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Quantitative DNA methylation analysis of genes coding for kallikrein-related peptidases 6 and 10 as biomarkers for prostate cancer

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Abbreviations: PCa, prostate cancer; PSA, prostate-specific antigen; GS, Gleason score; FFPE, formalin-fixed paraffin-embedded; KLK, kallikrein-related peptidases; HM, high methylation; LM, low methylation; ROC, receiver-operating characteristic; DAC, 5-aza-2'deoxycitidine

DNA methylation plays an important role in carcinogenesis and is being recognized as a promising diagnostic and prognostic biomarker for a variety of malignancies including prostate cancer (PCa). The human kallikrein-related peptidases (KLKs) have emerged as an important family of cancer biomarkers, with *KLK3*, encoding for Prostate Specific Antigen, being most recognized. However, few studies have examined the epigenetic regulation of KLKs and its implications to PCa. To assess the biological effect of DNA methylation on *KLK6* and *KLK10* expression, we treated PC3 and 22RV1 PCa cells with a demethylating drug, 5-aza-2'deoxycytidine, and observed increased expression of both *KLK*s, establishing that DNA methylation plays a role in regulating gene expression. Subsequently, we have quantified *KLK6* and *KLK10* DNA methylation levels in two independent cohorts of PCa patients operated by radical prostatectomy between 2007-2011 (Cohort I, n = 150) and 1998-2001 (Cohort II, n = 124). In Cohort I, DNA methylation levels of both *KLK*s were significantly higher in cancerous tissue vs. normal. Further, we evaluated the relationship between DNA methylation and clinicopathological parameters. *KLK*6 DNA methylation was significantly associated with pathological stage only in Cohort I while *KLK10* DNA methylation was significantly associated with pathological stage in both cohorts. In Cohort II, low *KLK*10 DNA methylation was associated with biochemical recurrence in univariate and multivariate analyses. A similar trend for *KLK*6 DNA methylation was observed. The results suggest that *KLK6* and *KLK10* DNA methylation distinguishes organ confined from locally invasive PCa and may have prognostic value.

Introduction

With an estimated 266,390 new cases and 37,820 deaths in 2011, prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer death in men in North America.[1,2] PCa is a remarkable disease because unlike many other cancers, it can follow a variable course in different patients. Most men die with, rather than from, the disease as a large subset of PCas are non-life threatening, often described as indolent PCa. Even histologically similar tumors may behave dramatically differently.

Prostate specific antigen (PSA), a protein encoded by the kallikrein 3 (*KLK3*) gene, is the most commonly used serum marker for PCa diagnosis. PSA is a well-established biomarker but its utility is limited by low diagnostic sensitivity (75%-85%) and specificity (25-35%), and it cannot reliably distinguish between life-threatening and indolent PCa.[3,4] The Gleason score (GS), the most widespread method of PCa tissue grading, is the single most important prognostic factor in PCa and is a strong determinant of a patient's specific risk of dying due to PCa.[5] However, there are limitations to the utility of GS as it is subjected to substantial inter-observer variation among pathologists. Because of incomplete biopsy sampling of the prostate, GS obtained on a biopsy may underestimate the GS in the corresponding prostatectomy specimen, and tumors with the same GS may show a variable biological behavior.[6]

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Table 1. Clinical characteristics of two Cohorts of prostate cancer patients.

Clinical characteristic	Cohort I 2007-2011	Cohort II 1998-2001
Gleason score	**No. of patients (%)**	**No. of patients (%)**
4	0	1 (0.8)
5	2 (1.3)	7 (5.6)
6	36 (24)	48 (38.7)
7	75 (50)	53 (42.7)
8	18 (12)	11 (8.9)
9	19 (12.7)	2 (1.6)
10	0	2 (1.6)
Pathological stage		
pT2	88 (58.7)	75 (60.5)
pT3[a]	42 (28)	30 (24.2)
pT3[b]	18 (12)	14 (11.3)
pT4	2 (1.3)	5 (4.0)
Age		
Average	61.2	61.2
Median	62.0	62.0
Range	38-75	41.5-75.9
Surgical margins		
Positive	29 (19.3)	30 (24.2)
Negative	121 (80.7)	94 (75.8)
Weight of prostate		
Average	50[a]	N/A
Range	15-191	N/A
Biochemical recurrence		
Recurrences	N/A	48 (38.7)
PSA		
Average	9.3[b]	9.1[c]
Range	0.21-165.43*	0.1-45.8
Total	150	124

[a]Data was available for 146 patients, [b]data was available for 142 patients, [c]data was available for 116 patients, *one patient had a PSA of 165.43, the remaining 141 patients PSA levels range from 0.21-56.16.

Therefore, the development of novel PCa biomarkers that can be used in conjunction with existing markers to improve the diagnosis and prognosis of the disease and distinguish aggressive and indolent forms of the disease remains an important and exciting challenge, in particular using the universally and readily available formalin-fixed paraffin-embedded (FFPE) specimens.

