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Cloning and characterization of novel isoforms of the human kallikrein 6 gene☆,☆☆,★

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Abstract

Human kallikrein 6 (protease M/zyme/neurosin) was originally identified based on its aberrant expression in tumor cells and is considered a biomarker for ovarian cancer. Here, we describe the identification, cloning, and tissue expression of three novel transcript variants of the *KLK6* gene that encode for wild-type kallikrein 6. Contrary to the classical form, transcript variants contain one untranslated exon, exploit intronic sequences, and are likely products of alternative promoters. In addition, we cloned splice variants 2 and 3 produced by splicing out exons 3 and 4, respectively. Given the potential diagnostic applications of kallikrein 6 at both the mRNA and protein levels, we developed a duplex RT-PCR, in order to differentially detect and quantitate mRNA species corresponding to splice variants. We show that in normal mammary epithelial cells and mammary tumor cell lines that overexpress the *KLK6* gene, splice variants account for approximately 10–20% of all mRNA species.

Keywords: Kallikrein 6; Protease M; Zyme; Neurosin; Transcript variant; Splice variant; Differential detection

Protease M was originally identified by differential display as being inactivated in metastatic breast and ovarian tumor cells, while overexpressed in corresponding primary tumors [1]. The same gene was later cloned from an adenocarcinoma cell line and was named neurosin [2], and from brains of Alzheimer's disease patients and was named zyme [3]. The gene encoding protease M (*KLK6*) was mapped on human chromosome 19q13.3-13.4 [1,4], a locus where all 15 kallikrein genes are tandemly clustered (reviewed in [5]). According to the new official nomenclature for kallikrein genes, protease M/zyme/neurosin was named human kallikrein 6 (hK6).

Based on its aberrant expression in tumor cells [1] and recent studies [6–9], hK6 is a potential circulating biomarker for the diagnosis and monitoring of ovarian cancer. However, the physiological function of hK6 remains unknown. Biochemical and structural studies have shown that hK6 is an active serine protease [10,11] that may play a physiological role in the central nervous system (CNS), since it cleaves and activates the amyloid precursor protein (APP) implicated in the pathogenesis of Alzheimer's disease [3] and may be exploited as a biomarker of this condition [12]. In addition, hK6 cleaves myelin, a protein implicated in multiple sclerosis and demyelinating diseases [10]. Interestingly, hK6 seems to play a significant role in physiological degradation of

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☆☆ The identified nucleotide sequences for *KLK6* variants were deposited in GenBank with the following Accession Nos. AY318867, transcript variant 1; AY318869, transcript variant 2; AY318870, splice variant 2; and AY318868, splice variant 3.

★ *Abbreviations: KLK*, human kallikrein gene; *KLK6*, gene encoding human kallikrein 6; hK6, human kallikrein 6 protein; PSA, prostate-specific antigen; HMECs, human mammary epithelial cells; RACE, rapid amplification of cDNA ends; RLM-RACE, RNA ligase-mediated RACE; TSS, transcriptional start site; UTR, untranslated region; ESTs, expressed sequence tags; cDNA, complementary DNA; bp, base pair(s).

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α-synuclein and may also be involved in the pathogenesis of Parkinson's disease and other synucleinopathies [13]. More recently, it was reported that hK6 participates in enzymatic cascades mediating CNS inflammatory disease and may represent a novel therapeutic target for the treatment of progressive inflammatory disorders [14].

While only the classical kallikrein genes *KLK1*, *KLK2*, and *KLK3*/*PSA* were studied in detail, several novel kallikrein genes encode for multiple isoforms [5]. Both *KLK3*/*PSA* and *KLK2* genes produce isoforms resulting from inclusion of intronic sequences located immediately after the first exon [15]. These unusual alternative splicing products were designated PSA-LM and K-LM and yield completely different proteins [15]. PSA isoforms produced by alternatively splicing of coding exons were also described [16–18]. The PSA-RPI isoform differs only in the C-terminal domain, including the active site Ser, and is produced by usage of an alternative acceptor site located within intron 4 [17]. This isoform contains PSA-like antigenic domains/epitopes and, thus, it could crossreact with PSA-specific antibodies used in established diagnostic immunoassays. Moreover, it was suggested that the free immunoreactive fraction of PSA in blood results from PSA-RPI [17]. An additional *KLK3*/*PSA* isoform with a deletion of 129 nucleotides in exon 3 was characterized and this protein may also crossreact with PSA-specific antibodies [18]. Finally, a distinct transcript variant of *KLK3*/*PSA* with longer 3′ UTR is produced by usage of an alternative polyadenylation signal [19]. Similarly, *KLK2* gene that is closely homologous to *KLK3*/*PSA* encodes for multiple isoforms, including transcript variants differing in the length of the 3′ UTR [19] that encode for truncated hK2 isoforms [20].

