J Korean Med Sci 2000; 15: 442-8
ISSN 1011-8934

Detection of Human Herpesvirus 8 DNA in Pemphigus and Chronic Blistering Skin Diseases

Increased incidences of Kaposi's sarcoma and lymphoid malignancies have been observed in patients with pemphigus, and the human herpesvirus 8 (HHV-8) is very strongly associated with these tumors. Because the virus may be one of the triggering factors of pemphigus, we undertook this study to screen for the presence of HHV-8 in chronic blistering skin diseases including pemphigus. A total of 45 paraffin-embedded specimens were studied using nested polymerase chain reaction (PCR) with primers to amplify a 160-base pair HHV-8 fragment. HHV-8 DNA could be detected in 7 of 9 patients with pemphigus vulagris, and 1 of 2 with pemphigus foliaceus. All specimens of other blistering skin diseases were negative for HHV-8. On sequencing PCR products, the sequences were almost identical with the prototypic sequence for HHV-8, and a few base- pair substitutions at 1086C→T and 1139A→C were detected. The results of our study suggests that HHV-8 might have trophism for pemphigus lesions. Further studies including comparison of HHV-8 DNA load in both lesional and normal skin in the same patient, serological and animal studies would be helpful to study the relationship between HHV-8 and pemphigus.

Key Words: *Herpesvirus, Kaposi Sarcoma-Associated; Polymerase Chain Reaction; Pemphigus; Blister*

Ho-Sun Jang, Chang-Keun Oh, Jae-Young Lim, Eun-Sook Jun*, Yu-Sun Kim, Kyung-Sool Kwon

Department of Dermatology, College of Medicine, Pusan National University, Busan, Korea
Medical Research Institute*, Pusan National University Hospital, Busan, Korea

Received: 2 May 2000
Accepted: 2 June 2000

Address for correspondence
Ho-Sun Jang, M.D.
Department of Dermatology, Pusan National University Hospital, 1-10, Ami-dong, Seo-gu, Busan 602-739, Korea
Tel: +82.51-240-7337, Fax: +82.51-245-9467
E-mail: hsjang@hyowon.cc.pusan.ac.kr

*This work was supported by the Medical Research Institute Grant from Pusan National University Hospital (1999).

INTRODUCTION

Human herpesvirus 8 (HHV-8) is a new herpesvirus first found in the tissue of acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma (KS) (1). Also, numerous other studies have confirmed the presence of HHV-8 in all four clinical forms of KS and in various geographical regions (1-3). Subsequent reports have also implicated HHV-8 in the development of premalignant or malignant skin tumors in immunosuppressed organ-transplant patients (4) and immunocompetent patients (5), body cavity-based lymphomas (6), multicentric Castleman's disease (7), and other lymphomas (8, 9).

Pemphigus is an autoimmune blistering skin disease mediated by antibodies against the autoantigens desmoglein 3 and desmoglein 1. An increased incidence of malignancies, especially KS and lymphoid malignancies including Castleman's disease, has been observed in patients with pemphigus (10, 11). These tumors were strongly associated with HHV-8, and the virus is one of a variety of exogenous factors triggering the lesions of pemphigus (12). The possible etiologic role of viral infections in the development and/or exacerbation of pemphigus has been reported in the literature (12-17). Recently, the presence of HHV-8 DNA was found in the lesional skin of pemphigus vulgaris and pemphigus foliaceus (18, 19). These observations raised the possibility that HHV-8 is involved in a broader range of skin lesions besides skin tumors and might be related to the pathogenesis of pemphigus. In order to assess whether the virus is associated with pemphigus and other blistering diseases, we looked for the presence of HHV-8 DNA in patients with pemphigus vulgaris, pemphigus foliaceus, bullous pemphigoid, epidermolysis bullosa acquisita, dermatitis herpetiformis, and Hailey-Hailey disease.

