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Research Paper

Molecular Characterization of Porcine *MMP19* and *MMP23B* Genes and Its Association with Immune Traits

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Abstract

MMP19 and *MMP23B* belong to the Matrix metalloproteases (*MMPs*) family, which are zinc-binding endopeptidases that are capable of degrading various components of the extracellular matrix. They are thought to play important roles in embryonic development, reproduction and tissue remodeling, as well as in cell proliferation, differentiation, migration, angiogenesis, apoptosis and host defense. However, they are poorly understood in pigs. Here, we obtained the full length coding region sequence and genomic sequence of the porcine *MMP19* and *MMP23B* genes and analyzed their genomic structures. The deduced amino acid sequence shares similar precursor protein domains with human and mouse MMP19 and MMP23B protein, respectively. Using IMpRH panel, *MMP19* was mapped to SSC5p12-q11 (closely linked to microsatellite DK) and *MMP23B* was mapped to SSC8q11-q12 (linked to microsatellite Sw2521). Quantitative real-time PCR showed that *MMP19* was abundantly expressed in the liver, while *MMP23B* was strongly expressed in the ovarian and heart. Furthermore, both genes were all expressed increasingly in prenatal skeletal muscle during development. Three SNPs were detected by sequencing and PCR-RFLP methods, and association analysis indicated that C203T at exon 5 of *MMP19* has a significant association with the blood parameters WBC (G/L) and IgG2 (mg/mL) ($P<0.05$), SNP C131T at exon 3 of *MMP23B* is significantly associated with the blood parameters HGB (g/L) and MCH ($P<0.05$), and A150G in exon 4 has no significant association with the economic traits in pigs.

Key words: pig; *MMP19*; *MMP23B*; chromosome mapping; expression; association analysis.

Introduction

Matrix metalloproteinases (*MMPs*), also called matrixins, are zinc-dependent endopeptidases that are involved in extracellular matrix (ECM) degradation by degrading a number of extracellular molecules and bioactive molecules, thereby playing a central role in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in cell proliferation, migration,

differentiation, angiogenesis, apoptosis and host defenses. *MMP19* and *MMP23B* are members of the Matrix metalloproteinases (*MMPs*) family.

The *MMP19* gene was cloned from a rheumatoid arthritis patient [1] and human liver cDNA library [2]. It is expressed widely in normal human tissues, including the mammary gland, placenta, lung, pancreas, ovarian, and other tissues [2]. Additionally, *MMP19* promotes proliferation, migration, and cell adhesion of keratinocytes [3-4], and it is a likely candidate to be the major *IGFBP-3* degrading MMP in the quiescent epidermis because *IGFBP-3* and *MMP19* are both expressed in the skin [5]. Since it is expressed in theca cells and granulosa cells, *MMP19* is suggested that functions during follicular growth, ovulation, and luteal regression [5-6]. Moreover, *MMP19* is highly expressed in proliferating astrocytoma/glioma cells, and its expression may facilitate the cell's invasion through brain extracellular matrix components [7]. In MDA-MB-231 cells, the decreased invasiveness appeared to be mediated by decreased transcript levels of *MMP19* [8]. *MMP19* also contributes to the integrin switch favoring epithelial migration and actively participates in the early stages of squamous cell cancer invasion [9]. Knock-out mice deficient in *MMP19* expression are viable and fertile and do not display any obvious abnormalities. However, they exhibit decreased susceptibility to skin tumors and adipocyte hypertrophy induced by diet [10]. Based on the phenotype of *MMP19*-deficient mice, researchers proposed that *MMP19* is an important factor in cutaneous immune responses and influences the development of T cells [11].

The human cDNA encoding *MMP23* was cloned from an ovarian cDNA library and mapped to chromosome 1p36. It is expressed predominantly in ovarian, testis and prostate tissues [12]. The rat homolog was cloned from gonadotropin-primed immature rat ovaries, and its expression is spatially and temporally regulated in a cell type-specific manner during follicular development [13]. *MMP23* was transcribed in chondrocytes and osteoblasts, suggesting a role in some aspect of cartilage or bone formation [14]. In addition, *MMP23* is expressed in the synovium and cartilage [15], at the cranial sutures [16] and in human amniochorion [17]. And *MMP23* is up-regulated in common bile duct ligation (CBDL) livers and therefore may be involved in hepatic bone marrow beta 2m(-)/Thy-1+ hepatic stem cells (BMHSCs) priming [18].

Current studies indicate that *MMP19* promotes proliferation, migration and cell adhesion and influences immunity, while *MMP23* may play important roles in follicular development and bone formation. However, little is known about the porcine *MMP19* and *MMP23* genes. To characterize the structures of porcine *MMP19* and *MMP23* genes and explore their possible functions in pig, multiple approaches were undertaken in our study. Porcine *MMP19* and *MMP23B* genes were firstly cloned and sequenced. Sequently, the genes were mapped using IMpRH panel. And then, quantitative real-time PCR was employed to analyze the temporal and spatial expression in different tissues and skeletal muscle at different periods. Finally, SNP detection and association analyses were undertaken to discover the relationship between the genetic variation and the economic traits of pigs.

Materials and Methods

Animal samples and traits data collection

Twenty-two tissues, including heart, liver, spleen, kidney, small intestine, ovarian, abdomen fat, oviduct, large intestine, limb muscle and longissimus dorsi muscle from adult Landrace pigs and heart, liver, spleen, kidney, small intestine, ovarian, abdomen fat, back fat, lung, tongue and stomach from adult indigenous Chinese Tongcheng pigs, were collected for spatial expression analysis. Prenatal skeletal muscle was collected at three developmental stages (33, 65 and 90 days post conception (dpc)) from pregnant Landrace and Tongcheng pigs. All the tissue samples were harvested, immediately frozen in liquid nitrogen, and then stored at -80°C until used. Unrelated individuals from 4 pure pig breeds (Landrace (n=3), Yorkshire (n=3), Tongcheng (n=3) and Wuzhishan (n=3)) were used to scan single-nucleotide polymorphism (SNP) sites. To identify variants, allele frequencies were estimated in a population composed of unrelated, randomly selected individuals representing Landrace, Yorkshire, Tongcheng and Laiwu pigs, as well as Wuzhishan, Bama and Guizhou mini pigs. The population used for association analysis contained 3 pure pig breeds (Landrace, Yorkshire and Tongcheng) and a further pair of 3-cross breeds: Landrace× (Large White × Tongcheng) (LYT) and Yorkshire × (Landrace × Tongcheng) (YLT) [19]. Blood samples and traits data from individuals were obtained using standard methods, as described by Wang *et al* [20].

