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Infection frequency of Epstein-Barr virus in subgingival samples from patients with different periodontal status and its correlation with clinical parameters*

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Abstract: Objective: To detect the infection frequencies of different genotypes of Epstein-Barr virus (EBV) in subgingival samples from chronic periodontitis (CP) patients, and to discuss the correlation between infection with EBV and clinical parameters. Methods: Nested-PCR assay was used to detect EBV-1 and EBV-2 in subgingival samples from 65 CP patients, 65 gingivitis patients and 24 periodontally healthy individuals. The amplicons were further identified by restriction fragment length polymorphism analysis (RFLP) with endonucleases *Afa* I and *Stu* I. Clinical parameters mainly included bleeding on probing (BOP), probing depth (PD), attachment loss (AL) in six sites of the dentition. Results: In CP patients, gingivitis and periodontally healthy individuals, the infection frequencies were 47.7%, 24.6% and 16.7% for EBV-1, and 15.4%, 7.7% and 0% for EBV-2, respectively. In 2 out of the 65 CP patients co-infection of EBV-1 and EBV-2 was found. The positive rate of EBV-1 in chronic periodontitis patients was higher than that in gingivitis patients (P=0.01) and periodontally healthy individuals (P=0.01). But no significant difference was shown in EBV-1 frequency between gingivitis patients and healthy individuals (P>0.05) or in EBV-2 frequency among the three groups (P>0.05). In CP patients, higher mean BOP value was found in EBV-1 or EBV-2 positive patients than that in EBV negative ones (P<0.01), but with no statistical difference in the mean PD or AL value between EBV positive and negative patients (P>0.05). After initial periodontal treatment, 12 out of the 21 EBV-1 positive CP patients did not show detectable EBV-1 in subgingival samples. Conclusion: nPCR plus RFLP analysis is a sensitive, specific and stable method to detect EBV-1 and EBV-2 in subgingival samples. Subgingival infection with EBV-1 is closely associated with chronic periodontitis. Infection of EBV in subgingival samples was correlated with BOP.

Key words: Periodontitis, Polymerase chain reaction, Epstein-Barr virus, Polymorphism, Restriction fragment length
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INTRODUCTION

Periodontal diseases are multifactorial infections elicited by a complex of bacterial species that interact with host tissues and cells causing the release of a broad array of inflammatory cytokines, chemokines, and mediators, some of which lead to destruction of the periodontal structures, including the tooth-supporting tissues, alveolar bone, and periodontal ligament (Socransky and Haffajee, 2002). Even though specific infectious agents are of key importance in the development of periodontitis, it is unlikely that a single agent or even a small group of pathogens are the sole cause or modulator of this heterogeneous disease.

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Since the mid 1990s, herpesviruses have emerged as putative pathogens in various types of periodontal disease (Contreras and Slots, 2000). In particular, Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) seem to play important roles in the etiopathogenesis of severe types of periodontitis. Genomes of the two herpesviruses occur at high frequency in progressive periodontitis in adults, localized and generalized aggressive (juvenile) periodontitis, HIV-associated periodontitis, acute necrotizing ulcerative gingivitis, periodontal abscesses, and some rare types of advanced periodontitis associated with medical disorders (Saygun *et al*., 2004a; Slots, 2002). EBV infects periodontal B-lymphocytes, and HCMV infects periodontal monocytes/macrophages and T-lymphocytes (Contreras *et al*., 1999a). Herpesvirus-infected inflammatory cells elicit tissue-destroying cytokines and may exert diminished ability to defend against bacterial challenge (Slots and Contreras, 2000). Herpesvirus-associated periodontal sites also tend to harbor elevated levels of periodontopathic bacteria, including *Porphyromonas gingivalis*, *Tannerella forsythia*, *Dialister pneumosintes*/*Dialister invisus*, *Prevotella intermedia*, *Prevotella nigrescens*, *Treponema denticola*, *Campylobacter rectus* and *Actinobacillus actinomycetemcomitans* (Saygun *et al*., 2004b; Slots *et al*., 2003; Slots, 2002). A novel infectious disease model for human periodontitis is described in which periodontal herpesvirus activation results in suppression of periodontal immune defenses, overgrowth of periodontal bacterial pathogens, release of pro-inflammatory cytokines and chemokines, initiation of cytotoxic or immunopathological events, and subsequently periodontal tissue breakdown (Slots and Contreras, 2000).

