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LATENT PROTEIN GENE SITE IN EPSTEIN BARR VIRUS (EBV) AND ITS DELETION

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ABSTRACT

In the present study, samples which were diagnosed as nasopharyngeal carcinoma, larynx carcinoma, Burkitt lympoma, hodgkin and nonhodgkin lympoma were investigated in terms of latent protein gene site in Epstein Barr Virus (EBV).
After DNA isolation from those samples, polimerase chain reaction was carried out at LMP (Latent Membrane Protein) gen sequences. Amplified samples were subjected restriction enzyme (Xhol, Msp I) analysis.
We were not observed any EBV-Xho-I variants. Heterodoublex formation was determined in one of the hodgkin samples. Deletion was observed at LMP 2 sequence in 11 of the same samples.

1. INTRODUCTION

The Epstein Barr Virus (EVB) is the first herpes virus whose genome has been completely cloned and which has been sequentially analyzed. The EBV infects the epithelial cells in vivo epithelial cells in vivo and the B lympocytes in vitro.

The EBV genome exhibits a linear structure and is composed of a DNA stand which has a double helix and which includes 172.000 base pairs. The EBV genome has two parts. The first one of these is the one which includes 15.000 base pairs; the second part includes 150.000 base pairs. There are some repeating sites in the second part (Bear et al., 1984).

Only a small part of the 80-100 genes that exist in the latent period of the EBV genome is expressed. This double helical DNA strand is covered with a protein sheath which is composed of 160 amino acids (Heller et al., 1982).

The EBV types are related to latent infections genes. Accordingly, two types of EBV have been defined as EBV-1 and EBV-2.

Apart from these, in a study which analyzed the EBV localization in nasopharyngeal carcinoma tumors in South China, the f variant of EBV has been defined. In that study, the polymerase chain reaction has been used and the f variant has been demonstrated by using the extra BamH I restriction site (Lung et al., 1992). Another variant is the EBV LMP-XhoI variant. As a result of the substitution of T for G in nucleotide no. 169 in exon 1 of the LMP-I gene, there occurs a dissection site for XhoI variant. Apart from the above-mentioned EBV types, there are some other EBV variants which occur during the laboratory studies.
In the latent period, the number of LMP mRNAs synthesized in the infected cells is ten times more than of the EBNA mRNAs synthesized. In the EBV genome of LMP, a gene labeled as BNF-1 is encoded. This gene exhibits an integral protein structure, with a molecular weight of 62-kD. The LMP gene is a viral oncogene. 30% of the cytoplasmic LMP is included in the membrane structure in the cytoplasm. The LMP of the Epstein-Barr virus has a short half-life (Baichwal and Sugden, 1987). The EBV –immortalized lodges itself to the membrane in B lymphocytes and becomes a part of the intracellular skeletal system by being phosphorized from serine amino acid residue (Liebowitz et al., 1987). The LMP localizes differently in the membrane and exhibits a ligand-binding property. In the infected B cells, it is localized to the plasmic membrane by the vimentin protein. The latent membrane protein activates two events in the cell; First, the viral protein causes a morphologic transformation and leads to development of tumor, second, it localizes in the cell membrane. The carboxy-terminal part of the LMP is the essential element of this transformation. It has been shown that the phenotypes of LMP lymphocyte cells has been changed by the B lymphocytes in cell cultures (Wang et al., 1988). The expression of LMP leads to increases in the level of intracellular free calcium, the membranous folding, the production of cell-surface CD23 receptor, the size of the cell and the number of LFA-1, LFA-3, ICAM-1 adhesion molecules. The increase in the number of these molecules is important, because the adhesion of B lympocytes facilitates the cellular development and, consequently, the development of tumor. In addition, it increases the contact inhibition (Wang et al., 1988).

A mutation (deletion or insertion) in BNLF-1 gene causes the EBV genome to be very aggressive. The mutant LMP is more stable in structure and does not become a part of the intracellular cytoplamic skeletal system (Wang et al., 1987).
The deletions which take part in this region are important for the definition of EBV types.

