Abstract. Cisplatin (CDDP) is one of the standard first-line chemotherapeutic agents for advanced non-small cell lung cancer (NSCLC). Unfortunately, prolonged exposure to CDDP results in acquired resistance which prevents the successful treatment of lung cancer patients. Thus, it is necessary to explore the mechanism underlying the resistance of NSCLC to CDDP. In the present study, a CDDP-resistant human lung cancer cell line A549/CDDP was established from the parental cell line A549. The results demonstrated that A549/CDDP cells acquired an epithelial-mesenchymal transition (EMT) phenotype, with morphological changes including acquisition of a spindle-like fibroblastic phenotype, downregulation of E-cadherin, upregulation of mesenchymal markers (vimentin, Snail and Slug), and increased capability of invasion and migration. Compared with A549 cells, the A549/CDDP cells showed decreased connexin43 (Cx43) expression. Overexpression of Cx43 reversed EMT and CDDP resistance in the A549/CDDP cells. Conversely, knockdown of Cx43 expression by siRNA initiated EMT and induced CDDP insensitivity in A549 cells. In summary, Cx43 reverses CDDP resistance in A549 CDDP-resistant cells by preventing EMT, making Cx43 a possible therapeutic target for lung cancer.

Introduction

Lung cancer is the leading cause of death from cancer in China. Non-small cell lung cancer (NSCLC) constitutes ~85% of all lung cancers (1), with adenocarcinoma comprising the major histological type. Platinum-based chemotherapeutic agents are the standard first-line chemotherapeutic agents for advanced NSCLC (2). Cisplatin (CDDP), for example, is widely used for the treatment of NSCLC. However, acquired resistance develops during treatment, leading to tumor recurrence and further progression. Therefore, understand the underlying mechanism of drug resistance to CDDP and increasing the sensitivity to therapeutic drugs are key steps towards the improved treatment of lung cancer patients.

Studies suggest a direct molecular and phenotypic association between resistance to chemotherapy and acquisition of epithelial-mesenchymal transition (EMT) characteristics in cancer cells (3-6). EMT is a cellular process during which epithelial polarized cells become motile mesenchymal-appearing cells (7-9) and gain increased cell motility and invasiveness (10,11). EMT involves loss of epithelial cell-cell junctions and expression of epithelial markers such as E-cadherin, and gain in the expression of mesenchymal markers such as vimentin, Snail and Slug.

Connexins are a group of homologous proteins that form the inter-membrane channels of gap junctions (12). An abnormal expression level and distribution of connexins are closely related to tumor formation (13,14). Studies indicate that Cx genes, including Cx43, are tumor-suppressor genes (15,16). Cx43 is one of the most common of the connexins and the major Cx homolog expressed in lung tissue (17-19). Studies have shown that Cx43 plays important roles in cancer development, cell proliferation, apoptosis, invasion and metastasis in lung cancer (20-26). Most importantly, Cx43 is able to sensitize NSCLC cells to CDDP and ionizing radiation (27,28).

In the present study, a CDDP-resistant human lung adenocarcinoma A549 cell line (A549/CDDP) was established. We found that Cx43 is involved in the acquisition of EMT in A549/CDDP cells. By overexpression of Cx43 in A549/CDDP cells, the mesenchymal phenotype was reversed and A549/CDDP-resistant cells by preventing EMT, making Cx43 a possible therapeutic target for lung cancer.

Materials and methods

Materials. CDDP, anti-Cx43, anti-β-actin and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell culture reagents and Lipofectamine™ 2000 were obtained from Invitrogen. Antibodies for E-cadherin,

Cx43 reverses the resistance of A549 lung adenocarcinoma cells to cisplatin by inhibiting EMT

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Vimentin, Slug and Snail were obtained from Cell Signaling Technology (Boston, MA, USA). Secondary antibodies for western blotting were obtained from Amersham Biosciences Corp. (Piscataway, NJ, USA). All other reagents were obtained from Sigma-Aldrich unless stated otherwise.

**Overexpression of Cx43 and inhibition of Cx43 expression by siRNA transfection.** Cx43 was expressed in A549/CDDP cells with a pcDNA3.1-Cx43 vector (Shanghai Jima Co. Ltd., Shanghai, China). Cells were transfected with siRNA targeting the human Cx43 gene (CAGUCUGCCUUUCGUGUA) or a non-specific control siRNA (NC control in the figures). Transfection into A549 cells was carried out using Lipofectamine 2000 according to the manufacturer’s instructions.

