Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2022.013

RESEARCH ARTICLE

Exploring the Genetic Basis of Xiangxi Cattle Ovary Reproduction: A Rudimentary Study of Ovary Transcriptome in Xiangxi and Angus Cattle

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ARTICLE HISTORY (21-439)

Received: November 04, 2021
Revised: January 25, 2022
Accepted: January 31, 2022
Published online: February 24, 2022

Key words:
Angus
Cattle
Ovary
Transcriptome
Xiangxi

A B S T R A C T

Basic understanding of molecular features of ovaries and comparison of biological events in ovaries of different beef cattle breeds with indigenous potential has been indicated to improve beef production. The objective of the study was to explore the basic molecular features of Xiangxi cattle ovary and to identify the biological processes generating the differences between the ovaries of Xiangxi and Angus cattle. For this purpose, RNA-seq technology was used to analyze transcriptome data between the ovaries of Xiangxi and Angus cattle. A total of 64.87 Gb clean data were obtained that contained 6.10 Gb clean data of each sample. A total of 109 differentially expressed genes were obtained from comparison of transcriptome analysis between Xiangxi and Angus cattle. Among the 109 differentially expressed genes, 60 genes were upregulated in Angus, whereas 59 genes were upregulated in Xiangxi cattle. Gene ontology and Kyoto Encyclopedia of Genes and Genomes Pathway Analysis indicated that many of these genes encode the proteins involved in signal transduction and ovarian development. In this study, the mRNA expression of *ASIP*, *IGLL1*, *FOSB* and *IL17RD* in ovary of Angus cattle was found to be upregulated. While, the mRNA expression of *GSTA3*, *TICAM1*, *CCDC6* and *TSPAN18* was upregulated in Xiangxi cattle. These observations could provide basic information to improve understanding of fertilization, ovarian development and immune cell functions in the Xiangxi cattle and other closely related cattle breeds.

To Cite This Article: Fang H, Ao S, Yun L, Jianbo L, Haobang L, Yang L, Sheng L, Xiong L, Baizhong Z and Kangle Y, 2022. Exploring the genetic basis of xiangxi cattle ovary reproduction: a rudimentary study of ovary transcriptome in xiangxi and angus cattle. Pak Vet J, 42(2): 266-270. http://dx.doi.org/10.29261/pakvetj/2022.013

INTRODUCTION

Xiangxi cattle, an important local cattle resources in southern China, are mostly distributed in the northwest region of the Hunan Province (Wang *et al.,* 2012). They are efficient utilizer of low-quality feed stuff and well renowned for stress resistance and excellent meat quality (Wang *et al.,* 2012; Mao *et al.,* 2016). Heifers of Xiangxi cattle reach puberty at 16 to 18 months of age compared to the heifers of Angus that reach maturity at 22 to 24 months of age (Mao *et al.,* 2016). It has been reported that Xiangxi heifers continues to have regular estrous cycles every 21 days after reaching puberty (the normal range is every 18 to 24 days) (Wang *et al.,* 2012). In the past, multi-level taxonomic research on Xiangxi cattle have been carried out and a lot of data have been obtained from phenotype, blood type, enzyme type, and blood protein type to chromosome characteristics, which provide some useful data for breed identification and mining of genetic markers (Luoreng *et al.,* 2014). However, these markers cannot fully explain the biological differences between Xiangxi cattle and other beef cattle breeds, and the molecular genetic mechanism that determines their excellent beef characteristics. The advent of high-throughput sequencing-based RNA-Seq technology has provided the basis for the application of transcriptomics in animal research (Ji *et al.,* 2018) and transcriptome technology could be main research direction for animal reproduction. The ovary, as the primary reproductive organ of female mammals, has the function of producing

structures like follicles and secreting substances like steroid hormones (Xu *et al.*, 2020). Thus, the analysis of the relationship between mammalian ovarian gene expression and its physiological function using transcriptome technology can open new horizons in animal reproduction research (Sha *et al*., 2018).