DNA methylation is the most common and best characterized epigenetic mechanism that involves the addition of a methyl group to a cytosine that precedes a guanine (CpG).[7] In mammals, enriched DNA stretches of CpG dinucleotides known as CpG islands are common in the promoter regions, but have also been found in other parts of the gene, including introns and exons. CpG island methylation has many important functions in normal cells including promotion of chromosomal stability and regulation of gene expression. Aberrant CpG island methylation is a well-recognized hallmark of many cancers.[8] DNA methylation patterns in cancer typically consist of global hypomethylation, in conjunction with localized hypermethylation at CpG islands that often lead to the inactivation of essential regulatory genes. Importantly, DNA methylation events seem to be involved in cancer development and progression, and are being recognized for their potential as promising diagnostic and prognostic biomarkers for a variety of malignancies including PCa. Among several hypermethylated genes identified in PCa, a few candidates including *GSTP1, APC, PITX2, RASSF1A* and *HOXD3* have been described as potentially promising biomarkers.[9-12] However, at present no single biomarker has the optimal degree of sensitivity and specificity and it is now accepted that a combination of markers is likely to prove more valuable as a prognostic and/or diagnostic panel for clinical use.

The human kallikrein-related peptidases, formerly known as kallikreins (KLKs) are a family of secreted serine proteases, encoded by 15 genes that have emerged as an important family of cancer biomarkers, with PSA being most recognized.[13,14] Many KLKs have been implicated in tumor invasion, metastasis, and promotion of tumor angiogenesis. Numerous studies have demonstrated that *KLK* genes show aberrant expression in a number of cancers such as prostate, ovarian and breast tumors.[15,16] However, only a few studies have examined the potential regulation of *KLK* gene expression by epigenetic mechanisms. Using biocomputational analysis, multiple CpG Islands, identified by the NCBI "relaxed" definition as DNA sequences (200 base window) with a GC base composition greater than 50% and a CpG observed/expected ratio of > 0.6, have been identified in the 5'UTR and coding regions of different *KLK* genes.[17]

No CpG islands are located in the major regions regulating *KLK3* gene expression including the proximal promoter and the distal enhancers. The largest CpG island located in the *KLK* locus is found in exon 3 of *KLK*10. Consequently, *KLK*10 exon 3 hypermethylation has been investigated in tumor samples and cell lines derived from several different cancers.[18-21]

Very few studies to date have systematically investigated *KLK* DNA methylation in primary prostate tissue and their potential contribution to PCa diagnosis or prognosis. By genome-wide differential methylation hybridization CpG Island microarray profiling of GS6 versus GS8 prostate tumors, we previously established tumor specific hypermethylation of several genes including *HOXD3* and *TGF*β*2*.[9,10,22] Using the same profiling platform we identified differential DNA methylation of *KLK*10 5'UTR but did not detect significant *KLK10* exon 3 hypermethylation in prostate tumors. We also chose *KLK6* as a candidate gene for analysis since its promoter is CpG rich and its promoter hypermethylation was found to be associated with gene downregulation in breast cancer.[23,24] Subsequently, in this study, we investigated for the first time the association between *KLK*6 and *KLK*10 DNA methylation status and clinicopathological parameters in two large independent cohorts of PCa patients operated by radical prostatectomy.

Results

Detailed characterization of *KLK*10 DNA methylation status by MassARRAY EpiTYPER. Quantitative DNA methylation of

Table 2. *KLK6* and *KLK10* median PMR values, proportion of HM cases and p values for PCa specimens and normal tissues in Cohort I

Cohort I 2007-2011	Median PMR	Wilcoxon signed-rank test p value	No. of HM (%)	X^2 p value
KLK 6				
Cancer	16.27	< 0.001	106 (72%)	<0.001
Normal	11.47		70 (48%)	
KLK 10				
Cancer	10.08	< 0.001	106 (71%)	<0.001
Normal	0.79		26 (18%)	

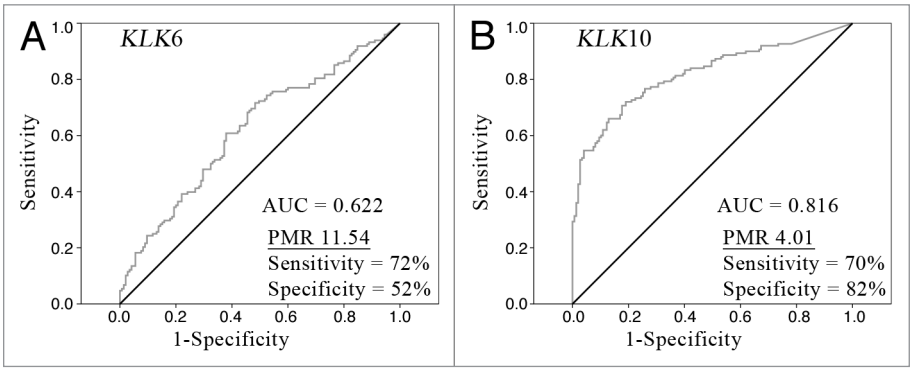

Figure 1. Receiver Operator Curve analysis used to determine the diagnostic performance of (A) *KLK6* and (B) *KLK10* percent of methylated reference values in Cohort I (2007–2011).

