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Prognostic value of circWWC3 as a novel survival-related biomarker for clear cell renal cell carcinoma

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Abstract. The aim of the present study was to assess the prognostic value of circular RNA WW and C2 domain-containing protein 3 (circWWC3) in patients with clear cell renal cell carcinoma (ccRCC). The clinicopathological data were collected for patients with ccRCC treated between January 1, 2012, and February 31, 2014, at The Fourth Hospital of Hebei Medical University Hospital (Shijiazhuang, China). A total of 150 patients who underwent nephrectomy were included in the study. Analysis of stored tissues and long-term follow-up data was performed. Fluorescence *in situ* hybridization was used to detect the relative circWWC3 expression in fresh-frozen cancerous and adjacent para-cancerous tissue samples from patients with ccRCC. A χ^2 test was used to analyze the association between circWWC3 expression levels and the clinicopathological parameters of the patients. A Cox proportional risk regression model was used to analyze the clinical factors affecting patient prognosis. The survival curve was plotted using the Kaplan-Meier method, and the association between circWWC3 expression levels and patient survival status was tested using the log-rank test. circWWC3 expression in cancerous tissues was higher than that in the adjacent normal tissues. Additionally, circWWC3 expression was significantly associated with T stage (P=0.005) and pathological grade (P=0.033). Univariate Cox regression analysis showed that overall survival (OS) was associated with T stage, pathological Fuhrman grade and circWWC3 expression levels (all P<0.05). Multivariate Cox regression analysis also yielded similar results in patients with ccRCC (P<0.05). Moreover, the OS time of patients with high circWWC3 expression was significantly shorter than that of patients with low circWWC3 expression. In conclusion, high circWWC3 expression is an independent risk factor affecting patient prognosis, and is expected to be an important prognostic biomarker and novel therapeutic target for patients with ccRCC.

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Introduction

Renal cell carcinoma (RCC) is the most common malignancy of all functional renal tissues, except for the renal urothelium. There are three subtypes of RCC: Clear cell RCC (ccRCC) (75-80%), papillary RCC (10-15%) and chromophobe RCC (5-10%) (1). ccRCC is the most common type of renal malignancy and it accounts for the majority of renal cancer-associated deaths. As the most common and lethal kidney tumor, ccRCC incidence is increasing and the prognosis is poor (2). Surgical removal is still the primary treatment option for ccRCC due to resistance to traditional chemotherapy and radiotherapy strategies (3). As there are few useful biomarkers and treatments for ccRCC, a poor prognosis and high mortality rates are common in patients at an advanced stage. However, the molecular mechanisms underlying ccRCC tumorigenesis remain unclear, and new therapies for ccRCC are difficult to develop (4). There is a requirement for researchers to determine the molecular mechanisms underlying the tumorigenesis of renal cancer and to engineer new therapeutics, including those aimed at specific molecular targets, in order to decrease the mortality rate associated with renal cancer.

Circular RNAs (circRNAs) are a family of non-coding RNAs (ncRNAs) that are circularized by joining the RNA 3' and 5' ends to form a circular structure (5). There are different types of circRNAs, including exonic, intronic and intergenic circRNA. Changes in circRNA expression induce the expression of tumor-related genes, which affect the occurrence and development of tumors (6,7). circRNAs have three main functions: i) Serving as microRNA (miR/miRNA) sponges to sequester and inhibit miRNA activity; ii) acting as regulators that interact with proteins; iii) serving as templates for protein synthesis (8). circRNAs play an important role in the development and progression of human tumors, according to accumulating evidence (9-11).

