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Porcine SLITRK1: Molecular cloning and characterization

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A B S T R A C T

The membrane protein SLITRK1 functions as a developmentally regulated stimulator of neurite out-growth and variants in this gene have been implicated in Tourette syndrome. In the current study we have cloned and characterized the porcine *SLITRK1* gene. The genomic organization of *SLITRK1* lacks introns, as does its human and mouse counterparts. RT-PCR cloning revealed two *SLITRK1* transcripts: a full-length mRNA and a transcript variant that results in a truncated protein. The encoded SLITRK1 protein, consisting of 695 amino acids, displays a very high homology to human SLITRK1 (99%). The porcine *SLITRK1* gene is expressed exclusively in brain tissues.

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1. Introduction

Tourette syndrome (TS) is a complex developmental neuropsy-chiatric disorder characterized by persistent multiple motor and vocal tics. Tics are often accompanied by comorbidities such as attention deficit hyperactivity disorder (ADHD) and obsessive–compulsive disorder (OCD). The prevalence of TS is uncertain, but ranges from 1 to 30 per 1000 populations [1–3].

The exact cause of TS is still unknown but both genetic and environmental factors seem to contribute to its development [4]. Neuroimaging studies have identified specific brain regions, i.e. prefrontal cortex, anterior cingulated cortex, somatosensory cortex, striatum and thalamus, being involved in the pathophysiology of TS [5]. Several twin and family studies have indicated that TS is highly heritable and very likely to be genetically related [6]. Recent genome wide complex trait analysis has confirmed this by identi-fication of genetic risk factors for TS [7]. Similarly, a TS genome wide association study has identified TS susceptibility variants [8].

Among the genes associated with TS are *SLITRK1*, *CNTNAP2* and *HDC* [9–13].

Two *SLITRK1* gene sequence variants have been implicated in TS: a rare frameshift mutation giving rise to a truncated form of the SLITRK1 protein and two independent occurrences of the iden-tical variant in the binding site for microRNA189, found in the 3′-untranslated region of the *SLITRK1* gene, were associated with TS [10,14]. Among those is the non-coding variant (var321) within a conserved binding site for microRNA189. The single nucleotide variation within the 3′UTR, appears to strengthen the binding of a miR-189 with consequent downregulation of *SLITRK1* expression [10]. However, subsequent sequence analyses of Tourette patients did not detect any of these binding site variants [15–18]. Further-more, a single study identified the same 3′UTR variants in unaf-fected individuals [19] indicating that *SLITRK1* may be of limited effect in TS. A fourth variation was found in one patient with famil-ial TS, a heterozygous for a novel 708C > T polymorphism resulting in a silent mutation Ile236Ile [15]. In conclusion, it might be diffi-cult to precisely establish a clear association with TS and rare vari-ants in *SLITRK1*. The *SLITRK1* gene is an intronless gene located on human chromosome 13q31.1 [20]. The gene encodes an integral membrane protein, SLITRK1, which is a member of the SLITRK (SLIT and NTRK-like family) protein family. Members of this family are characterized by the presence of two N-terminal leucine-rich repeats (LRR) in the extracellular domain, similarly to those found in the Slit family, and a C-terminal region that shares homology with trk neurotrophin (tyrosine kinase) receptors (Fig. 1). The SLIT-RK1 protein differs from other members lacking the region of homology to neurotrophin receptors. SLITRK1 is expressed pre-dominantly in the brain, more specifically in the cortex, thalamus and the basal ganglia [21]. SLITRK1 is involved in neurite out-growth and branching [22,23]. The aim of this study was to clone and characterize the porcine *SLITRK1* gene, compare its sequence with known SLITRK1 from other vertebrates and investigate its

spatial expression. Our data show that the porcine homologue of SLITRK1 is conserved during evolution and the expression profile is similar to that in human. Interestingly, two transcript variants of *SLITRK1* were identified of which one represent a full-length transcript and another encodes a truncated SLITRK1 protein.

2. Materials and methods

2.1. Animals and tissue collection

Pigs were housed and used in compliance with European Community animal care guidelines. Beforehand, the experimental procedures were submitted to the National Ethical Committee in Denmark. The pig cerebellum and parietal cortex used for RT-PCR cloning of *SLITRK1* and various other organs and tissues employed in the expression analysis were obtained from Danish Landrace pigs adult pigs (2–3 years old).

2.2. Nucleic acids

The pig cerebellum and parietal cortex tissue used for RT-PCR cloning of *SLITRK* and other pig organs and tissues employed in expression analysis were obtained from two adult pigs. The tissues were dissected and pulverized in liquid nitrogen after removal. Total RNA was isolated by the RNeasy method (Qiagen). The integrity of the RNA samples was verified by ethidium bromide staining of the ribosomal RNA on 1% agarose gels. DNA was isolated from liver, cerebellum, parietal cortex and occipital cortex according to standard purification protocols [24].

