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Molecular identification of Epstein-Barr virus in human placental tissue

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Abstract

Background: The Epstein-Barr virus (EBV) relates to the torch virus family and is believed to have a substantial impact on mortality and perinatal events, as shown by epidemiological and viral studies. Moreover, there have been documented cases of EBV transmission occurring via the placenta. Nevertheless, the specific location of the EBV infection inside the placenta remains uncertain.

Methods: The genomic sequences connected to the latent EBV gene and the levels of lytic EBV gene expression in placental chorionic villous cells are examined in this work. A total of 86 placentas from patients who had miscarriage and 54 placentas from individuals who had successful births were obtained for analysis.

Results: The research employed QPCR to detect the BRLF1 (Rta) EBV lytic gene and quantify DNA burden in miscarriage patients and controls. In miscarriage patients, endpoint PCR and Sanger sequencing validated a particular region of the EBER1 EBV latent gene. BRLF1 gene presence ranged from 2.5×10^2 to 9.3×10^4 copies/mL in 75 miscarriage patients. However, a sample of 5 people with healthy deliveries showed a range of 2.0×10^2 to 2.9×10^2 copies/mL. All miscarriage samples were observed EBER1. The sequencing results indicated full sequence identity to EBV strains.

Conclusion: The detection of EBV gene expression in placental tissues in Iraq is a novel finding. The examination of EBV is of utmost importance in pregnant women who are experiencing severe illness, since it has the potential to lead to mortality in both the mother and the developing fetus.



Introduction

Numerous infectious pathogens that could harm the fetus are exposed to pregnant women. When these infectious agents enter an embryo or fetus, they typically result in one of two outcomes: either severe congenital defects or early embryonic and fetal mortality, which can lead to miscarriages; long-term effects can also include developmental issues. They may also cause severe neonatal and/or perinatal illness or interfere with fetal growth, leading to intrauterine growth restriction [1,2]. The herpesvirus family includes the widespread EBV. One of the most widespread viruses identified in humans [3,4]. It is well known that it has a preference for B lymphocytes and particular kinds of epithelial cells [5,6]. Over ninety-five percent of individuals all over the globe have an EBV infection that is dormant in their B cells. Getting EBV on B cells as a child is linked to several diseases in humans, such as infectious mononucleosis, various types of malignant lymphomas, lymphoproliferative disorders, and systemic autoimmune diseases [7–10]. More than 86 open reading frames may be found in the genome of the EBV double-stranded DNA, which is around 170 kilobytes in size. The virus genome has terminal repeats (TR) and four significant internal repeats (IR1 to IR4). Nine latent proteins are encoded by genes located in certain parts of the genome. These are latent membrane protein 1 (LMP1) and LMP2A, -2B, EBV nuclear antigen 1 (EBNA1), EBNA2, -3A, -3B, and -3C, and EBNA-LP [11]. Different lytic proteins with different functions are also encoded by other open reading frames, along with transcription factors and capsid proteins such as (BRLF1, BZLF1, BALF2, BNLF2a, BcLF-1, BPLF1, BILF1, and BNLF2a) [12]. To make more EBV RNAs that don't code for proteins, the virus also makes microRNAs from BART and BHRF-1. These include EBV-encoded small RNAs 1 and 2 [13].

The life cycle of EBV includes two distinct phases: lytic replication and latency. It uses two different gene classes, each of which performs a very different function during its life cycle [14,15]. Many viruses exploit host cell manipulation-specifically, changes in cell cycle progression-as a means of accelerating their replication. Cells enter the S phase when exposed to little DNA tumor viruses. Herpesviruses might stop cells at any stage in the cell cycle, while small DNA tumor viruses can prompt cells to enter the S phase [16–19]. BRLF1 is also unknown as a replication transcription activator (Rta). It is a factor that controls the transcription that EBV expresses during the initial, early stage of the lytic cycle. Within two hours of the administration of an inducing stimulus, BRLF1 encodes and expresses it. The promoter just upstream of the BRLF1 ORF regulates the expression of the 4.0-kb mRNA that codes for the BRLF1 gene [20,21]. Rta is

