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Screening and Identification of mRNA Candidate Immunobiomarkers during Pregnancy in Sheep

Q.I. Dong-ming[1], Q.I.U. Feng-jiao[1], ZHOU Zheng-Kang[1], N.I. Guo-Chao[1], ZHAO Ying[1], WANG Juan-hong[1], CHANG Wei-Huag[1]

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ABSTRACT

Background: During pregnancy, the immune status of maternal animals undergoes significant changes. Effective monitoring of immunological biomarkers can provide valuable references for enhancing gestational management in livestock. This study aims to screen and identify mRNA-based candidate immunological biomarkers during ovine pregnancy, thereby establishing a scientific foundation for novel monitoring technologies to assess immune status in gestating animals.

Methods: High-throughput sequencing technology was used to perform sheep ovaries transcriptome sequencing in four stages: follicular phase, luteal phase (10 days after estrus), 30 days of pregnancy, and 45 days of pregnancy. Differential mRNAs were obtained by bioinformatics analysis. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed on the differential mRNAs.

Result: The experiment successfully constructed mRNA libraries for pregnant and non-pregnant sheep, with an average output of 11.50 G data per sample. The average alignment rate of each sample to the genome was 93.88%, and the average alignment rate to the gene set was 49.46%. A total of 23,219 genes were detected, of which 467 were differential genes. Data analysis, GO, and KEGG pathway studies showed that MX2, DDX58, IRF7, IFIT5, ISG15, RSAD2, and PLA2G1B were significantly upregulated in ovarian tissues during pregnancy, all of which are involved in processes such as bacterial and viral defense, immune regulation *et al.* QRT-PCR was used to detect the expression of seven different mRNAs in the blood of pregnant ewes, and the result confirmed that the expression was significantly elevated in the blood of pregnant ewes ($p<0.01$). The experiment indicates that the seven mRNAs are promising candidate biomarkers for immune monitoring, which is of great significance for the screening and early intervention of diseases during animal pregnancy.

Key words: Biomarker, Duration of pregnancy, Immunity, MRNA, Transcriptome.

INTRODUCTION

Animal immunity refers to the ability of an animal to resist invasion by pathogens, such as bacteria, viruses, parasites and fungi, as well as to cope with other harmful substances, which was provided by the animal's immune system. The immune system of a female animal faces a great challenge during pregnancy. Part of the immune function must be suppressed to protect the embryo from being excessively rejected in the parent body, which could lead to abortion or restricted growth and development. Meanwhile, the parent body also needs to maintain a certain level of immune function to protect itself against invasion by foreign pathogens and excessive invasion during embryo implantation. Interleukin 6 (IL-6) plays a crucial role in angiogenesis and induced parturition, while granulocyte-macrophage colony-stimulating factor plays a facilitating role in embryo implantation (Gan *et al.*, 2002). The interferon-γ (IFN-γ) and tumor necrosis factor-α secreted by uterine natural killer (NK) cells are believed to have a general regulatory effect on the pregnancy process. Wu *et al.* (2010) found that there was IFN-γ expression in the placenta during embryo implantation and late pregnancy. In non-pregnant animals, uterine NK cells expressed low levels of IFN-γ and as pregnancy progressed, the expression level of IFN-γ gradually increased, but no significant changes in the level of IFN-γ were observed in the peripheral blood circulation and the spleen. This indicates that the immunity of the parent body during pregnancy has a significant influence on the maternal-fetal.

Biomarkers are biochemical indicators that can mark changes or possible changes in the structure or function of systems, organs, tissues, cells and subcellular components, which have a wide range of clinical applications. The measurement of biomarkers provides insight into the ongoing biological processes within an organism and the examination of one or multiple specific biomarkers helps in the identification of diseases, early

[1]College of Animal Science, Xichang University, Xichang City, Sichuan, 615 000 PR China.

