

miR-21 Influence on Radiation Sensitivity of Cervical Cancer HeLa Cells

*Ling-Ping Meng**

Anesthesia Surgery Department, The Center Hospital of Xinxiang, Xinxiang City, 453000, Henan Province, China

ABSTRACT

Objective: This study discussed the relation of miR-21 and radiation sensitivity of cervical cancer HeLa cells, clarify the possible action mechanism of miR-21 on cell apoptosis and autophagy change. **Method** Cervical cancer HeLa cells were divided into sham-irradiated group and 4Gy radiotherapy group, respectively transfected miR-21 NC (negative control), miR-21 mimics, miR-21 inhibitor NC and miR-21 inhibitor. Real-time fluorescent quantitative PCR detection were performed to check the miR-21 expression level before and after the irradiation. Colony forming experiment was to observe the effect of miR-21 on radiation sensitivity of HeLa cells. Flow cytometry was to detect cell apoptosis and autophagy cell percentage. Bioinformatics method was adopted to predict the target genes of miR-21. The combination of miR-21 and target gene 3'UTR were verified by luciferase reporter gene analysis. Expression level of related protein beclin1 were detected by Western Blotting method. **Results:** compared to sham-irradiated group, the miR-21 expression level increased significantly in 4Gy radiotherapy group ($p < 0.05$). In colony forming experiment, compared to miR-21 NC group, the HeLa cells radiation sensitivity had no obvious changes in miR-21 mimics group ($p > 0.05$). Compared to miR-21 inhibitor NC group, the HeLa cells radiation sensitivity also had no evident changes in miR-21 inhibitor group ($p > 0.05$). Compared to miR-21 NC + 4Gy group, HeLa cell apoptosis rate increased significantly in miR-21 mimics + 4Gy group. Compared to miR-21 inhibitor NC group, HeLa cell apoptosis rate decreased apparently in miR-21 inhibitor group. Compared to miR-21 NC group, the autophagy rate of inR-21 mimics group could increase ($p < 0.05$). Compared to miR-21NC+4Gy group, the autophagy rate in miR-21 mimics+4Gy could increase ($p < 0.05$). Compared to miR-21 NC+4Gy group, the protein expression level increased in miR-21 mimics_4Gy group ($p < 0.05$). **Conclusion:** the target genes beclin1 of miR-21 were detected. Over expression of miR-21 promoted the radiation induced apoptosis and autophagy, but had no direct impact on cervical cancer HeLa cell radiation sensitivity.

Keywords: *miR-21, cervical tumor, HeLa cells, radiation sensitivity, cell apoptosis, autophagy.*

INTRODUCTION

In recent years, many studies confirmed that miR-21 had the cancer gene effect, with high expression in breast cancer, glioblastoma, prostate and colon cancer [1-4]. It was confirmed that miR-21 high expression in cervical cancer cells [5]. Radiation therapy was the conventional treatment method for cervical

Corresponding author: memglingping@163.com

cancer. Halimi et al observed that the miR-21 level in serum was significantly increased before radiotherapy, when the radiation therapy was performed on breast cancer patients [6]. They proposed that miR-21 level in serum could be the potential biological markers to identify whether exposure to ionizing radiation. While, it had be a study hotspot that miR-21 had impact on cancer cells radiation sensitivity. In malignant glioma cells, Chao et al discovered

that over expression of miR-21 could cause the increase of radiation resistance. The similar results also was observed in breast cancer cells [7,8]. However, miR-21 study was not yet widely researched on cervical cancer field, and its function mechanism on cell apoptosis and autophagy has not been reported. The cervical cancer HeLa cells were chosen as the research objective in this study, which discussed the close relation and its expression rules between miR-21 and radiation sensitivity of cervical cancer HeLa cells, and provided theoretical basis for clinical treatment of cervical cancer.

