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Molecular study of Epstein Barr virus among patients with malignant diseases in al Najaf and Diwaniyah governorates

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***Abstract*---**The present study is an effort to study the Epstein-Barr virus and it is relationship to the different types of cancers and their relationship to the virus and to the demographic distribution of the patients. our study started from Sep 2019 to Dec 2020 , blood samples were collected (2 ml of venous blood) were drawing by disposable syringe under a septic conditions. From patients with age grouped more 13 years old , represented by 47 specimens that subjected to molecular examination in Nested PCR technique detecting EBV LMP-1 in patients' blood as a main route. The study has included patients with different types of cancers from different areas. 47 specimens submitted to molecular examination, polymerase chain reaction (PCR) and nested PCR. Where EBV LMP-1 detected in 20 positive Specimens. All the virus isolates were sent to NCBI-BLAST to confirm the variation of the isolates. The study summarize that there is a strong relationship between EBV and some cancer diseases and show no significant difference according to age or gender or hereditary.

***Keywords*---**Epstein-Barr virus, cancer, NCBI-BLAST, lymphotropic

Introduction

The Epstein–Barr Virus (EBV), also called human herpesvirus 4, is a lymphotropic herpesvirus and the causative agent of infectious mononucleosis (IM) (1). It was first discovered in cells isolated from African Burkitt's lymphoma, later, it was recognized that it is highly prevalent worldwide (2). Similar to other herpesviruses, following a primary infection, the EBV has a latency phase where it infects epithelial cells, enters the circulating B lymphocyte, and persists for the life in a latent state (3).

Cancer is a malignant form of disease resulting from uncontrolled cell division and the resulting proliferating cells have the ability to invade other tissues through direct cell migration or through the vascular system, In general terms, carcinogenesis represents a complex, multistep process (4).

The understand of breast cancer etiology is that invasive cancers arise through a sequences of molecular alterations at the inside cell level, these alterations result in breast tissues with immortal features and uncontrolled raised (5)

There are two main EBV genotypes, type 1 and type 2, or A and B, respectively, distinguished by the differences in EBNA-2 gene, since the divergence in EBNA-2 reveals only 54% homology between the two types (6). EBV types 1 and 2 can further be subdivided into different virus strains (7). Most of the investigations concerning the genetic variability of EBV strains were based on studying the LMP-1 oncogene since it has a greater degree of polymorphism than most of the others EBV genes (8).

Variants in LMP-1 were classified into 7 main groups: B95-8, Alaskan, China 1, China 2, Med+, Med-, and NC (9). However, new LMP-1 strains were reported from different origins such as the Southeastern Asia 1 (SEA1), and Southeastern Asia 2 (SEA2) reported in Thailand (10). Interestingly, it was found that multiple EBV variants could be detected within one individual (7). Moreover, some LMP-1 variants were correlated with cancer progression such as CAO strain, which was isolated from NPC patient is China and has shown to carry atypical 10 amino acid deletion resulted in increased transforming ability (11).

Method

Specimen collection
Blood samples were collected by venipuncture according to the procedure from 47 patients (2 ml of venous blood) were drawing by disposable syringe under a septic conditions. Each blood sample was collected directly in a sterile tube one contains EDTA and the other Gel, Tubes with EDTA stored in deep freeze for later use in DNA extraction and nested PCR technique.

Viral DNA Extraction
Viral DNA was extracted from seropositive blood samples by using G-spinTM Total DNA Extraction Kit and done according to company instruction.

Genomic DNA estimation
The extracted DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm).

Nested PCR
Nested PCR technique was performed to detection Epstein-Barr virus from seropositive blood samples. This assay was carried out according to method described by (7).

PCR master mix preparation
PCR master mix was prepared by using (Maxime PCR PreMix kit Hotstar) and this master mix done according to company instructions.

PCR Thermocycler Conditions
PCR Thermocycler conditions were done by using convential PCR thermocycler system.

Nested PCR Master mix	Volume
PCR product	3 µl
Second Nested PCR Forward primer (10pmol)	1µl
Second Nested PCR Forward primer (10pmol)	1µl
PCR water	15µl
Total volume	20 µl

After that, these Nested PCR master mix components that mentioned above were placed in standard CR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler.

Phylogenetic Tree Analysis
Phylogenetic tree analysis based on LMP1 gene partial sequence of Epstein-Barr Virus that used for study of genetic relationship between local Isolates EBV virus clones and NCBI-BLAST Epstein-Barr Virus clones. The phylogenetic tree was constructed using the UPGMA method in (MEGA X version).