2.3. Cloning of the porcine SLITRK1 gene and cDNA

Initially we performed a blast search analysis of the porcine genome (*Sus scrofa* 10.2), with the human *SLITRK* cDNA sequence. The search was carried out with gapped alignment using NCBI Blastall with options blastn minimum value 10^{-8}. The porcine SLITRK1 sequences identified were subsequently used to derive oligonucleotide primers for cloning of the SLITRK1 gene and SLITRK cDNA. Samples of cerebellum and parietal cortex were dissected from two Danish Landrace pigs aged 2 and 3 years and homogenized in liquid nitrogen. Total RNA was isolated by the RNeasy method (Qiagen) and RNA integrity was verified by ethidium bromide staining of 1% agarose gels. Synthesis of cDNA was conducted with 5 μg of total RNA isolated from pig cerebellum and parietal cortex using SuperScript II RNase H⁻ reverse transcriptase (Invitrogen) and oligo(dT)$_{12–18}$ primers according to the manufacturer's recommendations. The PCR and RT-PCR reaction mix contained: 2.0 μL DNA (50 ng)/2.0 μL cDNA, 1.5 mM MgCl$_2$, 0.2 mM dNTP, 0.5 μM of primers SLITRK1-F: 5′-ATGCTGCTTTGGATTCTGTTGCTG-GAG-3′ and SLITRK1-R: 5′-GGGGTCTTAGTCTGAGAGCGAGTGGGA-3′ and 1 U Phusion DNA polymerase (Finnzymes), contained in a final volume of 25 μL. The PCR conditions were: 95 °C for 2 min., 10 touchdown cycles of 95 °C for 20 s, 60 °C for 30 s, 72 °C for 45 s, followed by 25 cycles of 95 °C for 20 s, 55 °C for 30 s, 72 °C for 45 s and finally an elongation at 72 °C for 5 min. Two PCR products of approx. 2100 bp were identified by agarose gel electrophoresis and ethidium bromide staining. The recovered cDNA amplicons were cloned directly into the pCR TOPO 2.1 vector (Invitrogen) and sequenced as previously described, employing the dideoxy chain termination method using BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase FS (PE Applied Biosystems [25]. The DNA sequencing analysis was carried out with an automated DNA sequencer (ABI PRISM™ Genetic Analyzer Model 3730xl, PE Applied Biosystems).

2.4. Methylation status of the SLITRK1 gene

Two male Danish Landrace pigs from unrelated families (no parents or grandparents in common) of the age of one year were used in this study. Methylation status of *SLITRK1* was determined by library preparation, sequencing, mapping and analysis as previously described [26]. In brief, the methylation status of SLITRK1 was performed by library preparation, sequencing, mapping and analysis. DNA from each sample was extracted and sheared to a size of 200–300 bp using the Covaris Adaptive Focused Acoustics™ (AFA) process (Covaris). Double-stranded DNA fragments were end repaired, A-tailed, and ligated to methylated Illumina adaptors. Ligated fragments were bisulfite converted using the EZ-DNA Methylation-Kit (Zymo research). Following PCR enrichment, fragments of 325–425 bp were size selected and sequenced using Hiseq 2000 Illumina sequencing system. We used Novoalign short read aligner (version 2.07.12 http://www.novocraft.com/) to align reads to a reference genome. Novomethyl (Beta.8.0 http://novocraft.com/main/page.php?s=novomethyl) was used to call the consensus sequence, identify cytosines and call their methylation state or percentage of cytosines methylated. For finding the methylation percentage of special genes or sequences from our methylome data file, we used Tabix [27].

2.5. Expression analysis

Expression analysis based ion RNAseq data was performed as previously described [28]. Ten tissues from two unrelated one year old Landrace boars were included in the study. Hence, total RNA was extracted from heart, spleen, liver, kidney, lung, *musculus longissimus dorsi*, occipital cortex, hypothalamus, frontal cortex, and cerebellum employing the mirVana™ RNA extraction kit (Ambion) according to manufactures protocol, yielding a total of 20 samples. RNA integrity of the individual RNA samples was assessed on a 2% agarose gel. Library preparation was performed using the mRNA-seq library prep kit from Illumina [28]. Mapping and assembly of fragments was carried out as described previously [28]. Relative abundance of each transcript for each animal for all tissues in the unit of fragments per kilobase of exon per million fragments mapped (FPKM) were estimated.

