Dysregulation of miR-200 family microRNAs and epithelial-mesenchymal transition markers in oral squamous cell carcinoma

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Abstract. MicroRNAs (miRNAs) are reported to function as a major component in the cellular signaling circuit, which regulates epithelial-mesenchymal transition (EMT). Dysregulation of the microRNA-200 (miR-200) family and EMT-associated genes enables tumor metastasis and resistance to therapy. The present study profiled miR-200 family members miR-200a, miR-200b, miR-200c, miR-141 and miR-429, and also several EMT-regulatory genes including zinc finger E-box-binding homeobox (ZEB)1, ZEB2, epithelial cadherin and vimentin in 40 oral primary tumors in order to understand their role(s) in oral squamous cell carcinoma (OSCC). The reverse transcription-quantitative polymerase chain reaction was used to analyze each sample. Results demonstrated a significant downregulation of miR-200 family members in tumors with a history of tobacco chewing/smoking ($P<0.0006$, $P=0.0467$, $P=0.0014$, $P=0.0087$ and $P=0.0230$, respectively) and undifferentiated pathology (miR-200a, $P=0.0067$; miR-200c, $P=0.0248$). EMT markers ZEB2 ($P=0.0451$) and vimentin ($P=0.0071$) were significantly upregulated in the oral tumors. Furthermore, ZEB2 antisense RNA1 was overexpressed in 50% of OSCC samples ($P=0.0075$). EMT-regulatory genes did not exhibit any association with clinical outcome. The present study also analyzed the expression of EMT-regulatory genes in 523 head and neck squamous cell carcinoma (HNSCC) samples from The Cancer Genome Atlas (TCGA) database, and the association with treatment outcome. Analysis of TCGA datasets also demonstrated no significant association in the expression of EMT markers with disease recurrence and treatment outcome. The results of the present study revealed dysregulation of miR-200 family miRNAs and EMT-regulatory genes in OSCC without any significant effect on treatment outcome.

Introduction

Oral squamous cell carcinoma (OSCC) is the 11th most common type of cancer worldwide, and is ranked the most common malignant tumor in males and fourth most common in females in India. Despite recent advances in cancer therapy, oral cancer remains a major health threat due to the lack of improvement in the 5-year overall survival (OS) rate (1). Treatment of OSCC includes surgery, radiotherapy, chemotherapy or a combination of different systemic therapies (2). However, an effective strategy to select suitable patients for these therapies does not currently exist owing to the complexities associated with radiation response, and also limitations regarding tolerance of normal surrounding tissues. The most effective radiotherapy regimens attain disease-free survival (DFS) rates of between 45 and 55% in patients with locally advanced head and neck cancer, and between 30 and 40% in oral cancer due to disease recurrence/metastasis (1). Oral carcinogenesis arises through the accumulation of genetic and epigenetic changes, including alterations in the expression of coding and non-coding RNAs (3,4). Oral cancer types express a specific miRNA portfolio, which contributes to maintain the epithelial characteristics of the cells (5,6).

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Abbreviations: OSCC, oral squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; TCGA, the Cancer Genome Atlas; EMT, epithelial-mesenchymal transition; DFS, disease-free survival; CSC, cancer stem-like cells; NAT, natural antisense transcript; ZEB2-AS1, zinc finger E-box-binding homeobox 2 antisense RNA1

Key words: non-coding RNA, microRNA-200 family, zinc finger E-box-binding homeobox 2 antisense RNA1, epithelial-mesenchymal transition, oral cancer

Epithelial-mesenchymal transition (EMT) is a process by which an epithelial cell adopts a mesenchymal phenotype and migrates to a different site and proceeds to colonize (7). A previous study demonstrated that cells with an EMT phenotype become rich sources for cancer stem-like cells (CSCs) (7). CSCs undergo self-renewal, initiate tumors development at distal sites and also serve an important role in chemo/radio-resistance (8-10). The presence of EMT and CSCs has been implicated in the increased resistance to radiotherapy through hypoxia, which assists in maintaining the CSC niche. In total, >90% of cancer-associated mortalities are due to metastatic events and are reported to be initiated by the dysregulation of microRNAs (miRNAs), particularly the microRNA-200 (miR-200) family (11).

miRNAs are a family of small non-coding RNAs that are between 21 and 25 nucleotides in length, and bind to target mRNAs through a 6-8-base seed sequence. miRNAs modulate gene expression at the post-transcriptional level by blocking translation or degrading the mRNA, depending on the extent of sequence complementarity with the target mRNA (12). The miR-200 family includes miR-200a, miR-200b, miR-200c, miR-141 and miR-429, and all share common seed sequences, which modulate EMT through the regulation of epithelial (E-)cadherin expression (11). Tumor invasion and metastasis have previously been demonstrated to be tightly controlled by the balanced expression of the miR-200 family members and zinc finger E-box-binding homeobox (ZEB) transcription factors (9,11). In previous studies, it has been demonstrated that miR-200 expression is downregulated within cancer cells, enabling a positive regulatory loop to maintain ZEB1/ZEB2 expression (11,13). Inhibition of miR-200 reduces E-cadherin expression, increases vimentin expression and induces the EMT mechanism (14). Long non-coding RNAs (lncRNAs) are non-protein coding transcripts >200 nucleotides in length, and have the potential to regulate gene expression through *cis* and *trans* mechanisms. ZEB2 antisense RNA 1 (ZEB2-AS1) is an lncRNA that overlaps the 5' splice site of an intron within the 5' untranslated region (UTR) of the ZEB2 gene. ZEB2 and ZEB2-AS1 expressed as bidirectional *cis*-natural antisense transcripts (NAT) are essential in downregulating E-cadherin during EMT (15).

