

Original Article

High expression of Zinc-finger protein X-linked is associated with reduced E-cadherin expression and unfavorable prognosis in nasopharyngeal carcinoma

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Abstract: Zinc-finger protein X-linked (ZFX), a novel transcription factor required for self-renewal of embryonic stem cells, has recently been implicated in the initiation and progression of various human malignancies. However, its clinical significance in cancer patients remains largely inconclusive and its role in nasopharyngeal carcinoma (NPC) has never been reported. In this study, quantitative real-time polymerase chain reaction, Western blot and Immunohistochemistry were performed to detect ZFX expression in NPC and normal nasopharyngeal tissues. As a result, we found ZFX expression was significantly elevated in NPC tissues compared with that in normal nasopharyngeal tissues. The statistical analysis based on immunohistochemical staining demonstrated that ZFX expression was significantly correlated with lymph node stage and clinical stage. Furthermore, we found NPC patients with high ZFX expression had lower 5-year overall survival rates, progression-free survival rates, loco-regional relapse-free survival rates and distant metastasis-free survival rates than those with low ZFX expression (all $P < 0.05$). The multivariate analysis indicated that ZFX expression was an independent prognostic factor for patients with NPC. More importantly, we also detected E-cadherin expression in NPC tissues and found it was inversely correlated with ZFX expression in NPC tissues, suggesting a potential involvement of ZFX in Epithelial-mesenchymal transition (EMT). Therefore, it is speculated that ZFX may promote NPC progression partly by regulating EMT. In summary, our study not only for the first time identified that ZFX could serve as an effective prognostic biomarker for NPC patients, but also suggested that targeting ZFX might be a novel therapeutic strategy for preventing NPC progression and metastasis.

Keywords: ZFX, E-cadherin, nasopharyngeal carcinoma, prognosis

Introduction

Nasopharyngeal carcinoma (NPC) is a relatively rare malignancy globally but commonly diagnosed in South-Eastern Asia, especially among Chinese and Malay populations [1]. Environmental factors, genetic susceptibility and Epstein-Barr virus infection have been identified as important contributors for NPC initiation and progression [2, 3]. Most patients with NPC are diagnosed at advanced stage due to non-specific clinical symptoms, which inevitably results in unfavorable prognosis [4]. Radiotherapy (RT) is the standard therapy for NPC and improved medical techniques have allowed

it to ensure high recurrence control rate with reduced radiation-related toxicities [5]. Furthermore, chemotherapy has also been increasingly applied in combination with RT to achieve long term local control and survival probability [6]. However, for patients with same pathological stage and/or therapeutic regimens, their outcome may vary with individual differences, although current TNM staging system plays a crucial role in selecting and determining treatment. Therefore, identifying novel biomarkers, which can function as sensitive diagnostic or prognostic indicators, is of great importance for the individualized treatment of NPC.