Given the diagnostic applications of *KLK3*/*PSA*, *KLK2*, and putatively other *KLK* genes and encoded proteins as disease biomarkers, the identification of novel *KLK* isoforms that may crossreact with specific antibodies or have aberrant expression patterns between normal and tumor cells should be of great interest. In this report, novel transcript and splice variants of the *KLK6* gene were identified and characterized. Transcript variants comprise a single untranslated exon in the 5′ UTR and encode for an intact/full-length hK6 protein. In contrast, two splice variants are produced by splicing out coding exons 1 and 2, respectively, and yield truncated proteins. Finally, we developed a duplex RT-PCR assay, in order to determine whether the high mRNA levels detected in certain breast tumor cells [1] account for transcripts encoding for full-length hK6 or mainly represent splice variants encoding for truncated proteins. It was shown that in both normal and tumor cell lines overexpressing the *KLK6* gene, splice variants account for approximately 10–20% of all *KLK6* mRNA species. These results should contribute to the development of improved molecular diagnostic assays.

Materials and methods

Materials. Total RNAs from normal human tissues were purchased from Clontech, restriction enzymes were from Invitrogen, [α-^{32}P]dCTP was from Amersham, and synthetic oligonucleotides were from ACGT or MWG-BIOTECH. All chemicals were of analytical grade.

Cell lines. The 76N normal mammary myoepithelial cell strain and the 21PT primary breast tumor cell line were established as described [1]. The MDA-MB-468 metastatic breast tumor cell line was obtained from the American Type Culture Collection.

RT-PCR. Total RNA was isolated using RNeasy (Qiagen). Total RNA (2 μg) was reverse-transcribed into first-strand cDNA in a 20 μl reaction using the Superscript preamplification system (Invitrogen). One microliter of cDNA template was amplified in a 50 μl reaction containing 2.5 pmol of each primer, 200 μM dNTPs, and 5 U HotStarTaq polymerase (Qiagen). Cycling conditions were as follows: 95 °C for 15 min, to activate HotStarTaq polymerase, followed by 35 cycles of 95 ° for 1 min, 62 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Equal volumes of PCR mixtures were resolved on 1.5% agarose gels and visualized by ethidium bromide staining.

5′ RACE. 5′ RACE was performed on total RNA (700 ng) isolated from 21PT cell line using 5′/3′ RACE (Roche). RT-R primer was used for reverse transcription, RACE1 gene-specific primer for PCR amplification, and RACE2 for nested PCR. Primers were designed to flank 6, 4, and 2 introns, respectively, to avoid amplification of products due to residual contamination of genomic DNA. Primer sequences and their annealing positions are given in Table 1. PCR products were resolved on a 1.5% agarose gel, transferred to Zetaprobe nylon membrane (Bio-Rad), and hybridized to a ^{32}P-radiolabeled probe of 956 bp corresponding to the 5′ upstream sequence of *KLK6* gene. Signals were visualized by autoradiography. Bands of interest were extracted and cloned into pCR4-TOPO vector (Invitrogen). Multiple individual colonies were selected by hybridization, plasmid DNA was isolated using Plasmid Isolation kit (Qiagen) and sequenced. Sequencing was performed by ACGT or MWG-BIOTECH .

RLM-RACE. RLM-RACE was performed on 5 μg of total RNA template from normal human mammary and testis tissues using GeneRacer (Invitrogen). Primers RLM1 and RACE1 were designed to flank 4 introns. Subsequently, nested PCR was performed on 1 μl of the resulting PCR products. Amplification conditions were as described above. PCR products were cloned, selected by hybridization, and sequenced.

Duplex RT-PCR. For resolution and quantification of *KLK6* splice variants, a duplex PCR was performed using OneStep RT-PCR (Qiagen) and primers E3 or E4 (forward) and RT-R (reverse) (Table 1). Reverse transcription was carried out at 50 °C for 30 min. Amplification conditions were as follows: 95 °C for 15 min, for inactivation of reverse transcriptases and heat-activation of HotStarTaq polymerase, followed by 35 cycles of 94 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Equal volumes of RT-PCR mixtures were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. Quantification of products was performed using KODAK EDAS 120 software.

