Original Article

Alteration of *OGG1*, *MYH* and *MTH1* genes expression in relapsing-remitting multiple sclerosis patients

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Abstract

Introduction: Previous studies revealed that oxidative stress is elevated in multiple sclerosis (MS). It can harm to biological macromolecules such as DNA. However, the molecular mechanism in protection of genetic information from DNA damages is not clear in MS disease. In this study the expression level of some important genes of *OGG1* and *MYH* involved in base excision repair pathway and, *MTH1* and *ITPA* as main cleaning genes of nucleotide pool from rough nucleotides are examined in MS patients in compared to healthy group.

Methods: Peripheral blood mononuclear cells were isolated from relapsing-remitting-MS patients and healthy subjects. After RNA extraction and cDNA synthesis, the expression levels of target genes were examined by RT-qPCR technique.

Results: The level of the *MTH1* and *MYH* genes expression were decreased, but the level of *OGG1* mRNA was higher in patients in comparison to the control group. Obtained result did not shown any correlation between expression of examined genes and clinical features of patients such as MS severity and disease duration.

Conclusion: These preliminary results provide more supportive evidences for involvement of oxidative damage and variation in expression of DNA repair genes in MS. Significant increase of *OGG1* suggest that the development and progression of pathogenesis in Iranian MS can be related to chronic and direct oxidative damage of genomic DNA not nucleotide pools.

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Introduction

Multiple sclerosis (MS) is a chronic neurologic disorder that estimated affects 2.5 million peoples in the worldwide. Different types have been identified in MS including relapsing-remitting (RR), primary progressive and secondary progressive (Chen M et al., 2013) that are categorized based on patterns of progression, as well as the intensity and frequency of generated symptoms. RR-MS is the most common form of MS disease. Environmental and genetic factors have been found to affect the development of MS. The disease is characterized by inflammation, damaged myelin sheaths in nerve cells, axonal degeneration, loss of oligodendrocyte and oxidative stress (Villoslada, 2016).

It has been suggested that oxidative stress and its by-products, reactive oxygen or nitrogen spices (ROS or RNS) are involved in pathogenesis of many types of human diseases such as cardiovascular, autoimmune and neurodegenerative diseases

(Behmanesh et al, 2009, Adamczyk and Adamczyk-Sowa, 2016).

It has been shown that by establishing active demyelination process, ROS-mediated injury by peripheral immune cells and central counterparts promotes progressive tissue damage in MS (Kremer et al. 2004, Adamczyk and Adamczyk-Sowa, 2016). Also, microglia and infiltrated macrophages can generate proinflammatory mediators and oxidizing radicals (Colton and Gilbert, 1993).

Free radicals are normally produced in physiological conditions but their level increased in oxidative stress conditions. In all of eukaryotic cells, adjusted levels of free radicals have a crucial role in regulating various cellular functions such as inflammatory response in which production of ROS and RNS attribute to monocyte interactions with brain endothelium (Tasset et al., 2012).

Uncontrolled production of free radicals may cause oxidation of different types of biomolecules such as free nucleotides and genomic DNA. Oxidative modification of genetic material and accumulation of oxidized bases, cause different types of mutation in the DNA and instability in RNA molecules (Behmanesh et al., 2005). Among biomarkers of oxidative DNA damage, 8-oxo-guanine has the crucial role in DNA alteration. It has been shown that this rough base has different quantity in the genomic DNA of blood and brain patients with neurodegenerative diseases (Sliwinska et al., 2016).

To protect the genetic integrity of the cells from endogenous and exogenous source of free radicals, all of living cells are equipped with DNA repair systems. Nucleotide pool cleaners and base excision repair (BER) pathway are among the major mechanisms which are responsible for cell protection from accumulation of oxidized lesions in genomic DNA or nucleotide pools (Nakabeppu et al., 2014).

