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Research Article

Cloning of full length sequence, expression profiling and polymorphism analysis of porcine *Wnt*10b gene

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Abstract

The wnt signaling transduction pathway is divided into two types, that are un-canonical kind and canonical kind, and *Wnt10b* is one of the member of canonical type. This pathway plays an important role in the formation of the fat deposition. In order to investigate the function of *Wnt10b* gene in pig, we acquired the full-length cDNA of porcine *Wnt10b* gene from the *longissimus dorsi* muscle by Rapid Amplification of cDNA of End (RACE) method. The expression level of *Wnt10b* in six tissues was also explored through qPCR. Moreover, one single nucleotide polymorphism (SNP) site was identified by PCR-RFLP, then its allele frequency and associations of genotypes with corrected back fat thickness were analyzed. The results showed that the full-length of *Wnt10b* gene was 2075 bp with 1170 bp of open reading frame (ORF) coding 389 amino acids. The pig *Wnt10b* gene shared more than 94% homology with the *Homo sapiens*, *Pan troglodytes*, *Equus caballus*, *Macaca mulatta* and *Ovis aries*. The deduced *Wnt10b* protein contained 20 phosphorylation sites and one WNT1 domain. The results of real-time polymerase chain reaction showed that the expression of *Wnt10b* gene in which Ningxiang pigs of lung was significantly different ($P<0.01$), other tissues had no significant difference in expression. The SNP of -607G>A locus was genotyped by PCR-RFLP method. Its associations with corrected back fat thickness were not significant ($P>0.05$). The objective of study was to provides an expression and structural basis for further study on functions of the *Wnt10b* gene in pigs.

Key words: Cloning, Expression profiles, Pig, SNP, *Wnt10b*

Highlights

- The full length sequence of *Wnt10b* gene was identified by RACE method.
- The expression of *Wnt10b* in different tissues of Ningxiang pigs was checked.
- One SNP was detected and its association with porcine back fat thickness was analyzed.

INTRODUCTION

The name Wnt is derived from the amalgamation of the name of the Drosophila segment polarity gene wingless and the name of the vertebrate homolog, integrated or int-1 (Wodarz and Nusse, 1998) It is an evolutionarily conserved, secretory glycoprotein family (Prestwich and Macdougald, 2007). Wnt protein is an important signaling molecule involved in cell proliferation, differentiation and migration (Nusse and Clevers, 2017). According to the Wnt protein signal transfer, the Wnt signal transfer method is divided into classical and non-classical forms, and *Wnt10b* is one of the typical ways. The study found that *Wnt10b* inhibits the development of white and brown fat tissue (Longo *et al*., 2004). Cho *et al* (2013) demonstrated that X-Box-binding protein 1 enhanced adipogenic differentiation in 3T3-L1 cells by down-regulating *Wnt10b* expression. Liu *et al*. (2013) found that *Wnt10b* inhibited the synthesis of zemocarp muscle fatty acids. In recent years, more investigations have concentrated on the meats, which consist of many components, including the amount of fat

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in the muscle. The amount of fat in the muscle of pork is one of the yardsticks for determining the quality of meat (Verbeke *et al*., 1999). We know that intramuscular fat has a high heritability (Na, 2016). The pig *Wnt10b* gene is on chromosome 5 (He *et al*., 2011). In the current study, obtaining full length sequence by RACE provides structural information about the gene based on which further experiments can be done. *Wnt10b* was identified and its expression level in six tissues of Ningxiang pigs was determined by quantitative real-time polymerase chain reaction. In this study we cloned the full-length sequence of porcine *Wnt10b* gene, checked expression profile in different tissues of pig. Also, one SNP was detected and its association with porcine back fat thickness was analyzed.

MATERIALS AND METHODS

Animals and samples: Ear tissue samples were collected from adult Ningxiang (N=67), Large white (N=325), Shaziling (N=57) and Daweizi (N=70) pigs. Longissimus dorsi muscle, lung, liver, heart, spleen and adipose were sampled from 30-day-old, 90-day-old, 150-day-old and 210-day-old full-sib Ningxiang pigs (both N=3), immediately frozen in liquid nitrogen, and then stored at -80ºC until further use. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University, Changsha, Hunan Province.

