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MicroRNAs-424 and 206 are potential prognostic markers in spinal onset amyotrophic lateral sclerosis

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ABSTRACT

Introduction: Skeletal muscle microRNAs (miRNAs) are potential candidate biomarkers for amyotrophic lateral sclerosis (ALS) that deserve further investigation.
Objectives: To identify miRNAs abnormally expressed in the skeletal muscle and plasma of patients with ALS, and to correlate them with parameters of disease progression.
Methods: Expression profile of miRNAs in muscle was evaluated using an array platform. Subsequently we assessed the plasmatic expression of candidate miRNAs in a set of 39 patients/39 controls. We employed generalized estimating equations to investigate correlations with clinical data.
Results: We identified 11 miRNAs differentially expressed in the muscle of ALS patients; of these, miR424, miR-214 and miR-206 were validated by qPCR in muscle samples. In plasma, we found only miR-424 and miR 206 to be overexpressed. Baseline expression of miR-424 and 206 correlated with clinical deterioration over time.
Conclusion: MiR-424 and miR-206 are potential prognostic markers for ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the prototypical motor neuron disease and the 3rd most frequent neurodegenerative disorder in adults [1]. The disease is characterized by progressive skeletal muscle weakness and atrophy that culminate in fatal respiratory paralysis within 3–5 years of disease onset [2]. There is no diagnostic test for ALS, and diagnosis still depends on clinical assessments [3]. This limitation results in a diagnostic delay of one year on average. In this setting, biomarkers are urgently needed to ease the specific diagnosis of ALS diagnosis and to help establish prognosis early in the disease course as well as to act as outcome measures in upcoming clinical trials [4].

MicroRNAs (miRNAs) are small molecules that can influence posttranscriptionally gene expression of a variety of transcript targets, and can be released into the circulation by normal and pathologically affected tissues [4]. Because miRNAs are stable in plasma and serum, they are promising potential biomarkers and have been extensively studied in several human illnesses, such as cancer [5], Alzheimer's disease [6–8], Parkinson's disease [9], multiple sclerosis [10], Huntington's disease [11] and multiple system atrophy [12].

Some studies investigated the role of miRNAs in amyotrophic lateral sclerosis. Most of them employed animal models of the disease and identified abnormal expression of miRNAs involved with skeletal muscle integrity [13–15]. However, there are few studies involving patients with ALS, and most of them focused on miRNA expression in tissue samples from deceased patients [4,15]. In two previous studies using serum samples of living patients, miRNA-206 and 338-3p were proposed as potential biomarkers for the disease [15–16]. However, some important issues were not properly addressed in these studies, such as the correlation with clinical parameters and longitudinal assessment.

Therefore, we designed the present study to investigate the expression profile of miRNAs in skeletal muscle and plasma of a representative cohort of patients with ALS. In addition, we assessed whether plasma expression of miRNAs differentially expressed in patients could be related to disease progression. To accomplish that, we correlated expression data with clinical parameters of disease progression over 1 year of follow-up.

2. Methods

2.1. Study design

We summarized the different steps of the study design in Fig. 1.

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2.2. Subjects' selection and protocol approvals

We enrolled patients with definite and probable ALS according to El Escorial criteria regularly followed at the Neuromuscular Outpatient Clinic at UNICAMP hospital between 2012 and 2013 [3]. Every patient underwent a comprehensive nerve conduction/EMG evaluation and fulfilled the Awaji neurophysiological criteria [17]. We did not include patients with exclusive upper (i.e., primary lateral sclerosis) or lower motor neuron (i.e., progressive muscular atrophy, flail syndromes) involvement, or with bulbar presentation. All patients were genotyped for GGGGCC expansions at the gene *C9orf72* but none of them tested positive.

Subjects with concomitant unrelated neurological diseases were excluded as well. We also selected a group of age and gender matched healthy subjects to be used as a control group.

This study was approved by our institution Ethics Committee and a written informed consent was obtained from all participants prior to any study-related procedure.

2.2.1. Expression analyses in skeletal muscle samples

We performed open biopsies of biceps brachii muscle of 5 patients with definite and probable ALS according to El Escorial criteria (mean age of 50.6 ± 14.3 years, 3 men). In all patients, the force of the biopsied muscle was graded as 4/5 (MRC scale). At the time of biopsy, patients were moderately disabled (mean ALSFRS score of 25, range 21–28), but had preserved respiratory function (forced vital capacity >70% of predicted). None of them had family history of motor neuron disease, and screening for GGGGCC expansions at the *C9orf72* gene proved negative in all 5 subjects. These samples underwent routine histological and enzymatic staining to assess the extent of denervation. They were then used in microarray and real-time PCR experiments to assess the differential expression of miRNAs between patients and controls (described below). These control samples of skeletal muscle came from five matched subjects that underwent biceps brachii biopsy for clinical investigation (mean age of 40.1 ± 15.1 years, 2 men). These individuals presented myalgia as the sole complaint (without weakness) without laboratorial abnormalities (normal CK levels) and entirely normal histology (seen by a board-certified neuropathologist).

