CXCR4 is involved in CD133-induced EMT in non-small cell lung cancer

ZHENBO TU[1*], SONGPING XIE[2*], MENG XIONG[1*], YINCHU LIU[3], XIANGYONG YANG[4], KINGSLEY MIYANDA TEMBO[1], JIE HUANG[2], WEIDONG HU[5], XIAOXING HUANG[1], SHAN PAN[1], PAN LIU[1], EHTISHAM ALTAF[1], GANJUN KANG[2], JIE XIONG[1] and QIUPING ZHANG[1,6]

[1]Department of Immunology, School of Basic Medical Sciences, Wuhan University; [2]Department of Thoracic Surgery, Renmin Hospital of Wuhan University; [3]School of Basic Medical Sciences, Wuhan University; [4]Engineering and Technology College, Hubei University of Technology; [5]Department of Thoracic Oncology, Zhongnan Hospital and [6]Hubei Provincial Key Laboratory of Developmentally Originated Disease, School of Basic Medical Sciences, Wuhan University, Wuhan, Hubei, P.R. China

Received September 2, 2016; Accepted November 3, 2016

DOI: 10.3892/ijo.2016.3812

Abstract. Metastasis is the major cause of death in patients with non-small cell lung cancer (NSCLC), and epithelial-mesenchymal transition (EMT) has been observed to be one of the key regulators of metastasis in certain cancers as it confers an invasive phenotype. CD133 is a widely used cancer stem cell (CSC) marker, and CD133-positive cancer cells are thought to be tumor-initiating cells with CSC characteristics, while CXCR4, a stromal-derived-factor-1 specific chemokine receptor, is highly expressed in NSCLC tissues and participates in cancer progression by regulating cell anti-apoptosis. We previously demonstrated that CXCR4 promotes NSCLC chemoresistance by upregulating CYP1B1, however, the relationship of CD133, CXCR4 and EMT processes in NSCLC metastasis are unclear. In this study, we detected a CD133 and CXCR4 high expression in tissue specimens from 64 NSCLC patients by immunohistochemistry, of which CD133 and CXCR4 were found to be positively associated with metastatic NSCLC patients. CD133 was found to promote NSCLC tumorigenesis and mediated the expression of CXCR4. Furthermore, CD133/CXCR4 co-expression was found to be an independent prognostic factor as shown by univariate and multivariate Cox regression analysis, and was observed to regulate the expression of EMT-related molecules and transcriptional factors in NSCLC. In addition, our results showed that E-cadherin and Vimentin were simultaneously downregulated and upregulated, in $CD133^+CXCR4^+$ A549 cells, respectively. While E-cadherin was upregulated and Vimentin was downregulated in metastatic NSCLC patients. Vimentin expression was also observed to have a positive correlation with CD133/CXCR4 co-expression in NSCLC patients and survival analysis results suggested that Vimentin high expression might be significantly associated with poor survival rates of the patients. Thus, these results suggest that the CD133/CXCR4/EMT axis may be a prognostic marker and may provide novel targets for combinational therapies in the treatment of NSCLC.

Introduction

Lung cancer is one of the increasingly potential causes of cancer deaths in both males and females worldwide (1). It was estimated that 595,690 Americans will die from cancer in 2016, with >27% being due to lung cancer (2). Cancer statistics in China indicated that an estimated 4,292,000 new cancer cases and 2,814,000 cancer deaths would occur in 2015, with lung cancer being the most common incident cancer and the leading cause of cancer death (3). The main lung cancer types include small cell lung cancer and non-small cell lung cancer (NSCLC), which together account for ~87% of all lung cancer cases (4). Most patients with NSCLC are observed to be in advanced stages of cancer at diagnosis, however even when diagnosed at an early stage and treated surgically, cancer recurrence has been observed in patients, with spread to the lymph node, liver and other tissues.

Metastasis is the major cause of death in patients with NSCLC. The process in which immobile epithelial cells transform to invasive cell types in what has been termed as epithelial-mesenchymal transition (EMT), is increasingly being accepted as having an instrumental role in tumor metastasis (5). EMT is characterized by the loss of cell-cell

Correspondence to: Professor Qiuping Zhang or Dr Jie Xiong, 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
E-mail: jiexiong@whu.edu.cn

*Contributed equally

Abbreviations: NSCLC, non-small cell lung cancer; EMT, epithelial-mesenchymal transition; CSC, cancer stem cell

Key words: non-small cell lung cancer, metastasis, CD133, CXCR4, epithelial-mesenchymal transition

adhesions, and the transition to spindle-like cells that are capable of invading the extracellular matrix (6). An increasing number of studies have demonstrated that cancer stem cell (CSC) molecule CD133 promotes tumor invasion and metastasis by inducing EMT processes (7-15).

Human CD133 is a five transmembrane single-chain glycoprotein that belongs to the prominin family containing two large extracellular and two small intracellular loops (16). Lee *et al* examined the effects of IL-6 on growth, EMT processes and metastatic ability of $CD133^+$ and $CD133^-$ cell subpopulations isolated from NSCLC cells. Of which they demonstrated the dual roles of IL-6 in regulating growth of $CD133^-$ and $CD133^+$ subpopulations of lung cancer cells, as well as the positive regulation of EMT/metastasis increase by IL-6 in $CD133^+$ cells, but not in $CD133^-$ cells (14). Some researchers have suggested that CSCs are present in both blood and tumor tissue of NSCLC patients, and that EMT-inducing transcriptional factors Bmi1 may play an important role in initiation and maintenance of CSCs and might be involved in vascular dissemination of NSCLC (12). Others have shown that elevated CD133 expression is the signature marker of EMT and CSC association in lung adenocarcinoma (17). Additionally, Bertolini *et al* showed that $CD133^+/CXCR4^+/EpCAM^-$ lung cancer-initiating cells sustain metastasis and correlate with poor prognosis (18).

