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Circ_0010235 confers cisplatin resistance in lung cancer by upregulating E2F7 through absorbing miR-379-5p

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Abstract

Background: Cisplatin (DDP) treatment is one of the most predominant chemotherapeutic strategies for lung cancer patients. Circular RNAs (circRNAs) have been revealed to participate in the chemoresistance in lung cancer. Hence, the role and mechanism of circ_0010235 in cisplatin resistance in lung cancer was investigated.

Methods: Expression levels of circ_0010235, microRNA (miR)-379-5p and E2F transcription factor 7 (E2F7) were analyzed using quantitative reverse transcription PCR (qRT-PCR) and western blot. Cell DDP sensitivity, proliferation, apoptosis, invasion, and migration were detected by cell counting kit-8 assay, 5-ethynyl-2′-deoxyuridine (EDU) assay, flow cytometry and western blot, respectively. The binding interaction was verified using dual-luciferase reporter assay. A murine xenograft model was established to investigate effects in vivo.

Results: Circ_0010235 was highly expressed in DDP-resistant lung cancer tissues and cells. Knockdown of circ_0010235 elevated DDP sensitivity, constrained proliferation, invasion and migration as well as fostered apoptosis in DDP-resistant lung cancer cells. Moreover, circ_0010235 silencing boosted DDP sensitivity and impeded tumor growth in lung cancer in vivo. Mechanistically, circ_0010235 acted as a sponge for miR-379-5p to elevate the expression of its target E2F7. Rescue experiments showed that miR-379-5p inhibition attenuated circ_0010235 knockdown-evoked reduction on DDP resistance of DDP-resistant cancer cells. In addition, miR-379-5p re-expression elevated DDP sensitivity and suppressed the malignant phenotype of DDP-resistant lung cancer cells through miR-379-5p.

Conclusion: Circ_0010235 knockdown reduced DDP resistance and tumor growth via miR-379-5p/ E2F7 axis in lung cancer, suggesting an effective therapeutic target for lung cancer patients.

KEYWORDS
circ_0010235, cisplatin resistance, E2F7, lung cancer, miR-379-5p

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths, ranking second in malignancies.[1] Globally, there are about 2.09 million new cases in 2018 with 1.76 million deaths.[2] Although tremendous efforts in improving the clinical management of lung cancer have been made, the survival rate after complete lung resection is still unsatisfactory.[3] Cisplatin (DDP)-based chemotherapy is typically first-line therapy for lung cancer, which is able to improve the survival rate of patients.[4,5] Nevertheless, patients undergoing repeated cisplatin therapy can develop chemoresistance, which in turn restricts the therapeutic effects.[6] Thus, an in-depth study of molecular mechanisms underlying cisplatin resistance is of great significance for the treatment of resistant lung cancer patients.

Circular RNAs (circRNAs) are noncoding molecules formed by a covalently closed loop that lack the 3′ and 5′ ends, thus, circRNAs are highly stable and resistant to the degradation mediated by exonuclease.[7] Moreover, circRNAs have been increasingly recognized to play key roles in modulating significant biological processes related to carcinogenesis, migration, and differentiation,[8,9] and are thus involved in the initiation and progression of various diseases, including cancers.[10] More importantly, numerous studies have uncovered that aberrantly expressed circRNAs participate in chemoresistance in cancer by impacting cancer cell survival, metastasis, and drug transport.[11,12] For example, circRNA_102272 conferred cisplatin-resistance in hepatocellular carcinoma by elevating RUNX2 through sponging microRNA (miR)-326.[13] CircRNA AKT3 has been demonstrated to reduce apoptosis and expedite DNA damage repair in gastric cancer via miR-198/PIK3R1 to increase cisplatin resistance.[14] Circ_0010235 is derived from its host gene ALDH4A1 (aldehyde dehydrogenase 4 family member A1), it is located at chr1:19201875–19 216 599 with a length of 14 724 bp. Recently, circ_0010235 was found to be highly expressed in NSCLC, and knockdown of circ_0010235 impaired cancer cell survival, thus reducing tumor growth.[15] However, the role of circ_0010235 in cisplatin resistance in lung cancer remain vague.

Hence, this study used the cisplatin-resistant lung cancer cells and xenograft in mice to investigate the action of circ_0010235 in regulating cisplatin sensitivity. Furthermore, how circ_0010235 regulates cisplatin resistance in lung cancer was also explored.

METHODS

Clinical samples

This work was approved by the Ethics Committee of Changzhou Third People's Hospital, and all subjects signed written informed consent. Fifty-five paired lung cancer tissues and adjacent normal tissues were collected by surgery at Changzhou Third People's Hospital. All patients were newly diagnosed by pathological examination and none had received chemotherapy or radiotherapy before DDP-based chemotherapy. Among them, 27 specimens were DDP-sensitive and 28 were DDP-resistant ($N = 28$). All tissues were stored at −80°C until used.

Cell culture and treatment

Human bronchial epithelial (HBE) cells and lung cancer cells (A549 and H1299) were obtained from Biotechnology, and cultured in RPMI-1640 medium (Life Technologies) plus 1% penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (FBS) (Life Technologies) with 5% CO_2 at 37°C.

Parental cell lines (A549 and H1299) were gradually exposed to medium with increased doses of DDP (Sigma) for 6 months to produce DDP-resistant lung cancer cell lines (A549/DDP and H1299/DDP). The initial concentration of DDP was 5 μM and the final concentration was 150 μM. DDP-resistant cells were cultured in the same medium with 25 μM cisplatin to maintain cell resistant property.

Cell transfection

The designed siRNAs for circ_0010235 (si-circ_0010235) and nontarget siRNA (si-NC), pCD5-ciR/circ_0010235 overexpressing vector (overexpressing vector) and empty pCD5-ciR (pCD5-ciR), pcDNA3.1/E2F7 overexpressing vector (E2F7) and empty pcDNA3.1 (pcDNA), miR-379-5p mimic or inhibitor (miR-379-5p or anti-miR-379-5p) and mimic or inhibitor negative control were procured from Genechem. Then, transient transfection was performed in A549/DDP and H1299/DDP cells using lipofectamine 2000 (Invitrogen) with 100 nM of siRNAs, 100 ng of plasmids, or 50 nM of miRNA mimics or inhibitors.

Quantitative reverse transcription PCR (qRT-PCR)

Nuclear and cytoplasmic separation was performed as per the protocol of the PARIS kit (Invitrogen) to determine circRNA localization. Total RNAs was prepared using the RNeasy mini kit according to the standard protocol. To validate the cyclization of circRNA, 2 μg of RNA extractions was incubated with RNase R (3 U/μg, Epicenter Technologies) for 1 h at 37°C. Thereafter, the first-strand of cDNA was synthesized using the PrimeScript RT polymerase (Qiagen) or miScript II RT kit (Qiagen), and quantitative PCR was then implemented using the SYBR Premix Ex Taq II (Qiagen). Fold changes were assessed using relative quantification ($2^{-\Delta\Delta Ct}$) with U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference control. The primer sequences were:

circ_0010235: F 5′-GGGCCTGTACTGTCTGTGTA-3′, R 5′-CTCCCACCCTCCCATTACCTT-3′;

E2F7: F 5′-GCAGTGGTTGTTTCTGTCAGG-3′, R 5′-AACCCTGGTCAGTGTAGGGC-3′;

ALDH4A1: F 5′-CTCAGCCTTCGAGTACGGTG-3′, R 5′-CCCGAAGATCTCCTTGGCAT-3′.