3. Results and discussion

3.1. Cloning and characterization of the SLITRK1 gene

Using SLITRK1primers derived from porcine genomic sequences and RT-PCR a SLITRK1 cDNA representing the complete open

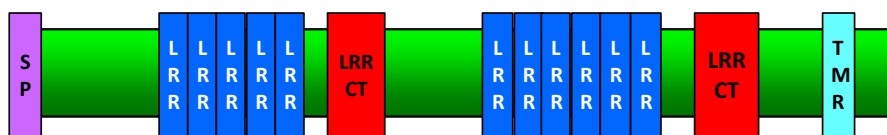**Fig. 1.** Schematic presentation of the SLITRK1 protein. In the extracellular domain, SLITRKs contains two leucine-rich repeat (LRR) domains, which are each composed of 13–17 LRRs, flanked by cysteine-rich domains. The characteristic domains of SLITRK1 are indicated by their respective names: SP, signal peptide; LRR, leucine-rich repeat; LRR-CT, LRR C-terminal domain; TMR, transmembrane region.

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                                                          ↓ SP                    LRR
SsSLITRK1    MLLWILLLLETSLCFAAGNVTGDVCKEKICSCNEIEGDLHVDCEKKGFTSLQRFTAPTSQF    60
HsSLITRK1    MLLWILLLLETSLCFAAGNVTGDVCKEKICSCNEIEGDLHVDCEKKGFTSLQRFTAPTSQF    60
MmSLITRK1    MLLWILLLLETSLCFAAGNVTGDVCKEKICSCNEIEGDLHVDCEKKGFTSLQRFTAPTSQF    60
             ************************************************************

                                    LRR
SsSLITRK1    YHLFLHGNSLTRLFPNEFANFYNAVSLHMENNGLHEIVPGAFLGLQLVKRLHINNNKIKS   120
HsSLITRK1    YHLFLHGNSLTRLFPNEFANFYNAVSLHMENNGLHEIVPGAFLGLQLVKRLHINNNKIKS   120
MmSLITRK1    YHLFLHGNSLTRLFPNEFANFYNAVSLHMENNGLHEIVPGAFLGLQLVKRLHINNNKIKS   120
             ************************************************************

                                    LRR
SsSLITRK1    FRKQTFLGLDDLEYLQADFNLLRDIDPGAFQDLNKLEVLILNDNLISTLPANVFQYVPIT   180
HsSLITRK1    FRKQTFLGLDDLEYLQADFNLLRDIDPGAFQDLNKLEVLILNDNLISTLPANVFQYVPIT   180
MmSLITRK1    FRKQTFLGLDDLEYLQADFNLLRDIDPGAFQDLNKLEVLILNDNLISTLPANVFQYVPIT   180
             ************************************************************

                                    LRR
SsSLITRK1    HLDLRGNRLKTLPYEEVLEQIPGIAEILLEDNPWDCTCDLLSLKEWLENIPKNALIGRVV   240
HsSLITRK1    HLDLRGNRLKTLPYEEVLEQIPGIAEILLEDNPWDCTCDLLSLKEWLENIPKNALIGRVV   240
MmSLITRK1    HLDLRGNRLKTLPYEEVLEQIPGIAEILLEDNPWDCTCDLLSLKEWLENIPKNALIGRVV   240
             ************************************************************

                                    LRR
SsSLITRK1    CEAPTRLQGKDLNETTEQDLCPLKNRVDSSLPAPPAQEETFAPGPLPTPSKTNGQEDHAT   300
HsSLITRK1    CEAPTRLQGKDLNETTEQDLCPLKNRVDSSLPAPPAQEETFAPGPLPTPFKTNGQEDHAT   300
MmSLITRK1    CEAPTRLQGKDLNETTEQDLCPLKNRVDSSLPAPPAQEETFAPGPLPTPFKTNGQDEHAT   300
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                            LRR
SsSLITRK1    PGSAPNGGTKIPGNWQIKIRPTAAIATGG-RNKPPANSLPCPGGCSCDHIPGSGLKMNCN   359
HsSLITRK1    PGSAPNGGTKIPGNWQIKIRPTAAIATGSSRNKPLANSLPCPGGCSCDHIPGSGLKMNCN   360
MmSLITRK1    PGAVPNGGTKIPGNWQLKIKPTPPIATGSARNKPPVHGLPCPGGCSCDHIPGSGLKMNCN   360
             **  *********** ** **  ****  ****   ******************

                                        ↓ LRR           ↓
SsSLITRK1    NRNVSSLADLKPKLSNVQELFLRDNKIHSIRKSHFVDYKNLILLDLGNNNIATVENNTFK   419
HsSLITRK1    NRNVSSLADLKPKLSNVQELFLRDNKIHSIRKSHFVDYKNLILLDLGNNNIATVENNTFK   420
MmSLITRK1    NRNVSSLADLKPKLSNVQELFLRDNKIHSIRKSHFVDYKNLILLDLGNNNIANIENNTFK   420
             ***************************************************  ******