RNA extraction, reverse transcription, cloning and sequencing: Total RNA was extracted from longissimus dorsi muscle of Ningxiang pigs according to the manufacturer instructions of an RNA extraction kit (Takara, Tokyo, Japan). The quality and concentration of RNA were evaluated by Nanodrop 2000 spectrophotometer. First strand cDNA was generated using the Superscript II RT reverse transcription system kit (Invitrogen, Carlsbad, USA). The procedure of PCR was as follows: 95ºC for 5 min; 40 cycles of 95ºC for 30 s, 59ºC for 30 s and 72ºC for 30 s; and, 72ºC for 5 min. After gel purification, RT-PCR products were cloned into a pMD18-T vector (Takara, Tokyo, Japan).

Rapid amplification of cDNA ends (RACE): To obtain full-length porcine *Wnt10b* cDNA, two primer pairs were designed to amplify 5′- and 3′- ends of *Wnt10b* gene by RACE, and obtained a coding sequence (CDS) of porcine *Wnt10b* (Table 1). Amplifications by 5′ - and 3′ -RACE PCR were performed using a SMART™ RACE cDNA amplification kit (Clontech, Madison, USA) and the manufacturer protocol. Amplified products were separated by electrophoresis on a 1.5% agarose gel. Following gel purification, RT-PCR products were cloned into a pMD18-T vector (Takara, Tokyo, Japan) and sequenced, using DNAStar7.1 software (http:// www.gpxz.com) to obtain full-length cDNA sequence. The NCBI-BLAST database was used for homology analysis with published sequences (http://blast. ncbi. nlm.nih.gov/ Blast. cgi).

Bioinformatics analysis: The cDNA and amino acid (aa) sequence were deducted from the Expert protein analysis system (http:// www.expasy.org/). Amino acid sequences were aligned using CLUSTALW version 2.0 (http:// www.simgene.com/ClustalW), and protein domain features was predicted using the simple modular architecture tool version 8.0 (http:// smart. embl- heidelberg. de/). Phosphorylation sites were analyzed by NetPhos2.0 (http:// www. cbs. dtu. dk/ services/ NetPhos/). A phylogenetic tree was constructed with *Wnt10b* full-length cDNA of different species using the neighbor-joining (NJ) method and MEGA version 7.0 software. The secondary structure of *Wnt10b* protein was predicted by SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/ npsa_automat.pl?page=npsa_sopma.html). Swiss-model(https://swissmodel.expasy.org/) was used to predict the tertiary structure of *Wnt10b* domain online.

Table 1. Primers sequences for cloning of *Wnt10b*

Primers	5′→3′	Annealing temperature/°C	Size (bp)
5′-RACE	F: CGTCTTGTTGGCTCATTATGCCTGGTTG R: TCAAGCAGACGGTGTTGGCG	69	288
Middle fragment	F: GTCTCCTGTTCCTGGCGTTG R: GTCCATGTCATGGTTACAGC	68	566
Middle fragment	R: GCTGTAACCATGACATGGAC F: ACAGCGCTCAACTCGTGTCT	60	500
3′-RACE	R: GATGGTTGTGGCAGCCTGTG F: GCATCCTGAAGCAATCCCTGCGAATAAG	70	79
Q-PCR	F: GCCCAGTGACATTATCATCCC R: CCGAGGCTCAGACCTTGTAGTA	59	155
GAPDH	F: ATTTGGCTACAGCAACAGGGT R: AAGTCAGGAGATGCTCGGTG	59	172
PCR-RFLP	F: CCGGGATTCGACATGCGGAA R: CACCCCGCAGTCCACCATT	63	732

Real-time quantitative PCR (qPCR) analysis: To evaluate the expression of porcine *Wnt10b* in different tissues, RNA was isolated from six different tissues using an RNA extraction kit and following the manufacturer instructions (Takara, Tokyo, Japan). Total cDNA was generated using a QuantiTect transcription kit (Takara, Tokyo, Japan). Primers of SYBR green-based real time PCR were designed using primer premier 5.0 (Table 1). Real-time PCR was carried out using a BIORAD iCycler Thermal Cycler w/iQ5 (Hercules, California, USA). Results were calculated using the relative quantification method and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control gene. Expression levels of *Wnt10b* were calculated using the $2^{-\Delta\Delta Ct}$ comparative Ct method ($\Delta\Delta Ct = \Delta Ct_{treatmentgroup}$ target gene $_- \Delta Ct_{control\ group}$) (Pfaffl, 2001). Amplification reactions were carried out in a final volume of 20 µL, comprising 10 µL 2 X qPCR Mix (Takara, Tokyo, Japan); 1 µL each primer (10 µM); 1 µL template cDNA; and 8 µL nuclease free water, according to the following program: 95ºC for 2 min, followed by 35 cycles of 95ºC for 10 s, 59ºC for 30 s and 72ºC for 30 s.