In the present study, the expression of miR-200 family and EMT-associated markers in OSCC was analyzed using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and paired with patients' clinicopathological characteristic data. Furthermore, head and neck cancer datasets from The Cancer Genome Atlas (TCGA) database were also analyzed in order to evaluate the concordance of the present study.

Materials and methods

Clinical specimens and RNA isolation. The present study was approved by the Institutional Ethics Committee (IEC), Madras Medical College (Chennai, India; no. 04092010) and was conducted within the ethical guidelines of IEC, Madras Medical College. OSCC tissue samples (n=40) were obtained from patients at the Royapettah Government Hospital (Chennai, India). Informed consent was obtained from each patient in the form of a standard questionnaire in accordance

with IEC guidelines. Patients' contextual and clinicopathological characteristics are presented in Table I. Tumor specimens were obtained under local anesthesia using punch biopsy immersed in RNAlater® solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and transported to the laboratory at Department of Genetics, University of Madras (Chennai, India) in cold storage. Stored tissues were washed twice with ice-cold PBS to remove residual RNAlater® solution, and homogenized using a MicroSmash MS-100 automated homogenizer (Tomy Digital Biology Co., Ltd., Tokyo, Japan) using zirconium beads. RNA was isolated using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) and miRNAs were isolated using an miRNeasy mini kit (Qiagen) according to the manufacturer's protocol.

Reverse transcription. DNA was reverse-transcribed from total RNA isolated from the OSCC samples using a custom-designed miRNA seed-specific stem-loop primer for miRNAs (Table II), oligo (dT) primer for ZEB2-AS1 [5'-CAG TGCAGGGTCCGAGGTACAGAGCCACCTGGGCAATTT TTTTTTTTTVN-3' with 3' wobble bases: V-(A, C, G), N-(A, C, G, T)] (16,17), and a random hexamer primer for coding genes. Prior to RT reactions, all samples were pre-incubated for RNA secondary structure denaturation and primer annealing at 65°C for 20 min. All cDNA conversions were performed using a Reverse Transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.) using the following reaction conditions: 55°C for 90 min, 72°C for 15 min and final hold at 4°C.

Quantitative polymerase chain reaction (qPCR). Relative quantification was performed using the TaqMan® custom designed assays (Thermo Fisher Scientific, Inc.). qPCRs were performed in triplicate (384-well optical plates) with 10 μl total volume using cDNAs (diluted 25-fold) with TaqMan® 2X Universal Master mix (No AmpErase® UNG; Thermo Fisher Scientific, Inc.), universal reverse primer, either universal reverse lncRNA, 5'-CAGTGCAGGGTCCGAGGT-3' or universal reverse miRNA, 5'-TCGTATCCAGTGCGTCGAGT-3', and the following specific forward primer: miR-200a forward, 5'-AGT AACACTGTCTGGTAACGA-3'; miR-200b forward, 5'-CGC AGTAATACTGCCTGGT-3'; miR-200c forward, 5'-AGTAAT ACTGCCGGGTAATGA-3'; miR-429 forward, 5'-CGCAGT AATACTGTCTGGTA-3'; miR-141 forward, 5'-CGCAGT AACACTGTCTGGT-3'; RNU44 forward, 5'-GCAAATGCT GACTGAACATGA-3'; ZEB1-AS1 forward, 5'-TGTGCATGA TGAATTTCTGGACTGGA-3'; GAPDH forward, 5'-GAA GAGGGGGAGGGGCCTAGG-3') and universal fluorescein amidite-labeled minor groove binder probe (5'-CAGAGC CACCTGGGCAATTTT-3') for lncRNA (ZEB2-AS1) and miRNAs. Expression of coding genes was analyzed using SYBR-Green master mix (Takara Bio, Inc., Otsu, Japan) using gene-specific primers including: GAPDH forward, 5'-AGG GCTGCTTTTAACTCTGGT-3' and reverse, 5'-CCCCACTTG ATTTGGAGGGA-3'; ZEB1 forward, 5'-CACTCCCTGCAG CAGAAGCTGA-3' and reverse, 5'-GCTCTTCTGCACTTG GTTGTGCT-3'; ZEB2 forward, 5'-AACTGGAGGAACGCG ATGGTCA-3' and reverse, 5'-GCAGTTGGGCAAAAGC ATCTGGA-3'; E-cadherin forward, 5'-GCTGCTGCAGGT CTCCTCTTG-3' and reverse, 5'-CCTTTGTCGACCGGT

Table I. Demographic and clinicopathological details of the 40 cancer patients.

Clinical parameters	No. of patients (%)
Age, years (mean ± SD, 50.53±10.63)	
≤52	19 (47.5)
>52	21 (52.5)
Sex	
Male	30 (75)
Female	10 (25)
Anatomical site	
Buccal mucosa	14 (35)
Tongue	8 (20)
Alveolar ridge	4 (10)
Lip	3 (7.5)
Other	11 (27.5)
Clinical stage	
≤T3	15 (37.5)
≥T4	25 (62.5)
Nodal metastasis	
≤N1	19 (47.5)
≥N2	21 (52.5)
Histological grade	
Well-differentiated	13 (32.5)
Moderately differentiated	19 (47.5)
Poorly differentiated	8 (20)
Risk habit profile	
Exclusive smokers	12 (30)
Exclusive tobacco-chewers	4 (10)
Mixed habits	
Smoking and alcoholic	5 (12.5)
Smoking and chewing	3 (7.5)
All three	9 (22.5)
None	7 (17.5)

SD, standard deviation; T, tumor size; N, nodal involvement.

