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Original Article

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

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KEYWORDS
Cytobrush;
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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: Cytobrushed 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.

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.[1,2] Upregulation of *miR-31* was observed in OPMD tissues and correlated with malignant transformation.[3] Increased salivary *miR-184* and decreased *miR-145* levels were also noted in OPMD patients and found to have significant diagnostic power.[4] 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.[5–7] A study also revealed that *miR-10b* expression is associated with chemoresistance in HNSCC cells.[8] Moreover, *miR-10b* could downregulate E-cadherin and trigger epithelial mesenchymal transition in the laryngeal SCC cell line, Hep-2,[9] which could be the initial step in oral carcinogenesis. Tu et al.[10] 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.[11,12] 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.[13] In laryngeal SCC, downregulated *miR-375* expression indicates poor prognosis, which might be mediated by targeting hepatocyte nuclear factor 1β.[14] 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,[15,16] 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 18MMHIS187e, 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

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

Characteristics		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 ([a]Total 34 lesions)	Hyperplasia	11
	Mild dysplasia	4
	Moderate dysplasia	16
	Severe dysplasia	2
	others	1

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

together with the tip were immediately immersed in RNA-later solution (Cat No AM7021, Ambion, Austin, TX, USA) and stored at 4 °C. RNA isolation was performed within 24 h.

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 rRNasin RNase Inhibitor (Cat No N2515, Promega) at 37 °C for 30 min to remove DNA. The reaction was terminated with 20 mM 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 $-\Delta Ct$ represents the difference in Ct values between the detected miRNAs and U6B. $-\Delta\Delta Ct$ 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 $p < 0.05$.

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 in OPMD were considered early events in oral carcinogenesis.

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 the normalization of paired samples, the sampling of 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.

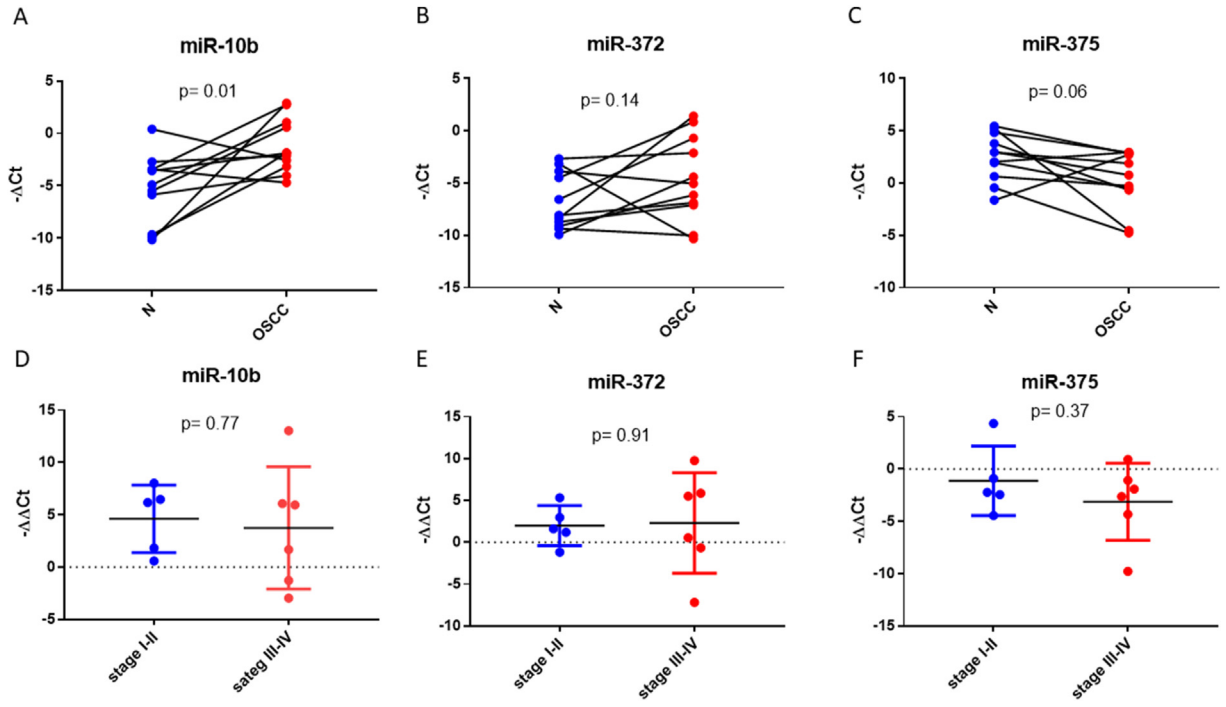

Figure 1 Expression of *miR-10b*, *miR-372*, and *miR-375* in OSCC. Altered *miR-10b* (A), *miR-372* (B), and *miR-375* (C) expression (−ΔCt) relative to that in adjacent normal tissue. Correlation of *miR-10b* (D), *miR-372* (E), and *miR-375* (E) expression (−ΔΔCt) with tumor staging (−ΔCt: difference in Ct values between detected miRNAs and *U6B*. −ΔΔCt: 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).