GAPDH: F 5′-TCACCACCATGGAGAAGGC-3′, R 5′-GCTAAGCAGTTGGTGGTGCA-3′;

miR-379-5p: F 5′-GCCGAGTGGTAGACTATGGAA-3′, R 5′-CTCAACTGGTGTCGTGGA-3′;

U6: F 5′-CTCGCTTCGGCAGCACA-3′, R 5′-AACGCTTCACGAATTTGCGT-3′.

Cell counting kit-8 (CCK-8) assay

For the assessment of IC50 value, transfected A549/DDP and H1299/DDP cells were maintained in cell medium with different concentrations of DDP (0, 5, 10, 20, 30, 40 or 50 μM) for 48 h. For cell viability detection following assigned transfection, cells were seeded into a 96-well plate with cell medium containing 25 μM cisplatin, then 10 μL CCK-8 (5 mg/mL) (Solarbio) was added to each well and incubated for another 1 h. The absorbance was measured at 450 nm to assess cell viability.

5-ethynyl-2′-deoxyuridine (EdU) assay

After 48 h of the indicated transfection, resistant cells were incubated with respective medium containing 50 μM EdU (RiboBio) and 25 μM cisplatin for 2 h. Afterwards, cells were fixed by 4% paraformaldehyde, and then interacted with 1 × Apollo staining solution for 30 min after washing with phosphate buffered saline (PBS). Cell nuclei was stained with 4', 6-diamidino-2-phenylindole (DAPI), and the EdU positive cells were determined using a fluorescence microscope.

Flow cytometric analysis

After transfection, resistant cells were cultured with 25 μM cisplatin for 48 h. Then cell apoptosis was analyzed as per the protocol of the annexin V-fluorescein isothiocyanate apoptosis detection kit I (BD Biosciences).

Transwell invasion assay

Transwell chambers (Costar) with matrigel-coated membrane (BD Biosciences) were used for cell invasion analysis. After transfection, A549/DDP and H1299/DDP cells with 200 μL serum-free medium were seeded onto the upper chamber containing 25 μM cisplatin. Then, 600 μL 10% FBS-contained culture medium was added to the lower chamber. After incubation for 48 h, cells on the bottom surface of the membranes were fixed in precooled methanol and stained with 0.1% crystal violet solution (Solarbio), and then counted using a microscope (CKX41, Olympus Corporation).

Wound healing assay

Transfected A549/DDP and H1299/DDP cells were placed in a six-well plate with complete medium containing 25 μM cisplatin, then a scratch in single-cell layer was generated using a sterile 200 μL pipette tip. The wound closure was photographed at 0 and 24 h, and cell migration was calculated. The relative migration ratio was calculated by (the initiation width of the scratches minus the endpoint width of the scratches)/the initiation width of the scratches, setting the ratio of the control group as 100%.

Western blot

Total proteins, extracted using radioimmunoprecipitation assay (RIPA) buffer (Beyotime), were separated by 10% SurePAGE, and then shifted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was probed with primary antibodies against cyclin D1 (1:10 000, ab134175), matrix metallopeptidase 9 (MMP-9) (1:2000, ab38898), MDR1 (1:1000, ab170904), GAPDH (1:10 000, ab181602), obtained from Abcam and E2F7 (1:1000, Cat# PA5-68912, Invitrogen) at 4°C overnight. After incubation with horseradish peroxidase (HRP)-conjugated antibody for 2 h, the protein bands were determined using the enhanced chemiluminescence system (ECL; Solarbio).

Dual-luciferase reporter assay

Circ_0010235 or E2F7 3'UTR fragments covering wild-type miR-379-5p binding sites or the mutated sequences at miR-185-5p binding sites were cloned into a pGL3-control luciferase reporter plasmid (Invitrogen). Then, resistant cells infected with miR-379-5p mimic or mimic NC were transfected with 50 ng pGL3 vector, and 10 ng pRL-TK Renilla for 48 h, and the Firefly and Renilla luciferase activity were assayed using a dual-luciferase assay kit (Promega).

Tumor formation experiments

Lentiviral particles carrying sh-NC or sh-circ_0010235 were generated by GeneChem and then transfected into H1299/DDP cells, followed by puromycin selection. Stable infected H1299/DDP cells were subcutaneously injected into the right flank of 24 BALB/c nude mice ($n = 6$/per group, 5-week-old, Charles River Labs, Beijing, China). When the tumor volume reached 100 mm^3, nude mice were intraperitoneally injected with PBS or DDP (4 mg/kg) twice a week. Tumor size was measured every 3 days and tumor volume calculated with the equation: Volume = (length × $width^2$)/2. Mice were killed at day 23, the tumors were separated, weighed and divided either for molecular detection using qRT-PCR and western blot, or fixed in formalin for E2F7 immunohistochemistry (IHC) analysis as described previously.[16] The protocols of this animal study were permitted by the ethics committee of Changzhou Third People's Hospital.

Statistical analysis

The data were derived from three replicate experiments and expressed as mean ± standard deviation. Student's *t*-test (two-sided) or analysis of variance was used to compare the differences. Pearson's correlation analysis was used to measure the linear correlation between two continuous variables. All statistical significance was calculated using GraphPad

Prism 6 software (GraphPad) with $p < 0.05$ as significant differences.

RESULTS

Circ_0010235 is highly expressed in DDP-resistant lung cancer tissues and cells

To explore the role of circ_0010235 in the resistance of lung cancer to DDP, the expression profile of circ_0010235 in clinical samples was first detected. As shown in Figure 1a, circ_0010235 expression was significantly higher in 28 DDP-resistant lung cancer tissues than those in 27 DDP-sensitive cancer tissues. Thereafter, DDP-resistant lung cancer cells were established. We found that the expression of circ_0010235 was overtly boosted in lung cancer cells (A549 and H1299) relative to the HBE cells, furthermore, circ_0010235 expression was apparently higher in A549/DDP and H1299/DDP cells in comparison to their parental cells (Figure 1b). Then the stability and localization of circ_0010235 was investigated. The results suggested that circ_0010235 was resistant to the degradation by RNase R in A549/DDP and H1299/DDP cells exhibiting that circ_0010235 stably acted as a typical circRNA (Figure 1c,d). In addition, the results of nuclear-cytoplasm separation implied that circ_0010235 was predominantly localized in the cytoplasm in A549/DDP and H1299/DDP cells (Figure 1e,f). Therefore, these results confirmed that circ_0010235 was a stable circRNA and might be associated with DDP resistance in lung cancer.

