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Downregulation of Human Kallikrein 10 *(KLK10/NES1)* by CpG Island Hypermethylation in Breast, Ovarian and Prostate Cancers

Michael Sidiropoulos[a, b] Georgios Pampalakis[c] Georgia Sotiropoulou[c]
Dionyssios Katsaros[d] Eleftherios P. Diamandis[a, b]

[a]Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, and [b]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada, [c]Department of Pharmacy, School of Health Sciences, University of Patras, Patras, Greece, and [d]Department of Gynecology, Gynecologic Oncology Unit, University of Turin, Turin, Italy

Key Words
Breast cancer · CpG islands · *KLK10/NES1* · Methylation · Ovarian cancer · Prostate cancer

Abstract
Objective: The human kallikrein 10 *(KLK10)*/normal epithelial cell-specific-1 *(NES1)* gene is highly expressed in normal mammary, ovary and prostate cells, but its expression is dramatically decreased in cancer cell lines. Recently, it has been shown that CpG island hypermethylation of the *KLK10* gene is responsible for the tumor-specific loss of *KLK10* gene expression in certain breast cancer cell lines. ***Method:*** We examined the role of CpG island hypermethylation in the tumor-specific loss of *KLK10* expression in breast, ovarian and prostate cancers. We treated cells with the demethylating agent 5-aza-2′-deoxycytidine (dC) and monitored changes in *KLK10* mRNA by RT-PCR and secreted hK10 protein expression by ELISA. The following cell lines were used: MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, T-47D and BT-474 (breast); BG-1, MDAH-2774, HTB-75, HTB-161, PA-1 and ES-2 (ovary), and LNCaP and PC-3 (prostate). ***Results:*** Upregulation of KLK10 mRNA levels, which was accompanied by an increase in secreted hK10 protein concentration, was observed for a subset of breast, ovarian, and prostate tumor cell lines after 5-aza-2′-dC. Genomic sequencing of sodium-bisulfite-treated DNA demonstrated that CpG sites within the *KLK10* gene exon 3 were highly methylated. Hypermethylation of exon 3 CpG regions was also detected in primary ovarian cancers. ***Conclusion:*** These data suggest that CpG island hypermethylation plays an important role in the downregulation of kallikrein 10 mRNA and protein expression, but it cannot explain the pattern of expression of this gene in all cell lines or tissue tested.

Introduction

Numerous mechanisms have been identified to account for the inactivation of gene expression during oncogenesis. Mutations, rearrangements or deletions within a gene or its regulatory regions, as well as a loss of essential transcriptional factors that are required for gene expression, could provide such a mechanism [1]. However, many genes do not show any gross rearrangements, muta-

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Dr. E.P. Diamandis
Department of Pathology and Laboratory Medicine, Mount Sinai Hospital
600 University Avenue
Toronto, Ont. M5G 1X5 (Canada)
Tel. +1 416 586 8443, Fax +1 416 586 8628, E-Mail ediamandis@mtsinai.on.ca

tions, or deletions within their sequences or a loss of transcription factors. Studying other mechanisms, therefore, seems plausible in order to account for the inactivation of certain genes in numerous cancer cells. Another prominent mechanism to silence genes is DNA methylation [2, 3].

Accumulating evidence has depicted that inactivation of genes in eukaryotes is often due to the presence of modified cytosine residues, specifically 5-methylcytosine. Approximately 3–5% of all the cytosine residues within the human genome are methylated [4]. DNA methylation occurs at cytosines located 5′ to guanosine residues as CpG dinucleotides. Seventy to eighty percent of these CpG dinucleotides are located in clusters, termed CpG islands. These CpG islands are further clustered within control regions of a gene, mainly in the regulatory and promoter regions, but often in other parts of the gene, including exons [2, 5].

Numerous studies have demonstrated that a close correlation exists between methylation and transcriptional inactivation, supporting the notion that not only genetic changes, but also epigenetic changes can contribute to the carcinogenic process [3, 6]. The methylation pattern in tumors consists of a global hypermethylation, in conjunction with localized hypermethylation at CpG islands [7, 8]. This regional hypermethylation at CpG islands is associated with the transcriptional inactivation of cancer-related genes. Recent studies have demonstrated that hypermethylation of CpG islands may be implicated in tumorigenesis, acting as a mechanism to inactivate specific gene expression of a diverse array of genes [9, 10]. Genes that have been reported to be regulated by CpG island hypermethylation include tumor suppressor genes, cell cycle-related genes, DNA mismatch repair genes, hormone receptors and tissue or cell adhesion molecules [6]. For example, tumor-specific deficiencies of expression of the DNA repair genes *MLHI* and MGMT [11, 12] and the tumor suppressors, p16, *CDKN2* and *MTS1*, have been directly correlated to hypermethylation [9, 10, 13]. Increased CpG island methylation can result in the inactivation of these genes, resulting in increased levels of genetic damage, predisposing cells to later genetic instability which then contributes to tumor progression [3, 6].

Human kallikrein 10 *(KLK10)*/normal epithelial cell-specific-1 *(NES1)* is a member of the human tissue kallikrein family of secreted serine proteases, encoded by a family of 15 genes clustered in tandem on chromosome 19q13.3–4. All kallikreins share important similarities, including significant sequence homologies at both the DNA and protein levels [14, 15]. Serine proteases are a homologous family of protein-degrading enzymes, involved in a variety of biological functions, such as coagulation/fibrinolysis, digestion, wound healing, tissue remodeling and activation of growth factors containing the kringle domain [16, 17]. The expression of serine proteases has been shown to correlate positively with invasiveness and metastatic potential of many tumor cells [18–20]. The kallikrein gene family has been under intensive study due to its implications in carcinogenesis and the application of many members as biomarkers for the diagnosis and monitoring of certain cancers [14, 15].

Human kallikrein 10 *(KLK10)* was discovered by subtractive hybridization between normal and immortalized breast epithelial cell lines [21]. The hK10 protein is composed of 276 amino acids and is found in diverse tissues, such as breast, ovary, and prostate, as well as in many biological fluids [22]. It has been suggested that hK10 functions as a tumor suppressor, as overexpression of *KLK10* in nude mice can suppress tumor formation [23]. *KLK10* mRNA is known to be downregulated in various cancer cell lines, including those from the prostate and breast [21, 24]. A previous study from our laboratory found no evidence for somatic mutations in the *KLK10* gene in cancers of the prostate, ovary, testis and breast [25]. Recently, it has been shown that CpG island hypermethylation of the *KLK10* gene is responsible for the tumor-specific loss of *KLK10* gene expression in certain breast cancer cell lines and in primary breast tumors [24]. We therefore hypothesized that epigenetic alterations may underlie the lack of *KLK10* expression in prostate and ovarian cancer cells.

In the present study, we examined the role of CpG island hypermethylation in the tumor-specific loss of *KLK10* mRNA and hK10 protein expression within prostate, ovarian and breast cancers. Sequence analysis revealed that exons 2, 3 and 4 were rich in CpG. Using sodium bisulfite DNA sequencing, we demonstrate tumor-specific CpG island hypermethylation within exon 3 of the *KLK10* gene in breast, ovarian and prostate cancer cell lines and primary ovarian tumors. CpG hypermethylation of exon 1 was also studied for BG-1, an ovarian cancer cell line. Furthermore, treatment of low- and non-expressing *KLK10* mRNA and hK10 protein cell lines with the demethylating agent 5-aza-deoxycytidine (5-aza-dC), which can induce de novo expression of stably repressed genes, restored *KLK10* mRNA and hK10 protein expression in the majority of cancer cell lines.

