Genome-wide association analysis of inguinal/scrotal hernia in pigs using specific length amplified fragment (SLAF) sequencing

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ABSTRACT

Inguinal and scrotal hernias are the most frequent congenital disorders in pigs, and they may cause severe economic loss in the pig breeding industry. Genetic factors play a significant role in the susceptibility to hernias, but the genetic mechanisms of inguinal/scrotal hernia are poorly understood. In this study, we performed a genome-wide association study (GWAS) with specific-locus amplified fragment sequencing (SLAF-seq) on 120 (59 cases and 61 controls) full and half sib pigs to identify genetic loci underlying variations in inguinal/scrotal hernias. A total of 218,460 high-quality SNPs were generated for further statistical analysis with case-controls and the farmCPU model. Based on these two methods, a total of 59 SNPs were significantly ($P < 0.01$) associated with inguinal/scrotal hernia. Finally, 14 novel candidate genes implicated in the defect were identified, and 5 genes (*CPNE5*, *DEGS1*, *PLCG2*, *PRKCE* and *NUAK1*) were related to cell apoptosis, which is one of the pivotal pathogenesis factors of inguinal/scrotal hernia. In addition, 4 of them were shown strong associations with the hernia were confirmed in 270 samples ($P < 0.05$). This finding provides new evidence that genes related to cell apoptosis may be associated with inguinal/scrotal hernias.

INTRODUCTION

Inguinal and scrotal hernias, which occur in the weak areas of the inguinal canal [1, 2] or the processus vaginalis [3, 4], are some of the most frequent congenital disorders observed in pigs and humans [5, 6]. They cause severe economic loss in the pig breeding industry [7]. Hernia development is caused by both multiple genes and environmental factors [8]. To date, candidate genes related to the development of inguinal/scrotal hernias have been inspected. A linkage map of 15 porcine STS markers was constructed and the sex determining region Y-box 9 (*SOX9*), which results in sex hormone deregulation, was identified

as a potential candidate gene of pig inguinal/scrotal hernias [9]. Moreover, the collagen type II alpha 1(*COL2A1*) and matrix metalloproteinase 2 (*MMP2*) genes have been found to be involved in pig scrotal hernias by promoting aberrant collagen metabolism processes [10]. However, only a few candidate genes have been reported and the molecular mechanism of the etiology remains unclear.

To obtain more effective susceptibility genes, a series of GWAS identifications of inguinal/scrotal hernias have been recently published in several different pig breeds. Four candidate genes (*ELF5*, *KIF18A*, *COL23A1* and *NPTX1*) for the defects on Sus scrofa chromosomes (SSC) 2 and 12 were identified from 6 commercial

pig breeds and there was no overlap in the significant SNPs from different pig breeds [11]; Ding et al. (2009) detected the most remarkable loci on SSC 7, 8, and 10 in a White Duroc×Erhualian resource population of F2 pigs [12]. Sevillano et al. (2015) identified five distinct QTL regions that strongly affected the inguinal/scrotal hernias detected in both Large White and Landrace datasets [13]. Conclusively, the current study findings showed the putative inheritance mode of inguinal/scrotal hernias was of polygenic effects. Nevertheless, the main genes with important roles were not found. Thus, more high-quality and high-density SNPs were studied among the whole genome to seek the markers observably associated with inguinal/scrotal hernias.

Next-generation sequencing (NGS) technologies have made it possible to obtain high-density SNP markers throughout the entire genome, which was precisely the basis of the GWAS [14–16]. Consequently, several reduced representation sequencing technologies, including restriction site associated sequencing (RAD-seq), genotyping by sequencing (GBS) were developed for SNP scanning [17]. Specific-locus amplified fragment sequencing (SLAF-seq) is a new efficient and high-resolution approach based on reduced representation sequencing for *de novo* SNPs discovery and large-scale genotyping [18]. Compared with other sequencing-based technologies, the SLAF-seq technology has more advantages in large populations, such as significant cost saving, high capacity of samples, less sequencing demand, and high genotyping accuracy without prior genomic information [19–22]. Thus, this method has now been successfully applied to screen for specific molecular markers in many species. Zhao et al. constructed a high-density haplotype map of SNP markers in soybean through SLAF-seq technology and 4 candidate genes were identified for resistance to *Sclerotinia sclerotiorum* [23]. Zhang et al. performed a GWAS using SLAF-seq to detect the SNP markers that were associated with growth traits in Jinghai Yellow chicken hens [24]. Li et al. developed a SLAF-seq approach to reveal the genetic differences among Landrace, Erhualian, and Meishan pigs [25]. Thus, we used SLAF-seq and GWAS to discover the candidate genes associated with pig inguinal/scrotal hernias in a set of 120 accessions (59 inguinal/scrotal hernia cases and 61 unaffected controls) from four pig populations including French Yorkshire, French Landrace, American Yorkshire and crossbred pigs. These results can be used for subsequent investigations of pig inguinal/scrotal hernias in the hope of eradicating the disease at a molecular level.