Knockdown of circ_0010235 overcomes DDP resistance in DDP-resistant lung cancer cells

Considering the upregulation of circ_0010235 in DDP-resistant lung cancer tissues and cells, we then explored the detailed function of circ_0010235 in the resistance of lung cancer cells to DDP using loss-of function assay. The siRNA targeting circ_0010235 was designed and transfected into A549/DDP and H1299/DDP cells. The results of qRT-PCR showed that the introduction of si-circ_0010235 overtly reduced circ_0010235 expression in A549/DDP and H1299/DDP cells compared with si-NC transfection (Figure 2a). Functionally, we analyzed the IC50 values of A549/DDP and H1299/DDP cells to DDP, which were almost 3.0- and 2.7-fold higher than that of A549 and H1299 cells (Figure 2b), while circ_0010235 knockdown boosted the sensitivity of A549/DDP and H1299/DDP cells to DDP and reduced the IC50 values of A549/DDP and H1299/DDP cells compared with the si-NC group (Figure 2c). Subsequently, the effects of circ_0010235 knockdown on the malignant phenotype of DDP-resistant lung cells in the presence of 25 μM DDP were analyzed and we proved that circ_0010235 knockdown reduced cell proliferation in A549/DDP and H1299/DDP cells, manifested by CCK-8 and EdU assays (-Figure 2d,e). Flow cytometry assay indicated that silencing of circ_0010235 promoted the apoptosis rate in A549/DDP and H1299/DDP cells (Figure 2f). Moreover, both the transwell and wound healing assays showed that circ_0010235 knockdown suppressed cell invasion and

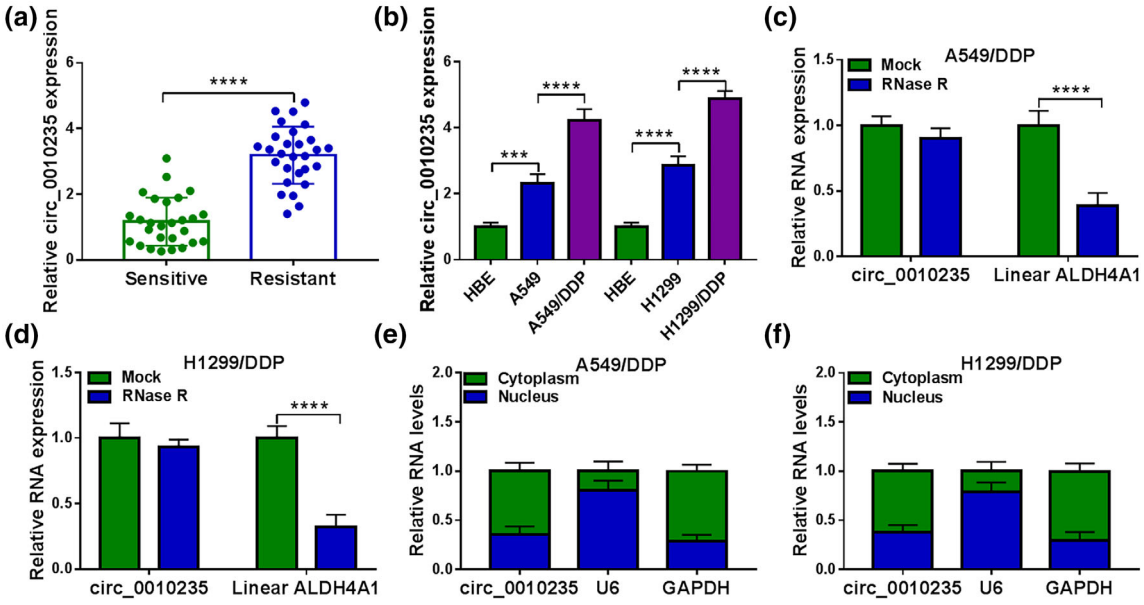

FIGURE 1 Circ_0010235 is highly expressed in DDP-resistant lung cancer tissues and cells. (a) Quantitative reverse transcription PCR (qRT-PCR) analysis of circ_0010235 expression in 27 DDP-sensitive and 28 DDP-resistant lung cancer tissues. (b) qRT-PCR analysis of circ_0010235 expression in normal human bronchial epithelial (HBE), A549, H1299, A549/DDP and H1299/DDP cells. (c, d) Detection of the expression of circ_0010235 and linear ALDH4A1 mRNA in A549/DDP and H1299/DDP cells after RNase R treatment. (e, f) qRT-PCR analysis of the levels of circ_0010235 in cytoplasm and nucleus of A549/DDP and H1299/DDP cells. ***$p < 0.001$ and ****$p < 0.0001$.

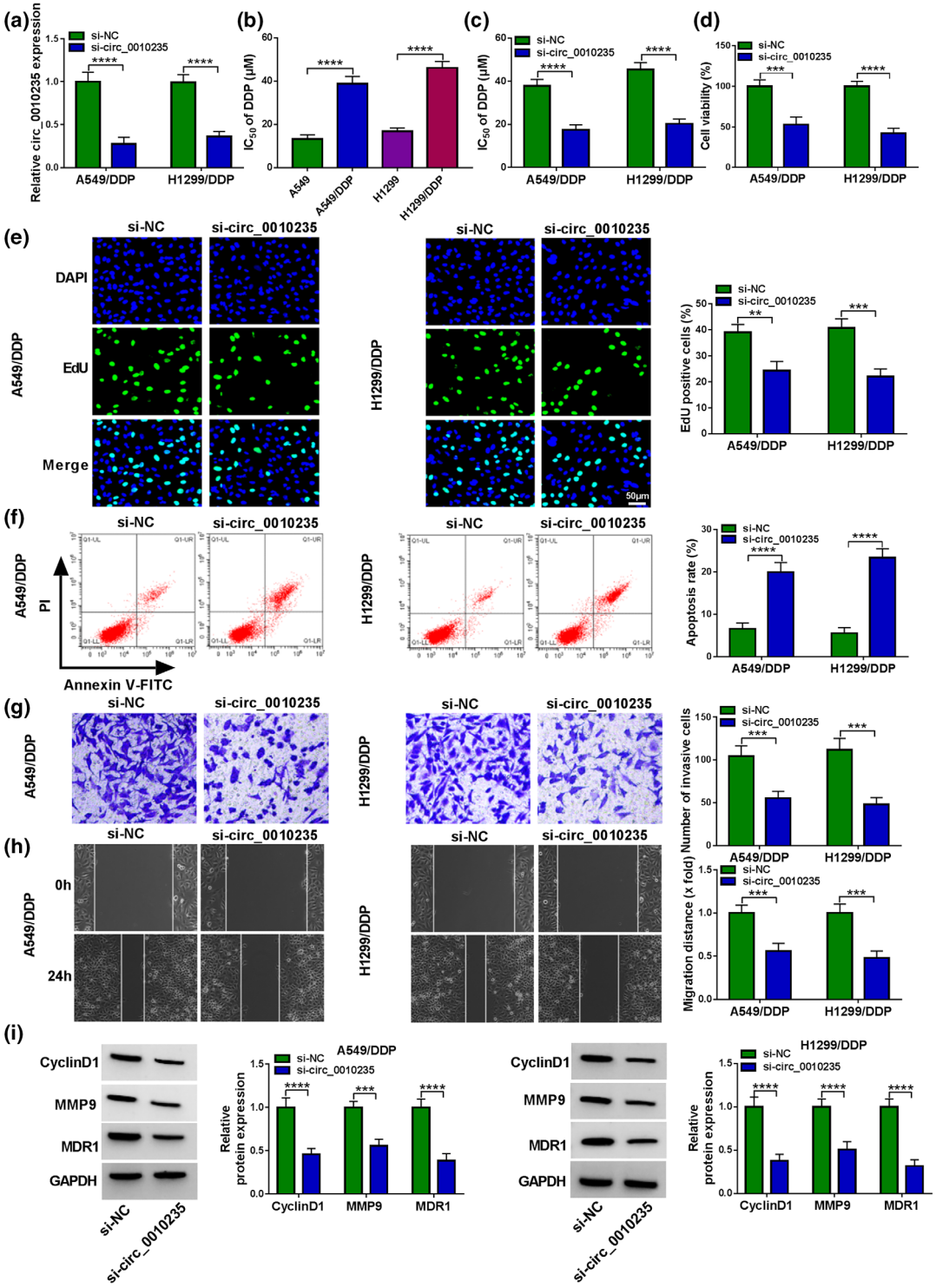

FIGURE 2 Knockdown of circ_0010235 overcomes DDP resistance in DDP-resistant lung cancer. (a) The interference efficiency of si-circ_0010235 or si-NC in A549/DDP and H1299/DDP cells using quantitative reverse transcription PCR (qRT-PCR). (b) Cell counting kit-8 (CCK-8) assay of the IC50 values of A549/DDP and H1299/DDP as well as their parental cells to DDP. (c) CCK-8 assay of the IC50 values of A549/DDP and H1299/DDP cells to DDP after the introduction of si-circ_0010235 or si-NC. The proliferation (d, e), apoptosis (f), invasion (g) and migration (h) of si-circ_0010235 or si-NC-transfected A549/DDP and H1299/DDP cells under 25 μM DDP treatment were determined using CCK-8 assay, 5-ethynyl-2′-deoxyuridine (EdU), flow cytometry, transwell, and wound healing assays, respectively. (i) Western blot analysis of cyclin D1, MMP9, and MDR1 protein levels in A549/DDP and H1299/DDP cells transfected with si-circ_0010235 or si-NC under 25 μM DDP treatment. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

