Long non-coding RNA HAND2-AS1/miR-106a/PTEN axis re-sensitizes cisplatin-resistant ovarian cells to cisplatin treatment

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Abstract. Cisplatin (DDP) resistance in patients suffering from ovarian cancer is a considerable hurdle to successful treatment. The present study aimed to identify a possible long non-coding RNA (lncRNA)-microRNA (miRNA)-mRNA axis participating in ovarian cancer DDP-resistance based on the critical roles of non-coding RNAs, including lncRNAs and miRNAs, in carcinogenesis. According to online data and experimental results, lncRNA HAND2-AS1 expression was significantly downregulated within ovarian carcinoma, especially within recurrent and DDP-resistant ovarian carcinoma. The expression of HAND2-AS1 was also shown to be markedly inhibited in SKOV3/DDP (DDP) cells with resistance to DDP. In SKOV3/DDP cells, HAND2-AS1 overexpression inhibited cell viability and promoted cell apoptosis upon DDP treatment through the Bcl-2/caspase-3 apoptotic signaling. It was hypothesized that PTEN mRNA expression was also markedly inhibited in SKOV3/DDP ovarian cancer cells, while HAND2-AS1 overexpression rescued PTEN proteins and blocked PI3K/AKT signaling activation. Moreover, miR-106a was found to bind directly to PTEN 3' UTR and HAND2-AS1. Upon DDP treatment, miR-106a overexpression in SKOV3/DDP cells promoted cell viability. It inhibited cell apoptosis through the Bcl-2/caspase-3 apoptotic signaling pathway and downregulated the protein levels of PTEN and upregulated PI3K/AKT signaling activity. Furthermore, miR-106a overexpression partially reversed the effect of HAND2-AS1 overexpression upon PTEN proteins and SKOV3/DDP cell proliferation upon DDP treatment. In conclusion, a lncRNA HAND2-AS1/miR-106a/PTEN axis that re-sensitizes DDP-resistant SKOV3/DDP cells to DDP treatment has been established.

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Introduction

Ovarian cancer is one of the leading causes of cancer-related deaths worldwide (1). Few patients present marked clinical symptoms of ovarian cancer at initial stages, resulting in late diagnoses. Although most patients with ovarian cancer are initially responsive to cisplatin (DDP) -based chemotherapy regimens, most patients with recurring ovarian cancer acquire DDP resistance (2). Exploring novel agents and targets aiming at curbing resistance to DDP-based chemotherapy is a matter of utmost urgency.

PTEN, located at 10q23, exerts a tumor-suppressive effect on numerous types of cancer by interfering in multiple pathogenic events (3). As a phosphatase, PTEN dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate, thus inhibiting PI3K/AKT signaling (4). PTEN deactivation in tumorigenesis subsequently increases the activity of PI3K/AKT signaling, therefore amplifying the ability of cancer cells to proliferate, migrate and invade, while inhibiting the apoptosis of these cells (5,6). Notably, PTEN downregulation is closely associated with the DDP-resistance of ovarian cancer (7,8). Concerning the PTEN downregulation mechanism, it has been reported that the demethylation agent fails to restore the expression of PTEN protein, indicating that PTEN is highly post-transcriptionally modulated (9). Notably, PTEN is identified as a direct downstream target of multiple microRNAs (miRs), a series of short non-coding RNAs, including miR-214 (7), miR-205 (10), miR-221/miR-222 (11), miR-93 (12) and miR-106a (13-16). Among them, miR-106a has been widely regarded as an oncogenic miRNA in types of cancer such as stomach cancer, liver cancer, non-small cell lung cancer, prostate cancer and ovarian cancers (13-17). However, its specific effect on ovarian cancer cell resistance to DDP remains to be elucidated. It is, therefore, hypothesized that the miR-106a/PTEN axis might appear in ovarian cancer and have an effect on the DDP-resistance of ovarian cancer.

Long non-coding RNAs (lncRNAs) are a type of RNA and are defined as being transcripts with lengths exceeding 200 nucleotides that are not translated into proteins (18). lncRNAs can genetically, epigenetically and post-transcriptionally modulate gene expression (19,20), thus participating in the occurrence and development of various types of cancer (21,22), such as ovarian cancer (23,24). By serving as competing endogenous RNAs (ceRNAs) for miRNAs, lncRNAs act as natural miRNA

decoys (25). Notably, miR-106a serves as a critical component of several lncRNA-miRNA-mRNA networks affecting PTEN expression. In gastric cancer, lncRNA-FER1L4 competes for miR-106a-5p to regulate PTEN expression through its miRNA response elements (26). In chronic myeloid leukemia, lncRNA-BGL3 serves as a miR-106a ceRNA, inhibiting PTEN expression from repressing Bcr-Abl-induced cell transformation (27). It was therefore hypothesized that lncRNAs could potentially be involved in ovarian cancer cell resistance to DDP in a miR-106a/PTEN axis-related manner.

To test the hypothesis, online data and microarray expression files were procured from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases. These data were analyzed for lncRNAs that were positively associated with PTEN and negatively associated with miR-106a in ovarian cancer. Among the candidates, lncRNA HAND2-AS1 was selected as it was downregulated in recurrent ovarian cancer and is hypothesized to be associated with DDP-resistance. The specific effects of HAND2-AS1 on ovarian cancer cells with resistance to DDP were subsequently investigated. In further confirmation of the predicted lncRNA-miRNA-mRNA network, the role of HAND2-AS1 in regulating PTEN/PI3K/AKT signaling and the binding of miR-106a to HAND2-AS1 and PTEN 3'UTR was examined. Finally, the dynamic effects of HAND2-AS1 and miR-106a on PTEN/PI3K/AKT signaling pathway and ovarian cancer cells with resistance to DDP were detected. In summary, a lncRNA-miRNA-mRNA network modulating ovarian cancer cell resistance to DDP was proposed.

Materials and methods

Clinical tissue samples. A total of 12 paired ovarian cancer tissues and adjacent non-cancerous tissues (at least 0.5 cm in distance) were harvested from patients (average age, 58.08±7.74 years old) diagnosed with ovarian cancer and underwent surgical resection in the Fourth Hospital of Changsha between January 2010 and May 2020. Patients who had received chemotherapy before surgery were excluded from this study. Ovarian cancer samples were histologically examined and the diagnosis was established by clinical pathologists. Clinical sampling was performed with the approval of the Ethics Committee of Hunan Normal University [approval no. 2019(188); Changsha, China] Signed written informed consent was obtained from all patients enrolled.

