Molecular and Immunological study of Epstein-Bar virus associated with leukemia patients in the Babylon province of Iraq

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Abstract. The first virus to be implicated in the causation of a human cancer was the Epstein–Barr virus (EBV), discovered in 1964 by Epstein, and Barr in a lymphoid cell line established from a biopsy of an African Burkitt lymphoma, the present study establishes for detection Epstein –Bar virus in thirty serum samples collected from Marjan hospital in the Babylon Province of Iraq. The present study included detection of virus by ELISA and polymerase chain reaction in leukemia patients. Twenty three of the thirty samples are positive for EBV association with leukemia patients, according to ELISA and PCR technique results produced 161 bp band referred to EBV gene noticed at gel electrophoresis technique.

Keyword: Epstein–Barr virus, Leukemia, PCR, ELISA.

Introduction

Epstein–Barr virus (EBV) is one of the herpesviridae family of the most common viruses in humans. The virus is approximately 122–180 nm in diameter and is composed of a double helix of DNA, which contains about 172,000 base pairs and 85 genes, the DNA is surrounded by a protein nucleocapsid [1]. This nucleocapsid is surrounded by a tegument made of protein, which in turn is surrounded by an envelope containing both lipids, and surface projections of glycoproteins which are essential to infection of the host cell [4] that best known as the cause of infectious mononucleosis (glandular fever). It is also associated with particular forms of cancer, such as Hodgkin's lymphoma, Burkitt's lymphoma, gastric cancer, nasopharyngeal carcinoma, and conditions associated with human immunodeficiency virus (HIV), such as hairy leukoplakia and central nervous system lymphomas [1, 2] is evidence that infection with EBV is associated with a higher risk of certain autoimmune diseases, especially dermatomyositis, systemic lupus erythematosus, rheumatoid arthritis [6].
Infection with EBV occurs by the oral transfer of saliva and genital secretions[3]. As previously stated there are few symptoms or asymptomatic when a person contracts EBV at the adolescence stage of life, but when EBV is contracted as an adult it may cause fatigue, fever, inflamed throat, swollen lymph nodes in the neck, enlarged spleen, swollen liver, or rash[7].

EBV can infect both B cells and epithelial cells. To enter B cells, viral glycoprotein gp350 binds to cellular receptor CD21 (also known as CR2). Then viral glycoprotein gp42 interacts with cellular MHC class II molecules. This triggers fusion of the viral envelope with the cell membrane, allowing EBV to enter the B cell. Most people become infected with EBV and gain adaptive immunity. In the United States, about half of all five-year-old children and about 90 percent of adults have evidence of previous infection. Infants become susceptible to EBV as soon as maternal antibody protection disappears [8].

Aims of the study
Detect the EBV gene with the PCR and ELISA techniques in leukemia patients in the Babylon province of Iraq.

Material and Methods:

Sample collection:
This study includes 30 patients with Chronic Myeloid Leukemia (CML) admitted to Marjan Hospital in Babylon Province of Iraq. Patients were diagnosed by specialist physicians and selected in the current study.

Blood Sampling:
About five milliliters of venous blood were collected from each patient in the study. The blood was divided into two parts: one part (about two milliliters) was collected into EDTA containing tubes for genetic part. The second part of the blood was placed in gel tube for thirty minutes, then transferred to plain tube and serum was obtained by centrifugation at 3000 rpm for 15 min; after that the serum collected and kept in the freezer (-20 ºC) until it was used for the immune and viral assay.

Promega Wizard genomic kits. The isolation of DNA depended on the 5 stage procedure utilizing salting out techniques [9]:
- Lysis of the RBCs in the Cell Lysis Solution.
- Lysis of the WBCs and their nuclei in the Nuclei Lysis Solution.
- The cellular proteins were then removed by a salt out precipitation step using the Protein Precipitation Solution.
- The genomic DNA was concentrated and desalted by Isopropanol precipitation.
- The genomic DNA was rehydrated using the DNA Rehydration Solution.

Isolation kit Components:

| Components                        | Amount  |
|-----------------------------------|---------|
| Cell Lysis Solution               | 500 ml  |
| Protein Precipitation Solution    | 125 ml  |
Nuclei Lysis Solution 250 ml DNA Rehydration Solution 100 ml

The Protocol for DNA Separation:

Procedure which provided with Promega kit recommend for DNA separation as reveled in bellow:

1. Cell Lysis Solution (900 l) was added to a clean 1.5 ml small scale rotator tube.

2. Gently shook the container of blood until it was altogether blended; then 300 l of blood was transfused to the tube which contained the cell lysis arrangement. The tube was modified 5-6 times to be blended.

3. The blend was hatched for 10 minutes at room temperature and modified 2-3 times amid brooding to lyse the RBCs. In addition, the tube centrifuged at 13000-14000 rpm/20 seconds at room temperature.

4. The supernatant was expelled and disposed of however much as could be expected without exasperating the obvious white pellet.