recognized to perform a wide range of biological tasks. The Rta-responsive element (RRE), a GC-rich motif presents in the viral promoters of various EBV lytic genes, including BMRF1, BMLF1, and BALF2, is where Rta attaches. Rta, however, also stimulates the transcription of viral genes whose promoters do not contain an RRE. For example, Rta indirectly turns them on by supporting the PI3-K and p38 mitogen-activated protein kinase-connected cell-signaling pathways. Rta may potentially use the regulation of E2F1 to activate certain viral genes [22–24]. Rta encourages growth arrest and starts a cellular senescence programme [25,26]. It is likely that Rta's impacts on the host cell environment increase the effectiveness of lytic virus replication. Numerous viruses use host cell environment manipulation, specifically targeting cell cycle progression, to establish the ideal circumstances for viral reproduction [27,28]. The most prevalent EBV transcripts in EBV-associated tumors are the short RNAs that EBV encodes (EBERs, EBER1 and EBER2) [29,30]. It has long been assumed different EBV isolates have a common set of EBER coding sequences. Therefore, the connection between EBV-associated illnesses and mutations in the EBER genes has received minimal study [31].

Scientific investigation and debate have focused on the link between EBV infection and a number of pathologic diseases that can occur during pregnancy, including depression, pre-eclampsia, abortion, and stillbirth. Although several studies have revealed a possible connection, the proof is not yet conclusive [32–34]. The current study examined the expression of the BRLF1 (Rta) gene to identify the EBV infection in the placenta and connect it with abortion reasons in Baghdad, Iraq. Furthermore, we investigated sequence variation in the EBER gene, the reliable method for identifying latent EBV infection in tissues and contrasted our results with those from earlier studies. This work is the first description of the expression patterns of lytic EBV genes in placental tissue, namely in chorionic villous cells. Our research also demonstrates the connection between EBV infection and EBV-related abortions.

Methods

Study population

In the present cohort research, placental tissues were collected from 86 female miscarriage patients (patients' group), whose ages ranged from 20 to 41 years, and 54 placental tissues from healthy deliveries (control group). Samples obtained from the teaching laboratories at AL-Alwaiya Maternity Hospital in Baghdad, Iraq, between June - August 2023 were selected for this research. This study was approved by the Human and Animal Research Ethics Committee of

the University of Baghdad (reference No. CSEC/0923/0060). All participants received verbal explanations of the study's methodology and goals before reading the consent form and signing it.

DNA extraction and sample preparation

Placentas were kept chilled at 4°C from the moment of delivery and processed for DNA as soon as they arrived at the laboratory. 25-50 mm³ in volume of placental tissue (chorionic villous) samples were collected and fresh frozen at -80°C for 2 hours before being proceeded for DNA extraction. During the pre-treatment phase, the tissue was homogenised using proteinase K and ATL tissue lysis buffer at 56°C. DNA samples were extracted using the New iGNatal Kit (iGNatal Biotech, Spain) according to the manufacture's protocol. A NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA) was used to assess the DNA quantity and quality.

Assessment of viral loads by qPCR

For PCR amplification, a primer set targeting the highly conserved BRLF1 (Rta) EBV lytic locus, gene: BRLF1-FOR 5'-GCACATCTGCTTCAACAGGA-3' (Forward) and BRLF1-REV 5'-TACAAGAATCGGGTGGCTTC-3' (Reverse) gene bank accession NC_007605.1 (89838-93925), was utilized to increase detection EBV lytic rates and decrease false negative results. In lytic infection, BRLF1 (Rta) is a crucial regulatory gene that cooperates with BZLF1 to start the lytic phase. It contributes to the lytic cycle's driving force by triggering the expression of several lytic genes [35]. As a "house-keeping" gene, the β -actin gene: Actin-FOR 5'-CACCTTCTACAATGAGCTGCGTGTG-3' (Forward) and Actin-REV 5'-ATAGCACAGCCTGGATAGCAACGTAC-3' (Reverse) was utilized to check for DNA integrity and control for PCR inhibitors [36]. Prior to the EBV QPCR, beta-actin PCR was performed on all samples. DNase/RNase free water (no template DNA) controls were included to monitor contamination.

