RESEARCH ARTICLE

Six Serum miRNAs Fail to Validate as Myotonic Dystrophy Type 1 Biomarkers

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Abstract

Myotonic dystrophy type 1 (DM1) is an autosomal dominant genetic disease caused by expansion of a CTG microsatellite in the 3' untranslated region of the DMPK gene. Despite characteristic muscular, cardiac, and neuropsychological symptoms, CTG trinucleotide repeats are unstable both in the somatic and germinal lines, making the age of onset, clinical presentation, and disease severity very variable. A molecular biomarker to stratify patients and to follow disease progression is, thus, an unmet medical need. Looking for a novel biomarker, and given that specific miRNAs have been found to be misregulated in DM1 heart and muscle tissues, we profiled the expression of 175 known serum miRNAs in DM1 samples. The differences detected between patients and controls were less than 2.6 fold for all of them and a selection of six candidate miRNAs, miR-103, miR-107, miR-21, miR-29a, miR-30c, and miR-652 all failed to show consistent differences in serum expression in subsequent validation experiments.

Introduction

Myotonic dystrophy type 1 (DM1) can appear at any time in life and is regarded as the human disease which probably has the most variable clinical presentation, somehow affecting virtually all body systems [1]. Although typically classified as a neuromuscular disease, besides its prominent muscular system defects (including cardiac, smooth, and skeletal muscle cell types), it also compromises cognitive, ocular, digestive, endocrine, respiratory, reproductive, cutaneous, haematopoietic, and immune systems to varying degrees [2]. Characteristic muscular symptoms include cardiac problems such as malignant arrhythmias and conduction defects, and involvement of facial (ptosis), bulbar (dysarthria, dysphagia), limb (steppage, gait troubles), and smooth (constipation) muscle with associated muscular atrophy and myotonia [1, 3, 4]. Patients also suffer from iridescent cataracts and insulin resistance with metabolic syndrome. Genetically, it is an autosomal dominant disease caused by unstable expansion of the CTG...
microsatellite in the 3′ untranslated region of the dystrophia myotonica-protein kinase (DMPK) gene and is a rare disease that afflicts one in 8000 people worldwide. Unaffected individuals carry between 5 and 37 CTG repeats whereas DM1 patients carry between 50 and thousands of CTG triplets [5]. Importantly, CTG trinucleotide expansions are unstable both in the somatic and germinal lines, likely contributing to the heterogeneity in clinical symptoms and age of onset, which inversely correlates with the size of the triplet expansion. A further increase in the size of the CTG microsatellite occurs in most intergenerational transmissions of the expanded allele, which correlates with genetic anticipation [6]. Despite the correlation between the size of the CTG expansions in blood cells with disease severity and age of onset, its predictive power is poor and it is not a good parameter for characterising the disease load. Forthcoming therapeutic trials urgently need good biomarkers to evaluate the therapeutic response to treatments.

Alternative splicing changes in skeletal muscle have been described as potential biomarkers of disease severity and therapeutic response, but they involve invasive techniques [7] and it would be difficult to routinely measure them in other sites (such as cardiac or cerebral tissues) which are strongly involved in DM1 pathophysiology.

Expanded RNA transcripts containing CUG repeats are retained in the cell nucleus as insoluble RNA aggregates known as ribonuclear foci [8]. These foci are able to sequester different RNA binding proteins that are prevented from performing their normal functions. The alternative splicing regulators Muscleblind-like1 (MBNL1) is among the recruited proteins, which result in its functional depletion [9]. CUGBP, Elav-like family member 1 (CELF1), a splicing factor antagonist of MBNL1 [10], is not sequestered in ribonuclear foci but becomes abnormally activated due to hyperphosphorylation [11]. As a consequence, several alternative splicing events are misregulated in DM1 and in some cases these splicing defects contribute to DM1 symptoms such as myotonia, insulin resistance, or muscle weakness [7, 12, 13, 14]. The molecular mechanism leading to DM1 pathogenesis is complex and, in addition to splicing defects, also includes mispolyadenylation of pre-mRNA, a process that is also regulated by MBNL proteins [15], repeat-associated non-ATG translation (RAN translation) [16], bidirectional transcription [17], defects in transcription and translation [18, 19], epigenetic changes [20], and the silencing of cardiac and muscle transcripts by changes in miRNA expression levels [21, 22, 23, 24]. miRNAs are endogenous non-coding RNAs, approximately 21 nucleotides long, that function as post-transcriptional gene expression regulators by targeting the 3′ untranslated region of their complementary target mRNA. miRNAs regulate RNA stability and translation rates via degradation or inhibition of protein translation, respectively (reviewed in [25]). Over 2000 miRNAs have been identified in the human genome [26] and have been implicated in numerous biological processes including development, proliferation, differentiation, and stress responses (reviewed in [27]). Because miRNAs can be readily detected in body fluids, and particularly in blood components [28], differences in serum miRNAs have been proposed as potential non-invasive biomarkers of disease progression for several conditions such as cancer, Alzheimer’s disease, hepatitis B infection, retinopathies, gestational diabetes mellitus, or Duchenne muscular dystrophy [29, 30, 31, 32, 33, 34].

