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ZFX is a Strong Predictor of Poor Prognosis in Renal Cell Carcinoma

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Manuscript Preparation E
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Background: This study was designed to assay the expression of zinc finger protein X-linked (*ZFX*) in renal cell carcinoma (RCC) tissues and evaluate the correlation between *ZFX* expression and prognosis of RCC patients.

Material/Methods: The expressions of *ZFX* mRNA in 53 RCC tissues and 51 normal tissues were determined by quantitative real-time polymerase chain reaction (qRT-PCR). Immunohistochemistry (IHC) technology was used to measure the expression of *ZFX* protein. Then chi-square test was conducted to verify the association between *ZFX* expression and clinical parameters. Next, we explored the overall survival rate of RCC patients with Kaplan-Meier analysis. Finally, the correlation between *ZFX* expression and the prognosis of RCC patients was evaluated by Cox regression analysis.

Results: The qRT-PCR result showed that the *ZFX* was significantly up-regulated in RCC tissues. As for the IHC consequence, the positive rate of *ZFX* expression in RCC specimens was 79.2%, while that in the normal control tissues was only 17.6%. Chi-square test showed that *ZFX* expression shared no close relationship with age, sex, or smoking ($P>0.05$), but was tightly associated with TNM stage, tumor size, and lymph node metastasis ($P<0.05$). Kaplan-Meier analysis showed that patients with *ZFX* positive expression had higher mortality than those with negative expression ($P<0.05$). Cox regression analysis revealed that *ZFX* expression had tight correlation with prognosis of RCC patients (HR=4.997, $P=0.045$, 95%CI=1.033–24.180).

Conclusions: Our findings show that *ZFX* could be considered as a predictor for prognosis of RCC patients.

MeSH Keywords: **Carcinoma, Renal Cell • Genes, X-Linked • Prognosis**

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Background

Renal cell carcinoma (RCC) is one of the most frequent malignancies in the urinary system and represents approximately 90% of all kidney cancers [1–3]. It is estimated that about 340 000 patients are diagnosed with RCC annually world-wide [4,5]. In the past 2 decades, the incidence of RCC has increased by 2% world-wide, with a steadily growing mortality rate [6]. After kidney transplantation, the patients are also likely to develop RCC because of the malignant tumor in the donor kidney [7]. At present, 20–30% of RCC patients have distant metastasis at diagnosis [8–11], which contributes to undesirable treatment effects and poor prognosis. In addition, about 40% of human RCC is diagnosed incidentally [12] and 5-year survival rate of RCC patients is only 12.3% [13]. Surgical resection is an effective way to cure RCC, but nearly 30% of patients have tumor recurrence after surgery [1,14]. Therefore, it is necessary to find novel and significant molecular markers for prognosis of RCC patients.

Zinc finger protein X-linked (*ZFX*) locates at X chromosome [15]. *ZFX* protein belongs to the zinc finger protein family, members of which are all conserved in vertebrates. The *ZFX* protein contains an acidic transcriptional activation domain (AD), a nuclear localization sequence (NLS), and a DNA binding domain (DBD) [16–19]. Existing reports have shown that *ZFX* can act as a transcriptional regulator for self-renewal in embryonic and adult hematopoietic stem cells [20–22]. Currently, emerging evidence indicates that *ZFX* plays an important role in the initiation and development of several malignancies. Overexpression of *ZFX* was observed in esophageal carcinoma cell lines [23] and *ZFX* was upregulated in prostate cancer and glioma [24–26]. Moreover, Fang et al. [18] demonstrated that knockdown of *ZFX* significantly inhibited renal cell carcinoma cell proliferation and cell cycle progression. However, few studies have investigated the prognostic role of *ZFX* in RCC.

In this study, we explored *ZFX* expression in RCC tissues and normal control tissues and evaluated its possible use as a prognostic biomarker for RCC patients. Our findings will contribute to providing timely treatments and improving the survival of RCC patients. Moreover, it is helpful for the use in individualized therapy.

Material and Methods

Patients and samples

A total of 53 patients with RCC were randomly selected in this study in the Second Hospital of Tianjin Medical University. Among them, 44 cases were males and 9 were females, aged 25–69 years with an average age of 43 years. All patients received no preoperative chemotherapy or radiotherapy. All 53 RCC tissues were included in the case group, and 51 adjacent normal tissues were chosen as a control group. The study was approved by the local ethics committee and all of the patients signed consent forms before surgery.

