INTERNATIONAL JOURNAL OF Experimental Pathology

Int. J. Exp. Path. (2017), 98, 34–39

ORIGINAL ARTICLE

Decreased expression of EphA5 is associated with Fuhrman nuclear grade and pathological tumour stage in ccRCC

Xiaolin Wang*[,a], Haifei Xu*[,a], Zhijun Wu[†], Xiao Chen[‡] and Jiandong Wang[‡]

*Department of Urology, Nantong Tumor Hospital, Nantong, Jiangsu, China, [†]Department of Radiotherapy, Nantong Tumor Hospital, Nantong, Jiangsu, China and [‡]Department of Pathology, Jinling Hospital, Nanjing University School of Medicine, Nanjing, China

INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY

doi: 10.1111/iep.12219

Received for publication: 22 August 2016
Accepted for publication: 23 December 2016

Correspondence:
Zhijun Wu
Department of Radiotherapy
Nantong Tumor Hospital
Nantong
Jiangsu 226361
China
E-mail: ntzlyyflk@163.com
and
Jiandong Wang
Department of Pathology
Jinling Hospital
Nanjing University School of Medicine,
Nanjing 210002
China
E-mail: jdwang1216@163.com
[a]These authors equally contributed to this work.

SUMMARY

The incidence of renal cell carcinoma is increasing all over the world. The molecular mechanisms for tumorigenesis, progression and prognosis are still unknown. The erythropoietin-producing hepatoma amplified sequence (Eph) receptors have been reported to be expressed aberrantly in many types of human cancers and in particular EphA5 may play a role in certain human cancers. In this study, a set of clear cell renal cell carcinoma (ccRCC) tissues were subjected to immunohistochemistry. The relationship between EphA5 protein expression and clinicopathological parameters was statistically analysed. Our data show that EphA5 protein was negatively (0) or weakly (1+) expressed in 48 of 78 (61.5%), moderately (2+) expressed in 15 of 78 (19.2%) and strongly (3+) expressed in 15 of 78 (19.2%) tumour samples of ccRCC. Decreased expression of EphA5 was detected more often in females than in males ($P = 0.017$, $r_s = -0.267$). Expression of EphA5 was related negatively to Fuhrman grade ($P = 0.013$, $r_s = -0.279$) and pathological tumour stage pT ($P = 0.003$, $r_s = -0.334$). No relation between the expression of EphA5 and age of patients was found ($P = 0.107$, $r_s = 0.184$). Fuhrman grade and pT stage are the most important factors used in prognosis of ccRCC. Hence this study may provide a new and useful prognostic marker in the clinical practice of ccRCC.

Keywords
clear cell renal cell carcinoma, EphA5, receptor tyrosine kinase, renal cell carcinoma

Renal cell carcinoma (RCC) is the most common malignancy derived from the adult kidney, accounting for 2–3% of all human tumour types in adults (Ricketts *et al.* 2016; Sun & Choueiri 2016). It has been increasing in incidence over recent decades, with 61,560 estimated new cases in 2015 (Siegel *et al.* 2015). This increased incidence of RCC has been observed in both developed and developing countries. According to the World Health Organization classification of RCC, clear cell RCC (ccRCC) is the most prevalent histological subtype and accounts for around 80% of all RCCs and more than 90% of advanced metastatic cases (Trpkov *et al.* 2013; Humphrey 2014; Dunnick 2016).

Even though a dramatic improvement has been made in the management of ccRCC, the clinical behaviours and long-term outcomes are highly variable (Fu *et al.* 2016; Kawano *et al.* 2016; Lopez *et al.* 2016). Although a number of traditional clinicopathologic parameters are used in current clinical practice, the ability to predict the outcome after surgical or systemic therapy is still limited. Thus, the identification of tumour-specific prognostic factors is of great importance, underscoring the need for new approaches or

novel prognostic markers to assist in the management of ccRCC, which could help guide the therapeutic intervention and follow-up strategies.

Both patient genetic background and environmental factors contribute to ccRCC pathogenesis. Loss of chromosome 3p is the most prevalent genetic alteration in ccRCC, while a mutation of the von Hippel–Lindau gene is observed in approximately two-thirds of patients with ccRCC (Kobayashi *et al.* 2016). Loss of the von Hippel–Lindau tumour suppressor function due to either gene alteration or promoter hypermethylation is the crucial event in the tumorigenesis for this tumour subtype.

