Detection and quantitative analysis of Epstein-Barr virus DNA and *Porphyromonas gingivalis* associated with Japanese chronic periodontitis patients

（日本人慢性歯周炎患者における Epstein-Barr virus DNA と *Porphyromonas gingivalis* の検出と定量解析）

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Preface

This article is based on a main reference paper, "Prevalence and quantitative analysis of Epstein-Barr virus DNA and *Porphyromonas gingivalis* associated with Japanese chronic periodontitis patients" in Clinical Oral Investigation, and a reference paper, "Higher Prevalence of Epstein-Barr Virus DNA in Deeper Periodontal Pockets of Chronic Periodontitis in Japanese Patients" in PLoS ONE.

Abstract

Periodontitis, a complex chronic inflammatory disease caused by subgingival infection, is among the most prevalent microbial diseases in humans. Although traditional microbiological research on periodontitis has focused on putative bacteria such as *Porphyromonas gingivalis* (*P. gingivalis*), the herpes virus is proposed to be involved in the pathogenesis of periodontitis because bacterial etiology alone does not adequately explain various clinical aspects. A number of studies have recently suggested Epstein–Barr virus (EBV) involvement in the pathogenesis of periodontitis. In the first study, we established that more EBV DNA is found in deeper periodontal pockets of Japanese chronic periodontitis (CP) patients. Subgingival samples were collected from 85 CP patients having two periodontal sites with probing pocket depth (PPD) of ≤3 mm

(shallow) or ≥5 mm (deep) and were subjected to a nested polymerase chain reaction (PCR). In the second study, we investigated the association between major periodontopathic bacteria *P. gingivalis* and EBV in Japanese CP patients. A group of 25 patients with CP participated in the study along with 13 individuals without periodontitis. Quantitative real-time PCR was used to detect EBV DNA and *P. gingivalis*.

EBV DNA was more frequently detected in patients with deeper PPD sites (66%) than in those with shallow PPD sites (48%) or healthy controls (45%). Coexistence of EBV DNA and *P. gingivalis* was significantly higher in deeper PPD sites (40%) than shallow PPD sites (14%) in the same patients or healthy controls (13%). Although no difference in clinical index for periodontitis, the odds ratio of EBV DNA in patients with deeper PPD sites was 2.36, which was 2.07-fold higher than that in those with shallow PPD sites. Interestingly, the odds of acquiring chronic periodontitis (PPD ≥5 mm) were higher in the presence of both EBV DNA and *P. gingivalis* compared with either EBV DNA or *P. gingivalis* only. In addition, we also observed that EBV-encoded small RNA (EBER) in positive cells of human gingival tissues.

In the CP patients, EBV DNA and *P. gingivalis* were detected in both 80% of sites with probing pocket depths (PPD) of ≥5 mm and in 40% and 36% of sites with PPD ≤3

mm, respectively. EBV DNA and *P. gingivalis* were detected in 50% and 27% of the sites in periodontally healthy individuals. Coexistence of EBV DNA and *P. gingivalis* was significantly higher in the deeper PPD sites of CP patients (68%) than in the PPD sites of the healthy controls (15%) and shallow PPD sites of CP patients (12%). PCR-positive deeper PPD sites of CP patients for EBV DNA and *P. gingivalis* ranges between $3.74 \times 10^3 \sim 2.83 \times 10^9$ and $2.73 \times 10^5 \sim 6.65 \times 10^9$ (copies/ml), respectively.

These results suggest that EBV DNA serve as a pathogenic factor leading to CP, and an association between EBV DNA, *P. gingivalis* and CP in Japanese individuals.

Introduction

The pathogenesis of periodontitis is multiple-step process involving complex interactions among specific bacterial factors, host factors, and a variety of environmental factors. Periodontitis affects the periodontium, and severe periodontitis can result in its destruction, occasional pain, alveolar bone resorption, and eventual tooth loss. It is now evident that host immune responses against infection with bacteria and the subsequent production of pro-inflammatory cytokines are of particular importance in destruction of periodontium [1, 2]. Although no single etiological agent has been identified, a number of putative bacteria are considered to be associated with

the disease and are used as diagnostic markers. *Porphyromonas gingivalis* and *Tannerella forsythia* are considered markers of adult chronic periodontitis, and *Aggregatibacter actinomycetemcomitans* is associated with aggressive periodontitis characterized by rapid alveolar bone loss [3, 4]. However, bacterial activity alone has not been able to explain the several clinical characteristics of periodontal diseases [5]. In addition, several reports have demonstrated the absence of putative periodontal bacteria in patients with periodontal disease, and there was no significant difference in the prevalence of bacteria between healthy and diseased periodontium [6-9]. Moreover, herpes virus has been suggested to be involved in the etiology of periodontal diseases.