At present, annotation of specific genes related to reproduction for some beef cattle breeds especially Xiangxi cattle has not been studied and no research data related to Xiangxi cattle transcriptome is available. Therefore, in this study, ovarian tissues from 18 months and 30 months old Xiangxi and Angus cattle were collected and mRNA transcriptome analysis was performed. High-throughput transcriptome sequencing analysis was performed using RNA-Seq technology to screen for expression of genes for ovarian development and related genes. This study could provide a basis for further understanding of the environmental adaptability, molecular breeding, and reproductive performance of Xiangxi cattle.

MATERIALS AND METHODS

Healthy Xiangxi cattle from the National Xiangxi Cattle Farms, Huayuan, Hunan Province, China and the Angus cattle from the National Breeding Farms in Lianyuan, Hunan Province, China were included in the study. A total of six animals of 18 months age, 3 from Xiangxi cattle, and 3 from Angus cattle were slaughtered. Ovarian tissues were immediately collected and placed in liquid nitrogen at -70°C for extraction of total RNA from the ovarian tissues of both species.

RNA quantification and qualification: A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. NEB Next Ultra TM Ribonucleic Acid Library Prep Kit for Illumina (NEB, USA) was applied to create seq libraries by following manufacturer's instruction. Polymerase chain reaction was carried out with Phuision High-Fidelitiy DNA polymerase, Index Primer and Universal PCR primers. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

Clustering and sequencing: RNA-Seq analysis was performed using BMK cloud (www.biocloud.net). TruSeq PE Cluster Kit v4-cBot-HS (Illumia, USA) was used as per manufacturer's guidelines, for clustering of the index-coded samples utilizing a cBot Cluster Generation System. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated.

Comparative analysis: Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In the next step, adaptor sequence and lower quality sequences reads were deleted from the processed raw data sets. After processing, the raw sequences obtained were further transformed into clean reads. After obtaining the clean read, the clean reads were then mapped against the referenced genome sequence. Furthermore, reads with exact match or at least one mismatch was again analyzed and annotated on the basis of referenced genome. To map with referenced genome Hisat2 tools soft were used.

Gene functional annotation and quantification of gene expression levels: Gene function was annotated as suggested in a recent study (Chen *et al.,* 2021) and based on the NCBI non-redundant protein sequence, NCBI non-redundant nucleotide sequences, protein family, KOG/COG of clusters of orthologous proteins, Swiss-Prot, KEGG Ortholog database, and Gene Ontology. Gene expression levels quantifications were estimated by fragments per kilobase of transcript per million fragments mapped.

Differential expression analysis: Differential expression analysis of two conditions was carried out using the D.E.-seq. The obtained P values after analysis were corrected using the Benjemini and Hochberg's methods for regulating the false discovery rate. Resultant genes with corrected P-value <0.01 were obtained by D.E.-seq and were designated as differentially expressed. E.B-seq was used for the expression analysis of Angus cattle and Xiangxi cattle ovarian tissue samples. The F.D.R <0.05 and Folded Changes ≥1.5 was considered as the edge for significantly differential expression.

GO enrichment and KEGG pathway enrichment analysis: Gene Ontology (GO) enrichment analysis for the genes expressed differentially (DEGs) was further carried out by the GO seq R package which relay on the Wallaenius non central hypergeometric distribution. KOBAS software was used to assess the statistical enrichment of DEGs in KEGG pathways.

RT-qPCR validation: To examine the DEGs explored by the ribonucleic acid (RNA)-Seq (Eight genes identified were differentially expressed), q-PCR validation was carried out by choosing DEGs randomly. The primers used were designed using software, Primer Express 3.0 software, and used for q-PCR analysis (Table 1) with PPP1R11 as a reference control. The qPCR reactions were carried out on an ABI Step OnePlus™ Real-Time PCR System (ABI, USA) using a SYBR® Premix Ex TaqTM II (Takara, Dalian, China) in accordance with the manufacturer's protocol. Relative quantification analysis was performed using the comparative cycle threshold (CT) method, and comparative genes expression level were calculated by using the $2^{-\Delta\Delta CT}$ method.

