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A Preliminary Study on the Molecular Identification of Herpes Simplex Virus Type 1 in Iranian Population

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Abstract

Herpes Simplex Virus Type 1 (HSV-1) is a member of the Herpesviridae family that causes herpetic disease in human. During the last 25-30 years, many investigations have been conducted on pathogenesis and molecular biology of this virus in several countries. In Iran HSV-1 has been isolated from patients and detected using immunological techniques. In this study, we investigated the molecular aspects of two Iranian isolated viruses by PCR-RFLP of HSV-1 DNA Polymerase gene. The result of Southern blot hybridization indicated that the Iranian isolates are HSV type 1. Moreover, the restriction pattern of the Iranian isolates is different from the KOS strain for *Dpn*I enzyme. It is proposed to use this difference as a molecular marker of the Iranian strain.

Keywords: Herpes simplex virus; Polymorphism; PCR-RFLP; DNAP Gene

Introduction

Herpes simplex type 1 is an infectious agent widespread in human population [1]. It is the first human herpes viruses to be discovered and among the most intensively investigated of all viruses [2]. With rapid replication and selection by the host, genetic variation of a virus is common. Features of the genomic variation differ among viruses. Viruses that require close contact for transmission between individuals, for example herpes viruses, tend to be divided into geographically isolated genotypes. HSV-1 strains from geographically separate countries or anthropologically different races have distinct pattern of restriction endonuclease (RE) cleavage, termed Restriction Fragment Length Polymorphism (RFLP), mostly due to gain or loss of an RE cleavage site. The RFLP is stable and serves as a physical marker of the HSV-1 genome [3].

The HSV-1 genome is a linear, double strand DNA of 152 kb that contains three origins of DNA replication and approximately 75 open reading frame (ORF). Of these ORFs, seven including *pol* gene (UL30) encode proteins that are required for origin-specific DNA replication. [4,10]. Some mutations in *pol* gene renders the virus resistant to Acyclovir (an antiviral drug) [5]. For the past 25-30 years, many investigations have been directed towards comparisons of RFLP patterns amongst HSV-1 strains in many countries including Japan and consequently, two predominant HSV-1 genotypes, F1 and F35, have been elucidated.

Further studies in Japan have enabled efficient detection and identification of HSV-1 strains using RFLP markers specific for the F35 genotype. This approach has also facilitated attempts to associate

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genotypes with clinical manifestations and identify HSV-1 strains that have been derived from a common ancestor [1,3,6].

Moreover, other investigations in Japan showed that sequence analysis of the HSV-1 thymidine kinase (TK) gene, on the basis of its polymorphism, was an easy and effective method which was comparable to genome RFLP analysis for the identification and discrimination of HSV-1 strains [7].

DNA variability in sequence among HSV-1 strains has been employed in the field to trace transmission of the virus in hospitals and other communities and to determine whether infection was resulted from reactivation of latent virus or from reinfection with exogenous virus [8].

In Iran, the study of HSV genome has not, so far, made use of molecular approaches. This research attempts to study the molecular aspects of HSV-1 isolates from Iranian patients using Southern blot hybridization and PCR-RFLP analysis of HSV-1 *pol* gene.

Materials and Methods

Viruses

Iranian isolates were prepared at the Virology Department of TMU, from seropositive patients using HSV-1 specific antibody.

DNA Extraction

HSV-1 DNA was prepared from Vero cells infected with HSV-1, as described below.

Vero cells, a continuous African green monkey kidney cell line, were grown as monolayer at 37°C in Dulbecco's Modified Eagle Medium (DMEM) containing 5% fetal calf serum. The cells were then infected with HSV-1 at multiplicity of infection of 0.1 per cell and incubated in DMEM at 37°C until the virus-infected cells showed 80% cytopathic effect. The culture media and the cells which were scraped from the plate were transferred to centrifuge tubes, and frozen in liquid nitrogen. After three freeze-thaw cycles, the cell debris were pelleted and discarded. Then, 1 µl/m1 DNase (5 mg/m1) was added to the supernatant for 1 h at 37°C. The viruses were precipitated by addition of 0.1 g/m1 polyethylene glycol (average molecular weight, 6000 Daltons) and NaCl (0.5 M) after standing for 2 h at 4°C. The precipitate was pelleted at 4000 g for 1 h. The pellet containing virus particles was suspended in lysis buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8, 0.6% w/v SDS) and incubated for 30 min at 37°C. The lysate was extracted with phenol and phenol/chloroform, and the viral DNA was precipitated with ethanol [9,10].

