

The Kallikrein Gene 5 Splice Variant 2 Is a New Biomarker for Breast and Ovarian Cancer

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Key Words

Human kallikrein gene 5 · *KLK5* · Splice variants · Ovarian cancer · Breast cancer · Cancer biomarkers

Abstract

The presence of more than one mRNA form for the same gene is common among kallikreins, and many of the kallikrein splice variants may hold significant clinical value. The human kallikrein gene 5 (*KLK5*) is a member of the human kallikrein gene family of serine proteases on chromosome 19q13.4. *KLK5* has been shown to be differentially expressed in a variety of endocrine tumors including ovarian, breast and prostate cancer. Utilizing Expressed Sequence Tag database analysis and reverse transcriptase polymerase chain reaction, we identified a new alternatively spliced form of *KLK5* (*KLK5-splice variant 2*, *KLK5-SV2*). This variant mRNA is 1,438 bp in length; formed of 195 bp of 5' untranslated region, 882 bp of protein coding sequence and a 3' untranslated region of 326 nucleotides. *KLK5-SV2* has 7 exons, the first 2 of which are untranslated, and 6 intervening introns. *KLK5-SV2* is different from the classic form of the *KLK5* mRNA in its 5' untranslated region, where the first 5' untranslated exon of the classic form is split into 2

exons with an intervening intron of 135 nucleotides. *KLK5-SV2* is expressed in a variety of tissues, with higher expression levels in the mammary gland, cervix, salivary gland and trachea. The steroid hormone receptor-positive breast cancer cell line BT-474 was used to examine the effect of different steroids on the expression levels of *KLK5-SV2*. Expression levels were significantly higher after stimulation with androgens, but not estrogens, progestins, aldosterone or corticosteroids. While relatively high levels of expression were found in all 10 normal breast tissues examined, no expression was detected in 16 breast cancer tissues, and expression was significantly lower than normal in the remaining 4 cancers. Expression levels comparable to normal were found in only 1 breast cancer cell line. Weak to no expression was detected in 3 other breast cancer cell lines. *KLK5-SV2* was not detectable in any of the 10 normal ovarian tissues examined. It was, however, expressed at relatively high levels in 10 out of 20 ovarian cancer tissues, and lower levels were found in 4 other cancers. No expression was detected in the remaining 6 cancers. High expression levels were also detected in the CAOV-3 ovarian cancer cell line. *KLK5-SV2* is a potential biomarker for breast and ovarian cancers.

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1010-4283/04/0256-221\$21.00/0

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Introduction

The mechanism by which a single gene gives rise to more than one mRNA transcript is referred to as differential splicing. This system is often tightly regulated in a cell-type or developmental stage-specific manner and increases genome complexity by generating different proteins from the same mRNA. The presence of more than one mRNA form for the same gene is common among kallikreins. These forms may result from alternative splicing, retained intronic segment or utilization of an alternative transcription initiation site [1].

Using the positional candidate approach, we have recently cloned the cDNA encoding the human kallikrein gene 5 (*KLK5*, according to the official kallikrein gene nomenclature) [2], which was previously named kallikrein-like gene 2 (*KLK-L2*) [3]. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis detected expression of *KLK5* primarily in the skin, brain, mammary gland and testis, with lower levels in other tissues [3, 4]. In the presence of estrogens and progestins, *KLK5* expression was up-regulated in the BT-474 breast cancer cell line [3].

Thus far, the *KLK5* gene is being investigated for its potential physiological role in the stratum corneum and in cancer. Kim et al. [5] reported a strong positive correlation between *KLK5* expression in ovarian cancer and tumor grade and disease stage, concluding that *KLK5* expression is associated with more aggressive forms of epithelial ovarian carcinoma. Similar data were found at the protein level [6]. A *KLK5* splice variant was found to be differentially expressed in ovarian and prostate cancer [7]. Kallikreins as biomarkers have recently been reviewed [8]. We have also recently shown that *KLK5* is differentially expressed, at the mRNA level, in other endocrine-related malignancies, including breast [9], prostate [10] and testicular cancers [11], and that it has the potential of being a cancer biomarker. At the protein level, we recently provided the first evidence that while the hK5 protein is almost undetectable in serum of normal individuals and patients with diverse malignancies, higher concentrations were found in a proportion of patients with ovarian (69%) and breast (49%) cancers [12]. High levels were also detected in ascites fluid from metastatic ovarian cancer patients and in ovarian cancer tissue extracts.

