Downregulation of long noncoding RNA FENDRR predicts poor prognosis in renal cell carcinoma

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Abstract. Long noncoding RNA FOXF1 adjacent non-coding developmental regulatory RNA (FENDRR) dysregulation associates with multiple types of human cancer. However, the biological functions of FENDRR in renal cell carcinoma are unresolved. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to determine the expression level of FENDRR in renal cell carcinoma tissues. An RNA interference assay and ectopic expression experiments were conducted to evaluate the effects of FENDRR on cell proliferation, migration, invasion and colony formation in vitro. RNA immunoprecipitation was conducted to identify proteins associated with FENDRR. It was observed that FENDRR is frequently downregulated in renal cell carcinoma and overexpression of FENDRR attenuated proliferation, migration, invasion and colony growth of renal carcinoma cells. Conversely, knockdown of FENDRR promotes proliferation and invasiveness of renal carcinoma cells. Downregulation of FENDRR associates with poor prognosis of renal cell carcinoma. Mechanistically, it was identified that FENDRR may bind to Polycomb Repressive Complex 2 and lysin methyltransferase 2A histone modifying complexes. In summary, FENDRR acts as an tumor suppressor in renal cell carcinoma and may serve as a candidate target for gene therapy.

Introduction

Renal cell carcinoma (RCC) comprises 90% of all kidney tumors and its incidence is increasing worldwide (1). Worldwide, approximately 30% of patients with RCC were diagnosed with metastatic disease, which decreased the 5-year survival rate to only 10% as reported in 2006 (2,3). Conventional radical nephrectomy is not able to cure metastatic RCC, which is resistant to radiotherapy and chemotherapy (4). In the past decade, tyrosine kinase inhibitors, also known as targeted therapies, were introduced and demonstrated relatively high rates of response but the 5-year survival rate of patients with metastatic RCC remains low (5). More efficient targets are urgently needed to improve long term survival of patients with advanced RCC.

FOXF1 adjacent non-coding developmental regulatory RNA (FENDRR), a long noncoding RNA lacking an open reading frame of significant length, was first identified as a gene that is specifically expressed in the posterior mesoderm of mouse embryos and is essential for heart and body wall development (6). FENDRR is a conserved transcript of ~3 kilobases, located on human chromosome 16 (6). Further characterization revealed that it is downregulated in patients with gastric and lung cancer (7,8). Further analysis has demonstrated that histone deacetylation is involved in downregulation of FENDRR and may serve as prognostic factor for human gastric cancer (7).

Although the key functions of FENDRR in embryogenesis and development have been well elucidated (6,9,10), the biological functions of FENDRR and whether aberrant expression of FENDRR occurs in RCC remains to be determined. In the present study, it was identified that FENDRR was frequently downregulated in RCC and other types of human cancer. Overexpression of FENDRR attenuates RCC cells proliferation, migration, invasion and colony formation capabilities, suggesting pivotal functions of FENDRR in RCC progression. Mechanistically, FENDRR physically associates with Polycomb Repressive Complex 2 (PRC2) and lysin methyltransferase 2A (MLL) histone modifying complexes. Importantly, downregulation of FENDRR predicts poor prognosis of RCC.

Materials and methods

Tissue samples. Samples of fresh frozen RCC cancer tissues, together with normal adjacent tissues (3 cm away from the tumor as in previously published studies) (11), were obtained during radical nephrectomy from Sun Yat-sen Memorial Hospital of Sun Yat-sen University (Guangzhou, China). All samples were
snap frozen using liquid nitrogen and transferred to -80°C until processed. Tissue samples were collected with written informed consent from patients, and approved by the Ethic Review Committees of Sun-Yat Sen Memorial Hospital. All samples had their diagnoses confirmed pathologically. The nuclear grade was assessed with the Fuhrman grade system (12).

**Cell lines and transfection.** Human RCC cell lines (769-P, 786-O, ACHN) used in the study were obtained from the American Type Culture Collection (Manassas, VA, USA). 769-P and 786-O cells were cultured in RPMI-1640 medium (Hyclone; GE Healthcare, Logan, UT, USA). ACHN cells were cultured in Dulbecco’s modified Eagle’s medium (Hyclone; GE Healthcare, Logan, UT, USA). All media were supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were cultured in a humidified air atmosphere of 5% CO₂ at 37°C. Routine tests for mycoplasma infection were negative. Cell transfection was performed using Lipofectamine 2000 or RNAlivMAX (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol for overexpression or knockdown experiments, respectively. Sequences of siRNAs were as follows: siFENDRR#1, 5'-CCAGCCAUUGAUUCCAA ATT-3'; siFENDRR#2, 5'-GGCAUUGACUGUCUUAUA ATT-3'; siControl, 5'-UUUCGGAACGUGUCAGTTT-3'.

