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Original Article

Elevated HBXIP expression is associated with aggressive phenotype and poor prognosis in esophageal squamous cell carcinoma

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Abstract: The oncoprotein hepatitis B virus X-interacting protein (HBXIP) has been suggested to play an essential role in several malignancies. However, the clinicopathological significance and prognostic value of HBXIP expression in esophageal squamous cell carcinoma (ESCC) is still unknown. Therefore the aim of this study was to characterize HBXIP expression and its prognostic value in ESCC. Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were performed to assess the mRNA and protein expression of HBXIP in ESCC tissues and cell lines. Immunohistochemistry (IHC) was conducted to characterize the expression pattern of HBXIP in 152 archived paraffin-embedded ESCC and matched nontumorous tissues. The mRNA and protein expression of HBXIP in ESCC tissues was significantly higher than those in adjacent nontumorous tissues. High HBXIP expression was associated with histological grade (P=0.016), depth of tumor invasion (P=0.012), lymph node metastasis (P<0.001) and TNM stage (P=0.002). Kaplan-Meier analysis indicated that ESCC patients with high HBXIP expression had poor disease-free survival (DFS) and overall survival (OS). Furthermore, multivariate Cox regression analyses demonstrated that HBXIP expression remained an independent prognostic factor for DFS and OS. Collectively, our present study demonstrated that HBXIP may be a candidate molecular prognostic marker for ESCC.

Keywords: Hepatitis B virus X-interacting protein, esophageal squamous cell carcinoma, prognosis

Introduction

In China, esophageal squamous cell carcinoma (ESCC) accounts for >90% of all esophageal cancer cases. ESCC is ranked the fourth most lethal cancer in China [1]. Despite the improvements in multimodal treatment strategies in the past decades, the prognosis of ESCC remains poor, with a 5-year survival of less than 30% [2]. The prognosis of ESCC patients is tumor-node-metastasis (TNM) stage specific, but the TNM stage is not sufficiently sensitive to predict the prognosis of ESCC patients [3]. Therefore, the identification of novel prognostic markers and new therapeutic targets is critical to improve the management of ESCC patients.

Hepatitis B virus X-interacting protein (HBXIP) is a conserved 18 kDa protein among mammalian species, which was originally identified for interacting with hepatitis B virus X proteins [4]. The expression of HBXIP mRNA is not limited to liver, it occurs in nearly all tissues [5]. HBXIP could interact with hSuv3 protein and the HBXIP binding domain was found to be important for mitochondrial import and stability of the Suv3 protein in vivo [6]. In addition, HBXIP also functions as a Ragulator component that is required for mTORC1 activation by amino acids [7]. Emerging evidence demonstrated that HBXIP has broad roles in several malignancies, including liver cancer, breast cancer and ovarian cancer. HBXIP acts as an oncoprotein in controlling cell growth, proliferation, angiogenesis, and migration [8-11]. HBXIP overexpression is also correlated with poor prognosis in breast cancer, cervical cancer, and ovarian cancer [12-14]. However, studies investigating the prognostic value of HBXIP in ESCC have not been reported.

In this study, we assessed the expression levels of HBXIP in ESCC using quantitative real-time polymerase chain reaction (qRT-PCR), western blot and immunohistochemistry (IHC). The correlation between HBXIP expression and clinicopathological parameters of ESCC patients was investigated. Univariate and multivariate Cox regression analyses were performed to evaluate the correlation of HBXIP expression with prognosis of ESCC patients.

Materials and methods

Patients and tissue specimens

Fresh ESCC tissues and corresponding nontumorous tissues (located more than 5 cm away from the ESCC) used for qRT-PCR and western blot were collected from 30 ESCC patients who received radical resection at the Department of Cardiothoracic Surgery, Tianjin Hospital, between January 2015 and December 2016. Immediately after resection, each tumor tissue and corresponding nontumorous tissue was immediately stored at -80°C until RNA and protein extraction.