                  ↓                  LRR
SsSLITRK1    NLLDLRWLYMDSNYLDTLSREKFAGLQNLEYLNVEYNAIQLILPGTFNAMPKLRILILNN   479
HsSLITRK1    NLLDLRWLYMDSNYLDTLSREKFAGLQNLEYLNVEYNAIQLILPGTFNAMPKLRILILNN   480
MmSLITRK1    NLLDLRWLYMDSNYLDTLSREKFAGLQNLEYLNVEYNAIQLILPGTFNAMPKLRILILNN   480
             ************************************************************

                                    LRR
SsSLITRK1    NLLRSLPVDVFAGVSLSKLSLHNNYFMYLPVAGVLDQLTSIIQIDLHGNPWECSCTLVPF   539
HsSLITRK1    NLLRSLPVDVFAGVSLSKLSLHNNYFMYLPVAGVLDQLTSIIQIDLHGNPWECSCTIVPF   540
MmSLITRK1    NLLRSLPVDVFAGVSLSKLSLHNNYFMYLPVAGVLDQLTSIIQIDLHGNPWECSCTIVPF   540
             ******************************************************** ***

                                    LRR          ↓          ↓
SsSLITRK1    KQWAERLGSEVLMSDLKCETPVNFFRKDFMLLSNDEICPQLYARISPTLTSHSKNSTGLA   599
HsSLITRK1    KQWAERLGSEVLMSDLKCETPVNFFRKDFMLLSNDEICPQLYARISPTLTSHSKNSTGLA   600
MmSLITRK1    KQWAERLGSEVLMSDLKCETPVNFFRKDFMLLSNEEICPQLYARISPTLTSHSKNSTGLA   600
             ********************************** ************************

                          LRR
SsSLITRK1    ETGTHSNSYLDTSRVSISVLVPGLLLVFVTSAFTVVGMLVFILRNRKRSKRRDANSSASE   659
HsSLITRK1    ETGTHSNSYLDTSRVSISVLVPGLLLVFVTSAFTVVGMLVFILRNRKRSKRRDANSSASE   660
MmSLITRK1    ETGTHSNSYLDTSRVSISVLVPGLLLVFVTSAFTVVGMLVFILRNRKRSKRRDANSSASE   660
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                          TMR     ↓
SsSLITRK1    INSLQTVCDSSYWHNGPYNADGAHRVYDCGSHSLSD                          695
HsSLITRK1    INSLQTVCDSSYWHNGPYNADGAHRVYDCGSHSLSD                          696
MmSLITRK1    INSLQTVCDSSYWHNGPYNADGSHRVYDCGSHSLSD                          696
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Fig. 2. Alignment of amino acid sequences of the porcine SLITRK1 protein (GenBank Access. No. KJ210858) with SLITRK1 sequences from human (NM_052910) and mouse (NM_199065). Sequence alignment was performed using the Clustal W program at Kyoto University Bioinformatics Center (http://www.genome.jp/tools/clustalw). The numbers represent the positions of the amino acids in the respective protein sequences. Identical amino acids in all three sequences are indicated by asterisks. The following abbreviations for species names are used: Ss, *Sus scrofa*; Hs, *Homo sapiens*; Mm, *Mus musculus*.

reading frame (ORF) was amplified, cloned and sequenced. The *SLIT-RK1* gene was identified by comparison of the nucleotide sequence and the deduced polypeptide sequence with human and other isolated SLITRK1 sequences. The porcine *SLITRK1* gene cloned (Fig. S1) consists of 2094 bp with the translation start found at nucleotide 1 and the TAA stop codon located at nucleotide 2086.

The ORF of porcine *SLITRK1* shows a G + C content of 51.2% and encodes a protein of 695 amino acids. The SLITRK1 polypeptide has an estimated molecular mass of 77.5 kDa and a pI of 6.0.

Amino acid sequence similarity between porcine SLITRK1 and its human and mouse counterparts was analyzed using the Clustal method (Fig. 2). The deduced porcine SLITRK1 and human SLITRK1

display significant sequence identity (99%). Multiple alignment of pig SLITRK1 with SLITRK1 sequences from other species also demonstrated significant amino acid identities e.g. mouse (97%) and rat (97%).

