miR-497 promotes the progression of cutaneous squamous cell carcinoma through FAM114A2

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Abstract. – OBJECTIVE: To explore the possible role and mechanism of miR-497 in cutaneous squamous cell carcinoma.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect miR-497 and FAM114A2 expression level in 38 cases of cutaneous squamous cell carcinoma (CSCC) and 22 normal skin tissues as well as in CSCC cell lines (A431, HSC-5) and normal cells (HaCaT). MiR-497 effects on cell proliferation and cell cycle were examined by CCK8 assays and flow cytometry. Dual luciferase reporter gene assay was performed to detect the regulating relationship between miR-497 and FAM114A2. In addition, the expression of FAM114A2 after overexpression or knockdown of miR-497 was detected by Western blot to evaluate whether miR-497 could regulate proliferation and cell cycle by regulating the expression of FAM114A2.

RESULTS: MiR-497mRNA expression in CSCC tissues and cell lines was markedly lower than that in normal tissues and cells. Meanwhile, FAM114A2 mRNA and protein levels in CSCC tissues were markedly higher when compared to than that in normal tissues. miR-497 overexpression or knockdown could inhibit or promote the cell proliferation and cell cycle of A431, HSC-5. The dual luciferase reporter gene assay suggested that FAM114A2 might be a direct target gene of miR-497, and that FAM114A2 expression had a significant negative correlation with miR-497. Overexpression of miR-497 could inhibit FAM114A2 protein expression. Besides, FAM114A2 knockdown reversed the inhibitory effect of low expression of miR-497 on proliferation rate of A431 or HSC-5 cells.

CONCLUSIONS: MiR-497 was lowly expressed in squamous cell carcinoma tissues and cells, which can participate in the regulation of cell proliferation through FAM114A2, thus promoting the progression of CSCC.

Key Words: Cutaneous squamous cell carcinoma, miR-497, Cell proliferation, FAM114A2.

Introduction

Cutaneous squamous cell carcinoma (CSCC) is one of the most common skin malignant tumors, whose incidence is increasing year by year¹.². CSCC often occurs in the exposed parts of the skin, where the cancer cells tend to have different degrees of keratinization. It is characterized by recurrence, lymph node and distant metastasis. At present, the main treatment of CSCC includes Mohs microsurgery, chemotherapy, photodynamic therapy, radiation therapy, biological therapy and immunotherapy. Early CSCC can be surgically removed, but the prognosis is poor after metastasis has occurred³. The pathogenesis of CSCC is complex and the concerning genes are still unclear. Afaq et al⁴ have shown that CSCC is associated with UV radiation, chemical carcinogens, human papillomavirus infection, chronic ulcers, and certain skin diseases. The relevant gene pathways currently analyzed include TP53 pathway, NOTCH pathway, RAS pathway, EGFR pathway, SRC family kinase pathway (SKF), CDKN2A pathway, NF-KB pathway, TGF-β pathway and KNSTRN pathway⁵. Therefore, the investigations of the pathogenesis of CSCC and the identification of key molecules in its pathogenesis have become the focus of skin tumor research. MiRNAs are a series of small non-coding single-stranded RNAs ranging from 18 to 25 nt⁶. miRNAs can bind to target mRNA by partial complementa-
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tion to degrade or inhibit post-transcriptional translation to regulate target gene expression, thereby regulating biological behavior such as cell proliferation and migration. Darido et al. have shown that miRNAs are involved in the development of CSCC. For instance, miR-21 expression was significantly increased in CSCC and the miR-21 oncogene could target the action of Grhl3 and PTEN to amplify PI3K/AKT/mTOR signaling to induce CSCC. miR-181a can inhibit cell proliferation by targeting the proto-oncogene KRAS resulting in slow down cutaneous squamous cell carcinoma. In addition, miR-365 can inhibit cell apoptosis by inhibiting BAX expression to promote the progression of cutaneous squamous cell carcinoma. As a tumor suppressor gene for tumors such as rectal cancer, breast cancer and NSCLC (non-small cell lung cancer), miR-497 can affect the expression of Nrdp1, cyclin E1 and insulin-like growth factor 1 receptor (IGF1R) to promote tumor cell growth, migration, invasion and apoptosis. However, the specific mechanism of miR-497 in regulating the progression of cutaneous squamous cell carcinoma remains to be explained. This research was to investigate the effects of miRNA-497 on the proliferation and cell cycle of CSCC and to verify whether miR-497 can exert its biological functions by targeting FAM114A2 expression.

