Altered Expression of miR-326 in T Cell-derived Exosomes of Patients with Relapsing-remitting Multiple Sclerosis

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ABSTRACT

Invasion of auto-reactive CD4+ T cells especially Th17 into central nervous system (CNS) is an underlying pathogenic mechanism in multiple sclerosis (MS). CD4+ T cells release exosomes which are enriched in microRNAs, reflective of cell's physiological or pathological condition. Thus exosomes could be potent agents to provide quantitative and qualitative information about involved cells in MS. We investigated the expression of pathogenic microRNAs in T cells-derived exosomes of MS patients or healthy controls.

Conventional T cells (Tconv) derived from relapsing-remitting (RR) MS patients (n=10) and healthy controls (n=10) were purified and cultured for 3 days by soluble anti-CD3/CD28. Exosomes were purified from cultured-T cells supernatants. The expression levels of exosomal miR-146a, miR-29a, miR-155, and miR-326 were quantified by real-time PCR.

A statistically significant increased expression of miR-326 in Tconv-derived exosomes was observed in RRMS patients as compared with controls (7.5±1.88vs 2.51±0.9 p=0.03), On the contrary, no differences were found in the expression levels of miR-155, miR-146a, and miR-29a, in Tconv-derived exosomes of patients as compared with controls (p>0.05).

Our results point to altered expression in exosome-derived microRNAs. MiR-326 was previously shown to play a role in the immunopathogenesis of MS by inducing TH17 differentiation and maturation. Therefore, miR-326 containing exosomes might also be a potential clinical target in course of MS. Moreover, the deregulation of this miRNA in exosomes may serve as a diagnostic and prognostic biomarker.

Keywords: Exosome; Lymphocyte; microRNA; Multiple sclerosis

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INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease characterized by the myelin degeneration of the central nervous system (CNS).[1] Although the etiology of MS remains unclear, several factors, including genetics and environmental factors, are involved in the onset of the disease.[2] Relapsing-remitting MS (RRMS) is the most common clinical form of the disease which manifests several periods of relapses and remissions.[3]

The immunopathology of multiple sclerosis is characterized by invasion of autoreactive CD4+ T cells, especially Th1 and Th17, into CNS leading to neuroaxonal degeneration and disability. In addition to CD4+ T cells, recent studies revealed that microRNAs, small regulatory non-coding RNAs, also play critical roles in the pathology of MS.[4-6] For example, studies have shown that miR-155 and mir-326 could induce development of Th1 and Th17 inflammatory cells. MicroRNA-155 deficient mice are resistant to experimental autoimmune encephalomyelitis (EAE).[4,7] In contrast, some microRNAs such as miR-146a and miR-29 are known to prevent autoimmune diseases. MicroRNA-146a suppresses the functions of Th1 cells by activating regulatory T cells[5,6]. Moreover, miR-29 is a powerful mediator in the suppression of inflammatory cytokines production including gamma interferon (IFNɣ). Production of IFNɣ in miR-29 deficient cells is much higher than in normal ones.[4] Additionally, studies have demonstrated that miRNAs profile in leukocytesbecome dysregulated in MS patients;[8,9] indeed, Keller et al showed 165 miRNAs were significantly up or down-regulated in patients with RRMS as compared to healthy controls.[10] Similarly, De Santis et al revealed that miR-106b and miR-25 which modulate the TGF-β signaling were down-regulated in regulatory T cells of MS patients.[11] Hence, altered miRNA expression may influence MS pathology.

The majority of microRNAs is released from cells in an encapsulated compartment named exosomes and affects the functioning of other cells.[12,13] Exosomes are nanovesicles (30-100 nm in diameter) which are released by various cell types including T cells.[13] MicroRNAs that included in exosomes are protected from degradation; therefore exosomal microRNAs seem more stable than free microRNAs.[14] Hence, exploration of microRNAs in leukocyte-derived exosomes could help us to find a potent agent to provide quantitative and qualitative information about MS pathogenesis.

