Cisplatin selects for multidrug-resistant CD133+ cells in lung adenocarcinoma by activating Notch signaling

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Abstract

Platinum-based chemotherapy is the first-line treatment for non-small cell lung cancer, but recurrence occurs in most patients. Recent evidence suggests that CD133+ cells are the cause of drug resistance and tumor recurrence. However, the correlation between chemotherapy and regulation of CD133+ cells has not been investigated methodically. In this study, we revealed that CD133+ lung cancer cells labeled by a human CD133 promoter-driven GFP reporter exhibited drug resistance and stem cell characteristics. Treatment of H460 and H661 cell lines with low-dose cisplatin (IC20) was sufficient to enrich CD133+ cells, to induce DNA damage responses, and to up-regulate ABCG2 and ABCB1 expression, which therefore increased the cross-resistance to doxorubicin and paclitaxel. This cisplatin-induced enrichment of CD133+ cells was mediated through Notch signaling as judged by increased levels of cleaved Notch1 (NICD1). Pretreatment with the γ-secretase inhibitor, DAPT, or Notch1 shRNAs remarkably reduced the cisplatin-induced enrichment of CD133+ cells and increased the sensitivity to doxorubicin and paclitaxel. Ectopic expression of NICD1 reversed the action of DAPT on drug sensitivity. Immunohistochemistry showed that CD133+ cells were significantly increased in the relapsed tumors in 3 of 6 lung cancer patients, who have received cisplatin treatment. A similar effect was observed in animal experiments as cisplatin treatment increased Notch1 cleavage and the ratio of CD133+ cells in engrafted tumors. Intratumoral injection of DAPT with cisplatin treatment significantly reduced CD133+ cell number. Together, our results demonstrated that cisplatin induces the enrichment of CD133+ cells, leading to multi-drug resistance by the activation of Notch signaling.
Introduction

Non-small cell lung cancer (NSCLC) represents approximately 80% of all lung cancers, and platinum-based chemotherapy is the standard first-line therapeutic approach to treat patients with NSCLC. However, the patients treated with single modalities are at a high risk for local regional recurrence and distant relapse (1). Over the past few decades, trials have evaluated the benefits of doublet chemotherapy by combination of cisplatin with other non-platinum drugs. Although the overall median survival of the patients who received platinum-based therapy has reached 9-12 months (2), the chemoresistance of tumor cells continues to be a considerable challenge in the management of NSCLC. Tumor cells often show initial sensitivity to chemotherapeutic drugs, but acquired resistance develops during the treatment, leading to tumor recurrence and further tumor progression. The most cited mechanism for the acquisition of drug resistance is the active efflux of chemotherapeutic agents via ATP-binding cassette (ABC) transporters, such as P-glycoprotein P-gp/MDR1/ABCB1, breast cancer resistance protein BCRP/ABCG2, and multi-drug resistance proteins (MRPs) (3). However, there is no effective treatment strategy to override these transporters for clinical therapy. In addition, several receptor-mediated survival signaling pathways, including mitogen-activated protein kinase (MAPK), Akt, mTOR, NF-κB and Notch pathways, have been linked to the drug resistance of conventional chemotherapy (4-6).

One emerging hypothesis that explains how cancer cells can withstand therapeutic assaults, acquire resistance and establish distant metastasis is the cancer stem cell (CSC) hypothesis. The CSC hypothesis states that CSCs possess similar biological properties of normal stem cells, such as unlimited self-renewal, asymmetric cell division, and resistance to toxic agents partially attributed to the elevated
expression of ABC transporters. A recent study showed that expression of CD133 is associated with the levels of resistance-related proteins in NSCLC patients (7). Furthermore, a combination of CD133 and ABCG2 can be used as an independent predictor of postoperative recurrence for patients with stage I NSCLC (8). The mechanisms causing drug resistance in CSCs are still poorly understood; CSCs may be intrinsically resistant to chemotherapeutic agents due to their low proliferation rate and resistance mechanisms, such as the expression of ABC transporters (9).

Even though the origin of CSCs is unknown, there is a consensus that CSCs reside in a niche that provides the cells with elementary signals. Notch signaling is critical for regulating cell-to-cell communication during embryogenesis, cellular proliferation, differentiation, and apoptosis (10). Activation of Notch signaling occurs when the Notch receptor undergoes a conformational change that allows proteolytic cleavage by γ-secretase, releasing an intracellular domain (NICD) that undergoes nuclear translocation and modulates Notch-specific gene expression. Recently, inhibition of γ-secretase-mediated Notch cleavage is a primary focus for the development of cancer therapeutics (11). Targeting CSCs with inhibitors of Notch signaling promotes cell differentiation, increases sensitivity to chemotherapy, and reduces metastasis (12).