Table 1. hK10 and actin primers

Gene name or *KLK10* exon position	Primer name/sequence (5′–3′)	Reference
Actin	forward: 5′-ACAATGAGCTGCGTGTGGCT-3′ reverse: 5′-TCTCCTTAATGTCACGCACGA-3′	26
KLK10/NES1	forward: 5′-GATCACCTGCTGCTTCTTC-3′ reverse: 5′-CACTCTGGCAAGGGTCCTG-3′	26
KLK10 CpG exon 1	forward: 5′-GGTAGGGTAGAGGTATGTTTGGG-3′ reverse: 5′-CCCTATCCCCCACCTTAC-3′	
KLK10 CpG exon 3	forward: 5′-GAATGTAGTTTAGTGTTATAGTTTAG-3′ reverse: 5′-CACACCTCCAACTATAAAAATTCC-3′	24

Materials and Methods

Cancer Cell Lines

Breast cancer cell lines (BT-474, MCF-7, MDA-MB-231, MDA-MB-468, T-47D and ZR-75-1), ovarian cancer cell lines (ES-2, HTB-75, HTB-161, MDAH-2774 and PA-1), prostate cancer cell lines (LNCaP and PC-3) and a cervical cancer cell line, HeLa, were obtained from the American Type Culture Collection. The BG-1 ovarian cancer cell line was provided by Dr. Henri Rochefort, Montpellier, France. Cells were cultured in RPMI media (Gibco, Gaithersburg, Md., USA) supplemented with glutamine (200 m*M*) and fetal bovine serum (10%), in plastic flasks. Cells were grown to 80% confluency, then harvested for genomic DNA extraction or seeded in dishes for subsequent 5-aza-dC treatment.

Total RNA Extraction

Total cellular RNA was isolated from 60–80% confluent cell monolayers using the RNeasy Midi Kit (Qiagen, Valencia, Calif., USA) following the manufacturer's instructions. Total RNA concentration and its purity were determined spectrophotometrically.

Reverse Transcriptase-Polymerase Chain Reaction

Two micrograms of total RNA were converted to cDNA with the Superscript^{TM} Preamplification Kit (Gibco), following the manufacturer's recommendations. The final volume was 20 μl. Two PCR primers were utilized to amplify the *KLK10* cDNA, as previously described [26]. Their sequences are depicted in table 1. The expected size of the PCR product is 383 bp. The actin primers amplify a PCR product that is 372 bp. PCR was performed in 50 μl of reaction mixture, containing 1 μl of cDNA, 10 m*M* Tris-HCl buffer (pH 8.3), 50 m*M* KCl, 200 μ*M* deoxynucleoside triphosphates, 100 ng primers and 2.5 units of HotstarTaq^{TM} DNA polymerase (Qiagen) on an Eppendorf MasterGradient Thermal Cycler. In all PCR reactions, a blank control was included (water as the template). The same PCR conditions were utilized for both actin gene and KLK10 gene amplification. They were kept at 95°C for 15 min to activate the polymerase, followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. The final extension was at 72°C for 10 min. Equal amounts of PCR products were then separated on a 2.5% agarose gel, stained with ethidium bromide and subsequently visualized under UV light.

Treatment of Cells with 5-Aza-2′-dC

Cells were seeded at a density of 5×10^5/100-mm dishes, cultured for 48 h, and treated with 0, 50 or 100 μ*M* 5-aza-dC (Sigma, St. Louis, Mo., USA) [24]. Forty-eight hours after treatment, cells were washed with PBS, and fresh medium was added. Cells were further incubated for another 48 h before we isolated total cellular RNA. For protein studies, cells were seeded also at a density of 5×10^5/100-mm dishes, cultured for 24 h, and treated with 0, 1, 2, 3, 5, and 10 μ*M* 5-aza-dC. After 5 days, cell supernatants were harvested and centrifuged at 1,800 rpm to pellet and remove any cell debris. Supernatants were transferred to a new tube and analyzed for hK10 protein concentrations or stored at –20°C.

Ovarian Cancer Patients and Specimens

Seven patients with primary ovarian cancer were examined in this study. These patients underwent surgery and treatment for ovarian cancer at the Department of Gynecology, University of Turin, Italy between July, 1991 and April, 1999. Investigations were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983, and were approved by the Institutes of Obstetrics and Gynecology (Turin, Italy, and Mount Sinai Hospital, Toronto, Canada).

Preparation of Cytosolic Extracts

Tumor specimens were snap-frozen in liquid nitrogen immediately after surgery and stored at –80°C until extraction. Frozen tissues (20–100 mg) were pulverized on dry ice to a fine powder and added to 10 volumes of extraction buffer (50 m*M* Tris, pH 8.0, 150 m*M* NaCl, 5 m*M* EDTA, 10 g/l of NP-40 surfactant, 1 m*M* phenylmethyl sulfonyl fluoride, 1 g/l of aprotinin and 1 g/l of leupeptin). The resulting suspensions were incubated on ice for 30 min, with repeated shaking and vortexing every 10 min. The mixtures were then centrifuged at 14,000 rpm at 4°C for 30 min, and the supernatant (cytosolic extract) was collected and stored at –80°C until further analysis. Protein concentration of the extracts was determined using the bicinchoninic acid method, with albumin as standard (Pierce, Rockford, Ill., USA).

Measurement of hK10

The concentration of hK10 in the cancer cell line supernatants and cytosolic extracts of primary ovarian cancers was quantified with a sensitive and specific noncompetitive immunoassay (ELISA). By utilizing two monoclonal anti-hK10 antibodies, a sandwich-type, one-step immunofluorometric assay for hK10 was used. This assay has been described and evaluated in detail elsewhere [27]. The detection limit is 0.05 μg/l and precision was <10% within the measurement range (0.05–20 μg/l).

All supernatants were analyzed in quadruplicate. Tumor extracts were measured in duplicate. hK10 concentrations in micrograms per liter were converted to nanograms of hK10/mg of total protein to adjust for the amount of tumor tissue extracted.

Analysis of DNA Methylation by Sequencing of Sodium-Bisulfite-Treated DNA

Genomic DNA was isolated using the Qiagen QIAamp DNA Mini Kit and subjected to sodium bisulfite treatment to modify unmethylated cytosine to uracil, using the CpGenome™ DNA Modification Kit (Intergen, Oxford, UK) following the manufacturer's instructions. Bisulfite-treated DNA was subjected to PCR using the primers shown in table 1 for the CpG islands located within the KLK10 gene. The conditions for PCR were as follows: 1 cycle at 95°C for 15 min; 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and 1 cycle of 72°C for 10 min. The product was purified using the Qiagen gel extraction kit and ligated to a TA cloning vector (Invitrogen, Grand Island, N.Y., USA). Five positive clones were sequenced for each sample using M13 primers (Invitrogen).

