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Original Article

Exploiting salivary miR-375 as a clinical biomarker of oral potentially malignant disorder

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Abstract *Background/purpose:* Oral potentially malignant disorder (OPMD) is an important premalignancy worldwide. MicroRNAs (miRNAs) are endogenously expressed non-coding RNAs that regulate the post-transcriptional levels of targeted mRNAs. *MiRNA-375* (*miR-375*) is markedly downregulated in oral carcinoma tissues and plays an oncogenic role in oral carcinogenesis. We explored the potential of the deregulated salivary *miR-375* levels in OPMD patients.
Materials and methods: . We analyzed the levels of *miR-375* in the saliva of patients with OPMD ($n = 45$) and healthy controls ($n = 24$) by quantitative RT-PCR. The cell lysates and supernatants were treated with the miR-375 mimic and inhibitor.
Results: Salivary *miR-375* levels were decreased markedly in the patients with OPMD, compared with the controls. OPMD patients with non-dysplasia showed a higher abundance of *miR-375* in the saliva than dysplasia patients, suggesting that salivary *miR-375* is a more sensitive marker for OPMD. Patients with malignant transformation during the follow-up period showed lower expression of saliva *miR-375* than the others. *MiR-375* expression was markedly decreased by treatment with the miR-375 inhibitor, and the supernatants of both NHOK and SAS cells showed a corresponding decline in *miR-375* expression.
Conclusion: Our results indicate the potential application of salivary *miR-375* as a biomarker for the detection and long-term follow-up of OPMD.

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Introduction

Oral potentially malignant disorders (OPMDs) are lesions that correlate with the risk of oral squamous cell carcinoma (OSCC). The overall OPMD transformation rate was estimated at 6.73% in previous studies in Taiwan.[1–4] A British report indicated that the average overall OPMD transformation rate was 2.6% in 12 years.[5] Interventions for OPMDs are important to prevent OSCC. However, among the diverse and large number of OPMDs, identifying the lesions with the 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 the alterations in 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.[6,7] Upregulation of *miR-31* was observed in OPMD tissues and correlated with malignant transformation.[8] Increased salivary *miR-184* and decreased *miR-145* levels were also noted in OPMD patients and found to have a notable diagnostic power.[9] Cervigne et al. stated that the tissue expression of *miR-21*, *miR-181b*, and *miR-345* is an early event in leukoplakia transforming into malignancy.[10] The altered expression of miRNAs could be a feasible biomarker for determining the potential of malignancy. However, not all progressive premalignant oral lesions were associated with the overexpression of these miRNAs.[10] Critical miRNA alterations occurring in OPMD require vigorous exploration to address the underlying pathogenetic process.

MiR-375 is a well-established tumor suppressor microRNA known to be downregulated in many cancer types, and its downregulation has been suggested to be correlated with tumor size and invasion in OSCC.[11,12] A low expression level of *miR-375* in tongue and laryngeal cancer patients was related to poor of prognosis.[13,14] Underexpression of *miR-375* could lead to uncontrolled CIP2A expression and extended stability of MYC, which contributes to the promotion of oral cancerous phenotypes.[15] As an OSCC suppressor, whether the downregulation of *miR-375* occurs in OPMD remains uncertain.

Since the collection and processing of saliva is simple, relatively non-invasive, and cost-effective, it has been used extensively to extract meaningful biological data in different localized and oral diseases.[16] Salivary miRNA biomarkers have recently emerged as a valuable diagnostic method for the detection of oral lesions.[9] The expression of *miR-31* significantly increased in the saliva of OPMD patients; *miR-31* and epithelial dysplasia are significantly associated with disease progression.[8]

In this study, we further explored the potential of the deregulated levels of *miR-375* demonstrated in saliva to serve as biomarkers for the early detection of, and prognostic indicators in OPMD patients.

