

DKC1 serves as a potential prognostic biomarker for human clear cell renal cell carcinoma and promotes its proliferation, migration and invasion via the NF- κ B pathway

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Received November 24, 2017; Accepted May 31, 2018

DOI: 10.3892/or.2018.6484

Abstract. DKC1, an X-linked gene encoding dyskerin at Xq28, is a crucial component of the telomerase complex and is indispensable for normal telomere function and the post-transcriptional modification of precursor rRNA. It has been revealed to exert diverse biological functions and prognostic values in numerous types of cancers. Our present study was aimed at examining DKC1 expression in normal renal tissues and clear cell renal cell carcinoma (ccRCC) samples and the prognostic value of DKC1 in ccRCC. We examined DKC1 protein expression levels in tissue microarrays including 307 cases of ccRCC tissues and in 75 pairs of ccRCC and paracancerous tissues with immunohistochemistry. The percentage of DKC1 expression in ccRCC (61.3%) was markedly higher than that in paracancerous tissues (34.7%) ($P=0.001$). Positive DKC1 expression tends to significantly be associated with unfavorable clinicopathological characteristics such as tumor diameter >7 cm ($P=0.002$) and tumor-node-metastasis (TNM) stage III or IV ($P<0.001$). Multivariate COX analysis revealed that positive DKC1 expression was an independent unfavorable factor for prognosis of ccRCC patients [hazard ratio (HR)=1.932, 95% CI,

1.290-2.893, $P=0.001$ for 5-year overall survival; HR=1.778, 95% CI, 1.150-2.748, $P=0.010$ for disease-free survival]. In the PROGgeneV2 platform, we also found that ccRCC patients with high DKC1 mRNA expression had a poorer prognosis than patients with low DKC1 expression in The Cancer Genome Atlas (TCGA). Furthermore, we found that knock-down of DKC1 inhibited proliferation, migration and invasion of ccRCC through regulation of the NF- κ B/MMP-2 signaling pathway *in vitro*. We also demonstrated that DKC1 regulated ccRCC proliferation and the expression of NF- κ B-p65 and MMP-2 *in vivo*. In summary, the expression of DKC1 was upregulated in ccRCC, which was associated with unfavorable clinicopathological characteristics and DKC1 may act as an independent prognostic indicator of ccRCC patients.

Introduction

Renal cell carcinoma (RCC) is accountable for ~90% of renal cancer patients and is the most prevalent malignancies of kidney cancer (1). Among all RCC histological subtypes, clear cell RCC (ccRCC) is the most malignant form and leads to the most cancer-related deaths (2). Although marked development has been achieved in the diagnosis and treatment of ccRCC, ~30% of patients have metastatic disease (3). Surgery is still the most effective treatment for localized primary ccRCC, but ccRCC is resistant to conventional treatments such as radiation, hormone treatment and chemotherapy. Despite the fact that specific targeted therapies with favorable clinical outcomes to a certain extent have been developed, individual differences in response and the risk of adverse effects restrict the use of these drugs (4). Therefore, appropriate ccRCC biomarkers may contribute to improve early diagnosis and patient therapy.

Dyskerin is a predominantly nucleolar protein encoded by the DKC1 gene and is involved in dyskeratosis congenital (5). It is a component of H/ACA small nucleolar ribonucleoprotein with various characteristics, such as bone marrow failure, mucocutaneous abnormalities and an increased tumor susceptibility (6). Several lines of evidence have demonstrated that DKC1 expression is significantly upregulated and associated with poor prognosis in some human cancers, such as prostate cancer (7), neuroblastomas (8) and hepatocellular carcinoma (9). Despite

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Abbreviations: ccRCC, clear cell renal cell carcinoma; IHC, immunohistochemistry; NF- κ B, nuclear factor κ B; MMPs, matrix metalloproteinases; TMA, tissue microarray; IRS, immunoreactive score; TCGA, The Cancer Genome Atlas; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ECM, extracellular matrix; HR, hazard ratio; CI, confidence interval

Key words: renal cell carcinoma, DKC1, NF- κ B, prognostic biomarker, tumorigenesis

these survey results, the significance of dyskerin expression in cancer has been disputed, with some research revealing that dyskerin may have a function as a tumor suppressor (10,11). In addition, the specific mechanism and significance of DKC1 in ccRCC progression are not fully known.

In this study, we sought to examine the expression levels of DKC1 in ccRCC, and then explored its association with the clinical characteristics, 5-year overall and disease-specific survival of ccRCC patients. In addition, we explored the difference in the expression levels of DKC1 in renal cancer and paracancerous tissues. Moreover, we also investigated how DKC1 regulated ccRCC cell proliferation, migration and invasion *in vitro*, and tumor growth *in vivo* as well as the molecular mechanisms.

Materials and methods

Patients and specimens. Two independent retrospective ccRCC cohorts with tissue microarrays (TMAs) were examined in the present study. The small ccRCC TMA was obtained from Shanghai Outdo Biotechnology (Shanghai, China), which contained 75 pairs of ccRCC tissues and matched paracancerous tissues. It contained 75 patients who underwent radical nephrectomy between November 2006 and September 2008. Another large TMA was composed of 310 ccRCC tissues from patients collected from the Affiliated Hospital of Xuzhou Medical University who underwent radical nephrectomy without prior treatment from February 2005 to December 2008, and their clinicopathological characteristics containing age, sex, tumor diameter, depth of invasion, lymph node and distant metastasis, as well as tumor-node-metastasis (TNM) stage were obtained. Due to specimen deficiency during antigen retrieval, finally 307 ccRCC patients in the large TMA were utilized to explore the association of DKC1 expression with clinicopathological features. Informed consents from all patients were obtained and institutional approval was obtained by the review board of the Affiliated Hospital of Xuzhou Medical University prior to this study.

Construction of TMAs and immunohistochemistry (IHC). Construction of the large TMA was performed by contract service at the National Engineering Center for Biochip (Shanghai, China). Each tissue microarray dot was cut 1.5 mm in diameter from the paraffin tumor block. The standard procedure for IHC of TMA was performed as previously described (12). The polyclonal rabbit anti-DKC1 (1:50, cat. no. ab64667; Abcam, Cambridge, MA, USA) was used for primary antibody incubation at 4°C overnight. The slides without primary antibody incubation were used as negative controls.

Assessment of immunostaining. The evaluation of DKC1 staining was blindly examined by two pathologists. Positive DKC1 immunostaining was defined mainly in the cytoplasm but could also be observed in the nucleus area. The tissues were scored according to both the intensity and percentage of cells with positive staining. The staining intensity of DKC1 was scored as 0-3 (0=negative; 1=weak; 2=moderate; 3=strong). The percentage of DKC1-positive stained cells was also scored into 4 categories: 1 (0-25%); 2 (26-50%); 3 (51-75%); and 4 (76-100%). In the case of a discrepancy between copied cores, the mean score from the two tissue cores was

selected as the final score. The level of DKC1 staining was evaluated by immunoreactive score (IRS), which is calculated by multiplying the scores of the staining intensity and the percentage of positive cells. For statistical analysis, scores of 0-5 were considered as low expression, while scores of 6-12 were considered as high expression; the cut-off value was determined by receiver operating characteristic curve analysis.