CXCR4, a seven-transmembrane G protein-coupled receptor, is a physiological receptor for stromal-derived-factor-1 (19) which is highly expressed in various types of human tumors (20-24). A number of studies have demonstrated the vital role of CXCR4 in cancer cell survival, proliferation, invasion and metastasis, and that CXCR4 promotes EMT processes in various cancers (25-29), with $CD133^+CXCR4^+$ cells showing a stronger invasiveness capacity (18,30-33).

Although researchers have suggested a possible role for CD133 and CXCR4 in NSCLC metastasis, the precise relationship of CD133, CXCR4 and EMT processes are unclear and requires further investigation. Therefore, in this study, we detected the expression of CD133 and CXCR4 in NSCLC patients and investigated the effects of CD133 in NSCLC tumorigenesis. We further investigated the CXCR4/CD133 interaction and explored the possible molecular mechanisms by which CXCR4/CD133 regulates NSCLC metastasis via the EMT process.

Materials and methods

GEO databases analysis. Gene expression data were obtained from the publicly available GEO databases. We chose GSE10072, GSE40275 and GSE63459 to identify the expression signature of CD133 because these sets included normal (n>30) and tumor samples (n>30). Original values were normalized by application of $\log_2$ method and comparisons were performed by two-tailed Student's t-test for GSE10072 and GSE40275, and paired t-test for GSE63459. We chose GSE30219 because it was the largest lung cancer dataset to identify the correlation between CD133 and CXCR4 by two-tailed bivariate analysis.

NSCLC tissue samples. A total of 64 specimens was collected from patients with primary NSCLC surgical resection at Renmin Hospital of Wuhan University from May 2012 to April 2015. Patients were followed-up by telephone until recurrence/metastasis or until May 2016. 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 (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), containing 100 U/ml penicillin and 100 mg/ml streptomycin. A549 cells were cultured in a 5% CO_2 air incubator at 37˚C and passaged using 0.25% trypsin-EDTA (Gibco) when they reached confluence.

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), anti-CD133 (1:200, orb99113; Biorbyt Ltd., UK), anti-E cadherin (1:500, ab40772; Abcam Corp.) or anti-vimentin (1:500, ab92547; 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. The final score is the average of the percentage of stained area [scored as 0 (without staining), 1 (<25%), 2 (25-50%) and 3 (>50%)] and intensity [scored as 0 (without staining), 1 (light yellow), 2 (yellow) and 3 (brown yellow)] of stained cells The expression levels were divided into low expression (mean score <2).

Magnetic cell sorting. A549 cells were washed and suspended in PBS at a maximal concentration of $1.0x10^8$ cells. Cells were incubated at 4˚C with 100 μl FcR blocking reagent and 100 μl CD133 Micro Beads (130-097-049, Miltenyi Biotec, Germany) for 30 min. Cells were isolated into $CD133^+$ and $CD133^-$ cells by using MS columns (130-042-201, Miltenyi Biotec) and LD columns (130-042-901, Miltenyi Biotec) according to the manufacturer's instructions.

Immunofluorescence assay. Cells were fixed and permeabilized with 2% paraformaldehyde and 0.5% Triton X-100. After overnight incubation with anti-CD133 (1:50, 18470-1-AP; Proteintech, USA), the specimens were rinsed thoroughly and treated with anti-goat antibodies (1:100, BA1032, Boster), respectively. Nuclei were stained using 0.3 μM DAPI (C1002, Beyotime Biotechnology, China). The digital images were then captured with a cooled CCD camera and processed with the help of photoshop (Adobe) software.

Clone formation assay. Cells (500 cells/well) were seeded in 6-well plate and allowed to grow for 2 weeks in 37˚C incubator. The cells were then washed twice with ice-cold PBS, fixed by methanol for 15 min and stained with Giemsa for 15 min. The

images of staining and clone spheres of cells were obtained by a charge coupled device camera.

Cell proliferation assay. Cells were seeded in a 96-well plate at the density of $1.0x10^4$ cells per well for 48 h, 10 μl CCK8 solution was added to each well at indicated times and incubated for another 2 h. The absorbance of each well was obtained from PerkinElmer 2030 Victor X multi-label plate reader (Perkin-Elmer, Waltham, MA, USA) at 450 nm.

SiRNA transfect assay. Cells were plated 24 h before transfection at 30-50% confluence, cells were transfected using Lipofectamine™ 2000 (Invitrogen, USA) with siRNA duplexes specific for human CD133 (Ribobio, China) or negative control (NC) siRNA. The following were the sequences of CD133 siRNA used in the study: sense-CUGGGAAGCU AUUUAAUAA; antisense-UUAUUAAAUAGCUUCCCAG. In addition, 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 quantitative RT-PCR and 72 h to analyse the protein expression level by western blot analysis.

Quantitative RT-PCR. Total RNA was extracted by using TRIzol (Invitrogen) according to the manufacturer's specifications and quantified by NanoDrop 2000 (Thermo Scientific, USA). Total 1 μg RNA was reverse-transcribed to cDNA with random primers using the Promega reverse transcriptase kit (USA) according to 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 (AB and Invitrogen) USA]. The relative levels were calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$). The sequences of specific primers are shown in Table I.

Semi-quantitative RT-PCR. To assess gene expression, cDNAs were amplified with the 2X Ex Taq™ master mix (CWBio, China) using the Mycycler thermal cycler PCR system (Bio-Rad, USA). The expression of each gene was captured by Gel imaging system (BiD-1D/VILBER, France). The sequences of specific primers are shown in Table I.