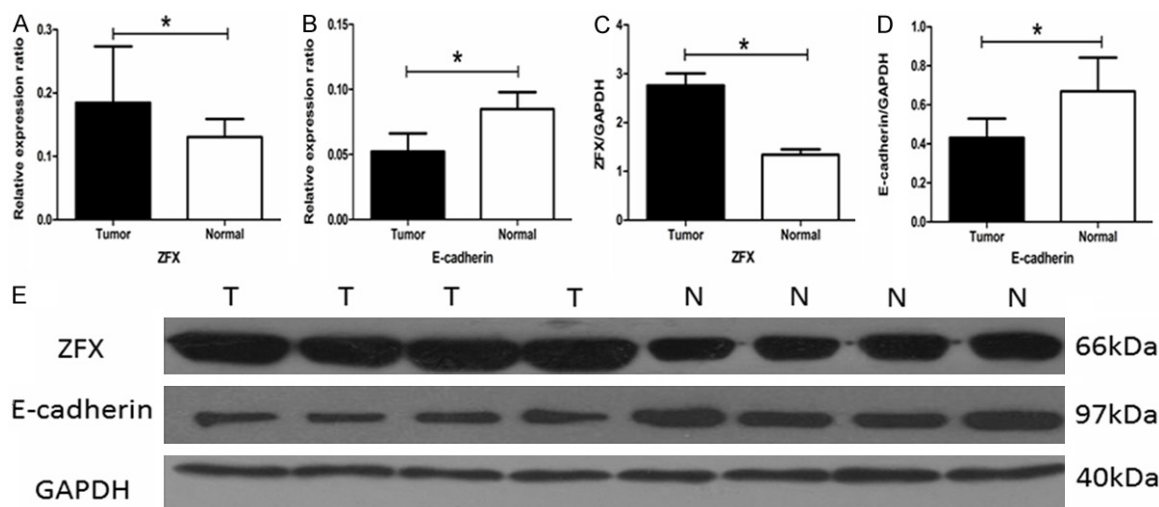


Figure 1. Expression of ZFX and E-cadherin detected by qRT-PCR and western blot in NPC and normal nasopharyngeal tissues. A: Relative mRNA expression of ZFX in tissues; B: Relative mRNA expression of E-cadherin in tissues; C: Relative protein expression of ZFX in tissues; D: Relative protein expression of E-cadherin in tissues.

Zinc-finger protein X-linked (ZFX), a member of Krueppel C2H2-type zinc finger protein family, is encoded on the X chromosome and contains an acidic transcriptional activation domain, a basic nuclear-localization signal and a carboxy-terminal zinc-finger domain [7]. Apart from its essential biological role in sex determination and stem cell self-renewal [8-10], emerging studies have suggested ZFX may also be implicated in malignant progression of several human malignancies. A recent work by Weisberg et al demonstrated that ZFX could maintain undifferentiated phenotype in two highly aggressive acute leukemia types [11]. In solid tumors such as hepatocellular, renal and breast carcinoma, ZFX expression has been found to be significantly elevated in tumor tissues compared with that in corresponding normal tissues [12-14]. Furthermore, silencing ZFX has been proved to dramatically inhibit the proliferation and migration of cancer cells, suggesting its great potential to be developed as a therapeutic target [15, 16].

Previously, our cooperative group has firstly proved that ZFX may promote proliferation and apoptosis resistance of glioma cells in vitro [17]. More recently, based on immunohistochemical assay and complete follow-up records, we found that ZFX may be a novel and effective prognostic predictor for patients with colorectal cancer [18]. However, to our knowledge, the expression and clinical significance of ZFX in NPC remains inconclusive. In this study, the

expression of ZFX between fresh NPC and normal nasopharyngeal tissues were compared by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. Additionally, immunohistochemical technique was also performed to detect the ZFX protein expression in paraffin-embedded NPC and normal nasopharyngeal tissues. Then, the correlation of ZFX expression with patient clinical characteristics and prognosis was statistically evaluated. Since epithelial-mesenchymal transition (EMT) is a well-established molecular mechanism in NPC development and loss of E-cadherin has been regarded as a hallmark of EMT [19], E-cadherin expression was also examined in NPC tissues and its potential association with ZFX expression was investigated.

Materials and methods

Patient data and tissue samples

In our study, 30 frozen NPC and normal nasopharyngeal tissues were prepared for qRT-PCR and Western blot. Additionally, 125 paraffin-embedded NPC tissues were prepared for immunohistochemistry assay and 40 paraffin-embedded normal nasopharyngeal tissues were used as controls. All the tissue samples were obtained from patients (median age of 41 years, ranging from 18 years to 75 years) using an electronic nasopharyngoscope at Department of otorhinolaryngology, Affiliated Sixth People's Hospital of Shanghai Jiao Tong

Table 1. Correlation between ZFX and E-cadherin in 125 NPC tissues

	Total	ZFX		r	P
		Low expression	High expression		
E-cadherin					
Low expression	97	38	59	-0.270	0.002
High expression	28	20	8		

