

## Original Article

# Co-expression of uPAR and CXCR4 promotes tumor growth and metastasis in small cell lung cancer

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**Abstract:** Urokinase-type plasminogen activator receptor (uPAR) and C-X-C-chemokine receptor-4 (CXCR4) are considered as key molecules in invasion and metastasis of several cancers via extracellular matrix degeneration and assist tumor metastasis to specific sites by chemotaxis. However, the combined effect of uPAR and CXCR4 on small cell lung cancer (SCLC), the most aggressive type of lung cancer, is not clear. In this study, we detected the expression of uPAR and CXCR4 in SCLC tissue samples (n = 50) by immunohistochemistry. The tumors with high expression of both uPAR and CXCR4 (12/50) had larger size, higher lymph node (LN) metastasis and worse prognosis of patients than those with low expression of uPAR and CXCR4 (38/50) ( $P < 0.05$ ). We further identified and isolated the both uPAR and CXCR4 positive expression subpopulation cells (uPAR<sup>+</sup>CXCR4<sup>+</sup> cells) from the SCLC cell line H446 by flow cytometry. The uPAR<sup>+</sup>CXCR4<sup>+</sup> cancer cells showed a higher invasive and migrating capacity in the transwell and wound healing assays compared with other subpopulation cells ( $P < 0.05$ ). uPAR<sup>+</sup>CXCR4<sup>+</sup> cells injected subcutaneously in nude mice markedly increased tumor growth and induced lung metastasis, while other subpopulation cells did not. In conclusion, these data suggest that uPAR and CXCR4 co-expression predicts worse prognosis of SCLC patients. uPAR<sup>+</sup>CXCR4<sup>+</sup> cells promote the tumor growth and play a potential role in metastasis of SCLC.

**Keywords:** uPAR, CXCR4, small cell lung cancer, metastasis

## Introduction

Small cell lung cancer (SCLC) is a type of highly aggressive neuroendocrine tumor exhibiting a uniformly poor prognosis because of its rapid growth and early metastasis. Therefore, elucidating the process of tumor initiation and metastasis of SCLC is necessary for novel therapy development. In our previous study, we identified a population of uPAR<sup>+</sup> sphere-forming cells that exhibited stem cell-like properties in H446 SCLC cells [1]. The urokinase plasminogen activator receptor [uPAR or cluster of differentiation 87 (uPAR)] is a glycoprotein 55 kDa to 60 kDa in size that belongs to the Ly-6 family [2]. The expression and activation of uPA system plays an important role in tumorigenicity, and high endogenous levels of uPAR are associated with advanced cancers [3]. These uPAR<sup>+</sup> cells may play an important role in SCLC initial and development.

SCLC possesses a high propensity for early and widespread metastases, particularly in the

bone and bone marrow, liver, adrenal glands, and brain [4, 5]. Stromal-derived factor-1 (SDF-1), the natural ligand for C-X-C-chemokine receptor-4 (CXCR4), can be found in these tissues [6]. Therefore, cancer cells expressing CXCR4 may play an important role in metastasis of SCLC. Evidence is growing on the CXCR4/SDF-1 axis regulation of the migration and metastasis of a variety of cancers [7, 8]. CXCR4 is a seven-transmembrane G protein-coupled receptor expressed by various solid and liquid tumors, such as breast cancer [6], prostate cancer [9], and acute and chronic leukemia [10, 11]. Hermann et al. [12] found that CD133<sup>+</sup>/CXCR4<sup>+</sup> as well as CD133<sup>+</sup>/CXCR4<sup>-</sup> CSC were both capable of inducing an orthotopic primary tumor. However, only the co-implantation of CD133<sup>+</sup>/CXCR4<sup>+</sup> cells induced metastatic spread of the primary tumor. Other investigators have demonstrated CXCR4-mediated cell migration, integrin activation, and adhesion to stromal cells by studying SCLC cell lines [13, 14]. For studies showed uPAR could interfere in CXCR4 activity, regulates the adhesive

and migratory ability of CXCR4-expressing cells [15]. We hypothesized that a subset of uPAR<sup>+</sup> cells that co-expresses CXCR4 capable of forming tumor metastasis may exist in SCLC.

In the present study, we investigated the significance of uPAR and CXCR4 expression in SCLC. In addition, we identified a subpopulation of uPAR<sup>+</sup>CXCR4<sup>+</sup> cells that plays a potential role in tumor metastasis.

### Materials and methods

#### *Patients*

This study included 50 primary human SCLC from patients who underwent surgical resection in General Hospital of Tianjin Medical University in China from 1999 to 2009. The patients included 40 men and 10 women; the median age of the patients was 56 years (range: 38 years to 76 years). A total of 41 patients were considered lymph node metastasis-positive and 9 as lymph node metastasis-negative. The pathological diagnosis was counter-checked by two senior pathologists; follow-ups were conducted by telephone, which were sent to obtain information on the patients' outcomes. The median follow-up time was 31.5 months (range: 8 months to 69 months). Overall survival was calculated from the time of surgery to the time of death or the date of the last follow-up. Patients who were alive at the last follow-up were censored. The entire survey was conducted with the approval of the Ethics Committee of Tianjin Medical University.

#### *Cell line*

SCLC cell line H446 was purchased from the Cell Resource Center (IBMS, CAMS/PUMC, Beijing China) and were cultured in RPMI-1640 medium (Neuronbc Laboratories Co., Ltd. Beijing) supplemented with 10% fetal bovine serum (Thermo Scientific HyClone), in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C.

