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Aberrant Promoter Methylation of PCDH17 (Protocadherin 17) in Serum and its Clinical Significance in 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: Current studies indicated that PCDH17 functions as a tumor suppressor, which is frequently inactivated by aberrant promoter methylation in urologic tumors. The main purpose of this study was to investigate the methylation status of PCDH17 in serum and its clinical significance in renal cell carcinoma (RCC).

Material/Methods: The methylation status of PCDH17 in serum samples of 142 RCC patients and 34 controls was evaluated by methylation-specific PCR (MSP). Then we correlated PCDH17 methylation status with the clinicopathologic features of RCC patients and patient outcomes.

Results: We found that PCDH17 was more frequently methylated in RCC patients than in controls. Moreover, PCDH17 methylation in serum was significantly correlated with advanced stage (p=0.044), higher grade (p=0.019), lymph node metastasis (p=0.008) and tumor progression (p<0.001). In addition, patients with methylated PCDH17 had shorter progression-free survival (p<0.001) and overall survival (p=0.017) than patients without, and PCDH17 methylation in serum was an independent prognostic factor for worse progression-free survival (HR: 4.215, 95% CI: 1.376–9.032, p<0.001) and overall survival (HR: 5.092, 95% CI: 1.149–12.357, p=0.046) of patients with RCC.

Conclusions: The present study indicates that PCDH17 methylation in serum is a frequent event in RCC and associated with risk factors of poor outcomes. Moreover, PCDH17 methylation in serum is a potential prognostic biomarker for patients with RCC after surgery.

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

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Background

Renal cell carcinoma (RCC) is one of the most commonly diagnosed urinary malignant diseases, with an estimated 61,560 newly diagnosed cases and 14,080 deaths in 2015 in the USA [1]. Histopathologically, clear cell RCC (ccRCC) is the most common subtype of renal tumor, accounting for approximately 80–90% of cases [2,3]. Currently, nephrectomy or partial nephron sparing resection represents the standard therapy for localized and locally advanced RCC. However, about 30% of RCC patients experience progression to metastatic or locally recurrent disease after nephrectomy for localized disease, and approximately 11% of patients will die of disease progression after surgery [4–6]. RCC is a heterogeneous disease with outcomes difficult to predict. Currently, some clinicopathological parameters are used as prognostic tools for RCC, but they fail to predict patient prognosis accurately, and novel predictors are needed [7].

Recent studies have shown that aberrant methylation of some tumor suppressor genes is involved in the initiation and progression of RCC [8–12]. Epigenetic silencing of tumor suppressor genes caused by DNA methylation, leading to increased tumor cell proliferation, invasion, and metastasis, and DNA methylation can be used as potential diagnostic or prognostic biomarkers [8–12]. Aberrant DNA methylation can be detected in tumor tissue and also in serum, and DNA methylation status in serum corresponds to those found in the tumor. Methylated DNA in serum can be used as potential biomarker for tumor diagnosis and prognosis, and for monitoring therapy response [13–15]. PCDH17, a member of protocadherin family, functions as a tumor suppressor in human tumors, including RCC [16–18]. Our previous study indicated that PCDH17 methylation occurred frequently in RCC tissues, and correlated with malignant clinicopathologic characteristics as well as poor outcomes of patients [19]. However, the methylation status of PCDH17 in serum and its clinical significance in RCC remains largely unknown.

In the present study, we examined the methylation status of PCDH17 in preoperative serum of patients with RCC, and then correlated with clinicopathological features as well as patient outcomes, so as to evaluate its clinical significance.

Material and Methods

Patients and serum samples

A total of 142 consecutive patients with ccRCC who underwent laparoscopic radical nephrectomy from January 2003 to January 2011 at the Department of Urology, Xuzhou Cancer Hospital, were included in this study. These patients were diagnosed as ccRCC histopathologically for the first time, and had not had any type of anticancer therapy performed before their surgery. The ccRCC tumor of each patient was staged according to the Union for International Cancer Control 2002 classification, and the tumor grading was assessed as previously described [19–21]. The main patient characteristics are shown in Table 1. The time to disease progression was defined as the point at which patients had either a local recurrence or a synchronous/metachronous metastasis as detected by computerized tomography (CT) scan [19–21]. The 5-year overall survival data was also collected. Peripheral blood (10 mL) was collected in clot activator tubes before surgery, and the serum was separated by centrifugation. Samples were subjected to two consecutive centrifugations at 1,800 g for 10 minutes at room temperature to remove the cellular components, and the serum was stored at –80°C. In addition, 34 serum samples from age- and sex- matched normal healthy volunteers were also included. This study was performed according to the Declaration of Helsinki and approved by the local ethics committee (XCH20080107x). Written informed consent was obtained from each participant.

