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The Possible Role of Epstein Barr Virus and Its Latent Proteins in Systemic Lupus Erythematosus Patients

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Abstract

Background Systemic lupus erythematosus is a chronic, systemic, idiopathic autoimmune disease. One of the suggested environmental factors that lead to development of systemic lupus erythematosus is infection with Epstein-Barr virus.

Objective First, detection and quantification of Epstein-Barr virus in peripheral blood of systemic lupus erythematosus patients compared to control. Second, estimation of mRNA level of latent and lytic genes and compare them with control groups.

Methods This a case-control study conducted on systemic lupus erythematosus patients during the period from (December 2018 to March 2019). A total of 40 patients were involved in this study. Blood samples were taken from Baghdad Teaching Hospital of Medical City. On the other hand, 40 blood samples were collected from apparently healthy subjects, as control samples from blood donor center in Al-Imamein Al-kadhimein Medical City.

Results Eleven of forty (27.5%) of systemic lupus erythematosus patients were positive with Epstein-Barr virus at mean viral load 815.72 copy/ml with (P value 0.59). And the rate of Epstein-Barr virus detection in blood of patients group was highest in severe cases rather than in less severe ones. Only 2/40 (5%) of control subjects were positive with Epstein-Barr virus at much lower mean viral load, 64.75 copy/ml (P Value 0.34). The expression of the latent genes in patients versus control groups, was 100% versus 77.5% for EBNA-2, 50% versus 15% for EBNA-3C, 82.5% versus 97.5% for late membrane protein -1, and 85% versus 75% for EA/D, respectively. EBNA-2 expression showed significant direct proportional correlation with viral load.

Conclusion Increased rate of Epstein-Barr virus DNA detection in systemic lupus erythematosus group compared to control group and higher rate of viral DNA detection within severe cases might indicate a possible defect in controlling viral infection and increased number of latent infected cell in systemic lupus erythematosus. Also increased positive EBNA-2 and EBNA-3C expression in systemic lupus erythematosus group rather than in control group indicates that these Epstein-Barr virus proteins might have ability to disrupt the normal immune system, and might trigger and/or promote the autoimmune status.

Keywords EBV, SLE, EBNA-2, EBNA-3C, LMP-1, EA/D

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List of abbreviations: EA/D = Early antigen/diffuse, EBV = Epstein-Barr virus, EBNA-2 = Epstein-Barr virus nuclear antigen 2, EBNA-3C = Epstein-Barr virus nuclear antigen 3C, LMP-1 = Late membrane protein -1, QRT-PCR = Quantitative Real Time polymerase chain reaction, SLE = Systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a chronic, systemic, idiopathic autoimmune disease [(1)]. SLE mainly

affects women in child bearing age. Female to male ratio is 9:1 to 15:1 [2]. The etiology of SLE is believed to be multifactorial including genetic and environmental factors that both take part in the development of this very complex disease.

Epstein-Barr virus (EBV) is a lymph trophic double stranded DNA virus with a172 kb genome. It is one of a human herpes virus belonging to the gamma herpes family [3]. After the primary lytic EBV infection, the virus existence continues in host through latent phase [4]. There are several lytic cycle antigens that participate in immune evasion. One of them EBV early antigen/diffuse (EA/D), which is vital for DNA polymerase to replicate the viral genome [5]. In the latent phase, EBV persists in memory B cells, in cryptic form [6]. To successfully escape from the immune response, EBV expresses many viral genes, one of them is EBV nuclear antigen 2 (EBNA2) [7]. It is one of the most essential transcription factors that transform infected B cells [8] to lymphoblastoid cell lines (LCLs). Also has capacity to control the expression of all other latent viral antigens [4]. EBV nuclear antigen 3C (EBNA3C) protein serves as a transcriptional co-regulator by cooperating with various cellular and viral factors [9]. It is responsible for B-cell immortalization. Late membrane protein -1 (LMP-1) is regarded as most important oncogenic EBV gene, that intermediates cell proliferation and inhibits apoptosis [10].