#### *Immunohistochemistry*

All human small cell lung cancer and xenograft tumors paraffin-embedded tissues were cut with a thickness of 4 µm. Antigen retrieval was accomplished by heat retrieval. Tissue sections were placed in a 0.01 M citrate buffer at pH 6.0 and then heated at a temperature ranging from 98°C to 100°C for 15 min in a microwave oven. Endogenous peroxidase activity was blocked

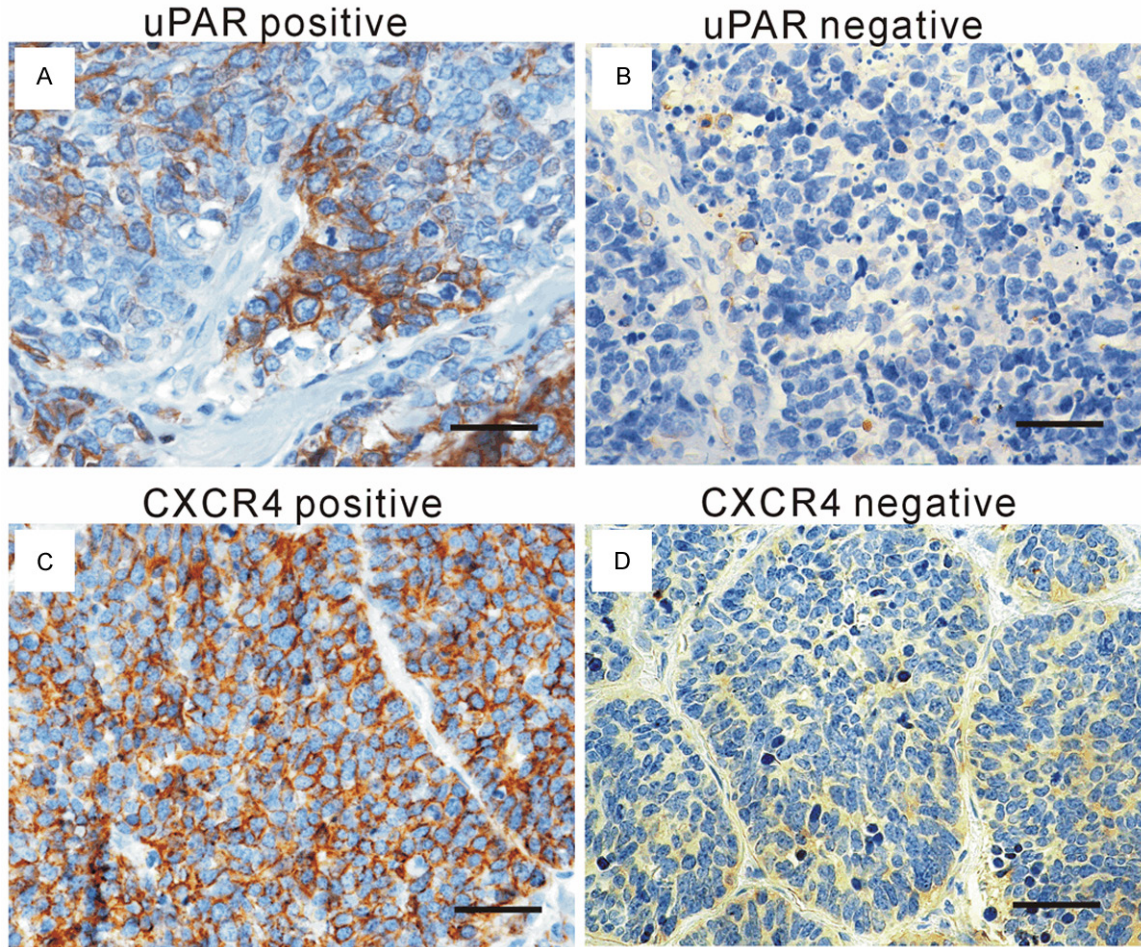
using 3% hydrogen peroxide (in fresh methanol) for 15 min at room temperature. Then tissue sections were stained for primary antibodies specific for uPAR (mouse monoclonal, 1:100, American Diagnostica, No. 3936), and CXCR4 (mouse monoclonal, 1:100, R&D, Clone 44716). As a secondary antibody, horseradish peroxidase (HRP) labeled rabbit anti-mouse IgG (Dako Envision plus System) was used. Positive staining was visualized with DAB. Images were captured by an Olympus BX41 light microscope. Tumor cells with cytoplasmic and/or membrane immunohistochemical expression was considered positive cells. The percentage of positive tumor cells was counted in three separate fields and at least 1000 adjacent cells in the area with the highest density of positive cells for each slide. The numbers of positively labeled tumor cells were scored as follows: 0, 0%; 1, 1%-10%; 2, 11%-33%; 3, 34%-66%; and 4, 67%-100%. The intensity of staining was also evaluated and graded from 1 to 3, where 1 indicates weak staining; 2, moderate staining; and 3, strong staining. The two values obtained were multiplied to calculate a receptor score (maximum value, 12). For statistical analysis, the samples were grouped into negative (score ≤ 2) or positive (score > 2). Slides were evaluated by two blinded observers.

