

**Short Communication**

## **Comparison of Salivary Cytokine Levels in Oral Cancer Patients and Healthy Subjects**

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### **Abstract**

In order to find informative salivary biomarkers specific to oral cancer we examined expression of 4 kinds of cytokine in saliva. Levels of interleukins (IL-1 $\beta$ , -6, -8) and osteopontin were measured by ELISA using whole saliva samples collected from 19 patients with oral cancer (9 men, 10 women; mean age, 60.9 years) and 20 healthy persons (15 men, 5 women; mean age, 32 years). Expression of the 4 cytokines was higher in patients with oral cancer than in healthy controls. The difference was significant in IL-6, in particular.

The results suggest that saliva offers a potential target for a screening test aimed at detection of precancerous lesions.

Key words: Saliva—Oral cancer—Screening test—IL-6

### **Introduction**

Despite growing success in tackling low-stage cases of oral cancer due to progress in treatment, the number such cases and the death rate have continued to increase each year in proportion with the increased aging of society<sup>3)</sup>. This suggests that health care facili-

ties are, as yet, not diagnosing and treating oral cancer at an early enough stage.

While oral cancer is quite often discovered in a patient during dental treatment, there are, as yet, no specific tumor markers that enable the effective and simple detection and diagnosis of oral cancer. In many cases of suspected cancer, a prompt diagnosis at the

primary medical institution is not made, and patients are referred for examination to specialists at secondary or tertiary medical institutions only after the disease has developed to an advanced stage. The result of this delay in diagnosis is a missed opportunity for early treatment that forces the patient to undergo extended treatment.

In response to this situation, we have attempted to find biomarkers for oral cancer in saliva that would allow the development of a screening test for oral cancer utilizing whole saliva samples, which can be collected non-invasively and repeated easily during the practice of routine clinical dentistry. The widespread employment of such a screening test at general dental clinics has the potential to greatly increase the early discovery and treatment of oral cancers. El-Naggar *et al.*<sup>2)</sup> reported a screening test for oral cancers utilizing saliva. Although the study pointed out a certain degree of genetic heterogeneity in saliva, it failed to detect any specific markers, making it difficult to apply this to a screening test. In this study, we compared IL-1 $\beta$ , IL-6, IL-8, and osteopontin, interleukins (herein-after referred to as IL), present in the saliva of both healthy subjects and patients with oral cancer, and identified proteins that might be useful as markers.

## Materials and Methods

### 1. Samples

Samples were obtained from 19 Japanese patients with T1- or T2-type tongue and gum cancer (9 men and 10 women; mean age, 60.9 years) who visited our department between April, 2003 and October, 2004. Informed consent was obtained from all patients and their families. We also recruited a control group of 20 healthy subjects (15 men and 5 women; mean age, 32 years). This study was approved by the Institutional Ethics Committee of Tokyo Dental College and the National Hospital Organization Tokyo Medical Center.

### 2. Comparison of IL-1 $\beta$ , -6, -8, and osteopontin in saliva of oral cancer patients

Saliva collection was performed using the 'draining (drooling)' method in accordance with the method of Li *et al.*<sup>7)</sup>, obtaining 3-ml whole saliva samples. Unstimulated saliva samples were collected between 9 am and 10 pm. Subjects were asked to refrain from eating, drinking, smoking, or oral hygiene procedures for at least 1 h prior to saliva collection. Saliva samples were subjected to centrifugation at 3,500 rpm for 15 min at 4°C. The fluid phase was then removed, and RNase inhibitor (Superase-In, RNase Inhibitor, Ambion Inc, Austin, TX, USA) and protease inhibitors (aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate) (Sigma, St Louis, MO, USA) were then added promptly on ice<sup>6)</sup>.

Measurements were performed by ELISA with the Human ELISA Kit (IL-1 $\beta$ , -6, -8, Pierce Endogen, IL, USA) and Human Osteopontin Kit (IBL Co. Ltd. Japan) in accordance with the manufacturer's protocol. Absorbency was 450 nm and measured by microplate reader.

### 3. Statistical analysis

The distribution of IL-1 $\beta$ , -6, -8 levels in saliva was computed and compared between the oral cancer patients and healthy subjects using 2 independent group *t*-tests.