Several studies are shown that DNA damage and other oxidative stress biomarkers are increased in peripheral blood samples of MS patients (Grecchi et al., 2012; Ibitoye et al., 2016; Tasset et al., 2012). Satoh et al. (2005) identified an aberrant expression of some DNA repair genes in peripheral blood mononuclear cells (PBMC) of MS patients. The associations of some DNA pathway repair genes polymorphisms with MS have been verified (Briggs et al., 2010; Karahalil et al., 2015), but there is not enough information about the essence of DNA damages types which are involved in MS pathogenesis.

To get this point the expression of 8-oxoguanine DNA glycosylase (*OGG1*) and MUTYH glycosylase (*MYH*) involved in BER pathway as well as nudix hydrolase 1 (*MTH1*) and inosine triphosphatase (*ITPA*) as the main nucleotide pool cleaner genes compared in PBMC samples from RR-MS patients and healthy subjects.

We evaluated *OGG1*, *MYH*, *ITPA* and *MTH1* genes expression based on previous information about the possible association of DNA repair system with the pathological features of disease, elevated levels of oxidative stress (Miller et al., 2011; Tasset et al., 2012) and abnormal oxidized base (Haider et al., 2011) in MS patients.

Materials and methods

Patients and PBMC collection

Thirty one eligible patients were recruited for this study that diagnosed according to McDonald criteria (McDonald et al., 2001) and MRI test was performed for all of the patients. Patient's age was 22-52 years and The Expanded Disability Status Scale (EDSS) values of patients were between 0-5.5. They had presented to the MS Research Center of Sina Hospital of Tehran University of Medical Sciences, Tehran, Iran from November 2012 to October 2013 (Naghavi Gargari et al., 2015). All of the patients had no familial relation and no other inflammatory or autoimmune disease. They were RR-MS subtype. Also, 27 healthy samples were collected from volunteers with no history of hospitalization for neurological and autoimmune diseases. All of precipitants were selected after interviewed by an experienced neurologist. Written informed consent was obtained from all participants prior to the blood sampling. The Ethics Committees of Tarbiat Modares University approved this study. Three milliliters of whole blood was taken and collected in the anti-coagulant EDTA tubes from each participant. PBMC were isolated by density gradient centrifugation on Ficoll-Paque solution (lympholyte, Cedarlane, Netherlands) according to manufacturer's instructions as described.

RNA extraction and cDNA synthesis

Total RNA of PBMC was extracted by acid

guanidinium-phenol-chloroform procedure using RNX™-plus solution (SinaClon co., Iran) according to the manufacturer's instructions. The isolated RNAs were treated with DNAaseI (Fermentas, Lithuania) for 20 min at 37°C to eliminate any genomic DNA contamination. Integration, concentration and purity of RNAs were verified by agarose gel electrophoresis and spectrophotometry. Three micrograms of purified RNA was used for cDNA synthesis with random hexamer and oligo $(dT)_{18}$ primers (MWG, Germany) through M-MuLV reverse transcriptase (Thermo scientific, USA) in total 20 µl reaction mixture according to manufacturer's instructions.