Table 2. *KLK10* mRNA and protein expression in various ovarian, prostate and breast cancer cell lines

Cancer cell line	*KLK10* mRNA expression	hK10 protein μg/l	Methylation status	Reference
BT-474	+	0.54	partial	24
MCF-7	+	6.4	partial	24
MDA-MB-231	–	0.08	partial/full	24
MDA-MB-468	+	10.0	–	
T-47D	+	3.5	partial	
ZR-75-1	–	<0.05	no	
HeLa	–	0.11	partial/full	24
BG-1	–	0.09	partial/full	
ES-2	–	<0.05	partial	
HTB-75	+	0.67	no	
HTB-161	+	2.1	partial/full	
MDAH-2774	+	1.9	partial	
PA-1	–	0.07	partial	
LNCaP	–	0.06	partial	
PC-3	+	0.22	partial/full	

Partial methylation is defined as <100% methylation within the region of interest (fig. 1a, ES-2). Full methylation is defined as 100% methylation within the region of interest (fig. 1a, PC-3).

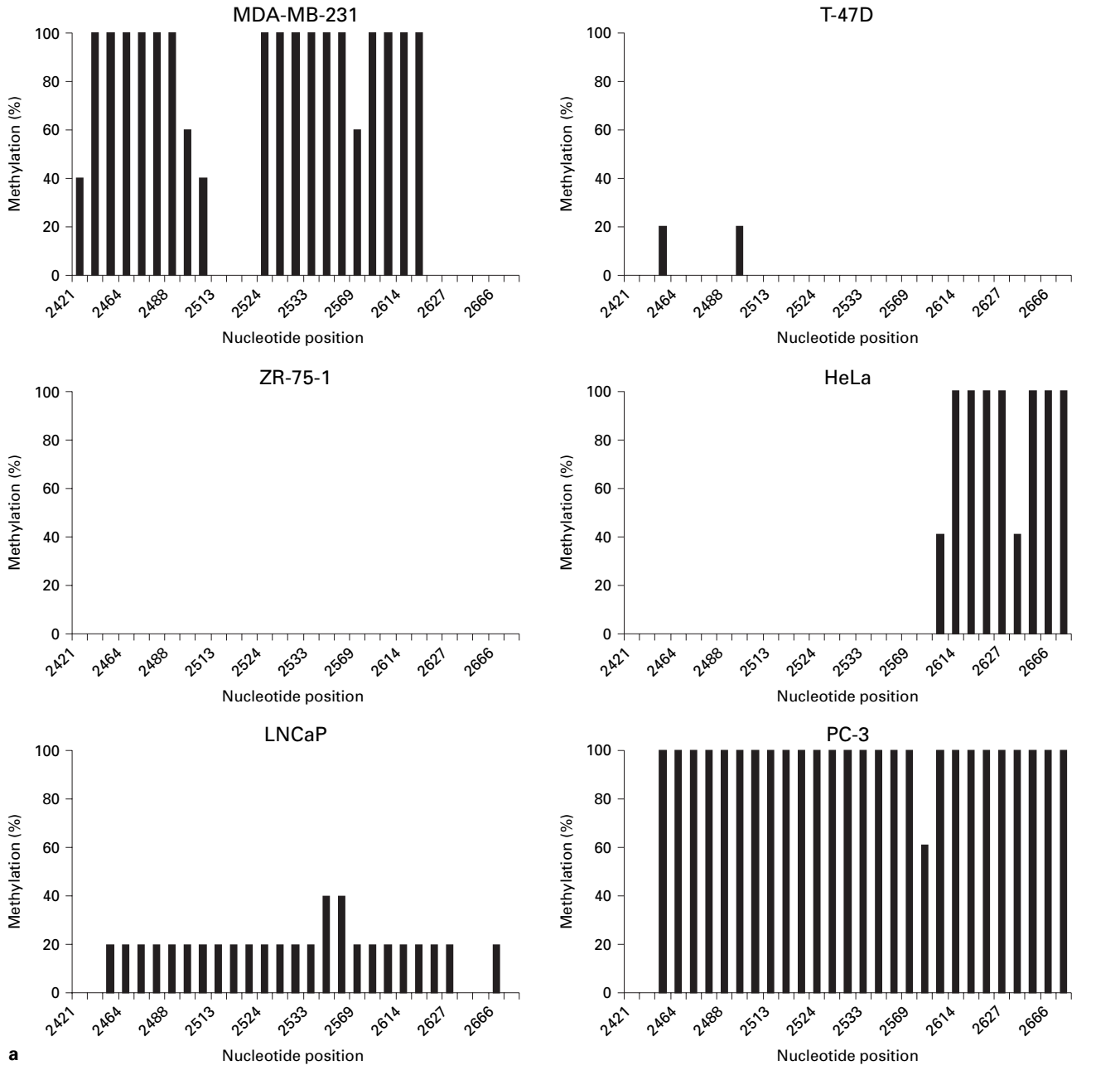
Results and Discussion

KLK10 *mRNA and Protein Expression in Various Breast, Ovarian and Prostate Cancer Cell Lines*

KLK10 mRNA expression in normal cells has been previously described, as well as the reduced or completely absent expression in most breast and prostate cancer cell lines [21, 23, 24]. However, *KLK10* mRNA expression in ovarian cancer cells and hK10 protein expression in breast, ovarian and prostate cancer cells have not been examined previously. We first examined *KLK10* mRNA expression and hK10 protein expression in a panel of cancer cell lines, using RT-PCR and an ELISA assay for hK10. For our study, we chose six human breast (BT-474, MCF-7, MBA-MD-231, MBA-MD-468, T-47D and ZR-75-1), one cervical (Hela), six ovarian (BG-1, ES-2, HTB-75, HTB-161, MDAH-2774 and PA-1) and two prostate (LNCaP and PC-3) carcinoma cell lines. As expected, *KLK10* mRNA levels were similar to the results previously reported (table 2). *KLK10* mRNA and hK10 protein were very low or undetectable in the majority of the cancer cell lines. In contrast, three breast (MCF-7, MDA-MB-468 and T-47D), and two ovarian cancer cell lines (HTB-161 and TBHTMDAH-2774) expressed easily detectable levels of hK10 protein. As expected, cells with positive *KLK10* mRNA expression had relatively high hK10 protein levels in their supernatants. All these cell lines were used to study CpG island hypermethylation as a mechanism of tumor-specific downregulation of kallikrein 10 mRNA and protein expression.

Tumor-Specific Hypermethylation of KLK10 *Exons 1 and 3*

To elucidate whether CpG island hypermethylation is associated with the down-regulation of *KLK10* gene expression, we examined the pattern of CpG abundance within the *KLK10* gene. CpG-rich regions were analyzed using the Cpgplot program from the European Bioinformatics Institute (www.ebi.ac.uk). DNA sequences with an observed overexpected ratio >0.60 and a >50% G+C content were defined as CpG-rich [28]. Analyses of the *KLK10* gene showed that exons 2–4, as previously shown by Li et al. [24], and introns 1, 2 and 4 were rich in CpG. Similarly to Li et al. [24], we focused on the analysis of methylation of exon 3 due to the particularly high CpG-rich structure of exon 3, showing a 70% G+C content and an observed-overexpected CpG frequency of 1.05.