Differences were considered significant for *p* values of less than 0.01. Because of the range of IL-1 $\beta$ , -6, and -8 levels, log transformations of these measures were also used in the analyses. Data were expressed as the mean  $\pm$  SD.

## Results and Discussion

We noted increased levels of IL-1 $\beta$  in oral cancer patients. While patients with oral cancer revealed an average of 158.9 pg/ml of IL-1 $\beta$ , only 14.1 pg/ml of IL-1 $\beta$  was detected in the control group. Oral cancer patients showed elevated values of IL-1 $\beta$  in significant differences (*t*-test: *p*<0.05) (Fig. 1). The amount

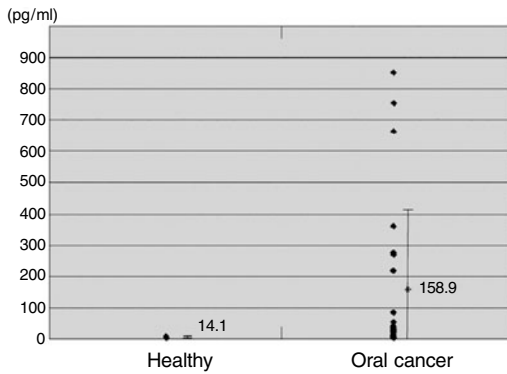
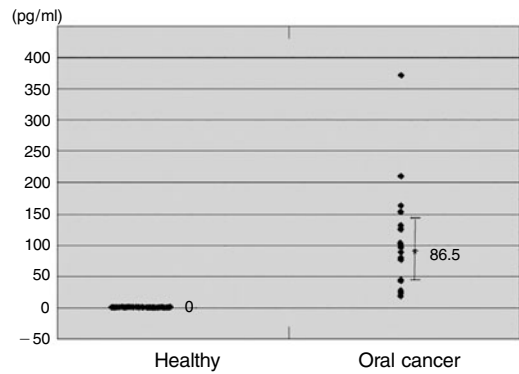
Fig. 1 IL-1 $\beta$  from whole saliva

Fig. 2 IL-6 from whole saliva

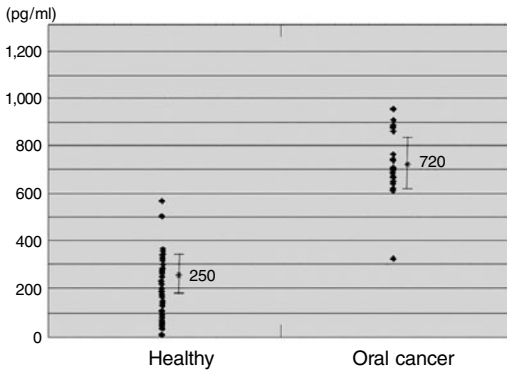


Fig. 3 IL-8 from whole saliva

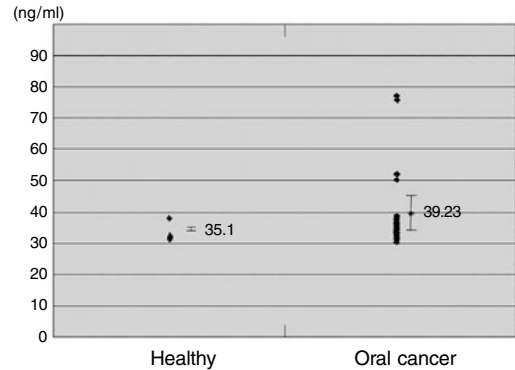


Fig. 4 Osteopontin from whole saliva

detected was minute in most cases; however, individual differences were significant. While 7 oral cancer patients showed increased levels, no connection was established with clinical findings.

Although an average 86.5 pg/ml IL-6 was detected in patients with oral cancer, it was not detected in any patients in the control group. These elevated IL-6 values in the oral cancer patients yielded a significant difference ( $t$ -test:  $p < 0.05$ ) (Fig. 2). These increased values, however, showed no correlation with clinical findings.

An average 720.0 pg/ml IL-8 was detected in patients with oral cancer, and an average 250.0 pg/ml was detected in the control group. This indicated high values in patients with oral cancer; however, no significant differences were noted (Fig. 3).

