Isolation and Characterization of the Porcine Tissue Kallikrein Gene Family

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Story in Brief

Kallikreins are members of a multigene family of serine proteases that are widespread throughout living organisms. They are found in diverse tissue specific patterns and are known to have highly diverse physiological functions. To gain insight into the structure and evolutionary origin of the kallikrein gene region in the pig, we isolated several BAC clones containing members of the porcine tissue kallikrein gene family and have characterized the porcine kallikreins. Sequence analysis of the greater kallikrein region has revealed the presence of 13 kallikrein genes in the porcine genome, among which 11 are novel porcine kallikrein genes. Furthermore, in an effort to understand the tissue specific expression profile of the porcine kallikrein genes family members, we have analyzed the expression pattern of the kallikrein genes across a wide array of porcine tissue.

Key Words: Kallikrein, Gene Expression, Serine Proteases, Porcine, Pig

Introduction

During the last three decades, extensive research on tissue kallikrein gene families of humans and rodents have been carried out (Evans et al., 1987). These studies have helped us better understand the structural and functional similarities among tissue kallikreins. Kallikreins are members of a multigene family of serine proteases that are widespread throughout living organisms (Gan et al., 2000). They show high degree of substrate specificity and demonstrate highly diverse physiological functions (Evans et al., 1987). Kallikreins show high degree of conservation at both gene and protein structure level, and co-localize or organize into the same chromosomal locus in all species they are studied in (Evans et al., 1987; Yousef and Diamandis, 2001; Yousef et. al. 2003).

Based on the conservation of the primary and tertiary structures and the enzymatic activities among kallikreins, it is hypothesized that all known serine proteases arose from a common ancestor through gene and/or chromosomal duplication during the course of evolution, which may have caused the kallikrein gene family to be clustered in the genome (Gan et al., 2000; Yousef and Diamandis, 2001). Comparison of the kallikrein family members among species reveal that there is a greater level of conservation within species than among orthologous genes across species, suggesting that recombination has occurred within a species leading to concerted evolution (Wines, 1991). Currently 15 kallikrein genes with diverse expression profiles have been identified in humans. All co-localize to the same locus on chromosome 19q13.3 – q13.4 (Riegman et al., 1992; Yousef and Diamandis, 2001). Several are implicated in breast, ovarian and other human cancers (Yousef and Diamandis, 2001).The mouse kallikrein gene family is the largest family of kallikreins known to date and consists of 24 members, among which, 14 code for active proteins while the remaining are pseudogenes (Evans et al., 1987; Yousef et al., 2003).

As in humans, mouse kallikreins too are clustered together in a single locus and maps to murine chromosome 7 in a region that is of conserved synteny to human chromosome 19q13.4 where human kallikrein gene family is located (Evans et al., 1987; Yousef et al., 2003). Rat kallikrein gene family consists of 13 members, of which 10 code for active genes, while the remaining 3 genes are pseudogenes (Wines, 1989; Southardsmith et al., 1994; Yousef et al., 2003) They are clustered together within a single 440 kb chromosomal region in chromosome 1.

The functional diversity among kallikreins is emphasized by their diverse, tissue specific expression patterns. Kallikreins are implicated in patho-physiology of brain, kidney, respiratory, gastrointestinal, and reproductive systems (Bhoola et al., 1992). The diverse expression pattern of kallikreins suggests that the functional role of this enzyme family is dependent upon cell type (Yousef and Diamandis, 2001). Although enzymatic activities and functional roles of most kallikreins are unknown, kallikreins are being used as potential biomarkers for cancers and other malignancies (Yousef and Diamandis, 2001; Corthorn et al., 1997; Rittenhouse et al., 1998) and seem to have chymotrypsin and trypsin like enzymatic activities (Yousef and Diamandis, 2001).

Unfortunately, little was known about the structural organization and expression of the porcine kallikrein gene family. Except for partial sequences of kallikrein 4 (genbank accession # U76256) and kallikrein 1 (genbank accession # NM_001001911) genes, no other porcine tissue kallikreins had been identified thus far. These novel kallikrein genes identified will help us to evaluate the expression of tissue kallikreins in the porcine endometrium and conceptus during the estrus cycle and pregnancy to help us better understand the role of the kininogen-kallikrein-kinin system in placental development and embryonic survival throughout gestation in the pig. We have isolated and characterized the kallikrein gene family in the pig. In the process, have sequenced the entire kallikrein gene region in porcine chromosome 6 and have isolated 11 novel porcine tissue kallikrein genes. We have also characterized the expression profile of kallikreins across a comprehensive array of porcine tissues. This information would be a valuable asset in understanding the contribution of this important gene family in various patho-physiological processes in the pig.