A total of 152 ESCC patients receiving radical surgery from January 2009 to December 2012 were enrolled for immunohistochemistry in the present study. The inclusion criteria include the following: (1) histologically confirmed ESCC after surgery and (2) R0 resection. The exclusion criteria include (1) patients who previously received preoperative chemotherapy or radiotherapy before surgery and (2) patients without data of complete follow-up information. The seventh American Joint Committee on Cancer (AJCC) staging system was used in this retrospective study [15]. According to the World Health Organization (WHO) classification of esophageal tumors, histological grade was defined as well differentiated (G1), moderately differentiated (G2), or poorly differentiated (G3) [16]. All patients provided informed consent. This study was approved by the ethics committees of Tianjin Hospital.

Cell line and culture

The human ESCC lines Kyse-450, Kyse-510, Kyse-30 and normal esophageal epithelial cell line Het-1A were purchased from the China Academy of Science cell library and maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco, Grand Island, NE, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

qRT-PCR

Total RNA was extracted with trizol reagent (Takara, Dalian, China) according to the manufacturer's instructions. After RNA intensity and purity were quantified, cDNA reversely transcribed from 1 ug RNA the PrimeScript RT reagent kit (Takara, Dalian, China) was used as templates for polymerase chain reaction (PCR) amplification using a SYBR Premix Ex TaqII kit (Takara, Dalian, China). Primer sequences used for HBXIP detection were as follows, forward: 5'-ATGGAGCCAGGTGCAGGTC-3'; reverse: 5'-TGGAGGGATTCTTCATTGTG-3'. GAPDH was used as an internal control, and primer sequences were as follows, forward: 5'-GTCGGAGGCAACATCACC-3'; reverse: 5'-GTCCAAATGCGGGAACAG-3'. The $2^{-\Delta\Delta CT}$ method was used to calculate expression relative to the GAPDH. Each reaction was performed in triplicate.

Western blot

The total protein was extracted from fresh ESCC tissues and cell lines using a protein extraction kit (Beyotime Biotechnology, China) and quantified using a BCA assay. Equal amounts of protein lysates were separated by SDS-PAGE gel by electrophoresis and transferred to PVDF membrane (Millipore, USA). The membrane was incubated with anti-HBXIP rabbit antibody (1:800 dilution; ProteinTech, USA) overnight at 4°C. Anti-GAPDH antibody (1:5000 dilution; Cell Signaling Technology, USA) was used as the loading control. After incubation with a horseradish peroxidase-conjugated secondary antibody, the signal was detected by ECL detection reagents (Millipore, USA).

Immunohistochemistry analysis

Briefly, after baking at 65°C for 1 h, slides were subjected to deparaffinization, antigen retrieval with citrate buffer (pH=6.0) at 98°C for 15 min, blocking endogenous peroxidase with 3% H_2O_2 for 15 min, reducing nonspecific binding with normal goat serum for 30 min, and subsequent incubation with anti-HBXIP antibody (1:50 dilution; ProteinTech, USA) overnight at 4°C. For negative control, the anti-HBXIP antibody was replaced with an unrelated rabbit IgG mAb. After washing three times with phosphate buffered saline, slides were incubated with corresponding secondary antibody for 1 h at room temperature, and then revealed with 3,

Table 1. The correlation between HBXIP expression and clinicopathologcial characteristics

Characteristics	No. (%)	HBXIP expression		*P* value
		Low	High	
Age				0.137
≤65	80 (52.6)	43	37	
>65	72 (47.4)	30	42	
Sex				0.952
Male	119 (78.3)	57	62	
Female	33 (21.7)	16	17	
Smoking status				0.756
No	44 (28.9)	22	22	
Yes	108 (71.1)	51	57	
Tumor location				0.197
Upper	11 (7.2)	8	3	
Middle	126 (82.9)	57	69	
Lower	15 (9.9)	8	7	
Tumor size(cm)				0.200
<4	73 (48.0)	39	34	
≥4	79 (52.0)	34	45	
Histological grade				0.016
G1	18 (11.9)	12	6	
G2	99 (65.1)	51	48	
G3	35 (23.0)	10	25	
Depth of tumor invasion				0.012
T1-T2	40 (26.3)	26	14	
T3-T4	112 (73.7)	47	65	
Lymph node metastasis				<0.001
Negative	91 (59.9)	55	36	
Positive	61 (40.1)	18	43	
TNM stage				0.002
I	10 (6.6)	8	2	
II	46 (30.3)	29	17	
III	96 (63.1)	36	60	

G1 was defined as well-differentiated, G2 was defined as moderately-differentiated and G3 was defined as poorly-differentiated.