RESULTS

SLAF sequencing and SNP marker screening

To genotype the 120 pig accessions, SLAF-seq was performed with Illumina HiSeq™ 2500 (Illumina,

Inc, USA) at Biomarker Technologies Corporation. The restriction enzymes RsaI and HaeIII were selected based on *in silico* digestion prediction, and it resulted in 332,425 predicted 314–344 bp SLAF tags that were evenly distributed across the whole pig genome. Basing on SLAF library construction and high-throughput sequencing, a total of 110 Gb raw data including 782.06 M reads were obtained, with each read 100 bp in length and an average depth of 12.81x from this experiment. The average Q30 ratio (a quality score of 30) and the average guanine-cytosine (GC) content of 85.34% and 44.36%, respectively, was indicative of good quality (Supplementary Table 1).

A total of 407,274 high-quality SLAF tags were selected to obtain high-quality polymorphic SLAFs after two rounds of sequencing and exclusion of low-quality fragments, and 217,408 polymorphic SLAFs were identified from these reads by sequence alignments with the *Sus scrofa* reference genome (GenBank Assembly ID GCA_000003025.4 ftp://ftp.ensembl.org/pub/release-80/fasta/sus_scrofa/dna/). According to the results, SLAF tag numbers were calculated on each chromosome (Supplementary Table 2), and the distribution diagram of SLAF tags involved each chromosome (Figure 1A). The SLAFs were well distributed on each chromosome with high integrity and accuracy. The average coverage in the samples revealed that the sequencing results are reliable for calling SNPs. In total, we initially called 2,514,779 SNPs for quality control, and a large proportion were found to have a minor allele frequency (MAF) of < 0.05 or low integrity (< 0.8) and should be removed. After filtration, 218,460 SNPs, which covered all 20 chromosomes, were used for inclusion in the GWAS (Supplementary Table 3, Figure 1B).

Population structure

Before conducting the genome-wide association study, the population structure assessed by ADMIXTURE [26] was performed using 218,460 SNPs of the 120 accessions to separate accessions from mixed populations and estimate the number of subgroups, which had great influence on the association analyses between phenotypes and molecular bands. This method is based on a clustering strategy to calculate the cross validation (CV) errors of the large sample sizes, and then the minimum K value could produce the best dataset. With the maximum membership probability, the best dataset was K = 3, which meant the samples were probably derived from 3 ancestors in our study (Figure 2). The result is highly consistent with the sample information, indicating that this swine population could be used for association analysis. Additionally, principal component (PCA) and neighbor-joining cluster analyses were set to verify the evolutionary relationships between the 120 samples. Both the PCA and cluster results showed that all the samples fell mostly into three groups,

with only a few outliers, which was in accordance with the above results (Figure 3A, 3B).

GWAS analysis

In an attempt to investigate inguinal/scrotal hernia loci, a GWAS was carried out to search for associated tags and allelic variations. We selected two popular methods (PLINK and farmCPU) to analyze the data.

In PLINK, 7 SNP loci ($P < 10^{-6}$) were found to be significantly related to inguinal/scrotal hernias according to the case-control mode, and the QQ-plot revealed that there was no obvious population stratification in this experiment. Two of the seven SNP loci (rs327702852, $P = 4.82\text{E-}06$ and rs340140110, $P = 8.44\text{E-}06$) overlapped the known genes *CPNE5* and *MAP7D2*, respectively (Table 1, Figure 4).

In farmCPU, a total of 52 significant association signals were identified, with $P < 8.91\text{E-}06$, from the disparate threshold (Table 2, Table 3). Both the default and suggestive p.threshold could make a great difference in the association results. Specifically, in trait1, the two significant SNPs (rs319767757, $P = 1.24\text{E-}06$ and rs320497311, $P = 2.19\text{E-}06$) confirmed in the two p.threshold were located in the known gene *AUH* (Figure 5A, 5B, 5G, 5H). In trait2, when p.threshold=0.01/N and $1\text{E-}05$, 9 and 6 remarkable SNPs overlapped the known genes, and two of those (rs331932518, $5.69\text{E-}08$ and rs344315078, $8.11\text{E-}10$) detected in both results were located on *VWA3B* and *NUAK1*, respectively (Figure 5C, 5D, 5I, 5J). In trait3, comparison of the two p.thresholds revealed no differences in 15 significant association signals and three genes (*TENM3*, *NOL10* and *PLCG2*) were located on the known susceptibility loci according to these genomic positions (Figure 5E, 5F, 5K, 5L). Furthermore, the Q-Q plots of all these arithmetic values

indicated that the GWAS with farmCPU could be used to accurately identify genes associated with inguinal/scrotal hernias. In summary, we observed 14 known genes related to the susceptibility SNPs ($P \leq 7.76\text{E-}06$) identified through GWAS, and four of them (*TENM3*, *PLCG2*, *PRKCE* and *NUAK1*) showed the strongest association signal ($P < 10^{-9}$) with the disease. From the results, the power of farmCPU was better than the case/control model, which cannot completely distinguish the real signals from the background noise. With PLINK, only 2 candidate genes were identified, but 13 associated genes were identified using farmCPU, suggesting this method may be more suitable for the discovery of complex and polygenic traits.