#### *Flow cytometry analysis*

For flow cytometry and cell sorting, H446 cells were collected and washed with PBS. Incubation with the antibody uPAR (mouse monoclonal, 1:100, American Diagnostic, No. 3936) was applied at 1:100 dilutions to the cells and the FITC conjugated rabbit anti-mouse IgG (Dako) was used as secondary antibody, and then CXCR4 antibody was added, which directly conjugated with the PE (PE-conjugated CXCR4 antibody from Biosynthesis Biotechnology Co., Ltd., Beijing. bs-1011R-PE). The cells were incubated for 30 min at room temperature in the dark, followed by three washes of PBS, and then resuspended in 600 µl of PBS. All samples were analyzed and sorted by a FACS Calibur flow cytometer (BD Bioscience) with Cell Quest software (BD Biosciences).

#### *Tumor cell invasion assay*

Invasion assay was performed with the Transwell chamber with 8 µm pores (Corning). Fifty microliters diluted matrigel (2 mg/ml, BD Biosciences, Bedford, MA) was placed on the



**Figure 1.** Immunohistochemistry of uPAR and CXCR4 in SCLC specimens. A: uPAR positive expression. B: uPAR negative expression. C: CXCR4 positive expression. D: CXCR4 negative expression. uPAR immunoreactivity was observed in the membrane and CXCR4 was observed in the membrane and cytoplasm of tumor cells ( $\times 400$ , Scale bar = 50  $\mu\text{m}$ ).

**Table 1.** Expression of uPAR and CXCR4 in SCLC specimens

	uPAR (%)	CXCR4 (%)	uPAR and CXCR4 (%)
Positive	17 (34%)	35 (70%)	12 (24%)
Negative	33 (66%)	15 (30%)	38 (64%)

inner surface. Isolated cells at a concentration of  $10^5/\text{ml}$  resuspended in RM1640 were placed on the top chamber. RM1640 was added to the bottom chamber. After 48 h, non-invading cells were removed from the top of the Matrigel with a cotton-tipped swab. Invading cells at the bottom of the Matrigel were fixed in methanol and stained with Crystal violet. The invasiveness was determined by counting the penetrated cells under a microscope at  $\times 200$  magnification of 5 random fields in each well. Each experiment was performed in triplicate.

#### Wound assay to assess cell migration

Isolated four subpopulation cells ( $1 \times 10^5$ ) for wound-healing assays (conventional scrape motility assays) were plated in twelve-well plates for 24 h and the cells reached 90% confluence, we used sterile pipette tips to scratch the wound uniformly. Cell motility was assessed by measuring the movement of cells into a scraped wound. The speed of wound closure was monitored after 48 h by measuring the distance of the wound from 0 h. Each experiment was conducted in triplicate.

#### Tumorigenicity assay in nude mice

All protocols were approved by Institutional Animal Care and Use Committee and carried out according to institutional guidelines. Animal experiments were performed on four weeks old

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**Table 2.** Correlations of uPAR and CXCR4 expression with clinicopathologic parameters

Variant	Patients No.	uPAR expression			CXCR4 expression			uPAR and CXCR4 co-expression		
		Positive	Negative	P value	Positive	Negative	P value	Co-positive	Single and Negative	P value
Gender										
M	40	12	28	0.232	28	12	1.000	9	31	0.619
F	10	5	5		7	3		3	7	
Age										
< 55	17	7	10	0.442	12	5	0.948	5	12	0.520
≥ 55	33	10	23		23	10		7	26	
Tumor diameter (cm)										
< 3	30	7	23	0.051	24	6	0.059	3	27	0.005*
≥ 3	20	10	10		11	9		9	11	
Lymph node metastases										
Present	36	15	21	0.066	27	9	0.216	12	24	0.013*
Absent	14	2	12		8	6		0	14	
TNM stage										
I + II	27	6	21	0.057	16	11	0.073	4	23	0.099
III + IV	23	11	12		19	4		8	15	

\* $P < 0.05$ .

Balb/c nude mice purchased from Beijing HFK Bio-Technology. Co, LTD (Beijing, China) with six animals per group. Sorted cells were implanted subcutaneously in the right flanks of nude mice with  $5 \times 10^5$  in 100  $\mu$ l RPMI1640 and 100  $\mu$ l of matrigel (BD Biosciences), making a 1:1 mixture. Tumors were measured once a week. After 8 weeks, mice were sacrificed and tumors were removed and then subjected to immunohistochemical analysis. Tumor volume =  $0.5 \times \text{length} \times \text{width}^2$ .

### Statistical analysis

All data in the study were evaluated with SPSS version 17 software (SPSS Inc.). Data are presented as means  $\pm$  SD. When two groups were compared, the Student t-test was used. The  $\chi^2$  test was performed to determine correlations among the various parameters. Cumulative survival rate was assessed by the Kaplan-Meier method and analyzed by log-rank test. Differences were considered significant at value of  $P \leq 0.05$ .