Although the drug action of cisplatin has been widely explored (13), whether cisplatin may regulate the ratio of CSCs and elicit further multi-drug resistance in NSCLC is still unknown. Here, we provide evidence showing that cisplatin treatment significantly increased the ratio of CD133+ cells through the Notch signaling pathway. The cisplatin-elevated CD133+ cells were resistant to paclitaxel and doxorubicin by expressing ABCB1 and ABCG2. In addition, the enrichment of CD133+ cells by cisplatin was demonstrated in a tumor-engrafted mouse model and clinical specimens.
Our data indicate that exposure to cisplatin induced CSCs, leading to the increase of multi-drug resistance in NSCLC, suggesting that a new therapeutic strategy may be necessary to prevent the production of CSCs while platinum-based chemotherapy is applied for the management of NSCLC.
Materials and Methods

Clinical Specimens

We enrolled 6 lung cancer patients diagnosed from 2010 through 2012 with adenocarcinoma or squamous cell carcinoma from Kaohsiung Medical University Chung-Ho Memorial Hospital (KMUH) with approval of Institutional Review Board (KMUH-IRB-20120068). These patients received combination chemotherapy of cisplatin with docetaxel, gemcitabine, vinorelbin, or pemetrexed for 4-6 cycles according to the guideline of KMUH. The initial response of these patients for the first-line chemotherapy was from partial response to stable disease, which tumor recurrence occurred within one year. All these patients received surgery or thoracoscopic biopsy for the relapsed tumor. The primary (before cisplatin treatment) and relapsed (after cisplatin treatment) tumors of each patient were paraffin-embedded, sectioned and used for evaluating CD133+ cells by immunohistochemistry.

Immunohistochemistry (IHC)

Tissue sections (5 μm) were dewaxed and rehydrated. Antigen retrieval was done by incubating the slides in 10 mM citric buffer (pH6.0) and microwaved for 15 min. After blocking, the slides were incubated with primary antibody against CD133 followed by biotin-conjugated secondary antibody, polymer-HRP and diaminobenzidine tetrahydroxychloride (DAB) solution.

Cell culture

The human NSCLC cell lines A549, H460, H1299, PC9 and H661 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). A549, H1299 and PC9 cell lines were maintained in Dulbecco's Modified Eagle
Medium (DMEM) supplemented with 10% FBS (Invitrogen). H460 and H661 cell lines were maintained in RPMI 1640 supplemented with 10% FBS. Cell lines were kept under a humidified incubator containing 5% CO₂ at 37°C. For long-term culturing, mycoplasma test was performed every month for all cell lines.

**Generation of plasmids and lentivirus preparation**

The shRNAs targeting ABCB1 and Notch1 were purchased from Invitrogen. The EF.hICN1.CMV.GFP plasmid was a gift from Dr. Linzhao Cheng (Addgene plasmid #17623). For generation of the human CD133 promoter construct (CD133-GFP reporter), chromosomal DNA was isolated from HEK293T cells. The 5’-promoter P1 region of the human prominin1 gene (CD133; GenBank accession number AY275524) was generated by PCR amplification cloned into lentiviral vector pLL3.7 to replace the CMV promoter, which drives EGFP expression.

**Isolation of CD133+ cells and sphere-forming assay**

H460 cells were infected by CD133 P1 promoter-driven GFP reporter lentivirus and cultured in complete medium. The GFP+ cells were sorted using a FACSAria cell sorter (BD Biosciences). The GFP+ cells were expanded as spheres in a 10-cm ultra-low adhesion culture dish (Corning, PA, USA) containing DMEM/F-12 with N2 supplement (Invitrogen), 20 ng/ml EGF and 20 ng/ml basic-FGF (PeproTech, Rocky Hill, NJ, USA), referred to stem-cell medium, for 2 weeks. The tumor-sphere formation efficiency was calculated as the ratio of sphere number to the plated cell number.

**Quantitative real-time PCR (Q-PCR)**

Total RNA isolation and reverse transcription were performed using the method as described previously (14). The mRNA expression of CD133, Oct4, β-catenin, Bmi-
1, Smo, Notch1, Nestin, Nanog, Sox2, ABCG2, ABCB1, hHes-1 and GAPDH was analyzed by Q-PCR. The polymerase chain reaction for each gene was performed for 20 seconds at 95°C, followed by 40 cycles at 95°C for 3 seconds and annealing at 60°C for 30 seconds. The results were normalized to those of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Flow cytometry**

After cisplatin treatment for 24h, cultures from the H460 and H661 cell lines were washed with PBS. For some experiments, single cells dissociated from tumor spheres were analyzed by this method. One million trypsinized cells were incubated with an anti-CD133 antibody (Cell Signaling Technology Inc., Beverly, MA, USA) or isotype control IgG (Upstate Biotechnology, Waltham, MA, USA) for 45 minute. After washing, the cells were incubated with an Alexa488/Alexa594-conjugated secondary antibody (Invitrogen) for 30 min and washed again before analysis using a BD FACScaliber flow cytometer (BD Biosciences). The fluorescence intensities were analyzed with Cell Quest Software (BD Biosciences).