MATERIALS AND METHODS

Study specimens

Twelve biopsy specimens from 9 pemphigus vulgaris, 3 from 2 pemphigus foliaceus, 5 bullous pemphigoid, 3 epidermolysis bullosa acquisita, 4 dermatitis herpetiformis, and 7 Hailey-Hailey disease patients with no clinical evidence of human immunodeficiency virus (HIV) infec-

tion were included in the study. These specimens were formalin fixed and paraffin embedded. The diagnosis was confirmed both histologically and by direct immunofluorescence tests. To differentiate bullous pemphigoid and epidermolysis bullosa acquisita, direct immunofluorescence test using salt-splited normal skin of patients and electron microscopy were also performed. All of them did not have cutaneous KS and lymphoid malignancies. They were not previously treated with immunosuppressive therapy, including steroids, cyclophosphamide, cyclosporin, and azathioprine. One AIDS-associated KS lesion served as positive control, and for negative control, 3 biopsy samples from herpes simplex virus skin infection, 3 psoriasis, and 5 normal skin were used to assess the specificity of PCR for HHV-8.

Polymerase chain reaction (PCR) amplification

Five sections (10 μM) were cut from the paraffin blocks, and DNA was extracted by proteolytic digestion. After deparaffinization twice with 1 mL xylene, the tissues were pelleted at 13,000 rpm, resuspended twice in 500 μL of absolute ethanol, pelleted again and dried. The tissues were then resuspended in 200 μL of 5% chelex® 100 DNA extraction reagent (Perkin-Elmer, U.S.A.) and incubated at 100℃ in a heat block for 30 min. The samples were then centrifuged at 13,000 rpm for 5 min and the supernatant was used as a DNA template in the PCR process. The DNA concentration and purity were assessed by reading the absorbance at 260 and 280 nm, using a Ultrospec 2000 UV/Visible spectrophotometer (Pharmacia, NJ, U.S.A.). First round PCR for HHV-8 DNA was done with the primers KS1 and KS2 for the 233-bp $KS330_{233}$-fragment of the HHV-8 (1). PCR product was obtained in reaction buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 5 μL of genomic DNA, 50 pmol of each primer, 200 μM each of dNTP, and 2.5 U of AmpliTag Polymerase (Perkin Elmer, CA, U.S.A.) per reaction mixture at a final volume of 50 μL. The conditions for PCR analysis were as follows: initial denaturation at 94℃ for 2 min (1 cycle), denaturation at 94℃ for 1 min, annealing at 58℃ for 1 min, extension at 72℃ for 2 min (40 cycles), and final extension at 72℃ for 5 min using an GeneAmp PCR system 2400 (Perkin Elmer, CA, U.S.A.). Second round PCR was also done with nested primers using primers NS1 and NS2, amplifying a 160-bp fragment internal to the KS1/KS2 product (20). In this analysis, 5 μL of first round PCR products was added to the PCR mixtures containing primers NS1 and NS2, and amplified as described above. After the last cycle, 10 μL of reaction mixture was separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and photographed.

Table 1. Sequence of the oligodeoxynucleotides used in the PCR

Primer	Deoxynucleotides sequence (5'-3')
KS1	AGC-CGA-AAG-GAT-TCC-ACC-AT
KS2	TCC-GTG-TTG-TCT-ACG-TCC-AG
NS1	ACG-GAT-TTG-ACC-CCG-TGT-TC
NS2	AAT-GAC-ACA-TTG-GTG-GTA-TA
PCO3	ACA-CAA-CTG-TGT-TCA-CTA-GC
PCO4	CAA-CTT-CAT-CCA-CGT-TCA-CC

The human β-globin gene DNA was also amplified with primers producing a 110-bp amplifier (PCO3, PCO4), under the same conditions described above. It was used as a positive internal control to demonstrate the integrity of the DNA preparations and the absence of PCR inhibitors (Table 1) (21). All primers were purified by PAGE and purchased from MicroNet Co. (Daejeon, Korea).

