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Research Paper
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Evaluation of saliva and plasma cytokine biomarkers in patients with oral squamous cell carcinoma

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Abstract. The aim of this study was to investigate potential biomarkers in human saliva and plasma to aid in the early diagnosis of oral squamous cell carcinoma (OSCC). Saliva and plasma samples obtained from OSCC patients (n = 41) and non-oral cancer patients (n = 24) were analyzed by Luminex Bead-based Multiplex Assay. Data were analyzed using the non-parametric Mann–Whitney U-test, Kruskal–Wallis test, and receiver operating characteristics curve (ROC) to evaluate the predictive power of 14 biomarkers individually for OSCC diagnosis. The plasma level of IP-10 in early OSCC differed significantly from that in controls. Among the salivary biomarkers, IL-1β, IL-6, IL-8, MIP-1β, eotaxin and IFN-γ and TNF-α showed significant differences between OSCC patients and controls. With respect to carcinogenesis, significant differences in plasma levels of eotaxin, G-CSF, and IL-6 were found between OSCC stages III/IV and OSCC stages I/II. The area under the curve (AUC) for OSCC vs. control was greater than 0.7 for plasma IP-10 and saliva IL-1β, IL-6, IL-8, and TNF-α. The study findings indicate that salivary biomarkers may serve a useful role as a complementary adjunct for the early detection of oral OSCC. With regard to the evaluation of tumour progression, plasma eotaxin, G-CSF, and IL-6 may help in the detection of advanced OSCC. However, the correlation between saliva and plasma biomarkers in OSCC was weak.

Key words: Luminex Bead-based Multiplex Assay; salivary biomarker; plasma biomarker; oral squamous cell carcinoma; ROC.

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According to the Health Promotion Administration of Taiwan, oral cancer is the fifth most prevalent cancer in the country (ranking fourth in males). When oral squamous cell carcinoma (OSCC) patients are diagnosed in the advanced stage, the prognosis is poor. Early diagnosis and early treatment are important, resulting in superior outcomes in OSCC patients. Risks factors for OSCC include alcohol consumption, betel nut chewing, and cigarette smoking. OSCC may also arise from a molecular mutation that leads to carcinoma[1].

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In head and neck squamous cell carcinoma (HNSCC), the tumour and surrounding lymphocytes may produce altered cytokine levels[2]. The interaction between the immune cells and tumour cells is not fully understood. Studies suggest that cytokines with proinflammatory, pro-angiogenic, and immunoregulatory activity can stimulate tumour cell proliferation[3,4]. The use of body fluids, i.e., saliva or serum/plasma, has shown considerable promise for the early diagnosis of cancers, including breast cancer, prostate cancer, and lung cancer, among others[5–7].

Human saliva is a multi-component oral fluid that may play a potential role in the diagnosis of cancer. Saliva contains water (95%), proteins, minerals, nucleic acids, and electrolytes[8]. Saliva in the oral tumour milieu may directly reflect tumour characteristics[9]. However, the correlation between saliva and plasma/serum cytokine levels remains unclear. Brailo et al. reported that altered cytokine levels produced in oral cancer in the oral cavity are not reflected in serum cytokine concentrations[10]. Some cytokines that could not be detected or were present at a low concentration in serum were found at a higher concentration in saliva. Although oral inflammation could result in a higher concentration of salivary cytokines, no significant differences were observed in periodontal disease[10]. OSCC patients often have periodontitis due to poor oral hygiene, and thus the influence of inflammation by cytokine expression is a concern.

Besides lymphocytes, tumour cells can also produce cytokines[10]. The proinflammatory cytokines can lead to up-regulation of positive cell cycle regulators in various pathways, such as nuclear factor kappa B (NF-κB), signal transducer and activator of transcription (STAT), and the mitogen-activated protein kinase/extracellular signal-regulated pathway[10]. Cytokines are intercellular signalling proteins that play a role in regulating growth, cellular proliferation, angiogenesis, and tissue repair[11]. Some are proinflammatory, such as interleukin (IL)-1β, IL-6, IL-8, and tumour necrosis factor alpha (TNF-α); others, such as IL-10, are anti-inflammatory[12].

