Identification of Single Nucleotide Polymorphisms in the Human Kallikrein 10 (*KLK10*) Gene and Their Association With Prostate, Breast, Testicular, and Ovarian Cancers

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BACKGROUND. The *KLK10* gene (also known as the normal epithelial cell-specific 1 gene) is a member of the expanded human kallikrein gene family. Recently, it has been reported that *KLK10* is a tumor suppressor gene and that its expression is downregulated in various forms of cancer and cancer cell lines. *KLK10* is also upregulated in ovarian cancer. We thus hypothesized that the *KLK10* gene may be a target for mutations in various cancers.
METHODS. We sequenced the five coding exons of the *KLK10* gene using genomic DNA from various tumors, normal tissues, and blood, by PCR amplification and automated sequencing.
RESULTS. In none of the tumor-derived DNAs, we identified somatic mutations that could inactivate this gene. However, we identified a prevalent germline single nucleotide variation at codon 50 (exon 3) of this gene [GCC (alanine) to TCC (serine)]. The GCC genotype was less prevalent in prostatic cancer patients in comparison to control subjects ($P = 0.027$) but no differences were seen with testicular, ovarian, and breast cancer. We also identified four genetic variations in exon 4, at codons 106 [GGC (glycine) to GGA (glycine)], codon 112 [ACG (threonine) to ACC (threonine)], codon 141 [CTA (leucine) to CTG (leucine)], and at codon 149 [CCG (proline) to CTG (leucine)]. None of these variations was significantly different between normal subjects and cancer groups.
CONCLUSIONS. We found no evidence for somatic mutations of the *KLK10* gene in cancers of the prostate, breast, ovary, and testis. The single nucleotide variation at codon 50 appears to be associated with prostate cancer risk. *Prostate 51: 35–41, 2002.* © 2002 Wiley-Liss, Inc.

KEY WORDS: single nucleotide polymorphisms; normal epithelial cell specific-1 gene; kallikreins; human kallikrein 10; cancer biomarkers; serine proteases

INTRODUCTION

Prostate cancer is one of the leading causes of death in men and accounts for approximately 30% of all newly diagnosed cancers [1]. Molecular genetic studies have revealed a few mutations that seem to be responsible for predisposition to prostate cancer or for early tumor progression [2,3]. With the advent of DNA amplification and new DNA variation detection technologies, germline polymorphisms have been identified in many genes and throughout the whole human genome. These polymorphisms have become

particularly attractive potential markers of cancer risk and/or disease progression. Some polymorphisms can alter the protein function or concentration in a biochemically relevant direction or show a population distribution that correlates with well-known ethnic cancer risk profiles [4]. Their presence in germline implies that they have the potential to affect early events that initiate carcinogenesis in many tissues, including the prostate, believed by many to occur at puberty or even earlier, during fetal life [5,6]. Also, they can affect decades-long progression of carcinogenesis, promoted by continued trophic stimulation and division of cells.

Serine proteases are a homologous family of protein degrading enzymes which are involved in a host of biological functions, such as coagulation and fibrinolysis, digestion and activation of the kringle domain containing growth factors, like human tissue plasminogen activator [7,8]. The expression of serine proteases, such as plasminogen activator, as well as other classes of proteinases, has been shown to correlate positively with invasiveness and metastatic potential of many tumor cells [9–14]. This is linked to the ability of the tumors to degrade extracellular matrix components, either directly, or indirectly, through the proteolytic activation of other zymogens.

Many serine proteases are secreted into the extracellular space in order to function [7]. Some of these enzymes can serve as tumor markers. Prostate-specific antigen (PSA), a serine protease, is widely used as a tumor marker for the diagnosis and monitoring of prostate cancer [15].