Animals and cell lines. A total of 20 female BALB/c nude mice weighing 14-18 g, 6-8 weeks old, were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China) for studies approved by the Animal Care Committee of Xuzhou Medical University and maintained under specific pathogen-free conditions. Human embryo kidney epithelial cell line HK-2 and human ccRCC cell lines ACHN, 786-O and OSRC-2 were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). HK-2 cells were cultured in keratinocyte serum free medium (K-SFM) supplemented with 10% fetal calf serum (FCS; both from Invitrogen; Thermo Fisher Scientific, Inc., Shanghai, China). ACHN cells were cultured in Minimum Essential Media medium (MEM) supplemented with 10% FCS (both from Invitrogen; Thermo Fisher Scientific, Inc.). 786-O and OSRC-2 cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Invitrogen; Thermo Fisher Scientific, Inc., Shanghai, China) supplemented with 10% FCS. These four cell lines were both incubated in a 37°C humidified incubator with 5% CO₂.

Plasmid, siRNA and shRNA transfections. The DKC1 siRNA and scrambled siRNA were purchased from Shanghai GenePharma Co., Ltd., (Shanghai, China). The p65 overexpression plasmids were obtained from Dr Yu Wu (Nanjing Medical University, Nanjing, China). DKC1 siRNA and scrambled siRNA were transfected into the ACHN and 786-O cells by siLentFect™ Lipid reagent (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's protocol. Transfection of p65 overexpression plasmid and control vector into the ACHN and 786-O cells was performed using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The DKC1 knockdown ACHN cell lines (DKC1^{KD}-ACHN) and control ACHN cell lines (Ctrl-ACHN) were established by transfecting with lentivirus packing DKC1 shRNA expression and control vector, respectively (Shanghai GenePharma). Target cells were transfected with the lentivirus for 48 h and then selected with puromycin (Santa Cruz Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at a concentration of 5 µg/ml for 3 weeks.

Cell proliferation assay. Complete medium (2 ml) containing 1x10⁵ stable DKC1 knockdown ACHN or 786-O cells and corresponding controls were seeded in 6-well plates and cultured for 24, 48, 72 and 96 h. At exact time-points, the number of cells in the DKC1 knockdown ACHN or 786-O cells and corresponding controls was counted respectively.

Cell migration and invasion assays. Cell migration and invasion assays were carried out using Transwell filter inserts (8.0-µm pore size with polycarbonate membranes) precoated with or without Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The detailed conditions were previously described (13).

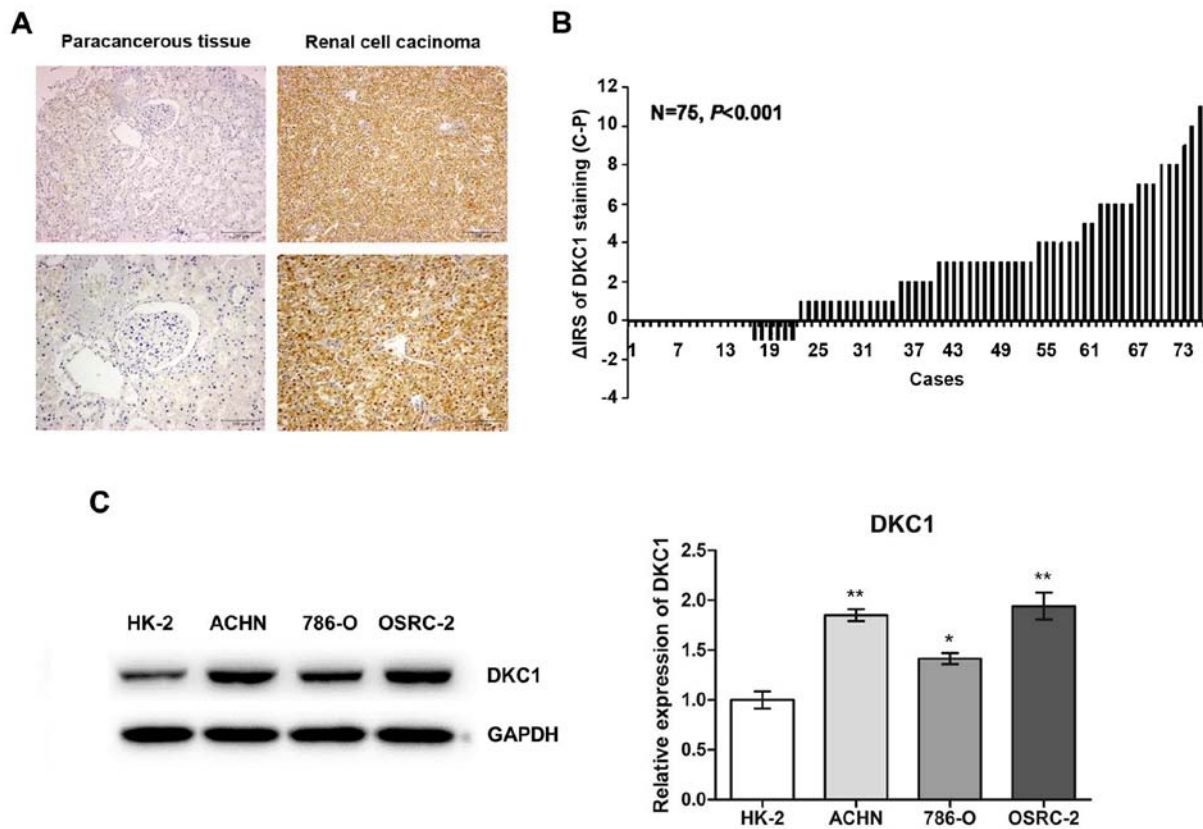


Figure 1. DKC1 expression is increased in ccRCC compared with normal renal tissues and cell lines. (A) Representative images of DKC1 immunohistochemical staining in TMA are shown. Top panel, magnification, x100; Bottom panel, magnification, x200. (B) The distribution of the difference in staining intensities of DKC1 in ccRCC tissues compared with paracancerous tissues. C, ccRCC tissues; P, paracancerous tissues; IRS, immunoreactivity score. (C) Western blot analysis of DKC1 expression in human embryo kidney epithelial cells HK-2 and renal cell carcinoma cell lines, including ACHN, 786-O and OSRC-2. Data are presented as the mean \pm SD, * $P<0.05$, ** $P<0.01$. ccRCC, clear cell renal cell carcinoma.