Materials and methods

Cell culture and reagents

The OSCC cell lines SCC25, SAS, and OECM-1, and normal human oral keratinocytes (NHOKs) were cultured as described previously.[17] The levels of miR-375 in the cultured cells and supernatants were analyzed. NHOKs and SAS were prepared for the further expression study. The miR-375 mimic (C-300682-05), miR-375 inhibitor (IH-300682-07), and negative control (CN-001000-01) were purchased from Dharmacon (Horizon Discovery, Lafayette, CO, USA). A total of 2×10^5 NHOK and SAS cells were seeded in a 6-well plate and transfected with 100 nmol/L miR-375 mimic/inhibitor/negative control using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 24 h. The levels of miR-375 in the cultured cells and supernatants were analyzed.

Saliva samples

These samples were collected after obtaining written informed consent from all the participants, and this study was approved by the Institutional Review Board (IRB) of the Mackay Memorial Hospital (IRB approval numbers 09MMHIS146 and 10MMHIS185). Saliva was collected before surgery from 41 patients with OPMD and 26 matched controls that provided written informed consent. Saliva specimens from 26 healthy individuals matched by age, sex, and oral habits served as the controls. All patients with OPMD underwent total excision and were confirmed by the same pathologist. They were then followed up for at least 5.5 years. No OPMD was diagnosed in the matched controls during the follow-up period. Clinicopathological parameters of the study subjects are listed in Table 1. Nearly all patients were male, and the vast majority was smokers and/or betel chewers. Eighteen patients were diagnosed as oral epithelial dysplasia, Patients with specific types of OPMD, including oral submucosal fibrosis and erosive lichen planus, were excluded from this study. During the following period there were 5 patients became as OSCC (Table 1).

Two milliliters of saliva were collected from the mouth floor after simple mouth rinsing.[18,19] After centrifugation, 400 μL of saliva was aliquoted and preserved at −80 °C before use, as described previously.[19,20]

Table 1 Clinicopathological characteristics of oral potentially malignant disorder (OPMD) patients.

Characteristics		Case	Control
Age	(years old, Mean ± SD)	47.89 ± 13.39	49.19 ± 10.5
Gender	Male	40	25
	Female	1	1
Follow-up period	(years, Mean ± SD)	6.81 ± 1.4	7.02 ± 2.1
Oral habits	Alcohol	10	5
	Betel quid	40	22
	Cigarette	38	20
Pathological diagnosis	Epithelial Hyperplasia	11	
	Mild oral epithelialdysplasia	3	
	Moderate oral epithelial dysplasia	11	
	Severe oral epithelialdysplasia	4	
Malignant transformation	[a]Others	12	
	Yes	5	
	Non	36	

[a] Others: acanthosis, hyperkeratosis, hyperparakeratosis.

miRNA extraction and quantitative-RT-PCR analysis

The supernatant of the cultured cells was collected and centrifuged at 1000 rpm for 5 min to remove potentially contaminating debris and floating cells. The supernatant (400 μL) was aliquoted and preserved at −80 °C. To analyze the cellular miRNAs, the attached cells were harvested by trypsinization and suspended in 400 μL of PBS.[21] miRNAs were then purified from 400 μL of the cell lysates, supernatants, and saliva using the *mir*Vana™ PARIS™ isolation kit (mirVana™, Cat No AM1556, Ambion, Austin, TX, USA). The *miR-375* levels in 5 μL of eluate (out of 100 μL) were measured using the TaqMan miRNA assay system (Applied Biosystems, Foster City, CA, USA). Briefly, a looped RT primer specifically paired with *miR-375* formed a primer/*miR-375*-chimera, and the extended oligonucleotide served as a template for subsequent quantitative assays.[22,23] Quantitative RT-PCR (qRT-PCR) was performed in triplicate or duplicate using the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The expression of *U6B* served as an internal control.[21,24] A negative control without a template was amplified in parallel to determine the specificity of the reactions. Ct is the cycle threshold of the signal amplification, and ΔCt represents the difference in Ct values between the *miR-375* and *U6B* levels. Extreme samples, i.e., those with Ct values for *U6B* beyond 40, were excluded from the analysis.[21]

