Deregulation of microRNAs in blood and skeletal muscles of myotonic dystrophy type 1 patients

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Abstract

**Introduction:** MicroRNAs (miRNAs) are short RNA molecules of approximately 22 nucleotides that function as post-transcriptional regulators of gene expression. They are expressed in a tissue-specific manner and show different expression patterns in development and disease; hence, they can potentially act as disease-specific biomarkers. Several miRNAs have been shown to be deregulated in plasma and skeletal muscles of myotonic dystrophy type 1 (DM1) patients. **Methods:** We evaluated the expression patterns of 11 candidate miRNAs using quantitative real-time PCR in whole blood (n = 10) and muscle biopsy samples (n = 9) of DM1 patients, and compared them to those of normal control samples (whole blood, n = 10; muscle, n = 9). **Results:** In DM1 whole blood, miRNA-133a, -29b, and -33a were significantly upregulated, whereas miRNA-1, -133a, and -29c were significantly downregulated in the skeletal muscles compared to controls. **Conclusions:** Our findings align to those reported in other studies and point towards pathways that potentially contribute toward pathogenesis in DM1. However, the currently available data is not sufficient for these miRNAs to be made DM1-specific biomarkers because they seem to be common to many muscle pathologies. Hence, they lack specificity, but reinforce the need for further exploration of DM1 biomarkers.

How to cite this article:

How to cite this URL:
Myotonic dystrophy type 1 (DM1) is an autosomal dominant, neuromuscular disease that affects a variety of organ systems in patients.[1] DM1 is caused by CTG repeats, which are expanded beyond the normal limits of between five and 50 at the 3′-untranslated region (UTR) of the dystrophymyotonica-protein kinase (DMPK) gene.[2] It is generally accepted that the larger the number of repeats, the earlier the age of disease onset and the more severe the disease manifestation. Although the disease causing mutation is located at the 3′-UTR, patients manifest a wide phenotypic array with multisystemic involvement.

MicroRNAs (miRNAs) are short RNA molecules of approximately 22 nucleotides. They function as post-transcriptional regulators of gene expression by degrading target mRNAs or inhibiting translation.[3] Several miRNAs have been shown to be deregulated in skeletal muscles of DM1 patients, including miRNAs that have been associated with splicing and ion conduction defects.[4] Animal models with the Dicer allele deleted, as well as selected miRNA knockout models, have shown severe pathogenic phenotypes which suggest that miRNAs are pertinent for development and normal physiological functioning.[5] Many miRNAs are expressed in a tissue-specific manner and show different expression patterns in development and disease.[6] A group of miRNAs has been distinguished to influence and regulate muscle-specific genes in both cardiac and skeletal muscles. These miRNAs, known as myomiRs, include miR-1, -133a/b, and -206.[7] They have been extensively studied and are seen to play key roles in the differentiation and proliferation of skeletal muscles. Studies on these myomiRs and other miRNAs in muscle pathologies suggest that the differential expression patterns observed for each disorder sufficiently discriminate one disease from another, and hence, they can potentially act as disease-specific biomarkers.[8]

MicroRNAs are also found to be deregulated in biological fluids such as blood, saliva, urine, breast milk, etc.[9] This has sparked interest in the development of these miRNAs as minimally invasive, disease-specific biomarkers. The plausibility of this role was hypothesized due to several attractive characteristics of these miRNAs [10]: (i) stability of miRNA even after being subjected to severe conditions, (ii) circulating miRNAs are helpful in distinguishing between diseased and normal samples with appropriate levels of sensitivity and specificity, (iii) miRNAs are relatively easy to be extracted and quantified, especially when compared to protein-specific biomarkers that require considerations for complex post-translational modifications. Given the fact that DM1 is a multisystemic disease with a history of rampant misdiagnosis, late diagnosis as well as unclear genotype–phenotype correlation, the identification of minimally invasive diagnostic, and prognostic biomarkers would be beneficial in the management of DM1 patients.

To our knowledge, there have been only four other publications investigating the role of miRNA in DM1.[11][12][13][14] In a slightly different approach, we studied the expression of selected miRNAs in whole blood samples of DM1 patients. The miRNAs were selected based on findings in literature that described their association with DM1 and other muscle disorders, as well as based on computational predictions. We next studied the expression of the same panel of miRNAs in DM1 skeletal muscle biopsy samples to see if the miRNA dysregulation in whole blood reflected the patterns observed in skeletal muscles. Here, we report the differential expression of miRNAs in whole blood and skeletal muscle samples of DM1 patients, and discuss the potential use of these miRNAs as DM1-specific biomarkers.

**Materials and Methods**

**Ethics statement**

Ethical approval for this study was obtained from the University of Malaya Medical Centre (UMMC)
ethics committee (Ref. no. 800.6).