**RNA isolation and reverse transcription-quantification polymerase chain reaction (RT-qPCR).** Total RNA from cells was isolated using Trizol® reagent (Life Technologies; Thermo Fisher Scientific, Inc.) and treated with an RNase-free DNase set (Qiagen China Co., Ltd., Shanghai, China) according to the manufacturer’s protocols. RNA electrophoresis on a denaturing agarose gel at 5-6 V/cm was performed to inspect RNA integrity. Intact total RNA run on a denaturing gel has sharp 28S and 18S rRNA bands, and the 28S RNA band is approximately twice as intense as the 18S RNA band. RT was performed using M-MLV reverse transcriptase (Life Technologies; Thermo Fisher Scientific, Inc.) and qPCR was performed with SYBR-Green master mix (Roche Diagnostics, Basel, Switzerland). Relative expression values were calculated using the 2^ΔΔCq method with GAPDH as a normalizer (13). The primer sequences used were as follows: FENDRR: Forward, 5’-AGAGTCCTCCACTCCCTA-3’ and reverse, 5’-CCCATTTGCCAAAGGCTACAT-3’; GAPDH, forward, 5’-GAAGGTGAAGTCCAGAGTGC-3’ and reverse, 5’-GAAGATGGTGATGGATTTC-3’.

**Cell proliferation, motility, invasion and colony formation assays.** Cell proliferation, motility, invasion and colony formation assays were performed as previously described (14). For the cell proliferation assay, 2x10⁴ cells were plated in quadruplicates in 96-well plates and incubated in a humidified air atmosphere of 5% CO₂ at 37°C. Cell number was calculated using an MTT assay kit (Roche Diagnostics). The cell motility assay was performed using Transwell chambers (BD Falcon; BD Biosciences, San Jose, CA, USA). A total of 1x10⁶ cells were plated in Transwell chambers and incubated in complete medium in a humidified air atmosphere of 5% CO₂ at 37°C for 8-24 h. Cells were stained with 0.1% crystal violet. For the colony formation assay, 100-500 transfected cells were placed in a fresh six-well plate and maintained for two weeks and colonies were fixed with methanol and stained with 0.1% crystal violet and counted. An in vitro cell invasion assay was performed using the BD BioCoat Matrigel Invasion Chamber (BD Falcon; BD Biosciences) according to manufacturer’s instructions. A total of 1x10⁴ cells were plated in BD BioCoat Matrigel Invasion Chambers and incubated in complete medium in a humidified air atmosphere of 5% CO₂ at 37°C for 12-36 h. Subsequently, crystal violet on the Transwell membrane was dissolved in 33% acetic acid solution and measured the absorbance of crystal violet (OD570 nm). Cells were visualized with inverted microscope (magnification, x200; Nikon Corporation, Tokyo, Japan). All experiments were performed in triplicate.

**5’-Ethynyl-2’-deoxyuridine (EdU) incorporation assay.** 786-O and 769-P cells in S-phase were measured using an EdU assay kit (Guangzhou Ribobio Co., Ltd., Guangzhou, China) according to the manufacturer’s protocol. Briefly, cells were incubated with 5 µM EdU in culture media supplemented with 10% FBS for 2 h at 37°C. Then cells were fixed with 4% formaldehyde for 30 min at room temperature and treated with 0.5% Triton X-100 for 20 min for permeabilization. Subsequent to washing with PBS for 30 seconds at room temperature for three times, the cells were incubated with 1x Apollo® reaction cocktail (Guangzhou Ribobio Co., Ltd.) at 37°C for 30 min. Subsequently, the DNA contents were stained with Hoechst 33342 (5 µg/ml) for 30 min at 37°C and visualized under a fluorescent microscope (magnification, x100; Nikon Corporation, Tokyo, Japan).

