Original Article

The evaluation of gene expression and enzyme activity of SIRT1 in peripheral blood mononuclear cells isolated from patients with relapsing-remitting multiple sclerosis

Solaleh Emamgholipour[1], Mohammad Ali Sahraian[2], Somayeh Shapourizadeh[3], Mohammad Ansari[1*]

[1] Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2] MS Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran
[3] Payame Noor University, Tehran, Iran

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Abstract

Background: Little in known regarding the clinical relevance of SIRT1 in multiple sclerosis (MS). Here, we aimed to evaluate mRNA expression, protein level and enzyme activity of SIRT1 in peripheral blood mononuclear cells (PBMCs) isolated from relapsing –remitting MS patients (RRMS) and healthy controls. **Materials and Methods:** Twenty patients with RR-MS and twenty two age- and sex-matched healthy subjects were enrolled in this case-control study. Following PBMCs isolation, mRNA expression was evaluated by real time-PCR. SIRT1 activity and SIRT1 protein level were measured using a fluorometric assay and an enzyme-linked immunosorbent assay (ELISA) respectively, in PBMC lysates. **Results:** There was no statistically significant difference in the mRNA expression of SIRT1 (p=0.56) and its protein levels (p=0.15) between MS patients and healthy subjects. By contrast, SIRT1 enzyme activity were significantly (p=0.008) lower in RRMS patients compared with that in healthy subjects. **Conclusion:** Our findings demonstrated that enzyme activity of SIRT1 is significantly lower in PBMCs of RRMS patients in comparison with healthy subjects. However, more investigations are essential to clarify the role of SIRT1 in MS pathogenesis.

Keywords*:* enzyme activity, multiple sclerosis, pathogenesis

***Corresponding Author:** Mohammad Ansari, Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences (TUMS), Tehran, Iran, Tel: (+98)-21 88991593, E-mail: ansarimo@sina.tums.ac.ir
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Introduction

Multiple sclerosis (MS) is a chronic inflammatory and demylinating disease of the central nervous system (CNS) which results into severe neurological disability with deleterious impacts on quality of life. The clinical heterogeneity is the characteristics of MS, however, most patients initially exhibit a relapsing-remitting course ,which is followed by a secondary progressive phase. There is substantial evidence that several pathological processes including inflammation, demylination , axonal loss and oxidative stress play an important role in MS pathogenesis(1-2). It is well-known that more than one mechanism may direct toward development of MS and also complicated and different mechanisms may prevail in various clinical course of disease(3).

Among complicated network of molecules

engaged in regulation of inflammation , immune responses and oxidative stress,SIRT1 might be involved in MS pathomechanism (4, 5).

SIRT1, a well-characterized member of mammalian sirtuin (silent mating type information regulation 2 homolog) family, is a nicotinamide adenine dinucleotide-dependent protein deactylase. SIRT1 through function on numerous protein targets, play important roles in several cellular pathways including metabolism ,apoptosis, inflammation, regulation of neuronal death and survival and oxidative stress (5-7).

There is accumulating evidence that SIRT1 activation exert the neuroprotective effects on several neurodegenerative diseases such as Alzhiemer,Parkinson, amyotrophic lateral sclerosis Huntington's disease, and MS (5, 8-9).

Moreover, it was reported that SIRT1 activation alleviated disease severity in experimental autoimmune encephalomyelitis (EAE) as animal model of MS through preserving axonal function, neurological dysfunction and reducing neuronal loss-induced oxidative stress and inflammation (10-12). Furthermore, SIRT1 was significantly expressed by inflammatory cells in both active and chronic MS plaques (13).

However, little is known regarding the SIRT1 expression and its enzyme activity in peripheral blood mononuclear cells (PBMCs) of MS patients. Hence,the purpose of the present study was to evaluate mRNA expression, protein level and enzyme activity of SIRT1 in PBMCs isolated from relapsing–remitting MS patients (RRMS) and healthy controls.

Methods

This case-control study was conducted on 20 patients with relapse-remitting MS (RR-MS) and 22 age- and sex-matched healthy subjects. All patients were definite MS diagnosed by a neurologist based on the MacDonald criteria. In details,12 patients were in relapse phase and rest were in rem ission course of disease. Also, no patients were newly diagnosed cases and had not received any immunomodulatory and immunosuppressive drugs (anti-inflammatory drugs)for at least 6 month prior to study entry. Also, no patients had received corticosteroid therapy within the previous 6 months or for more than 6 months before inclusion in the study. The Expanded Disability Status Scale (EDSS) was used to score degree of disability at time of blood sampling. The control group was randomly selected among age and sex matched volunteers who were genetically unrelated to the patients included in the study.