A 5-year follow-up survey was conducted in all the RCC patients. The information was obtained through a telephone or a questionnaire survey and updated every 3 months. The collected clinical parameters were recorded in a database.

RNA extraction and qRT-PCR

The expression levels of *ZFX* mRNA were determined with the use of quantitative real-time polymerase chain reaction (qRT-PCR). We extracted the total RNA from RCC tissues and noncancerous tissues by RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Then, reverse transcription was conducted with a high-capacity cDNA synthesis kit (Takara, China). After reverse transcription, we used qRT-PCR to evaluate the expression abundance of *ZFX* mRNA. The reaction was conducted under optimal conditions: 95°C for 3 min, followed by 40 cycles at 95°C for 6 s, and 60°C for 35 s. The relative mRNA expression value was calculated by $2^{-\Delta\Delta T}$ method. β-actin was utilized as the internal control. The test was done in triplicate.

Immunohistochemistry (IHC) assay

The expression of *ZFX* protein was measured by IHC test in both RCC tissues and normal tissues. Samples were cut into 4-μm-thick sections and baked at 65°C for 1 h. Deparaffinization and rehydration was performed with alcohols of gradient concentration. The sections were incubated with 0.01 M citric acid buffer (pH 6.0) at 98°C for 10 min and then air dried at room temperature. After that, the sections were mixed with primary antibody at 37°C for 1 h. PBS buffer was used to wash the sections 3 times, each for 3 min. Biotin-labeled second antibody was added to each section at 37°C for 30 min. Staining signaling was conducted with DAB. Samples treated by PBS, rather than primary antibody, were used as negative controls. We also performed positive controls by the sections with ZFX expression. Staining mainly showed brown in cytoplasm. The IHC result was expressed by the staining percentage of cells (0 to 100%). Staining of fewer than 10% of the cells or no staining was considered to be negative expression. Staining of 10–20% of cells was considered to be moderate immunopositivity, and staining of more than 20% of cells was considered to be strong immunopositivity. Both moderate and strong immunopositivity were classified as positive expression. The sections were blocked and preserved for further use.

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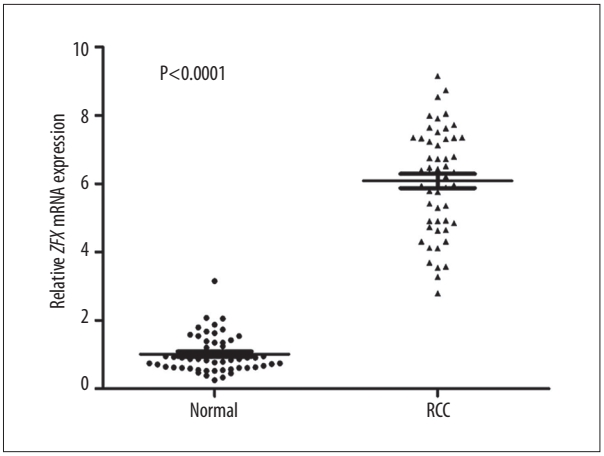

Figure 1. The expression of *ZFX* mRNA in RCC tissues and normal tissues. qRT-PCR was conducted to assay the expression of *ZFX* mRNA in RCC tissues and normal tissues. The result showed that the level of *ZFX* mRNA in RCC tissues was significantly higher than in normal tissues (*P*<0.0001).

Statistical analysis

Data collected in this study were analyzed by SPSS18.0 software (SPSS Inc., USA). The relationship between *ZFX* expression and clinical parameters of RCC patients was evaluated by chi-square test. Kaplan-Meier analysis was performed to detect the overall survival rate of RCC patients with positive *ZFX* expression and negative *ZFX* expression. Multivariate analysis was conducted to explore whether there was a correlation between *ZFX* and prognosis of RCC patients by use of Cox regression analysis. Statistical significance existed when *P* value was less than 0.05.

Results

Up-regulation of ZFX mRNA in RCC tissues

The expression level of *ZFX* mRNA was significantly higher in RCC tissues compared to normal tissues (Figure 1).

High expression of ZFX protein in RCC tissues

The positive expression rate of ZFX was 79.2% (42 out of 53) in the RCC tissues, but only 17.6% (9 out of 51) in the normal tissues (Table 1, Figure 2). Therefore, the expression level of ZFX was significantly higher in RCC tissues than in normal tissues (*P*<0.05).