**Western blot**

For Western blot analysis, cells were harvested and lysed in 1X RIPA buffer containing protease inhibitor cocktails (Roche, Mannheim, Germany). The detail protocol for Western blot and the antibodies used in this study were described in Supplementary Materials and Methods.

**Soft agar assay**

A soft-agar assay was performed on 6-well plates with a base layer of 0.5% agarose gel containing DMEM and a upper layer of 0.35% agarose gel with
DMEM/F12 medium containing N2 supplement, 20 ng/ml EGF and 20 ng/ml basic FGF.

**Xenograft tumor growth**

Nude mice (6–8 wks old, male, 20 to 25 g body weight) were obtained from the National Laboratory Animal Center. Animal care was provided in accordance with the *Laboratory Animal Welfare Act, Guide for the Care and Use of Laboratory Animals* approved by the Institutional Animal Care and Use Committee of National Cheng Kung University.

**Immunofluorescence assay**

After the tumors were removed from xenografted mice, half of each tumor was fixed in 4% formalin at 4°C overnight. The immunofluorescence was performed using anti-CD133 antibody and anti-cleaved Notch1 antibody.

**Statistical analysis**

Data are presented as the mean±SE. To analyze the triplicate results of the experiments, two-tail Student’s *t*-test was used to demonstrate significant differences.

Additional information was described in Supplementary Materials and Methods.
Results

**CD133+ cells isolated from the H460 cell line display the characteristics of cancer stem cells.**

Studies have shown that CD133+ cells in different lung cancer cell lines exhibit self-renewal and tumor-initiating abilities. To isolate CD133-expressing cells, we constructed a human CD133 P1 promoter-driven GFP reporter into the PLL3.7 lentiviral vector (Fig. 1A). After infection, 1.5±0.5% GFP+ cells were identified in the H460 cell line (Supplementary Fig. S1A). The GFP+ cells were sorted and cultured in ultra-low adhesion culture dishes containing stem-cell medium, which allowed the cells to grow as tumor spheres and maintain GFP expression (Fig. 1B). Higher expression levels of CD133 protein and mRNA in GFP+ cells were confirmed by Western blot and Q-PCR (Fig. 1C and 1D). Flow cytometry demonstrated that the CD133 antibody recognized the membrane-bound CD133 protein and identified over 70% GFP+ cells as CD133+ cells (Fig. 1E). Next, we analyzed the cellular properties of the GFP+ cells. The GFP+ cells proliferated slower than GFP- cells in complete medium containing 10% FBS (Fig. 1F), suggesting that differentiation of GFP+ cells may occur after serum stimulation. Indeed, the GFP+ cells cultured in serum-containing medium gradually lose their GFP expression within two weeks (Supplementary Fig. S1B). Functional assay showed that the GFP+ cells displayed greater sphere forming ability, cisplatin resistance and migration ability (Fig. 1G, 1H and Supplementary Fig. S1C). We next analyzed the expression of stemness-associated genes in GFP+ cells by Q-PCR. As shown in Fig. 1I, the GFP+ cells expressed higher mRNA levels of stem cell-associated genes, including *Oct4*, *Sox2*, *Nanog*, *Smo*, *Bmi1*, *β-catenin*, *Nestin*, and *Notch1*. After serum-triggered differentiation of GFP+ cells for two weeks, the GFP-diminished cells were sorted.
and subjected to Q-PCR analysis. The results showed that the expression of stemness-associated genes in differentiated GFP+ cells was significantly decreased when compared with undifferentiated GFP+ cells (Fig. 1J). Our data confirmed the existence of CD133+ cells in the H460 cell line with a CD133 antibody and hCD133 promoter-driven GFP reporter lentivirus. The CD133+ cells were also characterized as CSCs.

