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ORIGINAL ARTICLE

Expression of MiRNA-137 in oral squamous cell carcinoma and its clinical significance

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Summary

Purpose: *To investigate the expression of microRNA (miRNA-137) in oral squamous cell carcinoma (OSCC) and its effects on activity, invasion and proliferation of OSCC human squamous cell carcinoma cell lines 2 (HSC-2 cell line).*

Methods: *158 cases of pathologically-confirmed OSCC tissues and corresponding para-cancerous tissues were collected. The tissue mRNA was extracted and miRNA-137 expression in tissues was detected using real-time polymerase chain reaction (RT-PCR). With miRNA-137 mimics and scramble mimics transfected into OSCC HSC-2 cells, invasion and proliferation of cells were measured with Transwell assay, and cell viability was measured with methyl thiazolyl tetrazolium (MTT) assay. The relationship miRNA-137 expression in OSCC tissues and cell invasion, proliferation and viability with clinicopathological parameters were recorded and analyzed.*

Results: *The expression of miRNA-137 in OSCC tissues was significantly lower than that in adjacent tissues (p=0.0057). Compared with untreated OSCC HSC-2 cells, the expression of miRNA-137 was significantly increased in OSCC HSC-2 cells treated with miRNA-137 mimics, and the difference was statistically significant (p<0.05). No statistically significant difference was detected when comparing the miRNA-137 expression between the untreated OSCC HSC-2 cells and OSCC HSC-2 cells treated with scramble simulation (p>0.05). The cell viability, invasion and proliferation of OSCC HSC-2 treated with miRNA-137 mimics were significantly lower than those of untreated OSCC HSC-2 cells and scramble simulation-treated OSCC HSC-2 cells (p<0.05). The miRNA-137 expression in OSCC tissue was significantly relevant with World Health Organization (WHO) pathological grading and the history of areca nut use (p=0.044), but miRNA-137 expression showed no correlation with the patient's gender, age, TNM stage, smoking and lymph node metastasis (p>0.05).*

Conclusion: *miRNA-137 is lowly expressed in OSCC tissues, which is related to tumor differentiation. MiRNA-137 is expected to be a potential marker for early diagnosis of OSCC.*

Key words: *miRNA-137, MTT method, OSCC, PCR, Transwell method*

Introduction

OSCC is the sixth most common cancer in the world, accounting for more than 40% of head and neck tumors [1]. OSCC bears a poor prognosis due to its invasive capacity. Despite advances in treatment and diagnosis over the past 30 years, the 5-year survival rate is still below 50% without significant improvement of its incidence [2]. Frequently, before OSCC diagnosis is made, the tumor has invaded the underlying structures, leading to poor prognosis of patients. Therefore, in the present study, we tried to find an effective OSCC tumor marker for more efficient early diagnosis.

miRNAs are small non-coding single-stranded RNA molecules with approximately 22 nucleotides in length. By binding to specific sites within the 3'-untranslated region (3'UTR) of the transcription product, miRNAs regulate post-transcription expression of the target genes [3]. Nowadays, a large number of studies have shown that miRNAs play key roles in the development of hu-

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man cancers, and differential expression of miRNAs has been found in multiple cancers [4], including breast cancer [5], gastric adenocarcinoma [6] and liver cancer [7] , suggesting that expression and regulation of miRNA-mediated genes may be an important factor in the etiology of malignant tumors. miRNA-137 is related to the occurrence and development of thyroid tumors [8]. However, miRNA-137 has not been reported in OSCC. In the present study we collected cancer tissues and para-cancerous tissues of patients with OSCC and examined the expression of miRNA-137 to determine its expression in OSCC tissues.

Methods

Sample collection

158 pairs of OSCC tissues and corresponding para-cancerous tissues (>3 cm away from the cancer tissues) were collected from OSCC patients admitted to our hospital from March 2011 to July 2016 to be subjected to surgical resection. OSCC was confirmed by pathological examination. Ninety five males and 63 females were included, with an average age of 61±11.5 years. Clinical staging and grading of patients were performed according to TNM staging system and World Health Organization (WHO) criteria for histological classification (Table 1). The samples were placed in liquid nitrogen tank within 5 min for preservation at -80°C. The study was approved by the hospital's Ethics Committee and all the participants signed informed consent.

