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Analysis of RNA from brush cytology detects changes in B2M, CYP1B1 and KRT17 levels with OSCC in tobacco users

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SUMMARY

RNA expression analysis of oral keratinocytes can be used to detect early oral cancer, but a limitation is the inability to obtain high quality RNA from oral tissue without using biopsies. While oral cytology cell samples can be obtained from patients in a minimally invasive manner, they have not been validated for quantitative analysis of RNA expression. Earlier we showed RNA from brush cytology of hamster Oral Squamous Cell Carcinoma (OSCC) demonstrated differential expression of B2M and CYP1B1 using real time RT-PCR in a dibenz[a,l]pyrene, tobacco carcinogen, induced model of this disease. Here we show reproducibility of this approach to measuring gene expression in humans. Cytology brush samples from 12 tobacco and betel related OSCC and 17 nonmalignant oral lesions revealed B2M mRNA was enriched in tumor samples while CYP1B1 mRNA was reduced, similar to what was seen in the model system. Additionally, we showed that KRT17 mRNA, a gene linked to OSCC in another brush cytology study, was also enriched in OSCC versus nonmalignant lesions, again supporting the promise of using RNA from brush oral cytology to reproducibly monitor oral gene expression.

Introduction

Oral squamous cell carcinoma (OSCC) which includes over 90% of all oral cancers, is believed to usually develop from dysplasia a change in the mucosa visible microscopically as disorder in the epithelium and the appearance of aberrant cells. Dysplasia in turn can develop into invasive OSCC with expansion into the underlying stroma. Macroscopic mucosal changes of dysplasia and/or OSCC include color and texture changes which can be detected by a visual and tactile oral exam. Visual aids such as toluidine blue staining of oral mucosa may assist with lesion detection.[1] Typically if a lesion persists for longer than two weeks with no obvious causation the patient is offered, or referred for, a biopsy of the lesion to allow histopathological examination of the changes. This approach often delays diagnosis and potentially misses early OSCCs.

The major risk factor for OSCC in the United States is prolonged oral exposure to carcinogens such as tobacco leaf, or, in the case of immigrants from Asian countries, exposure to betel nut or leaf. An additional risk factor is alcohol usage especially in conjunction with tobacco. Tobacco and betel are mutagens which can increase oral cancer rates even after usage is ended by causing a large range of DNA changes, including deletions, rearrangements, and point mutations. P53 gene is often mutated while the p16 gene can be silenced by promoter methylation.[1,2] More recently there has been an increase in OSCC and oropharyngeal cancers in younger patients with tumors located in the lingual and palatine tonsilar regions with distinct risk factors: transforming human papilloma virus like HPV16 and multiple sexual partners, with less of a correlation with tobacco consumption.[3] SSCs in this subgroup show distinct gene expression changes, the tumor suppressors, p16 and p53 are seldom down regulated and/or mutated in tumor tissue[3] and global gene expression can differ.[4,5]

Global gene expression analysis of tissue obtained by surgical biopsy of oral squamous cell carcinoma and more generally head

Abbreviations: OSCC, oral squamous cell carcinoma; SCC, squamous cell carcinoma; B2M, beta 2-microglobulin; CYP1B1, cytochrome p450, Sub family 1, Polypeptide 1; KRT17, keratin 17.

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and neck tumors has defined a large group of genes which show changes in expression with this disease.[6–13] Related analyses have defined gene expression based classifiers that use the expression levels of a panel of genes to differentiate OSCC and normal tissue with varying degrees of success. This type of molecular analysis can improve on the standard diagnostic technique of histopathological analysis. However, the need for surgical acquisition of tissue limits the use of gene expression analysis for cancer screening.

Brush cytology offers a noninvasive method to obtain epithelial cells from oral and cervical mucosa which can be used to detect disease based on molecular analysis such as global gene expression measurement. Combining brush cytology with gene expression analysis has a potential to improve the accuracy and speed of cancer diagnosis. While both these tissues offer ready clinical access, there is evidence that RNA from brush cytology can be of varying quality.[14] There is no consensus on the optimal processing of samples from these tissues to minimize degraded RNA in the final sample.[15] Remarkably, for a method that allows multiple sampling there has been very little published on the reproducibility of gene expression measurement.[15] While characterization of gene expression in RNA from brush cytology showed promise in early studies progress has been slow in validating this approach.[16,17] Steinau et al. used RNA from brush cytology to detect cervical SCC. This large scale study used RT-PCR analysis of the expression of 40 genes involved in cervical SCC to reveal that the expression of five of these genes could be used to correctly differentiate OSCC from normal tissue at a rate of 71%, with 76% specificity and 60% sensitivity, indicating room for improvement.[18] A study of RNA from brush cytology of OSCC showed KRT17 is enriched in OSCC, and not in normal tissue, while KRT18 and 19 did not show significant changes.[19] Our study in hamsters with experimentally induced OSCC showed reproducibility of the approach and that B2M and CYP1B1 are potential markers for OSCC in humans.[20] In the current study we focused on patients with oral cavity SCC and non-pharyngeal tumors, who were tobacco and or betel users, and thus more likely to have had cancers of similar etiology. We tested differential expression of B2M, CYP1B1 and KRT17. With the goal of working toward a noninvasive method to differentiate malignant and non-malignant lesions we looked for differences in gene expression in RNA from brush cytology of OSCC versus nonmalignant but pathological tissue. We also tested the utility of using levels of these three mRNAs in these types of samples in a classifier to identify OSCC.