34 CpG dinucleotides within the 5'UTR CpG Island in *KLK10* was determined by MassARRAY EpiTYPER analysis of two fresh frozen PCa tissues: the first showed over 3-fold enrichment while the second showed less than 1.1-fold DNA methylation signal on the microarrays, as described previously.[22] The average methylation levels for these 34 CpG sites in sample 1 were 82.2% compared to 30.3% in sample 2 (**Fig. S1**). These results confirmed the DNA methylation profile of *KLK10* evident from the CpG Island microarrays. Additionally, these results were used to identify the most distinct, differentially methylated CpGs within *KLK10* 5'UTR to design DNA methylation specific primers and probe for subsequent MethyLight analysis. For *KLK6*, selection of differentially methylated CpGs for MethyLight analysis was achieved using a previously published bisulfite sequencing map of the promoter region.[23,24]

Analysis of the effect of 5-Aza-2'-deoxycitidine (DAC) on PCa cell lines. To assess the biological effect of DNA methylation on *KLK6* and *KLK10* gene expression in the PCa cell lines, PC-3 and 22RV1, we used the demethylating agent DAC, a cytidine analog that sequesters DNA methyltransferases after its incorporation into genomic DNA.[25] We chose PC-3 and 22RV1 cells because they displayed significant DNA methylation of *KLK6* and *KLK10* as determined by MethyLight (**Fig. S2**). Following treatment, 9.5-fold and 6-fold increase in *KLK10* expression was observed in PC-3 and 22RV1 cells, respectively, and a 4-fold increase in *KLK6* expression was observed in PC-3 cells (**Fig. S2**). *KLK6* expression was not detectable in 22RV1 cells. Hence, these cells were excluded from this analysis. To correlate the increase in *KLK6* and *KLK10* expression with DAC induced change in DNA methylation, we carried out MethyLight analysis. DAC treatment led to 57% and 49% decrease in percentage of methylated reference (PMR) in *KLK10* DNA methylation in PC-3 and 22RV1 cells, respectively, and 29% PMR decrease in *KLK6* DNA methylation in PC-3 cells.

Quantitative analysis of *KLK6* and KLK10 DNA methylation in primary PCa and normal tissue. We quantified the methylation levels of *KLK6* and *KLK10* in a test cohort (Cohort I) consisting of 150 PCa specimens and 147 normal tissues and subsequently in a validation cohort (Cohort II) consisting of 124 PCa specimens. **Table 1** displays the clinicopathological characteristics of both cohorts. Patients in both cohorts were of comparable median age and average pre-operative PSA levels. The pathological stage distribution was comparable between the two cohorts but GS distribution varied. In Cohort I tumors ranged from GS 5 to 9, with 25% GS ≤ 6, 50% GS7 and 25% GS ≥ 8, whereas tumors in Cohort II ranged from GS 4 to 10, with 45% GS ≤ 6, 43% GS7 and 12% GS ≥ 8. A total of 48 (38.7%) patients in Cohort II had biochemical recurrence with the mean follow-up time of 4.00 years (range 0.18–8.56). Due to the recent recruitment period of Cohort I, collection of follow up data is still in progress.

In Cohort I, median PMR values for both *KLK*s, given in **Table 2**, were significantly higher in PCa compared to normal tissue (Wilcoxon Signed-Rank Test p value <0.001). Additionally, we performed Receiver-operating characteristic (ROC) curve analysis, **Figure 1**, to determine the ability of DNA methylation of each *KLK* to accurately distinguish cancer from normal tissues. The area under the curve (AUC) for *KLK6* and *KLK10* were 0.622 (95% CI: 0.558–0.686), and 0.816 (95% CI: 0.767–0.865), respectively. We then established PMR threshold values using the ROC curves that allowed optimal separation between benign and malignant tissue with maximum combined sensitivity and specificity. The PMR threshold values were 11.54 for *KLK6* and 4.01 for *KLK10* as the maximized combined sensitivity and specificity were 72% and 52%, 70% and 82%, respectively. We further classified *KLK* DNA methylation into a high methylation (HM) group, which was equal or greater than the PMR threshold values determined by the ROC analysis, and a low methylation (LM) group, which accounted for the rest of the samples. χ^2 analysis showed a greater proportion of HM cases in

Table 3. *KLK6* and *KLK10* Median PMR, proportion of HM cases and p values stratified according to Gleason score and pathological stage, Cohort I and II

	Cohort I (2007–2011)				Cohort II (1998–2001)			
	Median PMR	n (%) HM	Median PMR	n (%) HM	Median PMR	n (%) HM	Median PMR	n (%) HM
Gleason Sscore	**KLK6**		**KLK10**		**KLK6**		**KLK10**	
≤ 6	15.2	26 (72%)	7.44	26 (68%)	28.55	49 (96%)	14.97	41 (73%)
7	18.36	55 (73%)	14.24	57 (76%)	22.74	43 (96%)	19.06	43 (81%)
≥ 8	13.23	25 (68%)	7.06	23 (62%)	28.32	11 (85%)	7.39	9 (60%)
Pathological stage	**KLK6**		**KLK10**		**KLK6**		**KLK10**	
pT2	14.37	54 (63%)	7.54	55 (63%)	26.49	62 (94%)	12.94	51 (68%)
pT3a	18.91	35 (83%)	13.1	34 (81%)	28.55	24 (96%)	22.12	27 (90%)
pT3b + pT4	20.71	17 (85%)	23.11	17 (83%)	22.44	17 (94%)	20.81	15 (79%)
pT3a + pT3b	19.67	50 (83%)	14.36	49 (82%)	23.88	36 (95%)	21.01	39 (80%)
Comparison	**Kruskal Wallis p value**	**X^2 p value**	**Kruskal Wallis p value**	**X^2 p value**	**Kruskal Wallis p value**	**X^2 p value**	**Kruskal Wallis p value**	**X^2 p value**
≤ 6 vs. 7 vs. ≥ 8	0.219	0.771	0.064	0.299	0.225	0.35	0.349	0.228
pT2 vs. pT3a vs. pT3b + pT4	0.0286	0.012	0.007	0.031	0.953	0.804	0.031	0.057

