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Identification and Molecular Characterization of Five Novel Kallikrein Gene 13 (KLK13; KLK-L4) Splice Variants: Differential Expression in the Human Testis and Testicular Cancer

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Abstract. *The kallikrein gene family is comprised of genes that have either established or potential applications in prostate and breast cancer diagnostics. New members of the human kallikrein gene family have been recently identified. By using the positional candidate gene approach, we were able to clone a novel human serine protease gene that maps to chromosome 19q13.3-q13.4, the location of the kallikrein gene family. We named this gene KLK-L4 (now also known as KLK13). Here, we describe the identification of five new KLK-L4 splice variants which are not expressed in any other tissue except the human testis. We have further established that these splice variants can be detected in normal testis but not in the adjacent matched testicular tumors. In addition, differential expression of the KLK-L4 gene was found in various histological types of testicular cancer. Our results suggest that the KLK-L4 gene is expressed in normal and cancerous testicular tissue; however, its five variants are all expressed in normal tissue but not in testicular tumors. The physiological relevance of these variants and the implications of their differential expression between cancerous and normal tissues are currently unknown.*

The kallikreins are a subgroup of the serine protease enzyme family. Serine proteases play important roles in many physiological processes including apoptosis, cell migration, tissue remodelling, coagulation and fibrinolysis. The biological role of kallikreins is thought to be the selective cleavage of high molecular weight substrates (kininogens) and release of peptides (kinins) with potent biological activity. The prostate specific antigen gene (KLK3, encoding for prostate specific antigen, PSA), is a member of this human tissue kallikrein gene family (1). PSA is an important biomarker for prostate cancer diagnosis and monitoring (2). Recently, new serine proteases with a high degree of homology to the three classical kallikrein genes were cloned (3-5). The clinical use of PSA in prostate cancer suggests that other related molecules could be used as markers for breast, ovarian and other cancers. For example, the zyme/protease M/neurosin gene (KLK6) is expressed in primary breast cancers but is down-regulated at metastatic sites (6). The normal epithelial cell-specific 1 gene (NES1, KLK10) seems to be a tumor suppressor and is down-regulated during breast cancer progression (7). Thus, further investigation of kallikreins and kallikrein-like genes is warranted to assess their diagnostic, prognostic and possibly therapeutic applications in various forms of cancer.

Recently, we studied a relatively large genomic area around chromosome 19q13.3-q13.4 and identified or mapped a number of novel kallikrein-like genes (1, 3-8). We have previously cloned and characterized a kallikrein-like gene named KLK-L4 (now also known as KLK13) (8). Our preliminary results indicated that KLK-L4 is differentially expressed in breast cancer tissues and breast cancer cell lines, and that its expression is regulated by steroid hormones in the breast cancer cell line BT-474 (8). We therefore postulated that KLK-L4 may be involved in the pathogenesis and/or progression of breast cancer and may represent a novel cancer biomarker. We have also noted the presence of a short as well as a longer mRNA form (the latter is referred to onwards as the classic form). The classic form is present in various tissues, but is predominantly expressed in prostate, breast, testis and salivary gland (8). We also identified a variant mRNA which was longer than the classic form, and was expressed exclusively in the testis. Thus, we hypothesized that several tissue-specific splice variants of this gene may exist. In this study, we describe the characterization of several novel KLK-L4 splice variants and examine their expression in various human tissues, and especially in testicular tumors and adjacent non-malignant tissues.

