

ORIGINAL ARTICLE

Dysregulation of miR-31 and miR-375 expression is associated with clinical outcomes in oral carcinoma

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OBJECTIVES: To identify differentially expressed miRNA between oral squamous cell carcinoma (OSCC) and non-cancer (NC) and to associate these with clinico-pathological parameters.

MATERIALS AND METHODS: miRNA microarray profiling was utilized to obtain the expression profile of miRNAs in four OSCC and four NC samples. The expression of miR-31 and miR-375 was further validated in 26 OSCC and three NC samples using real-time-PCR. The association between miRNA expression and clinico-pathological parameters was tested by univariate and multivariate analyses.

RESULTS: Microarray profiling demonstrated that 15 and four miRNAs were up-regulated and down-regulated, respectively, in OSCC as compared with NC. miR-31 and miR-375 were validated as up- and down-regulated miRNAs, respectively. In univariate analyses, expression of miR-31 was significantly elevated in early stage, tumours with no metastatic nodes and those from the buccal mucosa. By contrast, low miR-375 expression was significantly associated with late stage disease, larger tumour size and the non-cohesive type of pattern of invasion in OSCC. The association between miR-31 expression with tumour staging and site and miR-375 with tumour staging remained significant in multivariate analyses.

CONCLUSIONS: This study has identified 19 miRNAs significantly associated with OSCC, and expressions of miR-31 and miR-375 were significantly related with clinico-pathological parameters suggesting they could be important in driving oral tumourigenesis.

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Keywords: microRNA profiling; miR-31; miR-375; oral squamous cell carcinoma; clinico-pathological parameters

Introduction

Oral cancer is one of the most common malignancies and a major cause of cancer morbidity and mortality worldwide (Ferlay *et al.*, 2010). In 2008, the global burden of cancer (GLOBOCAN) has estimated that around 263 900 new cases and 128 000 deaths worldwide are due to oral cavity cancer (including lip cancer) (Jemal *et al.*, 2011), and the majority of these are oral squamous cell carcinoma (OSCC) (Beenken and Urist, 2003).

Despite the refinement of advanced surgical techniques and adjuvant therapies over the last 30 years, the incidence and mortality rates of this disease still remain alarmingly high. The reasons behind this high incidence were mainly due to the serious delay in the diagnosis of the cancer and the high recurrence rate (Laronde *et al.*, 2008).

While many efforts were focused on protein-coding genes that may contribute to OSCC development, current knowledge on the role of non-coding genes such as microRNA (miRNA) in carcinogenesis remains limited. miRNAs are small endogenous RNA molecules that are involved in the regulation of the gene's expression at the post-transcriptional level. Understanding the expression pattern of miRNAs in OSCC using high-throughput microarray profiling techniques may reveal novel cancer-related alterations, which would be beneficial in further studying the biological functional potential of miRNAs in relation to OSCC development.

There were several levels of evidence that miRNA dysregulation can play an essential role in human cancers including OSCC (Takamizawa *et al.*, 2004; Hayashita *et al.*, 2005; Langevin *et al.*, 2010; Schrammedei *et al.*, 2011). Further, previous studies also reported the

significant associations between miRNA expression and important clinical features of tumours (Blenkiron *et al*, 2007; Marcucci *et al*, 2008; Giovannetti *et al*, 2010; Lee *et al*, 2011; Rothe *et al*, 2011). A recent study demonstrated that up-regulated miR-21 is involved in promoting the growth of OSCC cell lines (Wang *et al*, 2012). This study subsequently reported that combination of an anti-cancer drug, cisplatin with down-regulation of miR-21 oligonucleotide, might be a potential approach to OSCC therapy (Wang *et al*, 2012).

This study aims to identify the differential expression of miRNA in OSCC as compared to NC tissue and to associate the expression of differentially expressed miRNAs with clinico-pathological characteristics. An understanding of this association of miRNAs with clinico-pathological parameters could provide insights into the role of these miRNAs in driving OSCC development and could lead to the development of miRNA-based prognostic biomarkers for OSCC in the future.