Direct sequencing of PCR products

The amplification products were purified from 2% agarose gel. The UV-illuminated DNA bands of interest (160 bp HHV-8 DNA fragment) were cut out with disposable scalpels and placed in DNA-collection tubes. The DNA fragments were recovered using the QIAquick Gel Extraction Kit (QIAGEN, Inc., CA, U.S.A.) according to the manufacturer's instruction and quantified by measuring absorption at 260 nm. The fragments were directly sequenced by cycle sequencing using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, CA, U.S.A.). For each reaction, 90 ng DNA were used as a template with 3.2 pmol forward nested primer (NS1) and 8 μL terminator ready reaction mix (A-, C-, G-, and T-Dye Terminator, dNTP, AmpliTag DNA polymerase, $MgCl_2$, Tris-HCl buffer, pH 9.0). The sequencing PCR, denaturation at 96℃ for 10 sec, annealing at 50℃ for 5 sec, and extension at 60℃ for 4 min (25 cycles), as well as extraction of its products by phenol chloroform and the preparation of the samples were carried out following the kit protocol. Finally, the samples were loaded onto the ABI PRISM 377 DNA Sequencer (Perkin-Elmer, CA, U.S.A.) according to the manufactures' instructions.

RESULTS

Result of PCR

A total of 45 clinical samples were analyzed for the presence of HHV-8 DNA. After first round PCR, none

Table 2. Result of PCR and direct sequencing in patients with pemphigus vulgaris and pemphigus foliaceus

Case No./ Age (yr)/Sex	Site	Diagnosis	Previous IST†	β-globin	HHV-8 DNA 160 base pair	DNA sequence variations	Amino acid substitutions
1/64/F	Shin	PV*	-	+	+	1086 C→T	Ala → Val
						1139 A→C	Lys → Gln
2/36/F	Back	PV	-	+	+	1082 G→C	Ala → Pro
						1086 C→T	Ala → Val
						1139 A→C	Lys → Gln
3a/41/F	Back	PV	-	+	+	1086 C→T	Ala → Val
						1130 G→T	Gly → Trp
						1139 A→C	Lys → Gln
3b	Back	PV	-	+	+	1086 C→T	Ala → Val
						1130 G→T	Gly → Trp
						1139 A→C	Lys → Gln
4/33/F	Back	PV	-	+	+	1086 C→T	Ala → Val
						1139 A→C	Lys → Gln
5a/44/M	Shoulder	PV	-	+	+	1060 A→C	Isocoding
						1139 A→C	Lys → Gln
						1187 G→C	Als → Pro
5b	Back (NS†)	PV	-	+	-		
6/60/F	Flank	PV	-	+	-		
7a/38/F	Abdomen	PV	-	+	-		
7b	Back	PV	-	+	+	1086 C→T	Ala → Val
						1139 A→C	Lys → Gln
8/53/M	Back	PV	-	+	-		
9/30/F	Back	PV	-	+	+	1086 C→T	Ala → Val
						1139 A→C	Lys → Gln
10/30/F	Arm	PF	-	+	-		
11a/62/M	Back	PF	-	+	+	1077 C→T	Ser → Phe
						1086 C→T	Ala → Val
11b/62/M	Chest	PF	-	+	-	1139 A→C	Lys → Gln
12/43/M	Neck	KS	-	+	+	1033 C→T	Pro → Leu

*PV, pemphigus vulgaris; PF, pemphigus foliaceus; BP, bullous pemphigoid; EBA, epidermolysis bullosa acquisita; KS, Kaposi sarcoma
†IST, immunosuppressive therapy; ‡NS, normal-appearing skin

of the 45 specimens were positive for 233-bp sized HHV-8 DNA. Seven of 9 patients with pemphigus vulgaris (8 of 12 specimens) and 1 of 2 patients with pemphigus foliaceus (1 of 3 specimens) were positive for 160-bp HHV-8 DNA by nested PCR (Table 2, Fig. 1). In 1 patient with pemphigus vulgaris, lesional skin was positive for HHV-8 but nonlesional, normal-appearing skin was negative (Case 5a, 5b, Table 2). In 1 patients with pemphigus vulgaris and 1 pemphigus foliaceus, 1 lesional skin of each patient was positive but another lesional skin was negative for HHV-8. In another patient with pemphigus vulgaris, both of lesional skins were positive (Case 3a, 3b). HHV-8 DNA screening by nested PCR on 3 epidermolysis bullosa acquisita, 4 dermatitis herpetiformis, 7 Hailey-Hailey disease, 3 herpes simplex virus-positive skin specimens, 3 psoriasis, and 5 normal