**MTT assay.** MTT assay was used to examine cell viability. Briefly, cells were seeded in a 96-well plate at a density of 5x10^3/well. After incubation in 5% CO₂ at 37°C for 24 h, cells were exposed to different concentrations of CDDP for 48 h. Then 20 µl of MTT (5 mg/ml in PBS) was added to each well and cells were incubated for 4 h at 37°C. After the medium was removed, the dark blue formazan that formed was dissolved in 200 µl DMSO. The absorbance at 570 nm was measured by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Data presented represent at least three separate repeated experiments.

**Western blotting.** Western blotting protocols were performed according to our previous study (29). Anti-Cx43, anti-E-cadherin, anti-vimentin, anti-Slug and anti-Snail were used. All western blotting exposures were in the linear range of detection, and the intensities of the resulting bands were quantified by Quantity One software on a GS-800 densitometer (Bio-Rad Laboratories).

**Wound-healing assay.** The cells were seeded into a 6-well plate and incubated until they reached 80% confluency. A 200-µl pipette tip was used to create a wound, and cells were washed twice with serum-free culture media to remove floating cells and then replaced with fresh medium without serum. Cells were subjected to the indicated treatment for 24 h, and cells migrating from the leading edge were photographed at 0 and 24 h.

**Matrigel invasion assay.** The assay for cell invasion was performed in a 24-well Transwell unit (8-µm pore size) which was coated with 1 mg/ml Matrigel matrix as described (30). Briefly, the cells were placed on the Matrigel-coated Transwell (the upper compartment of the invasion chamber) in the presence or absence of drugs. Conditioned medium (500 µl) was added to the lower compartment of the invasion chamber. After incubation at 37°C for 48 h, cells that had invaded the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. Cells in random fields were counted by light microscopy.

**Statistical analysis.** Statistical analysis between groups was considered to have statistical significance.

**Results**

**Establishing a CDDP-resistant A549 cell line.** We generated a CDDP-resistant cell line A549/CDDP by exposing these cells to cisplatin continuously. Cells were exposed to CDDP for more than a year, at an initial concentration of 2.5 µM CDDP reaching a final concentration of 40 µM CDDP. This cell line was maintained for 6 months. As shown in Fig. 1, the A549/CDDP cell line demonstrated a 5.8-fold higher resistance to CDDP than the A549 cell line.

**Acquired resistance of A549/CDDP cells to CDDP induces the cells to undergo EMT.** We first observed the morphological changes in A549/CDDP cells. As shown in Fig. 2A, the A549 cells, which were sensitive to CDDP, displayed classical epithelial morphology. In contrast, the A549/CDDP cells, which were resistant to CDDP, showed a spindle-like fibroblastic phenotype and increased formation of pseudopodia (Fig. 2A). These results suggest that the A549/CDDP cells gained a mesenchymal phenotype. To further determine the induction of EMT in A549/CDDP cells, we investigated the expression of EMT markers: epithelial markers such as E-cadherin, and mesenchymal markers such as vimentin, Snail and Slug by western blotting. As shown in Fig. 2B, the expression of E-cadherin was significantly less in the A549/CDDP cells than that in the parental A549 cells. In contrast, the expression levels of vimentin, Snail and Slug were largely increased. These results demonstrated that the acquired resistance to CDDP induced A549/CDDP cells to undergo EMT.

**A549/CDDP cells display increased potential for migration and invasion.** Cells that have undergone EMT display increased migratory and invasive behaviors. Thus, in the following experiment, we measured the invasive and migratory activity of the cells by Transwell and wound-healing assays. We found that the A549/CDDP cells displayed increased potential for migration and invasion. A549/CDDP cells demonstrated a 3.6-fold increase in invasive capability in the Matrigel-coated membrane when compared with the A549 cells (Fig. 3A). Also, as shown in Fig. 3B, the number of cells migrating across
the wound in the A549/CDDP cells was significantly increased relative to the A549 cells, suggesting that A549/CDDP cells showed enhanced migratory activity.

A549/CDDP cells show decreased expression of Cx43. To explore the effect of Cx43 on the resistance of lung adenocarcinoma cells to CDDP, we investigated the Cx43 expression...
Figure 4. A549/CDDP cells show decreased expression of Cx43. Western blotting was used to detect the expression of Cx43 in A549 and A549/CDDP cells. Bar graphs derived from the densitometric scanning of the blots. Columns, mean from three experiments; bars, SEM. *P<0.05, significantly different from A549 cells.

Figure 5. Overexpression of Cx43 reverses EMT in A549/CDDP cells. (A) Images of morphological changes of A549/CDDP cells following transfection by pcDNA-Cx43. (B) The effect of Cx43 overexpression on the expression of E-cadherin, vimentin, Slug and Snail was determined by western blotting in A549/CDDP cells. (C-G) Bar graphs derived from the densitometric scanning of the blots. Columns, mean from three experiments; bars, SEM. *P<0.05, significantly different from pcDNA-transfected group.
level in the A549 cells and A549/CDDP cells by western blotting. The expression level of Cx43 in the A549/CDDP cells was significantly decreased when compared with that in the A549 cells (Fig. 4). This suggests that Cx43 may be involved in CDDP-induced EMT in human lung cancer.