Chemokines may exert a direct effect on cellular transformation and an indirect effect on angiogenesis to promote tumour growth. The molecules responsible for this biological effect belong to a sub-family of CXC that has a conserved ELR amino acid motif[13]. One such chemokine is growth-related oncogene (GRO). This chemokine was initially identified and purified from serum-free culture supernatants of malignant melanoma cell lines and characterized as an autocrine growth factor[14]. Furthermore, several chemokines, such as interferon gamma-induced protein 10 (IP-10, or CXCL10), can inhibit angiogenesis. In lung cancer, the degree of malignancy has been correlated with the level of secretion of IP-10 by the tumour. Less progressive lung carcinoma secretes more IP-10[15].

Biomarker quantitation is a more objective evaluation modality compared to an optical examination in the adjunctive diagnosis of OSCC[16]. The enzyme-linked immunosorbent assay (ELISA) has been used widely in previous investigations of biomarker expression[1,3,11,17]. The major limitations of ELISA are the time-consuming protocol and the relatively high cost when analyzing a small number of analytes[1].

At present, no single biomolecule has met the clinical requirement for high accuracy in identifying early disease onset[18]. An assay for the simultaneous survey of multiple cytokine biomarkers in OSCC patients is still lacking[19]. The recent introduction of multiplex assays offers the potential for a large amount of data to be obtained from small samples. The use of such assays also provides optimum consistency, as all samples and cytokines are tested under the same conditions[20].

Although some studies in OSCC patients have investigated biomarkers in saliva or serum/plasma individually, few studies have analyzed both salivary and plasma biomarkers simultaneously. The purpose of this study was to detect diagnostic biomarkers in early OSCC and to identify biomarkers that may reflect tumour progression. A multiplex immune bead-based suspension system was used to detect 14 different cytokines at the same time. The correlation between saliva and plasma cytokine levels was also evaluated.

Materials and methods

Patients

All participants were recruited from the Department of Oral and Maxillofacial Surgery of Taichung Veterans General Hospital, Taiwan. The institutional review board-approved consent form (CE14342B) was signed by each participant before specimens were collected for the experiments. All OSCC patients underwent surgery, including wide tumour excision and neck lymph node dissection. The study population consisted of three groups: group 1 comprised non-oral cancer patients ($n = 24$) who served as controls, group 2 was composed of OSCC patients with stage I or II disease ($n = 22$), and group 3 patients had been diagnosed with OSCC stage III or IV ($n = 19$). No patient had a history of prior malignancy, and those with osteomyelitis, HIV infection, active infection, immunodeficiency, autoimmune disease, or hepatitis were excluded from the study.

Sample collection

Blood and saliva samples were collected from each subject before any surgery. For the collection of saliva, the donors had to avoid eating, drinking, smoking, and using oral hygiene products for at least 1 h before the procedure. A 3-ml blood sample was collected from each patient using a disposable vacuum blood collection needle and ethylenediaminetetraacetic acid (EDTA) blood collection tubes under aseptic conditions by venipuncture. The plasma was separated by centrifugation at 2000 rpm for 10 min at 4 °C. Saliva was separated by centrifugation at 3000 rpm for 20 min at 4 °C. The samples were then divided into 1.5-ml aliquots and immediately stored at −80 °C until the laboratory analyses.

Luminex Bead-based Multiplex Assay

The Human Cytokine/Chemokine Magnetic Bead Panel (MILLIPLEX MAP) was performed according to the manufacturer's protocol (EMD Millipore, Billerica, MA, USA). Specimens were centrifuged at 3000 rpm for 15 min at 4 °C prior to analysis by bead-based multiplex assay. First, the 96-well plate was prewashed with wash buffer. Twenty-five microlitres of assay buffer, 25 μl of matrix solution (saliva samples without matrix solution), 25 μl of sample, and 25 μl of mixed magnetic beads were added to each well: vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), eotaxin, granulocyte colony stimulating factor (G-CSF), GRO, interferon gamma (IFN-γ), IL-1α, IL-1β, IL-6, IL-8, IL-10, IP-10, macrophage inflammatory protein 1 beta (MIP-1β), and TNF-α. The plate was then sealed and incubated overnight at 4 °C with shaking. Afterwards, the plate was washed twice with wash buffer, following which 25 μl detection antibodies were added to each well. Next, 25 μl streptavidin–phycoerythrin was added to each well and incubated for 30 min at room temperature. Finally, the plate was washed twice and 150 μl sheath fluid was

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