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Sequence Characterization, Expression Profile, Chromosomal Localization and Polymorphism of the Porcine *SMPX* Gene

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ABSTRACT : The full-length cDNA of the porcine *SMPX* gene was obtained by the rapid amplification of cDNA ends (RACE). The nucleotide sequences and the predicted protein sequences share high sequence identity with both human and mouse. The promoter of *SMPX* was sequenced and then analyzed to find the promoter binding sites. The reverse transcriptase-polymerase chain reaction (RT-PCR) revealed that *SMPX* has a high level of expression in heart and skeletal muscle, a very low expression in lung and spleen and no expression in liver, kidney, fat and brain. Moreover, *SMPX* has a differential expression level in skeletal muscle, the expression in 65-day embryos being higher than other stages. The porcine *SMPX* was mapped to SSCXp24 by using a somatic cell hybrid panel (SCHP) and was found closely linked to *SW1903* using the radiation hybrid panel IMpRH. An A/G single nucleotide polymorphism (PCR-RFLP) in the 3'-untranslated region (3'-UTR) was detected in eight breeds. The analysis of allele frequency distribution showed that introduced pig breeds (Duroc and Large White) have a higher frequency of allele A while in the Chinese indigenous pig breeds (Qingping pig, Lantang pig, YushanBlack pig, Large Black-White pig, Small Meishan) have a higher frequencies of allele G. The association analysis using an experimental population (188 pigs), which included two cross-bred groups and three pure-blood groups, suggested that the SNP genotype was associated with intramuscular fat content. (**Key Words :** Sequence Analysis, Physical Mapping, Polymorphism, Promoter, Porcine, *SMPX*)

INTRODUCTION

The small muscle protein X-link (*SMPX*/*Csl*) gene was firstly identified from human striated muscle, and might play a role in the cardiac or muscular development (Patzak et al., 1999). *SMPX*, a muscle-specific gene, was dramatically up-regulated in response to passive stretch in *vivo*, so it could be involved with the regulation of muscle fiber development (Kemp et al., 2001). *SMPX* was prominently expressed in cardiac and skeletal muscle in embryonic and postnatal stage. Its down regulation in *Csx*/*Nkx2-5*-null embryonic heart suggests that it is a target gene for *Csx*/*Nkx2-5*. *SMPX* could enhance transcriptional activities of the myocyte-specific enhancer factor 2 (*MEF2*) and nuclear factor of activated T cells (NFAT) in an *IGF-1* signal-dependent manner (Palmer et al., 2001). Both *MEF2* and *NFAT* are important for the differentiation and hypertrophy of cardiac and skeletal muscles. When *SMPX* is expressed in C2C12 myoblasts, it associates with focal adhesion complexes and can modify cell shape and actin dynamics in a *Rac1* and p38 kinase-dependent manner (Schindeler et al., 2005). Furthermore, it is interesting that *SMPX* is highly expressed in red muscles than white muscles (Bai et al., 2003). In brief, the function of *SMPX* encode protein was not clear, but the expression and mapping results indicated that *SMPX* could play a critical role in the regulation of muscle fiber and heart development (Ervasti, 2003). *SMPX* which affects muscle fiber development may be a candidate gene related to muscle growth and quality in domestic pigs.

In this study, we obtained the porcine *SMPX* full-length cDNA, and part of *SMPX* promoter sequence. We then mapped it and analyzed its expression distribution in different tissues and at different stages. We also report an association of nucleotide variation of this gene with intramuscular fat content.

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Table 1. Primer pairs for porcine *SMPX* fragments isolation

Primer purpose	Primer name	Primer sequence (5'-3')	Banding region	Size (bp)	TM (°C)
RACE	3'RACE	GCGGTCAACCTATCGGAGATCCAGAACAT	CDS	571	68
	5'RACE	CTGGATCTCCGATAGGTTGACCGCAGGT	CDS	477	68
	5'NEST	GAATTGGCTTCTTCTCCTCATCT	CDS	405	60
5'-Promoter	P1L	CCAAGTGACCAAGCTAGGATTC	Promoter	489	59
	P1R	GTGTCCTCTGAGCTGCGATCT	5'UTR		
Expression	P2L	CAACCTATCGGAGATCCAGAACAT	CDS	330	61
Profiles	P2R	TCCCTCCTCAAAAACCACACC	3'UTR		
Mapping	P3L	CCTGAGACTCTAGCAGAAATGTCC	3'UTR	151	65
	P3R	TCCCTCCTCAAAAACCACACC	3'UTR		
Polymorphism	P4L	CGGAATGCCTGAGACTCTAGCAGAAATGTACC	3'UTR	158	64
	P4R	TCCCTCCTCAAAAACCACACC	3'UTR		

