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Identification of genes differentially expressed in Jining Grey and Liaoning Cashmere goats ovaries

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To search for genes controlling high prolificacy of Chinese indigenous goats, differential display reverse transcription-polymerase chain reaction (DDRT-PCR) was used to screen differentially expressed cDNA bands in the sexually matured ovaries of 3-year-old prolific Jining Grey goats and monotocous Liaoning Cashmere goats with 24 combinations of three anchored primers and eight arbitrary primers. 22 expressed sequence tags (ESTs) were proved to be the positive bands by Northern hybridization. They comprised 10 known ESTs and 12 ESTs without homologous sequences in the GenBank. These results indicate that several genes such as GATA-4, metallothionein-like protein, CAT genes and unknown ESTs (CV983340 and CV983341) were expressed only in Jining Grey goats.

Key words: Differential display reverse transcription-polymerase chain reaction, goat, ovary, prolificacy.

INTRODUCTION

The Jining Grey is a prolific indigenous goat breed, and Liaoning Cashmere is a monotocous indigenous goat breed in People's Republic of China. The two goat breeds have an obvious difference in litter size (2.94 and 1.18, respectively) (Feng et al., 2012; Tu, 1989), which made them possible models for elucidating the molecular mechanism of high prolificacy.

As an important reproductive and endocrine organ, ovary plays a pivotal role in determining female prolificacy. So, in the present study, we examined gene expression differences in the sexually matured ovaries of 3-year-old Jining Grey goats and Liaoning Cashmere goats. Differential display reverse transcription-polymerase chain reaction (DDRT-PCR), as a more powerful, sensitive and rapid method in detecting the differences in gene expression (Liang et al, 1994), was applied in the present study to search for genes or expressed sequence tags (ESTs) involved in high prolificacy of Chinese

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Abbreviations: **DDRT-PCR**, Differential display reverse transcription-polymerase chain reaction; **StAR**, steroidogenic acute regulatory protein; **MAPK**, mitogen-activated protein kinases.

indigenous goats.

MATERIALS AND METHODS

DDRT-PCR

Ovaries were collected from three healthy 3-year-old Jining Grey does (Jining Grey Goats Conservation Base, Jiaxiang County, Shandong Province, China) and three healthy 3-year-old Liaoning Cashmere does (Qingshui Town, Mentougou District, Beijing, China). Total RNA was extracted from goat ovary samples using Trizol kit (Invitrogen, Carlsbad, USA). Then, 25 µl of total RNA was treated with an equal volume of RNase-free DNase-I mixture (Promega, Madison, WI, USA). A pool was prepared by mixing equal amount of total RNA of three individuals from the same breed. The cDNA primers including three 3' end anchored primers of H-T11A (5'-AAGCTTTTTTTTTTTA-3'), H-T11G (5'-AAGCTTTTTTTTTTG-3') and H-T11C (5'-AAGCTTTTTTTTTTC-3') and eight 5' end arbitrary primers (P1: 5'-TGCCGAAGCTTTGGTGTC-3'; P2: 5'-TGCCGAAGCTTTGGTACC-3'; P3: 5'-TGCCGAAGCTTTGGTAGC-3'; P4: 5'-TGCCGAAGCTTTGGTATG-3'; P5: 5'-TGCCGAAGCTTTGGTCAC-3'; P6: 5'-TGCCGAAGCTTTGGTCAG-3'; P7: 5'-TGCCGAAGCTTTGGTCTG-3'; P8: 5'-TGCCGAAGCTTTGGTCTC-3') were designed according to the third generation primers of GenHunter Company. Total RNA (4 µg) treated with RNase-free Dnase was reverse-transcribed using 3' anchored primer and M-MLV Reverse Transcriptase (Invitrogen).

1 µl of the reverse transcription product was added to 24 µL of PCR solution containing 20 µmol/l dNTPs, 1.5 mmol/l $MgCl_2$, 1 U of *Taq* DNA polymerase (Promega), 20 µmol/l H-T11N (H-T11G/ H-T11C/H-T11A) primer and 10 µmol/l of each of the eight arbitrary primers. The PCR amplification was carried out on Mastercycler® 5333 (Eppendorf AG, Hamburg, Germany). The PCR parameters reported by Zhao et al. (1995) were adopted. The amplified fragments were then separated on a 8% polyacrylamide gels. Differential fragments from 100 to 1200 bp were excised from the gels, extracted by boiling in 100 µL of distilled water, purified with a DNA purification system (Promega), and reamplified using the same primer corresponding to cDNA display.