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Analysis of CpG island methylation was performed on sodium-bisulfite-treated DNA. In this method, sodium bisulfite is used to convert all unmethylated cytosines to uracils, which Taq polymerase subsequently recognizes as thymidines during the subsequent PCR step [29]. The CpG islands within exons 1 and 3 for the BG-1 cancer cell line, and exon 3 for all cancer cells were amplified from sodium-bisulfite-treated DNA using the primers shown in table 1. PCR products were subsequently cloned into TA cloning vectors, and five individual colonies were sequenced to identify methylated cytosine residues. Many cancer cell lines, expressing low or no *KLK10* mRNA,

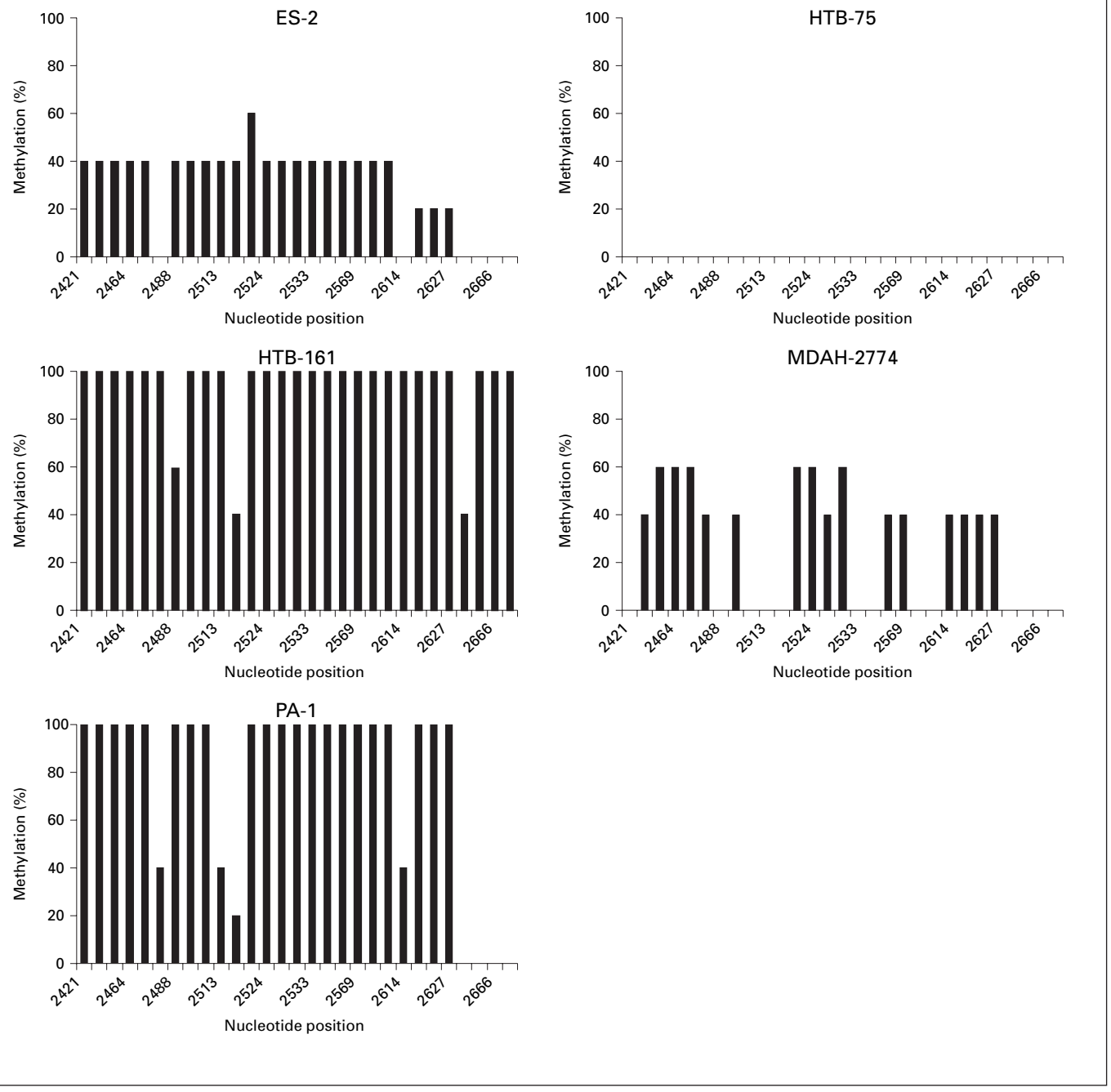


Fig. 1. Differential cytosine methylation of the kallikrein 10 gene locus. **a** Summary of 5-methylcytosine levels obtained by sodium bisulfite genomic sequencing of *KLK10* exon 3 in 11 cancer cell lines. Five cloned PCR products were sequenced to determine the percent methylation of the CpG sites in the regions analyzed.

(For fig. 1b see next page.)

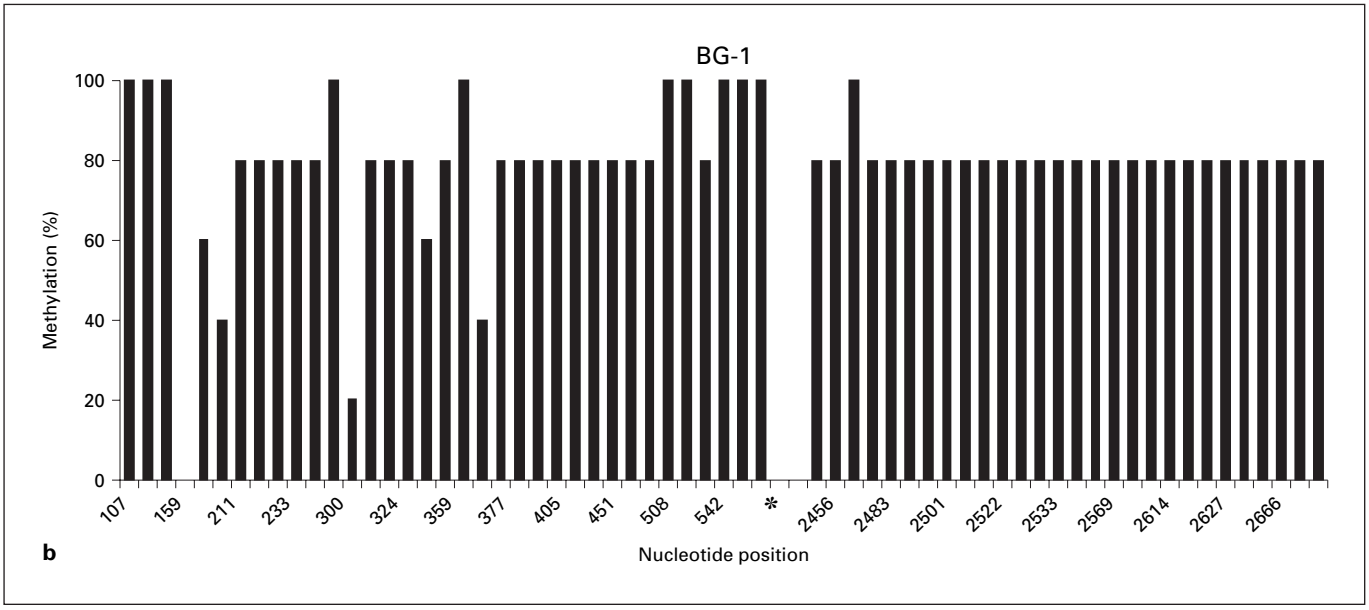


Fig. 1. b Summary of 5-methylcytosine levels of CpG sites spanning exons 1 and 3 of the 5′ end of the *KLK10* gene in the ovarian cancer cell line BG-1. Asterisk shows the position of regions not analyzed. The y-axis represents percent cytosine methylation and the x-axis represents the nucleotide position relative to the transcription start site.

contained partially or fully methylated CpG dinucleotides within exon 3 (fig. 1a). Exons 1 and 3 of the ovarian cancer cell line BG-1 demonstrated partially or fully methylated CpG dinucleotides (fig. 1b). The MDA-MB-231 and HeLa cancer cell lines were our positive controls, as the pattern of methylation of *KLK10* exon 3 has been described by Li et al. [24] previously. We were able to reproduce their results.

