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MiR-200c-3p and miR-485-5p overexpression elevates cisplatin sensitivity and suppresses the malignant phenotypes of non–small cell lung cancer cells through targeting RRM2

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Abstract

Background: This study intended to investigate the potential mechanism of microRNA-200c-3p (miR-200c-3p) and miR-485-5p in mediating the cisplatin (DDP) resistance in non–small cell lung cancer (NSCLC).

Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to measure the expression of miR-200c-3p, miR-485-5p, and ribonucleotide reductase regulatory subunit M2 (RRM2) messenger RNA (mRNA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to analyze the DDP resistance and the proliferation of NSCLC cells. Colony formation assay was used to assess cell proliferation. Transwell assays were used to evaluate cell migration and invasion. The target relationship between RRM2 and miR-200c-3p or miR-485-5p was verified using dual-luciferase reporter assay. The protein level of RRM2 was measured using Western blot assay. Animal experiments were conducted to analyze the roles of miR-200c-3p and miR-485-5p in the DDP resistance of xenograft tumors in vivo.

Results: MiR-200c-3p and miR-485-5p were both downregulated in DDP-resistant NSCLC tissues and cell lines. Overexpressing miR-200c-3p or miR-485-5p suppressed the DDP resistance and malignant behaviors of NSCLC cells. MiR-200c-3p played a synergistic role with miR-485-5p in regulating the chemo-resistance and biological behaviors NSCLC cells. RRM2 was confirmed as a target of miR-200c-3p and miR-485-5p. RRM2 silencing restrained the DDP resistance and progression of NSCLC. RRM2 overexpression partly reversed miR-200c-3p or miR-485-5p-induced influences in NSCLC cells. The overexpression of miR-200c-3p or miR-485-5p aggravated DDP-mediated suppressive effect on tumor growth in vivo.

Conclusion: MiR-200c-3p or miR-485-5p enhanced the DDP sensitivity and suppressed the malignant behaviors of NSCLC cells partly through targeting RRM2.

KEYWORDS
cisplatin, miR-200c-3p, miR-485-5p, non–small cell lung cancer, RRM2

INTRODUCTION

Although surgical resection and chemotherapy for non–small cell lung cancer (NSCLC) have been greatly improved, the 5-year survival is still far from satisfactory.[1,2] Cisplatin (DDP) is used as the chemotherapeutic drug for many malignancies, containing NSCLC.[3] The development of drug resistance becomes a big obstacle in cancer therapy. Many NSCLC patients experienced recurrence and metastasis because of the developed chemo-resistance.[4] Hence, it is imperative to explore the molecular targets that could sensitize NSCLC cells to DDP and to uncover associated signaling to develop effective treatment methods for DDP-resistant NSCLC patients.

Accumulating articles have pointed out that dysregulated microRNAs (miRNAs) are associated with the tumorigenesis

of malignancies.[5,6] Yang et al.[7] demonstrated that miR-200c-3p repression in epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) resistance NSCLC cell lines enhanced the drug resistance, and miR-200c-3p accumulation suppressed the malignant properties of EGFR TKI resistance cell lines. Huang et al.[8] found that miR-485-5p hampered NSCLC development via regulating insulin like growth factor 2 mRNA binding protein 2 (IGF2BP2). Cheng et al.[9] demonstrated that small nucleolar RNA host gene 11 (SNHG11) accelerated NSCLC progression through competitively upregulating Basigin (BSG) via sponging miR-485-5p. Nevertheless, the mechanism of miR-200c-3p and miR-485-5p in regulating DDP resistance of NSCLC cells remains to be illustrated.

Ribonucleotide reductase regulatory subunit M2 (RRM2) is important for the replication and repair of DNA.[10] The regulatory role of RRM2 on NSCLC progression has been reported by previous articles.[11–13] AKT elevated the tamoxifen resistance of breast cancer cells, and this effect was alleviated by RRM2 suppression,[14] demonstrating that high expression of RRM2 contributed to drug resistance of cancer cells. Huang et al.[15] found that actin filament-associated protein 1-antisense RNA1 (AFAP1-AS1) contributed to the growth and the drug resistance to DDP and 5-fluorouracil through up-regulating RRM2 partly via sponging miR-139-5p, suggesting that RRM2 elevated the chemo-resistance of NSCLC cells. Interestingly, RRM2 was a downstream partner of miR-200c-3p and miR-485-5p, and the role of RRM2 on the malignant phenotypes and DDP resistance of NSCLC cells was explored.

We investigated the functions of two miRNAs (miR-200c-3p and miR-485-5p) on the malignant behaviors and DDP resistance of NSCLC cells. The molecular mechanism behind the tumor suppressor roles of miR-200c-3p and miR-485-5p was subsequently explored.

MATERIALS AND METHODS

Clinical specimens

Thirty-nine DDP-sensitive NSCLC specimens and 31 DDP-resistant NSCLC specimens were collected at Jingmen No.1 People's Hospital. We followed the procedures for studies using human materials and received the permission by the ethics committee of Jingmen No.1 People's Hospital. Written informed consent had been provided by each subject before the surgery.

Cell lines

Human lung adenocarcinoma cell lines (A549 and H1299) and BEAS-2B were acquired from BeNa Culture Collection. A549/DDP and H1299/DDP were acquired from JRDUN Biotech. Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco) plus 10% fetal bovine serum (FBS) (Gibco), 1% penicillin (100 U/mL), and 1% streptomycin (100 μg/mL). Cell culture plates were placed in a 37°C incubator with 5% CO_2.

Quantitative real-time polymerase chain reaction

RNA was extracted from NSCLC specimens and cells with RNeasy Mini Kit (Omega). A total of 500 ng of RNA sample was used to synthesize complementary DNA (cDNA) with the Primer Script RT reagent kit (Takara, Dalian, China) and Mir-X miR First-Strand Synthesis Kit (Takara). The relative abundance of RRM2 messenger RNA (mRNA) was assessed using SYBR Green detection reagent (Cowin Biotech), whereas the levels of miR-200c-3p and miR-485-5p were determined with the miRNA quantitative real-time polymerase chain reaction (qRT-PCR) Detection kit (GeneCopoeia). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acted as the house-keeping gene for RRM2, whereas U6 small nuclear RNA was used as the house-keeping gene for miR-200c-3p or miR-485-5p. The relative expression was analyzed by the $2^{-\Delta\Delta Ct}$ method. Primers are listed in Table 1.

Cell transfection

MiR-200c-3p mimics (miR-200c-3p), miR-485-5p mimics (miR-485-5p), negative control miRNA (miR-NC), miR-200c-3p inhibitor (anti-miR-200c-3p), miR-485-5p inhibitor (anti-miR-485-5p), anti-miR-NC, RRM2 small interfering RNA (si-RRM2), si-NC, RRM2 overexpression plasmid (RRM2), and empty vector (Vector) were purchased from Genepharma. AgomiR-NC, AgomiR-200c-3p, and AgomiR-485-5p were acquired from Ribobio.

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

To evaluate the DDP resistance of NSCLC cells, the half maximal inhibitory concentration (IC50) value for DDP in NSCLC cells was analyzed through treating NSCLC cells with increasing concentrations of DDP. MTT reagent (Solarbio) was added to incubate with NSCLC cells for 4 hours, and formazan products were dissolved through adding 150 μL dimethyl sulfoxide (DMSO) (Sangon Biotech) to the wells. The optical density at 570 nm was determined.

To assess cell proliferation ability of NSCLC cells, NSCLC cells were transfected for 0, 24, 48, or 72 hours before incubate with MTT reagent (Solarbio) as described above.

Colony formation assay

NSCLC cells were detached by trypsin and re-inoculated into the wells of 6-well cell culture plates with ~200 cells/well. After culture for 14 days, colonies coming from surviving NSCLC cells were stained using 0.1% crystal violet (Sangon Biotech).

