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ORIGINAL ARTICLE

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MicroRNA-324-5p affects the radiotherapy response of cervical cancer via targeting ELAV-like RNA binding protein 1

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Abstract

Cervical cancer (CC) seriously threatens the health of women. Radiation therapy (RT) is the major treatment for CC. However, the recurrent CC can acquire resistance to RT. Thus, it is necessary to find a new method for reversing RT resistance in CC. It has been reported that microRNA-324-5p (miR-324-5p) can suppress the progression of multiple cancers. However, whether it can reverse resistance to RT in CC remains unclear. qRT-PCR and Western blotting were used to detect gene and protein expression in CC cells, respectively. Cell proliferation was tested by CCK-8 assay and colony formation assay. In addition, cell apoptosis was detected by flow cytometry. Transwell assays were performed to detect cell migration. Dual luciferase reporter assay and TargetScan were used to explore the targets of miR-324-5p. MiR-324-5p was downregulated in CC cells. Overexpression of miR-324-5p sensitized CC cells to RT. In addition, miR-324-5p mimics significantly induced apoptosis and inhibits the migration of CC cells in the presence of ^{137}Cs ionizing radiation. Furthermore, miR-324-5p sensitized CC cells to ionizing radiation by targeting ELAV-like RNA binding protein 1 (ELAVL1). MiR-324-5p overexpression affects the radiotherapy response of CC by targeting ELAVL1, which may serve as a new target for the treatment of CC.

KEYWORDS

apoptosis, cervical cancer, ELAVL1, miR-324-5p, radiation therapy

1 | INTRODUCTION

Cervical cancer (CC) has been considered to be one of the most frequent malignancies threatening the lives of women.[1–4] Despite the efforts scientists have made, the outcome of patients with advanced CC remains to be improved.[5] Radiation therapy (RT) is one of the major treatments for CC. However, some patients are not sensitive to RT, which notably affects the efficacy of RT.[5] Although it is generally recognized that radioresistance is closely associated with tumour heterogeneity resulting from genetic defects and different cell origins, the exact mechanism remains to be explored. Therefore, it is necessary better understand the underlying mechanism to find new strategies that improve the sensitivity of patients with CC to RT.

It has been confirmed that ELAV-like RNA binding protein 1 (ELAVL1) is located primarily in the nucleus and is translocated into the cytoplasm upon cellular perturbation.[6] The biological function of ELAVL1 is implicated in gametogenesis, placental growth,[7,8] and cell survival.[9] In addition, ELAVL1 can mediate the progression of inflammation and angiogenesis.[10] Moreover, previous studies have indicated that ELAVL1 can participate in the occurrence of malignant tumours.[11] However, knowledge on the role of ELAVL1 in the resistance of CC cells to ionizing radiation (IR) is very limited.

MicroRNAs (miRNAs) are a group of small, noncoding RNAs that suppress the translation of target mRNAs by binding to complementary sequences in the 3′-untranslated regions (3′-UTRs).[12–15] It has been confirmed that by inhibiting their target genes, miRNAs can regulate the progression of multiple diseases, especially malignancies.[16–21] Previously, a correlation between miR-324-5p and ELAVL1 expression in colorectal cancer was reported.[22] However, the function of the miR-324-5p/ELAVL1 axis in CC remains largely unknown.

In this research, we hypothesized that miR-324-5p binds with ELAVL1 and explored whether miR-324-5p can affect the sensitivity of CC cells to RT by targeting ELAVL1. The purpose of this study was to verify the therapeutic effect of miR-324-5p/ELAVL1 in CC and provide a new strategy for the treatment of CC.

2 | MATERIAL AND METHODS

2.1 | Cell culture and treatment

The normal human cervical cell line H8 and CC cell lines (C33A, ME-180, Hela and Caski) were purchased from American Type Culture Collection (ATCC, Manassas, Virginia). Cells were cultured in dulbecco's modification of eagle's medium (DMEM) or roswell park memorial institute (RPMI) 1640 medium supplemented with 10% foetal bovine serum (FBS, Thermo Fisher Scientific, Carlsbad, California), and 100 IU/mL streptomycin and penicillin (Thermo Fisher Scientific) in a humidified atmosphere and 5% CO_2 at 37°C.

For IR, the cell monolayer was irradiated by using a Gamma Cell 40 Exactor (Nordion International).

