miR-502-mediated histone methyltransferase SET8 expression is associated with clear cell renal cell carcinoma risk

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Abstract. Genetic variants may affect the interactions between microRNAs (miRNAs/miRs) and their target genes by modulating their binding affinity or by creating, or destroying a miRNA-binding site. SET domain containing (lysine methyltransferase) 8 (SET8) is the sole lysine methyltransferase that catalyzes the monomethylation of histone H4 lysine 20, and is associated with tumor growth, invasion and metastasis. In the present study, the rs16917496 polymorphism within the miR-502 binding site of the SET8 mRNA 3’ untranslated region (3’UTR) in patients with clear cell renal cell carcinoma (ccRCC) and healthy controls was genotyped. The SET8 CC genotype was associated with a decreased ccRCC risk compared with the CT [P=0.003; odds ratio (OR)=0.318; 95% confidence interval (CI), 0.186-0.736] genotypes. The CC genotype was associated with a decreased ccRCC risk compared with the CT [P=0.003; odds ratio (OR)=0.318; 95% confidence interval (CI), 0.186-0.736] genotypes. The SET8 CC genotype was associated with a decreased ccRCC risk compared with the CT [P=0.003; odds ratio (OR)=0.318; 95% confidence interval (CI), 0.186-0.736] genotypes. The SET8 CC genotype was associated with a decreased ccRCC risk compared with the CT [P=0.003; odds ratio (OR)=0.318; 95% confidence interval (CI), 0.186-0.736] genotypes. The SET8 CC genotype was associated with a decreased ccRCC risk compared with the CT [P=0.003; odds ratio (OR)=0.318; 95% confidence interval (CI), 0.186-0.736] genotypes. The SET8 CC genotype was associated with a decreased ccRCC risk compared with the CT [P=0.003; odds ratio (OR)=0.318; 95% confidence interval (CI), 0.186-0.736] genotypes.

Introduction

Renal cell carcinoma (RCC) occurs in 2-3% of adult malignancies, with ~337,860 new cases and 143,406 mortalities worldwide in 2012 (1). The global incidence of RCC increased gradually between 1997 and 2007 (2). In China, there are ~66,466 cases of RCC annually, making it the third most prevalent genitourinary cancer (3). Several risk factors have been associated with RCC, including smoking, alcohol use, hypertension and obesity (4). However, numerous individuals exposed to these risk factors in their lifetime do not develop RCC. Accumulating evidence has suggested that genetic and environmental factors perform important roles in the development of RCC (5). MicroRNAs (miRNAs/miRs) have recently been implicated in RCC tumorigenesis (6,7).

miRNAs are a class of small, single-stranded, noncoding RNAs that modulate gene expression by binding to the 3’ untranslated region (3’UTR) of their target genes, causing translational suppression and/or mRNA degradation (8,9). Accumulating evidence has suggested that miRNAs perform important roles in a broad range of biological processes, including cellular proliferation, apoptosis, differentiation and cancer development (10). To regulate mRNA and protein expression levels, miRNAs bind to the 3’UTR of their target mRNAs. Thus, single nucleotide polymorphisms (SNPs) in the 3’UTR may impede existing binding sites or create novel binding sites, resulting in the misregulation of target genes, which may affect the tumor risk of an individual (11,12). SET domain containing (lysine methyltransferase) 8 (SET8; also termed PR-Set7, SETD8 or KMT5A) is a SET domain-containing methyltransferase family member, and is modulated by miR-502 binding to its 3’UTR (13,14). SET8 encodes a histone H4 lysine 20 monomethyl transferase that has been implicated in modulating cell cycle progression and development (14-16). It was previously reported that SET8 is recruited to DNA replication foci through its interaction with proliferating cell nuclear antigen and is required for proper DNA replication (17,18). The precise modulation of SET8 levels is important for proper cell cycle progression, and the inability to modulate SET8 expression results in severe cell cycle defects (17-20). Previous studies have revealed that SET8 is highly expressed in several types of tumors, including breast cancer (21), small cell lung cancer (22), hepatocellular
carcinoma (11) and ovarian cancer (23). Furthermore, SET8 expression levels were identified to be associated with the rs16917496 SNP in the SET8 3'UTR (21).

In the present study, the rs16917496 SNP in patients with ccRCC in a case-control study was genotyped to assess its association with the risk of cancer. In addition, the association between this SNP and SET8 expression, and the roles of SET8 in renal carcinoma 786-O cell proliferation, migration and invasion were examined using RNA interference.

