Original Article

Inhibition of extracellular matrix metalloproteinase inducer expression reduces clear cell renal carcinoma cell invasion and metastasis

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Abstract: Background: Extracellular matrix metalloproteinase inducer (EMMPRIN), as a glycoprotein member enriching on surfaces of many malignant tumor cells, may play a certain role in promoting the tumor progression; furthermore, it also has something to do with tumor invasion, metastasis and angiogenesis. The study has been carried out to investigate the role of EMMPRIN in renal clear cell carcinoma, as well as its clinical significance. Methods: We have adopted a variety of methods, including Real-time quantitative PCR, Western blot and immunohistochemistry, to discover the expression of EMMPRIN in primary ccRCC clinical specimens and cell lines; as a result, EMMPRIN was knocked down in 786-O cells by siRNA (small interfering RNA). In addition, we have also adopted CCK-8 and Colony Formation to examine the biological functions and inhibitory effects of the knockdown investigated in 786-O. Results: The study found that the expression of EMMPRIN was higher in renal cancer than in normal tissues, which led to the down-regulation of MMP-2 and MMP-9, and the up-regulation of E-cadherin. The same result was found in the expression of EMMPRIN in renal cancer cell lines, being higher than that in normal renal epithelial cells. EMMPRIN knockdown by RNA interference led to cell proliferation, migration and invasion, conversely resulting in increased expression of E-cadherin in renal clear cell carcinoma, while the level of Vimentin remained unchanged. Conclusions: It is found in this study that EMMPRIN is over-expressed in kidney cancer, and EMMPRIN expression is related to tumor tissues and cell lines of renal cancer. It is therefore concluded that high EMMPRIN expression can be taken as a predictor of poor prognosis in patients suffering from kidney tumor, and EMMPRIN may be taken as a latent therapeutic gene of kidney cancer.

Keywords: EMMPRIN, RNA interference, ccRCC, tumor progression, potential therapeutic target

Introduction

Renal cell carcinoma (RCC), the most common neoplasm of adult kidney, accounts for about 3% of adult malignancies [1]. Clear cell renal cell carcinoma (ccRCC) of various histological subsets accounts for approximately 80%-95% of the malignancies of adult kidney [2]. Surgical resection is the best curative therapy for ccRCC, however, about 20%-40% patients would still suffer from tumor relapse after curative nephrectomy [3]. Since ccRCC has the feature of being chemotherapy and radiotherapy resistant, no adjuvant therapy is available in clinical routine [4]. Accordingly, biomarkers are required to be found for early detection, for the follow-up of disease would complicate the on-time diagnosis.

Extracellular matrix metalloproteinase inducer (EMMPRIN), a transmembrane glycoprotein encoded by a gene localized to 19p13.3, can activate the matrix metalloproteinase, including CD147/basigin, HT7, M6, and Neurothelin [5-8], etc., and it is also a tumor cell-derived collagenase stimulating factor [9]. EMMPRIN is a group of enzymes playing an important role in tumor invasion and metastasis formation of patients suffering from cancer and a few animal models [10]. Furthermore, it is recently shown that EMMPRIN also has the function of stimulating the expression of hyaluronan and vascular endothelial growth factor, which, respectively, leads to the anchorage-independent growth and angiogenesis. Indeed, it is reported that in a variety of human cancers, including breast cancer, oral squamous cell carcinoma, lympho-
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Table 1. EMMPRIN expression in cRCC and normal renal tissue group

<table>
<thead>
<tr>
<th>Number</th>
<th>EMMPRIN expression levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Normal</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>45 (2.1)</td>
</tr>
<tr>
<td></td>
<td>5 (7.9)</td>
</tr>
<tr>
<td>ccRCC</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>16 (15.7)</td>
</tr>
<tr>
<td></td>
<td>86 (84.3)</td>
</tr>
</tbody>
</table>

χ²=77.122, P < 0.01, EMMPRIN in renal cell carcinoma was significantly higher expression than the normal kidney tissue.

