

Profiling of microRNA from skeletal muscle of Bandur sheep using RNA sequencing

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Received: 6 November 2019; Accepted: 2 December 2019

ABSTRACT

MicroRNA profiling is a powerful approach for identifying key regulators of molecular functions which control skeletal muscle development, regeneration and function. Information on gene expression and the regulatory factors involved in myogenesis is very limited for Indian sheep. This study reports the identification and characterization of miRNAs from the skeletal muscles of Bandur sheep breed for the first time. Bandur is a consumer favoured, mutton type sheep of India, mainly distributed in Mandya district of Karnataka. Skeletal muscles from four animals of Bandur sheep of similar age, sex and reared under same management conditions were used for RNA sequencing. The total number of reads (15–36 bp) for each library of Bandur sheep ranged from 19,350,000 to 30,000,000. Highly expressed transcripts with an RPKM value of ≥ 1000 were observed to be 34%, whereas 38% transcripts exhibited RPKM between 100–1000 and 28% had RPKM < 100 in Bandur sheep. A total of 110 known mature miRNAs could be identified on comparison with known human and bovine sequences. All the identified miRNAs represented 32 miRNA families and 44 clusters. A total of 499 novel miRNAs were discovered in Bandur sheep. The miRNAs identified in our study were enriched for functions namely cell proliferation, cell differentiation, osteogenesis, lipid metabolism, muscle development, adipocyte differentiation and stress response. Potential gene targets for the identified miRNAs were predicted. Most relevant target genes predicted in our study included *MYO5A*, *SIN3B* and *NR2F2* which are mainly involved in myogenesis. This study provides information of miRNAs in the skeletal muscle tissue of Bandur sheep.

Keywords: Bandur, Indian sheep, miRNA, Muscle, RNA sequencing

MicroRNAs (miRNAs) are a type of endogenous, small ~22 nucleotides in length non-coding RNAs that regulate the expression of protein coding genes at post transcriptional level or enhance messenger RNA degradation (Hamilton and Baulcombe 1999). A single miRNA may regulate several mRNAs, thereby effecting gene expression networks. Therefore, study of expression patterns of few miRNAs may provide considerable biological information (Pritchard *et al.* 2012). Previous studies have led to the discovery of miRNAs that regulate specific muscle functions like myoblast proliferation, differentiation, contractility and stress responsiveness (Lee *et al.* 2003, Clop *et al.* 2006). The skeletal muscles of livestock eventually form meat, which is an economically important product. miRNAs are being hailed as a new class of molecules that control the post transcriptional gene expression. They play a significant role in myogenesis by controlling the process of myoblast proliferation and differentiation (Bartel 2004, Williams *et al.* 2009). As a consequence, they can affect

muscle mass, muscle fibre type and muscle-related diseases. Accumulated evidence indicates that miRNAs are important in the regulation of skeletal muscle development (Novák *et al.* 2013). miRNAs that are highly enriched in muscles either cardiac and/or skeletal muscles, are called MyomiRs. The knowledge of specific miRNAs in skeletal muscles has expanded our insight into regulation of muscle development. An improved understanding of the molecular mechanisms of muscle development in ovines may facilitate efficient muscle/meat productivity.

Sheep comprise one of the most important domestic livestock species worldwide that provide livelihood to a large proportion of small and marginal farmers. These animals are a good source of mutton and possess the ability to adapt to different climatic and grazing conditions. The contribution of sheep to the total meat production in India is 7.94% (BAHS 2018). It is exported mainly to Middle East countries like UAE, Kuwait, Oman, Iran etc. The demand for mutton and goat meat has been projected to increase to 8.36% by 2020 (Dastagiri 2004). Sheep breeds like Muzaffarnagri, Bandur (Mandya/Bannur), Deccani, Madgyal, Nellore etc. are major contributors of mutton production in the country.

