Int J Clin Exp Pathol 2014;7(6):3150-3157
www.ijcep.com /ISSN:1936-2625/IJCEP0000405

*Original Article*

# Zinc-finger protein X-linked is a novel predictor of prognosis in patients with colorectal cancer

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Received April 2, 2014; Accepted May 25, 2014; Epub May 15, 2014; Published June 1, 2014

**Abstract:** Zinc-finger protein X-linked (ZFX) has been demonstrated to play an important role in the development of human malignancies. However, its prognostic significance in cancer patients remains unclear and less is known about its role in colorectal cancer (CRC). In this study, we found that the expression of ZFX in CRC tissues was significantly higher than that in corresponding normal tissues by quantitative real-time polymerase chain reaction and Western blot. Using immunohistochemistry, we explored the associations between protein expression of ZFX and clinicopathological parameters in 120 CRC cases. The results showed that ZFX expression was significantly associated with tumor differentiation (P = 0.022), tumor size (P = 0.037), tumor invasion (P = 0.027), lymph node metastasis (P = 0.042), distant metastasis (P = 0.011), and Dukes' classification (P = 0.028). Moreover, according to Kaplan-Meier model, patients with high expression of ZFX had a significantly poorer prognosis compared to those with low expression of ZFX. Multivariate analysis suggested that high expression of ZFX was an independent prognostic factor for CRC patients. In conclusion, our findings for the first time demonstrated that ZFX expression may be associated with the progress of CRC and suggested that ZFX has the potential value to be an effective prognostic predictor for CRC patients.

**Keywords:** ZFX, colorectal cancer, prognosis

## Introduction

Colorectal cancer (CRC) is the third most frequently diagnosed cancer in males and the second in females , accounting for over 1.2 million new cancer cases and 608,700 cancer deaths in 2008 worldwide [1]. Despite encouraging progress in diagnosis and treatment in recent years, the prognosis of CRC patients in advanced stage is very poor, mainly due to local recurrence and distant metastases formation [2]. Clinicopathological parameters play a major role in determining the management of CRC, but are usually not reliable predictors of prognosis [3]. Therefore, it is hoped that novel biomarkers, which have the potential value of serving as prognostic predictors and therapeutic targets, should be identified and used in combination with classic clinicopathological staging.

Zinc-finger protein X-linked (ZFX) encoded on the X chromosome, is a member of zinc-finger protein family, all of which are highly conserved in vertebrates. It was originally identified as a factor that determined whether an embryo develops as a male or female [4]. Later, conditional gene targeting assays suggested that ZFX could transcriptionally regulate the self-renewal of embryonic and hematopoietic stem cells [5]. This result was then confirmed by a further study using genome-wide RNAi screen that ZFX could form a unique transcription network required for self-renewal in embryonic stem cells with Cnot3, Trim28 and c-Myc, suggesting a potential role of ZFX in cancer development [6]. Recently, emerging studies have indicated that ZFX can play a key role in the initiation and development of several human malignancies. Knocking down ZFX dramatically inhibited the proliferation and apoptosis resistance in gastric cancer and glioma cells [7, 8]. Furthermore, ZFX has been reported to be involved in the metastasis of gallbladder cancer and non-small cell lung cancer [9, 10]. An interesting study by Akiyoshi et al has showed that ZFX is a target of miR-144 and is associated