In addition to the 'classic' form of the gene, another splice variant with a short 5' untranslated region has been recently cloned [13] and was found to be highly expressed in ovarian cancer cell lines, but at very low levels in nor-

mal ovarian epithelial cells. More recently, the *KLK5*-splice variant 1 (*KLK5-SV1*) has been characterized [7]. This variant was found to be highly expressed in ovarian cancer tissues compared to normal ovarian tissues, where it was absent. Hormonal regulation experiments suggest that *KLK5-SV1* is regulated by steroid hormones in cancer cell lines. Furthermore, the variant had significantly higher expression in normal prostate tissues compared to their matched cancer tissue counterparts.

In the present study, we report the cloning of a new *KLK5* splice variant, *KLK5*-splice variant 2 (*KLK5-SV2*). We examined its expression pattern in normal tissues compared to other variants. In addition, we analyzed its expression in breast, ovarian and prostate cancers and its hormonal regulation pattern.

Materials and Methods

Expressed Sequence Tag Searching

Expressed sequence tag (EST) clones with >95% homology to the *KLK5-SV2* (table 1) were obtained from the IMAGE consortium [14] through Research Genetics Inc., Huntsville, Ala., USA. The clones were propagated, purified as described elsewhere [15] and sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from tissues using Trizol reagent (Invitrogen, Carlsbad, Calif., USA) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. Two micrograms of total RNA were reverse-transcribed into first-strand cDNA, using the Superscript™ pre-amplification system (Invitrogen). The final volume was 20 µl. To confirm the efficiency of RT-PCR, 1 µl of cDNA was subsequently amplified by PCR with primers specific for actin, a housekeeping gene (ActinS, ActinAS) (table 2) and were visualized on a 2% agarose gel stained with ethidium bromide.

Two variant-specific primers were designed for PCR amplification of the *KLK5-SV2* (*KLK5-AF*, *KLK5-RP*). Another primer set (*KLK5-BF*, *KLK5-RP*) was used to specifically amplify *KLK5-SV1*, and a third set (*KLK5-5F* and *KLK5-RP*) was used to amplify the classic form of the gene. A different primer set (*KLK5-VF*, *KLK5-RP*) was used to simultaneously amplify all three forms of the gene. All primer sequences are listed in table 2. All primers spanned more than 2 exons to avoid contamination by genomic DNA.

PCRs were carried out in a 25-µl reaction mixture containing 1 µl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM deoxynucleoside triphosphates, 100 ng of each primer and 2.5 units of HotStarTaq™ DNA polymerase (Qiagen Inc., Valencia, Calif., USA) on an Eppendorf master cycler. The cycling conditions were 95°C for 15 min, followed by 35–40 amplification cycles of 94°C for 30 s, annealing (57–60°C, according to primer pair) for 30 s, 72°C for 45 s, and a final extension step of 10 min at 72°C. PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining.

To verify the identity of the PCR products, they were cloned into the PCR 2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions. The inserts were sequenced from both directions, using vector-specific primers, with an automated DNA sequencer.

Tissue Expression

Total RNA, isolated from 39 different human tissues, was purchased from Clontech (Palo Alto, Calif., USA). cDNA was prepared as described above and amplified at various dilutions, using the mentioned sets of primers. Each experiment was repeated at least twice to ensure reproducibility.

Breast Cancer Cell Lines and Hormonal Stimulation

Experiments

The breast cancer cell lines BT-20, BT-474, MDA-468 and MCF-7 were purchased from the American Type Culture Collection (ATCC), Rockville, Md., USA. Cells were cultured in RPMI medium (Invitrogen) supplemented with glutamine (200 mmol/l), bovine insulin (10 mg/l), fetal bovine serum (10%), antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. Twenty-four hours before the experiments, the culture media were changed to phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of 10^{-8} M and ethanol at 0.1%. Cells stimulated with 100% ethanol (final concentration 0.1%) were included as controls. The cells were cultured for 24 h and then harvested for total RNA extraction as described above. Control genes (PSA, pS2 and actin) were amplified as previously described [3].