Epstein-Barr virus (EBV) is an enveloped herpes virus with double-stranded DNA that infects human only [10]. EBV is one of the most common herpesviruses in humans, infecting more than 90% of adult population worldwide [11, 12]. In primary human infection, cell free EBV in saliva infects naive B lymphocytes, causing them to become proliferating blasts [9]. It then establishes a latent infection in those lymphocytes, which are largely non permissive for virus replication [10]. Among immunocompromised hosts, EBV is frequently reactivated and can induce infectious mononucleosis, autoimmune diseases, several malignancies such as Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, and post-transplant lymphoproliferative disorders

[8-13]. Recently, a positive association has been reported between periodontitis and EBV infection [14-16]. EBV is frequently found in the gingival crevicular fluid, saliva, salivary glands, and gingival tissues [16-19]. In addition, higher levels of EBV DNA have been detected in the saliva of patients with chronic or aggressive periodontitis [20-22]. However, no studies have evaluated the prevalence and quantity of EBV DNA in chronic periodontitis (CP) among the Japanese.

The purpose of this study was to examine whether higher prevalence and quantity of EBV DNA and *P. gingivalis* are associated with deeper periodontal pocket found in Japanese CP patients.

Materials and Methods

Sampling

The subjects received dental care at Nihon University Hospital School of Dentistry at Matsudo, Japan. The Institutional Internal Review and Ethics Board at the Nihon University School of Dentistry at Matsudo approved the study (EC11-027, EC14-11-027-1).Written informed consent was obtained from each study subject after all procedures had been fully explained.

Periodontal status was assessed by probing pocket depth (PPD), clinical attachment

level (CAL), and bleeding on probing (BOP). The PPD and CAL were measured with a PCP11 probe (Hu-Friedy, Chicago, IL, USA). CP patients were defined as the presence of at least two sites with PPD ≥5 mm and attachment loss of more than 5 mm. The healthy controls (HC) showed no clinical signs of gingival inflammation or attachment loss, no detectable bone loss on radiographic examination, and a PPD of ≤3 mm. All subjects were systemically healthy and had no history of periodontal treatment or any type of antibiotic therapy for at least 3 months prior to present study.

Before sampling, supragingival plaque was removed with sterile cotton pellets. Sterile paper points were then inserted to the sample site and retained for 30 s, (three paper points used per sample sits). The paper points were pooled in microcentrifuge tubes and stored at -80°C until DNA extraction.

DNA extraction

DNA extraction from the clinical samples was performed using the High Pure Viral Nucleic Acid Kit according to the user manual (Roche Applied Science, Mannheim, Germany). After DNA extraction, the sample DNA were pooled in microcenrifuge tubes and stored at -80°C.

Nested and multiplex polymerase chain reaction (PCR)

In this study, 20 periodontally healthy individuals (HC) (mean age, 45.9 ± 17.0 years) and 85 CP patients (mean age, 57.4 ± 13.1 years) were included. A total of 170 subgingival plaque samples were collected from two periodontal PPD sites [≥5 mm (deep) and ≤3 mm (shallow)] in 85 CP patients, and 40 subgingival plaque samples were collected from two PPD sites (≤3 mm) in 20 periodontally healthy controls. For the detection and typing of EBV DNA in the samples, nested PCR protocols were used, modifying those described previously for amplification of the *EBNA2*[11]. DNA extracted from the cell lines Raji and AKATA were used as positive controls, and human placenta DNA was used as a negative control .

The first PCR amplified *EBNA2*, generating a DNA fragment of 237 bp for EBV-1 and 253 bp for EBV-2 and was performed using following primer sets: EBV first forward, 5'-GCGGGTGGAGGGAAAGG-3'; EBV first reverse, 5'-GTCAGCCAAGGGACGCG-3'. With second nested primers, the PCR product comprised of 168 bp for EBV-1 and 184 bp for EBV-2. The second PCR was performed using the following primer sets: EBV second forward, 5'-AGGCTGCCCACCCTGAGGAT-3'; EBV second reverse, 5'-GCCACCTGGCAGCCCTAAAG -3'. The amplification reactions were performed in

