Original Paper

Inhibition of MicroRNA-21-5p Promotes the Radiation Sensitivity of Non-Small Cell Lung Cancer Through HMSH2

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Key Words

MicroRNA-21-5p • HMSH2 • Non-small cell lung cancer • Radiation sensitivity

Abstract

Background: This study aimed to explore the effects of microRNA-21-5p (miR-21-5p) on the radiation sensitivity of non-small cell lung cancer (NSCLC) and the involvement of human MutS homolog 2 (hMSH2). **Methods:** Fourteen NSCLC patients at stage II or III who received surgery and postoperative radiotherapy were enrolled in this study. The patients were assigned into radiation-sensitive and -insensitive groups. NSCLC A549 cells were transfected to generate control, Negative control (NC), miR-21-5p inhibitor, miR-21-5p mimic, small interfering hMSH2 (sihMSH2), miR-21-5p inhibitor + sihMSH2 and hMSH2 overexpression groups. Immunohistochemistry was performed to detect the hMSH2 expression in the irradiated and non-irradiated cells. Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting were performed to evaluate A549 miR-21-5p and hMSH2 expression in transfected and irradiated cells. A colony formation assay was adopted for cell survival analysis. The relationship between miR-21-5p and hMSH2 was verified by a luciferase reporter assay. Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, and apoptosis was assessed by flow cytometry. Nude mouse models were established, and tumor volumes and tumor weights were recorded. **Results:** The radiation-sensitive group of patients exhibited lower miR-21-5p but higher hMSH2 expression than the insensitive group. For irradiated A549 cells, lower cell survival, higher apoptosis, increased miR-21-5p expression and decreased hMSH2 expression were observed at 6 and 8 Gy than at 0, 2 and 4 Gy; compared to 6 Gy, cell survival and hMSH2 expression were decreased and apoptosis and miR-21-5p expression were increased at 8 Gy. Additionally, miR-21-5p was found to target hMSH2. Compared with the control group, the cell survival rate was lower and the apoptosis rate higher in the miR-21-5p inhibitor group, whereas the opposite was observed for the miR-21-5p mimic and sihMSH2 groups. For the mouse model, decreased tumor volume and tumor weight and higher hMSH2 expression were

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found in the miR-21-5p inhibitor, radiation, hMSH2 overexpression, miR-21-5p inhibitor + radiation and hMSH2 overexpression + radiation groups compared with the control group. In addition, tumor volume and tumor weight were decreased and hMSH2 expression increased in the miR-21-5p inhibitor + radiation and hMSH2 overexpression + radiation groups compared with the radiation alone group. **Conclusion:** These findings indicate that inhibition of miR-21-5p can promote the radiation sensitivity of NSCLC by targeting hMSH2.

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Introduction

Non-small cell lung cancer (NSCLC) comprises the three most common types of lung cancer: squamous cell carcinoma (SCC), large cell carcinoma (LCC) and adenocarcinoma [1]. Due to its high incidence and mortality, lung cancer has for several decades been among the most frequent malignant cancers worldwide [2]. Approximately 85% of lung cancers are confirmed as NSCLC [3]. Risk factors of NSCLC are smoking (the leading cause), asbestos exposure, radon exposure and chronic interstitial pneumonitis [4]. Moreover, approximately two-thirds of NSCLC patients are already at a locally advanced or metastatic stage when diagnosed, and the 5-year survival rate is only 16.6% [5]. In addition to cigarette smoking, a well-known cause of lung cancer, complex interactions between genetic factors (e.g., *rs667282* TT/TC and CC genotypes) and environmental exposures (xenobiotics or inhaled exogenous carcinogens) contribute to NSCLC [6]. The major therapeutic methods for NSCLC are surgery, chemotherapy, radiotherapy and palliative care. Radiotherapy is regarded as a radical primary therapy with non-symptom-driven intention or as a consolidation therapy after initial chemotherapy, and it is reported that a biologically equivalent dose ≥ 53 Gy halts disease progression. Recently, increasing evidence has demonstrated the promising outcomes of radiotherapy for selected patients with advanced NSCLC, with a median overall survival (OS) ranging from 10 to 27 months [7].

MicroRNAs (miRNAs) are an abundant class of small non-coding RNAs, roughly 19~22 nucleotides in their mature form [8]. They posttranscriptionally regulate the expression of hundreds of target genes, thereby serving as oncogenes or tumor suppressors by modulating a wide range of biological functions such as cellular proliferation, differentiation, metastasis, apoptosis, angiogenesis [9–11]. As an oncomicroRNA involved in tumor progression, microRNA-21-5p (miR-21-5p) is a potential therapeutic target in lung cancer [12]. Overexpression of miR-21-5p has been found in many human malignancies, including NSCLC [13]. For example, high expression of miR-21-5p enhances viability, invasion, viability, and migration in NSCLC cell line (NCI-H157), and down-regulation of miR-21-5p significantly decreases the levels of certain protein in these cells [12]. Although radiation therapy is long regarded as a major treatment approach for NSCLC, its effects are limited by radioresistance, as NSCLC cells are often resistant. As one of the primary proteins in the mismatch repair (MMR) system, human MutS homolog 2 (hMSH2) forms heterodimers with other homologs to perform multiple repair processes in response to DNA damage [14]. Moreover, hMSH2 plays a different role in NSCLC, with a function that is associated with smoking status [15]. Although hMSH2 is a known target gene of the microRNA (miRNA) miR-21-5p, with an effect on cell viability and the cell cycle in lung cancer [16], the relationship between miR-21-5p and hMSH2 with regard to the radiation sensitivity of NSCLC has rarely been reported. Therefore, our study aimed to explore the effect of the targeting of hMSH2 by miR-21-5p on radiation sensitivity in NSCLC.