GCAATCT-3'; vimentin forward, 5'-AGCTGCAGGCTCAGA TTCAGG A-3' and reverse, 5'-CGGTTGGCAGCCTCAGAGA GGT-3'. The experiments were carried out using a 7900HT Real-Time PCR system (Thermo Fisher Scientific, Inc.) with the following reaction conditions: 50°C for 2 min, 95°C for 10 min for initial denaturation and enzyme activation followed by cyclic denaturation at 95°C for 15 sec and primer annealing and extension at 60°C for 1 min. A negative control without a cDNA template was included in parallel for all assays. GAPDH and RNU44 were used as endogenous controls for coding/non-coding genes and miRNAs, respectively. Relative expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (18). Each experiment was completed in triplicate, and the mean was used for analysis.

Clinical evaluation and TCGA data analysis. All patients with OSCC were treated using radiotherapy (50-60 Gy) alongside three rounds of chemotherapy (Cisplatin; Naprod Life Sciences Pvt., Ltd., Mumbai, India) and 5-fluorouracil (Celon Laboratories, Ltd., Telangana, India). Tumor responses to chemo/radio-therapy were evaluated following 4 weeks of treatment. Patients who exhibited a partial or complete response to treatment were categorized as treatment responders, and the remaining patients were categorized as either poor responders or resistant to treatment. Patients were monitored for 18 months following treatment to study the therapeutic outcome. Furthermore, a comparison study on head and neck cancer were completed using RNA sequencing datasets obtained from 523 head and neck cancer datasets from TCGA database using the cBioportal interactive genomics data portal. Expression levels of the genes obtained from TCGA datasets were quantified using reads/kb/10^6 mapped reads (RPKM).

Statistical analysis. All statistical analyses were performed using GraphPad Prism (version 6; GraphPad Software, Inc., La Jolla, CA, USA). Numerical data are presented as the mean ± standard error of mean. Differences between means were analyzed using Student's t-test for parametric data and Mann-Whitney for non-parametric data. A univariate analysis was performed for the association with clinical features. Survival curves were plotted using the Kaplan-Meier estimator method, and the fraction survival rate was tested using the log-rank test. All tests were two-tailed and P<0.05 was considered to indicate a statistically significant difference.

Results

miR-200 family miRNAs are downregulated in OSCC. Downregulation of the miR-200 family induces EMT and promotes metastasis in epithelial tumors (19-21). Expression levels of the miR-200 family in OSCC (n=40) and normal (n=8) tissues were determined using RT-qPCR. Results demonstrated a downregulation of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-429 and miR-141) in OSCC samples compared with normal tissue samples. Statistical analysis of expression levels demonstrated that each member of the miR-200 family [miR-200a 33/40 (82.5%), miR-200b 33/40 (82.5%), miR-200c 25/40 (62.5%) and miR-141 28/40 (70%)] were significantly downregulated (P=0.002, 0.0116, 0.0099 and 0.0035, respectively; Fig. 1A).

Downregulation of the miR-200 family miRNAs is associated with tobacco chewing/smoking risk habits. Tumors with distinct clinicopathological and demographic features may be unique due to their anatomical origin. Expression levels of individual miRNAs were categorized based on demographic features and analyzed using the univariate analysis. Results also demonstrated that expression levels of the miR-200 family were markedly influenced by risk factors including tobacco chewing and smoking. Results presented in Fig. 1B revealed that patients with OSCC and who are associated with a risk factor/habit (n=33/40) demonstrated a significant downregulation in miR-200a (82%; n=27; P=0.0006), miR-200b (79%; n=26; P=0.0467), miR-200c (57.5%; n=19; P=0.0014), miR-429 (57.6%; n=19; P=0.0087) and miR-141 (60.6%; n=20; P=0.0230). Furthermore, a significant downregulation in miR-200a (P=0.0067) and miR-200c (P=0.0248) was detected

Table II. MicroRNA-specific stem-loop primers used for cDNA synthesis.

| miRNA	Stem-loop primers (5'→3')
miR-200a	GTCGTATCCAGTGCGTCGAGTGACACGAGAGCCACCTGGGCAATTTGCACTGGATACGACACATCG
miR-200b	GTCGTATCCAGTGCGTCGAGTGACACGAGAGCCACCTGGGCAATTTGCACTGGATACGACTCATCA
miR-200c	GTCGTATCCAGTGCGTCGAGTGACACGAGAGCCACCTGGGCAATTTGCACTGGATACGACTCCATC
miR-429	GTCGTATCCAGTGCGTCGAGTGACACGAGAGCCACCTGGGCAATTTGCACTGGATACGACACGGTT
miR-141	GTCGTATCCAGTGCGTCGAGTGACACGAGAGCCACCTGGGCAATTTGCACTGGATACGACCCATCT
RNU44	GTCGTATCCAGTGCGTCGAGTGACACGAGAGCCACCTGGGCAATTTGCACTGGATACGACAGTCAG