Confirmation of association results in 270 samples

After the GWAS, 14 genes overlapping the candidate regions of 21 SNPs were discovered. To validate the SNPs associated with inguinal/scrotal hernias, we chose the most strongly associated SNPs and the genes that were shown to be related to cell apoptosis in previous studies for replication of the association signal. In total, 6 genes (*CPNE5*, *DEGS1*, *PLCG2*, *PRKCE*, *NUAK1* and *TENM3*) were confirmed for further genotyping in 270 individuals. Furthermore, two strong candidate genes *COL2A1* [27] and *MMP2* [28], which were proved to be associated with increased risk of inguinal/scrotal hernias in former studies were selected for the replication study to measure the relatedness with hernias in our group. Interestingly, the results in Table 4 show that 4 of 8 SNPs were significantly ($p < 0.05$) associated with the inguinal/scrotal hernia in 270 pigs. However, while using the purebred samples including the French Yorkshire and the French Landrace, the association was not significant all the time. In detail,

Figure 1: SLAF and SNP distribution on the 12 chromosomes of pigs. Horizontal lines represent chromosomes, with the chromosomal length on the x-axis. The scale in the top-right corner indicates the number of SLAFs or SNPs. All SLAFs (black lines) distributed on each chromosome (A). All SNPs (black lines) distributed on each chromosome (B). In each chromosome, the more the markers are, the darker the color is.

Table 1: Significant association SNPs in the genome-wide association by PLINK for inguinal/scrotal hernia

Chromosome	SNP name	Position (bp)	P value	Overlapped gene
1	rs330433880	167390400	5.48E-06	
6	rs324506705	84565388	7.33E-06	
6	rs332435472	84565401	3.95E-06	
6	rs705699393	117226150	5.14E-06	
7	rs327702852	37402882	4.82E-06	<i>CPNE5</i>
8	rs342486064	104820715	1.31E-06	
X	rs340140110	17669963	8.44E-06	<i>MAP7D2</i>

CPNE5 (rs327702852) showed an extremely significant association with hernias in all the groups with a chi-squared test *P* value of 3.241E-07, 0.001423 (*p* < 0.01) and 0.02066 (*p* < 0.05). Actually, *CPNE5* (rs327702852) was the only one that showed the strongest signals in different breeds in our study, which indicated the *CPNE5* gene may play an important role in the formation of an inguinal/scrotal hernia. Otherwise, three remarkable farmCPU-identified SNPs maintained genome-wide significant associations: *TENM3* rs693312052 (*p* = 0.003708 < 0.01), *PLCG2* rs336839455 (*p* = 0.02157

< 0.05), *PRKCE* rs328681942 (*p* = 0.01461 < 0.05). Specifically, *TENM3* and *PRKCE* were also suggestively associated with hernias (*p* = 0.04922 and 0.03025) even in the purebred groups, i.e., French Landrace and French Yorkshire, respectively. Moreover, SNPs within the *NUAK1* gene showed associations with only French Landrace but not with more pigs and it may be caused by the limited sample numbers. For the remaining three SNPs, the comparison in mixed or purebred groups did not uncover notable differences between inguinal/scrotal hernias and the polymorphisms of *DEGS1*, *COL2A1* and

Figure 2: Population structure analysis in 120 accessions of pigs. In the upper panel, each color represents a group and each line represents a cluster and different colours represent separate groups. The lower panel displays the sub-group value, where the value ranges from 1 to 10. The colored coefficient of variation (CV) value for minimum K value (K = 3) represents inferred membership in K genetic clusters.

MMP2 ($p > 0.05$) providing some evidence that numerous SNPs in pig inguinal/scrotal hernias were likely breed specific, which coincided with the former studies of pig inguinal/scrotal hernias [29–31].

DISCUSSION

The SLAF-seq strategy, a novel NGS-based method, has been applied widely in *de novo* SNP discovery and genotyping of large populations [18], such as high-density genetic map construction of cucumbers [32], candidate genes analysis of cotton [33], and high-quality SNP identification of rapeseed [34]. However, there have been few reports about the application of SLAF-seq technology in resistance breeding of livestock, and to our best knowledge, this study is the first report of pig inguinal/scrotal hernias at genome-wide significance using SLAF-seq technology. Traditionally, the porcine SNP60K chip of Illumina was utilized to genotype samples [35, 36]. Compared with the SLAF-seq technology, SNP chip cannot be used when a reference genome is unavailable, suggesting that only known SNP markers could be detected by SNP-arrays [37]. While the SLAF-seq is designed to be independent of the reference genome, the new SNPs may be investigated and developed by this method [38]. In our study, the sequencing depth among each sample was 12.81X, which revealed the overall coverage of the whole genome was more than 95% and the number of the called SNPs was 2,514,779, which was much higher than the number of the markers in previous studies that used the 60K SNP Beadchip (64,232 SNPs) [35].