Results

Identification of KLK6 transcript variants

In order to identify the transcriptional start site of the *KLK6* gene, 5′ RACE-PCR was performed using total RNA isolated from the *KLK6* overexpressing 21PT primary breast tumor cell line. The longest known *KLK6* transcript (classical transcript) was isolated pre-

Table 1
Primers used in this study

Primer	Sequence[a]	Binding position[b]
For 5′-RACE, RLM-RACE		
RACE1	CAC AGC CCG GAC AAC AGA ACT CTG	524–500
RLM1	GAT GTC CTG GTC ATG GCT GGC GGC	566–543
RACE2	CAT CAG CTT CTT CAT GGC CGC	260–240
For P1 product		
P1	ACG CTG TAG CTG TCT CCC CGG CTG G	97–121
For P2 product		
P2	CTG CAG CCC TGG GCT CTG CGG CCC CTG CG	965–993[c]
For isoform lacking exon 4		
E4	GTG CTG AGT CTG ATT GCT GCA GGA ATC T	262–446 (spans exon 3 and exon 5 junction)
For isoform lacking exon 3		
E3	CCC GGA GGC CTG CAG CAG CCT GGG	220–289 (spans exon 2 and exon 4 junction)
For KLK6 classical transcript		
RT-F	GGA GGA ATT CAG CAG GAG CGG CCA TG	223–248
RT-R	TGT CTC GAG TCA GGG TGA CTT GGC CTG	995–969

[a] All nucleotide sequences are given in the 5′ → 3′ orientation.
[b] Binding position relative to the transcriptional start site A^{+1} of *KLK6* gene that yields the classical transcript (GenBank Accession No. U62801).
[c] Relative to genomic sequence (GenBank Accession No. AF149289).

viously from a cDNA library derived from 76N normal mammary myoepithelial cell strain and contained a 5′ UTR of 245 bp with two untranslated exons (GenBank Accession No. U62801) [1]. This transcript would yield a PCR product of 270 bp. However, gene-specific RACE product(s) of approximately 140 bp were obtained. A novel transcript variant of the *KLK6* gene was revealed, which contains one untranslated exon, exploits intronic sequences, and is derived from the transcriptional start site C^{+1068} (please see below for details).

To exclude that partial cDNAs were synthesized during reverse transcription, as it often happens with CG-rich templates, 5′ RLM-RACE was performed on total RNA from testis and mammary normal tissues known to express the *KLK6* gene [4]. The advantage of this technique is that only full-length mRNAs are captured. 5′ RLM-RACE product(s) appeared as a single band that was cloned and sequenced. Nine clones from testis and seven clones from mammary tissue were sequenced. The results are depicted in Fig. 1. The *KLK6* classical transcript was not detected among the clones selected for sequencing. Three new transcriptional start sites were identified that are located within a 20 bp region inside the first intron of the *KLK6* gene. Transcript variant 1 of 1517 bp, derived from TSS1 (A^{+894}), was identified in one of nine clones isolated from testis, while the most abundant transcript variant 2 of 1503 bp was derived from TSS2 (A^{+908}) and was present in eight of nine clones from testis and five of seven clones from mammary tissue. Transcript variant 3 is derived from TSS3 (A^{+910}) and was detected in two of seven clones isolated from mammary tissue. The expression of *KLK6* transcript variants seems to be controlled by an alternative promoter located inside intron 1. The term P1 was used for the promoter yielding the *KLK6* classical transcript [1] and P2 for the intronic promoter, yielding *KLK6* transcript variants 1, 2, and 3, that all encode for full-length hK6 protein, since their sequences diverge only in the 5′ UTR.

