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MiR-214 promotes proliferation and inhibits apoptosis of oral cancer cells through MAPK/ERK signaling pathway

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Abstract. – OBJECTIVE: To study the influences of micro ribonucleic acid (miR)-214 on the proliferation and apoptosis of oral cancer cells through the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway.

MATERIALS AND METHODS: In this work, human oral cancer HB cell lines were cultured *in vitro* and then treated with phosphate-buffered saline (PBS) as Control group, with miR-214 mimics as miR-214 mimics group or with miR-214 mimics + ERK inhibitor U0126 as miR-214 mimics + U0126 group. The messenger RNA (mRNA) levels of miR-214 and ERK were determined using quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), and the protein expression levels of phosphorylated ERK (p-ERK), ERK, proliferating cell nuclear antigen (PCNA), p21, and tubulin were measured *via* Western blotting (WB). Besides, the proliferation and apoptosis of cells in each group were evaluated *via* methyl thiazolyl tetrazolium (MTT) assay and Hoechst staining, respectively.

RESULTS: Compared with Control group, miR-214 mimics group exhibited increased expression of miR-214 in oral cancer cells ($p<0.01$), extremely raised expression levels of p-ERK and PCNA, but an extremely decreased protein expression level of p21 ($p<0.01$), whereas miR-214 mimics + U0126 group had remarkably lower levels of p-ERK and PCNA, and a considerably higher protein level of p21 than miR-214 mimics group ($p<0.05$). The qRT-PCR results showed no significant differences in the mRNA level of ERK among the three groups ($p>0.05$). In addition, the proliferation ability was enhanced successively in Control group, miR-214 mimics + U0126 group and miR-214 mimics group, and the increase was more notable in miR-214 mimics group, with statistically significant differences ($p<0.05$). Finally, it was found through the Hoechst apoptosis assay that compared with that in Control group, the cell apoptosis was notably inhibited in miR-214 mimics group, and it was greatly increased in miR-214 mimics + U0126 group.

CONCLUSIONS: MiR-214 increases p-ERK level and p-ERK/ERK to activate the MAPK/ERK signaling pathway, raise PCNA level, and decrease p21 level, thereby promoting cell proliferation and inhibiting cell apoptosis.

Key Words:

Oral cancer, MiR-214, MAPK/ERK signaling pathway..

Introduction

Micro ribonucleic acids (miRNAs) are a kind of endogenous non-coding single-stranded small-molecule RNAs, generally measuring 19-22 nt in length, and they regulate the expression of target genes at the transcription level[1,2]. MiRNA molecules were first discovered in Caenorhabditis elegans in the 1920s, and as the research has been deepened, various miRNA molecules have also been found in drosophila, zebrafish, mammals, and human body[3]. Besides, these molecules directly degrade messenger RNAs (mRNAs) or inhibit translation mainly through complementary base pairing with the 3' un-translated region (3'UTR) of the target gene, thereby modulating the gene expression at the transcription level. MiRNAs play vital roles not only in the multiple processes in organisms, such as cell proliferation, differentiation, apoptosis, and aging, but also in the development and progression of tumors[4-6].

MiR-214, an osteoclast-derived miRNA molecule, can suppress bone formation[7]. According to the study findings, miR-214 is associated with the formation of such tumors as breast cancer, ovarian cancer, gastric cancer, and colon cancer and involved in tumor cell proliferation, apoptosis, and migration. MiR-214 can regulate the development of lung cancer through the miR-214-FGFR1-Wnt/mitogen-activated protein kinase (MAPK)/

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Akt signaling pathway[8]. However, there have been no relevant reports on whether miR-214 is related to the development of oral cancer now.

MAPK mainly involves phosphorylated c-Jun N-terminal kinase (p-JNK), p-p38, and p-extracellular signal-regulated kinase (ERK) signaling pathways. The MAPK/ERK signaling pathway is important and ubiquitous in inferior and superior creatures and participates in regulating cell proliferation, differentiation, and migration. It has been found in studies that the aberrantly active ERK signaling pathway increases cell proliferation and migration, but inhibits cell apoptosis[9-11]. Therefore, the present study explored whether miR-214 can affect the proliferation and apoptosis of oral cancer cells through the MAPK/ERK signaling pathway.