Materials and methods

Samples selection

Eight formalin-fixed paraffin-embedded (FFPE) tissue samples were utilized for miRNA microarray profiling. These samples consist of four histologically confirmed OSCCs (S1, S2, S3 and S4) and four NCs (mucocoele, fibroepithelial hyperplasia and normal oral mucosa; S5, S6, S7 and S8) from individuals with no potentially malignant lesions or cancer. An independent set of 26 OSCC and three normal oral mucosa (NOM) FFPE tissues were used for validation. All FFPE tissue samples were obtained from the Oral Pathology Diagnostic Laboratory, Faculty of Dentistry, University of Malaya. This study was approved by Medical Ethics Committee, Faculty of Dentistry, University of Malaya [MEC number: DFOP1007/0044(P)].

Socio-demographic and clinico-pathological data of the patients were obtained from the Malaysian Oral Cancer Database and Tissue Bank System (MOCDBTS) coordinated by Oral Cancer Research and Coordinating Centre, University of Malaya (OCRCC, UM) (Zain *et al*, 2012).

Sample processing and miRNA extraction

Four microns thick sections of FFPE tissues were stained with haematoxylin and eosin (H&E), and the percentage of tumour cells and normal epithelial cells were determined by an oral pathologist (RBZ). Only samples with $\geq 70\%$ of tumour or normal epithelial cells were selected for further processing. To obtain normal epithelium, the NOM tissues were cored using ATA-100 Advanced Tissue Arrayer (Chemicon, Temecula, CA, USA) following the procedure reported in Saleh *et al* (2010).

The macrodissected sections and tissue cores were deparaffinized with xylene. After 4 h of xylene incubation, total RNA was extracted using miRNeasy FFPE kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Quantification and purity of total RNA was assessed using NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and Agilent 2100 bioanalyzer RNA 6000 Nano Kit assay (Agilent Technologies,

Santa Clara, CA, USA). This was carried out to examine the integrity of total RNA and ensure that short RNA such as miRNAs were present in the sample. Only those samples with sufficient RNA integrity number (RIN) ≥ 2.0 , concentration $>50 \text{ ng } \mu\text{l}^{-1}$ and A260/A280 > 1.8 were selected for microarray profiling and validation. Due to the high level of degradation of nucleic acid in FFPE, generally, a RIN value of 2.0 was considered as a minimum cut-off value for successful execution of further analyses including RNA expression arrays (Schroeder *et al*, 2006; Ribeiro-Silva *et al*, 2007; Ludyga *et al*, 2012).

miRNA microarray profiling

miRNA profiling from total RNA was performed using the Agilent Technologies 8 \times 15K Human miRNA Microarray Kit version 2 (Agilent Technologies). This platform was based on Sanger miRBase 10.1, which consist of 723 human miRNA and 76 viral miRNA targets of 60-mer oligonucleotide that are synthesized directly onto the array.

Data and pathway analysis

Feature extraction data from the miRNA profiling were normalized and analysed using Partek Genomic Suite (Partek Incorporated, St. Louis, MO, USA). miRNA with a fold-change above 2.0 and below -2.0 between the two groups (OSCC and NC) were considered to be differentially expressed. The *P*-value <0.05 was considered as statistically significant.

The significantly differentially expressed miRNAs were further analysed in MetaCore Analytical Suite version 4.5 (GeneGo, Inc., St. Joseph, MI, USA) to determine their role in biological pathways that are associated with tumorigenesis. The significant association between miRNA and biological pathway was ranked by false discovery rate (FDR) <0.05 based on the hypergeometric distribution statistic.