Antibodies and western blotting (WB). WB was performed as previously reported (14). The following antibodies against the corresponding proteins were used for WB: DKC1 (1:1,000 for WB, cat. no. ab64667; Abcam); NF- κ B-p65 (1:1,000 for WB; 1:50 for IHC, cat. no. 8242P; Cell Signaling Technology, Inc., Beverly, MA, USA); MMP-2 (1:1,000 for WB, 1:50 for IHC, cat. no. 4022S; Cell Signaling Technology, Inc.); p-AKT (1:1,000 for WB, cat. no. 4060T; Cell Signaling Technology, Inc.); p-S6K (1:1,000 for WB, cat. no. 9234T; Cell Signaling Technology, Inc.); MMP-9 (1:1,000 for WB, cat. no. 3852S; Cell Signaling Technology, Inc.) and GAPDH (1:5,000 for WB, cat. no. sc-365062; Santa Cruz Biotechnology, Inc.). Then, HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (1:10,000, anti-mouse, cat. no. SA00001-1, anti-rabbit cat. no. SA00001-2; Proteintech Group, Inc., Rosemont, IL, USA) were applied to the blot for 1 h at room temperature.

Subcutaneous tumor model in vivo. To produce a subcutaneous tumor model *in vivo*, the BALB/c nude mice were randomly divided into two groups consisting of 15 mice each. Stable DKC1 knockdown (1×10^6) and control ACHN cells were suspended in 200 μ l phosphate-buffered saline (PBS) and subcutaneously injected through axillary fossa, respectively. After 2 weeks, the two groups of mice were sacrificed; their subcutaneous tumors were excised and fixed in 10% buffered formalin for statistical analysis and further histopathological analysis.

Statistical analysis. Paired Wilcoxon test was used to explore the difference of DKC1 staining between tumors and their matched non-tumor tissues. Fisher's exact test was carried out to assess the correlation between DKC1 expression and clinicopathological features. The Kaplan-Meier method with a log-rank test was used to investigate the differences of the 5-year survival and disease-specific survival between the positive DKC1 expression group and the negative expression group. Univariate and multivariate Cox proportional hazards regression analysis were used to evaluate the hazard ratios (HRs) and 95% confidence interval (CI) of HRs. Independent samples t-test was used in the proliferation, migration and invasion assays. All the statistical analyses were performed by SPSS 20.0 statistical software package (IBM Corp., Armonk, NY, USA). A P-value of <0.05 was defined as statistically significant, and all tests were two-sided.

Results

DKC1 expression is increased in ccRCC tissues and RCC cell lines. To explore whether the expression level of DKC1 was altered in RCC, a small TMA was used to investigate DKC1 protein expression in 75 pairs of ccRCC and paracancerous tissues using IHC. Our data revealed that DKC1 protein was localized in both the cytoplasm and nucleolus (Fig. 1A). As shown in Table I, high DKC1 expression was detected in 46 out of 75 (61.3%) ccRCC tissues and in 26 out of 75 (34.7%)

Table I. DKC1 expression in ccRCC and paracancerous tissues.

Tissues	DKC1 staining			P-value ^a
	Low (%)	High (%)	Total	
Paracancerous tissues	49 (65.3)	26 (34.7)	75	0.001
Renal cell carcinoma	29 (38.7)	46 (61.3)	75	

^a χ^2 test. ccRCC, clear cell renal cell carcinoma.

Table II. DKC1 staining and clinicopathological characteristics of 307 renal cancer patients.

Variables	DKC1 staining			P-value ^a
	Low (%)	High (%)	Total	
Age (years)				0.674
≤56	119 (80.4)	29 (19.6)	148	
>56	124 (78.0)	35 (22.0)	159	
Sex				0.460
Male	164 (80.4)	40 (19.6)	204	
Female	79 (76.7)	24 (23.3)	103	
Tumor size (cm)				0.002
≤7	115 (57.5)	85 (42.5)	200	
>7	41 (38.3)	66 (61.7)	107	
pT status				<0.001
pT ₁ -pT ₂	178 (74.8)	60 (25.2)	238	
pT ₃ -pT ₄	28 (40.6)	41 (59.4)	69	
pN status				0.041
pN ₀	154 (54.0)	131 (46.0)	285	
pN ₁ -pN ₃	9 (40.9)	13 (59.1)	22	
pM status				0.609
pM ₀	166 (57.6)	122 (42.4)	288	
pM ₁	8 (42.1)	11 (57.9)	19	
TNM stage				<0.001
I-II	221 (93.6)	15 (6.4)	236	
III-IV	22 (31.0)	49 (69.0)	71	

^aTwo-sided Fisher's exact tests. TNM, tumor-node-metastasis.

paracancerous tissues, and there was a significant expression difference between ccRCC and paracancerous tissues (Fig. 1B). Furthermore, western blot analysis revealed that the expression of DKC1 was significantly lower in HK-2, a type of human embryo kidney epithelial cell line, as compared with that in all 3 analyzed RCC cell lines, including ACHN, 786-O and OSRC-2 (Fig. 1C). Consequently, our results demonstrated that DKC1 was increased in RCC.

DKC1 expression is associated with clinicopathological characteristics in ccRCC patients. To further study the association between DKC1 expression and clinicopathological features, another TMA including 307 cases of ccRCC tissues

was used to investigate DKC1 protein expression. There were 204 male and 103 female patients. Their mean age was 55.8 years. The distribution of the TNM stage was as follows: 181 patients at stage I, 55 at stage II, 40 at stage III and 31 at stage IV. Table II contains the association between DKC1 and the clinicopathological features of ccRCC patients. The data revealed that there were significant correlations of DKC1 expression with tumor size ($P=0.002$), pT status ($P<0.001$), pN status ($P=0.041$) and TNM stage ($P<0.001$). However, no significance was found between DKC1 expression and other clinical characteristics, such as sex and age. These results indicated that high DKC1 expression tends to be associated with advanced clinicopathological parameters in ccRCC patients.

