Decreased microRNA-143 expression and its tumor suppressive function in human oral squamous cell carcinoma

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ABSRACT. MicroRNA-143 serves as a tumor suppressor in many human malignancies. However, its involvement in oral squamous cell carcinoma (OSCC) is still unclear. In this study, we investigated the effects of miR-143 in OSCC tumorigenesis and development. Using real-time quantitative reverse transcription-polymerase chain reaction, we detected miR-143 expression in 109 pairs of human OSCC and adjacent noncancerous tissues. The associations between miR-143 expression and clinicopathological factors and prognosis of OSCC patients were also statistically analyzed. Further, the effects of miR-143 on the biological behavior of OSCC cells were investigated. miR-143 expression was significantly downregulated in OSCC tissue samples and cell lines. Decreased miR-143 expression was significantly associated with advanced T classifications, positive N classification, advanced TNM stage, and shorter overall survival. In addition, upregulation of miR-143 in Tca8113 cells reduced cell proliferation, invasion, and migration, as well as promoted cell apoptosis *in vitro*. These findings

validate the clinical significance of miR-143 in OSCC and reveal that it may be an intrinsic regulator of tumor progression and a potential prognostic factor for this disease.

Key words: MicroRNA-143; Oral squamous cell carcinoma; Prognosis; Real-time reverse transcription-polymerase chain reaction

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity, with more than half a million new patients diagnosed annually (Chen et al., 2008). Smoking, alcohol abuse, and betel quid chewing are major risk factors of oral cancer (Zygogianni et al., 2011). Despite advances in the fields of oncology and surgery, the 5-year survival rates for patients with OSCC remain lower than 50% and have not improved over the last 3 decades (Brocklehurst et al., 2010). Previous studies have demonstrated diverse genetic alterations in OSCC, but the highly complex molecular mechanisms underlying OSCC formation and progression remain unknown. Identification of candidate molecules may lead to new diagnostic and therapeutic approaches for this disease, as well as improve the prognosis of OSCC patients.

MicroRNAs (miRNAs) are single-stranded, small noncoding RNAs that are 18-25 nucleotides in length (Osman, 2012). They can negatively regulate gene expression through base-pairing to the 3' untranslated region of target mRNA, resulting in translation inhibition or mRNA degradation (Mendell and Olson, 2012). It is clear that miRNAs play key roles in nearly all biological processes, including differentiation, development, gene regulation, cell proliferation, and apoptosis, as well as the development of various diseases, such as cancer. They can also function as tumor suppressors or oncogenes according to the roles of their target genes. Recent studies have shown that miRNA expression profiles differ between normal tissues and derived tumors and between tumor types in human cancer (Volinia et al., 2006). Furthermore, miRNA dysregulation has been confirmed to be associated with tumor recurrence, development of metastases, and patient survival (Nicoloso et al., 2009). In terms of OSCC, downregulation of miR-99a and upregulation of miR-155 may contribute to cell growth and survival (Rather et al., 2013; Yan et al., 2012). Knockdown of miR-29a promoted OSCC cell invasion and induced drug-resistance to cisplatin *in vitro* (Lu et al., 2013). Chang et al. (2013) found that miR-17 and miR-20a were downregulated in OSCC cell lines and clinical specimens, and overexpression of these miRs decreased the migratory ability of OSCC cells. Moreover, low miR-17/20a levels were correlated with advanced TNM stage, lymphatic metastasis, and poor prognosis. Shiiba et al. (2013) also reported an association between miR-125b expression and radiotherapy response and overall survival of OSCC patients. These findings suggest that miRNAs act not only as diagnostic and prognostic markers, but also as potential therapeutic targets of human OSCC.

miR-143 has been identified as one of the most lowly expressed miRNAs in various tumors, including non-small cell lung cancer (Ma et al., 2013), gastric cancer (Takagi et al., 2009), colorectal cancer (Zhang et al., 2012), pancreatic cancer (Pramanik et al., 2011), cervical cancer (Wang et al., 2008), prostate cancer (Ahmad et al., 2013), osteosarcoma (Osaki et al., 2011), and leukemia (Akao et al., 2009). In these cases, miR-143 has been consistently shown to act as a tumor suppressor. However, the role of miR-143 in human OSCC remains unknown. In the current study, miR-143 expression in paired OSCC and adjacent noncancer-

ous tissues was measured by quantitative real-time reverse transcription-polymerase chain reaction (PCR) assay. Next, we investigated whether miR-143 expression had any clinico-pathological and prognostic value in OSCC patients. The effects of miR-143 on the biological behavior of OSCC cells were also investigated.