Osteopontin was detected at an average of 39.23 ng/ml in patients with oral cancer and 35.1 ng/ml in the control group. Although a slight increase in patients with oral cancer was noted, this increase was not significant (Fig. 4). However, 4 patients with oral cancer exhibited remarkably high levels. The 2 patients exhibiting the highest values among these 4 patients were the T2 cases of lower gingival cancer, while the other 2 were T2 cases of tongue cancer. The results of this study did not, however, reach far enough to identify a relationship with clinical findings.

We have performed oral cancer screenings in our department in cooperation with dental associations in Chiba prefecture since 1992. A total of 2,070 patients underwent screening between 1992 and 2001. The detection rate of oral cancer among these subjects was

0.14%<sup>12)</sup>. Screening was carried out directly by oral surgeons specializing in oral cancer. Accordingly, the number of patients undergoing screening has been limited, and expanded screening is, at present, difficult to achieve. Meanwhile, 80% of the oral cancer patients who visit our department are referrals from dental clinics. Among these are patients who were seen at our department more than one month after their visit to the dental clinic, and whose advanced stage of oral cancer required major surgery. These circumstances led us to target saliva as a potential source of cancer markers. Saliva can be collected accurately, non-invasively and repeatedly; therefore, it is very convenient for both dental practitioners and patients.

Streckfus *et al.*<sup>11)</sup> and Bonassi *et al.*<sup>1)</sup> reported that certain proteins, which were biomarkers for diseases developing in organs other than the oral cavity, also appeared in saliva. However, no proteins that specifically indicate oral cancer have been detected in saliva, to date. In this study, we endeavored to detect and measure the quantity of IL-1 $\beta$ , -6, -8, and osteopontin<sup>4,6)</sup> in saliva, the levels of which are easy to detect using a kit. IL-1 $\beta$  and -8 were found in increased levels in patients with oral cancer. However, the increased levels of IL-1 $\beta$  cannot be considered specific to oral cancer due to the patterns of increase. In addition, IL-8 yielded higher values in patients with oral cancer than in the control group, and a wide range of increase was observed in the control group, although their overall values remained low. There were no differences in osteopontin levels between the control group and patients with oral cancer; however, 4 patients with oral cancer showed an increase greater than one SD. Two of those patients were T2 cases of gum cancer; however, we were unable to verify whether the increased levels were a result of the development of the cancer. Meanwhile, IL-6 was not detected in the control group, but we did observe significant differences in the increase of IL-6 among patients with oral cancer. This is consistent with the results for IL-6 and -8 levels in whole saliva in patients with oral or pharyngeal cancer reported by

St. John *et al.*<sup>10)</sup>.

Luz compared IL-6 and -2 $\beta$  in salivary gland and serum of young adults (20–40 yrs) with that in elderly subjects (60–91 yrs) and reported increased levels of IL-1 $\beta$  in elderly subjects<sup>8)</sup>. In addition, Fagiolo also measured IL-1 $\beta$  in serum in young adults and elderly subjects, and noted increased levels of IL-1 $\beta$  in elderly subjects<sup>3)</sup>. In this study there was an age difference between the control group and oral cancer subjects. Therefore, the present results do not definitively indicate how oral cancer contributes to elevated IL-1 $\beta$  and -6 levels. Supplemental verification regarding age differences in salivary cytokines will be necessary in future research.

Rosin *et al.*<sup>9)</sup> and Li *et al.*<sup>6)</sup> reported that IL-1 $\beta$ , -6, and -8 in saliva increased with periodontal infection; however, oral cancer-associated increase may be correlated with the development of tumor cells, with the subsequent increase being even greater than that induced by periodontal disease. St. John *et al.*<sup>10)</sup> reported that IL-6 and -8 in saliva reflected concentrations in the serum of cancer patients. In this study, IL-1 $\beta$  and -8 also showed an increase in the control group. IL-6 was not detected in the control group; however, an increase in patients with oral cancer was detected. Therefore, we believe that IL-6 is a potential biomarker. Examination via microarray is necessary to verify the causes of increase in IL-6 in saliva, and to investigate the influence of gum disease.

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