Materials and reagents

Human squamous cell carcinoma cells (HSC-2 cells) were purchased from the Cell Resource Center of Shanghai Institute of Life Sciences, Chinese Academy of Sciences. Polymerase chain reaction (PCR) kits and cDNA Synthesis Kit were purchased from Invitrogen (Waltham, MA, USA), primers and miRNA-137 mimics were all provided by GenePharma Corporation (Shanghai, China), and dimethyl sulfoxide (DMSO) was from Sigma-Aldrich (CA, USA).

Study methods

(1) Cell culture

Cells firmly clinging to the container wall were cultured in constant-temperature incubator containing 5% CO_2 at 37°C. Cells were cultured with Roswell Park Memorial Institute 1640 medium (RPMI-1640), and medium containing 10% fetal bovine serum (FBS) supplemented with 100 U/mL penicillin and 0.1mg/mL streptomycin. Adherent cells in the logarithmic growth phase were digested with 0.25% trypsin for passage.

(2) MTT assay

OSCC HSC-2 cells were inoculated into 96-well plates with 3.5×10^3 per well. After 24-hr culturing, cells in the logarithmic growth phase were used for the experiments. The cells were cultured again for 48 hrs to test the cell viability. After MTT was added to each well, cells were cultured for another 2 hrs in a thermostat incubator at 37°C. Finally, the supernatant was discarded by centrifugation followed by addition of DMSO. The absorbance was measured at the wavelength 570 nm using microplate reader.

(3) Transwell method

After Matrigel was placed in the regriferetor at 4°C overnight, 50 µl Matrigel (diluted at 1:9) was added to the upper Transwell chamber and stayed at 37°C for 4 hrs. Five hundred µl of medium containing 10% FBS and 200 µl of serum-free cell suspension were added to the lower and upper Transwell chambers, respectively. After culturing the cells for 48 hrs, Matrigel and the cells on the upper chamber were swabbed. The cells were fixed with formaldehyde for 20 min at room temperature, followed by DAPI (4',6-diamidino-2-phenylindole) staining for 5 min. The stained cells were counted with inverted microscope. Five optical fields (x100) were randomly selected to count the cells and calculate the average values.

RT-PCR detection of miRNA-137 expression

RNA in OSCC tissues and HSC-2 cells was extracted using Trizol reagent. Primer miRNA-137 was synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd, using reverse transcription primers 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACGCGT-3', upstream primer 5'-GGTTCCCGGTTCCTTATTGCTTAAGA-3', downstream primer

Table 1. Clinicopathological characteristics of 158 patients with OSCC

Characteristics	*n*	%
Age, years		
<60	89	56.33
≥60	69	43.67
Sex		
Female	95	60.13
Male	63	39.87
TNM staging		
I	47	29.75
II	68	43.04
III	20	12.66
IV	23	14.56
WHO classification		
I	55	34.81
II	68	43.04
III	5	3.16
Lymph node metastasis		
Yes	50	31.65
No	108	68.35
Smoking history		
Yes	117	74.05
No	41	25.95
History of areca nut use		
Yes	109	68.99
No	49	31.01

5'-AAAAGTTGCAGGGTCCGAGGTATT-3'. According to the instructions of reverse transcription kit, RNA was reversely transcribed into cDNA after 10 min at 37°C and 10 s at 85°C. The reaction system was composed of 10 μl of Maxima SYBR Green qPCR Master Mix (2X), 1 μl of PCR upstream primer (10 μmol/L), 1 μl of PCR downstream primer (10 μmol/L), 2 μl of template cDNA, and dH_2O was added till the total volume was up to 25 μl. PCR was performed by using the following cycling conditions: initial denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 2 min and 60°C for 30 s. The miRNA content in cells was expressed with U6 as internal reference, and the relative expression level of miRNA-137 was expressed with $2^{-\Delta\Delta CT}$ method.

Statistics

All data were statistically analyzed using the Statistical Package of Social Sciences (SPSS) 20.0 software. Non-parametric rank sum test and Student's *t*-test were preformed for independent samples. Enumeration data were expressed as mean±standard deviation ($\bar{x}$±s). Correlations between the expression level of miRNA-137 and clinical factors were analyzed with chi-square test. Difference with p<0.05 was considered statistically significant.