Therapeutic strategies for advanced ccRCC have been focused on the role of hypoxia signalling in this tumour type in view of its genetic/epigenetic silencing of the von Hippel–Lindau gene. This has led to the development of therapies targeting the vascular endothelial growth factor which induced by overexpression of hypoxia-inducible factor (HIF), which is part of a known family of transcription factors (Gao *et al.* 2013; Leisz *et al.* 2015; Messai *et al.* 2015).

The erythropoietin-producing hepatoma amplified sequence (Eph) receptor tyrosine kinase family is the largest family of tyrosine kinases, which was originally identified in the late 1980s in a human cDNA library screen for homologous sequences to the tyrosine kinase domain of the viral oncogene v-fps (Hirai *et al.* 1987). The Eph receptors make up the largest subgroup of the receptor tyrosine kinase (RTK) family. Eph receptors are divided into either the A or the B type based on their interactions with ephrin ligands. Eph receptors and their ephrin ligands have also been implicated in diverse developmental and neurological functions, including hindbrain development in vertebrates and tissue patterning. Particularly, Eph receptors are activated by ephrin ligands and appear to play important roles in axon guidance and cell migration during development of the nervous system (Tessier-Lavigne 1995; Holder *et al.* 1998; Zhang & Hughes 2006). Overexpression or constitutive activation of Eph receptors has been linked with increased proliferation in various tumours. Overexpression of EphA1 in NIH3T3 cells led to formation of foci in soft agar and promoted tumour formation in nude mice, suggesting a role of EphA1 as a classical oncogene (Maru *et al.* 1990). EphA2 was more highly expressed in gastric cancer tissues than in normal gastric mucosa tissues, and this was closely correlated with the depth of tumour invasion, tumour-node-metastasis stage and lymph node metastasis (Hou *et al.* 2012). The positive expression of EphA2 was negatively associated with E-cadherin expression and positively correlated with beta-catenin ectopic expression and vimentin expression in gastric cancer. These findings indicate that the EphA2 expression correlated with the loss of epithelial proteins and expression of mesenchymal proteins. EphA2 may be involved in epithelial mesenchymal transition in gastric cancer. EphA5 was first isolated from an adult mouse brain expression cDNA library and named as *bsk* (brain specific kinase) (Zhou *et al.* 1994). Zhou *et al.* reported that bsk was highly expressed in the brain with no detectable signals in other organs of mouse and was predominantly expressed in the hippocampus and other limbic structures. Increasing evidence shows that EphA5 plays an important function in the development of brain.

The expression and function of EphA5 in human cancers have not been intensively investigated. The role of EphA5 has been explored previously in human breast cancer, prostate cancer and lung cancer (Fu *et al.* 2010; Li *et al.* 2015; Staquicini *et al.* 2015). To the best of our knowledge, no study of EphA5 has been reported in ccRCC. In this study, we detected the expression of EphA5 protein in a set of ccRCC tissue specimens and statistically analysed the relationship between the expression of EphA5 and clinicopathological parameters.

Materials and methods

Patients and samples

The study included 78 patients (54 male and 24 female; aged 35–75 years, median age 53 years) with pathologically diagnosed ccRCC who had undergone radical or partial nephrectomy without any neoadjuvant treatment between January 2010 and December 2015 at Nantong Tumor Hospital (Nantong, China). All tissue samples were retrieved from the archive of the Department of Pathology, Nantong Tumor Hospital. All cases were classified according to World Health Organization Classification of Tumours (WHO), Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs (Lyon, 2004). Clinicopathological parameters of the patients with ccRCC, including age, sex, Fuhrman nuclear grade and pathological tumour stage pT, were reviewed. The protocols used in the study were approved by the institutional review board of the Nantong Tumor Hospital and were performed in accordance with international guidelines for the use of human tissues.

Histochemistry

The specimens were fixed in 10% buffered formalin and embedded in paraffin. A representative formalin-fixed, paraffin-embedded tissue block with viable tumour was selected from each case. From each block, serial 4-μm unstained sections were obtained and submitted for IHC staining. Immunohistochemical staining was performed by the standard method. Briefly, each tissue section was deparaffinized and rehydrated. The sections were autoclaved for antigen retrieval in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min after rehydration through a graded ethanol series. Then, they were cooled to 30°C and washed with phosphate-buffered saline (PBS, pH 7.3). The sections were blocked with 10% normal calf serum in phosphate-buffered saline for 10 min and then incubated with anti-EphA5 polyclonal antibody (ABGENT, San Diego, CA 92121, USA) at a dilution of 1:400 at 4°C overnight. The sections were incubated with secondary antibody (Dako REAL EnVision Detection System; Dako, UK) for 20 min at room temperature. This was followed by colour development with 3,3′-diaminobenzidine solution for 1 min

and counterstaining with haematoxylin for 3 min. Primary antibody was replaced with antibody diluent for negative controls. The colon mucosa with known positivity was used as a positive external control.

