The effect of IFN-β 1a on expression of MDA5 and RIG-1 in Multiple Sclerosis patients

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Running title: MDA5 and RIG-1 in MS patients

Abstract

The aims of this study were to compare mRNA levels of MDA5 and RIG-1 in multiple sclerosis (MS) patients in comparison to the healthy controls as well as investigating the effects of IFN-β 1a on the expression of these molecules. In this study, mRNA levels of MDA5 and RIG-1 in peripheral leukocytes of 30 new cases of MS patients and 35 healthy controls were evaluated using the Real-Time-PCR method. mRNA levels of MDA5 and RIG-1 were determined in the MS patients six months after treatment with standard doses of IFN-β 1a. mRNA levels of MDA5 and RIG-1 were significantly decreased in the MS patients in comparison to the healthy controls. The analysis also revealed that IFN-β 1a therapy leads to the up-regulation of RIG-1, but not MDA5, in the total MS patients and the female group. MS patients suffer from insufficient expression of MDA5 and RIG-1 and IFN-β 1a therapy results in the up-regulation of RIG-1 in the patients, especially in the female patients. Thus, it seems that IFN-β 1a not only decreased pathogenic inflammatory responses but also modulated the expression of RIG-1 to protect the patients from infectious diseases and up-regulation of IFN-I in a positive feedback.

Keywords: IFN-β 1a, Multiple Sclerosis, MDA5 and RIG-1.

MDA5 and RIG-1 are two intracellular sensors which induce production of type 1 interferons (IFN-I), including IFN-β 1a, which is a routine treatment strategy of MS patients.

MDA5 and RIG-1 levels were significantly decreased in MS patients compared to healthy controls and IFN-β 1a therapy leads to up-regulation of RIG-1, but not MDA5, in the total and female MS patients.

It appears that IFN-β 1a improves MS symptoms via up-regulation of several molecules such as RIG-1.

1. Introduction

Multiple sclerosis (MS) is an autoimmune pro-inflammatory disorder, in which the central nervous system is the target of immune responses [1, 2]. Epidemiological investigations demonstrated that women suffer from Relapse-Remitting MS (RRMS) twice as much as men [3, 4]. Increased incidences of MS have been observed in Iran and the prevalence of this disease in Kerman city is 57.3/100000 [5]. Previous investigations revealed that MS patients suffer from inappropriate immune responses to infectious diseases [6]. Thus, it has been hypothesized that uncontrolled immune responses to the central nervous system (CNS) may be associated with mal-production of some immune-related molecules which may be important against pathogens. Additionally, it has been documented that production of IFN-β 1a is disrupted in MS patients [7, 8]. Thus, it seems that some IFN-β 1a producing related pathways may be defective in the MS patients. Accordingly, our previous investigations revealed that IFN-β 1a up-regulated the receptor for advanced glycation end products (RAGE) in MS patients [9].

Melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene 1 (RIG-1) are two critical pathogen recognition receptors (PRRs), which detect intracytoplasmic intra-cellular dsRNA [10-13]. MDA5 and RIG-1 use the same pathway; they both use interferon promoter stimulator 1 (IPS-1) to activate two important transcription factors including interferon

regulatory transcription factor 3 (IRF3) and nuclear factor-kappaB (NF-κB) [14]. The activated transcription factors translocate to the nucleus and transcribe immune-related molecules which are important for fighting against pathogens [15]. Therefore, affected transcription of these molecules may be associated with defective immune responses against pathogens. Based on the fact that MS patients suffer from inappropriate expression of IFN-β 1a [7, 8] and therefore this interferon is used for the treatment of these patients [2], it has been hypothesized that the expression of MDA5 and RIG-1 may be defective in these patients.

Thus, the main aims of this study were to compare the expression of MDA5 and RIG-1 in MS patients and the healthy controls and also evaluate the effects of IFN-β 1a therapy on the expression of these molecules.

