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microRNA-21 and microRNA-375 from oral cytology as biomarkers for oral tongue cancer detection

Qianting He^{a,b}, Zujian Chen^a, Robert J. Cabay^c, Leitao Zhang^{a,d}, Xianghong Luan^e, Dan Chen^{a,b}, Tianwei Yu^f, Anxun Wang^{b,#}, and Xiaofeng Zhou^{a,g,#}

^aCenter for Molecular Biology of Oral Diseases, Department of Periodontics, College of Dentistry, University of Illinois at Chicago, Chicago, IL

^bDepartment of Oral and Maxillofacial Surgery, the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China

^cDepartment of Pathology, College of Medicine, University of Illinois at Chicago, Chicago, IL

^dDepartment of Oral and Maxillofacial Surgery, Nan Fang Hospital, Southern Medical University, Guangzhou, China

^eDepartment of Oral Biology, College of Dentistry, University of Illinois at Chicago, Chicago, IL

^fDepartment of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, GA

^gUIC Cancer Center, Graduate College, University of Illinois at Chicago, Chicago, IL

Abstract

Objective—We previously performed a meta-analysis of microRNA profiling studies on head and neck / oral cancer (HNOc), and identified 11 consistently dysregulated microRNAs in HNOc. Here, we evaluate the diagnostic values of these microRNAs in oral tongue squamous cell carcinoma (OTSCC) using oral cytology samples.

Materials and Methods—The levels of 11 microRNAs were assessed in 39 oral cytology samples (19 OTSCC and 20 normal subjects), and 10 paired OTSCC and adjacent normal tissues. The predictive power of these microRNAs was analyzed by receiver operating characteristic curve (ROC) and random forest (RF) model. A classification and regression trees (CART) model was generated using miR-21 and miR-375, and further validated using both independent oral cytology validation sample set (14 OTSCC and 11 normal subjects) and tissue validation sample set (12 paired OTSCC and adjacent normal tissues).

#Corresponding author: Anxun Wang, First Affiliated Hospital, Sun Yat-Sen University, 58 Zhongshan Road, Guangzhou, 510080, China. ; Email: anxunwang@yahoo.com, Xiaofeng Zhou, University of Illinois at Chicago, College of Dentistry, 801 S. Paulina Street, Chicago, IL 60612, USA. ; Email: xfzhou@uic.edu.

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Results—Differential expression of miR-21, miR-100, miR-125b and miR-375 was validated in oral cytology training sample set. Based on the RF model, the combination of miR-21 and miR-375 was selected which provide best prediction of OTSCC. A CART model was constructed using miR-21 and miR-375, and was tested in both oral cytology and tissue validation sample sets. A sensitivity of 100% and specificity of 64% was achieved in distinguishing OTSCC from normal in the oral cytology validation set, and a sensitivity of 83% and specificity of 83% was achieved in the tissue validation set.

Conclusion—The utility of microRNA from oral cytology samples as biomarkers for OTSCC detection is successfully demonstrated in this study.

Keywords

head and neck/oral cancer; squamous cell carcinoma; small non-coding RNA; biomarker; oral cytology; miR-21; miR-375

Introduction

Head and neck / oral cancer (HNOC) is the sixth most common cancer in the world [1]. Over 90% of HNOC cases are squamous cell carcinomas (HNSCCs), malignancies arising from the epithelia lining of the upper aerodigestive tract. Tongue squamous cell carcinoma (OTSCC) is one of the most aggressive form of HNOCs, which exhibits a propensity for rapid local invasion and spread [2], and shows a distinct nodal metastasis pattern [3, 4]. OTSCC patients also suffer from a high recurrence rate [5]. Despite the improvements in surgery, radiotherapy and chemotherapy over the last decade, the prognosis for OTSCC patients has only improved slightly because OTSCCs are frequently discovered late in their development. Improvement in patient survival requires better methods for cancer screening and early detection so that aggressive tumors can be detected early in the disease process and targeted therapeutic interventions can be deployed.

