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Molecular characterization, chromosome mapping, and expression profile of porcine *CDC16*

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ABSTRACT. Cell division cycle16 (CDC16) is a core component among the eight protein subunits of the anaphase-promoting complex (APC). APC is a cyclin degradation system that governs the exit of cells from mitosis. Not much information is available for *CDC16* in pig. In this study, a 2284-bp cDNA of porcine *CDC16* was obtained by rapid amplification of cDNA ends (RACE). Porcine *CDC16* was assigned to SSC11 q11-17, and was determined to be significantly linked with SW1452 by using somatic cell hybrid panel and radiation hybrid panel. One novel *A*/*G* SNP anchored in intron 7 of the gene was genotyped by restriction enzyme polymerase chain reaction (PCR)-restriction fragment length polymorphism-*Csp6*I. In five pig breeds, Shaziling, Taoyuan, *Duroc*, Landrace, and Yorkshire, the *A* allele frequency was dominant. Quantitative PCR revealed that porcine *CDC16* was expressed in ten selected tissues of 25-day-old Shaziling and Yorkshire piglets, and that the mRNA expression of *CDC16* in longissimus dorsi muscle of Shaziling was higher than that of Yorkshire. Expression levels of *CDC16* were highest in longissimus dorsi muscle followed by that in pancreas. CDC16 protein was detected in longissimus dorsi muscle of 25-day-old Shaziling and Yorkshire piglets by immunohistochemistry with

abundant protein expression index (P > 0.05). This study provides an insight into the role of porcine *CDC16* in the formation of meat.

Key words: Pig; *CDC16*; Chromosomal localization; Expression profile; Immunohistochemistry

INTRODUCTION

Cell division cycle 16 is a protein encoded by the gene *CDC16* in humans (Tugendreich et al., 1995). This protein is one of the components of anaphase-promoting complex (APC) which is composed of eight proteins, namely, BimE (APC1), APC2, CDC27 (APC3), APC4, APC5, CDC16 (APC6), APC7, and CDC23 (APC8), and it functions as a protein ubiquitin ligase (Yu et al., 1998). APC is a cyclin degradation system that governs the exit of cells from mitosis. Each component protein of the APC is highly conserved among eukaryotic organisms. CDC16 contains a tetratricopeptide repeat (TPR), a protein domain that may be involved in protein-protein interaction (Ollendorff and Donoghue, 1997; Kallio et al., 1998; Vodermaier et al., 2003). CDC16 has been demonstrated to interact with CDC27 and CDC20 (Gmachl et al., 2000; Nilsson et al., 2008).

A recent report suggested that CDC16 plays an important role in differentiation of epithelial cells in the hair follicle (Ollendorff and Donoghue, 1997). Immunofluorescence analysis revealed that, during mitosis, *CDC16* accumulated in the central body including the spindle (Tugendreich et al., 1995). In an RNA interference (RNAi) screen utilizing endoribonuclease-prepared short interfering (esi) RNA library, *CDC16* was demonstrated to be involved in cell division (Kittler et al., 2004). By fluorescence *in situ* hybridization, human *CDC16* was found to be localized on *HSA*13q34 (Kallio et al., 1998; Kallio et al., 2002). The gene was demonstrated to be located on mouse (*Mus musculus*) chromosome 8A1.1-1.2 (Skarnes et al., 2011) and on rat (*Rattus norvegicus)* chromosome 16q12.5 (Steen et al., 1999). The aim of the present study was to gain an insight into the function of porcine *CDC16* during processing of the meat.

MATERIAL AND METHODS

Isolation of cDNA

Total RNA was isolated from longissimus dorsi muscle of one, 25-day-old, weaned Shaziling piglet using Trizol reagent (Invitrogen, Karlsruhe, Germany). The isolated RNA was reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Promega, USA), according to the manufacturer instructions; the RT-reaction product was used as a template for further analysis. Several pig ESTs were initially identified using the cDNA sequence of human *CDC16* mRNA (NM_004661) by a BLASTN search against the GenBank EST databases by Sequence alignment. The porcine ESTs sharing at least 80% identity to the corresponding human mRNA were selected for designing gene-specific primers (Table 1). The PCR products were purified with a Gel Extraction Mini Kit (Qiagen, Hamburg, Germany), cloned into pGEMT plasmid (Promega, USA), sequenced by using random amplification of cDNA ends (RACE) methods.

Table 1. Primer sequences for PCR amplifications.

