

# CDH13 promoter methylation regulates cisplatin resistance of non-small cell lung cancer cells

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**Abstract.** Reversing cisplatin resistance of lung cancer cell line A549/DDP through recovering cadherin 13 (CDH13) expression by demethylation was investigated in the current study. RT-PCR was used to measure CDH13 expression in lung cancer A549 and A549/DDP cells with or without 5-Aza-CdR intervention. Methylation-specific PCR was used to detect CDH13 methylation. MTT assay and flow cytometry were used to measure the effects of cisplatin on inhibiting cell proliferation, apoptosis, and the reversal of cisplatin resistance. The IC<sub>50</sub> value of cisplatin for A549 and A549/DDP cells was 3.278±0.532 and 28.341±1.435 μmol/l, respectively (P<0.05). The cisplatin-resistance index of A549/DDP cells was up to 8.65. After 2.5, 10, or 40 μmol/l 5-Aza-CdR treatment, the apoptotic rates of A549/DDP cells were 9.4±0.86, 18.1±1.42 and 42±2.01%, respectively, which were significantly different to those of the control group (P<0.05). Methylation-specific PCR detected both methylation (M) and unmethylation (U) bands at CDH13 promoter region before 5-Aza-CdR intervention while it only detected an unmethylation band after the treatment with a higher concentration of 5-Aza-CdR, which indicates the transformation to unmethylation state. When 10 μmol/l 5-Aza-CdR was added, the IC<sub>50</sub> of cisplatin to A549/DDP cells was 8.472±0.415 μmol/l, and cisplatin resistance was reversed by 3.35-fold. CDH13 methylation is related to the cisplatin resistance of A549/DDP cells. 5-Aza-CdR can inhibit CDH13 methylation and recover CDH13 expression. With the increase in 5-Aza-CdR concentration, the unmethylation state of CDH13 is enhanced, which can strengthen the

function of cisplatin inhibiting proliferation and apoptosis in A549/DDP cells.

## Introduction

Recently, several studies have shown that cadherin 13 (CDH13; T-cadherin, H-cadherin) functioned as an anti-oncogene and that its polymorphisms were associated with the development of different cancers (1-3).

CDH13 is a close phylogenetic relative of classical cadherins and shares with them the typical organization and overall sequence similarity of the extracellular domain (4). CDH13 is encoded by a single gene in each vertebrate genome, and is thought to have appeared recently in evolution as a result of duplication of a gene of a classical cadherin (4). Yet, the EC1 subdomain of CDH13 shows replacements of several amino acid residues crucial for homophilic interactions, in particular the Trp2Ile replacement. Therefore, it was suggested that CDH13 may be unable to efficiently form homodimers, which was supported by NMR studies (5). However, recent findings have identified a novel mechanism of homophilic adhesion of CDH13 (and possibly other nonclassical cadherins) via an alternative interface near the EC1-EC2 calcium-binding sites (6). Another unique feature of CDH13 is that it is devoid of a transmembrane domain and is anchored to the exterior surface of the plasma membrane via a glycosyl-phosphatidylinositol (GPI) anchor. Similar to all cadherins, CDH13 is glycosylated on several sites (7,8). Structural and functional aspects of CDH13 have been covered in considerable detail in recent reviews (9,10). The highest CDH13 expression has been reported in the nervous and cardiovascular system (in endothelial and smooth muscle cells). In this respect, it resembles type II cadherins, which are primarily expressed in the nervous system and vasculature (6), although CDH13 is closer to type I classical cadherins in terms of sequence similarity (4).

Classical cadherins are often located at and contribute to the formation of intercellular junctions. Loss of intercellular adhesion facilitates cell motility and growth. By contrast, CDH13 is not strongly associated with the areas of cell-cell contacts in confluent cell cultures, and shows globular punctate distribution (9). In migrating cells, it is located primarily at the leading edge (9). Recently, its nuclear and centrosomal location has been reported in endothelial cells (11). In HEK 293A cells, CDH13 was detected in the centrosomes but not in

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the nucleus (11). Nuclear localization of cleaved intracellular domains of some other members of the cadherin superfamily, such as protocadherins a (12), c (13) and Fat1 (14,15), as well as classical E-cadherin (16), N-cadherin (17) has been reported. Cleaved intracellular domains may act as transcription factors or modulate gene expression (18). However, CDH13 has no corresponding intracellular domain and presents in the nucleus as a full-length molecule (11); therefore, functional parallels with other cadherins may not be relevant in this case.

