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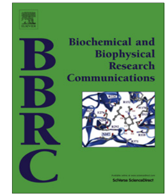

Aberrant methylation and loss of *CADM2 tumor suppressor* expression is associated with human renal cell carcinoma tumor progression

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ABSTRACT

Cell adhesion molecules (CADMs) comprise a protein family whose functions include maintenance of cell polarity and tumor suppression. In this report, we show that the *CADM2* gene is repressed in human clear renal cell carcinoma by DNA promoter hypermethylation and/or loss of heterozygosity. Moreover, the loss of *CADM2* expression is associated with a higher tumor pathology stage ($p < 0.05$). The re-expression of *CADM2* in the renal cancer cell line 786-O significantly suppressed tumor cell growth *in vitro* and in mouse xenografts by a G1 phase cell cycle arrest and the induction of apoptosis. Lentivirus-mediated *CADM2* expression also significantly suppressed cancer cell anchorage-independent growth and invasion. Furthermore, the inhibition of endogenous *CADM2* expression using siRNAs induced a tumorigenic phenotype in polarized non-tumorigenic MDCK cells. Thus, we conclude that *CADM2* functions as a novel tumor suppressor and may serve as a potential therapeutic target for human renal cell carcinoma.
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1. Introduction

Renal cancer accounts for approximately 2% of all cancers worldwide, and more than 200,000 new cases of renal cancers are diagnosed annually [1]. Approximately, 75% of renal cancers are classified as clear renal cell carcinoma (cRCC) [2]. Although surgery and targeted therapies improve the treatment of cRCC, the prognosis of cRCC remains poor [1]. Accurate prognostic tests as well as more effective treatment strategies require a better understanding of the molecular events that underlie cRCC progression [3].

Recent studies suggest that the expression of cell adhesion molecules (CADMs), a protein family that may have a role in cancer progression, is lost as a result of promoter hypermethylation [4,5]. Moreover, hypo-expression of *CADM2* gene expression has been observed in prostate cancer [6], ovarian cancer [7], lymphoma and melanoma [8,9]. Thus, this study sought to characterize *CADM2* expression in cRCC. Here, we showed that *CADM2* expression was reduced in cRCC, and re-expression suppressed cancer cell tumorigenicity *in vitro* and *in vivo*, implicating that *CADM2* is a novel tumor suppressor candidate in cRCC.

2. Materials and methods

2.1. Cell lines and tissue samples

The human renal cancer cell lines, 786-O, OS-RC-2 and CAKI-1, were purchased from the National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China), and maintained in RPMI1640 or McCoy's 5A containing 10% GIBCO FBS (Carlsbad, CA) with 1% antibiotics. HEK-293 cells were grown in DMEM/HG supplemented with FBS and antibiotics. The human kidney proximal tubular epithelial cells, HK-2, was purchased from ATCC (Manassas, VA) and cultured in KSF medium with bovine pituitary extract and epidermal growth factor (Invitrogen, Carlsbad, CA). All cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

Primary tumor tissues (T), paired adjacent normal kidney tissues (N) specimens and clinical data were obtained from 61 histo-

Abbreviations: CADMs, cell adhesion molecules; cRCC, clear renal cell carcinoma; RT-qPCR, reverse transcription quantitative polymerase chain reaction; MSP, methylation-specific polymerase chain reaction; BSP, bisulfite sequencing polymerase chain reaction.

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logically classified cRCC patients that received radical nephrectomies or partial renal resection in the Urology Department of the Peking University First Hospital. Informed consent was obtained from all patients, and this study was approved by the institutional review board of the Peking University First Hospital. The pathological stage was assessed by two experienced urological pathologists, according to the AJCC 2009 classification system.

2.2. Peptides synthesis and antibody generation

Peptides synthesis and antibody used for Immunohistochemistry and Western blot analysis were carried out as previously described [6]. The following synthetic peptide corresponding to amino acids 426–444 of CADM2 were produced: COOH-terminal CIINAEGSQVNAEEKKEYFI. After conjugation to keyhole limpet hemocyanin (KLH), the resulting antibodies were affinity purified using the immunizing peptide (Research Genetics, Huntsville, AL).

2.3. Immunohistochemistry and Western blot analysis

The immunohistochemistry was carried out as previously described [6]. Tissue sections were stained using a DAKO Immunohistochemistry Kit (DAKO, Carpinteria, CA) and visualized using an Olympus microscope. Protein lysates were prepared by homogenization in RIPA lysis buffer containing PSFM and 30 μg was separated by SDS–PAGE. The immunoreactive bands were visualized by Immobilon™ Western Kit (Millipore, Billerica, MA) using SYNGENE G:BOX imaging system (Frederick). Semi-quantification of the bands was performed using ImageJ software.

