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ORIGINAL RESEARCH

Evaluation of the Role of Salivary Transcriptome as a Diagnostic Aid in Oral Precancer (Homogeneous Leukoplakia) and Oral Cancer

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is considered to be the most common oral cancer that makes up 90% of all oral cancers. If diagnosed early, it has a 5-year survival rate of around 80 to 90%. Lowered mortality rate for oral cancer can be achieved only through early detection and management of potentially premalignant lesions that would enable to increase the survival rate of the population suffering from oral cancer. Saliva is the reservoir of innumerable biomolecules whose levels reflect systemic health and disease status. Hence, saliva reflects the body in health and disease. It is conceivable that OSCC-associated ribonucleic acid (RNA) can find its way into the saliva. Early diagnosis and intervention remain the best crux to achieve better prognosis and reduce functional loss. There is a need to develop a rapid and less invasive screening tool that will allow screening of oral cancer and precancer on a large scale. However, the final diagnosis can be conferred with a conventional biopsy procedure. Yet, a noninvasive procedure, such as saliva collection has gradually proven to be an effective diagnostic tool for screening of oral cancer and, more importantly, precancerous lesions.

Keywords: Biomarkers, Messenger ribonucleic acid, Saliva.

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INTRODUCTION

As per the International Classification of Diseases, oral cancer refers to a subgroup of head and neck malignancies that develop at the lips, tongue, salivary glands, gingiva, floor of the mouth, oropharynx, buccal surfaces, and other intraoral locations.[1] Oral squamous cell carcinoma is considered to be the most common oral cancer that makes up 90% of all oral cancers. If diagnosed early, it has a 5-year survival rate of around 80 to 90%.[2,3] The incidence of oral cancer has been witnessed to vary in different parts of the world. The high prevalence of oral cancer in India and other Asian countries is attributed to the risk habits and factors, such as tobacco smoking and betel quid chewing.

Lowered mortality rate for oral cancer can be achieved only through early detection and management of potentially premalignant lesions. This would enable the increase of the survival rates of the population suffering from oral cancer. Apart from standard visual and tactile examinations, newer techniques, such as vital staining, visualization adjuncts (VELscope and ViziLite), and transepithelial sampling of oral mucosa for cytologic analysis (OralCDx Brush Test system) have been proved to enhance the ability to diagnose the premalignant lesions with great ease.[4] Nevertheless, tissue biopsy and histopathological examination remain the gold standard for oral cancer diagnosis.

SALIVA AS A BIOMARKER

Saliva is the reservoir of innumerable biomolecules whose levels reflect systemic health and disease status. Hence, saliva reflects the body in health and disease.[5] The stages that conclude in oral carcinogenesis display histopathologic changes from mild-to-moderate hyperplasia, to severe dysplasia followed by carcinoma *in situ* and finally precipitating as invasive squamous cell carcinoma. It is a process accompanied by genetic mutations and expression changes of many genes at a molecular level that leads to uncontrolled cellular growth.[6]

Presently, OSCC is confirmatively diagnosed through tissue biopsy and histopathological examination. In order to achieve early detection of squamous cell carcinoma, unlike biopsy, we need a noninvasive screening tool (biomarker test).

In 1998, the National Institutes of Health defined the biomarker as a characteristic, i.e., an objectively measured and evaluated indicator of normal biologic processes, pathologic processes, or pharmacologic responses to therapeutic intervention.[7] Biomarkers are molecular signatures that are unique to a certain disease (e.g., oral cancer). Biomarkers of cancer could include a broad range of biochemical entities, such as nucleic acids, proteins, sugars, lipids, and small metabolites, cytogenetic and cytokinetic parameters as well as whole tumor cells found in the body fluid.

Salivary biomarkers for oral cancer can be studied as protein- and RNA-based biomarkers. Protein-based biomarkers include a group of biomarkers consisting of cytokines, fibroblast growth factor, cyfra 21-1, cancer antigen-125, tissue polypeptide antigen, endothelium, matrix metalloproteinases, glutathione transferase, and superoxide dismutase.[5] The RNA-based biomarkers are the recently discovered biomarkers, currently the topic of research, including messenger RNAs and micro RNAs.