Normal and Malignant Breast Tissues

Normal breast tissues were obtained from women undergoing reduction mammoplasties. Breast tumor tissues were obtained from female patients at participating hospitals of the Ontario Provincial Steroid Hormone Receptor Program. The normal and tumor tissues were immediately frozen in liquid nitrogen after surgical resection and stored in this manner until extracted. The tissues were pulverized with a hammer under liquid nitrogen, and RNA was extracted as described above.

Ovarian Cancer Tissues and Cell Lines

Normal ovarian and ovarian cancer tissues were obtained from the Department of Gynecology, Gynecological Oncology Unit, University of Turin, Italy. All tumor specimens were confirmed by histopathology. No patient received any treatment before surgery. The CAO-3 ovarian cancer cell line was obtained from ATCC.

Prostate Cancer Tissues

Prostate tissue samples were obtained from 29 patients who had undergone radical retropubic prostatectomy for prostatic adenocarcinoma at the Charité University Hospital, Berlin, Germany. The patients did not receive any hormonal therapy before surgery. The use of these tissues for research purposes was approved by the Ethics Committee of the Charité Hospital. Fresh prostate tissue samples were obtained from the cancerous and non-cancerous parts of the same prostates that had been removed. Small pieces of tissues were dissected immediately after removal of the prostate and stored in liquid nitrogen until analysis. Histological analysis of all tissue sam-

Table 1. EST clones with >95% similarity to the *KLK5-splice variant 2* gene

Clone ID	Library ID	Cell line	Tissue type
S13KMS5-15-C01	S13KMS5	KMS-5	Myeloma
S13KMS5-15-E01	S13KMS5	KMS-5	Myeloma
S13KMS5-7-F04	S13KMS5	KMS-5	Myeloma

Table 2. Primers used for RT-PCR analysis

Gene or variant	Primer name	Sequence ¹
<i>KLK5</i>	KLK5-AF	TGGGGGACAGGTGCCTGG
	KLK5-BF	CTGGGGGACAGGGTGCAG
	KLK5-5F	GGCAGGGAAGGAGAGGTGT
	KLK5-RP	TGAGGTCGTTAGAGTGGCCA
	KLK5-VF	TGCGGCTGAGCTGGGAGC
<i>pS2</i>	PS2S	GGTGATCTGCGCCCTGGTCCT
	PS2AS	AGGTGTCCGGTGGAGGTGGCA
<i>PSA</i>	PSAS	TGCGCAAGTTCACCCTCA
	PSAAS	CCCTCTCCTTACTTCATCC
<i>Actin</i>	ActinS	ACAATGAGCTGCGTGTGGCT
	ActinAS	TCTCCTTAATGTCACGCACGA

¹ All nucleotide sequences are given in the 5' → 3' orientation.

ples was performed as previously described [16]. The tissues were pulverized with a hammer under liquid nitrogen, and RNA was extracted as described above.

Results

Cloning of the *KLK5*-Splice Variant 2

Screening of the EST database revealed several EST clones that were highly similar to the *KLK5* mRNA sequence. Three of these clones (table 1) showed a different 5' untranslated region. Sequencing of these clones revealed a new splice variant of *KLK5*, tentatively named *KLK5-splice variant 2* (*KLK5-SV2*). Variant-specific primers were used to amplify the full length mRNA of this variant from different tissues, and the sequence was submitted to GenBank (accession No. AY279381). This variant is formed of 1,403 bp; 195 bp of 5' untranslated region, followed by 882 bp of protein coding sequence, then a 3' untranslated region of 326 nucleotides. The nucleotide sequence of this variant forms 7 exons, the first

2 of which are untranslated, and 6 intervening introns. The exact 5' transcription start site cannot be verified, and the possibility of the presence of more 5' extension still exists.

This variant is different from the classic form of the *KLK5* mRNA in its 5' untranslated region. The first 5' untranslated exon of the classic form is split into 2 exons with an intervening intron of 135 nucleotides. As this difference is in the 5' untranslated region, the protein product of this variant is not different from that of the classic form. *KLK5-SV2* differs from *KLK5-SV1* by having an extra 5' untranslated exon 68 nucleotides in length. Figure

1 shows a diagrammatic comparison of the three forms of *KLK5* mRNA identified so far. All exon-intron splice sites conform to the consensus AG acceptor site and the GT donor site, except for the donor site of the first exon, which is replaced by the less common GC dinucleotide.