2.2.2. Expression analyses in plasma

We collected blood samples from 39 patients and 39 healthy controls. These were used for plasma isolation and then for real time PCR experiments, as detailed below.

2.3. Clinical evaluation

All 39 patients underwent detailed clinical evaluation at baseline. After 6 and 12 months, we were able to reassess 19 and 12 patients, respectively. The remaining patients were lost to follow-up ($n = 15$) or died ($n = 12$). We employed the revised version of the ALS functional rating scale [18], ALS severity scale (ALSSS) [19] and Medical Research Council (MRC) sumscore to quantify disease severity at each point in time.

2.4. Laboratorial procedures and in silico analysis

2.4.1. RNA isolation

Tissue: MiRNAs enriched fraction was isolated with miRNeasy Kit (Qiagen®), following the manufacturer instructions. The extracted RNA was assessed for quality with Agilent 2100 Bioanalyzer™ (Agilent®, Santa Clara, CA) and quantity was determined using Nanodrop™ (Thermo Scientific®, Waltham, MA).

Plasma: Peripheral blood (4 ml) was drawn into EDTA tubes. Within 3 h, the tubes were subjected to centrifugation at 515 ×*g* for 10 min, 4 °C. Next, 1 ml aliquots of the plasma were transferred to 1.5 ml tubes and centrifuged at 16,000 g for 10 min, 4 °C to pellet any remaining cellular debris. Subsequently, the supernatant was transferred to fresh tubes and stored at −80 °C. Small RNA enriched fraction was extracted using the mirVana PARIS™ miRNA isolation kit from 625 μl of plasma sample following the manufacturer's instructions (Ambion®, Austin, TX). The final elution volume was 35 μl RNase-free water. The purity of extracted RNA was quantified using NanoDrop™ 1000 Spectrophotometer.

2.4.2. Gene Chip miRNAarray analysis

MiRNA microarray profiling in skeletal muscle samples was assessed with 300 ng of isolated enriched small RNA by Affymetrix® GeneChip™ platform miRNA array (Santa Clara, CA, USA, version 1.0), which

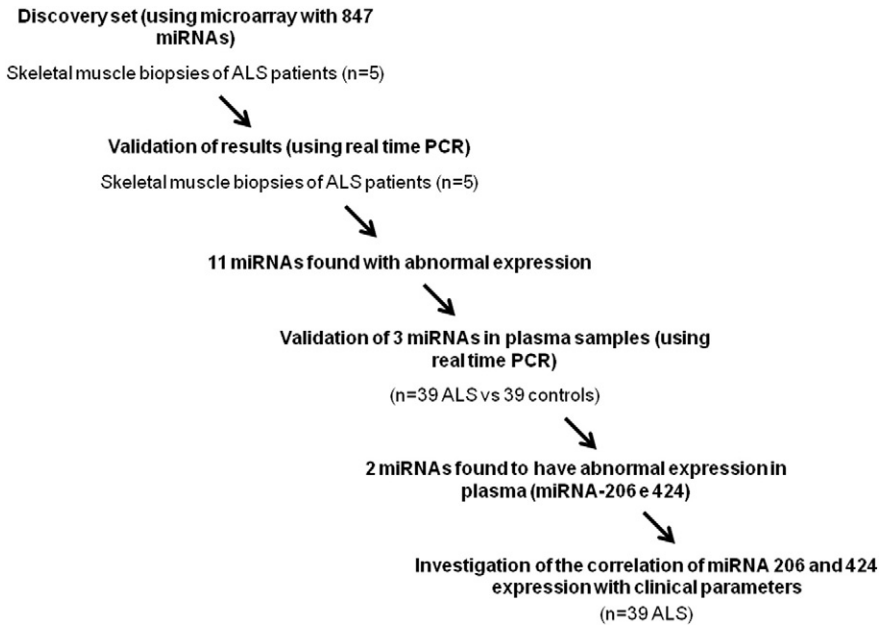

Fig. 1. Design of the study: Discovery phase done with a microRNA array in muscle tissue (n = 5), followed by validation in muscle and plasma (n = 39) and correlation with clinical parameters (n = 39).