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Differential expression profiles of miRNA in the serum of sarcopenic rats

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ABSTRACT

As the geriatric population and life expectancy increase, the interest in preventing geriatric diseases, such as sarcopenia, is increasing. However, the causes of sarcopenia are unclear, and current diagnostic methods for sarcopenia are unreliable. We hypothesized that the changes in the expression of certain miRNAs may be associated with the pathophysiology of sarcopenia. Herein, we analyzed the miRNA expression profiles in the blood of young (3-months-old) healthy rats, old sarcopenic (17-months-old) rats, and age-matched (17-months-old) control rats. The changes in miRNA expression levels were analyzed using Bowtie 2 software. A total of 523 miRNAs were detected in the rat serum. Using scatter plots and clustering heatmap data, we found 130 miRNAs that were differentially expressed in sarcopenic rats (>2-fold change) compared to the expression in young healthy and age-matched control rats. With a threshold of >5-fold change, we identified 14 upregulated miRNAs, including rno-miR-133b-3p, rno-miR-133a-3p, rno-miR-133c, rno-miR-208a-3p, and rno-miR434-5p among others in the serum of sarcopenic rats. A protein network map based on these 14 miRNAs identified the genes involved in skeletal muscle differentiation, among which *Notch1*, *Egr2*, and *Myocd* represented major nodes. The data obtained in this study are potentially useful for the early diagnosis of sarcopenia and for the identification of novel therapeutic targets for the treatment and/or prevention of sarcopenia.

1. Introduction

As the geriatric population and life expectancy increase, the interest in preventing and treating geriatric diseases is increasing. Aging is associated with various diseases, including metabolic disorders such as diabetes, obesity, and high blood pressure [1]. Maintaining muscle strength and development is important for physical fitness and exercise. However, the loss of muscle mass (sarcopenia) due to aging makes it difficult for the elderly to exercise. Several muscle-related disorders affect the geriatric population. These include diabetic neuropathy, muscular dystrophy, and sarcopenia. Among these, sarcopenia, which was first identified in 1989, is well-known owing to its prevalence in the geriatric population.

An increasing number of people are at risk of developing sarcopenia based on increased life expectancy, and 200 million people are estimated to develop sarcopenia by 2050 [2]. The current index for the diagnosis of sarcopenia is based on grip strength or walking speed [3,4]. However, these parameters vary with the physical condition of individuals, making them unreliable for accurate diagnosis.

MicroRNAs (miRNAs) are gene products that can block mRNA translation. Since each miRNA regulates the expression of hundreds of target mRNAs, miRNAs function as master mediators, efficiently regulating basic cellular processes, including proliferation, apoptosis, and development. In addition, miRNAs may represent useful diagnostic and therapeutic targets in various diseases [5]. Increased levels of certain miRNAs can inhibit protein synthesis and contribute to cardiovascular