The *CDH13* gene is a new member of the cadherin superfamily, which was isolated recently and has been mapped to 16q24 (19). Cadherins are transmembrane glycoproteins expressed on the epithelial cell surface that mediate intercellular  $\text{Ca}^{2+}$ -dependent adhesion, which is important for maintaining normal tissue structure. Abnormalities in the *CDH13* gene have been identified in human malignancies (20,21). Moreover, an association between the abnormal expression of *CDH13* and its promoter methylation in lung cancer has been demonstrated (22-24). Recent studies have reported that *CDH13* functioned as an anti-oncogene in lung (1), breast (25), ovarian (3), bladder (26), esophageal (27) and gastric cancer (28).

*CDH13* promoter methylation plays a key role in cancer development by promoting the inactivation of tumor suppressor genes, activation of oncogenes, and increase in chromosomal instability (29). This study investigated the mechanism between *CDH13* promoter methylation and the drug resistance of lung cancer cells during chemotherapy and aimed to clarify whether *CDH13* can serve as a molecular marker for predicting the efficacy of cisplatin treatment during adjuvant chemotherapy.

## Materials and methods

**Materials.** A549, a human lung adenocarcinoma cell line (obtained from the American Type Culture Collection and preserved by the Respiratory Department of the Second People's Hospital of Guangdong); A549/DDP, a drug-resistant cell line of lung adenocarcinoma (purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China); cisplatin (Qilu Pharmaceutical Co., Ltd., Jinan, China); 5-Aza-CdR (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); the Methylcode™ Bisulfite Conversion kit and the total RNA isolation reagent TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); the reverse transcription kit (Qiagen GmbH, Hilden, Germany). Cells were divided into 7 groups to measure *CDH13* mRNA expression level. Group 1 was A549 cells with no 5-Aza-CdR treatment, and groups 2-7 were the A549/DDP cells with different concentration (0, 0.5, 1, 5, 10, and 20  $\mu\text{mol/l}$ ) of 5-Aza-CdR treated in 48 h. The study was approved by the Ethics Committee of Guangdong Second Provincial General Hospital (Guangzhou, China).

## Methods

**Measurement of *CDH13* mRNA expression level by transcription-polymerase chain reaction (RT-PCR).** According to the principles of PCR primer design, *CDH13* and *GAPDH* primers were designed. *GAPDH* served as the positive control for RT-PCR. PCR primers were produced by the Beijing Genomics

Institute (Beijing, China). *CDH13* primers were F5'-AGTGTTCCATATCAATCAATCAGCCAG-3' and R5'-CGAGACCTCATAGCGTAGCTT-3'. *GAPDH* primers were F5'-GAAAGCTGCGCGGTGACTAA-3' and R5'-GCCCAATACGACCAAATCAGAG-3'.

The PCR solution (25  $\mu\text{l}$ ) contained 12.5  $\mu\text{l}$  2X PCR Master Mix, 0.5  $\mu\text{l}$  of each primer (25  $\mu\text{mol/l}$ ), 1  $\mu\text{l}$  DNA template, and DEPC water. The PCR reaction conditions for *CDH13* and *GAPDH*: 5  $\mu\text{l}$  cDNA, 10  $\mu\text{l}$  SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (2X Conc.) (Takara Bio, Inc., Otsu, Japan), 0.5  $\mu\text{l}$  of each primer, 4  $\mu\text{l}$  dH<sub>2</sub>O, 95°C 30 sec and 40 cycles of 95°C 3 sec and 60°C 34 sec. Each of 5  $\mu\text{l}$  PCR products was separated in 0.15% agarose gel by electrophoresis for 20 min.

**Detection of *CDH13* methylation in cell lines.** The EZDNA methylation kit (Zymo Research, Orange, CA, USA) was used to perform DNA methylation. A total of 900  $\mu\text{l}$  sterile ultrapure water, 50  $\mu\text{l}$  M-dissolving buffer, and 300  $\mu\text{l}$  M-dilution buffer were added into a CT conversion reagent tube. The solution was mixed by agitation. In each PCR tube, 20  $\mu\text{l}$  DNA was added into 130  $\mu\text{l}$  CT conversion reagent. The PCR tubes were placed into the PCR instrument and kept at 98°C for 10 min and then 64°C for 2.5 h. Then, 600  $\mu\text{l}$  M-binding buffer was added into the purification column which was inserted into a liquid collection tube. The sample was added into the purification column, and the tube was centrifuged at 10,000 x g for 30 sec at 4°C. The liquid in collection tube was discarded and 100  $\mu\text{l}$  M-wash buffer was added into the purification column. The tube was centrifuged at 10,000 x g for 30 sec at 4°C and the liquid in collection tube was discarded. A total of 200  $\mu\text{l}$  M-desulphonation buffer was added into the purification column and left at room temperature for 20 min. The tube was centrifuged at 10,000 x g for 30 sec at 4°C. A total of 200  $\mu\text{l}$  M-wash buffer was added and the tube was centrifuged at 10,000 x g for 30 sec at 4°C, and the step was repeat once. The liquid in collection tube was discarded and 20  $\mu\text{l}$  M-elution buffer was added to elute the DNA. The tube was centrifuged at 10,000 x g for 30 sec at 4°C, and the eluted solution was used for PCR amplification.

