Interferon regulatory factor 8 functions as a tumor suppressor in renal cell carcinoma and its promoter methylation is associated with patient poor prognosis

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ABSTRACT

Interferon regulatory factor 8 (IRF8), as a central element of IFN-γ-signaling, plays a critical role in tumor suppression. However, its expression and underlying molecular mechanism remain elusive in renal cell carcinoma (RCC). Here, we examined IRF8 expression and methylation in RCC cell lines and primary tumors, and further assessed its tumor suppressive functions. We found that IRF8 was widely expressed in human normal tissues including kidney, but frequently downregulated by promoter methylation in RCC cell lines. IRF8 methylation was detected in 25% of primary tumors, but not in adjacent non-malignant renal tissues, and associated with higher tumor nuclear grade of RCC. Ectopic expression of IRF8 inhibited colony formation and migration abilities of RCC cells, through inducing cell cycle G2/M arrest and apoptosis. IFN-γ could induce IRF8 expression in RCC cells, together with increased cleaved-PARP. We further found that IRF8 inhibited expression of oncogenes YAP1 and Survivin, as well as upregulated expression of tumor suppressor genes CASP1, p21 and PTEN. Collectively, our data demonstrate that IRF8 as a functional tumor suppressor is frequently methylated in RCC, and IRF8-mediated interferon signaling is involved in RCC pathogenesis.

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Original Articles

Introduction

Renal cell carcinoma (RCC) is the highest mortality rate of the genitourinary cancers [1], with a steadily increased incidence, which accounts for ~90% of kidney cancer cases in adults. Deciphering molecular mechanisms underlying RCC tumorigenesis is critical for developing better biomarkers for its early diagnosis and prognosis. The frequency of TSG methylation found in RCC (~10–30%), identifies alternative epigenetic abnormalities involving in renal tumorigenesis.

Epigenetic alterations, including promoter CpG methylation and histone modification, mediate oncogene activation and tumor suppressor gene (TSG) inactivation, thus contribute to tumorigenesis. Multiple TSGs with aberrant promoter methylation have been found in RCC, such as VHL, Ras association family 1A (RASSF1A), secreted frizzled-related protein 1 (SFRP1), cadherin 1 (CDH1) and p16 [8–12]. We have identified some TSGs silenced by promoter methylation in RCC, such as deleted in lung and esophageal cancer (DLEC1) and deleted in liver cancer 1 (DLC1), and DLEC1 could be a potential prognostic biomarker for RCC [13–15]. These findings not only uncover molecular heterogeneity of RCC, but provide an attractive strategy for RCC biomarker discovery. As relatively low frequency of TSG methylation found in RCC (~10–30%), identifying more novel TSGs inactivated by promoter methylation in RCC is needed.
Interferon regulatory factor 8 (IRF8), also known as interferon consensus sequence-binding protein (ICSBP), is a transcription factor of the interferon (IFN) regulatory factor (IRF) family [16], which as a central element of IFN-γ-signaling modulates multiple physiological processes [17–21]. Previous studies of IRF8 were mainly concentrated on cells of myeloid and lymphoid lineages. Recently, its expression and function in other types of human solid tumors have been demonstrated by us and others. Our previous studies showed that IRF8 was frequently silenced by promoter methylation in nasopharyngeal, esophageal, lung, colon and breast carcinomas, thus as a TSG [22]. However, its expression and biological function in RCC pathogenesis remain unclear.

In this study, we examined IRF8 expression and methylation in RCC cell lines and primary tumors, analyzed the relationship between its methylation and clinicopathological features in RCC patients. We also investigated its tumor suppressive function in RCC cells, including inhibition of clonogenicity and migration abilities, cell cycle arrest and apoptosis, as well as further identified its target genes.