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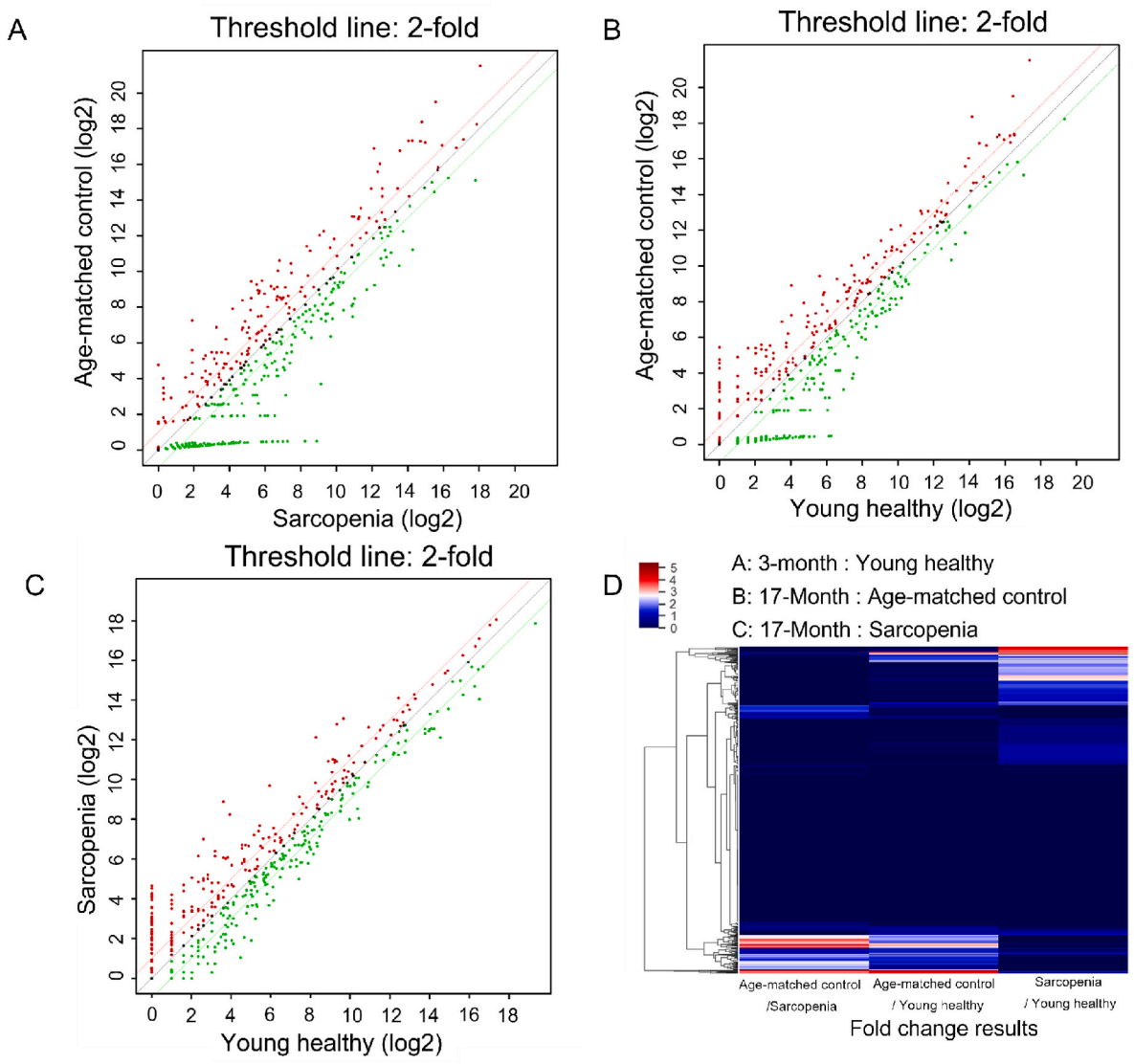


Fig. 1. Scatter plot and heatmap data of miRNA expression. Scatter plot data of miRNA expression for the young healthy, old sarcopenia, and the age-matched old control rat groups. (A) Comparison between sarcopenia and the age-matched control groups. (B) Comparison between the young healthy and aged-matched control groups. (C) Comparison between the young healthy and sarcopenia groups. Both the X and Y axes indicate gene expression levels, and genes that show altered expression by more than 2-fold (above the red or below the green line) around the center line (black line) can be identified. (D) Heatmap data of miRNA from each group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

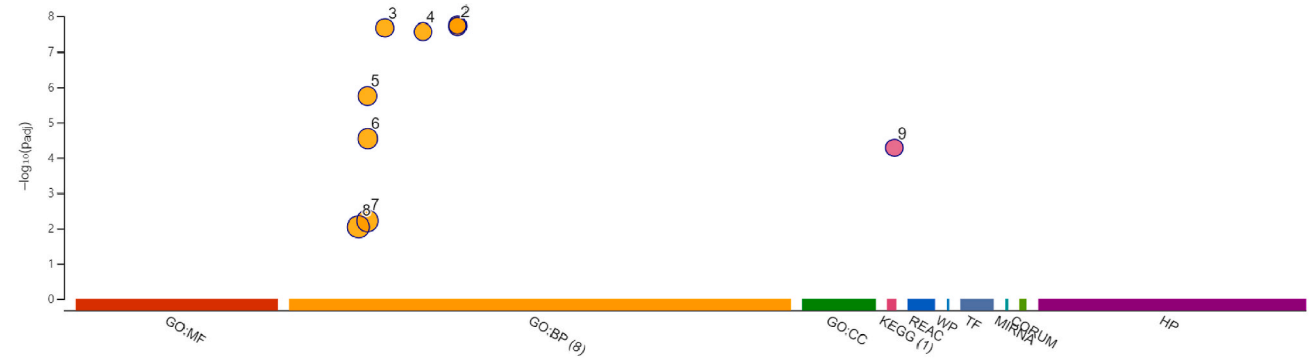
disease and muscle pathophysiology [6,7]. Furthermore, circulating miRNAs such as Myo-miRNA (c-miR-486) and c-miR-146a have been suggested to function as critical biomarkers of age-related sarcopenia [8]. Based on the findings, we compared the miRNA expression profiles in blood samples derived from muscle-reduced (sarcopenia) old rats, age-matched old rats (control), and young healthy control rats. We hypothesized that changes in levels of certain miRNAs may be related to the pathophysiology of sarcopenia.

