

Characterization of the porcine *CDKN3* gene as a potential candidate for congenital splay leg in piglets

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Abstract – Congenital splay leg is a hereditary disease observed in newborn piglets. The etiology and pathogenetic mechanism of the disorder are still unknown. The gene for cyclin-dependent protein kinase inhibitor 3 (*CDKN3*) was identified as a potential candidate gene in a differential display experiment. We analyzed the gene on sequence variations and compared its expression in *M. biceps femoris* between healthy and affected piglets. Comparative sequencing of the coding region of three healthy and four splay leg piglets revealed twelve single nucleotide polymorphisms (SNP) resulting in six amino acid exchanges in the deduced sequences. However, all polymorphisms were observed in healthy as well as in splay leg piglets thus excluding structural differences of the gene as a cause of the disease. Besides full length transcripts, we found a variety of aberrantly transcribed cDNA in clones derived from *M. biceps femoris* of healthy as well as of splay leg piglets. All alternative transcripts coexist with normal cDNA. Expression analysis revealed a trend towards higher values in *M. biceps femoris* of splay leg piglets supporting the results obtained from a differential display.

***CDKN3* gene / expression analysis / congenital splay leg / piglet**

1. INTRODUCTION

Splay leg in newborn piglets was first recognized as a hereditary disorder by Thurley *et al.* [19]. A sex-affected inheritance of the disorder is assumed since higher frequencies have been observed in male piglets than in female piglets [7, 15]. A monogenic hereditary path could be excluded by statistical analyses although the existence of one or more major genes for congenital splay

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leg seems possible [16]. Affected piglets are characterized by an obvious weakness of the hind limbs immediately after birth leading to impaired mobility [1]. Consequently, losses among splay leg piglets amount to 15–50% [6,17,21]. Despite intensive research, the pathological basis of the disease is still poorly understood. Several biochemical and histo-morphological investigations indicate an immaturity of the skeletal muscle in the affected piglets at birth [1,2,5,14]. We have recently reported the isolation of skeletal muscle EST differentially expressed in *M. biceps femoris* of healthy and affected piglets [9–12]. Among the fragments strongly displayed in the splay leg muscle, we identified the porcine *CDKN3* (cyclin-dependent kinase inhibitor 3) gene [13]. *CDKN3* belongs to a family of dual-specificity protein phosphatases and is involved in cell cycling [3,4]. Mutations of *CDKN3* as well as overexpression and aberrant transcripts are related to delayed cell cycle progression and carcinogenesis in humans, respectively [3,8,22]. Because of the physiological function of the gene product and the observed variability of gene expression in human tissues, we investigated the porcine *CDKN3* gene as a potential candidate for splay leg in piglets.

2. MATERIALS AND METHODS

2.1. Origin of samples

Total RNA was prepared from *M. biceps femoris* of twenty-eight piglets (fourteen splay leg piglets and fourteen healthy controls) immediately after birth by CsCl centrifugation and stored at -80°C until further processing. From these samples, seven were randomly chosen (four splay leg, three controls) and comparatively sequenced. The remaining twenty-one samples (ten splay leg and eleven controls) were analyzed on expression differences. These samples were derived from eleven males and ten females and almost evenly distributed in both experimental groups. All animals were sampled on a single commercial farm within three weeks. The healthy controls were unrelated to the splay leg piglets. However, the relationships within both experimental groups are unknown.

2.2. Sequence analysis

The *pCDKN3* cDNA was synthesized as described elsewhere [12]. For the amplification of the coding region of the porcine *CDKN3* gene, the following primer sets were used:

CdknF: 5'GTG AGA CTG CCA GCC ATG AAG3';
CdknR: 5'TTG ATG ATA GAT GTG CAG CTA ATT T3';
KAP3F: 5'ATC GAC AGC CTG AGA GAC C3';
KAP3R: 5'TTG CCA ATA AAA GCT TTA GGA A3'.

The PCR products were cloned into the pGEM-T vector (Promega) and both strands were sequenced on an automated sequencer (ALFexpress, Amersham Biosciences). Sequence alignments were performed with the DNASIS software (v2.1, Hitachi).

