MicroRNAs 181a and 125a are highly expressed in early diagnosed RRMS: a pilot case-control study

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Abstract

**Background:** Recently microRNAs (miRs) have been proposed as possible disease biomarkers in Multiple Sclerosis (MS) field. Among miRs, those secreted through microvesicles (MVs) may contribute to different disease phases.

**Methods:** This is a case-control study performed at the Department of Medical and Surgical Sciences, University of Foggia, Italy. Patients have not been yet exposed to disease modifying therapies (DMTs) and had received a confirmed diagnosis of relapsing remitting MS (RRMS). We analyzed the serum level of 6 MVs miRs by using Quantitative Real-Time PCR, comparing RRMS to HCs, with a 2:1 ratio. Subsequently, the differentially expressed miRNAs, were further tested with receiving operator curves (ROC). We aimed to explore possible role of MVs miRs as disease biomarker at the time of diagnosis.

**Results:** A total of 12 patients were enrolled. MiR-181a and miR-125a had higher levels in RRMS patients when compared to HCs (p<.01 and p<.05 respectively).

The ROC curve indicated that both miR-181a and 125a could be considered as biomarkers with an area under the curve of 0.896 (p=.014; 95% CI: 0.72–1.00) and 0.785 (p=.046; 95% CI:0.59–1.00) respectively.

**Conclusions:** Our study suggested MVs miR-181a and 125a as possible disease biomarkers in early diagnosed RRMS patients, not yet exposed to DMTs.

Introduction

Multiple Sclerosis (MS) is characterized by various degree of inflammatory, demyelinating, and degenerative processes, presenting with impressive inter- and intraindividual heterogeneity(1).

The application of clinical and imaging biomarkers does not allow individual characterization and prediction and only a few molecular biomarkers have so far been routinely used in clinical practice(2).

Recently attention has been posed on small noncoding RNA as microRNAs (miRs), is the most represented. MiRs act as posttranscriptional regulators of gene expression by base-pairing to specific sites(3).

Altered expression of some miRs may serve as valuable biomarkers to diagnose MS, and rapidly and effectively distinguish different clinical courses(4–8); other studies had also proposed a role in the evaluation of therapeutic response and risk stratification (9).

MiRs could be carried out also by extracellular vesicles (EVs), generated by most of mammalian cells and may hence behave as cell-to-cell molecular communication devices, under both physiological and pathological conditions, by specific cell uptake(10, 11). Among EVs, the submicron-size microvesicles (MVs; also known as ectosomes) are the larger ones ranging from 100 to 1000 nm in size. They bud off
the cytoplasmic membrane of the parent cell under normal physiological or pathophysiological conditions, including coagulation, inflammation, tumorigenesis, and differentiation (11).

In this study we aimed to explore a panel of miRs expressed in microvesicles in a cohort of early diagnosed RRMS patients, naïve to any disease modifying therapy (DMT), compared to sex-age matched healthy controls (HCs), to identify its possible role as a diagnostic biomarker.

**Methods**

**Setting**

A case-control study performed at the Department of Medical and Surgical Sciences, University of Foggia, Italy. Patients were consecutively admitted between January, 1, 2021 and June 30 2021.

**Participants**

Key eligibility criteria were patients (1) aged 18-55; 2) with a confirmed diagnosis of RRMS as per the revised 2010 McDonald criteria (2) naïve to any disease modifying treatments (DMTs).

Patients receiving other immunosuppressive/immunomodulant drugs for other diseases or exposed to steroids within 30 days of initial blood collection were excluded.

**Procedures and outcomes**

All patients underwent clinical and radiological evaluations; data were retrospectively collected within 12 months from enrolment.

Blood samples were collected at baseline. HCs were collected from blood donors of the transfusion centre, University of Foggia and they were randomly age and sex matched to the patient group, in a 2:1 ratio.

The Ethics Committee of Azienda Ospedaliero-Universitaria, Foggia Italy (14/CE/2022) approved the study and informed consent was obtained from all patients. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and with the appropriate national regulations.