MATERIAL AND METHODS

Patients and tissue samples

This study was approved by the Research Ethics Committee of School & Hospital of Stomatology, Wenzhou Medical University, China. Written informed consent was obtained from all patients. All specimens were handled and made anonymous according to ethical and legal standards.

A total of 109 pairs of primary OSCC and adjacent noncancerous tissues were collected at the time of surgery from OSCC patients who underwent surgical resection at Department of Oral and Maxillofacial Surgery, School & Hospital of Stomatology, Wenzhou Medical University between January 1, 2005 and December 31, 2008. All tissues were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Final diagnosis of OSCC was confirmed by histological examination following surgery. None of the patients had undergone chemotherapy or radiotherapy before surgery. Clinicopathological information including gender, age, tumor (T) classification, node (N) classification, and tumor, node, and metastases (TNM) stage are indicated in Table 1.

All patients were followed up periodically. They underwent a checkup every month during the first 6 months after surgery, every 2 months during the following 6 months, every 3 months during the second year, and every 6 months thereafter. Overall survival (OS) was defined as the time from primary surgery to death of the patient or, for living patients, the date of last follow-up.

Cell lines and transfection

Human OSCC cell lines (SCC-4, SCC-9, SCC-25, and Tca8113), and a human normal oral keratinocyte cell line (hNOK) were obtained from the Beijing Institute for Cancer Research (Beijing, China) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/ mL streptomycin. All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. For RNA transfection, the cells were seeded into each well of a 24-well plate and incubated overnight, then transfected with either miR-143 mimics (GenePharma, Shanghai, China) or negative control (NC) RNA-oligonucleotides (GenePharma) using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer procedure.

RNA extraction and quantitative real-time PCR

Total RNA from tissue samples was extracted using TRIzol® reagent (Invitrogen) according to manufacturer instructions. Reverse transcription was carried out starting with 10 ng total RNA using the looped primers. Real-time PCR was performed using the standard Taqman MicroRNA assays protocol on ABI7500 real-time PCR detection system. U6 small nucle-

ar RNA was used as an internal control. For miR-143, the primers were as follows: forward, 5'-CCG CTC GAG TTA GGC TGG AAT GCG CCA AG-3' and reverse, 5'- TGA GAT GAA GCA CTG TAG CTC-3'. For U6, the primers were as follows: forward 5'-CTC GCT TCG GCA GCA CA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. PCR amplification was initiated at 95°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 60 s. The threshold cycle (Ct) was determined as the fractional cycle number at which the fluorescence passed the fixed threshold. All experiments were repeated twice and, in each experiment, samples were assayed in triplicate. The relative amount of miR-143 to U6 was calculated using the equation $2^{-\Delta Ct}$, where $\Delta CT = (CT^{miR-143} - CT^{U6})$.

MTT assay

Cells were seeded into 96-well culture plates at a density of 2000 cells in 200 μL/well and incubated at 37°C after transfection. Next, 100 μL MTT solution (0.5 mg/mL; Sigma, St. Louis, MO, USA) was added to each well, and the cells were incubated for another 4 h. Next, the medium was replaced with 150 μL dimethyl sulfoxide. Spectrophotometric absorbance at 490 nm was measured using a microplate reader. Cell proliferation was assessed daily for 4 consecutive days, and the MTT assay was repeated 3 times.

Detection of apoptosis by flow cytometry

Apoptosis was detected by flow cytometric analysis. Briefly, the cells were washed and resuspended at a concentration of 1 x 10⁶ cells/mL. Next, the cells were stained with Annexin V and propidium iodide using the Annexin V apoptosis detection kit. After incubation at room temperature in the dark for 15 min, cell apoptosis was analyzed on a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA).