Cx43 is involved in the regulation of CDDP-induced EMT

Overexpression of Cx43 reverses EMT in A549/CDDP cells.

Based on the results described above, we hypothesized that Cx43 regulates EMT in A549 cells. Thus, the downregulation of Cx43 may initiate EMT during the development of the resistance of A549 cells to CDDP. To directly investigate the role of Cx43 in CDDP-induced EMT, we manipulated Cx43 expression levels in two ways: overexpression of Cx43 by transfection of pcDNA-Cx43 and knockdown of Cx43 expression with siRNA-Cx43. Firstly, we overexpressed Cx43 by transfection of pcDNA-Cx43 in A549/CDDP cells. Western blotting was used to confirm this effect (Fig. 5B). Consistent with the previous experiment (Fig. 2), the morphological changes and the marker proteins associated with EMT (epithelial marker such as E-cadherin, and mesenchymal markers such as vimentin, Snail and Slug) were observed in the subsequent experimental study. Fig. 5A shows that the pcDNA-transfected cells exhibited an elongated fibroblast-like morphology, whereas Cx43-A549/CDDP cells, which had a high Cx43 expression level, displayed epithelial morphology. Moreover, compared with the pcDNA-transfected cells, the expression level of epithelial marker E-cadherin was significantly increased, while levels of mesenchymal markers vimentin, Snail and Slug were decreased upon overexpression of Cx43 in the A549/CDDP cell line (Fig. 5B). These results demonstrate that upregulation of Cx43 by pcDNA-Cx43 converted EMT to mesenchymal-epithelial transition (MET) in the A549/CDDP cells.
Knockdown of Cx43 expression induces EMT in A549 cells. To further verify whether the EMT-associated phenotypes were specifically regulated by Cx43, we downregulated Cx43 expression in A549 cells which were sensitive to CDDP using siRNA (Fig. 6A and B). The results showed that A549 cells underwent EMT. Compared with the NC control cells, the cells displayed a spindle-like fibroblastic phenotype (Fig. 6C), and the expression of E-cadherin was markedly decreased, while the expression levels of vimentin, Snail and Slug were upregulated in A549 cells transfected with Cx43 siRNA (Fig. 6D). These results suggest that Cx43 is involved in the regulation of CDDP-induced EMT in human lung cancer cells.

Figure 7. Cx43 regulates the capability of invasion and migration in human adenocarcinoma cells. (A) The invasive ability of A549/CDDP cells following transfection by pcDNA-Cx43. Results represent the means ± SEM of 3 independent experiments; *P<0.05, significantly different from the pcDNA-transfected group. (B) The effect of Cx43 overexpression on the migratory ability of A549/CDDP cells by wound-healing assay. (C) The invasive ability of A549 cells following treatment with siRNA-Cx43. Results represent the means ± SEM of 3 independent experiments; *P<0.05, significantly different from the NC control. (D) The effect of Cx43 knockdown on the migratory ability of A549 cells by wound-healing assay.

Figure 8. Cx43 regulates the cytotoxicity of CDDP in human adenocarcinoma cells. (A) Overexpression of Cx43 reduced cellular resistance to CDDP in the A549/CDDP cells. The effect of Cx43 overexpression on the cytotoxicity of CDDP (20-320 µM) in the A549/CDDP cells was detected by MTT assay. Results represent the means ± SEM of 5 independent experiments; bars, SEM. *P<0.05, significantly different from the A549/CDDP cells. (B) Cx43 knockdown reduced CDDP sensitivity in the A549 cells. The effect of Cx43 knockdown on the cytotoxicity of CDDP (20-320 µM) in the A549 cells was detected by MTT. Results represent the means ± SEM of 5 independent experiments; bars, SEM. *P<0.05, significantly different from the A549 cells.
Cx43 regulates invasive and migratory properties of cells. To further investigate the effect of Cx43 on EMT in human lung cancer, we investigated the invasive and migratory properties of the cells. The results in Fig. 7A and B revealed that when A549/CDDP cells were transfected with Cx43, the capability of these cells to migrate and invade were obviously reduced relative to the pcDNA-transfected cells. In contrast, A549 cells transfected with Cx43 siRNA showed significant enhancement in their invasive and migratory properties (Fig. 7C and D). These results provide further evidence that Cx43 is involved in the regulation of CDDP-induced EMT in human lung cancer cells.