Real-time PCR analysis

The quantification of *OGG1*, *MTH1*, *MYH* and *ITPA* mRNAs expression was performed with Real-time PCR and Hot FIREPol EvaGreen qPCR Mix plus ROX (Solis BioDyne, Estonia). Relative levels of genes expression were detected by specific primers designed by Oligo software version 6 (USA). The sequence of used primers were: 5'-GTATATGGGCTGGCCTTGGAAG-3' and 5'-CTGTTGGCCCTGATACACACG-3' for *MYH*, 5'-ACCCTGGCTCAACTGTATCACCAC-3' and 5'-CCGCTCCACCATGCCAGTGATG-3' for *OGG1*, 5'-GGGCCAGATCGTGTTTGAGTTCGT-3' and 5'-TCGTCGGGCCACATGTCCTTG-3' for *MTH1* and 5'-AAGAAGCTGGAGGAGGTCG-3' and 5'-TCCAAGGGCATTGAAGCACA-3' for *ITPA* mRNAs amplification. The *GAPDH* primers sequences were forward: 5'-CCATGAGAAGTATGACAAC-3' and reverse: 5'-GAGTCCTTCCACGATACC-3' which was used as the internal control. Real-time PCR was carried out using Applied Biosystems StepOne™ Real-Time PCR Systems (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) in a final reaction volume of 20 µl with 10 ng cDNA, 4 µl of EvaGreen 5X master mix and 200 nM of each forward and reverse primers. The PCR condition was as follows: an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 60°C for 15sec and extension at 72°C for 30sec. Specificity of PCR products were confirmed by running of qPCR products on 12% poly acrylamide gel electrophoresis and melting curve analysis. All experiments were done at least in duplicate. Each gene expression of the individual samples was normalized with the *GAPDH* gene

expression and relative fold change was calculated by $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001).

Statistical analysis

Data obtained from this study was tested for normal distribution by calculating Kolmogorov–Smirnov test and performing the Shapiro–Wilk normality test. Based on the normal distribution Pearson and spearman rank was carried out to analyze correlations between gene expression and clinical features. The expression level of genes were compared to controls via independent T-test and Mann–Whitney U test using SPSS software (Version 21; SPSS Inc, Chicago, IL) and GraphPad Prism Software (Version 5.0, Inc., San Diego, CA) to compare parametric and nonparametric continuous variables, respectively. Data are presented as mean ± standard deviation (SD) and $p<0.05$ was defined as the statistical significance.

Results

In this study we analyzed *OGG1, MTH1, MYH* and *ITPA* mRNAs expression in 31 MS patients and 27 healthy samples. The EDSS score of patients was a mean 2.16±0.97. The patients consisted of 25 females and 6 males with a mean age of 30.81±7.30 years old and the mean of disease duration was 4.64±3.87 years. The control group was included 17 females and 10 males with a mean age of 27.88±4.04 years old.

The relative expression of *MTH1, ITPA, MYH* and *OGG1* were determined using quantitative real-time PCR analysis. As shown in Figure 1A, although the level of *MTH1* transcripts in patients was significantly lower than healthy control (p=0.04, 2.6-fold), the expression of *ITPA* mRNA level was not significant difference between MS patients compared to control subjects (p=0.43, 1.4-fold, Fig. 1B). Moreover, results showed, *MYH* expression was decreased in MS patients compared to healthy control (p=0.025, 3.03-fold, Fig. 1C). Interestingly, the expression of *OGG1* was up-regulated in patients (p=0.001, 4.2-fold, Fig. 1D) compared with control samples.