**Detection of DNA methylation using methylation-specific PCR (MSP).** The primer sequences for detecting *CDH13* methylation (the length of amplified fragments was 243 bp) were: F, 5'-TCGCGGGGTTTCGTTTTTCGC-3', R, 5'-GACGTTTTTCATTCATACACGCG-3'; and primer sequences for detecting *CDH13* unmethylation (the length of amplified fragments was 242 bp) were: F, 5'-TTGTGGGGTTTGTTTTTTGT-3', R, 5'-ACTTTTCATTCATACACACA-3'.

**PCR amplification:** in 20  $\mu\text{l}$  PCR solution, the blood genomic DNA of healthy subjects was treated by the methylase *SssI* and served as the positive control, and that not treated by *SssI* served as the negative control. Sterile double-distilled water served as the blank control. The amplification conditions were 12 cycles of 94°C 30 sec, 64°C 30 sec, and 72°C 40 sec. PCR product (5  $\mu\text{l}$ ) of methylation or unmethylation DNA template was mixed with 1  $\mu\text{l}$  loading buffer and then subjected to gel electrophoresis. The gel was stained by using a nucleic acid silver staining kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's protocol.

Methylation of blood genomic DNA from healthy subjects: the reaction system (50  $\mu$ l) contained 5  $\mu$ l 10X NBE buffer, 0.25  $\mu$ l SAM (32 mM), 2 U *Sss*I methylase, 2  $\mu$ l DNA, and 40.75  $\mu$ l sterile double-distilled water. It was placed in a 37°C water bath for 3 h and then 65°C water bath for 10 min. Then, 6  $\mu$ l PCR product was mixed with 2  $\mu$ l loading buffer and loaded into a 2% agarose gel containing a nucleic acid dye. Electrophoresis was performed at 120 V for 30 min. The gel was observed under a gel imaging system (UVP).

*Detecting the reversal of drug resistance in A549/DDP cells by MTT.* Concentrations of 0, 1.25, 2.5, 5, 7.5, and 10  $\mu$ mol/l of cisplatin were used to treat A549 cells, and 0, 10, 20, 30, 40 and 50  $\mu$ mol/l cisplatin was used to treat A549/DDP cells.

Following treatment with a non-toxic dose of 5-Aza-CdR (10  $\mu$ mol/l) for 48 h, A549/DDP cells were treated with cisplatin (the same concentrations as above) for 48 h. For the control group, an equal volume of DMSO was added. Each group had 3 repeats. After 48 h, 20  $\mu$ l MTT (5 mg/ml) was added to each well. After 4 h of culture, the culture medium was centrifuged at 2,000  $\times$  g for 10 min at 4°C. The supernatant was discarded, and 150  $\mu$ l DMSO was added into each well. After MTT was completely dissolved, OD 490 nm was measured in a microplate reader and cell viability was calculated. Inhibition rate (%) was calculated as: (A492 of control cells - A492 of intervened cells)/A492 of control cells  $\times$  100%. Reversal index of drug resistance = IC<sub>50</sub> of the drug-resistant cells only treated with cisplatin/IC<sub>50</sub> of the drug-resistant cells treated with both cisplatin and 5-Aza-CdR. Drug resistance index = IC<sub>50</sub> of drug-resistant cells/IC<sub>50</sub> of parental cells.

*Morphological changes of cisplatin-treated apoptotic A549/DDP cells before and after 5-Aza-CdR intervention detected by Hoechst 33258.* The cells in the logarithmic growth phase were inoculated into 6-well cell culture plates (3 $\times$ 10<sup>5</sup>/well). The cells were then divided into four groups: i) A549/DDP group; ii) A549/DDP + 5-Aza-CdR (10  $\mu$ mol/l; 48 h) group; iii) A549/DDP + DDP (20  $\mu$ mol/l) group; iv) A549/DDP + 5-Aza-CdR (10  $\mu$ mol/l; 48 h) + DDP (20  $\mu$ mol/l) group. The cells were cultured in 2 ml medium/well at 37°C and 5% CO<sub>2</sub> for 48 h. The culture medium was discarded, and 0.5 ml 4% paraformaldehyde solution was added to fix the cells for 10 min. The cells were washed twice with PBS for 3 min each time. The cells were treated with 1 ml Hoechst 33258 staining solution (10  $\mu$ g/ml) for 5 min. The staining solution was discarded, and the cells were rinsed 3 times with PBS for 5 min each time. One drop of anti-fluorescence quenching liquid was added onto the coverslip which was then observed under a fluorescence microscope (IX70, Olympus Corporation, Tokyo, Japan).

