Original Article

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Correlation between cell free DNA in gingival crevicular fluid and clinical periodontal parameters by using two collection techniques

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Objectives: The purpose of this study was to evaluate the correlation between cell free DNA (cfDNA) in gingival crevicular fluid (GCF) and clinical periodontal parameters and to compare the efficiency of the two GCF collection techniques.

Materials and Methods: Clinical periodontal parameters including pocket depth, clinical attachment level and bleeding on probing were recorded in forty teeth of twenty patients with moderate to severe periodontal disease. GCF samples were collected from each tooth by two collection techniques. First, it was randomly collected by either modified washing technique or absorbent paper strips. Following one week, the samples were recollected from the same tooth with another technique. The obtained samples were centrifuged to obtain cfDNA in the supernatant, from which DNA extraction was performed by using InstaGene Matrix. The concentration of cfDNA was measured by nanodrop spectrophotometer and processed to polymerase chain reaction with specific primers for 110, 536, and 2,000 bp of human β-globin gene.

Results: There was no significant difference in cfDNA concentration between the two collection techniques. Furthermore, these cfDNA concentrations were not significantly correlated with any clinical periodontal parameters. Interestingly, 2,000 bp PCR products showed positive correlation among all clinical periodontal parameters ($p<0.05$). The presence of this PCR product was significant to the level of severity of the clinical parameters.

Conclusion: The cell free DNA collected by absorbent paper strips was as good as by modified washing technique. Therefore, the absorbent paper strips could be used for GCF collection instead of the modified washing technique. The presence of 2,000 bp PCR product, in GCF may be related to the severity of periodontal diseases.

Keywords: absorbent paper strip, β-globin gene, cell-free DNA, gingival crevicular fluid, modified washing technique, periodontal clinical parameter

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Introduction

Chronic periodontitis is an infective inflammatory disease which affects connective tissue attachment and bone around teeth. Host response to the pathogenic infection causes tissue damage and leads to disease progression. [1] The severity of periodontitis is characterized by the formation of pockets, clinical attachment loss, bleeding and radiographic bone loss. [2] Gingival crevicular fluid(GCF) is an inflammatory exudate that gathers at the gingival sulcus. It is composed of cellular components, cell mediators, enzymes, and organic materials. Therefore, GCF has been useful to identify biomarkers in the pathogenesis of periodontal disease, disease activity and

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prognosis. The traditional diagnosis of periodontal disease is based on examining clinical parameters, probing pocket depth (PD), bleeding on probing (BOP), clinical attachment loss (CAL), plaque index (PI), and radiographs which display the past information of periodontal tissue destruction but they are not able to predict the pathogenesis of the disease. [3] Numerous biomarkers in GCF have been studied; however, none have been strongly correlated with clinical periodontal parameters.

Nowadays, cell free DNA(cfDNA) in the circulatory system and body fluids is well known in various medical fields for purposes such as to study the severity of cancer [4], fetal analysis [5] and circulating infection. [6] CfDNA was present in low concentration in healthy individual but was high in individual with cancer. [7] There are three possible sources of cfDNA which are apoptosis, necrosis, and NETosis. [8] In healthy conditions, a cell undergoes apoptosis and its DNA digestion is accomplished by endonuclease, resulting in DNA fragments representing multiples of short base pairs(bp). Large sized cfDNA fragments are often observed in cancer patients, indicating an origin from necrosis. [8] In case of a serious infection, neutrophils undergo NETosis which is another major source of cfDNA. Microorganisms are eliminated by trapping with host proteins and enzymes bound to DNA fragments released by the neutrophils. [9] In periodontal disease, neutrophils are the dominating leukocytes in periodontal tissue and crevicular exudate for maintaining homeostasis. [10] NETosis was one of mechanism for preventing bacteria adhering and colonizing gingival epithelium. DNA releasing from neutrophils with enzymes for trap the periodontal pathogens at site inflammation. [11]

The human beta globin (β-globin) gene was one of the genes that amplified cfDNA in polymerase chain reaction (PCR) to investigate diseases. [12-14] In periodontal diseases, cfDNA has been proposed to be a promising marker of disease activity.[15] The PCR product of β-globin gene size 100 bp used to determine as a positive control of human DNA. On the other hand, the PCR product sizes 536 and 2,000 bp were used to determine the relative length of cfDNA fragments in GCF. There was a significant difference in cfDNA fragment sizes among healthy, gingivitis, and periodontitis subjects. [15] The presence of longer cfDNA fragment sizes seemed to relate to periodontal diseases. Likewise, 2,000bp PCR product could serve as a biomarker for periodontal progression [15] and mucositis. [16] Regarding the method of GCF collection of the previous studies was the washing technique [15, 16] which was appropriate to collect cfDNA in the GCF and easy to separate it from the cellular component by centrifugation. However, this method required a well trained and experienced investigator therefore it may not convenient to do in the dental clinic. The paper strips technique is wildly used, easy to carry out with less chair time and minimal invasiveness. [17] For this reason, the paper strips technique was proposed to collect the GCF instead. However, there is no information about the cfDNA collection by absorbent paper strips. Therefore, the first aim of this study was to evaluate the correlation between cfDNA in GCF and clinical periodontal parameters and the second was to compare the cfDNA in GCF collected by absorbent paper strips and modified washing techniques.