**RNA immunoprecipitation assay.** RNA immunoprecipitation was performed using the EZ-MagnaRIP kit (EMD Millipore, Billerica, MA, USA) according to manufacturer’s protocols. Briefly, 5x10⁶ cells were harvested and lysed with radioimmunoprecipitation assay lysis buffer with one freeze-thaw cycle. Cell extracts were co-immunoprecipitated with anti-enhancer of zeste 2 polycomb repressive complex 2 (EZH2; 1:500 dilution; cat. no. ab195409, ChIP grade), suppressor of zeste 12 protein homolog (SUZ12; 1:500 dilution; cat. no. ab12073, ChIP grade) and WD repeat domain 5 (WDR5; 1:500 dilution; cat. no. ab195409, ChIP grade), with anti-enhancer of zeste 2 polycomb repressive complex 2 (EZH2; 1:500 dilution; cat. no. ab195409, ChIP grade), and incubated with agitation overnight at 4°C. The retrieved RNA was subjected to RT-qPCR analysis. Anti-snRNP70 (1:500 dilution; cat. no. CS20312; EMD Millipore) was used as a technical positive control, and normal mouse Immunoglobulin G was used as a negative control. For RT-qPCR analysis, U1 splicesome RNA was used as a non-specific control. Experiments were performed in triplicates.

**Bioinformatic analysis.** Data from The Cancer Genome Atlas (TCGA) were obtained from The Atlas of Noncoding RNAs in Cancer website (http://ibl.ndanderson.org/tanric/_design/basic/resources.html). Data from the Gene Expression Omnibus (GEO) were downloaded from the GEO website (https://www.ncbi.nlm.nih.gov/geo/).

**Statistical analysis.** All quantitative data were presented as the mean ± standard deviation from at least three independent experiments. The differences between two independent groups were analyzed using independent t-tests, and the differences between paired tumor and normal adjacent tissues were
analyzed with paired t-tests. The differences between multiple groups were assessed using one-way analysis of variance followed by Dunnett's multiple-comparison test. The association between FENDRR and clinical characteristics was analyzed using Pearson Chi-square test. All tests performed are two sided. Statistical tests were performed using SPSS software (version 10.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

**FENDRR is downregulated in multiple human cancers.** To explore the biological roles of FENDRR in human cancer, publically available databases were searched. As presented in Fig. 1, FENDRR is downregulated in multiple types of human cancer, including bladder and lung cancer, and pancreatic ductal adenocarcinoma. Interestingly, in...
Table I. Characteristics of renal cell carcinoma patients.

| Characteristic          | Patient frequency (%) | Low   | High  | Pearson $\chi^2$ | P-value |
|-------------------------|-----------------------|-------|-------|------------------|---------|
| Total                   | 56                    | 41 (73.2%) | 15 (26.8%) |                   |         |
| Sex                     |                       |       |       |                  |         |
| Male                    | 39 (69.6)             | 30    | 9     | 0.386            | 0.535   |
| Female                  | 17 (30.4)             | 11    | 6     |                  |         |
| Age, years              |                       |       |       |                  |         |
| <60                     | 32 (57.1)             | 25    | 7     | 0.918            | 0.338   |
| ≥60                     | 24 (42.9)             | 16    | 8     |                  |         |
| Tumor stage             |                       |       |       |                  |         |
| T1                      | 38 (67.9)             | 26    | 12    | 0.730            | 0.39    |
| T2-4                    | 18 (32.1)             | 15    | 3     |                  |         |
| Fuhrman grade           |                       |       |       |                  |         |
| 1-2                     | 34 (60.7)             | 21    | 13    | 5.785            | 0.016   |
| 3-4                     | 22 (39.3)             | 20    | 2     |                  |         |

FENDRR, FOXF1 adjacent non-coding developmental regulatory RNA.

Figure 2. FENDRR is downregulated in renal clear cell carcinoma and correlated with poor prognosis. Box-and-whisker diagrams presenting the expression level of FENDRR in (A) normal adjacent tissues and renal cell carcinoma tissues and (B) different Fuhrman grade RCC tissues. (C) The cancer genome atlas data presents that a lower expression level of FENDRR associates with poor prognosis of patients with renal clear cell carcinoma. FENDRR, FOXF1 adjacent non-coding developmental regulatory RNA.
the observation of multistep pancreatic carcinogenesis, the expression levels of FENDRR were downregulated in intraductal papillary mucinous adenoma, intraductal papillary mucinous carcinoma and invasive cancer of intraductal papillary mucinous neoplasm. More importantly, the expression levels of FENDRR were downregulated in the metastatic site compared with the primary tumor in colorectal cancer and prostate cancer.

Collectively, these data demonstrated that FENDRR serves important functions in carcinogenesis and cancer metastasis in multiple types of human cancer.