**Table 1**
Association of clinicopathological features with IRF8 methylation status in renal cancer.

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**Fig. 1.** IRF8 silencing by promoter methylation in RCC cell lines. (A, B) Detection of IRF8 expression in human adult normal tissues and a panel of RCC cell lines by semi-quantitative RT-PCR. M, methylated; U, unmethylated. (C) Bisulfite genomic sequencing (BGS) analysis of the IRF8 promoter in HEK293 cell line and RCC A498 cell line. Circles, CpG sites analyzed; row of circles, an individual promoter allele that was cloned, randomly selected, and sequenced; filled circle, methylated CpG site; open circle, unmethylated CpG site.
Materials and methods

Patients and tissue samples

Forty-four primary RCCs and matched adjacent non-malignant renal tissues were obtained from the urology department, Peking University First Hospital, Beijing, China, from January 2005 to March 2006. All samples were harvested after obtaining patients’ written consent. Clinicopathological features of patients were listed in Table 1. All specimens were collected from primary surgical resection without prior history of RCC and adjuvant therapy. Primary RCC was defined by two urological pathologists. Only tumor samples with greater than 80% tumor cells were selected. The histopathology of tumors was classified by 2002 AJCC TNM stage and Fuhrman nuclear grade. Normal human adult tissue RNA samples were purchased commercially (Stratagene, La Jolla, CA, or Millipore-Chemicon, Billerica, MA).

Cell lines and drug treatment

RCC cell lines studied included A498, ACHN, Caki, Caki-2, HH050, HH244, RCC52, RCC98 and 786-O. The human normal embryonic kidney cell line HEK293 was used. These cell lines were cultured at 37 °C in 5% CO₂, and routinely maintained in RPMI1640 or DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Paisley, Scotland), 1% penicillin G and 1% streptomycin. Cell lines were treated with 10 μM 5-aza-2’-deoxycytidine (Aza) for 3 days, or further treated with 100 nmol/L trichostatin A (TSA) for additional 24 h. Cells were treated with 250 U/ml recombinant human IFN-γ (Millipore, Billerica, MA) for 24 h, then harvested for analysis.

DNA and RNA extraction

Genomic DNA and total RNA were extracted from tissues and cell lines using TRI Reagent, as previously described [15].

Semiquantitative RT-PCR and quantitative real-time PCR

Semiquantitative RT-PCR was used for detected gene expression with GoTaq (Promega, Madison, WI) and AmpliTaq Gold (Invitrogen, Carlsbad, CA). GAPDH was used as an internal control. The primers used were IRF8F: 5’-TCCGGATCCCTTGGAAACAC and IRF8R: 5’-CCTCAGGAACAATTCGGTAA, which performed for 36 cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s). Quantitative real-time PCR (qRT-PCR) was performed to screen IRF8 target genes, according to the manufacturer’s protocol (HT7500 system, Applied Biosystems). The primers of target genes were: CASP1F1: 5’-CAGGCCTGCCGTGGTGACAG and CASP1R1: 5’-CAGTGGTGGGCATCTGCGCT; p21F: 5’-CAGCAGGGACTCAGAGGA and p21R: 5’-CTCGCTGAATATCGACGCT; SurvivinF: 5’-AGCCCTTTCTCAAGGACCAC and SurvivinR: 5’-GCCCTTTAGAGACAAGAA; PTEF: 5’-GATATCAAGAGGATGGATTCG and PTEFR: 5’-GTGCGGCTTCCAGAAAT.

Bisulfite treatment and promoter methylation analysis

Genomic DNA bisulfite promoter methylation was performed as described previously [10,14]. Methylation specific PCR (MSP) and bisulfite genomic sequencing (BGS) were used to detect methylation status of IRF8 promoter. For MSP, the bisulfite-treated DNA was amplified with the primers, IRF8m1: 5’-ATTTCGGGGGATGCTGTTT, IRF8m2: 5’-CAGCTAAATCCAAAAACGACC and IRF8u1: 5’-GATTATTCGCTGGGTATGTGGTTT, IRF8u2:
5′-CTCACACCTAAAATCCAAAAACAACA. MSP was performed for 41 cycles using AmpliTaq Gold (Invitrogen, Carlsbad, CA). The BGS primers were IRF8BGS1: 5′-TTTTGAAGTTGGGATTTTTTTGTTT and IRF8BGS2: 5′-TAAAATCCRAACCTCTTCTAAAACC. The PCR products were cloned into the PCRA-Topo vector (Invitrogen, Carlsbad, CA), and 5–8 colonies were randomly chosen and sequenced.

