

# CXCR4 promotes cisplatin-resistance of non-small cell lung cancer in a CYP1B1-dependent manner

SONGPING XIE<sup>1\*</sup>, ZHENBO TU<sup>2\*</sup>, JIE XIONG<sup>2\*</sup>, GANJUN KANG<sup>1</sup>, LINA ZHAO<sup>3</sup>, WEIDONG HU<sup>4</sup>, HAIYAN TAN<sup>5</sup>, KINGSLEY MIYANDA TEMBO<sup>2</sup>, QIANSHAN DING<sup>6</sup>, XINZHOU DENG<sup>2</sup>, JIE HUANG<sup>1</sup> and QIUPING ZHANG<sup>2</sup>

<sup>1</sup>Department of Thoracic Surgery, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060;

<sup>2</sup>Department of Immunology, School of Basic Medical Science, Wuhan University, Wuhan, Hubei 430071;

<sup>3</sup>Department of Pathology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060;

<sup>4</sup>Department of Thoracic Oncology, Zhongnan Hospital, Wuhan University, Wuhan, Hubei 430071;

Departments of <sup>5</sup>Gastrointestinal Surgery, and <sup>6</sup>Gastroenterology,

Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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**Abstract.** Chemoresistance is the main cause of treatment failure and high mortality in advanced lung cancer. Cisplatin, an important chemotherapeutic agent for lung cancer, has been observed to show enormously reduced chemotherapeutic efficacy owing to the development of chemoresistance. CXCR4, a stromal-derived-factor-1 specific chemokine receptor, is highly expressed in non-small cell lung cancer (NSCLC) tissues and participates in cancer progression by regulating cell growth, apoptosis or invasion. In this study, we therefore investigated whether CXCR4 plays a role in the cisplatin associated resistance in NSCLC. We detected the expression of CXCR4 in tissue specimens from 64 NSCLC patients by immunohistochemistry. Cisplatin-resistant NSCLC cells A549/DDP and its parental A549 cells were employed in this study. RNA interference was performed to silence the CXCR4. The influence of CXCR4 on tumor cell chemoresistance, apoptosis and growth, as well as the relationship between CXCR4 and the expression of cytochrome p450 associated molecule CYP1B1 in NSCLC were evaluated. Finally, we found CXCR4 was

significantly highly expressed in cisplatin-resistant NSCLC patients and the A549/DDP cell line. CXCR4 inhibition by siRNA reversed chemoresistance and decreased tumor cell proliferation. Bioinformatics analysis showed that the expression of CYP1B1 had a positive correlation with CXCR4, the CYP1B1 silencing significantly decreased CXCR4 expression levels and cisplatin resistance. Immunohistochemistry also verified that CYP1B1 was upregulated in NSCLC tissues of cisplatin-resistant patients. In conclusion, our results indicate that overexpression of CXCR4 in NSCLC promotes cisplatin resistance via CXCR4-mediated CYP1B1 upregulation. Thus, it can be used as a potential therapeutic target in NSCLC chemoresistance patients and be used as a clinical predictor of cisplatin response.

## Introduction

Lung cancer is one of the leading causes of cancer-related deaths worldwide in both males and females, with non-small cell lung cancer (NSCLC) accounting for ~87% of all lung cancer cases (1). At diagnosis, most patients present with advanced NSCLC stages, mainly due to the lack of effective early diagnosis techniques. Presently, chemotherapy is a major treatment to prolong disease-free survival for these patients. However, little success has been made in improving patient survival rates, as the 5-year survival rates have remained at ~18.2% (2,3). Researchers have reported the occurrence of intrinsic or acquired resistance, as the main challenge associated with chemotherapeutic resistance in cancer cells (4). Cisplatin-based chemotherapy is frequently used in the treatment of NSCLC. The resistance obtained following continuous therapy limits the benefit of cisplatin in cancer treatment, but the exact mechanism of cisplatin resistance in lung cancer remains unknown.

CXCR4, a seven-transmembrane G protein-coupled receptor, is a physiological receptor for stromal-derived-factor-1 (5) which is highly expressed in various types of human tumors, including lung cancer (6,7), esophageal

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*Correspondence to:* Professor Qiuping Zhang, Department of Immunology, School of Basic Medical Sciences, Wuhan University, 185 Donghu Road, Wuchang, Wuhan, Hubei 430071, P.R. China  
E-mail: qpzhang@whu.edu.cn

Professor Jie Huang, Department of Thoracic Surgery, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuchang, Wuhan, Hubei 30060, P.R. China  
E-mail: doctor\_huangjie@126.com

\*Contributed equally

**Abbreviations:** NSCLC, non-small cell lung cancer; CYP1B1, cytochrome P450 1B1

**Key words:** non-small cell lung cancer, chemoresistance, CXCR4, CYP1B1

cancer (8), gastric cancer (9), endometrial cancer (10) and breast cancer (11). A number of studies have demonstrated the vital role of CXCR4 in cancer cell survival, proliferation, invasion and metastasis, thus high expression of CXCR4 has been shown to be associated with a poor prognosis in cancer patients. Nakamura *et al* reported that CXCR4 signaling is crucial for drug resistance in bone marrow-disseminated tumor cells (12). While Li *et al* observed that the over-expression of CXCR4 was significantly associated with cisplatin-based chemotherapy resistance in epithelial ovarian cancer (13). Although this suggests a possible role for CXCR4 in development of chemotherapy resistance, the precise role of CXCR4 in cisplatin resistance of lung cancer remains largely unexplored.

