Restriction Enzyme Analysis of Thymidine Kinase Gene in Four Iranian Isolates of Herpes Simplex Virus Type 1

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Summary

The thymidine kinase (TK) gene of four Iranian herpes simplex virus-1 isolates was amplified and the electrophoretic patterns of the gene were compared after digestion with six restriction endonucleases including *Apa*I, *Ava*I, *Bgl*I, *Dra*II, *Eco*RV and *Pst*I. DN8A of each virus was first extracted separately by lysis buffer and phenol/chloroform method. Their TK genes were amplified using a pair of special primers, which were designed by Gene 8Runner software. The polymerase chain reaction product of each TK gene was digested with the restriction enzymes separately and electrophoresed in polyacrylamide gel. The results indicate that there was no difference among the electrophoretic patterns of the isolates TK gene.

Key words: herpes simplex virus-1, RFLP, thymidine kinase

Introduction

Herpes simplex virus type 1 (HSV-1) is a member of *Herpesviridae* family and is one of the most contagious agents, which infect human being (Handler *et al* 1996). The virus usually infects above the waistline, causes vesicles on the face and mucosal epithelium of mouth and nose, pharyngitis, keratitis and encephalitis. This

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virus is especially dangerous in immunocompermized patient and may cause fatal disease in them (Zuckerman 2000, Fenner *et al* 1994). Antiviral compounds are effective in shortening the duration of disease and often used as therapeutic and prophylactic agents (Pramod *et al* 2000, Gaudreaw *et al* 1998).

The thymidine kinase (TK) gene encoded by HSV-1 plays an important role in pathogenesis of the virus at least in antiviral models, because TK defective mutants are not able to reactivate as efficiently as the wild type virus. Furthermore it is also an important target for certain antiviral drugs, which are nucleoside analogs and is one of the key enzymes in the determination of susceptibility to acyclovir that is widely used for the treatment of HSV infections.. Mutant viruses without TK activity or with altered TK activity can become drug resistant. Based on this property the TK mutagenesis assay has been developed and applied to isolate TK mutants (Qiaosheng *et al* 2002). Previous studies have shown that the HSV-1 TK gene is a relatively highly polymorphic gene and the frequency of nucleotide substitution per 1kb of the TK gene is higher than the other virus genes (Nagamine *et al* 2000).

In this study we used restriction fragment length polymorphism (RFLP) technique that is based on the presence or absence of a restriction site in the polynucleotide sequence. At first, we amplified the entire sequence of the TK gene of each HSV-1 isolate and then evaluated the presence of any probable mutation in each gene by RFLP.

Materials and Methods

Cell culture. Established bovine kidney (BK) cell line (Khedmati *et al* 2001), a gift from Dr. Khedmati (Razi Institute) were used. The cells were grown in DMEM supplemented with 5-10% heat inactivated calf serum. After a complete and confluent monolayer of the cells was formed, they were passaged up to ten times. These cells could be kept for 30 days at 4°C.

Virus isolation and propagation. Samples were collected from vesicle fluid of the patients who referred to the virology lab during winter 2000. Each fluid sample was collected by a swab, which was clearly moisted by cell culture medium containing antibiotics and then centrifuged at 3000rpm for 20min at 4°C. The supernatant was then incubated onto the prewashed Hela cells and incubated at 37°C for 1h. Then the maintenance medium was added. All inoculated cultures were examined for the presence of CPEs daily. Any isolated virus was tested with monoclonal antibody against HSV-1 and the positive ones were identified and put separate at -70°C. All four isolated viruses as well as standard KOS strain of HSV-1 were propagated further in BK cell line. In order to do this, cells were inoculated with 0.1MOI (multiplicity of infection) of each virus separately. After obtaining 70% CPEs each inoculated cell culture was harvested and kept at -70°C for further steps.

Extraction of viral DNA. The harvested viruses were frozen and thawed several times. The virus suspension was then centrifuged at 4000rpm for 60min at 4°C. 1µg/ml of DNase was added to the virus suspension before being concentrated by 8%(w/v) PEG 6000. The DNA of the virus was isolated using lysis buffer (Tris-HCl 10mM, EDTA 12mM, NaCl 10mM, SDS 1.2%, proteinase K 250µg/ml) and phenol-chloroform method. The extracted DNA was electrophoresed in 0.8% agarose gel.