Taken together, in the current study, we asked the question as to whether the expression of exosomes derived microRNAs in MS patients might be altered in comparison to healthy controls. We isolated exosomes from MS or healthy control-derived conventional T cells and analyzed the expression of four microRNAs involved in MS pathogenesis (miR-146a, miR-155, miR-326, and miR-29a) in purified exosomes.

MATERIALS AND METHODS

Subjects

Present Study was performed on 10 patients (6 female, 4 male) with relapsing-remitting MS (mean age: 35.2±6.7) diagnosed according to McDonald's criteria. Patients were in the relapse phase at the time of sampling. None of the patients were under immunosuppressive and or immunomodulatory drugs for at least three months before the study. Disability was assessed by an expert neurologist using the expanded disability status scale (EDSS). The EDSS of all patients was less than 3. Patients who suffered from other forms of the MS or other immune-related diseases were excluded from the study.

Ten sex/age-matched individuals (5 female, 5 male; mean age: 37.6±5.5), who had no history of any autoimmune and inflammatory diseases, were enrolled as healthy controls. All participants were Iranian origin. The study was approved by the ethics committee of Tehran University of Medical Sciences (N.: IR.TUMS.REC.1394.1551). Written informed consent was gained from each individual. Sampling was performed from patients referred to the Iranian Center of Neurological Research in Imam Khomeini General Hospital, Tehran University of Medical Sciences, Tehran, Iran during 1 year, between June 2016 to September 2017.

Purification Cell Isolation and Culture

Fresh peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll density gradient centrifugation using Lymphodex (Innotrain, Germany). The CD4$^+$CD25$^-$ Conventional T cells (Tconv) were purified using Dynabead T cell kit (Invitrogen Dynal Carlsbad, CA, USA) according to its instructions. Purified Tconv were cultured in RPMI 1640 (Biosera, France) supplemented with 10% exosome-depleted fetal bovine serum (SBI System Bioscience, USA), penicillin (100 IU/mL) and streptomycin (100 μg/mL) and 1% L-glutamine100

mM for 72 h. Isolated Tconv were stimulated by soluble anti-CD3 (1 µg/mL) and anti-CD28 (5 µg/mL) (MabTech, Sweden).

Isolation and Characterization of Exosomes from Cell Culture Supernatant

The Total Exosome Isolation kit (Invitrogen, Carlsbad, CA, USA) was used for purification of exosomes from the supernatant of Tconv. Briefly, to remove cell debris, the culture supernatant was centrifuged at 2000g for 30 minutes and Total Exosome Isolation reagent was added to cell-free supernatant. After overnight incubation at 4C, exosomes were precipitate by centrifuging at 10,000×g for 1 hour at 2°C to 8°C. A CD63 ELISA kit (SBI System Bioscience, USA) was used for exosomes quantification with a standard curve of known CD63+ exosomes.

RNA Extraction and MicroRNA Quantification

Total RNA was extracted from exosomes pellet using GeneAll[R] RiboEx Total RNA extraction kit (GeneAll Biotechnology, Korea) according to manufacturer's instructions. The concentration and purity of RNA were analyzed by measuring absorbance at 260/280 nm by a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcription was performed using miScript Reverse Transcription kit (Qiagen, Germany) according to manufacturer's protocols. MicroRNA specific primers (miR-146a, miR-155, miR-326, and miR-29a) were purchased from Qiagen. Real-time PCR (RT-PCR) was carried out using the miScript SYBR Green PCR Kit (Qiagen, Germany) which included universal primer. The RNU6B gene was used as an internal control for normalization. All quantitative PCR measurements were performed using StepONE Real-Time PCR system (Applied Biosystem). The threshold cycles were normalized to RNU6B and the relative expression levels were calculated using the $2^{-\Delta\Delta ct}$ method.

Statistics

Statistical analysis was performed using Prism (GraphPad) Software. Mann Whitney U test was used for analysis of data. Data are presented as Mean ± SEM and p values<0.05 were considered significant.

RESULTS

Patients' characteristics

Ten MS patients (6 female; 4 male) and 10 healthy controls were examined in this study. Eighty percent of the patients exert lesion in brain periventricular region. About 70% of patients showed the sensory symptoms as the first clinical sign. Because our patients have not received any immunomodulatory/immunosuppressive drug at least in 3 months before sampling; their EDSS was less than 3. Disease duration was from 1 month to 7 years. Demographic and clinical characteristics of patients are given in Table 1.

