High Expression of PC4 is Associate With Lymphatic Metastasis and Predicts Poor Prognosis in Lung Adenocarcinoma Probably via CCR7/VEGF-C/VEGFR-3 Cascade

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Research Article

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Abstract

Purpose: PC4 is a novel marker for diagnosis and treatment of advanced human cancers metastasis. This study aimed to verify that high expression of PC4 is associated with lymphatic metastasis and predicts poor prognosis in lung adenocarcinoma probably via CCR7/VEGF-C/VEGFR-3 cascade.

Methods: PC4 protein expressions in 96 lung adenocarcinoma cases, CCR7/VEGF-C/VEGFR-3 protein expressions in 23 lung adenocarcinoma cases, and PC4 clinical outcome in 83 lung adenocarcinoma cases and TCGA as validation were evaluated, respectively. Small interfering RNA was used to explore the relationship of PC4 and the VEGF-C/VEGF-D/VEGFR-3 axis in A549 cells. The correlations between PC4 and CCR7, CCR7 and VEGF-C/VEGFR-3 were analyzed in A549 cells and adenocarcinoma tissues, respectively.

Results: The results shown PC4 protein highly expressed in tumor tissue compared with normal lung tissue. High expressions of PC4 were remarkably associated with advanced tumor stage (P=0.032), lymphatic metastasis (P=0.004) and poor clinical outcomes (the cohort: HR: 2.135, 95% CI: 1.279-3.562; TCGA: HR: 2.983, 95% CI: 1.249-7.127) in lung adenocarcinoma. CCR7 expressions were remarkably decreased after PC4 RNAi in A549 cells, and significantly correlated with the expressions of VEGF-C and VEGFR-3 in adenocarcinoma tissues.

Conclusion: CCR7/VEGF-C/VEGFR-3 expressions in lung adenocarcinoma were closely associated with lymphatic metastasis. Overexpression of PC4 is a predictor of lymphatic metastasis and poor prognosis in lung adenocarcinoma. PC4 plays important oncogenic roles probably via activation of CCR7/VEGF-C/VEGFR-3, which warrants further study.

Introduction

Human transcriptional positive cofactor 4 (PC4) is a highly abundant nuclear protein produced by human SUB1 gene and functions as a transcriptional coactivator through RNA polymerase II[1 2]. PC4 mediates activation of p53 binding to its cognate sites via PC4-p53 interaction[3], and activates p53-mediated apoptosis by enhancing the expression of the proapoptotic gene Bax[1], showing the pro-apoptotic roles of PC4 as a tumor suppressor. However, overexpression of PC4 was observed in several cancers e.g., primary colorectal carcinoma[4], invasive intraductal papillary mucinous neoplasm of the pancreas[5]and astrocytoma of the brain [6]. Moreover, PC4 RNAi in NSCLC cells (A549, H460 and H358) significantly induced cell cycle arrest and increased cell apoptosis[7], suggesting the oncogenetic roles of PC4 in promoting tumor growth. Our previous study demonstrated that PC4 RNAi reduced the expressions of VEGF-C/VEGFR-3 in A549 cells, inhibited lymphatic metastasis and reduced tumor lymphatic vessel density in nude mice[4].

CC-chemokine receptor 7 (CCR7) is a G-protein-coupled receptor that specifically combines with CC chemokine ligands 19 (CCL19) and CC chemokine ligands 21 (CCL21) [8]. CCR7 is mainly expressed in naive and central memory lymphocytes and mature dendritic cells and is reported to be a homing

receptor that regulates the migration of immune cells to secondary lymphoid organs[9]. Overexpression of CCR7 has been reported to be correlated with lymphatic metastasis in some malignancies, i.e., colorectal cancer [10], gastric cancer [11], esophageal squamous cell carcinoma[9]. CCR7 was found to play its roles via vascular endothelial growth factor (VEGF) axis[12]. In breast cancer MDA-MB-435s cell line, CCR7 and VEGF-C/VEGFR-3 act synergistically in promoting lymphatic invasions[13]. Levels of CCR7-mRNA were found to be significantly associated with the expressions of vascular endothelial growth factor-C (VEGF-C) in human breast cancer tissues. CCR7-RNAi significantly inhibited the expressions of VEGF-C in MDA-MB-231 breast cancer cells. Furthermore, activation of CCR7 promoted proliferation, migration, as well as tube formation of the primary human lymphatic endothelial cells (LEC) in vitro, which was significantly reduced by blocking VEGFR-3 [12].