3'-diaminobenzidine. Two investigators evaluated the samples independently and were unaware of the clinical data of all ESCC patients. Specific staining of HBXIP was mainly observed in the cytoplasm of the tumor cells. The IHC scoring was conducted according to the intensity and extent of staining [12]. In brief, the staining intensity was graded as follows: 0 (no staining, negative), 1 (light yellow, weak staining), 2 (yellow brown, moderate staining) and 3 (brown, strong staining). The extent of staining was scored as 0 (<5% positive tumor cells), 1 (5-25% positive tumor cells), 2 (26-50% positive tumor cells), or 3 (51-100% positive tumor cells). The immunoreactivity score was calculated by multiplying the staining intensity and staining extent. Based on the immunoreactivity score, HBXIP expression was divided into two groups: low expression (a total of score of 0~3) and high expression (a total of score of 4~9).

Statistical analysis

All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). Wilcoxon matched paired test was used to compare HBXIP mRNA levels in ESCC tissues and corresponding nontumorous tissues. Correlations between HBXIP expression and clinicopathological parameters were determined by Chi-square test or Fisher's exact tests. Survival rates were calculated using the Kaplan-Meier method, and differences in survival curves were analyzed by log-rank tests. Disease-free survival (DFS) was calculated from the time of surgery until the first progression or last follow-up. Overall survival (OS) was defined as the time from surgery to death or last follow-up. Cox regression analysis was conducted at both univariate and multivariate levels to analyze independent factors affecting prognosis. All statistical tests were two-sided, and *P* value <0.05 was considered statistically significant.

Results

Characteristics of patients

Table 1 shows the baseline characteristics of the 152 ESCC patients. The median age of the participants was 65 years, and the age range is 40 years to 80 years. A total of 119 patients (78.3%) were men, and 33 patients (21.7%) were women. Of these, 82.9% tumors were in the middle esophagus, 17.1% in the upper and lower area. Lymph node metastasis was found in 61 (40.1%) patients. According to the seventh edition of AJCC staging system, 10 patients were stage I, 46 patients were stage II, and 96 patients were stage III.

HBXIP expression in ESCC tissues and cell lines by qRT-PCR and western blot

qRT-PCR was performed to evaluate and compare HBXIP expression in 30 pair of ESCC fresh tissues along with matched nontumorous tis-