To estimate the heritability of inguinal/scrotal hernias, a GWAS of pig inguinal/scrotal hernias was carried out after SLAF sequencing on these population of pigs. Initially, both the general linear model (GLM) and the mixed linear model (MLM) were used to seek the associated markers

and allelic variation by TASSEL [39]. However, there was no clear signal associated with the hernia even when the cutoff P value was < 0.05 . In consideration of different statistical models that tend to perform differently from various datasets [40], a case-control model implemented in PLINK, which has been previously reported [41], was employed to ascertain the remarkable SNPs associated with inguinal/scrotal hernias, and 7 significant association loci and 2 candidate genes (*CPNE5*, *MAP7D2*) were identified. Some studies have reported that case-control analysis by PLINK controlled the population structure and kinship among individuals as covariates to reduce false positives, but the use of covariates and testing markers may cause confounding, which could produce false negatives [42]. Interestingly, in addition to these classical models, farmCPU is a modern tool that can be used to effectively control the false positives in a genome-wide association study and completely eliminate the confounding problem in current methods [43]. Specifically, this method is divided into two parts as follows: a Fixed Effect Model (FEM) and a Random Effect Model (REM). Pseudo markers were calculated as covariates in FEM and optimized by REM to be designed to control false positives and even false negatives simultaneously [43]. FarmCPU, as an original statistic strategy, is equipped to significantly improve statistical power and speed, which successfully detected more QTNs than MLM under a low false discover rate in *Arabidopsis thaliana* and pigs [28]. Concretely, three more loci were identified for rupture of the cranial (anterior) cruciate ligament (RCCL) of complex orthopedic traits in dogs by farmCPU, which were not identified for RCCL in the previous report [44]. Avjinder S. detected 17 never-before-reported putative SNPs which were associated with soybean canopy wilting by farmCPU and these remarkable markers has been successfully used for identifying parental genotypes in breeding programs [43]. As these examples

Figure 3: Characterization of the genetic structure of the 120 pig accessions. (A) The evolutionary tree of the 120 pig accessions based on the analysis of 218,460 SNPs. (B) PCA analysis of 120 pig accessions based on the analysis of 218,460 SNPs. The branch and point indicates each material. The blue line/point is materials who suffered inguinal/scrotal hernia, and red line/point is controls.

Table 2: Significant association SNPs by farmCPU with p.threshold = 0.01/N for inguinal/scrotal hernia

Trait	Chromosome	SNP name	Position (bp)	P value	Overlapped gene
1	12	rs324304114	34729146	1.19E-06	<i>LOC102167662</i>
1	13	rs344395079	202760816	1.74E-06	<i>LOC100623424</i>
1	14	rs319767757	3186015	1.24E-06	<i>AUH</i>
1	14	rs320497311	3186054	2.19E-06	<i>AUH</i>
1	8	rs342486064	104820715	6.23E-08	<i>LOC102167758</i>
1	8	rs344971120	124744876	6.46E-06	
2	11	rs333915086	79002243	8.91E-06	
2	12	rs707102499	7147387	4.56E-06	
2	13	rs345544978	216120419	6.90E-06	<i>TMPRSS3</i>
2	13	rs340959250	216123992	5.60E-06	<i>TMPRSS3</i>
2	13	rs703849339	216162734	6.90E-06	<i>UBASH3A</i>
2	13	rs327383802	216162735	6.90E-06	<i>UBASH3A</i>
2	13	rs340532657	216186419	2.33E-06	<i>UBASH3A</i>
2	13	rs329192909	216186440	8.91E-07	<i>UBASH3A</i>
2	14	rs691341365	96397595	7.76E-06	<i>PTPN20B</i>
2	16	rs695479999	3704632	2.78E-07	
2	3	rs331932518	58618400	2.55E-06	<i>VWA3B</i>
2	4	rs326799532	72683195	5.69E-06	
2	5	rs344315078	14224907	1.35E-06	<i>NUAK1</i>
2	9	rs339050967	134709123	3.64E-06	
3	10	rs341855439	45457399	3.49E-06	
3	10	rs342639148	49056928	1.63E-06	
3	13	rs327747621	41755529	5.31E-06	<i>LOC100737245</i>
3	14	rs711689020	12819127	2.68E-07	
3	15	rs693312052	50816340	2.26E-11	<i>TENM3</i>
3	16	rs329831629	13208012	1.29E-14	<i>LOC102166550</i>
3	3	rs345757418	36050138	3.16E-09	<i>LOC102163305</i>
3	3	rs326409933	60432354	2.01E-06	
3	3	rs343695952	134682302	4.60E-06	<i>NOL10</i>
3	3	rs337988450	134682458	4.60E-06	<i>NOL10</i>
3	4	rs320400918	62031612	6.60E-13	
3	6	rs336839455	6997544	6.27E-10	<i>PLCG2</i>
3	6	rs333396283	88961742	4.61E-06	
3	8	rs341737443	17618314	4.15E-07	
3	8	rs320838432	115656516	3.43E-06	

suggest, farmCPU showed a stronger test power in different samples. Because it can effectively distinguish the associated markers between background noise above a strict control of error rate [45, 46]. More importantly, farmCPU has a higher test speed and a dataset with tens of thousands of markers can be analyzed in less than a week [47]. Therefore, we also performed a farmCPU analysis in order to verify more novel susceptibility loci for this

complex disease. Based on this model, we confirmed 52 significant association loci and 12 candidate genes affecting inguinal/scrotal hernias in pigs utilizing the same data. The different results between these models further proved that farmCPU is an efficient tool for retrieving missed genes by other methods.