*Cisplatin treatment elevates the ratio of CD133+ cell*

To study whether cisplatin may elevate the ratio of CSCs in lung cancer, we first tested the cytotoxic effect of cisplatin in NSCLC cell lines. Two lung cancer cell lines, H460 and H661, were treated with different concentrations of cisplatin for 24h and 48h, and cell viability was determined by MTT assay (Fig. 2A). Cisplatin-induced DNA damage was confirmed by the activation of DNA damage sensors, including cleaved PARP and phospho-γH2AX as well as the transducer phospho-CHK1, which leads to the elevation of p53 expression (Fig. 2B). According to the results from the cytotoxic analysis of cisplatin, H460 and H661 cells were treated with 10 µM and 20 µM cisplatin for 24h, respectively, which are sufficient to induce DNA damage but not significant cell death (~IC20) for the following experiments. As shown in Fig. 2C, cisplatin treatment for 24h remarkably increased the percentage of CD133+ cells in H460 and H661 cell cultures. This cisplatin-induced enrichment of CD133+ cells was in a dose- and time-dependent manner (Supplementary Fig. S2A and S2B). In addition to H460 and H661, this phenomenon could be observed in another cell lines, including H1299, A549 and PC9 (Supplementary Fig. S2C). In addition to cisplatin, carboplatin is also used as a platinum-base chemotherapy agent for NSCLC, especially for the patients with chronic renal diseases. We found that carboplatin
treatment also triggered DNA damage and induced enrichment of CD133+ cells in H460 cell cultures (Supplementary Fig. S3).

Aldehyde dehydrogenase (ALDH) has been reported as another lung cancer stem cell marker (15), and we found that cisplatin treatment also increased the ratio of ALDH+ cells in H460 cell cultures (Supplementary Fig. S4). As expected, the stem cell-associated genes CD133, Oct4, Sox2, Nanog, and Notch1 and the multi-drug-resistant genes ABCG2 and ABCB1 were up-regulated in cisplatin-primed H460 and H661 cultures (Fig. 2D). Next, we tested whether cisplatin treatment could increase the ratio of the cells with stem cell properties. After cisplatin treatment for 24h, the remaining cells were subjected to the tumor-sphere and soft-agar colony forming assays. Compared with vehicle controls, the cisplatin-treated cells generated more tumor spheres (Fig. 2E) and colonies in soft-agar cultures (Fig. 2F). Next, to demonstrate whether the increase of stem cell-associated genes was specifically expressed in CD133+ cells, CD133- and CD133+ cells were sorted from the cisplatin-treated H460 cell line. The purity of the sorted cells was confirmed by flow cytometry and Western blot (Fig. 2G). Results from Q-PCR showed that the stem cell-associated genes were highly expressed in the CD133+ cells, while the CD133- cells showed a similar expression pattern with the parental cells (Fig. 2H). Collectively, these results indicated that cisplatin elevated the ratio of CD133+ cells, which displayed stem cell properties.

**Cisplatin-induced CD133+ cells display multi-drug resistance**

ABCB1 and ABCG2 mediate the drug resistance of cancers for a range of chemotherapy drugs, including paclitaxel and doxorubicin (16). In our results, cisplatin-induced CD133+ cells expressed higher levels of ABCB1 and ABCG2 mRNA (Fig. 2D and 2H). Thus, we hypothesized that the cisplatin-induced
enrichment of CSCs may display multi-drug resistance. By treating with doxorubicin at different concentrations for 48h, the vehicle control in H460 cells had an IC\textsubscript{50} of 90.9±8.2 nM, but cisplatin-treated cells showed an IC\textsubscript{50} of >500 nM (Fig. 3A). In addition to doxorubicin, cisplatin-primed cells also exhibited drug resistance to paclitaxel treatment (Supplementary Fig. S5A). Pretreatment with 5 μM pantoprazol, a specific inhibitor for ABCG2, significantly blocked cisplatin-induced protection to doxorubicin treatment in H460 cells (Fig. 3B). On the other hand, pretreatment with 5 μM verapamil, a pan-inhibitor for ABC transporters, completely inhibited cisplatin-induced doxorubicin resistance of H460 cells (Fig. 3C). These results suggested that cisplatin-induced ABCG2, but not ABCB1, expression in CD133+-enriched cultures leads to drug resistance to doxorubicin treatment. This conclusion was further confirmed by the observation that ABCB1 knockdown by specific shRNAs (Fig. 3D) did not reverse the cisplatin-induced doxorubicin resistance (Fig. 3E). In contrast to the role of ABCG2 in doxorubicin resistance, pantoprazol partially reversed cisplatin-induced drug resistance to paclitaxel, but verapamil completely inhibited the effect of cisplatin on H460 and H661 cells, suggesting that not only ABCG2 but also other ABC transporters may be involved in cisplatin-induced multi-drug resistance (Supplementary Fig. S5B and S5C). Interestingly, although doxorubicin alone at IC\textsubscript{20} concentration was sufficient to induced DNA damage, but little effect was observed on CD133+ cell enrichment (Fig. 3F). In addition, co-treatment of cisplatin and doxorubicin did not have an additional effect on regulating the ratio of CD133+ cell in the H460 culture, suggesting that the induction of CD133+ cells was mediated by the mechanisms specifically triggered by cisplatin but not by doxorubicin. On the other hand, cisplatin combined with paclitaxel is one of the currently therapies for NSCLC. However, paclitaxel alone or co-treatment of cisplatin and paclitaxel had no
further induction effect on the enrichment of CD133+ cells (Supplementary Fig. S5D). Collectively, we concluded that cisplatin treatment induced DNA damage and triggered CD133+ cell enrichment.