Quantitative real-time PCR validation

Selected miRNAs of interest were validated using quantitative real-time PCR. Expression levels of selected mature microRNAs were confirmed by TaqMan Human MicroRNA Assay kit (Applied Biosystems Incorporated, Foster City, CA, USA) according to the manufacturer's protocol. The expression of each miRNA was normalized to the housekeeping gene, RNU-44. RNU-44 was selected as endogenous control in this experiment because of its high abundance and low expression variability across normal human tissues (Lussier *et al*, 2011).

Statistical analysis

All statistical analyses were performed using SPSS[®] 12 (SPSS, Incorporated, Chicago, IL, USA). In univariate analysis, Mann-Whitney and Kruskal-Wallis tests were used to determine the association between miRNAs expression and clinico-pathological parameters (TNM staging, tumour size, lymph node metastasis, differentiation, pattern of invasion and tumour site). For those significant associations, multivariate analysis was

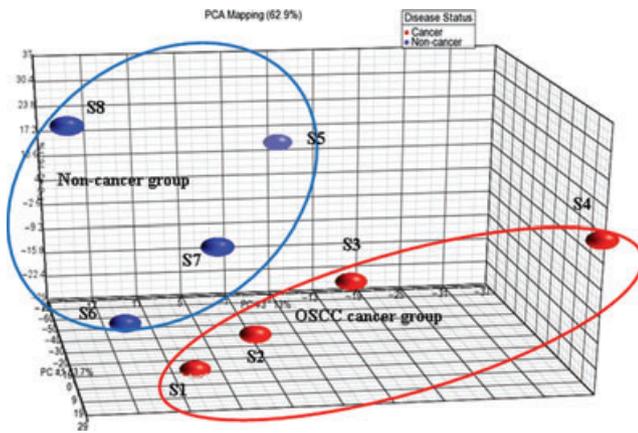


Figure 1 Principal component analysis (PCA) demonstrating that the OSCC samples are genetically distinct from the NC samples

subsequently applied to adjust for risk habits (cigarette smoking, betel quid chewing and alcohol drinking). As the socio-demographic variables (age and gender) were not significantly associated with miRNAs expression in univariate analysis, these factors were not further adjusted in multivariate analysis. The *P*-value of <0.05 was considered significant.

Results

Global microRNA expression pattern

Principal component analysis (PCA) demonstrated that all the four OSCC samples (S1–S4) were more similar to one another as compared to the four NC samples (S5–S8) (Figure 1), suggesting OSCC samples had distinct miRNA expression profile from NC samples. Focusing on the OSCC group, sample S4 was found to be farthest away from the main OSCC group (S1, S2, and S3). Within NC group (S5–S8), each sample was located slightly apart from one another.

miRNA expression in OSCC compared with NC

A total of 939 miRNAs were identified as differentially expressed between OSCC and NC samples. Upon filtering with fold-change less than –2.0 and more than 2.0 with

Table 1 List of 19 specific microRNAs (15 up-regulated and four down-regulated miRNAs) that are significantly (*P* < 0.05) differentially expressed in OSCC samples, when compared with NC samples with their average fold-change indicated, respectively

| MicroRNAs | Average fold-change (OSCC vs NC) |
|------------------|----------------------------------|
| hsa-miR-510 | –4.2 |
| hsa-miR-375 | –4.1 |
| hcmv-miR-UL70-3p | –2.3 |
| hsa-miR-100 | –2.2 |
| hsa-miR-105 | 2.1 |
| hsa-miR-155 | 2.1 |
| hsa-miR-183 | 2.2 |
| hsa-miR-501-5p | 2.2 |
| hsa-miR-629 | 2.2 |
| hsa-miR-182 | 2.3 |
| hsa-miR-1274a | 2.3 |
| hsa-miR-941 | 2.6 |
| hsa-miR-181b | 2.6 |
| hsa-miR-7 | 2.8 |
| hsa-miR-151-3p | 2.9 |
| hsa-miR-222 | 2.9 |
| hsa-miR-200c | 3.7 |
| hsa-miR-21 | 11.8 |
| hsa-miR-31 | 13.2 |

P-value <0.05, visualization using a volcano plot (Figure 2) unveiled a total of 19 specific miRNAs (28 probes), of which 15 were up-regulated and four were down-regulated in OSCC, respectively (Table 1).