To date, many theories have intended to explain the etiology of inguinal/scrotal hernias and the most typical

Table 3: Significant association SNPs by farmCPU with p.threshold = 1E-05 for inguinal/scrotal hernia

Trait	Chromosome	SNP name	Position (bp)	P value	Overlapped gene
1	11	rs321121582	51457751	1.64E-07	
1	12	rs324304114	34729146	8.64E-11	<i>LOC102167662</i>
1	13	rs344395079	202760816	1.89E-08	<i>LOC100623424</i>
1	15	rs341787989	123715674	4.81E-06	<i>LOC102168011</i>
1	2	rs321767225	120299079	5.86E-06	
1	3	rs340228447	128504973	9.02E-10	
1	4	rs331771053	133585924	9.05E-07	
1	8	rs342486064	104820715	4.06E-12	<i>LOC102167758</i>
1	9	rs326461723	120701827	4.26E-07	<i>LOC100625132</i>
2	1	rs340355827	228307603	3.88E-09	
2	10	rs342370930	14999738	1.71E-07	<i>DEGSI</i>
2	12	rs707102499	7147387	3.98E-11	
2	16	rs337170191	7611324	8.69E-06	
2	3	rs331932518	58618400	5.69E-08	<i>VWA3B</i>
2	3	rs322669192	100395213	6.05E-06	
2	3	rs328681942	100554717	1.53E-09	<i>PRKCE</i>
2	3	rs329076481	100667390	1.11E-06	<i>PRKCE</i>
2	4	rs326799532	72683195	2.56E-14	
2	5	rs344315078	14224907	8.11E-10	<i>NUAK1</i>
2	6	rs321426177	125417548	5.76E-06	
2	6	rs707167754	140966050	1.17E-06	
2	6	rs322990991	155603932	1.66E-06	
2	8	rs340232857	140425238	3.56E-06	<i>MEPE</i>
3	10	rs341855439	45457399	3.49E-06	
3	10	rs342639148	49056928	1.63E-06	
3	13	rs327747621	41755529	5.31E-06	<i>LOC100737245</i>
3	14	rs711689020	12819127	2.68E-07	
3	15	rs693312052	50816340	2.26E-11	<i>TENM3</i>
3	16	rs329831629	13208012	1.29E-14	<i>LOC102166550</i>
3	3	rs345757418	36050138	3.16E-09	<i>LOC102163305</i>
3	3	rs326409933	60432354	2.01E-06	
3	3	rs343695952	134682302	4.60E-06	<i>NOL10</i>
3	3	rs337988450	134682458	4.60E-06	<i>NOL10</i>
3	4	rs320400918	62031612	6.60E-13	
3	6	rs336839455	6997544	6.27E-10	<i>PLCG2</i>
3	6	rs333396283	88961742	4.61E-06	
3	8	rs341737443	17618314	4.15E-07	
3	8	rs320838432	115656516	3.43E-06	

hypotheses that were widely accepted were as follows: the apoptosis of muscle cells and aberrant collagen metabolism [30]. According to previous studies, the failure of incomplete closure of processus vaginalis and internal

inguinal ring was thought to play an important role in the development of inguinal hernias and scrotal hernias, respectively [48, 49]. Both pathological appearances were caused by dysfunction of smooth muscle, which was

Table 4: Results of the further association analyses in 270 samples by chi square test of independence

Gene symbol	SNP	All samples (n = 270)	French Yorkshire (n = 111)	French Landrace (n = 50)
		P value	P value	P value
<i>CPNE5</i>	C/T	3.241E-07	0.001423	0.02066
<i>TENM3</i>	G/T	0.003708	0.4291	0.04922
<i>PLCG2</i>	C/T	0.02157	0.3558	0.3371
<i>PRKCE</i>	A/G	0.01461	0.03025	0.9171
<i>NUAK1</i>	C/T	0.1589	0.1852	0.01644
<i>DEGS1</i>	C/T	0.2340	0.3735	0.3192
<i>COL2A1</i>	C/T	0.2011	0.1662	0.2463
<i>MMP2</i>	C/T	0.9347	0.5556	0.6776