Corresponding Author: CHANG Wei-huag, College of Animal Science, Xichang University, Xichang City, Sichuan, 615 000 PR China. Email: changweihua112@163.com

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diagnosis and physiological monitoring during treatment (Prasanta *et al.*, 2023). The emergence of new methods, such as transcriptome sequencing, metabolome sequencing, small RNA sequencing and DNA methylation sequencing, has provided scientists with additional options for in-depth research in life sciences. During pregnancy, the immunity of female animals undergoes certain changes and effective monitoring of immune biomarkers can provide a reference to improve the management of pregnant animals. In the study, we performed transcriptome sequencing to examine mRNAs and analysis of differential expression data, GO, KEGG pathway studies showed that seven mRNAs were significantly upregulated in ovarian tissues during pregnancy, all of which are involved in processes such as bacterial and viral defense, immune regulation and so. QRT-PCR was used to detect the expression of seven different mRNAs in the blood of pregnant ewes and the result confirmed that the expression was significantly elevated in the blood of pregnant ewes. The experiment indicates that the seven mRNAs are promising candidate biomarkers for immune monitoring, which is of great significance for the screening and early intervention of diseases during animal pregnancy.

MATERIALS AND METHODS

Experimental animals and collection of samples

The study was conducted at Xichang University between January 2023 and June 2024. Experimental animals and collection of samples were as same as Chang *et al.* (2024).

RNA extraction

Total RNA was extracted as same as Chang *et al.* (2024).

Library synthesis and high-throughput sequencing

Library construction, transcriptome sequencing were as same as Chang *et al.* (2024).

Sequencing data were filtered as same as Chang *et al.* (2024). The filtered data were mapped to the reference genome (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_002742125.1/) using HISAT2 (v2.0.4), followed by detection of differentially spliced genes using rMATS (v3.2.5). The filtered data were aligned to the database established by BGI using Bowtie 2 (v2.2.5), which includes known and novel, coding and non-coding transcripts. Gene expression levels were then calculated using RSEM (v1.2.12) and differential expression analysis was performed using DESeq 2.0 (v1.4.5) with a Q value of ≤0.05. To take insight to the change of phenotype, GO and KEGG enrichment analysis of annotated different gene expression was performed based on Hypergeometric test.

Clinical detection of mRNA

The study used approximately 0.1 μg of each RNA sample and reverse transcribed it into cDNA using an RT reagent. For the qRT-PCR reaction system (20 μL), 10 μL of 2xSYBR qPCR mix, 0.4 μL of the upstream and downstream primers (2 μmol/L each), 1.2 μL of cDNA and 8 μL of RNase-free dd H_2O were used, making up a total volume of 20 μL. Which was performed at 95°C for 10 min, then 95°C for 15 s, 60°C for 60 s for 42 cycles and 72°C for 20 s. All primers used in the qRT-PCR are shown in Table 1. Each qPCR experiment was performed in triplicate and the relative RNA expression values were calculated using the $2^{-\Delta\Delta CT}$ method. Differences were deemed statistically significant if $P<0.05$. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

Table 1: Real-time fluorescence-based quantitative PCR primer sequences and parameters.

Primer name	Primer sequence	Annealing temperature/°C	Amplification length/bp
MX2	F: GCTGGATGGCATCACGGACTC	55	170
	R:TTTGCTCGTTTCTGTTGTACATGAC		
DDX58	F: GCCTTAACTTGTTGCCATCCTTCC	55	274
	R:CCTTCTCCAGGGGGATCTTCCCAACC		
IRF7	F: CATCTTTGACTTCGGCACCTTCTTC	55	277
	R:CTCTACGAGGACCTGGAGCACTTC		
IFIT5	F: GAGATTGACTGTGAGAAAGGATGGGG	55	142
	R:CGGCTATGCCATCACAGTGTATC		
ISG15	F: ATGGGCGGGGGACCTGAAGG	55	409
	R:CGGCTATGCCATCACAGTGTATC		
RSAD2	F: CCAGCAGGGCAGAGGGAGAGAG	55	177
	R:CTGCTTCCTTCAGCATCAG		
PLA2G1B	F: CTGGACAGGTGCTGCCAGAC	55	195
	R:GCAGATGGCAGCATTACGGTC		
GAPDH	F: GTCGGAGTGAACGGATTTGGG	55	196
	R:CATTGATGACGAGCTTCCCG		

Table 2: Differential expression of 7 mRNAs.