In this study, we detected the expression of CXCR4 in NSCLC patients and cell lines, and demonstrated the possible effect of CXCR4 knockdown on cisplatin-induced apoptosis. In addition, we also explored the possible molecular mechanisms by which CXCR4 possibly regulates cisplatin resistance in NSCLC.

## Materials and methods

**NSCLC tissue samples.** A total of 64 lung cancer specimens were collected from patients with primary NSCLC surgical resection at Renmin Hospital of Wuhan University from May 2012 to April 2015. None of the subjects had received any chemotherapy and radiotherapy prior to surgery. All of these patients were treated with cisplatin-based chemotherapy after surgery according to the NCCN guideline every 3 weeks for four cycles. Patients were followed-up by telephone until recurrence/metastasis or until May 2016. All patients were monitored by chest computed tomography scan, abdominal ultrasonography and brain magnetic resonance imaging every 3 months in the first year after surgery, or immediately when a recurrence/metastasis was suspected. A whole-body fludeoxyglucose positron emission tomography/CT was performed when needed. The WHO grades and clinical features of these patients were summarized in Table I. Patients with disease progression or recurrence 12 months after surgery were defined as being chemoresistance, whereas those without recurrence or recurrence >12 months after surgery were defined as chemosensitive. There were 32 NSCLC patients who are sensitive to cisplatin treatment and 32 NSCLC patients with cisplatin chemoresistance. This study was carried out in accordance with the principles of the Helsinki Declaration and approved by the ethics committee of the Renmin Hospital of Wuhan University.

**NSCLC cell lines.** A549 was obtained from American Type Culture Collection, cisplatin resistant A549 cell line (A549/DDP) was obtained from Fudan Institutes of Biomedical Sciences Cell Center, and the cells were maintained in Dulbecco's modified Eagle's medium with high glucose (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA), containing 100 U/ml penicillin and 100 mg/ml streptomycin. Both cell lines were cultured in a 5% CO<sub>2</sub> air incubator at 37°C and passaged using 0.25% trypsin-EDTA (Gibco) when they reached confluence. Cisplatin was purchased from Sigma Chemical Co. (USA).

Table I. Comparison of clinical characteristics between patients with low and high CXCR4 cell content.

Clinical factor	Total patients (n=64)	High expression (n=46)	Low expression (n=18)	P-value
Age (years)				
≤65	15	12	3	0.424
>65	49	34	15	
Gender				
Male	44	35	9	0.043
Female	20	11	9	
T stage				
T2	43	26	17	0.004 <sup>b</sup>
T3	14	13	1	
T4	7	7	0	
N stage				
N0	36	24	12	0.490 <sup>b</sup>
N1	7	6	1	
N2	21	16	5	
M stage				
M0	62	46	16	0.076 <sup>a</sup>
M1	2	0	2	
Histology				
Adenocarcinoma	39	26	13	0.247
Squamous carcinoma	25	20	5	
Drug-resistance				
Yes	32	27	5	0.026
No	32	19	13	

<sup>a</sup>Fisher's exact test. <sup>b</sup>Likelihood ratio.

**Immunohistochemistry.** Paraffin-embedded tissue sections were dewaxed and rehydrated, and antigen retrieval was performed by microwaving in 10 mM sodium citrate buffer, pH 6.0, for 20 min. Sections were then incubated with 3% hydrogen peroxide for 30 min at room temperature to block endogenous peroxidase, then blocked in 10% normal goat serum for 0.5 h. Immunostaining was performed by incubating with anti-CXCR4 (1:500, ab124824, Abcam Corp., UK) or anti-CYP1B1 (1:1,000, ab32649, Abcam Corp.) at 4°C overnight. Slides were then washed in PBS and incubated with secondary antibody (anti-rabbit detection system; Boster, China) for 30 min at 37°C. Staining was visualized with 3,3'-diaminobenzidine and counterstained with hematoxylin.

**Quantitative RT-PCR.** Total RNA was extracted from A549 and A549/DDP cells using TRIzol (Invitrogen, USA) according to the manufacturer's instructions and quantified by Nanodrop 2000 (Thermo Scientific, USA). RNA (1 µg) was reverse-transcribed to cDNA with random primers using

the Promega reverse transcriptase kit (USA) according to the manufacturer's protocol. To assess gene expression, cDNAs were amplified with the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan) using the QuantStudio 6 Flex Real-Time PCR system (Life Technologies, USA). The relative level was calculated by the comparative Ct method ( $2^{-\Delta\Delta C_t}$ ). The sequences of specific primers were as follows: CXCR4, forward, 5'-CCGTGGCAAACCTGGTACTTT-3'; reverse, 5'-GACGCCAACATAGACCACCT-3'. CYP1B1, forward, 5'-AAGTTCTTGAGGCACTGCGAA-3'; reverse, 5'-GGCCGGTACGTTCTCCAAAT-3'.

**Western blot analysis.** Cells were washed with ice cold PBS and lysed by RIPA supplemented with protease inhibitor PMSF on ice. The extracted proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, USA). The membrane was blocked with 5% non-fat milk TBS-T (0.1% Tween-20, 100 mM Tris-HCl, 0.9% NaCl) and incubated with anti-CXCR4 (1:200, sc-6190, Santa Cruz, USA) or anti-CYP1B1 (1:1,000, ab32649, Abcam Corp.) with gentle shaking at 4°C overnight. After washing three times, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The protein bands were visualized with ECL plus western blotting detection reagents (Thermo Scientific).

