

Dysregulated expression profile of myomiRs in the skeletal muscle of patients with polymyositis

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ABSTRACT

MicroRNA (miRNA) research has intensively developed over the past decade. Characterization of dysregulated miRNA expression profiles could give a better understanding of the development of pathological conditions and clinical disorders, such as autoimmune diseases with polygenic etiology, including idiopathic inflammatory myopathies (IIMs). IIMs are a group of rare autoimmune disorders characterized by skeletal weakness and inflammation. Polymyositis (PM) is one of the conditions of autoimmune myopathies with proximal skeletal muscle weakness. A novel group of miRNAs, known as myomiRs are described as striated muscle-specific or muscle-enriched miRNAs. They are involved in myoblast proliferation/differentiation as well as muscle regeneration. To determine the role of myomiRs in the development and progression of PM, we performed an initial skeletal muscle miRNA profiling using microarray technique at diagnosis. The aim of the study was to examine myomiRs expression profile in patients with PM in order to remark the association between the dysregulated

myomiRs' expression and the development of the disease. As a results of microarray investigation, most of the myomiRs showed altered expression patterns in the muscle samples of PM patients compared to controls. These results suggest that myomiRs, especially miR-1, miR-133a, miR-208b, miR-486, and miR-499 function in a network, and are associated with the development of PM.



INTRODUCTION

MicroRNAs (miRNAs) are endogenous non-coding RNAs, playing critical roles in regulating gene expression, and they are important in a wide range of physiological processes such as cell development, differentiation and function at transcription, post-transcription and translation level (1) (2). MicroRNA research has intensively developed over the past decade. The miRNA database, called miRBase (www.mirbase.org) provides increasing number of newly identified miRNAs with 1917 human miRNAs according to its current version. Some miRNAs are ubiquitously expressed in tissue, while others are tissue-specific or tissue-enriched. Alterations in the expression of miRNAs provide valuable information on the development of pathological conditions and clinical disorders. Changes in miRNA expression profiles have been identified in different autoimmune diseases such as multiple sclerosis (MS) (3), systemic lupus erythematosus (SLE) (4), rheumatoid arthritis (RA) (5) (6), Sjögren-syndrome (pSS) (7) (8). A recently published work, that examines the whole expression profile of miRNAs in both SLE and pSS instead of only certain miRNA (9). A certain miRNA may have hundreds of different mRNA targets and a target might be regulated by multiple miRNAs. Thus, characterization of dysregulated miRNA expression profiles could give a better understanding of the development of immunological disturbances in autoimmune

diseases with polygenic etiology, including idiopathic inflammatory myopathies (IIMs). IIMs are a group of rare autoimmune disorders characterized by skeletal muscle weakness and inflammation (10) (11). PM is one of the five conditions of autoimmune myopathies predominantly with proximal skeletal muscle weakness. Not much was known about the pathogenesis of this condition. MiRNAs represent a new and potentially exciting pathway to the future research into idiopathic inflammatory myopathies as well. Identification of dysregulated miRNAs has led to a greater understanding of inflammation, muscle weakness/wasting and extra-muscular organ involvement in IIMs (12) (13) (14). For instance, five miRNAs, miR-146b, miR-221, miR-155, miR-214 and miR-222, have been found to be usually over-expressed across 10 primary muscle disorders including IIMs (12) (15). Up-regulation of immune-related miRNAs in muscle, for example, miR-155 and miR-146b, is closely related to autoimmunity (12). However, down-regulation of miRNAs such as miR-1 and miR-206 is associated with inhibition of muscle regeneration (16) (17). MiR-1 and miR-206 are members of a novel group of miRNAs, known as myomiRs (14) (18). MyomiRs are described as striated muscle-specific or muscle-enriched miRNAs (19). The group of myomiRs include eight miRNAs: miR-1, miR-133a/b, miR-206, miR-208a/b, miR-486, and miR-499 (Table 1). MyomiRs are expressed in both cardiac and skeletal muscle with the exception of miR-206, which is skeletal muscle-specific, and miR-208a, which is cardiac muscle-specific (14) (Table 1). Some studies have proved that not all myomiRs are exclusively expressed in a muscle-specific manner but may be detected in low levels in other tissues (20) (21). However, myomiRs main function is confined to muscle. MiR-486 is sometimes considered muscle-enriched rather than muscle-specific as it is also expressed in other tissues. Skeletal muscle development is a complex process requiring

coordination of multiple factors, which control the proliferation of myoblast, their exit from the cell cycle and subsequent differentiation into multinucleated myotubes (14). MyomiRs affluence is regulated by myogenic regulatory factors in a negative feedback loop, through influencing many aspects of myogenesis. For example, miR-1 stimulates differentiation of myoblast, miR-1 and miR-206 promotes myogenic differentiation (19).