Recently, EBNA-2 was found to act as a transcriptional activator for almost half of SLE risk genes [11]. Increasing evidence in field that EBV might trigger or promote SLE disease as part of the multifactorial etiology of SLE rather than a mere consequence of the disease itself [4]. Hence, the current study was carried out on SLE patients for estimating the role of EBV and its expressed genes in SLE compared to healthy population.

Methods

Patients and controls

The current study is a case control study which was conducted on SLE patients in the period from (December 2018 to March 2019). A total of 40 SLE patients were involved in this study, blood samples were taken from Baghdad Teaching Hospital of Medical City.

Inclusion criteria

All patients were diagnosed with SLE according to 1982 American College of Rheumatology (ACR) criteria for the classification of SLE [12], and also depending on immunological tests, anti-dsDNA and anti-nuclear antibody. Moreover, only newly diagnosed SLE patients or those at off therapy stage were included in order to avoid the effect of immune suppressive drugs (Mycophenolate mofetil 50 mg, Azathioprine 50 mg) on the results of the current study.

Exclusion criteria

Patients with other chronic autoimmune diseases and immune suppressant conditions, such as cancer, were excluded

The control subjects were apparently healthy and age-, sex- matched volunteers; were taken from blood donor center in Al-Imamein Al-kadhimein Medical City. Written informed consents were obtained from subjects.

This study was approved by the Ethical Committee of the College of Medicine/Al-Nahrain University. The current study was conducted in the laboratories of the Microbiology Department at the College of Medicine, Al-Nahrain University.

Disease severity assessment

SLE disease activity index (SLEDAI) score was calculated according to the scale of SLEDAI-2K [12] in SLE patients to determine disease severity, and to explore the correlation of disease severity and rate of EBV detection, gene expression levels with disease severity.

Collection and storage of samples

Forty blood samples were collected from SLE patients who are either during off therapy stage or taking just prednisolone (20 mg). On the other hand, 40 blood samples were taken from apparently healthy subjects, as control samples. From all participant, (3 ml) of whole blood were collected in EDTA tubes, up to (1.5 ml) for RNA extraction and (1.5 ml) for DNA extraction. RNA extraction and conversion to complementary DNA (cDNA) was carried out on the same day of blood collection, and the residual blood was stored at -20C for later step of DNA extraction.

Viral DNA extraction

DNA extraction was carried out by using DNA extraction kit (Bosphore, Turkey, Cat, # ABXVD1) and the method of extraction was according to the manufacturer guidelines.

Viral RNA extraction

RNA extraction was conducted using (Bosphore, Turkey, Cat. #ABXVR1) and extracted RNA was then converted to cDNA by using GO Script reverse transcription system (promega, U.S.A, Cat. #A5000), After DNA, RNA extraction and conversion to cDNA, the yield and purity of the EBV DNA and cDNA were measured using Nano Drop following instruction of the manufacturer (ActGene NAS99).

Detection of viral DNA

EBV quantification kit V1 (Bosphore, Turkey Cat, # ABEBV3) used to detect and quantify viral DNA. Reaction mixture consists of (15 µl) of PCR Master Mix, (0.1 µl) of Internal Control was added/reaction, (10 µl) of DNA sample, four standards with copy number ($2x10^3$-$1x10^6$ copies/ml), Negative/Positive Control were added/ reaction to reach final volume (2 5 µl). Real-time PCR instrument used was Stratagen MX 3005P. The thermal protocol consisted of an initial denaturation at 95 °C for 14:30 min, followed by 50 cycles each with denaturation at 97 °C for 00.30 sec, annealing at 53 °C for 1:30 sec and elongation at 72 °C for 00:15 sec. Moreover, the standard curve is plotted using the data obtained from the defined standards, with the axes (Y-axis) for Ct- Threshold Cycle and (X-axis) Log Starting Quantity.

Estimation of viral gene expression

After RNA extraction and conversion to cDNA; the gene expression for EBV genes was estimated by using Syber green master mix ("SINTOL", Russian, Cat, No M-427). Reaction mixture consists of 10ul of master mix, and 1ul of each forward and reverse primers were added/reaction. (1.5 µl) of $MgCl_2$ along with (8.5 µl) of dd H_2O were added / reaction. (3 µl) of template were added / reaction to reach final volume (25 µl).