WW and C2 domain-containing protein 3 (WWC3) is a member of the WWC scaffold protein family, with involvement in cellular transport processes that are necessary the migration, polarity and synaptic signaling of cells (12,13). circRNAs may be potential regulators at the RNA level, and the abnormal expression of circRNAs may be associated

with specific human diseases, especially cancer. WWC3 is a tumor suppressor that is downregulated in a number of malignancies, such as colorectal carcinoma, lung cancer and osteosarcoma (13-15). An association has been found between WWC3 downregulation and a poor prognosis in patients with cancer (16). A recent study has shown that linear WWC3 is associated with a good prognosis, and that it inhibits breast cancer cell growth and metastasis (17). However, the circular form of WWC3 (circWWC3) dominates and exhibits oncogenic functions in breast cancer, suggesting that circWWC3 competes with linear WWC3 to promote the progression of breast cancer (18).

circRNAs act as essential regulators of RCC tumorigenesis. Wang *et al* (19) revealed that hsa-circ_0035483 is highly expressed in RCC and enhances gemcitabine resistance by regulating the hsa-miR-335/CCNB1 signaling pathway. In RCC, ZEB2 expression is upregulated by circPCNXL2 and downregulated by miR-153 (20). The derivation of circPCNXL2 (circ_406752) is the PCNXL2 gene and the circRNA acts to augment *in vitro* and *in vivo* tumor growth, while miR-153 can reverse its effect. Some circRNA genome-wide transcriptional profiles have been reported in RCC (19). Using a circRNA microarray, the study by Ma *et al* (21) showed the aberrant expression of 542 circRNAs in ccRCC. Among these circRNAs, 324 exhibited significant downregulation, whereas 218 exhibited significant upregulation in ccRCC. The majority of circRNAs enhance RCC development and progression via circRNA-miRNA-mRNA interactions. Some circRNAs can also be used as prognostic biomarkers. For example, circ-ABCB10 promotes RCC cell growth and suppresses apoptosis *in vitro* (22).

While a number of advances have been made in circRNA biological research, the exact effect on patient prognosis and the regulation of circRNA function remain largely unclear. The present study aimed to predict the survival of patients with RCC by analyzing circWWC3 expression data and demographic and clinical variables. Therefore, the study was conducted on patients who had previously undergone kidney cancer resection and were undergoing follow-up visits. The study further aimed to identify a better prognostic molecular marker through a retrospective analysis of existing data.

Patients and methods

Ethical statement. Ethical approval was obtained from the Ethics Committee of The Fourth Hospital of Hebei Medical University (Shijiazhuang, China; approval no. 202159). This study conformed to the Declaration of Helsinki. Patients were followed up according to national clinical guidelines. All patients provided written informed consent.

Patients' inclusion and exclusion criteria. Patients who underwent radical nephrectomy in The Fourth Hospital of Hebei Medical University with a pathological diagnosis of ccRCC and who had both tumor and normal renal tissues available after surgery were included. All patients needed to be available for regular follow-up for ≥8 years. None of the included patients had received anticancer radiotherapy or chemotherapy before surgery. Other exclusion criteria were as follows: i) A history of other tumors (benign or malignant); ii) an age of <18 years; iii) rare pathological features; iv) data with incomplete information; and v) death from other causes. A flow chart illustrating the inclusion and exclusion criteria is shown in Fig. 1.

Collection of excised tissue samples. All kidney cancer tissue samples (ccRCC) and adjacent morphologically normal renal cortex (adjacent tissues) samples were collected from patients with ccRCC who met the inclusion criteria and underwent renal surgical resection at the Fourth Affiliated Hospital of Hebei Medical University between January 1, 2012, and February 31, 2014.

All samples were collected during surgical resection and either snap frozen in liquid nitrogen and stored at -80˚C or fixed in formalin and embedded in paraffin. After fixing the kidney tissue with 4% paraformaldehyde at room temperature for 24 h, it was dehydrated, embedded in paraffin, sectioned (4 μm) and subjected to hematoxylin and eosin (HE) staining. Formalin-fixed paraffin-embedded slides was stained with hematoxylin for 10 min and 1% eosin for 30 sec at room temperature. Using an optical microscope, the pathological changes in the kidney tissue after HE staining were examined. According to the Fuhrman grade (23) and tumor condition, pathologists in the Department of Pathology performed microscopic pathological observations of the tissue samples of all patients and recorded all pathological information.

All tissue samples were numbered for subsequent molecular histological examination and follow-up.

