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Characterization of microRNAs from goat (*Capra hircus*) by Solexa deep-sequencing technology

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ABSTRACT. MicroRNAs (miRNAs) are an important class of small noncoding RNAs that are highly conserved in plants and animals. Many miRNAs are known to mediate a myriad of cell processes, including proliferation and differentiation, via the regulation of some transcription and signaling factors, which are closely related to muscle development and disease. In this study, small RNA cDNA libraries of Boer goats were constructed. In addition, we obtained the goat muscle miRNAs by using Solexa deep-sequencing technology and analyzed these miRNA characteristics by combining it with the bioinformatics technology. Based on Solexa sequencing and bioinformatics analysis, 562 species-conserved and 5 goat genome-specific miRNAs were identified, 322 of which exceeded 100 in the expression levels. The results of real-time quantitative polymerase chain reaction from 8 randomly selected miRNAs showed that the 8 miRNAs were expressed in goat muscle,

and the expression patterns were consistent with the Solexa sequencing results. The identification and characterization of miRNAs in goat muscle provide important information on the role of miRNA regulation in muscle growth and development. These data will help to facilitate studies on the regulatory roles played by miRNAs during goat growth and development.

Key words: Goat; Solexa deep-sequencing technology; MicroRNA characteristics; Real-time quantitative PCR

INTRODUCTION

MicroRNAs (miRNAs) are members of a class of small noncoding RNA molecules, which are critical post-transcriptional regulators of gene expression (Gunel et al., 2011). miRNAs negatively regulate gene expression through complementary base pair binding of the miRNA "seed sequence" (nucleotides 2 to 7) to the 3'-untranslated region of target mRNA, degrading or destabilizing the RNA message, or inhibiting protein translation, depending on the quantity of complementary base pair matches or the number of miRNA targeting sites within the 3'-untranslated region (Sandberg et al., 2008). However, studies have shown that miRNAs could increase protein translation by binding to the complementary promoter sequences (Vasudevan et al., 2007; Place et al., 2008). Numerous cellular and developmental processes in various organisms have been reported to be regulated by miRNAs, such as cell proliferation, differentiation, development, apoptosis, and disease (Chiromatzo et al., 2007; Kole et al., 2011), viral defense (Pedersen et al., 2007), hematopoietic stem cell development (Georgantas et al., 2007), environmental stress (Jones-Rhoades and Bartel, 2004), tumorigenesis (Sini et al., 2009), and the morphogenesis of specific organs (Zhao et al., 2007).

With the development of sequencing technology and bioinformatics, more and more miRNAs have been identified in animals (Ji et al., 2012). The number of miRNAs found and deposited in miRBase has risen almost exponentially. There are basically 2 kinds of approaches to identify these miRNAs. One is to sequence size-fractionated cDNA libraries. This method allows the identification of both conserved and unconserved miRNAs, but a limitation is that some miRNAs are expressed at low levels, and expressed at very specific stages or in rare cell types (Sheng et al., 2011). In contrast, the Solexa deep-sequence technology (also known as high-throughput technology) is a large massively parallel sequencing technology that utilizes sequencing by synthesis with a simply operated automatic platform. The technology has become a powerful tool for discovering miRNAs and in small RNA (sRNA) research, because of its small requirements for sample quantity and its high throughput, repeatability, and accuracy, which have led to a sharp rise in the rate of novel miRNA discoveries (Ji et al., 2012).

China has many goat breeds, including nearly 50 native goat breeds. The rearing number reached 152 million in 2010, which has been playing an important role in meat supply and agricultural improvement (Jin et al., 2012). However, there is limited information about goat miRNAs. miRNAs, which are a key component in post-transcriptional gene expression regulation in a variety of organisms, are involved in certain pathways in animal physiology and development. Several miRNAs were reported to regulate the process of skeletal muscle

development; for example, miR-1 and -133 were found to contribute to muscular hypertrophy. We further demonstrated that miR-1 and -133 play central regulatory roles in myoblast proliferation and differentiation (Chen et al., 2010). In the present study, several miRNAs were isolated from the goat skeletal muscle, which might be researched as good candidates for taking part in the process of skeletal muscle development. Expression analysis for several miRNAs indicated that some of them were expressed in a specific tissue.