Statistical analysis

Data are shown as the means or means ± S.E. of triplicate analyses. The sample size of this study was estimated by accessing the PS: Power/Sample Size Calculator version 3.1 (https://biostat.app.vumc.org/wiki/Main/PowerSampleSize). SPSS software (version 20.0; IBM, Armonk, New York, USA) was used to perform the statistical analyses. The Mann–Whitney U test was used to compare the differences between the various clinical variants. Statistical significance was set at $P < 0.05$. To determine the extent to which the obtained −ΔCt could efficiently separate different clinical subsets, receiver operating characteristic (ROC) analysis was performed, and the area under the curve (AUC) was used as a measurement of the separating ability.

Results

Correlation of *mir-375* levels between cell lysates and supernatants

To explore whether the concentration of extracellular *miR-375* reflected the corresponding changes in NHOK, SCC25, SAS and OECM-1 cells. The results of the in vitro study revealed that the expression level of miR-375 was reduced in OSCC cell lines (SCC25, SAS, and OECM-1), compared with that in the NHOK cells (Fig. 1). qRT-PCR was used to evaluate the expression levels of *miR-375* in the cell lysates and supernatants. Transfection with *the miR-375* mimic increased *miR-375* levels in NHOK and SAS cells (Fig. 2A and C). Likewise, *miR-375* mimic-treated cells also had higher supernatant *miR-375* levels than the cells treated with the negative control (Fig. 2B and D). When the cellular *miR-375* level was significantly decreased by treatment with *miR-375* inhibitor (Fig. 2A and C), the *miR-375* levels in the supernatants from both the NHOK and SAS cells showed a corresponding decline (Fig. 2B and D).

Decreased *mir-375* levels in the saliva of OPMD patients

To investigate whether *miR-375* downregulation occurred not only in cytobrushed tumor tissues (as shown in our previous study),[25] but also in the secretome of saliva from patients with OPMD, we analyzed the expression level of *miR-375* in the saliva of patients with OPMD and healthy individuals. Regardless of pathological condition, the expression of *miR-375* in the saliva of patients with OPMD was significantly lower than that in healthy individuals, as revealed by a mean −ΔCt value of 8.97 in OPMD patients,

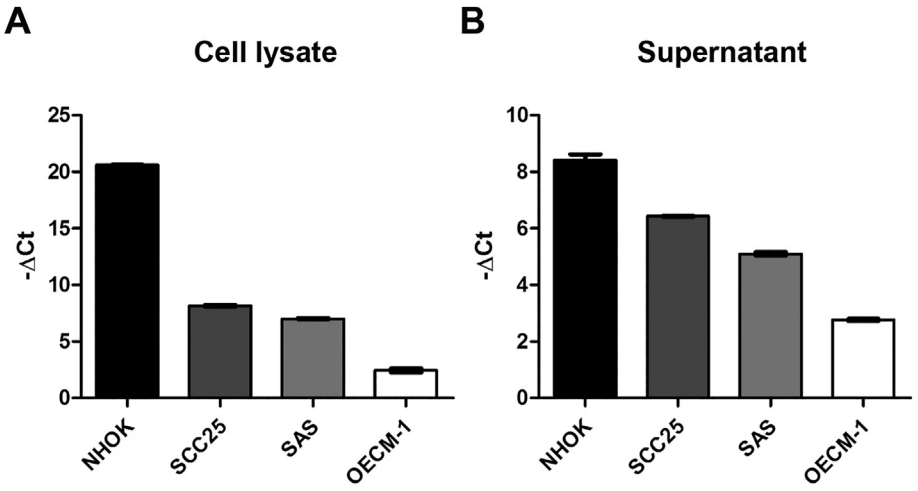

Figure 1 *miR-375* is underexpressed in OSCC cells. Q-RT-PCR analysis showed the expression level of *miR-375* in OSCC cell lines and NHOK cells.