**FENDRR is downregulated in renal cell carcinoma and associates with poor prognosis of RCC.** In order to determine whether dysregulation of FENDRR expression indeed occurs in renal cell carcinoma, the RNA level of FENDRR was quantified in renal cell carcinoma tissues and paired normal adjacent cortex tissues using RT-qPCR. Fold changes of <1.0 were designated as downregulated in cancer tissues compared with normal adjacent tissues. The results revealed that FENDRR was downregulated in 73.2% of renal cell carcinoma tissues (Table I; Fig. 2A). Statistical analysis revealed that lower FENDRR expression was associated with Fuhrman...
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As a result of the limited number of cases and the follow up period, TCGA database was searched to identify whether FENDRR expression associates with the prognosis of patients with renal cell carcinoma. The results demonstrated that lower FENDRR expression associates with shorter overall survival (P=0.00025; Fig. 2C).

FENDRR downregulation results in accelerated cell proliferation of RCC cells. Unlimited proliferation ability comprises one of the most important characteristics of malignant tumor. Reports have identified that downregulation of FENDRR promotes tumor growth and associates with poor prognosis in gastric cancer (7). To further determine the role of FENDRR in regulating RCC cell proliferation, FENDRR was overexpressed using expression vectors or depleted in RCC cell lines using siRNAs. As presented in Fig. 3A-D, knockdown of endogenous FENDRR accelerated cell proliferation in 786-O and 769-P RCC cells, compared with the non-specific control siRNA that shared no homology with the human genome. Conversely, overexpression of FENDRR significantly inhibited proliferation of RCC cells (Fig. 3E-F). These data suggested a function of FENDRR in regulating RCC cell proliferation.

To further validate the role of FENDRR in cell cycle modulation, an EdU incorporation assay was performed. EdU is a nucleotide analog of thymidine and is incorporated into DNA during DNA synthesis. Thus EdU positive cells indicate the cell is in S phase. Markedly increased EdU positive cells were observed following FENDRR depletion in 786-O and 769-P RCC cells (Fig. 3G), and overexpression of FENDRR decreased the proportion of cells in S phase, further indicating that FENDRR expression inhibited cell proliferation of RCC cells.

FENDRR overexpression inhibits the migration, invasion and colony formation abilities of RCC cells. The ability to invade the basement membrane and migrate from the primary site to a distant site is critical for malignant cancer cells to form metastatic lesions. Cell migration ability was evaluated using Transwell chambers and cell invasion was assessed using a Matrigel invasion assay which mimics the process of tumor cells invading the basement membrane. As presented in Fig. 4A-B, FENDRR knockdown promoted, while FENDRR overexpression inhibited, cell migration and invasion of RCC cells, suggesting that FENDRR serves a regulatory function in RCC cell migration and invasion.

Colony formation ability demonstrates the cell-population dependent proliferation capacity of cancer cells. Knockdown of endogenous FENDRR markedly promoted, and FENDRR overexpression inhibited, colony formation both in 786-O and 769-P cells (Fig. 4C-D). Together, these results indicate the critical function of FENDRR in the process of metastasis of renal cell carcinoma.

Potential targets of FENDRR in RCC. To identify potential target genes of FENDRR involved in the metastasis of RCC, an analysis of FENDRR and mRNA expression was performed using TCGA database. As presented in Fig. 5,
a positive association was identified between FENDRR expression and tumor suppressors protocadherin 17, sprouty RTK signaling antagonist 4, MYC target 1 and a negative association was identified between FENDRR and oncogene aurora kinase A, jupiter microtubule associated homolog 1 and poly(ADP-ribose) polymerase family member 3, suggesting that FENDRR may serve as a tumor suppressor by activating important tumor suppressor genes and inhibiting expression of oncogenes.

**FENDRR physically associates with PRC2 complex and MLL complex.** To further elucidate how FENDRR regulates down-stream target genes, we next performed RNA immunoprecipitation to identify FENDRR interacting proteins. Our RIP assay confirmed that FENDRR physically associates with EZH2, SUZ12 and WDR5 in RCC cells (Fig. 6), suggesting that FENDRR may exert its function at least partially by interacting with PRC2 complex and MLL complex.

**Discussion**

FENDRR has been suggested to serve important functions in vertebral development and is downregulated in numerous types of cancer (7-9,15). A low level of FENDRR expression promotes local invasion and lymphatic metastasis in gastric cancer, which are key steps for cancer progression (7). To interpret how this transcript exerts its biological function, potential mechanisms were proposed, including controlling epigenetic modifications of promoters of target genes critical for embryonic development (6), and promoting cancer invasion through regulating expression of matrix metalloproteinase (MMP)2/MMP9 (7). In the present study, it was identified that FENDRR downregulation occurs in the process of carcinogenesis and cancer metastasis through searching a publicly available database. Though a tumor suppressing role was identified in multiple types of human cancer, functional characterization of FENDRR in RCC remains undocumented. Consistent with observations in other cancers, the present data revealed that FENDRR downregulation is very common in RCC and associates with poor prognosis of patients with RCC. Importantly, overexpression of FENDRR significantly inhibited cell proliferation and invasion, pointing to the possibility of FENDRR as a therapeutic target for cancer treatment.

