

# MiR-214 regulates oral cancer KB cell apoptosis through targeting *RASSF5*

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**ABSTRACT.** Ras association domain family member 5 (*RASSF5*), a member of the Ras association domain family, induces cell apoptosis by phosphorylating FOXO3a, which triggers target gene *BIM* (pro-apoptotic factor) activation. MiR-214 is overexpressed in oral cancer tissue, indicating its possible involvement in oral cancer pathogenesis. Bioinformatics analysis has revealed a complimentary sequence between miR-214 and the 3'-UTR of *RASSF5* mRNA. However, whether miR-214 regulates *RASSF5* in oral cancer remains poorly understood. We aimed to investigate the role of miR-214 in *RASSF5* expression regulation in oral cancer. Tumor and paracarcinoma tissues were obtained from 48 oral cancer patients to examine miR-214 and *RASSF5* expression. The relationship between miR-214 and *RASSF5* was investigated by dual luciferase reporter gene assay. Oral cancer KB cells were cultured *in vitro* and divided into inhibitor NC, miR-214 inhibitor, Scramble-pMD18, *RASSF5*-pMD18, and miR-214 inhibitor + *RASSF5*-pMD18 groups. Caspase 3 activity, cell apoptosis, and

total protein expression were measured by spectrophotometry, flow cytometry, and western blot, respectively. MiR-214 expression was significantly increased, while that of *RASSF5* decreased in oral cancer tumor tissues compared to paracarcinoma tissues. Luciferase assay showed that miR-214 suppressed *RASSF5* expression by targeting its 3'-UTR. Down-regulation of miR-214 and/or enhancement of *RASSF5* expression markedly increased FOXO3a phosphorylation, *BIM* expression, caspase 3 activity, and apoptosis. In conclusion, miR-214 expression was elevated and *RASSF5* was down-regulated in oral cancer. Moreover, miR-214 regulated KB cell apoptosis through targeted inhibition of *RASSF5* expression, FOXO3a phosphorylation, and *BIM* expression, suggesting its possible application as a novel therapeutic oral cancer target.

**Key words:** MiR-214; *RASSF5*; Oral cancer; KB cell; Apoptosis

## INTRODUCTION

Oral cancer is a common type of cancer of the head and neck, and squamous cell carcinoma is its major histopathological type (Sankaranarayanan et al., 2015). The incidence of oral cancer ranks second, after nasopharyngeal carcinoma, among all cancers of the head and neck. Although there have been significant developments in comprehensive treatment strategies such as surgery, radiotherapy, and chemotherapy, the prognosis remains poor. In-situ recurrence, neck metastasis and recurrence, and distant metastasis are important factors affecting survival and prognosis (Koloutsos et al., 2014).

The Ras superfamily, composed of small molecular GTP-binding proteins, plays a key role in the regulation of cell proliferation, differentiation, and apoptosis. It can undergo self-activation by binding to GTP and does not need stimulation by external growth signals. Activated Ras can form a continuous stimulation signal to trigger cell proliferation and inhibit apoptosis for tumor occurrence (Calaf & Abarca-Quinones, 2016). Ras association domain family member 5 (*RASSF5*), a member of the *RASSF* family, is the human homologue of the mouse Ras receptor Nore1 (Donninger et al., 2016). *RASSF5* can bind to mammalian sterile 20-like kinase 1 (Mst1) through its C-terminal SARAH domain structure, leading to the translocation of Mst1 to the nucleus, phosphorylation of transcriptional factor FOXO3a, and subsequent initiation of cell apoptosis (Praskova et al., 2004). *RASSF5* levels have been reported to significantly decrease in tissues in multiple cancers, including osteosarcoma (Zhou et al., 2014), esophageal cancer (Guo et al., 2015), lung cancer (Hesson et al., 2003), liver cancer (Calvisi et al., 2009), and gastric cancer (Han et al., 2015), suggesting its inhibitory role in tumor occurrence.

MicroRNAs (miRNAs) are small non-coding single-stranded RNAs with a length of about 18-22 nucleotides. They participate in cell proliferation, cycle, differentiation, and apoptosis through complete or incomplete complementary binding to target genes to promote target gene mRNA degradation or inhibit mRNA translation. MiRNAs have been receiving increasing attention for their role in oral cancer (Manikandan et al., 2016). MiR-214 levels were revealed to be up-regulated in oral cancer tissues, indicating that miR-214 might be an oncogene involved in oral cancer (Scapoli et al., 2010). Furthermore, bioinformatics analysis

demonstrated that miR-214 could bind to the 3'-UTR of *RASSF5*. However, whether miR-124 regulates *RASSF5* in oral cancer remains poorly understood. This study aimed to investigate the role of miR-214 in the regulation of *RASSF5* expression in oral cancer.

