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Candidate Genes with Ovulation by Differential Display PCR in Small Tail Han Sheep*

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ABSTRACT : To find the candidate genes concerned with ovulation rate of sheep, Differential Display Reverse Transcription Polymerase Chain Reaction was employed to find the differently expressed cDNA controlling ovulation in the Small Tail Han sheep of polyembryony and in Tan sheep of single birth. Twenty-four primer pairs of three anchored primers and eight arbitrary primers were assembled to amplify the specialized bands from these sheep. Positive cross tests were applied to optimize the ascertainable PCR conditions in which different special bands can be identified by silver strain in one PCR tube. After eliminating the false positive PCR products by Northern hybridization, 24 differential display bands were acquired from the ovary in the Small Tail Han sheep. These EST bands were sequenced and 18 different ESTs were found in which five ESTs had several copies and 13 ESTs had only one copy. Comparing these ESTs with homologous sequences by BLAST in the GenBank, there were six ESTs with known open reading frame (ORF) and function, three ESTs with known ORF and no function, and 9 ESTs without homologous sequence. These ESTs partly represent several genes such as NOS2, tensin, TCRA, CDKN1A, ESR1 and ACTB which express especially in Small Tail Han sheep. (**Key Words :** Ovary, Little Size, Small Tail Han Sheep, DD-PCR)

INTRODUCTION

The Small Tail Han sheep is a celebrated high reproductive sheep line with early mature, perennial oestrus and polyembryony in the world, which come from a ramus of Mongolia sheep. The average lamb rate of breeding ewe are 286.5% and multiparous ewe are 304.3% (Du et al., 2003). Tan sheep is another ramus of Mongolia sheep in China characterized by single birth. These sheep lines had kept atretic breeding method since 300 years ago in their respective group from Mongolia sheep line. Therefore, the two sheep line had the similar heredity material except the difference of little size which made it possible to study the molecular mechanism of fetus quality.

Differential Display Reverse Transcription Polymerase Chain Reaction (DDRT-PCR) is an mRNA fingerprinting technique to identify differentially expressed genes by comparative display of arbitrarily amplified cDNA subsets (Liang et al., 1992). This attractively simple screening method was, however, followed by a labour intensive multistep identification procedure for DD-PCR products.

As an important reproductive organ determining reproductive performance of female animal, ovary has outer excrete function of ovary periodically and inner excrete function of hormone verifies greatly based on the animal species. Thus, it is likely that during the follicles development some other unknown genes are associated in regulation of proliferation and differentiation of ollicle. To better understand the molecular mechanism of this process, the difference of mRNA in mature or small follicles was determined by DDRT-PCR technique in this study.

MATERIALS AND METHODS

Reagent

TRIzol Reagent (Gibco BR L, Life Technologies, USA), DEPC (Sigma, MO, USA), Expand TM Reverse Transcriptase (Roche, Basel,Switzerland), PAGE gel

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reclaimed kit (Minipo, France), DIG DNA Labeling and Detection Kit (Roche, Basel, Switzerland).

Primer

The cDNA primers of three anchored primers of H-T11A, H-T11G and H-T11C and eight arbitrary primers of H-AP17~H-AP24 were designed according to the third-generation primers of GenHunter Company. Anchored primers were :

H-T_{11}A: 5'-AAGCTTTTTTTTTTA-3'
H-T_{11}G: 5'-AAGCTTTTTTTTTTG-3'
H-T_{11}C: 5'-AAGCTTTTTTTTTTC-3'

Arbitrary primers were :

H-AP17: 5'-AAGCTTACCAGGT-3'
H-AP18: 5'-AAGCTTAGAGGCA-3'
H-AP19: 5'-AAGCTTATCGCTC-3'
H-AP20: 5'-AAGCTTGTTGTGC-3'
H-AP21: 5'-AAGCTTTCTCTGG-3'
H-AP22: 5'-AAGCTTTTGATCC-3'
H-AP23: 5'-AAGCTTGGCTATG-3'
H-AP24: 5'-AAGCTTCACTAGC-3'

Collection of ovarian in the follicular phase

Ovaries were collected from three healthy euthanized multiparous Small Tail Han ewe which provides by institute of Small Tail Han Sheep of Shandong Province, and three euthanized multiparous Tan ewe which come from Tan Sheep Farm of Ningxia Municipality. These ovaries from all the sheep were at the early stage of oestrus and stored at liquid nitrogen.