Materials and Methods

Study subjects

One hundred fourteen NSCLC patients at stage II or III who were treated with surgery and postoperative radiotherapy at Zhangjiagang First People's Hospital (accumulated radiotherapy dose, 60 Gy; ^{60}Co photon

beam; weekly dose, 10 Gy) from May 2012 to July 2015 were evaluated. The selected patients did not receive any treatment prior to surgery. Radiotherapy sensitivity was assessed according to the OS rate and local or distant recurrence rate; additionally, all of the patients were divided into a sensitive group ($n = 61$) or an insensitive group ($n = 53$) [17]. Tissue samples from the selected patients were stored in liquid nitrogen until used. Among the included patients, 69 were male and 45 female; the age range was 33-75 years, with a mean age of 61.7 years. Among the patients, 61 had squamous cell carcinoma and 53 adenocarcinoma. 37 had high differentiation, 35 moderate differentiation, and 42 low differentiation; 50 of the patients had lymph node metastasis (LNM) and 64 did not. Regarding surgical procedures, 46 patients were treated with simple lobectomy, 38 with bilateral lung resection and 30 with total lung resection. The study was approved by the ethics committee of Zhangjiagang First People's Hospital, and all of the patients provided informed consent.

Cell culture and radiation regimen

NSCLC A549 cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in an incubator at 37°C with 5% CO₂ in RPMI 1640 medium (Invitrogen, Gibco, NY, USA) containing 10% fetal bovine serum (Invitrogen Gibco, NY, USA). Cells at 80–100% confluence were digested and collected for cell suspension (5×10^4 /ml). The cells were then seeded into 6-well plates at 2 mL per well, and all plates were γ -ray irradiated using the PHOENIX irradiator (Phoenix Contact, Japan). Doses were 0 Gy, 2 Gy, 4 Gy, 6 Gy or 8 Gy, and the medium was changed daily.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from NSCLC tissues, NSCLC tissues of patients receiving different radiation doses and cells in each group according to the manufacturer's instructions (QIAGEN, Valencia, CA). The optical density (OD) 260/280 value of the extracted RNA was measured using an ultraviolet radiation (UV) spectrophotometer; the concentration was calculated, and the RNA was stored at -80°C. Reverse transcription was carried out according to kit instructions (QIAGEN, Valencia, CA). Primers were designed based on gene sequences in the GenBank and NCBI database using Primer 5.0; the sequences are shown in Table 1. The designed primers were synthesized by Shanghai Sangon Company (Shanghai, China). PCR was carried out in a 25- μ L volume and included SYBR PremixExTaq (10 μ L), forward primer (0.8 μ L), reverse primer (0.8 μ L), ROX Reference Dye II (0.4 μ L), DNA template (2 μ L), and dH₂O (6 μ L). The reaction conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 15 s. U6 and β -actin were used as internal reference for miR-21-5p and hMSH2, respectively. A dissolution curve was added to evaluate the reliability of the results. Relative expression of the target gene was calculated using the $2^{-\Delta\Delta C_t}$ method [18].

Western blotting

Cells were lysed, and the protein was extracted, and the concentration was assessed using a bicinchoninic acid (BCA) kit (Wuhan Boster Bioengineering Co., Ltd., Wuhan, China) according to the manufacturer's instructions. Extracted proteins were combined with sample buffer and boiled at 95°C for 10 min, 30- μ g proteins in each lane. The protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Wuhan Boster Bioengineering Co., Ltd., Wuhan, China) at 80 V to 120 V. The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane at 100 mV for 45-70 min. After 1 h of blocking with 5% bovine serum albumin (BSA) at room temperature, a primary antibody against hMSH2 (1:1000 dilution; Abcam, Cambridge, England) was incubated overnight at 4°C. After rinsing 3 times each with Tris-buffered saline and Tween (TBST) for 5 min, corresponding secondary antibodies were added, followed by incubation at room temperature for 1 h. The membrane was washed as above, and chemiluminescence reagent and the Bio-Rad Gel Dol EZ

Table 1. Primer sequences for quantitative real-time polymerase chain reaction (qRT-PCR), mir-21-5p, microRNA-21-5p; hMSH2, human MutS homolog 2

Gene	Upstream primer (5'~3')	Downstream primer (5'~3')
Has-mir-21-5p	ACGTTGTGTAGCTTATCAGACTG	AATGGTTGTTCTCCACACTCTC
hMSH2	AATGACTTGGAAAAGAAGATGC	TTAAAGAAGTCAATTTGCTGTTG
β -actin	CAGAAGGAGATTACTGCTCTGGCT	TACTCCTGCTTGCTGATCCACATC
U6	CTCGGTTCCGCAGCACAC	AACGCTTCACGAATTGCGT

imaging instrument (GEL DOC EZ IMAGER, Bio-Rad, California, USA) were utilized for detection. β -Actin (1:3000 dilution; Becton, Dickinson and Company, USA) was used as an internal reference. The gray-scale values of target bands were analyzed using Image J software.