Fluorescence in situ hybridization (FISH). circWWC3 expression in ccRCC and normal tissues was detected using FISH. FISH was performed with probes specific for circWWC3 sequences. The Biotin-labeled circWWC3 probes were purchased from Qiagen GmbH. The probe sequences were as follows: 5'BiosG/TCAATGGCTTTGTTATCCTCTTTCT/3'Bio. The paraffin slides of tissue were first deparaffinized in xylene and ethanol solutions. Following prehybridization in PBS with 0.5% Triton X-100, the cells were hybridized with the aforementioned probes overnight at 37˚C. The fluorescence signal of circWWC3 was detected by Cy5-Streptavidin Conjugate (ZyMAXTM Grade; Invitrogen; Thermo Fisher Scientific, Inc.). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and the images were taken under a ZEISS LSM 710 confocal microscope (Zeiss AG). Based on the cytoplasmic expression intensity of circWWC3, tumor tissue samples were classified as follows: Negative or weak expression in most cells was defined as the negative group, weak expression in most cells or moderate expression in <50% of cells was defined as the low expression group, and moderate to strong expression in most cells was defined as the high expression group. Fluorescence intensity measurements were performed using ImageJ (National Institutes of Health). A mean fluorescence intensity (MFI) value of >90 was defined as high expression, a value of <70 was defined as low expression, and MFI values between these thresholds were defined as moderate expression. All of the results of the 150 tumor and normal tissue samples were recorded in a table for association and survival analysis.

Clinical information collection and patient follow-up. The admission and pathological information of all patients was