Polymeras chain reaction (PCR). A pair of primer was designed to amplify the TK gene of each virus. To use the proper primers the nucleotide sequence of the TK gene was obtained from Gene Bank. A 1.3Kb fragment encoding the gene of interest was amplified by PCR using the following primers: forward, 5GCC TTG TAG AAG CGC GAT TG3 and reverse, 5'CTT CGC TGT TTC AGT TAG CCT C3'. The primers were designed based on information obtained from Gene Runner software. The PCR reaction was carried out in 0.5ml microcentrifuge tube with a final volume of 25µl. The reaction solution contained 50mM KCl, 20mM Tris HCl (pH8.4), 1.5mM3 MgCl$_2$ and 0.2mM dNTPs. Both primers were used in 0.5pmol/ml concentration. About 0.5-1µg/µl of isolated DNA was added to each microtube. 1-

1.5unit of *Taq* DNA polymerase(Rosche, Switzerland) was added and the tubes were overlayed with a drop of mineral oil to prevent of evaporation. The amplification program consisted of 30 cycles. Reaction mixture were placed in a thermal cycler programmed for a three steps protocol, 5min at 94°C for one cycler (first denaturation) and then for 30 cycle at 94°C for 50sec (denaturation), 62°C for 1min (annealing) and 72°C for 1min (extension). The final extension step was allowed to continue for 5min. Then PCR product of TK gene was runed in 1% agarose gel containing 0.1mg/ml ethidium bromide in TBE buffer at 60 volt for about 50-60min. Standard molecular weight marker was used for each gel with rang of 100 to 3000bp. The gels were than visualized under UV light.

Digestion of the amplified DNA. A total of 6 restriction enzymes including *Apa*I, *Ava*I, *Bgl*I, *Dra*II, *Eco*RV and *Pst*I were used to digest the amplified DNA from each of the virus isolates as well as the standard virus strain. A mix with total volume of 20µl was prepared for each PCR product. Each mix contained 1µl of the amplified DNA, 1µl of one of the above enzymes, 2µl of 10X special buffer and 16µl DDW. Each mix was incubated at 37°C for 6h and then electrophoresed in 10% polyacrylamide gel.

Electrophoresis of the digested DNAs. All digested DNA were electrophoresed in 10% polyacrylamide gel. The standard molecular weight marker was used for each gel from range 100 to 3000bp. Sample with bromophenol blue was loaded onto gels. The gels were run in TBE buffer at room temperature for about 2h at 100 volts until the dye reached the end of the gels. Then gels were stained in 0.1% $AgNO_3$ in deionised water.

Results

Figure 1a shows the result of PCR of the TK gene of the KOS strain and isolates number 1-3. PCR product of TK gene of each isolate as well as KOS strain were electrophoresed in 10% polyacrylamide and their results are shown in figure 1b.
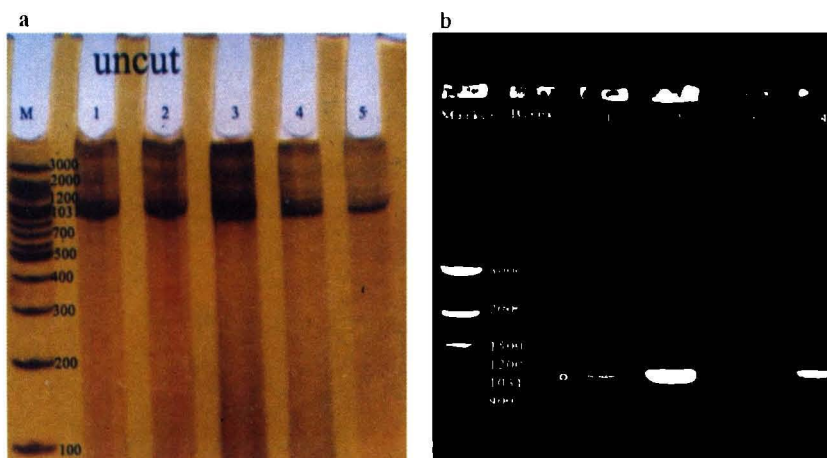
Figure 1. *a) Result of PCR of the TK gene of KOS strain and three other isolates in 1% of agarose gel. Lanes 1-3 are related to isolates 1-3 and lane 4 belong to KOS standard strain. b) Results of PCR products of isolates and KOS strai8n in 10% polyacrylamide gel. Lanes 1-4 belong to isolates 1-4 and lane 5 is related to KOS standard strain*

Table 1 shows the restriction enzymes used to digest the PCR product of each virus isolate. Each enzyme has it own site of activity resulting in production of certain fragments.