Cell line and cell transfection. SKOV3 cell line [American Type Culture Collection (ATCC)® HTB-77™] was obtained from ATCC and cultured in McCoy's 5a Medium Modified (ATCC) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). DDP-resistant SKOV3/DDP (DDP) cell line was purchased from the Chinese Academy of Medical Sciences and Peking Union Medical College. Briefly, the SKOV3/DDP cell line was established by exposing the original SKOV3 cell line to gradually increasing DDP concentrations. SKOV3 cells were cultured in a medium containing 0.02 mg/l DDP. The dose of DDP was steadily increased until the cells reached a stable growth phase with the medium containing 0.2 mg/l DDP. This induction culture lasted 6 months. All cells were cultured at 37˚C in 5% CO_2.

To generate lncRNA HAND2-AS1-overexpression or -knockdown cells, $2x10^5$ cells/ml target SKOV3 cells were transfected with 2 μg lncRNA HAND2-AS1-overexpressing vector (HAND2-AS1), HAND2-AS1 knockdown vector (sh-HAND2-AS1). The empty vector (pLVX-puro) or pLVX-shRNA2-puro containing a scramble sequence (sh-NC) were used as negative controls for overexpression vector or knockdown vector transfection. For miR-106a-5p overexpression or knockdown $2x10^5$ cells/ml target SKOV3 cells were transfected with 50 pmol miR106a-5p mimics (miR-106a-5p) or miR106a-5p inhibitor (anti-miR106a-5p). The mimics NC (miR-NC) or inhibitor NC (anti-NC) were used as negative controls. All the vectors or miRNAs were obtained from Shanghai GenePharma Co., Ltd. The sequences are listed in Table SI. Transfection was generated using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. After incubation with the DNA/RNA Lipofectamine 3000 complex for 6 h at 37˚C in the cell culture incubator, the SKOV3 cells were further cultured with fresh complete medium for 48 h. Then, cells were harvested for further experiments. The transfection efficiency was determined by reverse transcription-quantitative PCR (RT-qPCR).

Bioinformatics analysis. The GEO dataset GSE15709 (expression profiling of DDP-sensitive and resistant ovarian cancer cell lines, n=5 respectively) (28) and GSE14407 (expression profiling of ovarian surface epithelia and ovarian cancer epithelia, n=12 respectively) (29) were downloaded using R language GEOquery (version 3.12) (30) package and the differential expression genes were analyzed by Limma package (version 3.44.3) (31). The expression of lncRNA HAND2-AS1 in primary ovarian carcinoma (n=354) and recurrent ovarian carcinoma tissue samples (n=5) were obtained from TCGA-ovarian cancer (OV), download from Xena data hub: https://ucscpublic.xenahubs.net.

For co-expression genes selection, GSE15709 was used for analysis. Pearson's correlation coefficient analysis was performed by R language Psych package (version 2.1.3) (32) to identify genes positively correlated with HAND2-AS1 ($|r|>0.95$, P-value <0.01). Then, these co-expressed genes were analyzed by Gene Set Enrichment Analysis (GSEA) using R language ClusterProfiler package (version 3.16.1) (33) based on the tumor marker pathway gene set (Hall mark genesets: h.aLL.v7.2.entrez. GMT) from msigdb (https://www.gsea-msigdb.org).

For lncRNA and miRNA binding site prediction, online tool LncTar (http://www.cuilab.cn/lnctar) was used.

MTT assay. Following treatment and transfection, target cells were seeded in 96-well plates at a density of $2x10^4$ cells/well. MTT (0.5 g/l; Sigma-Aldrich; Merck KGaA) was added to each well. The cells were then incubated at 37˚C for 0 and 48 h. Following the removal of the medium, 50 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added and the cells were further incubated at 37˚C for 10 min. The absorbance of each sample was subsequently measured at 450 nm using a plate reader.

Flow cytometry analysis. Following treatment and transfection, target cells were seeded in 6-well plates at a density

of $1x10^5$ cells/ml medium for 24 h. An Annexin V-FITC Apoptosis Detection kit (KeyGen Biotech Co., Ltd.) was then used to determine cell apoptosis. Briefly, harvested cells were suspended in 500 μl binding buffer and incubated with 5 μl Annexin V at 4˚C for 10 min in the dark. Then, cells were further incubated with 5 μl PI solution at 4˚C for 10 min in the dark and then subjected to flow cytometry (BD FACSDiva Fusion; BD Biosciences). The early and late apoptotic rate was statistically analyzed by FlowJo software version 10.5 (FlowJo LLC).

RT-q)PCR. Total RNA from target cells with or without treatment or transfection was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and converted into cDNA using a PrimeScript® RT reagent kit (Takara Bio, Inc.) following the manufacturer's instructions and a previous method (34,35). qPCR was conducted using an SYBR Premix Ex Taq II (Takara Bio, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, and annealing and extension at 60˚C for 30 sec. U6 and GAPDH were used as the internal references for miRNA and mRNA expression determination, respectively. The $2^{-\Delta\Delta Cq}$ method was used for the calculation of relative expression (36). The primers are listed in Table SI.

Western blotting. Protein was extracted from target cells with or without treatment or transfection using RIPA lysis buffer containing PMSF (Beyotime Institute of Biotechnology). The protein concentration was determined by Bradford Protein Assay Kit (Beyotime Institute of Biotechnology). Protein was harvested, diluted, denatured at 100˚C for 5 min and then 30-50 μg protein were separated via 10% SDS-PAGE gel, and subsequently transferred to PVDF membranes (EMD Millipore). After blocking with 5% BSA (Beyotime Institute of Biotechnology) at room temperature for 2 h, the membranes were incubated overnight at 4˚C with the following primary antibodies (1:1,000) against cleaved-caspase-3 (cat. no. ab2302), caspase-3 (cat. no. ab13847), poly-ADP ribose polymerase (PARP; cat. no. ab74290), cleaved-PARP (cat. no. ab32064), Bax (cat. no. ab32503), Bcl-2 (cat. no. ab32124), PTEN (cat. no. ab32199), PI3K (cat. no. ab32089), phosphorylated (p-)PI3K (cat. no. ab182651), AKT (cat. no. ab179463), p-AKT (cat. no. ab81283) and GAPDH (cat. no. ab8245; all from Abcam). GAPDH was used as a visual loading control. After having been incubated with the HRP-conjugated secondary antibodies (1:5,000; cat. nos. SA00001-1 and SA00001-2; ProteinTech Group, Inc.) for 1 h at room temperature, protein signals were detected using enhanced chemiluminescence (ECL, Amersham; Cytiva) and visualized with an iBright™ CL1500 Imaging System (Invitrogen; Thermo Fisher Scientific, Inc.). Densitometry was performed using ImageJ software version 1.44 (National Institutes of Health).

