Circulating MicroRNAs in Duchenne Muscular Dystrophy

Nahla O. Mousa\textsuperscript{a,b}, Ahmed Abdellatif\textsuperscript{a,⁎,++}, Nagia Fahmy\textsuperscript{c}, Suher Zada\textsuperscript{b}, Hassan El-Fawal\textsuperscript{b}, Ahmed Osman\textsuperscript{d,e,***}

\textsuperscript{a} Biology Department, and Biotechnology Program, School of Sciences & Engineering, The American University in Cairo, School of Sciences and Engineering, 11835, Cairo, Egypt
\textsuperscript{b} Biotechnology Department, Faculty of Science, Cairo University, 12613, Giza, Egypt
\textsuperscript{c} Neuromuscular Unit, Neuropsychiatry Department, Faculty of Medicine Ain Shams University, 11566, Cairo, Egypt
\textsuperscript{d} Biotechnology Department, Basic and Applied Sciences Institute, Egypt-Japan University of Science and Technology, Borg Al Arab, 21934, Egypt
\textsuperscript{e} Biochemistry Department, Faculty of Science, Ain Shams University, 11566, Cairo, Egypt

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ABSTRACT

Objective: The diagnosis of Duchenne Muscular Dystrophy (DMD) currently depends on non-specific measures such as Creatine kinase (CK) levels. MicroRNAs (miRNAs) are a class of small, endogenous RNAs of 21–25 nucleotides, that are important regulators for numerous physiological and pathological processes. The aim of the current study is to assess the potential of miRNAs as non-invasive biomarker for the diagnosis of DMD and for identifying carriers.

Patients and methods: Thirty healthy subjects and 29 families with one member diagnosed with DMD were enrolled in the study. Peripheral blood samples were collected from all subjects where microRNAs were extracted from plasma followed by the quantification of miR-499, miR-103a-3p, miR-103a-5p, miR-206, miR-208a, miR-223 and miR-191-5p. MLPA and NGS were carried out as a gold standard technique to identify the mutations in the participants.

Results: Our data revealed that miR-499 was significantly upregulated in all DMD patients, and true carriers (mothers), while 78 % of potential carriers (sisters) exhibited high levels of this miRNA. Similarly, miR-103a-3p showed an increase in the patients’ families although to a lesser extent. On the other hand, miR-206 and miR-191-5p were significantly downregulated in the majority of the DMD patients and the tested female family members. MicroRNA miR-103a-3p and miR-208a followed a comparable trend in patients and mothers.

Conclusions: Our results suggest that the plasma levels of miRNAs have the capability to diagnose DMD patients and more importantly, miRNAs can be used to identify female carriers.

1. Introduction

Duchenne Muscular Dystrophy is a lethal X-linked recessive disorder that is caused by mutations in the dystrophin gene (DMD). Such mutations result in c-terminal truncation of the dystrophin protein due to premature translation termination (non-sense point mutations or frame shift mutations) mainly because of exon deletions. The disease occurs in about 1 in 5128 to 6289 newborn males and is characterized by progressive muscle wasting that eventually will involve respiratory and cardiac muscles [1,2].

DMD gene (Xp21.29: p21.1) encodes dystrophin which binds to the intracellular cytoskeleton via the association of its N-terminus with actin filaments. Whereas its C-terminus interacts with members of the dystrophin-glycoprotein complex (DGC), forming a link between cytoskeletal actin and the extra-cellular matrix, which stabilizes muscle sarcolemma [3,4].

Dystrophin is normally present in skeletal, cardiac, smooth muscles as well as in some neuronal cell types [3,5]. Absence of dystrophin-DGC complexing destabilizes the sarcolemma thus increasing intracellular calcium influx [6] causing proteases activation which results in proteins degradation [7] and eventually causing cell death. Excessive release of Creatine Kinase (CK) is a typical consequence of the apoptotic reaction of myocytes [8]. Activation of proliferative pathways as a response to cellular apoptosis causes the infiltration of muscle tissue with adipose
and fibrous tissues, which causes progressive muscle weakness [9–11]. Death usually occurs due to respiratory failure or cardiac complications [12].