The current topic of research focuses on the use of saliva as a mode of diagnostic tool for early detection of OSCC. Due to the simple, noninvasive, and inexpensive mode of collection, saliva should be considered as a diagnostic medium of choice for early detection of OSCC.

The aim of the following study was to establish and evaluate the role and utility of salivary transcriptome as a diagnostic aid in oral precancer (homogeneous leukoplakia) and oral cancer. The study also tries to compare the salivary transcriptomes in (a) oral cancer, (b) oral precancer (homogeneous leukoplakia), and (c) healthy individuals, free of oral cancer and precancer (homogeneous leukoplakia in our study).

MATERIALS AND METHODS

Patient selection: 30 subjects were divided into three groups, viz I., II, and III with 10 in each group (Table 1).

Subjects with a prior history of malignancy or secondaries, other immunodeficiencies, autoimmune conditions, hepatitis, or human immunodeficiency virus infection were excluded from the study. Patients in group I were interrogated for any previous treatment in the form of chemotherapy, radiotherapy, surgery, or any other treatment modalities for OSCC. Only those subjects who consented with the institutional review board-approved consent form and ready to participate in the study by donating saliva were included in the study.

Table 1: Three study groups

Group I	Clinically documented primary T1 or T2 OSCC
Group II	Clinically documented precancerous lesion (homogeneous leukoplakia)
Group III	Age- and gender-matched controls with no clinical evidence of cancer or precancerous lesion

Saliva Collection

A standardized protocol with a neatly designed program was used for collection, storage, and processing of the samples under similar conditions. Unstimulated saliva samples were collected between 9 and 10 A.M. with predetermined guidelines.[8] Subjects were asked to refrain from eating, drinking, smoking, or oral hygiene procedures for at least 1 hour before the collection. Collected saliva samples were centrifuged at 2600×g for 15 minutes at 4°C. The supernatant was removed from the pellet and treated with ribonuclease (RNase) inhibitor. Isolation of RNA from 560 µL of saliva supernatant was carried out with a specific RNA kit. Aliquots of isolated RNA were treated with RNase-free deoxyribonuclease as per the instructions mentioned by the manufacturer. The quality of isolated RNA was examined by reverse transcription polymerase chain reaction (PCR) for three cellular maintenance gene transcripts: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cytoplasmic beta actin (ACTB), and ribosomal protein S9 (RPS9). Only those samples exhibiting PCR products for all three mRNAs were used for subsequent analysis.

Microarray Analysis

Isolated RNA was subjected to linear amplification by RiboAmp RNA Amplification kit and compared with known quantity. The arrays were scanned and the fluorescence intensity measured with quantitative PCR (qPCR), which was performed on the subset identified by microarray assay. The selected samples were analyzed for sensitivity of the following seven biomarkers, viz. interleukin (IL)-8, IL-1B, DUSP-1, HA-3, OAZ-1, S-100P, and SAT.

RESULTS

All the 10 samples in the precancerous and oral squamous carcinoma groups showed presence of GAPDH, ACTB, and RPS9.

Chi-square = 0.69, Df = 6, p = 0.99990, Yates correction = 0.162, Yates p-value = 0.99991.

The present study comprised 30 patients, divided randomly into three study groups. It was observed that H3F3A, IL-1B, and SAT1 were the highest in proportion compared with the others, but this was not statistically significant (Tables 2 and 3). Similar to the studies carried out by Li et al,[9] we observed that there was a statistically significant increase in the presence of the seven biomarkers in oral cancer and precancer (homogeneous leukoplakia in our study).

Table 2: Distribution of the presence of the selected seven transcripts in saliva (n = 10) among cases of precancerous lesions (group II): Chi-square = 0, Df = 6, p = 1, Yates correction = 0.29, p values after Yates correction = 0.9995

Gene symbol	*Number*	*Percentage*
H3F3A	07	70
IL1B	07	70
IL8	06	60
OAZ1	05	50
SAT1	07	70
DUSP1	05	50
S-100P	06	60

Table 3: Distribution of the presence of the selected seven transcripts in saliva (n = 10) among cases of OSCC (group I): Chi-square = 0, Df = 6, p = 1, Yates correction = 0.237, Yates p-value = 0.9997

Gene symbol	*Number*	*Percentage*
H3F 3A	08	80
IL1B	08	80
IL8	07	70
OAZ1	07	70
SAT1	08	80
DUSP1	07	70
S-100P	07	70

