Association between protocadherin 8 promoter hypermethylation and the pathological status of prostate cancer

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Received May 5, 2015; Accepted October 26, 2016

DOI: 10.3892/ol.2017.6282

Abstract. Promoter hypermethylation of tumor suppressor genes has been confirmed to serve a pivotal role in tumorigenesis. Protocadherin 8 (*PCDH8*), a novel tumor suppressor gene, has been reported to be inactivated by promoter hypermethylation a number of cancer types, including bladder cancer and renal cell carcinoma. The aim of the present study was to investigate the occurrence of *PCDH8* hypermethylation in prostate cancer and its potential as a novel biomarker of prostate cancer. The transcriptional levels of *PCDH8* were examined by quantitative polymerase chain reaction (PCR) in 82 prostate cancer tissues as well as 30 prostate hyperplasia tissues, and verified the protein level by western blot analysis of representative samples. *PCDH8* expression levels were found to be reduced to 0.30±0.10 in 70.7% (58/82) of prostate cancer tissues. To identify the possible reason for mRNA down-regulation, the methylation status of the *PCDH8* promoter was assessed in prostate cancer tissues and prostate hyperplasia tissues by methylation-specific PCR (MSP). A total of 47 prostate cancer patients who exhibited reduced *PCDH8* expression (57.3%; 47/82) also showed promoter hypermethylation (47/58). None of the samples (0/30) in the benign prostate hyperplasia group were positive on MSP. Furthermore, the associations between the methylation status of the *PCDH8* promoter and various clinicopathological features of prostate cancer were analyzed, revealing that the methylation status of *PCDH8* was closely associated with tumor size, tumor shape (papillary/non-papillary), tumor stage and tumor grade (all $P<0.05$), while there were no correlations with the age of the patients or the number of tumors ($P>0.05$). Additionally, patients with hypermethylation of the *PCDH8* gene promoter had a relapse rate of 36.17% and a mortality rate of 29.79%, which were significantly higher than the hypermethylation-negative patients ($P<0.05$), indicating a poorer prognosis. Therefore, the methylation status of the *PCDH8* gene in prostate cancer may be an important marker for use in the early diagnosis and prediction of prognosis in prostate cancer.

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Key words: prostate cancer, protocadherin 8, promoter hypermethylation, prognosis

Introduction

Prostate cancer is the second leading cause of cancer-associated mortality in men worldwide, and the incidence of the disease is increasing year by year (1). Currently, the level of prostate-specific antigen, the clinical stage and the grade of the tumor (Gleason score) are the predominant methods for predicting the prognosis of prostate cancer (2). However, the epigenetic silencing of key tumor suppressors genes during prostate cancer tumorigenesis and progression may be translated into a promising clinical diagnostic marker (2). CpG islands at transcription initiation sites are generally poorly methylated. However, in certain cancer tissues, hypermethylation of CpG may silence gene transcription (3). In prostate cancer tissues, a number of large-scale genome-wide analyses have suggested that >30 key genes may be regulated through promoter hypermethylation (3). At present, there are still relatively few biomarkers for the prediction of prostate cancer (2).

Protocadherin 8 (*PCDH8*) is a member of the cadherin family and has been identified to be a novel tumor suppressor gene that is silenced in a number of cancer types, including gastric cancer, breast cancer, bladder cancer and renal cell carcinoma (4-7). Transcriptional silencing may occur via genetic mutation (5,6) or epigenetic promoter hypermethylation (4-7).

The present study analyzed the methylation status of the promoter of the *PCDH8* gene in prostate cancer tissues, with the aim of identifying a potential association between the methylation status and the diagnosis and prognosis of prostate cancer.