A total of 22 traits were measured. These traits included growth (average daily gain from birth to marketing, ADG), carcass (dressing percentage, DP; loin-muscle height, LH; backfat at the loin, XBF; eye muscle area, EMA; average backfat thickness at 3 points, BF; percentage of leaf and caul fat, PLCF; percentage of ham in the carcass, PHC), meat quality

(meat color score, MCS; mobling score, MS; muscle tender, MT; pH value at 45-60 min postmortem, pH45; muscle shear force, MSF; intramuscular fat content, IMF) and immune traits (white blood cell counts, WBC; hemoglobin, HGB; total erythrocytes, RBC; mean corpuscular volume hemoglobin, MCH; mean corpuscular volume hemoglobin concentration, MCHC; hematocrit, HCT; red cell distribution width, RDW; concentration of IgG, IgG2).

Cloning of porcine *MMP19* and *MMP23B* genes

The porcine *MMP19* and *MMP23B* genes were isolated using an in silicon cloning strategy. The human *MMP19* and *MMP23B* transcripts (NM_002429 and NM_006983, respectively) retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/) were used as entries to search for homologous sequence in the pig EST database (http://www.ncbi.nlm.nih.gov/genbank/GenbankSearch.html). The homologous EST sequence fragments showing above 85% identity and having a size of more than 100 bp were obtained and assembled using the SeqMan program (DNAStar, Inc., Madison, WI, USA). Primer pairs were designed from the assembled EST contigs with Primer 5.0 software to amplify porcine *MMP19* and *MMP23B* cDNA sequences.

The PCR 50 μL reaction consisted of 100 ng DNA or cDNA template, 5 μL 10x PCR buffer, 4 μL 2.5 mM dNTP mix, 1 μL sense primer (100 ng/μL), 1 μL antisense primer (100 ng/μL), 0.5 μL 5 U r-Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and sterile water. The PCR amplification was initiated with a 5-minute denaturation at 95°C and continued with the following parameters (35 cycles): 30 sec at 95°C (denaturation), 30 sec at Tm specific for primer (annealing) and 30 sec at 72°C (elongation), followed by 5 min at 72°C (elongation), and stored at 4°C until analysis. Rapid Amplification of cDNA Ends (RACE) was then employed to sequence the whole gene, as described in the user manual for RACE kit (Takara, Dalian, China). The total RNA and genomic DNA used for gene cloning were extracted from the blood samples of Wuzhishan mini pigs and the primer pairs used for PCR amplification are listed in Table 1.

Mapping of porcine *MMP19* and *MMP23B* genes

The chromosome assignment of porcine *MMP19* and *MMP23B* was performed with the INRA-University of Minnesota 7000 porcine radiation hybrid panel (IMpRH), consisting of 118 hamster porcine hybrid cell lines [21]. PCR reactions for IMpRH mapping were carried out in a volume of 20 μL containing 100 ng DNA, 2 μL 10x PCR buffer, 1.6 μL 2.5 mM dNTP mix, 0.5 μL sense primer (100 ng/μL), 0.5 μL anti-sense primer (100 ng/μL), 0.2 μL 5 U LA Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and sterile water. The PCR amplification was initiated with a 5-minute denaturation at 95°C, followed by 35 cycles of 95°C for 30 sec, Tm for 30 sec and 72°C for 30 sec, and a final extension 72°C for 4 min. PCR products were analyzed by 2% agarose gel electrophoresis and each clone in the IMpRH panel was amplified three repeats to get reliable results. Typing results were recorded and submitted to https://www-lgc.toulouse.inra.fr/pig/RH/IMpRH.htm for analysis. The primer pairs used for IMpRH mapping of porcine *MMP19* and *MMP23B* genes are shown in Table 1.

Expression patterns of porcine *MMP19* and *MMP23B* genes

Quantitative real-time PCR was employed to detect temporal and spatial expression of porcine *MMP19* and *MMP23B* genes. Total RNA was extracted from tissue samples using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was prepared using the reverse transcription kit (Takara, Dalian, China) according to the manufacturer's procedures. Each 20μL real-time PCR reaction contained 100 ng cDNA, 2.0 μL 10x PCR buffer, 0.4 μL 10 mM dNTP, 3.6 μL 5 μM sense primer, 1.2 μL 5 μM antisense primer, 0.2 μL 5 U/μL r-Taq DNA polymerase, 0.6 μL 5 μM probe, 0.5 μL DMSO, 4 μL 10 M BETAINE and sterile water. The PCR reaction was performed in triplicate and carried out at 95°C for 5 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C on a 7500 Real-Time PCR System (Applied Biosystems, USA). The gene expression levels were quantified relative to the expression of GAPDH using Gene Expression Macro software (BIO-RAD, USA) and the results were analyzed by the $2^{-\Delta\Delta Ct}$ method [22]. The primer pairs and hydrolysis probes for expression analysis are listed in Table 1.

SNP detection and association analysis

Primer pairs were designed for SNP scanning of our porcine *MMP19* and *MMP23B* genes, and SNP sites were identified by sequencing PCR products from 4 pig breeds: Yorkshire, Landrace, Tongcheng and Wuzhishan pigs. Consequently, three SNPs C203T in exon 5 of *MMP19* (*MseI* site), C131T in exon 3 (*MboI* site) and A150G in exon 4 of *MMP23B* (*Bsh1236I* site) were discovered.

