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Digital PCR quantification of miR-30c and miR-181a as serum biomarkers for Duchenne muscular dystrophy

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Abstract

Circulating microRNAs (miRs/miRNAs) are being used as non-invasive biomarkers for diagnosis, prognosis and efficiency of clinical trials. However, to exploit their potential it is necessary to improve and standardize their detection. In a previous study, we identified two microRNAs, miR-30c and miR-181a, that appear to be key regulators of muscular dystrophy. We hypothesized that they could represent useful biomarkers of Duchenne and Becker muscular dystrophies (DMD and BMD). The objective of this study was to assess the absolute levels of miR-30c and miR-181a in sera of DMD and BMD patients using digital PCR (a robust technique for precise and direct quantification of small amounts of nucleic acids without standard curves and external references), and investigate the correlation between miR-30c and miR-181a expressions and several clinical parameters. Our results show that the serum levels of miR-30c and miR-181a increased 7- and 6-fold respectively in DMD patients (n = 21, 2–14 years, ambulant), and 7-fold in BMD patients (n = 5, 9–15 years) compared to controls (n = 22, 2–14 years). No association between miRNA levels and age or corticosteroid treatment was detected in DMD. However, there was a trend towards higher levels of miR-30c in DMD patients with better preserved motor function according to various motor scales and timed tests. We demonstrate that digital PCR is a useful technique for accurate absolute quantification of microRNAs in sera of DMD/BMD patients. We propose miR-30c and miR-181a as reliable serum diagnostic biomarkers for DMD and BMD and miR-30c as a potential novel biomarker to assess disease severity in DMD.

Keywords: Digital PCR; MicroRNAs (miRs); Biomarkers; Serum; Muscular dystrophy; Outcome measurements

1. Introduction

Duchenne muscular dystrophy (DMD) is the most common childhood muscular dystrophy affecting 1 in 5000 male births [1] and it is caused by mutations in the X-linked *DMD* gene leading to the absence or severe reduction of the dystrophin protein. DMD is characterized by severe and progressive muscle weakness which is accompanied by skeletal muscle fiber degeneration, chronic inflammation, and fibrous and fatty replacement of muscle tissue. In frame deletions in the same *DMD* gene lead to the production of truncated, partially functional protein and a milder phenotype known as Becker muscular dystrophy (BMD) [2,3].

Although diagnosis of DMD and our knowledge of the pathophysiological mechanisms have improved due to the advance of genetic and molecular techniques, early detection, assessment of disease progression and response to treatments are still a challenge. Moreover, the number of active clinical trials for DMD is increasing and most of them are international and multicentric. Therefore, it is important to find and validate reliable biomarkers that can be used in a reproducible manner across several centers and that together with other tools can facilitate diagnosis and prognosis and comparison of clinical trial results. In addition, some of these novel biomarkers could also be used as potential therapeutic targets [4,5].

MicroRNAs (miRNAs or miRs) are small non-coding RNAs (19–22 nucleotides long) that act as post-transcriptional regulators of gene expression. It has been demonstrated that miRNAs regulate a wide range of biological processes in skeletal muscle

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in physiological and pathological states, such as myogenesis, maintenance of muscle mass, metabolism, aging, exercise, as well as muscular dystrophy [6,7]. The use of miRNAs as diagnostic and prognostic biomarkers and as clinical trials outcome measures is well established in various biomedical fields such as oncology and cardiology, due to their specificity, robustness, and their easy accessibility and stability in body fluids [8,9]. In the context of DMD, there is a group of microRNAs called dystromirs (miR-1, miR-133a, miR-133b, miR-31 and miR-206) that are muscle specific and were found to be elevated in serum of DMD patients [10,11]. A correlation between miR-1 and miR-133 levels and respiratory involvement was described in DMD patients but no significant association was found between the amount of dystromirs in serum and validated functional scores such as the North Star Ambulatory Assessment (NSAA) [11]. Studies in mice have identified some miRNAs (miR-29 and miR-21) that play an important role in the TGF-β signaling pathway and thus in the regulation of fibrosis, a hallmark of DMD [12–14].

