LncRNA ANRIL negatively regulated chitooligosaccharide-induced radiosensitivity in colon cancer cells by sponging miR-181a-5p

Chunfeng Sun[1,A,D,E], Chen Shen[2,B,C], Yaping Zhang[3,C,E], Chunhong Hu[4,E,F]

[1] Department of Radiology, The First Affiliated Hospital of Soochow University, Affiliated Hospital of Nantong University, Department of Nuclear Medicine, China
[2] Department of Gastrointestinal Surgery, Affiliated Hospital of Nantong University, China
[3] Department of Radiology, The First Affiliated Hospital of Soochow University, Institute of Medicine Imaging, Soochow University, Suzhou, China
[4] Department of Radiology, The First Affiliated Hospital of Soochow University, Suzhou, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Address for correspondence
Chunhong Hu
E-mail: gyk45663@126.com

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Abstract

Background. The radiosensitivity of colon cancer cells can be regulated by noncoding RNAs.

Objectives. In this study, the lncRNA antisense non-coding RNA in the INK4 locus (ANRIL) was selected to analyze its regulatory role in chitooligosaccharides (COS)-related radiosensitivity in colon cancer cells.

Material and methods. The ANRIL expression in colon cancer cell lines was examined using real-time quantitative polymerase chain reaction (RT-qPCR), based on which we selected the cell line that presented the highest expression of ANRIL for radiosensitivity research. The cells were exposed to X-rays (0 Gy, 2 Gy, 4 Gy, and 6 Gy) and evaluated for changes in ANRIL and miR-181a-5p expression using RT-qPCR. Cell viability was evaluated using the CCK8 method, while apoptosis was detected with flow cytometry assays. Dual luciferase assays validated the binding between ANRIL and miR-181a-5p. The cell survival rates after differential COS treatments (0 mg/mL, 1.0 mg/mL, 2.0 mg/mL, 3.0 mg/mL, 4.0 mg/mL, and 5.0 mg/mL) were rated using CCK8 assay. The cells with the strongest dosage of COS (5.0 mg/mL) were selected to further investigate the role of ANRIL/miR-181a-5p in modulating the radiosensitivity observed with CCK-8 and flow cytometry assays.

Results. The ANRIL was highly expressed in colon cancer cells lines, especially in the SW480 cell line. Irradiation significantly decreased cell viability and ANRIL expression in a dose-dependent manner. The overexpression of ANRIL reduced the cell apoptosis rate after irradiation. MiR-181a-5p directly bound to ANRIL and was upregulated by irradiation in a dose-dependent manner. The suppression of miR-181a-5p decreased cell apoptosis. The COS treatment notably downregulated cell survival and promoted apoptosis in cells exposed to irradiation. The overexpression of ANRIL partially reversed COS-induced apoptosis and the inhibition rate; the upregulation of miR-181a-5p could counteract the impact of ANRIL regulation in cells.

Conclusions. The ANRIL negatively regulated radiosensitivity induced by COS in colon cancer cells by sponging miR-181a-5p.

Key words: colon cancer, lncRNA ANRIL, chitooligosaccharides, miR-181a-5p

Introduction

Colon cancer has been reported as one of the most common malignant tumors of the digestive system, with high morbidity and death rates which account for about 10% of cancer-related deaths.[1] Globally, colon cancer ranks as the 2nd most common cause of death in cancers.[2] Though the overall incidence rate of colon cancer is falling in many high-income countries, the morbidity of this cancer is increasing in adults under the age of 50 based on analyses in countries like the USA, Australia and Canada.[3]

In China, colon cancer has already become the 4th leading cause of death among all kinds of malignant tumors, with about 140,000 cases confirmed per year.[4] Although surgery is the first choice, radiotherapy and chemotherapy are also important methods for blocking the progression of colon cancer. Furthermore, patients with local recurrence are often treated with radiotherapy, which can attack cancer cells by ionizing radiation.[5] Nevertheless, ionizing radiation results in nearly identical damage to normal cells as cancer cells. In addition, radiotherapy also leads to cell toxicity, cancer cell resistance and immunosuppression.[6] Hence, the dosage of irradiation should be kept beneath treatment levels in order to protect adjacent healthy tissues.[7] Therefore, radiation sensitizers are a great help for radiotherapy in cancer patients. Chitooligosaccharides (COS) have been reported to be an anticancer factor, which can also raise the radiosensitivity of colon cancer.[8] However, the mechanism of COS in mediating the radiosentivity of colon cancer is unclear. Therefore, functions of COS at the molecular level were explored in this study.

Recent studies have proven that noncoding RNAs play important roles in regulating the occurrence and progression of tumors. Among these noncoding RNAs, long noncoding RNAs (lncRNAs) have over 200 nucleotides that can encode the expression of genes without protein-coding abilities. miRNAs are endogenous, small noncoding RNAs with 19–22 nucleotides that can negatively regulate target gene expression by binding to mRNAs or suppressing their translation.[9] According to previous discoveries, the lncRNA antisense non-coding RNA in the INK4 locus (ANRIL), the antisense RNA1 of CDKN2B, is highly expressed in colon cancer tissues and cell lines.[10,11] Though the radiosensitivity of ANRIL has been mentioned in nasopharyngeal carcinoma,[12] its role in regulating radiosensitivity in colon cancer has seldom been investigated. On the other hand, miR-181a-5p was found to be downregulated in colon cancer and it negatively regulated MMP-14 to inhibit the migration and invasion of cancer cells.[13] Additionally, miR-181a-5p suppressed tumor growth in vivo and promoted apoptosis of colon cancer by binding to CCAT.[14] Therefore, miR-181a is known as a tumor suppressor. In addition, ANRIL was discovered in previous research to sponge miR-181a, facilitating proliferation and epithelial–mesenchymal transformation in laryngeal squamous cell carcinoma.[15] Therefore, we hypothesized that ANRIL and miR-181a-5p might play a part in mediating COS-induced radiosensitivity in colon cancer cells. The aim of this study was to explore the roles of ANRIL and miR-181a-5p in regulating the radiosensitivity of colon cancer cells.