2. Material and methods

2.1 Subjects

This experimental study was performed on 30 new cases of MS (22 females and 8 males) and 35 healthy controls (25 females and 10 males) from October 2018 to June 2019. The patients were not infected by HBV, HCV, and HIV since MS patients are routinely examined in Iran. The patients were evaluated regarding the expression of MDA5 and RIG-1 in the peripheral leukocytes before receiving any other medication and also 6 months after receiving IFN-β 1a (Cinnovex). The Revised McDonald criteria and radiology, laboratory and

clinical outcomes and physician neurologist observations were used for the diagnosis of MS [16]. The excluding criteria were as follows: other inflammatory disorders such as type 1-4 hypersensitivities, type 1 and 2 diabetes, heart failure, kidney diseases; also if the patients were not administrated with any anti-inflammatory medication they were excluded from the study. All of the MS patients and healthy controls signed an informed consent form prior to entering the study. A research permit was obtained from the Ethics Committee of Kerman University of Medical Sciences (Code: k/92 /587). IFN-β 1a was prepared by CinnaGen Company (Tehran, Iran), as the commercial name of Cinnovex. Before receiving any other drugs and after six months of treatment with standard doses of IFN-β 1a, 10 mL of whole blood was collected in EDTA pre-treated tubes to isolate peripheral leukocytes using Ficoll. After total RNA purification, cDNA was synthesized and the expression of MDA5 and RIG-1 were evaluated.

2.2 Real-Time PCR condition

Total RNA extraction, cDNA synthesize and the protocol of Real-time PCR were described in our previous investigations [12, 17]. β-actin was considered as the housekeeping gene to normalize amplification signals. Table 1 illustrates the primer sequences.

The relative expression of MDA5 and RIG-1 in untreated MS patients versus healthy controls and untreated MS patients versus six months treated ones were computed using the $2^{-\Delta\Delta CT}$ formula.

2.3 Data analyses

Data were analyzed using SPSS-18 software. The comparison of untreated MS patients and healthy controls as well as untreated MS patients and six months treated ones were performed using the Mann-Whitney U test and Paired-Samples t test, respectively. The effects of Cinnovex (IFN-β 1a) treatment on the male and female MS patients were also analyzed using Paired-Samples t test. The results regarding the differences between untreated MS patients and healthy controls were reported as MEAN RANK and the results regarding the expression of the molecules before and after treatment of total, male and female MS patients were reported as Mean ± SEM. P-value less than 0.05 was reported as the significance level.

3. Results

The results revealed that the mRNA levels of MDA5 ($p< 0.001$) and RIG-1 ($p< 0.001$) were significantly decreased in untreated MS patients in comparison to the healthy controls (Figure 1).

Results also revealed that expression levels of MDA5 in untreated and treated MS patients were 1.10 ± 60 and 0.63 ± 0.28, respectively. The statistical analysis demonstrated that the differences were not significant ($p= 0.457$, figure

2). Figure 2 also shows that IFN-β 1a therapy leads to significant down-regulation of MDA5 in males ($p< 0.001$) but not in females ($p= 0.286$).

The expression levels of RIG-1 were significantly increased in the IFN-β 1a treated MS patients (0.20 ± 0.09) compared to the untreated patients (0.76 ± 0.24, $p= 0.043$, figure 3). RIG-1 mRNA levels were also significantly increased in the female group ($p= 0.05$), but not the male group ($p= 0.490$), after 6 months of threatment with IFN-β 1a (Figure 3).

4. Discussion

The results demonstrated that MS patients suffer from down-regulated MDA5 and RIG-1 expression in their peripheral leukocytes, hence, it may be hypothesized that MDA5 and RIG-1 pathways may be defective in the MS patients and it may be a reason for inappropriate immune responses against pathogens in the MS patients. Additionally, it has been reported that the production of type 1 interferons (IFN-I) is disrupted in the MS patients [7, 8]. MDA5 and RIG-1 are the main molecules that activate IRF3, one of the main transcription factors for the production of IFN-I. Therefore, it may be concluded that the disrupted expression of MDA5 and RIG-1 might be a reason for the disrupted expression of IFN-I in the MS patients. To the best of our knowledge, there are no investigations regarding the status of MDA5 expression or the effects of IFN-β 1a therapy on the expression of this molecule. However, a

study by Wawrusiewicz-Kurylonek et al. revealed that the polymorphisms within the MDA5 gene had a significant association with susceptibility to MS [18]. Thus, it may be hypothesized that the down-regulation of MDA5 in our patients in comparison to healthy controls might be related to their differences regarding the genetic variations. And it may be the main cause of the down-regulation of IFN-I in the MS patients. An investigation approved the benefits of RIG-1 ligand in activating the RIG-1 pathway and the improvement of clinical presentation of MS [19]. Accordingly, it has been shown that using RIG-1 ligands leads to decreased Th1 and Th17 T-cell responses within the CNS [20]. Th1 and Th17 T-cell responses are the most responsible pathogenic immune responses in the MS patients [21]. Interestingly, another investigation revealed that mice lacking IPS-1, a down-stream molecule of MDA5 and RIG-1, develop exacerbated diseases [22]. Therefore, it seems that defected expression of RIG-1, and maybe MDA5, can be considered as crucial reasons for the deterioration of MS. In parallel with this conclusion, Varzari et al. reported that the polymorphisms within the RIG-1 gene are associated with an increased risk of MS in a German population [23].

