Research paper

The miR-495-UBE2C-ABCG2/ERCC1 axis reverses cisplatin resistance by downregulating drug resistance genes in cisplatin-resistant non-small cell lung cancer cells

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1. Introduction

Lung cancer is associated with high morbidity and mortality rates and its prevalence has increased in China and worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases; approximately 75% of these patients are diagnosed at a relatively late stage of the disease when secondary multiple-organ metastasis occurs; indeed, the 5-year survival rate is very low, thereby deterring the treatment of NSCLC [2, 3].

miRNA deregulation and dysfunction play a significant role in cancer pathogenesis in humans. miRNA is a class of short RNA molecules ~22 nucleotides in length. Aberrant miRNA expression may be associated with tumorigenesis, since miRNAs play important roles in various cellular processes [4–8]. Recent studies have reported advancements in unraveling the molecular mechanism underlying the pathogenesis of lung cancer. The activation of numerous miRNAs underlies lung cancer cell proliferation, apoptosis, migration, invasion, and epithelial–mesenchymal transition (EMT), and miRNAs are significantly deregulated in human lung cancer tissues [9–11]. Certain miRNAs serve as novel biomarkers and therapeutic targets for lung cancer [12–14]. miR-495 is associated with human tumorigenesis and cancer pathogenesis. Downregulation of miR-495 may promote cell proliferation and inhibit apoptosis, thereby accelerating the progression of lung cancer [15–18]. However, the mechanisms underlying the miR-495-mediated inhibition of lung cancer progression and drug resistance remain unknown and warrant further investigation.

Ubiquitin-conjugating enzyme E2 C (UBE2C; also known as UbcH10) is a member of the E2 family, which interacts with three or four different proteins, including ubiquitin, E1, E3, and the target protein [19–21]. The anaphase-promoting complex/cyclosome is the only E3 enzyme known to transfer ubiquitin molecules to target proteins via UBE2C [22]. UBE2C overexpression can promote cell proliferation...
and short interfering (si)RNA-mediated UBE2C knockdown in various cell lines decreases cell proliferation [23–26]. UBE2C expression is associated with the degree of malignancy in breast, lung, ovarian, and bladder cancers and lymphoma [21, 27]. UBE2C downregulation inhibited proliferation and clone formation and promote senescence in tumor cells. These effects are associated with cell cycle regulation [23]. However, UBE2C is associated with resistance to epirubicin treatment in mammary cancer cells [20]; furthermore, UBE2C is associated with chemotherapeutic resistance in NSCLC cells, although the underlying mechanism is unclear.

Enhanced cell proliferation and drug resistance are important features of tumor growth and metastasis resulting from drug resistance [28–30]. Radiotherapy in combination with chemotherapy is the prototypical treatment strategy for NSCLC; cisplatin (DDP), a commonly used metal-based chemotherapeutic agent, targets the cell cycle [31–33]. However, NSCLC patients frequently develop resistance to DDP, which is associated with a higher mortality rate. It is unclear whether UBE2C contributes to DDP resistance in NSCLC.

EMT is a crucial event in progression towards cancer metastasis. It triggers cellular mobility and induces tumor cell invasion. During EMT, epithelial cells stop expressing E-cadherin expression and lose cell-cell adhesion, alter their apical–basal polarity and transdifferentiate into mesenchymal cells. The most prominent characteristics of EMT are downregulation of E-cadherin and epithelial markers and upregulation of mesenchymal markers N-cadherin and vimentin. EMT in associated with drug resistance in tumor cells [28–30]. During EMT, the expression profiles of certain specific molecules is altered, followed by upregulation of drug resistance genes to induce chemotherapeutic resistance in cancer cells.

ABCG2 is one of at least three human ATP-binding cassette (ABC) transporters, which facilitate the efflux of various toxic xenobiotic substances and harmful agents in drug-resistant patients. This capacity for multidrug transport is not only a confounding factor in chemotherapy but is also one of the more perplexing phenomena in transporter biochemistry [34, 35]. However, the regulatory mechanism of ABCG2, especially its transcriptional regulation, is still unclear in DDP-resistant NSCLC cells. Platinum-based drugs damage DNA by introducing intra-strand and inter-strand crosslinks, resulting in cell death. Excision repair cross-complementing 1 (ERCC1) is a component of the nucleotide excision repair (NER) pathway which reverses such defects. Moreover, overexpression of ERCC1 mRNA is associated with DDP resistance in lung cancer in humans [36, 37], and its transcriptional regulation is also still unclear in DDP-resistant NSCLC cells. Therefore, lung cancer patients overexpressing ABCG2 and ERCC1 are potentially tolerant to DDP and result in failure of using DDP, thereby increasing the mortality rate of lung cancer. Development of ABCG2 and ERCC1 inhibitors for clinical use may allow for increased penetration of therapeutic agents, thereby prolonging survival and improving the quality of life.

To address this issue, this study aimed to investigate molecular mechanism of the miR-495-UBE2C-ABCG2/ERCC1 axis and the function of miR-495 and UBE2C in the progression of cisplatin resistant in NSCLC.

2. Materials and methods

2.1. Molecular biology

The pcDNA-Flag UBE2C, pcDNA-ABCG2 and pcDNA-ERCC1 constructs were made using the pcDNA 3.1 vector (Invitrogen, Carlsbad, CA, USA). Sequences encoding the Flag epitope (DYKDDDDK) were added by PCR through replacement of the first Met-encoding codon in the respective pcDNA clones. The PCR primers were:

UBE2C forward primer: 5′-GGTTACCCCGATTACAGCAGCATGA CAAGATGGCTTCCCACCCCGGCAACCT-3′
UBE2C reverse primer: 5′-GCTCTAGAGCTCGAAGCTGGTGTG AC-3′
ABCG2 forward primer: 5′-GGGTACCCCATGCGTCTTGGAGTCTGAC-3′
ABCG2 reverse primer: 5′-CCCTCGAGGTTCAAAATATTTCCTCGCC AG-3′
ERCC1 forward primer: 5′-GGGGTTACCCCGATTACAGCAGCATGA CAAGATGGCTTCCCACCCCGGCAACCT-3′
ERCC1 reverse primer: 5′-CCCTCGAGGTTCAAAATATTTCCTCGCC AG-3′

2.2. Cell lines and culture

Human NSCLC cell lines, A549, H1299, Calu6, H520 and the human lung normal control cell line, HBEC 3KT (HBEC) were purchased from American Type Culture Collections (Manassas, VA). Cell lines were cultivated in RPMI-1640 medium supplemented with 10% FBS (HyClone, USA), penicillin /streptomycin (100 mg/ml). Culture flasks were kept at 37 °C in a humid incubator with 5% CO2. The cisplatin resistant subline A549/DDP was gifted from the Resistant Cancer Cell Line (RCCl) collection (http://www.kent.ac.uk/stms/cmp/RCCl/RCClCulture.html). Other cisplatin resistant sub-lines H1299/DDP or Calu6/DDP had been established by adapting the growth of H1299 or Calu6 cells in the presence of increasing concentrations of cisplatin until a final concentration of 16 μg/ml on H1299 cells and Calu6 cells, then cultivated in RPMI-1640 medium supplemented with 10% FBS additionally contained 2 μg/ml cisplatin.

2.3. Over-expression and knockdown of genes

Overexpressing plasmid (2 μg) or siRNA (1.5 μg) of indicated genes were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for over-expression and knockdown of indicated genes, followed by analysis 48–72 h later. The selected sequences for knockdown of UBE2C, ABCG2 and ERCC1 as follows:

si UBE2C-1 were: 5′-CCUGCAAGAACCUACUCUA-3′
si ABCG2-1 were: 5′-CUUCUAGGAGAACCCAACA-3′
si ERCC1-1 were: 5′-AAGGAUCUCCGAAAAUGCUU-3′
si ABCG2-2 were: 5′-GGGAUACCGGCAAGCAGCCCC-3′
si ERCC1-2 were: 5′-AGGGUCGCUAAACCAGGCUUCC-3′
si ERCC1-2 were: 5′-GGUCACCGCCUCGGCACACCA-3′

2.4. Western blot analysis

Human lung cancer cells were transfected with the relevant plasmids and cultured for 36 h. For western blot analysis, cells were lysed in NP-40 buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 1 mM PMSF, and 0.5% NP-40) at 25 °C for 40 min. The lysates were added to 5× loading dye and then separated by electrophoresis. The primary antibodies used in this study were 1:1000 rabbit anti-Flag (sc-166,384, Santa Cruz, Dallas, TX, USA), 1:1000 Abcam (Cambridge, UK) antibody of UBE2C (ab12290),
2.5. Immunofluorescent staining

To examine the protein expression by immunofluorescent staining, lung cancer cells were seeded onto coverslips in a 24-well plate and left overnight. Cells were then fixed using 4% formaldehyde for 30 min at 25 °C and treated with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 min. The coverslips were incubated with rabbit anti-UBE2C, Ki67, Annexin V, ABCG2, ERCC1, Vimentin and mouse anti-E-cadherin monoclonal antibody (Abcam) at 1:200 dilution in 3% BSA. The coverslips were then incubated with an Alexa-Fluor 467 mouse anti-E-cadherin monoclonal antibody, and the cell cycle was cultured for 36 h, harvested and counted in 2 weeks. The resulting supernatant was discarded, and the pellets were nucleus. The resulting supernatant from the first round of differential centrifugation was sedimented for 30 min at 14000 rpm. The resulting pellets were membranes and the supernatant were cytoplasm.