DNA extraction, bisulfite treatment, and methylation-specific PCR (MSP)

The circulating DNA was extracted from 0.8 mL serum using the QIAmp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA); the isolated DNA was modified with bisulfite using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions as described previously [17,22]. The methylation status of the PCDH17 in serum was detected using MSP, and the primers were used as previously reported [17,19,23,24]. Sequences for unmethylation were: forward 5'-AGATTATTGGGTGTTGTAGTTT-3' and reverse 5'-AACCCTAACACAACATACACA-3'; and methylation: forward 5'-GATTATCGGGTGTCGTAGTTC-3' and reverse 5'-CCCTAACGCAACGTACGCG-3' [17,19,23,24]. The PCR amplification was carried out as reported previously [17]. *In vitro* methylated DNA and unmethylated DNA (New England Biolabs, Beverly, MA, USA) were used as controls as described previously. The PCR products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide; DNA bands were visualized by UV light. The methylation-positive samples were defined as methylated allele present in the methylated DNA lane or present both in the methylated and unmethylated DNA lanes and methylation-negative was defined as the band present only in the unmethylated DNA lane [17,19,23,24].

Statistical analysis

The differences of PCDH17 methylation status between ccRCC patients and controls was evaluated by Fisher's exact test. The relationship between PCDH17 methylation and clinicopathologic

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Table 1. The features of the patients with ccRCC (n=142) and its relationship with PCDH17 methylation in serum.

Features	Variables	No.	M (%)	U (%)	P
Sex	Male	97	58 (59.8)	39 (40.2)	0.468
	Female	45	24 (53.3)	21 (46.7)	
Age	<60	61	31 (50.8)	30 (49.2)	0.147
	≥60	81	51 (63.0)	30 (37.0)	
Pathological stage	T1/T2	123	67 (54.5)	56 (45.5)	0.044
	T3	19	15 (78.9)	4 (21.1)	
Fuhrman grade	I/II	96	49 (51.0)	47 (49.0)	0.019
	III/IV	46	33 (71.7)	13 (28.3)	
Lymph node metastasis	N0	133	73 (54.9)	60 (45.1)	0.008
	N1	9	9 (100.0)	0 (0.0)	
Progression	Presence	43	37 (86.0)	6 (14.0)	<0.001
	Absence	99	45 (45.5)	54 (54.5)	
Total		142	82 (57.7)	60 (42.3)	

M – methylation; U – unmethylation.

features was evaluated by chi square test. Kaplan-Meier survival analysis and log-rank test were used for survival analysis. Univariate and multivariate Cox proportional hazards model analysis was used to evaluate the prognostic value of PCDH17 methylation in ccRCC. The statistical analysis was carried out using SPSS 13.0 software. A p value <0.05 was considered statistically significant.

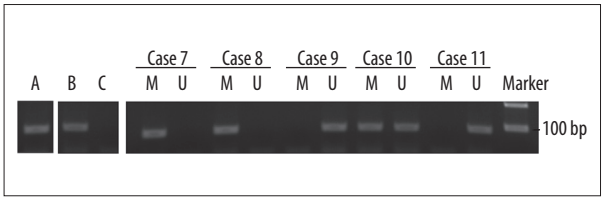

Figure 1. Representative MSP results of PCDH17 methylation in serum samples of ccRCC cases. A – Methylation positive control; B – unmethylation positive control; C – water. Case 7, 8, and 10 represented PCDH17 methylation. Case 9 and 11 represented PCDH17 unmethylation.

Results

Methylation status of PCDH17 in serum samples

The methylation status of PCDH17 in serum samples of 142 ccRCC patients and 34 controls was examined by MSP. We found that PCDH17 methylation was detected in 82 (57.7%) patients (Figure 1), but no methylation was detected in the controls. The difference of PCDH17 methylation status between ccRCC patients and controls was statistically significant (p<0.001).