Kruskal-Wallis one-way analysis of variance p value

PCa specimens compared with normal; *KLK6*: 72% vs. 48% (p value < 0.001), *KLK*10: 71% vs. 18% (p value < 0.001).

DNA methylation levels in each *KLK* gene were positively correlated with DNA methylation in the other gene, in both cohorts (Pearson correlation coefficient = 0.339, p value < 0.001, Pearson correlation coefficient = 0.182, p value = 0.047, respectively). Additionally, patients in Cohort I with one HM *KLK* were significantly more likely to have the other *KLK* also highly methylated (χ^2 p value < 0.001). This association was not observed in Cohort II (χ^2 p value = 0.336).

We found that the *KLK6* DNA methylation observed in normal tissue significantly correlated with age and with DNA methylation in the matched cancerous tissue (Pearson correlation coefficient = 0.216, p value = 0.009, Pearson correlation coefficient = 0.652, p value < 0.001, respectively, **Table S1**). However, such association was not observed for *KLK*10 DNA methylation in normal tissue. *KLK10*, but not *KLK6* DNA methylation levels significantly differed between normal tissue acquired from the transition zone, median PMR 0, compared to the peripheral zone, median PMR 1.40 (p value < 0.001).

DNA methylation and clinicopathological features. We then examined the association between *KLK*6 and *KLK*10 DNA methylation status and GS. The PCa specimens were separated into three groups based on GS; low (GS ≤ 6), intermediate (GS = 7), and high grade (GS ≥ 8). The median PMR values, proportion of HM cases and p values for each gene analyzed in both cohorts are given in **Table 3**. Overall, no significant association between the three GS groups and *KLK* DNA methylation was observed.

Similarly, we examined the relationship between quantitative *KLK* DNA methylation and pathological stage. The PCa specimens were separated into three groups; organ confined pT2 cases, pT3a cases that extended into the periprostatic tissue and pT3b + pT4 cases that infiltrated into the seminal vesicles or bladder musculature. In Cohort I, *KLK*6 DNA methylation levels and prevalence of HM cases were significantly different between the three pathological stage groups (p value = 0.029, 0.012, respectively). However, this association between *KLK*6 DNA methylation and pathological stage was not observed in Cohort II. *KLK10* DNA methylation PMR values were significantly associated with pathological stage in both cohorts (p value =0.007, 0.031). Further, in Cohort I, the proportion of *KLK10* HM cases significantly associated with pathological stage (p value = 0.031) and a similar trend was also observed in Cohort II (χ^2 p value = 0.057).

DNA methylation and biochemical recurrence. Univariate log-rank analysis of GS, stage, and surgical margin status showed that each variable is a significant predictor of biochemical recurrence (**Fig. 2A–C**), indicating that the series of patients in Cohort II is representative of other populations studied.[26,27] We next examined the relationship between biochemical recurrence and DNA methylation status of *KLK*6 and *KLK*10 genes in Cohort II. Patients with low levels of *KLK*10 DNA methylation showed a trend to shorter time to biochemical recurrence than patients with high levels of *KLK*10 DNA methylation (log-rank p value = 0.046, **Fig. 2E**). The overall proportion of biochemical recurrences in the LM group was 55%, whereas in the HM group it was only 33%. A similar trend for *KLK*6 DNA methylation status was observed but was not significant (p value = 0.107, **Fig. 2D**). Furthermore, we performed multivariate Cox regression analysis of our data (**Table 4**). Significant predictors of biochemical recurrence included GS, pathological stage, and surgical margin status. *KLK*10 low DNA methylation status was a borderline significant predictor of biochemical recurrence (HR = 2.108, p value = 0.028, **Table 4B**) and *KLK*6 DNA methylation status has also shown a trend (HR = 2.155, p value = 0.074, **Table 4A**).

Discussion

In this study, we have shown a significant, tumor specific DNA hypermethylation of *KLK*6 and *KLK*10 in PCa, as well as a