2.6. Cell flow cytometry assays

A549 and A549/DDP cells were transfected with the relevant plasmids culturing for 36 h, harvested and fixed with 70% ethanol. These cells were then stained using propidium iodide (PI) and the cell cycle stage assessed by flow cytometry. Data were collected and analyzed on a BD FACSC Flow Cytometer using FACS software (BD Biosciences, San Jose, CA, USA).

2.7. RNA isolation and real time reverse transcription (RT)-PCR assay

We used Trizol reagent (TransGen Biotech, Beijing, China) to isolate total RNA from the samples and cells. RNA was reverse transcribed into first-strand cDNA using a TransScript All-in-One First-Strand cDNA Synthesis Kit (TransGen Biotech). cDNAs were used in the RT-PCR assay with the human GAPDH gene as an internal control. Amplification of RT-PCR was performed as follows: a denaturation step at 94 °C for 5 min, followed by 30 cycles of amplification at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The reaction was stopped at 72 °C for 10 min and the product of PCR was kept at 4 °C. RT-PCR analysis of miR-495 was conducted with the Dream taq Green master mix (Fermentas, K1082) following the manufacturer’s protocols, then used the 4% agarose gel at 120 V for 70 min. We got the images of RT-PCR by Image Lab™ Software (ChemidocTM XRS+, Bio-RAD), and these images were TIF with reversal color format. The RT-PCR primers were:

**UBE2C forward primer:** 5′-GGATTTCTGCTTCCCTCTGAA-3′
**UBE2C reverse primer:** 5′-GATAGCCAGGGCGTGAGAAG-3′
**E-cadherin forward primer:** 5′-ACCATTAAACGAAGACAGG-3′
**E-cadherin reverse primer:** 5′-GATGCATTCTTCACTTCTCTGGT-3′
**Vimentin forward primer:** 5′-CGCAACTACATCGAAGACCTGC-3′
**Vimentin reverse primer:** 5′-GTGTCCTCCTCGCCGCGGGC-3′
**GAPDH forward primer:** 5′-CTCTCTGTCTTGCAGCTCAC-3′
**GAPDH reverse primer:** 5′-CCCCATAGCAAACTTCCGT-3′
**ABCG2 forward primer:** 5′-GGGTCCTCCTCTGAGCACC-3′
**ABCG2 reverse primer:** 5′-TGTTTTGAGATTGACCAACAGC-3′
**ERCC1 forward primer:** 5′-CTCAAGGAGCTGCTAAGATG-3′
**ERCC1 reverse primer:** 5′-CATAGCCCTTGTAGGTCG-3′

2.8. Subcellular fraction

Transfected A549 and A549/DDP cells were harvested in PBS and re-suspended for 10 min on ice in 500 µl CLB Buffer (10 mM Hepes, 10 mM NaCl, 1 mM KH₂PO₄, 5 mM NaHCO₃, 5 mM EDTA, 1 mM CaCl₂, 0.5 mM MgCl₂). Thereafter, 50 µl of 2.5 M sucrose was added to restore isotonic conditions. The first round of centrifugation was performed at 6300 x g for 5 min at 4 °C. The pellet washed with TSE buffer (10 mM Tris, 300 mM sucrose, 1 mM EDTA, 0.1% NP40, PH 7.5) at 4000 g for 5 min at 4 °C until the supernatant was clear. The resulting supernatant was discarded, and the pellets were nucleus. The resulting supernatant from the first round of differential centrifugation was sedimented for 30 min at 14000 rpm. The resulting pellets were membranes and the supernatant were cytoplasm.

2.9. SA-β-gal staining

SA-β-gal was detected using the Senescence (β-Galactosidase Staining kit (C0602; Beyotime) following the manufacturer’s instructions: In brief, the cells were washed twice with PBS and then fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 10 min. The cells were then incubated at 37 °C for 15 h with staining solution. After being washed twice with PBS, the SA-β-gal-positive cells were observed under an optical microscope (IX53; Olympus) and assessed using the Image J software.

2.10. Soft agar colony forming assay

For soft agar colony formation assay, A549 cells with indicated treatment were added to growth medium with 0.2% agar and layered onto 1% agar beds in six-well plates. Cells were fed with 1 ml of medium every three days. The colonies were stained with 0.01% crystal violet and counted in 2–3 weeks. The result were observed under an optical microscope (IX53; Olympus) and assessed using the Image J software.

2.11. MTT and CCK8 assays

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and CCK8 assays in 96-well plates in a manner. Cells were transfected with the relevant plasmids culturing for 36 h, followed by incubation with MTT and CCK8 for 4 h. Next 100 µl dimethyl sulfoxide was added to dissolve the formazan crystals for the MTT assay. Absorbance was read at 570 nm using a spectrophotometer (Tecan, Männedorf, Switzerland). Cell viability was calculated as relative absorbance compared to a DMSO-only control.

2.12. Luciferase reporter assay

To construct the core region of UBE2C, ABCG2 or ERCC1 promoter, the regions of UBE2C, ABCG2 or ERCC1 were amplified by PCR from the human cDNA of A549 cells and were inserted into the upstream of the pGL3-Basic vector (Promega, Madison, WI, USA) via KpnI and Xhol sites to generate UBE2C luc, ABCG2 luc and ERCC1 luc. For luciferase reporter Gene Assay Kit (Beyotime, RG005) to detect the promoter activities. The PCR primers were:

**UBE2C forward primer:** 5′-GATATGACCTTGTTGCTG-3′
**UBE2C reverse primer:** 5′-GGCTCGGCTCAGCTCCTTTACGG-3′
**ABCG2 forward primer:** 5′-TCAGGCTACAGCCACATCCCTTCAGCAAGG-3′
**ABCG2 reverse primer:** 5′-TGTTTTGAGATTGACCAACAGC-3′
**ERCC1 forward primer:** 5′-CTCAAGGAGCTGCTAAGATG-3′
**ERCC1 reverse primer:** 5′-CATAGCCCTTGTAGGTCG-3′

2.13. CHIP assay

ChIP experiments were performed according to the laboratory manual. Immunoprecipitation was performed for 6 h or overnight at 4 °C with specific antibodies. After immunoprecipitation, 45 µl protein A-Sepharose and 2 μg of salmon sperm DNA were added and the incubation was continued for another 1 h. Precipitates were washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1). Precipitates were then washed three times with TE buffer.
Xenografted mice were then intraperitoneally administered for three of the xenografted tumors had reached approximately 5 mm. The membrane was then fixed in 4% paraformaldehyde overnight and then embedded in paraffin wax. Four-micrometer thick sections were stained using hematoxylin and eosin (H&E) for histological analysis according to the laboratory manual.

### 2.15. Transwell migration assays

Transwell migration assays were performed using a 24-well chamber (Costar 3422; Corning Inc., Corning, NY, USA). The lower and upper chambers were partitioned by a polycarbonate membrane (8-μm pore size). Lung cancer cells (5 × 10⁴) were seeded into DMEM without FBS in the upper chamber. DMEM containing 10% FBS was added to the lower chamber. The cells were allowed to migrate for 36 h at 37 °C in a humidified atmosphere containing 5% CO₂. Cells remaining on the upper side of the membrane were removed using PBS-soaked cotton swabs. The membrane was then fixed in 4% paraformaldehyde for 20 min at 37 °C and then stained with crystal violet. Cells on the lower side of the membrane were counted under an Olympus light microscope (Olympus, Tokyo, Japan) and assessed using the Image J software.

### 2.16. Analysis of publicly available datasets

To analyze correlation between miR-495 and UBE2C expression level and prognostic outcome of patients, Kaplan-Meier survival curves of NSCLC patients with low and high expression of miR-495 and UBE2C were generated using Kaplan-Meier Plotter (www.kmplot.com/analysis) [38].

### 2.17. Human lung cancer specimen collection

A total of 50 human lung cancer (NSCLC) with their corresponding normal lung specimens and a total of 40 NSCLC subjects received anthracyclines-based neoadjuvant chemotherapy were collected in Affiliated Hospital of Binzhou Medical College with written consents of patients and the approval from the Institute Research Ethics Committee.

