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Aberrant Methylation of PCDH8 is a Potential Prognostic Biomarker for Patients with Clear Cell Renal Cell Carcinoma

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: PCDH8 is a tumor suppressor that regulates cell adhesin, proliferation, and migration. It is often inactivated by aberrant promoter methylation in several human cancers, including clear cell renal cell carcinoma (CCRCC). The clinical significance of PCDH8 methylation in CCRCC remains unclear. The aim of this study was to investigate the relationship between PCDH8 methylation and clinicopathological characteristics as well as outcome of patients with CCRCC.

Material/Methods: The methylation status of PCDH8 in 153 CCRCC tissues and 97 paired adjacent normal renal tissues were examined using methylation-specific PCR (MSP). Then the relationships between PCDH8 methylation and clinicopathological features as well as progression-free survival of CCRCC patients were evaluated.

Results: PCDH8 methylation was significantly more frequent in CCRCC tissues compared with normal renal tissues. Moreover, PCDH8 methylation was significantly correlated with advanced clinical stage (P=0.0141), higher grade (P=0.0190), and lymph node metastasis (P=0.0098). In addition, multivariate analysis showed that PCDH8 methylation was independently associated with poor progression-free survival (P=0.0316).

Conclusions: PCDH8 methylation is a frequent event in CCRCC and is correlated with unfavorable clinicopathological features. Moreover, PCDH8 methylation may be a useful biomarker to predict the progression of CCRCC.

MeSH Keywords: Cadherins • Carcinoma, Renal Cell • DNA Methylation • Tumor Markers, Biological

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Background

Renal cell carcinoma (RCC) is a common genitourinary tumor, with an estimated 63 920 new cases and 13 860 deaths in the United States in 2014, and the incidence of this malignancy has increased over the last 2 decades [1,2]. Histopathologically, clear cell renal cell carcinoma (CCRCC) accounts for approximately 75% of newly diagnosed RCC cases. Papillary (10–15%), chromophobe (5%), and other rare forms account for the remainder [2–4]. RCC is heterogeneous in clinical behavior, tumors with similar grade and stage may behave differently, and the outcome of RCC differs significantly among patients [2,3,5]. Currently, clinicopathological parameters, such as stage and grade, are commonly used to predict the outcome of patients with RCC. However, these clinicopathological parameters are unable to accurately predict the outcome of RCC patients [2,6]. Thus, new prognostic indicators are needed, in addition to common clinicopathological features, to guide the treatment of the individual patient and to predict outcome [7,8].

The initiation and progression of RCC are attributed to the accumulation of genetic and epigenetic changes, silencing tumor suppressor genes or activating oncogenes [9,10]. Epigenetic changes in human cancer include aberrant DNA methylation, histone modification, and RNA interference. Moreover, aberrant DNA methylation is the best-studied epigenetic alteration in human cancers [11, 12]. DNA methylation is an enzyme-induced chemical modification in the cytosine molecule, on which a methyl residue is transferred at 5' position, in CpG islands [12]. Aberrant methylation of normally unmethylated genes is associated with silencing gene expression, and may be used as a potential biomarker in human cancers [13,14]. In recent years, the association of PCDH8 with human cancers has been proposed. PCDH8 is a member of protocadherin family, which belong to the cadherin superfamily. The cadherins play important roles in calcium-dependent cell-cell adhesion, cell migration, and morphogenesis [15]. Recent studies revealed that PCDH8 is silenced by aberrant promoter methylation in several human cancers, and functions as a candidate tumor suppressor [16–19]. Aberrant methylation of PCDH8 in CCRCC has been reported, but the clinical significance of PCDH8 methylation in CCRCC needs to be further elucidated [20].

In the current study, we examined the methylation status of PCDH8 in primary CCRCC tumors and normal renal tissues using methylation-specific PCR (MSP). Then the correlation between PCDH8 methylation status and clinicopathological features was investigated in CCRCC cases. The relationship between PCDH8 methylation and the prognosis of patients with CCRCC was also examined to evaluate the clinical significance of PCDH8 methylation in CCRCC.

Table 1. Clinicopathological characteristics of patients with CCRCC (n=153).