Western blot analysis. Cells were washed with ice-cold PBS and lysed by RIPA buffer supplemented with protease inhibitor PMSF on ice. Total protein concentrations were measured by BCA kit (Thermo Scientific) and absorbance was measured by the PerkinElmer 2030 Victor X multi-label plate reader. The extracted proteins were separated by a 12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, USA). The membranes were blocked with 5% non-fat milk TBS-T (0.1% Tween-20, 100 nM Tris-HCl, 0.9% NaCl) and subsequently incubated with anti-CXCR4 (1:200, sc-6190; Santa Cruz, USA), anti-CD133 (1:500, orb99113; Biorbyt Ltd.), anti-E-cadherin (1:10,000, ab40772; Abcam Corp.) or anti-vimentin (1:2,000, ab92547; Abcam Corp.) respectively, 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 blot analysis detection reagents (Thermo Scientific).

Table I. The sequences of specific primers for PCR or qPCR in this study.

Gene	Primer sequences
β-actin	F: 5'-AGCGAGCATCCCCCAAAGTT-3'
	R: 5'-GGGCACGAAGGCTCATCATT-3'
CD133	F: 5'-GCACTCTATACCAAAGCGTCAA-3'
	R: 5'-CACGATGCCACTTTCTCACT-3'
CXCR4	F: 5'-ATCTGTGACCGCTTCTACCC-3'
	R: 5'-CGATGCTGATCCCAATGTAG-3'
E-cadherin	F: 5'-CGTAGCAGTGACGAATGTGG-3'
	R: 5'-CTGGGCAGTGTAGGATGTGA-3'
Vimentin	F: 5'-CGCCAACTACATCGACAAGG-3'
	R: 5'-GGCTTTGTCGTTGGTTAGCT-3'
Snail	F: 5'-TTTACCTTCCAGCAGCCCTACGA-3'
	R: 5'-GCCTTTCCCACTGTCCTCATCT-3'
Slug	F: 5'-TCAAGAAGCATTTCAACGCCTC-3'
	R: 5'-TGAGCTGAGGATCTCTGGTTGT-3'
Twist	F: 5'-AGCAACAGCGAGGAAGAGCC-3'
	R: 5'-CACAGCCCGCAGACTTCTTG-3'

F, forward. R, reverse.

Transwell migration assay. A total of $5x10^4$ cells was plated in the upper chamber, which had a 6.5-mm diameter polycarbonate membrane with an 8-μm pore size coated filter (Corning, USA). The lower chamber was filled with 600 μl DMEM solution containing 10% FBS, which acted as a chemoattractant. After 24 h of incubation at 37˚C, non-migrated cells were removed with cotton swabs and migrated cells were fixed in methanol and stained with 0.1% crystal violet. Images were obtained by light microscope with a charge coupled device camera. Cells from four different fields were counted to obtain the migration rates.

Statistical analysis. All assays were performed in triplicates. Statistical analysis was performed using SPSS 19.0 for Windows or GraphPad Prism 5 software. Data are reported as means ± SD. Statistical differences were analyzed by Student's t-test for data between control and treated groups, or a one-way analysis of variance (ANOVA) for data from multiple groups, with the level of significance set at $p<0.05$.

Results

CD133 and CXCR4 are highly expressed in NSCLC patients with metastasis. To explore the effect of CD133 in lung cancer, we downloaded GSE10072, GSE40275 and GSE63459 to identify the expression of CD133 in lung cancer and normal lung tissues. These results showed that CD133 was highly expressed in lung cancer compared to normal lung tissues

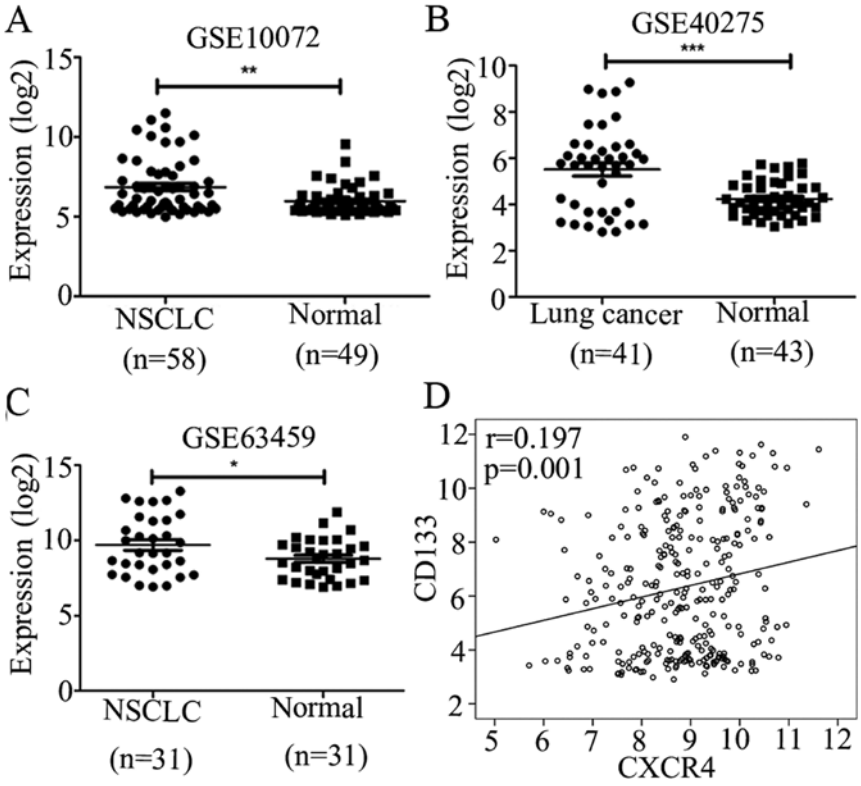


Figure 1. The expression of CD133 in lung cancer and normal lung tissues. Original values are presented as $\log_2$ ratios. Spots showing the level of CD133 mRNA in NSCLC and normal tissues. (A) GSE10072. (B) GSE40275. (C) GSE63459; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$. (D) Correlation between CD133 and CXCR4 in GSE30219.