RESULTS

Quality detection of Angus and Xiangxi cattle ovarian total RNA and library: Total RNA was extracted from the ovarian tissue of six cattle, the concentration was ≥10ng / μL, and the purity (260nm / 280nm) was above 2.0. This shows that the integrity of the RNA was preserved and can be used for the next test. The qPCR method was used to accurately quantify the effective concentration of the library (the effective concentration of the library was> 4nmol / L). The results showed that a high-quality cDNA library was generated, and the library was successfully obtained.

Table 1: List of primers used in the qPCR analysis

Target Name	Primer	
ASIP	F	ATTTCCCTTCAGTCTCTATCGTGG
	R	ACGTTCTTCATCGGAGCCTT
IGLL1	F	CTAACCATCAACTCGCTCCA
	R	AAACGGCAGTATTGACACT
FOSB	F	CCCTATGACATGCCAGGAACCAGT
	R	TAGCCACTCATGCCCGGTGT
IL17RD	F	CAGAAGATCCACGAGTCCCA
	R	TGCTTGTAGTTCTTCTCGTCCA
GSTA3	F	TGAAGAAAGCTAAAACCCGGTA
	R	AAAATCCTCTCCGTGGT
TICAM1	F	CCAGCCTCTCATCATACACC
	R	TTTCCCGCGTGTTGTCC
CCDC6	F	AGCCCGATCCCTTACACAC
	R	AGACATTCCGGCTCGAGTCA
TSPAN18	F	CACTACCAGGGCAGCAACGA
	R	CCACAGCAACCAAACGTGA
PPP1R11	F	CAGAAAAGACAGAAGGGTGCC
	R	CGTTTCCGAAGTTTGATGGTTA

Quality evaluation of Angus and Xiangxi cattle ovarian transcriptome sequencing: After the transcriptome analysis of the ovarian tissue samples, a total of 44.21 Gb Clean Data was obtained. The obtained Clean Data of each ovarian tissue sample reached to the 6.10 Gb, and the percentage of Q30 bases was above 92.42% (Table 2). The clean reads per ovarian tissue sample of both species were aligned and sequenced with the designated reference genome, and the efficiency obtained varied from 94.41 to 95.54%. Based on the comparison results, gene structure optimization analysis, alternative splicing prediction analysis, and discovery of new genes were performed.

Differential expression analysis of Angus and Xiangxi cattle tissue samples: Pearson Correlation Coefficient was utilized in the current study as the evaluation index of biological repeated correlation. The findings explored that the squares (r2) of the Pearson correlation coefficients among samples of the same species were greater than 0.93. The squares of the Pearson correlation coefficients (r2) between samples of two species were greater than 0.91 (Fig. 1), and the resemblance of the expression patterns between the samples of each group was high.

Screening for differential expression: After differential expression analysis using DESeq2_EBSeq, the differential gene set between two species/biological conditions was obtained; after the FDR (P<0.05) correction and the parameter setting of the absolute value of FC≥1, comparison results of Xiangxi and Angus cattle were obtained. Volcano plots are presented in Fig. 2 that express gene expression levels and statistically significant differences in the two groups. Functional annotation results showed that a total of 165 differential genes were annotated, of which 58 were annotated in the GO database (Fig. 3). Ten DEGs were randomly selected for qPCR analysis to validate the expression profiles obtained by RNA-Seq. The results verified that *ASIPASIP*, *IGLL1*, *FOSB* and *IL17RD* were upregulated in the Angus cattle. While *ASIP*, *IGLL1*, *FOSB* and *IL17RD* were downregulated in the Xiangxi cattle. *GSTA3*, *TICAM1*, *CCDC6* and *TSPAN18* were downregulated in Angus cattle as compared to Xiangxi cattle (Fig. 3).

