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Research paper

Exploiting RNA-sequencing data from the porcine testes to identify the key genes involved in spermatogenesis in Large White pigs

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ABSTRACT

Mammalian testis development and spermatogenesis play critical roles in male fertility. However, little genomic information is available for porcine sexually mature and immature testis. Presently, we detected approximately 76% of previously annotated genes that were expressed in the porcine testes by RNA sequencing. Taking an FDR of 0.001 and a |log2Ratio| of 1 as cutoffs, 10,095 genes were significantly differentially expressed between two stages, including 242 spermatogenesis-associated genes. These genes were significantly enriched to GO BP terms concerning spermatogenesis, male gamete generation, developmental process and sexual reproduction; to the KEEG pathways, including focal adhesion, ECM–receptor interaction, and phagosome. 186 extended transcripts, 1273 alternative splicing events and 2846 SNPs were detected in spermatogenesis-associated DEGs. Two *PIWIL4* SNPs were successfully validated and suggested to be the potential molecular markers for semen quality. This study will help identify the specific genes and isoforms that are active in porcine spermatogenesis and sexual maturity.

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1. Introduction

Spermatogenesis is a complex process of cellular divisions and developmental changes that occur within the seminiferous tubules of the testis (de Kretser et al., 1998). The process of spermatogenesis is composed of three major phases, including mitotic process of spermatogonia to form spermatocytes, meiosis to reduce the number of chromosomes to form spermatids, and spermiogenesis in which haploid spermatids develop into spermatozoa (de Kretser et al., 1998; Ro et al., 2007). Spermatogenesis involves many cellular and molecular events unique to germ cells, such as cell growth and development, cell adhesion, signaling and cell migration (Kleene, 2001). This complex process is developmentally regulated by thousands of genes that express specifically at both transcriptional and translational levels (Yu et al., 2003; Hecht, 1998; Eddy and O'Brien, 1998). For example, approximately half of the testis-specific genes are expressed in late stage spermatogenesis, sperm (30%) and spermatids (21%), whereas only a few are expressed in earlier stages, spermatogonia (3%) and spermatocytes (4%) (Djureinovic et al., 2014).

To our knowledge, there are three approaches to study gene expression during spermatogenesis. One uses cell sorting to separate mitotic, meiotic, and postmeiotic germ cells, and characterize their gene expressions compared with somatic cells or other germ cells (Yu et al., 2003; Chalmel et al., 2007; Fallahi et al., 2010). Using a mouse 1.2 cDNA expression arrays with 1176 unique gene probes, 260 were detected during spermatogenesis in at least one of the six cell types (Yu et al., 2003). Only 46% (120 of 260 genes) were expressed in all six types, but a number of genes showed a differential expression pattern (Yu et al., 2003). The second approach is based on examining gene expression in the testis at different time points during spermatogenesis (Margolin et al., 2014; Schultz et al., 2003; Shima et al., 2004; Laiho et al., 2013). By analyzing the SOLiD 4 next-generation sequencing data from the whole testes at 5 time points (days 7, 14, 17, 21 and 28), Laiho et al. (2013) identified 2494 differentially expressed genes associated mostly with meiosis, Piwi-interacting RNA metabolism. As the wild-type testis always contains a mixed population of germ cells at different stages of their differentiation process, it is impossible to make profiling gene expression within Sertoli cells or specific germ cells. Therefore, the third approach, developed very recently, synchronizes spermatogenesis without affecting fertility by manipulating retinoic acid levels within the neonatal testis from the RiboTag transgenic mouse line that allows for the isolation of polyribosomes from specific cell types using a Cre/Lox

Abbreviations: VOL, semen volume per ejaculate; SCON, sperm concentration; MOT, motility; ASR, abnormal sperm rate; DEGs, differentially expressed genes; RPKM, per million mapped reads; AS, alternative splicing; SNP, single nucleotide polymorphism; QTL, quantitative trait locus.

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system (Evans et al., 2014). A microarray analysis showed that 392 and 194 transcripts were significantly changed in expression in germ and Sertoli cells during a synchronized first wave of spermatogenesis in RiboTag/Stra8-cre or RiboTag/Amh-cre mice (Evans et al., 2014).

Onset of spermatogenesis appeared at boars of the typical US breeds including Large White pigs less than 120 days of age (Lunstra et al., 1997, 2003). Large White pig is one of the most common pig breeds used in the world-wide pork production. During the pre-pubertal period of development such as at 60 days of age, germ cells multiply and differentiate in a synchronous fashion in testis; but at 180 days of age, spermatozoa are produced asynchronously and the testis contains all kinds of germ cells. Therefore, the analysis of changes in gene expression based on major stages of development, especially in regard to male germ cell development, will provide a powerful tool to determine the cellular processes involved in the formation of spermatozoa. Several previous studies, based on expressed sequence tags (ESTs), microarray and RNA-sequencing (RNA-Seq), have identified an unusual and diverse set of genes expressed in testes (Margolin et al., 2014; Laiho et al., 2013). The high-throughput RNA-Seq approach has been used to detect the dynamic changes of various genes in a more sensitive and more robust way (Margolin et al., 2014). However, the transcriptomic profiles related to porcine sexual maturity are still not fully studied. Hence, we analyzed gene expression in the testis samples of 60-day-old (60-d) and 180-day-old (180-d) Large White boars using RNA deep sequencing in order to discover genes and isoforms that are active in porcine spermatogenesis and meiosis. The results from the study will allow us to ascertain differential dynamics of gene expression in swine testis, and gain a global understanding of the underlying functions of differentially expressed genes involved in spermatogenesis and sexual maturity.