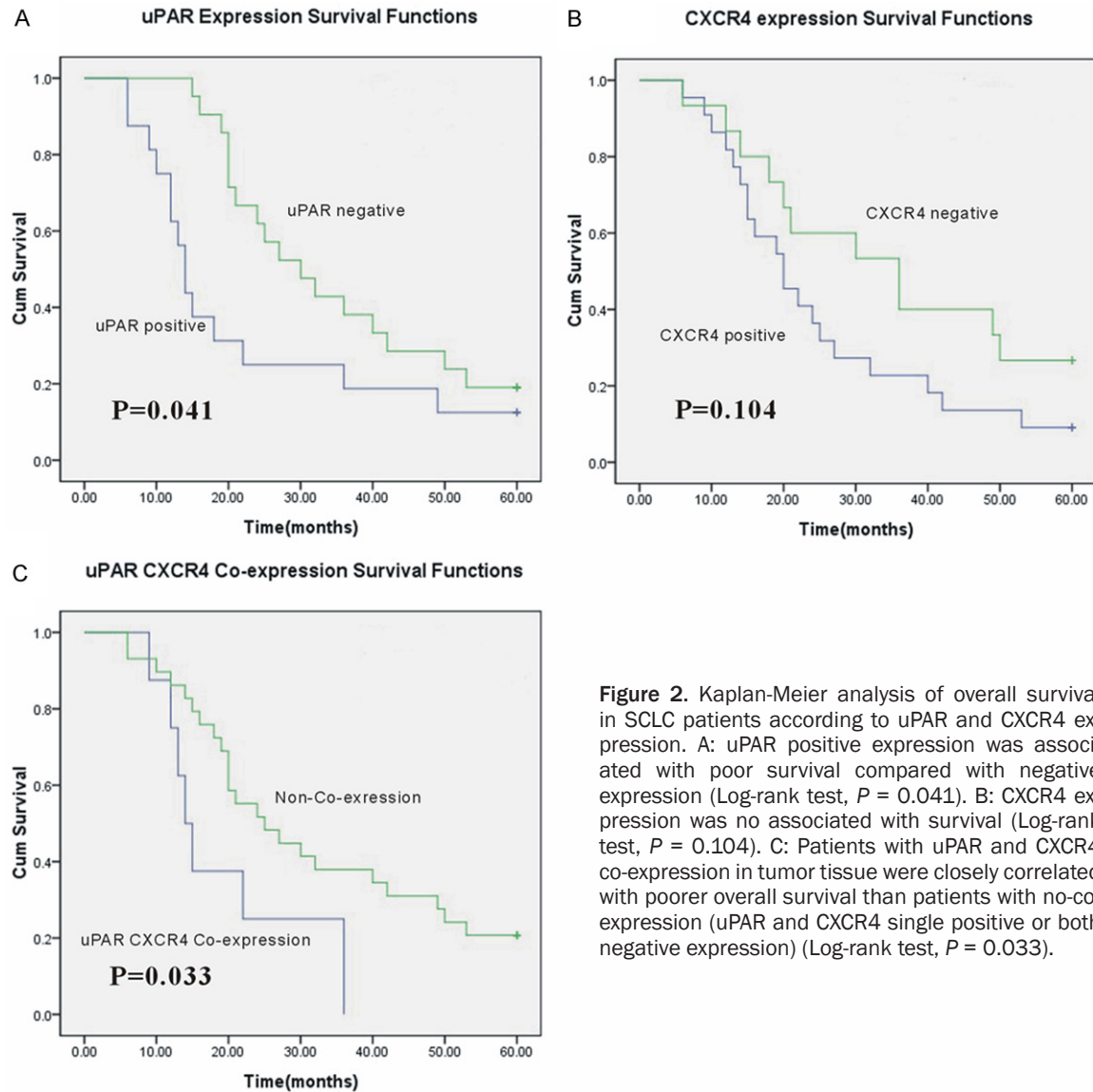
### Results

#### *uPAR and CXCR4 expressions in SCLC samples*

From our and other researches previous study we hypothesis uPAR and CXCR4 play important role in SCLC development. To determine the expression and clinical significance of uPAR and CXCR4 in SCLC, we analyzed the expres-

sion of uPAR and CXCR4 by using immunohistochemistry in 50 clinical specimens of SCLC. uPAR was positive in 17 (34%) of the SCLC cases. uPAR expression was mainly observed in the membrane of tumor cells and the uPAR positive expression were generally tended to exist in foci near the invasive front of the carcinoma (**Figure 1A**). CXCR4 immunoreactivity was observed in the membrane and cytoplasm of tumor cells (**Figure 1B**), a strong CXCR4 expression was observed in 35 (70%) SCLC cases. There were 12 (24%) cases co-expressed uPAR and CXCR4 (**Table 1**). We examined the relationship in uPAR, CXCR4 expression and clinicopathological factors. As shown in **Table 2**, the tumor diameter of the uPAR and CXCR4 co-expression group was markedly larger than that of the negative uPAR and/or CXCR4 expression group ( $P = 0.005$ ). uPAR and CXCR4 co-expression was correlation to lymph node metastases ( $P = 0.013$ ). No significant difference was found between uPAR or CXCR4 single positive expression and the clinical parameters studied. Moreover, the association of uPAR, CXCR4 expression with patients' overall survival was further evaluated. The uPAR expression observed correlation to survival, the mean survival time was  $22.250 \pm 4.468$  months in the positive uPAR expression group, but it was  $34.762 \pm 3.476$  months in the negative uPAR expression group ( $P = 0.041$ ; **Figure 2A**). There was no correlation between CXCR4 and survival ( $P = 0.104$ ; **Figure 2B**). The mean survival

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**Figure 2.** Kaplan-Meier analysis of overall survival in SCLC patients according to uPAR and CXCR4 expression. A: uPAR positive expression was associated with poor survival compared with negative expression (Log-rank test,  $P = 0.041$ ). B: CXCR4 expression was no associated with survival (Log-rank test,  $P = 0.104$ ). C: Patients with uPAR and CXCR4 co-expression in tumor tissue were closely correlated with poorer overall survival than patients with no-co-expression (uPAR and CXCR4 single positive or both negative expression) (Log-rank test,  $P = 0.033$ ).

time of the uPAR and CXCR4 Co-expression group was  $19.625 \pm 3.803$  months that significant shorter than the single and co-negative expression group ( $29.351 \pm 2.944$  months) ( $P = 0.033$ ; **Figure 2C**).