MATERIALS AND METHODS

Isolation of full-length cDNA of porcine *SMPX*

The porcine *SMPX* full-length cDNA was obtained by the rapid amplification of cDNA ends (RACE). BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/) was performed using the human *SMPX* cDNA sequence (GenBank accession number: 014332.1) in order to obtain porcine ESTs which shared at least 80% sequence homology. Then these ESTs were assembled into a contig for primers design (Yang et al., 2005). Total RNA of a mature Landrace pig was extracted from skeletal muscle tissue with a TRIzol Reagent Kit (Life Technologies, Grand Island, NE, USA), then treated with RNase-free DNase I (Promega, Madison, WI, USA) and precipitated with ethanol. RACE was performed according to the instructions of the SMART™ RACE cDNA Amplification Kit (Clontech Inc, Palo Alto, CA, USA). The PCR products were purified with Gel Extraction Mini Kit (Waston Biotechnologies Inc, Shanghai, China) and cloned into the pMD18-T vector (TaKaRa, Dalian, China), then sequenced by commercial service. Finally, the sequence was submitted to NCBI (GenBank accession no. DQ104414).

Isolation of the porcine *SMPX* promoter

Comparative anchor tagged sequences (CATS) is an important method to isolate the unknown sequences of genes (Kenealy et al., 1998). By using CATS, the forward (P1L) and reverse (P1R) primers were designed according to the human promoter and porcine *SMPX* exon 1 sequences respectively (Table 1). PCR was performed in 20 μl reaction mixture containing 1×PCR buffer, 1.5 mM $MgCl_2$, 75 μM dNTP, 0.3 μM of each primer (P1L, P1R), and 1 unit Taq DNA polymerase (TaKaRa). PCR amplification conditions were 95°C for 4 min, followed by 35 cycles of 94°C for 40 s, 59°C for 40 s, 72°C for 40 s, and a final extension step of 5 min at 72°C. Then PCR product were purified and sequenced. Finally, the sequence was submitted to NCBI (GenBank accession no. DQ104415).

Sequence analysis

The ORF of porcine *SMPX* gene were found and the amino acid sequences were deduced with the EditSeq program (DNA Star, Madison, WI, USA). The motifs of the putative protein were analyzed with PSORTII (http://www.nibb.ac.jp) and ScanProsite (http://au.expasy.org/tools/scanprosite/) programs. The putative promoter motifs were analyzed with the tools available on the web site, Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME) (Wingender et al., 2000).

Expression profile analysis

Total RNAs were extracted from adult porcine heart, skeletal muscle, lung, liver, spleen, kidney, fat and brain respectively. In addition RNAs were extracted from skeletal muscles at different stages: 33-day, 65-day, 90-day embryos, birth, 28-day and adult Tongcheng pigs. Reverse transcriptions were performed as described by Pan et al (2003) using the primer pairs P2L and P2R (Table 1). PCR conditions were 4 min at 95°C followed by 27 cycles of 30 s at 94°C, 30 s at 61°C, 30 s at 72°C, and a final extension of 5 min at 72°C. Amplification of *GAPDH* was performed as an internal control in the same conditions, and *GAPDH* primers were synthesized according to the reported sequence (Janzen et al., 2000). Finally 8 μl of each PCR products were used for the expression profile analysis on 2.0% agarose gels.

Somatic cell hybrid and radiation hybrid mapping

The somatic cell hybrid panel SCHP (Yerle et al., 1996) was used to cytogenetically map *SMPX* on porcine chromosomes and its position was then refine using the radiation hybrid panel IMpRH (Yerle et al., 1998). PCR reactions were performed in a volume of 10 μl of 1×PCR buffer (TaKaRa), containing 20 ng of hybrid DNA (either SCHP or IMpRH samples), 0.2 μM of each primer (P3L, P3R) (Table 1), 100 μM of each dNTP, 1.5 mM $MgCl_2$ and 2 units Taq DNA Polymerase (TaKaRa). The PCR conditions were 3 min at 94°C, followed by 35 cycles of 30