MATERIAL AND METHOD

Cells, main reagent and instrument

Cervical cancer HeLa cells and HEK-293 cells were provided by the lab of this research. miR-21 mimics, miR-21 inhibitor NC and miR-21 inhibitor were synthesized by Shanghai GenePharma Co., Ltd. RPMI-1640 medium and Lipofectamine™2000 were respectively purchased from Gibco company of the United States and Invitrogen company of the United States. Dual-luciferase report gene assay kit, Western Blotting antibody beclin1 and qPCR kits were respectively bought from Promega company, Cell Signal company and Kakara Dalian Biotechnology company. Mx3000 real-time fluorescent quantitative PCR were purchased from Stratagene company of the United States.

Irradiation conditions

X-ray irradiation condition: Philips deep X-ray apparatus, voltage for 180kV, current for 18mA, absorbed dose rate for $0.344\text{Gy}\cdot\text{min}^{-1}$, target skin distance for 60cm.

Cell cultivation and transfection

HeLa cells were cultivated in 10% FBS fetal bovine serum RPMI-1640 medium, 37°C , 5% CO_2 . 0.25% trypsin digestion substance were cultivated every 2 ~ 3 days. HeLa cells in logarithmic phase were digested, which inoculate into 6 orifice as 3×10^5 cells per hole, then continued

cultivation for 24 hours. Transfection were performed when cells developed 70% fusion on culture plate. Below operation was performed based on the manual, the Lipofectamine™2000 $7.5\mu\text{L}$, miR-21 inhibitor, miR-21 mimics and their negative control group sequences for $20\mu\text{mol}\cdot\text{L}^{-1}$ were mixed and added into the plate hole, then serum-free medium medium RPMI-1640 were added to make every final volume of pore solution to be 2 ml. The serum were replaced to RPMI-1640 culture medium containing 10% fetal bovine after 4 hours. 24 hours after transfection, cells were collected for further experiment.

miR-21 expression detection by q-RT PCR method

Primers were synthesized by Kakara Dalian Biotechnology company, according to miScript SYBR Green PCR Kit instruction. The reaction required reagents, miR-21 specific primers and cDNA samples were respectively added. U6RNA was adopted as internal gene for PCR amplification and expression detection of miR-21. Based on miScript SYBR Green PCR Kit instruction, reaction condition were below, 95°C , 30 seconds, 1 cycle; 35 cycles of 95°C , 20 seconds, and 60°C , 20 seconds. All were performed on Mx3000 real-time fluorescent quantitative PCR instrument. The miR-21 expression level in HeLa cells was times of the expression level of miR-21 in HEK-293.

Colony formation

Cells was transfected for 24 hours, then digested by pancreatic enzyme to be single cell suspension, and then vaccinated into 6 orifice, and then received different dose (0, 2, 4, 6 and 8Gy) irradiation. The vaccinated cells quantity of each dose point were respectively 500, 1000, 1500, 3000 and 5000 cells. Each dosage point were set 3 parallel samples. Then it was placed in the incubator for continue cultivation 14 days. Anhydrous methanol was utilized to fix cells, then for giemsa's staining. Clone quantity not less than 50 were calculated under

microscope. Results was the survival fractions. Survival fraction percentage equals to cloning cell quantity divides vaccinated cell quantity, then times 100%.

Cell apoptosis and necrosis detected by flow cytometry method

24 hours after the transfection, the collected cells were vaccinated into 6 holes culture plate. The inoculation density was 3.0×10^5 per hole. It were divided into irradiated group and sham-irradiated group, each group for 3 dual hole. When cells were transfected by miRNA for 24 hours, then conducted 4Gy irradiation for irradiation group. 24 hours after irradiation, cells were collected, $1500 \text{ r} \cdot \text{min}^{-1}$ centrifugal for 5 minutes, $0.01 \text{ mol} \cdot \text{L}^{-1}$ PBS washing cells for 2 times. The supernatant was removed and filtered. $5 \mu \text{L}$ each of Annexin V and PI were added into the tube, the mixed, and light-free reaction in room temperature for 20 minutes. Then it was detected on machine in 2 hours. Each samples took 1.0×10^4 cells. The results was marked as cell percentage in each phase.