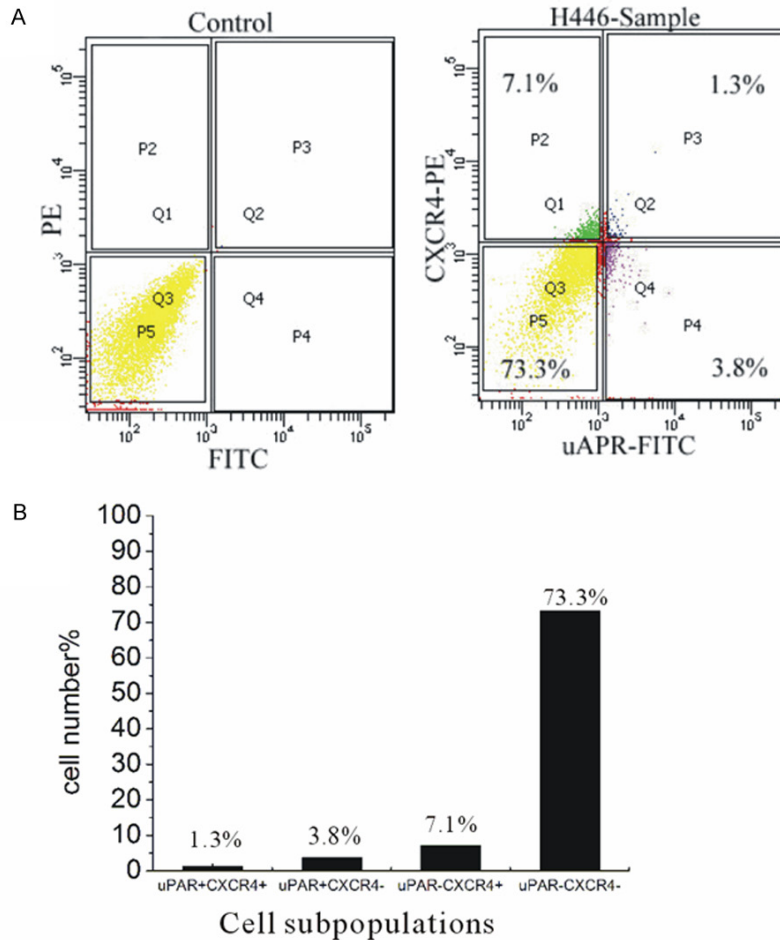
*Identify of uPAR<sup>+</sup>CXCR4<sup>+</sup> subpopulation exist in SCLC cell line H446*

The co-expression of uPAR and CXCR4 in SCLC tissues showed correlation with tumor metastasis. To further demonstrate uPAR and CXCR4 involve in metastasis in SCLC, We stained both uPAR and CXCR4 fluorescent antibodies in SCLC cell line H446, analyzed by using a high-speed fluorescence-activated cell sorter. Results showed there were contain uPAR<sup>+</sup>CX-

CR4<sup>+</sup>, uPAR<sup>+</sup>CXCR4<sup>-</sup>, uPAR<sup>-</sup>CXCR4<sup>+</sup> and uPAR<sup>-</sup>CXCR4<sup>-</sup> four subpopulations in cell line H446 (**Figure 3A**). The proportion of uPAR<sup>+</sup>CXCR4<sup>+</sup>, uPAR<sup>+</sup>CXCR4<sup>-</sup>, uPAR<sup>-</sup>CXCR4<sup>+</sup> and uPAR<sup>-</sup>CXCR4<sup>-</sup> four subpopulations in H446 were 1.3%, 3.8%, 7.1% and 73.3% respectively (**Figure 3B**).

*Invasion and migration capacity of uPAR<sup>+</sup>CXCR4<sup>+</sup> subpopulation sorted from SCLC cell line H446*

The isolated four subpopulation cells were compared for invasion using the transwell invasion assay. As demonstrated via the transwell assay, more significant number of uPAR<sup>+</sup>CXCR4<sup>+</sup> cells passed through the upper membrane pretreated with matrigel compared with than the



**Figure 3.** FACS analysis of H446 cell line double-labeled with uPAR-FITC and CXCR4-PE, antibody. A: H446 cells staining mouse IgG as isotype control analyzed by FACS (left). H446 cell line double-labeled with uPAR-FITC and CXCR4-PE antibody analyzed by FACS (right). B: The proportion of uPAR<sup>+</sup>CXCR4<sup>+</sup> (1.3%), uPAR<sup>+</sup>CXCR4<sup>-</sup> (3.8%), uPAR<sup>-</sup>CXCR4<sup>+</sup> (7.1%) and uPAR<sup>-</sup>CXCR4<sup>-</sup> (73.3%) four subpopulations in H446.

other groups, uPAR<sup>-</sup>CXCR4<sup>-</sup> cells showed the fewest cells migrated to the bottom chamber invasive capacity (**Figure 4A**). We further observed uPAR<sup>+</sup>CXCR4<sup>+</sup> cells ( $65.60 \pm 9.91$ ) showed higher invasion capacity than uPAR<sup>+</sup>CXCR4<sup>-</sup> cells ( $31.00 \pm 9.77$ ) ( $P < 0.05$ ; **Figure 3B**), uPAR<sup>-</sup>CXCR4<sup>+</sup> ( $21.80 \pm 4.81$ ) cells showed no significant difference in invasion capacity than uPAR<sup>-</sup>CXCR4<sup>-</sup> cells ( $16.60 \pm 7.09$ ) ( $P < 0.05$ ; **Figure 4B**). Cell migration capacity was evaluated by the wound healing assay, also known as the “scratch” assay. We observed the accordance results with invasive capacity that uPAR<sup>+</sup>CXCR4<sup>+</sup> cells showed the highest migration capacity than the other subpopulations (migration distance were  $100.50 \pm 8.63$ ,  $82.80 \pm 8.81$ ,  $64.80 \pm 6.97$  and  $53.4 \pm 8.96$   $\mu\text{m}$

respectively,  $P < 0.05$ , **Figure 4C**). These results supports that the strong invasive and migrate activity of cancer cells are mediated by uPAR and CXCR4, the function of CXCR4 maybe depend on uPAR triggered.

