Upregulation of UHRF1 promotes renal cancer metastasis and poor prognosis

Long-Sheng Wang1*, Yu-Chun Gu2*, Yang-Yang Hu1, Shao-Jun Chen1, Feng-Qiang Yang1,3, Jun-Hua Zheng1

1Department of Urology, Shanghai Tenth People’s Hospital, Tongji University, Shanghai, China; 2Department of Anesthesiology, Shanghai Tenth People’s Hospital, Tongji University, Shanghai, China; 3Department of Urology, The Fourth People’s Hospital of Changzhou, Suzhou University, Changzhou, China. *Equal contributors.

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Abstract: Background: Renal cell carcinoma (RCC) is one of the most common types of cancer in urological system worldwide. Recently, the ubiquitin-like with PHD and ring-finger domains 1 (UHRF1) was found to play critical regulatory roles in carcinogenesis. The aim of this study was to investigate the role of UHRF1 in RCC progression. Methods: The expression of UHRF1 in RCC tissue samples and cell lines was explored by real-time quantitative PCR, western blot and immunohistochemistry. Associations of UHRF1 expression level with clinicopathologic features and overall survival were also determined. Additionally, the effects of UHRF1 on RCC cells proliferation, migration and invasion were investigated by MTT assay, wound assay and matrigel invasion assay respectively in vitro. Results: Our data showed that UHRF1 expression was up-regulated in RCC tissues and cell lines. In addition, the increased expression of UHRF1 was correlated with the advanced tumor stage, lymph node metastasis, and poor overall survival (OS) of RCC patients. Multivariate analysis suggested that UHRF1 expression was an independent prognostic factor of OS in RCC patients. Moreover, in vitro assay demonstrated that decreased UHRF1 expression could inhibit the RCC cell proliferation, migration and invasion ability. Conclusions: Our findings indicated that UHRF1 might play a vital role in RCC progression and could represent a novel prognostic biomarker and potential therapeutic target in RCC patients.

Keywords: Renal cell carcinoma, UHRF1, prognosis, proliferation, migration, invasion

Introduction

Cancer is a major public health problem all over the world and renal cell carcinoma (RCC) ranks third in frequency of genitourinary cancer, which accounts for about 3% of all cancers in adults [1]. Every year, around 1000,000 patients died of RCC, and the incidence is increasing steadily over these years [2]. Among all the subtypes of RCC, a proportion of 80% is clear cell renal carcinoma (ccRCC) and approximate 30% of patients with RCC have encountered metastases before surgery [3-5]. Surgery is the only curative treatment for RCC and the 5-years survival rate is estimated to be 55%, however that of metastatic renal cell carcinoma is only 10% [6]. Currently, for lacking of fully understanding of the underlying mechanisms of metastasis, the progress in proper therapy for enhancing prognosis is limited. Therefore, it is urgent to identify the molecular mechanism of RCC metastasis, so as to develop some effective strategies for diagnosis and therapy.
Although the role of UHRF1 has been identified in various cancers, its properties in RCC have not been well illustrated. In the present study, we found that the expression of UHRF1 was significantly upregulated in the RCC tissues and cell lines. Then, we observed that UHRF1 overexpression was correlated with several clinico-pathological features and poor overall survival of RCC patients. Multivariate analysis indicated that UHRF1 expression was an independent prognostic factor of OS in RCC patients. When UHRF1 was knockdown in RCC cell lines (786-O and A498), the proliferation, migration as well as invasion were all significantly inhibited. Taken together, our results suggested that UHRF1 might play a role as a novel oncogenic factor in RCC and could act as a promising diagnostic biomarker and therapeutic target in the treatment of RCC.

**Materials and methods**

**Patients and specimens**

A total of 60 pathologically diagnosed RCC tissues and matched adjacent non-tumor renal tissues were collected from 2007 to 2009 in the Department of Urology, Shanghai Tenth People’s Hospital of Tongji University. None of the patients received radiotherapy or chemotherapy before surgery. All the tissues were collected after surgical reactions, parts of each tissue samples were fixed in formalin and then embedded in paraffin, the remains were stored in liquid nitrogen immediately until use. Clinical and pathological messages including histological grade, tumor stage, lymph node metastasis were collected and the details were summarized in Table 1. Prior patient’s consent was obtained from all patients and the study was approved by the Institute Research Ethics Committee of Tongji University.