Evaluation for immunoreactivity

The immunostaining results were evaluated independently by two pathologists. Any different results were verified by consensus. EphA5 immunoreactivity was scored on a scale of 0 to 3+ based on a semiquantitative system including the intensity and extent of staining. The tumour was assigned a score of 0 if there was no staining or if there was staining in <10% of the tumour cells; 1+ if there was only weak staining (light brown) in >10% of the tumour cells; 2+ if there was moderately intense staining (brown) in >10% of the tumour cells; and 3+ if there was intense staining (dark brown) in >10% of the tumour cells.

Statistical analysis

Statistical calculation was performed using SPSS version 15.0 for Windows software (SPSS Inc., Chicago, IL, USA). The Spearman test was used to analyse the possible association of WLS protein expression with clinicopathological parameters. A $P < 0.05$ was considered statistically significant.

Ethical approval statement

This investigation was performed following approval from the Ethics Committee of Nantong Tumor Hospital, China (2015-040).

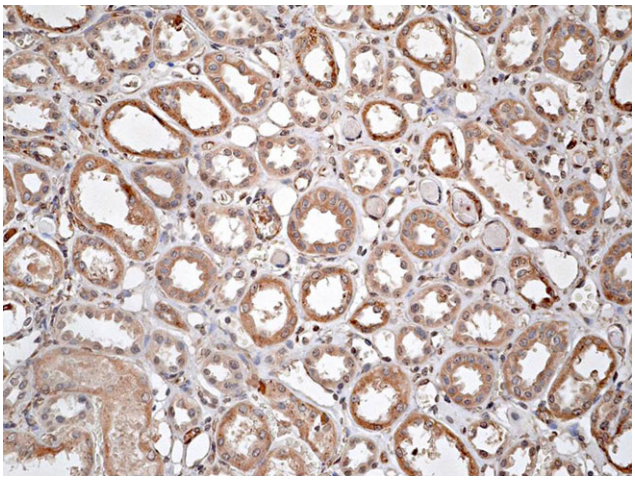

Figure 1 EphA5 protein was strongly expressed in the cytoplasm of normal tubular epithelial cells (3+). (400× original magnification). [Colour figure can be viewed at wileyonlinelibrary.com].

Results

Immunohistochemistry of EphA5 in ccRCC

EphA5 protein expression in human ccRCC and normal kidney was examined by immunohistochemistry. EphA5 protein was positively expressed in the cytoplasm of normal tubular epithelial cells of all specimens tested (Figure 1). The EphA5 was heterogeneously expressed in intersamples of tumour cells (Figure 2). The subcellular location of EphA5 protein in tumour cells was the cytoplasm and membrane. The EphA5 was