Each QPCR reaction mixture was prepared in a 200 μ L flat capped tube (PCR-certified colorless) (Thermo Fisher Scientific, USA) to a final 12 μ L volume, including 6 μ L of 2X iTaq Universal SYBR Green Supermix (BioRad, USA), 4 μ L of DNA elute, 0.25 μ L (10 μ M) of sense and antisense primers (Macrogen, South Korea), and 1.5 μ L of DNase/RNase free water. Each primer set underwent a QPCR cycling profile with two steps. After an initial activation/denaturation stage of 13 minutes at 98°C, followed by 40 cycles were performed, each involving of the following: 15 seconds at 94°C, followed by 30 seconds optimised annealing temperature at 60°C using Rotor-Gene Quantitative Real Time Analyzer (QIAGEN, USA). Rotor-Gene software was used to analyse the QPCR readings.

Between 55°C and 95°C, melt curves were measured at 0.5°C intervals. The cycle threshold (Ct) was set at 0.03 levels during the exponential amplification phase. DNA samples with no identifiable target DNA had a load of zero and were classified as negative.

The viral load calculations were evaluated based on the number of replicates tested, the final elution volume, and the DNA extraction volume. Ratios were maintained constant by extracting and eluting samples in equal amounts. To achieve the final measurement of DNA copies/mL of sample, the PCR volume of 4 μ L was multiplied by the elution volume (250) and divided by the number of replicates. Since the copies per microgram of DNA are closely correlated with this unit of measurement, normalisation to the amount of input DNA is not necessary [37]. Samples that did not contain any target DNA were marked as negative and given a load of zero.

EBV identification and Sanger sequencing

Fifteen placental aborted samples derived from population carriers underwent PCR amplification and subsequent Sanger sequencing to confirm the EBV region of the EBV-encoded small RNA 1 (EBER1) (coordinates 6776-7397). The sense primer (EBER1-FOR) and antisense primer (EBER1-REV) used for the endpoint PCR amplification are 5'-GTCTTCGGTCAAGTACCA-3' and 5'-GTAGAGTGGGAGTGCTATC-3', respectively [11]. 20 ng of gDNA was added to a 50 μ L reaction mixture, containing 5 μ L each of 10 μ M forward and reverse primers and 25 μ L of 2X Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA). Conventional PCR was performed in a T100TM Thermocycler (Bio-Rad, USA). The PCR condition was setup as follows: an initial DNA denaturation step at 98°C for 30 seconds, followed by 35 cycles at the temperatures: 98°C for 10 seconds, 59°C for 15 seconds, 72°C 15 seconds, followed by 72°C for 5 minutes.

All sequences were matched with the B95-8 prototype sequence, which was found in the GenBank database under the accession number V01555. In order to mitigate the potential impact of PCR-induced mismatched bases, the amplification and sequencing of samples containing mutations were repeated. The alignment of all EBER1 sequences was conducted using the programme Geneious 9.0.4.

EBV DNA verification

Direct gel analysis was carried out to find amplified PCR products. The amplification result was run on a 2 % agarose gel using a 10 μ L sample (PCR amplicon). PCR amplicon was stained with ethidium bromide (0.5 μ g/mL), and then it was examined using a GeneFlash UV transilluminator (Syngene, Cambridge, UK).

Statistical analysis

The GraphPad (Prism) software programme (version 5) was used. The correlations between the EBV DNA load in the miscarriage patients and healthy deliveries were evaluated using Spearman's (rho) correlation coefficient. *P* values below 0.05 were taken into consideration while analysing the results.

Results

EBV lytic rates are strongest associated with aborted women

In the miscarriage patients' group and the control group, BRLF1 DNA was detected in 87.2 % (75) and 2.7 % (5) of the samples, respectively (Table 1). In both sample groups, viral loads were detectable in the placental tissues at levels ranging from 2.0×10^2 to 9.3×10^4 copies/mL in both groups. Of the 75 BRLF1 DNA-positive miscarriage patient samples, 85.2% were detectable between 5.5×10^2 to 9.3×10^4 copies/mL. However, of the 5 BRLF1 DNA-positive control group samples, 80% were detectable between 2.0×10^2 to 2.5×10^2 copies/mL.

Samples	BRLF1 (copies/mL) positive (n)	Detectable range	Spearman correlation (p)
Miscarriage patients	87.2 % (75)	$2.5 \times 10^2 - 9.3 \times 10^4$	rho= 0.75, p < 0.001
Healthy deliveries (control group)	2.7 % (5)	$2.0 \times 10^2 - 2.9 \times 10^2$	rho= 0.11, p = 0.14

Abbreviations: EBV, Epstein Barr virus; BRLF1 (Rta)

Table 1: EBV DNA load in the population samples [miscarriage patients group n= 86 and healthy deliveries (control group n=54)].