Transwell migration and invasion assays

Migration and invasion assays were performed using 24-well transwell chambers (8 μm; Corning Life Sciences, Corning, NY, USA). For the migration assay, tumor cells were resuspended in DMEM and 2 x 10⁵ cells were seeded into the upper chambers. Next, 0.5 mL DMEM containing 10% fetal bovine serum was added to the bottom chambers. Following a 24 h-incubation, cells on the upper surface of the membrane were scrubbed off, and the migrated cells were fixed with 95% ethanol, stained with 0.1% crystal violet, and counted under a light microscope. The invasion assay protocol was similar to that of the migration assay except that the upper chambers were first covered with 1 mg/mL Matrigel.

Scratch migration assay

A scratch migration assay was also performed to confirm the influence of miR-143 on OSCC cell migration. When the cells transfected with miR-143 mimics or NC were grown to confluence, a scratch in the cell monolayer was made with a cell scratch spatula. After the cells were incubated under standard conditions for 24 h, pictures of the scratches were acquired using a digital camera system coupled to a microscope.

Statistics

Statistical analyses were carried out using the SPSS software (version 16.0, SPSS, Inc., Chicago, IL, USA). Data are reported as means ± standard deviation (SD). The differences between groups were analyzed using the Student *t*-test or chi-square test. Survival probabilities were described by Kaplan-Meier curves and compared using the log-rank test. Cox regression (proportional hazard model) was adopted to assess the independence of different prognostic factors. All tests were 2-tailed, and the significance level was set at P < 0.05.

RESULTS

Decreased expression of miR-143 in OSCC cell lines and tumor samples

Expression levels of miR-143 in OSCC cell lines and tumor samples were detected by quantitative reverse transcription-PCR and normalized to U6 small nuclear RNA. As shown in Figure 1A, the results showed that the expression levels of miR-143 were significantly lower in OSCC subjects (means ± SD: 12.2 ± 2.3) than those in the corresponding adjacent non-cancerous tissues (means ± SD: 23.4 ± 4.5; P < 0.001). The miR-143 expression in 4 OSCC cell lines was also clearly downregulated (Figure 1B). The Tca8113 cell line, which showed the lowest levels of miR-143 expression among all tested cell lines, was selected for further studies.
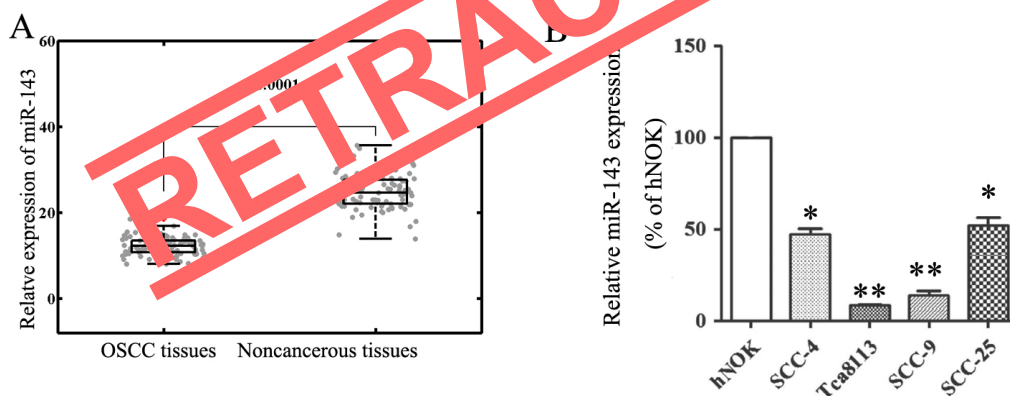

Figure 1. Expression of miR-143 in OSCC tissues and cell lines. (**A**) miR-143 expression was significantly lower in OSCC cancer tissues than in the corresponding non-cancerous tissues (P < 0.001, paired-samples *t*-test). miR-143 expression levels were calculated by the $2^{-\Delta Ct}$ method and normalized to U6 small nuclear RNA. (**B**) miR-143 expression was downregulated in OSCC cell lines SCC-4, SCC-9, SCC-25, and Tca8113 compared to human normal oral keratinocyte cells. *P < 0.05; **P < 0.01.

miR-143 expression and clinicopathological features in OSCC

The associations between miR-143 expression and various clinicopathological parameters of OSCC tissues are summarized in Table 1. Using the median miR-143 expression in all 109 OSCC patients as a cutoff, the patients were divided into a high miR-143 expression group (N = 55) and low miR-143 expression group (N = 54). As shown in Table 1, low expression of miR-143 was significantly correlated with the advanced T classifications (P = 0.001), positive

N classification (P = 0.01), and advanced TNM stage (P = 0.003). No significant difference was observed between miR-143 expression and patients' age, gender, and tumor differentiation.