2.2 | Cell transfection

Chemically synthesized miR-324-5p inhibitor, miR-324-5p mimics, and negative control (NC) were purchased from GenePharma (Shanghai, China). The pcDNA3.1 vector and pc-ELAVL1 were obtained from RiboBio (Guangzhou, China).

For transfection, miR-324-5p inhibitor, miR-324-5p mimics, pc-ELAVL1, and the corresponding control were transfected into CC cell by using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. The efficiency of transfection was detected by quantitative real-time PCR (qRT-PCR). After transfection, the cells were incubated at 37°C for 6 hours.

2.3 | Quantitative real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized from 5 to 10 μg of total RNA using the Super Master Mix synthesis kit PrimeScript RT reagent kit (Takara Bio, Tokyo, Japan). Then, we performed PCR assays using the SYBR premix Ex Taq II kit (Takara). The qRT-PCR conditions were as follows: 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. β-actin or U6 was used as the internal control. The primers used were as follows: miR-324-5p forward, 5′-CTT CCG CCA TGA TTG TGA GG-3′ and reverse, 5′-AAC AGT CCC TCC TTG GTC TC-3′; β-actin forward, 5′-AGC GAG CAT CCC CCA AAG TT-3′ and reverse, 5′-GGG CAC GAA GGC TCA TCA TT-3′; ELAVL1 forward, 5′-ATG CCA CAC TCA AGT CCC TCA-3′ and reverse, 5′-GTC TCG CCA GTC TCC ATG TTG-3′; and U6 forward, 5′-GCT TCG GCA GCA CAT ATA CT-3′ and reverse, 5′-GTG CAG GGT CCG AGG TAT TC-3′. The $2^{-\Delta\Delta Ct}$ method was utilized to measure the relative expression levels according to the reference levels.[23]

2.4 | Cell counting kit (CCK)-8 assay

Caski or Hela cells were seeded in 96-well plates (5×10^3 cells per well) and then treated with the following reagents: mimic NC, miR-324-5p mimics, miR-324-5p mimics plus pcDNA3.1 or miR-324-5p mimics plus pc-ELAVL1 for 1, 2, 3, 4, or 5 days. Then, the cells were incubated with 10 μL CCK-8 reagent (Beyotime, China) for another 2 hours at 37°C. Next, the absorbance of cells was measured at 450 nm using a microplate reader (Thermo Fisher Scientific).

2.5 | Colony formation assay

Cells were treated in the presence of irradiation. Fourteen days later, the cells were stained with 0.5% crystal violet in absolute ethanol, and colonies that contained more than 50 cells were counted under an inverted microscope (Olympus, Tokyo, Japan).

2.6 | Cell apoptosis analysis

Caski or Hela cells were seeded in 6-well plates (5×10^4 cells per well). The cell pellet was resuspended in 100 μL binding buffer after centrifugation at 1000 rpm for 5 minutes. Then, 5 μL of Annexin V-Fluoresceine isothiocyanate (FITC) and propidium (PI) were added to the cell solution for 15 minutes. The cell apoptotic rate was measured using a flow cytometer (BD Biosciences), and the results were analysed using the WinMDI 2.9 software (Thermo Fisher Scientific).

2.7 | Hoechst staining

Nuclei were counterstained with Hoechst 33342 (1:500; Sigma, Missouri). The stained cells were washed with phosphate buffered saline (PBS) and visualized by an inverted fluorescence microscope (Carl Zeiss, Jena, Germany). The number of immunostained cells was counted in each of three random fields per well, and the fluorescence images were selected randomly. Quantification of the immunofluorescence signal was performed by measuring the average intensity in individual cells according to the ImageJ software manual.

2.8 | Transwell assay

Briefly, 24-well Transwell plates (Corning, New York) were used for cell invasion and migration assays. For the cell migration assay, 5×10^4 CC cells were seeded into the upper chambers of the Transwell in 200 μL serum-free DMEM or RPMI 1640. The lower chamber contained DMEM or RPMI 1640 supplemented with 10% FBS. After 24 hours of incubation at 37°C, the nonmigrating cells were gently removed from the upper side of the chamber with a cotton swab, while the migrated cells were fixed with 95% alcohol for 10 minutes and stained with 1% crystal violet for 5 minutes.