While many studies have been devoted to identify molecular biomarkers for HNOC detection and early diagnosis, most efforts are focused on protein coding genes. The knowledge regarding non-coding genes (e.g., microRNA) and their potential as biomarkers for detecting HNOC is relatively limited. MicroRNAs are an abundant class of small (18–25 nucleotides long) single-stranded non-coding RNA molecules that control the target gene's expression at the post-transcriptional level. Several microRNAs have been functionally classified as proto-oncogenes or tumor suppressors and are aberrantly expressed in various cancer types, including HNOC [6–10]. Dysregulation (e.g., overexpression or loss of expression) of these "cancerous" microRNAs contributes to tumor initiation and progression by promoting uncontrolled proliferation, favoring survival, and/or promoting invasive behavior [11, 12]. Several recent studies suggested the potential of differentiating cancerous and normal tissues using microRNA markers with varying degrees of success [13, 14]. Recent studies also suggested that microRNA markers may have predictive values for the progression of oral potentially malignant disorders (OPMDs) [15–18]. Furthermore, microRNA dysregulation has also been detected in the field of cancerization [19–22]. As such, microRNA-based molecular analysis can enhance the standard histopathological analysis for early detection and monitoring of field of cancerization which have profound

implications for cancer prevention. However, the need for biopsy or surgical acquisition of tissue limits the use of microRNA analysis for cancer screening. Obtaining patient RNA without surgery would be an ideal way to facilitate cancer screening and simplify patient diagnosis. Brush cytology offers a minimally-invasive method to obtain exfoliated epithelial cells. This cell collection technique, popularized by George Papanicolaou in the first half of the twentieth century, has helped reduce cervical cancer incidence and mortality rates by 75%. Using a brush to collect cytologic samples is a technique that can also be applied to the oral cavity. In the last few years, the interest in oral cytology as a diagnostic and prognostic methodology has grown substantially, and the exfoliated cells acquired with this technique have been shown to be suitable for detecting HNOC based on molecular analysis [23–25]. As such, combining oral cytology with microRNA analysis has the potential to improve the accuracy and speed of HNOC diagnosis. In this study, we aim to assess the feasibility of utilizing microRNA from oral brush cytology samples as a biomarker for the detection of OTSCC. We found that, by using specific combination of microRNAs (miR-21 and miR-375), we were able to detect OTSCC using oral cytology samples with proficiency compatible with that using tissue samples.

Materials and Methods

Patient cohorts

We used clinical samples or existing data from 4 patient cohorts in our study, including: 1) Oral cytology training sample set: the oral cytology samples were obtained from 19 cases of OTSCC patients before tumor resection and 20 normal subjects as described [23, 24]; and 2) Oral cytology validation sample set: An independent set of oral cytology samples was obtained from 14 cases of OTSCC patients and 11 normal subjects for the validation study. 3) Tissue training sample set: The TaqMan-based qPCR results of microRNA expression and the clinical data on 10 cases of OTSCC and their matching adjacent normal tissues from our previous study [10] were used as the training set for analysis of the tissue samples; 4) Tissue validation sample set: The deep sequencing-based data on microRNA expression and clinical data on 12 cases of OTSCC and their matching adjacent normal tissues were downloaded from The Cancer Genome Atlas (TCGA) Data Portal (tcga-data.nci.nih.gov) for the validation analysis of the tissue samples. The demographics and clinical data of these patient cohorts were presented in Supplement Table S1. The study was approved by the Ethical Committee of the First Affiliated Hospital, Sun Yat-Sen University.

