WITHDRAWN: LncRNA RP3-326I13.1 Promoted Cisplatin Resistance of Lung Adenocarcinoma by Collaborating RNA Binding Protein HSP90B and Upregulating Downstream Molecule MMP13

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Abstract

Background: We previously obtained a lncRNA RP3-326I13.1, which significantly upregulated by cisplatin resistance in lung adenocarcinoma (LAD), but the biological function and molecular mechanism is unclear.

Methods: Expression levels of RP3-326I13.1 and HSP90B mRNA were estimated by qPCR from 57 pairs of LAD and NT samples without and with cisplatin. Knockdown and overexpression in A549/DDP and A549 cell lines by lentiviral- mediated techniques to observe changes in tumor behavior in A549/DDP and A549 cells, as well as tumorigenicity in experimental nude mice. The ranscriptome was sequenced to obtain downstream target molecules of RP3-326I13.1 and RNA-binding proteins were obtained using RNA pulldown.

Results: QPCR showed that the expression level of RP3-326I13.1 and HSP90B mRNA in A549/DDP cells, LAD tissues and progressive LAD tissues (cisplatin treatment was not effective) were tangibly higher than that of A549 cells, adjacent tissues, and complete remission (P=0.0037, P=0.0181; P=0.0027, P=0.009 and P=0.002, P=0.007). RP3-326I13.1 markedly enhanced the proliferation, migrate, invasion, clonal proliferation ability of LAD cell lines and speed and weight of tumorigenicity in nude mice experiment while increased the proportion of G1 phase cells (P=0.019). RNA-pull down and mass spectrometry obtained RNA binding protein HSP90B and HSP90B clearly decreased proliferation, invasive ability while increased the apoptosis of LAD cell lines after knocked down. We found matrix metalloproteinase-13 (MMP-13) was RP3-326I13.1 downstream target gene.

Conclusions: So, RP3-326I13.1 was a drug-resistant relative lncRNA promoted cisplatin resistance of lung adenocarcinoma by collaborating RNA binding protein HSP90B and upregulating downstream target molecule MMP13.

Background

Lung cancer is a highly metastatic and invasive cancer. Lung cancer ranks first in the incidence and mortality of cancer worldwide. Research shows that about 1.8 million people are diagnosed with lung cancer each year in the earth, nearly 1.6 million people die from lung cancer[1]. Non-small cell lung cancer (NSCLC) is the most common form of lung cancer, accounting for about 85 percent of all lung cancers[2], Lung adenocarcinoma (LAD) is the most common type of histology. Combination chemistry based on cisplatin occupied a key position in their comprehensive treatment plans[2, 3]. The widespread use of cisplatin could cause tumor cells to become resistant to it, resulting in a significant reduction in the effectiveness of chemotherapy [4]. Long-term use could lead to resistance to cisplatin, resulting in a relapse rate of more than 60%[5]. Once tumor cells became resistant to cisplatin, they would develop multidrug resistance to doxorubicin, vincristine and other first-line chemotherapy drugs, which was particularly harmful[6]. Therefore, cisplatin resistance would distinctly affect the efficacy of chemotherapy, cure rate and long-term survival of LAD, so it was particularly important to find specific biomarkers and

molecular targets associated with cisplatin resistance in LAD, to reverse its resistance, and to improve the prognosis of LAD patients and to avoid and overcome multidrug resistance.

In recent years, domestic and foreign studies had shown that cisplatin mainly acts by inhibiting tumor cell DNA synthesis [7] and inducing apoptosis[8]. The mechanism of resistance to cisplatin was extremely complex, involving multiple genes, and was currently thought to be achieved primarily through multiple mechanisms [7–11]. Despite of many advances in genomic and proteomic studies, the mechanism of cisplatin resistance remained elusive.

Abnormal expression of lncRNA caused a series of diseases, including the occurrence and development of tumors[12], lncRNA was mainly in the form of RNA in epigenetic regulation, transcription regulation, and post-transcriptional regulation to achieve equal regulation of gene expression at various levels[13–15]. It was known that lncRNA has many mechanisms of action. One of its important mechanisms was to influence the expression of downstream coding genes through its transcriptional process itself[16], or directly exert an enhancer-like effect on the expression of downstream protein-coding genes[17, 18].

We used high-throughput lncRNA microarray technology to compare LAD cisplatin-resistant A549/DDP cells with cisplatin-sensitive A549 cells to obtain a differential expression profile of lncRNAs for cisplatin-resistant LAD. It shown that RP3-326I13.1(also known as PINCR) was a very significantly up-regulated lncRNA by the gene chip and real-time fluorescent quantitative RT-PCR (qPCR) verification. The RP3-326I13.1 gene located at Xp11.3 and the RNA sequence length was 2228 bp. Currently, only Ritu shown that RP3-326I13.1 distinctly overexpressed in colon cancer, responding to the DNA damage of colon cancer cells through the p53/PINCR/Matrin3 axis[19]. Therefore, we speculated that RP3-326I13.1 might be a new lncRNA molecule that promotes cisplatin resistance in LAD. The study conducted a series of experimental studies and demonstrations on the effects and possible mechanisms of lncRNA RP3-326I13.1 in the LAD.

Materials And Methods

Cell culture

A549, A549/DDP cells were added to an appropriate amount of RPMI1640 medium containing 10% calf serum, gently aspirated into a single cell suspension, and the cell suspension was transferred to a cell culture flask using a pipette and placed at 37 °C and 5% CO2. A549/DDP was added to 2 μg/ml cisplatin to maintain drug resistance. Cells were observed to grow in good condition with a cell throughput density of 70–90%.

Human LAD tissue specimens

From August 2013 to August 2014, 57 samples of LAD and corresponding adjacent tissues were collected from the First Affiliated Hospital of Wenzhou Medical University, including 28 male and 29 female cases. The tissue types included 11 cases of low differentiation, 7 cases of poor differentiation, 17 cases of medium differentiation, 9 cases of high differentiation, and 13 cases of high differentiation. All were

confirmed to be LAD by histopathological examination. LAD and adjacent tissue samples were frozen in liquid nitrogen immediately after excision. This study was approved by the Institutional Ethics Review Committee of the First Affiliated Hospital of Wenzhou Medical University.

Cisplatin-treated LAD samples

Human LAD tissue specimens were collected from 2010 to 2015 at the First Affiliated Hospital of Wenzhou Medical University. These specimens were obtained by lung biopsy, surgical resection, and lymph node metastasis biopsy, and were rigorously identified by the Department of Pathology. All tissue samples had to meet the following criteria: patients with primary LAD and clinical stage IIIB-IV. The first-line chemotherapy regimen was cisplatin 25 mg/m^2 on 1-3days in combination with gemcitabine 1000 mg/m^2 or paclitaxel 80 mg/m^2 on days 1 and 8 for 21 days, with each patient receiving 3–4 cycles of treatment. Patients were divided into a "cisplatin-sensitive group" (complete remission + partial remission) and a "cisplatin-insensitive group" (progression) based on medical imaging tests such as CT and MRI, serum tumor markers, and RECIST criteria. There were 25 sensitive specimens and 32 insensitive specimens. Tissue specimens should be stored in liquid nitrogen before use. This study was approved by the Institutional Ethical Review Committee of the First Affiliated Hospital of Wenzhou Medical University.

Constructed lentivirus-mediated overexpression and shRNA vector

A549 cells were transfected with an overexpression vector (OE) targeting RP3-326I13.1 as well as a negative control (OE-NC) (Genechem, Shanghai, China). These groups were categorized into A549 OE-NC, A549 OE-NC + cisplatin, A549 OE, and A549 OE + cisplatin according to experimental requirements. a549/DDP cells were transfected with shRNA vector targeting RP3-326I13.1, siRNA HSP90B and siRNA MMP13 as well as negative control (siRNA NC) (Genechem Shanghai, China), shRNA and siRNA sequences are listed in Table 1. The best shRNA sequences were selected by three shRNA sequence experiments as subsequent experimental groups. These groups were classified as A549/DDP shRNA-NC or siRNA-NC, A549/DDP shRNA-NC or siRNA-NC + cisplatin, A549/DDP shRNA or siRNA, and A549/DDP shRNA or siRNA + cisplatin. The concentration value of cisplatin was 2ug/ml. 2×10^5 cells were added to 6-well plates at the time of transfection, and the medium was aspirated after 24 h and incubated with the transfection complex, adhering to the manufacturer's protocol and MOI values. These cells were infected with lentivirus for 72 h and treated with 2ug/ml puromycin and further tested by qPCR for overexpression or shRNA or siRNA efficiency.

Table 1
shRNA or siRNA sequences

Gene	shRNA or siRNA sequences	
RP3-326113.1	shRNA1	5’-GTTCCACTGAAATAGCGAGTAACTCGAGTTACTCGC TATTTCA GTGGAATTTTT-3’
	shRNA2	5’-GCAGGATAAACACTTACAAAGACTCGAGTCTTTGTAAGTGTTTATCCTGTTTTT-3
	shRNA3	5’-ATGGCAGG AGCAAGTGGTTAT-3’
	shRNA NC	5’-GCTCAACAGAAGCTGAGCAAATCTCGAGATTTGCTCAGCTTCTGTTGAGTTTTT-3’
HSP90B	siRNA1	5’-GAAGGAACGAGAGAAGGAATT-3’
	siRNA2	5’-GCAGAGGAAGAGAAAGGUGTT-3’
	siRNA3	5’-AGAGAAAGGUGAGAAAGAATT-3’
	SiRNA4	5’-GAGAAAGAAGAGGAAGAUATT-3’
	siRNA NC	5’-UUCUCCGAACGUGUCACGUTT-3’
MMP13	siRNA1	5’-AGAAAGACUGCAUUUCUCGGA-3’
	siRNA2	5’-AAGAAAGACUGCAUUUCUCGG-3’
	siRNA3	5’-UUUUUCAUGACAUCUAAGGUG-3’
	siRNA NC	5’-UUCUCCGAACGUGUCACGUTT-3’

Library preparation for Transcriptome sequencing and analysis

A total of 1 μg RNA per sample (including sh-RP3-326I13.1, sh-RNA NC group and OE-RP3-326I13.1, OE-NC) was used as input material for RNA. Sequencing libraries were generated using NEBNext® UltraTM. RNA library preparation kits for Illumina® (NEB, USA) were used, following the manufacturer's requirements. Suggestions and index codes were added to the attribute sequences for each sample. Briefly, mRNA was purified from total RNA using poly-T oligonucleotides. magnetic beads. Fragmentation was performed using divalent cations under elevated. Finally, PCR products purification (AMPure XP system) and to assess library quality at Agilent Bioanalyzer 2100 system. We screened the differential genes between sh-RP3-326I13.1 and sh-RNA NC group, and the differential genes between OE-RP3-326I13.1 and OE-NC, and then used the Wien method to obtain the two common differential mRNAs for follow-up PCR verification.

Quantitative PCR

Total RNA from cells and tissues was obtained using TRizol reagent (Invitrogen). Total RNA was reverse transcribed into cDNA using an RT kit (Shanghai Takara, China), following the manufacturer's instructions. Gene level expression was determined by qPCR in an ABI 7500 instrument. The primer sequences of the genes are shown in Table 2. 20 µl PCR reaction volume consisted of 6 µl double vaporized water, 10 µl SYBR Premix (2×), 1 µl PCR forward primer (10 mM), 1 µl PCR reverse primer (10 mM), and 2 µl cDNA template. qPCR reaction procedure included a denaturation step of 10 min at 95 °C. 40 cycles (5 s at 95 °C, 30 s at 60 °C) with a final extension step of 5 min at 72 °C. All samples were normalized to β-actin. Relative target gene concentrations were calculated using the triple median ($\triangle$Ct = Ct median target gene - Ct median β-actin) and $2^{-\triangle\triangle Ct}$ in expression was calculated.

Table 2
primer sequences of genes

Gene	upstream primer (5′-3′)	downstream primer (5′-3′)	PCR product length
RP3-326113.1	TCTTCTGCTGTACTTGCCCTT	CACCTCCAACATAGGGGATCG	158 bp
HSP90B	CAAACTCTATGTCCGCCGTG	AGATGTTCAGG GGCAGATCC	145 bp
MMP13	CCAGTTTGCAGAGCGCTACC	GACTGCATTTCTCGGAGCCT	111 bp
OAS3	CTTGGCCAGCTTCGAAAACC	TAAAGGAGGGCTGGCATCAC	182 bp
CP	CCAGCCTGGGCGAAAGAAA	AGATATTGGAATGTTCCGTGTCAAC	118 bp
ICAM1	GACCAGAGGTTGAACCCCAC	GCGCCGGAAAGCTGTAGAT	172 bp
PARP14	GTGTGCAGAATGCTAAGACCG	GGAGCTCTGGTCCAGCTTTT	111 bp
PLEKHA4	GCCCTCACTTAGGTCTTGGG	TGGTGTTCCTTGGACTTGGT	103 bp
CXCL8	GCTCTGTGTGAAGGTGCAGTT	AATTCTCAGCCCTCTTCAAAAACTT	244 bp
CDK18	GGTATAAGGAGCAAAGGACCCG	AAGTTCTCATTCCGCCGGTT	171 bp
β-actin	CATGTACGTTGCTATC CAGGC	CTCCTTAATGTCACGCACGAT	155 bp

Cell proliferation experiment

Prepared 100uL cell suspension in a 96-well plate and Pre-cultured the culture plate in the incubator for 24 hours (37 °C, 5% CO2), and added 10uL of the test substance of different concentrations to the culture plate. Incubated the culture plate in the incubator for an appropriate period (1–4 hours). Added 10uL of Cell Counting Kit-8 (Corning, UAS) solution to each well. Incubated the plate in the incubator for 1–4 hours. The absorbance at 450 nm was measured using a microplate reader. The experiment was carried out in tetraploid cells.