Material and methods

Cell culture

Human colonic epithelial cell line NCM460 and colon cancer cell lines SW480, SW620 and HCT116 were all acquired from American Type Culture Collection (ATCC; Manassas, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Waltham, USA) containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 mg/mL of streptomycin at 37°C with 5% CO_2. Cells in the log phase were selected for examination.

Cell transfection

The cell line SW480 was selected for further study because it showed the highest expression of ANRIL among the cell lines. The overexpressed ANRIL plasmid was synthesized with respect to pcDNA 3.1 vector (Invitrogen, Carlsbad, USA). In brief, the ANRIL lncRNA sequence was cloned into a pcDNA 3.1 vector, forming the overexpressed ANRIL plasmid (oeANRIL), while the empty pcDNA3.1 vector (oeNC) served as a negative control (NC). The cells were upregulated through transfection of the oeANRIL plasmid, with oeNC as its control. The upregulation of miR-181a-5p was achieved after the cells were transfected with miR-181a-5p plasmid mimics, while the negative control group was transfected with the NC plasmid mimics (Thermo Fisher Scientific, Waltham, USA). The miR-181a-5p inhibitor and inhibitor NC plasmids were purchased from Thermo Fisher. Lipofectamine™ 3000 Transfection Reagent (Invitrogen) was used during the transfection assays. In addition to the oeNC, oeANRIL, NC mimics, miR-181a-5p mimics, NC inhibitor, and miR-181a-5p inhibitor groups, a combined group was also generated through transfection using Lipofectamine™ 3000 Transfection Reagent (Invitrogen). This combined group was transfected with oeANRIL and miR-181a-5p mimics. To simplify, we named it the oeANRIL+miR-181a-5p mimic in this study. All the groups were examined with real-time quantitative polymerase chain reaction (RT-qPCR) for expression of ANRIL or miR-181a-5p.

RT-qPCR

Beyozol (Beyotime, Shanghai, China) was applied to extract total RNA from the incubated NCM460, SW480, SW620, and HCT116 cells. cDNA was then created through reverse transcription of RNA and utilized as templates for

amplification through PCR. The following conditions were used in the RT-qPCR: pre-denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C. The sequences of primers were as follows: ANRIL forward, 5'-GGGCCTCAGTGGCACATACC-3' and reverse, 5'-TGCTCTATCCGCCAATCAGG-3'[16]; miR-181a-5p, forward, 5'-CCGCGAACATTCAACGCTGTCG-3' and reverse, 5'-ATCCAGTGCAGGGTCCGAGG-3'[17]; GAPDH, forward, 5'-CCACATCGCTCAGACACCAT-3' and reverse, 5'-ACCAGGCGCCCAATACG-3'[16] and U6, forward, 5'-CAAATTCGTGAAGCGTTCCATAT-3' and reverse, 5'-GCTTCACGAATTTGCGTGTCATCCTTGC-3'.[17] The relative expressions of ANRIL and miR-181a-5p were quantified using the $2^{-\Delta\Delta Ct}$ method, normalized to GAPDH and U6.

CCK-8

Normal SW480 cells and SW480 cells transfected with oeNC, oeANRIL and a mixture of oeANRIL and miR-181a-5p mimics were seeded into a 96-well plate at a density of 1×10^4 cells per well and incubated at 37°C with 5% CO_2. Later, normal SW480 cells were divided into groups and treated with COS (0 mg/mL, 1.0 mg/mL, 2.0 mg/mL, 3.0 mg/mL, 4.0 mg/mL, and 5.0 mg/mL) and SW480 transfected with oeNC, oeANRIL, and compounds of oeANRIL and miR-181a-5p mimics were grouped and treated by COS (5.0 mg/mL). A linear accelerator (Varian, Palo Alto, USA) was then used to irradiate normal SW480, transfected SW480, SW480+COS (5.0 mg/mL) and transfected SW480+COS (5.0 mg/mL) at different dosages (0 Gy, 2 Gy, 4 Gy, and 6 Gy). For cell proliferation detection, 10 µL of CCK-8 was added into wells at 24 h, 48 h and 72 h. After the CCK-8 was added, the cells were kept incubating for another 2 h at 37°C. A Thermo Scientific Varioskan™ LUX Microplate Reader (Thermo Fisher Scientific) then was performed to check the optical density (OD) values at a wavelength of 450 nm. As for toxicity, 10 µL of CCK-8 was mixed after the SW480 cells were treated with COS for 72 h. The cells were also incubated for another 2 h and the OD values were checked at a wavelength of 570 nm with a Varioskan™ LUX Microplate Reader.

Apoptosis

Normal SW480 cells and the cell groups after transfection were seeded into a six-well plate with 1×10^6 cells per well. Then, SW480 cells in normal condition and SW480 cells transfected with oeNC, oeANRIL and oeANRIL with miR-181a-5p mimics were treated with 5.0 mg/mL of COS. Next, different doses of X-rays (0 Gy, 2 Gy, 4 Gy, and 6 Gy) were used to irradiate normal SW480 and transfected SW480 cells. An Annexin V-FITC Apoptosis Detection Kit (Beyotime) was used to check for apoptosis. Cells after irradiation were stained with Annexin V and PI, and incubated for 15 h without light. The relative apoptosis rate was detected with a BD Biosciences FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, USA).