Here, we evaluated the entire miRNA profiles (523 miRNAs) and identified 14 miRNAs whose expression was increased more than 5-fold in the sarcopenia group, which was supported with a network map of related proteins. We believe that this information can be used not only for the early diagnosis of sarcopenia, but also for the identification of novel therapeutic targets for the treatment and/or prevention of sarcopenia.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (SAMTACO BIO KOREA, Osan, Korea) were used in the experiments. All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of animals, and the Institutional Animal Care and Use Committee of Konkuk University. Rats were euthanized via exposure to an increasing concentration of carbon dioxide or exsanguinated by cutting the carotid arteries under deep ketamine-xylazine anesthesia. Experiments were performed using 3- and 17-month-old Sprague-Dawley rats: 3-month-old young healthy controls (n = 2); 17-month-old age-matched controls (n = 2); 17-month-old muscle-reduced sarcopenia group (n = 2). The 17-month-old rats weighed less than the 3-month-old rats (330 ± 10 g vs. 600 ± 20 g). The tibialis anterior muscle mass is reportedly decreased in patients with sarcopenia and is correlated the most with the development of sarcopenia [6,7]. Based on these reports, old rats in which the tibialis anterior muscle/body weight ratio belonged to the



ID	Source	Term ID	Term Name	Padj (query_1)
1	GO:BP	GO:0035195	gene silencing by miRNA	1.753×10^{-8}
2	GO:BP	GO:0035194	post-transcriptional gene silencing by RNA	1.945×10^{-8}
3	GO:BP	GO:0016441	posttranscriptional gene silencing	2.117×10^{-8}
4	GO:BP	GO:0031047	gene silencing by RNA	2.713×10^{-8}
5	GO:BP	GO:0010608	posttranscriptional regulation of gene expression	1.801×10^{-6}
6	GO:BP	GO:0010629	negative regulation of gene expression	2.882×10^{-5}
7	GO:BP	GO:0010605	negative regulation of macromolecule metabolic p...	6.092×10^{-3}
8	GO:BP	GO:0009892	negative regulation of metabolic process	9.069×10^{-3}
9	KEGG	KEGG:05206	MicroRNAs in cancer	5.198×10^{-5}

Fig. 2. GO enrichment analysis. g:Profiler enrichment results with the 14 target miRNAs. Where the x-axis shows the feature terms grouped by color code from the source database used and the y-axis shows the enriched-adjusted p-values on a negative decimal logarithmic scale. The dots on the graph represent all enriched terms that meet the significance criterion of $p < 0.01$, term size between 268 and 10,000. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

lower 50% were considered sarcopenic, and the rats in which the ratio belonged to the upper 50% were considered as the age-matched control group. The grip strength of the age-matched control group was 273.5 ± 7.5 g and that of the sarcopenia group was 241.5 ± 5.5 g. The body weight of the age-matched control rats was 615 ± 20 g and that of the sarcopenic rats was 585 ± 5 g. The tibialis anterior weight/body weight ratio was 1.67 ± 0.36 g/kg and 1.48 ± 0.15 g/kg in the age-matched control group and sarcopenia group, respectively. Blood samples were obtained from the rats. The serum was separated from the blood samples and stored at −80 °C immediately after separation.

