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Review Article

miR-92b regulates the proliferation and apoptosis of head and neck squamous cell carcinoma through regulating the expression of EZH2

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Abstract: Background: MiR-92b acts as a carcinogen in various malignant tumors, but its exact role in head and neck squamous cell carcinoma (HNSCC) remains to be clarified. Objective: To study the miR-92b expression in HNSCC tissues and its influence on cell proliferation and apoptosis. Methods: Fifty specimens of HNSCC tissues and normal tumor-adjacent tissues confirmed based on pathology and obtained through resection from The Central Hospital of Jia Mu Si City were collected, respectively, CCK-8 assay and flow cytometry were adopted to determine the proliferation ability and apoptosis ratio of the cells, respectively, and then RT-qPCR was employed to quantify miR-92b in the cells and tissues and Western Blot was used to quantify EZH2 protein. Dual luciferase reporter was adopted for the determination of the correlation between miR-92b and EZH2. Results: HNSCC tissues showed higher expression of miR-92b and EZH2 proteins than normal tumor-adjacent tissues, and they were positively correlated. MiR-92b was related to neoplasm staging, pN+, and high pathological stage of advanced HNSCC patients. The miR-92b-mimic group showed significantly higher optical density (OD) and EZH2 protein expression, and significantly lower cell apoptosis rate than the miR-92b-N group. The miR-92b-inhibitor group presented much lower OD and EZH2 protein expression and higher cell apoptosis rate than the miR-92b-NC group. Dual luciferase report assay determined the targeting relationship between miR-92b and EZH2. Conclusion: MiR-92b is highly expressed in HNSCC tissues, and it may affect the proliferation and apoptosis of HNSCC cells by regulating EZH2 expression.

Keywords: HNSCC, miR-92b, EZH2, proliferation, apoptosis

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant tumor worldwide; which usually occurs in the oropharynx, oral cavity and laryngeal cavity [1]. HNSCC is a special complex in its etiology, pathogenesis, morphological characteristics, clinical characteristics, natural course, and is closely related to smoking, heavy drinking, betel nut chewing, and poor oral hygiene [2]. HNSCC is characterized with a high incidence and mortality, and more than 500,000 people suffer from it each year. In America, more than 40,000 new patients are reported to have HNSCC, of which nearly 12,000 patients died from it [3]. Early stage HNSCC patients (at I stage or II stage) can be treated through surgery or radiation therapy, and advanced stage HNSCC patients (at stage III or stage IV) have a high mortality [4]. Advanced stage HNSCC patients suffer a 3-year survival rate of only 30-50% after standard treatment, and most of patients (nearly 40%-60%) may have local recurrence or distant metastasis [5]. Despite advances in treatment strategies, the survival

rate of advanced stage HNSCC patients has not increased significantly [6]. Therefore, it is especially important to understand the pathogenesis of HNSCC and seek out specific targeted molecular therapies.

MicroRNA (miRNAs), are a kind of endogenous non-coding RNA molecular, it can silence genes by binding to complementary sequences of target genes [7]. MiRNAs accounts for about 1% of human gene expression, and may be able to regulate the expression of various human protein coding genes [8]. Some studies have shown that up-regulation or down-regulation of miRNAs may play a key role in cancer development for its influence on proliferation and apoptosis [9]. Earlier studies have confirmed that miR-92b is dysregulated in many cancers [10]. As reported by Liu et al., miR-92b can promote tumor growth and activation of NF-κB signaling transduction through regulating neuroleukin (NLK) in oral squamous cell carcinoma [11]. Another study by Wang et al. has revealed that miR-92b can control the proliferation and invasion of gliomas by regulating the Wnt/β-catenin signaling pathway through nemo-like kinase [12]. These studies implied that miR-92b may induce cancer, but there are few studies on miR-92b in HNSCC.

This study explored the expression and clinical value of miR-92b in HNSCC and biological functions of HNSCC cells, with the goal of providing a clinical reference for diagnosis, treatment and prognosis of HNSCC.

Materials and methods

General materials

Fifty specimens of HNSCC tissues confirmed based on pathology and obtained through resection from The Central Hospital of Jia Mu Si City from April 2014 to August 2017 were collected, and specimens of normal tumor-adjacent tissues (with distance from tumor focus >2 cm) were taken during operation. Inclusion criteria: Patients who had not received any radiotherapy, chemotherapy and immunotherapy, patients treated surgically in the Central Hospital of Jia Mu Si City and patients whose HNSCC tissues and normal tumor-adjacent tissues were confirmed based on pathology [13], patients whose tissue specimen were marked and saved in liquid nitrogen immediately after surgery, and patients with detailed clinical data. Exclusion criteria: Patients with comorbid hepatic renal dysfunction, hematological diseases, mental diseases, nervous system diseases, systemic autoimmune diseases or other malignant tumors. This study was carried out with the permission from the Ethics Committee of The Central Hospital of Jia Mu Si City and filed on record, and all subjects signed an informed consent form.