Table 1. *The restriction enzymes used to digest th8e PCR product of the TK gene of each HSV-1 isolate*

Enzyme	No. of restriction site	Restriction site	Length of fragment
*Apa*I	3	GGGCC/C	638, 259, 217, 44
*Ava*I	3	C/YCGRG	640, 261, 223, 35
*Bgl*I	2	GCCNNNN/NGGC	579, 432, 147
*Dra*II	2	RG/GNCCY	634, 262, 262
*Eco*RV	2	GAT/ATC	806, 245, 104
*Pst*I	1	CTGCA/G	766, 392

Results of the electrophoresis in 10% polyacrylamide gel of each PCR product related to TK gene are shown in figure 2 (a to f). As indicated in the figures there are4 (638nt., 217nt., 259nt. and 44nt.), 4 (640nt., 261nt., 223nt. and 35nt.), 3 (579nt., 432nt. and 147nt.), 3 (634nt. and 2×262nt.), 3 (804nt., 248nt. and 104nt.) and 2

(766nt. and 392nt.) fragments obtained from digestion of the PCR products with *Apa*I, *Ava*I, *Bgl*I, *Dra*II, *Eco*RV and *Pst*I, respectively. A very important point to be mentioned here is that the TK gene of each isolate had the same restriction sites for the enzym8s used which best correlated with the standard strain virus.

Discussion

Herpes simplex virus infections are treated with acyclovir or other sorts of
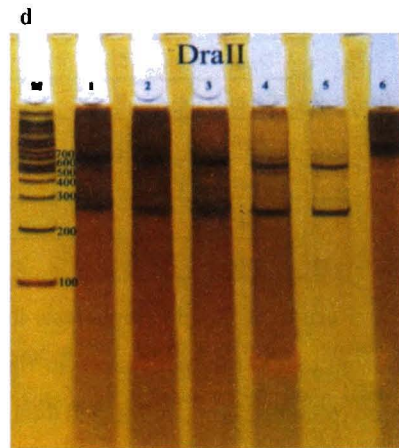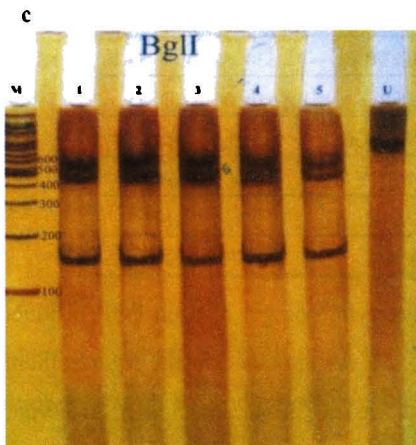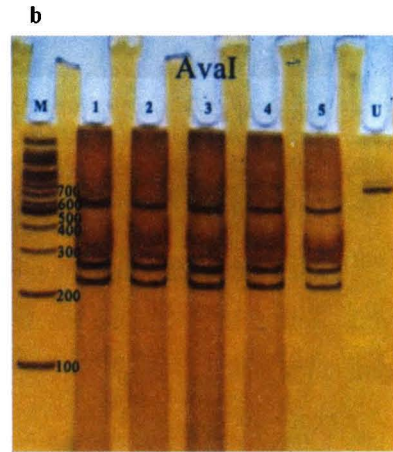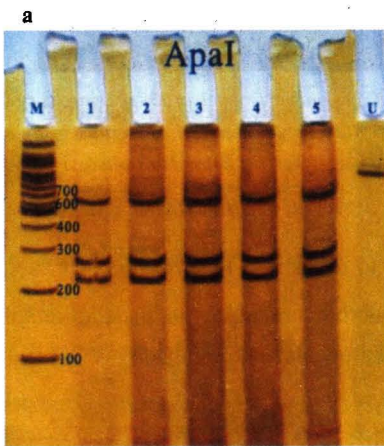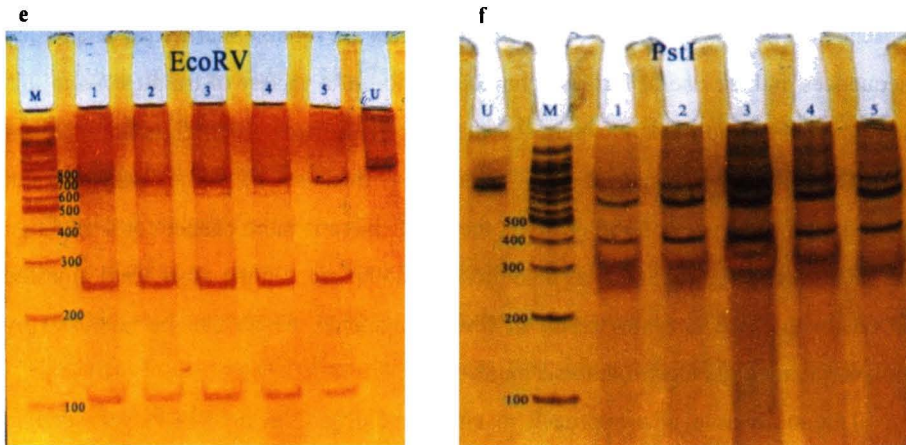
Figure 2. *Result of electrophoresis in 10% polyacrylamide gel of PCR products of the gene digested with Apal (a), Aval (b), Bgl I(c), DraII (d), EcoRV (e) and PstI (f). M, DNA marker, Lanes 1-4 are related to viruses 1-4 and lane 5 belong to standard virus (KOS). U, undigested DNA*

