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*Original Article*

# EphA4 protein promotes invasion in clear cell renal cell carcinomas

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**Abstract:** *E*rythropoietin-*p*roducing *h*epatocellular carcinoma cell receptor A4 (EphA4) is one of the largest superfamily of human Eph receptor tyrosine kinases. The roles of EphA4 receptor in the development of nervous system have been well documented. More recently, functions of EphA4 receptor in several types of human cancer were reported with a paradoxical result. The expression and clinicopathological significance of EphA4 receptor in clear cell renal cell carcinoma (ccRCC) have not been well investigated and are unknown. In this study, a set of formalin-fixed paraffin-embedded ccRCC tissue specimens were subjected to immunohistochemistry using a specific anti-EphA4 polyclonal antibody. The relationship between EphA4 expression and clinicopathological parameters was statistically analyzed. EphA4 receptor was differentially expressed inter-specimens, which was negative (score 0) or week (score 1) staining in 34 out of 56 (60.7%), moderate (sore 2) in 12 out of 56 (21.4%) and strong (score 3) in 10 out of 56 (17.9%) ccRCC specimens. Expression level of EphA4 was positively associated with primary tumor (pT) stage ($P$<0.001, $r_s$=0.611) and tumor-node-metastasis (TNM) stage ($P$<0.001, $r_s$=0.661). Strikingly, strong expression of EphA4 was observed in tumors that invading into renal veins. No relationship between EphA4 expression and Fuhrman nuclear grade, tumor size, age and sex was found. Our data suggest that EphA4 protein promotes ccRCC tumor cell invasion and may function in progression and metastasis of ccRCC. EphA4 may be used as a potential molecular marker for prognosis.

**Keywords:** Receptor tyrosine kinase, EphA4, TNM stage, metastasis, ccRCC

## Introduction

Clear cell renal cell carcinoma (ccRCC) is the most prevalent cancer of the kidney, accounting for approximately 75% of renal cell carcinoma (RCC) [1, 2]. It has a relative unfavorable prognosis compared to other common renal cell carcinomas, such as papillary and chromophobe renal cell carcinoma. The incidental diagnoses of RCC have become frequent due to the widespread use of abdominal imaging and ~60% patients are diagnosed at early stage [3-5]. When ccRCC is diagnosed at an early stage, the optimal treatment of localized tumors is surgical resection by radical or partial nephrectomy. Unfortunately about 20% of patients were diagnosed at advanced stage and one-third of patients will develop metastasis after initial surgery. Approximately ~30% of them would recur after surgery with poor 5-year survival and mortality rates of ccRCC have been climbing steadily [6, 7].

In the last decade, a significant improvement in the understanding of ccRCC carcinogenesis has resulted in the development of a predictive panel and target agents [8-13]. Though interleukin-2 and interferon-alpha have been widely used as first-line treatment of metastatic diseases, there is a low level of response. New target therapy have favorable results, which including bevacizumab targeting vascular endothelial growth factor (VEGF) and small molecular tyrosine kinase inhibitors targeting VEFG or platelet-derived growth factor (PDGF) such as sorafenib or sunitinib.

Receptor tyrosine kinases (RTKs) are transmembrane proteins, which mediating signals control embryonic development, physiologic and pathological processes. Eph receptors are the largest RTK with nine members of A-type (EphA1-8, 10) and four members of B-type (EphB1-4, 6). Similar to other RTKs, Eph receptors and their ephrin (Eph family receptor inter-