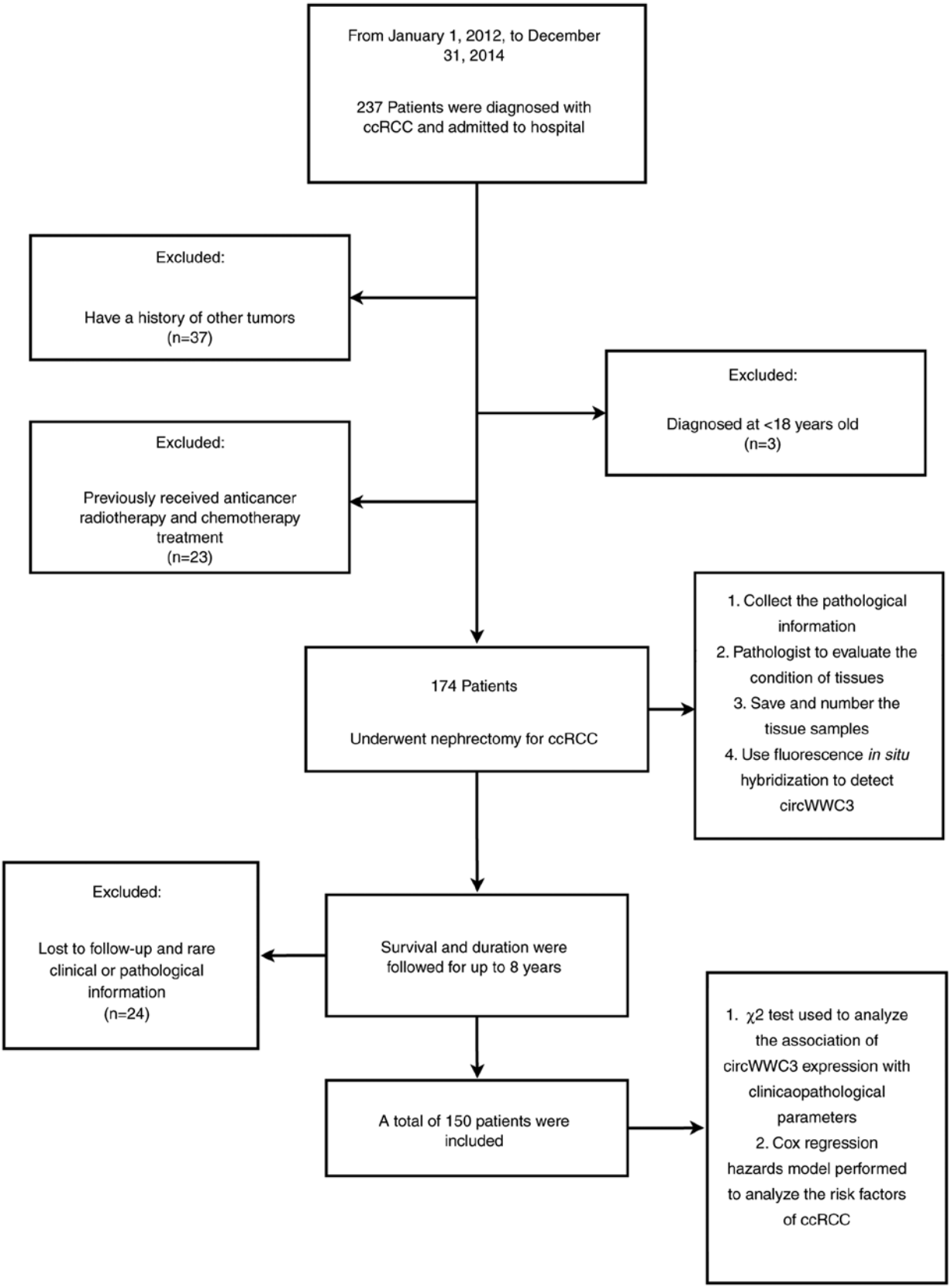

Figure 1. Flowchart showing the enrollment process for the population in this prospective study, inclusion and exclusion criteria, and statistical analysis method. ccRCC, clear cell renal cell carcinoma; circWWC3, circular RNA WW and C2 domain-containing protein 3.

collected. Clinical data collected on admission included age, sex, tumor stage (T stage) (24), Fuhrman grade, circWWC3 expression in tumor samples and tumor size. Due to the limition of sample size, T stage was classified into T1/T2 and T3/T4. All patients were followed up for 8 years after discharge. Patients were followed up via telephone every