Southern Blot Hybridization

Southern hybridization was carried out as described [11]. Briefly, 5 µg of total viral DNA from one of the isolates was electrophoresed in a 1% agarose gel for 12 h. The gel was treated with 0.2 M HCl for 10 min and then with a solution of 0.5 M NaOH and 1.5 M NaCl for one h at room temperature. Subsequently, it was subjected to 0.5 M Tris HCl pH 8) and 1.5 M NaCl for one h at room temperature. The nitrocellulose filter was prewashed in 1xSSC and the contents of the gel were transferred on to the nitrocellulose filter overnight. The filter was washed in 2xSSC the next day and air-dried.

The DNA fragment used as probe was prepared from pNN2 plasmid [10] containing *pol* gene of HSV-1 KOS strain. This plasmid was constructed by cloning the *pol* gene in pUC18 plasmid as previously described [10]. The probe was labeled using DIG DNA labeling and Detection kit and the hybridization was carried out according to the manufacturer's instructions [11].

Selection of Oligonucleotide Primers

The primers were designed for the 5′ and the 3′ end of *pol* gene (3730 bp) using DNASIS software and were prepared by Faza Pazhouh Co., Tehran, Iran.

The sequence of forward primer was 5′-AACAACCGCGATGTTTTCCG-3′ and reverse primer was 5′-ATGAAGGGACATCAGCTTCG-3′.

PCR Condition for Amplification from HSV-1 DNA

DNA amplification was carried out in a 50 µl reaction mixture containing 1 µl of sample, 5 µl PCR buffer (containing 1.5 µM $MgCl_2$), 10 µl of dNTP (mixture of dTTP, dCTP, dGTP, dATP), 2.5 µl of each primer (20 µM), 5 µl GC rich solution (Cinnagen Co., Tehran, Iran) and 3.5 units of Taq Polymerase (Cinnagen Co.).

The reaction mixture then underwent 1 cycle of initial denaturation at 94°C for 8 min, 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, and extension at 72°C for 4 min, followed by a final single 10 min extension at 72°C [12].

Analysis of PCR Products

After PCR, 7 µl of the reaction mixture was subjected to electrophoresis in a 1% agarose gel and

examined by ultraviolet fluorescence of the ethidium bromide stained gel. λ DNA digested with *Hind* III was used as a molecular weight standard to identify the presence of the 3730 bp product.

Restriction Endonuclease (RE) Digestions

Several RE have been found suitable for the analysis of the PCR products, because they cleave the DNA into a relatively small number of fragments that can be separated by electrophoresis according to size. The REs used in this study were *Eco*RI, X*ho*I, *Hae*III, *Hinf* I and *Dpn*I (Roche, Germany).

Cleavage of Viral DNA with RE

PCR products (<1 µg) were incubated with 4 units of the appropriate RE in the presence of 2 µl enzyme-specific buffer (10x) and 1 µl BSA (Sigma, USA) for 8 h at 37°C.

Polyacrylamide Gel Electrophoresis

The digests of PCR products (KOS and Iranian isolates) were loaded on to 8% polyacrylamide gel and electrophoresed and stained with silver nitrate.

Results

The result of Southern blot hybridization using a total of 5 µg extracted viral DNA from one of the Iranian isolates and probed with pNN2 plasmid carrying the HSV-1 *pol* gene is depicted in Figure 1 (lane 2). A positive control DNA (pNN2, 1 µg) has been included in lane 1 and a negative control, pUC19 DNA, in lane 3.

