Circulating microRNA after autologous bone marrow mononuclear cell (BM-MNC) injection in patients with ischemic stroke

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ABSTRACT

Previous studies have shown the potential of microRNAs (miRNA) in the pathological process of stroke and functional recovery. Bone marrow mononuclear cell (BM-MNC) transplantation improves recovery in experimental models of ischemic stroke that might be related with miRNA modifications. However, its effect on circulating miRNA has not been described in patients with stroke. We aimed to evaluate the circulating levels of miRNAs after autologous BM-MNC transplantation in patients with stroke. We investigate the pattern of miRNA-133b and miRNA-34a expression in patients with ischemic stroke included in a multicenter randomized controlled phase IIb trial (http://www.clinicaltrials.gov; unique identifier: NCT02178657). Patients were randomized to 2 different doses of autologous intra-arterial BM-MNC injection (2×10^6/kg or 5×10^6/kg) or control group within the first 7 days after stroke onset. We evaluate plasma concentration of miRNA-113b and miRNA-34a at inclusion and 4, 7, and 90 days after treatment. Thirteen cases (8 with 2×10^6/kg BM-MNC dose and 5 with 5×10^6/kg dose) and 11 controls (BM-MNC non-treated) were consecutively included. Mean age was 64.1±12.3 with a mean National Institutes of Health Stroke Scale score at inclusion of 14.5. Basal levels of miRNA were similar in both groups. miR-34a-5p and miR-133b showed different expression patterns. There was a significant dose-dependent increase of miRNA-34a levels 4 days after BM-MNC injection (fold change 3.7, p<0.001), whereas miRNA-133b showed a significant increase in the low-dose BM-MNC group at 90 days. Intra-arterial BM-MNC transplantation in patients with ischemic stroke seems to modulate early circulating miRNA-34a levels, which have been related to precursor cell migration in stroke and smaller infarct volumes.

INTRODUCTION

Stroke is the leading cause of disability in the adult. Although new treatments such as thrombolysis and thrombectomy in acute stroke have made a revolution in stroke management, there are still no neuroprotective or neurorestorative treatments approved for established neurological deficits after stroke. Cell-based therapy is a potential new approach in the treatment of ischemic stroke. Promising preclinical studies have shown improvement of neurological recovery in animal stroke models, especially when administered within the first days after stroke.[1 2] In addition, preliminary clinical trials in patients with stroke have shown the safety of this approach.[3] The main mechanism of recovery seems to be a paracrine effect that modulates the neurogenesis, microglia response, neoangiogenesis, but also the systemic inflammatory response.[4]

Previous studies have shown the importance in stroke of microRNAs (miRNA), which are a group of short non-coding RNA molecules, and their potential involvement in the pathological process of stroke and functional recovery.[5] Some specific miRNAs, such as miRNA-113b and miRNA-34a, have been proven that can be modulated after cell therapy in stroke and myocardial infarction preclinical models.[6 7]

We aimed to evaluate the circulating levels of miRNAs after autologous bone marrow mononuclear cell (BM-MNC) transplantation in a phase IIb multicenter controlled clinical trial in middle cerebral artery (MCA) ischemic stroke.

MATERIALS AND METHODS

The Intra-arterial Bone marrow mononuclear cells transplantation in acute Ischemic Stroke clinical trial (IBIS trial) is an ongoing phase IIb randomized controlled multicenter clinical trial. Twenty-three patients with MCA ischemic stroke were randomized to BM-MNC group or

control group. Criteria for inclusion were age between 18 and 80 years, an MCA ischemic stroke with a National Institutes of Health Stroke Scale (NIHSS) score of 6–20, and treatment window within 7 days of stroke onset. Further inclusion and exclusion criteria are described previously.[8]

Transplantation was done 1–7 days after stroke onset. Bone marrow was obtained by puncture in the iliac crest. The aspirate was centrifuged on a Ficoll density gradient to isolate the mononuclear cells and BM-MNCs were injected in the M1 segment of the infarct-related MCA at low pressure (rate of infusion was 0.5–1 mL/min). No bone marrow aspiration or sham injection was performed in the control group.

Blood samples were drawn before (t0) and 4, 7, and 90 days after transplantation. Plasma was obtained from EDTA tubes after centrifugation (1500× *g*). They were aliquoted and stored at −80°C until analysis. Total miRNA was extracted from plasma (200 µL) using miRNeasy Serum/Plasma Kit in the QIAcube extractor (Qiagen, Hilden, Germany) adding an *Arabidopsis thaliana* sequence (nth-miR-159a) as spike-in and exogenous control up to final concentration at 10:00 PM. All miRNAs were amplified in a single reverse transcription reaction adding a poly(A) tail to the miRNA-3′-end and an adapter to 5′-end. cDNA was synthetized using TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher, Cambridge, MA) according to the manufacturer's instructions. All products were quantified in Qubit-3.0 fluorometer (Thermo Fisher). TaqMan Advanced miRNA assay (Thermo Fisher) was performed for hsa-miR-34a-5p and hsa-miR-133b and nth-miR-159a in a ViiA 7 Real-Time PCR System (Thermo Fisher) and analyzed with SW Web CLOUD (Thermo Fisher).

