Aberrant Methylation of the 16q22.1 Tumor Suppressor Gene CDH11 Promotes Tumorigenesis and Progression of Renal Cell Carcinoma through NF-κB Pathway

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Primary research

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Abstract

**Background:** To discover new epigenetic biomarkers for early cancer detection. The *CDH11* gene has been reported as a critical tumor suppressor gene (TSG) for multiple tumors, although it has not yet been reported in renal cell carcinoma (RCC). We explored its epigenetic alteration in RCC and analyzed the possible biological function and mechanism in tumorigenesis and progression of RCC.

**Materials and Methods:** We examined *CDH11* gene expression and methylation using semiquantitative reverse transcriptase PCR (RT-PCR) and methylation-specific polymerase chain reaction (MSP) in RCC cell lines before and after treatment with 5-aza-2'-deoxycytidine (5-Aza). MSP was further applied to 93 RCC primary tumors, and the relationship between *CDH11* gene methylation and clinicopathological features was discussed. A selection of the cell lines and specimens was subsequently examined using bisulfite genomic sequencing (BGS) and real-time PCR. Meanwhile, assays of cell viability, colony formation, migration and invasion, wound healing, and western blot were performed to confirm the tumor-suppressive function and mechanism of *CDH11* gene.

**Results:** *CDH11* gene methylation was detected in 4 of 5 RCC cell lines with silenced expressions. Treatment with 5-Aza reversed methylation and restored *CDH11* gene expression. Aberrant methylation was further detected in 41 of the 93 (44.1%) primary tumors. Furthermore, *CDH11* gene methylation was significantly associated with tumor stage and nuclear grade in patients with RCC (*p*<0.05). Due to the phenomenon of aberrant methylation, ectopic low-level expression of *CDH11* gene could result in promotion of tumorigenesis and progression in RCC cell lines, which might be mediated through NF-kB pathway.

**Conclusions:** The *CDH11* gene is often down-regulated by aberrant promoter methylation in RCC cell lines and primary tumors, indicating its critical role as a TSG in RCC. It may be involved in the tumorigenesis and progression in RCC through NF-kB pathway, thus which could potentially serve as a novel biomarker and therapeutic target for RCC.

1. **Background**

RCC is a malignant tumor with a relatively poor prognosis and high rates of metastasis to distal organs, accounting for 2% of all adult tumors [1]. The detailed molecular mechanisms that underlie RCC development remain poorly understood. However, an increasing number of studies have determined that the inactivation of TSG is frequently involved in the tumorigenesis of RCC as a result of epigenetic abnormalities in DNA methylation [1-6].

Currently, many TSGs in a wide range of cancers have been found to be inactivated by the methylation of promoter regions of DNA. This methylation frequently occurs at 5'-CpG islands, which are regions with a high density of CG dinucleotides found in approximately 70% of the coding genes in mammals [7]. It has been proposed that the aberrant promoter methylation status of specific TSGs could be a potentially sensitive marker for use in RCC diagnosis and prognosis prediction. However, the rate of aberrant
promoter methylation of most TSGs is relatively lower in RCC than in other solid tumors, correspondingly to only approximately 10% to 30%. Therefore, identifying a critical TSG with a higher methylation rate in RCC is critical. Our team has always been devoted to this research and a series of highly aberrant methylation TSGs of RCC had been explored [8-12].

The CDH11 gene, located in the 16q22.1 region and belonging to the E-cadherin family, is often involved in an important group of cell-cell adhesion molecules that mediate intercellular adhesion by Ca\(^{2+}\)-dependent hemophilic interactions [13]. Because cadherins have been widely documented in cancer development [14], the promoter CpG island hypermethylation-associated silencing of the CDH11 gene, a component of this superfamily, is consistent with the hypothesis that epigenetic inactivation of this gene could be involved in the tumorigenesis and progression of carcinomas. L Li et al [15] found that the CDH11 gene is frequently methylated in a variety of tumor tissues including esophageal squamous cell carcinoma (13/14, 93%), nasopharyngeal carcinoma (17/18, 94%), hepatocellular carcinoma (28/42, 66%), breast carcinoma (11/12, 91%), gastric carcinoma (13/13, 100%), colon carcinoma (10/11, 90%) and other carcinomas.