Materials and Methods

Materials

Human oral cancer HB cell lines (China Cell Resource Seed Bank, Shanghai, China), cell radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China), bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA), polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), p-ERK, ERK, p21, cell nuclear antigen (PCNA) and tubulin monoclonal primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies (CST, Danvers, MA, USA), TRIzol (Invitrogen, Carlsbad, CA, USA) and complementary deoxyribonucleic acid (cDNA, TaKaRa, Otsu, Shiga, Japan).

Cell Culture, Treatment and Grouping

The human oral cancer HB cell line was cultured using Roswell Park Memorial Institute-1640 (RPMI-1640) complete medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 0.1% P/S double antibodies and 0.1% glutamine in an incubator with 5% CO_2 at 37°C in accordance with the standard operation manual, in which aseptic operation was required to avoid contamination. Then, HB cells were sub-cultured in a 6-well plate, and when growing to 75% density, the cells in 3 wells were added with phosphate-buffered saline (PBS), miR-214 mimics and miR-24 mimics + U0126 separately to harvest cells in Control group, miR-214 mimics group and miR-214 mimics + U0126 group. Then, the three groups of cells were used for detection in subsequent experiments.

Detection of Protein Expression Via Western Blotting (WB)

The three groups of cells in the 6-well plate were added with lysis buffer, lysed on a shaking table at 4°C for 30 min, and centrifuged at 12,000 g and 4°C for 10 min. Next, the supernatant was collected. The concentration of total proteins was determined with reference to the instructions of the BCA protein assay kit. Then, 10 μg of protein samples were added into loading buffer, and the mixture was boiled at 98°C for 10 min, followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins were transferred onto a PVDF membrane, and the membrane was washed using Tris-Buffered Saline with Tween-20 (TBST) for 10 min × 3 times. After that, the membrane was incubated with p-ERK, ERK, p21, PCNA, and tubulin monoclonal primary antibodies overnight at 4°C, and washed again using TBST for 10 min × 3 times, followed by incubation with the horseradish peroxidase-conjugated secondary antibodies for 1 h. Subsequently, the resulting membrane was rinsed using TBST as above. Finally, enhanced chemiluminescence (ECL) mixture was added, and protein bands were acquired using the fluorescence development technique.

Determination of MiR-214 Expression Level Via Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from the cells in Control group, miR-214 mimics group, and miR-214 mimics + U0126 group using TRIzol and then reversely transcribed into complementary deoxyribose nucleic acids (cDNAs). The primer sequences of miR-214 and the internal reference gene U6 are listed as follows: miR-214: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTGCC-3' and U6: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAA TATGGAAC-3'. Subsequently, real-time PCR was performed using SYBR Green dye (TaKaRa, Otsu, Shiga, Japan). Reaction procedures: pre-denaturalization at 95°C × 10 min, denaturalization at 95°C × 1 min, annealing at 60°C × 34 s, and extension at 74°C × 30 s, using the primer sequences shown in Table I. Finally, the relative

Table I. List of real-time PCR primer sequences.

Name of gene	Forward (5′-3′)	Reverse (5′-3′)
MiR-214	GGACAGCAGGCACAGACA	CAGTGCAGGGTCCGAGGT
U6	TGCGGGTGCTCCGCTTCGGCAGC	CAGTGCAGGGTCCGAGGT

expression level of miRNAs was calculated based on the amplification cycle threshold (Ct).