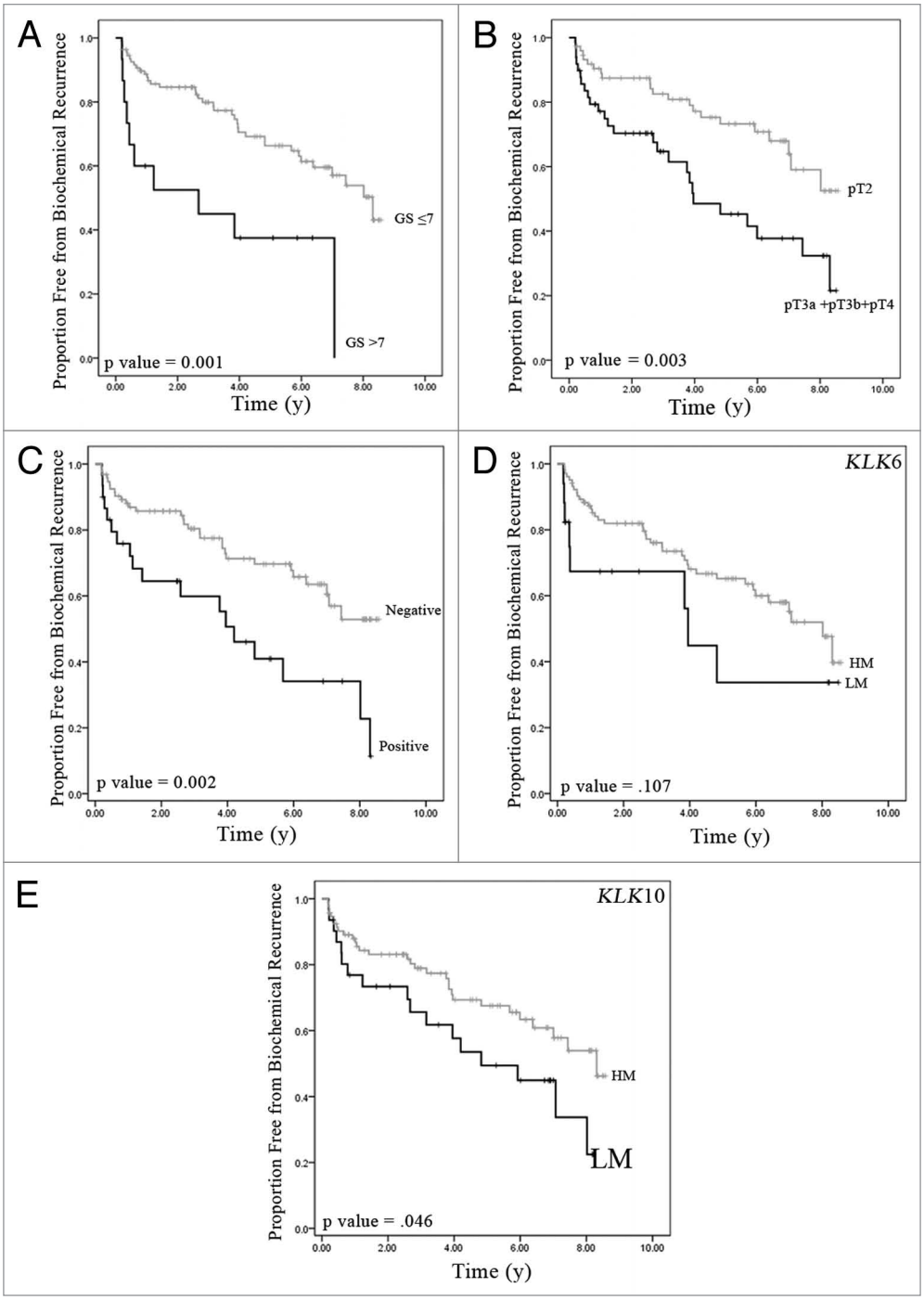

Figure 2. Kaplan-Meier Curves of biochemical progression-free probability for (A) Gleason score, (B) stage, (C) surgical margin status, (D) *KLK*6 methylation status and (E) *KLK*10 methylation status in Cohort II (1998–2001). HM, high methylation; LM, low methylation.

significant association between *KLK*10 DNA methylation, pathological stage, and biochemical recurrence. We have chosen to investigate *KLK*6 promoter DNA methylation, which has been previously shown to be differently methylated in breast cancer, and *KLK*10 5'UTR, which we identified as differently methylated by genome-wide profiling using CpG island microarrays.[22] We then used EPITYPER analysis to verify the *KLK*10 5'UTR DNA methylation within individual CpG sites and for the purpose of designing primers and probe for MethyLight analysis. For these purposes, analysis of two samples was sufficient.

Although earlier studies have focused on *KLK*10 exon 3 DNA methylation, the 5'UTR region may have more biological significance because several studies have shown that abnormal methylation of CpG islands within gene promoters and 5'UTR may be associated with transcriptional gene silencing, thus potentially contributing to cancer pathogenesis.[17-19,21,28-30]

Table 4. Two independent multivariate Cox regression analyses of biochemical recurrence with Gleason score, pathological stage, surgical margins, (A) *KLK6* and (B) *KLK10* methylation status, Cohort II

Cohort II (1998–2001)			
A	Hazard ratio	95% CI	LRT p value
Gleason score	5.08	2.37–10.88	< 0.001
Pathological stage	2.41	1.33–4.36	0.004
Surgical margin status	3.07	1.63–5.76	0.001
Low *KLK6* methylation	2.15	0.99–4.70	0.074
B			
Gleason score	4.19	1.94–9.02	0.001
Pathological stage	3.00	1.61–5.57	0.001
Surgical margin status	2.61	1.40–4.87	0.004
Low *KLK10* methylation	2.11	1.10–4.02	0.028