Because several miRNAs have been detected to be altered in DM1 cardiac and muscle tissues [21, 22, 23, 24, 35], and there are numerous drugs that work in DM1 animal models pending accurate pharmacological development and clinical testing in humans [36, 37, 38, 39], we explored the possibility that misexpression of specific serum miRNAs could be identified as non-invasive DM1 biomarkers. To this end, we profiled 175 miRNAs in the peripheral blood serum of DM1-affected individuals and healthy controls by real time qPCR. Even though none of them showed expression differences greater than 2.6 fold, the six miRNAs with the highest fold-change score (miR-103, miR-107, miR-21, miR-29a, miR-30c, and miR-652) were further investigated.
investigated but no significant differences between the control and DM1 conditions were found for any of them.

**Materials and Methods**

**Sample collection and serum isolation**

This study was approved by the Ethics Committee at the University of Valencia. All blood samples were taken after specific written informed consent to participate in the present study. All individuals were Subjects with DM1 were ambulatory adults with proven CTG expansions. Peripheral blood samples were obtained by venous punctures with a fine bore needle (21 G ¾") of 26 DM1 and 22 healthy individuals (Tables 1 and 2) and placed in serum collection tubes (BD VACUTAINER SST II ADVANCE). After 10 min centrifugation at 1200 g at room temperature, the serum was aliquoted and kept at -80°C until use. For CTG repeat size determination, genomic DNA was isolated from peripheral blood leucocytes [40] and was processed for Southern blotting with a^32_P-labelled cDNA25 probe (S1 Fig) or, alternatively, the CTG-repeat region was amplified by PCR using DM101 and DM102 as primers [41, 42, 43, 44].

**RNA extraction and cDNA synthesis**

We assayed for the presence of oxyhaemoglobin in the serum samples because haemolysis has been described to affect the levels of certain miRNAs [45]. The absorbance at 414 nm was determined spectrophotometrically and samples with an absorbance higher than 0.2 were discarded, as this is the cutoff at which samples have previously been considered to be haemolysed [45]. Independent total RNA extraction was performed for each serum sample using the miRNAeasy Mini kit (Qiagen). Briefly, 500 μL of serum was thawed on ice, centrifuged for 5 min at 3000 g at 4°C and 200 μL of the supernatant serum was taken and mixed with 750 μL QIAzol containing 1.25 mg/mL bacteriophage MS2 RNA as a carrier. The extraction was performed according to the manufacturer’s instructions, except that the final wash (with RPE buffer) was performed three times instead of once. Total RNA was eluted with 50 μL water, and cDNA synthesis was performed with 4 μL of total RNA using the Universal cDNA synthesis II kit (Exiqon).

**MicroRNA expression profiling and validation**

The miRCURY LNA™ Universal RT microRNA PCR assay and the Serum/Plasma Focus microRNA PCR Panel (Exiqon) was used for miRNA expression profiling. These panels
contain primers for the detection of the 175 most-expressed miRNAs in human serum (S1 Table). Each 384-well plate contained 2 complete panels of primers and 2 negative controls; real-time PCR was performed according to the manufacturer’s instructions, and cDNAs from a DM1 patient and a control sample were amplified in parallel in each plate. Expression values were calculated using the $2^{-\Delta\Delta C_{t}}$ method [46] using the mean Ct of miRNAs detected (Ct $<34$) for normalisation (S1 Table). During the validation step, the analysis of expression of these miRNAs used real-time PCRs with specific miRCURY LNA microRNA PCR primers (Exiqon). The GeNorm and Normfinder algorithms were used to find optimal reference genes to normalise the expression of the miRNAs being validated [47, 48]. Expression level determinations were performed using an Applied Biosystems 7900HT Fast Real-Time PCR System.