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Materials and Methods

Identification of ESTs related to KLK-L4 gene. The cDNA sequence of the KLK-L4 gene (8) was subjected to homology search using the BLASTN (9) algorithm at the National Center for Biotechnology Information Web Server (http://www.ncbi.nlm.nih.gov/BLAST) against the human EST database (dbEST). Clones with >95% homology were obtained from the I.M.A.G.E. consortium (10) through Research Genetics Inc., (Huntsville, AL, USA). The clones were propagated, purified and sequenced from both directions using insert-flanking vector primers. Based on the combined information obtained from the KLK-L4 genomic sequence (Genbank accession #AF135024) and the ESTs, two gene-specific forward primers were designed, L4-D and L4-E (Table I and Figure 1). The reverse primer L4-R1 binds to exon 5 (Table I and Figure 1). In addition, a second reverse primer, named L4-R2, was designed to bind to exon 2 of the KLK-L4 classic form (Table I and Figure 1). Screening of testicular cDNA with L4-X1 (a forward primer binding to exon 1; see Figure 1 and Table I) and L4-R2 was then performed. All selected primers were designed to amplify specific alternatively spliced forms, as further exemplified below.

Reverse transcriptase-polymerase chain reaction. Total RNA isolated from 26 different human tissues was purchased from Clontech, (Palo Alto, CA, USA). Two mg of total RNA was reversed-transcribed into first strand cDNA using the Superscript preamplification system (Gibco BRL, Gaithersburg, MD, USA). The final volume was 20 ml. Using the three gene-specific forward primers with reverse primer L4-R1, the tissue panel was screened by PCR. PCR was carried out in a reaction mixture containing 1 ml cDNA, 2.5 mM $MgCl_2$, 25mM PCR buffer, 200 mM dNTPs (deoxynucleoside triphosphates), 150 ng primers and 1 unit of HotStar Taq polymerase (Qiagen, Valencia, CA, USA) on a Eppendorf Mastercycler gradient system (Eppendorf, Westbury, NY, USA). The cycling conditions were 95°C for 15 minutes to activate the HotStar Taq polymerase, followed by 35 cycles of 94°C for 30 seconds, 63°C for 1 minute and a final extension at 72°C for 10 minutes. The amplified splice variants were separated using 1.5% agarose gels. The isolated bands were then purified using the Qiagen gel purification kit. The PCR products were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) in order to verify their identities. Inserts were sequenced from both directions using vector-specific primers with an automated DNA sequencer.

Screening for the KLK-L4 splice variants in testicular cancer tissue. Included in this study were tissue samples from patients who had undergone radical orchiectomy for testicular cancer at the Charite University Hospital, Germany and the National University Hospital, Denmark. The patient age ranged from 23-60 years with a median of 36. Matched testicular tissue samples were obtained from the tumors and adjacent morphologically normal parts of the same testis. All patients had a histologically-confirmed diagnosis of primary testicular cancer and had received no treatment before surgery. Of the ten matched samples, five tumors were seminomas, three were categorized as embryonal carcinomas and two were teratocarcinomas. Other tissue samples included one Leydig cell tumor and two samples of testicular tissue containing tubules with pre-invasive carcinoma-*in situ* (CIS) within morphologically normal parenchyma. In addition, the expression of KLK-L4 and its splice variants was examined in two embryonal cell lines derived from a human teratocarcinoma; one pluripotent (NTERA-2) and one nullipotent (2102Ep).

Tissue specimens were minced with a scalpel, on ice and immediately transferred into a 2ml polypropylene tube. Tissue samples were then homogenized, and RNA extracted using the RNeasy Mini Spin column method from Qiagen, according to the manufacturer's recommendations. The RNA concentration was determined spectrophotometrically and 2 mg of total RNA was reverse-transcribed into first strand cDNA as described above.

