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Host *β-globin* gene fragments in crevicular fluid as a biomarker in periodontal health and disease

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Background and Objective: Leukocytes and epithelium are the first line of defense in preventing bacterial invasion into periodontium. Some of these cells die in gingival crevicular fluid, whereupon their DNA is spilled out. The present study was designed to investigate the profile of host *β-globin* gene fragments in the gingival crevicular fluid of various periodontal conditions.

Material and Methods: Gingival crevicular fluid from 40 teeth with chronic periodontitis, 30 with gingivitis and 22 that were clinically healthy were centrifuged (3000*g*, 10 min). The supernatant (cell-free gingival crevicular fluid) was centrifuged again (13,000*g*, 10 min), resulting in the pellet and the supernatant as debris and debris-free fractions, respectively. Specific primers for amplifying 110 bp, 536 bp and 2 kb amplicons of human *β-globin* gene were used to investigate host DNA by quantitative and qualitative polymerase chain reaction.

Results: The periodontitis group showed the largest amount of host *β-globin* gene fragments, while the healthy group had the lowest. In the debris and debris-free fractions, the 536 bp and 2 kb amplicons were more often detected in the periodontitis group than in the other groups. Interestingly, the presence of 2 kb amplicon in the debris fraction could be used to discriminate periodontitis from gingivitis and healthy groups because we found it in 85% of periodontitis samples but only in 13% of gingivitis samples, and it was absent in the healthy group.

Conclusion: This study shows the different DNA profiles of cell-free gingival crevicular fluid in periodontal health and disease. It suggests that the quantity and quality of host DNA are dependent on the disease conditions. Therefore, the *β-globin* gene fragments in cell-free gingival crevicular fluid may be a potential biomarker of periodontal disease progression.

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Periodontal disease is an infective and inflammatory disorder that damages tissue through the complex interactions between periodontopathic bacteria and host defense systems (1). Neutrophils and epithelium play an important role in defense against bacterial plaque at the free gingival margin to the base of the sulcus/pockets (2). In periodontal disease, neutrophils present in the sulcus at chronically elevated levels can mediate tissue damage and destruction

of the periodontium. The predominant and more powerful mechanism of plaque control may be the degranulation of neutrophils, which results not only in the extracellular killing of pathogens but also of the adjacent host cells. It seems to be the principal mechanism of bacterial attenuation used by neutrophils in the gingival crevice, especially when confronted by a high concentration of bacterial cells, resulting in the appearance of cells that die by necrosis (3,4). Crawford *et al*. (5) have shown that approximately 30% of neutrophils in periodontal pockets and gingival crevice die by necrosis, whereas < 1% of them die by apoptosis. Lohinai *et al*. (6) have studied murine models of ligature-induced periodontitis, and proposed that in periodontitis, oxidative stress induces cell dysfunction and ultimately necrosis. Recently, Chapple & Matthews (7) have exhaustively reviewed the presence and role of reactive oxygen species (ROS) in periodontal tissue damage. It is likely that DNA could be released from apoptotic or necrotic cells, and DNA size distribution may be used to determine the origin of DNA from either apoptotic or necrotic cells (8). Apoptotic cells generate small and uniform DNA fragments. In contrast, random and incomplete digestions of the DNA in necrotic cells result in a spectrum of DNA fragments with different strand lengths. Many studies have shown that cancer patients have not only a larger amount of DNA but also more long DNA fragments in cell-free specimens than in the control group (8–13).

The human *β-globin* gene is one of the genes that is successfully amplified in the qualitative and quantitative polymerase chain reaction (PCR) of human DNA in various samples such as plasma, serum, buccal cells and saliva (14–19). The 100–130 bp amplicons of this gene fragment are usually used to determine the total amount of DNA or as a positive control of human DNA (16–20). The amplicons of this gene that are longer than 200 bp (200–2000 bp) are usually used to determine the relative length of DNA fragments in the sample (14,15).

As mentioned above, cell damage takes place in periodontal tissues and in gingival crevice; therefore the gingival crevicular fluid in periodontal health and disease may contain different amounts of DNA fragments. Our study was designed to investigate the quantity and quality of *β-globin* gene fragments of cell-free gingival crevicular fluid in periodontal health and disease. We propose that *β-globin* gene fragments might be used as a biomarker for periodontal disease progression.