**Table 1.** The relation between EphA4 expression and clinicopathological parameters in ccRCC

| Parameter | All cases | EphA4 expression score | | | *P*-value | $r_s$ |
|---|---|---|---|---|---|---|
| | | 0/1 | 2 | 3 | | |
| pT stage | | | | | | |
| pT1 | 40 | 31 | 6 | 3 | <0.001 | 0.611 |
| PT2 | 5 | 3 | 2 | 0 | | |
| PT3+4 | 11 | 0 | 4 | 7 | | |
| TNM stage | | | | | | |
| I | 40 | 31 | 6 | 3 | <0.001 | 0.661 |
| II | 4 | 3 | 1 | 0 | | |
| III+IV | 12 | 0 | 5 | 7 | | |
| Nuclear grade | | | | | | |
| 1 | 21 | 14 | 2 | 5 | 0.990 | 0.002 |
| 2 | 26 | 14 | 8 | 4 | | |
| 3+4 | 9 | 6 | 2 | 1 | | |
| Tumor size | | | | | | |
| ≤7.0 cm | 46 | 31 | 6 | 9 | 0.130 | 0.205 |
| >7.0 cm | 10 | 3 | 6 | 1 | | |
| Age | | | | | | |
| <55 years | 28 | 16 | 8 | 4 | 0.824 | -0.030 |
| ≥55 years | 28 | 18 | 4 | 6 | | |
| Sex | | | | | | |
| Male | 44 | 25 | 11 | 8 | 0.353 | -0.127 |
| Female | 12 | 9 | 1 | 2 | | |

acting proteins) ligands have been demonstrated play important roles in embryonic development and human diseases including cancers. EphA4 was firstly identified from a 10 day chicken embryo library with a DNA probe corresponding to the related kinase Cek4, previously named as Sek, Sek1, Cek8, Hek8, and Tyro1 [14]. EphA4 has been demonstrated having important functions in the development of nervous system. Canty *et al* found EphA4 is dynamically expressed in the developing corticospinal tract (CST) of mice, high at the time of forelimb branching and down-regulated before hindlimb branching [15]. These findings suggest that EphA4 regulates topographic mapping of the CST by controlling the branching of CST axons in the spinal cord. Molecules involved in axon guidance play a role in blood vessel guidance. To examine whether EphA4 receptor plays a role in development of the central nervous system, Goldshmit *et al* examined wild-type and EphA4 mutant mice. They found EphA4-/- mice exhibited an abnormal CNS vascular structure and EphA4 was expressed on endothelial cells. EphA4 was not expressed in the adult. In wild-type mice after spinal cord injury, expression of EphA4 was markedly up-regulated on activated astrocytes that associated with blood vessels. Their data support EphA4 play role in CNS vascular formation and guidance during development [16]. EphA4 expression and its roles in carcinogenesis have been investigated in several types of human cancers with paradoxical results. Takahashi *et al* examined EphA4 mRNA expression in 205 cases of primary colorectal carcinoma fresh tissues with quantitative real-time RT-PCR [17]. They found the relative expression of EphA4 transcript was higher in the presence than in the absence of liver metastasis. Their data suggest that overexpression of EphA4 might promote liver metastasis in colorectal cancer. EphA4 mRNA and protein were detected in eleven gastric carcinoma cell lines, 24 paired surgical fresh specimens of gastric adenocarcinoma and adjacent nontumor tissue and 74 formalin-fixed paraffin embedded tumor specimens and a tissue microarray including 55 specimens by using RT-PCR and immunohistochemistry [18]. EphA4 mRNA was detected in 73% of gastric cancer cell lines and upregulation in 42% of gastric cancer tissue samples. EphA4 protein was positive in 48% of gastric cancer tissue, which was significantly associated with depth of invasion and recurrence. Very interestingly, Sun *et al* recently reported that EphA4 receptor reduced in breast cancer compared with matched normal luminal cells [19]. They found lower and reduced EphA4 expression are related to advanced TNM stage, lymph node metastasis and poor survival of patients. These data suggest that EphA4 receptor may play an organ-specific role in the development and progression of human cancers. Till now, according to our knowledge, the expression and clinicopathological significance of EphA4 receptor in clear cell renal cell carcinoma (ccRCC) have not been well investigated and is unknown.