Primers

- nth-miR-159a: 5′- UUUGGAUUGAAGGGAGCUCUA-3′.
- hsa-miR-34a-5p: 5′-UGGCAGUGUCUUAGCUGGUUGU-3′.
- hsa-miR-133b: 5′-UUUGGUCCCCUUCAACCAGCUA-3′.

Statistical analysis

We used univariate analysis for comparisons between groups. The relative expression was obtained using the ΔΔCt method: ΔCt=Ct value (miRNA 34a or 133b)-Ct value (miR-159a) and ΔΔCt=ΔCt (treated)-ΔCt (control). All experiments were performed in duplicate and ΔCts were computed using the spike-in miRNA-159a. Fold change was calculated as $2^{-\Delta\Delta Ct}$. miRNA levels at each time point were analyzed by repeated measures analysis of variance with Bonferroni correction for multiple comparisons. We used SPSS V.25.0 (IBM) and GraphPad Prism V.7 (La Jolla, CA), with $p<0.05$ as statistically significant.

RESULTS

Thirteen cases and 11 controls were included. Baseline characteristics were similar in both groups (table 1).

Mean age at inclusion was 64.1±12.3. Most of patients were treated with intravenous thrombolysis in the acute phase of stroke (72.7% of controls vs 84.6% of BM-MNC group, p=0.63) and thrombectomy (72.7% of controls and 76.9% of BM-MNC group, p=1.0). However, patients were severely disabled at inclusion, as randomization was done after a mean 3.3 days after stroke onset with a mean NIHSS of 14.5±3.3. Mean infarct volume was 134.5 mL (84.9–153.0) with no differences between groups (p=0.57). Eight patients were allocated to the low cell dose (2×10^6/kg BM-MNC) and 5 to the high dose (5×10^6/kg BM-MNC). BM-MNC transplantation was done at 6.4±1.3 days after stroke onset.

Table 1 Baseline characteristics

	Control group (n=11)	BM-MNC group (n=13)	p Value
Age (y)	65.4±12.7	62.9±12.3	0.63
Sex (men)	5 (50%)	8 (61.5%)	0.68
Vascular risk factors			
Hypertension	6 (54.5%)	8 (61.5%)	1.0
Diabetes	3 (27.3%)	2 (15.4%)	0.63
Dyslipidemia	6 (54.4%)	6 (46.2%)	0.68
Coronary artery disease	2 (18.2%)	1 (7.7%)	0.57
Current smoker	2 (18.2%)	6 (46.2%)	0.18
Baseline NIHSS score (at inclusion)	15.3 (3.9)	13.9 (2.6)	0.33
Intravenous thrombolysis	8 (72.7%)	11 (84.6%)	0.63
Thrombectomy	8 (72.7%)	10 (76.9%)	1.0
Time from stroke to inclusion (d)	3.6 (1.4)	3.1 (1.9)	0.44

Data are number (%) or median (SD).
BM-MNC, bone marrow mononuclear cell; NIHSS, National Institutes of Health Stroke Scale.

Circulating plasma levels of miR-34a-5p and miR-133b were similar between groups at baseline, except for the miRNA133b in the 2×10^6/kg BM-MNC group that were significantly higher than control group (figure 1).

After BM-MNC transplantation, a marked increase was observed in miR-34a-5p levels 4 days after BM-MNC injection in the high cell dose group (fold change 3.7, p<0.001), with a trend towards higher levels in the low dose (p=0.1).

During follow-up, at 90 days after BM-MNC injection, there was also a significant increase in miR-34a-5p and miR-133b plasma levels in the low-dose BM-MNC group (fold change 2.3, p<0.01 and 2.0, p=0.04, respectively).

DISCUSSION

To the best of our knowledge, we describe for the first time the miRNA plasma level changes after cell therapy in patients with ischemic stroke. As stressed in the stem cells as an emerging paradigm in stroke (STEPS) guidelines publication, there is a great need for markers to gauge the biological activity of cell therapy.[9] The selection of the cell dose or timing of therapy might depend, at least in part, on the intended mechanisms of action of the cell therapy.