Results

1.DNA detection using PCR
1.1.hereditary aspects with viral infection

Table 2
Distribution according to hereditary aspects by PCR technique

Inheritance	No. of total samples	Positive samples No. (%)	Chi-square= 0.381 with 1 degrees of freedom. (P = 0.537) (No significant difference)
Hereditary	14	5 (35.71)	
Not hereditary	33	15 (45.45)	
Total	47	20 (42.55)	

2.Relation of virus infection with cancer stage

Table 3
Distribution according to Cancer stage by PCR technique

Cancer stage	No. of total samples	Positive samples No. (%)	
1st	0	0 (0)	Chi-square= 5.218 with 3 degrees of freedom. (P = 0.074) (No significant difference)
2nd	15	3 (20)	
3rd	19	9 (47.36)	
4th	13	8 (61.53)	
Total	47	20 (42.55)	

Target gene in EBV (LMB-1)

Most of the recent investigations on the EBV strain variation were based on studying the LMP-1 oncogene sequence, because it has shown to have a greater degree of polymorphism than most of EBV genes between different strains (9). LMP-1 is a 356-amino acid protein, which consists of a short cytoplasmic N-terminus, six membrane-spanning domains, and a long cytoplasmic C-terminal domain (8).

Agarose gel electrophoresis

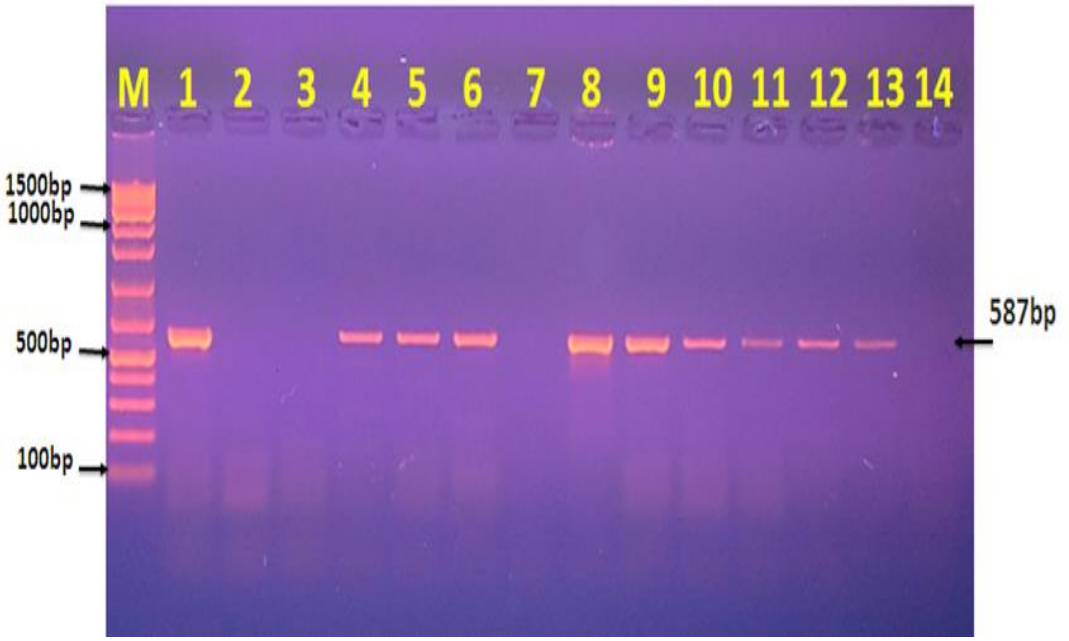

Figure 1: Agarose gel electrophoresis image that show the Nested PCR product analysis of EBV, where (M) DNA marker ladder (1500-100bp), lane (1-14) was showed some positive samples at (587bp) Nested PCR product size.

Phylogenetic analysis

Phylogenetic tree analysis based on LMP1 gene partial sequence of Epstein-Barr Virus that used for study of genetic relationship between local Isolates EBV virus clones and NCBI-BLAST Epstein-Barr Virus clones. The phylogenetic tree was constructed using the UPGMA method in (MEGA X version).