(Fig. 1A-C), and we chose GSE30219, which is the largest dataset from GEO datasets to identify the correlation of CD133 and CXCR4, it showed that CD133 positively correlated with CXCR4 (Fig. 1D). Further, to investigate the role of CD133 and CXCR4 in NSCLC metastasis, we first detected the expression levels of CD133 and CXCR4 in 64 NSCLC patients by immunohistochemistry, the clinical and pathological characteristics of the patients are shown in Table II. These results showed that CD133 and CXCR4 levels significantly increased in NSCLC metastatic patients as compared to those in non-metastatic NSCLC patients (Fig. 2A). We categorized NSCLC patients with metastasis into low (34.8%) and high group (75.6%), and patients with non-metastasis into low (65.2%) and high group (24.4%) for CD133 expression (Fig. 2B), then we categorized NSCLC patients with metastasis into low (47.8%) and high group (65.9%) and patients with non-metastasis into low (52.2%) and high group (34.1%) for CXCR4 expression, respectively (Fig. 2C). The staining scores were 1.57±0.24 in metastasis group and 0.95±0.14 in non-metastasis group for CD133 expression (Fig. 2D), were 1.61±0.22 in metastasis group and 1.17±0.17 in non-metastasis group for CXCR4 expression (Fig. 2E), respectively.

These results show a significant association between CD133 and CXCR4 expression levels with NSCLC metastatic state in patients. A positive correlation between CD133 and CXCR4 expression was observed as shown in Fig. 2F (r=0.419, p=0.001).

To further study the association between CD133/CXCR4 expression and cancer patient prognosis, we divided the patients into two groups which including CD133/CXCR4

Table II. The clinical and pathological characteristics of the patients.

Features	Patients (n=64)
Age (years)	
≤65	15
>65	49
Gender	
Male	44
Female	20
T stage	
T2	43
T3	14
T4	7
N stage	
N0	36
N1	7
N2	21
M stage	
M0	62
M1	2
Histology	
Adenocarcinoma	39
Squamous carcinoma	25

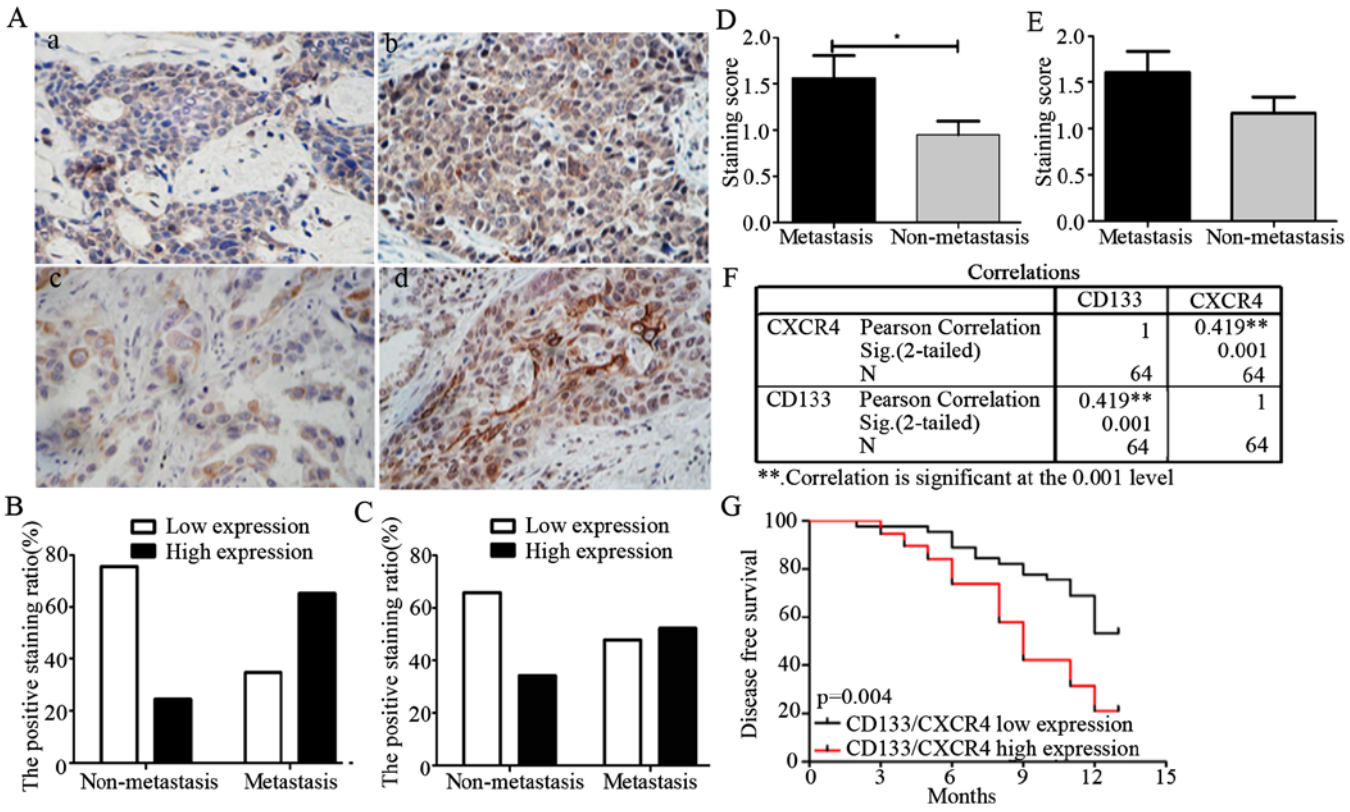


Figure 2. CD133 and CXCR4 are highly expressed in NSCLC patients with metastasis. (A) Representative images of immunohistochemistry staining (magnification, x400) of NSCLC patients. (a) CD133 low expression; (b) CD133 high expression; (c) CXCR4 low expression; (d) CXCR4 high expression. Positive staining ratio of CD133 (B)/CXCR4 (C) in metastatic and non-metastatic NSCLC patients. Positive staining score of CD133 (D)/CXCR4 (E) in metastatic and non-metastatic NSCLC patients. (F) Correlation results of CD133 and CXCR4. (G) Correlation analysis between CD133/CXCR4 co-expression and disease-free survival (red and black curves indicate high and low CD133/CXCR4 co-expression groups of patient death, respectively; *p<0.05).