EBV, a double-stranded DNA virus, is one of the eight human herpes viruses (HHV4). It is transmitted by salivary contact and establishes a lifelong latent infection, usually asymptomatic. EBV could be arbitrarily divided into two types according to the allelic polymorphisms in the latent gene sequences encoding EBV nuclear antigen 2 (Moss *et al*., 2001). Infection of EBV is frequent in healthy individuals and most EBV serum positive healthy Caucasians are infected with EBV-1, while EBV-2 has a high infection rate in individuals exposed to immunosuppression (HIV-infected and transplant patients) (Cohen, 2000; Contreras *et al*., 2001). In vitro study showed that EBV-2 causes more lysis of B-lymphocytes than EBV-1 (Buck *et al*., 1999). Therefore, different genotypes of EBV might differ in their pathogenicity.

The purpose of this study was to investigate the subgingival infection frequency of EBV in Chinese patients with different periodontal status. A nested-PCR method was established to detect EBV-1 and EBV-2 in subgingival plaque samples from 65 CP patients and in sulcular samples from 65 gingivitis patients and 24 periodontally healthy individuals. The amplicons were further analyzed with restriction endonuclease digestion, and the relationship between infection of EBV in subgingival samples and clinical parameters was also analyzed.

MATERIALS AND METHODS

Subjects

The subjects were 65 Chinese CP patients (26 males aged 20 to 74 years, mean age was (43.9±8.9) years; and 39 females aged 21 to 75 years, mean age was (45.3±6.7) years), 65 gingivitis patients (30 males aged 17 to 58 years, mean age was (38.2±7.9) years; and 35 females aged 18 to 60 years, mean age was (37.1±5.4) years) and 24 periodontally healthy individuals (10 males aged 21 to 62 years, mean age was (37.3±5.9) years; and 14 females aged 22 to 63 years, mean age was (36.3±6.3) years) who were referred to the dental clinic in the Second Affiliated Hospital of the School of Medicine of Zhejiang University for dental or periodontal treatment or health monitoring. All the subjects were non-smokers without any systemic disease, and with at least 14 teeth remaining. Those who had received professional cleaning or had history of antibiotic therapy during the preceding 3 months were excluded. All of the patients and the healthy individuals underwent full-mouth examination. The criteria of diagnosis for chronic periodontitis and gingivitis were based on the Classification of the Periodontal Diseases issued by the American Academy of Periodontology in 1999 (Armitage, 1999). Briefly, the 65 generalized CP patients had >30% sites showing periodontal probing depth ≥3 mm, clinical attachment loss >1 mm and radiographic evidence of alveolar bone loss. The 65 plaque-induced gingivitis patients showed inflamma-

tion of gingiva, with periodontal probing depth no more than 3 mm, without any clinical attachment loss or bone loss on X-ray examination. These individuals were considered periodontally healthy with periodontal probing depth <3 mm, without any clinical attachment loss or radiographic evidence of bone loss and without inflammation of gingiva. All the subjects received detailed information concerning the nature of the study and the procedures involved, and their informed consent was obtained.

Sample collection

Each CP patient contributed four subgingival samples from the 4 deepest periodontal pockets of the dentition, preferably one pocket from each quadrant. Each of the gingivitis patients or periodontally healthy individuals provided 4 sulcular samples from four sites in each quadrant. Prior to subgingival sampling, supragingival plaque was removed with sterile cotton rolls. Three sterile endodontic paper points were placed into the depth of each site for 30 s and than transferred to a microcentrifuge tube containing 200 μl TE buffer (10 mmol/L Tris-HCl, 1.0 mmol/L EDTA, pH 8.0) and stored at −70 °C. The full-mouth clinical parameters recorded included bleeding on probing (BOP), probing pocket depth (PD) and attachment loss (AL) at six sites per tooth. Subgingival samples were also collected after initial periodontal treatment in 21 out of the 65 CP patients (9 males aged 41 to 57 years, mean age was (45.6±5.8) years; and 12 females aged 37 to 59 years, mean age was (44.6±4.9) years). The 2~4-month-longitudinal periodontal treatment consisted mainly of oral hygiene instruction, and scaling and root planning.