**Table 1.** Correlation between ZFX expression and clinicopathological Characteristics

| Characteristics | Total | ZFX expression | | *P* value |
|---|---|---|---|---|
| | | High expression | Low expression | |
| Gender | | | | |
| male | 63 | 37 | 26 | 0.104 |
| female | 57 | 25 | 32 | |
| Age | | | | |
| ≤60 | 54 | 27 | 27 | 0.741 |
| >60 | 66 | 35 | 31 | |
| Tumor location | | | | |
| colon | 73 | 41 | 32 | 0.219 |
| rectal | 47 | 21 | 26 | |
| Tumor differentiation | | | | |
| well | 40 | 14 | 26 | |
| moderate | 37 | 20 | 17 | 0.022 |
| poor | 43 | 28 | 15 | |
| Tumor size | | | | |
| ≤5 cm | 69 | 30 | 39 | 0.037 |
| >5 cm | 51 | 32 | 19 | |
| Tumor invasion | | | | |
| T1 | 18 | 4 | 14 | |
| T2 | 41 | 22 | 19 | 0.027 |
| T3 | 49 | 27 | 22 | |
| T4 | 12 | 9 | 3 | |
| Lymph node metastasis | | | | |
| Absent | 63 | 27 | 36 | 0.042 |
| Present | 57 | 35 | 22 | |
| Distant metastasis | | | | |
| Absent | 56 | 22 | 34 | 0.011 |
| Present | 64 | 40 | 24 | |
| Dukes' stage | | | | |
| A/B | 60 | 25 | 35 | 0.028 |
| C/D | 60 | 37 | 23 | |

with chemotherapy sensitivity to 5-fluorouracil [11].

Our colleagues have previously proved that ZFX is important for the malignant characteristics of glioma cells [12]. However, to our knowledge, none of studies have reported the association between ZFX and cancer prognosis. Moreover, the role of ZFX in CRC remains unclear. In this study, we found the expression of ZFX is higher in CRC tissues than that in normal tissues. Then statistical studies suggested that high expression of ZFX was closely correlated with several clinicopathological parameters of CRC patients. Finally, we indicated that ZFX was an independent prognostic factor of CRC patients. Our findings not only provide a novel insight into the clinical significance of ZFX, but also suggest a potential value of ZFX in the targeted treatment of CRC.

## Materials and methods

### *Patients and specimens*

In our study, a total of 30 fresh primary CRC tumor tissues and corresponding normal tissues were prepared for quantitative real-time polymerase chain reaction and western blot. In addition, a total of 120 paraffin-embedded primary CRC tumor tissues and corresponding normal tissues were prepared for immunohistochemistry assay. All the specimens were collected from patients with CRC undergoing surgery at Department of General Surgery, The Sixth People's Hospital affiliated to Shanghai Jiao Tong University between 2002 and 2007. All the patients were diagnosed of CRC clinicopathologically and have complete follow-up records. The basic clinical characteristics of patients were shown in **Table 1**. Tumor-Node-Metastasis (TNM) staging was determined according to the criteria of the World Health Organization classification. None of the patients have received preoperative chemotherapy or radiotherapy. The study was approved by the ethics committee of The Sixth People's Hospital affiliated to Shanghai Jiao Tong University. The written informed consents were obtained from patients for using their tissue specimens in our study.

### *Quantitative real-time polymerase chain reaction*

Total RNA was isolated from tissues using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The obtained RNA was used to synthesize cDNA by Superscript III Reverse Transcriptase (Promega, USA). Real-time PCR