The sequences and amplicon size of primers listed in table (1).

Table 1. The primers used along with sequence and amplicon size

Gene		Primer sequence	Product size (bp)	Reference
EBNA2	F	-GTCTGGCACATGCAAGACA	154 bp	(11)
	R	TCTGCCACCTGCAACACTAA		
EBNA3c	F	GGCACATTGTCTTCCGTGTC	220 bp	(11)
	R	-TACAGACTACCGGCGAGCAT		
LMP1	F	CCAATAGAGTCCACCAGTT	78 bp	designed by sigma
	R	TCTTCCTAGCCTTCTTCCTA		
EA/D	F	TAAGGTGACACTCAATCC	83 bp	designed by sigma
	R	TCAGAGGCTTGTAGTCTA		
GADPH	F	GCACCGTCAAGGCTGAGAAC	138 pb	(11)
	R	TGGTGAAGACGCCAGTGGA		

Thermal profile for EBNA-2 includes initial denaturation 95 °C for 5min, followed by 40 cycles of denaturation 95 °C for 30 sec, annealing &extension 62 °C for 30 sec, followed by melting curve analysis. And thermal profile for EBNA-3C &GADPH, as reference gene, includes initial denaturation 95 °C for 5 min, followed by 40 cycles of denaturation 95 °C for 30 sec, annealing 58 °C for 30 sec, extension 72 °C for 30 sec, followed by melting curve analysis. The thermal profile for LMP-1 and EA/D includes initial denaturation 95 °C for 5 min, followed by 40 cycles of denaturation 95 °C for 30 sec, annealing and extension 60 for 30 sec, followed by melting curve analysis. The cycle threshold ΔΔCT method for mRNA was used to measure the relative level of genes expression and make comparison between SLE and control groups at gene expression level for the target genes.

Statistical analysis

The data of the current study was processed and analyzed using SPSS version 16.0.02. Quantitative data were first assessed in terms of normality tests and accordingly parametric (student t-test) or non-parametric tests (Mann-Whitney) were used. For nominal qualitative data, chi-square and Fisher Exact tests were used to measure the significance of association among different qualitative variables of the study. Correlation coefficient, or r, and linear regression analysis were also included. P values <0.05 were considered significant.

Results

This case control study included 40 blood samples of SLE patients with mean age 32 years (Std. error 1.51), and with mean duration of disease 3.5 years (Std. error 0.48), the mean age for controls 25 years (Std. error 2.0).

The positive detection and mean viral load of EBV in SLE versus control group

The findings of the current study showed that the rate of positive EBV, detected by real-time qPCR, was much higher in SLE patients than in control group (P < 0.01), as shown in table (2). In addition, interestingly the mean blood viral load of EBV was found to be 384.11 folds higher in SLE patients than in control subjects.

Table 2. Rate of EBV detection in SLE versus control group

			Case-control		Total
			Case	Control	
Detection of EBV	Negative	Count	29	38	67
		% within Detection of EBV	43.3%	56.7%	100%
		% within Case-control	72.5%	95.0%	83.8%
		% of Total	36.2%	47.5%	83.8%
	Positive	Count	11	2	13
		% within Detection of EBV	84.6%	15.4%	100%
		% within Case-control	27.5%	5.0%	16.2%
		% of Total	13.8%	2.5%	16.2%
Chi-square: P = 0.006 (highly significant), Odds ratio = 7.2, P = 0.014					

The expression of EBNA-2 in SLE compared to control groups

The results of the current study on the positive/negative gene expression of viral EBNA-2 gene, using relative real-time PCR, showed that all of SLE patients were with positive EBNA-2 gene expression (100%) while only (77.5%) of control subjects showed positive EBNA-2 gene expression (P < 0.01).The odds of SLE patients to have a detected

positive expression of EBNA-2 was extremely high, 24.22 times more than control subjects to have a positive EBNA-2 expression, as shown in table (3) .