### 2.18. In vivo experiments

To assess the in vivo effects of UBE2C, 3 to 5-week old female BALB/c athymic (NU/NU) nude mice were housed in a level 2 biosafety laboratory and raised according to the institutional animal guidelines of Binzhou Medical University. All animal experiments were carried out with the prior approval of the Binzhou Medical University Committee on Animal Care. For the experiments, mice were injected with 5 × 10⁶ lung cancer cells with stable expression of relevant plasmids and randomly divided into two groups (five mice per group) after the diameter of the xenografted tumors had reached approximately 5 mm. Xenografted mice were then intraperitoneally administered for three times a week and tumor volume and body weight were measured every second day. Tumor volume was estimated as 0.5 × a² × b (where a and b represent a tumor's short and long diameter, respectively). Mice were euthanized after six weeks and the tumors were measured at a final time. Tumor and organ tissue were then collected from xenograft mice and analyzed by immunohistochemistry.

### 2.19. Immunohistochemical analysis

Tumor tissues were fixed in 4% paraformaldehyde overnight and then embedded in paraffin wax. Four-micrometer thick sections were stained using hematoxylin and eosin (H&E) for histological analysis according to the laboratory manual.

### 2.20. Ethics approval and consent to participate

The experimental protocol was approved by the Research Ethics Committee of Binzhou Medical University, China (No. 2017–016-01 for human lung cancer specimen and No. 2017–009-09 for mouse experiments in vivo) and the written informed consent was obtained from all subjects. Informed consent was obtained from all individual participants included in the study. All patients were staged based on the criteria of the 7th Edition of the AJCC Cancer Staging Manual: Stomach (2010).

### 2.21. Statistical analysis

Each experiment was repeated at least three times. The statistical analyses of the experiment data were performed by using a two-tailed Student’s paired t-test and one-way ANOVA. Statistical significance was assessed at least three independent experiments and the P-value <.05 was considered statistically significant and highlighted an asterisk in the figures, while P-values <.01 were highlighted using two asterisks and P-values <.001 highlighted using three asterisks in the figures.

### 3. Results

#### 3.1. miR-495 was downregulated in lung cancer and inhibited cancer cell proliferation, migration, invasion and EMT in lung cancer cells

Recent studies have reported that miRNA contributes to tumorigenesis and cancer development in humans. To determine whether miR-495 was associated with the occurrence of lung cancers and drug resistance, we performed reverse transcription polymerase chain reaction (RT-PCR) assay and found that miR-495 levels were lower in human lung cancer tissues than in their normal adjacent lung tissues, indicating that miR-495 significantly contributes to lung cancer as a tumor suppressor (Fig. 1a, b). Fifty samples were obtained from patients who underwent a lung resection surgery at the Affiliated Hospital of Binzhou Medical College (Binzhou, China) between January 2014 and January 2016. Each sample was examined, and the clinicopathological findings are summarized in Table 1. Moreover, RT-PCR assay revealed that miR-495 expression was lower in lung cancer cells than in normal HBEC controls (Fig. 1c). Publicly available datasets (http://www.kmplot.com/analysis/index.php?p=service&cancer) [38] were filtered and used to analyze the prognostic correlation between lung cancer patient survival and miR-495 expression. Kaplan-Meier analyses revealed that miR-495 expression level was positively correlated with survival. High expression levels are associated with longer overall survival (OS) (n = 1926, P = 2.2 × 10⁻⁶) (Fig. 1d). Further, we analyzed miR-495 expression in the DDP non-resistant (NR) and resistant (R) NSCLC tissues via treatment with anthracycline-based neoadjuvant chemotherapy (UBE2C, ABCG2, and ERCC1 expression is shown in these panels) (Fig. 1e, f). We found that miR-495 expression levels were significantly higher in the non-resistant tissues than in their DDP-resistant lung cancer tissues (Fig. 1e). These data suggested that miR-495 was associated
with NSCLC DDP resistance. Furthermore, the miR-495 mimics and miR-495 inhibitors were used to determine whether miR-495 tumor suppressor activation underlies lung cancer cell initiation, progression, and metastasis (Fig. 1g). In A549 cells, the miR-495-mimics decreased cell growth (Fig. 1h), Ki67 protein levels (Fig. 1i), clonal formation (Supplementary Fig. S1a), cell migration (Supplementary Fig. S1b) and cell invasion (Fig. 1j) but increased apoptosis (Supplementary Fig. S1c), Annexin V protein levels (Fig. 1k) and cleaved caspase-3 protein levels (Fig. 1l) in A549 cells. The opposite effects were observed for all these factors in A549 cells with ectopically expressing miR-495 inhibitors (Fig. 1h-o and Supplementary Fig. S1 a-c). Moreover, cell cycle profiling in lung cancer cells treated with miR-495 revealed that treatment of miR-495 mimics induced significant G2 arrest in A549 cells; however, no alterations were observed in the cell cycle upon treatment with miR-495 inhibitors, compared to the control group; meanwhile, the number of cells in the S phase increased (Fig. 1m). Furthermore, we confirmed whether E-cadherin and Vimentin were analyzed by RT-PCR (n) and western blot assay (o). Results were presented as mean ± SD, and the error bars represent the SD of three independent experiments. *p < .05; **p < .01 vs control group.
A549 cells upon assessing the effects of miR-495 inhibitors in A549 cells via RT-PCR and western blotting (Fig. 1 n, o). These data indicate that miR-495 was downregulated in lung cancer and promoted cancer cell proliferation, migration, and invasion in lung cancer cells.

### Table 1
Patient's demographics and tumor characteristics and association of UBE2C and miR-495 levels with clinicopathological features in lung cancer population.

| Characteristics                  | No. of patients, N = 50 (%) | P value |
|----------------------------------|-----------------------------|---------|
| Patients parameter               |                             |         |
| Age (years)                      |                             | 0.192   |
| Average [range]                  |                             |         |
| <55                              | 55 [30–81]                  |         |
| ≥55                              | 22 (44.0)                   |         |
| Gender                           |                             | 0.0681  |
| Male                             | 32 (64.0)                   |         |
| Female                           | 18 (36.0)                   |         |
| Tumor characteristics            |                             |         |
| Tumor size (cm)                  |                             |         |
| ≤4                              | 10 (20.0)                   | 0.019*  |
| ≥4                              | 40 (80.0)                   |         |
| Differentiation                  |                             | 0.086   |
| Poor                             | 39 (78.0)                   |         |
| Well-moderate                    | 11 (22.0)                   |         |
| Lymph node metastasis            |                             |         |
| N−                               | 15 (30.0)                   | 0.024*  |
| N+                               | 35 (70.0)                   |         |
| Distant metastasis               |                             |         |
| M−                               | 16 (32.0)                   | 0.014*  |
| M+                               | 34 (68.0)                   |         |
| Level of miR-495                 |                             |         |
| mRNA level (Fig. 1A)             |                             |         |
| High                             | 4 (8.0)                     | 0.101   |
| Median                           | 8 (16.0)                    | 0.013*  |
| Low                              | 38 (76.0)                   | 0.001** |
| Level of UBE2C                   |                             |         |
| mRNA level (Fig. 3A)             |                             |         |
| High                             | 35 (70.0)                   | 0.001** |
| Median                           | 9 (18.0)                    | 0.041*  |
| Low                              | 6 (12.0)                    | 0.102   |
| Protein level (Fig. 3B)          |                             |         |
| High                             | 34 (68.0)                   | 0.001** |
| Median                           | 10 (20.0)                   | 0.004*  |
| Low                              | 6 (12.0)                    | 0.063   |

Differences between experimental groups were assessed by Student’s t-test or one-way analysis of variance. Data represent mean ± SD. *p < .05; **p < .01.