TABLE 1 Primers used in qRT-PCR assay

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
miR-200c-3p	GCCGAGTAATACTGCCGGGT	CTCAACTGGTGTCGTGGA
miR-485-5p	GCCGAGAGAGGCTGGCCGTG	CTCAACTGGTGTCGTGGA
RRM2	GCCACACCATGAATTGTCCG	ATGGTAAGTCACAGCCAGCC
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	CTGGGCTACACTGAGCACC	AAGTGG TCGTTGAGGGCAATG

Transwell migration and invasion assays

Transwell chambers with inserts (Costar) were used in transwell migration assay to assess cell migration ability. NSCLC cells were serum-starved for 24 hours to block the proliferation ability, and then these cells were treated using trypsin and re-suspended in medium without serum. 100 μL of cell suspension was pipetted into the upper chambers, and the lower chambers were filled with complete medium. Migrated cells on the lower surface of the inserts were dyed using 0.1% crystal violet (Sangon Biotech). The number of migrated cells in five random fields was counted at the magnification of 100×. Inserts pre-coated with Matrigel (BD Biosciences) were used in transwell invasion assay to evaluate cell invasion ability.

Flow cytometry

After transfection for 72 hours, NSCLC cells were collected through centrifuging. Annexin V-fluorescein isothiocyanate (annexin V-FITC) (Jiancheng Biotech) and propidium iodide (PI) (Jiancheng Biotech) were incubated with NSCLC cells for 15 minutes in a dark room. The apoptosis rate ($FITC^+/PI^-$ and $FITC^+/PI^+$) was immediately assessed by the FACS CantoII flow cytometer (BD Biosciences) within 1 hour of cell cultivation.

Bioinformatics analysis

Bioinformatics software (StarBase database) was used to predict the binding relationship between miR-200c-3p and RRM2, whereas the target interaction between miR-485-5p and RRM2 was predicted by TargetScan software.

Dual-luciferase reporter assay

The wild-type (WT) 3′ untranslated region (3′UTR) sequence of RRM2, containing the WT binding sites with miR-200c-3p or miR-485-5p, was inserted into luciferase reporter plasmid psiCHECK2 (Promega), termed as RRM2 3′UTR WT. The fragment of RRM2 3′UTR, including the site-directed mutation in the binding sites with miR-200c-3p or miR-485-5p, was inserted into psiCHECK2 (Promega) to generate RRM2 3′UTR mutant type (MUT). Cells were co-transfected with RRM2 3′UTR WT or RRM2 3′UTR MUT and miR-200c-3p (miR-485-5p) or miR-NC. Cells were subsequently subjected to the determination of luciferase activities using dual-luciferase reporter assay system (Yingrun). Renilla luciferase intensity was used as the control.

Western blot assay

Tissues and cells were disrupted using radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime) plus protease inhibitor cocktail (Roche). A total of 30 μg of protein samples were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto 0.22 mm polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked using 2% bovine serum albumin (BSA) to seal the non-specific binding sites and probed with primary antibodies, containing anti-RRM2 (ab57653) (Abcam) and anti-GAPDH (ab9485) (Abcam). The immunoblots were then probed with horseradish peroxidase (HRP)-labeled secondary antibody (Abcam) for 1 hour. Protein signals were determined using the enhanced chemiluminescent (ECL) system (Beyotime).

Tumorigenicity in vivo

Animal manipulates were conducted with the permission of Animal Research Ethics Committee of Jingmen No.1 People's Hospital. BALB/c nude mice (4–6 weeks old; $n = 5$) were purchased from Vital River Laboratory Animal Technology. Mice were subcutaneously injected with 2×10^6 A549/DDP cells on their right flank. After injection for 7 days, DDP (4 mg/kg) or phosphate-buffered saline (PBS) (Sangon Biotech) was intraperitoneally injected to the mice twice per week. Meanwhile, a total of 10 nmol AgomiR-200c-3p, AgomiR-485-5p, or AgomiR-NC in 100 μL saline buffer was injected into the tumor mass twice per week. Tumor volume was measured as volume $=$ length $\times$ width^2 $\times$ 0.5. The expression of miR-200c-3p, miR-485-5p, and RRM2 mRNA and protein was measured by qRT-PCR or Western blot assay.

Statistical analysis

All data were represented as mean ± standard deviation (SD). In vitro assays were repeated for three times. Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's

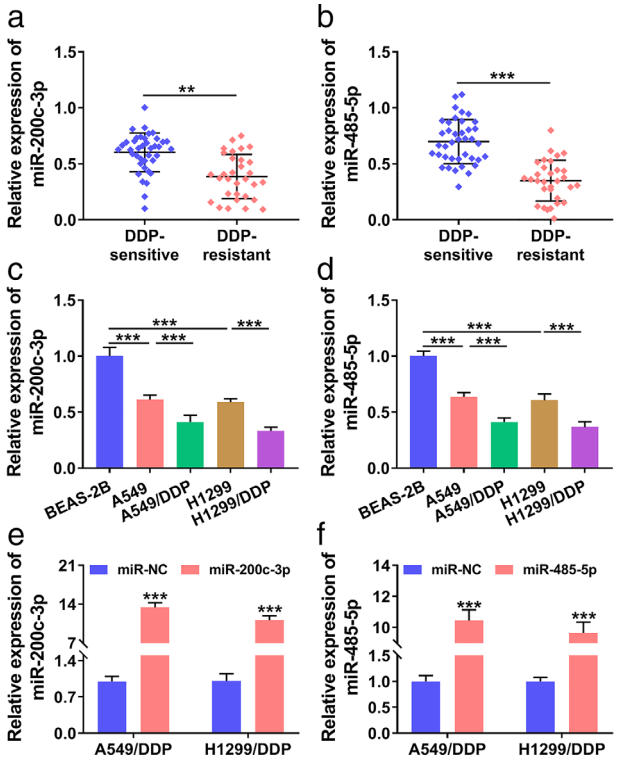

FIGURE 1 MiR-200c-3p and miR-485-5p are downregulated in DDP-resistant NSCLC tissues and cell lines. (a),(b) The expression of miR-200c-3p and miR-485-5p was determined in DDP-sensitive and DDP-resistant NSCLC tissues by qRT-PCR. (c),(d) The levels of miR-200c-3p and miR-485-5p were measured in BEAS-2B, A549, DDP-resistant A549 cell line (A549/DDP), H1299, and DDP-resistant H1299 cell line (H1299/DDP) by qRT-PCR. (e) A549/DDP and H1299/DDP cells were transfected with miR-NC or miR-220c-3p, and qRT-PCR was conducted to measure the level of miR-220c-3p in transfected NSCLC cells. (f) A549/DDP and H1299/DDP cells were transfected with miR-NC or miR-485-5p, and the expression of miR-485-5p was determined in transfected NSCLC cells by RT-qPCR. $^{**}p < 0.01$, $^{***}p < 0.001$

test was used to analyze the statistical significant as appropriate. $p < 0.05$ was identified to be statistically significant.

RESULTS

MiR-200c-3p and miR-485-5p are downregulated in DDP-resistant NSCLC tissues and cell lines

MiR-200c-3p and miR-485-5p were significantly downregulated in DDP-resistant NSCLC tissues compared with that in DDP-sensitive NSCLC tissues (Figure 1(a),(b)). The levels of miR-200c-3p and miR-485-5p were reduced in A549 and H1299 cells compared with BEAS-2B cells, and the expression of miR-200c-3p and miR-485-5p was further downregulated in DDP-resistant NSCLC cells (A549/DDP and H1299/DDP) than that in their parental cells (A549 and H1299) (Figure 1(c),(d)). The overexpression efficiencies of miR-200c-3p and miR-485-5p were high in A549/DDP and H1299/DDP cells (Figure 1(e),(f)). Taken together, miR-200c-3p and miR-485-5p were downregulated in DDP-resistant NSCLC tissues and cell lines, which implied that these two miRNAs might be implicated in the DDP resistance of NSCLC.