Cell migration experiments

Used a Marker pen on the back of the 6-well plate and compared with a ruler, and evenly drawn horizontal lines, approximately every 0.5-1 cm, across the holes, each hole through at least 5 lines. Added about 5×10^5 cells to the holes, which could be covered overnight. The next day, used the pipette tip to compare the ruler. Washed the cells with PBS 3 times, removed the cells, added serum-free medium. Put it in a 37 °C 5% CO2 incubator cultivate and took pictures at 0, 48 h.

Cell invasion assays

The cells were digested, resuspended in serum-free 1640 to prepare a cell suspension, and counted on a hemocytometer. The upper chamber of the transwell chamber of the 24-well plate was inoculated with 5000 cells of the above cell suspension (the number of cells depends on different cells. After 24 hours, the medium in the well plate was sucked dry, fixed with 4% paraformaldehyde for 15 minutes, and crystal violet stained for 20 minutes., washed with PBS and taken pictures under the microscope.

Clone formation experiment

After 48 hours of incubation, the cells in the 6-well plate were digested, then the cell number was adjusted (300 cells), and 50 μL was added to the 6-well plate and incubated at 37 °C for 14 days. The resulting colonies were fixed with glutaraldehyde (6.0% v/v), stained with crystalline violet (0.5% w/v), and counted by microscope; the rate of clone formation was calculated. Clone formation rate=(number of clones/numbers of inoculated cells) × 100%.

Flow cytometry to detect apoptotic rate

After a certain time of incubation, cells were harvested, suspended by adding 300 μL of culture binding solution, and labeled with 1 × Annexin. Added 50 μL of 1 × Annexin and mixed well, incubated for 15 min at room temperature and labeled with PI. The 5 μL of PI staining and 200 μL of culture buffer were added 5 min before entering the machine. The excitation light wavelength of the flow cytometer was 488 nm, used a bandpass filter with a wavelength of 515 nm for fluorescence detection and a filter with a wavelength greater than 560 nm for PI detection. CytExpert/FlowJo software was used for data processing and analysis.

Cell cycle assay

Harvested the cells in a flow analysis tube, added 3–4 ml of PBS to wash the cells, followed by centrifugation at 1000 rpm for 10 min and discard the supernatant. Add 5 ml of 70–80% ethanol, mix the cells and protect them from light overnight at 4 °C (> 18 hours). The cells were centrifuged at 1000–1500 rpm for 10 minutes and the supernatant was discarded. Cells were then washed twice to remove all ethanol and resuspended in 0.5 ml of PI/RNAase staining solution. Incubate for 15 minutes in the dark at room temperature. Stored the samples at 4 °C and avoided light before analysis. Detection was performed on a flow cytometer.

Nude mouse tumor formation experiment

The experimental materials were BALB/c mice, and each group was performed with 5 mice. The experimental grouping of the cisplatin drug injection concentration was 5 mg/kg/mouse and included (1)RP3-326I13.1 overexpression lentivirus A549 (+ cisplatin), (2) RP3-326I13.1 overexpression control lentivirus A549 (+ cisplatin),(3) RP3-326I13.1 interferes with the lentivirus A549/DDP (+ cisplatin),(4) RP3-326I13.1 interferes with the control lentivirus A549/DDP (+ cisplatin), (5) A549/DDP (+ cisplatin) as a positive control.

Experimental procedure: first prepare the groups of tumor cells to be transplanted; fitted with a tumor cell syringe, place the needle in the groin at a 45-degree oblique angle. Then insert the needle almost completely under the skin in a nearly horizontal position, inject tumor cells under the skin (tumor cell count is about 1 × 106), quickly withdraw the needle, and gently press the needle hole with the left index finger for about 1 minute, then put the mice back in the cage, taking care to keep the mice in the nest. When the tumor grew to about 5 mm, cisplatin was injected into the tail vein of the mice at an injection dose of 5 mg/kg/mouse. The relative volume size of tumors in each mouse was measured and counted at 7, 14, 21 and 28 d after the onset of tumorigenesis, respectively.

Tumors were dissected from nude mice in an ultra-clean bench, and tumors were removed with surgical scissors and photographed. The tumor tissue samples of each group were fixed in 4% paraformaldehyde and then stained with HE, and three mice from each group were taken for HE staining, and photographed under 40x, 100x, 200x and 400x microscope, respectively. Tumor tissues were obtained for subsequent qPCR of HSP90B and MMP13 mRNA levels.

RNA pull-down

Designed primers and PCR amplification to obtain DNA template. Sense primer: PF: taatacgactcactatagggattcaatctgtgaggtggatgcgtgttg, R: ataaaaataaaagttttatttggttcacag; anti-sense primer: PF: taatacgactcactatagggataaaaataaaagttttatttggttcacag, PR: attcaat ctgtgaggtggatgcgtgttg. Labeled the RP3-326I13.1 transcript with biotin, and then mixed it with total cell protein. Some proteins could form a RP3-326I13.1-RBP complex with RP3-326I13.1; the complex was obtained, and then RBP was obtained by mass spectrometry.

Statistical methods

For comparison of data between multiple groups, one-way ANOVA was used for normally distributed data and Kruskal-Wallis test was used for non-normally distributed data; t-test or LDS was used for comparison between two groups and Mann-Whitney U test was used for non-normally distributed data. Statistical analysis was performed using SPSS 23.0 software, and $P < 0.05$ was considered statistically significant.

Results

QPCR preliminary verification of RP3-326I13.1 and level expression in LAD

QPCR showed that the expression level of RP3-326I13.1was consistent with the gene chip result (change fold = 121.053) and the expression level of RP3-326I13.1 and HSP90B mRNA of A549/DDP cells were tangibly higher than that of A549 cells (t = 4.599 and 7.333, P = 0.0037 and P = 0.0181) (Fig. 1A, B). We further showed that the RP3-326I13.1 and HSP90B mRNA expression level in LAD tissues were apparently higher than that in adjacent tissues (t = 4.356 and 2.985, P = 0.0027 and P = 0.009, Fig. 1C, D). The RP3-326I13.1 and HSP90B mRNA expression level of tissues in progressive LAD patients (cisplatin treatment was not effective) were significantly higher than that in patients with complete remission (t = 3.816 and 2.676, P = 0.002 and P = 0.007, Fig. 1E, F). These results indicated that RP3-326I13.1and HSP90B might be closely related to cisplatin resistance of LAD.

Construction of RP3-326I13.1 OE, shRNA and HSP90B siRNA vectors and PCR verification

Compared with OE-RP3-326I13.1 NC group, RP3-326I13.1 level expression of OE- RP3-326I13.1 group apparently increased (P< 0.001, Fig. 2A) and hinted RP3-326I13.1 overexpression vector was successful constructed. We found that the expression level of RP3-326I13.1 in the shRNA-2 group was significantly lower than that of shRNA-1 group, shRNA-3 and the control group (P< 0.05, P< 0.05, P< 0.05, Fig. 2B), so the shRNA-2 group had the lowest relative expression level, so shRNA-2 was selected for subsequent experiments. Compared with the control group si-NC and si-HSP90B-4, the expression levels of si-HSP90B-1, si-HSP90B-2, si-HSP90B-3 decreased apparently (P< 0.001, P< 0.001 and P< 0.001). Among them, HSP90B mRNA level of si-HSP90B-3 was the lowest and was chosen for follow-up tests (Fig. 2C).

RP3-326I13.1 plainly promoted the proliferation of LAD cell lines

Compared with 0 h, 450 nm OD values were significantly increased in each group at 24 h (P< 0.001, P< 0.001, and P< 0.001) and 48 h (P< 0.001, P< 0.001, P< 0.001, and P< 0.001). The 450 nm OD values were significantly increased in the OE-RP3-326I13.1 group at 24 h (P< 0.05) and 48 h (P< 0.05) compared to the OE-control group. The OE-RP3-326I13.1 + cisplatin group still significantly promoted the proliferation of A549 cells at 24 h (P< 0.05) and 48 h (P< 0.05) in a 2ug/ml cisplatin environment. Compared with the OE-control + cisplatin group, the 450 nm OD value of OE-RP3-326I13.1 + cisplatin group also increased significantly at 24 h (P< 0.05) and 48 h (P< 0.01).Compared with 0 h, 450 nm OD value of 24 h (P< 0.001, P< 0.01, P< 0.05 and P< 0.05) and 48 h (P< 0.001, P< 0.001, P< 0.001 and P< 0.001) increased plainly. Compared with sh-RNA NC group, the sh-RP3-326I13.1 group showed a significant increase in the 24 h (P < 0.05) and 48 h (P< 0.01), the 450 nm OD values decreased significantly. Similarly, the sh-RNA NC + cisplatin group also showed a significant decrease in 450 nm OD values at 24 h (P< 0.05) and 48 h (P< 0.01) compared to the sh-RNA NC + cisplatin group in a 2ug/ml cisplatin environment, as shown in Fig. 3.

RP3-326I13.1 markedly enhanced the migrate ability of LAD cell lines

The migrate distance of the OE- RP3-326I13.1 group was markedly higher than the OE-control group (t = 41.46, P < 0.0001). The migrate distance of the OE- RP3-326I13.1 + cisplatin group was lower than the OE-RP3-326I13.1 (t = 81.28, P < 0.0001). The migration distance was still significantly increased in the OE-RP3-326I13.1 + cisplatin group compared to the OE-control + cisplatin group (t = 31.98, P < 0.0001), as shown in Fig. 4A and 4C. The migration distance of the sh-RP3-326I13.1 group decreased (t = 6.90, P = 0.0027) compared to the sh-RNA NC group. The migration distance of the sh-RP3-326I13.1 + cisplatin group lower than that of the of sh-RP3-326I13.1 (t = 17.66, P < 0.0001). Compared with the sh-RNA NC + cisplatin group, the migration distance of sh-RP3-326I13.1 + cisplatin group was still significantly increased (t = 6.538, P = 0.0028), as shown in Fig. 4B and 4D. Therefore, RP3-326I13.1 can significantly enhance the migration ability of LAD cell lines.

RP3-326I13.1 clearly promoted the invasion ability of LAD cell lines

The relative invasion rate was significantly higher in the OE-RP3-326I13.1 group than in the OE-control group (t = 48.14, P < 0.0001). The overall invasion rate was lower in the OE-RP3-326I13.1 + cisplatin group than in the OE-RP3-326I13.1 under 2ug/ml cisplatin treatment (t = 72.99, P < 0.0001). The relative invasion rate of the OE-RP3-326I13.1 + cisplatin group was still significantly increased compared with the OE-control + cisplatin group (t = 37.63, P < 0.0001), as shown in Fig. 5A,5B. Therefore, overexpression of RP3-326I13.1 could significantly enhance the invasive ability of A549. knockdown of RP3-326I13.1 could significantly inhibit the invasive ability of A549/DDP. The relative invasion rate was decreased in the sh-RP3-326I13.1 group compared with the sh-RNA NC group (t = 0.0141, P = 0.0144). The overall invasion rate was lower in the OE-RP3-326I13.1 group than in the OE-RP3-326I13.1 group under 2ug/ml cisplatin treatment (t = 38.37, P < 0.0001). The relative invasion rate of the sh-RP3-326I13.1 + cisplatin group was significantly lower than that of the sh-RNA NC + cisplatin group (t = 25.82, P = 0.002) in the presence of 2ug/ml cisplatin, as shown in Fig. 5A,5C. Therefore, RP3-326I13.1 knockdown could significantly inhibit the invasive ability of A549/DDP.

Cell cycle changes of A549 and A549/DDP cells after regulating RP3-326I13.1 expression in vitro

The proportion of G1 phase cells was increased in the OE-RP3-326I13.1 group compared to the OE-control group (P = 0.019), but there was no significant difference in G2 + S phase (P = 0.06). The proportion of G1 phase cells was significantly increased in the OE-control + cisplatin group compared to the OE-control group (P < 0.01), while the addition of 2ug/ml cisplatin showed no significant difference in the proportion of G2 + S phase cells (P > 0.05). Compared with the OE-RP3-326I13.1 group, the OE-RP3-326I13.1 + cisplatin group had no significant difference in the proportion of G1 phase cells after the addition of cisplatin (P > 0.05), while the proportion of G2 + S phase cells was significantly decreased (P < 0.01), as shown in Fig. 6A-E.

Compared with the sh-RNA NC group, the sh-RP3-326I13.1 group had an increased proportion of G1-phase cells ($P < 0.001$) and a decreased proportion of G2 + S-phase cells ($P < 0.001$). Compared with the sh-RNA NC group, the addition of 2ug/ml cisplatin significantly increased the proportion of G1 phase cells and decreased the proportion of G2 + S phase cells in the sh-RNA NC + cisplatin group ($P < 0.001$, $P < 0.001$); similarly, compared with the sh-RP3-326I13.1 group, the addition of sh-RP3-326I13.1 + cisplatin significantly increased the proportion of G1 phase cells and decreased the proportion of G2 + S phase cells in the sh-RNA NC + cisplatin group ($P < 0.001$, $P < 0.001$). The proportion of G1 phase cells increased significantly after cisplatin, while the proportion of G2 + S phase cells decreased significantly ($P < 0.001$, $P < 0.001$), as shown in Fig. 6F-J.