## Materials and methods

### *Patients and specimens*

A total of 56 patients with ccRCC who underwent radical or partial nephrectomy between January, 2007 and December, 2013 at Jinling Hospital, China were included in the present retrospective study. All specimens were re-eval-

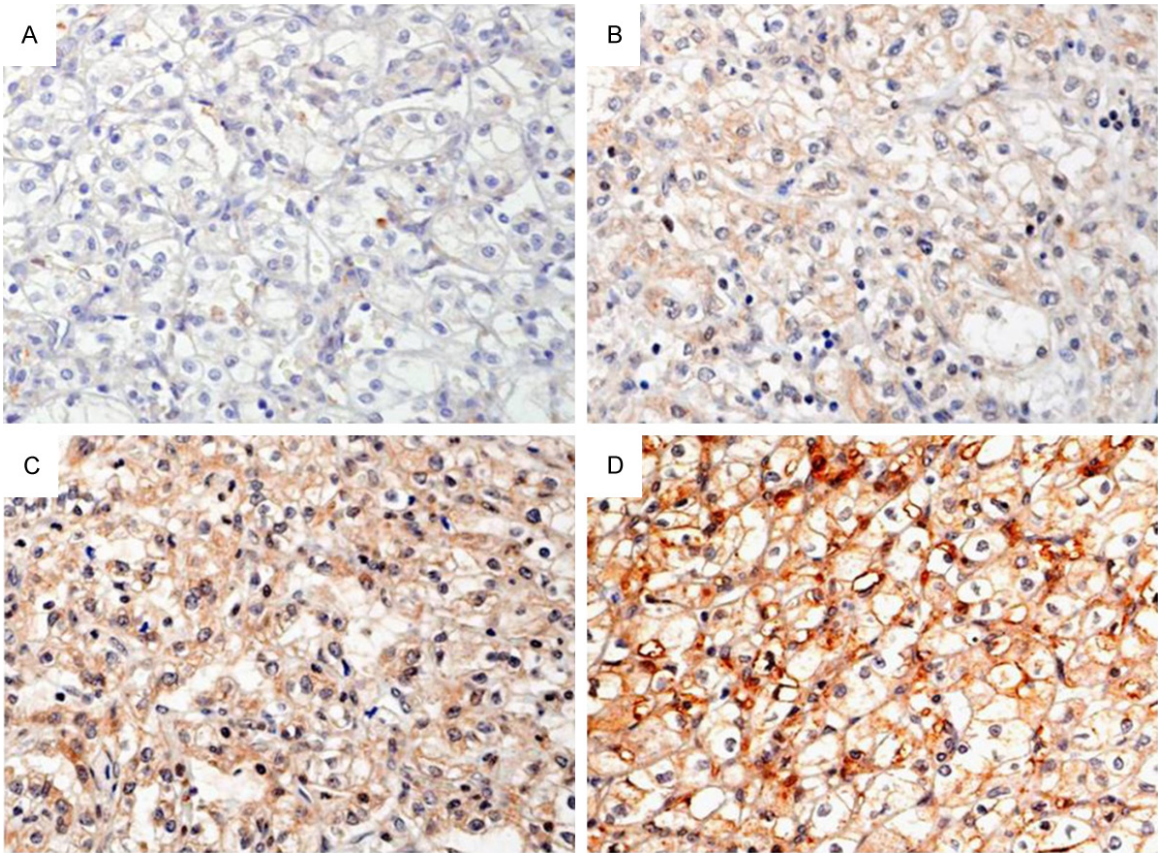


**Figure 1.** Representative examples of immunohistochemistry of EphA4 in ccRCC. A: Negative staining (score 0); B: Weak staining (score 1); C: Moderate staining (score 2); D: Strong staining (score 3). All magnification, 400×.

uated regarding to carcinoma type, the nuclear grade, and the stage of tumor. Nuclear grading was evaluated by Fuhrman grade method. Tumor stage was defined according to the 2010 TNM classification system. The detailed information of patients was listed in **Table 1**. This retrospective study was approved by the ethics committee of Jinling Hospital, Nanjing University School of Medicine, China.

