ANTICANCER RESEARCH *35*: 2715-2724 (2015)

Identification, Molecular Characterization and Alternative Splicing of Three Novel Members of the Canine Kallikrein (*Klk*)-related Peptidase Family

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Abstract. *Background/Aim: Kallikrein-related peptidases (KLKs) comprise a serine protease family with prominent roles in tissue physiology and disease pathogenesis, including cancer. Previously, we have characterized canine Klk4-10 and -14. Herein, we continue our efforts by characterizing three novel members of the canine family, i.e. Klk11-13, and investigating their expression in mammary cancer. Materials and Methods: Reverse transcription-polymerase chain reaction (RT-PCR) and DNA sequencing were used for investigating the expression and determining the nucleotide sequence of all transcripts identified, respectively. Results: It was demonstrated that (i) unlike other Klks, (CANFA)Klk12 probably possesses a non-AUG translation initiation codon, (ii) all three Klks undergo alternative splicing, with exon 2 and 3 concurrent elimination serving as the most prominent event, (iii) all transcripts identified were detected in both tumor and normal tissues, yet with different frequencies. Conclusion: Having completed this work, Klk15 is the only gene remaining to experimentally resolve the entire canine Klk family. Our data lay sufficient groundwork for validation studies and await further incorporation into genetic/evolutionary studies with translational impact.*

The human kallikrein-related peptidases (KLKs) comprise a family of 15 serine proteases, encoded by a contiguous *KLK* gene cluster (1-3). The genes encode single-chain pre-proenzymes, which carry a signal peptide, a short propeptide and the catalytic domain. Proteolytic cleavage of these peptides eventually allows for secretion and generation of the active enzymes (4).

Earlier experimental and *in silico* investigations have successfully described *Klk* gene families in the mouse, rat, pig, chimpanzee, dog and opossum (5-7). However, the most detailed breakthroughs on the structural characterization of this family in various animal species have risen from studies addressing their evolutionary perspective (8-10). It is now presumed that the kallikrein locus is unique to all mammals and the majority of tissue KLKs is highly conserved among species. Quite expectedly, certain structural properties within the *KLK* gene sequences are particularly conserved. For instance, all *KLKs* have 5 coding exons and 4 intervening introns with identical patterns of intron phases. In addition, their catalytic domain comprises three residues, namely His, Asp and Ser, whose codons are strictly positioned within the second, third and fifth exon, respectively (5).

The expression pattern of most human KLKs expands to the majority of cell types and tissues, where they cooperate in proteolytic cascade pathways to regulate physiological processes (11-13) of which skin desquamation and homeostasis, dental enamel formation and regulation, as well as seminal plasma liquefaction, constitute a few notable examples. The complex enzymatic circuitries elicited by KLKs in various tissues are also seen as mediators of cellular responses during disease onset and progression, including inflammation and cancer (4, 11, 14). Furthermore, the levels of multiple KLKs are disturbed in both the gene and protein expression levels in various malignancies, an observation that also provides rationale for assessing these molecules as putative biomarkers (15).

Following earlier studies on the characterization of canine *Klk1* (16) and *Klk2* (17, 18) mRNA sequences, our group experimentally characterized many of the remaining

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Key Words: kallikrein-related peptidases, gene expression, alternative splicing, Canis familiaris, mammary cancer.

0250-7005/2015 $2.00+.40

Table I. *Primers used for PCR amplification.*

Primer	Sequence[a]	Primer pair	Amplicon size (bp)
KLK11-F	AAGAACCTGGAGCCTGCC	KLK11-F/R	834[b]
KLK11-R	GCCATGTTGCCAAGTAGGGA		
KLK12-Fa	ATGGAGGACCCAGCAGGC	KLK12-Fa/Ra	795[b]
KLK12-Fb	ATGACAGGAAGGCAGGTAGTG	KLK12-Fb/Ra	860
KLK12-Fc	ACCTGGAGGTGGGTCACC**A**[c]	KLK12-Fc/Ra	765
KLK12-Fd	ACCTGGAGGTGGGTCACC**C**[c]	KLK12-Fd/Ra	765
KLK12-Ra	TTAGTTGTTCCTCATGACCAT		
KLK13-F	CCCGGAGTCGCCATGTG	KLK13-F/R	869[b]
KLK13-R	CAGGTGAGCCAACCTAGCAT		

[a]Primer sequences are presented in 5'-3' direction. [b]Sequence that harbors the predicted coding region of the corresponding Klk. [c]The different nucleotide at the 3'-end of each forward primer used in the allele-specific PCR is denoted in bold

members of the family (*i.e. Klk4-10* and *-14*) for the first time using normal and neoplastic canine mammary tissues (19-21). Our published data have collectively pointed-out certain inaccuracies between *in silico* predicted and experimentally verified mRNA sequences, as in the case of *Klk4, -9, -10* and *-14*, while also denoted alternative splice variant forms for *Klk8*, *-9* and *-14* genes (19-21). Additionally, certain *Klks* and/or their variants demonstrated differential expression between normal and neoplastic tissues (19-21), which, on occasion, showed to be consistent with relevant human breast cancer studies (22). Our findings, therefore, support the notion that the dog could be a useful animal model for *in vivo* studies of *Klk* expression and function in breast cancer. To complement and finalize our efforts on the structural delineation of the entire canine *Klk* transcriptome, here, we sought to investigate the expression of three additional canine *Klks*, namely *Klk11*, *-12* and *-13*, in canine mammary cancer.

