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Original Article

Expression profiling and clinicopathological significance of DNA methyltransferase 1, 3A and 3B in sporadic human renal cell carcinoma

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Abstract: Purpose: This study aimed to evaluate the expression of DNA methyltransferase (DNMT) family proteins in renal cell carcinoma (RCC) and to assess the clinical significance and prognostic value of their expression patterns. Methods: A total of 97 renal cell carcinoma and 52 no-tumor tissues were recruited for immunohistochemical analysis of their expression. Results: DNMT1, DNMT3A and DNMT3B proteins were highly expressed in clear cell RCC, papillary RCC and chromophobe RCC tissues than that of no-tumor tissues (all $P < 0.05$). DNMT1, DNMT3A and DNMT3B expression was significantly associated with tumor size (P=0.003, 0.001 and 0.003, respectively), tumor pathology stage (P=0.039, 0.034 and 0.037, respectively), histopathological grading (P=0.042, 0.026 and 0.031, respectively), lymph node metastasis (P=0.022, 0.030 and 0.020, respectively) and vascular invasion (P=0.042, 0.031 and 0.044, respectively). The Kaplan-Meier survival analysis demonstrated that expression of DNMTs protein in RCC was significantly associated with shorter over all survival and disease-free survival (all $P < 0.05$). Furthermore, multivariate analysis showed that the expression of DNMT1 was an independent prognostic factor for overall survival (OS) (P=0.036), and the expression of DNMT3A or DNMT3B was an independent prognostic factor for disease-free survival (DFS) in the patients (P=0.031 and P=0.023, respectively). Conclusions: DNMTs were higher expressed in RCC than no-tumor tissues, and the expression of DNMTs were strongly associated with RCC tumor size, tumor pathology stage, histological grading, lymph node metastasis, vascular invasion, recurrence, and prognosis. DNMTs may thus serve as prognostic markers and novel therapeutic targets for RCC patients.

Keywords: Renal cell carcinoma, DNA methyltransferase, progression, prognosis

Introduction

Renal cell carcinoma (RCC) is a common urologic malignancy and accounts for about 3% of adult malignancies and causes about 90,000 deaths worldwide annually [1]. The rate of RCC has increased by over 2% per year for the past 3 decades [2]. Because RCC has a highly resistant phenotype to conventional therapeutic modalities, including chemotherapy and radiation, surgical resection of localized disease has been regarded as the only curative treatment. However, 20-30% of these patients experience local and/or distant disease recurrence [3]. Moreover, up to 30% of patients have metastases at the time of the initial diagnosis [4]. The prognosis of RCC is very poor, with the highest mortality rate among all the genitourinary tract tumors and a third of patients dying from their disease [5].

Epigenetics refers to stable alterations in gene expression with no underlying modifications in the genetic sequence. Several epigenetic mechanisms regulate gene expression, including DNA methylation, chromatin remodeling, histone variants, and the epigenetic function of non-coding RNA [6]. Among these, DNA methylation is a covalent modification of DNA that plays a significant role in the regulation of gene transcription [7]. Nevertheless, abnormal DNA methylation does also play an important role in human cancer development, and most cancer cells show a global hypomethylation of the genome that induces abnormal expression of genes but a local hypermethylation that silences tumor suppressor genes [6].

Methylation changes to the epigenome are controlled by DNA methyltransferases (DNMTs), which catalyze the transfer of a methyl group from the methyl donor S-adenosyl methionine onto the 5-position on the cytosine ring. Three catalytically active DNMTs have been identified in mammals, DNMT1, DNMT3A, and DNMT3B [8]. The levels of DNMT1, DNMT3A, and DNMT3B mRNA are reportedly elevated in various malignancies, including breast, liver and prostate tumors [9-11].

Similar to other malignancies, aberrant DNA methylation on CpG islands is also an important mechanism for human renal cell carcinoma development. Recently *in vitro* studies have shown DNMTs inhibitor can induce apoptosis in RCC cells [12]. In addition, another study suggest that DNMTs inhibitor could suppress RCC cell proliferation by inducing G2/M cell cycle arrest and strikingly increase the susceptibility of RCC to paclitaxel [13], leading to using DNMT inhibitors in clinical trials of renal cancers [14-16]. Surprisingly, the specific role of the different DNMT isoforms in carcinogenesis and tumor progression of renal cell carcinoma is not well understood.

In this study, we collected 97 cases of renal cell carcinoma samples and 52 cases of adjacent non-tumor tissues for detection of DNMT family protein expressions in order to determine the role of DNMT proteins in renal cancer and clinical significance.

Patients and tissue specimens

Renal cell carcinoma tissue was collected from radical nephretomy specimens performed between January 2004 and January 2012 at Department of Urology, Shengjing Hospital of China Medical University. The tumor cases included 97 cases with histologically confirmed malignant carcinoma and 52 cases of adjacent non-tumor tissues. The criteria for study enrollment were as follows: patients with histopathologically diagnosed RCC who were newly diagnosed, untreated without a history of other tumors, and subsequently underwent radical nephrectomy. Histological diagnosis was established according to the guidelines of the World Health Organization [17]. Cases were selected according to tissue availability and were not stratified for any known preoperative or prognostic factor. None of the patients underwent chemotherapy, radiotherapy, or adjuvant treatment before surgery. We obtained the written informed consent from all the patients. The Institutional Review Board of China Medical University approved the research protocol. The patients were carefully followed up by consulting their case documents and through telephone monitoring.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue samples were cut into 4-mm thick sections and mounted onto poly-L-lysine-coated glass slides. For immunohistochemical staining, the sections were deparaffinized in xylene, rehydrated in a series of alcohol, and washed in the tap water. The sections were then cooked in 10 mM sodium citrate buffer, pH 6.0, for 10 min in an autoclave for antigen retrieval. Endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 at 37°C for 20 min. After that, the sections were blocked to avoid nonspecific binding by addition of a 10% normal goat serum at 37°C for 30 min and then incubated for 4°C overnight with the polyclonal antibody against DNMTs (DNMT1, sc-20701, 1:250 dilution; DNMT3a, sc-20703, 1:250 dilution; DNMT3b sc-130740, 1:250 dilution; Santa Cruz Biotechnology, USA). The specificity of antibodies had been confirmed by using Western blot analysis (data not shown). In the next day, the sections were washed five times with 0.01 mol/L phosphate-buffered saline (PBS; pH 7.4) for 15 min and then incubated with a biotinylated secondary antibody for 30 min at 37°C in the dark. After that, the sections were incubated with a streptavidin horseradish peroxidase solution for another 30 min (LSAB kit; Dako, Glostrup, Denmark), washed in PBS, and stained with DAB (3, 3-diaminobenzidine). Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. Negative controls were run in parallel, and were generated by PBS replacing the anti-DNMTs antibody.

