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Knockdown of *ZFX* suppresses renal carcinoma cell growth and induces apoptosis

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The *ZFX* (zinc finger protein, X-linked) gene located on the human X chromosome controls the self-renewal of embryonic and hematopoietic stem cells as a transcriptional regulator. Recently, studies have affirmed that *ZFX* is associated with several human cancers, including lymphoma, laryngeal squamous cell carcinoma, prostate cancer, and liver cancer, which suggests *ZFX* as a potential therapeutic target in cancer. However, the functional role of *ZFX* in human renal cancer remains unclear. Herein, we detected the expression of ZFX in 42 patients with renal cancer and found the expression of *ZFX* was specifically upregulated in cancer tissues at the mRNA and protein levels. Moreover, we employed lentivirus-mediated short hairpin RNA (shRNA) to knock down *ZFX* expression in two human renal cell carcinoma cell lines, 786-0 and ACHN. Functional analysis indicated that *ZFX* silencing significantly inhibited renal cell carcinoma cell proliferation and cell cycle progression, probably because of suppression of CDK4 and cyclin D1, and induced apoptosis via activation of Bax, Caspase 3, and PUMA in a p53-dependent manner. Our findings suggest that knockdown of *ZFX* by shRNA may be a potential therapeutic approach for the treatment of renal cancer.

Keywords *ZFX*, renal carcinoma, short hairpin RNA, growth, apoptosis

Renal cell carcinoma (RCC), the most common form of kidney cancer in adults (1), accounts for about 209,000 new cases and 102,000 deaths per year worldwide (2). It is capable of spreading to the lungs, lymph nodes, and many other areas of the body. The genetics of renal cancer is dominated by inactivation of the *VHL* tumor suppressor gene in clear cell RCC (ccRCC), the most common histological subtype. However, known cancer-related genes that are frequently mutated in other adult epithelial cancers, such as the *RAS* genes, *BRAF*, *TP53*, *RB*, *CDKN2A*, *PIK3CA*, *PTEN*, *EGFR,* and *ERBB2*, make only a small contribution to ccRCC. A large-scale screen of approximately 3,500 genes by PCR-based exon resequencing identified several new cancer-related genes in ccRCC, including *UTX* (also known as *KDM6A*), *JARID1C* (also known as *KDM5C*), and *SETD2* (3). Recently, Varela et al. have sequenced the protein-coding exome in a series of primary ccRCC and identified the SWI/SNF complex gene *PBRM1* as a second major ccRCC cancer gene (4), which highlights the role of mutations in components of the chromatin modification machinery in RCC. Arterial embolization and biological therapy are two emerging technologies that are gaining popularity (5). Targeted silencing of cancer genes has been identified as a potent therapeutic possibility to treat various types of cancers. Therefore, better understanding of the genetic carcinogenic effect has led to a new era of targeted therapy in the management of RCC (6).

The zinc-finger (ZNF) protein family is one of the most common families of DNA-binding transcription factors, which regulate transcription of genes essential for cell growth, differentiation, and apoptosis (7,8). ZFX, a zinc-finger protein, contains an acidic transcriptional activation domain, a nuclear localization sequence, and a DNA-binding domain consisting of 13 Cys-Cys/His-His zinc fingers (9). *ZFX* is located on the X chromosome and is structurally similar to *ZFY*, a related gene on the Y chromosome (10,11). *ZFX* and *ZFY* have been speculated to function in sex differentiation, spermatogenesis, and Turner syndrome (12). Previous

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studies showed that *ZFX* was required as a transcriptional factor for self-renewal of embryonic and hematopoietic stem cells (13,14). Recently, studies have shown that *ZFX* is associated with several human cancers. *ZFX* was upregulated in cancer stem-like cells in esophageal carcinoma cell lines (15). Overexpression of *ZFX* was also observed in prostate cancer (8), diffuse large B-cell lymphoma, and follicular lymphoma (16). Moreover, knockdown of *ZFX* could inhibit cell proliferation and induce apoptosis in human laryngeal squamous cell carcinoma (17), suggesting that *ZFX* may serve as a therapeutic target in cancers. However, the functional role of *ZFX* in human kidney cancer remains unclear.

In this study, the expression of ZFX was firstly analyzed in 42 patients with renal cancer by real-time PCR and immunoblot assays. Moreover, we employed lentivirus-mediated short hairpin RNA (shRNA) to knock down *ZFX* expression in human RCC cell lines 786-0 and ACHN. Functional analysis showed that *ZFX* silencing significantly alleviated RCC cell growth by inducing cell cycle arrest and apoptosis, which provides a novel insight for the function of *ZFX* and a potential therapeutic target for the treatment of RCC.