The mutton quality is largely dependent on the muscle

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growth and development. Since miRNA profiling is a powerful approach for identifying key regulators of molecular functions, it will help to understand the molecular factors which control skeletal muscle development, regeneration and function. Several miRNAs have been identified as key regulators of skeletal muscle development as well as hypertrophy (Clop *et al.* 2006, Kim *et al.* 2006). In view of the fact that microRNAs can affect muscle growth and metabolism, they present great potential as molecular markers (Güller and Russell 2010). Skeletal muscle development is a complex process that requires coordination of multiple factors which govern the proliferation of myoblasts, their exit from cell cycle and subsequent differentiation into multinucleated myotubes (Buckingham 2006). The size and number of muscle fibers affect muscle mass and meat quality (Hou *et al.* 2016). The key priority is to identify the mechanisms which influence the processes regulating skeletal muscle function. The skeletal muscles of sheep are economically valuable, therefore, it is pertinent to have knowledge of the molecular drivers involved in muscle development. Although several SNPs have been identified in candidate genes for mutton quality in Indian sheep (Arora *et al.* 2014) and the skeletal muscle expression of mRNA of Indian sheep have been analysed (Arora *et al.* 2019), the miRNA profile is not yet available. Therefore, the main aim of this study was to identify and characterize miRNAs from skeletal muscle of Bandur sheep breed to get an insight into the regulatory factors relevant in myogenesis.

MATERIALS AND METHODS

Samples: Animals were selected with prior consultation from butchers. Four rams of Bandur sheep were identified and selected for sampling. All the selected animals were in the two-tooth stage (12–19 months). The animals were slaughtered according to standard commercial ‘halal’ procedures.

RNA sequencing: Total RNA was extracted from the skeletal muscle tissue of Bandur sheep using TRIzol reagent as per the manufacturer’s instructions and treated with DNase 1 (Qiagen) to remove genomic DNA contamination. The concentration and quality of RNA was assessed by calculating the RIN using Bioanalyzer and gel electrophoresis. The miRNA was isolated from the extracted total RNA using miRNeasy Mini Kit (Qiagen). Illumina TruSeq small RNA Sample Prep kit was used to generate sequencing libraries as per manufacturer’s protocol. The libraries were normalized to a concentration of 2 nM using Tris-HCl 10 mM, pH 8.5. All the samples were purified, indexed, diluted and sequenced on Illumina HiSeq 2000 platform.

Data analysis: FastQC (v0.11.5) software was used to check the quality of the miRNAseq raw reads obtained followed by the removal of the adapter (Illumina TruSeq Small RNA 3’ Adapter- AGATCGGAAGAGCACACG-TCT) using FastX-ToolKit (v0.0.13). The results obtained were visualized using the R language (v3.4.1). Target

prediction of expressed miRNAs against Human and bovine database was done by using TargetScan- 7.2 (Agarwal *et al.* 2015), an online web portal. Novel miRNAs identification was done by using miRDeep-Star software (v37) (An *et al.* 2012). Minimum free energy for novel miRNAs was calculated by using RNA fold tool (v2.4.3) from Vienna RNA package (v2.0) (Lorenz *et al.* 2011). The target genes were used for Gene Ontology predictions using DAVID (Huang *et al.* 2008a, 2009) and Consensus Pathway Database (Kamburov *et al.* 2009, 2010).

RESULTS AND DISCUSSION

Summary of RNA Seq data: The total number of reads (15–36 bp) for each library of Bandur sheep ranged from 19,350,000 to 30,000,000 (Table 1). The raw sequence data has been submitted to the NCBI Sequence Read Archive with Accessions SRR6346737- SRR6346740. Expression levels were evaluated by counting the number of RPKM (Reads per Kilobase of transcript per Million mapped reads). The reads were mapped to Human as well as Bovine reference assemblies. Highly expressed transcripts with an RPKM value of ≥ 1000 were observed to be 34%, whereas 38% transcripts exhibited RPKM between 100–1000 and

Table 1. MicroRNA data statistics of skeletal muscles of Bandur sheep

Sample name	Read length	Percentage GC	Number of raw reads	Number of clean reads
B1	15–36	46	30,000,000	29,993,152
B2	15–36	46	19,350,000	19,344,412
B3	15–36	46	20,750,000	20,742,504
B4	15–36	46	19,503,030	19,496,891