The relationship between PCDH17 methylation and clinicopathologic features of ccRCC

The main purpose of this study was to evaluate the clinical significance of PCDH17 methylation in serum of patients with ccRCC. Then we correlated PCDH17 methylation status to clinicopathological features. The results suggested that PCDH17 methylation in serum was significantly correlated with advanced stage (p=0.044), higher grade (p=0.019), lymph node metastasis (p=0.008) and tumor progression (p<0.001). However, no correlation was found between PCDH17 methylation and age or sex. These findings are shown in Table 1.

The prognostic value of PCDH17 methylation in serum of ccRCC patients

The main challenge in clinical practice for ccRCC is to predict patient outcomes accurately. For this reason, the relationship between PCDH17 methylation in serum and patient outcomes was evaluated. During the follow-up time, 43 (30.3%) patients experienced disease progression and 15 (10.6%) patients died. The Kaplan-Meier survival analysis and log-rank test suggested that patients with PCDH17 methylation in serum had shorter progression-free survival (p<0.001, Figure 2) and overall survival (p=0.017, Figure 3) than patients with

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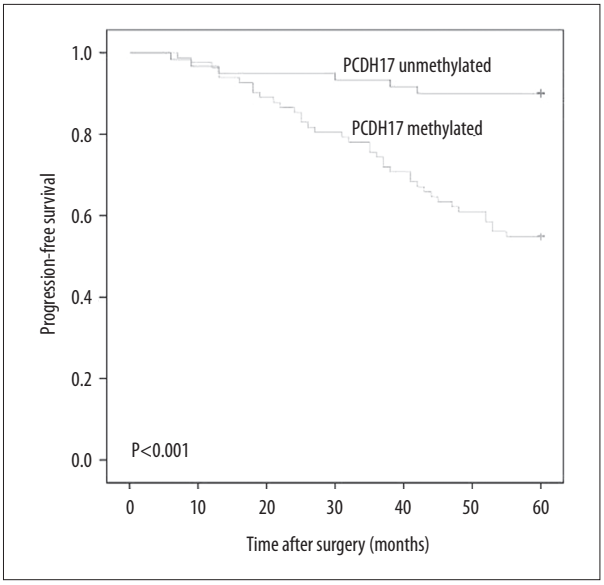

Figure 2. Patients with PCDH17 methylated showed significantly shorter progression-free survival than those without (log-rank test, p<0.001).

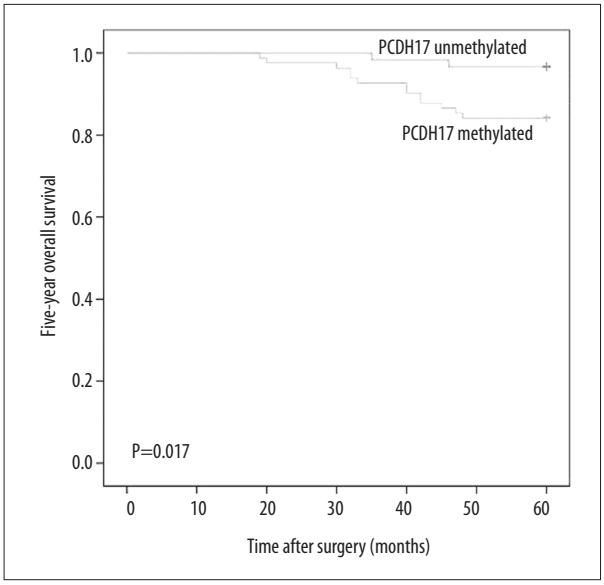

Figure 3. Patients with PCDH17 methylated showed significantly shorter overall survival than those without (log-rank test, p=0.017).

Table 2. Predictive value of protocadherin17 methylation in serum for the progression-free survival in univariate and multivariate analyses.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Sex	0.894	0.765–5.013	0.721			
Age	1.024	0.783–3.762	0.351			
Pathological stage	1.638	1.113–6.327	0.037	1.133	0.832–4.759	0.216
Fuhrman grade	3.752	1.217–8.105	0.007	3.143	1.362–7.531	0.003
Lymph node metastasis	2.561	1.071–10.815	0.011	1.763	1.042–5.321	0.026
PCDH17 methylation	4.215	1.376–9.032	<0.001	4.011	1.137–8.043	<0.001

HR – hazard ratio; M – methylated; U – unmethylated.

