Aberrant DNA methyltransferase 1 expression in clear cell renal cell carcinoma development and progression

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Objective: To better understand the contribution of dysregulated DNA methyltransferase 1 (DNMT1) expression to the progression and biology of clear cell renal cell carcinoma (ccRCC).

Methods: We examined the differences in the expression of DNMT1 in 89 ccRCC and 22 normal tissue samples by immunohistochemistry. In addition, changes in cell viability, apoptosis, colony formation and invading ability of ccRCC cell lines (786-0 and Caki-1) were assessed after transfection with DNMT1 siRNA.

Results: We found DNMT1 protein was significantly higher expressed in ccRCC than that of in no-tumor tissues (56.2% and 27.3%, respectively, P=0.018). The expression of DNMT1 was strongly associated with ccRCC tumor size, tumor pathology stage, histological grading, lymph node metastasis, vascular invasion, recurrence and prognosis. Moreover, knockdown of DNMT1 expression significantly inhibited ccRCC cell viability, induced apoptosis, decreased colony formation and invading ability.

Conclusions: Expression of DNMT1 protein is increased in ccRCC tissues, and DNMT1 expression is associated with poor prognosis of patients. Experiments *in vitro* further showed DNMT1 played an essential role in proliferation and invasion of renal cancer cells. Moreover, targeting this enzyme could be a promising strategy for treating ccRCC, as evidenced by inhibited cell viability, increased apoptosis, decreased colony formation and invading ability.

Keywords: Clear cell renal cell carcinoma (ccRCC); DNA methyltransferase 1 (DNMT1); immunohistochemistry; siRNA

Introduction

Renal cell carcinoma (RCC) is the most lethal urologic tumor and the sixth leading cause of cancer deaths in Western countries. Each year, around 200,000 patients are diagnosed with this malignancy resulting in approximately 100,000 deaths, and its incidence is increasing steadily in recent years (1). Nearly 25-30% of patients with RCC have evidence of metastases at initial presentation (2). RCC is represented by 80% by clear cell renal cell carcinoma (ccRCC), originating from the renal proximal tubule (3). ccRCC is resistant to both radiation and chemotherapy, and the prognosis remains poor. Although standard pathological features such as tumor grade and stage can be used to decide on treatments, to explore novel molecular markers, which could be served as prognostic factors and therapeutic targets for ccRCC is still an important issue.

During carcinogenetic development, epigenetic changes occur at least as frequently as genetic mutations and deletions. Aberrant DNA methylation plays a key role in carcinogenesis, leading to the epigenetic silencing of the expression of tumor-suppressor genes involved in cell cycle regulation, apoptosis, and DNA repair (4,5). In such cases, DNA methylation is typically mediated by DNA methyltransferases, particularly DNA methyltransferase

1 (DNMT1), DNMT3A, and DNMT3B (6). DNMT1 is significantly overexpressed in several tumor types (7,8). In a variety of cancers, DNMT1, DNMT3A, and DNMT3B were reported to be highly expressed and associated with poor prognosis, including breast, liver, lung, colorectum, stomach, bladder, prostate, cervical cancers, lymphomas and other malignancies (9-20). Until now, there is minimal amount of report on DNMT1 in RCC. A report in 2006 (21) found that the incidence of nuclear immunoreactivity for DNMT1 tended to be higher in proximal tubules from non-tumorous renal tissue than in those from normal renal tissue, and was significantly higher in RCCs, however, there have been no reports regarding biological behavior alterations of ccRCC cells after knockdown of DMNT1 expression and the underlying molecular events *in vitro*. Thus, in this study, we analyzed the expression of DNMT1 protein in ccRCC tissue specimens and investigated their association with clinicopathological features and patient survival. Then, we knocked down DNMT1 expression using siRNA to assess the effects on the regulation of biological behavior of ccRCC cells and the underlying molecular mechanisms.

