Original Article
EphB4 knockdown suppresses renal cancer cells growth and inhibits the activity of ERK and STAT3 in vitro and in vivo

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Abstract: Objective: Erythropoietin producing human hepatoma (EphB4) promotes cell viability, contributes to migration, invasion and angiogenesis in cancers of many origins. In this study, we investigated the impact of EphB4 on the oncogenic potential of renal cell tumor cells in vitro and in vivo. Methods: We examined the impact of EphB4 knockdown on cell proliferation and cell death using CCK-8 and annexin V-FITC/PI staining assays, respectively. The effect of Eph4 knockdown on cell invasion and migration was determined using Boyden chamber assay and wound healing assay. To investigate the downstream consequences of Eph4 knockdown, the activities of ERK and STAT3 were examined. Furthermore, the effect of EphB4 knockdown in vivo was investigated using xenograft tumor model in nude mice. Results: EphB4 downregulation suppresses growth, invasion and migration of renal cancer cells and promotes cell apoptosis. EphB4 knockdown also decreases the levels of p-ERK and p-STAT3. In vivo, EphB4 knockdown significantly suppresses tumor growth and decreases the levels of p-ERK and p-STAT3 in xenografts tumor. Conclusion: Our study demonstrates that EphB4 knockdown efficiently inhibits proliferation and induces apoptosis in human renal cancer cells in vitro and in vivo.

Keywords: EphB4, renal cancer, proliferation, apoptosis

Introduction

Renal cell carcinoma (RCC), accounts for about 3% of adult malignancies and is the most lethal of the urological cancers [1]. More importantly, the incidence rate of RCC has been increasing worldwide over the past 20 years [1]. At present, the mainstream treatment for patients with RCC is partial or radical nephrectomy. However, 25%-30% patients with metastatic disease [2], have a poor survival rate, despite receiving conventional therapies. Therefore, novel therapeutic agents against RCC that are able to provide long-term clinical benefits are urgently needed.

Many solid tumors frequently over-express receptor tyrosine kinases (RTKs) such as Epidermal Growth Factor Receptor (EGFR) and its related family members [3, 4]. RTKs are trans-membrane proteins that can be phosphorylated and activated by each other, which in turn control cell aggregation, migration, development, maturation, angiogenesis and vascular remodeling [5, 6]. Similarly, erythropoietin-producing hepatocyte (EPH) receptors, another group of RTKs, are also over-expressed in cancers of many origins. The EPH family is functionally and structurally divided into A- and B-classes based on sequence similarity and ligand-binding affinity. It was reported that over-expression of EphB4 and its ligand ephrin-B2 is linked to tumor development and progression [7-9]. Activation of EphB4 by ephrinB2 promotes endothelial adhesion, cell proliferation, tube formation, migration and cytoskeletal organization [10]. Moreover, high expression of EphB4 has been found in many kind of solid tumors and is associated with hyper-angiogenesis and with poor prognosis [11, 12].
Interestingly, EphB4 is strongly expressed in type II papillary RCC and oncocytoma in a majority of samples, however, it exhibits weak expression in other RCC subtypes including clear cell, chromophobe, sarcomatoid, type I papillary and angiomyolipoma [13]. This huge variation in Eph4 levels in different kinds of RCC cases promoted us to investigate its role in RCC.

Methods

Cell lines and culture conditions

Renal cancer cell lines 786-O were purchased from ATCC and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Cell transfection

786-O cells were transfected with EphB4 cDNA or siRNA reagents in serum-free DMEM using lipofectamine 2000 according to the manufacturer’s instructions. After transfection for 20 h, the medium was replaced with complete culture medium. The efficiency of transfection was assessed by western blotting. Cells were analyzed at optimal time-points by different assays. The EphB4 siRNA sequences were as follows: forward: 5’-GUACUAAGGUCUAACGAdTdT-3’ and reverse: 5’-UCGAUGUAGACCUUAGUACTd-Td-3’.