Cell culture

HNSCC cell strains (HN4 and HN12) purchased from Shanghai Yu Bo Biotech Co., Ltd. (China) and normal human oral keratinocytes (HOK) from the ScienCell Research Laboratories (Carlsbad, CA, USA) were all tested based on STR genotype and incubated in Dulbecco's modified eagle medium (DMEM, Yu Bo Biotech Co., Ltd., Shanghai, China) containing 10% fetal bovine serum, penicillin (100 U/mL) or streptomycin (100 U/mL) in a constant temperature incubator with saturated humidity.

Transfection of cells

Cells in a logarithmic growth phase were transferred to a 6-well plate and transfected in accordance with the instructions of Lipofectamine 2000 when the cell confluency was cultured to 80%-85%. Lipofectamine 2000 (22 μL) and 5 nmoL miR-92b mimic, corresponding negative control (miR-92b-NC), 5 nmoL miR-92b-inhibitor or corresponding negative control (miR-92b-NC) (Invitrogen, Carlsbed, CA, USA) were added into a serum-free medium for transfection after being fully mixed according to volume ratio of 1:50, washed with phosphate buffer (PBS) three times after 6 hours, and then placed in a normal medium for culture in a constant temperature incubator for 48 hours. Non-transfected cells were taken as a blank group and cultured for 48 hours. Transfection efficiency of cells was verified by RT-qPCR. The primer sequences were all designed by Thermo Fisher Scientific-CN, and they are as follows: miR-92b-mimic: 5'-UAUUGCACUCGUCCCGGCCUCC-3'; miR-92b-inhibitor: 5'-GGAGGCCGGGACGAGUGCAAUA-3'; miR-92b-NC: 5'-CAGUACUUUUGUGUAGUACAA-3'.

RT-qPCR assay

Total RNA was acquired from tissues and cultured cells with an extraction kit Trizol (Simgen Biological Reagent Development Co., Ltd.,

Table 1. Primer sequences

Gene	Upstream	Downstream
miR-92b	5'-TATTGCACTCGTCCCGGCCTCC-3'	5'-CAGTGCGTGTCGTGGAGT-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

Hangzhou, China), and the RNA was reversely transcribed into cDNA with reverse transcription kit Takara (Yuduo Biological Technology Co., Ltd., Shanghai, China). U6 was taken as an internal reference, and the primer sequences are shown in **Table 1**. The primers were all synthesized by Thermofisher Scientific (China) Co., Ltd. The reaction was carried out on ABI Real-time fluorescent quantitative PCR instrument (Thermofisher Scientific Co., Ltd., China). The conditions of PCR amplification: 90°C for 5 minutes, followed by 90°C for 5 seconds, 60°C for 30 seconds and 72°C for 5 seconds. The relative expression of genes was expressed after being calculated based on $2^{-\Delta CT}$.

Cell proliferation detection by CCK-8 assay

A total of 100 μL cell suspension (5×10^5 cells/mL) was seeded into a 96-well plate. Three duplicate wells were set for each group at 1×10^3 cells per well. The plate was incubated in an incubator at 37°C. Then CCK-8 value was measured at specific time points after culture. During detection, the original medium was transferred out, and detection solution was prepared with CCK-8 solution and culture solution according to the ratio of 1:9. Each well was given 100 μL detection solution, and incubated for 2 h. After 2 h, the OD of each well was detected at 450 nm on BIOBASE4000 Enzyme-linked Immunosorbent Assay (BIOBASE Biological Industry Co., Ltd., Shandong, China). The ODs of all specimens were measured three times, and then the mean value was taken.

Apoptosis detection by flow cytometry

Cells transfected for 48 hours were taken and digested with trypsin, washed with PBS twice, and placed into an eppendorf (EP) tube. The cells were resuspended through 200 μL AnnexinV binding buffer, added with 5 μL Annexin-V/FITC solution (BestBio Co., Ltd., Shanghai, China), then added with 5 μl PI staining solution, and incubated after being mixed well. The cells were placed in a flow tube and detected using a BD FACSCanto II flow cytometer (Delica Biotechnology Co., Ltd., Beijing, China) within 1 hour.