Patients and Methods

Patients

38 cases of CSCC tissue specimens were derived from specimens of CSCC patients in the Third Hospital of Ji’nan from October 2015 to October 2017. 22 cases of normal skin tissue specimens were removed by orthopedic surgery. Patients were newly diagnosed with typical lesions and confirmed by histopathology as CSCC. Patients had no distant organ metastasis, no autoimmune disease and history of important organ dysfunction, and had not received anti-tumor treatment such as radiotherapy, chemotherapy or immunotherapy. All of the above tissue samples were collected after patient consent and the study was approved by the Ethics Committee.

Cell Culture and Transfection

Normal skin cells (HaCaT) and skin squamous cells (A431, HSC-5) were purchased from ATCC (American Type Culture Collection) (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% inactivated newborn bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin in a 37°C, 90% relative humidity, 5% CO₂ incubator. Cells reaching about 50% to 60% confluency were used for transfection by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The si-FAM114A2, miR-497 mimic/inhibitor and the corresponding negative controls were transfected for further analysis.

Cell Counting Kit-8 (CCK-8) Assay

24 h after transfection, cells were digested and inoculated into 96-well plates at 4*10³ per well. 6 replicate wells were set. The viability of the cells was determined by CCK-8 (Dojindo, Kumamoto, Japan) at 0 h, 24 h, 48 h, and 72 h after seeding, respectively. 2 h before the test, 10 µL of CCK-8 solution was added and then cells were placed at 37°C for 2 h. The absorbance at 450 nm was measured by a microplate reader.

Cell Cycle

Cells treated differently for 24 hours were routinely digested into single cell suspensions with phosphate-buffered saline (PBS) and 70% alcohol (pre-cooled) was added overnight. After that, cells were centrifuged with PBS for about 8 minutes to discard the supernatant.100 µL of RNase A were added; cells were stained with 400 µL of propidium iodide (PI) staining solution at 37°C for 30 min in the dark. Cell cycle was detected by recording the red fluorescence intensity of the flow cytometer at wavelength of 488 nm.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total RNA of the cells or tissues was extracted using a TRizol kit (Invitrogen, Carlsbad, CA, USA), and a reverse transcription reaction system was prepared on ice using a PrimeScript RT reagent Kit (TaKaRa, Code No. RR037A, Otsu, Shiga, Japan). Quantitative PCR operations were performed according to the SYBR Green PCR Kit instructions. The total reaction system was 10 µL. The primers were listed below: miR-497 (F: 5’-CTCAACTGGTGTCGTGGAAGTCGGCAATTCAGTTGAGAACA-3’, R: 5’-ACACTCCAGCTGGGCAAGCACACTGTTGG-3’, U6 primer (F: 5’-CTCGCTTCGGCAGAACA-3’).
CATATA-3', downstream of the kit universal primer). GAPDH (F: 5'-CACCCACTCCTC-CACCTTTG-3', R: 5'-GCTCAATTCAACGGGATAAGTC-3'), FAM114A2 (F: 5'-CAAGTTTCATGGCTAGTTGTC-3', R: 5'-GTCCTCTCCTTTCCCTGCTT-3').

Western Blot
After extracting each group of cellular proteins, the amount of protein was determined by bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA), and the protein loading per well was adjusted to 80 μg. Total proteins from each sample were applied to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for electrophoresis. After blocked for 2 hours with skim milk, the proteins were incubated with the specific primary antibody separately overnight. On the next day, the proteins were incubated with the secondary antibody. Protein bands were visualized with the enhanced chemiluminescence (ECL) luminescence (Thermo Fisher Scientific, Waltham, MA, USA).