The main purpose of this research is to investigate the existance of any deletion in the gen region of latent membrane protein in Epstein Barr virus.

2. MATERIALS AND METHODS

Various tumor tissues have been used in this study. Nasopharyngeal carcinoma, larynx carcinoma, Burktit's lymphoma, Hodgkin and non-hodgnin lymphoma and the following samples have been used as materials:

- Blood samples (lymphocyte)
- Biopsy materials (fresh tissue)
- DNA isolation from cell cultures
- Tissue samples embedded in paraffin blocks and cross-sections mounted on slide.

2.1. DNA Isolation

2.1.1. DNA Isolation from Blood Samples (Lymphocyte)

The blood samples that had been taken into heparinized injectors were decomposed into mononuclear cells by being sread onto Histopaque 1077 and then being centrifuged at 1350 rpm for 30 min. Proteinase K with a final cancentration of 100 xx 10% lauryl sulfate with a final concentration of 0,5% and STE solutions (0.1 M NaCI; 0.05M Tris Base: 1 mM EDTA) were added to the mononuclear cell samples. This new mixture was kept under 37^0C for overnight and then by using the phenol-chloroform/ethanol method, the mononuclear cells were isolated (Maniatis et all., 1989a).

2.1.2. DNA Isolation from Biopsy Material (Fresh Tissue)

The tumor tissues, after being pulled to pieces with a sterile scalpel so that it has a diameter of 0.5 cm, were transferred into a sterile tube, mixed with 500 xx sterile water and stirred in tissue homogenizer at a speed of 120000 rpm. After that, Proteinase K (sigma) with a final concentration of 250 µg / mg ; 10% lauryl sulfate with a final concentration of 0.5% and STE solution (0.1 M Na C1; 0.05 M Trisma chloride, pH 7.5; 1mM EDTA, pH 7.4) with a total volume of 2 ml were added to the cells obtained from the above-mentioned mixture (Mainiatis et al., 1989b).

The new mixture was kept under 37^0C for overnight. On the following day, phenol (phenol-chloroform-isoamyl alcohol) with a volume equal to the total volume of the mixture was added to it. The mixture was shaken for 10 min., embedded in ice for 10 min. And centrifuged at $+4^0$C at a speed of 5000 rpm for 2.5 minutes. It was then taken into a supernatant tube, and 2 M sodium acetate that was 10% of the amount of the mixture was added to it; 95% ethanol which was twice as much the new amount was then added.

The prepared solution was kept at -20^0C for one night. After it was precipitated by centrifugation at 400 rpm, it was washed with 70% ethanol DNA was solved in mM THE (Tris, EDTA) solution by being kept at 37^0C for overnight.

2.2 Polymerase Chain Reaction

The LMP-1 and LMP-2 sites in EBV genome were amplified by polymerase chain reaction. Each of these sites has a concentration of 300 pg/ml and specific primers have been used for each site. In addition, by using dATP, dCTP, dGTP, dTTP (promega); Taq DNA polymerase (Promega) and 100 mM Tris-HCI, 200 mM KC1, 15 mM MgC1 and 0.01% gelatin, the required mixture for polymerase chain reaction has been obtained. Two drops of mineral is added to the mixture before PCR is activated (Saiki et al., 1988).

The PCR conditions for the gene site encoding EBV latent membrane protein is given in Table 2.1.

Table 2.1 PCR conditions

Gene Site	First Loop	Adhesion Heat	Last Loop	Number of Loops
LMP-2	94^0C/4 min	60^0C/2 min	72^0C/2 min	35
LMP-2	94^0C/4 min	60^0C/2 min	72^0C/2 min	35

The amplified products were controlled by using 4% agarose gel electrophoresis or 9% polyacrilamid gel electrophoresis (PAGE) of the PCR.