In order to determine whether the disease severity affected on the DNA repair genes expression, the patients divided into two groups based on EDSS scores: EDSS ≤ 2 (n=18) and EDSS > 2 (n=13). We observed that expression of *OGG1* (p=0.01, 5.2-fold)
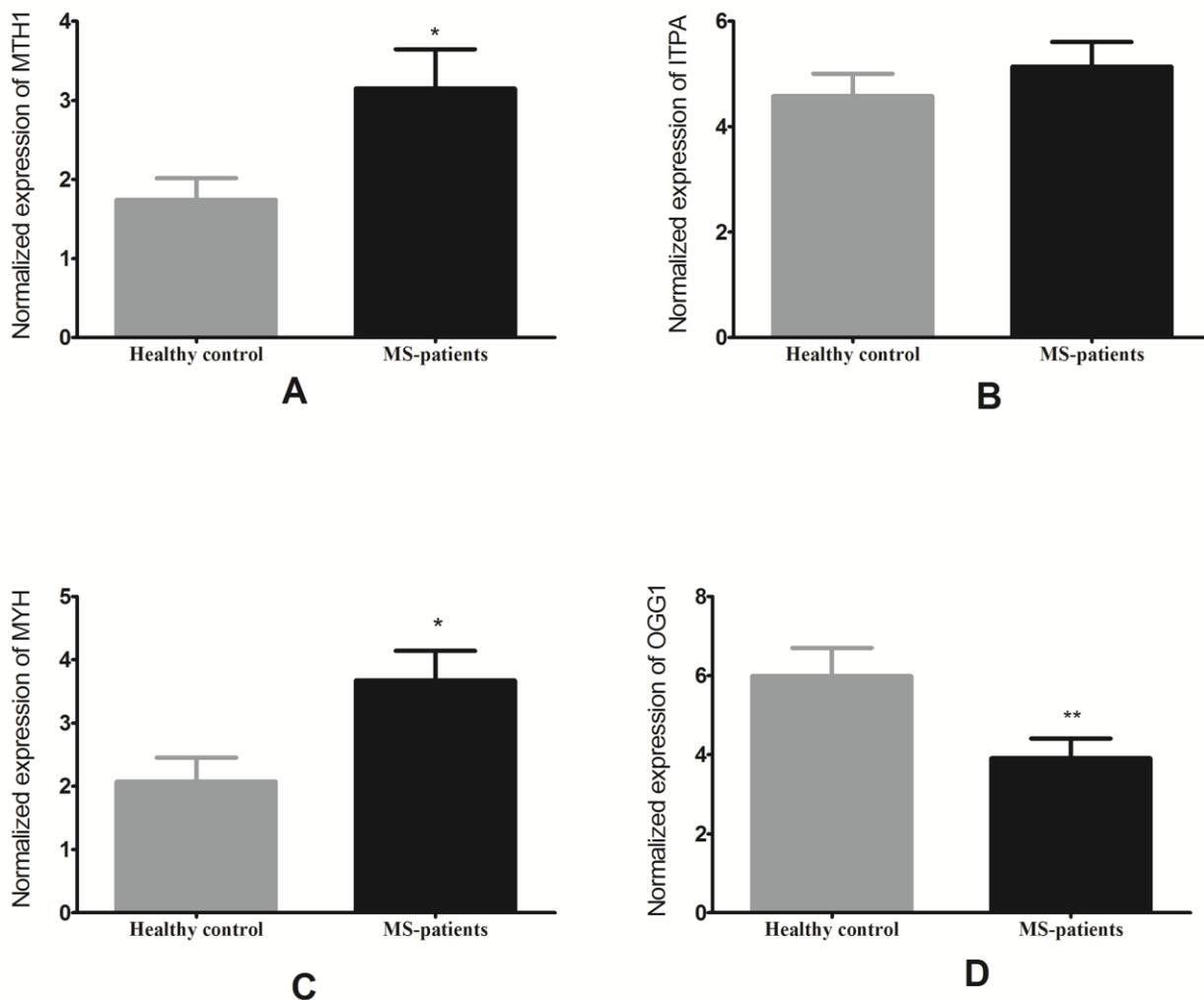
Fig.1. Comparison of normalized genes expression levels between healthy controls and MS patients. Normalized expression of *MYH* (*p*=0.025), *MTH1* (*p*=0.04) and *OGG1* (*p*=0.001) mRNA levels were statistically different in RR-MS patients in comparison with healthy subjects, while the expression of *ITPA* (B) did not show any difference between two groups. The genes expression levels were normalized relative to the GAPDH expression as an internal control. p-value≤0.05 was considered statically significant. The expression shown as mean ΔCt ± SED.