Colony formation assay

A549 cells at the logarithmic growth phase were digested with pancreatin to generate single-cell suspensions and then seeded into 6-well plates. The suspensions were divided into 0 Gy, 2 Gy, 4 Gy, 6 Gy and 8 Gy groups, with respective cell numbers of 500, 2,000, 4,000, 6,000, and 10,000; three parallel samples were prepared for each group. After adherence, cells were γ -ray irradiated for 2 h and incubated at 37°C with 5% CO₂ for 2 weeks; fresh medium was added as appropriate. After 2 weeks, the culture medium was removed, followed by washing with phosphate-buffered saline (PBS), fixation in 75% methanol for 1 min, and staining in 0.5% crystal violet staining solution for 20 min. The cells were washed with water, and the number of cells was counted (colonies with more than 50 cells by microscopic observation were recorded as a clone). The multi-target single-hit model [SF = 1 - (1 - eD/D₀)^N] in SigmaPlot 8.0 software was used to produce a cell survival curve to obtain the survival fraction (SF) under different radiation doses.

Cell transfection

Sequences for hMSH2 overexpression as well as miR-21-5p inhibitors and mimics were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences were as follows: miR-21-5p inhibitor: 5'-UCAACAUCAGUCUGAUAAGCUA-3'; miR-21-5p mimic: 5'-AACAUCAUCUGAUAAGCUAUU-3'; negative control (NC), 5'-CAGUACUUUUGUGUAGUACAA-3'; human small interfering RNA (siRNA) (siRNA): forward sequence, 5'-CCACGUUCAUGGCUGAAAUTT-3'; reverse sequence, 5'-AUUUCAGCCAUGAACGUGGTT-3'. Cells were divided into control, miR-21-5p NC, miR-21-5p mimic, sihMSH2, miR-21-5p inhibitor + sihMSH2 and hMSH2 overexpression groups. Liposome Lipofectamine 2000 was used for transient transfection. One day before transfection, cells were digested and seeded into 6-well plates; when the cell density reached 90-95%, 50 μ L of serum-free culture medium was used to dilute 1.0 μ g of each construct and 1 μ L of Lipofectamine 2000 transfection reagent. The transfection reagent was mixed with the diluted construct within 5 min and incubated at room temperature for 20 min. After changing the medium, this mixture was added to each well; the culture plate shaken to mix well, followed by culture in an incubator containing 5% CO₂ at 37°C for 48 hours later, the medium was replaced. After transfection for 12 h, γ -ray radiation at different doses (0, 2, 4, 6 and 8 Gy, respectively) was carried out. The cells were then continuously cultured for 24 h before subsequent experiments.

Luciferase reporter gene assay

The online miRNA target gene prediction tool Targetscan (<http://www.targetscan.org/>) was used to screen target genes of miR-21 and to predict the binding site of miR-21 and in the 3' untranslated region (UTR) of hMSH2. Based on the predicted binding site and the human genome sequence, the hMSH2 3'UTR sequence containing the miR-21 binding site was amplified by PCR. The primer sequences: Forward, 5'-CCGCTCGAGATCCCAGTAATGGAATGAAG3'; Reverse, 5'-TAATGCCACCGCCATCACTTATTATTGCCTATGT3'. After XhoI and NotI digestion, the fragment was inserted upstream of the promoter of the psiCHECK-2 vector (Promega Corporation, USA). The hMSH2 3'UTR wild-type (WT) plasmid was constructed and named phMSH2-Wt. Based on this plasmid, a mutated primer was used to induce mutation in the miR-21 binding site to construct the hMSH2 3'UTR mutant (MUT) plasmid phMSH2-Mut (Primer sequences: Forward, 5'-CCGCTCGAGTGAAGGTAATATTGTGAAGCTATTGTCT3'; Reverse, 5'-ATAAGAATGCGCGCCATCACTTATTATTGCCTATGT3'). Sequencing was performed to confirm successful cloning. A plasmid extraction kit (Promega Corporation, USA) was used to generate four groups: miR-21 mimic + p hMSH2-WT (WT + mimic group), miR-21 mimic + p hMSH2-MT (MT + mimic group), miR-21 NC + p hMSH2-WT (WT + NC group) and miR-21 NC + p hMSH2-MT (MT + NC group). The reporter gene vector used in the experiment was pcDNA3.1-luc (GenePharma company, Shanghai, China), encoding firefly luciferase. To assess the transfection efficiency, pRL-TK, which encodes *Renilla* luciferase, was used as an internal control. Fluorescent STOP & Glo Reagent was preheated to establish a zero value for fluorescence measurement. Thereafter, 40 μ L of LARII and 40 μ L of STOP & Glo Reagent were added to a tube, and fluorescence was measured and recorded using a fluorescence meter.