Materials and Methods

Isolation of BAC Clones. 40 bp long overgo probes were developed for all known human kallikrein genes using sequence information available at GenBank and publicly available software (http://www.genome.wustl.edu/tools/?overgo=1). These probes were dual-labeled with [α-32P]dCTP and [α-32P]dATP, pooled, and were used to screen a high-density gridded BAC library with 6.3X coverage of the porcine genome (Pieter de Jong; http://www.chori.org/bacpac/) using protocols previously described by Sambrook et al., (Sambrook and Russell 2001). Clones identified from the initial screen were tested for the presence of individual kallikrein genes using a protocol previously described by DeSilva et al. (1998).

"In Silico" Gene Prediction. Sequence information generated for clone 69G11 was analyzed using GENSCAN sequence analysis software available at MIT

(http://genes.mit.edu/GENSCAN.html ; Burge and Karlin, 1997) to predict the coding sequences and peptides present in the BAC clone. Each open reading frame identified was compared to the non-redundant sequence database of GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and the complementary mRNA-Genomic DNA alignment program, Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/).

Expression Analysis. Tissue samples were collected from ovary, heart, prostate, kidney, salivary glands, central nervous system (CNS), liver, lung, skin, pancreas, stomach, large intestine, small intestine, lymph node, brain, spleen, trachea, uterus, testis, endometrium and thymus. Total RNA was extracted using TRIzol™ reagent (Invitrogen, Carlsbad, CA) according to manufactures protocols.

PCR Primers were developed based on open reading frames identified for the kallikrein genes using the primer 3 software (Rozen and Skaletsky, 2000). Each primer set developed was optimized and used to evaluate kallikrein gene expression.

Results and Discussion

Identification of Novel Kallikreins. The screening of high density porcine BAC library with overgo probes designed using all known human kallikreins resulted in 8 BAC clones that contained putative kallikrein gene fragments. Subsequent sequence analysis of BAC insert ends and overgo-based hybridization experiments with human kallikreins resulted in a BAC-based physical map of the porcine kallikrein region (Fernando, et al., 2004).

Based on the physical map constructed and the overgo-based hybridization and restriction enzyme-based fingerprint analysis, clone 69G11 was selected to contain all porcine tissue kallikrein genes identified by us so far. Clone 69G11 was sequenced to completion. The resulting sequence was subjected to in-silico gene predictionusing GENSCAN gene structure prediction software MIT. (Figure 1).

The resulting analysis suggested that the clone 69G11 contained 13 kallikrein genes (KLK1, KLK4, KLK5, KLK6, KLK7, KLK8, KLK9, KLK10, KLK11, KLK12, KLK13, KLK14, and KLK15) among which 11 are newly identified porcine kallikrein genes. We further confirmed the presence of these 13 open reading frames by using an independent mRNA - genomic DNA alignment program –Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). This analysis confirmed the presence of the 13 kallikrein open reading frames in the BAC sequence.

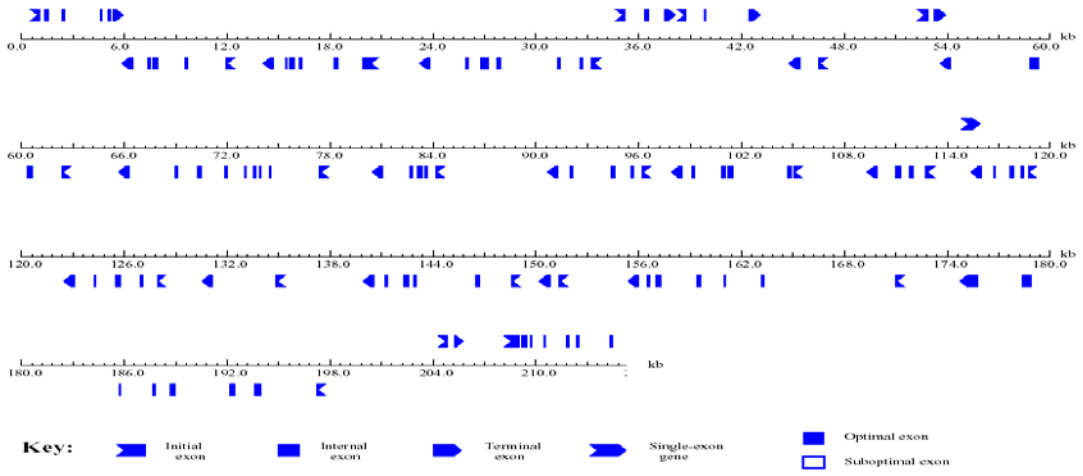

Figure 1. GENSCAN predicted coding sequences and peptides. The GENSCAN results suggests the pig kallikrein gene family to have 13 members.