The vascular endothelial growth factor (VEGF) family has an integral role in many physiological and pathological processes including angiogenesis, lymphangiogenesis, vasculogenesis and vascular permeability [14]. VEGF-C, as an important member of the family, binds to VEGF receptor-3 (VEGFR-3) and plays central roles in lymphangiogenesis[15-17]. In malignant diseases, alteration of VEGF-C/VEGFR-3 axis often leads to progression and metastasis of the primary tumor[18 19]. Thus far, little is known about PC4 expression and clinicopathological features and clinical outcomes of lung adenocarcinoma. Based on the close correlation between the expressions of PC4 and VEGF-C/VEGFR-3 [4] and that between the expressions of CCR7 and VEGF-C/VEGFR-3[12] respectively, we presume that high expression of PC4 subsequently induced the over-expressions of CCR7/VEGF-C/VEGFR-3 and caused lymphatic metastasis in lung cancer. In this study, we aimed to evaluate the high expressions of PC4 and clinical outcomes of lung adenocarcinoma, and clarify that PC4 may promote lymphatic metastasis via CCR7/VEGF-C/VEGFR-3 signaling cascade.

Materials And Methods

Patients and samples

The study protocol was reviewed and approved by the Research Ethics Board in Daping hospital (Chongqing City, P.R.China) [TMMU-DPH/2013-039], and informed consent was written and obtained from all the patients.

The study included three steps as follows:

Step 1: we included 96 cases with tumor and paired normal lung tissues (5-10 cm from the primary tumor) for analysis of PC4 expression and clinicopathological features between 2013 and 2014.

Step 2: 83 cases were included with tumor tissues for the evaluation of PC4 expression and clinical outcome between 2006 and 2009.

Step 3: we included 23 cases with tumor and paired normal lung tissues, and tumor-positive and -negative lymph nodes between 2008 and 2010. The expressions of CCR7, VEGF-C and VEGFR-3 and

clinicopathological features were analyzed.

All the pulmonary adenocarcinomas were confirmed by pathology. None of the patients had received treatment before enrollment in the study. All the patients underwent curative resection. Postoperative adjuvant chemotherapy was given as needed. Postoperative follow-up was conducted by telephone or letter interview, and 9 cases lost to follow-up due to the invalid contact information.

The Cancer Genome Atlas (TCGA) Dataset

The mRNA-seq data for lung adenocarcinoma was downloaded from TCGA websites (http://tcga-data.nci.nih.gov/tcga/findArchives.htm). Both clinical and PC4 expression were retrieved from the datasets. The mRNA expression data was processed level 3 data (RNA-seq V2), which normalized gene count to a fixed upper quantity value of 1,000. We used log2 (normalized count) for the analysis.

Immunohistochemistry (IHC) and scoring

The sections were incubated with serum blocking solution and anti-PC4 (Abcam, USA, 1:100), anti-CCR7 (Abcam, USA, 1:100), anti-VEGF-C (Abcam, USA, 1:50), anti-VEGFR-3 (Abcam, USA, 1:100) antibodies, biotinylated secondary antibody, and streptavidin-horseradish peroxidase. Diaminobenzidine solution was used as a chromogen. The slides were then counterstained in a hematoxylin solution. The intensity and percentage of IHC staining in tumor cells were recorded and counted manually and analyzed using ImageJ software 1.48 (National Institutes of Health, Bethesda, MD, USA). The intensity was scored from 0 to 3+ and defined as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining. Additionally, Q score was adopted for IHC scoring by multiplying the percentage of positive cells by the intensity (Q = percentage × intensity; maximum = 300). Three observers scored the slides and the mean score was used for final analysis. And the expressions of protein were defined as high (Q<150) and low (Q>=150) accordingly.

RNA Interference of PC4

Firstly, the following oligoribonucleotide pairs were used: 5’-ACAGAGCAGCAGCAGCAGA-3’ and 5’-TCTGCTGCTGCTGCTCTGT-3’. Entire sequences were derived from the sequence of human PC4 mRNA. The oligonucleotides were obtained from Sunbio Medical Biotechnology CO., Ltd (Shanghai City, P.R.China). The complementary two strands (each at 20 μM) in 60 μl of annealing buffer (Sunbio Medical Biotechnology CO., Ltd, Shanghai City, P.R.China) were heated for 5 min at 95°C and then incubated for 1h at room temperature. Thereafter, the GFP-tagged lentiviral vector pLVT351.LV for PC4-RNAi was constructed by inserting the annealing nucleotides into the Age I+EcoR I site of pMAGic 4.1 (Sunbio Medical Biotechnology CO., Ltd, Shanghai City, P.R.China).

Secondly, A549 cell line was plated at 2.3×105 cells per well of 24-well cell culture plate and infected with lentivirus at a multiplicity of infection (MOI) of 10. Cells infected with pLVT351-L.V. and CMV-GFP-L.V. (blank lentiviral vector, pMAGic 4.1) was named as A549pGCL-siRNA and A549pGCL-siRNA control respectively.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from cells or tissues using Trizol reagent (Invitrogen). First-strand cDNA was synthesized with M-MLV transcriptase (Promega) and oligo dT. Real-time PCR was performed using SYBR Green PCR master mix (TAKARA) and the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). PCR primers were used as listed in Online Table1. The specificity of detected signals was confirmed by a dissociation curve consisting of a single peak. All samples were run in duplicate in each experiment. Values were normalized by human β-actin.