25 µl of final reaction mixture containing: 2 × KAPA Taq Extra HotStart Ready Mix (KAPA Biosystems, Buenos Aires, Argentina); 5 µM forward and reverse primers; and 100 ng (1µl) DNA. The thermal cycling condition (1st and 2nd PCR) was 95°C for 3 min, 35 cycles at 95°C for 15 s, 63°C for 15 s, and 72°C for 30 s, with a final extension at 72°C for 1 min. We counted EBV-1 and EBV-2 together to quantify total EBV. The PCR-amplified product was analyzed by 1.5% agarose gel stained with ethidium bromide upon preparation. For detecting *P. gingivalis*, we used PCR primers against 16S rDNA as follows, forward, 5'-TGTAGATGACTGATGGTGAAAACC-3'; and reverse primer, 5'-ACGTCATCCCCACCTTCCTC -3' [27, 28]. The amplification reaction was the same as EBV nested PCR. The thermal cycling condition was 95°C for 3 min, 35 cycles at 95°C for 15 s, 59°C for 15 s, and 72°C for 30 s, with a final extension at 72°C for 1 min. The PCR-amplified product (*P. gingivalis*; 197 bp) was analyzed by 2% agarose gel stained with ethidium bromide upon preparation.

Real-time PCR

Thirteen periodontally healthy individuals (mean SD age 52.9 ± 18.0) and 25 CP patients (mean SD age 54.2 ± 13.8) were included in this study. A total of 50 subgingival plaque samples were collected from two periodontal sites of PPD (≥5 mm

and ≤3 mm) of 25 CP patients, and 26 subgingival plaque samples were collected from two sites of PPD (≤3 mm) of 13 periodontally healthy individuals. For quantitative detection of EBV DNA and *P. gingivalis* in the samples, real-time PCR was used. DNA extracted from the cell lines AKATA and *P. gingivalis* TDC60 were used as positive control. PCR was performed using the following primer sets: EBV forward, 5'-CCTGGTCATCCTTTGCCA-3'; EBV reverse, 5'-TGCTTCGTTATAGCCGTAGT-3'; *P. gingivalis* forward, 5'-AGGCAGCTTGCCATACTGCG-3'; *P. gingivalis* reverse ,5'-ACTGTTAGCAACTACCGATGT-3'; GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3'; GAPDH reverse, 5'-ATGGTGGTGAAGACGCCAGT-3', using the SYBR Premix Ex Taq in a TP800 thermal cycler dice real-time system (Takara-bio, Tokyo, Japan). PCR products comprised 95 bp for EBV, 404 bp for *P. gingivalis*, and 142 bp for GAPDH, respectively. The amplification reactions were performed in a final volume of 25 μl [12.5 μl 2× SYBR Premix Ex Taq, 0.2 μl forward and reverse primers (0.4 μM) and 12.3 μl DNA sample (300 ng / well)]. The thermal cycling conditions were at 95°C, 10 s and 45 cycles at 95°C, 5 s and 60°C, 30 s. Post-PCR melting curves confirmed the specificity of single-target amplification, and the expressions of EBV DNA and *P. gingivalis*

relative to GAPDH were determined. The dynamic range of the real-time PCR assays were determined through serial dilution of DNA extracts either AKATA cells or *P. gingivalis* TDC60 of the standards in the range of 10^1~10^{10}copies per ml. Positive and negative DNA controls were included in all runs. DNA-free water served as a negative control in each run.

Histological Examinations and In-situ Hybridization

Gingival tissues obtained during periodontal flap surgery from CP patients were used in this study. Gingival tissues were fixed in 10% neutral formaldehyde solution. There specimens were embedded in paraffin and stained with hematoxylyn-eosin (HE) for histological examinations. EBV was detected by in-situ hybridization (ISH) with EBV-encoded small RNA (EBER) probes. The immunohistochemical staining of CD19 (diluted at 1:250, DAKO) was performed using streptavidin-biotine-peroxidase, and then visualized with 3,3'-diaminobenzidine trahydrochloride. The sections were then counterstained with Mayer's hematoxylin.

Statistical analysis

One-way ANOVA was used to determine whether individual pathogens was

associated with CP and Chi square for independence test confirmed by Fisher's exact probability test to calculate odds ratio.

Results

Nested and Multiplex PCR

The age, gender, and PPD and BOP distributions of the patients are listed in Table 1. The average PPD of the HC (PPD ≤3 mm) was 2.73 ± 0.45 mm. In CP patients (n=85), the average depth of the two periodontal PPD sites (≤3 mm and ≥5 mm) was 2.91 ± 0.36 mm and 6.18 ± 1.04 mm, respectively. The prevalence of EBV DNA in the HC and CP patients is listed in Table 2. The periodontopathic bacterium *P. gingivalis* was also evaluated. EBV DNA was detected in 18 (45%) periodontal pockets of HC and 41 (48%) and 56 (66%) of the shallow (≤3 mm) and deeper PPD sites (≥5 mm) of CP patients, respectively. No difference in EBV DNA detection rate between males and females. EBV DNA occurred at significantly higher frequencies in deeper PPD sites of CP patients than in PPD sites of patients with chronic periodontitis (P <0.05). The occurrence frequency of *P. gingivalis* was similar to that of EBV DNA in both the healthy controls and in CP patients. Coexistence of EBV DNA and *P. gingivalis* was significantly higher in the deeper PPD sites of CP patients (40%) than in the PPD sites

of HC (13%) and shallow PPD sites of CP patients (14%) (P <0.01). These results suggested that there may be a correlation between the presence of EBV DNA and a deeper PPD (≥5 mm).