MATERIAL AND METHODS

Animal and sample preparation

Three 6-month-old Boer goats were obtained from Hefei Bo Da Livestock Technology Development Co., Ltd., which were under the same rearing condition. The animals were allowed access to feed and water *ad libitum* under normal conditions and were sacrificed humanely to minimize suffering. After the slaughter, the longissimus muscle tissue was surgically removed and frozen immediately in liquid nitrogen for further use.

Total RNA isolation

The longissimus muscle tissue of Boer goat was collected and used to generate sRNA libraries. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer instructions. After the column purification, we used the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) to analyze the concentration. The quality of the total RNA was checked using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Palo Alto, CA, USA) and the samples were stored at -70°C until analysis.

sRNA library construction and sequencing

Total RNA was used for library preparation and sequencing by pooling equal quantities (30 μg) of total RNA isolated from 3 Boer goats. Briefly, total RNA (~90 μg) was separated on a 10% denaturing polyacrylamide gel, and sRNAs of 18 to 26 nt in length were recovered. Then, proprietary adapters were ligated to the 5' and 3' termini of the RNAs, and the samples were used as the templates for cDNA synthesis. The reverse transcription was performed, followed by PCR amplification. The PCR products were subsequently subjected to the proprietary Solexa sequencing-by-synthesis method using the Genome Analyzer (Agilent Technologies). Sequencing was carried out at the Beijing Genomics Institute, Shenzhen, China.

Real-time quantitative PCR (q-PCR)

Expressions of 8 randomly selected miRNAs identified by deep sequencing were validated by real-time q-PCR. The miRNA primers used are presented in Table 1. The q-PCR was performed using the High-Specificity miRNA qRT-PCR Detection Kit (Qiagen, Germany) on the Roche LightCycler 480 II Real-Time PCR Detection System (LC480 II; Roche, Basel, Switzerland). The ΔΔCt method was used to determine the relative expression levels of miRNAs in the goat muscles. Each sample from each individual animal was analyzed

in triplicate. Normalized factors of the internal control gene (U6 snRNA) and the relative quantities of the miRNAs were analyzed using the qBase software (Deo et al., 2011).

Table 1. Primer sequences of miRNAs for q-PCR.

Control gene and miRNA	TaqMan probe sequences (5'-3')	Expression of Solexa sequencing
U6 snRNA	CAAGGATGACACGCAAATTCG	-
miR-26a	TTCAAGTAATCCAGGATAGGCT	75,806
miR-206	TGGAATGTAAGGAAGTGTGTGG	614,734
miR-27a	TTCACAGTGGCTAAGTTCCGC	3,767
miR-383	AGATCAGAAGGTGATTGTGGCT	24
miR-320	AAAAGCTGGGTTGAGAGGGCGA	20,291
miR-187	TCGTGTCTTGTGTTGCAGCCGGA	11
miR-27b*	AGAGCTTAGCTGATTGGTGAAC	667
JR-m0005	TAAAGTGCTTATAGTGCAGGTAG	336

Data analysis

According to the principle of bioinformatics analysis, low-quality reads were removed from the raw reads. After trimming the 3' adaptor sequence, removing 5' adaptor contaminants, and counting the total, unique, and length of reads, all valid sequences were obtained and used for further analysis. Sequences in the libraries with identical or related sequences to goat or other mammals were identified as conserved miRNAs. The unannotated sequences were used to predict potential novel miRNA candidates and their target genes. The SOAP software was used to analyze sRNA expression and distribution on the genome. Gene ontology analysis was performed on the predicted target gene candidates of the novel miRNAs using 3 ontologies: molecular function, cellular components, and biological process (Ji et al., 2012). All of the predicted target genes were classified according to KEGG functional annotations, to identify the pathways actively regulated by miRNAs in goat (Kanehisa et al., 2008). The databases and software used in this article are shown in Table 2.