Primer's purpose	Primer name	Primer sequence (5'-3')	Tm (°C)	Product size (bp)
Cloning	RC11-1F	GGAAATGTCACAGTCCTCAATA	58	1086
	RC11-1R	CAGAAAATGTATCATCTCGCCT		
	RC11-2F	TGAGGTGACAGTTGACAAATGGGA	58	816
	RC11-2R	CCATAGGCTACATCCATAGTCTGA		
Gene mapping	M-F	GCGCAGTATC ATAGAGCAGC	58	743
	M-R	GGTAGCGACA CGCTTCATAC		
Polymorphism	Genomic-F	GACACGGCAACATGCTAG	50.5	696
	Genomic-R	TTGGGATGGAAGAAGACC		
Expression profile	CDC16-F	CCGAGACACTGAATTTTG	60	51
	CDC16-R	ACTGAGGTTAGTCTGCTAM		
Internal control	GAPDH-F	ATTTGGCTACAGCAACAGGGT	59	172
	GAPDH-R	AAGTCAGGAGATGCTCGGTGT		

Mapping of porcine *CDC16*

A somatic cell hybrid panel (SCHP) was used for chromosomal mapping and an INRA-University of Minnesota porcine radiation hybrid (IMpRH) was employed for ascertaining the precise location of porcine *CDC16*. Primers, corresponding to exon 4 and 5 and intron 4 of pig *CDC16*, were designed (Table 1). A 743 bp PCR product for *CDC16* was used to type SCHP and IMpRH panel (Yerle et al., 1999, 2001). The PCR products were visualized on 2% agarose gel stained with ethidium bromide. The PCR results were statistically analyzed using SCHP and IMpRH mapping tools, accessible through the websites, http://www2. toulouse. inra.fr/lgc/pig/pcr/pcr.htm and http://www. imprh. toulouse. inra.fr, respectively (Milan et al., 2000).

Novel SNP identification using PCR-RFLP test

Samples were collected from eight breeds of pig viz. *Duroc* (N = 62), Landrace (N = 45), Yorkshire (N = 443), Shaziling (N = 62), Daweizi (N = 63), Taoyuan (N = 70), Ningxiang (N = 67), and Wuzhishan (N = 54) provided by Hunan Xinwufeng Breeding Company in Pig (Xiangtan City, Hunan Province, China) and Resource Field of Chinese native pig. Genomic DNA extracted using EasyPure Genomic DNA Kit (Transgen Biotech, Beijing, China) was amplified and sequenced directly for SNP identification. For genotyping, the PCR was performed in a 20 µL mixture consisting of 100 ng genomic DNA, 25 pmol primers, 75 µM dNTPs, 1.5 mM $MgCl_2$, and 0.5 U *Taq* DNA polymerase. The PCR conditions were: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 50.5°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. PCR product (4 µL) was digested overnight with 3 U *Csp6*I (Thermo Fisher Scientific, USA) at 37°C, electrophoresed on a 2.0% agarose gel and visualized by staining with ethidium bromide.

Expression profile of *CDC16*

Total RNA was isolated from heart, liver, spleen, lung, kidney, intestine, cecum, pancreas, crureus, and longissimus dorsi muscle of three, 25-day-old, weaned Yorkshire piglets and three weaned Shaziling piglets of the same age. These two pig breeds are full-sib. The cDNA from the above ten tissues obtained after reverse transcription was used as template in quantitative PCR, using SYBR Green I method (Livak and Schmittgen, 2001). The reaction mixture (25 µL) contained 2 µL RT-PCR product, 12.5 µL 2X SYBR Premix Ex Taq II, 0.5 µL each *CDC16*-F and *CDC16*-R

primers, and 9.5 µL RNase-free double-distilled H_2O. The reaction conditions were: 95°C for 30 s followed by 40 cycles of 95°C for 50 s and 60°C for 30 s and 72°C for 30 s. Average results from three technical replicates per test sample were used for analysis. The $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) was used to calculate the relative expression of the target gene. GAPDH (glyceraldehydes-3-phosphate dehydrogenase) gene was used as an internal control. The cecum sample from Yorkshire was defined as the reference, and internal control primers were designed according to the pig mRNA (Table 1).

Immunohistochemical experiment

For immunohistochemical experiments (Fregly and Graybiel, 1968; Nishiwaki et al., 2000), the longissimus dorsi muscle tissue was embedded in paraffin after fixing in 4% paraformaldehyde. Sections (4 µm thick), were dehydrated by passing through an alcohol series. They were incubated at 4°C overnight with bs-7832R monoclonal antibody (1:100 dilution) specific for CDC16 protein and washed 3 times with phosphate buffered saline (PBS) for 5 min. Thereafter, the sections were stained with hematoxylin and eosin, as described previously (Cerri and Sasso-Cerri, 2003). Meat tissue micrographs were taken at a magnification of 400X with an Axio Imager Z1 (ZEISS, Germany).

