Biol. Chem., Vol. 387, pp. 795–799, June 2006 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2006.100

Short Communication

The epigenetic basis for the aberrant expression of kallikreins in human cancers

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Abstract

The tissue kallikrein gene family consists of 15 genes tandemly arranged on human chromosome 19q13.4. Most kallikrein genes are characterized by aberrant expression patterns in various human cancers, a feature that makes them ideal cancer biomarkers. In the present study, we investigated the effect of the epigenetic drug compound 5-aza-2′-deoxycytidine on the expression of downregulated kallikrein genes in prostate, breast, and ovarian cancer cell lines. Reactivation of multiple kallikrein genes was observed, although some of these genes do not contain CpG islands in their genomic sequence. Epigenetic regulation provides a new mechanism for the pharmacological modulation of kallikreins in human cancers with putative therapeutic implications.

Keywords: aberrant expression; cancer; DNA methylation; epigenetic regulation; human tissue kallikreins.

The human tissue kallikrein gene (*KLK*) family was discovered in the 1980s and was believed to contain three genes: *KLK1*, encoding for pancreatic/renal kallikrein (hK1), *KLK2*, encoding for human glandular kallikrein 2 (hK2), and *KLK3*, encoding for PSA (prostate-specific antigen or hK3) (Evans et al., 1988; Riegman et al., 1989, 1992; Qin et al., 1991; Richards et al., 1991). The major interest in human kallikreins lies in the restricted tissue expression of hK2 and hK3 in the prostate, which qualifies them as biomarkers for prostatic diseases (Wang et al., 1977, 1979, 1981; Papsidero et al., 1980; Rittenhouse et al., 1988). Improved techniques for differential expression cloning and the availability of biocomputational analysis tools led to the remarkable expansion of the human kallikrein gene family. The entire kallikrein locus is now known to span a finely mapped and sequenced region of 300 kb on chromosomal locus 19q13.4 (Gan et al., 2000; Harvey et al., 2000; Yousef et al., 2000). All 15 genes share significant structural similarities and sequence homologies.

Many kallikrein genes display aberrant expression patterns in various forms of cancer, such as breast, ovarian and prostate cancers (reviewed by Borgono and Diamandis, 2004). For example, it has been shown that *KLK4*, *KLK5*, *KLK7* and *KLK8* are concomitantly overexpressed in subsets of ovarian carcinomas. *KLK10* is downregulated and *KLK5* is upregulated in aggressive forms of prostate cancer. *KLK6* is overexpressed in subsets of primary breast cancer and downregulated in the corresponding metastases. *KLK5*, *KLK8* and *KLK10* are downregulated in breast cancer. This cancer-related deregulated expression of *KLK*s is well established and makes them candidate biomarkers for various forms of cancer; however, the underlying molecular mechanisms remain unknown.

Recently, it has become clear that genomic DNA methylation provides an alternative mechanism for regulation of gene expression (Jones and Baylin, 2002). Cancer-associated hypermethylation of known tumor suppressor genes correlates with loss of their expression in cancer cell lines and primary tumors. Interestingly, it was shown that *KLK10* is silenced in breast and other cancers by DNA hypermethylation of its exon III CpG island (Li et al., 2001; Sidiropoulos et al., 2005). Similarly, in acute lymphoblastic leukemia (ALL), downregulation of *KLK10* occurs through methylation in both exon III CpG island and in the *KLK10* promoter (Roman-Gomez et al., 2004).

Genes silenced by DNA methylation are amenable to reactivation by demethylating agents, which are potent DNA methyltransferases (DNMT) inhibitors (reviewed by Lyko and Brown, 2005). The potential anticancer activities of DNMT inhibitors have been extensively studied in recent years. DNA hypomethylation induced by DNMT inhibitors, such as 5-aza-cytidine (5-aza-C, Vidaza®) and 5-aza-2′-deoxycytidine (5-aza-dC, Decitabine), results in reactivation of tumor suppressor genes that are epigenetically silenced. Recently, the combination of demethylating agents with other chemotherapeutics has attracted increasing interest as a possible molecularly targeted therapeutic strategy. In particular, the combination of histone deacetylase (HDAC) inhibitors with demethylating agents has become attractive, since histones are connected to DNA by both physical and functional interactions (Johnstone, 2002). Increasing experimental evidence has confirmed the synergistic effect of com-

Regarding a recommendation for future nomenclature of kallikrein gene-derived proteases, see the article 'A comprehensive nomenclature for serine proteases with homology to tissue kallikreins' by Lundwall et al., this issue pp. 637–641.