Figure 1. Part of putative promoter sequence 381 bp and putative transcription factor binding sites are underlined and in boldface type. The MEF-2 binding site is in italics and boldface type.

s at 94°C, 30 s at 65°C, 15 s at 72°C, and an extra 5 min extension at 72°C. In addition, PCR reactions were carried out with pig genomic DNA (as positive control) and without DNA (as negative control). Mapping results were analyzed using the tools available at (http://www.toulouse.inra.fr/lgc /pig/hybrid.htm) (Chevalet et al. 1997) and (http://imprh. toulouse.inra.fr/) (Milan et al., 2000) for SCHP and RH mapping respectively.

Genetic variation analysis and traits association analysis

A 158 bp genomic fragment was amplified using the primers P4L and P4R. Polymorphism was detected in *SMPX* by PCR-RFLP and analysis on 3.0% agarose gels. PCR fragments from different genotypes were cloned and sequenced. DNA samples of 199 unrelated pigs from eight breeds (Duroc, Large White, Min pig, Qingping pig, Lantang pig, YushanBlack pig, Large Black-White pig and Small Meishan) were genotyped for genetic variation analysis and χ^2 test on the allele frequencies was performed with population genetic structure comparison performed using SAS version 8.1. An experimental population (188 pigs) including two cross-bred groups and three pure-blood groups, Large White×(Landrace×Tongcheng) (43 individuals), Landrace×(Large White×Tongcheng) (51 individuals), Tongcheng breed (33 individuals), Landrace (29 individuals) and Large white (32 individuals), were selected for association analysis. The association between genotype and traits (carcass traits and meat quality) was performed with the least square method (GLM procedure, SAS version 8.1). The model used to analyze the data was assumed to be: $Y = \mu+B_i+P_j+G_k+e_{ijk}$. Where, Y is the observation of the trait; μ is the least square mean; B_i is the effect of breed, P_j is the effect of batch (j = 1 to 8); G_k is the effect of genotype (k = AA, AG, GG); e_{ijk} is the random residual.

RESULTS

***SMPX* full-length cDNA isolation**

Based on bioinformatics analysis, 10 porcine ESTs (Acc.No: BX664815, BX667327, BX666546, BX664976, BE014221, AJ654762, CO938466, AJ656431, BE013988 and CA778404) were obtained and these overlapping ESTs were assembled into one contig covering 842 bp. The primers for RACE were designed in the contig. The size of the 5'RACE and 3'RACE products were 405 bp and 571 bp respectively. Bioinformatics analysis of the combined nucleotide sequence revealed that there was a 261 bp ORF flanked by a 209 bp 5'-UTR and a 450 bp 3'-UTR. A polyadenylation signal (AATAAA) was detected in the 3'-UTR region. Sequence comparisons revealed that the porcine *SMPX* sequence is respectively 85% and 79% identical to the human (NM_014332) and mouse (NM_025357) corresponding sequences. PSORTII analysis showed that the *SMPX* gene encoded 86 amino acids with a calculated molecular mass of 9.34 kDa and isoelectric point

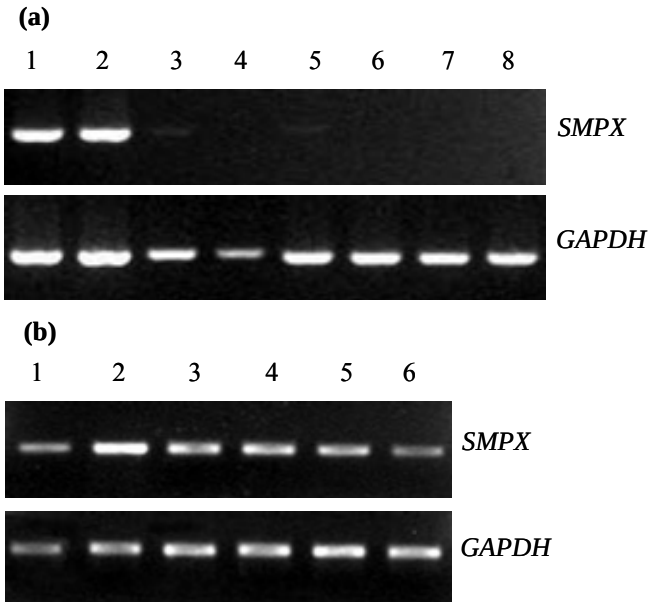

Figure 2. Expression profile analysis of porcine *SMPX*. (a) RT-PCR expression analyses for *SMPX* and *GAPDH* (positive control) at different tissue. From lane 1 to lane 8: heart, skeletal muscle, lung, liver, spleen, kidney, fat and brain of adult Landrace pig respectively. (b) RT-PCR expression analyses for *SMPX* and *GAPDH* (positive control) at different stages. From lane 1 to lane 6: 33-day embryo, 65-day embryo, 90-day embryo, birth, 28-day and adult Tongcheng pig respectively.