Study subjects

Whole blood samples from 10 patients were obtained after taking informed consent from DM1 patients visiting the Neurology or Genetics clinics in UMMC. Control blood samples were obtained from 10 healthy anonymous blood donors. RNAlater was added to the samples in ethylenediaminetetraacetic acid tubes upon collection to ensure that the integrity of the RNA species is maintained. Nine skeletal muscle tissue samples each for both DM1 patients and controls were obtained from an archived collection of tissue samples. The tissue samples had been processed upon receipt, snapped frozen, and embedded in the optimal cutting temperature (OCT) compound before being archived in a −80°C refrigerator. When RNA extraction was to be performed, the frozen samples were sectioned in a microtome-cryostat to obtain the required amounts and dropped into tubes containing RNAlater. The samples had been archived for varying periods of between 6 months and 18 years and were either from the biceps brachii, gastrocnemius, or deltoid region. The diagnosis of DM1 in patients was based on the results of either a molecular genetic, electromyography (EMG), or muscle biopsy test, or a combination of these. The nine skeletal muscle control samples were all found to be within normal limits without evidence of a well-defined myopathy by a pathologist. [Table 1] and [Table 2] briefly outline the demographic details of the participants of this study.

MicroRNA expression analysis

MicroRNA isolation was performed on the whole blood samples using the Ribo-Pure Blood Kit (Life Technologies, USA) according to an alternate protocol for enrichment of small RNAs. MicroRNA was purified from the skeletal muscle tissue samples using the mirVana miRNA Isolation Kit (Life Technologies, USA) according to the manufacturer's instructions. The purity (OD260/280) and concentration of the RNA samples were measured using the NanoDrop. Ten nanograms of each RNA sample was reverse transcribed to cDNA with specific miRNA primers using the TaqMan Small RNA Assays Kit (Life Technologies, USA) according to the manufacturer's protocol. Following cDNA synthesis, real-time PCR amplification was performed for each sample and target using the TaqMan Fast Advanced Master Mix Kit in the 7500 Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocol. The miRNAs analysed were hsa-miR-1 (Assay ID: 002222), hsa-miR-133a (Assay ID: 002246), hsa-miR-148a (Assay ID: 000470), hsa-miR-152 (Assay ID: 000475), hsa-miR-206 (Assay ID: 000510), hsa-miR-29b (Assay ID: 000413), hsa-miR-29c (Assay ID: 000587), hsa-miR-335 (Assay ID: 000546), hsa-miR-365 (Assay ID: 001020), hsa-miR-33a (Assay ID: 002135), and hsa-miR-7 (Assay ID: 000386). The endogenous controls used were hsa-miR-183 (Assay ID: 002269) for blood samples, and hsa-let-7a (Assay ID: 000377) for skeletal muscle samples after their stable expression across the respective sample groups was validated.

The Ct values obtained for each assay were exported into the DataAssist Software, version 3.01 (Life Technologies Corp., 2012) for relative gene expression analysis using the 2(−ΔΔCt) method. Ct values above 35 were omitted and outliers in technical replicates were automatically excluded. Analyses were performed at two levels. For comparison of miRNA expression in individual patient samples against the mean of control samples, the 2(−ΔΔCt) was calculated to determine relative fold change of each miRNA expression. For analysis between groups, a two-sample, two-tailed Student's t-test comparing the ΔCt values of the patient and control groups was performed. The fold change of the miRNA expression in DM1 patients was obtained using the 2(−ΔΔCt) formula, and the P value for each miRNA
was calculated and adjusted using the Benjamini–Hochberg False Discovery Rate. Statistical significance was set at \( P < 0.05 \).

**Results**

Levels of circulating miR-133a, -29b, and -33a are increased in myotonic dystrophy type 1 patients

Expression levels of 11 candidate miRNAs were profiled in whole blood samples of 10 DM1 patients relative to 10 controls. Three miRNAs, miR-133a, -29b, and -33a were found to be significantly upregulated in DM1 patients compared to normal controls. [Figure 1] shows the expression of the three miRNAs in individual samples relative to the mean expression in normal controls. MiR-133a showed an average increase of 1.61 fold \( (P = 0.0360) \), with eight out of the ten samples showing upregulation. MiR-29b showed an average increase of 2.09 fold \( (P = 0.0344) \), with seven samples showing upregulation at various levels. A larger relative difference was noted in the expression of miR-33a in DM1 patients. The average fold change was 2.13 \( (P = 0.0097) \), with all but one sample showing upregulation. [Table 3] lists all the miRNA assayed and their regulation in DM1 whole blood samples. [Figure 1] [Table 3]

MiR-29c and two myomiRs are downregulated in skeletal muscle biopsies of myotonic dystrophy type 1 patients