analogs. Several mutant strains, which are resistant to antiviral therapy, have emerged due to widespread application of such drugs. One of the main reasons for such resistance is related to mutation of thymidine kinase gene. Analysis of the nucleotide sequence of the TK gene in serial isolates from a patient could reveal the 2appearance of an acyclovir resistant mutant (Kudo *et al* 1998, Pramode *et al* 2000). The aim of this study was to detect the possible sequence polymorphism in TK gene of four Iranian HSV-1 isolates.

There are several methods to study to the probable mutation in gene specially in TK gene, for example sequencing (Nagamine *et al* 2000), denaturing gradient gel electrophoresis (DGGE) (Qiaosheng *et al* 2002), single strand conformation polymorphism (SSCP) (Kudo *et al* 1998), Rnase-A mismatch and RFLP (Sakaoka *et al* 1994, Umene *et al* 1991 and 1994, Roizman *et al* 1983 and 1979), which the last was used in this study. An ideal analysis for genomic polymorphism would be to co9mpare nucleotide sequence data directly. However, this approach is labour intensive and cumbersome and it is not practical for a large number of isolates. Our comparison among the isolates was performed on the basis of the presence or

absence of the restriction sites, because these sites are reliable marker for nucleotide sequences and are useful for easy characterization of a variety of isolates. So in this approach the amplified TK gene of the HSV-1 isolates were digested with six restriction endonuclease enzymes separately. Because of these enzymes cut palindrome sequences of 6bp or more, therefore, there is more chance for a mutation to occur in such restriction sites than those with 4bp are. On the other hand digested DNAs with these enzymes result in fewer fragments, which can be analyzed by polyacrylamide gel electrophoresis much easier.

This study is a new approach to analyze TK gene using RFLP technique in different HSV-1 isolates and indicate that there is no difference in RFLP of the TK gene of the isolates with the restriction endonuclease enzymes. One method to compare the nucleotide sequence of this gene in different isolates is sequenceing the whole gene. Parts of this gene have sequenced (Nagamine *et al* 2000) the whole nucleotide sequence of that has not sequenced. The next project is sequenceing the entire nucleotide sequence of TK gene of all the HSV-1 isolates to study the likely difference between them.

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