2.2. RNA isolation

miRNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted miRNA was characterized using an RNA 6000 Pico Kit and reagents (Agilent Technologies, Santa Clara, CA, USA). miRNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Preparation and sequencing of the library

Extracted miRNAs were tested and used to construct a library using the NEBNext Multiplex Small RNA Library Prep Kit (New England BioLabs, Ipswich, MA, USA). We used 1 μg of total RNA from each sample to build the library. RNA was modified using an adapter, and adapter-specific primers were used to synthesize cDNA using reverse transcriptase. PCR was performed for amplification, and library cleanup was performed using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and AMPure XP beads (Beckman Coulter, Brea, CA, USA). The Agilent 2100 Bioanalyzer (Agilent Technologies) was used to perform high-sensitivity DNA analysis and confirm the yield and size distribution of the miRNA library. High-throughput sequences were generated using the NextSeq500 system using 75 single-ended sequences.

2.4. Relation of miRNA to predicted target gene and co-regulatory networks between TFs and miRNAs

We investigated mRNA associated with sarcopenia. First, by using the target miRNA, the related mRNA was sorted through Targetscan (http://www.targetscan.org/vert_71/) (Supplementary Data 1). The high-confidence miRNA-mRNA interactions were used to construct the miRNA-mRNA network using Cytoscape software (version 3.9.1; http://www.cytoscape.org) (Supplementary Fig. 1). Enrichment analysis of the selected miRNA was performed using the differentially expressed genes (DEGs) g:Profiler (http://biit.cs.ut.ee/gprofiler/) (Fig. 2). miRNA pathway enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) mapper (Fig. 3). Finally, the relationship between Transcription Factor (TF) and miRNA was investigated. It was confirmed through the NCBI search and the database of Bioinformatics and Systems Biology (http://www.cuilab.cn/transmir). We obtained the TF information of 6 genes out of a total of 14 genes. For the remaining 8 miRNAs, related TFs were investigated by searching NCBI papers and the network mapping between the TFs and miRNA was drawn (Supplementary Fig. 2).

2.5. Protein gene network mapping

Proteins associated with the miRNAs were identified using TargetScanVert. Target proteins related to skeletal muscle differentiation were determined using quickgo (https://www.ebi.ac.uk/QuickGO/). Network mapping was performed using STRING (https://string-db.org/). We performed k-means clustering at the STRING website and classified it into three clusters (Fig. 4).

2.6. Data analysis

The sequences generated were mapped using Bowtie 2 software (v2.3.4.3/USA/Ben Lanmead et al., university of Maryland). The number of reads mapped to the miRNA array was extracted from the alignment file using bedtools (v2.26.0/USA/Quinlan laboratory at the