migration in A549/DDP and H1299/DDP cells (Figure 2g,h). Additionally, the levels of cyclin D1, MMP9, and MDR1 in A549/DDP and H1299/DDP cells were found to be decreased after circ_0010235 knockdown (Figure 2i). Altogether, circ_0010235 knockdown sensitized DDP-resistant lung cancer cells to DDP.

MiR-379-5p is a target of circ_0010235

Based on the results that circ_0010235 was predominantly localized in the cytoplasm in DDP-resistant lung cancer cells, we searched the latent miRNAs with complementary base pairing with circ_0010235 using circBank and starBase databases, and both showed that miR-379-5p, miR-34a-5p, and miR-34c-5p had base pairs complementary to circ_0010235 (Figure 3a). Next, qRT-PCR analysis suggested that only miR-379-5p expression was significantly elevated by circ_0010235 knockdown in A549/DDP and H1299/DDP cells (Figure 3b,c). The binding sites between miR-379-5p and circ_0010235 are shown in Figure 3d. After confirming the elevation efficiency of miR-379-5p mimic (Figure 3e), further dual-luciferase reporter assay implied that miR-379-5p overexpression strongly decreased the luciferase activity of wild-type circ_0010235 reporter in A549/DDP and H1299/DDP cells, while the mutated circ_0010235 reporter group exhibited no luciferase activity difference (Figure 3f,g), verifying the direct interaction between miR-379-5p and circ_0010235.

In addition, miR-379-5p expression was discovered to be decreased in DDP-resistant lung cancer tissues (Figure 3h), which was negatively correlated with circ_0010235 expression (Figure 3i). Similarly, its expression was also lower in A549/DDP and H1299/DDP cells compared with their parental cells (Figure 3j). Moreover, circ_0010235 overexpression vector significantly elevated circ_0010235 expression in A549/DDP and H1299/DDP cells (Figure 3k), while miR-379-5p expression was decreased in circ_0010235-overexpressed A549/DDP and H1299/DDP cells (Figure 3l). Therefore, we verified that circ_0010235 targetedly suppressed miR-379-5p expression.

Knockdown of circ_0010235 promotes DDP-resistant lung cancer cell sensitivity to DDP via targeting miR-379-5p

To further investigate whether miR-379-5p was a functional target of circ_0010235, rescue experiments were performed. We observed that miR-379-5p inhibitor reduced the elevation of miR-379-5p level caused by circ_0010235 knockdown in A549/DDP and H1299/DDP cells (Figure 4a). CCK-8 assay showed that the decrease of IC50 values of A549/DDP and H1299/DDP cells mediated by circ_0010235 knockdown were partly reversed by miR-379-5p inhibition (Figure 4b). Moreover, in the presence of 25 μM DDP, miR-379-5p inhibition attenuated

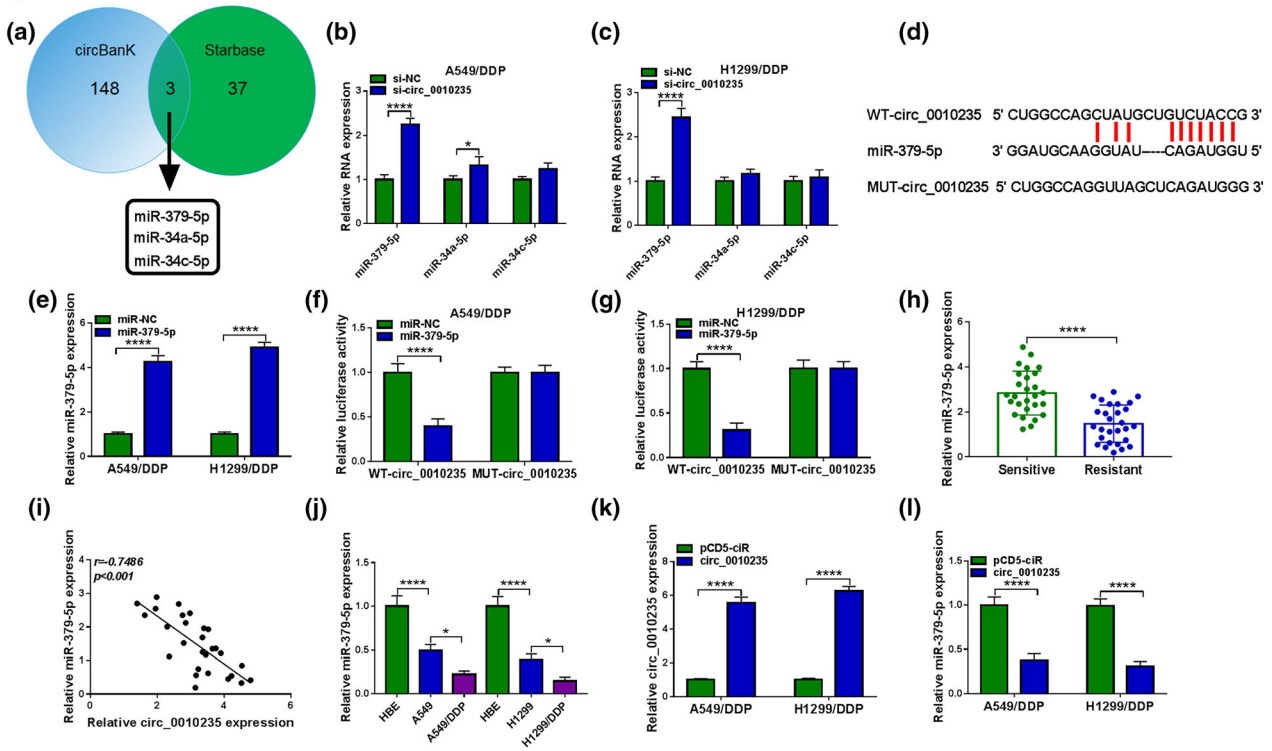

FIGURE 3 MiR-379-5p is a target of circ_0010235. (a) Schematic illustration of the overlap of the target miRNAs of circ_0010235 predicted by circBank and starBase databases. (b, c) Quantitative reverse transcription PCR (qRT-PCR) analysis of miR-379-5p, miR-34a-5p, and miR-34c-5p expression levels in A549/DDP and H1299/DDP cells transfected with si-circ_0010235 or si-NC. (d) The binding sites between miR-379-5p and circ_0010235 are exhibited. (e) qRT-PCR analysis of miR-379-5p expression in A549/DDP and H1299/DDP cells transfected with miR-379-5p mimic or mimic negative control. (f, g) Dual-luciferase reporter assay for the luciferase activity of wild and mutated circ_0010235 reporter after miR-379-5p overexpression in A549/DDP and H1299/DDP cells. (h) qRT-PCR analysis of miR-379-5p expression in 27 DDP-sensitive and 28 DDP-resistant lung cancer tissues. (i) MiR-379-5p expression was negatively correlated with circ_0010235 expression in DDP-resistant lung cancer tissues. (j) qRT-PCR analysis of miR-379-5p expression in normal human bronchial epithelial (HBE), A549, H1299, A549/DDP and H1299/DDP cells. (k) Detection of the elevation efficiency of circ_0010235 or pCD5-ciR vector in A549/DDP and H1299/DDP cells. (l) qRT-PCR analysis of miR-379-5p expression in circ_0010235-increased A549/DDP and H1299/DDP cells. $^{*}p < 0.05$ and $^{****}p < 0.0001$.