Materials and Methods

In this study, 20 periodontitis patients were recruited from the Department of Oral Medicine and Periodontology, Faculty of Dentistry, Mahidol University and Golden Jubilee Medical Center, Mahidol University. Subjects were excluded if they had any history of smoking, systemic disease affecting periodontal disease progression (e.g. diabetes, immune deficiency disease), pregnancy and lactation at the time of the study, or medical

condition that required antibiotics in the previous 3 months. Subjects were over 30 years old with the presence of at least four teeth (the third molar not included) and with a diagnosis of moderate to severe chronic periodontitis. To be included in the study, the sampled tooth had at least three bleeding on probing (BOP) sites with the deepest periodontal pocket as per chart. Two teeth were selected from each subject, one with the deepest pocket as probing depth (PD) > 4 mm and clinical attachment loss (CAL) ≥ 4 mm with radiographic evidence of bone loss and the other one with less severity than the first one was selected from a different quadrant with at least three BOP sites which classified as gingivitis or periodontitis. [18]

All subjects signed an informed consent prior to their enrollment into the study. There were three consecutive visits for each participant. At the first visit, each patient was examined and clinical parameters were measured. A periodontal probe (PCP UNC-15, Hu-Friedy) was used to measure PD, CAL and BOP. At the second visit, GCF sample was collected from the selected tooth by either modified washing technique[15] or absorbent paper strips (Periopaper®; Oraflow Inc., Smithtown, NY, USA) by using the simple random sampling with number table and followed by the remaining technique on the third visit. The time interval between the second and the third visits was 7 to 10 days. Data were recorded in a periodontal chart.

Before sample collection, the selected tooth was separated by a sterile cotton roll and dried with gentle air spray. A Supragingival plaque was removed without irritating the marginal gingiva while avoiding saliva contamination. In the modified washing technique, a 5 µL aliquot of sterile phosphate buffer saline (PBS, pH 7.2) was used to gently flush at the entrance of the sulcus and the sample was drawn by a micropipette 10 µL. The aspirated fluid was collected in 1.5 mL sterile plastic microtube. The procedure was repeated 5 times at the buccal aspect and another 5 times at the lingual aspect of the selected tooth and then phosphate-buffered saline (PBS) solution was added up to 100 µL. [15] In an absorbent paper strip technique, the top three depths of pocket from the same tooth were selected. The absorbent paper strips were gently placed into each selected site until light resistance was felt and the paper was left for 30 seconds. GCF was collected with three absorbent paper strips. All of them were placed in 1.5 mL microtubes containing 100 µL PBS at 4°C. Three pieces of absorbent paper strip were trapped to the cap of the tube and the tube was vortexed for 1 minute for eluting GCF. The GCF sample from each collection technique was centrifuged at 3,000 g for 10 minutes at 4°C. [19] Seventy µL of supernatant was kept in a new microtube as a cell free sample for DNA extraction. DNA was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA, USA) according to the instruction's recommendations and stored at -20°C. DNA concentration was measured by NanoDrop 2,000c UV-Vis spectrophotometer (Thermo Scientific, USA). The DNA samples were amplified by conventional polymerase chain reaction (PCR) using three sets of primers specific to human β-globin gene to obtain three PCR products including 110, 536, and 2,000 bp. PCR assay was done as in a previous study. [16] The PCR product size 110 bp was positive control to prove human cfDNA in the samples. The experiments were repeated three times to confirm the results of PCR products.

Statistical analysis

The data were evaluated for normal distribution of variable by Shapiro-Wilk test. The data was divided according to PD of ≤4 mm and >4mm Cell free DNA concentration between two GCF collection techniques were compared with Wilcoxon signed-rank test. Correlation of clinical parameters of periodontitis and cfDNA concentration with the two techniques were investigated using Pearson's correlation coefficient. Correlation of clinical parameters of

periodontitis and PCR product 536, 2,000 bp with the two techniques were investigated using Spearman's rank correlation coefficient. The positive of each PCR product 536, 2,000 bp in PD, CAL, and BOP sites were evaluated by Chi-square test. Statistical level of significance was set at p-value <0.05.