The normal epithelial cell-specific 1 gene (*NES1*) was identified by subtractive hybridization, by virtue of its downregulation in breast cancer cell lines [16]. The gene has been predicted to encode for a secreted serine protease. The *NES1* gene, now officially known as human kallikrein 10 (*KLK10*) [17], has high sequence homology with three protease families, including the trypsin family, the kringle family, and the human kallikrein family [16]. The *KLK10* gene spans 5.5 kb of genomic DNA, contains five coding exons and one non-coding exon, and resides on chromosome 19q13.4 in the same region where the human kallikrein family is located [7,18]. This family is now known to contain 15 different genes [7].

The *KLK10* gene is downregulated in breast and other cancer cell lines [16,19]. Its expression has been associated with tumorigenic progression, suggesting that the products of *KLK10* proteolytic cleavage are likely to be involved in the negative growth regulation of epithelial cells. Thus, *KLK10* may encode for a tumor suppressor protein [19].

Recently, we have shown that the protein encoded by the *KLK10* gene (hK10, human kallikrein 10) is secreted and found in many biological fluids [20]. Moreover, we found high levels of hK10 in ovarian tumors; these levels correlate positively with ovarian tumor aggressiveness [21]. Also, serum levels of hK10 may have value for ovarian cancer diagnosis and monitoring [22]. However, the mechanism of hK10 upregulation in ovarian cancer is still obscure.

A number of tumor suppressor genes are inactivated by mutations and many single nucleotide polymorphisms are associated with malignant diseases [23–26]. Given the previous information that *KLK10* may be a tumor suppressor [19] and that the expression of this gene is altered malignancies [16,19–22], we hypothesized that *KLK10* may be a target for mutations or cancer-predisposing polymorphisms. We thus examined the mutational and polymorphic status of this gene in DNA isolated from prostate, breast, ovarian, and testicular tumors as well as from normal tissues.

MATERIALS AND METHODS

Prostate and Testicular Cancer Tissues

Prostate tissue samples were obtained from 13 patients who had undergone radical retropubic prostatectomy for prostate adenocarcinoma at the Charite University Hospital, Berlin, Germany. The patients did not receive any hormonal therapy before surgery. The use of these tissues was approved by the ethics Committee of the Charite Hospital. Fresh tissue samples were obtained from the cancerous and non-cancerous parts of the same prostates (matched) that had been removed. Small pieces of the samples were dissected immediately and stored in liquid nitrogen until analysis. Testicular samples were procured similarly.

Whole Blood From Healthy Controls and Prostate Cancer Patients

Fifty-two healthy males who had no evidence of prostate cancer and total PSA < 2 μg/L and 49 age-matched patients with histologically confirmed prostate cancer, treated with radical prostectomy, were also included in this study. Blood was collected in heparinized tubes and DNA was extracted as described below.

Ovarian and Breast Cancer Specimens

Thirty-four ovarian cancer tissues were obtained from patients who underwent surgery for primary ovarian carcinoma at Mount Sinai Hospital. Twenty-seven breast cancer tissues were obtained from women who had undergone surgical treatment for primary breast carcinoma at Mount Sinai Hospital.

Our protocols have been approved by the Institutional Review Boards of the Charite University Hospital, Berlin, Germany and the University of Toronto, Ontario, Canada.

DNA Extraction

DNA was extracted from tissues and whole blood using the Qiagen QIAmp blood and tissue DNA extraction kit (Qiagen, Chatsworth, CA). Approximately 25 mg of tissue or 200 μL of whole blood was used for each extraction. Tissues were pulverized into a fine powder on dry ice. Otherwise, we followed the manufacturer's recommendations.