Evaluation of immunohistochemistry

The immunostained sections were evaluated by two investigators who were blinded to the patients' clinicopathological characteristics. For each slide, the number of DNMTs positive cells was counted in 10 fields at ×200 magnification, and the percentage of positively stained cells was determined. The percentage of positively stained cells was graded semi-quantitatively according to a four-point scoring system as follows: negative (-), 0; weakly positive (+), < 25%; moderately positive (++), 26-50%; and strongly positive (+++), > 50%.

Table 1. Patient characteristics

Feature	Categories	Number	%
Gender			
	Female	37	38.1
	Male	60	61.9
Age, years			
	≤ 55	59	60.8
	> 55	38	39.2
Histological type			
	Clear cell RCC	60	61.9
	Papillary RCC	23	23.7
	Type1	10	10.3
	Type2	13	13.4
	Chomophobe RCC	14	14.4
Tumor size (cm)			
	≤ 4	20	20.6
	> 4	77	79.4
Tumor pathology stage			
	pT1a	20	20.6
	pT1b	25	25.8
	pT2a	17	17.5
	pT2b	11	11.3
	pT3a	16	16.5
	pT3b	2	2.1
	pT4	6	6.2
Grading			
	G1	25	25.8
	G2	33	34.0
	G3	27	27.8
	G4	12	12.4
Lymph node metastasis			
	pNx	21	21.6
	pN0	62	63.9
	pN1/2	14	14.4
Vascular invasion			
	No	91	93.8
	Yes	6	6.2
Tobacco smoking			
	No	41	42.3
	yes	56	57.7

Statistical analysis

Statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, USA). Comparison of DNMTs expression between samples was analyzed by using the Mann-Whitney U-test. Chi-square tests were applied to assess associations between expression of DNMTs and clinicopathological parameters. Univariate survival analysis was carried out according to Kaplan-Meier, differences in survival curves were assessed with the log rank test. Cox regression analysis was used for the multivariate analysis. *P*-values < 0.05 were considered significant.

Results

Patient characteristics

The clinicopathological data from the patients are shown in **Table 1**. The mean age of the patients at surgery was 53 years (rang 15-84), and 59 (60.8%) of the patients were diagnosed before 55 years old. 60 (61.9%) were male. 60 (61.9%) patients had clear cell RCC (ccRCC), 23 (23.7%) papillary RCC (pRCC) and 14 (14.4%) chromophobe RCC (chRCC). Clinical follow-up data, as annually assessed survival time was available for all patients. The median follow-up time of all cases was 50 months, ranging from 12 to 118 months. 38 (39.2%) patients exhibited recurrence and 29 (29.9%) patients died from renal cancer during follow up. The pT status was as follows: pT1a-20 (20.6%), pT1b-25 (25.8%), pT2a-17 (17.5%), pT2b-11 (11.3%), pT3a-16 (16.5%), pT3b-2 (2.1%), and pT4-6 (6.2%). 14 patients (14.4%) had pathologically confirmed nodal metastases. 62 (63.9%) patients had no nodal metastases (pN0). In 21 (21.6%) patients lymph nodes were not examined (pNx). Tumor grades, according to Fuhrman, were G1-25 (25.8%), G2-33 (34%), G3-27 (27.8%) and G4-12 (12.4%), respectively.

Expression of DNMTs in renal cell cancer and no-tumor tissues

Differential expression of DNMT proteins in renal cell carcinoma and no-tumor tissues according to histological type is summarized in **Table 2**. In our study, DNMT proteins were significantly highly expressed in different subtypes of renal cell cancer tissues than that of no-tumor tissues (Mann-Whitney U-test, all $P < 0.05$). Briefly, The positive rates for DNMT1, DNMT3A, and DNMT3B expression in the ccRCC tissues were 56.7%, 63.3%, and 65.0%, respectively, which were significantly higher than those of no-tumor tissues (27.3, 31.8%, and 36.4%, respectively); the positive rates for DNMT1, DNMT3A, and DNMT3B expression in the pRCC tissues were 60.9%, 65.2%, and 69.6%, respectively, which were

Table 2. DNMTs expression in RCC and non-tumor tissues

			-	+	++	+++		
		n	N (%)	N (%)	N (%)	N (%)	PR, %	*P*-value
Clear cell RCC	DNMT1							
	Tumor	60	26 (43.3)	22 (36.7)	9 (15.0)	3 (5.0)	56.7	0.019
	non-Tumor	22	16 (72.7)	3 (13.6)	2 (9.1)	1 (4.5)	27.3	
	DNMT3A							
	Tumor	60	22 (36.7)	20 (33.3)	13 (21.7)	5 (8.3)	63.3	0.012
	non-Tumor	22	15 (68.2)	4 (18.2)	2 (9.1)	1 (4.5)	31.8	
	DNMT3B							
	Tumor	60	21 (35.0)	24 (40.0)	11 (18.3)	4 (6.7)	65.0	0.021
	non-Tumor	22	14 (63.6)	4 (18.2)	3 (13.6)	1 (4.5)	36.4	
Papillary RCC	DNMT1							
	Tumor	23	9 (39.1)	7 (30.4)	4 (17.4)	3 (13.0)	60.9	0.037
	non-Tumor	18	13 (72.2)	2 (11.1)	2 (11.1)	1 (5.6)	27.8	
	DNMT3A							
	Tumor	23	8 (34.8)	7 (30.4)	5 (21.7)	3 (13.0)	65.2	0.019
	non-Tumor	18	13 (72.2)	3 (16.7)	2 (11.1)	0 (0)	27.8	
	DNMT3B							
	Tumor	23	7 (30.4)	6 (26.1)	7 (30.4)	3 (13.0)	69.6	0.003
	non-Tumor	18	14 (77.8)	3 (16.7)	1 (5.6)	0 (0)	22.2	
Chomophobe RCC	DNMT1							
	Tumor	14	5 (35.7)	4 (28.6)	3 (21.4)	2 (14.3)	64.3	0.041
	non-Tumor	12	10 (83.3)	1 (8.3)	1 (8.3)	0 (0)	16.7	
	DNMT3A							
	Tumor	14	3 (21.4)	5 (35.7)	5 (35.7)	1 (7.1)	78.6	0.022
	non-Tumor	12	8 (66.7)	2 (16.7)	1 (8.3)	1 (8.3)	33.3	
	DNMT3B							
	Tumor	14	3 (21.4)	6 (42.9)	3 (21.4)	2 (14.3)	78.6	0.007
	non-Tumor	12	9 (75.0)	2 (16.7)	1 (8.3)	0 (0)	25.0	

