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# Molecular characterization, chromosomal localization, expression profile and association analysis with carcass traits of the porcine *dickkopf homolog1* gene

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**Abstract** *DKK1* (dickkopf homolog 1) is a potent inhibitor of the canonical Wnt/$\beta$-cantenin signalling pathway, which plays a pivotal role in myogenesis, adipogenesis, and many other crucial biological processes. In this study, *DKK1* was assigned to porcine 14q25-26 by using the radiation hybrid (IMpRH) panel. A G1757A single nucleotide polymorphism site by *Csp*6I PCR-RFLP was identified. Association analysis showed that different genotypes were associated with loin muscle area ($P = 0.0281$). Semi-quantitative-RT-PCR analysis revealed that *DKK1* was highly expressed in spleen and lymph node at two developmental stages, while in skeletal muscle, further real-time PCR quantified that *DKK1* was down-regulated in Large White pigs compared to Tongcheng pigs, accompanied by the down-regulation of *CTTNB1* and *TCF4*, the up-regulation of *LRP6*, suggesting that the phenotypic difference between lean and obese pigs might be correlated with the activity of Wnt/$\beta$-cantenin signalling pathway.

**Keywords** Association analysis · Loin muscle area · Chromosomal localization · *DKK1* · Expression profiles · Pigs

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## Introduction

Extensive studies have shown that the canonical Wnt/$\beta$-cantenin signaling pathway regulates a number of critical events during cell growth and differentiation both in embryos and adults. Activation of this multifunctional cascade depends on the nucleic$\beta$-cantenin, which functions as the co-activator of TCF/LEF to promote the transcription of target genes [1]. The Wnt/$\beta$-cantenin pathway is subject to strict control, and DKK1 (the secreted protein Dickkopf1) is a potent negative modulator, which is from a multi-gene family of glycoproteins of 225–350 amino acids. In vertebrates, there are four members, named DKK1, DKK2, DKK3 and DKK4, respectively, all of which are synthesized as precursor proteins with an N-terminal signal peptide and two conserved cysteine-rich domains, separated by a linker region [2]. Among the DKKs, DKK1 is most extensively studied. The carboxyl terminal cysteine-rich region of DKK1 binds the co-receptor of Wnts—LRP-5/-6[3], which functions as a switch from non-canonical to canonical signalling and is essential to trigger the Wnt/$\beta$-catenin pathway [4]. In the promoter region of *DKK1*, there is a binding site for p53 protein [5], which plays a role in DNA damage.

DKK1 negatively modulates the canonical Wnt/$\beta$-catenin pathway by cooperating with a high-affinity single transmembrane receptor—Kremen, promoting the endocytosis of LRP-5/-6. Ke Wang et al. showed that DKK1 residues Arg (197), Ser (198), and Lys (232) were specifically involved in its binding to Kremen [6]. It is of note that the internalization of LRP-5/-6 is context-dependent for Kremens: Kremens act as DKK1 receptors and synergize with it to inhibit Wnt signalling in cells with a quantity of DKK1 above a critical concentration, while enhance the sensitivity to Wnts and potentiate the signal transduction in cells with a quantity of DKK1 below the threshold value

[7]. *DKK1* is a direct target gene of Wnt/β-catenin signalling [8, 9], indicating that DKK1 may act as a negative feedback-loop mechanism.

Previous work shows that DKK1 plays an important role in tissue development: DKK1 affected lung development, and cultured organs treated with DKK1 display morphogenetic defects [10]; In mouse, DKK1 participates in the head development and knockout of this gene causes absence of anterior head structures [11]; Dkk1 is capable of inducing cardiac gene expression, and exogenous administration of DKK1 to posterior lateral plate mesoderm promotes heart muscle formation [12].

Numerous studies have elucidated the effect of Wnt signalling on myogenesis and adipogenesis (reviewed by Constantinos [13]), which are closely related to growth rate, carcass traits and meat quality of pigs. However, so far little work has been carried out in the pig industry. To address the potential functions of *DKK1* by way of Wnt/β-cantenin signalling in this field, we implemented experiments in pigs, involving in the expression pattern of *DKK1* gene its chromosomal location, SNP detection ranging the whole gene and the association analysis between the variations and the traits we concerned.