Material and methods

Study population

The study population consisted of patients being treated at the periodontal surgery clinic, Faculty of Dentistry Mahidol University, Bangkok, Thailand, and dental staff with clinically healthy periodontium as control subjects. All of them were examined by one periodontist and categorized into three groups based on the gingival index (GI; 21), probing depth, clinical attachment loss (CAL) and radiographic evidence of bone loss. Radiographic bone loss with periapical radiographs using long cone technique was recorded as positive or negative to differentiate chronic periodontitis from other groups. After a full-mouth periodontal probing with PCP-UNC 15 probe (Hu-Friedy, Chicago, IL, USA), probing depth, probing CAL and bleeding on probing (BOP) were recorded. The periodontitis group consisted of 25 patients (17 women, 8 men; 35–60 years of age) with generalized chronic periodontitis (22) having at least four molar teeth (third molar not included). They had signs of clinical inflammation, GI > 1, probing CAL ≥ 4 mm, probing depth ≥ 5 mm and radiographic evidence of bone loss. One or two molar teeth from different quadrants with GI > 1, probing CAL ≥ 4 mm, probing depth ≥ 5 mm, and with at least four sites of BOP per tooth were selected from each patient for gingival crevicular fluid sampling. Fifteen patients (12 women, 3 men; 25–35 years of age) with signs of gingival inflammation, GI ≥ 1, probing depth ≤ 3 mm and no probing CAL or radiographic bone loss were recruited for the gingivitis group. Two molar teeth from different quadrants with GI ≥ 1, probing depth ≤ 3 mm and with at least three sites of BOP per tooth, and without probing CAL or radiographic bone loss from each patient were selected for gingival crevicular fluid sampling. For the control group, 11 dental staff (9 women, 2 men; 22–30 years of age) with clinically healthy periodontium, GI = 0, probing depth ≤ 3 mm and without probing CAL or radiographic bone loss were recruited. Two molar teeth from different quadrants with GI = 0, probing depth ≤ 3 mm, no BOP and without probing CAL or radiographic bone loss were selected from each subject for gingival crevicular fluid sampling. All subjects in the gingivitis and healthy control groups had at least 24 remaining teeth (third molar not included). Exclusion criteria were: history of systemic diseases, history of smoking, pregnancy at the time of the study, and those who had taken medication within 2 months prior to the study. Subjects were enrolled in the study from May 2006 to July 2007. The experimental protocol was approved by the ethical committee of Mahidol University Bangkok, Thailand. Informed consent was obtained from all subjects who agreed to participate in this study.

Collection of gingival crevicular fluid

Gingival crevicular fluid samples were collected from the subjects 1 week after clinical baseline measurement. Before sample collection, the selected tooth was separated by sterile cotton rolls and dried with gentle air syringe. Supragingival plaque was removed without irritating the marginal gingiva. Gingival crevicular fluid samples were collected from 40 teeth with periodontitis, from 30 teeth with gingivitis and from 22 healthy teeth with a modification of the washing technique of Skapski & Lehner (23). Briefly, a 5 μL aliquot of sterile phosphate-buffered saline (PBS), pH 7.2, was gently introduced at the entrance of the pocket or sulcus using a micropipette. The solution was aspirated after a short interval and collected in a 1.5 mL

sterile plastic microtube. This process was done five times along the buccal side and five times along the lingual or palatal sides of the selected tooth. Phosphate-buffered saline solution was added to each gingival crevicular fluid sample to make the volume up to 100 μL. Then, host cells were precipitated by centrifugation at 3,000*g* for 10 min at 4°C. The supernatant (70 μL) was collected into another microtube and designated as cell-free gingival crevicular fluid. Phosphate-buffered saline (30 μL) was added to the supernatant, and this was centrifuged again at 13,000*g* for 10 min at 4°C. The resulting supernatant (70 μL) was collected into another tube. The remaining 30 μL and the supernatant were labeled as the debris and the debris-free fractions, respectively. To avoid contamination of the pellet in each step during transferral of the supernatant into a new tube, the remaining 30 μL was not touched. Each fraction was mixed with 99.5% ethanol to make a final concentration of 80% ethanol and kept at −20°C until DNA extraction could be performed within 3 months.