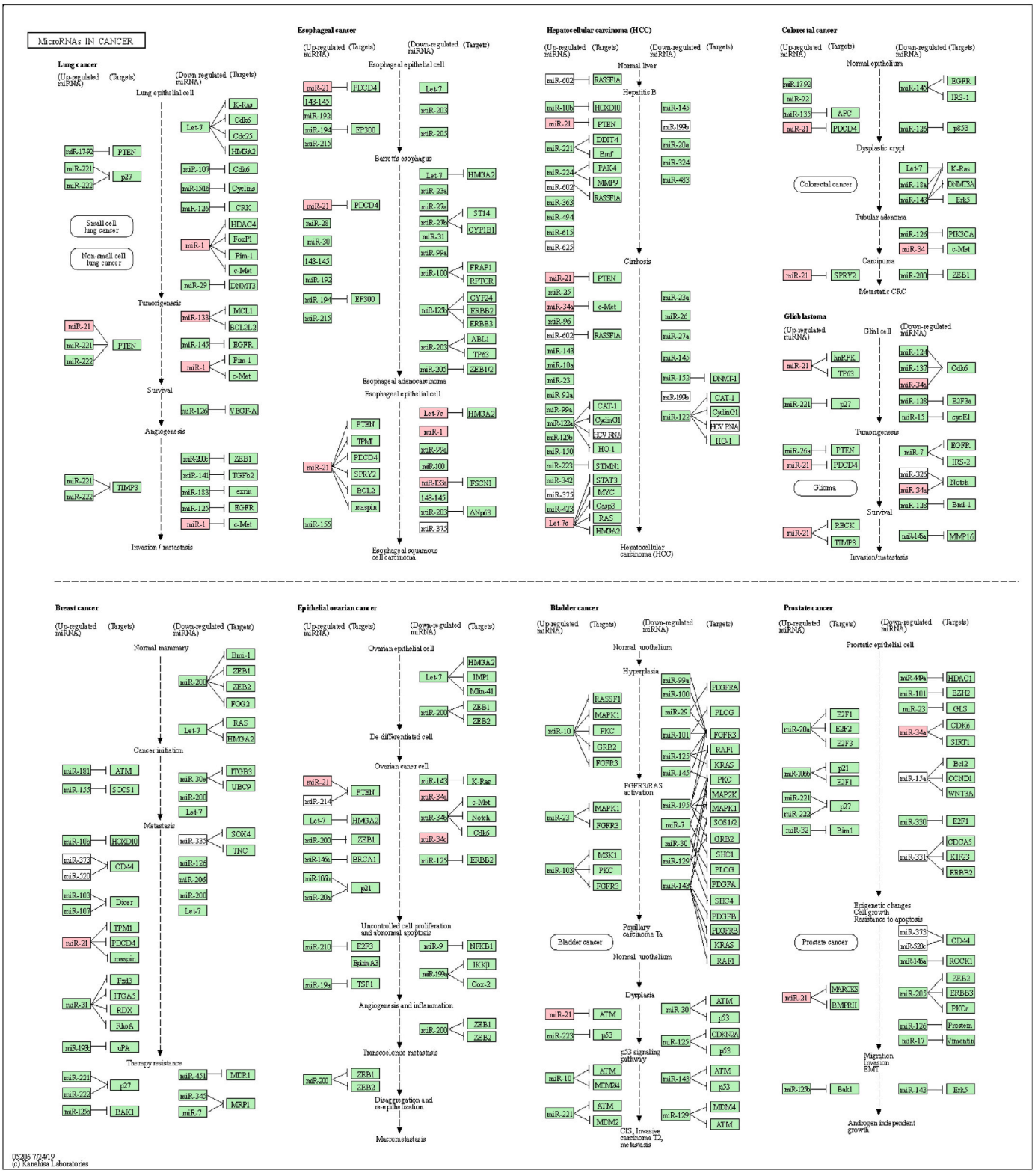


Fig. 3. KEGG mapping for the target miRNAs. The target miRNA signaling pathways are shown in various cancer. Target miRNAs associated with sarcopenia are marked in red on the map. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

university of Utah) and Bioconductor (R Development Core Team, 2011), using R statistical programming language (version 3.2.2). miRWalk2.0 (v2.0/Germany/university of Heidelberg) was used in miRNA analyses.

3. Results

3.1. Scatter plot and cluster heatmap data

The expression levels of miRNAs were determined in serum samples, and 523 genes were profiled (Supplementary Table 1). Correlation