Gene ID	Gene symbol	log2 (FP/LP)	Q value (FP/LP)	log2 (P30/LP)	Q value (P30/LP)	log2 (P45/LP)	Q value (P45/LP)	log2 (P30/FP)	Q value (P30/FP)	log2 (P45/FP)	Q value (P45/FP)	log2 (P45/P30)	Q value (P45/P30)
780441	MX2	0.147025348	0.998404944	0.919260209	0.997492653	3.362679263	0.008732585	0.773016169	0.999817318	3.202230783	2.16E-28	2.434454935	0.029167078
101107257	DDX58	0.963030966	0.998404944	0.494730426	0.997492653	1.766418395	0.174083252	1.457605517	0.999817318	2.714912255	1.16E-08	1.262241698	0.478939843
101103683	IRF7	0.430244389	0.998404944	0.614652874	0.997492653	2.061944345	0.099732542	1.04507191	0.987343844	2.479587928	4.96E-08	1.440342032	0.183391818
101102441	IFIT5	0.376972654	0.998404944	0.112862905	0.997492653	0.836147884	0.639953259	0.490514948	0.999817318	1.19871403	0.001028486	0.713022825	0.797244823
443057	ISG15	0.901777893	0.998404944	0.380002428	0.997492653	4.224637512	0.024287685	1.279840416	0.999817318	5.111671909	6.54E-25	3.837646692	0.189274277
101115013	RSAD2	0.345185234	0.998404944	1.060304833	0.997492653	3.314822879	0.030772138	1.404836279	0.999817318	3.644755927	2.36E-09	2.245568366	0.219269664
101117700	PLA2G1B	4.085468541	8.35E-04	3.084887577	0.392248776	4.147936611	0.003049594	1.001653302	0.999817318	0.075387864	0.996009745	1.07212844	0.875155713

Statistical analysis

One-way ANOVA was used to determine the significance of differences in expression of both genes during different physiological stages of sheep bloods.

RESULTS AND DISCUSSION

Library construction and high-throughput sequencing

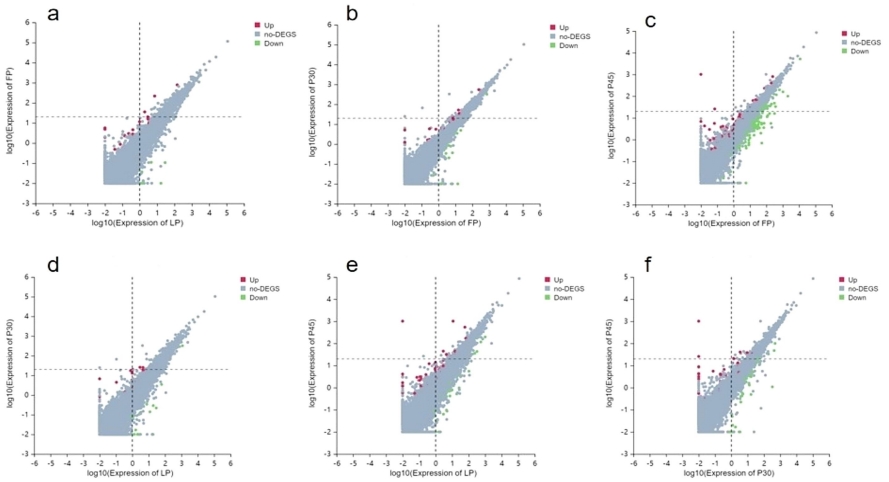
Sequencing was performed on 12 samples in four time periods (FP, LP, P30 and P45) using the DNBSEQ platform, yielding an average output of 11.50 Gb per sample. The average alignment rate of the samples to the reference genome was 93.88%. In the experiment, a total of 23,219 genes were detected, of which 467 were found to be differentially expressed. Compared to the LP sample, 26 genes were upregulated in the FP, 24 genes were upregulated in the P30. Compared to the FP,82 genes were upregulated in the P45. Compared to the P30, 47 genes were upregulated in the P45 (Fig 1). Compared to LP and FP, gene MX2 was extremely significantly upregulated at P45; compared to FP, gene DDX58, IRF7 and PLA2G1B were extremely significantly upregulated at P45; IFIT5, ISG15 and RSAD2 were significantly upregulated at P45 (Table 2).