University, from 2002 to 2013. None of patients have received radiotherapy or chemotherapy before biopsies. All the NPC patients were classified to the criteria of the Union for International Cancer Control (UICC) staging system and had complete follow-up records. The written informed consents were obtained from patients for using their tissues and this study was approved by the ethics committee of Affiliated Sixth People's Hospital of Shanghai Jiao Tong University. The basic clinicopathologic characteristics of patients were demonstrated in **Table 2**.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from 30 frozen NPC and normal nasopharyngeal tissues using Trizol reagent (Invitrogen, USA) according to manufacturer's instructions. The obtained RNA was then synthesized into cDNA using MMLV reverse transcriptase (Promega, USA). Real-time polymerase chain reaction was performed using SYBR Green Mix (Thermo, USA). The cycling conditions were used as follows: 10 min at 95°C, 15 sec at 95°C and 45 sec at 60°C for 40 cycles, 15 sec at 95°C, 1 min at 60°C, 15 sec at 95°C, 15 sec at 60°C. GAPDH was employed as an internal control and the $2^{-\Delta\Delta T}$ method was used to calculate the relative mRNA expression level. The following sequences of the primers were used: ZFX forward: 5'-ACCCCTAGTGGAGTGTGGCT-3' and reverse: 5'-TGAACCACTGAAGGGAGTCG-3'; E-cadherin forward: 5'-TCATGAGTGTCCTCCCGGTAT-3' and reverse: 5'-TCTTGAAGCGATTGCCCAT-3'; GAPDH forward: 5'-CACCACTCCTCCACCTTTG-3' and reverse: 5'-CCACCACCCTGTTGCTGTAG-3'. Each experiment was repeated three times independently.

Western blot

Tissue samples were lysed in RIPA buffer (Jrdun Biotechnology, China). The lysates containing

20 µg protein were separated on 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, USA). The membranes were then blocked in 5% skim milk for 1 h at room temperature. After that, the membranes were incubated with following

primary antibodies overnight at 4°C: anti-ZFX (1:1000, Abcam, UK), anti-E-cadherin (1:1000, Abcam, UK) and anti-GAPDH (1:1500, Cell Signaling Technology, USA). After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 37°C for 1 h. Protein expression was visualized using an enhanced chemiluminescence method and quantified by Quantity One software. The optical density of each protein was normalized against the optical density of GAPDH.

Immunohistochemistry and staining evaluation

4 µm-sections of paraffin-embedded tissues were prepared for Immunohistochemistry. Briefly, each section was dewaxed in xylene and rehydrated in gradient alcohol (100% alcohol, 95% alcohol, 85% alcohol and 75% alcohol). Antigen retrieval was performed by 15-min pressurized steam in 0.01 M citrate buffer solution and endogenous peroxidase activity was blocked by 10-min incubation in 0.3% hydrogen peroxidase solution. The sections were then incubated with primary antibodies against ZFX (1:150) and E-cadherin (1:100) overnight at 4°C. After three washes with PBS, the sections were incubated with secondary antibody for 20 min at 37°C. Color reactions were developed using diaminobenzidine solution. The sections were finally counterstained with hematoxylin, dehydrated and sealed. Negative controls were the sections incubated with PBS instead of primary antibodies.

The immunohistochemical staining was evaluated by two independent researchers who were blind to patient clinicopathological characteristics. The discrepant cases were determined by a pathologist and a consensus was obtained. For each section, five microscopic fields were randomly selected for scoring. The scoring system was based on Staining Intensity (SI) and Percentage of Positive cells (PP). SI was categorized as follows: 0 (negative), 1 (weak), 2 (mod-

