HERV-K18 Env Expression and the Potential Association With TGF- β and Epstein–Barr Virus Infection in the Presence and the Absence of Vitamin D in Multiple Sclerosis Patients

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Research Article

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Abstract

Previous studies have reported that Human endogenous retroviruses (HERVs) such as HERV-K18 is associated with an increased risk of multiple sclerosis (MS). The present study aimed to evaluate the association between the expression of HERV-K18 *env*, TGF- β , and in vitro effect of EBV infection on the expression level of HERV-K18 *env* in PBMC of MS patients in both presence and absence of vitamin D (1,25-OHD). The levels of HERV-K18 *env* was measured in peripheral blood mononuclear cells (PBMCs) from 20 MS patients and 20 healthy controls by quantitative real-time PCR. PBMCs were further treated with EBV in the presence or the absence of vitamin D. After 72 hours, cells were collected for measurement of HERV-K18 *env* expression using Real-time PCR. While the expression of HERV-K18 *env* was significantly higher in MS patients than healthy controls, the expression of TGF- β was found to be significantly lower in MS patients compare to healthy controls. Interestingly, an inverse correlation was found between HERV-K18 *env* expression and TGF- β expression in MS patients but not for healthy controls. Although in vitro stimulated PBMCs with EBV showed no significant differences in terms of HERV-K18 expression, EBV infected cells revealed different patterns in the presence and the absence of vitamin D treatment. These findings not only support the important role of HERV-K18 *env* expression but also highlight the plausible interactions with different risk factors in MS.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that occurs mainly in young adults aged 20–40 years [1]. While both genetic and environmental factors are attributed as risk factors for MS development [2], it is largely unclear how different risk factors act in this scenario [3]. The interplay between different environmental risk factors particularly Epstein–Barr virus (EBV) infection, vitamin D deficiency, and human endogenous retroviruses (HERVs) have recently gained much attention in the field of autoimmune diseases [3, 4]. The plausible interaction between EBV infection and vitamin D status has been previously highlighted [5, 6]. Indeed, increased anti-EBNA-1 IgG antibodies and reduced 25(OH)D levels have been described prior to the first clinical manifestation of MS [7]. Furthermore, a seasonal fluctuation has been observed between anti-EBNA-1 IgG levels and 25(OH) D levels in relapsing remitting MS (RRMS) patients [8].

HERVs are a family of ancient viruses, which consist around 8% of our genome [9, 10]. While their existence has been known for years, the biological implications of HERVs in different settings including MS have recently gained more attention [11]. Certain HERV families including MRSV/HERV-W, HERV-H, and HERV-K have been reported to play an important role in MS although it is largely unclear if these risk factors act independently or jointly [12–15]. It has been previously described that EBV infection can transactivate the expression of HERV-K18 *env* gene [16, 17], which has a superantigen activity with strong capability of stimulating T cells [18].

While the plausible interaction of viral infections particularly EBV and HERVs has been well acknowledged in the field of autoimmune diseases including MS [19], less data are available with regards

to HERVs and vitamin D status. In line with our previous findings, the present study aimed to first evaluate of HERV-K18 *env*, TGF- β and then further delineate the in vitro effects of EBV infection on the expression levels of HERV-K18 *env* and TGF- β in PBMCs of MS patients and healthy controls in the presence and the absence of 1,25-OHD.

Methods And Martials:

Study population. Twenty MS patients (15 females and 5 males; mean age 35 ± 5 years) and 20 healthy control subjects (15 females and 5 males; mean age 34.1 ± 5 years) were recruited. All MS patients had been diagnosed based on McDonald's criteria [20] and assessed for clinical picture (Expanded Disability Status Scale: EDSS) by clinicians at the MS Research Centre, Sina Hospital, Tehran, Iran. All study participants gave informed consent to provide 10 ml blood samples for the study, which was approved by the local research ethics committee (approval code: IR.TUMS.SPH.REC.1395.756) of Tehran University of Medical Sciences (TUMS).