Stacked histogram of expression levels

To visually represent the number of genes in different fragments per kilobase of transcript per million mapped reads (FPKM) ranges for each sample, gene counts were tabulated for three FPKM conditions (FPKM≤1, FPKM 1-10, FPKM≥10). The experimental data analysis showed that the sample genes were predominantly expressed at high, moderate and low levels, with genes at extremely low expression levels accounting for only 30.57% to 33.97% (Fig 2).

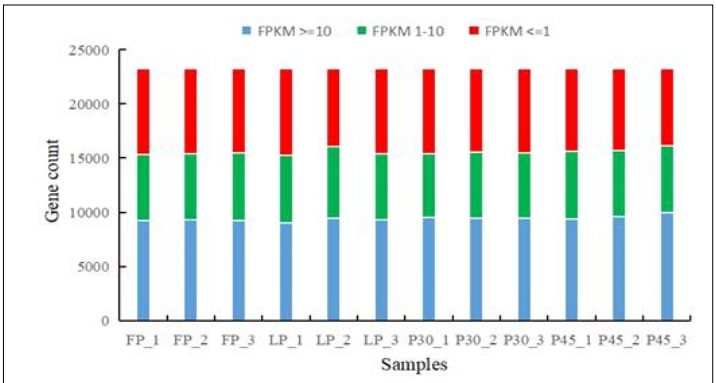
Table 3: Top 5 ranked GO terms.

Level 1 GO classification	Level 2 GO classification	Amount
Biological process	Cellular process	220
	Biological regulation	181
	Regulation of biological process	171
	Response to stimulus	152
	Metabolic process	150
Cellular component	Cell	201
	Cell part	199
	Membrane	134
	Organelle	131
	Membrane part	109
Molecular function	Binding	197
	Catalytic activity	118
	Molecular function regulator	31
	Transporter activity	22
	Molecular transducer activity	19



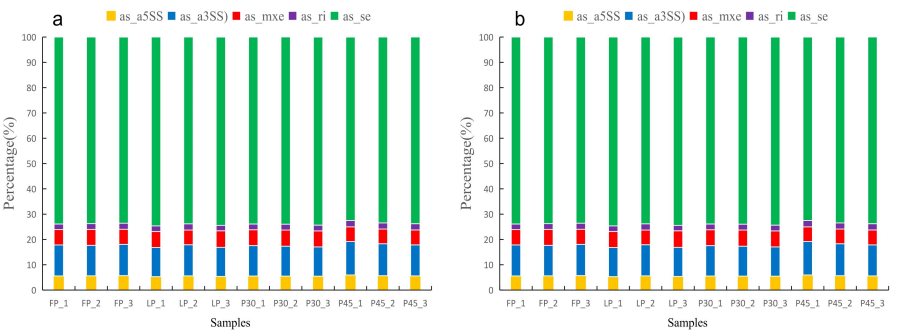
The X- and Y-axes represent the logarithmic values of gene expression levels. Red represents upregulated differential genes, blue represents downregulated differential genes and gray represents non-differential genes. a: FP vs LP; b: P30 vs FP; c: P45 vs FP; d: P30 vs LP; e: P45 vs LP; f: P45 vs P30.

Fig 1: Scatterplot.



The X-axis represents the sample names, while the Y-axis indicates the number of genes. Different colors indicate different expression levels: Genes with FPKM d" 1 indicate extremely low expression, those within the FPKM range of 1-10 represent low expression and genes with FPKM e" 10 indicate moderate to high expression.

Fig 2: Stacked histogram.



(a) Alternative splicing events. (b) Differential alternative splicing events. The X-axis represents the samples and groups, while the Y-axis indicates the proportion of different alternative splicing types in the corresponding sample. Each color represents a different alternative splicing type.

Fig 3: Alternative splicing events.

Alternative splicing

The experiment detected five types of alternative splicing events: skipped exon (SE), alternative 5' splicing site (A5SS), alternative 3' splicing site (A3SS), mutually exclusive exons (MXE) and retained introns. Among the alternative splicing and differential alternative splicing events, the SE category held the highest proportion, constituting 72.51%-74.64% and 37.04%-66.67%, respectively (Fig 3).

