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ORIGINAL ARTICLE

Increase of microRNA *miR-31* level in plasma could be a potential marker of oral cancer

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BACKGROUNDS: Oral squamous cell carcinoma (OSCC) is a worldwide disease. MicroRNAs are endogenously expressed non-coding RNAs that have important biological and pathological functions. *miR-31* was found markedly up-regulated in OSCC and several other malignancies. However, *miR-31* expression was also down-regulated in the metastasis process of breast carcinoma.

MATERIALS AND METHODS: Using quantitative RT-PCR analysis, we identified plasma *miR-31* in OSCC patients (n = 43) and case controlled individuals (n = 21). Nine OSCC patients saliva were also analyzed. The Mann–Whitney test and Wilcoxon matched pairs test were used to compare the differences among the various clinical variants.

RESULTS: *miR-31* in plasma was significantly elevated in OSCC patients relative to age and sex-matched control individuals. This marker yielded a receiver operating characteristic curve area of 0.82 and an accuracy of 0.72 defined by leave-one-out cross-validation. In addition, the plasma *miR-31* in patients was remarkably reduced after tumor resection suggesting that this marker is tumor associated. Our preliminary analysis also demonstrated the feasibility of detecting the increase of *miR-31* in patient's saliva.

CONCLUSION: This study concluded that plasma *miR-31* could be validated a marker of OSCC for diagnostic uses.
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Keywords: carcinoma; *miR-31*; marker; oral; plasma; saliva

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most frequent carcinoma worldwide (Lin *et al*, 2005; Shieh *et al*, 2007; Chang *et al*, 2008a; Wong *et al*, 2008b; Liu *et al*, 2009a). MicroRNAs (miRNAs) are 19–24 nucleotides non-coding RNAs that regulate the translation and degradation of target mRNAs (Bartel, 2004, 2009). Evidences indicate that miRNA may play important roles in carcinogenesis (Dalmay and Edwards, 2006; Bartel, 2009). It is predicted that there may be more than 1000 miRNAs in the human genome (Yu *et al*, 2006; Bartel, 2009). Since each miRNA has the potential to regulate multiple mRNAs, it is possible that very few biological pathways are not impacted on by miRNAs. The abundance of miRNAs and their apparent pluripotent actions suggest that the identification of miRNAs involved in oncogenesis might yield targets or networks that are suitable for diagnostic marker or therapeutic intervention (Dalmay, 2008; Bartel, 2009).

An association has also been identified between *miR-211* expression and the vascular invasion of OSCC (Chang *et al*, 2008a). *miR-21* and *miR-184* were found to be oncogenic in OSCC (Chang *et al*, 2008b; Wong *et al*, 2008b). *miR-31* was found up-regulated in a wide variety of neoplasms including head and neck cancer, hepatocellular carcinoma (HCC), and colorectal carcinoma (CRC), and this miRNA appeared oncogenic for these neoplasms (Bandres *et al*, 2006; Slaby *et al*, 2007; Tran *et al*, 2007; Kozaki *et al*, 2008; Wong *et al*, 2008a,b; Liu *et al*, 2009b). However, a recent study demonstrated the up-regulation of *miR-31* in metastasis-free breast carcinoma and it drives inhibitory effects on breast carcinoma metastasis by targeting multiple genes (Valastyan *et al*, 2009). Several markers were found in the OSCC blood samples (Cheng *et al*, 2005; Garcia-Olmo *et al*, 2006; Nakamoto *et al*, 2006; Bijian *et al*, 2009). In our previous study, we have used the serum levels of MMP-9 and vascular epidermal growth factor (VEGF) from pretreatment patients to evaluate the prognosis of OSCC (Liu *et al*,

2009a). Recent studies have detected the circulatory miRNA in the plasma or saliva of cancer patients (Li *et al*, 2004; Chen *et al*, 2008; Gilad *et al*, 2008; Mitchell *et al*, 2008; Wong *et al*, 2008b; Ng *et al*, 2009a; Park *et al*, 2009). This study is tested if the plasma and saliva level of *miR-31* could be a potential non-invasive marker for OSCC.

