AMPLIFICATION AND SEQUENCING OF VARICELLA-ZOSTER VIRUS (VZV) GENE 4: POINT MUTATION IN A VZV STRAIN CAUSING CHICKENPOX DURING PREGNANCY

V.T.K. CHOW, K.P. LIM

Department of Microbiology, Faculty of Medicine, National University of Singapore, Kent Ridge, Singapore 119260, Republic of Singapore

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Summary. – The varicella-zoster virus (VZV) causes chickenpox (varicella) as the primary disease and shingles (zoster) as a recurrent manifestation of infection, both being generally benign and self-limiting. While these infections may be severe in adults and even life-threatening in immunosuppressed individuals, they may be amenable to effective antiviral drugs or varicella-zoster immune globulin, provided the treatment is administered early. The prompt diagnosis of VZV infections may be accelerated by rapid, sensitive and specific molecular techniques such as amplification by polymerase chain reaction (PCR), compared with slower and more cumbersome tissue culture and serological procedures. Based on the VZV gene 4 which encodes a transcriptional activator, primers were designed for use in PCR to amplify a target fragment of 381 bp. Distinct diagnostic bands were observed by agarose gel electrophoresis of PCR products of VZV strains isolated from 11 varicella and 7 zoster patients in Singapore, as well as of the Japanese vaccine Oka strain. The detection sensitivity of this PCR assay was determined to be 1 pg of purified VZV DNA equivalent to about 7,000 viral DNA copies. No target bands were amplified from negative control templates from five related human herpesviruses and from human DNA. The specificity of the PCR products was ensured by direct cycle DNA sequencing, which revealed complete identity of the 18 VZV isolates with the published European Dumas strain. The strong sequence conservation of the target fragment renders this PCR assay highly reliable for detecting the VZV sequence. Only one VZV strain isolated from a patient with varicella during pregnancy exhibited a GGA to GAA point mutation at codon 46 of gene 4, culminating in the non-conservative substitution of Ser with Phe. The predicted secondary structure of the mutant polypeptide portrayed a radical alteration, which may influence its function in transcriptional activation.

Key words: varicella-zoster virus; gene 4; polymerase chain reaction; sequencing; mutation

Introduction

VZV is one of the human pathogenic viruses belonging to the family Herpesviridae. The virus has a linear, double-stranded DNA genome of approximately 125 kbp, the genome of the Dumas strain having been completely sequenced (Davidson and Scott, 1986; Ostrove, 1990). VZV is the causative agent of primary varicella and zoster, the latter being a secondary reactivation of a latent VZV infection. While these clinical conditions are often benign and self-limiting in the immunologically competent host, varicella or herpes zoster may be serious in adults and even life-threatening in immunocompromised patients (Weller, 1992). Effective antiviral therapies are available, which should ideally be administered early in the infection process, e.g. acyclovir, related antiviral agents and varicella-zoster immune globulin for severe VZV infections in immunosuppressed hosts (Brunell, 1991; Wallace et al.,

Abbreviations: aa = amino acid; CMV = cytomegalovirus; CPE = cytopathic effect; EBV = Epstein-Barr virus; EDTA = ethylenediamine tetraacetate; HSV-1, HSV-2 = herpes simplex virus type 1,2; IE = immediate early; nt = nucleotide; ORF = open reading frame; PCR = polymerase chain reaction; SDS = sodium dodecyl sulphate; VZV = varicella-zoster virus
1992; Saltzman et al., 1994). In addition, a prompt recognition of viral infection allows measures to be taken to minimize further viral transmission, e.g. patient counselling and health education.

Clearly there is a need for more rapid, specific and sensitive techniques to improve the conventional methods of viral diagnosis. The molecular procedures of nucleic acid hybridization with gene probes, and PCR technology are increasingly being applied to microbial diagnosis, including viral detection (Chow et al., 1989). The PCR amplification technique has the important attributes of sensitivity, specificity, speed and simplicity. In this study, primers targeting VZV gene 4 (which encodes a transcriptional activator) were employed for DNA amplification to detect the presence of VZV DNA, and for direct cycle DNA sequencing of the PCR products.