Western Blot assay detection

Total protein of collected cells was acquired with RIPA (radio immunoprecipitation assay) lysate (Shanghai BestBio Co., Ltd., China) using the BCA method. Specimen (15 μg) was added in each lane for constant voltage electrophoretic deposition (100 V), and subjected to 10% SDS-PAG electrophoretic separation. The protein was transferred to a PVDF membrane, and then immersed in 5% skim milk at room temperature for 2 hours, added with rabbit anti-human EZH2 polyclonal antibody (Invitrogen, Carlsbed, CA, USA, dilution rate: 1:1000) and rabbit anti-human β-actin monoclonal antibody (Invitrogen, Carlsbed, CA, USA, dilution rate: 1:1000), and blocked and incubated at 4°C overnight, washed three times with TBST to wash the antibody off, then added with horseradish peroxidase to label goat anti-rabit IgG (Shanghai LMAIBio Engineering Co., Ltd., China, dilution rate: 1:1000), cultured at room temperature for 2 hours, and then washed three times. ECL luminescence was performed for development. The band's gray value was analyzed in Quantity one 4.0 software (Beijing EASYBIO Technology Co., Ltd., China), and the measurement was repeated 3 times.

Luciferase assay

Wild-type and mutant EZH2 gene vectors were constructed by Shanghai GenePharma Co., Ltd., and named Lut-EZH2-WT and Lut-EZH2-MUT, respectively. Cells were seeded into a 24-well plate at a density of 5×10^4/well, and transfected with miR-92b-mimic and miR-92b-NC 24 hours later. After 48 hours, 0.5 μg wild-type or mutant recombinant vector and 0.02 μg pRL-TK plasmid (Ke Lei Biological Technology Co., Ltd., Shanghai, China) were transfected into each well of cells, and then relative luciferase activity was detected on a Elx-800 enzyme-linked immunosorbent assay appliance according to the double luciferase activity detection

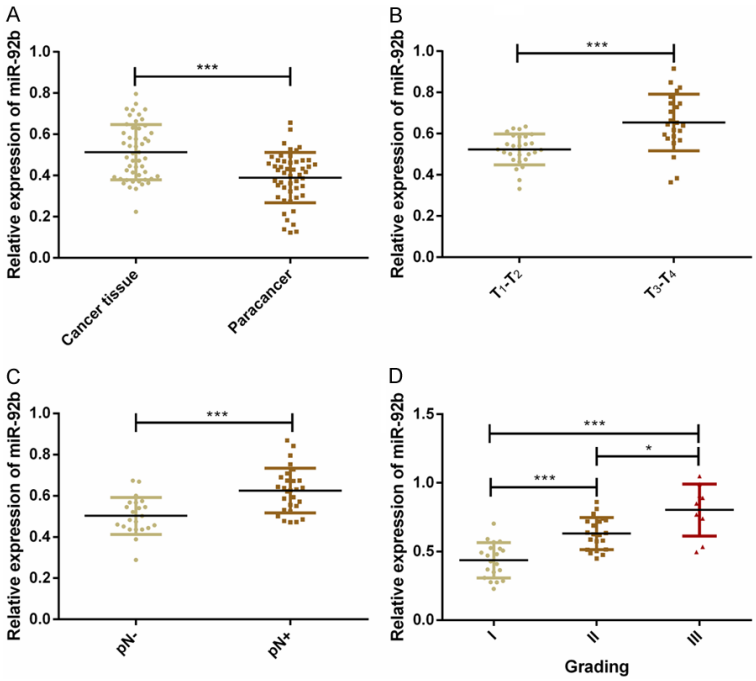

Figure 1. Expression of miR-92b in tissues of HNSCC patients. A. The expression of miR-92b in HNSCC tissues was significantly higher than that in normal tumor-adjacent tissues (P<0.001); B. The expression of miR-92b in tissues of HNSCC patients at stage T_3-T_4 was significantly higher than that of HNSCC patients at stage T_1-T_2 (P<0.001); C. The expression of miR-92b in tissues of pN+ HNSCC patients was significantly higher than that of pN- HNSCC patients (P<0.001); D. The expression of miR-92b in tissues of HNSCC patients in grade II and grade III in pathological grade was significantly higher than that of HNSCC patients in grade I (P<0.001) and the expression of miR-92b in tissues of HNSCC patients in grade III in pathological grade was significantly higher than that of HNSCC patients in grade II (P<0.05). Note: *P<0.05; ***P<0.001.

kit (Promega, Madison, WI, USA) 36 hours after transfection. Relative luciferase activity = luciferase activity of glowworm/luciferase activity of sea pansy.