The PCR 20 μL reaction consisted of 20 ng DNA template, 2 μL 10x PCR buffer, 1.6 μL 2.5 mM dNTP mix, 0.5 μL sense primer (100 ng/μL), 0.5 μL antisense primer (100 ng/μL), 0.2 μL 5 U r-Taq DNA polymer-

ase (Fermentas, Vilnius, Lithuania) and sterile water. The PCR reaction comprised of the initial denaturation at 95 °C for 5 min, 33 cycles of 30 sec at 94 °C, 30 sec at 60°C, and 30 sec at 72°C, followed by a final extension at 72 °C for 5 min. The RFLP reaction mixture consisted of 1 μL 10x buffer, 10 U restriction enzyme (NEB, Ipswich, MA, USA), 5 μL PCR products and sterile water. Samples were incubated at the specific temperature for restriction enzyme overnight. The digested products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV illumination. The primer pairs for SNP genotyping are displayed in Table 1.

Table 1 Primers used in this study

Gene	Primer symbol	Primer sequence (5'-3')	Tm (℃)	Used for
MMP19	*MMP19*.L	ACAGGGTCCGTATGAGGCA	65	cDNA cloning
MMP19	*MMP19*.R	GGAAATCCGAGGCTCAACG		
MMP19	5'RACE.*MMP19*.R	GGTTCAAGATGCGGAAGGTCAGGTGC		
MMP19	3'RACE.*MMP19*.L	CCACGCCGTTGGATGGGGATACTTC		
MMP19	3'RACE nest	TCTCCACCACCCACTCATTC		
MMP23B	*MMP23B*-CDS.L	GACGCCGCTACACGCTGACC	65	cDNA cloning
MMP23B	*MMP23B*-CDS.R	CGCGGACCCCCCAGGAGTA		
MMP23B	MIB2.3003	GCTACCGGCAGATGGAGGAACGC		
MMP23B	*MMP23B*.258	GGTCAGCGTGTAGCGGCGTCTTC		
MMP23B	3'RACE.MMP23B.R	TACCCCGTCAACCACACCGACTGCC		
MMP23B	3'RACE nest	GACTTCTGCTACGAGTTCCC		
MMP19	19.116	GGCTTCCTACTCCCCATGAC	60	Intron 1 Intron 2
MMP19	19.336	CTCATACGGACCCTTGTGGC		
MMP19	19.311	GATGATGCCACAAGGGTCCG	58	Intron 3
MMP19	19.601	GTTATTGGAGCAGTATGGGC		
MMP19	19.501	GTGGCTCCCTTGACTTTCCG	56	Intron 4
MMP19	19.775	CTGGGTGTATCGGGAGTG		
MMP19	19.715	CATCATTGCAGCCCACGAAC	59	Intron 5,6,7
MMP19	19.1085	CTTCCCAAAGGGCAGACAC		
MMP19	19.1031	ACCGTGACAGATTCAGGGC	59	Intron 8
MMP19	19.2114	AATCCGAGGCTCAACGACG		
MMP19	19map1	TGCTCAACGGCACCCTAC	58	mapping
MMP19	19map2	AGGAAGGCGGTCAGAGTC		
MMP23B	23map1	CAAAGAGCGGCACCGAAAGC	60	
MMP23B	23map2	GTGGTCCCAACGTAGCCTG		
MMP19	*MMP19*V1-F	TCGGACACAATGGATTCACTTC		expression analyses
MMP19	*MMP19*V1-R	GCCCGGAGACATCTTGAAATT		
MMP19	*MMP19*V1-P	FAM-CGTAGCGCCACACCTTGTCTCCCT-TAMRA		
MMP23B	*MMP23B*-F	TCGCAGGACGAGCTGTGG		
MMP23B	*MMP23B*-R	GCGTCACAGAAGCCCCTC		
MMP23B	*MMP23B*-P	FAM-CACACGAACAGCCGGTCCAGG-TAMRA		
β-actin	*ATCB*-F	TCCAGAGGCGCTCTTCCA		
β-actin	*ATCB*-R	CGCACTTCATGATCGAGTTGA		
β-actin	*ATCB*-P	FAM-CCTCCTTCCTGGGCATGGAGTCCT-TAMRA		
MMP19	19mor3023:exon 5	ACTCTGACCGCCTTCCTC	60	SNP genotyping
MMP19	19mor3367:exon 5	TCAAGCCCGAAGAGGTGC		
MMP23B	23mor2.1:exon 3	AGAGCGGCACCGAAAGCG	60	
MMP23B	23mor2.2:exon 3	CTGCTTGACCCCAGACCC		
MMP23B	23mor260:exon 4	CGGCGGCATCCACTTTGACG	60	
MMP23B	23mor2.2:exon 4	CTGCTTGACCCCAGACCC		

The association analysis was performed using the GLM procedure (SAS, 2006). Yijk =μ+ Bi + Gj +εijk [20], Where Yijk is the phenotypic value of a target trait; μ represents the population mean, Bi is the combination effect, Gj is the genotype effect and εijk represents the random error.

Results and Discussion

Molecular cloning and sequence analysis of porcine *MMP19* and *MMP23B* genes

A 2258 bp cDNA contig of porcine *MMP19* gene was assembled after sequencing the PCR and RACE products. This contig contains an ORF of 1623 bp, flanked by an 87 bp 5'UTR and a 548 bp 3'UTR. A consensus AATAAA polyadenylation signal was identified at 21 bp upstream of the poly(A) stretch. The porcine *MMP19* was predicted to encode a protein of 540 amino acid residues (http://genes.mit.edu/GENSCAN.html) with a calculated molecular mass of 61.03218 kDa and an isoelectric point of 6.13 (http://weblab.cbi.pku.edu.cn/program.inputForm.do?program=pepstats(v6.0.1)). The deduced amino acid sequence shares 84% and 76% sequence similarity with human (NP_002420.1) and mouse (NP_067387.1) homologues, respectively. However, the porcine MMP19 has additional 32 and 13 amino acids more than human and mouse, respectively (shown in Fig. 1). Amino acid sequence analysis of pig MMP19 revealed that the precursor protein contains zinc-dependent metalloprotease (ZnMc) and hemopexin-like repeats (HX), similar to the domains of the human (Q99542) and mouse (Q9JHI0) MMP19 proteins (shown in Fig. 1 and Fig. 3).