Methods

Subjects

Samples were collected from former and current tobacco and betel users who presented with oral lesions necessitating a biopsy to rule out malignancy in the Oral and Maxillofacial Surgery Clinic and the Otolaryngology Clinic in the University of Illinois Medical Center. Diagnoses were determined by biopsy and histopathological analysis unless noted. Three normal samples were from lesion free patients with no pathology detectable by biopsy. Excluded were subjects with prior history of head and neck cancer chemotherapy or irradiation treatment. All subjects provided consent to participate in accordance with guidelines of the Institutional Review Board of the University of Illinois at Chicago.

Brush cytology

Brush cytology was performed as described earlier taking care to minimize tissue damage.[20]

Quantitative real-time-PCR

RNA was collected from the brush directly in Trizol and frozen until further purified using the RNAeasy Mini Kit (Qiagen, Valencia, CA) with removal of DNA using column purification. The cDNA synthesis was as described earlier with approximately 70 nanograms RNA per reaction and also oligo dt primers. Quantitative real time PCR was carried out using the iCycler iQ (Bio-Rad, Hercules, CA) and SYBR Green fluorescence to detect double stranded DNA.[20] Values were normalized to the geometric mean of the controls. GAPD, RPLPO and RPL4 were selected as internal controls as these mRNAs showed similar relative expression levels in each sample.[21–23] Primers for these mRNAs and those to detect, B2M, CYP1B1, KRT17, SPINK5 and ECM1 were designed to give products of approximately 100 bases and are included in the supplemental data section.

Statistical analysis and class prediction

Analysis of variance (ANOVA) was used for the determination of the intraclass correlation coefficient (ICC) for mRNA measurements from two separate samples of the same oral site in a subject for SPINK5, and also for ECM1. For class comparison, due to the non-normal distribution of B2M, CYP1B1 and KRT17 expression levels the Wilcoxon test was used to determine the statistical significance of the differences. A gene expression based classifier to differentiate OSCC versus non-malignant oral tissue using RNA from cytology samples was developed and tested using BRB array tools (http://linus.nci.nih.gov/BRB-ArrayTools.html.[24] After log2 transformation of the normalized expression levels of mRNAs, B2M, CYP1B1 and KRT17, the data was imported into the program to undergo simultaneous testing of six different algorithms, including compound covariate predictors (CCP), k -nearest neighbor, nearest centroid, support vector machine (SVM), and diagonal linear discriminant analysis (LDA). These algorithms use different aspects of the data to perform classification, with k -nearest neighbor and nearest centroid both being nonlinear and nonparametric methods.[25,26] Leave-one-out-cross-validation (LOOCV) was used to simultaneously develop a classifier using an algorithm and to test the misclassification rate. Predictive performance was compared to the prevalence of the more common sample type, nonmalignant, and an error rate assuming 100% assignment to that larger group.[27]

Results

Reliability of quantitation of RNA from brush cytology in duplicate samples

It was important to first demonstrate the reliability of gene expression measurements of RNA from brush cytology of human subjects. Duplicate brush cytology samples from one site were obtained from six subjects with no obvious pathology and two additional subjects with OSCC. RT-PCR analysis revealed three optimal housekeeping genes (data not shown). Based on the absorbance at 260 nm there was as much as a 20× variation in levels of the total RNA content between replicates (data not shown). In order to detect the expression of individual mRNAs in the duplicate samples we focused on genes known to show high expression levels in oral mucosa but vary in expression in surgical samples from oral cancer and other diseases.[28–33] For example, Spink5 mRNA, encoding a serpin, is highly expressed in epithelium of the oral mucosa and skin and can show variable expression depending on inflammation levels. The RNA was purified and converted to cDNA and subjected to real time RT-PCR. Relative expression levels of SPINK5 were