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay

The MTT reagent kit (DOJINDO Company, Japan) was used for detecting cell viability. Cells of the different groups at the logarithmic growth phase were digested with 0.25% pancreatin and seeded into 96-well plates at a cell density of 5000/cell, with each well containing 100 μ L. After adherence, the cells were γ -ray irradiated at 0, 2, 4, 6 and 8 Gy for 2 h and then cultured for 24 h before 10 μ L MTT in medium was added to each well. The culture plate was incubated at 4°C for 1-4 h, after which the absorbance at 490 nm was detected using a microplate reader (Thermo Fisher Scientific, Waltham, USA). Cell survival rate = $(\text{OD value}_{\text{treatment group}} - \text{OD value}_{\text{control group}}) / (\text{OD values}_{\text{NC group}} - \text{OD value}_{\text{negative blank group}}) \times 100\%$.

Flow cytometry

The Annexin V reagent kit (Beyotime Biotechnology, Beijing, China) was utilized for detecting apoptosis. Cells of the different groups were cultured for 12 h, γ -ray irradiated at 0, 2, 4, 6 and 8 Gy and cultured for another 48 h. The cell culture medium in the 6-well plate was collected and placed into a centrifuge tube and then analyzed for cell lysis. The cells were washed with PBS, and 0.25% pancreatin was used for digestion. This sample was subjected to centrifugation at $12000 \times g$ for 5 min at 4°C, after which the supernatant was discarded. The pellet was collected, and the cells were prepared as a dense cell suspension containing 50,000-100,000 cells with PBS, followed by centrifugation for another 5 min; the supernatant was discarded. A 195- μ L aliquot of Annexin V-fluorescein isothiocyanate (FITC) was added to the cell suspension, to which 5 μ L of Annexin V-FITC was added, followed by mixing and incubation in the dark for 10 min at room temperature. After centrifugation for 5 min, the supernatant was collected. Next, 190 μ L of the Annexin V-FITC sample and 10 μ L of propidium iodide solution were added. After mixing well, the new mixture was incubated in an ice bath, followed by flow cytometry analysis (BD FACSCalibur II, USA). Early apoptosis was detected in the lower right quadrant, advanced apoptosis was detected in the upper right quadrant, and surviving cells were detected in the lower left quadrant. The apoptosis rate = early apoptosis rate + late apoptosis rate.

Mouse model establishment

This animal experiment was approved by the ethics committee of Zhangjiagang First People's Hospital. BALB/c male nude mice aged 6 weeks and weighing 18 ± 2 g purchased from Xipuer-Bikai Experimental Animal Co., Ltd. (Shanghai, China) were used in the experiment and raised under specific pathogen-free (SPF) conditions. After adaptive feeding for one week, the mice were randomly divided into model, miR-21-5p inhibitor, hMSH2 overexpression, radiation group, miR-21-5p inhibitor + radiation and hMSH2 overexpression + radiation groups. Subcutaneous injection was performed in the right hind legs, as follows: 100 μ L of A549 cells for the model group; 1×10^7 cells/ml miR-21-5p inhibitor cells for the miR-21-5p inhibitor and miR-21-5p inhibitor + radiation groups; and 1×10^7 cells/mL hMSH2-overexpressing cells for the hMSH2 overexpression + radiation group. Tumor growth was observed. When the tumor volume reached 100 mm³, the mice in the latter two groups were γ -ray irradiated at a total dose of 32 Gy (8 Gy/week). A Vernier caliper was used to measure tumor volume. On the 28th day, all of the mice were killed (no mice died for the study died before the end of the experiment), and the tumor tissues were collected for further immunohistochemical analysis.

Immunohistochemistry

All tissue samples were fixed and embedded using conventional paraffin. The samples were cut into 5- μ m-thick slices, dehydrated with xylene and an alcohol gradient, and subjected to antigen retrieval using citrate antigen repair solution at a pH of 7.2-7.4. The primary anti-hMSH2 antibody (1:1000; Cell Signaling Technology, Inc, Massachusetts, USA) was added, and the tissue samples were incubated overnight at 4°C. The samples were then incubated for 1 h at 37°C and washed with PBS (0.01 mol/L); a biotin-labeled secondary antibody (Wuhan Boster Bioengineering Co., Ltd., China) was added dropwise. The samples were incubated at 37°C for 30 min and stained for 10 min with diaminobenzidine (DAB). The cells were then stained with hematoxylin, dehydrated by an alcohol gradient, sealed with neutral balsam and observed under a light microscope. The average optical density was calculated using Image-Pro Plus software for pathological image analysis, and statistical analysis was conducted.

The judgment criteria for the experimental results were that the hMSH2 protein signal was located in the nucleus, and positive staining was displayed as yellow or dark-brown granules. Integration was achieved

in accordance with the percentage of the positive area of the total area [15]: 0 points (0%), 1 point (1-10%), 2 points (11-50%) and 3 points (>50%). The staining intensity was scored and stratified as follows: 0 points (negative), 1 point (weakly positive), 2 points (moderate) and 3 points (strong positive). Sum of the integration of staining intensity and range ≥ 4 was defined as high expression; that < 4 was defined as low expression.

