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 (IC50) was sufficient to enrich CD133+ cells, to induce DNA damage responses, and to upregulate 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, N-[N-(3,5-diﬂuorophenacetyl)-1-alamyl]-S-phenylglycine t-butyl ester (DAPT), or Notch1 short hairpin RNAs (shRNA) 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 three of six patients with lung cancer 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 showed that cisplatin induces the enrichment of CD133+ cells, leading to multidrug resistance by the activation of Notch signaling. Cancer Res; 73(1): 406–16. ©2012 AACR.

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 NSCLCs. 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 to 12 months (2), the chemoresistance of tumor cells continues to be a considerable challenge in the management of NSCLCs. 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 multidrug resistance proteins (MRP; ref. 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 biologic 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 patients with NSCLCs (7). Furthermore, a combination of CD133 and ABCG2 can be used as an independent predictor of postoperative recurrence for patients with stage I NSCLCs (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 ß-secretase, releasing an intracellular domain (NICD) that undergoes nuclear translocation and modulates Notch-specific gene expression. Recently, inhibition of ß-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 multidrug resistance in NSCLCs 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 shown in a tumor-engrafted mouse model and clinical specimens. Our data indicate that exposure to cisplatin induced CSCs, leading to the increase of multidrug resistance in NSCLCs, suggesting that a new therapeutic strategy may be necessary to prevent the production of CSCs, whereas platinum-based chemotherapy is applied for the management of NSCLCs.

Materials and Methods

Clinical specimens

We enrolled 6 patients with lung cancer diagnosed from 2010 through 2012 with adenocarcinoma or squamous cell carcinoma from Kaohsiung Medical University Chung-Ho Memorial Hospital (KMUH; Kaohsiung, Taiwan) with approval of Institutional Review Board (KMUH-IRB-20120068). These patients received combination chemotherapy of cisplatin with docetaxel, gemcitabine, vinorelbine, or pemetrexed for 4 to 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 when tumor recurrence occurred within 1 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

Tissue sections (5 µm) were dewaxed and rehydrated. Antigen retrieval was done by incubating the slides in 10 mmol/L citric buffer (pH 6.0) and microwaved for 15 minutes. After blocking, the slides were incubated with primary antibody against CD133 followed by biotin-conjugated secondary antibody, polymer horseradish peroxidase, and diaminobenzidine tetrahydroxychloride (DAB) solution.

Cell culture

The human NSCLC cell lines A549, H460, H1299, PC9, and H661 were purchased from American Type Culture Collection. 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% CO$_2$ at 37°C. For long-term culturing, mycoplasma test was conducted every month for all cell lines.

Generation of plasmids and lentivirus preparation

The short hairpin RNAs (shRNA) targeting ABCB1 and Notch1 were purchased from Invitrogen. The EF.hICN1. CMV.GFP plasmid was a gift from Dr. Linzhao Cheng (Aldgene 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) containing DMEM/F-12 with N2 supplement (Invitrogen), 20 ng/mL EGF, and 20 ng/mL basic fibroblast growth factor (FGF; PeproTech), 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

Total RNA isolation and reverse transcription were conducted using the method as described previously (14). The mRNA expression of CD133, Oct4, ß-catenin, Bmi-1, Smo, Notch1, Nestin, Nanog, Sox2, ABCG2, ABCB1, hHes-1, and GAPDH was analyzed by quantitative real-time PCR (qPCR). The PCR for each gene was conducted 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 24 hours, 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.) or isotype control IgG (Upstate Biotechnology) for 45 minutes. After washing, the cells were incubated with an Alexa488/Alexa594-conjugated secondary antibody (Invitrogen) for 30 minutes and washed again before analysis using a BD FACScaliber flow cytometer (BD Biosciences). The fluorescent intensities were analyzed with Cell Quest Software (BD Biosciences).

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

Soft agar assay
A soft agar assay was conducted on 6-well plates with a base layer of 0.5% agarose gel containing DMEM and an upper layer of 0.35% agarose gel with DMEM/F-12 medium containing N2 supplement, 20 ng/mL EGF, and 20 ng/mL basic FGF.

Xenograft tumor growth
Nude mice (6–8 weeks 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 (Tainan, Taiwan).

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 conducted 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, 2-tailed Student t test was used to show significant differences. Additional information is described in Supplementary Materials and Methods.

Results
CD133⁺ cells isolated from the H460 cell line display the characteristics of CSCs
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 blotting and qPCR (Fig. 1C and D). Flow cytometry showed that the CD133 antibody recognized the membrane-bound CD133 protein and identified more than 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 2 weeks (Supplementary Fig. S1B). Functional assay showed that the GFP⁺ cells displayed greater sphere-forming ability, cisplatin resistance, and migration ability (Fig. 1G and 1H; Supplementary Fig. S1C). We next analyzed the expression of stemness-associated genes in GFP⁺ cells by qPCR. 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 2 weeks, the GFP-diminished cells were sorted and subjected to qPCR 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 CD133 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 24 and 48 hours, 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 μmol/L and 2 μmol/L cisplatin for 24 hours, respectively, which are sufficient to induce DNA damage but not significant cell death (~IC₅₀) for the following experiments. As shown in Fig. 2C, cisplatin treatment for 24 hours 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 line, including H1299, A549, and PC9 (Supplementary Fig. S2C). In addition to cisplatin, carboplatin is also used as a platinum-based chemotherapy agent for NSCLCs, 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 CSC 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
Cisplatin Enriches CD133\(^+\) Cells via Notch Signaling

