Research Article

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The Screening of Ovarian Candidate Proteomics Biomarkers of Dolang Sheep at Different Physiological Periods Based on iTRAQ Technology

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Abstract To screen the differentially expressed ovary proteins in Dolang sheep with oestrus, anestrus and pregnancy using isobaric tag for relative and absolute quantitation (iTRAQ) technology, which will provide the basis for the research of regulation and identification of oestrus, the diagnosis of early pregnancy. In total, we identified 4847 proteins, 470 of which were significantly different proteins. Compared with anestrus, 18 proteins were up and 102 proteins were down in oestrus, 20 proteins were up and 60 proteins were down in pregnancy; Compared with oestrus, 50 proteins were up and 24 proteins were down during pregnancy. Such as cyclin-Y isoform X1, cleavages stimulation factor subunit 1 isoform X1, follistatin-related protein 1 isoform X2, nicotinate phosphoribosyl transferase isoform X1 at up-regulated, hemopexin isoform X1, apolipoprotein A-II, nucleophosmin at down-regulated, which were related to hormones generation, regulation of oestrus, zygote production and apoptosis, early embryo attachment and embryo development. Of which, cyclin-Y isoform X1 and nicotinate phosphoribosyl transferase isoform X1 significant increase in pregnancy, compared with oestrus and anestrus. The study identified different proteins at oestrus and pregnancy of Dolang sheep based on iTRAQ proteomics technology, which provide new thoughts and methods to further exploring the candidate biomarkers of sheep estrous identification and diagnosis of early gestation.

Keywords Dolang sheep; Ovary; iTRAQ; Proteins; Biomarkers

Dolang sheep can be estrus all the year round, and it is less affected by the law of long and short sunshine. It is a very excellent local sheep breed in southern Xinjiang. It can be bred according to "two production in one year" or "three production in two years". Most of the sheep breeds are seasonal estrus, which is greatly affected by the law of sunshine. Generally, it only oestrus in summer and autumn, which seriously affects the reproductive efficiency. At present, animal husbandry has been large-scale, intensive and modern. In this mode, the maximum potential of livestock reproduction can ensure the best economic benefits. According to the literature reports, the regulation mechanism of major genes of perennial estrus and seasonal estrus in sheep is not very clear, and the early pregnancy diagnosis technology and disease screening still need to be improved. From prokaryotes to eukaryotes, from lower animals to higher animals, the function of genetic materials is realized by proteins, and the spatial structure, function and activity of proteins determine all aspects of animal body activities (Shi et al., 2015)

iTRAQ (isobaric tags for relative and absolute quantification) is a new protein research method developed by American biotechnology company. Due to its powerful function, good operability, high accuracy and repeatability, iTRAQ has been widely used in animal and plant research and Molecular Biology (Ma et al., 2017; Bai et al., 2017). Guo et al. (2018) studied the differentially expressed proteins between bovine and human *Streptococcus agalactiae* strains by iTRAQ technology, and identified 350 differentially expressed proteins, covering 28 biological functions and 14 pathways; Miao et al. (2016) analyzed the ovarian differential proteins of Small Tail Han sheep and hornless Dorset by iTRAQ technology, and found many proteins and cytochrome c oxidase through GO and KEGG pathway analysis. It is related to the activities of oxidoreductase and carrier. Wang et al. (2019) used iTRAQ technology and LC-MS / MS to analyze the differential proteins between *Eimeria tenella*

spore infected and uninfected primary chicken embryo fibroblasts. In the experiment, 259 differential proteins were obtained, of which 145 were up-regulated and 114 were down-regulated. According to GO and KEGG pathway analysis, differential proteins were mainly associated with glycolysis / gluconeogenesis, extracellular matrix receptor interaction, adhesion plaque, cysteine Acid is related to methionine metabolism and amino acid biosynthesis. ITRAQ technology has been reported more and more in the regulation mechanism of animal physiological functional proteins and the screening of candidate biomarkers (Zhang et al., 2018; Wang et al., 2019). However, it has not been reported in the screening and identification of ovarian related proteins in different physiological periods of Dolang sheep. In this study, we used iTRAQ technology to explore the differentially expressed proteins in ovarian tissues of Dolang sheep in different physiological periods, which provided the basis for the study of the regulation mechanism of perennial estrus protein and the identification of estrus, early pregnancy examination and screening of biomarkers for disease diagnosis.