Western Blotting analysis

The lysates from tissues and cell lines were prepared in a RIPA buffer comprising 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid and 0.02% sodium azide. The protein concentrations were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL). Proteins were denatured at 95°C for 5 min, and 50 μg protein per lane was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel. Proteins were blotted on polyvinylidene difluoride (PVDF) membranes (Thermo), which were then blocked with 5% skim milk for 1h at room temperature. The proteins were immunoblotted using anti-PC4 (Abcam, USA, 1:250), anti-CCR7 (Abcam, USA, 1:250), anti-VEGF-C (Abcam, USA, 1:250), anti-VEGFR-3 (Abcam, USA, 1:200) antibody. An anti-β-actin (Sigma) or GAPDH (Sigma) antibody served as the control. Gray scales of immunoblotting of the samples were quantitatively analyzed by using image acquisition and analysis software (Image Lab software, BIORAD, USA) according to the manufacturer's instructions.

Statistical Analysis

The statistical analyses were performed using the Student's t test to analyze mRNA and protein differential expression between two groups, $\chi 2$ or Fisher test for comparison of rates, Pearson Correlation for continuous variables, e.g., correlation of CCR7/VEGF-C. Prognostic factors were examined by multivariate analyses using a Cox proportional hazards model and Kaplan-Meier curves with log-rank test. All of the aforesaid calculations were performed using SPSS Version 18.0 software (SPSS, Inc., Chicago, IL) and GraphPad Prism 5.0 software (GraphPad Software, CA). $P<0.05$ (two-sided) was considered statistically significant. Overall survival (OS) and progression-free survival (PFS) were respectively calculated for analysis. OS represented the time from surgery to death, and patients lost to follow-up or still alive at the last follow-up were defined as censored values. PFS was measured from the date of surgery to the date of recurrence, metastasis or death, whichever occurred earlier, and was censored for patients lost to follow-up or still alive without any progression at the last follow-up [21].

Results

High expression of PC4 is associated with advanced tumor stage, lymphatic metastasis and poor prognosis

Immunohistochemistry staining showed that PC4 protein expressions were significantly higher in adenocarcinoma compared with normal lung tissue (Fig. 1). As listed in Table 1, high expression of PC4 significantly correlated with advanced tumor stage (p=0.032). Additionally, high expression of PC4 was observed in 57.1% of tumor-positive lymph nodes, compared with only 27.5% of tumor-negative lymph nodes (p=0.004). No significant correlations were observed between PC4 expression and other clinicopathological features (Table 1). The Kaplan-Meier curves showed that patients with highly expressed PC4 generally had remarkably poorer OS or PFS, compared to those with low expressions (21 vs. 39 months and 21 vs. 33 months, respectively; Fig. 2). Either lymphatic metastasis or tumor stage was independent prognostic factor for both OS and PFS. In univariate Cox model, PC4 protein expression was found to be an independent prognostic factor for both OS (HR: 2.135, 95% CI: 1.279-3.562) and PFS (HR: 1.848, 95% CI: 1.138-3.001). However, there was no significant association between PC4 expression and OS or PFS in multivariate Cox model, suggesting its prognostic power was covered by other variables, i.e., lymphatic metastasis and tumor stage. TCGA database also revealed that PC4 was an independent risk factor for poor prognosis (HR: 2.983, 95% CI: 1.249-7.127), among the current smokers and those who quit smoking for less than 15 years.

PC4 plays the key roles in lung cancer probably via CCR7/VEGF-C/VEGFR-3 cascade

We established A549 cells that stably expressed pGCL-siRNA-L.V. and NC-GFP-L.V. through use of a lentivirus system. The cell lines were observed under the fluorescence microscope (Fig. 3). At least an 80% reduction in mRNA and protein levels of PC4 in A549-PC4-siRNA cells was confirmed by qRT-PCR (Fig. 4A) and Western Blot (Fig. 4B), respectively. Simultaneously, we could see the inhibition of CCR7 along with PC4 knockdown, in mRNA and protein levels, respectively (Fig. 4). Our previous study showed that VEGF-C and VEGFR-3 were significantly reduced following PC4 RNAi (Data not shown) [8], demonstrating PC4 take the oncogenetic roles in lung cancer probably via CCR7/VEGF-C/VEGFR-3 cascade. By using western blot in the tumor tissues and gray scale analysis, we found that CCR7 expression was significantly associated with VEGF-C and VEGFR-3 ($r=0.782$ and 0.958, $P<0.05$).

The expression of CCR7, VEGF-C and VEGFR-3 were correlated to lymphatic metastasis.

In both mRNA (Fig. 5A) and protein (Fig. 5B&C) levels, the expressions of CCR7, VEGF-C, and VEGFR-3 were significantly higher in adenocarcinoma than in normal lung tissues ($P<0.05$). Additionally, CCR7, VEGF-C and VEGFR-3 expressions were significantly higher in metastatic lymph nodes than in normal lymph nodes. Combined with the above IHC test, the result showed that overexpression of CCR7, VEGF-C and VEGFR-3 were positively correlated with lymphatic metastasis ($\chi 2$ test, $P<0.05$, table 1).