**Cisplatin-induced enrichment of CD133+ cells is mediated by Notch1 signaling**

A number of studies have demonstrated that activation of Notch signaling maintains stemness and the self-renewal ability of normal stem cells and CSCs (15, 17). Inhibition of Notch signaling promotes differentiation of CSCs (18). Although cisplatin-induced Notch1 expression and activation have been discussed in some cancer types (19), studies on the correlation between cisplatin and Notch signaling in lung cancer are lacking. Thus, we were interested in investigating whether the increase of CD133+ cells by cisplatin treatment is mediated by Notch signaling in lung cancer cells. As shown in Fig. 4A, treatment of cisplatin dose-dependently increased Notch1 cleavage and activated it downstream target gene, Hes-1 (Fig. 4C). In contrast, the level of cleaved Notch3 was not altered by cisplatin treatment, suggesting that Notch1 activation may play a major role on cisplatin-induced enrichment of CD133+ cells (Supplementary Fig. S6A). Pretreatment with γ-secretase inhibitor, DAPT, abolished cisplatin-induced Notch1 activation (Fig. 4B) as well as the mRNA expression of Hes-1 (Fig. 4C). DAPT only did not alter the ratio of CD133+ cells in the H460 and H661 cell lines as compared with the control. However, pretreatment of DAPT significantly inhibited cisplatin-induced enrichment of CD133+ cells in H460 (Fig. 4D) and H661 cells (Supplementary Fig. S7A). On the other hand, shRNAs specifically targeting Notch1 greatly blocked cisplatin-induced enrichment of CD133+ cells (Supplementary Fig. S6B and S6C). These results indicated that Notch1 plays an important role in mediating the cisplatin-induced enrichment of CD133+ cells. Next, we analyzed whether inhibition of Notch signaling
could block cisplatin-induced multi-drug resistance to paclitaxel. Treatment of DAPT did not affect paclitaxel-induced cell death in H460 cells (vehicle: IC$_{50}$=4.2±0.3 nM; DAPT: IC$_{50}$=2.7±1.0 nM), but pretreatment of DAPT significantly blocked cisplatin-induced paclitaxel resistance (cisplatin: IC$_{50}$>50 nM; DAPT: IC$_{50}$=38.1±11.0 nM) in H460 cells (Fig. 4E). This DAPT-induced blockage of paclitaxel resistance could also be observed in H661 cells (Supplementary Fig. S7). To confirm the importance of Notch1-mediated signaling in cisplatin-induced multi-drug resistance, we transiently transfected the human Notch1 intracellular domain construct (hICN1) into H460 cells (Fig. 4F). Ectopically expressed hNICD1 did not change the cytotoxicity of paclitaxel in H460 cells (Fig. 4G). However, the blockage of cisplatin-induced paclitaxel resistance by DAPT was greatly reversed by ectopically expressed hNICD1 (Fig. 4G).

**Cisplatin treatment increases the ratio of CD133+ cells in lung tumor of patients and in H460-xenografted nude mice**

Accumulating evidences have demonstrated a tumor re-growth cell hierarchy originating with the survived CSCs after chemotherapy. According to the results from our in vitro study, we assumed that CD133+ cells might increase in relapse tumors of the patients who received cisplatin as their first chemotherapy. We enrolled 6 lung cancer patients with resected tumor (before) and who received cisplatin as their first chemotherapy. All these patients experienced tumor recurrence and received surgery or endoscopy for the relapsed tumors (after). Tumor samples, before and after cisplatin treatment, were paraffin-embedded and sectioned. The expression of CD133 was examined by immunohistochemistry. As shown in Fig. 5A, CD133 staining signal can be detected in the tumor region but not in the surrounding normal tissue. Few of CD133+ cells could be detected in the primary tumor (before cisplatin treatment), but the CD133 intensity and the number of CD133+ cells dramatically
increased in the recurrent tumors (after cisplatin treatment) in 3 out of 6 enrolled patients (Fig. 5A).