Measurement of ERK MRNA Expression Level Via QRT-PCR

Total RNAs were extracted from the three groups of cells using TRIzol. Then, the concentration of the total RNAs was measured. Subsequently, the mRNAs were reversely transcribed into cDNAs according to TaKaRa's specifications. Specifically, 500 ng of RNAs were added into the mixture of 2 μL of 5 × PrimeScript RT Master Mix, 0.5 μL of OligodT, and 0.5 μL of reverse transcriptases to obtain an overall reaction system (10 μL), followed by PCR amplification. According to the instructions, the reaction system consisted of 2 μL of cDNAs , 10 μL of SYBR Premix Ex TaqII (2×), 0.8 μL each of forward and reverse primers, and 0.4 μL of ROX reference dye II (50×). Finally, ddH_2O was added to make the system reach 20 μL. The relative expression level of mRNAs was calculated based on the Ct value, with β-actin as an internal reference. The primer sequences are shown in Table II.

Detection of Cell Proliferation Via Methyl Thiazolyl Tetrazolium (MTT) Assay

The three groups of cells in the 6-well plate were digested using 0.25% trypsin, and paved onto a 96-well plate. At 24 and 48 h after the cells covered the whole plate, they were added with MTT powder and reacted for 3 h. Then, with the medium removed, the cells in each well were added with 200 μL of dimethyl sulfoxide (DMSO) and placed at 37°C. After 30 min, the optical density (OD) of cells at the wavelength of 490 nm was read using a microplate reader. The OD value is positively correlated with the number of cells. In other words, the larger OD indicates more cells and vice versa. The proliferation ability of cells in the three groups was determined using the above method.

Evaluation of Cell Apoptosis Using Hoechst Staining

The human oral cancer HB cells were fixed in paraformaldehyde and stained with 1 μg/mL of Hoechst 33342, a cell membrane-permeable dye. Then, the cells were observed under an Olympus fluorescence microscope. Next, the apoptotic cells were identified based on nuclear condensation and fragmentation, and their percentage in the total was calculated. Five stained cell fields at least were randomly selected for each sample.

Statistical Analysis

GraphPad Prism 6.0 software (La Jolla, CA, USA) was used for statistical analysis of data, and all the results were expressed as mean ± standard error of the mean (SEM), and analyzed using two-tailed *t*-test. $p<0.05$ denoted a statistically significant difference.

Results

Overexpression of MiR-214 in Human Oral Cancer HB Cell Lines Detected

The human oral cancer HB cell lines were first treated with PBS or miR-214 mimics to form Control group and miR-214 mimics group. It was found through qRT-PCR that miR-214 mimics group exhibited increased expression of miR-214 in the HB cell lines compared with Control group (Figure 1), suggesting that the miR-214 overexpression model was established successfully.

Table II. List of gene primer sequences

Name of gene	Forward (5′-3′)	Reverse (5′-3′)
ERK	AGAGTTGAAGGATGATGACT	CACTCATGCAGCACCTGCAG
β-actin	GCAGAAGGAGATTACTGCCCT	GCTGATCCACATCTGCTGGAA

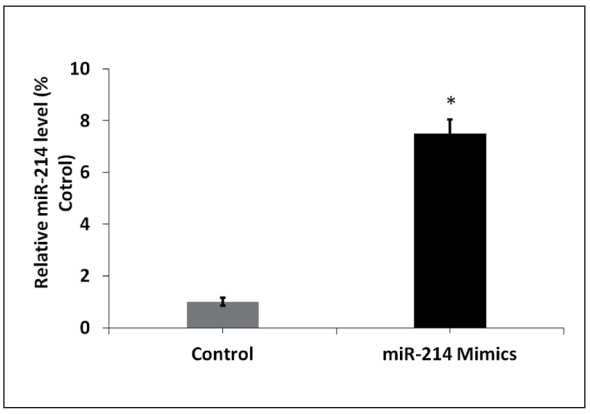

Figure 1. Detection of miR-214 lentivirus-mediated overexpression. The expression of miR-214 is detected *via* qRT-PCR, and data are presented as mean ± SEM. *p<0.01: miR-214 mimics group *vs.* Control group.