In this study, we believe EMMPRIN is a potential therapeutic biomarker for kidney cancer. We have found out that EMMPRIN is expressed in ccRCC with positive associations at the primary tumor stage and nuclear grade, and we have also determined the mechanism of tumor progression. In addition, down-regulation of EMMPRIN expression in ccRCC could inhibit the invasion and metastasis of cells through matrigel in vitro.

Materials and methods

Patients and tissue samples

The Department of Urology, First People’s Hospital, Shanghai Jiao Tong University kindly provided us with 102 fresh kidney cancer tissues and 50 normal tissues. In the study, none patients had chemotherapy or radiotherapy prior to surgery. All the patients received resection of kidney cancer from January 22, 2008 to December 29, 2010 from Tissue Paraffin Block Bank of Pathology. The normal kidney tissues were the ones over 6 cm cutting from the tumor tissues. The pathological diagnosis was performed before surgery and confirmed after surgery. In March, 2012, we reviewed all the patients and re-examined all specimens. We determined the tumor stage in accordance with the regulations of American Joint Committee on Cancer (AJCC), and classified the nuclear grading in accordance with the method described by Fuhrman et al [23]. Two senior pathologists of being experienced in diagnosis of kidney cancer examined TNM grade, Furherman Score and histological grade. According to the ethical and legal standards, clinical data and summaries (Table 1), all samples were handled and made in an anonymous manner.

Cell culture and reagents

RPMI-1640, DMEM/F12 (1:1) and FBS (fetal bovine serum) were gained from Invitrogen (Hyclone, CA). RNeasy RNA kits were bought from TaKaRa Biotechnology Co. Ltd (Dalian, China). The neutralizing anti-human EMMPRIN monoclonal antibody and anti-GAPDH antibody were bought from Abcam, Inc. (CA, USA). siRNA-EMMPRIN primer was synthesized by Ruibo Biological Co. Ltd (Guangzhou, China). Immunohistochemistry kit was bought from Changdao Biological Co (Shanghai, China).

Immunohistochemistry

EMMPRIN and E-cadherin were stained with the two-step staining method of EnVision™, and
the major steps are described previously [24]. Cells having brown particles in cytoplasm and/or on membrane are regarded as positive for EMMPRIN, and those having brown particles in membrane are regarded as positive for E-cadherin. For each case, randomly select ten high-power observation fields, if the average rate of positive cells is equal to or larger than 25%, the corresponding ones are considered as positive cases, and if the same rate is less than 25%, the corresponding ones are considered as negative cases. The kit-provided positive slide was taken for positive control, and that stained with PBS (pH 7.4), other than primary antibody, was served for negative control.

Cells were studied using Immunohistochemistry staining with antibodies that are directed against EMMPRIN. Wash the cells with PBS and then, at room temperature, fix them in 4% paraformaldehyde for 15 minutes. Wash the cells with 0.1% Triton X-100 (Sigma) in PBS for three times, and 5 minutes each. Conjugate serum blocking with 5% normal goat serum in PBS for 20 minutes. Dilute primary antibody (rabbit anti-EMMPRIN; Epitomics) into an appropriate concentration (1:50), and incubate cells with this antibody over night at 4°C. Wash the cells with the PBS solution for another three times, 5 min each. Dilute the species-specific secondary antibody (goat anti-rabbit; Invitrogen) to 1:200 in PBS, and incubate the cells with the secondary antibody for 60 min. At the end of secondary incubation, wash the slides with 1% Triton X-100 in PBS and then quickly rinse them with dH2O, and finally it can be observed that the color brown is aborted by using DAB color dark for 1-2 minutes. Capture the images with an inverted microscope Nikon Eclipse TE2000-S (Tokyo, Japan) with 200×magnification, and then qualitatively analyze the presence of green fluorescence indicative of α-SMA by two raters, naive to the study. According to Fourier scoring system, the positive percentage of counted cells was semiquantitatively graded: negative (-), 0-5%; weakly positive (+), 6-25%; moderately positive (++), 26-50%; and strongly positive (+++), 51-100%. Two independent pathologists have performed the histological and immunohistochemical assessments.