The deduced porcine SLITRK1 amino acid sequence contains several primary structural characteristics; two leucine-rich repeats (LRR) at amino acids 29–322 and 344–578, respectively (Fig. 2). The LRR domains, consisting of approx. 230 amino acids, are flanked by cysteine-rich regions. Both LRR domains are composed of three to four characteristic motifs LxxLxLxxN/GxL, where x is any amino acid [29,30]. The amino acid sequences of pig, human and mouse SLITRK1 are extremely conserved with the LRR domains (Fig. 2). The two LRR domains in SLITRK1 are connected by an 81 amino acid intervening sequence. Within this sequence the non-conserved amino acid residues are found. Of notice is that an amino acid residue is missing in the pig SLITRK1 sequence compared with the human and mouse counterparts. The amino acid homology is also highly conserved in the transmembrane region (amino acids 614–641), the sequence being 100% identical between the three compared species (Fig. 2). Also, the carboxy-terminal end of SLITRK1 is highly homologous. The hydrophobicity profile of the porcine SLITRK1 protein, shown in Fig. S2, is highly similar to those of human and mouse SLITRK1 proteins [20].

3.2. Cloning and characterization of the SLITRK1 cDNA

Two SKLITRK1 cDNA were identified by RT-PCR cloning from cerebellum and frontal cortex isolated from two pigs of the age of two and three years, respectively.

Both transcripts amplified were approx. 2100 bp of size. One transcript, designated SsSLITRK1-wt in Fig. 3, displayed 100% nucleotide identity with the SLITRK1 gene. The SsSLITRK1-wt clone consisted of a coding region of 2088 nucleotides and six nucleotides of 3′UTR sequence. In addition to the full-length SLITRK1 cDNA a variant encoding a truncated SLITRK1 polypeptide was found, SsSLITRK1-tv1 (Fig. 3). The first 44 amino acids of SsSLITRK1-tv1 are completely identical with the wt amino acids sequence. Deletion of an adenosine at position 133 in the genomic SLITRLK1 sequence (GenBank ID: KJ210858), i.e. at the triplet encoding Lys45, results in a change in reading frame creating a truncated protein. The predicted amino acid sequence of the mutated SLITRK1 shows 61 nonsynonymous substitutions followed by a premature stop codon (TGA). The truncated porcine SLITRK1 protein lacks a substantial portion of the full-length protein, 651 out of 695 amino acids, including the two LRR repeats, LRRCT, LRRNT and the transmembrane region (Fig. 1). The truncated SLITRK1 protein has an estimated molecular mass of 12.1 kDa and a pI of 5.2. The carboxy terminal end of the truncated SLITRK1 protein of 107 amino acids shows a very low homology (around 40%) to cytochrome b-c1 complex subunit from horse. The truncated SLITRK1 may therefore very likely play a different role than the mature protein. However, there is presently no evidence for a different function.

A frameshift mutation in the human SLITRK1 gene was reported in an individual diagnosed with TS and ADHD [10]. A single-base deletion caused a frameshift resulting in a truncated SLITRK1 protein, which lacks a great portion of the second LRR repeat as well as its transmembrane and intracellular domains (Fig. 1). This particular variant has not been identified in pigs.

3.3. Sequence analysis of the 5′ flanking region of the porcine SLITRK1 gene

We have PCR cloned a DNA fragment containing the porcine SLITRK1 gene promoter and exon 1, and performed a sequence analysis (GenBank ID: KJ210859). The 1756-bp fragment of the porcine SLITRK1 gene contains 847 nucleotides of a putative promoter sequence (nucleotide 1-847), a 5′-untranslated sequence (5′UTR) (nucleotides 848–1750) and a short coding sequence (nucleotides 1751–1756). Using the Promoter 2.0 prediction software (http://www.cbs.dtu.dk) we confirmed the promoter nature of the 5′-flanking sequence. The putative promoter sequence of 800 bp aligned with the human SLITRK1 promoter is shown in Fig. S3. The nucleotide sequence of the genomic DNA 800 bp upstream of the transcription start site (TSS) of the porcine SLITRK1 gene was analyzed for transcription factor binding sites using the computer-based MatInspector and TFSEARCH program (http://molsun1.cbrc.aist.go.jp/htbin/nph-tfsearch) and using the transfac database. The analysis revealed neither a TATA box nor any CCAAT box in the 800 bp 5′-flanking sequence of porcine SLITRK1 (Fig. S3). However, two Sp1 binding sites (TGCACC and CCCTCC, respectively) were identified close to the TSS at positions −60 and −15, respectively. These sequences were completely conserved between the porcine and human SLITRK1 promoters. Another Sp1 site was identified at pos. −425. In addition, the sequence search demonstrated presence of putative transcription-binding sites for CdxA (TTTAATGC), and GATA-1 (CCAGATGGAT) (Fig. S3). The porcine and human SLITRK1 promoter sequences were compared by alignment of 800 nucleotides upstream TSS. A high degree of sequence homology was observed in two separate regions. A nucleotide identity of 82% was seen in the region −800 to +10 relative to the TSS. Within this region, the recognition sites for the transcription factors GATA-1 (−112) is completely conserved between the porcine and the human SLITRK1 promoters. The high sequence similarity between human and porcine SLITRK1 could indicate the existence of similar mechanisms for regulation of expression.