Materials and methods

Patients and specimens. According to the prostate cancer diagnostic criteria of the World Health Organization and National Cancer Institute for prostate cancer (8), 82 patients with prostate cancer and 30 hospitalized patients with benign prostatic hyperplasia at Yantai Yuhuangding Hospital (Yantai, China) were enrolled between May 2010 and May 2012. The

inclusion criteria were as follows: i) no radiation therapy, chemotherapy or hormone therapy administered prior to surgery; ii) general information, including clinical stage and histological grade, is available; iii) diagnosis of primary prostate cancer; iv) confirmed prostate cancer by pathological report. The exclusion criteria were as follows: i) history of other malignant tumors; ii) incomplete clinical data; iii) presence of remote metastatic or recurrent prostate cancer. The prostate cancer patients were aged between 37 and 77 years, with a mean age of 58.1±3.9 years. There were 34 cases with a single tumor, and 48 cases with multiple tumors. With regard to tumor size, 48 cases had a tumor diameter of ≤3 cm, and 34 cases had a diameter of >3 cm. A total of 57 cases were papillary and 25 cases were non-papillary. There were 55 cases of grade (G)1-G2, and 27 cases of G3. Regarding tumor stage, a total of 49 cases were Ta-T1 and 33 cases were T2-T4 (9,10).

DNA extraction. All surgical specimens were placed immediately in liquid nitrogen following the surgery. The specimens (~25 mg) were ground in liquid nitrogen and DNA was extracted using QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany; lot 130217) according to the manufacturer's instructions. The purity and concentration of DNA was measured using a DU800 ultraviolet (UV) spectrophotometer (Beckman-Coulter, Inc., Brea, CA, USA).

Methylation-specific polymerase chain reaction (MSP). The methylation status of *PCDH8* was determined by MSP (11). A total of 1 μg genomic DNA was modified by methylation using an EZ DNA Methylation-Gold Kit (UZymo Research Corporation, Irvine, CA, USA; lot 130522) according to the manufacturer's instructions. In this method, following the genomic DNA methylation reaction, unmethylated cytosine is converted to uracil, whereas methylated cytosine remains unchanged. Therefore, specific primers may be used to differentiate the methylation status. The primers were designed as previously described (12). All primers were synthesized by BayGene Biotech Company Limited (Beijing, China). The primers for the methylated reaction were as follows: Sense, 5'-CGGTTATTGGTTATTCGGTTCC-3'; and antisense, 5'-ACGAACTCTAAAAACGCGCG-3'; product size, 94 bp. The primers for the unmethylated reaction were as follows: Sense, 5'-GGTGGTTATTGGTTATTTGGTTT-3'; antisense, 5'-CCAACAAACTCTAAAAACACACA-3'; product size, 97 bp. Water was used as a blank control in each assay. PCR products (10 μl) were separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination. Specimens were scored as methylation-positive when a specific band was amplified by methylated reaction-specific primers, and negative when amplified only by unmethylated reaction-specific primers. The thermocycling conditions were as follows for a total of 40 cycles: Denaturing at 95˚C for 30 sec, annealing at 60˚C for 5 sec and extension at 72˚C for 45 sec. The $2^{-\Delta\Delta Cq}$ method (13) was used for the quantification of the PCR results.

Western blotting. Western blotting was performed to study the protein expression levels of PCDH8 in the clinical samples. A total of 1 mg tissue was solubilized in 100 μl lysis buffer (30 mM Tris, 2 M Thiourea, 4% CHAPS and 7 M urea; pH 8.5) on ice for 30 min. The lysis buffer was subsequently centrifuged at 1,000 x *g* for 15 min and the supernatant was collected for further testing. Primary antibodies were used for incubation at 4˚C overnight and secondary antibodies at room temperature for 1 h. Primary antibodies targeting PCDH8 (1:1,000 dilution; catalog no. ab85561; Abcam, Cambridge, UK) and actin (1:1,000; rabbit; polyclonal; cat. no. ab8227; Abcam, Cambridge, UK) were used. The following secondary antibodies were used: Anti-rabbit IgG-biotin (cat. no. BA1020, Boster Biological Technology, Wuhan, China). Bands were detected using the DAB Chromogenic Reagent kit (cat. no. AR1021; Boster, Biological Technology).

Postoperative follow-up. Following surgery, patients were followed up for 6-24 months for the analysis of recurrence and survival.