miRNA/miR, microRNA.
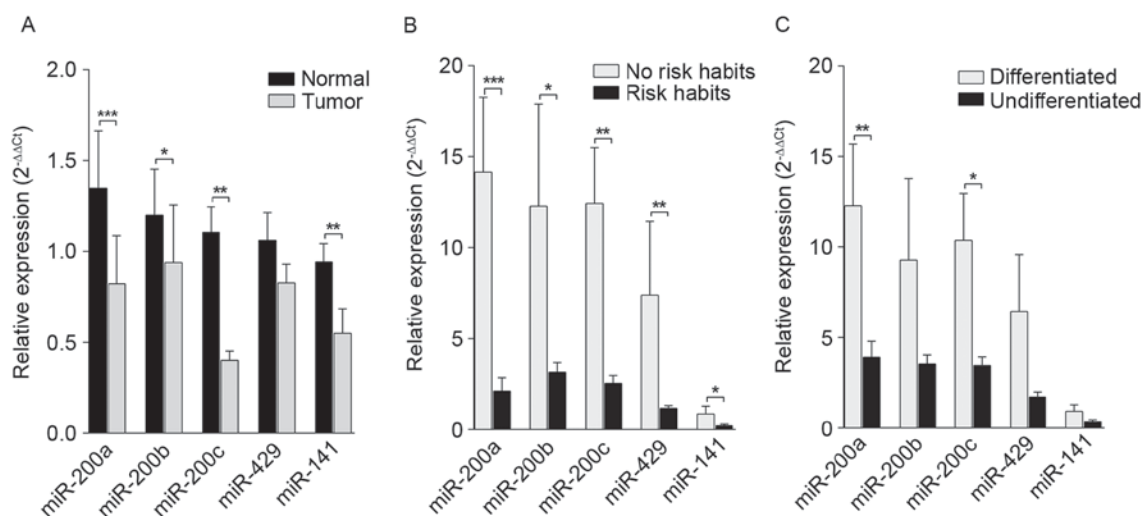
Figure 1. Expression profiling of miR-200 family members in OSCC. (A) Expression of miR-200 family members in OSCC and adjacent normal tissues. Results demonstrate that miR-200a, miR-200b, miR-200c and miR-141 are significantly downregulated in OSCC. (B) miR-200 family members are significantly downregulated in patients with oral cancer with risk habits including tobacco chewing and smoking. (C) miR-200 family members are downregulated in undifferentiated oral tumors. The decrease in miR-200a and miR-200c expression was statistically significant. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method. miR, microRNA; OSCC, oral squamous cell carcinoma. *P<0.05, **P<0.001 and ***P≤0.0001.

in undifferentiated tumors. No significant associations were observed between expression levels of miR-200 family members, tumor grade and nodal stage of tumor samples (Fig. 1C).

EMT-regulatory genes are overexpressed in OSCC. Expression of the EMT driver genes ZEB1 and ZEB2, and the epithelial marker E-cadherin and mesenchymal marker vimentin were analyzed in OSCC. Results demonstrated an overexpression of ZEB1 (36.5%; P=0.7733) and ZEB2 (50%; P=0.0451) in tumor samples compared with normal tissue samples. E-cadherin was downregulated in 50% of tumors, whereas vimentin was significantly upregulated in 54.5% of tumors (P=0.0071; Fig. 2A). However, there was no significant association between EMT-regulatory gene expression with the clinicopathological features of oral cancer samples (Fig. 2B-F).