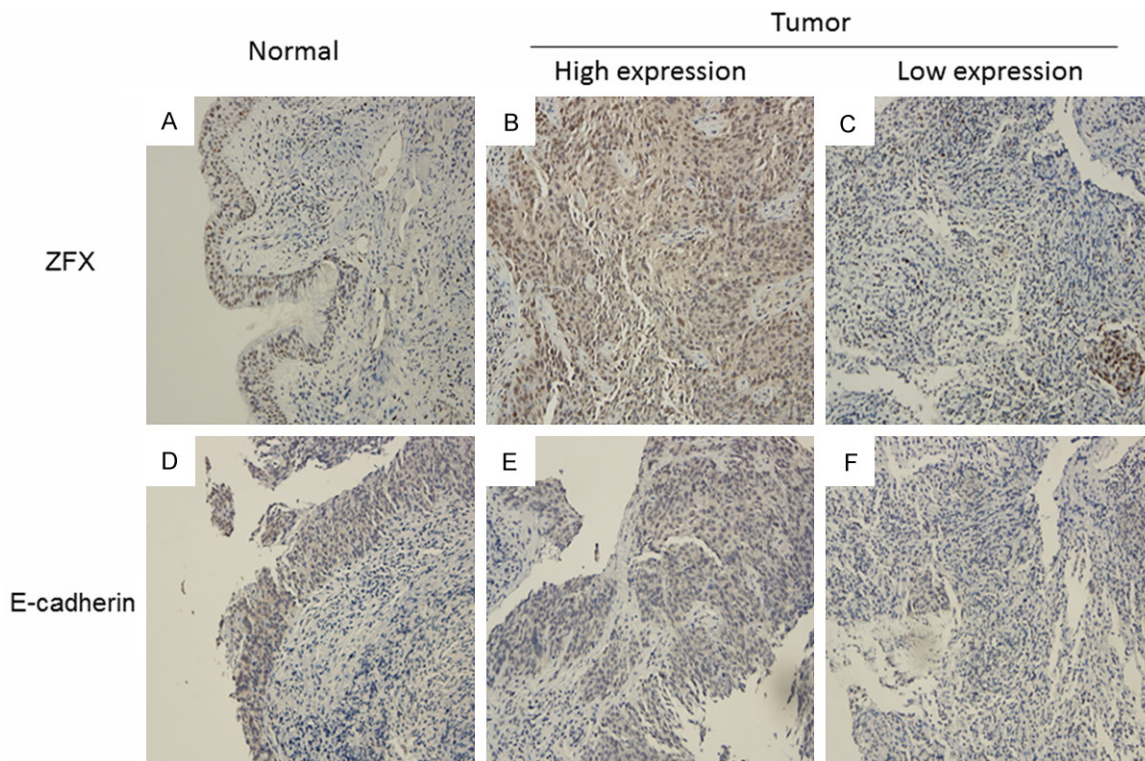


Figure 2. Representative results of immunohistochemical staining. A: Expression of ZFX in normal nasopharyngeal tissue; B: High expression of ZFX in NPC tissue; C: Low expression of ZFX in NPC tissue; D: Expression of E-cadherin in normal nasopharyngeal tissue; E: High expression of E-cadherin in NPC tissue; F: Low expression of E-cadherin in NPC tissue.

erate) and 3 (strong). PP was categorized as follows: 0 (none), 1 ($\leq 10\%$), 2 (11-50%), 3 (51-80%) and 4 ($>80\%$). The final score of each section was calculated as a product of SI and PP. For statistical analysis, the sections scored at least 3 points were recorded as high expression.

Statistical analysis

Data were present as mean \pm standard deviation. All the statistical analyses were performed using 19.0 SPSS statistical software. The Student's *t* test was used to analyze the data of qRT-PCR and western blot. The Chi-square test was employed to assess the correlations between ZFX expression and clinicopathological parameters of NPC patients. Survival curves were constructed based on the Kaplan-Meier model and intergroup differences were analyzed by the log-rank test. The univariate and multivariate analysis were carried out on the Cox proportional hazard model. The correlation between ZFX expression and E-cadherin expression was determined by nonparametric

Spearman's rank correlation coefficient. For each analysis, a *P*-value <0.05 was considered statistically significant.

Results

Expression of ZFX and E-cadherin in NPC and normal nasopharyngeal tissues

Firstly, qRT-PCR was employed to determine the mRNA expression of ZFX and E-cadherin in NPC and normal nasopharyngeal tissues. As a result, the mRNA expression of ZFX in NPC tissues was significantly higher than that in normal nasopharyngeal tissues (0.18 ± 0.09 vs. 0.13 ± 0.03 , $P < 0.001$, **Figure 1A**), while the mRNA expression of E-cadherin in NPC tissues was significantly lower than that in normal nasopharyngeal tissues (0.05 ± 0.01 vs. 0.08 ± 0.01 , $P < 0.001$, **Figure 1B**). The above results were then further confirmed by western blot (ZFX: 2.76 ± 0.24 vs. 1.34 ± 0.10 , $P < 0.001$, **Figure 1C**; E-cadherin: 0.43 ± 0.10 vs. 0.67 ± 0.17 , $P < 0.001$, **Figure 1D**). The representative bands of western blot were shown in **Figure 1E**.