Results

There were 40 samples from 20 patients that were successful in GCF collection by both techniques during the experiment. The distribution of age was normal while the PD, BOP, CAL and cfDNA concentration were not. The 110 bp PCR products were present in all samples which indicated human cfDNA in the samples. Demographic data and clinical parameters of the subjects were shown in Table 1. There was no statistically significant difference in cfDNA concentration between the two GCF collection techniques in both groups of PD in Table 2. To determine the cfDNA concentration in PD between two techniques from the fourteen pairs of teeth which, were collected from individuals to reduce confounding factors. There was no significant difference in PD≤4 mm and PD>4mm in Table 3.

Table 4 showed no statistically significant correlation between clinical parameters (PD, BOP, and CAL) with cfDNA concentrations. In contrast, a significant correlation was observed between 2,000bp PCR products and clinical periodontal parameters as shown in Table 5. Table 6 elucidated a significant difference the presence 2,000bp PCR products between the groups of PD ≤4mm and >4mm. In addition, the group CAL<4mm also showed significantly lower number of the positive 2,000bp PCR products than the group with ≥4 mm in Table 7. On the other hand, the group of BOP 3-4 and 5-6 sites had no significant difference in the presence of 2,000bp PCR products in Table 8. Mean PD, CAL and BOP in 2,000 bp groups were 8.33±2.38mm, 9.75±2.41mm and 5.5±0.99 sites, respectively. They were higher level than the reports on Table1.

Table 1 Demographic and clinical periodontal parameters

Gender	Male 9 Female 11
Age (years; Mean+SD)	50.95±4.83
Pocket depth per tooth (mm; Mean+SD)	6.03±2.64
BOP sites per tooth (Mean+SD)	4.85+1.16
CAL per tooth (mm; Mean+SD)	7.55±2.91

Table 2 Cell free DNA concentrations in two groups of periodontal pocket depth collected by washing technique and absorbent paper strip

Periodontal pocket depth (mm)	N (Teeth)	Washing technique (ng/µl; mean±SD)	Absorbent paper strip (ng/µl; mean±SD)	P-value
≤4	14	13.21±11.37	11.51±9.18	0.311
>4	26	16.96±11.47	16.11±7.62	0.869

Table 3 Cell free DNA concentrations between shallow and deep pocket depth group

GCF collection tecnique	N	Pocket depth≤4mm (ng/µl; mean±SD)	Pocket depth >4mm (ng/µl; mean±SD)	p-value (0.05)
Washing technique	14	13.21±11.37	17.00±10.63	0.42
Absorbent paper stri[p]	14	11.51±9.18	15.17±6.85	0.10

Table 4 Correlation between clinical periodontal parameters and cell free DNA concentration

Clinical parameters	DNA concentration	CC	P Value (P<0.05)
PD	Washing technique	0.101	0.535
	absorbent paper strip	0.138	0.396
BOP site	washing technique	0.038	0.816
	absorbent paper strip	0.130	0.423
CAL	washing technique	0.078	0.634
	absorbent paper strip	0.070	0.668

CC = coefficient of correlation.

Table 5 Correlation between clinical periodontal parameters and PCR products of 536 and 2,000 bp

Clinical parameter	PCR products	GCF collection technique	CC	P Value (P<0.05)
Pocket Depth	536	Washing	0.181	0.265
		paper strip	0.053	0.743
	2000	washing	0.567*	0.00
		paper strip	0.567*	0.00
BOP sites	536	Washing	0.048	0.770
		paper strip	0.045	0.784
	2000	Washing	0.358*	0.023
		paper strip	0.448*	0.004
CAL	536	Washing	0.203	0.209
		paper strip	0.053	0.746
	2000	Washing	0.503*	0.001
		paper strip	0.500*	0.001

*P<0.05.

Table 6 Comparison the presence of PCR products between two groups of periodontal pocket depth (N=40)

Cell free DNA (bp)	Pocket depth (mm)		GCF collection technique	P Value (P<0.05)
	≤4	>4		
536	12	25	washing	0.276
	11	24	paper strip	0.222
2000	1	11	washing	0.021*
	1	10	paper strip	0.036*

*P<0.05.

Table 7 Comparison the presence of PCR products between two groups of clinical attachment level (N=40)

Cell free DNA (bp)	Clinical attachment level (mm)		Technique	P Value (P<0.05)
	<4	≥4		
536	8	29	washing	0.545
	7	28	paper strip	0.311
2000	0	13	washing	0.017*
	0	12	paper strip	0.025*

*P<0.05.