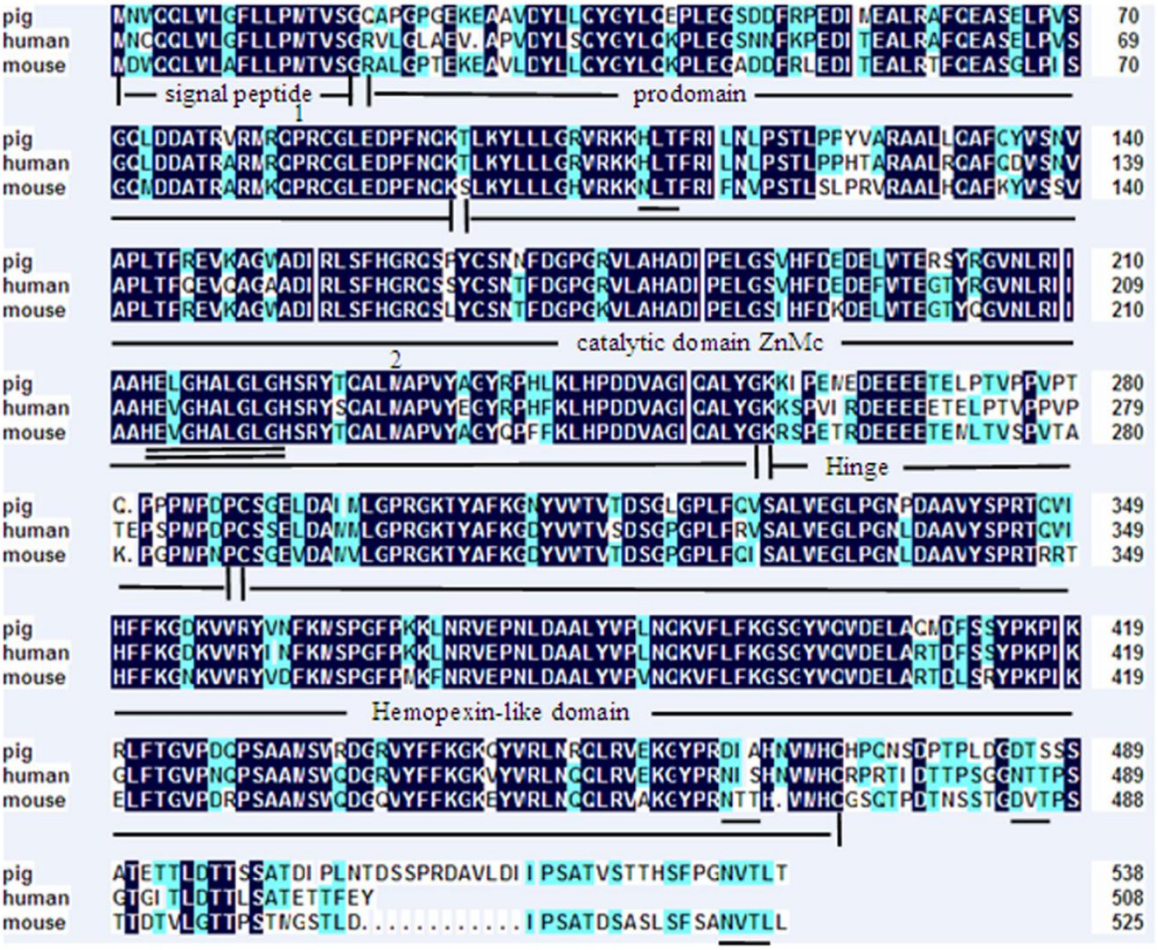
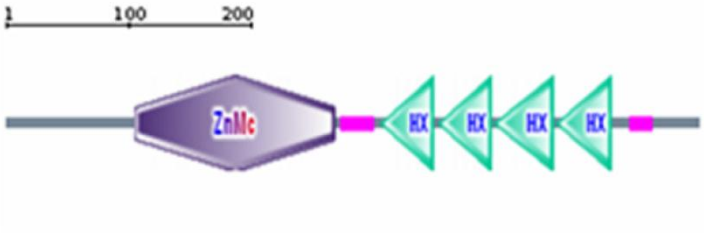


Fig. 1 Amino acid sequence alignment of porcine, human and mouse *MMP19*. Note: Shading shows identical amino acid residues among the three species. Common structural domains are underlined. The *MMPs* consensus zinc-binding motif, HEXGHXXGXXH, is double underlined. Potential N-linked glycosylation sites are underlined. The conserved pro-domain cysteine residue is marked by the number 1 and the methionine residue is marked by the number 2.

Fig. 2 Amino acid sequence alignment of porcine, human and mouse *MMP23B*. Note: Shading shows identical amino acid residues among the three species. Common structural domains are underlined. The *MMPs* consensus zinc-binding motif, HEXGHXXGXXH, is double underlined. Potential N-linked glycosylation sites are underlined. The Flynn cleavage site is framed. The conserved pro-domain cystein residue is marked by the number 1 and the methionine residue is marked by the number 2.



Name	Begin	End	E-value
ZnMc	101	258	1.51e-49
low complexity	261	288	-
HX	293	335	9.33e-06
HX	337	378	2.72e-07
HX	380	427	2.14e-05
HX	429	472	7.34e-13
low complexity	485	503	-

Fig. 3 Domain of porcine MMP19 protein. Note: The porcine MMP19 precursor protein contains zinc-dependent metalloprotease (ZnMc) and hemopexin-like repeats (HX).