Materials and methods

Patients and tissue samples

ccRCC tissue was collected from radical nephrectomy specimens performed between January 2004 and January 2012 at Department of Urology, Shengjing Hospital of China Medical University. The tumor cases included 89 cases with histologically confirmed ccRCC and 22 cases of adjacent non-tumor tissues. The criteria for study enrollment were as follows: patients with histopathologically diagnosed ccRCC 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 (22). 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 Institute Research Medical Ethics Committee of Shengjing Hospital 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 ℃ for 20 min. After that, the sections were blocked to avoid nonspecific binding by addition of a 10% normal goat serum at 37 ℃ for 30 min and then incubated for 4 ℃ overnight with the polyclonal antibody against DNMT1 (sc-20701, 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 ℃ 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-DNMT1 antibody.

The immunostained sections were evaluated by two investigators who were blinded to the patients' clinicopathological characteristics. For each slide, the number of DNMT1 positive cells was counted in ten fields at ×200 magnifications, 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%.

ccRCC cell line and culture

786-0 human ccRCC cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1,640 supplemented with 10% FCS. Caki-1 human ccRCC cell line was obtained from Tiancheng Technology Co. Ltd (Shanghai, China) and cultured in DMEM supplemented with 10% FCS. Cells

were harvested when they were in the logarithmic phase of growth for use in the following experiments.

RNA isolation and quantitative-reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from transfected cells as well as untransfected cells after transfection, using a trizol reagent (Invitrogen) and was then reversely transcribed into cDNA using a PrimeScriptTM RT kit (TakaRa, Dalian, China). The expression of DNMT1 was detected by qPCR in ABI7500 (Applied Biosystems, Foster City, CA, USA). The primer sequences were 5'-GTTCTTCCTCCTGGAGAATGTCA-3' and 5'-GGGCCACGCCGTACTG-3' for DNMT1.The program had an initial denaturation at 95 ℃ for 1 min followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 15 s. The relative quantity (RQ) was determined using the $2^{(-\Delta\Delta C_t)}$ method, a β-actin primer was used as a loading control, and each experiment was repeated six times.

Construction of DNMT siRNA vector and gene transfection

The DNMT1 specific siRNA was chosen from the GenBank sequences (accession #NM_001379.1) and synthesized by Ambion Inc. (Austin, TX, USA). The DNMT1 siRNA sequences were 5'-GGAUGAGAAGAGACGUAGAtt-3' and 5'-UCUACGUCUCUUCUCAUCCtg-3'. Negative control siRNA was also purchased from Ambion Inc. (Catalog #4,390,843). Final concentrations were 7 nM. The OptiMEM (Gibco BRL Inc. USA) and Lipofectamine 2,000 Transfection Kits (Invitrogen) were used for siRNA transfections. Cells were plated at 1×10^5 cells per well in culture dishes for overnight growth and transfected by siRNA on the following day. Six hours after transfection, the medium was removed and replaced with RPMI 1,640 or DMEM, and the cells were allowed to grow for 48 h. Then, viability, apoptosis, colony formation and invading ability were assayed.

Protein extraction and western blot

Total cellular protein was extracted from cells using an M-PER mammalian protein extraction buffer (Pierce, Rockford, IL) containing 0.5 mm PMSF. The protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%)

and electronically transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated with an anti-DNMT1 antibody (sc-20701, 1:250 dilution Santa Cruz Biotechnology, USA) or an anti-β-actin (1:5000, Sigma, St Louis, MO, USA) antibody at 4 ℃ overnight, followed by a secondary antibody for 2 h at room temperature. The protein bands were detected with enhanced chemiluminescence (ECL) reagent (Invitrogen).

Cell viability Tetrazolium Salt-8 (WST-8) assay

Briefly, cells were transfected with DNMT1 or negative control siRNA oligonucleotides. Seventy-two hours later, 10 μL of WST-8 reagent was added to each well and incubated for an additional 4 h. The absorbance rate was then measured at 450 nm with an ELISA plate reader (Thermo ELISA Reader, USA).