Relapsing-remitting course of MS was ascertained according to McDonald criteria in all these patients. All patients were recruited from Sina MS Center, Sina Hospital, Tehran University of Medical Sciences, Tehran, Iran.

The exclusion criteria for all participants included a history of diabetes mellitus, malignancy, acute or chronic infection, cardiovascular disease, current smoking, any autoimmune disease and clinically significant systemic disease, treatment with immunosuppressive and immunomudulatory drugs and antioxidant supplements in previous 6 months.

This case-contol study was approved by Tehran University of Medical Sciences (TUMS) Ethics Committee, and written informed consent was obtained from all participants.

PBMC isolation

Venous blood sample was collected from all participants after an overnight fast and was aliquoted into in heparinized tubes to separate PBMCs. PBMCs were isolated from freshly heparinized blood using Ficoll-Hypaque (Lympholyte-H; Cedarlane Laboratories, Hornby, ON, Canada) gradients centrifugation. After washing with PBS at 4°C, isolated PBMCs were counted and viability was tested using the Naebaur counting chamber after staining with Trypan blue. To analysis mRNA expression,protein expression and enzyme activity assay of SIRT1, PBMCs were aliquoted into separate sterile 2-ml eppendorf tubes.

SIRT1 mRNA expression

To analysis mRNA expression, total RNA was extracted from PBMCs using a Total RNA Extraction Miniprep kit (Viogene, Taiwan) following the manufacturer's instruction. The concentration of RNA was quantified with a NanoDrop ND-1000 (Thermo Fisher Scientific) and RNA purity was determined by the 260/280 nm absorbance ratio. RNA integrity was

assessed by agarose gel electrophoresis. The cDNA was synthesized from 1 µg of DNase-treated RNA using Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific, Fermentas, USA).

mRNA levels of target gene SIRT1 (Qiagen, Hilden, Germany) and housekeeping gene β-actin (Qiagen, Hilden, Germany) measured using quantitative real time -PCR in a Rotor Gene real-time thermocycler (Qiagen, Hilden, Germany) using SYBR Green detection kit (Takara Bio, Ostu, Japan). To determine the linear range of the real-time PCR assay, standard curves were generated for SIRT1 and β-actin prior to performing the assay on test samples. The product specificity was confirmed by both melting curve analysis and gel electrophoresis. Relative gene expression was normalized to β-actin and calculated as 2-ΔCT using the formula: 2-(Ct target gene-Ct β-actin) .

The measurement of SIRT1 protein

Quantitative measurement of SIRT1 protein in PBMCs was determined using a commercial available ELISA assay (ab123457, Abcam, Cambridge, MA, USA) according the manufacturer's instructions. Briefly, PBMCs were lysed in a cold extraction buffer (provided with kit), which was supplemented with protease inhibitor cocktail and PMSF. Following centrifugation at 16000×g for 20 min at 4°C, the supernatant was collected and the protein concentration of the resulting cell lysate was determined using the Bradford assay. SIRT1concentration was normalized for the total protein concentration of samples and expressed as ng/ µg of total protein. The sensitivity of the SIRT1 ELISA was 8 ng/ml SIRT1 standards. The mean inter- and intra-assay coefficient of variance were 7.5% and 7%, respectively.

The assessment of SIRT1 activity

SIRT1 activity was measured in PBMCs using the Cyclex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit (CycLex, Nagano, Japan). This kit is based on the NAD-dependent deacetylase activity of SIRT1 on the specific fluero-substrate peptide, in the presence of trichostatin A, a potent inhibitor of SIRT1-independent histone deacetylases. The nuclear and cytoplasmic extracts were prepared from isolated PBMCs using an EpiSeeker Nuclear Extraction Kit (Abcam) according to the manufacturer's instructions. It should be noted that protease inhibitor-free extracts was used to determine SIRT1 activity based on the kit protocol. Fluorescence intensity was determined by reading fluorescence using a microplate fluorometer (Synergy H4 Bio Tek) with an excitation wavelength 340 nm and an emission wavelength of 440 nm using a microplate fluorometer (Synergy H4 Bio Tek) every 1 min for 1h. Value of SIRT1 activity was expressed as fluorescence intensity change [arbitrary fluorescence unit (AFU)] per minute and was normalized to the protein concentration, determined by the Bradford method.