**Immunofluorescence assay.** Cells were fixed and permeabilized with 2% paraformaldehyde and 0.5% Triton X-100. After overnight incubation with anti-CXCR4 (1:100, sc-6190, Santa Cruz), the specimens were rinsed thoroughly and treated with anti-goat antibodies, respectively, conjugated with Alexa Fluor 488 and Alexa Fluor 594 (Invitrogen), diluted 1:400 (1 h, 37°C). Nuclei were stained using 0.3  $\mu$ M DAPI (Molecular BioProbes, USA). The digital images were then captured with a cooled CCD camera and processed with the help of Photoshop (Adobe) software.

**siRNA transfect assay.** Cells were plated 24 h before transfection. At 30-50% confluence, cells were transfected using Lipofectamine™ 2000 (Invitrogen) with siRNA duplexes specific for human CYP1B1 (RiboBio, China) or control negative control (NC) siRNA. The following were the sequences of siRNAs used in the study, CXCR4 siRNA: sense, GUGGCAACUGGUACUUUG; antisense, CAAAGUACCAGUUUGCCAC. CYP1B1 siRNA: sense, GCAUGAUGCGCAACUUCUU; antisense, AAGAAGUUGCGCAUCAUGC. In addition, mock control was included where cells were treated with Lipofectamine 2000 alone. The siRNA experiment was carried out for 48 h to analyse the RNA expression level by qRT-PCR and 72 h to analyse the protein expression level by western blot analysis.

**Apoptosis assay.** Cells were dual stained with the Annexin V-FITC apoptosis detection kit (BD Biosciences, USA) according to the manufacturer's protocol. Stained cells were immediately analyzed by flow cytometry (FACS Aria III, BD Biosciences).

**Cell proliferation assay.** A549 and A549/DDP cells were seeded in 96-well plate at the density of  $1.0 \times 10^4$  cells per

well overnight for adherence. The next day cells were transfected with CXCR4-siRNA or CYP1B1-siRNA. After 48 h of transfection, 10  $\mu$ l CCK-8 solutions was added to each well at indicated times and incubated for another 2 h. The absorbance of each well was obtained from Perkin-Elmer 2030 Victor x multilabel plate reader (Perkin-Elmer, Waltham, MA, USA) at 450 nm.

**Gene expression omnibus dataset correlation analysis.** We downloaded GSE30219 (14) and GSE41271 (15,16) which were the largest NSCLC datasets from Gene expression omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) to verify the correlation of CXCR4 and CYP1B1. GSE30219 included 272 NSCLC patients and GSE41271 included 263 NSCLC patients.

**Statistical analysis.** Each assay was performed in triplicate and repeated a minimum of three times. Statistical analysis was performed using SPSS 19.0 or GraphPad Prism 5 software. Data are reported as means  $\pm$  SD. Statistical differences were analyzed by Student's t-test for data between control and treated groups, or a one-way analysis of variance for data from multiple groups, with the level of significance set at  $P < 0.05$ .

## Results

**CXCR4 expression closely correlated with NSCLC cisplatin resistance.** To investigate the association between CXCR4 and cisplatin resistance in NSCLC, we first detected the expression levels of CXCR4 in 64 NSCLC patients by immunohistochemistry. We found that the levels of CXCR4 were significantly increased in cancer samples from cisplatin-resistant patients, compared to that in samples from cisplatin-sensitive patients. We categorized the NSCLC-sensitive group into negative (40.6%), weak (28.1%), moderate (18.8%) and strong (12.5%) and resistance group into negative (15.6%), weak (34.4%), moderate (21.9%) and strong (28.1%), respectively (Fig. 1A and B). The staining scores being  $1.63 \pm 1.07$  in the cisplatin-resistant group and  $1.03 \pm 1.06$  in the sensitive group (Fig. 1C). Furthermore, we investigated the expression levels of CXCR4 in A549 and A549/DDP cells by qPCR, western blot analysis and immunofluorescence. As shown in Fig. 1D and E, the expression levels of CXCR4 mRNA and protein in A549/DDP were significantly higher than that in A549 cells. These results indicate that CXCR4 may be involved in NSCLC cancer cisplatin resistance.

**Clinical characteristics associated with CXCR4 expression in NSCLC patients.** We carried out a comparative analysis of clinicopathological characteristics and associated CXCR4 expression. As shown in Table I, 75% (n=49) of the patients were >65 years old, with ~68% (n=44,  $P=0.043$ ) being male patients, who accounted for ~76% (n=35) of all patients with high CXCR4 expression. CXCR4 high expression was observed to be mostly associated with T2N0M0 cancer stages, indicating an increased tumor size at T2 stage ( $P=0.004$ ), with no nearby lymph node invasion and no metastatic spread to other regions. CXCR4 high expression was also associated with a slightly higher percentage prevalence in adenocarcinoma as compared to squamous carcinoma and a higher drug resistance levels ( $P=0.026$ ). This therefore suggests that high