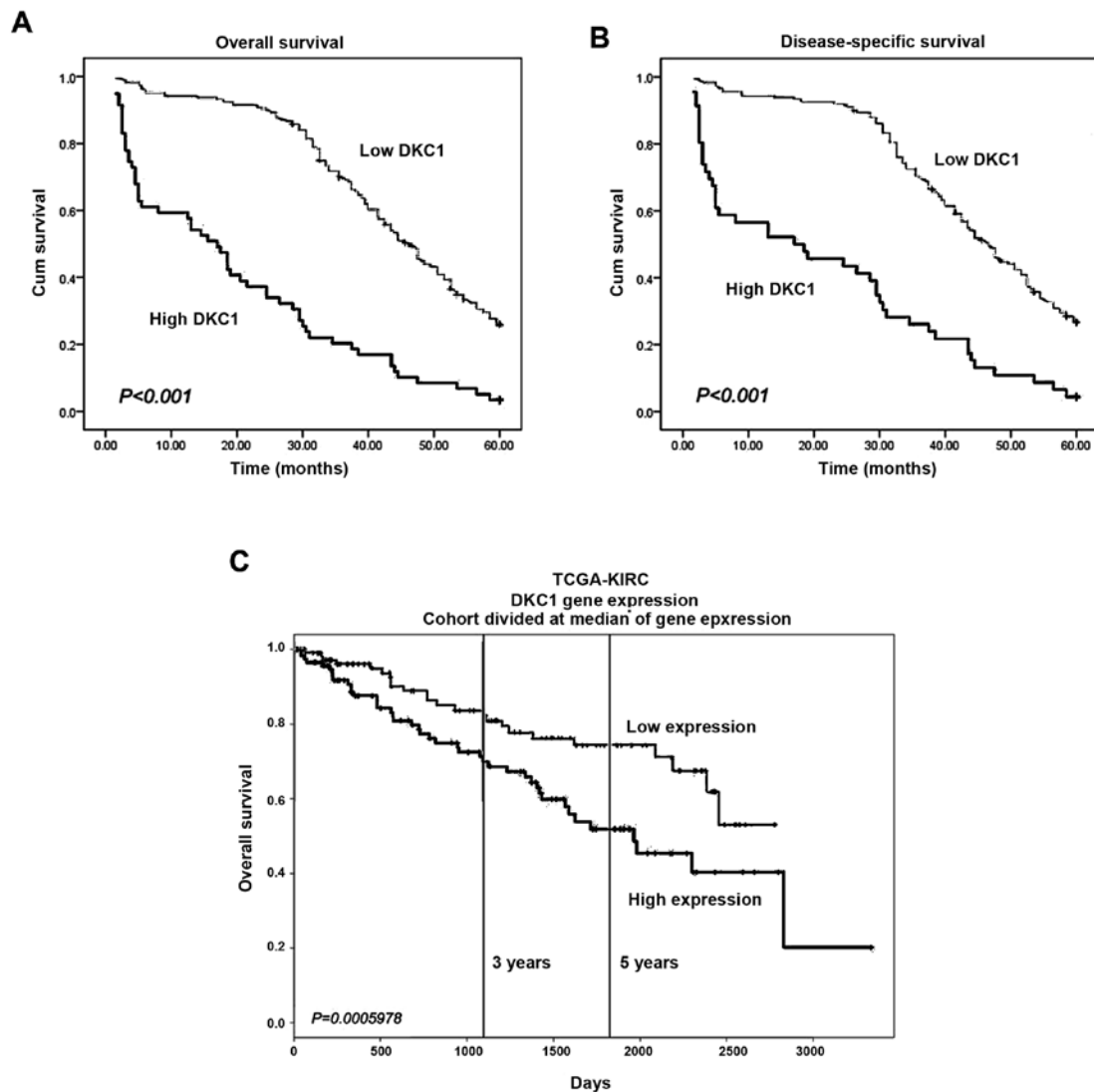


Figure 2. DKC1 expression is correlated with prognosis in ccRCC patients. (A and B) Kaplan-Meier curves revealed the difference of cum survival in the 5-year overall survival and the disease-specific survival of the ccRCC patients with high/low DKC1 expression. (C) Increased DKC1 mRNA expression resulted in poor survival of ccRCC patients in TCGA. Survival data were searched for and analyzed from PROGeneV2 (<http://watson.compbio.iupui.edu/chirayu/progene/database/?url=progene>). ccRCC, clear cell renal cell carcinoma; cum survival, cumulative survival; TCGA, The Cancer Genome Atlas.

DKC1 expression is correlated with the prognosis of ccRCC patients. To evaluate the prognostic value of DKC1 expression in ccRCC, the log-rank test combined with Kaplan-Meier survival curves was established. Our data revealed that ccRCC patients with high DKC1 expression were correlated with greater unfavorable 5-year overall and disease-specific survival than the rest of the patients with low DKC1 expression ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 2A and B). Furthermore, to investigate whether DKC1 was an independent prognostic marker in ccRCC, univariate and multivariate COX analysis was performed. In the univariate COX analysis, our data suggested that increased DKC1 expression was significantly correlated with a worse 5-year overall and disease-specific survival, as well as with other prognostic markers, such as tumor size, pT status, pN status, pM status and TNM stage (Table III). In addition, both increased DKC1 expression (HR=1.932, 95% CI, 1.290-2.893, $P=0.001$ for 5-year overall survival; HR=1.778, 95% CI, 1.150-2.748, $P=0.010$ for disease-free survival) and TNM

stage (HR=2.790, 95% CI, 1.861-4.184, $P < 0.001$ for 5-year overall survival; HR=2.689, 95% CI, 1.746-4.140, $P < 0.001$ for disease-free survival) were independent adverse prognostic markers for the 5-year overall and disease-specific survival of ccRCC patients in multivariate COX analysis (Table IV). By searching the PROGeneV2 platform, we found that ccRCC patients with high DKC1 expression had significant poorer prognosis than those with low DKC1 expression in The Cancer Genome Atlas (TCGA) which was consistent with our findings (Fig. 2C).

Knockdown of DKC1 suppresses ccRCC cell proliferation, migration and invasion in vitro. To explore the role of DKC1 in ccRCC progression, ACHN and 786-O cells were transiently transfected with control siRNA and DKC1 siRNA, respectively (Fig. 3A and B). Then the cell proliferation assays were carried out and the data revealed that DKC1 depletion led to a significant decrease in cell proliferation (Fig. 3C and D). Since high DKC1 expression was relevant

Table III. Univariate Cox proportional regression analysis on the 5-year overall and disease-specific survival of ccRCC patients.

Variables	Overall survival			Disease-specific survival		
	Hazard ratio	95% CI ^b	P-value ^a	Hazard ratio	95% CI ^b	P-value ^a
DKC1						<0.001
Low	1.000		<0.001	1.000		
High	3.525	2.574-4.828		3.126	2.213-4.414	
Age (years)						0.728
≤56	1.000		0.637	1.000		
>56	1.067	0.810-1.410		1.053	0.786-1.411	
Tumor size (cm)						0.021
≤7	1.000		0.003	1.000		
>7	1.676	1.194-2.352		1.549	1.069-2.244	
pT status						0.014
pT ₁ -pT ₂	1.000		0.002	1.000		
pT ₃ -pT ₄	1.574	1.178-2.102		1.473	1.081-2.007	
pN status						<0.001
pN ₀	1.000		<0.001	1.000		
pN ₁ -pN ₃	3.421	1.842-6.352		3.046	1.686-5.502	
pM status						0.021
pM ₀	1.000		<0.001	1.000		
pM ₁	4.162	2.282-7.594		2.870	1.172-7.027	
TNM stage						<0.001
I-II	1.000		<0.001	1.000		
III-IV	4.084	2.972-5.613		3.713	2.628-5.247	

^aP-values are from log-rank test; ^bCI, confidence interval. ccRCC, clear cell renal cell carcinoma; TNM, tumor-node-metastasis.

Table IV. Multivariate Cox regression analysis on the 5-year overall and disease-specific survival of ccRCC patients.

