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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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S U M M A R Y

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.

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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 nonmalignant 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

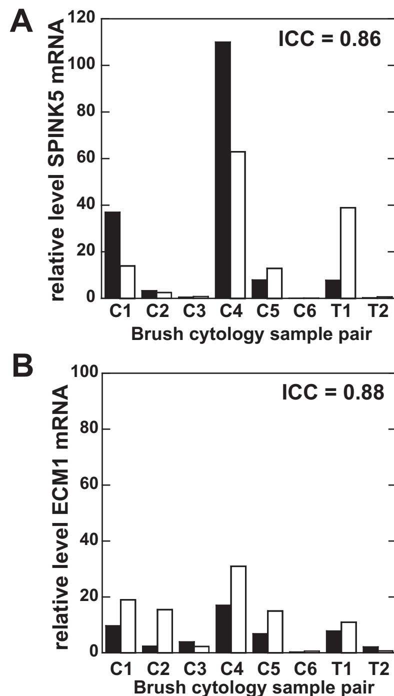

Figure 1 Reproducibility of gene expression measured from independent cytology samples. Two brush cytology samples were consecutively harvested from the same site in patients C1-C8 without lesions, and T1 and T2 with OSCC. RT-PCR analysis of levels of (a) SPINK5 mRNA and (b) ECM1 mRNA revealed an intraclass correlation coefficient (ICC) of 0.81 and 0.86 respectively for SPINK5 and ECM1 mRNA between the first and second samplings.

Table 1
Tumor samples patient data and OSCC details.

Sample	Site[a]	Sex	Age	Tobacco/Betel[b]	TNM Classification	Grade
OSCC1	UG	M	45	Tob/Bet	T1N0M0	Gr 1
OSCC2	LG	M	46	F-Tob	T4aN0M0	Gr 2
OSCC3	UG	M	65	F-Tob/F-Bet	Ta1N1M0	Gr 1
OSCC4	FOM	M	55	Tob	T2N0M0	Gr 2
OSCC5	Bu	M	64	Tob	T4aN0M0	Gr 2
OSCC6	LG	M	38	Tob	T4aN2bM0	Gr 2
OSCC7	FOM	M	64	Tob	T1N0M0	Gr 2
OSCC8	T	M	81	Tob	T2N0M0	Gr 1
OSCC9	Bu	M	53	Tob	T2N0M0	Gr 2
OSCC10	FOM-T	M	60	Tob	T4aN0M0	Gr 1
OSCC11	T	M	64	F-Tob	T1N0M0	Gr 3
OSCC12	P	M	61	Tob	T3N0M0	Gr 2

[a] T, tongue; P, palate; LG/UG, lower/upper gingival; FOM, floor of mouth; Bu, buccal mucosa, LM, lip mucosa.
[b] Tob, Tobacco user; Bet, betel nut user, if former user than F-Tob or F-Bet.

calculated in relationship to mRNA levels for the three housekeeping genes. Despite the variability in total RNA in each brush cytology pair, as measured by the level of housekeeping gene expression, we found a strong intraclass correlation between expression level in the two samples, (Fig. 1). Similar consistency for duplicates was seen with a second mRNA, which encodes another extracellular protein, ECM1, (Fig. 1).

Differential expression in tobacco and betel users of B2M, CYP1B1 and KRT17 as measured in RNA from brush cytology

Risk factors for the 12 patients with OSCC, the 17 patients with nonmalignant lesions and the three additional controls with no

Table 2
Pathological appearance of nonmalignant lesions and description of nonpathological controls.

	Site[a]	Sex	Age	Tobbaco/betel	Lesion diagnosis[b]
BL1	UG	M	49	Tob	Leukoplakia
BL2	T	F	40	Tob	Leukoplakia
BL3	LG	M	22	Tob	Ameloblastoma
BL4	T	F	68	Bet	Ulceration
BL5	LG	M	70	Tob/Bet	Leukoplakia
BL6	UG	M	53	Tob	Leukoplakia
BL7	LM	M	61	F-Tob	Mucocele
BL8	Bu	F	54	Tob	Lipoma
BL9	P	F	62	Tob	fibroma
BL10	Bu	M	22	Tob	Lichen planus
BL11	UG	F	83	F-Tob	Pyogenic granuloma
BL12	P	F	56	Tob	Leukoplakia
BL13	UG	M	43	Tob	Leukoplakia
BL14	T	M	25	Tob	Granular cell tumor
BL15	Bu	M	59	Tob	Epulis fisularum
BL16	FOM	F	50	Tob	Sialolithiasis
BL17	T	M	61	Tob	Traumatic Ulcer
C1	UG	M	42	Tob	Normal
C2	UG	M	45	Tob	Normal
C3	UG	M	70	F-Tob	Normal