**Cell lines and UHRF1 knockdown**

Human renal cancer cell lines 786-O and A498 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CCCAS, China). Immortalized normal human proximal tubule epithelial cell line HK-2 was obtained from the American Type Culture Collection (ATCC, USA). RCC cell lines 786-O and A498 were cultured in RPMI-1640 medium (HyClone), HK-2 cells were cultured in KSFM medium (Gibco). All media were supplemented with 10% fetal bovine serum (Gibco). All cells were cultured in a humidified incubator with 5% CO₂ at 37°C.

siRNA that targeted UHRF1 RNA (si-UHRF1) and scrambled negative control (si-NC) was provided by Life Technologies. The target siRNA oligos sequences for si-UHRF1 was got from the previous literature [13]. Cells were transfected with the si-UHRF1 or si-NC according to the manufacturer’s instructions. After 48 hours, the mRNA and protein were extracted from the transfected cells to determine the transfection efficiency.

**Real-time quantitative PCR**

The total RNA was collected by the Trizol reagent according to the manufacturer’s protocol (Invitrogen). For qPCR assay, RNA was reverse transcribed to cDNA using SuperScript First-Strand cDNA System (Invitrogen). The expression of UHRF1 was measured by real-time PCR using the SYBR EX TAQ (Takara). The PCR amplification was conducted for 40 cycles of universal conditions (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s), on a Applied Biosystems 7900HT (Applied Biosystems) with 1.0 μl

### Table 1. Correlation between clinicopathological features and UHRF1 expression

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group</th>
<th>Total n=60 (%)</th>
<th>UHRF1 staining intensity</th>
<th>P value</th>
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<td></td>
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<td>High (++/++++)</td>
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<tr>
<td></td>
<td>Female</td>
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<td>7</td>
<td></td>
</tr>
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<tr>
<td></td>
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<tr>
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<td></td>
<td>≥4 cm</td>
<td>28 (47)</td>
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<td></td>
<td>III-IV</td>
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<tr>
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<td></td>
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<td></td>
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<td>20 (33)</td>
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</tbody>
</table>

**Materials and methods**

**Patients and specimens**

A total of 60 pathologically diagnosed RCC tissues and matched adjacent non-tumor renal tissues were collected from 2007 to 2009 in the Department of Urology, Shanghai Tenth People’s Hospital of Tongji University. None of the patients received radiotherapy or chemotherapy before surgery. All the tissues were collected after surgical reactions, parts of each tissue samples were fixed in formalin and then embedded in paraffin, the remains were stored...
The primers used in real-time quantitative PCR are as the following: UHRF1 sense, 5’CCCAAATGCGAGTTTCGC 3’, reverse, 5’TGGGGATGGCGATGAAACC 3’; GAPDH sense 5’GTAAGACCCCTGGACCA 3’, reverse, 5’CAAGGGGTCTACATGGCAACTC 3’.

Western blot assay

Total protein of tissues or cells were extracted using precooled RIPA lysis buffer with protease inhibitors. The concentration of total protein was measured using a Bio-Rad protein assay system. Equal amount of protein was separated by 9% SDS-PAGE for electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). Afterward, the membrane was incubated at 4°C for 12 hours with specific primary antibodies (Bioworld, Nanjing, China). After incubation with secondary antibodies for 2 hours at room temperature, signals were visualized by ECL detection reagent (Amersham LifeScience, Piscataway, NJ).

Immunohistochemical staining

All RCC tissues and adjacent normal tissues were formalin-fixed and embedded in paraffin blocks. Each 4 μm sections was dewaxed in xylene and rehydrated in grading alcohol. Antigen retrieval was performed by pre-heated TrisEDTA for 20 min. Methanol containing 0.3% H₂O₂ was used to block endogenous peroxidase activity for 8 min. And then, bovine serum albumin was used to block the sections for 30 minutes. The sections were incubated with primary anti-UHRF1 antibody (1:50, Bioworld, Nanjing, China) overnight at 4°C. The sections were reacted with HRP link and horseradish peroxidase-conjugated rabbit-anti-mouse IgG for 30 min. Followed with the DAB+ EnVision System and counterstaining with Mayer/hematoxylin.

Evaluation of immunohistochemical staining

The area of staining was evaluated and recorded as a percentage: 0, less than 5%; +, 5%-25%; ++, 26%-50%; 3+, 51%-75% and 4+, more than 75%. The combined scores were recorded and graded as follows: -, 0; +, 1-2; ++, 3-5; ++++, 6-7. Additionally, for statistical analysis, the- and 1+ cases were pooled into the low-expression group, and the 2+ and 3+ cases were pooled into the high-expression group.

MTT assay

Two groups of RCC cells (si-UHRF1 group and si-NC group) were plated in 96-well culture plates at a concentration of about 5×10³/well. After incubated for different time (12, 24, 48, 72 h), 20 μl MTT solution was added to each well. Then, cells were incubated at 37°C for 4 hours and the medium was discarded from each well. After that, 150 μl DMSO was used and thoroughly mixed for 15 min. The optical density (OD) of each well was measured at of 490 nm using a micro-plate reader (Bio-Rad). All experiments were performed three times and three replicates in each repeat.