Flow cytometry assay

Apoptosis levels were detected using flow cytometry (Becton Dickinson) with an Annexin V-FITC Kit (Jingmei Biotech Company, Shanghai, China) according to the manufacturer's protocol.

In vitro colony formation assay

Cells were suspended in 0.1 mL of culture medium with 10% FBS, and 1,000 cells were plated in culture dishes with 1 mL of methylcellulose-containing culture medium supplemented with 15% FBS. The number of colonies was counted on day 14. Colonies were stained and counted by applying diff-quick staining kit (Siemens, Munich, Germany).

Matrigel invasion assay

Invasion of tumor cells into Matrigel was examined with a BD BioCoat Matrigel Invasion Chamber (BD Biosciences). Cells were seeded in culture medium without FCS in the Matrigel invasion upper chamber and cultured for 72 h. The lower chamber contained culture medium with 10% FBS. Invading cells were stained with a diff-quick staining kit (Siemens, Munich, Germany). The number of invading cells was counted in four microscopic fields per well at a magnification of 20, and the extent of invasion was expressed as the average number of cells per square millimeter.

Table 1 Patient characteristics		
Feature	Number	%
Gender		
Female	35	39.3
Male	54	60.7
Age, years		
≤55	50	56.2
>55	39	43.8
Tumor size (cm)		
≤4	15	16.9
>4	74	83.1
Tumor pathology stage		
pT1-pT2	67	75.3
pT3-pT4	22	24.7
Grading		
G1	23	25.8
G2	30	33.7
G3	24	27.0
G4	12	13.5
Lymph node metastasis		
pNx	21	23.6
pN0	55	61.8
pN1/2	13	14.6
Vascular invasion		
No	80	89.9
Yes	9	10.1
Tobacco smoking		
No	32	36.0
Yes	57	64.0

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 years), and 50 (56.2%) of the patients were diagnosed before 55 years old. Fifty-four (60.7%) were male. Clinical follow-up data, as annually assessed survival time was available for all patients. The median follow-up time of all cases was 49.5 months, ranging from 14 to 118 months. Twenty-five (28.1%) patients exhibited recurrence and 21 (23.6%) patients died from ccRCC during follow up. The pT status was as follows: pT1 and pT2-67 (75.3%), pT3 and pT4-22 (24.7%). Thirteen (14.6%) patients had pathologically confirmed nodal metastases. Fifty-five (61.8%) patients had no nodal metastases (pN0). In 21 (23.6%) patients lymph nodes were not examined (pNx). Tumor grades, according to Fuhrman, were G1-23 (25.8%), G2-30 (33.7%), G3-24 (27.0%) and G4-12 (13.5%), respectively.

Expression of DNMT1 in ccRCC and no-tumor tissues

The expression of DNMT1 protein in clear cell renal cell cancer and no-tumor tissues is summarized in *Table 2*. In our study, DNMT1 protein was significantly highly expressed in clear cell renal cell cancer tissues than that of no-tumor tissues (Mann-Whitney U-test, P=0.018). Briefly, the positive rates for DNMT1 expression in the ccRCC tissues were 56.2%, which was significantly higher than those of no-tumor tissues (27.3%). Representative expression patterns of immunohistochemical staining of DNMT1 in ccRCC and non-tumor tissues were shown in *Figure 1*.

Association of DNMT1 expression with clinicopathological characteristics

The correlation analysis of DNMT1 with clinicopathological factors in ccRCC was shown in *Table 3*. Our data showed that DNMT1 expression was significantly associated with tumor size (P=0.040), tumor pathology stage (P=0.022), histopathological grading (P=0.004), lymph node metastasis (P=0.029) and vascular invasion (P=0.037). However, there were no association between DNMT1 expression with gender (P=0.772), age (P=0.695), or tobacco smoking (P=0.379).