Features	Variables	No. (%)
Age (years)	Min. to max.	33–86
	Median	65
Sex	Male	102 (66.7)
	Female	51 (33.3)
Tumor stage	pT1	102 (66.7)
	pT2	26 (17.0)
	pT3	25 (16.3)
Lymph node metastasis	N0	140 (91.5)
	N1	13 (8.5)
Distant metastasis	M0	153 (100.0)
	M1	0 (0.0)
Tumor grade	G1	77 (50.3)
	G2	51 (33.3)
	G3	25 (16.7)
Clinical stage	I	97 (63.4)
	II	22 (14.4)
	III	34 (22.2)
Progression	Presence	51 (33.3)
	Absence	102 (66.7)

Material and Methods

Patients and samples

A total of 153 CCRCC tissues and 97 paired adjacent normal renal tissues samples were included in this study. These tissues were obtained from radical nephrectomy (n=120) or partial nephrectomy (n=33) at the Third Hospital of Hebei Medical University between March 2004 and March 2008. These patients were histopathologically diagnosed with CCRCC for the first time, and they did not receive any anticancer therapy before surgery. Adjacent normal renal tissues were isolated with minimum of 0.5 cm to 2 cm distance from the primary tumor lesion. These tissues were examined pathologically to exclude the tumor; tissues were obtained from the clinical stage I subgroup. All tissue samples were immediately frozen in liquid nitrogen and stored at –80°C until used. All the patients underwent chest X-ray, bone scan, and computerized tomography (CT), and staged according to 2002 TNM staging system of Union for International Cancer Control (UICC) [21]. Clinical stage I was defined as pT1, and lymph node and distant metastasis negative (N0/M0); Clinical stage II was defined as pT2 and N0/M0; Clinical stage III was defined as pT1 or pT2 and N1/M0, or pT3 and N0 or N1/M0 [21]. Tumors were

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Table 2. Associations between PCDH8 methylation and clinicopathologic parameters of CCRCC patients (n=153).

Features	Variables	No.	M (%)	U (%)	P
Age	≤65	77	51 (66.2)	26 (33.8)	0.6424
	>65	76	53 (69.7)	23 (30.3)	
Sex	Male	102	66 (64.7)	36 (35.3)	0.4752
	Female	51	38 (70.4)	16 (29.6)	
Pathological stage	pT1/pT2	128	84 (65.6)	44 (34.4)	0.1588
	pT3	25	20 (80.0)	5 (20.0)	
Grade	G1/G2	128	82 (64.1)	46 (35.9)	0.0190
	G3	25	22 (88.0)	3 (12.0)	
Lymph node metastasis	N0	140	91 (65.0)	49 (35.0)	0.0098
	N1	13	13 (100.0)	0 (0.0)	
Clinical stage	I/II	119	75 (63.0)	44 (37.0)	0.0141
	III	34	29 (85.3)	5 (14.7)	

M – methylation; U – unmethylation.

pathologically graded as G1, G2, and G3 on the basis of UICC classification too [22,23]. The 97 paired adjacent normal renal tissues samples were obtained from clinical stage I subgroup because they were better used as normal control. Therefore, the paired adjacent normal renal tissues samples form other clinical stage subgroups were not used as controls in our study, but this did not mean that the resection was inadequate and did not affect the survival times. The patients were followed up at intervals, and the time to progression was defined as the time at which patients demonstrated either a local recurrence or a synchronous/metachronous metastasis by CT scan, as reported previously [24].

The current study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Third Hospital of Hebei Medical University (No. HMU20030377E). Written informed consent was obtained from each participant. The clinical and histopathological characteristics of CCRCC patients are summarized in Table 1.

DNA isolation, bisulfite conversion of DNA and MSP

DNA isolation from frozen tissues was carried out as described previously [25,26]. Genomic DNA from the tissue samples was isolated using DNeasy Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The isolated DNA was treated with bisulfite using EpiTect Bisulfite Kit (Qiagen, Valencia, CA) according to the manufacture's protocol. The bisulfite-modified DNA was then used for MSP. The methylation status of PCDH8 was detected using primers specific for PCDH8 unmethylated and methylated sequences, respectively, as previously reported [16,27]. The following primers were used: methylated: forward 5'- CGGTTATTGGTTATTCGGTTCC-3' and reverse 5'- ACGAACTCTAAAAACGCGCG -3'; unmethylated: forward 5'- GGTGGTTATTGGTTATTTGGTTT-3' and reverse 5'- CCAACAAACTCTAAAAACACACA-3'. The PCR amplification of the modified DNA was performed as reported before [16,27]. *In vitro* methylated DNA and unmethylated DNA (New England Biolabs, Beverly, MA, USA) was used as methylation and unmethylation positive control, and water blanks were included with each assay. The MSP products were separated in 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination. Samples were scored as methylation-positive when methylated alleles were present in the methylated DNA lane and as unmethylation-postive when bands were present in the unmethylated DNA lane only [16,25–27].