Additionally, the results demonstrated that IFN-β 1a therapy led to the up-regulation of RIG-1, but not MDA5, in the total and female MS patients. Based on the fact that the number of male patients in the current study was low, hence a larger sample size is needed to confirm the results. In agreement with our

results, a study by Hundeshagen et al. revealed that IFN-β 1a therapy leads to the up-regulation of RIG-1 in a German population [24]. Accordingly, it seems that increased expression of RIG-1, but not MDA5, is the main mechanism used by IFN-β 1a to overcome MS.

Collectively, it may be hypothesized that the down-regulation of MDA5 and RIG-1 is a reason for the decreased expression of IFN-β 1a as well as the defective immune responses against pathogens in MS patients. IFN-β 1a therapy can improve the expression of RIG-1 but is unable to alter the expression of MDA5. According to the fact that MDA5 is also significantly decreased in the immune cells of MS patients, hence, it appears that a combination therapy which targets the expression of this molecule can be considered for the treatment of MS patients.

The results also showed that IFN-β 1a can increase mRNA levels of RIG-1 in the female, but not in the male MS patients. It appears that female patients show different responses to IFN-β 1a regarding the expression of RIG-1 and it may be due to various sexual hormones which can modulate the immune responses [25, 26]. Interestingly, the sex-dependent responses to IFN-β 1a were also seen in the male patients to produce MDA5 which has been decreased. Thus, it appears that in the treatment of MS patients, sex can be considered as an intervention factor.

Finally, the main limitations of the project were the sample size, limited evaluated PRRs and using the technique that evaluated mRNA, but not MDA5 and RIG-1 protein levels. Accordingly, more investigations with more participants may be associated with significant results. Additionally, investigations which use powerful techniques, such as western blotting and flowcytometery, to evaluate the protein levels of MDA5 and RIG-1 may be more valuable than the evaluation of mRNA. Furthermore, assessment of other internal RNA sensors, including TLR3, TLR7 and TLR8, in the MS patients following IFN-β 1a therapy can elucidate the molecular mechanisms of the drug in improving the clinical presentation of the patients.

5. Acknowledgment

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6. Conflict of interest

Authors have no conflict of interest to declare.

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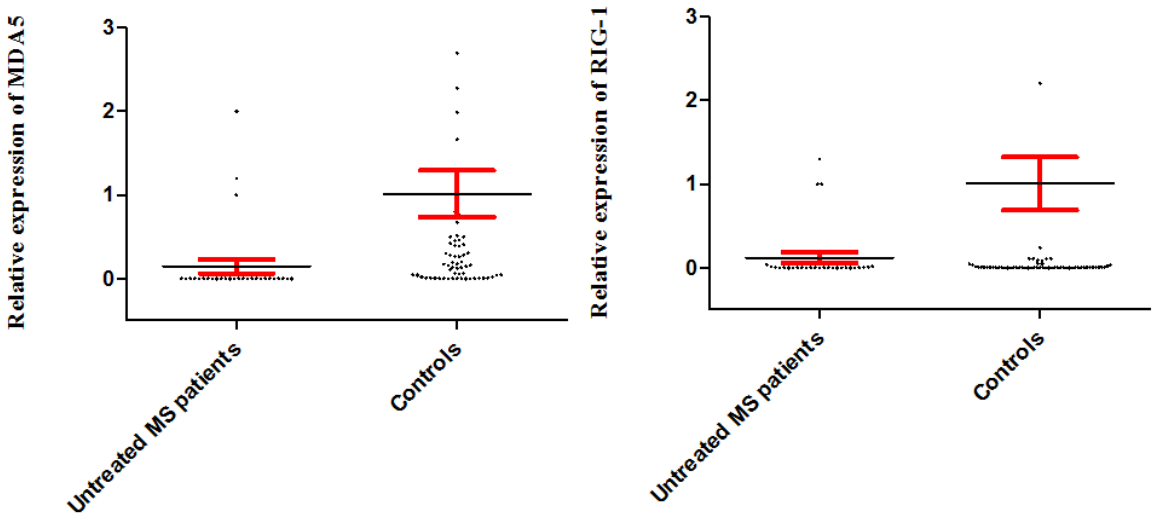

Figure 1: Expression levels of MDA5 and RIG-1 in the untreated MS patients compared to healthy controls. Both mRNA levels of MDA5 ($p < 0.001$) and RIG-1 ($p < 0.001$) significantly decreased in the MS patients in comparison to healthy controls.