Dual Luciferase Reporter Gene Assay
FAM114A2 3'UTR wild-type and mutant plasmids were constructed based on the sequence. The miR-497 mimics/inhibitor was co-transfected with the constructed FAM114A2 wild type or mutant plasmid. The relative dual luciferase values were detected by a standardized method after plasmids transfection.

Statistical Analysis
Data was analyzed using Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) and expressed as mean ± standard deviation. Comparisons between the two independent samples were performed using two-sided independent t-tests. Differences between FAM114A2 expression and miR-497 expression were analyzed using Pearson correlation analysis. The difference was statistically significant at p < 0.05.

Results
miR-497 is Decreased in CSCC
In order to investigate the role of miR-497 in cutaneous squamous cell carcinoma, we detected miR-497 expression by quantitative RT-PCR in 38 cases of CSCC tissues and normal skin tissues. We found that miR-497 expression was markedly reduced in the skin tissues of the CSCC patients (Figure 1A). At the same time, FAM114A2 showed a remarkable increase in mRNA and protein expression in CSCC tissues (Figure 1B, 1C). In addition, miR-497 was significantly down-regulated in squamous cell carcinoma cell lines (A431, HSC-5) compared to normal cells (HaCaT) (Figure 1D). The above results indicated that miR-497 was lowly-expressed in CSCC and may be involved in the progression of it.

Low Expression of miR-497 Can Promote the Proliferation of CSCC
To further validate the specific regulation of miR-497 on cell proliferation and cell cycle in squamous cell carcinoma cells (A431, HSC-5), miR-497 mimics or inhibitors were transfected to achieve miR-497 overexpression or knockdown. Transfection efficiency was evaluated by miR-497 expression detection (Figure 2A, 2B). In addition, cell proliferation was significantly inhibited after overexpression of miR-497, whereas miR-497 knockdown resulted in the promotion in cell proliferation (Figure 2C, 2D). After miR-497 overexpression in A431and HSC-5 cells, G0/G1 phase time was markedly increased while the S phase time was significantly decreased, suggesting the cell cycle arrest (Figure 2E, 2F). However, miR-497 knockdown significantly decreased the G0/G1 phase time but the G2/M phase was not significantly changed and the cell cycle was promoted (Figure 2E, 2F). These results demonstrated that miR-497 played a role in the regulation of cell proliferation and cell cycle in CSCC.

miR-497 Can Selectively Regulate FAM114A2 Expression
MiRNAs can bind to 3’ untranslated region (UTR) of the target genes to regulate their expression and participate in the occurrence of disease15. We used bioinformatics methods to predict possible target gene of mir-497 and constructed the FAM114A2 wild-type sequence FAM114A2-WT 3’UTR and the mutant sequence FAM114A2-MUT 3’UTR plasmids (Figure 3A). Furthermore, we analyzed whether there was a correlation between the differentially expressed expression levels of miR-497 and FAM114A2 in CSCC. The results showed a significant negative correlation between miR-497 and FAM114A2 expression (Figure 3B). After miR-497 mimics transfection, the lucifer-
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Figure 1. miR-497 is lowly-expressed in CSCC. A, The expression level of miR-497 in squamous squamous cell carcinoma tissue is significantly lower than that in normal skin tissue. B, The mRNA expression level of FAM114A2 in squamous cell carcinoma is significantly higher than that in normal skin tissue. C, The protein expression level of FAM114A2 in squamous cell carcinoma tissues is significantly higher than that in normal skin tissues. D, miR-497 is expressed in normal skin cells (HaCaT) cells higher than skin squamous carcinoma cells (A431, HSC-5).

ase activity in the FAM114A2-WT 3’UTR group was decreased; in contrast, FAM114A2-MUT 3’UTR group showed no clear change. Besides, miR-497 inhibitor transfection increased the FA- M114A2-wild type group luciferase activity, while no significant difference in luciferase intensity was observed in the FAM114A2 mutant group (Figure 3C, 3D). Thus, miR-497 could target the expression of the target gene FAM114A2 to inhibit the progression of CSCC.