Reversal of DNA Methylation with 5-Aza-2′-dC Induces Kallikrein 10 mRNA and Protein Expression in Low- and Non-Expressing Tumor Cells

Numerous studies have demonstrated that methylation-regulated genes are released from repression or silencing after treatment of cells with 5-aza-dC [30]. 5-Aza-dC is a cytidine analog that sequesters DNA methyltransferase after its incorporation into genomic DNA [31]. To assess whether 5-aza-dC treatment induces re-expression of kallikrein 10 mRNA and protein in tumor cells, we treated numerous kallikrein 10 mRNA low-expressing (BT-474, MCF-7, MDA-MB-468, T-47D, HTB-75, HTB-161, MDAH-2774 and PC-3) and non-expressing (MDA-MB-231, ZR-75-1, HeLa, BG-1, ES-2, PA-1 and LNCaP) cells (table 2) with different concentrations of 5-aza-dC and performed RT-PCR to detect *KLK10* mRNA expression (fig. 2). Non-treated cells served as negative controls. Co-amplification of β-actin was performed to demonstrate that the changes seen were not due to the amount of RNA used. In each case, the control actin levels were roughly equivalent in samples obtained from cells treated with various levels of 5-aza-dC. Cell numbers were also counted in order to form a baseline for comparison of expression.

A specific and sensitive hK10 immunoassay was utilized to evaluate changes in protein expression (table 2, fig. 3). Treatment of low- and non-expressing cells resulted in a dose-dependent increase in kallikrein 10 expression at both the mRNA and protein levels for the following cells: MCF-7, MDA-MB-231, ZR-75-1, BG-1, MDAH-2774 and PC-3. In contrast, an increase in mRNA levels was only seen for the cancer cell line HTB-161. No changes in protein expression were seen with the 5-aza-dC concentrations utilized for this cell line. No change in mRNA or protein expression was observed in the following low- and non-expressing kallikrein 10 cancer cells: BT-474, HeLa, ES-2, HTB-75, LNCaP, PA-1 and T47-D. All experiments were repeated three times. The cell lines MCF-7, MDA-MB-231 and HeLa were used as positive controls. We were able to reproduce the results demonstrated by Li et al. [24] for these cell lines.

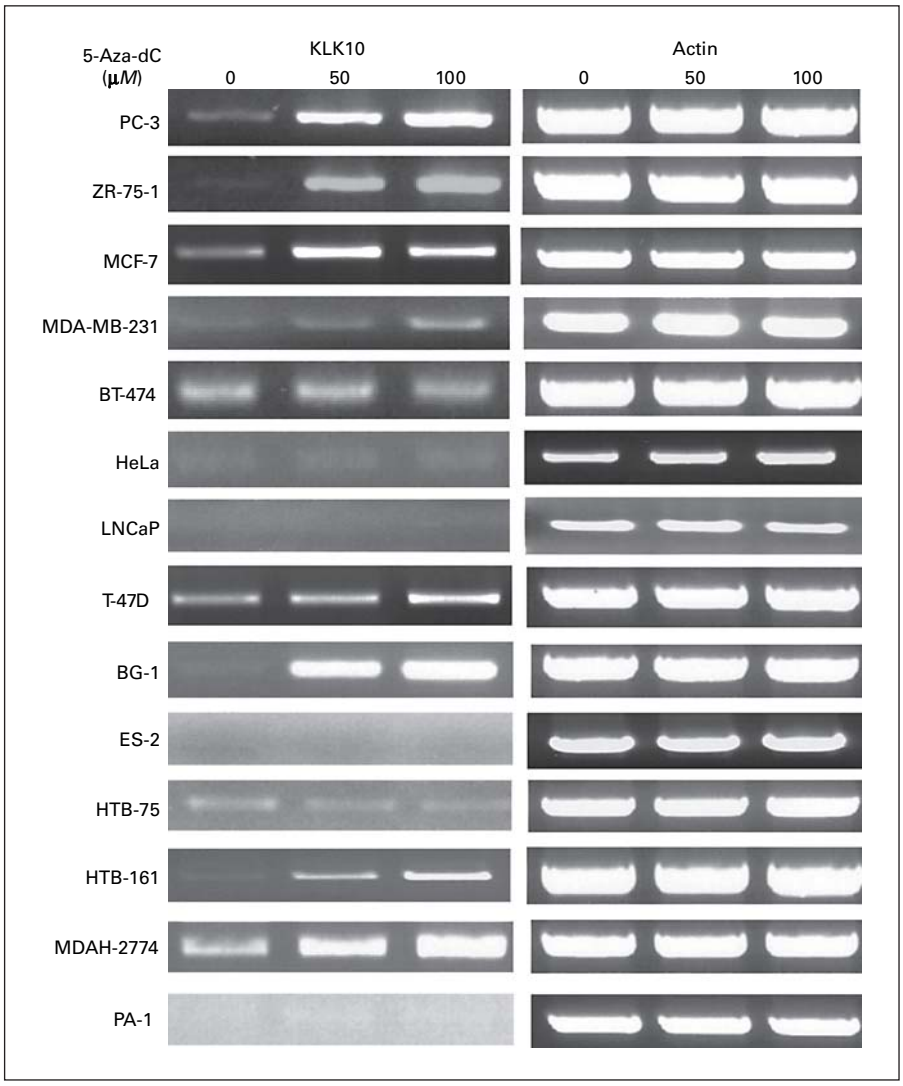


Fig. 2. Kallikrein 10 mRNA expression analysis by RT-PCR following treatment of cancer cells with 0, 50 and 100 μ*M* of 5-aza-dC. Forty-eight hours after treatment, cells were washed with PBS, and fresh medium was added. Cells were incubated for another 48 h before total cellular RNA was isolated. *KLK10* mRNA was then analyzed by RT-PCR using the primers described in table 1. Actin was co-amplified as an internal control. Water was used as a negative control (not shown). For data analysis, see the Results.

Exon 3 CpG Island Hypermethylation Status and Kallikrein 10 Protein Expression in Primary Ovarian Tumor Specimens

We further examined the methylation status and kallikrein 10 protein expression in 7 microdissected ovarian tumor specimens. Sodium-bisulfite-treated DNA from 7 tumor and 2 cancer-free samples was subjected to PCR amplification of exon 3 to identify the pattern of CpG methylation (fig. 4). As seen in figure 4, 6 of the 7 (86%) tumor samples showed partially methylated CpG dinucleotides within exon 3. The 2 cancer-free ovarian samples displayed no methylated CpG dinucleotides.