achieved by cell apoptosis [50]. Compared with smooth muscle samples from controls, smooth muscle samples from patients with inguinal hernias failed to reveal the nucleolus of apoptotic cells and had lower levels of Ca^{2+} , which is the key to programmed cell death [51]. In addition, abnormal collagen metabolism is strongly associated with hernia disease [52]. Collagen is the main protein for resisting stress because of its tensile strength and it is composed of some specific anatomical structures such as the internal inguinal ring and gubernaculum [30, 53]. Compared with the patients, more type III collagen, which is an immature collagen form of connective tissue, can be attributed to the development of hernias [53]. Thus, we propose that the genes related to collagen metabolism and apoptosis may be hernia-associated genes that contribute to the weakening of the abdominal wall and the failure of obliterative processus vaginalis. Further, to evaluate the association between the two genes detected in a previous study (*COL2A1* and *MMP2*) and inguinal/scrotal hernias in our samples, a chi-squared test of independence on 270 individuals was carried out. As the results showed, there was no association between the two certified candidate genes (*COL2A1* and *MMP2*) and inguinal/scrotal hernias in our samples, implying that the molecular mechanisms of pig inguinal/scrotal hernias

may be different in diverse pig breeds and lines. However, gene function classification of these loci revealed that five of the candidate genes (*CPNE5*, *DEGS1*, *PLCG2*, *PRKCE* and *NUAK1*) were concerned with cell apoptosis, which was one of the possible pathogenesis factors for hernias in current studies. Thus, to evaluate the associations between the interesting genes in our study and inguinal/scrotal hernias in pigs, a chi-squared test of independence on 270 individuals were carried out. In contrast, the genes confirmed in our study showed a strong association with inguinal/scrotal hernias and, more importantly, these genes were certified to take part in the apoptotic pathways in various reports. To be specific, the *CPNE5* gene is in a novel family of ubiquitous Ca^{2+} -dependent phospholipid-binding proteins and plays a key role in many processes of Ca^{2+} -mediated signal transduction, especially apoptosis [54]. It has been reported that the overexpression of the *CPNE5* gene in HEK293 cells can cause cell death [55] and in early apoptosis of SH-SY5Y cells. The *Cpne5* protein expression was significantly changed [56]. Similarly, the *DEGS1* gene encoded a member of the membrane fatty acid desaturase family [57] and was proved to participate in the activation of caspase 9 and caspase 3, which were marker proteins of cell apoptosis [58]. Moreover, *PLCG2* and *PRKCE* genes

Figure 4: Manhattan plot (A) and quantile-quantile plot (B) of genome-wide association by PLINK for susceptibility to inguinal/scrotal hernia. The observed $-\log_{10} p$ -values (Y-axis) of the association between the SNPs and susceptibility for inguinal/scrotal hernia are shown. All SNP are represented by dots and displayed per chromosome (X-axis). The blue line shows the potential significance threshold: $-\log_{10} (E-05)$.

Figure 5: Manhattan and Quantile-quantile plots of genome-wide association by farmCPU for susceptibility to inguinal/scrotal hernia. Manhattan plots with p -threshold = $0.01/N$ in trait1 (A), trait2 (C), trait3 (E). Manhattan plots with p -threshold = $1E-05$ in trait1 (G), trait2 (I), trait3 (K). The observed $-\log_{10} p$ -values (Y-axis) of the association between the SNPs and susceptibility for inguinal/scrotal hernia are shown. All SNP are represented by dots and displayed per chromosome (X-axis). The blue line shows the potential significance threshold: $-\log_{10} (E-05)$, while the red shows the potential significance genome-wide significance threshold: $-\log_{10} (E-7.5)$. Quantile-quantile plot with p -threshold = $0.01/N$ in trait1 (B), trait2 (D), trait3 (F). Quantile-quantile plot with p -threshold = $1E-05$ in trait1 (H), trait2 (J), trait3 (L).

encoded phospholipase C-(PLC) γ 2 and protein kinase C(PKC)- ϵ , respectively, and both proteins were important elements of cellular signal transduction pathways that could regulate a chain of events either in physiological or pathological status [59–61]. Kajstura found that angiotensin-converting enzyme (ACE) II could induce the translocation of PKC- ϵ of myocytes due to an increase in intracellular free Ca^{2+} , which could lead to the initiation of apoptosis [62], while the translocation of PLC- γ 2 was certified to transmit the TPA signal to its downstream molecule PKC and directly promote the process of apoptosis in MGC80-3 cells [59]. Analogously, NUA1, also known as AMPK-related protein kinase 5, was the downstream molecule of Akt, which participated in the classical signaling pathway with extensive consequences [63]. Numerous studies have confirmed that Akt-ARK5 was closely related to cancer development and metastasis through blocking cell apoptosis by the inhibition of caspase 8 activation [64, 65]. Based on the above information, the loci that we identified were more likely to be the true causal variant. Nonetheless, further investigation is needed to examine exactly how these SNPs affect disease or health by formal verification tools of biomedical research. For instance, We found that TENM3 gene is significantly correlated to inguinal/scrotal hernias ($P < 0.01$) in our samples and according to the JASPAR database, the SNP within TENM3 is found to be coincided with the DNA motifs to which the RUNX2 transcription factor bind. Therefore, a series of molecular biology assay are used to study the functions of TENM3 gene, hoping to confirm whether the TENM3 could be a candidate marker in marker-assisted selection breeding. Furthermore, some new methods (such as ISIS EMBLASSO) could be used in our dataset for detecting more significant loci of inguinal/scrotal hernias.