Results

miRNA-137 expressions in normal tissues and OSCC tissues

The expression of miRNA-137 in normal tissues was significantly higher than that in OSCC tissues, and the difference was statistically significant (p=0.0057; Figure 1).

Relationship between miRNA-137 expression and clinicopathological features of OSCC

The miRNA-137 expression was significantly relevant with WHO classification and the history of areca nut use (both p<0.05). Correlation with miRNA-137 expression of the patient's gender, age, TNM staging, smoking and lymph node metastasis showed no statistically significant differences (p>0.05; Table 2).

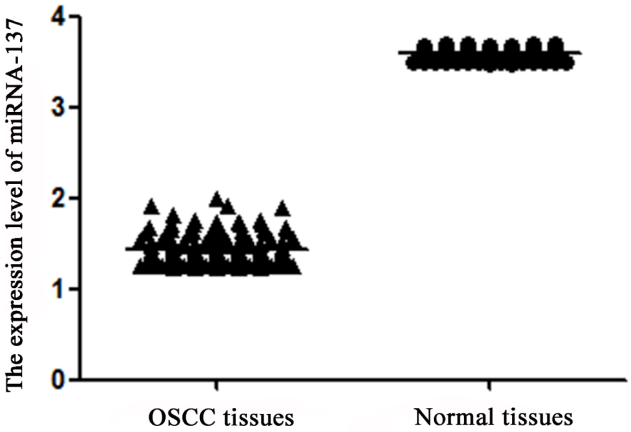

Figure 1. Comparison of miRNA-137 expression in paracancerous tissues and tumor tissues of OSCC patients, showing that the expression of miRNA-137 in normal tissues was significantly higher compared with OSCC tissues (p<0.05).

Comparison of the expression of miRNA-137 in OSCC HSC-2 cells treated with miRNA-137mimics and scramble mimics

Compared with untreated HSC-2 cells, miRNA-137 level in OSCC HSC-2 cells treated with miRNA-137 mimics was increased significantly, and the difference was statistically significant (p<0.05). No statistically significant difference was observed by comparing the miRNA-137 expression between untreated HSC-2 cells and OSCC HSC-2 cells treated with scramble mimics (p>0.05; Figure 2).

Detection of viability of OSCC HSC-2 cells with MTT method

The cell viability measured in OSCC HSC-2 cells treated with miRNA-137 mimics was 1.04±0.08, significantly lower than that in untreated OSCC HSC-2 cells (1.61±0.11) and OSCC HSC-2 cells treated with scramble mimics (1.58±0.10), and the difference was statistically significant (p<0.05; Figure 3).

Table 2. Relationship between the expression of miRNA-137 and clinicopathological characteristics in OSCC

Characteristics	*n (%)*	*Expression level*	*p value*
Age, years			0.714
<60	89 (56.33)	1.47±0.58	
≥60	69 (43.67)	1.42±0.51	
Sex			0.841
Female	95 (60.13)	1.38±0.47	
Male	63 (39.87)	1.42±0.57	
TNM staging			0.057
I	47 (29.75)	1.62±0.55	
II	68 (43.04)	1.49±0.67	
III	20 (12.66)	1.38±0.84	
IV	23 (14.56)	1.25±0.71	
WHO classification			0.044
I	55 (34.81)	1.56±0.69	
II	68 (43.04)	1.44±0.71	
III	5 (3.16)	1.29±0.64	
Lymph node metastasis			0.614
Yes	50 (31.65)	1.25±0.84	
No	108 (68.35)	1.61±0.64	
Smoking history			0.072
Yes	117 (74.05)	1.43±0.66	
No	41 (25.95)	1.57±0.59	
History of areca nut use			0.048
Yes	109 (68.99)	1.33±0.67	
No	49 (31.01)	1.59±0.64	