### Association of DNMT1 expression with survival of the patients

The correlation between DNMT1 expression and

Statistical analysis

Statistical analysis was performed with SPSS version 19 (SPSS Inc., Chicago, IL, USA). Comparison of DNMT1 expression between samples and the difference of the variables (such as expression of DNMT1 mRNA *vs*. tumor cell viability, apoptosis, colony formation and invading ability) between the differential siRNA transfected groups, were analyzed with a Mann-Whitney U test. Chi-square tests were applied to assess associations between expression of DNMT1 and clinicopathological parameters. Univariate survival analysis was performed with a Kaplan-Meier log-rank test, while multivariate survival analysis was performed with a Cox Regression test. A two-tailed P<0.05 was considered statistically significant.

Table 2 DNMT1 expression in ccRCC and non-tumor tissues

	n	– n (%)	+ n (%)	++ n (%)	+++ n (%)	PR, %	P value
Tumor	89	39 (43.8)	44 (49.4)	5 (5.6)	1 (1.1)	56.2	0.018
Non-tumor	22	16 (72.7)	3 (13.6)	2 (9.1)	1 (4.5)	27.3	

–, negative; +, weak; ++, moderate; +++, strong staining; PR, positive rate; ccRCC, clear cell renal cell carcinoma; DNMT1, DNA methyltransferase 1. P value obtained from Mann-Whitney U test.
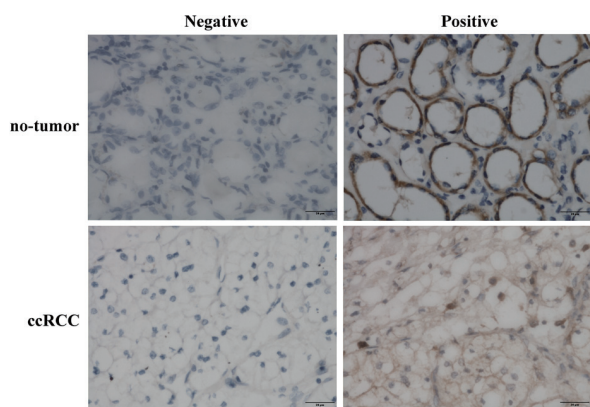
Figure 1 Nuclear immunostaining of DNMT1 protein in no-tumor and ccRCC tissues (Original magnification: ×400). ccRCC, clear cell renal cell carcinoma; DNMT1, DNA methyltransferase 1.

prognosis in ccRCC patients was analyzed with the Kaplan-Meier method. We observed that the expression of DNMT1 in ccRCC was significantly correlated with overall survival (OS) and disease free survival (DFS) (P=0.002 and 0.026, respectively, *Table 4*). The log-rank test further demonstrated that the OS and DFS time were both significantly different between groups with and without expression of DNMT1, which indicated expression of DNMT1 was correlated with a shorter survival time (*Figure 2*). Other clinicopathologic parameters, including tumor size (P=0.003 and 0.015, respectively), tumor pathology stage (P=0.002 and 0.006, respectively), histological grade (P=0.003 and 0.022, respectively), lymph node metastasis (P=0.024 and 0.036, respectively), and vascular invasion (P=0.007 and 0.003, respectively) were also significantly correlated with OS and disease-free survival in univariate analysis (*Table 4*). In addition, multivariate analysis using the Cox proportional hazards model showed that the expression of DNMT1 was an independent

Table 3 Correlation between DNMT1 expression and clinicopathological factors of ccRCC

Parameter	n	DNMT1 expression		P value
		Negative	Positive	
Gender				
Female	35	16	19	0.772
Male	54	23	31	
Age at diagnosis				
≤55	50	21	29	0.695
>55	39	18	21	
Tumor size				
≤4	15	10	5	0.040
>4	74	28	46	
Tumor pathology stage				
pT1-pT2	67	34	33	0.022
pT3-pT4	22	5	17	
Grading				
G1	23	16	7	0.004
G2	30	15	15	
G3	24	5	19	
G4	12	3	9	
Lymph node metastasis				
pNx	26	8	18	0.029
pN0	50	28	22	
pN1/2	13	3	10	
Vascular invasion				
No	80	38	42	0.037
Yes	9	1	8	
Tobacco smoking				
No	32	16	16	0.379
yes	57	23	34	

ccRCC, clear cell renal cell carcinoma; DNMT1, DNA methyltransferase 1.