Table1. Demographic characteristics and MRI scan of multiple sclerosis (MS) patients

Patient number	Sex	age	Brain stem	Perivent ricular	Subcortical	Corpus callosum	Brachium pontis	Medulla	Centrum	*Cervical MRI*	*Symptoms& sign at attacks*
						Brain MRI					
P1	M	36	*	*	*					Plaques in C1,C2,C3,C5,C6	Vertigo, brain stem sign, Disequilibrium
P2	F	26	*	*	*					Normal	Sensory
P3	M	26			*	*	*			Normal	Visual
P4	F	43		*				*		Normal	Vertigo, brain stem sign
P5	M	29	*	*		*	*			Plaques in C2	Sensory, brain stem sign
P6	F	44	*		*	*	*			Normal	Visual, sensory
P7	F	33		*		*			*	Plaques in C2	sensory
P8	M	43		*						Normal	sensory
P9	F	37		*						Normal	sensory
P10	F	35		*						Normal	Sensory, Visual Disequilibrium

*The mean age of patients was 35 ± 6.7. Eighty percent of patients had plaques in the brain Periventricular area. About 70% of patients show sensory symptoms as the first clinical symptom. F: Female; M: Male; C: Cervical Vertebra

miR-146a

a

miR-29a
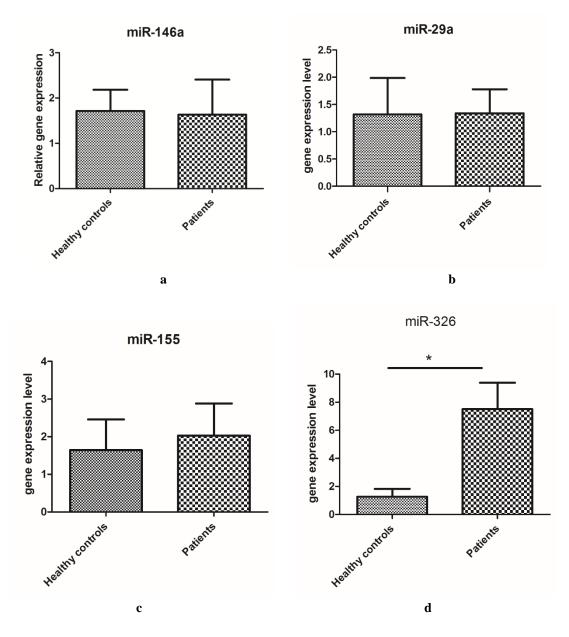

b

miR-155

c

miR-326

d

Figure 1. miR-326 is up-regulated in conventional T cells (Tconv)-derived exosomes. Conventional Tcells were isolated from relapsing-remitting multiple sclerosis (RRMS) patients or healthy controls; the isolated cells were cultured for 3 days and exosomes were purified from cell culture supernatants. RNA was extracted from exosomes. The expression levels of miR-146a, miR-29a, miR-155, and miR-326 were quantified by RT-PCR. Data are shown as means ± SEM. a: expression levels of miR-146a; b: expression levels of miR-29a; c: expression levels of miR-155; d: expression levels of miR-326; Mann Whitney U test was used for analysis of data. Data are presented as Mean±SEM and $p<0.05$ were considered significant.

miRNA Expression in T Cell-Isolated Exosomes

The purity of the isolated conventional (CD4$^+$CD25$^-$) T cells was 95.4 as examined by flow cytometry. The purified exosomes were quantified using a CD63 ELISA kit. CD63 is a well-characterized marker for detection of exosomes. On average, 8.7×10^8 CD63+ exosomes were extracted from Tconvs.

We used RT-PCR to study the expression of miR-146a, miR-155, miR-29a and miR-326 in conventional T cells from RRMS patients and healthy controls. As shown in Figure 1 we found, miR-155 and miR-326 were upregulated in MS patients compared to healthy

controls reaching statistical significance only for miR-326(p=0.03). Otherwise, miR-146a and miR-29a were down-regulated in MS patients; which were not statistically significant (p>0.05).

