Original Article

EphrinB2: Expression of a novel potential target in renal cell carcinoma

Chhavi Gupta[1,2], Akash Pramod Sali[1,2]*, Alexandra Jackovich[1,3], Binyun Ma[3], Sarmad Sadeghi[3], David Quinn[3], Parkash Gill[1,3], Inderbir Gill[1]

[1]Department of Urology, Keck School of Medicine, University of Southern California, [3]Department of Medicine, USC Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA, [2]Department of Pathology, Karkinos Healthcare Private Limited, Navi Mumbai, Maharashtra, India

*E-mail: aakki1910@gmail.com

ABSTRACT

Introduction: Renal cell carcinoma (RCC) is primarily managed by surgery with the use of systemic targeted therapy in a metastatic setting. Newer targeted therapeutic options are evolving; Eph-ephrin is a potential new pathway. The therapeutic potential of targeting the EphB4-EphrinB2 pathway has been demonstrated in many solid tumors; however, its expression in RCC has only been evaluated in a few studies with limited cases. We herein determine the immunohistochemical expression of EphrinB2 in RCC.

Methods: A tissue microarray comprising 110 cases of different histological subtypes of RCC and 10 normal kidney tissues were stained with monoclonal anti-EphrinB2 antibody (Abcam, AB201512). The tumor and endothelial cells expressing the EphrinB2 were examined and its expression was correlated with sex, histological subtypes, and tumor nodes metastasis (TNM) stage.

Results: Twenty cases of urothelial carcinoma and two unsatisfactory conventional clear cell RCC cases were excluded, and EphrinB2 expression was interpreted in the remaining 88 tumors. EphrinB2 was expressed in 42 out of 88 tumors (47.7%) and was negative in the normal renal parenchyma. There was a statistically significant difference in the expression of EphrinB2 in males (55%) and females (32%). However, no such difference of expression was noted for the histological subtypes and the stages. Half (51%) of Stage 1 (n = 30) and Stage 2 (n = 11) tumors showed EphrinB2 positivity.

Conclusions: EphrinB2 is expressed in approximately half of RCC cases. EphrinB2 expression in the early stage cancer might indicate its induction as an early event.

INTRODUCTION

Cancer of the kidney and renal pelvis is the sixth-most common cancer in men and the eighth-most common cancer in women in the United States.[1] Among renal cell carcinoma (RCC), clear cell RCC (ccRCC) is the most common subtype, accounting for 65%–70% of all renal cancers.[2] Surgery has been the standard of care for most patients with localized and locally advanced RCC. Nonetheless, the risk of recurrence remains as high as 10%.[3] On the other hand, metastatic RCC is treated with systemic therapy, with or without cytoreductive nephrectomy, and remains an active area of investigation.[4,5] Apart from the distinct advantages of each category of systematically administered drugs, many patients relapse, requiring novel therapies.[4-6] Hence, the identification of novel targets has led us to study the Eph-ephrin pathway.

Eph-ephrin is a receptor tyrosine kinase-ligand system that has many cellular and physiological functions in embryonic

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life and is also involved in certain physiologic and pathologic conditions in adults. Eph, a receptor tyrosine kinases, forms the largest family with 14 members divided into Classes A and B based on the sequence homology.[7] Both classes of Eph receptors have a similar structure with extracellular, transmembrane, and intracellular domains. Their ligands, the ephrins, are also divided into Classes A and B. Of the ligands, Ephrins-A are glycosylphosphatidylinositol (GPI) -anchored surface proteins, while Ephrins-B are transmembrane proteins.[8] The receptor-ligand interactions are bi-directional, with forward signaling through receptor activation and reverse signaling through ligand activation.[9] Both the receptors and ligands have varying roles during embryonic development and their dysregulation in adults is associated with various pathological conditions, including cancers, where they play important roles in neoangiogenesis, tumor progression, invasion, and metastasis.[9-11]

This pathway and its proteins are thus attractive targets for cancer therapy.[10,12-15] The expression of various Eph and ephrin proteins (EphA in particular) have been studied in RCC;[16-18] however, the EphB4-EphrinB2 pathway has been evaluated in a limited number of cases.[19,20] The importance of this pathway is underscored by recent studies, wherein the overexpression of EphrinB2 in other solid tumors was associated with poor prognosis and response to therapy.[21] Several studies have even validated the therapeutic potential of targeting EphrinB2.[22-24] In light of the above findings, the expression of EphrinB2 has even been evaluated in other genitourinary organs like the prostate.[25] It thus becomes essential to evaluate this potentially therapeutic pathway in RCC. We herein studied the immunohistochemical expression of EphrinB2 in RCC.