Autophagy incidence rate detected by MDC staining method

24 hours after the transfection, the collected cells were vaccinated into 6 holes culture plate. The inoculation density was 3.0×10^5 per hole. It were divided into irradiated group and sham-irradiated group, each group for 3 dual hole. When cells were transfected by miRNA for 24 hours, then conducted 4Gy irradiation for irradiation group. 24 hours after irradiation, cells were collected, $1500 \text{ r} \cdot \text{min}^{-1}$ centrifugal for 5 minutes, $0.01 \text{ mol} \cdot \text{L}^{-1}$ PBS washing cells for 2 times. The supernatant was removed and filtered. Then it was cultivated by $0.05 \text{ mmol} \cdot \text{L}^{-1}$ MDC in 37°C for 30 minutes. 4% paraformaldehyde was adopted to fix 15 minutes, PBS was utilized to wash 2 times on machine. The results was marked as cell percentage.

Target gene of miR-21 predicted by bioinformatics

Bioinformatics software Target Scan 6.0, Union of miRBase Targetv4 and PicTar 4.0 were applied to predict the target gene of miR-21.

Luciferase reporter gene analysis

HeLa cells were inoculated inot 24 orifice with inoculation density of 4.0×10^4 per hole. Each hole performed the transfection of 30ng pMIR-beclin 3'UTR and 5 ng pRL-SV40. At the same time, $100 \text{ nmol} \cdot \text{L}^{-1}$ of miR-21 NC or miR-21 mimics was also transfected. Each group were transfected in 4 dual holes. After 48 hours of transfection, the dual luciferase reporter gene detection was applied to detect the kits.

beclin1 protein expression detected by Western Blotting method

Cell total protein were extracted. BCA method was adopted to detect protein concentration. $40 \mu \text{g}$ (Protein sample) were taken from sample of each group. 10% SDS-PAGE gel was used for electrophoresis. Protein electricity was removed to NC membrane. 5% skimmed milk powder was sealed for 1 hours in room temperature. First antibody was incubated at 4°C over the night, second antibody (1:1000) was incubated for 1 hour in room temperature over the night.

Statistical analysis

SPSS 18.0 software was employed for statistical analysis. $\bar{x} \pm s$ was adopted for cell survival rate, cell apoptosis and autophagy. *t* test was utilized for the mean comparison between the two groups samples. One-way ANOVA analysis was conducted for the mean of multiple sets.

RESULTS

miR-21 expression detected by qRT-PCR

Through the qRT-PCR detection, compared to HEK-293 cells (1.00 ± 0.00), miR-21 had high expression (4.99 ± 0.41) in HeLa cells ($p < 0.05$). After the 4Gy irradiation, the expression level (8.53 ± 0.03) of HeLa cells significantly increased than sham-irradiated group ($p < 0.05$).

Table 1: Radiation sensitivity of Hela cells after transfection with miR-21 mimics (n=3, $\bar{x} \pm s$)

Group	Survival fraction				
	(D/Gy) 0	2	4	6	8
miR-21 NC	1	0.43±0.02	0.29±0.01	0.10±0.01	0.04±0.01
miR-21 mimics	1	0.48±0.02	0.19±0.02	0.09±0.01	0.04±0.01

Table 2: Radiation sensitivity of Hela cells after transfection with miR-21 inhibitor (n=3, $\bar{x} \pm s$)

Group	Survival fraction				
	(D/Gy) 0	2	4	6	8
miR-21 NC	1	0.48±0.03	0.31±0.02	0.09±0.01	0.03±0.01
miR-21 mimics	1	0.43±0.02	0.29±0.02	0.11±0.01	0.04±0.01

Cellular radiosensitivity detected by colony forming experiments

Compared to control group, mimics and inhibitor transfection had no statistical significance on the change of cellular radiosensitivity ($p>0.05$), as seen in table 1 and table 2.