*Growth and metastatic capacity of uPAR<sup>+</sup>CXCR4<sup>+</sup> subpopulation from SCLC cell line H446 in vivo*

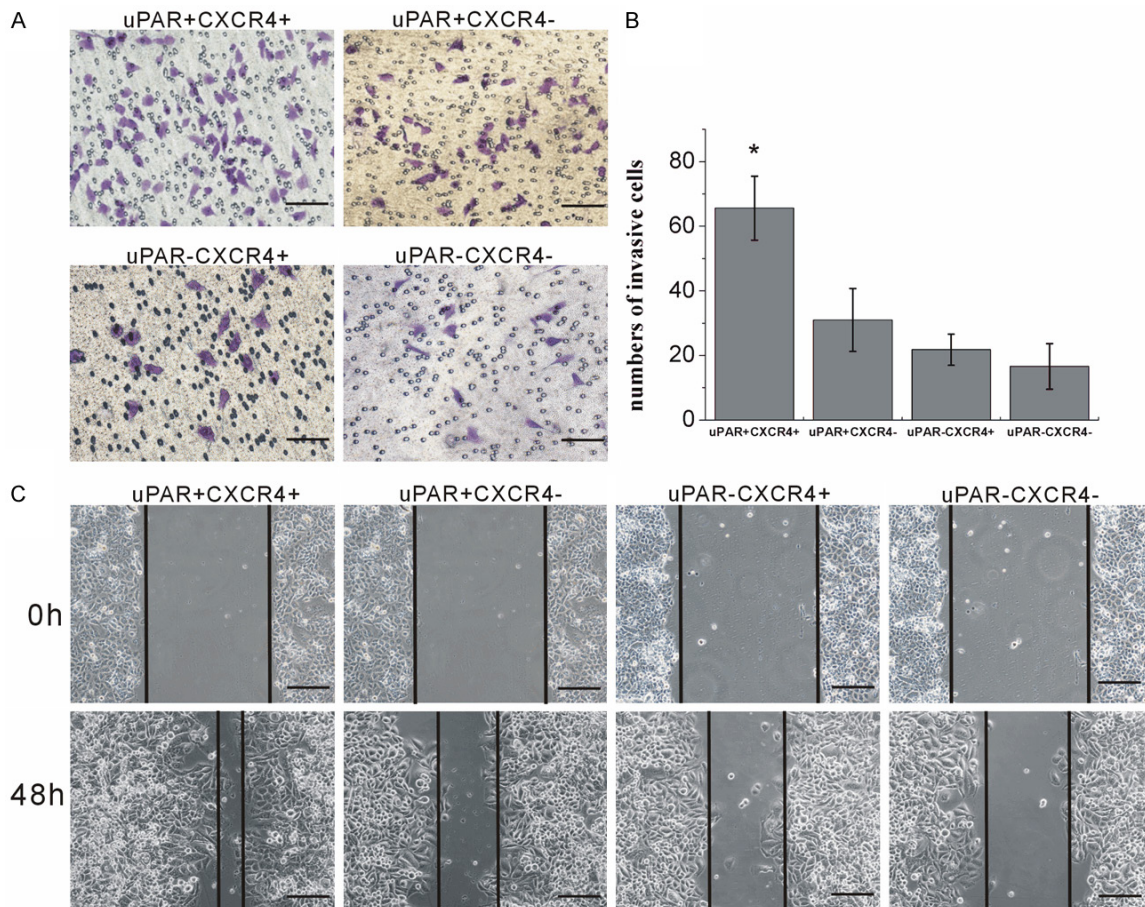
To further confirm the growth and metastatic capacity of uPAR<sup>+</sup>CXCR4<sup>+</sup>, we used an in vivo experiments.  $5 \times 10^5$  uPAR<sup>+</sup>CXCR4<sup>+</sup> or other subpopulation cells (including uPAR<sup>+</sup>CXCR4<sup>-</sup>, uPAR<sup>-</sup>CXCR4<sup>+</sup> and uPAR<sup>-</sup>CXCR4<sup>-</sup> three subpopulations) were subcutaneously injected into BALB/c nude mice, which were divided into two groups, with six mice each group. Eight weeks later, both uPAR<sup>+</sup>CXCR4<sup>+</sup> cells and other subpopulation cells formed tumors (6/6 and 5/6 respectively) (**Figure 5A**). uPAR<sup>+</sup>CXCR4<sup>+</sup> group tumor volume ( $2227.6 \pm 211.35$   $\text{mm}^3$ ) much larger than the other subpopulation cells group ( $1201.0 \pm 170.36$   $\text{mm}^3$ ) ( $P < 0.01$ ,

**Figure 5B**). Three mice in the uPAR<sup>+</sup>CXCR4<sup>+</sup> cells group developed lung metastases (**Figure 5C**) whereas mice that received other subpopulation cells showed no trace of metastasis (**Figure 5D**). The lung metastatic tumor high expressed uPAR and CXCR4 (**Figure 5E, 5F**). These data support the hypothesis that uPAR<sup>+</sup>CXCR4<sup>+</sup> cells may represent a distinct, migrating cell population.