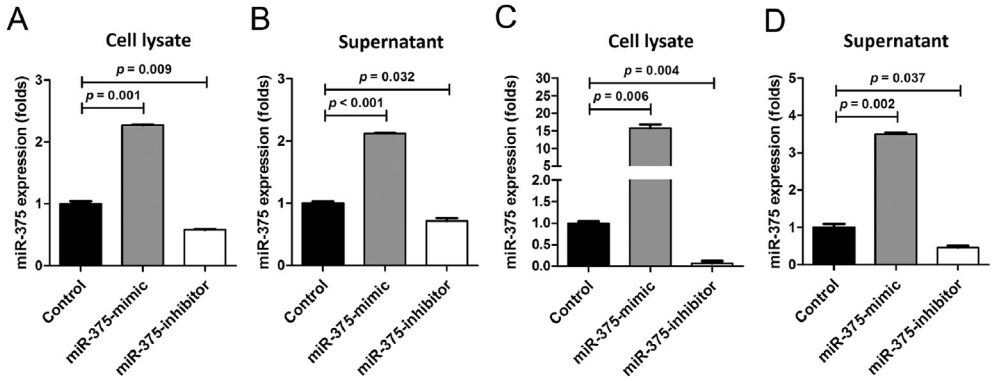

Figure 2 The cellular expression levels of *miR-375* in the supernatant were consistent with the *miR-375* levels in the supernatant. NHOK and SAS cells were transfected with the *miR-375* mimic/inhibitor/negative control. Q-RT-PCR analysis was performed to detect the expression levels of *miR-375* in NHOK (A) and SAS cells (B) and the *miR-375* levels in the supernatants of NHOK (C) and SAS cells (D).

compared with a value of 10.17 in the controls (Fig. 3A, Table 2). Differences in the *miR-375* levels were found between clinical subsets of patients, such as those with or without dysplasia; this difference was statistically significant (Fig. 3B). In the ROC analyses, when a value of −ΔCt of −10.3 was used as a cutoff to differentiate the healthy state from the OPMD state, a discrimination power of 0.82 was achieved, with a sensitivity of 0.80 and a specificity of 0.68 (Fig. 3D). In the ROC analyses, when a −ΔCt value of −8.9 was used as a cutoff to differentiate the non-dysplasia state from the dysplasia state, a discrimination power of 0.77 was attained, with a sensitivity of 0.71 and specificity of 0.83 (Fig. 3E) (see Table 3).

Decreased *mir-375* levels in the saliva of patients with malignant transformation

We found that 12.2% (5/41) of the OPMD patients presented with OSCC during the follow-up period (Fig. 3C). The mean −ΔCt value of the *miR-375* levels declined from a −8.3 to −10.2. The discrimination power for differentiating a patient's operative status using the ROC analysis was 0.71 when a −ΔCt of − 9.5 was used as the cutoff (Fig. 3F).

Discussion

It was found that hazard ratio of the malignant transformation rate was 8.19 times higher in the OPMD group than in the comparison cohort.[3] Data suggested that anyone diagnosed with OPMDs should be closely followed up.[3] The development of new, specific biomarkers is critical to improving the diagnosis and prognosis for patients with such OPMDs. Saliva is in constant contact with oral tissues and is easily obtained using non-invasive methods. These properties make saliva an advantageous source of specimens for the search for potential biomarkers although it may contain serum and desquamated epithelial cells from the oral mucosa, including some leucocytes, especially if there is ulceration or mucositis, the latter being inevitable if a neoplasm is present.[26,27] Because of the stability of their structures, miRNAs may exist stably even in harsh biological