Discussion

PC4 plays an important role in a variety of cellular processes including transcription, replication, chromatin organization and cell cycle progression, and also participates in normal cellular growth by preventing mutagenesis or killing by oxidative DNA damage[3 20]. Thus far, reports on PC4 in cancer are inconsistent. On one hand, PC4 was observed to enhance p53 function and inhibit self-repression of

transcriptional factor AP-2 in ras-transformed human teratocarcinoma PA-1 cells, thus acting as a putative tumor suppressor[21]. On the other hand, PC4 overexpression was correlated with the tumor development and progression in human prostate, breast and lung cancer[22], showing the oncogenetic roles of PC4 [22]. PC4 RNAi in A549, H460 and H358 cells significantly inhibited the growth of cancer cells by inducing cell cycle arrest and increasing cell apoptosis in vitro[7].

In our study, overexpression of PC4 protein was associated with advanced TNM stage, lymphatic metastasis and poor prognosis, demonstrating the potential carcinogenetic roles of PC4 in lung cancer. However, to our knowledge, the underlying mechanism and downstream signaling pathway has not been clarified yet. Our study indicated PC4 takes oncogenetic roles in lung cancer probably via CCR7/VEGF-C/VEGFR-3 cascade. CCR7 was reported to be involved in the development and progression of a variety of cancers, e.g., bladder and lung cancer [23 24], and overexpression of CCR7 was closely correlated with lymphatic metastasis and poor prognosis in gastric cancer [11]. Meanwhile, VEGF-C/VEGFR-3 was a well-known regulatory axis and confirmed to be critical factors in lymphangiogenesis and prognosis in many malignancies [25 26]. CCR7 RNAi significantly decreased VEGF-C expression in MDA-MB-231 breast cancer cells, indicating the close association between CCR7 and VEGF-C, which specifically binds to VEGFR-3[12]. In this study, the close association of the expressions of CCR7/ VEGF-C/VEGFR-3 was proved in lung adenocarcinoma tissues. Importantly, CCR7, VEGF-C and VEGFR-3 protein expressions were significantly correlated with lymphatic metastasis in adenocarcinoma patients. Moreover, we observed a significant decrease of CCR7 protein and mRNA expressions following PC4 RNAi in A549 cells, indicating the potential interaction between PC4 and CCR7.

Collectively, we found high expression of PC4 predicted poor prognosis in lung adenocarcinoma cases. We identified the decreased CCR7 expression following PC4 RNAi in A549 cells. Interestingly, PC4, CCR7 and VEGF-C/VEGFR-3 were closely related to lymphatic metastasis in lung adenocarcinoma. Based on the definite correlation and regulation of CCR7 on VEGF-C/VEGFR-3 axis, we postulate that high expression of PC4 can enhance the lymphatic metastasis in lung adenocarcinoma via CCR7/VEGF-C/VEGFR-3 cascade.

Declarations

The authors declare no competing interests.

Funding

The authors declare no competing financial and non-financial interests in relation to the work described in the manuscript.

Conflicts of interest/Competing interests

The authors declare no competing interests.

Availability of data and material

The authors understand that this manuscript and associated personal data will be shared with Research Square for the delivery of the author dashboard.

Code availability

All data, models, and code generated or used during the study appear in the submitted article.

Authors' contributions

ShaoLin Tao and Xuemei Wang designed the study, performedthe data search, interpreted the data, and wrote the manuscript.LiCheng Wu participated in the literature search and critically weighed interpretation of data. Cheng Shen,QunYou Tan critically revised the work,supervised the whole project and proofread the manuscript.

Ethics approval

This study was approved by Ethics Committee of Daping hospital of Army Medical university NO.14(2021)

Consent to participate

Written informed consent for publication was obtained from all participants.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