To evaluate the effect of cisplatin on the regulation of CD133+ cell number in vivo, H460 cells were subcutaneously injected into 22 nude mice. When the tumor grew to an appropriate size, the mice were divided into 4 groups according to the treatments described in Methods. The mice were sacrificed at day 4, and the ratio of CD133+ cells and expression of cleaved Notch1 within the tumors were analyzed using double immunofluorescence. As shown in Fig. 5B, CD133+ cells and cleaved Notch1 expression were rarely seen in vehicle- and DAPT-treated tumors. Consistent with the results from in vitro study, CD133 and cleaved Notch1 signals were dramatically increased in cisplatin-treated tumors. In addition, the CD133 signals were co-localized with cleaved Notch1 signals. The induction of CD133+ cells and Notch1 activation by cisplatin treatment was inhibited by co-treatment with DAPT (Fig. 5B). Quantification analysis by flow cytometry showed that cisplatin significantly elevated the number of CD133+ cells as compared with HBSS control groups, (19.5±3.8% vs 5.2±1.5%, p<0.01). Furthermore, co-treatment of DAPT blocked cisplatin-induced CD133+ cells and resulted in a similar ratio of CD133+ cells as compared with DAPT control groups (7.1±1.9% vs 7.0±0.8%, p=0.42; Fig. 5C and 5D). Together, our studies demonstrated that cisplatin elevated the ratio of CD133+ cells through Notch1 signaling, leading to multi-drug resistance.
Discussion

Platinum-based combination chemotherapy is the standard first-line treatment for advanced NSCLC. Here, we provide direct evidence that cisplatin treatment elevated the ratio of CD133+ cells, which exhibited stem-like properties and cross-drug resistance to paclitaxel. Our in vitro and in vivo studies also suggested that Notch signaling is involved in this cisplatin-triggered CSC transition. Our data partially explained the high recurrence rate and drug resistance to second-line chemotherapy in cisplatin-treated patients with NSCLC.

Accumulating evidence has demonstrated that CSCs may not only originate from the transformation of normal stem cells but also arise from the de-differentiation of cancer cells (22). An impressive observation has shown that CSCs exist in single cancer cell-derived cultures, which reveals the flexibility of the cancer cell status in tumors due to genomic instability in cancer cells (23, 24). In addition, the de-differentiation of cancer cells can be induced by different environmental cues, such as inflammation, hypoxia, and serum deprivation through epigenetic or genetic regulation (25). Recently, epithelial-to-mesenchymal transition (EMT) has been broadly demonstrated to convert mature cancer cells into CSCs (26, 27), which is mediated by EMT-associated genes and microRNAs (28-30). In our study, we demonstrated that treatment with cisplatin elevated CD133+ cells in the H460 and H661 cell lines. This induction effect could not be resulted from eliminating CD133-cells, which is more sensitive to cisplatin, and preserving the existing CD133+ cells before treatment because limited cells were killed by a low dose of cisplatin around the IC20 for each cell line during treatment. On the other hand, a high-dose treatment (>IC50) of paclitaxel, which induced significant cell death, showed no effect on
regulating CD133+ cell number in the H460 and H661 cell lines. These results indicated that cisplatin might induce de-differentiation of NSCLC cells.

Cancer cells are considered to be more genetically unstable, which can be accelerated by exogenous and endogenous exposures that cause DNA damage, such as irradiation and chemotherapy (31). A previous study showed that DNA damage induced by UV light and mitomycin C increased the side population in human nasopharyngeal carcinoma and CD133+ cells in human neuroblastoma SKN-SH cells (24). In our study, we demonstrated that cisplatin-induced DNA damage could be another driving force that triggers the enrichment of H460 and H661 cells. In addition, the biological relevance of the DNA damage recognition proteins may not be limited to the induction of apoptosis. Exposure to cisplatin elicits pro-survival and pro-apoptotic signals simultaneously, suggesting that the DNA damage induced by cisplatin may trigger different downstream signaling pathways to determine the final fate of cells.

So far, the cisplatin-activated signaling pathways have not been fully addressed. Two phosphatidylinositol-3-related kinases, ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3-related), are located at the top of checkpoint signal cascades. Among of these two kinases, cisplatin preferentially activates ATR kinase and its downstream CHK1 kinase (32). Recently, CHK1-mediated activation of mitogen-activated protein kinase (MAPK) has been linked to cisplatin-induced cellular effects. All three members of MAPK, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase, are activated following the exposure of tumor cells to cisplatin, and ERK activation is the most critical for cisplatin-induced apoptosis (33). However, additional research suggested that the activation of ERK and the JNK-MAPK cascade by cisplatin prevents tumor cells from undergoing apoptosis.
(34), reflecting that different cell contexts and the degree of DNA damage may cause different cellular effects. Furthermore, activation of ERK signaling has been demonstrated to be involved in the de-differentiation of myogenic lineage-committed human myoblasts (35).