2.2.1. Agorose Gel Electrophoresis

Agarose was so weighed as to obtain the desired percentrage, and distilled water and Tris-Borat-EDTA (TBE) 5X obtained solution which was from Tris-Borat-EDTA (TBE) IX solution was added to it. 54 g. Of Tris base, 27.5 g. of boric acid, 0.5 m 20 ml EDTA was prepared and compeled to 1 it. With deionized water. This solution was added on to the agarose, and 6 µl ethidium bromide was added. It was kept in a microwave oven for 5 minutes. In the meantime, it was stirred for 5 or 6 times. The agarose was poured onto the electrophoresis plate. 30-40 minutes had to elapse for the freezing of agarose, and then the samples were loaded. The power

supply (Pharmacia) was so adjusted as to be 0.5 V/cm. 50 minutes had to elapse fo rthe forming of bands on the gel. After that, the anticipated bands were photographed under ultraviolet light (Spectroline) (Maniatis et al., 1989c).

2.2.2. Polyacrilamid Gel Electrophoresis (PAGE)

First, a 30% acrylamide (29 g acrylamide: 1 g bisacrylamide/ 100 ml) solution was used as a stock solution (Maniatis et al., 1989d). Polyacrilamid gel was shaken in the beaker by adding the desired amount of TBE 5x (tris-borad-EDTA) buffer, ammonium Per sulfate and TEMED, and was poured between two previously prepared glasses before it polymerized. After time has elapsed for the freesing of the gel, a 200 V current was applied to homogenize the gel. The samples were loaded were loaded and were photographed after 4-6 hours, during which a 150 V current was applied, had elapsed for the forming of the bands.

2.6. Restriction Enzyme Analysis

In this analysis, the determination of LMP was made by using the XhoI enzyme. It was treated with XhoI 10U/ml (Promega) enzyme by being taken from the amplified LMP samples. Buffer D was used as the buffer. The samples underwent agarose gel electrophoresis after they were kept at 37^0C for 24 hours.

Msp I (Biolabs) enzyme was used as a second enzyme. It was treated with Msp I enzyme by being taken from the amplified LMP samples. The buffer of the enzyme was Buffer 2. After the samples were kept at 37^0C for 24 hours. They underwent agarose gel electrophoresis.

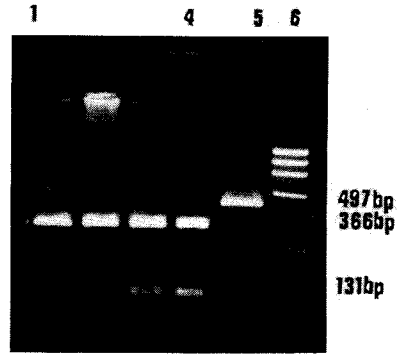

Figure 3.1. Determination of LMP-XhoI variant in EBV with PCR
Line 1-4 PCR product cut with Xho-I
Line 5 Uncut PCR product
Line 6 OX 174 Hae III (Marker)

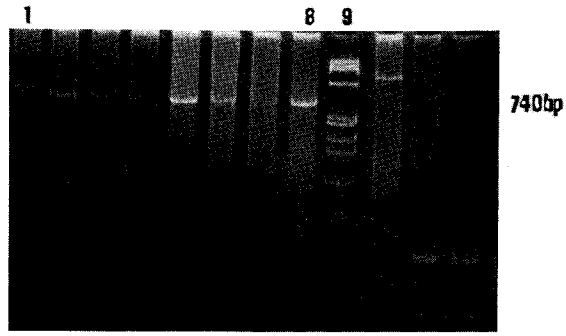

Figure 3.2. Non-observation of heterodublex band with PAGE in LMP-2
Line 1-8 Samples of PCR product
Line 9 OX 174 Hae III (Marker)

When these samples were analyzed with 12% PAGE, heteroduplex formation, as in the second line had occured (Fig 3.3).