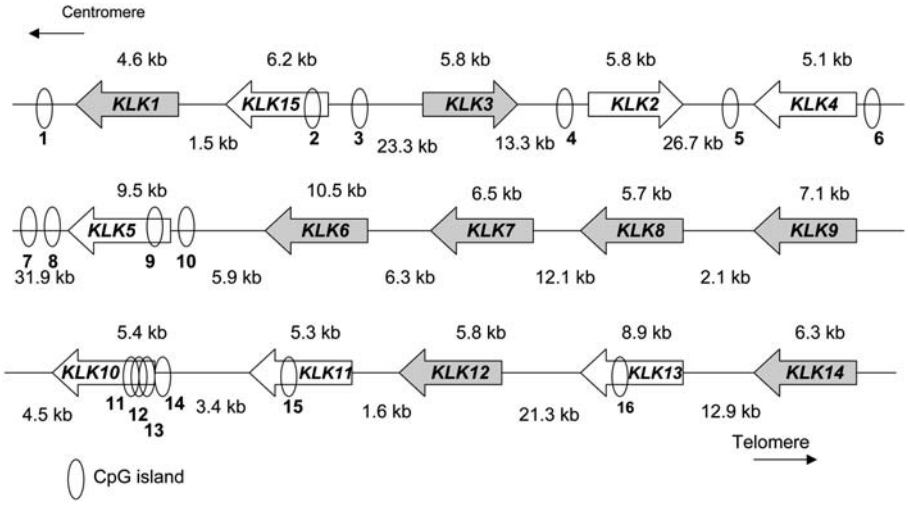

Figure 1 Location of predicted CpG islands in the kallikrein locus.
Using CpG plot (European Bioinformatics Institute, http://www.ebi.ac.uk), 16 putative CpG islands were identified (open ovals). The parameters for CpG island search were: length of CpG region more than 200 bp, C+G content more than 50% and an observed/expected ratio {(No. of CpGs)/[(No. of Cs)×(No. of Gs)]×length} greater than 0.6. Arrows indicate the direction of transcription. *KLK* genes with no CpG islands are indicated with gray arrows and *KLK* genes containing CpG islands with white arrows. Numbering of CpG islands is indicated in boldface numbers.

bined HDAC and DNMT inhibition in inducing apoptosis, differentiation and/or cell growth arrest in human lung, breast, thoracic, leukemia and colon cancers.

The aim of this study was to examine if genomic DNA methylation is involved in the downregulation of novel human kallikreins in prostate, breast and ovarian cancer cell lines. So far, hormonal regulation by various steroids has been proposed as a common mechanism for the parallel regulation of kallikrein gene expression (reviewed by Yousef and Diamandis, 2001). Here, we found that additional transcriptional mechanisms underlie the cancer-associated downregulation or complete inactivation of certain *KLK* genes that can be pharmacologically reactivated upon demethylation of DNA by 5-aza-dC.

Using the CpGplot program, we first analyzed the presence of putative CpG islands in the kallikrein locus that would suggest that epigenetic mechanisms play an important role in the transcriptional regulation of the corresponding genes (Jones and Baylin, 2002). The sequences analyzed with CpGplot are available in the GenBank database with the following accession numbers: AY094609 (*KLK1*, including an extended 3′ downstream region), AF243527 (*KLK2–KLK12*), AF135024 (*KLK13)*, and AF283669 (*KLK14*). The locations and other characteristics of the CpG islands identified are shown in Figure 1 and Table 1. CpG islands were not identified upstream nor in the exon-intron sequences of *KLK1*, *KLK3*, *KLK6*, *KLK7*, *KLK8*, *KLK9*, *KLK12* and *KLK14*. The largest CpG islands were No. 2 and No. 14 (Table 1) located 2.5 kb upstream of *KLK15* and at exon III of *KLK10*, respectively. These consisted of 57 and 61 CpG dinucleotides, respectively, and were predicted with a high score for the observed/expected ratio. Two kallikrein members, *KLK5* and *KLK10*, were found to have four CpG islands. *KLK5* had one CpG island in its proximal upstream region, one inside the first intron and the other two 3′ downstream. In *KLK10*, the second and third CpG island are separated by only 25 bp and the first and sec-