1 Results and Analysis

1.1 SDS PAGE analysis of ovarian protein

The ovarian proteins in estrus, estrus and pregnancy were extracted by ultrasonic fragmentation method, and the protein integrity was detected by SDS-PAGE. The results showed that the total proteins of 6 samples with molecular weight ranging from 25 to 200 kDa were effectively separated. The total protein amount was more than 400 μ g, and the bands were complete, clear, and without degradation. The quality inspection results were all class A. for the specific results, please refer to the previous literature published by the research group (Qian et al., 2018)

1.2 Identification of proteins and analysis of differentially expressed proteins

After enzymolysis, peptide quantification and labeling, the ovarian protein samples were graded by SCX. The software (proteome discoverer 1.4) was used to quantitatively analyze the reported ion peak intensity values of peptide segments. A total of 27943 peptides and 4845 proteins were obtained in the experiment (Table 1).

Set the threshold condition of $FC = \text{fold change of } 1.2$, $p < 0.05$, and then compare the two groups, $FC \geq 1.2$ is up-regulated, $FC \leq 0.83$ is down-regulation, which is considered as no significant change in expression, so as to obtain differential protein. 470 differential proteins were obtained. Compared with the estrus interval, 18 proteins were up-regulated and 102 proteins were down-regulated in estrus; 20 proteins were up-regulated and 60 proteins were down-regulated in pregnancy; compared with estrus, 50 proteins were up-regulated and 24 proteins were down-regulated in pregnancy (Table 1). The main up-regulated or down-regulated proteins related to reproductive physiology and diseases are shown in Table 2, Table 3 and Table 4. Data analysis showed that cyclin-y isoform X1 and nicotinate phosphoribosyl transferase isoform X1 were significantly up-regulated during pregnancy, which provided a basis for screening biomarkers for early pregnancy diagnosis.

Table 1 The protein datas statistics of mass spectrometry

Peptide	Protein	Contrast of Groups	Up	Down
27943	4845	oestrus VS anestrus	18	102
		pregnancy VS anestrus	20	60
		pregnancy VS oestrus	50	24

Table 2 Main proteins of up or down regulation at oestrus compared to anestrus

Accession	Protein	MW/PI	Coverage	P value	Up/Down
XM_101118196	Carbonic anhydrase 3	29.4/7.84	15.38	0.04	Up
XM_101120455	Histone H2B type 2-F	13.9/13.2	73.02	0.027	Up
XM_101118330	Follistatin-related protein 1 isoform X2	35/5.39	53.57	0.048	Up
XM_101119326	Cleavage stimulation factor subunit 1 isoform X1	53.1/7.14	32.2	0.04	Up
XM_101118334	Cyclin-Y-like protein 1 isoform X1	41.2/6.07	30.58	0.01	Down
XM_101116802	Cyclin-dependent kinase 16 isoform X1	63.3/7.97	32.11	0.01	Down
XM_101117872	Hemopexin isoform X1	51.8/7.12	52.94	0.04	Down
XM_101118551	Phospholipase D3	54.6/6.65	28.57	0.01	Down

Table 3 Main proteins of up or down regulation at pregnancy compared to oestrus

Accession	Protein name	MW/PI	Coverage	P value	Up/Down
XM_101107301	Tubulin alpha chain-like 3	49.3/5.69	35.43	0.03	Up
XM_101113505	Nicotinate phosphoribosyltransferase isoform X1	37.7/7.02	17.61	0.03	Up
XM_101122455	Cyclin-Y isoform X1	33.3/7.74	30.66	0.04	Up
XM_101121603	Immunoglobulin J chain isoform X1	18/6.18	44.3	0.02	Up
XM_101117273	Apolipoprotein C-III isoform X1	10.8/4.93	52.58	0.003	Down
XM_101118587	Glycerol kinase isoform X1	63.7/5.92	29.3	0.04	Down
XM_101110712	Leukocyte elastase inhibitor	42.2/6.57	53.85	0.02	Down
XM_101104619	Apolipoprotein A-II	11.1/8.1	35	0.03	Down