Clinical indices such as average PPD and frequency of BOP in deeper PPD sites of CP patients in which EBV DNA was detected alone (20 sites), *P. gingivalis* alone (19 sites), and coexistence of EBV DNA and *P. gingivalis* (36 sites) are shown in Table 3. In 10 of the deeper PPD sites (total 85 sites), neither EBV DNA nor *P. gingivalis* were detected. Although the frequency of BOP in areas with EBV DNA alone (65%), *P. gingivalis* alone (58%), and EBV DNA and *P. gingivalis* (61%) was higher than in sites where these microorganisms were not detected (50%), the difference was not significant. In addition, there was no association between the average PPD and the detected microorganisms in patients with CP (Table 3). Although no difference in clinical indices for periodontitis, the odds ratio of EBV was dependent on depth of periodontal pockets (Table 4). To calculate the odds ratios of qualitative risk factors for chronic periodontitis, the findings of periodontitis groups were compared with that of the HC group. In the shallow PPD sites (≤3 mm) of patients with CP, the odds ratios for EBV DNA alone and *P. gingivalis* alone were approximately 1.0. The presence of both EBV DNA and *P. gingivalis* did not affect the odds ratios in the shallow PPD sites. In contrast, in deeper

PPD sites (PPD ≥5 mm) of patients with CP, the odds ratios for EBV DNA and *P. gingivalis* were 2.07 and 2.75-fold higher than the odds ratio for shallow PPD sites (≤3 mm), respectively. Interestingly, the odds ratio of having CP (PPD ≥5 mm) was higher (approximately 1.82-fold) in the presence of both EBV DNA and *P. gingivalis* compared with the odds ratios associated with the solitary presence of either EBV DNA or *P. gingivalis*.

Histological examination

Subsequently, we attempted to detect EBV in the gingival tissue of patients with CP in whose periodontal pockets we had previously detected EBV DNA presence. The results of B-cell marker CD 19 immunostaining showed that a large number of B cells had infiltrated in the connective tissue subjacent to the gingival epithelium (Fig. 1B). Interestingly, based on the ISH results, EBV EBER showed a large number of cells in the same location that were CD19-positive (Fig. 1C).

Real-time PCR

Characteristics of patients and clinical data are summarized in Table 5. The average PPD (n=26) of the HC was 2.77 ± 0.43 mm. Among CP patients (*n*=25), the two

periodontal sites of PPD (≤3 and ≥5 mm) were 2.84 ± 0.37 and 6.28 ± 1.28 mm, respectively. BOP was detected 3.8% in HC and 4 or 72% in shallow (≤3 mm) or deep PPD (≥5 mm) sites from CP patients.

Table 6 describes clinical data and counts of EBV DNA and *P. gingivalis* in the CP patients. Two PCR-positive periodontial sites of PPD (≤3 and ≥5 mm) of CP patients for EBV DNA range from 4.37×10^4~9.13×10^6 copies/ml (≤3 mm) and 3.74×10^3~2.83×10^9 copies/ml (≥5 mm), and for *P. gingivalis* were 3.97×10^6~2.13×10^9 copies/ml (≤3 mm) and 2.73×10^5~6.65×10^9 copies/ml, respectively. Table 7 shows clinical data and counts of EBV DNA and *P. gingivalis* in the HC. PCR-positive sites of PPD (≤3 mm) of HC for EBV DNA range from 1.27×10^4~2.66×10^8 copies/ml and for *P. gingivalis* were 4.16×10^6 ~ 6.62×10^9 copies/ml, respectively. The occurrence frequencies of EBV DNA and *P. gingivalis* in the HC and patients with CP are listed in Table 8. EBV DNA was detected in 13 (50%) periodontal pockets of HC and in 10 (40%) and 20 (80%) of the shallow (≤3 mm) and deeper PPD sites (≥5 mm) of patients with CP, respectively. *P. gingivalis* was detected in 7 (27%) periodontal pockets of HC and in 9 (36%) and 20 (80%) of the shallow (≤3 mm) and deeper PPD sites (≥5 mm) of CP patients, respectively. EBV DNA and *P. gingivalis* were detected with higher frequencies in deeper PPD sites of CP patients than in PPD sites of HC. Additionally, EBV DNA and *P.*

