ISSN: 0067-2904

Human β-Defensin 2 as a Link between EBV Infection and Multiple Sclerosis

Aya Refaat*, Hula Y. Fadhil
Department of biology, College of science, University of Baghdad, Baghdad, Iraq

Received: 9/4/2023 Accepted: 2/9/2023 Published: 30/10/2024

Abstract

Multiple sclerosis (MS) is the result of a complex interaction between environmental factors and genetic predisposition.

Epstein-Barr virus (EBV) is a significant environmental risk factor associated with MS. Human- beta defensin2 (HβD-2) is a crucial innate immune response proteint that as an inflammatory marker protects humans from pathogens invading the body like viruses. Therefore, a case-control study was conducted on 90 subjected (48 patients with MS and 42 healthy controls) to examine the role of HβD-2 in EBV viral load infection and MS patients. An enzyme-linked immunosorbentassay kit was used to measure HβD-2 levels in serum. The viral load of EBV was determined using a real-time PCR. The findings revealed that median HβD-2 levels in patients were significantly lower than in controls. (96.62 *vs.*124.8 ng/mL; $p < 0.01$). According to the receiver operating characteristic curve analysis, HβD-2 is good predictor for MS disease (area under the curve=0.739; $p < 0.001$). EBV frequency was lower in MS patients compared to the controls, although difference was not statistically significant (25 vs. 38.1%; $p = 0.181$).On other hand, the median (EBV) load was substantially greater in patients than in healthy controls (8.55 vs. 1.4 DNA copy/100 cells). This study concluded that HβD-2 levels showed no significant differences in each age group, sex, Expanded Disability Status Scale (EDSS) of multiple sclerosis patients, rather showed an inverse significant correlation (correlation coefficient = - 0.432) with EBV load, which protect against human viral infections.

Keywords: Autoimmune disease; Human beta defensin-2; Viral infection; Viral quantity.

علاقة β–Defensin 2 البشري بأصابة فيروس ابشتاين –بار والتصلب المتعدد

اية رفعت* ، حلا يونس
قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

يحدث التصلب المتعدد (MS) بسبب التفاعل المعقد بين العوامل البيئية والاستعداد الوراثي . فيروس ابشتاين بار هو عامل بيئي خطر مرتبط بشدة بمرض التصلب العصبي المتعدد . Human– beta defensing (HβD–2) هو بروتين مهم للاستجابة المناعية الفطرية كعلامة للالتهاب، الذي يوفر الحماية ضد مسببات الامراض مثل الفايروسات. لذلك، اجريت الدراسة على90 عينه (48 مريضا بالتصلب المتعدد و 42 من الاصحاء كمجموعة سيطرة) لتحديد دور HβD–2 في عدوى الحمل الفيروسي EBV ومرضى ا

*Email: ayarufat88@gmail.com

لتصلب المتعدد. تم تقييم مستويات مصل HβD−2 باستخدام مجموعة المقايسة المناعية المرتبطة با لأنزيم (ELISA) ، بينما يتم تحديد الحمل الفيروسي ل EBV بأستخدام تفاعل البلمرة المتسلسل اللحظي. بينت النتائج ان وسيط مستويات HβD−2 كان اقل بشكل ملحوظ في المرضى عنها في مجموعة السيطرة (96.62 مقابل 124.8 نانو غرام/ مل ؛ الاحتمالية< 0.01).اشار تحليل المنحنى المميز لأداء المستقبل الى ان HβD−2 هو مؤشر جيد للتصلب المتعدد (المنطقة الواقعة تحت المنحنى =0.739؛ الاحتمالية <0.001). سجل وجود EBV بدرجة اقل في المرضى منه في السيطرة لكن هذا الانخفاض لم يبلغ مستوى المعنوية (25 مقابل38.1% ;الاحتمالية =0.181)، بينما كان وسيط حمل EBV أعلى معنوياً في المرضى مقارنة بالسيطرة (8.55مقابل 1.4 نسخة من الحمض النووي /100 الخلايا).ايضا" تم الاستنتاج من هذه الدراسة ان ،أظهرت مستويات HβD−2عدم وجود فروق ذات دلالة أحصائية في كل من الفئة عمرية ، والجنس ومقياس حالة الاعاقة الموسع (EDSS) لمرضى التصلب العصبي المتعدد ،لكن هناك علاقة عكسية معنوية (معامل الارتباط =−0.432) مع حمولة EBV، التي تحمي ضد الفيروسات البشرية.