**Statistical analysis**

A logarithmic transformation (log2) was used to normalise the expression data in the profiling experiment. Expression differences were analysed using the Student t-test and different methods for multiple-testing correction were applied, including Bonferroni, Benjamini-Hochberg (False Discovery Rate), Westfall-Young, and Benjamini-Yekutieli corrections. Cluster software was used for hierarchical clustering analysis of genes and samples. Euclidean distances and the average linkage method were selected, using the normalised expression values of each miRNA to represent clusters.

**Table 2. Information about the samples used in qPCR.**

| sample | sex | age | (CTG)n | sample | sex | age | (CTG)n |
|--------|-----|-----|--------|--------|-----|-----|--------|
| P_1    | male| 48  | 667    | C_11   | male| 40  | -      |
| P_2    | male| 41  | 500    | C_12   | male| 53  | -      |
| P_4    | male| 50  | 333    | C_13   | male| 44  | -      |
| P_5    | male| 54  | 333    | C_14   | male| 54  | -      |
| P_6    | male| 56  | 333    | C_15   | male| 45  | -      |
| P_7    | male| 48  | 333    | C_16   | male| 42  | -      |
| P_8    | male| 57  | 1333   | C_17   | male| 43  | -      |
| P_9    | male| 56  | 1000   | C_19   | male| 41  | -      |
| P_10   | male| 50  | 333    | C_20   | male| 46  | -      |
| 21     | male| 56  | 70     |        |      |      |        |
| 22     | female| 61  | 333   | 51     | male| 58  | -      |
| 23     | female| 44  | 1000  | 52     | male| 49  | -      |
| 24     | female| 53  | 730   | 53     | male| 52  | -      |
| 25     | female| 41  | 500   | 54     | female| 59 | -      |
| 26     | female| 42  | 833   | 55     | female| 59 | -      |
| 28     | female| 46  | 667   | 57     | female| 57 | -      |
| 30     | female| 45  | 667   | 58     | female| 65 | -      |
| 32     | female| 48  | 1333  | 60     | female| 61 | -      |
| 33     | male| 26  | 1000  | 61     | female| 55 | -      |
| 34     | male| 38  | 1333  | 62     | female| 51 | -      |
| 35     | male| 37  | 1000  | 64     | male| 34  | -      |
| 36     | male| 39  | 1333  | 65     | female| 25 | -      |
| 37     | male| 31  | 333   |        |      |      |        |
| 38     | male| 36  | 400   |        |      |      |        |
| 39     | male| 30  | 333   |        |      |      |        |
| 40     | male| 37  | 333   |        |      |      |        |

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Results

MicroRNA expression profiling in human myotonic dystrophy type 1 serum

A total of 175 miRNA expression levels were obtained from each peripheral blood serum total-RNA sample using commercial microRNA PCR panels (Exiqon; S1 Table). miRNA profiling was initially carried out with peripheral blood from 10 male DM1-affected individuals (aged 51.3 ± 1.6; P01-P10), expressing between 333 and 1333 CTG repeats (in blood samples), and 10 sex and age-matched controls (aged 46.1 ± 1.7; C11-C20) that did not display any neuromuscular disorders (Table 1). The absorbance at 414 nm was measured in all the samples to discard the possibility of haemolysis, which can occur during blood collection and has a potentially substantial impact on serum miRNA content [49] (S2 Table). Because two samples, P3 and C18, generated a positive result for this parameter (absorbance > 0.2) they were discarded during data analysis (S3 Table). Expression data from each sample was initially normalised to the mean values of all 175 miRNAs. Statistical analysis of the results (Student t-test) showed 35 miRNAs with a $P$-value lower than 0.05, of which 24 miRNAs were up- and 11 were downregulated when compared to controls (Fig 1A, S3 Table). However, only miR-21 was significantly downregulated in DM1 according to three different statistical corrections (Bonferroni, Benjamini-Hochberg (False Discovery Rate) and Westfall-Young, S3 Table). It is worth mentioning that all the differences in expression levels detected between controls and DM1 patients were relatively low (below 2.6-fold) compared to other described biomarkers [50, 51, 52]. Given the controversy regarding the most appropriate way to normalise data when determining miRNA expression values, in addition to mean normalisation, we also normalised the data to specific miRNA expression levels [53]. For that purpose we used two different algorithms, NormFinder and geNorm [47, 48], to identify the most stable miRNAs from our study cohort (S4 Table). Therefore we normalised the miR-15a, miR-23a, miR-28-3p, and miR-484 expression levels to the mean of miR-15a, miR-23a, and miR-484 and the mean of miR-15a and miR-28-3p (S3 Table). In most cases, miR-21 was the only miRNA with significantly different expression between the controls and patients.