3.2. miR-495 reverses DDP resistance by downregulating drug resistance genes ABCG2 and ERCC1 in DDP-resistant NSCLC cells

To determine whether miR-495 is associated with DDP resistance in lung cancer cells, we performed RT-PCR assay and found that miR-495 levels were lower in DDP-resistant NSCLC cells (A549/DDP and H1299/DDP and Calu6/DDP) than in their parent cells, indicating that miR-495 significantly contributes to DDP resistance in DDP-resistant NSCLC cells (Fig. 2 a). The miR-495-mimics combined treatment with DDP were used to determine whether miR-495 activation underlies proliferation, apoptosis, migration, invasion, and EMT in DDP-resistant NSCLC cells. These effects remained almost unchanged in A549/DDP cells upon treatment with DDP compared with control treatment of PBS, indicating that A549/DDP cells are DDP-resistant (Fig. 2 b–I). However, comparing co-treatment of miR-495 mimics and DDP with co-treatments of miR-495 mimics and PBS in A549/DDP cells, we found that the co-treatment of miR-495 mimics and DDP decreased cell growth (Fig. 2 b), Ki67 protein levels (Fig. 2 c), clonal formation (Fig. 2 d), cell migration (Supplementary Fig. S2 a), and cell invasion (Fig. 2 e), but increased cleaved caspase-3 protein levels (Fig. 2 f) and Annexin V protein levels (Fig. 2 g) in A549/DDP cells. Moreover, we performed RT-PCR, western blotting and immunofluorescence staining to confirm whether miR-495 regulates E-cadherin and vimentin. Co-treatment of miR-495 mimics and DDP upregulated E-cadherin but downregulated vimentin compared with co-treatment with miR-495 mimics and PBS in A549/DDP and H1299/DDP cells (Fig. 2 h, i and Supplementary Fig. S2 b). These data suggest that miR-495 reverses DDP resistance in DDP-resistant NSCLC cells. To determine the molecular mechanism by which miR-495 reverses DDP resistance in lung cancer cells, we performed a RT-PCR assay to confirm whether miR-495 regulates drug resistance genes HER2, MRPI, KRAS, BRCA1, MDR1, ABCG2, and ERCC1 in A549/DDP cells (Supplementary Fig. S2 c). However, miR-495 only regulated ABCG2 and ERCC1 (Fig. 2 j and Supplementary Fig. S2 c). The mRNA and protein levels of ABCG2 and ERCC1 were measured 48 h after transfection with miR-495 mimics and miR-495 inhibitors. ABCG2 and ERCC1 mRNAs and proteins were significantly downregulated after miR-495 overexpression. These inhibitory effects were suppressed when miR-495 was downregulated (Fig. 2 j, k and Supplementary Fig. S2 d, e). Moreover, miR-495 reduced the mRNA and protein levels of ABCG2 and ERCC1 in a dose- and time-dependent manner in A549/DDP and H1299/DDP cells (Fig. 2 l, m). Collectively, these data indicated that miR-495 reverses DDP resistance by downregulating drug resistance genes ABCG2 and ERCC1 in DDP-resistant NSCLC cells. However, miR-495 did not directly bind to the 3’ - untranslated region of ABCG2 and ERCC1 by crosslinking-ligation and sequencing of hybrids assay (data not to shown). There would possibly be additional molecules mediating the interaction between miR-495 and ABCG2/ ERCC1. Hence, the molecular mechanism underlying miR-495 and ABCG2/ERCC1 should be further explored.

3.3. Aberrant activation of UBE2C in lung tumors from patients and dysfunction of UBE2C affected cell proliferation, invasion, and EMT

UBE2C plays a principal role in cell cycle progression and is aberrantly expressed in various cancers including human lung cancer, ovarian cancer, bladder cancer, and lymphoma [39–42]. Moreover, UBE2C, as a regulatory factor of its target genes, promotes tumor occurrence and development in many human cancers. Furthermore, UBE2C downregulation enhances the chemosensitivity of dual drug-resistant breast cancer cells to epirubicin and docetaxel [43], suggesting that UBE2C potentially contributes significantly to chemotherapeutic resistance. However, the molecular function of UBE2C in the occurrence, development, and DDP resistance in human NSCLC is unclear. Therefore, we assumed that UBE2C mediates the interaction of miR-495 and ABCG2/ERCC1 and we should further investigate whether aberrant activation of UBE2C promotes cell growth, migration, invasion and induces DDP resistance in NSCLC. To examine endogenous mRNA and protein expression of UBE2C in human lung cancer tissues, we performed RT-PCR and western blot analyses, respectively. UBE2C mRNA and protein levels were higher in human lung cancer tissues than in normal adjacent lung tissues (Fig. 3 a, b). Moreover, the aforementioned 50 samples obtained from patients who underwent a lung resection surgery at Affiliated Hospital of Binzhou Medical College were examined, and the clinicopathological findings are summarized in Table 1. Furthermore, UBE2C mRNA and protein levels were higher in lung cancer cells than in normal human bronchial epithelial cell (HBEC) controls (Fig. 3 c). As shown in Fig. 3d, UBE2C protein levels were also significantly elevated in human lung tumor tissues compared to those in adjacent normal lung tissues upon immunohistochemical analysis of frozen sections. Cytoplasmic UBE2C was lower in these lung tumor samples than in adjacent normal lung tissues. An immunoblotting assay indicated that UBE2C proteins accumulated to higher concentrations in the nuclei of NSCLC cells than in those of normal adjacent lung tissues (Fig. 3 e). Publicly available datasets were screened and used to determine the prognostic correlation between UBE2C expression and lung cancer patient survival. Kaplan-Meier analyses indicated that UBE2C upregulation was highly correlated with shorter overall survival (OS) (n = 1926, P = 1.0 × 10^{-6}) [31, 38, 39] (Fig. 3 f). These data indicate that UBE2C, a transcription factor, promoted human tumorigenesis and cancer development. siRNA-mediated UBE2C silencing (siUBE2C-1 and siUBE2C-2)
Fig. 2. miR-495 reverses DDP resistance by downregulating drug resistance genes ABCG2 and ERCC1 in DDP-resistant NSCLC cells. (a) Gel-based RT-PCR densitometric quantitation demonstrating reduced mRNA expression of miR-495 in DDP-resistant NSCLC cells compared with their parent cells. (b) A549/DDP, H1299/DDP or Calu6/DDP cells were transfected with control mimics or miR-495 mimics and then were treated with PBS or DDP 6 μg/ml for 60 h, respectively. The cellular proliferation and cell growth was analyzed by CCK8. (c-h) A549/DDP cells were transfected with control mimics or miR-495 mimics then treated with PBS or DDP 6 μg/ml for 60 h, respectively. (c) The protein of Ki67 was analyzed by immunofluorescent staining. (d) Colony formation density was analyzed by colony formation assay. (e) Cellular migration and invasion ability was analyzed by transwell assay. (f) The protein of cleaved Caspase3 was analyzed by immunoblotting assay. (g) The protein of Annexin V was analyzed by immunofluorescent staining. (h, i) The expression of E-cadherin and Vimentin were analyzed by RT-PCR and western blot in A549/DDP (h) cells and H1299/DDP (i) cells. (j, k) A549/DDP (j) and H1299/DDP (k) cells were transfected with miR-495 inhibitors. The mRNA and protein levels of miR-495, ERCC1 and ABCG2 were analyzed were analyzed by RT-PCR and immunoblotting. (l, m) A549/DDP (l) and H1299/DDP (m) cells were transfected with miR-495 mimics. RT-PCR and Western blot result shows that miR-495 dose-dependently and time-dependently decreased the mRNA and protein levels of ERCC1 and ABCG2. Results were presented as mean ± SD, and the error bars represent the SD of three independent experiments. **p < .01 vs control group.
Aberrant activation of UBE2C in lung tumors from patients and dysfunction of UBE2C affected cell proliferation, invasion, and EMT. (a, b) RT-PCR and western blot indicated that the mRNA (a) and protein levels (b) of UBE2C were higher in human lung cancer tissues compared with their normal adjacent lung tissues. Statistical analysis of the mRNA and protein level of UBE2C (n = 20). (c) Gel-based RT-PCR and immunoblotting with densitometric quantitation demonstrating elevated mRNA and protein expressions of UBE2C in human lung cancer cells compared with their normal control cell HBEC. (d) Immunohistochemistry with frozen sections indicated that increased the protein level of UBE2C and UBE2C accumulated in nuclear in lung cancer samples while more UBE2C was localized in cytoplasm of those normal adjacent lung tissues. (e) Immunoblotting showing increased UBE2C in nuclear in human lung cancer tissues compared with their normal adjacent lung tissues. (f) Kaplan Meier overall survival (OS) curves of UBE2C (n = 1926, p = 1E-16 by log-rank test for significance) for human lung cancers. (g-o) A549 cells were transfected with siUBE2C or Flag-UBE2C to decrease or increase the protein of UBE2C. (g) The mRNA and protein levels of UBE2C were analyzed by RT-PCR and Western blot assay. (h) The cellular proliferation was analyzed by CCK8 assay. (i, j) Cell cycle profile (i) and the apoptosis (j) were analyzed by cell flow cytometry. (k) The protein of cleaved Caspase3 was analyzed by immunoblotting assay. (l) Cellular migration and invasion ability was analyzed by transwell assay. (m) Cell senescence was analyzed by SA-β-gal staining. (n, o) The expression of E-cadherin and Vimentin were analyzed by RT-PCR (n) and western blot (o). Results were presented as mean ± SD, and the error bars represent the SD of three independent experiments. *p < .05; **p < .01 vs control group.
and UBE2C overexpression using pcDNA.Flag-UBE2C were carried out to determine whether UBE2C oncogene activation underlies lung cancer cell proliferation, apoptosis, migration, invasion and EMT (Fig. 3 g). The knockdown efficiency of UBE2C was better using the siUBE2C-2 (Supplementary Fig. S3 a, b); therefore, siUBE2C-2 was only used for the subsequent experiment for knockdown of UBE2C. Moreover, for convenient assessment and to compare different expression levels of UBE2C in lung cancer cell proliferation, apoptosis, cell senescence, migration, invasion, and EMT, we setup control (co-transfection of pcDNA vector and si control), siUBE2C (co-transfection of siUBE2C and pcDNA vector) and Flag-UBE2C (co-transfection of pcDNA Flag-UBE2C and siControl) in the same experiment, simultaneously. These approaches are also applicable to the following experiment for ABCG2, ERCC1, or miR-495. According to a CCK8 and MTT assay (Fig. 3 h and Supplementary Fig. S3 c), UBE2C knockdown and overexpression decreased and increased A549 cell proliferation, respectively, siUBE2C-induced significant G2 arrest and Flag-UBE2C increased the number of A549 cells in the S phase, as shown in histograms representing cell cycle distribution and quantitation analysis (Fig. 3 i). siUBE2C induced apoptosis (Fig. 3 j) and increased cleaved caspase-3 protein levels (Fig. 3 k) in A549 cells. These inhibitory effects were reversed when Flag-UBE2C was transfected into A549 cells (Fig. 3 g-o and Supplementary Fig. S3 c-e). Moreover, in A549 cells, siUBE2C decreased clonal formation (Supplementary Fig. S3 d), cell migration (Supplementary Fig. S3 e), and cell invasion (Fig. 3 i), but increased cell senescence (Fig. 3 m) in A549 cells. The opposite effects were observed for all these factors in A549 cells upon ectopic expression of Flag-UBE2C (Fig. 3 l, m). Furthermore, we confirmed whether E-cadherin and Vimentin were regulated by UBE2C. siUBE2C increased the mRNA and protein levels of E-cadherin, but decreased those of Vimentin; the opposite effect was observed for each of these factors in A549 cells, wherein treatment of A459 cells with Flag-UBE2C via RT-PCR and western blotting (Fig. 3 n, o). These data showed that aberrant activation of UBE2C in lung tumors from patients and dysfunction of UBE2C affected cell proliferation, invasion, and EMT.