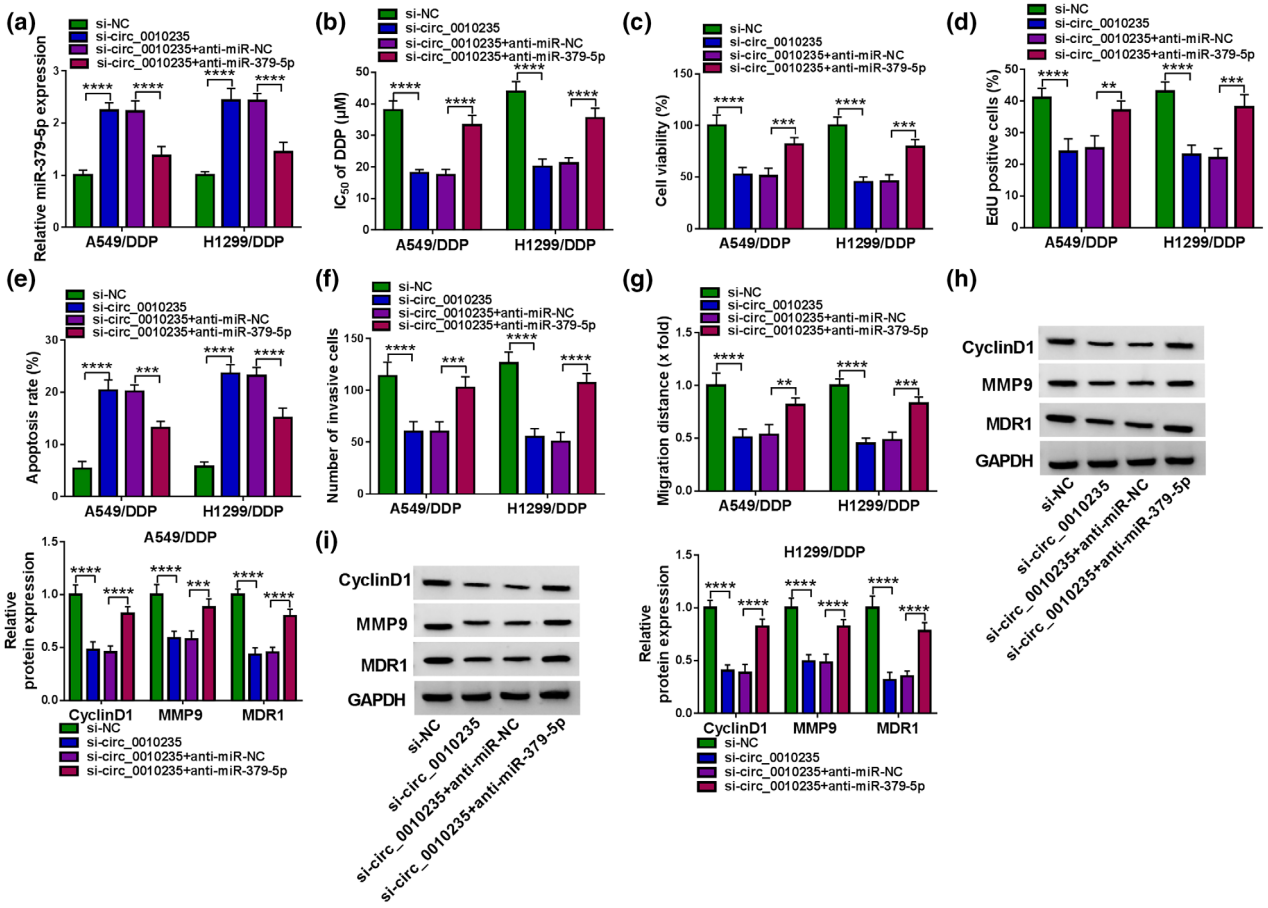

FIGURE 4 Knockdown of circ_0010235 promotes DDP-resistant lung cancer cell sensitivity to DDP via targeting miR-379-5p. (a–i) A549/DDP and H1299/DDP cells were cotransfected with si-NC, si-circ_0010235, si-circ_0010235 + anti-miR-NC or si-circ_0010235 + anti-miR-379-5p. (a) Measurement of miR-379-5p expression levels in A549/DDP and H1299/DDP cells using quantitative reverse transcription PCR (qRT-PCR). (b) Cell counting kit-8 (CCK-8) assay of the IC50 values of A549/DDP and H1299/DDP cells to DDP. The proliferation (c, d), apoptosis (e), invasion (f) and migration (g) of transfected A549/DDP and H1299/DDP cells under 25 μM DDP treatment were determined using CCK-8, 5-ethynyl-2′-deoxyuridine (EdU), flow cytometry, transwell, and wound healing assays, respectively. (h, i) Western blot analysis of cyclin D1, MMP9, and MDR1 protein levels in A549/DDP and H1299/DDP cells under 25 μM DDP treatment. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

circ_0010235 silencing-evoked suppression of cell proliferation (Figure 4c,d), enhancement of apoptosis (Figure 4e), reduction of cell invasion and migration (Figure 4f,g) in A549/DDP and H1299/DDP cells. In addition, the decrease of cyclin D1, MMP9 and MDR1 in circ_0010235-downregulated A549/DDP and H1299/DDP cells was rescued by the introduction of miR-379-5p inhibitor (Figure 4h,i). Thus, we demonstrated that the circ_0010235/miR-379-5p axis was responsible for DDP resistance in lung cancer.

E2F7 is a target of miR-379-5p

According to the prediction of the starBase database, miR-379-5p also had base pairs complementary to E2F7 (Figure 5a). The results of the dual-luciferase reporter assay exhibited that miR-379-5p overexpression strongly reduced the luciferase activity of wild-type E2F7 reporter but not the mutated one in A549/DDP and H1299/DDP cells (Figure 5b,c), implying the binding between miR-379-5p and E2F7. Then the expression pattern of E2F7 was investigated. The mRNA of E2F7 was increased in DDP-resistant lung cancer tissues (Figure 5d), and was negatively correlated with miR-379-5p expression (Figure 5e). Also, western blot analysis showed an increase of E2F7 protein level in DDP-resistant lung cancer tissues and cells (A549/DDP and H1299/DDP cells) (Figure 5f,g). In addition, we observed that miR-379-5p inhibition reduced miR-379-5p expression level in A549/DDP and H1299/DDP cells (Figure 5h). Furthermore, miR-379-5p inhibition elevated E2F7 expression, while miR-379-5p mimic reduced E2F7 expression in A549/DDP and H1299/DDP cells (Figure 5i). In all, miR-379-5p directly targeted E2F7 and negatively regulated its expression.