Luciferase reporter assay. The wild type (wt) or mutant (mut) type of PTEN 3'UTR or HAND2-AS1 was inserted to the multiple cloning site of the psiCHECK-2 vector (Promega Corporation). Mut reporter vector contained a 6 or 8 bp mutation in the predicted miR-106a binding site. 293T cells (ATCC) were co-transfected with wt-PTEN-3'UTR/mut-PTEN-3'UTR or wt-HAND2-AS1/mut-HAND2-AS1 and miR-106a/anti-miR-106a. Transfection was performed using Lipofectamine® 3000 in accordance with the manufacturer's instructions. After 48 h, the *Renilla* luciferase activity and firefly luciferase activity was ascertained using a Dual-Luciferase Reporter Assay System (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analyses. All data were processed and analyzed using GraphPad version 6.0 software (GraphPad Software, Inc.) and expressed as the mean ± standard deviation. The experiments were repeated at least three times. The differences between two groups were analyzed using an unpaired Student's t-test. Whereas, differences between the ovarian cancer tissues and paired normal ovarian tissues were analyzed using a paired Student's t-test. Differences among more than two groups were analyzed using a one-way analysis of variance, followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Selection of lncRNA associated with ovarian cancer resistance to DDP. As confirmed by GSE14407, the expression of lncRNA HAND2-AS1 was shown to be significantly downregulated within epithelial tissue samples of ovarian cancer, compared with that within non-cancerous ovarian epithelial tissue samples (Fig. 1A). According to GSE15709, the expression of lncRNA HAND2-AS1 was markedly downregulated in ovarian carcinoma cell line A2780 with resistance to DDP, compared with that in A2780 with sensitivity to DDP (Fig. 1B). According to TCGA-OV database, the expression of lncRNA HAND2-AS1 was markedly downregulated within recurrent ovarian carcinoma tissue samples, compared with that in primary ovarian cancer tissue samples (Fig. 1C). According to TCGA database, the expression of lncRNA HAND2-AS1 was markedly downregulated within ovarian carcinoma tissue samples, compared with that in non-cancerous tissue samples (Fig. 1D). Additionally, lncRNA HAND2-AS1 expression in 12 paired non-cancerous and ovarian carcinoma tissue samples was evaluated; Fig. 1E showed that lncRNA HAND2-AS1 expression was markedly downregulated within ovarian carcinoma tissue samples, compared with that in normal tissue samples.

In vitro effects of HAND2-AS1 on the DDP-resistance of ovarian cancer cells. After confirming that HANDs-AS1 was downregulated within ovarian carcinoma, particularly in DDP-resistant and recurrent ovarian carcinoma, its specific effects on ovarian cancer DDP-resistance were examined. Regular and DDP-resistant SKOV3/DDP cells exposed to 0.125, 0.5, 2, 8 and 32 mg/l DDP were analyzed for cell viability through MTT assays; the IC_{50} value for SKOV3/DDP cells with resistance to DDP was much higher compared with that for regular SKOV3 cells (IC_{50}=2.963 and 15.80, respectively; Fig. 2A). Consistent with *in vitro* results, lncRNA HAND2-AS1 expression was shown to have markedly decreased within SKOV3/DDP cells with resistance to DDP compared with that in regular SKOV3 cells (Fig. 2B).

To evaluate the effect of HAND2-AS1 on ovarian cancer cell resistance to DDP, HAND2-AS1 overexpression

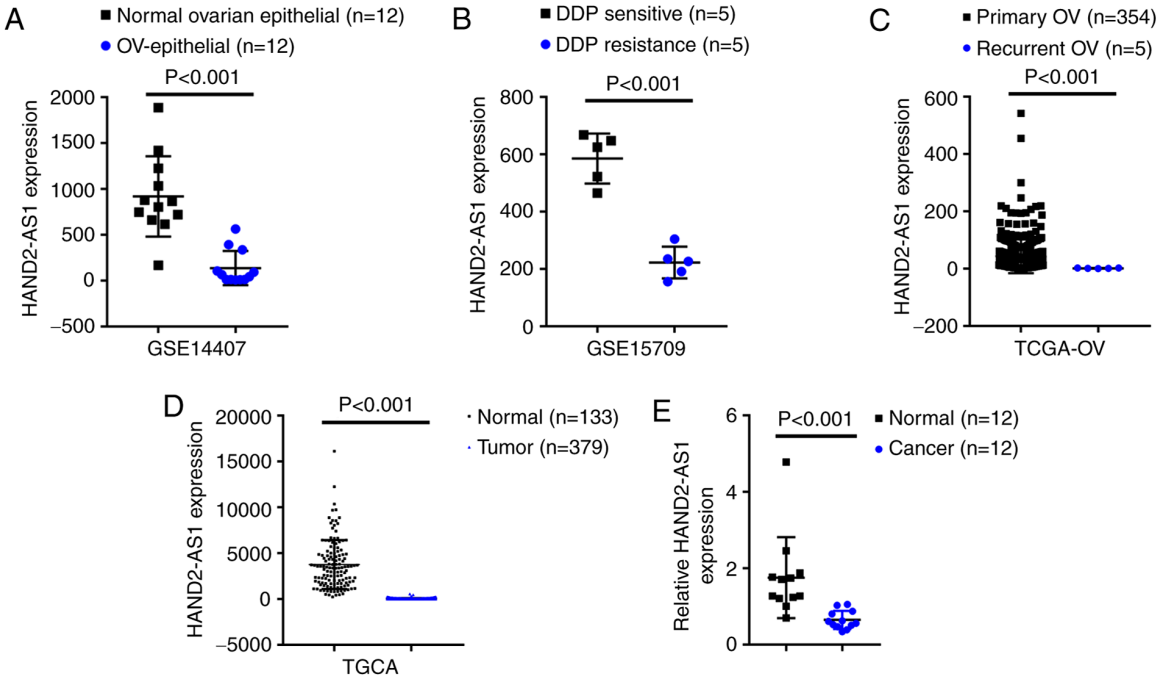

Figure 1. Selection of lncRNA associated with DDP-resistance of ovarian cancer. (A) lncRNA HAND2-AS1 expression in normal ovarian epithelial tissues and ovarian cancer epithelial tissues according to GSE14407. (B) lncRNA HAND2-AS1 expression in DDP-sensitive ovarian cancer cell line A2780 and DDP-resistant A2780 according to GSE15709. (C) lncRNA HAND2-AS1 expression in primary ovarian cancer tissues and recurrent ovarian cancer tissues according to TCGA-OV database. (D) lncRNA HAND2-AS1 expression in non-cancerous tissues and ovarian cancer tissues according to TCGA database. (E) lncRNA HAND2-AS1 expression in 12 paired non-cancerous tissues and ovarian cancer tissues determined by reverse transcription-quantitative PCR. lncRNA, long non-coding RNA; TCGA, The Cancer Genome Atlas; OV, ovarian cancer; DDP, cisplatin.