Table 8 Comparison of the PCR products presence between two groups of bleeding on probing sites (N=40)

Cell free DNA (bp)	BOP (sites)		GCF collection technique	P Value (P<0.05)
	3, 4	5,6		
536	15	22	washing	0.652
	15	20	paper strip	0.323
2000	2	10	washing	0.05
	2	9	paper strip	0.083

Discussion

The cfDNA sample was extracted within 3 hours to avoid genomic DNA contamination which was present at 72 hours due to lysis of white blood cells. [20] Neutrophils were essential in innate immunity for maintaining homeostasis in periodontal disease. Polymorphonuclear neutrophils (PMNs) can eliminate pathogens by phagocytosis and intracellular killing through oxidative and proteolytic means and by extracellular mechanisms, such as degranulation and release of neutrophil extracellular traps (NETs). It is estimated that 30,000 PMNs transit through periodontal tissues every minute and their presence in the GCF is physiological. In fact, more than 90% of GCF cells are PMNs and they form a barrier between the junction epithelium and subgingival biofilm, preventing its apical migration. [10] Therefore, dead cell of neutrophils and NETs in periodontal disease can be one of the major sources of cfDNA in GCF. The presence 2,000 bp PCR products used to be a biomarker for periodontitis group, which classified with PD>4mm. [15] Our study, the PD classification conformed to the previous study, [15] supported that 2,000 bp PCR product correlation to deep pockets. CAL was classified to mild-moderate group(<4mm) and moderate-severe group (>4mm). [2] The presence of long DNA fragments in CAL>4mm group was significant. GCF volume collecting with flushing or absorbent technique was total cfDNA solution in the gingival sulcus. In this study, PD and CAL level were examined 6 sites. However, the highest PD and CAL levels represent the severity condition [2] of each tooth.

As mention earlier, the absorbent paper strip was selected to compare with the washing technique [15], because it was easy to carry out and less chair time than other techniques. [17] The results of the present study showed both GCF collection techniques were effective for collecting cfDNA in periodontal pockets depth ≤4mm or >4mm as there was no significant difference in DNA concentration between the two techniques. In addition, cfDNA from both techniques showed the same correlation with the clinical periodontal parameters. Therefore, using three absorbent paper strips for GCF collecting with 30 seconds may be equivalence to washing technique.

The cfDNA concentrations in GCF failed to correlate with any periodontal clinical parameters. This was consistent with the finding that an intensity of NETs was not difference between gingivitis and periodontitis. [21] In some systemic disease, such as pulmonary arterial hypertension, has also shown that there was no difference in plasma cfDNA concentration between mild and severe disease. [22] In contrast, cfDNA in cancer [23], liver disease [24], and bacteremia [25] showed a significant correlation between clinical parameters and high concentration of cfDNA, which shows its importance as a potential diagnostic tool.

Regarding cfDNA fragment length, there was a significant correlation in the presence of 2,000bp PCR product with all three clinical parameters (PD, CAL, or BOP). CAL is an indicator of cumulative tissue destruction, including past periodontal disease, while PD is an indicator of current disease status. [26] Using CAL alone as a parameter would lead to the overestimation of periodontitis presence since attachment loss can be due to non-inflammatory causes. [27] In this study, the samples were collected before periodontal treatment, therefore the clinical attachment level relates to the pocket depth. The long DNA fragments were significantly high in deep PD, due to lipopolysaccharides of periodontal microorganisms and TNF-α triggered the neutrophil to prolong its lifespan which produced NETs for trapping pathogens. [11], [28], [29] Impaired NETs degradation and NETs persistence at sites of periodontal inflammation may be associated with failure of disease resolution, that caused periodontitis. [10, 11] However, NETs were degraded from DNase, [30]

that may produce the 536 bp PCR products in both shallow and deep PDs. Other than that, short DNA fragments were released from the apoptosis of healthy cells. [7]

Although the number of BOP site were significantly correlated with the presence of 2,000 bp PCR products, the group with a higher number of BOP (5-6 sites) had a trend of higher presence of 2,000bp PCR product than the group with a lower number (3-4 sites). In a previous study [31], the severity and extent of gingival bleeding are often associated with the degree of bacterial plaque accumulation, that induced NETs. [32]

The limitation of this study was a small subject that could not be classified to more specific clinical parameters. Second; the clinical parameters (PD CAL and BOP) were examined for the periodontal chart by post-graduated Periodontology students. Subjects were recruited to collect GCF next visit by a researcher. However, there were not calibrated among examiners that may have to be error measurement. Further study, the presence of 2,000bp PCR product may compare amounts between pre- and post-periodontal treatment to evaluate the resolution.

In conclusion, the presence of 2,000 bp PCR product correlates to clinical periodontal parameters that might be a biomarker representing the severity of periodontal disease. Using absorbent paper strips were as effective in collecting GCF as the washing method for the determination of cfDNA in shallow and deep periodontal pockets. However, these findings should be validated in further studies that should be more subjects and consider a wider range of clinical parameters.

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