Changes in Ovary Transcriptome and Alternative splicing at estrus from Xiang pigs with Large and Small Litter Size

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Abstract

Background/Aims: Litter size is one of the most important reproductive traits in pig breeding, which is affected by multiple genes and the environment. Ovaries are the most important reproductive organs and have a profound impact on the reproduction efficiency. Therefore, genetic differences in the ovaries may contribute to the observed differences in litter size. Although QTLs and candidate genes have been reported to affect the litter size in many pig breeds, however, the findings cannot elucidate the marked differences of the reproductive traits between breeds. The aim of present work is to elucidate the mechanisms of the differences for the reproductive traits and identify candidate genes associated with litter size in Xiang pig breed. ***Methods***: The changes in ovary transcriptome and alternative splicing were investigated at estrus between Xiang pigs with large and small litter size by RNA-seq technology. The RNA-seq results were confirmed by RT-qPCR method. ***Results***: We detected 16,219 - 16,285 expressed genes and 12 types of alternative splicing (AS) events in Xiang pig samples. A total of 762 differentially expressed genes were identified by XL (Xiang pig group with larger litter size) vs XS (Xiang pig group with small litter size) sample comparisons. A total of 34 genes were upregulated and 728 genes were downregulated in XL ovary samples compared with the XS samples. Alternative splicing (AS) rates in XL samples were slightly lower than that observed in XS samples. Most of differentially expressed genes were differentially regulated on AS level. Eleven candidate genes were potentially identified to be related to Xiang pig fecundity and litter size, which may be closely related to the gonad development, oocyte maturation or embryo quality. ***Conclusion***: The significant changes in the expression of the protein-coding genes and the level of alternative splicing in estrus ovarian transcriptome between XL and XS groups probably are the molecular mechanisms of phenotypic variation in litter size.

Introduction

In pig breeding, reproduction performance, particularly female reproductive performance, is a very important economic trait [1]. Reproductive traits are complex, and desirable reproductive phenotypes, such as litter size, are true polygenetic traits affected by interactions between multiple genes and the environment [2-5]. Each individual gene contributes to the overall variation in these traits [3, 6]. Litter size is one of the most important reproductive traits and has a great impact on the profit of pig producers [7]. As ovaries are the most important reproductive organs, they directly mediate ovulation and have a profound impact on the reproduction efficiency. Therefore, genetic differences in the ovaries may contribute to the observed differences in litter size [8-10].

To find the quantitative trait loci (QTL) and causal genes for these traits, several studies for linkages [11-13] and candidate genes ([14-17] have been conducted by using modern molecular information. Many of the major genes involved in the prolificacy of pig, such as *ESR* , *FSHB*, *RBP4*, *PRLR*, *MTNR1A*, *OPN*, *BMP* families, and *GDF9* genes have been characterized [18, 19]. Genetic studies have revealed an increasing number of associated QTLs and candidate genes involved in pig litter size [20-22]. Recently, several studies of the transcriptome for reproductive organs reveals differentially expressed genes affecting reproduction and candidate genes for litter size in European pig breeds [9, 15, 17] and Chinese indigenous pig breeds [14, 23]. Although QTLs and candidate genes have been reported to affect the litter size in many pig breeds, however, the studies were only focused on specific species or breeds and the findings cannot elucidate the marked differences of the reproductive traits between Asian and European pig breeds and the variations of litter size between pigs.

Chinese indigenous Xiang pig is one of the miniature pig breed originated from the mountain region in south east of Guizhou province. Small size, early sexual maturity, disease resistance, good meat quality and crude feed tolerance are the basic distinctively biological characteristics [24, 25]. Large phenotypic variation in litter size was observed within Xiang pig sows [26]. To analyze changes in gene expression across the entire transcriptome and to identify key genes relating to litter size, in the present study, we collected ovary samples of Xiang pigs having previously a large or small litter size. Then at estrus after weaning, a genome wide analysis of transcriptome and alternative splicing events of transcripts using RNA-seq technology was performed. Our results will give an insight of understanding on the molecular mechanisms about Xiang pig fecundity and litter size.

Materials and Methods

Ethics statement

Xiang pigs were obtained from Guizhou Dachang pig breeding Farm, in Congjiang county, Guizhou province, China. All animal procedures were approved by the Institutional Animal Care and Use Committee of Guizhou University (GZU-201709), and were conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals.

Animals and ovary collection

A total of forty Xiang pig gilts were used in this study from the sows that previously had a large litter size (XL: n=20, TNB > 12), and a small litter size (XS: n =20, TNB < 8), representing pigs with high and low fecundity, respectively. The gilts were selected after their first estrus cycle. The animals were reared in the same environment and fed the same diet under a standardized feeding regimen with free access to water during the experimental period. Information on the pedigree, breeding and performance of breeding animals was obtained from the farm records. After weaning, estrous detection was carefully performed twice to three

times daily at proestrous and tested by a boar. Standing reflex was assessed by the back-pressure test. Reddening and swelling of the vulva were measured visually. The expression of estrus was scored according to a standard scoring system [14, 27]. In this study, we randomly chose six Xiang gilts from the XL (n=3; TNB mean=16.16) and XS (n=3; TNB mean=6.78) groups to perform the genome-wide analysis of transcriptomes by RNA-seq. The ovarian samples from 6 Xiang pig gilts were collected with surgery at the day of the third estrous when the gilts exhibited strong expression of reddening and swelling of the vulva, and standing reflex. Ovaries were dissected and collected with better ovulation points or preovulatory follicles on the surfaces of the ovaries. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until total RNA was isolated.