Single nucleotide polymorphism (SNP) identification and allele frequency analysis of porcine *Wnt10b*: Genomic DNA was extracted by the phenol/chloroform method from ear tissue samples of pigs, and used as template DNA for amplification using primer pairs (Table 1) to detect SNPs. Genomic DNA was amplified by PCR, and amplicons were directly sequenced. A polymorphism site was identified analyzing the sequence with the DNAStar 7.1 software (http:// www. gpxz.com). Genotyping was based on genomic DNA of all breeds using the PCR-restriction fragment length polymorphism (RFLP) with Sal I restriction enzyme (Fermentas, Beijing, China). Amplified PCR products were digested at 37ºC for 10 h in a mixture (20 µL) containing 10 µL PCR product; 7.8 µL nuclease-free water; 2 µL 10X Buffer Tango (Promega, Madison, USA), and 0.2 µL Sal I (10 U).

Statistical analysis: Excel was used for data statistics and sorting. Data from experiments with multiple treatment groups were subjected to a one-way ANOVA followed by Duncan's multiple comparison test of significance using SPSS 25.0 software (IBM, Armonk, NY, USA). Data were presented as the mean ± standard deviation (SD). $P< 0.05$ or $P< 0.01$ was considered statistically significant.

RESULTS

cDNA cloning and sequence analysis of porcine *Wnt10b*: The full-length cDNA of *Wnt10b* from Shaziling pig was obtained by RACE and was 2075 bp in length (Supply supportive gel picture). Porcine *Wnt10b* sequence was deposited to GenBank (accession No. KF569219). The total cDNA length and deduced amino acid sequences were shown in