PCR Amplification of the *KLKl0* Gene

The primer sequences flanking exons 1 through 6 were designed using Oligo 5.0 software (National Biosciences, Plymouth, MN) according to the *KLK10* genomic DNA sequence deposited in GenBank (accession #AF055481). A detailed description of the primers used to amplify each of the six exons is shown in Table I. PCR amplification was performed in a final volume of 25 μL, containing approximately 100–150 ng of template DNA, 10 mmol/L Tris-HCl buffer (pH 8.3), 50 mmol/L of KCl, 2.5 U of Hot Star Taq polymerase (Qiagen), 250 μmol/L of deoxynucleoside triphosphates, 1.5 mmol/L $MgCl_2$, and 1 μmol/L of each primer. The thermal cycling profile consisted of an initial 15 min denaturation at 95°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 50 sec for exons 1 and 5, 60°C for exons 3 and 6, and 62°C for exons 2 and 4, respectively, and extension at 72°C for 50 sec. A final extension step at 72°C for 5 min completed the reaction. The success of the PCR was verified by gel electrophoresis of 5 μL of the amplified PCR product on a 1% agarose gel containing ethidium bromide.

DNA Sequencing

Both strands of the PCR products were sequenced on the Open Gene LR Automated DNA Sequencer (Visible Genetics Inc., Toronto, Ontario Canada), following a general protocol described elsewhere [27]. The sequencing primers were labeled at the 5′ end with the fluorescent dye Cy 5.5 (Visible Genetics, Inc.). The detailed sequences of the nested labeled primers used for sequencing are depicted in Table I.

PSA Immunoassay

The determination of total PSA in all serum samples was performed by a time-resolved immunofluorometric assay as described elsewhere [28]. All specimens were measured in duplicate.

hKl0 Immunofluorometric Assay

The assay for hK10 protein in tumor extracts was performed as previously described [20].

Statistical Analysis

Statistical analysis was performed by using the χ^2 test or the Fisher's exact test where applicable. All tests were two-sided. A *P* value of ≤ 0.05 was considered as statistically significant.

RESULTS

Genomic DNA from 13 high grade prostate tumors and the surrounding normal cells from the same individuals were sequenced for exons 2 through 6 of the

TABLE I. PCR and Sequencing Primer Sequences of *KLKl0* Gene Exons

Exon	Primer code	PCR primers	Size	Sequencing primers[a]	Size
1	5 × 1s	TTGGGGTCAAAAGGGAAGGT	20	TCAAAAGGGAAGGTCCCGCCA	21
	3 × 1s	TGGAGAAACCGAGGCTCTAA	20	AACCGAGGCTCTAAGCCGGCA	21
2	5 × 2s	CTCTCTAACGGAGCATCTGT	20	AACGGAGCATCTTTTTAACCC	21
	3 × 2s	CTTGGTGACGGGAACACATT	20	GTGACGGGAACACATTGCCCT	21
3	5 × 3s	GCTCCAACCCCAAACCCAGA	20	CAACCCCAAACCCAGACCCT	20
	3 × 3s	ACCTCCAGCTGTGGGGAGTT	20	CCAGCTGTGGGGAGTTCCGA	19
4	5 × 4s	ACCTGCTTTCCCAGTCCCTGT	21	TGCTTTCCCAGTCCCTGTGAA	21
	3 × 4s	TCCTGAGGTTCTGGCCTCAGA	21	GTTCTGGCCTCAGAGCCCCA	20
5	5 × 5s	GGGCTTAGGTATGAAACGGT	20	TAGAAACGGTCTTGTATCCTGGAC	24
	3 × 5s	TGGGAATGGAGTTTGGGATC	20	ATGGAGTTTGGGATCGGGCACA	24
6	5 × 6s	CCTCGTCTTTATCCCAACCCA	21	CGTCTTTATCCCAACCCAA	19
	3 × 6s	AGGAAGAGGATGGACGATGGA	21	GAGGATGGACGATGGAGCCT	20

[a]Sequencing primers were 5′-labeled with Cy 5.5 fluorescent dye.

KLK10 gene, using an automated sequencer. The non-coding exon 1 was not sequenced. In all samples, exons 2 and 5 had wild-type sequence (no mutations or polymorphisms were detected). However, we identified a single nucleotide variation in codon 50 (exon 3), GCC (alanine) to TCC (serine) (Fig. 1). The distribution of this sequence variation in the DNA from tumors and adjacent normal tissues is shown in Table II. Of the 13 tumors, only 1 (8%) was wild-type (GCC), according to the sequence reported previously [16,18], 7 (54%) were heterozygotes (G/TCC) and 5 (38%) were homozygotes (TCC). The DNA from the adjacent normal cells of the same individuals had the same genotype, implying no somatic changes.