MiR-200c-3p plays a synergistic role with miR-485-5p in suppressing the chemo-resistance and malignant behaviors of DDP-resistance NSCLC cells

To explore the roles of miR-200c-3p and miR-485-5p in the DDP resistance and malignant properties of NSCLC cells, gain-of-function experiments were performed. The overexpression of miR-200c-3p or miR-485-5p alone significantly reduced the IC50 value of DDP in A549/DDP and H1299/DDP cells (Figure 2(a),(b)), suggesting that miR-200c-3p or miR-485-5p overexpression suppressed the DDP resistance of DDP-resistant NSCLC cells. MTT assay showed that cell proliferation was restrained by the overexpression of miR-200c-3p or miR-485-5p (Figure 2(c),(d)). The number of colonies was significantly decreased by the overexpression of miR-200c-3p or miR-485-5p (Figure 2(e)), which further demonstrated that miR-200c-3p and miR-485-5p suppressed the proliferation of NSCLC cells. Transwell assays revealed that the overexpression of miR-200c-3p or miR-485-5p notably reduced the numbers of migrated and invaded NSCLC cells (Figure 2(f),(g)), suggesting that miR-200c-3p and miR-485-5p inhibited the motility of NSCLC cells. The percentage of apoptotic NSCLC cells was increased by the overexpression of miR-200c-3p or miR-485-5p (Figure 2(h)), suggesting that miR-200c-3p and miR-485-5p overexpression induced the apoptosis of NSCLC cells. The overexpression of miR-200c-3p or miR-485-5p significantly reduced the phosphorylation levels of PI3K and AKT (Figure 2(i),(j)), suggesting that miR-200c-3p and miR-485-5p might function by inactivating PI3K/AKT signaling in NSCLC cells. Taken together, both miR-200c-3p and miR-485-5p restrained the DDP resistance and the malignant phenotypes of NSCLC cells in vitro.

Furthermore, we intended to explore if miR-200c-3p and miR-485-5p were synergistic in regulating the chemo-resistance and biological behaviors of NSCLC cells, and DDP-resistance NSCLC cells were co-transfected with miR-200c-3p and miR-485-5p. The results revealed that miR-200c-3p and miR-485-5p together played greater effects in DDP-resistant NSCLC cells than either of them alone (Figure 2(a)–(j)), suggesting that miR-200c-3p played a synergistic role with miR-485-5p in NSCLC cells.

MiR-200c-3p silencing elevates the DDP resistance and malignant behaviors of parental NSCLC cells

To further elucidate the biological role of miR-200c-3p, we silenced the expression of miR-200c-3p in parental NSCLC cells. qRT-PCR assay confirmed that the knockdown efficiency of anti-miR-200c-3p was significant in A549 and

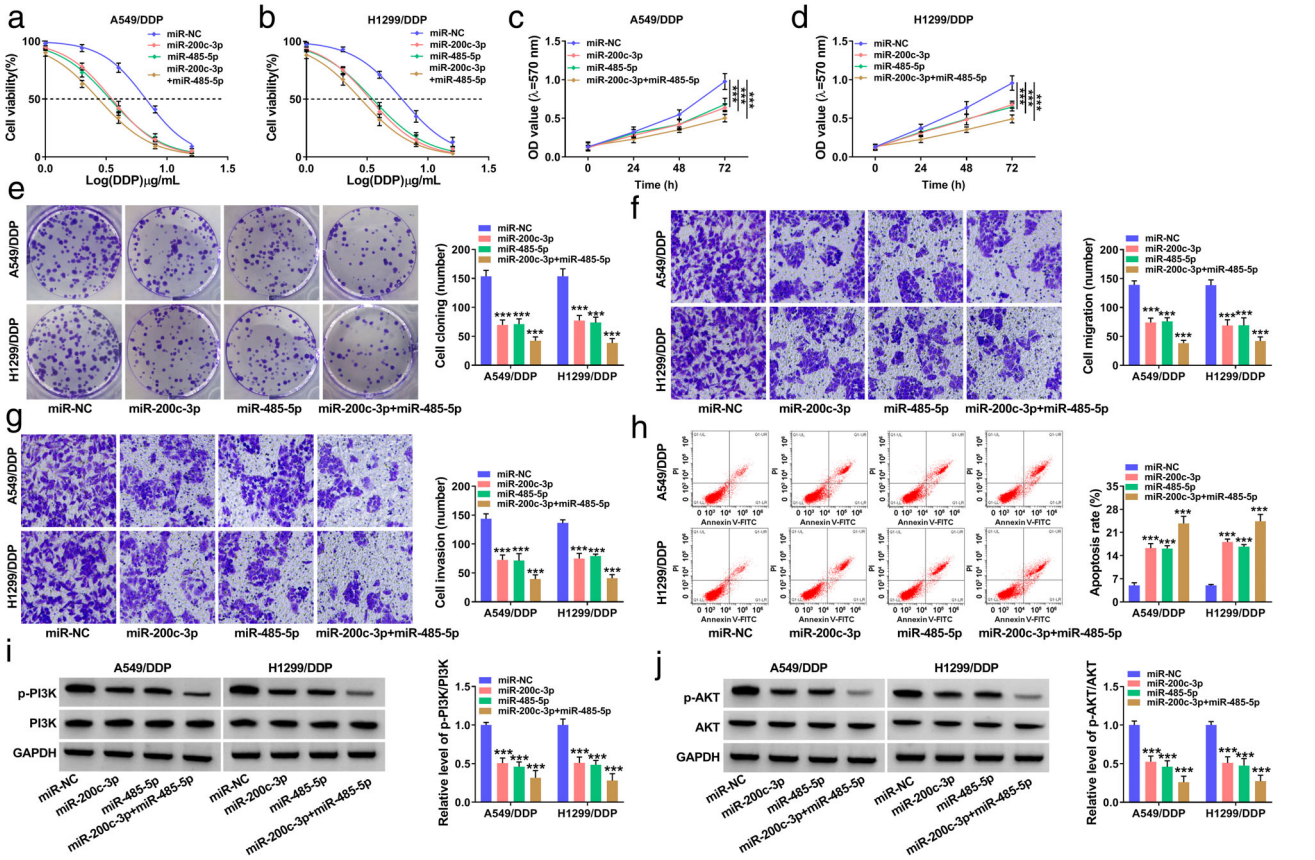

FIGURE 2 MiR-200c-3p plays a synergistic role with miR-485-5p in suppressing the chemo-resistance and malignant behaviors of DDP-resistance NSCLC cells. (a)–(j) A549/DDP and H1299/DDP cells were transfected with miR-NC, miR-200c-3p, miR-485-5p, or miR-200c-3p + miR-485-5p. (a),(b) The chemo-resistance of transfected cells was assessed by MTT assay. (c),(d) MTT assay was conducted to analyze cell proliferation ability. (e) The number of colonies was analyzed to assess cell proliferation ability. (f),(g) The numbers of migrated and invaded cells were analyzed to assess cell migration and invasion abilities. (h) Cell apoptosis rate was analyzed by flow cytometry. (i),(j) The levels of PI3K, p-PI3K, AKT, and p-AKT were measured by Western blot assay. ***$p < 0.001$

H1299 cells (Figure 3(a)). MiR-200c-3p silencing elevated the IC50 value of parental NSCLC cells to DDP (Figure 3(b),(c)), suggesting that miR-200c-3p knockdown promoted the chemo-resistance of DDP-sensitive parental NSCLC cells. Additionally, we found that miR-200c-3p silencing promoted the proliferation (Figure 3(d)–(f)), migration (Figure 3(g),) and invasion (Figure 3(h)) and restrained the apoptosis (Figure 3(i)) of parental NSCLC cells. These results together demonstrated that the level of miR-200c-3p might be crucial for the DDP resistance and malignant potential of NSCLC cells.

MiR-485-5p knockdown promotes the DDP resistance and malignant potential of parental NSCLC cells

We conducted loss-of-function experiments in parental A549 and H1299 cells to further assess the role of miR-485-5p in the chemo-resistance and progression of NSCLC. High silencing efficiency of anti-miR-485-5p was verified by qRT-PCR (Figure 4(a)). MiR-485-5p interference promoted the DDP resistance of parental NSCLC cells (Figure 4(b),(c)). Moreover, miR-485-5p silencing facilitated the proliferation (Figure 4(d)–(f)), migration (Figure 4(g)), and invasion (Figure 4(h)) and inhibited the apoptosis (Figure 4(i)) of parental NSCLC cells. Taken together, we found that the level of miR-485-5p might be pivotal for the DDP resistance and malignant potential of NSCLC cells.