Cisplatin-induced CD133\(^+\) cells display multidrug 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 H). Thus, we hypothesized that the cisplatin-induced enrichment of CSCs may display multidrug resistance. By treating with doxorubicin at different concentrations for 48 hours, the vehicle control in H460 cells had an IC\(_{50}\) of 90.9±8.2 nmol/L, but cisplatin-treated cells showed an IC\(_{50}\) of more than 500 nmol/L (Fig. 3A). In addition to doxorubicin, cisplatin-primed cells also exhibited drug resistance to paclitaxel treatment (Supplementary Fig. S5A). Pretreatment with 5 \(\mu\)mol/L pantoprazol, a specific inhibitor for ABCG2, significantly

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blocked cisplatin-induced protection to doxorubicin treatment in H460 cells (Fig. 3B). On the other hand, pretreatment with 5 \( \mu \text{mol/L} \) 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 varapamil 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 multidrug resistance (Supplementary Fig. S5B and S5C). Interestingly, although doxorubicin alone at IC\(_{20}\) concentration was sufficient to induce DNA damage but little effect was observed on CD133\( ^+ \) cell enrichment (Fig. 3F). In addition, cotreatment of cisplatin and doxorubicin did not have an additional effect on regulating the ratio of CD133\( ^+ \) cells 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 current therapies for NSCLCs. However, paclitaxel alone or...
Cotreatment 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 shown 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 \(\gamma\)-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 paclitaxel resistance to paclitaxel. Treatment of DAPT did not affect paclitaxel-induced cell death in H460 cells (vehicle: IC\(_{50}\) = 4.2 ± 0.3 nmol/L; DAPT: IC\(_{50}\) = 2.7 ± 1.0 nmol/L), but pretreatment of DAPT significantly blocked cisplatin-induced paclitaxel resistance to paclitaxel.

Figure 3. Cisplatin treatment results in multidrug 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 48 hours, and then the cell viability was determined by MTT assay. B and C, vehicle- or cisplatin-treated H460 cells were cultured in 96-well plates and pretreated with dimethyl sulfoxide (DMSO), 5 \(\mu\)mol/L pantoprazole (panto), or 5 \(\mu\)mol/L 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 blotting. E, control and ABCB1-knockdown H460 cells were treated with cisplatin for 24 hours followed by different concentrations of paclitaxel treatment for 48 hours. The cell viability was determined by MTT assay. F, vehicle- or cisplatin-treated H460 cells were treated with 20 nmol/L doxorubicin (dox) for 24 hours, and the number of CD133\(^+\) cells was analyzed by flow cytometry.
resistance (cisplatin: IC_{50} > 50 nmol/L; DAPT: IC_{50} = 38.1 ± 11.0 nmol/L) 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 multidrug resistance, we transiently transfect 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 shown a tumor regrowth 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 chemother-apy. We enrolled 6 patients with lung cancer with resected tumor (before) and who received cisplatin as their first chemother-apy. 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 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 Materials and Methods. The mice were sacrificed at day 4, and the ratio of CD133+ cells and

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 patients with lung cancer. 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 4',6-diamidino-2-phenylindole (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). Cotreatment of DAPT blocked the induction effect of cisplatin on the percentage of CD133+ cells. *P < 0.05 compared with vehicle control.
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 colocaled with cleaved Notch1 signals. The induction of CD133+ cells and Notch1 activation by cisplatin treatment was inhibited by cotreatment 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, cotreatment 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 D). Together, our studies showed that cisplatin elevated the ratio of CD133+ cells through Notch1 signaling, leading to multidrug resistance.

Discussion

Platinum-based combination chemotherapy is the standard first-line treatment for advanced NSCLCs. Despite the relevance of first-line chemotherapy, tumor recurrence occurs in most cases (20). In addition, the use of second-line agents, such as docetaxel and pemetrexed, is not efficacious in patients who had already received these drugs in first-line chemotherapy (21). 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 NSCLCs.

Accumulating evidence has shown that CSCs may not only originate from the transformation of normal stem cells but also arise from the dedifferentiation 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 dedifferentiation 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 showed to convert mature cancer cells into CSCs (26, 27), which is mediated by EMT-associated genes and microRNAs (28–30). In our study, we showed that treatment with cisplatin elevated CD133+ cells in the H460 and H661 cell lines. This induction effect could not have resulted from eliminating CD133+ cells, which are 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 IC50 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 dedifferentiation of NSCLCs.

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 SK-N-SH cells (24). In our study, we showed that cisplatin-induced DNA damage could be another driving force that triggers the enrichment of H460 and H661 cells. In addition, the biologic relevance of the DNA damage recognition proteins may not be limited to the induction of apoptosis. Exposure to cisplatin elicits prosurvival 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. Of these 2 kinases, cisplatin preferentially activates ATR kinase and its downstream CHK1 kinase (32). Recently, CHK1-mediated activation of MAPK has been linked to cisplatin-induced cellular effects. All 3 members of MAPK, extracellular signal–regulated kinase (ERK), c-jun-NH2 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 shown to be involved in the dedifferentiation 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.

ABC1 and ABCG2 are 2 well-known multidrug resistant genes, which were upregulated in CD133+ cells. Our study showed that cisplatin-induced multidrug resistance was through the expression of ABCG2 and ABC1 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 patients with NSCLCs 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 I NSCLCs (8). In vitro study showed that ABCG2-overexpressing human NSCLC cell lines were more resistant to chemotherapy (44, 45). Inhibition of ABCG2 and/or ABCB1 by selective inhibitors sensitized NSCLCs to chemotherapy (46, 47). 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 showed 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 patients with lung cancer but also suggested that blocking Notch signaling during first-line treatment may reduce the recurrence of NSCLCs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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