Viral nucleic acid extraction

The specimens resuspended in 200 μl TE buffer were vigorously vortex mixed. The nucleic acid extraction technique was based on preferential binding to silica particles in the presence of a high concentration of guanidium thiocyanate (GuSCN) (Parra and Slots, 1996). Briefly, 200 μl of sample was mixed with 50 μl of silica particles (Sigma) in 800 μl of lysis buffer (120 g of GuSCN, 100 ml of 0.1 mmol/L Tris-HCl pH 6.4; 22 ml of 0.2 mol/L EDTA, pH 8.0; 2.6 g of Triton X-100), mixed on a vortex for 10 s and kept at room temperature for 10 min. Nucleic acid/silica complexes were recovered by centrifugation at 12000×g for 1 min, washed twice in buffer (GuSCN-Tris-HCl), twice in 70% ethanol and once in acetone. The sample was then dried at 37 °C. The nucleic acid pellet was resuspended in 100 μl of TE buffer containing 0.5 U/ml Rnasin (Promega), and DNA/RNA was separated from the silica particles by incubation at 56 °C for 10 min. After centrifugation at 12000×g for 2 min, the supernatant was stored at −70 °C.

PCR procedures

A nested-PCR method was used to detect viral DNA of EBV-1 and EBV-2. The primers had demonstrated high specificity and no cross-reactivity with the human genome, other viruses or various microorganisms by other researchers (Parra and Slots, 1996; Contreras and Slots, 1996). The sequences of EBV outer primers were (Parra and Slots, 1996): (F) 5′-AGG GAT GCC TGG ACA CAA GA-3′, (R) 5′-TGG TGC TGC TGG TGG TGG CAA-3′. The inner primers for EBV-1 were (Parra and Slots, 1996): (F) 5′-TCT TGA TAG GGA TCC GCT AGG ATA-3′, (R) 5′-ACC GTG GTT CTG GAC TAT CTG GAT C-3′. The inner primers for EBV-2 were (Parra and Slots, 1996): (F) 5′-CAT GGT AGC CTT AGG ACA TA-3′, (R) 5′-AGA CTT AGT TGA TGC TGC CCT AG-3′.

The first round PCR reaction was carried out in a volume of 40 μl total mixture that included 5 μl of the template, 1×PCR buffer (pH 8.3) and 1.25 U Ex-Taq DNA polymerase (TaKaRa), 0.2 mmol/L of each dNTP, 1.5 mmol/L $MgCl_2$, and 50 pmol/L of the EBV outer primers. PCR amplification, performed in a Perkin Elmer DNA cycler 2400, included an initial denaturation step at 94 °C for 5 min followed by 30 cycles of denaturation steps at 94 °C for 1 min, primer annealing at 60 °C for 1 min and an extension step at 72 °C for 1 min, and then a final extension step at 72 °C for 7 min. In a new tube, a second round of amplification was performed using 2 μl of the first round PCR product, 50 pmol of the inner primers for EBV-1 or EBV-2, and PCR buffer, Ex-Taq DNA polymerase, dNTP and $MgCl_2$ in concentrations described above. The program for the second PCR was 35 cycles of denaturation step at 94 °C for 1 min, an annealing step at 55 °C for 1 min and an extension step at 72 °C for 1.5 min, and then a final extension step at 72 °C for 7

min.

EBV-1 and HCMV DNA were gifts from the Department of Microbiology of Anhui Medical College, which were used as positive and negative controls respectively. For sensitivity test, 20 ng/μl of the EBV-1 DNA was serially 10-fold diluted and 5 μl of each dilute containing 10^{-6}~100 ng DNA was used as template in PCR assay. Amplicons were detected by electrophoresis of 10 μl of sample in a 1.5% agarose gel containing 1 μg/ml of ethidium bromide. The expected sizes of target fragments from EBV-1 and EBV-2 were 497-bp and 165-bp respectively. PCR assay was repeated once more for each clinical sample.

Restriction endonuclease digestion analysis

The amplicons were further identified by restriction fragment length polymorphism analysis (RFLP) with endonucleases *Afa* I and *Stu* I (TaKaRa). *Afa* I digested the 497-bp amplicon of EBV-1 in 355- and 142-bp fragments, while *Stu* I digested the 165-bp amplicon of EBV-2 in 118- and 47-bp fragments. Restriction enzyme digests were resolved in 2.5% agarose gel containing 1 μg/ml of ethidium bromide. 100-bp marker (BioAsia) was used as molecular weight marker.