Table 4 Main proteins of up or down regulation at pregnancy compared to anestrus

Accession	Protein name	MW/PI	Coverage	P value	Up/Down
XM_101102720	Diacylglycerol kinase beta isoform X1	90.5/7087	32.5	0.01	Up
XM_101116698	TBC1 domain family member 24 isoform X1	61.7/8.46	33.76	0.02	Up
XM_101113505	Nicotinate phosphoribosyltransferase isoform X1	37.7/7.02	17.61	0.03	Up
XM_101122455	Nyctin-Y isoform X1	33.3/7.74	30.66	0.04	Up
XM_443217	Integrin alpha-M precursor	127.5/7.49	19.01	0.008	Down
XM_101115576	Serpin A3-5	39.2/5.59	35.82	0.03	Down
XM_101102605	Nucleophosmin	32.7/4.77	49.66	0.04	Down
XM_494432	Cofilin-1	18.5/8.0	77.71	0.04	Down

1.3 Go analysis

Go analysis of differential proteins showed that biological process was the most abundant in biological process, followed by cellular process and metabolic process, followed by single biological process and organic process (Figure 1a). In terms of cell composition, cellular component was the most abundant, followed by cell part, cell and intracellular, the third was intracellular part, and the fourth was organelle (Figure 1b). In terms of molecular function, molecular function items are the most abundant, followed by binding, catalytic activity and protein binding (Figure 1c).

Figure 1 GO annotation of differential proteins

1.4 KEGG analysis

KEGG pathway analysis was performed on the enriched differential proteins. The results showed that the most items of differential protein pathway were accumulated in metabolic pathways, followed by phagosome, and the third was viral carcinogenesis, pertussis, complement and coagulation cascade the fourth pathway is systemic lupus erythematosus and Staphylococcus aureus infection (Figure 2). The pathway shows that differential proteins participate in the body defense, which is related to the improvement of body resistance during pregnancy and estrus.

Figure 2 KEGG annotation of differential proteins

2 Discussion

With the rapid development of science and technology and molecular biotechnology in China, the research on the regulation mechanism of estrus, estrus identification, pregnancy diagnosis and other molecular aspects of sheep has become more and more in-depth. The research of sheep reproductive diseases and reproductive physiology has developed from the initial study of a small number of major genes to the level of miRNA, transcriptomics, metabonomics and proteomics, which are biomarkers, it provides a new way of thinking for the screening and research. At present, two-dimensional differential gel electrophoresis (2D-DIGE) is widely used in traditional proteomics research, but 2D-DIGE has some drawbacks. Its separation effect is related to the relative molecular mass, protein abundance, isoelectric point and hydrophobicity. Its application is limited. Moreover, 2D-DIGE is time-consuming and laborious, and it is difficult to automate. It is difficult to achieve direct coupling with mass spectrometry (Xie et al., 2011). ITRAQ protein quantitative technology can be used for quantitative analysis of any type of protein (Unwin et al., 2010). At the same time, iTRAQ combined with liquid chromatography (LC) separation technology makes up for some shortcomings of 2D-DIGE (Huang et al., 2011). Compared with 2D-DIGE and ICA-MS / MS, iTRAQ-MS / MS was more sensitive in the detection of low abundance proteins (Wu et al, 2006). Therefore, this experiment took Dolang sheep as the research object, and studied the differential expression of ovarian proteins in estrus, diestrus and pregnancy by iTRAQ quantitative technology. 27943 peptide segments and 4845 proteins were obtained, including 470 differential proteins. Compared with the estrus interval, there were 18 up-regulated and 102 down regulated proteins during estrus. Compared with the estrus interval, 20 proteins were up-regulated, and 60 proteins were down-regulated during pregnancy; compared with estrus, 50 proteins were up-regulated, and 24 proteins were down-regulated during pregnancy. In estrus, the up-regulated proteins were carbonic anhydrase 3, histone H2B type 2-F, follistatin related protein 1 isoform X2, cleavage stimulation factor subunit 1 isoform X1; while in estrus, tubulin alpha chain like 3, nicotinate phosphoribosyl