Endpoint PCR and genomic analysis of EBV isolated from placental tissues

Miscarriage DNA samples were used for endpoint PCR amplification and Sanger sequencing to confirm a region of the EBER haplotype (coordinates 6776-7397). The endpoint PCR findings revealed that all samples tested positive for EBER1 (Figure 1). Positive identification, the electrophoretic sizes of the PCR products were identical to the sizes that were expected based on the annotated sequencing. This confirmed that EBV was present in the placental tissues.

Fifteen of the PCR amplicon samples were exposed to commercial Sanger sequencing to confirm the PCR results. Figure 2 displays a chromatogram of sequencing data that represents various samples. We compared our results with globally published EBV genomes because this work takes into account the first sequencing of EBV genomes isolated from patients in Iraq. It was possible to directly check the PCR products by comparing them to herpesvirus genomic DNA sequences from the National Library of Medicine/National Institutes of Health. The 653-base pair fragments were amplified using endpoint PCR to

target EBER1 region, specifically covering coordinates 6776 to 7397. A single-nucleotide change was identified by comparative analysis with the EBV reference sequence B95-8 (No. V01555) and other spontaneous alterations (synonymous mutations). Of fifteen sequenced samples, only two were revealed synonymous mutations from T to A. Interestingly, the viral loads for these two samples (mismatch sequences) were below 2.8×10^2 copies/mL. There was a weak association (not statistically significant) between BRLF1 DNA load and mismatch sequence (Spearman's rho = 0.11, p = 0.11). No nucleotide deletions, insertions, or premature stop codons were observed in the investigated versions of EBER1, indicating a lack of evidence for such genetic alterations.

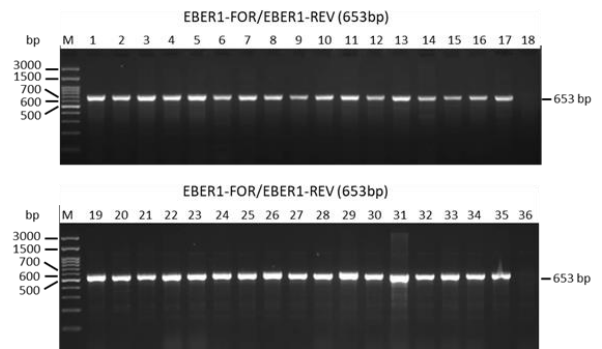


Figure 1: Gel electrophoresis image following endpoint PCR amplification of EBV (EBER1) DNA of patient samples. Lane M: 100bp DNA ladder (GeneDirex, South Korea). Lane 1 - 17, 19 - 35 placental DNA and 18, 36 no template control. PCR amplicons were electrophoresed onto 2 % agarose gel containing ethidium bromide (0.5 µg/mL) and visualized by a GeneFlash UV transilluminator. PCR fragment size (653 bp) is represented on the right side of the panel.

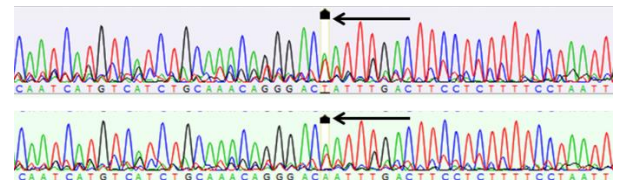


Figure 2: Sanger sequencing chromatogram data analysis for the forward primer (EBER1-FOR) reveals genotyping of the EBER region. A black arrow and white highlight denote the area of interest, which is the predicted synonymous mutation region.

Discussion

Genital HSV can cause a serious neonatal condition, particularly in primary infections, which puts the newborn at risk if infected during birth. Even though they don't happen very often, intrauterine infections can cause abortion or stillbirth, skin scars (called cutaneous symptoms), eye problems (like chorioretinitis and microphthalmia), and neurological problems (like brain damage) [1,38,39]. The EBV-encoded BRLF1 (Rta) and EBER1 genes, which trigger the lytic cycle, and the EBER1 gene, which participates

in the interaction between the virus and its host cells, respectively, during virus infection, have been verified in this study as an indicator for lytic and latent virus infections [40,41]. The findings of the current investigation reveal a noteworthy incidence of EBV in placental tissues. In this study, a total of 86 placental samples were examined to identify the presence of the BRLF1 gene associated with EBV using the quantitative polymerase chain reaction (QPCR) method. The results revealed that 75 out of the 86 samples, accounting for 87.2 %, tested positive for the presence of the BRLF1 gene (which serves as an indicator for EBV lytic replication). The aforementioned high proportion indicates a significant prevalence of EBV infection in the placental tissues being examined.