*Measurement of cisplatin-induced A549/DDP cell apoptosis before and after 5-Aza-CdR intervention by flow cytometry.* Cells in the logarithmic growth phase were cultured at 37°C and 5% CO<sub>2</sub> for 48 h, and 1 ml cell suspension (2.5-5 $\times$ 10<sup>5</sup>/ml) was transferred into a 5 ml centrifuge tube which was centrifuged at 1,000  $\times$  g at 4°C for 5 min. The supernatant was discarded and the precipitate was rinsed twice with PBS. The precipitate was dissolved with 200  $\mu$ l binding buffer, and the solution was treated with 10  $\mu$ l FITC-labeled Annexin-V and 5  $\mu$ l PI

in the dark for 15 min. After adding 300  $\mu$ l binding buffer, the solution was measured by flow cytometry. At the same time, three control groups were conducted respectively as follows: i) unstained cells; ii) the cells stained only by AnnexinV-FITC; iii) the cells stained only by PI. The concentrations of cisplatin treatment for A549/DDP cells were 0, 2.5, 10 and 40  $\mu$ mol/l respectively, for 48 h. After 48 h of 5-Aza-CdR (10  $\mu$ mol/l) and A549/DDP, the concentration of cisplatin treatment for A549/DDP cells and the time were the same as above.

*Statistical analysis.* Data were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). The quantitative data following a normal distribution are presented by mean  $\pm$  standard deviation. Analysis of variance and post hoc test (Least Significant Difference) was used to compare more than two groups of data following a normal distribution. The t-test was used to compare the data between two groups. P<0.05 was considered to indicate a statistically significant difference. IC<sub>50</sub> was calculated using linear regression.

## Results

*CDH13 mRNA expression levels in A549 cells and A549/DDP cells.* CDH13 mRNA expression levels in A549 cells and A549/DDP cells were measured by RT-PCR. The OD value of GAPDH served as the internal reference. The relative CDH13 mRNA expression level was represented by the ratio of CDH13 OD value to GAPDH OD value. The PCR products of A549 cells and A549/DDP cells showed a CDH13 band with the size of 243 bp (Table I; Fig. 1). The CDH13 mRNA expression level in A549 cells was significantly higher than that of A549/DDP concentration. CDH13 mRNA expression level was upregulated, indicating that at a certain range, the relative expression of CDH13 mRNA increased with the increase of 5-Aza-CdR concentration. The CDH13 mRNA expression level in A549/DDP cells with 10.0  $\mu$ mol/l or 20.0  $\mu$ mol/l 5-Aza-CdR treatment showed no significant difference (P>0.05), indicating that 10.0  $\mu$ mol/l 5-Aza-CdR treatment can already induce a high expression level of CDH13 mRNA in A549/DDP cells.

*CDH13 promoter methylation in A549 and A549/DDP cells before 5-Aza-CdR intervention and that in A549/DDP cells after 5-Aza-CdR intervention.* MSP was used to measure CDH13 methylation level in A549 and A549/DDP cells. The results showed that A549 cells only had an unmethylation band (U) of CDH13 (Figs. 2 and 3). Drug-resistant A549/DDP cells had both methylation (M) and unmethylation (U) bands of CDH13, which suggests the partial methylation of CDH13. When treated with a non-toxic dose of 5-Aza-CdR (10  $\mu$ mol/l), A549/DDP cells showed an unmethylation band. The average OD values before and after 5-Aza-CdR treatment were calculated by the gel electrophoresis software QuantityOne and showed a statistically significant difference (P<0.05).

*Results of reversal of drug resistance of A549/DDP before and after 5-Aza-CdR intervention detected by MTT.* MTT results showed that the IC<sub>50</sub> value of A549 cells with 48 h cisplatin treatment was 3.278 $\pm$ 0.532  $\mu$ mol/l, while that of A549/DDP cells was 28.341 $\pm$ 1.435  $\mu$ mol/l (8.65-fold) (Fig. 4).

Table I. Relative CDH13 mRNA expression levels in A549 and A549/DDP cells treated with different concentrations of 5-Aza-CdR (mean  $\pm$  standard deviation).

No.	Relative CDH13 mRNA expression levels
1	0.8395 $\pm$ 0.0045 <sup>a</sup>
2	0.2797 $\pm$ 0.0041 <sup>b</sup>
3	0.3421 $\pm$ 0.0037 <sup>b</sup>
4	0.4735 $\pm$ 0.0016 <sup>b</sup>
5	0.6383 $\pm$ 0.0051 <sup>b</sup>
6	0.8381 $\pm$ 0.0034 <sup>b</sup>
7	0.8393 $\pm$ 0.0056

<sup>a</sup>P<0.05, comparison with the group 2. <sup>b</sup>P<0.05, six groups were compared pairwise. Group 1 was A549 cells with no 5-Aza-CdR treatment, groups 2-7 were the A549/DDP cells with different concentration (0, 0.5, 1, 5, 10, and 20  $\mu$ mol/l) of 5-Aza-CdR treated in 48 h. CDH13, cadherin 13.