To our knowledge, we are the first throughout the world to explore the CDH11 gene methylation status in RCC cell lines and primary tumors. Besides, its roles in RCC suppression remain unclear up to present. Based on the current situation above, this investigation will aid in elucidating whether the CDH11 gene is a potential novel biomarker and therapeutic target for early detection and prognosis of RCC.

2. Materials And Methods

2.1 Patient and tissue samples

All human primary RCCs (93 cases) and adjacent normal tissues (20 cases) were obtained from the Urology Department, Peking University First Hospital, Beijing, from September 2012 to May 2013. The specimens were collected via radical nephrectomy, and the RCC diagnoses were confirmed according to the pathological findings. The adjacent normal tissues were resected at least 2cm from the tumors. Additionally, 3 similarly aged normal renal parenchyma specimens were collected during non-cancer-related kidney surgeries that were performed during the same time period for use as normal controls. All of the resected tissues were snap frozen in liquid nitrogen and stored at -80°C. Patients with localized lymph node or distant metastases detected by a preoperative computed tomography scan were excluded from this study. The participants gave informed written consent for participation in this research study and all of the research procedures were approved by the Institutional Review Board of Peking University First Hospital. The study was also strictly conducted according to the principles defined in the Declaration of Helsinki.

The tumor set was composed of samples taken from 64 males and 29 females ranging from 28-78 years of age, with a median age of 58.3 years at diagnosis. Of the samples, 54 primary tumors were located on the left side, and 39 on the right. The average preoperative diameter of the primary tumors was 5.5cm
(range 2.1-12.5 cm). The classification of the tumors was based on the staging system of the 2018 American Joint Committee on Cancer (AJCC). Additionally, the nuclear grade of the tumors was determined during postoperative pathological analysis.

2.2 Cell line preparation and treatment of RCC cell lines with 5-Aza

Five RCC cell lines (A498, Caki-2, Ketr-3, Osr, and 786-O) obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were prepared to validate the \( CDH11 \) gene methylation status. The HEK293 human normal embryonic kidney cell line and HK-2 human kidney proximal tubular epithelial cell line were both routinely cultured and served as "normal" controls. All cell lines were maintained in DMEM supplemented with 2 mM glutamine and 10% fetal bovine serum at 37\(^\circ\)C with 5% CO\(_2\). The demethylating agent 5-Aza (Sigma\(^\circ\)) was freshly prepared in ddH\(_2\)O and filtered sterilized. Afterwards, the RCC cell lines were treated with 10 \( \mu \)M 5-Aza for 3 days. The media was changed every 24 hrs. After treatment, the cells were pelleted and washed with PBS, and the DNA/RNA was extracted.

2.3 DNA extraction from the RCC tissue samples

DNA was extracted from the RCC primary tumors and adjacent normal tissues. Genomic DNA was isolated from the tissues using the TIANamp Genomic DNA Kit (TIANGEN\(^\circ\)) protocol. The quality of the isolated DNA was subsequently assessed by electrophoresis.

2.4 RT-PCR

\( CDH11 \) gene expression in the RCC cell lines before and after treatment with 5-Aza was determined via RT-PCR using an RNA PCR kit (Applied Biosystem\(^\circ\)) and Go Taq\(^\circ\). The 12.5 \( \mu \)l RT-PCR mixture contained 2.5 \( \mu \)l of RT product, 0.75 \( \mu \)l of 5′-primers (10 \( \mu \)M), 0.75 \( \mu \)l of 3′-primers (10 \( \mu \)M), 2 \( \mu \)l of 5x Flexi buffer, 0.5 \( \mu \)l of MgCl\(_2\) (25 mM), 1.0 \( \mu \)l of Promega Go Taq\(^\circ\) (5U/\( \mu \)l) and 5.9 \( \mu \)l of ddH\(_2\)O. GAPDH was used as an internal control.