**Clinical assessment and neuroimaging**

The following data were collected: a) demographical (age, gender, smoking status, comorbidities), b) clinical (disease duration, disability assessed by the Expanded Disability Status Scale (EDSS) and number of relapses in the year before diagnosis), and c) radiological, as number of brain and spinal cord lesions in T2 and T1 gadolinium-weighted sequence on baseline Magnetic Resonance Imaging (MRI). The MRI obtained within 6 months of a confirmed diagnosis was considered as baseline MRI; all scans were obtained using the same 1.5-Tesla machine.
All information were retrieved from a database, iMed© software (iMed©, Merck Serono SA, Geneva).

**Blood sample collection and MVs isolation**

Twenty millilitres of peripheral blood were collected from each subject (MS and HC) in Vacutainer® tubes by standard venipuncture and processed within two hours.

Serum was obtained by centrifugation protocol.

MVs from each serum sample were separated and collected by subsequent differential centrifugation steps, as described elsewhere(12). Biological samples were diluted 1:2 in Dulbecco's Phosphate Buffer Saline (DPBS) w/o Calcium w/o Magnesium (Euroclone) and were centrifuged at 2,000 x g for 30 min at 4°C to remove dead cells and large debris. The supernatant was filtered by gravity through 0.8 µm filters to remove particles >800 nm and centrifuged at 12,000 x g for 45 min at 4°C to pellet MVs. The MV-enriched pellets were re-suspended and lysed in DPBS to MVs quantification and characterization or in TRIzol Reagent (Invitrogen) for the isolation of high-quality total RNA and stored at -80°C.

MVs were characterized by Western blotting (see Appendix e1).

**Total RNA Isolation and Quantitative Real-Time PCR miRNAs Analysis**

Total RNA was isolated from MVs using TRIzol Reagent, according to the manufacturer’s instructions and measured by absorbance at 260 nm with a NanoDrop 1000 Spectrophotometer. RNA was reverse transcribed into cDNA using Universal cDNA synthesis kit II (Exiqon). The resulting cDNA transcript were used for PCR amplification using ExiLENT SYBR® Green Master Mix II (Exiqon) and miRNA specific primer set (miRCURY LNA™ Universal RT microRNA PCR, Exiqon) for miR-223-3p, miR-181a-5p, miR-146a-5p, miR-125a-5p, miR-30c-5p and miR-23a-3p. The relative miRNA levels were calculated using the comparative Ct method, using miR-16-5p reference gene as endogenous control.

miRNAs were chosen accordingly to previously published works in our research team(13).

**Statistical analysis**

Categorical variables are presented with counts and proportions, while continuous ones as the mean±SE of the mean (SEM) or median with IQR. Data distribution was assessed using the Kolmogorov–Smirnov test. Mann–Whitney test was used to compare variables of interest. A receiver-operator characteristic (ROC) curve was used to test the utility of miRNA statistically different as a biomarker for the diagnosis of MS. Significance was settled to .05. SPSS statistical software version 21.0 (IBM, Armonk, NY) was used for all analyses.

**Results**

**Clinical and radiological characteristics**
From a cohort of 27 patients, 12 were eligible, 8 of which females. Baseline characteristics are shown in Table 1.

Table 1. Baseline characteristics of the enroled cohort

<table>
<thead>
<tr>
<th></th>
<th>RRMS (n=12)</th>
<th>HC (n=6)</th>
<th>P**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female n (%)</td>
<td>8 (66.6%)</td>
<td>4 (66.6%)</td>
<td>ns</td>
</tr>
<tr>
<td>Age (year)</td>
<td>32.7±8.4</td>
<td>34.2±7.9</td>
<td>ns</td>
</tr>
<tr>
<td>Smokers n (%)</td>
<td>3 (25%)</td>
<td>2 (33.3%)</td>
<td>ns</td>
</tr>
<tr>
<td>Patients with comorbidities n (%)</td>
<td>3 (25%)</td>
<td>2 (33.3%)</td>
<td>ns</td>
</tr>
<tr>
<td>Relapses within 12 months before diagnosis</td>
<td>1.6±0.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>EDSS at baseline (median, q1-q3)</td>
<td>1.5 (1.5-2.5)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N. brain lesions on MRI T2 weighted sequences</td>
<td>18.5±9.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N. brain lesions on MRI T1 gad+ weighted sequences</td>
<td>1.1±3.0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N. spinal cord lesions on MRI T2 weighted sequences</td>
<td>0.2±0.3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N. spinal cord lesions on MRI T2 weighted sequences</td>
<td>1.9±0.9</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*data are expressed as mean±standard deviation when otherwise specified

**via Mann-Whitney or Fisher exact test

EDSS, Expanded Disability Status Scale, N., number; MRI, magnetic resonance imaging

miRs expression levels in serum MVs

Figure 1a reports box plot comparing the levels of expression of miR-223, miR-181a, miR-146a, miR-125a, miR-30c and miR-23a.