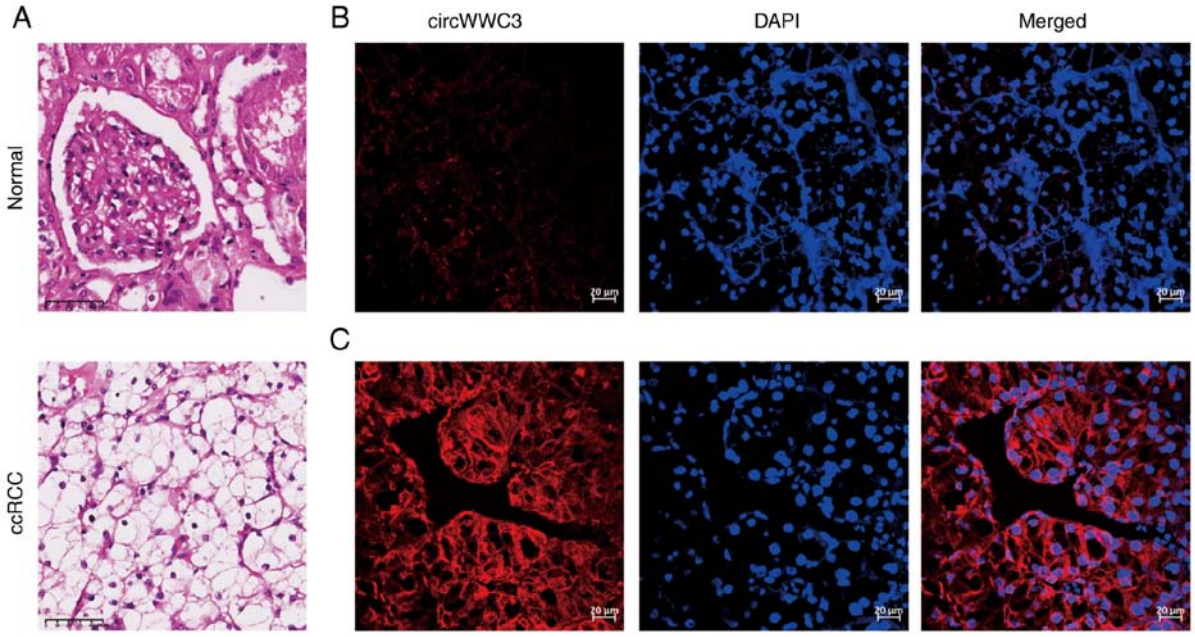

Figure 2. HE staining and FISH results in tumor and normal tissues. (A) HE staining results in normal and ccRCC tissues (x40 magnification). FISH results in (B) normal tissue and (C) ccRCC tissue (x400 magnification). Red fluorescence indicates protein expression of circWWC3 and DAPI-emitted blue fluorescence indicates the cellular nuclei; >98% overlap (purple) of red and blue fluorescence was observed following merging in ccRCC tissue. There was almost no circWWC3 expression observed in the normal group. DAPI, 4',6-diamidino-2-phenylindole; circWWC3, circular RNA WW and C2 domain-containing protein 3; ccRCC, clear cell renal cell carcinoma; HE, hematoxylin and eosin; FISH, fluorescence *in situ* hybridization.

year. Meanwhile, the patients' survival status and time were recorded for survival analysis.

Statistical analysis. Statistical analyses were performed using SPSS for Windows (version 26.0; IBM Corp.). Clinicopathological prognostic factors were compared with regard to circWWC3 expression using the χ^2 test. Mean fluorescence intensity was analyzed using one-way ANOVA, followed by SNK-q test. Univariate and multivariate analyses were performed using a Cox regression hazard model. Kaplan-Meier curves were created and log-rank tests were used for analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Demographic characteristics. The present study included 150 patients; 48.7% were women and 51.3% were men. Patients with ccRCC whose tumor diameter was measured after resection were divided into two groups; one group had tumor diameters ≥4 cm and the other had tumor diameters <4 cm. The results of all followed up patients were classified according to the pathologist's examination report. Pathological grades I and II were considered to be the low differentiation group, and patients with grades III and IV were considered to be the high differentiation group.

circWWC3 expression in para-cancer and cancer tissue samples. circWWC3 expression in ccRCC tissues was examined using FISH in 150 tumor and adjacent normal tissues (Fig. 2). Using FISH, DAPI staining of the nuclei was indicated in blue and circWWC3 staining of the cytoplasm was indicated in red. circWWC3 and DAPI together were indicated by a purple color in merged images. circWWC3 expression was higher in all the examined tumor samples compared with that in the normal samples using ImageJ. Subsequently, the 150 ccRCC tissue samples were divided into three circWWC3 expression levels based on their mean fluorescence intensity: Low, moderate and high (Fig. 3A). Among all the followed up patients, 51 had low circWWC3 expression, 50 had moderate circWWC3 expression and 49 had high circWWC3 expression.

Associations between circWWC3 expression and clinicopathological parameters. Associations between circWWC3 expression and other clinicopathological parameters were evaluated using the χ^2 test (Table I). The results revealed that tumor samples with high circWWC3 expression were significantly associated with advanced tumor stage, including T stage (P=0.005) and pathological grade (P=0.033). There were no associations between expression level and age, sex or tumor diameter.

Survival analyses. The results of the univariate and multivariate Cox regression and prognostic factor analysis with regard to mortality based on the final Cox model are shown in Table II. Univariate survival analyses were employed to determine the differences between patients with ccRCC with different circWWC3 expression levels. The possible prognostic factors for patients with ccRCC who were included in the univariate analysis were determined to be the pathological grade of the tumor (P<0.001), T stage (P<0.001) and the circWWC3 expression level (P<0.05). In the multivariate analysis, the pathological grade of the tumor (P<0.05), T stage (P<0.001) and the circWWC3 expression level (P<0.05) were determined to be independent risk factors. The results showed that, in the univariate analysis, moderate circWWC3