Statistical analyses

Data were statistically processed with SPSS 20.0 (Beijing EASYBIO Technology Co., Ltd., China) and figures were drawn using GraphPad Prism 6 (Shenzhen APSGO Network Co., Ltd., China). Measurement data were presented as the mean ± standard deviation (x ± SD), and compared between groups using the independent sample t-test, and comparison of data in multiple time points was carried out using the variance of repeated measures. Comparison among multiple groups in means was analyzed using single factor analysis of variance and comparison between any two means was carried out using the LSD-t test. Pearson test was performed for correlation analysis. P<0.05 indicated a significant difference.

Results

MiR-92b is highly expressed in HNSCC tissues

RT-qPCR detection showed that the miR-92b expression in HNSCC tissues and normal tumor-adjacent tissues was (0.526±0.124) and (0.384±0.112), respectively, revealing HNSCC tissues showed higher miR-92b expression than normal tumor-adjacent tissues. MiR-92b expression was not significantly different in HNSCC tissues from patients with different clinicopathological features including; smoking history, drinking history, lesion sites, vessel embolism, perineural invasion, and diffuse infiltration. The miR-92b expression in tissues of HNSCC patients at stage T_3-T_4 was higher than that of HNSCC patients at stage T_1-T_2; the miR-92b expression in tissues of pN+ HNSCC patients was higher than that of pN-HNSCC patients; the miR-92b expression in tissues of HNSCC patients at grade II or grade III in pathological grade was higher than that of patients at grade I, and the miR-92b expression in tissues of HNSCC patients at grade III in pathological grade was higher than that of HNSCC patients at grade II **Figure 1** and **Table 1**.

MiR-92b was highly expressed in HNSCC cell strains

RT-qPCR detection revealed that the miR-92b expression in HN4, HN12 and HOK cells were (1.436±0.113), (2.124±0.115) and (1.031±0.102), respectively, revealing that the miR-92b expression in HN4 and HN12 cells was higher than that in HOK cells, and the miR-92b expression in HN12 cells was higher than that in HN4 cells. HN4 cells were transfected with miR-92b-mimics, and HN12 cells were transfected with miR-92b-inhibitor. It turned out that the

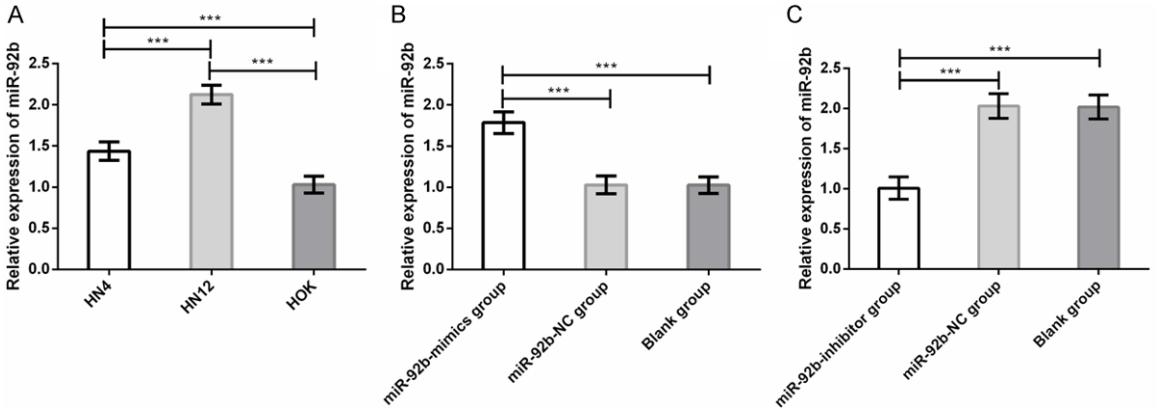

Figure 2. Expression of miR-92b in HNSCC cell strains. A. HN4 and HN12 cells showed significantly higher miR-92b expression than HOK cells, and HN12 cells showed significantly higher miR-92b expression than HN4 cells; B. The miR-92b-mimic group showed significantly higher miR-92b expression than the miR-92b-NC group and the blank group; C. The miR-92b-inhibitor group showed significantly lower miR-92b expression than the miR-92b-NC group and the blank group. Note: ***P<0.001.