**Cell culture and transfection**

We got human renal carcinoma cells 786-O and Caki-1 from the Institute of Cell and Biochemistry, Chinese Academy of Sciences (Shanghai, China), and purchased 769-P, human renal carcinoma cell line, and HK-2, normal renal proximal tubule epithelial cell line, from American Type Culture Collection (ATCC). We cultured 786-O and 769-P cells in RPMI-1640 medium (HyClone) of 90%, which was supplemented by FBS (Gibco) of 10% and penicillin/streptomycin (Invitrogen) of 100 U/ml; cultured Caki-1 cells McCoy’s 5A medium (Gibco) of 90%, which was supplemented by FBS of 10%; and cultured HK-2 cells in DMEM/F12 medium (HyClone) of 90%, which was supplemented by FBS of 10%. All cells were incubated in a humidified incubator at 37°C in an atmosphere containing 5% of CO₂.

In order to elucidate silencing experiments, 786-O cells were transfected to Control-siRNA and EMMPRIN siRNA (siRNA1, siRNA2, siRNA3). 786-O cells, at a density of 2.0×10⁵, were seeded into 6-well plate and incubated (37°C and 5% CO₂) for 24 h before co-transfection. siRNA EMMPRIN was synthesized by Ribobio and Lipofectamine 2000 reagents (Invitrogen) in accordance with instructions of the manufacturer’s. All cells were collected at 48 hours after transfection with siRNA, and the efficiency of siRNA transfection was confirmed by Western blot and RT-PCR analysis.

**Real-time quantitative PCR Assay**

Total RNA was isolated from cells and tissue specimens with Trizol (Invitrogen), reversely transcribed into cDNA through using the Superscript first strand synthesis kit (Invitrogen) in accordance with the protocol of the manufacturer, and amplified by qRT-PCR (Quantitative Real-time Reverse Transcripase polymerase chain reaction) with the help of Mini-Opticon real-time PCR detection system (Bio-Rad, CA, USA) in SYBR Green II master mix (TakaRa, Dalian, China) in accordance with the instruction of the manufacturer. All data were analyzed by the Opticon Monitor software (Bio-Rad; version 3.1). The order of PCR primers are shown as follows:

**EMMPRIN** (Forward: 5'-AGATACTCCTCCTACCTG-3', Reverse: 5'-ACGACTCCAGCCCTCCACT-3');
**E-cadherin** (Forward: 5'-AGAACGCATTGCACTCAC-3', Reverse: 5'-AAGAACCTTCCATGAAGAC-3');
**Vimentin** (Forward: 5'-CCGTGGAGATCTTCTTCT-3', Reverse: 5'-CCTCGTATACGCATCAATCT-3');
**MMP-2** (Forward: 5'-CCGTTGGAAGAT TTCTTCT-3', Reverse: 5'-CCTCGTATACC-
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CGCATCAATCT-3'); MMP-9 (Forward: 5'-TGTACC-GCTATGTTACACTCG-3', Reverse: 5'-GGCAGG-GACAGTTGCTTCT-3'); and GAPDH (Forward: 5'-AGGTCCGAGTCAACGGATTGG-3', Reverse: 5'-GTGATGGCATGGACTGTGGT-3'). The PCR conditions are shown as follows: 2 min at 94°C, 5 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C. Each sample's mRNA level, normalized to the level of GAPDH mRNA, was presented as the unit values of $2^{\Delta\Delta C_T}$: $2^{\Delta C_T(GAPDH) - C_T(EMMPRIN)}$. The amplified products were analyzed with 1.5% gel by agarose gel electrophoresis, and then the ethidium bromide staining was conducted. Each experiment was replicated in triplicate.