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

| Variable	n	Overall survival time	P	Disease-free survival time	P
Gender					
Female	35	85.647±5.428	0.435	81.369±6.339	0.296
Male	54	82.408±7.128		78.919±7.546	
Age, years					
≤55	50	85.289±5.364	0.141	80.235±5.888	0.224
>55	39	80.583±7.627		77.429±8.166	
Tumor size (cm)					
≤4	15	113.000±4.714	0.003	107.442±6.919	0.015
>4	74	62.831±2.823		59.125±2.999	
Tumor pathology stage					
pT1-pT2	67	109.409±5.706	0.002	105.564±6.668	0.006
pT3-pT4	22	61.803±3.016		57.605±3.199	
Grading					
G1	23	101.421±7.149	0.003	97.042±7.829	0.022
G2	30	79.333±6.647		79.519±6.059	
G3	24	57.177±3.935		48.145±3.686	
G4	12	39.175±3.140		37.023±4.098	
Lymph node metastasis					
pNx	21	61.590±4.182	0.024	61.572±4.648	0.036
pN0	55	95.946±6.365		90.654±6.870	
pN1/2	13	51.496±6.478		47.138±8.417	
Vascular invasion					
No	80	90.184±5.859	0.007	89.374±5.706	0.003
Yes	9	50.000±7.751		45.667±9.858	
Tobacco smoking					
No	32	88.905±7.336	0.668	81.334±8.117	0.825
Yes	57	76.277±4.170		73.948±4.376	
DNMT1 expression					
Positive	50	59.166±3.918	0.002	57.427±4.390	0.026
Negative	39	99.020±6.433		93.495±6.885	

OS, overall survival; ccRCC, clear cell renal cell carcinoma.

prognostic factor for OS in patients with ccRCC (P=0.031), the traditional tumor size, tumor pathology stage, histological grading, lymph node metastasis, and vascular invasion were independent predictors of both OS and disease-free survival (all P<0.05) (*Table 5*).

Effects of DNMT1 silencing on cell viability, apoptosis, colony formation and invading ability in ccRCC cell line

The DNMT1 were knocked down in ccRCC cell lines 786-0 and Caki-1. As shown in *Figure 3*, qRT-PCR and Western blot data showed that transfection of DNMT1 siRNA significantly reduced expression of their corresponding mRNA and protein. We found that transfection of DNMT1 siRNA significantly inhibited the viability of ccRCC cells and induced apoptosis (*Figure 4A,B*).Then, the ability for colony formation and invasion *in vitro* was evaluated. As shown in *Figure 4C,D*, when DNMT1 was inhibited by siRNA, clonies and invasive cell number reduced in 786-0 and Caki-1 cell lines.
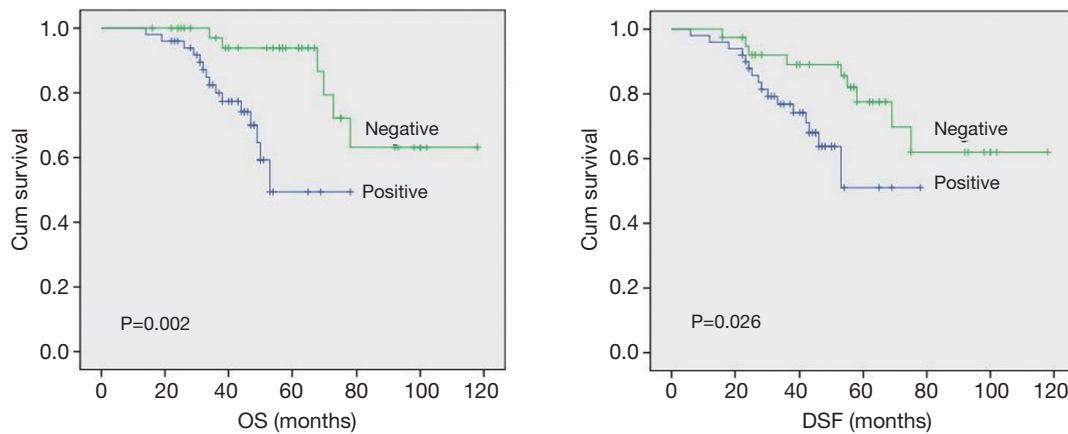
Figure 2 Univariate analyses of OS and DSF in patients with ccRCC using the Kaplan-Meier method according to DNMT1 expression. The log rank test was used to calculate P values. OS, overall survival; DFS, disease-free survival; ccRCC, clear cell renal cell carcinoma; DNMT1, DNA methyltransferase 1.