After molecular identification of the Iranian isolate by Southern blot hybridization, HSV-1 *pol* gene was amplified by PCR for RFLP studies (Fig. 2). The absence of 3730 bp fragment in lane 2 indicated lack of contamination in the reaction mixtures. This 3730 fragment was apparent in other lanes (3-6) by ethidium bromide staining, indicating PCR amplification of HSV-1 *pol* gene. The digestion pattern for the *pol* gene in the Iranian isolate and the KOS strain following treatment with the four restriction endonucleases is shown in Figures 3-7. Results in Figures 3, 4, 5 and 6 indicate that the position of RE sites and fragments for *Eco*RI, *Xho*I, *Hae*III and *Hinf* I, respectively, in *pol* gene of HSV-1 KOS strain and the Iranian isolates are similar. It is to be noted that in digestions with *DpnI*, both isolates are used (Fig. 6). *Dpn*I restriction digestion (Fig. 7) indicates that the position of RE site in *pol* gene of the Iranian isolates and the KOS strain are different. On the polyacrylamide gel, DNA from the Iranian isolates digested with *Dpn*I show a fragment approximately 3400 bp while digestion from KOS strain DNA produced two bands of approximately 1850 and 1450 bp in length. Therefore, the difference in the *Dpn*I recognition site is evident between KOS strain and the Iranian isolates.

Data in Figure 8 reconfirms *Dpn*I map of KOS strain in the agarose gel stained with ethidium bromide.

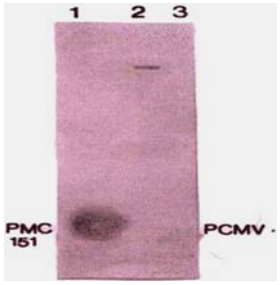

Figure 1. Southern blot hybridization. 1-Positive control; 2-Total viral DNA; 3-Negative control. See text for details.

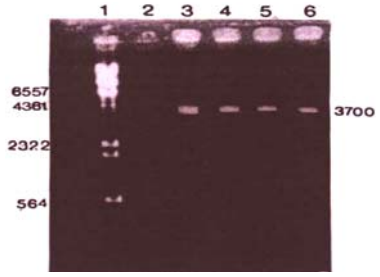

Figure 2. Agarose gel for PCR products: Marker (λ*Hind*III). 2-Negative control (PCR reaction mixture without DNA); 3-PCR product of Iranian isolate no. 1; 4-PCR product of Iranian Isolated virus 2; 5-PCR product of pNN2 plasmid (for testing the primer); 6-PCR product of KOS strain.

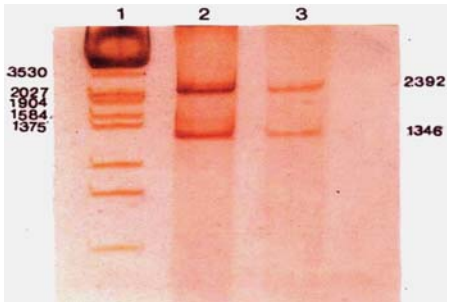

Figure 3. *Eco*RI map: It was used for comparison KOS and Iranian isolated virus. 1-Marker; 2-Iranian isolated virus; 3-KOS strain.

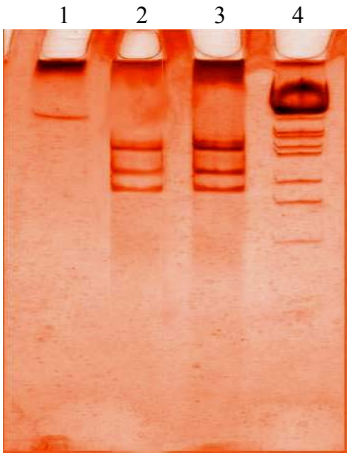

Figure 4. *Xho*I map: 1-No digested DNA (as a control); 2-KOS strain; 3-Iranian isolated virus; 4-Marker.

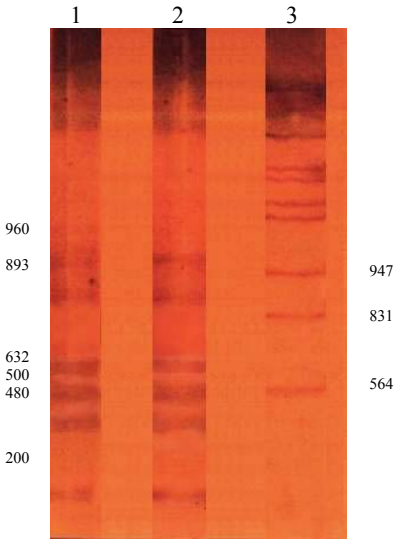

Figure 5. *Hinf* I map, 1- Iranian isolated virus. 2-KOS strain. 3-Marker.