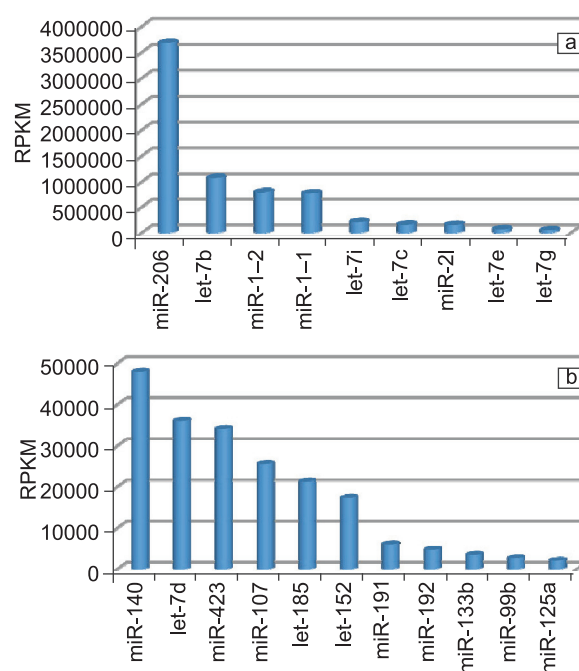


Fig. 1. Top 20 expressed miRNAs identified in Bandur sheep skeletal muscle with (a) RPKM > 50000 and (b) RPKM < 50000.

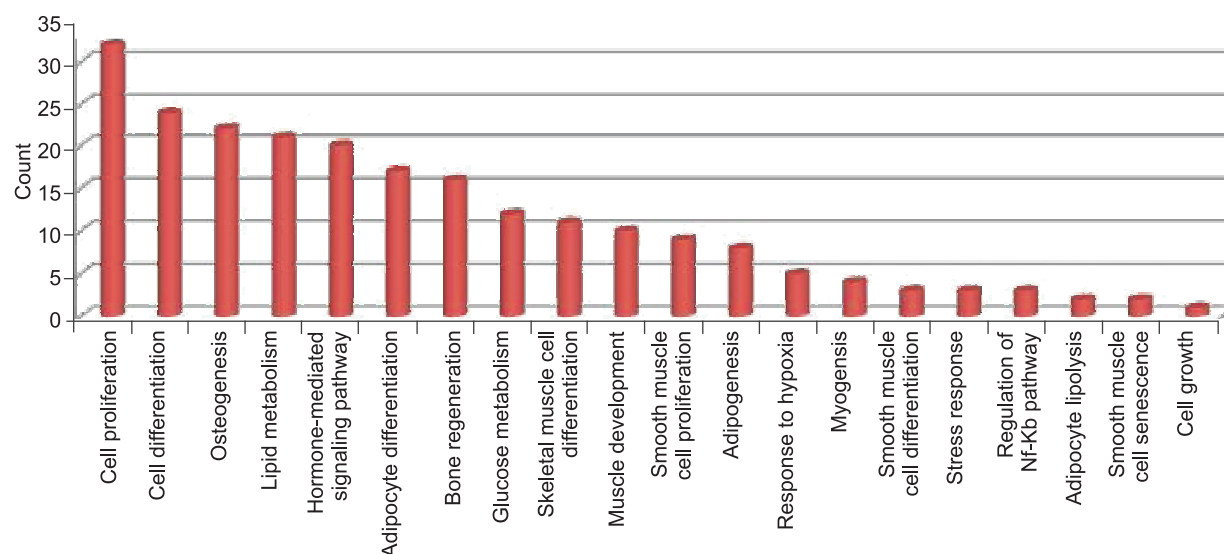


Fig. 2. Important functional terms identified in miRNAs from Bandur sheep skeletal muscles.

28% had RPKM <100 in Bandur sheep.

Identification of miRNAs: A total of 110 known mature miRNAs could be identified on comparison with known human and bovine sequences. The top 20 most abundant miRNAs are shown in Fig. 1. miRNAs with RPKM >50,000 were miR-206, let-7b, miR-1-2, miR-1-1, let-7i, let-7c, miR-21, let-7e and let-7 g, while miR-140, let-7d, miR-423, miR-107, miR-185, miR-152, miR-191, miR-192, miR-133b, miR-99b and miR-125a showed expression of <50,000 RPKM. All the identified miRNAs represented 32 miRNA families and 44 clusters. A total of 499 novel miRNAs were identified in Bandur sheep.