Cell viability assay

The growth of 786-O cells after transfection were checked by CCK-8 assays. Untreated cells and cells treated with the NTC-siRNA were used for control. Cell suspensions (at 5 × 10⁸/mL) were transferred to 96-well plates in quintuplicate and cultured for 24, 48 and 72 hours. Then, CCK-8 (10 μl) was added to each well, and cells were cultured for an additional 2 hours. The values of each well were measured by microplate reader at 450 nm.

Apoptosis detection

The apoptosis of cells was examined by annexin V-FITC/PI staining using flow cytometry. After transfection and culturing for 3 days, cells were harvested, centrifuged and washed with phosphate buffered saline (PBS) for three times. Subsequently, binding buffer was added to each tube, and cells were resuspended. After incubation with 5 μl annexin V-FITC and PI for 15 min at room temperature, 786-O cells (10⁴ counts) were analyzed by flow cytometry (BD FACSCalibur, USA).

Matrigel invasion assays

In vitro invasive ability of renal cancer cells was examined by Boyden chamber assay. 100 μl matrigel was added into the upper chambers of the transwell inserts. Transfected cells were transferred on the upper transwell chamber which contained serum-free medium, and the lower chamber was filled with complete medium. After 24 h incubation, the cells on the upper surface of the insert were erased by swab, and the cells on the lower surface of the insert were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of invaded cells were counted under 400 × microscope at 5 random selected fields.

Wound healing assays

Cells were transferred in the plates after transfection at a density of 40%. After 24 h, cell monolayers were wounded by scratching using a sterile 200 μl pipette tip. Cells were then rinsed third times with PBS to remove floated cells, and incubated in serum-free medium. Images of the wounds were acquired at 0 h and 24 h under a light microscope. Cell migration was quantified by evaluated the degree of wound closure.

Western blotting analysis

Proteins were extracted from whole cell lysates and separated by SDS polyacrylamide gel electrophoresis, then transferred to a PVDF membrane. The following primary antibodies were used: rabbit polyclonal anti-EphB4 (1:1500; Cell Signaling Technology), rabbit monoclonal anti-p-STAT3 (1:2000; Cell Signaling Technology), rabbit monoclonal anti-p-ERK1/2 (1:2000; Cell Signaling Technology) and rabbit monoclonal anti-GAPDH (1:2000; Cell Signaling Technology). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibodies (1:4000; Abcam) for an hour. Membranes were subsequently washed three times with TBST, and then visualized using DAB detection system.
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Animal models

All animal experiments were carried out with the approval of the Animal Ethics Committee of Huazhong University of Science and Technology. 4-week old male athymic nude mice (BALB/c-nu/nu mice) were inoculated subcutaneously with 1 × 10^7 786-O cells on shoulder. After about one week, all the mice grew visible tumors. The mice were randomized and assigned to the control group or the experimental group, and 5 mice in each group. Mice in the experimental group received intratumoral injection of EphB4 siRNA (0.1 mL, 80 nM) every 2 days for a total of 14 days. Control mice were injected with control siRNA. The tumor sizes were measured from the first day until 40 days post-cell injection, using calipers and the formula: V (volume) = LW^2 × 0.52, where “L” represents the greatest length and “W” represents the perpendicular width. Mice were sacrificed after injection of cells about 4 weeks, and the tumors were isolated respectively. The apoptotic cells in tumor tissues were detected by TUNEL assay. Staining procedure was carried out according to the protocol. Apoptotic cells were evaluated by counting the positive cells as well as the total number of cells at 10 random selected fields at 400 × magnification in a blinded manner.

Immunohistochemistry (IHC)

Paraformaldehyde-fixed and paraffin-embedded tissue sections (5 µm) were dewaxed with xylene and rehydrated through an ethanol gradient into water. Following inactivation of endogenous peroxide with 0.3% H_2O_2 for 10 min, the slides were washed with PBS 3 times and incubated with either p-ERK or p-STAT3 primary antibody at 1:1000 dilution in a humidified chamber at 4°C over-night. After washing with PBS 3 times, slides were incubated with secondary antibody for 1 h at 37°C, and then with horseradish peroxidase labeled streptavidin for 30 min at 37°C. Diaminobenzidine (DAB) was used as the chromogen and the slides were subsequently counterstained with hematoxylin, then dehydrated, cleared and mounted.