and *MYH* (*p*=0.03, 3.6-fold) were significantly increased and decreased, respectively in patients with EDSS ≤ 2 compared with healthy control, but other studied genes have no statistically different from control samples (*MTH1*: p=0.06, *ITPA*: *p*=0.66). Also, in EDSS > 2 group, there were no significant differences between the mRNA level of interested genes in MS patients versus healthy controls (*MYH*: *p*=0.052, *ITPA*: *p*=0.24, *OGG1*: p=0.15) but interestingly normalized expression of *MTH1* decreased in this group (*p*=0.04, fold change-2.1). Additionally, genes expression did not show any significant difference between two classified groups (EDSS > 2 and EDSS ≤ 2 to each other) (*MYH*1: *p*=0.49, *MTH1*: *p*=0.59, *OGG*1: *p*=0.64 and *ITPA*: *p*=0.65). Obtained results did not reveal any correlations between the normalized expression of

OGG1, MTH1, MYH and *ITPA* and clinical features such as MS severity, disease duration and patient age (data not shown).

Correlation analysis was conducted that the expression level of *OGG1* was correlated with nucleotide pool enzymes (*ITPA*: *p*=0.01, r= 0.43, *MTH1*: *p*=0.01, r=0.45, Fig. 2). In addition, significant positive correlation was observed between *MYH* mRNA level with *ITPA* and *MTH1* (*p*<0.0001, r=0.7, *p*<0.0001, r= 0.8, respectively, Fig. 3).

Discussion

MS is a neurodegenerative disorder at which oxidative stress plays a major role in neuronal and axonal deterioration and inflammation in the CNS. Investigation of the molecular mechanism for
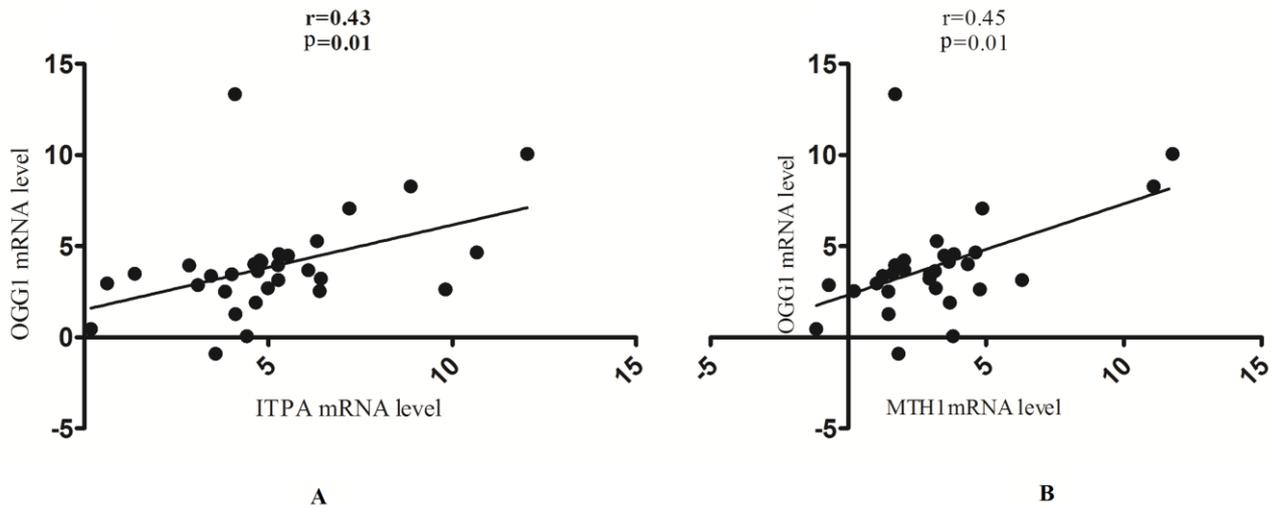
Fig.2. Correlation analysis of normalized expression levels of *OGG1* with *ITPA*(A) and *MTH1* (B) as the main nucleotide pool cleaner encoding genes. The result of Pearson correlation test showed a positive correlation between the expression levels of *OGG1* with *ITPA* (A) and *MTH1* (B) genes in PBMCs of MS patients.
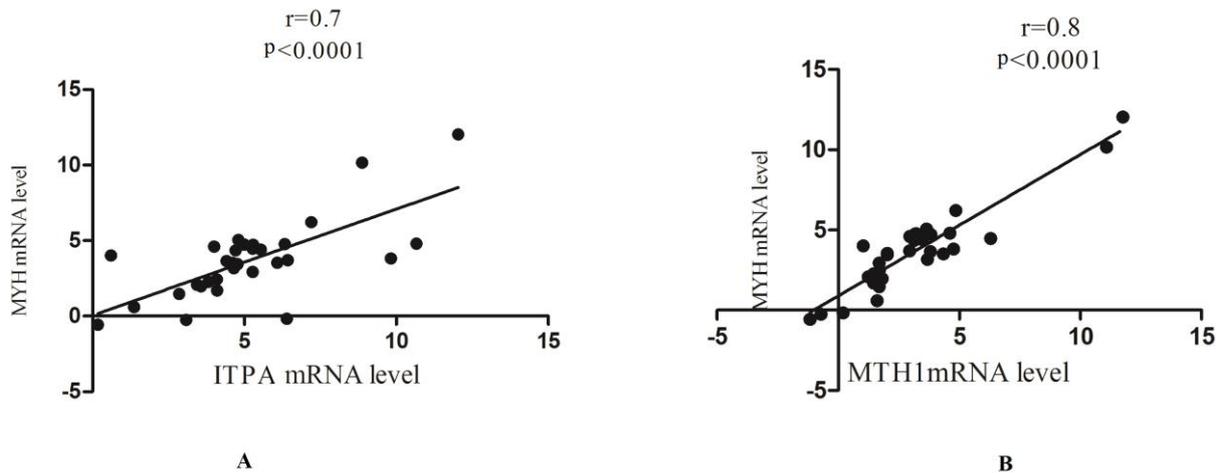
Fig.3. Correlation analysis between normalized expression of MYH with *ITPA* and *MTH1* genes. The MYH level correlated significantly with *ITPA* (A) and *MTH1* (B) mRNA level in PBMCs of MS patient.