Statistical analysis. All data were statistically analyzed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). The different mRNA and protein expression between cancer tissues and controls were analyzed with the Student's t-test. For survival data, differences between two groups were assessed with the log-rank test. Kaplan-Meier curves of ovarall survival were constructed. The differences between clinicopathological features and the status of methylation were analyzed using the χ^2 test. The data were presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of PCDH8 is reduced in prostate cancer tissues. As a tumor suppressor, the expression of *PCDH8* is reduced in various cancer types (4-7). Therefore, the present study investigated the expression levels of *PCDH8* in prostate cancer tissues by quantitative polymerase chain reaction (qPCR). The results revealed that the mRNA level of *PCDH8* was reduced to 0.30±0.10 in 70.7% (58/82) of prostate cancer tissues (Table I). Cases 22-25 are shown as representative samples for the mRNA results in Fig. 1. This result was also verified at the protein level by western blot analysis (Fig. 2). The mRNA and protein levels in cases 22 and 24, but not in cases 23 and 25, were reduced compared with the control (Table I; Figs. 1 and 2).

Methylation status of PCDH8 gene promoter. To identify the possible reason for the mRNA downregulation, the methylation status of the *PCDH8* promoter was examined. The results revealed that the benign prostatic hyperplasia and prostate cancer samples that did not exhibit *PCDH8* reduction were not methylated (Tables I and II). The 47 cases in which methylation was detected were all prostate cancer samples that exhibited *PCDH8* reduction (Table I; Fig. 3A). Representative results from cases 22-25 are shown in Fig. 3B.

Analysis of the association between methylation status of PCDH8 and various clinicopathological features. The detailed clinicopathological features of patients with prostate cancer are shown in Table III. The results demonstrated that *PCDH8* methylation was significantly associated with a larger

Table I. Relative *PCDH8* expression and methylation summary for patients with prostate cancer (n=82) or benign prostatic hyperplasia.

		Relative mRNA expression			
Group	Effect	Mean	SEM	P-value	MSP-positive
Control (C1-C3)	-	1.00	0.11	-	-
Cases with no *PCDH8* reduction (n=24/82; 29.3%)					
P3	N	1.03	0.21	N.S.	n
P4	N	1.21	0.14	N.S.	n
P10	N	1.17	0.13	N.S.	n
P14	N	1.08	0.22	N.S.	n
P15	N	1.22	0.32	N.S.	n
P23	N	1.12	0.21	N.S.	n
P25	N	0.91	0.09	N.S.	n
P31	N	1.08	0.28	N.S.	n
P38	N	0.86	0.14	N.S.	n
P39	N	0.85	0.23	N.S.	n
P40	N	1.08	0.27	N.S.	n
P45	N	0.88	0.14	N.S.	n
P46	N	0.93	0.13	N.S.	n
P47	N	1.21	0.37	N.S.	n
P50	N	0.79	0.32	N.S.	n
P55	N	0.86	0.09	N.S.	n
P58	N	0.81	0.16	N.S.	n
P59	N	1.08	0.11	N.S.	n
P64	N	1.32	0.44	N.S.	n
P73	N	1.21	0.13	N.S.	n
P76	N	1.24	0.21	N.S.	n
P78	N	0.86	0.16	N.S.	n
P80	N	1.21	0.28	N.S.	n
P81	N	0.94	0.08	N.S.	n
Cases with *PCDH8* reduction (n=58/82; 70.7%)					
P1	R	0.27	0.03	<0.005	y
P2	R	0.33	0.18	<0.050	y
P5	R	0.41	0.13	<0.050	n
P6	R	0.39	0.18	<0.050	y
P7	R	0.35	0.11	<0.005	y
P8	R	0.31	0.13	<0.005	y
P9	R	0.25	0.18	<0.050	n
P11	R	0.32	0.09	<0.010	y
P12	R	0.28	0.07	<0.050	y
P13	R	0.39	0.13	<0.050	n
P16	R	0.33	0.13	<0.050	y
P17	R	0.37	0.18	<0.005	y
P18	R	0.17	0.04	<0.010	y
P19	R	0.42	0.03	<0.050	n
P20	R	0.40	0.18	<0.050	y
P21	R	0.38	0.11	<0.005	y
P22	R	0.18	0.08	<0.010	y
P24	R	0.31	0.07	<0.010	y
P26	R	0.24	0.18	<0.005	y
P27	R	0.22	0.07	<0.010	n
P28	R	0.39	0.07	<0.050	y

Table I. Continued.