Statistical analysis

All statistical analyses were carried out using GraphPad Prism version 6. The differences among multiple groups were analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison tests, two groups were analyzed by an unpaired Student’s t-test (two-tailed). All data are expressed as the mean values of three independent replicates ± SD; differences were considered to be statistically significant when P < 0.05. All experiments were performed at least three separate times.

Results

EphB4 knockdown decreases cell growth in RCC cells

Initially we over-expressed EphB4 (Figure 1A), which increased the proliferation of RCC cells (measured using CCK-8 assay) (Figure 1B-D). Similarly, when EphB4 was knocked-down (Figure 1A), cell proliferation decreased significantly (Figure 1B-D). Those results showed that EphB4 positively regulates cell proliferation.

EphB4 knockdown promote cell apoptosis in RCC cells

The apoptotic effect of EphB4 on RCC was detected by annexin V-FITC/PI double staining assay. Stained cells were immediately analyzed by flow cytometry. The results showed that EphB4 downregulation promoted cell apoptosis. With Annexin V-FITC staining, early apoptosis was obviously detectable in the 786-O cells treated with transfection of EphB4 siRNA (Figure 1E, 1F). Compared to the control group, the apoptotic rates of cells transfected with EphB4 siRNA increased significantly (P < 0.05).

EphB4 knockdown inhibit cell invasion on RCC

In order to check whether EphB4 affect cell invasion and motility, we utilized matrigel in vitro invasion assay. EphB4 downregulation profoundly influenced cell invasion capability of RCC cells. Compared with the control cells, 786-O cells transfected with EphB4 siRNA, showed an observably lower invasion ability (Figure 2A, 2B). These data indicate that the decreased expression of EphB4 inhibits the invasive potential of these cells.

EphB4 knockdown inhibit cell migration

Similarly, downregulation of EphB4 in 786-O cells inhibited migration in wound healing assays compared to the control groups (P < 0.05). Also, decreased migration was observed in the EphB4 groups compared to control groups (Figure 2C, 2D).
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EphB4 knockdown inhibit the activity of ERK and STAT3

To investigate the underlying mechanism of EphB4 downregulation which leads to the inhibition of 786-O cell growth, the levels of p-ERK and p-STAT3 were analyzed. Strikingly, following transfection of EphB4 siRNA, the expression of p-ERK and p-STAT3 declined significantly (Figure 3), indicating that EphB4 siRNA inactivates ERK and STAT3 by inhibiting their phosphorylation.

Antitumor effect of EphB4 downregulation in vivo

We further examined the effect of EphB4 downregulation on tumor xenografts growth in vivo. Our data shows that the growth of 786-O tumor xenografts are inhibited by the injection of EphB4 siRNA (Figure 4A). The average tumor volume in the control mice were nearly 2 fold (P < 0.05) bigger than that of EphB4 downregulated mice (Figure 4C, 4D). Furthermore, the average bodyweight of the control group were a...
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little bit heavier than the treatment groups (Figure 4B). The apoptosis of tumor cells was evaluated using TUNEL stain. The number of apoptotic cells was more prominent in the EphB4 knockdown tumors than that of the control group (Figure 5A, 5B). We also checked the expression levels of p-ERK and p-STAT3 by IHC, and verified that EphB4 siRNA injection also influenced the activity of ERK and STAT3 in vivo (Figure 5C, 5D). Together, these results confirm our cellular data showing that EphB4 downregulation decreases the oncogenic potential of RCC cells.