Flow cytometry detection

Flow cytometry detection was performed on apoptosis and autophagy of HeLa cells. Compared to sham-irradiated group, the cellular apoptosis of radiotherapy group evidently increased ($p<0.05$). After mimics transfection, compared to transfected NC group, although the cellular apoptosis rate

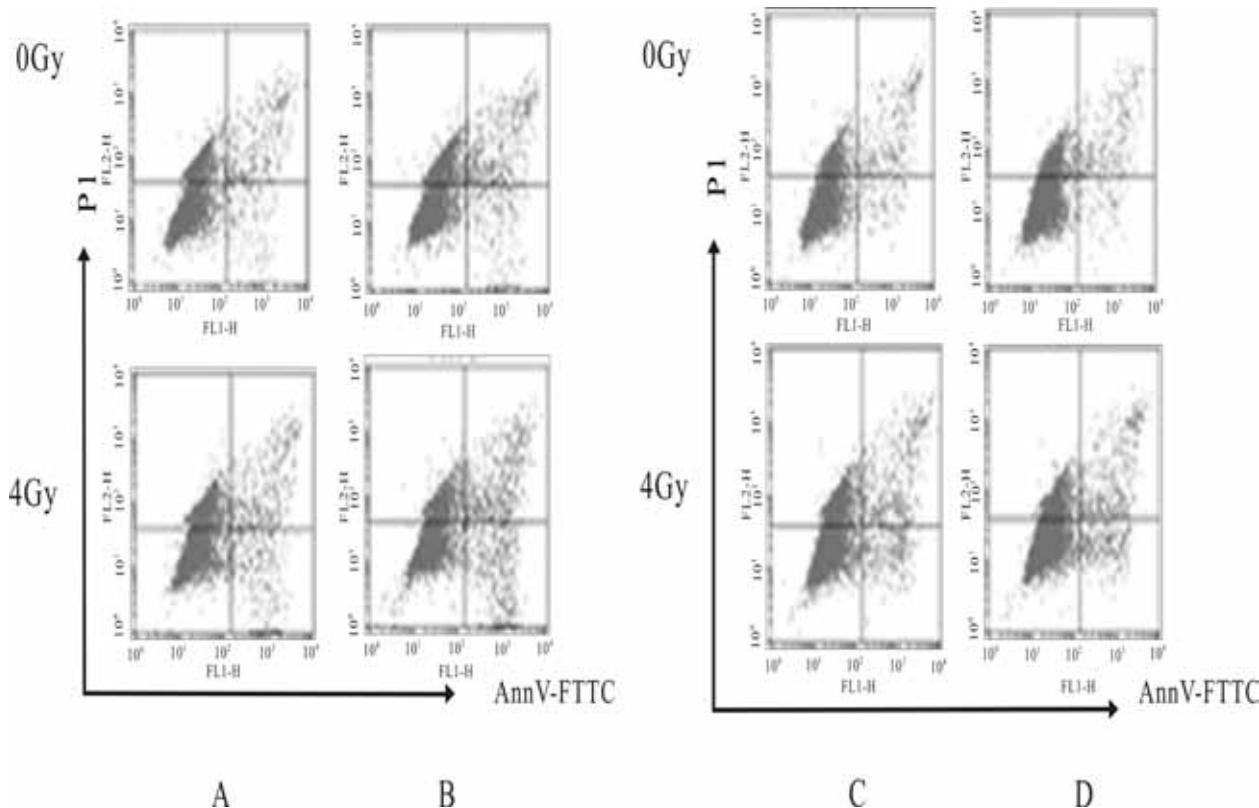


Fig.1: Apoptotic rates of Hela cells in various groups detected by flow cytometry

A: miR21 NC group; B: miR-21 mimics group; C: miR-21 inhibitor NC group; D: miR-21 inhibitor group