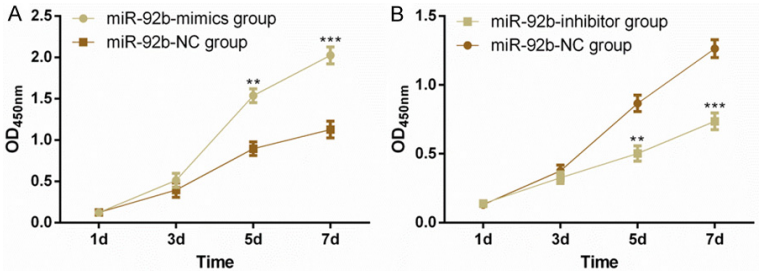

Figure 3. Effect of miR-92b on proliferation of HN4 and HN12 cells. At different times within 3 days after transfection, (A) The miR-92b-mimic group showed significantly higher OD value than the miR-92b-NC group; (B) The miR-92b-inhibitor group showed significantly lower OD value than the miR-92b-NC group. Note: In comparison with the miR-92b-NC group at 5 days, **P<0.01; in comparison with the miR-92b-NC group at 7 days, ***P<0.001.

miR-92b-mimics group showed higher miR-92b expression than the miR-92b-NC group and the blank group, and the miR-92b-inhibitor group showed lower miR-92b expression than the miR-92b-NC group and the blank group **Figure 2**.

Influence of miR-92b on the proliferation of HN4 and HN12 cells

CCK-8 assay detection showed that at different times within 3 days after transfection, the miR-92b-mimic group showed significant higher OD value than the miR-92b-NC group and showed significantly strengthened cell proliferation ability. While the miR-92b-inhibitor group showed significantly lower OD value than the miR-92b-NC group and showed significantly weakened cell proliferation ability **Figure 3**.

Influences of miR-92b on apoptosis of HN4 and HN12 cells

Flow cytometry revealed that the cell apoptosis rate in the miR-92b-mimic group and the miR-92b-NC group was (6.89±1.02)% and (11.83±1.36)%, respectively. The apoptosis rate in the miR-92b-inhibitor group and the miR-92b-NC group was (22.37±1.49)% and (8.01±1.09)%, respectively, revealing that the miR-92b-mimic group showed significantly lower cell apoptosis rate than the miR-92b-NC group, which indicated significantly reduced ability of apoptosis. The miR-92b-inhibitor group showed significantly higher apoptosis rates than the miR-92b-NC group, which indicated significantly increased ability of apoptosis **Figure 4**.

Regulation on EZH2 expression by miR-92b

TargetScan Release7.2 online software (http://www.targetscan.org) was adopted for target gene predication to miR-92b. EZH2 was a potential target of miR-92b. Dual luciferase reporter gene showed that the relative fluorescence activity of EZH2 in the miR-92b-mimic group was higher than that in the miR-92b-NC