Materials and Methods

Tissue samples. Tissue specimens were obtained from surgically removed mammary gland tumors from 30 pet dog cancer patients that were admitted to the Companion Animal Clinic, Department of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki. For 20 of them, tissue samples were taken during surgery from the center of the tumor, whereas tissue samples were taken from both the tumor and a site 2-5 cm from the visible tumor margin (normal tissues adjacent to the tumor) for the other 10. All specimens were immediately immersed in an RNAlater solution (TAKARA, Shiga, Japan) and stored at-80˚C until further processing. Histological analysis was performed to verify that the tissues were either malignant or normal.

Table II. *Homologies of canine kallikrein-related peptidases 11-13 to their human counterparts.*

	% homology with *KLK*	
	Coding sequence	Amino acid sequence
Klk11	85.7	82.0
Klk12-model 1	76.7	71.9
Klk12-model 2	81.6	77.0
KlkK13	86.1	83.8

Table III. *Expression of canine Klk11, Klk12 and Klk13 mRNA transcripts in tumor and adjacent normal mammary tissues.*

	Tumor samples (n=20)	Matched samples (n=10)	
		Tumor	Normal
Klk11			
Classical form	16	9	9
Variant 1	19	9	7
Variant 2	19	9	5
Klk12			
Classical form	19	9	5
Variant 1	15	5	3
Klk13			
Classical form	12	5	3
Variant 1	19	9	4
Variant 2	9	6	6

RNA extraction and cDNA synthesis. Total RNA was extracted from tissue samples using the NucleoSpin Total RNA Isolation kit (Macherey-Nagel, Duren, Germany) and reverse transcription was carried out using the PrimeScript 1st strand cDNA synthesis kit (TAKARA), according to the manufacturers' instructions. One μg of total RNA was used as starting material for cDNA synthesis.

Polymerase chain reaction (PCR) amplification. The primers used for PCR amplification, along with the sizes of the amplicons produced for each primer pair, are presented in Table I. Primers were designed based on: (i) *in silico* predicted sequences available in GenBank (accession numbers-*Klk11*: XM_005616254, *Klk12*: XM_849479, *Klk13*: XM_003638808) and (ii) alignments of the corresponding human *KLK* mRNAs (GenBank accession numbers-*KLK11*: NM_006853, *KLK12*: NM_145894, *KLK13*: NM_015596) with *Canis familiaris* "whole genome shotgun sequences"

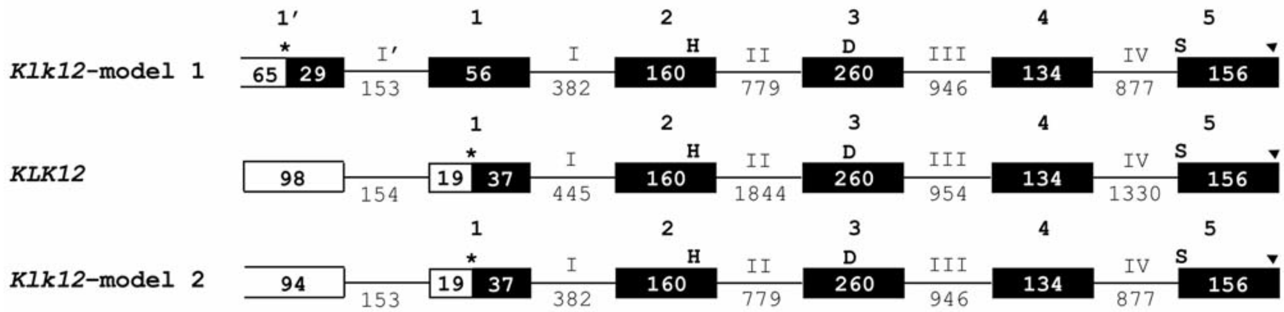

Figure 1. *Structural organization of canine and human Klk12. Model 1 uses the start codon indicated in the predicted Klk12 sequence available in GenBank (XM_849479); model 2 uses the alternative (CTG) translation initiation codon proposed in the present study. Boxes represent exons with the coding regions shown in black. Boxes with their left side open indicate exons with partially characterized sequence. Lines represent intervening introns. Exon and intron identities are indicated by arabic and roman numbers, respectively. The numbers inside the boxes and below the lines indicate exon and intron lengths, respectively, in base pairs (bp). The asterisk denotes translation start codon. The arrowhead depicts translation termination codon. The positions of the residues of the catalytic triad are shown: H, Histidine; D, aspartic acid; S, serine. The figure is not drawn to scale.*

(GenBank accession numbers: AAEX03000771.1 for *KLK11* and *KLK13*; AOCS01189798.1 for *KLK12*).