Extraction of DNA

The debris fraction was centrifuged at 13,000*g* for 10 min at 4°C. The pellet was washed with 500 μL of cold 99.5% ethanol and centrifuged again at 13,000 *g* for 10 min at 4°C. The supernatant was poured out and the tube was placed upside-down on a paper towel to remove excess ethanol. The pellet was dried in an incubator at 37°C for 1 h. For the debris-free fraction, it was evaporated under negative pressure in desiccators overnight at room temperature and then dried in an incubator at 37°C for 1–2 h. To extract DNA, 30 μL of sterile deionized water was added to the dried sample and mixed well by a vortex mixer. Two hundred microlitres of DNA extracting solution (InstaGene Matrix; Bio-Rad, Hercules, CA, USA) was added to each sample, as indicated by the manufacturer's protocol. Total DNA concentration and purity were determined by spectrophotometer (UV-1200V; Shimadzu, Kyoto, Japan) at 260 and 280 nm.

Polymerase chain reaction amplification of host DNA fragments

We decided to amplify three PCR amplicons (110 bp, 536 bp and 2 kb) of human *β-globin* gene based on previous studies (9,11,14,15,24). It has been reported that 100 and 200 bp amplicons could be amplified from DNA in cell-free specimens of both diseased and non-diseased subjects. In contrast, the 400 bp and 1.8 kb amplicons were more difficult to amplify from the DNA in the specimens of non-diseased than of diseased subjects. The DNA sequences of these PCR amplicons were nested together in the DNA fragment. Although the Roche Company (Roche, Tokyo, Japan) could not disclose the sequences of primers specific to 110 bp amplicon, this primer set was confirmed by successfully amplifying with template of 536 bp and 2 kb amplicons.

The 110 bp amplicon was amplified in quantitative real-time PCR (qPCR) by the primer/probes set of human *β-globin*, GenBank accession no. U01317 (LightCycler; Roche, Tokyo, Japan). The reaction mixture consisted of 25 μL of PCR solution (iQ SYBR Green Supermix; Bio-Rad), 1 μL each of forward and reverse primers, 1 μL of DNA sample and 22 μL of sterile deionized water to make up a total reaction volume of 50 μL. The human *β-globin* standard was serially diluted in standard dilution buffer to 4000, 400 and 40 copies/μL for making the standard curve. Human *β-globin* positive control (2000 copies/μL) and sterile deionized water were used as positive and negative controls, respectively, in each experimental run. All test and control specimens were assayed in triplicate. The real time amplification was performed with precycling heat activation of iTaq DNA polymerase at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 54°C for 20 s, and extension at 72°C for 30 s, using iCycler MyiQ real-time PCR (Bio-Rad). The copy number of human *β-globin* in each sample was determined by reference to a standard curve.

Another two PCR amplicons, 536 bp and 2 kb, were amplified in qualitative PCR for human genomic DNA (14). The sequences of forward and reverse primers were as follows: for 536 bp 63360F (GenBank accession number U01317), 5′-GCA GCT ACA CAG CTA CCA TTC TGC-3′ and 63865R 5′-GCA GCC TCA CCT TCT TTC ATG GAG T-3′; and for 2 kb 61992F (GenBank accession number U01317), 5′-GAA GAG CCA AGG ACA GGT AC-3′ and 63958R (GenBank accession number U01317), 5′-CCT CCA AAT CAA GCC TCT AC-3′, respectively. Ten nanograms of DNA extracted from neutrophils of healthy volunteers and sterile deionized water were used as positive and negative controls, respectively, in the PCR assay. The assays of 50 ng DNA samples were carried out with 25 μL of 2× PCR solutions (Premix Taq; Takara Bio, Shiga, Japan), 2 μL each of forward and reverse primers (0.16 μM, final concentration) and sterile deionized water to make the total volume 50 μL. The PCR reactions were performed on a DNA thermal cycler (I Cycler; Bio-Rad). The cycling conditions for both PCR products were 94°C for 4 min, initial denaturizing; 36 cycles of 94°C for 1 min, denaturizing; 54°C for 1 min, annealing; and 72°C for 2 min, extension. The PCR products (110 bp from qPCR; 536 bp and 2 kb from qualitative PCR) were visually inspected following 1.8% agarose-gel electrophoresis and ethidium bromide stain.

Statistical analysis

The mean of total DNA concentration or copy number of host β-globin in each fraction among the samples were analyzed by one-way analysis of variance (ANOVA) after natural logarithm transformation for homogeneity of variance. Then the difference of means between the two groups of samples was analyzed by Tukey's honestly significant difference (HSD) test. For discrete data, the positive detection of each amplicon of these groups was analyzed by Fisher's exact test. According to the non-normal distribution of the data, Spearman's rank correlation (r) test was used to relate between two parameters within the samples. The level of statistical significance was set at 0.05. All data

were analyzed on website http://faculty.vassar.edu/lowry/VassarStats.html.