was generated in SKOV3/DDP cells by transfection of HAND2-AS1-overexpressing vector, and confirmed by RT-qPCR (Fig. 2C). Next, the changes in the viability and apoptosis of cells and Bcl-2/caspase-3 apoptotic signaling were examined in transfected SKOV3/DDP cells upon DDP treatment. As shown in Fig. 2D and E, the overexpression of HAND2-AS1 downregulated SKOV3/DDP cell viability, while upregulating SKOV3/DDP cell apoptosis. As for the signaling pathway involved, HAND2-AS1 overexpression led to significant increases in cleaved-caspase-3/caspase-3 ratios, cleaved-PARP/PARP ratios and Bax proteins, while reducing Bcl-2 protein levels (Fig. 2F). These data indicated that HAND2-AS1 overexpression could potentially re-sensitize DDP-resistant SKOV3 cells to DDP.

PTEN expression is positively correlated with HAND2-AS1 in ovarian cancer. To further investigate the underlying mechanism, differentially-expressed genes that were positively associated withHAND2-AS1 were further analyzed. According to GSE15709, 124 genes were positively correlated and 78 were negatively correlated with HAND2-AS1 (r >0.95; Fig. S1A). Gene Set Enrichment Analysis analyzed these co-expressed genes and showed that signaling of 'hallmark_PI3K_AKT_MTOR_signaling' was found to be negatively associated with HAND2-AS1 (Fig. S1B and C). As the natural inhibitor of the PI3K/AKT pathway, PTEN has been widely reported to modulate cell chemosensitivity of several cancer types (37,38), including ovarian cancer (8). According to GSE14407, the expression of PTEN was downregulated within epithelial tissue samples of ovarian carcinoma, compared with that within non-cancerous ovarian epithelial tissue samples (Fig. 3A). According to GSE15709, the expression of PTEN was distinctly downregulated within ovarian carcinoma cell line A2780 with resistance to DDP, compared with that in A2780 with sensitivity to DDP (Fig. 3B). Notably, HAND2-AS1 and PTEN expression was strongly positively correlated in GSE15709 (Fig. 3C). As with HAND2-AS1, PTEN expression was markedly downregulated in SKOV3/DDP cells, compared with that in SKOV3 cells (Fig. 3D). SKOV3/DDP cells were then transfected with HAND2-AS1 and examined for protein levels of PTEN/PI3K/AKT signaling factors, such as PTEN, p-PI3K, PI3K, p-AKT and AKT. As shown in Fig. 3E, HAND2-AS1 overexpression in SKOV3/DDP cells significantly increased PTEN protein levels, while reducing p-PI3K/PI3K ratios and p-AKT/AKT ratios. In summary, PTEN could potentially participate in HAND2-AS1 effects on ovarian cancer DDP-resistance.

miR-106a directly binds to PTEN 3'UTR and HAND2-AS1. As aforementioned, miR-106a serves as an oncogenic miRNA by targeting PTEN (15,17,39). Notably, online tools LncTar predicted that miR-106a could directly bind to HAND2-AS1 (Fig. 4E). The potential of the competitive binding of HAND2-AS1 to miR-106a to counteract miR-106a-mediated suppression on PTEN was then investigated. Consistent with previous studies on the association between miR-106a expression and cancer DDP-resistance (14), miR-106a expression was demonstrated to be markedly upregulated in SKOV3/DDP cells with resistance to DDP, compared with that in regular SKOV3 cells (Fig. 4A). miR-106a/**anti-miR-106a was transfected** to generate miR-106a expression in SKOV3/DDP cells with resistance to DDP; RT-qPCR was performed to verify