RRM2 is a common target of miR-200c-3p and miR-485-5p in NSCLC cells

To investigate the working mechanism of miR-200c-3p and miR-485-5p on the development of NSCLC, the interacted partners of miR-200c-3p and miR-485-5p were predicted by bioinformatics software. The complementary sites between miR-200c-3p and RRM2 were predicted by StarBase database (Figure 5(a)). We constructed WT and MUT luciferase plasmids that contained the WT and MUT fragments of RRM2 to test the interaction between RRM2 and miR-200c-3p. Transfection with miR-200c-3p notably reduced the luciferase activity in RRM2 3′UTR WT group rather than RRM2 3′UTR MUT group compared with their matched control group (Figure 5(b),(c)), suggesting that miR-200c-3p interacted with RRM2 3′UTR in NSCLC cells. The potential binding sites between miR-485-5p and RRM2 were predicted by

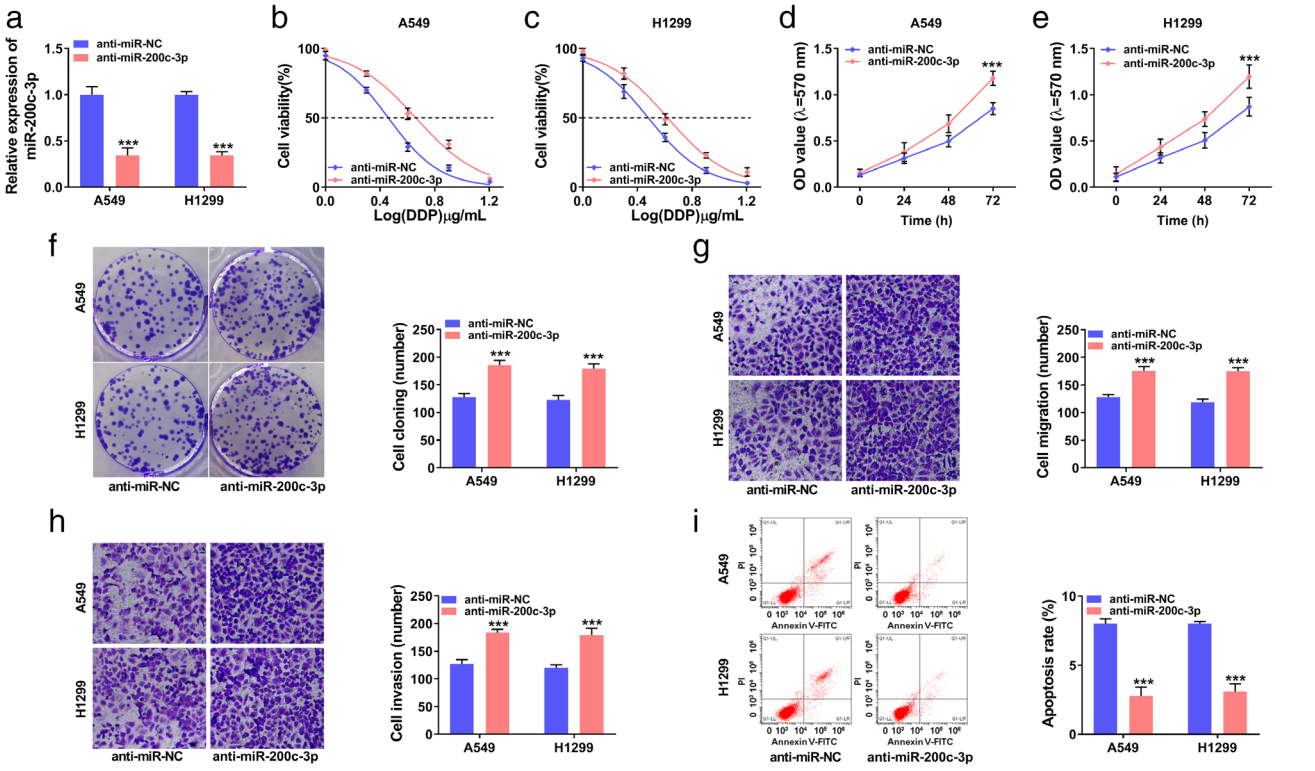

FIGURE 3 MiR-200c-3p silencing elevates the DDP resistance and malignant behaviors of parental NSCLC cells. (a)–(i) A549 and H1299 cells were transfected with anti-miR-NC or anti-miR-200c-3p. (a) The level of miR-200c-3p was determined by qRT-PCR. (b),(c) MTT assay was performed to analyze the chemo-resistance of NSCLC cells to DDP. (d),(e) MTT assay was conducted to assess the proliferation ability of NSCLC cells. (f) Colony formation assay was conducted to measure the proliferation capacity of NSCLC cells. (g),(h) The migration and invasion abilities of NSCLC cells were assessed by transwell assays. (i) Flow cytometry was implemented to analyze the apoptosis rate ($FITC^+/PI^-$ and $FITC^+/PI^+$) of NSCLC cells. ***$p < 0.001$

TargetScan software (Figure 5(d)). Luciferase activity was dramatically reduced with the co-transfection of miR-485-5p and RRM2 3′UTR WT compared with miR-NC and RRM2 3′UTR WT group (Figure 5(e),(f)), suggesting that there was interaction between miR-485-5p and RRM2 in NSCLC cells. The mRNA and protein expression of RRM2 was upregulated in DDP-resistant NSCLC tissues than that in DDP-sensitive NSCLC tissues (Figure 5(g),(h)). RRM2 mRNA and protein levels were elevated in A549 and H1299 cells than that in BEAS-2B cells, and the expression of RRM2 mRNA and protein was further elevated in DDP-resistant NSCLC cells (Figure 5(i),(j)). MiR-200c-3p overexpression reduced the mRNA and protein expression of RRM2 in A549/DDP and H1299/DDP cells (Figure 5(k),(l)). The mRNA and protein expression of RRM2 was downregulated with the accumulation of miR-485-5p in DDP-resistant NSCLC cells (Figure 5(m),(n)). Taken together, miR-200c-3p and miR-485-5p bound to RRM2 in NSCLC cells.

RRM2 interference suppresses DDP resistance and progression of NSCLC in vitro

We silenced RRM2 in NSCLC cells using si-RRM2 to test its role in NSCLC progression. The transfection of si-RRM2 significantly decreased the mRNA and protein expression of RRM2 in NSCLC cells (Figure 6(a)). RRM2 silencing restrained the DDP resistance of A549/DDP and H1299/DDP cells via MTT assay (Figure 6(b),(c)). The proliferation ability was notably inhibited with the silencing of RRM2 in NSCLC cells (Figure 6(d),(e). RRM2 interference also hampered the colony formation of NSCLC cells (Figure 6(f)). The migration and invasion abilities were both suppressed with the knockdown of RRM2 in NSCLC cells (Figure 6(g),(h)). Cell apoptosis was triggered with the silencing of RRM2 compared with si-NC group (Figure 6(i)). Overall, RRM2 knockdown inhibited the DDP resistance and malignant properties of DDP-resistant NSCLC cells.

RRM2 accumulation partly reverses miR-200c-3p-induced effects in DDP-resistant NSCLC cells

To test if miR-200c-3p exerted a tumor suppressive role through targeting RRM2, we conducted rescue experiments through transfecting miR-200c-3p alone or together with RRM2 plasmid. Transfection with RRM2 plasmid notably increased the mRNA and protein expression of RRM2 in NSCLC cells (Figure 7(a),(b)). RRM2 overexpression counteracted the suppressive effect of miR-200c-3p overexpression on the DDP resistance of A549/DDP and H1299/