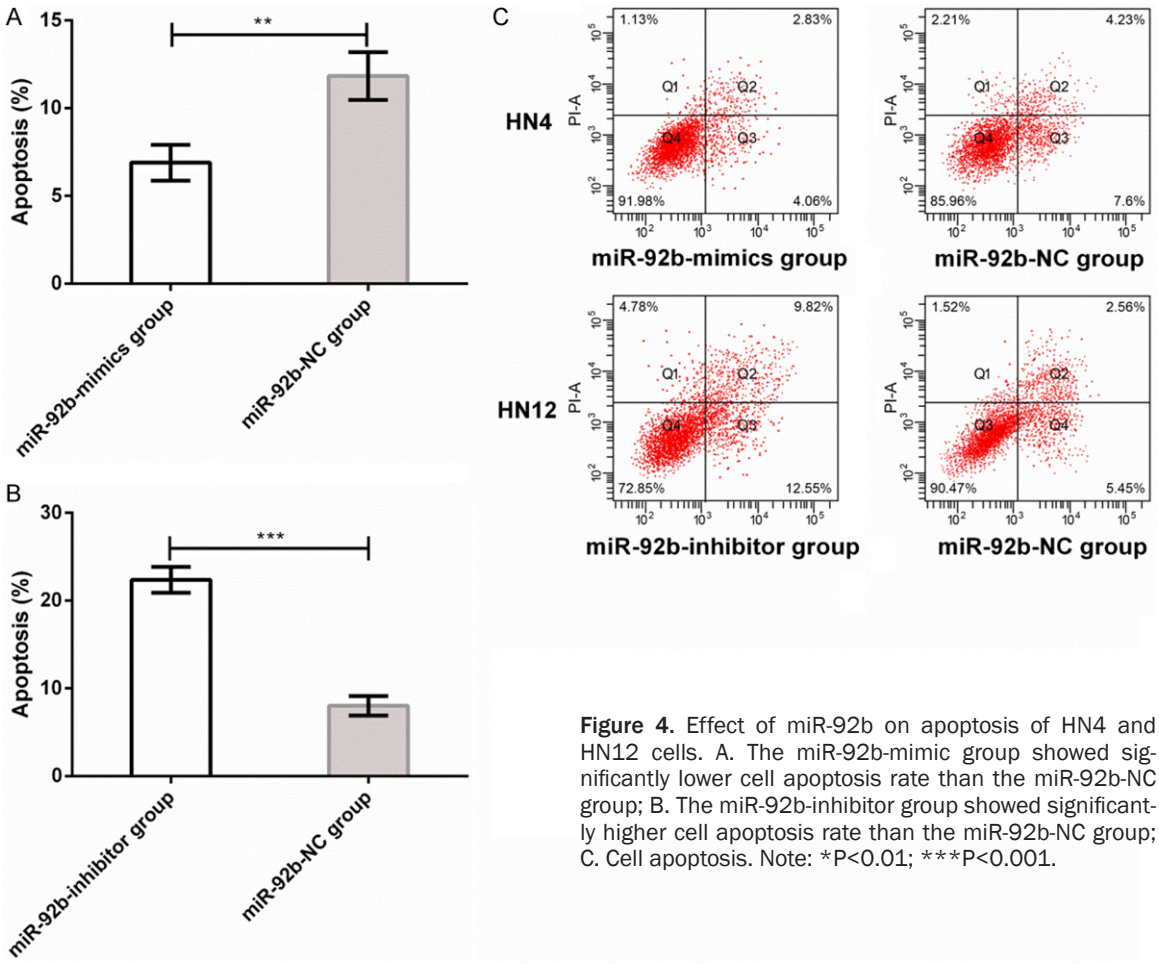

Figure 4. Effect of miR-92b on apoptosis of HN4 and HN12 cells. A. The miR-92b-mimic group showed significantly lower cell apoptosis rate than the miR-92b-NC group; B. The miR-92b-inhibitor group showed significantly higher cell apoptosis rate than the miR-92b-NC group; C. Cell apoptosis. Note: *P<0.01; ***P<0.001.

group, and there was no remarkable difference in relative fluorescence activity between the miR-92b-mimic group and the miR-92b-NC group. The WB assay revealed that the expression of EZH2 protein in the miR-92b-mimic group was higher than that in the miR-92b-NC group, while the expression of EZH2 protein in the miR-92b-inhibitor group was lower than that in the miR-92b-NC group **Figure 5**.

Expression of EZH2 protein in HNSCC tissues and its correlation with miR-92b

Western Blot detection showed that the expression of EZH2 protein in HNSCC tissues and normal tumor-adjacent tissues was (0.946±0.083) and (0.647±0.075), respectively; revealing that HNSCC tissues had significantly higher expression of EZH2 protein than normal tumor-adjacent tissues. Pearson test showed that the miR-92b expression in HNSCC tissues was positively correlated with EZH2 protein (r= 0.662) **Figure 6**.

Discussion

HNSCC is a common malignant tumor, which is mainly treated through surgery, radiotherapy and chemotherapy, currently. Although great progress has been made in surgery and level of repair, moderate and advanced HNSCC patients still showed non-ideal overall survival [14]. Thus, it is of great value to investigate the molecular pathogenesis of HNSCC and seek for effective targeted therapy methods for this disease.

MiRNA is abnormally expressed in a variety of malignant tumors, which can prevent the synthesis of target gene proteins or degradation of target molecule mRNA by recognizing and regulating target molecules, thereby playing a role in cancer promotion or cancer inhibition in tumors [15, 16]. MiR-92b is strongly linked to the development of various malignant tumors [17, 18]. One study by Song et al. has indicated that miR-92b regulates the biological behaviors