3.4. miR-495 reduced cellular proliferation and invasion by regulating UBE2C mRNA stability in DDP-resistant NSCLC cells

miRBase, miRanda, and TargetScan analysis predicted the same single binding site at the 3′-UTR of UBE2C. Therefore, miR-495 may directly target UBE2C (Fig. 4 a). To confirm this interaction between miR-495 and UBE2C, we constructed luciferase reporter plasmids containing either the sequences of the 3′-UTR of UBE2C (UBE2C-WT) or the miR-448 response element mutant (UBE2C-Mut) (Fig. 4 a). Co-transfection of UBE2C-3′-UTR-WT and miR-495 mimics into A549/DDP cells significantly decreased luciferase activity, compared to co-transfection with scramble miRNA. This reduction was reversed in cells transfected with UBE2C-3′-UTR-MUT or miR-495 inhibitor. Therefore, miR-495 directly targets UBE2C (Fig. 4 b). To determine whether miR-495 endogenously regulates UBE2C, UBE2C mRNA and protein levels in A549/DDP cells were determined 48 h after transfection of miR-495 mimics or miR-495 inhibitor. UBE2C mRNA and protein were significantly downregulated in the A549/DDP cells upon miR-495 overexpression. These inhibitory effects were suppressed when miR-495 was downregulated (Fig. 4 c, d). Moreover, miR-495 mimics downregulated UBE2C mRNA and protein in a dose- and time-dependent manner (Fig. 4 e, f). These data indicate that miR-495 regulated UBE2C by binding the 3′-UTR of UBE2C. To confirm whether miR-495 inhibits cell proliferation, migration, invasion, and EMT by targeting complementary sites in the 3′-UTR of UBE2C, we co-transfected miR-495 mimics and UBE2C or miR-495 inhibitor and siUBE2C into A549/DDP cells. We separately transfected miR-495 mimics or miR-495 inhibitors into A549/DDP cells and performed RT-PCR and western blotting to detect miR-495 and UBE2C in them. UBE2C levels were reduced upon transfection with miR-495 mimics and increased upon treatment with miR-495 inhibitors. However, the opposite effects were observed for each of these factors in A549/DDP cells ectopically transfected with UBE2C or siUBE2C (Fig. 4 g). Moreover, a CK8 assay in A549/DDP cells indicated that lung cancer cell proliferation significantly increased after transfection of an miR-495-inhibitor; however, this inhibition was reversed upon co-transfection with siUBE2C. Similarly, lung cancer cell proliferation decreased significantly upon transfection with miR-495 mimics; however, this inhibitory effect was suppressed upon UBE2C co-transfection (Fig. 4 h). Similarly, protein expression of cleaved caspase-3 (Fig. 4 i), Annexin V (Supplementary Fig. S4 a), cell senescence (Supplementary Fig. S4 b), colony formation (Fig. 4 j), cell migration (Supplementary Fig. S4 c), and cell invasion (Fig. 4 k), displaying similar effects with cell proliferation. Since miR-495 inhibitor promotes cell invasion and migration, we performed RT-PCR and western blotting to confirm whether it regulates E-cadherin and vimentin via regulation of UBE2C. miR-495 overexpression upregulated E-cadherin but downregulated vimentin. In contrast, A549/DDP cells co-transfected with miR-495 mimics and UBE2C had EMT marker levels that were significantly reverse to those in only miR-495 mimics–transfected cells (Fig. 4 l, m). The opposite effects were observed for cells co-transfected with miR-495-inhibitor and siUBE2C (Fig. 4 l, m). Therefore, by targeting the 3′-UTR of UBE2C, we verified that miR-495 reduced proliferation, migration, invasion and EMT by regulating UBE2C mRNA stability in DDP-resistant NSCLC cells.

3.5. UBE2C was expressed at higher levels in DDP-resistant NSCLC cells and positively correlated with DDP resistance

To investigate the relationship between UBE2C and the DDP resistance in NSCLC, we generated the DDP-resistant NSCLC cell lines H1299/DDP and Calu6/DDP. The A549/DDP cells were gifted from the Resistant Cancer Cell Line (RCCL) collection (http://www.kent.ac.uk/stms/cmp/RCCL/RCLLabourt.html). UBE2C mRNA and protein levels were determined 48 h after transfection of an miR-495 mimic or an miR-495 inhibitor. Therefore, miR-495 directly targets UBE2C (Fig. 4 b). To determine whether miR-495 endogenously regulates UBE2C, UBE2C mRNA and protein levels in A549/DDP cells were determined 48 h after transfection of miR-495 mimics or miR-495 inhibitor. UBE2C mRNA and protein were significantly downregulated in the A549/DDP cells upon miR-495 overexpression. These inhibitory effects were suppressed when miR-495 was downregulated (Fig. 4 c, d). Moreover, miR-495 mimics downregulated UBE2C mRNA and protein in a dose- and time-dependent manner (Fig. 4 e, f). These data indicate that miR-495 regulated UBE2C by binding the 3′-UTR of UBE2C. To confirm whether miR-495 inhibits cell proliferation, migration, invasion, and EMT by targeting complementary sites in the 3′-UTR of UBE2C, we co-transfected miR-495 mimics and UBE2C or miR-495 inhibitor and siUBE2C into A549/DDP cells. We separately transfected miR-495 mimics or miR-495 inhibitors into A549/DDP cells and performed RT-PCR and western blotting to detect miR-495 and UBE2C in them. UBE2C levels were reduced upon transfection with miR-495 mimics and increased upon treatment with miR-495 inhibitors. However, the opposite effects were observed for each of these factors in A549/DDP cells ectopically transfected with UBE2C or siUBE2C (Fig. 4 g). Moreover, a CK8 assay in A549/DDP cells indicated that lung cancer cell proliferation significantly increased after transfection of an miR-495-inhibitor; however, this inhibition was reversed upon co-transfection with siUBE2C. Similarly, lung cancer cell proliferation decreased significantly upon transfection with miR-495 mimics; however, this inhibitory effect was suppressed upon UBE2C co-transfection (Fig. 4 h). Similarly, protein expression of cleaved caspase-3 (Fig. 4 i), Annexin V (Supplementary Fig. S4 a), cell senescence (Supplementary Fig. S4 b), colony formation (Fig. 4 j), cell migration (Supplementary Fig. S4 c), and cell invasion (Fig. 4 k), displaying similar effects with cell proliferation. Since miR-495 inhibitor promotes cell invasion and migration, we performed RT-PCR and western blotting to confirm whether it regulates E-cadherin and vimentin via regulation of UBE2C. miR-495 overexpression upregulated E-cadherin but downregulated vimentin. In contrast, A549/DDP cells co-transfected with miR-495 mimics and UBE2C had EMT marker levels that were significantly reverse to those in only miR-495 mimics–transfected cells (Fig. 4 l, m). The opposite effects were observed for cells co-transfected with miR-495-inhibitor and siUBE2C (Fig. 4 l, m). Therefore, by targeting the 3′-UTR of UBE2C, we verified that miR-495 reduced proliferation, migration, invasion and EMT by regulating UBE2C mRNA stability in DDP-resistant NSCLC cells.
treatment as compared to the single treatment groups (Fig. 5l). We also investigated whether siUBE2C influences DDP-mediated apoptosis in A549/DDP cells via analysis of the expression of pro-apoptosis marker cleaved caspase-3. The protein level of cleaved caspase-3 was higher in A549/DDP cells co-treated with siUBE2C and DDP than in those subjected to individual treatments (Fig. 5m). Flow cytometry analysis