Data analysis

Chi-square test and Fisher's exact test were employed to compare the positive rates of EBV-1 and EBV-2 in patients and periodontally healthy individuals. Association between the infection frequencies of EBV-1 or EBV-2 and clinical parameters was examined using Kruskal-Wallis test with Stata 8.0 software. Differences in clinical parameters before and after the initial periodontal treatment were analyzed using Student's *t*-test. *P*-value equal to or below 0.05 was considered statistically significant.

RESULTS

Sensitivity and specificity of nested-PCR assay

Using 10^{-6}~100 ng EBV-1 DNA as template in PCR assay, the nPCR could detect as low as 0.01 ng of EBV-1 DNA (Fig.1). Repeated PCR results were identical in all the clinical samples. All the EBV-1 products could be digested in 355- and 142-bp fragments by *Afa* I and *Stu* I could digest all the EBV-2 amplicons in 118- and 47-bp fragments (Fig.2).

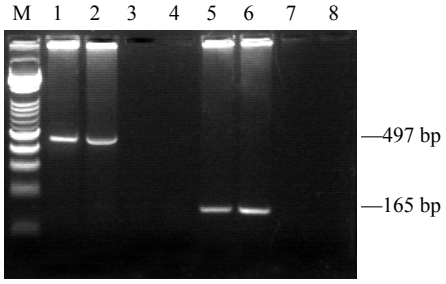

Fig.1 Nested-PCR results of EBV-1 and EBV-2 detection in subgingival samples from CP patients
M: 100-bp marker; Lane 1: EBV-1 positive control; Lane 2: EBV-1 positive result in subgingival samples from patient; Lanes 3 and 7: Negative control (HCMV) for EBV-1 and EBV-2 detection; Lanes 4 and 8: Blank control for EBV-1 and EBV-2 detection; Lane 5: EBV-2 positive control; Lane 6: EBV-2 positive result in subgingival samples from patient

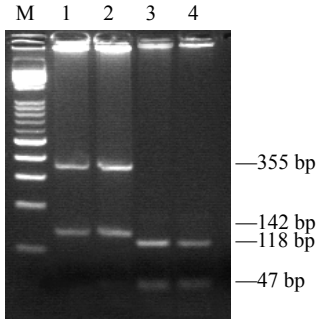

Fig.2 Restriction endonuclease digestion analysis of fragments of EBV-1 and EBV-2
M: 100-bp marker; Lane 1: Restriction endonuclease digestion analysis of PCR fragment from EBV-1 positive control by *Afa* I; Lane 2: Digestion of EBV-1 positive fragment from subgingival sample from patients by *Afa* I; Lanes 3 and 4: Digestion of two EBV-2 positive fragments from subgingival samples from patients by *Stu* I

Infection frequency of EBV-1 and EBV-2 in subgingival samples

Ninety-five out of the 260 subgingival samples from CP patients had detectable EBV, among which 74 (28.5%) were EBV-1 positive and 21 (8.1%) were positive for EBV-2. In the 260 sulcus samples from gingivitis patients, 44 (16.9%) and 8 (3.1%) were positive for EBV-1 and EBV-2, respectively. In the samples from periodontally healthy individuals, 14.6% (14/96) were EBV-1 positive, but none was EBV-2 detectable. The frequencies of EBV-1, EBV-2 and total EBV in CP samples were all higher than

those in healthy samples (χ^2=7.26, P=0.01; P=0.00; χ^2=15.91, P=0.00) or those in gingivitis samples (χ^2=9.87, P=0.00; χ^2=6.17, P=0.01; χ^2=17.54, P=0.00), respectively. But no significant difference could be found in the positive rates of EBV-1, EBV-2 and total EBV between gingivitis and healthy samples (χ^2=0.28, P=0.60; P=0.11; χ^2=1.36, P=0.24).

Infection frequency of EBV-1 and EBV-2 in patients and healthy individuals

The distribution of different genotypes of EBV in patients and healthy individuals are shown in Table 1. Forty-three out of the 65 (66.2%) CP patients were infected with EBV, among which 31 (47.7%) were infected with EBV-1 only, 10 (15.4%) with EBV-2 only, and 2 (3.1%) with both EBV-1 and EBV-2. In 21 out of the 65 (32.3%) gingivitis patients, 16 (24.6%) cases revealed EBV-1 and 5 (7.7%) had EBV-2. No co-infection of EBV-1 and EBV-2 was found in gingivitis patients. In the 24 periodontal healthy individuals, only 4 (16.7%) cases showed EBV-1 and no EBV-2 was detected (Table 1). CP patients were more frequently infected with EBV than gingivitis patients (χ^2=12.33, P=0.00) and periodontally healthy individuals (χ^2=15.10, P=0.00). The EBV-1 positive rate in CP patients was significantly higher than that in gingivitis patients (χ^2=7.50, P=0.01) and that in healthy individuals (χ^2=7.07, P=0.01). But no statistical difference was found in EBV or EBV-1 frequency between gingivitis patients and healthy individuals (χ^2=2.12, P=0.15; χ^2=0.64, P=0.43) or in EBV-2 frequencies among the three groups (χ^2=1.88, P=0.17; P=0.11; P=0.32).