Touchdown PCR protocols were adopted to achieve high specificity, as previously described (19-21). RNA integrity was verified through PCR amplification of canine β-actin (Actb) housekeeping gene, as previously described (19-21).

DNA sequencing, GenBank accession numbers and in-silico analysis. PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel) and sent to a commercial sequencing facility (VBC-Biotech Service GmbH, Vienna, Austria) for DNA sequencing. The nucleotide sequences obtained, either corresponding to classical forms or splicing variants of *Klk11, Klk12* and *Klk13,* were then submitted to GenBank and assigned accession numbers KJ831037 through KJ831044. Sequence homology searching/analyses and signal peptide predictions were performed as previously described (19-21).

Results

Structural characterization of Klk12. Since *Klk12* nucleotide sequence has never been experimentally determined, the primers required for investigating its expression by RT-PCR, were designed based on an in-silico identified sequence (predicted) available in GenBank (accession No. XM_849479). Primers KLK12-Fa/KLK12-Ra (Table I) align at the very ends of the coding region (795 bp) -as allocated within the predicted sequence -and yielded a PCR product, which, upon DNA sequencing, revealed 99.75% homology with the predicted *Klk* (2/795 nucleotides difference). In order to determine the structural characteristics of this gene -*i.e.* exon/intron boundaries, exon/intron sizes, catalytic triad codons *etc*.- the obtained sequence was aligned with both *KLK12* (GenBank accession No. NM_145897) and the canine genome. The alignments, however, revealed the following discrepancies to the well-known *KLK* features: (i). *Klk12* has 6 instead of 5 coding exons (Figure 1, *Klk12*-model 1); (ii) the first 48 nucleotides of the coding region have no homology to the human *KLK12* coding region (Figure 2A); and (iii) only the last four exons exhibit similarity in terms of size and sequence with human *KLK12* (Figure 1, *Klk12*-model 1 and Figure 2A). For that, we kept the numbering of these exons the same as in the human *KLK12*, *i.e.* 2, 3, 4 and 5, and designated the first two exons as 1' and 1.

We hypothesized that the additional coding exon identified (*i.e.* exon 1 or 1') might have been the consequence of an incorrectly predicted translation initiation codon within the *in silico* identified canine *Klk12* (XM_849479). In this case, if the real start codon resides downstream of the one predicted, the additional coding exon might simply constitute an untranslated exon. On the other hand, if the start codon is located upstream of the predicted one, then, the additional coding exon might be the result of an insertion, possibly due to partial intron retention, as previously found in other canine *Klks* (19-21). Since no start codon was identified further downstream of the predicted one and around the area of high homology to the human *KLK12* translation initiation site (Figure 2A), we searched for alternative start codons upstream of the one proposed in GenBank. An ATG codon obeying to the Kozak rules (23) was identified 65 nucleotides upstream and a new primer was designed to incorporate it (Table I, primer Fb). PCR amplification using primers KLK12-Fb/KLK12-Ra resulted in the expected 860 bp product, which, upon sequencing, showed 99.77% homology to the predicted sequence (2/860 nucleotides difference). Importantly, no product of smaller size that would indicate absence of the extra coding exon described above was detected (data not shown).

While searching for alternative start codons, we rationalized that if nucleotide C at position +49 of the predicted *Klk12*