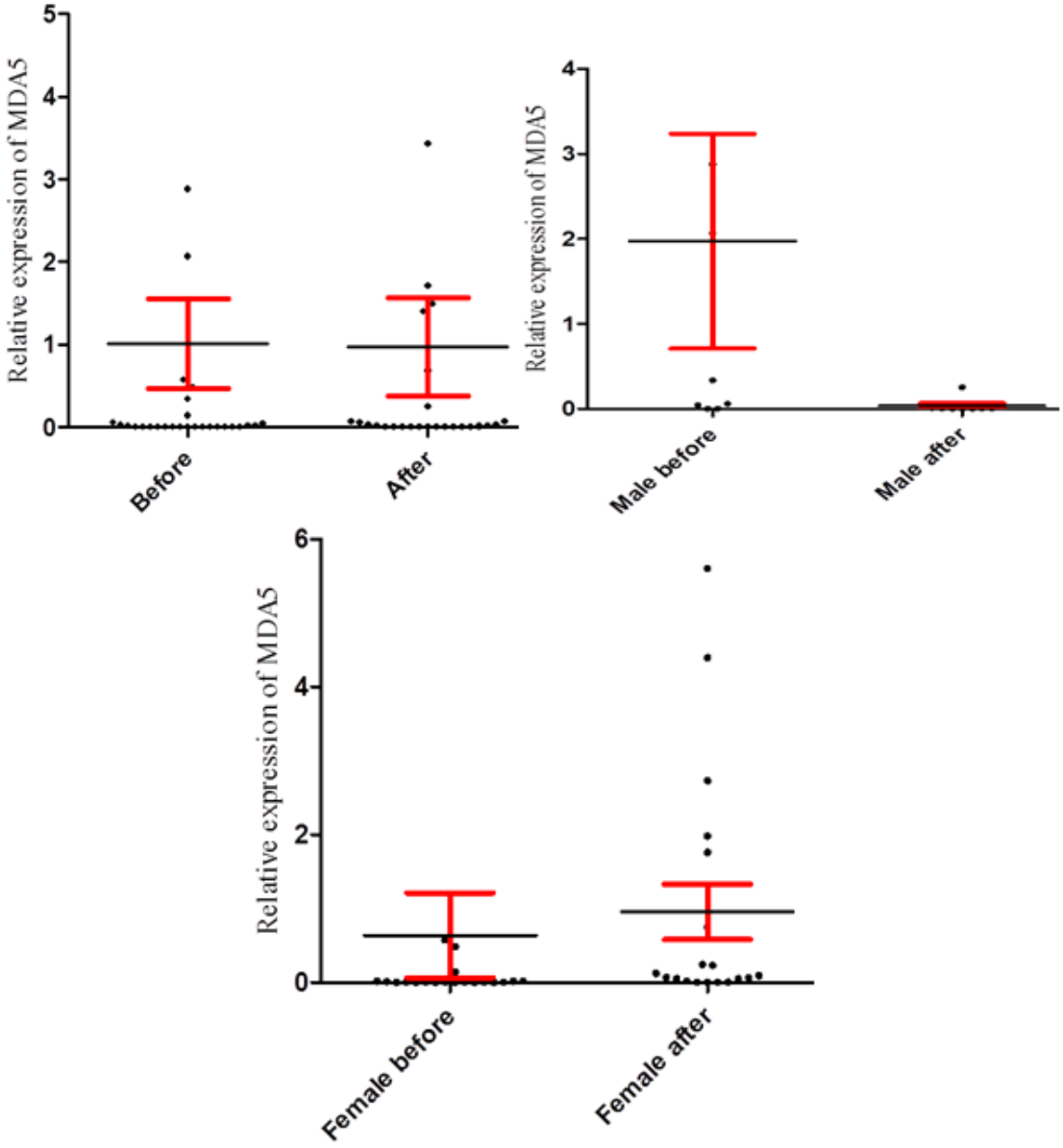

Figure 2: Expression levels of MDA5 before and after treatment with IFN-□ 1a in total, male and female groups. MDA5 mRNA levels did not alter after 6 months therapy with IFN-□ 1a in total, male and female MS groups. The data are reported as Mean ± SEM.