Materials and Methods

DNA preparation from viral isolates. The characterized VZV strains included varicella isolates which represented “new” strains, while zoster isolates exemplified “old” VZV strains according to the Hope-Simpson model which asserts that VZV remains latent in an individual following chickenpox, but may reactivate later in life resulting in zoster (Croen and Straus, 1991). VZV strains were propagated in human embryo lung MRC5 cells grown in Eagle’s Minimal Essential Medium supplemented with 10% foetal calf serum for 3-4 days or until 90% cytopathic effect (CPE) was observed. Cell-associated VZV DNAs of isolates obtained from 9 varicella and 4 zoster patients between 1990 and 1991 (Chow et al., 1993a), and from the vaccine Oka strain (Takahashi et al., 1974) were extracted from infected cells by a modified method of Straus et al. (1981). In addition, DNAs from cells infected with VZV strains derived from 2 varicella and 3 zoster patients in 1994 – 1995, were individually prepared using 0.3 mol/l sodium acetate, 0.5% sodium dodecyl sulphate (SDS), 5 mmol/l ethylenediaminetetraacetate (EDTA) pH 7.0 buffer, phenol/chloroform/isoamylalcohol extraction and ethanol precipitation. DNAs of other related human herpesviruses and of the human SiHa cervical carcinoma cell line served as negative controls. DNAs were isolated also from cells infected with herpes simplex virus types 1 and 2 (HSV-1, HSV-2), and cytomegalovirus (CMV), from lymphoid B95-8 cells which express Epstein-Barr virus (EBV), and from lymphocytes infected with the Hashimoto strain of human herpesvirus type 6.

PCR amplification. Based on VZV gene 4, two oligonucleotide primers, VZV4U (5'-ATGCGTGCATATCCAGTCTC-3') and VZV4D (5'-TACAGGCAACTGCAAACACG-3'), were designed and their sequences screened in the GenBank database to exclude high homology with known gene sequences of primate or other viruses. VZV4U (nt 3804-3823) and VZV4D (nt 4184-4165) primers flank a 381 bp target fragment which encodes codons 1-112 at the amino terminus of the gene 4 product (Davidson and Scott, 1986). One ng of each DNA preparation including negative controls was subjected to PCR. The sensitivity of VZV PCR was evaluated by amplifying serial ten-fold dilutions of purified VZV DNA ranging from 1 ng to 1 fg. The PCR was performed in a 50 µl volume containing 1 x PCR buffer, 4 dNTPs (0.2 mmol/l each), primers (0.2 µmol/l each), Taq DNA polymerase (1.25 U), and DNA sample. The reaction mixture was subjected to an initial denaturation of 95°C for 1 min, and to 30 thermal cycles each consisting of 95°C for 0.5 min, 62°C for 0.5 min and 72°C for 1 min. Each PCR product (10 µl) was electrophoresed in 2% agarose gels stained with ethidium bromide and visualized on an UV-transilluminator. Stringent pre- and post-PCR laboratory procedures and equipment were adopted to prevent cross-contamination.

Cycle sequencing and computer analysis. The PCR products were purified for sequencing by chloroform/ether extraction and polyethylene glycol/NaCl precipitation (Chow et al., 1993b). Direct sequencing was carried out with [32P]-end-labelled VZV4U and VZV4D primers using a dSNA Cycle Dideoxy Sequencing kit (Gibco BRL) through 20 thermal cycles of linear amplification. The secondary structures of polypeptides were predicted according to the model of Rose (1978) using the PROSIS computer software program (Hitachi).

Results

PCR amplification of all the 19 VZV DNA samples from 11 varicella and 7 zoster patients, and of the vaccine Oka strain yielded distinct bands of diagnostic size of 381 bp, clearly scoring as positive for VZV DNA (Fig. 1). Visible diagnostic bands were observed after agarose gel electrophoresis of PCR products from small quantities of VZV DNA (1 ng to 1 pg), whilst no target bands were obtained for all the negative control samples (Fig. 2).