Correlation between frequencies of EBV-1 and EBV-2 in CP patients and clinical parameters

Table 2 describes the full-mouth clinical parameters in EBV positive and negative patients. In patients infected with EBV-1 or EBV-2 or both EBV-1 and EBV-2, the mean BOP index of the six sites of the dentition was significantly higher than that in EBV negative patients (P=0.00, 0.00, 0.04). Although in EBV positive patients the mean PD or AL value was higher than that in EBV negative ones, there were no statistical differences among them (P=0.18, 0.29, 0.22; P=0.14, 0.94, 0.71). No correlation was found between EBV infection and age or gender of the patients (P=0.12, 0.76, 0.53; P=0.68, 0.81, 0.30).

Twenty-one EBV-1 positive CP patients were compared before and after initial periodontal treatment. Periodontal status improved following periodontal treatment (Table 3). Only 9 out of the 21 patients were still positive for EBV-1 in subgingival samples after 2 to 4 month initial periodontal treatment. The mean BOP, PD and AL values in the 9 positive patients post-treatment (BOP (29.64±4.54)%, PD (2.83±0.48) mm, AL (3.12±0.26) mm) were slightly higher than those in the 12 negative patients (BOP (28.39±2.42)%, PD (2.74±0.17) mm, AL (2.96±0.44) mm).

Table 1 Distribution of different nuclear antigen 2 genotypes of EBV in CP, gingivitis patients and periodontal healthy individuals

Groups (n)	EBV-1 positive cases	EBV-2 positive cases	EBV-1 and EBV-2 positive cases	Total positive cases
CP patients (65)	31*	10	2	43**
Gingivitis patients (65)	16	5	0	21
Periodontal healthy individuals (24)	4	0	0	4

*P<0.05, **P<0.01 vs gingivitis patients or healthy individuals

Table 2 Detection of EBV in CP patients and its association with clinical parameters

Variables	Positive			Negative (n=22)
	EBV-1 (n=31)	EBV-2 (n=10)	EBV-1 and EBV-2 (n=2)	
BOP (%)	58.60±11.98**	52.34±8.75**	47.50±3.54*	33.50±7.71
PD (mm)	3.76±0.78	3.64±0.39	3.75±0.21	3.50±0.33
AL (mm)	4.18±0.62	4.09±0.73	4.15±0.63	3.98±0.53
Age (years)	46.52±12.11	43.70±11.09	45.50±6.36	42.64±9.80
Gender (male/female)	12/19	4/6	0/2	10/12

*P<0.05, **P<0.01 vs EBV negative patients

Table 3 Changes in clinical parameters and EBV-1 in CP patients before and after treatment

Variables	Before	After
EBV-1 positive	21	9
BOP (%)	58.61±10.50	29.64±4.54**
PD (mm)	3.84±0.59	2.83±0.48**
AL (mm)	4.19±0.33	3.12±0.26**

**P<0.01 vs parameter before treatment

DISCUSSION

In this study, a nested-PCR method was established to detect EBV in subgingival samples with a detection limit of 0.01 ng of EBV DNA template, which provided sufficient sensitivity to allow meaningful data to be obtained from small specimen volume removed from periodontal sites. Restriction endonuclease digestion analysis also confirmed the specificity of the method. *Afa* I and *Stu* I could digest all the EBV-1 and EBV-2 amplicons into predicted fragments. Moreover, repeated PCR results also showed a high degree of reproducibility of the method.