Cx43 regulates CDDP-induced cytotoxicity in human adenocarcinoma cells. To observe the effect of Cx43 on the cytotoxic effect of CDDP, we manipulated Cx43 expression in two ways: overexpression of Cx43 by transfection of pcDNA-Cx43 in A549/CDDP cells and knockdown of Cx43 expression with siRNA-Cx43 in A549 cells. The results showed that compared with the pcDNA-transfected cells, overexpression of Cx43 in the A549/CDDP cell line significantly reversed resistance of the cells to CDDP (Fig. 8A). Conversely, knockdown of Cx43 expression with siRNA-Cx43 resulted in insensitivity of A549 cells to CDDP (Fig. 8B). These results suggest that downregulation of Cx43 which induces EMT may underlie the resistance of A549 cells to CDDP.

Discussion

Emerging evidence suggests that chemo-resistance is associated with the acquisition of EMT in cancer cells (3-5,31,32). For example, gemicitabine-resistant pancreatic cancer (31), tamoxifen-resistant MCF7 breast cancer (5), oxaliplatin-resistant colorectal cancer (4), paclitaxel-resistant ovarian cancer (3) and gefitinib-resistant lung cancer cells (32) acquire EMT characteristics. Novel research has demonstrated that targeting EMT is a promising new therapeutic strategy (33-36). Tan et al (33) reported that Par-4 downregulation confers CDDP resistance in pancreatic cancer cells by inducing PI3K/Akt pathway-dependent EMT; knockdown of miR-134/487b/655 inhibited the EMT process and reversed TGF-β1-induced resistance to gefitinib in lung adenocarcinoma cells (34); downregulation of the PDGF-D pathway reversed EMT in gemicitabine-resistant hepatocellular cancer cells (35); and knockdown of Snail and Slug reversed CDDP resistance in ovarian cancer (36). Thus, regulating EMT is a new way to overcome drug resistance in cancer cells and to promote a better treatment outcome for cancer patients.

Consistent with the above studies, when the human lung adenocarcinoma cell line A549 became resistant to CDDP, these cells underwent EMT with significant morphological changes such as acquisition of a classical mesenchymal phenotype, downregulation of epithelial marker E-cadherin, and upregulation of mesenchymal markers vimentin, Snail and Slug. However, the exact mechanism underlying the CDDP-induced EMT phenotype of A549 cancer cells is still unclear.

Cx43 has been reported to play important roles in cancer development, cell proliferation, apoptosis, invasion and metastasis in lung cancer (20-26). A study showed that concurrent reduction in the expression of Cx43 and E-cadherin may contribute to the development of lung cancer. For example, Cx43 may induce E-cadherin expression and inhibit cell proliferation and progression of lung cancer (20). Another report also confirmed that transfection of Cx43 induced E-cadherin overexpression (23). Since E-cadherin is a typical epithelial marker, based on these reports, Cx43 may influence lung cancer development by regulating EMT. Indeed, the expression level of Cx43 was significantly less in A549/CDDP cells than that in the parental A549 cells. To further investigate the effect of Cx43 expression on CDDP-induced EMT in the human adenocarcinoma cell line, firstly, Cx43 was overexpressed in A549/CDDP cells. This resulted in A549/CDDP cells displaying classical epithelial morphology, increased E-cadherin expression, decreased expression of mesenchymal markers, reduced invasive and migratory activity and re sensitization to CDDP. This suggests that overexpression of Cx43 reversed CDDP-induced EMT and enhanced the cytotoxicity of CDDP. Secondly, when Cx43 expression was knocked down in A549 cells sensitive to CDDP exposure, the cells underwent EMT. Thus, the present study is the first report to show that Cx43 is involved in the regulation of CDDP-induced EMT in human lung adenocarcinoma cells.

Cx43 could sensitize cancer cells to chemotherapeutic agents, including CDDP (37,38). Although the mechanisms underlying these effects are still not clear, we can speculate the following scenarios: i) Cx43 could improve cell resistance to CDDP which may be mediated by the suppression of Src activity (37); ii) Cx43 could enhance the cytotoxic effect of various chemotherapeutic agents (etoposide, paclitaxel, doxorubicin) by promoting apoptosis (39); and iii) Cx43 enhanced the efficiency of CDDP in tumor testicular cells by gap junctional intercellular communication (GJIC)-mediated toxic bystander effects (38). The present study provides the first evidence that Cx43 enhances the cytotoxicity of CDDP by regulating EMT in human lung cancer cells.

In summary, the present study showed that Cx43 plays a critical role in promoting lung cancer progression and CDDP resistance by regulating EMT. Overexpression of Cx43 in CDDP-resistant A549 cells reversed EMT to MET and enhanced the cytotoxicity of CDDP. Thus, Cx43 may be a potential target for overcoming CDDP resistance in lung cancer therapy.

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