Their network has a main role in the regulation of skeletal muscle plasticity by organizing changes in fiber type and muscle mass in response to altered contractile activity (Table 1) (19). They are involved in myoblast proliferation/differentiation, muscle regeneration, or fiber type specification. The tissue specificity of myomiRs is scheduled either for the genomic location of their coding DNA within introns of myosin heavy

Table 1 Group of skeletal muscle myomiRs: locations, tissue specificity, summary the function of myomiRs, and their host genes*

MyomiRNAs	Location on chromosome	Genome context	Tissue specificity	Function	Host gene
miR-1	20q13.33 (miR-1-1) 18q11.2 (miR-1-2)	intragenic	heart/skeletal muscle	Stimulation of myoblast differentiation, regeneration, angiogenesis regulation	<i>MIB1</i>
miR-133a	18q11.2 (miR-133a-1)	intragenic	heart/skeletal muscle	Promotion of myoblast proliferation, differentiation, fusion, regeneration, muscle fiber shift	<i>MIB1</i>
miR-133b	6p12.2	intergenic	skeletal muscle	Promotion of myoblast differentiation and fusion, regeneration	intergenic
miR-206	6p12.2	intergenic	skeletal specific	Promotion of myoblast differentiation, regeneration, regeneration of neuromuscular synapses	intergenic

miR-208a	14q11.2	intragenic	heart	Muscle fiber shift, promotion of muscle growth	MYH6 MYH7
miR-208b	14q11.2	intragenic	heart/skeletal specific	Muscle fiber shift, promotion of muscle growth	MYH7
miR-486	8p11.21	intragenic	heart/skeletal specific	Promotion of myoblast differentiation and fusion	ANK1
miR-499	20q11.22	intergenic	heart/skeletal specific	Muscle fiber shift, promotion of muscle growth	MYH7B

*Data are from M. Horak et al. (14).

chain genes or for transcriptional factors such as MyoD, Mef2, or muscle-specific transcriptional factors such as MyoD, Mef2, or Srf (22).

To determine myomiRs role in the development and progression of PM, we performed skeletal muscle miRNA profiling using microarray technique in PM patients at diagnosis before treatment. Improved understanding of the role of miRNAs and their targets therefore could help to elucidate the pathogenesis of PM. Although, there are some reports about the changes of miRNA level in muscle of PM patients, microarray profiling of all muscle-related miRNAs, called myomiRs is not available in the literature.

The aim of our study was to examine myomiRs expression profile in patients with PM in order to remark the associations between the dysregulated myomiRs' expression and the development of the disease. Since the pathogenesis of PM characterized by symmetric muscle weakness, elevated serum creatine kinase levels, typical myopathic features, we paid a special attention on myomiR expression levels in PM patients compared to controls.

To our best knowledge, this is the first study to investigate all the eight myomiRs in the initial skeletal muscle of patients with PM.

PATIENTS AND CONTROL INDIVIDUALS

Out of five hundred and forty patients with IIM myositis patients, 4 patients were selected for this study at the Division of Clinical Immunology, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Hungary (10) (23). They all were diagnosed with the clinicopathological subgroup PM and had a definitive diagnosis according to Bohan and Peter (11).