MATERIALS AND METHODS

Kidney tissue specimen

Kidney cancer tissue microarrays (TMA) were obtained from a commercial supplier (US Biomax, Rockville, MD; TMA catalog number BC07115a). The TMA comprised formalin-fixed paraffin-embedded tissue samples from 120 patients consisting of 79 ccRCC, 20 urothelial carcinomas, 6 sarcomatoid carcinoma, 3 papillary RCC, 2 chromophobe RCC, and 10 normal kidney tissues with single-core per case. Individual tissue cores were 1.0 mm in diameter and 5 μm in thickness. The clinicopathologic characteristics provided by the commercial supplier for each case were: age, sex, diagnosis, and tumor nodes metastasis (TNM) stage. The TMA slide was stained for the Hematoxylin and eosin (H and E) stain and the slides were evaluated by an experienced pathologist (AS). The diagnosis and tissue adequacy were confirmed for each case.

Antibodies

Anti-EphrinB2, a monoclonal antibody produced in rabbit was obtained from Abcam PLC (San Francisco, CA; clone AB201512). The antibody was validated for use in immunohistochemistry (IHC) as follows: isogenic Chinese hamster ovary (CHO) cell lines were prepared by stable expression of EphrinB2, EphrinB1, and EphrinB3. CHO/EphrinB2 showed membranous staining for this antibody, while the wild-type CHO did not express EphrinB2. Further, the human normal tissue included in the array showed the absence of expression, confirming the embryonic expression of this protein.

Immunohistochemical staining

IHC was performed using the above-mentioned antibody clone. TMA slide was deparaffinized using xylene and rehydrated in graded alcohol. Antigen retrieval was accomplished by using citrate buffer (pH 6.5) and heat plate (100°C). The EphrinB2 antibody was used in 1:500 dilution. Slides were incubated with horseradish peroxidase polymer secondary antibodies and the antigen-antibody reaction was visualized using DAB chromogen. Slides were then counterstained with H and E stain and IHC results were scored by an experienced pathologist (AS). Immunohistochemical staining of EphrinB2 was studied in tumor cells and endothelial cells of tumor blood vessels. The component stained (cytoplasm, membrane, and nucleus) and intensity of the staining (weak, 1+; moderate, 2+, and strong, 3+) were noted. Staining was defined as positive when at least 10% of tumor or endothelial cells displayed membrane expression of any intensity.

Statistical analysis

For all statistical analyses, SPSS (IBM, Armonk, NY, USA) statistics software version 24.0 was used. The Chi-squared test and Fisher's exact test were used to find an association between the categorical variables. Statistical significance was defined as a $P < 0.05$.

RESULTS

The diagnosis was confirmed in all 110 cases. Apart from the 20 confirmed urothelial carcinoma cases, two ccRCC cases were excluded from the analysis as these showed only necrosis without any viable tumor. Thus, 88 RCC cases formed the final study cohort. The age of the patients ranged from 26 to 82 years (median 58 years). EphrinB2 expression was noted in 42 (47.7%) cases. Most of these cases ($n = 30$, 71.4%) displayed moderate intensity staining, while weak and strong intensities were noted in 6 (14.3%) cases each [Table 1]. The staining was restricted to the endothelial cells in most cases (n = 39/42, 92.8%), with expression in tumor cells observed in 3 (7.2%) cases [Table 1 and Figure 1]. There was a statistically significant difference between the expression of EphrinB2 in both sexes (P = 0.046), with 55% of males showing EphrinB2 expression against only 32% of females [Table 2]. EphrinB2 was expressed in 53.6% Stage 1 tumors and 45.8% Stage 2 tumors. There was no statistical correlation between the expression of EphrinB2 and histological subtypes ($P = 0.1$) or TNM stage