*Immunohistochemistry analysis*

Immunohistochemical staining was carried out using a standard protocol. Briefly, 4-μm sections from formalin-fixed and paraffin-embedded tissues mounted on poly-L-lysine coated slides. The slides were deparaffinized in xylene and rehydrated through a graded ethanol series. The sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min for antigen retrieval, then cooled to 30°C and washed with phosphate-buffered saline (PBS, pH 7.3). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Slides were incubated overnight at 4°C with primary polyclonal anti-EphA4 antibody (Abgent, San Diego, CA, USA) at a 1:100 dilution in antibody diluent solution (Zymed, Invitrogen, California, USA) and then washed with PBS. Next, the sections were incubated with secondary antibody (Dako REAL EnVision Detection System, Dako, Glostrup, Denmark) for 30 min at room temperature. Color development was performed with 3, 3'-diaminobenzidine (DAB).

The primary antibody was replaced by PBS to validate the specificity of negative controls.

The immunostaining results were evaluated independently by two pathologists. The different results were verified by consensus. EphA4 immunoreactivity was scored on a scale of 0 to 3. The score of EphA4 expression was made semi-quantitatively by assessing the percentage of stained cells and the staining intensity in both tumor tissue and normal mucosa. The tumor was assigned a score of 0 if there was no staining or if there was staining in <10% of the

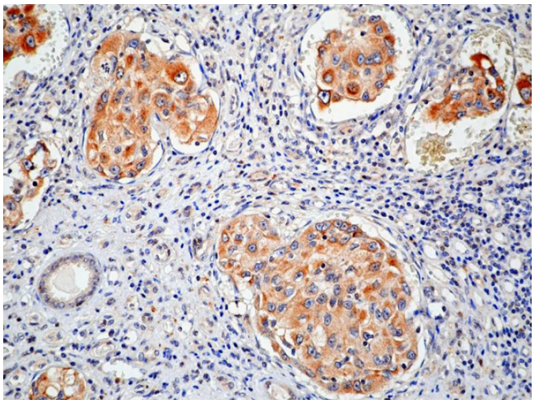

**Figure 2.** ccRCC tumor cells invaded into renal veins and showed strong expression of EphA4 protein. Magnification, 400×.

tumor cells; 1 if there was only weak staining in >10% of the tumor cells; 2 if there was moderately intense staining in >10% of the tumor cells; and 3 if there was intense staining in >10% of the tumor cells. The expression of the EphA4 protein in colorectal carcinomas was categorized into low expression (0~1), moderate expression (2), and high expression (3).

*Statistical analysis*

The correlation between EphA4 expression and clinicopathological parameters was evaluated by a Spearman's rank correlation test. All statistical analyses were performed by use of SPSS 15.0 software (SPSS, Chicago, IL, USA). A *P*-value <0.05 was considered statistically significant.

## Results

*Expression of EphA4 in ccRCC*

The expression of EphA4 protein was evaluated in 56 cases ccRCC tissue specimens. As shown in **Table 1**, EphA4 protein was negatively or weakly (score 0/1) detected in 60.7% (34/56), moderately (score 2) detected in 21.4% (12/56), and strongly (score 3) detected in 17.9% (10/56) of ccRCC. EphA4 protein was subcellularlly located in the cytoplasm (**Figure 1**). Heterogeneous expression of EphA4 was observed intra- and inter-specimens.

*The clinical significance of EphA4 protein*

The relationship between the expression level of EphA4 protein and clinicopathological parameters was statistically analyzed. Expression of EphA4 was positively associated with primary tumor stage pT ($P<0.01$, $r_s=-0.611$) and Tumor-node-metastasis TNM stage ($P<0.001$, $r_s=0.661$). No significant relationship between EphA4 expression and sex, age of patients, tumor size and Fuhrman nuclear grade was found (**Table 1**).

