Analysis and Identification of miRNA Expression in the Skeletal Muscle of Sichuan White Rabbits

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Abstract [**Objectives**] miRNAs play an important role in the proliferation and differentiation of different myoblasts. This study was conducted to elucidate the complex genetic mechanisms that affect the meat production performance of Sichuan white rabbits and reveal the regulatory role of miRNAs in their muscle growth and meat quality formation. [**Methods**] Three constructed skeletal muscle libraries of Sichuan white rabbits aged six months were sequenced by the solexa technology to identify known miRNAs, predict new miRNAs and construct an expression profile of muscle miRNAs. [**Results**] A total of 511 known miRNAs and 42 miRNAs were detected in 34 089 472 pure sequences, and the proportion of miRNAs with a length of 22 nt was the highest. The number of known miRNA sequences accounted for 71.38% of pure sequences, which was much higher than the proportion of other types of RNAs. The proportion of sequences from exons was 0.38%, indicating a low degree of mRNA degradation in the samples. Base U had the highest proportion at the first position, and the bases with the highest proportions at positions 8 and 10 were U and A, respectively. Muscle-specific miRNAs (miR-1, miR-133, and miR-206) ranked in the top 10 in terms of expression level. The number and expression levels of new miRNAs were lower than those of known miRNAs. The length distribution, base bias at different positions and expression profile characteristics of miRNAs might be related to the biological function of miRNAs in regulating muscle proliferation and differentiation and the action mechanisms with target genes. [**Conclusions**] The identification and expression of miRNAs in muscle tissues of Sichuan white rabbits will help to understand the complex molecular mechanisms of meat production performance and provide a theoretical basis for the functional research of miRNAs in meat rabbits.

Key words Meat rabbit; Muscle; High-throughput sequencing; Nucleotide bias

miRNAs are a class of small non-coding molecules with a length of approximately 22 bp, widely present in animal tissues and organs. miRNAs inhibit the expression of target genes through RNA-induced gene silencing complexes (RISCs), which are formed by the interaction of Argonaute protein, siRNA, and Dicer enzyme[1]. One miRNA can regulate multiple target genes, and one target gene can also be regulated by multiple miRNAs. Through the establishment of complex miRNA target gene interaction networks, miRNAs participate in important biological processes such as cell proliferation, differentiation, apoptosis, and signal transduction[2]. Studies in mice and humans have shown that miRNAs are almost involved in all processes of muscle cell proliferation and differentiation, regulating skeletal muscle development by targeting key factors at various stages. For livestock and poultry, miRNAs have been reported to regulate the proliferation and differentiation of the skeletal muscle. MiRNA-1 and miRNA-206 can promote the differentiation of skeletal muscle cells in Hereford and Limousin cattle, while there are differences in expression between breeds[4]. Clop *et al.*[5] found that a G of the mRNA3'-UTR encoding myostatin changed to A by combining the research methods of quantitative genetics and molecular genetics, and this mutation produced a target site of miR-1 and miR-206, which caused the muscle overdevelopment of Texel sheep. MiRNA-199b regulates the proliferation of Landrace muscle satellite cells by inhibiting the target gene JAG1[6]. In addition, overexpression of fibroblast growth factor 4 can reduce the expression abundance of miRNA-206, leading to changes in somites during chicken embryonic development[7]. It mainly involves bioinformatics prediction of miRNAs and differential expression of miRNAs in the retina. Compared with other livestock and poultry, the research on rabbit miRNAs is still in its infancy at home and abroad[8].

Sichuan white rabbits are an excellent local breed in China, which has the characteristics of roughage resistance, early maturity, high fecundity, good meat quality and strong disease resistance. Clarifying the complex genetic mechanisms that affect the meat production performance of Sichuan white rabbits and revealing the regulatory role of miRNAs in their muscle growth and meat quality formation have extremely important practical significance for the conservation and development of Sichuan white rabbits. Identification and expression profiling of miRNAs in muscle tissues of Sichuan white rabbits are beneficial for understanding the regulatory mechanisms of miRNAs during muscle development. Therefore,

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in this study, sRNAs in muscle tissues of Sichuan white rabbits were sequenced by the solexa technology to identify known miRNAs and predict new miRNAs and construct an expression profile of muscle miRNAs, hoping to provide a theoretical basis for the functional research of miRNAs in meat rabbits.