## Materials and methods

### Chromosomal localization

The INRA University of Minnesota porcine radiation hybrid (IMpRH) panel was employed for the chromosomal localization of *DKK1* [14]. Pig specific primers (Table 1) were designed to amplify porcine genomic DNA within the porcine IMpRH panel. PCR was performed in a volume of 10 μl consisting of 20 ng of genomic DNA, 1 × PCR buffer, 20 pmol of each primer, 750 pmol of each dNTP, 150 pmol of $MgCl_2$, and 0.5 unit of Taq DNA polymerase. The PCR protocols were 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 64°C, 30 s at 72°C, and a final extension of 5 min at 72°C. The PCR results were recorded and analyzed on http://imprh.toulouse.inra.fr/for RH mapping [15].

### SNP identification and association analysis

Pooled genomic DNA from Large White, Landrace and Tongcheng pigs (Chinese native pigs) was amplified and directly sequenced to identify SNPs (Single Nucleotide Polymorphism). Based on the *Sus scrofa DKK1* genome sequence obtained from NCBI (GenBank Accession number: NC_010456), specific primers were designed as shown in Table 1, which were employed in the following PCR-RFLP method. The PCR for genotyping was performed in a volume of 10 μl consisting of 20 ng of genomic DNA, 1 × PCR buffer, 20 pmol of each primer, 750 pmol of each dNTP, 150 pmol of $MgCl_2$, and 0.5 unit of Taq DNA polymerase (Takara, Dalian, China). The PCR parameters were 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 56°C,15 s at 72°C, and a final extension of 5 min at 72°C. 7 μl PCR product was digested overnight with 1 unit of *Csp6*I (Jingmei, Carlsbad, CA, USA) at 37°C, then separated on a GelRed stained 2.5% agarose gel.

The animals used in the association analysis included 140 pigs: Tongcheng(27), Large White (27), Landrace (29), and crossbreeds of Large White × (Landrace × Tongcheng) (29) and Landrace × (Large White × Tongcheng) (28). Meat quality, growth, carcass and immune traits were recorded. The GLM (General Linear Model) procedure in the SAS software package (SAS Inst. Inc., Cary, NC) was used to reveal the associations between genotypes and traits. The linear model is as follows:

$$Y_{ijkl} = \mu + G_i + B_j + C_k + \varepsilon_{ijkl}$$

where $Y_{ijkl}$ is the phenotypic value of target traits; $\mu$ is the mean value of the population; $G_i$ is the effect of the $i$th genotypes; $B_j$ is the effect of $j$th batch; $C_k$ is the effect of $k$th combination (breed) and $\varepsilon_{ijkl}$ is random residual [16, 17]. $P < 0.05$ was considered as the statistically significant criterion.

**Table 1** Primers for gene mapping, polymorphism and semi-RT-PCR

| Primers purpose | Primers number | Primer sequences(5′–3′) | Product size (bp) | Annealing temperature (°C) |
|---|---|---|---|---|
| Polymorphism | DSNP1S | AAGTTTTTGAAAGTGAACGACT | 192 | 56 |
| | DSNP1A | ACTTTCTACTGTTAAAGCAAAACT | | |
| Mapping | DRH1S | CAACGCCATCAAGAACCT | 283 | 64 |
| | DRH1A | TGCCCACCTCTCCAAAAG | | |
| Semi-RT-PCR | DE1S | CTGTTCGAGGGTGGCAACA | 234 | 60 |
| | DE1A | CATCCAAGGTGCTATGGTCATT | | |
| Internal control | pRPL32S | TAAGCGGAACTGGCGGAAAC | 284 | 60 |
| | pPRL32A | TGGGATTGGTGACCCTGATG | | |

### Analysis of expression profile

Two Tongcheng pigs and two Large White pigs at different developmental stages (35 days and adult) were slaughtered, and immediately total RNA was extracted from the heart, liver, spleen, lung, kidney, skeletal muscle, fat and lymph node. Subsequently, RNA was reverse-transcribed into cDNA, and semi-quantitative RT-PCR was performed to reveal the differential expression of *DKK1* in these two breeds.

Intron-spanning primers were designed to amplify *DKK1* in different tissues (Table 1). Amplification of the gene *RPL32* served as an internal control under the same conditions. PCR amplification was done in a total volume of 10 μl consisting of 20 ng of cDNA, 1 × PCR buffer, 20 pmol of each primer, 750 pmol of each dNTP, 150 pmol of $MgCl_2$, and 0.5 unit of Taq DNA polymerase (Takara, Dalian, China). The PCR procedure was 5 min at 94°C followed by 32 cycles of 30 s at 94°C, 30 s at 60°C, 15 s at 72°C, and a final extension of 5 min at 72°C. Finally, PCR products were analyzed on 1.5% agarose gel stained with GelRed [18].