To elaborate on these findings, we extended this study with DNA from the peripheral blood leucocytes of 52 healthy individuals (controls) who had no history of prostate cancer and total PSA < 2 μg/L and another 49 age-matched individuals who had histologically confirmed prostate cancer. The distribution of this genetic variation in these subjects is summarized in Table II. Of the 52 controls, 26 (50%) had the GCC genotype, 17 (32%) were heterozygotes (G/TCC), and 9 (17%) were homozygotes (TCC). In the cancer group, of the 49 tested individuals, 13 (26%) had GCC genotype, 28 (57%) were heterozygotes (G/TCC), and 8 (16%) were homozygotes (TCC) ($P = 0.027$ by the Fisher's exact test).

The codon 50 polymorphism was also examined in 11 high-grade testicular tumors and 34 high-grade ovarian tumors. Four testicular tumors (36%) had GCC genotype, five tumors (45%) were heterozygotes (G/TCC), and two tumors (18%) were homozygous TCC. In the case of the ovarian tumors, 17 (50%) had GCC genotype, 14 tumors (41%) were heterozygotes, and 3 tumors (9%) had homozygous TCC genotype (Table II). The hK10 protein concentration (encoded by the *KLK10* gene) in 26 available ovarian cancer tissue extracts was not significantly different ($P = 0.23$ by *t*-test) in tumors with GCC genotype (mean = 4.3 ng/mg of total protein; N = 11)) or G/TCC and TCC genotypes (mean = 2.3 ng/mg of total protein, N = 15), although the majority of the tumors with the latter genotypes had less hK10 protein than tumors with GCC genotype. Of the 27 breast tumors analyzed, 6 (22%) had GCC genotype, 14 (52%) were heterozygotes with G/TCC genotype, and 7 (26%) were homozygotes with a TCC genotype (Table II).

Exon 4 of this gene had a number of single nucleotide polymorphisms (SNPs). The genetic changes identified in the paired normal/cancerous prostatic tissues were located at codon 106 [GGC (glycine) to GGA (glycine)], codon 112 [ACG (threonine) to ACC (threonine)], codon 141 [CTA (leucine) to CTG (leucine)], and finally, at codon 149 [CCG (proline) to CTG (leucine)]. The changes at codons 106, 112, and 141 did not affect the encoded amino acid. The variation at codon 149 changes the amino acid from proline to leucine (Table III). All these SNPs were identical in tumor as well as the corresponding normal tissue
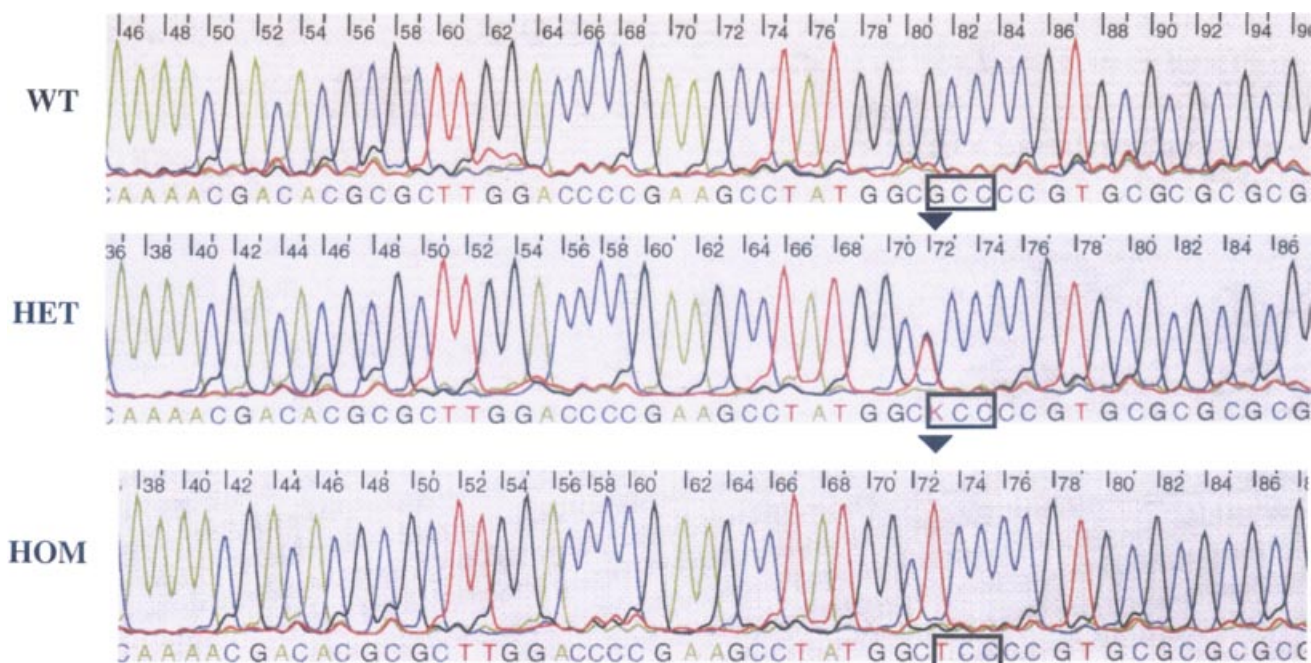