Table 5 Multivariate analysis of prognostic factors in patients with ccRCC

Factor	Overall survival			Disease-free survival		
	RR	95% CI	P	RR	95% CI	P
Gender						
Male/Female	1.095	0.658-2.927	0.742	1.253	0.720-2.916	0.786
Age, years						
>55/≤55	1.369	0.615-2.874	0.613	1.387	0.584-2.887	0.651
Tumor size, cm						
≤4/>4	7.085	4.102-13.023	0.037	7.713	4.423-14.018	0.027
Tumor pathology stage						
T3-T4/T1-2	8.684	5.103-14.053	0.024	8.501	5.012-13.958	0.023
Grading						
3,4/1,2	3.341	2.324-6.573	0.021	2.896	2.021-3.989	0.019
Lymph node metastasis						
Yes/no	3.531	2.530-6.604	0.017	3.701	2.672-6.714	0.013
Vascular invasion						
Yes/no	2.437	1.987-6.021	0.027	2.887	1.906-5.134	0.022
Tobacco smoking						
Yes/no	1.848	0.893-4.283	0.593	1.659	0.993-3.984	0.682
DNMT1 status						
Positive/negative	1.876	0.926-2.021	0.031	1.962	1.053-2.416	0.213

RR, relative risk; 95% CI, 95% confidence interval; ccRCC, clear cell renal cell carcinoma.

