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QUANTITATIVE ANALYSIS OF HIPPOSTASIN/KLK11 GENE EXPRESSION IN CANCEROUS AND NONCANCEROUS PROSTATIC TISSUES

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ABSTRACT

Objectives. Hippostasin/kallikrein 11 (KLK11) is a member of the human kallikrein gene family, which includes prostate-specific antigen (PSA), human kallikrein 2 (hK2), and another 12 members, all localized on chromosome 19q13.4. Hippostasin has two alternative splicing isoforms, known as the brain type and prostate type. We have previously reported that the prostate-type isoform is not expressed in human prostate cancer cell lines.

Methods. We compared the expression of hippostasin/KLK11 isoforms in 76 matched pairs of human normal and prostate cancer tissues by quantitative reverse transcriptase-polymerase chain reaction.

Results. The expression of both isoforms of KLK11 was 25% to 45% higher in cancer tissues compared with their normal counterparts. Regarding prostate-type KLK11, we identified a significant association between lower expression and higher tumor stage, Gleason score, and tumor grade. No such association was seen with the brain-type isoform.

Conclusions. The expression of the prostate-type isoform of KLK11 is increased in prostate cancer. This parameter should be examined further as a new prognostic indicator of prostate cancer. UROLOGY **61:** 1042–1046, 2003. © 2003, Elsevier Inc.

Prostate cancer is the most common cancer of North American men. Prostate-specific antigen (PSA), also known as human kallikrein 3 (hK3), according to the approved new nomenclature of the human kallikrein family,[1] is used for early detection and monitoring of prostate cancer.[2,3] However, nonmalignant prostatic diseases, especially benign prostatic hyperplasia and acute prostatitis also cause serum PSA elevation, thus complicating the diagnosis of prostate cancer by PSA measurement alone.[4] Analysis of the molecular forms of PSA improves the specificity for prostate cancer. In particular, the determination of free PSA and its ratio to total PSA is now clinically established and is used to reduce the number of unnecessary prostate biopsies.[5,6] Other structurally similar kallikrein genes may also be related to prostate cancer.[7] Human glandular kallikrein gene 2 (hK2) has also been proposed as an adjuvant tumor marker in subgroups of patients with moderate elevations of serum PSA.[8–10] KLK4 is highly expressed in the prostate and is under steroid hormonal regulation in prostate and breast cancer cell lines.[11,12]

Hippostasin/KLK11, also known as trypsin-like serine protease (TLSP),[13] was cloned from hippocampus and prostatic tissues and called brain-type and prostate-type hippostasin/TLSP, respectively.[14] The gene encoding for hippostasin/TLSP is located on chromosome 19q13.4 between KLK10/NES-1 and KLK12.[1,7] Alternative splicing is responsible for the prostate type, which has an additional 32 amino acids at the *N*-terminus of brain-type hippostasin.[14] Recently, we examined the expression of KLK11 in benign prostatic hyperplasia tissues and human prostate cancer cell lines. The prostate-type isoform was not detected in the cancer cell lines.[15] More recently, hK11 protein

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was found to be elevated in the serum of patients with prostate and ovarian cancer.[16]

The aim of this study was to investigate the quantitative expression of the KLK11 gene in paired normal and cancerous prostatic tissues. We also examined the relationship between KLK11 expression and standard clinicopathologic variables of prostate cancer.

MATERIAL AND METHODS

Study Group

Included in this study were 76 patients who had undergone radical retropubic prostatectomy for prostate adenocarcinoma at the Charite University Hospital, Berlin, Germany. Patient age ranged from 48 to 75 years (mean 62.6, median 63). Six of the 76 patients received hormonal therapy for at least 2 to 4 weeks before surgery. Stage and grade were established using the TNM and World Health Organization classification systems.[17,18]

Prostatic Tissues

Fresh prostatic tissue samples were obtained from the cancerous and noncancerous parts of the same prostates. Small pieces of tissue were dissected immediately after removal of the prostate and stored in liquid nitrogen. Histologic analysis was performed as previously described[19] to ensure that the tissues were either malignant or benign. The ethics committee of the Charite Hospital approved the use of these tissues for research purposes.

