Original Article

Aberrant miR-10b, miR-372, and miR-375 expression in the cytobushed samples from oral potentially malignant disorders

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Abstract  Background/purpose: MicroRNA (miRNA) alterations play important roles in the neoplastic process of oral squamous cell carcinoma (OSCC). Upregulation of miR-10b and miR-372 and downregulation of miR-375 are frequent events in OSCC. The aberrances of these miRNAs in oral potentially malignant lesions (OPMD) were studied to determine their status during the establishment of OSCC.

Materials and methods: Cytobushed sampling was used to collect epithelial cells from 11 OSCC and 34 OPMD lesions and matched normal mucosa. The expression levels of miR-10b, miR-372, and miR-375 were analyzed using quantitative reverse transcription polymerase chain reaction analysis. The clinical implications of these aberrances were further investigated.

Results: Both miR-10b and miR-372 were upregulated in OPMD, but only miR-10b expression was upregulated in OSCC comparing to control. miR-375 was downregulated in OPMD and tended to be downregulated in OSCC. Dysplastic OPMD could be distinguished based on miR-372 expression level; miR-375 expression levels facilitated discrimination between OPMD and OSCC. The combined analysis of miR-375 and miR-372 remarkably enhanced the accuracy of differentiating OPMD from OSCC.

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Conclusion: Aberrant miR-10b, miR-372, and miR-375 expression occurs early during oral carcinogenesis. The detection of miR-372 and miR-375 expression using cytobrush samples may assist in differentiating between OPMD and OSCC.

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Introduction

Oral potentially malignant disorders (OPMDs) are lesions that correlate with the risk of oral squamous cell carcinoma (OSCC). Interventions for OPMDs are important to prevent OSCC. However, among the diverse and large number of OPMDs, identifying the lesions with greatest potential for malignant transformation is not easy but remains very important. Epigenetic changes, especially noncoding RNA alterations, have drawn more attention in recent cancer research. Compared to other noncoding RNAs, microRNA (miRNA) alterations are more intensively characterized in OSCC and head and neck squamous cell carcinoma (HNSCC). However, in the progression of OPMDs, limited miRNA alterations have been reported in malignant transformations. miR-21 and miR-31 were found to be important oncomirs in oral carcinogenesis. Upregulation of miR-31 was observed in OPMD tissues and correlated with malignant transformation. Increased salivary miR-184 and decreased miR-145 levels were also noted in OPMD patients and found to have significant diagnostic power. The altered expression of miRNAs could be a feasible biomarker for determining the potential of malignancy. However, critical miRNA alterations occurring in OPMD require vigorous exploration to address the pathogenetic process.

In breast cancer, hepatocellular carcinoma, and lung cancer, miR-10b upregulation has been found in invasive and metastatic disease. A study also revealed that miR-10b expression is associated with chemoresistance in HNSCC cells. Moreover, miR-10b could downregulate E-cadherin and trigger epithelial mesenchymal transition in the laryngeal SCC cell line, Hep-2, which could be the initial step in oral carcinogenesis. Tu et al. demonstrated that upregulation of miR-372 is associated with lymph node metastasis and poor prognosis in OSCC. Functional assays also demonstrated that miR-372 could target p62 and ZBTB7A, leading to enhanced migration and drug resistance. The expression profiles of miR-10b and miR-372 in the OPMD stage of oral carcinogenesis remain unknown.

Compared to adjacent normal tissues, HNSCC tissues harbor lower miR-375 expression. In laryngeal SCC, downregulated miR-375 expression indicates poor prognosis, which might be mediated by targeting hepatocyte nuclear factor 1α. As an OSCC suppressor, whether the downregulation of miR-375 occurs in OPMD remains obscure. This study explored the expression of miR-10b, miR-372, and miR-375 in patients with OPMD and OSCC. Although the detection of circulating or salivary miRNAs would be a convenient and less or non-invasive approach, this study adopted a cytobrush strategy to acquire cells from lesion and control sites. Quantitative analysis demonstrated, for the first time, miR-10b and miR-372 upregulation and miR-375 downregulation in the early oral neoplastic stage.