Fig. 1. Codon 50 genetic polymorphism in exon 3 of the *KLK10* gene. WT, wild-type sequence, as reported in GenBank Accession # AF055481. HET, heterozygous, HOM, homozygous variant sequence. For more details, see text. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II. *KLKI0* Codon 50 Genotype in Various Groups of Patients

Type of specimen	N	GCC (Ala)	GCC/TCC (Ala/Ser)	TCC (Ser)
Prostate cancer tissue	13	1 (8%)	7 (54%)	5 (38%)
Normal prostatic tissue	13	1 (8%)	7 (54%)	5 (38%)
Peripheral blood, healthy males	52	26 (50%)	17 (32%)	9 (18%)
Peripheral blood, prostate cancer patients	49	13 (26%)	28 (57%)	8 (16%)
Testicular cancer tissue	11	4 (36%)	5 (45%)	2 (18%)
Ovarian cancer tissue	34	17 (50%)	14 (41%)	3 (9%)
Breast cancer tissue	27	6 (22%)	14 (52%)	7 (26%)

DNA from the same individuals. It is interesting to note that in three prostatic tumors and their matched normal counterparts, all polymorphisms at codons 106, 112, 141, and 149 were linked and present simultaneously in each DNA specimen. Only one tumor had codon 112 SNP alone. The distribution of these SNPs are summarized in Table III. The percentages of these polymorphisms between healthy controls and prostate cancer patients were approximately the same ($P = 0.5$; data not shown).

There were no changes observed in the coding region of exon 6 of the *KLK10* gene. However, a prevalent polymorphism was detected at nucleotide 4779 (based on the numbering of GenBank Accession # AF055481) in the intron preceding exon 6. There was a substitution of thymine with cytosine (T to C). The polymorphism was homozygous (T) in half of these tumor DNAs and heterozygous (T/C) in the other half. No C homozygotes were found in this group of patients. The distribution of this polymorphism was similar between healthy controls and prostate cancer patients (data now shown).

DISCUSSION

The *KLK10* gene (previously known as the normal epithelial cell-specific 1 gene) is a new member of the human kallikrein gene family [7,17]. Recent data suggest that *KLK10* may function as a tumor suppres-sor [19]. The expression of this gene in hormonally regulated tissues [16] and its regulation by steroid hormones [29] suggest that it may have a specific role in the development of hormonally related cancers, such as those of breast and prostate.