Colony formation assay

Tumor cell clonogenicity was assessed by colony formation assay. pcDNA3.1-IRF8 plasmid was constructed as previously described [10]. Briefly, cells (1.5 × 10^5 per well) were plated in 12 well-plate and transfected with IRF8 expression construct or the empty vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfecants were replated in a 6-well plate after 48 h, and selected for 10–15 day with G418 (0.4 mg/ml). Visible colonies (≥50 cells) were counted after methanol fixation and gentian violet staining.

Western blot

Whole cell lysates were prepared with RIPA (Radio-Immunoprecipitation Assay) lysis buffer, supplemented with 1 mM Na3VO4, 1 mM NaF, 1X protease inhibitor and 1 mM PMFS before use. Cellular lysates were resolved using SDS-PAGE gels and transferred onto nitrocellulose membranes, which were incubated with primary antibodies overnight at 4°C followed by incubation with secondary antibody. Immunoreactive bands were visualized using Western blot Luminol reagent according to the manufacturer’s protocol. Antibodies used were: cleaved PARP (#9541), α-tubulin (#2144), goat anti-rabbit IgG-HRP (#7074), goat anti-mouse IgG-HRP (#7076) (Cell Signaling Technology Inc., Beverly, MA); IRF8 (C-19) (sc-6058), rabbit anti-goat IgG-HRP(sc-2922, Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Wound-healing assay

To assess the cell motility, a scratch wound-healing assay was used. The 2–3 × 10^5 cells were plated in 6-well plate 1 day before transfection so that cells would be 90–95% confluent at the time of transfection. The cell layers were carefully scratched using sterile tips and washed with PBS after 24 h of transfection. The cells were further incubated for 12 and 24 h, and caught photos. The assays were repeated in triplicate.

Statistical analysis

Statistical analyses were done using the two-tailed t-test, Fisher exact test or chi-square test to determine p-value, which are <0.05 considered significant.

Results

IRF8 is frequently downregulated by promoter methylation in RCC cell lines

We firstly examined IRF8 expression in a panel of normal adult tissues and RCC cell lines by semiquantitative RT-PCR. As shown in Fig. 1, IRF8 was widely expressed in normal tissues including kidney (Fig. 1A), and immortalized normal renal epithelial cell line HEK293, but frequently reduced or silenced in RCC cell lines (Fig. 1B), suggesting that IRF8 is a candidate TSG for RCC.

Fig. 3. Tumor suppressive functions of IRF8 in RCC cells. (A) IRF8 inhibits tumor cell growth by colony formation assay in A498 and 786-O cells. Quantitative analysis of colony numbers in three replicates are shown in the right as values of mean ± S.D, *p < 0.05. (B) RT-PCR showed IRF8 expression in IRF8- or vector-transfected cells, with GAPDH as a control. (C) Ectopically expressed IRF8 suppressed RCC cell migration in 786-O cells by wound healing assay. Pictures captured at 0, 12, or 24 h represented width of open wound compared with 0 h (**p < 0.01).
As both genetic and epigenetic alterations cause TSG silencing, we checked IRF8 gene mutation in COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/), and found that there is no point mutation reported in 475 RCC tumor samples tested. We next assessed whether promoter methylation is responsible for IRF8 downregulation in RCC cells. MSP analysis showed that IRF8 was methylated in 6 of 9 RCC cell lines, but rarely detected in immortalized normal kidney cell line HEK293 (Fig. 1B), correlated with its downregulation. To confirm MSP results, high-resolution bisulfite genomic sequencing (BGS) was performed to examine methylation status of 43 CpG sites within the IRF8 promoter. High density methylated alleles were detected in IRF8-silenced A498 (89% of methylated CpG sites) and 786-O cell lines (73% of methylated CpG sites), while more unmethylated alleles were detected in immortalized HEK293 cell line (Fig. 1C and Fig. 2B). These data indicated that IRF8 is frequently downregulated by promoter methylation in RCC cell lines. Pharmacological demethylation restored IRF8 expression in RCC cells

To check whether promoter methylation directly mediates IRF8 reduction in RCC, RCC cell lines with methylated and silenced IRF8 were treated with DNA methyltransferase inhibitor Aza, alone or combined with the HDAC inhibitor trichostatin A. Results showed that Aza treatment could restore IRF8 expression in RCC cells along with decreased methylated alleles, and the same results were observed in A + T treated-RCC cells (Fig. 2A). BGS analysis showed that the percentage of IRF8 methylation decreased from 73% to 36% in Aza-treated, and to 51% in Aza combined TSA-treated 786-O cells, further confirmed demethylation of the IRF8 promoter in representative RCC cells after demethylation treatment (Fig. 2B). These results indicate that promoter methylation directly leads to IRF8 silencing in RCC.