2.9 | Western blot

Cells were lysed using radio immunoprecipitation assay (RIPA) lysis buffer to obtain total proteins. The protein concentration was then detected using a bicinchoninic acid (BCA) protein kit (Thermo Fisher Scientific). Proteins (40 μg per lane) were separated on a 10% sodium salt (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred onto polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific). Subsequently, the membranes were blocked with 5% skim milk in tris-buffered saline and Tween 20 (TBST) for 1 hour at room temperature and then incubated with primary antibodies against ELAVL1 (Abcam, 1:1000), Bax (Abcam, 1:1000), Bcl-2 (Abcam, 1:1000), or β-actin (Abcam, 1:1000) overnight at 4°C. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature. Finally, the protein bands were detected using an enhanced

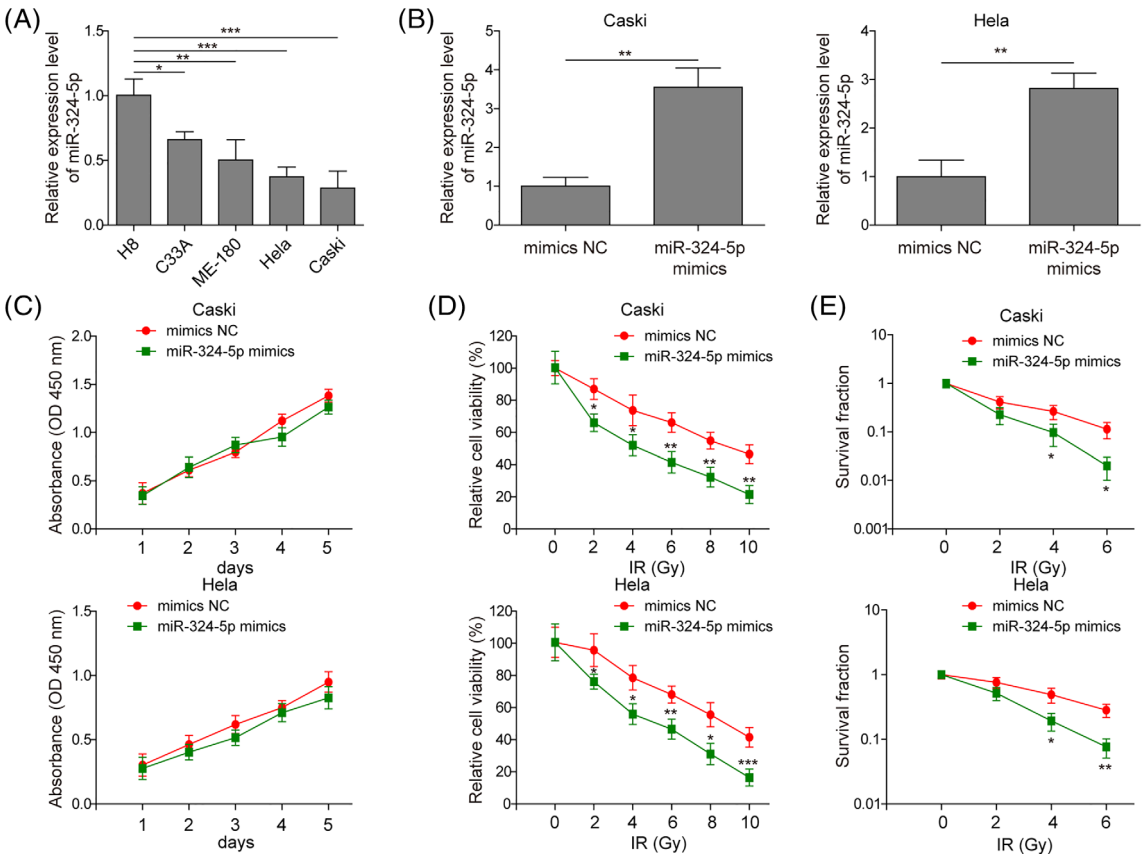

FIGURE 1 MiR-324-5p significantly sensitized CC cells to IR. A, The expression of miR-324-5p in H8, C33A, ME-180, Hela and Caski cells was detected by qRT-PCR. B, Hela and Caski cells were transfected with NC or miR-324-5p mimics for 24 hours. Then, the transfection efficiency was confirmed by qRT-PCR. C, After incubation for 1, 2, 3, 4, and 5 days, CC cell proliferation was tested by CCK-8 assay. D, Hela and Caski cells were transfected with NC or miR-324-5p mimics for 24 hours and then treated with different dosages of IR for 72 hours. Cell proliferation was tested by CCK-8 assay. E, The survival fraction of CC cells was detected by colony formation assay. $^{*}P < .05$, $^{**}P < .01$. CC, cervical cancer; IR, ionizing radiation; NC, negative control; qRT-PCR, quantitative real-time PCR;

chemiluminescence kit (Thermo Fisher Scientific). β-actin was used as an internal control. IPP 6.0 (Image-Pro Plus 6.0) was used for the densitometry analysis.