The diagnosis of DMD currently depends on CK levels, however CK is non-specific and its levels could significantly increase in other medical conditions that involve excessive muscle destruction, and hence CK levels could be misleading to some extent [13,14]. To accurately diagnose DMD, molecular techniques along with muscle biopsy should be performed in addition to clinical assessment. However, molecular techniques are expensive and have some limitations in the

Fig. 1. Levels of studied microRNAs in plasma of DMD children compared to controls. Panel (A) to (G); mir-206, mir-103a-3p, mir-223, mir-208a, mir-191-5p, mir-103a-5p and mir-499 respectively.
diagnosis process [15,16] and muscle biopsy is not informative due to its heterogenic nature. Also, muscle strength will significantly vary from patient to patient and in different muscles within the same patient [17].

MicroRNAs (miRNAs) are small, endogenous RNA molecules around 21 nucleotides in length, which are epigenetic regulators that act through binding specific mRNAs and thus, target them for degradation and hence block the protein translation [18,19]. MicroRNAs could be present in tissues and different bio-fluids such as plasma [20] and their levels change dramatically in different cellular contexts [21]. Thus, due to association with various diseases, circulating miRNAs have gained momentum for their promising use as biomarkers that can aid disease diagnosis and/or have prognostic values to monitor disease progression as well as having the potential for therapeutic intervention [22].

Some microRNAs were shown to be abundantly expressed in skeletal and cardiac muscle cells like miR-499, miR-206, miR-208a, miR-103a, miR-109-5p. These muscle-specific miRNAs could be detected in minute amounts in other tissues [23–27]. A recent study by Li et al. [28] found that the serum levels of a group of miRNAs, which include mir-1/133/206/208a/208b/499 have significant diagnostic and prognostic values for DMD [28].

Likewise, other X-linked disorders, DMD affects mainly male patients, who show disease symptoms. However, in some instances, female carriers may exhibit variable degree of symptoms, but the majority are asymptomatic. Thus identification of carriers is one of the major concerns in genetic counseling [29].

In our study, we attempted to investigate the diagnostic/prognostic potential of some microRNA species for detection of DMD. In the meantime, we wanted to evaluate the value of the selected miRNAs in identifying disease carriers.

Table 1

Results for Mann-Whitney Test showing the difference between microRNA levels between patients and control group.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>p-value (DMD compared to control group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir 103a-3p</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>mir 223</td>
<td>.883</td>
</tr>
<tr>
<td>mir 208a</td>
<td>.013</td>
</tr>
<tr>
<td>mir 103a-5p</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>mir 206</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>mir 191-5p</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Values in bold are statistically significant (p < 0.05).

2. Patients and methods

2.1. Ethical declaration

The human study (DMD patients, families and controls) was guided by the principles of the Declaration of Helsinki for medical research and it was approved by the Institutional Review Board of the American University in Cairo and Ain Shams University medical school, Cairo, Egypt. Informed consent was acquired from enrolled participants and guardians of DMD patients.

2.2. Human subjects

Thirty healthy subjects and 29 families (who have one member diagnosed with DMD) were enrolled in the study. Subjects were grouped into 4 groups (DMD patients, mothers, sisters and the control group). The control group was comprised of healthy individuals with similar age and sex of the other participants; 10 subjects of same age range and sex served as controls for each study group. Venous peripheral blood samples were collected on EDTA from all subjects for miRNA extraction [30].

Inclusion Criteria: 29 DMD patients, 3–17 years old males, along with some of their female family members were enrolled from the Neuromuscular unit, Ain Shams University Hospital, Faculty of Medicine, Ain Shams University, Cairo, Egypt between 2017-2018. Patients’ diagnosis was confirmed with Multiplex Ligation dependent probe amplification (MLPA) to detect large deletions or duplications [31]. Alternatively, targeted Sequencing of DMD gene was performed in cases of non-deletion /duplication mutations.

Exclusion Criteria: Patients with active cancer, muscle trauma, active infection or amputation were excluded from the study.