Materials and Methods

Experimental animals

Three female Sichuan white rabbits with the same birth date were selected as the experimental subjects and raised in the same environment at National Sichuan White Rabbit Genetic Resources Conservation Farm. The white rabbits were raised until 6 months old for slaughter, after which the longissimus dorsi was immediately collected and stored in liquid nitrogen for total RNA extraction.

Construction of library and sequencing

The total RNA was extracted according to the operation steps of TRIZOL kit, and agarose gel electrophoresis (1%) was carried out at 180 V to simply identify the quality and integrity of RNA. Next, 3 μg of total RNA was taken from each library to build a library that met quality requirements. According to the operation guidelines of Illumina TruSeqTMRNA Sample Preparation Kit (Illumina, San Diego, USA), different index labels were selected to build the library and test the quality of the library. The library samples were amplified using TruSeq PE Cluster Kit v3-c Bot-HS (Illumina) reagent, and cloned clusters were generated on the c Bot through isothermal amplification. Finally, sequencing was performed on the Hiseq 2000 sequencing platform, resulting in 50 bp single-read reads.

Processing and analysis of sequencing data

The original data contained a large amount of adapter contamination, polyA/T/G/C, and low-quality fragments. After filtering these fragments, clean reads were obtained, and the clean reads within the target range were subjected to length statistics. The sequenced sRNAs were located onto the rabbit genome using Bowtie software, including its intron and exon locations. Other types of RNAs (tRNA, rRNA, snRNA, snoRNA) were identified and removed by comparing with GenBank and Rfam databases. The repetitive sequences of sRNAs were analyzed by RepeatMask software. Finally, we identified known miRNAs and their expression levels by comparing with mature miRNAs and precursor sequences in the miRase database based on the conservatism of miRNA sequences, and we also predicted new miRNAs using MIREAP and simultaneously calculated the distribution of bases at different positions.

Results and Analysis

Quality and length screening of sequencing data

By removing low-quality reads and 5' and 3' adapter fragments from the original sequencing data, 34 089 472 pure sequences were obtained, accounting for 95.76% of the original data. The sRNA lengths were distributed from 18 to 35 nt, mainly in the range of 20 – 23 nt, of which 22 nt accounted for 67.2%, followed by 23 nt (18.54%), 21 nt (5.77%) and 20 nt (4.14%).

Classification annotation of Samle RNA

The classification annotation results of various RNAs (Table 1) indicated that the number of known miRNAs was the highest, accounting for 71.38%. The proportion of other types of RNAs was only 0.19%. The proportion of rRNAs was 0.07%, which was much lower than 0.5%, indicating that the constructed sRNA sequencing library had relatively high quality. The proportion of repetitive sequences was 0.71%. The proportion of sequences from exons was 0.38%, indicating a low degree of mRNA degradation in the samples.

Table 1 Annotation of small RNAs among different categories

Category	Quantity	Proportion//%
Pure sequence	34 089 472	100.00
Known miRNA	24 334 405	71.38
Repetitive sequence	240 360	0.71
Exon sequence	130 779	0.38
Intron sequence	137 131	0.40
tRNA	3 942	0.01
rRNA	23 790	0.07
snRNA	2 268	0.01
snoRNA	35 630	0.10

Distribution of bases at different positions

The distribution of bases at different positions is shown in Fig. 2. It can be seen that the distribution of the first base had a strong bias, with base U being the main base, followed by base A, and the proportions of bases G and C being the lowest. The 8^{th} position was rich in base U, and the 10^{th} position was rich in base A. The base distribution at other positions was basically consistent.

Expression analysis of known and novel miRNAs

A total of 511 known miRNAs were detected in the skeletal muscle of Sichuan white rabbits, and 42 novel miRNAs were identified. As shown in Table 2, the expression levels of ocu miR-1a-3p, ocu miR-1b-5p and ocu miR-133a-3p were among the top 10 known miRNAs. Among them, the expression level of ocu miR-1a-3p miRNA was higher, while the expression level of ocu miR-148a-3p miRNA was the lowest, with a copy number of over 50 000. Newly discovered miRNAs had a low expression level, and the highest expression level was found in novel_4 with a copy number of 1 614.