Tissue Expression Pattern

As determined by RT-PCR analysis, *KLK5-SV2* is expressed in a variety of tissues, with higher levels of expression in the mammary gland, cervix, salivary gland and trachea; lower levels were seen in the brain, vagina,

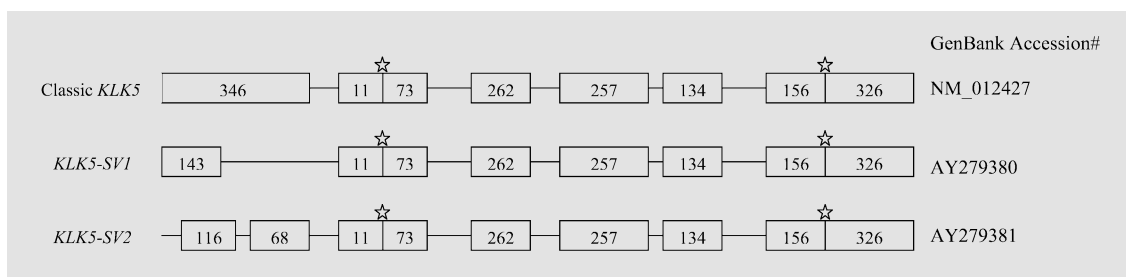


Fig. 1. Schematic representation of *KLK5*, *KLK5-SV1* and *KLK5-SV2* splice variants. Boxes represent exons, and numbers inside boxes are the lengths in nucleotides. Introns are represented by the connecting lines. Asterisks indicate start and stop codons.

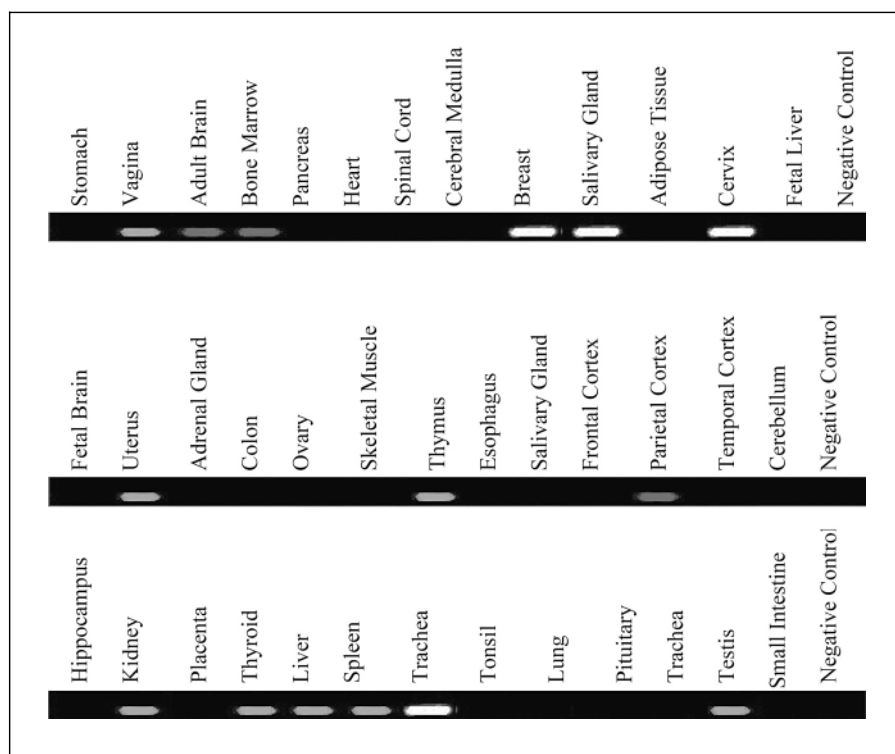


Fig. 2. The tissue expression pattern of *KLK5-SV2* as determined by RT-PCR. *Actin* (not shown), a housekeeping gene, was used as a control gene. Highest levels of expression were found in the breast, salivary gland, cervix and trachea.

bone marrow, uterus, kidney, thyroid gland, liver, thymus, parietal cortex, spleen and testis (fig. 2). The expression pattern is different from that of the classic variant, which shows highest levels of expression in the skin, mammary gland and testis, but is more comparable to the *KLK5-SV1* which shows higher levels in the cervix, salivary gland, kidney and skin.