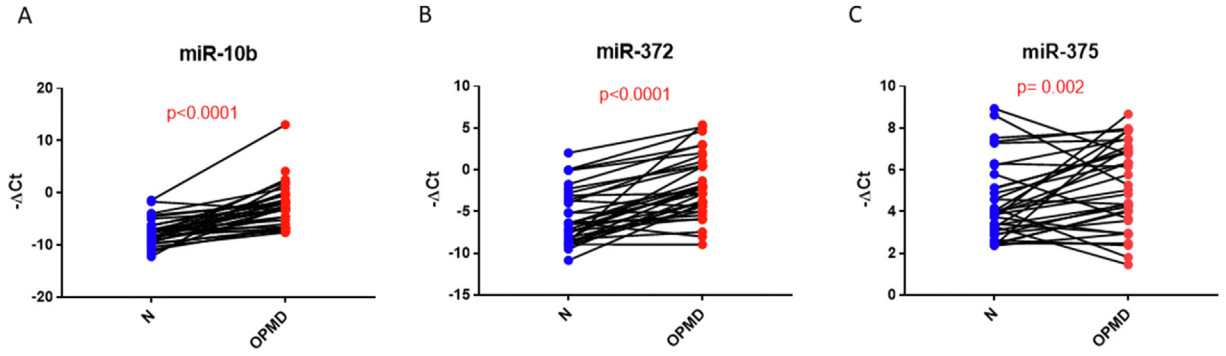

Figure 2 Expression of *miR-10b*, *miR-372*, and *miR-375* in OPMD tissue pairs. Altered *miR-10b* (A), *miR-372* (B), and *miR-375* (C) expression (−ΔCt) relative to that in adjacent normal tissue (paired t-test, p-values are shown in the figure).

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 and found lower −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,

