DIFFERENTIAL EXPRESSION OF KALLIKREIN GENE 5 IN CANCEROUS AND NORMAL TESTICULAR TISSUES

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ABSTRACT

Objectives. Kallikrein gene 5 (KLK5; formerly designated as kallikrein-like gene 2, or human stratum corneum tryptic enzyme) is one of the new members of the human kallikrein gene family on chromosome 19q13.4. Although it is expressed at low levels in various tissues, KLK5 expression is highest in the human mammary gland and testis. Previous investigations have established that the expression of KLK5 is estrogen and progesterone driven in the BT-474 breast cancer cell line. In this study, we focused on KLK5 expression in normal and cancerous testicular tissue.

Methods. Fourteen matched testicular tumor and adjacent normal tissue samples were minced on dry ice and homogenized. Total RNA was extracted and mRNA was reverse transcribed. The cDNA samples were amplified by real-time quantitative polymerase chain reaction with KLK5-specific primers to compare the relative KLK5 levels in normal and cancerous testicular tissues.

Results. In 13 (93%) of the 14 patients, KLK5 expression in the cancerous area was significantly lower than in the adjacent, histologically confirmed, normal testicular tissue samples (P < 0.001). The KLK5 level was 9.0 ± 3.9 (mean ± standard error, arbitrary units) in the noncancerous tissue and 4.5 ± 2.9 in the cancerous tissue. We noted significantly lower KLK5 expression in seminomas than in nonseminomas (P = 0.009), as well as in late-stage (II/III) tumors versus early-stage (I) tumors (P = 0.026). KLK5 expression was also associated with the extent of primary tumor, with tumors with vascular/lymphatic invasion (T2/T3) expressing lower KLK5 message than did tumors limited to the testis and epididymis (T1) (P = 0.008).

Conclusions. We found significantly lower expression of KLK5 in testicular tumors than in normal testicular tissue. More studies are necessary to investigate the mechanism behind this finding. UROLOGY 60: 714–718, 2002. © 2002, Elsevier Science Inc.

Testicular cancer has an annual incidence rate of 3 to 4/100,000 men in North America. Although testicular cancer-related mortality rates have decreased by approximately 70% since the early 1970s, this disease remains the most common form of cancer in young males between the ages of 15 and 35 years. Although both seminoma and nonseminoma are highly treatable (greater than 95%) if diagnosed early, the available standard treatments, which include orchiectomy, chemotherapy, and radiotherapy, carry considerable morbidity for men of reproductive age. Moreover, survival rates remain poor in cases in which initial high-dose chemotherapy fails. Although serum tumor markers such as alpha-fetoprotein (AFP), the beta-subunit of human chorionic gonadotropin (beta-hCG), and lactate dehydrogenase may help in the diagnosis and follow-up, the discovery of molecular markers that can detect cancer-promoting changes may help in better understanding this disease.

The recently discovered kallikrein gene 5 (KLK5; formerly designated as kallikrein-like gene 2 [KLK-L2] and human stratum corneum tryptic enzyme [HSCTE]) is a new member of the human kallikrein family of serine proteases. This gene
has been cloned and mapped to chromosome 19q13.4 by the positional candidate gene approach. It codes for a secreted protein of 293 amino acids and putative trypsin-like serine protease activity. Similar to the other members of the kallikrein gene family, KLK5 is also composed of five coding exons and four introns, has the catalytic triad His-Asp-Ser in exons 2, 3, and 5, respectively, as well as conserved exon-intron splice junctions. In addition, this gene is now known to possess a 5' untranslated exon (GenBank Accession No. AF135028), a common feature of many kallikrein genes. KLK5 mRNA is highly expressed in the human brain, mammary gland, and testis, with lower expression levels in various other tissues. The expression of KLK5 has been shown to be estrogen and progesterin driven in the breast carcinoma cell line BT-474. The biologic function of KLK5 is currently unknown, but there is evidence for its aberrant expression in ovarian carcinoma. Because the testis is the male gonad, and KLK5 is known to be highly expressed in the normal testes, we hypothesized that this gene may be differentially expressed in normal versus cancerous testicular tissue and that this expression may be related to tumor aggressiveness.

MATERIAL AND METHODS

NORMAL AND CANCEROUS TESTICULAR SAMPLES AND PATIENTS

Fourteen testicular tumor samples were obtained from patients who had undergone radical orchiectomy at the University Hospital Charite, Berlin, Germany. Histologically confirmed normal testicular tissue samples adjacent to malignant tissue were obtained from each patient. The tissues were dissected immediately and stored in liquid nitrogen until analysis. Histologic analysis was performed to determine whether the tissue was malignant or benign. All patients had primary testicular germ cell cancer. The samples included six seminomas and eight nonseminomas (teratomas, chorio-carci-nomas, and embryonal carcinomas). The median age of the patients was 37 years (range 17 to 60). Follow-up information and hCG and AFP levels were available for all patients. The Ethics Committee of the Charite Hospital approved the use of these tissues for research purposes.