To better understand the relevance of the findings from the blood sample analysis, we next studied the expression of the same panel of miRNAs in DM1 skeletal muscle biopsy samples. Two myomiRs, miR-1 and -133a, were found to be downregulated in muscle biopsies of DM1 samples. The average decrease of miR-1 was 1.93 \( (P = 0.0009) \) and of miR-133a was 1.26 \( (P = 0.0339) \). Individually, six out of the nine muscle biopsies recorded at least a two-fold decrease for miR-1, compared to only four for miR-133a. Intriguingly, the skeletal muscle specific miR-206 failed to show any significant deregulation in the muscle biopsies. A rather erratic pattern of regulation was seen for this miRNA, with three samples showing slight downregulation, and the remainder being slightly upregulated. In addition to the two myomiRs, miR-29c also showed significant downregulation in DM1 muscle biopsies with an average fold-change of 2.47 \( (P = 0.0220) \). Five of the samples reached the two-fold mark. [Figure 2] shows the miRNA modulation in skeletal muscle biopsies of DM1 patients. [Table 4] lists the results of all the miRNAs assayed in DM1 muscle biopsies. [Figure 2] [Table 4]

**Discussion**

The 11 candidate miRNAs that were selected for this study were picked based on findings in literature that described the deregulation of these miRNAs in DM1 and in other muscle disorders,[12],[13] as well as based on computational predictions of miRNA candidates that are likely to bind to the DMPK, MBNL1, and CELF1 genes based on their seed sequences (TargetScan Human, Release 6.2). Due to funding constraints, we did not perform a global miRNA screen using the microarray technique to look for deregulated miRNAs prior to validating it via qPCR, as is usually done.

Out of the 11 candidate miRNAs analyzed, miR-133a, -29b, and -33a were found to be upregulated in DM1 whole blood samples compared to healthy controls. In DM1 skeletal muscle samples, miR-1,
133a, and -29c were found to be downregulated instead. The increased levels of the three miRNAs in the blood samples in our study may be attributed to the leakage or secretion from skeletal muscle fibers or an excessive expression in the dystrophic skeletal muscle, which then influences blood expression levels. However, studies have also shown that erythrocytes contain abundant miRNAs despite the absence of nucleus and DNA in these cells, and hence, there is a possibility that the miRNAs are deliberately highly expressed in erythrocytes as part of a mechanism in response to the DM1 pathology.

A study by Perfetti et al.,[11] which reported for the first time the deregulation of miRNA in DM1 plasma, showed that eight miRNAs were upregulated in their samples, one of which is miR-133a. In our study, whole blood samples were extracted for miRNAs as opposed to plasma or serum that were used in other similar studies. Observation of miRNA expression changes in whole blood would be beneficial in the development of biomarkers because whole blood is easier to collect and has 200–1000 times more RNA content than plasma. Whole blood consists of white blood cells as well as the anucleated red blood cells and platelets. Although devoid of nucleus and DNA, studies have shown that red blood cells and platelets are rich sources of miRNA.[15] Keller et al., have demonstrated that the miRNome of blood cells can reflect the presence of diseases that are not necessarily blood borne.[16] Our findings suggest that the increased levels of miR-133a, -29b, and -33a seen could possibly be produced in blood itself because of a yet to be ascertained physiological or pathological response, in addition to the alternative hypothesis that upregulated circulating miRNAs are a result of leakage from dystrophied muscle tissues.[17]

Our analyses also show that miRNAs are indeed deregulated in DM1 muscle samples, as seen in those reported elsewhere for DM1, as well as other muscle disorders. Our results of downregulated miR-1, -133a, and -29c in DM1 skeletal muscle biopsies is comparable to those reported in the study by Perbellini et al.,[12] which recorded the downregulation of miRNA-29c and -33. However, in addition, the study also reported the upregulation of miRNA-1 and -335 and the down-regulation of miRNA-29b. Another study by Gambardella et al.,[13] reported an upregulation in the expression of miR-206 in DM1 skeletal muscle biopsies, compared to controls; whereas, a third study by Rau et al.,[14] reported the misregulation of miR-1 processing in cardiac muscle samples from DM1 patients.

Apart from miR-1 and -133a, the muscle specific miR-206 was also expected to be deregulated in DM1 muscle biopsies, given its important role in the regulation of muscle development.[18] MIR-206 is expressed only in skeletal muscles, and promotes muscle differentiation if induced by MyoD and myogenin during myogenesis.[19],[20] These muscle-specific miRNAs seem to participate in muscle diseases, including cardiac hypertrophy, heart failure, cardiac arrhythmias, congenital heart disease, and muscular dystrophy.[8],[21] Gambardella et al.,[13] reported no differences in the expression of miR-1 and -133 between DM1 and control muscles, and instead observed an increase in the expression of miR-206. The differences of this result with those observed in our study may be attributed to low patient numbers in both studies or to the different skeletal muscle analyzed.