Dual luciferase report assay

The online tool Starbase (http://starbase.sysu.edu.cn) was used to predict the potential target genes of ANRIL. Then, wild-type and mutant-type ANRIL containing miR-181a-5p binding sites were inserted into pGLO plasmids (Bio-Rad, Hercules, USA); the recombinant plasmids were named pGLO-ANRIL-wt and pGLO-ANRIL-mut. Then, NC mimics and miR-181a-5p mimics were co-transfected with pGLO-ANRIL-wt or pGLO-ANRIL-mut into the SW480 cells using Lipofectamine™ 3000. A GloMax® Discover Microplate Reader (Promega, Madison, USA) was used to detect luciferase activity.

Statistical analysis

All data is presented as mean ± standard deviation (SD) and was analyzed in GraphPad Prism 7 (GraphPad Software, La Jolla, USA). Each experiment was repeated 3 times. Comparisons among the groups were processed with Student's t-test and one-way analysis of variance (ANOVA).

Results

The ANRIL was highly expressed in the colon cancer cells and upregulating it resulted in less apoptosis.

In order to confirm the role of ANRIL, RT-qPCR was first done to analyze the expression of ANRIL. We found that ANRIL expression was much higher in the colon cancer cell lines than in the normal NCM460 cell line. Moreover, the SW480 cell line displayed the highest ANRIL expression (Fig. 1A). Therefore, the SW480 cell line was selected for subsequent experiments. The cell viability of SW480 cell groups with radiation treatment at different dosages was determined, revealing that cell viability was significantly decreased in a dose-dependent manner after irradiation (Fig. 1B). Meanwhile, ANRIL expression was significantly inhibited as the radiation dosage increased (Fig. 1C). Overexpression of ANRIL was achieved with transfection assay. The RT-qPCR results indicated significantly upregulated expression of ANRIL in the SW480 cells transfected with the oeANRIL plasmid (Fig. 1D). Moreover, the apoptosis rate notably declined in comparison with the negative control group after irradiation when ANRIL was upregulated (Fig. 1E, Fig. 4 [Supplement 1]).

MiR-181a-5p was a target of ANRIL in colon cancer cells and the inhibition of miR-181a-5p suppressed the radiosensitivity of the cells.

Using an online bioinformatics tool, putative binding sites between miR-181a-5p and ANRIL were displayed (Fig. 2A).

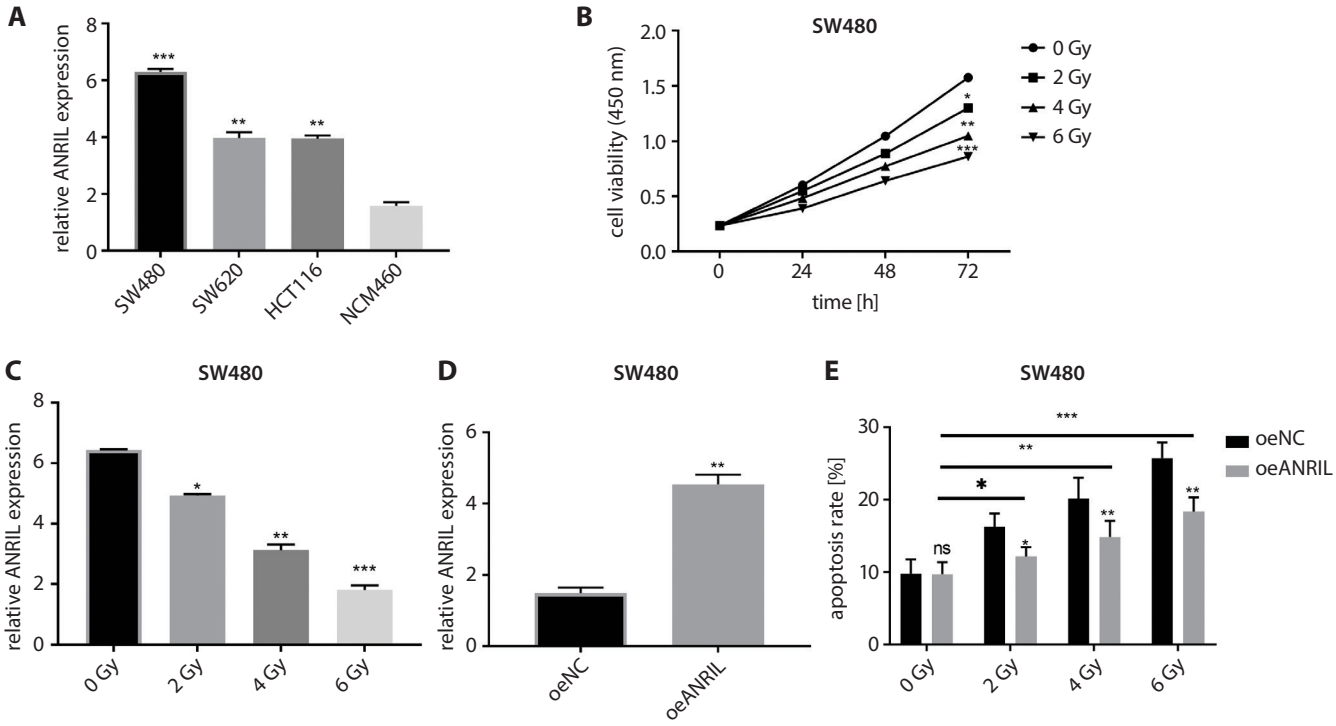

Fig. 1. ANRIL expression was higher in colon cancer cells with downregulating radiosensitivity and apoptosis