Sample Preparation. Peripheral blood mononuclear cells (PBMCs) were separated from fresh whole blood by Ficoll- Paque centrifugation (GE Healthcare Life Sciences, Massachusetts, USA), resuspended in RPMI 1640 (Gibco Life Technologies, USA) supplemented with 10 % FCS (Gibco), and used fresh.

EBV and vitamin D preparation. EBV stock was prepared as described previously [21]. Briefly, B95-8 cells were grown 3–4 weeks at 37°C with 5% CO_2 in RPMI-1640 supplemented with 10 % FCS, penicillin (100 IU) and streptomycin (100 Ig/ml) (Biosera, Ringmer, East Sussex, UK) as completed medium 10% (CM-10). After removal of remained cells and debris by centrifugation at 600g for 6 min at 4°C , the supernatants were passed through a $0.45\ \mu\text{m}$ membrane filter (Millipore, Billerica, USA) and concentrated by Beckman ultracentrifuge (L8-80 M; Beckmann, Palo Alto, CA, USA) at 30,000 g for two hours at 4°C . The pellets containing concentrated virus were re-suspended with CM-10 in 1/100 of original volume, passed through a $0.22\ \mu\text{m}$ filter, aliquoted and stored in cryovials at -80°C .

As described previously [22] the infectious capability and the absolute amount of EBV genome was determined. To optimize the virus concentration, PBMCs (with $1\ \mu\text{g/ml}$ cyclosporine) and B cells were transformed by different titers of virus and 15×10^6 copies included further in all experiments to minimize inter-assay variations. To determine the optimal vitamin D concentration, the expression of VDR and CYP24A1 was checked after PBMCs treatment with different concentration of vitamin D and 100 nM of vitamin D showed the best expression of VDR and CYP24A1.

EBV stimulation in the absence and the presence of vitamin D. PBMCs were infected with EBV and seeded into 24-well plates (1×10^6 cells/well) (SPL Life Science Co, Phcheon-si, Gyeonggi-do, Korea) and incubated at 37°C in a 5% CO_2 atmosphere in the presence and the absence of 100 nM recombinant 1,25-(OH) $_2$ D (Enzo Life Sciences, Inc., AnnArbor, MI.). After 72 hours, supernatants and cells were collected and stored at -80°C until further use. Our previous study [22] showed 72 hours' post-infection was considered as optimal time for cells harvesting for the both EBV activity and vitamin D metabolism.

RNA extraction and cDNA synthesis. RNA from PBMCs (both treated and untreated cells) were manually extracted by Trizol according to the manufacturer's instructions (Roche, Mannheim, Germany). The final RNA pellet were dissolved in 30 µl of RNase and DNase water (Invitrogen). The purity and concentration of total RNA were determined using a Nano Drop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). 1000 ng of total RNA was used for cDNA synthesis according to the manufacturer's instructions (Roche, Mannheim, Germany).

Determination of HERV-K18 env and TGF-β and level in PBMCs. Relative quantitative (RQ) RT-PCR was applied to quantify the expression of HERV-K18 *env* (before and after treated) and TGF-β by using SYBR® GreenER™ qPCR SuperMix Universal (Roche, Mannheim, Germany) on the on the Rotor-Gene® 6000 (Corbett Research, Sydney, Australia). PCR amplification reactions were performed in 25µl reaction mixtures containing cDNA (1:10 fold diluted), 12µl SYBR Green, and 10 pmol of each primer. The reactions were incubated at 95°C for 10 min, followed by of 95°C for 15 s, 57°C for 30 s, and 70°C for 30 s. A melting curve analysis was performed to confirm single gene-specific peaks. The linearity and accuracy of real-time RT-PCR were evaluated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a reference gene, and standard curve derived from amplifying serially diluted pooled cDNA. Biomarker expression patterns were analyzed according to the $2^{-\Delta C_t}$ method [23]. The relative values of each biomarker were expressed as change fold to compare mRNA levels between treatment groups.

Statistical analysis. GraphPad Prism software, version 8 (GraphPad software, Inc, La Jolla, California) was used for plotting graphs and statistical analysis. p values ≤ 0.05 were regarded as significant. Data are presented either with median or mean $\pm$ SEM.