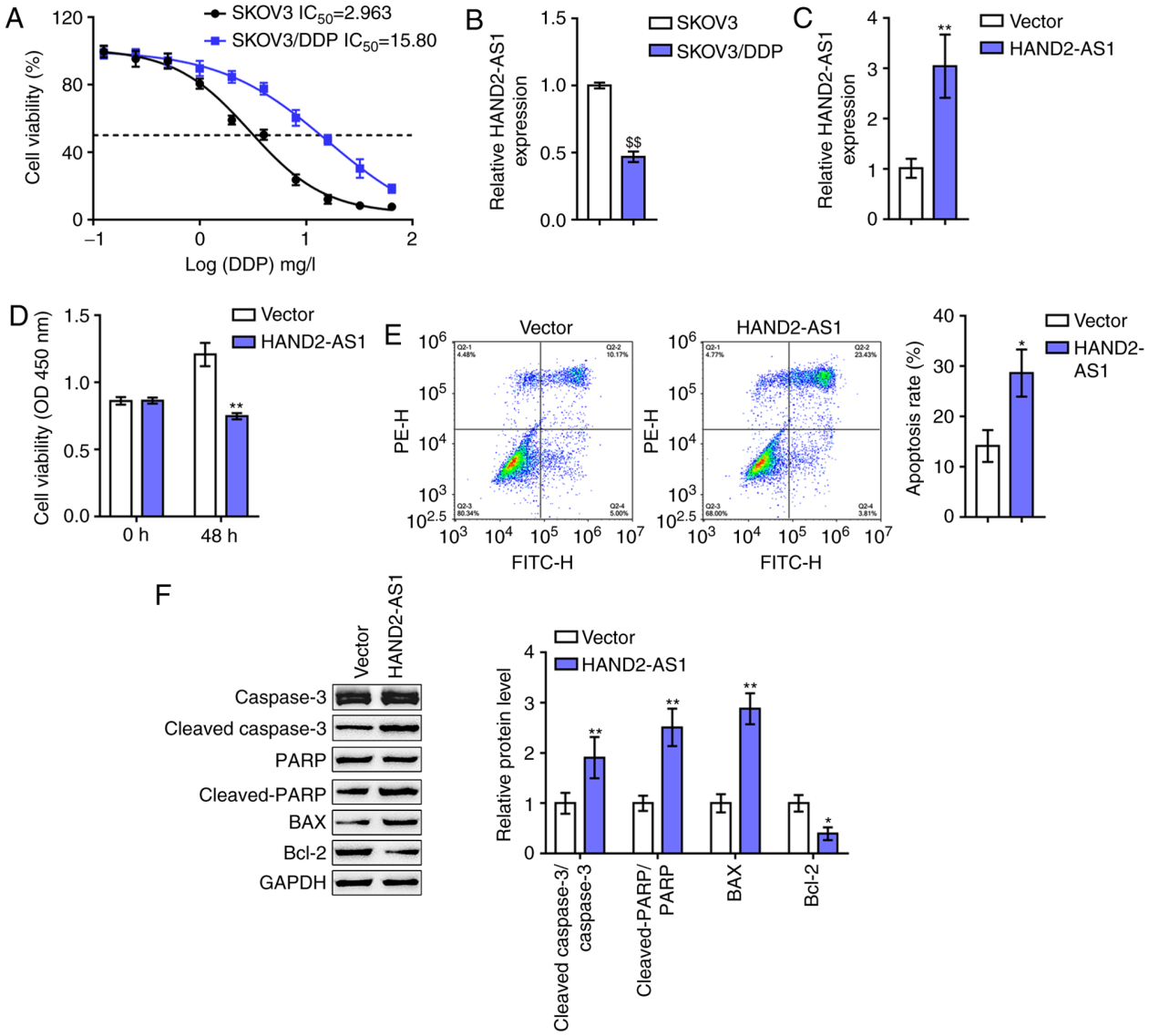

Figure 2. *In vitro* effects of HAND2-AS1 on ovarian cancer cell resistance to DDP. (A) Regular SKOV3 cells and DDP-resistant SKOV3/DDP cells were exposed to 0.125, 0.5, 2, 8 and 32 mg/l DDP and examined for cell viability via MTT assays. Data are shown as IC_{50} values. (B) lncRNA HAND2-AS1 expression in regular SKOV3 cells and DDP-resistant SKOV3/DDP cells determined by RT-qPCR. (C) HAND2-AS1 overexpression generated in SKOV3/DDP cells by transfection of HAND2-AS1-overexpressing vector, as confirmed by RT-qPCR. Next, SKOV3/DDP cells were transfected with HAND2-AS1 upon DDP treatment and examined for (D) cell viability by MTT assay and (E) cell apoptosis by flow cytometry. (F) The protein levels of cleaved-caspase-3, caspase-3, cleaved-PARP, PARP, Bax and Bcl-2 were determined via western blotting. $^{\$\$}$P<0.01 vs. SKOV3 group; *P<0.05, **P<0.01 vs. vector. DDP, cisplatin; lncRNA, long non-coding RNA; RT-qPCR, reverse transcription-quantitative PCR; PARP, poly-ADP ribose polymerase.

the transfection efficiency (Fig. 4B). SKOV3/DDP cells with resistance to DDP were subsequently transfected with miR-106a/anti-miR-106a and the protein levels of PTEN were examined. As presented in Fig. 4C, it was demonstrated that the overexpression of miR-106a significantly decreased, while the inhibition of miR-106a increased, PTEN proteins.

To verify the reported and predicted bindings of miR-106a and PTEN 3'UTR and HAND2-AS1, luciferase reporter assays were performed. As described above, two different types of PTEN 3'UTR and HAND2-AS1 luciferase reporter vectors, wt and mut, were constructed (Fig. 4D and E). These vectors were co-transfected with miR-106a/anti-miR-106a in 293T cells and the luciferase activity was determined and showed that miR-106a was significantly suppressed. At the same time, miR-106a inhibition enhanced luciferase activity in both the wt PTEN 3'UTR and HAND2-AS1 vectors. Mutating the putative miR-106a binding site could eliminate the luciferase activity alterations (Fig. 4D and E). In SKOV3/DDP cells, miR-106a-5p decreased the levels of HAND2-AS1 and anti-miR-106a-5p increased levels of HAND2-AS1 (Fig. 4F), whereas HAND2-AS1 overexpression or knockdown successfully increased or reduced the levels of HAND2-AS1 (Fig. 4G) and also negatively regulated miR-106a-5p expression (Fig. 4H). In brief, miR-106a directly targeted PTEN 3'UTR and HAND2-AS1.

Dynamic effects of HAND2-AS1 and miR-106a on ovarian cancer cells. After confirming the binding of miR-106a to PTEN 3'UTR and HAND2-AS1, the dynamic effects of these factors on SKOV3/DDP cell resistance to DDP were subsequently investigated. SKOV3/DDP cells were co-transfected with HAND2-AS1 and miR-106a and were first examined for