Discussion

EphB4 is over-expressed in venous endothelial cells. Binding of EphB4 with its ligand ephrinB2 induces bi-directional signaling and regulates diverse endothelial functions in various diseases. Upon engagement of EphB4 with its ligand, EphB4 becomes tyrosine phosphorylated through auto-phosphorylation on its kinase domain, thereby activating kinase-dependent forward signaling, whereas the reverse signaling is activated upon ephrinB2 tyrosine phosphorylation through recruitment of itself [14]. Both the receptor and the ligand are membrane-bound but usually expressed on neighbouring cells. Alterations in normal Eph-ephrin balance, such as high EphB4 and low ephrin-B2, disrupts normal ligand-dependent signalling and promotes ligand-independent-mediated mechanisms that drive tumorigenesis [15, 16]. In most situations, the EphB4/ephrin-B2 balance in many cancer cells is disrupted by over-expression of the EphB4 receptor. EphB4 is reported as a frequently over-expressed RTK in numerous types of cancer, including lung, esophagus, ovary, breast, thyroid, cervix and prostate [17-21]. But the underlying mechanisms that drive EphB4 over-expression in cancer cells have not been determined. Knockdown
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or over-expression experiments using tumor cell lines have shown that EphB4 increases cancer cell viability, and contributes to migration, invasion and angiogenesis [22, 23]. A specific EphB4 polyclonal antibody, which targeted a region of 200 amino acids in the extracellular portion of EphB4, showed potent in vitro anti-cancer effects [24]. With roles in regulating and modifying the important cancer progression hallmarks of viability, migration and invasion and common over-expression in up to 82% of epithelial cancers, EphB4 will be an important target for the development of anti-cancer agent [24].

EphB4 has previously been reported to be high expressed in the endothelium of renal, bladder, and prostate cancer tissues than in corresponding normal tissues [25, 26]. However, another study reported that type II papillary RCC and oncocytoma demonstrated greater EphB4 expression in a majority of samples, while the other RCC subtypes (clear cell, chromophobe, sarcomatoid, type I papillary) and angiomylipoma exhibited weaker expression in tumor tissues [13]. It is interesting that EphB4 in RCC exhibits unique biology behavior relative to its other solid tumor counterparts. In order to reveal the role of EphB4 in RCC, we designed this study.

In the present study, we over-expressed and knocked-down EphB4 in 786-O cells to detect its biological function. As revealed by CCK-8 assay, down-regulation of EphB4 possessed an inhibitory effect on cell viability, while up-regulation of EphB4 promoted the cell growth. Decreased expression of EphB4 increased the proportion of apoptotic cells. Furthermore down-regulation of EphB4 also could reverse cell invasion in 786-O cells. All of these changes of biological behavior suggest that EphB4 is a tumorigenic gene in RCC. In order to validate our cellular data in a more pathological model, we investigated the effect of EphB4 in vivo. We observed significant increase in TUNEL-positive apoptotic cells in tumors from mice that were treated with EphB4 siRNA. As we expected, the tumor volume of mice treated with EphB4 siRNA was significantly reduced. Together, these results suggest that downregulation of EphB4 has anticancer effect in RCC.
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In our study, EphB4 downregulation decreases the contents of p-ERK and p-STAT3, which has been suggested as the downstream target for EphB4 in RCC. We also confirmed that EphB4 siRNA could inhibit the activity of ERK and STAT3 in vivo. Therefore, we speculate that ERK and STAT3 signaling pathways are involved in the oncogenic potential of EphB4 in RCC.

In conclusion, the results of the current study suggest that EphB4 serves a key role in the pathogenesis of RCC. Our study provide an insight into the function of EphB4 and may provide a novel therapeutic strategy for the suppression of RCC.

Disclosure of conflict of interest

None.

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Figure 5. EphB4 knockdown induced cell apoptosis and inhibited the activity of ERK and STAT3 in vivo. A. Representative results of the TUNEL staining of tumor sections. B. The percentages are displayed showing the apoptosis cells. C. IHC analysis of p-ERK and p-STAT3 expression in control and EphB4 siRNA treated tumor. D. The percentages are displayed showing the positive cells. *, P < 0.05 for EphB4 siRNA vs. control.
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