RP3-326I13.1 obviously enhanced the clonal proliferation ability of LAD cell lines

Overexpression of RP3-326I13.1 significantly enhanced the clonogenic proliferation of A549 cells. The rate of clone formation was significantly increased in the OE-RP3-326I13.1 group compared to the OE-control group ($P < 0.001$). In the presence of 2ug/ml cisplatin, the clone formation rate of the OE-RP3-326I13.1 + cisplatin group was also significantly higher than that of the OE-control + cisplatin group ($P < 0.001$), as shown in Fig. 7A,7B.

Knockdown of RP3-326I13.1 significantly inhibited the clone proliferation ability of A549/DDP cells. The clone formation rate of sh-RP3-326I13.1 group was significantly reduced compared to sh-RNA NC group ($P < 0.001$). In the presence of 2ug/ml cisplatin, the clone formation rate of the sh-RP3-326I13.1 + cisplatin group was also significantly lower than that of the sh-RNA NC + cisplatin group ($P < 0.01$), as shown in Fig. 7A,7C.

RP3-326I13.1 could promote tumor volume and weight in nude mice and increased sensitivity to cisplatin chemotherapy

On the 7d, 14d, 21d, and 28d after tumor formation, the relative tumor volume of each mouse was measured and counted, and the tumor growth curve was drawn based on the obtained data, as shown in Fig. 8A-8C. HE staining showed that tumor cell necrosis clearly increased in the RP3-326I13.1 knockdown group using cisplatin compared to the RP3-326I13.1 knockdown group using cisplatin. Compared with the RP3-326I13.1 overexpression group, tumor cell necrosis significantly increased in the RP3-326I13.1 knockdown using cisplatin group, but lower than in the RP3-326I13.1 knockdown using cisplatin group (Fig. 8A). Compared with the 7th day of each group, the volume of tumors on 14d, 21d, and 28d of the 5 groups gradually increased ($P < 0.001$). Compared with the OE-NC + cisplatin control group, there was no statistically significant difference in tumor growth and volume on the 7th day in the OE-RP3-326I13.1 + cisplatin group ($P > 0.05$), while the tumor grew clearly faster and the volume significantly increased on the 14d ($P < 0.01$), 21d ($P < 0.01$) and 28d ($P < 0.001$). Compared with the sh-control + cisplatin group, the sh-RP3-326I13.1 + cisplatin group had no significant difference in tumor growth and volume on the 7d ($P > 0.05$), while tumor growth and volume decreased clearly on the 14d ($P < 0.01$), 21d ($P < 0.01$), 28d ($P < 0.001$)

Further research had shown that the tumor weight of sh-RP3-326I13.1 + cisplatin group was lighter than that of sh-control + cisplatin group($P < 0.001$) and control + cisplatin group($P < 0.001$) while tumor weight of OE-RP3-326I13.1 + cisplatin group was heavier than OE-control + cisplatin group($P < 0.001$) and control + cisplatin group($P < 0.001$) (Fig. 8E). These findings hinted that RP3-326I13.1 could promote tumor volume and weight in nude mice and decreased sensitivity to cisplatin chemotherapy.

RNA-pulldown experiment and MS identification results

PCR amplified the sense and antisense strands of RP3-326I13.1 to obtain an in vitro transcribed RNA template of the A549 cell line. The transcribed RNA electrophoresis results are shown in Fig. 9A. To find the proteins that interact with RP3-326I13.1, this study attempted to directly use the transcribed RNA by RNA-pulldown technique, labeling biotin and then performing silver staining to detect proteins that bind to RP3-326I13.1. There were many enriched proteins, but no obvious difference bands were visible to the naked eye (Fig. 9B), so it was necessary to bind the mass spectra were further analyzed for differential proteins. The experimental results of mass spectrometry showed that when the confidence level of conf ≥ 95% and unique peptide ≥ 1 was set, the number of secondary spectra produced by the mass spectra of the samples of just and antisense chains were 17868 and 17712, respectively, and the number of resolved secondary spectra were 5545 and 5517, respectively. The common contaminating proteins and matching peptides were filtered out, and the total number of peptides and proteins obtained by mass spectrometry experimental identification is shown in Table 3. Partial The information related to the top ranked proteins is shown in Table 4. There are two samples in this experiment, and the proteins identified in each sample are not only different in number, but also different proteins may be present in different samples, or the same protein may be present in different samples at the same time. Figure 9C shown a Venn diagram of the differential protein sets between the samples.

Table 3
Summary table of protein identification information

Sample name	Total number of spectra	Number of identification spectra*	Number of identified peptides*	Number of identified proteins*	Unique-2**
Sense	17868	5545	3530	785	519
Antisense	17712	5517	2965	705	512

Note: * indicates that the reliability is at least 95%, ** indicates the number of identified proteins containing at least 2 unique peptides.

Table 4
Some protein related information sheet

Protein ID	Coverage (%)	Mass (Da)	Unique Peptide	Identified by
sp|Q13085|ACACA_HUMAN	46.55	265551.7	100	Sense
sp|P11498|PYC_HUMAN	53.82	129632.6	74	
sp|P08238|HS90B_HUMAN	51.52	83263.5	27	
sp|P04406|G3P_HUMAN	72.24	36053	45	
sp|P07437|TBB5_HUMAN	68.24	49670.5	5	
sp|Q13085|ACACA_HUMAN	37.68	265551.7	87	Antisense
sp|P11498|PYC_HUMAN	46.26	129632.6	48	
sp|P08238|HS90B_HUMAN	42.27	83263.5	19	
sp|P14618|KPYM_HUMAN	57.44	57936.4	28	
sp|P07437|TBB5_HUMAN	55.86	49670.5	7	

A total of 916 proteins were identified from both just and antisense chain samples, with 574 proteins identified simultaneously in two samples, and 211 and 131 unique proteins identified in the just and antisense chain samples, respectively. Combining the combined analysis of unused (score) and unique peptides in mass spectrometry identification, we finally obtained the RBP with protein ID: sp|P08238| HSP90B_HUMAN as RP3-326I13.1. Thus, we obtained HSP90B as the RBP of RP3-326I13.1.

Knock down of HSP90B decreased the proliferation ability of RP3-326I13.1

Overall cell proliferation capacity was lower with cisplatin intervention than in the corresponding group without cisplatin intervention ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$). Compared with OE-RP3-326I13.1 + si-NC-DDP, the cell proliferation capacity of OE-RP3-326I13.1 + si-HSP90B was significantly decreased in both groups with or without cisplatin intervention at 24 h and 48 h after HSP90B knockdown ($P < 0.01$, $P < 0.01$ and $P < 0.05$, $P < 0.05$, $P < 0.05$, respectively). as shown in Fig. 10.

Knock down of HSP90B decreased the invasive ability of RP3-326I13.1

The overall cell invasion capacity was significantly lower with cisplatin than in the corresponding group without cisplatin intervention ($P < 0.001$, $P < 0.001$). Compared with OE-RP3-326I13.1 + si-NC-DDP, the cell proliferation capacity of OE-RP3-326I13.1 + si-HSP90B was significantly decreased after knockdown of HSP90B in both groups with or without cisplatin treatment ($P < 0.001$, $P < 0.001$), as shown in Fig. 11.

Knock down of HSP90B increased the apoptosis of the RP3-326I13.1 overexpression group

Under the intervention of cisplatin, the overall apoptosis rate of cells was visibly higher than the corresponding group without cisplatin intervention ($P < 0.001$, $P < 0.001$). After HSP90B was knocked down, compared with the OE-RP3-326I13.1 + si-NC-DDP, in the two groups with or without cisplatin intervention, the apoptosis rate of OE-RP3-326I13.1 + si-HSP90B group increased visibly ($P < 0.001$, $P <$ 00.01) and as shown in Fig. 12.

Analysis of downstream regulatory mRNAs of RP3-326I13.1

We obtained multiple differential mRNA expression genes through transcriptome sequencing (Fig. 13A, B, C). These genes as matrix metalloproteinase-13 (MMP-13), oligoadenylate synthase 3 (OAS3), ceruloplasmin, intercellular cell adhesion molecule-1 (ICAM1), poly(ADP-ribose) polymerase family member 14 (PARP14), family A (phosphoinositide binding specific) member 4(PLEKHA4), C-X-C motif chemokine ligand 8(CXCL8), cyclin dependent kinase 18 (CDK18) might be downstream mRNAs of RP3-326I13.1. Further qPCR testing showed the expression of MMP13, CP, ICAM1, CDK18 mRNA had a reverse relationship in OE-RP3-326I13.1 and sh-RP3-326I13.1 group (Fig. 13D), which was consistent with the results of mRNAs expression profile sequencing. This verification result of qPCR indicated that these four genes were downstream mRNAs of the RP3-326I13.1.

The role of MMP13 in cisplatin in lung adenocarcinoma was further investigated considering the literatures [20–22]. Compared to 0 h, OD450nm absorbance values were significantly increased in each group at 24 h ($P < 0.001$, $P < 0.001$ and $P < 0.001$) and 48 h ($P < 0.001$, $P < 0.001$, $P < 0.001$ and $P < 0.001$). oE-MMP13 group at 24 h ($P < 0.05$) and 48 h ($P < 0.05$), The OD450nm absorbance value was significantly increased compared to OE-control group. The OE-MMP13 + cisplatin group was still able to significantly promote the proliferation of A549 cells at 24 h ($P < 0.05$) and 48 h ($P < 0.05$) in 2ug/ml cisplatin environment. The OD 450 nm absorbance values of the OE-MMP13 + cisplatin group were higher at 24 h ($P < 0.05$) and 48 h ($P < 0.01$) compared to the OE-control + cisplatin group. Compared with 0 h, OD 450 nm absorbance values increased significantly at 24 h ($P < 0.001$, $P < 0.01$, $P < 0.05$ and $P < 0.05$) and 48 h ($P <$ 0.001, $P < 0.001$, $P < 0.001$ and $P < 0.001$). Compared with sh-RNA NC group, sh-MMP13 group showed a significant increase in 24 h ($P < 0.05$) and 48 h ($P < 0.01$) and a significant decrease in OD 450 nm absorbance value. Similarly, the sh-RNA NC + cisplatin group also showed a significant decrease in the OD 450 nm absorbance value at 24 h ($P < 0.05$) and 48 h ($P < 0.01$) compared to the sh-RNA NC + cisplatin group in the 2ug/ml cisplatin environment, as shown in Fig. 14.

Further qPCR was used to detect the expression levels of HSP90B and MMP13 mRNA in the tumor-forming tissues of nude mice. It shown that the mRNA expression level of MMP13, HSP90B of sh-RP3-326I13.1 + cisplatin group was lower than that of sh-control + cisplatin group($P < 0.01$ and $P < 0.01$) and control + cisplatin group($P < 0.01$ and $P < 0.01$) while the mRNA expression level of MMP13, HSP90B of OE-RP3-326I13.1 + cisplatin group was higher than OE-control + cisplatin group ($P < 0.001$ and $P < 0.001$) and

control + cisplatin group($P < 0.001$ and $P < 0.001$) (Fig. 15). These findings hinted that MMP13, HSP90B synergized with RP3-326I13.1 to promote tumorigenesis and cisplatin resistance in a nude mouse model of LAD.

Discussion

Studies had shown that some lncRNAs may be associated with resistance to cisplatin in lung cancer [23, 24].Our results showed that the expression levels of RP3-326I13.1 and HSP90B mRNA in cisplatin-resistant A549/DDP cells were significantly higher than those in cisplatin-sensitive A549 cells. In addition, the expression levels of RP3-326I13.1 and HSP90B mRNA in LAD tissues were also significantly higher than those in adjacent tissues. the expression levels of RP3-326I13.1 and HSP90B mRNA in LAD patients were also significantly higher than those in patients in complete remission. The above results indicated that RP3-326I13.1 and HSP90B were most likely closely related to cisplatin resistance in LAD, but the exact mechanism was still unclear.

Furtherly, the CCK8 experimental results showed that RP3-326I13.1 might enhance the proliferation ability of LAD cells by reducing the sensitivity of cisplatin. It showed that RP3-326I13.1 might also affect the resistance to cisplatin by increasing the migrate and invasive ability of LAD cells by reducing the sensitivity of cisplatin. Cell cycle experiment shown that RP3-326I13.1 reduced the proportion of G1 phase cells while increased the proportion of G2 + S phase cells. The clone formation shown that RP3-326I13.1 evidently enhanced the clonal proliferation ability of LAD cells by reducing the sensitivity of cisplatin. These experiments demonstrated that RP3-326I13.1 affected the biological function of lung adenocarcinoma cell lines by reducing cisplatin sensitivity, thereby enhancing cisplatin resistance. The tumorigenesis in nude mice experiment shown that RP3-326I13.1 could enhance evidently enhanced tumorigenic speed and volume in nude mice.

There were many literatures showing that lncRNA could exert its own function by interacting with some specific proteins[25]. An RNA-pulldown screen was used to obtain proteins associated with RP3-326I13.1 and to select differentially expressed HSP90B as RBP of RP3-326I13.1 by mass spectrometry. HSP90B was a member of the HSP90 family and a marker located in the endoplasmic reticulum. the HSP90 family was closely related to human tumors and was known for its key role in regulating cell proliferation, apoptosis, migration, and invasion, as well as regulating EMT and angiogenesis [26–29]. Our findings shown HSP90B enhanced the invasion and proliferation ability while decreased the apoptosis rate of LAD cells thereby enhancing cisplatin resistance.