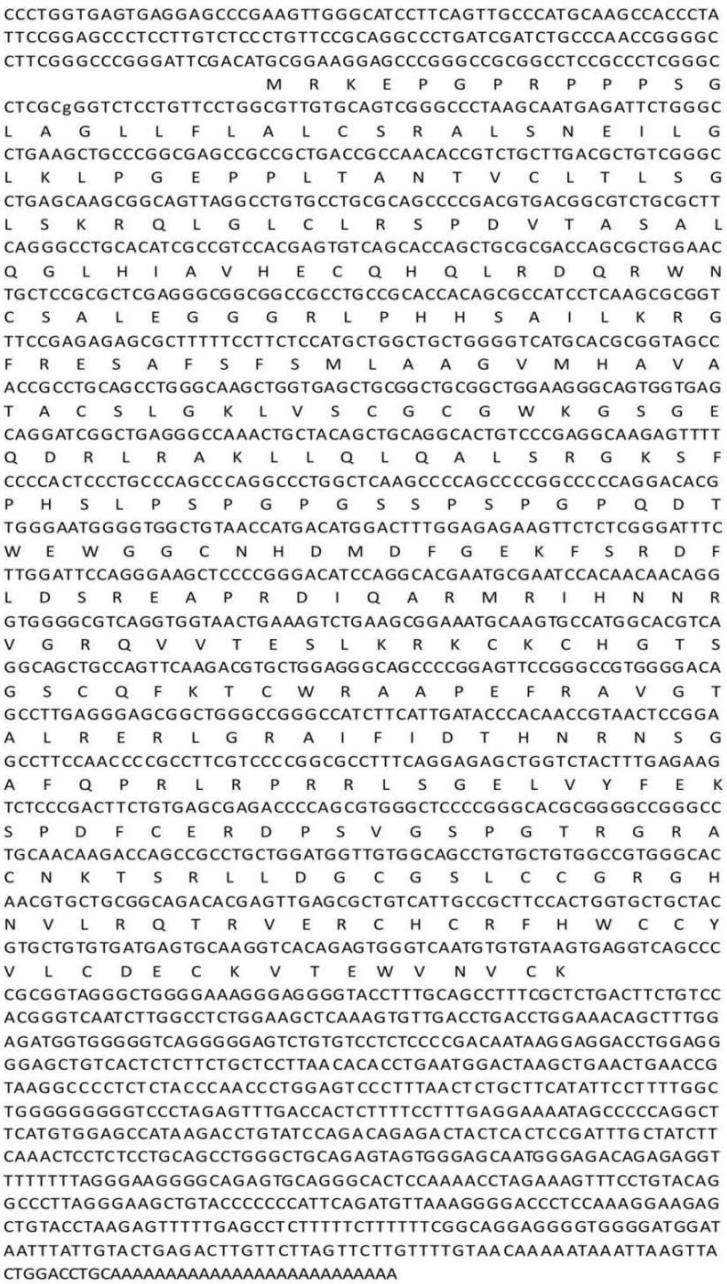

Fig. 1. Nucleotide and amino acid sequence of pigs *Wnt10b* gene

Fig. 1. The ORF of *Wnt10b* was 1170 bp and encoded 389 amino acids, and the 5′ - and 3′ - untranslated region (UTR) were 910 and 268 bp in size, respectively. The predicted molecular weight of *Wnt10b* was 42873.2 Da and the theoretical isoelectric point (PI) was 9.46. Among 20 amino acids encoded by the *Wnt10b*, Leu was in the highest proportion and accounted for 11% of amino acid. Analysis of the primary protein structure revealed that *Wnt10b* comprised a single peptide, eight putative transmembrane domains, and a low complexity region with 20 phosphorylation sites (Ser 16 and Thr 4) (Fig. 2). The Smart prediction demonstrated that along porcine *Wnt10b*, aa residues 1 to 28 represented a hormone receptor (HormR) domain, while aa residues 50 to 389 represented PFAM: WNT1 domain (Fig. 3).

Deduced protein sequence analysis, putative secondary and tertiary structures, and phylogenetic tree analysis: Secondary and tertiary protein structure predictions are depicted in Fig. 4 and Fig. 5, respectively. The proportions of α-helix, extended chain and random curlof *Wnt10b* protein were 26.74%, 18.51% and 54.76%, respectively (Fig. 4). The Wnt10b domain constitutes a curved spiral structure. Homology was compared between Sus scrofa *Wnt10b* gene and other 10 different species' *Wnt10b* gene nucleotide sequences (*Homo sapiens*, *Ovis aries*, *Bostaurus*, *Danio vrerio, Mus musculus*, *Rattus norvegicus*, *Pan troglodytes*, *Macaca mulatta*, *Equus caballus, Xenopuslaevis*) downloaded from the NCBI database. Then BLAST software was used for sequence alignment. It revealed that the coding nucleotide sequence of *Rattus norvegicus, Equus caballus, Ovis aries, Danio vrerio, Macaca mulatta, Homo sapiens, Pan troglodytes, Mus musculus Wnt10b* gene is 89%, 95%, 94%, 93%, 95%, 95%, 94%, 90%, respectively; amino acid homology is 96%, 98%, 82%, 94%, 97%, 98%, 97%, 96%, respectively. Phylogenetic tree analysis using