Fig. 4. miR-495 reduced cellular proliferation and invasion by regulating UBE2C mRNA stability in DDP-resistant NSCLC cells. (a) Putative miR-495 binding sites in the 3'-UTR sequences of UBE2C. (b) Luciferase activity of A549/DDP cells transfected with plasmids carrying a wild-type or mutant 3'UTR of UBE2C, in response to miR-495 mimics or inhibitor. (c, d) The A549/DDP cells were transfected with miR-495 mimics or miR-495 inhibitor. The mRNA and protein levels of UBE2C were analyzed by RT-PCR, immunoblotting (c) and immunofluorescent staining (d). (e, f) RT-PCR and Western blot result shows that miR-495 dose-dependently (e) and time-dependently (f) decreased the mRNA and protein levels of UBE2C. (g-m) A549/DDP cells were transfected with miR-495 mimics or miR-495 inhibitor. UBE2C or siUBE2C were used for upregulating or downregulating the protein level of miR-495 target genes, respectively. (g) The mRNA and protein expression levels of miR-495 and UBE2C were analyzed by RT-PCR and immunoblotting. (h) The cellular proliferation was analyzed by CCK8 assay. (i) The protein of cleaved Caspase3 was analyzed by immunoblotting assay. (j) Colony formation density was analyzed by colony formation assay. (k) Cellular migration and invasion ability was analyzed by transwell assay. (l, m) The mRNA and protein levels of E-cadherin and Vimentin were analyzed by RT-PCR, immunoblotting (l) and immunofluorescent staining (m). Results were presented as mean ± SD, and the error bars represent the SD of three independent experiments. *p < .05; **p < .01 vs control group.
Fig. 5. UBE2C was expressed at higher levels in DDP-resistant NSCLC cells and positively correlated with DDP resistance. (a, b) RT-PCR (a) and western blot (b) showed that the mRNA and protein levels of UBE2C were higher in cisplatin resistant cells with the increased adapting DDP concentration than their parent cells. (c) Immunofluorescent staining showing increased UBE2C in A549/DDP and H1299/DDP cells than in the parent cells. (d) The activities of UBE2C promoter was examined by luciferase reporter gene assays in A549 and A549/DDP cells. (e) A549/DDP cells were treated with DDP, siUBE2C and cotreatment with siUBE2C and DDP at 6 μg/ml for 60 h. The protein expression levels of UBE2C was analyzed by immunoblotting. (f) A549 and H1299 cells were treated with DDP or A549/DDP and H1299/DDP cells were treated with DDP, siUBE2C and cotreatment with siUBE2C and DDP at 6 μg/ml for 60 h. The cellular proliferation was analyzed by CCK8 assay. (g) A549/DDP cells were treated of DDP, siUBE2C or co-treatment of siUBE2C and DDP at 6 μg/ml for 60 h. (g) The protein level of Ki67 was analyzed by immunoblotting. (h) The soft gel colony formation density was analyzed by colony formation assay. (i, j) Cell cycle profile and the apoptosis were analyzed by cell flow cytometry. (k) Cellular morphology was analyzed by phase contrast microscope assay. (l) The protein level of cleaved Caspase3 was analyzed by immunoblotting assay. (m) Cell migration and invasion growth were analyzed by transwell assay. (o) The mRNA and protein levels of EMT relevant molecular protein E-cadherin and Vimentin were analyzed by RT-PCR and Western blot assay. Results were presented as mean ± SD, and the error bars represent the SD of three independent experiments. *p < .05; **p < .01 vs control group.
(Fig. 5 j) and immunofluorescence detection of Annexin V (Supplementary Fig. S5 c) confirmed that DDP-mediated apoptosis was increased in A549/DDP cells upon co-treatment with siUBE2C and DDP. EMT, including increased cell invasion and migration, is a defining feature of cancer cells, implicated in chemotherapeutic resistance. UBE2C promoted EMT and stimulated A549 cell invasion and migration (Fig. 5 n, o and Supplementary Fig. S5 d, e). We investigated the relationship between UBE2C-mediated EMT and chemotherapeutic resistance via a wound healing assay and found that treatment with 6 μg/ml DDP for 36 h reduced A549 cell migration relative to A549/DDP cells. However, migration was arrested in A549/DDP cells co-treated with siUBE2C and 6 μg/ml DDP (Supplementary Fig. S5 d). The Matrigel invasion and migration assay indicated that UBE2C knockdown combined with DDP administration suppressed A549/DDP cell invasion and migration to a greater extent than either single treatment (Fig. 5 n). We also examined whether UBE2C silencing influenced the DDP-mediated phenotypic transformation in lung cancer cells by evaluating the expression of EMT-associated markers. Co-treatment of A549/DDP cells with siUBE2C
and DDP upregulated E-cadherin and downregulated Vimentin mRNAs and proteins, compared to single treatments, as determined via RT-PCR, western blotting, and immunofluorescence staining analyses (Fig. 5 o and Supplementary Fig. S5 e). These results indicate that UBE2C was greatly upregulated in DDP-resistant NSCLC cells and positively correlated with DDP resistance.

3.6. UBE2C directly binds to the promoter of ABCG2 and ERCC1 to regulate their transcriptional activity, result in DDP resistance

