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Impact of EBV on Multiple in a Sample of Iraqi Females: Immunological and Molecular Study

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Abstract

This study was designed to highlight the role of Epstein Barr viruses (EBV) as a possible causative effect in multiple sclerosis (MS) through testing the viral load along with both biochemical and immunological parameters in female MS patients. We also aimed at finding the effects of different types of treatment line on the various study aspects. The results of the enzyme-linked immunosorbent assay (ELISA) revealed first an increased level of EBV EBNA1IgG IL-17A (96.13±27.60 pg/ml) in sera of female MS patients (0.05±0.01 U/ml, n=50) compared with the control group (n=40). We confirmed this result using real-time polymerase chain reaction (RT-PCR) which also showed a significantly higher EBV load in MS patients (22.61±12.72 copies/ml) as compared to the control. This was associated with an increased level of IL-17A (96.13±27.60 pg/ml) in the patients, while levels of both melatonin hormone (0.63±0.19 pg/ml) and vitamin D3 (29.90±12.41 ng/ml) showed a significant decrease as compared to the normal values of the control group. Analysis of the relation between each of the studied parameters with the level of disability in the patients, as reflected by the expanded disability status score (EDSS), revealed no significant differences between the two categories of EDSS <3 and ≥3 patients in terms of EBV EBNA1IgG levels, whereas they were slightly different in terms of IL-17A and vitamin D3 ($p < 0.001$). With respect to differences between the treatment lines, the results from the showed analysis of variance (ANOVA) test showed no significant differences in all the tested parameters between patients treated with the first line (Rebif, Avonex, Betaferon) and second-line (Gilenya, Tysabri). However, the only significant difference in comparison to the control group was found in levels of IL-17.

It could be concluded that EBV has a crucial role in the initiation and targeting MS disease, leading to increasing levels of IL-17A, and decrease serum levels of melatonin hormone for female patients.

Keywords: Multiple Sclerosis, Epstein Barr virus, Expanded Disability Status Score, Interleukin -17A, Melatonin.

تأثير EBV على التصلب المتعدد في بعض الإناث العراقيات: دراسة مناعية وجزيئية

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الخلاصة

تم تصميم هذه الدراسة لتسليط الضوء على دور فيروسات ابشتاين بار (EBV) كأثر سببي محتمل في التصلب المتعدد (MS) من خلال اختبار الحمل الفيروسي جنبا إلى جنب مع المعلمات البيوكيميائية والمناعية

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في مرضى التصلب المتعدد الإناث. كما استهدفنا العثور على آثار أنواع مختلفة من خطوط العلاج على جوانب الدراسة المختلفة. أظهرت نتائج مقايسة الممتص المناعي المرتبط بالإنزيم (ELISA) أولاً زيادة مستوى EBV EBNA1IgG IL−17A (96.13 ± 27.60 جزء من الغرام / مل) في مصل مرضى التصلب العصبي المتعدد الإناث (0.05 ± 0.01 وحدة / مل ، ن = 50) مقارنة مع المجموعة الضابطة (ن = 40). أكدنا هذه النتيجة باستخدام تفاعل سلسلة البلمرة في الوقت الحقيقي (RT−PCR) والذي أظهر أيضًا حمل EBV أعلى بشكل ملحوظ في مرضى التصلب المتعدد (22.61 ± 12.72 نسخة / مل) مقارنة بالمجموعة الضابطة. ارتبط هذا بزيادة مستوى IL−17A (96.13 ± 27.60 جزء من الغرام / مل) في المرضى، بينما أظهرت مستويات كل من هرمون الميلاتونين (0.63 ± 0.19 جزء / مل) وفيتامين D3 (29.90 ± 12.41 نانوغرام / مل) انخفاض معنوي مقارنة بالقيم الطبيعية للمجموعة المسيطرة. تحليل العلاقة بين كل من المعلمات المدروسة مع مستوى الإعاقة في المرضى، كما يتضح من درجة حالة الإعاقة الموسعة (EDSS)، كشف عن عدم وجود فروق ذات دلالة إحصائية بين فئتان من مرضى EDSS <3 و ≤3 من حيث مستويات EBV EBNA1IgG، في حين كانت مختلفة قليلاً من حيث IL−17A وفيتامين (p <0.001) D3. فيما يتعلق بالاختلافات بين خطوط العلاج، أظهرت نتائج تحليل التباين الموضح (ANOVA) عدم وجود فروق ذات دلالة إحصائية في جميع المعلمات المختبرة بين المرضى الذين عولجوا مع الخط الأول (ريبيف ، أفونيكس ، بيتافيرون) والخط الثاني (جيلينيا ، تيسابري). ومع ذلك، تم العثور على الفرق الوحيد مقارنة بالمجموعة الضابطة في مستويات IL−17.