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disorder of the central nervous system in which cell-mediated immunity damages the myelin sheath surrounding neurons [1]. The blood-brain barrier (BBB) is penetrated via autoreactive lymphocytes, that enter the central nervous system and generate a localized inflammatory response leading to demyelination, gliotic scarring, and axonal loss [2]. Among neurological disabilities, MS is the most prevalent as around 2.5 million people throughout the world are impacted, with most affected being between ages of 20 and 40 years [3]. Women are more likely to develop MS than men [4].

Two main subtypes of multiple sclerosis are clinically recognized, namely relapsing-remitting MS (RRMS) and progressive MS [1]. The present analysis demonstrated that the cause of MS is multifactorial which includes genetic predisposition with environmental variables, even though the pathophysiology and etiology are still unknown and mentioned that MS is related to various geographical, physiological factors, and infectious agents such as high latitudes, vitamin D deficiency, low sunlight exposure, obesity in adolescence, tobacco smoking, and EBV infection [5]. Epstein Barr virus(EBV) is a double-stranded DNA virus, which infects B lymphocytes. It is a herpesvirus that was identified in 1964 [6] and is the most prevalent virus linked to MS. Those who test negative for EBV have a lower risk of developing MS, whereas individuals with a history of infectious mononucleosis face an highly elevated risk [7].

Defensins can prevent viral infection by directly affecting the virion or through the target cell and indirectly preventing viral infection [8].

Human-defensin 2 (HβD-2) is one of the defensin family members. Its expression can be stimulated by bacteria, viruses, and pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1β) [9]. HIV-1 stimulates mRNA expression of HβD-2 with a manner analogous to the cytokine induction, which happens like an early innate immune response for viral infection except HβD 1, in healthy human oral epithelial cells, even in the absence of HIV-1 duplication [10]. In addition, HβD-2, and HβD-3 expression, excluding HβD-1, when exposed to the human rhinovirus, is activated in bronchial epithelial cells, is independent of IL-1, and is mediated via nuclear factor-κB (NF-κB) activation [11]. In addition to its activity in diverse epithelial tissues, HβD-2 can also play a role in the chemotaxis of immune cells and the stimulation of Toll-like receptors (TLRs) that are found on their surface [12]. Pathogen-associated molecular patterns (PAMP) activation Toll-like receptors (TLRs), particularly TLR2 and TLR4, on certain cells is also important for the

activation of HβD-2expression during infection. [13].

Astrocytes among various kinds of nerve cells in the brain are sensitive to the production of HβD-2mRNA and HβD-2 protein liberation in vitro in response to lipopolysaccharide and cytokines (TNF- and IL-1). Chlamydophila pneumoniae infection boosts HβD-2 secretion in brain capillary endothelial cells (both mRNA expression and peptide synthesis increase), thus indicating that defensins could be crucial for immune protection in cerebral blood vessels [14]. Development of pathological diseases in the central nervous system (CNS), such as changes in cell maturation (like dendritic cells), and overexpression of proinflammatory cytokines , has been hypothesized to be influenced by alterations in antimicrobial peptide levels, particularly HβD-2[15].

The current information available might not give a complete picture of the association between HβD-2 and MS, as this current study may have been the first of its kind. Assessing serum HβD-2 levels in both MS patients and HC was the first step in the initial investigation to help in this area (HC). Viral load and EBV DNA were also assessed. The examination of the association between HβD-2 and sex, age, EBV load, Expanded Disability Status Scale (EDSS), and therapy were added to the understanding.