Table I. *Primers used for the RT-PCR analysis and identification of the novel mRNA variants.*

Gene	Primer name	Sequence[1]
KLK-L4	L4-X1	CTAGTGATCGCCTCCCTGAC
	L4-R1	TTATTGTGGGCCCTTCAACC
	L4-R2	CCCTTGCACTAGTAGGCCAG
	L4-D	AAGACTTCAAGGAGCCAAGC
	L4-E	GACCCTTCACCTCCCAAAAT
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT
	ACTINAS	TCTCCTTAATGTCACGCACGA

1. All primer sequences are in the 5'→3' direction.

Results

Identification of the KLK-L4 splice variants. We cloned a novel kallikrein-like gene, KLK-L4, as previously described (8). Two EST clones with >97% homology with this new gene were identified (Genbank accessions #AA846771 and AI002101); both of them were cloned from the testis. These EST clones were obtained and inserts were sequenced from both directions. Sequences were then compared with the known genomic sequence of the region (see our Genbank accession #AF135024) and selection of the intron/exon splice sites were made according to EST sequences. Intron/exon splice sites conformed to the consensus sequences (AG...GT).

In our previous study (8), we had obtained indications that there might be various splice variants of the KLK-L4 gene, since: 1) multiple PCR bands were observed with primers L4-X1 and L4-R1, which amplify the whole coding sequence of KLK-L4 and 2) we have identified two ESTs with some exons which were different from those of our previously reported gene (8). Preliminary information revealed that at least part of this alternative splicing occurred between exons 1 and 2 of the classic KLK-L4 form.

We therefore designed primers L4-D and L4-E, binding to this region (please refer to Figure 1 for areas of primer binding). Screening of cDNAs from 26 different human tissues by RT-PCR, using gene-specific primers L4-D or L4-E (forward) and L4-R1 (reverse), was then performed. Only testis cDNA produced several PCR bands (data not shown). Sequencing of these bands indicated the presence of four mRNA variants: two of these were characterized from L4-D/L4-R1 screening and two from L4-E/L4-R1 screening (Figure 1). In comparison to the classic KLK-L4 form, the L4-D and L4-E variants contain additional exons that we have tentatively named exon D and E, respectively, that are not present in any of the previously reported splice variants (8). We also noted (Figure 1) that exon 3 of the classic form was spliced differently in these L4-D and L4-E splice variants. Exon 3 for each of the splice variants was either shorter or