transferase isoform X1, cyclin-y isoform X1, immunoglobulin were significantly up-regulated during pregnancy. Compared with the estrus interval, the up-regulated proteins in pregnancy were diacylglycerol kinase beta isoform X1, TBC1 domain family member 24 isoform X1, nicotinate phosphoribosyl transferase isoform X1, cyclin-y isoform X1. The screening of differentially expressed proteins provides the basis for estrus identification, estrus regulation mechanism and early pregnancy biomarkers screening. Cui et al. (2018) studied the effects of weaning stress on intestinal metabolism, structure and function of lambs by iTRAQ technology, and obtained 389 differential proteins, including 143 up-regulated proteins and 246 down-regulated proteins. According to GO and KEGG pathway analysis, most of the differential proteins were related to intestinal morphology and immune function. In 2020, Wang et al. (2020) reported the use of iTRAQ in this study, a large number of candidate biomarkers related to reproduction were obtained, which accelerated the application of iTRAQ technology in animal reproduction regulation technology or theoretical research field.

Through GO and KEGG pathway analysis, the differential proteins were highly expressed in biological processes, cells, organelles and membrane components. The pathway analysis showed that these differential proteins were mainly involved in the metabolism and cell processes of the body, and had the functions of immunity, enhancing the body resistance and binding, mainly in the estrus and pregnancy to improve the body resistance and enhance the new. Compared with estrus and diestrus, cyclin-y isoform X1 (cyclin-y isoform x1) and nicotinate phosphoribosyl transferase isoform X1 (nicotinate phosphoribosyl transferase isoform x1) proteins were significantly increased during pregnancy. Cyclin-y is a new cell cycle protein discovered in recent years. It contains 341 amino acids and has a molecular weight of 39 kD. CCNY, as its coding gene, is located on chromosome 10, with a length of 3968 bp and contains 10 exons and 9 introns (Zhao et al., 2013). Studies have confirmed that cyclin-y gene plays an important regulatory role in embryonic growth and development, adhesion, cell cycle process, immunity and cell apoptosis (Yue et al, 2010; Li et al., 2015). Nicotinic acid phosphoribosyl transferase assisted in the transformation of quinolinic acid (QA) to nicotinic acid (NA). Chen et al. (2012) amplified the gene fragment encoding nicotinic acid phosphoribosyl transferase by PCR, and successfully constructed a prokaryotic expression plasmid for expression in *E. coli*, realizing the 2-site selective decarboxylation of quinolinic acid, which is conducive to the production of nicotinic acid (Nathan et al., 2019). And nicotinic acid is one of the essential vitamins for the body, which can promote the health of the digestive system. It can be seen from the literature that both cyclin y and nicotinate phosphoribosyl transferase are related to the occurrence and development of animal diseases and immunity. The cyclin y subtype X1 and nicotinic phosphoribosyl transferase subtype X1 obtained in this experiment are two protein subtypes respectively. Compared with estrus and diestrus, the expression patterns of cyclin y and nicotinate phosphoribosyl transferase X1 are significantly up-regulated during pregnancy, which can be inferred Cyclin y subtype X1 and nicotinic acid phosphoribosyl transferase subtype X1 should be related to the improvement of maternal immunity during pregnancy and the prevention of diseases.

In this study, iTRAQ proteomics, LC-MS/MS and bioinformatics were used to analyze the differentially expressed proteins during oestrus, anestrus and pregnancy of Dolang sheep, 470 differentially expressed proteins were obtained. Among them, cyclin y subtype X1 and nicotinic acid phosphoribosyl transferase subtype X1 proteins are related to immunity, which are significantly up-regulated during pregnancy. It is speculated that they may maintain the body's health and avoid the occurrence of pregnancy diseases by improving the body's immune function. They are expected to become candidate biomarkers for pregnancy diagnosis and disease screening, and their mechanism needs further study.