## Results and discussion

### Molecular characterization and bioinformatics analysis

Porcine *DKK1* gene is composed of 4 exons, encoding a 266-amino acid protein (GenPept Accession number: XP_001926143) with a molecular weight of 28.75 K Da and a theoretical isoelectric point of 8.78 (http://cn.expasy.org/tools/). As DKK1 was a secreted protein, a cleavage site was predicted at the position between the 28th amino acid and the 29th amino acid (http://www.cbs.dtu.dk/services/SignalP/), which indicated that a 28-amino acid signal peptide was likely to be trimmed away from the precursor protein during post-transcription modification.

To analyses the phylogenesis of *DKK1* gene, we constructed the phylogenetic tree from all the species which had a complete gene sequence, that included *Homo sapiens* (NC_000010), *Rattus norvegicus* (NC_005100), *Mus musculus* (NC_000085), *Macaca mulatta* (NC_007866), *Sus scrofa* (NC_010456), *Oryctolagus cuniculus* (NC_013686), *Danio rerio* (NC_007123) and *Bos taurus* (NC_007327). In Fig. 1a, two primates were neatly clustered into one branch, and two artiodactylas into another. Successively, the two branches fused to form a big group. And then, the rabbit joined, followed by the mouse, and the fish came last. Herein, we could conclude that the phylogenesis of *DKK1* was in consensus with the evolutionary track of these species.

As DKK1 is a conserved secretory protein, to investigate its conservatism we downloaded protein sequences of *Homo sapiens* (AAQ89364), *Mus musculus* (AAH50189), *Rattus norvegicus* (NP_001099820), *Oryctolagus cuniculus* (AAS91587), *Sus scrofa* (ABF68758), *Bos taurus* (DAA14986), *Gallus gallus* (XP_421563), *Xenopus laevis* (NP_001079061), *Danio rerio* (AAI65086). DNAMAN software was employed to align these sequences. Alignment of DKK1 CRD-N (N-terminal cysteine-rich domain) and CRD-C (C-terminal cysteine-rich domain) fragments were exhibited in Fig. 1b. All the cysteine residuals were