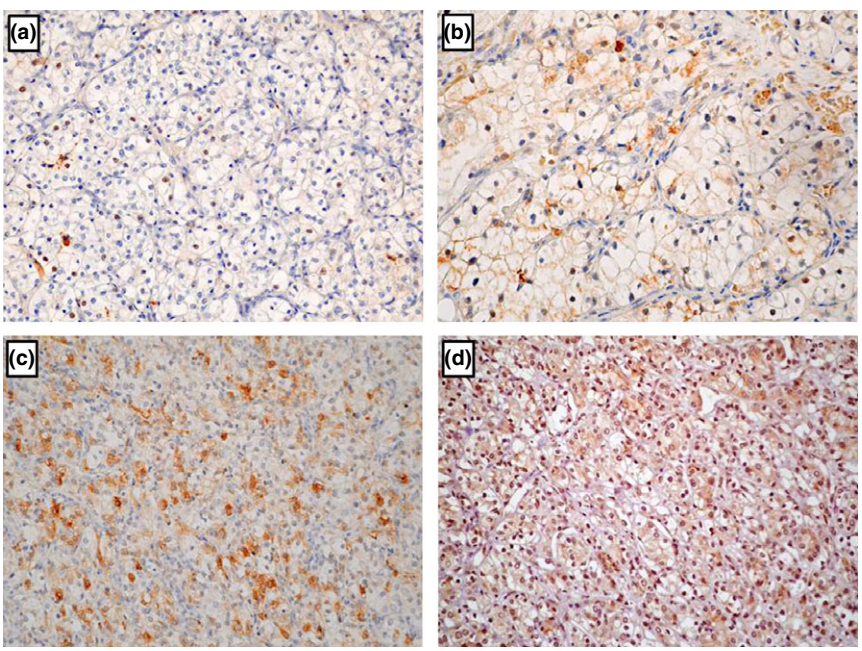

Figure 2 a. EphA5 protein was negatively expressed in the cytoplasm of ccRCC cells (0). b. EphA5 protein was weakly expressed in the cytoplasm of ccRCC cells (1+). c. EphA5 protein was moderately expressed in the cytoplasm of ccRCC cells (2+). d. EphA5 protein was strongly expressed in the cytoplasm of ccRCC cells (3+). (400× original magnification). [Colour figure can be viewed at wileyonlinelibrary.com].

Table 1 The relationship between the expression of EphA5 and clinicopathological parameters

		EphA5 protein				
Parameters	**No.**	**0/1+**	**2+**	**3+**	***P***	r_s
	78	48	15	15		
Gender						
Male	54	28	14	12	0.017	−0.269
Female	24	20	1	3		
Age (years)						
<=50	30	21	7	2	0.107	0.184
>50	48	27	8	13		
Fuhrman Grade						
1/2	27	12	6	9	0.013	−0.279
3/4	51	36	9	6		
Tumour stage						
T1	48	23	13	12	0.003	−0.334
T2~T4	30	25	2	3		

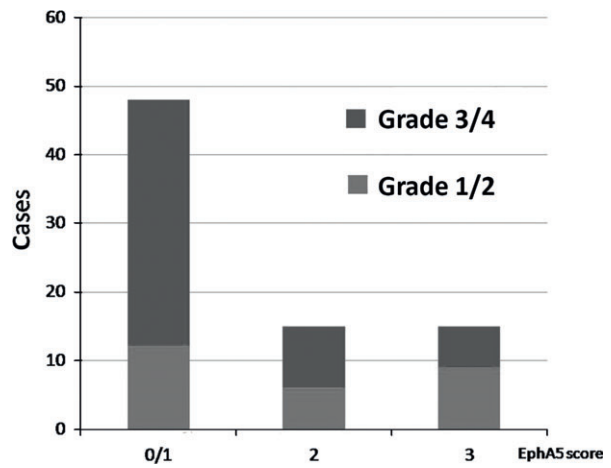

Figure 3 The expression of EphA5 was negatively related to Fuhrman grade.

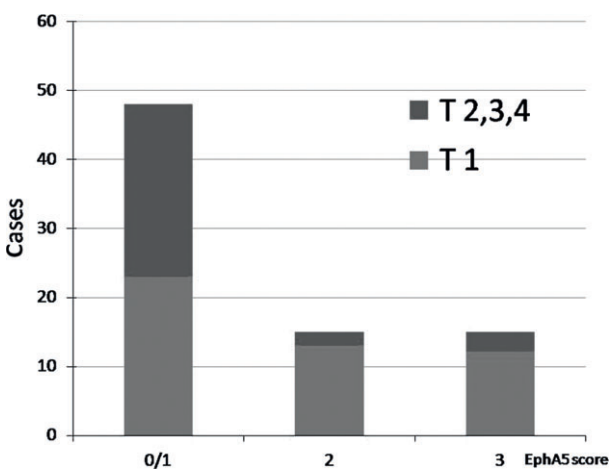

Figure 4 The expression of EphA5 was negatively related to pathological tumour stage pT.

negatively (0) or weakly (1+) expressed in 48 of 78 (61.5%), moderately (2+) expressed in 15 of 78 (19.2%) and strongly (3+) expressed in 15 of 78 (19.2%) tumour samples of ccRCC.

The relationship between the expression of EphA5 and clinicopathological parameters

EphA5 expression was evaluated by immunohistochemistry analysis in 78 ccRCC specimens. The relationship between the expression of EphA5 protein and clinicopathological parameters was statistically analysed. As shown in Table 1, the decreased expression of EphA5 was often detected in females than in males ($P = 0.017$, $r_s = -0.267$). The expression of EphA5 was negatively related to Fuhrman grade ($P = 0.013$, $r_s = -0.279$; Figure 3) and pathological tumour stage pT ($P = 0.003$, $r_s = -0.334$; Figure 4). No relation between the expression of EphA5 and age of patients was found ($P = 0.107$, $r_s = 0.184$).