The sequences of the porcine SLITRK1 gene, promoter and the SLITRK6 gene have been submitted to DDBJ/EMBL/GenBank under the accession numbers GenBank: KJ210858, GenBank: KJ210859 and GenBank: KJ210857, respectively.

3.4. The SLITRK gene localizes to chromosome 11

Recently, we have used Blat software to localize the SLITRK1 gene in the S. scrofa 10.2 genome [31]. The SLITRK1 gene maps to SsChr11: 60,166,124-60,169,737 (Table S1). The human and mouse SLITRK1 genes have been mapped to chromosomes 13q31.1 and 14 of these species, respectively [21]. In silico analysis demonstrated that the chromosomal organization of the SLITRK family in human is conserved in pig. The porcine SLITRK1, SLITRK5 and SLITRK6 genes

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SsSLITRK1-wt    MLLWILLLETSLCFAAGNVTGDVCKEKICSCNEIEGDLHVDCEKKGFTSLQRFTAPTSQF  60

SsSLITRK1-tv1   MLLWILLLETSLCFAAGNVTGDVCKEKICSCNEIEGDLHVDCEKRALQVCSVSPPRLPSF  60


SsSLITRK1-wt    YHLFLHGNSLTRLFPNEFANFYNAVSLHMENNGLHEIVPGAFLGLQL---          695

SsSLITRK1-tv1   TIYFCMAIPSLDFSLMSSLTFIMRLVCTWKTMACMKSFLGLFWDCSW*           107
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Fig. 3. Splice variant of porcine SLITRK1. Amino acid sequence alignment of porcine SLITRK1 (wt) and a splice variant, SLITRK1-Sp1, hereof. (A). The SLITRK1-Sp1 encodes a truncated version of the full length protein.

are located on chromosome 11, *SLITRK3* is found on chromosome 13 and *SLITRK2* and *SLITRK4* are present on chromosome X. This conservation is also extended to the SLITRK gene family in mouse [14]. The pig *SLITRK1* gene is located approx. 850 Kb from the *SLITRK6* gene and 1.6 Mb from the *SLITRK5* gene.

3.5. Methylation status of the SLITRK1 gene

To establish global methylation profiles and obtain a quantitative measurement of the methylation status of CpG sites in porcine brain and liver tissues we applied high throughput bisulfite sequencing on the Illumina HiSeq platform. Two male Danish Landrace pigs from unrelated families (no parents or grandparents in common) of the age of one year were used in this study. Sequencing of bisulfite converted *S. scrofa* genomic DNA yielded a dataset of 1926 and 1302 million reads, equal to 194.5 and 131.5 Gbp of paired-end sequence data for liver and brain, respectively. Prior to mapping, reads with an average base quality of less than 20 (Phred score) were discarded from the dataset. To ensure accuracy only CpGs covered by at least three reads were used for further analysis and determination of methylation levels and status. For our analysis the methylation level of a particular CpG could range continuously from 0 to 100 percent, hence methylation level of CpGs or average of genomic features could assign as frequency (β). The *SLITRK1* gene and its promoter include 132 and 32 CpG sites, respectively. Our methylation dataset covers 131-128-130-131 CpGs of SLITRK1 gene and 23-22-23-21 CpGs of SLITRK1 promoter in two liver and two brain tissues, respectively, with 128 common CpGs (97%) in the gene and 21 common CpGs (66%) in the promoter were selected for further analysis. Every CpG covers by some reads which show that this CpG is methylated or unmethylated. For example one CpG covers by 10 reads that eight of them show that this CpG is unmethylated and two of them show methylated, so the methylation percentage of this CpG is 20%. For the entire gene this also is the same. Approximately 3.6 kb of the *SLITRK1* gene body, including the coding sequence and the 5′ and 3′ untranslated regions, was investigated for two different porcine tissues: occipital cortex and liver. In occipital cortex 219 methylated CpG reads were detected out of a total of 7059 reads yielding a methylation degree of 3.1% (Table S2). Similarly, in liver tissue 282 methylated reads were seen in a total of 7183 reads, i.e. a methylation degree of 3.9%. In conclusion, the methylation degree is not significantly different in liver and in brain tissue. A 849 bp DNA stretch in the *SLITRK1* promoter was also examined for methylation. In brain tissue 44 reads out of 641 were found to be methylated yielding a methylation degree of 6.9%, i.e. higher than in the gene body. Similarly, 13 reads out of 604 reads were identified in liver tissue yielding a methylation degree of 2.2% which in significantly lower than that found in brain (using the chi-square test (*P*-value <0.001)).