GO annotation and KEGG pathway analysis

The study performed GO and KEGG pathway analyses for 467 differentially expressed genes. In terms of biological processes, the 467 genes were enriched in 261 entries of biological processes, 258 entries of cellular components and 260 entries of molecular function. The top five ranked GO terms are listed in (Table 3). 467 genes primarily classified into 231 pathways, with the top 10 KEGG entries being signal transduction, immune system, infectious diseases (viral), signaling molecules and interactions, cancers (overview), endocrine system, global and overview maps, cell growth and death, transport and catabolism and cellular community-eukaryotes.

Through GO and KEGG pathway analysis, seven mRNAs were found to be involved in processes related to defense against bacterial and viral infections and regulation of body's immunity (Table 4).

QRT-PCR

The results indicated a significant increase in the expression of seven mRNAs in the blood of pregnant ewes, with very low expression levels in the blood of non-pregnant ewes ($p<0.01$) (Fig 4).

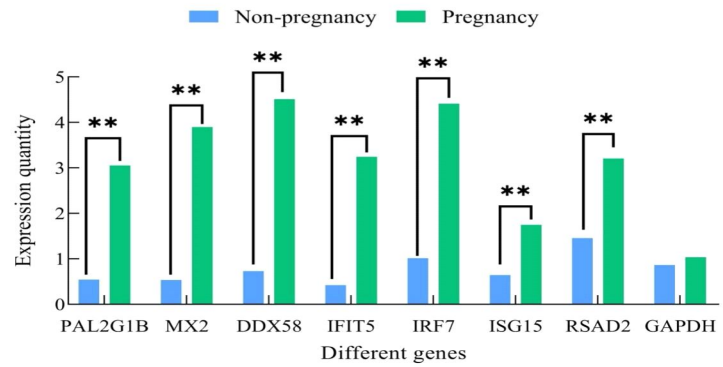
Finding and discovering valuable biomarkers has become an important focus of current research. High-

Table 4: Partial GO and KEGG pathways of 7 mRNA target genes.

mRNA	GO classification	KEGG pathway
MX2	GTPase activity (GO:0003924), GTP binding (GO:0005525), immune system process (GO:0002376), response to virus (GO:0045087), response to interferon-alpha (GO:0045087), innate immune response (GO:0045087), defense response to virus (GO:0051607), regulation of cell cycle (GO:0051726)	----
DDX58	Cytoplasm (05169), cytosol (GO:0005829), bicellular tight junction (GO:0005829), actin cytoskeleton (GO:0015629), ruffle membrane (GO:0032587)	NF-kappa B signaling pathway (04064), RIG-I-like receptor signaling pathway (04622), Cytosolic DNA-sensing pathway (04623), hepatitis C (05160), influenza A (05164), herpes simplex infection (05168), Epstein-Barr virus infection (05169) toll-like receptor signaling pathway (04620), NOD-like receptor signaling pathway (04621), hepatitis C (05160), measles (05162), influenza A (05164), herpes simplex infection (05168), Epstein-Barr virus infection (05169), viral carcinogenesis (05203)
IRF7	Nucleus (GO:0005634), nucleoplasm (GO:0005654), cytoplasm (GO:0005737), cytosol (GO:0005829)	
IFIT5	Plasma membrane (GO:0005886), IkappaB kinase complex (GO:0008385), actin cytoskeleton (GO:0015629), ruffle membrane (GO:0032587), intracellular membrane-bounded organelle (GO:0043231), apical part of the cell (GO:0045177)	----
ISG15	Response to bacterium (GO:0009617), regulation of interferon-gamma production (GO:0032649), response to type I interferon (GO:0034340), defense response to bacterium (GO:0042742), negative regulation of viral genome replication (GO:0045071), defense response to virus (GO:0051607), interleukin-10 secretion (GO:0072608), interferon-gamma secretion (GO:0072643)	RIG-I-like receptor signaling pathway (04622), human papillomavirus infection (05165), Epstein-Barr virus infection (05169)
RSAD2	Fibrillar center (GO:0001650), mitochondrion (GO:0005739), mitochondrial outer membrane (GO:0005741), mitochondrial inner membrane (GO:0005743), endoplasmic reticulum (GO:0005783), endoplasmic reticulum membrane (GO:0005789), lipid droplet (GO:0005811)	Hepatitis C (05160), influenza A (05164)
PLA2G1B	Innate immune response in the mucosa (GO:0002227), anti-bacterial humoral response (GO:0019731), defense response to Gram-positive bacterium (GO:0050830), antimicrobial humoral immune response mediated by antimicrobial peptide (GO:0061844)	Ether lipid metabolism (00565), arachidonic acid metabolism (00590), linoleic acid metabolism (00591), metabolic pathways (01100), biosynthesis of secondary metabolites (01110), vascular smooth muscle contraction (04270), pancreatic secretion (04972)