Table 2. Databases and softwares used in this article.

Name	Purpose	Address
miRBase 18.0 database	Known miRNA reference sequences	http://www.mirbase.org
Hircine EST database	Download EST sequences	http://www.ncbi.nlm.nih.gov/nucest
BLASTN software	Sequence alignment	http://blast.ncbi.nlm.nih.gov
Mireap software	Novel miRNA prediction	http://sourceforge.net/projects/mireap
SOAP software	Their expression and distribution on the genome	http://soap.genomics.org.cn
RNAhybrid software	Target gene prediction	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid
KEGG database	Functional pathway analysis	http://www.genome.jp/kegg

RESULTS

Extraction of goat RNA

The sRNA was extracted with the mirVana miRNA Isolation Kit (Ambion). After removal of the small fragments of RNA, the products were detected on 1% agarose gel electrophoresis. Compared with total RNA of ovarian tissue extracted with the Trizol method, the 5S strip could barely be seen. The 18S and 28S strips were clear, indicating that the RNA was

not degraded and the quality of the extracted RNA was good.

Data processing and length distribution

After gradually removing the low-quality reads and eliminating reads with 5'primer contaminants, reads without the 3'primer, reads without the insert tag, reads with poly(A), and reads shorter than 18 nt, the clean reads were obtained. Moreover, the sRNA species and the quantity were recorded, and then the length distribution statistics was done. In general, the interval of sRNA length is 18-30 nt, and the peak of length distribution can help us determine the sRNA type, such as miRNA focused on 21 or 22 nt, and siRNA concentrated in 24 nt (Figure 1).

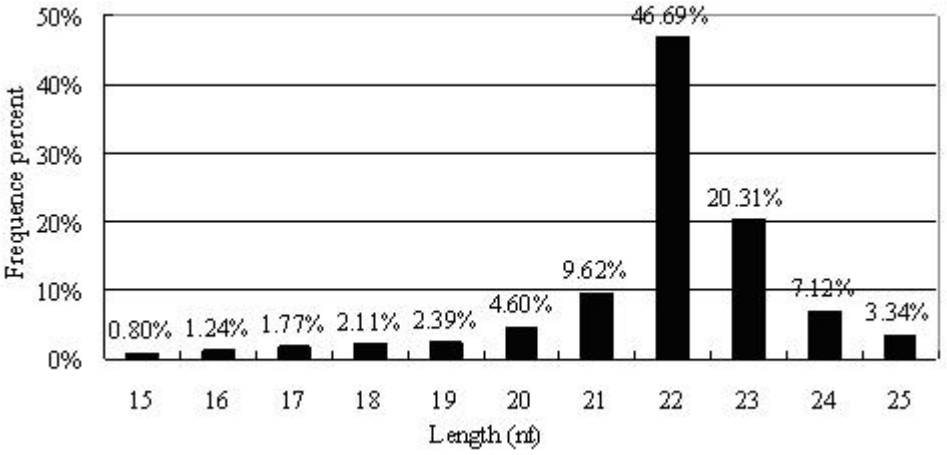

Figure 1. Length distribution of tags produced by small RNA sequencing.

Genome positioning of sRNA

The sRNA clean reads were mapped to the goat genome using the SOAP software to analyze expression and distribution of sRNAs on the genome. In this study, a total of 8,079,677 clean reads representing 402,585 unique sequences were obtained and used for further analysis. Among the sequencing data, there were 51,765 unique sRNAs matching to the genome (12.86%), and there were 659,596 total sRNAs mapping to the genome (8.16%) (Table 3).

Table 3. Mapping statistics of sample mapping to genome.