Table I. Association between circWWC3 expression and the demographic and clinicopathological parameters of patients with primary clear cell carcinoma of the kidney.

	circWWC3, n (%)					
Variable	Low	Moderate	High	Total no.	χ^2	P-value
Age, years					1.535	0.464
<60	31 (36.9)	29 (34.5)	24 (28.6)	84		
≥60	20 (30.3)	21 (31.8)	25 (37.9)	66		
Sex					0.817	0.665
Female	25 (34.2)	22 (30.1)	26 (35.6)	73		
Male	26 (33.8)	28 (36.4)	23 (29.9)	77		
Tumor diameter, cm					3.13	0.209
<4	32 (35.2)	34 (37.4)	25 (27.5)	91		
≥4	19 (32.2)	16 (27.1)	24 (40.7)	59		
Pathological grade					6.835	0.033
I/II	43 (36.8)	42 (35.9)	32 (27.4)	117		
III/IV	8 (24.2)	8 (24.2)	17 (51.5)	33		
T stage					10.459	0.005
T1 and T2	41 (40.2)	36 (35.3)	25 (24.5)	102		
T3 and T4	10 (20.8)	14 (29.2)	24 (50.0)	48		

circWWC3, circular RNA WW and C2 domain-containing protein 3.

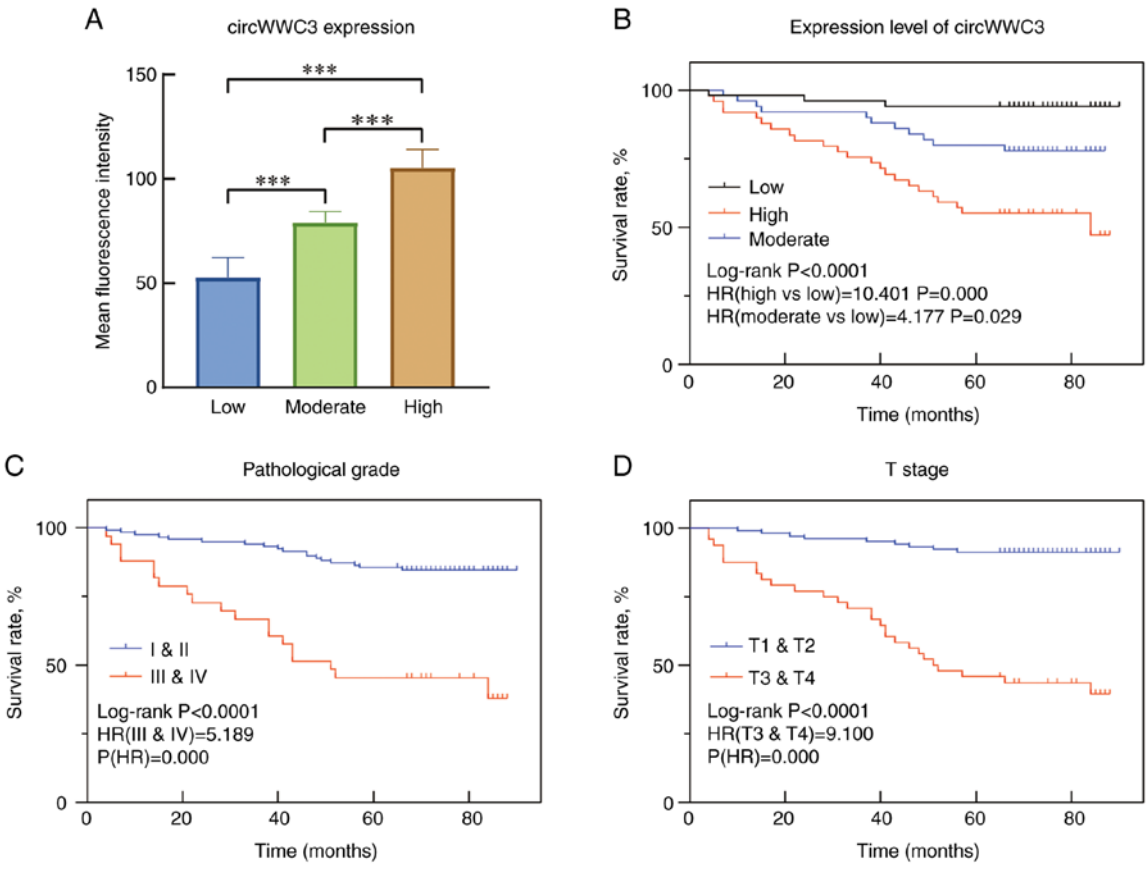

Figure 3. (A) MFI levels in the three different circWWC3 expression level groups (***P<0.001). (B) Kaplan-Meier survival curves for the different levels of circWWC3 expression, (C) pathological grades and (D) T stages. (A) shows that the MFI is different among the low, moderate and high expression level groups. (B) shows that high circWWC3 expression leads to a poor prognosis. (C) and (D) show that grades III and IV and T stages 3 and 4 cause a poor prognosis. These three factors can become risk factors for the prognosis of patients with clear cell renal cell carcinoma. Additionally, circWWC3 can be a novel biomarker for prognosis in the future. MFI, mean fluorescence intensity; circWWC3, circular RNA WW and C2 domain-containing protein 3.

Table II. Univariate and multivariate analysis of overall survival in patients with primary clear cell carcinoma of the kidney.

	Univariate analysis			Multivariate analysis		
Parameters	HR	95% CI	P-value	HR	95% CI	P-value
Age, years						
<60	1.000	-	-	-	-	-