We have demonstrated that the DNA methylation levels and prevalence of *KLK*6 and *KLK*10 were significantly higher in PCa tissue compared to normal prostate tissue in Cohort I. KLK6 and KLK10 protein expression has been previously shown to be downregulated in PCa compared to non-malignant prostatic tissue.[31] These results combined with our DAC treatment data suggest that DNA methylation plays a role in regulating their expression in PCa. This is consistent with previous reports regarding the epigenetic regulation of *KLK*6 and *KLK*10 in breast cancer.[17,23,24,32] However, among these two DNA methylation markers, only *KLK*10 methylation shows good diagnostic characteristics and can most efficiently distinguish malignant from benign prostate tissue. This is consistent with one previous study that have identified promoter *KLK*10 DNA methylation as a PCa associated epigenetic event on a microarray platform.[33]

A limitation of the study is that is that the benign tissue used, was from the vicinity of the tumor. These normal appearing tissues may show field cancerization effect or other changes secondary to the nearby cancer consequently skewing the results. Future work is necessary to examine DNA methylation in *KLK*10 5'UTR in normal tissue from a large independent cohort of non-PCa patients to better define its diagnostic potential in PCa.

In this study, we observed that *KLK*10 DNA methylation in normal tissue from prostates harboring tumors was associated with the zonal anatomy of the prostate, with significantly lower DNA methylation status in the transition zone. It was previously shown that different anatomic regions of the prostate have diverse biological characteristics including difference in gene expression for such genes as ASPA, and difference in DNA methylation for such genes as *TIMP3* and *S100A2*.[34,35] It is possible that the epigenetic mechanisms regulating *KLK*10 expression are different between the prostate zones as is *KLK*10 expression, also contributing to the biological differences between the zones. Further quantitative DNA methylation and expression analyses in different prostate anatomical regions of normal tissue from non-PCa patients are required to investigate this possibility. On the other hand, *KLK6* DNA methylation in normal tissue seems to be age-related. These results are not surprising since it has been previously shown that DNA hypermethylation of numerous genes, such as *GSTP1*, increases with age in the normal prostate tissue.[36] Additionally, *KLK6* DNA methylation in the normal tissue was associated with higher DNA methylation in the matched cancerous tissue. This may possibly be explained by the field cancerization effect, which refers to molecular abnormalities in histologically benign epithelium contiguous to cancerous tissue.[37] This has been previously shown for other genes such as *GSTP1* and *RARβ2*.[38,39] However, it is necessary to analyze *KLK6* DNA methylation in normal prostatic tissue to support this possibility.

Our results show that DNA methylation of *KLK*6 and *KLK*10 in PCa is not correlated with GS but is associated with pathological stage. We have observed a significant increase in *KLK*10 DNA methylation for pathological stages pT3 + pT4 versus pT2 in both cohorts. This observation may have important implications for PCa detection and management as high *KLK*10 DNA methylation levels detected in prostate tissue may mark clinically relevant disease and could be incorporated in predictive models for preoperative prostate cancer staging. Additionally, these results imply that the tumor suppressive effect of this protease is linked to tumor invasion rather than gland differentiation. However, a previous publication has found no significant association between KLK mRNA or protein expression and pathological stage in cancerous prostate tissues.[31,40] Therefore, future work is necessary to elucidate the biological significance of *KLK*10 DNA methylation to protein expression and other epigenetic mechanisms that could potentially regulate these proteins expression. Similarly, we have demonstrated a significant increase in *KLK*6 DNA methylation from pathological stage pT2 to pT3 + pT4 in Cohort I. However, this significant finding generated in Cohort I was not confirmed in Cohort II. This can be possibly explained by differences in patient cohort characteristics or a spurious correlation in Cohort I. This discrepancy needs to be further investigated in a larger independent cohort.

On multivariate Cox regression analyses we found that low *KLK*10 DNA methylation in Cohort II was associated with shorter time to biochemical recurrence, but not more significant than GS, pathological stage or surgical margin status. Yet, the methylation of *KLK*10 might be used in a panel of methylation markers, which together will accurately predict PCa disease course and/or outcome. Prior studies have already shown the added value of a multi-gene DNA methylation panels for diagnosis and prognosis of PCa in comparison to single gene DNA methylation.[41,42]

In addition, low *KLK*10 DNA methylation was also found to be associated with tumors of lower pathological stage. This paradoxical observation is intriguing and may have several possible explanations. First, *KLK*10 DNA methylation may have two phases in PCa; an initial increase in DNA methylation from organ confined pT2 to invasive pT3 + pT4 stages, followed by a decrease in DNA methylation after tumor progression. Second, perhaps most cells in a prostate tumor have an increase in *KLK*10 DNA methylation during the progression from organ confined to locally invasive PCas but a subset of organ confined tumours that have a unique epigenetic identity including low DNA methylation of *KLK*10 are more prone to be involved in biochemical recurrence of PCa. Since previous studies have demonstrated no statistically significant difference in the biochemical recurrence

rate between patients with KLK10 protein positive and negative tumors, it is possible that *KLK*10 DNA methylation is just an epiphenomenon associated with these processes.[31,40]

In this study, we have used MethyLight technology for KLK methylation analysis. This approach has several advantages. It is a high-throughput, sensitive, specific and quantitative assay that requires very small amounts of DNA thus making it is suitable to be used in clinical laboratories. However, this approach also has certain limitations including reliance on bisulfite-treated DNA as a template for PCR, and inability to recognize heterogeneously methylated molecules and their significance to PCa.