[a] Same as Table 1.
[b] No dysplasia observed excepted BL6 which showed mild dysplasia.

lesions, are shown in Tables 1 and 2. All subjects had a history of usage of the oral carcinogens, tobacco, or betel. We focused on CYP1B1 and B2M, genes that we had earlier shown to be differentially expression in a hamster model of oral cancer induction by a tobacco and environmental carcinogen, dibenz[a,l]pyrene, and KRT17 shown to be enriched in surgically obtained tissue from OSCC, and versus normal tissue in a recent brush cytology study.[19] Fig. 2 reveals expression of both KRT17 and B2M was enriched in the OSCC samples. This is consistent with the potential for these mRNAs to serve as markers for this disease. CYP1B1 was expressed at decreased levels in OSCC brush cytology samples as predicted by our earlier hamster study, also making it a potential OSCC marker.[20]

Classification of OSCC versus non-tumor using RNA from brush cytology

In order to test the utility of quantitation of these three, or a subset of these three, mRNAs in RNA from brush cytology to classify OSCC and non-malignancies, we tested a small group of supervised classification algorithms. Of these, K-nearest neighbor, nearest centroid, SVM and LDA all showed an 81% rate of correct classification as derived by external LOOCV based on the level of all three mRNAs. This compared favorably to the baseline classification rate of 63% (20 non-malignancies/32 total samples) if one selected all samples as non-malignancies, the more prevalent sample type.

Discussion

RNA obtained from the oral cavity by brush cytology offers a noninvasive method to obtain multiple samples and we took advantage of that to show reproducibility of mRNA quantification with the method. It should be noted that in theory quantification of genes expressed differentially in the different layers of the epithelium, poorly expressed in epithelium or particularly sensitive to degradation, may not show reliable quantification in RNA from brush cytology as opposed to surgical samples. Two genes chosen for their expression in the epithelium, and the possibility of

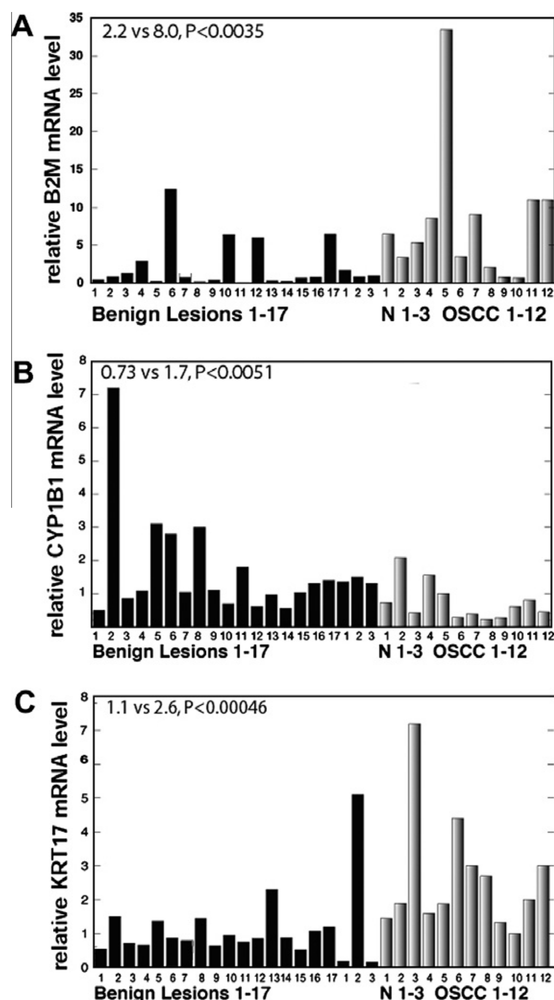

Figure 2 Relative expression of B2M and KRT17 in RNA from brush cytology from OSCC and nonmalignant oral lesions and normal controls. Each bar represents the relative mRNA level for (a) B2M, (b) KRT17, (c) CYP1B1, measured by RT-PCR and normalized to the geometric mean of GAPD, RPLO and RPL4 levels. The samples are grouped according to the diagnosis. In the left corner is the mean level of expression of the OSCCs versus the benign lesions and normals and the significance of this difference based on the Wilcoxon test.