Western blot analysis

The protein of cells was lysed for 30 minutes on ice in cell lysis buffer. The cell lysates' protein concentrations were determined by the BCA method (Beyotime). The proteins were placed on gradient gels of 10% and then separated by SDS-PAGE (Bio-Rad, Hercules, CA). Proteins resolved were transferred into PVDF membranes (Invitrogen) electrophoretically, and then blocked for 1 h at room temperature with the non-fat dry milk of 5% in TBST with 0.1% Tween 20. The membranes were probed with rabbit anti-GAPDH (mAb) (1:1000) or rabbit anti-EMMPRIN monoclonal antibody (mAb) (1:1000) overnight at 4°C. Then, they were washed with 1×TBST and incubated in the dilution of secondary antibodies of 1:2000 that was conjugated to horse-radish peroxidase (Santa Cruz, CA, USA) for 2 h at room temperature. With enhanced chemiluminescence reagents to detect the proteins (Boster, Wuhan, China). The membrane protein bands were imaged using X-film (Kodak Co) and scanned. All Western blots were performed for three times at least.

CCK-8 assay

786-O cells were transfected with EMMPRIN siRNA 3 or 50 nM of Control short interfering RNA (siRNA). 48 h after transfection, 3,000 cells were seeded in duplicate into each well of the 96-well plate, in which, 90 μl of culture medium was added with 10 μl CCK-8 (Dojindo, Kumamoto, Japan). After 4 h of incubation at 37°C, absorbency A450 was measured with a microplate reader (Bio-Rad). Each experiment was in five replicates on triplicate.

Colony formation assay

SiRNA-control and siRNA-EMMPRIN cells were plated on a 0.6% agarose base, and then put in a 6-well plate (1.0×10^3 cells per well), every 1 ml of medium contained FBS of 10%, and agarose peptides of 0.3%. 48 h later, transfection cells were collected and replated in a 6-well plate, and selected with G418 (0.4 mg/ml) for 2 weeks. After staining with gentian violet, the surviving colonies were counted. The rate of colony formation can be calculated with the following formula: the rate of colony formation = number of colonies/10^7 cells. Each treatment was carried out in triplicate.

In vitro migration and invasion assays

With the modified Boyden chamber method, migration was performed, and cells were seeded on 8-μm polycarbonate filters coated with 4.5 μg/cm^2 gelatin, which were in the upper compartment of the Transwell chambers, and the cells were allowed to migrate for 6 h. Invasion assays with 786-O transfectants were conducted in a similar manner, except that the filters were coated with reconstituted Matrigel (35 μg/cm^2) and conditioned medium was used as chemoattractant. After incubation for 24 h at 37°C, for scrapping off the non-migrating cells, the membranes were fixed and stained in dye solution that contains the crystal violet of 0.1% and methanol of 20%. The cells, migrated through membrane, were quantified by counting the cells in visual fields (×200 magnification) being randomly chosen. The cells having been invaded were counted and imaged with an inverted microscope Axio Scop-A1 (Nikon Corp).

Statistical analysis

All experimental data were analyzed by the SPSS17.0 statistical software, and all results were expressed in the manner of Mean ± SD. For statistical analysis, Fisher's exact test was conducted for any 2×2 tables, Pearson χ^2 test was conducted for non-2×2 tables, and chi-square trend test was conducted for ordinal datum. When the value $P$ was no more than 0.05, we considered the difference statistically significant.