DISCUSSION

Oral cancer, being a global health problem, is afflicting millions of people worldwide. It is well known that the most common epithelial malignancy is OSCC with a significant morbidity and mortality. Early diagnosis and intervention remain the best crux to achieve better prognosis and reduce functional loss. There is a need to develop a rapid and less invasive screening tool that will allow screening of oral cancer and precancer on a large scale, although the final diagnosis can be conferred with a conventional biopsy procedure. Yet, a noninvasive procedure, such as saliva collection has gradually proven to be an effective diagnostic tool for screening of oral cancer and, more importantly, precancerous lesions.

Recent observations indicate that the clinical and histological appearance of oral mucosa may not truly depict the damage occurring at the genetic level. This genetic disparity may reflect in the failure to establish effective screening procedures based on conventional procedures like clinical and microscopic examinations.[9] Carcinogenesis is a multistep process involving initiation, promotion, and progression, and evidence indicates that these are driven by accumulation of specific gene alterations.[10] An understanding of the molecular mechanisms involved in OSCC is helpful in providing a more complete picture of the ways in which tumors arise and advance and a rationale for novel strategies of cancer detection. The oral cavity is particularly conducive to such strategies, given the ease with which saliva and exfoliated cells can be collected.[11]

Notable alterations in deoxyribonucleic acid (DNA) served as biomarkers from saliva and were explored for oral cancer detection. The presence of human mRNA in saliva expands the spectrum of research in the field of oral cancer detection. Ribonucleic acid being more labile than DNA is presumed to be susceptible to degradation by RNases. Moreover, it is reported that the RNase activity is comparatively elevated in patients with cancer.[12] Studies using advanced diagnostic aids, such as PCR, qPCR, and microarray assays have concluded that human mRNA does not easily get degraded extracellularly.

The RNase inhibitors added to freshly collected saliva samples enabled us to facilitate RNA preservation and transportation of samples.

The source of information is largely derived from the variety of DNAs, RNAs, and proteins present in the saliva. Salivary DNA represents the genetic information of the hosting human body, the oral microbiota, and the infecting DNA viruses. Salivary RNA provides information on the transcription rates of the host genes and those of oral microbiota. Salivary proteins represent genetic information and help to understand the translational regulation of the host body and the oral microbiota.[13]

The University of California, Los Angeles research group has recently found that there are approximately 3,000 human mRNAs in the cell-free saliva of normal subjects.[9] Furthermore, there is a core signature of 186 mRNAs present in all normal subjects, which provides the rationale for the use of salivary transcriptome for disease diagnosis.

Research with the positive feedback stating that a large panel of human RNA can be reliably detected in saliva gives rise to the potential of this novel clinical approach.

For oral cancer patients, the detected cancer-associated RNA signature is likely to originate from the matched tumor and/or a systemic response (local or distal) that further reflects itself in the whole saliva coming from each of the three major sources (salivary glands, gingival crevicular fluid, and oral mucosal cells).[9] It is conceivable that disease-associated RNA can find its way into the oral cavity via the salivary gland or circulation through the gingival crevicular fluid. A good example is the elevated presence of HER-2 proteins in saliva of breast cancer patients.[14] For oral cancer, the local tumor is the source of elevated salivary mRNAs.

CONCLUSION

Studying oral cancer is a challenging subject. Oral cancer, being the biggest cause of morbidity and mortality, especially in developing countries, needs immediate attention and early detection. Detection of tumors at an early stage

is the only way to decrease the mortality rate owing to cancer and, hence, is termed the goal of all cancer screening tests. Along with high specificity and high sensitivity of the screening tool, noninvasiveness and inexpensive nature of the tool impart greater usability to the tool.

It can be concluded that the above study provides credential evidence that with an attentive approach toward research of salivary markers for oral as well as systemic diseases, saliva shall be termed as a very useful diagnostic tool for improving the prognosis of the diseases. Thus, it can give the patient a chance for a better quality-of-life to those suffering from oral cancer and precancerous lesions.

It needs to be acknowledged that the suggested salivary analysis should be regarded as an aid and not as a total replacement for other well-established diagnostic tools available for OSCC and precancerous lesions.

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