Library preparation and sequencing

Total RNA was extracted from the ovarian samples in the two groups with Trizol reagent (TIANGEN, China) following the manufacturer's instructions, and then the DNase I was used to degrade any possible DNA contamination. The quantity and integrity of the total RNAs were assessed using the Agilent 2100 Bio-analyzer (Agilent Technologies, CA, USA). Total RNAs were stored at −80 °C until subsequent analysis. The same sample was used to both the sequencing and RT-qPCR analysis. The oligo (dT) magnetic beads were used to enrich mRNA. The purified mRNA was broke into short fragments by adding fragmentation buffer in the condition of appropriate temperature, and then cDNA was synthesized using random hexamer primers and mRNA fragments as templates. After adenylation of 3’ ends, DNA fragments were ligated to adapters. The cDNA fragments with 100–200 bp in length were selected for PCR amplification to generate cDNA libraries. The library preparations were sequenced on an Illumina HiSeqTM 2500 platform and generated 90 bp paired-end reads according to the manufacturer's protocols at the Beijing Genomies Institute (BGI), Shenzhen, China.

Data analysis

The raw sequence reads were generated by the Illumina platform and saved as a format of fastq. After removing low quality reads (more than 5% unknown nucleotides) and sequencing adapters and being assessed by the FastaQC software (Q20 < 20%), the clean reads were obtained. The pig reference genomes (Ssc 11.1) and the annotation files were downloaded from ENSEMBL database (http://www.ensembl.org/index.html). The genome index was built by the Bowtie2 (v2.2.3) software. TopHat2 (v2.0.12) software was used to align the clean reads onto the pig reference genome, which the maximum mismatch was not more than 2 bp. The Cufflinks software was used to assemble transcripts and to predict gene model. The HTSeq software was used to count the number of reads which aligned to each gene and exon. FPKMs (Fragments Per Kilobase of transcript per Million mapped reads) were then calculated to estimate the expression values of genes and alternative splicing variants. Asprofile (v1.0.4) software was used to classify and count the alternative splicing events in each sample. Venny online tool

(http://bioinfogp.cnb.csic.es/tools/venny/index.html) was used for the statistics of the number of genes in each sample.

Identification of differentially expressed genes and differential alternative splicing events

The Bio-conductor package limma [28] was used for analysis of differential gene expression and differential alternative splicing between two samples with biological replicates using a model based on the negative binomial distribution. The P-value had been assigned to each gene or AS and adjusted by the Benjamini and Hochberg's approach for controlling the false discovery rate. P-value ≤ 0.01, adj P-value≤0.05 and the absolute value of log2 Ratio ≥1 were employed as the threshold to determine the differential expression genes or differential alternative splicing events.

GO enrichment analysis

Enrichment analysis of Gene ontology (GO) annotation was conducted on differentially expressed genes at gene or isoform level using Gene Ontology Consortium online (http://geneontology.org/) .

RT-qPCR validation

RNA samples from the 6 gilts in the RNA-seq experiment were used to validate the results by quantitative real-time RT-PCR (qPCR). After removing genomic DNA by RNase-free DNase (New England BioLabs, USA), the total cDNA was synthesized using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Real-time PCR reactions were performed as described by the manufacturer in triplicate with Power SYBR Green PCR Master Mix on the BIO-RAD CFX98 Real-Time System (BIO-RAD, US). The reaction was performed in a 96-well plate. Each reaction contained 5 μL of SYBR Green PCR Master Mix, 0.4 μL of the forward and reverse primers (10 pM/μL), 1.0 μL of cDNA (5 ng/μL) and 3.6 μL of distilled water. Thermal cycling conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and then a 55–95 °C melting curve detection. The *GAPDH* gene was used as a control in the experiments. All amplifications were followed by dissociation curve analysis of the amplified products. Relative expression levels of the selected target genes were calculated with the 2(-Delta Delta C[T]) method [29]. The statistical difference in gene expression between groups was analyzed by SPSS software (v21.0). The results were presented as mean ± standard deviation. The correlation between the results of RNA-seq and qPCR was calculated using correlation test (Pearson correlation) by SPSS Software (v21.0). Primer informations can be found in additional file: Table 5.

Results

Transcriptome sequencing and assemble

We performed an RNA-seq analysis on ovary transcriptome from Xiang pigs with large and small litter size. The cDNA libraries from 6 samples were sequenced using Illumina HiSeqTM 2500 platform. The total number of clean reads with maximum of 90 base-pair (bp) ranged from 49.9 to 50.39 million were produced after quality control and filtering. The reads were mapped by TopHat2 (version 2.0.12) to *Sus scrofa* reference genome (Ssc11.1). 81.5% to 84.6% clean reads were mapped onto the *Sus scrofa* reference genome respectively for each sample (Table 1). The unique mapped reads were used for subsequent analysis. FPKM was used to estimate the expression at gene and isoform levels.

Changes of ovary transcriptome in XL and XS

After mapping onto *Sus scrofa* reference genome, in total, 16,454 genes were detected in all transcriptomes, including 13,899 known genes and 2,555 un-annotated genes (Supplementary Table S1, Fig 1). Among of those, 16,219 and 16,285 genes were detected from the XL and XS libraries, respectively. 16,050 genes were co-expressed in both XL and XS libraries, One hundred and sixty nine genes were exclusively expressed in XL libraries, and two hundred and thirty five genes were preferentially expressed in XS libraries (Fig 1). The number of un-annotated gene in each library from XL to XS was 2,400, and 2,441, respectively. The expression levels of those genes varied greatly, ranging from less than 0.1 FPKM to more than hundreds of thousands of FPKM (Supplementary Table S1). There were 27 top expressed genes in XL and/or XS samples respectively (FPKM>1,000) (Table 2). 17 of 27 genes including *TPT1*, *EEF1A1*, *TMSB10*, *FTH1* and 11 members of ribosomal protein genes, were overlapped between XL and XS samples. Five genes (*GPX3, ACTG1, TIMP1*, *COL1A2*, and *VIM)* were dominantly expressed in XL libraries. Two genes (*CLU*, and *OVGP1*) were the top expressed genes in XS libraries. The sequencing frequency of the top expressed genes constituted 11.7 ~ 15.5% of the total expressed values in XL and XS samples, respectively. Most of the genes specific for XL or XS were showed low or very low expression levels.