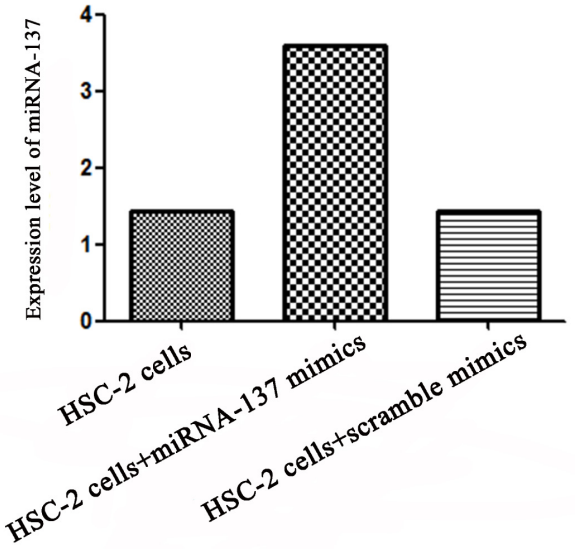

Figure 2. Comparison of miRNA-137 expression in OSCC HSC-2 cells after adding miRNA-137 mimics and scramble mimics, showing that miRNA-137 level in OSCC HSC-2 cells treated with miRNA mimics was significantly increased compared with that of untreated cells ($p<0.05$). No statistically significant difference was observed by comparing the miRNA-137 expression between untreated HSC-2 cells and OSCC HSC-2 cells treated with scramble mimics ($p>0.05$).

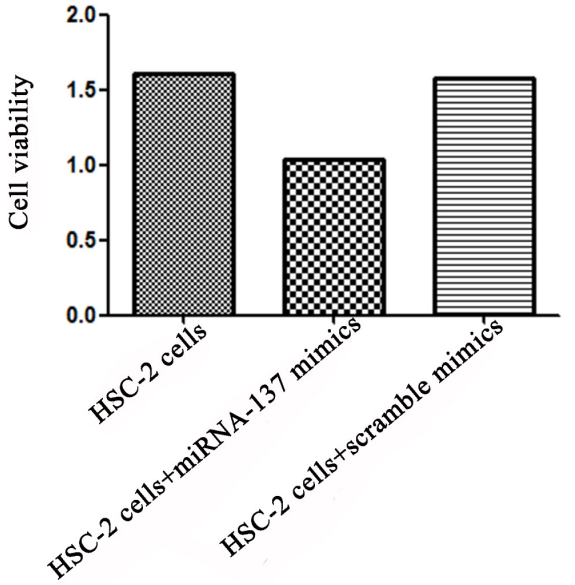

Figure 3. Viability of OSCC HSC-2 cells by MTT method, showing that the cell viability measured in OSCC HSC-2 cells treated with miRNA-137 mimics was significantly lower than in untreated OSCC HSC-2 cells and OSCC HSC-2 cells treated with scramble mimics ($p<0.05$).

Detection of cell invasion and proliferation of OSCC HSC-2 cells with Transwell method

The cell invasion and proliferation measured in OSCC HSC-2 cells treated with miRNA-137 mimics was 7.64±1.53, significantly lower than that in untreated OSCC HSC-2 cells (25.14±2.34) and OSCC HSC-2 cells treated with scramble mimics (19.47±1.83), and the difference was statistically significant ($p<0.05$; Figure 4).

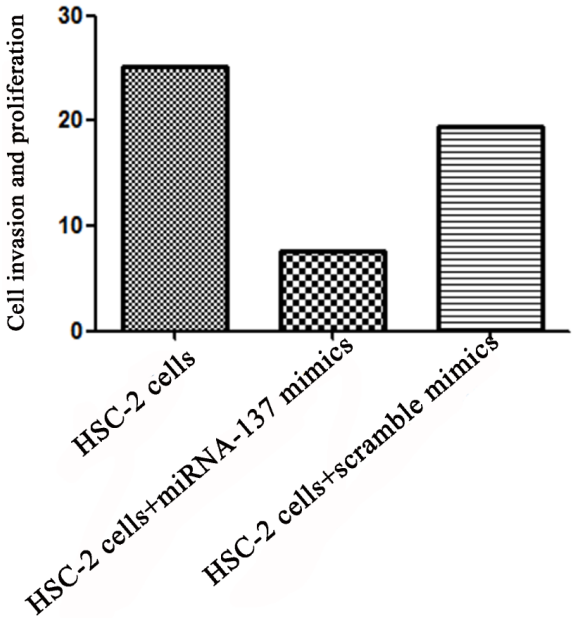

Figure 4. Detection of cell invasion and proliferation of OSCC HSC-2 cells using Transwell method, showing that the cell and proliferation measured in OSCC HSC-2 cells treated with miRNA-137 mimics was significantly lower than that in untreated OSCC HSC-2 cells and OSCC HSC-2 cells treated with scramble mimics ($p<0.05$).