It is reported in other populations that 30%~46% (Konstantinidis *et al*., 2005; Kubar *et al*., 2005; Contreras *et al*., 1999b; Contreras *et al*., 2000) of the samples from CP and 17% (Contreras *et al*., 1999a) from gingivitis patients are EBV positive, while only 10%~25% (Contreras *et al*., 2000) of specimens from periodontally healthy individuals reveal EBV. A study on EBV in sites with different periodontal status from CP patients in the Chinese population show that EBV is detected in 58%, 22.6% and 19.4% from active, rest periodontitis sites and gingivitis sites, respectively (Li *et al*., 2004). The results obtained in this study from periodontitis, gingivitis patients and periodontally healthy individuals revealed that EBV frequency was 36.5%, 20.0% and 14.6%, respectively, was consistent with previous reports. It was also showed that EBV positive rates in CP samples were higher than that in gingivitis samples (P<0.01) or in healthy controls (P<0.001), which indicated that EBV infection might be associated with CP in the Chinese population.

Although EBV-1 was detectable in subgingival samples from gingivitis patients and periodontally healthy individuals, it was significantly more frequently found in periodontitis samples (P=0.01), which suggested that EBV-1 infection might be associated with the pathogenesis of CP (Contreras *et al*., 1999b; Kubar *et al*., 2005). Viruses exert higher pathogenicity in their active rather than latent phase (Ahmed *et al*., 1996). Latent viruses could be reactivated spontaneously or by emotional stress, fever, exposure to ultraviolet light, tissue trauma, drugs, and immunosuppression (Ahmed *et al*., 1996). Most EBV-1 seropositive individuals secret low levels of virus continuously or intermittently for prolonged periods (Khanna *et al*., 1995). It is intriguing to speculate that the episodic clinical nature of human periodontitis with periods of exacerbation and remission may in part be related to a local reactivation of EBV-1 and possibly other viruses not examined for in this study (Contreras and Slots, 1996).

The association between EBV-2 infection and periodontitis remains to be established. EBV-2 could be detected in gingival biopsy from HIV positive periodontitis patients while it is not detectable in HIV negative periodontitis patients. Moreover, in HIV patients a high percentage of co-infection of EBV-1 and EBV-2 is found (Contreras *et al*., 2001). Other reports indicate that EBV-2 might also be found in subgingival samples from HIV negative periodontitis patients while in gingivitis and periodontal healthy individuals it is negative (Contreras *et al*., 1999b; 2000). The present study showed that 8.1% or 3.1% samples from CP or gingivitis patients were positive for EBV-2, while all periodontally healthy samples were negative. Although statistical analysis did not indicate any difference in EBV-2 frequency among these three groups of subjects, it was demonstrated that the positive rate of EBV-2 was higher in CP samples than in gingivitis and periodontal healthy samples (P<0.05). EBV-2 infection is closely associated with immunosuppression and EBV-1 and EBV-2 might differ in their pathogenecity (Contreras *et al*., 2001; Buck *et al*., 1999; Khanna *et al*., 1995), taken together, which implied that EBV-2 might also play a role in the pathogenesis and development of CP, especially in patients with local periodontal immune suppression. Further study should focus on the immune status of local periodontal tissue to define the correlation of EBV-2 with CP.

Periodontal herpes virus activation would destroy the normal periodontal defense barrier and im-

pair the local immune system which promotes subgingival colonization and proliferation of periodontal bacterial pathogen and finally results in periodontal tissue destruction (Slots, 2004; Kubar *et al*., 2005; Sugano *et al*., 2004; Kamma and Slots, 2003). In this study, the mean BOP value in EBV positive patients was significantly higher than that in negative patients. Previous research indicated that the frequency of EBV is associated with BOP (Idesawa *et al*., 2004), and is explained by the fact that EBV can be found in B lymphocytes from CP patients (Contreras *et al*., 1999a), and that the number of B lymphocytes in sites with active periodontitis is significantly higher when compared to stable sites (Zappa *et al*., 1991). Follow-up study also revealed that after initial periodontal treatment, 12 out of the 21 EBV-1 positive patients did not show detectable subgingival EBV-1, which indicated that eliminating inflammation might contribute to a decrease in subgingival EBV (Idesawa *et al*., 2004). These results implied the possible correlation of EBV-1 with inflammation of periodontal tissue or perhaps activity of periodontitis. Reports on the association between the presence of EBV in subgingival samples and PD or AL are not consistent (Idesawa *et al*., 2004; Slots *et al*., 2002). In this study, although no statistical difference was found in the mean PD and AL values between EBV positive and negative patients, they were slightly higher in EBV positive patients than in negative ones. Furthermore, in the 9 EBV-positive patients post-treatment, the mean PD and AL values were also slightly higher than those in the negative patients. Further investigation is needed to reach a conclusive result.

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