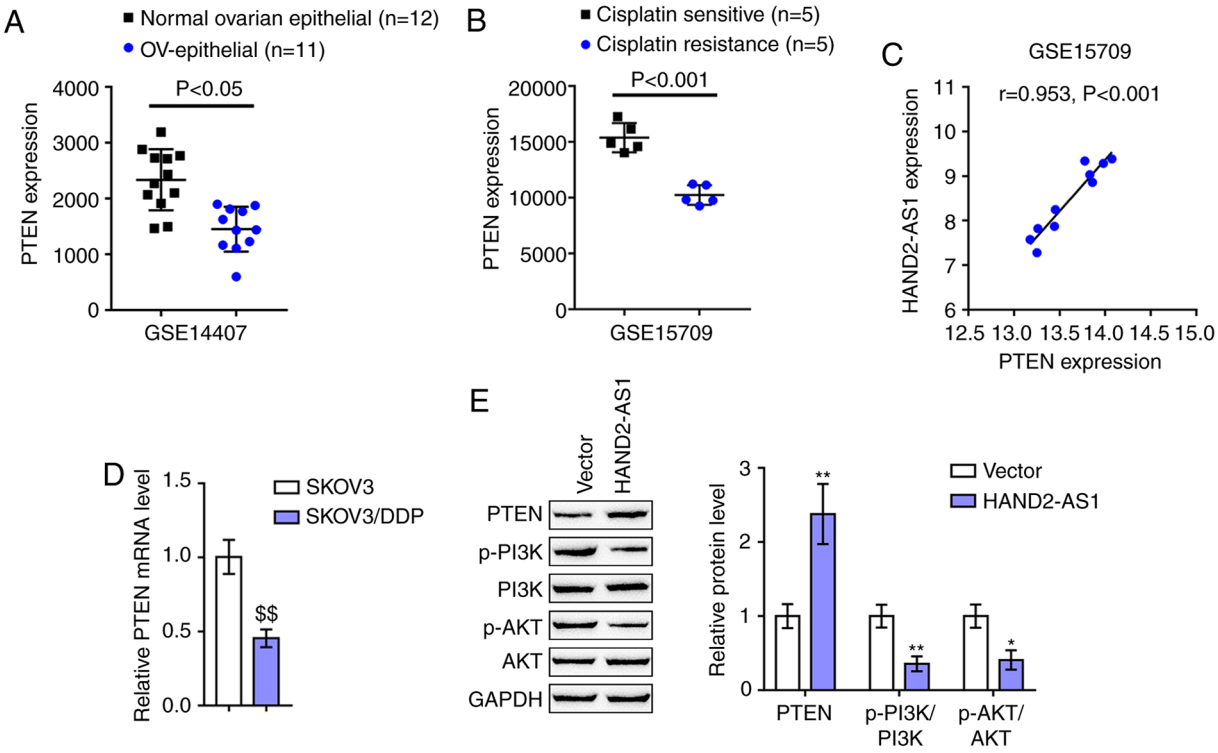

Figure 3. PTEN expression is positively associated with HAND2-AS1 in ovarian cancer. (A) PTEN expression in normal ovarian epithelial tissues and ovarian cancer epithelial tissues according to GSE14407. (B) PTEN expression in DDP-sensitive ovarian cancer tissues and DDP-resistant ovarian cancer cell line A2780 according to GSE15709. (C) The correlation of HAND2-AS1 and PTEN expression was analyzed by Pearson's correlation analysis. (D) PTEN mRNA expression in SKOV3 and SKOV3/DDP cells determined by reverse transcription-quantitative PCR. (E) SKOV3/DDP cells were transfected with HAND2-AS1 and examined for the protein levels of PTEN, p-PI3K, PI3K, p-AKT and AKT by western blotting. $^{\$\$}$P<0.05 vs. SKOV3 group; *P<0.05, **P<0.01 vs. vector group. p-, phosphorylated; DDP, cisplatin; OV, ovarian cancer.

the mRNA expression of PTEN. PTEN mRNA expression was significantly upregulated by HAND2-AS1 overexpression, but downregulated by miR-106a overexpression. The effects of HAND2-AS1 overexpression were partially reversed by miR-106a overexpression (Fig. 5A).

As for the dynamic effects of HAND2-AS1 and miR-106a on SKOV3/DDP cells with resistance to DDP, cleaved-caspase-3, caspase-3, cleaved-PARP, PARP, Bax, Bcl-2, PTEN, p-PI3K, PI3K, p-AKT and AKT cell viability, cell apoptosis and protein levels were then examined. HAND2-AS1 overexpression significantly reduced cell viability, while it increased cell apoptosis, whereas miR-106a exerted the opposite effects. Notably, the effects of HAND2-AS1 overexpression were markedly reversed by miR-106a overexpression (Fig. 5B and C).

Regarding the Bcl-2/caspase-3 apoptotic signaling pathway, HAND2-AS1 overexpression significantly increased the cleaved-caspase-3/caspase-3 ratios, the cleaved PARP/PARP ratios and Bax proteins, while it decreased Bcl-2 protein levels (Fig. 5D). miR-106a overexpression exerted opposite effects on these factors (Fig. 5D). The overexpression of miR-106a could significantly attenuate the effects of HAND2-AS1 overexpression (Fig. 5D). The HAND2-AS1/miR-106a axis modulated Bcl-2/caspase-3 apoptotic signaling to affect the viability and apoptosis of SKOV3/DDP cells.

Regarding the PTEN/PI3K/AKT signaling pathway, HAND2-AS1 overexpression significantly increased PTEN protein levels, while it decreased p-PI3K/PI3K ratios and p-AKT/AKT ratios (Fig. 5E). miR-106a overexpression exerted opposite effects on these factors (Fig. 5E). Similarly, the overexpression of miR-106a could significantly attenuate the effects of HAND2-AS1 overexpression on PTEN/PI3K/AKT signaling (Fig. 5E). In summary, HAND2-AS1 counteracted miR-106a-mediated suppression on PTEN through its role as a ceRNA.

Discussion

The present study observed that lncRNA HAND2-AS1 expression was significantly downregulated within ovarian carcinoma, especially within recurrent and DDP-resistant ovarian carcinoma. The expression of HAND2-AS1 was also markedly inhibited in SKOV3/DDP cells with resistance to DDP. In SKOV3/DDP cells, HAND2-AS1 overexpression inhibited cell viability and promoted cell apoptosis upon DDP treatment through Bcl-2/caspase-3 apoptotic signaling. In agreement with the hypothesis of the present study, PTEN mRNA expression was also markedly inhibited in SKOV3/DDP ovarian cancer cells, while HAND2-AS1 overexpression rescued PTEN proteins and blocked PI3K/AKT signaling activation. In addition, miR-106a was directly bound to PTEN 3'UTR and HAND2-AS1. Upon DDP treatment, miR-106a overexpression in SKOV3/DDP cells promoted cell viability, inhibited cell apoptosis through Bcl-2/caspase-3 apoptotic signaling, downregulated the protein levels of PTEN and upregulated PI3K/AKT signaling activity. Notably, miR-106a overexpression partially reversed the effect of HAND2-AS1 overexpression upon PTEN proteins and SKOV3/DDP cell proliferation upon DDP treatment.