Pathway analysis of significantly differentially expressed miRNAs in OSCC

The involvement of the 19 differentially expressed miRNAs in cancer-related biological pathways was determined using pathway analysis. Eleven miRNAs were mapped to the top five most significant biological pathways as indicated in Table 2. As miR-31 and miR-375 were involved in the top 5 most significant pathways, they were selected for validation, and subsequently, their expressions in OSCC were associated with clinico-pathological parameters.

Validation using real-time PCR

Both miR-31 and miR-375 were validated using real-time PCR. miR-31 was found to be up-regulated in OSCC

Figure 2 Volcano plot that was used to filter significant differentially expressed miRNAs. Every single dot within the plot represents a single miRNA probe which is differentially expressed in the miRNA microarray experiment. There are 28 probes (19 miRNAs) which are shown to be significantly differentially expressed. Among them, 15 miRNAs which are differentially induced with >twofold change (up-regulated expression) are represented in grey-shadowed area, and four miRNAs were repressed with >2 fold change (down-regulated expression) are represented in green-shadowed area

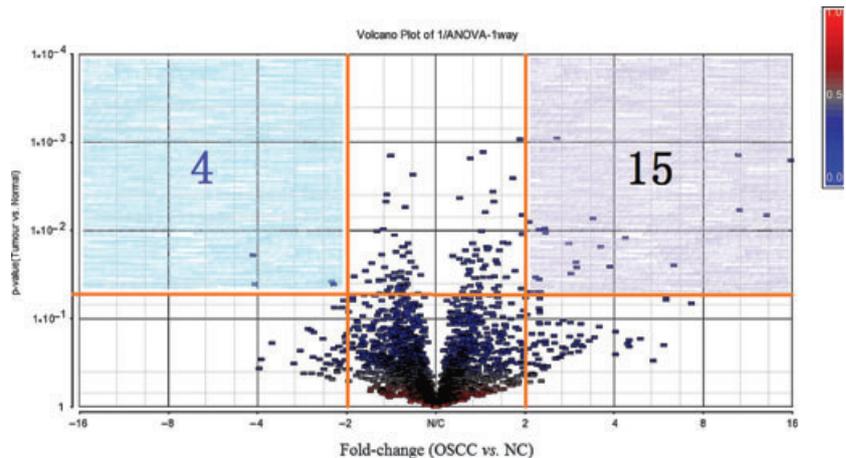


Table 2 Pathway analysis revealed that seven of 19 differentially expressed miRNAs were mapped to the top five most significant biological pathways. Highlighted miR-31 and miR-375 (with their targeted mRNA respectively) were selected for validation as they were demonstrated to be statistically enriched which is highly suggestive of biological potential and involvement in carcinogenesis

| No | Pathway | P-value | MicroRNAs involved |
|----|---|----------|---|
| 1 | Cell cycle: Regulation of G1/S transition | 1.23E-13 | miR-222, miR-182, miR-183, miR-31 (p16INK4), miR-21, miR-155, miR-375 (SP1) |
| 2 | Cytoskeleton: Remodelling TGF, WNT and cytoskeletal remodelling | 2.30E-13 | miR-155, miR-31 (Frizzled), miR-222, miR-181-b2, miR-200c, miR-21, miR-100, miR-182, miR-375 (SP1; Caveolin-1) |
| 3 | Development: Regulation of epithelial-to-mesenchymal transition (EMT) | 1.97E-12 | miR-7, miR-21, miR-200c, miR-155, miR-31 (PDGF-B; Frizzled), miR-181-b, miR-375 (ZFHX1B; SP1) |
| 4 | Development: GM-CSF signalling | 2.10E-09 | miR-155, miR-375 (14-3-3 zeta/delta; JAK2), miR-183, miR-222, miR-181b, miR-182 |
| 5 | Cytoskeleton: Cytoskeletal remodelling | 3.34E-09 | miR-155, miR-21, miR-31 (alpha-3/beta-1 integrin; alpha-V/beta-3 integrin), miR-200c, miR-182, miR-375 (Caveolin-1) |