Another potential limitation of the study is that the higher percentage of GS > 8 cases in Cohort I compared to Cohort II. Having more GS > 8 cases in Cohort I vs. Cohort II may introduce a bias, skew the survival analysis of our study and skew association analysis between *KLK* DNA methylation and Gleason score. However, no follow-up data is available for Cohort I and no significant association between *KLK* DNA methylation and Gleason score was found in the study.

In conclusion, we have for the first time characterized *KLK*6 and *KLK*10 DNA methylation in two independent cohorts of, 274 PCa specimens and 147 normal prostatic tissues. We have shown significant, tumor specific DNA hypermethylation of both *KLK*s, and in particular of *KLK10* implying the diagnostic potential of *KLK*10 DNA methylation. We demonstrated that *KLK*6 DNA methylation is a potential marker of field cancerization in normal peripheral zone prostate tissue. Further, we validated the significant association of increased *KLK*10 DNA methylation with advanced pathological stage. In biochemical recurrence probability analyses of Cohort II, we found that low DNA methylation of *KLK*10 can potentially further predict patient biochemical recurrence risk. Future work is necessary to further elucidate both the clinical utility and functional relevance of *KLK*10 DNA methylation as a progression biomarker.

Material and Methods

Patients and pathology. The test cohort (Cohort I) included in the study consists of 150 patients diagnosed with PCa and who underwent radical prostatectomy between 2007 and 2011 at the University Health Network (UHN) in Toronto. Due to the recent recruitment period of this cohort, collection of follow up data is still in progress. The validation cohort (Cohort II) is made up of 124 patients diagnosed with PCa who underwent radical prostatectomy between 1998 and 2001 at UHN in Toronto, with available follow-up data. In Cohort II, biochemical recurrence was defined as a rise in PSA levels above 0.05 following radical prostatectomy. Patients who received neo-adjuvant or hormone therapy before radical prostatectomy were excluded from the study. The clinicopathological characteristics of both cohorts are listed in **Table 1**. Patient consent was obtained for specimen accrual following radical prostatectomy into the UHN tissue bank. All samples and clinical and pathological follow-up information were obtained according to the protocols approved by the Research Ethics Board at Mount Sinai Hospital and UHN, Toronto. The complete set of hematoxylin and eosin (H&E)-stained slides from each prostatectomy was collected and reviewed by an expert pathologist (TVDK) to confirm GS (WHO/ISUP criteria), stage (TNM), and surgical margin status. For each patient in Cohort I, a subset of slides was selected based on the presence of carcinoma with specific GS. Additionally, a subset of slides was selected for each of these patients based on the presence of normal prostatic tissue containing at least 50% glandular content. In Cohort II, tumor areas of each Gleason pattern representing the overall GS were marked on the H&E-stained slides. All of the marked tumors were corresponding to an area of at least 80% neoplastic cellularity.

Cell lines and DNA extraction. Human PCa cell lines PC-3 [American Type Culture Collection (ATCC) # 59500] and 22RV1 (ATCC # CRL-2505) were obtained from Dr Diamandis and cultured as monolayers in F-12 and RPMI 1640 media (Life Technologies), respectively, and supplemented with 10% fetal bovine serum. All cell lines were grown in humidified atmosphere with 5% CO_2 at 37°C. DNA was extracted after harvesting the cells by trypsinization followed by DNA extraction using QIAamp DNA mini kit (Qiagen Inc.), as per manufacturer's instructions.

5-aza-2'-deoxycitidine treatment and qRT-PCR. A 250 mg/ml stock solution of 5-aza-2-deoxycitidine (DAC) was prepared in water and kept at -80°C until use. PC-3 and 22RV1 cells were plated in 10cm dishes, cultured for 24 hours, and treated with 4 μM DAC for 2 days as previously established in our laboratory.[22] After treatment, cells were washed with PBS, and fresh medium was added. Cells were then further incubated for another 2 d. Cells were harvested and subsequently genomic DNA along with total RNA were extracted simultaneously using AllPrep DNA/RNA mini kit (Qiagen Inc.), as recommended by the supplier. Primer sequences for RT-PCR of *KLK*6 and *KLK*10 are as follows: (*KLK*6 forward) 5'-CCT GCA GCA GGA GCG GCC-3', (KLK6 reverse) 5'-TGT GAG GAC CCA CAG TGG ATG GAT A-3'; (KLK10 forward) 5'-TAC AAC AAG GGC CTG ACC TGC T-3', (KLK10 reverse) 5'-GTC ACT CTG GCA AGG GTC CTG-3'; (β-actin forward) 5'-ACA ATG AGC TGC GTG TGG CT-3', (β-actin reverse) 5'-TCT CCT TAA TGT CAC GCA CGA-3'. The real-time RT-PCR was performed on Applied Biosystems 7500 Real Time PCR instrument. A standard curve for calibration of amplification rates was generated by serial dilutions of 22RV1 cDNA and resulted in a scale of arbitrary units of gene expression. All paired samples were measured in one run.