RNA isolation and quantitative RT-PCR analysis

Brush cytology was performed on subjects as previously described using a cervical cytology brush [23, 24]. The total RNA was immediately isolated using miRNeasy Mini kit (Qiagen), and quantified by spectrophotometer. The levels of miR-21, miR-155, miR-130b, miR-223, miR-31, miR-7, miR-34b, miR-100, miR-99a, miR-375, and miR-125b were determined using TaqMan microRNA assays per the manufacturer's protocol (Applied Biosystems). Quantitative PCR reactions were performed in duplicates using a 7900HT Fast Real-Time PCR system (Applied Biosystems). Since it has been showed previously that using housekeeping genes as internal reference can sufficiently control for the variability in the RNA yield from each oral cytology samples [24], we also measured the level of U6 snRNA

for each samples using TaqMan assay (Applied Biosystems). The relative microRNA levels were computed using the $2^{-\Delta\Delta C_t}$ analysis method [26], where U6 was used as an internal reference.

Statistical analysis

Data was analyzed using the S-plus 6.0. Wilcoxon Signed Rank Test was used to compare differences between groups. The receiver operating characteristics (ROC) curve analysis was used to evaluate the predictive power of each microRNA biomarker. Due to its capability to adapt to non-linear response surfaces, tolerate outliers, and provide predictor importance and potential interactions, the Random Forest model was used to determine the combination of microRNA biomarkers that provide best prediction. The classification and regression trees (CART) model was constructed to validate the selected microRNA biomarkers as predictors. The performance of the model for classification was assessed by identifying the cut-off value of the prediction probability, which yielded the largest sum of sensitivity and specificity. For all analyses, $p < 0.05$ was considered statistically significant.

Results and Discussion

Our recent meta-analysis on 13 published microRNA profiling studies on HNOCC (comprising 215 tumor and 121 corresponding normal control samples) revealed 11 most consistently differentially expressed microRNAs, including miR-21, miR-155, miR-130b, miR-223, miR-34b, miR-31, miR-7, miR-100, miR-99a, miR-375, and miR-125b [10]. We further confirmed the differential expression of 8 of these 11 microRNAs in an independent set of OTSCC tissue samples using TaqMan-based quantitative RT-PCR (up-regulation of miR-21, miR-155, miR-130b, miR-223 and miR-31, and the down-regulation of miR-100, miR-99a and miR-375) in a recent study [10]. Here, the TaqMan-based quantitative PCR was performed on oral cytology samples from a cohort consists of 19 OTSCC and 20 normal subjects to measure the levels of these 11 microRNAs. As shown in Figure 1 and Table 1, differential expression of miR-21, miR-100, miR-375 and miR-125b was validated in oral cytology samples. The other microRNAs tested were not validated, suggesting that there is potentially inherited difference between oral cytology samples and tissue samples. This difference between two sample types was also observed previously with mRNA gene expression analysis [27]. The apparent discrepancy between oral cytology and tissue samples may be due to several possible reasons. First, this discrepancy may be a reflection of the difference in study design. While many of the earlier studies, including ours [10], compared microRNA expression in surgically obtained tumor vs. histopathologically normal tissues from the same subject, the oral cytology samples were obtained from OTSCC patients and normal matching healthy subjects. Also, the difference in microRNA expression maybe reflects the makeup of the cells in oral cytology samples vs the surgically obtained tissue samples. It has been demonstrated that oral cytology samples contain almost exclusively exfoliated mucosa epithelial cells [23, 28, 29]. However, the oral cytology samples from OTSCC patients may contain blood cells, even with extra precaution was taken to avoid bleeding. In contrast, surgically obtained tissue samples contain, in addition to epithelium, a variety of stromal cells, including fibroblasts, immune cells, endothelial cells, and blood cells. While laser capture microdissection procedure can greatly enhance the

homogeneity of the cells procured from tissue samples, it is difficult to totally eliminate the stromal cell. These differences may all contribute to the observed difference in microRNA expression of oral cytology and tissue samples. Nevertheless, the differential expression of microRNAs that consistently observed in both oral cytology and tissue samples (up-regulation of miR-21, and down-regulation of miR-100, miR-375 and miR-125b) are highly reproducible molecular measurements and may be utilized as robust biomarkers for OTSCC detection.