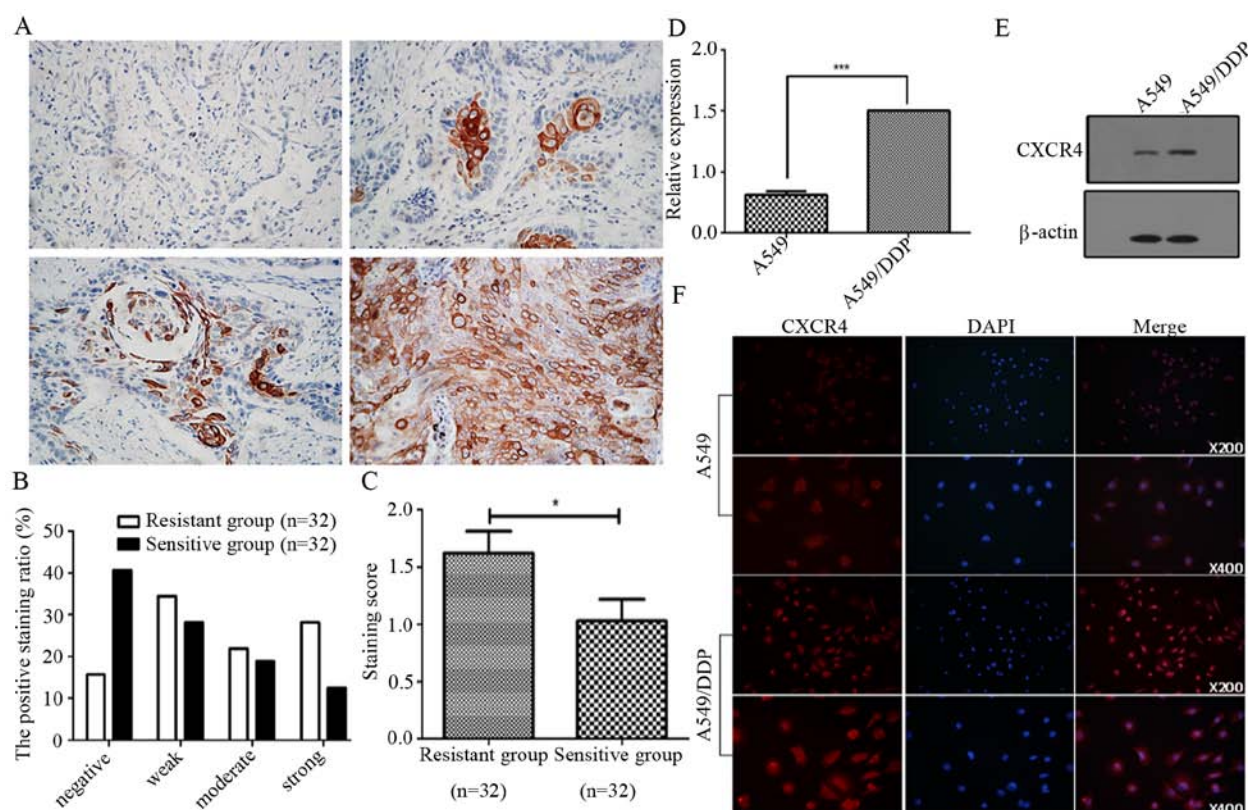


Figure 1. CXCR4 expression correlates with NSCLC cisplatin resistance. (A) Representative images of NSCLC patients by immunohistochemistry (magnification, x40). (B) Positive staining ratio of cisplatin-resistant and sensitive NSCLC group. (C) Immunohistochemistry staining score of cisplatin-resistant and sensitive group. (D) qPCR analysis of CXCR4 expression in A549 and A549/DDP cells. (E) Western blot analysis for the expression of CXCR4 in A549 and A549/DDP cells. (F) Immunofluorescent assay for the expression of CXCR4 in A549 and A549/DDP cells. \*\*\* $P < 0.001$ .