Notch1 is important for the maintenance and self-renewal of cancer-initiating cells in different malignancies (15, 36, 37). Inhibition of Notch signaling promotes differentiation and the radio- and chemotherapy of CSCs (38). In contrast, overexpression of Notch1 promotes EMT and the number of CSCs in pancreatic cancer. We found that inhibition of Notch signaling by pretreatment with the γ-secretase inhibitor, DAPT, significantly blocked the cisplatin-induced elevation of CD133+ cells in vitro and in vivo. Although the mechanism of how cisplatin-induced DNA damage activates Notch signaling is unclear, there is evidence showing that the expression and activation of Notch1 can be controlled by p53 (39). In addition, Notch1 exerts a p53-dependent protective function against DNA damage in response to UVB light through suppression of Forkhead box O3 (FOXO3), a key pro-apoptotic gene (40). Accordingly, these studies designate that the cisplatin-induced upregulation and activation of Notch1 may be the consequence of elevated p53 caused by DNA damage.

ABCB1 and ABCG2 are two well-known multidrug resistant genes, which were up-regulated in CD133+ cells. Our study showed that cisplatin-induced multidrug resistance was through the expression of ABCG2 and ABCB1 in CD133+ cells. Consistently, it has been reported that high expression level of ABCG2 protein is correlated with poor survival and lower response rate of the NSCLC patients, who received platinum-based chemotherapy (41-43). In addition, dual CD133+/ABCG2+ status can be an independent predictor of tumor recurrence for the patients with stage
In vitro study showed that ABCG2-overexpressing human NSCLC cell lines were more resistant to chemotherapy (45, 46). Inhibition of ABCG2 and/or ABCB1 by selective inhibitors sensitized NSCLC cells to chemotherapy (47, 48). More studies are required to investigate whether blockage of Notch signaling may decrease the expression of ABCG2 and ABCB1 and re-sensitize cancer cells to platinum-based chemotherapy.

Collectively, we provided evidence showing that cisplatin-induced DNA damage enriched CD133+ cells. Elevation of CD133+ cells is related to the increase in cross-drug resistance to paclitaxel. We also demonstrated that the induction of CD133+ cells by cisplatin was mediated by Notch signaling. Thus, our data not only provided the possible mechanism of tumor recurrence in cisplatin-treated lung cancer patients but also suggested that blocking Notch signaling during first-line treatment may reduce the recurrence of NSCLC.
Acknowledgments:

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References


Figure legends

**Figure 1. Identification of CD133+ cells in the H460 cell line.** (A) Illustration of the GFP reporter lentiviral construct driven by the human CD133 P1 promoter. (B) Representative image of tumor sphere. Scale bar = 50 μm (C) Cell lysates of parental H460 cells, GFP- and GFP+ cells were analyzed by Western blot. (D) Expression of CD133 mRNA was analyzed by Q-PCR. *p<0.01 compared with GFP- cells. (E) Parental H460 cells and GFP+ cells that dissociated from tumor spheres were labeled with isotype control or anti-CD133 antibody for flow cytometry assay. (F) Cell proliferation assay was performed to determine the growth rate of GFP- and GFP+ cells. *p<0.05 compared with GFP- cells. (G) Sphere-forming ability of GFP- and GFP+ cells after serial passage. The sphere number was counted and calculated as the number of spheres per 100-seeded cells. *p<0.01 compared with relative GFP- cells. (H) Transwell migration assay for parental H460, GFP- and GFP+ cells. *p<0.01 compared with parental H460 cells. (I) Total RNA isolated from GFP- and GFP+ cells were analyzed by Q-PCR for the indicated genes. (J) GFP+ cells were differentiated in serum-containing medium for 2 weeks. Cells losing GFP expression were sorted. Total RNA was isolated from GFP+ cells, and differentiated cells were analyzed by Q-PCR for the indicated genes.

**Figure 2. Cisplatin treatment elevates the ratio of CD133+ cells in H460 and H661 lung cancer cell lines.** (A) H460 and H661 cell lines were treated with different concentrations of cisplatin for 24h and 48h. The cell viability was determined by an MTT assay. (B) Cell lysates of H460 and H661 cells treated with different concentrations of cisplatin for 24h were analyzed by Western blot. The arrowhead indicated the cleaved PARP. (C) CD133+ cells in cisplatin-treated H460 and H661 cultures were detected by flow cytometry. (D) H460 and H661 cells were
treated with 10 μM and 20 μM cisplatin, respectively, for 24h. The mRNA levels of the indicated genes were analyzed by Q-PCR. (E) Cisplatin-treated H460 and H661 cells were subjected to the tumor-sphere forming assay. The arrow indicates the large spheres counted. Scale bar=200 μM. *p<0.05 compared with vehicle controls. (F) Cisplatin-treated H460 and H661 cells were subjected to the soft-agar colony-forming assay. The inset showed the higher magnification of the colony morphology *p<0.05 compared with vehicle controls. (G) H460 cells were treated with 10 μM cisplatin for 24h. CD133- and CD133+ cells were sorted. The purities of CD133- and CD133+ cells were evaluated by flow cytometry and Western blot. (H) The total RNA isolated from parental H460, CD133- and CD133+ cells were analyzed by Q-PCR.

