S100A8 and S100A9 in Saliva, Blood and Gingival Crevicular Fluid for Screening Periodontitis

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Abstract

Background: Periodontitis is one of major oral diseases, which has no consensus on early screening tool. This study aimed to compare the association and screening ability of S100A8 and S100A9 in saliva, blood and gingival crevicular fluid (GCF) for periodontitis.

Methods: We recruited 149 community Koreans, 50 healthy and 99 periodontitis. Using clinical attachment loss and a panoramic radiograph, stage II-IV of new classification of periodontitis proposed at 2018 was considered as periodontitis. Enzyme linked immunosorbent assay kit was used to quantify S100A8 and S100A9. T-test, ANCOVA, Mann-Whitney test and correlation analysis were applied to compare the relationship of S100A8 and S100A9 in saliva, blood, and GCF for periodontitis. Receiver operating characteristic curve was applied for screening ability.

Results: Among S100A8 and S100A9 in saliva, blood and GCF, S100A8 in saliva was significantly higher in periodontitis participants than in healthy participants ($p < 0.05$) and showed highest screening ability of 0.73 for periodontitis. However, S100A8 and S100A9 in GCF were significantly higher in healthy participants ($p < 0.05$). Salivary S100A8 was positively correlated to blood S100A8 ($r = 0.21$, $p < 0.05$).

Conclusion: Salivary S100A8 could be a potential diagnostic marker for periodontitis. Thus, S100A8 salivary kit will be useful for screening periodontitis.

Summary sentence: Salivary S100A8 kit produced appropriate diagnostic ability for periodontitis and would be an efficient tool for early screening of periodontitis risk.

Introduction

Periodontitis, the major oral disease, is a polymicrobial infectious disease that is related with systematic inflammation, destroys supporting tissue around the tooth and ultimately leads to tooth loss [1, 2]. Systematic disease like diabetes, cardiovascular and stroke has been shown to be associated with periodontitis [3–5]. Polymicrobial biofilm interacts with periodontal tissue and the biofilm triggers the host response, which leads to elevate systemic inflammation through change in proteins, immunoglobulins and inflammatory mediators [6, 7]. Upon activation of inflammatory mediators, various degradation pathways are activated that causes secretion of destructive cellular molecules like protease, reactive oxygen species, chemokines and cytokines [8].

Early detection of periodontitis is necessary for public health in a preventive dimension, because it leads to tooth mobility, tooth loss, mastication deficiencies and digestive problems [9]. Periodontitis is conventionally identified by dentist, inspecting the tissues around the teeth and using radiograph to determine bone loose around the teeth. However, the clinical procedures of present diagnostic measures are time consuming and too delayed to be restored.

Biofluids like blood, saliva, urine, tears has been used as source of biomarkers for certain disease [10]. Scientists are focusing much attention on biofluids, compared to use of tissue because of several factors like ease of accessibility, low cost of obtainment, avoiding risk of biopsies, and availability of multiple sampling [11]. Saliva contains proteins, peptides, organic and inorganic salts, electrolytes from blood with additional contribution from mucosal transudates and GCF [12]. Therefore, Saliva has been study and used for diagnostic tools over last decade. Recently salivary biomarkers have been applied for cardiovascular disease, autoimmune diseases, diabetes, HIV, oral cancer, caries and periodontal diseases [13].

S100A8 and S100A9 are a subgroup of molecules within the broader family of S100 calcium-binding protein and it has ability to bind with zinc. These proteins are mostly expressed on neutrophils and monocytes or macrophages [14]. Previously it has been reported that increased concentration of S100A8/A9 in saliva and serum were associated with periodontitis patients [15, 16]. Similarly, GCF fluid contains S100A8 and S100A9 were associated with periodontitis [17]. Thus salivary S100A8 and S100A9 have been specific targets for researcher and practitioners who are interested to identify periodontitis using robust and cost-effective method [18]. One study reported that salivary calprotectin (S100A8/9) was compared with that in serum, which had only 100 participants [16]. There has been no comparative evidence on S100A8 and S100A9 among saliva, blood and GCF. Hence more evidence is needed to compare salivary S100A8 and S100A9 with those in blood and GCF from more sufficient number of participants.