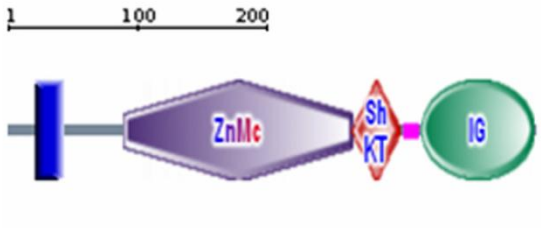
For the porcine *MMP23B* gene, a 1254 bp cDNA contig was assembled after sequencing the PCR and RACE products. This contig contains a 69 bp 3'UTR and an 1182 bp ORF encoding a protein of 393 amino acids. The predicted protein has a calculated molecular mass of 44.06404 kDa and an ioselectic point of 10.052. The deduced amino acid sequence shares 91%, 83%, 82%, 89%, 93% and 67% sequence identity with bovine (NP_001033645.1), rat (NP_446058.1), mouse (NP_036115), human (NP_008914.1), dog (XP_848890.1) and chicken (XP_417569.1) homologs, respectively (shown in Fig. 3 and Fig. 5). The amino acid sequence analysis showed that the porcine MMP23B precursor protein contains a transmembrane segments, zinc-dependent metalloprotease (ZnMc), ShK toxin domain (ShKT) and immuno-

globulin (IG), similar to the domains of the human (O75900) and mouse (O88676) MMP23 proteins (shown in Fig. 3 and Fig. 4). The MMP23 ShKT domains from humans to hydra exhibit remarkable sequence conservation [23], indicating that it contributes critical role throughout the plant and animal kingdoms. Online analysis of the approximate 2000-bp fragment upstream of the pig *MMP23B* gene exon 1 indicated that this sequence contains no TATA box or CAAT box, but a classical CpG island promoter. This CpG island is 506bp in length, with 70.75% GC content and is rich in transcription factors binding sites including Sp1+, Myod+, Snail+, MZF1-, c-Ets+ and c-FOS (shown in Fig. 6).

The genomic sequence of the porcine *MMP19* gene is 6369 bp containing 9 exons and 8 introns, while the *MMP23B* gene is 2343 bp with 8 exons and 7 introns. Based on the analysis of the genomic structure, the exon/intron junction of *MMP19* and *MMP23B* all conform to the GT-AG rule (shown in Table 2).

Chromosome assignment of porcine *MMP19* and *MMP23B* genes

The data analysis of the somatic cell hybrid panel revealed that porcine *MMP19* was mapped to the region of swine chromosome 5p12-q11 (LOD score=18.02) and closely linked to marker DK, the *MMP23B* gene was mapped to 8q11-q12 (LOD score=11.69) and tightly linked to the microsatellite marker Sw2521 (shown in Table 3 and Fig. 7). The mapping information of these genes is in consistent with our isolated porcine genome sequence. In addition, the *MMP19* gene was assigned to chromosome 12q14 in human and chromosome 10 D3 in mouse, while *MMP23B* was assigned to chromosome 1p36.3 in human and chromosome 4 E2 in mouse. Our results agree with the comparative mapping data, as human chromosome 12q14 is a syntenic region of porcine chromosome 5 [24]. Therefore, our result gives a precise location of porcine *MMP19* and *MMP23B* and enriches the comparative map information between HSA 12 and SSC 5.



Name	Begin	End	E-value
transmembrane	20	42	-
ZnMc	86	255	1.24e-41
ShKT	254	290	1.97e-08
low complexity	292	304	-
IG	306	389	6.97e-03

Fig. 4 Domain of porcine MMP23B protein. Note: The porcine MMP23B precursor protein contains a transmembrane segments, zinc-dependent metalloprotease (ZnMc), ShK toxin domain (ShKT) and immunoglobulin (IG).

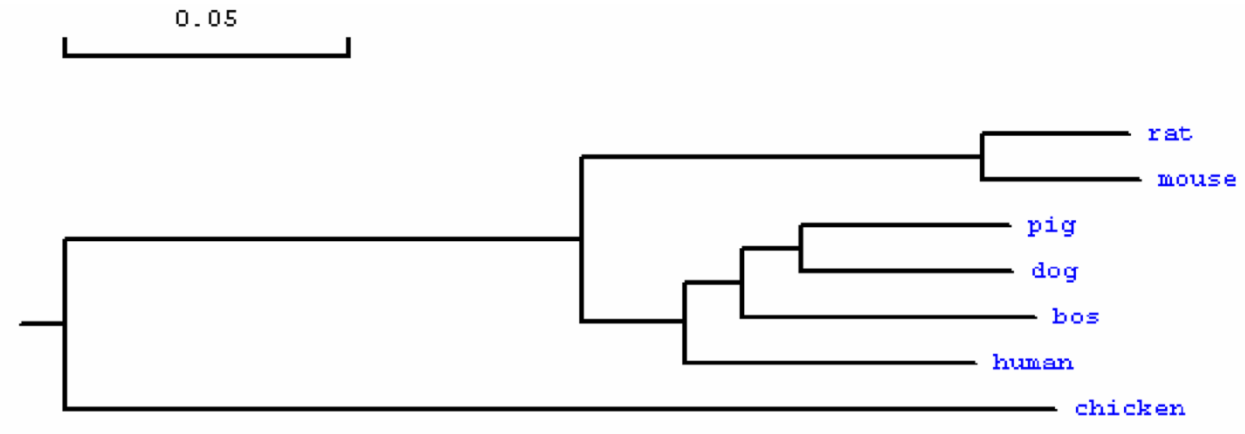


Fig. 5 Phylogenetic tree of MMP23B in different species. Note: The GenBank accession numbers of those sequences are as follows: human: NP 008914.1, bos: NP 001033645.1, rat: NP 446058.1, mouse: NP 036115, dog: XP 848890.1, chicken: XP_417569.1. The porcine MMP23B amino acid sequence was deduced from the full cDNA sequence in this study.

Fig. 6 Structure of pig *MMP23B* promoter. Note: The CpG island of the pig *MMP23B* promoter is shaded. The binding sites of the transcription factor are framed, and the corresponding transcription factors are shown beneath the binding sites. The transcription start site is indicated by triangles ▼. The start codon of the pig *MMP23B* gene is underlined. The symbol + represents transcription factor binding on the sense strand. The symbol – refers to the transcription factor binding on the antisense strand.