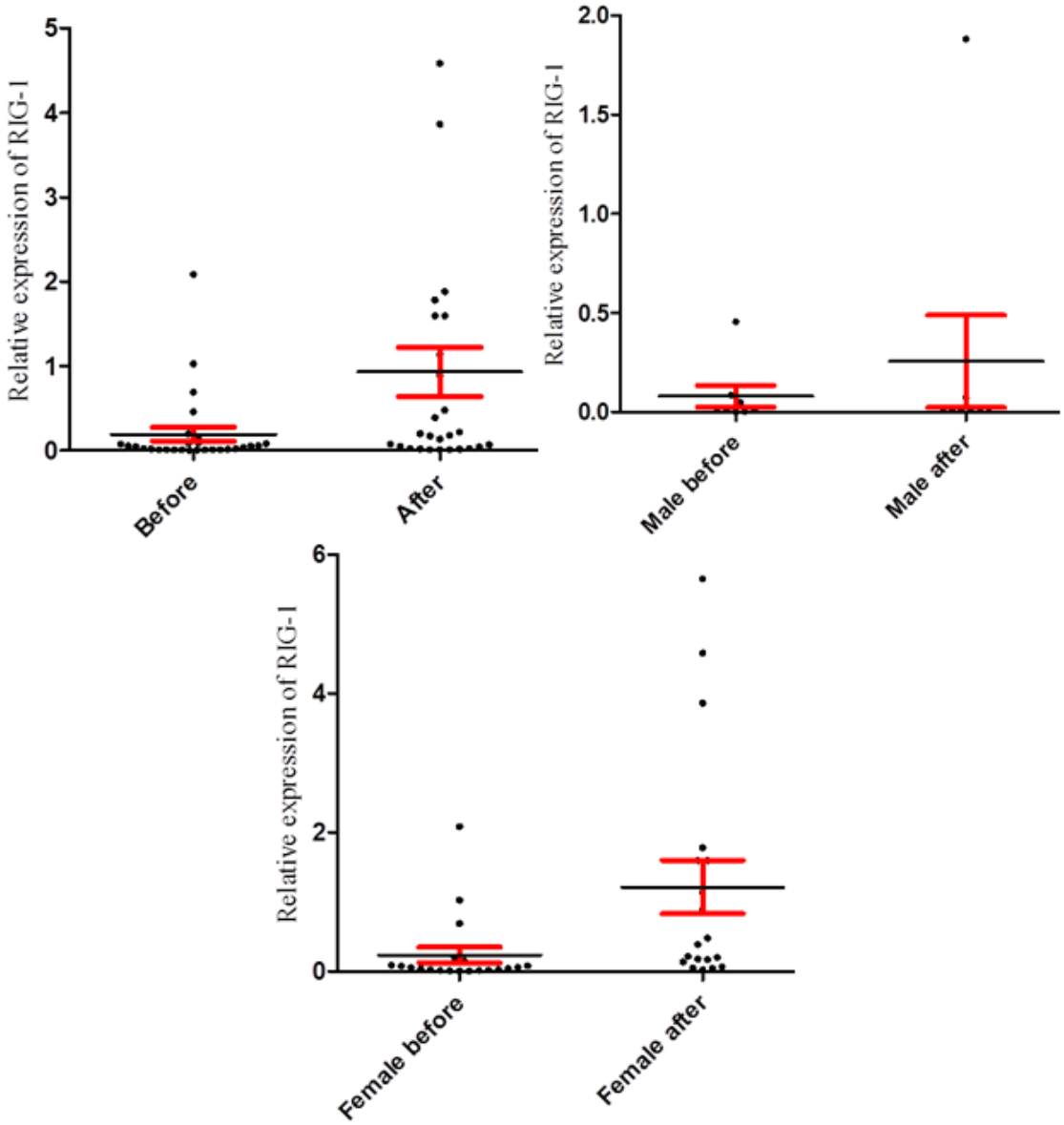

Figure 3: Expression levels of RIG-1 before and after treatment with IFN-□ 1a in total, male and female groups. RIG-1 mRNA levels significantly increased in total and female groups, but not male group, after 6 months therapy with IFN-□ 1a. The data are reported as Mean ± SEM.