Discussion

In the present study, a comprehensive knowledge about the pathogenic characteristics of DNMT1 involved in ccRCC tumorigenesis was gathered and our data indicate that overexpression of DNMT1 plays a key role in ccRCC tumorigenesis and progression. Consistent with the previous studies in other types of cancer (10-12,23-26), we
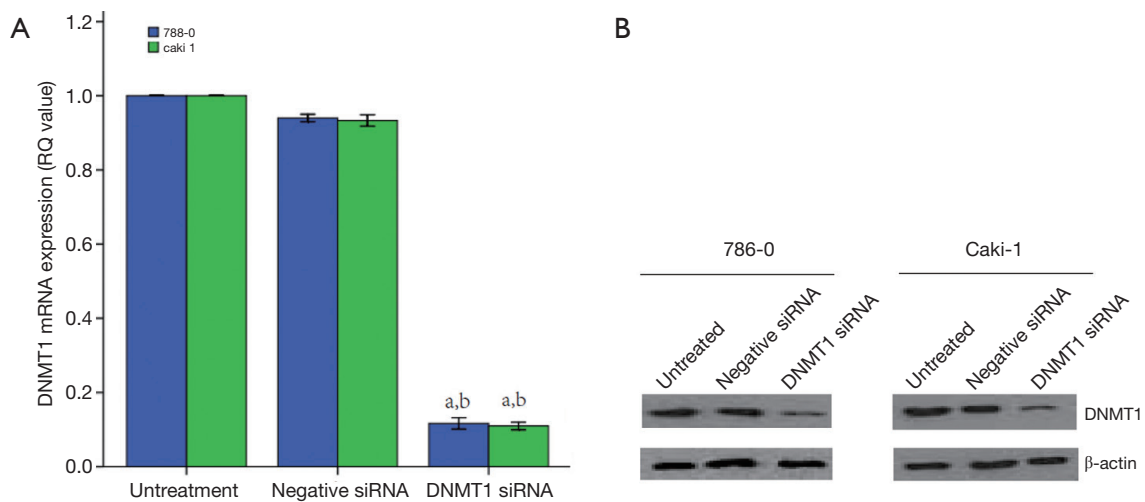
Figure 3 Konckdown of DNMT1 expressions in ccRCC cells. DNMT1 siRNA was transiently transfected into ccRCC cell lines and then subjected to qRT-PCR (A) and western blotting (B) analysis for DNMT1 expression. a, $p < 0.05$ compared with the untreated cells. b, $p < 0.05$ compared with the negative siRNA cells. The dates are shown as the mean ± SD of six independent experiments. DNMT1, DNA methyltransferase 1; ccRCC, clear cell renal cell carcinoma.
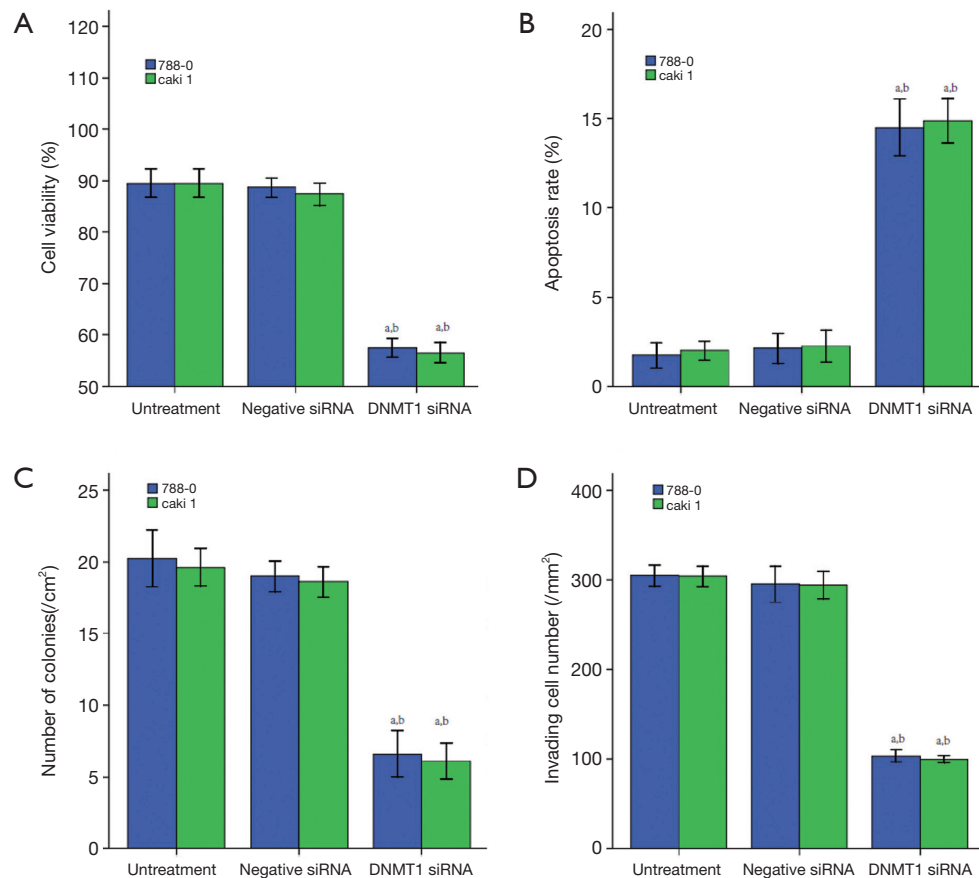
Figure 4 Effects of DNMT1 siRNA on cell viability (A), apoptosis (B), colony formation (C) and invading ability of invation (D). a, $p < 0.05$ compared with the untreated cells. b, $p < 0.05$ compared with the negative siRNA transfected cells. The data are shown as the mean ± SD of eight independent experiments. DNMT1, DNA methyltransferase 1.