Table 2 Structure of pig *MMP19* and *MMP23B* genes

Gene	Number	Exon size(bp)	Intron size (bp)	5′ splice donor	3′ splice acceptor
MMP19	1	177	283	GTG/GTGAG	TCATAG/GATTAT
	2	86	1467	TGAG/GTGAGA	TTTTCAG/AGCTT
	3	131	244	GCTGG/GTGA	ACCAG/GCC
	4	216	451	CTG/GTAGGT	TCCAG/GGAGGG
	5	246	444	CTATG/GTCAG	TGCAG/GCAAG
	6	126	684	TGG/GTAAGAC	CTCAG/GGCC
	7	165	350	TTAAGG/GTAAC	CTCTCAG/GAG
	8	128	188	TAAG/GTACA	TACCTAG/GGCT
	9	953			
MMP23B	1	162	336	CAA/GTGAG	GAAG/GG
	2	137	79	CAG/GTGCG	CCCAG/GATCC
	3	140	77	ATAG/GTGGG	CCTAG/GCTTCT
	4	168	368	AG/GTGAC	CGCAAG/GCGT
	5	165	77	ACG/GTGAGT	TCCCGCAG/GCT
	6	111	78	TACG/GTGAG	AGCAG/AGTTC
	7	127	78	AGTGTA/GTGA	CCCGCAG/CTGG
	8	216			

Table 3 RH mapping results of *MMP19* and *MMP23B* genes

Genes	Cytogenetic position	Linked markers	Retention	Breakage frequency	RH Dist (Ray)	LOD score
MMP19	SSC5	DK	44	0.19	0.21	18.02
MMP23B	SSC8	Sw2521	26	0.28	0.33	11.69

Table 4 Allele frequency in different pig breeds

SNPs of MMP19	Pig breeds						
	Tongcheng pig	Yorkshire pig	Landrace pig	LYT pig	YLT pig		
C203T in exon 5							
NO. of CC individuals	2	12	3	8	18		
NO. of CT individuals	13	1	6	23	10		
NO. of TT individuals	14	0	0	6	0		
Frequency of C	0.29	0.96	0.57	0.53	0.82		
Frequency of T	0.71	0.04	0.33	0.47	0.18		
SNPs of *MMP23B*	Pig breeds						
	Tongcheng pig	Yorkshire pig	Landrace pig	Wuzhishan pig	Bama pig	Laiwu pig	Guizhou pig
C131T in exon 3							
NO. of CC individuals	4	12	0	6	0	0	7
NO. of CT individuals	13	3	8	25	7	11	20
NO. of TT individuals	18	0	3	8	25	31	15
Frequency of C	0.30	0.90	0.37	0.47	0.11	0.87	0.41
Frequency of T	0.70	0.10	0.63	0.53	0.89	0.13	0.59
A150G in exon 4							
NO. of GG individuals	16	0	2	0	33	32	15
NO. of AG individuals	12	5	9	39	11	10	20
NO. of AA individuals	5	13	0	0	0	0	6
Frequency of G	0.66	0.14	0.59	0.50	0.88	0.12	0.61
Frequency of A	0.34	0.86	0.41	0.50	0.12	0.88	0.39

Table 5 Association analysis of different genotypes with traits

traits	genotypes/number			P value			
C203T in exon 5 of MMP19							
WBC	27.71±3.275	32.72±2.986	19.67±5.23	0.0472*	0.278	0.2236	0.0183*
IgG2	46.919±2.85	55.06±2.287	45.81±3.99	0.0195*	0.0459*	0.8368	0.0256*
C131T in exon 3 of MMP23B							
	CC/27	TC/77	TT/35				
HGB	58.263±9.492	54.322±5.73	29.298±8.322	0.0189*	0.7371	0.0326	0.0074**
MCH	152.94±25.71	147.33±15.52	82.86±22.54	0.0304*	0.8598	0.0558	0.0108*

Temporal and spatial expression of porcine *MMP19* and *MMP23B* gene

Quantitative real-time PCR analysis showed that while the porcine *MMP19* gene was highly expressed in the liver of both breeds, it is much higher in Tongcheng pigs than in Landrace pigs. In both breeds, *MMP19* was weakly expressed in other tissues. Porcine *MMP23B* was expressed at high levels in the heart and ovarian of both Landrace and Tongcheng pigs. Otherwise, it was expressed ubiquitously at low levels in both breeds. In ovarian tissue, however, the expression level of *MMP23B* in the Tongcheng pig was much higher than in the Landrace pig, while this expression pattern is reversed in the heart tissue. However, the expressions of porcine *MMP19* and *MMP23B* are partial consistency with those of the human *MMP19* and *MMP23B* [2, 12], respectively. These may be attributed to the distribution characteristics and biological function of these genes in different species.

The tissue expression patterns correspond with our mapping results that the porcine *MMP23B* gene was mapped to the Sus scrofa chromosome 8, where it is strongly linked to the microsatellite marker SW252. Within this region, several QTLs associated with reproduction traits, such as ovulation rate, teat number, as well as the FSH content in blood, are found. Moreover, previous reports indicated that *MMPs* play key roles in follicle rupture and they are up-regulated and activated in coordination with ovarian follicle rupture before ovulation [25].

By using RT-PCR, the temporal expression patterns of porcine *MMP19* and *MMP23B* were examined in Tongcheng and Landrace pigs. As shown in Fig. 8c and Fig. 8d, porcine *MMP19* and *MMP23B* genes were expressed at three stages and had the similar trend of expression: increasing during the development stages. The expression patterns indicated that *MMP19* and *MMP23B* could play roles in porcine prenatal skeletal muscle development, the result is consistency with the *MMPs* functions of development and normal physiology [26]. Moreover, the myoblast proliferation is an important molecular event in development of porcine skeletal muscle. This suggested that porcine *MMP19* and *MMP23B* are involved in cell proliferation in myogenesis. Notably, the expression level in Tongcheng pigs is significantly higher than that in Landrace pigs. According to our previous study, Tongcheng pigs have a slower muscle growth rate than Landrace pigs [27], and the difference expression level of *MMP19* and *MMP23B* may be associated with the differences between Tongcheng and Landrace pigs in the intensity and timing of myoblast fusion during prenatal myogenesis.

SNP detection, allele frequency and association analysis

Three SNP sites were discovered through scanning DNA sequence of porcine *MMP19* and *MMP23B* genes. The three SNPs, C203T in exon 5 with an *MseI* site in *MMP19*, C131T in exon 3 with an *MboI* site and A150G in exon 4 with a *Bsh1236I* site in *MMP23B*, were genotyped by PCR-RFLP technique in the more than 200 pigs in our experimental population. After digestion by *MseI*, the 354 bp PCR products of *MMP19* were digested into 246 bp and 108 bp fragments (allele T) (Fig. 9a). In addition to the *MboI* site at 131 bp in exon 3 of *MMP23B*, the fragment used for genotyping contains another *MboI* site, and after digestion, the 825 bp PCR amplicon produced three fragments of 342 bp, 356 bp and 127 bp for allele T or two fragments of 483 bp and 342 bp for allele C. However, the 342 bp and 356 bp fragments were not distinguished in our study (Fig. 9b). For exon 4 of *MMP23B*, the 163 bp PCR amplicon can be digested by *Bsh1236I*, producing 114 bp and 49 bp fragments for allele G (Fig. 9c).