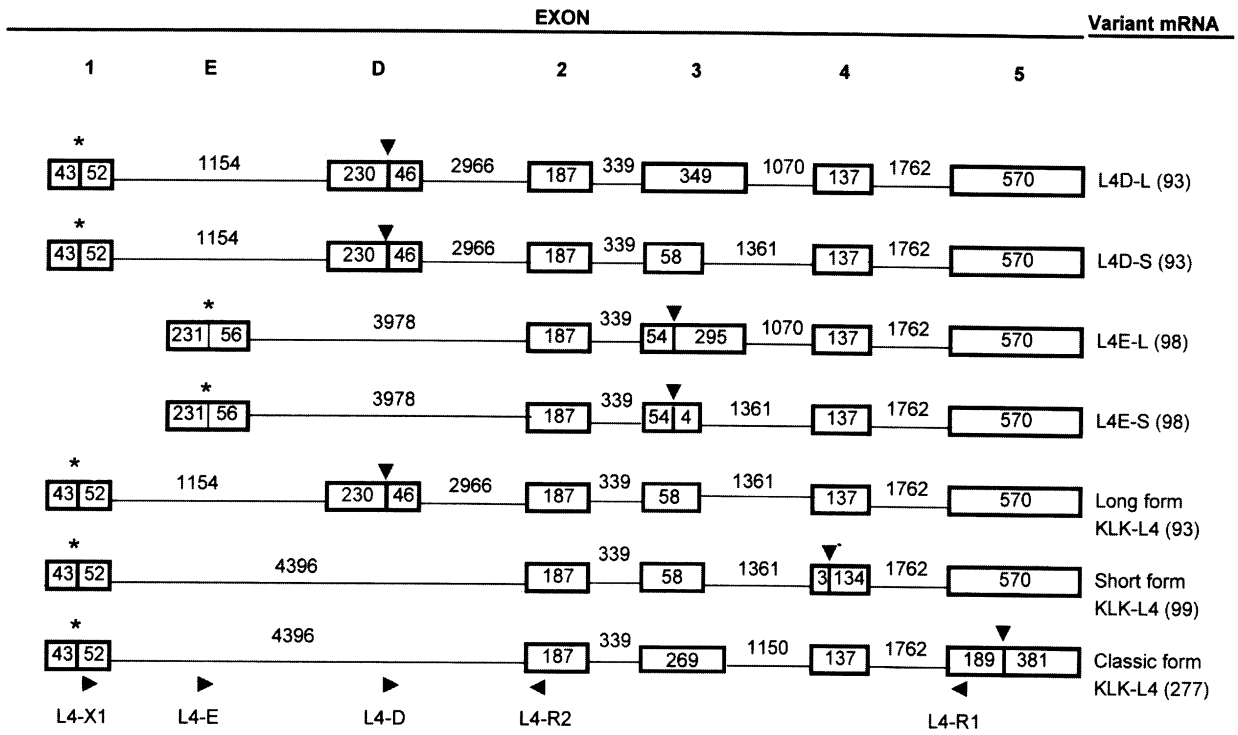

Figure 1. *Comparative genomic structure of the KLK-L4 variants L4D-L, L4D-S, L4E-L, L4E-S, KLK-L4 long and short forms and of the classic KLK-L4 gene. Exons are represented by solid bars and introns by the connecting lines. Numbers in the boxes and above the lines represent the sizes of exons and introns, respectively, in base pairs. The arrowheads represent predicted stop codons and the asterisks start codons. Numbers in parenthesis indicate length of predicted polypeptides in amino acids. Primers and their directions are illustrated on the bottom. For details see text.*

longer than exon 3 of the KLK-L4 classic form. However, exons 2, 4 and 5 of the classic KLK-L4 gene, are identical in all splice variants, as shown in Figure 1. We decided to name the four new variants L4D-S, L4D-L, L4E-S, L4E-L, for L4D short, L4D long, L4E short and L4E long, respectively. To verify that these splice variants, identified by RT-PCR, can also be defined precisely on the genomic sequence of KLK-L4, the BLASTN algorithm (9) was used. Exon identification was further confirmed based on the consensus exon-intron splice sites.

In order to precisely characterize the long KLK-L4 variant which was identified previously (8), we designed a reverse primer binding on exon 2 of the classic form (L4-R2) (Figure 1). Screening of the testis cDNA with L4-X1 and L4-R2 primers revealed the presence of exon D, but not exon E, in this variant. In addition, exon 3 of this variant was shorter than that of the KLK-L4 classic form (Fig. 1). Thus, the structure of the testicular long form, which was observed previously (8), has now been precisely defined. We have previously observed three KLK-L4 variants (8): the long variant (described above), the classic form, and the short variant, which is spliced earlier in exon 3 and has a putative stop codon at exon 4 (see Figure 1). The short variant does not contain exons D or E.

All seven mRNA forms transcribed from the KLK-L4 gene (four novel forms described here and the three previously described forms) are shown diagrammatically in Figure 1.

Screening for KLK-L4 variants in testicular cancer tissues. Total RNA from matched tumor and adjacent morphologically normal tissue samples from patients with various testicular cancers was screened by RT-PCR for the newly-characterized variant mRNAs . The samples were first screened with L4-X1 and L4-R1, then with L4-D and L4-R1, and finally with L4-E and L4-R1 primers. Primers L4-X1 and L4-R1 amplified the short, classic and long forms of KLK-L4 as well as L4D-L and L4D-S (Figure 2), while the L4-D and L4-E primers, in combination with L4-R1, detected specifically the L4D, long form of KLK-L4 and L4E splice variants (Figure 3), respectively. These data are summarized in Table II and representative RT-PCR gels are presented in Figures 2 and 3. In general, the variant mRNAs are detectable in a fraction of testicular normal tissues but not in their adjacent cancerous counterparts.