Preparation of RNA and reverse transcription

The ovaries samples were harvested and stored at liquid nitrogen for RNA isolation, ovary which stored in liquid nitrogen were cut out and ground into flour, then extracted by Trizol (GIBCO BRL) according to the manufacturer's instructions. The total RNA samples were purified byOligotex Kit (QIAGEN, Inc, USA) and quantitated by absorbance at 260 nm and evaluation on formaldehyde gels. For reverse transcription of an mRNA subset, 0.2 μg of RNA was mixed first with 50 pmol anchor primer in a volume of 7 μl, kept for 10 min at 65°C and immediately put on ice. Following addition of 300 U MLV-reverse transcriptase, it was incubated for 1 h at 37°C in reverse transcription buffer (40 mM KCl, 50 mM Tris–HCl pH 8.3, 6 mM $MgCl_2$) containing 20 mM dNTP, 10 mM DTT and 40 U RNA in a total volume of 20 μl. After 5 min at 95°C the cDNA was stored at -20°C until use in DD-PCR.

DD-PCR

DD-PCR was performed with 2 μl of 20 μl cDNA in a Eppendorf thermal cycler 480 using PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2.5 U ExTaq DNA Polymerase (Takara Bio Inc, Japan), 1.25 mM $MgCl_2$, 4 μM dNTP, 2.5 μM of the respective downstream anchor primer and 0.5 μM of one upstream DD-primer from the 8 arbitrary primers using the following conditions: 5 min incubation at 95°C followed by 40 cycles of 94°C for 30 s, 40°C for 2 min and 72°C for 30 s and incubated at 72°C for 10 min. The PCR products were resolved on a 10% polyacrylamide gel at 4°C with 150V for 10-14 h. The gel was fixed and stained by silver stain and then analyzed (Weaver, et al., 1994). The differentially displayed bands were cut out from a wet polyacrylamide gel. The DNA was eluted and purified with the QIAGEN Gel Extraction Kit (QIAGEN) according to the protocol of the supplier. With 6 μl of the 20 μl eluation the DD-PCR was repeated as before. The reamplification product was resolved on a 2% TBE agarose gel, stained with ethidium bromide, recovered with the QIAGEN Gel Extraction Kit and resuspended in 20 μl ddH_2O. This amplification was used for (i) direct sequencing and (ii) generation of a labeled probe.

Reverse northern

The reverse Northern test was performed using a transcription as probe labeled by ovary RNA extracted from Small Tail Han sheep or Tan sheep according to the kit manuscript. Briefly, the differential bands of Tan or Han sheep were resolved by 6×SSC and diluted to 50 μg/μl. Each sample was dotted in the same location on two nylon membranes .The genome of Tan and Han sheep were used as positive and 6×SSC solution as negative. After denaturalization, neutralization, desiccation and torrefaction, the nylon membranes were hybridized with probes labeled with the first cDNA strand of Tan or Han sheep at 68°C a night. On next day the membranes were washed and added into antibody and its substrate to inspect the results at room temperature.

Sequence and analysis of differential bands

The recovered differential bands were ligated with pGEM-T easy vector and transfermated to complement cells of DH5α. The positive clones were sequenced by Boya CTD and analyzed by BLAST in GenBank.

Some software of BLASTn and FASTA3.1 were operated on these sequences with GenBank and EMBL. If the score of BLASTn overcome than 100, the E value of ESTs was less than 1e-10 and the identity overran 90%, we contributed these sequences as one family (Table 1). To forecast the open reading frame (ORF), these sequences were screened by GenScan (http://genes.mit.edu/GENSCAN.html) to acquire the possible nucleotide and amino acid sequence. Those sequences which score outcome than 100 will annotated on its function.

Table 1. Differential expression genes database of DDRT-PCR between ovaries in Small Tail Han Sheep and Tan Sheep