MiR-379-5p re-expression elevates DDP-resistant lung cancer cell sensitivity to DDP via targeting E2F7

Next, whether miR-379-5p/E2F7 axis was engaged in DDP resistance in lung cancer was determined. Western blot analysis showed that E2F7 transfection rescue miR-379-5p-

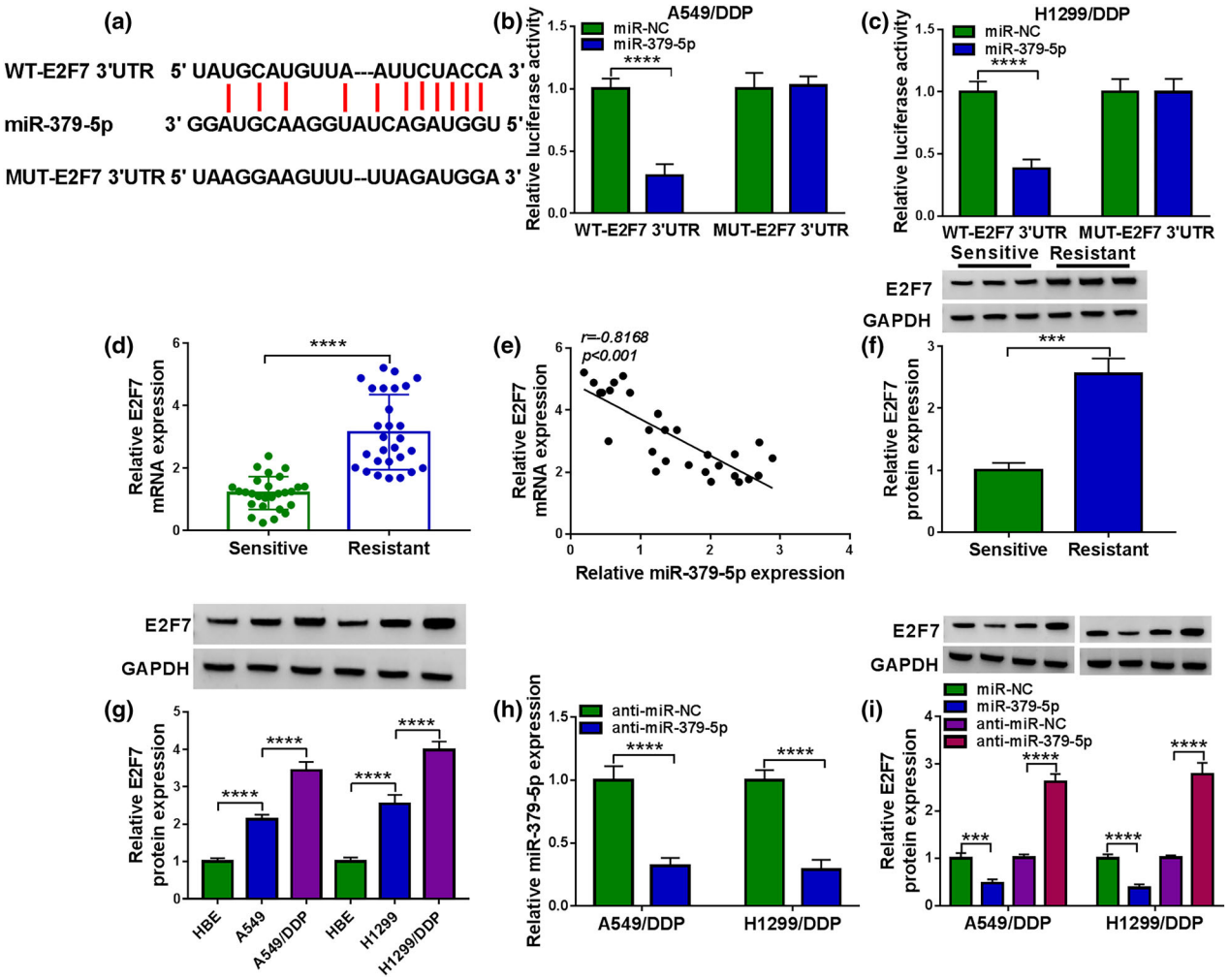

FIGURE 5 E2F7 is a target of miR-379-5p. (a) The binding sites between miR-379-5p and E2F7 are shown. (b, c) Dual-luciferase reporter assay for the luciferase activity of wild and mutated E2F7 reporter after miR-379-5p overexpression in A549/DDP and H1299/DDP cells. (d) Quantitative reverse transcription PCR (qRT-PCR) analysis of E2F7 mRNA expression in 27 DDP-sensitive and 28 DDP-resistant lung cancer tissues. (e) E2F7 mRNA expression was negatively correlated with miR-379-5p expression in DDP-resistant lung cancer tissues. (f) Western blot analysis of E2F7 protein levels in 27 DDP-sensitive and 28 DDP-resistant lung cancer tissues. (g) Western blot analysis of E2F7 protein levels in normal HBE, A549, H1299, A549/DDP and H1299/DDP cells. (h) The interference efficiency of miR-379-5p inhibitor or inhibitor NC in A549/DDP and H1299/DDP cells using qRT-PCR. (i) Western blot analysis of E2F7 protein levels in miR-379-5p up- or downregulated A549/DDP and H1299/DDP cells. ***$p < 0.001$ and ****$p < 0.0001$.

induced reduction of E2F7 level in A549/DDP and H1299/DDP cells (Figure 6a). Further function experiments demonstrated that miR-379-5p upregulation reduced the IC50 values of the A549/DDP and H1299/DDP cells, which was reversed by E2F7 overexpression (Figure 6b). In addition, re-expression of miR-379-5p impeded the proliferation ability (Figure 6c,d), enhanced the apoptosis rate (Figure 6e), and suppressed the invasion and migration capacities (Figure 6f,g) in A549/DDP and H1299/DDP cells under 25 μM DDP treatment, while this condition was attenuated by E2F7 upregulation (Figure 6c–g). Moreover, E2F7 upregulation abolished miR-379-5p re-expression-evoked decreases of cyclin D1, MMP9 and MDR1 protein levels in A549/DDP and H1299/DDP cells after 25 μM DDP treatment (Figure 6h,i). Collectively, miR-379-5p increased cell sensitivity to DDP in DDP-resistant lung cancer cells via targeting E2F7.

Circ_0010235/miR-379-5p axis can regulate E2F7 expression

Thereafter, we detected whether there was a feedback loop among circ_0010235, miR-379-5p and E2F7. As shown in Figure 7a,b, knockdown of circ_0010235 led to a reduction of E2F7 expression, which was rescued by the inhibition of miR-379-5p in A549/DDP and H1299/DDP cells. Therefore, the circ_0010235/miR-379-5p/E2F7 axis was identified.