Wound assay

To determine cell migration, RCC cells transfected with si-UHRF1 or si-NC were seeded into 12-well plates and cultured overnight. Similar sized wounds were made by scraping with a sterilized 200 μl white pipette tip, and wounded monolayer cells were rinsed with PBS for three times. To measure the speed of wound healing, photographs were obtained after 48 hours by a phase contrast microscope (Olympus). Each experiment was carried out three times independently.

Matrigel invasion assay

For invasion assays, matrigel-coated invasion chambers with a pore size of 8 μm (Costar, NY, USA) were used according to manufacturer's protocol. Cells were collected after transfection with si-UHRF1 or si-NC for 48 hours and equal numbers of the indicated cells were transferred to the upper Matrigel chamber in 200 μl serum free medium. The bottom chamber was filled with medium which supplied with 10% fetal bovine serum. After incubated for 24 hours, the non-invaded cells on the surface of the upper membrane were removed using a cotton swab, and the invasive cells were stained using 0.1% crystal violet and counted in five randomly
selected high power fields under a microscope. The experiment was performed in triplicates.

Statistical analysis

SPSS version 18.0 software was used for all statistical analyses of this study. Data are expressed as mean ± SD from at least three independent experiments. The differences between each experimental group was analyzed by Student’s t-test or chi-square test. Survival analysis was calculated by Kaplan-Meier survival analysis and Log-rank test. Variables were used in multivariate analysis on the basis of the Cox proportional hazards model. P-value of <0.05 was treated as statistically significant.

Results

Upregulated of UHRF1 in RCC tissues and cell lines

To measure the expression of UHRF1 in mRNA and protein levels in RCC tissues and cell lines, real-time quantitative PCR (qRT-PCR) and western blot assay were performed. The results indicated that the expression of UHRF1 was significantly upregulated in RCC tissues compared to the matched adjacent normal tissues (Figure 1A). Furthermore, we explored the expression of UHRF1 in RCC cell lines, our data suggested that the expression level of UHRF1 in RCC cells (786-O and A498) were higher than normal human proximal tubule epithelial cell line HK-2 (Figure 1B). Taken together, these results suggested that UHRF1 was upregulated in RCC both in the mRNA and protein levels.

Relationship between UHRF1 expression and clinicopathological features in RCC patients

Except for western blot, the protein level of UHRF1 expression in 60 RCC tissues and adjacent non-tumor tissues were analyzed by immunohistochemistry. The UHRF1 immunostaining was more substantial in tumor tissues than the adjacent normal tissues. Furthermore, our data showed that the UHRF1 was mainly stained in
Among all the RCC tissue samples, 41 (68.3%) cases showed high UHRF1 expression (UHRF1 ++ or UHRF1 +++), and 19 (31.7%) cases exhibited low UHRF1 expression (UHRF1 - or UHRF1 +).

Based on relative expression of UHRF1 defined by immunostaining, the relationship between UHRF1 expression and clinicopathological features of RCC patients was determined. As shown in Table 1, UHRF1 overexpression was correlated with the tumor stage and lymph node metastasis of RCC patients, but not correlated with patients’ gender, age, tumor size and histological grade. These results indicated that increased UHRF1 expression was related to the development and progression of RCC.

Relationship between UHRF1 expression and overall survival of patients with RCC

As we have demonstrated above, UHRF1 expression was upregulated in RCC and its upregulation was associated with tumor progression,
which may influence the prognosis of patients with RCC. Therefore, the relationship between UHRF1 expression and overall survival (OS) of RCC patients was investigated. Survival analysis was performed by Kaplan-Meier survival curve and log-rank test showed that RCC patients with high UHRF1 expression had obviously poorer prognosis than those with low UHRF1 expression group (Figure 3). Furthermore, multivariate analysis indicated that relative UHRF1 expression level, tumor stage and lymph node metastasis were each determined to be independent prognostic indicators for the overall survival of patients with RCC (Table 2).

**Knockdown of UHRF1 inhibited cell proliferation**

To further detect the role of UHRF1 in RCC cell proliferation, UHRF1 was silenced by si-UHRF1 in 786-O and A498 cells. The transfection efficiency was confirmed by qRT-PCR and western blot assay. Compared with the si-NC group, the expression of UHRF1 was obviously downregulated in si-UHRF1 group both in mRNA and protein levels (Figure 4A, 4B). MTT assay was used to measure the effect of UHRF1 on RCC cells proliferation. Our date showed that the survival fraction of si-UHRF1 cells were significantly decreased compared with si-NC cells (Figure 4C). These results suggested that UHRF1 might play an important role in RCC cell proliferation.