Total RNA Extraction and cDNA Synthesis

Tissues were minced with a scalpel, on dry ice, and transferred immediately to 2-mL polypropylene tubes. They were then homogenized, and total RNA was extracted using the Rneasy total RNA isolation system, following the manufacturer's instructions (Qiagen, Valencia, Calif). The concentration and purity of total RNA were determined spectrophotometrically. Two micrograms of total RNA were reverse transcribed into first-strand cDNA using the Superscript preamplification system and oligo-dT as primer (Gibco BRL, Gaithersburg, Md). The final volume was 20 μL.

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction Analysis

On the basis of the published genomic sequence of KLK11 (GenBank Accession AB041036, AB013730 and AF164623), two isoform-specific primer sets were designed. For prostate-type, Fp: 5′-CTG CCT TGC TCC ACA CCT G-3′ and Rp: 5′-CAG GTT CCT TGG CTG CTG TG-3′ and brain-type, Fb: 5′-GGA CTC AAG AGA GGA ACC TG-3′ and Rb: 5′-CAC TCG AAC CCC TTG ATG AT-3′). These primers spanned more than 1 exon to avoid contamination by genomic DNA (Fig. 1).

Real-time monitoring of polymerase chain reactions (PCRs) was performed using the LightCycler system (Roche Molecular Systems, Indianapolis, Ind) and the SYBR green I dye, which binds preferentially to double-stranded DNA. Fluorescence signals, which are proportional to the concentration of the PCR product, are measured at the end of each cycle and immediately displayed on a computer screen, permitting real-time monitoring of the PCR.[20] The reaction is characterized by the point during cycling when amplification of PCR products is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting quantity of the template, the earlier a significant increase in fluorescence is observed.[21] The threshold cycle is defined as the fractional cycle number at which fluorescence passes a fixed threshold above the baseline.[22]

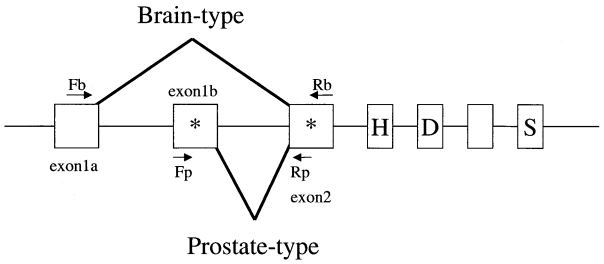

FIGURE 1. *Genomic organization and splicing of the two isoforms of the KLK11 gene. The brain-type isoform uses an upstream untranslated exon (exon 1a) and the prostate-type isoform uses a different untranslated exon 1 (exon 1b). *Two potential translation initiation sites. Also shown are exons harboring the catalytic amino acids histidine (H), aspartic acid (D), and serine (S). The primers used for PCR amplification of the two isoforms are shown. Their sequences are described in the text.*

Endogenous Control

For each sample, the amount of the target and an endogenous control (beta-actin, a housekeeping gene) were determined using a calibration curve. The amount of the target molecule was then divided by the amount of the endogenous reference to obtain a normalized target value.[22]

Calibration Curves

Separate calibration (standard) curves for beta-actin and both isoforms of KLK11 were constructed using serial dilutions of total cDNA from healthy human prostatic tissue (purchased from Clontech, Palo Alto, Calif). The standard curve samples were included in each run. The LightCycler software automatically calculates the standard curve by plotting the starting dilution of each standard sample versus the threshold cycle, and the sample concentrations were then calculated accordingly. Standards for both KLK11 and beta-actin RNAs were defined to contain an arbitrary starting concentration, because no primary preparations exist. Hence, all calculated concentrations are relative to the concentration of the standard.