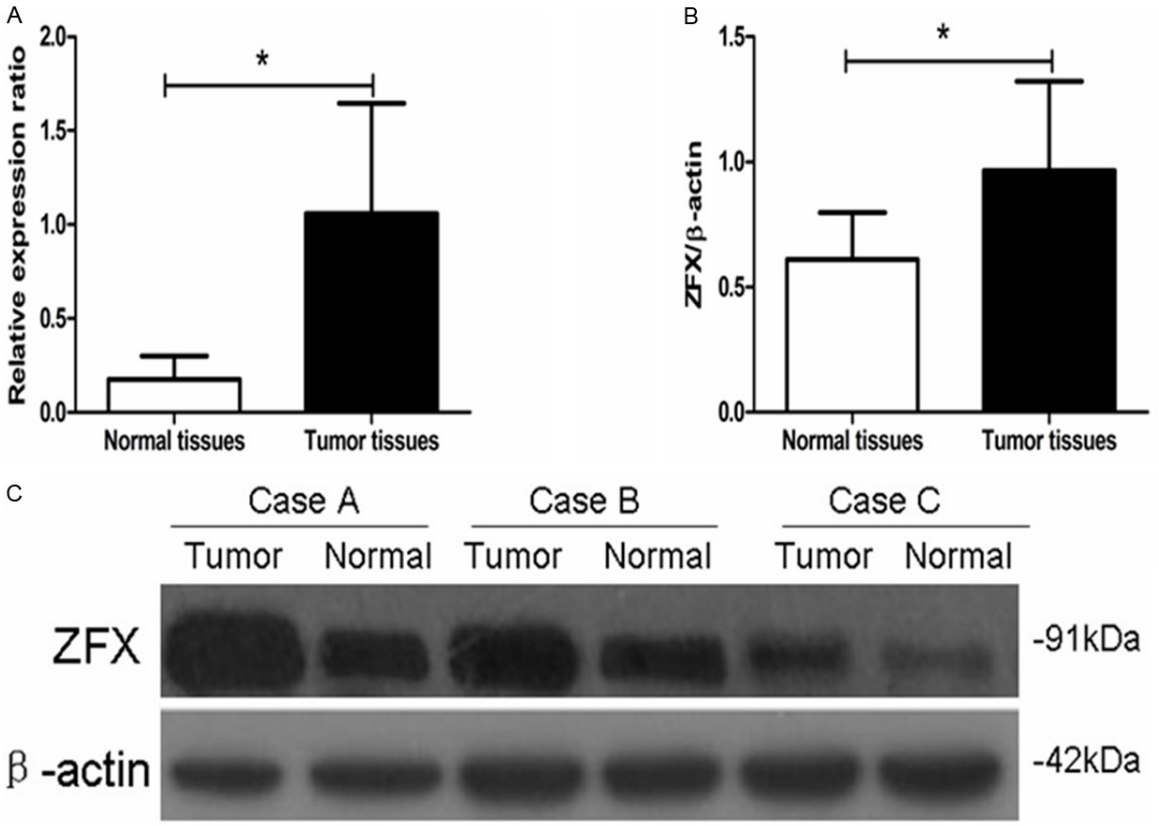


**Figure 1.** Expression of ZFX in CRC and corresponding normal tissues. Quantitative Real-Time Polymeras Chain Reaction (qRT-PCR) was employed to detect the mRNA expression of ZFX in CRC and corresponding normal tissues. The relative expression level of ZFX in tumor tissues was significantly higher than that in corresponding normal tissues (1.05 ± 0.58 vs 0.17 ± 0.12, **P* = 0.0048, A). The result was confirmed by Western blot (0.97 ± 0.37 vs 0.61 ± 0.18, **P* = 0.017, B and C).

reaction mixes were prepared using SYBR Green (TaKaRa, Japan) and run on the StepOne Plus Real-time PCR System (Applied Biosystems, USA) with the following conditions: 95°C for 5 minutes, 95°C for 5 seconds, 60°C for 30 seconds, for 40 cycles. The relative mRNA expression value was calculated by $2^{-\Delta\Delta T}$ method. β-actin was utilized as the internal control. The following primers were used: ZFX: forward: 5'-ACCTCTTGGCAGTCCACAGCAA-3'; reverse: 5'-CTGGCATTGGTACGGCTTCTCC-3'; β-actin: forward: 5'-CCTCCATCGTCCACCGCAAATG-3'; reverse: 5'-TGCTGTCACCTTCACCGTTCCA-3'. Experiments were repeated in triplicate.

*Western blot*

Total protein extracted with lysis buffer from tissues was prepared. The primary antibody and the dilution were used as follows: ZFX (Santa Cruz Biotechnology) 1:400, β-actin (Abmart) 1:2000. HRP-labeled Goat Anti-Rabbit IgG (1:2000) was used as secondary antibody. Briefly, 20 μg protein were separated by electrophoresis on a 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was then incubated with the diluted primary antibody overnight at 4°C. After three washes, the membrane was incubated with secondary antibody for 2 h at room temperature. Chemiluminescence reagent (Santa Cruz Biotechnology) was used to detect protein expression according to the manufacturer's instructions. Quantitative analysis of Western blot was performed by Image J 1.43 software.