revealing the MS pathophysiology has been the aim of some researches. The presence of oxidative stress markers, including oxidative DNA damage and 8-oxoG were reported in MS patients. Ljubisavljevic et al. (2016) demonstrated that the level of 8-oxoG was significantly higher in RR-MS patients in comparison to controls. Furthermore, Polachini et al. (2016) found a significant increase of damaged DNA in leukocytes of RR-MS patients. Although, Oxidative damage to DNA is not only a feature of MS disease, it can cause a pathological cascade of the damages which suggests the involvement of DNA repair system in the pathogenic mechanisms of this neurodegenerative disease. Concerning the potential role of oxidative stress and DNA repair system in neurodegenerative disease progression, we speculated whether the

expression genes involved in DNA damage repairs (*MYTH, MTH1, OGG1* and *ITPA*) are implicated in MS progression. Over 150 genes are involved in the known nuclear DNA and mitochondrial DNA repair pathways (Briggs et al., 2010). The current study focused on expression of four genes derived from two DNA repair pathways: base excision repair and nucleotide pool cleaner. We observed that the expression levels of *MTH1* RNA was down-regulated in RR-MS patients in comparison to healthy control. Moreover, we found expression of *ITPA* mRNA was not significant difference in MS patient.

Increased level of oxidative stress and oxidized bases (Miljković and Spasojević, 2013; Ohl et al., 2015; Tasset et al., 2012) can cause mis-incorporation of modified bases into DNA/RNA during

replication and transcription that cause changing in the content of genetic information and functional abnormality in proteins level which may result in carcinogenesis or neurodegeneration (Nunomura et al., 2007; Gon et al., 2011). A possible explanation for our result might be that the substrate-level has significant effect on the cell economy (Peterson et al., 2016). So, reduction in expression may be related to absence of enzyme substrate. An alternative explanation for this result is that epigenetic modifications may regulate these gene expression in MS patients or dysregulation in upstream genes may affect studied genes expression. Deficiency of studied genes may lead to accumulation of mutant bases during replication and transcription in DNA and RNA respectively. Analysis of data demonstrated that the expression level of *MYH* mRNA was down-regulated in RR-MS patients in comparison to healthy control.