Materials and methods

Subjects

Thirty-one patients with OPMD lesions and 11 patients with OSCC were enrolled in this study. The samples were collected from the National Yang Ming Chiao Tung University Hospital and Taipei Mackay Memorial Hospital between October 2019 and February 2021. This study was approved by the ethics review committee with approval numbers RD-2019-003 and 18MH15187, and all participants signed informed consent forms. The clinicopathologic features of the study subjects are listed in Tables 1 and 2. Nearly all patients were male, and the vast majority were smokers and/or betel chewers. Specific types of OPMD, including oral submucosal fibrosis and erosive lichen planus, were excluded from this study.

Cytobrushing

Before biopsy, the lesion site and its contralateral or adjacent normal site were brushed using a Libo specimen collection swab (Cat No. 30221.3, Iron Will Biotech, New Taipei City, Taiwan). Each focus was brushed 30 times to ensure adequate cell collection. The brushed samples

| Table 1 | Clinicopathological characteristics of OSCC (oral squamous cell carcinoma) patients. |
|---|---|
| Characteristics | Case number |
| Age (years, Mean ± SD) | 59.0 ± 7.9 |
| Gender | |
| Male | 9 |
| Female | 2 |
| Oral habits | |
| Alcohol | 5 |
| Betel quid | 10 |
| Cigarette | 10 |
| Tumor size | |
| T1 | 1 |
| T2 | 4 |
| T3 | 2 |
| T4 | 4 |
| N status | |
| N = 0 | 8 |
| N > 0 | 3 |
| Stage | |
| I | 1 |
| II | 4 |
| III | 2 |
| IV | 4 |
| LVI (lymphovascular invasion) | |
| (+) | 0 |
| (−) | 11 |
| PNI (perineural invasion) | |
| (+) | 2 |
| (−) | 9 |
Three patients have two lesion sites and there are totally 34 lesions and their matched controls. Cycle threshold of the signal amplification, and miRNAs and U6B.

represents the difference in Ct values between the detected expression in OPMD were considered early events in oral carcinogenesis. Statistical significance was set at p < 0.05.

### Results

**Expression of miR-10b, miR-372, and miR-375 in paired OSCC lesions**

Among the 11 patients with OSCC, the tumor lesion and adjacent normal part were brushed, and the exfoliated cells were analyzed. Expression levels of miR-10b, miR-372, and miR-375 were measured. miR-10b was significantly upregulated in tumor samples (Fig. 1A), while miR-375 was non-significantly downregulated in tumor samples (Fig. 1C). In addition, there was no significant difference noted between the normal and tumor samples in miR-372 expression (Fig. 1B). Increased miR-372 expression has been reported to be related to worse survival.10 We compared miR-10b, miR-372, and miR-375 expression between early stage (I–II) and late stage (III–IV) subjects. Due to the limited number of cases, there was still no difference found between early and late-stage OSCC (Fig. 1D–F). miR-10b was upregulated in OSCC, and miR-375 expression tended to be decreased in OSCC tumors.

**Aberrant miR-10b, miR-372, and miR-375 expression in OPMD**

Tissue pairs were analyzed from 31 patients with 34 OPMD lesions. miR-10b and miR-372 were significantly upregulated in OPMD lesions (Fig. 2A and B), while miR-375 was significantly downregulated in OPMD lesions (Fig. 2C). Since the comparison was made between paired normal and lesion tissues in the same individuals, individual discrepancies could be eliminated. Although normal-looking tissues may harbor molecular changes due to carcinogenic stimulation, aberrances in miR-10b, miR-372, and miR-375 expression between early stage (I–II) and late stage (III–IV) subjects. Due to the limited number of cases, there was still no difference found between early and late-stage OSCC (Fig. 1D–F). miR-10b was upregulated in OSCC, and miR-375 expression tended to be decreased in OSCC tumors.