2.3. MicroRNA extraction and reverse transcription

MicroRNA extraction was carried out using 200 μl plasma using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions. Isolated microRNAs were subjected to a reverse transcription reaction to synthesize cDNAs using miScript II RT Kit (Qiagen, Germany) following the recommended manufacturer’s instructions.

2.4. Quantification of miRNAs

MicroRNAs were selected based on their involvement in the regulation of vital physiological processes in muscle fibers and myocytes [23,32–34]. The selection was further confirmed with the help of data mining using specific databases such as miRSearch v. 3.0, miRBase and Target Scan v. 7.2 [35]. For miRNAs normalization, miR-16 was used as
describe previously by Lange et al. 2017 [36]. We further compared miR-16 to RNU6-6P to determine which one should be used as Normalizer. The microRNA, miR-16, showed lower standard deviation than RNU6-6P. miRNA stability was assessed through quantifying the levels of miR-16 and RNU6-6P in different periods; after 1-week storage in −20 °C and −80 °C and after long-term storage at −20 °C and −80 °C. Data analysis revealed that the levels of the microRNAs were unchanged even after 6 months of storing the samples at −20 °C or 9 months at −80 °C.

Real-time PCR using miScript SYBR Green PCR kit (Qiagen,
Germany) along with miRNA-specific primers (0.25 pmole/reaction; Eurofins, Belgium) was performed to determine the circulating levels using miRNA-16 as a normalizer [37–39]. Ten-fold diluted miRNA cDNA reactions of miRNAs (1 μl) were used as templates in the qPCR reactions using CFX96 thermal cycler (BioRad, USA). Reactions were incubated at 92 °C for 15 min, followed by 45 cycles of 95 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s. Data of melt curve analysis was obtained to confirm reactions’ specificity. Amplification reactions were performed in triplicate. No template control reaction in addition to -RT reactions were included in every assay.

2.5. Data and statistical analysis

Quantification cycle (Cq) values were calculated with the CFX manager software (California, United States). Data normalization was performed using the most stable miR-16. Fold change was calculated using Livak analysis (2 ΔΔCt) method [40]; stringent cutoffs were used (fold change values greater than 1.5 were considered over expression and values less than -1.5 were considered downregulation) [41]. Raw data could be obtained from the Supplementary excel file.

Statistical analysis was performed using IBM SPSS® Statistics version 23 (IBM® Corp., Armonk, NY, USA). For Numerical data, mean ± standard deviation or median and range were used for data expression. Qualitative data were expressed as frequency and percentage. Mann-Whitney test (non-parametric t-test) was used for quantitative data that are not normally distributed. Spearman-rho method was used to test correlation between numerical variables. Wilcoxon signed ranks test (non-parametric paired t-test) was used for correlation analysis (DMD patients and their mothers and sisters). The Receiver Operating Characteristic (ROC) curve was used for prediction of cut off values for differentiating patients with DMD and their mothers form the control healthy persons and presented as area under the curve and its 95 % confidence interval. All tests were two-tailed. A p-value at α < 0.05 is considered significant. No missing values were reported.

3. Results

3.1. Levels of miRNAs in the plasma of DMD patients

In an attempt to investigate the role of the selected miRNAs in the onset and progression of DMD, plasma levels of miRNAs -499, -103a-3p, -223, -208a, -103a-5p, -206 and -191-5p were quantified in the study subjects (n = 29) and compared to those of the healthy controls (n = 10). Data was presented as fold change of each miRNA level relative to that of the healthy controls.

Interestingly, miR-499 was not detected in the plasma of control subjects, while it could be detected in the plasma samples of all the patients (for calculation purposes, we assumed that the Ct cycle of the control, calibrator, is 50). Similarly, miR-103a-3p exhibited a significant up-regulation in 27 out of 29 patients (2-23 folds). On the other hand, the levels of miR-206 were significantly decreased in 23 patients (1.66–67 folds), 3 patients showed a marginal increase while the remaining 3 patients had comparable levels to that of the controls. Likewise, the plasma levels of miR-191-5p were downregulated in 21 patients (1.83–53 folds), while 7 patients showed levels comparable to those of the controls. The levels of miR-103a-5p were also downregulated in 16 patients (1.56–58.89 folds), 5 patients had higher levels than controls, and 8 patients had the same levels as that of the controls. Cardiac muscle specific miRNA 208a, was also significantly downregulated in 16 DMD patients (1.6–100 fold decrease), 4 patients showed higher levels and in the rest of the patients, levels were similar to that of the control subjects. The plasma levels of miR-223 levels did not show a specific trend in the patients since 11 patients had increased levels (1.5–25.6 folds), while 11 patients had lower levels than that of the controls (1.88–79 folds) (Fig. 1, Supplementary Fig. 1, Tables 1 and 2).