Table 1. *ZFX* expression in RCC and normal tissues.

Tissue	Case No.	Expression		Positive rate	*P* value
		Positive	Negative		
RCC	53	42	11	79.2%	*P*<0.05
Normal	51	9	42	17.6%	

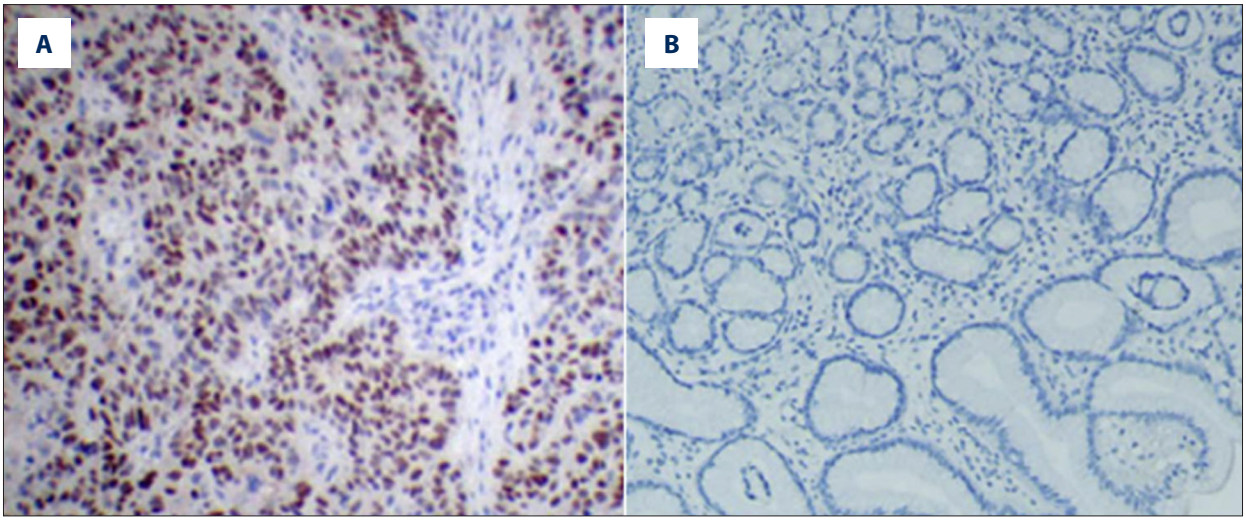

Figure 2. IHC analysis of *ZFX* expression. (**A**) Positive expression of *ZFX* in RCC tissues; (**B**) Negative expression of *ZFX* in normal tissues. Original magnification: ×200.

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Table 2. Relationship of clinical parameters and *ZFX* expression.

Characteristics	Case No.	Protein expression		χ^2	*P* value
		Positive	Negative		
Age (years)				0.279	0.597
≤35	23	19	4		
>35	30	23	7		
Gender				0.014	0.905
Male	44	35	9		
Female	9	7	2		
Smoking				1.817	0.178
Yes	29	21	8		
No	24	21	3		
TNM stage				4.322	0.038
I,II	34	24	10		
III	19	18	1		
Tumor size (cm)				4.220	0.040
≤5	24	16	8		
>5	29	26	3		
Lymph node metastasis				4.681	0.031
Yes	25	23	2		
No	28	19	9		

Association between ZFX expression and clinical parameters of RCC patients

The result showed that *ZFX* expression was significantly related with TNM stage, tumor size, and lymph node metastasis ($P<0.05$). However, no statistical correlation was observed between *ZFX* expression and age, sex, or smoking ($P>0.05$) (Table 2).

Correlation between ZFX and prognosis of RCC patients

A postoperative follow-up was an average of 46.8 months. During the survey, 28 (66.7%) patients with positive expression died, but only 2 (9.1%) died among the RCC patients with negative *ZFX* expression. Kaplan-Meier survival analysis demonstrated that patients with positive *ZFX* expression had statistically higher mortality than those with negative expression (Figure 3). Cox regression analysis was conducted to evaluate the correlation between *ZFX* expression and the prognosis of RCC patients. The result confirmed that *ZFX* expression had a significant correlation with the prognosis of RCC patients, and positive *ZFX* expression predicted poor prognosis (HR=4.997, $P=0.045$, 95% CI=1.033–24.180) (Table 3).