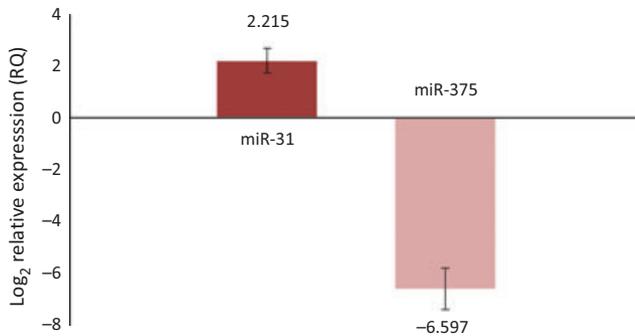


Figure 3 Expression of miR-31 and miR-375 determined by quantitative real-time PCR to validate miRNAs' expression in 26 OSCC samples compared with three NC samples

samples with an average fold-change of 2.2, while miR-375 showed a down-regulation in OSCC samples with an average fold-change of 6.6 in OSCC samples after normalizing with NC samples (Figure 3).

Association between miR-31 and miR-375 with clinico-pathological parameters

Socio-demographic and clinico-pathological characteristics of the independent set of 26 OSCC samples were summarized in Table 3.

Upon validation of the expression of miR-31 and miR-375, their association with clinico-pathological parameters of OSCC was examined. As shown in Table 4 (univariate analysis), differential expressions of both miR-31 and miR-375 were significantly associated with TNM staging. miR-31 has a significantly higher expression in early stage OSCC as compared to late stage OSCC ($P = 0.018$). By contrast, a very significantly lower level of miR-375 was observed in late stage OSCC as compared to the early stage ($P = 0.006$). Expression of miR-31 was also significantly associated with the lack of lymph node metastasis ($P = 0.025$) and tumour subsites ($P = 0.006$). Interestingly, the expression of miR-375 was strongly associated with tumour size and pattern of invasion in OSCC where the down-regulation of miR-375 was observed in OSCC of larger size at diagnosis ($P = 0.025$) and non-cohesive pattern of invasion at the invasive front ($P = 0.024$). Following multivariate analyses, miR-31 expression remained significantly associated with tumour

Table 3 Clinico-pathological data and characteristics of 26 OSCC samples involved in the validation experiment

| Characteristics | No. of sample | % |
|---------------------------------|---------------|------|
| Gender | | |
| Male | 4 | 15.4 |
| Female | 22 | 84.6 |
| Age | | |
| ≤ 45 | 3 | 11.5 |
| >45 | 23 | 88.5 |
| Tumour site | | |
| Tongue | 9 | 34.6 |
| Gingival | 8 | 30.8 |
| Buccal mucosa | 8 | 30.8 |
| Lip | 1 | 3.8 |
| TNM staging | | |
| I + II (early stage) | 12 | 46.2 |
| III + IV (late stage) | 14 | 53.8 |
| Tumour size | | |
| T ₁ + T ₂ | 15 | 57.7 |
| T ₃ + T ₄ | 11 | 42.3 |
| Lymph node metastasis | | |
| No | 21 | 80.8 |
| Yes | 5 | 19.2 |
| Differentiation | | |
| Well | 11 | 42.3 |
| Moderate | 15 | 57.7 |
| Pattern of invasion | | |
| Cohesive | 8 | 30.8 |
| Non-cohesive | 18 | 69.2 |

staging and site, while miR-375 expression remained significantly associated with tumour staging (highlighted in Table 4).