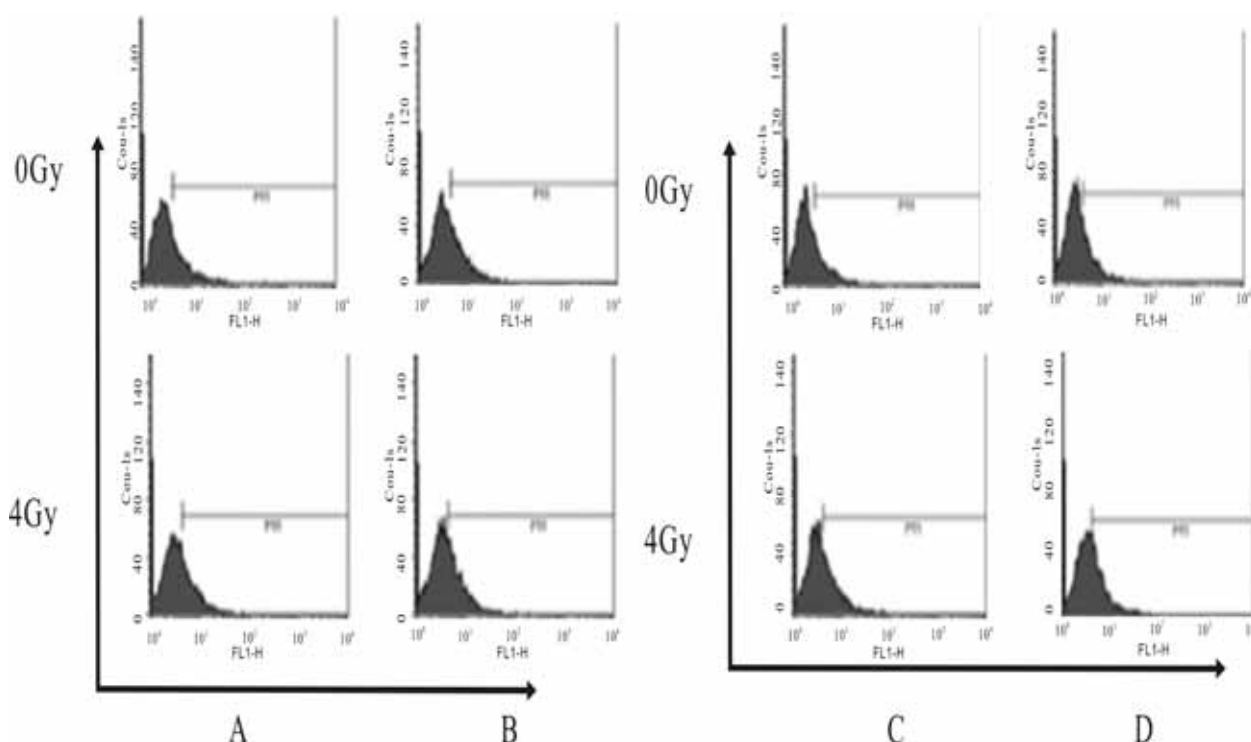


Fig.2: Autophagy rates of Hela cells in various groups detected by flow cytometry

A: miR21 NC group; B: miR-21 mimics group; C: miR-21 inhibitor NC group; D: miR-21 inhibitor group.

Table 3: Apoptotic rates and autophagy rates of cells in various group ($n=3, \bar{x} \pm s, \eta/\%$)

Group	Apoptotic rate	Autophagy rate
miR-21 NC	2.36±0.39	22.13±0.99
miR-21 mimics	3.07±0.35	27.00±2.26*
miR-21 NC + 4Gy	5.09±0.53	27.24±1.07
miR-21 mimics + 4Gy	7.05±0.93 ^Δ	34.58±2.15 ^Δ
miR-21 inhibitor NC	2.00±0.30	17.30±2.33
miR-21 inhibitor	1.46±0.120 [#]	19.70±2.49
miR-21 inhibitor NC + 4Gy	4.82±0.24	27.24±0.52
miR-21 inhibitor + 4Gy	4.51±0.24	29.75±1.39 ^Δ

* $p<0.05$ compared with miR-21 NC group;