2.10 | MiR-324-5p target gene prediction

MiR-324-5p target gene prediction was performed using a publicly available program (TargetScan). The results were selected for further analysis.

2.11 | Dual luciferase reporter assay

The fragment of the 3′-UTR of ELAVL1 containing the putative binding sites of miR-324-5p was synthetized and obtained from Sangon Biotech Co., Ltd. (Shanghai, China) and then cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation) to construct the wild-type (wt) ELAVL1 reporter vector. The ELAVL1 3′-UTR sequence containing the putative binding sites of miR-324-5p was mutated using the Q5 Site-Directed Mutagenesis kit (New England Biolabs, Inc.) and then cloned into the pmirGLO vector

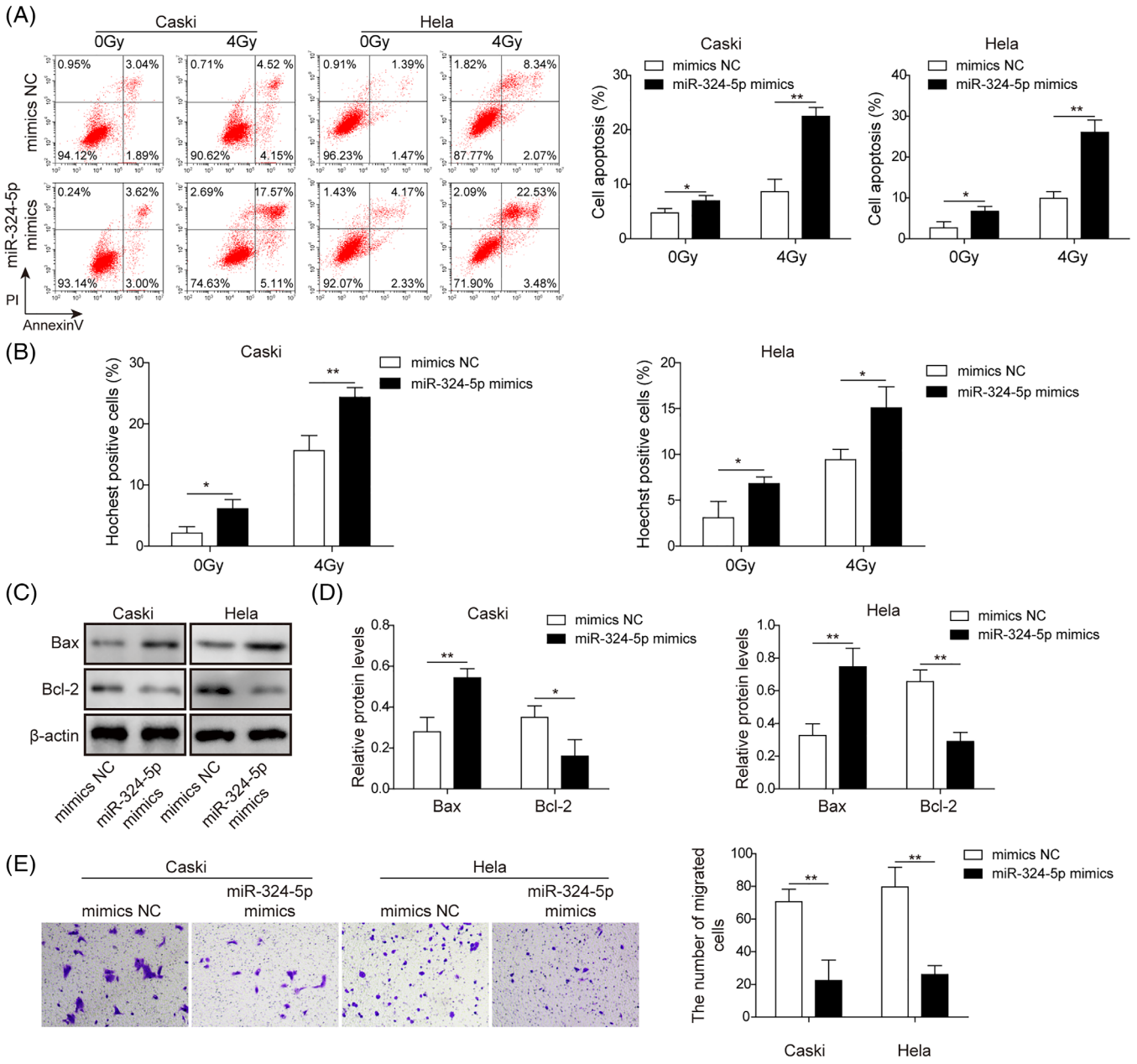

FIGURE 2 MiR-324-5p notably increased the inhibitory effect of IR on the growth of CC cells. A, Cells were treated with 4 Gy IR for 72 hours. Then, the rate of apoptotic cells was detected by fluorescence activating cell sorter (FACS) after double staining with Annexin V and PI. X axis: The level of Annexin-V FITC fluorescence; Y axis: The PI fluorescence. B, NC- or miR-324-5p mimic-transfected Hela and Caski cells were treated with IR and detected by Hoechst staining. The positive rate of Hoechst staining was calculated. C, The protein expression of Bax and Bcl-2 in CC cells was detected by Western blot. D, The relative protein expression was quantified by normalizing to β-actin expression. E, Cell migration was tested by the Transwell assay. $^{*}P < 0.05$, $^{**}P < 0.01$. CC, cervical cancer; IR, ionozing radiation; NC, negative control;

to construct the ELAVL1 mutant (mut) reporter vector. Subsequently, the ELAVL1 (wt) or ELAVL1 (mut) vector was transfected into CC cells together with vector-control (NC) or miR-324-5p mimics. The relative luciferase activity was analysed by the Dual-Glo Luciferase Assay System (Promega Corporation).

2.12 | Statistical analysis

Data are presented as the mean ± SD. Comparisons between two groups were analysed using Student *t*test. Comparisons among multiple groups were made using ANOVAs followed by Tukey's test using GraphPad Prism7 (GraphPad Software, Inc.). $P < .05$ was considered to indicate a statistically significant difference.

3 | RESULTS