Table 1. Correlation between miR-143 expression and different clinicopathological features in oral squamous cell carcinoma.

Clinicopathological features	No. of cases	miR-143 expression		P
		High (N, %)	Low (N, %)	
Age				
<60	53	31 (58.5%)	22 (41.5%)	0.127
≥60	56	24 (42.9%)	32 (57.1%)	
Gender				
Male	80	41 (51.2%)	39 (48.8%)	0.831
Female	29	14 (48.3%)	15 (51.7%)	
Histology/differentiation				
Well + Moderate	75	35 (46.7%)	40 (53.3%)	0.302
Poor	34	20 (58.8%)	14 (41.2%)	
T classification				
T1 + T2	41	29 (70.7%)	12 (29.3%)	0.001
T3 + T4	68	26 (38.2%)	42 (61.8%)	
N classification				
Positive	39	13 (33.3%)	26 (66.7%)	0.010
Negative	70	42 (60.0%)	28 (40.0%)	
TNM stage				
I + II	31	23 (74.2%)	8 (25.8%)	0.003
III + IV	78	32 (41.0%)	46 (59.0%)	

Correlation between miR-143 expression and prognosis of OSCC patients

Next, we evaluated whether miR-143 expression has prognostic potential for OS of OSCC patients. Using the Kaplan-Meier method and log rank test, the OS of patients with low miR-143 expression was significantly shorter than in those with high miR-143 expression (P = 0.002; Figure 2). In addition, survival benefits were also observed in those with early T classification (P = 0.016), negative N classification (P = 0.005) and early TNM stage (P < 0.001; Table 2).
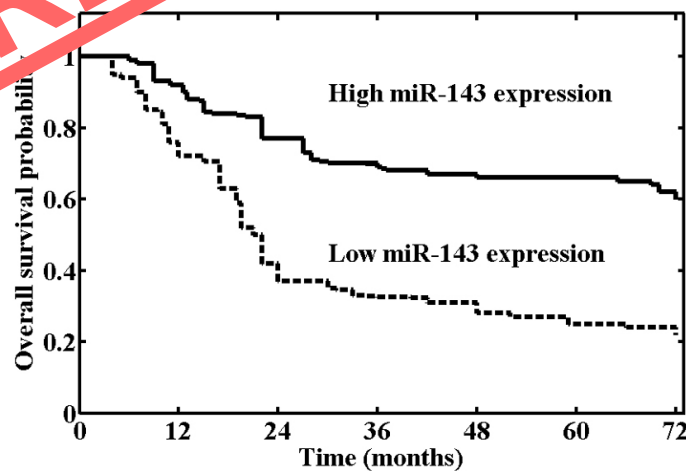

Figure 2. Overall survival curves for 2 groups defined by low and high expression of miR-143 in patients with OSCC. Lower miR-143 expression levels were significantly associated with poor outcome (P = 0.002, log-rank test).

Table 2. Univariate and multivariate analysis of overall survival in 109 patients with OSCC.

Variables	Univariate log-rank test (P)	Cox multivariable analysis (P)	Relative risk (RR)
Age at diagnosis (years)			
<60 *vs* ≥60	0.36	-	-
Gender			
Male *vs* Female	0.55	-	-
T classification			
T1-2 *vs* T3-4	0.016	0.028	3.465
N classification			
Positive *vs* negative	0.005	0.012	5.266
TNM stage			
I-II *vs* III-IV	<0.001	0.001	9.252
MiR-143 expression			
High *vs* low	0.002	0.005	7.332

Multivariate Cox regression analysis for significant parameters revealed that miR-143 expression [relative risk (RR) 7.332; P = 0.005], T classification (RR 3.465; P = 0.028), N classification (RR 5.266; P = 0.012), and TNM stage (RR 9.252; P = 0.001) were independent prognostic markers for OS of OSCC patients (Table 2).