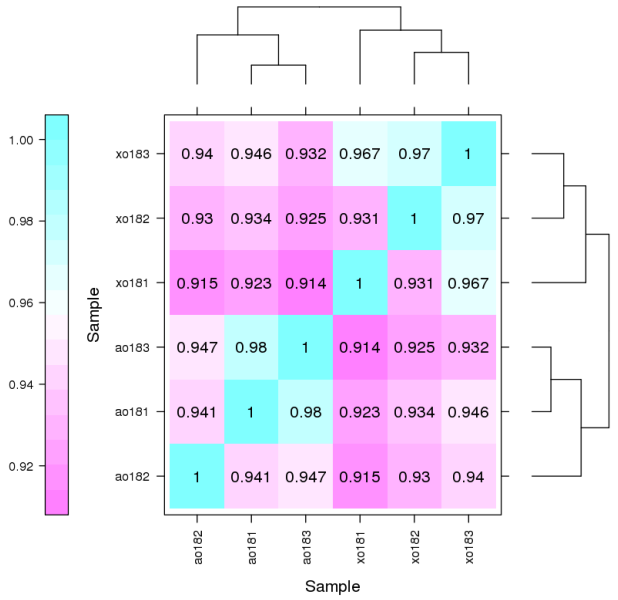

Fig. 1: Distribution of RNA Seq correlation examination in all transcriptional samples. The encode plan recommends that square of Pearson correlation coefficient is greater than 0.9 (under ideal sampling and experimental conditions). In specific project operations, the square of Pearson correlation coefficient is required to be is greater than 0.8. The results showed that r2 values between samples of the species was greater than 0.90. It indicates that the similarity of the expression patterns between the samples of each group was high. Please describe results here.

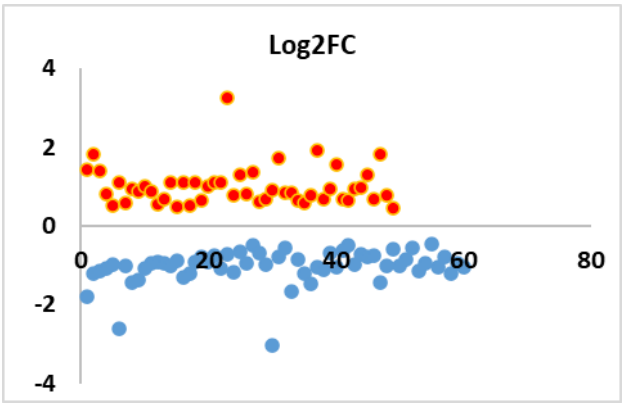

Fig. 2: The differentially expressed genes of Ovary in Xiangxi and Angus Cattle during puberty. The red dots showed that gene level was upregulated and the blue dots was down-regulated in ovary from Angus compared with Xiangxi Cattle.

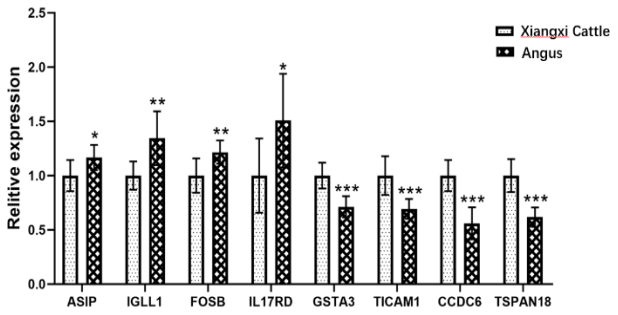

Fig. 3: Validation of the RNA-Seq (Quantification) results by qPCR. Results represent the mean (±SD) of three experiments. P≤0.05. Based on functional annotation results ten DEGs were randomly selected for qPCR analysis to validate the expression profiles obtained by RNA-Seq. The results are in consonance with GO analysis.