2.3. Analysis of gene expression

The comparative analysis of *CDKN3* expression was done with a PCR-ELISA test (Roche) essentially as described by the manufacturer and Van den Hemel-Groten *et al.* [20]. Briefly, a 307 bp fragment of the *pCDKN3* cDNA was amplified with the following primers:

Cdkn3F: 5'GGT TTA TGT GCT CTT CCA GGT TGT A3';

Cdkn3R: 5'TCC CAA GTC CTC CGT AAC AGT GTA T3'.

In a first step, the range of quantitative (linear) amplification was determined by reducing the cycle number gradually starting with 40 cycles. The internally DIG-labeled PCR product was then bound to the streptavidin coated surface of a microplate by use of a biotin-labeled, gene specific capture probe (Cdkn3CAP: 5'BioGCT CCT GAC ATA GCC AGC TGC TGT G3'). The bound, DIG-labeled PCR products were detected with an anti-DIG-peroxidase conjugate and the substrate ABTS. The colorimetric signal allows semi-quantitative determination of the amount of PCR product. A fragment of the β -actin gene was amplified in a separate reaction for each sample and used as the external standard (primers: ActinF: 5'GGG CAG GTC ATC ACC ATC GGC AA3'; ActinR: 5'GTC CCG CCA GAC AGC ACC GTG TT3'; capture probe; ActinCAP: 5'CTA CAG AGG CGG GGG CGG GC3'). The values for individual *CDKN3* expression are given as the ratio between the absorbance units for *CDKN3* and β -actin, respectively.

Additionally, we analyzed the gene expression by Real-time PCR (ABI PRISM 7000 Sequence Detection System, Applied Biosystems). For this experiment, we used the primer pairs for *CDKN3* and β -actin described above. The fluorescent dye SybrGreen[®] was used for detection of the PCR products. For both genes, standard curves were calculated from dilution series and each individual sample was normalized to the standards. Again, the obtained normalized values for *CDKN3* expression were given as a ratio to the individual, normalized β -actin expression. The statistical analysis was performed with the General Linear Model option of the Statistica software (v6.0, StatSoft Inc.). The fixed effects health status (splay leg, control) and sex were included in the model to analyze the variables birthweight and expression of β -actin and *CDKN3* with PCR-ELISA and Real-time PCR, respectively.

3. RESULTS

3.1. Comparative sequencing of the coding region of the porcine *CDKN3* gene

The initial sequencing of the cDNA in four piglets revealed only one polymorphism in a poly(A) stretch of the 3'-untranslated region of the gene [13]. In order to detect potential differences on DNA and protein level, the coding region of the *pCDKN3* gene was sequenced in four healthy and three splay leg piglets. Sequence differences were found at twelve nucleotide positions which would lead to six amino acid substitutions in the deduced sequence (Fig. 1). All observed SNP are located in the exons 2, 3, 4 and 5, whereas the sequences of exons 1, 6, 7 and 8 were completely identical in all investigated animals. However, none of the observed SNP were exclusively found in either healthy or splay leg piglets. Moreover, sequence analysis revealed the occurrence of aberrant transcripts in healthy as well as in affected piglets. In three out of four investigated splay leg samples and in one out of three control samples, we found a total of six cDNA with either deletions of partial or complete exons or insertions of intronic sequences. All of the aberrant transcripts coexist with full length transcripts. A conceptual translation of the sequences (Fig. 2) demonstrated that the deletions observed in two splay leg samples (tr1a and tr2) result in deletions of complete amino acid blocks from an otherwise normal amino acid sequence. In all other cases the deletions/insertions led to premature stop codons and/or significant changes of the deduced amino acid sequences. In 5 out of 6 transcripts the catalytic core motif (HCXXXXGR) of the enzyme was altered or deleted.

3.2. Expression of the porcine *CDKN3* gene in *M. biceps femoris* of healthy and splay leg piglets

Gene expression was investigated in skeletal muscle samples of healthy and affected piglets of both genders. The birth weights of all groups did not differ significantly thus excluding a bias caused by this trait. Furthermore, no effect of the factor "sex" was observed on any of the expression data obtained with PCR-ELISA and Real-time PCR, respectively. The range of linear amplification was between PCR cycles 21 and 24 for β -actin as derived from PCR-ELISA and Real-time PCR analyses. This confirmed the results of Te Pas *et al.* [18] obtained with comparable reaction conditions. The corresponding cycle numbers for *CDKN3* were 31 and 33, indicating the existence of fewer amounts of *CDKN3* mRNA in the muscle samples compared to β -actin mRNA. Furthermore, the standard curves as well as the melting curve analysis confirmed the generation of a single, specific PCR product for β -actin and *CDKN3* under the applied reaction conditions, respectively.