	Unique sRNAs	Percent	Total sRNAs	Percent
Total RNA	402,585	100.0%	8,079,677	100.0%
Mapping to genome	51,765	12.86%	659,596	8.16%

Expression profiling of miRNAs

For the goat miRNAs not recorded in miRBase 18.0, all unique sequences were used

to search the ncRNA data (GenBank, Repeat sequence, and Rfam) with BLASTN to remove non-miRNA sequences (rRNAs, tRNAs, snRNAs, snoRNA, etc.). Subsequently, the remaining sequences were analyzed using a BLAST search against miRBase 18.0. Sequences in the libraries with identical or related sequences (G-U base pairing and 1-2 nucleotide substitutions were permitted) to *Ovis aries* or other mammals (*Bos taurus*, *Sus scrofa*, *Canis familiaris*, and *Equus caballus*) were identified as conserved miRNAs.

There were 562 miRNAs species-conserved and 5 goat genome-specific miRNAs, 322 of which exceeded 100 in the expression levels. The expression levels of the top 20 are shown in Table 4.

Table 4. Expression level of the top 20 miRNAs in goat sample.

miRNA family	Expression level	Sequence
miR-133a	1,505,108	TTGGTCCCCTTCAACCAGCTGT
miR-133c	1,502,394	TTGGTCCCCTTCAACCAGCTG
miR-133b	1,426,436	TTTGGTCCCCTTCAACCAGCTGT
miR-1	1,155,248	TGGAATGTAAAGAAGTATGTAT
miR-378	969,945	ACTGGACTTGGAGTCAGAAGGC
miR-206	614,734	TGGAATGTAAGGAAGTGTGTGG

Classification and annotation of sRNA

To further evaluate the efficiency of Solexa deep-sequencing for miRNA detection, all of the sRNA sequences were annotated and classified using the tag2 annotation software. Because some sRNA tags may be assigned to more than one category, to assign each unique sRNA to only one annotation, we followed the following priority rules: rRNA, etc. (GenBank > Rfam3) > known miRNA > repeat > exon > intron (Ji et al., 2012). The results of taxonomic annotation showed that the total rRNA might be regarded as a standard: the total rRNA in better quality in animals should be less than 40% (Table 5).

Table 5. Distribution of small RNA among different categories.

Type	Unique sRNAs	Percent	Total sRNAs	Percent
Total	402,585	100.0%	8,079,677	100.0%
miRNA	40,533	10.07%	6,180,517	76.49%
rRNA	48,111	11.95%	762,586	9.44%
repeat	2,104	0.52%	2,581	0.03%
snRNA	2,709	0.67%	14,215	0.18%
snoRNA	3,082	0.77%	15,332	0.19%
tRNA	9,749	2.42%	74,474	0.92%
unann	296,297	73.60%	1,029,972	12.75%

Prediction and expression profiling of candidate miRNAs

For the characteristic hairpin structure of miRNA precursors that could be used to predict novel miRNAs, we developed the Mireap software (http://sourceforge.net/projects/mireap/) to predict novel miRNAs by exploring the secondary structure, the Dicer cleavage site, and the minimum folding-free energy of the unannotated sRNA tags that could be mapped to the goat genome. Although we are currently short of substantial goat genome sequencing

data, it is feasible to identify novel miRNAs by means of alignment with Hircine expressed sequence tags (ESTs). Therefore, we used the above software to predict the goat-specific miRNAs through extracting 50-70 nt of the ESTs (Table 6).

Table 6. Novel miRNA prediction in muscles of goat.

Sample	Expression level	Location	Sequence
JR-m0001	57	3'	AAAATCCGAACGAACTTTTTGG
JR-m0002	576	5'	CAAAGTGCTTACAGTGCAGGTAG
JR-m0003	28	5'	TAAGGTGCATCTAGTGCAGA
JR-m0004	575	3'	TGTGCAAATCTATGCAAAACTGA
JR-m0005	336	5'	TAAAGTGCTTATAGTGCAGGTAG

q-PCR validation of miRNAs identified in the goat muscle

The expression levels of 8 randomly selected miRNAs were determined in the goats using q-PCR. The results from Figure 2 showed that the 8 miRNAs were expressed in the goat muscle. The expression patterns were consistent with the Solexa sequencing results (Table 1).