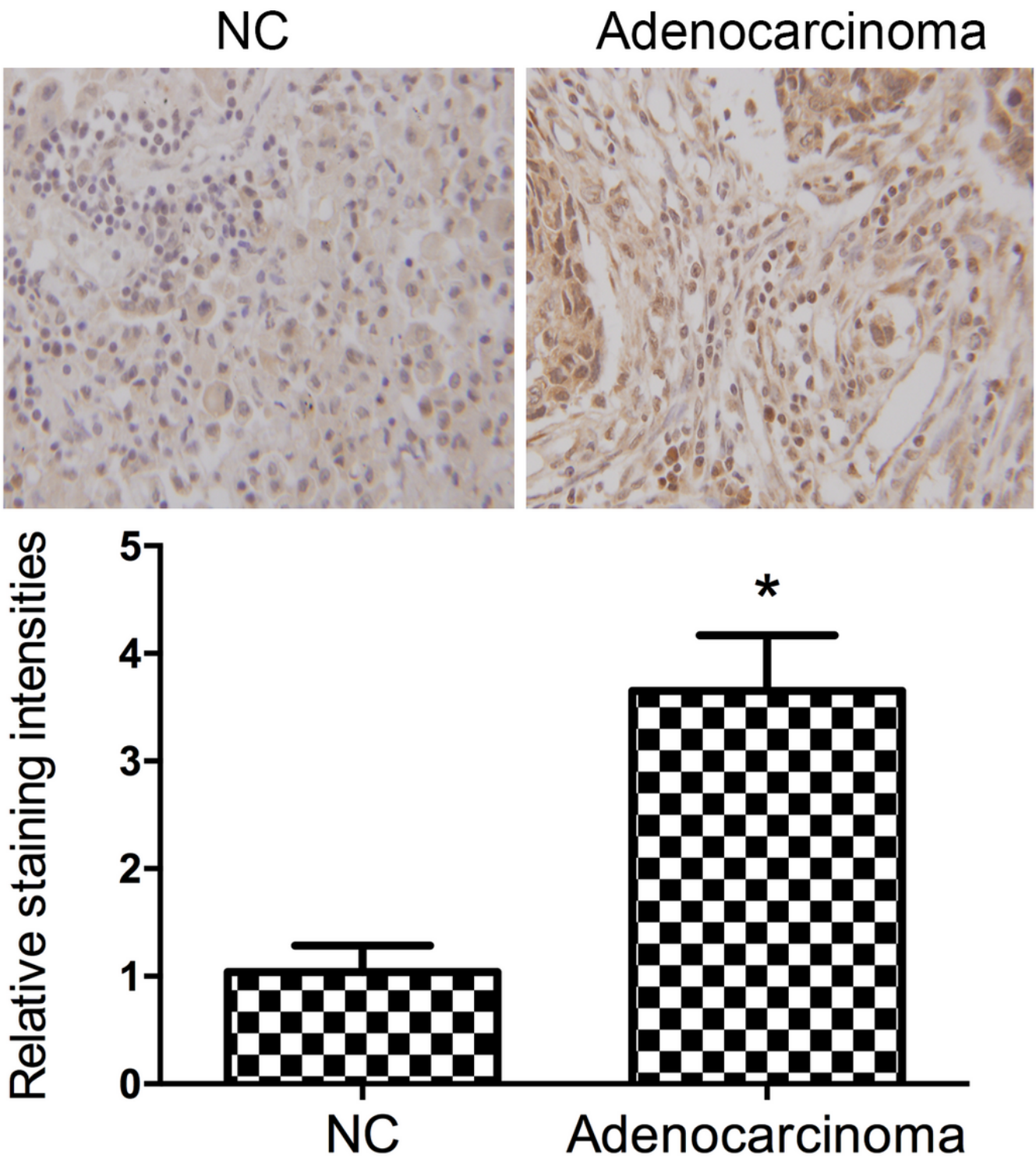

Figure 1

PC4 protein expressions were detected by immunohistochemistry in lung adenocarcinoma and normal lung tissue (×200). Relative staining intensities, the average positive stained cells percentage to evaluate the slice immunohistochemistry result, measured by ImageJ software.