Table 2. Relationship between radiation sensitivity and clinicopathological characteristics in NSCLC patients in the sensitive and insensitive groups, NSCLC, non-small cell lung cancer

Clinicopathological characteristic	n	Sensitive group (n, %)	Insensitive group (n, %)	P
Gender				
Male	69	37 (60.7%)	32 (60.4%)	1.000
Female	45	24 (39.3%)	21 (39.6%)	
Age				
< 58	48	22 (36.1%)	26 (49.1%)	0.000
≥ 58	66	39 (63.9%)	27 (50.9%)	
Pathologic type				
Squamous-cell carcinoma	61	32 (52.5%)	29 (54.7%)	0.852
Adenocarcinoma	53	29 (47.5%)	24 (45.3%)	
Degree of differentiation				
High differentiation	37	21 (34.4%)	16 (30.1%)	0.769
Moderate differentiation	35	17 (27.9%)	18 (33.3%)	
Low differentiation	42	23 (37.7%)	19 (35.6%)	
Lymphatic metastasis				
Yes	50	27 (44.3%)	23 (43.4%)	0.926
No	64	39 (63.9%)	25 (46.6%)	
Operation process				
Simple lobectomy	46	25 (40.3%)	22 (41.5%)	0.961
Bilateral lung resection	38	21 (34.4%)	17 (32.1%)	
Total lung resection	30	16 (26.3%)	14 (26.4%)	

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) version 18.0. The measurement data are reported as the mean $\pm$ standard deviation (SD). The t-test and paired t-test were used for comparison between two groups of measurement data conforming to a normal distribution. One-way analysis of variance (ANOVA) was used for multiple groups. Measurement data are expressed as the percentage and rate, as analyzed using the χ^2 test. A value of $P < 0.05$ was regarded as statistically significant.

Results

Relationship between radiation sensitivity and clinicopathological characteristics of NSCLC patients

No obvious correlation between radiation sensitivity and clinicopathological characteristics, including age, gender, pathologic type, degree of differentiation, lymphatic metastasis and surgical intervention, was found for the examined NSCLC patients (all $P > 0.05$) (Table 2).

Correlation between tissue expression of miR-21-5p and hMSH2 between sensitive and insensitive NSCLC patients

RT-PCR was used to detect mRNA levels of miR-21-5p and hMSH2 in lung cancer tissues. The level of miR-21-5p mRNA was significantly decreased ($P < 0.05$) and that of hMSH2 markedly increased ($P < 0.05$) in the sensitive group ($n = 61$) compared with the insensitive group ($n = 53$) (Fig. 1A). Western blotting was used to detect the protein levels of hMSH2 in lung cancer tissues. Compared with the insensitive group, the level of hMSH2 protein in the sensitive group was notably increased, and the difference was statistically significant ($P < 0.05$) (Fig. 1B).

Correlation of miR-21-5p and hMSH2 expression levels with radiation sensitivity in A549 cells under different γ -ray radiation doses

The cell clone formation method was adopted for detecting the sensitivity of A549 cells under different doses of γ -ray radiation. The A549 cell survival rate gradually decreased with increasing dose. Moreover, the survival rates at 6 and 8 Gy were both significantly

Fig. 1. Expression levels of miR-21-5p and hMSH2 in lung cancer tissues between sensitive and insensitive groups. A, mRNA levels of miR-21-5p and hMSH2 mRNA in the sensitive group (n = 67) and insensitive group (n = 53), as detected by qRT-PCR. B, Protein level of hMSH2 between the sensitive and insensitive groups, as detected by western blotting; *, $P < 0.05$, compared with the insensitive group. miR-21-5p, microRNA-21-5p; hMSH2, human DNA MutS homolog 2; qRT-PCR, quantitative real-time polymerase chain reaction.

Fig. 2. Relationship between levels of miR-21-5p and hMSH2 expression and radiation sensitivity in NSCLC A549 cells under different doses of γ -ray radiation. A, Survival curve of A549 cells based on the colony formation assay. B, Relationship between mRNA level of miR-21-5p and radiation dosage, as detected by qRT-PCR. C, Relationship between mRNA level of hMSH2 and radiation dosage, as detected by qRT-PCR. D, Relationship between protein level of hMSH2 and radiation dosage, as detected by western blotting; *, $P < 0.05$, compared with 0 Gy; #, $P < 0.05$, compared with 2 Gy; Δ , $P < 0.05$, compared with 4 Gy; Δ , $P < 0.05$, compared with 6 Gy. miR-21-5p, microRNA-21-5p; hMSH2, human MutS homolog 2; NSCLC, non-small cell lung cancer; qRT-PCR, quantitative real-time polymerase chain reaction.

decreased compared with those at 0, 2 and 4 Gy (all $P < 0.05$), and the survival rate at 8 Gy was markedly decreased compared with that at 6 Gy ($P < 0.05$) (Fig. 2A). The relative expression levels of miR-21-5p and hMSH2 under various doses of γ -ray irradiation were detected by qRT-PCR and western blotting, with the results showing that the level of miR-21-5p mRNA increased and the levels of hMSH2 mRNA and protein both decreased with increasing γ -ray radiation dose. Compared with γ -ray radiation at 0, 2 and 4 Gy, 6 and 8 Gy resulted in an increased level of miR-21-5p mRNA but decreased levels of hMSH2 mRNA and protein (all $P < 0.05$). Compared with γ -ray radiation at 6 Gy, the mRNA level of miR-21-5p was significantly increased but the mRNA and protein levels of hMSH2 markedly decreased at 8 Gy (all $P < 0.05$) (Fig. 2B, C).