Circ_0010235 silencing boosts tumor sensitivity to DDP in vivo

The action of circ_0010235 on the resistance of lung cancer cells to DDP in vivo was further elucidated. Compared to those of control xenografts, circ_0010235 knockdown

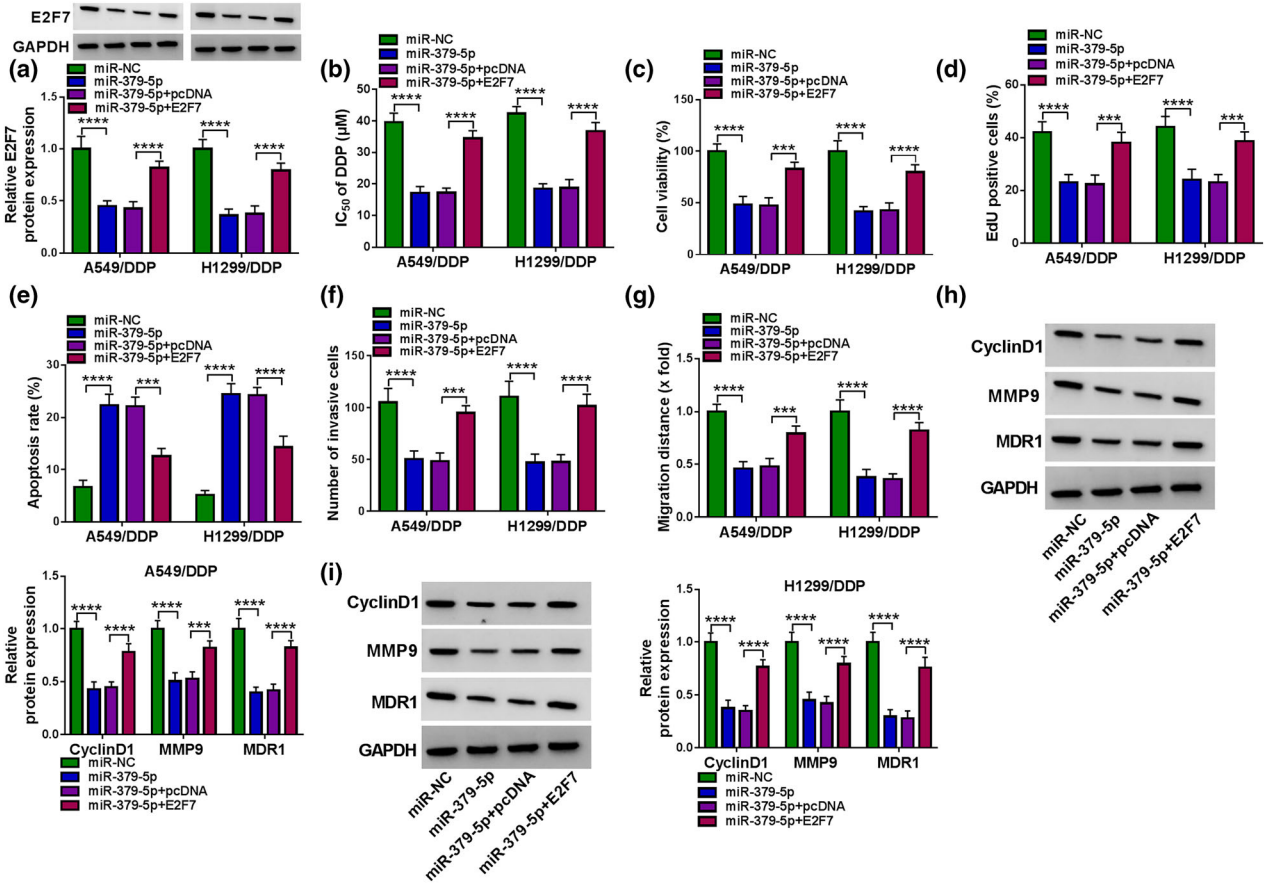

FIGURE 6 MiR-379-5p re-expression elevates DDP-resistant lung cancer cell sensitivity to DDP via targeting E2F7. (a–i) A549/DDP and H1299/DDP cells were transfected with miR-NC, miR-379-5p, miR-379-5p + pcDNA, or E2F7. (a) Western blot analysis of E2F7 protein levels in A549/DDP and H1299/DDP cells. (b) Cell counting kit-8 (CCK-8) assay of the IC50 values of A549/DDP and H1299/DDP cells to DDP. The proliferation (c, d), apoptosis (e), invasion (f) and migration (g) of transfected A549/DDP and H1299/DDP cells under 25 μM DDP treatment were determined using cell counting kit-8 (CCK-8) assay, 5-ethynyl-2′-deoxyuridine (EdU), flow cytometry, transwell, and wound healing assays, respectively. (h, i) Western blot analysis of cyclin D1, MMP9, and MDR1 protein levels in A549/DDP and H1299/DDP cells under 25 μM DDP treatment. ***$p < 0.001$ and ****$p < 0.0001$.

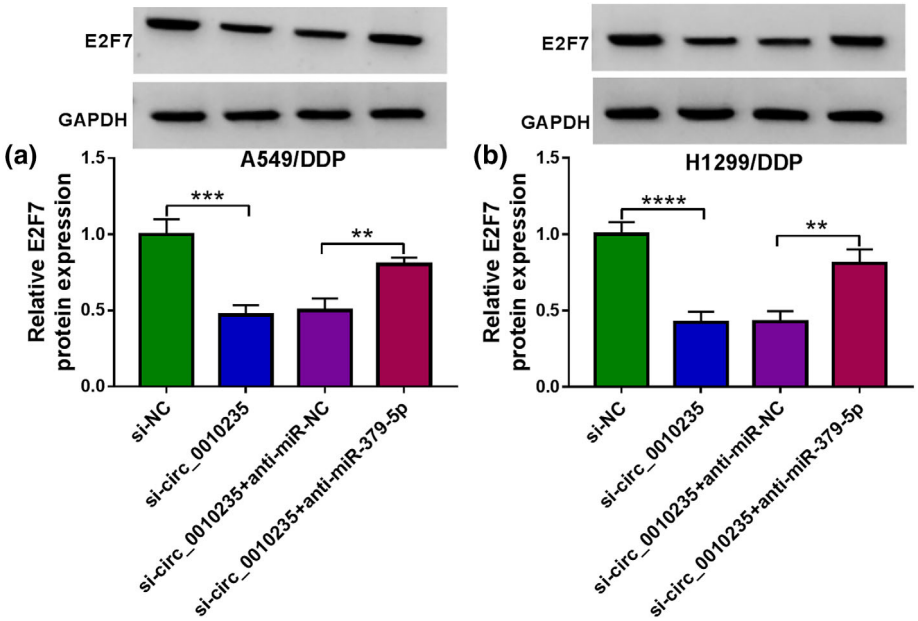

FIGURE 7 Circ_0010235/miR-379-5p axis can regulate E2F7 expression. (a, b) Western blot analysis of E2F7 expression in A549/DDP and H1299/DDP cells transfected with si-NC, si-circ_0010235, si-circ_0010235 + anti-miR-NC or si-circ_0010235 + anti-miR-379-5p. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

