Short communication

Effect of fingolimod treatment on circulating miR-15b, miR23a and miR-223 levels in patients with multiple sclerosis

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ARTICLE INFO

Article history:
Received 14 June 2016
Received in revised form 20 July 2016
Accepted 30 August 2016
Available online xxxx

Keywords:
Multiple sclerosis
MicroRNA
Fingolimod
Biomarker

ABSTRACT

MicroRNAs (miRNAs) have recently found to be dysregulated in serum from multiple sclerosis (MS) patients. Cell free circulating miR-15b, -23a and 223 levels were analyzed by Real Time PCR in a cohort consisting of 30 serum samples from Relapsing Remitting MS patients at baseline (T0) and after three, six, nine and twelve months (T1, T2, T3, T4) after starting the treatment. A down-regulation of miRNA levels in patients at T0 compared with controls was present (p < 0.001). MiRNA levels slightly increased at T1 and this trend reached the statistical significance at T2 vs T0 and remains stable at T3 and T4. Our preliminary results suggest that aberrant levels of circulating miRNAs are recovered in fingolimod treated MS patients. Circulating miRNAs profiling could thus represent an easy detectable biomarker of disease and response to treatment.

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1. Introduction

Fingolimod (marketed as Gilenya®) is the first orally available compound recently approved in Europe and USA for the treatment of Relapsing Remitting Multiple Sclerosis (RRMS), first in class of S1P receptor modulators. S1P expressed on lymphocyte regulates the normal egress of lymphocytes from lymphoid tissues, whereas S1P receptors expressed in the Central Nervous System (CNS) can modulate neurogenesis, neural function and migration. Fingolimod exerts a double action: immune modulating in the periphery, and protective and reparatory in the CNS (Chun and Hartung, 2010). Fingolimod has demonstrated good efficacy in reducing MS clinical activity compared to first-line disease modifying treatments (DMTs) and showed a unique central capacity in reducing brain atrophy progression (Cohen et al., 2010). Furthermore, fingolimod has demonstrated the ability to modify the expression level of several proteins (Chun and Hartung, 2010). MicroRNAs (miRNAs) are small noncoding RNA molecules of ~22–25 nucleotides in length which are synthesized by enzymatic cleavage of RNA precursor. They act as negative regulators of gene expression interfering with mRNA stability and translation (Abdellatif, 2012).

De-regulation of miRNAs have been associated with autoimmune diseases, including MS, and they have been proposed as potential biomarkers of disease (Fenoglio et al., 2012, 2013). A growing body of evidence support a modulation of specific miRNA levels by common drugs used in MS, highlighting a possible role of miRNAs as biomarkers of treatment response (Fenoglio et al., 2012; Muñoz-Culla et al., 2014; De Felice et al., 2014).

Recently, it was demonstrated that natalizumab modified the expression levels of three specific miRNAs (let-7c, miR-125a-5p and miR-642) after 6-month treatment, while miR-320, miR320b and miR-629 showed different expression levels in patients who occurred in PML compared with patients who did not develop PML (Muñoz-Culla et al., 2014).

Considering Interferon treatment, a different expression of miR-26a-5p in patients at 3 months treatment was shown, keeping stable at 6 months treatment (De Felice et al., 2014). There is instead no information so far about a possible influence of fingolimod treatment on miRNA levels. Given this premise, we investigated the effect of fingolimod treatment on the expression of selected cell free circulating miRNAs, miR-15b, miR-23a and miR-223, previously described to be differentially expressed in MS (Fenoglio et al., 2013).

2. Material and methods

2.1. Patients

This study was approved by the Institutional Review Board (IRB) of the Fondazione Cà Granda, IRCCS Ospedale Maggiore Policlinico and all subjects provided written informed consent.

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http://dx.doi.org/10.1016/j.jneuroim.2016.08.017
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Thirty patients underwent the standard work up for MS (medical history, physical and neurological examination, screening laboratory tests, brain Magnetic Resonance Imaging) and diagnoses were based on the McDonald’s 2010 criteria (Polman et al., 2011). All subjects underwent blood withdrawal for serum isolation, before starting fingolimod treatment (T0) and after 3, 6, 9 and 12 months therapy (T1, T2, T3, T4 respectively). Patients were not treated at the time of blood sampling.

Eleven age and gender matched healthy blood donors, consisting mainly in students and personnel working at the University Department, were enrolled as well (Table 1).

2.2. miR-23a, miR-15b and miR-223 isolation and expression analysis

Serum was obtained as previously described (Fenoglio et al., 2013). Briefly, for miRNA extraction, 400 µl of human serum was thawed on ice and lysed with equal volume of 2× Denaturing Solution (Ambion). To allow sample-to-sample normalization, synthetic C. elegans miRNAs cel-mir-39 (Qiagen) was added (as a mixture of 25 fmol of each oligonucleotide in 5 µl total volume) to each denatured sample. RNA was isolated using mirVANA PARIS kit following the manufacturer’s protocol for liquid samples (Ambion). Reverse transcription and RT-PCR were performed as previously described (Fenoglio et al., 2013).