Hence, the present study aimed to compare levels of salivary S100A8 and S100A9 with those in blood and GCF according to healthy and diseased periodontitis patients.

Materials And Methods

Study design and ethical consideration

This cross-sectional study randomly selected participants from public advertisement. All of the participants voluntarily provided a written informed consent. The Institutional Review Board for Human Subjects of Seoul National University Dental Hospital reviewed and granted the ethical consideration for this study (CRI17009).

Sample size estimation

The results of pilot test using ten participants (each five cases and controls) showed that MMP-9 (mean $\pm$ standard deviation [SD], ng/ml) was 8.0 ± 10.2 for periodontitis patients versus 6.1 ± 8.3 for healthy participants. Under the condition of type I error of 0.05, type II error of 0.8 and ratio of 2 between periodontitis patients and control, 49 patients and 98 controls were estimated.

Study participants

The following inclusion criteria were set for this study, (1) agreed to have periodontal examination including clinical alveolar loss (CAL) and panoramic radiograph according to the new international periodontal classification guideline by American Academy of Periodontology (AAP) and European Federation of Periodontology (EFP) [19], (2) aged over 20 years, (3) having no medication during previous three months (4) having idea to donate adequate sample of blood, GCF and saliva for analysis (5) having no missing data used in the final analysis.

The total of 149 adults (50 healthy and 99 periodontitis) were participated in this study.

Assessment of periodontitis

CAL and radiographic bone loss of each natural tooth were examined by trained dentists using panoramic radiograph (Pax-Primo, Vatech Global, Seoul, Korea). CAL was measured in all natural teeth except 3rd molar by using a UNC-15 probe. Periodontal status was categorised according to the guidelines of 2017 AAP-EFP workshop in Periodontology [19]. The participants diagnosed as Stage II-IV periodontitis were considered as periodontitis ('yes') and the other participants are non-periodontitis ('no'). Stage II-IV periodontitis is CAL ≥ 3 mm or extraction due to periodontitis or radiological bone loss $>15\%$ of coronal third or pocket depth >5 mm [19].

Saliva sampling

Each participant has information about standard sampling protocol for saliva collection. Patients have information that they do not have to brush tooth, drink or eat one hour before sampling. Unstimulated whole saliva was collected using passive drool or spitting method for 10 minutes in a 50ml conical tube in order to maintain consistency of samples. Collected saliva in tubes was centrifuged (2600 x g for 15 minutes at 4°C) and supernatants were collected and stored at -80 °C for further analysis.

GCF collection:

Radiographic evaluation was performed for each participant and GCF samples were obtained from the deepest pocket of the tooth using panoramic radiograph. GCF samples were collected before any clinical measurements to avoid bleeding. After isolating the tooth with cotton rolls, three absorbent paper points (#25, Meta Biomed Inc., Chungbuk, Korea) were gently inserted in the pocket for 30 seconds. Paper points were immediately placed in a cryovial containing 1 ml of phosphate buffer saline (PBS) in pH 7.4. GCF samples were stored at stored at -80 °C for further analysis.

Blood collection

Blood of 4 ml was drawn by venepuncture by a trained medical technologist. The blood samples were immediately aliquotted into 1 ml in sterilised 1.5 ml Eppendorf tube. The tubes were then stored at -80 °C for further analysis.

Quantification of salivary S100A8 and S100A9

S100A8 and S100A9 protein concentrations were determined from saliva, blood and GCF using enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MN, USA) following manufacturer's instruction. Standard curve was drawn using standard S100A8 and S100A9 supplied by the manufacturer. GCF and saliva samples were diluted on concentration dependent using reagent diluent provided by manufacture (1, 1/2, 1/4, 1/8, 1/16, 1/32) and diluted sample concentration for S100A8 and S100A9 were calculated from standard curve of S100A8 and S100A9. Similarly, blood samples were diluted on concentration dependent manner with reagent diluent (1/10, 1/20, 1/40, 1/80, 1/160, 1/320) and concentrations of S100A8 and S100A9 were estimated using standard curve. We decided the standard dilution rate that falls on the range of 500 pg/ml to 1000 pg/ml on pilot study.