Name	GenBank ID	bp	Copies	BLASTn homologous sequence	Function	Score	E value	Identities
O05[1]	C0001054	104	2		Unknown gene			
O08	C0001055	245	1		Unknown gene			
O10	C0001056	187	1	gi|21637524|gb|AC091133.11| Length=68613	Homo sapiens chromosome 17, clone RP11-501C14	168	2e-39	128/141 (90%)
O13		113	2	gi|25992264|gb|AF520959.1| Length = 482	Bos taurus ubiquitin-like/S30 ribosomal fusion protein	174	2e-41	91/92 (98%)
O14	C0001057	373	1		Unknown gene			
O21	C0001058	428	1	gi|13383951|gb|AF333248.1|AF333248 Length=8504	Bos taurus inducible nitric oxide synthase (NOS2) gene	71.9	9e-10	132/164 (80%)
O26[2]	C0001059	326	3	gi|11275670|gb|AF225897.1|AF225897 Length=7164	Bos taurus tensin mRNA	377	e-102	255/273 (93%)
O32	C0001060	108	1	gi|28403988|gb|AY227782.1| Length=97500	Bos taurus T cell receptor alpha (TCRA) gene	87.7	3e-15	71/80 (88%)
O34	C0001061	146	2	gi|26097379|dbj|AK077497.1| Length = 3014	Mus musculus 8 days embryo whole body cDNA, RIKEN full-length enriched library, clone: 5730422C08, product: p21 (CDKN1A)-activated kinase 1	87.7	5e-15	102/120 (85%)
O36	C0001062	194	1		Unknown gene			
O37[3]	C0001063	201	2		Unknown gene			
O39	C0001064	290	1		Unknown gene			
O42	C0001065	177	1		Unknown gene			
O43	C0001066	401	1	gi|37499470|gb|AY425004.1| Length=299637	Homo sapiens estrogen receptor 1 (ESR1) gene	77.8	1e-11	70/79 (88%)
O44	C0001067	178	1	gi|28189253|dbj|AB098782.1| Length = 700	Bos taurus mRNA for similar to tumor protein, translationally-controlled 1	278	3e-72	153/156 (98%)
O45	C0001068	182	1	gi|33504457|emb|Z86064.2|HS435D1 Length=184975	Human DNA sequence from clone RP3-435D1 on chromosome Xq25	95.6	3e-17	81/92 (88%)
O46	C0001069	246	1		Unknown gene			
O48	C0001070	118	1	gi|22655315|gb|AY141970.1| Length = 1804	Bos taurus beta-actin (ACTB) mRNA	155	2e-35	87/90 (96%)

[1] O05 sequence of EST same as O06. [2] O26 sequence of EST same as O28, O41. [3] O37 sequence of EST same as O38.

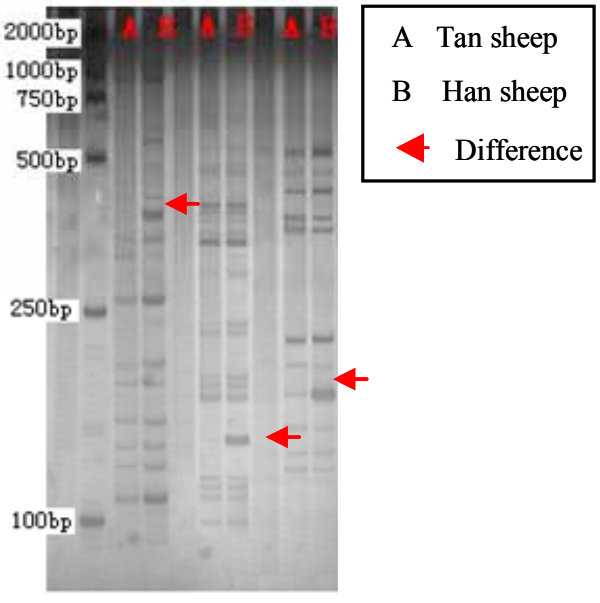

Figure 1. Polyacrylamide gel electrophoresis of DDRT-PCR (partial).

RESULTS

Extraction and reverse transcription of total RNA of sheep ovary

High quality RNA was found to be imperative for the success of the subsequent steps. To stabilize the similarity of Tan and Han sheep, we collected the ovary at the same follicles phages to extract RNA. The gel profile of RNA showed two clarity bands of 28S and 18S which were accord with the requirement of reverse transcription. These RNA were used as template to transcript for next step of DD-PCR.

Ovary DD-PCR of Tan and Han sheep

To optimize the differential display condition of Tan and Han sheep ovary, we performed 3 anchored primers and 8 arbitrary primers comprising of 24 pairs to react with two different kinds of RNA. Most of bands centralized at 100 bp to 1,000 bp. Experimental group of Han sheep as template got 632 bands while Tan sheep as template got 589 bands (Figure 1). At last 54 bands were choosen to recover and sequence according to the lack, density and size from both Han and Tan sheep ovary.

The recovered differential bands were reamplificated with the same anchor primer and arbitraryprimer using recovered products as template. However, some of bands (6/54) couldn't amplify the same size band as before. Other bands ranged from 200 bp to 1,000 bp.