In summary, we identified 59 SNPs significantly associated with inguinal/scrotal hernias, including 14 novel genes (*CPNE5*, *MAP7D2*, *AUH*, *TPRSS3*, *UBASH3A*, *PTPN20B*, *VWA3B*, *NOL10*, *DEGS1*, *MEPE*, *TENM3*, *PLCG2*, *PRKCE* and *NUAK1*). To our knowledge, no studies investigating the associations of these SNPs with inguinal/scrotal hernias have been published. However, some SNPs that were previously identified as susceptibility loci were not detected. Moreover, the replication analyses supplied credible evidence for the association between gene polymorphisms concerning cell apoptosis and the risk of inguinal/scrotal hernias. Our findings could be useful for supplementing past studies of the etiology in pig inguinal/scrotal hernias and offer new evidence of genetic variation in this disease.

MATERIALS AND METHODS

Ethics statement

All animal experiments were conducted according to the regulations for the Administration of Laboratory

Animals of China. Animal experiments of our study were approved by the Animal Welfare Committee of Huazhong Agricultural University.

Animals and sample preparation

A total of 270 full and half sib pigs including 111 French Yorkshire pigs, 59 French Landrace pigs, 43 American Yorkshire pigs, 57 Duroc $\times$ Landrace $\times$ Yorkshire or Landrace $\times$ Yorkshire crossbred pigs, were obtained from the Kaisheng Pig Farm in Zhejiang, China. The samples consisted of 85 inguinal/scrotal hernia cases and 185 controls. In general, inguinal hernia and scrotal hernia cannot be easily distinguished without clinical examination. Both of them were diagnosed by palpation. For instance, an aberrant protrusion of abdominal or scrotum contents was found in pigs, and the protrusion may disappear when put up the legs slowly. Thus, the affected pigs were recorded as one kind of trait and the hernia phenotype was diagnosed carefully with palpation done by veterinarians. Finally, we selected 120 individuals consisting of 59 affected cases and 61 controls for SLAF sequencing.

The genomic DNA of all animals was extracted from porcine venous blood with a standard protocol with a Blood Genprep DNA Kit (Qiagen, USA), and all the DNA samples were diluted to a similar concentration. The quality and quantification of the genomic DNA were then estimated using 0.8% gel electrophoresis and a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA), respectively.

SLAF library construction and sequencing

One hundred and twenty pigs, including 59 cases and 61 controls, were used for SLAF library construction and sequencing, as described by Sun et al. [18]. Sequencing libraries of the samples were prepared through double digestion with the restriction enzymes *Rsa*I and *Hae*III (New England Biolabs, NEB, USA), which can digest pig genomic DNA into more than 300,000 sequencing tags of length 314–344 bp. Klenow Fragment (3'→5'exo-) (NEB) and dATP were used to add a single nucleotide (A) overhang to the digested fragments at 37°C. Subsequently, T4 DNA ligase (NEB) was used to ligate the A-tailed fragments and the Duplex Tag-labeled Sequencing adapters (PAGE purified, Life Technologies, USA) [40]. Next, the DNA fragments were purified by Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK). Finally, according to the manufacturer's recommendations, the gel purified product was sequenced using an Illumina HiSeq 2500 system (Illumina, Inc., San Diego, CA, USA) at Biomarker Technologies Co Ltd. in Beijing.

SLAF-seq data analysis and SNP calling

A total of 2,514,779 SNPs were remained for genome-wide association analysis. Before the association

Table 5: SNP name, primers and restriction enzymes for PCR-RFLP and AS-PCR assays

Gene	SNP	Primers sequence (5'-3')	Genotyping
<i>CPNE5</i>	C/T	Forward: TGTAATAATTGTCCCTCCTATGCTAC; TAATAATTGTCCCTCCTATGCAGT Reverse: CCTGGCTATTGTGAATAGTGCT	AS-PCR
<i>TENM3</i>	G/T	Forward: GATCCTCTTATGGGAAAA Reverse: AACCTACTGACTGGGAGA	Sequencing
<i>PLCG2</i>	C/T	Forward: GCCAGCTGCAGCTCAGCCCA; TTTTAAGCCAGCTGCAGCTCAGAACG Reverse: TTTGGTTGTGTCAGAGAGGAAAGGGTAAGAG	AS-PCR
<i>PRKCE</i>	A/G	Forward: ACCCTCAACGCTCTGTCC Reverse: TAGGCCGCTTAACCAAAA	TaqI ^a
<i>NUAK1</i>	C/T	Forward: ACACCCCAGATGCTATCAGAACCACC; CACACCCCAGATGCTATCAGAACCACT Reverse: ACAATGGCTGGGGATCTAGAAGTGACAAG	AS-PCR
<i>DEGS1</i>	C/T	Forward: TGACATGAGGCATCTTAGAGT Reverse: GGAAGGGAAGTTCTTGGTAT	FokI ^a
<i>COL2A1</i>	C/T	Forward: CCCATGCTGAGAAGCACTGA Reverse: GTGACCCACACTCCTGAAAG	BclI ^a
<i>MMP2</i>	C/T	Forward: AAAATGCTCTTCAGGCAGGA Reverse: AGGAGATGGGACTGGGAGTT	BstAPI ^a