5. The tube was vortexed enthusiastically until the WBCs were re-suspended (10-15 seconds).

6. Nuclei Lysis Solution (300 l) was added to the re-suspended cells; the arrangement was pipetted 5-6 times to lyse the WBCs. The arrangement ought to have turned out to be exceptionally gooey .If clusters of cells were unmistakable in the wake of blending, the arrangement ought to have been brooded at 37 oC until the bunches interruption; if clusters are still obvious following 60 minutes, extra Nuclei Lysis Solution (100 l) ought to be included; in addition, the hatching ought to be rehashed.

7. Protein Precipitation Solution (100 l) was added to the atomic lysate and vortex enthusiastically for 10-20 seconds. Little protein bunches may be obvious.

8. The tube was centrifuged at 13000-14000 rpm for 3 minutes at room temperature until dull cocoa protein pellet was noticeable.

9. The supernatant was exchanged to a clean 1.5 ml smaller scale rotator tube which contained 300 l of room temperature Isopropanol; in addition, the arrangement was tenderly blended by reversal while waiting for the white string approximating features of DNA frame noticeable mass.

10. Centrifugation was directed at 13000-14000 rpm for 1 minute at room temperature; the DNA was obvious as a little white pellet.

11. The supernatant was emptied, and one specimen volume of room temperature 70% Ethanol was added to the DNA. The tube was delicately modified little periods to wash-down the pellet of nucleic acid and the adjacent of the smaller scale rotator tube. Centrifugation as is specified in step 10.

12. The Ethanol was deliberately suctioned utilizing a micropipette .The DNA pellet was free by then; along these lines, care must be taken to abstain from suctioning the pellet into the pipette. The tube was upset on clean retentive paper and air-dried the pellet for around 10-15 minutes.

13. The DNA Rehydration Solution (100 l) was added to the tube and the DNA was rehydrated by bringing forth its at 65°C for an hour. Irregularly, the game plan was mixed by tapping the tube carefully. Then again, the DNA was rehydrated by hatching the arrangement overnight at room temperature or at 4 oC.

14. DNA was put away in a cooler at -20 oC.
The Estimation of DNA Concentration and Purity:

The DNA concentration of samples was estimated by using the Nano drop by putting 2.5μl of the extracted DNA in the machine to detect concentration in ng/μL and the purity detected by noticing the ratio of optical density (OD) 260/280 nm to detect the contamination of samples with protein. The accepted 260/280 ratio for purifying DNA was between 1.7-1.9 [10].

Electrophoresis of Agarose Gel:

Agarose gel electrophoresis was embraced to affirm the nearness and uprightness of the separated DNA after genomic DNA extraction in 1.5% agarose with 100 V for 10 min. [9].

Gel Electrophoresis Reagents:

- Powder of Agarose
- TBE Buffer with 1X concentration
- Loading dye
- Ethidium Bromide
- DNA Ladder Marker

Protocol of Gel Electrophoresis:

Tris Borate EDTA Buffer preparation (1X TBE)

This solution was prepared by adding 900 ml Distill water to 100 ml 10X TBE (Promega/ Germany) ,forming 1 liter of( 1x) TBE buffer [9].

Preparation of agarose gel:

1- The amount of 1 X TBE (100 ml) was taken in a beaker
2- Agarose powder (1.5 gm) was added to the buffer
3- The solution was heated to boiling using a microwave oven for 2 min.
4-Ethidium Bromide (1 l) of (10mg/ml) was added to the agarose solution.
5- The agarose was stirred in order to be mix and avoid making bubbles.
6- The solution was left to cool down at 50 – 60 oC.

DNA Loading & Electrophoresis:

DNA (3 l) was mixed with (2 l) loading dye. The samples loaded carefully into the individual wells of the gel, and then electrical power was turned on at 70 volt for 1 hour, afterwards the DNA moved from cathode (-) to anode (+) poles. The Ethidium Bromide stained bands in the gel were visualized using UV. Transilluminator at 350 nm and photographed.

PCR Technique:
In this study, conventional PCR was used to detect virus presence by using one primer as shown in the following table. The primer was supplied by Bioneer (Korea) Organization as a lyophilized result of various picomols fixations. Lyophilized preliminary was disintegrated in a free DNase/RNase water to give a final concentration of 100 pmol/l and kept as a stock in -20°C, to prepare 10 M concentration as work primer re suspended 10 pmol/l in 90 l of free DNase/RNase to reach a final concentration 10 M.

Amplification of EBV-LMP1
EBV-LMP1-DNA sequences were amplified by Polymerase Chain Reaction (PCR): Forward: 5’CGGAAGAGGTTGAAACAAA’
Reverse: “5’GTGGGGGTCGTCATCATCTC3. [11].

Table 1: Detection of EBV Virus by PCR

| Step       | Temperature | Time/min | Cycles |
|------------|-------------|----------|--------|
| Initial denaturation | 94          | 5        | 1      |
| Denaturation | 94          | 1        | 35     |
| Annealing   | 55          | 1        | 35     |
| Extension   | 72          | 2        | 35     |
| Final extension | 72         | 7        | 1      |
| Storage     | 4           |          |        |

Virus identification using ELISA kit
The ELISA method applies to the in vitro quantitative determination of EBV. The procedure was done as manufacture company leaflet "Biotechnology Co., Ltd (Eabscience)

Results
Twenty-three from thirty samples are positive for EBV association with leukemia patients by PCR technique.