Results

EMMPRIN expression is high in kidney cancer, and E-cadherin expression is high in normal tissues of kidney. EMMPRIN-positive expression was found in cytoplasm and cell membrane, and E-cadherin-positive expression in cytoplasm, being strongly expressed in cell membranes of collecting duct and distal tubule epi-
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The epithelium, but the cytoplasm is short of immunoreactivity. It has been reported that E-cadherin expression is related to metastasis of kidney cancer [25]. EMMPRIN immunostaining heterogeneous areas were discovered in the 102 specimens of kidney cancer and 50 specimens of normal tissues, and see details in respective image (Figure 1). Positively, although it was weakly stained on a few stromal cells, scattered areas of EMMPRIN immunostaining weak heterogeneous epithelial cell membrane were found in 5/50 (10.0%) normal tissue specimens (Table 1), while 87/102 (85.3%) tissues were positive for EMMPRIN (score 1-3). For sections of EMMPRIN positive primary kidney cancer, heterogeneous weak-moderate (score 1-2), and strong staining (score 3) were respectively found in 52/102 (50.9%) and 31/102 (30.4%) (Table 1). The EMMPRIN expression in tumors is higher than in normal tissue.

The expression of EMMPRIN and E-cadherin with clinicopathological characteristics of kidney cancer

The relation between expression of EMMPRIN and that of E-cadherin with pathological features of kidney cancer patients is shown in (Table 2). A high expression of EMMPRIN tended to be related to the tumor stage (P=0.021),

Figure 1. Immunohistochemical staining for EMMPRIN and E-cadherin in differentiated kidney tissues (original magnification x 200). Aa. EMMPRIN staining in non-tumor kidney tissues; b. A non-invasive case showing superficial malignant cells positive for EMMPRIN. c. An invasive case showing the strong immunoreaction of malignant cells stained for EMMPRIN in the cytoplasmic membranes; d-f. E-cadherin staining in non-tumor kidney tissues, non-invasive case and invasive case. B. Following transfection with or without NC, 786-O cells suspended in RPMI-1640 medium without fetal bovine serum (FBS) were added to the insert. RPMI 1640 medium with 20% FBS were added to the well out of the insert. After 48 h, the cells on the lower surface of the insert were fixed with 95% ethanol and stained with crystal violet (DAB, ×200).
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Table 2. The expression of EMMPRIN and E-cadherin Clinic pathological parameters in kidney cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>NO. of Patients</th>
<th>EMMPRIN expression levels</th>
<th>P value</th>
<th>E-cadherin expression levels</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative (%)</td>
<td>Positive (%)</td>
<td></td>
<td>Negative (%)</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>&lt; 60</td>
<td>60</td>
<td>12 (20.0)</td>
<td>48 (80.0)</td>
<td>0.456</td>
<td>19 (31.7)</td>
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<td>≥ 60</td>
<td>42</td>
<td>4 (9.5)</td>
<td>38 (90.5)</td>
<td></td>
<td>20 (47.6)</td>
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</tr>
<tr>
<td>Male</td>
<td>63</td>
<td>9 (14.3)</td>
<td>54 (85.7)</td>
<td>0.879</td>
<td>30 (47.6)</td>
</tr>
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<td>39</td>
<td>7 (17.9)</td>
<td>32 (82.1)</td>
<td></td>
<td>20 (51.3)</td>
</tr>
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<td>Tumour stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>24</td>
<td>7 (29.2)</td>
<td>17 (70.8)</td>
<td></td>
<td>15 (62.5)</td>
</tr>
<tr>
<td>II</td>
<td>32</td>
<td>8 (25.0)</td>
<td>24 (75.0)</td>
<td>0.021*</td>
<td>28 (87.5)</td>
</tr>
<tr>
<td>III</td>
<td>27</td>
<td>2 (7.4)</td>
<td>25 (82.6)</td>
<td></td>
<td>25 (92.6)</td>
</tr>
<tr>
<td>vI</td>
<td>19</td>
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<td>19 (100)</td>
<td></td>
<td>17 (89.4)</td>
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<tr>
<td>Grade</td>
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<td>LMP</td>
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<td>23 (69.7)</td>
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<td>LG</td>
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<td>46 (90.2)</td>
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<td>1 (5.6)</td>
<td>17 (94.4)</td>
<td></td>
<td>16 (88.9)</td>
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<tr>
<td>Tumor size (cm)</td>
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<td></td>
<td></td>
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<tr>
<td>&lt; 7</td>
<td>85</td>
<td>10 (11.8)</td>
<td>75 (88.2)</td>
<td>0.015*</td>
<td>67 (78.8)</td>
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<tr>
<td>≥ 7</td>
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<td>6 (35.3)</td>
<td>11 (64.7)</td>
<td></td>
<td>15 (88.2)</td>
</tr>
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<td>Lymph node metastasis</td>
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</tr>
<tr>
<td>Negative</td>
<td>85</td>
<td>24 (28.2)</td>
<td>61 (71.8)</td>
<td>0.012*</td>
<td>60 (70.6)</td>
</tr>
<tr>
<td>Positive</td>
<td>17</td>
<td>0 (0)</td>
<td>17 (100)</td>
<td></td>
<td>16 (94.1)</td>
</tr>
</tbody>
</table>