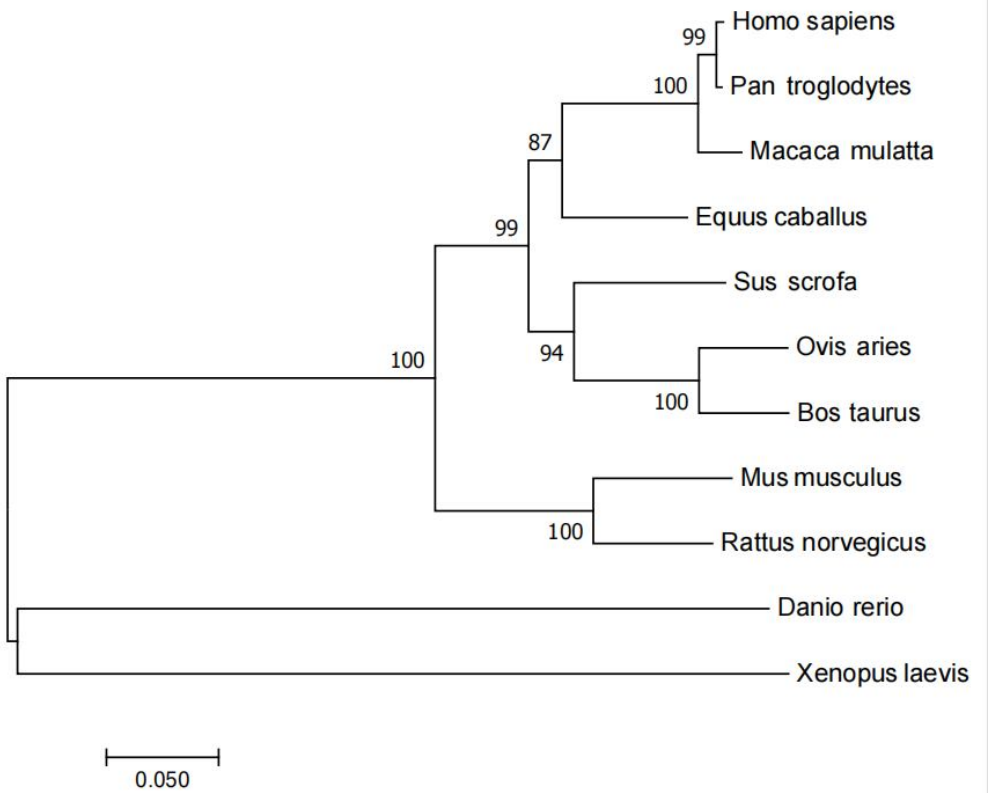

Fig. 6. Phylogenetic tree of *Wnt10b* gene

Molecular characterization of porcine *Wnt10b*

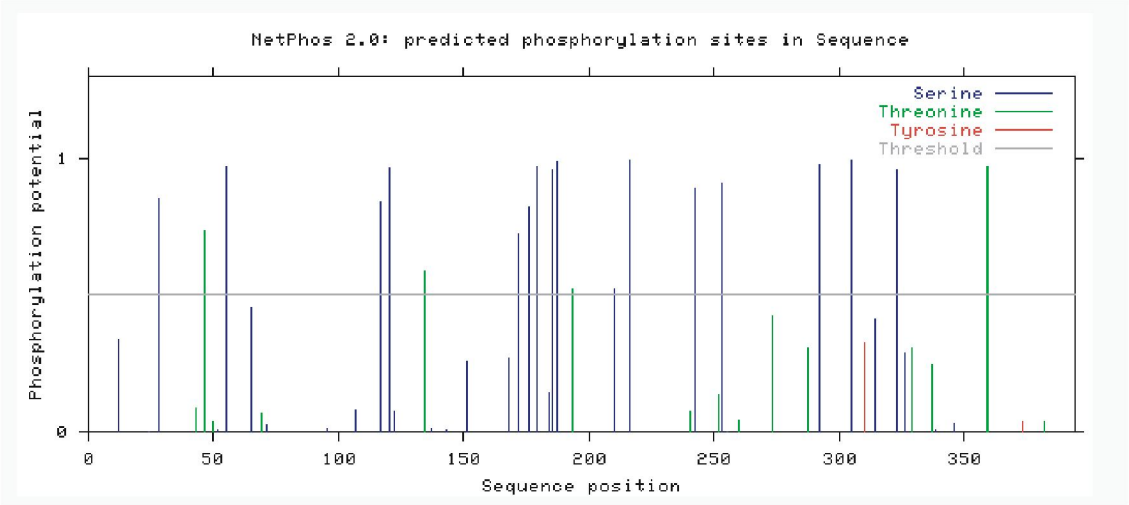

Fig. 2. *Wnt10b* phosphorylation site analysis

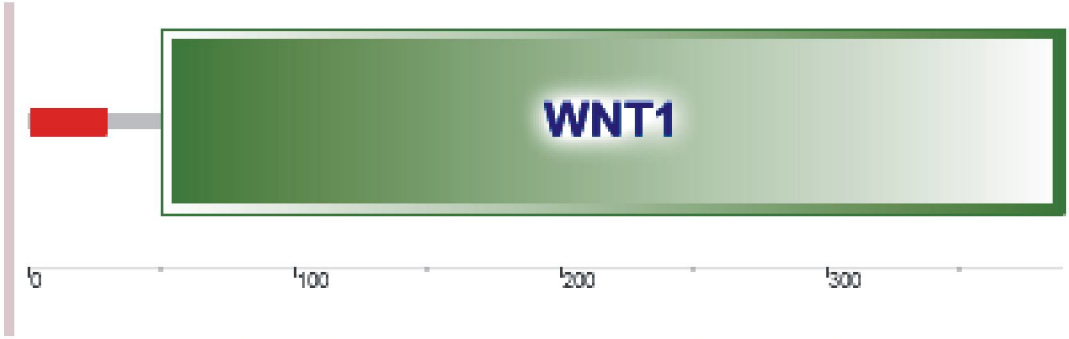

Fig. 3. *Wnt10b* conservative structure domain analysis

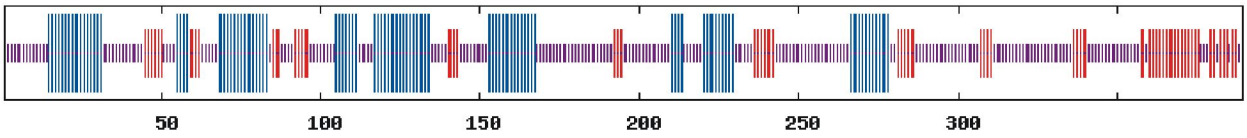

Fig. 4. Predicted secondary structure for protein encoded by pig *Wnt0b* gene

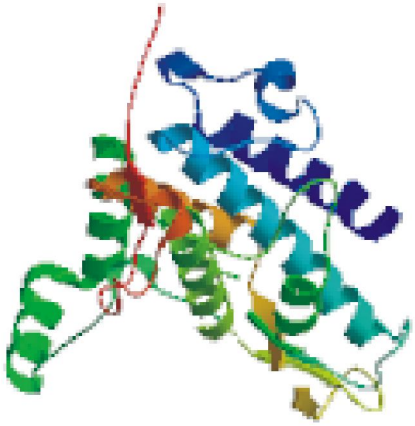

Fig. 5. Predicted tertiary structure of *Wnt10b* protein

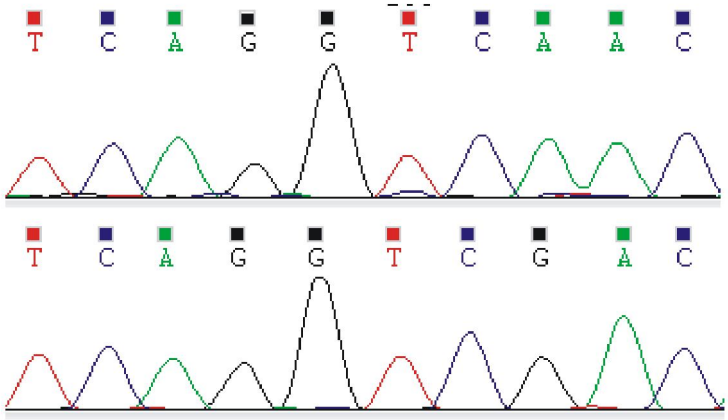