The putative tumor suppressor activity of *KLK10*, in analogy to other tumor suppressor genes that are inactivated by mutations (e.g., *p53*, *BRCA1*, *BRCA2*, etc.) [23–26,30–34], prompted us to speculate that this gene may also be a target for either somatic or germline mutations that predispose to cancer development or progression. We have thus undertaken this study to examine in detail the polymorphic and mutational status of this gene using DNA isolated from normal tissues or from cancers of the prostate, breast, ovary, and testis.

Sequencing of the five coding exons of this gene revealed a relatively prevalent genetic variation at codon 50 in exon 3. This variation (GCC → TCC) changes the amino acid at this position, from alanine to serine. Since the signal and activation peptides of hK10 protein are 33 and 9 amino acids, respectively [7]; this amino acid change will be present in the mature hK10 protein. The two forms of this enzyme are present in significant proportions of individuals, as shown in Table II.

Examination of this genetic variation in adjacent normal and cancerous prostatic tissues from the same patients revealed no somatic changes in the tumors.

TABLE III. Genetic Variability of Exon 4 of the *KLKI0* Gene*

Codon	Genotype change[a]	Amino acid	Homozygous (wild-type)	Heterozygous	Homozygous (variant)
106	GGC → GGA	Gly-Gly	10 (GGC)	3	0
112	ACG → ACC	Threo-Threo	9 (ACG)	2	2 (ACC)
141	CTA → CTG	Leu-Leu	10 (CTA)	3	0
149	CCG → CTG	Pro-Leu	10 (CCG)	3	0

*Data obtained by sequencing 13 pairs of DNA from normal and cancerous prostatic tissues. No somatic changes were identified. Numbers represent individual patients.
[a]Wild-type, based on the sequence deposited in GenBank, Accession #AF 055481.

The originally reported codon 50 sequence (GCC) by both Liu et al. [16] and Luo et al. [19] is represented in the minority of the subjects examined. Furthermore, lowest frequency of this allele is seen in patients with prostate cancer, in comparison to matched controls. This difference was statistically significant ($P = 0.027$). Relatively low frequencies of this allele were also seen in breast cancer patients (Table II). However, since the number of patients in each of these categories is relatively small, it will be necessary to confirm these preliminary findings with larger clinical investigations. Since information about specific substrate recognition by hK10 is lacking, and functional analysis of the two putative hK10 isoforms has not been reported, it is difficult to speculate on a biological explanation of these findings.

We further report four genetic variations of this gene in exon 4, three of them being single nucleotide polymorphisms, and one resulting in an amino acid substitution. These polymorphisms seem to co-segregate within individuals and are less prevalent than the codon 50 variation reported above. Furthermore, we identified a prevalent single nucleotide polymorphism in intron 5 of the *KLK10* gene. The significance of these polymorphisms with cancer susceptibility has not been examined in detail.

The protein encoded by the *KLK10* gene has been recently shown to be overexpressed in subsets of ovarian cancer patients and hK10 appears to be a novel serum biomarker for ovarian cancer diagnosis and monitoring [21,22]. Similarly, other members of this gene family have been found to be overexpressed or underexpressed in various malignancies [7]. The role of inactivating mutations or polymorphisms of these genes in cancer is currently unknown.

In conclusion, we here describe novel genetic variations of the *KLK10* gene and report the frequencies of these changes in DNA from normal individuals and patients with various cancers. Our preliminary data indicate that the codon 50 variation seems to be associated with prostate cancer.

CONCLUSION

The *KLK10* gene, although overexpressed in ovarian cancer, does not seem to be a target for somatic mutations in either ovarian, prostate, breast or testicular cancer. We identified a single nucleotide variation at codon 50, which seems to be significantly associated with prostate cancer risk. This finding requires verification with larger clinical studies.

ACKNOWLEDGMENTS

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