يمكن استنتاج أن EBV له دور حاسم في بدء واستهداف مرض التصلب المتعدد، مما يؤدي إلى زيادة مستويات IL−17A ، وانخفاض مستويات هرمون الميلاتونين في الدم للمرضى الإناث.

Introduction

In multiple sclerosis, the immune system destroys myelin which protects the nerve fibers and causes communication problems of the human nervous system, resulting from neurological and neurodegeneration disability of the nervous system [1]. Young adults are most commonly affected by MS while women are more susceptible and men have worse progression [2,3]. It is assumed that both genetic and environments factors are effective on this disease. Understand this complex disease took more than 30 years of research. Previous genetic studies contributed in the provision of sufficient information about the disease. MS is characterized by the existence of white and grey matter with focal lesions in the peripheral immune cell's infiltration [4]. In MS the presence of perivascular and parenchymal inflammatory infiltrates, extreme demyelination and neurodegeneration of the nervous system are correlated with the composition of T and B lymphocytes [5]. Kurtzke calculated the Expanded Disability Status Scale (EDSS) [6], for which there is some evidence of relation with MS inflammatory markers [7]. This scale is composed of three classes with a total range of 0 to 10 as a measure of the extent and development of impairment. Higher scores suggest more severe disability, a score of 4 reflects reduced walking capacity, but an ability to walk more than 500 m without help or rest, a score of 6 reflects the ability to walk not more than 100 m with unilateral aid and without rest, and a score of 7 refers to patients who are not able to walk more than 10 m without rest [8]. Among the various environmental factors, EBV infection was widely studied and strongly linked to disease risk [9]. EBV is also referred to as the human gamma herpesvirus, which is one of eight recognized human herpesvirus forms in the herpes family and is one of the most prevalent viruses in humans. It is asymptomatic in childhood, but it becomes symptomatic in adolescents. The frequency of MS is nearly 15 times higher in early childhood with EBV and about 30 times higher among adolescent and later-life patients who have EBV infections [10]. In a previous study, anti-EBV antibodies were correlated with MS patients, but not all patients required to have EBV [11]. However, the variation in MS risk between migrants coming from elevated to low MS prevalence regions indicates that there are infectious or non-infectious factors other than EBV might be involved [9]. Viruses might be a non-specific autoimmunity trigger. It is feasible for EBV to mimic a self-antigen [12]. It is interesting that EBV antigens are similar to myelin antigen and can be targeted for the same antibody response [13]. Additional analytical support for EBV's causative role in MS pathogenesis results from postmortem MS tissue studies. Although this finding was lately questioned, brain-infiltrating B-cells in MS

patients [14] demonstrate arguments of EBV infection. Recently, CD8 + T-cell responses to lytic EBV antigens were also shown to be predominant in patients with severe MS. On the other hand, CD8 + T-cell responses to latent EBV Ag appear to be predominant in patients with inactive MS [15]. It is believed that MS is an autoimmune disease and that it is, like other autoimmune diseases, linked to disease severity [16-19].

The aims of the current study is to investigate the role of EBV in MS patients and estimate serum levels of the pro inflammatory cytokine IL17A, melatonin hormone, and vitamin D3. We also aim at estimating the viral EBNA1 antibody titer as well as the viral load of EBV by using the very sensitive RT-PCR technique to confirm the existence of the virus. Finally, we designed our approach to find the effects of difference treatment lines and different disability status scores on all study aspects.