Table III. Univariate and multivariate Cox regression analyses in NSCLC patients.

	Univariate model			Multivariate model		
Variables	HR	95% CI of HR	P-value	HR	95% CI of HR	P-value
Gender	0.858	0.422-1.745	0.672	0.924	0.385-2.218	0.860
Age	1.007	0.969-1.046	0.729	0.999	0.952-1.048	0.965
Histology	0.754	0.388-1.463	0.404	1.363	0.532-3.488	0.519
T stage	1.475	0.953-2.282	0.081	1.514	0.917-2.499	0.105
N stage	1.659	1.182-2.329	0.003	1.672	1.107-2.526	0.014
M stage	2.610	0.617-11.047	0.192	2.291	0.412-12.732	0.343
CD133/CXCR4 co-expression	2.722	1.400-5.294	0.003	2.480	1.077-5.709	0.033

FDR, false discovery rate; HR, hazards ratio; CI, confidence interval.

high expression (score ≥4) and low expression (score <4) group according to the immunohistochemistry staining score average (stained area and intensity) and we analyzed the correlation between CD133/CXCR4 co-expression and cancer patient disease-free survival, and we observed that patients with high expression of both CD133 and CXCR4 presented a significantly poor survival rate (Fig. 2G). Using univariate and multivariate Cox regression models, we also observed that N stage and CD133/CXCR4 co-expression correlated with a shorter disease-free survival. These results therefore suggest that CD133/CXCR4 co-expression may be a potential independent predictor of NSCLC patient survival (Table III).

CD133 promotes NSCLC tumorigenesis. To investigate the role of CD133 in NSCLC tumorigenesis, $CD133^+$ and $CD133^-$ A549 cells were sorted by magnetic cell sorting and verified by immunofluorescence assay. Our results indicated that CD133 glycoproteins were generally expressed in the $CD133^+$ as compared to the $CD133^-$ groups (Fig. 3A). Furthermore, colony

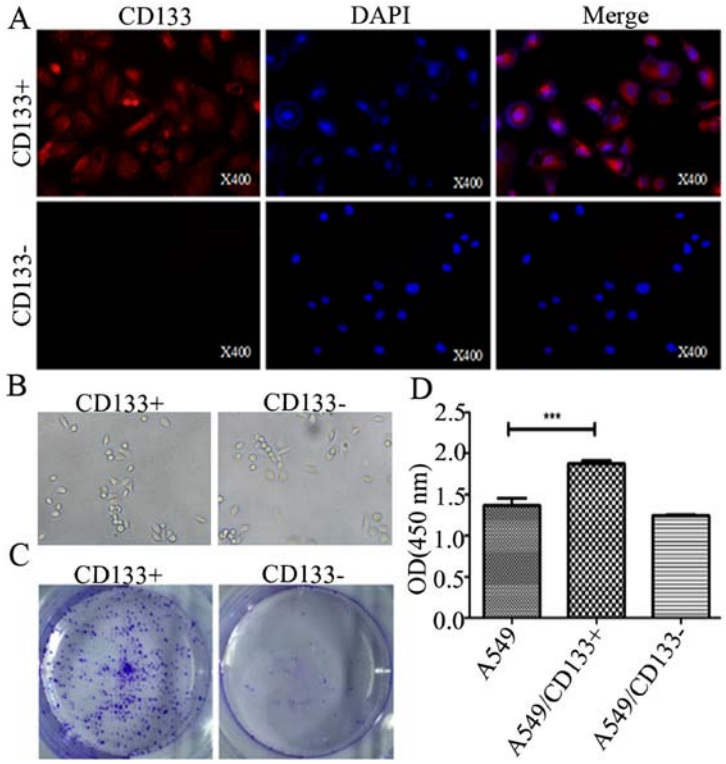


Figure 3. CD133 enhances A549 cells proliferation. (A) Effectiveness of magnetic cell sorting was verified by immunofluorescence assay (magnification, x400). Red and blue indicate CD133 expression and cell nucleus, respectively. (B) Formation of colonies by A549 cell lines after 2-week incubation. (C and D) Cell proliferation capacity was determined in A549 cells, CD133$^+$ A549 cells and CD133-A549 cells by CCK8 assays.

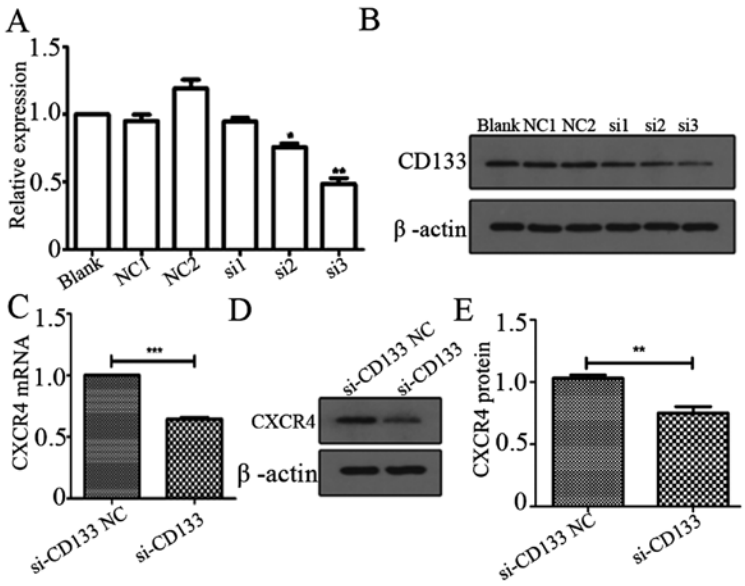


Figure 4. CXCR4 is upregulated by CD133. Silencing effectiveness of CD133 siRNA was verified by qPCR (A) and western blot analysis (B). (C) Expression of CXCR4 after treatment with CD133 siRNA (50 nM, 48 h) by qPCR analysis. (D) Western blot analysis for the expression of CXCR4 after treatment with CD133 siRNA (50 nM, 72 h). β-actin as a loading control. (E) The statistics of (D). All experiments were performed in triplicate. ***p<0.001.

formation assay and CCK8 assay results revealed that CD133$^+$ cells had a significant growth advantage over the control cells as shown in Fig. 3B and C.