## MATERIAL AND METHODS

### Main reagents and materials

Human oral cancer cell line KB, Lipofectamine 2000, Horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from ATCC (Manassas, VA, USA), Invitrogen (Thermo Fisher Scientific Corp., Carlsbad, CA, USA), and Jackson ImmunoResearch, Inc. (West Grove, PA, USA), respectively. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and G418 were obtained from Gibco Laboratories (Gaithersburg, MD, USA). The E. Z. N. A. total RNA extraction, Annexin V/PI apoptosis detection, and caspase-3 detection kits were bought from Omega Bio-Tek, Inc. (Norcross, GA, USA), Yeasen Corp. (Shanghai, China), and Beyotime Institute of Biotechnology (Haimen, China), respectively. ReverTra Ace qPCR RT kit and SYBR green Realtime PCR Master Mix were obtained from Toyobo Co., Ltd (Osaka, Japan). Rabbit anti human *RASSF5*, p-FOXO3a, and Bim primary antibodies were obtained from Abcam (Cambridge, UK). Dual-Luciferase Reporter Assay System and pGL3-promoter plasmid were obtained from Promega Corp. (Fitchburg, WI, USA).

### Clinical information

A total of 48 oral cancer patients undergoing treatment in the Fourth Hospital of Hebei Medical University between June 2015 and May 2016 were recruited for the study. Tumor and paracarcinoma tissue samples were obtained during surgery. All the samples were confirmed by pathology for tongue cancer (28 cases), oral floor carcinoma (6 cases), carcinoma of the buccal mucosa (4 cases), gum cancer (5 cases), and other types (5 cases). There were 26 male and 22 female study participants with a mean age of 49.7 (36-71) years. None of the patients had received chemotherapy, radiotherapy, or any other special cancer therapy. The present study was approved by the Hospital Ethics Committee, and all the subjects provided signed informed consent.

### Cell culture

The human oral cancer cell line KB was maintained in DMEM, containing 10% FBS and 1% penicillin-streptomycin, and cultured at 37°C with 5% CO<sub>2</sub>. Cells in the logarithmic phase of growth were used for the following experiments.

### Luciferase reporter gene vector construction

The 3'-UTR of *RASSF5* was amplified based on the human embryonic kidney 293 (HEK293) cell genome. Polymerase chain reaction (PCR) products were recycled and connected to the pGL-3M luciferase vector to transform competent DH5 $\alpha$  cells. The products were screened for positive clones by colony PCR, and plasmids bearing the correct sequence

were used for cell transfection. In addition, the 3'-UTR of *RASSF5* was mutated to investigate whether miR-214 directly binds to *RASSF5*.

### Luciferase reporter gene assay

The luciferase reporter gene assay was performed using the Dual-Luciferase Reporter Assay System according to the kit manufacturer's instructions. Briefly, HEK293 cells were transfected with 500 ng pGL3-*RASSF5*-3'UTR, 30 nM miRNA nucleotide fragments, and 30 ng pRL-TK plasmid using the reagent Lipofectamine 2000. Following incubation for 6 h, Opti-MEM was replaced with DMEM supplemented with 10% FBS and 1% penicillin-streptomycin, and the cells were incubated for another 48h. After washing the cells twice with phosphate buffered saline (PBS), 100  $\mu$ L passive lysis buffer was added, and the cells were incubated for 30 min at 37°C and centrifuged at 1000 g for 10 min. LAR II (100  $\mu$ L) was then added into the supernatant, which was tested for fluorescence I immediately using a chemiluminescence apparatus. Stop & Glo reagent (100  $\mu$ L) was then added to the supernatant to test for fluorescence II. The relative luciferase activity levels were calculated by the ratio of fluorescence I to fluorescence II values. The sequences used for this assay were as follows: mimic NC, 5'-UUCUCCGAACGUGUCACGUUU-3'; miR-214 mimic, 5'-UUGUGCUUGAUCUAACCAUGUAUGGUUAGAUAAGCACA AUU-3'; inhibitor NC, 5'-UUCUCCGAACGUGUCACGUUU-3'; miR-214 inhibitor, 5'-UUGUGCUUGAUCUAACCAUGUAUGGUUAGAUAAGCACA AUU-3'.