Assessment of confounding variables

Sociodemographic factors (sex and education), behavioural factors (smoking and drinking) and systemic health information (diabetes) hypercholesterolemia, hypertension and obesity) were considered as confounders. Data for all confounders were collected using face to face interview, laboratory blood analysis and physical examination. Dichotomised variables were as follows, sex: male and female, education: until middle school and above high school, smoking: smoker and non-smoker, drinking: alcohol drinker and non-drinker. Four systemic health components were as follows: 1) diabetes: high plasma glucose level (>126 g/dl) or having anti-diabetic medication, 2) hypercholesterolemia: high plasma cholesterol level (>240 mg/dl) or having anti-hypercholesterolemia medication, 3) hypertension: systolic >130 mmHg or diastolic >85 mmHg or having anti-hypertensive medication and 4) obesity: body mass index (BMI) calculated as kg of body weight divided by square meter of height ≥ 25 . Physicians measure the blood pressure and diagnosed the hypertension. The blood pressure was measure in the sitting position using mercury manometer. For biochemical variable, 12 hour fasting blood samples were drawn at recruitment.

Statistical analysis

The distribution of characteristic variables by periodontitis (no versus yes) were addressed using mean values with standard deviations (mean $\pm$ SD) for continuous variables and frequencies and proportion for categorical variables. Chi-square test was applied for categorical variables. Kolmogorov-Smirnov (K-S) test was applied to evaluate the normal distribution for continuous variables. When variables were in normal distribution, parametric tests were applied, otherwise non-parametric tests were applied. T-test were performed to evaluate difference in continuous variable with normal distribution. Mann-Whitney (M-W) test were applied for continuous variable without normal distribution. The relationships between values in the blood, GCF and saliva were analysed with Spearman's correlation test. Since number of participants were big ($n=149$), Analysis of covariance (ANCOVA) was applied to estimate adjusted means with standard errors (mean $\pm$ SE) of S100A8 and S100A9 levels after controlling for age, sex, education, smoking, drinking, diabetes, hypercholesterolemia, hypertension and obesity. The receiver operating characteristic (ROC) curve was applied for estimating c-statistics (area under the curve: AUC) as screening ability of S100A8 and S100A9 for periodontitis. Statistical significance was set at p-value

<0.05. Data were analysed using Statistical Package for Social Sciences (SPSS) version 25 (SPSS inc, Chicago, IL, USA).

Results

Characteristic of participants

One hundred forty-nine participants aged from 21 to 77 years were included in this study. The participants with periodontitis were older, more males, more hypertensive and more obese than healthy participants (Table 1).

S100A8 and S100A9 in saliva, blood, GCF by periodontitis

S100A8 and S100A9 in saliva, blood and GCF were not in normal distribution (K-S test, $p < 0.05$).

The representative level of S100A8 in saliva was higher in periodontitis participants than in healthy participants (M-W test, $p < 0.05$) (Figure 1). Although blood showed no difference in S100A8 according to periodontitis, GCF showed lower S100A8 in periodontitis participants than in healthy participants (M-W test, $p < 0.05$). However, S100A9 in GCF was lower in periodontitis participants than in healthy participants (K-S test $p < 0.05$). S100A9 in saliva and blood showed no difference.

The crude value of S100A8 in saliva was higher by two times in periodontitis participants than in healthy participants (T-test $p < 0.05$) (Table 2). After controlling for confounders, the adjusted value of S100A8 in saliva was also higher by 1.6 times in periodontitis participants than in healthy participants (ANCOVA, $p < 0.05$). In blood, the adjusted value of S100A8 was higher by 1.2 times in periodontitis participants than in healthy participants (ANCOVA, $p < 0.05$). However, both crude and adjusted values of S100A8 in GCF were lower by 70% in periodontitis participants than in healthy participants ($p < 0.05$). In terms of S100A9, GCF showed lower values in both crude and adjusted levels ($p < 0.05$).