Association analysis results indicated that C203T in exon 5 of *MMP19* has a significant association with the blood parameters WBC (g/L) and IgG2 (mg/mL) ($P<0.05$). Furthermore, the WBC in the genotype CT individuals (32.72 ± 2.986) is significantly more than in the genotype TT individuals (19.67 ± 5.23) ($P<0.05$). Genotype CT individuals (55.06 ± 2.287) have signifi-

cantly higher levels of IgG2 than the genotype TT (45.81 ± 3.99) and CC (46.919 ± 2.85) individuals (P<0.05). Consistent with our mapping results, the porcine *MMP19* gene was mapped to the Sus scrofa chromosome 5 and is strongly linked to the marker DK; in this region, a QTL exists for IgG2 of K88 *E. coli*. Moreover, the results also agree with the immunity function of *MMP19* gene involving breast cancer, nasopharyngeal carcinoma, brain, lung, ovarian cancer and so on [28-30]. *MMP23B,* C131T in exon 3 is associated with the blood parameters HGB (g/L) and MCH significantly (P<0.05). SNP A150G in exon 4 has no significant association with the economic traits in pigs.

Overall, the temporal expression patterns and the association results of *MMP19* and *MMP23B* indicated that the genes may play roles in porcine immunity and embryonic development. The results correspond with the *MMPs* functions involving regulate both development and immunity in the Tribolium Model Insect [31].

In conclusion, we cloned and characterized the porcine *MMP19* and *MMP23B* genes. Chromosome localization by the INRA IMpRH panel assigned the porcine *MMP19* gene to SSC5p12-q11, closely linked to marker DK, and the *MMP23B* gene to SSC8q11-q12, tightly linked to microsatellite marker Sw2521. We have also analyzed their tissue expression differences and skeletal muscle development stages by real-time PCR in Landrace and Tongcheng pigs. We provided initial evidence that porcine *MMP19* and *MMP23B* genes were ubiquitously expressed in tissues and similarly expressed in prenatal skeletal muscle development. Genotyping and association analyses were performed on the SNPs *MseI*, *MboI* and *Bsh1236I* and revealed that *MMP19* has a significant association with the blood parameters WBC (g/L) (P<0.05) and IgG2 (mg/mL) (P<0.05), *MMP23B* was significantly associated with the blood parameters HGB (g/L) (P<0.05) and MCH (P<0.05). Our information provides an important molecular basis for further studies on the functions of porcine *MMPs* genes and the identified polymorphisms that may serve as genetic markers for immune traits in animal breeding.

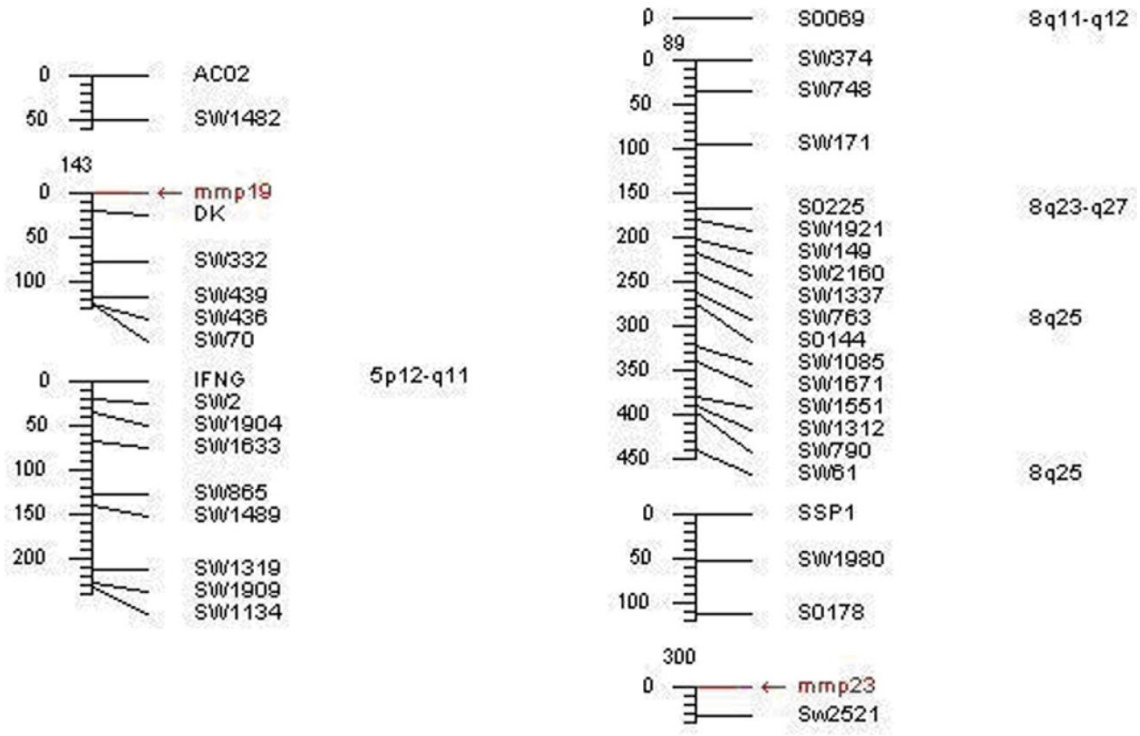


Fig. 7 Mapping results of porcine *MMP19* and *MMP23B* genes.Note: The left refers to the porcine *MMP19* gene location, and the right represents the pig *MMP23B* gene mapping result.