Expression of EMT activators, their antisense transcripts and the clinical outcomes in OSCC. ZEB1-AS1, a natural antisense transcript has previously been reported to upregulate the expression of ZEB1 in tumor cells (22). The present study investigated the association between the expression of ZEB1 and its antisense transcript ZEB1-AS1 in head and neck datasets obtained from TCGA and identified that majority of the tumor samples co-expressed ZEB1 and ZEB1-AS1 (data not shown). Therefore, further analysis was performed to assess whether ZEB2-AS1, an antisense transcript of ZEB2 gene, was similarly able to also upregulate ZEB2. Notably, the majority of the samples co-expressed ZEB2 and ZEB2-AS1, and a high-level expression of the ZEB2-AS1 in 16/40 OSCC tumors (40%; P=0.0075) compared with normal tissues (Fig. 3A). Further comparisons were made regarding expression levels of ZEB2-AS1 with EMT activators. Notably, the expression of ZEB2 and vimentin were upregulated 40 and 60%, respectively, in tumor samples, which overexpressed ZEB2-AS1. Results demonstrated a statistically significant difference in ZEB2 expression level between the low (54.5%) and high (45.5%) ZEB2-AS1-expressing tumors (Fig. 3B; Table III; P=0.0303). Furthermore, the analysis of expression levels of ZEB1, ZEB2, E-cadherin and vimentin genes with treatment response demonstrated no significant difference between responder and non-responder groups (Fig. 2C). Survival rates of patients in association with the expression
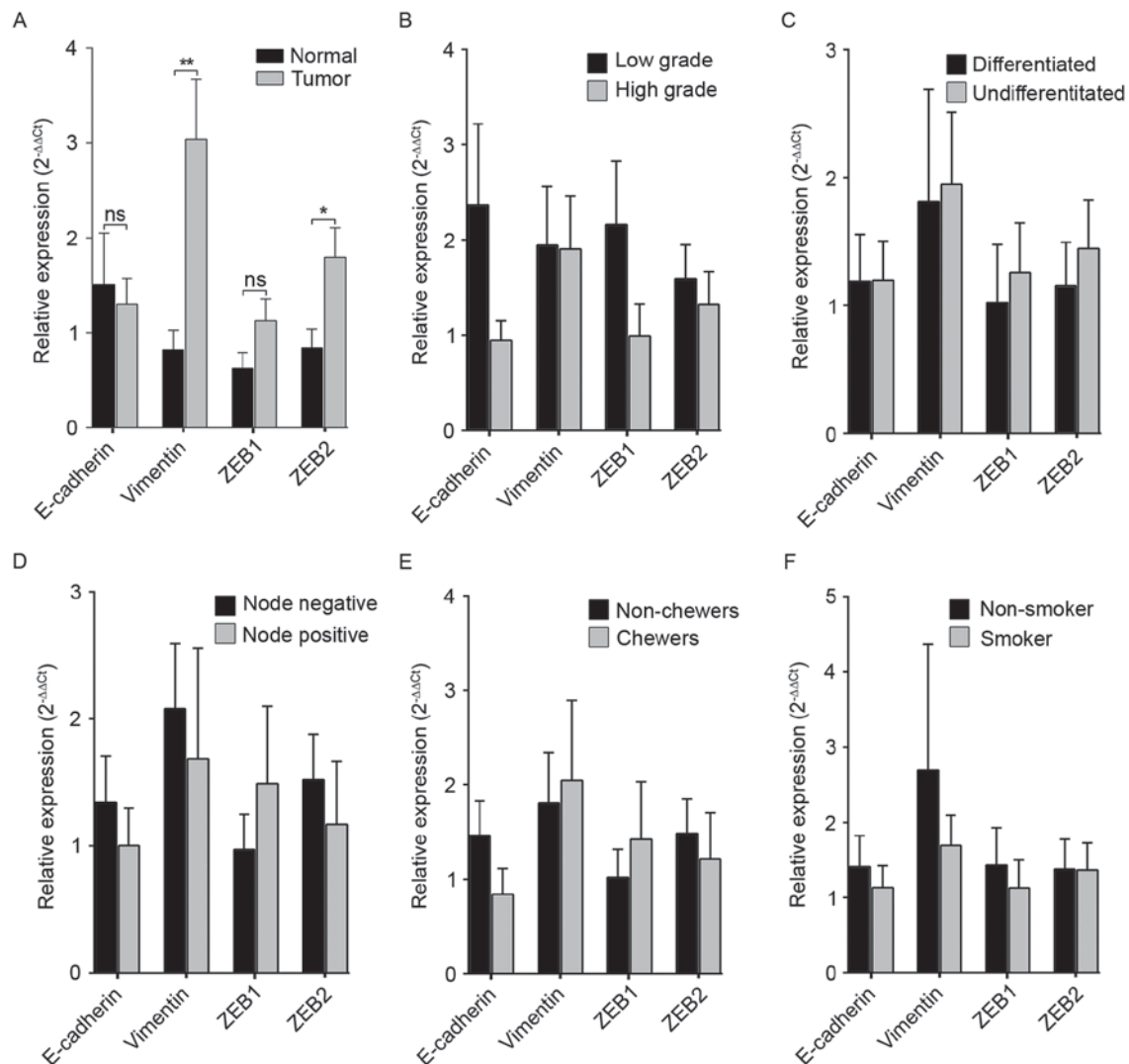
Figure 2. Expression of EMT markers in OSCC and normal tissues. (A) The mesenchymal markers vimentin, ZEB1 and ZEB2 were overexpressed in OSCC, whereas E-cadherin was downregulated in oral tumors. Expression of vimentin and ZEB2 were statistically significant. Expression of EMT-regulatory genes in (B) lower-tumor grade (<T3) compared with high-grade (≥T3) tumors, (C) differentiated tumor pathology compared with undifferentiated tumors, (D) node-negative compared with node-positive OSCC, (E) non-tobacco-chewers compared with tobacco-chewers and (F) non-smokers compared with smokers. No statistically significant association between clinical characteristics and expression was identified. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method. ZEB, zinc finger E-box-binding homeobox; OSCC, oral squamous cell carcinoma; EMT, epithelial-mesenchymal transition; E-cadherin, epithelial cadherin. *P<0.05 and **P<0.001.

level of ZEB2-AS1 did not demonstrate any statistical significance (Fig. 3D; P=0.6515).

EMT markers are not associated with clinical outcomes in head and neck squamous cell carcinoma (HNSCC) in TCGA database. The expression levels of ZEB1 and ZEB1-AS1 were analyzed in 523 HNSCC samples in TCGA database; results identified an overexpression of ZEB1 in samples, which also expressed a high level of ZEB1-AS1. However, the results of the present study could not confirm the association between ZEB2-AS1 and ZEB2 expression levels as it is yet to be annotated in TCGA database. Notably, HNSCC samples obtained from TCGA dataset identified ZEB1 (36%) and ZEB2 (37.3%) overexpression in tumors that were significantly hypomethylated (P<0.001 and P<0.001 respectively) at their promoters (data not shown). Furthermore, the expression levels of EMT-regulatory genes ZEB1, ZEB2, E-cadherin and vimentin did not demonstrate any association with tumor stage

(Fig. 4A). In addition, the role of other EMT markers including Twist-related protein 1 (TWIST1), snail family transcriptional repressor 1 (SNAIL1) and B lymphoma Mo-MLV insertion region 1 homolog (BMI1) were analyzed and no association with tumor stage was observed (Fig. 4B). Similarly, the EMT-regulatory genes demonstrated no significant change in expression levels between disease-free and disease-recurrence groups (Fig. 4C and D). Survival analysis of datasets of patients with HNSCC obtained from TCGA database did not demonstrate any association with ZEB1, ZEB2, ZEB1-AS1 and ZEB2-AS1 expression (data not shown); these results are consistent with the present study.

Discussion

The miR-200 family is a group of tumor suppressor miRNAs involved in the regulation of EMT, repression of self-renewal and differentiation of CSCs, and reversal of chemoresistance (10). In

Table III. Expression values of EMT marker genes in ZEB2-AS1-low and -high expressed oral cancer samples.