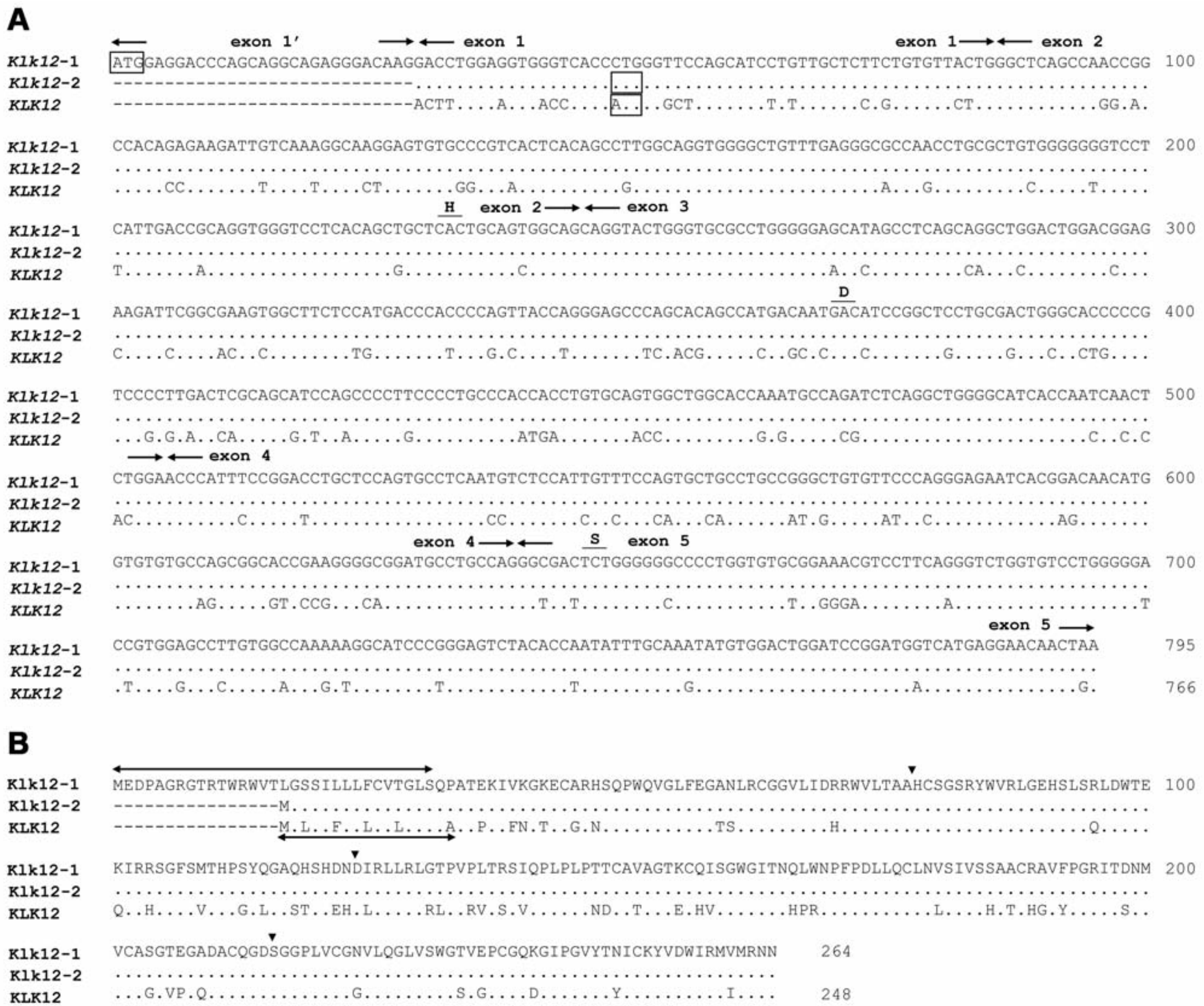

Figure 2. *Alignments of canine and human KLK12 mRNA (A) and amino acid sequences (B). Klk12-1 and Klk12-2 represent models 1 and 2, respectively, as described in Figure 1. Only nucleotides/residues differing from Klk12-1 are depicted. Dots indicate identity with Klk12-1; dashes represent gaps. (A) The start codons are enclosed in open boxes. Exon boundaries are denoted by arrows above the sequences. Numbers at the right-end of the sequences represent size in base pairs (bp). The codons for the amino acids of the catalytic triad are shown: H, Histidine; D, aspartic acid; S, serine. (B) The double-headed arrow above the alignment shows the signal peptide of Klk12-1; the one below the alignment delineates the signal peptide of Klk12-2 and KLK12. Numbers at the end of the sequences denote length in amino acids. The residues that constitute the catalytic triad are depicted by arrowheads.*

coding region was substituted by an A, an ATG start codon would be created, at the exact same site as the translation initiation codon of the *KLK12* (Figure 2A). This would give rise to a coding region with 5 exons and high homology to human *KLK12*. However, all our sequencing data revealed a C at that position. To exclude the possibility that there might be some transcripts with an A at that position, we performed an allele-specific PCR using two new primers (Table I, KLK12-Fc and KLK12-Fd) differing only by the last nucleotide (A or C, respectively) at their 3'-end. PCR amplification both with KLK12-Fc/KLK12-Ra and KLK12-Fd/KLK12-Ra pairs revealed products only for the latter combination, thus verifying the absence of A at that position.