MiR-181a (23.2± 19.4 vs 3.7 ± 4.9, p=0.013) and miR-125a (11.7 ± 11.9 vs 3.2 ± 3.4, p=.045) had higher levels in RRMS patients when compared to HCs.

The ROC curve indicated that both miR-181a and 125a could be considered as biomarkers with an area under the curve of 0.896 (p = .014; 95% CI: 0.72–1.00) and 0.785 (p =.046; 95% CI:0.59–1.00) respectively (Figure 1b).

Discussion
Our study suggested miR 181a and 125a expressed in MVs as possible disease biomarkers in early diagnosed RRMS patients, not yet exposed to DMTs. To our knowledge, not similar data exist about c-miRs, generally evidence has been provided from miRs expressed in peripheral blood cells, as monocytes and T-cells(6, 7, 13, 14).

In the context of autoimmune neuroinflammation, miR-181a has been supposed to influence differentiation of T helper cell and activation of macrophages, providing potential therapeutic options for controlling inflammation in MS(14).

In the counterpart, miR125a has been investigated as potential biomarker of therapeutic efficacy. In detail, it has been reported that miR-125a is down-regulated in B-cells after six months of natalizumab administration(9).

The major strength of this study is to suggest MVs as an excellent source of miR biomarkers because of their easy extraction from body fluids. Additionally, MVs miRs are enclosed in membrane vesicles and are extremely stable in the extracellular environment characterized by high RNAse activity. High stability means that these miRs are long-lived in bio-fluids and are consequently proposed as attractive diagnostic and prognostic biomarkers also for non-invasive, less expensive, and less time-consuming methods than classical protein biomarkers employed so far.

Limits of the study is the relatively small sample size that reduces power of analysis and the absence of longitudinal clinical data after the beginning of first DMT and the lack of comparison with other neurological non inflammatory conditions.

However, these results although pivotal and preliminary, encourage further studies with largest sample size and prospective design to validate our findings.

**Declarations**

**Funding**

The researchers were independent from funders and sponsors. All researchers could access all the data.

**Conflicts of interest**

Authors have nothing to disclose related to the manuscript.

**Protocol Approvals Standard, Registrations, and Patient Consents**

The local Ethics Committee approved the study and informed consent was obtained from all patients.

**Data availability**
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Authors contribution

ED. and CA wrote the main manuscript text; AZ drafted the file and made statistical analysis, table, and figure 1. VM, AM made biomolecular analyses. CA drafted the manuscript and revised it for intellectual content

All authors reviewed the manuscript.

References


12. https://orca.cardiff.ac.uk/50139/1/Thery RCPpAoJ.


**Figures**

(a) Box plot of miRs expression in RRMS and HCs

miRNAs expression levels in serum MVs of RRMS patients and HCs by Real-Time PCR. Quantitative Real-Time PCR analysis of miRNAs was performed. The relative expression levels were calculated using the comparative Ct method, with miR-16-5p as endogenous control. Data are expressed as mean ± SD of fold change values (*p < 0.05; **p < 0.01), Mann Whitney U test.

(b) Logistic regression analyses with ROC curve output of patients with RRMS plotted against the HC group.
AUC, with 95% CI, is given for each parameter. The surface expression of each parameter for patients with RRMS (n = 12) are combined as true positives and plotted against HC as true negatives (n = 6). The diagonal dividing the ROC space represents the random event. A logistic regression analysis with combined parameter results has been performed for “all parameters”, parameters with AUC > 0.70, and AUC > 0.75. ROC, receiver operating characteristic; AUC, Area under the curve; RRMS, relapsing-remitting MS; HCs, healthy controls

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Appendixe1MVscharacterization.docx