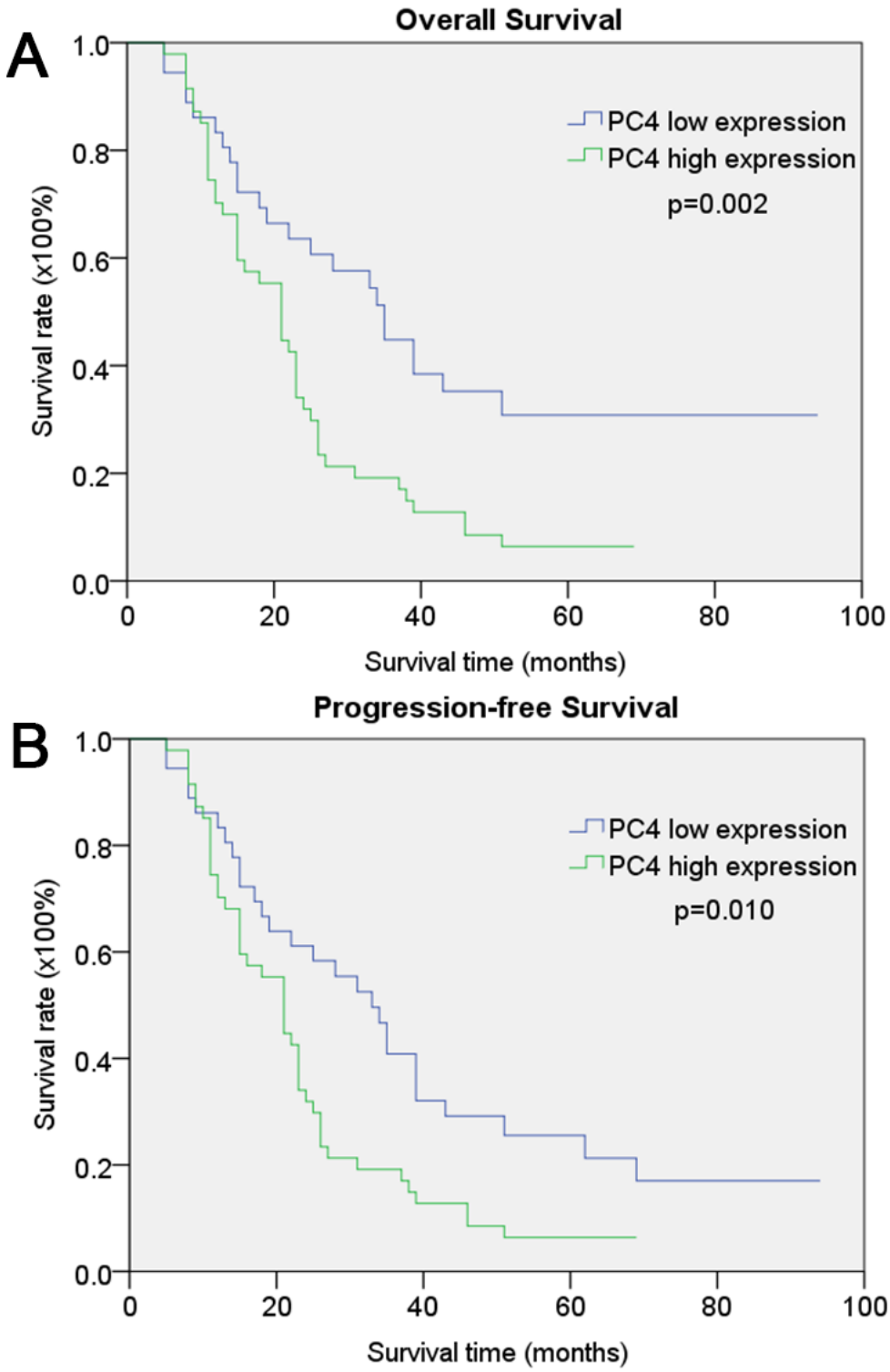

Figure 2

The expression of green fluorescence protein in A549 cell. A&B. Infected the siRNA PC4 recombinant lentiviral vector (×200). C&D. Infected control recombinant lentiviral vector (×200). E&F. A549 cell (×200).