Table 2. Correlations between ZFX expression and clinicopathologic parameters

Characteristics	Total	ZFX expression		P value
		high	low	
Age				
<45	84	40	44	0.060
≥45	41	27	14	
Gender				
Male	94	50	44	1.000
Female	31	17	14	
T classification				
T1-T2	75	36	39	0.145
T3-T4	50	31	19	
N classification				
N0	15	2	13	0.002
N1- N3	110	65	45	
Clinical stage				
I-II	31	8	23	<0.001
III-IV	94	59	35	

The protein expression of ZFX and E-cadherin in NPC tissues were also investigated by immunohistochemistry. The expression of ZFX and E-cadherin were mainly located on nucleus and membrane of tumor cells respectively. High expression of ZFX was found in 67 of 125 NPC tissues (53.6%), while high expression of E-cadherin was only detected in 28 of 125 NPC tissues (22.4%). As shown in **Table 1**, the correlation analysis indicated that ZFX expression was negatively correlated with E-cadherin expression in 125 NPC tissues ($r=-0.270$, $P=0.002$). The representative results of immunohistochemistry were shown in **Figure 2**.

Correlations between ZFX expression and clinicopathological parameters

The correlations between ZFX expression and clinicopathological parameters were demonstrated in **Table 2**. ZFX expression was found to be significantly associated with lymph node stage ($P=0.002$) and clinical stage ($P<0.001$). However, no significant associations were observed between ZFX expression and other clinicopathological parameters, including age ($P=0.060$), gender ($P=1.000$) and primary tumor stage ($P=0.145$).

Prognostic significance of ZFX expression in NPC patients

Survival curves based on the Kaplan-Meier model were plotted to illustrate the prognostic

significance of ZFX in NPC patients. As shown in **Figure 3**, patients with high ZFX expression had significantly lower 5-year overall survival (OS) rates, progression-free survival (PFS) rates, loco-regional relapse-free survival (LRRFS) rates and distant metastasis-free survival (DMFS) rates than those with low ZFX expression ($P=0.030$, $P=0.015$, $P=0.040$ and $P=0.034$). The univariate and multivariate analysis were performed to identify independent prognostic factors for the OS of NPC patients. As shown in **Table 3**, univariate analysis suggested ZFX expression, age, lymph node stage and clinical stage were significantly correlated with the OS of NPC patients ($P=0.035$, $P=0.003$, $P=0.034$ and $P=0.027$), while multivariate analysis suggested only ZFX expression, age and clinical stage were independent prognostic factors for the OS of NPC patients ($P=0.046$, $P=0.021$ and $P=0.029$).

Discussion

Despite encouraging advances in medical techniques, a large number of cancer patients continue to experience poor prognosis after curative treatment, mainly due to genetic mutations, environmental factors and presence of cancer stem cells (CSCs) [20]. CSCs are a subtype of cancer cells characterized by uncontrolled self-renewal and high pluripotency. Accumulating studies have suggested that CSCs are implicated in many hallmarks of cancer, especially in metastasis formation [21]. CSCs markers, such as Oct4 and Nanog, are self-renewal regulators that have already been reported to participate in malignant progression of various tumors [22, 23]. ZFX, as a crucial transcription factor in embryonic stem cell self-renewal, has recently been identified as a novel CSCs marker in hepatocellular carcinoma [12]. Furthermore, a comprehensive study based on 7 cancer cell lines and 20 tumor samples with different histological origins, has demonstrated that ZFX significantly expressed in bladder, prostate, and colon cancer tissues/cell lines, suggesting its potential to function as a diagnostic or prognostic biomarker [24]. This speculation was then confirmed by our recent study that high expression of ZFX might be associated with pathological development and predict unfavorable prognosis in colorectal cancer [18]. However, to our knowledge, none of studies have investigated the expression of ZFX in NPC and its clinical significance in NPC patients is still unclear.