≥60	1.597	0.836-3.051	0.156	-	-	-
Sex						
Female	1.000	-	-	-	-	-
Male	1.011	0.530-1.926	0.974	-	-	-
Tumor diameter, cm						
<4	1.000	-	-	-	-	-
≥4	1.772	0.902-3.275	0.100	-	-	-
Pathological grade						
I/II	1.000	-	-	1.000	-	-
III/IV	5.189	2.715-9.920	<0.001	2.318	1.125-4.775	0.023
T stage						
T1/T2	1.000	-	-	1.000	-	-
T3/T4	9.100	4.281-19.343	<0.001	5.117	2.220-11.800	<0.001
circWWC3 expression						
Low	1.000	-	-	1.000	-	-
Moderate	4.177	1.162-15.010	0.029	3.918	1.084-14.153	0.037
High	10.401	3.113-34.750	<0.001	6.216	1.830-21.117	0.003

circWWC3, circular RNA WW and C2 domain-containing protein 3.

expression increased the risk of ccRCC-related mortality by 4.177 (95% CI, 1.162-15.010; P=0.029) compared with low circWWC3 expression. Furthermore, high circWWC3 expression increased the risk of ccRCC-related mortality by 10.401 (95% CI, 3.113-34.750; P<0.001). A Kaplan-Meier plot of mortality was constructed to illustrate the differences between the groups (Fig. 3B-D). The pathological grade of the tumor and T stage were also incorporated into the multivariate Cox proportional hazards model (grades III/IV: HR, 5.189; 95% CI, 2.715-9.920; P<0.0001; stage T3/T4: HR, 9.100; 95% CI, 4.281-19.343; P<0.0001). circWWC3 expression was shown to be an independent risk factor upon multivariate analysis (moderate: HR, 3.918; 95% CI, 1.084-14.153; P=0.037; high: HR, 6.216; 95% CI, 1.830-21.117; P=0.003).

Overall, the results showed that patients with high circWWC3 expression in cancer tissues had a shorter survival time (P<0.05), suggesting that circWWC3 was negatively associated with patient prognosis. The analysis revealed that circWWCC3 was an independent prognostic risk factor for ccRCC. In addition, statistical analysis showed that the pathological grade of the tumor (P<0.005) and T stage (P<0.005) were risk factors for the prognosis of patients with ccRCC.