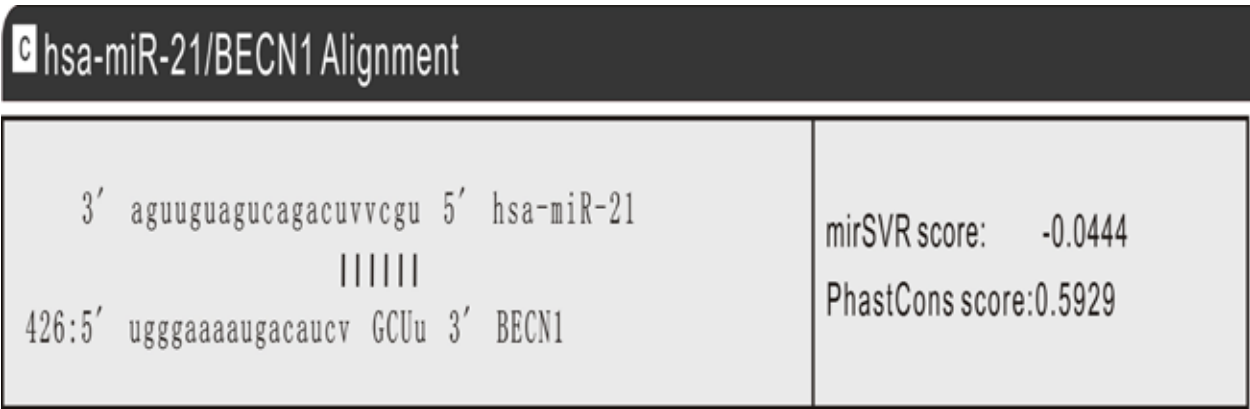
^Δ $p<0.05$ compared with miR-21 NC + 4Gy group;

[#] $p<0.05$ compared with miR-21 inhibitor NC group;

^Δ $p<0.05$ compared with miR-21 inhibitor NC + 4Gy group.

increased somehow, there was no statistical difference ($p>0.05$). After the combination of 4Gy irradiation, the cell apoptosis rate in over-expressed group was obviously higher than that of control group ($p<0.05$). After inhibitor transfected, the cell apoptosis in control group

decreased ($p>0.05$). And after the irradiation combination, the comparison of apoptosis rate had no statistically significant difference compared with control group ($p>0.05$), as seen in figure 1 and table 3. Flow cytometry detection demonstrated that the cellular



Mouseover a miRNA maturt name to see the miRNA/BECN1 alignment.

Fig.3: Prediction of target gene of miR-21 beclin1

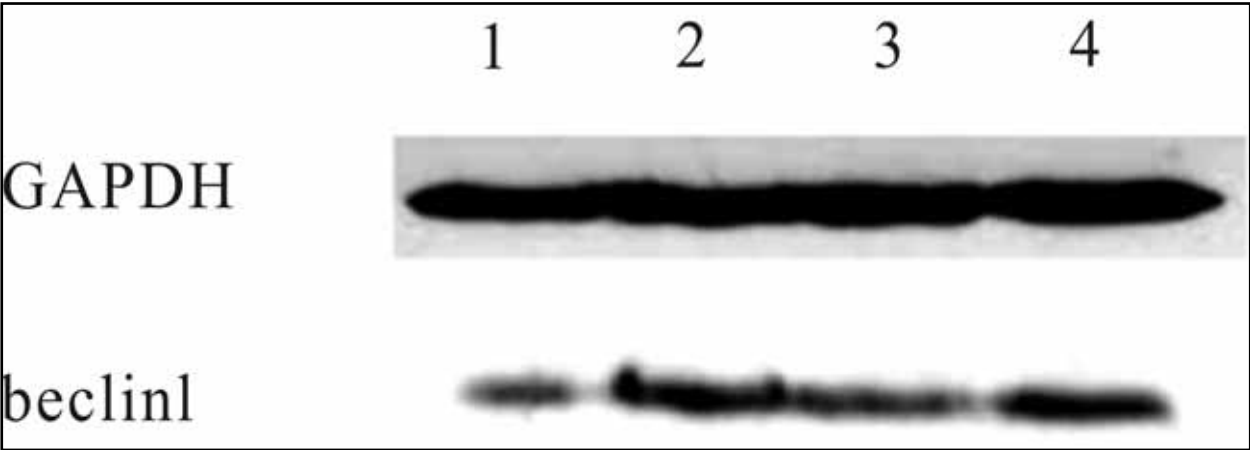


Fig.4: Electrophoregram of expressions of beclin1 protein in Hela cells in various groups
Lane 1: miR-21 NC group; Lane 2: miR-21 mimics group; Lane 3: miR-21 NC + 4Gy group;
Lane 4: miR-21 mimics + 4Gy group.