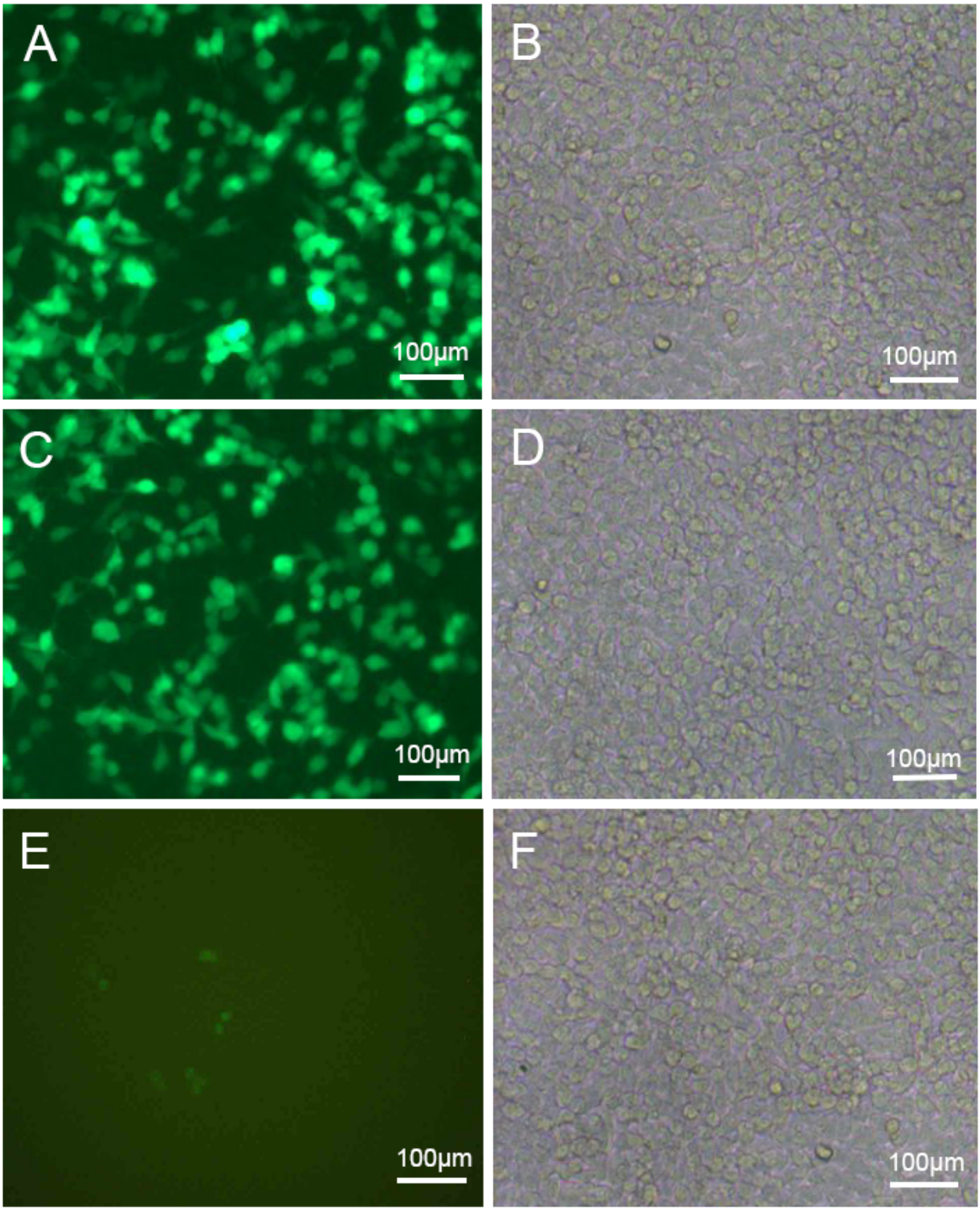

Figure 3

Patients were divided into high expression group (n=47) and low expression group (n=36). Kaplan-Meier survival analysis with log rank test was adopted for overall survival (A) and progression-free survival (B). PC4 high expression group had significantly poorer clinical outcome than PC4 low expression group in both OS and PFS analyze.

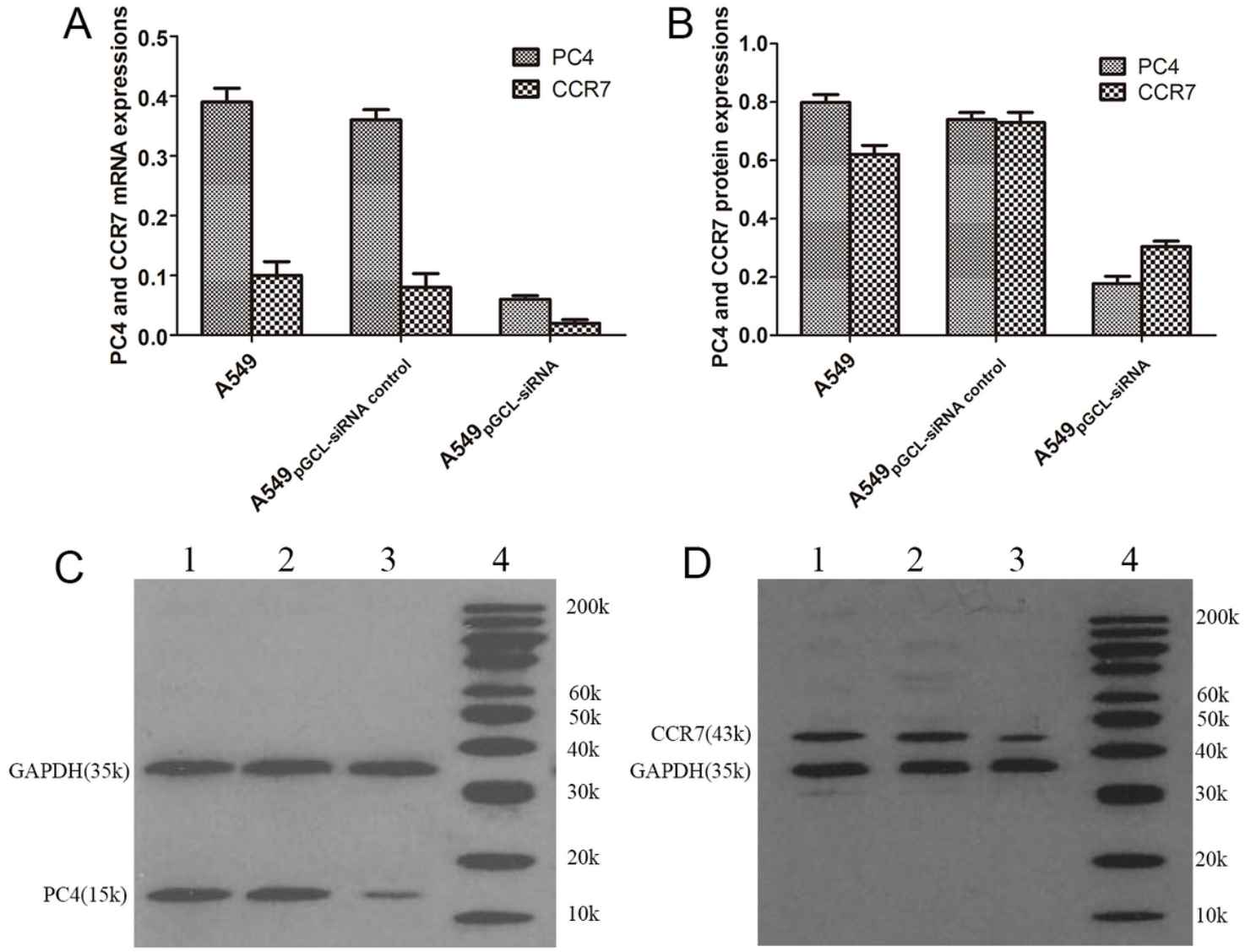

Figure 4

PC4 and CCR7 expressions were detected by qRT-PCR (A) and western blot (B). Western blot showed obvious reduction of PC4 (C) and CCR7 (D) expressions in A549pGCL-siRNA group. PC4 expression was significantly correlated with CCR7 expression in mRNA (r=0.661, P=0.026) and protein (r=0.589, P=0.001) levels. 1: A549 group 2: A549 pGCL-siRNA control group 3: A549pGCL-siRNA group 4: Marker.