of 9.16. The deduced porcine protein sequence is 94% and 82% identical to human and mouse corresponding sequences respectively. Cytoplasmic/nuclear discrimination (PSORTII) predicted that *SMPX* might exist predominantly in the nuclear with a probability of 94.1%. Prediction of protein sorting signals (PSORTII) identified a pat 7 nuclear localization motif (PPRRKECT) (Hicks and Raikhel, 1995). Conservation of the casein kinase phosphorylation site and of the pat 7 nuclear localization signal was also observed in pig, mouse and human sequences, which suggest that these putative sites were genuine.

Analysis of the 5' *SMPX* promoter

By the means of comparative anchor tagged sequences (CATS), the *SMPX* upstream sequence (381bp) was obtained (Figure 1). The PCR product was obtained using genomic DNA as template. Analysis of the 5'promoter region revealed that there were no typical TATA box and cap motifs. However, the upstream sequence contain putative binding sites for several muscle-specific transcription factors, such as MyoD, E12, E47, MEF-2, RSRFC4 and Nkx2.5 which also existed in human *SMPX* promoter. Sequence comparisons between pig and human *SMPX* upstream sequences showed that the sequences shared 88% identity which was higher than that observed between the porcine and human coding sequences even in

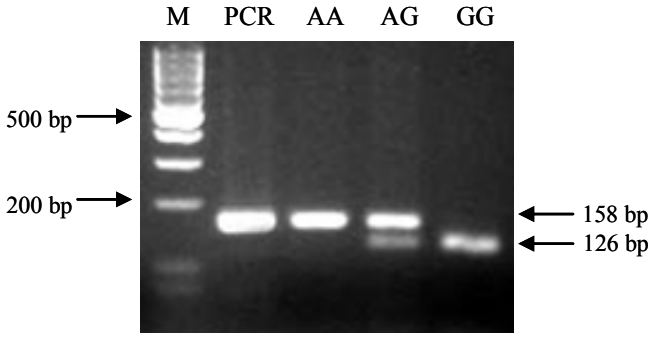

Figure 3. Three per cent agarose gel showing the PCR-RFLP analysis and genotypes of porcine *SMPX*. The genotypes are indicated on the top of the lanes (M, 100 bp DNA ladder; PCR, undigested PCR product).

the ORF region. The result showed the conservation of *SMPX* cis-site is very high.

Expression profile analysis

RT-PCR was performed to detect the porcine *SMPX* expression profile in eight different tissues and six different stages (Figure 2). The result showed that the expression was predominant in heart and skeletal muscles, very low in lung and spleen, and no expression was detected in liver, kidney, fat and brain. Moreover, *SMPX* presented different expression levels in skeletal muscle in these different stages. The expression levels of 33-day embryo and adulthood were lower than other four stages.

SCHP and RH mapping

Using the somatic cell hybrid panel, *SMPX* was assigned to porcine Chr Xp24 (probability of regional localization 0.8875, Correlation 1.0000 and error risk <0.1%). The mapping was further precised by using the radiation hybrid panel IMpRH. The statistical analysis revealed that *SMPX* is significantly linked to microsatellite *SW1903* with a distance of 55cR and LOD score of 7.04.