A. RNA levels of ANRIL in SW480, SW620, HCT116, and NCM460 were assessed with RT-qPCR, with NCM460 group as a negative control group (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$); B. The cell viability of SW480 with X-ray irradiation (0 Gy, 2 Gy, 4 Gy, and 6 Gy) was evaluated using CCK-8, with 0 Gy group as a control group (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$); C. The expression of ANRIL at X-ray dosages of 0 Gy, 2 Gy, 4 Gy, and 6 Gy was evaluated using RT-qPCR, with 0 Gy group as a control group (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$); D. Overexpressed ANRIL expression was evaluated using RT-qPCR (** $p < 0.05$); E. SW480 cells after transfection with oeNC and oeANRIL plasmids were exposed to radiation (0 Gy, 2 Gy, 4 Gy, and 6 Gy) and then measured using flow cytometry for apoptosis rates. In the same irradiation groups, the oeANRIL group was compared to the oeNC group (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$). In addition, the overexpressed groups were compared when they were exposed to different dosages of radiation, with 0 Gy as a negative control (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$). Each experiment was run in triplicate.

Dual luciferase reporter assay was used for verification at the binding sites. It was found that the combined group of miR-181a-5p mimics and wild-type ANRIL had significantly low luciferase activity in comparison with the remaining groups, confirming that miR-181a-5p was targeted by ANRIL (Fig. 2B). Then, the expression of miR-181a-5p was examined when the cells were exposed to radiation; the results revealed that miR-181a-5p was significantly upregulated with increasing X-ray doses (Fig. 2C). Therefore, a transfection assay was conducted to suppress miR-181a-5p in the SW480 cells (Fig. 2D). Apoptosis was also dramatically reduced by inhibiting miR-181a-5p in cells. In general, apoptosis rates were elevated with increasing dosages of X-ray treatment (Fig. 2E, Fig. 5 [Supplement 2]).

The upregulation of ANRIL inhibited COS-induced radiosensitivity in colon cancer cells by targeting miR-181a-5p.

The cell toxicity of COS in the SW480 cells was checked and the results showed that the survival rate significantly decreased as the concentration of COS increased (Fig. 3A). The SW480 cells treated with 5.0 mg/mL of COS were chosen for the following analysis. The cells with or without 5.0 mg/mL COS treatment underwent different irradiation and were then examined using flow cytometry for apoptosis; it was found that COS treatment induced more apoptosis and led to higher sensitivity to higher dosages of X-ray exposure (Fig. 3B, Fig. 6 [Supplement 3]). The functions of ANRIL and miR-181a-5p in modulating COS were also investigated. Upregulated ANRIL significantly downregulated the cell inhibition rate, while miR-181a-5p mimics partially counteracted this (Fig. 3C). Meanwhile, apoptosis inhibited by ANRIL upregulation was reversed by miR-181a-5p mimics dose-dependently to a certain degree (Fig. 3D, Fig. 7 [Supplement 4]). As Fig. 3B depicts, COS induced apoptosis in cells dose-dependently. Taking the results from Fig. 3D and Fig. 3B together, we decided that ANRIL could inhibit COS-induced apoptosis and deteriorate the radiosensitivity stimulated by COS by targeting and suppressing miR-181a-5p.

Discussion

Though medical technology has developed with the rapid growth of economy, the risks of cancers have not been eliminated. The occurrence rate of colon cancer in China has risen by more than 2 times the international rate.[18] Under such circumstances, it is urgent