TOTAL RNA EXTRACTION

Tissues were minced with a scalpel on dry ice. The tissue pieces were transferred immediately to 2-mL polypropylene tubes and homogenized. Total RNA was extracted using the RNAeasy total RNA isolation method per the manufacturer's instructions (Qiagen, Valencia, Calif). The concentration and purity of RNA were determined using spectrophotometry.

MEASUREMENT OF KLK5 EXPRESSION LEVELS BY QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Preparation of cDNA. Two micrograms of total RNA was reverse transcribed into first-strand cDNA using the SuperScript premplification system (Gibco BRL, Gaithersburg, Md). To test the success of cDNA synthesis, 1 μL of the reverse transcription product was amplified using polymerase chain reaction (PCR) with primers specific for actin. Product was visualized on a 1.5% agarose gel stained with ethidium bromide.

PCR Amplification. On the basis of the published genomic sequence of KLK5 (GenBank Accession No. AF135028), two gene-specific primers were designed (5'-TAAGACCCGCCCT- GGATGTG-3' and 5'-AGTCTTAAGAGTCGTCGG-3'). These primers spanned more than two exons to avoid contamination by genomic DNA. The PCR reaction was carried out using the LightCycler system (Roche Molecular Systems, Indianapolis, Ind) and the SYBR green I dye, which binds preferentially to double-stranded DNA. 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 MgCl2. 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 follows: initial denaturation at 94°C for 10 minutes, followed by 45 cycles of denaturation at 94°C for 0 seconds, annealing at 60°C for 5 seconds, and extension at 72°C for 16 seconds. The temperature transition rate was set at 20°C/s. Fluorescent product was measured by a single acquisition mode at 86°C after each cycle (see below).

Data Acquisition. In the LightCycler system, 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 product. The reaction is characterized by the point during cycling when amplification of PCR products is first detected, rather than by 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. The threshold cycle is defined as the fractional cycle number at which fluorescence passes a fixed threshold above the baseline. For distinguishing specific from nonspecific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 70°C for 30 seconds followed by a gradual increase in temperature to 98°C at a rate of 0.2°C/s, with the signal acquisition mode set at step, as described.

Quantification of PCR Product. For each sample, the amount of KLK5 (target) and amount of an endogenous control (ie, beta-actin, with its own pair of gene-specific primers) were determined using calibration curves. Separate calibration (standard) curves were constructed for beta-actin and KLK5 using serial dilutions of total cDNA from healthy human testicular tissue (Clontech, Palo Alto, Calif), as described by Bieche et al. 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 KLK5 and beta-actin 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 and expressed in arbitrary units. In determining a normalized KLK5 value for each sample, the amount of target molecule was divided by the amount of target molecule plus the amount of endogenous reference, in accordance with a previously established method.

PCR Product Verification. To verify the melting curve results, representative samples of the PCR products were run on 1.5% agarose gels, purified, and cloned into the pCR 2.1-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 using Statistical Analysis System software (SAS Institute, Cary, NC). The analyses of differences between KLK5 expression in noncancerous and cancerous tissues were performed with the nonparametric McNemar test and Wilcoxon signed rank sum test. The binomial distribution was used to compute the significance level of the McNemar test. Relationships between the KLK5 levels and the different variables (AFP, beta-hCG, patient age) were assessed by the Mann-Whitney U test and Spearman correlation.

RESULTS
KLK5 EXPRESSION IN MATCHED TESTICULAR TISSUES
The quantitative PCR results indicated that KLK5 mRNA is present in both normal and cancerous testicular tissues. KLK5 levels of 9.0 ± 3.9 (mean ± standard error, arbitrary units) were present in the noncancerous tissues; in the cancerous tissues, KLK5 expression was 50% lower (4.5 ± 2.9; Table I). Because the KLK5 levels in normal and cancerous testicular tissues did not follow a gaussian distribution, the median KLK5 values between cancerous (0.44) and normal tissues (2.41) were compared (P < 0.01, Wilcoxon signed rank sum test; Table I). With the exception of one pair, KLK5 expression was always lower in the cancerous tissue (P = 0.008) than in the adjacent nonmalignant tissue (Fig. 1).

KLK5 EXPRESSION AND CHARACTERISTICS OF TESTICULAR MALIGNANCY
Table II summarizes the distribution of KLK5 expression in the 14 testicular cancer samples in relation to histotype, tumor size, nodal status, and disease stage. A significantly lower percentage of cancerous tissues from patients with advanced stage (P = 0.026) and larger tumor size (P = 0.008) expressed the KLK5 message compared with patients with early-stage disease and smaller tumor size, respectively. KLK5 mRNA levels were also lower in seminomas than in nonseminomas, and this result was highly significant statistically (P = 0.009; Table II and Fig. 2). KLK5 mRNA levels were not different in node-positive patients versus node-negative patients (Table II). No significant correlation was found between KLK5 expression in tissues and serum AFP or hCG levels, nor was there any relation between KLK5 expression and patient age (data not shown).