**Discussion**

In human malignancies, uPAR overexpression is associated with an increased propensity for cancer progression and metastasis [16, 17]. Studies indicated intact uPAR and its cleaved forms are associated with poor prognosis in

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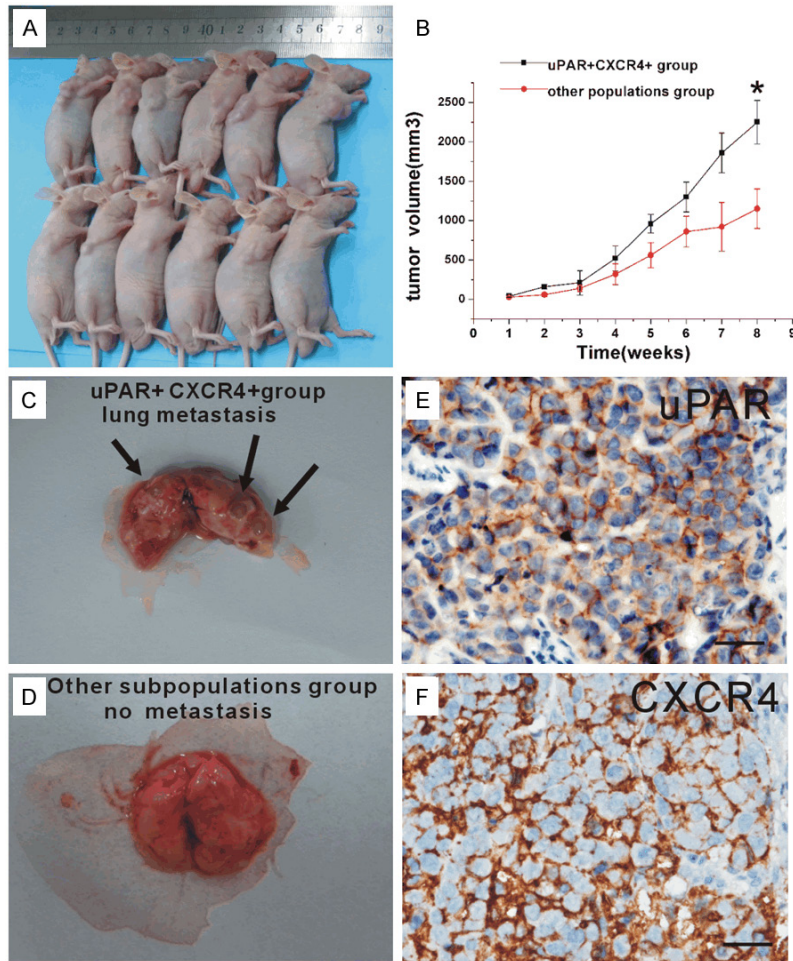
**Figure 4.** Cell invasion and migration capability in vitro was detected by transwell and wound healing assays. A: The transmembrane cells of uPAR<sup>+</sup>CXCR4<sup>+</sup>, uPAR<sup>+</sup>CXCR4<sup>-</sup>, uPAR<sup>-</sup>CXCR4<sup>+</sup> and uPAR<sup>-</sup>CXCR4<sup>-</sup> group ( $\times 100$ , Scale bar = 50  $\mu\text{m}$ ). B: The transmembrane numbers of uPAR<sup>+</sup>CXCR4<sup>+</sup>, uPAR<sup>+</sup>CXCR4<sup>-</sup>, uPAR<sup>-</sup>CXCR4<sup>+</sup> and uPAR<sup>-</sup>CXCR4<sup>-</sup> group were  $86.50 \pm 11.32$ ,  $40.25 \pm 10.15$ ,  $21.34 \pm 5.35$  and  $15.45 \pm 7.82$  respectively. The transmembrane number of uPAR<sup>+</sup>CXCR4<sup>+</sup> group cells was significant more than the other subpopulations group cells ( $P < 0.05$ , respectively). Furthermore, the transmembrane cells of uPAR<sup>+</sup>CXCR4<sup>+</sup> group was significant more than the uPAR<sup>+</sup>CXCR4<sup>-</sup> group ( $P = 0.01$ ). However, the difference between uPAR<sup>+</sup>CXCR4<sup>+</sup> and uPAR<sup>-</sup>CXCR4<sup>-</sup> group was not significant ( $P = 0.212$ ). C: The cell migration distances of uPAR<sup>+</sup>CXCR4<sup>+</sup>, uPAR<sup>+</sup>CXCR4<sup>-</sup>, uPAR<sup>-</sup>CXCR4<sup>+</sup> and uPAR<sup>-</sup>CXCR4<sup>-</sup> group were  $100.50 \pm 8.63$ ,  $82.80 \pm 8.81$ ,  $64.80 \pm 6.97$  and  $53.4 \pm 8.96$   $\mu\text{m}$  respectively. The cell migration distances of uPAR<sup>+</sup>CXCR4<sup>+</sup> group cells was significant farther than the other subpopulations group cells ( $P < 0.05$ , respectively). ( $\times 100$ , Scale bar = 50  $\mu\text{m}$ ).

NSCLC and SCLC [18, 19]. However, the association was mainly proved by detection of high levels of uPAR in patient blood but not in tumor tissue. In the present study, immunohistochemical staining showed 17 (17/50, 34%) cases were positive for the uPAR expression in SCLC tissues. High expression of uPAR in SCLC patients was significantly correlated with shorter survival times than that of uPAR negative expression ( $P = 0.041$ ). Thus, uPAR may be an independent prognostic indicator in SCLC.