Differential gene expression from ovaries of Xiang pig gilts with large and small litter size were calculated by applying the cutoff of expression values at FPKM+0.001, padj-value≤0.05, and log2 fold-change ≥1 or ≤-1. The significance scores were corrected for multiple testing using Benjamini-Hochberg correction. A total of 762 genes differentially expressed between XL and XS samples. Compared with XS samples, 34 genes were up-regulated and 728 genes were down-regulated in XL samples (Supplemental Table S2). Most of the genes with greatest changes in expression were down-regulated genes. The range of log2 fold change values for DEGs was from 5.94 to -5.96. The scatter plots used to demonstrate the up- and down-regulated genes identified in the ovary tissues from XL and XS gilts (Figure 2). Of these genes, 47 were highly upregulated or down-regulated genes with a fold change of more than 4 (log2>2) or 30 (log2<-5) in expression between XL and XS, including *STAR, NR5A1*, *MORN5, TSPAN1, FRK*, *TEKT1, LHCGR, MTHFD2*, *ZSWIM2* (Table 3). *STAR* (P-adj=9.5e-03) was the most up-regulated gene in XL group, whereas *ZSWIM2* showed the highest down-regulation (P-adj = 0.01 and logFC = -5.96).

Alternative splicing events and differentially spliced genes in XL and XS

We used ASprofile program to detect 12 types of alternative splicing (AS) events in XL and XS data sets. The results were showed in Table S3. In total, 65,238 AS events from 15,047 genes and 66,086 AS events from 15,282 genes were detected in XL and XS data sets respectively. Approximately, 92.8~93.8% of the total expressed genes underwent alternative splicing in XL and XS samples. The ovary transcriptome between XL and XS showed similar AS patterns (Table 4). Three types of AS events (TSS, TTS and SKIP) were predominant in pig ovary transcriptome, which accounted for more than 80% of AS events in each library.

The differentially spliced genes (DSGs) were investigated by using the same cutoff with DEGs. In total, 808 DSGs, which represented 2,039 differentially splicing events, were identified between XL and XS samples (Table S4). Among these DSGs, 602 genes were both differentially regulated on the expression and AS level. Thirty seven of the differentially splicing events from 26 genes (e.g. *TTLL8*, *STAP2*, *ZUFSP*, *LNX1*, *ARNTL*, *SPAG9* etc.) showed large (log2 fold-change >10-fold) differences in AS level between XL and XS (Table S5). Furthermore, we found the DSGs were enriched in several gene families (Table S6), for example, cilia and flagella associated protein family (CFAP), intraflagellar transport family (IFT), kinesin family member (KIF), MORN repeat-containing protein family (MORN), NIMA related kinase family (NEK), solute carrier family member (SLC), spermatogenesis associated protein (SPATA), tektin family (TEKT), transmembrane protein family (TMEM), tubulin tyrosine ligase like protein (TTLL), ubiquitin specific peptidase (USP), zinc finger protein (ZNF) and so on.

Gene ontology

Enrichment analysis of annotation was performed on the DEGs and DSGs using Gene Ontology (GO) Consortium online (http://geneontology.org/). The DEGs and DSGs were enriched in 120 and 110 GO terms ($p < 0.05$) (Table S7) based on biological process, molecular function, and cellular component ontologies. Most of the GO terms were in common between DEGs and DSGs. For the biological processes GO terms, “process”, “assembly” and “organization” were the predominant categories. For the GO terms of molecular function, most of DEGs and DSGs were annotated with the term “binding” and “activity”. For the cellular component category, the DEGs and DSGs were mainly enriched in GO terms of the “cilium” and “organelle”.

Validation

To validate the RNA-seq results, nine genes（*SCARB1*, *LDLR*, *CYP11A1*, *HSD3B1*, *STAR*, *AKR1C1*, *SERPINE2*, *RARRES1*, *LRP8*） were randomly selected for quantitative PCR (qPCR). In the same RNA samples of the RNA-seq experiment, the expression fold changes ($2^{-\Delta\Delta Ct}$) of these nine genes were tested by using qRT-PCR. The expression patterns of these nine genes were in agreement with the RNA-seq results (Table 5). This suggested that the results from the RNA-seq experiments were accurate and efficient.

Discussion

In this study, we constructed two libraries prepared from Xiang pig gilt samples with extremely large and small litter size. We compared the ovarian transcriptome and alternative splicing of Xiang pig gilts at estrus with large (XL) and small (XS) litter sizes using Solexa sequencing technology. A total of 16,219 and 16,285 expressed genes were detected in ovaries of large and small litter size pigs, respectively. Among of those, 16,050 genes were co-expressed in both libraries. The expression levels of those genes varied greatly, ranging from less than 0.1 FPKM to more than hundreds of thousands of FPKM. The 27 top highly expressed genes (FPKM>1,000) were identified in the ovaries of large and/or small litter size pig samples (Table 2). Of these top highly expressed genes, *GPX3* and *TIMP1* were upregulated and *OVGP1* and *CLU* genes were downregulated in the high litter size samples compared with small litter size ovary samples. Most of the genes specific for XL or XS showed low or very low expression values. The sequencing frequency of the top expressed genes constituted 11.7~15.5% of the total expressed values in XL and XS samples. The strong expression of these genes in the ovaries suggests that they are important for ovaries development or specific reproductive processes. For example, more than half of those genes were ribosomal protein genes. Ribosomal proteins (RPs) are ubiquitous RNA binding proteins, the functions of which are not only to participate jointly with ribosome RNA (rRNA) in the synthesis of protein, but also to take part in the regulation of gene transcription and the regulation of cell proliferation and apoptosis through its extra-ribosomal functions [30]. The abnormal expression of RPs often results in serious diseases such as anemia and tumor [31]. *GPX3* gene (glutathione peroxidase 3) and *TIMP1* gene (metalloproteinase inhibitor 1) were proved to be related with oocyte maturation or embryo quality in previous reports [32, 33]. *TPT1* gene is thought to promote cell survival by enhancing the anti-apoptotic response and suppressing proapoptotic activities [34]. The expression of *CLU* gene appears to correlate with cell remodeling or differentiation that occurs during estrus [35].