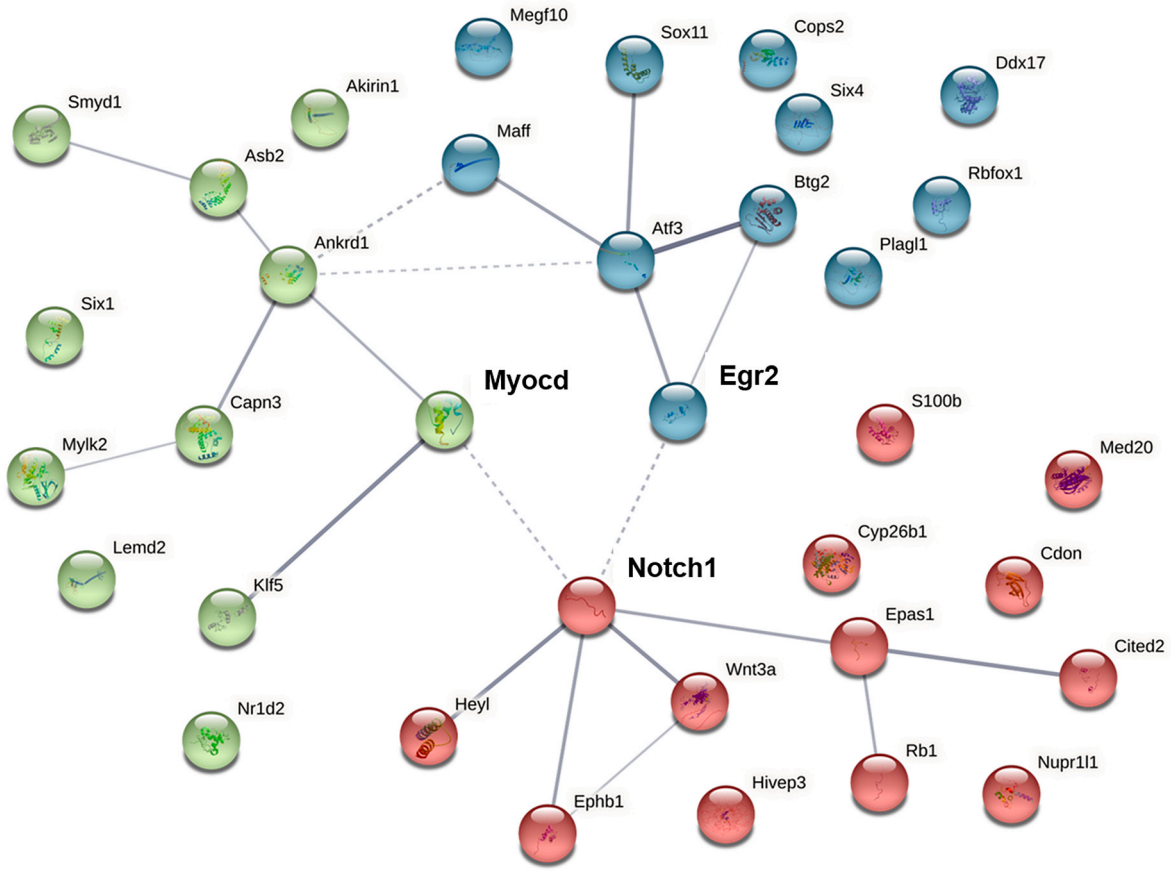


Fig. 4. Protein network mapping. A network map was generated from 14 miRNA genes that have been differentially expressed (>5-fold, Table 1) in the sarcopenic rat serum compared to the expression in young and age-matched controls, targeting the genes involved in the differentiation of skeletal muscle cells. *Notch1*, *Egr2*, and *Myocd* are in the central nodes.

(variance) plots for gene expression levels in young (age, 3 months) healthy, age-matched (age, 17 months) control, and sarcopenia (age, 17 months) groups are shown in Fig. 1A–C. The differences between the age-matched control and sarcopenia groups are shown in Fig. 1A. The expression levels of certain miRNAs were considerably increased in the sarcopenia group. The correlation of miRNA gene expression levels between the age-matched control and young healthy group and between sarcopenia and young healthy group is shown in Fig. 1B and C, respectively. The expression level of certain miRNAs was considerably increased in the sarcopenia group (Fig. 1C) compared to that in the young healthy group. A heatmap was generated to determine the differences in gene expression among the three groups. We found that 130 miRNAs were differentially expressed (>2-fold change) in the sarcopenia group compared to the expression in the young healthy and age-matched control groups (Fig. 1D and Supplementary Table 1).

3.2. Candidate target miRNAs associated with pathogenesis of sarcopenia

Among the 523 miRNAs identified, a comparative analysis of sarcopenic, age-matched control, and young healthy rats revealed 130 differentially expressed miRNAs (>2-fold change) (Supplementary Table 1). Since the number of miRNAs was relatively high, we raised the threshold for differential expression (>5-fold change) to identify miRNAs whose expression was considerably altered in sarcopenia. Consequently, 14 miRNAs were identified whose expression was increased in sarcopenia compared to that in the age-matched control and young healthy groups (Table 1). Notably, no miRNAs were found to be downregulated in sarcopenia within the 5-fold change threshold.

3.3. miRNA on gene network

We investigated the mRNA genes involved in 14 miRNAs (Supplementary data 1). There are 16,374 mRNAs related to 14 miRNAs, and references registered in targetscan website were searched. As a result, 8 miRNAs and 954 mRNAs were found to be related. From this, we drew a miRNA-mRNA network map using genetic information (Supplementary Fig. 1.). Next, we performed Gene Ontology enrichment analysis with the 14 miRNAs to find related cell signaling through the target miRNA. 8 biological process (GO.BP) and 1 KEGG signal were found to be related to the 14 miRNAs (Fig. 2). In KEGG, the miRNAs were found to be linked to cancer (Fig. 2). Therefore, we further analyzed the KEGG pathway with the 14 miRNAs. Fig. 3 shows the associated cancer signaling pathways, in which the red colors are the miRNAs we targeted (Fig. 3). Next, we searched for the transcription factor associated with the 14 target miRNAs. TFs play a biologically important roles together with miRNAs. miRNAs and TFs regulate gene expression together or play a role in regulating the expression of each other. 17 related TFs were found, and network mapping was drawn from them (Supplementary Fig. 2).