Results

Quantitative analysis of host DNA fragments

Total DNA extracted from each sample contained DNA both of host and of microorganisms, and was calculated in nanograms per microlitre (Table 1). The mean copy number of host *β-globin* fragments in the debris and debris-free fractions of the teeth with periodontitis was much greater than in the teeth with gingivitis and healthy teeth (Table 1). The total DNA concentration of the teeth with periodontitis was significantly higher than that of the healthy teeth but not of the teeth with gingivitis in both the debris and debris-free fractions ($p < 0.01$; Tables 2 and 3). The significant differences of the copy number of host *β-globin* fragments in the debris and debris-free fractions between two groups were $p < 0.01$, respectively except between gingivitis and health (Table 3). With Spearman's rank correlation test, the copy number of host *β-globin* fragments in debris and debris-free fractions showed a good correlation within the same groups, especially in the periodontitis group ($r = 0.761$, $p < 0.001$). In addition, the copy number of host *β-globin* fragments in the debris fraction showed a positive correlation to the deepest site of probing depth in the teeth with periodontitis ($r = 0.603$, $p < 0.005$) but not in the debris-free fraction ($p > 0.05$). The correlation between the copy number of host *β-globin* fragments and total DNA in debris or debris-free fraction was not significant in all groups ($r < 0.5$, $p > 0.05$; Table 4).

Qualitative analysis of host DNA fragments

In this study, the 110 bp amplicon was detected in both debris and debris-free fractions of all samples (Fig. 1A,B). Therefore, the method we used was good enough to investigate host *β-globin* gene fragments in cell-free gingival crevicular fluid. The positive detection of the 536 bp and 2 kb amplicons showed a significant difference ($p < 0.005$) among the groups in both debris (Fig. 1A) and debris-free fractions (Fig. 1B). The teeth with periodontitis showed the highest prevalence of the 536 bp amplicon in the debris fraction (100%) and the debris-free fractions (95%), while the healthy teeth showed the least prevalence in the debris (59.09%) and the debris-free fractions (36.36%). In the teeth with gingivitis and in healthy teeth, positive detection of the 536 bp occurred more often in the debris fraction than in the debris-free fraction. Positive detection of the 2 kb amplicon in the debris fraction showed highly significant difference among the groups. It could be detected in 85% of the teeth with periodontitis and only in 13.33% of the teeth with gingivitis, while it was absent in the healthy teeth. In the debris-free fraction, the 2 kb amplicon could be detected in only a minority of the teeth with periodontitis (20%) and was never detected in the teeth with gingivitis and healthy teeth. Figure 2 shows the most common profiles of the two amplicons, 536 bp and 2 kb, of host *β-globin* fragments from the periodontitis and gingivitis samples. Periodontitis samples often showed positive results of both amplicons in the debris fraction (lane 3) and only of 536 bp in the debris-free fraction (lane 4). In contrast, the gingivitis samples were usually positive only for the 536 bp amplicon in the debris and the debris-free fractions (lanes 6 and 7, respectively). The intensities of the 536 bp bands in the debris and debris-free fractions of the periodontitis samples were much stronger than those of the gingivitis samples.

Table 1. Descriptive statistics of clinical parameters and DNA contents of the study teeth

	Periodontitis ($n = 40$)	Gingivitis ($n = 30$)	Health ($n = 22$)
GI	1.82 ± 0.56	1.20 ± 0.27	0
Probing depth (mm)	5.81 ± 1.47	2.83 ± 0.46	1.92 ± 0.36
CAL (mm)	4.24 ± 1.02	0	0
Debris fraction			
Total DNA (ng/μL)	10.85 ± 7.33	6.84 ± 4.11	2.56 ± 3.01
CNG (copy number/μL)	149.49 ± 151.05	35.84 ± 17.91	23.47 ± 10.71
Debris-free fraction			
Total DNA (ng/μL)	12.27 ± 6.30	11.39 ± 6.18	6.01 ± 3.98
CNG (copy number/μL)	154.43 ± 142.12	49.27 ± 32.04	16.86 ± 12.26

Data are expressed as means ± SD.
Abbreviations: GI, gingival index; CAL, clinical attachment loss; and CNG, copy number of host *β-globin*.