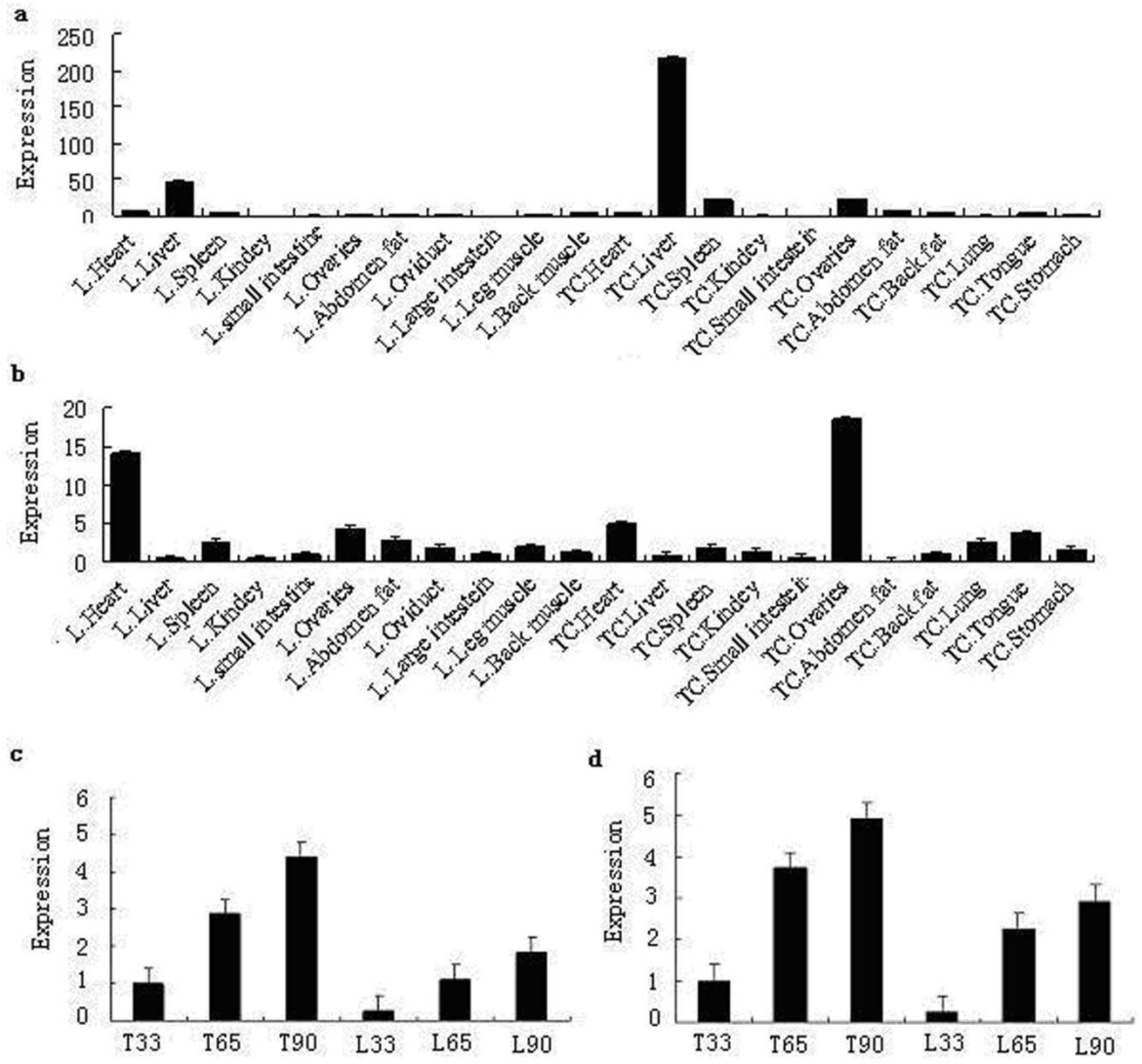


Fig. 8 Expression pattern analysis by real-time PCR method. Note: **a** and **b** are the tissue expression results of porcine *MMP19* and *MMP23B* in Tongcheng and Landrace pigs, respectively. **c** and **d** are the period expression patterns during prenatal skeletal muscle development in Tongcheng and Landrace pigs. L and TC refer to Landrace and Tongcheng pig for porcine *MMP19* and *MMP23B* genes, respectively. 33, 65 and 90 indicate days post coitus (dpc), respectively.

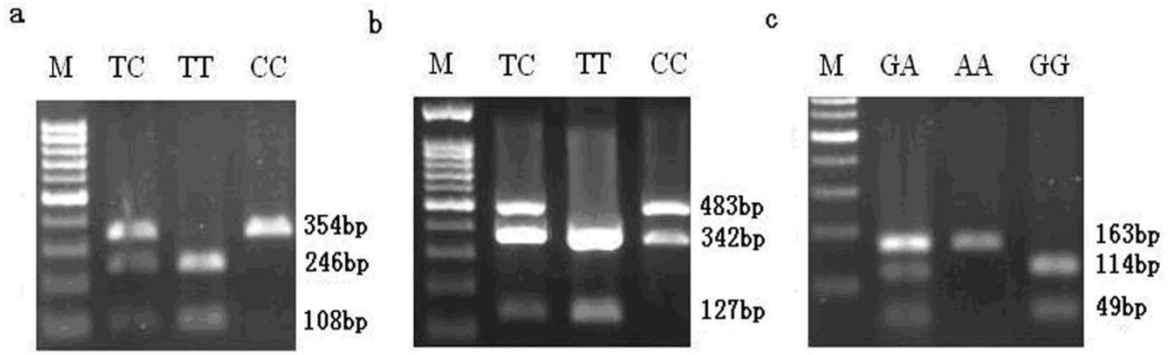


Fig. 9 SNP genotyping results by PCR-RFLP method. Note: **a** is the PCR-*MseI*-RFLP analysis result of SNP at C203T in exon 5 of the *MMP19* gene; **b** is the PCR-*MboI*-RFLP analysis at C131T in exon 3 of the *MMP23B* gene; **c** is the PCR-*Bsh1236I*-RFLP analysis at A150G in exon 4 of the *MMP23B* gene. The genotypes are shown on the top lanes; M refers to the DNA molecular weight marker.

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Conflict of Interests

No competing financial interests exist.

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