Previous studies of goat ovaries suggest that some specific differentially expressed genes identified by RNA-Seq are likely to be important for improving litter size [8, 36]. Similar studies on litter size in Yorkshire pigs indicate that some of the top 10 most differentially expressed genes are suggested to be candidate genes involved in specific reproductive processes [19]. In this study, 762 genes were differentially regulated in the ovaries of large and small litter size pigs (Table S2). A number of highly upregulated genes with a fold change of more than 4 (log2>2) involved in steroid hormone metabolism and biosynthesis, cell adhesion, organic substance metabolism, Ion transport and signaling transduction [Table 3]. Even though the functions of a lot of genes are still unclear, some of the highly upregulated genes found in our study have been reported to be candidate genes involved in reproduction. For example, the *STAR* gene is required for cholesterol transport into mitochondria to initiate steroidogenesis in the adrenal and gonads [37]. The NR5A1 protein regulates the transcription of key genes involved in sexual development and reproduction, including *STAR*, *CYP17A1*, *CYP11A1*, *LHB*, *AMH*, *CYP19A1*, and *INHA* [38]. NR5A1 is expressed in multiple cell types in

the fetal, postnatal, prepubertal, and mature ovary [39]. The inactivation of Nr5a1 specifically in mouse granulosa cells causes infertility associated with hypoplastic ovaries. Nr5a1-/- ovaries have follicles but lack corpora lutea, a finding that indicates impaired ovulation [38]. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are gonadotropins (GTHs) that signal through their cognate receptors, FSH receptor (FSHR) and LH/choriogonadotropin receptor (LHCGR), to control major gonadal events in vertebrates, including folliculogenesis and steroidogenesis in the ovary [40]. MTHFD2 is likely responsible for mitochondrial production of both NADH and NADPH in rapidly proliferating cells [41]. *PAPSS2* gene plays an important role in modulating ovarian function and female fertility by control of the bioavailability of ovarian androgen [42]. Adrenodoxin, a versatile ferredoxin, is involved in steroid hormone biosynthesis and vitamin D and bile acid metabolism [43].

The most significantly downregulated genes with a fold change of more than 30 (log2<-5), is presented in Table 3. A lot of downregulated genes are directly involved in cilium movement, receptor signaling pathway, hormone secretion, protein modification and regulation of apoptotic process. For example, the fyn-related kinase (FRK) is a non-receptor tyrosine kinas, which represses cell proliferation, migration and invasiveness by suppressing epithelial to mesenchymal transition [44]. *TEKT1* is a spermatid specific gene and plays an important role in spermatogenesis [45], but its function in ovary is unclear. TGFβ family control follicle development. MORN5 is involved in TGFβ signaling at all levels. BMP signaling is required for MORN5 expression and reduction of MORN5 derepresses several genes in the BMP and TGFβ signaling pathways [46]. TSPAN1, a new member of the tetraspanin family, could inhibit cell proliferation and migration. TSPAN1 is positive related to PTEN in both clinical specimen and mouse models [47].

In this study, a great number of alternative splicing (AS) events were predicted from the expressed genes in XL and XS data sets. Approximate 92.8% and 93.8% of the total expressed genes underwent alternative splicing in XL and XS samples. This result was similar to the AS rates in human [48]. We found that AS rates in XL samples were lower than that observed in XS samples. Alternative splicing is an important mechanism for regulating gene expression and generating proteome diversity [49]. An increasing number of evidence has revealed that AS influences gene functions in animals and plays a decisive role in the generation of receptor diversity and regulation of growth and development [50]. Many genetic diseases have been closely linked to higher than normal rates of AS [51]. Therefore, we speculated the higher AS rates in Xiang pigs with small litter size might be involved in the progression of lower reproductive capacity. In addition, we identified 808 differentially spliced genes (DSGs)(P-adj<0.05) and found that 602 genes were differentially regulated on both the expression and AS level. The DEGs exhibited diverse and specific splicing patterns and events between the XL and XS samples. Most of top differentially regulated genes, including *STAR*, *TSPAN1, FRK, PAPSS2, MORN5, MTHFD2, LHCGR* and *TEKT1*, also exhibited highly differentially splicing on AS levels. These results indicate that changes in ovary transcriptome at estrus from Xiang pigs with large and small litter size were differentially regulated on the expression and AS level.

Finally, by analyzing the most highly expressed genes, the top DEGs and top DSGs in the large and small litter size samples, we identified a total of 11 candidate genes (*STAR*, *TSPAN1, FRK, PAPSS2, MORN5, MTHFD2, LHCGR*, *GPX3*, *FDX*1*, NR5A1* and *TEKT1*) relating to porcine fecundity and litter size (Table 6). The functions of *MTHFD2, GPX3, STAR, FDX1, NR5A1, LHCGR* and *PAPSS2* involve in cell proliferation, response to oxidative stress, ovarian steroid hormone synthesis and secretion which may be closely related to the gonad development, oocyte maturation or embryo quality. These genes increasingly expressed in ovary of Xiang pigs with large litter size and promoted ovarian steroidogenesis and oocytes quality. They may be associated with high fecundity in Xiang pigs. The functions of *TSPAN1*, *FRK* and *MORN5* may be related to repress cell proliferation, migration and invasiveness. Reduction of TSPAN1, FRK and MORN5 promotes cell proliferation. They may also be associated with high fecundity in Xiang pigs. *TEKT1* involves cell motility. It is a spermatid specific gene which plays an important role in spermatogenesis, but its function in pig ovary is unknown. In ovary of Xiang pigs with small litter size, the expression of *TEKT1* was found to be higher at estrus stage, which might have specific roles in gonadal physiology. Previous study identified 11 candidate genes for high litter size in Yorkshire pigs using the RNA-Seq method [19]. Interestedly, in our study, two of them (*GPX3, STAR*) were also identified as candidate genes that may be associated with high litter size in Xiang pigs. However, other candidate genes in Yorkshire pigs were completely different from that in Xiang pigs. The results suggest that litter size traits were not only affected by common genes, but regulated by different genes due to genetic differences between pig breeds.