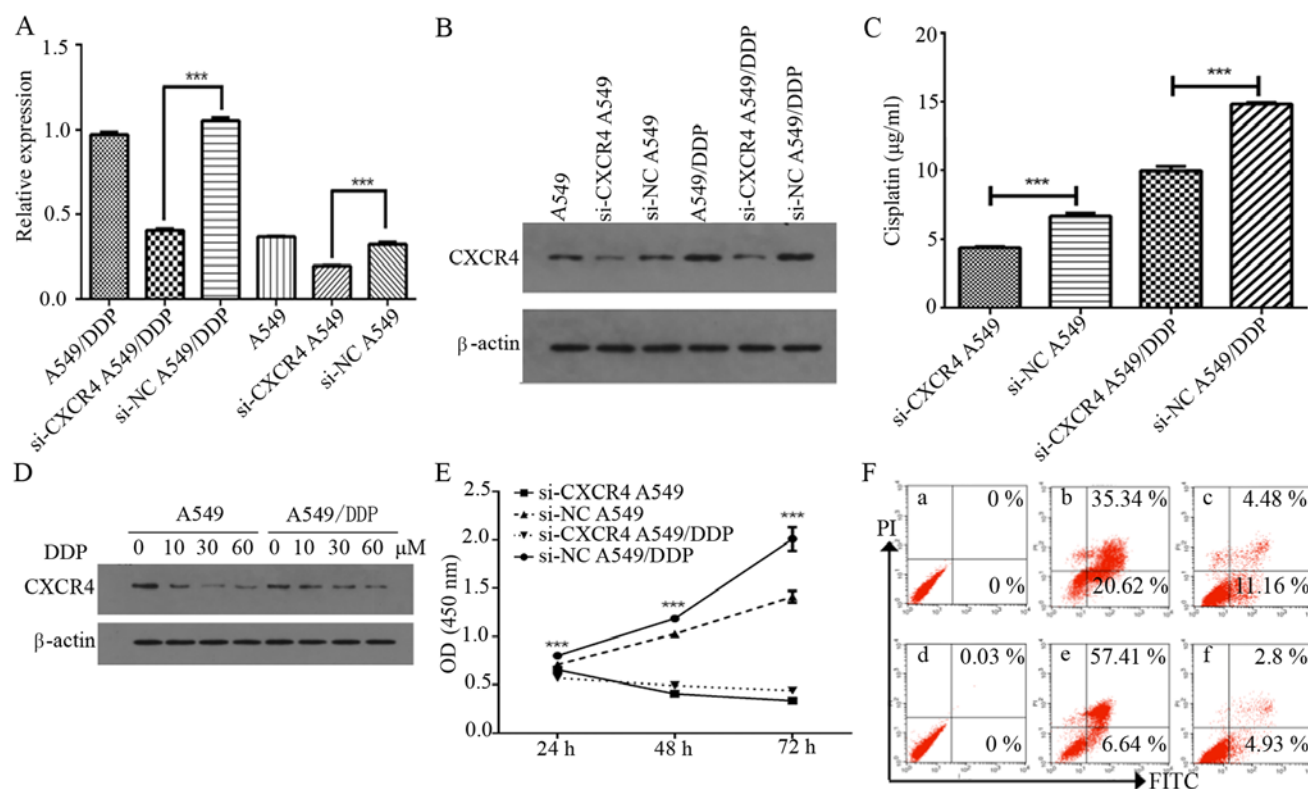


Figure 2. Effect of cisplatin in A549 and A549/DDP cells. (A and B) After CXCR4 siRNA transfection, CXCR4 mRNA was determined by qPCR (A) and protein expression was measured by western blot analysis (B). (C) Chemosensitivity assay was conducted at 48 h after cisplatin treatment. Values indicate mean  $IC_{50} \pm SEM$ . (D) CXCR4 protein was determined after treated with the different concentrations of cisplatin by western blot analysis. (E) Growth curves from siRNA transfected cells assessed by CCK8 assay. (F) Ratio of apoptotic cells was determined by flow cytometry.

Table II. The correlation of CXCR4 and cytochrome p450 molecules.

	GSE219 (n=72) r, P-value	GSE271 (n=263) r, P-value
CYP1A1	0.014, 0.819	0.027, 0.667
CYP1A2	0.022, 0.721	-0.07, 0.255
CYP1B1	0.616, 0.000	0.538, 0.000
CYP2C8	0.058, 0.337	-0.114, 0.064
CYP2C9	-0.134, 0.027	0.028, 0.657
CYP2C18	-0.135, 0.026	-0.028, 0.654
CYP2C19	-0.25, 0.000	-0.173, 0.005
CYP2E1	-0.135, 0.026	-0.087, 0.161
CYP3A4	0.063, 0.3	0.033, 0.597
CYP3A5	-0.409, 0.000	-0.118, 0.055
CYP3A7	-0.264, 0.000	0.025, 0.689
CYP3A43	-0.287, 0.000	-0.204, 0.001

CXCR4 expression levels may be influenced by patient age and sex, thereby having an effect on the tumor staging, cancer subtype and drug resistance characteristics.

**CXCR4 knockdown enhances cisplatin-induced apoptosis and inhibits cell growth.** To investigate whether CXCR4 mediates chemotherapy resistance in A549 and A549/DDP cells, we investigated the effect of CXCR4 siRNA on CXCR4 expression. CXCR4 siRNA was observed to inhibit the mRNA and protein expression levels of CXCR4 in A549 and A549/DDP cells ( $P<0.0001$ ) (Fig. 2A and B). We further investigated whether CXCR4 knockdown using CXCR4 siRNA had an effect on cisplatin sensitivity in NSCLC cells, of which it was observed that the half-maximal inhibitory concentration ( $IC_{50}$ ) after 48 h was  $6.665\pm0.215$  and  $4.373\pm0.077$   $\mu\text{g/ml}$  for A549 and si-CXCR4 A549 cells, respectively ( $P<0.0001$ ), while it was found to be  $14.837\pm0.099$  and  $9.969\pm0.318$   $\mu\text{g/ml}$  for A549/DDP and si-CXCR4 A549/DDP cells, respectively ( $P<0.0001$ ). Both the A549 and A549/DDP si-CXCR4 transfected cells were more sensitive to cisplatin treatment than the non-transfected A549 and A549/DDP cells, indicating that CXCR4 knockdown increases sensitivity to cisplatin (Fig. 2C).