Materials and methods

Tissue specimens and DNA extraction. Blood samples were obtained from 140 patients with ccRCC, which were all treated at The Fourth Affiliated Hospital of Hebei University (Hebei, China) between December 2006 and December 2010. The ccRCC patients included 59 males and 81 females, mean age 56.8 years (range, 36-79 years). Blood samples were also acquired from age-matched healthy controls. Total DNA was extracted using a Wizard Genomic DNA Extraction kit (Promega Corporation, Madison, WI, USA; cat. no. SC-47778A). Membranes were blocked in TBS-Tween-20 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. SC-5200) for 1 h at room temperature and paraffin-embedded. The thickness of tissue. RCC tissues were fixed in 10% formalin overnight at room temperature and paraffin‑embedded. The thickness of the RCC tissue sections was 5 µm. The primary antibody against SET8 (Abcam, Cambridge, UK; cat. no. ab3798) was applied to sections at a dilution of 1:100 overnight at 4°C, and sections were subsequently incubated with a biotinylated secondary antibody at a dilution of 1:10,000 (ProteinTech Group, Inc., Chicago, IL, USA; cat. no. SA00003-1) for 1 h at room temperature. The sections were then incubated with horseradish peroxidase (HRP)‑conjugated streptavidin (Origene Technologies, Inc., Beijing, China; cat. no. K156617 J) at 37°C for 30 min and developed using 3,3-diaminobenzidine.

Stained slides were scored by two independent experienced pathologists without knowledge of the patients' clinical data. Immunostaining results were semi-quantified using HSCORE as reported previously (24,25) under a light microscope at magnification of ×200. Briefly, the score was calculated based on estimates of the percentage of positively stained renal cells in each intensity category (0, 1+, 2+, 3+, 4+). The intensity of staining of the antibody was analyzed by HSCORE. The HSCORE was calculated using the following equation: HSCORE=(i+1) π, where i=1, 2, 3 or 4, and π varies between 0 and 100%. A score of >100% was defined as high expression and ≤100% was defined as low expression (Fig. 1).

Cell culture and transfection. The renal carcinoma 786-O cell line was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 mg/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a standard humidified incubator containing 5% CO₂.

The renal carcinoma 786-O cells were transfected with psi-H1-SET8 small interfering (si)RNA or psi-H1 plasmids (GeneCopoeia, Inc., Rockville, MD, USA) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The target sequences of four siRNAs against SET8 were as follows: SET8-siRNA1, 5'-CAGAUAUCAGGAAACCUCUACCC AG-3'; SET8-siRNA2, 5'-GAAUGAGAAUAGUCCUCUA UCG-3'; SET8-siRNA3, 5'-GCCUAGGAAGACUGAUCAU AU-3'; and SET8-siRNA4, 5'-GGCCGCUCACUGAGUUGA UG-3'. Successful knockdown of SET8 was confirmed by western blot analysis using an anti-SET8 antibody (Abcam; cat. no. ab3798).

Western blot analysis. Radioimmunoprecipitation assay lysis buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, Ph 7.9, 10 mM NaF, PMSF, and 1X protease inhibitors (Roche Diagnostics, Basel, Switzerland) was used to isolate total protein from all the experimental renal carcinoma 786-O cells. The Bradford assay was used to determine protein concentration. Western blot analysis was performed as described previously (26). Briefly, 40 µg of total protein for each lane was separated on a 10% denaturing polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Roche Diagnostics). Immunoblots were probed with a mouse monoclonal anti-SET8 antibody at 1:500 (Abcam; cat. no. ab3798) or β-actin at 1:20,000 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. SC-47778A). Membranes were blocked in TBS-Tween-20 containing 5% nonfat dry milk for 1 h at room temperature and incubated overnight with primary antibody at 4°C. A HRP-conjugated anti-mouse IgG antibody was used as the secondary antibody (ProteinTech Group, Inc, Chicago, IL, USA; cat. no. SA00003-1) for 2 h at room temperature. Signals were detected using FluorChem® HD2 (Alpha-InnoTec, San Leandro, CA, USA).
RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cultured cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) one-step method according to the manufacturer's protocol. RNA (2 µg) was used for reverse transcription to synthesize template cDNA using RevertAid First-Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

GAPDH gene was used as an endogenous control. The primer sequences are listed in Table I. PCR was performed using the Quantstudio™ 3kD PCR instrument (Thermo Fisher Scientific, Inc.) and iQ™ SYBR-Green Super mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), which contained 5 ng cDNA and 10 pM of each primer. PCR reaction started with 1 cycle of 95°C for 10 min, followed by 40 cycles of three steps as 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec. The PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide staining, and the data were normalized to the endogenous control gene GAPDH using the 2^ΔΔCq method (27).