PCDH17 unmethylated. Moreover, Cox proportional hazards model analysis suggested that PCDH17 methylation in serum was an independent prognostic factor for worse progression-free survival (p<0.001, Table 2) and overall survival (p=0.046, Table 3) of patients with ccRCC.

Discussion

Genetic and epigenetic changes are involved in the initiation and progression of RCC [8]. Epigenetic changes include DNA methylation, histone modification, and miRNA regulation. However, aberrant DNA methylation is the most common epigenetic change, which can result in the inactivation of important tumor suppressor genes, and associates with tumorigenesis and progression [8]. Aberrant DNA can be analyzed by MSP technique, which allows for the identification of a single methylated allele among hundreds of unmethylated alleles. Recent studies indicate that MSP is a rapid, simple, and cost effective method, which allows for rapid examination of multiple samples, and is convenient for clinical or basic research [12].

DNA methylation can be detected not only in tumor tissues, but also in body fluids, especially in the serum, and aberrant DNA methylation in serum appears to be a very consistent feature of cancer [13,25–27]. This consistency makes it amenable to the design of widely applicable clinical assays.

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Table 3. Predictive value of protocadherin17 methylation in serum for the overall survival in univariate and multivariate analyses.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Sex	0.873	0.788–3.684	0.762			
Age	1.006	0.841–2.719	0.473			
Pathological stage	2.761	1.108–9.362	0.124			
Fuhrman grade	3.744	1.063–8.456	0.048	1.643	0.748–10.251	0.365
Lymph node metastasis	4.625	1.133–10.962	0.040	2.895	0.832–11.072	0.074
PCDH17 methylation	5.092	1.149–12.357	0.032	3.93	1.354–9.143	0.046

HR – hazard ratio; M – methylated; U – unmethylated.

Currently, there is a very large amount of literature describing DNA methylation patterns in tumor tissue and their potential use for diagnosis and prognosis, and disease monitor after surgery [28,29]. However, research that focuses on DNA methylation serum is relatively scarce.

In the present study, we focused on the methylation status of PCDH17 in serum of RCC patients and its clinical significance. PCDH17 is a tumor suppressor in several human cancers, and plays crucial roles in cell adhesion, signal transduction, proliferation, and invasion [16]. Previous studies indicated that PCDH17 was frequently inactivated by aberrant promoter methylation in RCC, and this is the first study to research PCDH17 methylation in serum of patients with RCC [19,30]. We have found a significantly high frequency of PCDH17 methylation in serum of cases with RCC. This is in accordance with previous studies on PCDH17 methylation status in bladder cancer and prostate cancer [17,31]. Accordingly, we explored the possible association between PCDH17 methylation in serum and clinicopathological factors. The results confirmed that PCDH17 methylation significantly correlated with advanced stage, higher grade, and lymph node metastasis. These are all risk factors for poor outcomes of RCC patients. On the basis of these observations, we decided to explore the possibility of using PCDH17 methylation in serum as a prognostic biomarker in RCC patients. Subsequently, Kaplan-Meier survival analysis and log-rank test were performed; the result suggested that patients with PCDH17 methylation in serum had shorter progression-free survival and overall survival than patients with PCDH17 unmethylated. Moreover, multivariate Cox proportional hazards model analysis suggested that PCDH17 methylation in serum was an independent prognostic factor for worse progression-free survival and overall survival for patients with RCC. These data indicated that the detection of PCDH17 methylation in serum can be a potential biomarker for predicting RCC patient prognosis and formonitoring tumor progression after surgery. However, the number of patients in our study was relatively small, and further study in a large sample size is needed to confirm our findings and to optimize the strategy and protocol for this purpose. In any event, the most remarkable characteristics of PCDH17 methylation detection in serum are that it is efficient, rapid, low cost, and non invasive, and as such demonstrate potential application in the future.

Conclusions

In conclusion, the results of the present study indicate that PCDH17 methylation in serum is a frequent event in RCC and associated with risk factors of poor outcomes. Moreover, PCDH17 methylation in serum is a potential prognostic biomarker for patients with RCC after surgery.

Competing of interests

The authors had no conflicts of interest to declare in relation to this article.

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