Detection of hepatocyte growth factor in oral rinses using water for possible periodontal diagnosis

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Abstract: The aim of this study is to analyze the relationship between Hepatocyte Growth Factor (HGF) levels in oral rinses using water and clinical parameters of periodontitis; and furthermore, to evaluate the potential of a prototype HGF immunochromatographic paper test strip (HGF-TS) for screening of periodontitis, in comparison with a commercially-available occult blood (hemoglobin) test strip (Hb-TS). Clinical periodontal parameters were recorded, and oral rinses were collected, from 125 subjects. Then, the presence of HGF, and hemoglobin (Hb), in each sample was detected using a prototype HGF-TS and an Hb-TS. In addition, the concentrations of HGF and Hb were also determined in each sample by ELISA. The positive rate and read value on HGF-TS showed significantly higher values in cases of severe periodontitis compared to healthy subjects. Hb-TS showed generally higher positive rates than HGF-TS; however, it showed false positive results in healthy subjects. The concentration of HGF in oral rinses showed close association with the severity of periodontitis, suggesting that the prototype HGF-TS has potential for use in the diagnosis of periodontitis, although further refinement of the test strip is required to increase the sensitivity.

Keywords: hepatocyte growth factor, immunochromatographic paper, mass-screening test, occult blood test, periodontitis

Introduction

Periodontitis is a chronic inflammatory disease, accompanied by bacterial infection, that is characterized by degradation of the periodontium; including connective tissue, cementum, and alveolar bone [1,2]. Monitoring and management of periodontitis has become important because almost half of the adult population has periodontitis [3-5], and a close association between periodontal and systemic diseases has been demonstrated [6].

Currently, the clinical diagnosis of periodontitis is mainly performed by assessment of probing pocket depth, bleeding on probing, and tooth mobility, in addition to radiographic examination of alveolar bone loss [2,7,8]. While these conventional procedures are beneficial for precise diagnosis of individual patients [9], they are impractical for mass-screening to identify subjects with periodontitis because pocket probing takes time and skill, and radiographic examination entails radiation exposure. Thus, an easier, patient-friendly, screening method is desirable for mass-screening, in particular, mass medical examinations.

Previously, Ohshima et al. [10-12] invented a salivary occult blood test using an immunochromatographic paper strip for detecting periodontal disease. The salivary occult blood test can efficiently detect oral hemoglobin (Hb), in saliva or oral rinses using water, using a special paper strip coated with anti-human hemoglobin monoclonal antibody. This Hb test is effectively used for detecting gingival and periodontal inflammation; however, it produces false positive results, even in healthy patients. Thus, a novel specific indicator of periodontitis is required for mass-screening of periodontitis.

Hepatocyte Growth Factor (HGF), also known as Scatter Factor, is a multifunctional protein secreted by mesenchymal cells, activated epithelial cells, and endothelial cells [13,14]. HGF regulates mitogenesis, morphogenesis, cell proliferation, angiogenesis, cell motility and matrix invasion [15,16]. Ohshima et al. [17,18] previously demonstrated that HGF level in gingival crevicular fluid (GCF) and saliva was well correlated with clinical parameters of periodontitis.

Based on these previous findings, it was hypothesized that HGF could be a promising indicator of periodontitis, and efforts were made to develop a laboratory screening method for periodontal diagnosis. Regarding clinical samples to be taken from subjects, it was considered that oral rinses using water would have the advantage of being non-invasive, as well as being quick and simple to collect, in a wide variety of clinical situations. However, the relationship between the HGF level in oral rinses, and periodontal status, has not yet been demonstrated.

Thus, the aim of this study is to first examine the relationship between the concentration of HGF in oral rinses using water and the progression of periodontitis, and subsequently to evaluate the effectiveness of a prototype HGF immunochromatographic test strip as a screening method for periodontitis, compared with the conventional salivary Hb test strip.

Materials and Methods

Subjects and clinical evaluation of periodontal status

A total of 125 subjects (60 male and 65 female) participated in this study. The subjects with periodontal disease were patients of the Department of Periodontics, Dental Hospital of Tokyo Medical and Dental University (TMDU). The healthy subjects were volunteers consisting of dentists in the Department of Periodontology, TMDU, as well as dentists and hygienists from private dental offices. Written informed consent for participation was obtained from all individuals. This study was approved by the Ethics Committee of the Faculty of Dentistry, TMDU (#1209), and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013, and the ethical guidelines for epidemiology research of 2002, as revised in 2008 (Ministry of Health, Labor and Welfare, Japan).