**Aberrant miR-372 expression could be a diagnostic marker of dysplasia**

Dysplasia is a strong indicator of malignant changes in OPMDs.17 No significant difference was noted in miR-10b, miR-372, or miR-375 expression in relation to epithelial dysplasia (Fig. 3A–C). However, ROC analysis demonstrated that the level of miR-372 expression could discriminate dysplastic states in OPMD lesions (Fig. 3E), with a sensitivity of only 57.1% and a high specificity of 92.3%. The expression of miR-10b and miR-375 could not distinguish the dysplastic state of OPMD (Fig. 3D and F).

Although the miRNA expression level is acquired from normal counterparts in OPMD or OSCC patients could be clinically unavailable, especially in a clinical screen. Therefore, examining the data of lesion sites across different individuals provides invaluable practical insights.

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**Table 2** Clinicopathological characteristics of OPMD (oral potentially malignant disorder) patients.

| Characteristic                        | Case number |
|--------------------------------------|-------------|
| Age (years, Mean ± SD)              | 52.9 ± 9.6  |
| Gender                               |             |
| Male                                 | 30          |
| Female                               | 1           |
| Oral habits                          |             |
| Alcohol                              | 9           |
| Betel quid                           | 22          |
| Cigarette                            | 28          |
| Pathological diagnosis               |             |
| Hyperplasia                          | 11          |
| Mild dysplasia                       | 4           |
| Moderate dysplasia                   | 16          |
| Severe dysplasia                     | 2           |
| Other                                | 1           |

*a* Three patients have two lesion sites and there are totally 34 OPMD lesions.

RNA isolation

The brushed tip was immersed in PBS at 4 °C and vortexed for 5 s. The procedure was repeated three times and the tip was discarded. The PBS was centrifuged at 1400 g for 10 min at 4 °C, and the cell pellet was collected. RNA was extracted using the mirVana™ PARIS™ kit (mirVana™, Cat No AM1556, Ambion).

**Quantitative reverse transcription polymerase chain reaction analysis (Q-RT-PCR)**

Approximately 2000 ng RNA was treated with RQ1 RNase-Free DNase (Cat No M6101, Promega, Madison, WI, USA) and rNasIn RNase Inhibitor (Cat No N2515, Promega) at 37 °C for 30 min to remove DNA. The reaction was terminated with 20 μl ethylenediaminetetraacetic acid (EDTA) at 65 °C for 10 min. The purified RNA was reverse transcribed to cDNA using MMLV High Performance Reverse Transcriptase (Cat No RT80125K, Lucigen, Middleton, WI, USA). The TaqMan™ MicroRNA Reverse Transcription Kit (mirVana™, Cat No 4366596, Ambion) was used for miRNA reverse transcription. TaqMan miRNA assay kit (Applied Biosystems, Foster City, CA, USA) was used to quantify the expression of miR-10b, miR-372, and miR-375 according to the manufacturer’s protocols. Q-RT-PCR was performed in triplicate with small nuclear RNA U6B as an internal control. Ct is the cycle threshold of the signal amplification, and −ΔΔCt represents the difference in Ct values between the detected miRNAs and U6B. −ΔΔCt is the difference in ΔCt between lesions and their matched controls.

**Statistical analysis**

Paired and unpaired t-tests were used to compare the differences between samples. Receiver operating characteristic (ROC) analysis was performed to determine the diagnostic power. The combined effect of the different variants was determined by the coefficient obtained from multiple regression for further ROC analysis. Statistical significance was set at p < 0.05.
By analyzing the −ΔCt values of OPMDs, significantly lower miR-372 expression was noted in dysplastic lesions relative to non-dysplastic lesions (Fig. 4B). In addition, ROC analysis confirmed a high specificity for the −ΔCt of miR-372 to discriminate dysplastic and non-dysplastic OPMD (Fig. 4E). −ΔCt miR-10b and miR-375 were not able to distinguish dysplasia from non-dysplasia (Fig. 4A, C, D, F).