gingivalis were significantly more frequent in deeper PPD sites than in shallow PPD sites of CP patients. The occurrence frequency of EBV DNA (50%) was higher than *P. gingivalis* (27%) in PPD sites of HC. However, EBV DNA and *P. gingivalis* were detected at almost similar frequencies in shallow PPD sites (40 and 36%) and in deeper PPD sites (80 and 80%) of CP patients. Coexistence of EBV DNA and *P. gingivalis* was significantly higher in the deeper PPD sites of CP patients (68%) than in the PPD sites of the HC (15%) and shallow PPD sites of CP patients (12%).

Discussion

Although a number of putative bacteria play an essential role in the etiology of periodontal disease, it has become increasingly clear that herpes viruses, especially EBV, are involved in the etiology of several types of periodontal disease because bacterial activity alone does not adequately explain some clinical characteristics of periodontal disease [5,44]. In fact, a purely bacterial cause of aggressive periodontitis does not explain why the disease tends to develop bilaterally symmetric and site-specific and why vertical bone resorption can advance at one tooth while barely affecting the periodontium of an adjacent tooth sharing the interproximal space [5,50]. Junctional

epithelial cells may serve as an oral reservoir of latent EBV infected cells.

In this study, we examined whether higher EBV DNA prevalence is associated with deeper periodontal pocket found in Japanese CP patients. As expected, we detected high level of EBV DNA in deeper periodontal pockets. The results reveal an association between the presence of EBV DNA and CP lesions (PPD $\geq$5 mm). The results correlated with that of previous studies that showed statistically significant levels of EBV DNA in patients with CP compared with that in HC [14, 26, 31]. Slots *et al.* discovered more EBV DNA in the gingival crevicular fluid and saliva of patients with periodontal diseases than in the saliva of an otherwise healthy control group [14, 26]. The same group demonstrated a correlation between EBV DNA prevalence in periodontal patients and periodontal pocket depth [18, 20, 22]. Because EBV DNA detection using nested PCR is a qualitative test, we also attempted to quantitative tests using real-time PCR for some samples to support the results of nested PCR. The results showed that real-time PCR data was consistent with nested PCR data. Real-time PCR did detect the presence of EBV DNA in those sites in which nested PCR had detected the presence of the DNA. EBV DNA was detected at ~300-fold higher copy numbers in the PCR-positive deep PPD compared to shallow PPD sites(Table 6). Moreover, *P. gingivalis* was detected at ~ 3-fold higher copy numbers in the PCR-positive deep PPD compared to shallow PPD

sites. Althogh there were many EBV DNA- and *P. gingivalis*-negative shallow PPD sites in both patients with CP and HC (Tables 6 and 7). These results suggest that high copy numbers of EBV DNA and *P. gingivalis* reflect the severity of inflammation. In the previous report, range of counts in PCR-positive sites of periodontitis patients and periodontally normal subjects for EBV DNA (positive %; 60 and 13%) were 2.1×10^{3}~ 8.3×10^{8} and 2.4×10^{3}~3.2×10^{4} copies/ml, and for *P. gingivalis* (positive %; 87 and 13%) were 5×10^{3}~1×10^{10} and 2.1×10^{4}~3.1×10^{6} copies/ml [41]. The results showed that copy numbers of EBV DNA and *P. gingivalis* in the periodontal lesions were almost the same as in our study (Table 6). However, their copy numbers in the normal subjects were lower compared to our data (Table 7). EBV DNA was detected at higher rate in the PPD sites of HC (50%) than in the shallow PPD sites of CP (40%). On the other hand, *P. gingivalis* was detected at higher rate in the shallow PPD sites of CP (36%) than in the PPD sites of HC (27%) (Table 8). These results might be caused by higher latent infection rate of healthy Japanese by EBV. Results of in situ hybridization using EBV-encoded small RNA (EBER) showed a large number of cells in the same location that were CD19-positive [44]. Results suggest that EBV copy numbers in the subgingival plaque samples may relate to the severity of inflammation and the numbers of inflammatory cell infiltration in the gingiva. The mechanisms of EBV reactivation