Conclusion

This work presented a genome-wide view of gene expression and alternative splicing in estrus ovarian between Xiang pig gilts with large and small litter size. The significant changes in the expression of at least 4.7% of all expressed genes were found in estrus ovarian between XL and XS groups. AS rates in XL samples were lower than that observed in XS samples. Most of differentially expressed genes were differentially regulated on both expression and AS level. After analyzing the function of these genes, 11 candidate genes were potentially identified to be related to Xiang pig fecundity and litter size. The significant changes in the expression of the protein-coding genes and the level of alternative splicing in estrus ovarian transcriptome between XL and XS groups probably are the molecular mechanisms of phenotypic variation in litter size.

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Disclosure Statement

The authors have no Disclosure Statement to declare.

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Table 1. Summary of read mapping

samples	Raw Reads	Clean Reads	BasePairs	Mapped read pairs	Percentage of mapped reads
XS12	51850632	50031430	4502828700	41776244	83.50%
XS15	51851262	50390108	4535109720	42630031	84.60%
XS16	51851248	50338918	4530502620	42586725	84.60%
XL13	51843247	49981708	4498353750	42034616	84.10%
XL17	51841443	50375286	4533774540	42214490	83.80%
XL18	51840932	50368172	4533135480	41050060	81.50%
Total	311078764	301485622	27133704810	252292166	83.68%

Table 2. The top highly expressed genes (FPKM>1,000)

Gene ID	Gene symbol	XL FPKM	XS FPKM	LogFC	P.Value	adj.P.Val	Main Function
ENSSSCG00000036438	GPX3	1396.32	326.93	2.0945	0.0039	0.0597	oocyte maturation, embryo quality
ENSSSCG00000028355	ACTG1	1412.21	817.74	0.7882	0.0077	0.0826	cellular response to interferon-gamma
ENSSSCG00000009668	CLU	253.88	1125.66	-2.1485	0.0238	0.1477	extracellular chaperone
ENSSSCG00000035997	RPL10	1092.14	1392.33	-0.3503	0.0399	0.1978	translation, embryonic development
ENSSSCG00000004489	EEF1A1	3534.45	4202.53	-0.2497	0.0471	0.2134	protein biosynthesis
ENSSSCG00000013597	RPS28	2076.56	2321.82	-0.1611	0.0498	0.2195	translation
ENSSSCG00000037674		1544.21	1855.19	-0.2646	0.0577	0.2356	unknown
ENSSSCG00000014540	FTH1	2907.67	1863.75	0.6416	0.0606	0.2418	iron ion transport
ENSSSCG00000006791	OVGP1	128.61	6752.92	-5.7145	0.0684	0.2565	carbohydrate metabolic process
ENSSSCG00000011033	VIM	1430.62	759.72	0.9131	0.0731	0.2653	astrocyte development
ENSSSCG00000015326	COL1A2	1192.61	634.94	0.9094	0.0801	0.2773	blood vessel development
ENSSSCG00000024260	RPL11	961.26	1061.56	-0.1431	0.0877	0.2911	translation
ENSSSCG00000012277	TIMP1	1498.91	235.32	2.6711	0.0917	0.2972	positive regulation of cell proliferation
ENSSSCG00000040929	TPT1	2546.01	3172.08	-0.3171	0.1172	0.3364	cell differentiation
ENSSSCG00000035904	RPL7A	1121.11	1273.71	-0.1841	0.1337	0.3607	ribosome biogenesis
ENSSSCG00000038507	RPS29	2071.41	2258.68	-0.1248	0.1569	0.3919	translation
ENSSSCG00000008245	TMSB10	1434.32	1046.39	0.4549	0.1745	0.4142	actin filament organization
ENSSSCG00000003042	RPS19	957.37	1023.71	-0.0966	0.2905	0.5415	translation
ENSSSCG00000036014	RPLP0	1191.31	1365.74	-0.1971	0.2993	0.5503	ribosome biogenesis
ENSSSCG00000003930	RPS8	924.33	1012.12	-0.131	0.3136	0.5641	translation
ENSSSCG00000001502	RPS18	1205.32	1150.56	0.0671	0.4464	0.6793	translation
ENSSSCG00000039544	RPS24	1170.68	1222.17	-0.0621	0.4762	0.7047	translation
ENSSSCG00000035768	RPS15A	1440.37	1395.94	0.0451	0.5956	0.7892	regulation of cell cycle, translation
ENSSSCG00000012119	TMSB4	1138.57	1164.31	-0.0322	0.6229	0.8079	organization of the cytoskeleton
ENSSSCG00000022176		1262.61	1418.36	-0.1678	0.6416	0.8203	unknown
ENSSSCG00000027358	RPS17	1417.91	1448.65	-0.0309	0.6752	0.8411	translation
ENSSSCG00000020817	RPS16	1294.51	1315.45	-0.0231	0.7698	0.8973	translation