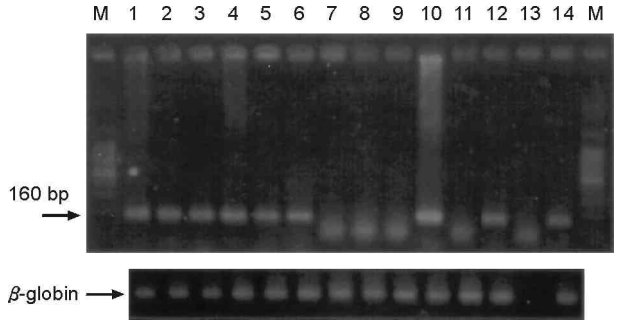

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction products of HHV-8 DNA with nested primers in pemphigus. M, molecular weight marker (DNA ladder, 100 bp); lane 1-12, pemphigus vulgaris (Case 1, 2, 3a, 3b, 4, 5a, 5b, 6, 7a, 7b, 8, 9, respectively); lane 13, no human DNA; lane 14, AIDS-associated Kaposi's sarcoma.

Table 3. Detection of HHV-8 DNA in other blistering skin diseases and control samples

Diagnosis	Number of tested samples	Number of positive samples
Bullous pemphigoid	5	0
Epidermolysis bullosa acquisita	3	0
Hailey-Hailey disease	7	0
Herpes simplex	3	0
Psoriasis vulgaris	3	0
Normal skin	5	0
Total	26	0

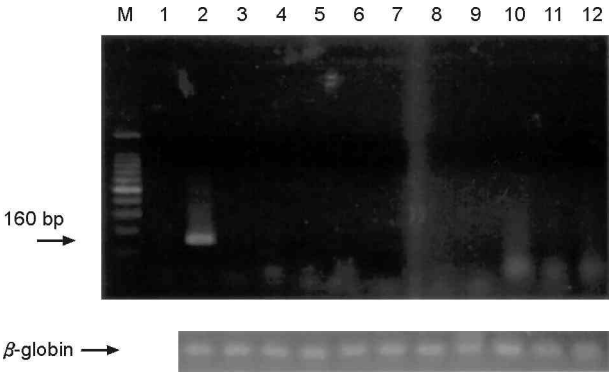

Fig. 2. Nested-PCR amplification of HHV-8 DNA in normal skin, herpes simplex, and psoriasis. M, molecular weight marker (DNA ladder, 100 bp); lane 1, no human DNA; lane 2, AIDS-associated Kaposi's sarcoma; lanes 3-6, normal skin; lanes 7-9, herpes simplex; lanes 10-12, psoriasis.

skin samples were negative for HHV-8 (Table 3, Fig. 2). All specimens that were evaluated for the presence of HHV-8 were positive for amplification of 110-bp sized β-globin DNA as internal control, suggesting the absence of major PCR inhibitors in DNA samples.

Result of directing sequencing

Positive PCR products from pemphigus vulgaris and pemphigus foliaceus were sequenced directly. The sequences were almost identical with the prototypic sequence for HHV-8 DNA and only a few base-pair substitutions in our DNA samples revealing multiple sequence variability from the originally described sequence (1) were found. Substitutions at 1086C→T and 1139A→C were detected frequently and translated into amino acid substitutions, resulting in an alanine to valine and lysine to glutamine replacement, respectively. Two specimens from one patient with pemphigus vulgaris (case 3a, 3b, Table 2) showed the same sequence variation. Four of 8 HHV-8 positive pemphigus vulgaris specimens had the same DNA sequence variation, while other pemphigus vulgaris and pemphigus foliaceus specimens had different DNA sequence variations (Table 1, Fig. 3).