Variables ^a	Overall survival			Disease-specific survival		
	Hazard ratio	95% CI ^b	P-value ^a	Hazard ratio	95% CI ^b	P-value ^a
DKC1	1.932	1.290-2.893	0.001	1.778	1.150-2.748	0.010
Age (years)	1.189	0.897-1.576	0.228	1.126	0.838-1.513	0.432
Tumor size	1.319	0.930-1.871	0.120	1.293	0.885-1.888	0.184
TNM stage	2.790	1.861-4.184	<0.001	2.689	1.746-4.140	<0.001

^aCoding of variables, DKC1 was coded as 1 (low), and 2 (high). Age was coded as 1 (≤56 years), and 2 (>56 years). Tumor size was coded as 1 (≤7 cm), and 2 (>7 cm). TNM stage was coded as 1 (I-II), and 2 (III-IV); ^bCI, confidence interval. ccRCC, clear cell renal cell carcinoma; TNM, tumor-node-metastasis.

to poor prognosis in the ccRCC patient cohort, we further explored the metastatic role of DKC1 in ccRCC cells. The Transwell assays were performed and our data revealed that knockdown of DKC1 decreased the abilities of cell migration in ACHN and 786-O cells when compared with the corresponding controls (Fig. 4A and B). In accordance with these consequences, the abilities of cell invasion were significantly decreased in ACHN and 786-O cells by DKC1 siRNAs when compared with the respective controls (Fig. 4C and D).

Knockdown of DKC1 inhibits ccRCC cell proliferation, migration and invasion via regulation of the NF-κB/MMP-2 signaling pathway. NF-κB is a crucial transcriptional factor and plays an important role in tumorigenesis. It regulates cell proliferation, metastasis, angiogenesis and survival, thus it is not surprising that NF-κB has been demonstrated to be activated in many human cancers (15). Its κB site was found in the promoters of genes encoding MMP-2 which plays a critical role in cancer metastasis (16). In order to investigate the possible mechanism of DKC1 regulation of proliferation and metastasis in ccRCC

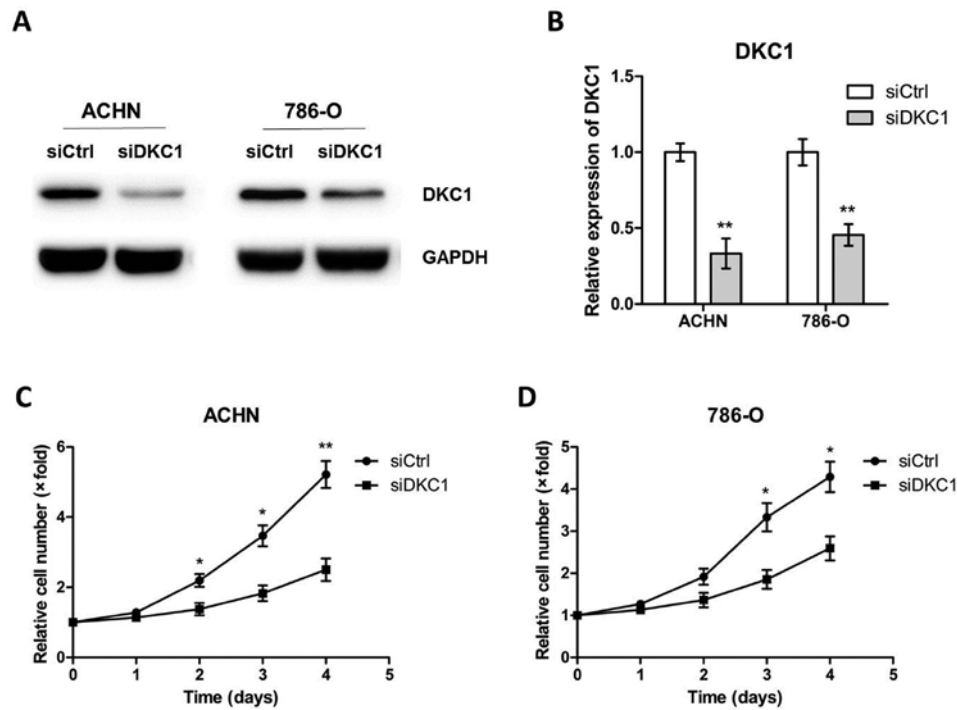


Figure 3. Knockdown of DKC1 inhibits cell proliferation of ccRCC cells *in vitro*. (A and B) Western blot analysis revealed that DKC1 expression in ACHN and 786-O was markedly knocked down by siRNA when compared with corresponding controls. (C and D) Cell proliferation assays were carried out to assess the cell proliferation rates of ACHN and 786-O cells with different DKC1 expression levels. Data are presented as the mean \pm SD, * P <0.05, ** P <0.01. ccRCC, clear cell renal cell carcinoma.

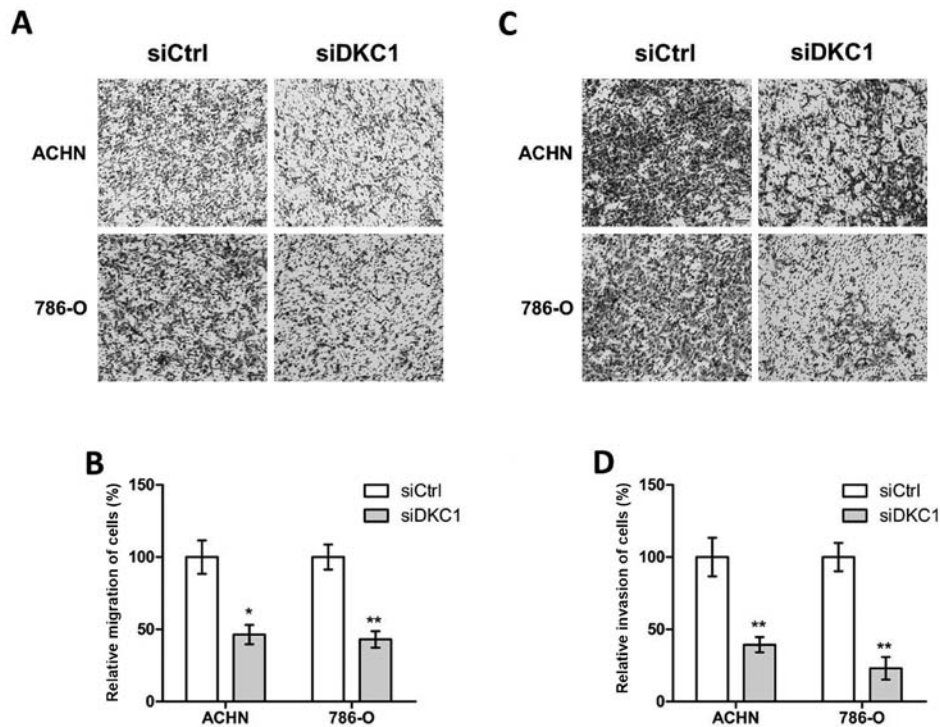


Figure 4. Knockdown of DKC1 inhibits the migration and invasion of ccRCC cells *in vitro*. (A) The migration of ACHN and 786-O cells with different DKC1 expression levels. (B) The cell migration number per field was counted in three random fields in ACHN and 786-O cells. (C) The invasion of ACHN and 786-O cells with different DKC1 expression levels. (D) The cell invasion number per field was counted in three random fields in ACHN and 786-O cells. Data are presented as the mean \pm SD, * P <0.05, ** P <0.01. ccRCC, clear cell renal cell carcinoma.