3.2. Levels of miRNAs in the plasma of Female Carriers

Data of the circulating plasma levels of tested miRNAs in DMD true carriers (mothers of DMD patients, n = 26), were compared to that of female controls of comparable age (n = 10). Like DMD patients, miR-499 could be detected in plasma samples of all mothers included in the study, while it was undetectable in the controls (here, we also considered the Ct value of the calibrators to equal to 50 for calculation purposes). Following the same pattern of DMD patients, miR-103a-3p, showed elevated levels in 19 out of 26 mothers (73.0 %; 2–17 folds), while 6 mothers had levels comparable to that of the controls and one mother had slight decrease in this miRNA’s level. On the other hand, the plasma levels of miRNAs -206, -191-5p, -208a, -103a-5p and -223 showed variable degrees of downregulation of their plasma levels in the majority of mothers (22, 21, 19, 18 and 13 out of 26 participating

Table 3

<table>
<thead>
<tr>
<th>microRNA</th>
<th>p-value (mothers compared to control group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir 103a-3p</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>mir 223</td>
<td>.073</td>
</tr>
<tr>
<td>mir 208a</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>mir 103a-5p</td>
<td>.010</td>
</tr>
<tr>
<td>mir 206</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>mir 191-5p</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Values in bold are statistically significant (p < 0.05).

Table 4

Correlations between levels of microRNAs in true carriers.

<table>
<thead>
<tr>
<th>age</th>
<th>miR-499</th>
<th>miR-103a-3p</th>
<th>miR-103a-5p</th>
<th>miR-223</th>
<th>miR-208a</th>
<th>miR-206</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
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<td>-.274</td>
<td>-.280</td>
<td>.599</td>
<td>.721</td>
<td>.880</td>
</tr>
<tr>
<td>P</td>
<td>.274</td>
<td>.029</td>
<td>.280</td>
<td>.999</td>
<td>.007</td>
<td>.001</td>
</tr>
<tr>
<td>r</td>
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<td>.428</td>
<td>.529</td>
<td>.488</td>
<td>.488</td>
<td>.693</td>
</tr>
<tr>
<td>P</td>
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<td>.093</td>
<td>.066</td>
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<td>.043</td>
<td>.031</td>
</tr>
<tr>
<td>r</td>
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<td>.663</td>
<td>.578</td>
<td>.878</td>
<td>.878</td>
<td>.878</td>
</tr>
<tr>
<td>P</td>
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<td>.002</td>
<td>.693</td>
<td>.001</td>
<td>.683</td>
<td>.683</td>
</tr>
<tr>
<td>r</td>
<td>.220</td>
<td>.229</td>
<td>.280</td>
<td>.073</td>
<td>.073</td>
<td>.073</td>
</tr>
<tr>
<td>P</td>
<td>.01</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
<td>.017</td>
<td>.017</td>
<td>.017</td>
</tr>
<tr>
<td>r</td>
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<td>.073</td>
<td>.073</td>
<td>.413</td>
<td>.520</td>
<td>.455</td>
</tr>
<tr>
<td>P</td>
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<td>.002</td>
<td>.036</td>
<td>.036</td>
<td>.006</td>
<td>.019</td>
</tr>
<tr>
<td>r</td>
<td>-.284</td>
<td>-.284</td>
<td>-.284</td>
<td>-.086</td>
<td>-.086</td>
<td>-.052</td>
</tr>
<tr>
<td>P</td>
<td>.487</td>
<td>.013</td>
<td>.634</td>
<td>.036</td>
<td>.036</td>
<td>.036</td>
</tr>
<tr>
<td>r</td>
<td>-0.31</td>
<td>-0.31</td>
<td>-0.31</td>
<td>-.545</td>
<td>-.545</td>
<td>-.545</td>
</tr>
<tr>
<td>P</td>
<td>.618</td>
<td>.129</td>
<td>.455</td>
<td>.763</td>
<td>.739</td>
<td>.782</td>
</tr>
</tbody>
</table>