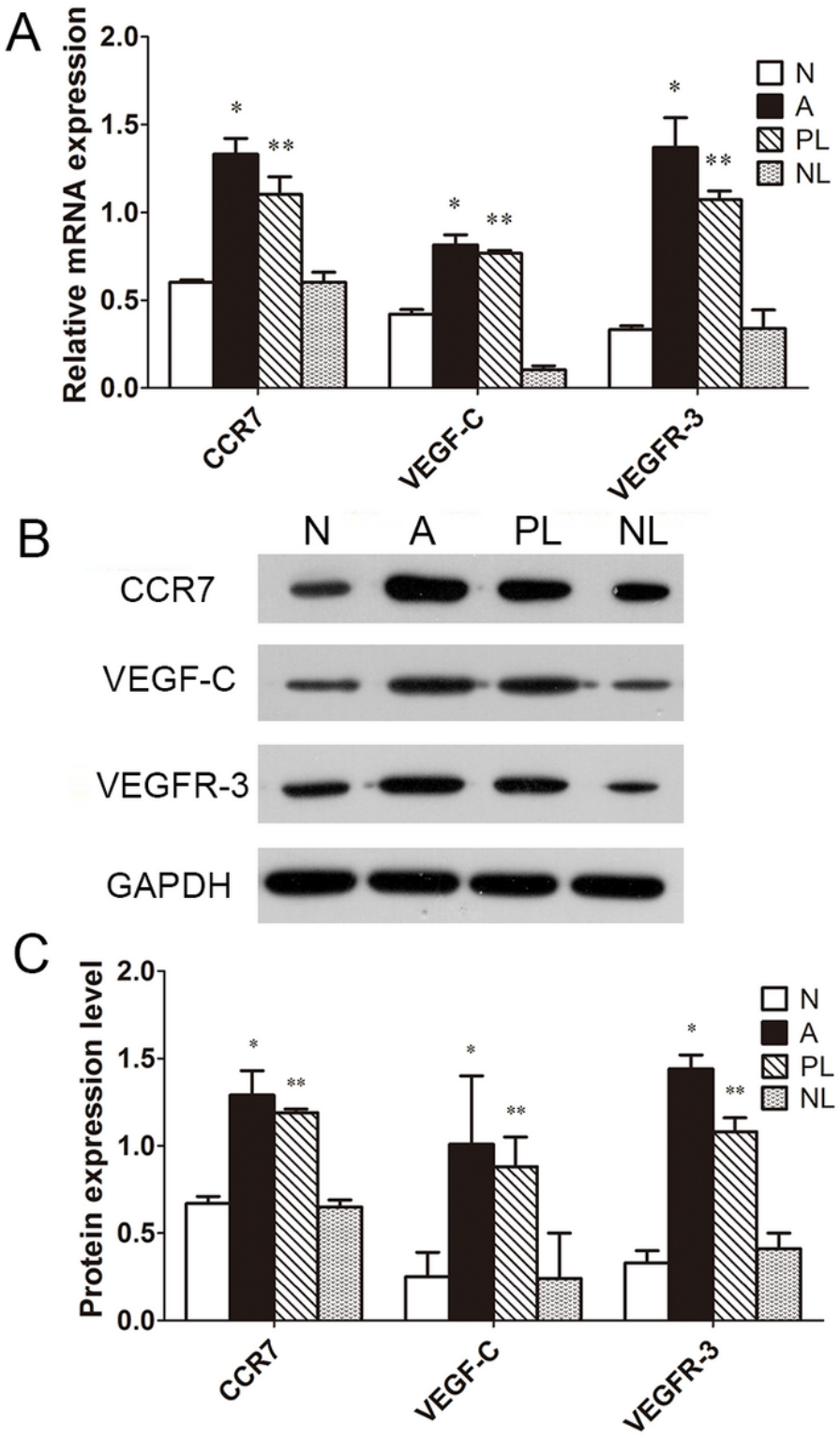

Figure 5

CCR7, VEGF-C and VEGFR-3 mRNA (A) and protein (B, C) expressions were detected by qRT-PCR and western blot respectively. *CCR7, VEGF-C and VEGFR-3 expressions were significantly higher in adenocarcinoma tissues than in normal lung tissues. **CCR7, VEGF-C and VEGFR-3 expressions were significantly higher in tumor-positive lymph nodes than in -negative lymph nodes. N: normal lung tissue; A: lung adenocarcinoma tissue; PL: tumor-positive lymph node; NL: tumor-negative lymph node.

Supplementary Files

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- Table1.pdf