We further verified that MMP13, CP, ICAM1, CDK18 were consistently expressed in the RP3-326I13.1 group by both interference and overexpression, and further indicating that these four mRNA molecules were key mRNAs downstream of RP3-326I13.1. MMP13 had been reported to be involved in the mechanism of cisplatin resistance in some tumors [20–22]. Further studies shown that MMP13 could enhance the ability of lung adenocarcinoma cell lines to add value and cisplatin resistance. The tumor-forming experiments in nude mice confirmed that RP3-326I13.1 promoted cisplatin in LAD and MMP13, HSP90B synergized with RP3-326I13.1 to promote tumorigenesis and cisplatin resistance.

Conclusions

In summary, the present study demonstrated that the expression of RP3-326I13.1 and HSP90B were distinctly up-regulated in LAD cell lines and cisplatin-resistant tissues. Cytological experiments demonstrated that RP3-326I13.1 could enhance the proliferation, invasion, and migration of tumor cells and thus promote the development of LAD resistance to cisplatin. Animal experiments also confirmed the tumor-promoting effect of RP3-326I13.1 overexpression. In addition, the RNA-binding protein HSP90B was obtained by RNA pulldown and mass spectrometry, and further correlation experiments showed that RP3-326I13.1 together with HSP90B and key downstream molecules MMP13 promoted the development of cisplatin resistance in LAD. This suggested the possibility of using RP3-326I13.1 was a therapeutic target to reverse LAD cisplatin resistance in the future.

Abbreviations

NSCLC
Non-small cell lung cancer; LAD:lung adenocarcinoma; RHBDD1
qPCR
quantitative RT-PCR; FBS:Fetal bovine serum; NC:Negative
control; CCK-8
Cell Counting Kit-8. OE:overexpression vector

Declarations

Acknowledgements

Not applicable.

Authors' contributions

All authors have reviewed the data analyses. HZ, HX, JC, JW, KH and YM designed and conducted experiments, analyzed data, and wrote the manuscript. WS, XH, JC, JW, KH and YM drafted and revised the manuscript. LH, FZ, JC, JW, KH and YM designed the study, supervised the research, and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All datasets used in this study are included in the manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Institutional Ethics Review Committee of the First Affiliated Hospital of Wenzhou Medical University. All patients agreed to participate in this study.

Consent for publication

Not applicable.

Competing interests

The authors confirm that there are no conflicts of interest.

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Figures

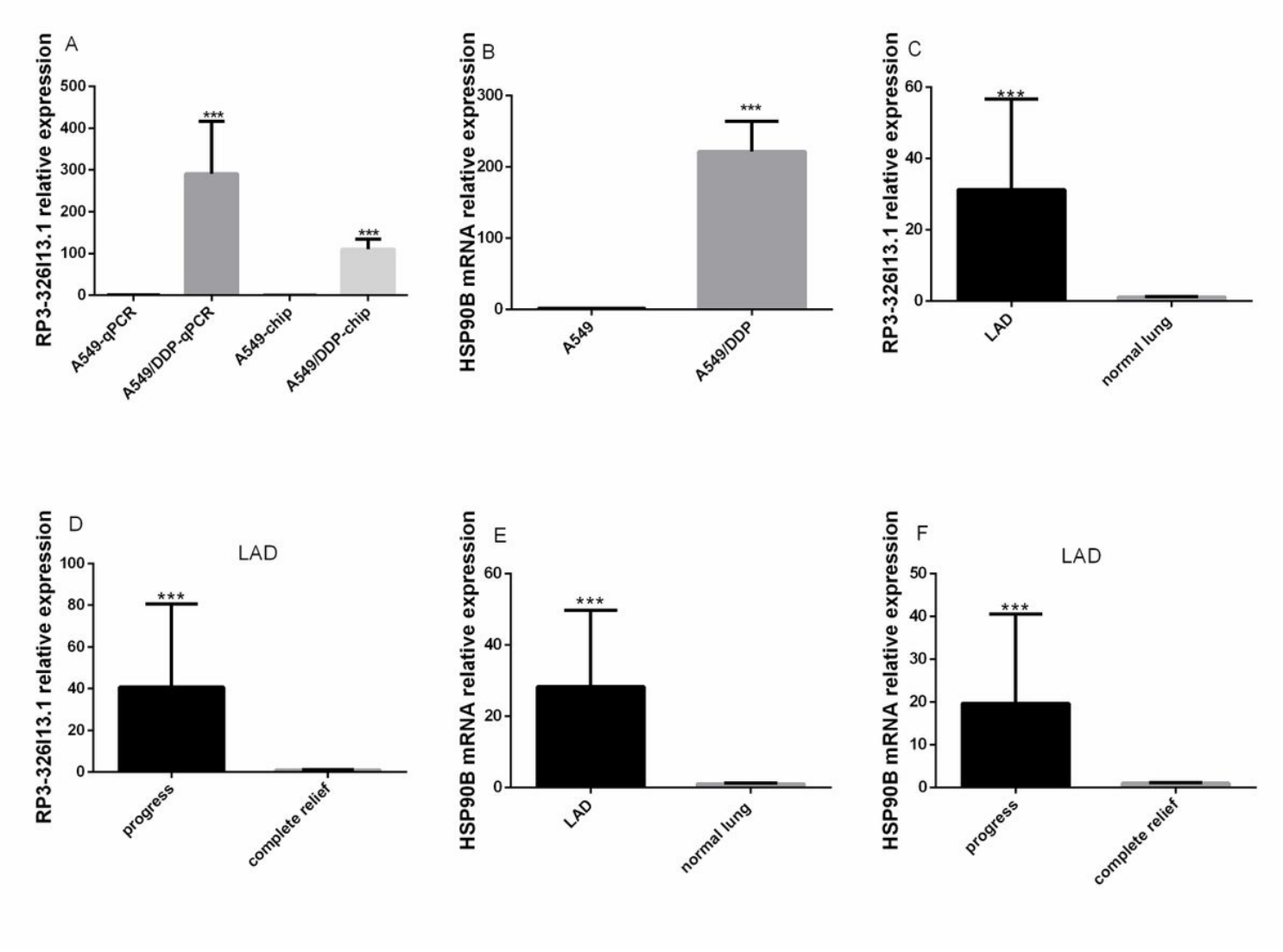

Figure 1

QPCR preliminary verification of RP3-326I13.1 and level expression in LAD. (A) QPCR showed that the PCR results were consistent with the gene chip results (change fold=121.053), and the expression level of RP3-326I13.1 was significantly higher in A549/DDP cells than A549 cells (t=4.599, P=0.0037). (B) The expression level of HSP90B mRNA was significantly higher in A549/DDP cells than in A549 cells (t=7.333, P=0.0181). (C) We further showed that the expression level of RP3-326I13.1 in LAD tissues was significantly higher than that in adjacent tissues (t=4.356, P=0.0027). (D) We further showed that the expression level of HSP90B mRNA in LAD tissues was significantly higher than that in neighboring tissues (t=2.985, P=0.009). (E) The expression level of RP3-326I13.1 in the tissues of patients with progressive LAD (cisplatin treatment-naïve) was significantly higher than that of patients in complete remission (t=3.816, P=0.002). (F) HSP90B mRNA expression levels in tissues of patients with progressive LAD (cisplatin treatment-naive) were significantly higher than those of patients in complete remission (t=2.676, P=0.007). ***P<0.001.

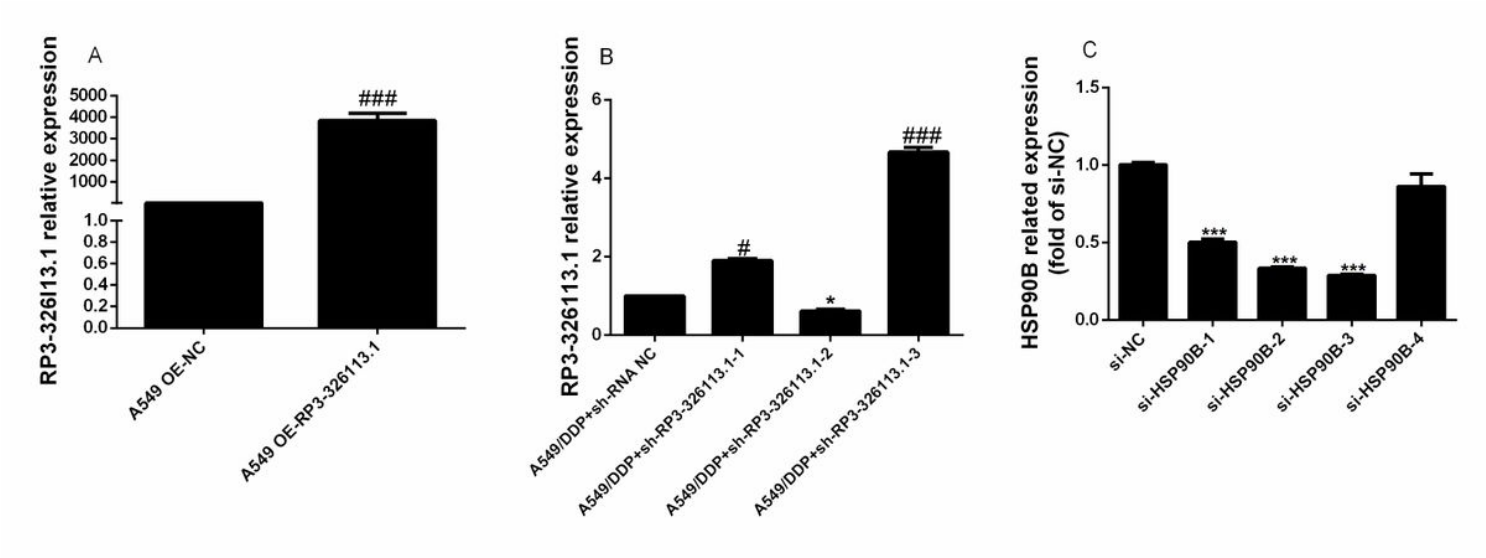

Figure 2

Construction of RP3-326I13.1 OE, shRNA and HSP90B siRNA vectors and PCR verification. (A) Compared with the OE-RP3-326I13.1 NC group, the expression of RP3-326I13.1 level was significantly increased in the OE-RP3-326I13.1 group (P<0.001). (B) We found that the expression level of RP3-326I13.1 in the shRNA-2 group was significantly lower than that in the shRNA-1, shRNA-3 and control groups (P<0.05, P<0.05 and P<0.05, respectively). (C) The expression levels of si-HSP90B-1, si-HSP90B-2, and si-HSP90B-3 were significantly decreased compared with those of control si-NC and si-HSP90B-4 (P<0.001, P<0.001, P<0.001, P<0.001). *P<0.05, ***P<0.001; P<0.001.