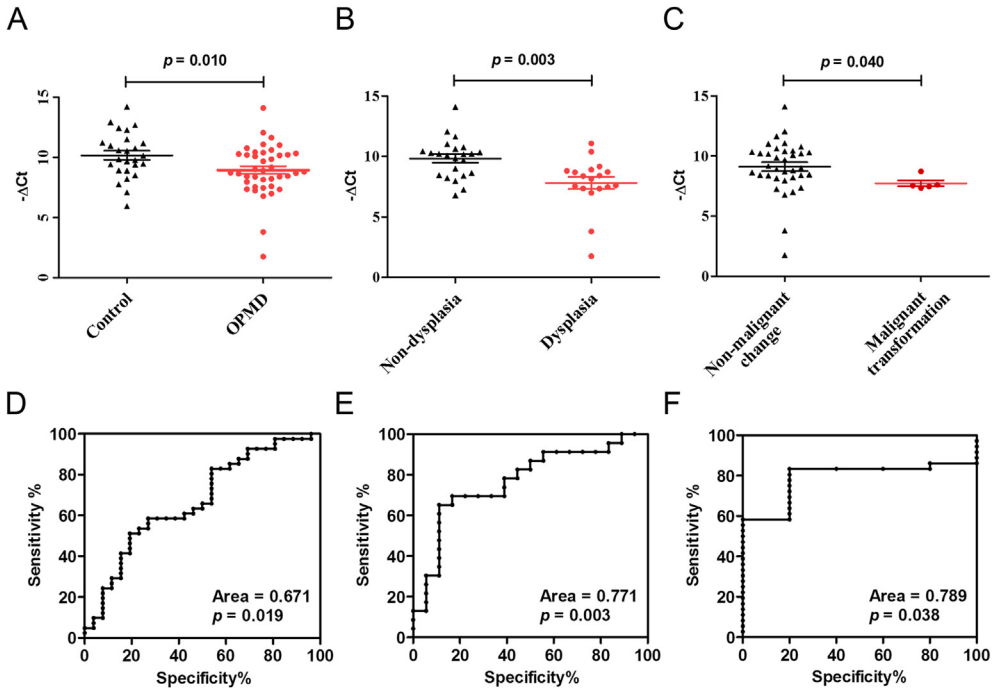

Figure 3 Salivary *miR-375* expression in controls and patients with oral premalignant disorder (OPMD). Q-RT-PCR analysis was performed to detect the expression levels of *miR-375* in saliva samples from control subjects and OPMD patients (A), subjects with non-dysplasia and dysplasia (B), and subjects with non-malignant change and malignant transformation (C). Receiver operating characteristic (ROC) analysis of saliva samples from control subjects and OPMD patients (D), subjects with non-dysplasia and dysplasia (E), and subjects with non-malignant change and malignant transformation (F).

Table 2 -ΔCt values of *miR-375* expression in the saliva of OPMD patients and controls.

	OPMD (n = 41)	Control (n = 26)	*p* value[a]
	-ΔCt (Mean ± SE)	-ΔCt (Mean ± SE)	
All	8.97 ± 0.37	10.17 ± 0.33	0.020[b]
Dysplasia(n = 18)	7.87 ± 0.5		<0.001[c]

Abbreviations: OPMD, oral potentially malignant disorder.
-ΔCt: difference in cycle threshold values between detected miRNAs and U6B.
[a] Mann–Whitney analysis.
[b] Comparison of salivary *miR-375* levels across OPMD patients and controls.
[c] Comparison of salivary *miR-375* levels across dysplasia patients and controls.

Table 3 -ΔCt values of *miR-375* expression in the saliva of OPMD patients.