cells, we carried out western blotting to explore the protein levels of NF- κ B and MMPs in ACHN and 786-O cells. Our data revealed that DKC1 knockdown significantly inhibited

p65 and MMP-2 protein expression in ACHN and 786-O cells compared with the corresponding controls, but not MMP-9 (Fig. 5A). Since the PI3K/AKT pathway is also an important

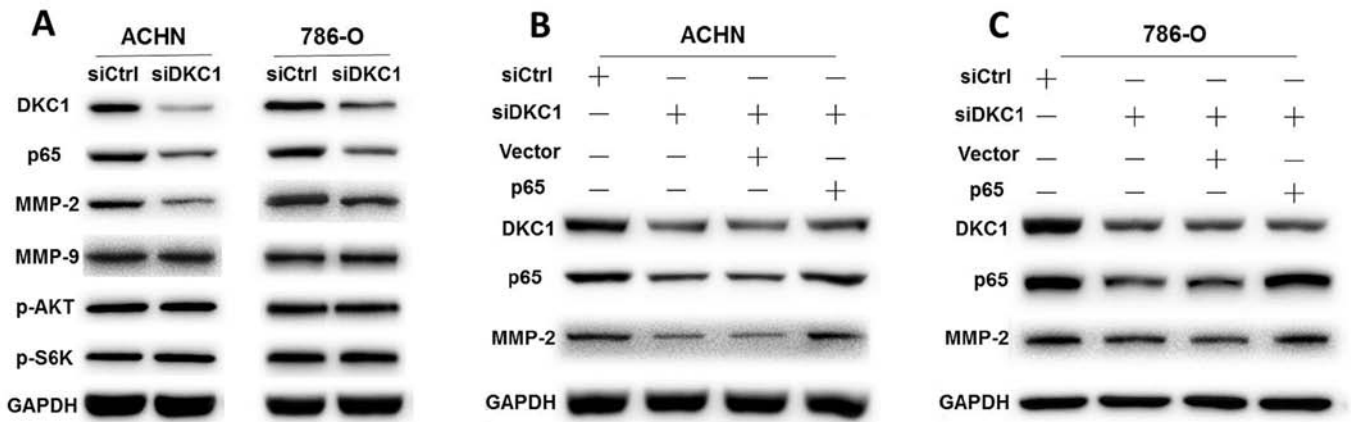


Figure 5. DKC1 regulates the expression of p65 and MMP-2. (A) Western blotting was performed to investigate the expression of cell proliferation and metastasis-related proteins, such as p65, MMP-2, MMP-9, p-AKT, p-S6K in ACHN and 786-O with different DKC1 expression levels. (B and C) Western blot analysis revealed that the p65 and MMP-2 expression in DKC1-knockdown ACHN and 786-O cells was significantly rescued by p65 expression plasmid when compared with the corresponding controls.

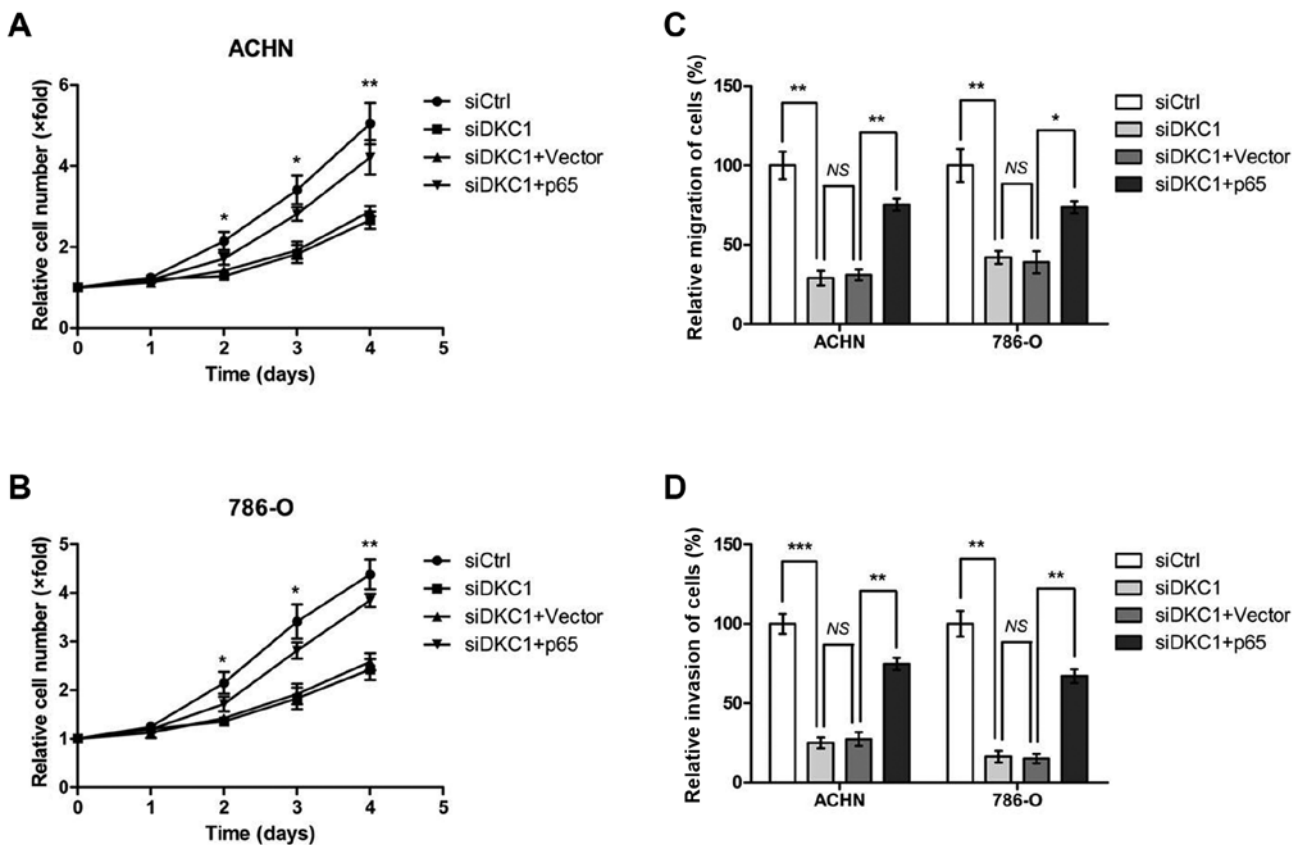


Figure 6. p65 rescues the decreased abilities of proliferation, migration and invasion of ccRCC cells induced by knockdown of DKC1. (A and B) p65 overexpression rescued the reduced abilities of proliferation in DKC1-knockdown ACHN and 786-O cells. (C and D) p65 overexpression rescued the reduced abilities of migration and invasion in DKC1-knockdown ACHN and 786-O cells. The cell migration and invasion number per field was counted in three random fields in ACHN and 786-O cells. Data are presented as the mean \pm SD, * P <0.05, ** P <0.01, *** P <0.001. ccRCC, clear cell renal cell carcinoma.

factor of cell proliferation and survival (17), we detected the expression levels of p-AKT and p-S6K. However, the results revealed that the protein levels of p-AKT and p-S6K were similar regardless of the expression levels of DKC1 (Fig. 5A).