Of the 13 kallikrein genes identified in this study, 11 are novel and are reported here for the first time. Similar to the observation in rodents (Yousef and Diamandis, 2001; Yousef et al. 2003), we too were unable to find any porcine orthologs for human kallikrein 2 and 3. This suggests that, the recently identified kallikrein genes in humans may have evolved after the artiodactyls radiation between 40 and 55 million years ago, and may have arisen as a result of gene duplication and/or exon shuffling events over time.

Expression Profiling of Porcine Kallikreins. Expression patterns of most kallikreins in humans have been extensively studied using Northern blotting and RT-PCR techniques (Gan et al., 2000). But, except for the expression of KLK4, the expression patterns of porcine kallikreins have not being investigated. We assayed the expression pattern of porcine tissue kallikreins over a comprehensive set of tissues using RT-PCR (Fig. 3). This approach was only intended to identify the expression profile of individual kallikrein genes and was not intended to be a quantitative measure of relative expression levels among kallikreins. All 13 kallikrein genes were expressed in the thymus while no kallikrein expression was detected in the large intestine. KLK1, KLK4, KLK6, KLK7, KLK11, KLK14 and KLK15 were expressed in a wide array of tissues while KLK5, KLK8, KLK9, KLK10, KLK12 and KLK13 were only expressed in a few tissues.

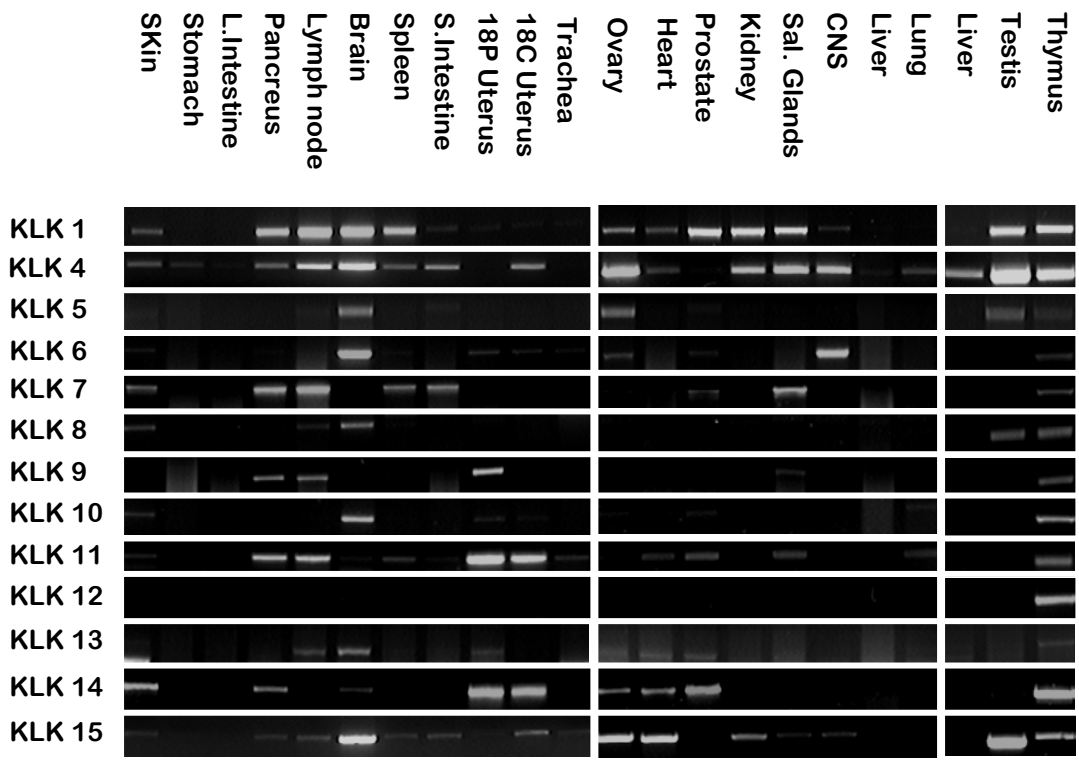

Figure 2. Global expression of porcine kallikrein genes

The profiles generated for porcine kallikrein genes suggests that all kallikrein genes are transcribed. The expression profile generated looks similar to the expression pattern found in humans. All kallikreins were expressed in the thymus suggesting that the thymus would be a good candidate tissue to study functional roles of kallikreins. The expression of kallikreins in an array of tissues suggests the involvement of tissue kallikreins in diverse physiological roles. The involvement of kallikrein genes in reproductive events in humans and rodents have been well documented (Brann et al. 1995; Chan et al., 1999; Yousef and Diamandis, 2001). The observation of high levels of expression of several kallikreins in porcine reproductive tissue suggests that kallikreins may play a similar role in pigs as well (Fernando et al., unpublished). Identification and availability of the total repertoire of porcine kallikrein genes would be a valuable asset in studying important physiological events that occur during embryonic development in the pig.

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