MyomiRs or muscle specific miRNAs, influence multiple facets of muscle development and function through their regulation of genes controlling myogenesis (Chen *et al.* 2006, Rao *et al.* 2006, Güller and Russell 2010). The muscle-specific miRNAs, miR-206, miR-1 and miR-133, are among the best characterized miRNAs in skeletal muscle differentiation. The MyomiRs affect several processes such as myogenesis, cell differentiation and regeneration, as well as muscle fibre specification. The high expression of the MyomiRs in our study also confirms previous reports. Many studies have confirmed the role of miR-133b, miR-133a-1, miR-133a-2 and miR-206 in skeletal muscle differentiation, as they were the most significantly up-regulated miRNAs (Chen *et al.* 2006, Huang *et al.* 2008b, Güller and Russell 2010, Dey *et al.* 2011, Sheng *et al.* 2011). miR-1, miR-133b and miR-206 have been reported to be highly enriched in skeletal muscle tissue (Güller and Russell 2010). These three miRNAs form the same cluster and are transcribed simultaneously (Liu and Olson 2010). miR-133a is known to increase myoblast proliferation, via its repression of serum response factor (SRF), while miR-1 stimulates myoblast differentiation by inhibition of histone deacetylase 4 (Chen *et al.* 2006). In addition, a mutation in the 3' untranslated region (3'-UTR) of the myostatin gene in the Texel sheep creating a target site for the miR-206 and miR-1 leads to inhibition of myostatin expression,

which is likely to cause the muscular hypertrophy phenotype of this breed of sheep (Clop *et al.* 2006).

Some other important miRNAs like miR-21, miR-29, miR-221, and miR-222, were also expressed in Bandur sheep. MicroRNA-21 has been reported to regulate the vascular smooth muscle cell function (Wang *et al.* 2011). Previous studies have reported that miR-29 is a critical regulator in skeletal muscle development (Wang *et al.* 2014), while the role of miR-221 and miR-222 has been indicated in differentiation and maturation of skeletal muscle cells (Cardinali *et al.* 2009). Many non-muscle-specific miRNAs are also required for the differentiation of muscle through interaction with myogenic factors. Changes in the regulation of some miRNAs may alter the intracellular signalling networks, causing disease conditions (Eisenberg *et al.* 2009).

The miRNAs identified in our study were enriched for functions namely cell proliferation, cell differentiation, osteogenesis, lipid metabolism, muscle development, adipocyte differentiation, stress response etc. (Fig. 2). Previous study on mRNA of Bandur sheep also reported several upregulated genes associated with muscle development, lipid metabolism and response to stress (Arora *et al.* 2019). Our results indicate that the enriched molecular functions in Bandur skeletal muscle may affect the muscle characteristics.

Target gene prediction: Genes with an aggregate probability of conserved targeting (PCT) >0.9 were selected as targets of the identified miRNAs using TargetScan 7.2 (Agarwal *et al.* 2015). A total of 154 target genes could be predicted. The predicted target genes were analyzed for biological process, cellular component and molecular functions. The functional analysis of the predicted genes revealed CRD mediated mRNA stabilization, skeletal muscle tissue development, regulation of GTPase activity as significant biological processes ($P < 0.05$) (Fig. 3). Actomyosin, cytoplasmic stress granule, transcriptional repressor activity and metal ion binding were some of the