Cycle sequencing of the VZV gene 4 PCR products of 18 VZV strains revealed 100% homology with the 5’ to 3’ rightward DNA sequence of the prototype Dumas strain. An exception was strain 1242V which exhibited a G to A mutation at nt 4005 within codon 46 of gene 4 which has a leftward coding orientation of the deduced aa sequence (Davidson and Scott, 1986). As confirmed by direct sequencing repeated four times, this point mutation resulted in a non-conservative substitution of Ser (an aa with an uncharged polar side chain) by Phe (a hydrophobic aromatic aa), as illustrated in Fig. 3. A comparison of the predicted protein secondary structure of the prototype versus mutant VZV gene 4 products indicated a drastic change in the structure of the mutant polypeptide in which β-sheets and a turn replaced a stretch of coils in the prototype (Fig. 4).

Discussion

The clinical diagnosis of VZV infections is often relatively obvious, but may occasionally be confused by other vesicular eruptions, e.g. herpes simplex. Conventional methods for the laboratory diagnosis of VZV infections include tissue cultures, and immunological detection of viral antigens and antibodies. While these methods have proven use-
ful, they suffer from certain disadvantages. A tissue culture is cumbersome and time-consuming, the interpretation of CPE is sometimes subjective and lacks type-specificity unless combined with indirect immunofluorescent identification (Nahass et al., 1992). Although a definitive serological diagnosis can be achieved by observing a four-fold or greater increase in antibody titer between an acute and convalescent serum sample taken about 10-14 days apart, such an approach is slow. Furthermore, infected individuals may be negative for viral antibodies during the “window period” between viral acquisition and seroconversion.

The PCR assay using VZV gene 4 primers described in this study could detect VZV DNA from varicella and zoster cases, and from the unrelated vaccine Oka strain with a high detection sensitivity of 1 pg of VZV DNA equivalent to approximately 7,000 viral genome copies. Furthermore, the specificity of the amplification reaction was validated by the absence of target bands specific of other related members of
the herpesvirus family and human cellular DNA. A conventional PCR may be combined with other complementary techniques to enhance its sensitivity and specificity, or for epidemiological and evolutionary analyses of viral diseases. Such techniques may include Southern blot analysis of PCR products and internal oligonucleotide hybridization, oligomer restriction (Kúdelová et al., 1996), restriction fragment length polymorphism, and DNA sequencing. The authenticity of the PCR products of genomes of all the VZV strains tested was verified by cycle sequencing which revealed complete homology with the published Dumas strain sequence, with exception of strain 1242V. The highly conserved nature of gene 4 among different local VZV strains, as well as of the Dumas and Oka strains, attests to the reliability of these gene 4 primers for the amplification of even geographically and temporally distant VZV strains.

The exquisite sensitivity of PCR is potentially one of its greatest drawbacks in view of the danger of false positives arising from contamination with extraneous or "carryover" DNA. Notwithstanding this, rigorous quality control
measures (Kitchin and Bootman, 1993) can be instituted to avoid this apparent limitation, e.g. valid negative and positive controls in every batch of PCR samples for trustworthy results, and mandatory repetition of PCR for doubtful cases. The absence of a diagnostic band may be interpreted as a negative result for VZV DNA or as a failure of amplification ("dropout"). To exclude the latter possibility, an additional pair of primers should be included in the PCR mixture to co-amplify a conserved human gene, e.g. β-actin or ras gene (Tham et al., 1991).

The application of the PCR assay under study to the molecular diagnosis of VZV infections may be realized by subjecting appropriate clinical samples such as vesicular fluid or throat swabs to DNA extraction and PCR procedures. Other researchers have exploited PCR for the detection of various VZV genes (Kido et al., 1991; Koropchak et al., 1991; Dlugosch et al., 1991; Puchhammer-Stokl et al., 1991; Ito et al., 1995).