Gene	ZEB2-AS1 low expression		ZEB2-AS1 high expression		P-value[a]
	Mean	SD	Mean	SD	
ZEB1	0.7477	1.102	0.6926	0.1721	0.3268
ZEB2	0.4551	0.3479	2.348	2.214	0.0303[a]
E-cadherin	0.5882	0.5379	0.9788	0.9756	0.7706
Vimentin	0.7374	0.6573	4.355	3.704	0.1775

[a]P<0.05. ZEB, zinc finger E-box-binding homeobox; AS1, antisense RNA1; SD, standard deviation.

the present study, the role of each member within the miR-200 family in OSCC was analyzed through the observation of expression levels, and results identified a significant downregulation in miR-200 family members in OSCC compared with levels detected in normal tissues. Several previous studies established that miR-200 family members are critical for the maintenance of the epithelial phenotype, and has been demonstrated to be deregulated in metastatic cells in several types of cancer (19,23). Expression levels of miR-200 family genes have been revealed to inhibit EMT and decrease invasion and metastasis in tumors (19-21). Environmental risk factors including areca nut, slacked lime, betel quid, alcohol consumption, and tobacco chewing and smoking were frequently associated with oral cancer in India (24). The present study identified a significant association between the expression levels of the miR-200 family members and history of tobacco chewing and smoking. In particular, miR-200a and miR-200c were identified to be significantly deregulated and associated with tumor clinicopathological features. In our previous study, miRNAs deregulation was reported in tobacco-chewing-associated oral cancer (25). In addition, another study established the role of tobacco-specific nitrosamine in methylating gene promoters (26). Tellez *et al* (27) reported that tobacco carcinogens induced the transformation of human lung epithelia through epigenetic silencing of miR-205 and miR-200, which led to EMT and stem cell-like properties. miR-200 family miRNAs regulate EMT through the modulation of E-cadherin expression by targeting E-cadherin repressors ZEB1 and ZEB2 (21). Inhibition of miR-200 has been demonstrated to decrease E-cadherin expression, increase the expression of vimentin and induce EMT (11). The present study therefore investigated the expression of ZEB1 and ZEB2 in order to determine the effect(s) of the miR-200 downregulation in OSCC, and upregulation of EMT inducers, namely ZEB1 and ZEB2, was observed in the tumor samples compared with the normal tissue, suggesting the existence of EMT in oral tumors. These results are consistent with those of several previous studies (11,19,21). E-cadherin, an epithelial cell marker, was reported to be downregulated in the metastatic tumors during EMT, and is a proven target of ZEB1/2 transcription factors (11). Despite detecting a trend, but no marked change in E-cadherin expression, results identified a significant overexpression in the

mesenchymal marker vimentin, confirming EMT in OSCC. Therefore, the downregulation of the miR-200 family and the upregulation of ZEB1 and ZEB2 suggest the induction of EMT, which may lead to the maintenance and release of circulating tumor cells in oral tumors, as reported in several types of cancer with epithelial origin (28,29).

During EMT, ZEB1 and ZEB2 levels may be influenced by the level of their antisense transcripts ZEB1-AS1 and ZEB2-AS1. Therefore, analysis of the expression of ZEB2-AS1 in OSCC was performed and a significant upregulation was identified. In addition, the expression levels of ZEB2 in the samples that also expressed increased levels of ZEB2-AS1 were determined, and it was identified that the expression level of ZEB2 was consistent with the high-level expression of its natural antisense transcript. In epithelial cells, ZEB2-AS1 was demonstrated to prevent splicing of the ZEB2 5'UTR, and promote levels of ZEB2 protein in colon adenocarcinomas (30). However, an increased level of ZEB2 in the cytosol was revealed to downregulate E-cadherin, and enable cells to undergo EMT (31). Similarly, ZEB1-AS1 upregulation has been reported to increase the promoter activity of the ZEB1 gene resulting in the repression of E-cadherin, and induction of EMT in hepatocellular carcinoma (22). The results of the present study identified a co-expression pattern in ZEB2 and ZEB2-AS1 in oral tumor samples. Owing to the limitation of sample size and no annotation of ZEB2-AS1 in TCGA, the present study could not derive a conclusion that ZEB2 overexpression is due to the ZEB2-AS1. Therefore, the association between the expression of ZEB1 and its natural antisense transcript ZEB1-AS1 in 523 TCGA HNSCC datasets was analyzed and identified that the majority of the samples co-expressed ZEB1 and ZEB1-AS1. Therefore, our results suggest that ZEB2 levels may be regulated by its natural antisense transcript by increasing promoter activity or increasing the levels of ZEB2 protein through the stabilization of its 5'UTR. In addition, the methylation signature of the ZEB family gene promoters from TCGA HNSCC cases were examined, and a high level of ZEB1 and ZEB2 gene expression was observed in tumors with a hypomethylated promoter region, suggesting that gene regulation is achieved by altering promoter methylation events. Chaffer *et al* (32) reported that the induction of transforming growth factor β (TGFβ) expression leads to the hypomethylation of the ZEB1 promoter region. Therefore, a similar response may hypomethylate the ZEB2 promoter region and induce the expression level. Hypomethylation of the ZEB transcription factor promoter region may enable the overexpression of ZEB transcription factors (32). The miR-200 family post-transcriptionally regulates the EMT marker genes including ZEB1 and ZEB2. The ZEB transcription factors may downregulate the expression of miR-200 family miRNAs. In addition, natural anti-sense transcript ZEB1-AS1 was demonstrated to enhance the transcription of ZEB1 and increase mRNA stability within the cytosol (30,22). Similarly, ZEB2 stability may also be enhanced by ZEB2-AS1 expression. Thus, the enhanced expression of ZEB1 and ZEB2 may downregulate miR-200 family miRNAs more effectively than normal expression levels, leading to the activation of EMT.