Group	Effect	Relative mRNA expression		P-value	MSP-positive
		Mean	SEM		
Cases with *PCDH8* reduction (n=58/82; 70.7%)					
P29	R	0.27	0.13	<0.005	y
P30	R	0.40	0.02	<0.010	n
P32	R	0.33	0.13	<0.005	y
P33	R	0.30	0.05	<0.005	y
P34	R	0.32	0.07	<0.010	y
P35	R	0.39	0.08	<0.050	y
P36	R	0.30	0.13	<0.005	y
P37	R	0.17	0.04	<0.005	n
P41	R	0.30	0.13	<0.010	y
P42	R	0.21	0.13	<0.010	y
P43	R	0.19	0.13	<0.010	y
P44	R	0.24	0.02	<0.005	y
P48	R	0.27	0.09	<0.005	y
P49	R	0.36	0.18	<0.010	n
P51	R	0.27	0.09	<0.010	y
P52	R	0.39	0.04	<0.050	y
P53	R	0.30	0.05	<0.005	y
P54	R	0.33	0.11	<0.010	y
P56	R	0.34	0.08	<0.050	y
P57	R	0.24	0.04	<0.010	y
P60	R	0.24	0.13	<0.010	n
P61	R	0.40	0.05	<0.005	y
P62	R	0.37	0.08	<0.010	y
P63	R	0.22	0.09	<0.050	y
P65	R	0.15	0.03	<0.010	y
P66	R	0.34	0.14	<0.050	y
P67	R	0.31	0.05	<0.050	y
P68	R	0.21	0.05	<0.010	n
P69	R	0.33	0.08	<0.010	y
P70	R	0.17	0.07	<0.005	y
P71	R	0.32	0.08	<0.005	y
P72	R	0.24	0.18	<0.010	y
P74	R	0.27	0.04	<0.010	n
P75	R	0.15	0.03	<0.050	y
P77	R	0.32	0.09	<0.050	y
P79	R	0.24	0.13	<0.005	y
P82	R	0.33	0.08	<0.010	y
Mean	-	0.30	0.10	-	-

Samples P1-P82 were from 82 prostate cancer patients, and C1-C3 were from controls with benign prostatic hyperplasia. *PCDH8*, protocadherin 8; SEM, standard error of the mean; MSP, methylation-specific polymerase chain reaction; N, not affected; R, reduced; N.S., not significant; n, MSP-negative; y, MSP-positive.

tumor diameter (>3 vs. ≤3 cm; P=0.016), non-papillary shape (P=0.023), advanced tumor stage (T_2-$T_{4\ vs.\ Ta\text{-}T1;\ P=0.016}$) and advanced pathological grade ($G_{3\ vs.\ G1\text{-}2;\ P=0.009}$). By contrast, no significant associations were identified between *PCDH8* methylation and age (P=0.842) or number of tumors (P=0.500).

Postoperative follow-up to assess the association between PCDH8 methylation and prognosis. Survival data were collected for 6-24 months after surgery. The data revealed that the methylation status of *PCDH8* is associated with the overall survival time of patients with prostate cancer; those

Table II. Relative protocadherin 8 expression and methylation summary for controls with benign prostatic hyperplasia (n=30).

Case no.	Effect	Relative mRNA expression		P-value	MSP-positive
		Mean	SEM		
Control[a]	-	1.00	0.11	-	-
C1	N	1.07	0.13	N.S.	n
C2	N	0.96	0.08	N.S.	n
C3	N	1.17	0.12	N.S.	n
C4	N	1.24	0.08	N.S.	n
C5	N	1.22	0.22	N.S.	n
C6	N	1.05	0.13	N.S.	n
C7	N	1.21	0.21	N.S.	n
C8	N	1.20	0.09	N.S.	n
C9	N	1.06	0.16	N.S.	n
C10	N	1.08	0.14	N.S.	n
C11	N	1.17	0.14	N.S.	n
C12	N	0.84	0.22	N.S.	n
C13	N	1.04	0.28	N.S.	n
C14	N	1.21	0.08	N.S.	n
C15	N	1.24	0.21	N.S.	n
C16	N	0.86	0.23	N.S.	n
C17	N	1.21	0.19	N.S.	n
C18	N	0.94	0.16	N.S.	n
C19	N	1.10	0.11	N.S.	n
C20	N	0.91	0.08	N.S.	n
C21	N	1.23	0.20	N.S.	n
C22	N	1.11	0.07	N.S.	n
C23	N	0.89	0.19	N.S.	n
C24	N	1.24	0.28	N.S.	n
C25	N	1.20	0.10	N.S.	n
C26	N	1.02	0.19	N.S.	n
C27	N	0.98	0.05	N.S.	n
C28	N	1.26	0.23	N.S.	n
C29	N	1.14	0.16	N.S.	n
C30	N	1.24	0.19	N.S.	n

[a]Average of C1-C3. SEM, standard error of the mean; MSP, methylation-specific polymerase chain reaction; N, not affected; N.S., not significant; n, MSP-negative.