This means that they had progressive symmetric muscle weakness of the proximal upper and lower extremities and neck flexors; had no skin lesions pathognomic or characteristic for dermatomyositis (DM); had elevated serum creatine kinase levels; had typical myopathic features on electromyography; and had positive muscle biopsy features (endomysial infiltration of mononuclear cells surrounding, but not invading, myofibers). Muscle biopsies were taken from the weaker deltoid muscle or quadriceps femoris muscle, in local anaesthesia, by surgeon

specialists at the Surgical Department, Faculty of Medicine, University of Debrecen, Hungary. Muscle biopsy was taken in every single case before steroid or any other immunosuppressive therapy not to suppress inflammation. The biopsy-samples were frozen in liquid nitrogen until used for preparation. The average age of the patients was 60.75 years, while the female:male ratio was 3:1. No patient had any internal organ manifestations; their PM was localized only in muscle. The control group consisted of three age-matched (average age: 54.3 years, female:male ratio was 2:1) healthy female and male volunteers who underwent surgery for total hip replacement, and the muscle biopsy was taken and collected, and kept in liquid nitrogen then. No patients or controls enrolled in this study had ongoing infections, either viral or bacterial.

This study meets, and is in compliance with, all ethical standards of medicine. Informed consent was obtained from all of the subjects enrolled in the investigation, and the study has been approved by the Ethics Committee of our University and the Policy Administration Services of Public Health of the Government Office (protocol number: HBR/052/00766-2/2014.). This study is ethically compliant and was carried out in compliance with the Declaration of Helsinki.

METHODS

Sample handling

After taking the muscle biopsies of PM patients and healthy individuals samples obtained from each study subject were collected and kept in liquid nitrogen until used.

miRNA processing

MiRNAs were extracted from frozen biopsy samples, using the mirVana miRNA Isolation Kit (Ambion) in accordance with the manufacturer's instructions.

miRNA microarray and data analysis

Integrity of RNA samples was checked on Agilent BioAnalyzed using RNA Nano chips (Agilent Technologies), samples with > 7 RNA integrity number were used for the further experiments. To obtain global miRNA expression data Affymetrix miRNA 3.0 arrays (Affymetrix) were processed. Total RNA samples were labelled using Affymetrix FlashTag Biotin HSR RNA Labeling Kit according to the manufacturer's protocol. Briefly, 500 ng of total RNA samples were poly (A)-tailed using poly A polymerase enzyme and ATP at 37°C for 15 minutes, then biotinylated by ligating biotin-labeled fragment to the 3' end. Labeled samples were hybridized on miRNA 3.0 arrays at 48°C and on 60 rpm for 16 hours. After that, arrays were washed and stained by standard Affymetrix protocol using Affymetrix Hybridization, Wash and Stain Kit on FS 450 fluidic station instrument then the arrays were scanned on Affymetrix GeneChip Scanner 3000 7G instrument.

Quality of miRNA arrays were checked in miRNA QC Tool (Affymetrix) software and raw intensity values were exported with annotations as a text file. Further data analysis was performed in GeneSpring GX 12.0 software (Agilent Technologies). First, a custom technology was created based on the exported text file then data were normalized using quantile normalization algorithm. To determine differentially expressed miRNAs between diseased and healthy groups moderated T-test was executed and $p < 0.05$ was considered as statistically significant difference.

RESULTS

We carried out analysis to evaluate the expression patterns of miRNAs focusing on levels of myomiRs (miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486, and miR-499). For this purpose, initial skeletal muscle biopsies of 4

patients with active PM have been investigated and compared to muscle samples of healthy individuals. MiR-1, miR-133a, miR-208b, miR-486, and miR-499 were differentially expressed in PM skeletal muscle. All the myomiRs's expression were significantly altered in the muscle samples of PM patients compared to healthy controls (Table 2) ($P < 0.05$). In specific, miR-1, miR-499 and miR-208b were significantly down-expressed in the affected muscle of PM patients compared to controls. At the same time, miR-133a and miR-486 were significantly up-regulated in the same patient samples compared to control samples.

In contrary, miR-133b and miR-206 expression levels were the same in both groups. In PM patients both up-regulated myomiRs, namely miR-133a and miR-486 had less than 2-fold change in their expression compared to controls (Table 2). On the contrary, miR-1, miR-208b, and miR-499 were significantly down regulated in PM group (Table 2), miR-1 showed nearly 2-fold change, but miR-208b had over 2-fold change compared to controls. However, miR-499 expression

exceeded 5-fold change in patients compared to controls (Table 2).