2.5 Bisulfite modification of specimen DNA and MSP

Bisulfite modification of the specimen DNA was carried out as described previously \cite{16}. To examine the presence of epigenetic alterations in the \( CDH11 \) gene of the RCC primary tumors, bisulfite-modified DNA (50 \( \mu \)g) was used in MSP with specifically methylated and unmethylated primers. All of the primers were provided by Sangon Biotech\(^\circ\) Co. Ltd. (Shanghai) and were previously shown to not amplify non-bisulfited DNA. The 12.5 \( \mu \)l MSP mixture contained 0.5 \( \mu \)l of bisulfate-treated genomic DNA, 1 \( \mu \)l of dNTPs (2.5 mM), 0.75 \( \mu \)l of 5′-primers (10 \( \mu \)M), 0.75 \( \mu \)l of 3′-primers (10 \( \mu \)M), 2 \( \mu \)l of 5xFlexi buffer, 1 \( \mu \)l of MgCl\(_2\)
(25mM), 0.1μl of AmpliTaq Gold® (5U/μl) and 6.4μl of ddH₂O. The MSP products were checked on a 2% agarose gel stained with ethidium bromide.

### 2.6 Immunohistochemistry (IHC) assays

IHC assays were performed with an UltraSensitive SP Kit (Maixin-Bio, Fujian, China) following the manufacturer’s instructions. Tissue sections were dewaxed, rehydrated, processed for antigen retrieval, and blocked. The sections were incubated with a primary antibody against CDH11 overnight at 4°C. Following treatment with secondary antibodies, 3,3′-diaminobenzidine, and hematoxylin staining, the staining was evaluated with Image-Pro Plus, version 6.0 (Image Pro, Silver Spring, MD).

### 2.7 Quantitative real-time PCR

Total RNA from 10 pairs of patient tumors and adjacent normal tissue samples was isolated using the TRIZOL reagent (Invitrogen), and 2μg of total RNA was reverse-transcribed using a reverse transcription system. Real-time PCR was performed using an ABI Prism 7500™ instrument ( Applied Biosystems) system with SYBR green PCR mix purchased from Sangon Biotech® Co. Ltd. (Shanghai). GAPDH was used as an internal control. The relative expression levels of the CDH11 gene were calculated according to the 2^-△△CT method [17].

### 2.8 BGS of the CDH11 gene in the cell lines and specimens

BGS was then performed to analyze the detailed methylation status of the CDH11 gene in a selection of the cell lines and specimens, and this method was performed according to the previous report [18]. The amplified BGS products were TA cloned, and 6 colonies were randomly chosen and sequenced.

### 2.9 Establishment of stable cell lines

CDH11 full-length cDNA-expressing vector pcDNA3.1(+)–CDH11 was generated. To establish stably transfected tumor cells with CDH11 expression, cell lines (A498, 786-O) were transfected with CDH11 expression plasmids using Lipofectamine™ 2000 (Invitrogen), and further screened a monoclonal cell population by G418.

### 2.10 Cell viability assay

Cells were replated into 96-well plates after being transfected with CDH11 expression vector and empty vector for 48h, and further measured using the Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China) as described
2.11 Colony formation assay

Monolayer culture was used for colony formation assay. Cells were selected for ~14 days with presence of G418 after transfection for 48h. Surviving colonies (≥ 50 cells/colony) were counted and stained with gentian violet. All experiments were performed in triplicate wells with three times. The detailed procedures could be traced by HY Xu et al [21].

2.12 Migration and invasion assays

Cell motility and invasive abilities were assessed by Transwell® and Matrigel™ invasion assays (Corning Life Sciences, Bedford, MA) as described previously [22-23]. Cells were migrated and invaded to the lower side of the membrane, and further stained with 0.1% crystal violet. Cells were then counted in five microscopic fields, and the mean values were counted.

2.13 Wound healing assay

Wound healing assay was performed as described previously [19-20]. A mechanical wound was created after scratching with a pipette tip, and images were taken at different time points. The distance between the wound edges was measured and quantified.

2.14 Western blot

Protein Extraction Reagent (Thermo Scientific, Rockford, IL) was used for protein extraction. Western blot analysis was performed as described previously [20-22]. Antibodies included: CDH11, pp65, MMP2 and GAPDH.