Analysis of polymorphism in different breeds and association analysis

An A/G (611) single nucleotide polymorphism (PCR-RFLP) was found by sequence analysis in 3'-untranslated region (3'-UTR). No restriction enzyme was available to directly genotype the SNP. So a *Taa*I RFLP test was developed by using a mismatch forward primer. The *Taa*I enzyme recognizes "ACNGT" sequence and the genomic sequences have "CCCGT" or "CCCAT". The forward primer sequence was designed to include the C↔A substitution at the position 608 and ended at the position 610 (sequence: 5'-CGGAATGCCTGAGACTCTAGCAG AAATGTACC-3'). After *Taa*I digestion, allele A was characterized by an uncut fragment of 158 bp and allele G

Table 2. Allele frequencies of the porcine *SMPX* gene in different breeds

Breeds	Samplesize	Genotype			Allele frequency	
		AA	AG	GG	A	G
Duroc	26	26	0	0	100	0
Large White	12	9	2	1	83.33	16.67
Min pig	29	9	18	2	62.07	37.93
Qingping pig	28	4	11	13	37.5	62.5
Lantang pig	18	1	10	7	33.33	66.67
YushanBlack pig	27	3	6	18	22.22	77.78
Large black-white pig	30	0	12	18	20	80
Small Meishan	29	0	0	29	0	100

Table 3. x^2 test results for the allele frequency distribution among different populations of *Taa*I-RFLP for the porcine *SMPX* gene

	Duroc	Large White	Min pig	Qingping	Lantang	Yushanhei	Large black-white pig
Large White	7.0571*	-	-	-	-	-	-
Min pig	28.1773**	7.3478*	-	-	-	-	-
Qingping	40.1143**	14.1941**	8.9455*	-	-	-	-
Lantang	40.0165**	15.6597**	9.4041**	0.9512	-	-	-
Yushanhei	37.3333**	16.9485**	21.7563**	4.3947	5.25	-	-
Large black-white pig	42.2375**	28.9579**	22.9897**	5.6674	3.2232	4.8556	-
Small Meishan	55**	36.3306**	50.5161**	25.0902**	23.1373**	11.5177**	14.5617**

* p<0.05; ** p< 0.01. $x^2_{0.05}$ (df = 2) = 5.99, $x^2_{0.01}$ (df = 2) = 9.21.

Table 4. T-test results for the association analysis of *Taa*I-RFLP for the porcine *SMPX* gene in the experimental population

Genotype	Number (No.)	Dressing percent (%) (l±SE)	Eye-muscle area (CM^2)	Percentage of ham (%)	Muscle pH value	Water lossing percentage (%)	Mucle drip loss (%)	Intramuscular fat content (%)
AA	111	75.88±0.19	32.27±0.47	30.34±0.20	6.50±0.03	5.37±0.23	10.37±0.29	2.23±0.08
AG	23	75.77±0.36	31.96±0.90	29.82±0.38	6.49±0.05	5.70±0.45	11.28±0.56	2.15±0.13
GG	54	75.38±0.31	31.53±0.78	30.49±0.33	6.49±0.05	4.81±0.39	10.32±0.49	2.57±0.13
P value AA-AG		0.8154	0.7519	0.2139	0.9302	0.5056	0.1462	0.5911
AA-GG		0.2511	0.4789	0.7424	0.8718	0.2930	0.9355	0.0244*
AG-GG		0.4070	0.7137	0.1823	0.9438	0.1355	0.1968	0.0141*

* p<0.05.

by two fragments of 126 bp and 32 bp (the 32 bp fragment was not detected on agarose as it produced a too weak band) (Figure 3).

The analysis of allele frequency distribution revealed that introduced pig breeds (Duroc and Large white) present a high frequency of A allele while the G allele is more frequently presented in the Chinese indigenous pig breeds (Min pig, Qingping pig, Lantang pig, YushanBlack pig, Large Black-White pig, Small Meishan) (Table 2). The x^2 test results for the allele frequency distribution indicates that there is a significant difference between Chinese native breeds and exotic breeds Duroc (p<0.01) and Large white (p<0.05) (Table 3). Traits association analysis was done in an experimental population. In the association analysis, the *Taa*I-RFLP genotype was significantly associated with intramuscular fat content. The intramuscular fat content of pigs with GG genotype was significantly higher than those of pigs with the AA genotypes (p = 0.0141) and the AG genotypes (p = 0.0244) (Table 4).