*EphA4 protein is strongly expressed in tumors invading into renal veins*

Strikingly, we observed that EphA4 protein was strongly expressed in tumor cells invading into renal veins in all 4 cases out of 67 ccRCC tissue samples (**Figure 2**).

## Discussion

The poor diagnosis of early stage ccRCC and resistance to both traditional chemotherapy and radiation therapy are the cause of treatment failure in patients. Therefore, understanding the molecular mechanisms during the initiation and the development of ccRCC and the identification of tumor-specific prognostic factors are of great importance, and findings could be used to help identify therapy strategies, guide the therapeutic intervention and follow-up strategies in ccRCC. Renal cell carcinoma is notorious for its potential to metastasize, often before the primary renal tumor has become symptomatic. The histologic diagnosis of metastatic RCC may be difficult, particularly in small biopsies owing to the morphologic heterogeneity of RCC and the significant morphologic overlap with nonrenal neoplasms in many different anatomic sites. Prediction of prognosis in ccRCC is traditionally based on clinicopathological variables, especially tumor-node-metastasis stage (TNM), Fuhrman nuclear grade, the presence of a sarcomatoid component, tumor necrosis, and invasion of the renal or hilar fat by tumor cells.

EphA4 receptor promotes tumor metastasis has been reported in several human cancers, especially in colorectal cancer and gastric cancer [17, 18, 20]. Recently, Jing *et al* reported their research explored the possible mechanism for EphA4 promoting tumor cell metastasis [21]. They isografted 4T1 breast cancer cell into both EphA4-knockout and control wild-type female littermate mice. Their results show that the EphA4-deleted host could inhibit breast

cancer cell growth and metastasis. They found that the level of insulin-like growth factor 1 (IGF1) in the circulation and local tissues was decreased. The inhibition of tumor growth can be reversed by supplying the mice with IGF1. They deduced that EphA4-deleted microenvironment and delayed tumor development reduced the production of granulocyte colony-stimulating factors (G-CSF) resulting in the decrease of splenomegaly and leukemoid reaction including myeloid-derived suppressor cells (MDSCs), which leading to inhibit the tumor growth. de Marcondes *et al* found that progeny of irradiation survivor human colon cancer cell line HT-29 cells develop a more aggressive phenotype with increased EphA4 activity [22]. EphA4 interacted with E-cadherin in the progeny of radiation survivor HT 29 cells and EphA4 can induce adherens junction disorganization including E-cadherin internalization and down-regulation. They demonstrated AKT and ERK1/2 signaling pathways active in the progeny of radiation survivor HT-29 cells is dependent on EphA4. Their results indicate that EphA4 can promote migration, invasion, and metalloproteease-2 activation in the progeny of radiation survivor HT-29 cells.

In this study, we found that EphA4 expression is associated with primary tumor pT stage and TNM stage in ccRCC. Very interestingly, tumors invaded into renal veins presented strong expression of EphA4 protein. The important factors evaluated for pT stage include tumor size and invasion into adrenal gland, perinephric tissues, renal sinus (peripelvic) fat, renal veins, and directly invade beyond Gerota fascia. Our data indicate that EphA4 may play a role in promoting ccRCC tumor cell invasion. This is a preliminary report for EphA4 expression file and clinical significance of EphA4 in ccRCC. In the next project, we will explore the molecular mechanisms for EphA4 promoting invasion of ccRCC cells *in vitro* and *in vivo*.

## Conclusion

EphA4 expressed in part of ccRCC tumors and EphA4 expression is associated with pT and TNM stage. EphA4 receptor promotes invasion and may function in progression and metastasis of ccRCC. EphA4 may be used as a potential molecular marker for prognosis.

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## Disclosure of conflict of interest

None.

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