Table 1 Relative location and characteristics of CpG islands in the kallikrein locus.

CpG island	Gene	Position relative to gene	No. of CpGs	Score	Size (bp)	C+G content (%)
1	*KLK1*	Downstream	21	0.75	265	65
2	*KLK15*	Upstream	57	0.89	565	69
3	*KLK15*	Intron II-exon III	28	0.93	306	65
4	*KLK2*	Upstream	16	0.77	235	60
5	*KLK4*	Upstream	23	0.83	228	65
6	*KLK4*	Downstream	17	0.90	305	59
7	*KLK5*	Upstream	17	0.89	239	57
8	*KLK5*	Intron I	17	0.77	305	54
9	*KLK5*	Downstream	18	0.73	298	58
10	*KLK5*	Downstream	16	0.77	240	56
11	*KLK10*	Intron I	26	0.80	287	67
12	*KLK10*	Intron I-exon II-intron II	23	0.67	274	71
13	*KLK10*	Exon II-intron II	36	0.86	494	60
14	*KLK10*	Intron II-exon III	61	0.84	822	62
15	*KLK11*	Intron IV	7	0.99	216	51
16	*KLK13*	Intron I	12	0.78	201	56

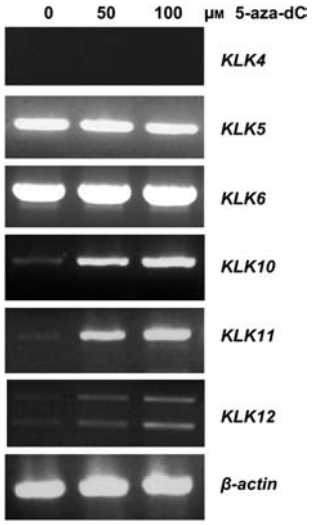

Figure 2 Reactivation of *KLK*s in prostate cancer cells.
PC-3 cells were maintained in RPMI medium supplemented with 10% FBS. Cells were plated onto 100-mm dishes 24 h before treatment. When cells reached approximately 30% confluence, they were treated with 5-aza-dC (Sigma Chemical Co, St. Louis, MO, USA) for 48 h. Then, fresh medium without 5-aza-dC was added and cells were maintained for another 48 h before total RNA was isolated (RNeasy, Qiagen, Valencia, CA, USA). The expression of *KLK*s was analyzed by semi-quantitative RT-PCR carried out on 250 ng of total RNA template with OneStep (Qiagen). Cycling conditions were as follows: 50°C for 30 min and 95°C for 15 min, followed by 35 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min, and a final extension step at 72°C for 10 min. Equal volumes of PCR products were electrophoresed on agarose gels and visualized by ethidium bromide staining. β-Actin was used as a control. The presence of multiple bands (e.g., *KLK12*) indicates multiple gene isoforms. Primers were designed to amplify the cDNA that encodes pro-hK or pre-pro-hK (*KLK6*).

ond CpG islands by 99 bp. Therefore, all these three CpG islands may be regarded as one large CpG island with a length of 1175 bp. The island located in the last intron of *KLK11* contains seven CpG dinucleotides, which makes it the smallest in the kallikrein locus.

Treatment of the prostate cancer cell line PC-3 with 5-aza-dC reactivated *KLK10*, *KLK11* and *KLK12* (Figure 2), despite the fact that only *KLK10* contains CpG islands and *KLK11* has a small CpG island that is not located in a regulatory region. Levels of *KLK5* and *KLK6* expression were unaffected, while 5-aza-dC could not restore the expression of *KLK4*, indicating that mechanisms other than DNA methylation account for *KLK4* inactivation in these cells. All primer sequences for the RT-PCR amplifications are shown in Table 2.