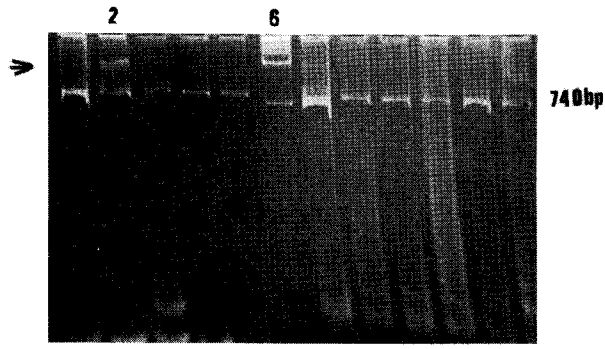

Figure 3.3. 12% PAGE heteroduplex formation in EBV-LMP-2
Line 2 heteroduplex formation
Line 6 OX 174 Hae III (Marker)

There seperate bands in each of the four columns were observed when LMP-2 site was agin multiplied and samples treated with MspI enzyme were run in 8% PAGE gel (Fig. 3.4.).

A site that includes 240 base pairs from LMP-2 was multiplied and treated with MspI enzyme (Fig. 3.5).

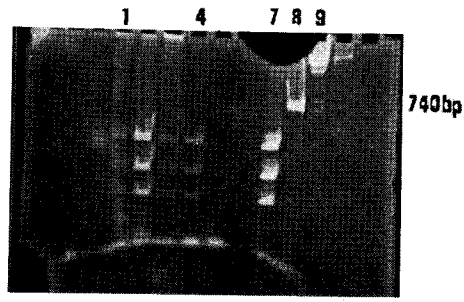

Figure 3.4. Treament of EBV-LMP-2 site with MspI enzyme
Line 1-4 cut sample
Line 7 Cut sample
Line 8 PCR product not treated with the enzyme
Line 9 OX 174 Hae III (Marker)

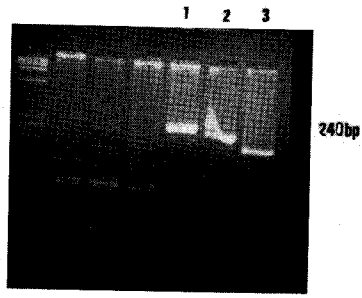

Figure 3.5. Deletions in bands with were obtained by the cutting of the 240 base pair-site in LMP-2 with MspI
Line 1 B 958
Line 2 AG 876
Line Patient sample

In our studies on the patient samples in Turkey, we did not detect the XhoI variant that was defined in China (Hu and et al., 1991). LMP-2 deletion is shown in 11 of the 15 samples analyzed.

3. RESULTS

The tumor samples that had EBV were analyzed in terms of LMP/XhoI polymorphism and LMP second site deletions. It was reported that, of the 15 samples with hodgkin lymphoma only, 11 had a possibility of deletion. The other

samples were found to have no deletions. Seven Burkitt samples, 10 nasophorynx samples, 20 nondodgkin samples and 18 samples with hodgkin lymphoma were analyzed, and in none of these ssample groups was LMP/XhoI polymorphism detected (Fig 3.1). The heteroduplex formation was observed in the PCR product of LMP-2 site (Fig. 3.2.).

4. DISCUSSIONS

The former studies suggest that there was no correlation between the EBV type and the malign Iymphoma, and that it had the property of forming tumors on the basis of EBV-LMP-DEL immunodeficiency. However, in this study, there is deletion in EBV of the samples with hodgkin. Knecht et al., (1993) reported that there are deletions in LMP-1 site in both hodgkin and nasopharyngeal carcinoma. There were deletions only in the hodgkin group of samples that we took from the LMP-2 site of EBV. Knecht (1993) has established that the frequency of mutational points in LMP-1 plays a role in EBV-related Iymphogenesis and that this point has a clinaical improtance.

Hu et al. (1991) has reported that the EBV type responsible for the nasopharyngeal carcinoma in China was EBV-LMP1-XhoI variant and that it was widespread in China. However, we did not detect the EBV-LMP1-XhoI variant an our samples. Therefore, another type of EBV might be responsible for the nasopharygeal carcinoma.

Apart from these EBV types, Hamid et al. (1992) has defined the EBV type Mediterranean variant, and Lung (1992) has defined the BamHI-F/f variant.
These two variants have not been searched thoroughly yet in Turkey.

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