Materials and Methods

Patients and control

A case-control study was conducted in Iraqi female MS patients and controls in order to identify the role of cytokines, such as IL_17A, and the existence of EBNA1 IgG of EBV virus by enzyme linked immunosorbent assay (ELISA) and RT- PCR technique [20]. We also detected the correlation with serum vitamin D3 ratio and the levels of melatonin hormone. In alignment with the McDonald guidelines [21], fifty females with an age range of 39.21 ± 9.57 years who were clinically defined as MS patients were enrolled in the present study in the period between Nov. 2018 and April 2019. They were admitted to the Multiple Sclerosis Clinic of the Neuroscience hospital in Baghdad. The duration of the disease was between three months to 11 years. Most patients were diagnosed as having the RRMS type. The EDSS score was also calculated according to Kurtzke [6]. Patients were distributed into two groups with relevance to therapy line; the first line group (Rebif, Avonex, Betaferon) included 36 cases and the second (Gilenya, Tysabri) included 14 cases. Female patients were also distributed according to EDSS. Characteristics of MS patients and their distributions are given in Table-1. Forty healthy females were also included as control, with a mean age of 36.65±8.03 years.

Table 1-Details and distributions of multiple sclerosis female patients

MS Female Patients	Females (No. = 50)	
Age Mean ± SD (Years)	39.21 ± 9.57	
Duration of disease (Years)	3.65 ±1.53	
Extended Disability Status Score	≤3 (No. = 26)	>3 (No. = 24)
Characteristics	First line treatment (No. = 36)	Second line treatment (No. = 14)

Specimen Collection

Venous blood was collected with a five ml sterile syringe and was spread over 2 aliquots. A gel tube was filled with 3 ml of blood and, after clot formation; the tubes were centrifuged at 4000 rpm for 15 minutes. The serum was stored in 1.5 ml Eppendorf tube and frozen at -20 ^{0}C until all the serum tests were conducted. The remaining blood (2 ml) was frozen at -20 ^{0}C in ethylene diamine tetra acetic acid (EDTA) tube until DNA isolation and viral load measurement.

Methods

Measurements of parameters in the sera of patients and control subjects were performed using ELISA kits. For IL-17A assessment, Human IL-17A ELIZA; Elabscience, USA kit was used, while Human Melatonin ELIZA; Elabscience, USA kit was used for the competitive-ELISA principle for the assessment of melatonin hormone. The kit icroma vitamin D; Boditech, Korea, which applies a competitive immunodetection approach was used for vitamin D3 assessment, while for the assessment of serum levels of Epstein-Barr EBNA IgG , the kit of EPSTEIN-BARR EBNA ELIZA IgG; Vircell, Spain was used. The isolation of virus DNA was performed using a ready kit (Viral Nucleic Acid Extraction Kit II; Geneaid, Taiwan), which provides a fast and simple technique for the preparation of

purified and intact DNA from mammalian blood. Finally, Bosphore EBV Quantification v1; Anatolia, Turkey kit was used to measure Epstein-Barr Virus (Human Herpes Virus-4/HHV-4) DNA in human blood samples, identifying all EBV subtypes through RT-PCR technique.

Statistical analysis

The Data were analyzed using the SPSS statistical package for the Social Sciences (SPSS, version 20.0 for windows, Chicago, IL, USA). Shapiro–Wilk normality test was used to determine whether the studied parameters followed a Gaussian distribution. Categorical variables were expressed as proportions which were compared using the Chi-square test (χ^2). Data were expressed as mean ± standard deviation (SD) for continuous variables. Differences between groups were analysed by the student's t test. Tukey's and Scheffee Post Hoc tests for multiple comparisons were applied after ANOVA tests. The association degrees between variables were analysed by Pearson correlation analysis. A two-tailed p-value of less than 0.05 ($p<0.05$) was considered as significant [22].

Results and Discussion

To the best of our knowledge, this is the first study in Iraq that detects the correlation of EBV with IL-17A, melatonin, and vitamin D3 levels in sera of MS female patients.

Interleukin-17A

The results demonstrated consistency with previous research showing that the levels of the pro-inflammatory cytokine IL-17A in patients with MS are significantly elevated [23]. In this study, the mean serum level of IL-17A showed a significant increase (p <0.001) in female patients compared to female healthy subjects, Table -2. Along with the alteration in cytokine production in the periphery, these data demonstrate persistent pro-inflammatory activation even during the clinically stable period of the disease, although most patients enrolled in this study are in the RRMS stage [24]. Furthermore, previous research detected lower levels of IL-17A in female MS patients [25], while other reports showed an opposite increase in the levels of this cytokine [26].