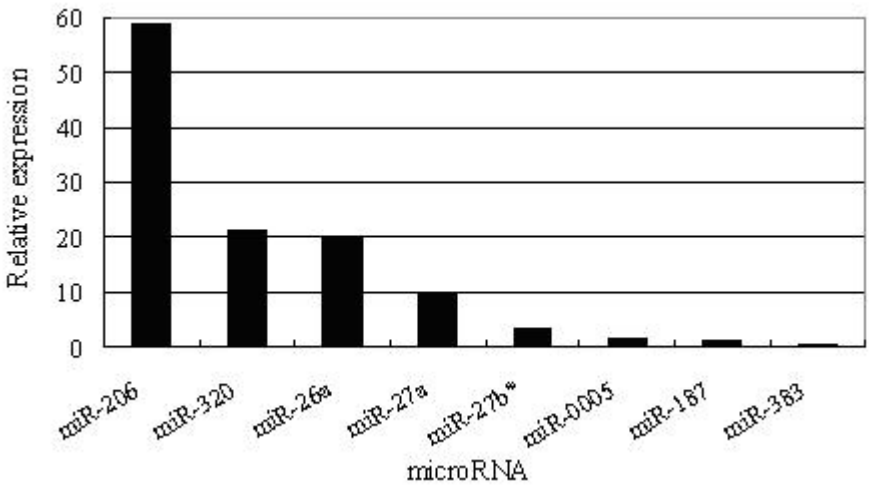

Figure 2. Relative expressions of 8 randomly selected miRNAs.

miRNA target prediction and pathway analysis

The RNAhybrid program was applied to predict the potential target sites of the identified goat miRNAs. Then, KEGG pathway analysis was also used for target gene candidates and revealed the main pathways in which the target gene candidates were involved (Allen et al., 2005; Schwab et al., 2005).

A total of 2,561,325 putative target sites for 562 miRNAs were identified in the goat muscle (data not shown). All of the predicted target genes were classified according to KEGG

functional annotations, to identify pathways actively regulated by miRNAs in the muscle of goats. Among them, a total of 18,344 target gene sites associated with different functional pathways were identified (data not shown).

For instance, 217 target gene sites were associated with the Wnt signaling pathway, which plays an important role in many processes of life, regulating early embryonic development, cell differentiation, and cell proliferation and growth. In addition, many highly expressed miRNAs were involved in the relevant signaling pathways of growth and development. For example, the miR-133 family (miR-133a, -133b, -133c), the most highly expressed miRNA in the muscle of goat, was predicted to bind the target sequences and repress expression of the serum response factor genes. Low expression of the serum response factor genes could promote myoblast proliferation. These miRNAs were in the top 10 of expression levels, including miR-133, -1, -378, -206, and -143, which play an important role in muscle growth and development.

DISCUSSION

miRNAs and muscle growth and development

Muscle growth and development result from the increase of muscle fibers and the expansion of cell volume, which will entirely depend on the muscle cell proliferation and differentiation. Some miRNAs are reported to be enriched in muscle tissues and play an important role in the regulation of myoblast proliferation and differentiation. In recent years, the genomics of relevant growth and development has gradually been elucidated from gene structure and function analyses, to determine the gene expression regulation mechanism. miRNAs perform critical roles in various biological and metabolic processes, by regulating gene expression at the post-transcriptional level.

Moreover, miRNAs are known to mediate a myriad of cell processes, including proliferation and differentiation, through the regulation of some transcription and signaling factors, which are closely related to muscle development and disease (Ge et al., 2011; Huang et al., 2012). The discovery of miRNAs points out a new path for determining the post-transcription regulatory mechanism of muscle cell proliferation and differentiation. Besides studying protein-coding genes, we also need to study the regulatory role of myogenic miRNAs, so that the molecular regulatory mechanism of skeletal muscle growth and development can be elucidated.