DISCUSSION

Viral infections, in particular herpesvirus infections, have been identified as a possible triggering factor for pemphigus. Also, in other acantholytic diseases, such as Hailey-Hailey disease, herpes simplex virus (HSV) may be able to induce or exacerbate Hailey-Hailey disease lesions (22, 23). There are a few reports describing the activation and/or exacerbation of pemphigus by viral infection (12, 13, 18, 19). The development of pemphigus vulgaris (PV) following HSV infection was first mentioned in 1974 by Krain (14). Thereafter, besides HSV, herpes zoster virus (15), cytomegalovirus (16), and Epstein-Barr virus (17) were also reported to exacerbate or trigger pemphigus. Recently, Schlupen et al. (24)

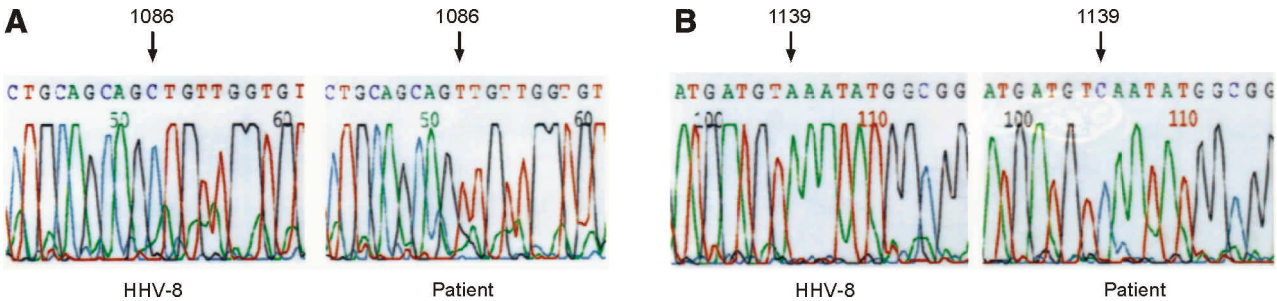

Fig. 3. Direct sequencing of PCR product from pemphigus vulgaris reveals C to T transition at position 1086 (A) and A to C at 1139 (B).

reported three cases of pemphigus exacerbated by a HSV infection, which was detected by polymerase chain reaction. The treatment with acyclovir led to a noticeable improvement of lesions in all three patients and a stop to longer amplification of HSV-specific DNA by PCR. Tufano et al. (25) also demonstrated the presence of DNA sequences of herpes simplex virus 1/2 (50% in peripheral mononuclear cells and 71% in skin lesions), Epstein-Barr virus (15% in peripheral mononuclear cells and 5% in skin lesions) and human herpesvirus 6 (20% in peripheral mononuclear cells only) in patients with pemphigus. However, the inability to detect herpesvirus DNA consistently in their cases suggested that viral infection may only be an occasional factor triggering the outbreak or exacerbation of the pemphigus. In addition, there are other reports demonstrating the association between pemphigus and viral infection in which the onset of pemphigus preceded that of the viral infection (26-30). Until now, there has been no definitive evidence confirming a significant association between HSV and pemphigus, although viruses are a major contributing factor at least in some cases of pemphigus.

In 1997, Memar et al. (18) have first reported HHV-8 DNA in a pemphigus vulgaris patient without HIV infection or Kaposi's sarcoma, and they subsequently reported the presence of HHV-8 DNA in skin lesions from six patients with pemphigus foliaceus and four of six patients with pemphigus vulgaris irrespective of immunosuppressive therapy (19). The presence of HHV-8 DNA in peripheral blood mononuclear cells and lesional skin, but not in normal skin from patients with pemphigus vulgaris and normal healthy controls (18, 19), suggests that HHV-8 may be lesion specific and involved in certain aspects of the pathogenesis of pemphigus. Moreover, in situ hybridization on some of the pemphigus vulgaris lesions showed that HHV-8 DNA was present in the perilesional endothelial cells and basal keratinocytes (19). The results of our study showing the presence of HHV-8 DNA in 8 of 12 pemphigus vulgaris and 1 of 3 pemphigus foliaceus specimens and the lack of HHV-8 DNA in other blistering skin disorders and control skin samples are in agreement with other studies (18, 19). Therefore, HHV-8 might be an alternative virus contributing to the activation or exacerbation of pemphigus. However, the crucial question to be resolved is whether this association is causal, with the virus triggering the disease, or whether it is merely an incidental finding and/or an associated phenomenon (25).