and activated EBV progressing to periodontal disease have not been determined. Latent EBV in B cells can be reactivated to switch to lytic replication. EBV reactivation can be induced in vitro by a variety of stimuli, including 12-O-tetradecanoylphorbol-13-acetate and anti-immunoglobulin, but a causal relationship between a co-infection with EBV and periodontopathic bacteria and the disruption of viral latency is not well understood. We have previously reported that the culture supernatant from *P. gingivalis*, which contains high concentrations of butyric acid, inhibits histone deacetylase and thus increases histone acetylation and transcriptional activity of the EBV BZLF1 gene, which encodes the master regulator protein (ZEBRA) for the transition from latency to the lytic replication cycle[23]. Given that regulation of the switch from latency to reactivation is an initial key step in EBV infection, these observations suggest that butyric acid producing periodontopathic bacteria, such as *P. gingivalis*, have the potential to trigger EBV reactivation in the oral cavity of infected individuals [23]. EBV-infected inflamed periodontal sites tend to harbor elevated levels of periodontopathic bacteria [16, 19, 44, 47]. Furthermore, bacterial and viral coinfections were also reported more frequently in deep periodontal pockets [14, 46, 19, 47]. EBV-1, EBV-2, and *P. gingivalis* were detected in 72.5, 10, and 95% of sites with PPD ≥6 mm, respectively [19]. We also reported that EBV and *P. gingivalis* were detected in 66 and

65% of sites with PPD ≥5 mm, and EBV DNA and *P. gingivalis* coinfection was found in 42% of sites with PPD ≥5 mm [44]. These observations suggest that a "negative chain reaction" by EBV and periodontopathic bacteria may contribute to the etiopathogenesis of periodontitis [36].

In summary, we performed quantitative analysis of EBV DNA and *P. gingivalis* in Japanese chronic periodontitis patients which to our knowledge is the first such attempt. EBV DNA and *P. gingivalis* were detected in higher copy numbers PPD and showed a higher incidence of the coexistence in deep PPD as compared to shallow PPD sites. Taking into account that periodontopathic anaerobic bacteria may increase the virulence of EBV via reactivation of EBV through butyric acid, their suppression or eradication may become an effective treatment to block EBV reactivation for early treatment or prevention of chronic periodontitis.

Further studies to establish EBV as an etiologic or co-etiologic agent of periodontitis are required. New treatments and superior prevention methods can be developed with enhanced understanding of the pathogenesis of periodontitis involving EBV infections.

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Figure legend

Fig. 1 Detection of EBER inflamed gingival connective tissue of patients with chronic periodontitis. Serial sections of periodontitis lesion were stained with HE (A), anti-CD19 antibody (B) and (C) EBER ISH, respectively.

Table 1 Patient characteristics for nested and multiplex PCR

	Healthy Control (n=20)	Chronic Periodontitis (n=85)
Age	45.9±17.0	57.4±13.1
Males	3 (15%)	36 (42%)
Females	17 (85%)	49 (58%)
PPD	2.73±0.45 (n=40)	2.91±0.36 (≤3 mm)
		6.18±1.04 (≥5 mm)
BOP	1 (2.5%) (n=40)	9 (11%) (≤3 mm)
		51 (60%) (≥5 mm)

Table 2 Occurrence of EBV DNA and *P. gingivalis* in the subgingival samples from HC and CP

	Detection frequency			Significance (*P*-value)		
Infectious agents	HC	CP(≤3 mm)	CP(≥5 mm)	HC vs CP(≤3 mm)	HC vs CP (≥5 mm)	CP(≤3 mm) vs CP(≥5 mm)
EBV	18 (45%)	41(48%)	56 (66%)	0.44	0.022*	0.015*
P. gingivalis	16 (40%)	34 (40%)	55 (65%)	0.58	0.008**	0.001**
EBV + *P. gingivalis*	5 (13%)	12 (14%)	34 (40%)	0.52	0.0013**	0.0001**

Statistically significant; $P<0.01$**, $P<0.05$* HC, healthy control; CP, chronic periodontitis.

Table 3 Average PPD and frequency of BOP in the chronic periodontitis with PPD of ≥5 mm

Infectious agents	Number of sites (n=85)	Average PPD (mm)	Frequency of BOP (%)
EBV(-), *P. gingivalis*(-)	10	5.90 ± 0.94	50
EBV(+)	20	5.85 ± 0.73	65
P. gingivalis(+)	19	6.47 ± 0.99	58
EBV(+), *P. gingivalis*(+)	36	6.25 ± 1.14	61

Table 4 Association between EBV, periodontopathic bacteria and CP

Infectious agents	CP (PPD≤3 mm) odds ratio	CP (PPD≥5 mm) odds ratio
EBV	1.14	2.36
P. gingivalis	1.0	2.75
EBV + *P. gingivalis*	1.15	4.67