Materials and methods

Plasma samples

The blood samples were collected from 43 OSCC patients in the week prior to operation and 4–6 weeks after operation. Patients received their operation in 2008–2009 (Table 1), who provided written informed consent. Twenty-one age- and sex-matched subjects served as controls. All the tumor underwent stages classification according to the American Joint Committee on Cancer system. None of the patients had received adjuvant chemotherapy or radiotherapy before surgery. Five milliliter of whole blood was collected in a heparin-coated tube from the patients. Patients have been followed for an average period of about 6 months.

Saliva samples

The saliva from nine patients was collected in the week prior to operation and 6 weeks after operation. The saliva collected from eight normal individuals was used as control. Patients received their operation in 2009, who provided written informed consent. Five milliliter of saliva was collected from the floor of mouth after cleaning of oral cavity by mouth rinsing (Park *et al*, 2006; Hu *et al*, 2008). The saliva samples were centrifuged to separate the fluid fraction from the cell fraction and debris. The supernatant phase of whole saliva was collected as described previously (Li *et al*, 2004; Hu *et al*, 2008). Aliquots of fluids were stored at −80°C until use.

miRNA extraction and qRT-PCR analysis

miRNA was purified from 600 μl plasma or saliva using *mir*Vana™ PARIS™ isolation kit (Ambion, Austin, TX, USA). Around 5 μl aliquots from a total of 100 μl eluted miRNA solution was used to quantify the expression of *miR-31* using a TaqMan microRNA assay system according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). In brief, looped RT primer specific for *miR-31* was used to produce a primer/*miR-31*-chimera. The extended oligonucleotide presented a template amenable to a subsequent quantitative assay (Chang *et al*, 2008a). TaqMan quantitative-RT-PCR (abbreviated as qRT-PCR) was done in triplicate. qPCR reactions were done on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). The comparative threshold cycle (ΔC_t) method was used to measure the *miR-31* expression level relative to the expression of internal control. A previous study specified the absence of difference in C_t value of *miR-16* between the plasma of controls and of CRC patients (Ng *et al*, 2009), the expression of *miR-16* was used as an internal control. A negative control without a template was run in parallel to assess the overall specificity of the reaction. $-\Delta C_t$ represents the normalized *miR-31* expression level achieved by deducing the C_t of *miR-31* from C_t of *miR-16*.

Table 1 The clinical parameters of the subjects

	OSCC patients	*Controls*
Age (years)	53.9 ± 9.4	51.4 ± 8.4
Sex (M/F)	41/2	20/1
Site		
Gingiva	10	
Buccal mucosa	17	
Tongue	11	
Others	5	
TNM staging		
T1–2	19	
T3–4	24	
N0	27	
N+	16	
Stage I–II	15	
Stage III–IV	28	

Statistical analysis

The Mann–Whitney test and Wilcoxon matched pairs test were used to compare the differences among the various clinical variants. Binary logistic regression analysis was used to achieve an adjusted odds ratio and a 95% confidence interval (CI). A P-value of < 0.05 was considered statistically significant. To evaluate the extent to which the obtained qRT-PCR data could separate the different clinical settings, receiver operating characteristic (ROC) analysis was performed and the area under curve was used as a measurement for the level of separation. The leave-one-out cross-validation (LOOCV) model was also used to compute the accuracy of the analysis in differentiating various settings.

Results

Plasma miR-31 as potential diagnostic marker

qRT-PCR analysis indicated that the OSCC patients had significantly higher levels of *miR-31* in their preoperative plasma than the controls (Figure 1a). The plasma levels of *miR-31* in T1–2 patients, patients without nodal involvement (N0) or stage I–II patients were also significantly higher than controls (Figure 1a). When using $-\Delta C_t$ of -16.0 as the cutoff, this marker yielded an area of 0.82 under ROC and an accuracy of 0.72 as defined by LOOCV (Figure 1b). Multivariate logistic regression analysis indicated an adjusted odds ratio 24.06 (95% CI = 3.93 - 177.58; $P = 0.003$).