We then examined expression of KLK-L4 and its variants in a sample of morphologically normal testicular parenchyma (adjacent to overt tumors), two with carcinoma *in situ* tubules, one without carcinoma *in situ* (CIS), one Leydig-cell tumor as

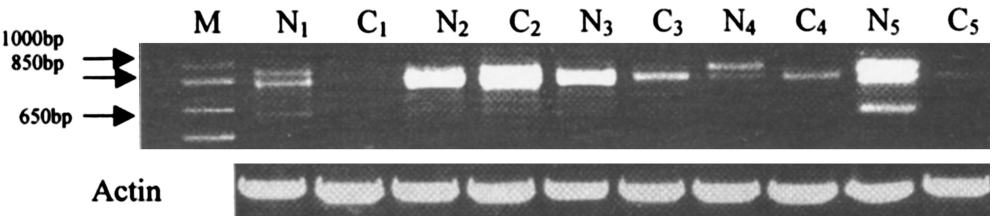

Figure 2. *Expression of the KLK-L4 variants KLK-L4 long (upper band; clearly shown in lanes N_1, N_4 and N_5), KLK-L4 classic form (most intense band; clearly shown in lane N_3) and KLK-L4 short form (lower band, clearly shown in lane N_5), in matched, paired cancerous and non-cancerous testicular tissues, using primers L4-X1 and L4-R1 (primer location is shown in Figure 1). N= normal tissue, C=cancerous tissue, M= marker (1 kb molecular weight ladder), -ve= negative control. Numbers shown on the left side correspond to the marker size. Expected band sizes are: 877bp for the KLK-L4 long variant, 819bp for the KLK-L4 classic form, and 608bp for the KLK-L4 short variant. Figure depicts a sample of 5 out of the 10 tissue-pairs tested. For discussion and intrepretation see text.*

well as in two human embryonal carcinoma cell lines. We found that only the normal parenchyma expressed the long KLK-L4 variant (Figure 4) and the L4D-S, L4D-L, L4E-S, L4E-L variants (data not shown). However, these variants were not present in any of the tumors.

Discussion

Although testicular cancer accounts for only 1% of all tumors in males, it is the most common malignancy between 15 to 34 years of age (11). The incidence of testicular cancer in the United States has almost doubled since the 1930s and continues to climb, while more effective treatments have led to a decline in mortality (11). Early diagnosis of testicular cancer is important because the doubling time of testicular tumors is relatively short (10 to 30 days) (11). Current testicular tumor markers include human chorionic gonadotropin (hCG), alpha-fetoprotein (AFP), lactate dehydrogenase (LDH), and placental alkaline phosphatase (PAP) (12); however, sensitivity and specificity of these markers are not high. For example, AFP is not specific for testicular neoplasms, and its high serum concentration has been documented in lung, gastric, pancreatic and hepatocellular cancers (12). AFP and hCG are currently the most widely applied markers for testicular tumor staging, despite their relatively low specificity and sensitivity (13); however, they are only useful in about half of the germ cell tumor cases, because seminomas usually do not secrete AFP or hCG. Hence, new tumor markers could be valuable tools for both diagnosing and staging of various types of testicular cancer. As KLK-L4 and its newly-identified splice variants appear to be preferentially expressed in the testis, we investigated their expression in a series of testicular tumors.

Table II: *Detection and relative abundance of KLK-L4 mRNA variant forms in ten pairs of normal and cancerous testicular tissues.*

	Relative mRNA Abundance				
Splice mRNA variant [1]	Detected in normal tissue [2]	Detected in cancer	Approx Equal	Higher in normal	Higher in cancer
Classic form	9	9	5	4	1
Long form	3	0	-	3	-
L4D-S	4	0	-	4	-
L4D-L	4	0	-	4	-
L4E-S	4	0	-	4	-
L4E-L	4	0	-	4	-
Short form	2	0	-	2	-

1. For splice variant definition and structure, please see text and Figure 1.
2. We used 10 pairs of normal/cancerous tissues from patients with testicular cancer. Please refer to *Material and Methods* for more details.