Table 2. The concentration of total DNA and copy number of host *β-globin* in the debris and debris-free fractions of the cell-free gingival crevicular fluid of the teeth with periodontitis, teeth with gingivitis and healthy teeth, analyzed by one-way ANOVA after logarithmic transformation

Source of DNA	Periodontitis ($n = 40$)	Gingivitis ($n = 30$)	Health ($n = 22$)	p-value
Debris fraction				
Total DNA (ng/μL)	2.12 ± 0.79	1.69 ± 0.74	0.97 ± 0.75	< 0.0001*
CNG (copy number/μL)	4.67 ± 0.84	3.46 ± 0.49	3.09 ± 0.40	< 0.0001*
Debris-free fraction				
Total DNA (ng/μL)	2.31 ± 0.58	2.28 ± 0.56	1.69 ± 0.72	< 0.001*
CNG (copy number/μL)	4.71 ± 0.88	3.66 ± 0.74	2.56 ± 0.74	< 0.0001*

Transformed data are expressed as means ± SD.
Abbreviation: CNG, copy number of host *β-globin*.
*Statistically significant difference at $p < 0.05$.

Discussion

In the present study, the DNA profiles in cell-free gingival crevicular fluid (debris and debris-free fractions) showed significant differences among the groups. The mean of total DNA

Table 3. Tukey's honestly significant difference test to compare mean of total DNA or of copy numbers of host *β-globin* between two groups

	PS and GS	PS and HC	GS and HC
Debris fraction			
Total DNA	NS	< 0.01*	< 0.01*
CNG	< 0.01*	< 0.01*	NS
Debris free fraction			
Total DNA	NS	< 0.01*	< 0.01*
CNG	< 0.01*	< 0.01*	< 0.01*

Values are expressed as *p*-values.
Abbreviations: NS, not significant; PS, periodontitis ($n = 40$); GS, gingivitis ($n = 30$); HC, healthy control ($n = 22$); and CNG, copy number of host *β-globin*.
*Statistically significant difference at $p < 0.05$.

Table 4. Spearman's rank order correlation (*r*) test comparing copy number of host *β-globin*, total DNA and the deepest probing depth within the groups

	Periodontitis ($n = 40$)	Gingivitis ($n = 30$)	Health ($n = 22$)
CNG in debris and debris-free fractions	0.761 (0.0006)*	0.5152 (0.1275)	0.620 (0.0554)
CNG in debris fraction and DPD	0.603 (0.0037)*	0.3482 (0.3243)	ND
CNG in debris-free fraction and DPD	0.326 (0.1474)	−0.522 (0.1218)	ND
CNG and total DNA in debris fraction	0.483 (0.0262)	0.079 (0.7281)	−0.321 (0.3651)
CNG and total DNA in debris-free fraction	0.276 (0.2264)	0.127 (0.7281)	−0.279 (0.4359)

Values are expressed as *r* (*p*-values).
Abbreviations: ND, not determined; CNG, copy number of host *β-globin*; and DPD, deepest probing depth.
*Statistically significant difference at $p < 0.05$.

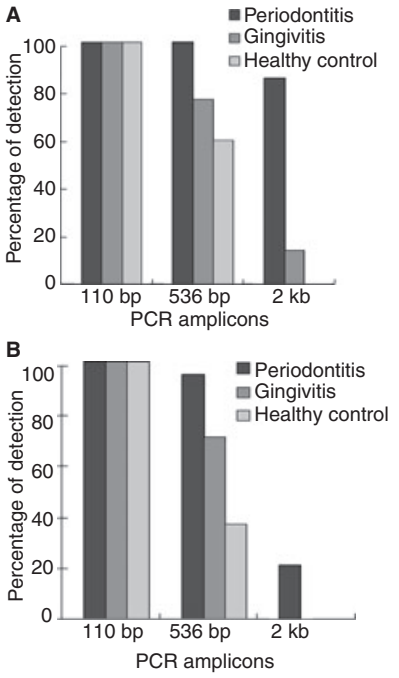

Fig. 1. Percentage of positive detection of PCR amplicons (110 bp, 536 bp and 2 kb) in the debris (A) and debris-free fractions (B) of the teeth with periodontitis or gingivitis and in healthy teeth.