Correlation between S100A8 and S100A9 in saliva, blood, GCF

Scatter plot showed that S100A8 in saliva was positively correlated to that of blood ($n=149$, $r=0.21$, $p < 0.05$) (Figure 2). This correlation was highlighted in healthy participants ($n= 50$, $r=0.32$, $p < 0.05$). However, S100A9 in saliva was negatively correlated to that in GCF among periodontitis patients ($n= 99$, $r= -0.20$, $p < 0.05$).

Screening ability of S100A8 and S100A9 in saliva, blood, GCF for periodontitis

ROC curve showed that salivary S100A8 had highest screening ability for periodontitis among S100A8 and S100A9 in saliva, blood, and GCF (Figure 3). S100A8 in saliva showed highest screening ability (c-statistics of 0.73, $p < 0.05$). S100A8 and S100A9 in GCF showed significant screening ability (c-statistics of 0.26 in S100A8 and 0.38 in S100A9 for periodontitis, $p < 0.05$), which addressed that its screening ability was 0.74 in S100A8 and 0.62 in S100A9 for non-periodontitis.

Table 1
Characteristics of the participants according to periodontitis status (n = 149)

Variable	Periodontitis		<i>p</i> -value
	No (n = 50)	Yes (n = 99)	
Age, mean ± SD	42.1 ± 15.1	55.8 ± 11.6	< 0.001 ^a
Sex, n(%)			0.005
Male	15 (30.0)	54 (54.5)	
Female	35 (70.0)	45 (45.5)	
Education, n(%)			0.054
Middle school	0 (0.00)	7 (7.1)	
High school or higher	50 (100.0)	92 (92.9)	
Smoking, n(%) [*]			0.262
No	48 (96.0)	90 (90.9)	
Yes	2 (4.0)	9 (9.1)	
Drinking, n(%) [†]			0.326
No	30 (60.0)	51 (51.5)	
Yes	20 (40.0)	48 (48.5)	
Diabetic [‡]			0.269

Bold denotes statistical significance at $p < 0.05$.

P-values: obtained by chi-square test for categorical variables and T-test ^afor continuous variables.

^{*}Smoking: No = never smoked, Yes = past and current smoker.

[†]Alcohol intake: No = never drunken, Yes = past and current drinker.

[‡]Diabetic: Yes = fasting plasma glucose >126 or taking diabetes medication.

[§]Hypercholesterolemia: Yes = plasma cholesterol > 240 or taking hypercholesterolemia medication.

[#]Hypertension: Yes = systolic blood pressure $\geq$ 140 mmHg or diastolic blood pressure $\geq$ 90 mmHg or taking hypertension medication.

[♦]Obesity: Body mass index (body kg/ height m²) $\geq$ 25.

Variable	Periodontitis		<i>p</i> -value
	No (n = 50)	Yes (n = 99)	
No	49 (98.0)	93 (93.9)	
Yes	1 (2.0)	6 (7.0)	
Hypercholesterolemia [§] , n(%)			0.102
No	49 (98.0)	90 (90.9)	
Yes	1 (2.0)	9 (9.1)	
Hypertension [#] , n (%)			0.005
No	49 (98.0)	81 (81.8)	
Yes	1 (2.0)	18 (18.2)	
Obesity [♦] , n (%)			0.031
No	40 (80.0)	62(62.6)	
Yes	10 (20.0)	37 (37.4)	
Bold denotes statistical significance at $p < 0.05$.			
<i>P</i> -values: obtained by chi-square test for categorical variables and T-test ^a for continuous variables.			
*Smoking: No = never smoked, Yes = past and current smoker.			
[†] Alcohol intake: No = never drunken, Yes = past and current drinker.			
[‡] Diabetic: Yes = fasting plasma glucose >126 or taking diabetes medication.			
[§] Hypercholesterolemia: Yes = plasma cholesterol > 240 or taking hypercholesterolemia medication.			
[#] Hypertension: Yes = systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg or taking hypertension medication.			
[♦] Obesity: Body mass index (body kg/ height m ²) ≥ 25 .			