2.15 Statistical analysis

Clinicopathological data of the patients were obtained from the institutional database of Peking University First Hospital. The CDH11 gene methylation status and the clinicopathological features of the RCC patients were analyzed. Continuous data were shown as means ± standard deviation (SD). All of the statistical analyses were performed using Student’ s t test, Fisher’s exact test or the chi-square test with the SPSS17.0 software (StatSoft Inc., Tulsa, OK, USA). A p-value <0.05 was considered statistically significant.
3. Results

3.1 Down-regulated CDH11 gene expression is aberrantly methylated in RCC cell lines

Figure 1A listed the primer sequences and cycling parameters for the RT-PCR and MSP. CDH11 gene expression was silenced in all of the 5 RCC cell lines as shown in Figure 1B. As expected, it was aberrantly methylated in 4 of the 5 RCC cell lines (80.0%), including 786-O, A498, Osr and Ketr-3.

After 5-Aza treatment, CDH11 gene expression levels were restored in 4 of the 5 (80%) RCC cell lines. Meanwhile, MSP also showed that the CDH11 gene was partially or totally demethylated following pharmacological demethylation. These changes confirmed that CDH11 gene expression silencing was directly mediated by aberrant promoter methylation.

3.2 Down-regulated CDH11 gene expression is aberrantly methylated in RCC primary tumors, which is significantly associated with tumor stage and nuclear grade

We subsequently used this validated system to analyze the CDH11 gene methylation status in a series of 93 RCC samples and 20 adjacent normal tissues. Aberrant promoter methylation was detected in 41 of the 93 (44.1%) RCC primary tumors and in 3 of the 20 (15.0%) adjacent normal tissues (p<0.05), and representative results were shown in Figure 2A.

To confirm that the methylation of the CDH11 gene indeed correlated with the down-regulation of its gene expression, we performed real-time PCR to detect the mRNA expression levels of the CDH11 gene in 10 primary tumors paired with their adjacent normal tissues (10T/N, 11T/N, ..., 18T/N, 19T/N), as demonstrated in Figure 2B. As expected, in 8 out of the 10 (80%) RCC primary tumors (10T, 11T, 12T, 14T, 15T, 16T, 18T, and 19T), the expression levels of the CDH11 gene were significantly lower than that of the adjacent normal tissues, which was consistent with our MSP results. Because both 13T and 13N were methylated, there was no significant difference in the expression level of 13T/N. However, MSP-negative results were identified for the 11T/N, 16T/N and 18T/N tumors, which also had significantly different expression from their normal tissue pairs. By the IHC assays, the discrepancy of the protein expression levels in the CDH11 gene could be also directly reflected in Figure 2C.

Figure 2D listed the patients’ clinicopathological features. The CDH11 gene methylation status was not significantly associated with gender, age, tumor location or tumor diameter. However, there existed a significant association between AJCC pathological stage and nuclear grade in the patients with different CDH11 gene methylation status (p<0.05). The percentage of methylated RCC tumors increased dramatically with more advanced stages or grades.
3.3 The methylation status of the CDH11 gene is confirmed by BGS analysis

BGS was performed to confirm the methylation status of the CDH11 gene in HEK293 and A498/Ketr-3 cell lines (before and after 5-Aza treatment) and three methylated tumors along with their adjacent normal tissues. The BGS primers were listed in Figure 1A. The results were consistent with the MSP results, as a high density of methylated CpG sites were detected in the A498/Ketr-3 cell lines and in the methylated tumor samples (Figure 3A-3B). The BGS results for the HEK293 and A498/Ketr-3 cell lines after 5-Aza treatment and for the matched adjacent normal tissues (Figure 3A-3B) were consistent with CDH11 gene down-regulation, which was observed in RCC cell lines and primary tumors.

3.4 Aberrant methylation of the CDH11 gene can promote tumorigenesis and progression of RCC, which might be mediated through NF-kB pathway

We further assessed tumor-suppressive functions of CDH11 gene. Initially, real-time PCR confirmed CDH11 gene overexpression in CDH11-transfected RCC cell lines (Figure 4A). Colony formation and CCK8 assays showed that overexpression of CDH11 gene had significantly detectable effect on the proliferation of RCC cell lines (p<0.05) (Figure 4B-4C). Scratch wound healing assays also revealed that CDH11-transfected cells showed slower closure of the scratched wound in RCC cell lines (p<0.05) (Figure 4D). In addition, we measured cell migration and invasion using Transwell® assays (with or without Matrigel) in CDH11-transfected and the respective control cells. Results showed that CDH11 overexpression significantly decreased cell migration and invasion in RCC cell lines (p<0.05) (Figure 4E).