Statistical Analysis

All data was analyzed using SPSS 19 (SPSS Inc., Chicago, IL, USA) . The normality was assessed by the Shapiro–Wilk test for data. Comparisons between RR-MS patients and healthy subjects were performed by the Mann-Whitney U test . Results were presented as median (interquartile range(IQR)). Comparative CT method (14) was used for analysis of the gene expression. Statistical significance was considered at a p-value <0.05.

Results

The results of mRNA level of SIRT1 in PBMCs from MS patients and healthy subjects were shown in Figure1. As depicted in this figure, the difference between healthy subjects and patients with RRMS was not statistically significant (p=0.56). Similarly, protein levels of SIRT1 in RRMS patients were not significantly (p=0.15) decreased compared with healthy controls (Median (IQR); RRMS patients: (0.04393 ng/µg of protein (0.03199 ng/µg of total protein -0.06073 ng/µg of protein); healthy subjects: (0.08843 ng/µg of protein (0.02799 ng/µg of protein -0.1124 ng/µg of protein) (Figure 2).

By contrast, SIRT1 enzyme activity were significantly (p=0.008) lower in RRMS patients (Median (IQR); 293 AFU/mg of protein (207 AFU/mg of protein -397.2 AFU/mg of protein)) compared with that in healthy subjects (541.6 AFU/mg of protein (379.5 AFU/mg of protein -745.5 AFU/mg of protein)) (Figure 3).

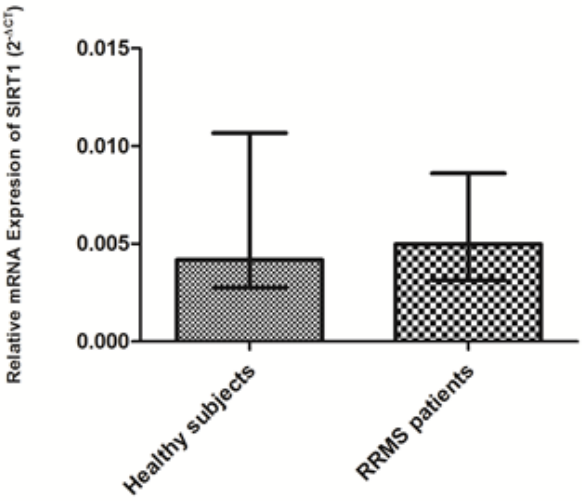

Fig. 1. TAFI polymorphism Thr 325 Ile of 8 fetuses. L:Ladder 100bp, UD: undigested product, 1,7: unmutant, 2,3,4,5,8: homozygote, 6: heterozygote

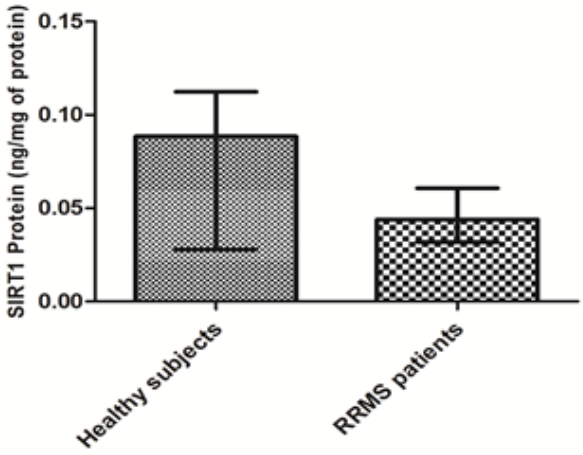

Fig. 2. Protein level of SIRT1 in PBMCs from patients with relapsing-remitting multiple sclerosis (RRMS) and healthy subjects. Data are expressed as median (IQR)