Fig. 8. The sequencing maps of SNP for *Wnt10b* gene

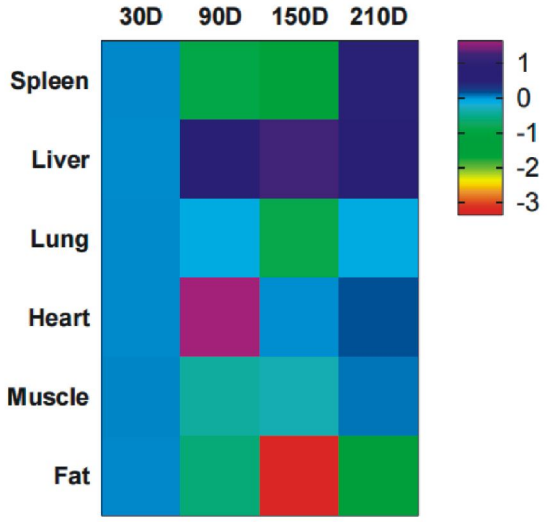

Fig. 10. Heat maps of *Wnt10b* in six tissues of Ningxiang pigs of different ages

Table 2. The frequency of allele and genotype of *Wnt10b* of *Sal* I-PCR-RFLP

Breed	Sample size (No.)	Genotype frequency			Allele frequency		$\chi 2$
		AA	*AG*	*GG*	*A*	*G*	
Large white	325	0.4523	0.4308	0.1169	0.6677	0.3323	73.3446
Daweizi	70	0.6429	0.3143	0.0429	0.8000	0.2000	60.0571
Shaziling	57	0.3509	0.4211	0.2281	0.5614	0.4386	3.1404
Ningxiang	67	0.4627	0.4478	0.0896	0.6866	0.3134	19.3881