throughput sequencing technologies, such as transcriptomics and proteomics, have emerged as an important means of screening small-molecule biomarkers Mann *et al. (*2021). The study successfully constructed RNA libraries for sheep in the early stages of pregnancy, yielding an average output of 11.50 G of data per sample. The average alignment rate of the samples to the reference genome was 93.88%, exceeding the sequencing yield of 10 G with an average alignment rate of 81.22% as reported by Wu *et al.* (2020). The data output rate of this study was higher than the sequencing results of the study mentioned above, demonstrating the reliability of the sequencing data and ensuring a solid foundation for further in-depth analysis in subsequent experiments. The experiment detected 467 differential genes, comprising 418 differential mRNAs. Differential expression analysis revealed markedly different gene expression patterns in different physiological periods in animals. These findings further confirmed the involvement of genes in the regulation of animal physiological activities, which was consistent with the reports in the existing literature (Chang *et al.,* 2017; Chang *et al.,* 2022).

GO and KEGG pathway analysis combined with differential expression analysis revealed that the expression of gene MX2 was significantly elevated at P30 and P45. Additionally, it was involved in biological processes such as immune system functions, antiviral responses, interferon responses, innate immune responses and defense responses against viruses. The study by Fang *et al.* (2023) confirmed an association between MX2 expression levels and complement C3 and C4, which was consistent with the findings of this study. The expression level of gene DDX58 was extremely significantly elevated in P45, which was involved in the regulation of influenza A virus pathway, positive regulation of the host defense response to viruses, viral response, positive regulation induced by interleukin-6/8, positive regulation of interferon secretion and innate immune responses. Lu *et al.* (2022) demonstrated that the cytoplasmic DNA-sensing pathway may play an important role in the pathogenesis of sepsis, with DDX58 being one of its key functional targets. The analysis in this study indicates that DDX58 plays an important role in the regulation of animal immunity, consistent with the aforementioned literature. The expression level of gene IRF7 was significantly elevated in P45; it was involved in the regulation of adaptive immune responses, immunoglobulin-mediated immune responses, positive regulation of type I interferon secretion, interferon-α secretion, positive regulation of interferon-α/β secretion, regulation of MyD88-dependent/non-independent toll-like receptor signaling pathways, type I interferon biosynthetic process, antiviral defense responses and type I interferon signaling pathways. Studies have confirmed that the IRF7 gene plays an important role in host resistance, body defense, maintenance of maternal animal health and its regulation. As a key regulator factor for type I interferon, it is regulated by multiple pathways (Yu *et al.,* 2018) and plays a crucial role in the host's antiviral innate immune response (Chen *et al.,* 2019). Studies have reported the absence of the transcription factor IRF3 in poultry and the production of type I interferon is mainly induced by IRF7 (Adam *et al.,* 2011; Diwakar *et al.,* 2017), demonstrating that IRF7 is an important regulator of interferons, proinflammatory cytokines and interferon-stimulated genes, highlighting its significant contribution to innate immunity. The expression level of gene IFIT5 was extremely significantly elevated at P45 and was involved in biological processes such as positive regulation of I-kappaB kinase/NF-kappaB signaling, negative regulation of viral genome replication and response to viruses, playing an important role in host resistance, body defense, maintenance of maternal animal health and its regulation. The research demonstrated through transcriptomic studies that the mRNA transcription levels of the IFIT5 gene in lung and intestinal tissues of 6-week-old ducks increased more than 1000-fold 24 h after a*vian* influenza infection (Hillary *et al.,* 2012). The expression level of gene ISG15



The X-axis represents the internal reference gene and different mRNAs, while the Y-axis represents the gene expression levels. Different colors represent samples from different time periods.