To date studies of serum/plasma miRNAs in the context of muscular dystrophies have employed qRT-PCR using standard curves for absolute quantification and normalization to endogenous miRNAs or exogenous spike-in miRNAs [10,11,15–17]. As miRNAs are more widely used as biomarkers, it becomes necessary to improve and standardize the methodologies to detect them accurately and reproducibly in biological fluids and correlate their levels with clinical function [4]. Digital PCR (dPCR) is a technique that allows absolute quantification of low abundance nucleic acids without the need for an external reference or standard curve, and is more reproducible than real-time PCR since it is less affected by variations in PCR efficiency [18,19]. dPCR is based on the partitioning of the target molecule into many small parts at limiting dilution so that PCR reactions contain either one or zero molecules. In case of droplet digital PCR (ddPCR), partitions are made of nanoliter-sized, water–oil emulsion droplets containing target and background DNA in a random distribution [20]. Hindson and colleagues compared the performance of both methods to measure expression levels of miRNAs in serum samples from patients with prostate cancer. They found that ddPCR showed greater precision, reproducibility, and significantly reduced variation across replicates; therefore, ddPCR is more efficient resolving patients from controls than qRT-PCR, showing superior diagnostic performance [21].

In a previous publication we identified two miRNAs by gene network analysis, miR-30c and miR-181a, which seem to be key players in muscular dystrophy. Our previous data showed that these miRNAs were significantly increased in tissue and serum from a small group of patients with collagen VI defects and other dystrophies including DMD, and that their levels correlated with some histopathological and biochemical variables including the extent of fibrosis and circulating adiponectin concentrations [22]. We hypothesized that miR-30c and miR-181a could be potential diagnostic and prognostic biomarkers of DMD. Therefore, the objective of this study was to assess the absolute levels of miR-30c and miR-181a in sera of DMD and BMD patients using ddPCR and investigate the correlation between their expressions and various parameters including age, corticosteroid treatment and muscle function using validated functional scores. In the context of muscular dystrophies, this is the first report investigating the potential use of ddPCR for precise and sensitive quantification of miRNAs in sera of DMD/BMD patients. Our results show that miR-30c and miR-181a can serve as reliable serum diagnostic biomarkers for DMD and BMD and miR-30c could be a suitable biomarker to monitor disease severity in DMD patients.

2. Patients and methods

2.1. Ethics statement

This work has been approved by the Ethical Committee of "Fundació Sant Joan de Déu". Written informed consent for research was obtained from all patients and controls (or their parents/legal guardians) according to the Hospital Sant Joan de Déu forms and regulations.

2.2. Study participants

The DMD patients (n = 21, mean age = 8.0 years, range 2–14 years) and BMD patients (n = 5, mean age = 12.7 years, range 9–15 years) were recruited from the clinical population at Hospital Sant Joan de Déu, Barcelona. Patient inclusion criteria were genetically confirmed diagnosis of DMD or BMD, still ambulant, and no severe or moderate learning difficulties or behavioral problems. Nine DMD patients (mean age = 5.1 years, range 2–6 years, except one patient who was 10 years old) were not on corticosteroid treatment and 12 DMD patients (mean age = 10.2 years, range 7–14 years) were taking daily corticosteroids (prednisone 0.75 mg/kg/day or deflazacort 0.9 mg/kg/day).

Age-matched control samples from healthy subjects who came to the Hospital Sant Joan de Déu for minor medical interventions (n = 22, mean age = 8.0 years, range 2–14 years) were collected.

2.3. Outcome measures

The following functional outcome measures were performed in ambulant DMD patients (n = 11–16, mean age = 8.5 years, range 5–14 years). These tests provide a measure of physical ability in ambulatory DMD patients.

6-Minute Walk Test (6MWT) measures the distance a patient can walk in 6 minutes on a hard, flat surface, according to the ATS guidelines [23].

NSAA is a scale consisting of 17 items, ranging from standing (item 1) to running (item 17) and including several abilities such as head raise, hopping or standing on heels. Each item is scored on a 3 point scale using the following criteria: 2 – Normal achieves goal without any assistance; 1 – Modified method but achieves goal independent of physical assistance from another person; 0 – Unable to achieve independently. A total score can be obtained by summing the scores for all the individual items. The score can range from 0 (absence of ambulation) to 34 (normal ambulation) [24].