3.1 | MiR-324-5p significantly sensitized CC cells to IR

First, qRT-PCR was used to investigate the expression of miR-324-5p in CC. As indicated in Figure 1A, miR-324-5p was significantly downregulated in CC cells (C33A, ME-180, Hela and Caski) compared with normal cervical cells (H8). In addition, miR-324-5p downregulation in Hela and Caski cells was more significant than that in C33A or ME-180 cells. Thus, Hela and Caski were selected for use in subsequent experiments. Furthermore, the expression of miR-324-5p in Hela and Caski cells was notably upregulated in the presence of miR-324-5p mimics (Figure 1B). This result suggested that miR-324-5p was stably transfected into CC cells. Moreover, the CCK-8 assay data demonstrated that miR-324-5p mimics had a limited effect on the proliferation of CC cells (Figure 1C). However, miR-324-5p significantly enhanced the inhibitory effect of IR on CC cell proliferation (Figure 1D). Similarly, as shown in Figure 1E, the survival fraction of CC cells was greatly decreased by IR, while miR-324-5p enhanced the inhibitory effect of IR. Since IR exhibited a significant inhibitory effect on the growth of CC cells when the dose was no less than 4 Gy, a 4 Gy dose was selected for our study in subsequent experiments. Altogether, these results indicated that miR-324-5p significantly sensitized CC cells to IR.

3.2 | MiR-324-5p notably increased the inhibitory effect of IR on the growth of CC cells

To further investigate the effect of miR-324-5p on IR-treated CC cells in vitro, flow cytometry was performed. The results revealed that 4 Gy IR notably induced the apoptosis of CC cells. In addition, the apoptotic effect of IR was further increased in the presence of miR-324-5p overexpression (Figure 2A). Similarly, the Hoechst positive rate of CC cells in the presence of 4 Gy IR was notably enhanced by overexpression of miR-324-5p (Figure 2B). Then, Western blotting was used to detect the protein expression in CC cells. As shown in Figure 2C, D, overexpression of miR-324-5p increased the expression of the apoptotic protein Bax and decreased the expression of the anti-apoptotic protein Bcl-2 in CC cells treated with 4 Gy IR. In addition, the migration of CC cells in the presence of 4 Gy IR was notably