Dysregulation of chromatin modifications including H3K4 and H3K27 methylation is implicated in the carcinogenesis of RCC (16). The MLL complex is responsible for
H3K4 methylation (17) and polycomb group proteins (PRC1 and PRC2) are epigenetic silencers implicated in cancer development (18). EZH2, a core component of PRC2, directly controls the histone H3 lysine 27 trimethylation and interacts with DNA methyltransferases promoting DNA methylation, and EZH2 is critical for these processes (19,20) and predicts cancer specific survival of RCC (16). Studies have uncovered that numerous long intergenic noncoding RNAs (lincRNAs) associate with epigenetic modifying complexes, and >20% of lincRNAs interact with PRC2 complex (21). In the present study, the gathered data suggested that FENDRR is able to physically associate with WDR5, EZH2 and SUZ12 in renal carcinoma cells. It is of note that certain studies provide evidence that EZH2 promotes the progression of RCC by modulating vascular endothelial growth factor and E-cadherin expression (22,23). However, a more precise mechanism detailing how FENDRR affects the targeting specificity of PRC2 complex and MLL complex to promote carcinogenesis and cancer metastasis remains unresolved and warrants further investigation.

It is a common phenomenon that genes associated with developmental process also serve important functions in cancer progression. Recent studies have demonstrated that long noncoding RNAs serve important functions in the process of limb development and lymph node metastasis of multiple types of cancer (HOXA transcript at the distal tip) (24,25), skeletal development and cancer metastasis (HOX transcript antisense RNA) (24), muscle differentiation and cancer metastasis (H19) (24,26). Specifically, FENDRR is dispensable for lung and heart development, and others and the present experiments suggested that FENDRR binds to PRC2 and MLL complexes (6,9). Markedly, the present study identified that FENDRR is not only critical for RCC but also serves a function in the development of multiple cancers. However, the precise molecular mechanisms by which FENDRR affects cellular phenotypic alterations, including cell proliferation and migration, is largely unexplored, which requires further investigation in future studies.

To conclude, the present study reports that the long noncoding RNA FENDRR acts as a tumor suppressor in RCC. It was identified that FENDRR is frequently downregulated in cancerous tissues of RCC, promoting cell migration and invasion. Furthermore, overexpression of FENDRR decelerates cell migration, invasion and colony formation of RCC cells. 

Figure 6. FENDRR physically associates with enhancer of zeste 2 polycomb repressive complex 2 and WDR5 protein. Histograms presenting an RNA immunoprecipitation assay using antibodies to EZH2 and SUZ12 as baits to co-immunoprecipitate RNAs that bind to these proteins in (A) 786-O and (B) 769-P cells. The achieved RNAs were subjected to reverse transcription-quantitative polymerase chain reaction to assess the quantity of FENDRR. A radioimmunoprecipitation assay was performed using an antibody against WDR5 in (C) 786-O and (D) 769-P cells. Statistical significance was assessed using one-way analysis of variance followed by Dunnett’s tests for multiple comparisons. FENDRR, FOXF1 adjacent non-coding developmental regulatory RNA; WDR5, WD repeat domain 5; EZH2, enhancer of zeste 2 polycomb repressive complex 2; SUZ12, suppressor of zeste 12 protein homolog; HOTAIR, HOX transcript antisense RNA; HOTTIP, HOXA transcript at the distal tip; Ig, immunoglobulin. *P<0.01.
These findings suggest that targeting FENDRR may help controlling RCC progression and metastasis.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the TCGA repository, http://ibl.mdanderson.org/tanric/design/basic/index.html.

Authors' contributions

WH and JH participated in the study design. GZ, PW and NJ carried out in vitro cell experiments and data analysis. PW and CJ performed the PCR experiments and data analysis. WH conceived the study and wrote the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

Tissue samples were collected with written informed consent from patients, and approved by the Ethics Review Committee of Sun Yat-sen Memorial Hospital.

Patient consent for publication

All samples were collected with written informed consent from patients. No identifying information, including names, initials, date of birth or hospital numbers, images or statements were included in the manuscript.

Competing interests

The authors declare that they have no competing interests.

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