and copy number of host *β-globin* fragments were highest in the teeth with periodontitis and lowest in the group of healthy teeth, with intermediate values for the teeth with gingivitis. They increased progressively from health to gingivitis to periodontitis, both in debris and in debris-free fractions. This was confirmed by a significant correlation between the copy number of host *β-globin* fragments and the deepest probing depth in the debris fraction of the teeth with periodontitis. Our results are in accord with other studies that have found a significantly higher DNA concentration in the plasma of severely injured patients and cancer patients compared with healthy individuals (10,11,16–18,25,26). This may imply that there are more dead cells in diseased than in normal states. These data are consistent with the information that Delima & Van Dyke (2) have reviewed concerning the cellular components in gingival crevicular fluid. The inflammatory cells will migrate through junctional epithelium into the periodontal pocket/sulcus, and some of them die in the gingival crevicular fluid. The numbers of these cells increase with the severity of the inflammation in the periodontal tissue.

Most of the host DNA in gingival crevicular fluid usually results from neutrophil death. The interaction of neutrophils with microorganisms is of particular importance in the progression of periodontitis (27). Neutrophils are short-lived cells and die in great numbers at acute inflammatory sites (28). Crawford *et al*. (5) have shown that 30% of neutrophils isolated from healthy sulci, periodontal pockets and whole saliva are necrotic while < 1% are apoptotic. According to the previous sentence shows that both the periodontal pockets and healthy sulci contain the same percentage (30%) of dead neutrophils. Therefore, the periodontal pocket which is larger volume than the sulci should contain more dead cells than the sulci. The larger volume of the gingival crevicular fluid sample from the teeth with periodontitis compared with the teeth with gingivitis and healthy teeth (29,30) also affects the total DNA content in gingival crevicular fluid.

The source of DNA in cell-free gingival crevicular fluid is physically different from the DNA in plasma. The volume of gingival crevicular fluid is very small compared with the blood volume, and the inflammatory cells die in the gingival crevicular fluid. In contrast to the blood, the cells die at the pathological sites and the DNA is released into the blood circulation. Therefore, most of them are degraded into free DNA in the blood circulation, while the DNA in gingival crevicular fluid is simply spilled out from dead cells. Some high-molecular-weight DNA will aggregate with debris in the gingival crevicular fluid and some low-molecular-weight DNA will be free DNA. To investigate the DNA in cell-free gingival crevicular fluid, the present study was designed to fractionate it into debris and debris-free fractions by

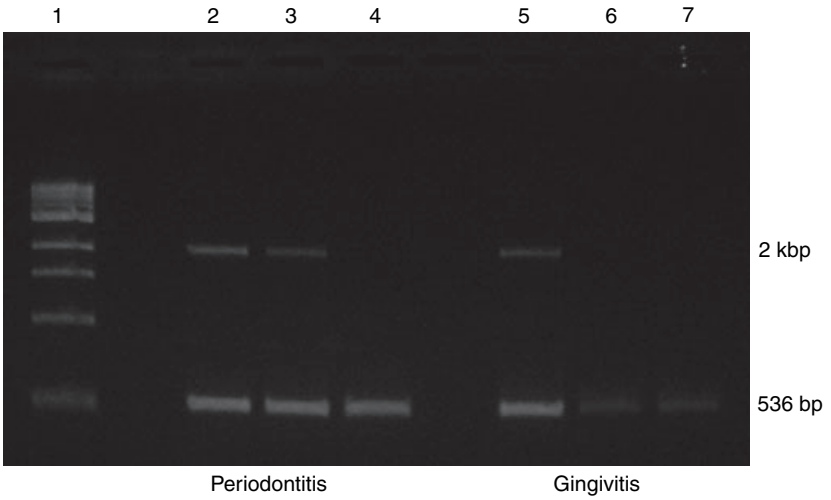

Fig. 2. The most common profiles of the two PCR amplicons, 536 bp and 2 kb, of *β-globin* fragments from the periodontitis and gingivitis samples, running on a 1.8% agarose gel with ethidium bromide staining. Lane 1 is a 1 kb DNA ladder. Lanes 2 and 5 are the positive controls. Lanes 3 and 4 are PCR amplicons from the debris and the debris-free fractions of the periodontitis sample. Lanes 6 and 7 are PCR amplicons from the debris and the debris-free fractions of the gingivitis sample.