Fig. 3. Relative luciferase activity of hMSH2 3'UTR, miR-21-5p mimic and miR-21 NC. A, B, Two binding sites for miR-21-5p are present in the hMSH2 3'UTR. C, Analysis of the relative luciferase activity of hMSH2 3'UTR, miR-21-5p mimic and miR-21-5p NC after co-transfection; **, $P < 0.01$, compared with that before transfection. miR-21-5p, microRNA-21-5p; hMSH2, human DNA MutS homolog 2; NC, negative control.

Fig. 4. Cell viability in each group under different doses of γ -ray radiation, as detected by the MTT assay. miR-21-5p, microRNA-21-5p; hMSH2, human DNA MutS homolog 2.

The relations between hMSH2 and miR-21-5p

The online miRNA target gene prediction tool Targetscan (<http://www.targetscan.org/>) was used for predicting target genes of miR-21. hMSH2 was found to be a target gene of miR-21, with two binding sites for miR-21-5p in the 3'-UTR of the hMSH2 mRNA (Fig. 3A, B). Detection of luciferase reporter gene activity showed notably decreased relative luciferase

Table 3. Effect of miR-21-5p on cell viability in A549 cells of each group under different doses of γ -ray irradiation, *, $P < 0.05$, compared with the control group, #, $P < 0.05$, compared with 0 Gy, 2 Gy and 4 Gy, Δ , $P < 0.05$, compared with 6 Gy; miR-21-5p, microRNA-21-5p; NC, negative control; hMSH2, human MutS homolog 2

Group	0 Gy	2 Gy	4 Gy	6 Gy	8 Gy
Control group	0.91 ± 0.14	0.86 ± 0.13	0.82 ± 0.12	$0.68 \pm 0.07^\#$	$0.53 \pm 0.09^\# \Delta$
miR-21-5p NC group	0.92 ± 0.15	0.85 ± 0.16	0.81 ± 0.14	$0.66 \pm 0.04^\#$	$0.52 \pm 0.06^\# \Delta$
miR-21-5p inhibitor group	$0.63 \pm 0.13^*$	$0.54 \pm 0.12^*$	$0.51 \pm 0.13^*$	$0.37 \pm 0.08^\#$	$0.22 \pm 0.13^\#$
miR-21-5p mimics group	$1.28 \pm 0.12^*$	$1.18 \pm 0.13^*$	$1.13 \pm 0.15^*$	$0.97 \pm 0.13^\#$	$0.81 \pm 0.09^\#$
sihMSH2 group	$1.26 \pm 0.14^*$	$1.16 \pm 0.12^*$	$1.10 \pm 0.04^*$	$0.95 \pm 0.02^\#$	$0.80 \pm 0.08^\#$
miR-21-5pinhibitor + sihMSH2 group	0.89 ± 0.13	0.84 ± 0.11	0.83 ± 0.03	$0.69 \pm 0.06^\#$	$0.51 \pm 0.07^\#$
hMSH2 overexpression group	$0.63 \pm 0.13^*$	$0.54 \pm 0.12^*$	$0.51 \pm 0.13^*$	$0.37 \pm 0.08^\#$	$0.22 \pm 0.13^\#$

Fig. 5. Cell apoptosis in each group under different doses of γ -ray radiation, as detected by flow cytometry.

activity of co-transfection of wild-type hMSH2 3'UTR-WT and miR-21-5p mimic compared with that of the untransfected miR-21-5p NC group ($P < 0.01$). Changes in relative luciferase activity were not found with transfection of mutant hMSH2 3'UTR-MUT or miR-21-5p mimic and miR-21-5p NC (Fig. 3C). The results were consistent with the bioinformatic prediction, further confirming that miR-21-5p can bind to the seed region in the hMSH2 mRNA 3'-UTR.

miR-21-5p promotes A549 cell viability

The viability of different groups of cells under various doses of γ -ray radiation was detected by the MTT assay. The cell viability at 0, 2, 4, 6 and 8 Gy showed no apparent difference among the miR-21-5p NC, miR-21-5p inhibitor + sihMSH2 and control groups (all $P > 0.05$). The cell viability of the miR-21-5p inhibitor group under γ -ray radiation at 0, 2, 4, 6 and 8 Gy was significantly decreased compared with that of the control group (all $P < 0.05$), whereas largely increased in the miR-21-5p mimic and sihMSH2 groups (all $P < 0.05$). Decreased cell viability at both 6 and 8 Gy was found in comparison with those at 0, 2 and 4 Gy (all $P < 0.05$). Compared with 6 Gy, cell viability in every group at 8 Gy was also notably decreased (all $P < 0.05$). The results suggest that NSCLC radiation sensitivity can be enhanced by the effect of miR-21-5p on cell viability (Fig. 4, Table 3).