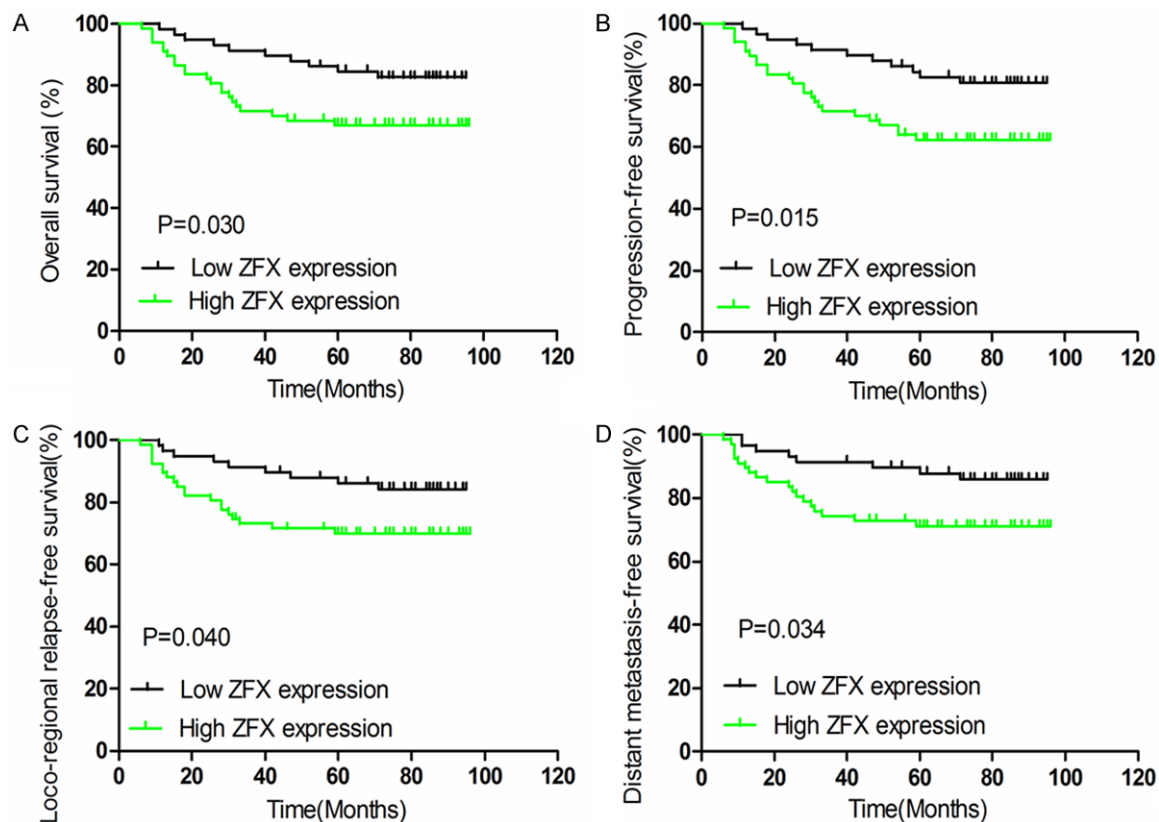


Figure 3. Kaplan-Meier survival curves for NPC patients with high and low expression of ZFX. A: Overall survival; B: Progression-free survival; C: Loco-regional relapse-free survival; D: Distant metastasis-free survival.

In this study, qRT-PCR and western blot were performed to compare the expression of ZFX between NPC and normal nasopharyngeal tissues. The results demonstrated that both the mRNA and protein expression of ZFX were remarkably up-regulated in NPC tissues, compared with those in normal nasopharyngeal tissues. This important finding implied a possible involvement of ZFX in nasopharyngeal carcinogenesis. To further elucidate its clinical significance in NPC patients, immunohistochemical staining for ZFX expression was performed on 125 NPC tissues and the result was statistically analyzed. In fact, the clinical significance of ZFX in cancer patients remains inconclusive, although recent studies have sufficiently explored the molecular mechanisms ZFX regulates in carcinogenesis. For example, in breast cancer, ZFX expression was shown to be only correlated with lymph node metastasis [14]. Using qRT-PCR on gastric cancer tissue, ZFX expression was reported to be correlated with tumor types and grades [25]. However, Wu et al found that ZFX expression was only significant-