DNA extraction and bisulfite modification. Formalin-fixed, paraffin-embedded tissue blocks matching the selected H&E slides were sectioned at a thickness of 10 μm. These tissue slides were then superimposed on H&E slides and each area of cancer or normal tissue was outlined. The circled areas of tissue slides were then scraped with a scalpel and placed into 1.5 ml tubes. DNA was extracted from tissues using QIAamp DNA Mini Kit (Qiagen) with a modified protocol as described previously.[9] For tumors containing multiple foci, two patterns were combined in equal amounts to represent the overall GS. Next, 100–400 ng of extracted DNA was converted using the EZ DNA Methylation Gold Kit (Zymo Research) according to the manufacturer's protocol and eluted to a final concentration of 20 ng/μl.

MassARRAY EpiTYPER analysis. MassARRAY EpiTYPER analysis (Sequenom) is a time of flight matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) based method that provides a quantitative view of CpG dinucleotide methylation status to single or multiple dinucleotide resolution.[43] DNA from two fresh frozen samples was bisulfite modified, tagged with a T7 promoter, and transcribed into RNA. RNA was cleaved with RNase A and the products were resolved by the MS instrument. Analysis was performed in triplicate by the Analytical Genetics Technology Centre (AGTC), Princess Margaret Hospital. The *KLK*10 region analyzed by EpiTYPER is chromosome 19: 51,522,948 to 51,523,410 (NCBI37).

MethyLight. DNA methylation analysis was performed on tumor and normal tissue using semi-quantitative MethyLight assay, a Taqman based technique that assesses percent DNA methylation at a defined gene locus, as described earlier.[44] In brief, 20 ng of bisulfite-converted genomic DNA was amplified using locus specific PCR primers flanking an oligonucleotide probe with a 5' fluorescent reporter dye and a 3' quencher dye. Primers and probe sequences used for the *KLK* 6 and *KLK*10 assayed regions were as follows: *KLK*6 (Forward) 5'-AGG AAG TTA TTG ATG TAA TCG TTT TTT CG-3', (Reverse) 5'-AAA ACA ATC GAA CTT TAT CCG CC-3', (Probe) 5'-ACT CCG ACC TCA ACC TCT CTT CCG ACG AAC A-3', *KLK*10 (Forward) 5'-GAG GGG GAA ATT TCG GGC GC-3', (Reverse) 5'-CCC TCG CGA CAT CTT CCC G-3', (Probe) 5'-ACC CGA ATA AAA CGC TCT CCG CGC CCC A-3', ALU-C4 (Forward) 5'-GGT TAG GTA TAG TGG TTT ATA TTT GTA ATT TTA GTA-3', (Reverse) 5'-ATT AAC TAA ACT AAT CTT AAA CTC CTA ACC TCA-3'and (Probe) 5'-CCT ACC TTA ACC TCC C-3'. A PMR score was calculated for each *KLK* gene locus by dividing the *KLK gene:Alu-C4* ratio of a sample by the *KLK gene:Alu-C4* ratio of commercially available fully methylated DNA (Millipore) and multiplying by 100. The *Alu-C4* PCR products (generated from a consensus CpG-devoid region of the *ALU* repetitive element) were used as controls to normalize for input DNA. Samples were analyzed in duplicates in 96-well plates on an ABI 7500 RT-PCR thermocycler.[45]

Statistical analysis. PMR scores for each sample analyzed were obtained from averaging duplicate runs. *KLK* DNA methylation was separated into those with high methylation (HM) and those with low methylation (LM) using PMR threshold values determined by ROC analysis as described earlier.[46,47] Briefly, data for all PCa and normal specimens was split based on PMR values above the threshold value determined by ROC analysis vs. those below. Those PMRs above the ROC threshold value were classified as HM, whereas those below this value were considered LM. Association between *KLK* PMR values and GS or pathological stage was analyzed using the Kruskal-Wallis test. Further pairwise comparisons were performed using the Mann-Whitney U-test regardless of the results of the Kruskal-Wallis test due to the exploratory nature of the study. DNA methylation in PCa and normal specimen pairs was assessed using the Wilcoxon signed-rank test. ROC curves were constructed in SPSS software (PASW 18) using *KLK* PMR values as the test variable to predict tissue diagnosis (cancer vs. normal tissue). Pearson $\chi 2$ tests were used to analyze proportional differences between HM cases in each cohort, each gene and in each category of GS and pathological stage. The Fisher exact method was used to replace the Pearson tests when spreadsheet cell counts were < 5. Pearson correlation was used to analyze association between *KLK* PMR values in normal and cancerous tissue as well as their relation to age. Univariate biochemical recurrence-free probabilities were estimated and plotted using Kaplan–Meier method and compared using the log-rank test. Multivariate Cox proportional hazards regression analysis was used to analyze individual contributions of each variable to biochemical recurrence-free probability. We used the likelihood ratio test (LRT) and implemented the backwards model selection to analyze the data. The LRT tests for significance of variables by comparing the full model to a reduced model, with the reduced model missing the variables being tested for significance. The criteria for staying in the reduced model were set to p value ≤ 0.1. Two multivariate analyses, one with *KLK*6 DNA methylation and one with *KLK*10 DNA methylation included in the model, were performed. For all described methods, two-sided *P*-values of ≤0.05 were considered significant. No adjustments to *P*-values were made for multiple comparisons.[48] Statistics were performed using SPSS software (PASW 18)

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/21524

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