Influence of MiR-214 on the MAPK/ERK Signaling Pathway

HB cells in miR-214 mimics group were treated using the MAPK/ERK pathway inhibitor U0126 as miR-214 mimics + U0126 group. The protein expression levels of p-ERK, ERK, and tubulin in each group of cells were measured *via* WB. The results revealed that the p-ERK or p-ERK/ERK was extremely raised in miR-214 mimics group compared with that in Control group (p<0.01), whereas miR-214 mimics + U0126 group exhibited a significant decline in p-ERK or p-ERK/ERK in comparison with miR-214 mimics group (Figure 2), implying that miR-214 mimics can activate the MAPK/ERK signaling pathway, and such activation can be suppressed by U0126.

Effect of MiR-214 on ERK mRNA Level

Subsequently, mRNAs were extracted from each group, and detected using qRT-PCR. The results revealed that there were no statistically significant differences (N.S.) in ERK mRNA level among the three groups (Figure 3), illustrating that miR-214 mimics decrease the level of p-ERK to suppress the MAPK/ERK signaling pathway, while miR-214 mimics can activate this pathway, not due to the differential expression of ERK mRNA.

Influences of MiR-214 on the Proliferation-Associated Protein Levels

The protein expression levels of p21 and PCNA in each group of cells were measured using WB, and the results showed that miR-214 mimics group had an extremely lower level of p21 and an extremely higher level of PCNA than Control group (p<0.01), while compared with those in miR-214 mimics group, the levels of p21 and PCNA were considerably elevated and decreased, respectively, in miR-214 mimics + U0126 group (p<0.05; Figure 4). These suggest that miR-214 mimics promote the proliferation of oral cancer cells, which is suppressed by U0126.

Impact of MiR-214 on Cell Proliferation

The proliferation of the differently treated cells in each group was evaluated *via* MTT assay in this study. It was discovered that the number of proliferating cells at 24 and 48 h in miR-214 mimics group was remarkably larger than that in Control group. Compared with that in miR-214 mimics group, the proliferation ability of cells was weakened at 48 and 72 h in miR-214 mimics

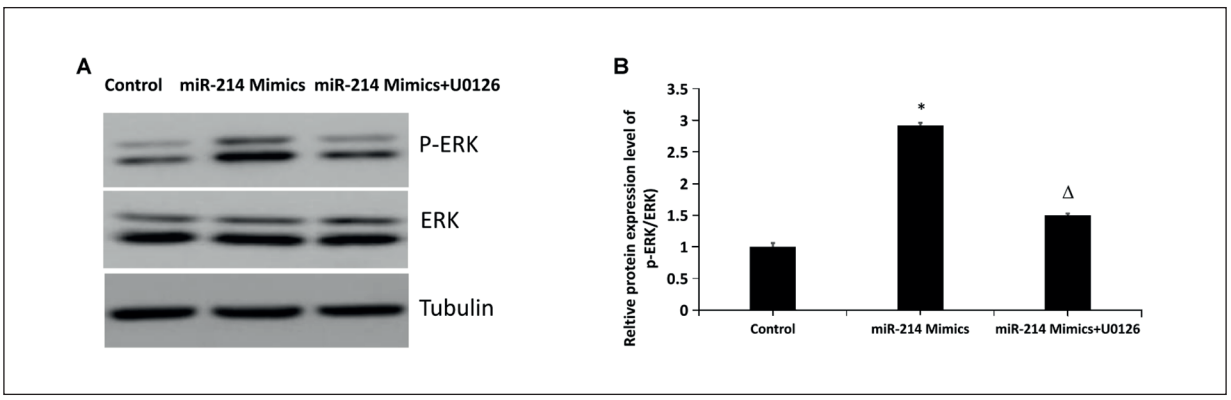

Figure 2. Influence of miR-214 on the MAPK/ERK signaling pathway. **A,** Protein levels of p-ERK, ERK and tubulin determined *via* WB. **B,** P-ERK/ERK value. Data are presented as mean ± SEM. *p<0.01: miR-214 mimics group *vs.* Control group. $^{\Delta}p$<0.05: miR-214 mimics + U0126 group *vs.* miR-214 mimics group.