Table 2
Differences in S100A8 and S100A9 by periodontitis (n = 149), unit = pg/ml

Type	Periodontitis		<i>p</i> -value
Protein	No	Yes	
Sample	(n = 50)	(n = 99)	
Crude mean [*]			
S100A8, Saliva	4635.53 ± 4621.86	8828.58 ± 5374.95	< 0.001
GCF	428.15 ± 456.15	150.34 ± 279.38	< 0.001
Blood	512142.2 ± 282315.06	587921.35 ± 188171.77	0.091
S100A9, Saliva	1293.8 ± 1891.66	1542.93 ± 2681.56	0.558
GCF	234.58 ± 323.48	88.39 ± 157.12	< 0.001
Blood	164681.43 ± 34912.69	169607.53 ± 22123.06	0.366
Adjusted mean [†]			
S100A8, Saliva	5373.44 ± 794.8	8455.9 ± 536.43	0.003
GCF	417.17 ± 55.84	155.89 ± 37.68	< 0.001
Blood	498796.49 ± 35611.9	594661.6 ± 24035.31	0.038
S100A9, Saliva	1437.91 ± 392.94	1470.15 ± 265.2	0.949
GCF	230.49 ± 36.31	90.46 ± 24.51	0.003
Blood	162294.02 ± 4141.63	170813 ± 2295.28	0.112
Bold denotes statistical significance at p < 0.05.			
<i>P</i> -values: obtained by T-test for crude mean and ANCOVA for adjusted mean.			
*Mean ± standard deviation.			
†Adjusted mean ± standard error: Values were adjusted for age, sex, education, smoking, drinking, obesity, diabetes, hypercholesterolemia, and hypertension by ANCOVA in GLM.			

Discussion

Our data showed that salivary level of S100A8 and S100A9 was validated by that of Blood according periodontitis in Korean adults, while those level of GCF did not validate salivary level of S100A8 and S100A9. Salivary S100A8 was positively correlated to that of blood, especially in healthy adults. However, salivary S100A9 was negatively correlated to that of GCF, especially in periodontitis patients. To the best of our knowledge, this is the first evidence that salivary S100A8 could be the best maker for screening periodontitis after comparing among S100A8 and S100A9 in saliva, blood and GCF. This result was

supported by the previous evidence that salivary S100A8/9 (calprotectin) was a significant marker, but that in serum was not [16].

Compare to the previous study, our study had a lot of advantage. Firstly, this study compared S100A8 and S100A9 levels among saliva, blood and GCF. Secondly, sufficient 149 number of participants were randomly recruited from the general population and there was no selection bias. Thus our results could be generalized. Thirdly, age, sex, smoking, drinking, education, diabetic, hypercholesterolemia, hypertension and obesity were considered as confounders for the adjustment. Fourthly, physical and dental examination were performed by physicians and trained dentists using UNC-15 probes and a panoramic radiograph. Fifthly, periodontitis was classified according to the recent New international classification of periodontitis [19]. Finally, concentrations of S100A8 and S100A9 were quantified using ELISA kits at picogram level.

Our data showed that elevated levels of S100A8 in saliva were significantly associated with periodontitis in Korean adults. A recent Korea study reported that salivary S100A8 levels were higher by 70% in periodontal disease than that of healthy participants [20]. S100A8 expression is up-regulated by oxidative stress, cytokine and growth factors [21] followed by activation of FcγRI and FcγRIV on macrophages through TLR-4 [22, 23], and aggrecanase enzymes from chondrocytes suggesting a role in pericellular matrix degradation [24]. Chinese and Swiss human studies [17, 25] reported positive results in GCF. An English study also showed that S100A8 in GCF was significantly higher in inflammatory gingival tissue than that of normal tissue [26]. However, our data showed that S100A8 in GCF was significantly higher in healthy participants than in periodontitis participants. Hence, more study on GCF S100A8 should be indicated to make certain the discrepancies of the results.