In this study, the goat miRNA expression profiles were systematically screened, and miRNAs were identified and characterized using Solexa deep-sequencing technology. The results indicated that sRNA libraries of the goat muscle tissue were mostly conserved miRNAs. Meanwhile, miRNAs deposited in the goat unigene database were subjected to the predicted target gene and functional analyses. We found that many highly expressed miRNAs were involved in the relevant signaling pathways of growth and development.

Database selection and analysis

Because the full goat genome has not yet been published, the complete goat miRNA information would also not be included in miRBase version 18.0 (Kozomara and Griffiths-

Jones, 2011). The goat miRNAs were analyzed using mammalian miRNAs closely related to goat and the Hircine ESTs.

In this study, 562 species-conserved miRNAs were found, but these miRNAs were from the homologous comparison and depended on the quantity and quality of data deposited in miRBase version 18.0. Theoretically, candidate miRNAs are needed for q-PCR validation (Mestdagh et al., 2008). The expression levels of 8 randomly selected miRNAs were validated by q-PCR. The 8 miRNAs were expressed in the goat muscle, and the expression patterns were consistent with the Solexa sequencing results. These results indicated the reliability of the data.

In addition, the goat EST information was used to predict the novel miRNAs, but only 5 goat-specific miRNAs were predicted (Wei et al., 2009). Fu et al. (2011) agreed with the viewpoint that this result was caused by the scarcity of goat EST information in GenBank. If the goat genome sequence information were being constantly improved, we could take advantage of the genomic information to research the goat miRNAs, which makes the obtained results much richer and perfect.

Expression profiling of muscle miRNAs

To date, many miRNAs have been reported for their important roles in development processes in various animals (*B. taurus*, *S. scrofa*, and *O. aries*), but there is limited information about goat miRNAs. Sheng et al. (2011) used the cDNA library and bioinformatics methods to identify sheep miRNAs and analyzed their expression in different tissues to discover novel miRNAs. In addition, the network bioinformatics software was used to predict new sheep miRNA target genes and analyze their function. Studies indicated that a large number of miRNAs were generally coexpressed with their host genes (Drummond et al., 2011), and expression of miRNAs had tissue specificity (Zhou et al., 2010). McDaneld et al. (2009) isolated miRNAs from pig skeletal muscle tissues and found that miR-206 had a high expression in skeletal muscle. Many miRNA expression profiles have been reported in sheep, cow, chicken, and other domestic animals (Huang et al., 2010; Li et al., 2010; Wang et al., 2012), but there is limited information about goat muscle miRNAs.

In animals, miRNAs negatively regulate gene expression post-transcriptionally by translational repression and target mRNA degradation (Wienholds and Plasterk, 2005). Many miRNAs have been shown to play crucial roles in muscle development and in regulation of muscle cell proliferation and differentiation. In our study, the highly expressed miRNAs of the goat muscle may inhibit some target gene expression related to muscle cell proliferation and differentiation, thereby inhibiting the goat growth and development. In the present research, among the 10 most highly expressed miRNAs were miR-133a, -133c, -133b, -1, -378, -20, and -143, which are common miRNAs in pigs, mice, and other animal skeletal muscle. The highest expression level of miR-1 was in the pig fattening period, and the expression level in the other periods was moderate; miR-133 in the fattening period showed moderate expression, whereas expression in the embryonic period and in newborn piglets was low.

To evaluate the roles of miRNAs in porcine skeletal muscle, miRNA expression profiles were investigated using longissimus muscle tissue from pigs at embryonic day 90 (E90) and postpartum day 120 (PD 120) (Zhou et al., 2010). During later fetal development at E90, miR-206 and let-7 were highly expressed, whereas miR-1a, -133a, -26a, and -1826 showed the highest abundance during the fast growing stage at PD 120. Studies have shown that miR-143

can promote normal adipose cell differentiation and fat deposition (Wang et al., 2011; Yi et al., 2011). In addition, miR-143 may be involved in the mammalian gonad endocrine and in maintenance of pregnancy (Huang et al., 2012). This means that miR-143 may play an important role in mammalian development and reproduction, showing the diversity and complexity of miRNA functions, which will bring us a new challenge in the further study of miRNAs.

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