3.4. Protein network mapping

We compared the genes encoding the 14 miRNAs and identified genes involved in the differentiation of skeletal muscle cells. We found a correlation between a network of 35 proteins, in which *Notch1*, *Egr2*, and *Myocd* represented major nodes (Fig. 4).

Table 1
List of differentially expressed (>5 fold) miRNAs in the sarcopenic rat serum compared to the expression in young healthy and age-matched controls.

Gene symbol	Fold change		
	Age-matched control/Sarcopenia	Age-matched control/Young healthy	Sarcopenia/Young healthy
rno-miR-133b-3p	0.003	0.115	38.715
rno-miR-133a-3p	0.005	0.092	19.751
rno-miR-133c	0.011	0.228	20.827
rno-miR-208a-3p	0.057	1.31	22.998
rno-miR-434-5p	0.068	1.297	19.194
rno-miR-133a-5p	0.071	0.642	9.06
rno-let-7c-1-3p	0.096	0.629	6.532
rno-miR-493-5p	0.108	1.252	11.547
rno-miR-1b	0.134	1.92	14.379
rno-miR-21-3p	0.158	1.21	7.683
rno-miR-3068-5p	0.166	1.204	7.25
rno-miR-34c-5p	0.176	1.198	6.817
rno-miR-34a-5p	0.176	1.198	6.817
rno-miR-208b-3p	0.187	1.191	6.382

4. Discussion

Previous studies have suggested that certain miRNAs serve as the biomarkers of sarcopenia. Therefore, we evaluated the entire miRNA profile (523 miRNAs) in sarcopenic rats in this study and found 14 miRNAs whose expression was markedly increased (>5 folds increase) in sarcopenia compared to that in young healthy and age-matched control rat serum samples. We also built a network map of proteins that are regulated by the 14 miRNAs and were found to be involved in skeletal muscle differentiation. *Notch1*, *Egr2*, and *Myocd* were identified as major nodes in the protein network.

4.1. miRNAs associated with skeletal muscle

The 14 miRNAs that were upregulated in sarcopenia included rno-miR-133b-3p, rno-miR-133a-3p, rno-miR-133c, rno-miR-208a-3p, and rno-miR434-5p among others. Zheng et al. (2018) reported age-related changes in both human skeletal muscles and many miRNAs [9]. rno-miR-133b-3p, which has been found to be increased >30 folds in sarcopenia compared to those in the age-matched and young control (Table 1), is associated with skeletal muscle recovery [10,11]. Rno-miR-133a, −133b and rno-miR-1 are reportedly associated with muscle damage [12]. rno-miR-133c, rno-miR-133a-3p, and rno-miR-133a-5p are also related to hypertension and reportedly represent one of the genes that regulate muscles in C2C12 cell studies [13,14]. rno-miR-434-5p is associated with aging and plays an important role in skeletal muscle metabolism [15]. Reportedly, the rno-miR-434-5p gene can be used as a biomarker of muscle damage because this gene is associated with muscle loss [16]. A recent study revealed that rno-miR-34c-5p is associated with neuronal nitric oxide synthase and causes muscle loss [17]. The expression of miR-34a-5p is increased in the skeletal muscles of aged mice, which is similar to our findings [18]. However, miR-34a-5p is also known to prevent muscle aging [9]. The miR-34a-5p gene and rno-miR-1b/rno-miR-1-3p reportedly regulate skeletal muscle differentiation [11,19]. rno-miR-208b-3p is also reportedly involved in muscle growth and is associated with aerobic exercise [20]. Taken together, from the results of the present study and previous reports, we suggest that rno-miR-133b-3p, −133a, −133b-1, -133c, −133a-3p, −133a-5p, -434-5p, −34c-5p, 34a-5p, -1b/-1-3p, and rno-miR-208b-3p are potential diagnostic targets that are directly related with pathogenesis of sarcopenia.