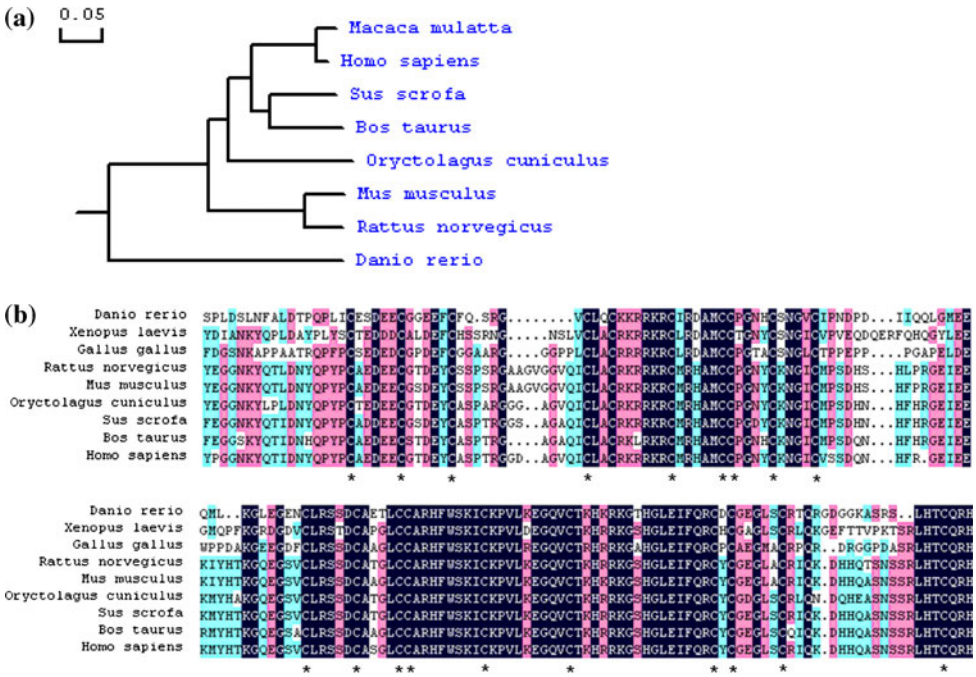


**Fig. 1** Bioinformatics analysis of nucleotide and protein sequences of DKK1 from different species. **a** Phylogenetic tree based on nucleotide sequences. **b** Multisequence alignment of DKK1 N-terminal cysteine-rich domain (*top panel*) and C-terminal cysteine-rich domain (*bottom panel*). All the cysteine residuals are labeled with an *asterisk*

conserved. What's more, high similarity was observed among the species we investigated, and especially CRD-C contained several fragments with 100% similarity, implying its vitally important functions during evolution. As Barbara reported, CDR-C was responsible for binding to LRPs and was sufficient to inhibit Wnt signalling [19]. Therefore, this highly-conserved feature is likely to indicate pivotal significance of DKK1 inhibition to Wnt pathway.

## Chromosomal localization

The 283 bp porcine-specific PCR product was successfully amplified in the IMpRH panel. The analysis of IMpRH results confirmed the assignment of *DKK1* to SSC14 and narrowed its position. Two-point analysis showed that *DKK1* was closely linked to SW1552 (LOD = 6.74). Compared with the linkage mapping, the region could be assigned to SSC14q25-26.

In human, *DKK1* was mapped to 10q11.2, which was in agreement with our result, as human chromosome 10 shared homologous regions with porcine chromosome 14. (https://www-lgc.toulouse.inra.frpig/compare/SSC.htm). In this study we found that *DKK1* was closely linked to SW1552, around which quite many meat quality-related QTLs (Quanttitative Trait Loci) can be found according to the Pig QTL Database, including loin muscle area, average daily gain, fat thickness at shoulder, average backfat thickness, body weight, and so on. (http://www.animalgenomeorg/cgi-bin/QTLdb/SS/draw_chromap).

## SNP identification and association analysis

Two pairs of primers were employed to detect SNPs almost covering the whole gene. One G1757A SNP in the 2nd intron was identified and could be recognized by the *Csp6*I restriction enzyme. Association analysis revealed that there was a significant association between this SNP and LMA (Loin Muscle Area) ($P = 0.0281$). The phenotype value of LMA in pigs with AG genotype was markedly higher than that in pigs with AA genotype, whereas the difference between AG and GG was not significant. We could conclude that heterozygous allele AG was an indicator for a larger loin muscle area. The relevant data is shown in Table 2.

It was suggested that different Wnt molecules could initiate myogenesis by activating expression of *Myf5* and *MyoD* [20, 21], whereas Wnt signalling could be inhibited by DKK1 as mentioned above. The effect of SNP on the phenotype could be explained by the fact that the fate of mRNA could be regulated by the splicing system, which could be affected by the SNPs occurring in introns [22]. Together with the result of chromosomal localization, association analysis exhibited that *DKK1* could be a candidate gene for LMA and may serve as a marker for MAS (Marker Assisted Selection).

**Table 2** Association analyses of *DKK1 Csp6I*-RFLP genotypes with loin muscle area

| Genotype | Number of animals | Loin muscle area ($cm^2$) |
|---|---|---|
| AA | 62 | 28.92 ± 0.66 |
| AG | 39 | 31.77 ± 0.84 |
| GG | 39 | 30.58 ± 0.86 |
| *P* value (AA-AG) | | 0.0077** |
| *P* value (AA-GG) | | 0.1287 |
| *P* value (AG-GG) | | 0.2503 |

*Note*: Phenotype values are presented as mean ± standard error. Comparisons significant at 0.01 level are indicated by **

## Analysis of expression profile

Semi-quantitative RT-PCR analysis of total RNA showed that *DKK1* was widely expressed in examined tissues in both Tongcheng and Large White pigs (Fig. 2) because of the wide target spectrum of Wnt/β-cantenin signalling. *DKK1* was mainly expressed in immune-related organs: in Tongcheng pigs mRNA of *DKK1* was most abundant in the spleen and lymph node; while in Large White pigs *DKK1* was expressed in the spleen at the highest level, while quite weakly in the lymph node. The significance of abundant expression of *DKK1* in theses two tissues was unknown (Table 3).