Fig. 4. IRF8 induced cell cycle G2/M arrest and apoptosis in RCC cells. (A) Increased numbers of IRF8-expressing A498 and 786-O cells in G2-M transition and reduced numbers of IRF8-expressing A498 and 786-O cells in S phase compared with controls. **p < 0.01. (B) Western blot showed that IFN-γ enhanced cleaved-PARP expression in vector-, IRF8-transfected 786-O and HEK293 cells. α-tubulin was used as a control. *Non-specific band. Densitometry analysis of western blots showed quantitation of cleaved-PARP levels. **p < 0.01.
Frequent IRF8 methylation in primary RCC tumors is associated with poor prognosis

We further examined IRF8 methylation in primary RCC samples and their adjacent non-malignant renal tissues. Results showed that IRF8 methylation was detected in 25% (11/44) of RCC tumors, but not in adjacent non-malignant renal tissues (Fig. 2C), indicating tumor-specific methylation of IRF8 in RCC. We further analyzed the correlation between IRF8 methylation and clinicopathological features of RCC patients. As shown in Table 1, IRF8 methylation was significantly associated with higher tumor grade of RCC (G1 vs G2, p = 0.001), while no significant correlation was found between its methylation and gender, tumor location and TMM stage. These data suggest that IRF8 methylation is frequent event in RCC pathogenesis and is associated with poor prognosis.

Ectopic expression of IRF8 inhibited the abilities of RCC cell clonogenicity and migration

Frequent silencing of IRF8 by promoter methylation in RCC suggested that it was likely a tumor suppressor. Thus, tumor suppressive functions of IRF8 were assessed by colony formation and wound-healing assays. IRF8-expressing vector and its empty vector were transfected to IRF8-silenced A498 and 786-O RCC cells. Semiquantitative RT-PCR confirmed its expression (Fig. 3B). Compared with controls, colonies were significantly reduced in IRF8-expressing A498 and 786-O cells (Fig. 3A). Furthermore, the effect of IRF8 on cell migration was examined by scratch wound healing assay. Results showed that IRF8-expressing 786-O cells closed the wound slower than controls (Fig. 3C). These data indicate that IRF8 inhibits tumor cell clonogenicity and migration thus as a functional TSG in RCC.

IRF8 induced cell cycle G2/M arrest and apoptosis of RCC cells

We next examined the effects of cell cycle and apoptosis induced by IRF8 on RCC cells. Flow cytometry analysis showed ~7–25% of cells dramatically increased in G2/M phase, and ~3–13% of cells significantly reduced in S phase in IRF8-expressing A498 and 786-O cells, compared with controls (Fig. 4A), indicating that IRF8 causes cell cycle G2/M arrest in RCC cells.

We then checked the change of classical apoptotic marker cleaved-PARP by IRF8. Western blot showed obviously upregulated cleaved-PARP expression in IRF8-expressing 786-O and HEK293 cells (Fig. 4B). We further treated cells with IFN-γ for 24 h, and found that IFN-γ could enhance the expression of IRF8 and cleaved-PARP in IRF8-expressing 786-O and HEK293 cells (Fig. 4B). These results suggest that IFN-γ promotes IRF8-induced apoptosis in RCC cells.

Downstream target genes of IRF8 in RCC cells

IRF8 as a transcription factor regulates multiple downstream target genes. To investigate possible target genes of IRF8 in RCC cells, we screened several genes involved in cell proliferation and apoptosis by qRT-PCR. Results showed that ectopic expression of IRF8 significantly downregulated the expression of oncopgenes, MYC and Survivin, as well as upregulated TSGs, p21, PTEN and CASP1 in RCC A498 and 786-O cells (Fig. 5A–C), and enhanced CASP1 expression in immortalized HEK293 cells with or without IFN-γ induction (Fig. 5D).