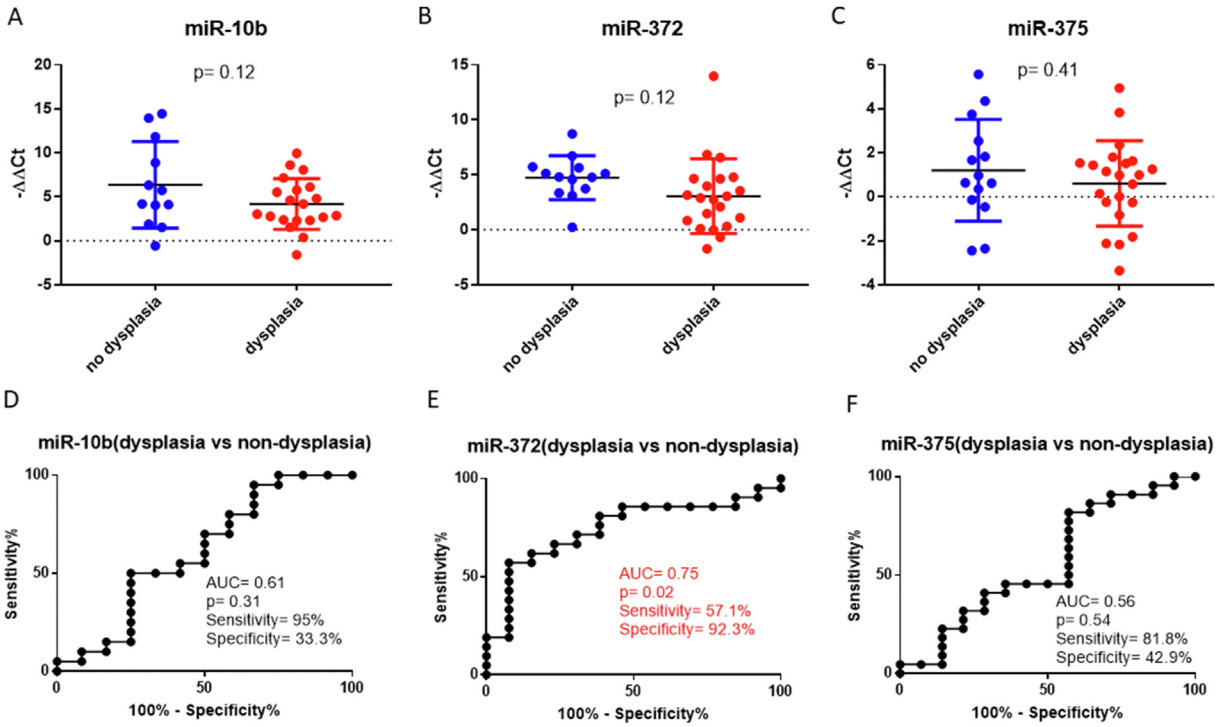

Figure 3 Expression of *miR-10b*, *miR-372*, and *miR-375* related to dysplasia states in OPMD lesions. Altered *miR-10b* (A), *miR-372* (B), and *miR-375* (C) expression (−ΔΔCt) in dysplastic and non-dysplastic OPMD tissues. The diagnostic power for dysplasia determined using ROC analysis of −ΔΔCt expression 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).

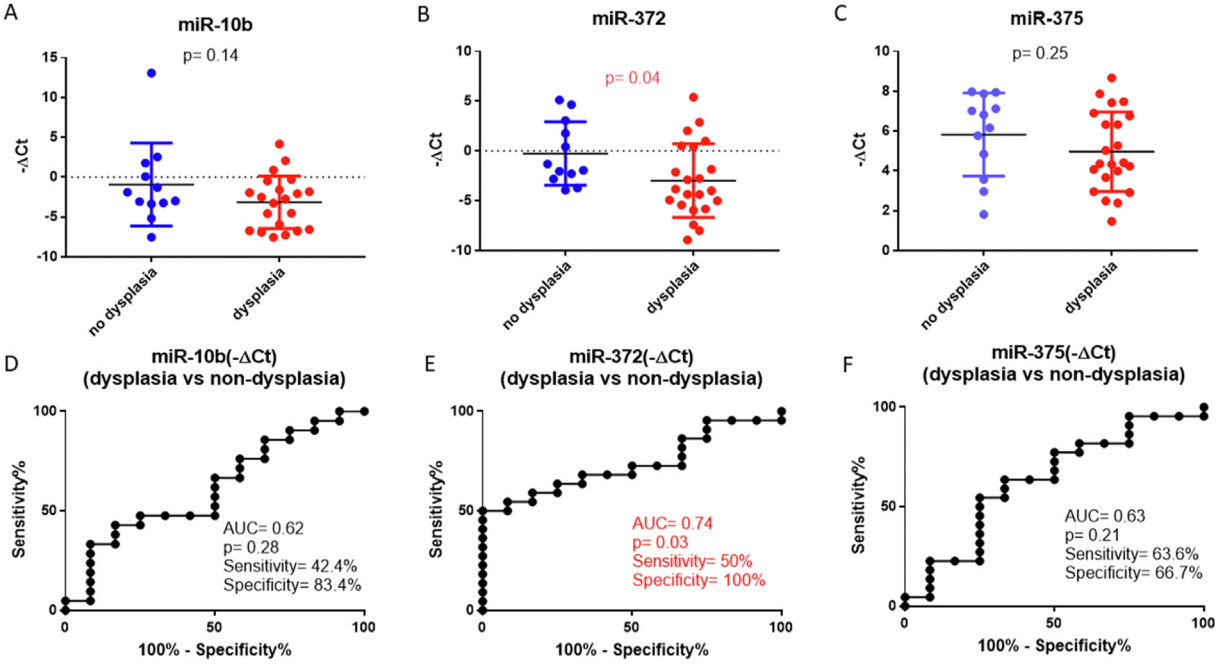

Figure 4 Expression of *miR-10b*, *miR-372*, and *miR-375* as related to dysplastic states in OPMD lesions. Altered *miR-10b* (A), *miR-372* (B), and *miR-375* (C) expression (−ΔCt) between dysplastic and non-dysplastic OPMD tissues. The diagnostic power for dysplasia determined using ROC analysis of −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).