The calculated area under the receiver operator characteristics (ROC) curve for miR-21, miR-100, miR-375 and miR-125b were 0.78, 0.75, 0.90, 0.69 for oral cytology samples, and 0.91, 0.83, 0.91, 0.69 for tissue samples, respectively (Table 1). These microRNAs were then selected for our following analysis to test the feasibility of utilizing microRNA as biomarkers for OTSCC detection.

To determine the combination microRNA biomarkers that provide best prediction, the Random Forest (RF) model was constructed and the relative importance of variables was assessed. As shown in Supplement Table S2, miR-21 and miR-375 are the highest ranked variables. For oral cytology samples, the combination of miR-21 and miR-375 yielded an out-of-bag (OOB) prediction error rate of 12.8% (5/39). For tissue samples, the combination of miR-21 and miR-375 yielded an OOB prediction error rate of 5% (1/20). It is worth noting that the combination of miR-21 and miR-375 has been suggested as either diagnostic or prognostic biomarker in both laryngeal SCC [30–32] and esophageal cancers (both esophageal SCC and esophageal adenocarcinoma) [33–35] by multiple studies. Interestingly, the consistent up-regulation of miR-21 and down-regulation of miR-375 not only observed in cell-based samples (e.g., oral cytology and tissue samples), but also as circulating microRNAs [33, 36, 37]. Taken together with our observation, it appears that the combination of miR-21 and miR-375 may represent a robust biomarker set for carcinomas of the upper aerodigestive tract. Biologically, miR-21 is one of the most well-studied oncomiRs [38, 39], and miR-375 is a functional tumor suppressor in multiple types of cancer [40, 41]. Downregulation of miR-375 has also been suggested contributing to the progression of oral potentially malignant disorders (OPMDs) [42]. In the following study, we will focus our analysis on the combination of miR-21 and miR-375 for the OTSCC detection.

To demonstrate the utility of miR-21 and miR-375 as markers for OTSCC discrimination, we recruited an independent subject cohort (14 OTSCC and 11 matching healthy controls), and collected their oral cytology samples as the oral cytology validation sample set. The levels of miR-21 and miR-375 for each sample were determined using the TaqMan-based qRT-PCR method as described above (Supplement Figure S1). A Classification and Regression Trees (CART) model was generated (Figure 2). MiR-21 was chosen as the initial split and produced 2 child groups from the parent group containing the total 25 samples, and 13 samples were assigned into “Normal-1” and 12 were assigned into “Cancer-1”. The “Normal-1” group was further partitioned by miR-375, resulting subgroup “Normal-2” (7 samples) and “Cancer-2” (6 samples). Consequently, the 25 oral cytology samples involved in our validation study were classified into “Normal” and “Cancer” by CART analysis. There are 7 samples assigned to “Normal” group, and all 7 are from normal subjects. There

are 18 samples assigned to “Cancer” group, and 14 are from OTSCC patients and 4 are from normal subjects. Thus, by using the combination of miR-21 and miR-375 for OTSCC prediction, the sensitivity is 100% (14/14), and the specificity is 64% (7/11). The overall error rate is 16% (Table 2).

For validating the microRNA biomarkers in tissue samples, we downloaded the microRNA data (deep sequencing-based microRNA profiling dataset) on 12 cases of paired OTSCC and adjacent normal tissues from The Cancer Genome Atlas (TCGA) Data Portal. The values of miR-21 and miR-375 were extracted from the TCGA dataset (Supplement Figure S1). Similar to the validation analysis using oral cytology samples, a CART model was generated based on miR-21 and miR-375 (Figure 3). The initial split with miR-21 led to 92% sensitivity (11/12), and 92% specificity (11/12). While the tree should not be grown to a level that decreases the classification rate, for the purpose of comparing the model performance between tissue samples and oral cytology samples, we continued our CART analysis by using miR-375 for the additional partitioning, and led to a sensitivity of 83% (10/12), and specificity of 83% (10/12). The overall error rate is 17% (Table 2).