RESULTS

Cloning and characterization of *CDC16* cDNA

The 2284 bp porcine *CDC16* cDNA (GenBank accession No. KC795824) was obtained by RACE. It contained an 1863 bp open reading frame (ORF) flanked by a 38 bp 5'-untranslated region (UTR) and a 383 bp 3'-UTR, which encoded the deduced 620 amino acids. The molecular weight of CDC16 was about 188.3 kDa, and its calculated isoelectric point (PI) was 4.92. Sequence alignment showed that the complete cDNA sequence shared 92 and 83% identity with human (NM_003903.3) and mouse (NM_027276) sequences, respectively.

Map assignment of porcine *CDC16*

By the somatic cell hybrid panel, *CDC16* was mapped to *Sus scrofa* chromosome (SSC) 11q11-q17, with the probability of regional localization being 0.9998, correlation 0.8563 and error risk < 0.1%. Further mapping was conducted using the radiation hybrid panel IMpRH. The statistical analysis revealed that *CDC16* was significantly linked to microsatellite SW1452 with a distance of 56cR and LOD score of 16.08 on chromosome 11 (Figure 1). Human *CDC16* was assigned to HSA 13 (Johansson et al., 1995). Our results were consistent with previous results because human chromosome 13 is homologous with porcine chromosome 11 [http://www2.toulouse.inra.fr/lgc/pig/compare/SSC.htm; Chevalet et al. (1997)].

Polymorphism detection

A 696 bp genomic fragment spanning the intron 7 of *CDC16* was amplified and one novel SNP was genotyped with PCR-RFLP-*Csp*6I (696 bp for allele *A*, and 570 and 126 bp for allele *G*; Figure 2). The results indicated that the frequency of allele *A* was greater than 0.500, in Yorkshire, Landrace, Duroc, Taoyuan, and Shaziling pigs (Table 2). In Daweizi, Ningxiang, and Wuzhishan

pig, the frequency of allele *G* was lesser than 0.500, and in the three European breeds, *Duroc*, Landrace and Yorkshire, alleles *A* was dominant. In Taoyuan pig, the frequency of allele *A* was highest (0.7570).

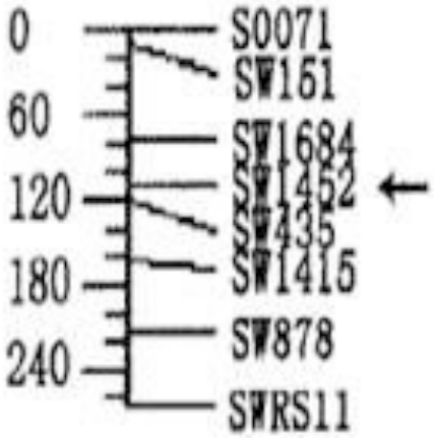

Figure 1. Radiation hybrid mapping of pig CDC16 onto SSC11.

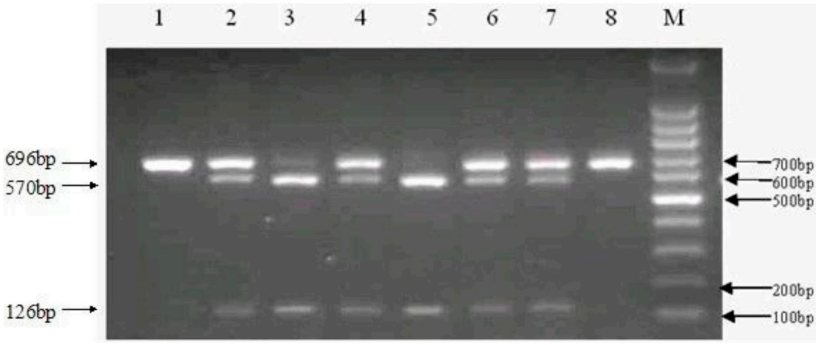

Figure 2. Novel single nucleotide polymorphism (SNP) detected in CDC16. Polymerase chain reaction (PCR) products were digested with *Csp*6I restriction enzyme to distinguish different alleles. The picture shows electrophoresis of different genotype samples on a 2% agarose gel (*lanes 1-8*). M: 100 bp DNA ladder.

Table 2. Frequency of allele and genotype of CDC16 about PCR-RFLP-Csp6 I among different pig breeds.