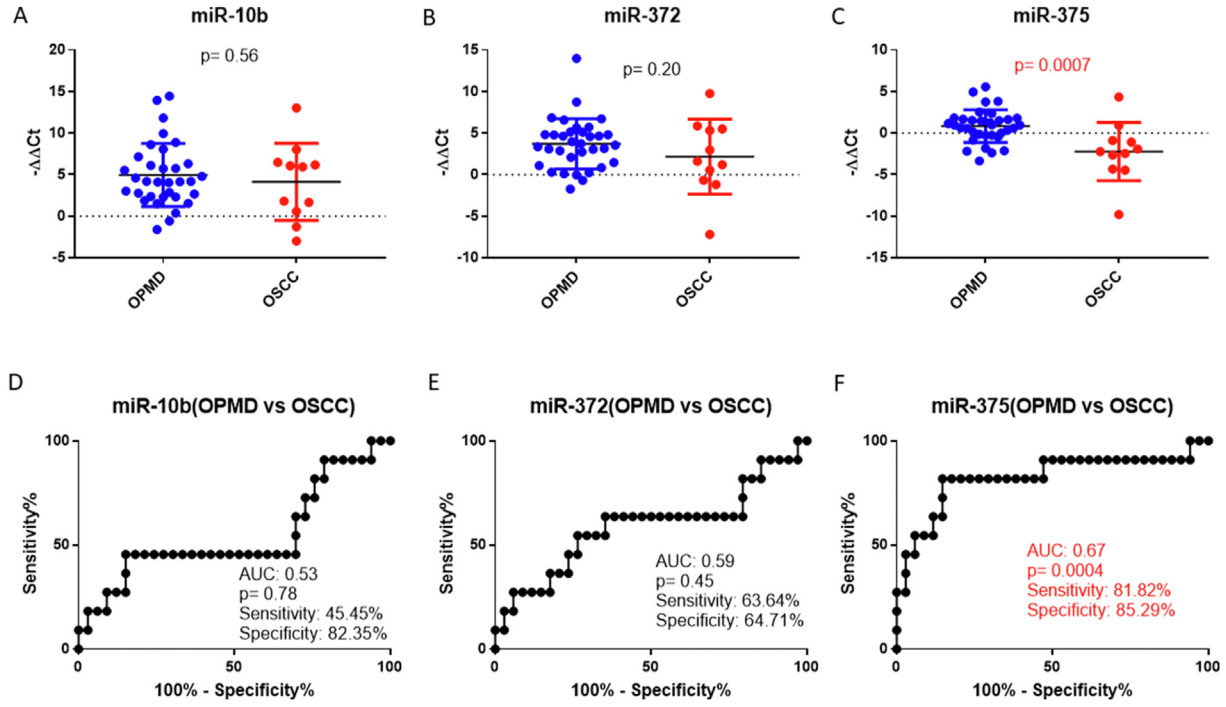

Figure 5 Expression of *miR-10b*, *miR-372*, and *miR-375* among OPMD and OSCC tissues. Altered *miR-10b* (A), *miR-372* (B), and *miR-375* (C) expression (−ΔΔCt) between OPMD and OSCC tissues. The diagnostic power to discriminate OSCC from OPMD determined using ROC analysis of −ΔΔCt expression of *miR-10b* (D), *miR-372* (E), and *miR-375* (F) (unpaired t-test for data in A–C, p-values are shown in the figure).