No differences were found between IL-17A levels of first and second lines of treatment in female MS patients. However, the mean level of IL-17A was significantly increased in patients from both treatment lines as compared to the control groups (p <0.001), as shown in Table-3. Female MS patients with EDSS < 3 and EDSS ≥ 3 revealed a significant difference in comparison to the control groups (p <0.001) (Table4). In addition, patients with EDSS ≥ 3 were observed to have a significantly increased level of IL-17A compared with the < 3 EDSS patients. The findings of the relationship between the EDSS score and cognitive performance may reflect the progression of pathological immune-mediated changes in the central nervous system. No correlation was observed between EDSS scores and IL-17A expression in female patients, as also observed by other studies [23,24]. In fact, many chronic inflammatory disorders such as rheumatoid arthritis, psoriasis, and MS are associated with high IL-17A levels but, according to the current best knowledge, variations in IL-17 were not previously reported, which could be important for the various phases of the disease and its heterogeneity [26]. Another study that detected the relationship between IL-17 and insulin resistance found a crucial role of IL-17 in autoimmune disease [27].

Serum Level of vitamin D3

Various previous studies reported that vitamin D3 levels in patients with MS were lower than those in the controls [28], which support the current result. Serum mean level of vitamin D3 showed a significant decrease in female MS patients compared to the controls (p <0.001) (Table-2). In addition, vitamin D3 levels in patients of the first and second lines of treatment demonstrated no differences (Table-3). Female MS patients with EDSS < 3 and EDSS ≥ 3 revealed a significant difference in comparison to the control groups (p <0.001) (Table-4). In addition, patients with EDSS ≥ 3 were observed to have a significantly increased level of vitamin D3 compared with the < 3 EDSS patients. The increased disorder is highly associated with lower levels of D3 and lower levels of sun exposure. Almost half of the excess risk of vitamin D3 is statistically based on low exposure to sun for those with greater disabilities compared to healthy subjects [29].

Serum Level of melatonin hormone

In agreement with a previously published study [30], the serum mean level of melatonin show a significant decrease in female MS patients in comparison with the control group (p <0.001), as shown in Table-2. In other studies, no significant difference existed among the two groups of patients and healthy subjects in terms of levels of saliva melatonin (p=0.417), but when age was considered, a

significant difference was observed (p=0.022). Patients with various clinical features such as disease duration, first attack, types of treatment and EDSS demonstrated no differences in melatonin levels.

Female patients of the first-line treatment showed no significant differences compared with those of the second-line treatment, as demonstrated in Table-3. In addition, as found in another study, there was no significant correlation between melatonin levels and EDSS [31]. Also, with respect to EDSS. There was no significant difference between the mean level of melatonin between female patients with EDSS < 3 and those with EDSS ≥ 3 (Table-4).

Serum Level of Epstein-Barr EBNA IgG

In Table-2, we show that serum mean level of EBV in the present study had a significant increase in female patients as compared to the healthy control (p <0.001), which agrees with the findings of previous studies [32]. However, some previous studies confirmed that anti EBNA 1 IgG does not constitute an accurate indicator for the development of MS clinic disease, where there was no variation in serum anti-EBNA-1 IgG statistical analysis data among patient categories and no correlation with phenotypic characteristics, including onset duration (r = 0.17, p = 0.16), disease span (r = 0.03, p = 0.78), and EDSS (r = 0.03, p = 0.78) that indicated the IgG titer for the anti-EBNA-1 is probably not a good marker of disease activity in patients using medications for disease modification [33].

Female patients with the first-line and second-line treatment showed no significant differences in EBNA 1 IgG levels between each other, while the levels in female healthy subjects were low (Table-3). Moreover, with respect to EDSS, there was no significant differences between mean EBV levels in female patients with EDSS < 3 and ≥ 3, in contrast to highly significant higher values in these two groups as compared to the control (p <0.001), as shown in Table-4. This result agrees with that reported by a previous study [34] and implies that EBV reactivation in patients with MS is higher, since late EBV-infected B cells are present. This rise in EBV reactivation was found to be significantly evident in MS patients. Furthermore.