The result of semi-quantitative RT-PCR was ambiguous due to its poor sensitivity. Hence, we further carried out real-time PCR to quantify the relative mRNA expression of *DKK1* in skeletal muscle and fat tissue at two developmental stages in two breeds. As exhibited in Fig. 3a, e, *DKK1* was less transcribed in Large White pigs in skeletal muscle, while the difference in adipose tissue was not significant compared to Tongcheng pigs (data not shown). Conclusively, down-regulation of *DKK1* in skeletal muscle of the lean breed correlated with its more muscle mass. It was in agreement with the inducible myogenesis of Wnt/β-cantenin signalling, and the inhibitory effect of DKK1 to this signalling.

Considering the myogenesis role aforementioned in association analysis, we were curious to investigate whether Wnt/β-cantenin signalling contribute to differential muscle mass in these two breeds, therefore β-cantenin-encoding gene *CTTNB1* and *TCF4* were quantified. As the transcriptional activation of target genes of Wnt/β-cantenin signalling depends on the binding of TCF/LEF to their promoter regions, and the nucleolus accumulation of β-cantenin, which interacts with TCF/LEF to stimulate the transcription.

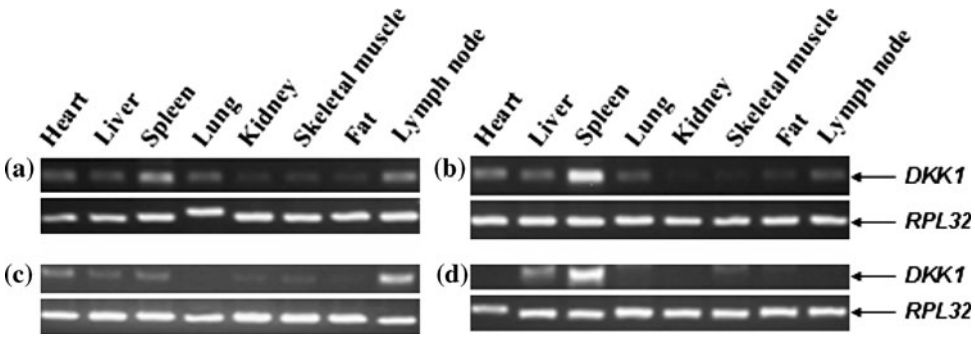


**Fig. 2** Expression profiles of *DKK1* in Tongcheng pigs and Large White pigs. Expression profiles of *DKK1* in postnatal 35 days Tongcheng pig (**a**) and Large White pig (**b**), adult Tongcheng pig (**c**) and Large White pig (**d**). Gene *RPL32* was amplified as internal control in all the four panels as the *arrows* indicate

**Table 3** Primers used in real-time PCR

| Gene | Primer sequences(5′–3′) | Product size (bp) | T (°C) |
|---|---|---|---|
| DKK1 | AGGGGAAATTGAGGAAACCA | 148 | 62 |
| | GGCACAGTCTGATGATCGGA | | |
| CTTNB1 | ATGCCATCATGCGTTCTCCT | 131 | 60 |
| | CAGCAAGCCCTCACGATGA | | |
| TCF4 | ATCAGCAAGCACTGCCGACT | 167 | 60 |
| | CCAACATTCCTCCGTAGCCA | | |
| LRP6 | AACGGGACCATGAGGAAGA | 180 | 60 |
| | TGGCCAACCAAGAGAAGTGT | | |

In vitro experiments depicted that over-expression of $\beta$-cantenin or the expression of dominant-negative mutant of TCF4E was determinant of Wnt/$\beta$-cantenin signalling [8]. To investigate whether Wnt/$\beta$-cantenin signalling contribute to differential muscle mass in these two breeds, $\beta$-cantenin-encoding gene *CTTNB1* and *TCF4* were quantified. In Large White pigs, mRNA levels of *CTTNB1* and *TCF4* were dramatically lower at 35 days (Fig. 3b, f) and adult (Fig. 3c). Furthermore, we examined the expression of *LRP6*, which was the target of DKK1 protein and was essential to Wnt/$\beta$-cantenin signalling. Fig 3h revealed *LRP6* was more transcriptionally active in adult Large White pigs, which was likely to imply more active pathway.