In T47D and MDA-MB-231 breast cancer cells lines, 5-aza-dC reactivated the expression of *KLK6* and *KLK11*. In MDA-MB-231, *KLK4* and *KLK10* were also reactivated. In addition, *KLK5* was reactivated in T47D cells, but remained unaffected in MDA-MB-231 (Figure 3).

In ovarian cancer cell lines ES-2 and HTB-161, *KLK4* was downregulated by 5-aza-dC treatment (Figure 4). *KLK4* has been correlated with poor prognosis and with paclitaxel resistance of ovarian cancer (Xi et al., 2004). Furthermore, downregulation of *KLK4* by 5-aza-dC suggests that distinct epigenetic mechanisms underlie the

Table 2 Oligonucleotide primers used in this study.

Primer	Sequence (5′→3′)
ACTIN-S	ACA ATG AGC TGC GTG TGG CT
ACTIN-AS	TCT CCT TAA TGT CAC GCA CGA
VDR-S	CCA GTT CGT GTG AAT GAT GG
VDR-AS	GTC GTC CAT GGT GAA GGA
KLK4-S	GGC TCG AGA AAA GAG GTA GCT GCA GCC
KLK4-AS	TCC GCG GCC GCA GAG TTA ACT GGC CTG GAC
KLK5-S	GGC TCG AGA AAA GAG CCC GGT CGG ATG AC
KLK5-AS	GTG CGG CCG CCT GGG ATG ACT CAG GAG TTG GCC
KLK6-S	GGA GGA ATT CAG CAG GAG CGG CCA TG
KLK6-AS	TGT CTC GAG TCA GGG TCA CTT GGC CTG
KLK9-S	GGC TCG AGA AAA GAG CAG ACA CCC GTG CC
KLK9-AS	CCG TGG AAT TCG GGC TCA GTT CTC CAT GAT TTC
KLK10-S	GGC TCG AGA AAA GAC AAA ACG ACA CGC
KLK10-AS	CGT GAA TTC TGG ATC AGT TGG AGC G
KLK11-S	GGC TCG AGA AAA GAG AGA CCA GGA TCA TCA AGG G
KLK11-AS	GGT GAA TTC AGT CTA ATT GTT CTT CAT CGT CTC CTG
KLK12-S	GGC TCG AGA AAA GAG CAG CCA CAC CGA AG
KLK12-AS	GGA GAA TTC AGG TCA GTT GTT CCT CAT GAT CAT CCG

S, sense; AS, antisense.

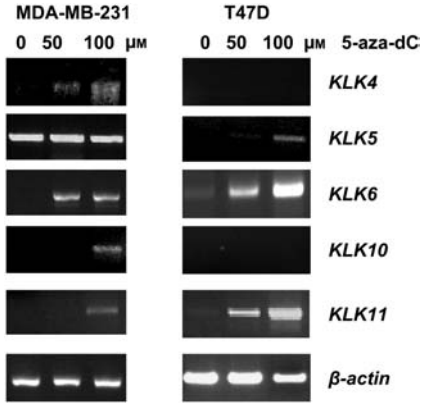

Figure 3 Reactivation of *KLK*s in breast cancer cells.
T47D and MDA-MB-231 cell lines were treated with 5-aza-dC. Treatment and RT-PCR conditions as described in Figure 2.

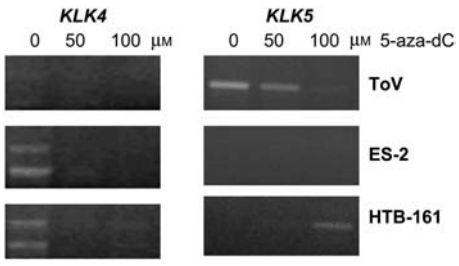

Figure 4 Expression of *KLK4* and *KLK5* in ovarian cancer cell lines.
Treatment and RT-PCR conditions were as described in Figure 2.