Identification of KLK6 splice variants

Among the 5′ RLM-RACE products, two novel mRNA species were identified and named splice variant 2 and splice variant 3. As summarized in Fig. 2A, splice variant 2 (929 bp) lacks exon 3 that contains the translational start site $A^{246}TG$ of the *KLK6* classical transcript [1]. Whether this mRNA is translated to a novel protein is currently unknown. Computational translation of the nucleotide sequence of splice variant 2 revealed an open reading frame initiating at $A^{+567}TG$ which lies within a Kozak consensus and yields a predicted protein of 137 amino acid residues that corresponds to the C-terminal domain of hK6 (M^{108}–K^{244}). Splice variant 3 (820 bp) lacks exon 4 and encodes for a predicted polypeptide of 40 amino acid residues, the first thirteen being identical to the hK6 signal peptide (Fig. 2B). A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) revealed no significant homology of splice variant 3 to any protein sequence in GenBank. Since homology between hK6 and splice

P1

AGGCGGACAA AGCCCGATTG TTCCTGGGCC CTTTCCCCAT CGCGCC<u>TGGG</u>
<u>CCTGCTCCCC AGCCCGGGGC A</u>GGGGCGGGG GCCAGTGTGG TGACACACGC
TGTAGCTGTC TCCCCGGCTG GCTGGCTCGC TCTCTCCTGG GGACACAGAG
GTCGGCAGGC AGCACACAGA GGGACCTACG GGCAG

gtgtg tgagtcaccc
caaccgcact gaacctgggc… intron 1 …ggacctgaga gacaggggtt
aaaaggacgt tcc

P2 **A[1]**gaagca tctgggg**A[2]**c**A[3]** gaaccagcct ctt<u>ccaggga</u>
ggcctgggag ctgggggtgt gtgtctggca gtccctgcag ccctgggctc
tgcggcccct gcgtcctccg cttggctctg ccactgcatc tgagtgtctt
ctctcctcac ggctccccgc atttctaact ctttctgcct cctcgtctca
aag

CTGTTCC TTCCCCCGAC TCAAGAATCC CCGGAGGCCC GGAGGCCTGC
AGCAGgtgag atcacagaca … intron 2…taatggacca tgtggcttcc
cctctcag**GA GCGGCCATGA AGAAGCTGAT GGTGGTGCTG AGTCTGATTG**
CTGCAGGTGG……

Fig. 1. The 5′ upstream sequence of the *KLK6* gene. Sequences of introns 1 and 2 are depicted in lowercase letters and sequences of untranslated exons 1, 2, and part of exon 3 in capital letters. Arrows indicate binding positions of forward primers used for amplification of P1 and P2 transcripts. Nucleotides in bold superscripts were numbered arbitrarily to specify transcriptional start sites identified within intron 1: TSS1, A^{+894}; TSS2, A^{+908}; and TSS3, A^{+910}. The asterisk indicates the most abundant site identified by 5′ RLM-RACE in normal mammary and testis tissues. The site identified by classical 5′ RACE is depicted in boldface-underlined letter.