Discussion

Most of RCCs are diagnosed in the advanced metastatic stage, resulting in dramatic decrease of patient survival. Thereby, early detection and monitoring of this disease may improve prognosis and treatment results. DNA methylation is recognized as a hallmarker of cancer, which helps to uncover the molecular mechanisms underlying tumor development and provide molecular screening strategies to identify cancer-specific diagnostic and prognostic tools.

In this study, we identified IRF8 as a functional TSG regulated by promoter methylation in RCC, which could serve as a potential prognostic biomarker in RCC. Like other IRFs, IRF8 contains a conserved DNA-binding domain in its N-terminal, and regulates gene expression stimulated by IFNs or other genes, further mediating apoptosis in tumor cells, through binding to the IFN-stimulated response element (ISRE) [23]. According to the analysis of human cancer genomics database, IRF8 is significantly focally deleted across the entire dataset of 3131 tumors, which suggested that IRF8 is likely a tumor suppressor in human cancers (http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf). Studies have shown that IRF8 expression was frequently reduced in hematopoietic cells of human myeloid leukemia patients. IRF8 mediated apoptosis and further suppressed myelogeneous leukemia, thus as a myeloid leukemia suppressor [24–26]. In addition, IRF8 silencing by promoter methylation has been reported in other multiple nonhemopoietic carcinomas including nasopharyngeal, esophageal, gastric and colon cancers by ours and others [22,27–29], suggesting that it could be an epigenetic biomarker for molecular diagnosis and prognosis prediction of these tumors [30]. IRF8 has also been found to play a critical role in histone deacetylase inhibitor-mediated antitumor activity [31].

We found that IRF8 is frequently silenced by promoter methylation in RCC cell lines, but not in normal kidney tissue and human embryonic kidney HEK293 cells. IRF8 methylation was further detected in RCC samples, but not in adjacent non-malignant renal tissues, indicating that IRF8 methylation is a tumor-specific event involved in RCC tumorigenesis. Moreover, IRF8 methylation was associated with higher nuclear grade, indicating its potential as prognostic predictor of RCC. Of note, we couldn't detect IRF8 protein expression in HEK293 cells, which is consistent with other reports about its expression in HEK293 cells [32], although it showed relatively high mRNA expression level. Emerging evidence has demonstrated that mRNA level of a gene cannot necessarily predict its protein level [33,34], thus further investigation of IRF8 protein expression and modulation in RCC tumorigenesis is needed.

IRF8 as an essential mediator of Fas-mediated apoptosis mediates apoptosis especially in metastatic tumor cells [28,35]. We further found that IRF8 exerts tumor suppressive function in RCC through inhibiting cell proliferation, migration and inducing apoptosis. IFN-γ induced apoptosis in RCC cells, especially in IRF8-expressing renal carcinoma and immortalized HEK293 cells, indicating that IFN-γ promotes IRF8-mediated apoptosis in RCC pathogenesis, in line with other studies. Recent studies showed that HEK293 cell line has the tumorigenic potential [36], thus our data suggest that IRF8 may play a role in early stage of RCC tumorigenesis.

IRF8 as a transcription factor exerts its tumor suppressive function through upregulating TSGs and downregulating oncogenes. It reported that Fas, Bax, FLIP, and STAT1, which played important roles in cell apoptosis, have been identified as target genes of IRF8 in non-hematopoietic cancer cells [28,35,37]. In myeloid cells, IRF8 regulates the expression of Bax and Fas to regulate apoptosis [38]. Here, we found that IRF8 could repress oncogenes (MYC and Survivin) and upregulate TSGs (p21, PTEN and CASP1), which was involved in multiple tumor processes, such as cell proliferation, apoptosis and migration.

In summary, we found that IRF8 is a functional TSG in RCC, its frequent methylation is associated with tumor poor prognosis in RCC, and could serve as a potential prognosis prediction for RCC.
Conflict of Interest

The authors have nothing to disclose.

Acknowledgements

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References


Fig. 5. (A, B) A panel of target genes expression examined by quantitative real-time PCR in vector-, IRF8- transfected 786-O and A498 cells. *p < 0.05. (C) Detection of YAP1 and Survivin expression by semi-quantitative RT-PCR in vector-, IRF8-transfected 786-O and A498 cells. (D) A panel of target genes expression examined by quantitative real-time PCR in vector-, IRF8- transfected HEK293 cells with or without treatment with IFN-γ. *p < 0.05.