-, negative; +, weak; ++, moderate; +++, strong staining; PR, positive rate. *P*-value obtained from Mann-Whitney U test.

significantly higher than those of no-tumor tissues (27.8%, 27.8%, and 22.2%, respectively); and the positive rates for DNMT1, DNMT3A, and DNMT3B expression in the chRCC tissues were 64.3%, 78.6%, and 78.6%, respectively, which were significantly higher than those of no-tumor tissues (16.7%, 33.3%, and 25.0%, respectively). Representative expression patterns of immunohistochemical staining of DNMTs in RCC and non-tumor tissues were shown in **Figures 1**-**3**, respectively.

Association between expression of DNMT proteins and clinicopathological parameters

The correlation analysis of DNMTs protein with clinicopathological factors in RCC was shown in **Table 3**. Our data showed that DNMT1, DNMT3A and DNMT3B expression was significantly associated with tumor size (*P*=0.003, 0.001 and 0.003, respectively), tumor pathology stage (*P*=0.039, 0.034 and 0.037, respectively), histopathological grading (*P*=0.042, 0.026 and 0.031, respectively), lymph node metastasis (*P*=0.022, 0.030 and 0.020, respectively), and vascular invasion (*P*=0.042, 0.031 and 0.044, respectively). However, there were no association between DNMT1, DNMT 3A and DNMT3B expression with gender (*P*=0.244, 0.533 and 0.155, respectively), age (*P*=0.889, 0.975 and 0.949, respectively), or tobacco smoking (*P*=0.481, 0.095 and 0.980, respectively).

Association of DNMT protein expressions with survival of the patients

The correlation between DNMTs protein expression and prognosis in RCC patients was analyzed with the Kaplan-Meier method. We

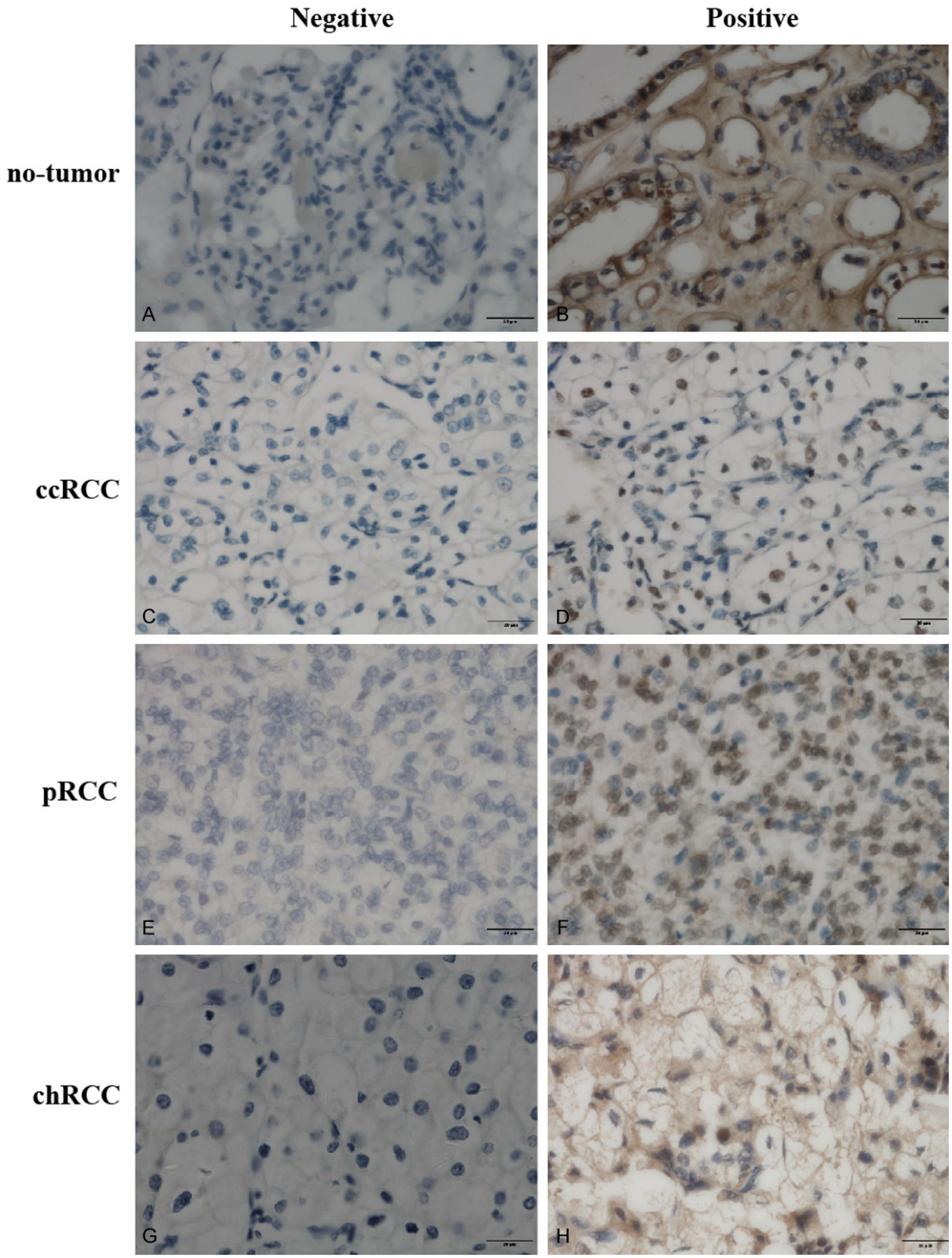

Figure 1. Representative immunohistochemical staining of DNMT1 protein in tissue samples (original magnification, ×400 in A-H).

observed that the expression of DNMT1, DNMT3A and DNMT3B proteins in RCC was significantly correlated with DFS and OS (all $P < 0.05$; **Table 4**). The log-rank test further demon-