1 Table 3. The most significantly upregulated and downregulated genes in ovaries between XL and XS

Gene ID	Gene	XL FPKM	XS FPKM	logFC	P.Value	adj.P.Val	Function
ENSSSCG00000004147	ECT2L	0.4911	17.2465	-5.1341	3.36E-06	0.007373	regulation of Rho protein signal transduction
ENSSSCG00000040973	HYDIN	0.7709	26.672	-5.1125	4.16E-06	0.007373	cilium movement,epithelial cell development
ENSSSCG00000002817	DRC7	0.5791	24.2991	-5.3911	1.65E-05	0.007981	flagellar motility
ENSSSCG00000033394	VWA3B	0.1207	4.4461	-5.2026	1.89E-05	0.007981	apoptosis pathway
ENSSSCG00000002504	AK7	1.5712	52.6944	-5.0676	1.86E-05	0.007981	nucleoside triphosphate biosynthetic process
ENSSSCG00000034942	STAR	101.3265	1.6502	5.9401	4.35797E-05	0.009582	steroid biosynthetic process
ENSSSCG00000016030	ZSWIM2	0.1006	6.2815	-5.9638	5.04E-05	0.010794	regulation of extrinsic apoptotic signaling
ENSSSCG00000005588	NR5A1	25.0387	5.3585	2.2242	0.003041	0.01082	female gonad development
ENSSSCG00000010100	LRRC74B	0.1608	5.3494	-5.0559	5.21E-05	0.010921	unknown
ENSSSCG00000017884	TEKT1	1.2328	46.2493	-5.2293	8.77E-05	0.012834	flagellated sperm motility
ENSSSCG00000009428	FAM216B	1.5967	51.5777	-5.0135	9.03E-05	0.012848	unknown
ENSSSCG00000005529	MORN5	1.4651	48.9129	-5.0611	0.000181	0.016821	follicle development
ENSSSCG00000003823	C1orf87	0.5735	22.2651	-5.2786	0.000222	0.017979	unknown
ENSSSCG00000017344		0.1175	3.8177	-5.0214	0.000233	0.018189	unknown
ENSSSCG00000030800		0.0978	4.2525	-5.4412	0.000255	0.018992	unknown
ENSSSCG00000028471	DTHD1	0.7056	26.3466	-5.2224	0.000271	0.019146	signal transduction
ENSSSCG00000001594		0.0673	2.6978	-5.3241	0.000301	0.019961	microtubule motor activity
ENSSSCG00000008908	PDCL2	0.1407	5.0321	-5.1595	0.000356	0.020796	queuosine biosynthetic process
ENSSSCG00000015696	MAP3K19	0.2522	8.5697	-5.0864	0.000387	0.021837	regulation of mitotic cell cycle
ENSSSCG00000020990	DNAH12	0.3121	10.7355	-5.1044	0.000458	0.023934	cilium movement
ENSSSCG00000007236	TTLL9	0.3516	11.9891	-5.0916	0.000487	0.024699	cellular protein modification process
ENSSSCG00000039903	SPATS1	0.2478	8.0353	-5.0187	0.000511	0.025256	testis development
ENSSSCG00000000983	IL17REL	0.0978	3.6634	-5.2264	0.000669	0.028073	inflammatory responses
ENSSSCG00000015741	CFAP221	0.2738	8.8253	-5.0103	0.000657	0.028073	cilium movement
ENSSSCG00000034242	HNF4G	0.0508	1.6365	-5.0079	0.000666	0.028073	positive regulation of transcription
ENSSSCG00000009809	MORN3	0.4031	15.8584	-5.2982	0.000682	0.028118	unknown
ENSSSCG00000011078	ARMC3	0.5553	23.2969	-5.3905	0.000687	0.028154	signal transduction, development
ENSSSCG00000012638	GRIA3	15.8909	2.9806	2.4145	0.000735	0.028781	glutamate-gated ion channel activity
ENSSSCG00000010478	FFAR4	0.2932	10.1441	-5.1123	0.000982	0.031997	hormone secretion
ENSSSCG00000003908	TSPAN1	5.3101	252.0776	-5.5689	0.001237	0.035091	cell proliferation
ENSSSCG00000003819	ANGPTL3	0.0813	3.8734	-5.5734	0.001327	0.035857	angiogenesis,cholesterol metabolic process
ENSSSCG00000033462		0.7444	27.9106	-5.2284	0.001334	0.035866	unknown
ENSSSCG00000017495	GRB7	0.1653	5.9526	-5.1701	0.001377	0.036561	negative regulation of translation
ENSSSCG00000032691	ANKRD66	0.5455	22.5371	-5.3683	0.001424	0.037318	unknown
ENSSSCG00000008421	LHCGR	20.6097	2.8525	2.8529	0.001452	0.037422	folliculogenesis and steroidogenesis
ENSSSCG00000031023		143.2986	14.9674	3.2591	0.001471	0.037631	cell adhesion
ENSSSCG00000004434	FRK	0.0985	3.8885	-5.3015	0.001674	0.039844	cell differentiation
ENSSSCG00000008289	MTHFD2	38.5153	7.4155	2.3768	0.001724	0.040391	tetrahydrofolate metabolic process
ENSSSCG00000003048	cxcl17	1.3079	44.9787	-5.1038	0.001762	0.040612	unknown
ENSSSCG00000011850	mucin 4	0.4179	21.0885	-5.6571	0.002031	0.042575	cell-matrix adhesion
ENSSSCG00000007510	C20orf85	4.4515	160.3676	-5.1709	0.002089	0.043155	unknown
ENSSSCG00000004195	ARG1	1.6843	0.3098	2.4425	0.002122	0.043722	urea cycle and Nitrogen metabolism.
ENSSSCG00000010437	PAPSS2	88.1918	19.4349	2.1819	0.002234	0.044691	folliculogenesis，bioavailability of androgen

ENSSSCG00000002734	MARVELD3	0.2771	9.8266	-5.1483	0.002641	0.047701	regulation of epithelial cell proliferation
ENSSSCG00000003733	KLHL14	0.2122	6.8489	-5.0121	0.003203	0.053298	protein ubiquitination
ENSSSCG00000036438	GPX3	1396.32	326.93	2.0945	0.0039	0.0597	oocyte maturation or embryo quality
ENSSSCG00000035945	FDX1	99.9512	18.5901	2.4266	0.008821	0.088508	steroid biosynthetic process