Effects of miR-143 on the proliferation, apoptosis, invasion and migration of Tca8113 cells

Further, we assessed the biological role of miR-143 in Tca8113 cells. As shown in Figure 3A, the expression level of miR-143 in miR-143 mimic transfected cells was significantly higher compared to that in NC transfected cells (P < 0.001). The MTT assay showed that cell proliferation was significantly impaired after miR-143 mimic transfection (Figure 3B). We also observed an increased cell apoptosis in miR-143 mimic transfected cells (Figure 3C).

Cell invasion is a significant event in cancer progression, involving the migration of tumor cells into contiguous tissues and the dissolution of extracellular matrix proteins. Transwell invasion and migration assays were performed to investigate whether miR-143 directly influences Tca8113 cell migration and invasion. As shown in Figure 3D and 3E, upregulation of miR-143 inhibited cell invasion/migration compared with control cells. The scratch migration assay also confirmed the inhibitory effect of miR-143 on Tca8113 cell migration (Figure 3F).

DISCUSSION

OSCC is a global health problem with increasing incidence and mortality rates. It is important to investigate molecular and cellular mechanisms of OSCC, as well as to identify novel genetic or protein markers for accurate diagnosis and prediction of prognosis. The discovery of miRNAs has substantially altered the view on gene regulation, and new findings over the past few years have catapulted miRNAs to the center stage of cancer molecular biology. However, their potential roles in OSCC remain largely uncharacterized. In the current study, we first showed that miR-143 was downregulated in OSCC cell lines and primary tumor samples. Next, low-miR-143 expression was found to be correlated with aggressive clinicopathological features and unfavorable survival. Furthermore, transfection of miR-143 mimics in Tca8113 cells reduced cell proliferation, invasion, and migration and promoted cell apoptosis *in vitro*. This is the first report regarding the clinical significance and functional attributes of miR-143 in OSCC.
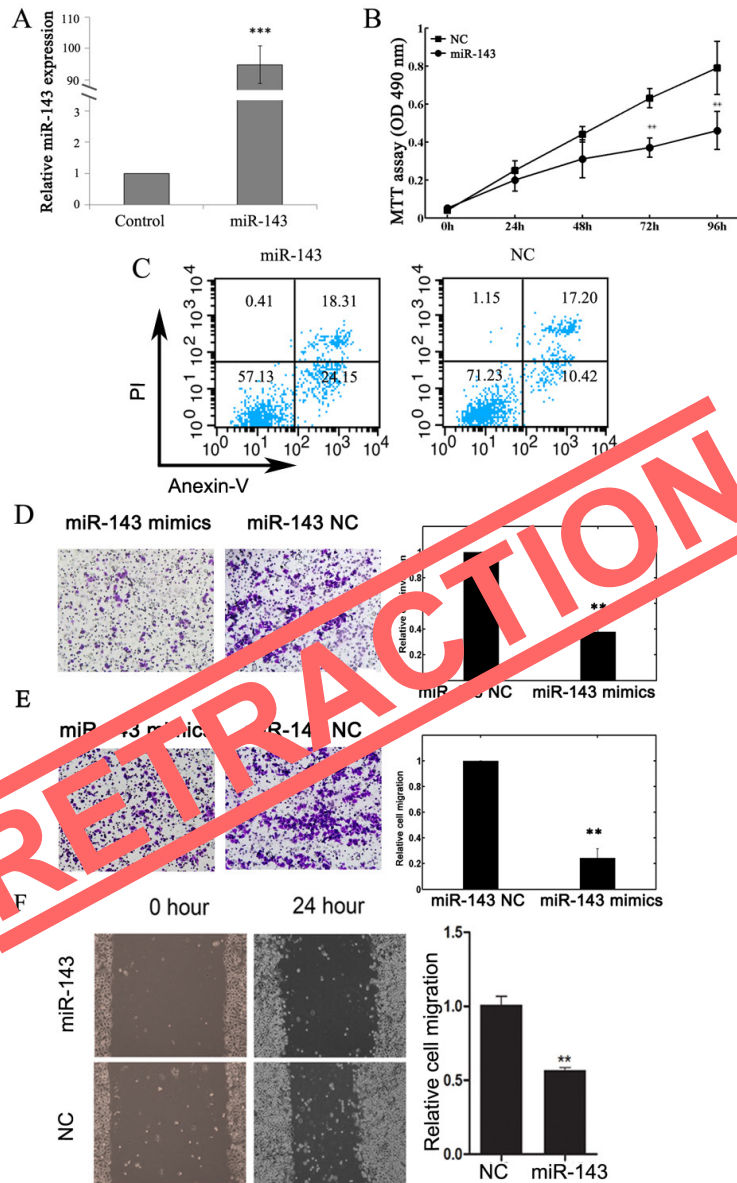
Figure 3. Effects of miR-143 mimics transfection on cell proliferation, apoptosis, invasion, and migration of Tca8113 cells. (**A**) The expression level of miR-143 in miR-143 mimic-transfected cells was significantly higher compared with NC transfected cells. Quantitative reverse transcription-PCR was conducted to detect the expression of miR-143. U6 RNA was used as an internal control. ***P < 0.001. (**B**) Cell proliferation was measured by MTT assays in Tca8113 cells transfected with miR-143 mimics or negative control. Data are reported as means ± SD of the experiments performed in triplicate. **P < 0.01. (**C**) Apoptosis of Tca8113 cells was detected by flow cytometric analysis after transfection with miR-143 mimics or negative control. (**D**, **E**) miR-143 ressed Tca8113 cell invasion and migration *in vitro*. Matrigel invasion and migration assays showed that the number of invaded or migrated cells was significantly lower in the miR-143-transfected group than in the NC-transfected group. **P < 0.01. (**F**) Scratch migration assay confirmed the inhibitory effect of miR-143 on Tca8113 cell migration.