We also simultaneously compared expression levels of all three splice variants of the gene, using another primer set (*KLK5-VF* and *KLK5-RP*; table 2). Expression levels of the classic form of the gene were mostly higher than, and in few occasions comparable to, *KLK5-SV1*. *KLK5-SV2* consistently displayed lower expression than the two other forms in all tissues examined (data not shown).

Hormonal Regulation of the *KLK5*-Splice Variant 2

The steroid hormone receptor-positive breast cancer cell line BT-474 was used to examine the effect of different steroids of the expression levels of *KLK5-SV2*. Expression levels were significantly higher 24 h after stimulation with androgens, but not estrogen, progestins, aldosterone or corticosteroids (fig. 3). No significant changes were found after hormonal stimulation in the BT-20 receptor-negative cell lines (data not shown). *KLK3* (PSA) was used

as a control, known to be up-regulated by androgens and progestins and *pS2* as an estrogen-up-regulated control (data not shown).

KLK5-Splice Variant 2 Expression in Malignancy

Expression levels of *KLK5-SV2* was analyzed in 20 breast cancer tissues, 10 normal breast tissues and 4 breast cancer cell lines. While high levels of expression were found in all normal breast tissues, no expression was detected in 16 cancer tissues, and expression was significantly lower than normal in the remaining 4 cancers (fig. 4). No expression was detected, under the same conditions, in the BT-20 and BT-474 (unstimulated) breast cancer cell lines, and only weak expression was observed in the MCF-7 cell line. Only the MDA-468 cells showed expression levels that are comparable to normal tissues (data not shown).

KLK5-SV2 was not detectable in any of the 10 normal ovarian tissues examined. The variant was, however, expressed at high levels in 10 out of 20 ovarian cancer tissues, and lower levels were found in 4 other cancers. No expression was detected in the remaining 6 cancers (fig. 5). High expression levels were also detected in the CAOV-3 ovarian cancer cell line.

Fig. 3. Hormonal regulation of *KLK5-SV2* in the BT-474 cell line, as determined by RT-PCR. This splice variant is up-regulated only after androgen (dihydrotestosterone, DHT) stimulation.



Fig. 4. Representative PCR gel showing differential expression of *KLK5-SV2* in normal breast (N) and breast cancer (C) tissues. *KLK5-SV2* is significantly down-regulated in breast cancer. Neg = negative control.

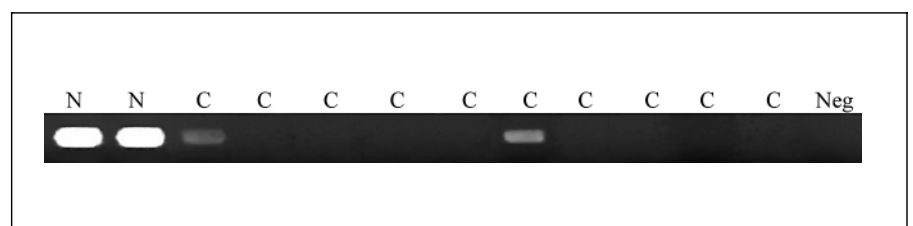
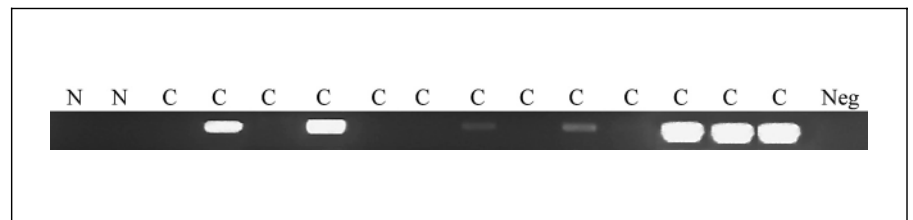


Fig. 5. Representative PCR gel showing differential expression of *KLK5-SV2* in normal ovarian (N) and ovarian cancer (C) tissues. *KLK5-SV2* is up-regulated in a subgroup of ovarian cancer patients. Neg = negative control. For more details, see text.



No significant difference was found in *KLK5-SV2* expression levels between normal and malignant prostate tissues (data not shown).

Discussion

Ovarian cancer is the second most common and the most lethal gynecologic malignancy among women [17], with the average 5-year survival rate of 39%. The low survival rate is mainly due to the lack of sensitive tests for detection of early stage disease, which is often asymptomatic. Therefore, identification of reliable diagnostic biomarkers would greatly improve the outcomes of ovarian cancer by enabling early detection. Furthermore, identification of prognostic and predictive biomarkers would aid in the optimal management of patients. Genes which display altered expression in cancer cells are generally considered as candidate tumor biomarkers and should be evaluated for possible correlation between their expression and patient prognosis.