3.6. Evolutionary relationship of SLITRK1

To evaluate the evolutionary relationship of porcine SLITRK1 with homologues from other species we constructed phylogenetic tree using the computer software MEAGALIGN program (ClustalW method). The phylogenetic analysis demonstrated that the phylogeny of porcine SLITRK1 and human SLITRK1 is most related (Fig. S4). All The topology of the dendrogram is basically in agreement with the classic taxonomic structure of the animal kingdom.

3.7. Spatial expression of SLITRK1 mRNA

In our RNAseq expression analysis totally, 223 million fragments were sequenced, allowing us to cover approximately 80% of the genes expressed. The fragments were mapped to the high quality *S. scrofa* reference genome build 10.2 [9] using TopHat enabling the downstream isoform construction. A total of 192 million reads were aligned to the reference genome yielding an overall mapping percentage of 86% with a standard deviation of 9.4%. Following mapping of the RNA-seq reads, transcripts were assembled using Cufflinks, which also reconstructed the various isoforms present in the different porcine tissues. Moreover, the relative abundance of each transcript in fragments per kilobase of exon per kilobase of fragments mapped (FPKM) was estimated by Cufflinks. Furthermore, the transcripts were annotated to the human Refseq database. The *SLITRK1* mRNA expression was also examined by RNAseq in various selected organs and tissues from two adult pigs. *SLITRK1* transcript was detected in all examined brain tissues and a differential expression was observed (Fig. 4). A very high expression was seen in brain tissues such as occipital cortex, frontal cortex, cerebellum and hypothalamus. Very low, or no, expression was detected in non-brain organs. The equality of *SLITRK1* expression levels between different organs and tissues were tested for statistical significance using the Relative Expression Software tool (REST). In occipital cortex the *SLITRK1* expression was significantly higher compared to lung ($P = 2.0 \times 10^{-5}$), liver ($P = 1.8 \times 10^{-5}$) and spleen ($P = 5.1 \times 10^{-5}$). This was also the case when comparing cerebellum with lung ($P = 0.01$), liver ($P = 0.01$) and spleen ($P = 0.01$). Similar values ($P = 0.02$) were obtained when comparing frontal cortex and lung, liver and spleen. Non-significant differences in SLITRK1 expression when comparing other tissues and organs. The obtained expression results are very similar to those found for human and mouse *SLITRK1* [20,21]. Expression profiling of human *SLITRK1* mRNA revealed differential transcript levels in various brain tissues with highest values detected in the cerebral cortex. In addition, SLITRK1 mRNA was found to be differentially expressed in various brain tumours [21].

3.8. Identification of a potential miRNA recognition site in the 3′UTR of SLITRK1

Using the Target Scan (http://www.targetscan.org) a recognition site sequence for miR24-1 was identified in the 3′UTR of the human *SLITRK1* gene. The recognition sequence for miR189 (miR24-1) was located 675–696 nucleotides downstream the TAA stop codon of the human *SLITRK1* gene. The position of the recognition sequence was very similar to that for the porcine counterpart (pos. 681–702 downstream the TAA stop codon). The nucleotide identity within a 22 bp stretch of the porcine and the
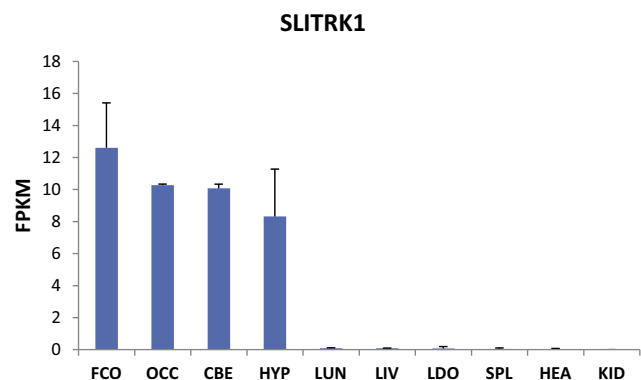

Fig. 4. Expression of porcine *SLITRK1* mRNA determined by RNAseq. Relative abundance of *SLITRK1* is reported in FPKM units. The tissues presented are: occipital cortex (OCC), frontal cortex (FCO), cerebellum (CBE), hypothalamus (HYP), heart (HEA), lung (LUN), musculus longissimus dorsi (LDO), liver (LIV), kidney (KID) and spleen (SPL). The error bars represent the biological variation between the two animals employed.