Discussion

In the present study, we detected the expression of EphA5 protein in a set of ccRCC tissue samples and analysed the association of expression level of EphA5 with clinicopathological parameters. To the best of our knowledge, this is the first time that the expression and clinical significance of EphA5 protein in ccRCC has been explored.

EphA5 receptor tyrosine kinase, similar to the other members of the Eph family, not only is involved in the human development, but also plays an important role in tumorigenesis and progression of human cancers. Staquicini *et al.* reported their research results of EphA5 in human lung cancer (Staquicini *et al.* 2015). They searched for new molecular targets through screening a phage display library and found a cell-binding peptide that exhibited targeting specificity for human lung cancer cells. They identified EphA5 as a molecular target of lung cancer. EphA5 was highly expressed in human lung cancer cell lines NCI-H522, NCI-H460 and NCI-H1299, weakly expressed in NCI-H23 and A549 and lost in NCI-H226. Functional studies found that, in the absence of EphA5, lung cancer displayed a defective G1/S cell cycle checkpoint and became radiosensitive. EphA5 was transported into the nucleus and interacted with activated ataxia-telangiectasia mutated (ATM) for DNA repair after cells were irradiated. They demonstrated that EphA5 is a novel regulator of IR-induced cell cycle checkpoint and DNA damage repair with unexpected roles in the resistance of lung cancer to radiotherapy. Signalling of Eph receptors and ephrin ligands (Eph-ephrin) is involved in human cancer development and progression. Eph receptors were thought to play an oncogenic role in human cancer. However, increasing evidence suggests that the role of Eph receptors is far more complex. Li *et al.* found that EphA5 mRNA was downregulated or lost in 62.2% prostate carcinomas, 5.1% hyperplasias and six prostate cancer cell lines, while EphA5 protein was reduced in 76.9% of carcinoma samples. They

demonstrated that downregulation of EphA5 in prostate carcinoma was due to the hypermethylation of CpG island at promoter region of the gene (Li *et al.* 2015). The status of EphA5 methylation was associated with Gleason score and tumour stage. Prostate cancer cells' invasion and migration were significantly inhibited by introduction of EphA5 *in vitro*. Fu *et al.* examined the EphA5 expression in a set of breast carcinoma specimens and found that the level of EphA5 mRNA was dramatically decreased in breast carcinoma compared with normal tissue. They also demonstrated that aberrant methylation of EphA5 was the molecular mechanism leading to downregulation of EphA5 in breast carcinoma (Fu *et al.* 2010).

In the present study, we found EphA5 was strongly expressed in normal tubular epithelial cells of all specimens tested, while negatively or weakly expressed in 61.5% ccRCC specimens. EphA5 protein was significantly reduced in ccRCC. We deduce that aberrant methylation of CpG island may be the molecular mechanism leading to downregulation of EphA5 in ccRCC. We will check the methylation status of promoter region of EphA5 gene in future studies. The expression of EphA5 was negatively related to Fuhrman grade and pathological tumour stage. Histological grade and pT stage of renal cell carcinoma have been thought the most powerful prognostic indicators. The Fuhrman grading system used by pathologists around the world is based on nuclear size, nuclear shape, nucleolar size and nuclear pleomorphism (Trpkov *et al.* 2013; Humphrey 2014) without consideration of any molecular markers. To our knowledge, the association of EphA5 protein and Fuhrman grade has not been reported. Interestingly, we found that decreased expression of EphA5 was detected more often in females than in males. Emerging evidence suggests there is an interplay between EphA2, a member of Eph A-type receptors, and oestrogen receptors (ER). Oestrogen regulates EphA2 expression in breast cancers (Zelinski *et al.* 2002; Lu *et al.* 2003) and thus we hypothesize that there may be an interaction between EphA5, ER, and other hormones or their receptors.

In summary, we found that EphA5 protein decreased in ccRCC cells compared to normal renal tubular epithelial cells. The molecular mechanism of downregulation of EphA5 in ccRCC may be hypermethylation of CpG island. Decreased expression of EphA5 is associated with Fuhrman nuclear grade and pT stage of ccRCC. Our data indicate that EphA5 protein could be used as a new prognosis marker in the clinical practice of ccRCC.

Conflict of interests

The authors declare that they have no competing interests.

Funding source

This work was supported in part by the National Natural Science Foundation of China (81371611).

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