Previous studies reported dysregulated miR-143 expression in many human malignancies and its functions as a tumor suppressor by targeting a number of oncogenic genes. For example, Noguchi et al. (2011) reported that miR-143 negatively contributed to cell proliferation and induced apoptosis in bladder cancer T24 cells. Ma et al. (2013) confirmed decreased miR-143 expression in non-small cell lung cancer tissues and cell lines, indicating that miR-143 can significantly suppress the migration and invasion of non-small cell lung cancer cells *in vitro* by targeting CD44v3. Downregulation of miR-143 was also responsible for invasion and migration of bladder cancer and human osteosarcoma via regulation of cyclooxygenase-2 and matrix metalloprotease-13 (Osaki et al., 2011; Song et al., 2011), respectively. In colorectal cancer, low miR-143 expression was associated with colorectal cancer growth and progression (Chen et al., 2009; Ng et al., 2009; Zhang et al., 2012), and predicted poor progression-free and cancer-specific survival (Pichler et al., 2012). For application of miR-143 in tumor treatment, Xu et al. (2011) showed that over-expression of miR-143 in prostate cancer cells increased their sensitivity to docetaxel by targeting the endothelial growth factor receptor/RAS/mitogen-activated protein kinase pathway. Akao et al. (2010) administered miR-143 to nude mice bearing DLD-1 tumors by intravenous injection. At the time of evaluation, the tumors had dose-dependently decreased in size. Pramanik et al. (2011) used a lipid-based nanoparticle for systemic delivery of miR-143/145 to subcutaneous and orthotopic pancreatic cancer xenografts for cancer therapy, and found that systemic miRNA-143/145 delivery inhibited the growth of subcutaneous and orthotopic pancreatic cancer xenografts. Thus, miR-143 may not only be useful as a marker but also serve as a target for the development of novel therapeutic strategies for human malignancies.

The mechanisms by which miR-143 expression affects OSCC carcinogenesis and development are complex, and the functional targets of miR-143 in OSCC require further clarification. Several genes have been suggested to be direct targets of miR-143 over the past few years. However, there is no one-to-one connection between miRNAs and target mRNAs. An average miRNA can have more than 100 targets (Brennecke et al., 2005). In contrast, several miRNAs can converge on a single transcript target. Thus, the potential regulatory circuitry afforded by miR-143 may be enormous, and identifying the molecular characteristics of miR-143 should be examined in future studies.

In conclusion, our results revealed that miRNA-143 was down-regulated in OSCC cell lines and clinical samples. Low-level expression of miR-143 was significantly associated with a more aggressive and poor prognostic phenotype of patients. Restored miR-143 expression in Tca8113 cells exhibited anti-tumor effects *in vitro*. Our data suggest an important role for miR-143 in the molecular etiology and gene therapy of OSCC.

Conflicts of interest

The authors declare no conflict of interest.

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