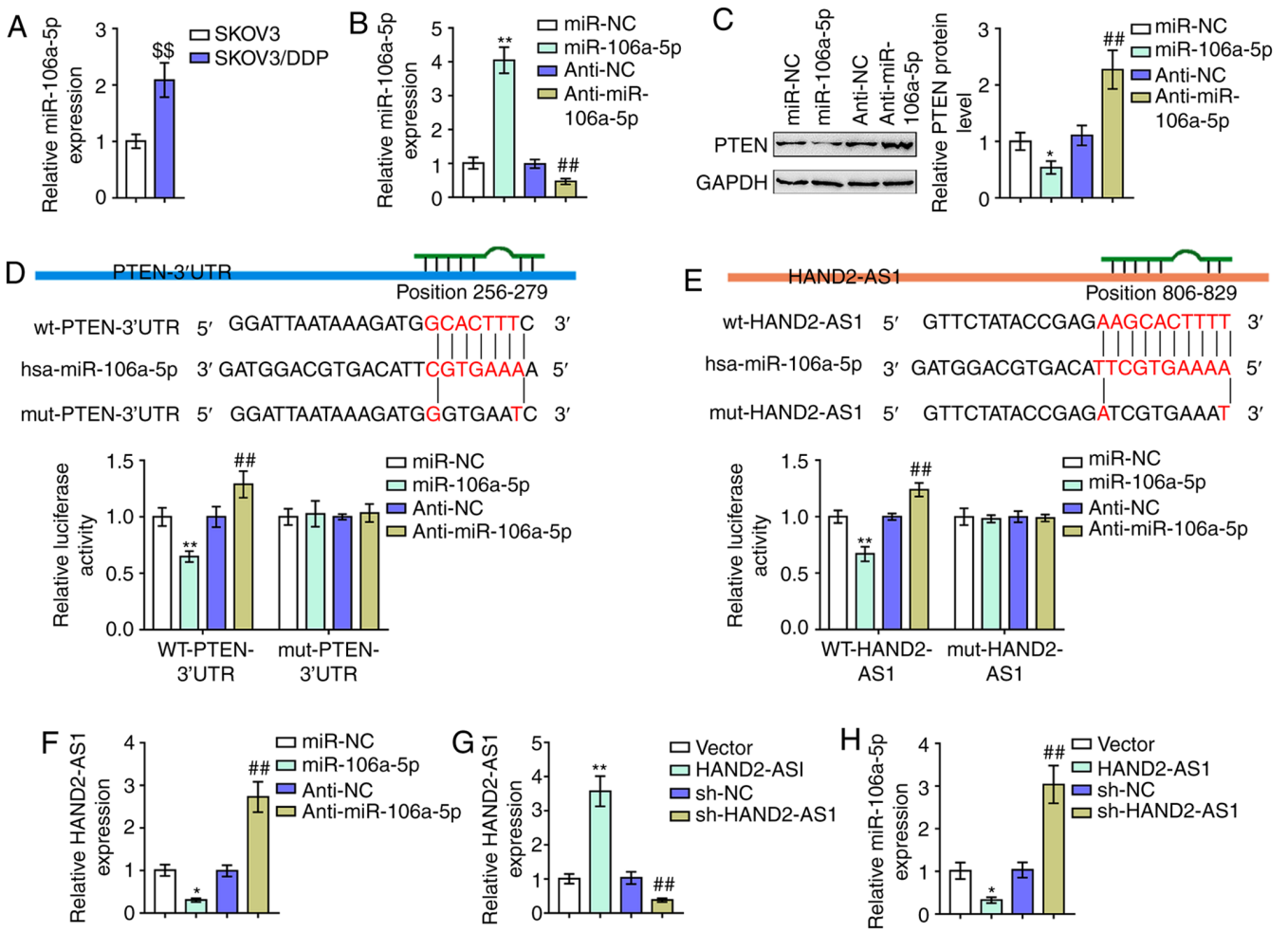

Figure 4. miR-106a directly binds to PTEN 3'UTR and HAND2-AS1. (A) Expression of miR-106a in normal SKOV3 cells and DDP-resistant SKOV3/DDP cells determined by RT-qPCR. $^{\$\$}$P<0.01 vs. SKOV3 group. (B) miR-106a expression generated in DDP-resistant SKOV3/DDP cells by transfection of miR-106a or anti-miR-106a, as confirmed by RT-qPCR. (C) DDP-resistant SKOV3/DDP cells were transfected with miR-106a or anti-miR-106a and examined for the protein levels of PTEN. wt and mut (D) PTEN 3'UTR and (E) HAND2-AS1 luciferase reporter vectors were constructed. These vectors were co-transfected with miR-106a or anti-miR-106a in 293T cells and luciferase activity was determined. (F) DDP-resistant SKOV3/DDP cells were transfected with miR-106a or anti-miR-106a and examined for the levels of HAND2-AS1. DDP-resistant SKOV3/DDP cells were transfected with HAND2-AS1 or sh-HAND2-AS1 and examined for the levels of (G) HAND2-AS1 and (H) miR-106-5p. *P<0.05, **P<0.01 vs. miR-NC group; $^{\#\#}$P<0.01 vs. anti-NC group. miR, microRNA; DDP, cisplatin; RT-qPCR, reverse transcription-quantitative PCR; wt, wild-type; mut, mutant type; NC, negative control; sh-, short hairpin RNA.

HAND2-AS1 is a lncRNA transcribed antisense adjacent to HAND2 in chromosome 4q33-34. HAND2-AS1 is considered to be tumor-suppressive on a variety of types of cancer (40-42). In endometrioid endometrial carcinoma, lncRNA HAND2-AS1 inactivates neuromedin U to suppress tumor invasion and metastasis (42). Through interacting with TGFβ1, lncRNA HAND2-AS1 can suppress the invasion and metastasis of non-small cell lung cancer and maintain stem cell activity (43). Serving as a sponge for micRNA-1275, lncRNA HAND2-AS1 suppresses cancer cell proliferation and enhances cell apoptosis in chronic myeloid leukemia cells (44). lncRNA HAND2-AS1 overexpression inhibits the capacity of esophagus squamous cell carcinoma cells to proliferate, migrate and invade (45). According to GSE data (28,29), lncRNA HAND2-AS1 is significantly inhibited within ovarian cancer, particularly in recurrent and DDP-resistant ovarian cancer. Based on the experimental results of the present study, lncRNA HAND2-AS1 expression was markedly reduced in SKOV3/DDP cells with resistance to DDP, compared with regular SKOV3 cells, suggesting its underlying effect on ovarian cancer cell resistance to DDP. As hypothesized, lncRNA HAND2-AS1 overexpression re-sensitized SKOV3/DDP cells to DDP treatment by inhibiting the viability and the enhancement of cell-apoptosis through Bcl-2/caspase-3 apoptotic signaling.

PTEN downregulation and consequent increase in PI3K/AKT signaling activity are critical events within ovarian cancer DDP-resistance (10-12,46). Consistent with these previous studies, the expression of PTEN is markedly lower in ovarian carcinoma with resistance to DDP according to online GSE data (28) and notably reduced within SKOV3/DDP cells, compared with regular SKOV3 cells according to experimental results from the present study. lncRNA HAND2-AS1 overexpression in SKOV3/DDP cells significantly rescued PTEN proteins and suppressed PI3K/AKT activity, suggesting that PTEN could be involved in lncRNA HAND2-AS1 regulation of ovarian cancer DDP-resistance. Considering the common mechanisms of lncRNAs exerting their roles in types of cancer, namely serving as ceRNAs for miRNAs to counteract