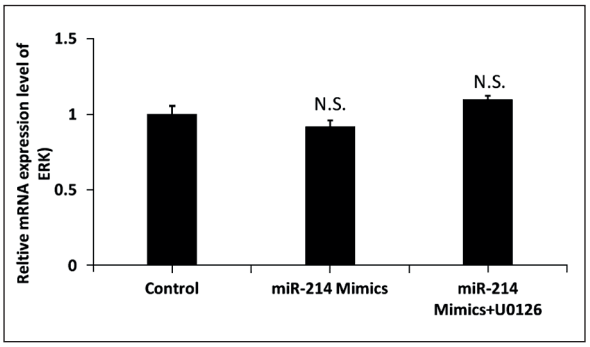

Figure 3. Effect of miR-214 on ERK mRNA level. The expression level of ERK mRNA is determined using qRT-PCR, and data are presented as mean ± SEM. N.S.: there are no statistically significant differences among the three groups.

+ U0126 group (Figure 5), implying that miR-214 mimics can enhance the proliferation ability of oral cancer cells, which can be repressed by the MAPK/ERK pathway inhibitor U0126, consistent with the WB results.

Influence of MiR-214 on Oral Cancer Cell Apoptosis

The cell apoptosis was evaluated using Hoechst staining, and it was found that miR-214 mimics group exhibited an extremely considerable decline in the number of apoptotic cells compared with Control group, whereas the cell proliferation was increased in miR-214 mimics + U0126 group compared with that in miR-214 mimics group (Figure 6). It can be seen that miR-214 mimics can inhibit the apoptosis of oral cancer cells, and such an anti-apoptosis effect can be repressed by the MAPK/ERK pathway inhibitor U0126.

Discussion

MiRNAs, a kind of endogenous non-coding single-stranded small-molecule RNAs with a length of 19-22 nt[12], are mainly involved in regulating transcription and suppressing the expression of the target gene by binding to the 3'UTR of the target gene. The current studies have reported that the maturation of miRNAs involves the following two steps: Drosha and DGCR8 proteins first process nuclear pri-RNA molecules to 70 nt-long pre-miRNAs, and then the pre-miRNAs are transported by the exportin5/RanGTP complex from the nucleus to the cytoplasm, in which they are further processed into mature miRNA molecules with a length of about 21 nt under the action of Dicer enzyme[13,14]. Later, miRNA molecules form a silencing complex with the mRNA of the target gene to inhibit or degrade the protein expression of the target gene[15,16].

MiR-214, a widely studied miRNA molecule, is derived from osteoclasts and can suppress bone formation. According to the findings in the current studies, miR-214 is associated with the formation of several human tumors. Notably, miR-214 inhibits the epithelial-mesenchymal transition by down-regulating RNF8 in breast cancer[17], and it targets PDK2 and PHF6 to repress cell proliferation, migration, and metabolism in liver cancer[18]. All the above reports suggest that miR-214 is negatively correlated with the development of cancers, but studies have also demonstrated that miR-214 is positively associated with the development of cancers. MiR-214 activates the MAPK signaling pathway to induce drug resistance to radiotherapy for non-small cell lung cancer. However, there have