CD133 mediates the expression of CXCR4 in NSCLC. We previously found that CD133 and CXCR4 were highly expressed in A549 cells, so here to investigate the effect of CD133 on CXCR4 expression, we first demonstrated that CD133 mRNA and protein levels were significantly inhibited by CD133 siRNA (Fig. 4A and B). We then observed the effect of CD133 on the mRNA and protein expression of CXCR4 by CD133 silencing using CD133 siRNA (si3). We observed that CXCR4 mRNA and protein levels were significantly downregulated as a result of CD133 silencing (Fig. 4C-E). Taken together, these results

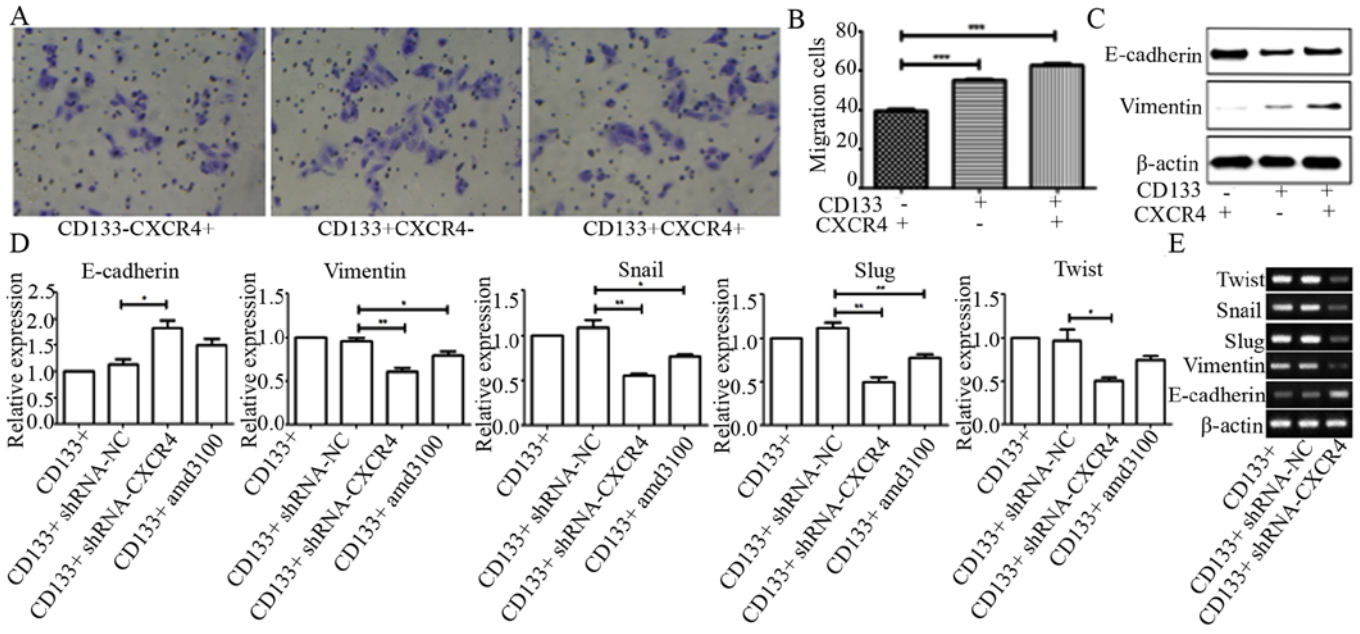
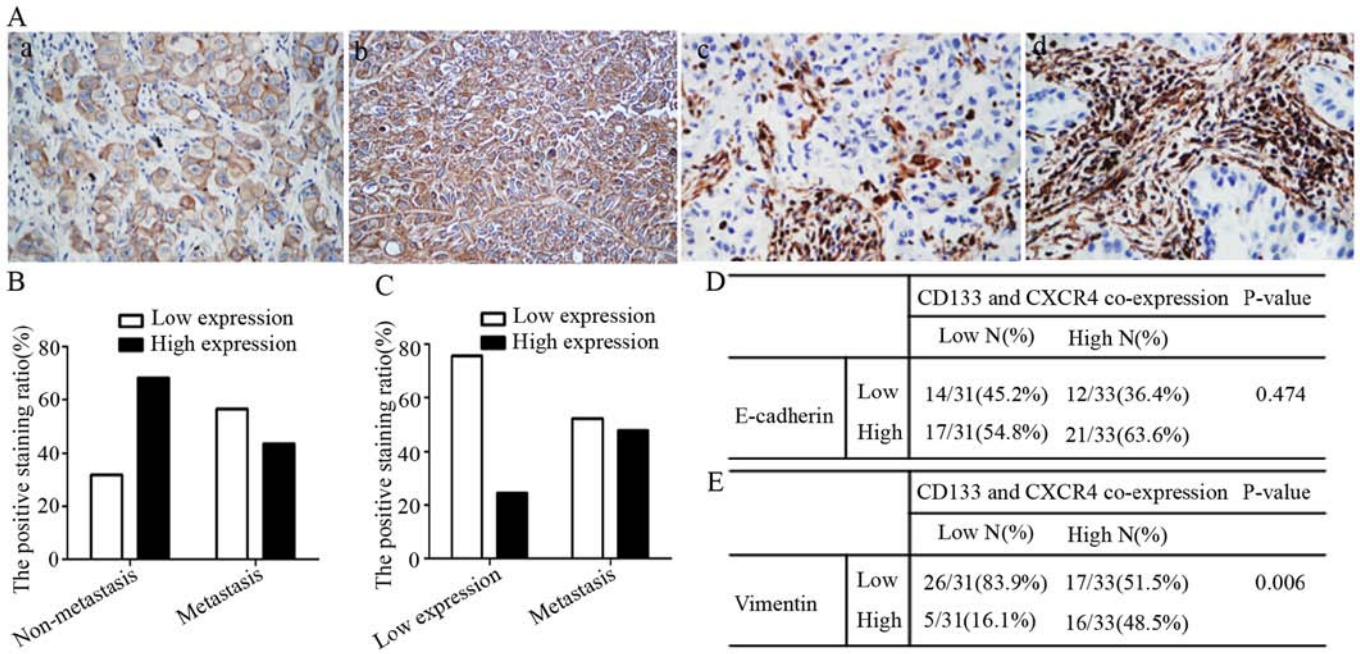