Table 4. The pattern of alternative splicing events in XL and XS

Type	XL	%	XS	%
AE	1366	2.093872	1376	2.082135
IR	2579	3.953217	2605	3.941833
MIR	401	0.614672	408	0.617377
MSKIP	2990	4.583218	3002	4.542566
SKIP	15393	23.59514	15470	23.40889
TSS	21449	32.87808	21793	32.97673
TTS	16317	25.0115	16543	25.03253
XAE	878	1.345841	928	1.404231
XIR	981	1.503725	1006	1.522259
XMIR	155	0.237592	156	0.236056
XMSKIP	518	0.794016	547	0.827709
XSKIP	2211	3.389129	2252	3.407681
Total	65238	100	66086	100

Table 5. Validation of selected RNA-seq based gene expression by real-time RT-PCR analysis

Gene ID	Gene	Item	XL	XS	Pvalue	q-RT PCR primers
ENSSSCG00000009759	SCARB1	RNA-seq	98.9841	30.1584	0.016858	F:GATGTCATCAACCCCAACGAGA
		qRT-PCR	0.656813	0.284241		R:GACTTGTGAGGCTGGAACTGGT
ENSSSCG00000028512	LDLR	RNA-seq	177.9738	8.64681	0.047969	F:TGTCGCCTTGCTCATTGCT
		qRT-PCR	0.126744	0.103311		R:CGGTGTTCCCATCACAAATC
ENSSSCG00000025273	CYP11A1	RNA-seq	393.1201	22.7851	0.045689	F:TCCACCCCATCTCCGTGAC
		qRT-PCR	2.347796	0.872615		R:CCCAGCCAAAGCCCAAGTT
ENSSSCG00000006719	HSD3B1	RNA-seq	218.4446	26.9416	0.027875	F:AAACCAGAAGTTCGGGAGGAAT
		qRT-PCR	0.452955	0.173151		R:CGTTGACCACGTCGATGATAGA
ENSSSCG00000034942	STAR	RNA-seq	101.3265	1.650261	0.0000436	F:CTGGAAGTCCCTCAAAGACCAA
		qRT-PCR	1.215981	0.638847		R:GGGCTGAGCTTTAACACCTGG
ENSSSCG00000011147	AKR1C1	RNA-seq	709.6603	66.287643	0.022607	F:AACGACGGTCACTTCATTCCTG
		qRT-PCR	1.782164	0.073004		R:ACTTCTATGGCATATTTGGTGGC

ENSSSCG00000016233	SERPINE2	RNA-seq	424.70167	116.3289	0.042519	F:ATCATCCCTCACATCAGCACCA
		qRT-PCR	3.675488	1.242115		R:ATCAAACATCTCGGTGACGCC
ENSSSCG00000028623	RARRES1	RNA-seq	934.2826	425.4131	0.026966	F:CGCAGCTCTGCACTTCTTCAAC
		qRT-PCR	2.176589	0.475644		R:ACGTGCCTTACCTTCCTCTGTG
ENSSSCG00000003848	LRP8	RNA-seq	78.4194	2.227203	0.023706	F:CCAATCGCATCTACTGGTGTGAC
		qRT-PCR	0.198645	0.015375		R:GGAGAGTGCAGCTGCTCATCAAT
	GAPDH					F:GTCCACTGGTGTCTTCACGA
						R:GCTGACGATCTTGAGGGAGT

Table 6. the candidate genes relating to porcine fecundity and litter size

Gene ID	Gene symbol	XL FPKM	XS-FPKM	logFC	P.Value	adj.P.Val	function
ENSSSCG00000003908	TSPAN1	5.3101	252.0777	-5.5689	0.001237	0.035091	cell proliferation
ENSSSCG00000004434	FRK	0.0986	3.8886	-5.3015	0.001674	0.039844	cell differentiation
ENSSSCG00000005529	MORN5	1.4651	48.9129	-5.0611	0.000185	0.016821	follicle development
ENSSSCG00000005588	NR5A1	25.0387	5.3585	2.2242	0.003044	0.010822	sexual development and reproduction
ENSSSCG00000008289	MTHFD2	38.5153	7.4155	2.3768	0.001724	0.040391	cell proliferation
ENSSSCG00000008421	LHCGR	20.6097	2.8525	2.8529	0.001452	0.037422	folliculogenesis and steroidogenesis
ENSSSCG00000017884	TEKT1	1.2328	46.2493	-5.2293	0.000087	0.012834	cell motility
ENSSSCG00000034942	STAR	101.3265	1.6503	5.9401	0.000043	0.009582	steroidogenesis
ENSSSCG00000010437	PAPSS2	88.1918	19.4349	2.1819	0.002234	0.044691	folliculogenesis，bioavailability of androgen
ENSSSCG00000035945	FDX1	99.9513	18.5901	2.4266	0.008821	0.088508	synthesis of various steroid hormones
ENSSSCG00000036438	GPX3	1396.3267	326.9341	2.0945	0.003981	0.059721	oocyte maturation or embryo quality

Figures with legends:

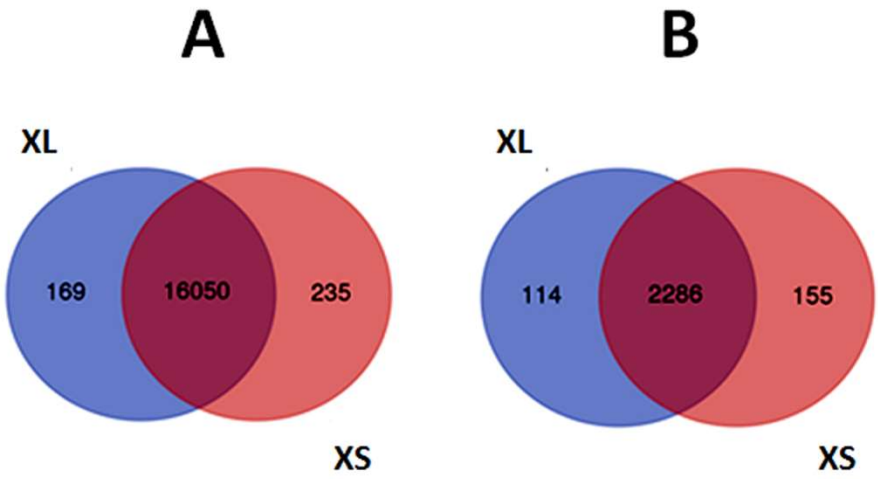

Figure 1. Profile of the gene expression between the ovaries of Xiang pigs with large (XL) and small (XS) litter size. Blue color represent genes only expressed in the XL group, red color show genes only expressed in the XS group, and common to both groups (intersection). A: Total of the expressed genes. B: Uncharacterized protein genes.