EBV has the potential to induce placental infection during pregnancy, leading to subsequent difficulties for the developing fetus. During pregnancy, it is possible for both primary and secondary EBV infections to take place. Nevertheless, initial infections with EBV by transplacental transmission are few, although secondary maternal EBV infections are often seen [1]. Since more than two decades the nucleic acid testing (NAT) techniques have been more widely available, quantifying EBV DNA in blood has shown to be helpful in both monitoring and diagnosing as Burkitt's lymphoma (BL), chronic active EBV infection, nasopharyngeal carcinoma (NPC), infectious mononuclear (IM), EBV infection in HIV-positive patients, and posttransplant lymphoproliferative disorder (PTLD) [42–49].

According to the study conducted by Avgil and Ornoy, the findings indicate that in 22.5% of instances (9 out of 40), there was observed nuclear localization of EBV-Viral Capsid Antigen (VCA) expression. This finding indicates that a significant proportion of EBV infections occurring in placental tissues during pregnancy exhibited a degree of severity ranging from mild to severe [1,50]. Our findings suggest that the QPCR method, which is well-known for its high sensitivity and accuracy in detecting viral nucleic acids, was employed in order to determine and quantify the quantity of the BRLF1 gene, which acts as an indication for EBV lytic replication. This is in contrast to the approach that was utilised by Avgil and Ornoy [1]. A study conducted by Wang and his colleagues has shown the notable preservation seen in the BHRF-1 gene across different samples associated with the EBV. The substantial degree of conservation implies an intriguing possibility - mutations in the BHRF1 gene may not have a direct correlation with disorders connected to EBV [40]. This discovery is crucial for our present emphasis on identifying EBV infection by detecting the BHRF-1 gene. By focusing on a gene that is recognised for its stability and conservation in EBV-

related situations, we may uncover subtle details in the diagnosing process. There is little knowledge about the associations between EBER1 mutations and the advancement of disorders related with EBV. In addition, there is currently a lack of documented information pertaining to the variances of the EBER1 gene across various geographical regions. It is imperative and time-sensitive to address and resolve these issues. Based on clinical data obtained from Shandong Province in North China, it has been observed that EBER1 mutations are not linked to the development of leukemia and myelodysplastic syndrome (MDS) [31]. The conservation of coding sequences among different EBV strains in EBERs has been a longstanding belief. Hence, the investigation of mutations occurring in the EBER genes and their correlation with disorders linked with EBV has received little scholarly focus [31,41]. The most prevalent transcripts in tumors linked with EBV are EBERs [29,30]. Mutations in EBER1 have the potential to impact its biological functions, carcinogenic properties, and the immunological responses of the host. Further investigation is needed to determine the correlation between mutations in the BHRF-1 gene and EBV-related illnesses across different geographical locations.

To evaluate the potential clinical repercussions of EBV infection in this tissue and to better understand the link between EBV and the placenta during pregnancy, more study is required. In order to enhance our comprehension of the virus's involvement in pregnancy and its potential influence on fetal development, more investigation is imperative. The results emphasise the possible influence of EBV on the well-being of the placenta and justify the need for more research to explore the consequences of EBV infection during pregnancy. The comprehension of the frequency of EBV in placental tissues enhances our understanding of viral dynamics in the context of reproductive health, potentially impacting the health and well-being of both the mother and the fetus. It is advisable to conduct more investigations and detailed research in order to investigate the unique processes and implications of EBV infection within the setting of pregnancy.

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Ethical Declaration

This study was approved by the Human and Animal Research Ethics Committee of the Department of Biology, College of Science, University of Baghdad (reference No. CSEC/0923/0060). All participants received verbal explanations of the study's methodology and goals before reading the consent form and signing it.

Conflicts of interest

The authors declare no conflict of interest.

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