Figure 5. CD133$^+$CXCR4$^+$ promotes EMT process in NSCLC cells. (A) Transwell assay for the invasion of CD133$^-$CXCR4$^+$, CD133$^+$CXCR4$^-$ and CD133$^+$CXCR4$^+$ cells. (B) Western blot assay for the expression of E-cadherin and Vimentin in CD133$^-$CXCR4$^+$, CD133$^+$CXCR4$^-$ and CD133$^+$CXCR4$^+$ cells. (C) qPCR for the expression of E-cadherin, Vimentin, Snail, Slug and Twist in A549/CD133$^+$, A549/CD133$^+$ shRNA-NC, A549/CD133$^+$ shRNA-CXCR4 and A549/CD133$^+$ with amd3100. (D and E) RT-PCR for the expression of E-cadherin, Vimentin, Snail, Slug and Twist in A549/CD133$^+$, A549/CD133$^+$ shRNA-NC and A549/CD133$^+$ shRNA-CXCR4 cells.



D

		CD133 and CXCR4 co-expression		P-value
		Low N(%)	High N(%)	
E-cadherin	Low	14/31(45.2%)	12/33(36.4%)	0.474
	High	17/31(54.8%)	21/33(63.6%)	

E

		CD133 and CXCR4 co-expression		P-value
		Low N(%)	High N(%)	
Vimentin	Low	26/31(83.9%)	17/33(51.5%)	0.006
	High	5/31(16.1%)	16/33(48.5%)	

Figure 6. Vimentin is positively associated with CD133/CXCR4 co-expression. (A) Representative images of immunohistochemistry staining (magnification, x400) of NSCLC patients. (a) E-cadherin low expression; (b) E-cadherin high expression; (c) Vimentin low expression; (d) Vimentin high expression. Positive staining ratio of E-cadherin (B)/Vimentin (C) in metastatic and non-metastatic NSCLC patients. Correlation analysis for E-cadherin (D) or Vimentin (E) with CD133/CXCR4 co-expression in NSCLC patients.

demonstrate that CD133 might play a significant role in the CXCR4 regulation.

CD133$^+$CXCR4$^+$ promotes EMT process in NSCLC. To investigate whether CXCR4 plays a vital role in CD133-induced EMT processes, we first sorted A549 cells into CD133$^-$CXCR4$^+$, CD133$^+$CXCR4$^-$ and CD133$^+$CXCR4$^+$ groups. Our results showed remarkably higher migration rates in CD133$^+$CXCR4$^+$ group as compared to the CD133$^-$CXCR4$^+$ group as revealed by Transwell migration assay (Fig. 5A

and B). We also detected the differential protein expression of E-cadherin and Vimentin in $CD133^{-}CXCR4^{+}$, $CD133^{+}CXCR4^{-}$ and $CD133^{+}CXCR4^{+}$ groups by western blot analysis, and results showed that E-cadherin was decreased, while Vimentin was increased in $CD133^{+}CXCR4^{+}$ as compared to the $CD133^{-}CXCR4^{+}$ (Fig. 5C).

We then went a step further and detected the expression of EMT-related molecules (E-cadherin, Vimentin) and transcriptional factors (Snail, Slug and Twist) in $CD133^{+}$A549 cells in the presence of CXCR4 inhibitors (amd3100) or CXCR4 siRNA. Our findings indicated that E-cadherin was significantly upregulated, while Vimentin, Snail, Slug and Twist were significantly downregulated as a result of CXCR4 silencing or presence of CXCR4 inhibitors (Fig. 5D and E). These results demonstrate that CD133 potentially regulates the expression of CXCR4 in inducing EMT processes.

Expression of Vimentin was positively associated with CD133/CXCR4 co-expression. To investigate the role of CD133, CXCR4 and EMT-related molecules (E-cadherin and Vimentin) in NSCLC metastasis, we detected the expression levels of CD133 and CXCR4, and investigated the correlative expression of EMT-related molecules (E-cadherin and Vimentin) with CD133/CXCR4 co-expression in 64 NSCLC patients by immunohistochemistry. Our results revealed that there was a significant increase in the Vimentin levels, while the expression levels of E-cadherin decreased as shown in Fig. 6A. NSCLC metastatic patients were categorized as either low (56.5%) or high (43.5%) E-cadherin expression groups and non-metastatic patients were categorized as either low (31.6%) or high (68.4%) E-cadherin expression groups. In terms of Vimentin expression, NSCLC metastatic patients were categorized as either low (52.2%) or high (47.8%) expression groups, while non-metastatic patients were further categorized as either low (75.6%) or high (24.4%) expression groups (Fig. 6B and C).