Table 3. The positive/negative expression of EBNA-2 gene in SLE versus control groups

			Case-control		Total
			Case	Control	
Expression of EBNA2	Negative	Count	0	9	9
		% within Detection of EBNA2	0%	100%	100%
		% within Case-control	0%	22.5%	11.2%
		% of Total	0%	11.2%	11.2%
	Positive	Count	40	31	71
		% within Detection of EBNA2	56.3%	43.7%	100.0%
		% within Case-control	100.0%	77.5%	88.8%
		% of Total	50.0%	38.8%	88.8%
Fisher Exact: P = 0.002 (highly significant) Odds ratio = 24.22, P = 0.029					

The expression of EBNA-3C in SLE compared to control groups

Half (50%) of SLE patients were with positive expression of EBNA-3C versus only 15% in control group. Hence, the expression of EBNA-3C in SLE patients was much higher than in control group (P < 0.01), as shown in table (4).

Table 4. The expression of EBNA3C gene in SLE group versus control group

			Case-control		Total
			Case	Control	
Expression of EBNA3C	Negative	Count	20	34	54
		% within Detection of EBNA3C	37.0%	63.0%	100%
		% within Case-control	50.0%	85.0%	67.5%
		% of Total	25.0%	42.5%	67.5%
	Positive	Count	20	6	26
		% within Detection of EBNA3C	76.9%	23.1%	100%
		% within Case-control	50.0%	15.0%	32.5%
		% of Total	25.0%	7.5%	32.5%
Chi-square: P = 0.001 (highly significant) Odds ratio = 5.66, P = 0.001					

The expression of LMP-1 and Early antigen/D (EA/D) in SLE compared to control groups

On contrary to the expression of EBNA-2 and -3C, the present study revealed that the positive expression of LMP-1 gene was 82.5% in SLE patients compared to 97.5% in control subjects (P > 0.05). In regard to EA/D, although the positive expression of EA/D was a bit higher in SLE patients (85%) than in control (75%) group, the difference was non- significant (P > 0.05).

The quantitative assessment of the relative EBV genes expression in SLE patients

By calculating the relative gene expression of EBNA2, EBNA3, LMP-1, and EA/D, it was shown that EBNA2 was the highest gene expressed in

SLE patients in relative to control group, 22.45 folds, then EBNA3C, 5.68 folds, then EA/D, only 3.35 folds (P < 0.05). On the other hand, LMP-1 gene expression was lower in SLE than in control group, 0.16 folds.

The correlation between EBV load and the relative expression of EBV studied genes

The findings of this study showed that the relative expression of EBV in fold change, was positively correlated with that of EBNA-2 (correlation coefficient or r = 0.82), LMP-1 (r = 0.9), and EA/D (r = 0.86) (P < 0.01). The relative expression of EBNA2 was found to be positively correlated with that of EBNA3C (r = 0.6), LMP-1 (r = 0.85), and EA/D (r = 0.94) (P < 0.01).

Regression analysis between EBV viral load and the relative expression of EBV genes

Linear regression analysis was tested between the viral load of EBV, in folds, as independent variable and the relative expression of EBV genes, EBNA2, EBNA3C, LMP-1, and EA/D. Except for EBNA-3C, all other genes reached significance as dependent variables on EBV load (P<0.01). EBNA-2, LMP-1, and EA/D expression and EBV load fold regression analysis results show that the expression of these three genes was highly dependent on EBV load.

The Expression of EBNA2 gene as an indicator for the presence of EBV infection

Based on the results of the current study, the expression of EBNA2 gene was detected positively in all of the SLE patients while only a portion of SLE patients were with detected EBV via absolute qPCR. Therefore, EBNA-2 expression seems to be more sensitive for the presence of underlying EBV infection in SLE patients. Accordingly, the receiver operator characteristic (ROC) curve was used to assess the feasibility of using the relative expression of EBNA-2 gene as a reliable indicator for the presence of EBV infection. The accuracy, or the usefulness of using this test, was found to significantly high, 0.708 (P < 0.05) and the ROC curve showed that it is accurate to measure EBNA-2 relative expression in folds at cut-off value of 11 folds for the detection of an underlying active EBV infection without measuring directly EBV load, and the use of this cut-off value guarantees an acceptable sensitivity and specificity for the detection of underlying EBV infection, up to 72% and 66%, respectively.