Table 2: Summary of clean data of each sample

Samples	Clean reads	Clean bases	GC Content	%≥Q30
ao181	26,868,265	7,995,921,996	50.04%	92.42%
ao182	28,370,966	8,446,828,776	50.39%	92.82%
ao183	27,227,839	8,084,942,616	50.02%	92.56%
xo181	22,843,285	6,803,055,060	50.68%	92.83%
xo182	22,750,192	6,774,880,420	49.67%	92.99%
xo183	20,483,196	6,102,515,852	49.49%	92.65%

DISCUSSION

In beef cattle production system, the fertility traits are the main important determinants of economic profitability. In indigenous beef cattle like Xiangxi cattle, determination of reproductive efficiency using various tools could enhance the total profitability. There are many factors that influence heritability, breeding cycle and have the complex relationship with the growth traits. Therefore, in order to increasing reproduction rate and mining special genetic traits in Xiangxi cattle, it is vital to evaluate the molecular process regulating the development process of ovary, particularly focusing on the function of ovary-specific transcription factors and signaling pathways.

In this experiment, Xiangxi and Angus cattle were slaughtered at early puberty (18 months of age) as early puberty is an important economic trait in bovine production (Valente *et al.,* 2015). The main objective of the current experiment was to screen for differential expression of mRNAs, and screening for expression of genes related for bovine ovarian development. The results of current study implied that a total of one hundred and nine genes were differentially expressed in both Xiangxi and Angus cattle. Among the differentially expressed genes, 60 genes were up-regulated in Angus, whereas 59 genes were upregulated in Xiangxi cattle. Results further explored that the mRNA expression of *ASIP*, *IGLL1*, *FOSB* and IL-17RD in ovary of Xiangxi cattle were downregulated as compared to Angus cattle.

The *ASIP* gene upregulation in Angus cattle reflects higher production of agouti signaling protein that is related to obesity (Albrecht *et al.,* 2012). The key role of *ASIP* gene in adipocyte development makes it more attractive in beef animals raised for fattening purpose. These findings reflect that Xiangxi cattle have lower potential to produce high quality beef as compared to Angus cattle. Angus cattle are well known for good immunity and the upregulation of *IGLL1* (immune system–related genes) in Angus as compared to Xiangxi cattle represent its superiority on dense ration. It has been reported that *IGLL1* has positive impact on B cells development which are known key factors for the adaptive immunity (Davoodi *et al.,* 2016). Therefore, it could be speculated that survival of fetus in the Angus cattle could be higher as compared to Xiangxi cattle.

FOSB is related to activator protein 1 of transcription factors and is responsible for diverse cellular processes, including differentiation, proliferation, apoptosis, angiogenesis, hypoxia, and steroidogenesis (Choi *et al.,* 2018), especially in the trophoblast cells (Renaud *et al.,* 2014). The higher gene expression of *FOSB* in Angus reflects strong differentiation, proliferation, apoptosis, angiogenesis, hypoxia and steroidogenesis in the Angus cattle as compared to Xiangxi cattle. It has been reported in western blotting, both in-vitro and in-vivo models, and DNA binding-electrophoretic mobility shift assays that *FOSB* play important role in follicular growth and terminal differentiation to luteal cells. Nevertheless, expression of *FOSB* in both Angus and Xiangxi cattle may play role in follicular growth and differentiation to luteal cells in both Angus and Xiangxi cattle but the potential of Angus cattle will be more as compared to Xiangxi cattle. Similar with *FOSB*, *IL-17RD* expression was more in Angus cattle as compared to Xiangxi cattle. Furthermore, expression of IL-17RD, a member of IL-17 signaling system in both species is assumed to perform a key role in the homeostasis of ovarian tissues in both species especially for disease recovery and better health rather the immune system.