To further investigate the relationship between UBE2C and the DDP resistance in NSCLC, we performed RT-PCR to confirm whether UBE2C regulates drug resistance genes HER2, MRPI, KRAS, BRCAn1, MDRI, ABCG2, and ERCC1 in A549/DDP cells. As shown in Supplementary Fig. S6 a, UBE2C only regulates ABCG2 and ERCC1. DDP resistance is closely associated with the expression of drug-resistance genes including ERCC1 and ABCG2 in the DDP resistant NSCLC cells. Moreover, ALGENN and JASPAR analysis predicted the binding site in the 5′ untranslated region (5′-UTR) of ABCG2 and ERCC1. UBE2C may directly target ABCG2 and ERCC1. To confirm the interaction between UBE2C and ABCG2/ERCC1, we further identified the promoter core region in ABCG2 and ERCC1. Various lengths of the ABCG2 5′-flanking region, including -1200/+105 (pGL3-1350), -1200/-450 (pGL3-750), -180/+150 (pGL3-330), and -450/-180 (pGL3-270) were cloned and transiently transfected into A549/DDP cells to determine promoter activity. The luciferase reporter gene assays revealed that pGL3-270 exhibited the maximum luciferase activity among pGL3-1350, pGL3-750, and pGL3-330 (Fig. 6 a), indicating that the region of -450/-180 is the promotor core region of ABCG2, which interacts with UBE2C. We further observed that UBE2C increased pGL3-270 activity in A549/DDP cells in a dose-dependent manner (Fig. 6 b), suggesting that the region -450/-180 of ABCG2 promoter may be the target promoter mediated by UBE2C. Similarly, the region -110/+180 of ERCC1 promoter may activate ERCC1 promoter, mediated by UBE2C (Fig. 6 a, b). We performed a deletion scan to determine the ABCG2 promoter region regulated by UBE2C. Deletion of nucleotides -344 to -274 abolished activation by UBE2C (Fig. 6 a). Within this region, we identified a putative UBE2C-response element spanning positions -314 to -296 (TGTGTA TTTCCAAGTTT) for ABCG2 (Fig. 6 c). Similarly, we identified a putative UBE2C-response element of ERCC1 spanning positions -98 to -80 (AAGGACTGTTTCAATA) (Fig. 6 c). Furthermore, we constructed luciferase reporter plasmids containing either wild type 5′-UTR of ABCG2/ERCC1 (ABCG2WT and ERCC1WT) or UBE2C response element mutant (ABCG2Mut and ERCC1Mut) sequences (Fig. 6 c). Co-transfection of ABCG2WT 5′-UTR and UBE2C into A549/DDP or H1299/DDP cells significantly increased luciferase activity to a greater extent than upon co-transfection with control plasmid and decreased the luciferase activity in A549/DDP or H1299/DDP cells upon co-transfection of ABCG2WT 5′-UTR and siUBE2C than co-transfection with control plasmid. Relative to the control group, luciferase activity was altered in either A549/DDP nor H1299/DDP cells after co-transfection of ABCG2Mut-5′-UTR and UBE2C or siUBE2C. Therefore, UBE2C directly targets the ABCG2 promoter (Fig. 6 d). Likewise, we get the similar luciferase activity results of ERCC1 in the DDP resistant NSCLC cells (Fig. 6 d). Quantitative chromatin immunoprecipitation (ChIP) assays were performed to determine whether UBE2C directly binds to ABCG2/ERCC1 5′-UTR. Consistent with the results of the luciferase activity assay, the ChIP assay indicated that ABCG2WT 5′-UTR and UBE2C co-transfection into A549/DDP or H1299/DDP cells significantly promoted UBE2C binding to the ABCG2WT 5′-UTR, whereas co-transfection with siUBE2C did not. Therefore, UBE2C directly targets the ABCG2 promoter (Fig. 6 e). Similar ChIP assay results for ERCC1 were obtained using A549/DDP and H1299/DDP cell lines (Fig. 6 e). These data indicate that UBE2C upregulates ABCG2 and ERCC1 by directly targeting their 5′-UTR region. Furthermore, we investigated the relationship between UBE2C and these two factors via RT-PCR and western blotting. The mRNA and protein levels of ERCC1 and ABCG2 were increased in A549 cells overexpressing UBE2C (Fig. 6 f). The opposite effects were observed for all these factors in A549/DDP cells upon knockdown of UBE2C (Fig. 6 g). ERCC1 and ABCG2 upregulation by UBE2C was confirmed via immunofluorescence analysis (Fig. 6 h). Moreover, UBE2C increased ABCG2 and ERCC1 mRNA and protein levels in a dose- and time-dependent manner in A549 cells (Fig. 6 i, j). Furthermore, ERCC1 and ABCG2 transcript and protein levels were significantly higher in A549/DDP cells than in the parent A549 cells (Fig. 6 k). The luciferase activity of ABCG2 and ERCC1 was also significantly higher in A549/DDP cells than in A549 cells, thereby contributing to DDP resistance in A549/DDP cells (Fig. 6 l). To further confirm the function of ABCG2 and ERCC1 in DDP-resistant A549/DDP cells, A549 cells were transfected with ABCG2 or ERCC1, while A549/DDP or H1299/DDP cells were transfected with siABC2 (siABC2−1 and siABC2−2) or siERCC1 (siERCC1−1 and siERCC1−2) to detect the proliferation of these cells upon treatment with DDP (Fig. 6 m and Supplementary Fig. S6 b-e). Knockdown of ABCG2 or ERCC1 with siABC2−1 or siERCC1−2 was better (Supplementary Fig. S6 b-e); therefore, siABC2−1 or siERCC1−2 was only used for the subsequent experiment for knockdown of ABCG2 or ERCC1. The result showed that the cellular proliferation was increased in DDP-treated A549 cells with the transfection of ABCG2 or ERCC1 but decreased in DDP-treated A549/DDP cells with knockdown of ABCG2 or ERCC1 compared with their control groups (Fig. 6 n). Therefore, ABCG2 and ERCC1 indeed played an important role in DDP-resistant NSCLC cells. Moreover, UBE2C knockdown combined with DDP treatment reduced ERCC1 and ABCG2 mRNA and protein levels as compared to the individual treatments (Fig. 6 o). Meanwhile, UBE2C silencing combined with DDP administration suppressed ERCC1 and ABCG2 expression compared to siUBE2C or DDP treatment alone via a luciferase reporter assay (Fig. 6 p). Spearman rank correlation analysis revealed positive correlations between UBE2C and ABCG2 or ERCC1.
3.7. miR-495 reverses DDP resistance by regulating ABCG2 and ERCC1 by directly targeting UBE2C

miR-495 inhibited lung cancer cell proliferation, apoptosis, migration, invasion, and EMT, which reversed DDP resistance via regulation of ABCG2 and ERCC1 (Figs. 1, 2). Similarly, siUBE2C reversed DDP resistance in NSCLC cells (Fig. 5). Moreover, miR-495 did not directly bind to the 3′-UTR of ABCG2 and ERCC1 (Fig. 2). Furthermore, miR-495 directly bound to the 3′-UTR of UBE2C (Fig. 3) and UBE2C directly bound to the 5′-UTR of ABCG2 and ERCC1 (Fig. 6). Therefore, we hypothesized that miR-495 reverses DDP resistance via regulation of ABCG2 and ERCC1 by directly targeting UBE2C, and these molecules participate in the miR-495-UBE2C-ABCG2/ERCC1 axis to reverse DDP resistance in DDP-resistant NSCLC cells.

To investigate this hypothesis, we separately transfected miR-495 mimics or miR-495 inhibitors into A549/DDP cell lines and performed WB to detect ABCG2 and ERCC1 in these cells. ABCG2 or ERCC1 mRNA and protein levels were reduced upon transfection of miR-495 mimics and increased by miR-495 inhibitors. However, the opposite effects were observed for each of these factors in A549/DDP cells ectopically transfected with UBE2C to miR-495 mimics or siUBE2C to miR-495 inhibitors (Fig. 7 a). These data indicate that miR-495 downregulated ABCG2 and ERCC1 via regulation of UBE2C. It is unclear whether the transcriptional relationship between miR-495-UBE2C and ABCG2/ERCC1 is applicable to A549/DDP cells treated with DDP for reversing DDP resistance. Furthermore, miR-495-mimics combined with co-treatment of DDP and siUBE2C were used to investigate whether miR-495 activation affected DDP-resistant NSCLC cells upon treatment with DDP and their ABCG2/ERCC1 expression, proliferation, apoptosis, migration, invasion, and EMT via regulation by UBE2C. These effects were obviously altered in A549/DDP cells with co-treatment with miR-495 mimics and siUBE2C upon treatment of DDP compared with treatment of PBS: the mRNA and protein levels of ABCG2 and ERCC1 (Fig. 7 b-d), cell growth (Fig. 7 e), Ki67 protein levels (Fig. 7 f), cleaved caspase-3 protein levels (Fig. 7 g), clonal formation (Fig. 7 h), cell migration (Fig. 7 i), and cell invasion (Fig. 7 j). Moreover, we performed RT-PCR, western blotting, and an immunofluorescence staining assay to confirm whether co-treatment of miR-495 mimics and DDP regulates E-cadherin and vimentin. Treatment with DDP upregulated E-cadherin but downregulated vimentin compared with treatment of PBS in A549/DDP cells with transfection of miR-495; however, these effects remained unchanged in A549/DDP cells co-transfected with miR-495 mimics and siUBE2C upon treatment with DDP compared with treatment of PBS (Fig. 7 k and Supplementary Fig. S6). These data indicate that UBE2C mediated miR-495 reverses DDP resistance via regulation of ABCG2 and ERCC1.

3.8. miR-495 inhibits DDP-resistant tumor growth in vivo

Based on the aforementioned function of miR-495 and UBE2C in cisplatin resistance, miR-495 directly bound to the 3′-UTR of UBE2C and UBE2C directly bound to the 5′-UTR of ABCG2 and ERCC1; we believe that miR-495 should play a much more important role in the miR-495-UBE2C-ABCG2/ERCC1 axis to reverse DDP resistance. To confirm the relationship between miR-495 and DDP resistance in human lung cancer, we generated a A549/DDP cell line with stable overexpression of miR-495 and used these cells to generate a mouse xenograft model of DDP resistance. First, the mRNA and protein levels of UBE2C, ABCG2, and ERCC1 were analyzed by RT-PCR and western blotting, respectively, to validate our generated cell lines (Fig. 8 a). UBE2C, ABCG2, and ERCC1 proteins were markedly downregulated in miR-495-overexpressing cells than in control cells, which is applicable in this study. Approximately 2 weeks after subcutaneous implantation of control and miR-495 cells into a concave niche of the ECM in mice, followed by treatment with 1.5 mg/kg DDP, those in the miR-495 group had a greater body weight (Fig. 8 b) and prolonged survival (Fig. 8 c) compared to control mice. Moreover, larger tumors were observed in the control group as compared to miR-495 mice (Fig. 8 d-f). Semi-quantitative immunohistochemical analysis (Fig. 8 g) of UBE2C (Fig. 8 h), ERCC1 (Fig. 8 i), ABCG2 (Fig. 8 j), Ki67 (Fig. 8 k), and Vimentin (Fig. 8 l) expression in xenografts revealed that the levels of these proteins were lower in the miR-495 group than in control mice. However, the opposite effects of E-cadherin were observed in xenografts ((Fig. 8 g, m). These results indicate that miR-495 reduces DDP-induced growth of tumors derived from DDP-resistant human lung cancer cells.