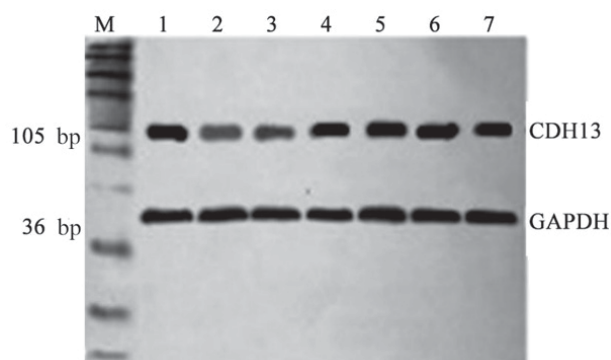


Figure 1. The RT-PCR results of A549 and A549/DDP cells. Lane 1 was the RT-PCR results of CDH13 mRNA in A549 cells; lanes 2-7 were the RT-PCR results of CDH13 mRNA in A549/DDP cells treated with 0, 0.5, 1, 5, 10, and 20  $\mu$ mol/l 5-Aza-CdR, respectively. CDH13, cadherin 13.

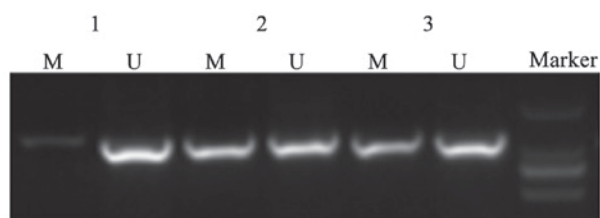


Figure 2. Electrophoresis results of MSP products. MSP, methylation-specific PCR.

It demonstrated that cisplatin could inhibit A549/DDP cell proliferation. However, A549/DDP cells showed a stronger cisplatin tolerance than that of A549 cells. After 48 h of 10  $\mu$ mol/l 5-Aza-CdR treatment, the  $IC_{50}$  value of A549/DDP cells was 8.472 $\pm$ 0.415  $\mu$ mol/l, and the reversal index of cisplatin resistance was 3.35. These results indicated that 10  $\mu$ mol/l 5-Aza-CdR treatment can increase the cisplatin sensitivity of A549/DDP cells. However, A549/DDP cells still showed a significantly lower cisplatin sensitivity than that of A549 cells (P<0.05).

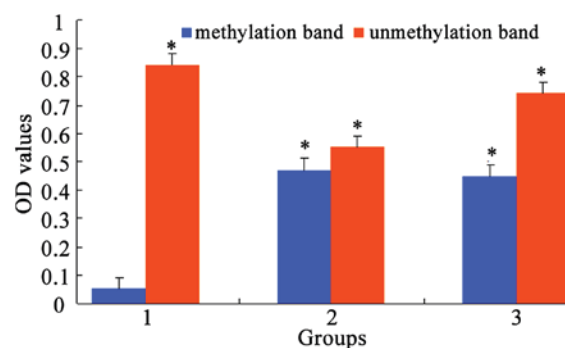


Figure 3. MSP method for detecting the average OD values of the methylation status of the *CDH13* gene. Group 1, A549/DDP cells treated with 40  $\mu$ mol/l 5-Aza-CdR; group 2, A549/DDP cells treated with 20  $\mu$ mol/l 5-Aza-CdR; group 3, A549/DDP cells. M, methylation band; U, unmethylation band. CDH13, cadherin 13; MSP, methylation-specific PCR. \*P<0.05, compared with methylation band of Group 1.

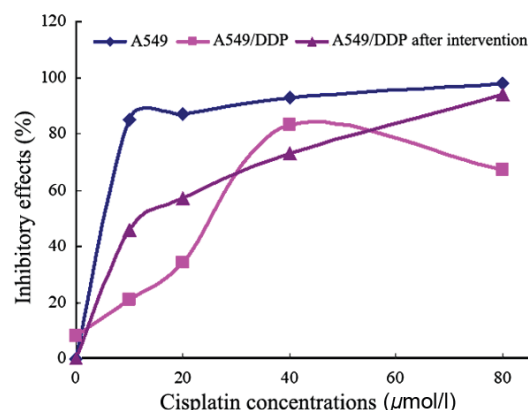


Figure 4. The inhibitory effects of different concentrations of cisplatin on A549 and A549/DDP cells.

Table II. Comparison of apoptotic ratios of cisplatin-treated A549/DDP cells before and after 5-Aza-CdR intervention.