2.4. Quantitative real-time PCR (qPCR)

Total RNA from cultured cells and frozen specimens was isolated using TRIZOL reagent (Invitrogen), and 2 μg total RNA was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI). qPCR was performed using the Applied Biosystem 7500-fast with SYBR Green PCR Mix (Roche, Indianapolis, IN). Each reaction was performed in triplicate with each primer sets (Supplementary Table 1). *CADM2* expression was normalized to human *β-Actin* expression using the ΔΔCT method.

2.5. Methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP)

Genomic DNA was extracted using a DNA extraction kit (Qiagen, Gaithersburg, MD); 1 μg was used for bisulfite treatment as previously described [6]. Ten ng modified genomic DNA was amplified with MSP primer sets (Supplementary Table 1) using a Hotstart PCR Mix (Promega) and the following reaction conditions: 94 °C for 6 min; 40 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s; and 72 °C for 5 min. PCR products were analyzed in 1.5% agarose gels stained with ethidium bromide. The BSP reaction was performed as previously described [6] using primers in Supplementary Table 1. The PCR products were purified and cloned into PCR2.1-TA cloning vector (Invitrogen), and a minimum of six positive clones from each product were selected for sequencing.

2.6. Demethylation analysis

786-O cells were seeded in six-well plates at a concentration of 1×10^5 cells per well, grown for 24 h, and then treated with 0, 2, 5, or 10 μM 5-Aza-2′-deoxycytidine (5-Aza-dC, Sigma, St. Louis, MO) for 4 days. Cells were cultured with or without 300 nM Trichostatin A (Sigma) for the final 24 h. RNA was isolated for RT-PCR analysis, and DNA was extracted for *CADM2* MSP.

2.7. Loss of heterozygosity (LOH) analysis

Genomic DNA (50 ng) from the paired tumor and adjacent normal tissues were amplified by PCR for LOH analysis as previously described [10], using fluorescent-labeled primers (Supplementary Table 2). Five human polymorphic short tandem sequence markers within a 17 Mb region at 3p12.1–3 around the *CADM2* were chosen. Molecular size and peak height were analyzed with Genetic Profiler v.1.0 software. LOH was determined using the calculation method described by Powell et al. [11], which defined LOH using a normalized allele ratio $\leqslant$0.6.

2.8. Construction of CADM2-expressing lentivirus

Previous report showed that the *CADM2* gene encodes several mRNA isoforms [6], including *CADM2* missing exon 8 isoform (*CADM2-M8*) and the full-length isoform (*CADM2-FL*). *CADM2-M8*- and *CADM2-FL*-expressing lentiviruses were generated using the GV177 system (Genecheme, Shanghai, China). The recombinant virus was purified, characterized, and titrated by qPCR, and its infectivity was detected after transduction at increasing multiplicity of infection (MOI).

2.9. In vitro cell proliferation, invasion, apoptosis, cell cycle and soft agar colony formation assays

After infection, 786-O cell proliferation was determined using a cell counting kit-8 (Keygen) following manufacturer's instructions. This assay was performed in triplicate. For the invasion assay, infected 786-O cells were seeded on transwells with 8-μm pores (Millipore, Billerica, MA), coated with Matrigel (BD, San Antonio, TX). After 48 h, adherent cells on the top surface of the filter were removed. Cells were fixed with 4% paraformaldehyde and stained with crystal violet, and the number of cells on the lower surface was counted.

To detect apoptosis, 786-O cells (1×10^5 cells per well) were seeded in six-well plates and infected with lentivirus at MOI 40. After 3 days, cells were stained with Annexin V-FITC (Keygen) and analyzed using BD Influx™ cell sorter (BD). Cell cycle was analyzed using the cell cycle detection kit (Keygen). For colony formation analysis, 1×10^4 infected 786-O cells were mixed with 0.7% top agar (Sigma) in 60 mm plates coated with 1.2% base agar. After 2 weeks, the cells were stained with crystal violet. The assay was performed in triplicate.

2.10. In vivo tumorigenesis assay

BALB/$c^{nu/nu}$ mice, which were 4–6 weeks-old and 18–25 g (Vitalriver, Beijing, China), were maintained in a specific pathogen-free environment and randomly divided into the following four treatment groups: control (786-O cells), empty mock (786-O cells infected with an empty vector control), M8, and FL (CADM2-expressing 786-O cells, at MOI 40 respectively) groups, four mice for each group. Mice received subcutaneous cell injections (5×10^6 cells in 100 μL PBS) in the armpit. Tumor diameter was measured every third day after day 10, and tumor volume was calculated using the following formula: length $\times$ (width)^2/2. Mice were sacrificed after 30 days. Animals were maintained and experiments were carried out according to the institutional guidelines established for the Animal Facility Center at Peking University.

2.11. CADM2 siRNA knockdown

The CADM2 target sequence (5′-AGCGGCTGCTTCAAAGAATAA-3′) and predesigned siRNA inserts were obtained using the

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