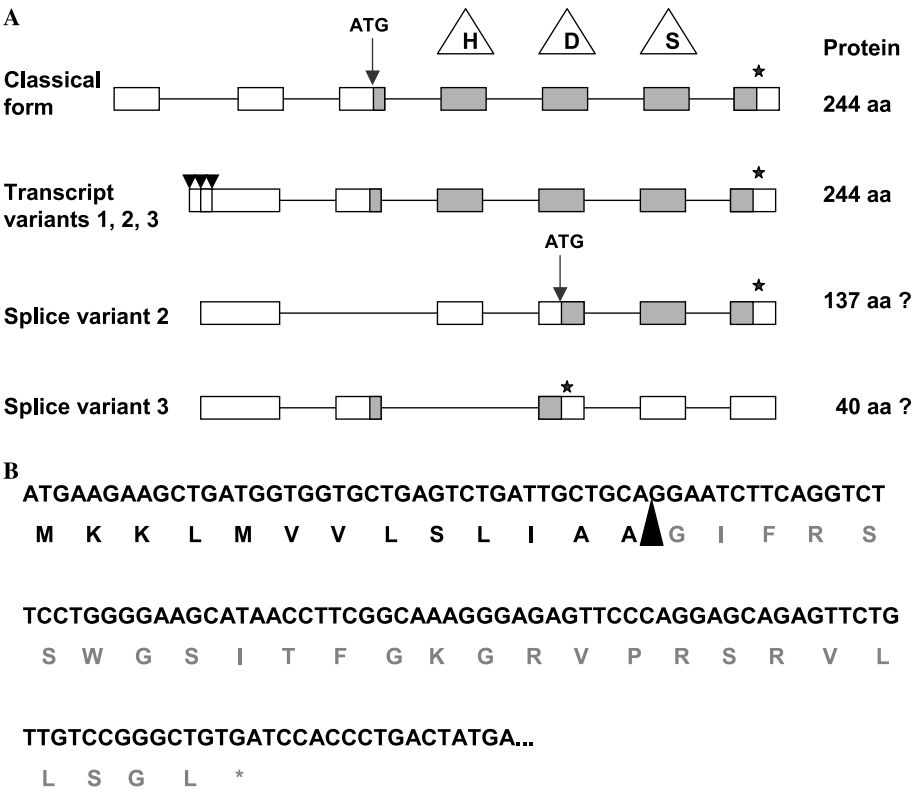

Fig. 2. (A) Schematic representation of isoforms encoded by the *KLK6* gene. The classical transcript contains two untranslated exons (GenBank Accession No. U62801) [1]. Transcript variants 1, 2, and 3 contain one untranslated exon and encode for full-length hK6 protein. Splice variant 3 (E4) lacks exon 4 and yields a novel putative protein of 40 amino acid residues. Splice variant 2 (E3) lacks exon 3, which contains the hK6 start codon (A^{246}TG). Gray boxes represent coding regions, white boxes represent untranslated sequences, and intervening lines represent introns. Stop codons are marked with stars. H, D, and S represent catalytic amino acids. (B) Nucleotide and predicted amino acid sequences encoded by *KLK6* splice variant 3. The arrow indicates the residue where sequences of classical/full-length hK6 and splice variant 3 diverge.

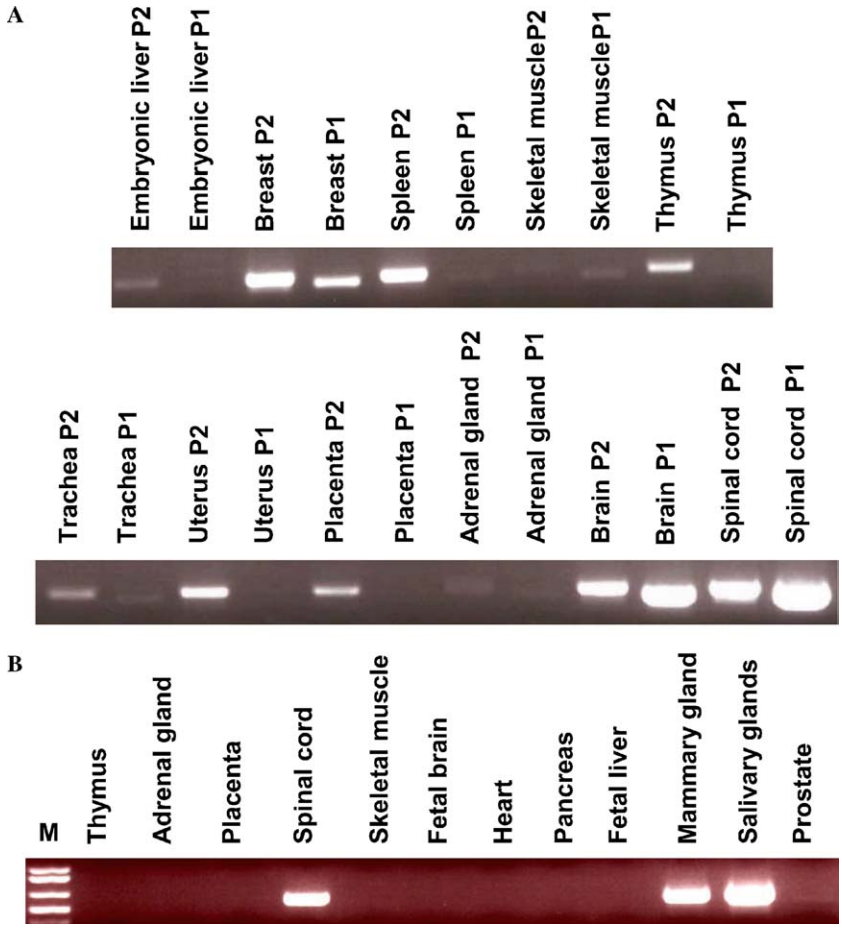

Fig. 3. (A) Tissue specificity of mRNA species derived from P1 and P2. RT-PCR was carried out with forward primer P1 (or P2) and reverse primer RACE1. One hundred nanograms of total RNA from various normal human tissues (Clontech) was used as template. P1-primer specifically amplifies the classical transcript and P2-primer the transcript variants. (B) Tissue-specific expression of splice variant 3. Expression was measured in 12 normal human tissues with primers E4 and RACE1. High expression was observed in mammary and salivary glands and in spinal cord.

variant 3 is restricted to only 13 of 16 amino acids of the hydrophobic signal peptide, we hypothesize that the resulting protein would be unlikely to crossreact with hK6-specific antibodies used in established immunoassays [6–9].