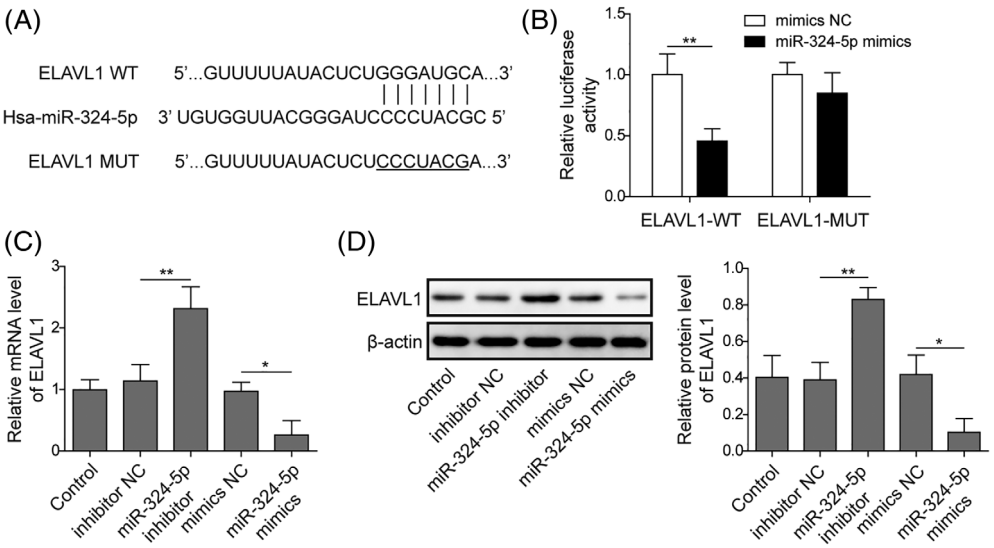

FIGURE 3 ELAVL1 was a direct target of miR-324-5p. A, Predicted miR-324-5p target sequences in the 3′-UTR of ELAVL1. B, The luciferase activity was measured after cotransfection of CC cells with the wt/mut ELAVL1 3′-UTR plasmid and miR-324-5p mimics or NC using the dual luciferase reporter assay. C, The expression of ELAVL1 in CC cells was detected by using qRT-PCR. β-Actin was used as an internal control. D, The expression of ELAVL1 in CC cells was detected by Western blot. The densities of the protein bands were normalized to the β-actin density. $^{*}P < 0.05$, $^{**}P < 0.01$. CC, cervical cancer; ELAVL1, ELAV-like RNA binding protein 1; mut, mutant; NC, negative control; qRT-PCR, quantitative real-time PCR; UTR, untranslated region; wt, wild type