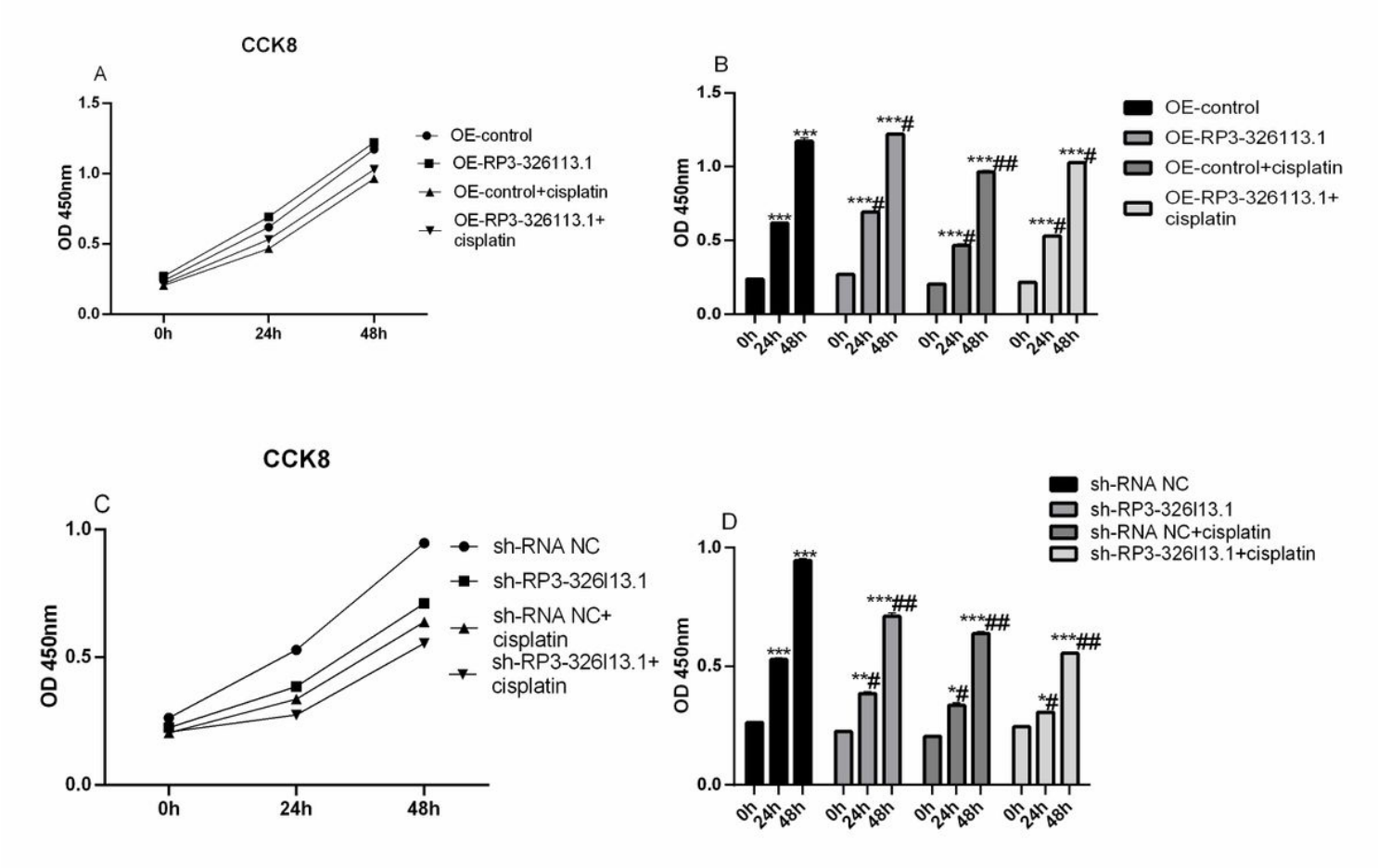

Figure 3

RP3-326I13.1 clearly promoted the proliferation of LAD cell lines (A) Compared with 0h, 450 nm OD values were significantly increased in each group at 24h (P<0.001, P<0.001, P<0.001, P<0.001) and 48h (P<0.001, P<0.001, P<0.001, P<0.001). The OE-RP3-326I13.1 group had significantly increased 450 nm OD values at 24h (P<0.05) and 48h (P<0.05) compared to the OE-control group. The OE-RP3-326I13.1 + cisplatin group could still significantly promote the proliferation of A549 cells at 24h (P<0.05) and 48h (P<0.05) in a 2ug/ml cisplatin environment. The OE-RP3-326I13.1+cisplatin group also significantly increased 450 nm OD values at 24h (P<0.05) and 48h (P<0.01) compared with the OE-control+cisplatin group. (B) Compared with 0h, 450 nm OD values were significantly increased at 24h (P<0.001, P<0.01, P<0.05, P<0.05) and 48h (P<0.001, P<0.001, P<0.001). Compared with the sh-RNA NC group, the sh-RP3-326I13.1 group had significantly decreased 450 nm OD values at 24h (P<0.05) and 48h (P<0.01). Similarly, the sh-RNA NC+cisplatin group also showed a significant decrease in 450 nm OD values at 24h (P<0.05) and 48h (P<0.01) compared to the sh-RNA NC+cisplatin group at 2ug/ml cisplatin. *P<0.05, ***P<0.001; P<0.05, P<0.001.

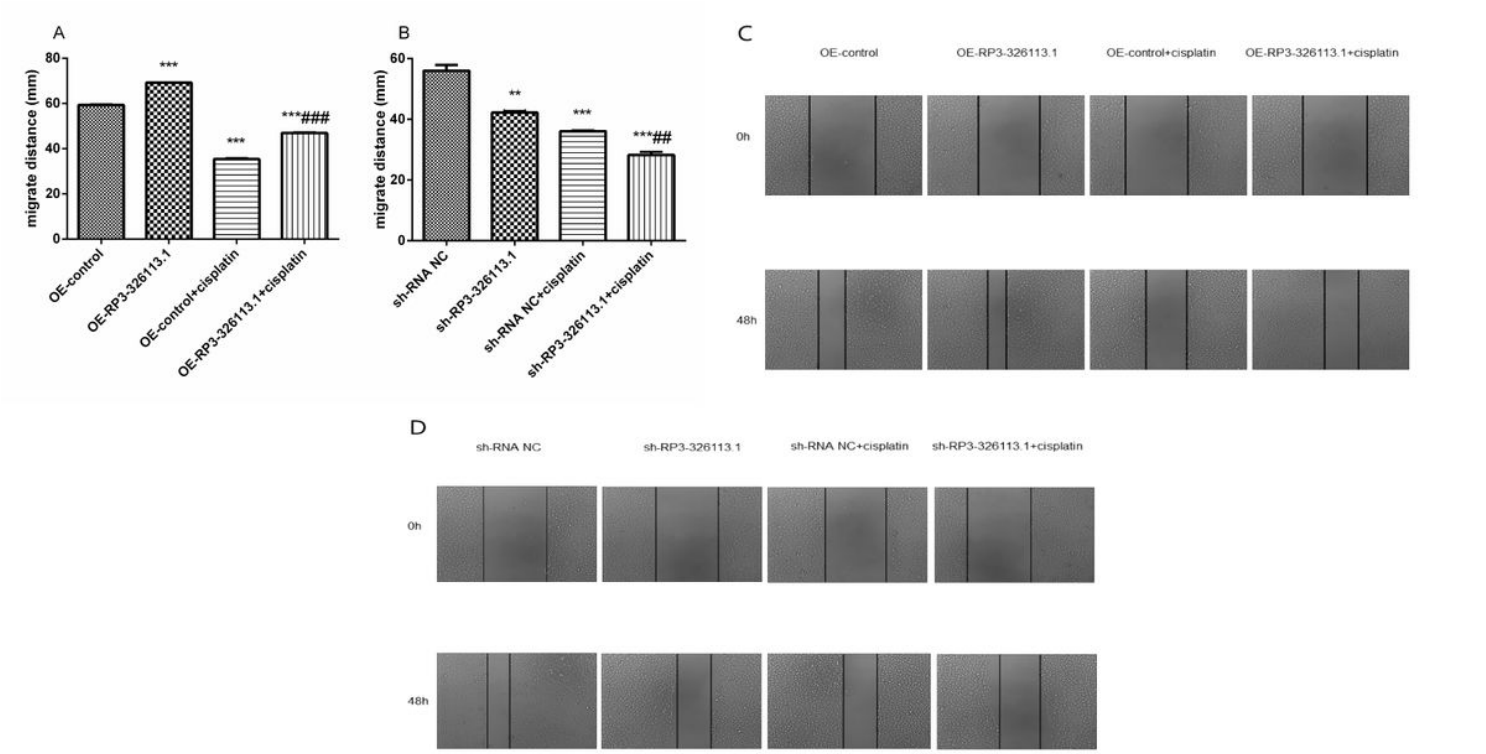

Figure 4

RP3-326I13.1 clearly enhanced the migrate ability of LAD cell lines. (A) The migration distance of the OE-RP3-326I13.1 group was obviously higher than the OE-control group (t=41.46, P<0.0001). The migration distance of the OE- RP3-326I13.1+ cisplatin group was lower than that of the OE-RP3-326I13.1 group (t=81.28, P<0.0001). (B) The migration distance of sh-RP3-326I13.1 group was decreased compared with sh-RNA NC group (t=6.90, P=0.0027). sh-RP3-326I13.1+cisplatin group had lower migration distance than sh-RP3-326I13.1 group (t=17.66, P<0.0001). sh-RP3-326I13.1+cisplatin group had lower migration distance than sh-RP3-326I13.1 group (t=17.66, P<0.0001). (C) The migration distance was still significantly increased in the OE-RP3-326I13.1+cisplatin group compared to the OE-control+cisplatin group (t=31.98, P<0.0001). (D) The migration distance was still significantly increased in the sh-RP3-326I13.1+cisplatin group compared with the sh-RNA NC+cisplatin group (t=6.538, P=0.0028). **P<0.01, ***P<0.001; P<0.01, P<0.001.

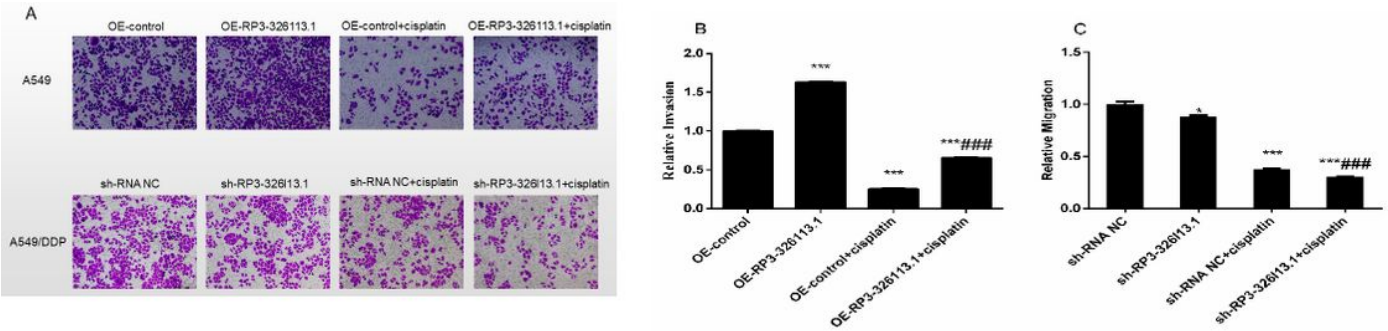

Figure 5

RP3-326I13.1 significantly enhanced the invasion ability of LAD cell lines. (A) Plots of cell invasion experiments in each group. (B) The relative invasion rate of the OE-RP3-326I13.1 group was significantly higher than that of the OE-control group (t=48.14, P<0.0001). The overall invasion rate was lower in the OE-RP3-326I13.1+cisplatin group than in the OE-RP3-326I13.1 under 2ug/ml cisplatin treatment (t=72.99, P<0.0001). (C) The relative invasion rate in the OE-RP3-326I13.1+cisplatin group was still significantly increased compared to the OE-control+cisplatin group (t=37.63, P<0.0001). Under 2ug/ml cisplatin treatment, the overall invasion rate was lower in the OE-RP3-326I13.1 group than in the OE-RP3- 326I13.1 group (t=38.37, P<0.0001). The relative invasion rate was decreased in the sh-RNA NC group compared to the sh-RP3- 326I13.1 group (t=0.0141, P=0.0144). In the presence of 2ug/ml cisplatin, the relative invasion rate of sh-RP3- 326I13.1+cisplatin group was significantly lower than that of sh-RNA NC+cisplatin group (t=25.82, P=0.002). Therefore, RP3-326I13.1 knockdown could significantly inhibit the invasive ability of A549/DDP. *P<0.05, ***P<0.001; P<0.001.

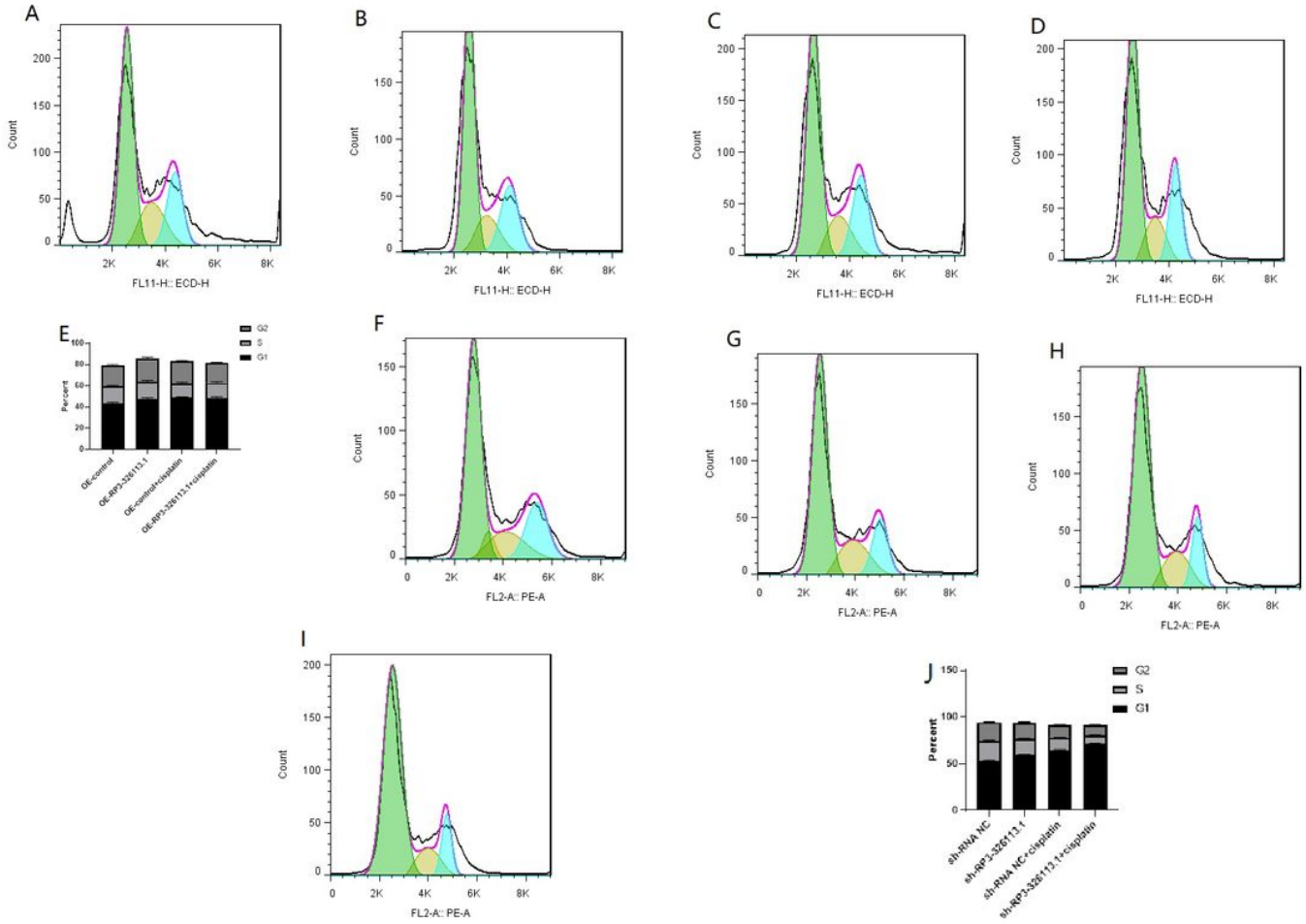

Figure 6

Cell cycle changes of A549 and A549/DDP cells after regulating RP3-326I13.1 expression in vitro (A) OE-control group. (B) OE-control + cisplatin group. (C) OE-RP3-326I13.1 group. (D) OE-RP3-326I13.1 + cisplatin group. (E) The proportion of G1 phase cells was increased in the OE-RP3-326I13.1 group compared to the OE-control group (P=0.019), but there was no significant difference in G2+S phase (P=0.06). The addition of 2ug/ml cisplatin significantly increased the proportion of G1 phase cells in the OE-control + cisplatin

group compared to the OE-control group (P<0.01), but there was no significant difference in the proportion of G2+S phase cells (P>0.05). Compared with the OE-RP3-326I13.1 group, the addition of cisplatin to the OE-RP3-326I13.1+cisplatin group resulted in no significant difference in the proportion of G1-phase cells (P>0.05), while the proportion of G2+S-phase cells was significantly reduced (P<0.01). (F) sh-RNA NC group. (G) sh-RNA NC+cisplatin group. (H) sh-RP3-326I13.1 group. (I)sh-RP3-326I13.1+cisplatin group. (J) The sh-RP3-326I13.1 group had more G1-phase cells (P<0.001) and a decreased proportion of G2+S-phase cells (P<0.001) compared to the sh-RNA NC group. Compared with the sh-RNA NC group, the addition of 2ug/ml cisplatin significantly increased the proportion of G1 phase cells and decreased the proportion of G2+S phase cells in the sh-RNA NC+cisplatin group (P<0.001, P<0. 001); similarly, compared with the sh-RP3-326I13.1 group, the sh-RP3- 326I13.1+cisplatin group with the addition of the proportion of G1-phase cells increased significantly after cisplatin, whereas the proportion of G2+S-phase cells decreased significantly (P<0.001, P<0.001). *P<0.05, **P<0.01, ***P<0.001.