A refers to the immunostaining grades observed using light microscopy. The criteria used for assessment were as previously reported. B Pearson $\chi^2$ test for non-2×2 tables was used. *, $P < 0.05$.

EMMPRIN mRNA and protein expression in primary ccRCC tissue samples and ccRCC cell lines

We examined EMMPRIN mRNA expression in 102 tumor tissues and 50 non tumor tissues (the normal kidney) of clinical samples from ccRCC patients through (real-time quantitative) qRT-PCR. The results showed a statistically higher elevation of EMMPRIN mRNA expression in tumors than that in non-tumor tissues ($P < 0.05$, Figure 2A). For investigating whether EMMPRIN was elevated at the protein level, the ccRCC clinical samples were performed by western blot. We found the protein level of EMMPRIN in non-tumor tissues was much lower than that in tumor tissues, as show in Figure 2B, which conformed to the results of qRT-PCR. We also detected EMMPRIN mRNA and expressions of protein in ccRCC cell lines, and a line of immortalized normal human proximal tubule epithelial cells. The cell lines of Caki-1, 769-P and 786-O showed higher EMMPRIN transcript levels than the HK-2 cell line ($P < 0.05$, Figure 2C). Similarly, the expression of EMMPRIN protein was elevated higher in those ccRCC cell lines than in the HK-2 cell line (Figure 2D).

Knock-down of EMMPRIN in 786-O cells inhibited proliferation

We measured the inhibition efficiency of EMMPRIN siRNAs (siRNA1, siRNA2, and siRNA3) through the methods of qRT-PCR and Western blot (Figure 3A, 3B). We selected EMMPRIN siRNA (siRNA3) with the highest efficiency to further research the functional consequences EMMPRIN expression loss on cell growth. After 48 h transfection, the in vitro proliferative ability of deficient cells of EMMPRIN was determined by CCK-8 assay (Figure 3C). In
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Figure 2. The expression of EMMPRIN mRNA and protein in the human renal tissue and kidney cancer cell lines, as evaluated by qRT-PCR and western blot. A. The relative mRNA expression of EMMPRIN was higher in RCC tissues than in matched non-tumorous tissues (*\(P < 0.01\)). B. Expression of EMMPRIN protein in three representative pairs of RCC tissues is presented. N, non-tumorous tissues; T, ccRCC tissues. C. RT-PCR of EMMPRIN expression in kidney cancer cell lines. D. The EMMPRIN protein expression in human RCC cell lines was higher in the Caki-1, 769-P and 786-O cells, particularly in the 786-O cells, compared with the normal proximal tubule epithelial cell line HK-2.