Our previously identified kallikrein-like gene, the KLK-L4 classic form (official nomenclature, KLK13), was found to be differentially expressed in five out of ten matched (normal/tumor) testicular tissues (Table I). Using expressed sequence tag (EST) analysis, sequencing and PCR, we have characterized several novel testis-specific KLK-L4 mRNA variants, tentatively named L4D-S, L4D-L, L4E-S, L4E-L and KLK-L4 long form, and noted the frequent presence of these

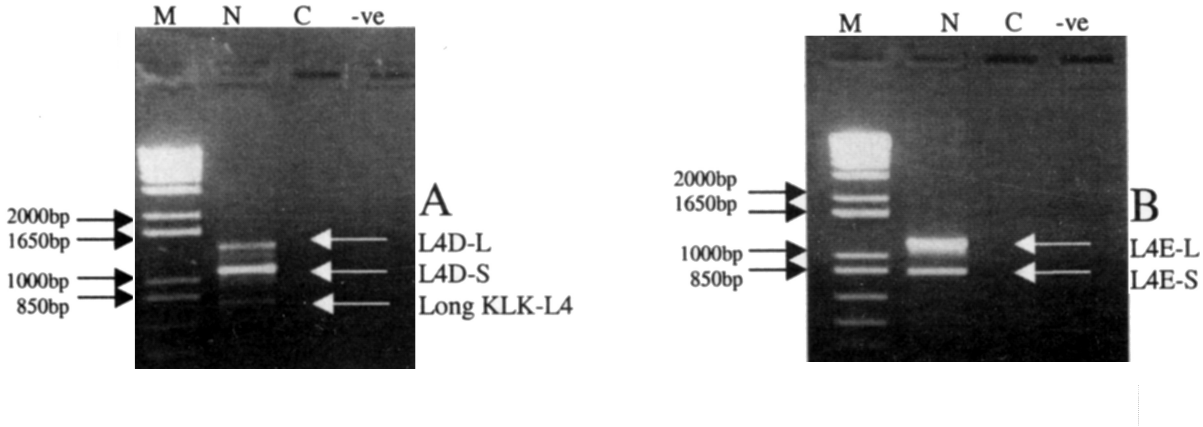

Figure 3. *Expression of the KLK-L4 mRNA variants, L4D-S, L4D-L, and long KLK-L4 form (panel A); L4E-S, L4E-L (panel B), in matched, paired cancerous and non-cancerous testicular tissues using (A) primers L4-D and L4-R1 and (B) primers L4-E and L4-R1. The PCR products of L4D-S and long KLK-L4 forms are identical. Depicted in panels A and B is an example of a matched tissue pair illustrating isoforms L4D-S, L4D-L, L4E-S, L4E-L. N= normal tissue, C= cancerous tissue. M= marker (1 kb molecular weight ladder), -ve= negative control. Numbers shown on the left side correspond to the marker size. Expected band size for L4D-S is 837bp (the lower band, panel A); L4D-L is 1136bp (the middle band, panel A). We occasionally observed a PCR band that is larger than L4D-L which could not be identified (top band, panel A). Expected band size for L4E-L is 1080bp (upper band, panel B) and L4E-S is 781 bp (lower band, panel B).*