DISCUSSION

Recently, miRNAs have appeared as new elements in skeletal muscle myogenesis by participating in arranged gene regulation processes. They have essential role in skeletal muscle development. MicroRNAs which are exclusively or preferentially expressed in striated muscle are called myomiRs. The group currently includes eight miRNAs: mi-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486, and miR-499 (Table 2). Some myomiRs are located on chromosomes in bicistronic clusters and are thus transcribed together; this includes miR-1-1/miR-133a-2, miR-1-2/miR-133a-1 and miR-206/miR-133b families placed in humans on chromosomal regions 20q13.33, 18q11.2 and 6p12.2, respectively (14). MiR-206/miR-133b is intergenic. Other myomiRs are monocistronic and situated in protein coding genes. MiR-208a, miR-208b and miR-499 are encoded within the

Table 2 Expression profile of myomiRs in muscle biopsies of patients with active PM*

miRNA	Fold change in expression	P-value	Regulation
miR-1	1.69	0.026	down
miR-133a	1.46	0.021	up
miR-133b	none	-	-
miR-208a	none	-	-
miR-208b	2.91	0.042	down
miR-486	1.8	0.036	up
miR-499	7.58	0.038	down

*Fold changes of myomiRs in PM patients compared to healthy individuals. MiR-1, miR-208b, and miR-499 were significantly down regulated, while miR-133a and miR-486 were significantly up regulated. $P < 0.05$ was considered as statistically significant difference.

introns of the myosin heavy chain genes (*MYH6*, *MYH7*, and *MYH7B*). MiR-486 is encoded in the intronic region of the *ANK1* (14). MyomiRs arrange a subset of miRNAs participating in myogenesis as a network.

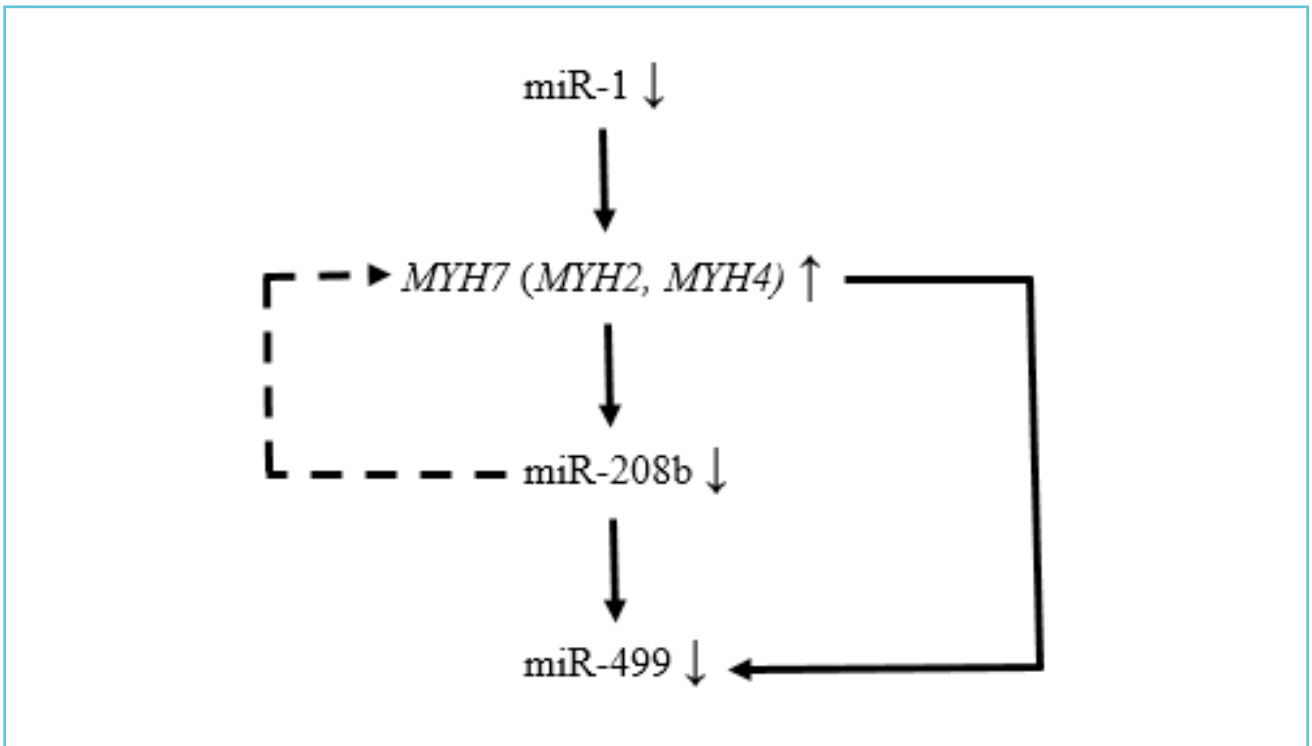
In our study, we revealed alterations in the expression patterns of myomiRs in patients with PM investigating initial muscle biopsies taken from the weaker deltoid or quadriceps femoris muscle. Analyzing myomiRs expression profiles including diseased muscle of patients and healthy skeletal muscle, we hypothesized to get better insights into the disease-specific changes. To determine differentially expressed miRNAs between diseased and healthy groups moderated T-test was executed and $p < 0.05$ was considered as statistically significant difference. In