4. Discussion

In recent decades, substantial improvements have been made in the early diagnosis and treatment of lung cancer (1). This has increased the survival rates and the quality of life in lung cancer patients. Nevertheless, NSCLC is highly aggressive and malignant and has one of the lowest survival rates [44, 45]. To date, almost all NSCLC patients with advanced and unresectable disease are presented with very few treatment alternatives except for chemotherapy and radiotherapy, both of which are potentially associated with severe side effects [46]. Hence, there is a high demand for novel tumor-selective drugs with low- or no toxicity. The present study evaluated the specificity and efficacy of the miR-495-UBE2C-ABCG2/ERCC1 signaling axis in regulating NSCLC progression and drug resistance (Fig. 8 n), which, based on our findings, was determined to be a novel candidate for NSCLC therapy. There is growing evidence that miRNAs regulate target genes in lung cancer cells. Therefore, our study aimed to elucidate the molecular mechanism underlying lung cancer pathogenesis. The miR-495 participates in lung cancer progression. We performed RT-PCR to assess miR-495 expression in lung cancer tissues and in matched normal tissues adjacent to the tumors. We showed that the miR-495 expression was significantly higher in tumor-adjacent tissues than in lung cancer tissues. In addition, miR-495 was expressed at significantly lower levels in lung cancer cell lines than in normal cells. Cell migration and invasion assays in lung cancer cells overexpressing miR-495 indicated that the
overexpression of miR-495 in lung cancer cells significantly suppressed cell proliferation, migration, and invasion. Additionally, miR-495 expression was lower in DDP-resistant NSCLC cells than in its parent cells and miR-495 can reverse DDP resistance, indicating that miR-495 contributes to cisplatin resistance. Moreover, miR-495 downregulated anti-drug genes ABCG2 and ERCC1; however, miR-495 did not directly regulate these genes. Hence, other molecules may mediate the interaction of miR-495 and ABCG2/ERCC1.

UBE2C belongs to the E2 gene family and encodes a 19-kDa protein provided with ubiquitin-dependent proteolysis. Overexpression of
UBE2C leads to chromosomal mis-segregation and alteration of the cell cycle profile, thereby facilitating cell proliferation and inhibition of cellular apoptosis. UBE2C is reportedly upregulated in various types of cancers including metastatic prostate carcinoma, ovarian carcinoma, thyroid anaplastic carcinoma, breast cancer, hepatocellular carcinoma, and lung cancer [19]. Moreover, some studies have suggested a close association between drug resistance and protein ubiquitination and degradation; for instance, C-X-C chemokine receptor type 4 promotes ubiquitin-mediated degradation of cyclooxygenase 1, leading to aspirin resistance [22]. UBE2C induced the transcription of ubiquitin-specific peptidase 1 to reduced DDP-associated cytotoxicity in response to DNA damage in NSCLC. Moreover, UBE2C inhibition blocked caspase-3/8 signaling to regulate c-Jun N-terminal kinase activity, increasing the sensitivity of chemotherapeutic drugs and improving treatment outcome in NSCLC [47]. Ubiquitination is an important cellular mechanism for targeted degradation of proteins, which are instrumental for various cellular processes including, but not limited to, transcription, cell cycle progression, programmed cell death, and antigen presentation. However, no information is available regarding the direct involvement of UBE2C in ubiquitination.

As reported previously, UBE2C primarily serves as a growth factor to promote cell proliferation and migration in human cancers [19, 46, 48]. For instance, expression of UBE2C is required for the destruction of mitotic cyclins, such as cyclin B, to promote cell cycle progression from M to G1 phase; UBE2C expression was accompanied by that of other biomarkers such as prolactin-inducible protein, AZGP-1, and S100A8 or with pituitary tumor-transforming 1 (PTTG1). Survivin and thymidylate kinase 1, which might improve the prediction of outcomes in human cancer [49]. Hence, overexpression of UBE2C contributes to increased cell proliferation and migration and UBE2C as a biomarker of efficacy of cancer chemotherapy should be explored. Moreover, our data indicated that UBE2C serves as a transcription factor for anti-drug genes ABCG2 and ERCC1 to induce cisplatin resistance in lung cancer, which does not occur via the proteasome pathway. However, it is unclear whether UBE2C participates in the proteasome pathway in other tumors. Hence, this question will be further explored in our follow-up study.

Our data also revealed that UBE2C mRNA and protein levels were upregulated in lung cancer tumor tissues, compared to those in adjacent normal tissues and that UBE2C overexpression promoted cell growth and invasion. UBE2C was overexpressed in DDP-resistant NSCLC cells and its knockdown reversed DDP resistance in human lung cancer cells. UBE2C directly bound to the 5′-UTR of ABCG2 and ERCC1 to increase the expression of these anti-drug genes. Combined UBE2C silencing and DDP treatment induced cell senescence and apoptosis and reduced invasiveness in A549/DDP cells via regulation of anti-drug genes and reduced EMT via E-cadherin and Vimentin. Therefore, miR-495 and UBE2C play important roles in lung cancer tumorigenesis and drug resistance. However, the relationship between miR-495 and UBE2C remains unknown. We showed that miR-495 directly binds to the 3′-UTR of UBE2C and miR-495 regulates the UBE2C mRNA stability. Therefore, miR-495 reverses DDP resistance via regulation of ABCG2 and ERCC1 by directly target of UBE2C. Finally, our colonic xenograft model showed that siUBE2C deficiency suppressed DDP-resistant human lung cancer growth. Thus, the miR495-UBE2C-ABCG2/ERCC1 axis reverses DDP resistance via downregulation of anti-drug genes and reducing EMT in cisplatin resistant of human lung cancer.

The emergence of DDP resistance in NSCLC leads to poor prognosis. EMT is thought to play a critical role in drug resistance by promoting cancer cell invasion and metastasis. Our results indicate that miR-495 inhibitors and UBE2C promote EMT and cancer cell invasion and migration; The miR-495 inhibitors and UBE2C knockdown combined with DDP treatment inhibited cell invasion and EMT and reduced DDP resistance in human lung cancer cells. Therefore, we conclude that miR-495 inhibitors and UBE2C induces DDP resistance by upregulating anti-drug genes and EMT and promoting cell proliferation and invasion (Fig. 8 n), although the specific mechanism remains unclear. The present results suggest that miR495-UBE2C-ABCG2/ERCC1 axis is a promising therapeutic target for overcoming DDP resistance in NSCLC and improving prognosis.

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Competing interests

The authors declare no competing financial interests.

Author contributions

Jiwei, Guo designed the experiments. Jiwei Guo, Yan Wu, Dan Jin, Lijuan Yang, Jing Du, Kaikai Gong, Wewei Chen, Juanjuan Dai, Shuang Miao, Sichuan Xi performed the work. Jiwei, Guo and Dan, Jin analyzed the data and competed the figures. Jiwei, Guo wrote the manuscript.

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Fig. 8. miR-495 inhibits DDP-resistant tumor growth in vivo. (a) The mRNA and protein levels of UBE2C, ABCG2 and ERCC1 were analyzed by RT-PCR and Western blot assay in the A549/DDP cell line with stable overexpression of miR-495 mimics and control mimics. (b) The DDP treatment significantly alleviate body weight loss of the nude mice injected by A549/DDP cells with stable overexpression of miR-495 mimics and control mimics. (c) Kaplan-Meier overall survival (OS) curves of mice injection with control and miR-495 treated with DDP (d-f). Microscopy of tumor nodules of mouse (d) and overall tumor sizes (e) and growth curves (f). (g-m) H&E microscopy of tumor nodules from primary A549/DDP cells with stable overexpression of miR-495 mimics and control mimics in the subcutaneous xenografts of nude mice treated with DDP. Immunohistochemical staining shows that UBE2C, ERCC1, ABCG2, Ki67, Vimentin of miR-495 mimics groups were decreased in xenograft tumor tissues after treatment with DDP. In contrast, E-cadherin was higher in the miR-495 mimics groups than the control mimics mice. Statistical analysis (n=23) of the protein levels of UBE2C (h), ERCC1 (i), ABCG2 (j), Ki67 (k), Vimentin (l) and E-cadherin (m). (n) The diagram of miR495-UBE2C-ABCG2/ERCC1 axis reverses DDP resistance in cisplatin resistant NSCLC cells. Results were presented as mean ± SD, and the error bars represent the SD of three independent experiments. *p < .05, **p < .01 vs control group.
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