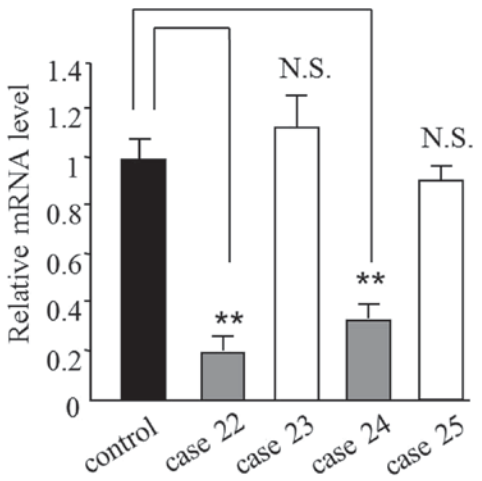

Figure 1. Representative mRNA levels of *PCDH8* from 4 patients with prostate cancer and the control. The mRNA level of *PCDH8* was reduced in cases 22 and 24. **P<0.01 compared with control; N.S., not significant compared with control. PCDH8, protocadherin 8.

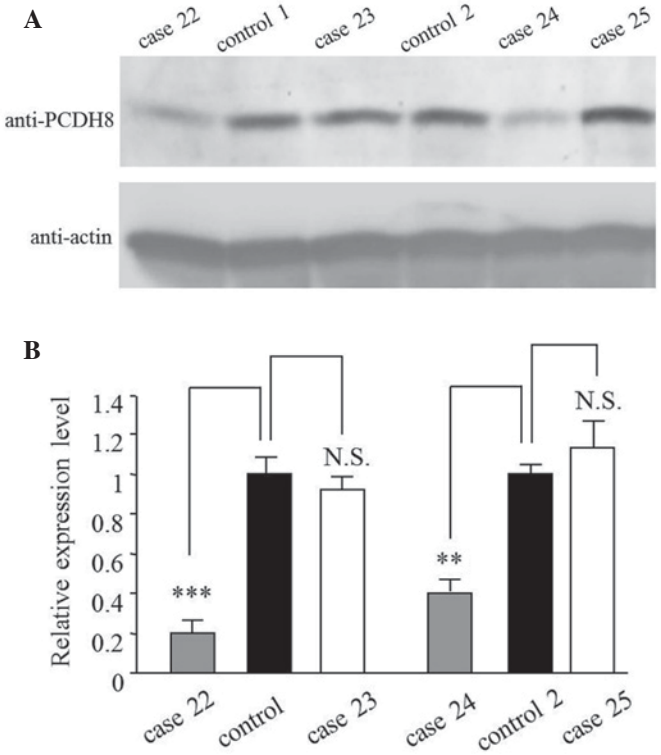

Figure 2. Protein levels of PCDH8 from 4 representative patients with prostate cancer and controls. (A) Western blot and (B) quantification of western blot. The protein level of PCDH8 was reduced in cases 22 and 24. **P<0.01 and ***P<0.005 compared with control; N.S., not significant compared with control. PCDH8, protocadherin 8.

with unmethylated *PCDH8* have a better prognosis (recurrence rate, 14.29; and mortality rate, 8.57%) compared with those with methylated *PCDH8* (recurrence rate, 36.17%; mortality rate, 29.79%). These differences were statistically significant (recurrence rate, P=0.027; mortality rate, P=0.019) (Table IV; Fig. 4).

Discussion

In recent years, the incidence of prostate cancer has been increasing. At present, a comprehensive treatment program consisting of radioactive seed implantation combined with radical prostatectomy has achieved remarkable results (14), particularly in those patients diagnosed early. However, a clinical survey found that distant metastasis occurs in 17-51% of prostate cancer cases, and local recurrence in 6-21% (15). Furthermore, the treatment of patients with metastasis and recurrence is often challenging, and the 5-year survival rate is <77% for such patients (16). Clinical studies have demonstrated

Table III. Association between protocadherin 8 methylation and pathological features of patients with prostate cancer.