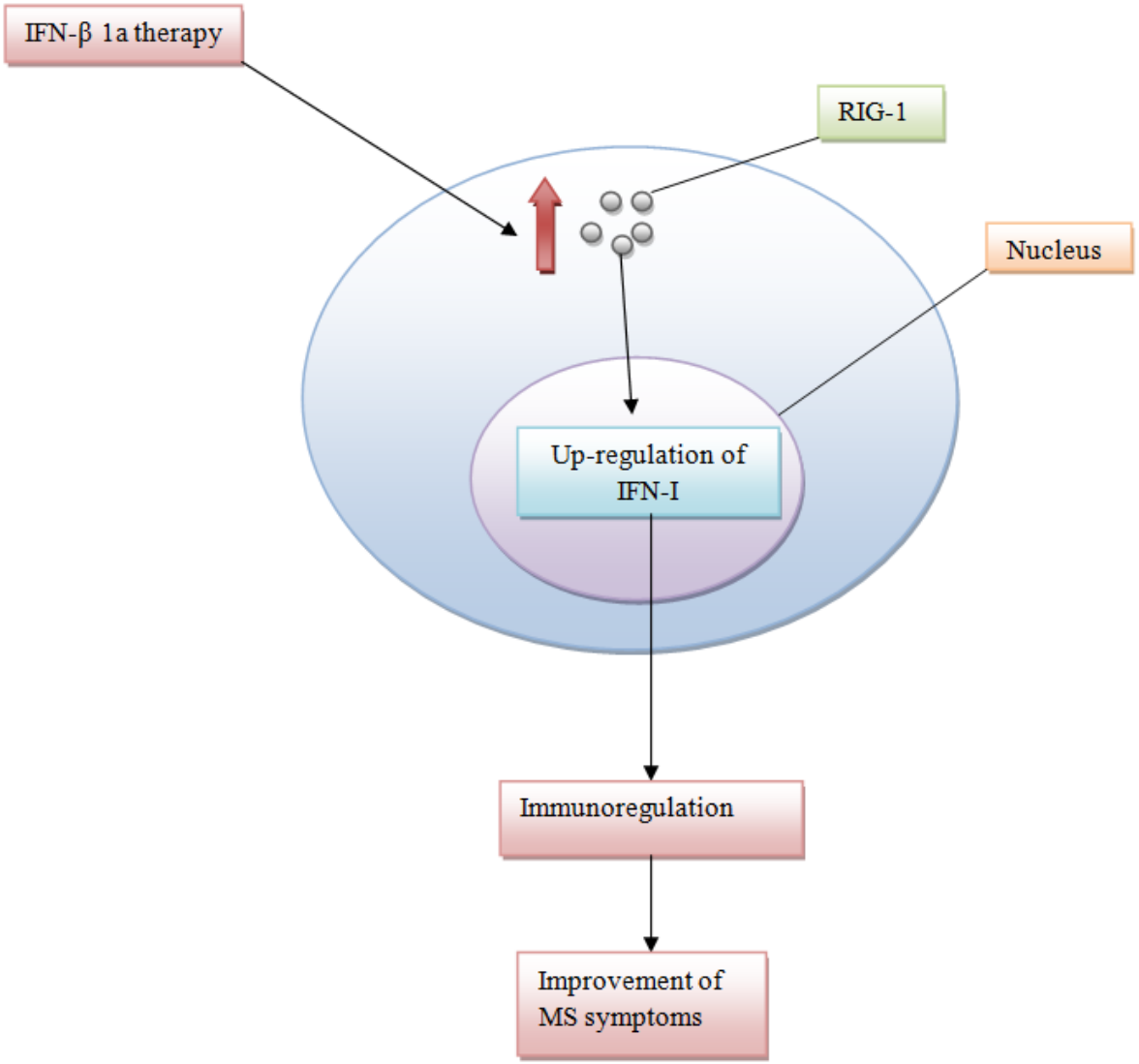

Table 1. Primer sequences of MDA5 and RIG-1.

MDA5	F	GCAGAGGTGAAGGAGCAGA
	R	AAACGATGGAGAGGGCAAG
RIG-1	F	CACACCAAGAGCCCAAAC
	R	TGACCCGATAGCAACAGC
Beta-actin	F	GGCACCCAGCACAATGAAG
	R	CCGATCCACACGGAGTACTTG