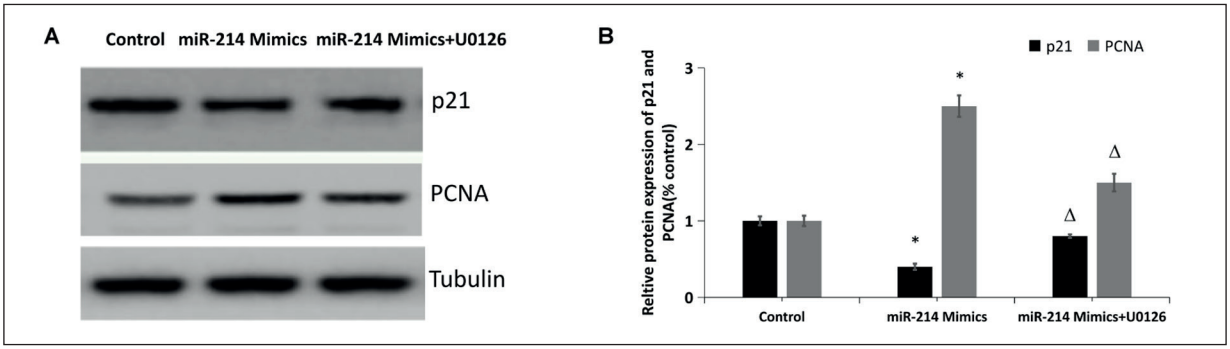

Figure 4. Influences of miR-214 on the proΔΔliferation-associated expression. **A,** Protein expressions of p21 and PCNA in each group determined using WB. **B,** Quantification of results in Figure a. Data are presented as mean ± SEM. *p<0.01: miR-214 mimics group *vs.* Control group. $^{\Delta}p$<0.05: miR-214 mimics + U0126 group *vs.* miR-214 mimics group.

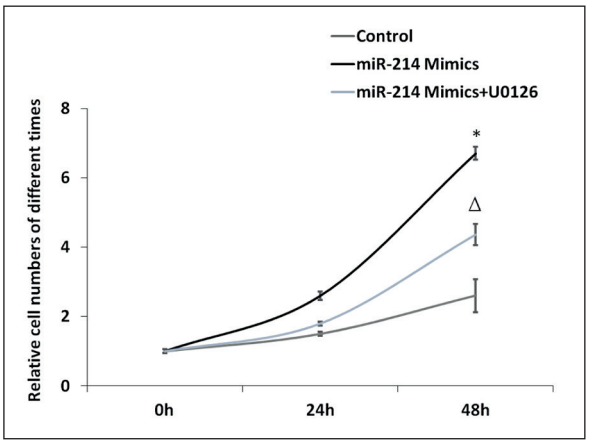

Figure 5. Impact of miR-214 on cell proliferation in each group. The number of cells at 0, 24, and 48 h is measured *via* MTT assay in each group, and the proliferation is compared. Data are presented as mean ± SEM. *p<0.01: miR-214 mimics group *vs.* Control group. $^{\Delta}p$<0.05: miR-214 mimics + U0126 group *vs.* miR-214 mimics group.

been no relevant reports that verify whether miR-214 is related to the development of oral cancer now.

MAPKs belong to a class of Ser/Thr protein kinases in cells[19]. Multiple parallel MAPK signaling pathways have been discovered in the cells of inferior prokaryotes, and superior mammalians through studies, mainly including the p-JNK, p-p38, and p-ERK signaling pathways, and they mediate different cellular biological responses. The MAPK/ERK signaling pathway is an important one that plays a vital role in various cell processes, such as proliferation, migration, and apoptosis[20].

Therefore, the present study explored the influences of miR-214 on the proliferation and apoptosis of oral cancer cells through the MAPK/ERK signaling pathway. Human oral cancer HB cell lines were cultured *in vitro* and treated with PBS as Control group, miR-214 mimics as miR-214 mimics group or miR-214 mimics + ERK inhibitor U0126 as miR-214 mimics + U0126 group. In this study, the overexpression of miR-214 in oral cancer HB cells was first analyzed *via* qRT-PCR, and the results suggest that miR-214 mimics can overexpress miR-214. Then, the WB results showed that compared with those in Control group, the level of p-ERK in miR-214 mimics group was increased, and the MAPK/ERK signaling pathway was activated in miR-214 mimics group, but the activation was repressed by U0126. This study only investigated the influence of miR-214 on the ERK signaling pathway,