Discussion

Formalin-fixed paraffin-embedded tissue specimens can be used for the analyses of miRNA study as they are not significantly affected by formalin fixation compared to other longer strands of RNA (Xi *et al*, 2007; Glud *et al*, 2009). This insusceptibility of miRNA to degradation in FFPE tissues is thought to be due to its small size (approximately 20 nucleotides long) and stability of its secondary structure (Xi *et al*, 2007). In addition, FFPE tissue represents excellent specimen as they are associated with documented clinico-pathological records (Glud *et al*, 2009). Due to its advantages, this study has embarked on miRNA profiling of OSCC using FFPE specimens.

Table 4 Association between miR-31 and miR-375 expression and socio-demographic and clinico-pathological parameters in OSCC tissues

| Socio-demographic and clinico-pathological parameter | No. of samples | miR-31 | | | Adjusted P-value ^b | miR-375 | | | Adjusted P-value ^b |
|--|----------------|-----------------|-----------------|--------------------------|-------------------------------|-----------------|----------------|--------------|-------------------------------|
| | | Median RQ (IQR) | Mean RQ (s.d.) | P-value ^a | | Median RQ (IQR) | Mean RQ (s.d.) | P-value | |
| Gender | | | | | | | | | |
| (i) Male | 4 | 7.040 (11.771) | 8.347 (6.306) | 0.776 | | 0.0622 (0.152) | 0.073 (0.082) | 0.669 | |
| (ii) Female | 22 | 4.712 (16.828) | 11.711 (15.218) | | | 0.001 (0.063) | 0.116 (0.279) | | |
| Age | | | | | | | | | |
| (i) ≤ 45 | 3 | 9.654 (–) | 11.677 (4.503) | 0.356 | | 0.015 (–) | 0.178 (0.289) | 0.185 | |
| (ii) >45 | 23 | 4.474 (16.626) | 11.130 (15.044) | | | 0.001 (0.066) | 0.100 (0.259) | | |
| TNM staging | | | | | | | | | |
| (i) I + II | 12 | 13.150 (11.732) | 16.100 (16.516) | 0.018 | 0.036 | 0.0898 (0.348) | 0.227 (0.350) | 0.006 | 0.023 |
| (ii) III + IV | 14 | 2.635 (7.103) | 6.987 (10.683) | | | <0.001 (0.001) | 0.008 (0.020) | | |
| Lymph node metastasis | | | | | | | | | |
| (i) Yes | 5 | 1.034 (3.296) | 1.864 (1.814) | 0.025 | 0.144 | <0.001 (0.033) | 0.0134 (0.029) | 0.625 | |
| (ii) No | 21 | 8.539 (14.939) | 13.415 (14.948) | | | 0.0071 (0.142) | 0.132 (0.283) | | |
| Tumour size | | | | | | | | | |
| (i) T ₁ +T ₂ | 15 | 8.539 (12.528) | 13.430 (15.664) | 0.113 | | 0.015 (0.237) | 0.182 (0.324) | 0.025 | 0.088 |
| (ii) T ₃ +T ₄ | 11 | 2.306 (17.412) | 8.143 (11.867) | | | <0.001 (0.003) | 0.010 (0.022) | | |
| Differentiation | | | | | | | | | |
| (i) Well | 11 | 5.540 (15.968) | 14.258 (19.658) | 0.856 | | 0.040 (0.166) | 0.193 (0.367) | 0.077 | |
| (ii) Moderate | 15 | 4.474 (15.354) | 8.946 (8.356) | | | <0.001 (0.008) | 0.047 (0.114) | | |
| Pattern of invasion | | | | | | | | | |
| (i) Non-cohesive | 18 | 4.115 (16.069) | 8.737 (10.190) | 0.243 | | <0.001 (0.0263) | 0.044 (0.105) | 0.024 | 0.084 |
| (ii) Cohesive | 8 | 12.565 (13.232) | 16.720 (20.363) | | | 0.0790 (0.422) | 0.256 (0.419) | | |
| Site ^c | | | | | | | | | |
| (i) Tongue | 9 | 8.540 (13.839) | 10.240 (7.370) | 0.006^d | 0.004 | 0.009 (0.316) | 0.130 (0.200) | 0.36 | |
| (ii) Gingival | 8 | 0.920 (2.153) | 3.019 (5.980) | | | <0.001 (0.030) | 0.013 (0.025) | | |
| (iii) Buccal | 8 | 16.824 (27.121) | 21.359 (20.398) | | | 0.033 (0.154) | 0.194 (0.412) | | |