In addition, cisplatin effectively inhibited CXCR4 expression in both cells in a dose-dependent manner (Fig. 2D). CCK-8 assay was further applied to confirm the effect of CXCR4 knockdown on proliferation in A549 and A549/DDP cells. As shown in Fig. 2E, si-CXCR4 significantly suppressed cell proliferation in A549 and A549/DDP from 24 to 72 h ( $P<0.0001$ ). Flow cytometry results indicated that A549 and A549/DDP transfected with negative control siRNA had an apoptotic cell percentage of  $\sim 11.16$  and  $4.93\%$ , respectively, while the percentage of apoptotic cells in the CXCR4 siRNA cells was  $20.62$  and  $6.64\%$ , respectively (Fig. 2F). These results also suggest that knockdown of CXCR4 with siRNA-

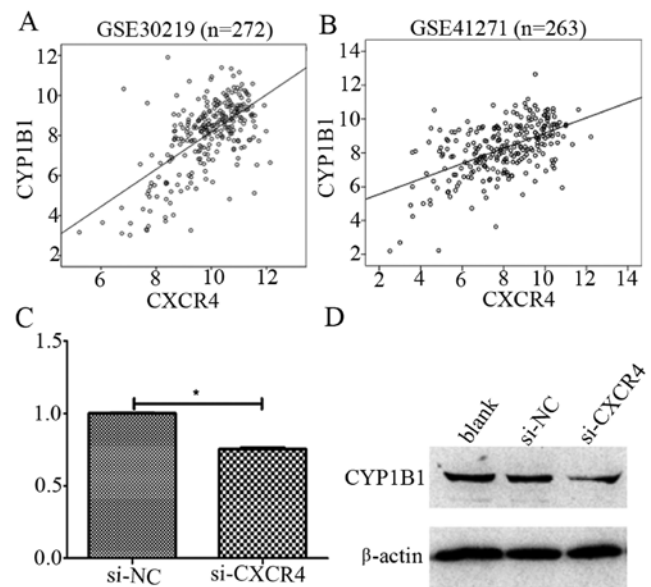


Figure 3. CXCR4 is upregulated with the expression of CYP1B1. (A) Expression of CYP1B1 associated with CXCR4 in GEO30219 dataset. (B) Expression of CYP1B1 associated with CXCR4 in GEO41271 dataset. (C) qPCR analysis of the expression of CYP1B1 after treatment with CXCR4 siRNA (50 nM, 48 h). (D) Western blot analysis for the expression of CYP1B1 after treatment with CXCR4 siRNA (50 nM, 72 h). GAPDH was detected as a loading control. All experiments were performed at least in triplicate. \*\*\*\* $P<0.001$ .

contributes to the recovery of cisplatin-induced apoptosis in A549 and A549/DDP cells.

**CXCR4 promotes cisplatin resistance through upregulation of CYP1B1.** Increasing evidence has shown that many cytotoxic drugs are metabolized by cytochrome p450 (CYP) enzymes (17-25), we therefore explored the correlation of CXCR4 with CYP-related molecules (CYP1A1, CYP1A2, CYP1B1, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2E1, CYP3A4, CYP3A5, CYP3A7 and CYP3A43) by bioinformatics (Table II). Our results indicated a positive correlation between CXCR4 and CYP1B1 with  $r=0.616$  ( $P<0.0001$ ) and  $r=0.538$  ( $P<0.0001$ ) in GSE30219 and GSE41271 datasets, respectively (Fig. 3A and B). Furthermore, we investigated whether CXCR4 plays an important role in cisplatin resistance in NSCLC by targeting CYP1B1, by examining the CYP1B1 mRNA and protein expression levels after CXCR4 knockdown by siRNA in A549/DDP cells. It was observed that CYP1B1 mRNA and protein level were significantly downregulated as a result of CXCR4 knockdown (Fig. 3C and D). Taken together, these results demonstrate CXCR4 may promote cisplatin resistance by regulating the expression levels of CYP1B1.

**CYP1B1 regulates NSCLC cell chemotherapy resistance.** To evaluate the effects of CYP1B1 on NSCLC cell survival and resistance to chemotherapy, we examined whether reduction of CYP1B1 levels affects NSCLC cell survival. Transfection with CYP1B1 siRNA resulted in a marked reduction in endogenous levels of CYP1B1 in A549/DDP by western blot analysis (Fig. 4A). Knockdown of CYP1B1 significantly decreased the survival rate of A549/DDP cells, but did not decrease the survival rate of A549 cells (Fig. 4B). A549 and A549/DDP transfected with negative control siRNA were observed to have