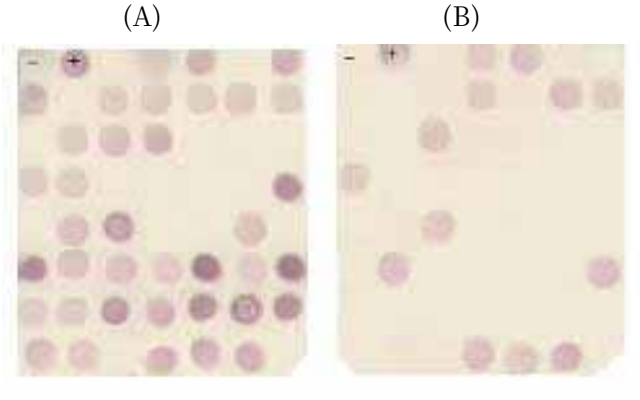

Figure 2. Detection of different samples with cDNA probe of the ovary. (A) Small Tail Han Sheep, (B) Tan sheep.

Reverse northern

In order to eliminate the disruption of error positive samples, reverse northern were carried out to validate differential bands. Fifty-four differential bands of PCR amplifications were blotted on two nylon membranes and hybridized with probes of Han ovary or Tan ovary. The results shown that a membrane had 37 positive blot hybridized with Han ovary probe while the other membrane had 13 positive blot hybridized with Tan ovary probe (Figure 2). Based on the origin of these differential bands which derived from Tan or Han sheep ovary, 24 bands were special expression genes in Small Tail Han sheep ovary while other bands were expressed both in Han and Tan sheep ovary.

Sequence and analysis of differential bands

The 23 positive bands of Small Tail Han sheep ovary were cloned and sequences. It was obvious that these sequences were ESTs ranged from 100 bp to 500 bp and the ratio of 100 bp to 200 bp bands was 54.2%. When found these positive bands of Han sheep ovary, we wanted to assess the background of these sequences. Table 1 demonstrated that 5 ESTs have several copies and 13 ESTs have only one copy among 24 different ESTs. Comparing these ESTs with homologous sequences by BLAST in the GenBank, there are 6 ESTs with known ORF and function, 3 ESTs with known ORF and un-function, and 9 ESTs without homologous sequence. And these ESTs are the part represent of several genes such as NOS2 (Bayir H, et al., 2005), tensin (Sanchez et al., 2005), TCRA (Ramiro, et al., 2001), CDKN1A (Rishi et al., 1997), ESR1 (Kato et al., 1994) and ACTB (Dahlen et al., 2004) which display specially in Small Tail Han sheep.

DISCUSSION

In this experiment, the primers were the third generation DDRT-PCR primers which both anchored and arbitrary primers have Hind enzyme site. The number of primers also reduces to three anchored primers and eight arbitrary primers, while the number of bases increases to16 and13. This primer recombination had proved by computer analysis to have the ability to include all mRNAs in certain stage of some cell development which make it easier to next operation and treatment.

However, it is obvious that the high sensitivity of DDRT-PCR will increase problems such as high background, low reproducibility and very high proportion of false positives. According to the published results and personal communications, it appeared to be a common problem. In avoid of these problems, all the RNA samples were treat ed with DNase I in our study and the RNA template of reverse transcription should outrun 3 μg. In order to reduce the disturbances of error positive bands, all the positives from Han sheep ovary were confirmed by reverse Northern which only 24 bands proved to be true. The reverse Northern assay also save a lot of outlay and time for its need of two cDNA probes compared to classical Northern blot assay.

The length of ESTs ranged from 100 bp to 500 bp lied at the 3' UTR which could distinguish the different genes in one gene family. More researches had proved that the length of 150 bp to 400 bp had available information of cDNA identification and chromosome location. Although our ESTs may not include intact ORF of some gene, it represent the gene expression profiles of some cells or organs in certain stages.

In this study, we have acquired 24 differential ESTs expressed in the ovary of the Small Tail Han sheep. Comparing these ESTs with homologous sequences by BLAST in the GenBank, there are six ESTs with known ORF and function, threeESTs with known ORF and un-function, and nine ESTs without homologous sequence. These known ESTs are the part represent of several genes such as NOS2, tensin, TCRA, CDKN1A, ESR1 and ACTB which display specially in Small Tail Han sheep. The relationships between these genes and sheep reproduction trait did not arouse up attention except ESR1 gene. Our studies further demonstrated that ESR gene may have a great effect on little size. Furthermore, the selected genes by differential display need to be studied and would promote the research of candidate genes which affected high ovulation of Small Tail Han sheep.

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