Fig 4: Differential expression of mRNA genes in the blood of non-pregnant and pregnant ewes.

was significantly elevated at P45 and it was involved in response reactions to bacteria and viruses, IL-10 secretion and interferon secretion, among other processes. It was related to the body's defense against viruses and bacteria, as well as improved resistance. The research have shown that ISG15 inhibits encephalomyocarditis virus (EMCV) replication *in vitro* and *in vivo* and knockout of the ISG15 gene can increase susceptibility to EMCV (He *et al.,* 2023). The gene RSAD2, also known as Viperin, is an interferon-induced antiviral protein. The analysis of this experiment showed that the expression level of RSAD2 was significantly elevated at P45 and it was involved in response to viruses, negative regulation of protein secretion and positive regulation of Th2 cell cytokine secretion, contributing to increased viral resistance and immune responses within the body. The research suggested that IL-6 and RSAD2 are synergistically involved in the immune response of the host to the Orf virus (Jiang *et al.,* 2023). The biological functions of RSAD2 analyzed in this experiment were consistent with those reported in the literature (Jiang *et al.,* 2023). The expression level of gene PLA2G1B was extremely significantly elevated at P45 and the analysis revealed that it was involved in mucosal innate immune responses, antimicrobial reaction, defense against Gram-positive bacteria and antimicrobial peptide-mediated humoral immune response, which were related to the defense against bacteria and immunity of the body. It was found that patients with lung adenocarcinoma with low expression of PLA2G1B exhibited a poorer prognosis and this result was related to the fact that patients with low expression presented stronger immune rejection and less immune cell infiltration, thus affecting immune cytotoxic function and consequently impacting overall patient prognosis (Zhu *et al.,* 2022).

The qRT-PCR results showed that the seven mRNAs were expressed at low levels in the blood of non-pregnant ewes, while their expression increased significantly in the blood of pregnant ewes. Seven mRNAs were involved in processes such as immune response, secretion of immune factors and defense against bacteria/viruses. Combined with differential expression patterns in pregnant and non-pregnant ewes, it is conceivable that these seven mRNAs, including MX2, DDX58, IRF7, IFIT5, ISG15, RSAD2 and PLA2G1B, could serve as feasible immunobiomarkers. In infectious diseases, C-reactive protein and procalcitonin are commonly used inflammatory markers [Li *et al.,* 2024]. The research demonstrated that ABCA3 expression was associated with a prognosis and immune cell infiltration in LUAD patients, making it a potentially valuable biomarker to predict the prognosis of non-small cell lung cancer Zhang *et al.* (2024). MicroRNA sequencing analysis revealed a significant upregulation of miR-26a in bovine plasma during early pregnancy, suggesting its potential as a biomarker for early pregnancy (Jason *et al.*, 2017). However, its application in pregnancy diagnosis needs to be further investigated. By detecting and analyzing immunobiomarkers, it is possible to better understand the immune status of diseased or healthy animals, enabling the development of individualized treatment or healthcare plans, which are of significant importance for diagnosis, treatment and physiological monitoring. Seven mRNAs, including MX2, DDX58, IRF7, IFIT5, ISG15, RSAD2 and PLA2G1B, emerged as crucial small molecules for immune monitoring, identified by high-throughput sequencing, bioinformatic analysis and validation of quantitative expression patterns in clinical blood samples. However, due to the differences in sample types, sample collection and processing, detection methods, animal breed and sex and personnel operations, more in-depth research is warranted to assess the specificity and accuracy of the seven mRNAs as immunobiomarkers for immune monitoring.

CONCLUSION

The RNA library for sheep was successfully constructed using high-throughput sequencing technology and in combination with bioinformatics analysis, a total of 467 differentially expressed genes were identified. Among them, MX2, DDX58, IRF7, IFIT5, ISG15, RSAD2 and PLA2G1B exhibited significantly elevated expression during pregnancy. GO and KEGG pathway analyses revealed that these candidate genes are functionally associated with antibacterial and antiviral defense mechanisms as well as immune system regulation. QRT-PCR validation further indicated that these genes exhibit threshold-dependent expression patterns, suggesting their potential utility as immunobiomarkers for pregnancy monitoring. These findings have important implications for developing screening protocols and early intervention strategies for pregnancy-associated disorders in livestock.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Laboratory Animal Welfare and Ethics Committee of Xichang University(No.xcc2023001) and in accordance with the "Guidelines for Use of Experimental Animals" of the Ministry of Agriculture.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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