total, there were three myomiRs (miR-1, miR-208b, and miR-499) down regulated significantly, while miR-133a and miR-486 were up regulated significantly in the muscle biopsies of the PM patients investigated.

Based on work from our laboratory and results of published literature, we assume the hypothesis of a regulatory network including myomiRs and myosin heavy chain genes in PM. Based on our findings, dysregulated expression of myomiRs might be in association with the symptoms, the clinical conditions and the course of PM.

Reduced miR-1 expression is the starting point of our hypothesis. However, up to this point the reasons for why miR-1 expression level is reduced are unknown.

Figure 1 Regulatory network of myomiRs and myosin heavy chain genes in PM*



*MiR-1 is a key element with reduced expression level in the regulation of myosin heavy chain genes' expressions (*MYH7*, *MYH2* and *MYH4*) (24). *MYH7* gene is over-expressed as well as the expression of *MYH2* and *MYH4* genes based on preliminary results. Expression level of myomiRs, which are located in intronic regions of *MYH7* (19) and *MYH7B* has been changed; more precisely, expression level of miR-208b and miR-499 decreased. In addition, miR-208 also regulates stress-dependent myosin heavy chain gene (for example, *MYH7*, *MYH2*, and *MYH4*) expressions and down-regulation of miR-208b promotes further upregulation of myosin heavy chain genes by positive feedback (20).

According to the literature data available, miR-1 is a key element in the regulation of myosin heavy chain genes' expressions (*MYH7*, *MYH2* and *MYH4*) (24). Literature has confirmed that the *MYH7* gene is over-expressed as well as the expression of *MYH2* and *MYH4* genes based on preliminary results (we have not yet published our own results). It is already acknowledged that miR-208b and miR-499 are placed in the intronic regions of *MYH7* gene (19). As a result of changes in the pattern of *MYH7* expression, the expression level of myomiRs, which are located in intronic regions of *MYH7* and *MYH7B* has been changed; more precisely, expression level of miR-208b and miR-499 decreased; measuring 2.91-fold change for miR-208b and 7.59 fold change for miR-499, respectively (Table 2). However, as far as we know, miR-208 controls miR-499 gene expression as well (25) and this condition makes miR-499 expression level even lower. In addition, miR-208 also regulates stress-dependent myosin heavy chain gene (for example, *MYH7*, *MYH2*, and *MYH4*) expressions (20) and down-regulation of miR-208b promotes further upregulation of myosin heavy chain genes by positive feedback.

There are several studies to date that reported the function of myomiRs. These reports established that miR-1, miR-208b, and miR-499 stimulate myoblast differentiation and regeneration; promote angiogenesis regulation; affect the muscle fiber shift; and encourage muscle growth in healthy conditions. Their reduced expression levels could influence their functions resulting in muscle weakness, muscle atrophy, which are typical symptoms of PM. Present data suggest that muscle levels of certain myomiRs might be associated with PM. However, the number of patients and controls are relatively low in our study and miRNA microarray results have not been validated yet on the tested and/or independent samples by real-time qPCR.

Validation of miRNA microarray results as well as our hypothesis also need further studies.

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