Cell proliferation assay. Cell proliferation was analyzed by MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) assay as described previously (26). Cells were seeded in sextuplicate at 1x10^4 cells/well on 96-well microplates and transfected with SET8 siRNA-2 or negative control plasmids using Lipofectamine 2000. The cells were incubated with 100 µl MTT (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) for 4 h at 37°C after various time periods of SET8-knockdown (0, 12, 24, 36, and 48 h). Following centrifugation (room temperature, 10 min at 1,000 x g), 100 µl of 0.04 mol/l HCl-isopropanol was added to the cells. The absorbance was measured at 490 nm using an ELISA microplate reader. The experiment was repeated three times.

Colony formation assay. The renal carcinoma 786-O cells were seeded on 6-well plates at a density of 500 cells/well at 48 h post-transfection. After 10 days at 37°C in 5% CO₂, the cells were fixed with 95% methanol and stained with 0.1% crystal violet for 15 min at room temperature. Colonies of >50 cells were scored under a light microscope at a magnification of x100. The colony formation ratio (%) was calculated as follows: (Number of cell colonies/500) x100.

Wound healing assay. At 48 h post-transfection, the cell monolayer was scraped in a straight line using a 200-µl pipette tip to create a scratch once they reached 100% confluence. The medium was removed and the cells were washed twice in PBS. Wound healing results were observed under a light microscope at a magnification of x100. Images were captured at 0 and 12 h after scratching. At least five fields were analyzed for each scratch, and the migration index was calculated as the width of a scratch divided by the initial width of the same scratch, as previously described (28).

Cell invasion assay. Cell invasion assays were performed using a Transwell assay (pore size, 8 µm; Corning Incorporated, Corning, NY, USA). The insert was coated with 30 µl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) mixed with RPMI-1640 serum-free medium in a 1:5 dilution for 30 min at 37°C. At 24 h post-transfection, 100,000 cells were resuspended in 100 µl serum-free medium and plated in the upper chamber. The bottom chamber was filled with 500 µl RPMI-1640 containing 10% FBS. Following incubation for 24 h at 37°C, the upper chamber was removed, and non-penetrating cells were gently wiped away. The remaining cells were stained with 0.1% crystal violet for 15 min at room temperature and counted under a light microscope (Axio Observer D1, Gemney) in five representative areas at 400x, irrespective of staining intensity or cell number. The experiment was repeated three times. The cell invasion inhibition rate (%) = [1-(the number of invasive cells in the experimental group / the number of invasive cells in the control group)] x100.

Statistical analysis. Data are presented as the mean ± standard deviation. A χ² test was used to analyze dichotomous values. The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression model. Student's t-test was performed to analyze results of MTT, colony formation, migration and invasion assays. All statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered statistically significant for all statistical tests.

Results

SET8 genotype is associated with ccRCC risk. A total of 140 patients with ccRCC and 130 controls were genotyped for the rs16917496 SNP. The SET8 CC, CT and TT genotype frequencies in patients with ccRCC, and control samples were 14, 47 and 79 and 30, 32 and 68, respectively. The distribution of the rs16917496 genotype followed a Hardy-Weinberg equilibrium. The overall frequencies and genotype distributions of the rs16917496 polymorphism in patients with ccRCC and controls are presented in Table II. The C allele frequencies of rs16917496 in patients with ccRCC (26.79%) were significantly

| Gene | Primer sequence | Amplicon, bp |
|------|-----------------|-------------|
| c-Myc | F: 5'-CCTACCCCTCTCAAC GACGC-3' | 179 |
| | R: 5'-TTCCTCCTAGAGT CGCTGC-3' |
| MMP-7 | F: 5'-TTGGAAACAGGA GCTC AGGACTAT-3' | 413 |
| | R: 5'-AAATGGCTAGGATC CCCATGA-3' |
| GAPDH | F: 5'-CAAAGGTCATCCATGA CAACTTGTG-3' | 496 |
| | R: 5'-GTCCACCACTGTC TGCTGTAG-3' |

MMP-7, matrix metalloproteinase-7; F, forward; R, reverse.
lower compared with that in controls (35.38%) (P=0.031), and the presence of the C allele significantly decreased the risk of developing ccRCC (OR=0.668; 95% CI, 0.463-0.964).

The CC genotype was associated with a decreased risk of ccRCC compared with the CT (P=0.003; OR=0.318; 0.146-0.691), TT (P=0.011; OR=0.402; 0.197-0.819) and CT+TT (P=0.004; OR=0.370; 0.186-0.736) genotypes.