MiR-1 and miR-133 are specifically expressed in adult cardiac and skeletal muscle tissues.[7],[18] They modulate skeletal muscle cell proliferation and differentiation by working antagonistically. MiR-1 strongly enhances myogenic differentiation, as indicated by an increase in the expression of myogenic markers in cultured muscle cells. In contrast, overexpression of miR-133 represses the expression of the same myogenic markers and hence promotes myoblast proliferation.[22] A study to test the effects of these two miRNAs on skeletal muscle and heart development in vivo suggested that correct temporal expression as well as amounts of both miR-1 and -133 are required for proper skeletal muscle and heart development.[22] Predicted targets of miR-1 include the HDAC4 protein, which has been shown to inhibit skeletal muscle gene expression and muscle differentiation by repressing MEF2C, an essential muscle-related transcription factor.[18],[22] Two conserved miR-133 binding sites have been found in the 3'-UTR of the mammalian gene encoding serum response factor (SRF), known to be important in muscle proliferation and differentiation in vitro and in vivo. A construct of the 3'-UTRs of mouse SRF and HDAC4 fused to a luciferase reporter gene and transfected into mammalian cells resulted in a strong repression of HDAC4 in the presence of miR-1 overexpression, and inhibition of SRF whenever miR-133 was over-expressed. Expression levels of miR-1 and 133a were also found to be decreased in a mouse model of skeletal muscle and cardiac hypertrophy.[21] In addition, the miRNA pair have also been found to regulate the expression of cardiac conduction-system components and are arrhythmogenic. These findings suggest that a substantial role is played
Muscle fibrosis is a common finding in dystrophic processes. Biopsy findings for DM1 include various degrees of chronic myopathic changes, frequently with dystrophic features such as fibrosis and fatty tissue replacement. In general, the DM1 heart is most prone to fibrosis but skeletal muscles have also been found to be affected, albeit to a lesser extent.[23],[24] Wang et al.,[25] described a novel cellular mechanism for the occurrence of muscle fibrogenesis through the negative regulation of miR-29 by TGF-β signaling. They demonstrated that the downregulation of miR-29 stems from the myoblast compartments, which causes suppression of myogenic differentiation and promotion of fibrogenic differentiation through the overproduction of collagens. This loss of miR-29 is caused by high levels of transforming growth factor (TGF) - β in dystrophic muscles. Promising findings have also been seen in the usage of miR-29 as an antifibrotic therapeutic agent.[25] Systemic delivery of miR-29 mimics led to the significant improvement of diaphragm muscle pathology in mouse mutant deficient in dystrophin (mdx) mice by reducing existing fibrosis and increasing regeneration. This finding could potentially be extended to DM1 patients.

The patterns of miRNA expression in DM1 whole blood observed in this study were different from those in the skeletal muscle samples. The up-regulation of blood miR-133a, -29b, and -33a were not reciprocated in skeletal muscles, which displayed downregulation of miR-1, -133a, and -29c. This incongruence between the miRNA expression profile of skeletal muscle and whole blood may suggest that extracellular miRNA do not simply leak from damaged muscle but may constitute a specific biological response. In addition, circulating miRNAs hold the potential to reflect the overall clinical state of the patient, rather than only that of the muscle or other specific tissue. This may have been reflected in the upregulation of miR-33a, an miRNA, associated with cholesterol metabolism.[26] There is, hence, an enormous need to study the aberrations of miRNA expression and how they contribute toward pathogenesis.

The data obtained from this study were limited by the small sample size due to the rare nature of the disorder, as well as the differences in the types of muscles analyzed. These muscles were of three types as listed in [Table 2]. The results may have been slightly affected by this variability due to the fact that muscle weakness associated with DM1 does not affect all muscles equally.

In conclusion, our study reinforces previously reported findings of miRNA deregulation in DM1 skeletal muscle, and reports, for the first time, evidences of aberrant miRNA expression in DM1 whole blood samples. We suggest that the miR-1/133–HDAC4/SRF pathway as well as the TGF-β–miR-29 pathway–could potentially be involved in the pathogenesis of DM1. The deregulation of miRNAs in whole blood also reinforces the need for this area of study to be further explored as we move toward diagnostic, prognostic, and therapeutic marker development by using less invasive means, which are economical, disease-specific, and timely.

Financial support and sponsorship

This study was supported by grants from the Ministry of Higher Education, Malaysia (Fundamental Research Grant Scheme; grant number: FG029/2010A), University of Malaya (Postgraduate Research Fund; grant number: PV126/2012A, UM HIR grant number UM.C/625/1/HIR/MOHE/EDU/27).

Conflicts of interest

There are no conflicts of interest.

References