Statistical analysis

Fisher's exact test was used to assess the difference of PCDH8 methylation status between CCRCC tissues and paired adjacent normal renal tissues. Chi-square test or Fisher's exact test was used to assess the relationship between PCDH8 methylation and clinicopathologic features of CCRCC patients. Kaplan-Meier survival analysis and log-rank test were used to assess the difference of progression-free survival between CCRCC patients with PCDH8 methylated and unmethylated. Multivariate Cox proportional hazard model analysis was used to assess the independent prognostic effect of PCDH8 methylation for progression-free survival. A 2-sided p value <0.05 was considered statistically significant. The statistical analysis was conducted using SAS version 8.0 (SAS Institute, Cary, N.C., USA) for Windows.

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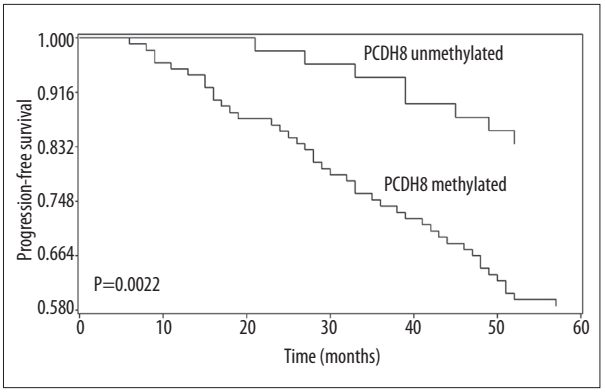

Figure 1. The relationship between PCDH8 methylation and progression-free survival in patients with clear cell renal cell carcinoma. Patients with PCDH8 methylated showed significantly shorter progression-free survival than patients without (P=0.0022, log-rank test).

Results

PCDH8 methylation in CCRCC

In the current study, we examined the methylation status of PCDH8 in CCRCC samples and paired adjacent normal renal tissue samples using MSP, and found that PCDH8 methylation was detected in 104 (68.0%) CCRCC samples, while PCDH8 methylation was only detected in 7 (7.2%) normal renal tissues. Moreover, the difference between these 2 groups was significant (P<0.0001).

Relationship between PCDH8 methylation and clinicopathological features of CCRCC

The relationship between PCDH8 methylation status and clinicopathological features of CCRCC is summarized in Table 2. We found that PCDH8 methylation was significantly correlated with advanced clinical stage (P=0.0141), higher grade (P=0.0190), and lymph node metastasis (P=0.0098). However, no correlation was found between PCDH8 methylation and age, sex, or pathological stage.

PCDH8 methylation and patients' outcome

The follow-up information was available from all the patients. The follow-up time ranged from 6 months to 60 months. We found that 51 patients had tumor progression during the follow-up period. Kaplan-Meier survival analysis and log-rank test results suggested that patients with PCDH8 methylated had significantly shorter progression-free survival (Figure 1, P=0.0022) than patients with PCDH8 unmethylated. This result indicates that PCDH8 methylation was significantly associated with unfavorable prognosis of patients with CCRCC. In addition, multivariate Cox proportional hazard model analysis was performed to determine whether PCDH8 methylation was an independent prognostic factor, after controlling for potential factors. Interestingly, the findings indicated that PCDH8 methylation was an independent predictor for progression-free survival. These findings are summarized in Table 3.

Discussion

RCC is a common cancer in humans, has the highest mortality rate of the genitourinary cancers, and approximately one-third of patients with RCC will die from the disease [2,28,29]. In recent years, although great progress has been made in the diagnosis and treatment of RCC, some patients will have disease progression after initial curative surgery and inevitably die from the disease [2,30]. Thus, novel prognostic biomarkers are required to more appropriately guide therapy of the individual patient and to predict outcome [31]. In the last decades, knowledge on epigenetic changes in the progress of RCC has significantly increased, and epigenetics research may deliver novel diagnostic and prognostic biomarkers [32]. Aberrant

Table 3. The predictive value of PCDH8 methylation for the progression-free survival in CCRCC (n=153).