Discussion

Nowadays, malignant tumors of the head and neck are the sixth most common cancers worldwide, seriously endangering people's life, health and safety [9]. OSCC is the most common head-neck tumor, accounting for 1-2% in all human malignancies [10]. The survival rate of OSCC remains low despite of many improvements in diagnosis and treatment over the past several decades [11]. The fundamental biological features of cancer cells include invasion and metastasis, which are related to tumor progression, leading to variable survival rates. The identification of molecular changes in cancer has been a topic of global research, so as to develop potential new therapies. MiRNAs may play significant roles in invasion, metastasis and angiogenesis of multiple tumors acting either as oncogenes or tumor suppressor genes [12], suggesting that miRNAs may also serve as molecular markers for OSCC, having clinical value in early diagnosis, treatment and prognosis of this disease.

MiRNAs are a class of non-coding small RNAs containing around 1000 short nucleotide fragments and 18-25 long nucleotide fragments widely existing in animals and plants [13]. One-to-multiple and multiple-to-one relationship between miRNAs and their target gene have been described [14]. MiRNAs act as post-transcriptional regulators of their messenger RNA (mRNA) targets by binding to the target mRNA in the un-

translated region for mRNA degradation and/or translational repression [15,16].

Only few studies have reported the relationship between miRNA and clinicopathological features of OSCC. The present study was performed to investigate the potential relationship between miRNA-137 and OSCC. Using RT-PCR we found that, compared with that in para-cancerous tissues, miRNA-137 was significantly down-regulated in tumor tissues, and the difference was statistically significant. Meanwhile, as reported by Bemis et al. [17], the down-regulation of miRNA-137 in melanoma further indicated that it contributes to invasion and metastasis of OSCC cells. By comparing with clinical factors, we also found that miRNA-137 expression was significantly relevant with WHO classification and the history of areca nut use (both $p<0.05$). In the present study we also studied the cell viability, invasion and proliferation ability of OSCC HSC-2 cells. Compared with untreated OSCC HSC-2 cells and OSCC HSC-2 treated with scramble mimics, cell viability was significantly increased and the invasion and proliferation were significantly decreased in OSCC HSC-2 cells treated with miRNA-137 mimics ($p<0.05$). A study conducted by Sun et al. [18] found that the overexpression of miRNA-137 *in vitro* leads to inhibition of proliferation of miRNA-137 analog by chemical synthesis, which arrests the cell cycle to G1/G0 and induces apoptosis. This further demonstrates that miRNA-137 plays a key role in the occurrence of OSCC. Taken together, the low expression of miRNA-137 is closely related to the occurrence and development of OSCC.

Li et al. [19] reported that miRNA-137 is closely related to small cell lung cancer multidrug resistance (SCLC MDR), in which miRNA-137 expression partially reduces the cisplatin resistance in H446/CDDP cells by interrupting the KIT expression. In addition, Sakaguchi et al. [20] suggested that miRNA-137 is capable of inhibiting the tumorigenesis of colon cancer stem cells (CSCs) and inhibiting also double-cortin like kinase 1 (DCLK1), resulting in expression of miRNA-137 in normal colon stem cells (NCSCs), thereby inhibiting the proliferation of cells out of control.

A number of limitations inherent in the present study should be noted. Receiver operating characteristic curve (ROC) analysis was not performed in this study; the strength of the study was limited by insufficient sample size; more samples should be collected from different regions to minimize the effect of regional restrictions and to provide more powerful evidence for enhancing the significance of the experiments.

In summary, the results of the present study showed that miRNA-137 expression is significantly down-regulated in OSCC tissues, which is significantly correlated with WHO classification and the history of areca nut use. Up-regulation of miRNA-137 may inhibit the development of OSCC. These indicate that the expression level of miRNA-137 has potential clinical significance in OSCC.

Conflict of interests

The authors declare no confict of interests.

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