Breed	Sample size	Genotype			Frequency of genotype			Frequency of allele		χ^2
		AA	AG	GG	AA	AG	GG	A	G	
Ningxiang	67	13	41	13	0.1912	0.6029	0.2059	0.4926	0.5074	2.8897
Daweizi	63	2	38	23	0.0317	0.6032	0.3651	0.3333	0.6667	8.0357
Shaziling	62	17	31	14	0.2742	0.5000	0.2258	0.5242	0.4758	0.0003
Taoyuan	70	38	30	2	0.5429	0.4286	0.0285	0.7570	0.2430	1.9143
Wuzhishan	54	5	34	15	0.0926	0.6296	0.2778	0.4074	0.5926	4.9897
Yorkshire	443	231	185	27	0.5215	0.4176	0.0609	0.7302	0.2698	1.5945
Landrace	45	24	17	4	0.5333	0.3778	0.0889	0.7222	0.2778	0.1538
Duroc	62	35	18	9	0.5646	0.2903	0.1451	0.7097	0.2903	5.4122

Expression profile analysis

Quantitative PCR showed that the porcine *CDC16* expressed in ten tissues of 25-day-old Yorkshire and Shaziling pigs (Figure 3). The constitutively expressed *GAPDH* was used as an endogenous reference in the analysis. The level of the *CDC16* expression in intestine of Yorkshire pig was defined as 100 and the mRNA expression level in different tissues was quantified relative to it. *CDC16* mRNA expressed in all the ten tissues, including intestine, caecum, heart, liver, kidney, lung, spleen, pancreas, crureus, and longissimus dorsi muscle. Highest expression was observed in the longissimus dorsi and the lowest in the lung and kidney of the Shaziling and Yorkshire pigs, respectively ($P < 0.01$). *CDC16* expression in the cecum and heart of Yorkshire and Shaziling pigs was significantly different ($P < 0.01$). *CDC16* was widely expressed in the assessed organs of Yorkshire and Shaziling pigs. It may be related to porcine cytokines and hormones involved in intracellular signal transduction.

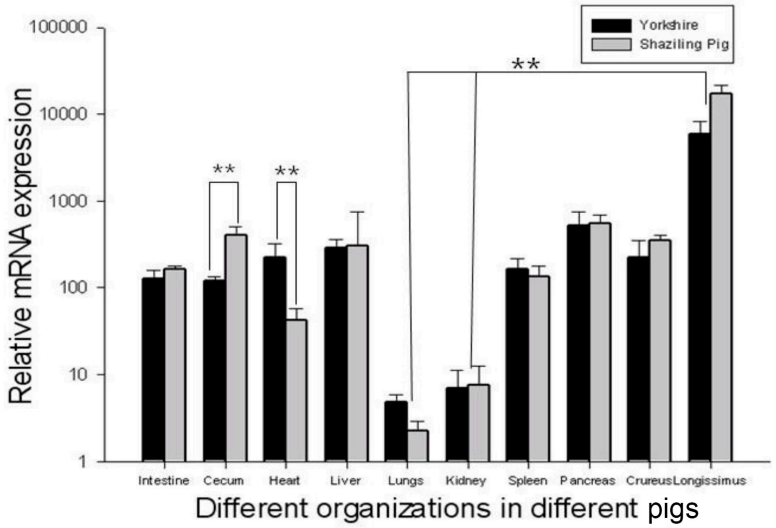

Figure 3. CDC16 expression in ten tissues of 25-day-old Yorkshire and Shaziling pigs evaluated by qRTPCR. The expression level was normalized to GAPDH and measured by $2^{-\Delta\Delta Ct}$. Results from three independent replicates in different tissues were averaged. **Significant difference between the two breeds ($P < 0.01$).

Histochemistry of CDC16 protein in longissimus dorsi

In this study, CDC16 protein was confirmed as a membrane protein and was detected in longissimus dorsi muscle tissue of Shaziling and Yorkshire piglets by immunohistochemistry which revealed abundant protein expression index (PEI) ($P > 0.05$). It is interesting that qPCR results for *CDC16* expression were contrary to that of histochemistry. This signified that the expression of *CDC16* was different from that of its protein in longissimus dorsi (Figure 4). In Yorkshire piglets, average optical density was 0.34, whereas in Shaziling, it was 0.37. According to the statistical analysis, no significant difference was observed between the two average optical densities ($P > 0.05$).