FAM114A2 Reverses the Inhibitory Effect of miR-497 on CSCC

In order to further clarify the regulation of miR-497 on FAM114A2 in A431, HSC-5 cells, we detected the expression of FAM114A2 protein after overexpression of miR-497. FAM114A2 expression was down-regulated after miR-497 overexpression. In contrast, miR-497 knock- down increased the expression of FAM114A2 (Figure 4A, 4B). Furthermore, we verified the role of FAM114A2 on cell proliferation and cell cycle progression after miR-497 inhibition in CSCC cells. After knockdown of miR-497, we silenced FAM114A2 and found that low expression of FAM114A2 reversed the promotion of miR-497 on cell proliferation of A431 and HSC-5 (Figure 4C, 4D). These above results indicated that miR-497 can inhibit the proliferation of CSCC by reducing the expression of FAM114A2.
Figure 2. Low expression of miR-497 can promote the proliferation of CSCC. A-B, A431, HSC-5 cells transfection with mimic and inhibitor. C, cell viability was enhanced after knockdown of miR-497, and cell viability decreased after overexpression of miR-497. D, In HSC-5 cells, cell viability was enhanced after knockdown of miR-497, and cell viability decreased after overexpression of miR-497. E, In A431 cells, the cell cycle was accelerated after knockdown of miR-497, and cell cycle arrest was observed after overexpression of miR-497. F, In HSC-5 cells, the cell cycle was accelerated after knockdown of miR-497, and cell cycle arrest was observed after overexpression of miR-497.
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Discussion

Cutaneous squamous cell carcinoma (CSCC) is a malignant tumor originating from the epidermal or accessory keratinocytes. It is more common in men than women, mostly occurring in the face, hands and forearms. Cutaneous squamous cell carcinoma is the leading cause of death from non-melanoma skin tumors, and its incidence is increasing year by year. Although the treatment of CSCC is becoming more and more mature, further research on molecular models and targeted therapies is needed. Recently, miRNAs in the development and progression of malignant tumors and targeted miRNA gene therapy have become hotspots in oncology research. It has been showed that a large number of miRNAs are significantly altered in CSCC. miRNAs, such as miR-21, miR-135b, miR-205, miR-365, are up-regulated in CSCC. On the other hand, the expression of miR-20a, miR124, miR-34a, miR-125b, miR-214, miR-199a, etc. are significantly down-regulated in CSCC. Research have found that downregulation of miR-125b expression in CSCC inhibits proliferation, migration and invasion of skin SCC cells by matrix metalloproteinase 13 (MMP-13). In addition, miR-214 can regulate tumor cell proliferation and differentiation by targeting ERK1 and participate in tumor progression. We found that miR-497 was down-regulated in squamous cell carcinoma tissues and cells, suggesting that miR-497 might be closely related to the progression of CSCC. The role of miR-497 in tumors has now attracted widespread attention. Zhong et al. have found that miR-497 is down-regulated in breast cancer and can be used as an important indicator for breast cancer diagnosis. In addition, in laryngeal squamous cell carcinoma, miR-497 expression is down-regulated, and tumor invasion is regulated by PlexinA4. The anti-cancer effect of miR-497 was also observed in cervical cutaneous squamous cell carcinoma.
miR-497 negatively regulates MAPK/ERK signaling pathway through RAF-1, promotes tumor cell apoptosis, and inhibits tumor cell proliferation, migration and invasion. We found that overexpressing miR-497 significantly inhibited the proliferation and cycle progression of A431 and HSC-5 cells. Furthermore, it was found that miR-497 can participate in the proliferation of CSCC by inhibiting the expression of FAM114A2 and regulating the proliferation of A431 and HSC-5 cells.

**Conclusions**

We showed that miR-497 was down-regulated in squamous cell carcinoma tissues and cells, which can inhibit the expression of FAM114A2 and regulate the cell proliferation of CSCC to promote its progression.

**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**References**

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