Figure 3. Inhibition of 786-O cells proliferation and tumorigenicity by down-regulation of EMMPRIN. A, B. 786-O cells were transfected with siRNA-control or three independent EMMPRIN siRNAs (siRNA1, siRNA2, and siRNA3). The EMMPRIN expression was determined at the mRNA and protein levels by qRT-PCR and Western blot after transfection for 48 h (*\(P < 0.05\)). C. 786-O cells were transfected with siRNA or EMMPRIN siRNA2. After 48 h transfection, and cell proliferation was measured by CCK-8 assay. Decreased growth ability was detected in siRNA-EMMPRIN cells compared with RNAi control cells. Values were means ± SD of five duplications (*\(P < 0.05\)). D, E. Colony formation numbers were compared between transfected and control-siRNA cells. Significant down-regulation of colony formation numbers was confirmed in EMMPRIN-siRNA cells compared with siRNA-control cells (*\(P < 0.05\)). The cell colonies were photographed (×200).
addition, compared with the controls \((P < 0.05)\), the transfection with EMMPRIN inhibitor significantly reduced the growth of 786-O cells (Figure 3D). About 50% inhibition rate of EMMPRIN-siRNA cells at 72 h is shown in (Figure 3A). Meanwhile, the growth of 786-O cells that were transfected with siRNA control was not influenced. Then we performed the subsequent soft agar colony formation assay to assess the capacity of siRNA-treated cells, and further form the macroscopically visible colonies. The rates of colony formation were respectively 28%, 31% and 13% in parental 786-O, control-siRNA, and EMMPRIN-siRNA. Compared with the control, EMMPRIN-siRNA cell clones showed less amount and smaller size of colonies.

Decreased expression of MMP-2, MMP-9 and vimentin, and increased expression of E-cadherin by knock-down of EMMPRIN in 786-O
cells can affect tumor invasion and metastasis.

MMPs are believed as a critical regulator of tumor invasion, so we want to determine whether EMMPRIN knock-down would change the expression of MMPs. The data in Figure 4A showed that EMMPRIN knock-down down-regulated the expression of MMPs at mRNA level. qRT-PCR was performed to detect mRNA expression of MMP-2, MMP-9, vimentin and E-cadherin enjoying specific common primer pair, but only MMP-2 and MMP-9 were observed and decreased significantly in EMMPRIN knock-down 786-O cells (Figure 4). Further, we found that E-cadherin expression was also significantly increased in 786-O transfected with EMMPRIN siRNA. The above results were also confirmed by qRT-PCR (Figure 4A). EMMPRIN silencing restricted the expression and activity of MMP-2, MMP-9 and VEGF, which are thought to be significantly involved in the processes of migration, invasion and metastasis of tumor cells, so we tested the effect of deletion of EMMPRIN on migration and invasion of cancer cells. In the assay on scratch migration, down-regulation of EMMPRIN largely suppressed 786-O cell migration (Figure 4B). The assay on matrigel invasion showed that down regulation of EMMPRIN largely inhibited the 786-O cancer cell invasiveness (Figure 4C). In one high power field, the average number of cells crossing matrigel-coated membrane was 78.6 ± 4.1, 82.7 ± 3.5 for siRNA control group and 37.6 ± 3.8 for siRNA EMMPRIN group of 786-O cells (P < 0.01).

Discussion

As we all know, tumor metastasis and invasion, as a complicated process, consists of disruption of basement membrane, intravasation, stromal infiltration, extravasation and tumor cell invasion into a target organ. EMMPRIN, expressed on surfaces of tumor cells, can stimulate the fibroblasts and endothelial cells nearby. With soluble or cell-bound factors stimulating the production of MMPs, tumor cells can interact with stromal cells [15]. It is demonstrated that EMMPRIN, with the characteristics of an adhesive molecule, can critically affect tumor metastasis and invasion, and mediate between cell to cell or cell to matrix [26]. It is revealed through Multivariate analysis that the high EMMPRIN expression, lymph node metastasis, extrathyroidal invasion, and pathologic grading of tumor invasion are seemingly the independent prognostic indicators [27]. EMMPRIN’s proposed role in cell-cell interaction, as well as its ability of inducing MMPs production in neighboring cells, implies that there are counter-receptors on the opposing cells, although none of which has been identified yet. However, it is quite important to identify other biological markers with better sensitivity and specificity owing to low sensitivity or specificity of these molecules [28].