COMMENT
The diagnosis, treatment, and follow-up of testicular cancer rely heavily on the use of serum markers. A poor prognosis is expected by any one or a combination of the following: liver, bone, or brain metastases or a high number of lung metastases, very high levels of any of the three serum markers, AFP, beta-hCG, or LDH, and the presence of primary mediastinal nonseminoma.12 Testicular cancer is classified into seminoma and non-
seminoma (embryonal carcinoma, teratoma, yolk sac carcinoma, choriocarcinoma, and combinations of these). Radiotherapy is more successful against seminomas, and cure rates approach 100% if diagnosed at an early stage.\textsuperscript{16} Although our knowledge of how to diagnose and treat testicular cancer is extensive, little is known about the initiation and progression of normal germ cells to germ cell tumors, which make up 90% of testicular cancer cases.\textsuperscript{17} To shed light on early events of cancer initiation and progression, research has been focusing on changes in gene expression at the tissue level. Examples of such genes that have been identified to date include the cell cycle-regulating proteins, p16\textsuperscript{18} and pRB,\textsuperscript{19} as well as the tumor suppressor Chk2\textsuperscript{20}; all three appear to be downregulated in testicular cancer cells compared with levels in normal testicular tissue.

In this study, we investigated the expression of KLK5 in testicular cancer and in matched normal testicular tissue. KLK5 seemed to be a reasonable candidate for this investigation because the testis is among the few organs in which KLK5 is expressed in high amounts\textsuperscript{8} and because several other members of the kallikrein gene family appear to be associated with cancer progression.\textsuperscript{21,22} Most notable is the kallikrein gene 10 (NES1), a putative tumor suppressor gene,\textsuperscript{23} which was also found to be downregulated in testicular cancer.\textsuperscript{24}

On the basis of our results, it appears that KLK5 expression is significantly downregulated in cancerous testicular tissue compared with matched normal testicular tissue. KLK5 expression was also significantly lower in seminomas, the more radiotherapy-responsive form of this cancer, and in more invasive tumors. These data seem to indicate that higher KLK5 expression is associated with less aggressive forms of testicular cancer. In two tissue pairs (No. 6 and 8, Fig. 2), KLK5 was downregulated to almost undetectable levels in the cancerous tissues compared with their normal counterparts. In a previous study, we found that KLK5 was expressed in higher amounts in ovarian cancer relative to normal ovarian tissue.\textsuperscript{10} This finding suggests that KLK5 expression may be influenced by different factors in ovarian versus testicular cancer. It should be noted that a similar opposing trend of gene expression in ovarian versus testicular cancer was also found for NES1 (KLK10).\textsuperscript{24}

Although this study was not designed to establish a cause-and-effect relationship between a lack

\begin{table}[h]
\centering
\caption{KLK5 expression in subgroups of patients with testicular cancer}
\begin{tabular}{|l|c|c|c|c|}
\hline
 & \textbf{Patients (n)} & \textbf{Mean} & \textbf{Standard Error} & \textbf{Median} & \textbf{P Value} \\
\hline
\textbf{Histotype} & & & & & \\
Seminoma & 8 & 0.36 & 0.16 & 0.20 & 0.009 \\
Nonseminoma & 6 & 10.0 & 6.3 & 3.8 & \\
\hline
\textbf{Tumor size} & & & & & \\
T1 & 10 & 6.3 & 3.9 & 1.1 & 0.008 \\
T2–T3 & 4 & 0.082 & 0.047 & 0.055 & \\
\hline
\textbf{Nodal status} & & & & & \\
Negative & 6 & 8.9 & 6.5 & 0.54 & 0.49 \\
Positive & 8 & 1.2 & 0.64 & 0.44 & \\
\hline
\textbf{Stage} & & & & & \\
I & 7 & 8.6 & 5.5 & 2.2 & 0.026 \\
II–III & 7 & 0.35 & 0.17 & 0.20 & \\
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of tumor progression and expression of KLK5, we speculate that KLK5 may negatively regulate cell growth. The putative tumor suppressor NES1, a close relative of KLK5, demonstrated a similar pattern of expression in testicular cancer. As yet, we do not have the data to establish whether lower KLK5 expression in cancerous testicular tissues is reflected at the protein level because of the unavailability of hK5 antibodies. Because mRNA expression levels do not always correlate with protein levels, more extensive studies are needed to evaluate the behavior of KLK5 at the protein level in normal versus testicular tissues.

CONCLUSIONS

The results of this study have demonstrated, for the first time, that KLK5 expression is downregulated in testicular tumors compared with matched normal testicular tissue. The expression of KLK5 message in later stage tumors, more invasive tumors, and seminomas is lower. It would be interesting to evaluate whether these altered mRNA levels are also reflected at the protein level in tissues and serum.

REFERENCES