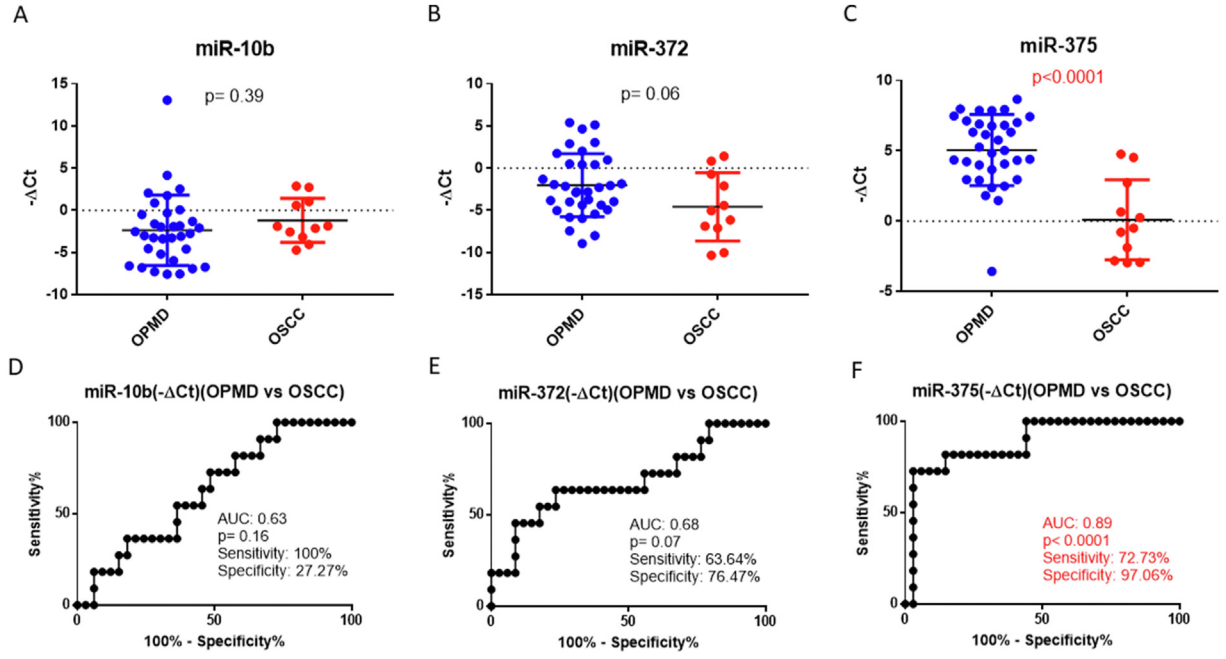

Figure 6 Expression of *miR-10b*, *miR-372*, and *miR-375* in OPMD and OSCC tissues. Altered *miR-10b* (A), *miR-372* (B), and *miR-375* (C) expression (−ΔCt) in OPMD and OSCC tissues. The diagnostic power to discriminate OSCC from OPMD determined using ROC analysis of −ΔCt expression of *miR-10b* (D), *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 x −ΔΔCt of *miR-372* + coefficient x −ΔΔCt 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 −ΔCt of *miR-372* and *miR-375* revealed a more accurate and 100% sensitive test (Fig. 7B) compared to the solitary analysis of the −ΔCt 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.[15,16] However, *miR-16* expression is not consistent in certain maliganacies[18] and the expression of *miR-16* could change over time, even under proper storage.[19] Exogenous *cel-miR-39* and *cel-miR-54* could solve this problem but produce technical complexities.[20] 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.[21] 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[21] and for methylation detection among OPMD lesions.[22] Liu et al.[23] 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.[24] 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.[25] 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,[12] which is reported to be correlated with dysplasia in colon cancer.[26] This study also revealed a borderline decrease in *miR-372* expression and similar −ΔCt of *miR-372* in OPMD and OSCC. These findings may be in conflict with our previous findings in tissues, plasma, and saliva.[11,12] The sample size limitation could be a reason for this. Hypoxia and many transcriptional signals may modulate the expression of *miR-372*.[11] In addition, family members in the *miR-371/372/373* gene cluster mediate complementary effects on oncogenic activation.[27] 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 −ΔCt of *miR-375*, representing the absolute *miR-375* expression level, was found to have a strength in discriminating OSCC from OPMD. The combination of −ΔCt 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%.[28] 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

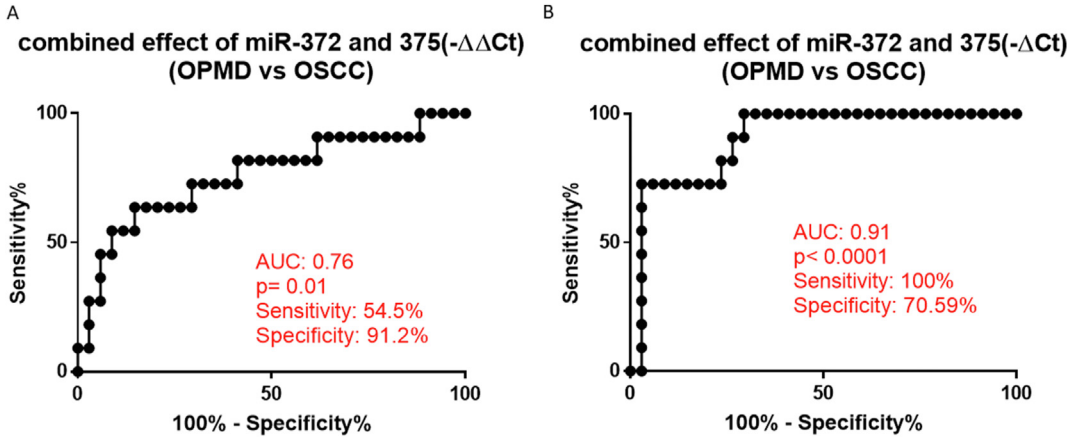

Figure 7 Combined effect of *miR-372* and *miR-375* in diagnosing OSCC. (A) −ΔΔCt expression of combined effect determined using ROC analysis. (B) −ΔCt expression of combined effect determined using ROC analysis (p-values are shown in the figure diagrams).

controls, the direct assay of −ΔCt 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 −ΔCt 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.

Acknowledgements

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