Feature	Total, n	Unmethylated, n (%)	Methylated, n (%)	χ^2 value	P-value
Age, years				0.040	0.842
≤65	50	27 (54.00)	23 (46.00)		
>65	32	18 (56.25)	14 (43.75)		
Tumor number				0.455	0.500
Single	34	16 (47.06)	18 (52.94)		
Multiple	48	19 (39.58)	29 (60.42)		
Tumor diameter				5.828	0.016
≤3 cm	46	25 (54.35)	21 (45.65)		
>3 cm	36	10 (27.78)	26 (72.22)		
Tumor shape				5.131	0.023
Papillary	57	29 (50.88)	28 (49.12)		
Non-papillary	25	6 (24.00)	19 (76.00)		
Tumor stage				5.803	0.016
Ta-T1	51	27 (52.94)	24 (47.06)		
T2-T4	31	8 (25.81)	23 (74.19)		
Pathological grade				6.888	0.009
G1-G2	55	29 (52.73)	26 (47.27)		
G3	27	6 (22.22)	21 (77.78)		

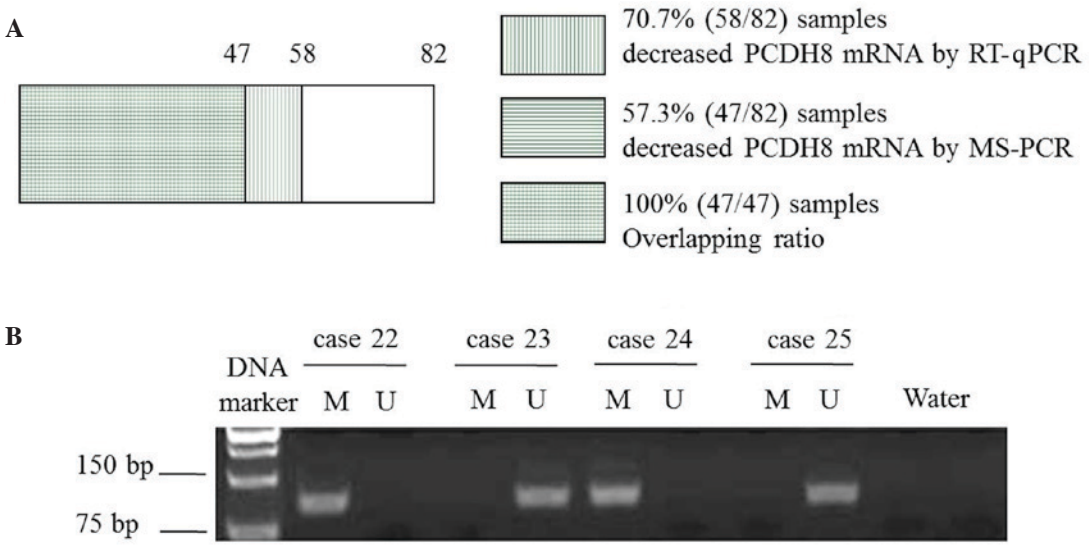

Figure 3. (A) Summary of RT-PCR and MS-PCR results. 47 samples with PCDH8 methylation all showed decreased PCDH8 expression. The overlapping ratio was 100%. (B) Methylation statuses of PCDH8 from 4 representative patients with prostate cancer. Cases 22 and 24 exhibited *PCDH8* promoter methylation. PCDH8, protocadherin 8; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MS-PCR, methylation-specific polymerase chain reaction; M, methylated; U, unmethylated.

that molecular markers, including growth hormone, vascular endothelial growth factor and transforming growth factor β1 may provide information relevant for prostate cancer diagnosis and treatment (10,15). For instance, studies have shown that patients with lymph node metastases had significantly higher expression of VEGF-C than patients without lymph node metastases (17). DNA methylation is a common form of epigenetic modification and changes in its status have been identified in multiple types of cancer. Hypermethylation of a gene inactivates its expression, which is important in the process of tumorigenesis (18). Therefore, the study of DNA methylation is significant for the diagnosis and prognosis of cancer.