### Construction of the vector for overexpression and cell transfection

pMD18-T (Sino Biological Inc., Beijing, China) was selected as the plasmid vector, and EcoRI and SalI were the restriction endonucleases used to amplify *RASSF5*. Primers for *RASSF5* were designed according to the cDNA sequence of *RASSF5* in GenBank and were as follows: forward, 5'-GACGAATTCATGGCAAAGCAACCT-3', reverse, 5'-GACGTCGACTTAATCAGGTGGAAG-3'. Gel electrophoresis was performed to determine the target fragment (519 bp). The target fragment was recycled, connected to the vector, and used to transform competent BJ5183 cells. Monoclonal antibodies were screened using an ampicillin resistant solid board and amplified to extract plasmids containing the target fragment. The extracted plasmid was sequenced to confirm its accuracy. Negative control (Scramble-pMD18-T vector) or *BIM* overexpression plasmids (*RASSF5*-pMD18-T vector) were transfected into KB cells using the calcium phosphate transfection method. Stable transfected positive cell clones were screened using G418. Transfected KB cells were divided into the five following groups: inhibitor NC group, miR-214 inhibitor group, Scramble-pMD18 group, *RASSF5*-pMD18 group, and miR-214 inhibitor + *RASSF5*-pMD18 group. After 72 h of incubation, the cells were used for successive experiments.

### Real-time reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from cells using the E. Z. N. A. total RNA extraction kit. RNA was then reverse transcribed to cDNA at reaction parameters of 37°C for 15 min and 98°C for 5 min. The reverse transcription system contained 1 mg total RNA, 2  $\mu$ L RT Buffer (5X), 0.5  $\mu$ L oligo dT + Random primer Mix, 0.5  $\mu$ L RT Enzyme Mix, 0.5

$\mu\text{L}$  RNase inhibitor, and  $\text{ddH}_2\text{O}$ . The PCR primers used were as follows: miR-214PF: 5'-GGACAGGACGCACAGTCA-3', miR-214PR: 5'-CAGACGAGGCTCCGTGGT-3'; *U6*PF: 5'-ATTGGAACGATACAGAGAAGATT-3', *U6*PR: 5'-GGAACGCTTCACGAATTTG-3'; *RASSF5*PF: 5'-GGGCATGAAACTGAGTGAAGA-3', *RASSF5*PR: 5'-TGGCATCATAGATG GACTGGG-3'; *BIM*PF: 5'-ATCTCAGAGCAATGGCTTCC-3', *BIM*PR: 5'-ATTCGTGGGTG GTCTTCG-3';  $\beta$ -*actin*PF: 5'-GAACCCTAAGGCCAAC-3',  $\beta$ -*actin*PR: 5'-TGTCACGCACGA TTTCC-3'. The PCR system was composed of 4.5  $\mu\text{L}$  2X SYBR green Realtime PCR Master Mix, 1  $\mu\text{L}$  2.5  $\mu\text{M}$  forward and reverse primers, 1  $\mu\text{L}$  cDNA, and 3  $\mu\text{L}$   $\text{ddH}_2\text{O}$ . The reaction was performed in the Bio-Rad CFX96 (Hercules, CA, USA) system at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

### Western blot

Total protein was extracted from the cells by ice-cold radioimmunoprecipitation assay buffer at 4°C, and centrifuged at 10,000 g for 10 min. After protein quantification by the bicinchoninic acid assay, 50 mg protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, run for 3 h, followed by transfer of the proteins to a polyvinylidene difluoride membrane for 1.5 h. Skim milk (5%) was added for membrane blocking, and the membrane was incubated at room temperature for 60 min. The membrane was incubated in primary antibody at 4°C overnight (*RASSF5* 1:300, p-FOXO3a 1:100, *BIM* 1:200,  $\beta$ -actin 1:1000). After a wash by phosphate buffered saline Tween-20, the membrane was incubated in HRP-labeled secondary antibody at room temperature for 60 min (1:10,000). Finally, it was treated with enhanced chemiluminescence reagent and developed. Quantity One software was used for quantitative analysis of the protein bind.