	Apoptotic ratios/% (mean $\pm$ standard deviation)			
	0 $\mu$ mol/l	2.5 $\mu$ mol/l	10 $\mu$ mol/l	40 $\mu$ mol/l
A549/DDP cells after 5-Aza-CdR intervention	0.80 $\pm$ 0.10	9.40 $\pm$ 0.86	18.10 $\pm$ 1.42	42.00 $\pm$ 2.01

*Morphological changes of cell apoptosis detected by Hoechst 33258.* The results showed that in cell apoptosis, the uptake of Hoechst 33258 by cell membrane was increased (Fig. 5). Since the chromosomes were in a highly condensed state, their binding with Hoechst 33258 was strong, and the stained chromosomes showed a strong blue fluorescence. The chromosomes of normal cells only showed a weak fluorescence, and the dead cells were not stained. After 48 h of 10  $\mu$ mol/l 5-Aza-CdR intervention, A549/DDP cells showed normal (round) morphologies and weak blue fluorescence, and there was few apoptotic cells. After cisplatin treatment, part of the A549/DDP cells showed apoptosis. Their nuclei and

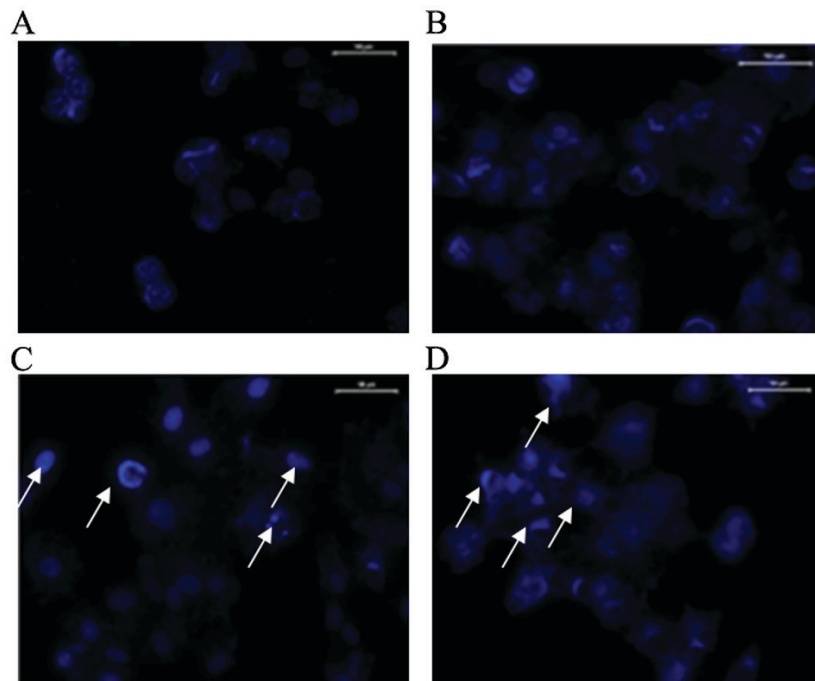


Figure 5. Cell apoptosis detected by Hoechst 33258. (A) A549/DDP cells with no treatment. (B) A549/DDP cells treated with 10  $\mu\text{mol/l}$  5-Aza-CdR. (C) A549/DDP cells treated with 20  $\mu\text{mol/l}$  cisplatin for 48 h. (D) A549/DDP cells treated with 10  $\mu\text{mol/l}$  5-Aza-CdR and then 20  $\mu\text{mol/l}$  cisplatin for 48 h.