To further investigate whether DKC1 regulated ccRCC cell proliferation and metastasis through the NF- κ B/MMP-2 signaling pathway, p65 rescue assays were performed. We

co-transfected p65 overexpression plasmids and DKC1 siRNAs in ACHN and 786-O cells, respectively. Our data revealed that p65 overexpression significantly rescued the expression of MMP-2 (Fig. 5B and C). The cell proliferation assays revealed that the decreased abilities of cell proliferation in the DKC1-knockdown ACHN and 786-O cells could be markedly accelerated by p65 overexpression (Fig. 6A and B).

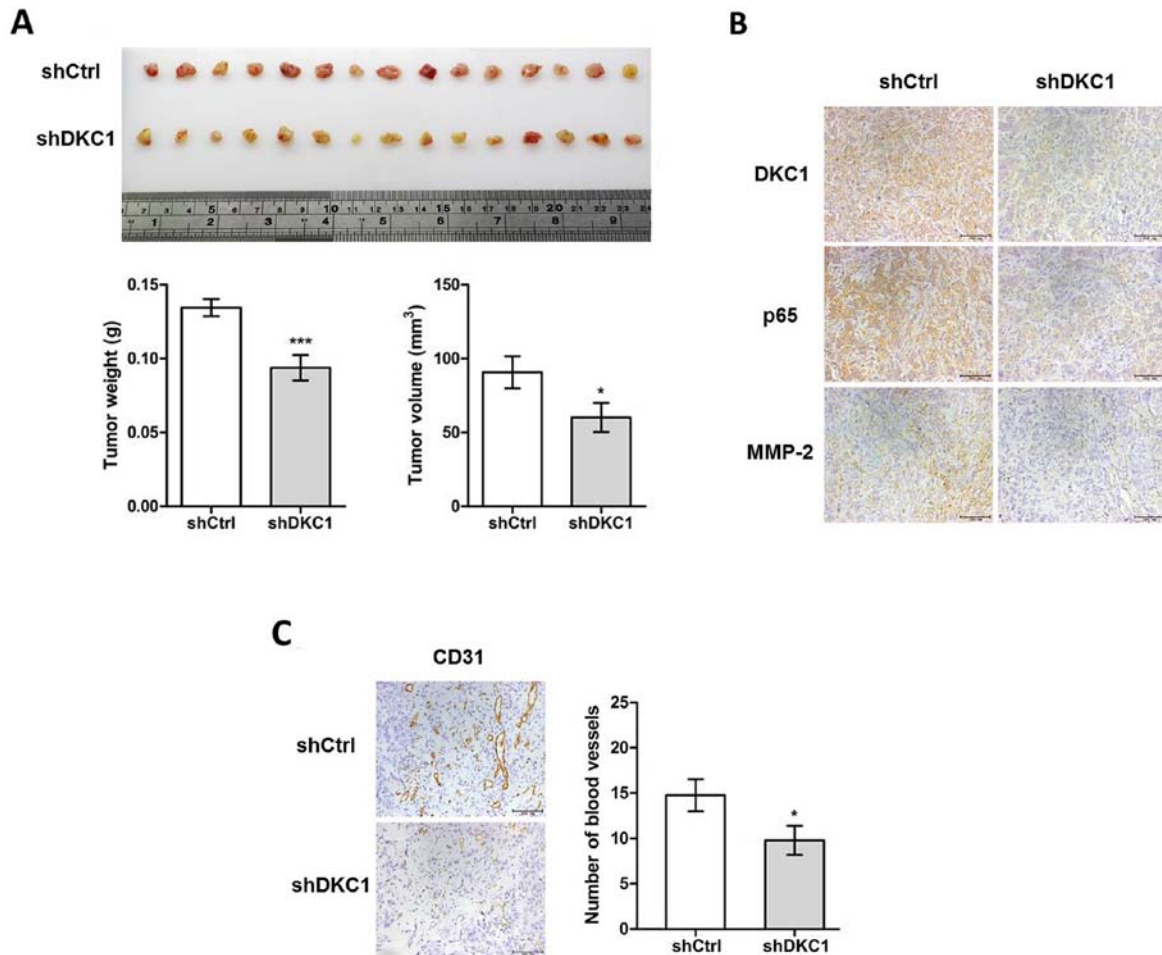


Figure 7. DKC1 promotes proliferation and angiogenesis of ccRCC *in vivo*. (A) Images of subcutaneous tumors resected from mice after 2 weeks of growth *in vivo*. (B) Immunostaining of DKC1, p65 and MMP-2 in subcutaneous tumors of DKC1^{KD} and control ACHN groups. DKC1, p65 and MMP-2 expression in DKC1^{KD} group were much lower than the control group. (C) Immunostaining of CD31 in subcutaneous tumors of DKC1^{KD} and control ACHN groups and the number of blood vessels marked by CD31 per field was counted in three random fields. Data are presented as the mean \pm SD, *P<0.05, ***P<0.001. ccRCC, clear cell renal cell carcinoma.

Moreover, the results of cell migration and invasion revealed that p65 overexpression markedly rescued the DKC1-knockdown-decreased cell migration and invasion abilities (Fig. 6C and D). These results indicated that p65 and MMP-2 functioned as the downstream targets of DKC1 in ccRCC cell proliferation, migration and invasion.