### Caspase-3 activity detection

The standard substrate pNA (10 mM) was diluted to 0, 10, 20, 50, 100, and 200  $\mu\text{M}$ , concentrations. The absorbance values of these solutions at 405 nm were used to prepare the standard curve. The cells were digested and then centrifuged at 600 g and 4°C for 5 min. After a PBS wash,  $2 \times 10^6$  cells were added to 100  $\mu\text{L}$  lysis buffer and incubated at 4°C for 15 min, followed by centrifugation at 18,000 g for 10 min. The supernatant was transferred to a precooled Ep tube, and 10  $\mu\text{L}$  Ac-DEVD-pNA (2 mM) was added to it. The solution was incubated at 37°C for 120 min, and absorbance was measured at 405 nm.

### Detection of cell apoptosis by flow cytometry

The cells were digested by trypsin and resuspended in 100  $\mu\text{L}$  binding buffer. They were then incubated in the dark at room temperature for 10 min after the addition of Annexin V-FITC and propidium iodide, 5  $\mu\text{L}$  each. Cell apoptosis was detected using the Beckman FC 500 MCL flow cytometry system (Beckman Coulter, Inc., Brea, CA, USA).

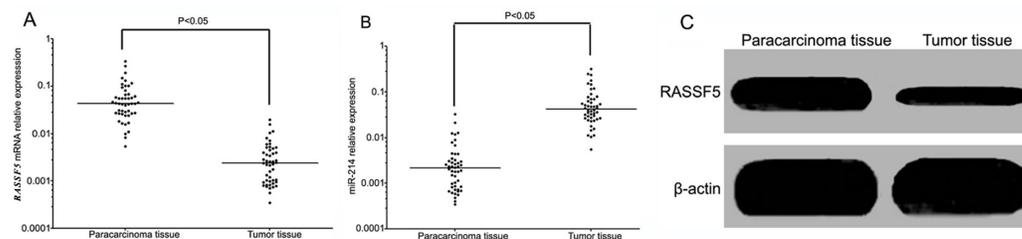
### Statistical analysis

The SPSS 18.0 software was used for data analysis. Data are reported as means  $\pm$  standard deviation and were compared by the student *t*-test.  $P < 0.05$  indicated a significant difference.

## RESULTS

### Elevation of miR-214 expression levels in oral cancer

To measure the expression levels of *RASSF5* and miR-214, RT-qPCR was performed, and the results demonstrated that *RASSF5* mRNA expression was significantly reduced (Figure 1A) and miR-214 levels were enhanced in oral cancer tissues than in paracarcinoma tissues (Figure 1B). Western blot analysis revealed that *RASSF5* expression was markedly lower in oral cancer tissues than in paracarcinoma tissues (Figure 1C), suggesting that miR-214 and *RASSF5* dysregulation may be related to oral cancer occurrence. Interestingly, a significantly negative correlation was observed between miR-214 and *RASSF5* in oral cancer tissue ( $r = 0.76$ ,  $P < 0.001$ ), suggesting miR-214 might be involved in the regulation of *RASSF5* expression.



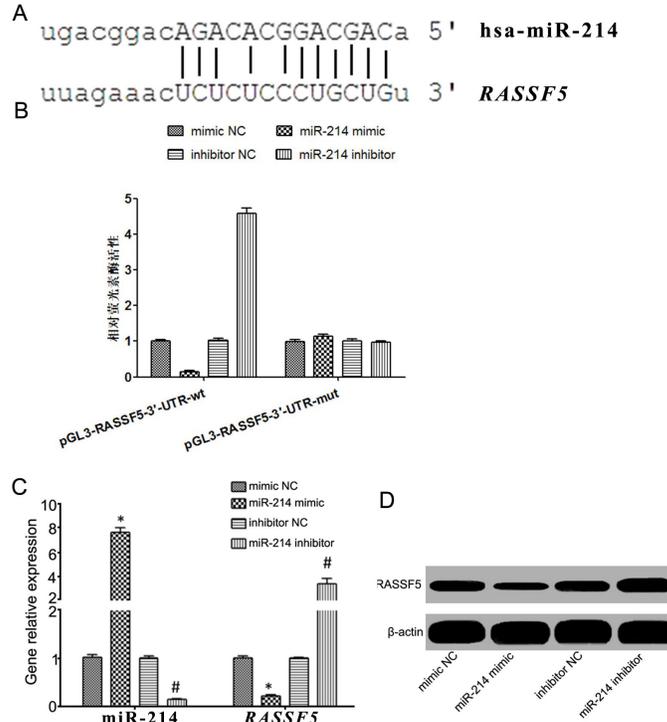
**Figure 1.** MiR-214 elevated and *RASSF5* decreased in oral cancer tissue. **A.** qRT-PCR detection of *RASSF5* mRNA expression. **B.** qRT-PCR detection of miR-214 expression. **(C)** Western blot detection of *RASSF5* expression.