Reverse Northern dot blotting and sequencing

Reverse Northern dot blotting was carried out according to the instruction manual of DIG-HIGH Prime DNA labeling and detection starter kit I (Roche, Penzberg, Germany). 1 µg of cDNAs from goat ovaries was denatured by heating in a boiled water bath for 10 min and quickly chilled in an ice/water bath, then DIG-HIGH Prime was mixed and incubated at 37°C for 1 h and the reaction was stopped by adding 2 µL of 0.2mol/L EDTA (pH 8.0). Subsequently, the cloned cDNAs were fixed on the nylon membranes positively charged by UV cross-linking, and then hybridized with DIG Easy Hyb (10 ml/100 cm^2) containing DIG-labeled DNA probe at appropriate hybridization temperature for 20 h. After hybridization and stringency washes, the membranes were incubated in 100 ml blocking solution for 30 min, 20 ml antibody solution for 30 min and detected with freshly color substrate solution in the dark after two washes with 100 ml washing buffer for 15 min. The reaction was completed after 16 h and terminated by 50 ml TE buffer for 5 min.

The positive clones detected by reverse Northern dot blotting were sequenced in both directions using an ABI3730 automatic sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China).

RESULTS AND DISCUSSION

We analyzed the mRNA expression differences in ovaries of the two goat breeds using 24 combinations of three anchored primers and eight arbitrary primers. The partial results are shown in Figure 1. At last, 1195 bands were detected on statistics, and 51 bands were recovered based on their differences (lack, density and size). However, some of the recovered bands could not be perfectly reamplified because of the false positive. Finally, 22 positive blots were detected by reverse Northern dot blotting (Figure 2). The sequencing and BLAST results are shown in Table 1. These ESTs were found to be the part representing several genes such as *Bos taurus* GATA binding protein 4 (GATA-4), YidC (60 kD inner-membrane protein), UMC-bof_0A01-002-g05 Ovarian follicle bof *B. taurus* cDNA, UMC-bof_0A01-002-g06 Ovarian follicle bof *B. taurus* cDNA, etc., which were expressed only in Jining Grey goats.

In recent years, more and more studies proved that GATA-4 was emerging as a critical player in mammalian reproductive development and function (Bennett et al., 2012; Lowry and Atchley, 2000; Tevosian et al., 2002). The cell culture data also support a requirement of GATA-4 for the induction of genes mediating critical biosynthetic steps in steroidogenesis (Tremblay and Viger, 2001). The *GATA-4* gene is correlated with proteins transporting cholesterol to the mitochondria, steroidogenic acute regulatory protein (StAR), proteins involving androgen synthesis p450c17, and proteins involved in estrogen synthesis and aromatase (Silverman et al., 1999; McCoard et al., 2002). Some researchers reported that GATA-4 factor was essential for activity of the neuron-specific enhancer of the gonadotropin-releasing hormone gene by activating the conserved GATA regulatory elements, and further regulates the FSH and LH through this pathway (Lawson et al., 1996; Vaskivuo et al., 2002). On the other hand, it was regarded that GATA-4 activity was positively regulated via phosphorylation at serine 105 by mitogen-activated protein kinases (MAPK), ERK1/ERK2 and P38 MAPK (Tremblay and Viger, 2003). Further researches are required to uncover its function in the prolificacy of goats.

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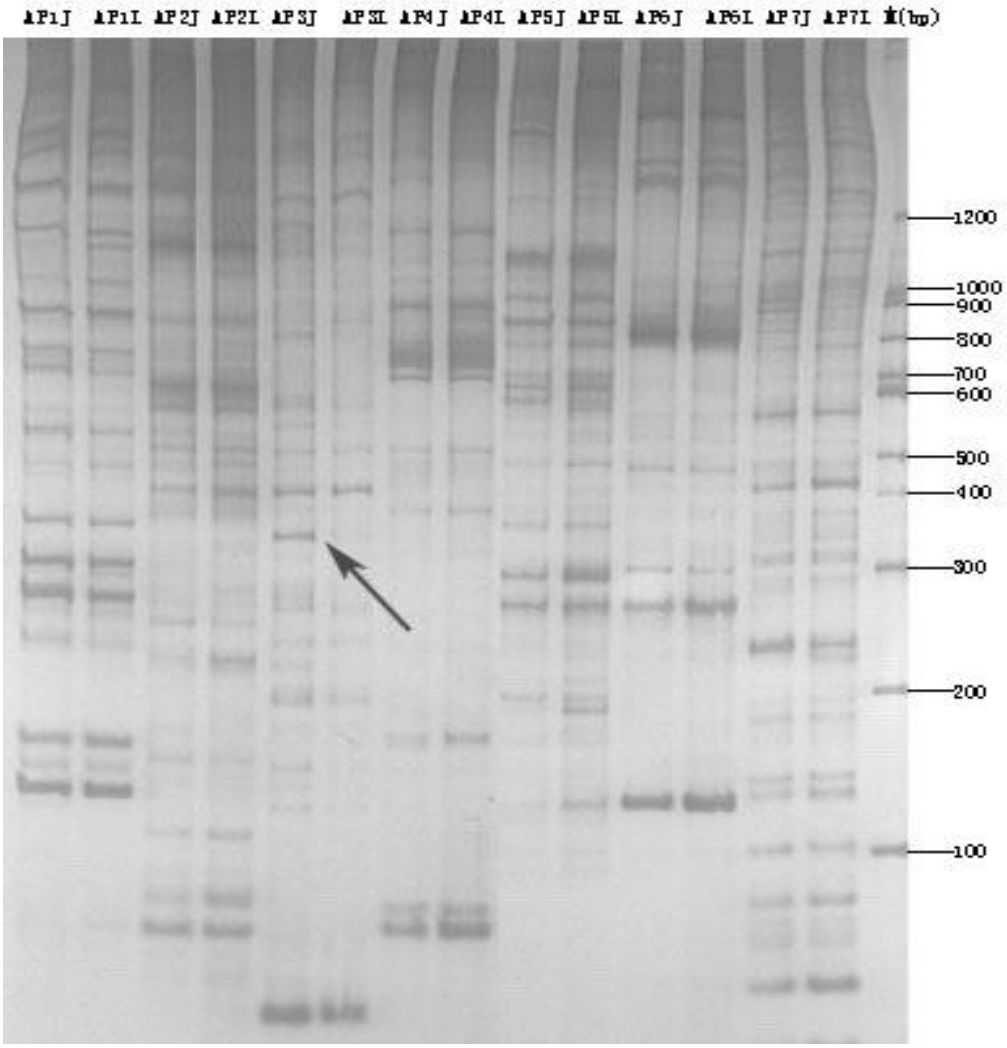