Tissue specificity of KLK6 variants

Human normal tissues were examined by RT-PCR for expression of *KLK6* transcripts. As shown in Fig. 1 and Table 1, two sense primers were designed: P1-specific primer anneals to exon 1, therefore, it can specifically amplify the *KLK6* classical transcript while P2-specific primer anneals to intron 1, thus, it can only amplify transcript variants 1, 2, and 3 (Fig. 3A). For specific amplification of splice variant 3, which lacks exon 4, RACE1 reverse primer was used in combination with a forward primer that spans the junction of exons 3 and 5. Splice variant 3 is preferentially expressed in spinal cord, mammary, and salivary glands, and to a lesser extent in prostate (Fig. 3B).

Differential detection of KLK6 splice variants

A duplex PCR was developed, in order to specifically co-amplify splice variant 2 (E3) and splice variant 3 (E4). The sizes of the expected products were 728 and 575 bp, respectively (Fig. 4A). Primers RT-F and RT-R (Table 1) are expected to amplify transcript variants that encode for full-length hK6 (743 bp) plus splice variant 3 (575 bp). PCR products were resolved and quantified (Fig. 4). About 90% of *KLK6* mRNA species for 76N, 80% for 21PT, and 90% for MDA-MB-468 encode for full-length hK6 and the remaining accounts for *KLK6* splice variants that encode for truncated isoforms of hK6.

Discussion

Human kallikrein 6 was identified based on its aberrant expression in tumor cells [1] and may have potential applications in clinical diagnosis and monitoring of

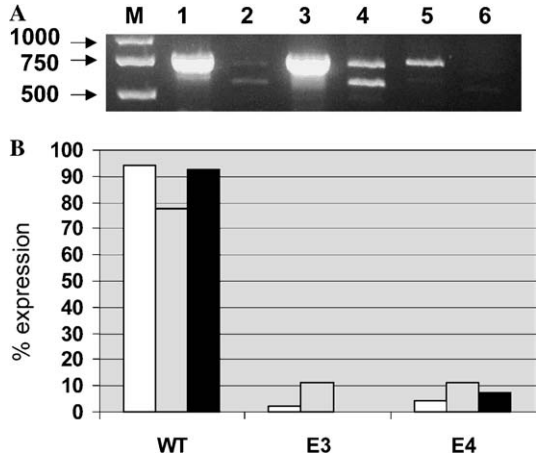

Fig. 4. (A) Differential detection of *KLK6* splice variants by duplex RT-PCR. Expression of all *KLK6* mRNA species was measured in 76N, 21PT, and MDA-MB-468 cells (lanes 1, 3, and 5, respectively). Differential detection of *KLK6* splice variant 2 (E3) and splice variant 3 (E4) is shown in 76N, 21PT, and MDA-MB-468 (lanes 2, 4, and 6, respectively). M, molecular size marker in bp. (B) Quantification of *KLK6* splice variants. The percentage amount of mRNAs corresponding to splice variants is shown in 76N (white bars), 21PT (gray bars), and MDA-MB-468 (black bars). One hundred percentage accounts for all *KLK6* transcripts. Approximately 90% (76N, MDA-MB-468) or 80% (21PT) of *KLK6* mRNAs encode for full-length hK6 protein (wt). Total RNA templates used: 200 ng (76N normal myoepithelial breast cell strain); 0.5 ng (21PT primary breast tumor cell line); and 1 ng (MDA-MB-468 metastatic breast tumor cell line). Data shown represent mean values of three independent experiments.