Detection of miRNA expression in cytobrush samples facilitates OSCC screening

Aberrant miRNA expression in OPMD and OSCC was further dissected to determine the disparities. The expression of miR-10b and miR-372 was similar between OPMD and OSCC patients (Fig. 5A and B). The expression of miR-375 suppressor-mir decreased from OPMD to OSCC patients (Fig. 5C). ROC analysis demonstrated that miR-375 expression was able to discriminate OSCC from OPMD lesions (Fig. 5F), but not miR-10b and miR-372 (Fig. 5D and E).

To mimic the actual clinical situation, we studied −ΔCt of miR-375 in OSCC relative to that in OPMD (Fig. 6C). ROC analysis demonstrated greater specificity when analyzing miR-375 expression to diagnose OSCC (Fig. 6F). The t-test and ROC analysis revealed that the −ΔCt of miR-372 was marginally different between OSCC and OPMD (Fig. 6B and E). −ΔCt of miR-10b was not different between OSCC and OPMD cells (Fig. 6A and D).

The combined effect of miR-372 and miR-375 expression in OSCC diagnosis was determined using multiple regression,

Figure 1  Expression of miR-10b, miR-372, and miR-375 in OSCC. Altered miR-10b (A), miR-372 (B), and miR-375 (C) expression (−ΔCt) relative to that in adjacent normal tissue. Correlation of miR-10b (D), miR-372 (E), and miR-375 (E) expression (−ΔCt) with tumor staging (−ΔCt: difference in Ct values between detected miRNAs and U6B. −ΔCt: difference in ΔCt between lesions and matched controls. Paired t-test for data in A−C, unpaired t-test for data in D-F, p-values are shown in the figure).

Figure 2  Expression of miR-10b, miR-372, and miR-375 in OPMD tissue pairs. Altered miR-10b (A), miR-372 (B), and miR-375 (C) expression (−ΔCt) relative to that in adjacent normal tissue (paired t-test, p-values are shown in the figure).
Figure 3  Expression of miR-10b, miR-372, and miR-375 related to dysplasia states in OPMD lesions. Altered miR-10b (A), miR-372 (B), and miR-375 (C) expression (–ΔΔCt) in dysplastic and non-dysplastic OPMD tissues. The diagnostic power for dysplasia determined using ROC analysis of –ΔΔCt expression of miR-10b (D), miR-372 (E), and miR-375 (F) (unpaired t-test for data in A–C, p-values are shown in the figure).

Figure 4  Expression of miR-10b, miR-372, and miR-375 as related to dysplastic states in OPMD lesions. Altered miR-10b (A), miR-372 (B), and miR-375 (C) expression (–ΔCt) between dysplastic and non-dysplastic OPMD tissues. The diagnostic power for dysplasia determined using ROC analysis of –ΔCt expression of miR-10b (D), miR-372 (E), and miR-375 (F) (unpaired t-test for data in A–C, p-values are shown in the figure).
which generated coefficients for miR-372 and miR-375 to measure the weight. ROC analysis using the sums (coefficient $\times -\Delta \Delta Ct$ of miR-372 + coefficient $\times -\Delta \Delta Ct$ of miR375) indicated that the combined expression of miR-372 and miR-375 yielded a slightly higher accuracy (Fig. 7A) than that of solitary analysis using miR-375 expression. The combined
analysis of $-\Delta\text{Ct}$ of miR-372 and miR-375 revealed a more accurate and 100% sensitive test (Fig. 7B) compared to the solitary analysis of the $-\Delta\text{Ct}$ of miR-375.

Discussion

Among studies to detect miRNA expression, selecting an appropriate internal control to normalize data is always an issue. In circulating miRNAs, endogenous miR-16 is usually adopted as an internal control for plasma or saliva. However, miR-16 expression is not consistent in certain malignancies and the expression of miR-16 could change over time, even under proper storage. Exogenous cel-miR-39 and cel-miR-54 could solve this problem but produce technical complexities. Small nuclear RNA U6 family members are widely used for normalization in intracellular miRNA expression analysis; however, low expression of U6 in body fluid limits its application in circulating miRNA analysis. In this study, we detected intracellular miRNA expression in cytobrush samples, and U6 expression among samples was consistent. Although analysis of the excised tissues was not performed, the analysis of paired cytobrush samples may substantiate the presence of aberrant miRNA expression in OPMD.