Table 2 Expression of known and novel miRNAs (top 10)

Name	Number of copies	Name	Number of copies
ocu-miR-1a-3p	6 358 990	novel_4	1 614
ocu-miR-1b-5p	6 348 435	novel_236	209
ocu-miR-133a-3p	322 051	novel_270	42
ocu-miR-206-3p	286 006	novel_176	40
ocu-miR-26a-5p	156 585	novel_91	29
ocu-miR-378a-3p	141 259	novel_170	14
ocu-miR-27b-3p	97 396	novel_118	14
ocu-let-7f-5p	71 539	novel_106	13
ocu-miR-101a-3p	66 011	novel_210	10
ocu-miR-148a-3p	58 920	novel_108	8

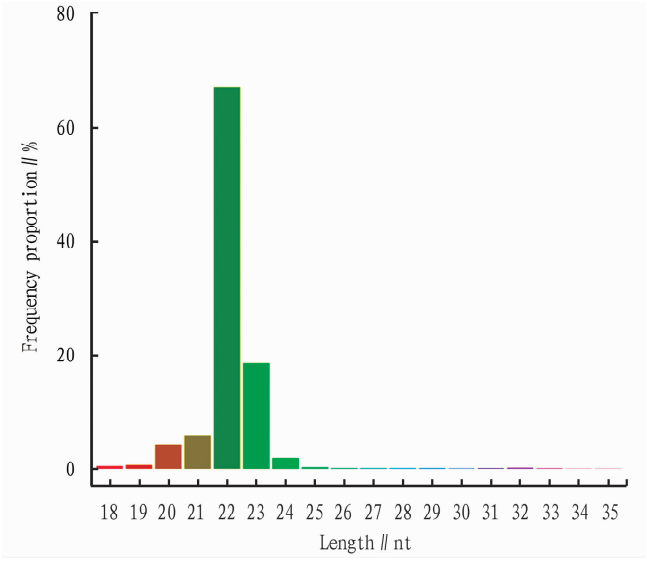

Fig. 1 Length distribution of sRNAs in muscles

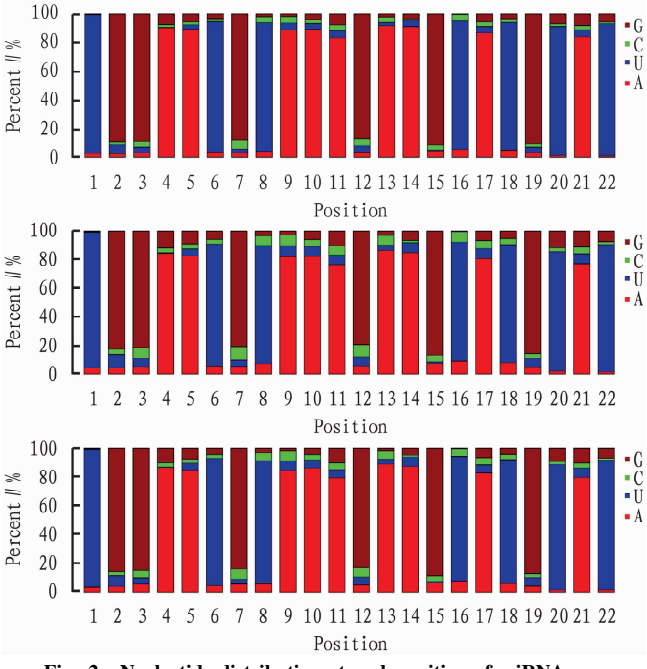

Fig. 2 Nucleotide distribution at each position of miRNAs

Discussion and Conclusions

The solexa sequencing technology belongs to the second-generation sequencing technologies, which have the characteristics of high throughput, large amount of information, good repeatability, and high sensitivity. It can not only avoid the problem of being limited by the whole genome information of a species, but also detect transcripts with low expression abundance, making it an important tool for comprehensive and accurate analysis on the transcriptome of a species[9]. At present, this technology has successfully identified miRNAs in different tissues of domestic animals such as pigs[10], cows[11], sheep[12-13], horses[14], and donkeys[15]. In this study, the SOLEXA sequencing technology was applied to sequence three constructed skeletal muscle libraries of Sichuan white rabbits, generating a total of 34 089 472 pure sequences and detecting a total of 511 known miRNAs and 42 miRNAs. The lengths of detected small RNAs were mainly distributed in the range of 20 – 23 nt, with 22 nt being the most concentrated, which is consistent with the length characteristics of miRNAs, indicating that the sequencing data had a high quality and the results were accurate and reliable. The miRNAs with the lowest expression abundance were detected as only one sequence (ocu miR-377-5p, ocu miR-542-5p, ocu miR-551b-3p, *etc.*). These miRNAs will provide a theoretical basis for further functional research.