demonstrated that DNMT1 expression was higher in the ccRCC than that in adjacent non-tumor tissues, suggesting that DNMT1 may be involved in the malignancy of ccRCC. This finding is also agreeable to previous study reporting that DNMT1 expression was significantly higher in RCCs (21). In any events, due to the complex mechanisms responsible for regulation of DNMTs expressions and functions of DNMTs in carcinogenesis, the altered expression and effects of DNMTs should be further investigated in ccRCC although their aberrant expressions were found to be because of methylation of their gene promoters in different cancers, such as gliomas and embryonic tissues (27,28). In addition, microRNAs (miRs), which are noncoding RNAs, are also involved in regulation of DNMT expression (29-32).

Our current study further associated the relevance of DNMT1 protein expression with clinicopathological features from ccRCC patients. The data showed that DNMT1 protein expression was positively correlated with tumor size, clinical stage, histological grade suggesting that DNMT1 may be markers for tumor progression. In addition, tumor invasion is considered to be an important risk factor for metastatic ccRCC. In our study, the positive expression of DNMT1 protein was associated with a highly malignant phenotype and could be considered as a poor prognostic index for ccRCC. This observation was confirmed by the study *in vitro*, we found that knockdown of DNMT1 expression significantly inhibited the viability, colony formation and invading ability, induced apoptosis in ccRCC cells. Moreover, our multivariate analysis showed that patients with ccRCC exhibiting positive DNMT1 protein expression had a significantly poor over-all survival, suggesting that DNMT1 is a biologic predictor of poor prognosis of ccRCC patient. However, to date, we don't know why these happened or the implication of these associations. Previous studies have demonstrated that hypermethylation of tumor suppress gene (TSG) promoters is crucial for cancer initiation and progression (33). Hypermethylation of some TSG promoters that affect the prognosis can partially explain the poor prognosis associated with DNMT overexpression (34-38).

Hypermethylation of tumor suppressor genes is caused by aberrant up-regulation of DNMT protein expression and enzyme activity. Thus, many researchers have targeted DNMT as a novel treatment strategy for tumors. Cytosine analogues capable of trapping DNMTs onto DNA, inhibition of DNMT mRNA expression through siRNA (39,40), antisense (40), irradiation (41) and mediation through the Hedgehog pathway (42) have been explored in previous studies. Recently *in vitro* studies have shown DNMTs inhibitor can induce apoptosis in RCC cells (43,44). In addition, another study suggest that DNMTs inhibitor could suppress RCC cell proliferation by inducing G2/M cell cycle arrest and strikingly increase the sensitivity of RCC to paclitaxel (45), leading to using DNMT inhibitors in clinical trials of renal cancers (46-48). Our data showed that knockdown of DNMT1 expression significantly inhibited cell viability, induced apoptosis, decreased colony formation and invading ability in ccRCC cells. These results were in agreement with previous studies in bladder cancer (13), pancreas cancer (49), colorectal cancer (50), ovarian cancer (51), cholangiocarcinoma (52), as well as in lung, esophageal cancer, and malignant pleural mesothelioma cells (53). Moreover, some studies have also investigated the synergistic knockdown of DNMT1 and DNMT3b and shown a synergistic effect in CP70 ovarian cancer cell line (51), QBC-939 cholangiocarcinoma cell line (52) and a colorectal cancer cell line (50). Thus, further studies are needed to confirm whether there are synergistic effects after combination of DNMT1 and DNMT3a or DNMT3b siRNA in ccRCC.

In summary, the present study demonstrated that DNMT1 was higher expressed in ccRCC than no-tumor tisues, and the expression of DNMT1 was strongly associated with ccRCC tumor size, tumor pathology stage, histological grading, lymph node metastasis, vascular invasion, recurrence, and prognosis. Knockdown of DNMT1 expression significantly inhibited the viability and induced apoptosis of ccRCC cells, as well as inhibited the colony formation and invading ability. DNMT1 may thus serve as a potential prognostic marker and a novel therapeutic target for ccRCC patients. Nevertheless, further studies with large samples are warranted to confirm the present findings.

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