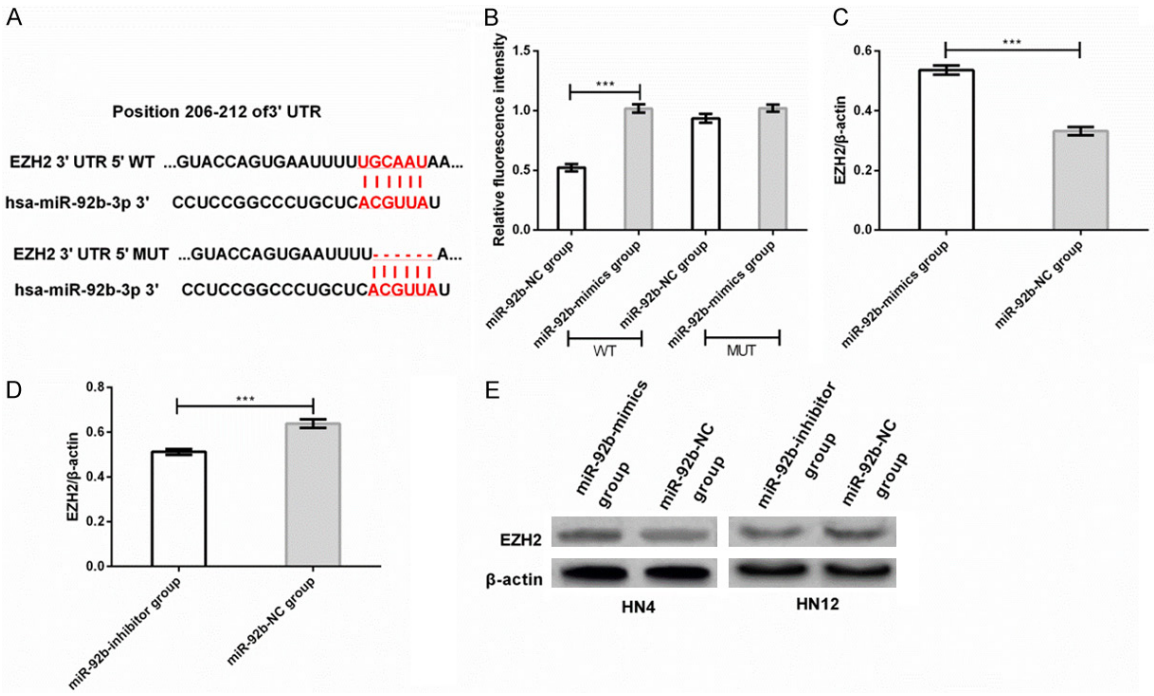

Figure 5. Regulation of EZH2 expression by miR-92b. A. There were potential miR-92b binding sites on the 3'UTR of EZH2; B. The relative fluorescence intensity of the miR-92b-mimic group was significantly higher than that of the miR-92b-NC group, and after mutation of miR-92b binding site on 3'UTR of EZH2 gene, there was no significant difference in relative fluorescence intensity between the miR-92b-mimic group and the miR-92b-NC group. C. The miR-92b-mimic group showed significantly higher expression of EZH2 protein than the miR-92b-NC; D. The miR-92b-inhibitor group showed significantly lower expression of EZH2 protein than the miR-92b-NC group. E. Protein band. Note: ***P<0.001.

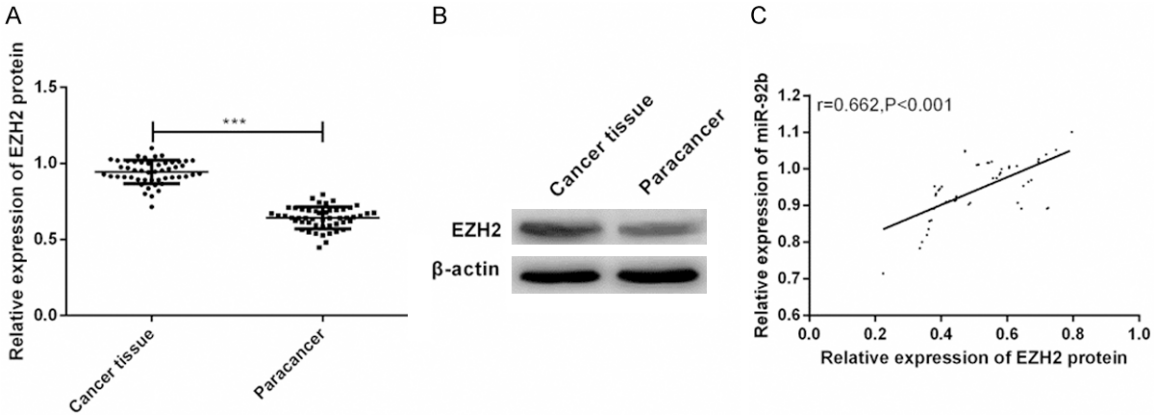

Figure 6. Expression of EZH2 protein in HNSCC tissues and its correlation with miR-92bs. A. The expression of EZH2 protein in HNSCC tissues was significantly higher than that in normal tumor-adjacent tissues. B. Protein band. C. The expression of miR-92b in NSCC tissues was positively correlated with EZH2 protein. Note: ***P<0.001.