Table 5 Characteristics of subjects and clinical data for real-time PCR

	HC (13 healthy individuals)	CP (25 CP patients)
Age	52.9 ± 18.0	54.2 ± 13.8
Males	2 (15%)	8 (32%)
Females	11 (85%)	17 (68%)
PPD	2.77 ± 0.43 (n=26)	2.84 ± 0.37 (≤3 mm; n=25)
		6.28 ± 1.28 (≥5 mm; n=25)
BOP	1 (3.8%) (n=26)	1 (4%) (≤3 mm; n=25)
		18 (72%) (≥5 mm; n=25)

Table 6 Clinical data and counts of EBV DNA and *P. gingivalis* in the CP patients

Subject No.	Gender	Age	PPD (≤ 3)	BOP	EBV (Copies/ml)	*P. gingivalis* (Copies/ml)	PPD (≥ 5)	BOP	EBV (Copies/ml)	*P. gingivalis* (Copies/ml)
1	Male	56	3 mm	-	ND	ND	8 mm	+	4.69×10^4	7.65×10^5
2	Male	57	3 mm	-	1.09×10^6	ND	8 mm	+	7.16×10^4	4.26×10^7
3	Female	58	3 mm	-	ND	2.13×10^9	6 mm	-	ND	2.86×10^8
4	Female	62	3 mm	-	1.22×10^6	ND	7 mm	+	8.85×10^5	2.46×10^6
5	Female	52	2 mm	-	4.37×10^4	3.97×10^6	7 mm	+	2.72×10^5	2.22×10^6
6	Female	40	3 mm	-	ND	4.10×10^7	6 mm	+	ND	1.29×10^7
7	Male	59	3 mm	-	ND	ND	6 mm	+	1.23×10^7	2.79×10^7
8	Female	29	3 mm	-	9.13×10^6	ND	8 mm	+	8.60×10^5	2.11×10^6
9	Female	63	3 mm	-	4.20×10^5	8.95×10^6	5 mm	-	1.05×10^6	1.71×10^7
10	Female	27	3 mm	-	9.33×10^5	ND	5 mm	-	3.70×10^7	ND
11	Female	63	3 mm	-	ND	7.75×10^7	8 mm	+	3.06×10^4	2.21×10^7
12	Male	82	3 mm	-	4.85×10^6	1.32×10^8	6 mm	+	2.83×10^9	6.65×10^9
13	Male	58	3 mm	-	2.89×10^6	ND	5 mm	-	1.67×10^7	2.13×10^8
14	Female	49	3 mm	-	ND	ND	6 mm	+	3.74×10^3	1.09×10^8
15	Female	72	3 mm	-	ND	ND	6 mm	+	6.65×10^3	2.79×10^7
16	Female	39	2 mm	-	ND	ND	5 mm	+	ND	ND
17	Male	54	3 mm	-	ND	7.81×10^6	6 mm	+	8.79×10^3	3.73×10^6
18	Female	42	3 mm	-	ND	1.33×10^8	10 mm	+	4.93×10^4	1.15×10^9
19	Female	60	3 mm	-	ND	ND	5 mm	-	3.22×10^6	ND
20	Female	33	3 mm	-	ND	2.45×10^7	6 mm	+	4.35×10^4	5.64×10^8
21	Male	49	3 mm	-	7.96×10^5	ND	5 mm	-	1.96×10^5	ND
22	Female	61	2 mm	-	ND	ND	6 mm	+	ND	3.56×10^6
23	Male	79	2 mm	-	ND	ND	6 mm	+	1.72×10^4	1.64×10^8
24	Female	59	3 mm	+	1.41×10^5	ND	6 mm	+	1.21×10^4	2.73×10^5
25	Female	52	3 mm	-	ND	ND	5 mm	-	ND	ND

not detectable (ND)