Table 3. Effect of three genotype of the *Wnt10b* on growth traits in pigs

Traits	Least squares means ± SD		
	AA(120)	AG (115)	GG(29)
Correction back fat thickness (mm)	12.0267±3.5278	12.8332±3.5400	12.9713±3.6172

MEGA version 7.0 software was showed in Fig. 6. *Susscrofa* with this gene is the most closely related to *Bos Taurus* and *Ovis aries.*

Polymorphism detection and allele frequency: In this study, a nucleotide substitution of A for G recognized with Sal I restriction endonuclease existed in the amplified 552 bp fragment (Fig. 7). Three genotypes, AA (732 bp), AG (180 bp, 552 bp, 732 bp) and GG (180 bp, 552 bp), were generated (Fig. 8). Allele A was predominant in Ningxiang and Daweizi pigs. In the Shaziling A and G allele frequencies were almost equal. (Table 2). The gene A was the dominant allele in the large white pigs, and the gene frequency was 0.6677. According to the Chi-square test, it can be known that there is no significant difference in Shaziling pigs. The least square mean of AA, AG and GG genotypes was estimated by general linear model (Table 3). There was no significant difference between AA, AG and GG genotypes and corrected back fat thickness of large white pigs.

Tissue expression pattern of porcine *Wnt10b*: The expression of *Wnt10b* gene in six tissues of Ningxiang pigs at 210 days of age was analyzed by qPCR (Fig. 9). The expression level of *Wnt10b* in Ningxiang pig lung was significantly different from that in other 6 tissues ($P<0.01$), *Wnt10b* expression in other tissues was not significantly different ($P>0.05$), but expressed in liver, spleen and fat. In Fig. 10, the expression levels of *Wnt10b* in six tissues of Ningxiang pigs at 30, 90, 150 and 210 days of age were analyzed, and there were differences in the expression levels at different time periods in the same tissue.

DISCUSSION

Bui *et al*. (1997) cloned human *Wnt10b* gene for the first time. Hardiman *et al*. (1996) first isolated *Wnt10b* gene from mouse lymphoid tissue, and Lee *et al*. (1995) again verified the presence of *Wnt10b* gene at the insertion site of mouse tumor mammary virus (MMTV). He *et al*. (2011) cloned the porcine *Wnt10b* gene, but did not find the 5′-UTR sequence. In this study, the full-length sequence of porcine *Wnt10b* gene was obtained by RACE, which laid a foundation for further study of its function and application in breeding. This study did not involve the molecular function and molecular mechanism of *Wnt10b* gene, and further study of its functional mechanism will provide reference for the application of *Wnt10b* in production practice.

Wnt10b is a soluble protein. In our study, bioinformatic analysis showed that 1-38 aa of porcine *Wnt10b* contained a signal peptide,

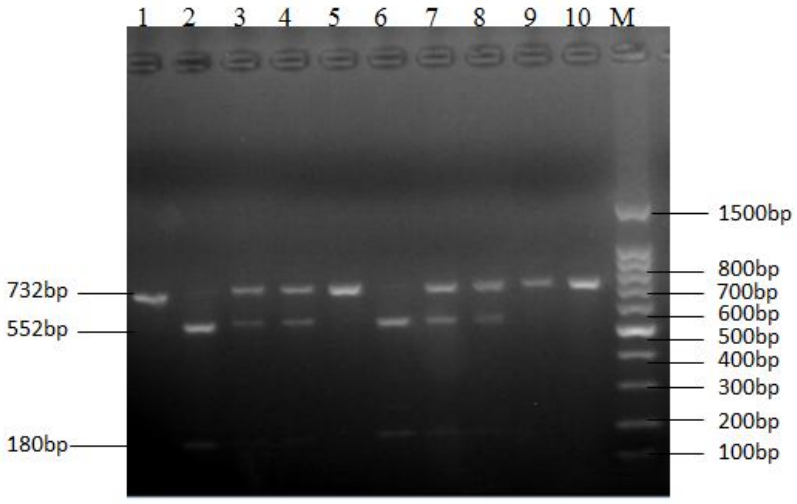

Fig. 7. The electrophoresis patterns obtained from digestion with *Sal* I restriction enzyme