To explore whether differences in hK10 protein expression levels are the result of the methylation status of exon 3 CpG dinucleotides, we prepared cytosolic extracts from the primary tumor samples and measured hK10 protein levels using an hK10-specific immunoassay (table 3, fig. 5). There seems to be no simple relationship between the degree of methylation of CpG dinucleotides and protein expression. Interestingly, tumor sample M269, which had no methylation of CpG dinucleotides, expressed the highest level of kallikrein 10 protein (fig. 5).

The human kallikrein 10 *(KLK10)*/normal epithelial cell-specific-1 *(NES1)* gene is a member of the human kallikrein gene family of serine proteases [14]. The hK10 protein is encoded by a gene designated as *KLK10*, which maps on chromosome 19q13.3–4, spans about 5.5 kb of genomic sequence and contains 5 coding exons and 1 untranslated exon [15]. hK10 is secreted by breast and oth-

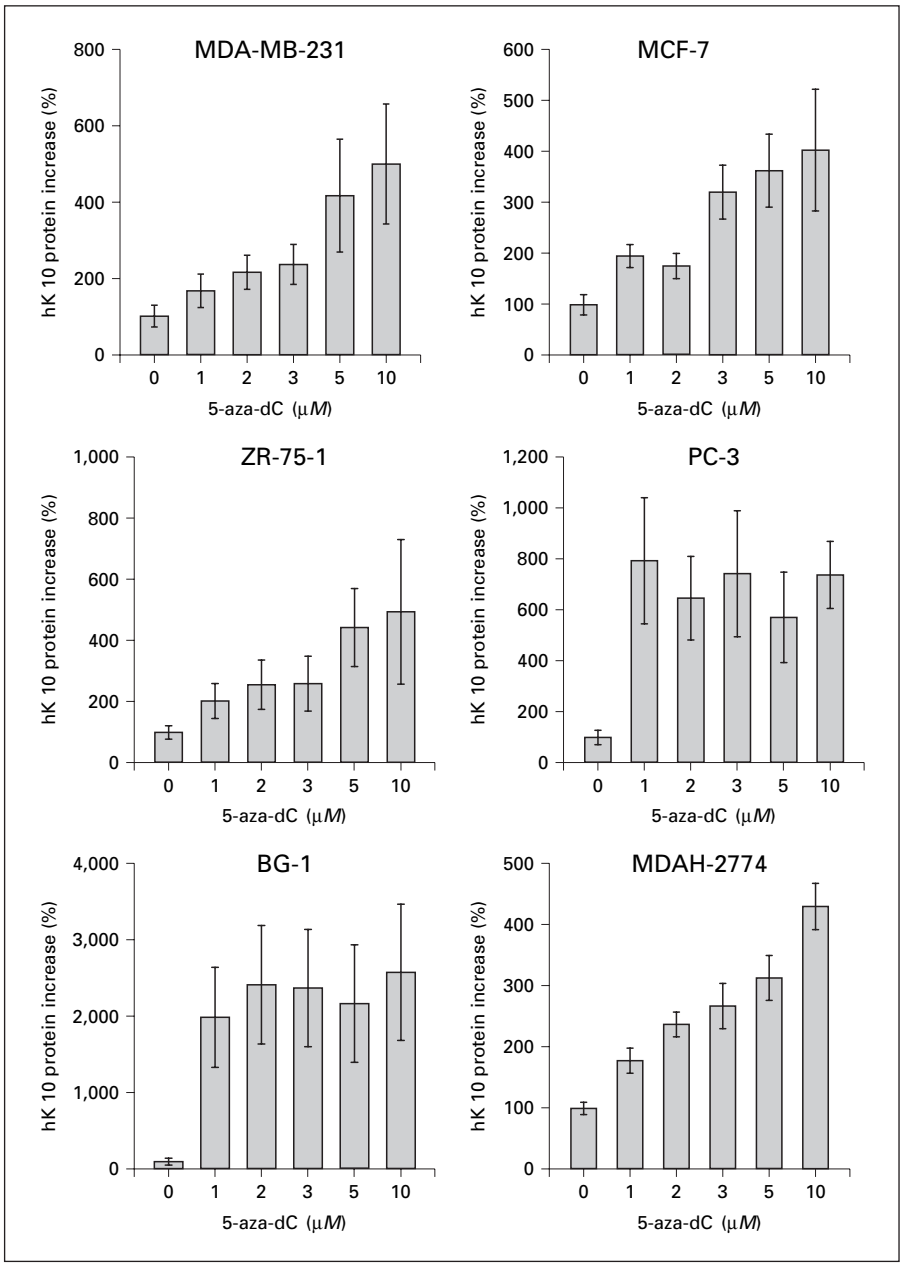


Fig. 3. Effects of 5-aza-dC on hK10 protein expression. Cancer cell lines were treated with 0, 1, 2, 3, 5 and 10 μ*M* 5-aza-dC for 5 days, after which cell line supernatants were analyzed with a highly sensitive and specific noncompetitive immunoassay for hK10. Protein levels are expressed as percentage values relative to the protein levels in the untreated cells (100%).

er epithelial cells, and *KLK10* is expressed in many tissues, including the ovary, breast, prostate, colon and testes.

The physiological function of hK10 is still unclear. Recent data have suggested that *KLK10* may have tumor suppressor functions, as it is downregulated in breast and prostate cancer cell lines, and its overexpression in nude mice can suppress tumor formation [21, 23]. This putative tumor suppressor activity prompted us to speculate that this gene may be a target for either somatic mutations or hypermethylation, in analogy to other tumor suppressor genes that are inactivated by such mechanisms. A previous study from our laboratory examined the polymorphic and mutational status of the *KLK10* gene using DNA isolated from normal tissues and from cancers of the breast, ovary, prostate and testes [25]. We found that the *KLK10* gene is not a target for somatic mutations in any of these cancers. However, a single nucleotide variation at codon 50 may be associated with the risk of prostate cancer. Recently, Li et al. [24] have suggested an important role for CpG island methylation in the loss of

KLK10 gene expression in breast cancer. We thus hypothesized that the *KLK10* gene may also be a target for methylation in ovarian and prostate cancer. In this study, we examined in detail the methylation status of this gene using the demethylating agent 5-aza-dC and sodium-bisulfite-treated DNA from 15 cancer cell lines from breast, ovarian and prostate cancers, and primary ovarian tumor samples.

Analysis of the *KLK10* gene through the use of a Cpgplot program revealed that its promoter was CpG poor, while exons 2–4, and introns 1, 2 and 4 were rich in CpG. Exon 3 had a particularly high density of CpG dinucleotides. Thus, our studies focused mainly on exon 3, although limited analysis of CpG dinucleotides within exon 1 was also performed.