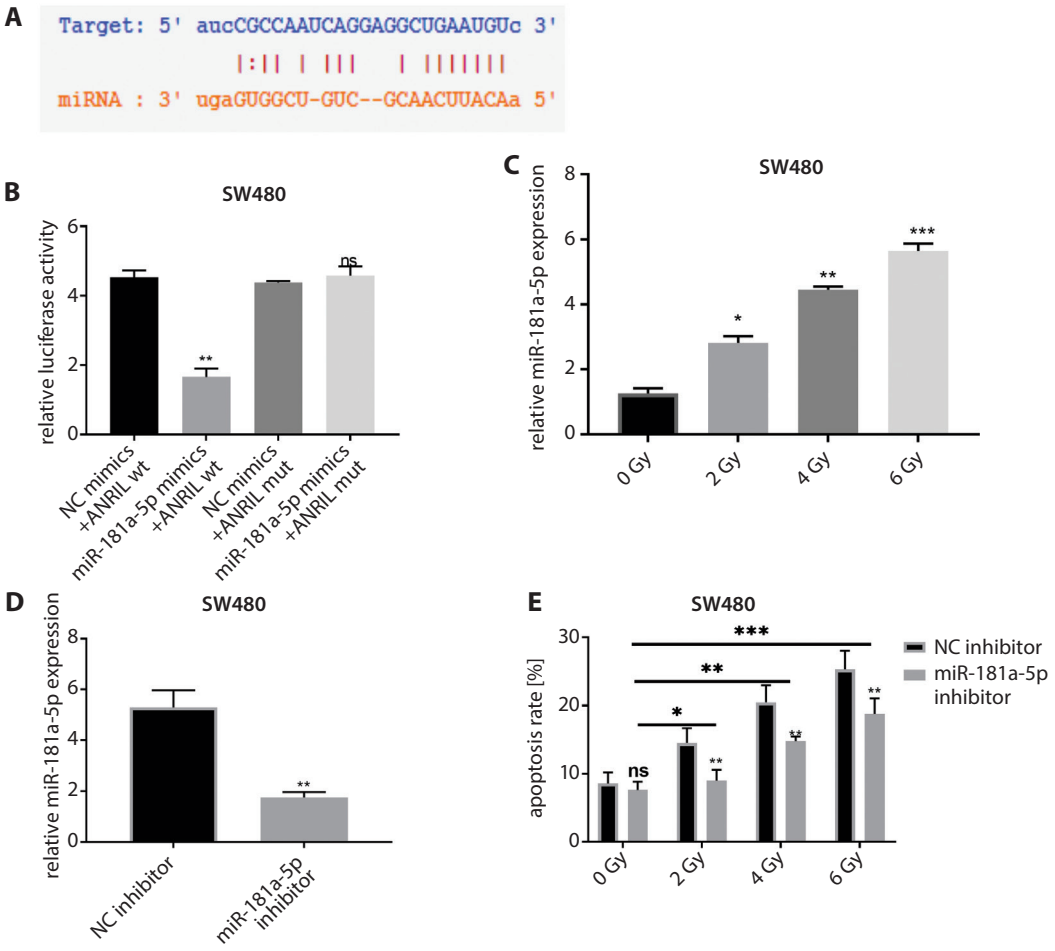

Fig. 2. miR-181a-5p was a target of ANRIL in colon cancer cells and the inhibition of miR-181a-5p suppressed the radiosensitivity of the cells

A. Putative binding sites of miR-181a-5p and ANRIL were predicted using Starbase (http://starbase.sysu.edu.cn); B. Luciferase reporter assays were performed to verify the binding between ANRIL and miR-181a-5p (** $p < 0.05$); C. miR-181a-5p expression was measured using RT-qPCR in SW480 cells exposed to X-rays (0 Gy, 2 Gy, 4 Gy, and 6 Gy), with 0 Gy group as a negative control (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$); D. miR-181a-5p expression was detected using RT-qPCR after the cells were transfected with negative control (NC) inhibitor and miR-181a-5p inhibitor plasmids (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$); E. Apoptosis rates were validated using flow cytometry (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$). In the groups exposed to the same X-ray dosage (NC inhibitor and miR-181a-5p inhibitor), the miR-181a-5p inhibitor group was compared to the NC inhibitor group, while in groups exposed to different dosages, we mainly compared the apoptosis rates in miR-181a-5p inhibitor groups with the 0-Gy treatment group as a control group (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$). All experiments were carried out 3 times.

to find efficient treatments for colon cancer. Radiotherapy is widely used in various stages of tumor treatment, which can shrink the tumor before surgery and remove residues after surgery to better prevent recurrence.[19] However, radiotherapy also causes damage to normal cells, which places a burden on patients. Therefore, increasing the radiosensitivity of tumor cells is a key issue in improving the effects of radiotherapy in cancer. This study aimed to explore the regulatory roles of the lncRNA ANRIL and miR-181a-5p in COS-induced radiosensitivity in colon cancer cells. In our study, we first selected the colon cancer cell lines SW480, SW620 and HCT116, and the normal colonic epithelial cell line NCM460 for ANRIL expression. The ANRIL has been found to be abnormally expressed in many kinds of cancers, such as gastric cancer, lung cancer, hepatocellular carcinoma, etc.[20] In colon cancer, ANRIL was also reported to be an oncogene that can accelerate the progression of colon cancer.[21] Therefore, we studied ANRIL and its regulation of radiosensitivity in colon cancer cells. The ANRIL expression was higher in colon cancer cell lines than in normal NCM460, and was significantly downregulated as radiation dosages increased. The overexpression of ANRIL reduced the rate of apoptosis induced by COS and as the radiation dose increased, the reverse effect was more significant. Therefore, ANRIL was primarily proven to block radiosensitivity in colon cancer cells.

As a potential target gene of ANRIL, miR-181a-5p was widely known as a tumor suppressor, including colon cancer.[22,23] However, regulation of miR-181a-5p in radiosensitivity in cancers was seldom mentioned before. In this study, miR-181a-5 was confirmed to have binding sites for ANRIL and it directly bound to wild-type ANRIL. Moreover, miR-181a-5p was significantly upregulated