DISCUSSION

The porcine *SMPX* full-length cDNA and promoter sequence

The porcine *SMPX* was identified, cloned and characterized. It generated a mRNA of 920 bp and expressed prominently in skeletal and cardiac muscles. *SMPX* mRNA contains A/T-rich regions which are associated with instability. And there are several ATTTA or ATTTT motifs in the 3'-UTR which were identified as the highly conserved motif in several short-lived lymphocytes (Shaw and Kamen, 1986). These results suggest that *SMPX* mRNA is probably unstable and should have a short-life within skeletal muscle. The porcine *SMPX* transcript codes for an 86 amino acid protein and there are two overlapping casein kinase II (*CK2*) phosphorylation sites. It is interesting that the two *CK2* phosphorylation sites are conserved among human, mice and pig. *CK2* is a serine/ threonine kinase that has been implicated in cell growth and proliferation. And *CK2* is required for *Myf-5* activity though

CK2-mediated phosphorylation of *Myf-5* (Bai et al., 2003). It suggests that the function of *SMPX* is possibly related to *CK2* and *Myf-5* in muscle growth.

We obtained the *SMPX* promoter sequence. Although the porcine *SMPX* doesn't contain TATA or CCAAT boxes, but there are several banding sites of muscle-specific transcription factors (*MyoD*, E12, E47, *MEF-2*, *RSRFC4* and *Nkx2.5*) which generally found in muscle-related gene promoters. *MyoD* can activate the muscle-specific genes that play an important role in the myoblast proliferation (Valdez et al., 2000). The *MEF2* are members of the MADS (MCM1, agamous, deficiens, and serum response factor) gene family and specific transcription factors which cooperate with *MyoD* in the process of muscle-specific gene transcription (Jeffery et al., 1996). *RSRFC4* (related to *SRF*) protein can bind to AT-rich promoter regions which were recognized by other muscle-specific transcription factors (Pollock and Treisman, 1991). *Nkx2-5* is an interrelated factor of cardiac chamber formation and development which been shown to increase during adrenergic and pressure-induced cardiac hypertrophy (Saadane et al., 1999). The basic helix-loop-helix (bHLH) transcription factors E12 and E47 regulate cell type-specific transcription and growth by binding to the E box (CANNTG) on target genes (Kho et al., 1997). The result suggested that *SMPX* might be implicated in muscle development and growth.

Expression pattern and mapping of porcine *SMPX* gene

RT-PCR analysis demonstrated that porcine *SMPX* gene expressed mainly in skeletal and cardiac muscles, while at a weak level in spleen and lung. It was similar to the situation observed in human and mouse. Moreover, it is interesting that the expression is stage-dependant. It is up-regulated from 33-day embryo to 65-day embryo and down-regulated from 28-day to adult. As report, there are three climaxes of the generations of myogenic cells, 35 days of foetal life, 55 days of foetal life and 0 to 15 days of postnatal life (Lefaucheur et al., 1995). Our result was consistent with the study. So we suggested *SMPX* maybe involve with generations of myogenic cells. The results also are consistent with the corresponding reports in human and mouse (Patzak et al., 1999; Palmer et al., 2001).

SMPX was mapped to porcine chromosome Xp24 and was linked to *SW1903*. *SMPX* have been mapped to chromosome X in mouse and to chromosome Xp22.1 in human (http://www.ncbi.nlm.nih.gov). The information is in agreement with comparative mapping data between porcine chromosome X and human chromosome X (McCoard et al., 2002). There are several of papers reported quantitative trait loci (QTL) on porcine chromosome X. Cepica et al. (2003) identified QTL for carcass traits and meat quality in the proximal part of SSCX between *SW949* and *SW2126*(0-30 cM). And *SW1903* is located between *SW949* and *SW2126*.

Polymorphism and association analysis

An A→G transition was found within the 3'-UTR of *SMPX* PCR specific fragments which were subjected to RFLP analysis. Allele frequency studies indicated that all the breeds present a polymorphism except the Duroc breed. The allele distribution revealed that introduced pig breeds present a high frequency of allele A whereas the Chinese indigenous breeds have a high frequency of allele G except Min pig.

As reported, *SMPX* links to the cardiac developing and myoblast proliferation. Overexpression of *SMPX* in C2C12 myblasts induced lamellipodia formation and differentiation into large myosacs (Kemp et al. 2001). And there was a high correlation between the muscle fiber and intramuscular fat content (Larzul et al., 1997). Moreover Cepica et al. (2003) discovered there was a QTL for carcass traits and meat quality in the region nearby *SMPX* gene. Here, we provide evidence that a polymorphism in the *SMPX* gene is associated with intramuscular fat content. Although data sets for some of the individual *SMPX* genotypes were limited, these results suggest that *SMPX* or a closely linked gene to *SMPX* may be important in intramuscular fat content in swine. And it is necessary to repeat this association analyses in other population of pigs.

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