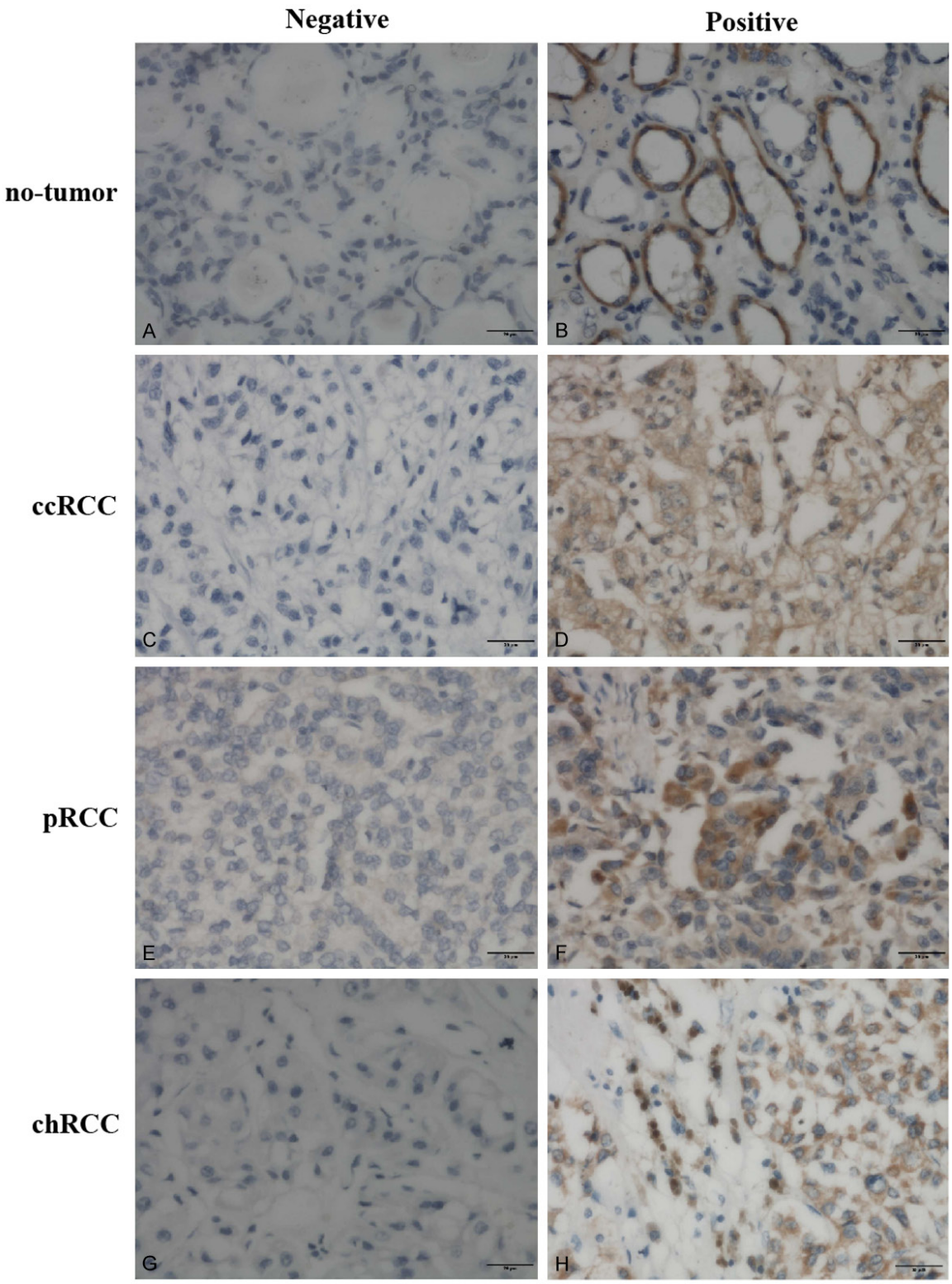

Figure 2. Representative immunohistochemical staining of DNMT3A protein in tissue samples (original magnification, ×400 in A-H).

strated that the OS and DFS time were both significantly different between groups with and without expression of DNMTs protein, which indicated expression of DNMTs protein was cor-