autophagy in radiotherapy group distinctly elevated compared to sham-irradiated group ($p<0.05$). After mimics transfection, compared to NC transfection, cellular autophagy increased significantly ($p<0.05$). After the combination irradiation, the autophagy rate in group of mimics plus 4Gy was obviously higher than group of NC plus 4Gy ($p<0.05$). After the inhibitor transfection, compared to control group, the autophagy rate slightly increased. But there was no statistically significant difference ($p>0.05$). After the combination irradiation, the cell autophagy in transfected inhibitor group evidently rose compared to control group ($p<0.05$). The results above proved that

miR-21 over expression could increase cellular autophagy, as seen in figure 2 and table 3.

miR-21 target gene detected by bioinformatics methods

The luciferase report gene analysis experiment was conducted in this part. miR-21 mimics/ NC and pMIR-beclin1 carrier was combined transfected into HeLa cells. The relative value of dual luciferase report gene was detected 8 hours after transfection. Compared to NC group (1.00 ± 0.05), the relative value of pMIR-beclin1 luciferase activity (1.33 ± 0.04) obviously increased after miR-21 over expressed ($p<0.05$). miR-21 clearly promoted the pMIR-beclin1

luciferase activity, as seen in Fig.3. Western Blotting gray analysis results reported that, the beclin1 expression increased after miR-21 mimics being transfected. And after the combination irradiation of 4Gy, beclin1 expression increased compared to sham-irradiation group, and further verified and promoted the occurrence of autophagy, as seen in Fig.4 .

DISCUSSION

microRNAs was a human small fragment and conservatively evolutionary noncoding single-stranded RNA, had the function of regulation of gene expression in translation level, and caused mRNA degradation or transcription inhibition through the combination of base pair match and target mRNA sequence 3'UTR or combined coding regions, thus inhibited the target protein synthesis. miR-21 located on 17q23.2 chromosome of FRA17B fragile region, and with independent miRNA transcription unit. Research [9] in recent years reported that miR-21 played an important role in variety of blood system and solid tumors. miR-21 was the only miRNA that over expressed in solid tumors. At present, miR-21 was to be one tumor markers, which had vital clinical significance on tumor staging, treatment and prognosis. Studies [10] stated that miR-21 could protect the cell apoptosis induced by chemotherapeutic medicine temozolomide. Experiment from Wu et al [11] demonstrated that miR-21 could down regulated Bcl-2 expression and promote apoptosis of DC cells in HEK-293 cells. The above results suggested miR-21 biological function in different tissue cells was depend on the type and specificity of located tissue cells.

Bioinformatics method is one of the important measure to predict miRNAs target genes,. It could be verified by experimental method through website prediction. The experimental verified miR-21 related target genes had programmed cell death factor-4 (PDCD4),

tropomyosin-1 (TPM1), phosphatase and tensin homolog (TPM1), maspin, etc [12-16]. Beclin1 as the miR-21 target gene did not have such related reports.

Radiation therapy had become one of the main treatment measure of cervical cancer. Approximate 80% of cervical cancer patients received the comprehensive therapy of single radiation or combined radiation, especially for cervical cancer patients in late stage or above inoperable second stage. Radiotherapy could obtain ideal effect. Many scholars at home and abroad had relate miRNAs and tumor radiosensitivity, tried to figure out the potential link to create a new pathway for tumor radiation therapy.

beclin1 was the homologous genes of yeast autophagy genes Atg6/Vps30, also known as BECN1, and lead an vital role in the process of autophagy and tumor occurrence. beclin1 involved in the formation of autophagy body and contributed to the mature of autophagy body, and interacted with regulatory proteins of upstream and downstream, constituted the multiply signal regulating circuit, and played counted function on the occurrence and development of autophagy and tumors. This results showed that miR-21 highly expressed in cervical cancer HeLa cells, and firstly researched beclin1 was the target genes of miR-21. beclin1 was key factor of mediated autophagy protein located at former autophagosome. miR-21 up-regulated the expression of beclin1 gene in cervical cancer HeLa cells, and promoted occurrence of cellular autophagy. This research proposed that over-expressed miR-21 promoted the increase of radiation induced cellular apoptosis, while, the radiation sensitivity had no changes, which might due to the autophagy promoting the survival function, which offset the effects of cell apoptosis.