Figure 1. Partial differential display profiles from different primer combinations of goats. M, 100 bp molecular ladder; arrowhead, differential expression fragments.

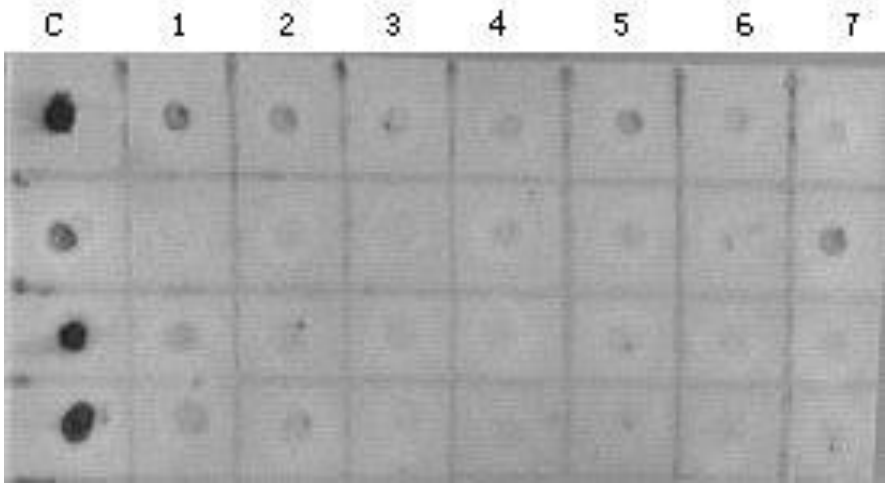

Figure 2. Partial results of reverse Northern dot blots for differential display bands. C represents positive control. Colum 1-7 all represent interested bands.

Table 1. Differential expression genes of DDRT-PCR between ovaries in Jining Grey and Liaoning Cashmere goats.

Number	GenBank accession number	Size (bp)	Copy	BLAST result	Score	E-value	Identity
1		396	2	Unknown gene			
2		226	1	Unknown gene			
3	2878485	369	3	Aspartyl/glutamyl-tRNA amidotransferase subunit A	180 bits (91)	2e-42	247/299 (83%)
4	AJ316283	416	1	Bos taurus partial mRNA for gata4 protein	665 bits (360)	0.0	402/421 (95%)
5	948214	387	2	YidC (60 kD inner-membrane protein)	737 bits (372)	0.0	378/380 (99%)
6	DR092343	564	1	Unknown gene	996 bits (539)	0.0	553/560 (99%)
7	CV983340	666	1	UMC-bof_0A01-002-g05 Ovarian follicle bof Bos taurus cDNA, mRNA sequence	1112 bits (602)	0.0	632/645 (98%)
8	CV983341	802	1	UMC-bof_0A01-002-g06 Ovarian follicle bof Bos taurus cDNA, mRNA sequence	1190 bits (644)	0.0	651/654 (99%)
9		246	1	Unknown gene			
10		283	1	Unknown gene			
11	X64410	463	1	Plasmid pBLCAT2 gene for beta-lactamase and CAT gene for chloramphenicol acetyltransferase	848 bits (428)	0.0	428/428 (100%)
12		455	1	Unknown gene			
13		792	1	Unknown gene			
14	CO729306	957	1	Metallothionein-like protein type 3	416 bits (210)	e-113	210/210 (100%)
15		227	2	Unknown gene			
16		148	1	Unknown gene			
17		791	1	Unknown gene			

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