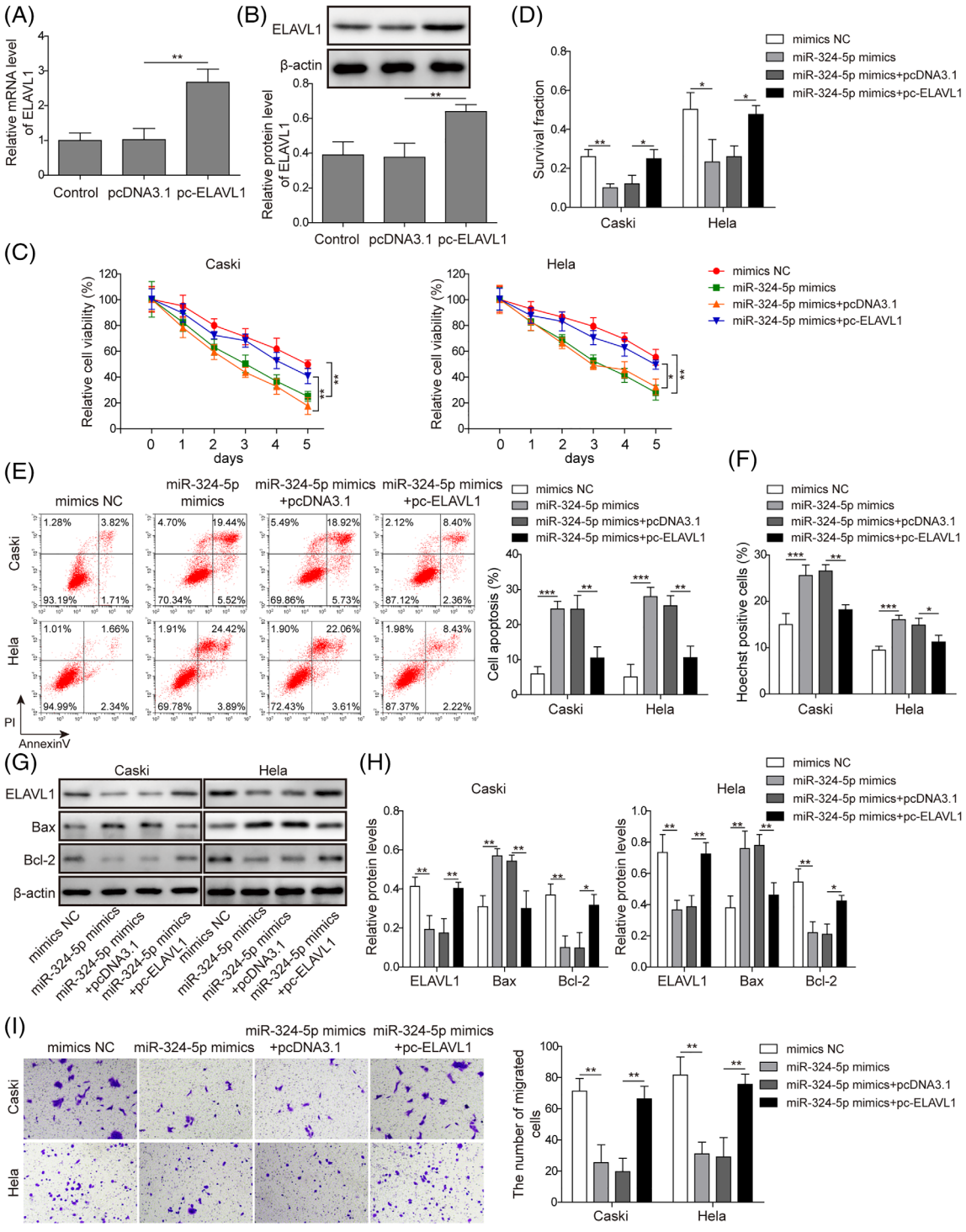

FIGURE 4 MiR-324-5p suppressed the growth of CC cells in the presence of IR by targeting ELAVL1. CC cells were transfected with NC or the ELAVL1 overexpression plasmid for 24 hours. Then, the transfection efficiency was detected by A, qRT-PCR and B, Western blot. The densities of the protein bands were normalized to the β-actin density. C, After incubation for 1, 2, 3, 4, and 5 days, cell proliferation was detected by CCK-8 assay. D, The survival fraction of CC cells was detected by colony formation assay. E, Cells were treated with 4 Gy IR for 72 hours. Then, the rate of apoptotic cells was detected by FACS after double staining with Annexin V and PI. X axis: The level of Annexin-V FITC fluorescence; Y axis: The PI fluorescence. F, Cells were detected by Hoechst staining. The positive rate of Hoechst staining was calculated. G, The protein expression of ELAVL1, Bax, and Bcl-2 in CC cells was detected by Western blot. H, The relative protein expression was quantified by normalizing to β-actin expression. I, Cell migration was tested by the Transwell assay. $^{*}P < 0.05$, $^{**}P < 0.01$. CC, cervical cancer; ELAVL1, ELAV-like RNA binding protein 1; NC, negative control; qRT-PCR, quantitative real-time PCR

inhibited by miR-324-5p (Figure 2E). Overall, miR-324-5p notably increased the inhibitory effect of IR treatment on the growth of CC cells.

3.3 | ELAVL1 was the direct target of miR-324-5p

To explore the target gene of miR-324-5p, TargetScan (http://www.targetscan.org/vert_71/) analysis and dual luciferase reporter assays were performed. The results suggested that ELAVL1 might be a downstream target of miR-324-5p (Figure 3A, B). To verify this prediction, qRT-PCR and Western blotting were used. As indicated in Figure 3C,D, the expression of ELAVL1 in CC cells was significantly upregulated by downregulation of miR-324-5p but inhibited in the presence of miR-324-5p mimics. In summary, miR-324-5p directly targeted ELAVL1.