ly correlated with the age of gastric cancer patients, although they found it was gradually increased from normal tissues, premalignant lesions to cancer tissues [26]. Similar contradictory observations were also found in hepatocellular carcinoma that ZFX expression was not correlated with any clinicopathological parameters, despite the fact that patients in advanced stage exhibited significantly higher ZFX expression than those in early stage [12]. Interestingly, in our study, ZFX expression was found to be significantly correlated with lymph node stage and clinical stage. However, no significant correlation was observed between ZFX expression and other parameters including age, gender and primary tumor stage. This finding suggested that ZFX might be involved in the development of NPC. We also deduced that the diverse findings about ZFX expression in cancer patients may be probably caused by several common uncertain factors, such as tumor heterogeneity, experimental methods and sample difference.

Table 3. Univariate analysis and multivariate analysis for prognostic factors in NPC patients

Variables	Univariate analysis			Multivariate analysis		
	RR	95% CI	P value	RR	95% CI	P value
ZFX expression	2.234	1.057-4.723	0.035	2.174	1.013-4.664	0.046
Age	2.867	1.430-5.746	0.003	2.308	1.135-4.694	0.021
Gender	0.983	0.442-2.189	0.967			
T classification	1.494	0.455-4.905	0.508			
N classification	2.129	1.058-4.282	0.034	0.925	0.255-3.362	0.906
Clinical stage	3.833	1.167-12.593	0.027	2.207	1.083-4.498	0.029

Although the overall survival rate has dramatically increased in recent years, approximately 20 percent of NPC patients continue to suffer from local/regional recurrence and distant metastases after standard treatment [27]. This highlights the importance of accurate prognostic evaluation for NPC patients. In this study, we found patients with high ZFX expression had a significantly lower OS, PFS, LRRFS and MFS rate than those with low ZFX expression. Moreover, the multivariate analysis demonstrated that ZFX expression, together with age and clinical stage were independent prognostic indicators for the OS of NPC patients, suggesting that combining detection of ZFX expression with current evaluation systems may be a novel approach for predicting accurate prognosis and improving personalized therapeutic regimens.

To preliminarily explain why high ZFX expression may be associated with NPC development and poor patient outcome, E-cadherin expression was also examined in NPC and normal nasopharyngeal tissues. E-cadherin is a transmembrane glycoprotein that inhibits tumor invasion and metastasis by mediating cell-cell junctions. Loss of E-cadherin has been widely regarded as a hallmark of EMT, a well-established molecular mechanism in tumor progression. In this study, we found high membranous expression of E-cadherin was only detected in 28 of 125 NPC tissues (22.4%). This result was consistent with a previous study, which reported reduced E-cadherin expression was detected in most NPC tissues and could serve as a prognostic indicator for NPC patients [28]. The correlation analysis demonstrated ZFX expression was negatively correlated with E-cadherin expression. This finding was indirectly supported by Luo et al, who found that high expression of other stem cell markers (such as OCT4 and Nanog) might be associated with reduced

E-cadherin expression in NPC [29]. Furthermore, although none of the current evidences have directly linked ZFX with EMT, ZFX has already been proved to drive the migratory potential of cancer cells [15, 16]. Therefore, considering previous studies and our results, it is reasonable to speculate that ZFX may promote the develop-

ment of NPC by regulating EMT program. However, the specific regulatory mechanisms of ZFX in EMT still need to be elucidated by further experiments in vitro and in vivo.

In summary, our study for the first time demonstrated that ZFX expression was significantly increased in NPC tissues and might be associated with NPC progression. We also reported that ZFX expression could act as an independent prognostic indicator for NPC patients. Finally, we found the expression of ZFX and E-cadherin were inversely correlated in NPC tissues, suggesting a possible involvement of ZFX in EMT. All these findings provide a novel insight into the clinical significance of ZFX in cancer and further studies are required to verify whether targeting ZFX could prevent NPC invasion and metastasis by reversing EMT process.

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Disclosure of conflict of interest

None.

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