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Elevated salivary endothelin levels in oral cancer patients——A pilot study

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Summary The analysis of saliva has been proposed as a potentially rapid, non-invasive method to monitor and diagnose patients with oral disease. In this study we measured salivary endothelin-1 (ET-1) levels in patients diagnosed with oral squamous cell carcinoma (SCC) prior to treatment. We demonstrate significantly elevated salivary ET-1 levels in the oral SCC group (4.37 ± 1.35 pg/ml), relative to the control group (1.16 ± 0.29 pg/ml). ET-1 and ET-1 mRNA were also measured in oral SCC tissue specimens and compared to normal oral epithelial controls. The concentration of ET-1 in the oral SCC specimens was 17.87 ± 4.0 pg/ml and in the normal epithelial controls the concentration of ET-1 was 5.43 ± 2.5 pg/ml. ET-1 mRNA was significantly overexpressed in 80% (8/10) of the oral SCC specimens. Our results demonstrate the potential utility of salivary analysis for ET-1 levels to monitor patients at risk for oral SCC.

Introduction

Salivary analysis is theoretically ideal for the diagnosis of oral or systemic disease given that saliva is readily available. The identification and measurement of a salivary biomarker would be particularly beneficial for screening patients at risk for oral squamous cell carcinoma (SCC). Patients who have had oral SCC have a 26—47% of developing a recurrence within two years of surgical resection and an annual 5% chance of developing a second oral primary SCC.[1] Screening patients with a history of oral cancer, those at risk because of alcohol and tobacco use, or those who have a potentially malignant epithelial lesion could be significantly improved with non-invasive salivary analysis.

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In this study we evaluated untreated oral cancer patients for salivary endothelin-1 levels (ET-1). ET-1, a vasoactive peptide, is normally synthesized by human keratinocytes,[2] and over-produced by a number of malignancies, including prostate,[3] lung,[4] breast,[5] and colorectal carcinoma.[6] Patients with bronchial carcinoid[7] and hepatocellular carcinoma[8] have elevated tissue expression and elevated plasma ET-1 levels. Salivary ET-1 levels have not been measured in patients with cancer of any primary and ET-1 production by oral SCC has not been reported. However, because ET-1 is easily measured in saliva[9,10] it is an attractive biomarker for oral cancer. The purpose of the present study was twofold: (1) to determine whether oral SCC overexpresses ET-1 and (2) whether salivary ET-1 levels in oral SCC patients are higher than salivary ET-1 levels in normal controls.

Materials and methods

Oral SCCs and normal epithelial control specimens

For ELISA, fresh, unfixed specimens, consisting of eight oral SCCs and three normal epithelial controls were used. Ten oral SCC specimens were studied for real time RT-PCR. All specimens were randomly selected from the UCSF Oral Cancer Research Center Tissue Bank. The UCSF Committee on Human Research approved collection of oral epithelium. Patient consent was obtained for use of all specimens. All cases were reviewed to confirm the histological diagnosis based on established histological criteria.

Saliva collection

Whole saliva was collected from eight patients, diagnosed with oral squamous cell carcinoma who had not been treated, and eight healthy individuals serving as controls. The samples were collected between 6:30 AM and 8:00 AM. All subjects had nothing by mouth since midnight the previous day. Patient consent was obtained for use of all samples. Upon collection, saliva samples were centrifuged at 14,000 rpm for 20 min at 4 °C. The clear supernatants were removed, aliquoted, and immediately stored at −80 °C until further usage.

ELISA measurement of ET-1 in oral SCCs, normal epithelial controls, and saliva

ET-1 levels were quantified in oral SCCs, normal epithelial controls, and saliva by ELISA. The samples were prepared as follows. 20—40 mg of frozen tissue was homogenized in the T-PER Reagent (Pierce Biotechnology, Inc., Rockford, IL). The lysates were centrifuged at 13,000 rpm for 5 min. The supernatants were removed, aliquoted and stored at −80 °C. The extraction of ET-1 was performed using a BondElut® C_{18}, 200 mg column (Varian, Inc., Lake Forest, CA). The column was activated by addition of 1 ml of methanol followed by 1 ml of water, and 1 ml of 10% methanol. 500 μl of tissue lysates or saliva samples were acidified with an equal volume of 20% acidic acid and centrifuged at 14,000 rpm for 10 min. The supernatants were removed and applied to a column. The cartridge was washed with 2 ml of ethyl acetate. The peptides were eluted with 1 ml of methanol/0.5 M ammonium bicarbonate (80/20 v/v). The eluants were evaporated to dryness using a centrifugal concentrator under vacuum (Eppendorf, Westbury, NY), reconstituted with assay buffer, and assayed immediately. The concentration of ET-1 was measured by ELISA using a TiterZyme® Immunoassay Kit (Assay Designs, Inc., Ann Arbor, MI). The concentrations were calculated from the standard curve using a four-parameter logistics data reduction software (Bio-Rad Laboratories, Inc., Hercules, CA). The standard curve was generated using the following concentrations: 100, 50, 25, 12.5, 6.25, 3.1, 1.56, and 0.78 pg/ml. The optical density of the standards and samples was read at 450 nm wavelength using a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA). Results are reported as mean ± standard error.

Real-time quantitative RT-PCR measurement of ET-1

ET-1 mRNA expression was measured in oral SCCs and normal oral epithelial controls. mRNA expression was assessed using real-time quantitative RT-PCR as previously described.[11,12] Total RNA was extracted from frozen tissues using RNeasy-®Mini Kit (Qiagen, Inc., Valencia, CA). 15—25 mg of tissue was homogenized in the lysis buffer provided in the kit. The lysate was then applied to an RNeasy mini spin column, and total RNA was eluted according to the manufacturer's instructions. Reverse transcription was performed using Gibco BRL Reverse Transcriptase kit (Life Technologies, Carlsbad, CA). We purchased Assays-on-Demand Expression assays from Applied Biosystems (Foster City, CA). Samples were run on an ABI 7700 Prism (PE Biosystems, Foster City, CA). Relative expression of ET-1 mRNA was calculated using the comparative Ct method.[11] This method of analysis was selected because the slopes of the dilution standard curves for ET-1 and the reference gene β-*N*-acetyl-glucosaminidase (β-GUS) were comparably similar across a range of input RNA. Thus differences in relative abundance for RNA species expressed at low levels would not distort the analysis. Moreover, absolute threshold cycle (Ct) values for each sample were found to lie within the range of RNA quantities used for standard curve generation. Analysis was carried out using the software supplied with the ABI 7700 Prism (PE Biosystems). ET-1 mRNA overexpression was defined as expression >1.0 relative to normal oral epithelial controls.

Immunohistochemistry

Immunohistochemistry for ET-1 was performed on 5 μm formalin-fixed paraffin-embedded tissue sections as we have described.[11] Briefly, the sections were deparaffinized and incubated with trypsin for 20 min at 37 °C for antigen retrieval. The endogenous peroxidase was quenched by immersing the sections into the freshly made 3% hydrogen peroxide solution for 5 min. The non-specific binding was blocked with normal goat serum for 20 min. The sections were then incubated with the ET-1 mouse monoclonal primary antibody (Affinity Bioreagents, Golden, CO), 1:250 overnight at 4 °C. Incubation with the secondary antibody was performed using a Vectastain®Elite ABC Kit (Vector Laborato-