### MiR-214 targeted regulation of *RASSF5* expression in the oral cancer cell line KB

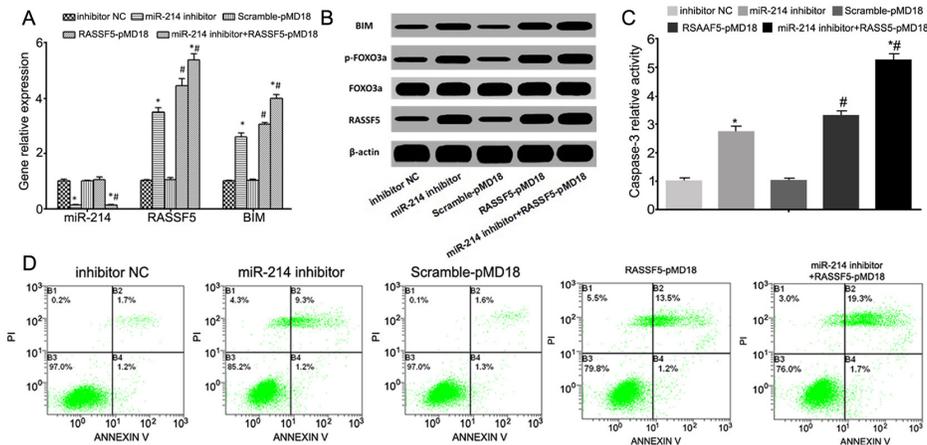
Target gene prediction using the microRNA.org resource showed a complementary binding site between miR-214 and the 3'-UTR of *RASSF5* mRNA (Figure 2A). To confirm this relationship further, dual luciferase reporter assay was performed, and the results showed that miR-214 mimic or inhibitor transfection significantly reduced or enhanced, respectively, the relative luciferase activity in HEK293 cells (Figure 2B), suggesting that *RASSF5* is the target gene of miR-218. Meanwhile, no luciferase activity was observed when the 3'-UTR of *RASSF5* was mutated (Figure 2B), indicating that miR-214 has the ability to directly bind to *RASSF5*. MiR-214 mimic or inhibitor transfection markedly decreased or elevated, respectively, the *RASSF5* mRNA (Figure 2C) and protein (Figure 2D) expression levels in KB cells, further verifying that miR-214 may suppress *RASSF5* expression levels by binding to the 3'-UTR of *RASSF5*.

### Reducing miR-214 expression promoted KB cell apoptosis

MiR-214 inhibitor and/or *RASSF5* overexpression plasmid transfection enhanced *RASSF5* mRNA and protein expression levels (Figure 3A and B), FOXO3a phosphorylation (Figure 3B), downstream *BIM* expression (Figure 3A and B), and caspase-3 activity (Figure 3C) and triggered cell apoptosis (Figure 3D) in KB cells.



**Figure 2.** MiR-214 regulated *RASSF5* expression in oral cancer KB cell line. **A.** The complementary binding site between miR-214 and the 3'-UTR of *RASSF5* mRNA. **B.** Dual luciferase reporter assay using wild type and mutated *RASSF5* (3'-UTR was mutated). **C.** qRT-PCR detection of miR-214 and *RASSF5* expression. **D.** Western blot detection of *RASSF5* expression. \* $P < 0.05$ , compared with mimic NC. # $P < 0.05$ , compared with inhibitor NC.



**Figure 3.** Reducing miR-214 expression promoted KB cell apoptosis. **A.** qRT-PCR detection of miR-214, *RASSF5*, and *BIM* expression. **B.** Western blot detection of protein expression. **C.** Spectrophotometric detection of caspase-3 activity. **D.** Flow cytometric detection of cell apoptosis. \* $P < 0.05$ , compared with inhibitor NC. # $P < 0.05$ , compared with scramble-pMD18.