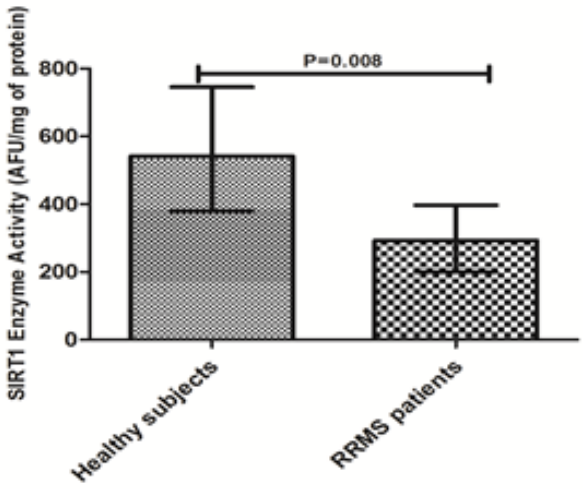

Fig. 3. Enzyme activity of SIRT1 in PBMCs from patients with relapsing-remitting multiple sclerosis (RRMS) and healthy subjects. Data are expressed as median (IQR)

Discussion

In present study, we found that mRNA levels and protein expression of SIRT1 tended to be higher (albeit not significantly) in PBMCs of RRMS patients in comparison with those in healthy controls. Interestingly, we demonstrated that SIRT1 activity were significantly lower in PBMCs of MS patients than in control group. As far as we know this the first report of decreased SIRT1 activity in PBMCs of MS patients compared to controls.

Compared to our previous study in which all patients were in relapse phase , we included RRMS patients in both relapse and remission phase of disease in current study.

With regard to mRNA levels and protein expression of SIRT1, our findings are inconsistent withPenissi et al. in which observed that protein levels of SIRT1 in plasma from MS patients was significantly increased when compared to levels of control subjects (15).

However, it was demonstrated that mRNA levels and protein expression of SIRT1 were significantly reduced in PBMCs of MS patients during relapse compared to those in healthy subjects (13).

It is evident that SIRT1 downregulates NF-Kb-dependent gene expression by deacetylating RelA/p65 at lysine 310. More specifically, NF-Kb activation caused upregulation of pro-inflammatory cytokines as well as several chemokines (16-18). Accordingly,they suggested that high expression of SIRT1 in MS patients may be an adaptive response to confront impaired inflammatory response in MS (15).

It is generally accepted that infiltration of inflammatory cells including T-lymphocytes and monocyte-derived macrophages into CNS and consequently production of large amounts of ROS and inflammatory mediators have an important role in neuronal damage, oligodendrocyte loss and myelin

phagocytosis (19, 20). Hence, targeting SIRT1 in PBMCs as a mixed population of immunocompetent cells can be helpful in the context of MS treatment.

Given the key role of SIRT1 in modulating immune responses, inflammatory signaling, apoptosis and oxidative stress, it is tempting to speculate that low activity of SIRT1 contributes to MS pathomechanism as an inflammatory, autoimmune and oxidative stress abnormality. Although further studies are needed to establish this concept, this explanation is supported by several lines of evidence. Firstly, treatment with SIRT1 activators attenuates neural loss during optic neuritis, an inflammatory demyelinating optic nerve lesion with high frequency in MS and its animal models (9) . Protection against myelin breakdown in the rodents models accompanying with reduced disease activity are also other evidence for this concept (8, 10). Secondly, it has been shown that loss of SIRT1 results to increased cell activation, defective maintenance of T cell tolerance and subsequent severe disease in EAE (4). Additionally, SIRT1 overexpressionin in chronic EAE was associated with reduction in inflammation and neuronal loss (8, 10). Notably, apoptosis in immune cells such as jurkat cells,CD4+ and CD8+ was observed in MS patients following SIRT1 inhibition (13) .

Significant decreased activity of SIRT1 in MS patients is probably due to post-translationl modification of SIRT1 which is mediated in response to intensified chronic inflammatory and oxidative stress milieu in MS patients (21). This explanation is supported by both in vivo and in vitro studies showing that post-translational modification such as SUMOylation, phosphorylation and carbonylation can lead to altered activity of SIRT1 (22, 23).

In conclusion,our results demonstrate that enzyme activity of SIRT1 is significantly lower in PBMCs of RRMS patients in comparison with healthy subjects. However,more investigation with a large sample size and further studies on other MS subtypes are essential to clarify the role of SIRT1 in MS pathogenesis.

Conflicts of Interest

The authors declare that there is no conflict of interest in this study.

Acknowledgment

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