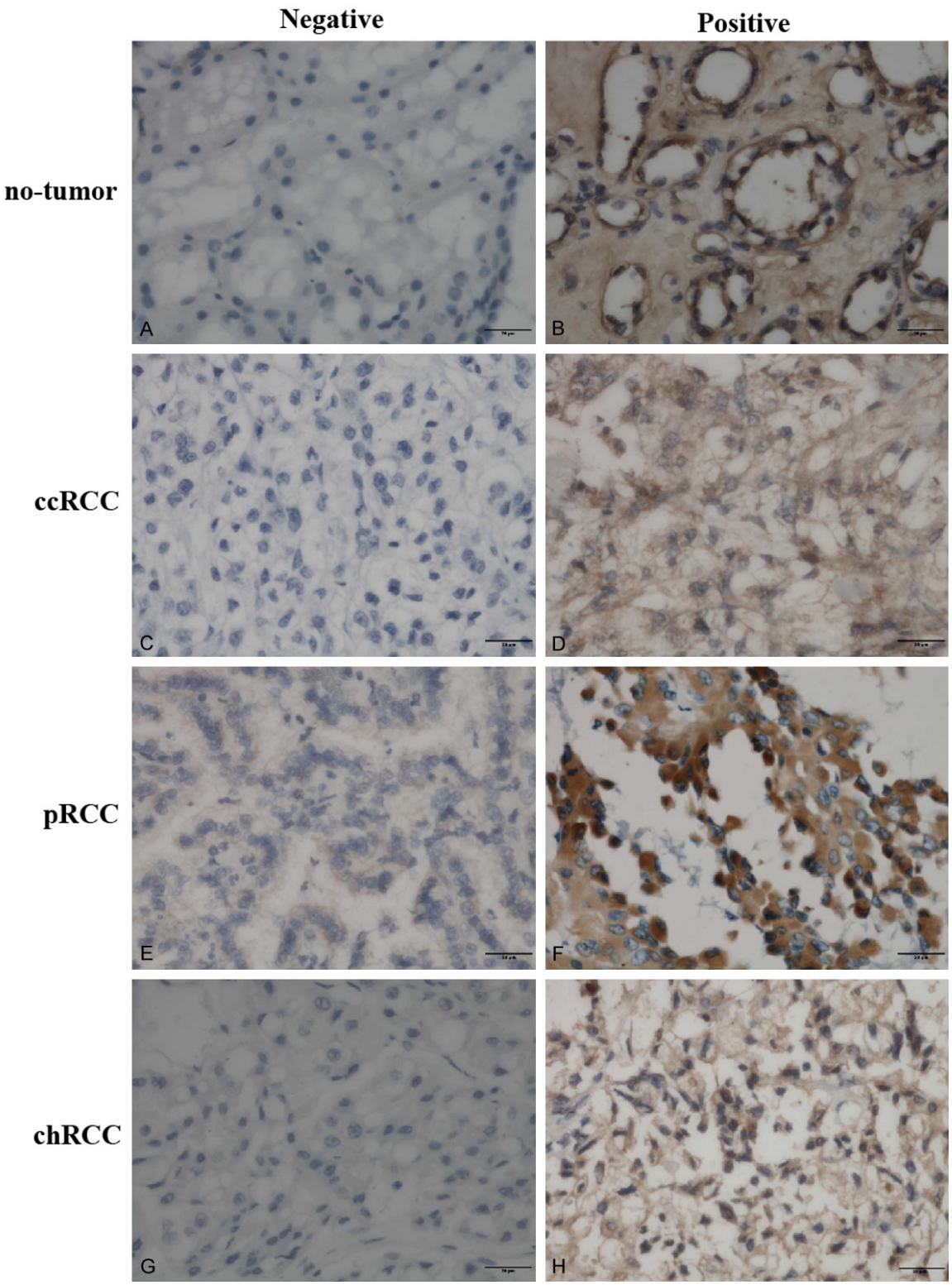

Figure 3. Representative immunohistochemical staining of DNMT3B protein in tissue samples (original magnification, ×400 in A-H).

related with a shorter survival time (**Figure 4**). Other clinicopathologic parameters, including histological type (P=0.003 and 0.026, respectively), tumor size (P=0.006 and 0.021, respec-

Table 3. Correlation between DNMTs expression and clinicopathological factors of RCC

Parameter		n	DNMT1 n (%)[a]	DNMT3a n (%)	DNMT3b n (%)
Gender					
Female		37	19 (51.4)	23 (62.2)	22 (59.5)
Male		60	38 (63.3)	41 (68.3)	44 (73.3)
	P[b]		0.244	0.533	0.155
Age at diagnosis					
≤ 55		59	35 (59.3)	39 (66.1)	40 (67.8)
> 55		38	22 (57.9)	25 (65.8)	26 (68.4)
	P		0.889	0.975	0.949
Tumor size					
≤ 4		20	6 (30.0)	7 (35.0)	8 (40.0)
> 4		77	51 (66.2)	57 (74.0)	58 (75.3)
	P		0.003	0.001	0.003
Tumor pathology stage					
pT1		45	20 (44.4)	23 (51.1)	24 (53.3)
pT2		28	18 (64.3)	21 (75.0)	22 (78.6)
pT3		18	14 (77.8)	15 (83.3)	15 (83.3)
pT4		6	5 (83.3)	5 (83.3)	5 (83.3)
	P		0.039	0.034	0.037
Grading					
G1		25	9 (36.0)	12 (48.0)	13 (52.0)
G2		33	20 (60.6)	20 (60.6)	21 (63.6)
G3		27	19 (70.4)	21 (77.8)	21 (77.8)
G4		12	9 (75.0)	11 (91.7)	11 (91.7)
	P		0.042	0.026	0.031
Lymph node metastasis					
pNx		21	16 (76.2)	17 (81.0)	18 (85.7)
pN0		62	30 (48.4)	35 (56.5)	36 (58.1)
pN1/2		14	11 (78.6)	12 (85.7)	12 (85.7)
	P		0.022	0.030	0.020
Vascular invasion					
No		84	46 (54.8)	52 (61.9)	54 (64.3)
Yes		13	11 (84.6)	12 (92.3)	12 (92.3)
			0.042	0.031	0.044
Tobacco smoking					
No		69	39 (56.5)	42 (60.9)	47 (68.1)
yes		28	18 (64.3)	22 (78.6)	19 (67.9)
	P		0.481	0.095	0.980

[a]Numbers in parentheses are percentage. [b]*P*-value obtained from Chi-Square test.

tively), tumor pathology stage (*P*=0.000 and 0.000, respectively), histological grade (*P*= 0.024 and 0.004, respectively), lymph node metastasis (*P*=0.000 and 0.001, respectively), and vascular invasion (*P*=0.019 and 0.006, respectively) were also significantly correlated with overall survival and disease-free survivals in univariate analysis (**Table 4**). In addition, multivariate analysis using the Cox proportional hazards model showed that the expression of DNMT1 was an independent prognostic factor for over all survival (*P*=0.036), and the DNMT3A or DNMT3B expression were independent predictors of disease-free survival in patients with RCC (*P*=0.031 and 0.023, respectively), the traditional tumor size, tumor pathology stage, lymph node metastasis, and vascular invasion were independent predictors of both over all survival and disease-free survival (all $P < 0.05$) (**Table 5**).

Discussion

In the present study, we immunohistochemically determined the expression of DNMT family proteins in RCC and adjacent non-tumor tissues, additionally, we analyzed the association with expression of DNMTs protein to the survival. The data showed that DNMT1 family protein expression was higher in the subtype of ccRCC, pRCC and chRCC than that in adjacent non-tumor tissues, which was consistent with the previous studies in other types of cancer [10, 18-20]. Until now, there is minimal amount of report on DNMT protein in RCC. A report in 2006 [21] found that the incidence of nuclear immunoreactivity for DNMT1 tended to be higher in proximal tubules from nontumorous renal tissues than in those from normal renal tissues, and was significantly higher in ccRCCs. However, there were no DNMT3A and DNMT3B. In our study,

Table 4. Univariate analysis of OS and DFS in patients with RCC (months, mean ± SE)