Invasion and metastasis are not random, but, rather, highly organ-specific and pathological

processes. Chemokine receptors are deemed crucial in the homing mechanisms of hematopoietic cells and metastasis of solid tumors such as breast and ovarian cancers [20, 21]. Recent reports suggested that CXCR4 may be a key regulator of tumor invasiveness leading to local progression and tumor metastasis [22]. In SCLC, the distant organ sites most commonly affected are the lymph nodes, bone marrow, and brain, all of which exhibit high SDF-1 contents [23]. The specific receptor of SDF-1, CXCR4, is also involved in SCLC metastasis. Previous immunohistochemical studies showed that SCLC cells expressed CXCR4 [24]. Here, we

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**Figure 5.** The xenograft and lung metastasis forming of uPAR<sup>+</sup>CXCR4<sup>+</sup> and other subpopulation cells in vivo. A: Subcutaneous implantation of  $5 \times 10^5$  uPAR<sup>+</sup>CXCR4<sup>+</sup> cells or other subpopulation cells in BALB/c nude mice led to tumor formation (6/6, 5/6, respectively). B: BALB/c nude mice shows that after 8 weeks injected, growth curve of xenograft tumors volume of uPAR<sup>+</sup>CXCR4<sup>+</sup> group exhibit significant larger than other subpopulation group ( $2227.6 \pm 211.35$ ,  $1201.0 \pm 170.36$  mm<sup>3</sup>, respectively,  $P < 0.01$ ). C: After 8 weeks, mice were sacrificed, lung metastasis foci were founded in uPAR<sup>+</sup>CXCR4<sup>+</sup> cells group (arrow indicated). D: Other subpopulation cells group showed no trace of metastasis. E: Immunohistochemistry showed uPAR positive expressed in lung metastasis tumor tissue. F: Immunohistochemistry showed CXCR4 positive expressed in lung metastasis tumor tissue ( $\times 400$ , Scale bar = 50  $\mu$ m).

detected CXCR4 in SCLC specimens. There were no significant relationship between CXCR4 expression and clinical data. But interestingly, we found evidence in SCLC specimens that links several conventional clinical factors to uPAR and CXCR4 co-expression. Tumor mean diameter of uPAR and CXCR4 co-expression group was larger than that of single or negative expression group ( $P = 0.005$ ), indicating that uPAR and CXCR4 positive cells may lead to faster tumor growth. High expression of uPAR in

SCLC patients was also significantly correlated with lymph node metastases indicating that uPAR and CXCR4 positive cells may play an important role in tumor metastasis. uPAR and CXCR4 co-expression group also showed shorter survival times than that of single and co-negative uPAR or CXCR4 expression ( $P = 0.033$ ). Therefore, uPAR and CXCR4 co-expression might be a reliable prognostic biomarker for tumor growth or metastasis in SCLC patients.

Studies analyzing uPAR and CXCR4 at the cellular and molecular level have revealed multiple functions of them in tumor characteristics. A study performed by Gutova and colleagues [25] provided evidence that the uPAR<sup>+</sup> subpopulation of cancer cells is resistant to traditional chemotherapies. Using urokinase receptor antibody or transfected with antisense uPAR vectors in cell line can inhibit in vivo tumorigenicity and metastases [26, 27]. There was also research confirmed that uPAR interference in CXCR4 activity in cells isolated from prostate carcinoma. In the present study, we successfully isolated a subpopulation of

uPAR<sup>+</sup>CXCR4<sup>+</sup> cells from cell line H446. The uPAR<sup>+</sup>CXCR4<sup>+</sup> cells, although a minor proportion, demonstrated stronger migratory and metastatic capacities than other subpopulation cells. Particularly we observed uPAR<sup>+</sup>CXCR4<sup>+</sup> cells showed higher invasion capacity than uPAR<sup>+</sup>CXCR4<sup>-</sup> cells ( $P < 0.05$ ), and uPAR<sup>-</sup>CXCR4<sup>+</sup> cells showed no significant difference in invasion capacity with uPAR<sup>-</sup>CXCR4<sup>-</sup> cells ( $P < 0.05$ ). These results confirmed our hypothesis that CXCR4<sup>+</sup> cell motility maybe triggered by



uPAR. Through in vivo experiments, we found that both uPAR<sup>+</sup>CXCR4<sup>+</sup> subpopulation and other subpopulation cells were capable of inducing orthotropic primary tumors. However, the uPAR<sup>+</sup>CXCR4<sup>+</sup> cells were capable of spreading from the primary tumor to form metastatic lesions, while the other subpopulation cells, although containing uPAR or CXCR4 positive cells could not form metastatic lesions, suggesting that both uPAR and CXCR4 expression are essential for metastatic spread. In the present study, we provide evidence for a metastatic subpopulation uPAR<sup>+</sup>CXCR4<sup>+</sup> existence in the SCLC cell line H446. The interaction of uPAR and CXCR4 in SCLC should be investigated in future studies.

In summary, uPAR and CXCR4 co-expression plays a critical role in tumor development and progression in SCLC. The existence of the uPAR<sup>+</sup>CXCR4<sup>+</sup> cell subpopulation is possibly responsible for tumor metastasis. Further examination of such cell subpopulation in SCLC cells will provide important clues on the malignant progression and therapy target of SCLC cancer.

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### Disclosure of conflict of interest

None.

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