To assess the status of the CpG islands within the *KLK10* gene, we sequenced exon 3 from sodium-bisulfite-modified DNA. The CpG dinucleotides in CpG islands are normally protected from methylation, but their aberrant methylation can promote the binding of proteins that recognize methyl-CpGs and lead to alterations in the chromatin structure and repression of transcription [32]. The CpG dinucleotides within *KLK10* exon 3 were partially or fully methylated in the majority of cancer cell lines, in both kallikrein 10 mRNA and protein low- and non-expressing cells. Furthermore, for the ovarian cancer cell line BG-1, exons 1 and 3 were sequenced. These analyses revealed a relatively high level of methylation within the CpG islands of both exons. Human kallikrein 10 protein-expressing cancer cell lines, such as MDAH-2774 and HTB-161, and low-expressing hK10 cells, MDA-MB-231 and PC-3, had partially or fully methylated CpG dinucleotides. In the breast cancer cell line, MDA-MB-231, Li et al. [24] demonstrated that treatment with the demethylating agent 5-aza-dC was able to induce expression of *KLK10* mRNA, and this was associated with demethylation of *KLK10* exon 3.

This approach was further applied to a set of primary ovarian tumors. The traditional ovarian cancer biomarker, CA 125, is not efficient for diagnosis of early ovarian cancer [33]. It has low sensitivity for early disease, and also suffers from low specificity. For optimal cancer diagnosis and management, it is conceivable that no single cancer marker will provide all the necessary information [27]. Current efforts focus on the identification of groups of biomarkers that can be used in combination. One potential early detection biomarker is DNA methylation of certain cancer-associated genes, which results in gene inactivation [34]. Examination of serum for circulating tumor DNA with abnormal methylation patterns offers a possible method for early detection of several cancers and serves as a point for early intervention and prevention strategies [35].

We therefore studied a small panel of primary ovarian tumors. Six of 7 (86%) tumor samples displayed methylation of the CpG island within exon 3. The two normal samples displayed no methylation. We further analyzed the hK10 protein expression levels, using an hK10-specific immunoassay within these tumors and correlated our results with the methylation status of exon 3. We did not find a relationship between the status of methylation and hK10 protein expression. However, the only tumor sample which had no presence of 5-methylcytosines, M269, expressed the highest level of hK10. In contrast, Li et al. [24] found that very low levels of methylation were seen in breast tumor samples that expressed *KLK10* mRNA and high levels of methylation were seen in tumors that did not express any detectable *KLK10* mRNA. Future studies including a larger panel of tumor samples should facilitate a stronger analysis.

The basis for a downstream CpG island methylation with the lack of kallikrein 10 expression remains unknown. In the present study, we focused on exon 3; however, exons 2 and 4 were also rich in CpG. Possibly, these other CpG-rich regions may also contribute to kallikrein repression or silencing. Specifically, exon 1, a region within the limits of regulatory CpG islands, located –500 to +1,500 bp of the transcriptional start site [36], was studied for the ovarian cancer cell line BG-1. We found that CpG dinucleotides within exon 1 were partially or completely methylated, similar to the patterns seen within exon 3. Larger studies examining the role of all the CpG-rich regions, together with the CpG-poor promoter region, should help to elucidate the role of CpG island methylation and tumor-specific downregulation of kallikrein 10 expression.

The inactivation of gene expression by CpG island hypermethylation is supported by studies using the demethylating agent 5-aza-2′-dC. Application of this agent to cancer cell lines has been shown to demethylate CpG islands and reactivate gene expression of the previously silenced genes [6, 24]. We demonstrated that treatment with 5-aza-dC led to a dose-dependent increase in kallikrein 10 mRNA and protein expression. After treatment with 5-aza-dC, expression of *KLK10* mRNA was restored in seven of the fourteen cell lines, and protein expression was restored in six of the fifteen cell lines. There was a concordance between expression of mRNA and protein, but protein expression was not restored in the HTB-161 cell line, having only enhanced mRNA expression. Cancer cell lines are known to vary in their sensitivity towards