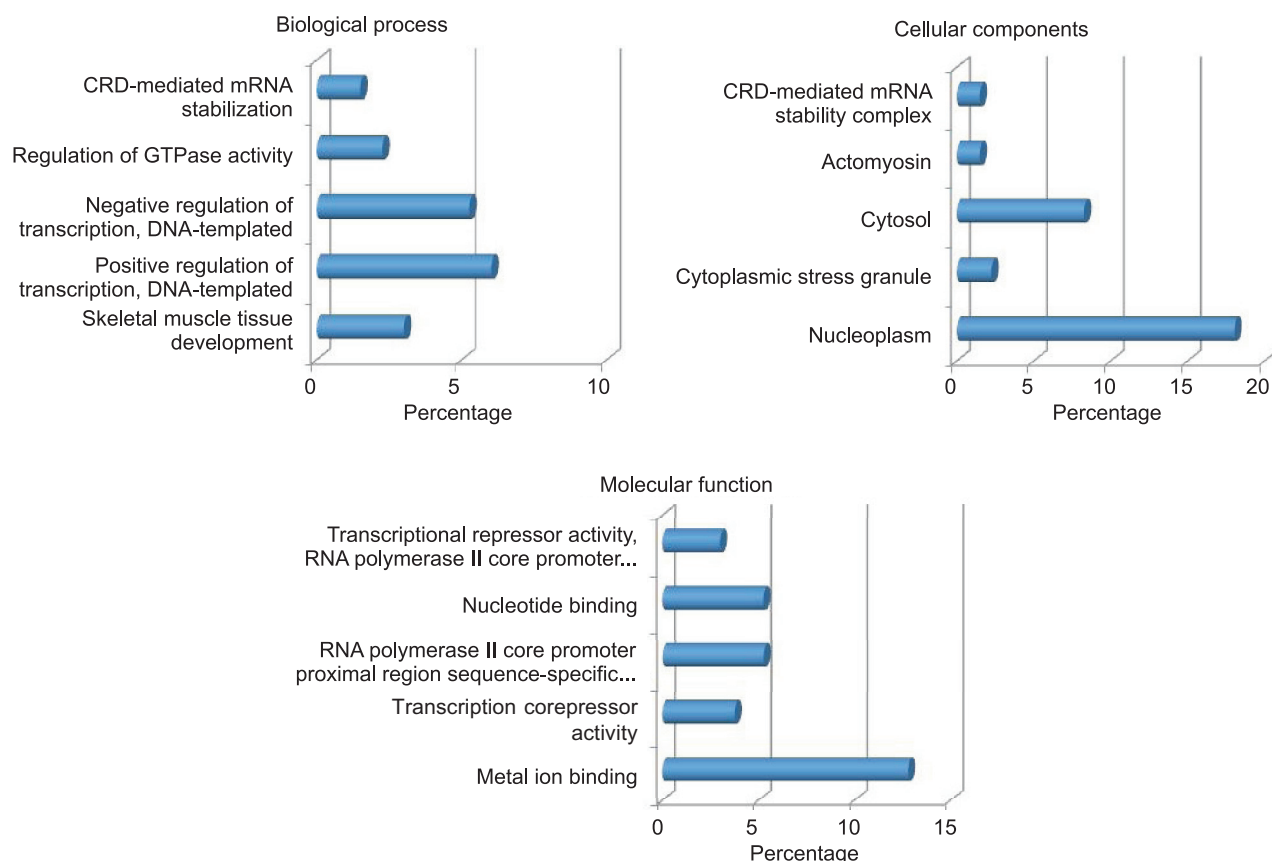


Fig. 3. Gene ontology terms for different categories and pathways for target genes in Bandur sheep.

significant cellular and molecular functions of the predicted target genes. Predicted target genes most relevant in muscle biology included *MYO5A*, *SIN3B* and *NR2F2*. *MYO5A* belongs to the myosin gene superfamily and is involved in cytoplasmic vesicle transport and anchorage. *NR2F2* represses myogenesis (Lee *et al.* 2017) while *SIN3B* is associated with maintenance of differentiated muscle cells (Van *et al.* 2010).

Since many miRNAs are species and tissue specific, they may be essential for certain biological processes. Previous studies on livestock RNA profiling have established that miRNAs regulate muscle development, immune response as well as metabolism (Liu *et al.* 2010). Hundreds of miRNAs have been found to be encoded in most eukaryotic genomes, and it is likely that many miRNAs are yet to be discovered. The role of microRNA causing muscular hypertrophy in Texel sheep has been demonstrated (Clop *et al.* 2006). SNPs in miR206 in pigs have been reported to be associated with muscle fibre composition, meat quality, and lean meat production (Lee *et al.* 2013). The porcine miR-208b SNP differentially inhibits the expression of *SOX-6*, which in turn affects the expression of *MYH7* and the characteristics of muscle fibre and meat quality (Kim *et al.* 2015). MicroRNAs play an important role in muscle proliferation and differentiation by regulating a number of transcription factors and signaling molecules involved in myogenesis. The profiling of miRNA expression will allow an in depth understanding of the role of miRNAs in various

physiological processes. Our study provides information on miRNAs in the skeletal muscle tissue of Indian sheep. It is a stepping stone in understanding the role of miRNAs in molecular pathways relevant to muscle development. Further studies on these miRNA are required to elucidate their precise regulatory mechanism.

ACKNOWLEDGEMENTS

This work was financially supported by the Indian Council of Agricultural Research (ICAR, New Delhi). The authors are grateful to Director, ICAR- National Bureau of Animal Genetic Resources (ICAR-NBAGR, Karnal) for providing necessary facilities. We acknowledge the help rendered by the sheep rearers and butchers for their help in collection of samples. We thank Mr. Rakesh Kumar, Technical Officer for laboratory assistance.

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