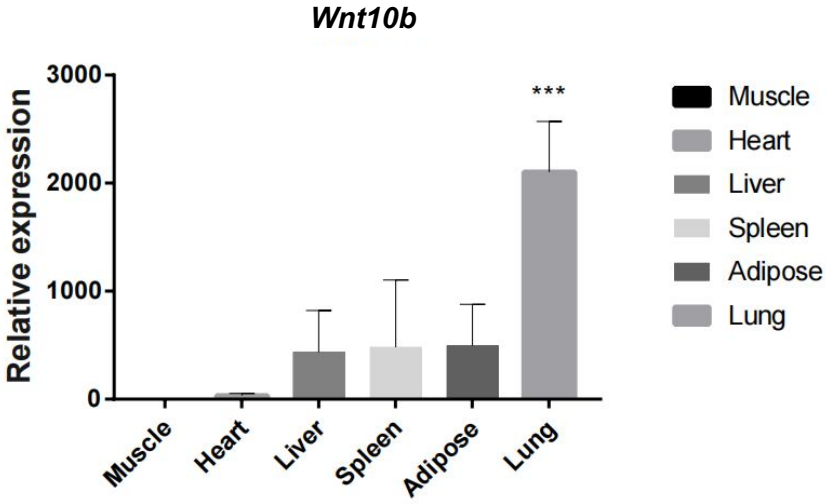

Fig. 9. Detection of *Wnt10b* expression in different tissues of Ningxiang pigs at 210 days of age

eight trans-membrane regions were distributed among 45-67, 87-106, 121-140, 216 - 238, 253 - 275, 312-334, 354-373 and 488-510 aa; furthermore, there was a low complexity region in 415 - 426 aa. We observed 16 serine (Ser) and 4 threonine (Thr), with a total of 20 potential phosphorylation sites. The amino acid coding ability of *Wnt10b* was analyzed, and its structural characteristics were further explored, including eight transmembrane domains, hormone receptor domains and phosphorylation modification sites, which may be related to Wnt transduction in cell signaling pathways. Studies have found that *Wnt10b*/α-catenin signaling pathway affects the maintenance of spermatogonial stem cells (Yu *et al*., 2022), proliferation and apoptosis of breast cancer cells (Chen *et al*., 2022), and progression of liver cancer (Zhang *et al*., 2022).

Wnt10b gene is highly homologous in many animals and highly conserved during evolution, indicating that it plays an important physiological role in animal growth and development. This is consistent with previous studies (Yinjuan and Huixia, 2008).

Wnt10b is highly expressed in liver, fat,

spleen and lung tissues of pigs. Liver is an important organ for lipid synthesis and decomposition, suggesting that *Wnt10b* may play an important role in physiological processes such as fat metabolism. Martinez, the study found that *wnt10b* present time-series sexual expression in different tissues (Martínez *et al*., 2017). Studies have found that *Wnt10b* knockout mice inhibit fat deposition (Li *et al*., 2022). Overexpression of *Wnt10b* can accelerate cartilage to bone remodeling and thus promote fracture healing (Hu *et al*., 2022). Previous studies have found that *Wnt10b* inhibits adipogenesis and promotes osteogenic differentiation by activating the WNT signaling pathway (Bennett *et al*., 2005; Ross *et al*., 2000).

This study also analyzed the role of *Wnt10b* gene polymorphism in molecular breeding. We compared the G>A mutation of *Wnt10b* among fatty (Ningxiang, Shaziling and Daweizi), and lean (large white) pigs, but found no significant difference in back fat thickness among different genotypes, which may be due to the small number of samples collected. Van *et al*. (2012) showed that *Wnt10b* mononucleotide was correlated with body mass index. Kim *et al*. (2011) found that the correlation between -607-G>A genotype and total abdominal fat and abdominal fat skin area was not significantly different. If the G>A mutation of *Wnt10b* is verified to be significantly correlated with backfat thickness through large samples, it will greatly improve the breeding process of pig growth and fat improvement.

In conclusion, we have characterized the *Wnt10b* gene. Our research provides an expression and structural basis for further study on functions of the *Wnt10b* gene in pigs. Also, this study indicated that this gene may simply be used as a genetic marker with effects on fatness traits and could be applied to breeding projects.

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Conflict of interest: Authors have no conflict of interest in this study.

Author contributions: NN, SG: Conception and design; NN, XL, ZG, BL, HM, SG: Performed acquisition of data; NN, SQ, YG, YB, SG: Performed statistical analysis of data; NN, YL, HM, SG: Performed interpretation of the results.

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