The present study demonstrated increased expression levels of EMT-associated genes in tumors compared with normal tissues, suggesting the existence of EMT in OSCC. Recently, EMT has
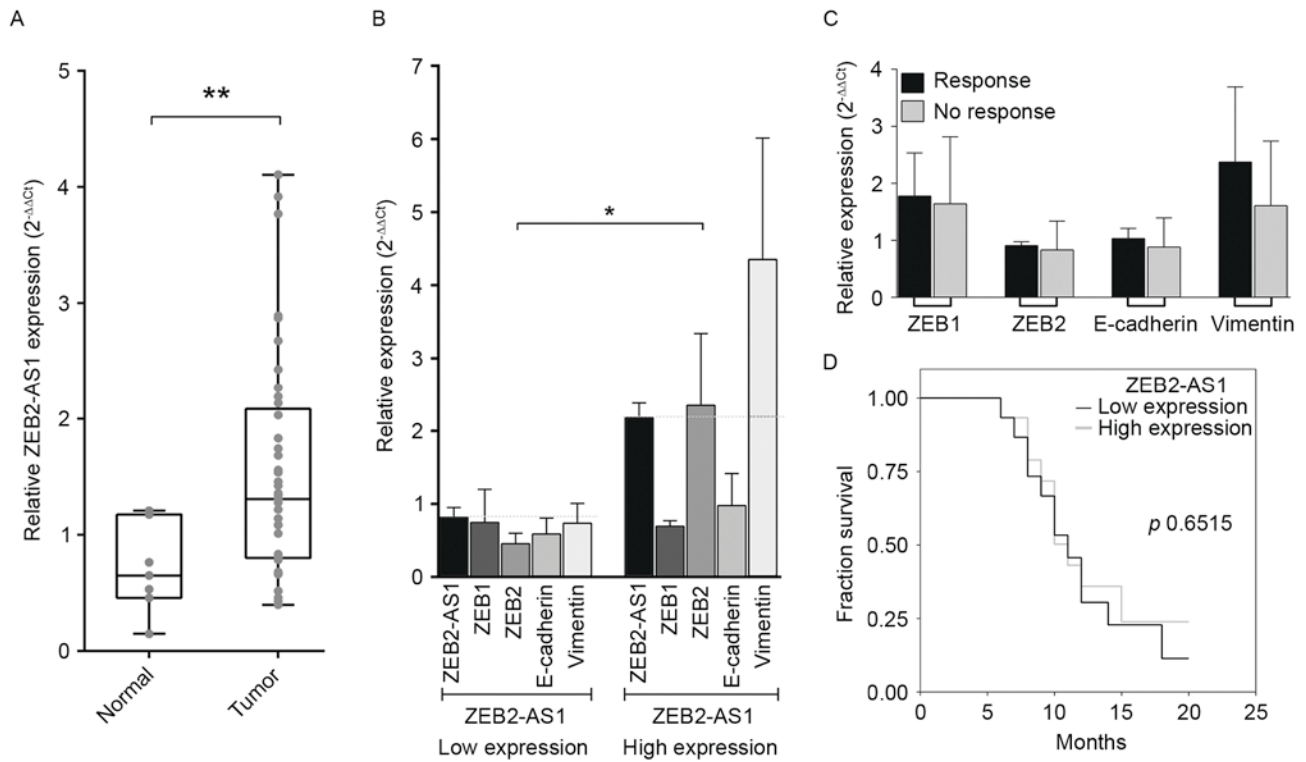
Figure 3. Association of ZEB2-AS1 expression and EMT marker genes with treatment response and overall survival. (A) The lncRNA ZEB2-AS1 was significantly overexpressed in OSCC compared with normal tissues. (B) ZEB2-AS1-overexpressed OSCC samples also expressed the mesenchymal markers ZEB2 and vimentin at increased levels, whereas ZEB1 levels remained unchanged. ZEB2 expression was statistically significant. (C) Expression of EMT markers ZEB1, ZEB2, E-cadherin and vimentin did not demonstrate any significant change in expression between treatment responders and non-responders. (D) Kaplan-Meier estimator curves presenting the overall survival rate of the patients with oral cancer with expression of ZEB2-AS1. There was no significant change in the survival rate between lower and higher ZEB2-AS1 expression levels. The relative expression levels were calculated using the 2^-ΔΔCq method. *P<0.05 and **P<0.001. lncRNA, long non-coding RNA; ZEB, zinc finger E-box-binding homeobox; AS1, antisense RNA1; EMT, epithelial-mesenchymal transition; E-cadherin, epithelial cadherin.
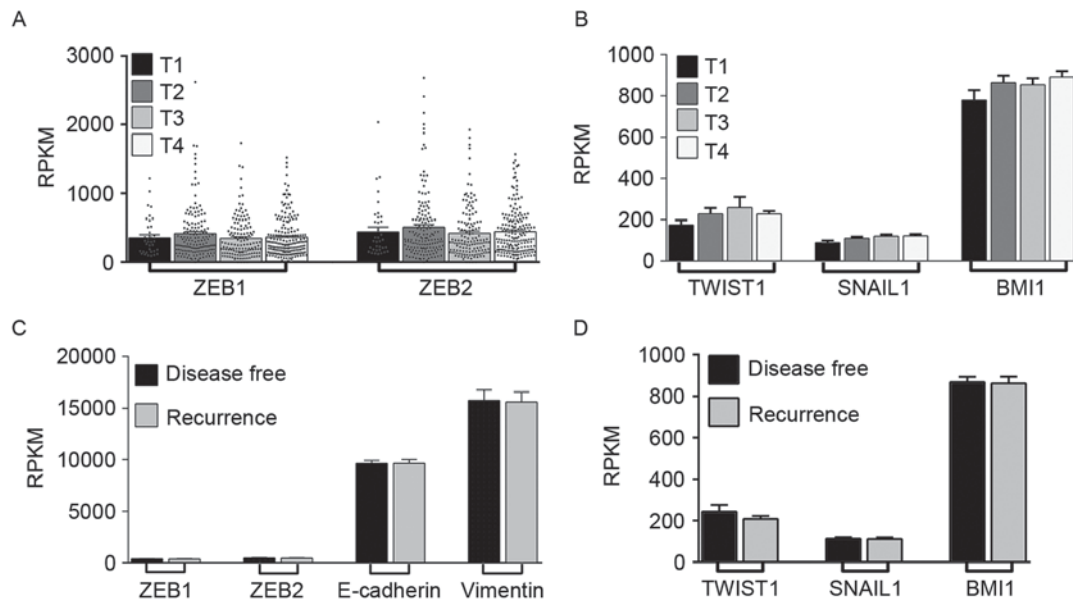