Sampling from lesion sites by brushing is an intuitive and direct method. This method has been applied in cytology and HPV detection in HNSCC and for methylation detection among OPMD lesions. Liu et al. used cytobrush to detect cyclin D1 gene copy number alterations in OSCC tissues. This study confirmed that miR-10b is an oncogenic miRNA, and its expression is upregulated in the precancer stage and remains higher in OSCC tissues. Recent study revealed its role in cancer stemness by targeting the actin- and tubulin cytoskeleton-associated protein DIAPH2, which could facilitate metastatic colonization. Thus, the consistent increase of miR-10b in OPMD and OSCC may hinder its use as a good separator of disease progression. In contrast, miR-375 expression was slightly downregulated in OPMD. However, it showed robust downregulation in OSCC cells. This disparity renders it a potent marker to differentiate OPMD from OSCC. miR-375 targets multiple oncogenes, such as AEG-1, YAP1, IGF1R, and PDK1, and its downregulation in cancer might be due to transcription control or promoter methylation. More studies are needed to uncover the mechanism related to miR-375 suppression and more clinical samples are needed to clarify its diagnostic power.

Although miR-372 expression is remarkably upregulated in OPMD, it is surprising that the expression level in cases with dysplasia is lower than that in non-dysplasia cases. Previous findings in our laboratory indicated that miR-372 could target ZBTB7A, which is reported to be correlated with dysplasia in colon cancer. This study also revealed a borderline decrease in miR-372 expression and similar $-\Delta\text{Ct}$ of miR-372 in OPMD and OSCC. These findings may be in conflict with our previous findings in tissues, plasma, and saliva. The sample size limitation could be a reason for this. Hypoxia and many transcriptional signals may modulate the expression of miR-372. In addition, family members in the miR-371/372/373 gene cluster mediate complementary effects on oncogenic activation. The overall effects of oncogenic stimuli and concordant regulation of miR-372 with other family members according to the evolution of pathogenic severity requires precise definition.

The identification of OSCC from other lesions is an important issue in oral cancer screening. The $-\Delta\text{Ct}$ of miR-375, representing the absolute miR-375 expression level, was found to have a strength in discriminating OSCC from OPMD. The combination of $-\Delta\text{Ct}$ of miR-372 further enhanced diagnostic accuracy and sensitivity. The nationwide oral cancer screening database revealed that from 2010 to 2013, more than 3.3 million people were enrolled in screening and more than 155 thousand people were clinically diagnosed with OPMD. The prevalence of OPMD is approximately 4.5% in screened individuals, and the malignant transformation rate is approximately 7%. Biopsy cannot be performed in all OPMD lesions to identify high-risk lesions, but using the cytobrush method, we could intuitively identify suspicious foci for further analysis. Therefore, a high specificity of the test is required to avoid false negatives among OPMD lesions. Compared to the detection of miR-375 expression after normalization to

**Figure 7** Combined effect of miR-372 and miR-375 in diagnosing OSCC. (A) $-\Delta\text{Ct}$ expression of combined effect determined using ROC analysis. (B) $-\Delta\text{Ct}$ expression of combined effect determined using ROC analysis (p-values are shown in the figure diagrams).
controls, the direct assay of $-\Delta$Ct of miR-375 from brushed samples yielded high diagnostic accuracy with a 97.06% specificity. The results suggest that the control counterpart tissue might be influenced by field cancerization, which carries insidious abnormalities. This study also demonstrates that the combination of $-\Delta$Ct of miR-375 and miR-372 exhibits enriched diagnostic power, although the biological basis remains to be uncovered.

In summary, by analyzing brushed samples, this study found aberrant expression of miR-10b, miR-372, and miR-375 in OPMD. A cytobrush sampling strategy, together with miR-375 assay, can detect at high risk of OPMD. Coupling this sampling system with the analysis of a broader spectrum of candidate miRNAs might be a feasible system for OPMD classification.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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