ovarian cancer [7–9]. The mechanism(s) of transcriptional regulation of the gene encoding hK6 are currently unknown. Here, we describe the identification and cloning of three novel transcript variants and two splice variants of the *KLK6* gene. Similar to *KLK11* gene [21], *KLK6* transcript variants are likely transcribed through the action of alternative promoters (P1 and P2) that may account for the tissue-specific expression of *KLK6*. Multiple sites for initiation of transcription were identified by 5′ RLM-RACE. P2 promoter is located inside intron 1 and utilizes three distinct sites contained within 20 bp. The sizes of transcripts derived from P1 and P2 are 1512 and 1503 bp, respectively. Therefore, they could not be resolved on Northern blots [1]. The *KLK6* classical transcript (P1 product) likely represents the full-length cDNA [1], since search in human EST databases for sequences homologous to its 5′ end revealed only shorter transcripts containing two untranslated exons (Table 2). Interestingly, all ESTs derived from P1 were isolated from brain where *KLK6* is abundantly expressed [6]. Multiple ESTs displayed homology to transcript variants 1, 2, and 3 (P2 products), two of them extending 37 or 25 bp to the 5′ upstream region (Table 2). This indicates that additional sites are likely utilized by the P2 promoter for initiation of transcription.

Most human kallikrein genes give rise to multiple splice variants [5]. Several distinct mRNA species are

Table 2
The longest sequences found in human EST databases with 5′ end homology to *KLK6* transcripts produced by P1 or P2

Transcript[a]	5′ end	GenBank Accession No.	Tissue
P1	+73	AL532786	Adult brain
	+74	BI757376	Brain
	+74	BI488726	Brain
	+95	BI488911	Brain
P2	−36	BG468256	Colon
	−24	BM763868	Myeloma
		BM763657	
	+3	BM761360	Stomach

[a] P1 represents *KLK6* classical transcript (GenBank Accession No. U62801). None of the ESTs are derived from a full-length cDNA. P2 represents transcript variant 2 (GenBank Accession No. AY318869).

transcribed from classical kallikrein genes (*KLK1*, *KLK2*, and *KLK3*/*PSA*) [15–20,22,23], as well as from certain novel kallikreins [5,24–31]. Production of *KLK* isoforms involves the utilization of intronic sequences [15] or alternative polyadenylation signals [19,26] or divergent 5′ UTRs [26], although most of known *KLK* splice variants are derived by alternative splicing of coding exons [28]. An additional *KLK6* transcript variant of about 1100 bp was detected previously in human pancreas and is likely produced by utilization of an alternative polyadenylation signal located at nt +1095 [1]. Specific *KLK* isoforms may have potential applications in the diagnosis of cancer. For instance, certain *KLK13* isoforms have been detected only in normal testis and not in testicular tumors, providing a potential tumor-specific biomarker [27]. Recently, a *KLK5* transcript variant with a shorter 5′UTR and a *KLK7* transcript variant alternatively spliced at the 3′UTR were described as biomarkers for serous carcinoma of epithelial origin [26].

A subset of breast tumor cell lines, such as 21PT and MDA-MB-468, expresses *KLK6* mRNA levels up to 50-fold higher than corresponding normal breast cells [1]. For example, the MDA-MB-468 cell line produces hK6 at concentrations 35-fold higher than the protein produced by normal HMECs (not shown). However, the amount of hK6 protein produced by 21PT cells did not correlate with the *KLK6* mRNA levels detected by Northern blot analysis [1] or RT-PCR (Fig. 4A, lane 3). This discrepancy could be explained by the presence of mRNA species, e.g., *KLK6* splice variants that encode for truncated proteins not recognized by hK6-specific antibodies. To test this hypothesis, we developed a duplex RT-PCR assay to specifically and selectively amplify *KLK6* splice variants 2 and 3. In 21PT tumor cells, the expression of both splice variants was low (Fig. 4A, lane 4) compared with transcripts encoding for full-length hK6 (classical transcript and transcript variants) (Fig. 4A, lane 3). Similarly, the expression of *KLK6* splice variants was low both in MDA-MB-468 breast

tumor cell line (Fig. 4A, lane 6) and in 76N normal cell strain (Fig. 4A, lane 2).

In conclusion, we showed for the first time that the *KLK6* gene encodes for various isoforms produced by the utilization of intronic sequences and alternative promoters with multiple transcriptional start sites (transcript variants) or by splicing out coding exons (splice variants). A duplex RT-PCR assay was developed to distinguish between transcripts that encode for full-length hK6 and those encoding for truncated isoforms or divergent protein sequences. We showed that transcripts encoding full-length hK6 protein are the predominant mRNA species both in normal and tumor cells.

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