Table 7 Clinical data and counts of EBV DNA and *P. gingivalis* in the HC

Subject No.	Gender	Age	PPD (≤ 3)	BOP	EBV (Copies/ml)	*P. gingivalis* (Copies/ml)	PPD (≤ 3)	BOP	EBV (Copies/ml)	*P. gingivalis* (Copies/ml)
1	Female	64	3 mm	-	ND	ND	3 mm	-	ND	ND
2	Female	72	3 mm	-	ND	ND	3 mm	-	1.30×10^7	ND
3	Female	72	3 mm	-	ND	ND	3 mm	-	ND	ND
4	Female	40	3 mm	-	1.09×10^5	ND	3mm	-	ND	ND
5	Male	28	2 mm	-	ND	ND	3 mm	-	ND	ND
6	Female	26	2 mm	-	ND	ND	3 mm	-	ND	ND
7	Female	64	3 mm	-	8.15×10^7	1.41×10^9	3 mm	-	2.58×10^7	8.34×10^7
8	Female	64	3 mm	-	ND	6.62×10^9	3 mm	-	2.20×10^6	ND
9	Female	46	3 mm	-	2.66×10^8	ND	2 mm	-	3.59×10^7	ND
10	Male	73	3 mm	-	2.33×10^5	ND	3 mm	-	1.27×10^4	5.96×10^8
11	Female	56	2 mm	-	5.81×10^5	ND	2 mm	+	ND	1.61×10^7
12	Female	25	3 mm	-	2.46×10^6	ND	2 mm	-	2.55×10^6	ND
13	Female	58	3 mm	-	2.56×10^5	4.16×10^6	3 mm	-	ND	9.17×10^6

not detectable (ND)

Table 8 Occurrence of EBV DNA and *P. gingivalis* in the subgingival samples from HC and CP patients

Infectious agents	Detection frequency			Significance (*P*-value)		
	HC (n=26)	CP(≤3 mm) (n=25)	CP(≥5 mm) (n=25)	HC vs CP(≤3 mm)	HC vs CP (≥5 mm)	CP(≤3 mm) vs CP(≥5 mm)
EBV	13 (50%)	10 (40%)	20 (80%)	0.33	0.025*	0.0043**
P. gingivalis	7 (27%)	9 (36%)	20 (80%)	0.35	0.00017**	0.0018**
EBV + *P. gingivalis*	4 (15%)	3 (12%)	17 (68%)	0.52	0.00015**	0.00006**

Statistically significant; $P<0.01$**, $P<0.05$*

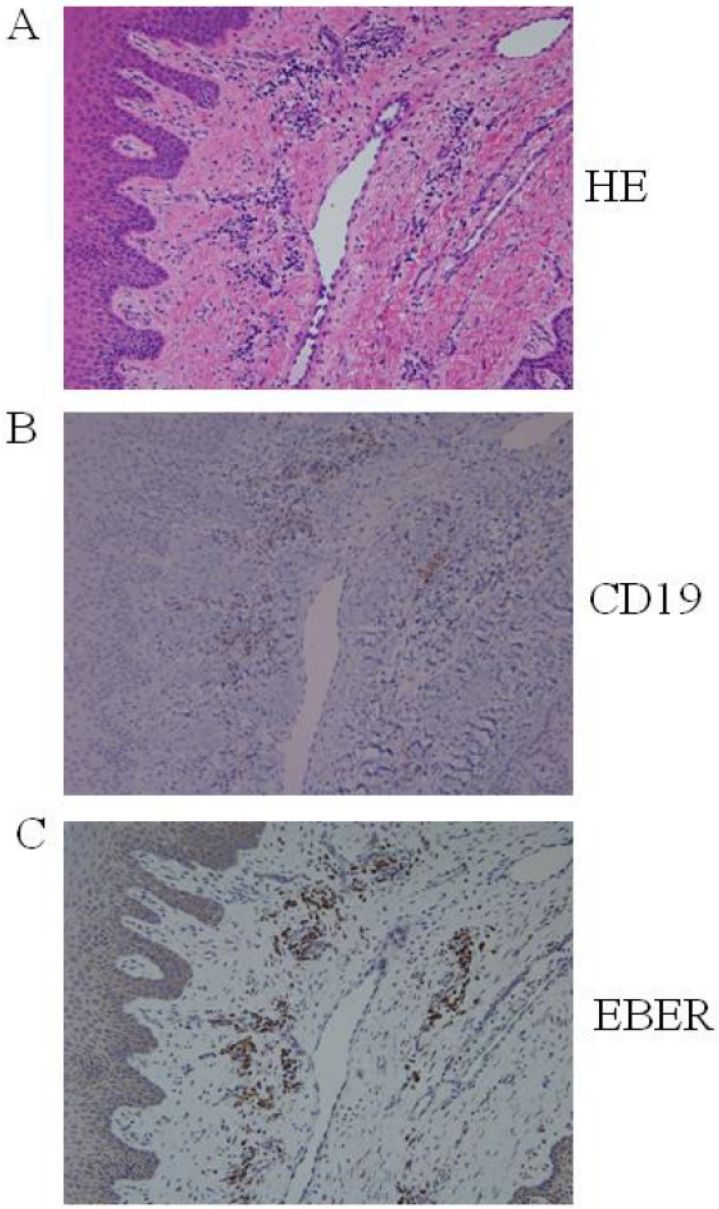

Fig.1