There may be several possible explanations for the association of pemphigus and HHV-8. First, HHV-8 could simply be an opportunistic infection. Pemphigus patients are usually treated with immunosuppressive therapy for a long-term period, and in some cases of virus-associated pemphigus, viral infection occurs under glucocorticoid therapy and develops in preexisting pemphigus lesions (29, 30). This means that the risk of herpetic infection might be potentiated by immunosuppressive therapy. However, the pemphigus patients we analyzed did not have the previous immunosuppressive therapy, and the results of another study (19) were irrespective of immunosuppressive therapy. Second, there is the possibility of correct positive results without any pathogenetic relevance, meaning the persistence of herpes virus or only viral DNA remained independently intact as well as pathogenic viruses in the skin lesions (24). Third, viruses might trigger pemphigus in a selective group of patients. Herpesvirus infections could potentially mediate the tissue damage by upregulating and increasing production of humoral and cellular factors that contribute to the development of pemphigus (25) or might enhance the presentation of autoantigens in genetically prediposed patients (19). Interferon-γ produced by virus-responding T cells induces the HLA expression of class II on keratinocyte cell membranes, forming the structural site of pemphigus antigen immunologically active. In case of chronic viral infection or reactivation, the cytokine 'switch' Th1→Th2 occurs, with the production of IL-4 and IL-10, which further stimulates the antibody response. In this manner, the autoimmune cascade is activated, and by means of cytotoxic effectors and pathogenic autoantibodies, it can induce the appearance of pemphigus (25, 31). Although the number of published cases of pemphigus induced by a viral infection is small, we cannot exclude the possibility that in the past virus-associated pemphigus has been overlooked because of a lack of knowledge concerning viral etiology, absence of molecular technology, and no discovery of new virus.

However, other studies (32, 33) did not detect HHV-8 DNA in lesions of patients with pemphigus vulgaris and pemphigus foliaceus, using PCR and in situ hybridization (32). Furthermore, analysis of serum samples did not reveal circulating antibodies against the lytically induced HHV-8 antigens, applying an immunofluoresence assay on pemphigus vulgaris and paraneoplastic pemphigus patients (32). They excluded HHV-8 as a major effect in pemphigus. There may be several possible explanations for the discrepant findings among studies concerning the rate of HHV-8 DNA sequences detection in non-KS lesions of the skin. Difference of patient population with or without HIV infection, sampling error such as insufficient number of cases, laboratory contaminations, differences of detection techniques, and the geographical difference in distribution of HHV-8 infection in different countries can be sources for the discrepant results (34, 35).

The sequence variations from our samples were differ-

ent from the results of Memar et al. (19). In their study, 1033 C→T, 1084 A→G, 1170 A→G substitutions appeared in most of the samples sequenced. In our study, HHV-8 DNA sequences revealed novel substitutions at positions 1086 and 1139, leading to C-to-T and A-to-C substitution, and alanine to valine and lysine to glutamine replacement, respectively. The significance of different nucleotide substitutions between HHV-8 DNA sequences derived from pemphigus vulgaris and pemphigus foliaceus esions is not known.

Our findings together with others (12, 18, 19, 25) have practical clinical importance, particularly in view of the various effective antiviral drugs now available. These drugs may help prevent the appearance of the disease and treat some patients. Also, unnecessary and potentially hazardous treatments may be avoided in others.

One of disadvantages of our study was the retrospective analysis of clinical samples by using formalin-fixed and paraffin-embedded materials. In order to make clear associations between pemphigus and HHV-8 or understand the role of HHV-8 in pemphigus, larger numbers of pemphigus patients need to be screened prospectively for HHV-8 in peripheral blood mononuclear cell, lesional skin and normal skin. Additional assays to detect virus, such as in situ hybridization, serologic assays, or electron microscopy, and animal studies should be included.

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