4.2. miRNAs associated with cardiac and aging

Among the miRNAs identified to be altered in the sarcopenic rat serum, some miRNAs were previously reported to be associated with cardiac diseases and aging: rno-miR-133b-3p reportedly affects the myocardium and is known to be associated with aging [21]. rno-miR-133 is associated with heart disease [22]. rno-miR-133c, rno--miR-133a-3p, and rno-miR-133a-5p are primarily associated with cancer development and are associated with the regulation of the myocardium in muscles [23,24]. rno-miR-208a-3p affects the myocardium and is associated with heart disease and cardiac hypertrophy [25]. rno-miR-21-3p is associated with cardiac hypertrophy [26] and muscle diseases [27]. rno-miR-208b-3p is associated with metabolic regulation of the myocardium [28]. Moreover, rno-miR-493-5p is expressed in aged myocardium and skeletal muscles and is reportedly associated with aging [29].

It is interesting that many of the miRNAs (including rno-miR-133 genes and −280a-3p) determined to be up-regulated in sarcopenic rats in the present study are also reportedly involved in the cardiovascular diseases such as heart failure and hypertension: sarcopenia is highly prevalent in patients with heart failure and heart failure may induce sarcopenia through common pathogenetic pathways such as hormonal changes, malnutrition, and physical inactivity [30,31].

4.3. miRNAs associated with other diseases such as cancer, frailty, diabetes, and obesity

Among the 14 miRNAs identified here, rno-miR-493-5p is reported to be associated with cancer cells [32–34]. In addition, our KEGG pathway analysis also indicated that miRNA21, 1, 34a, 34c, 133, 133a, and Let-7c also take part in the cancer pathway (Fig. 3). One of the features of cancer patients is weight loss (i.e., cachexia), which may implicate that above mentioned miRNAs may play important roles in the muscle loss as well as cachexia observed in patients with cancer. In addition to cancer, the 14 miRNAs are also reportedly found to be closely associated with diabetes, facility and obesity, which is summarized in Supplementary Table 2. Considering that the incidence of sarcopenia is closely related with diabetes and obesity is one of the major risk factors for diabetes, it is not surprising that the 14 miRNAs identified in this study are also associated with cancer, diabetes, and obesity as well as sarcopenia. Moreover, it has been recently suggested that the presence of sarcopenia is a critical prognostic factor in patients with cancer [35].

Although we do not present direct evidence that the 14 miRNAs are involved in the pathogenesis of sarcopenia, together with the aforementioned reports, our data suggest that the 14 miRNAs, or few of them, are likely to play important roles in the pathogenesis of sarcopenia. Further studies should evaluate the role of these 14 miRNAs in the pathogenesis of sarcopenia and skeletal muscle differentiation.

The network map of proteins that are regulated by the 14 miRNAs suggested that *Notch1*, *Egr2*, and *Myocd* may play important roles in sarcopenia. Notch protein is reportedly related to Wnt3a protein and is an important genetic material for muscle loss [36]. The Egr2 protein, which is related to the Notch protein, is a tendon-related protein involved in tendon differentiation. Tendons are associated with continuous muscle strength enhancement and differentiation [37]. Myocd, another protein associated with the Notch protein, plays an important role in cardiac muscle formation and lineage differentiation

of vascular smooth muscle cells. The origin of skeletal muscle cell differentiation is regulated by Myocd [38]. Heyl, which is also directly associated with Notch1 (Fig. 4), has been reported to be involved in muscle differentiation [39]. Heyl induces proliferation of muscle satellite cells by inhibiting *MyoD* expression in muscle. Muscle satellite cells are involved in muscle regeneration or overload, and the role of the Heyl protein is important [40]. In addition, the Ephb1 protein, which is also directly associated with Notch1 (Fig. 4), is an important protein in the cell proliferation of skeletal muscles [41]. We did not examine all proteins in the network map. However, the genes and related proteins identified were all proteins related to muscle regeneration, generation, and differentiation, and it can be inferred that these are related to muscle loss.

In conclusion, this study evaluated the miRNA profiles (523 miRNAs), and the expression of 14 miRNAs was markedly increased in sarcopenia, which was supported with a network map of related proteins. The 14 miRNAs, including rno-miR-133b-3p, rno-miR-133a-3p, rno-miR-133c, rno-miR-208a-3p, and rno-miR434-5p among others, and related proteins such as Notch1, Egr2, and Myocd may be useful not only in the early diagnosis of sarcopenia, but also in the development of novel therapeutic targets for the treatment and/or prevention of sarcopenia.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101251.

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