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
PCDH8 methylation (M *vs.* U)	3.061	1.439–6.513	0.0037	2.352	1.544–6.031	0.0316
Age (>65 vs. ≤65)	0.613	0.473–4.165	0.7621			
Sex (male vs. female)	0.835	0.651–3.681	0.5724			
Pathological stage (pT3 *vs.* pT1/pT2)	1.352	0.817–7.522	0.0668			
Lymph node metastasis (N1 *vs.* N0)	1.627	1.271–11.608	0.0369	1.044	0.896–3.577	0.0872
Grade (G3 *vs.* G1/ G2)	3.792	1.563–10.524	0.0017	1.873	1.332–9.723	0.0392
Clinical stage (III *vs.* I/II)	2.652	1.393–5.972	0.0145	1.336	1.072–7.413	0.0461

HR – Hazard Ratio; M – methylated; U – unmethylated.

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DNA methylation is the best-studied epigenetic changes in RCC, and is becoming increasingly important in cancer research. Though several reports have suggested the presence of numerous methylated genes in RCC, prognostic value has only been investigated in a few of them [33–35].

Accumulating evidence suggests that protocadherins can function as tumor suppressors in a range of tumor types [15]. PCDH8 is one of the best-studied protocadherins; it is located on chromosome 13q14.3, and is frequently inactivated by aberrant promoter methylation in several human cancers. Moreover, exogenous expression of PCDH8 can suppress tumor cell proliferation and migration [16–19]. A recent study found that PCDH8 is frequently silenced by aberrant promoter methylation in CCRCC. However, the prognostic value of PCDH8 methylation in CCRCC has not been elucidated [20]. MSP is a sensitive and specific method for detecting DNA methylation in tumor samples, and allowing the rapid examination of multiple samples, which is convenient for experimental and clinical use [36,37]. In this study, we examined the methylation status of PCDH8 in CCRCC tissues using MSP and evaluated its possible value as a prognostic biomarker.

In the present study, we analyzed the promoter methylation of PCDH8 in CCRCC tumor tissues and corresponding adjacent normal renal samples, and found that PCDH8 methylation occurred more frequently in CCRCC samples than in adjacent normal renal tissues. Moreover, PCDH8 methylation also occurred in early-stage CCRCC tumors and some adjacent normal renal tissues. These findings indicate that PCDH8 methylation may be correlated with the initiation and progression of CCRCC, and prompted us to investigate its clinical significance thoroughly. Then, we correlated PCDH8 methylation with clinicopathological features of CCRCC. We found that PCDH methylation was statistically correlated with unfavorable clinical and pathological characteristics of CCRCC such as presence of lymph node metastasis and advanced stage, as well as poor differentiation of tumor cells. These findings are consistent with those in bladder cancer, gastric cancer, nasopharyngeal carcinoma, and breast cancer [16–19]. Our results suggest that PCDH8 methylation is a frequent event in the development of CCRCC and may affect the clinical course of the disease. To evaluate this hypothesis, the progression-free survival of patients with CCRCC was examined in terms of PCDH8 methylation status in tumor samples. Interestingly, Kaplan-Meier survival analysis and log-rank test indicated PCDH8 methylation was correlated with worse progression-free survival. Moreover, multivariate Cox proportional hazard model analysis revealed that PCDH8 methylation is an independent prognostic factor for progression-free survival.

Aberrant DNA methylation can be reversed by demethylating agents. Previous studies reported that the methylation status of PCDH8 can be reversed in breast cancer and gastric cancer, and restored PCDH8 expression [17,19]. Moreover, ectopic expression of PCDH8 can suppress tumor cell proliferation and migration, which are the main malignant features of tumors [17,19]. The evidence presented above suggests that PCDH8 methylation may be a useful biomarker for individualized therapy and a target for anticancer therapy. This needs to be confirmed by further CCRCC research.

Conclusions

We found that PCDH8 methylation is a frequent event in CCRCC. Moreover, PCDH8 methylation was correlated with advanced stage, high grade, lymph node metastasis, and progression. In addition, this study indicated PCDH8 methylation as a potential biomarker for independent prognosis of progression-free survival. Our findings indicate that aberrant methylation of PCDH8 may be a potential prognostic biomarker for patients with CCRCC. For patients with PCDH8 methylated, more aggressive adjunctive should be performed after surgery to achieve better outcome.

Conflicts of interest

None.

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