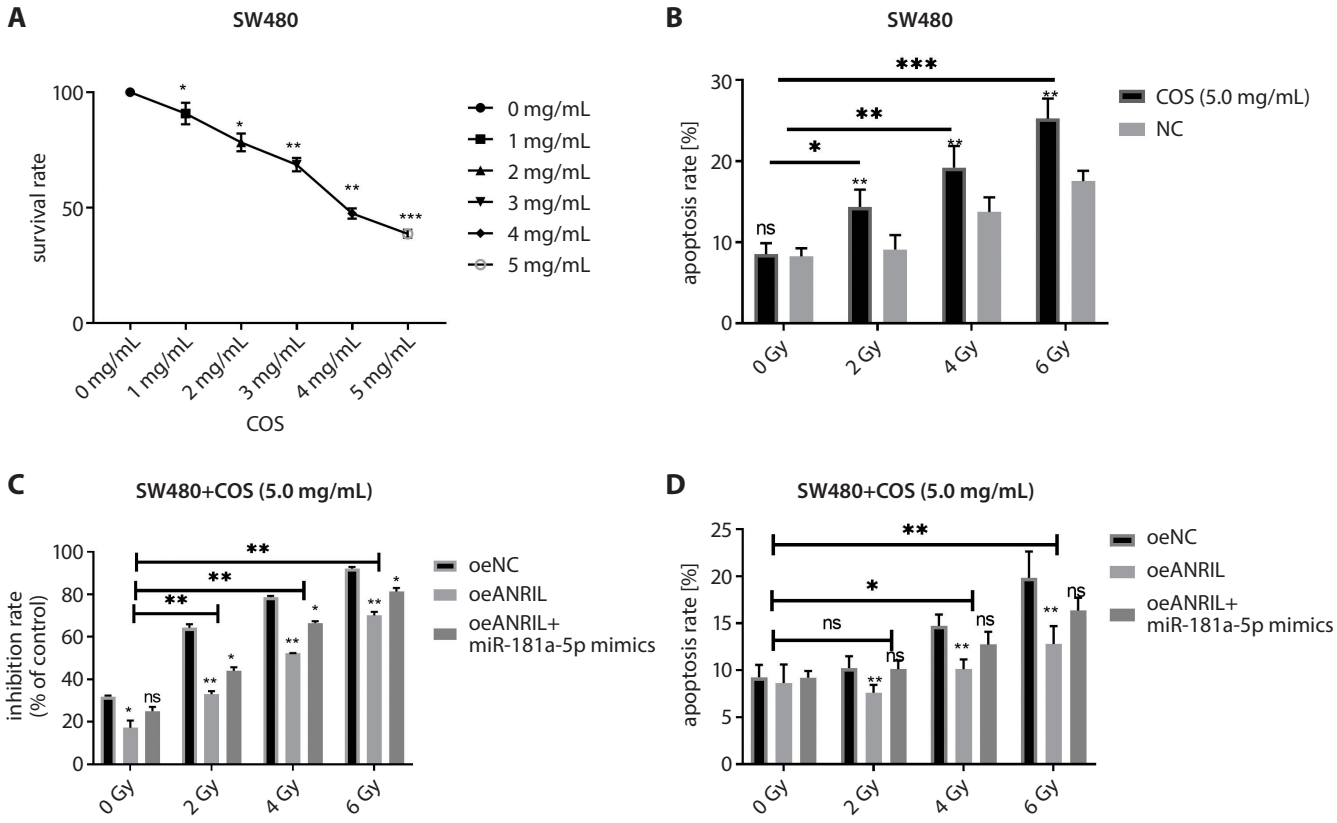

Fig. 3. Overexpressed miR-181a-5p upregulated COS-induced radiosensitivity in colon cancer cells by suppressing ANRIL expression

A. Cell survival rates were evaluated using CCK8 assays when the cells were treated with COS (0 mg/mL, 1.0 mg/mL, 2.0 mg/mL, 3.0 mg/mL, 4.0 mg/mL, and 5.0 mg/mL) (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$); B. SW480 cells after COS treatment (5.0 mg/mL) were exposed to X-rays (0 Gy, 2 Gy, 4 Gy, and 6 Gy) and apoptosis was evaluated using flow cytometry, with the 0 Gy treatment group serving as a control group (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$); C. SW480 cells transfected with oeNC, oeANRIL and oeANRIL+miR-181a-5p mimics underwent COS treatment (5.0 mg/mL) and were then exposed to X-rays (0 Gy, 2 Gy, 4 Gy, and 6 Gy). The inhibition rates were assessed with CCK-8. Within the same X-ray treatment group, the oeANRIL group was compared to the oeNC group, while the combined group was compared with the oeANRIL group (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$). Meanwhile, the general differences when cells were exposed to different dosages of X-rays were compared with 0 Gy group as a control group (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$); D. Apoptosis rate was evaluated in each subgroup. Within the same X-ray treatment group, the oeANRIL group was compared to the oeNC group, while the combined group was compared with the oeANRIL group (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$). Meanwhile, the general differences between cells exposed to different dosages of X-rays were compared with 0 Gy group as a control group (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$). Each experiment was carried out 3 times.

by irradiation and upregulating miR-181a-5p partially alleviated the suppressive function of ANRIL against COS in apoptosis and radiosensitivity in colon cancer cells. Therefore, in this in vitro model, we have proven that miR-181a-5p can serve as a biomarker that might contribute to radiosensitivity by reversing the functions of ANRIL in colon cancer cells.

The COS are of a chitinous substance which is reported to be an anti-tumor factor.[24] In various cancers, COS are well-known as a radiation sensitizer which could help to deter cell proliferation.[25,26] The COS have been proven to promote radiosensitivity by accelerating cell apoptosis in colon cancer.[8] However, the underlying molecular mechanisms of COS are rarely mentioned. We first measured the toxicity of COS to SW480 cells, revealing that survival rates decreased significantly as concentrations of COS grew. Moreover, SW480 cells with COS treatment promoted apoptosis, compared to untreated cells after irradiation. Additionally, the reduced cell inhibition rate and apoptosis provoked by ANRIL upregulation can be restored by miR-181a-5p mimics after COS treatment. Taken together, we concluded that ANRIL upregulation could inhibit COS-induced radiosensitivity by targeting miR-181a-5p.

Conclusions

The lncRNA ANRIL was highly expressed in colon cancer cells and suppressed radiosensitivity by binding to miR-181a-5p and reversing the functions of COS, suggesting that those 2 genes might be involved in sensitivity regulation in radiotherapy of colon cancer. However, in vivo and clinical studies are needed to further validate this finding.

ORCID iDs

Chunfeng Sun https://orcid.org/0000-0002-4602-7706
Chen Shen https://orcid.org/0000-0002-7854-6877
Yaping Zhang https://orcid.org/0000-0002-0730-5689
Chunhong Hu https://orcid.org/0000-0002-2548-2654

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Fig. 4. Supplement 1. Flow cytometry images for Fig. 1E. The images (from left to right and up to down) were put in the order of the groups in Fig. 1E