2. Materials and methods

2.1. Animals

Whole testes were removed from 3 young Large White boars at 60 days of age (sexually immature) and 180 days of age (sexually mature) Large White boars (Luo et al., 2010; Egbunike, 1979). Total RNAs were extracted by Trizol reagent (Invitrogen). The association analyses were conducted in Duroc (n = 186), Large White (n = 87) and Landrace (n = 50) pigs from Yangxiang Group Corporation. Genomic DNAs were isolated using standard phenol/chloroform extraction from sperm. Semen volume per ejaculate (VOL), sperm concentration (SCON), motility (MOT) and abnormal sperm rate (ASR) of above boars were recorded.

2.2. Construction and sequencing of cDNA libraries

After the total RNA extraction and DNase I treatment, the pools were made up of equally mixed RNAs of 3 testes at the same age. Magnetic beads with Oligo (dT) were used to isolate mRNAs. Simply mixed with the fragmentation buffer (Ambion), the mRNA was fragmented into short fragments of 200–300 nt. Then cDNAs were synthesized, purified and resolved with EB buffer for end reparation and single nucleotide A (adenine) addition. After that, the short cDNA fragments were connected with Illumina PE adapters. After size-selected on agarose gel, the libraries were amplified by 15 cycles of PCR with Phusion DNA polymerase (New England Biolabs) and primers containing barcode sequences to distinguish different libraries during sequencing and data analysis. During the quality control steps, Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System were used in quantification and qualification of the sample library. Finally, the library was sequenced using Illumina HiSeqTM 2000 sequencer.

Raw reads were filtered into clean reads which were then aligned to the reference swine genome Sscrofa10.2 with SOAPaligner/SOAP2. No more than 5 mismatches were allowed in the alignment. After that, we proceeded with the deep analyses including gene expression, gene structure refinement, alternative splicing (AS) and SNP analysis.

2.3. Expression analysis

Gene expression levels were measured as numbers of reads per kilobase of exon model in a gene per million mapped reads (RPKM). Differentially expressed genes (DEGs) between 60-d and 180-d testes were identified with an R package named DEGseq. The genes with false discovery rate (FDR) ≤0.001 and |log2Ratio| ≥ 1 were taken as DEGs.

2.4. Functional enrichment analysis

To annotate the function of these DEGs, we firstly mapped all DEGs to GO terms in the database (http://www.geneontology.org/), calculating gene numbers for every term. Then in order to find significantly enriched GO terms in the input list of DEGs, we used a strict in house algorithm, developed by BGI which was based on GO::TermFinder (Boyle et al., 2004) (http://smd.stanford.edu/help/GO-TermFinder/GO_TermFinder_help.shtml/), to do hypergeometric test. KEGG was used to perform pathway enrichment analysis of DEGs (Kanehisa et al., 2014). Only GO-BP terms or KEGG pathways with a *P* value less than 0.05 were considered as significant.

2.5. Gene structure refinement

We assembled transcripts with the reads using Cufflink (Trapnell et al., 2010). Through comparisons of assembled transcripts and gene annotation from swine genome assembly Sscrofa10.2, the assembled transcript(s) that extended 5′ or 3′ end of an annotated gene were found, and therefore that gene structure was refined.

2.6. Alternative splicing

SOAPsplice (v1.1) was used for genome-wide ab initio detection of both known and novel splice junction sites from RNA-Seq (Huang et al., 2011). All initially unmapped reads were searched to find a junction alignment. Splice junctions were utilized to identify alternative spliced transcripts.

2.7. SNP identification

SOAPsnp is the tool to detect SNPs (Langmead et al., 2009). The SNPs were identified on the consensus sequence through comparisons with the reference. We then compared the SNPs between 60-d and 180-d, and got the age-specific SNPs. As we focused on the spermatogenesis-associated mutations, we filtered with the chromosomal positions of the age-specific SNPs against those of spermatogenesis associated DEGs, and retained those variants which mapped to spermatogenesis associated DEGs.

2.8. Real-time RT-PCR

Total RNAs were reverse transcribed into cDNAs by the M-MLV Reverse Transcriptase (Promega). Each 15 μl real-time RT-PCR reaction included 7.5 μl SYBR Green Real-time PCR Master Mix, 1 μl cDNAs, 0.4 μl primers (S1 Table). PCR conditions consisted of 1 cycle at 94 °C for 5 min, followed by 40 cycles at 94 °C for 40 s, 57 °C for 30 s, and 72 °C for 30 s, with fluorescence acquisition at 74 °C in single mode. The relative expression level was determined using the $2^{-\Delta\Delta Ct}$ method with β-actin as the control.

2.9. SNP validations and population association analyses

SNPs were validated by PCR-RFLPs using the primers listed in S1 Table. The associations between genotypes and semen quality traits in

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