Values in bold are statistically significant (p < 0.05).
Fig. 3. Comparison of the levels of microRNAs in the plasma of potential carriers (sisters of DMD patients) and the controls. Panel (A) to (G): mir-103a-3p, mir-223 mir-208a, mir-206, mir-191-5p and mir-499 respectively.
DMD patients’ sisters may pass defective DMD genes to their male children if they receive a defective X-chromosome from their mothers. In this context, identification of true carriers among patients’ sisters, when associated with pre-natal diagnosis, is considered of utmost importance towards decreasing the magnitude of the disease as an essential mother in DMD disease control. In our study, we tackled this issue in the attempt to evaluate the diagnostic potential of the tested miRNAs. Eighteen sisters (ages 1–28 years) were enrolled in the study and the data was compared to female controls of matched age range (n = 10). Regarding miR-499, whose circulating levels were exclusively detected in DMD patients and their mothers, displayed a similar pattern in 14 out of 18 sisters, while 4 sisters did not show any detectable levels of this miRNA. In the meantime, as compared to the controls, miRNA-103a-3p also showed elevated levels in 16 out of 18 sisters (2.2–54 folds). Conversely, miR-206 and miR-191-5p showed lower levels in 16 out of 18 sisters. Other miRNAs (miR-208a, miR-103a-5p and miR-223) did not exhibit clear pattern in the sisters, where some had high levels, others had low levels and the rest did not show any change than the control (Fig. 3 and Supplementary Fig. 1).

3.4. Assessment of the diagnostic potential of circulating miRNAs

Receiver operating characteristics curve (ROC) analysis was carried out to assess the capacity of the circulating levels of the selected miRNAs to diagnose DMD and identify female carriers (Fig. 4a and b). Our data highlighted the potential of miR-499 as a remarkable marker with 100% specificity and sensitivity, capable of detecting the disease and identifying the female carriers. Likewise, miR-206 and miR-191-5p showed comparable levels of specificity and sensitivity in distinguishing DMD patients (AUC = 0.887 and 0.887 respectively; p < 0.0001) and participating mothers (AUC = 0.918 and 0.932 respectively; p < 0.0001). However, miR-208 and miR-103a-5p were less effective in the
Fig. 5. Comparison of the levels of microRNAs in plasma of the 4 groups recruited in this study; DMD patients, mothers, sisters and Control subjects. Panel (A) to (G); mir-499, mir-103a-3p mir-103a-5p, mir-208a, mir-223, mir-191-5p and mir-206 respectively.
Table 5

<table>
<thead>
<tr>
<th>microRNA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir499</td>
<td>.002</td>
</tr>
<tr>
<td>mir 103a-3p</td>
<td>.038</td>
</tr>
<tr>
<td>mir 223</td>
<td>.248</td>
</tr>
<tr>
<td>mir 208a</td>
<td>.469</td>
</tr>
<tr>
<td>mir 103a-5p</td>
<td>.545</td>
</tr>
<tr>
<td>mir 206</td>
<td>.004</td>
</tr>
<tr>
<td>mir 191-5p</td>
<td>.849</td>
</tr>
</tbody>
</table>

Values in bold are statistically significant (p < 0.05).

3.5. Correlation of miRNAs levels among family members of DMD patients and patient’s clinical status

No significant correlation could be found between the miRNAs’ levels and the age of the patients, as an indication of progressive muscle weakness. Similarly, no correlation could be found between miRNA levels and the clinical condition of the patients (ambulant or non-ambulant). However, paired comparisons showed that three microRNA species (miR-499, miR-103a-3p and miR-206) could be correlated between DMD patients and their mothers (Fig. 5 and Table 5).