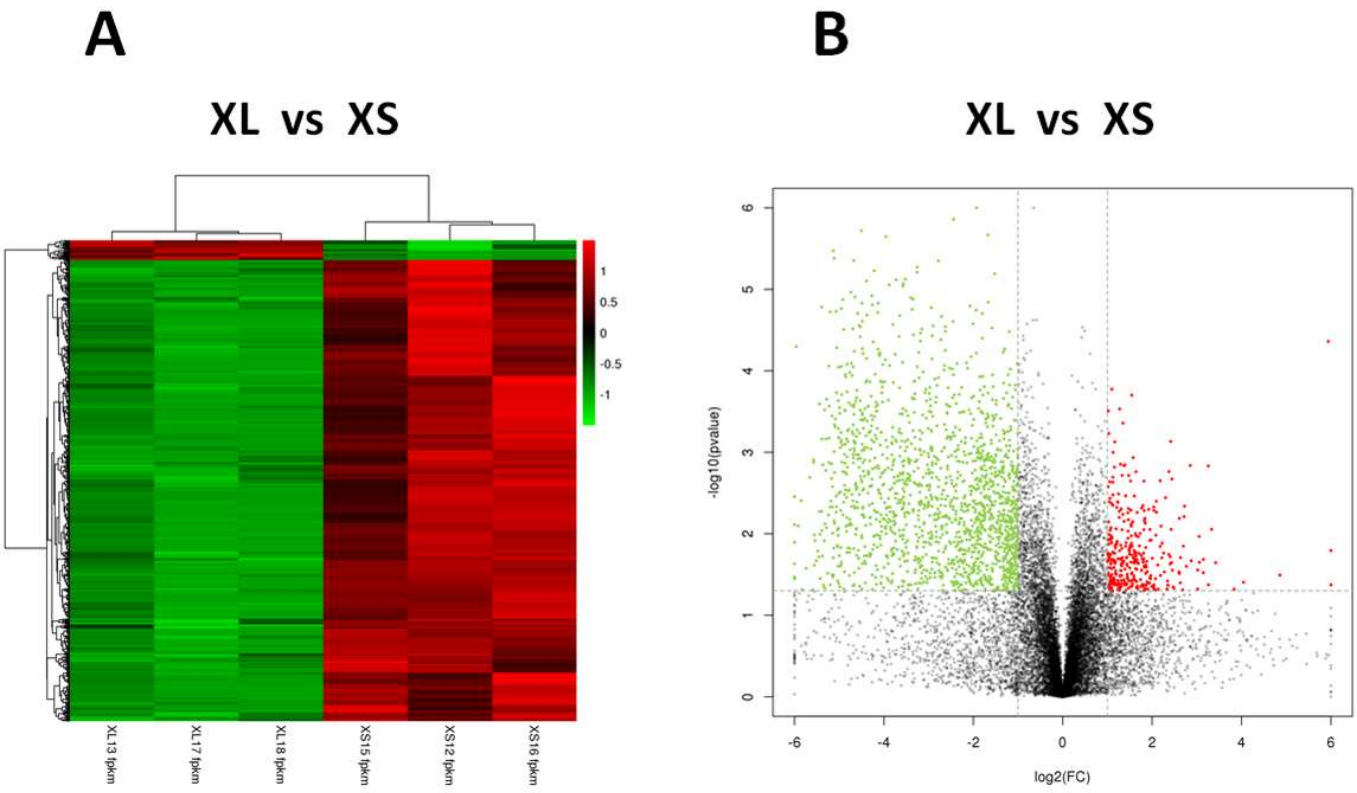

Figure 2. Differential expression of the genes between XL and XS groups. A: Scatter plot of differentially expressed genes (XL vs. XS). Each point in the figures represented one gene. Red points represent upregulated genes; Green points denote downregulated genes. Black points represent genes without significant difference. B: Heatmaps illustrate the most significantly upregulated and downregulated genes between two groups.

COVER LETTER

Feb 12, 2019

The present manuscript focused on the **ovary transcriptome and alternative splicing** of Xiang pig, a miniature indigenous pig breed in China. The distinctively biological characteristics of Xiang pig are small body size together with early sexual maturity. A large phenotypic variation in litter size was observed within Xiang pig sows. Genetic differences in the ovaries may contribute to the observed differences in litter size of Xiang pig. Although QTLs and candidate genes have been reported to affect the litter size in many pig breeds, however, the findings cannot elucidate the marked differences of the reproductive traits between breeds. Ovary samples of Xiang pigs were collected at estrus to perform a genome-wide analysis of transcriptome and alternative splicing events of transcripts using RNA-seq technology. The outstanding findings are:

(1) we detected more than 16,000 expressed genes and 12 types of alternative splicing (AS) events from Xiang pig samples. A total of 762 differentially expressed genes were identified based comparison between XL (Xiang pig group with larger litter size) vs XS (Xiang pig group with small litter size) groups.

(2) Alternative splicing (AS) rates in XL samples were slightly lower than that observed in XS samples. Most of differentially expressed genes were differentially regulated on AS level.

(3) Eleven candidate genes were identified to be related to Xiang pig fecundity and litter size, which may be closely related to the gonad development, oocyte maturation or embryo quality.

Our results will give an insight of the molecular mechanisms about Xiang pig fecundity and litter size.

All of authors agreed to submit the manuscript to the journal, eLIFE, by the platform bioRxiv. And the simplest supplement files together with the manuscript would be submitted. In which, seven supplement tables contracted to 63 pages from the original 1837 pages.