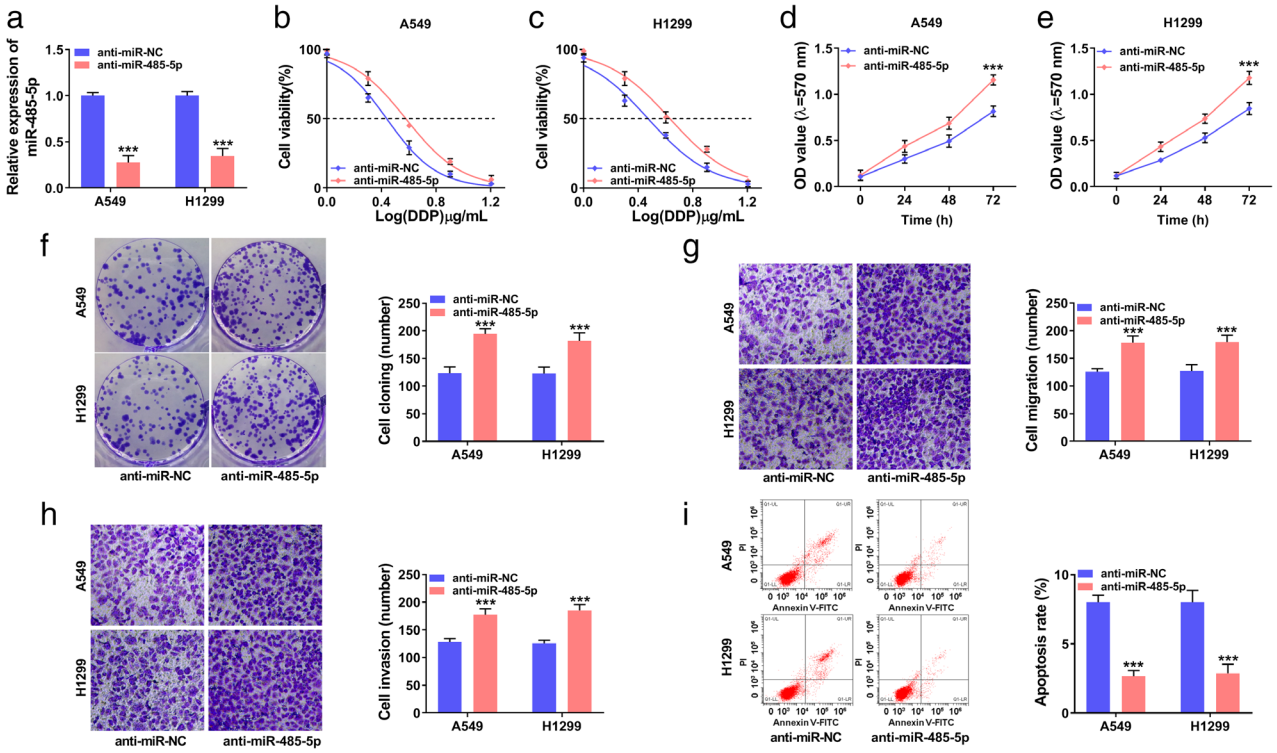

FIGURE 4 MiR-485-5p knockdown promotes the DDP resistance and malignant potential of parental NSCLC cells. (a)–(i) A549 and H1299 cells were transfected with anti-miR-NC or anti-miR-485-5p. (a) qRT-PCR was applied to detect the level of miR-485-5p in transfected NSCLC cells. (b),(c) The chemo-resistance of NSCLC cells to DDP was assessed by MTT assay. (d),(e) The proliferation ability of NSCLC cells was analyzed by MTT assay. (f) Colony formation assay was carried out to evaluate cell proliferation ability. (g),(h) Transwell assays were performed to analyze the migration and invasion abilities of NSCLC cells. (i) The apoptotic NSCLC cells ($FITC^+/PI^-$ and $FITC^+/PI^+$) were identified by flow cytometry, and the apoptosis rate was analyzed.
$^{***}p < 0.001$

DDP cells (Figure 7(c),(d)). MiR-200c-3p overexpression suppressed cell proliferation, and this inhibitory effect was attenuated by the overexpression of RRM2 in A549/DDP and H1299/DDP cells (Figure 7(e),(f)). RRM2 accumulation largely rescued the colony formation ability in miR-200c-3p-overexpressed A549/DDP and H1299/DDP cells (Figure 7(g),(h)). Cell migration and invasion abilities were restrained with the overexpression of miR-200c-3p, and these inhibitory influences were partly reversed by the addition of RRM2 plasmid in A549/DDP and H1299/DDP cells (Figure 7(i)–(l)). MiR-200c-3p overexpression-induced apoptosis in A549/DDP and H1299/DDP cells was attenuated by the accumulation of RRM2 (Figure 7(m),(n)). These findings suggested that miR-200c-3p suppressed NSCLC progression and DDP resistance partly through targeting RRM2.

MiR-485-5p-induced effects in DDP-resistant NSCLC cells are partly rescued with the addition of RRM2 plasmid

We also performed rescue experiments through transfecting miR-485-5p alone or together with RRM2 plasmid into A549/DDP and H1299/DDP cells. RRM2 overexpression partly recovered the DDP resistance in miR-485-5p-overexpressed A549/DDP and H1299/DDP cells (Figure 8(a),(b)). Through analyzing the results of MTT assay and colony formation assay, we found that miR-485-5p overexpression-induced suppressive effect on cell proliferation was partly alleviated by the accumulation of RRM2 in A549/DDP and H1299/DDP cells (Figure 8(c)–(f)). RRM2 overexpression also recovered the migration and invasion abilities in miR-485-5p-overexpressed A549/DDP and H1299/DDP cells (Figure 8(g)–(j)). MiR-485-5p promoted the apoptosis of A549/DDP and H1299/DDP cells, and cell apoptosis was inhibited in miR-485-5p and RRM2 co-transfected group (Figure 8(k),(l)). Taken together, miR-485-5p mediated suppressive effects on the DDP resistance and progression of NSCLC largely through targeting RRM2.

The overexpression of miR-200c-3p or miR-485-5p aggravates DDP-induced suppressive effect in tumor growth in vivo

To test the in vivo role of miR-200c-3p and miR-485-5p on the DDP resistance of NSCLC tumors, we established xenograft tumor model. DDP alone suppressed the growth of xenograft tumors, and tumor growth was further restrained with the overexpression of miR-200c-3p or miR-485-5p