All the above led us to propose that for *Klk12* translation may be initiated at a CTG rather than the classical ATG codon (Figures 1 and 2, model 2). Although relatively rare, initiation of translation can occur at non-ATG codons that differ from ATG by a single nucleotide, with CTG being the most efficient one in mammals (24). For initiation at non-ATG codons, the presence of a good Kozak context is still

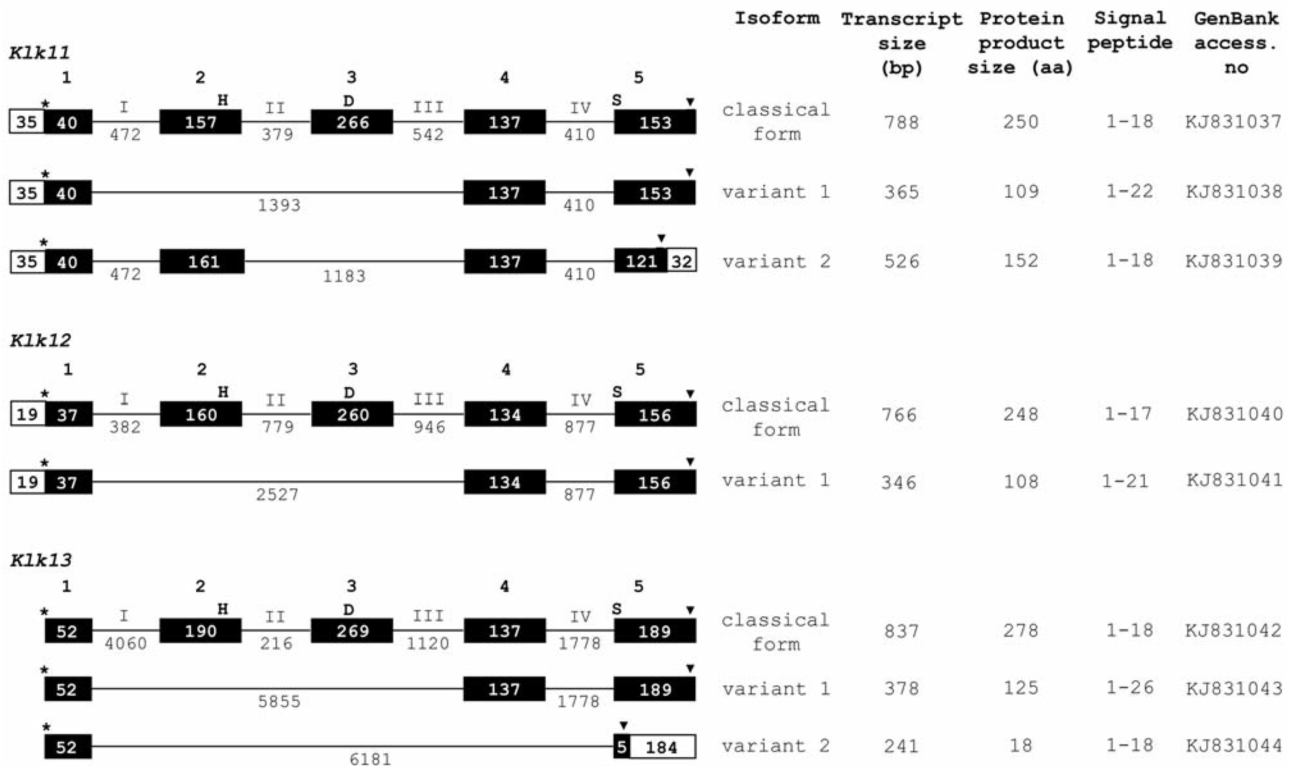

Figure 3. *Schematic representation of Klk11, Klk12 and Klk13 mRNA transcripts (for Klk12, only model 2 is presented). Boxes represent exons with the coding regions shown in black. Lines represent intervening introns. Exon and intron identities are indicated by arabic and roman numbers, respectively. The numbers inside the boxes and above the lines represent exon and intron lengths, respectively, in base pairs (bp). The asterisk denotes translation start codon. The arrowhead depicts translation termination codon. The residues of the catalytic triad are indicated: H, Histidine; D, aspartic acid; S, serine; aa, amino acids. The figure is not drawn to scale.*

crucial. Indeed, the hereby proposed CTG codon has a purine (A) at position -3 and a G at position +4, as dictated by the Kozak motif (23). The following observations further support model 2 as the prevailing one: (i) the start codon resides on nucleotides 20-22 of exon 1, consistently with the observation that all *KLKs* have a 5' non-translated sequence within their first exon (4); (ii) there is a purine (A) at position -3 (23), whereas in model 1 there is a pyrimidine (C) at that position; (iii) the coding region includes 5 exons with the exact same sizes with the one in *KLK12*; (iv) the coding region exhibits high homology to the corresponding human *KLK12* sequence (81.6% *vs.* 76.7% of model 1; Table II); (v) when translated, it produces a protein with high similarity to its human counterpart (77.0% *vs.* 71.9 % of model 1; Table II) and a signal peptide that, as in human KLK12, spans amino acids 1-17 (Figure 2B).