PCR Amplification

The PCR was carried out on the LightCycler system. For each run, a master mixture was prepared on ice, containing 1 μL of cDNA, 2 μL of LC DNA Master SYBR Green I mix, 50 ng of primers, and 1.2 μL of 25 mM $MgCl_2$. The final volume was adjusted with water to 20 μL. After the reaction mixture was loaded into a glass capillary tube, the cycling conditions were carried out, as shown in Table I. For example, the prostate-type isoform was amplified with an initial denaturation at 95°C for 10 minutes, followed by 38 cycles of denaturation at 95°C for 1 second, annealing at 64°C for 5 seconds, and extension at 72°C for 20 seconds. The temperature transition rate was set at 20°C per second. Fluorescent product was measured by a single acquisition mode at 85°C after each cycle.[20] No primer-dimer product was detected under the outlined experimental conditions.

TABLE I. *Summary of quantitative reverse transcriptase polymerase chain reaction conditions*

	Target Temperature (°C)	Incubation Time
Prostate-type isoform		
Denature (1 cycle)	95	10 min
PCR (38 cycles)	95	1 s
	64	5 s
	72	20 s
Data collection	85	
Brain-type isoform		
Denature (1 cycle)	95	10 min
PCR (38 cycles)	95	1 s
	62	5 s
	72	15 s
Data collection	83	
Beta-actin		
Denature (1 cycle)	95	10 min
PCR (35 cycles)	95	0 s
	62	5 s
	72	40 s
Data collection	85	

KEY: PCR = polymerase chain reaction.

MELTING CURVE

To distinguish the specific from the nonspecific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 72°C for 30 seconds followed by a gradual increase in temperature to 97°C at a rate of 0.2°C per second, with the signal acquisition mode set at step. To verify the melting curve results, representative samples of the PCR products were run on 1.2% agarose gels, purified, and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

STATISTICAL ANALYSIS

Statistical analysis was performed with Statistical Analysis System software (SAS Institute, Cary, NC). The analyses of differences between KLK11 expression in noncancerous and cancerous tissues were performed with Wilcoxon signed ranks test. Relationships between different variables were assessed by the Mann-Whitney *U* test and Spearman correlation.

RESULTS

The expression levels of KLK11 were determined using arbitrary units, according to a standard curve that was constructed by using serial dilutions of a cDNA obtained from normal prostatic tissue. Results were then normalized using the ratio of KLK11/beta-actin concentration for each sample. We then compared the expression of both isoforms of the KLK11 gene in 76 pairs of prostatic tissues (normal versus cancer) obtained from the same patient. The results are shown in Table II. The mean and median values of both isoforms of KLK11 were lower in the normal tissues by approximately 23% to 26% (prostate-type, $P = 0.056$) and 45% to 24% (brain-type, $P = 0.003$), respectively.

The association of the KLK11 mRNA level with clinicopathologic parameters in cancerous tissues is shown in Table III. The prostate-type KLK11 isoform was significantly associated with an earlier stage (I-II versus III), lower Gleason score (6 or less versus greater than 6), and lower tumor grade (G1-G2 versus G3). On the other hand, the brain-type isoform did not show any significant association with these parameters.

COMMENT

Kallikreins are a subgroup of serine proteases. Three adjacent members of the human kallikrein family, KLK3 (PSA), KLK2, and KLK4, are localized within a 57-kb region of chromosome 19q13.4, are highly expressed in prostatic tissue, and have been used as, or are candidate, prostate cancer biomarkers.[11,23,24]

Hippostasin/KLK11 is a new human kallikrein gene that is also located on chromosome 19q13.4. We have previously shown that this gene is under steroid hormone regulation in cancer cell lines[25] and that it is expressed in two alternative splicing isoforms.[14] Recently, we reported alternative splicing variant expression of KLK11 in prostate cancer cell lines.[15] Prostate-type KLK11 was expressed only in normal and benign prostatic hyperplasia tissues but not in prostate cancer cell lines. Furthermore, we demonstrated that serum levels of hK11 are elevated in patients with ovarian and prostate cancer.[16]