PCDH8 is a recently identified member of the cadherin family (19). The protein is composed of an intracellular domain, a transmembrane domain and six repeating extracellular regions. Previous studies have revealed that *PCDH8* is important in cytoskeleton formation, intercellular signaling,

Table IV. Postoperative follow-up analysis (n=82).

Status	Total, n	Recurrence rate, n (%)	Mortality rate, n (%)
Methylated	47	17 (36.17)	14 (29.79)
Unmethylated	35	5 (14.29)	3 (8.57)
χ^2 value		4.894	5.495
P-value		0.027	0.019

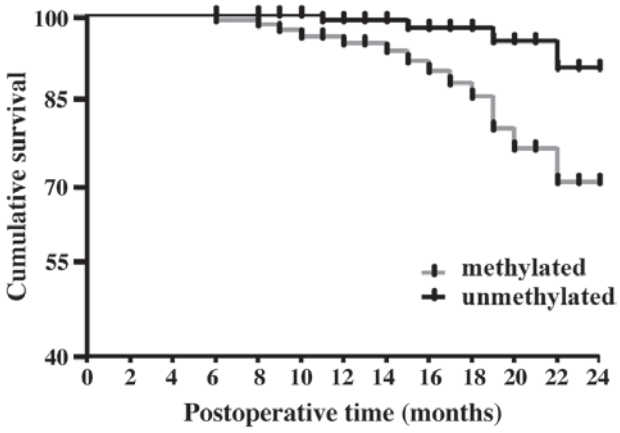

Figure 4. Kaplan-Meier survival curves for patients with different *PCDH8* methylation statuses. The curves show the overall survival time.

cell growth and differentiation (20). Létoquart *et al* found that the methylation of *PCDH8* was higher in patients with breast cancer than in those with breast hyperplasia, and that the methylation status of *PCDH8* is associated with the stage and metastasis of breast cancer (21). Heichman and Warren found that *PCDH8* methylation occurs in pancreatic cancer, whereas there is no occurrence in normal human pancreatic tissues (22). These findings indicate that *PCDH8* gene methylation may be closely associated with the occurrence and development of cancer (23). In the present study, the methylation status of the *PCDH8* gene promoter was examined and the correlation between methylation status and prostate cancer progression was analyzed. The results revealed that in 82 cases of prostate cancer, *PCDH8* gene promoter methylation was present in 57.32%, while no such methylation was present in benign prostatic hyperplasia samples; this is consistent with the results of clinical studies (24). These results demonstrate that the methylation of *PCDH8* may be associated with the occurrence of prostate cancer and could potentially be used as a molecular marker for the early diagnosis of prostate cancer.

PCDH8 gene is a tumor suppressor gene that is downregulated in a variety of malignant tumors. To date, studies have found that the methylation of the *PCDH8* promoter is an important factor for its downregulation (25,26). Inactivation of *PCDH8* weakens its ability to suppress tumorigenesis. In the present study, in cases in which *PCDH8* promoter hypermethylation was identified, a reduced mRNA level of these gene was also found. However, in certain cases that exhibited a reduced mRNA level, the promoter of *PCDH8* was not hypermethylated, indicating that other mechanisms may regulate the expression of *PCDH8*.

PCDH8 was found to be methylated in prostate cancer patients with a larger tumor diameter (>3 cm) and advanced tumor stage (T2-T4) and pathological grade (G3) (27), indicating that *PCDH8* methylation is significantly associated with the degree of differentiation and depth of invasion of the cancer. Furthermore, the data obtained in the present study revealed that the methylation of *PCDH8* is highly associated with tumor shape, with the non-papillary form more common than the papillary form in cases showing promoter methylation. This difference may be related to the geographical location, test methods, sample size, individual differences and other factors (28).

In the present study,postoperative follow-up of the 82 patients revealed that patients with methylated *PCDH8* had a poorer outcome compared with those with unmethylated PCDH8, indicating that methylation status may be associated with the prognosis of patients with prostate cancer. Considering the limited sample size of this study, a large multi-center study is necessary to confirm the findings.

In summary, the methylation of the *PCDH8* gene promoter in prostate cancer is associated with the development and prognosis of prostate cancer and may be used as a molecular marker to determine the early diagnosis and prognosis of prostate cancer.

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