*Immunohistochemistry and staining evaluation*

Paraffin-embedded tissue specimens were cut into 4 μm- thick sections. The sections were then dewaxed, rehydrated and heated by microwaving for antigen retrieval, followed by 25 min-incubation with 0.3% hydrogen peroxidase and methanol to block endogenous peroxidase

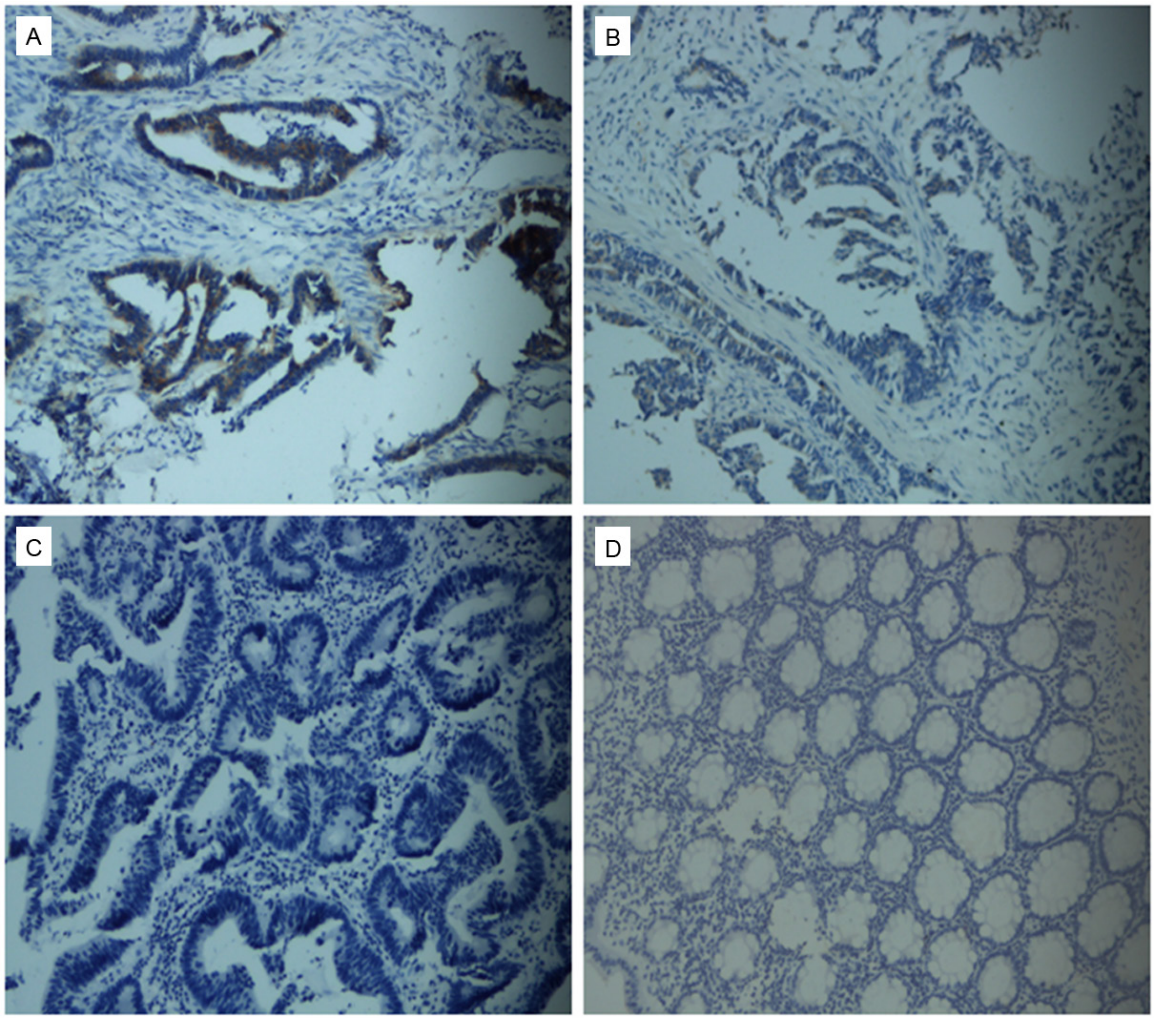


**Figure 2.** Representative results of immunohistochemical staining. A: High expression of ZFX in CRC tissues. B: Low expression of ZFX in CRC tissues. C: Negative control in CRC tissues. D: Negative expression of ZFX in normal tissues. Original magnification ×200.

activity. Subsequently, the sections were incubated with the primary antibody against ZFX (1:150) at 4°C overnight. After that, the sections were incubated with secondary antibody for 30 minutes. After extensive washing with PBS, all the sections were incubated with diaminobenzidine for 5 minutes and counterstained with hematoxylin. Finally, a semi-quantitative staining evaluation was performed under a light microscope. Negative controls were sections incubated with PBS instead of primary antibody.