Serine proteases, including kallikreins, serve several functions during tumor progression, including stimulation of cellular growth, angiogenesis as well as degradation of extracellular matrix. In particular, the last function is thought to be critical for the invasiveness and metastatic potential of cancer cells.

Many of the kallikrein splice variants may hold significant clinical value. Slawin et al. [18] reported the prognostic significance of a splice-variant-specific RT-PCR for *KLK2*, in detecting prostate cancer metastasis. Nakamura et al. [19] reported differential expression of the brain and prostate types of *KLK11* between benign, hyperplastic and malignant prostate cancer cell lines. A novel, ovarian cancer-specific variant of *KLK5* has been recently reported [13]. This transcript, with a short 5' untranslated region, and another *KLK7* variant with a longer 3' untranslated region were highly expressed in the ovarian cancer cell lines, but were expressed at very low levels in normal ovarian epithelial cells, and thus might be useful as tumor markers for epithelial-derived serous carcinomas [13]. Our results show a differential expression pattern of *KLK5-SV2* in breast and ovarian cancer that should be further investigated in a large-scale study.

The *KLK5-SV2* shows a different tissue expression profile than its classic counterpart. This is not unprecedented. Some of the kallikrein alternatively spliced forms were also found to be tissue specific. A 1.5-kb transcript of *KLK14* was only found in the prostate, and another 1.9-kb

transcript only in skeletal muscle [20]. Several splice variants of *KLK13* were found to be testis specific [21]. Type 2 neuropeptide (*KLK8*) is preferentially expressed in human adult brain and hippocampus [22], and a new splice variant of *KLK4* was isolated from prostatic tissue [23]. A *KLK6* splice variant was strongly expressed in adult brain compared to fetal brain. It is also important to mention that some of these splice variants were found to be translated [24, 25].

KLK5-SV2 has a weak variant GC donor site in its first alternatively spliced exon. This intrinsically weak site is reported in other genes including kallikreins, e.g., intron 3 for *KLK10* transcript variant 2 (GenBank accession NM_145888). Interestingly, a recent analysis of splice variants showed that 1 in every 20 alternatively spliced introns was found to be a GC-AG intron, and 3 of every 5 observed GC-AG introns are alternative isoforms [26].

Since the difference in the *KLK5-SV2* and other forms is only in the 5' untranslated region, the predicted protein structure should be the same for all forms. The impact of the 5' untranslated region on protein production and/or stability is yet to be analyzed. Previous reports show that the 5' UTR of the maternal mRNA is important in post-transcriptional regulation by controlling mRNA stability, localization and translational activity or repression [27]. Recent data suggest that differentiation of the 5' UTR can be associated with the development or progression of many diseases including cancer [28]. A deletion in a critical segment in the 5' UTR may lead to the deregulation of expression of various critical proteins regulating growth or differentiation in the normal tissue. This may be relevant in many alternatively spliced genes which affect the untranslated regions. In TGF- β , an alternative transcript with a 5' UTR truncation was found to be associated with breast cancer, and a single nucleotide polymorphism in the 5' UTR of RAD51 protein which interacts with BRCA1 and BRCA2 breast cancer suppressor proteins is reported to modulate breast cancer risk [29]. The association between 5' UTR changes and cancer is apparent in the androgen receptor (AR) gene. This event can affect the protein-binding region and can result in enhanced or suppressed transcription, termination of transcription, alteration in translation or change in mRNA stability, causing an up-regulation in AR and predisposing men to developing prostate cancer [28]. Therefore, the association between differential expression of *KLK5-SV2* in ovarian and breast cancers and the deleted region in the 5' UTR should be further investigated.

Unlike the classic form of *KLK5*, which was reported to be up-regulated by estrogens [3], *KLK5-SV2* was found to be regulated mainly by androgens. This difference might reflect different mechanisms of regulation of these forms in physiological and pathological conditions.

In conclusion, we identified a new *KLK5* splice variant, *KLK5-SV2*, and showed a differential expression pattern of this variant in breast and ovarian cancer. Our results show that *KLK5-SV2* is under steroid hormonal regulation in breast cancer cell lines.

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