Effects of rs16917496 SNP on SET8 expression. To identify the association between the rs16917496 SNP and SET8 expression, SET8 expression was measured by immunostaining in 140 ccRCC tissues. All samples were analyzed for SET8 staining and an HSCORE was calculated. Patients with the SET8 CC genotype had lower SET8 expression compared with patients with the CT (χ²=4.238; P=0.038) or TT (χ²=5.741; P=0.017) genotypes (Table III).

Associations between SET8 expression and clinicopathological variables. SET8 was diffusely distributed throughout the nucleus of ccRCC tumor cells, as determined by immunostaining (Fig. 1B). Among all of the samples analyzed, 99 cases (70.7%) demonstrated high SET8 protein expression, while 41 samples (29.3%) exhibited low expression. In addition, the χ² test was used to assess the association between SET8 protein expression and various clinicopathological variables (Table IV). Notably, low SET8 protein expression was negatively associated with ccRCC Tumor-Node-Metastasis (TNM) (29) staging (P=0.002), tumor size (P=0.039) and lymph node metastasis (P=0.014). However, no significant association was observed between SET8 expression and age or gender (P>0.05). These results demonstrated that SET8 expression is highly induced in human ccRCC, indicating a potential role for SET8 in ccRCC development and progression.

SET8-knockdown inhibits proliferation, colony formation, migration and invasion of renal carcinoma 786-O cells. For SET8-knockdown, four psi-H1-SET8 siRNAs were transfected into renal carcinoma 786-O cells. As presented in Fig. 2, SET8 siRNA2 in the psi-H1 plasmid markedly reduced SET8 protein levels compared with the other SET8

Table II. Association between the rs16917496 single nucleotide polymorphism and clear cell renal cell cancer risk.

| Type     | Case, n (%) | Controls, n (%) | χ²   | P-value | OR   | 95% CI   |
|----------|-------------|----------------|------|---------|------|----------|
| Genotype |             |                |      |         |      |          |
| CC       | 14 (10.0)   | 30 (23.1)      | 8.659| 0.003a  | 1.00 |          |
| CT       | 47 (33.6)   | 32 (24.6)      | 6.515| 0.011b  | 0.402| 0.197-0.819|
| TT       | 126 (90.0)  | 100 (76.9)     | 8.451| 0.004c  | 0.370| 0.186-0.736|
| CT+TT    | 126 (90.0)  | 100 (76.9)     | 6.515| 0.011b  | 0.402| 0.197-0.819|
| Allelotype|             |                |      |         |      |          |
| C        | 75          | 92             |      |         | 1.00 |          |
| T        | 205         | 168            | 4.666| 0.031d  | 0.668| 0.463-0.964|

aComparison between CC and CT genotype; bcomparison between CC and TT genotype; ccomparison between CC and CT+TT genotype; dcomparison between C and T allele. OR, odds ratio; CI, confidence interval.
Therefore, the SET8 siRNA2 construct was selected for subsequent analyses.

SET8 was reported to be associated with the development of several tumors (23,30,31). So, we next examined whether SET8-knockdown affects renal carcinoma 786-O cell proliferation. Renal carcinoma 786-O cells were transfected with psi-H1-SET8 siRNA, psi-H1 (empty vector) or blank control and MTT assays were performed to determine the proliferation capacity of the cells. Compared with the psi-H1-transfected cells and blank control cells, the proliferation rate of renal carcinoma 786-O cells was significantly decreased between 36 and 72 h following SET8 siRNA-2 transfection (P<0.05; Fig. 3). The effects of SET8-knockdown on cell colony formation were studied in vitro. As presented in Fig. 4, SET8-knockdown significantly inhibited colony formation as compared with empty psi-H1 or the blank control. To investigate the underlying mechanisms by which SET8 regulates proliferation and colony formation, RT-PCR was performed to examine the expression of proliferation-associated genes. Compared with cells transfected with empty psi-H1, c-Myc mRNA levels significantly decreased upon SET8 knockdown (Fig. 5). These results indicated that SET8-knockdown inhibits renal carcinoma 786-O cell proliferation and colony formation. It was speculated that SET8-knockdown suppresses cell growth by decreasing c-Myc mRNA expression.