Variable	n	Overall survival time	*P*	Disease-free survival time	*P*
Gender					
Female	37	70.527±5.960	0.461	67.001±6.542	0.811
Male	60	84.772±6.662		73.614±6.481	
Age, years					
≤ 55	59	83.286±6.505	0.810	75.783±6.511	0.709
> 55	38	72.984±5.755		63.374±6.242	
Histological type					
Clear cell RCC	60	82.807±6.102	0.003	74.754±6.219	0.026
Papillary RCC	23	58.625±8.444		53.610±8.642	
Chomophobe RCC	14	92.333±7.893		82.833±8.982	
Tumor size (cm)					
≤ 4	20	104.778±7.894	0.006	91.703±8.859	0.021
> 4	77	67.498±4.322		61.987±4.609	
Tumor pathology stage					
pT1a	20	96.489±8.586	0.000	83.074±9.445	0.000
pT1b	25	81.086±6.106		73.404±6.367	
pT2a	17	70.261±7.119		55.833±6.716	
pT2b	11	57.659±5.723		52.773±5.175	
pT3a	16	50.500±4.450		45.269±4.621	
pT3b	2	48.000±8.000		29.500±0.500	
pT4	6	28.500±5.374		24.167±7.295	
Grading					
G1	25	83.484±9.098	0.024	77.446±9.586	0.004
G2	33	80.976±5.847		75.247±6.307	
G3	27	70.065±7.626		61.312±7.579	
G4	12	37.661±4.288		34.333±4.667	
Lymph node metastasis					
pNx	21	72.742±7.890	0.000	68.129±8.470	0.001
pN0	62	89.848±6.287		78.247±6.116	
pN1/2	14	47.449±6.461		32.643±4.840	
Vascular invasion					
No	91	84.366±5.300	0.019	75.496±5.276	0.006
Yes	6	48.625±10.085		31.333±4.148	
Tobacco smoking					
No	41	91.782±6.990	0.082	76.597±7.049	0.440
yes	56	66.319±4.705		63.857±5.480	
DNMT1 expression					
Positive	57	61.117±4.768	0.004	82.823±6.375	0.004
Negative	40	92.260±6.174		57.251±5.098	
DNMT3A expression					
Positive	64	73.291±8.061	0.016	68.915±8.213	0.034
Negative	33	85.291±4.383		76.700±5.095	
DNMT3B expression					
Positive	66	71.837±7.326	0.011	63.623±7.690	0.003
Negative	31	87.223±4.534		80.382±5.157	

we revealed for the first time that the expression of DNMT1, DNMT3A and DNMT3B are highly expressed in ccRCC, pRCC and chRCC, and demonstrated that the expression of DNMT proteins is associated with a more aggressive behavior of RCC. In addition, we validated the expression of DNMTs was the independent risk factor of prognosis for RCC. Collecting duct RCC was a significant and important group of epithelial tumor of the RCC. We have also collected collecting duct RCC but the number of collecting duct RCC available in our study were only 2, so we do not analyzed the collecting duct RCC in this study, and we will continue to collect more collecting duct RCC for future study.

Our current study further associated the relevance of DNMT family protein expressions with clinicopathological features from RCC patients. We found that the DNMTs protein expression was positively linked to tumor size, pathological stage, histological grade, lymph node metastasis, and vascular invasion in RCC. Moreover, in our univariate analysis showed that the expression of DNMT1, DNMT3A or DNMT3B protein, tumor size, histological type, tumor pathology stage, lymph node me-

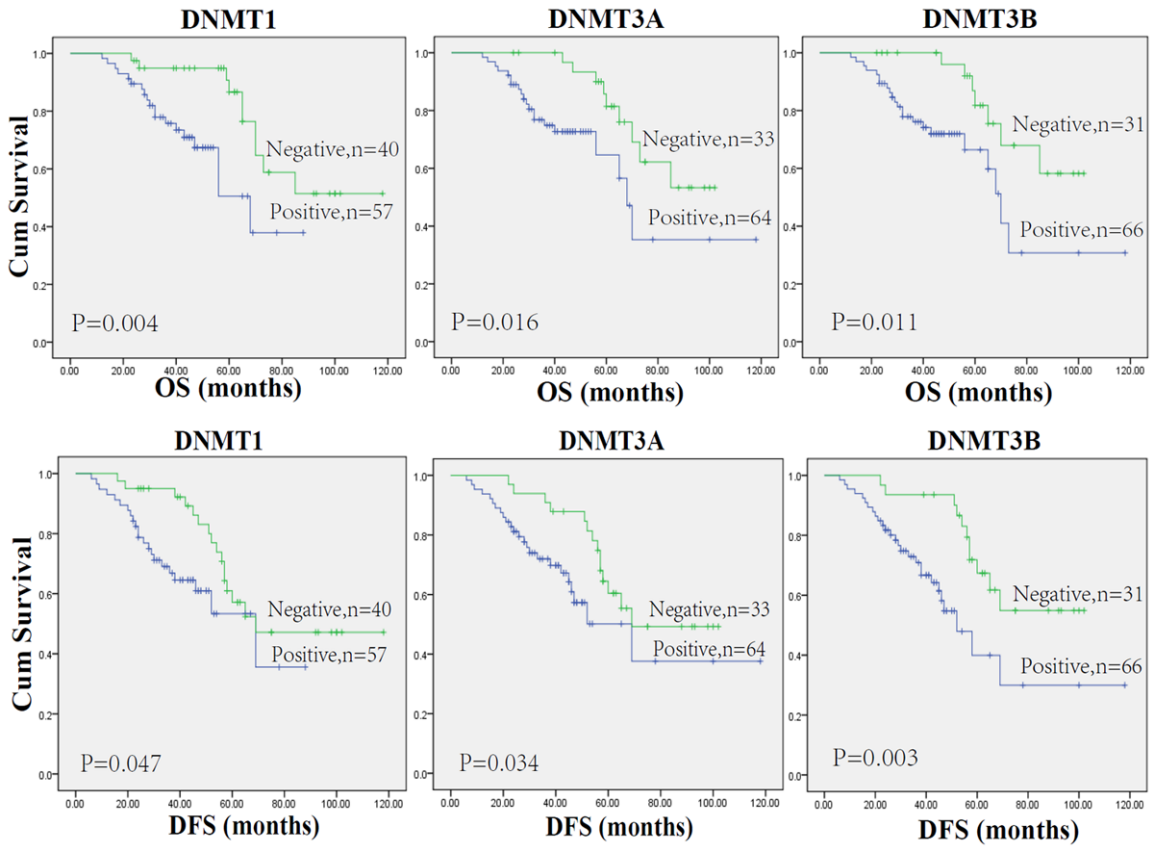

Figure 4. Kaplan-Meier estimates of patients with renal cell carcinoma stratified by DNMTs protein expression.

tastasis, and vascular invasion were associated with prognosis in RCC patients. The Overall survival (OS) and disease-free survival (DFS) of RCC patients were worse with expression of DNMT1, DNMT3A or DNMT3B compared with the patients with out expression of DNMT proteins. In addition, our multivariate analysis showed that the expression of DNMT1 was an independent prognostic factor of OS, and the expression of DNMT3A or DNMT3B was independent risk factor of DSF for RCC patients. In a variety of cancers, DNMT1, DNMT3A, and DNMT3B were reported to be highly expressed and associated with poor prognosis, including bladder, prostate, breast, lung, liver, cervical cancers [9, 10, 18, 22-24]. The prognostic association between DNMT family and RCC patients may be because of the function role of "maintenance methylation" of DNMT1 and the "de nove methylation" of DNMT3A and DNMT3B.