To further explore molecular mechanisms responsible for CDH11-mediated tumor-suppressive functions, we analyzed the effects on NF-kB signaling pathways. As expected, CDH11-transfected A498 and 786-O cell lines both displayed lower levels of pp65 and MMP2 than the control cells (Figure 4F).

These findings suggested that CDH11 may act as an antagonist of NF-kB signaling pathway in RCC.

4. Discussion

RCC is often distinguished by a set of genetic and epigenetic abnormalities. It is well known that inactivation of TSGs frequently occurs in RCC via DNA methylation and deletions, but only a few genes with lower rates of methylation in RCC have been reported, which may not be biologically relevant. Thereafter, the identification of genes with higher rates of methylation in RCC is critical. In this study, we validated the methylation status of a critical 16q22.1 TSG, the CDH11 gene, in RCC cell lines and primary tumors. More importantly, we explored its possible biological function and mechanism in tumorigenesis and progression of RCC.
Similar to other classical cadherin family members (CDH1, CDH3, CDH5, CDH8 and CDH13), the CDH11 gene protein can cluster through a zipper-like mechanism while their intracellular domain is anchored to the actin cytoskeleton through α/β-catenin [24]. These interactions have crucial roles in maintaining tissue architecture and cell polarity and in limiting cell movement and proliferation, thus resulting in tumor inhibition [25]. Recent studies have shown that the CDH11 gene meets several of the criteria of classic TSGs: (1) it shares the same locus of 16q22 as CDH1 and CDH13, which has been identified as involved in the inhibition of cell proliferation and invasiveness and the promotion of apoptosis; and (2) it is capable of inhibiting the growth of various types of tumor cells and colony formation, followed by frequent deletion and frequent methylation in multiple cancers.

The CDH11 gene was first identified as a TSG candidate in a murine retinoblastoma model by Marchong et al in 2010 [26]. Our investigation is the first study to report the prevalence of CDH11 gene methylation in a large set of RCC cell lines and tumor samples, although in the year of 2019 Chunsheng Li et al [27] has suggested a hypothesis that CDH11 gene may be a potential core gene in metastatic RCC using bioinformatics analysis without objective experimental verification. Using MSP, we discovered that the CDH11 gene is aberrantly methylated both in RCC cell lines and primary tumors, indicating that the CDH11 gene, a novel functional TSG, is frequently epigenetically inactivated in RCC. MSP revealed that the CDH11 gene promoter is methylated in 80% of RCC cell lines and in 44.1% of RCC primary tumors. The higher methylation rate of RCC cell lines compared to RCC primary tumors indicates that some RCC cell lines may have acquired the CDH11 gene methylation property during the establishment or maintenance process. Certainly, there were unmethylated alleles in the Caki-2 RCC cell line, and no expression was detected, which suggests that other transcriptional regulatory mechanisms, such as histone modification or transcriptional repressors, may also contribute to the silencing of this gene.

The AJCC pathological tumor stage and nuclear grade are commonly used for prognostic diagnoses in patients with RCC. Our statistical analysis of clinicopathological features in patients with RCC showed a positive correlation between the CDH11 methylation status and the tumor stage or nuclear grade, which was consistent with the conclusions obtained from gastric cancer and non-muscle invasive bladder cancer [28-31]. Our data in the subsequently functional trails also showed that ectopic expression of CDH11 gene inhibited the proliferation, migration and invasion in RCC cell lines. Moreover, this study appears to be also the first to reveal its underlying mechanism. We found that the inhibition of tumor migration and invasion by CDH11 might be associated with deregulation of NF-kB signaling pathway. Above all, these results all suggest that CDH11 gene functions a tumor suppressor through suppressing proliferation, migration and invasion through NF-kB pathway in RCC.