Significant *p* - value were highlighted in bold.

^aMann–Whitney test (univariate analysis) was used in gender, age, TNM staging, tumour size, lymph node metastasis, differentiation and pattern of invasion.

^bMultivariate analysis was applied to adjust the confounders [risk habits (cigarette smoking, betel quid chewing and alcohol drinking)] for those significant relationships in univariate analysis.

^cIn comparison with expression of miRNA in different tumour sites, one case of lip OSCC sample was eliminated due to its small sample size.

^dKruskal–Wallis test (univariate analysis) was used in tumour subsites.

In unsupervised PCA (Figure 1), among the OSCC sample group, sample S4 was observed to be slightly separated from other three OSCC samples (S1, S2, and S3). Histologically, the tumour cells in sample S4 appeared to be less differentiated and infiltrated into the underlying connective tissue in single cells as compared to the other three OSCC samples (S1–S3). Furthermore, a higher degree of mitotic activity was observed in sample S4 with 15–16 mitotic figures per 10 high-power fields (HPF), compared to 5–7 mitotic figures per 10 HPF in samples S1–S3. All these features are suggestive of an aggressive phenotype of sample S4 that probably reflected in it being separated from the other three OSCC samples (S1–S3) in PCA. Among the non-cancer sample group, the slight separation of each sample could be due to the heterogeneous presence of inflammatory cell observed within these samples (S5–S8; data not shown).

Among the significantly differentially expressed miRNAs reported in this current OSCC study, miR-21 and miR-200c were well studied previously in several cancers including oral cancer. The highly up-regulation of miR-21 reported in this study is in line with other previous OSCC studies (Cervigne *et al*, 2009; Li *et al*, 2009; Yu *et al*, 2009). Notably, the inhibition of miR-21 reduced the survival and anchorage-independent growth of oral cancer cells. Further, Li *et al* (2009) demonstrated that miR-21

inhibits apoptosis through TPM1-dependent mechanisms reiterating that miR-21 plays a major role in driving oral cancer development. Apart from this, miR-200c was well documented in a very recent study by Shiiba *et al* (2012), where its up-regulation was reported to dysregulate apoptotic activity to promote OSCC progression.

The current study has identified miR-31 up-regulation and miR-375 down-regulation as among the most significantly differentially expressed miRNAs in OSCC. These findings were in concordance with a recent study by Lajer *et al* (2011), which also identified the up-regulation miR-31 and down-regulation miR-375 in OSCC frozen tissue. Consistently, miR-31 and miR-375 have also been frequently reported to be differentially expressed in many non-oral squamous cell carcinomas (SCCs) (Mathe *et al*, 2009; Hui *et al*, 2010; Liu *et al*, 2010a; Wang *et al*, 2010).

Association of miRNAs expression with OSCC clinico-pathological parameters is essential for the better understanding of how miRNAs are involved in OSCC development. Although the miRNAs investigated here (miR-31 and miR-375) had previously been implicated in various cancers, yet their functions are largely unknown especially in OSCC. From the univariate analysis in this study (Table 4), miR-31 expression was significantly higher in the early stage as compared to the late stage of OSCC.