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human 1  MEPPSSIQTS  EFDSSDEEPI  EDEQTPIHIS  WLSLSRVNCV  QFLGLCALPG
porcine 1  *K*****  *****  *****Q**  **P*****Y*  *****
variant 1  *K**V***  *****  *****Q**  **P*****C*  *****

human 51  CKFKDVRNV  QKDTEELKSC  GIQDIFVFCT  RGELSKYRVP  NLLDLYQQCG
porcine 51  *****I  *****  ***V*****  *****  *****H*Y*
variant 51  *****F  *****  ***VC*G *  *****  *****H*Y*

human 101  IITHHHPIAD  GGTPDIASCC  EIMEELTTC  KNYRKTLLHC  YGGLGRSCLV
porcine 101  *****P  **A*****  *****EI**  Q*N*****  *****
variant 101  *****P  **A*****  *****EI**  Q*N*****  *****

human 151  AACLLLYLSD  TISPEQAIDS  LRDLRGSGAI  QTIQYNYLH  EFRDKLAAHL
porcine 151  *****  *V*Q*****  *****  *****  *****
variant 151  *****  *V*Q*****  *****  *****  *****

human 201  SSRDSQSRV  SR#
porcine 201  ***E*L***  **#
variant 201  ***E*L***  **#
    
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Figure 1. Multiple alignment of the deduced amino acid sequences of *CDKN3* in human and swine. The porcine consensus sequence is shown in the second line. The sequence referred to as the “variant” combines all amino acid exchanges observed in seven animals. Asterisks denote identical amino acids and the stop codon is marked by “#”. The catalytic core motif (HCXXXGR) is underlined.

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porcine 1  MKPPSSIQTS  EFDSSDEEPI  EDEQTPIQIS  WLPLSRVNYV  QFLGLCALPG
tr1a(S) 1  *****  *****  *****  -----  -----
tr2 (S) 1  *****  *****  *****  *****C*  *****_
tr3a(C) 1  *****  *****  *****  *****  *****
tr3b(C) 1  *****  *****  *****  CAAPAAYGS*  *ARDQTG TSA
tr1b(S) 1  *****  *****  *****  *****C*  *****A
tr4 (S) 1  *****  *****  *****  #

porcine 51  CKFKDVRNV  QKDTEELKSC  GIQDVVFCT  RGELSKYRVP  NLLDLYHQYG
tr1a(S) 51  -----  -----  -----  -----  -----
tr2 (S) 51  -----  -----  *****  *****  *****
tr3a(C) 51  *****  *****  *****  *****  *****
tr3b(C) 51  ASLHHSTIKF  *IPRLFWRNL  EEISKIQKN  #
tr1b(S) 51  VTEDLGDLVL  #

porcine 101  IITHHHPIPD  GGAPDIASCC  EIMEELEICL  QNNRKTLLHC  YGGLGRSCLV
tr1a(S) 101  -----  -----  -----  -----C*  *****
tr2 (S) 101  *****  *****  *****  *****  *****
tr3a(C) 101  *****  *****  *****  *****  *****A

porcine 151  AACLLLYLSD  TVSPQQAIDS  LRDLRGSGAI  QTIQYNYLH  EFRDKLAAHL
tr1a(S) 151  *****  *****  *****  *****  *****
tr2 (S) 151  *****  *****  *****  *****  *****
tr3a(C) 151  GHRQPERPER  VW*HTDHKAI  #

porcine 201  SSRESLSRV  SR#
tr1a(S) 201  *****  **#
tr2 (S) 201  *****  **#
    
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Figure 2. Multiple alignment of porcine *CDKN3* transcript variants. The porcine consensus sequence is given in the upper line. Asterisks denote identical amino acids and the stop codon is marked by “#”. The catalytic core motif is underlined. Dashes mark missing amino acids. Premature stop codons are marked by “#” in bold face type. Transcripts (tr) derived from one animal share identical numbers with lowercase letters. The uppercase letters in brackets assign the samples to either the control (C) or splay leg piglets (S).