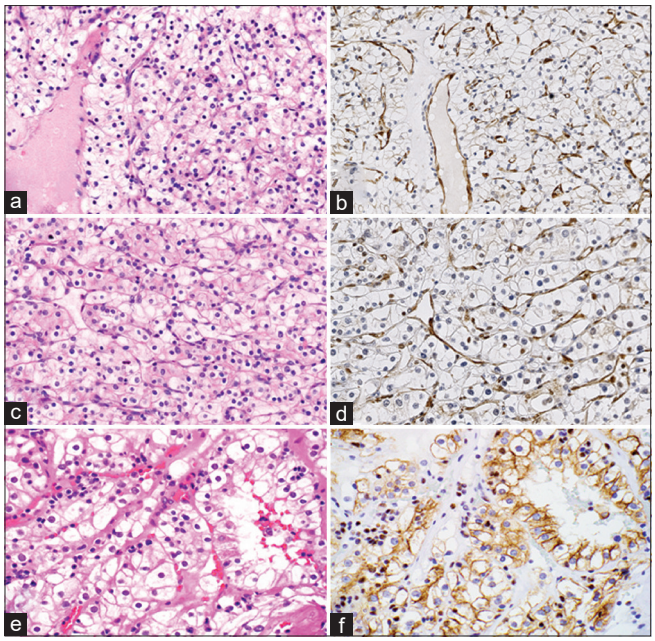

Figure 1: Expression of EphrinB2 in clear cell renal cell carcinoma (H and E, immunohistochemistry), (a-d) Expression of EphrinB2 in tumor vessel endothelial cells, ×400, (e and f) Membranous expression of EphrinB2 in tumor epithelial cells, ×400

Table 1: Distribution of cases according to different variables (*n*=88)

	Number of cases, *n* (%)
Sex	
Female	28 (31.8)
Male	60 (68.2)
RCC tumor subtypes	
Clear cell	77 (87.5)
Sarcomatoid	6 (6.8)
Papillary	3 (3.4)
Chromophobe	2 (2.3)
Stage	
Stage 1	56 (63.6)
Stage 2	24 (27.3)
Stage 3	6 (6.8)
Stage 4	2 (2.3)
EphrinB2 expression result	
Positive	42 (47.7)
Negative	46 (52.3)
EphrinB2 expression location	
Tumor vessels endothelium	39 (92.8)
Tumor cells	3 (7.2)
Intensity of IHC expression	
Weak	6 (14.3)
Moderate	30 (71.4)
Strong	6 (14.3)

RCC=Renal cell carcinoma, IHC=Immunohistochemistry

[P = 0.099, Table 2]. EphrinB2 expression was not seen in the normal kidney tissue.

DISCUSSION

Various Eph and ephrin proteins, particularly Class A proteins, have been studied for their expression in renal tumors.[26] Accordingly, in ccRCC, lack of EphA3 is associated with higher-stage cancer, loss of EphA1 connotes a favorable prognosis, while high expression of EphA2 is associated with poor disease outcome.[16-18] The literature on EphrinB2 expression in renal tumors, however, is scarce.[19,20] Selective expression of EphB4 and EphrinB2 in the veins and the arteries, respectively, during the embryonal phase of development enables vascular remodeling and veno-arterial segregation.[27,28] In adults, the expression of these proteins is diminished, only to re-express in tumor cells and tumor blood vessels. Over the years, several cancer therapeutic agents have been developed to target the EphB4-EphrinB2 pathway, including a soluble monomeric derivative of the extracellular domain of EphB4 (sEphB4), EphB4 kinase domain inhibitors, and EphB4 small interfering RNA and antisense oligodeoxynucleotides.[29-32] Of particular importance, the sEphB4 molecule binds EphrinB2 and blocks both forward and reverse signaling by blocking the activation of both receptor and ligand, respectively. sEphB4 has been extensively studied in murine tumor xenograft models and tumor cell lines and has been shown to inhibit tumor growth.[14,29,33] This EphrinB2 decoy has demonstrated impressive results in head–neck squamous cell carcinoma, where it has not only been shown to delay tumor growth but has also shown to radiosensitize this tumor and enhance the response to cetuximab-radiotherapy combination.[21-23] Recent clinical trials have established the safety of this drug, and several trials, including one with RCC patients (NCT02767921), are evaluating its efficacy in different solid tumors.[24,34] In light of the above-mentioned fact, we felt it pertinent to evaluate the expression of EphrinB2 in RCC.