Table 4. Effect of miR-21-5p on cell apoptosis in A549 cells of each group under different doses of γ -ray irradiation, *, $P < 0.05$, compared with the control group, #, $P < 0.05$, compared with 0 Gy, 2 Gy and 4 Gy, ▲, $P < 0.05$, compared with 6 Gy; miR-21-5p, microRNA-21-5p; NC, negative control; hMSH2, human MutS homolog 2

Group	0 Gy	2 Gy	4 Gy	6 Gy	8 Gy
Control group	0.14 ± 0.07	0.15 ± 0.03	0.17 ± 0.05	0.24 ± 0.06 [#]	0.33 ± 0.12 ^{#▲}
miR-21-5p NC group	0.07 ± 0.02	0.12 ± 0.05	0.14 ± 0.04	0.23 ± 0.08 [#]	0.32 ± 0.09 ^{#▲}
miR-21-5p inhibitor group	0.23 ± 0.07*	0.24 ± 0.07*	0.25 ± 0.07*	0.33 ± 0.11 ^{#*}	0.47 ± 0.13 ^{#*}
miR-21-5p mimics group	0.04 ± 0.01*	0.06 ± 0.01*	0.09 ± 0.02*	0.15 ± 0.04 ^{**}	0.23 ± 0.07 ^{**}
sihMSH2 group	0.05 ± 0.02*	0.06 ± 0.01*	0.07 ± 0.01*	0.15 ± 0.03 ^{**}	0.24 ± 0.08 ^{**}
miR-21-5p inhibitor + sihMSH2 group	0.06 ± 0.03	0.10 ± 0.04	0.12 ± 0.04	0.21 ± 0.09 [#]	0.31 ± 0.11 [#]
hMSH2 overexpression group	0.23 ± 0.07*	0.24 ± 0.07*	0.25 ± 0.07*	0.33 ± 0.11 ^{#*}	0.47 ± 0.13 ^{#*}

Fig. 6. Histogram showing apoptosis rate in each group under different doses of γ -ray radiation, as detected by flow cytometry.

miR-21-5p inhibits A549 cell apoptosis

Flow cytometry was used to detect apoptosis in cells of each group under different doses of γ -ray radiation. No apparent difference in apoptosis under γ -ray radiation at 0, 2, 4, 6 and 8 Gy was found for the miR-21-5p NC and miR-21-5p inhibitor + sihMSH2 groups compared with the control groups (all $P > 0.05$). However, at 2, 4, 6 and 8 Gy, apoptosis in the miR-21-5p inhibitor group was significantly enhanced compared with that in the control group (all $P < 0.05$), though that in the miR-21-5p mimic and sihMSH2 groups was markedly decreased (all $P < 0.05$). Comparison of different doses of γ -ray radiation showed significantly increased apoptosis in each group at 6 and 8 Gy compared with that at 0, 2 and 4 Gy (all $P < 0.05$). Relative to 6 Gy, apoptosis was markedly increased at 8 Gy (all $P < 0.05$). The above results indicate that miR-21-5p enhanced the radiation sensitivity of NSCLC through its effect on apoptosis (Fig. 5 and 6, Table 4).

Fig. 7. Tumor volume and tumor weight in nude mice in each group. A, Comparison of the tumor volume of nude mice in each group; *, $P < 0.05$, compared with the model group; #, $P < 0.05$, compared with the radiation group; B, Comparison of the tumor weight of nude mice in each group; *, $P < 0.05$, compared with the model group; #, $P < 0.05$, compared with the radiation group. miR-21-5p, microRNA-21-5p; NSCLC, non-small cell lung cancer.

Comparison of tumor volume and tumor weight in nude mice

Tumor volume and tumor weight of NSCLC were measured. The results indicated lower tumor volume and tumor weight in the miR-21-5p inhibitor, radiation, hMSH2 overexpression, miR-21-5p inhibitor + radiation and hMSH2 overexpression + radiation groups compared with the model group (all $P < 0.05$). However, no significant differences in tumor volume and tumor weight were found in the miR-21-5p inhibitor group compared with the radiation group ($P > 0.05$), while those in the miR-21-5p inhibitor + radiation group and hMSH2 overexpression + radiation group were both decreased (all $P < 0.05$). The above results indicate that miR-21-5p expression can increase radiation sensitivity in the NSCLC mouse model (Fig. 7).

hMSH2 expression in nude mice

The level of hMSH2 expression in each group was detected by immunohistochemistry. The staining scores in the model, miR-21-5p inhibitor, hMSH2 overexpression, radiation, miR-21-5p inhibitor + radiation and hMSH2 overexpression + radiation groups were 1.12 ± 0.52 , 2.76 ± 0.21 , 3.58 ± 0.11 , 3.82 ± 0.25 , 5.63 ± 0.29 and 5.48 ± 0.16 , respectively. For the miR-21-5p inhibitor, hMSH2 overexpression, radiation, miR-21-5p inhibitor + radiation and hMSH2 overexpression + radiation groups, positive hMSH2 staining in the nucleus appeared as a brownish-yellow color. Only a small number of positive cells were in the model group. Compared with the model group, the level of hMSH2 protein was notably increased in the miR-21-5p inhibitor, radiation, hMSH2 overexpression, miR-21-5p inhibitor + radiation and hMSH2 overexpression + radiation groups (all $P < 0.05$). In contrast, compared with the radiation group, no obvious difference in the level of hMSH2 protein was observed in the miR-21-5p inhibitor group ($P > 0.05$), whereas that in the miR-21-5p inhibitor + radiation and hMSH2 overexpression + radiation groups was significantly increased ($P < 0.05$) (Fig. 8).