	-ΔCt (Mean ± SE)	*p* value[a]
Dysplasia (n = 18)	7.87 ± 0.5	0.003[b]
Non-dysplasia (n = 23)	9.83 ± 0.34	
Malignant transformation(n = 5)	7.54 ± 0.25	0.04[c]
Non- malignant transformation (n = 36)	9.11 ± 0.37	

Abbreviation: OPMD, oral potentially malignant disorder.
-ΔCt: difference in cycle threshold values between detected miRNAs and U6B.
[a] Mann–Whitney analysis.
[b] Comparison of salivary *miR-375* in dysplasia patients and non-dysplasia patients.
[c] Comparison of salivary *miR-375* levels in OPMD patients with malignant transformation and Non-malignant transformation.

environments, for example, within the saliva, plasma, or other body fluids.[28,29] This characteristic makes salivary miRNAs an emerging field for monitoring not only oral diseases but also systemic diseases and general health.[8,16,20,30,31] The lack of well-characterized or matched clinical groups and lack of suitable endogenous

controls for extracellular miRNA detection in saliva and the need for normalization are among the major restrictions associated with utilizing salivary miRNAs for biomarker discovery. Therefore, we used gender, age, and oral habit-matched controls from various populations to identify appropriate salivary miRNA biomarkers, so that population-specific and habit-specific miRNA signatures in OPMD patients could be elucidated.

Studies on salivary miRNA expression in OPMD are sparse, given that miRNA expression studies associated with oral cancer began only in 2009.[32] Several studies on salivary miRNAs were performed in OPMD, and several miRNAs were found to be deregulated in OPMD.[8,16,32–34] *MiR-21*, *miR-31*, and *miR-184* were overexpressed in OPMD,[6,8,16,34] in contrast with *miR-145*, which was underexpressed in OPMD.[34] There have been no definite studies to evaluate *miR-375* expression in the saliva of OPMD patients. Previous studies have shown a marked decrease in *miR-375* expression in tissues and plasma from patients with OSCC, compared with that in healthy individuals.[12,13,15,35,36] In tissue samples, the expression level of *miR-375* was significantly higher in nonprogressive premalignant lesions than in progressive premalignant lesions. Moreover, *miR-375* expression was significantly lower after the progression to OSCC in all the samples.[37] This is the first time that salivary *miR-375* was found to be expressed in OPMD. Aberrant salivary *miR-375* expression could be detected in OPMD patients and distinguish them from matched control volunteers and dysplasia patients from subjects without dysplasia, suggesting a potential clinical application for oral lesion-specific miRNA signatures in saliva. This is a convenient and less invasive method that highlights *miR-375* as a biomarker for OPMD.

Since salivary fluid consists of cellular debris and degraded RNAs, the supernatant is probably a better sample for assaying cell-free nucleic acids. Moreover, exosome enrichment followed by miRNA isolation further reduced the variability in expression profiles among samples due to the removal of degraded RNAs. In a previous cytobrushing study, *miR-375* was derived directly and mainly from tumor tissue. This was supported by direct evidence from our in vitro study, which showed that the expression of *miR-375* in the supernatant decreased after the inhibition of intracellular *miR-375* expression in cells. These results suggest the potential application of salivary *miR-375* in monitoring or detecting residual or recurrent OSCC.

Downregulated *miR-375* levels in saliva could be detected even from different types of oral lesions, including dysplasia, and the sensitivity of detection of such lesions was not different from the detection of the severity of the dysplasia. This suggests that because of its abundance, salivary *miR-375* could potentially be exploited to detect and diagnose insidious oral precancerous lesions in at-risk populations.[19]

A recent study showed a notable decrease in the levels of *miR-200a* and *miR-125a* in the saliva of patients with tongue cancers.[32] Although the origin and clinical significance of these two salivary miRNAs showing decreased expression in OSCC remain to be determined, measuring the levels of salivary miRNAs in patients with OSCC by qRT-PCR appears to be a promising approach to identify new biomarkers in saliva.[20] The discovery and combination of multiple salivary miRNAs may provide a better power of discrimination for the diagnosis of OPMD and follow-up of OPMD patients. Considering that the origins of salivary and plasma miRNAs may be similar, decreases in plasma and salivary *miR-375* levels may have clinical or pathological implications. However, the high correlation between them suggests that the combined use of both salivary and plasma markers may also enable a more powerful prediction of OPMD.[16]

Declaration of competing interest

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

Acknowledgments

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