Subjects were selected according to the following criteria: absence of systemic diseases, no systemic antibiotics taken in preceding 3 months, and no pregnancy or lactation. Participants underwent a full-mouth periodontal examination including probing pocket depth (PD) and bleeding on probing (BOP) at all six sites of each tooth, as well as tooth mobility. Examinations were conducted by each patient’s dentist. Radiographic examination (bisection angle technique) was performed for some of the periodontitis patients and healthy subjects, and bone crest level (BCL) was determined. BCL was defined as the vertical distance (mm) from cemento-enamel junction (CEJ) to the most crestal point of marginal bone [19-21], and was calculated at the mesial and distal of all teeth on a calibrated computer screen. Since a standardized method for taking radiographs was not employed in the present study, the BCL ratio (%) was determined by...
deviation. *< 0.001 compared to H group (Kruskal-Wallis test with Steel-Dwass)
P < 0.01; ***
BGI, biofilm-gingival interface; H, healthy; G, gingivitis; P1, PD ≥ 4 mm and BOP <10
Occult blood detection in oral rinses was performed using a commercial kit (Quantikine human HGF immunoassay, R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Hb concentration in oral rinses
The oral rinses stored at −80°C were thawed on ice and diluted (2.86 to 3 folds, depending on the residual volume of each sample) with distilled water. Hb concentration in rinsed water was measured using a commercially available kit for latex agglutination turbidimetry (LZ test-Eiken HbAo, Eiken Chemical Co., Tokyo, Japan) according to the manufacturer’s instructions [24,25]. With this system, Hb measurement in saliva is performed after diluting saliva with a specific buffer solution which stabilizes the Hb before measurement. However, in the present study, the samples were already diluted during rinsing (and later in the lab); consequently the specific buffer solution was not employed.

Table 1 Characteristics of the groups according to BGI classification

| BGI classification | H | G | P1 | P2 | P3 |
|--------------------|---|---|----|----|----|
| number of subjects | 19| 3 | 32 | 50 | 21 |
| age (years)        | 45.3 ± 13.4 | 56.0 ± 25.2 | 57.2 ± 15.0* | 59.3 ± 13.7*** | 53.2 ± 15.9 |
| gender (male/female, number) | 7 | 1 | 2 | 19 | 26 |
| gender (male/female, %) | 7/2 | 1/1 | 3/2 | 19/26 | 14/7 |
| number of teeth | 27.2 ± 3.6 | 30.8 ± 3.9 | 25.4 ± 2.6 | 23.9 ± 4.4** | 25.1 ± 4.7 |
| mean PD (mm) | 1.9 ± 0.2 | 1.9 ± 0.1 | 2.4 ± 0.4*** | 2.9 ± 0.6*** | 4.2 ± 1.3*** |
| BOP positive sites (%) | 2.4 ± 2.6 | 12.2 ± 2.1 | 4.2 ± 3.1 | 24.7 ± 11.1*** | 69.8 ± 13.2*** |
| PD ≥ 4 mm (%) | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| PD ≥ 5 mm (%) | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| PD ≥ 6 mm (%) | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| mean tooth mobility | 0.12 ± 0.19 | 0.25 ± 0.27*** | 0.62 ± 0.70*** |

**Globin-gingival interface; H, healthy; G, gingivitis; P1, PD ≥ 4 mm and BOP <10; P2, PD ≥ 4 mm and BOP 10% to 50%; P3, PD ≥ 4 mm and BOP 50%; Data are presented as mean ± standard deviation. *P < 0.05; **P < 0.01; ***P < 0.001 compared to H group (Kruskal-Wallis test with Steel-Dwass).**

HGF concentration in oral rinses
HGF concentration in oral rinses was measured using HGF ELISA kit (Quantikine human HGF immunoassay, R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Hb detection by immunochromatographic paper test strip
HGF detection in oral rinses was performed using a prototype HGF paper test strip (HGF-TS), which is a newly-developed strip type assay kit, approximately 5 × 60 mm in size, using an immunochromatographic method [23] to allow visual determination of positive or negative results (Hokudo Corp., Sapporo, Japan). The HGF-TS is coated with anti-human HGF monoclonal antibody conjugated with latex on the test band area, so that the blue-color development induced by immunoreaction depends on HGF concentration. On the control band area, anti-mouse IgG antibody is coated for capture of unbound latex conjugate. The clearly visible control band serves as an indicator of assay validity.