We carried out additional statistical analyses using G*C Power software to select additional candidate miRNAs to validate by qPCR. We chose miRNAs with the highest fold-change and with a Power value ~ 1, which included miR-21. Considering these parameters, we selected the six most promising miRNAs for validation: miR-103, miR-107, miR-21, miR-29a, miR-30c, and miR-652 (Fig 1B and 1C).

Expression quantification of six candidate miRNAs in serum

We experimentally determined individual expression levels of miR-103, miR-107, miR-21, miR-29a, miR-30c, and miR-652 in the same nine DM1 and nine control serum samples used during the initial profiling. The data were normalised to miR-15a, the most and second-most stable miRNA from all of the samples, according to geNorm and NormFinder, respectively (Fig 2A and 2B; S4 Table). However, we did not detect statistically-significant differences between DM1 and control samples, not even for miR-21, the only miRNA that was positive after the profiling (Fig 2C).

Considering that from among all of our results, data supporting miR-21 misexpression was the strongest, we decided to carry out further analyses on it. To prevent potential false-negative results because of the miRNA selected as a normaliser, we normalised miR-21 to the expression of the miRNA with the strongest alteration in the opposite direction because this ratio would be independent of any endogenous control. Taking into account data from the profiling
miR-21 expression was downregulated 2.4 times in DM1 samples while miR-130a was upregulated, with a 2.5-fold change (Fig 3A). Next, we used serum samples from 21 DM1 male and female individuals and 17 counterpart controls to quantify expression levels of miR-21 and miR-130a. We confirmed the absence of haemolysis in all the samples by measuring absorbance at 414 nm (S2 Table). However, again, we were unable to detect any significant difference in the miR-21 to miR-130a ratio between controls and DM1 samples (Fig 3B).

Discussion

The only method available for monitoring the progression of DM1 is clinical assessment provided by semiquantitative scales, which correlates poorly with underlying biological defects [54]. A more targeted strategy which characterises muscle involvement is the transcriptomic analysis of muscle biopsies via invasive techniques. These analyses have led to the recent proposal that suggests that alternative splicing events in skeletal muscle can serve as valid biomarkers for quantifying the severity of DM1 and its likely response to therapy [14]. Nevertheless, the different patterns of muscle involvement in DM1, and the invasive nature of the approach, inherently limits this proposal as a good measurement of outcome. An alternative method for neuromuscular diseases is to use blood miRNAs as biomarkers. Cacchiarelli et al. described three miRNAs that correlated with disease severity in Duchenne muscular dystrophy where, as a consequence of muscle-fibre damage, muscle miRNAs are released into the bloodstream [34]. However, cell membranes remain undamaged in DM1 muscle fibres [55] and, consequently, (S1 Table), miR-21 expression was downregulated 2.4 times in DM1 samples while miR-130a was upregulated, with a 2.5-fold change (Fig 3A). Next, we used serum samples from 21 DM1 male and female individuals and 17 counterpart controls to quantify expression levels of miR-21 and miR-130a. We confirmed the absence of haemolysis in all the samples by measuring absorbance at 414 nm (S2 Table). However, again, we were unable to detect any significant difference in the miR-21 to miR-130a ratio between controls and DM1 samples (Fig 3B).
levels of myomiRs in blood in these patients are not expected to be as dramatically increased as a result of the disease as in Duchenne muscular dystrophy.