Table 2-The clinical parameters of female patients and control groups.

Characteristics	Female (n=90)		*P* value
	patients' group(n=50)	Control group(n=40)	
IL17A (pg/ml)	96.13±27.60	11.00±4.10	<0.001
Vitamin D3 (ng/ml)	29.90±12.41	45.58±11.65	<0.001
EBV (U/ml)	0.05±0.01	0.03±0.01	<0.001
Melatonin (pg/ml)	0.63±0.19	1.17±0.17	<0.001

Table 3-The studied parameters of female control and female patients according to treatments

Characteristics	Female control (n=40)	Female patients with first-line treatment (n= 36)	Female patients with second-line treatment (n=14)	*P* value
IL17A (pg/ml)	11.00±4.10[a]	94.01±29.22[b]	101.64±15.52[b]	<0.001
Vitamin D3 (ng/ml)	45.58±11.65[a]	29.95±7.75[b]	29.84±6.64[b]	<0.001
EBV (U/ml)	0.03±0.01[a]	0.053±0.006[b]	0.053±0.007[b]	<0.001
Melatonin (pg/ml)	1.17±0.17[a]	0.64±0.20[b]	0.62±0.13[b]	<0.001

Different small letter(s) denote significant differences

Table 4-The studied parameters of female control and female patients' sub-groups depending on Expanded Disability Status Score

Characteristics	Female control (n=40)	Female patients EDSS < 3 (n= 24)	Female patients EDSS ≥ 3 (n=26)	*P* value
IL17A (pg/ml)	11.00±4.10[a]	83.86±27.02[b]	107.50±33.26[c]	<0.001
Vitamin D3(ng/ml)	45.58±11.65[a]	34.76±16.41[b]	25.45±7.30[c]	<0.001
EBV (U/ml)	0.03±0.01[a]	0.052±0.005[b]	0.054±0.007[b]	<0.001
Melatonin (pg/ml)	1.17±0.17[a]	0.57±0.10[b]	0.69±0.23[b]	<0.001

Different small letter(s) denote significant differences

PCR results of EBV load

The viral load of EBV in PBMC was also identified in this study as an estimate of viral lytic infection and to assess whether the peripheral viral activity is consistent with disease activity. RT-PCR results demonstrated that the mean values of EBV load ere significantly increased in MS female patients in comparison to the control subjects who had no viral load. Our results are similar to those reported by previous studies [20, 34] in terms of positive EBNA-1 antibody in the peripheral blood of MS patients. Female MS patients showed no significant differences according to both first and second line treatment (p = 0.86, neither between the two groups of EDSS, as shown in Table-5. In summary, an easy-to-use and highly reproducible technique for assessing the significance of EBV DNA in plasma samples in immunosuppressed patients is available using RT-PCR. Inhibition of the amplification reaction by heparin may be removed, based on the isolation process used. The analysis also confirms the data provided by others that there is a link between plasma EBV DNA levels and diseases associated with EBV [35]. For patients with no EBV-related clinical disease, however, active replication could also be observed. It can then be used to control antiviral therapies on EBV by infusing the donor T cells, modifying immunosuppression, or supplying nucleoside analogs to prevent EBV replication [36, 37].

Table 5-Data of Real time PCR for Epstein Barr viruses

EBV Real time PCR (CT)		*P* value
Female patients	Female control	<0.001
22.61±12.72	0.00	
Female patients with first-line treatment	Female patients with second-line treatment	0.863
19.65±14.17	22.00±15.28	
Female patients with EDSS<3	Female patients with EDSS≥3	0.251
20.78±13.68	19.88±15.24	

Conclusion

This study concludes that Iraqi female MS patients have risk factors identical to those found in patients in the western countries, as with the older age which is associated with increased serum levels of anti-EBNA-1. The pathogenesis of EBV has a crucial role for the initiation of MS disease in female patients. Real time PCR procedure is effective in the detection and management of EBV symptomatic infections. This data, taken together, indicates that hormones possibly play a major role in the secretion of the pro inflammatory cytokine IL-17A, which showed an increased level. Also, melatonin hormone seems to have a low level in female MS patients, which indicates that hormones play a major role in this disease.

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