Staining evaluation was performed by two independent researchers, who were blind to the clinicopathological parameters of patients. The evaluation principle was quantified based on the immunoreactive score (IRS), which was calculated as a product of Staining Intensity (SI) and Percentage of Positive cells (PP). SI is determined as follows: negative (score 0), weak (score 1), moderate (score 2), strong (score 3). PP is determined as follows: none (score 0), ≤10% (score 1), 11-50% (score 2), 51-80% (score 3), >80% (score 4). Five random fields in each section were selected for the evaluation. The sections scoring at least 3 points in our study were indicating high expression of ZFX.

*Statistical analysis*

The results were presented as mean ± SD. All the statistical analyses were performed by 17.0 SPSS statistical software. The data of qRT-PCR

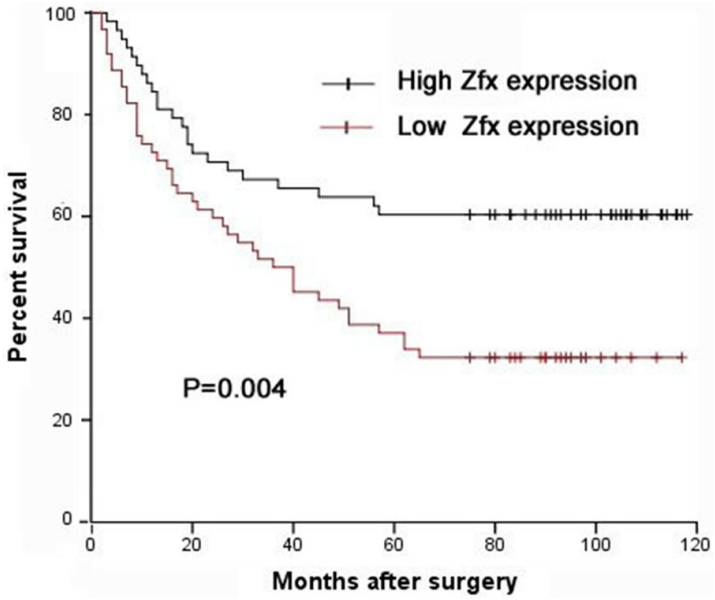


**Figure 3.** Kaplan-Meier graph showing the overall survival for the patients with CRC based on the immunohistochemical staining results of ZFX. Patients with high expression of ZFX had a significant lower overall survival rate than those with low expression of ZFX ($P$ = 0.004).

was analyzed by Student's t test. The correlations between ZFX protein expression and clinicopathologic parameters were analyzed by chi-square test. Survival curves were performed by the Kaplan-Meier model, and intergroup differences were determined by the log-rank test. The independent prognostic factors were identified by multivariate analysis based on the Cox proportional hazard model. A $P$ value <0.05 was considered to be statistically significant.

## Results

### *Expression of ZFX in CRC tissues and corresponding normal tissues*

To detect the mRNA expression of ZFX in 30 primary CRC tissues and corresponding normal tissues, quantitative Real-Time RT-PCR was applied. According to the result, 23 of the 30 patients (76.7%) exhibited a higher mRNA expression of ZFX in tumor tissues than in corresponding normal tissues. The relative expression level of ZFX in tumor tissues was significantly higher than that in corresponding normal tissues (1.05 ± 0.58 vs 0.17 ± 0.12, $P$ = 0.0048, **Figure 1A**). Western blot was then performed and showed the similar results with qRT-PCR (relative expression level: 0.97 ± 0.37 vs 0.61 ± 0.18, $P$ = 0.017; **Figure 1B** and **1C**).