Discussion

Many studies showed similar findings to these of the present study that there was a relation between EBV infection and development of SLE [12-15]. Increased viral load was related with disease activity and it was independent of intake of immunosuppressive medication [4,13]. The cause of elevated viral load in SLE patients might be due to impairment in controlling of infected cells, that leads to increased frequency of latent infection of memory B-cell and also increase in number of the replicating virus [16], and also, impairment of cytotoxic CD8+ T- cell function [14]. These immune defects might enable virus to proceed in persistent latent state and causes viral disease and may further propagate disease status [4]. Another study found that increased virally infected cells in severe cases of SLE rather than mild to moderate ones suggesting that EBV could highly be related to the disease flare in cause-effect manner [16].

The findings of the present study reveal that all SLE patients had positive EBNA-2 expression versus 77% in control group. These findings agree with a recent study showing that EBV can cause autoimmune disease by EBNA-2 transcription factor that help changing how infected B cells to act, and how body employment to those cells [12]. And this study also agrees with another study which showed that almost half of SLE risk alleles are occupied by the EBNA2 protein, triggering their transcription, indicating that an important mechanism by which EBV can perturb immunity [17].

Moreover, a correlation study for the relative expression of viral genes of EBV indicated that increased EBNA-2 expression is positively correlated with other latent genes, namely (EBNA-3C, LMP-1, EA/D) because EBNA-2 has

ability to control the expression of all other latent genes, and also the correlation between increased expression of EBNA-2 and that of EBNA-3C was due to the fact that EBNA-3C works as a co–activator for EBNA-2 [8]. This is exactly what was shown in the present study confirming the pivotal role of EBNA2 in SLE disease.

In the current study, the regression analysis for EBNA-2 expression showed clearly that it is dependent on the viral load and could use the expression of EBNA-2 as an indicator for the viral activity. This indicates several key points; first, EBNA2 and to a lesser extent EBNA3C are consistently and abundantly produced in the latent EBV infection in SLE patients while this phenomenon is not seen in healthy population. Second, the higher viral load observed in SLE patients is uniquely coupled with consistent high activity of latent cycle enabling EBV to possibly exert certain promoting or exaggerating effect on SLE condition. Actually, this shed light on a key point that the more deregulated immune system in severe cases of SLE is expected to have reactivation rather than an active latent cycle; however, this is not the case in SLE where both latent antigens and reactivation antigens are activated with stark favor to latent proteins. This shift in EBV cycle activity seen in SLE requires more investigation.

The findings of the current study on EBNA-3C expression reveal that half (50%) of SLE patients were with positive expression of EBNA-3C, which was much higher than in control group (15%). This result agrees and approach with another study that measured EBNA-3C expression from infected B-lymphocyte that induce lymphoma [18]. This study, besides our study, might explain the role of EBNA3C in contributing in disease pathogenesis through transformation and immortalization of lymphocytes. Hence, the association of the expression of these genes with the disease may be due to severe immune impairment during the flare of disease and that may enhance viral activity or may be the virus itself one of the factors for the flare up and progression of the disease, or both scenarios could be true.

The findings of EA/D expression in the current study showed that although the positive expression of EA/D was a bit higher in SLE patients (85%) than in control (75%) group, but the result was not significant. Anyhow, the high level of expression of EA/D means that there is some sort of EBV reactivation in SLE patients resulting in creation of new virions and ensure spread infection to other epithelial cell and B-cell. These findings may approach to other study findings; they found higher IgA antibody against EA/D in SLE patients compared to normal; this points to viral reactivation and endeavor of immune system to control of reactivation [13].

Overall, according to the findings of the current study, EBV seems to play an active role in SLE pathogenesis and it is not just a consequence of the immune impairment.

Lastly, the positive expression of EBNA-2 in all of systemic lupus erythematosus patients studied, even in cases with negative detection of virus, refers to that it is feasible to use detection of EBNA-2 expression as an indicator for viral infection.

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Author contribution

All authors contributed to this manuscript. Dr. Abdulamir: designed, interpreted and arranged this manuscript, Abdullah conducted the research, and Dr. Isho supervised the clinical aspect of this research.

Conflict of interest

There is no conflict of interest.

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