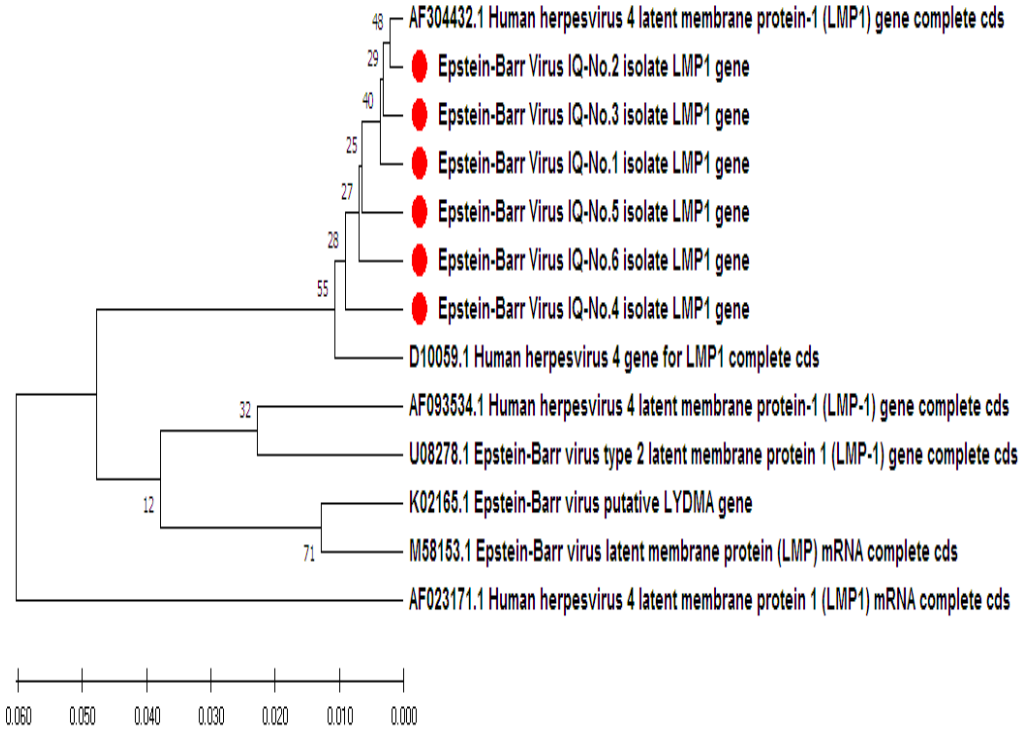

Figure 2: Phylogenetic tree analysis based on LMP1 gene partial sequence of Epstein-Barr Virus isolates that used for genetic analysis. The phylogenetic tree was constructed using the UPGMA method in (MEGA X version).

Table 4
The NCBI-BLAST Homology Sequence identity (%) between Epstein-Barr Virus IQ isolates and NCBI-BLAST submitted Epstein-Barr Virus isolates

EBV isolate No.	NCBI-BLAST Homology Sequence identity (%)		
	Identical NCBI BLAST EBV isolates	Genbank Accession number	Identity (%)
EBV IQ No.1	Human herpesviruses 4 latent membrane protein-1 (LMP1) gene	AF304432.1	99.29%
EBV IQ No.2	Human herpesviruses 4 latent membrane protein-1 (LMP1) gene	AF304432.1	99.59%
EBV IQ No.3	Human herpesviruses 4 latent membrane protein-1 (LMP1) gene	AF304432.1	99.79%
EBV IQ No.4	Human herpesviruses 4 latent membrane protein-1 (LMP1) gene	AF304432.1	98.58%
EBV IQ No.5	Human herpesviruses 4 latent membrane protein-1 (LMP1) gene	AF304432.1	98.84%
EBV IQ No.6	Human herpesviruses 4 latent membrane protein-1 (LMP1) gene	AF304432.1	98.83%

5.The NCBI-blast homology sequence identity
Represent the registry of the NCBI-GenBank and the genetic identical between the EBV IQ isolates and NCBI-BLAST isolates. Where the identity 99.29% , 99.59% and 99.79% for EBV IQ (No.1,No.2 and No.3) respectively with NCBI-BLAST identical isolates (AF304432.1), the identity 98.58%,98.84% and 98.83% for EBV IQ isolates (No.4 , No.5 and No.6) respectively with NCBI-BLAST identical isolate (AF304432.1) as shown in table (4)

Table 5
The total percent % of substitution mutations between local EBV IQ isolates and NCBI-BLAST genetic related isolates.

EBV IQ isolate	Total number of Substitution mutations	Single nucleotide polymorphism (SNP)	Percent %
EBV IQ No.1	5	C/T, T/A, A/T	0.71%
EBV IQ No.2	2	T/A, T/A	0.41%
EBV IQ No.3	1	T/A	0.21%
EBV IQ No.4	6	T/C, T/C, T/A, T/A, C/T, A/T	1.42%
EBV IQ No.5	5	T/C, T/C, T/A, A/T, C/G	1.16%
EBV IQ No.6	6	C/T. A/T, T/A, G/A, A/T, G/C	1.17%

Discussion

1.According to hereditary aspects

the results shows higher percentage with PCR test for non-hereditary infection transmission with 45.45% for fifteen positive samples ,it is more transmitted through direct contact , bodily fluids (saliva , blood transfusion and sexually transmitted) and personal things as EBV main route of transmission is the oral route and it agrees with (12, 13, 1).

2.According to cancer stage

PCR technique analysis according Cancer stage, results recorded in (Table 3) that showed gradually increasing as the cancer progress started with 20% for the 2nd stage , 47.36% for 3rd stage and 61.53% for 4th stage and there is no significant difference at (P = 0.074) and it is agreed with (14 , 15) as they mention that EBV infections increases with cancer progress.

3.Phylogenetic tree

The Epstein-Barr Virus IQ isolates (No.1- No.6) were showed closed genetic related to NCBI-BLAST Human herpesviruses 4 latent membrane protein-1 (LMP1) gene complete cds (AF304432.1) Whereas other NCBI BLAST Epstein-Barr Virus isolates were showed genetic variants at total genetic changes (0.050-0.010%).

Then confirmed by NCBI-BLAST Homology Sequence identity (**Table 3)** after that EBV isolates were deposited into NCBI-GenBank to get GenBank accession number for EBV isolates. Where the GenBank accession number was obtained.

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