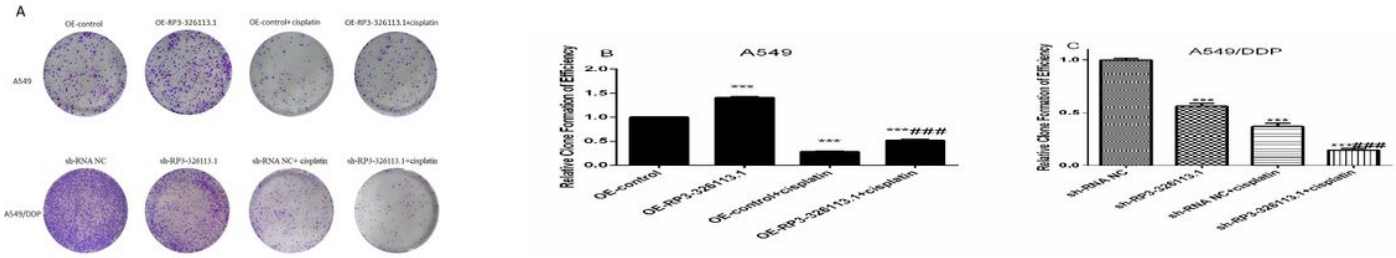

Figure 7

RP3-326I13.1 significantly enhanced the clonal proliferation ability of LAD cell lines. (A) Clonal proliferation map of the LAD cell line. (B) Overexpression of RP3-326I13.1 significantly enhanced the clonogenic proliferation of A549 cells. The clone formation rate was significantly increased in the OE-RP3-326I13.1 group compared to the OE-control group (P<0.001). In the presence of 2ug/ml cisplatin, the clone formation rate was also significantly higher in the OE-RP3-326I13.1+cisplatin group than in the OE-control+cisplatin group (P<0.001). (C) Knockdown of RP3-326I13.1 significantly inhibited the clonogenic proliferative capacity of A549/DDP cells. The rate of clone formation was significantly reduced in the sh-RNA NC group compared to the sh-RP3-326I13.1 group (P<0.001). In the presence of 2ug/ml cisplatin, the clone formation rate of sh-RP3-326I13.1+cisplatin group was also significantly lower than that of sh-RNA NC+cisplatin group (P<0.01). ***P<0.001; P<0.001.

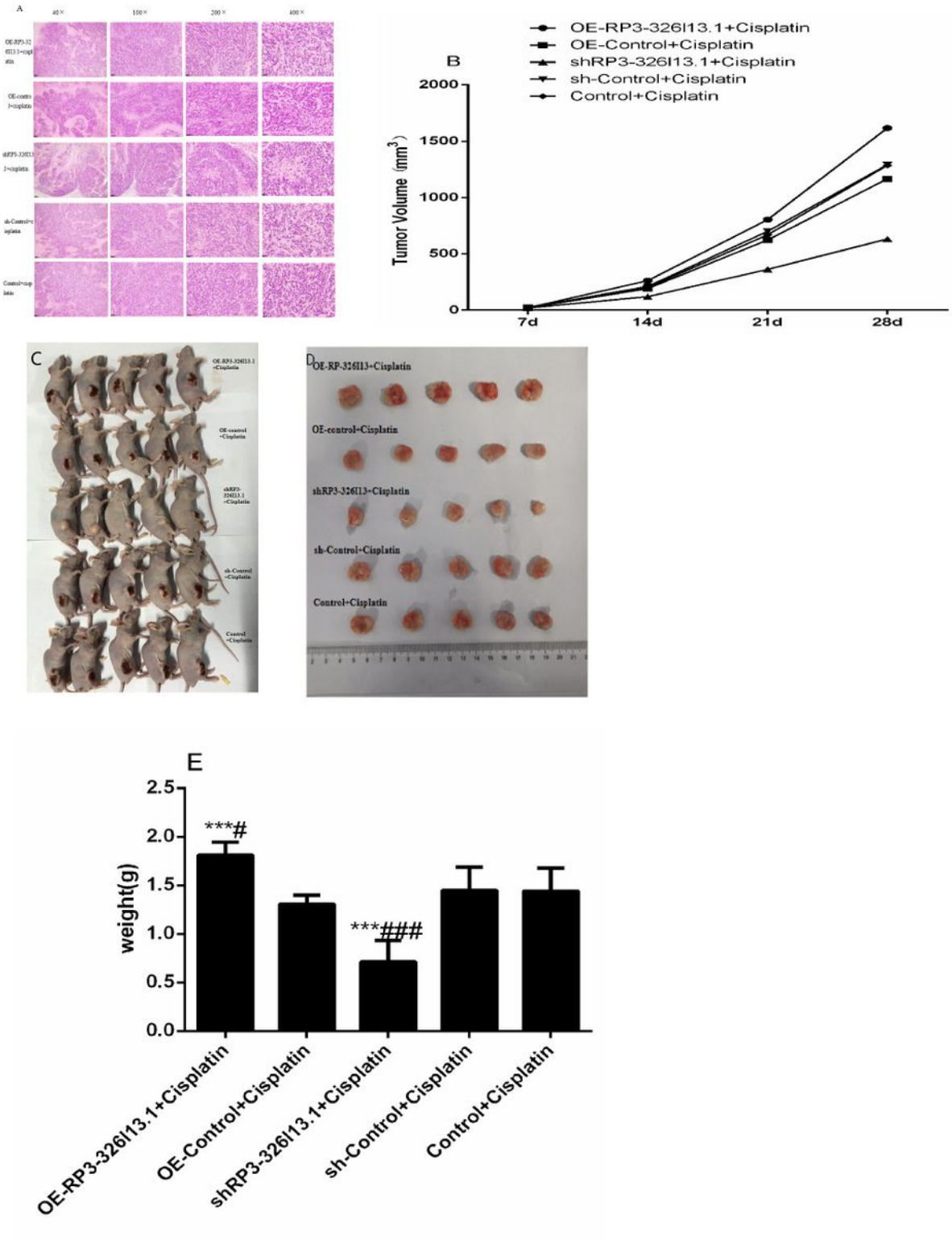

Figure 8

The relative tumor volume changes in nude mice after regulating the expression of RP3-326I13.1. (A)HE staining showed that tumor cell necrosis markedly increased in the RP3-326I13.1 knockdown group using cisplatin compared to the RP3-326I13.1 knockdown group using cisplatin. Compared with the RP3-326I13.1 overexpression group, tumor cell necrosis markedly increased in the RP3-326I13.1 knockdown using cisplatin group, but lower than in the RP3-326I13.1 knockdown using cisplatin group.(B) On the 7d,

14d, 21d, and 28d after tumor formation, the relative tumor volume of each mouse was measured and counted, and the tumor growth curve was drawn based on the obtained data. Compared with the 7th day of each group, the volume of tumors on 14d, 21d, and 28d of the 5 groups gradually increased (P<0.001). Compared with the OE-NC+cisplatin control group, there was no statistically significant difference in tumor growth and volume on the 7th day in the OE-RP3-326I13.1+cisplatin group (P>0.05), while the tumor grew markedly faster and the volume distinctly increased on the 14d (P<0.01), 21d (P<0.01) and 28d (P<0.001). Compared with the sh-control + cisplatin control group, the sh-RP3-326I13.1+ cisplatin group had no significant difference in tumor growth and volume on the 7d (P>0.05), while tumor growth and volume decreased distinctly on the 14d (P<0.01), 21d (P<0.01), 28d (P<0.001) .(C) Pictures of 5 groups of nude mice with tumor appearance.(D) Anatomy of tumor-forming nude mice in 5 groups. Data are presented as mean ± SEM. **P < 0.01, ***P < 0.001.

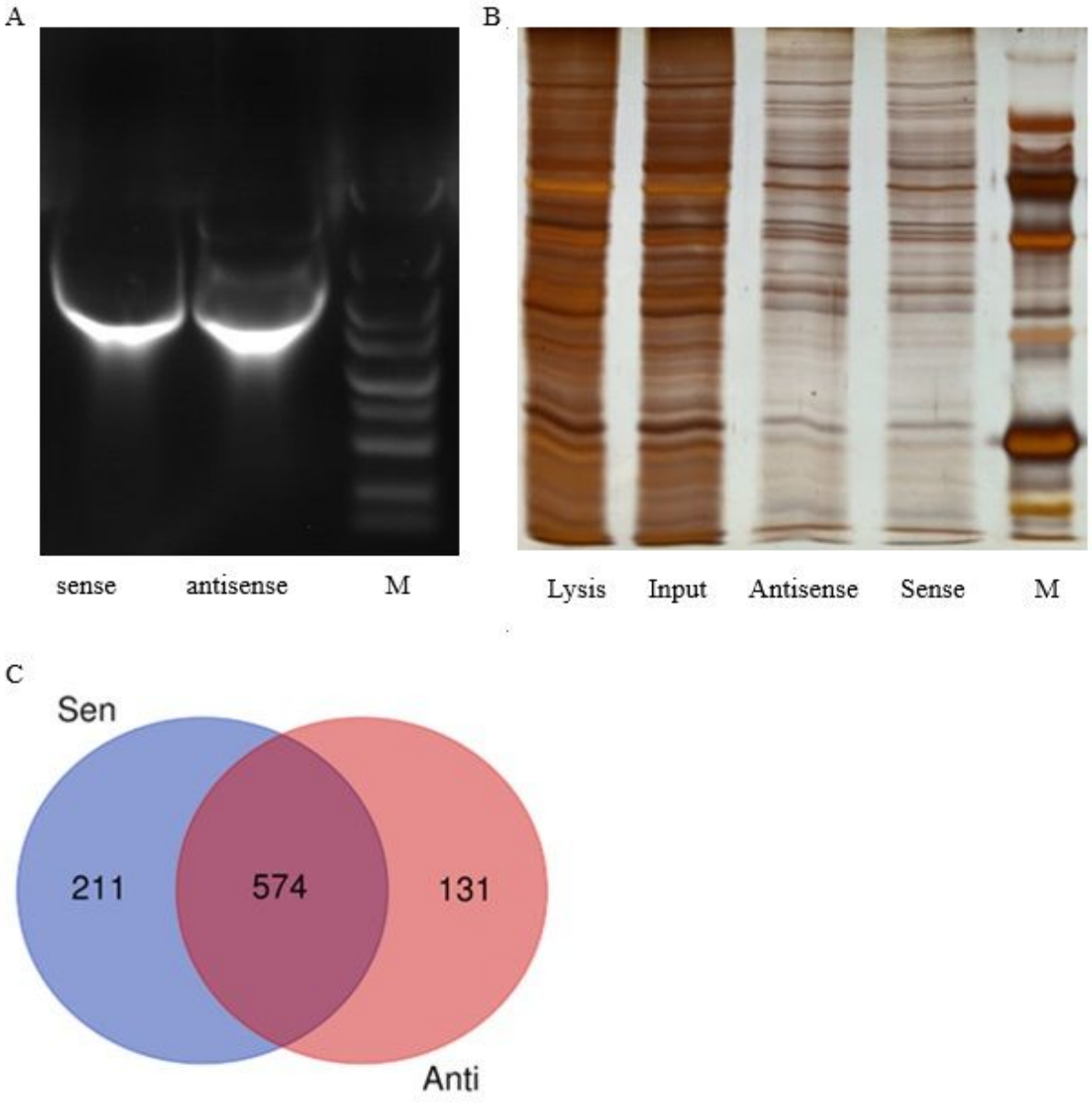

Figure 9

RNA-pulldown experiment and MS identification results (A)The transcribed RNA electrophoresis results. (B)There were many enriched proteins, but no obvious difference bands were visible to the naked eye. (C)Venn diagram of the differential protein set between the samples.