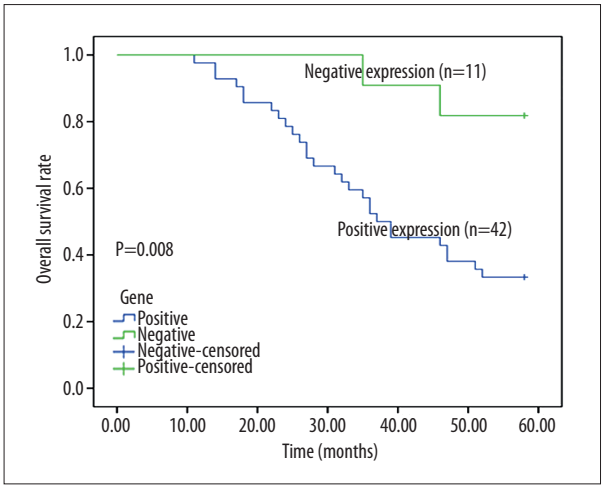

Figure 3. The overall survival rate of RCC patients was estimated by Kaplan-Meier analysis. The result showed that the overall survival rate of RCC patients with positive *ZFX* expression was significantly lower than in those with negative expression.

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Table 3. Multivariate analysis of prognostic factors.

Clinical parameters	P value	HR	95% CI
Age	0.093	2.107	0.883–5.028
Gender	0.074	0.377	0.129–1.099
TNM stage	0.612	1.232	0.550–2.758
Lymph node metastasis	0.743	0.849	0.320–2.258
ZFX expression	0.045	4.997	1.033–24.180

Discussion

Zinc finger proteins are broadly distributed in eukaryotic genomes and play important roles in genomic regulation by interaction with DNA and proteins (27). Recently, studies have found a close linkage between zinc finger proteins and human tumorigenesis, such as zinc finger 280B protein and prostate cancer, and zinc finger 703 protein and gastric cancer [28,29]. Similar to these proteins, ZFX is a member of the zinc finger protein family. Previous studies have demonstrated that ZFX can function as an oncoprotein in various types of cancers, including gastric cancer [16], prostate cancer [24,25], glioma [26], hepatocellular cancer [30], gallbladder cancer [31], tongue squamous cell carcinoma [32], non-small cell lung cancer [33], and laryngeal squamous cell carcinoma [34]. However, few studies have focused on the prognostic significance of *ZFX* in RCC.

ZFX is best known for its role in regulating the renewal and differentiation of stem cells. Wu et al. [16] reported that knockdown of *ZFX* inhibited gastric cancer cell growth *in vitro* and *in vivo*. Fang et al. [18] concluded that knockdown of *ZFX* suppressed renal carcinoma cell growth and induced cell apoptosis. Thus, our study focused on investigating the prognostic role of *ZFX* in RCC. qRT-PCR and IHC assay suggested higher expression level of *ZFX* in RCC tissues, which indicated that *ZFX* might play an important role in the pathogenesis of RCC. Further analysis showed the tight relationship between *ZFX* expression and TNM stage, tumor size, and lymph node metastasis. *ZFX* was confirmed as a prognostic biomarker in RCC by Kaplan-Meier survival and Cox regression analysis in a colorectal cancer study [35]. To the best of our knowledge, the present study is the first to report the correlation between *ZFX* expression and the prognosis of RCC patients.

According to previous studies, the involvement of *ZFX* in pathogenesis of RCC might be related to several possible molecular mechanisms. *ZFX* may impact the development of RCC by regulating cancer-associated signal pathways, such as the ERK-MAPK pathway, which was found in gastric cancer cell growth [16]. Fang et al. suggested that *ZFX* could regulate RCC cell proliferation and apoptosis through modifying the expression of certain downstream genes, such as Caspase-1, AKT, Survivin, and Ki-67 [36]. Further studies are needed to explore the molecular mechanisms of *ZFX* in RCC.

Conclusions

Our study showed that *ZFX* was significantly upregulated in RCC tissues. Kaplan-Meier and Cox regression analysis demonstrated that *ZFX* might act as a molecular marker for prognosis of RCC patients. However, how *ZFX* exerts an effect on the prognosis of RCC patients was not explored, and further research is needed to determine this.

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