Results:

Demographic characteristics. Twenty MS patients with different forms of disease including RRMS (n = 9), SPMS (n = 5), and PPMS (n = 6) were included for the purpose of this study. Demographic and clinical features of MS patients are summarized in Table 1. As controls, 20 age and sex matched healthy subjects were also included in this study. All patients received treatment except 3, which considered as 'drug naïve'. Among patients 7 have received rituximab and the rest received Tysabri, Dalfira, and Fingolimod either alone or in combination. All patients in this study were non-smokers and, except 3, all received different doses of 25-hydroxy-vitamin D supplements.

Table 1
Demographic and clinical characteristics of MS patients.

ID	Type	EDSS score	Age (years)	Sex	Vitamin D supplement	Treatment
MS1	RRMS	0	23	F	No	Drug naïve
MS2	RRMS	1	38	M	5000U/10day	Drug naïve
MS3	PPMS	6.5	33	F	5000/2week	Rituximab/Dalfira
MS4	RRMS	1.5	28	F	5000/month	Tysabri
MS5	SPMS	6.5	44	F	5000/2week	Rituximab
MS6	PPMS	6.5	41	F	5000/2week	Dalfira/Bioten
MS7	SPMS	5	34	F	5000/2week	Tysabri
MS8	PPMS	6.5	41	M	No	Rituximab
MS9	PPMS	6	34	F	5000/2week	Rituximab
MS10	PPMS	6	34	M	5000/2week	Rituximab
MS11	SPMS	4.5	38	F	5000/2week	Rituximab/Dalfira
MS12	RRMS	1	34	F	No	Recigene
MS13	RRMS	3	31	F	5000/2week	Tysabri
MS14	SPMS	6	33	F	4000/day	Rituximab
MS15	RRMS	1	40	F	5000/2week	Fingolimod
MS16	RRMS	1	40	F	5000/2week	Fingolimod
MS17	RRMS	1	38	M	50000/10Day	Recigene
MS18	SPMS	6	33	F	4000/Day	Rituximab
MS19	RRMS	0	23	F	No	Drug naïve
MS20	RRMS	6	40	M	5000/2week	Fingolimod
RRMS (Relapsing Remitting MS), PPMS (Primary Progressive MS), SPMS (Secondary Progressive MS) F: Female, M: Male EDSS: Expanded Disability Status Scale						

HERV-K18 *env* expression on PBMCs. To ascertain the expression of HERV-K18 *env* in MS, we assessed HERV-K18 *env* expression in PBMCs taken from MS patients and healthy individuals, as control group. The expression level of HERV-K18 *env* was found to be significantly higher in MS patients compared to healthy individuals. Patients who received rituximab, data were further analyzed

based on rituximab treatment. As shown in Fig. 1B, MS patients with no rituximab showed significantly higher levels of HERV-K18 *env* when compared to healthy controls ($p = 0.01$) but not with those who received rituximab ($p > 0.05$).

We further aimed to check whether there is correlation between HERV-K18 *env* and MS disease severity. As indicated in Figs. 1C and 1D, no significant differences were found when data obtained from HERV-K18 *env* analyzed based on either EDSS score or MS forms ($p > 0.05$).

TGF- β expression on PBMCs. Given the importance of TGF- β in CD4 regulatory T cells and MS patients [24], we further analyzed the expression of TGF- β on PBMCs of all study subjects. As shown in Fig. 2A, the expression of TGF- β was significantly lower in MS patients than healthy controls ($p = 0.002$). TGF- β expression was significantly lower in those patients who received rituximab compared to healthy controls ($p = 0.01$) although the difference between 2 groups of patients did not reach statistically significant ($p > 0.05$) (Fig. 2B). TGF- β expression was also significantly lower in patients with high and low EDSS scores compared to healthy controls ($p = 0.001$ and $p = 0.004$ respectively) (Fig. 2C). Analyzing data based on MS forms, no significant difference was found in terms of TGF- β expression ($p > 0.05$) (Fig. 2D).