One mL of oral rinse was added into a tube with buffer reagent, mixed gently for 5 min and filtered to remove mucus component. Then, 150 µL of the treated sample was dropped onto the end of the HGF-TS, and the liquid migrated upward along the strip through capillary action. After 30 min, visual judgement of the test band appearance was classified as positive (+, ++, ++++) or negative (−, − −, − − −) by 3 blinded examiners (AA, SK, and SM). The results were also evaluated by immunochromato-reader [23] to quantify the color intensity of the test band.

Statistical analysis
Data distribution was assessed using the Shapiro-Wilk test. Kruskal-Wallis test and Steel-Dwass was used to evaluate differences among the groups. Mann-Whitney U test with Bonferroni correction was performed to test the differences in proportions among the groups. Spearman’s rank-order correlation was applied for the evaluation of correlations. Statistical analyses were performed using JMP ver. 9.0 software (SAS Institute Inc., Cary, NC, USA). A P-value < 0.05 was considered statistically significant.

Results
Demographic data
A total of 125 subjects (age: 55.3 ± 15.4, male/female: 60/65) participated in this study. Demographic data for five groups of subjects categorized according to BGI classification, including age, gender, numbers of present teeth, and periodontal status are shown in Table 1. The Gingivitis group (n = 5) was excluded from statistical analysis because of the very low number of subjects. Data distribution about normality was evaluated with the Shapiro-Wilk test. All data did not allow normal distribution. Therefore, statistical methods for nonparametric statistics were applied. There was no significant difference in the percentage of male/female among the groups. Mean age was higher and number of teeth was lower in P1 and P2 compared with the Healthy group. Mean PD and percentages of PD ≥ 4, 5, and 6 mm were significantly different among the four groups (P < 0.001 vs. Healthy group). The percentage of BOP positive sites also showed significant differences among the four groups except between the Healthy group and P1 (P < 0.001 vs. Healthy group). The mean tooth mobility also showed significant differences among the four groups except between the Healthy group and P1 (P < 0.001 vs. Healthy group).

BGI in the subjects categorized by BGI classification is shown in Table 2. Compared with the Healthy group, BCL and BCL ratio were significantly increased in P1 (P < 0.01 and P < 0.001, respectively), as well as in P2 and P3 (P < 0.001 for both).
Correlation between HGF concentration and periodontal status

HGF concentrations in oral rinses in subjects classified according to BGI classification are shown in the Fig. 1A. HGF concentration in oral rinses in P2 was significantly increased as compared with the Healthy group (P = 0.03). Furthermore, HGF concentration in oral rinses in P3 was dramatically increased (P < 0.001 vs. all groups). HGF concentration was positively correlated with mean PD (P < 0.0001, ρ = 0.59) (Fig. 1B), percentage of BOP positive sites (P < 0.0001, ρ = 0.58) (Fig. 1C), mean tooth mobility (P < 0.0001, ρ = 0.49) (Fig. 1D), and BCL ratio (P < 0.0001, ρ = 0.49) (Fig. 1E).

Evaluation of HGF-TS

In the Healthy group, HGF-TS showed no detection of HGF in oral rinses. HGF-TS positive rates were 25.0%, 26.0% and 42.9% in P1, P2, and P3 groups, respectively, with a significant difference between P3 and the Healthy group (P < 0.01) (Fig. 2A). Sensitivity and specificity were 0.29 and 1.00, respectively. Positive and negative predictive values were 1.00 and 0.21, respectively.

The read values on HGF-TS by immunochromato-reader in the P3 were also significantly higher vs. the Healthy group (P = 0.04) (Fig. 2B). The read values on HGF-TS showed weak but positive correlation with HGF concentration (P < 0.0001, ρ = 0.37) (Fig. 2C). Although the read value did not show correlation with mean PD (Fig. 2D), mean tooth mobility (Fig. 2F) and BCL ratio (Fig. 2G), it showed significant but weak correlation with percentage of BOP positive sites (P = 0.02, ρ = 0.21) (Fig. 2E).

Correlation between Hb concentration and periodontal status

Hb concentration in oral rinses was significantly increased in subjects of groups P1 (P < 0.05), P2 (P < 0.001), and P3 (P < 0.001), compared with the Healthy group (Fig. 3A). In addition, Hb concentration in P3 was dramatically increased compared with P1 group (P < 0.001) and P2 (P < 0.001). Hb concentration was positively correlated with mean PD (P < 0.0001, ρ = 0.55) (Fig. 3B), percentage of BOP positive sites (P < 0.0001, ρ = 0.55) (Fig. 3C), mean tooth mobility (P < 0.0001, ρ = 0.48) (Fig. 3D), and BCL ratio (P < 0.0001, ρ = 0.48) (Fig. 3E).