Hypermethylation of some tumor suppress gene (TSG) promoters that affect the prognosis can also explain the poor prognosis associated with patients with DNMT overexpression [25-27]. In RCC, a series of investigations about the identification of methylated tumor suppressor gene candidates have been carried out. The tumor suppressor genes silenced by methylation in RCC include RASSF1A [28], VHL [29]. In addition, a recent report demonstrated that BNC1, PDLIM4, RPRM, CST6, SFRP1, GREM1, COL14A1 and COL15A1 showed frequent tumor-specific promoter region methylation and hypermethylation was associated with transcriptional silencing of TSG [30]. DNA methylation and histone modifications are intricately connected with each other. Histone deacetylase may play an important role in cooperation with DNA methyltransferases, in maintaining tumor suppressor gene silencing [31]. Thus, the use of a combination of inhibitors of DNMTs and histone deacetylase is an attractive therapeutic strategy in cancer [32]. In addition, microRNAs are also involved in regulation of DNMT expression [33, 34]. Recent evidence showed that microRNA (miRs), which are non-coding RNAs, can be involved in the promoter

Table 5. Multivariate analysis of prognostic factors in patients with RCC

Factor	Overall survival			Disease-free survival		
	RR[a]	95% CI[b]	*P*	RR[a]	95% CI[b]	*P*
Gender						
Male/Female	1.083	0.672-2.817	0.853	1.214	0.713-2.901	0.879
Age, years						
> 55/≤ 55	1.348	0.621-2.674	0.506	1.268	0.603-2.593	0.623
Tumor size, cm						
≤ 4/> 4	7.325	4.267-12.383	0.031	7.865	4.612-13.013	0.019
Tumor pathology stage						
T3-T4/T1-2	8.455	5.216-13.478	0.017	8.633	5.341-13.652	0.014
Grading						
3, 4/1, 2	3.231	2.216-6.485	0.022	2.975	2.014-3.880	0.015
Lymph node metastasis						
yes/no	3.452	2.421-6.533	0.012	3.623	2.583-6.632	0.008
Vascular invasion						
yes/no	2.286	1.783-5.043	0.017	2.632	1.937-5.232	0.011
Tobacco smoking						
yes/no	1.476	0.843-3.246	0.683	1.547	0.891-3.359	0.579
DNMT1 status						
positive/negative	1.598	0.926-2.021	0.036	1.683	1.053-2.416	0.121
DNMT3A status						
positive/negative	1.662	0.985-2.376	0.208	1.717	1.097-2.502	0.031
DNMT3B status						
positive/negative	1.713	1.035-2.681	0.192	1.845	1.106-2.583	0.023

[a]RR, relative risk; [b]95% CI, 95% confidence interval.

methylation of CpG islands by targeting DNMTs 3'UTR [33-35]. In any events, due to the complex mechanisms responsible for regulation of DNMT expressions and functions of DNMTs in carcinogenesis, the altered expression and effects of DNMTs should be further investigated in RCC.

The development of molecular biomarkers is clinically important in that they can help identifying tumors and predicting the recurrence and progression of tumors, which could potentially be benefit for effective treatments. Our data suggested that the expression of DNMT proteins is correlated with more aggressive tumors. Moreover, DNMTs could potentially be added to the current prognostic methods to improve the accuracy of clinical outcome predictions and the choice of appropriate therapy for RCC patients who might have disease recurrence and progression. The findings are potentially important in practice, because the expression of DNMTs could be a potential indicator for the use of DNMT inhibtor in the treatment of metastatic RCC.

In summary, we demonstrated that DNMT family proteins were higher expressed in RCC than no-tumor tissues, and the expression of DNMTs were strongly associated with RCC tumor size, tumor stage, histological grading, lymph node metastasis, vascular invasion, recurrence, and prognosis. DNMTs may thus serve as prognostic markers and novel therapeutic targets for RCC patients. However, this was a retrospective cohort research, so further multicenter studies are needed to confirm these findings and provide more evidence about the role and significance of DNMTs in renal cell carcinoma.

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Disclosure of conflict of interest

None.

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References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[2] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of