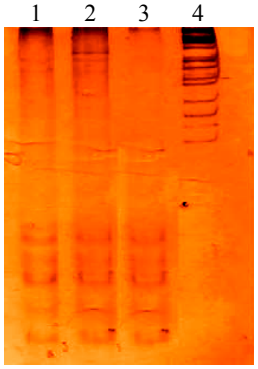

Figure 6. *Hae*III map, 1-Iranian isolated virus No.1; 2-Iranian Isolated virus No.2; 3-KOS strain; 4-Marker.

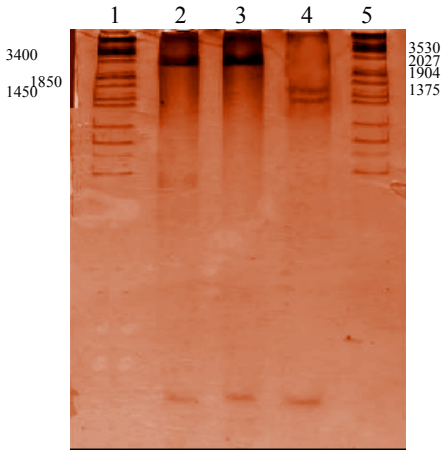

Figure 7. *Dpn*I map, 1-Marker; 2-Iranian isolated virus No.1; 3-Iranian isolated virus No.2; 4-KOS strain; 5-Marker.

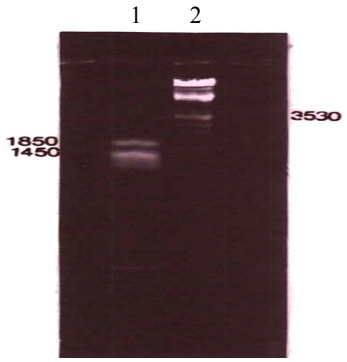

Figure 8. Agarose gel for *Dnp*I *m*ap: 1-KOS strain; 2-Marker.

Discussion

This study was aimed at analyzing the molecular identity of two Iranian HSV-1 isolates using Southern blot hybridization and PCR-RFLP for HSV-1 DNA *pol* gene.

The extracted viral DNA from the Iranian isolate showed homology with a plasmid containing HSV-1 KOS strain *pol* gene thus confirming the identity of the isolates as herpes simplex type 1 virus. Several studies have examined genomic variation in HSV-1 by the RFLP analysis [3]. However, the RFLP assay of the genome is relatively difficult and cumbersome as it requires identification of numerous DNA fragments that range in size from a few hundred base pairs to over 10 kb and must be analyzed on a single gel [7]. We considered PCR-RFLP for the *pol* gene of HSV-1 an easier method for molecular study [4].

Also, a similar molecular epidemiological technique, by which the vaccine strain of VZV can easily be distinguished from the circulation wild-type strains, was developed to accurately evaluate the protective efficacy of varicella vaccine. In the present study, RFLPs of DNA fragments covering ORF 1-37 of the VZV genome which had not been previously studied, was examined. A new genetic marker in ORF6, specific for the Oka vaccine strain, was established by PCR-RFLP analysis of ORF 6 [15].

Other investigators reported that the acquisition of three restriction sites in ORF 62 of VZV is specific for the Oka vaccine strain, and RFLP methods using these loci as markers enabled differentiation of the Oka strain from the Oka parent and other wild-type strains [16-18].

The molecular study of the *pol* gene was selected because Acyclovir interacts specifically as well as sequentially with the proteins encoded by *tk* gene and the *pol* gene [13] in which some groups of single point mutations exist that confer resistance to Acyclovir [5]. Furthermore, analysis of the nucleotide sequence of these genes in serially isolated HSV-1 virus from patients could reveal the presence of an Acyclovir-resistant mutant and its origin [5].

The validity of the *pol* gene selection by our group is reconfirmed by the difference in the *Dpn*I restriction pattern between the two Iranian isolates and the European KOS strain.

If this difference can also be found in other Iranian isolates, it can be used as a molecular epidemiological marker to distinguish the Iranian genotype.

Moreover, large-scale sequencing of the *pol* from clinical isolates can be utilized to monitor the transmit-ssion of Acyclovir-resistant mutants among individuals.

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