of glioma cells via the PTEN/Akt signaling pathway [19], and one study by Zhou et al. has confirmed that miR-92b is significantly up regulated in osteosarcoma, and it is closely related to the poor prognosis of patients with osteosarcoma [20]. However, one study by Lei et al. has indicated that the miR-92b expression in non-small cell lung carcinoma and cell strains increase significantly, and inhibition of miR-92b can strongly suppress the proliferation ability of non-small cell lung carcinoma [21]. Thus, it seems that miR-92b might act as a tumor-pro-

moting gene in different tumors. This study found that the miR-92b expression in HNSCC tissues greatly increased in patients at later TNM stages and higher pathological grades, which indicated that miR-92b may be dysregulated in the development of HNSCC and increase in expression with disease progress. One study by Liu et al. has indicated that the miR-92b expression in oral squamous cell carcinoma is up-regulated, and the high miR-92b expression is strongly associated with tumor size, advanced tumor stage, and poor prognosis [22]. HNSCC includes oral squamous cell carcinoma [23], which is similar to our study. The study by Liu et al. has also revealed that miR-92b can regulate NLK expression in oral squamous cell carcinoma, promote tumor growth, and activate NF-κB signal transduction. For the purpose of understanding the biological function of miR-92b in HNSCC, we further transfected HN4 with miR-92b-mimics and transfected HN12 cells with miR-92b-inhibitor. HN4 is composed of primary head and neck squamous cell carcinoma tissues [24], while HN12 cells are composed of lymph node metastasis of head and neck squamous cell carcinoma tissues [25]. The two cells were up-regulated and down-regulated, respectively, in this study, and it turned out that HN4 showed significantly increased cell proliferation ability and significantly decreased apoptosis ability after being transfected with miR-92b-mimics and showed significantly decreased cell proliferation ability and significantly increased apoptosis ability after being transfected with miR-92b-inhibitor, so miR-92b may become a biological index for HNSCC targeted therapy

To further analyze the regulation mechanism of action of miR-92b on HNSCC, target gene prediction analysis was carried out to miR-92b and it turned out that there was a potential miR-92b binding locus on the 3'UTR of EZH2. EZH2, a member of polycomb genome, is over-expressed or activated in most human cancers, including HNSCC. Analysis of HNSCC data based on The Cancer Genome Atlas (TCGA) has showed that EZH2 over-expression is linked to high tumor grade and unfavorable prognosis [26]. There are many studies on the function of EZH2 in HNSCC. For example, Chang et al. have conducted a study and have pointed out that EZH2 is associated with poor prognosis of HNSCC patients based on regulation of epithelial-mesenchymal transition and chemosensitivity [27]. Additionally, in invasive squamous cell carcinoma, rap1GAP can be silenced by miR-101-mediated over-expression of EZH2 [28]. However, it has not been studied whether miR-92b can target and regulate EZH2 and is involved in HNSCC cell proliferation and apoptosis. This study found that the miR-92b-mimic group showed significantly higher relative fluorescence intensity of EZH2 than the miR-92b-NC group, which indicated that EZH2 was a direct target of miR-92b. Furthermore, the miR-92b-mimic group presented higher expression of EZH2 protein than the miR-92b-NC group, while the miR-92b-inhibitor group showed lower expression of EZH2 protein than the miR-92b-NC group, and the relative expression in HNSCC tissues was positively correlated with EZH2 protein, which indicated that miR-92b might take a part in proliferation and apoptosis of HNSCC cells by regulating EZH2. One study by Liu et al. has indicated that miR-92b promotes autophagy and inhibits viability and invasion of breast cancer through targeting EZH2 [29]. It can be seen that miR-92b may play a biological role based on direct targeting to EZH2 in tumors. However, the mechanism remains to be further studied and analyzed.

This study has confirmed the role of miR-92b in HNSCC and has preliminarily explored its mechanism on HNSCC cell proliferation and apoptosis. However, there are still some shortcomings. We have not observed the function of miR-92b in the migratory and invasive ability of cell strains of HNSCC, and we have not conducted tumor formation in nude mice, so the effects of injecting miR-92b on tumor formation in nude mice are still unclear. In addition, we have not transfected miR-92b-mimics, miR-92b-inhibitor, and corresponding negative control into HN4 and HN12 cells to analyze the biological functions of the cells, These shortcomings need to be addressed in future research.

To sum up, miR-92b is highly expressed in HNSCC tissues, and may be involved in the development and progression of HNSCC, and miR-92b might take a part in the proliferation and apoptosis of HNSCC cells by regulating EZH2 expression.

Disclosure of conflict of interest

None.

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