For the reason that EMMPRIN is usually expressed on human tumor cells, and taken as the factor increasing tumor cell invasion, most of the studies having been conducted so far only attached importance to its role in cancer progression. Lots of researches have studied that the expression of EMMPRIN can be frequently found in most human malignancies and subsets of benign tumors. Many investigators have demonstrated the up-regulation of MMPs (MMP-1, MMP-2 and MMP-9) through interactions of tumor cell-fibroblast, and concluded that the up-regulation of MMPs can promote the tumor progression [29]. EMMPRIN can play an important role in the invasion and metastasis of tumor cells.

Moreover, as shown in the crosstabs analysis, EMMPRIN expression has a higher relative risk ratio to different urological carcinomas than histological subtypes, indicating that EMMPRIN, as compared with the others, is a better prognostic indicator [30]. As for MMPs production by endothelial cells, EMMPRIN also has a paracrine effect, which suggests that tumor-produced EMMPRIN has a potential implication in angiogenesis under regulation of MMPs by cells of kidney cancer. The transfection of tumor cells by EMMPRIN or the treatment of tumor cells by recombinant protein may increase the expression of MMPs in tumor cells, while there is no co-culturing with fibroblasts [31]. Through taking the transcriptome analysis on individual tumor cells obtained from bone marrow of patients with cancer with the comparative genomic hybridization technique, it is showed that EMMPRIN is the protein most frequently expressed in primary tumors, as well as in micrometastatic cells, which means that it plays a core role in progression and early metastasis of tumor [32]. The role of EMMPRIN in invasiveness of tumor cell and progression of cancer is most directly demonstrated by the vivo experimental approach; EMMPRIN cDNA
may transfect into breast cancer cells, which then will significantly accelerate the tumor growth in nudemice [33]. It is related to the increasing of expression in EMMRIN transfect-ed tumors of the gelatinases MMP-2 and MMP-9. In this paper, we have gained a consistent result through experiments with the methods of CCK-8, soft agar colony formation, and migration assays: EMMRIN depletion suppresses the proliferation of 786-O cells; EMMRIN silencing significantly reduces the tumor-metastasis, and decreases the lymph node metastases in vivo.

The recent findings show that low EMMRIN expression can be taken as a predictor of favorable prognosis for kidney cancer patients, and EMMRIN can be taken as a potential therapeutic biomarker of kidney cancer. Interestingly, EMMRIN is closely related to two members of the family of proton-coupled MCT, namely MCT1 and MCT4. EMMRIN can also act as an essential chaperone for the correct expression of plasma membrane, as well as the catalytic activity [34, 35]. These findings extend the role of EMMRIN to an angiogenic promoter from an MMP stimulator, highlight the importance of tumor-stroma interactions in cancer, and suggest a novel tumor angiogenesis mechanism driven by tumor cell-associated EMMRIN expression. It is of further interest to investigate the relationship of EMMRIN and MMPs expression and tumor angiogenesis in clinical situations, and decide if targeting EMMRIN may represent a feasible approach to managing or treating cancer. Accordingly, these results may start the view for the therapeutic approaches of taking EMMRIN-specific siRNA as a new cancer strategy. In general, EMMRIN siRNA inhibition can affect the cell proliferation, migration and invasion with the help of MMP-2 and MMP-9 expression, which in turn increases the E-cadherin level in renal cell carcinoma.

Conclusion

In conclusion, we have identified that EMMRIN stimulates tumor angiogenesis by elevating the level of MMP-2 and MMP-9 in tumor and stromal compartments. The reduced EMMRIN expression resulted in reductions of tumor migration, and perturbation of this molecule may have potential therapeutic application in preventing cancer metastasis. What is of particular interest is that the down-regulation of EMMRIN may lead to up-regulation of E-cadherin, but no effect of Vimentin. Accordingly, The EMMRIN might contribute to novel biomarkers for ccRCC.

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Disclosure of conflict of interest

None.

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