CONCLUSION

In conclusion, miR-21 could promote radiation

induced cellular apoptosis and autophagy in cervical cancer HeLa cells. Its mechanism may be related to the up-regulated target gene beclin1 of miR-21, and had no function on cellular radiation sensitivity changes. The specific mechanism remained to be further researched.

Conflict of Interest: None

REFERENCES

1. Bloomstone M, Frankel WL, Petrocca F, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis[J]. *JAMA*, 2007, 297(17):1901-1908.
2. Ciafre SA, Galardi S, Mangiola A, et al. Extensive modulation of a set of microRNAs in primary glioblastoma[J]. *Biochem Biophys Res Commun*, 2005, 334(4):1351-1358.
3. Lorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer[J]. *Cancer Res*, 2005, 65(16):7065-7070.
4. Schetter AJ, Leung SY, Sohn JJ, et al. MicroRNA expression profiles associated with prognosis and the therapeutic outcome in colon adenocarcinoma[J]. *JAMA*, 2008, 299(4):425-436.
5. Lui WO, Pourmand N, Patterson BK, et al. Patterns of known and novel small RNAs in human cervical cancer[J]. *Cancer Res*, 2007, 67(13):6031-6043.
6. Halimi M, Parsian H, Asghari SM, et al. Clinical translation of human microRNA 21 as a potential biomarker for exposure to ionizing radiation[J]. *Transl Res*, 2014, 163(6):578-584.
7. Chao TF, Xiong HH, Liu W, et al. MiR-21 mediates the radiation resistance of glioblastoma cells by regulation PDCD and hMSH2[J]. *J Huazhong Univ Sci Technol Med Sci*, 2013, 33(4):525-529.
8. Anastasov N, Hofig I, Vasconcellos IG, et al. Radiation resistance due to high expression of miR-21 and G2/M checkpoint arrest in breast cancer cells[J]. *Radiat Oncol*, 2012, 7. doi:10.1186/1748-717x-7-206.
9. Lu Z, Liu M, Stribinskis V, et al. MicroRNA-21 promotes cells transformation by targeting the programmed cell death 4 gene[J]. *Oncogene*, 2008, 27(31):4373-4379.
10. Shi L, Chen J, Yang J, et al. Mir-21 protected human glioblastoma U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase-3 activity[J]. *Brain Res*, 2010, 1352:255-264.
11. Wu Z, Lu H, Shen J, et al. Inductive microRNA-21 impairs anti-mycobacterial responses by targeting IL-12 and Bcl-2[J]. *FEBS Lett*, 2012, 586(16):2459-2467.
12. Cao Z, Yoon JH, Nam SW, et al. PDCD4 expression inversely correlated with miR-21 levels in gastric cancer[J]. *J Cancer Res Clin Oncol*, 2012, 138(4):611-619.
13. Meng F, Henson R, Wehbe-Janek H, et al. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer[J]. *Gastroenterology*, 2007, 133(2):647-658.
14. Torres A, Torres K, Paszkowski T, et al. Highly increased maspin expression corresponds with up-regulation of miR-21 in endometrial cancer: a preliminary report[J]. *Int J Gynecol Cancer*, 2011, 21(1):8-14.
15. Zhu S, Si ML, Wu H, et al. MicroRNA-21 targets the tumor suppressor gene tropomyosin I (TPMI)[J]. *J Biol Chem*, 2007, 282(19):14328-14336.
16. Zhang BG, Li JF, Yu BQ, et al. MicroRNA-21 promotes tumor proliferation and invasion in gastric cancer by targeting PTEN[J]. *Oncol Rep*, 2012, 27(4):1019-1026.