2. Materials and Methods

2.1. Populations Studied

A total of 90 people (48 patients and 42 HC) took part in this study. MS patients were diagnosed between November 2022 and February 2023 at the MS clinic at Baghdad General Hospital for Neurological Sciences. Patients with MS were split into two groups; <3.0 and ≥3.0, based on the results of the EDSS which was used to measure physical disability. This scale ranges from 0 indicating a typical ambulatory condition, to 10 for patients with total disability then death [16,17]. First_line (IFN beta 1-alpha) or second-line (fingolimod or natalizumab) treatment was administered to all patients. The healthy control sample consisted of age- and sex-matched blood donors and health-care workers. They did not have any neurological or immunological diseases. All participants were divided into two groups based on age (40 and > 40 years) and gender: male and female. Multiple sclerosis patients were divided into two groups based on their (EBV) status if it was positive or negative, the EDSS group (<3.0 and ≥3.0), and treatment kind (first-line and second-line). The protocol of the study was approved by the ethics committee of the Iraqi Ministry of Health and Environment and the Department of Biology/ College of Science/University of Baghdad. All participants provided their written consent.

2.2 Laboratory Tests

Five milliliters of blood was drawn from each participant and distributed into ethylene diamine-tetraacetic acid (EDTA) tubes. Following the manufacturer's instructions, genomic DNA was extracted from EDTA blood using the gSYNC DNA extraction kit (Geneaid Biotech Ltd, Taiwan).

Isolated DNA was subjected to RT-PCR analysis for detecting EBV qualitatively (positive or negative)and quantitatively (viral load). This analysis was carried out by using the Real-TM Quant kit in accordance with manufacturer’s instructions (Sacace Biotechnologies Srl, Italy). EBV DNA copy/100 cells served as a measure of viral load using an enzyme linked immunosorbent assay (ELISA) kit from SunLong Biotech, China (Catalogue Number: SL3278Hu). Serum levels serum were measured in accordance with the manufacturer's directive. The assay has sensitivity of 1 ng/mL and a detection range of 5-150 ng/mL.

2.3. *Statistical Analysis*

A Welch corrected t-test was used to assess if there were any significant differences between the means and standard deviations for a continuous variable. For categorical variables that were given as numbers and percentages, significant differences were found using the Fisher exact test or Pearson Chi-square test. The area under the curve (AUC), 95% confidence interval (CI), cut-off value, sensitivity, and specificity for assessing the validity of significant parameter as HβD-2 for the healthy control in prediction of MS disease progression were estimated using ROC curve analysis. The association between HβD-2 and EBV viral load in MS and healthy controls was investigated using Spearman's rank-order correlation. Statistical significance was defined as a probability (*P*) value of ≤ 0.05. These analysis were carried out using the statistical packages IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.) and GraphPad Prism version 8.0.0 (San Diego, California, USA).

3. Results and Discussion

3.1. Main Characteristics

The results revealed that the distribution of age (34 vs.36 years; p = 0.565), age groups (≤40 years 70.8 vs. 59.5 %;> 40 years 29.2 vs. 40.5%; p = 0.260) did not have any significant differences between MS patients and HC, although significant differences were observed in sex (males 33.3 vs. 54.8%; females 66.7 vs. 45.2%; p = 0.041). EBV prevalence in MS patients was lower than in healthy controls, although the difference was not statistically significant (25 vs.38.1%; p = 0.181). Contrary to these results, EBV load revealed that patients with EBV-positive cases had considerably greater viral loads than those with HC (8.55 vs. 1.4) DNA copy/100 cells; p = 0.033). In terms of EDSS groups, 45.8% of MS patients were assigned to group < 3.0, whereas 54.2% were assigned to group ≥ 3.0. Most patients undergoing therapy were getting first-line treatment. (79.2%) p < 0.001 (Table 1).

Table 1: Main characteristics of (MS) patients versus healthy controls.