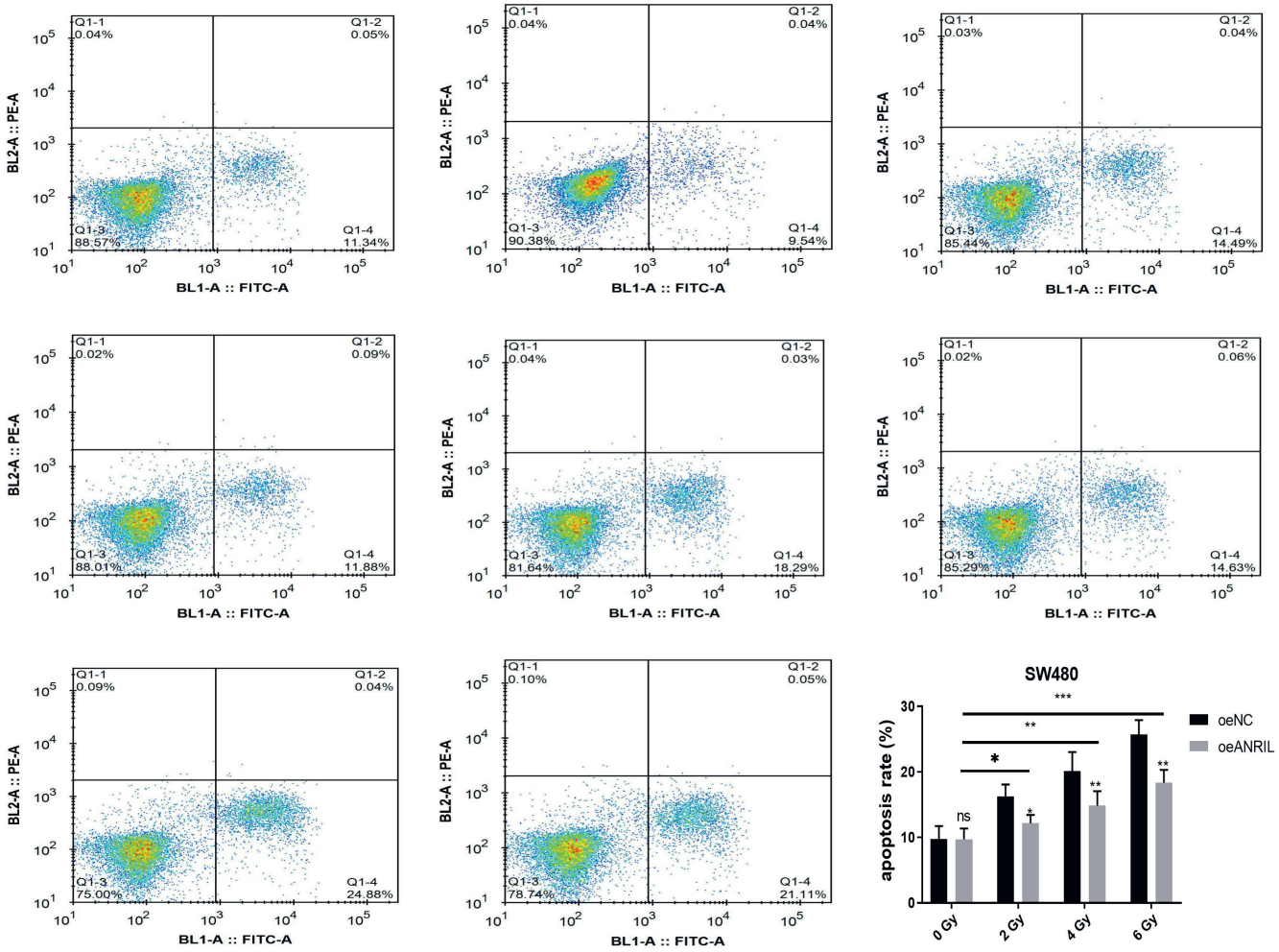

Fig. 5. Supplement 2. Flow cytometry images for Fig. 2D. The images (from left to right and up to down) were put in the order of the groups in Fig. 2D