In a study for expression profiles of Ephs and ephrins in various human benign and cancerous tissues using reverse transcription polymerase chain reaction and IHC, Hafner *et al.* found expression of EphrinB2 in both normal as well as cancerous renal tissue, with increased expression in the latter.[19] Ozgür *et al.* studied the IHC expression of EphB4 and EphrinB2 in various normal and malignant urogenital tissues.[20] The authors observed expression of EphrinB2 in arterial blood vessels in normal as well as RCC; however, the intensity of expression was higher in RCC and was noted in arterial endothelial and vascular smooth muscle cells as well as tumor epithelial cells. The IHC findings were complemented by immunofluorescence test results. The expression of EphB4 and EphrinB2 in tumor epithelial cells suggested the involvement of these proteins in the regulation of tumor angiogenesis and progression. Both the above-mentioned studies, although being pivotal, comprised only a limited number of samples.[19,20] In our study, expression of EphrinB2 was observed in approximately 48% of RCC samples. Further, the lack of staining in the normal kidney tissue highlights the role of this molecule in tumor pathobiology and angiogenesis. There was a statistically significant difference in the expression of EphrinB2 in males (55%) and females (32%) (P = 0.046).

Table 2: Cross-tabulation of cases based on the sex, stage and renal cell carcinoma subtypes

Variable	Number of positive cases, *n* (%)	Number of negative cases, *n* (%)	*P*
Sex			
Female	9 (32.1)	19 (67.9)	0.046
Male	33 (55.0)	27 (45)	
Stage			
Stage 1	30 (53.6)	26 (46.4)	0.099
Stage 2	11 (45.8)	13 (54.2)	
Stage 3	0	6 (100)	
Stage 4	1 (50)	1 (50)	
RCC subtypes			
Clear cell	40 (51.9)	37 (48.1)	0.1
Sarcomatoid	2 (33.3)	4 (66.7)	
Papillary	0	3 (100)	
Chromophobe	0	2 (100)	

RCC=Renal cell carcinoma

Kidney cancers are almost twice more common in males as compared to females, leading to speculation that drivers of the RCC may also have a larger impact on EphrinB2 gene expression. Although there was no statistically significant difference in the expression of EphrinB2 based on the stage ($P = 0.099$) or RCC subtypes ($P = 0.1$), the same cannot be said conclusively as the higher stage (Stage 3 and 4) and RCC subtypes (other than ccRCC) had limited representation in the cohort. Interestingly, the expression of EphrinB2 was evident in around 51% of Stage 1 ($n = 30$, 53.6%) and Stage 2 ($n = 11$, 45.8%) cases, implying that there is a possibility of therapeutic intervention at an early stage.

RCC is a highly angiogenic tumor, and the discovery of the involvement of vascular endothelial growth factor (VEGF) and mammalian/mechanistic target of rapamycin (mTOR) pathways in the development of RCC has led researchers to study therapeutic agents targeting these pathways, resulting in multiple Food and Drug Administration (FDA) approvals of drugs in this category, particularly for metastatic RCC. These drugs were associated with improved response and decreased risk of toxicities; however, a durable complete response is uncommon.[4] Hence, there is a need for the continued search for novel targets and drugs in addition to already existing targeting agents. Studies have shown that VEGF induces EphrinB2 and represses EphB4 while blocking the EphB4-EphrinB2 pathway inhibits tumor angiogenesis leading to hypoxia and VEGF induction.[14,20] Hence, the simultaneous targeting of these two pathways holds a potential therapeutic strategy.[35] Further, it has been noted that the EphrinB2 overexpressed tumors, compared to VEGF overexpressed tumors, have more efficient tumor vasculature that might facilitate efficient chemotherapeutic drug delivery to the tumor tissue.[8]

This study, however, does have certain limitations. The sample used was in the form of TMA; hence, the true expression of EphrinB2 would be higher than that reported in this study. The sample size was relatively small, with a lesser representation of higher-stage tumors (Stages 3 and 4), subtypes of RCC other than ccRCC, and female patients.

CONCLUSIONS

It was found that nearly half of RCC samples show EphrinB2 expression, primarily in tumor vascular endothelial cells, and more commonly in males. EphrinB2 expression in RCC is of interest for further investigation for its role in tumor development and therapy, especially in combination with immune checkpoint inhibitors, which are now used in the first line of advanced disease.

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