Structural characterization of Klk11 and) Klk13. Like *Klk12*, *Klk11* and *Klk13* of the canine family have never been experimentally characterized. Therefore, in order to study their expression in canine mammary tissues we designed primers (Table I), again, based on computationally identified sequences available in GenBank (accession numbers-XM_005616254 and XM_003638808, respectively). Primer pairs were selected to amplify the entire coding region of the genes. DNA sequencing of the amplified products revealed nucleotide sequences exhibiting 100% homology to the corresponding predicted *Klks* (data not shown), as well as high similarity rates (85.7% and 86.1%, respectively) to the corresponding human *KLKs* (Table II). Alignments of the obtained *Klk* sequences with both canine "whole-genome shotgun sequences" (GenBank accession No. AAEX03000771.1) and the respective *KLKs* (GenBank accession Nos.: NM_006853 and NM_015596, respectively) revealed several structural characteristics of these genes like exon/intron sizes, exon-intron boundaries and the positions of the codons of the catalytic triad (H, D, S) (Figure 3, classical forms). When translated, these nucleotide sequences gave protein products with high homologies

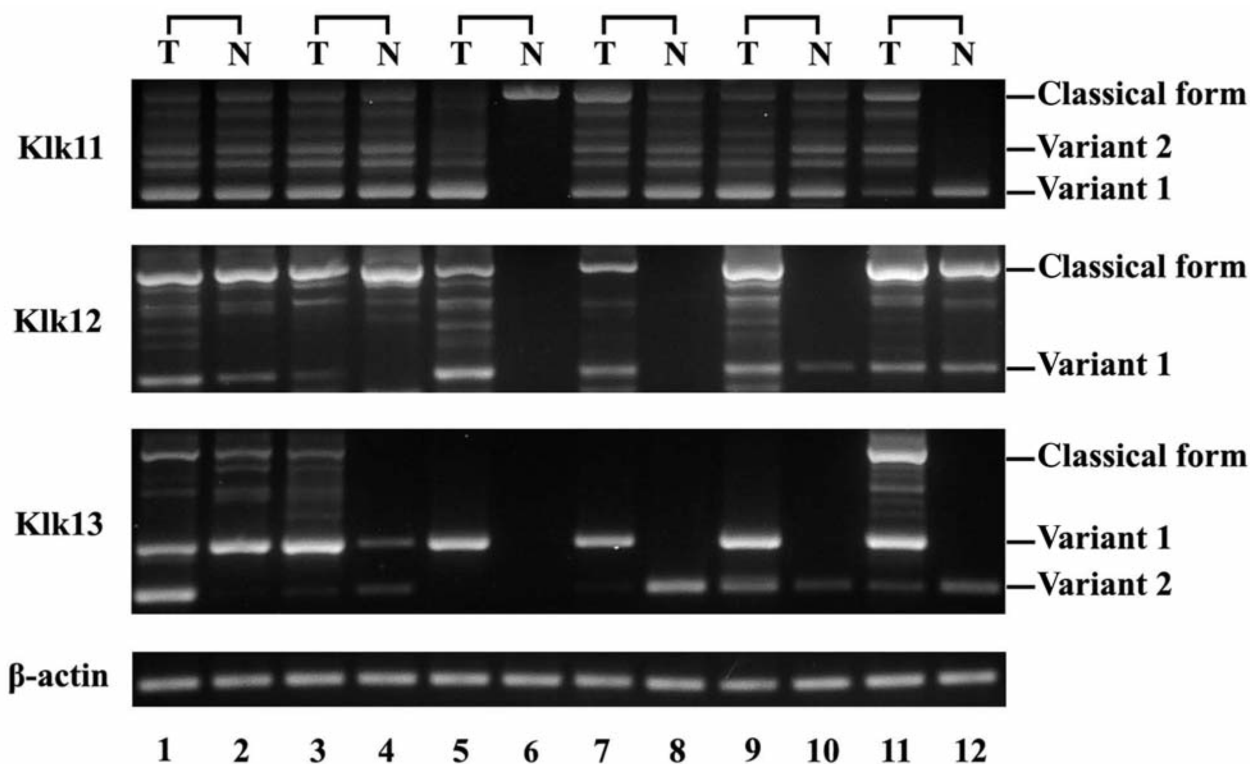

Figure 4. *Detection of Klk transcripts in paired tumor and adjacent normal mammary tissues. Each odd lane corresponds to a tumor tissue (T) and each even lane to the matched adjacent normal tissue (N). The different transcript variants are indicated.*

(82.0% and 83.8%, respectively) to their human counterparts (Table II) bearing an 18-amino-acid signal peptide on their N-terminal domain. The above observed characteristics are in complete agreement with those described for all human and other canine *Klks*.

