). Results obtained from these studies on various breast cancer cell lines and tumors isolated from patients with breast cancer suggest that full demethylation of the uPA promoter is associated with high levels of uPA expression and tumor cell invasive capacity. However, these changes in uPA promoter methylation and uPA gene expression did not always correlate with the ER status of the patients. Detailed analysis of grade 2 and grade 3 patients showed that uPA production is independent of the ER status of these tumors. Based on these novel findings, we suggest that the demethylation status of the uPA promoter can provide the earliest possible tool for predicting induction of uPA expression, independent of ER status of the patients. Therefore, methylation analysis of the uPA promoter in breast cancer patients can be used as a reliable method to predict disease progression into the invasive stage of the disease. These findings are of particular significance because

it is now well established that determination of suitable prognostic markers such as uPA can be highly reliable in identifying breast cancer patients who are at high risk of disease recurrence and because high uPA concentrations in primary breast cancers correlate with poor prognosis (8, 32). Although different types of assays such as catalytic activity assays, immunohistochemistry, and *in situ* hybridization have been used in studies to examine levels of uPA expression in primary breast cancers, only ELISA has been clinically used for determination of uPA production levels as a prognostic marker (8, 9, 34, 35). Detergent extract of breast cancer tissue has been shown to be most suitable for determining tumor uPA levels (36). The clinical significance of demethylation of the uPA promoter as a prognostic marker remains to be determined by further in-depth studies such as parallel and simultaneous measurements of uPA levels by ELISA and examination of methylation status of the uPA promoter by bisulfite sequencing analysis of fine needle biopsy samples from patients with breast carcinoma. Using laser capture microscopy, further analysis of methylation of the uPA promoter in both tumor cells and tumor-surrounding stroma is also required to determine the value of this technique in clinics.

Due to the established role of uPA in promoting tumor progression, this approach can also be applied to predict the induction of uPA by tumor cells in several other malignancies such as glioma, prostate, lung, and colon cancer (37–40). Indeed, methylation analysis has been shown to be highly beneficial in predicting the production of several tumor-associated genes (41, 42). Determination of the methylation status of such genes in tumoral extract, blood, saliva, and other body fluids provided the first reliable evidence of production of these genes; results that could not be reliably obtained by other methods (17, 41, 42). Collectively, the results obtained in this study provide new opportunities for the development of methylation-specific PCR-based analysis of uPA promoter methylation status, which can provide the earliest possible tool for predicting induction of uPA gene expression and thus subsequent progression of the disease into the aggressive stages (34, 35, 43). Additionally, determination of these changes in tumor cell behavior and the process of uPA gene demethylation will allow additional studies leading to the development of new therapeutic strategies to selectively silence the transcriptional activity of the uPA gene to block the process of tumor progression in patients with breast cancer (31, 44).

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