To determine whether SET8 affects cell migration or invasion, wound healing and Transwell assays were performed, respectively. As presented in Fig. 6, SET8-knockdown

**Table IV. Association between SET8 expression and clear cell renal cell carcinoma clinicopathological features.**

| Characteristics                        | No. of cases | Low | High | χ²   | P-value |
|----------------------------------------|--------------|-----|------|------|---------|
| Age, years                             |              |     |      |      |         |
| <55                                    | 57           | 19  | 38   | 0.761| 0.383   |
| ≥55                                    | 83           | 22  | 61   |      |         |
| Gender                                 |              |     |      |      |         |
| Male                                   | 59           | 20  | 39   |      |         |
| Female                                 | 81           | 21  | 60   |      |         |
| TNM classification                     |              |     |      |      |         |
| I                                      | 96           | 36  | 60   | 9.952| 0.002   |
| II+III+IV                              | 44           | 5   | 39   |      |         |
| Size of tumor (diameter, cm)           |              |     |      |      |         |
| <5                                     | 95           | 33  | 62   | 4.241| 0.039   |
| ≥5                                     | 45           | 8   | 37   |      |         |
| LN metastasis                          |              |     |      |      |         |
| Negative                               | 99           | 35  | 64   | 6.010| 0.014   |
| Positive                               | 41           | 6   | 35   |      |         |

LN, lymph node; TNM, tumor-node-metastasis; SET8, SET domain containing (lysine methyltransferase) 8.
significantly decreased cell migration capacity compared with cells transfected with empty vector or blank control cells (P<0.05). Cell invasion was examined using Transwell assays and it was revealed that SET8-knockdown markedly decreased cell invasiveness compared with cells transfected with empty vector or blank control cells (P<0.05; Fig. 7). To determine the mechanisms by which SET8 regulates invasion and migration, RT-PCR was performed. Compared with cells transfected with control-siRNA, SET8-knockdown cells exhibited significantly decreased matrix metalloproteinase-7 (MMP-7) levels (Fig. 5). These results indicated that SET8-knockdown inhibited cell migration and invasion. It was suggested that SET8-knockdown suppresses cell migration and invasion by decreasing MMP-7 mRNA expression.

Discussion

In the present study, the association between the rs16917496 SNP in the miR-502 binding site of the SET8 3’UTR and SET8 expression was assessed, and its implications in ccRCC development were investigated. Logistic regression analysis revealed that the rs16917496 SNP was associated with ccRCC risk. Therefore, the association between the rs16917496
SNP and SET8 expression was examined. Consistent with the previous study by Song et al (21), the present study revealed that the SET8 CC genotype was associated with low SET8 protein expression. Furthermore, the association between SET8 expression and clinicopathological features was explored, and it was revealed that SET8 expression was associated with TNM staging, tumor size and lymph node metastasis. Finally, SET8-knockdown inhibited proliferation and invasion of renal carcinoma 786-O cells, potentially through Wnt/β-catenin signaling. The present data suggested that altering SET8 expression, at least partially by the rs16917496 SNP in the miR-502 binding site of the SET8 3'UTR, was associated with ccRCC development and progression. SET8-knockdown inhibited renal carcinoma 786-O cell proliferation, migration and invasion potentially through Wnt/β-catenin signaling.

Accumulating evidence has suggested that polymorphisms within miRNA-binding sites may affect miRNA regulation of target gene expression and consequently modify cancer risk and outcome (11,32-35). The rs16917496 SNP in the miR-502 binding site of SET8 has been associated with several tumor types, including hepatocellular carcinoma (10), small cell lung cancer (20) and non-Hodgkin’s lymphoma (30). Consistent with the study by Jørgensen et al (18), the results of the present study revealed that the CC genotype was associated with low protein expression and low ccRCC risk. The present results also revealed that SET8 expression was associated with TNM staging, tumor size and lymph node metastasis of patients with ccRCC. These data demonstrated that the rs16917496 SNP in the miR-502 binding site of SET8 mediated SET8 expression and consequently modified ccRCC cancer risk.

As a methyltransferase, SET8 may regulate several signaling pathways by modulating protein lysine methyltransferases. Of note, the major signaling pathways affected by SET8 are Wnt/β-catenin signaling. Therefore, SET8 may modify cancer development and stability through Wnt/β-catenin target genes c-Myc and MMP-7 in renal carcinoma 786-O cells. However, expression of other Wnt target genes, including Axin2, nked cuticle homolog 1 and lymphoid enhancer-binding factor 1, was not detected. The full mechanism of SET8 regulation of renal carcinoma cell proliferation and invasion and its associated signaling pathways should be explored further.

The present results indicated that SNPs of a miRNA binding site were associated with ccRCC risk, but the results require validation in other populations and in laboratory-based functional studies. SET8 may modify cancer development and progression through its effects on proliferation and invasion, potentially via Wnt/β-catenin signaling. Therefore, SET8 may be a novel target for ccRCC therapy.

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