Evaluation of Hb-TS

Positive rates of Hb with Hb-TS were 15.8%, 68.8%, 70.0% and 100.0% in Healthy, P1, P2, and P3, respectively (Fig. 4A) with a significant difference in P1 (P < 0.01), P2 (P < 0.001), and P3 (P < 0.001) compared to the Healthy group. The positive rates of P3 were significantly higher compared to P1 (P = 0.04) and P2 (P = 0.04). The sensitivity and specificity were 0.76 and 0.84, respectively. Positive and negative predictive values were 0.96 and 0.39, respectively.

The mean read values on Hb-TS are shown in Fig. 4B. There were significant differences in all the intergroup comparisons except for P1 versus P2. The read values showed strong positive correlation with Hb concentration in the oral rinses (P < 0.0001, ρ = 0.75) (Fig. 4C).

### Table 2 Bone crest level (BCL) of the groups according to BGI classification

| BGI classification | H | G | P1 | P2 | P3 |
|-------------------|---|---|----|----|----|
| BCL (mm)          | 1.2 ± 0.4 | – | 2.1 ± 0.4** | 2.9 ± 1.3*** | 4.4 ± 2.6*** |
| BCL ratio (%)     | 6.7 ± 1.1 | – | 13.9 ± 6.0*** | 19.5 ± 8.4*** | 29.1 ± 13.7*** |

*H*, healthy; *G*, gingivitis; *P1*, PD ≥ 4 mm and BOP < 10%; *P2*, PD ≥ 4 mm and BOP 10% to 50%; *P3*, PD ≥ 4 mm and BOP ≥ 50%. Data are presented as mean ± standard deviation. **P < 0.01; ***P < 0.001 compared to H group (Kruskal-Wallis test with Steel-Dwass).
The read values showed positive correlations with mean PD ($P < 0.0001, \rho = 0.59$) (Fig. 4D), percentage of BOP positive sites ($P < 0.0001, \rho = 0.56$) (Fig. 4E), mean tooth mobility ($P < 0.0001, \rho = 0.49$) (Fig. 4F), and BCL ratio ($P < 0.0001, \rho = 0.46$) (Fig. 4G).

**Correlation between HGF concentration/read value and Hb concentration/read value**

Although HGF concentration showed positive correlation with Hb concentration in oral rinses ($P < 0.0001, \rho = 0.62$) (Fig. 5A), read value on HGF-TS showed a weak positive correlation with Hb concentration ($P = 0.003, \rho = 0.27$) (Fig. 5B). In contrast, Read value on Hb-TS showed positive correlation with HGF concentration ($P < 0.0001, \rho = 0.63$) (Fig. 5C). However, read value on HGF-TS showed weak positive correlation with read value on Hb-TS ($P < 0.0001, \rho = 0.43$) (Fig. 5D).

**Discussion**

In the present study, for categorizing subjects, a new periodontal disease classification was used; the Biofilm-gingival interface (BGI) proposed by Offenbacher et al. [22]. In their epidemiologic study investigating subject-level factors and biologic phenotype associated with increasing severity of periodontal disease, they demonstrated that the combination of BOP and PD scores could be applied to represent distinct inflammatory, immune, and microbial characteristics that resulted in separate biologic phenotypes. BGI classification has been utilized in a number of clinical studies [26,27], and was considered helpful for effective and quick classification of patients into meaningful clinical subgroups [28].

According to BGI criteria, the subjects in the present study were successfully categorized into H, G, P1, P2, and P3 groups. Mean PD and percentages of sites with PD ≥ 4, 5, and 6 mm were significantly different among the four groups [not including Gingivitis (G) group]. All 4 groups considered, this clearly exhibited the progression of disease severity among groups.

**Currently, the diagnosis and classification of periodontitis mainly relies on traditional clinical assessment methods such as manual probing and radiographic examination. The evaluation of these clinical parameters, except for radiography, largely depends on the experience and skill of the operator, and there could be disagreement in clinical judgment. Thus, adjunctive or alternative biological diagnostic methods, based on objective, quantitative measures, are needed to assist in the diagnosis of periodontitis, as well as for use as a screening method for large populations.**