S100A9 involved in the regulation of inflammatory processes and immune response [27]. Calprotectin (S100A8/A9) is the marker for gingivitis and periodontitis [28, 29]. Down regulation of S100A9 protein could indicate insufficient immunity stimulated by the infection [30]. This protein also promotes apoptosis and modulate the inflammatory response in periodontal ligament cells so its downregulation could suggest a suppression of inflammation [31, 32]. Antimicrobial activity of S100A9 also have been reported. The mechanism behind antimicrobial activity is the monomeric form of amyloid beta (Aβ) 1–42 that is negatively regulated by the innate immune system by downregulating the secretion of S100A9 [33]. However, our data showed that only GCF S100A9 level was significantly lower in periodontitis patients than in healthy adults. Recently, a Korean study reported that S100A9 was decreased in periodontitis patients compared to healthy participant [20]. However, our S100A9 data did not show significant difference in both saliva and blood. Thus, more studies are indicated to clarify these discrepancies.

Our data showed that S100A8 in saliva and blood was positively correlated each other. This link was highlighted in healthy adults. This results showed the evidence that saliva represented systemic inflammation via blood. Similarly, salivary S100A9 was negatively correlated to that of GCF, especially in

periodontitis patients. As to periodontitis, saliva showed the changes in GCF due to inflammation. Further studies are needed to elucidate the mechanism of these results.

Our data showed that the screening ability of S100A8 for periodontitis was 0.73 of c-statistics, which was higher than the previous Korean study [20] with 0.6 of c-statistics and a bit lower than Austrian [16] calprotectin study with 0.86 of c-statistics. Among S100A8 and S100A9 in saliva, blood and GCF. Salivary S100A8 had the best screening ability for periodontitis. Since salivary S100A8 could be the best marker for periodontitis, a rapid test kit using salivary S100A8 could be effective on promoting periodontal health for general public. The next step of Salivary S100A8 research will be whether salivary S100A8 could be the prognostic marker for periodontitis. The intervention of periodontitis using periodontal treatment will elucidate the role and fate of S100A8 on periodontitis prognosis.

There are some limitations of this study. Firstly, samples were analysed using ELISA were stored more than one months. Long term storage of saliva might influence on the detection of salivary protein [34]. Secondly, elevated level of S100A8 and S100A9 observed in cancer and other inflammatory diseases. This could degrade diagnostic ability for periodontitis. Notwithstanding these limitations, our data is appropriate to meet the objectives of this study.

Conclusion

This study showed that elevated level of salivary S100A8 protein concentration could be a valid marker for the periodontitis screening. Thus S100A8 salivary kit will be useful for screening periodontitis. Further prospective studies including periodontal treatment will be indicated for elucidating the prognostic effect of salivary S100A8 for the promotion of periodontal health.

Declarations

Conflicts of Interest

There is no conflict of interest among the authors.

Author contributions

HD Kim designed and performed this study; ST Kim recruited participants; S Karna did ELISA for S100A8 and S100A9; YJ Shin and HJ Cho analyzed the data; HD Kim, S Karna and HJ Cho wrote the draft; H Vu carried out draft revision; HD Kim, S Karna, YJ Shin, HJ Cho and ST Kim proofread and finalized the manuscript.

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Figures

(A) S100A8

(B) S100A9

Figure 1

Distribution of S100A8 and S100A9 according to periodontitis. (a) S100A8 in saliva, blood and GCF. (b) S100A9 in saliva, blood and GCF.

(A) Spearman's correlation of salivary S100A8 with blood and GCF

(B) Spearman's correlation of S100A9 between saliva and GCF

Figure 2

Correlation of salivary S100A8 and S100A9 with blood and GCF according to periodontitis. (a) Salivary S100A8 with blood, GCF, blood in healthy and periodontitis participants. (b) Salivary S100A9 with blood, GCF, GCF in healthy and periodontitis participants.

Figure 3

Receiver operating characteristic (ROC) curve for screening ability of S100A8 and S100A9 in saliva, blood and GCF.