centrifugation. The results showed that the longest PCR amplicon in this study, the 2 kb amplicon, could be amplified from the DNA in the debris fraction of the teeth with periodontitis substantially more frequently than from the teeth with gingivitis. Interestingly, it was absent in the healthy teeth. The 2 kb amplicon was more difficult to amplify in the debris-free than in the debris fractions. It could be amplified in only 20% of the debris-free fraction compared with 85% of the debris fraction of the teeth with periodontitis and it could not be amplified from the teeth with gingivitis or the healthy teeth. Similarly, the prevalence of 536 bp amplicon significantly decreased from periodontitis to gingivitis to healthy teeth, not only in the debris but also in the debris-free fraction. This might suggest that the amount of DNA from necrotic cells increased from healthy to diseased states. This finding agrees with the study by Boynton *et al.* (9) that showed substantially greater amounts of the 1.8 kb amplicon in stool samples from colorectal cancer patients compared with colonoscopy-negative patients. In addition, Wang *et al.* (11) have shown that the band intensity of a 400 bp amplicon is much stronger in neoplastic plasma samples than in non-neoplastic plasma controls.

One limitation of this study was the gingival crevicular fluid washing technique, which has the major disadvantage that not all fluid can be recovered and thus, accurate quantification of gingival crevicular fluid volume or composition is not possible because the precise dilution factor cannot be determined (31). This may affect the concentration of DNA in the gingival crevicular fluid. However, this technique is particularly valuable for harvesting cells, which is appropriate in this study to prepare the cell-free gingival crevicular fluid. Another limitation was the high prevalence of periodontitis in the Thai population over 35 years old. We aimed to avoid contamination of the DNA fragments via saliva from periodontitis sites that may be present in the same mouth of a patient with gingivitis. Even a few contaminated DNA fragments can cause a false positive result in the PCR technique. Therefore, we excluded patients with periodontitis sites from the gingivitis and healthy groups. It was very difficult to find patients with gingivitis who had no radiographic evidence of bone loss in the older group, so that the ages of the study population could not be matched. Although age is clearly a co-founder for oxidative stress, causing damage to cells and the extracellular matrix (7), the contamination of DNA fragments among the different periodontal conditions may have a more serious effect. Gender of the study group may be another limitation. Although Borden *et al.* (32) have shown that the relationship between gingival inflammation and crevicular fluid flow was not affected by age or sex, it is at present not known whether or not gender affects the DNA content. However, the present results showed large differences in the copy numbers of host *β-globin* gene fragments between periodontitis, gingivitis and clinical health. This implies that DNA in the cell-free gingival crevicular fluid depended on the disease conditions of the tooth rather than age and gender. Moreover, our pilot study showed that both the copy numbers and the positive detection of 536 bp and 2 kb amplicons of host *β-globin* gene fragments in cell-free gingival crevicular fluid were related to periodontal inflammation and were not affected by age or gender (33). We found that the copy numbers of host *β-globin* gene fragments decreased fourfold, and the 2 kb amplicon could not be detected after initial phase of periodontal treatment. In contrast, inflammation induced by periodontal surgery caused a fourfold increase in the copy numbers of host *β-globin* gene fragments, and the positive detection of 2 kb amplicon was increased from 0% immediately before operation to 90% 1 day after the operation.

Until now, most of the biomarkers that have been identified as potential candidates for diagnosis of periodontal disease are measures of inflammation and mediators of connective tissue breakdown (34,35), such as interleukin-1β, prostaglandin E_2, matrix metalloproteinases, neutrophil elastase and β-glucoronidase. However, there is no ideal marker that can predict when gingivitis is progressing into periodontitis or when periodontitis is in the active state with continuing attachment loss. Host DNA in cell-free gingival crevicular fluid may be an alternative marker that directly represents host

cell death in disease conditions. In conclusion, this study suggests that the quantity and quality of host *β-globin* gene fragments in cell-free gingival crevicular fluid were dependent on the disease conditions. If this is the case, it may be more valuable to use qualitative than quantitative analysis because DNA quantity is easily affected by various factors previously mentioned. Furthermore, qualitative PCR may be a better diagnostic tool because it is more simple to analyze and cheaper than quantitative PCR. Based on these results, host *β-globin* gene fragments in the cell-free gingival crevicular fluid may be a biomarker for periodontal disease progression. However, this evidence has to be investigated in gender and age-matched subjects using other genes and other amplicon lengths. In addition, longitudinal studies should be undertaken to validate these findings.

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