In this study, we assessed the expression of both types of KLK11 in human prostate cancer tissues and matched normal tissues using quantitative reverse transcriptase-PCR. Our results have demonstrated that both brain-type and prostate-type isoforms are expressed at lower levels in normal tissues compared with the cancerous tissues (Table II). In cancerous tissues, prostate-type KLK11 isoform levels were significantly associated with clinicopathologic parameters. In particular, prostate-type KLK11 expression was significantly lower in advanced stage and in less-differentiated prostate tumors (Table III). These data collectively suggest that KLK11 prostate isoform expression is increased in cancer tissue compared with normal tissue, but it is again lowered when the tumor progresses further. However, the changes seen were relatively small (Table II) and could have been due to variations of expression of the housekeeping gene used (beta-actin), in addition to variations of expression of the KLK11 gene. Some other kallikrein genes are differentially regulated

TABLE II. ***Descriptive statistics for KLK11 mRNA expression levels in noncancerous and cancerous prostatic tissues from 76 patients***

	Mean*	Standard Error*	Median*	Range*	*P* Value†
Prostate-type isoform					
Cancerous tissues	25.7	8.9	2.9	0.02–410	
Noncancerous tissues	19.9	4.7	2.1	0.02–192	0.056
Lower in normal‡ (%)	23	—	26	—	
Brain-type isoform					
Cancerous tissues	1.61	0.35	0.50	0.00–43	
Noncancerous tissues	0.89	0.16	0.38	0.00–10	0.003
Lower in normal‡ (%)	45	—	24	—	

* *These values are corrected for actin expression and are unitless ratios.*
† *Calculated by the Wilcoxon signed ranks test.*
‡ *Compared with cancer and assuming that value in noncancerous tissue is 100%.*

TABLE III. ***KLK11 expression in cancerous prostatic tissues from 76 patients classified by stage of disease, Gleason score, and tumor grade***

	Total	Mean*	Standard Error*	Median*	*P* Value†
Prostate-type isoform					
Stage					
I–II	39	39	14	8.4	0.043
III	37	24	9	1.6	
Gleason score					
≤6	33	44	17	10.4	0.011
>6	37	25	9	1.2	
Unknown	6				
Grade					
G1–G2	44	38	13	6.9	0.004
G3	32	23	10	0.8	
Brain-type isoform					
Stage					
I–II	39	2.4	1.1	0.8	0.38
III	37	2.2	0.8	0.5	
Gleason score					
≤6	33	2.8	1.3	0.8	
>6	37	2.2	0.8	0.6	0.30
Unknown	6				
Grade					
G1–G2	44	2.3	1.0	0.9	0.21
G3	32	2.3	0.9	0.5	

* *These values are corrected for actin expression and are unitless ratios.*
† *Calculated by the Mann-Whitney U test.*

between cancer and their normal counterparts and are associated with clinicopathologic parameters. KLK15, another member of human kallikrein family, is upregulated in prostate cancer tissues.[26] KLK5 is reported to be an indicator of poor prognosis in ovarian cancer.[27] Many kallikreins have alternative splicing variants,[7] but their quantitative assessment has not been reported. PSA and KLK2 also have alternative splicing variants but the association with disease is not known.[28,29]

We have recently reported the expression of KLK11 at the protein level.[16] HK11 protein was expressed at the highest levels in prostatic tissues followed by stomach tissue. Almost 60% of patients with prostate cancer have high hK11 levels in serum. It should be noted, however, that the tissue expression levels might not reflect the serum levels of the protein. Although levels of PSA and hK2 are elevated in the serum of patients with prostate cancer, their tissue concentration is lower in cancer versus normal tissue.[10] The elevation of the serum concentration may be attributed to angiogenesis, destruction of the tissue architecture, and leakage of these proteins into the general cir-

culation.[7] The same comments may apply to the KLK11 gene and the hK11 protein.

CONCLUSIONS

It has been shown in the past that many proteolytic enzymes may play a role in cancer invasion and metastasis. To this end, hK11 should also be considered, along with many other kallikreins found in the prostate (eg, hK2, hK3, hK6, hK10, hK15), in this context. It seems likely that the kallikrein system represents an enzymatic cascade pathway operating in many organs, including breast, ovary, and prostate.[30] Additional studies are needed to examine these possibilities further.

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