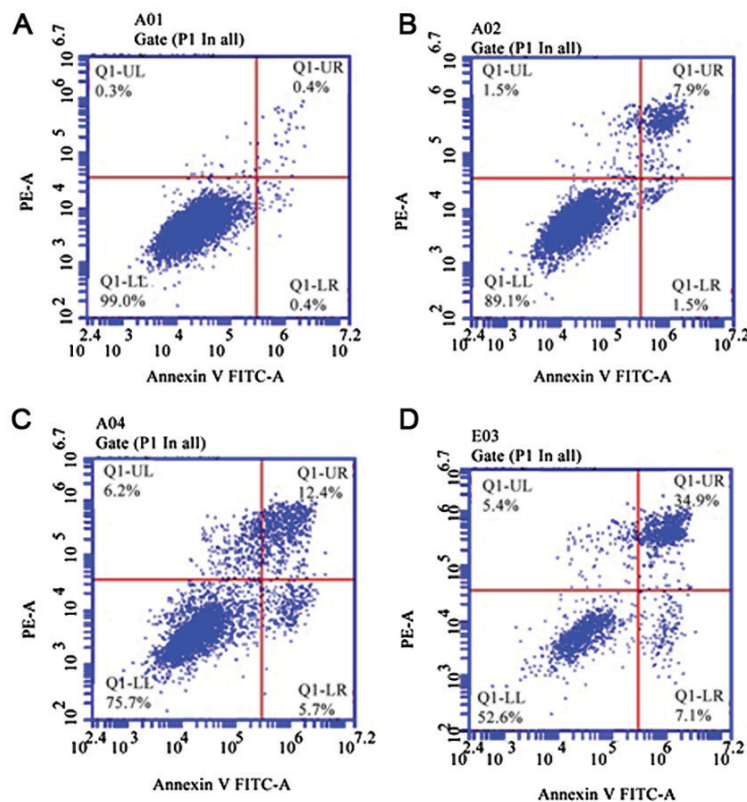


Figure 6. The effects of 5-Aza-CdR and different concentrations of cisplatin on A549/DDP cell apoptosis. A549/DDP cells treated with 0, 2.5, 10 or 40  $\mu\text{mol/l}$  cisplatin. (A) A549/DDP cells without treatment of cisplatin; (B) A549/DDP cells treated with 2.5  $\mu\text{mol/l}$  cisplatin; (C) A549/DDP cells treated with 10  $\mu\text{mol/l}$  cisplatin; (D) A549/DDP cells treated with 40  $\mu\text{mol/l}$  cisplatin.

cytoplasm had dense fluorescent signals with different sizes and irregular or plum blossom-like shape. Apoptotic bodies could be observed. After 5-Aza-CdR intervention, apoptotic A549/DDP cells were significantly increased.

*Cell apoptosis detected by flow cytometry.* Flow cytometry results showed that A549/DDP cells had a low apoptotic ratio of  $0.8 \pm 0.10\%$  after 10  $\mu\text{mol/l}$  5-Aza-CdR intervention, which indicates there is no cell apoptosis caused by 10  $\mu\text{mol/l}$

5-Aza-CdR intervention after 48 h. The apoptotic ratios of A549/DDP cells after 2.5, 10, or 40  $\mu\text{mol/l}$  cisplatin and 10  $\mu\text{mol/l}$  5-Aza-CdR treatments were  $9.40\pm 0.86$ ,  $18.10\pm 1.42$ , and  $42.00\pm 2.01\%$ , respectively (Fig. 6; Table II), which were significantly higher than that of the A549/DDP cells only treated with 5-Aza-CdR ( $P<0.05$ ).

## Discussion

General chemotherapy is one of the major means of treating non-small cell lung cancer (NSCLC) patients. Platinum drugs are a widely used class of chemotherapy drugs and have good treatment efficacy. Drug resistance in cancer cells is one of the most common causes of failure in tumor chemotherapy. CDH13 is closely related to the occurrence and development of cancers. Previous studies found that CDH13 expression is downregulated in bladder, breast, cervical, and colon cancers, which is closely related to CDH13 methylation (30-34).

DNA methylation is a covalent modification to cytosine at the CpG sites of gene promoter region and exon 1. Abnormal methylation of CpG at promoter region can lead to silencing of gene transcription, e.g., the extremely low or no expression of many important genes, such as tumor suppressor genes, cell cycle regulatory genes, apoptosis-related genes (35,36), which promotes the formation of tumor cells. However, DNA methylation is reversible, which provides a theoretical basis and new direction for reversal of tumor drug resistance. The drugs targeting this reversible process can be developed to enhance the drug sensitivity of tumor cells during chemotherapy. 5-Aza-CdR is a DNA methyltransferase inhibitor. Previous findings showed that non-toxic low doses of 5-Aza-CdR can reverse drug resistance in pancreatic cancer, neuroblastoma, ovarian cancer, and colon cancer (37). 5-Aza-CdR was first approved by the U.S. to be applied in chemotherapy of blood system tumors. It is also a first-line drug for chemotherapy of acute nonlymphocytic leukemia in patients older than 65 years. Currently, it has also been used for treating solid tumors, such as ovarian cancer (38-42).

In this study, we used the cisplatin-resistant NSCLC cell line A549/DDP and its parental cell line A549 (as the control group). We discovered that CDH13 mRNA was expressed in both cell lines, but its expression level in A549 cells was significantly higher than that of the cisplatin-resistant A549/DDP cells, which indicates that the cisplatin resistance in lung cancer cells may be related to CDH13 silencing and protein expression deficiency. MSP results showed that 5-Aza-CdR could convert the partial methylation state of CDH13 to unmethylation state in A549/DDP cells and induce CDH13 expression in cisplatin-resistant A549/DDP cells. These results suggest that 5-Aza-CdR can reverse the cisplatin resistance in lung cancer cells.