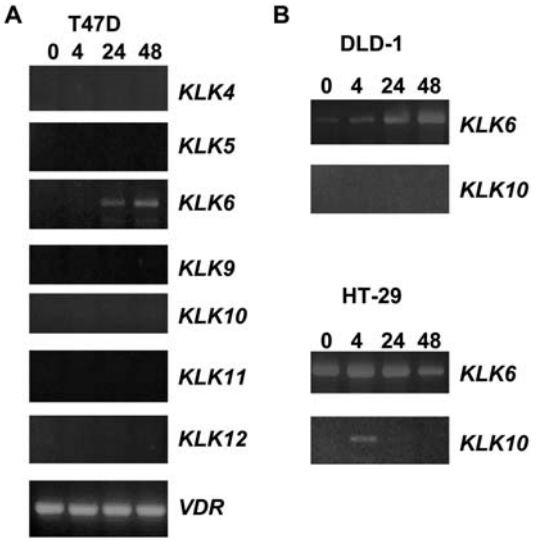

Figure 5 Reactivation of *KLK*s by the vitamin D3 analog EB1089.
(A) Treatment of the T47D breast cancer cell line with 10^{-7} M EB1089 (Leo Pharma, Ballerup, Denmark) for the indicated times given in hours resulted in upregulation of *KLK6* expression. Expression of the vitamin D receptor (*VDR*) was not affected. (B) EB1089 reactivated *KLK6* expression in DLD-1, but not in the HT-29 colon cancer cell line. A transient upregulation of *KLK10* was observed in HT-29 after 4 h of treatment with EB1089.

regulation of *KLK4* in breast compared to ovarian cancer. *KLK5* was also downregulated in the ovarian cancer cell line ToV-21G.

Although DNA methylation has been considered a mechanism regulating the activity of gene promoters that contain CpG islands (CpG-rich promoters), during recent years it became evident that an increasing number of genes with CpG-poor promoters are also subject to regulation by DNA methylation (Jones and Baylin, 2002). Here, we showed that although *KLK6* and *KLK12* do not contain CpG islands, they are strongly reactivated in cancer cells by 5-aza-dC. This suggests that upregulation is either a downstream effect of 5-aza-dC, probably through the activation of a specific transcriptional activator, or that specific non-CpG island cytosines are involved in the regulation of transcription. We showed that silencing of *KLK6* in breast cancer cells is associated with hypermethylation of specific CpG dinucleotides located in the *KLK6* proximal promoter (Sotiropoulou, personal communication).

Transcriptional profiling identified *KLK6* and *KLK10* as key target genes that are strongly induced upon induction of differentiation of squamous cell carcinoma (Lin et al., 2002) and colon cancer cells (Palmer et al., 2003) by the vitamin D3 analog EB1089. Previously, a vitamin D analog has been demonstrated to induce differentiation in T47D cells (Lazzaro et al., 2000). Here, we examined by RT-PCR analysis whether EB1089 treatment reactivates other *KLK*s. We found that only *KLK6* could be reactivated dose-dependently in T47D cells (Figure 5A) and in the DLD-1 colon cancer cell line. A transient reactivation of *KLK10* was observed in HT-29 colon cancer cells (Figure 5B).

Multiple *KLK* genes display similar expression patterns, indicating that common regulatory mechanism(s) most likely exist. Previous studies indicated that hormonal regulation, through the action of various steroids, is a prevalent mechanism accounting for the transcriptional regulation of *KLK*s (reviewed by Yousef and Diamandis, 2001). However, recent studies indicate that other mechanisms likely co-operate in *KLK* regulation. These include the production of multiple splice variants and the use of alternative promoters, as previously reported for *KLK6* (Christophi et al., 2004; Pampalakis et al., 2004) and *KLK11* (Nakamura et al., 2001). The present study indicates that DNA methylation is a mechanism that underlies the aberrant expression of specific *KLK* genes in cancer cells.

Acknowledgments

This study was funded by the Greek Secretariat of Research and Technology and the European Union through research project PENED2001 (01EΔ557) of operational program competitiveness.

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Received November 4, 2005; accepted March 28, 2006