human *SLITRK1* recognition sequence for miR24-1 was 91% (Fig. S5). A porcine homologue of miR24-1 has previously been identified in our laboratory [32]. Diseases associated with miR24-1 include familial breast cancer, and cervical cancer. miR24-1 is conserved in various species, and is clustered with miR-23 and miR-27, on human chromosome 9 and 19 [33]. The porcine miR24-1 is located on chromosome 2 (Sscrofa10.2:2:65581874:65582545). A mutation in the 3′UTR of the human SLITRK gene, named variant 321, has been detected in two Tourette patients [10]. This mutation, a G to A transition, is located within the recognition site for miR189, has not been found in the porcine sequence. Of notice is that this particular nucleotide in not conserved between the pig and human sequences as shown in Fig. S5.

Recently, miR-24 has been shown to suppress expression of two crucial cell cycle control genes, *E2F2* and *Myc* in hematopoietic differentiation [34] and also to promote keratinocyte differentiation by repressing actin-cytoskeleton regulators PAK4, Tsk5 and ArhGAP19 [35].

3.9. Porcine SLITRK6

A blast search of the porcine genome (*S. scrofa* 10.2) with the human *SLITRK6* gene sequence revealed a homologue. The genomic organization of the *SLITRK6* gene is similar to that of the human homologue comprising two exons of which one contains the coding sequence. The deduced amino acid sequence predicted a protein with a molecular weight of 95.3 kDa and a pI value of 6.1. A multiple alignment of porcine SLITRK6 with other SLITRK6 proteins, shown in Fig. S6, demonstrated a very high sequence homology. The amino acid identity between pig SLITRK6 and the human and mouse counterparts was 91% and 87%, respectively.

Expression profiling of *SLITRK6* by RNAseq revealed high expression in cerebellum, hypothalamus and lung and also moderate expression in spleen and heart (Fig. S7). No expression was detected in liver, kidney and FCO. The equality of SLITRK6 expression levels between different organs and tissues were tested for statistical significance using the Relative Expression Software tool (REST). In cerebellum the SLITRK6 expression was significantly higher compared to occipital cortex ($P = 0.017$), heart ($P = 0.02$) and spleen ($P = 0.02$). Non-significant differences in SLITRK6 expression when comparing hypothalamus and lung (P-values = 0.14–0.17) and lung (P-values 0.11–0.15) with other tissues and organs. This differential *SLITRK6* expression pattern is similar to that observed in human and also partly to that of mouse, where expression is restricted to few areas of the central nervous system and a few non-brain organs such as lung and liver [20,21,36]. As for human and mouse *SLITRK6*, no expression is seen in cortex, neither frontal nor occipital (Fig. S7). The highest human *SLITRK6* expression was detected in putamen and transcript was also found in fetal brain, fetal liver, adult lung and adult brain [21]. No expression of human SLITRK6 was detected in fetal kidney and adult kidney and liver [21]. Spleen was not included in this expression analysis. In mice, a highly compartmentalized expression of SLITRK6 is observed in developing mouse brain [36–38]. SLITRK6 expression is detected in the eye, in the olfactory system, in septum, in the diencephalon (thalamus and hypothalamus), in the Purkinje layer of the cerebellum and in the spinal cord. The *SLITRK6* expression profile is different from the other *SLITRK* family members with the restricted expression in brain and expression in lung [21].

In conclusion, our study provides fundamental molecular information about the porcine *SLITRK1* gene. The *SLITRK1* gene was RT-PCR cloned and characterized and two splicing variants were found. *SLITRK1* transcript displayed a brain-specific expression. Finally, the methylation status for the *SLITRK1* gene, including a putative promoter region, was examined. The high degree of similar molecular

properties between human and pig SLITRK1 might indicate that the pig could serve as a potential model to study TS. Null mutants, i.e. knock-outs, of the *SLITRK1* gene might reproduce the genetic predispositions that favor the onset and progression of TS. By generating transgenic pigs with eliminated expression of SLITRK1 we hope to contribute to the understanding of TS etiology. Hence, in further studies we will focus on generation of transgenic knock-out pigs with no or reduced expression of SLITRK1. Porcine models of TS may help to provide information about the underlying cellular and molecular mechanisms of the disease, and for the development of more effective treatment therapies. A SLITRK1-knockout mouse model of was developed by Katayama et al. [36]. The SLITRK1deficient mice did not precisely recapitulate TS as they exhibited no motor stereotypies or tics. However, behavioral studies revealed elevated anxiety-like and depressive-like symptoms and also alterations in the noradrenergic system [36].

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2014.10.001.

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