The results of this study showed that the identified miRNAs had a U-base bias at the first position, which is consistent with the results detected in the skeletal muscle of New Zealand white rabbits and fat and ovarian tissues of sheep[16-17]. Argonaute protein is more prone to binding to base U, which is beneficial for RNA-induced silencing complexes to specifically cleave mRNA targets[18]. The 8th and 10th bases had U and A biases, respectively, which is consistent with the identification results of miRNAs in the skeletal muscle of Qinchuan cattle[19]. Generally speaking, the " seed region" where miRNA binds to target genes is located at bases 2 – 8 and highly conserved, with the 10th base being the cleavage site[20]. Bases A and U are beneficial for the stability and shear of the complex. The distribution of bases at different positions of miRNAs detected in the three samples was basically consistent, indicating that the probability of base occurrence at different positions of the same species had certain regularity. It might be related to the conservation of known miRNAs, and the number and expression level of conserved miRNAs were much higher than those of novel miRNAs.

High-throughput sequencing has been widely used to identify miRNA expression profiles in livestock. The expression levels of miR-133a, miR-378, miR-1 and miR-206 in the longissimus dorsi of Anhui white goats aged 6 months, miR-1-3P, miR-378-3P, miR-133a-3P and miR-148a-3P in the longissimus dorsi and psoas major muscle of 210-day-old Landrace pigs, miR-1, miR-26, miR-206-3P, miR-133 and miR-27 in the gluteus maximus of yaks of different ages, and miR-1, miR-378, miR-133a and miR-26 in the longissimus dorsi of 24-month-old Wutou and Sanfen donkeys were all in the top 10, which is consistent to the results of this study[21-24]. It can be seen that miR-1, miR-133, miR-206 and miR-26 are highly expressed in muscle tissues of different varieties, ages, and parts. These highly-expressed miRNAs play an important regulatory role in muscle growth and development. miR-1, miR-133 and miR-206 are only expressed in muscles and belong to muscle-specific miRNAs. miR-1/206 inhibits the proliferation of mouse skeletal muscle satellite cells and promotes myogenic differentiation by interacting with Pax7 target genes[25-26]. miR-1, miR-133 and miR-206 regulate myoblast differentiation and proliferation by participating in the Wnt signaling pathway, P38/MAPK signaling pathway, JNK/MAPK signaling pathway, and p53 signaling pathway. miR-1/206 mainly regulates the differentiation of myoblasts, while miR-133 plays a role in myoblast proliferation[27]. Therefore, highly-expressed miRNAs may play an important role in the development of skeletal muscle in Sichuan

white rabbits. Among the predicted new miRNAs, only the expression level of one miRNA reached over 1 000. Compared with known miRNAs, the expression levels of new miRNAs were significantly lower, which is consistent with that of insect cephalochordates and vertebrates. Perhaps known miRNAs play a more important regulatory role in the body, making them conserved and exhibiting high expression[28].

In this study, the solexa sequencing technology was applied to sequence the longissimus dorsi of three 6-month-old Sichuan white rabbits. Five hundred and eleven known miRNAs and 42 new miRNAs were identified, with a length of mainly 22 nt and multiple positions exhibiting base bias. Muscle-specific miRNAs such as miR-1, miR-133 and miR-206 were highly expressed in the skeletal muscle, and the number and expression level of novel miRNAs were much lower than those of known miRNAs. These highly-expressed miRNAs playing an important regulatory role in the muscle differentiation and proliferation of other livestock and poultry may also be important factors affecting the proliferation and differentiation of muscle cells in meat rabbits. The next step is to conduct research on the function and mechanism of candidate miRNAs in important muscles, so as to provide new ideas for the breeding and molecular breeding for the meat production performance of rabbits.

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