Taking all the results of mRNA expression into account, we concluded that in skeletal muscle of Large White pigs, the expression of *DKK1*, an antagonist of Wnt/$\beta$-cantenin signalling was inactive; while the inducible components, including *CTTNB1*, *TCF4* and *LRP6* were not unanimous. To explain the relationship between Wnt/$\beta$-cantenin pathway and the distinct phenotypes, it was reasonable to speculate that the low-level expression of *CTTNB1* and *TCF4* exerted robust capacity to activate myogenesis in Large White pigs, because the repressive force was weak in

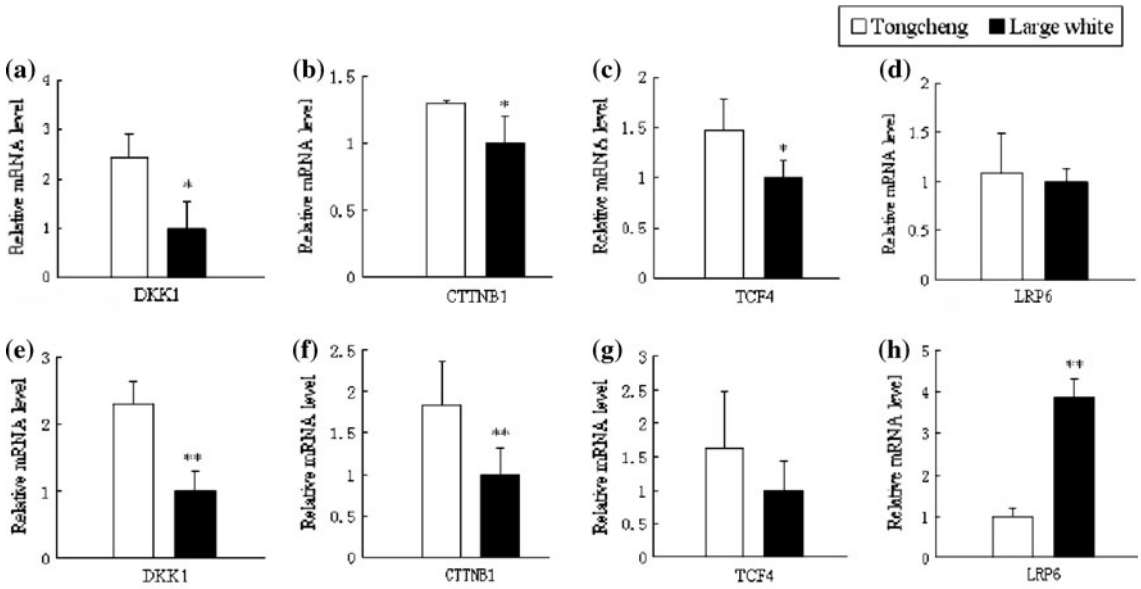


**Fig. 3** Differential expression of *DKK1* and other Wnt pathway associated genes in skeletal muscle from Tongcheng and Large White pigs. Real-time PCR was performed in 35 days (**a**–**d**) and adult (**e**–**h**) pigs. For each stage, three Tongcheng pigs and three Large White pigs were used and each sample was amplified in triplicate. The fluorescence signals of genes were adjusted by the internal gene *RPL32*. Data was shown as mean ± standard deviation. * $P < 0.05$; ** $P < 0.01$

terms of the down-regulation of antagonist *DKK1*, of which the inhibitory function was further counteracted by the up-regulation of *LRP6*. All of these events led to the activation of Wnt/$\beta$-cantenin pathway and eventually the more muscle mass in lean pigs.

In conclusion, we assigned porcine *DKK1* to SSC14q25-26 where a QTL of LMA could be found, which was in agreement with the identification of SNP (G1757A) and association analysis with LMA. Therefore, *DKK1* could act as a genetic marker for LMA. Analysis of expression profiles in Tongcheng and Large White pigs showed that *DKK1* was most abundantly present in immune-related organs. Compared with Tongcheng pigs, *DKK1*, *CTTNB1* and *TCF4* were down-regulatedly expressed, while *LRP6* was up-regulated in Large White pigs. The differential expression of these four crucial genes for Wnt/$\beta$-cantenin signalling indicated that this pathway might exert determinant functions in the phenotypic difference between Chinese obese breed and western lean breed. Obviously, more direct evidences are required to bridge the pathway and the phenotypic differences, as well as the precise mechanism underlying it.

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