## DISCUSSION

Oral cancer is a common cancer of the head and neck, and squamous cell carcinoma is its main histopathological type. It may invade surrounding tissues as invasive growth, and metastasis to the cervical lymph nodes occurs easily. Smoking and alcohol consumption, especially (Zygianni et al., 2011), among other factors such as viral infections, malnutrition, poor diet are reportedly involved in the incidence of esophageal cancer (Sankaranarayanan et al., 2015). The incidence of oral cancer accounts for 1.9-3.5% of systemic malignant tumors and 4.7-20.3% of head and neck malignant tumors, second only to nasopharyngeal carcinoma. More than 399,000 new cases of oral cancer are reported every year (Ferlay et al., 2015), along with a mortality rate of 22.8/100,000 (Johnson et al., 2011). Surgical resection is still the main approach adopted in the treatment of oral cancer. However, due to inconspicuous symptoms in the early stage of disease, most patients are diagnosed with oral cancer at the middle-late stages of disease progression and obtaining satisfactory clinical results by simple surgical treatment is difficult. In addition, because of the complicated anatomy of the disease, the tumor cannot be resected radically. The recurrence rate of oral cancer has been reported to be as high as 35% (Hsieh et al., 2012). In spite of the progress obtained in comprehensive treatment strategies such as surgery, radiotherapy, and chemotherapy, the survival and prognosis of oral cancer appear to be disappointing with lower survival rates in patients with lymph node metastasis (Koloutsos et al., 2014).

The small GTP-binding protein Ras is an important component of the growth and differentiation signal pathways which are activated by cell surface receptors. Once activated, Ras can initiate the activation of different signaling cascades and participate in the regulation of a variety of biological processes, including cell growth, survival, migration, apoptosis resistance, differentiation, and malignant transformation (Vos et al., 2003). In addition, activated Ras also plays a critical role in maintaining cell homeostasis by regulating cell aging, differentiation, cycle arrest, and apoptosis (Vos et al., 2003). *RASSF5* is a member of the Ras receptor family located on chromosome 1q32.1 (Donninger et al., 2016) and contains a Ras association domain and a C-terminal SARAH domain (Salvador, Rassa, Hippo domain). *RASSF5*, a tumor suppressor gene, was found to be down-regulated in multiple tumor tissues and cell lines in cancers (Hesson et al., 2003; Lee et al., 2010) such as osteosarcoma (Zhou et al., 2014), esophageal cancer (Guo et al., 2015), lung cancer (Hesson et al., 2003), liver cancer (Calvisi et al., 2009), and gastric cancer (Han et al., 2015). In this study, we showed that *RASSF5* expression was significantly decreased in oral cancer tissues, consistent with results of a previous study showing down-regulation of *RASSF5* expression in 54 cases of head and neck cancer patients, including oral cancer (Steinmann et al., 2009). Moreover, we also observed elevated expression levels of miR-214 in oral cancer tissues, consistent with another study (Scapoli et al., 2010). Unsurprisingly, miR-214 levels were reported to be significantly elevated in cisplatin resistant tongue squamous carcinoma cell line Tca/cisplatin compared to those in cisplatin sensitive Tca8113, indicating the role of miR-214 in tumor promotion in oral cancer (Yu et al., 2010).

*RASSF5* has been reported to have a role in inhibiting cell proliferation and promoting cell apoptosis (Zhou et al., 2014). To determine whether aberrant expression of *RASSF5* could affect the apoptosis of oral cancer cells, *RASSF5* overexpression plasmid was constructed, and the results showed that *RASSF5* overexpression significantly increased oral cancer cell apoptosis. Similar results were also obtained for cells transfected with the miR-214 inhibitor. It

has been demonstrated previously in a study that FOXO3a and BIM are involved in the *RASSF5*-induced cell apoptosis (Rahmani et al., 2009). Therefore, we also measured the expression of FOXO3a and *BIM* after *RASSF5* overexpression or miR-214 down-regulation and showed that FOXO3a phosphorylation and *BIM* expression were markedly enhanced. Therefore, miR-214 regulates cell apoptosis through suppressing *RASSF5*, which inhibits FOXO3a phosphorylation and *BIM* expression. The exact mechanism of *RASSF5* down-regulation in oral cancer remains unclear, though some other studies have demonstrated that hyper-methylation of the *RASSF5* promoter might be involved in the down-regulation of *RASSF5* in several cancers (Hesson et al., 2003; Volodko et al., 2014). However, the hyper-methylation of *RASSF5* promoter was not measured in our study and is the main study limitation. Further investigations on the epigenetic regulation of *RASSF5* in cancers are required.

## CONCLUSION

Our study demonstrated that miR-214 expression was elevated and *RASSF5* was down-regulated in oral cancer. Moreover, miR-214 suppressed KB cell apoptosis through down-regulation of *RASSF5* expression and subsequent inhibition of FOXO3a phosphorylation and *BIM* expression, suggesting that miR-214 could be used as a novel therapeutic target in the treatment of oral cancer.

## Conflicts of interest

The authors declare no conflict of interest.

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