Even though the ovarian tissue's gene expression were different in both species but *GSTA3* was identified as the most pronounced up-regulated gene in the ovary of both species where Xiangxi cattle was dominant over Angus cattle. *GSTA3* is known to play a major role in the detoxification of electrophilic decomposition products produced by metabolism of xenobiotics and reactive oxygen species (Ilic *et al.,* 2010; Kensler *et al.,* 2014). It has been reported that oxidative stress due to reactive oxygen species and xenobiotics increase embryonic mortality as well as alter uterine function and are responsible for severe gynecological diseases (Celi *et al.,* 2011; Loset *et al.,* 2011). We suppose that the Xiangxi cattle may have more possibility of detoxification and elimination of xenobiotics and reactive oxygen species, therefore, embryonic mortality in Xiangxi cattle could be speculated less as compared to Angus cattle. Previous study also reported that *GSTA3* also play a significant role in biosynthesis of steroids (Johansson *et al.,* 2001) and animals *GSTA3* gene upregulation show more endometrial epithelial cell proliferation, ad-enogenesis and uterine gland development (Gray *et al.,* 2001; Filant *et al.,* 2012; Hayashi et al. 2017).

TICAM1 is responsible for production of Interferon beta (IFN-β) (Bolivar *et al.,* 2018). IFN-β is well known cytokine formed by innate immune cells as well as non-immune cells (Ivashkiv and Donlin, 2014). It has been reported that IFN-β have multiple functions that includes anti-inflammatory and pro-inflammatory responses (Bolivar *et al.,* 2018). It has also been reported that IFN-β regulates the activation and development of all innate and adaptive immune effector cells (Ivashkiv and Donlin, 2014). Higher expression of *TICAM1* in Xiangxi cattle represents its greater potential for placental immunity as compared to Angus cattle.

TSPAN 18 gene were expressed in both Xiangxi and Angus cattle, however, TSPAN 18 gene expression in Xiangxi cattle was higher. It has been reported that *TSPAN 18* genes are responsible for the production of tetraspanins protein that are important, if not essential, in mammalian fertilization (Kaji *et al.,* 2000; Le *et al.,* 2000; Miyado *et al.,* 2000). Higher *TSPAN 18* gene expression in Xiangxi cattle confirm higher fertilization ability in Xiangxi cattle as compared to Angus cattle. *CCDC6* gene function is to depress the tumor progression in the organs. Previous study has reported that low levels of *CCDC6* protein is correlated with tumor prognosis (Morra *et al.,* 2015). Keeping in view the higher expression of *CCDC6* in Xiangxi cattle, it could be assumed that tumor

formation possibilities in Xiangxi cattle ovaries tissue are lesser as compared to Angus cattle.

Conclusions: In this study, Illumina II high-throughput sequencing technology was used to sequence transcriptomes of Xiangxi and Angus cattle ovarian tissues during puberty. Using transcriptome data, it was observed that *ASIP*, *IGLL1*, *FOSB* and *IL17RD* were upregulated in the Angus cattle as compared to Xiangxi cattle, while *GSTA3*, *TICAM1*, *CCDC6* and *TSPAN18* were downregulated in Angus cattle as compared to Xiangxi cattle. Based on findings it could be concluded that Xiangxi cattle have potential to be beef animals with better fetal survival, immunity and fertility rate. Furthermore, these observations may provide basic information to improve understanding of immune cell functions in the Xiangxi cattle and other local species.

Acknowledgements: This work was supported by 'Key Research and Development Project of Hunan Province, China (No. 2020NK2066)','Construction of Modern Agricultural Industrial Technology System in Hunan Province (Hunan Financial Agriculture Guide 2019 [97])', 'Xiangxi Yellow Cattle Engineering Technology Center (2019TP2010)','Research and Demonstration on Key Technologies of Straw Resources Utilization in Feed (2021NK4171)', 'Study on Breeding of Ramie with High Protein and Low Anti-Nutritional Factor and Key Technology of Feeding Xiangxi Cattle(2022NK2017)' and 'Construction and Demonstration of Key Technology System for Large-Scale Green Breeding of High-Quality Beef Cattle(2022NK2025).

Authors contribution: BZ and YK designed and conducted the study. FH, AS, and YL managed the cattle, collected, preserved and analyzed the ovarian tissue samples. JL, LS, and LH performed RNA extraction and RNA SEQ high-throughput sequencing. LY, XL and AS performed Pyrosequencing data analyses.

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