Discussion

ccRCC is the most common type of renal cancer among adults. The survival rate of patients with ccRCC remains unsatisfactory, as they present with insidious symptoms during the earliest stages and exhibit little sensitivity to chemotherapy and radiotherapy (25). The outcome of patients with ccRCC depends on several factors, including the type of surgery, the treatment method and the genetic heterogeneity of the disease. Of these factors, surgery and treatment methods can be controlled; however, genetic heterogeneity cannot (26). Recent studies have reported the role of circRNA in ccRCC. For example, Gui *et al* (27) reported that circCHST15 promotes ccRCC cell proliferation and metastasis through the miR-125a-5p/EIF4EBP1 axis, Zhang *et al* (28) proposed the circME1/ME1 pathway as involved in ccRCC progression and sunitinib resistance development (29) and Wang *et al* (29) presented circDVL1 as exerting a tumor-suppressive function during ccRCC progression through the circDVL1/miR-412-3p/PCDH7 axis. The upregulation of circWWC3 expression was observed in ccRCC tissues in the present study, and patients with high circWWC3 expression levels exhibited worse overall survival OS times.

In the present study, circWWC3 expression was higher in ccRCC tissues than in normal renal tissues. circWWC3 was demonstrated to predict survival in patients with ccRCC and may prove to be a new prognostic biomarker. Univariate regression analysis showed that T stage, Fuhrman stage and circWWC3 expression levels affected patient survival. The follow-up assessment for circWWC3 may be used as an index for monitoring patients, which can be an invaluable reference for medical practice. circWWC3 has seven open reading

frames, and 76 RNA-binding protein sites and 40 microRNA response elements can be observed in the circWWC3 structure according to the Cancer-Specific CircRNA Database (http://gb.whu.edu.cn/CSCD/).

Currently, ccRCC transmission and recurrence risk assessment are limited to the tumor stage, pathological classification and clinical characteristics (30,31). These shortcomings and lack of risk factors may lead to an unsatisfactory prognosis prediction. The study of effective postoperative adjuvant therapy is also necessary, as ccRCC is not sensitive to chemotherapy and radiotherapy. circRNAs can serve as a novel and attractive class of ncRNA biomarkers for liquid biopsy due to their resistance to RNase R digestion and stability within the blood circulation (32). Body fluids contain abundant circRNAs, which can be detected using reverse transcription-polymerase chain reaction assays, which are relatively inexpensive. circRNAs have been shown to be abnormally expressed in hepatocellular carcinoma, lung cancer and breast cancer, to have higher disease specificity and to exhibit clinical relevance in clinical RCC samples, making them ideal candidates for RCC diagnosis (19,33,34).

In clinical practice, when conducting a pathological diagnosis of a renal tumor biopsy, it is often impossible to obtain sufficient tissue for a definitive pathological diagnosis. However, if the circWWC3 expression level in tissues is measured during this process, it will likely help pathologists diagnose tissues and improve diagnostic accuracy. The circWWC3 expression level is associated with pathological grade and T stage. High circWWC3 indicates a higher pathological grade and a greater probility of T3/T4 stage disease. However, there were a limited number of samples in the present study, and the inclusion of further patient samples is required in future investigations to study the association between circWWC3 and T stage progression. In addition, circWWC3 expression is significantly associated with the overall survival for patients with ccRCC; therefore, it can also be used as a prognostic indicator.

In conclusion, the present clinical study was conducted to evaluate the prognostic value of circWWC3 in patients with ccRCC. It was observed that circWWC3 could be a novel prognostic biomarker due to the longer survival time of patients with low circWWC3 expression.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XN provided all patient information and experimental results, revised the manuscript critically and given final approval of the version to be published. HW and XM performed the statistical analysis and wrote the manuscript. SL performed the statistical analysis and plotted the tables and images. All the authors have read and approved the final manuscript. HW and XN confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China). All participants provided written informed consent before inclusion in the study.

Patient consent for publication

The patients provided written informed consent for the publication of any data and/or accompanying images.

Competing interests

The authors declare that they have no competing interests.

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