Characteristic		MS Patient (n =48)	Healthy Control (n=42)	*p*-value
Age; Year		34 (28 – 42)	36 (27 – 45)	**0.565**
The Age Group	≤ 40	34 (70.8)	25 (59.5)	**0.260**
	> 40	14 (29.2)	17 (40.5)	
	p-value	**0.004**	0.217	
Sex	Male	16 (33.3)	23 (54.8)	**0.041**
	Female	32 (66.7)	19 (45.2)	
	p-value	**0.021**	0.537	
EBV	Positive	12 (25)	16 (38.1)	**0.181**
	Negative	36 (75)	26 (61.9)	
EBV Load (Positive Subject); DNA Copy/100Cell		8.55 (2.09 – 15.51)	1.4 (6.09 – 2.94)	**0.033**
EDSS Group	< 3.0	22 (45.8)	**NA**	**0.564**
	≥ 3.0	26 (54.2)		
Therapy	First-line	38 (79.2)	**NA**	**< 0.001**
	Second-line	**10 (20.8)**		

Values of age are given as median with interquartile range (continues variables) or number and percentage (categorical variables); NA: Not applicable; *p*: probability of Mann-Whitney U test(to compare continues variables), two-tailed Fisher exact test or Pearson Chi-square test

(to compare categorical variables).

3.2. Human Beta Defensin-2 Level

The HβD-2 median levels in MS patients were significantly lower compared to healthy controls. [96.62 (IQR: 81.7 − 115.1) vs. 124.8 (IQR: 91.9 − 145.3) ng/mL; $p < 0.01$], (Figure 1). Higher levels of HβD-2 in HC compared with MS patients indicated that it was a protective factor against diseases.

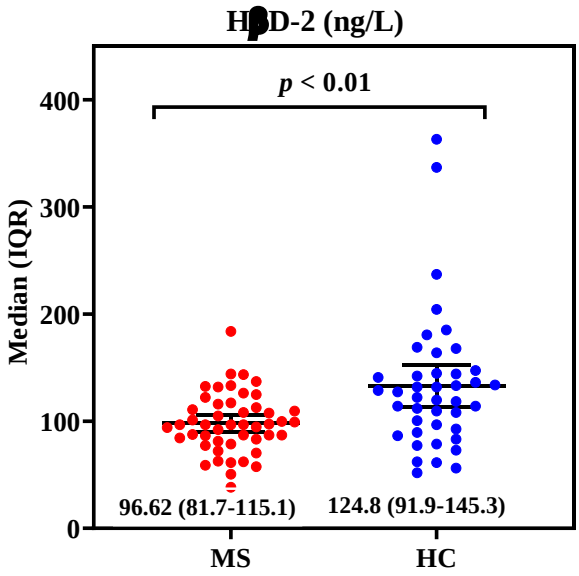

Figure 1: HβD-2 levels in multiple sclerosis patients and healthy controls are shown as a scatter dot plot. Horizontal and vertical lines represent the median and interquartile range (IQR) respectively.

Moreover, the human β defensin-2 level in MS patients and HC associated with EBV infection still higher in HC compared to MS [(95.66 (IQR: 84.12 − 100.8) vs. 111.7 (IQR: 82.84 − 141.8) ng/mL); $p > 0.05$] (Figure 2). The median difference of the HβD-2 in EBV among patient and the control group was non-statistically significant due to differences in sample size between the patients and the healthy control.

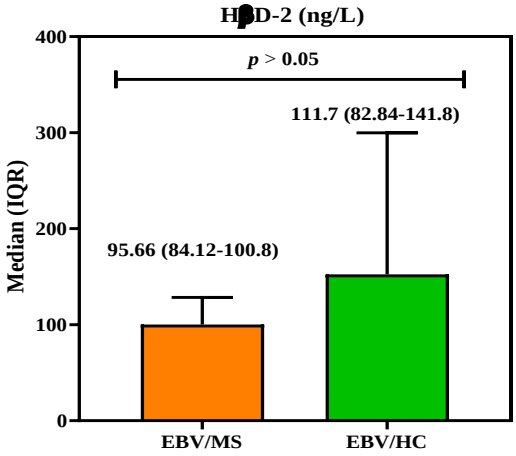

Figure 2: Human β defensin-2 levels in multiple sclerosis patients and healthy controls. Associated with EBV infection.