The VZV immediate early (IE) gene encoding open reading frame (ORF) 4 comprises 1356 nt encoding a protein product of 452 aa with a predicted Mr of 51,540. This protein possesses an aa sequence similarity of 28% with the IE trans-regulatory gene product ICP27 of HSV-1. This similarity is especially marked at their Cys- and His-rich carboxyl termini (Defechereux et al., 1993; Moriuchi et al., 1994; Perrera et al., 1994). Although their amino termini display a limited aa homology, both are highly acidic, contribute to transactivation of target genes, substitute for each other in transient expression assays and are functionally homologous (Moriuchi et al., 1995). The indispensable ORF4 encodes an essential protein that appears to be a direct transcriptional transactivator which can stimulate transcription of target genes involved in the IE phase of viral infection (Inchauspe et al., 1989; Inchauspe and Ostrove, 1989; Kinchington et al., 1994). Furthermore, Kinchington et al. (1995) have demonstrated that the ORF4 protein is associated with purified virus particles, most likely as a part of the tegument. This structural localization presumably enables the protein to fulfil its transcriptional regulatory function during the IE events of infection.
The complete identity of the gene 4 nt and deduced aa sequences of the 17 Singapore VZV strains and the Japanese vaccine Oka strain with those of the prototype European Dumas strain indicates that the amino terminus of the gene 4 product is extremely conserved and implies a vital role. It is thus particularly noteworthy that only a solitary VZV strain harboured a point mutation leading to a non-conservative aa replacement, and consequently to a radical alteration in the predicted secondary structure and possibly in the activity of the protein. Interestingly enough, this mutant strain was isolated from a patient who contracted varicella during pregnancy. While the degree of virulence of this mutant virus and the significance of its causative effect during pregnancy remains unclear, varicella is generally considered to be more severe in pregnant women (Brunell, 1992; Chapman and Duff, 1993; Dave et al., 1995) who may transmit the infection to the foetus and may incur a relatively small risk of foetal abnormalities (Pastuszak et al., 1994).

Singapore has been experiencing an upsurge of varicella since 1984. In 1990 to 1996, 18,934, 17,930, 29,976, 43,876, 39,558, 23,224 and 49,763 cases were reported yearly out of a population of approximately 3 million. This epidemic could be attributed to a low level of herd immunity and/or the emergence of novel strains of VZV. A seroepidemiological survey conducted between 1989 and 1990 revealed that only 43% of the cohort had anti-VZV IgG antibodies, with only 14% of those below 15 years of age being seropositive (Ooi et al., 1992), confirming the insufficient herd protection. The restriction endonuclease profiles of genomes of representative VZV strains from 9 varicella and 4 zoster patients were essentially identical, suggesting that the chronic epidemic of varicella in Singapore most likely stems from closely related VZV genotypes infecting a susceptible population with inadequate herd immunity. However, the Sfl-D, Sfl-E and XbaI-I fragments exhibited significant size variations between the Singapore and Dumas genotypes of VZV, which represent geographic VZV variants.

Nt and/or deduced aa sequence analyses of heterogenous genes also represent discriminatory tools for studying genomic diversity among viral isolates. Epidemiologically distinct viral strains can be differentiated by their unique sequences, i.e. those from the same cluster have identical or similar sequences, which are conspicuously different among strains from epidemiologically unrelated clusters. Such strategies have been exploited in the molecular epidemiology of other double-stranded DNA viruses, such as CMV (major IE gene, Brytting et al. (1992)) and human papillomavirus type 16 (long control region (Ho et al., 1991)). Dissimilarities between viral strains can emerge even when phenotypically silent mutations occur within conserved genes, e.g. the non-structural 3 gene of dengue viruses with RNA genomes resulting in different nt sequences (Chow et al., 1993b, 1994, 1997). DNA sequencing of the highly conserved gene 4 fragments of VZV strains would be insufficiently discerning for molecular epidemiological research. The elucidation of nt disparities by PCR and direct sequencing of a polymorphic region of the VZV genome, such as the inverted repeat sequenc- es, may permit a finer discrimination of VZV genotypes (Chow et al., 1992).

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References