We further studied the inhibition of A549/DDP cell proliferation and A549/DDP cell apoptosis after the reversal of CDH13 methylation by 5-Aza-CdR treatment. We measured the reversal index of cisplatin resistance in A549/DDP cells after non-toxic low-dose 5-Aza-CdR treatment and analyzed the correlation between CDH13 methylation and cisplatin resistance in lung cancer cells. After 10  $\mu\text{mol/l}$  5-Aza-CdR intervention, the  $\text{IC}_{50}$  value of A549/DDP cells to cisplatin was significantly decreased, which was positively correlated

with cell apoptosis and the inhibition of cell proliferation. With the increase in 5-Aza-CdR concentration, the cisplatin sensitivity in A549/DDP cells was significantly enhanced, which indicates that the cisplatin resistance in lung cancer cells may be closely related to methylation of CDH13 promoter region. Although the cisplatin resistance in A549/DDP cells after 5-Aza-CdR intervention was reversed by 3.41-fold, it still had a significant difference with that of the parental A549 cells, which indicates the involvement of other genes in chemotherapy resistance. These drug-resistant genes may induce the drug resistance in lung cancer cells through different targets and mechanisms.

To compare the differentially expressed proteins between A549 cells and DDP (cisplatin)-resistant A549/DDP cells, A549 cells were exposed to DDP for developing the DDP-resistant A549/DDP cells. Differentially expressed proteins in the A549 and A549/DDP cells were separated and identified by proteomics approach. A part of the differentially expressed proteins were validated by RT-PCR, western blot analysis and immunocytochemistry. The functions of these proteins were further explored. There were 8 differentially expressed proteins including POTE, FH (fumarate hydratase), PDE (phosphodiesterase), AKR1C1 (aldo-keto reductase family 1 member C1), DDH2 (dihydrodiol dehydrogenase 2), S100A10, prefoldin subunit 2, and karyopherin  $\beta$ 2-Ran GppNHp nuclear transport complex between A549 and A549/DDP cells, and the difference in expression was  $>5$ -fold. These proteins were related with cellular metabolism, apoptosis, cell proliferation, detoxification and signal transduction (43-45).

CDH13, a member of the cadherin family, has been considered as a tumor suppressor, and the introduction of CDH13 in human tumor cells can reduce their invasive potential. In addition, CDH13 can induce the reversion of morphology from an invasive to a normal cell-like type. In a previous study, downregulated CDH13 protein expression was found in bladder TCC (26). However, the effects and mechanisms of downregulated CDH13 expression on bladder TCC invasion need to be further elucidated. The role of CDH13 in cancer invasion is not fully understood. In the current study, we assessed the role of CDH13 in bladder TCC invasiveness by silencing its expression using siRNA and assessed its effects on *in vitro* invasiveness, migration, and adhesion. We examined the CDH13 expression in CDH13 siRNA-transfected cells, blank control cells and negative control cells using qRT-PCR and western blotting. CDH13 expression was significantly decreased in CDH13 siRNA-treated cells compared with blank and negative controls at the mRNA and protein level (46).

DNA methylation is a DNA modification method. It regulates gene expression mainly through the methylation of cytosine in CpG sequence but does not change the DNA sequence. CpG island is a dispersed or highly aggregated sequence in DNA. Mutation or methylation of CpG island in the promoter region of tumor and suppressor gene CDH13 will affect CDH13 transcription, reduce CDH13 expression, and thus promote tumor cell proliferation and induce lung cancer development (21,47,48). When CDH13 promoter region was methylated, the ability of CDH13 to recognize DNA damage was weakened, and DNA continued to be replicated, which resulted in the formation of drug resistance. This theory was consistent with the finding in our study that CDH13

methylation was associated with cisplatin resistance in lung cancer cells. However, whether the underlying mechanisms were the same still needs further study.

Early discovery is critical for cancer treatment. During the early stage of tumorigenesis, abnormal DNA methylation occurs and can be detected, which provides a basis for early diagnosis of cancer. Since DNA methylation is a reversible gene-modification process, demethylation treatment before tumor generation can restore gene expression and achieve tumor prevention and treatment. In this study, our results showed that methylation of CDH13 promoter region in drug-resistant A549/DDP cells is an important cause of the reduction of CDH13 transcription. Inhibition of CDH13 methylation can restore CDH13 expression, reverse tumor cell biologic activity and change drug resistance phenotype in NSCLC cells. Therefore, this study provided a new target for NSCLC chemotherapy in the future.

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### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

YW, LZ and JW conceived and designed the study. YW, LZ, JY, BL and JW were responsible for the collection and analysis of the experimental data. YW and LZ interpreted the data and drafted the manuscript. JY and JW revised the manuscript critically for important intellectual content. All authors read and approved the final study.

### Ethics approval and consent to participate

The study was approved by the Ethics Committee of Guangdong Second Provincial General Hospital (Guangzhou, China).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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