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

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SUMMARY

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).

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.

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.

Abbreviations: 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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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

Patent 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 down loaded 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 $2^{-\text{delta delta Ct}}$ 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 HNOC (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 Fig. 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