To determine any plausible correlation between HERV-K18 *env* expression and TGF- β , regression linear analysis was applied (Fig. 3). Interestingly, an inverse correlation was found between HERV-K18 *env* expression and TGF- β expression in MS patients ($p = 0.04$). Such a correlation was not observed for healthy controls (Fig. 3A and 3C respectively).

In vitro stimulation of PBMCs with EBV in the presence and the absence of vitamin D. An inverse correlation has been recently reported between EBV DNA load and serum concentration of vitamin D in MS patients [9, 17]. Given the fact that EBV has the capability to transactivate HERV-K18 *env*, we further aimed to evaluate the in vitro effects of EBV infection on HERV-K18 *env* expression in the presence and the absence of vitamin D on PBMCs. However, we could not find significant differences in treated groups with regards to HERV-K18 *env* expression ($p = 0.05$; Fig. 4) although EBV infected cells were found to exert different transcriptional patterns in the presence and the absence of vitamin D treatment (Table2).

Table 2
The expression of HERV-K18 *env* in the stimulation groups.

Stimulation groups	HERV-K18 <i>env</i> expression in MS (Mean $\pm$ SD)	HERV-K18 <i>env</i> expression in healthy controls (Mean $\pm$ SD)
PBMC	1.131 $\pm$ 0.97	1.118 $\pm$ 0.98
PBMC + vitamin D	1.075 $\pm$ 0.74	0.974 $\pm$ 0.54
PBMC + EBV	1.273 $\pm$ 0.89	0.906 $\pm$ 0.73
PBMC + EBV + vitamin D	0.977 $\pm$ 0.72	1.145 $\pm$ 0.67

Discussion:

Multiple sclerosis is a chronic inflammatory disease of CNS; whose etiology remains poorly defined. In this study, we have shown that 1) the expression of HERV-K18 *env* is significantly higher in MS patients than healthy controls; 2) TGF- β expression was significantly lower in MS patients than healthy controls 3) a negative correlation was found between the expression of HERV-K18 *env* and TGF- β expression in MS patients but not in healthy controls; and 4) in-vitro EBV infected PBMCs were found to exert different transcriptional patterns of HERV-k18 *env* after 72h in the presence and the absence of vitamin D treatment although the difference did not reach statistically different between treated groups.

While differential expression of HERVs have been reported in certain autoimmune diseases such as MS, their clinical significance is poorly defined. Moreover, it is not clear whether they are indicative of disease inheritance or act as markers of environmental/endogenous stress [25]. There are few studies with regards to the HERV-K family including HERV-K18 *env* in MS patients [26–28]. In this study, the expression of HERV-K18 *env* in MS patients was significantly higher than healthy controls as reported previously. Tai *et al*, have reported HERV-K18 *env* as a risk factor in MS [29]. Furthermore, a meta-analysis study evaluating the link between HERV-K18 polymorphisms and autoimmune diseases found a significant association between the haplotype HERV-K18.3 (97Y154W) and the American and Spanish MS population with an OR of 1.22 (95%CI:1.09–1.38) [15]. It has been reported that treatment can also impact the expression of HERVs [30]. Indeed, previous reports indicate that patients with pemphigus vulgaris who received rituximab treatment showed a decreased expression of HERV-K (HML-2) *env* compared to healthy controls [31]. However, in this study we could not find a significant difference when looking at the expression of HERV-K18 *env* based on rituximab treatment. Rituximab may play a role in decreasing the expression of HERVs through B cell depletion that co-express EBV and HERV proteins [32]. Given the fact that HERVs can be expressed by different cells [33]. It has been shown that the increased DNA copy number of MSRV (HERV-W) in patients with MS can also be influenced by gender and disease severity [34] although we could not find such a difference for HERV-K18 *env* in this study.

TGF- β is thought to play a major role in the development and function of CD4 regulatory T cells in MS patients [24]. In line with our previous finding [5], the expression of TGF- β was found to be significantly lower in MS patients compared to healthy controls. It may show the presence of lower frequency of Treg cells in these patients [35] although yet to be documented. Given the association of TGF β 1 SNPs with a good response to rituximab therapy in patients with rheumatoid arthritis [33] and increased level of TGF- β expression following treatment with rituximab in lupus nephritis [36], we further analyzed the expression of TGF- β based on rituximab treatment in our study subjects. However, the difference observed between MS patients who received rituximab treatment and other groups was not statistically significant.