These results clearly indicated that tissue sample-based miR-21 and miR-375 measurements have a better specificity in predicting OTSCC, while oral cytology-based miR-21 and miR-375 measurements demonstrated a better sensitivity for OTSCC classification. However, the study designs are different in these 2 sets of analysis. The major difference is that for oral cytology study, the normal group consists of matching healthy subjects, and for the tissue sample study, the normal group are histologically normal adjacent tissues from the same cancer patients. It is worth noting that microRNA dysregulation has also been detected in oral potentially malignant disorders (OPMDs) [15–18], and in the field of cancerization [19–22]. The concept of field cancerization, initially introduced by Slaughter et al in 1953 to describe the histologically normal epithelium adjacent to oral cancer that harbors early genetic changes [43], which explains the development of multiple primary tumors and local recurrent cancer. Oral cytology-based application offers a minimally-invasive method to survey large area of oral cavity and to explore the extent of field of cancerization. Thus, early detection and monitoring of field of cancerization with oral cytology-based method may have profound implications for cancer prevention. One of our future aims is to expand our study by using matched oral cytology samples (from the tumor site and from histologically normal epithelium adjacent to tumor) and surgically acquired tissue samples (as well as blood and saliva samples) from the same subjects, which will enable us to fully explore the potential of utilizing microRNAs as diagnostic markers for OTSCC.

Conclusions

In summary, our results indicated that dysregulated microRNAs can be utilized as biomarkers for the detection of OTSCC. Specific combination of microRNAs (e.g., miR-21 and miR-375) can achieve excellent outcomes in discriminating OTSCC from normal control subjects. While using tissue samples as source of microRNA biomarkers leads to better specificity and overall error rate, in our study, using microRNA markers from oral cytology samples provide a better sensitivity. Thus, this application could be exploited to a non-invasive and robust tool for cancer screening and early detection. We envision a two-

stage screening/diagnosis approach based on our observations. First, oral cytology-based microRNA biomarkers can be used as screening tools for the high risk population (e.g., heavy smokers). This oral cytology-based method is relatively noninvasive and inexpensive, which will allow widespread applicability. Once a specious lesion was identified, a biopsy sample can be taken for additional microRNA biomarker analysis, in conjunction with standard histopathological tests. Future studies with larger sample size will be needed to fully explore the feasibility of this novel application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

HNOC	head and neck / oral cancer
SCC	squamous cell carcinoma
HNSCC	head and neck squamous cell carcinoma
OTSCC	oral tongue squamous cell carcinoma
ROC	operating characteristic curve
RF	random forest
TCGA	The Cancer Genome Atlas
CART	classification and regression tree

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Highlights

- microRNA differential expression can be detected in oral cytology samples
- microRNA from oral cytology samples can be used as biomarkers
- miR-21 and miR-375 have diagnostic value for oral cancer detection

Figure 1. MicroRNAs differential expression detected in oral cytology samples from OTSCC patients

The TaqMan-based qPCR was performed to assess the levels of miR-21 (A), miR-100 (B), miR-375 (C), and miR-125b (D) in the oral cytology samples from OTSCC patients (n = 19) and match normal subjects (n = 20). The boxes represent 25th to 75th percentile of the observations, and the lines in the middle of the box represent the median. The whiskers represent maximum (or minimum) observations below (or above) the 1.5 times of the interquartile range, respectively. Outliers are also indicated in the plots as black circles.

Figure 2. Classification and regression trees (CART) model assessing the microRNA biomarkers in oral cytology samples

MiR-21 was used for the initial split of the validation sample set containing a total of 25 oral cytology samples (14 OTSCCs and 11 normal) and produced 2 child groups: Normal-1 and Cancer-1. Normal-1 group was further partitioned by miR-375. The 25 samples were classified into the final cancer and normal group by CART. The overall sensitivity is 100% (14 of 14) and the specificity is 64% (7 of 11) for OTSCC classification

Figure 3. Classification and regression trees (CART) model assessing the microRNA biomarkers in tissue samples