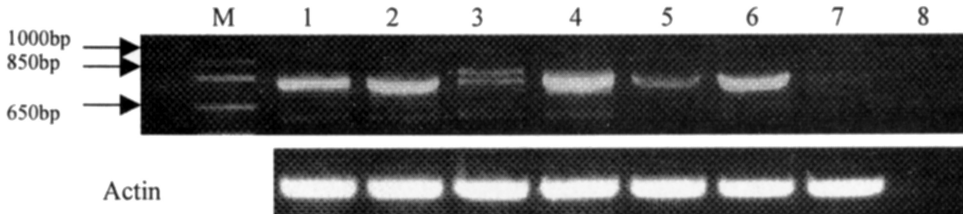

Figure 4. *Expression of the KLK-L4 variants KLK-L4 long, classic and short form, in testicular tumors and embryonal carcinoma-like (EC-like) cell lines. Primers used were L4-X1 and L4-R1 (primer location is shown in Figure 1). For further details see Figure 2. 1. Testicular carcinoma in situ; 2. Leydig-cell tumor; 3. Normal parenchyma; 4. Seminoma; 5. Non-seminoma; 6. Pluripotent EC-like cell line NTERA-2; 7. Nullipotent EC-like cell line 2102Ep; 8. Negative control. Numbers shown on the left side correspond to the marker size. Expected band sizes are mentioned in Figure 2. For discussion see text.*

splice variants in normal tissues but not in the adjacent tumors (Table I and Figures 2 & 3). The lack of expression of the KLK-L4 splice variants in testicular germ cell tumors provides information concerning the biology of these tumors. It is now commonly accepted that testicular germ cell tumors originate from a common pre-invasive precursor, the CIS cell (14), and that CIS in turn, is derived from gonocytes transformed early in life (15). It is not yet known whether or not CIS cells express the KLK-L4 splice variants, but it is unlikely, because seminoma has nearly identical phenotypic features, including expression of the number of markers. Further studies will be necessary to confirm whether the mRNA variant absence is related to tumor progression or aggressiveness.

By direct sequencing and alignment strategies, we were able to define precisely the exon composition for each of the putative splice variants, further confirmed by the presence of AG...GT splice sites that are well-conserved among vertebrates (16). It is clear that the variants arise from alternative splicing of exon 3 at three different locations, as well as from two additional exons, D and E (Figure 1). We did not investigate experimentally if the variant mRNAs are translated but open reading frame analysis indicated that if the mRNAs are translated, they will lead to production of truncated proteins of various lengths, as shown in Figure 1. None of the variant mRNAs (with the exception of the classic KLK-L4 form) encodes for a serine protease with a conserved catalytic triad (1); however, they are predicted to be secreted

proteins due to conservation of their hydrophobic signal peptide sequences (8).

Other kallikreins have been also noted to have splice variants of unknown physiological relevance (18-21). For example, Henttu *et al.* noted that the amount of aberrant PSA mRNAs which would produce variant proteins (if translated) was about 18% to 38% (21). These mRNA variants were noted to have extended 3' untranslated regions (UTR), which are speculated to be essential for mRNA stability or targeting of mRNA into cells (17). Differences in the transcriptional regulation of the PSA gene between benign prostatic hyperplasia and prostate carcinomas, resulting in a 3-fold variation in PSA amount in carcinoma tissues (vs. normal or benign hyperplasia) have been reported (22). A mRNA variant of PSA, which lacked the critical serine residue necessary for catalytic activity, was noted to produce a protein that would cross-react with anti-PSA antibodies used in clinical assays (19). Another PSA mRNA variant was reported to be expressed predominantly in the prostate tissue, but not in blood (20). Such a mRNA splice variant, if translated, would also produce a variant protein that may offer enhanced clinical utility (20). Recently, a highly sensitive splice variant-specific RT-PCR assay for human glandular kallikrein 2 (hK2) was reported, which was able to detect metastatic disease in men with clinically localized prostate cancer. Thus, further examination of our own reported splice variants is warranted to determine their potential diagnostic and therapeutic applicability.

In summary, we have cloned and characterized five new KLK-L4 mRNA variants, named L4D-S, L4D-L, L4E-S, L4E-L and KLK-L4 long form. Unlike their two other counterparts (the KLK-L4 classic and short form), our new variants do not seem to be present in any other tissue except the testis. Our preliminary results suggest that these variants are not expressed in testicular tumors. More studies will be necessary to elucidate the biological role of these mRNA variants in the testis.

Acknowledgements

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