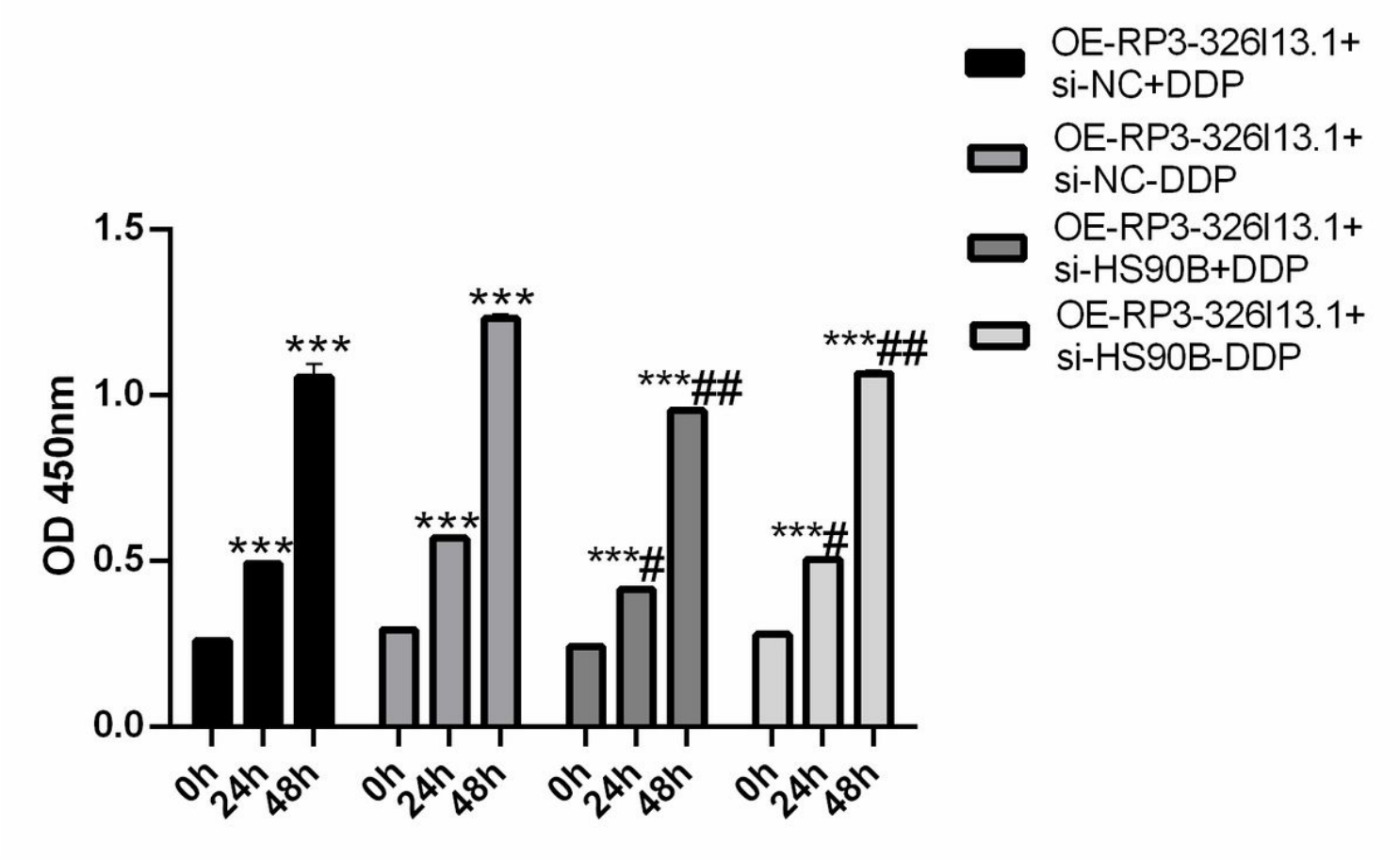

Figure 10

Knock down of HSP90B decreased the proliferation ability of RP3-326I13.1. (A) Pictures of cell invasion in different HSP90B treatment groups. (B) Overall cell proliferation capacity was lower with cisplatin intervention than in the corresponding group without cisplatin intervention ($p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$). Compared with OE-RP3-326I13.1+si-NC-DDP, the cell proliferation capacity of OE-RP3-326I13.1+si-HSP90B was significantly decreased in both groups with or without cisplatin intervention at 24h and 48h after HSP90B knockdown ($P<0.01$, $P<0.01$ and $P<0.05$, $P<0.05$). ***$P<0.001$; $P<0.05$, $P<0.01$.

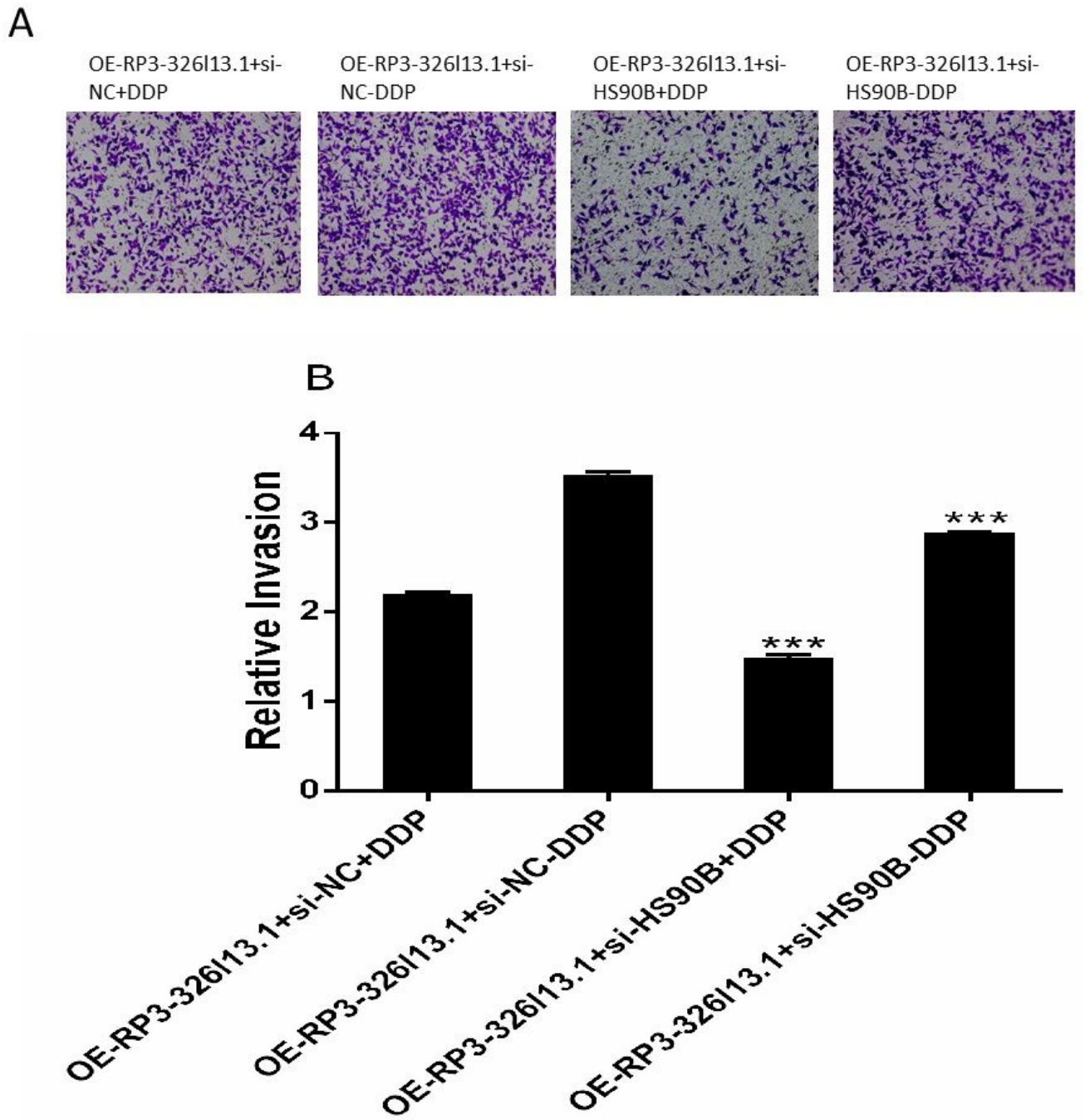

Figure 11

Knock down of HSP90B decreased the invasive ability of RP3-326I13.1. (A) Pictures of cell invasion in different HSP90B treatment groups. (B) The overall cell invasion capacity was significantly lower with cisplatin intervention than in the corresponding group without cisplatin intervention ($p<0.001$, $p<0.001$). Compared with OE-RP3-326I13.1+si-NC-DDP, the cell proliferation capacity of OE-RP3-326I13.1+si-HSP90B was significantly decreased after knockdown of HSP90B in both groups with or without cisplatin treatment ($P<0.001$, $P<0.001$). ***$P<0.001$.

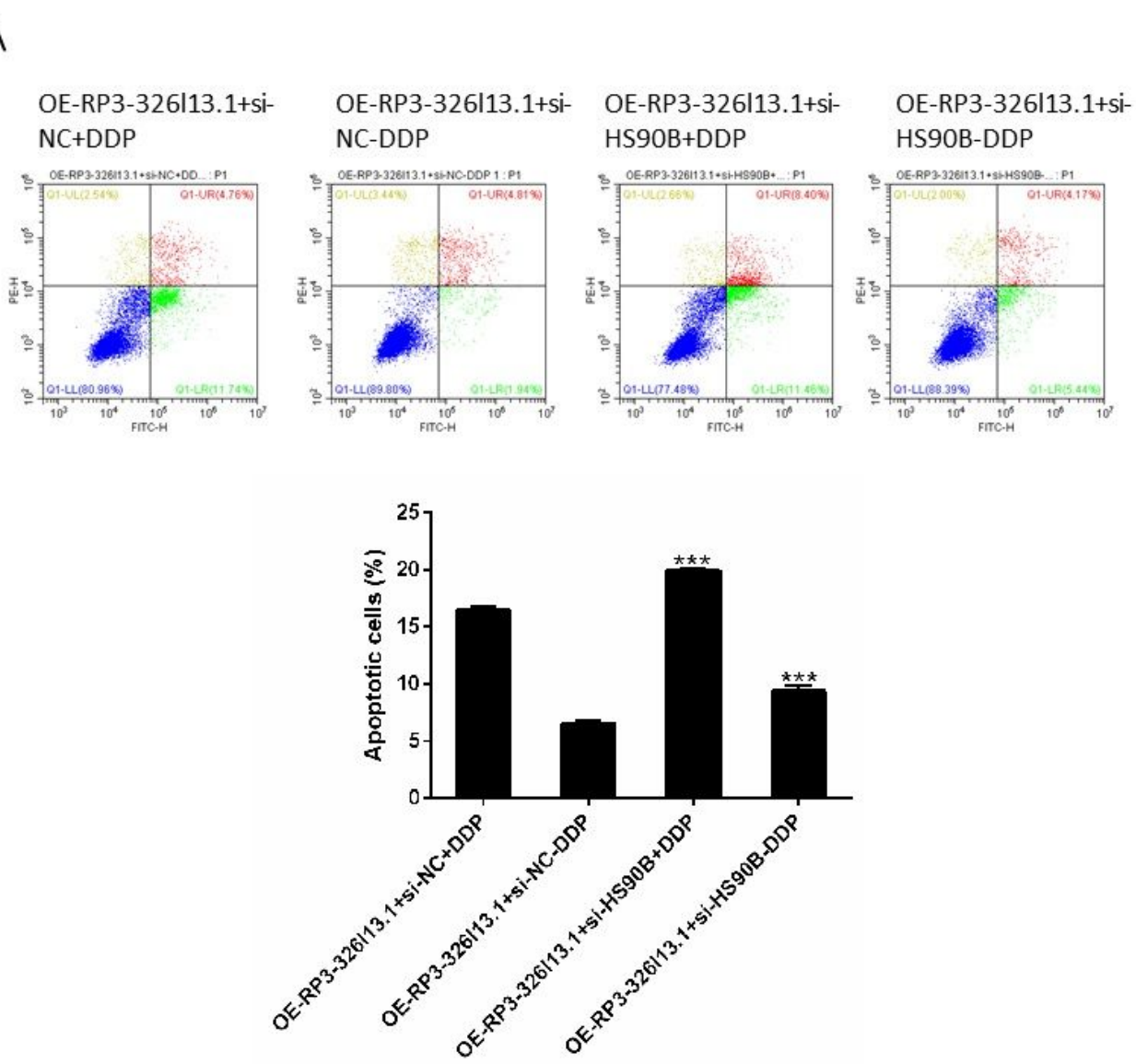

Figure 12

Knock down of HSP90B increased the apoptosis of the RP3-326I13.1 overexpression group. (A) Flow cytometric pictures of apoptosis in different HSP90B treatment groups. (B)Under the intervention of cisplatin, the overall apoptosis rate of cells was significantly higher than the corresponding group without cisplatin intervention (P<0.001, P<0.001). After HSP90B was knocked down, compared with the OE-RP3-326I13.1+si-NC-DDP, in the two groups with or without cisplatin intervention, the apoptosis rate of OE-RP3-326I13.1+si-HSP90B group increased distinctly (P<0.001, P<00.01). ***P < 0.001.