Many different miRNAs seem to have a possible therapeutic or prognosis role in stroke,[10] however, very few have been also associated to be modified by stroke cell therapy.[5–7] Thus, our aim was to evaluate these specific miRNAs in patients with stroke, to replicate the findings of preclinical stroke cell therapy studies and improve our understanding of the possible mechanisms of action of cell therapy after stroke.

Similar to preclinical studies, our group has previously described a strong negative correlation between dose of cells and disability after ischemic stroke.[11] The finding of

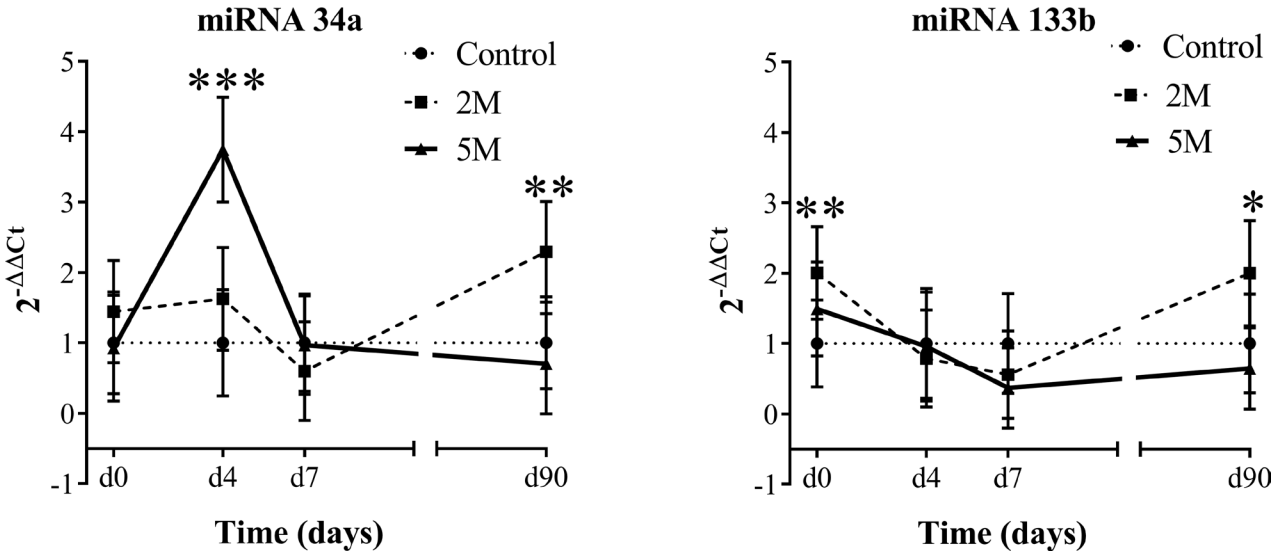

Figure 1 miR-133b and miR-34a levels before and after bone marrow mononuclear cell (BM-MNC) transplantation. Values are expressed as means±SD. *p<0.05; **p<0.01; ***p<0.001.

a different profile of miRNA expression after BM-MNC transplantation may have an impact in dose selection for larger phase III clinical trials.

The changing expression of miR-34a-5p levels in blood samples has been correlated with brain samples obtained from rat models, which demonstrates the ability of miR-34a-5p as a potential biomarker for acute ischemic stroke.[12] miR-34 is a brain-enriched miRNA family, which upregulates with neuronal differentiation and maturation.[13] In a recent study of miR-34a-5p expression, a negative association between miRNA-34a-5p and NIHSS scores (r=–0.692, p<0.05) and infarct volume (r=–0.719, p<0.05) was described.[12] In line with these findings, we detected a significant increase of miR-34a-5p expression within the first week after BM-MNC transplantation. However, whether this finding is related to less disability in the long-term needs to be proven. miR-133b has been implicated in functional recovery and improve neural plasticity after stroke.[5 7] However, we did not find a significant change in the early period after transplantation, but basal levels of the low-dose group were significantly different from control group and could influence in our findings. In addition, the long-term changes (ie, 90 days) of miRNA have not been previously studied in humans or preclinical stroke models, so its significance is unclear.

The main limitation of this study is the low number of patients included, but these preliminary findings enhance the hypothesis of the miRNA modulatory properties of cell therapy in patients with stroke. Another limitation is that there are many other miRNAs that could be relevant for stroke but not evaluated in our study.

CONCLUSION

In summary, our results suggest that autologous BM-MNC intra-arterial transplantation in ischemic stroke modulates circulating miRNA-34a levels, which has been related to precursor cell migration in stroke and smaller infarct volumes. This might become a surrogate marker to guide stroke cell therapy, although further studies are needed to replicate these findings and evaluate the relation of miRNA and stroke outcomes in cell therapy trials.

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