In addition, 88% (38/43) of the patients showed a varying degree of decrease in plasma *miR-31* after resection. The difference was statistically significant (Figure 2a). ROC analyses indicated the dissection power of 0.71 in using the cutoff to differentiate patient's operative status (Figure 2b).

Saliva miR-31 was increased in OSCC patients

qRT-PCR analysis of saliva samples indicated that the OSCC patients had significantly higher levels of *miR-31* in saliva than the controls (Figure 3). This was com-

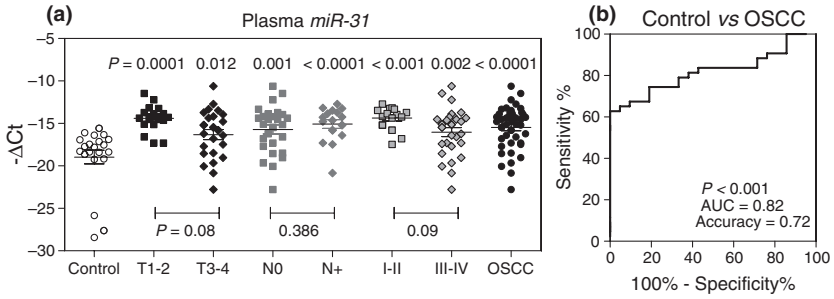

Figure 1 Plasma *miR-31* in controls and preoperative patients. **(a)** Scatter dot plot with mean ± s.e. of the $-\Delta C_t$ in controls and patient's preoperative samples. Mann–Whitney test. **(b)** ROC and LOOCV analysis across control and preoperative samples

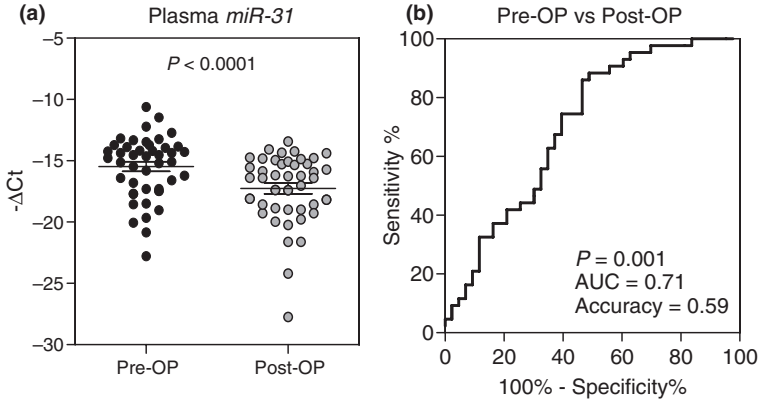

Figure 2 Plasma *miR-31* in preoperative and postoperative samples. **(a)** Scatter dot plot with mean ± s.e. of the $-\Delta C_t$ in patient's preoperative and postoperative sample pairs. Wilcoxon matched pairs test. **(b)** ROC and LOOCV analysis across preoperative and postoperative samples

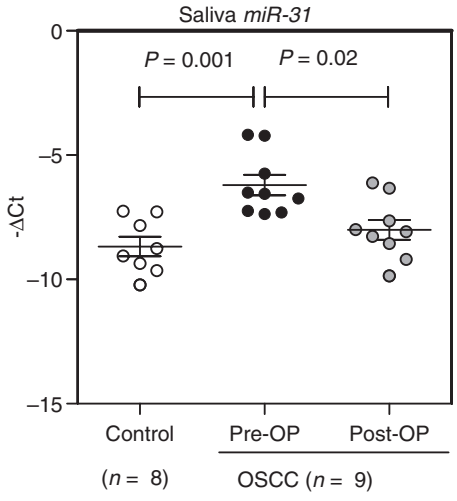

Figure 3 Saliva *miR-31*. Scatter dot plot with mean ± s.e. of the $-\Delta C_t$ in controls, and patient's preoperative and postoperative samples. Mann–Whitney test or Wilcoxon matched pairs test

plemented by a similar decrease of $-\Delta C_t$ for around 2.8 for patients after tumor resection (Figure 3). Eight of nine patients exhibited the decrease of *miR-31* in saliva after tumor resection. At this stage, the dissection power of saliva *miR-31* is undetermined because of the limited number of samples available. In both patients and controls, the saliva *miR-31* level was higher than plasma *miR-31* level as indicated by higher $-\Delta C_t$ values.