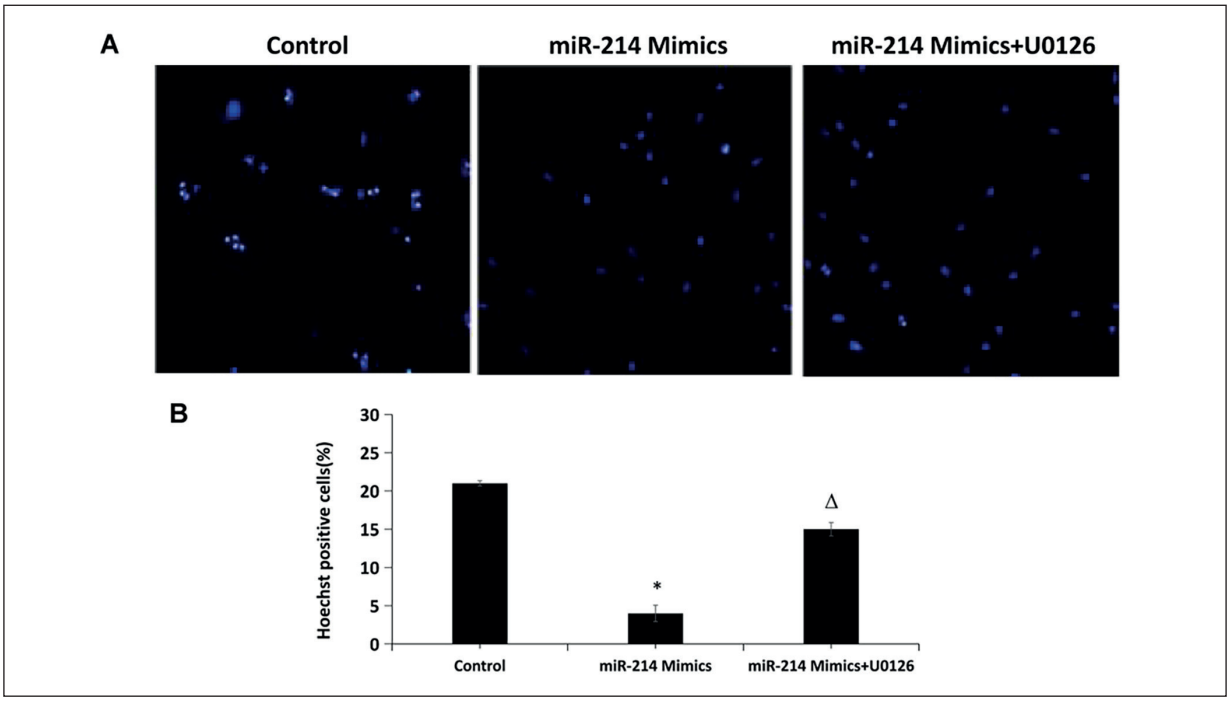

Figure 6. Influence of miR-214 on oral cancer cell apoptosis in each group. **A,** Cell apoptosis detected *via* Hoechst staining (magnification: 100×). **B,** Quantification of results in Figure A. Data are presented as mean ± SEM. *p<0.01: miR-214 mimics group *vs.* Control group. $^{\Delta}p$<0.05: miR-214 mimics + U0126 group *vs.* miR-214 mimics group.

not on the p-JNK and p38 signaling pathways. Moreover, it was found through the mRNA assay that different treatments had no significant influences on ERK mRNA. Besides, based on the WB results, miR-214 mimics group had an extremely higher level of PCNA protein and an extremely lower level of P21 protein than Control group, whereas PCNA protein was decreased and p21 protein was increased to inhibit cell proliferation in miR-214 mimics + U0126 group. Afterwards, the proliferation ability of cells in each group was detected *via* MTT assay, and the results showed that the proliferation ability of cells in miR-214 mimics group was enhanced compared with that in Control group, while the cell proliferation was inhibited in miR-214 mimics + U0126 group, which accorded with the WB results. Finally, it was found through the apoptosis assay that compared with that in Control group, the cell apoptosis was inhibited in miR-214 mimics group, but promoted in miR-214 mimics + U0126 group.