Detection and characterization of alternatively spliced variants. PCR amplifications revealed, besides the expected for each *Klk* product (Table I), amplicons of lower molecular weights (Figure 4). Gel extraction and DNA sequencing of these products verified that they comprise transcripts of the corresponding canine *Klks*. Transcript variants that lack the entire exon 2-3 sequence were detected in all three *Klks* and were designated as variant 1 in all cases (Figure 4). For *Klk11*, one more transcript (variant 2), bearing exon 3 deletion and a 4 bp insertion between exons 2 and 4, was identified. Searching within the *Klk11* genomic DNA, we found that the insertion encompasses the first 4 nucleotides of the 5'-end of intron II. A second transcript, missing exons 2, 3 and 4, was also identified for *Klk13* (variant 2).

The generation of these transcript variants could be explained either by complete omission of the regular splice sites or by employment of alternative splicing sites (Figure 5). The alternative splicing events that lead to exon 2-3 deletion do not disturb the open reading frame in any of the three *Klks* (Figure 3). The predicted protein products, however, do not encode for active serine proteases as they lack both His and Asp of the catalytic triad. On the other hand, both exon 2 3'-extention /exon 3 deletion and exon 2-3-4 skipping cause a frame shift and lead to premature termination of translation in *Klk11* and *Klk13*, respectively. The truncated polypeptides possess either only one or none of the amino acids of the catalytic triad and, as a result, they cannot act as serine proteases. All isoforms encode for a signal peptide and can, thus, be secreted (Figure 3).

Expression in tumor and normal mammary tissues. The expression of all three *Klks* was examined in 20 mammary tumors and 10 paired tumor and adjacent to the tumor normal mammary tissues. The results are presented in Table III. Among the 30 tumor samples analyzed in total, positivity rates ranged from (93.3%) for *Klk11* variants 1 and 2, the classical form of *Klk12* and *Klk13* variant 1 (28/30) to 50% for *Klk13* variant 2. All *Klk* transcripts were detected in normal tissues as well, yet with lower frequency, except for the classical form of *Klk11*, which was found in 9/10 tissues.

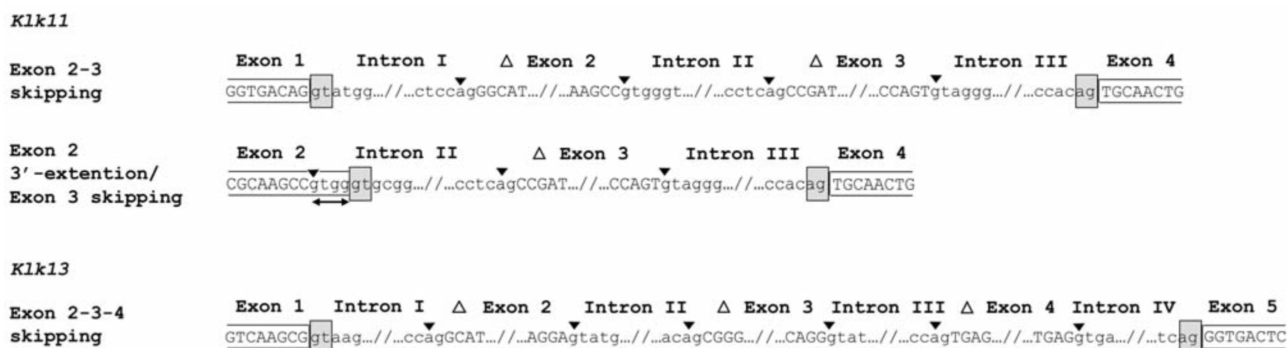

Figure 5. *Alternative splicing events. Exon sequences are denoted by upper-case letters and intron sequences by lower-case letters. Splice-donor and splice-acceptor sequences are marked by grey boxes. The arrowheads show skipped splicing sites. Unspliced sequences are depicted by white boxes. The double-headed arrow indicates the 4 bp insertion. The symbol Δ is used to denote deletion. The splicing events that lead to exon 2-3 skipping are presented only for Klk11.*

In the 10 matched samples, the positivity of *Klk11* variant 2, the classical form of *Klk12* and *Klk13* variant 1 in normal tissues dropped to approximately 50% of the one in the corresponding tumors (Figure 4).

Discussion

Despite the fact that the increasing availability of sequencing data in genomic databases has enabled *in silico* identification of the Klk family within the genome of several organisms (5, 8-10), studies on their experimental characterization and expression in non-human tissues are still limited. Such information, however, would be of great importance to assist in the development of appropriate animal models for investigating KLK functions in both physiology and disease. Lately, we have proposed the dog as a promising animal model for *in vivo* KLK studies and we sought to examine the *Klk* expression profile in both neoplastic and non-neoplastic tissues (19-21). In the present report we investigated the expression of canine *Klk11, Klk12* and *Klk13* in mammary tissues.