To further investigate the protein expression of ZFX in CRC tissues, immunohistochemistry was performed. We found that the positive staining of ZFX was mainly located in the cytoplasm of tumor cells in CRC tissues. According to the established evaluation principle for immunostaining, high expression of ZFX was found in 51.7% (62/120) of tumors. **Figure 2** shows the representative immunohistochemistry results.

### *Associations between the protein expression of ZFX and clinicopathological parameters*

The associations between ZFX expression and clinicopathological parameters of CRC patients were summarized in **Table 1**. The expression of ZFX was demonstrated to have a statistical association with tumor differentiation ($P$ = 0.022), tumor size ($P$ = 0.037), tumor invasion ($P$ = 0.027), lymph node metastasis ($P$ = 0.042), distant metastasis ($P$ = 0.011), and Dukes' classification ($P$ = 0.028). However, there was no correlation between ZFX expression and other clinicopathological parameters, including age ($P$ = 0.741), gender ($P$ = 0.104) and tumor location ($P$ = 0.219).

### *Prognostic significance of ZFX expression in CRC*

The Kaplan-Meier method was performed to investigate the influence of ZFX expression on the prognosis of CRC patients. As shown in **Figure 3**, patients with high expression of ZFX was shown to have a lower overall survival rate than patients with low expression of ZFX ($P$ = 0.004). To determine whether ZFX could be used as an independent risk factor for poor prognosis of CRC patients, conventional clinicopathological factors and ZFX protein levels were analyzed by Cox's univariate and multivariate hazard regression model. As shown in **Table 2**, univariate analysis suggested that ZFX expression, together with tumor differentiation, tumor invasion, lymph node metastasis, distant metastasis and Dukes' classification, were significantly correlated with overall survival of CRC patients ($P$ = 0.005, $P$ = 0.026, $P$ = 0.048,

**Table 2.** Univariate analysis and multivariate analysis for factors influencing the overall survival rate of CRC patients

| Variables | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | RR | 95% CI | *P* value | RR | 95% CI | *P* value |
| ZFX expression | 2.082 | 1.251-3.465 | 0.005 | 1.784 | 1.030-3.091 | 0.039 |
| Age | 1.433 | 0.869-2.363 | 0.159 | 1.009 | 0.590-1.728 | 0.973 |
| Gender | 1.103 | 0.678-1.795 | 0.692 | 1.259 | 0.757-2.093 | 0.375 |
| Tumor location | 1.041 | 0.634-1.711 | 0.872 | 1.414 | 0.822-2.432 | 0.210 |
| Tumor differentiation | 1.743 | 1.067-2.845 | 0.026 | 1.075 | 0.631-1.834 | 0.789 |
| Tumor size | 1.295 | 0.794-2.112 | 0.301 | 1.640 | 0.952-2.824 | 0.075 |
| Tumor invasion | 1.650 | 1.003-2.712 | 0.048 | 1.150 | 0.657-2.010 | 0.625 |
| Lymph node metastasis | 1.733 | 1.060-2.836 | 0.028 | 1.870 | 1.060-3.297 | 0.031 |
| Distant metastasis | 2.067 | 1.248-3.426 | 0.005 | 1.425 | 0.780-2.603 | 0.249 |
| Dukes' classification | 2.294 | 1.383-3.805 | 0.001 | 2.037 | 1.182-3.511 | 0.010 |

*P* = 0.028, *P* = 0.005, and *P* = 0.001), while multivariate analysis indicated that ZFX expression, Lymph node metastasis and Dukes' classification were independent prognostic factors for overall survival of CRC patients (*P* = 0.039, *P* = 0.031, and *P* = 0.010).

## Discussion

Zinc finger (ZNF) represents one of the most abundant DNA-binding motifs in eukaryotic transcription factors, and the Cys2His2 ZNF motif is known as the most canonical type [13]. Recently, increasing studies have linked ZNF proteins to human tumorigenesis closely. For example, ZNF 280B protein promotes the growth and survival of prostate cancer cells by regulating p53 [14]. In gastric cancer, ZNF 703 protein has been identified as a novel oncoprotein regulating cell proliferation and migration [15]. Furthermore, an interesting study in breast cancer has suggested that ZNF proteins may contribute to tumor progression by inducing epithelial-mesenchymal transition (EMT), a well-established molecular mechanism by which transformed epithelial cells can acquire enhanced abilities of anti-apoptosis, invasion and migration [16]. Similar to the mentioned proteins, ZFX is a member of ZNF protein family and may also be important for tumorigenesis. Previous studies have indicated that ZFX can function as an oncoprotein in various types of cancer such as gastric cancer [7], gallbladder cancer [9] and hepatocellular cancer [17]. However, limited data are available about the prognostic significance of ZFX in cancer, and less is known about its role in CRC.