Conclusions

In summary, miR-214 increases p-ERK level and p-ERK/ERK to activate the MAPK/ERK signaling pathway, raises PCNA level, and decreases p21 level, thereby promoting cell proliferation and inhibiting cell apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Cai Y, Yu X, Hu S, Yu J. A brief review on the mechanisms of miRNA regulation. Genomics Proteomics Bioinformatics 2009; 7: 147-154.
2) Masliah-Planchon J, Garinet S, Pasmant E. RAS-MAPK pathway epigenetic activation in cancer: miRNAs in action. Oncotarget 2016; 7: 38892-38907.
3) Hammond SM. An overview of microRNAs. Adv Drug Deliv Rev 2015; 87: 3-14.
4) Wu G, Yu W, Zhang M, Yin R, Wu Y, Liu Q. MicroRNA-145-3p suppresses proliferation and promotes apotosis and autophagy of osteosarcoma cell by targeting HDAC4. Artif Cells Nanomed Biotechnol 2018; 46: 579-586.
5) Miao J, Wang W, Wu S, Zang X, Li Y, Wang J, Zhan R, Gao M, Hu M, Li J, Chen S. MiR-194 suppresses proliferation and migration and promotes apoptosis of osteosarcoma cells by targeting CDH2. Cell Physiol Biochem 2018; 45: 1966-1974.
6) Yin Z, Ding H, He E, Chen J, Li M. Up-regulation of microRNA-491-5p suppresses cell proliferation and promotes apoptosis by targeting FOXP4 in human osteosarcoma. Cell Prolif 2017; 50. doi: 10.1111/cpr.12308.
7) Hrdlicka HC, Lee SK, Delany AM. MicroRNAs are critical regulators of osteoclast differentiation. Curr Mol Biol Rep 2019; 5: 65-74.
8) Yang Y, Li Z, Yuan H, Ji W, Wang K, Lu T, Yu Y, Zeng Q, Li F, Xia W, Lu S. Reciprocal regulatory mechanism between miR-214-3p and FGFR1 in FGFR1-amplified lung cancer. Oncogenesis 2019; 8: 50.
9) Sun Y, Liu WZ, Liu T, Feng X, Yang N, Zhou HF. Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. J Recept Signal Transduct Res 2015; 35: 600-604.
10) Wang G, Sun J, Liu G, Fu Y, Zhang X. Bradykinin promotes cell proliferation, migration, invasion, and tumor growth of gastric cancer through ERK signaling pathway. J Cell Biochem 2017; 118: 4444-4453.
11) Tanimura S, Takeda K. ERK signalling as a regulator of cell motility. J Biochem 2017; 162: 145-154.
12) Vishnoi A, Rani S. MiRNA biogenesis and regulation of diseases: an overview. Methods Mol Biol 2017; 1509: 1-10.
13) Ha M, Kim VN. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 2014; 15: 509-524.
14) Treiber T, Treiber N, Meister G. Regulation of microRNA biogenesis and function. Thromb Haemost 2012; 107: 605-610.
15) Kobayashi H, Tomari Y. RISC assembly: coordination between small RNAs and Argonaute proteins. Biochim Biophys Acta 2016; 1859: 71-81.
16) Kim Y, Kim VN. MicroRNA factory: RISC assembly from precursor microRNAs. Mol Cell 2012; 46: 384-386.
17) Min L, Liu C, Kuang J, Wu X, Zhu L. MiR-214 inhibits epithelial-mesenchymal transition of breast cancer cells via downregulation of RNF8. Acta Biochim Biophys Sin (Shanghai) 2019; 51: 791-798.
18) Yu Q, Zhou J, Jian Y, Xiu Z, Xiang L, Yang D, Zeng W. MicroRNA-214 suppresses cell proliferation and migration and cell metabolism by targeting PDK2 and PHF6 in hepatocellular carcinoma. Cell Biol Int. 2019 Jul 22. doi: 10.1002/cbin.11207. [Epub ahead of print]
19) Chen SX, Zhao F, Huang XJ. [MAPK signaling pathway and erectile dysfunction]. Zhonghua Nan Ke Xue 2018; 24: 442-446.
20) Xiong T, Zhang Z, Zheng R, Huang J, Guo L. N-acetyl cysteine inhibits lipopolysaccharide-induced apoptosis of human umbilical vein endothelial cells via the p38MAPK signaling pathway. Mol Med Rep 2019; 20: 2945-2953.