DISCUSSION

Multiple sclerosis is a heterogeneous disease in which Th1 and Th17 cells play a critical role in pathogenesis. Moreover, microRNAs play fundamental roles in differentiation and maturation of these immune cells.[4] Recent studies showed that CD4+ T cells release exosomes which are highly enriched in microRNAs, transfer between cells and affect the cellular function in the new site.[15] Thus, microRNAs containing exosomes could be potent agents to provide quantitative and qualitative information about immune cells involved in MS pathogenesis. In the present study, we analyzed the expression of four microRNAs involved in the MS pathogenesis (miR-146a, miR-155, miR-326, and miR-29a) in T cell-derived exosomes in patients with RRMS.

We found that mir-326a was over-expressed in T cell-derived exosomes of MS patients as compared with healthy controls. It is well established that mir-326 play critical roles in the pathogenesis of immune-mediated diseases especially MS. In 2009 Junker et al have shown that miR-326 was up-regulated in active multiple sclerosis and targets the CD47 molecule brain resident cells. The CD47 molecule, in turn, inhibits the phagocytic activity of macrophages, consequently reducing its expression which could increase the degradation of myelin.[16] Likewise, another study showed that expression of miR-326 in peripheral blood mononuclear cells (PBMCs) of RRMS patients was significantly higher than in normal subjects. Du and colleagues demonstrated that miR-326 by targeting Ets-1, a negative regulator of the Th17 differentiation, could differentiate T naïve into Th17; therefore, increasing the severity of MS and EAE.[7]

The main discrepancy of the present study is examining of microRNAs in T cell-derived exosomes, which has more stability than free miRNA. This approach seems to have more clinical efficacy.

Since microRNA-containing exosomes may have physiological and pathological roles, it is plausible that exosomes could be manipulated and used as a therapeutic vehicle for the treatment of patients. Indeed, manipulated exosomes have been previously

used in different context. Briefly, in 2014 Okoye and colleagues produced Let-7d+exosomes by transfecting regulatory T cells with Let-7d mimic, these armed exosomes could reduce the proliferation of Th1 cells in a mouse model of colitis.[13] In another study, Rivoltini et al produced TRAIL+ exosomes which could induce a higher degree of apoptosis in lymphoma and melanoma cells.[17] Considering that miR-326 contributes to the pathogenesis of MS; it is possible that inhibition of miR-326 expression in T cell-derived exosomes or engineer them to carry selected miRNA may be considered as a promising therapeutic approach for the treatment of MS.

Along with the pathologic role of miR-326, its altered expression in T cell-derived exosomes of patients can be considered as a biomarker for prognosis and monitoring of multiple sclerosis. Exosomes released by CD4+T cells carry unique microRNA set, which may resemble inflammation in MS. Hence, these vesicles are of considerable interest as a biomarker for CNS-related diseases.[18]

In the case of other microRNAs, although results were not statistically significant, we found, miR-146a and miR-29a were down-regulated in MS patients. Further, we observed that miR-155 was up-regulated in MS patients compared to healthy controls. This is inconsistent with previous studies that have demonstrated the dysregulation of microRNAs such as miR-155 was highly correlated with RRMS and EAE.[19] In addition, other studies showed that expression level of miR-29a decreased significantly in CNS related disorders. However, none of these studies have examined microRNAs expression in exosomes.

This study has some limitations: First, choosing the patients with strict inclusion and exclusion criteria; second, the high volume of peripheral blood (25-30cc) required for isolation the exosomes. Therefore, we could work on the minimum sample size; third, we compared the expression level of microRNAs in patients during the relapse phase in comparison to healthy control. It was better to have a remission group along with relapse and control groups.

Taken together, we showed over-expression of miR-326 in T CD4+ derived exosomes in MS patients. Although the physiological functions of exosomes are not clear yet, manipulation of such vesicles to modify their activity might be considered as a potential therapeutic approach for autoimmune diseases. Moreover, investigation of miRNAs profile in

leukocyte-derived exosomes might also serve as a beneficial biomarker for assessing disease activity.

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