Discussion

It is reported that more than 50% of cancer patients receive radiotherapy during their treatment course, and radioresistance remains a major problem in the treatment of

Fig. 8. Effect of miR-21-5p on hMSH2 expression levels in nude mice of each group, as detected by immunohistochemistry ($\times 200$). A, results of immunohistochemistry in each group ($\times 200$). Red arrow, positive expression level of hMSH2 (brownish-yellow staining). B, evaluation of hMSH2 expression level in each group by immunohistochemistry; *, $P < 0.05$, compared with the model group; #, $P < 0.05$, compared with the radiation group.

NSCLC [19]. Thus, effective treatment that sensitizes radioresistant NSCLC to radiotherapy is urgently needed [13]. Recent research has reported that the many miRNAs affect the radiosensitivity of NSCLC cells, which may be associated with apoptosis inhibition [17, 20]. In this study, we targeted hMSH2 to explore the effects of miR-21-5p on the radiation sensitivity of NSCLC.

According to our results, miR-21-5p expression was significantly decreased and the mRNA and protein expression of hMSH2 was markedly increased in radiation-sensitive patients compared with radiation-insensitive patients. These findings suggest that miR-21-5p and hMSH2 might play roles in the radiation sensitivity of NSCLC and that inhibition of miR-21-5p might promote the radiation sensitivity of NSCLC through hMSH2. hMSH2 is an important gene the dysfunction of which may cause malignant transformation of tumor cells [21]. Consistent with our results, Zhong Z reported that miR-21-5p is a key regulator of hMSH2 and modulates the cell cycle and viability by targeting hMSH2 in human lung cancer [16]. Additionally, Chao et al. demonstrated that miR-21-5p might mediate the resistance of glioblastoma cells against radiation via its target genes PDCD4 and hMSH2 and that miR-21-5p and its target genes may be used for clinical radiotherapy sensitization in the future [22]. Additionally, Valeri et al. revealed that miR-21-5p induces resistance to 5-fluorouracil by down-regulating hMSH2 [23].

Furthermore, our findings suggest that miR-21-5p enhances the radiation sensitivity of NSCLC through its effect on cell viability and apoptosis. Research conducted by Zhu et al. indicated that down-regulation of miR-21 suppresses the viability of radioresistant cells and promotes elevated radiation sensitivity in CNE-2-1 cells [24]. Previous studies have indicated that changes in cytochrome c oxidase (COX) activity induce apoptosis [25, 26]. In addition, Guo et al. demonstrated that enhanced COX activity inhibits cell apoptosis and inhibition

of miRNA-21 suppresses NSCLC cell viability by promoting cell apoptosis via the decrease of COX-19 expression [27]. For further verification, we established an NSCLC nude mouse model and evaluated tumor volume and tumor weight as well as hMSH2 protein expression by immunohistochemistry. Compared with the model group, the tumor volume and tumor weight in the miR-21-5p inhibitor, radiation and miR-21-5p inhibitor + radiation groups were reduced; moreover, compared with the radiation group, the tumor volume and tumor weight in the miR-21-5p inhibitor + group were smaller, suggesting that miR-21-5p expression can improve radiation sensitivity in the NSCLC nude mouse model. Additionally, we found that compared with the model group, the protein expression of hMSH2 was notably increased in the miR-21-5p inhibitor, radiation and miR-21-5p inhibitor + radiation groups. Expression of hMSH2 protein in the miR-21-5p inhibitor + radiation group was increased significantly compared with the radiation group, suggesting that miR-21-5p perhaps mediate radiation sensitivity by down-regulating hMSH2.

Additionally, our results demonstrated an effect of radiation and miR-21-5p inhibitor. A previous study reported that beneficial effects of crizotinib on ALK-positive NSCLC elicited in combination with radiotherapy [28]. miRNAs play a critical role in the development of NSCLC and are considered as potential biomarkers for NSCLC diagnosis and therapy [29, 30]. Overexpression of miR-124, miR-148a, miR-3619-5p and miR-148a-3p suppresses the growth, proliferation, metastasis and invasion of NSCLC cells [31–33]. Moreover, one previous study indicated that miR-21 can stimulate growth and invasion in NSCLC [34]. Anastasov et al. also observed that miR-21 knockdown promotes apoptosis induced by radiation, with the viability of radiation-resistant cells being nearly the same as that of sensitive cells [35]. Another study also reported that inhibition of miR-21 significantly enhances the radiosensitivity of NSCLC [36]. According to our results, miR-21-5p enhanced the radiation sensitivity of NSCLC by promoting cell viability and inhibiting apoptosis.

Conclusion

In summary, our study indicates that inhibition of miR-21-5p can promote the radiation sensitivity of NSCLC by targeting hMSH2. Additionally, inhibiting miR-21-5p expression, which leads to upregulation of hMSH2 expression, may serve as a therapeutic target for clinically enhancing the radiation sensitivity of NSCLC. However, there are limitations in this study. It is possible that miR-21 regulates other genes related to cell cycle arrest, and the exact mechanism by which miR-21-5p mediates radiation sensitivity in NSCLC remains unclear. Thus, further studies should be performed to rule out the influence of other target genes of miR-21-5p and to clarify the specific mechanisms by which miR-21-5p mediates radiation sensitivity in NSCLC by targeting hMSH2.

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Disclosure Statement

The authors have declared that no competing interests exist.

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