In contrary, we noted the level of *OGG1* in the PBMC of the RR-MS patients was significantly higher than the control group. Additionally, *OGG1* mRNA expression was significantly up-regulated in EDSS ≤2 patients group versus healthy individuals. These observations suggest that decreased *MYH* and *MTH1* expression may contribute to the elevated 8-oxodG level (Eshtad et al., 2016) in the genomic DNA not nucleotide pool of peripheral blood samples of MS patients that leads to *OGG1* up-regulation for removing the mutant base, specifically in the early and middle stages of pathogenesis. The direct oxidation dame to the genomic DNA can be the other possible explanation for increscent of *OGG1* expression. This result stays in agreement with significantly increased in DNA-damage biomarkers of MS PBMCs (Grecchi et al., 2012). Our finding is contrary to Tajouri et al. (2003) studies which have shown DNA repair genes such as *OGG1* mRNA expression is down-regulated in MS patients. Moreover, a study involving 13 RR-MS and 5 systemic lupus erythematosus (SLE) patients demonstrated that DNA repair genes were deregulated only in SLE patients (Mandel et al., 2004). Differences in our results with other studies may be due to the disease subtype and sample resource or methods of measurement (Array versus Real-time PCR).

Additionally our result showed positive correlation between *OGG1* and *MYH* with the expression of

MTH1 gene as a nucleotide pool cleaner. These results are in agreement with the study's findings that noted the h*MYH* and h*MTH1* functionally cooperate for effective repair (Eshtad et al., 2016). Despite the positive correlation between OGG1 and *MTH1*, surprisingly, *OGG1* expression was increased and versus *MTH1* expression level was reduced in patients in comparison with control group. A possible explanation for this might be that the high *OGG1* expression level could be a compensatory mechanism in the low expression of *MTH1* for maintaining DNA integrity.

Gene mutations can cause hereditary-forms of the neurodegenerative diseases, although, the majority of the patients with neurodegenerative disorder have sporadic disease (Nunomura et al., 2007; Gon et al., 2011). Previously Studies have confirmed the association between single nucleotide polymorphisms of DNA repair genes and the risk of disease occurrence in the lymphocytes of MS patients (Briggs et al., 2010; Karahalil et al., 2015). Moreover, Selvaraj et al. (2009) observed a complete inhibition of PARP1 (DNA single-stranded break repair) function in EAE mice that led to an earlier onset and developed a more severe EAE in compared with wild-type animals. Based on indirect immunofluorescence technique, Grecchi et al. demonstrated that poly (ADP-ribose) (PAR) value, which occurs under stress conditions, increased in MS PBMCs, but PARP1 expression did not show any significant difference between MS patients and healthy control. Also, they found more positive cells for γH2AX as a DNA-damage biomarker in MS patients in comparison to healthy donors (Grecchi et al., 2012). These findings imply the efficacy of DNA repair to remove the mutation bases and may be an important factor for preventing the development of different types of diseases such as MS. Cell type, disease subtype and sample size can be limitation of our study and may affect experimental results. Evaluation of protein level in combination with mRNA level and oxidative biomarker such as oxidized base measurement in large-scale is suggested for robust assessment in future studies.

Conclusion

In conclusion, the present study indicated that the DNA repair genes might involve in the

pathophysiology of MS disease. This is a basis for future research and further studies are needed to confirm the function of several DNA repair systems in the progression of MS disease.

Conflict of interest

The authors declare that they have no conflict of interest.

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