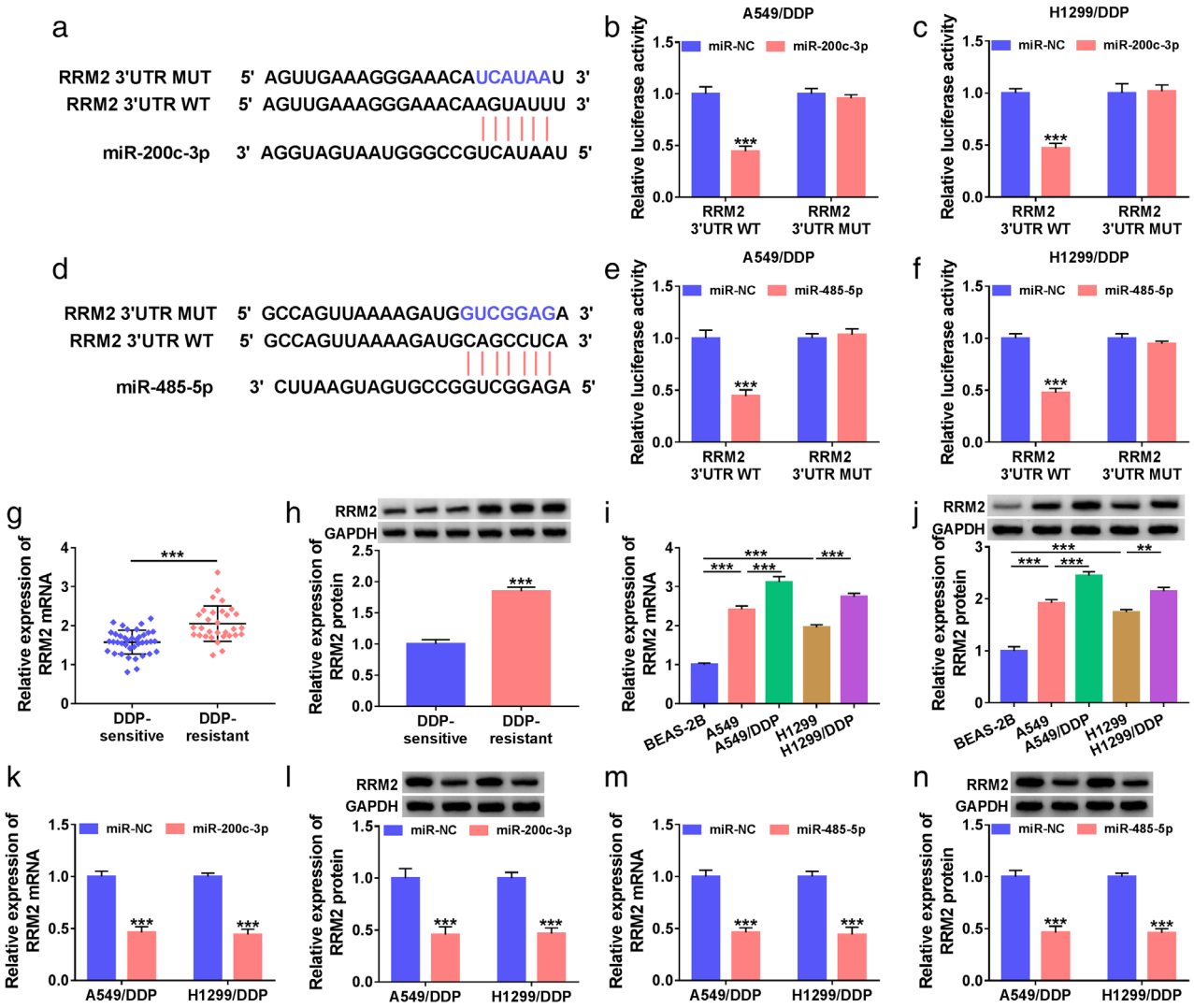

FIGURE 5 RRM2 is a common target of miR-200c-3p and miR-485-5p in NSCLC cells. (a) The binding sites between miR-200c-3p and RRM2 were predicted by StarBase database. (b),(c) Dual-luciferase reporter assay was performed to test the interaction between miR-200c-3p and RRM2. (d) The complementary sequence between miR-485-5p and RRM2 was predicted by TargetScan database. (e),(f) The target interaction between RRM2 and miR-485-5p was confirmed by dual-luciferase reporter assay. (g) The mRNA level of RRM2 in DDP-sensitive and DDP-resistant NSCLC tissues was determined via qRT-PCR. (h) Western blot assay was performed to analyze the protein expression of RRM2 in three pairs of DDP-sensitive and DDP-resistant NSCLC tissues. (i),(j) qRT-PCR and Western blot assay were used to measure the mRNA and protein abundance of RRM2 in BEAS-2B, A549, H1299, and two DDP-resistant NSCLC cell lines (A549/DDP and H1299/DDP). (k),(l) The mRNA and protein levels of RRM2 in A549/DDP and H1299/DDP transfected with miR-NC or miR-200c-3p were measured by qRT-PCR and Western blot assay. (m),(n) qRT-PCR and Western blot assay were performed to examine the mRNA and protein expression of RRM2 in A549/DDP and H1299/DDP cells with or without the overexpression of miR-485-5p. $^{**}p < 0.01$, $^{***}p < 0.001$

(Figure 9(a),(e)). Tumor weight was reduced with DDP treatment, and the overexpression of miR-200c-3p or miR-485-5p further reduced tumor weight (Figure 9(b),(f)). The expression of miR-200c-3p was elevated in AgomiR-200c-3p + DDP group compared with AgomiR-NC + DDP group (Figure 9(c)). The mRNA and protein expression of RRM2 exhibited an opposite tendency to miR-200c-3p (Figure 9(c),(d)). The injection with AgomiR-485-5p and DDP significantly enhanced the expression of miR-485-5p compared with AgomiR-NC + DDP group, whereas the expression of RRM2 mRNA and protein exhibited a reverse phenomenon to miR-485-5p (Figure 9(g),(h)). These results demonstrated that miR-200c-3p or miR-485-5p overexpression elevated the DDP sensitivity of xenograft tumors in vivo.

DISCUSSION

Accumulating articles found that aberrant expression of miRNAs was associated with tumorigenesis and the development of chemo-resistance. For instance, miR-210 silencing accelerated pancreatic cancer progression partly through enhancing the level of E2F3.[16] MiR-491-5p interference contributed to the motility of gastric cancer cells through targeting SNAIL and FGFR4.[17] Lv et al.[18] found

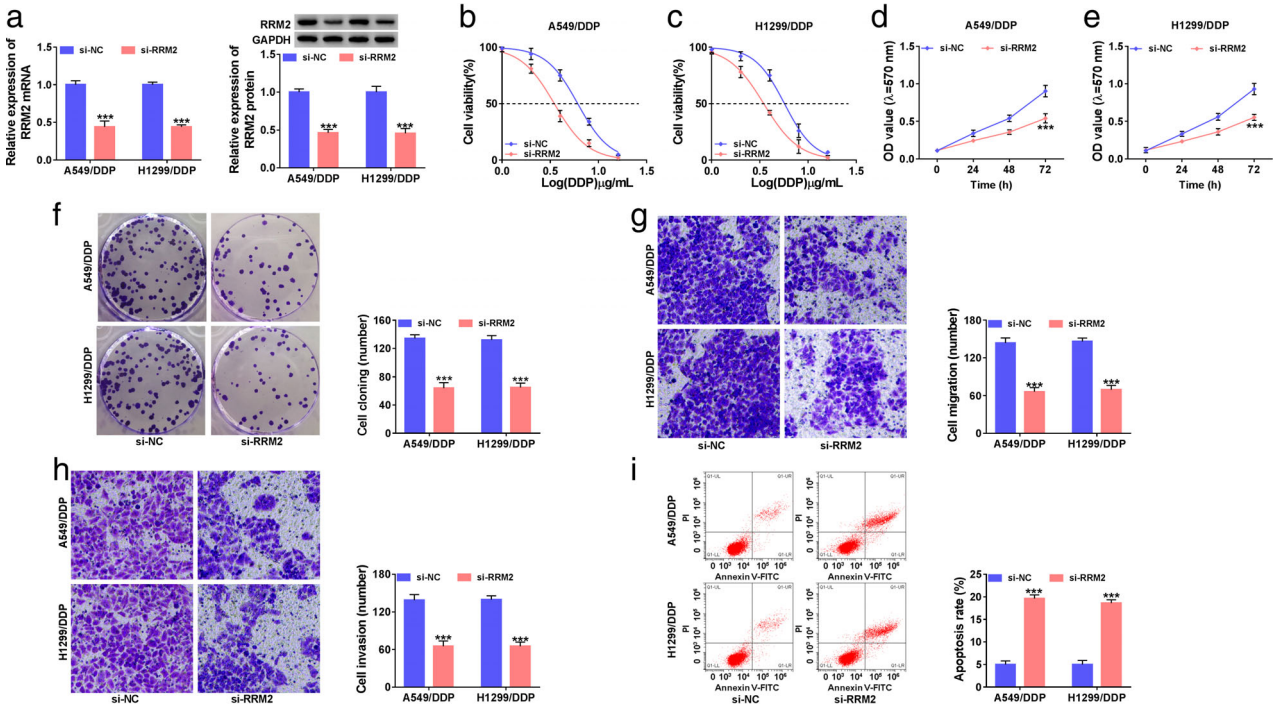

FIGURE 6 RRM2 interference suppresses DDP resistance and progression of NSCLC in vitro. (a)–(i) A549/DDP and H1299/DDP cells were transfected with si-NC or si-RRM2. (a) The mRNA and protein expression of RRM2 was determined by qRT-PCR and Western blot assay. (b),(c) MTT assay was performed to assess the DDP resistance of DDP-resistant NSCLC cells with the silencing of RRM2. (d),(e) MTT assay was conducted to analyze cell proliferation ability. (f) Colony formation assay was used to evaluate cell proliferation ability. (g),(h) The numbers of migrated and invaded cells were analyzed to assess the migration and invasion abilities of NSCLC cells with the silencing of RRM2 via transwell assays. (i) The apoptosis rate ($FITC^+/PI^-$ and $FITC^+/PI^+$) was assessed by conducting flow cytometry. $^{***}p < 0.001$