The results of current study revealed gene (161bp) of Epstein–Bar virus in leukemia patients (figure 1)

Figure (1) Detection of Epstein–Bar virus in leukemia patients by polymerase chain reaction technique.
Viral identification; Detection of EBV by ELISA

Twenty three from thirty samples are positive for EBV association with leukemia patients by ELISA technique.

In the present study, EBV contamination, as characterized by the nearness of particular EBV IgM antibodies in the serum was measurably fundamentally connected with the improvement of leukemia. The consequences of this study uncovered a relative relationship between EBV serum levels and the unending leukemia.

Discussion

EBV turned into the prime case of a human tumor infection that is etiologically connected to an assorted scope of malignancies. EBV has been connected with an assortment of lymphoid and epithelial malignancies [12].

Leukemia, as various tumors, results from physical changes in the DNA. Certain progressions produce leukemia by inciting oncogenes or deactivating tumor silencer qualities, and thusly disquieting the bearing of cell end, partition or division.

These progressions may happen all of a sudden or as an outcome of prologue to radiation or malignancy bringing about substances, and are inclined to be affected by inherited segments [13].

Among adults, the known causes are typical and fake ionizing radiation, several contaminations, for instance, Human T-lymphotropic disease, Epstein-Bar contamination, Cytomegalovirus and a couple of chemicals, prominently benzene and alkylating chemotherapy masters for past malignancies [14]. The ingenuity of EBV and its relationship to tumorigenesis was set up [15, 16].

Three main pieces of evidence have been used to show, separately or in conjunction with each other, that EBV must have been present in tumor progenitor cells before their malignant conversion. First, in some rare instances, EBV has actually been demonstrated in pre-malignant lesions related to a specific cancer [17] showing that infection is an early event in the development of the minor. Second, all malignant cells within a tumor contain EBV DNA Third; the EBV DNA represents a homogeneous episomal population demonstrating that the malignancy is likely a clonal development of a solitary EBV-infected ancestor cell [18].

References

[1] Maeda E, Akahane M, Kiryu S, (2009). "Spectrum of Epstein–Barr virus-related diseases: a pictorial review". Jpn J Radiol. 27 (1): 4–19.
[2] Cherry-Peppers, G; Daniels, CO; Meeks, V; Sanders, CF; Reznik, D (2003). "Oral manifestations in the era of HAART". Journal of the National Medical Association. 95 (2 Suppl 2): 21S–32S.
[3] Amon, Wolfgang; Farrell (2004). "Reactivation of Epstein–Barr virus from latency". Reviews in Medical Virology. 15 (3): 149–56
[4] Odumade, O. A.; Hogquist, Balfour (January 2011). "Progress and Problems in Understanding and Managing Primary Epstein–Barr Virus Infections". American Society for Microbiology. 24 (1): 193–209
[5] Toussirot E, Roudier J (October 2008). "Epstein–Barr virus in autoimmune diseases". Best Practice & Research. Clinical Rheumatology. 22 (5): 883–96
[6] About Epstein–Barr Virus (EBV. Centers for Disease Control and Prevention, 14 Sept. 2016. Web. 23 Oct. 2016
[7] CDC. "Epstein–Barr Virus and Infectious Mononucleosis". CDC. Retrieved 2011-12-29
[8] Sambrook J and Maniatis T. (1989) Molecular cloning: a laboratory manual. Second edition. New York: Cold Spring Harbor Laboratory Press.
[9] Sambrook J and Rusell (2001).Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
[10] Hussain Gadelkarim Ahmed, Shakir Idris Osman and Ibraheem M. Ashankty (2012). Incidence of Epstein-Barr Virus in Pediatric Leukemia in the Sudan.
[11] L. F. Lopes; M. M. Bacchi; D. Elgui-de-Oliveira; S. G. Zanati; M. Alvarenga and C. E. Bacchi (2004).Epstein-Barr virus infection and gastric carcinoma in Sao Paulo State, Brazil, Brazilian Journal of Medical and Biological Research, vol. 37, no. 11, pp. 1707–1712.
[12] Lesty C; Baudet S and Charlotte F (2010). A study of bone marrow neoangiogenesis in chronic lymphocytic leukemia patients. Anal Quant Cytol Histol 32: 11-23.
[13] Leonard and Barry (1998). Leukemia: A Research Report. DIANE Publishing 14.
[14] Thorley-Lawson, D. A. and A. Gross. (2004). Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med 350:1328-37.
[15] Cader FZ; Kearns P and Young L (2010). The contribution of the Epstein-Barr virus to the pathogenesis of childhood lymphomas. Cancer Treat Rev 36:348- 53.
[16] Pathmanathan R.; Prasad U.; Sadler R.; Flynn K.; and Raab-Traub N. (1995). Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. N. Engl. J. Med. 333, 693–698.
[17] Quoted in Laurent Degos; John Hughes Bennett; Rudolph Virchow; Raab-Traub N. and Flynn K. (1986). The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. Cell 47, 883–889.