This study further aimed to check if there is any association between HERV-K18 *env* expression with TGF- β . Interestingly, an inverse correlation was observed between HERV-K18 *env* and TGF- β expression. Although our finding supports a potential role of TGF- β in HERV-K18 *env* induction in MS patients, further studies are warranted to delineate the mechanisms behind the involvement of TGF- β and HERVs. While

studies investigating the plausible association of TGF- β and HERVs are scarce particularly in autoimmune diseases, it has been suggested that TGF- β 1 may play an important role in mediating HERV signaling in breast cancer [37]. Different transcriptional patterns of TGF- β with regards to HERV-K18 *env* may also exert complex interactions of different risk factors in MS development. Although yet to be defined, activation of human B cells by EBV [38] and the ability of B cells to negatively regulates TGF- β production [39] are the events that may impact HERV-K18 *env* expression.

It has been previously revealed that some viral agents can activate the expression of HERVs in vitro [17]. Indeed, EBV was shown to induce the super antigenic activity of HERV-K18 *env* in latently infected cells [18]. In vitro stimulation of PBMCs with EBV for 72 hours did not show statistically significant difference in terms of HERV-K18 *env* expression although EBV infected cells of MS patients and healthy controls showed opposite patterns in the absence or the presence of vitamin D treatment. Vitamin D appears to exert an effective role on HERV-K18 *env* expression but apparently difference time points would provide better indication of in-vitro effects of different risk factors in these patients. Further studies with greater number of subjects is necessary to validate the results reported in the current work. Also, additional studies are recommended to investigate the expression level of other HERVs involved MS pathogenesis.

Conclusion

Our results further highlight the mysterious role of HERV-K18 *env* in MS pathogenesis. However, the complex interactions of different risk factors with immune system in patients with MS would be imperative and future studies are warranted to delineate the missing links in this scenario.

Abbreviations

HERVs
Human endogenous retroviruses
MS
multiple sclerosis
PBMCs
peripheral blood mononuclear cells
TGF- β
Transforming Growth Factor Beta
EBV
Epstein–Barr virus

Declarations

Acknowledgments

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Declarations

Not applicable

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Figures

Figure 1

The expression level of HERV-K18 env. A) The expression level of HERV-K18 env in PBMCs of MS patients compared to healthy controls (p= 0.01). Results from MS patients were further subdivided according to treatment with Rituximab, EDSS score, and MS type, respectively (B–D). Data are presented as 2- Δ Ct value. Solid lines indicate mean values and P-value for indicated pairwise comparison was determined using Student's T test analysis. One-way ANOVA comparison test was performed for multiple comparisons. *, **, and ns indicate p values <0.01, p values <0.001, and non-significant, respectively.

Figure 2

The expression level of TGF- β . A) The expression level of TGF- β in PBMCs of MS patients compared to healthy controls ($p = 0.002$). Results from MS patients were further subdivided according to treatment with Rituximab, EDSS score, and MS type, respectively (B–D). Data are presented as $2^{-\Delta\text{Ct}}$ value. Solid lines indicate mean values and P-value for indicated pairwise comparison was determined using Student's T test analysis. One-way ANOVA comparison test was performed for multiple comparisons. *, **, and ns indicate p values < 0.01 , p values < 0.001 , and non-significant, respectively.

Figure 3

Correlation between HERV-K18 env and TGF- β expression. A, B) An inverse correlation between HERV-K18 env and TGF- β expression in MS patients ($p = 0.04$, $r = -0.4$) ($p = 0.8$, $r = -0.05$) but not for healthy controls. Data are presented as ΔCt values.

Figure 4

The HERV-K18 *env* expression represented as log10 of fold changes in different stimulating groups with EBV in the presence and the absence of vitamin D in MS patients and healthy controls. ns indicates non-significant. P values was determined with repeated measure ANOVA.