Discussion

The high stability of miRNA in blood enables circulating miRNA to be used as a potential marker for malignancies (Chen *et al*, 2008; Gilad *et al*, 2008; Mitchell *et al*, 2008; Wong *et al*, 2008b; Ng *et al*, 2009). The presence of a modest amount of miRNA may be sufficient to allow the non-invasive detection of malignancies using body fluid. Ng *et al* (2009) validated that the plasma levels of *miR-17-3p* and *miR-92*, members of the *miR-17-92* polycistron, can be used as screening markers for CRC. Our tests showed that there was a high level of plasma *miR-31* in OSCC patients compared to controls. Since plasma *miR-31* level in patients declined after surgery in most patients, it is highly likely that the circulatory *miR-31* is derived from the tumor tissue (Wong *et al*, 2008b). It should be noted that our results suggest that this assay has potential for the detection of early neoplasms (Ng *et al*, 2009). Thus, plasma *miR-31* levels might be useful as a systemic marker that distinguishes OSCC from non-cancerous status. A study is being carried out across various medical centers to test the predictive power for OSCC using plasma *miR-31* as a marker.

Saliva meets the demands of an inexpensive, non-invasive, and accessible bodily fluid to act as an ideal diagnostic medium. Specific and informative markers in saliva are greatly needed to serve for diagnosing disease and monitoring human health. Knowing the constituents in saliva is essential for using this body fluid to identify potential markers for disease diagnostics (Hu *et al*, 2008). Although OSCC cells are immersed in the

saliva and it is difficult to distinguish the presence of molecules in saliva as being originated from contaminated tumor cells or being released from tumor cells, salivary analysis of OSCC patients represents a potentially promising approach to validate potential markers for the disease (Li *et al*, 2004). Although our preliminary analysis indicated that *miR-31* was highly abundant in saliva, the estimation of this marker's diagnostic power was limited by the small sample size. Expanded number of samples should be tested to increase the confidence of our findings. Nevertheless, the saliva *miR-31* might be useful in the future for the detection of OSCC and the validation of this new opportunity for non-invasive preneoplastic diagnosis seems important (Hu *et al*, 2008). In future, analysis will also be laid to validate the discriminating power of the combined plasma test and saliva test for a new diagnostic approach of OSCC. It would also be important to know the sensitivity of salivary *miR-31* as a marker for screening insidious malignancy in risk population of OSCC.

Although it is very likely to extend plasma *miR-31* test to diagnostic uses for malignancies including HCC and CRC, which exhibited the increased *miR-31* expression in tissues similar to OSCC (Bandres *et al*, 2006; Slaby *et al*, 2007; Wong *et al*, 2008a,b; Chen *et al*, 2009), its uses in breast cancer and gastric cancer would be restricted (Valastyan *et al*, 2009; Zhang *et al*, 2009). Additional studies using a large cohort of samples are required to evaluate if the plasma *miR-31* can be a marker annotating multiple types of neoplasm. It worth noting that the plasma *miR-184* level was also significantly higher in tongue cancer patients (Wong *et al*, 2008b). The power of the combined use of *miR-31* with *miR-184*, MMP-9 or VEGF as plasma markers for diagnosis or prognostic prediction requires further evaluation (Wong *et al*, 2008b; Liu *et al*, 2009a). Since we have developed the early detection of genetic lesions in brushed samples of OSCC risk population using quantitative assays (Lin *et al*, 2005), it would be important to test the feasibility of using *miR-31* expression to signify the OSCC risk using plasma, saliva, or brushed oral samples. Till now, there is no widely accepted plasma or salivary marker specific for OSCC being available. Despite that the quantitative methodologies proposed in the present study could still be immature for routine clinical uses as restricted by cost and technical feasibility, the design of rapid and convenient practices will improve OSCC diagnosis.

Conflict of interest

None declared.

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