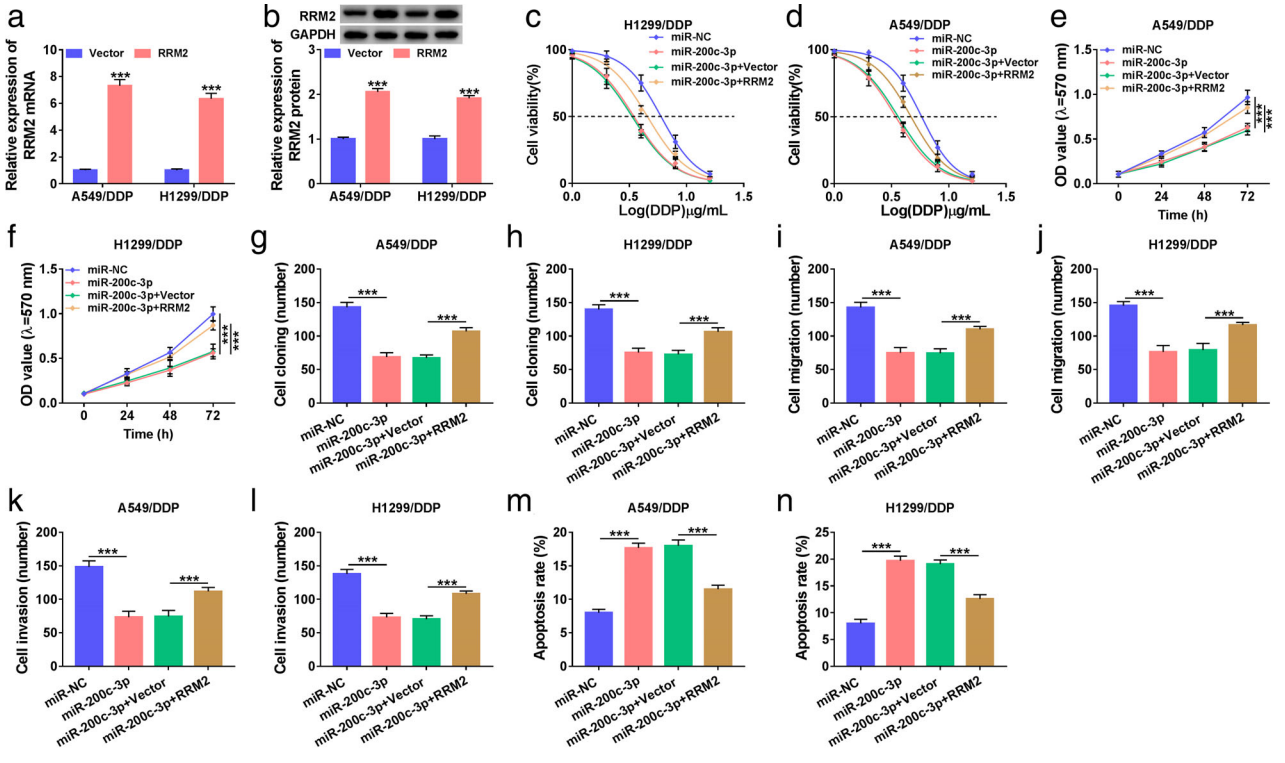

FIGURE 7 RRM2 accumulation partly reverses miR-200c-3p-induced effects in DDP-resistant NSCLC cells. (a),(b) qRT-PCR and Western blot assay were used to measure the mRNA and protein abundance of RRM2 in A549/DDP and H1299/DDP cells transfected with Vector or RRM2. (c)–(n) A549/DDP and H1299/DDP cells were transfected with miR-NC, miR-200c-3p, miR-200c-3p + Vector or miR-200c-3p + RRM2. (c),(d) MTT assay was used to analyze the IC50 value for DDP in transfected NSCLC cells. (e),(f) MTT assay was performed to assess cell proliferation ability. (g),(h) The number of colonies was analyzed to assess cell proliferation ability via colony formation assay. (i)–(l) Transwell assays were used to analyze the migration ability and invasion ability of NSCLC cells. (m),(n) Flow cytometry was performed to assess the apoptosis rate ($FITC^+/PI^-$ and $FITC^+/PI^+$) in NSCLC cells. $^{***}p < 0.001$

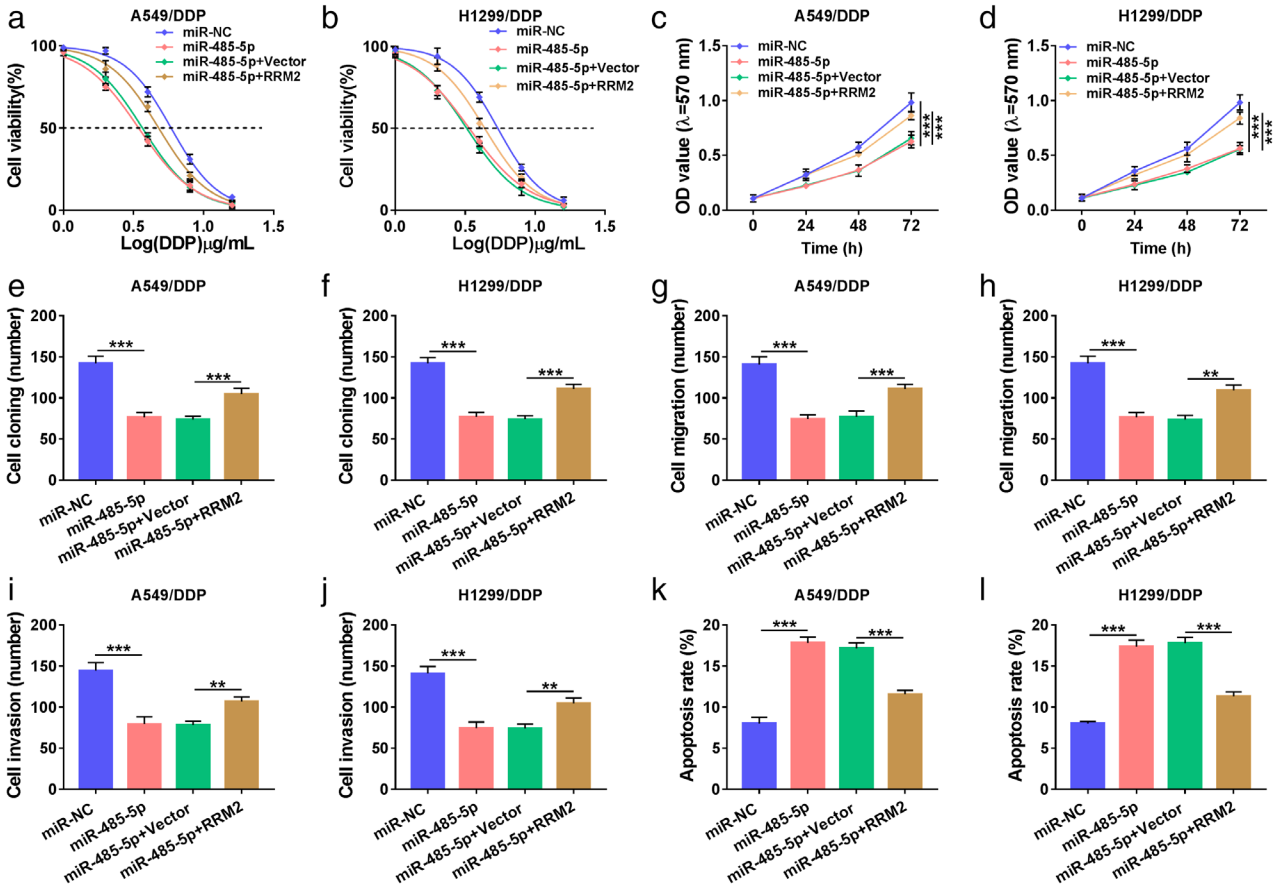

FIGURE 8 MiR-485-5p-induced effects in DDP-resistant NSCLC cells are partly rescued with the addition of RRM2 plasmid. (a)–(l) A549/DDP and H1299/DDP cells were transfected with miR-NC, miR-485-5p, miR-485-5p + Vector or miR-485-5p + RRM2. (a),(b) MTT assay was performed to analyze cell viability of transfected NSCLC cells treated with increasing concentrations of DDP to assess DDP resistance. (c),(d) MTT assay was conducted to measure cell viability after transfection for 0, 24, 48, or 72 hours to assess cell proliferation. (e),(f) Colony formation assay was used to analyze cell proliferation ability. (g),(h) Transwell migration assay was used to analyze cell migration ability. (i),(j) Cell invasion capacity was analyzed by transwell invasion assay. (k),(l) The apoptosis rate ($FITC^+/PI^-$ and $FITC^+/PI^+$) was assessed by flow cytometry. $^{**}p < 0.01$, $^{***}p < 0.001$