variance with disease, Spink5 and ECM1, were tested in RNA from brush cytology samples, and replicates taken immediately afterward from the same site. While total RNA recovered varied over 20× we found close agreement in levels for these two mRNAs between the two replicates, after normalization to the housekeeping genes.

Our study of human tobacco associated OSCC replicated what we saw in the hamster dibenz[a,I]pyrene induced model of the disease, B2M was enriched in RNA from OSCC brush cytology samples. This is further supported by the recent report that B2M protein is enriched in a subset of OSCCs based on immunostaining of tumor tissue samples and is also enriched at least on the RNA level in surgically obtained tumor samples from cervical and oral tumors, though not in stomach or colorectal cancer tumors.[34–37] Enrichment of B2M in cancer cells has been associated with a worse prognosis for OSCC and lymphocytic cancers while in contrast B2M down regulation is associated with colon cancer aggressiveness.[38–40] With the limited number of tumors sampled for this study, twelve, we saw no connection between stage and B2M expression level.

Our findings with CYP1B1 in the human tobacco/betel associated OSCCs concurred with the reduction in expression we saw in the hamster dibenz[a,I]pyrene tumor model. Certain variants of the CYP1B1 gene have been associated with different cancers, though less has been reported on changes in mRNA expression levels in tumor tissue. The CYP1B1 protein is a mono-oxygenase which plays a role in the Phase I metabolism of xenobiotics. It is possible CYP1B1 enzyme may be an anti-oncoprotein or oncoprotein depending on what pro-carcinogens are the frequent cancer causing agents in these tissue types and whether CYP1B1 serves to activate or inactive them. That may explain why CYP1B1 mRNA is found at decreased levels in some cancers, such as mesothelioma and melanoma, while it was increased with prostate and non small cell lung cancer.[41–44] It is important to note that smoking has a long term inductive effect on CYP1B1 in brush cytology cells from the airway epithelium and on punch biopsied tissue from the buccal mucosa,[45,46] though it is not clear if brush cytology of the oral mucosa would show the same increase. Thus it is possible we would not see the CYP1B1 decrease with OSCC if we compared the expression to nonmalignant samples from controls not matched for smoking or betel exposure.

Gene expression analysis of surgically obtained oral cavity SCC has revealed in several studies the induction of KRT17 with this disease.[9,28,47,48] The one study that looked at KRT17 using RNA from brush cytology revealed increases in this mRNA with OSCC versus normal appearing tissue from the same patients.[19] Our study instead used predominately as controls RNA from brush cytology of benign but pathological oral lesions that in some situations might be mistaken for OSCC. This shows the robustness of mRNA analysis using RNA from brush cytology of OSCC at least for some mRNAs and its potential for usage in differential diagnosis. There are a large number of genes which show differences in expression in surgical samples of OSCC versus normal tissue over multiple studies.[49] A subset of these will be differentially expressed in RNA from brush cytology from OSCC and nonmalignant oral tissue with pathology. The three gene classifier developed and validated with LOOCV is a start toward gene expression based classification of OSCC versus nontumor using RNA from brush cytology. The challenge is to identify using RNA from brush cytology sufficient numbers of genes differentially expressed in OSCC and other pathologies so to allow the formation of a more powerful gene expression based classifier for OSCC, and OSCC at the various stages.

Conflict of interest statement

The University of Illinois has applied for patent rights to the usage of differential expression of CYP1B1 and B2M in the identification of OSCC. Dr. Adami and Dr. Schwartz are named on this patent application. These and the other authors have no other actual or potential conflict of interest including any financial, personal or other relationships with people or organizations that could inappropriately influence (bias) this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.oraloncology.2011.03.029.

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