cancer in 2008. GLOBOCAN 2008. Int J Cancer 2010; 127: 2893-2917.
[3] Rini BI, Rathmell WK, Godley P. Renal cell carcinoma. Curr Opin Oncol 2008; 20: 300-6.
[4] Janzen NK, Kim HL, Figlin RA, Belldegrun AS. Surveillance after radical or partial nephrectomy for localized renal cell carcinoma and management of recurrent disease. Urol Clin North Am 2003; 30: 843-52.
[5] O'Rourke CJ, Knabben V, Bolton E, Moran D, Lynch T, Hollywood D, Perry AS. Manipulating the epigenome for the treatment of urological malignancies. Pharmacol Ther 2013; 138: 185-196.
[6] Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007; 128: 683-692.
[7] Matouk CC, Marsden PA. Epigenetic regulation of vascular endothelial gene expression. Circ Res 2008; 102: 873-887.
[8] Jeltsch A. Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. Chembiochem 2002; 3: 274-293.
[9] Girault I, Tozlu S, Lidereau R, Bieche I. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin Cancer Res 2003; 9: 4415-4422.
[10] Saito Y, Kanai Y, Nakagawa T, Sakamoto M, Saito H, Ishii H, Hirohashi S. Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. Int J Cancer 2003; 105: 527-532.
[11] Patra SK, Patra A, Zhao H, Dahiya R. DNA methyltransferase and demethylase in human prostate cancer. Mol Carcinog 2002; 33: 163-171.
[12] Konac E, Varol N, Yilmaz A, Menevse S, Sozen S. DNA methyltransferase inhibitor mediated apoptosis in the Wnt β catenin signal pathway in a renal cell carcinoma cell line. Exp Biol Med 2013; 238: 1009-1016.
[13] Shang D, Ito N, Kamoto T, Ogawa O. Demethylating agent 5-aza-2'-deoxycytidine enhances susceptibility of renal cell carcinoma to paclitaxel. Urology 2007; 69: 1007-1012.
[14] Stewart DJ, Donehower RC, Eisenhauer EA, Wainman N, Shah AK, Bonfils C, MacLeod AR, Besterman JM, Reid GK. A phase I pharmacokinetic and pharmacodynamic study of the DNA methyltransferase 1 inhibitor MG98 administered twice weekly. Ann Oncol 2003; 14: 766-774.
[15] Winquist E, Knox J, Ayoub JP, Wood L, Wainman N, Reid GK, Pearce L, Shah A, Eisenhauer E. Phase II trial of DNA methyltransferase 1 inhibition with the antisense oligonucleotide MG98 in patients with metastatic renal carcinoma, a National Cancer Institute of Canada Clinical Trials Group investigational new drug study. Invest New Drugs 2006; 24: 159-167.
[16] Amato RJ, Stephenson J, Hotte S, Nemunaitis J, Bélanger K, Reid G, Martell RE. MG98, a second-generation DNMT1 inhibitor, in the treatment of advanced renal cell carcinoma. Cancer Invest 2012; 30: 415-421.
[17] Eble JN, Sauter G, Epstein JI, Sesterhenn IA. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs. Lyon: IARC Press; 2004.
[18] Lin RK, Hsu HS, Chang JW, Chen CY, Chen JT, Wang YC. Alteration of DNA methyltransferases contributes to 5'CpG methylation and poor prognosis in lung cancer. Lung Cancer 2007; 55: 205-213.
[19] Yang J, Wei X, Wu Q, Xu Z, Gu D, Jin Y, Shen Y, Huang H, Fan H, Chen J. Clinical significance of the expression of DNA methyltransferase proteins in gastric cancer. Mol Med Report 2011; 4: 1139-1143.
[20] Xing J, Stewart DJ, Gu J, Lu C, Spitz MR, Wu X. Expression of methylation-related genes is associated with overall survival in patients with non-small cell lung cancer. Br J Cancer 2008; 98: 1716-1722.
[21] Arai E, Kanai Y, Ushijima S, Fujimoto H, Mukai K, Hirohashi S. Regional DNA hypermethylation and DNA methyltransferase (DNMT) 1 protein overexpression in both renal tumors and corresponding nontumorous renal tissues. Int J Cancer 2006; 119: 288-296.
[22] Wu CT, Wu CF, Lu CH, Lin CC, Chen WC, Lin PY, Chen MF. Expression and function role of DNA methyltransferase 1 in human bladder cancer. Cancer 2011; 117: 5221-5233.
[23] Gravina GL, Ranieri G, Muzi P, Marampon F, Mancini A, Di Pasquale B, Di Clemente L, Dolo V, D'Alessandro AM, Festuccia C. Increased levels of DNA methyltransferases are associated with the tumorigenic capacity of prostate cancer cells. Oncol Rep 2013; 29: 1189-1195.
[24] Sawada M, Kanai Y, Arai E, Ushijima S, Ojima H, Hirohashi S. Increased expression of DNA methyltransferase 1 (DNMT1) protein in uterine cervix squamous cell carcinoma and its precursor lesion. Cancer Lett 2007; 251: 211-219.
[25] Wu D, Xiong L, Wu S, Jiang M, Lian G, Wang M. TFPI-2 methylation predicts poor prognosis in non-small cell lung cancer. Lung Cancer 2012; 76: 106-111.
[26] Xu L, Li X, Chu ES, Zhao G, Go MY, Tao Q, Jin H, Zeng Z, Sung JJ, Yu J. Epigenetic inactivation of BCL6B, a novel functional tumor suppressor for gastric cancer, is associated with poor survival of gastric cancer. Gut 2012; 61: 977-985.

[27] Zhang Q, Chen L, Helfand BT, Jang TL, Sharma V, Kozlowski J, Kuzel TM, Zhu LJ, Yang XJ, Javonovic B, Guo Y, Lonning S, Harper J,Teicher BA, Brendler C, Yu N, Catalona WJ, Lee C. TGF-b regulates DNA methyltransferase expression in prostate cancer, correlates with aggressive capabilities, and predicts disease recurrence. PLoS One 2011; 6: e25168.

[28] Battagli C, Uzzo RG, Dulaimi E, Ibanez de Caceres I, Krassenstein R, Al-Saleem T, Greenberg RE, Cairns P. Promoter hypermethylation of tumor suppressor genes in urine from kidney cancer patients. Cancer Res 2003; 63: 8695-8699.

[29] Alleman WG, Tabios RL, Chandramouli GV, Aprelikova ON, Torres-Cabala C, Mendoza A, Rogers C, Sopko NA, Linehan WM, Vasselli JR. The in vitro and in vivo effects of re-expressing methylated von Hippel-Lindau tumor suppressor gene in clear cell renal carcinoma with 5-aza-2'deoxycytidine. Clin Cancer Res 2004; 10: 7011-7021.

[30] Morris MR, Ricketts C, Gentle D, Abdulrahman M, Clarke N, Brown M, Kishida T, Yao M, Latif F, Maher ER. Identification of candidate tumor suppressor genes frequently methylated in renal cell carcinoma. Oncogene 2010; 29: 2104-211.

[31] Ting AH, Jair KW, Suzuki H, Yen RW, Baylin SB, Schuebel KE. Mammalian DNA methyltransferase 1: inspiration for new directions. Cell Cycle 2004; 3: 1024-1026.

[32] Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003; 349: 2042-2054.

[33] Braconi C, Huang N, Patel T. MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology 2010; 51: 881-890.

[34] Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C, Volinia S, Guler G, Morrison CD, Chan KK, Marcucci G, Calin GA, Huebner K, Croce CM. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci U S A 2007; 104: 15805-15810.

[35] Duursma AM, Kedde M, Schrier M, le Sage C, Agami R. miR-148 targets human DNMT3b protein coding region. RNA 2008; 14: 872-877.