First, the experimentally identified nucleotide sequences were in agreement with the ones predicted *in silico* to represent canine orthologs of human *KLK11, KLK12 and KLK13*. *Klk11* and *Klk13* were found to share high homology to their human counterparts and abide by all KLK-defining characteristics. For Klk12, however, it was noted that the start codon, according to GenBank, generates a coding sequence and a protein product with absence of fundamental KLK features and no homology to the 5'- and NH_2-termini, respectively, of the KLK12 nucleotide and amino acid sequences. These discrepancies have never been observed in any other Klk either of the same or of a different species.

Having excluded the possibility that this *Klk12* transcript might constitute an alternatively spliced variant, we noticed a CTG codon further downstream of the *in silico* predicted start codon, which, upon acting to initiate translation, it could lead to a gene and a protein product with all KLK features and high homology to human *KLK12*. It is possible that an A to C substitution during evolution might have abolished the ATG initiation site at this position. Alternatively, the coding region may begin with a non-ATG codon. Tikole and Sankararamakrishnan (25) have demonstrated that coding regions of about 0.1% of the total mRNA sequences begin with a non-AUG codon. In mammals, CUG (CTG in the DNA sequence) appears to be the most efficient non-AUG start codon, whereas AAG and AGG are the least efficient ones (24). Searching within the "non-AUG" bioinformatic database (http://bioinfo.iitk.ac.in), we found that there are 5 such sequences within the canine genome, which all initiate translation at a CTG codon. Out of these, 3 have a purine at position -3 and a G at +4, as identified in the CTG codon, hereby suggested to constitute *Klk12* start codon. All the above led us to propose two potential models for the structure of the canine *Klk12* coding region; one beginning with the ATG codon predicted in the computationally determined sequence available in GenBank (model 1) and one beginning with the CTG codon presented in this study (model 2). For reasons explained above, the most probable -to our opinion- one is model 2. This, however, remains to be confirmed with *in vitro* translational studies.

As previously demonstrated for other canine *Klks* (19-21), *Klk11-13* were also shown to undergo alternative splicing. A splice variant lacking both exons 2 and 3 was detected for all three of them (variant 1). This was the sole variant identified for *Klk12*. *Klk11* had one more transcript, which combined 3'-extention of exon 2 and deletion of exon 3 (variant 2), and

Klk13 an additional variant bearing only exons 1 and 5 (variant 2). In *Klk11* variant 2 and *Klk13* variant 2, alternative splicing led to a frame shift and the subsequent generation of a premature termination codon. As a result, both variants, if translated, would produce truncated peptides. On the other hand, exon 2-3 elimination in variant 1 of all three *Klks* does not disrupt the reading frame but, still on translation, smaller polypeptides will be produced. None of these peptide isoforms is expected to encode functional serine proteases as they lack critical residues of the catalytic triad. In *Klk11* variant 2, the sequence encoding for the signal peptide is retained, whereas in variant 1 of all three *Klks*, it is disrupted by the deletion. The new sequence generated on the 5'-end of the transcripts, however, also encodes for a signal peptide in all three variants. These observations imply that all the alternatively spliced transcripts identified are likely secreted upon translation.

The investigation of the expression of all mRNA transcripts of the three *Klks* in mammary tumor tissues revealed almost ubiquitous expression of the classical form of *Klk12*, both variants 1 and 2 of *Klk11* and variant 1 of *Klk13*. With the exception of *Klk11* variant 1, the other three transcripts were detected in almost all tumors and in only half of the corresponding normal tissues. It is also worth mentioning that the predominant -in terms of expression levels- transcript for both *Klk11* and *Klk13* was the one with missing exons 2 and 3 (variant 1) and not the classical form of the genes. Moreover, *Klk13* variant 1 was detected in almost all tumor tissues, whereas the corresponding classical form in only half of them. It may, thus, be possible that the elimination of exons 2-3 *via* alternative splicing may be the mechanism utilized to abolish Klk11 and Klk13 function.

This study has experimentally characterized canine *Klk11*, *Klk12* and *Klk13*, along with their alternatively spliced variants, and demonstrated their expression in neoplastic and non-neoplastic mammary tissues. Having completed this work, *Klk15* is the only gene remaining to experimentally resolve the entire canine *KLK* family. Overall, the preliminary evidence from our current and past reports lays sufficient groundwork for validation studies with larger cohort sizes. These data await deeper mining, interpretation and, even, incorporation in both genetic/evolutionary and clinical studies with translational impact.

Acknowledgments

This work was funded by the "Support of Research Activity in A.U.TH-2012" institutional program (A.U.TH Research Committee no. 89290).

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Received January 26, 2015
Revised February 3, 2015
Accepted February 6, 2015