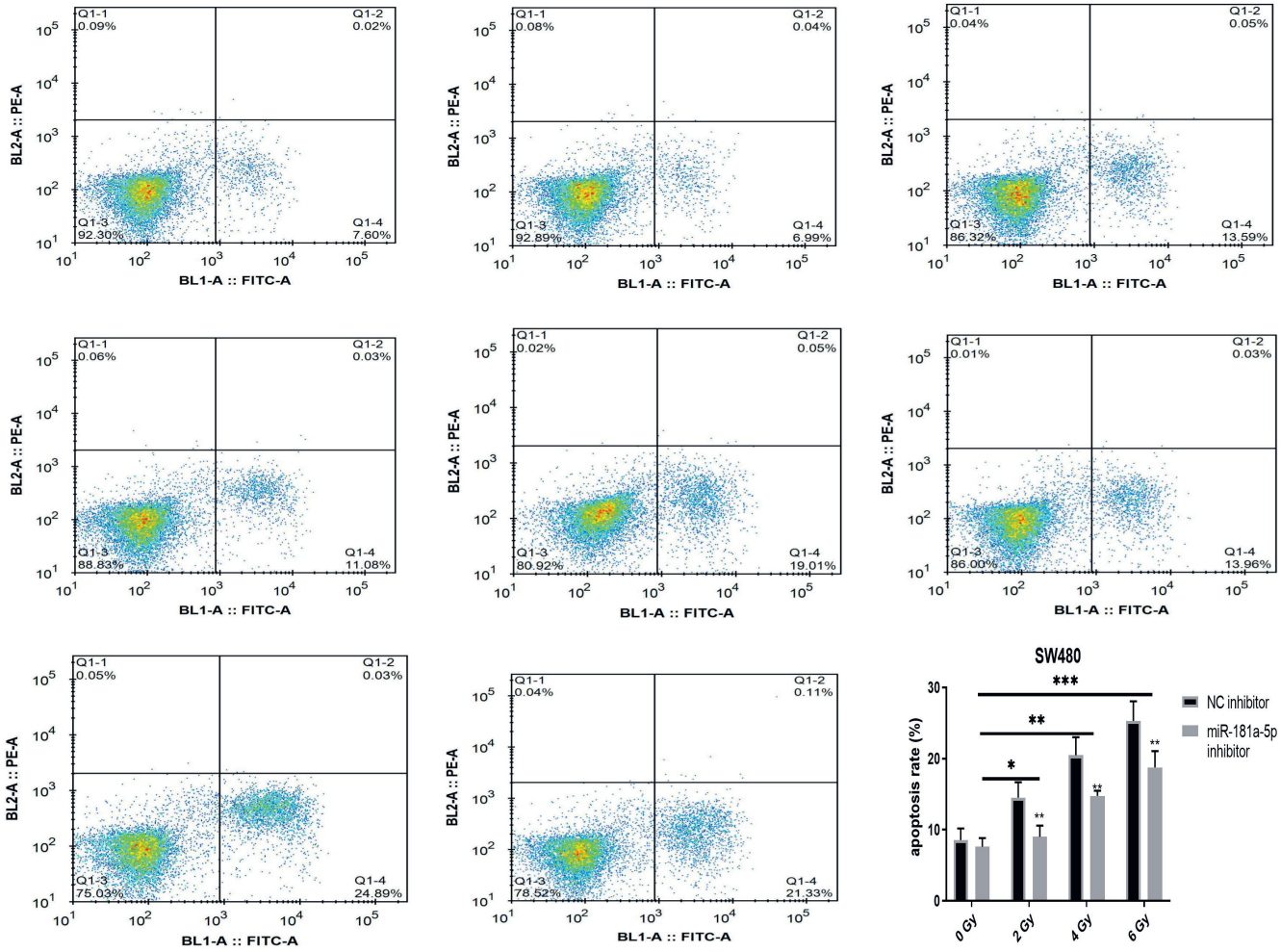

Fig. 6. Supplement 3. Flow cytometry images for Fig. 3B. The images (from left to right and up to down) were put in the order of the groups in Fig. 3B

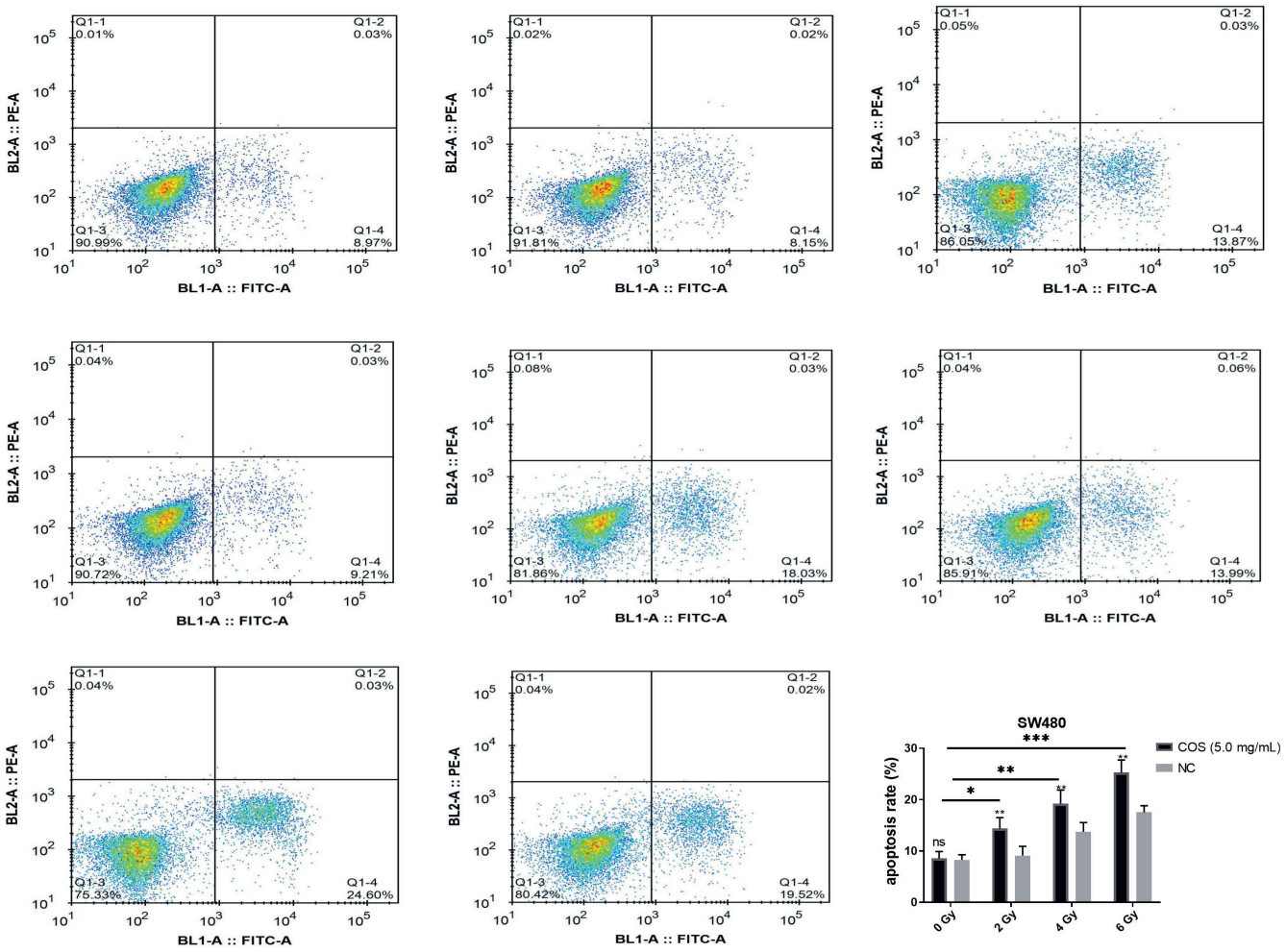

Fig. 7. Supplement 4. Flow cytometry images for Fig. 3D. The images were put in the order of the groups in Fig. 3D