^aRestriction enzymes used for PCR-RFLP genotyping.

analysis, a systematic quality control (QC) procedure of SNPs was applied as follows: $Q = -10 \cdot \log_{10} e$. Where Q is the quality value, and e is the error rate; if the error rate of sequencing is 0.001, the quality of the quality value should be 30. Thus, we selected $Q \geq 30$ to inspect the quality of our sequencing. Meanwhile, the Short Oligonucleotide Alignment Program (SOAP) was used to map raw paired-end reads onto the reference genome [66]. The SLAFs mapped to the same position suggested high accuracy of the sequencing [67]. Then the consistent SNPs were called by the GATK [68] and SAMtools packages [67]. Ultimately, the intersection of these two methods were deemed as a true result and SNPs were selected with the criteria of minor allele frequency (MAF) > 0.05 and integrity > 80%.

Evolutionary genetics analysis

Based on the SNP genotype data identified in this study, phylogenetic trees were performed in MEGA5 software based on the neighbor-joining algorithm [66, 69], principal components analysis (PCA) was used by cluster software [68], and the population structure matrix was constructed in ADMIXTURE program using the maximum-likelihood method with 10,000 iterations [26]. K, conducted by SPAGeDi [70] software, is the number of subgroups varying from 1 to 10, and the minimum value according to the model choice criteria was considered the most likely value of ancestors from each K subgroup of each accession [71].

GWAS analysis

GWAS was carried out using two strategies based on case/control data. One was conducted by the PLINK v1.07 [72] software based on a case/control model. The genome-wide significance threshold, employed by Bonferroni-corrected *P* values, was set as $0.1/N$, where *N* is the number of total SNPs used for GWAS.

The other strategy used a novel method, fixed and random model circulating probability unification (farmCPU), by farmCPU v1.0 [45]. Actually, farmCPU is a modified mixed linear model (MLM) that was designed to avoid the confounding problem of population structure and kinship. An additional function of farmCPU was to change the threshold by the parameter “p.threshold”, when the genotypic markers are large. We set the p.threshold value as $1E-05$, when the 95% quantile value of each experiment is recommended for p.threshold. This threshold value is more stringent than that reported in other pig GWAS studies. Furthermore, to identify the common significant SNPs present in more than one trait, a default Bonferroni-corrected threshold value of $P = 0.01/N$ (where *N* is the number of total SNPs) was used. Both thresholds were used to indicate the association with the related traits. To obtain more high-quality statistical results, we divided the samples into three classes, named trait1, trait2 and trait3. In all three kinds of traits, the 120 individuals were divided into 2 parts: cases and controls. Specifically, diseased or not could be the only factor

considered in trait1. Moreover, trait2 and trait3 were also classified by pig breeds: trait2 (Yorkshire, Landrace and crossbred) and trait3 (French lines and American lines). In sum, we analyzed the data using different classifications to get much greater statistical power by farmCPU.

Quantile-quantile (Q-Q) plots and Manhattan plots were implemented to check the veracity of these statistic parameters and obtain the visual results by R soft packages [73]. In this plot, each point represented a marker and the majority of the points should lie on the diagonal line, revealing no association with the trait. Only the point on the upper right section of the graph deviating from the diagonal could indicate that the SNP markers were most likely associated with the trait under study.

Genotyping in 270 samples and statistical analysis

To further validate the relevance between the identified SNPs and inguinal/scrotal hernias, genotyping of the screened SNPs of 270 pig samples was carried out by means of PCR-RFLP [74] AS-PCR [75] and a direct DNA sequencing method. Table 5 lists the oligonucleotide primers specific for candidate genes polymorphisms and the genotyping methods of each gene. For the RFLP analysis, we performed PCR reactions using 2×Taq PCR MasterMix (Aidlab, China) and the PCR temperature profile was as follows: 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by 72°C for 5 minutes. Then, the PCR products were digested with 10 units of each corresponding restriction endonuclease (RE) for more than 2 hours. The AS-PCR was performed with two sets of primers and the proportion of each of the components of the PCR mixture were the same as in the PCR method of PCR-RFLP. Finally, PCR and digestion products were separated directly by electrophoresis on 2% agarose gels (Biowest, Spain) and visualized under ultraviolet light.

The differences in the allele frequency among cases and controls were analyzed with a chi-squared test of independence via R 3.1.1 software [76]. If $p < 0.05$, the results were considered statistically significant.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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