4. Discussion

Duchenne Muscular Dystrophy (DMD), is a monogenic disorder characterized by progressive muscle wasting due to the absence of one of the fundamental muscular proteins; dystrophin [42]. Currently, creatine kinase (CK) is the only affordable non-invasive biomarker that is used for the diagnosis of DMD. However, in the best-case scenario, CK can detect only 70 % of the DMD cases. In addition, the enzymatic activities greatly vary with the disease progression and may also significantly increase in other pathological conditions that involve excessive muscle destruction such as myocardial infarction. Thus, CK could be a misleading marker [43-46]. Therefore, identifying novel non-invasive, affordable and specific biomarkers that can detect DMD is crucial for the diagnosis and management of the disease.

In this study, we investigated the plasma levels of a group of miRNAs in order to assess their capacities to diagnose the disease and to identify female carriers. The tested miRNAs were selected based on their roles in regulating vital muscle genes [23,32-34]. These roles were further confirmed by searching specific databases [miRBase [47,48]; miRSearch v.3.0, Exiqon, Germany and TargetScan v. 7.2 [35]]. Our hypothesis was that the levels of muscle-related miRNAs in DMD patients will be different than that of the healthy subjects due to progressive death of muscle cells or modifications of their expression by myocytes. Similarly, disease carriers may have variations in the circulating levels of those miRNAs due to presence of mutated copy of the DMD gene which may result in partial functional impairment.

MiR-499 is one of the Myosin-related myomiRs that play important roles in the regulation of muscle function during exercise [49]. This microRNA is expressed in the cardiac muscles as well as slow skeletal muscles and it plays a critical role in switching fibers of the muscle into type I myofibers [27,50]. Our data shows that miR-499 was exclusively found in DMD patients along with their mothers, while it was completely undetected in respective controls. Li et al. [28] reported that miR-499 was highly elevated in DMD patients. Consistently, Khanghiaei et al. [51] found positive correlation between miR-499 levels and serum cardiac Troponin I (cTnI) levels indicating cardiac damage. In addition, elevation in miR-499 levels was also shown to affect cardiac gene expression and is responsible, in part, for predisposition to cardiac stress-induced dysfunction [52]. Thus, our data can be interpreted in the light of the above reports which indicates that the elevation of miR-499 levels is due to the accelerated muscle atrophy in DMD patients and/or involvement of muscle weakness in disease carriers.

Apart from miR-499, when compared to controls, miR-206 exhibited significant downregulation in its circulating levels in 23 out of 29 DMD patients (79.3 %). In fact, our data is contradictory with that of Wong et al. [53] and Hu et al. [54], who reported an increase in miR-206 in DMD patients. However, our finding can be explained on the basis of the loss of the regulatory roles of miR-206 in muscle differentiation and regeneration of myoblasts and neuromuscular synapses [55-57]. Thus, the reduction in the levels of miR-206 is an expected consequence to the dystrophic nature of muscle cells in DMD patients. In the meantime, the contradiction between our data and other studies could be explained on the condition of transient increase of myomarkers in early stages of the disease due to the activation of compensatory mechanisms by muscle fibers. However, mounting such responses in usually followed by shutting down the unsuccessful mechanisms with concomitant deposition of fats and connective tissues in the muscle fibers [58]. Furthermore, miR-206 was shown to promote the regeneration of muscle cells in mdx mouse model of DMD [59]. Consistently with our hypothesis, Liu et al. [59] reported that knocking out miR-206 gene delayed muscle regeneration and accelerated the dystrophic phenotype of the mouse since it acts to promote stem cell differentiation and inhibit myogenesis suppressing factors.

Also, miR-206 expression was shown to enhance the stimulation of muscle-specific mRNA and proteins, in particular miR-206 enhances the expression Utrophin, one of the fundamental proteins in the sarclemma [60].

Although miR-103 (miR-103a-3p and miR103a-5p) is not a muscle-specific miRNA. It was previously shown to be associated with inflammatory conditions like ulcerative colitis [61], and is required for muscle maintenance and is expressed in fast and white muscles, however its expression is higher in the slow muscles compared to fast muscles [62]. In the current study a significant upregulation in the levels of miR-103a-3p was detected in 93 % of the DMD patients. Also, the true carriers showed significantly higher levels, compared to the control group, and 88 % from the sisters group had an elevated level of this miRNA. Regarding 103a-5p, 55 % of the patients had low levels of this miRNA, 70 % of the mothers and 44 % of the sisters showed downregulation of this marker.