Surprisingly, CDH11 gene methylation was also detected in some adjacent normal tissues. Methylation of the CDH11 gene promoter was not detected in normal renal parenchyma specimens, although our investigation revealed that 15.0% of the adjacent normal tissues were methylated. More importantly, the tumor samples of these 3 adjacent normal tissues were also found to be aberrantly methylated, indicating that aberrant promoter methylation might be caused as part of a premalignant field effect [32].
Unfortunately, the limited sample size made it difficult to draw an accurate conclusion concerning the significance of \textit{CDH11} gene methylation, and the in-depth upstream regulatory mechanisms underlying the tumor-suppressive biological functions still remain fuzzy. These parameters will be improved in our subsequent investigation.

5. Conclusions

We report for the first time that the CDH11 gene is often down-regulated by aberrant promoter methylation in RCC cell lines and primary tumors, indicating that it plays a critical role as a TSG in RCC. Additionally, we conclude that CDH11 gene methylation may be involved in the tumorigenesis and progression of RCC through NF-\textit{kB} pathway, thus which could potentially serve as a novel biomarker and therapeutic target for RCC. However, further investigations are still required.

Declarations

1. Ethics approval and consent to participate

Not applicable

2. Consent for publication

Yes

3. Availability of data and materials

Yes

4. Competing interests

Not applicable

5. Funding

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6. Authors' contributions
Ben Xu and Haifeng Song carried out the design of this research, analysis and interpretation of data, and drafted the manuscript. Cheng Luo and Lei Liang participated in the collection of data and data analysis. Qian Zhang assisted in the design of this research and project development. Ben Xu conceived the study, reviewed all of the statistical analysis of the data, and revised the manuscript. All authors read and approved the final manuscript.

7. Acknowledgements

Not applicable

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**Figures**

![Figure 1](image)

Down-regulated CDH11 gene expression is aberrantly methylated in RCC cell lines. (A) The primer sequences and cycling parameters for the RT-PCR, real-time PCR, MSP and BGS. (B) The analysis of CDH11 gene expression and aberrant methylation in the cell lines before and after 5-Aza treatment with GAPDH as a control. M, methylated. U, unmethylated.
Down-regulated CDH11 gene expression is aberrantly methylated in RCC primary tumors, which is significantly associated with tumor stage and nuclear grade. (A) Representative methylation status of the CDH11 gene in RCC primary tumors and adjacent normal tissues, where 3 non-cancer normal tissues were used as a negative control. M, methylated. U, unmethylated. T, RCC primary tumors. N, adjacent normal tissues. (B) The relative mRNA expression levels of the CDH11 gene for 10 pairs of RCC primary tumors and adjacent normal tissues.
tumor samples compared to their adjacent normal tissues. *p<0.05, #p<0.01. (C) The protein expression levels of the CDH11 gene in the representative 8T/N and 11T/N by IHC assays. (D) The clinicopathological features of RCC patients and CDH11 gene methylation status.

Figure 3

BGS high-resolution mapping of the methylation status of each CpG site (ovals) in the CDH11 gene promoter region. (A) BGS results of the HEK293, A498 and Ketr-3 cell lines. (B) BGS results of the RCC primary tumors and adjacent normal tissues. Rows represent individually analyzed alleles of the CDH11 gene promoter. Arrows indicate MSP primer sites. Filled ovals indicate methylation. Open ovals indicate unmethylation.
Figure 4

Effects of ectopic CDH11 on tumorigenesis and progression of A498 and 786-O cell lines (*P < 0.05 and **P < 0.01). (A) Real-time PCR analyses of CDH11 mRNA expression in CDH11 overexpressing cell lines. (B) Representative colony formation assay and quantitative analysis. The numbers of G418-resistant colonies in vector-transfected controls were set to 100%, values were expressed as the mean ± SD. (C) CCK-8 assay for cell proliferation on vector- and CDH11-transfected cell lines. (D) Migration of CDH11-transfected or vector-transfected tumor cells by scratch wound healing assays. The width of the remaining open wound was measured in relation to time 0h. Original magnification, 100×. Significant reduction in cell migration was observed in cells expressing CDH11 compared with control cells. Representative images in each group were shown. (E) Cells at the lower surface of the Transwell® chamber were counted. Original magnification, 100×. (F) Ectopic expression of CDH11 disrupted NF-κB signaling, which was confirmed using antibodies against pp65 and MMP2 by western blotting. GAPDH was used as a control.