3 Materials and Methods

3.1 Experimental materials

The ovaries of Dolang sheep (raised by animal experimental station of Tarim University) during estrus (10 days after estrus), estrus (12 hours after estrus) and pregnancy (about 45 days of pregnancy) were slaughtered, and the ovaries were washed with PBS buffer (pH7.4). After that, liquid nitrogen was put into rapid freezing and stored at - 80°C in the laboratory.

3.2 Main drugs and reagents

The procurement of urea, SDS, thiourea, G-250, Coomassie brilliant blue, acrylamide, β -mercaptoethanol, agarose, protease, iTRAQ kit, inhibitor mixture, Bradford reagent, NH_4HCO_3 , BSA and protein marker reagents were the same as those in the literature (Qian et al., 2018).

3.3 Methods

3.3.1 Extraction, quantification and detection of ovarian tissue protein in different periods

Take 0.1 g ovarian tissue of Dolang sheep in estrus, diestrus and pregnancy respectively, break them by ultrasound, and then extract the protein. The protein is quantified and detected by Bradford method. The process is shown in the literature published by the research group (Qian et al., 2018).

3.3.2 ITRAQ tag

100 μg peptide was taken from each sample and labeled according to iTRAQ Kit: oestrus group was labeled with 115 and 116, anestrus group with 117-118 and pregnancy group with 119 and 121, with 2 replicates in each group. After labeling, a small number of samples were mixed and detected by mass spectrometry.

3.3.3 High performance liquid chromatography

Each sample was separated by easynlc 1000 liquid phase system. The buffer solution was 0.1% formic acid aqueous solution and B was 0.1% formic acid acetonitrile solution. The sample was loaded on a 95% liquid an equilibrium column by an automatic sampler to the pre column C18 tray column (C183 μm 100 $\mu\text{m} \times 2$ cm). The sample was separated by C18 column (C183 μm 75 $\mu\text{m} \times 15$ cm) with a flow rate of 300 NL / min. The liquid gradient is shown in Table 5.

The samples were separated by capillary high performance liquid chromatography, and then analyzed by mass spectrometry (Q-Enactive HF).

3.3.4 Mass spectrometry analysis

The raw data were raw files. The software Sequest and ProteomeDiscoverer were used for qualitative and quantitative analysis. The relevant parameters are shown in Table 6.

Results the filter parameters were as follows: Peptide FDR $\leq$ 0.01 (Sandberg et al., 2012).

Table 5 The elution gradient parameters of liquid chromatography

Time (min)	A	B	Flow velocity (nL/min)
0	96%	4%	300
4	92%	8%	300
67	70%	30%	300
77	20%	80%	300
80	20%	80%	300

Table 6 The parameters of library searching

Item	Value
Database	protein.selected.fasta
Enzyme	Trypsin/P
Max Missed Cleavages	2
Fixed modifications	Carbamidomethyl C
Variable modifications	Oxidation(M)
Peptide Mass Tolerance	± 20 ppm
Fragment Mass Tolerance	0.02
Peptide confidence	High

3.3.5 Bioinformatics analysis

Open the go database, map the differential protein to each term, calculate the protein obtained by each term, and find out the go items which are significantly enriched in the differential protein compared with all protein background. Calculation formula:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$$

Where, M: the number of proteins annotated to a certain GO term in all proteins; m: the number of differential proteins in M; N: the number of proteins with GO annotation information in all proteins; N: the number of differential proteins in n.

With $P \leq 0.05$ as the boundary, the term reaching this condition is defined as go term which is significantly enriched in the differential protein.

In order to find out the difference of Gog pathway, we calculated the difference between the two pathways. KEGG enrichment can analyze the main regulatory mechanism of differential proteins and draw regulatory network.

Authors' contributions

CWH participated in the experimental design and results analysis of the study; CZL and WHY were the executors of the experimental research, and participated in some processes of the study; YXL and MTT completed the later revision of the paper; meanwhile, CZL participated in the collection process of the whole experimental sample. All authors read and approved the final manuscript.

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