Consistently, miR-31 was demonstrated to be highly expressed in tumour with absence of lymph node metastasis reiterating the fact that miR-31 may play a role in the early stages of cancer development. Previous studies reported that miR-31 functions in enhancing early stage events such as proliferation and tumorigenicity of OSCC cancer cells largely by inhibiting negative regulators of oncogenic pathways when expressed aberrantly (Liu *et al*, 2010b). For example, miR-31 has been demonstrated to be involved in regulation of lung cancer cell growth (Liu *et al*, 2010a). From the multivariate analysis, it is clear that miR-31 is an independent factor that is associated with tumour stage suggesting that this miRNA functions at a specific phase of cancer development.

In relation to tumour subsite, both univariate and multivariate analyses indicated that miR-31 was significantly expressed higher levels in OSCC of the buccal mucosa as compared to tongue and gingiva. Based on pathway analysis (Table 2), miR-31 negatively regulates the tumour suppressor p16. The loss of p16 gene has been recently reported to play a role in tumour development by increasing malignant potential and conferring higher metastatic potential in buccal SCC (Dong *et al*, 2012). In this study, the highly expressed miR-31 found in buccal mucosa might be responsible to suppress p16 to promote the OSCC tumour cell development. However, the metastatic effect of miR-31 in buccal SCC is yet to be determined as all OSCC samples validated were derived from buccal mucosa with no metastatic lymph node, and no buccal SCC with positive lymph node was included in this study.

miR-375 was observed to be highly suppressed in late stage as compared to early stage tumours. Coincidentally, miR-375 was also highly suppressed in tumours greater than 4 cm (T3 and T4) in tumour size suggesting that this could be a contributing factor in upstaging OSCCs under-expressing this particular miRNA. This observation suggests that down-regulation of miR-375 promotes tumour growth, and this is consistent with previous reports that miR-375 down-regulation in HNSCC could deregulate proliferation and apoptosis, leading to uncontrolled tumour cell growth (Poy *et al*, 2004; Hui *et al*, 2010). In addition, Ding *et al* (2010) reported that miR-375 is a tumour suppressor that negatively regulates the expression of oncogenic JAK2 in promoting cell proliferation in gastric cancer (Ding *et al*, 2010). Following multivariate analysis, miR-375 remained significantly associated with tumour staging suggesting that this miRNA could have a role in determining disease aggressiveness.

In conclusion, we demonstrated that differential expression of miR-31 and miR-375 in OSCC and their relationship with clinico-pathological features of OSCC. These findings suggested that different miRNAs may play a role in the different stage of oral carcinogenesis. While some miRNAs such as over-expression of miR-31 was found to be important in early stage, the under-expression of miR-375 appears to play a role in late stage of disease. Subsequent analysis suggested that the expression of both miRNAs may be influenced by risk habits in inducing several clinico-pathological outcomes. Thus, a larger sample size with sufficient number of each habit group would be necessary to address this relationship. Further studies on the biological function of

miR-31 and miR-375 and how they regulate their targeted genes in cancer-related pathways to promote the tumorigenesis of OSCC is needed as well to strengthen the findings of current study. As miRNAs have been shown to contribute significantly to tumorigenesis, knowledge of miRNA-based regulatory mechanisms in initiating and maintaining malignancy is essential to be able to design effective strategies for cancer management.

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Author contributions

MY Siow conducted the experimental works and drafted the manuscript. MY Siow, LP Karen-Ng, VK Vincent-Chong, Yi-Hsin Yang and Marhazlinda J have made contribution in the conception of manuscript framework and interpretation of data analysis. ZAA Rahman and MT Abraham participated in the study design conception and coordination and provided the clinical expertise in this study. SC Cheong critically revised the manuscript. RB Zain and TG Kallarakkal have provided the pathological expertise in sample acquisition and selection in the study. RB Zain supervised and coordinated the study/research group and critically revised the manuscript. All authors read and revised the manuscript.

Competing interests

The authors have declared that no competing interests exist.

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