**Figure 3. Cisplatin treatment results in multi-drug resistance to doxorubicin.** (A) Vehicle- or cisplatin-treated H460 cells were seeded into 96-well plates. The cells were treated with different concentrations of doxorubicin for 48h, and then the cell viability was determined by MTT assay. (B-C) Vehicle- or cisplatin-treated H460 cells were cultured in 96-well plates and pretreated with DMSO, 5 μM pantoprazole (panto) or 5 μM verapamil (vera) for 30 minutes before the application of doxorubicin. The cell viability was determined by an MTT assay. (D) The knockdown efficiency of shRNA lentiviruses (shRNA #1 and shRNA #2) was examined by Western blot. (E) Control and ABCB1 knockdown H460 cells were treated with cisplatin for 24 h followed by different concentrations of paclitaxel treatment for 48 h. The cell viability was determined by MTT assay. (F) Vehicle- or cisplatin-treated H460 cells were treated with 20 nM doxorubicin for 24h, and the number of CD133+ cells was analyzed by flow cytometry.
**Figure 4. Cisplatin-induced enrichment of CD133+ cells and multi-drug resistance is mediated by Notch signaling.** (A) The cell lysate of cisplatin-treated H460 cells was analyzed by Western blot. (B) H460 cells were pretreated with DMSO or 10 μM DAPT for 30 minutes and then exposed to vehicle or 10 μM cisplatin for 24h. The cell lysate was analyzed by Western blot. (C) Total RNA was isolated from vehicle- and cisplatin-treated H460 cells with or without DAPT pre-treatment. Human Hes-1 mRNA levels were analyzed by Q-PCR. *p<0.05 compared with vehicle control. (D) DMSO or DAPT-treated H460 cells were exposed to vehicle or 10 μM cisplatin for 24h. The CD133+ cells in the cultures were detected by flow cytometry. (E) Vehicle- or cisplatin-treated H460 cells were seeded into 96-well plates. The cells were pretreated with DMSO or 10 μM DAPT for 30 minutes and then exposed to different concentrations of paclitaxel for 48h. Cell viability was determined by an MTT assay. (F) H460 cells were transiently transfected with the hICN1 plasmid. The expression levels of Notch1 were analyzed by Western blot. (G) H460 cells with indicated treatments were treated with different concentrations of paclitaxel for 48 h, and cell viability was determined by MTT assay.

**Figure 5. Cisplatin elevates the ratio of CD133+ cells within H460-xenografted tumors via Notch signaling.** (A) Representative images showed the immunohistochemistry of CD133 staining in the clinical specimens, before and after cisplatin treatment, from 3 lung cancer patients. (B) After cisplatin and/or DAPT treatment, the xenografted tumors were harvested and analyzed by double immunofluorescence using CD133 (red) and cleaved Notch1 (green) antibodies. The nucleus was stained by DAPI. Scale bar=100 μm. (C) Xenografted tumor was dissociated into single cells and stained with anti-CD133 antibody and an Alexa488-conjugated secondary antibody for flow cytometry analysis. (D) Quantitative analysis
showed the significant increase of CD133+ cell within the cisplatin-treated tumors (Cis). Co-treatment of DAPT blocked the induction effect of cisplatin on the percentage of CD133+ cells. *$p<0.05$ compared with vehicle control.
Figure 3 (Liu et al.)

A

B

C

D

E

F
Figure 4 (Liu et al.)
Figure 5 (Liu et al.)

Panel A: Images before and after treatment for patients 1, 2, and 3.

Panel B: Immunohistochemical staining for CD133 and cleaved Notch1 in control, cisplatin, DAPT, and cisplatin/DAPT conditions. Merged images with DAPI are also shown.

Panel C: Histogram showing the ratio of CD133+ cells for control, cisplatin, and cisplatin/DAPT treatments.

Panel D: Bar graph comparing the ratio of CD133+ cells across different treatment conditions.
Cisplatin selects for multidrug-resistant CD133+ cells in lung adenocarcinoma by activating Notch signaling

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