Examination of miR-223 in DMD patients and their families, showed 11 patients with higher circulatory levels and another 11 patients with lower levels. In the case of the mothers group, 53 % had low levels, and 30 % had high levels and similar results were obtained upon investigating the sisters group. Greco et al. carried out a study to search for correlation between microRNAs associated with skeletal muscle damage in DMD and repair mechanism during the disease. They found that the expression of miR-223 was modulated in DMD children and DMD mice animal model as well [63]. The expression of miR-223 was found in areas of the muscles where infiltrating inflammatory cells and necrosis or regeneration took place. It was strongly induced in the dystrophic myofibrils [63,64].

Our data analysis showed that 21 patients had downregulated levels of mir-191-5p. In addition, 21 mothers and 16 sisters had low circulatory levels compared to the control groups. Cameron et al. showed that miR-191-5p is positively associated with extension strength and cross-sectional area of the muscles [65]. Also, miR-191-5p was found to be involved in Interleukin-6 signaling and serine/threonine protein phosphatase PP1-beta catalytic subunit (PP1CB) which is abundant in skeletal muscles and is responsible for myogenesis [66,67]. mir191-5p also targets Stat3 pathway, which is crucial for muscle repair mechanism [68]. Such finding explains the detected levels of such miRNA in our samples which might be associated with the muscle wasting and the failure of the muscle repair mechanism.

Quantification of heart specific miR-208a in our study showed that
DMD patients and female carriers had low levels miR-208a. MiR-208a was originally discovered along with miR-208b and miR-499 in a study by Rooj et al. [25]. Rooj et al., found that miR-208a, miR-208b and miR-499, are encoded by MYH6, MYH7 and MYH7B, respectively, and they control the content of myosin inside the muscles, as well as muscular behaviour [27]. Rooj et al., found that miR-208a binds to MYH7 (slow myosin) suppressors in the heart, and it is crucial for the upregulation of MYH7 and miR-208b as a result of stress dependent regulation [27]. Also, miR-208a is known to regulate the expression of miR-499 through regulating MYH7B [27].

5. Conclusion

In the quest to identify new and non-invasive biomarkers to diagnose DMD and to stratify female disease carriers from non-carriers, we found remarkable increase in the levels of miR-499 and miR-103a-3p in DMD patients and disease carriers. Our data revealed that both miRNAs have the potential to diagnose the disease with high specificity and sensitivity and both species have the capacity to identify female carriers. In addition, there was an overall trend of downregulation of other tested miRNAs in the plasma of patients and carriers. In conclusion, miRNAs carry the promise of being effective diagnostic biomarkers for the diagnosis of DMD and the identification of carriers, a finding that may represent a cornerstone towards better disease management and to reduce the societal impact of DMD. Currently we are testing the therapeutic potential of these miRNAs to further understand the underlying mechanisms of their roles.

Funding details

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Data availability statement

Supplementary data are available on request.

CRediT authorship contribution statement

Nahla O. Mousa: Investigation, Data curation, Writing - original draft. Ahmed Abdelatif: Supervision, Writing - review & editing. Nagia Fahmy: Conceptualization, Methodology, Supervision, Validation. Suher Zada: Supervision, Validation. Hassan El-Fawal: Supervision, Validation. Ahmed Osman: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Supervision, Validation.

Declaration of Competing Interest

The authors have no conflict of interest to report.

Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version, at doi:https://doi.org/10.1016/j.clineuro.2019.105634.

References

Supplementary Figure 1: A) A Graph representing the number of DMD patients (total; n=29) with modulation in the levels of different microRNAs detected in the plasma using qRT-PCR. B) A Graph representing the number of mothers/ True carriers (total; n=26) with modulation in the levels of different microRNAs. C) A Graph representing the number of sisters/ potential carriers (total; n=18) with modulation in the levels of different microRNAs.