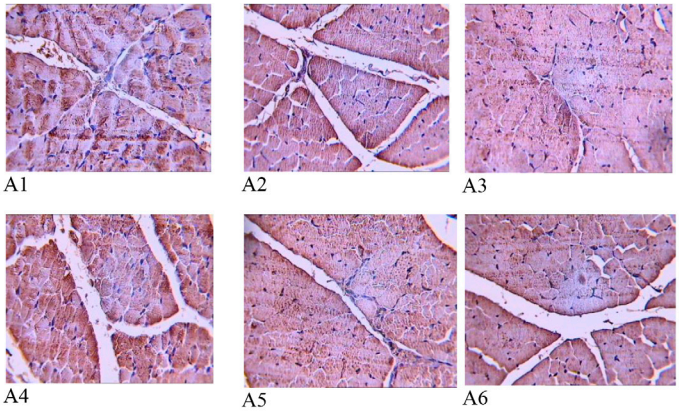

Figure 4. Expression of CDC16 protein in longissimus dorsi muscle tissue. A1-A3 indicate the expression in Yorkshire piglets. A4-A6 indicate expression in Shaziling piglets. Blue specks in the pictures denote the location of the CDC16 protein. All pictures were taken at a magnification of 400X.

DISCUSSION

Human *CDC16* is made up of 18 exons and 17 introns and its 5'-UTR is 186 bp, 3'-UTR is 232 bp, full length of the gene is 43.8 kb, and full-length of cDNA is 2461 bp (Tugendreich et al., 1995). Mouse *CDC16* consists of 7 exons and 6 introns with 110 bp 5'-UTR, 299 bp 3'-UTR, gene length 12676 bp, cDNA length 2253 bp, and ORF length 1763 bp. In this study, we performed RACE-PCR to obtain a 2284 bp full-length porcine *CDC16* cDNA having 1863 bp ORF flanked by a 38 bp 5'-UTR and 383 bp 3'-UTR that putatively encoded for 675 amino acids.

Our report indicates that allele *A* was predominant in the three European and Chinese indigenous breeds of Taoyuan pig, and the highest *A* gene frequency was 0.7570 in Taoyuan pig, whereas the highest *G* gene frequency was 0.6667 in Daweizi pig. The *AA* genotype frequency of Chinese breeds was superior to that of European breeds, whereas in native pigs, heterozygous *AG* genotype frequency was superior, except for the Taoyuan pigs.

The relative mRNA expression of *CDC16* varied in different tissues. *CDC16* mRNA expression was observed in the intestine, caecum, heart, liver, kidney, lung, spleen, pancreas, crureus, and longissimus dorsi, and significant differences existed among the different organs ($P < 0.05$). Highest expression was observed in the longissimus dorsi and the lowest was in the lung and kidneys of the Shaziling and Yorkshire pigs. Rest of the organs, especially the cecum and heart of Yorkshire and Shaziling pigs has high expression that was significant different ($P < 0.01$). *CDC16* in Yorkshire and Shaziling pigs was widely distributed in tissues, and it could be related to porcine cytokines and hormones in the body, that function during intracellular signal transduction. *CDC16* has been reported to play a key role in cell division. In the present study, *CDC16* mRNA expression was estimated in ten tissues of Shaziling and Yorkshire. Its expression in longissimus dorsi was higher than in other tissues, and the lowest expression was in lungs and kidney, indicating that *CDC16* could be associated with muscle growth, playing an important regulatory role during muscle development in pigs. The cecum and heart showed highly significant differences between Yorkshire and Shaziling piglets, which may be caused by the discrepancy in the initial development in the different breeds. It has an important significance to account for the genetic differences between native Chinese and European pigs.

Immunohistochemistry was carried out to quantitatively characterize the expression of proteins related to the meat quality. In histological examination, muscle phenotype variation was apparent at the 25-day stage. Moreover, there is fiber type-transition and muscle maturation process from the birth till the age of two months (Lefaucheur and Vigneron, 1986). Thus, *CDC16* might contribute to the formation of muscle fibers in Yorkshire and Shaziling pigs.

CONCLUSIONS

In summary, the complete cDNA of pig *CDC16* was isolated and characterized and one novel SNP in intron 7 was identified in *CDC16*. It was closely linked with microsatellite SW1452 of the SSC11. The spatial expression profiles of pig *CDC16* mRNA displayed its abundance in longissimus dorsi with significant differences between Shaziling and Yorkshire pig. However, the expression of CDC16 protein in longissimus dorsi was not significantly different between these two breeds. This suggests that the expression of *CDC16* was different at the level of transcription and translation in longissimus dorsi.

Conflicts of interest

The authors declare no conflict of interest

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