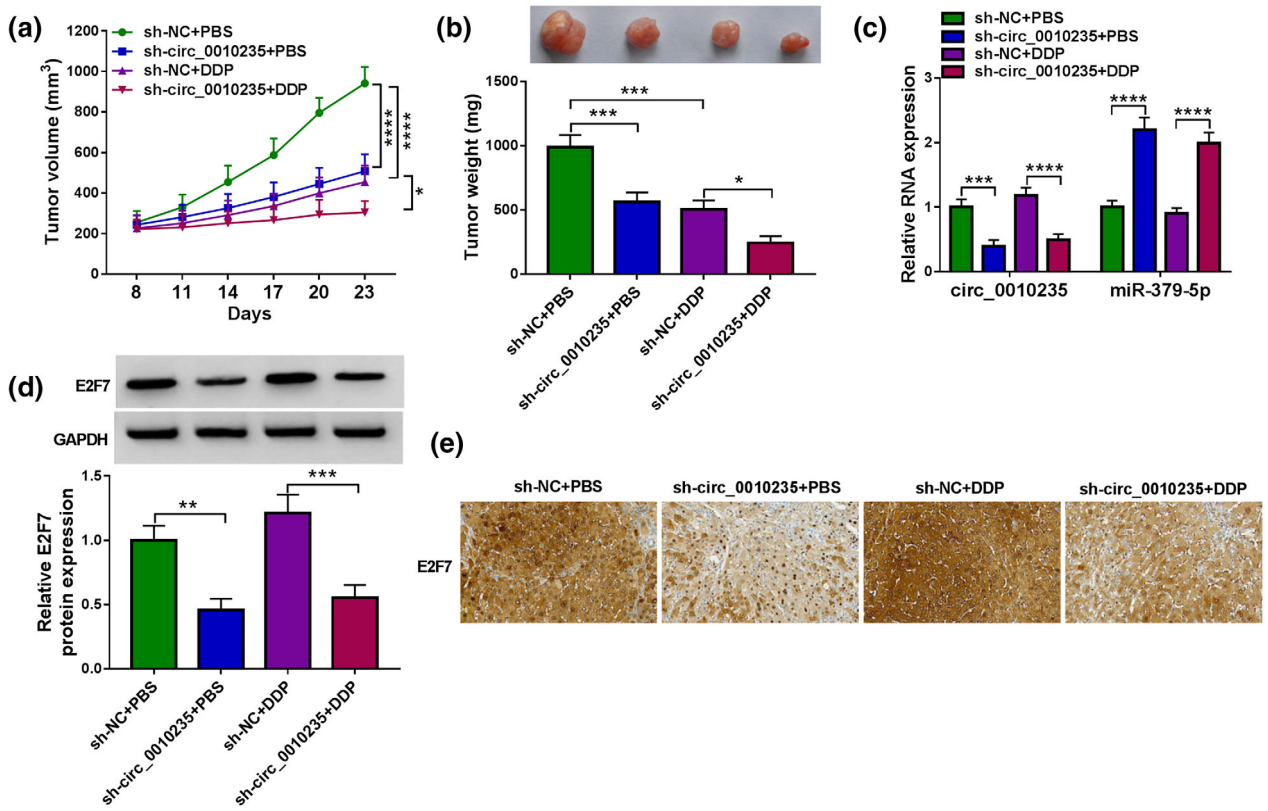

FIGURE 8 Circ_0010235 silencing boosts tumor sensitivity to DDP in vivo. (a) A caliper was used to measure tumor volume of mice in each group. (b) Typical lung cancer tumors were present, and tumor weight of mice in each group was detected. (c) The expression of circ_0010235 and miR-379-5p in tumor tissues of mice in each group was analyzed using quantitative reverse transcription PCR (qRT-PCR). (d, e) Western blot analysis and immunohistochemistry (IHC) staining for E2F7 protein level in tumor tissues of mice in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

slowed down the tumor growth in nude mice by size and weight without DDP treatment, moreover, circ_0010235 knockdown increased tumor sensitivity to DDP and further suppressed tumor growth (Figure 8a,b). After that, circ_0010235 and E2F7 expression were overtly decreased, and miR-379-5p expression was distinctly increased in tumor tissues of mice in the sh-circ_0010235 group with or without DDP treatment compared with the sh-NC group with or without DDP treatment (Figure 8c–e). Collectively, circ_0010235 silencing elevated tumor sensitivity to DDP in vivo.

DISCUSSION

Cisplatin is a well-recognized DNA-damaging cytotoxic agent in the treatment of cancer, and the resultant biological process in response to cisplatin therapy is the activation of the apoptotic pathway and destruction of malignant cells.[17] Cisplatin-based adjuvant chemotherapy plays a significant role in lung cancer treatment; however, acquired drug resistance after several cycles of cisplatin-based chemotherapy, which results in the limitation of overall clinical efficacy in patients, remains a significant clinical challenge in lung cancer patients.[18]

Recently, circRNAs have been revealed to be involved in cisplatin resistance in lung cancer. For instance, circRNA CDR1as,[19] hsa_circRNA_103809,[20] and circ-ABCB10[21] have been demonstrated to inhibit cisplatin sensitivity in lung cancer by affecting the malignant phenotype of resistant cancer cells. Accordingly, targeting circRNAs may be a potential strategy to overcome cisplatin resistance in lung cancer. In the current review, we focused on the role of circ_0010235 in lung cancer cisplatin resistance. It was found that circ_0010235 expression was higher in cisplatin-resistant lung cancer tissues and cells. Functionally, knockdown of circ_0010235 led to enhanced apoptosis and repressed the proliferative, migratory, and invasive capacities in cisplatin-resistant lung cancer cells after cisplatin treatment; furthermore, circ_0010235 silencing also boosted tumor sensitivity to DDP in vivo, suggesting that circ_0010235 silencing sensitized lung cancer cells to cisplatin.

Previous studies have revealed that circRNAs can serve as a competing endogenous RNA (ceRNA) to sponge miRNAs and subsequently repress their functions.[22,23] MiRNAs are short (~ 22 nt) noncoding RNAs, which have been found to participate in tumorigenesis and progression of various malignancies, including lung cancer.[24,25] In addition, miRNAs have also been shown to play a vital role in cisplatin resistance in lung cancer through regulating a wide

range of intracellular pathways.[26] In this study, we verified that circ_0010235 directly sponged miR-379-5p. MiR-379-5p has been discovered to act as a tumor suppressor, which induces lung cancer cell apoptosis.[27] In addition, in their study, Guo et al. found that miR-379-5p increased paclitaxel sensitivity and suppressed cell tumorigenesis in lung cancer.[28] Therefore, we assumed that miR-379-5p might be implicated in cisplatin resistance in lung cancer. This study showed a decrease of miR-379-5p expression in cisplatin-resistant tissues and cells, and further functional analyses demonstrated that miR-379-5p suppressed cell proliferation and mobility, but evoked apoptosis under cisplatin treatment, indicating the promoting role of miR-379-5p in forecasting the responses to cisplatin in lung cancer patients. More importantly, we also discovered that miR-379-5p inhibition reversed the inhibitory effects of circ_0010235 on cisplatin-resistant lung cancer cells.

MiRNAs can act as post-transcriptional regulators of target mRNAs via direct base pairing to target sites in mRNAs.[29,30] E2F family members are considered to play an important role in cell cycle control.[31] E2F7 is an atypical E2F factor, and has been reported to be involved in drug resistance in many types of cancers, such as breast cancer and squamous cell carcinoma.[32,33] In lung cancer, E2F7 has been recognized to function as an oncogene to promote cancer progression.[34,35] In the present study, we confirmed that miR-379-5p could directly bind to E2F7, and negatively modulate its expression level. E2F7 was highly expressed in cisplatin-resistant lung cancer tissues and cells. Moreover, E2F7 upregulation attenuated the promoting action of miR-379-5p on cisplatin sensitivity in lung cancer. In addition, it was also observed that circ_0010235 could upregulate E2F7 expression by miR-379-5p.

In conclusion, this study demonstrated that circ_0010235 knockdown reduced cisplatin resistance and tumor growth via the miR-379-5p/E2F7 axis in lung cancer, highlighting the potential of circ_0010235 as a therapeutic target for overcoming cisplatin resistance in lung cancer patients.

AUTHOR CONTRIBUTIONS

Lifei Wang conceived and designed the study, and drafted the first draft of the manuscript. All experiments were completed by all authors. Dongchang Wang, Zhen Xu, Yali Qiu, Gang Chen, Furong Tan analyzed and collated the results. All authors reviewed and critiqued the manuscript, and agreed to the final submission of the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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