We found E-cadherin expression did not correlate with CD133/CXCR4 co-expression (Fig. 6D), while Vimentin had a positive correlative expression with CD133/CXCR4 co-expression in NSCLC patients ($p=0.006$, Fig. 6E). Taken together these results suggest that Vimentin is involved in CD133/CXCR4 induced NSCLC metastasis.

Discussion

Lung cancer is one of the leading causes of cancer mortality worldwide. Being the most dominating type of lung cancer, NSCLC cases are being diagnosed at more advanced stages and hence has been associated with a poor prognosis in patients (4). So far, little success has been made in significantly improving patient survival rates, as the 5-year survival rates have remained around 18% (34,35).

The main challenges for the NSCLC patient prognosis are tumor metastasis and chemoresistance. Metastasis is a multi-step process, which begins when primary tumor cells break away from their neighboring cells invading the basement membrane, subsequently entering the circulation, either directly or via the lymphatics and eventually homing in distant organs. Tumor cells that successfully adapt to the new microenvironments proliferate from micro-metastases into clinical detectable metastatic tumor (36). The CSCs in tumor microenvironment are vital for the acquisition of the metastatic potential as they initiate, drive carcinogenesis and differentiation. In essence contributing to tumor cellular heterogeneity through the deregulation of the self-renewal processes, thus CSC populations may be a risk factor for carcinogenesis (37).

Previous studies have identified a number of cellular markers which include CD133 (38-40) and CXCR4 (41,42), that may contribute to the properties exhibited by lung CSCs. Chemokines are a family of small secreted proteins of ~70-80 amino acids. CXCR4 is an α-chemokine receptor specific for the CXCL12. Unlike other chemokine receptors that have several ligands, CXCL12 is the only known specific legend for the receptor CXCR4 (43). We previously reported that CXCR4 was upregulated in NSCLC patients, and that specific downregulation of CXCR4 inhibited cell growth, invasiveness and migration. Additionally, we also reported that MMP2 and MMP9 could be upregulated after treatment with CXCL12 in A549 cells (20,21), and that CXCR4 was highly expressed in chemo-resistant NSCLC patients and induced anti-apoptosis to cisplatin in a CYP1B1 manner (data not shown).

In this study, we found that CD133 and CXCR4 are highly expressed in NSCLC metastatic patients as compared to non-metastatic patients. We further used Cox regression analysis to verify whether CD133/CXCR4 could be a potential prognostic marker for NSCLC patients, of which univariate Cox regression results indicated that the N stage, M stage and CD133/CXCR4 co-expression significantly associated with the patient prognosis. Moreover, multivariate Cox regression results indicated that only the N stage and CD133/CXCR4 co-expression significantly correlated with the patient prognosis, with a CD133/CXCR4 co-expression hazards ratio of 2.48, thus suggesting that CD133/CXCR4 may be an independent prognostic marker in NSCLC patients. In the same line, other researchers have also shown that CD133/CXCR4 co-expression is highly associated with poor prognosis in patients (18). We also investigated the role of CD133 in promoting NSCLC tumorigenesis as a potential CSCs marker, as in some types of tumors, the stem cells exert increased proliferation (44,45), furthermore, CD133-positive cells showed proliferative characters in various tumors (46-48), which are consistent with our results, so these results indicated that CXCR4 might be modulated in part via CD133, as shown by the CXCR4 mRNA and protein expression downregulation upon CD133 silencing, but, CXCR4 silencing did not influence the CD133 expression after transfected with siCXCR4 or siNC for 48 h in A549 cells.

In recent years, EMT has emerged as a potential mechanism underlying cancer progression and metastasis. EMT processes are accompanied by profound changes in cell characteristics, which enable the epithelial cell to detach from tight junctions, change their shape and polarity, delaminate and migrate. As a result, mesenchymal cells exhibit a front-rear polarity, become flat and spindle-shaped, and become loosely associated with neighboring mesenchymal cells (49). We therefore also explored the role of CD133/CXCR4 as a CSCs marker in mediating EMT processes and observed that $CD133^{+}CXCR4^{+}$ cells had a stronger migration ability accompanied by a lower E-cadherin expression and a higher Vimentin expression as

compared to $CD133^{-}CXCR4^{+}$ and $CD133^{+}CXCR4^{-}$ A549 cells. Knapp *et al* also used immunohistochemical analysis of CXCR4 and E-cadherin on a melanoma tissue microarray which included 110 primaries, 73 local/regional metastatic and 44 distant metastatic patients, and observed that there was no significant E-cadherin expression (50).

Furthermore, we detected the expression of EMT-related molecules (E-cadherin, Vimentin) and transcription factors (Snail, Slug and Twist) after CXCR4 silencing or inhibition by a CXCR4 antagonist. Our results showed that E-cadherin was upregulated, while Vimentin, Snail, Slug and Twist were downregulated as a result of CXCR4 knockdown or inhibition, thus suggesting a potential role for CXCR4 in regulating EMT processes.

In addition, Vimentin expression was observed to have a positive correlation with CD133/CXCR4 co-expression in NSCLC patients and survival analysis results suggested that Vimentin high expression might be significantly associated with poor patients' survival rates, thus suggesting that Vimentin may be involved in CD133/CXCR4 induced NSCLC metastasis. Vimentin expressing metastatic lung cancer cells have been reported to be more motile and invasive while acting via a VAV2-Rac1 pathway to control focal adhesion kinase activity (51). In conclusion, this study shows that CXCR4 is involved in CD133-induced EMT processes in NSCLC. Thus, the CD133/CXCR4/EMT axis could be used as a potential therapeutic target in NSCLC metastasis.

Acknowledgements

This study was supported by National Natural Science Foundation of China (nos. 81270607 and 81541027) and Natural Science Foundation of Hubei (nos. 2014CFA070 and 2015CFB653).

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