that miR-133b restrained the stemness and drug resistance to 5-fluorouracil and oxaliplatin in colorectal cancer cells through regulating DOT1L. Chen et al.[19] demonstrated that miR-1307 accelerated the proliferation and chemo-resistance of ovarian cancer cells to Taxol and inhibited the apoptosis through negatively regulating ING5. We observed that miR-200c-3p and miR-485-5p levels were abnormally decreased in DDP-resistant NSCLC specimens and cell lines. MiR-200c-3p exhibited a tumor suppressor role in a variety of malignancies. For instance, miR-200c-3p hampered nephroblastoma development through modulating FRS2.[20] MiR-200c-3p blocked the motility of clear cell renal cell carcinoma cells.[21] The functional association between miR-200c-3p expression and chemo-resistance has also been reported. Zhang et al.[22] found that XIST facilitated doxorubicin resistance of breast cancer cells via elevating ANLN expression via sponging miR-200c-3p, suggesting that miR-200c-3p suppressed the chemo-resistance. The anti-tumor role of miR-485-5p in many malignancies has also been reported by previous works. MiR-485-5p restrained osteosarcoma development through CX3CL1.[23] Gao et al.[24] found that miR-485-5p blocked hepatocellular carcinoma development via in-activating WBP2/Wnt signaling. Wang et al.[25] claimed that miR-485-5p inhibited breast cancer development and elevated the chemosensitivity through regulating survivin. The overexpression of miR-200c-3p or miR-485-5p suppressed the DDP resistance, proliferation, migration, invasion, and triggered cell apoptosis of DDP-resistant NSCLC cells. Moreover, miR-200c-3p plays a synergistic role with miR-485-5p in suppressing the chemo-resistance and malignant behaviors of DDP-resistance NSCLC cells. The silence of miR-200c-3p or miR-485-5p promoted the DDP resistance and malignant behaviors of parental NSCLC cells. These results demonstrated that the levels of miR-200c-3p and miR-485-5p might be crucial for the chemo-resistance and progression of NSCLC.

MiRNAs are implicated in the regulation of cancer progression through interacting with the 3′UTR of downstream mRNAs. For instance, miR-195 restrained the proliferation and motility of colon cancer cells through regulating WNT3A.[26] MiR-24 contributed to the motility of NSCLC cells through modulating ZNF367.[27] MiR-187 blocked the

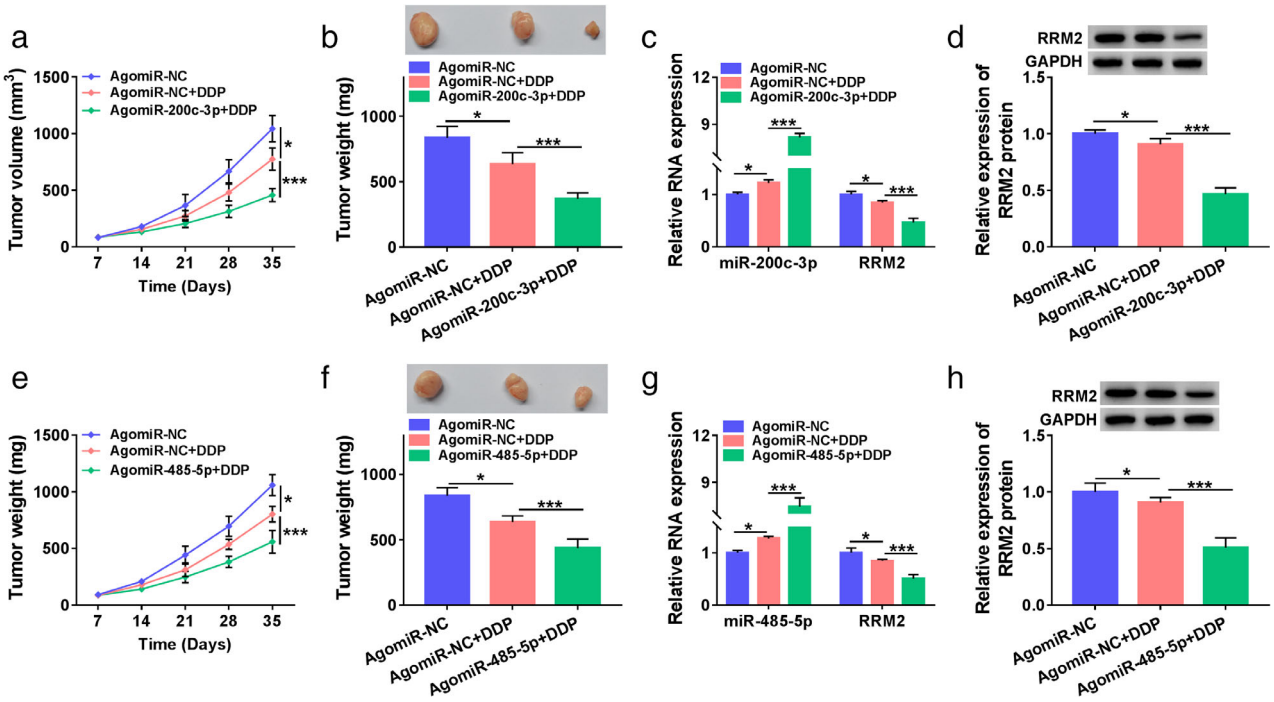

FIGURE 9 The overexpression of miR-200c-3p or miR-485-5p aggravates DDP-induced suppressive effect in tumor growth in vivo. (a),(e) Tumor volume was measured every week for 5 weeks. (b),(f) Tumor weight was analyzed after 5-week inoculation. (c),(g) The expression of miR-200c-3p and RRM2 mRNA was determined by qRT-PCR. (d),(h) Western blot assay was performed to measure the protein level of RRM2 in tumor tissues. $^{*}p < 0.05$, $^{***}p < 0.001$

proliferation of cervical cancer cells through negatively regulating FGF9.[28] Here, RRM2 was verified as a common target of miR-200c-3p and miR-485-5p. RRM2 mRNA and protein expression was enhanced in DDP-resistant NSCLC tissues and cell lines than that in DDP-sensitive NSCLC tissues and parental NSCLC cell lines. Furthermore, a reverse modulatory interaction between RRM2 and miR-200c-3p or miR-485-5p was observed in NSCLC cells.

RRM2 exerted an oncogenic role in many malignancies. RRM2 overexpression accelerated the proliferation, migration, and invasion and inhibited the apoptosis of glioblastoma cells.[29] RRM2 accumulation contributed to the invasion ability of gastric cancer cells through targeting AKT/NF-κB signaling.[30] RRM2 overexpression accelerated multiple myeloma progression through modulating Wnt/β-catenin signaling.[31] Huang et al.[15] demonstrated that AFAP1-AS1 contributed to the proliferation and chemo-resistance of NSCLC cells through targeting miR-139-5p/RRM2 axis. Consistent with above study,[15] we found that RRM2 interference caused significant suppression on the chemo-resistance, proliferation, migration, and invasion and notable promotion on cell apoptosis in DDP-resistant NSCLC cells. To further explore if miR-200c-3p or miR-485-5p-mediated effects on NSCLC cells were partly based on its negative regulatory relationship with RRM2, we conducted rescue experiments. The results revealed that RRM2 overexpression partly reversed miR-200c-3p or miR-485-5p-mediated effects in NSCLC cells, suggesting that miR-200c-3p or miR-485-5p functioned in NSCLC partly through targeting RRM2.

Animal experiments were conducted to explore if miR-200c-3p or miR-485-5p overexpression elevated the DDP sensitivity of xenograft tumors in vivo. DDP treatment alone suppressed the growth of xenograft tumors, and the growth of xenograft tumors was further inhibited with the overexpression of miR-200c-3p or miR-485-5p, demonstrating that miR-200c-3p or miR-485-5p overexpression contributed to DDP-mediated suppression on tumor growth in vivo.

There were several limitations of our study that should be noted. We found that miR-200c-3p played a synergistic role with miR-485-5p in DDP-resistant NSCLC cells, implying that there might be other signaling pathways downstream of miR-200c-3p and miR-485-5p. Further studies are needed to investigate the potential molecular mechanism. In addition, the signaling pathway by which miR-200c-3p/miR-485-5p-RRM2 axis functioned needs to be explored. Finally, the association between the expression of miR-200c-3p, miR-485-5p, or RRM2 and the clinicopathologic features of NSCLC patients needs to be analyzed to assess the diagnostic, therapeutic, and prognostic values of miR-200c-3p/miR-485-5p-RRM2 axis.

Taken together, our study found that miR-200c-3p and miR-485-5p suppressed NSCLC development and elevated the DDP sensitivity of NSCLC cells partly through targeting RRM2. MiR-200c-3p/miR-485-5p-RRM2 axis might be a novel target for NSCLC treatment.

CONFLICT OF INTEREST

The authors declare that no financial conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available at https://datadryad.org/stash/share/pCXCC8CZWTU1QGDTENoYVZXHNOpu1bFnAV_zffPU0SI

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