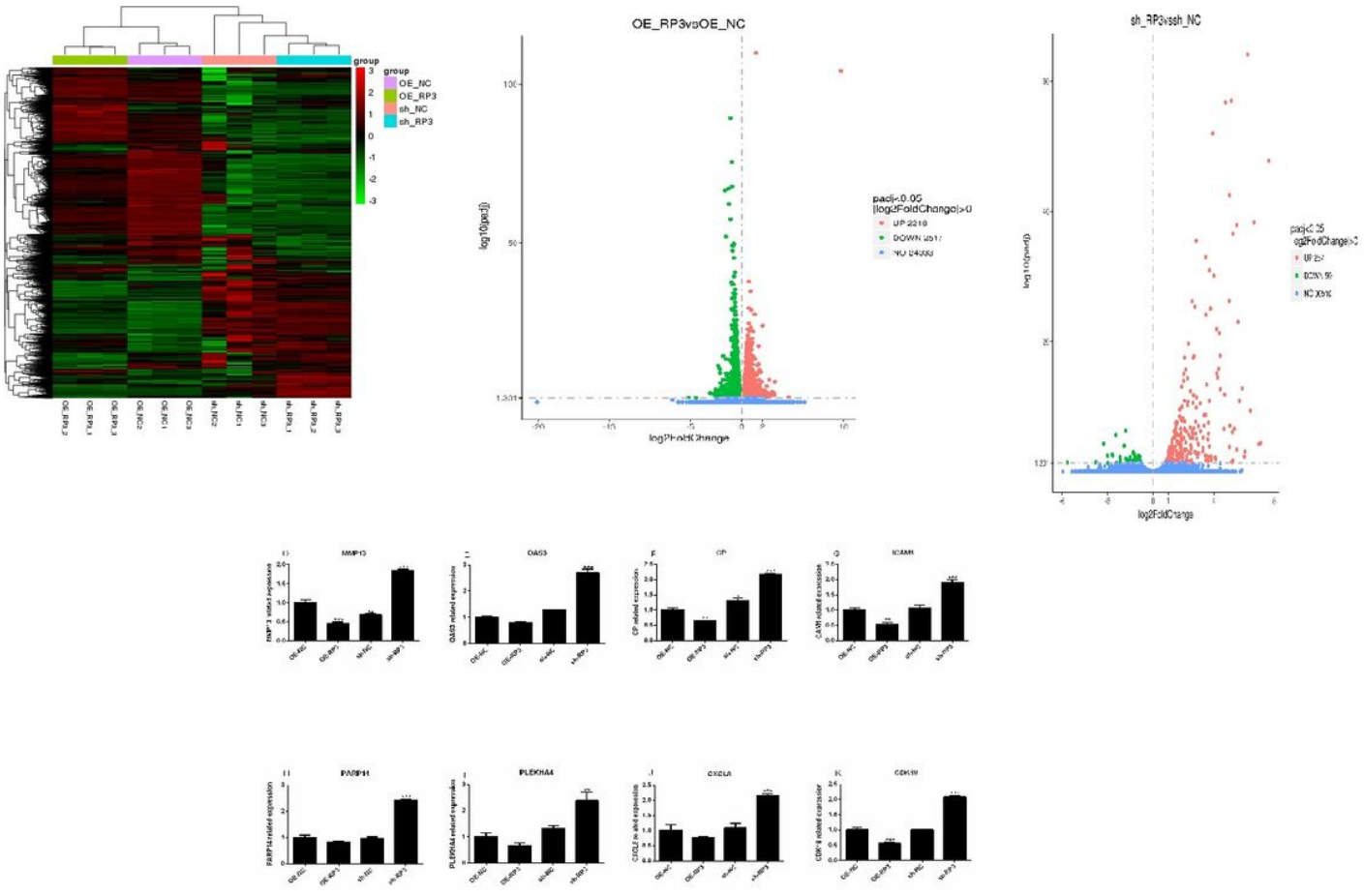

Figure 13

Analysis of downstream regulatory mRNAs of RP3-326I13.1. (A) Cladogram of mRNA sequencing. (B) Cladogram of mRNA sequencing differences between OE-RP3-326I13.1 A549 and OE-NC A549. (C) Cladogram of mRNA sequencing differences between Sh-RP3-326I13.1 A549 and Sh-NC A549. (D) qPCR assays showed that the expression of MMP13 mRNA (P<0.001, P<0.001) in the OE-RP3-326I13.1 group and sh-RP3-326I13.1 groups were inversely correlated.(E) oligoadenosine synthase 3 (OAS3) (F) qPCR assays showed that the expression of CP mRNA(P<0.01, P<0.001) in the OE-RP3-326I13.1 group and sh-RP3-326I13.1 groups were inversely correlated, which was consistent with the results of mRNAs expression profiling . (G) Further qPCR assays showed that the expression of ICAM1 (P<0.01, P<0.001) mRNA in the OE-RP3-326I13.1 group and sh-RP3-326I13.1 group were inversely correlated, which was consistent with the results of mRNAs expression profiling. (H) Poly (ADP-ribose) polymerase family member 14 (PARP14) (I) A family (phosphoinositide-binding specific) member 4 (PLEKHA4) (J) C-X-C group chemokine ligand 8 (CXCL8) (K) qPCR assays showed that the expression of CDK18 mRNA (P<0.001, P<0.001) in the OE-RP3-326I13.1 group and sh-RP3-326I13.1 group were inversely correlated. *P< 0.05, **P< 0.01, ***P <0.001.

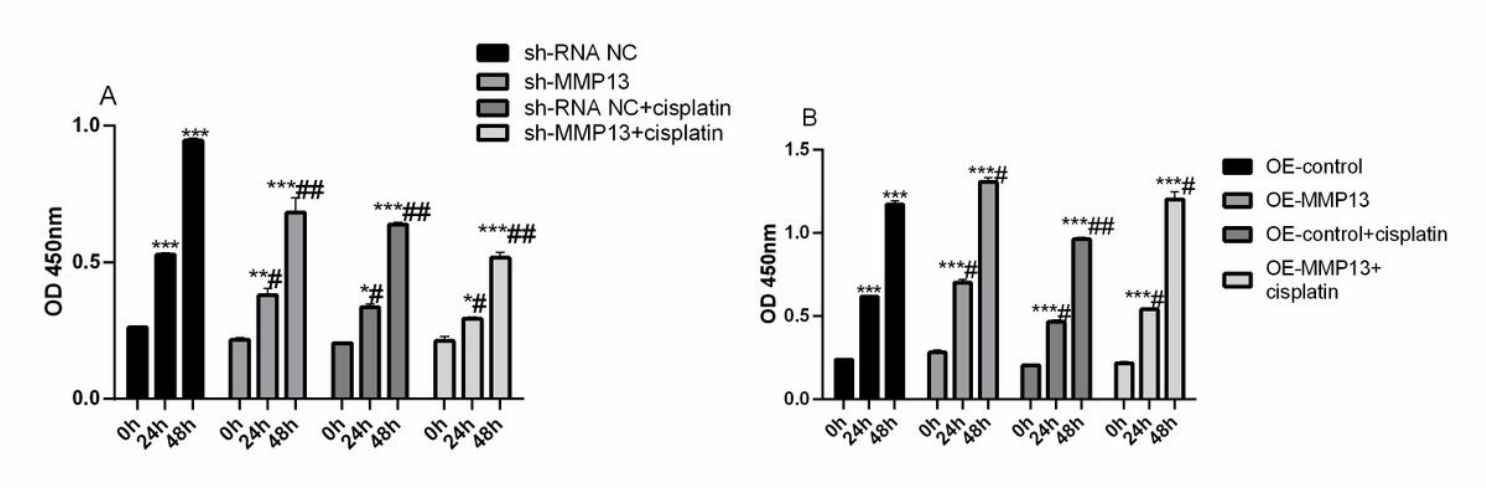

Figure 14

MMP13 enhanced the value-added capacity and cisplatin resistance of lung adenocarcinoma cell lines. (A)Compared to 0h, OD450nm absorbance values were significantly increased in each group at 24h (P<0.001, P<0.001 and P<0.001) and 48h (P<0.001, P<0.001, P<0.001 and P<0.001). oE-MMP13 group at 24h (P<0.05) and 48h (P<0.05), The OD450nm absorbance value was significantly increased compared to OE-control group. The OE-MMP13+cisplatin group was still able to significantly promote the proliferation of A549 cells at 24h (P<0.05) and 48h (P<0.05) in 2ug/ml cisplatin environment. The OD 450nm absorbance values of the OE-MMP13+cisplatin group were higher at 24h (P<0.05) and 48h (P<0.01) compared to the OE-control+cisplatin group. (B)Compared with 0h, OD 450nm absorbance values increased significantly at 24h (P<0.001, P<0.01, P<0.05 and P<0.05) and 48h (P<0.001, P<0.001, P<0.001 and P<0.001). Compared with sh-RNA NC group, sh-MMP13 group showed a significant increase in 24h (P<0.05) and 48h (P<0.01) and a significant decrease in OD 450nm absorbance value. Similarly, the sh-RNA NC+cisplatin group also showed a significant decrease in the OD 450nm absorbance value at 24h (P<0.05) and 48h (P<0.01) compared to the sh-RNA NC+cisplatin group in the 2ug/ml cisplatin environment. *P< 0.05, **P< 0.01, ***P <0.001.

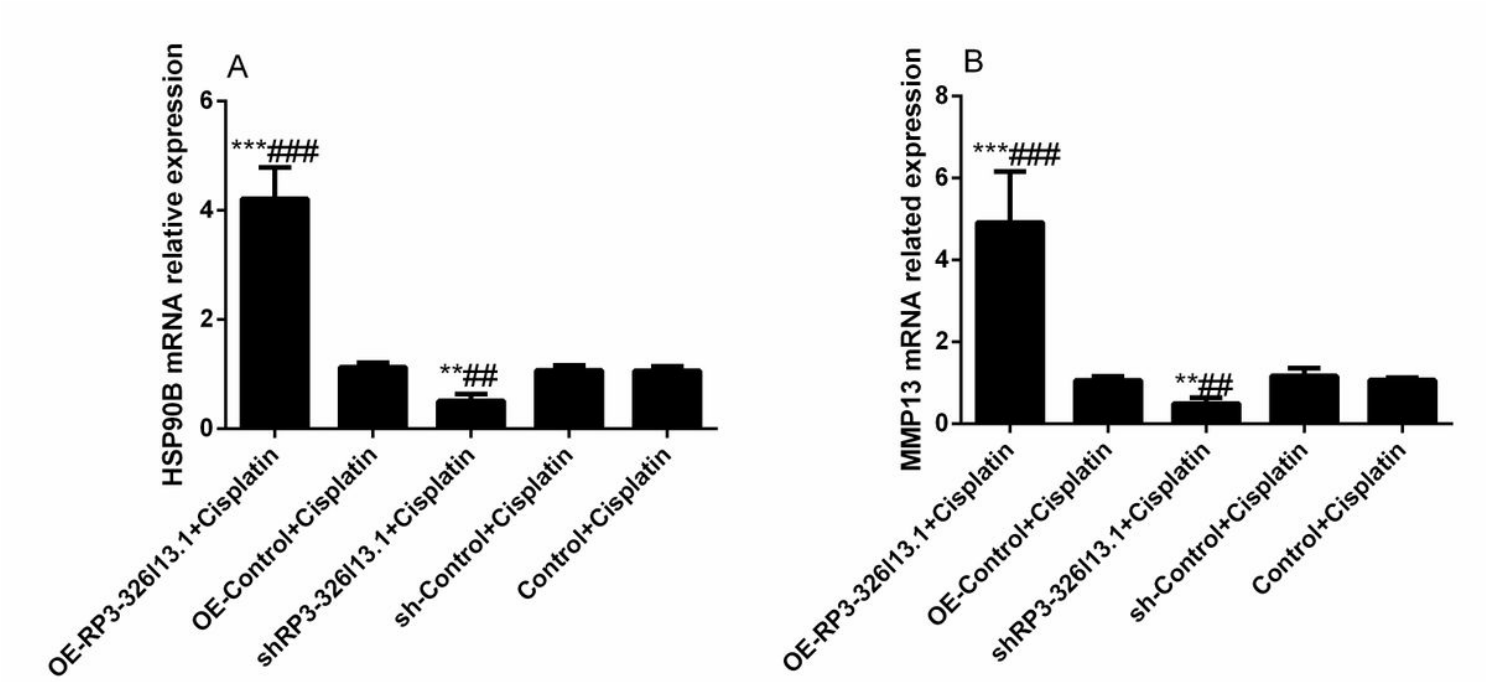

Figure 15

MMP13, HSP90B synergized with RP3-326I13.1 to promote tumorigenesis and cisplatin resistance in a nude mouse model of LAD. (A)It shown that the mRNA expression level ofHSP90B of sh-RP3-326I13.1+ cisplatin group was lower than that of sh-control + cisplatin group (P<0.01) and control+ cisplatin group (P<0.01) while the mRNA expression level of HSP90B of OE-RP3-326I13.1+ cisplatin group was higher than OE-control + cisplatin group (P<0.001) and control + cisplatin group(P<0.001).(B) It shown that the mRNA expression level of MMP13 of sh-RP3-326I13.1+ cisplatin group was lower than that of sh-control + cisplatin group (P<0.01) and control+ cisplatin group (P<0.01) while the mRNA expression level of MMP13 of OE-RP3-326I13.1+ cisplatin group was higher than OE-control + cisplatin group (P<0.001) and control + cisplatin group(P<0.001). *P< 0.05, **P< 0.01, ***P <0.001.