In the present work we profiled 175 miRNAs in serum samples but we did not observe differences greater than 2.6-fold between DM1 patients and healthy individuals. Owing to the novelty in the use of miRNAs as biomarkers, there is a lack of consensus regarding different technical aspects such as sample quality [56]. However, it was demonstrated that haemolysis can occur during blood collection and this can have a substantial effect on miRNA content in plasma or serum [49]. This fact shows the relevance of good sample quality control for the results obtained when searching for miRNA biomarkers. Initially, we performed the profiling with ten control and ten DM1 patient samples, however one sample from each group was removed because of unacceptable haemolysis levels. After the profiling we identified 35 altered candidate-miRNAs. However, after applying Bonferroni correction only one, miR-21, was statistically different.
It was recently published that four muscle-specific miRNAs, miR-1, miR-133a, miR-133b, and miR-206, were altered in serum from DM1 patients [57]. Of these, miR-1, miR-133a, and miR-133b were included in our profiling panel, however, we did not detect differences in their expression levels. There are two aspects that should be considered: Firstly, that in this aforementioned work the data were normalised to miR-16 expression, however other work has shown that levels of this miRNA vary as a function of haemolysis levels because it is one of the most abundant miRNAs in red blood cells [45, 49, 58], and thus miR-16 levels may be unacceptably influenced by haemolysis. Moreover, it was recently observed that miR-16 is sequestered by long CUG repeats [59] and consequently the amount of free miR-16 in the bloodstream may not be equal in healthy and DM1-affected individuals. Secondly, different methods may identify different sets of altered miRNAs. Indeed, a recent review showed that plasma and serum miRNAs described as breast cancer biomarkers in different publications in the literature do not overlap with each other [60], and although the exact reasons remain unclear, methodological differences in experimental procedures may be one major cause [61].

Another group, using a similar approach to ours, identified nine miRNAs that were differentially expressed between healthy controls and DM1 individuals [62]. Of those, seven were included in our profiling panel, however, none of them were positive. Of note, Perfetti et al. used plasma samples in their research, whereas we used serum; this is worth mentioning because differences in miRNA and RNA levels in serum vs. plasma have been reported [28]. The authors suggested that miRNAs are released from blood cells into serum during the
coagulation process, although they did not identify the reason for this. Therefore, results regarding the biomarkers identified using serum vs. plasma are not comparable. It is also noteworthy that we used different measurement platforms to those employed by Perfetti et al.: the Taqman and Exiqon miRNA qPCR panels, respectively. In this regard, Wang et al. demonstrated that the consistency between results obtained using both platforms is low [28], finding that from 358 miRNAs, only ~19% were detected by both platforms, and that Taqman measurements were 6.7 Ct-values higher than those from Exiqon [28].

After miRNA profiling we identified only one miRNA that was differentially expressed: miR-21. We tried to validate five additional miRNAs by qPCR, although the differences between controls and DM1 were not statistically significant, and we did not observe differential expression in any case. qPCR data were normalised to miR-15a because two different algorithms identified it as the most stable miRNA from among all of the samples. In addition, miR-21 expression was assessed as a ratio to miR-130a, however, neither of these analyses revealed significant differences. We used serum samples from males in the profiling, and samples from both genders during the validation where a higher number of samples was needed. However, it is unlikely this had any significant effect on our results because the qPCR results for each gender were similar to the results obtained from the combined sample analysis.

In summary, we conclude that, under our reported conditions, the miRNAs miR-103, miR-107, miR-21, miR-29a, miR-30c, and miR-652 are not useful serum biomarkers for DM1. Although the successful use of miRNAs from body fluids as disease-severity and progression biomarkers in other studies represents an encouraging advance, several technical aspects must first be standardised because methodological differences in the experimental procedures seem to be the main reason that data from different studies do not coincide [61].

Supporting Information

S1 Fig. Representative example of Southern blot used for CTG repeat size determination.
Arrows indicate wild type alleles. Mutant alleles in patients 1,3, 4 and 5 present more than 1000 CTG repeats while in patient 2 the mutant allele has a a repeat size of ~600 repeats.
(TIF)

S1 Table. Cts values for 175 miRNAs in DM1 and controls serum.
(XLS)

S2 Table. Absorbance at 414 nm for sera used in the assay.
(XLS)

S3 Table. Statistical analyses.
(XLS)

S4 Table. Genorm and Normfinder algorithms output for most stable miRNAs in the study.
(XLS)

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Author Contributions

Conceived and designed the experiments: JMFC BL MCAA MPA ALM ALC RA. Performed the experiments: JMFC BL. Analyzed the data: JMFC BL ALC RA. Contributed reagents/
materials/analysis tools: ALM. Wrote the paper: AB JMFC. Managed clinical data and processed human samples: MZ.

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