Materials and methods

Patient samples

We collected samples from 42 consecutive patients who underwent laparoscopic radical nephrectomy in our department from 2010 to 2012; the patients had a median age of 61 years (range, 35−78 y) and included 29 males and 13 females. Real-time PCR and immunoblot assays were performed on these tissue specimens to detect the expression of *ZFX* at the mRNA and protein levels. All patients provided informed consent. The pathological diagnoses of all enrolled patients were confirmed by two different pathologists. This study was approved by the tissue committee and research ethics board, Southwest Hospital, Third Military Medical University in Chongqing, China.

Cell culture

Human RCC cell lines 786-0 and ACHN were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cell lines were routinely maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Construction of *ZFX* shRNA lentivirus vector and cell infection

The small interfering RNA (siRNA) (5′-GTCGGAAATTGATCCTTGTAA-3′) targeting the human *ZFX* gene (NM_003410.3) and a nonsilencing siRNA sequence (5′-TTCTCCGAACGTGTCACGT-3′) were transformed into stem-loop-stem oligos (short hairpin RNAs, shRNAs) and cloned into the pFH-L vector (Shanghai Hollybio, China), respectively. To rule out a possible off-target effect of the shRNA, another shRNA (5′-CCAATCAGTCTCATTCACATACTCGAGTATGTGAATGAGACTGATTGGTTTTT-3′) was used to get comparable results. The generated plasmids were transfected into 293T cells, together with two lentiviral packaging vectors. After 3 days of incubation, the supernatants containing either the lentivirus expressing the *ZFX* shRNA (shZFX) or the control shRNA (shCTRL) were harvested and concentrated using Millipore Centricon-Plus-20 (Merck, Darmstadt, Germany).

For lentivirus infection, 786-0 and ACHN cells were incubated with shZFX or shCTRL at a multiplicity of infection of 30. As the lentivirus carries the green fluorescence protein (GFP), the infection efficiency could be determined by counting the GFP-expressing cells under a fluorescence microscope 72 hours post transduction. The knockdown efficiency was validated with real-time PCR and western blot at day 5 posttransduction. Each experiment was performed in triplicate and repeated three times.

Quantitative real-time PCR

Total RNA was extracted using Invitrogen's Trizol reagent (Thermo Fisher Scientific, Waltham, MA) and reverse transcribed using M-MLV-RTase (Promega, Madison, WI), according to the manufacturer's instructions. The resulting cDNA was used for real-time PCR using the Applied Biosystems SYBR Green Master PCR Mix (Thermo Fisher Scientific) on the TP800 qPCR System (Takara Bio, Otsu, Japan). All quantitations were normalized to an internal gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The relative quantitation value for the target gene compared with that of its calibrator was expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing to *GAPDH*). Primers used in real-time PCR were as follows: *GAPDH*, 5′-TGACTTCAACAGCGACACCCA-3′ (forward) and 5′-GGAGTGTTGGAGAAGTCATATTAC-3′ (reverse); *ZFX*, 5′-GGCAGTCCACAGCAAGAAC-3′ (forward) and 5′-TTGGTATCCGAGAAAGTCAGAAG-3′ (reverse). Each experiment was performed in triplicate and repeated three times.

Western blot analysis

To detect the knockdown efficiency of ZFX at the protein level, Western blot analysis was performed with the GAPDH protein as an internal standard. Cell lysates were separated on 12% SDS PAGE gels and transferred onto polyvinylidene fluoride membranes (Merck). After blocking, the membranes were incubated with primary antibodies—rabbit anti-ZFX (1:1,000; HPA003877) (Sigma-Aldrich, St. Louis, MO), rabbit anti-p21 Waf1/Cip1 (1:200; 2947) (Cell Signaling Technology, Danvers, MA), mouse anti-CDK4 (1:500; 2906) (Cell Signaling Technology), mouse anti−Cyclin D1 (1:2,500; 60186-1-1g) (Proteintech Group, Chicago, IL), rabbit anti-phospho-p53 (1:1,000; 2528) (Cell Signaling Technology), rabbit anti-PARP (1:1,000; 9542) (Cell Signaling Technology), and rabbit anti-GAPDH (1:5,000; 10494-1-AP) (Proteintech Group)—followed by horseradish peroxidase-conjugated goat anti−rabbit immunoglobulin G (IgG) or goat anti-mouse IgG (Santa Cruz Biotechnology, Dallas, TX). The protein levels were detected with an enhanced chemiluminescence reagent (Santa Cruz Biotechnology). Each experiment was repeated three times.