Low levels of HBD-2 were good indicator of MS, according to ROC analysis, which used 96.9 ng/L as the ideal cut-off value for distinguishing between MS patients and healthy controls. Area Under the Curve (AUC) was = 0.739; 95% CI = 0.632-0.846, $p < 0.001$, sensitivity = 70.7%, specificity = 71.4%) in patients(Figure 3).

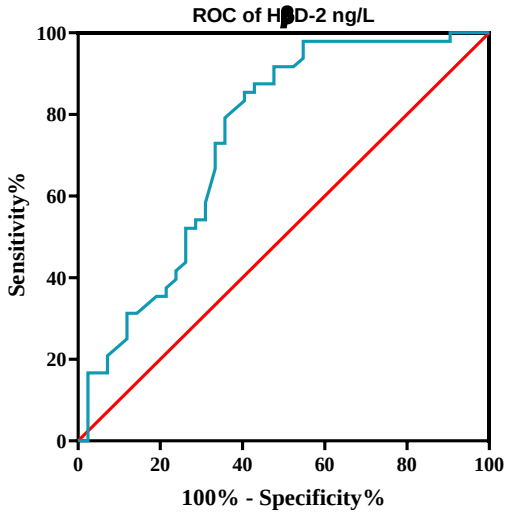

Figure 3: Receiver operating characteristic curve analysis of HβD-2 in multiple sclerosis patients *versus* healthy controls. A HβD-2 level of less than 96.9 ng/L can distinguish MS patients from HC (AUC = 0.739; 95% confidence interval = 0.632 − 0.846; $p < 0.001$; sensitivity = 70.7%; specificity = 71.4%).

Age group, sex, EBV positive, EDSS, and therapy were used to stratify HβD-2 median levels in MS patients and the healthy controls. No significant differences were found in any of the strata (Table 2).

Table 2: Median levels of HβD-2 stratified according to characteristics of multiple sclerosis patients and healthy controls.

Characters		Human β defensin-2 median (IQR); ng/L	
		Patient (No. 48)	**Control (No.42)**
Age Group	**≤ 40** **> 40**	96.94 (82.68 – 122.9) 93 (78.36 – 110.2)	119.7 (87.97 – 142.5) 118.4 (76.76 – 144.7)
	***p*-value**	$p = 0.483$	$p = 0.815$
Sex	**Male**	108.8 (68.1 – 115.1) 95.66 (81.72 – 115.5)	118.4 (78.68 – 136.4) 128.7 (96.6 – 144.1)
	Female		
	***p*-value**	**$p = 0.045$**	$p = 0.313$
EBV	**Positive**	95.66 (84.12 – 100.8) 96.94 (77.7 – 121)	111.7 (82.84 – 141.8) 128 (85.57 – 144.2)
	Negative		
	***p*-value**	$p = 0.837$	$p = 0.586$
EDSS Group	**< 3.0**	98.23 (80.4 – 109.8) 94.38 (80.6 – 122.9)	NA
	≥ 3.0		
	***p*-value**	$p = 0.98$	
Therapy	**First-line**	96.62 (80.6 – 111.2) 100.8 (83.3 – 124.8)	NA
	Second-line		
	***p*-value**	$p = 0.487$	

IQR: Interquartile range; NA: Not applicable; EBV: Epstein-Barr virus; EDSS: Expanded Disability Status Scale; IQR: Interquartile range; MS: Multiple sclerosis; HC; Healthy

controls;*p*: probability.; *p*: Mann-Whitney *U* test probability.

HβD-2 and Epstein-Barr virus correlation was investigated in individuals with multiple sclerosis using Spearman's rank correlation coefficient (rs). The findings showed that there was an inverse significant correlation between them (r_s = -0.432) (Figure 4), which provides protection for the human against viral.