Figure 4. Expression of EMT markers with tumor features and overall patient survival from TCGA HNSCC datasets. (A) Expression of EMT markers ZEB1, ZEB2, E-cadherin and vimentin between the grade of the tumor (T1, T2, T3 and T4) from TCGA HNSCC datasets (n=523). There was no significant change in expression between the groups. (B) Expression of EMT-associated genes TWIST1, SNAIL1 and BMI1 were unchanged in tumor stages of patients with HNSCC. (C) Expression levels of ZEB1, ZEB2, E-cadherin and vimentin were unchanged with disease-free survival and disease recurrence in patients with HNSCC. (D) Expression of EMT-associated genes TWIST1, SNAIL1 and BMI1 compared with disease-free and disease recurrence of patients with HNSCC from TCGA datasets. The expression levels from TCGA datasets were expressed as RPKM. EMT, epithelial-mesenchymal transition; ZEB; zinc finger E-box-binding homeobox; TCGA, The Cancer Genome Atlas; HNSCC, head and neck squamous cell carcinoma; TWIST1; Twist-related protein 1; SNAIL1, snail family transcriptional repressor 1; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; RPKM, reads/kb/10^6 mapped reads; E-cadherin, epithelial cadherin.

been demonstrated to serve a critical role in drug resistance and distal metastasis, which accounts for tumor recurrence (33). The present study analyzed the expression of EMT activator genes including TWIST1, SNAIL1 and BMI1, in addition to ZEB family members and phenotypic marker genes E-cadherin and vimentin in HNSCC cases obtained from TCGA database. Notably, there were no significant differences in expression levels of the EMT markers with reference to the tumor stage, disease recurrence and treatment response. Survival rate analysis also demonstrated no association between the expression of EMT-activating genes and patient survival rate. Differential roles of EMT have been previously reported in various organ-specific human cancers (34,35). The expression of TGFβ may also positively regulate ZEB1/2 levels, thereby promoting metastasis in tumor cells (11). During embryonic development, Wnt signaling, fibroblast growth factors, Ras, phosphoinositide 3-kinase/protein kinase B, bone morphogenetic proteins, c-myeloblastosis, eomesodermin, mesoderm posterior proteins and msh homeobox-1-mediated signaling pathways either in combination or individually are able to induce EMT (36-38). As metastatic cancer cells behave by way of embryonic stem cells through the reactivation of embryo-specific genes, EMT in cancer cells may be initiated by this alternative means of activation. Despite regular EMT activation, epithelial cells under inflammatory stress are able to initiate partial EMT to detach from the epithelium and accumulate at the basal membrane where they switch to a fibroblast phenotype (36).

Despite EMT activation through the upregulation of classical EMT-associated transcription factors, deregulation of miRNAs targeting EMT regulators may also be able to initiate EMT in cancer. Upregulation of miRNAs including miR-106b-25 cluster, miR-491-5p, miR-661 and miR-24 leads to decreased cell-cell adhesion and downregulation of miR-31, miR-124, miR-205 and miR-34, resulting in the derepression of translation of EMT-activating transcription factors (38). The low-level transcripts of EMT-activating factors, through decreased or lack of post-transcriptional regulation of deregulated miRNAs, may confer an advantage for a longer half-life in the cytosol, and may maintain the steady-state level of EMT factors. Furthermore, non-coding RNAs are also reported to be major components of the cellular signaling circuit that regulates EMT (39,40). Regardless of studies reporting the dysregulation of various factors involved in regulation of EMT, it has been demonstrated that EMT is not crucial for metastasis to occur in lung cancer; however, it has been identified to contribute to chemoresistance (34). Furthermore, knockdown studies demonstrated that EMT genes (TWIST1 and SNAIL1) were not required for metastasis; however, they contribute to treatment resistance in pancreatic cancer (35).

In conclusion, the results of the present study identified a dysregulation of miR-200 family miRNAs and the upregulation of EMT-inducer genes in OSCC. Results demonstrated an association between deregulated expression of miR-200 family miRNAs, tobacco chewing/smoking and the cellular differentiation status of oral tumors. To the best of our knowledge, the present study is the first to identify an association between the expression of ZEB2-AS1 and ZEB2 in oral cancer, and to demonstrate the association of ZEB2 levels with its natural antisense transcript ZEB2-AS1 level. Furthermore, the present study was not able to confirm any association between the EMT-regulatory genes and treatment response. A similar result was also observed in HNSCC cases obtained from TCGA database. The present study was performed with a limited number of oral cancer samples as the majority of patients with cancer visited the hospital at an advanced stage of the disease, and a number of participants discontinued treatment following one or two rounds of chemotherapy. Further sequencing genomes and protein estimation will add to the evidence obtained in the present study. The primary limitation of the present study was the nature of tissue sample collection as punch biopsies are only sufficient for histopathological analysis and RNA isolation. Therefore, genome sequencing and protein-based experiments were not permitted. Further studies with an increased sample size and functional dissection are warranted to confirm the differential role(s) of EMT observed in different types of human cancer.

Acknowledgements

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