**Knockdown of UHRF1 inhibited cell migration and invasion**

Metastasis is the main reason of death in most human cancers, thus, we explored the effect of UHRF1 on RCC cells migration and invasion ability. Wound assay showed that RCC cells (786-O and A498) transfected with si-UHRF1 displayed lower migration capacity than si-NC group (Figure 5A). Matrigel invasion assay showed that knockdown of UHRF1 reduced cell invasion abilities of RCC cells compared with control group (Figure 5B). These results indicated that knockdown of UHRF1 significantly suppressed the migration and invasion ability of renal cancer cells.

**Discussion**

Renal cell carcinoma (RCC) is one of the deadliest urogenital malignancies. The morbidity of RCC is increasing annually and the causes are multifactorial [17]. In addition, RCC has some unique feature, such as a relatively high rate of late recurrence (LR) after surgical procedure [18]. Therefore, earlier diagnosis and remedy based on fully understand of molecular pathways are of great importance for RCC patients.

Various molecular parameters have been estimated for prognostic value in RCC. Among these, UHRF1 was an important factor that took part in the development of cancers. As a significant epigenome regulator factor, UHRF1 played regulatory role in the maintains of DNA and protein methylation [7]. Achour et al. reported that UHRF1/DNMT1 would inhibit the expression of VEGF via downregulating the methylation of anti-oncogene p16INK4A in Jurkat and HVTs-SM1 cell lines [19]. Hopfner et al. showed that UHRF1 is particularly overexpressed in proliferative tissue, whereas it is not expressed in highly differentiated tissues [20]. They also reported that there was no UHRF1 expression in chronic pancreatitis and normal pancreatitis tissues [21]. In addition, the expression of UHRF1 didn’t influence the growth of human lung fibroblasts and mouse embryonic stem cell. It means that UHRF1 is not an essential factor in the development of nontumorous cells [15]. A recent study demonstrated that UHRF1 was overexpressed in ccRCC which suppress p53 pathway activation and help ccRCC cells to escape from p53-depen-dent apoptosis [22]. However, the biology functions of UHRF1 in RCC still remain elusive.

In this study, we confirmed that UHRF1 was upregulated in RCC tissues and cell lines. UHRF1 overexpression was correlated with the tumor stage and lymph node metastasis, but had no significant correlation with patients’ gender, age, tumor size and histological grade. In addition, UHRF1 high expression was corre-

<table>
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<td>1.251-4.958</td>
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<td>2.583</td>
<td>1.629-5.714</td>
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</table>
UHRF1 in RCC


related with lower overall survival rates and could be an independent prognostic factor in patients with RCC. To further understand the biological function of UHRF1 in RCC cell process, in vitro experiments were conducted. Small interfering RNA was used to suppress UHRF1 expression both in A498 and 786-O cell lines. In vitro assay revealed that the siRNA-mediated knockdown of UHRF1 significantly reduced proliferation and metastasis capability of RCC cells compared with control group. These findings demonstrated that UHRF1 might act as a new oncoprotein in renal cancer progression. However, as a limitation of this study, the potential detailed mechanism of UHRF1 functions in RCC is required to reveal in the future.

In conclusion, we verified the overexpression of UHRF1 in renal cancer tissues and cell lines. Elevated UHRF1 expression was associated with poor prognosis, likely due to the ability of UHRF1 to induce invasive and metastatic behavior in RCC cells. Cumulatively, these findings indicate that UHRF1 might play a vital role in RCC progression and could represent a novel prognostic biomarker and potential therapeutic target in RCC patients.

Disclosure of conflict of interest

None.

Address correspondence to: Feng-Qiang Yang, Department of Urology, The Fourth People’s Hospital of

Figure 4. Down-regulated expression of UHRF1 inhibits cell proliferation of 786-O and A498 cells. (A, B) Decreased expression of UHRF1 in transfected renal cancer cells were demonstrated by qRT-PCR (A) and western blot (B) analysis. (C) There were significant decreases in cell proliferation of UHRF1 reducing cells as compared with control cells. *P<0.05.
Figure 5. Down-regulated expression of UHRF1 inhibits cell migration and invasion of 786-O and A498 cells. A. Cell migration was assessed using scratch-healing assays. Confluent monolayer of 786-O and A498 cells were scratched and healing was monitored by taking photographs at the indicated time points. B. Cell invasion was determined in 786-O and A498 cells using Biocoat matrigel invasion assay. The invaded cells were counted under a microscope. The experiments were repeated thrice. *P<0.05.