DKC1 accelerates the proliferation and angiogenesis of ccRCC cells in vivo. To further validate the function of DKC1 in the regulation of ccRCC proliferation *in vivo*, DKC1^{KD}-ACHN cell lines and Ctrl-ACHN cell lines were established. The stable DKC1 knockdown and control ACHN cells were subcutaneously injected into BALB/c nude mice. After 2 weeks, the two groups of mice were sacrificed and their subcutaneous tumors were excised (Fig. 7A) and statistical analysis was performed on the differences in tumor weight and volume between the two groups of mice. Our data revealed that a significant decrease of tumor weight and volume was observed in the DKC1^{KD} group when compared with the control group (Fig. 7A). Then, we carried out immunohistochemical staining on paraffin-sectioned neoplastic tissues. The data revealed that the expression of DKC1, p65 and MMP-2 were markedly reduced in the knockdown group compared with the control

group (Fig. 7B). In addition, the visual examination revealed that neovessels in the subcutaneous tumors were reduced in the DKC1^{KD} group when compared with those in the control group (Fig. 7A). Previous research had demonstrated that NF- κ B promoted angiogenesis (18). To further validate the difference of angiopoiesis between the two groups, we detected the expression of CD31 in the two groups. The results revealed that the number of vessels labeled by CD31 were significantly fewer in the DKC1^{KD} group than the control group (Fig. 7C). These results further confirmed our conclusion which had been previously demonstrated *in vitro*.

Discussion

Updated research has demonstrated that functional genes which play a role in tumorigenesis can be regarded as potential biomarkers for the diagnosis and prognosis of ccRCC patients (19). DKC1, an X-linked gene encoding dyskerin at Xq28, is a crucial ingredient of the telomerase complex and is indispensable for normal telomere function and the post-transcriptional modification of precursor rRNA (20,21). It has been revealed to exert diverse biological functions and have prognostic value in numerous types of cancer (22-24). In the

present study, we examined the prognostic value and biological role of DKC1 in ccRCC. Using retrospective cohorts of ccRCC patients with TMAs, we discovered that DKC1 expression was significantly upregulated in ccRCC compared with normal renal tissues, and positively associated with TNM stage. Moreover, ccRCC patients with increased DKC1 expression had an unfavorable survival, and multivariate COX regression analysis revealed that positive DKC1 expression was an independent hazardous indicator for the prognosis of ccRCC patients. Our findings from survival analysis conformed with the data obtained in The Cancer Genome Atlas (TCGA) collected from PROGeneV2 platform. These results revealed that DKC1 was a prognostic factor for ccRCC patients and may act as an oncogene in ccRCC progression.

RCC is the predominant malignancy of kidney cancer and is greatly resistant to chemotherapy and radiation. The status of the p53 tumor-suppressor gene has been associated with the efficacy of chemotherapy and radiation, where aberrant p53 function can be attributed to defective responsiveness to treatment (25,26). However, p53 is rarely mutated in RCC, which suggests that other genes may be involved in the regulation of tumorigenesis in RCC (27). To date, few studies have reported the potential role of DKC1 in tumorigenesis and these results are disputable. von Stedingk *et al* (28) revealed that a potential function of DKC1 was to increase telomerase activity and contribute to advanced tumors, suggesting an oncogenic role of DKC1. However, Montanaro *et al* (29) reported that low DKC1 expression was correlated with tumor progression, thus revealing that DKC1 may serve as a tumor suppressor. The molecular mechanism and significance of DKC1 in ccRCC progression are still unclear. Our results demonstrated that DKC1 expression tends to be associated with adverse clinicopathological characteristics in ccRCC patients, which supports its role in tumor promotion.

Tumor development and progression require six necessary changes to normal cell physiology: An independent growth signaling pathway; sustained angiogenesis; resistance to growth inhibition; escape of apoptosis; tissue invasion and metastasis; and cell immortality (30). In the present study, we investigated the role of DKC1 in several important processes, such as proliferation, migration, invasion and angiogenesis. Our data revealed that DKC1 expression regulated ccRCC cell proliferation *in vitro* and *in vivo*, which was consistent with our clinical data that DKC1 expression was associated with tumor diameter. Moreover, we demonstrated that knockdown of DKC1 markedly inhibited ccRCC cell migration and invasion *in vitro* and angiogenesis *in vivo*. These findings could explain our previous assertion that positive DKC1 expression was associated with enhanced lymph node metastases.

Nuclear factor of κ B (NF- κ B) is a transcriptional factor and it has been most extensively studied for its function in immunity and inflammation (31). Until recent decades, NF- κ B has been characterized by its critical role in cancer development and progression and induces the expression of MMPs (32,33). Matrix metalloproteinases (MMPs) can break down the extracellular matrix (ECM) in numerous malignant tumors and are crucial for metastasis-promoting genes (34). Except for their role in the extracellular matrix and migration of cancer cells, MMPs also regulate signaling pathways which control cell growth and angiogenesis (35).

In the present study, we investigated whether DKC1 regulated ccRCC proliferation and migration via NF- κ B and MMPs, and we demonstrated that knockdown of DKC1 significantly inhibited the protein expression of p65 and MMP-2, which may well account for our proliferation, migration and invasion results. However, in our study, MMP-9 expression did not appear to be activated by p65 in ccRCC cells. The functions of p65 and MMP-2 in DKC1-mediated ccRCC cell proliferation, migration and invasion were further explored by co-transfection of both DKC1 siRNAs and p65 overexpression plasmids, which rescued the decreased cell proliferation, migration and invasion after DKC1 knockdown. Moreover, our subcutaneous tumor model *in vivo* revealed that knockdown of DKC1 significantly suppressed proliferation and angiogenesis of tumors and the expression of p65, MMP-2 and CD31 in tumor tissues, which conformed to the *in vitro* results and cohort of ccRCC patients. Unfortunately, we did not explore the potential mechanism between DKC1 and the NF- κ B pathway, and therefore whether the regulation of NF- κ B by DKC1 is direct or indirect is not clear. However, we did determine a possible relationship between them. Recently, it has been established that DKC1 is a direct target of c-Myc in several types of cancer (36). Thus, we speculated that NF- κ B could also directly activate DKC1 expression, and p65 was regulated by DKC1 via negative feedback. In future research, we will explore the potential molecular mechanisms between DKC1 and the NF- κ B signaling pathway in ccRCC cells consistently.

In conclusion, DKC1 expression was markedly enhanced in ccRCC compared with normal renal tissues. Positive DKC1 expression in ccRCC tissues was markedly associated with unfavorable clinicopathological characteristics and a dismal prognosis of patients, which can to some extent be explained by the NF- κ B/MMP-2 signaling pathway which regulated proliferation, migration, invasion and angiogenesis of ccRCC cells. Therefore, these results demonstrated that DKC1 may act as a significant prognostic indicator and therapeutic target for ccRCC.

Acknowledgements

Not applicable.

Funding

The present study was funded by grants from the National Natural Science Foundation of China (nos. 81472663, 81502280 and 81672845), the Education Department of Jiangsu Province (no. 15KJA320006) and the Project of Invigorating Health Care through Science, Technology and Education from Jiangsu Province.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

MZ, JB and YP conceived and designed the experiments; YP, RJ and FC conducted the experiments; MZ and PH carried

out the statistical analysis; HS and TJ supported the experiments and helped to draft the manuscript. All authors have read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Informed consents from all patients were obtained and institutional approval was obtained by the Review Board of the Affiliated Hospital of Xuzhou Medical University prior to this study. The animal studies were approved by the Animal Care Committee of Xuzhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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