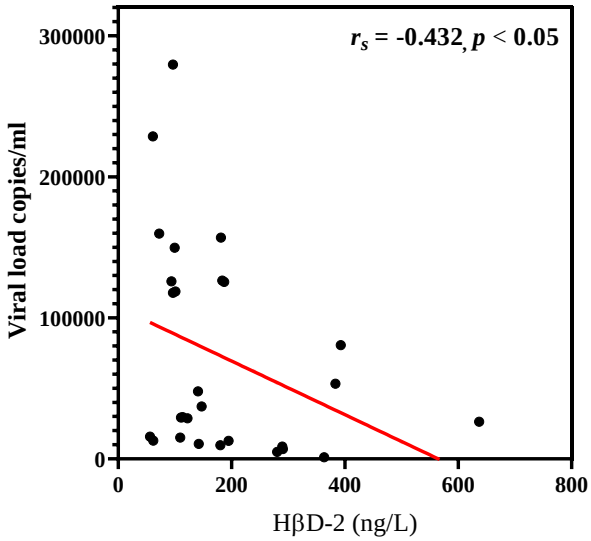

Figure 4: Spearman rank correlation coefficient (rs) between HβD-2 along with Expanded Disability Status Scale (EDSS) and Epstein-Barr virus (EBV) load in MS patients.

Discussion

Observational studies showed that (MS) is a central nervous system illness that causes demyelination and chronic inflammation. The cause is unclear, and the symptoms and course vary. Infectious agents were first suspected in the etiology of MS directly after its classification as a discrete clinical entity in the late 1800s . Although the adaptive immune system plays a role in MS by stimulating myelin-specific auto-reactive lymphocytes, mainly IFN- γ secreting T helper 1 cells or interleukin 17(IL-17) producing cells [19] in the periphery. They then migrate to the CNS causing demyelination, axonal loss, and subsequently neurological disability [20]. Recent research suggests that the innate immune system plays a critical role in both the onset and development of MS. HβD-2 is a crucial innate immune response protein that protects the human body from invading bacterial, viral, and fungal infections [21]. Since HβD-2 is a protective factor, its levels decreased in serum of MS patients compared with healthy controls, The current study, however revealed an increase in HβD-2 level when EBV viral load was high.The AUC value of 0.739 indicated that lower levels of HβD-2 were a good predictor for distinguishing MS patients from HC individuals. The ages of research participants, both patients and healthy controls, had a significant impact on the disease and disease progression where younger participants (< 40 years) were more affected than older participants. MS is mostly diagnosed in ages between 20 and 49 years. However, in scarce situations, MS is detected in infancy and adolescence prior to the age of 18 years, or between the ages of 50 and beyond [22].

With the start of MS at the age of 50 or older, it is referred to as late onset MS (LOMS). As compared to traditional MS, LOMS is distinguished by a progressive course, a longer time in the diagnosis and a greater rate of motor disability.

Increased age hassignificant impact on the main aspects of the MS: Clinical course, the therapeutic choices, the immunological and pathogenic mechanisms, all of which lead to a significant difference in HβD-2 level between two age groups [23]. MS is widely observed to be more frequent in women than in men. Extensive research has uncovered the fact that this cause is due to immune system differences between men and women which may be related to the influence of genetic variants or gonadal hormones, as well as differences in modern lifestyle and environmental exposures in men and women [24]. The month of birth has also been observed to be linked with MS susceptibility, suggesting that seasonal environmental factors such maternal exposure to UV or viral infection during the fetal period may influence on the chances of developing MS later in life [25]. In healthy controls, the findings showed an increase in HβD-2 level in females compared than males confirming females have a better immune system than males [26]. The small sample size in MS patients, could reduce the significance of this variation in HβD-2 levels difference between male and female patients. Also, the results showed no significant differences according to EDSS and lines of therapy groups among MS patients.