MiR-21 was used for the initial split of validation sample set containing a total of 24 tissue samples (12 OTSCCs and 12 normal) and produced 2 child groups: Normal-1 and Cancer-1. This initial split achieved sensitivity of 92% (11/12) and specificity of 92% (11/12) for OTSCC classification. The child groups, Normal-1 and Cancer-1, were further partitioned by miR-375 and led to 4 subgroups: Normal-2, Cancer-2, Normal-3 and Cancer-3. The 24 samples were classified into the final cancer and normal group by CART. The overall sensitivity is 83% (10/12) and the specificity is 83% (10/12) for OTSCC classification. While better prediction outcome was achieved after initial split with miR-21, further partitioning with miR-375 was continued (below the red dot line) for the purpose of comparing the model performance between tissue samples and oral cytology samples (see Figure 2).

Table 1

Receiver operator characteristic (ROC) curve analysis of OTSCC associated microRNAs in tissue samples and oral cytology samples

	Tissue samples				Cytology samples			
	Normal median (25%–75%)	OTSCC median (25%–75%)	Wilcoxon (p value)	ROC (AUC)	Normal median (25%–75%)	OTSCC median (25%–75%)	Wilcoxon (p value)	ROC (AUC)
miR-21	1.33 (0.84–1.53)	5.95 (4.05–16.39)	0.001	91	0.79 (0.54–1.35)	2.79 (1.38–7.31)	0.002	78
miR-99a	0.97 (0.87–1.04)	0.37 (0.24–0.69)	0.002	89	0.28 (0.14–1.52)	0.16 (0.02–0.76)	0.509	56
miR-100	0.95 (0.64–1.35)	0.39 (0.23–0.82)	0.011	83	1.14 (0.26–4.76)	0.1 (0.02–0.31)	0.006	75
miR-155	1.02 (0.88–1.14)	2.16 (1.43–3.39)	0.002	90	1.25 (0.24–6.69)	0.88 (0.28–2.5)	0.784	53
miR-130b	0.45 (0.14–1.19)	12.24 (2.82–21.60)	0.015	86	0.18 (0.001–1.72)	0.03 (0.01–0.07)	0.639	55
miR-223^a	0.53 (0.3–1.03)	2.73 (1.86–4.23)	0.009	84	1.14 (0.26–4.76)	0.08 (0.02–0.2)	0.002	79
miR-375	0.7 (0.32–1.02)	0.03 (0.003–0.07)	0.002	91	0.78 (0.32–2.18)	0.0003 (0.0001–0.05)	<0.001	90
miR-125b	1.06 (0.79–1.53)	0.49 (0.28–1.11)	0.165	69	0.79 (0.45–1.66)	0.19 (0.07–0.69)	0.041	69
miR-7[†]	0.78 (0.76–0.81)	0.79 (0.5–1.32)	0.968	51	2.81 (0.08–31.19)	0.03 (0.02–0.1)	0.005	76
miR-31	0.68 (0.31–0.99)	3.18 (1.51–8.08)	0.003	88	1.11 (0.42–6.26)	2.34 (0.55–5.33)	0.46	57
miR-34b	0.23 (0.22–0.46)	0.23 (0.12–1.59)	0.968	51	0.74 (0.17–1.56)	0.16 (0.08–0.63)	0.072	67

^aThe expressional changes for miR-223 and miR-7 in oral cytology samples were opposite of the observed changes in tissue samples from OTSCC and normal control.

Table 2

The performance of miR-21 and miR-375 as biomarkers to predict OTSCC in independent oral cytology and tissues samples ^a

	Sensitivity	Specificity	Overall error rate
Oral cytology samples	100% (14/14)	64% (7/11)	16%
Tissue samples	83% (10/12)	83% (10/12)	17%
Tissue samples (miR-21 only) ^b	92% (11/12)	92% (11/12)	8%

^a Analysis was based on CART model.

^b For the tissue samples, while better prediction outcome was achieved after initial split with miR-21, further partitioning with miR-375 was continued for the purpose of comparing the model performance between tissue samples and oral cytology samples.