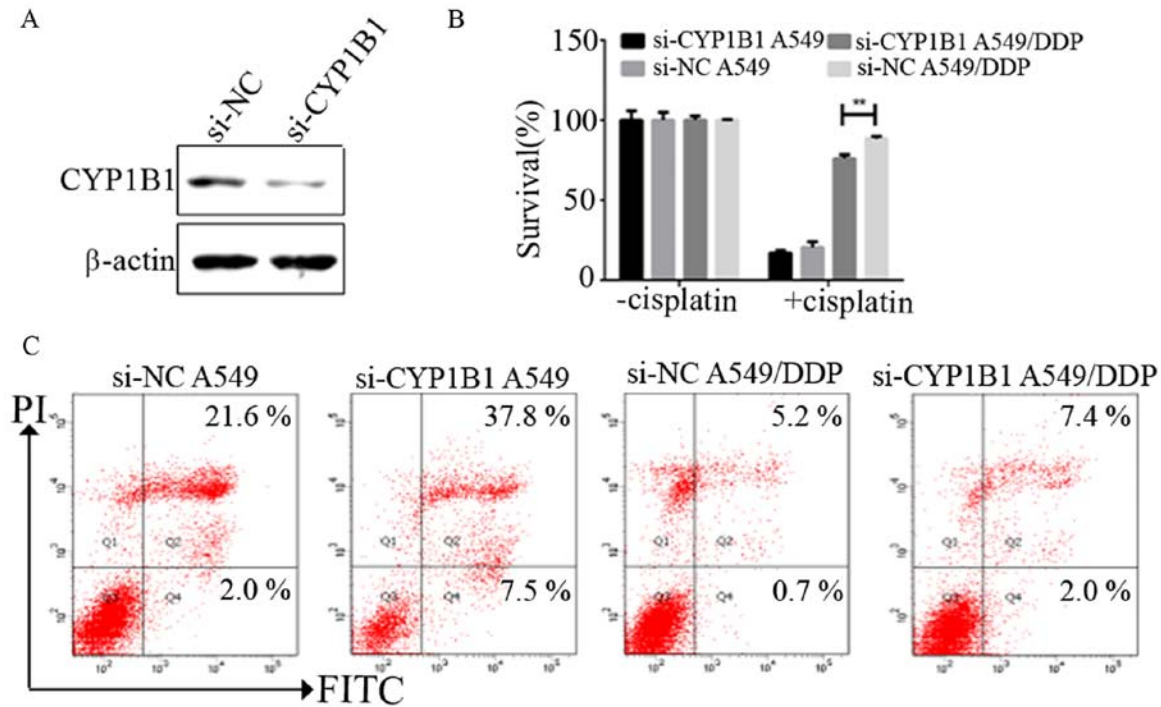


Figure 4. CYP1B1 knockdown sensitizes NSCLC cells to cisplatin *in vitro*. (A) Western blot analysis of the interference efficiency of CYP1B1 siRNA in A549 and A549/DDP. (B) CCK8 assay for the percentage of survival cells in A549 or A549/DDP cells when treated with/without si-CYP1B1. (C) Flow cytometry assay for the ratio of apoptotic cells in A549 or A549/DDP cells when treated with/without si-CYP1B1.

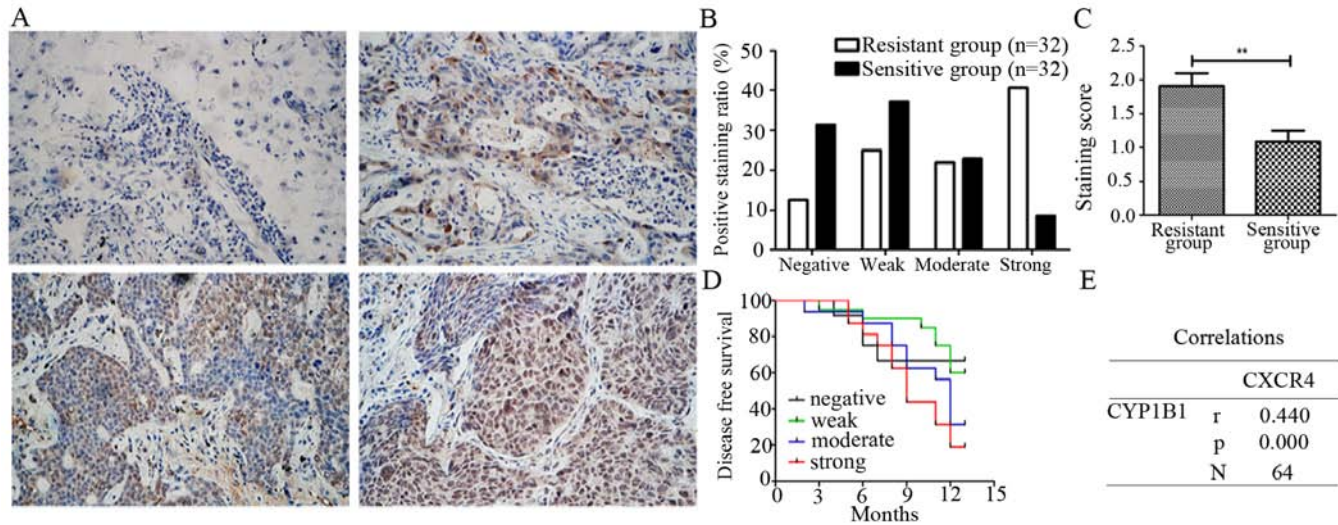


Figure 5. CYP1B1 overexpression was associated with poor survival in cisplatin-resistant NSCLC. (A) Representative images of NSCLC by immunohistochemistry (magnification, x40). (B) Positive staining ratio of cisplatin-resistant and sensitive group. (C) Immunohistochemistry staining score of cisplatin-resistant and sensitive group. (D) Kaplan-Meier estimate of disease-free survival for 64 patients with NSCLC according to the expression of CYP1B1. (E) Correlation of CXCR4 and CYP1B1 expression in 64 NSCLC by immunohistochemistry.

apoptotic cell percentages of 2.0 and 0.7%, respectively, while the percentage of apoptotic cells was observed to increase to ~7.5 and 2.0% in CYP1B1 knockdown cells, respectively (Fig. 4C).

Taken together, it was observed that the percentage of apoptosis was significantly increased in A549 and A549/DDP cells after inhibiting CYP1B1 expression by siRNA, thus suggesting that CYP1B1 may play a significant role in NSCLC cell survival and resistance to chemotherapy.