In several tissues that are affected by autoinflammatory or autoimmune diseases, including those with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), type 1 diabetes (T1D), psoriasis, and (MS), several studies have revealed that the expression of antimicrobial peptides is dysregulated. It is believed that AMPs in their course of influence on these illnesses. The most commonly reported is that inflammation is promoted and the autoimmune response is favored by abnormal synthesis of AMPs derived from epithelial and neutrophil cells [27]. The neutrophil extracellular traps (NETs) which are formed of self-nucleic acids from the nucleus connected to granular cytoplasmic proteins full in AMPs, are synthesized when neutrophils are activated in the tissue [28].

NETs are often created in an infectious environment to first immobilize and then kill microorganisms [29]. Type I interferons (IFNs) play an essential role in autoimmune disorder by activating antigen presentation via DCs and creation of autoantibodies via B lymphocyte, and are produced by pDCs by TLR7 and TLR9 in response to the formation of aberrant NETs in sterile conditions and the damage clearance of these NETs [30]. Conversely, new research has demonstrated that AMPs generated via particular nonimmune cells have shown immunoregulatory characteristics on various innate and adaptive immune cell types which stimulate Treg cells and prevent the onset of autoimmune illness. [31]. After searching a lot in previous studies, we unable to find a study on the relationship of HβD-2 and multiple sclerosis, as we interpreted the decrease in the level of HβD-2 with the disease as being a protection factor against the development of multiple sclerosis, In other study that was conducted compare type 2 diabetes patients or healthy people, T1D patients were found to have lower levels of cathelicidin and HβD-2 in their blood.This confirmed that AMPs may also prevent autoimmune diseases[32]. This result was consistent with the results of the current study, but there may have been another possibility that the protein level decreased in patients after taking the treatment as it happened with psoriasis patients who had significantly higher blood levels of HβD-2 protein than healthy individuals, although these levels decreased quickly after receiving a single 300-mg subcutaneous dosage of secukinumab [33]. It is however difficult to confirm as the specimens taken were all from patients who under went treatment. Hence, this topic will be important for subsequent researchers to determine the main reason for its decrease during the disease if it is due to it being a protective factor or because of the treatment.

In the current study HβD-2 was lower in MS patients than in HC (Figure1). HβD-2 is a member of the defensins family that has antiviral properties towards both enveloped and non-enveloped viruses. A variety of antiviral mechanisms are explained by the diversity of defensin-sensitive virus species, including post-entry neutralization, viral fusion inhibition and direct defensin targeting viral envelopes, glycoproteins, and capsids. In addition to, indirect antiviral mechanisms are exhibited by the ability of defensins to act as chemokines in increasing and modifing adaptive immune responses. Defensins can also disrupt intracellular signaling and modulation, binding host cell surface receptors to prevent viral multiplication. Also, HβD-1 and HβD-2 have been found in monocytes, macrophages, and dendritic cells (DCs) generated from monocytes, and they exhibit potent neutralizing action against several IAV strains [34].Determining the role of HβD-2 in EBV-negative and EBV- positive patients in MS or HC is important as EBV is a risk factor that is linked to greater disease susceptibility. According to certain studies, a virus may serve as an adjuvant in the development of autoimmunity [35]. Although the frequency of EBV in the present research was lesser in MS patients compared to healthy controls (HC), the EBV load was noticeably greater than in HC because in HC becomes a latent infection. As in a previous study, compared to controls, RA patients had a 10-fold higher (EBV) DNA load in mononuclear cells from the peripheral blood [36]. This suggests that the development of MS is associated with the latent reactivation of EBV [37]. Additionally, correlation analysis showed that when HβD-2 levels were high, EBV load was also low and vice versa (Figure 4).

This study had certain potential limitations. Firstly, it was difficult to detect a clear relationship due to the comparatively small sample size of MS patients and healthy controls. Secondly, neither EBV-infected nor healthy individuals had HβD-2 expression measured in their monocytes.

Conclusion

HβD-2 levels showed no significant differences in each age group, gender, (EDSS) of MS patients, rather an inverse significant correlation (rs = - 0.432) with EBV load, which protects against human viral infections.

Conflicts of interest

The authors have no conflicts of interest to declare

Acknowledgments

The authors would like to thank the medical personnel in Baghdad Medical City for their gracious cooperation.

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