Table I. Birthweight and expression of β -actin and *CDKN3* in *M. biceps femoris* of healthy and splay leg piglets (LSM \pm SE) measured with PCR-ELISA and Real-time PCR. The values are comparable only within a method and for a single gene.

Group	N	Birth-weight (grams)	β -Actin (a)	PCR-ELISA <i>CDKN3</i> (b)	Ratio (b/a)	β -Actin (c)	Real-time PCR <i>CDKN3</i> (d)	Ratio (d/c)
Control	11	1.132 \pm 76	0.130 \pm 0.017	0.161 \pm 0.018	1.541 \pm 0.381	0.361 \pm 0.050	0.354 \pm 0.073	1.087 \pm 0.177
Splay leg	10	1.235 \pm 79	0.087 \pm 0.018	0.191 \pm 0.019	2.672 \pm 0.398	0.314 \pm 0.052	0.417 \pm 0.076	1.382 \pm 0.185
<i>P</i> -value		0.36	0.10	0.26	0.06	0.53	0.56	0.26

With PCR-ELISA, a statistically non-significant trend towards lower β -actin and higher *CDKN3* gene expression was observed in splay leg piglets (Tab. I). This could be confirmed by Real-time PCR and was in accordance with the more intense fragment observed in the differential display experiment for *CDKN3* [10]. The calculated relative *CDKN3* gene expression (related to the individual β -actin expression values) was slightly higher in the splay leg piglets compared to the healthy ones (Tab. I).

4. DISCUSSION

The hereditary disorder “splay leg in newborn piglets” is suspected to be caused by a delayed maturation of limb muscles [1,2] although final proof is still lacking. Thus, a potential involvement of factors related to cellular development and differentiation can be assumed. We analyzed one of these factors derived from a previous Differential Display/Reverse Transcriptase experiment [9] on its relation to congenital splay leg on the structural as well as on the expression level. Mutations in the cyclin-dependent kinase inhibitor 3 (*CDKN3*) gene were shown to have an impact on the development of several human cell types. The detected SNP in the porcine *CDKN3* gene however, were not related to congenital splay leg in piglets. None of the SNP were located within or in close vicinity to the nucleotides coding for the catalytic active site, respectively. Whether the amino acid changes have any functional impact on the function of the enzyme remains unclear. Therefore, we can exclude single nucleotide polymorphisms in this gene as the primary cause for the disease. We observed a total of six aberrant transcripts of the *CDKN3* gene in our limited sample of three healthy and four affected piglets. All but one of the observed

transcripts resulted in a destruction or a deletion of the catalytic active site suggesting a loss of function of the enzyme. Investigations on human cancerous and healthy liver tissue have revealed the occurrence of aberrant transcripts of *CDKN3* in fourteen out of twenty-seven cancerous samples and two control samples [22]. The alterations are comprised of deletions of complete exons, frame shift mutations as well as premature stop codons. Functional analyses demonstrated the inactivity of most of the derived products [22]. Although, in our study no variant was found that is identical with the human transcripts, all described types of aberrations were observed in the porcine samples, too. The coexistence of full length with aberrant transcripts in our samples was also noticed in all investigated human samples. Yeh *et al.* [22] assume a competition mechanism between the wild type and aberrant transcripts. Lee *et al.* [8] demonstrated an increased expression of the *CDKN3* gene in breast and prostate cancer. This overexpression results in a dysregulation of the cell cycle by an unknown mechanism. Direct behavior as an oncogene cannot be excluded although the coexpression of *CDKN3* and specific oncogenes seems possible. The comparative analysis of *CDKN3* expression in healthy and splay leg piglets revealed no significant differences. The observed trend towards higher values in splay leg muscle was reproducible with two independent assays and furthermore, supported by the initial results of the differential display. However, a general overexpression of *CDKN3* can be ruled out as the primary cause for congenital splay leg based on our data. Since the PCR primers used for the expression studies are both located in exon 8, no differentiation between the expression of full length and variant transcripts is possible. Therefore, further experiments should include more animals and the generation of transcript specific hybridization probes analysis allowing the separate quantification of each type of transcript. In our experiment we could detect several SNP and aberrant transcripts of the porcine *CDKN3* gene. The expression of the gene was not significantly affected in splay leg piglets. Whereas the SNP alleles are not related to the splay leg syndrome, the role of transcriptional aberrations of the gene in the pathogenesis deserves further investigations.

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