3.4 | MiR-324-5p suppressed the growth of CC cells in the presence of IR by targeting ELAVL1

To determine the transfection efficiency, qRT-PCR and Western blotting were used. The data showed that the ELAVL1 overexpression vector significantly elevated ELAVL1 expression in CC cells (Figure 4A,B), revealing that ELAVL1 was stably transfected into CC cells. Next, the CCK-8 assay was performed to test cell proliferation. As demonstrated in Figure 4C, the proliferation of CC cells in the presence of 4 Gy IR was obviously suppressed by miR-324-5p mimics, which was partially reversed by overexpression of ELAVL1. Moreover, the colony formation results suggested that ELAVL1 overexpression significantly suppressed the antiproliferative effect of miR-324-5p upregulation on 4 Gy IR-treated CC cells (Figure 4D). Moreover, the flow cytometry and Hoechst staining data confirmed that the inhibitory effect of miR-324-5p on the growth of CC cells in the presence of IR treatment was notably suppressed by overexpression of ELAVL1 (Figure 4E,F). Additionally, the expression levels of ELAVL1 and Bcl-2 in IR-treated CC cells were greatly suppressed by miR-324-5p mimics, while their levels were partially restored in the presence of ELAVL1 overexpression. In contrast, miR-324-5p mimics significantly increased the expression of Bax in CC cells treated with 4 Gy IR, while the promoting effect of miR-324-5p mimics on Bax was obviously inhibited by ELAVL1 upregulation (Figure 4G,H). Similarly, the invasive effect of miR-324-5p mimics on IR-treated CC cells was partially reversed by ELAVL1 overexpression (Figure 4I). Altogether, the results demonstrated that miR-324-5p suppressed the growth of CC cells in the presence of IR by targeting ELAVL1.

4 | DISCUSSION

Downregulated miR-324-5p expression has been found in some types of malignancies, including gallbladder cancer, gastric cancer, non-small cell lung carcinoma (NSCLC), and bladder cancer.[24–27] In these malignant tumours, miR-324-5p could act as a tumour suppressor by targeting different genes. In gallbladder cancer, the restoration of miR-324-5p expression could significantly inhibit the migration, invasion, and epithelial-mesenchymal transition of cells in vitro by directly targeting transforming growth factor (TGF)-β.[24] In NSCLC, miR-324-5p has been confirmed to potentiate resistance to cisplatin by targeting FBXO11.[26] In colon cancer, miR-324-5p could induce colon cancer cell apoptosis by targeting FGFR3.[27] In bladder cancer, miR-324-5p has been considered to inhibit cancer cell proliferation by targeting FOS.[25] Therefore, miR-324-5p has a complex regulatory network, and its targets might be cancer specific. However, the exact expression and biological function of miR-324-5p in CC radiotherapy sensitivity and radiotherapy resistance remain unclear. In the present study, we demonstrated that miR-324-5p was downregulated in CC and that restoring its expression could mediate the sensitivity of radiation-induced cell death. Based on the confirmed regulatory role of miR-324-5p in the sensitivity to RT, we further explored its downstream targets in CC.

It has been previously reported that ELAVL1 is expressed in various organs and tissues and is regarded as an important molecular target of inflammatory responses.[6] Additionally, the upregulated expression of ELAVL1 has been found in several human malignant tumours.[8,11] ELAVL1 usually acts as a tumour promoter due to its proliferative and antiapoptotic effects.[28] In fact, ELAVL1 is a key mediator during the growth and metastasis of cancer.[29,30] In colorectal cancer, a previous study demonstrated that ELAVL1 could be negatively regulated by miR-324-5p during the apoptosis of cancer cells.[22] However, the effect of the miR-324-5p/ELAVL1 axis in CC remains to be explored. As we expected, our findings were consistent with the reported biological function of miR-324-5p/ELAVL1, suggesting that ELAVL1 could act as a key tumour promoter during the progression of CC and further confirming that ELAVL1 was negatively modulated by miR-324-5p. However, it is important to consider that our study is limited to cell experiments, and we will conduct in vivo experiments for further verification. Nevertheless, this research provides a new potential strategy to enhance the sensitivity of CC cells to RT.

In conclusion, this study further verified that ELAVL1 is a novel target of miR-324-5p and that miR-324-5p has two binding sites in the ELAVL1 3′-UTR. MiR-324-5p could directly regulate the expression of ELAVL1 and increase the radiotherapy sensitivity of CC.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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