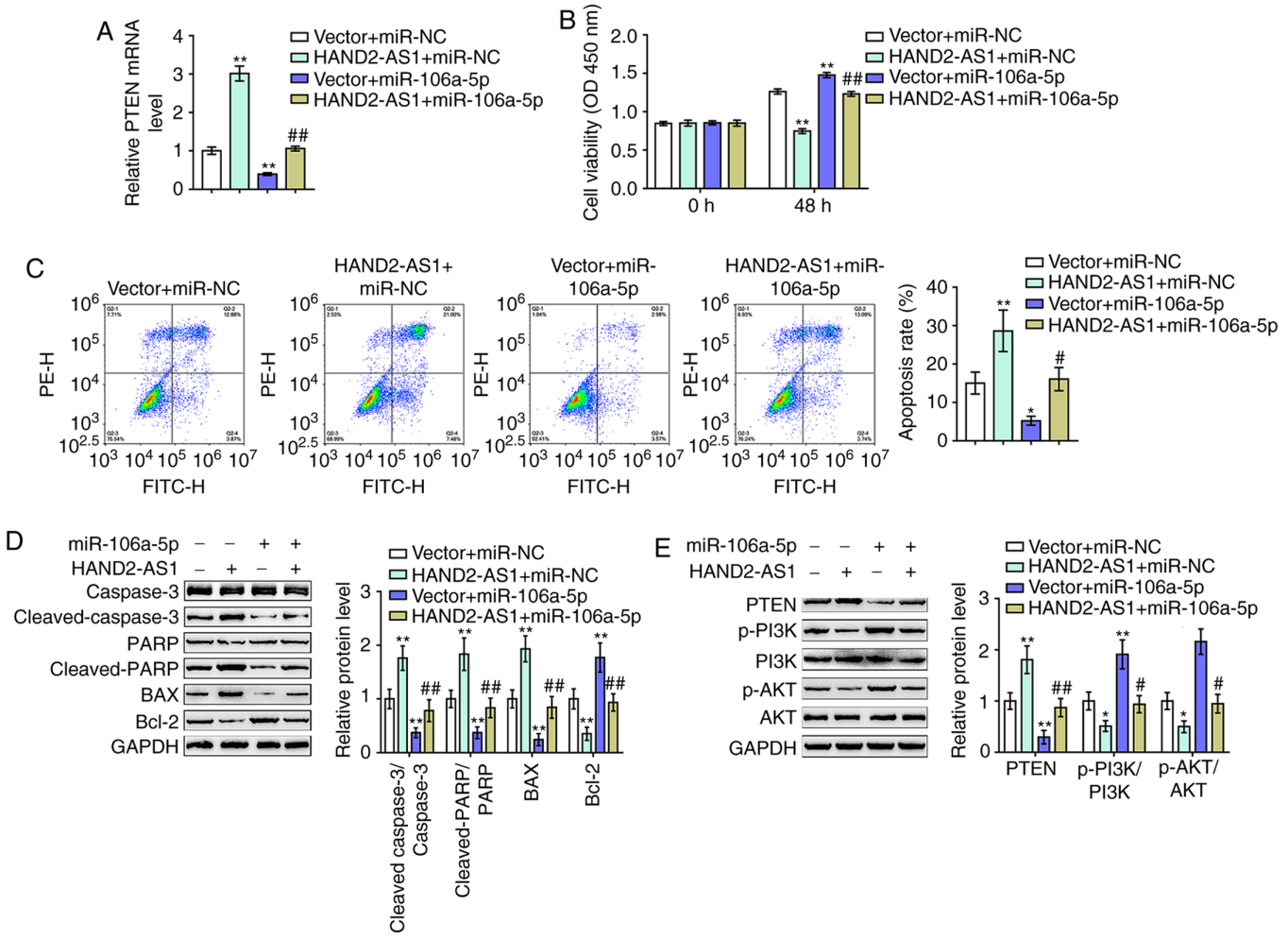

Figure 5. Dynamic effects of HAND2-AS1 and miR-106a on ovarian cancer cells. DDP-resistant SKOV3/DDP cells were co-transfected with HAND2-AS1 and miR-106a and examined for (A) mRNA expression of PTEN by reverse transcription-quantitative PCR. (B) Cell viability was assessed using an MTT assay. (C) Cell apoptosis was determined by flow cytometry. (D) The protein levels of caspase-3, cleaved-caspase-3, PARP, cleaved-PARP, Bax and Bcl-2 were measured by western blotting. (E) Protein levels of PTEN, p-PI3K, PI3K, p-AKT and AKT were determined by western blotting. *P<0.05, **P<0.01 vs. vector + miR-NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. vector + miR-106a group. miR, microRNA; DDP, cisplatin; p-, phosphorylated; PARP, poly-ADP ribose polymerase; NC, negative control.

miRNA-mediated suppression on target mRNAs (26,47,48), the involvement of miRNAs is also hypothesized.

Based on analysis of the GSE and TCGA-OV data and online tool LncTar prediction, attention was drawn to an oncogenic miRNA, miR-106a. Although there have been numerous reports that miR-106a exerts cancer-promoting effects on a variety of malignant tumors, only a few studies focus on the mechanisms of miR-106a enhancing ovarian cancer cell resistance to medication (49,50). It was reported by Li *et al* (49) that miR-106a reduces ovarian cancer cell sensitivity to DDP via binding to PDCD4. Huh *et al* (50) revealed that the increase in miR-106a is associated with the ovarian cancer cell and human tumor sample resistance to paclitaxel. Consistent with the observation by Li *et al* (49) that miR-106a expression is increased in ovarian cancer OVCAR3/CIS cells with resistance to DDP, compared with parental OVCAR3 cells, the present study also found that the expression of miR-106a is markedly upregulated in SKOV3/DDP cells with resistance to DDP, compared with regular SKOV3 cells. lncRNAs serve as ceRNAs to sponge miRNAs, thereby regulating gene expression (51). miRNAs have been shown to bind and regulate lncRNA stability and induce miRNA-mediated decay (52). In the present study, miR-106a formed a lncRNA-miRNA-mRNA network with lncRNA HAND2-AS1 and PTEN to regulate PTEN protein levels. miR-106a overexpression reduced HAND2-AS1 levels and further increased the SKOV3/DDP cells resistance to DDP upon DDP treatment; in addition, the overexpression of miR-106a markedly reversed the effects of HAND2-AS1 overexpression on SKOV3/DDP cells upon DDP treatment, indicating that miR-106a served as an oncogenic miRNA through enhancement of ovarian cancer DDP-resistance. Notably, the mechanism was shown to be similar to a previous study (49).

In conclusion, a lncRNA HAND2-AS1/miR-106a/PTEN axis re-sensitized DDP-resistant SKOV3/DDP cells to DDP treatment. Notably, one study reported that miR-106a inhibits ovarian cancer A2780 cell resistance to DDP via binding to myeloid cell leukemia-1 (53), which differs from the present findings. This might be attributed to the different targets of miR-106a. Since miRNAs target multiple downstream targets, the present findings require further *in vivo* and clinical investigations to extend their application scope.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LijL and LiL were responsible for the acquisition and analysis of data. LH and TL performed the bioinformatics and data analyses. DX and XL were responsible for collecting the clinical samples and analysis of data. LijL wrote the manuscript. LiL supervised the present study. LH and TL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Clinical sampling was performed with the approval of the Ethics Committee of Hunan Normal University (Changsha, China). Signed written informed consent was obtained from all patients enrolled.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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