In this study, we performed RT-PCR and western blot to detect the expression of ZFX in CRC tissues and corresponding normal tissues. We found that the expression of ZFX in tumor tissues was significantly higher than that in corresponding normal tissues, implying a possible involvement of ZFX in CRC development. To further explore the role of ZFX, immunohistochemistry assay was performed. We found the expression of ZFX was significantly associated with prognosis-related clinical parameters, including tumor differentiation, tumor size, TNM staging and Dukes staging (all $P<0.05$). The results shown here have been supported by previous studies in other tumors, which have also suggested that over-expression of ZFX is essential for the progress and metastasis of malignancies [7-9]. In clinical management, prognostic molecular biomarkers have been regarded as useful tools for predicting the progression of disease in patients. To investigate whether ZFX is a significant clinical predictor of CRC, we preformed Kaplan-Meier method as well as Cox's univariate and multivariate hazard regression model. Interestingly, the results showed that CRC patients with high expression of ZFX had a lower survival rate, and ZFX expression was a significant independent prognostic factor for CRC patients. To the best of our knowledge, this is the first report demonstrating the prognostic significance of ZFX in cancer patients, and also the first study investigating its role in CRC.

According to previous studies and our findings, we speculate that ZFX may be involved in malignant progression of CRC through several possible molecular mechanisms. Firstly, ZFX may

act as an oncoprotein by conferring stem cell characteristics to CRC cells. A striking research by Ben-Porath et al has previously proved that embryonic stem (ES) cell-associated transcription regulators may contribute to aggressive tumor behavior by influencing stem-like phenotype [18]. ZFX was raised to prominence following the reports about its facilitation in ES cell self-renewal [5, 6]. And, this possibility has been recently been identified in hepatocellular carcinoma cells that ZFX was able to regulate the expression of cancer stem cell (CSC) markers, such as Nanog and SOX-2 [17]. Thus, it is possible that ZFX could promote the survival of CRC cells partly by inducing stemness. Secondly, ZFX may promote the development of CRC by regulating several cancer-associated signaling pathways including ERK-MAPK pathway, PI3K/AKT pathway and STAT3 pathway, which have been preliminarily explored in other tumors [7, 10]. Finally, it is reasonable that ZFX may drive EMT program in CRC. It is widely accepted that tumor cells must alter their shapes and lose cell-cell junction before invasion and metastasis, which commonly appears to involve EMT. There have been several lines of evidence to imply the association between ZFX and EMT. For example, in non-small cell lung carcinoma cell line H1299, ZFX has been reported to promote cell migration by regulating matrix metalloproteinase 2 (MMP-2) [10], which has already been identified as an important inducer for EMT [19]. Furthermore, both CSC characteristics and signal pathways ZFX regulate are closely linked to EMT [20, 21], also indicating a possible involvement of ZFX in EMT.

In conclusion, we present novel data to demonstrate that ZFX is over-expressed in CRC patients and is associated with the progression of CRC. Moreover, we found high expression of ZFX is an independent prognostic factor for the poor survival of CRC patients. Our study may also lay the foundation for further studies of the role of ZFX in CRC. Cellular and animal model studies will be continued to investigate the specific molecular mechanisms that ZFX regulates in the development of CRC.

## Acknowledgements

This work was partly supported by the funding of Science and Technology Commission of Shanghai Municipality (NO.124119a7202). We thank Professor Yuping Gao (Department of Pathology, Renji Hospital Affiliated to Shanghai Jiao Tong University) for her kind assistance in immunohistochemistry assay.

## Disclosure of conflict of interest

None.

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