*CYP1B1 overexpression is associated with poor survival and cisplatin resistance in NSCLC.* To further investigate the association between CYP1B1 and cisplatin resistance in NSCLC, we examined the expression of CYP1B1 in 32 cisplatin-sensitive NSCLC tissues and 32 cisplatin-resistant NSCLC tissues by immunohistochemistry. The expression levels of CYP1B1 in most cisplatin-sensitive tissues was observed to range from weak to undetectable, while that in cisplatin-resistant tissues ranged from weak to strong. Thus we categorized the NSCLC

cisplatin-sensitive group into negative (25.0%), weak (37.5%), moderate (28.1%) and strong (9.4%), while the cisplatin-resistant group was categorized into negative (12.6%), weak (25.0%), moderate (21.9%) and strong (40.6%) (Fig. 5A and B), the staining scores being  $1.69 \pm 1.19$  in the cisplatin-resistant group and  $1.08 \pm 0.97$  in cisplatin-sensitive group (Fig. 5C).

Having analyzed the CYP1B1 expression levels in cisplatin-sensitive and resistance groups, we further went on and analyzed CYP1B1 expression with associated patient clinical outcomes of all 64 NSCLC tissues. It was observed that expression of CYP1B1 correlated with disease-free survival and strong expression thus staining of CYP1B1 suggested poor survival as compared to moderate, weak and negative CYP1B1 expression and staining (Fig. 5D). Lastly, we confirmed that CXCR4 has a positive correlation with CYP1B1 ( $r=0.44$ ,  $P<0.001$ ) in NSCLC patients (Fig. 5E). These results suggest that CYP1B1 overexpression is associated with poor survival and cisplatin resistance in NSCLC.

## Discussion

Cisplatin-based chemotherapy is one of the effective methods used in treatment of malignancies, however, cisplatin resistance has become a major therapeutic obstacle in clinical practice, and as such, much attention has been paid to the underlying mechanisms of chemotherapy resistance. In recent years, increasing evidence has revealed that CXCR4 is involved in chemoresistance in several types of cancers, including mantle cell lymphoma (26), chronic myelogenous leukemia (27) and breast cancer (28). However, the function of CXCR4 in NSCLC chemoresistance remains unknown. In this study, we explored the potential role of CXCR4 in NSCLC chemotherapeutic responses.

We investigated the possible association of CXCR4 high expression with cancer progression and poor prognosis in NSCLC tissues. Thus, we examined the expression of CXCR4 in 64 NSCLC patient samples. We observed that patient samples with a high CXCR4 expression significantly associated with a shorter disease-free patient survival as seen in the cisplatin-resistant group, when compared with the cisplatin-sensitive group, thereby suggesting that CXCR4 high expression might be closely related with chemoresistance. This was further amplified when we analyzed the clinicopathological characteristics in relation to the CXCR4 expression, in which we observed that CXCR4 was mostly expressed in male patients >65 years old and associated with the tumor size and drug resistance levels. Other researchers have also reported the possible association of CXCR4 expression levels with patient sex and tumor staging (29,30).

Some studies have previously revealed the association between CXCR4 overexpression and chemoresistance in some cancers. Nakamura *et al* observed that CXCR4 overexpression promoted drug resistance in cancer cells by upregulating transforming growth factor- $\beta 2$  (12). Similarly, Sison *et al* reported that CXCR4 overexpression protected pediatric acute lymphoblastic leukemia cells from chemotherapy-induced apoptosis, thus enhancing chemoresistance when the cells were co-cultured with bone marrow stroma. Treatment with a CXCR4 inhibitor plerixafor diminished the protective effect of apoptosis, thus conferring chemosensitivity (31). High CXCR4

expression levels have also been significantly associated with cisplatin-based chemotherapeutic resistance in epithelial ovarian cancer (13).

In this study, by using cisplatin-resistant NSCLC cells A549/DDP and its parental A549 cells, we demonstrated that CXCR4 might play an important role in regulating the chemoresistance in NSCLC, as it was observed that CXCR4 was highly expressed in A549/DDP cells and CXCR4 knockdown resulted in reduced proliferation and cell apoptosis induction. Analyses of the  $IC_{50}$  values also indicated that CXCR4 knockdown resulted in increased chemosensitivity of A549/DDP cells to cisplatin.

Therefore, in order to define the mechanisms of CXCR4 associated NSCLC chemoresistance, we further downloaded two largest datasets of NSCLC patients from gene expression omnibus and performed a correlation analysis with CYP related-molecules. We found only CYP1B1 had a positive correlation with CXCR4. CYP1B1 belongs to cytochrome p450 family, which is a multi-gene family of enzymes implicated in the metabolism of a diverse range of xenobiotics and endogenous compounds (24). Several researchers have proposed interesting concepts, in explaining the mechanisms of CYP1B1 induced cytotoxic effect reduction of chemotherapeutic drug (20,21,23,25), as other have mainly focused on CYP1B1 polymorphism in cancer process (22,32,33). Of particular interest, Patel *et al* reported that CYP1B1 may be regulated by cytokines, as interleukin-6 was observed to mediate of CYP1B1 upregulation via DNA methylation in colorectal cancer (34). Therefore, we further investigated the differential expression of CYP1B1 between the chemosensitive and chemoresistance groups, and we observed that CYP1B1 levels were significantly increased in the chemoresistance group. We also observed that CYP1B1 knockdown decreased the cell survival rates, enhanced cell apoptosis and partially reversed the chemoresistance of cisplatin in cancer cells. Additionally, we also observed that CXCR4 mediated cisplatin resistance via regulating CYP1B1 expression.

In conclusion, this study shows that CXCR4 mediates cisplatin chemoresistance in NSCLC in a CYP1B1-dependent manner. Thus, it could be used as a potential therapeutic target in NSCLC chemoresistance patients and as a clinically useful predictor of cisplatin response.

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