2.3. Sample size calculation and statistical analysis

The sample size needed to reach the statistical power was calculated according to the Pocock’s formula and considering our previous data (Mehta and Pocock, 2011; Fenoglio et al., 2013). According to that we expected an average of 5 fold modulation of miRNA levels (maximum effect previously observed) due to fingolimod treatment. Using these parameters we obtained that the minimum number of patients to be included was 14 when considering a standard deviation equal at 4. However, given the possibility that this estimation could be wrong, we included 30 patients in order to assure the achievement of an adequate statistical power also for fold change differences <5.

GraphPad Prism 6.0 software was used for statistical analysis. Comparison of miRNA levels between patients at T0 and controls were performed by using the Mann-Whitney test. Further comparisons among miRNA levels in MS group at different time points treatment were performed using the Friedman test. The correction for multiple comparisons using the Dunn’s test was applied. Spearman test was used for correlations with clinical data.

3. Results

Free circulating miR-15b, miR-23a and miR-223 expression levels were analyzed in serum obtained from 30 fingolimod-treated MS patients at baseline, and at 3, 6, 9 and 12 months (T0, T1, T2, T3, T4 respectively) after fingolimod treatment initiation. Clinical characteristics of patients are shown in Table 1. miRNA levels at baseline were compared to levels obtained from a cohort of healthy subjects as well as to the levels measured during fingolimod treatment.

In line with previous results (Fenoglio et al., 2013), miRNA levels were decreased in patients at T0 as compared with controls (miR 15b: 0.56 vs 1.8, p = 0.001, miR 23a: 0.26 vs 1.14, miR 223: 0.19 vs 0.69 relative expression levels, p < 0.0001, Fig. 1). MiRNA relative expression levels slightly increased at T1 as compared with T0 (miR-15b: 0.77 ± 0.15 versus 0.56 ± 0.15, miR-23a: 0.46 ± 0.07 versus 0.26 ± 0.07, miR-223: 0.31 ± 0.07 versus 0.19 ± 0.05, p > 0.05). This trend reached the statistical significance at T2 (miR-15b: 1.35 ± 0.23, p = 0.001; miR-23a: 0.71 ± 0.12, p < 0.0001; miR-223: 0.47 ± 0.06, p < 0.0001, Fig. 1) as compared with T0. miRNA levels remained stable at T3 (miR-15b: 1.18 ± 0.17, p = 0.001; miR-23a: 0.50 ± 0.08, p = 0.01; miR-223: 0.52 ± 0.07, p < 0.0001, Fig. 1) and after 12 months at T4 (miR-15b: 1.01 ± 0.15, p < 0.001; miR-23a: 0.63 ± 0.10, p = 0.001; miR-223: 0.48 ± 0.05, p < 0.0001, Fig. 1).

The correlations between miRNA levels and demographic data (age at sampling and disease duration) did not give significant results. In the same way no significant result was found when correlations between miRNAs levels and clinical variables such Expanded Disability Status Scale (EDSS), disease duration and lymphocyte counts were performed.

4. Discussion

Herein, the effect of fingolimod treatment on selected miRNAs, which were already found to be dysregulated in MS patients (Fenoglio et al., 2013), is reported considering the first year of treatment. Our results suggest that aberrant levels of miR-15b, miR23a and miR-223 are recovered, towards normality levels, in fingolimod treated MS patients. In particular, the effect of the treatment on these miRNAs was appreciable after 6 month-treatment, and remained stable over 12 months. Actually, the first three months of treatment might be enough to trigger the modification in miRNA levels, although it is just after six months that the recovery effect becomes statistically significant.

However, these observations need to be corroborated by functional data as it is not possible to state, at the moment, if the recovery of miRNA levels has to be considered a mechanism of action of fingolimod or a biomarker reflecting the partial recovery upon treatment.

Interestingly, miR-23a is known as brain-specific/enriched and it is involved in oligodendrocyte differentiation highlighting its possible role in the biomarker investigation research field (Galloway and Moore, 2016). Nevertheless, target prediction based upon miRWalk2.0 led to the identification of several target genes relevant to MS pathology and to fingolimod treatment response, suggesting their possible involvement in fingolimod mechanism of action.

In particular, among miR-223 targets, STAT5, that is involved in inflammatory processes would recover particular interest since its protein level is modulated by miR-223 (Pinatel et al., 2014). Fingolimod, through the activation of PP2A, can inhibit the activity of STAT5 in vitro and in vivo possibly resulting in a modulation of inflammation (Samy et al., 2007).

Both miR-15b and miR-23a target FGF-2 gene, a member of the fibroblast growth factor family, whose protein levels are reported elevated in CSF of MS patients, particular those with active disease (Sarchielli et al., 2008). FGF-2 was found to be differentially expressed in active and chronic MS lesions in postmortem tissues (Sarchielli et al., 2008) suggesting FGF-2 as marker of inflammation in MS lesions.

Besides a better knowledge of the role of miRNAs in the pathogenesis of MS, this research may also results in the identification of biomarkers for predicting the response to the treatment. To this aim, clinical and radiological data are going to be collected over time, to test whether the restoration of miRNA levels may predict a better outcome.

In summary, we observed a recovery in miRNA levels, previously found to be downregulated in MS, after 6 months of fingolimod treatment. Although a confirmatory study involving a larger population is needed to draw definitively conclusions, these preliminary data suggest that miR-15b, miR-23a and miR-223 might serve as treatment response biomarkers. However, the biological mechanism underneath the
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