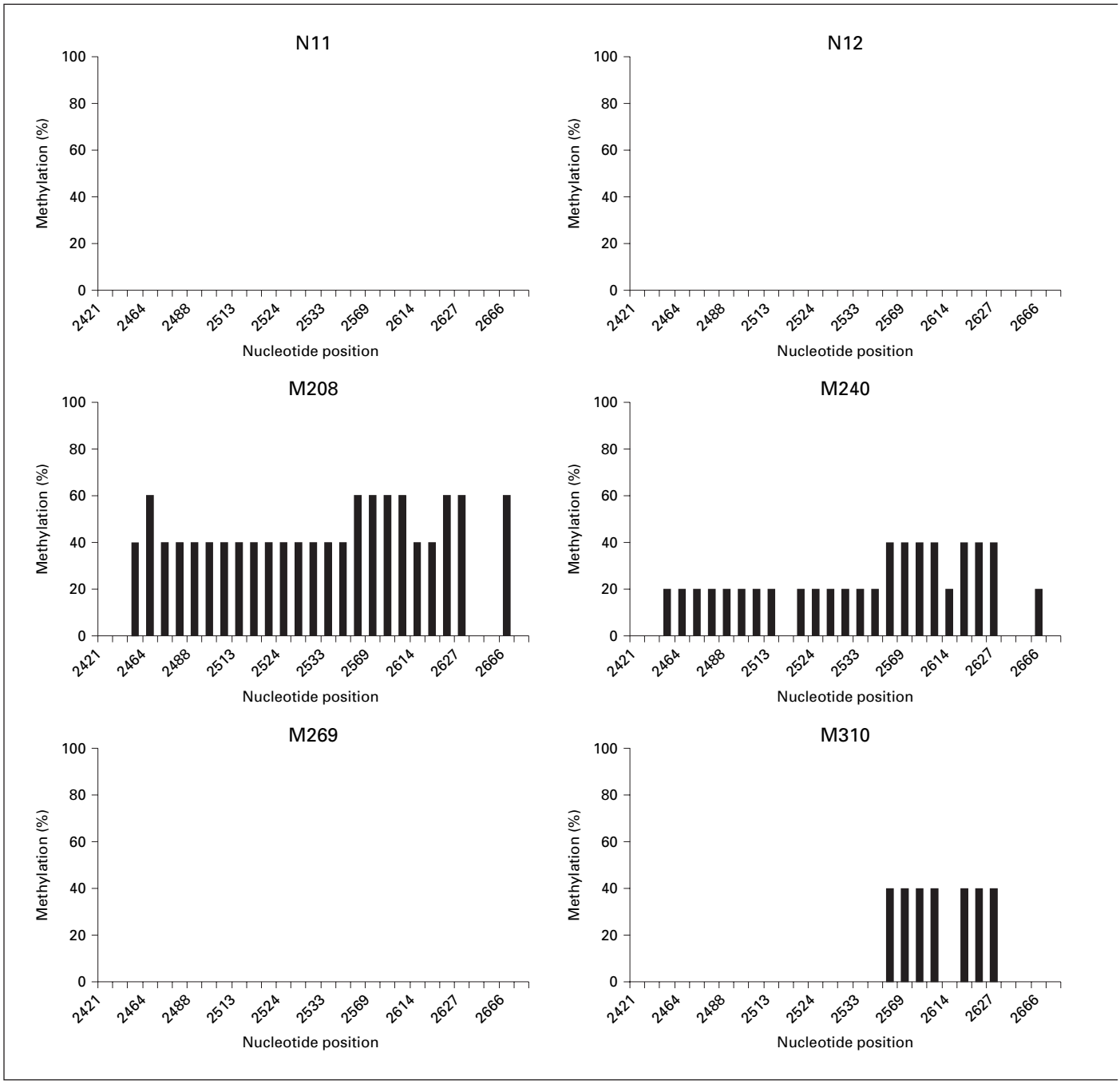
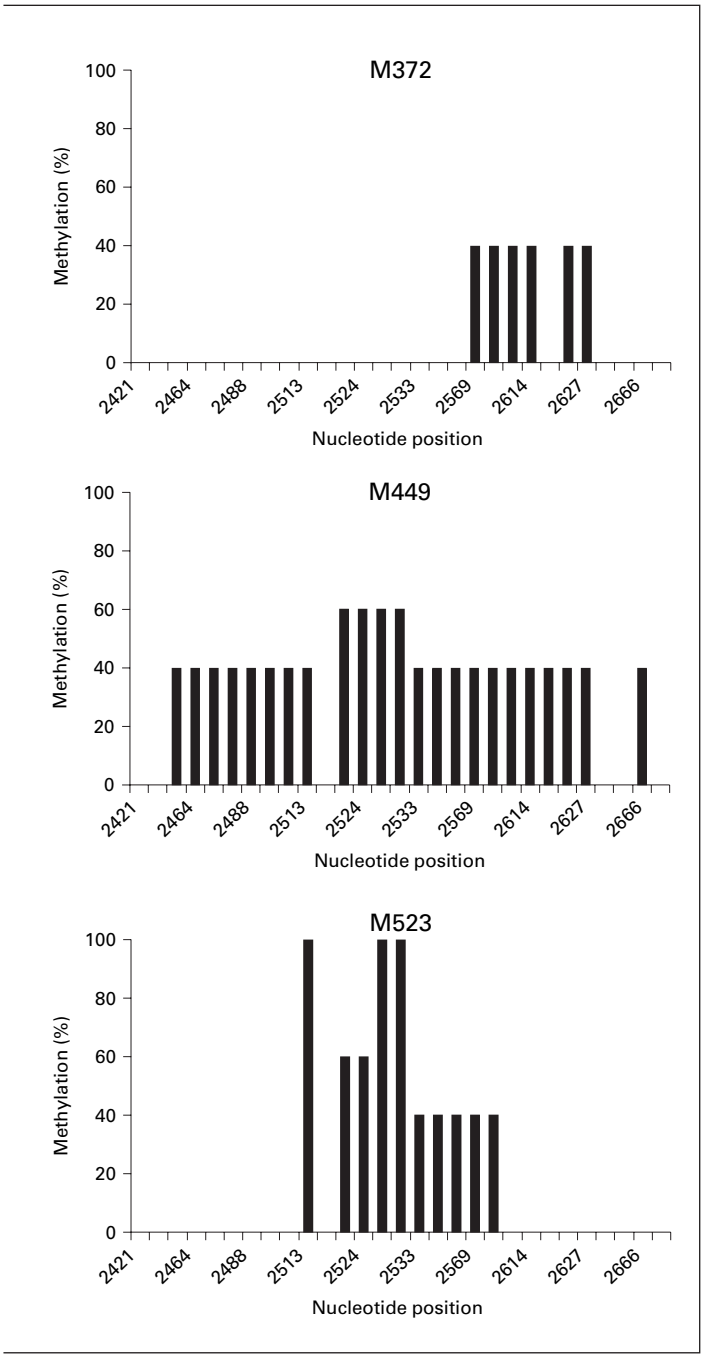


Fig. 4. Methylation status of the *KLK10* gene in primary ovarian cancers. Bisulfite-modified nucleotide sequencing analysis of *KLK10* exon 3 of DNA from primary ovarian cancers. The results of 7 ovarian tumors and 2 normal ovarian samples are illustrated. Five cloned PCR products were sequenced to determine percent methylation of the CpG sites in the regions analyzed. The y-axis represents percent cytosine methylation, and the x-axis represents the nucleotide position relative to the transcription start site. M = Malignant ovary; N = normal ovary.



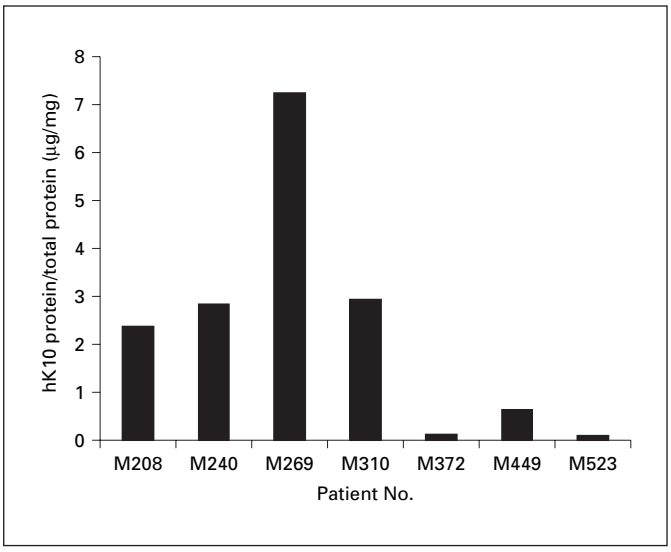


Fig. 5. Kallikrein 10 protein expression in primary ovarian cancers. Kallikrein 10 protein concentrations (μg/mg of total protein) were measured from cytosolic extracts using an hK10 ELISA.

Table 3. Analysis of protein expression and methylation status in a panel of primary ovarian tumors

Sample	hK10 protein, μg/mg	Methylation status
N11	0.32	no
N12	0.51	no
M208	2.38	partial
M240	2.84	partial
M269	7.24	no
M310	2.93	partial
M372	0.11	partial
M449	0.63	partial
M523	0.09	partial/full

N = Normal; M = malignant. No methylation is defined as 0% methylation within the region of interest (fig. 1a, HTB-75). Partial methylation is defined as <100% methylation within the region of interest (fig. 1a, ES-2). Full methylation is defined as 100% methylation within the region of interest (fig. 1a, PC-3).

been tested as a potential mechanism for inactivation. Many of these genes are downregulated in malignancy, and it is possible that some of these genes are epigenetically suppressed or silenced through CpG island methylation [14]. Future studies, focusing on the identification of CpG islands within other kallikrein members and performing sodium bisulfite treatment and sequencing of in-the demethylating agent, 5-aza-dC. For example, there was no increase in protein expression upon 10 μ*M* 5-aza-dC treatment within the HTB-161 cell line. However, at 50 and 100 μ*M* of 5-aza-dC treatment, *KLK10* mRNA was re-expressed.

For the remaining 11 human kallikrein genes that have been recently identified, in none of them has methylation

dividual DNA strands from cancer cells, as well as assessing gene re-expression from 5-aza-dC-treated cells, should further identify the role that epigenetic regulation plays within the kallikrein locus.

Our present study supports the notion that *KLK10* expression may be partially dependent on its methylation status. These results justify larger follow-up studies to evaluate *KLK10* methylation as a screening tool for the detection of cancer and the potential of *KLK10* methylation as a tumor marker.

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