For mass-screening, saliva has been employed for biological analysis of periodontitis, since it can be collected by a non-invasive, simple, rapid, and low-cost procedure. Saliva contains gingival crevicular fluid (GCF), a periodontal tissue exudate which contains a variety of proteins and nucleic acids including growth factors, cytokines and microRNAs [29,30]. These
provide much useful information for the diagnosis of periodontal diseases. Thus, various substances in GCF have been suggested as useful biomarkers for detecting periodontitis such as host-derived enzymes and their inhibitors, inflammatory mediators and host-response modifiers, and byproducts of tissue breakdown [31]. However, most of them have not been studied applied except for a few systems such as aspartate aminotransferase enzyme kit (Periogard) [32], neutral proteases detection kit (Periocheck) [33], and occult blood test paper (Perioscreen) [10-12]. In the present study, as a novel biomarker, focus was placed on HGF production in periodontal pockets. Previously Ohshima et al. [34] revealed that HGF, or a closely related factor which is produced by periodontal ligament fibroblasts and gingival fibroblasts, is a predominant chemotractant for gingival epithelial cells. They suggested that HGF could be involved in the process of epithelial apical migration (epithelial downgrowth) in the progression of periodontitis. In fact, significant correlation of HGF level in GCF and saliva with the severity of periodontitis has been previously demonstrated [17,18,35].

To detect HGF in the current study, oral rinses using water were employed instead of saliva since oral rinses are much more easily collected from subjects than saliva, especially during mass examinations. The concentration of HGF in oral rinses has not yet been investigated. Furthermore, there is concern that the concentration of a target substance in GCF might not be precisely represented by its subsequent concentration in oral rinses. These disparities may be due to variations, during sampling, among patients’ secretion amount and methods of mouth-rinsing. Nevertheless, in the current study it was successfully demonstrated that the concentration of HGF in oral rinses increased proportionally according to periodontitis severity. HGF concentration was significantly higher in moderate and, in particular, severe periodontitis, where it showed positive correlations with clinical parameters such as PD, BOP, and tooth mobility. Furthermore, HGF concentration in oral rinses showed positive correlation with BCL ratio, suggesting a significant association between alveolar bone loss in periodontitis. Thus, it was confirmed that determination of HGF concentration in oral rinses may be useful for evaluating periodontal status. With HGF-TS, the positive rate in oral rinses was generally low, showing no or low correlation with clinical parameters. On the other hand, the positive rate and read values were significantly increased in severe periodontitis. Thus, the HGF-TS may have potential for diagnosis of periodontitis especially if further refinement of the test strip can increase its sensitivity.

On the other hand, recently the usefulness of Hb detection in saliva for periodontal diagnosis has been increasingly reported [11,36]. However, currently there is a lack of consensus with regard to salivary Hb detection due to differences in Hb measuring methods, and the definition of periodontitis employed in Hb studies. Thus, clinical application of Hb detection has not gained widespread use for periodontal examinations.

In the present study, Hb in oral rinses was the product of direct mixing of saliva with water in the oral cavity resulting in Hb concentration values that may have been considerably reduced. Despite this, Hb concentration in oral rinses showed significant positive correlations with clinical parameters and bone loss. Furthermore, the reaction of the Hb-TS to rinse samples showed significant correlation with clinical parameters. Nonetheless, Hb-TS was very sensitive and false positive results were produced in healthy subjects for both positive rates and read values. Ultimately, Hb-TS alone could not selectively detect periodontitis patients. Thus, adjunctive, or alternative, application of other biomarkers to Hb are required to allow Hb-TS to better discriminate periodontitis patients in mass screening. Regarding the role of HGF in periodontitis, Ohshima et al. [37,38] reported that co-culture of primary human gingival fibroblasts from severe periodontitis patients, with gingival epithelial cells, using a novel three-dimensional collagen gel-culture system, degraded the collagen gel significantly. HGF, one of the up-regulated genes in the periodontitis-associated fibroblasts [37,38], was involved in the collagen degradation (manuscript in preparation), suggesting that HGF may reflect the progression of periodontitis in periodontal pockets. HGF has also been reported to be one of the macrophage colony-stimulating factor (M-CSF) substitutes and can also influence canonical [receptor activator of NF-kB ligand (RANKL) /M-CSF-induced] osteoclast formation [39]. Also, in osteoarthritis, elevated HGF levels in osteoblasts reportedly contribute to the osteoblasts’ altered response to bone morphogenetic protein-2 and reduced mineralization capacity [40]. Since HGF may also be involved in alveolar bone resorption in periodontitis, the detection of HGF in saliva would reflect the alveolar bone loss as indicated in the present study.

In conclusion, the concentration of HGF in oral rinses using water showed close association with the progression of periodontitis, suggesting that the prototype HGF-TS has potential for use in the diagnosis of periodontitis, although further refinement of the test strip is required to increase the sensitivity.

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Conflict of interest

The authors declare no conflict of interest in this study.

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