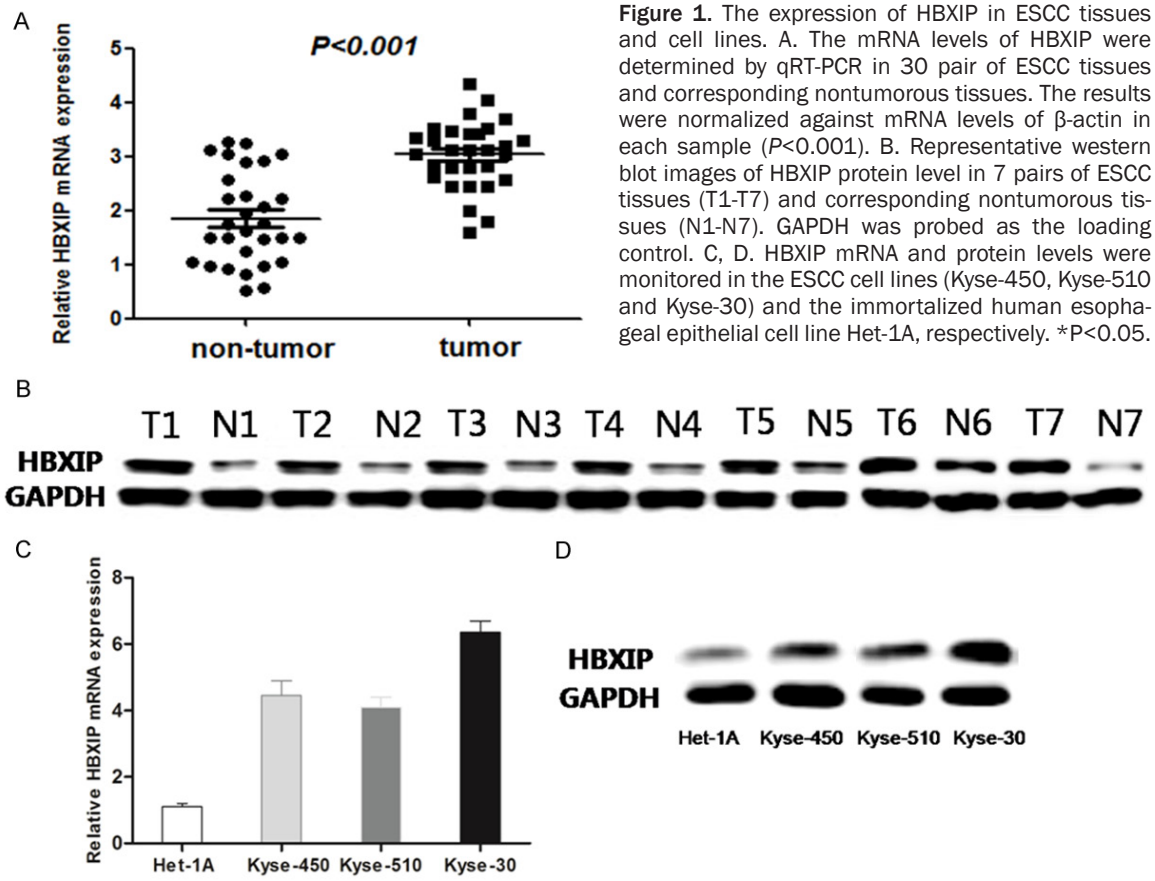

Figure 1. The expression of HBXIP in ESCC tissues and cell lines. A. The mRNA levels of HBXIP were determined by qRT-PCR in 30 pair of ESCC tissues and corresponding nontumorous tissues. The results were normalized against mRNA levels of β-actin in each sample (P<0.001). B. Representative western blot images of HBXIP protein level in 7 pairs of ESCC tissues (T1-T7) and corresponding nontumorous tissues (N1-N7). GAPDH was probed as the loading control. C, D. HBXIP mRNA and protein levels were monitored in the ESCC cell lines (Kyse-450, Kyse-510 and Kyse-30) and the immortalized human esophageal epithelial cell line Het-1A, respectively. *P<0.05.

sues. There were 3 (10.0%) patients well differentiated, 21 (70.0%) cases moderately differentiated, and 6 (20.0%) cases poorly differentiated. The mRNA level of HBXIP in ESCC tissues was significantly higher than that in the nontumorous tissues (**Figure 1A**). Furthermore, western blot was used to detect the protein level of HBXIP on these 30 pair of ESCC fresh tissues and matched nontumorous tissues. In consistent with the qRT-PCR results, expression of HBXIP protein was elevated in ESCC tissues (**Figure 1B**). In parallel, the mRNA and protein levels of HBXIP were highly expressed in the three ESCC cell lines, Kyse-450, Kyse-510, Kyse-30, when compared to immortalized normal human esophageal epithelial cell line Het-1A (**Figure 1C**, **1D**).

HBXIP expression in ESCC by IHC and its relationship with the clinicopathological characteristics

IHC was conducted to detect the expression and localization of HBXIP in 152 archived paraffin-embedded ESCC specimens. We found that specific staining of HBXIP was mainly observed in the cytoplasm of the tumor cells in ESCC tissues. As shown in **Table 1**, a total of 79 of the 152 cancer lesions (51.9%) were scored as high expression, while 20 of the 152 cases (13.1%) were scored as high expression in adjacent normal esophageal mucosal tissues, and their difference was significant (P<0.05). Therefore, our results demonstrated that HBXIP expression was upregulated in ESCC tissues. The representative results of HBXIP staining are shown in **Figure 2**.

The relationship between HBXIP expression and different clinicopathological characteristics was shown in **Table 1**. High HBXIP expression was associated with histological grade (P=0.016), depth of tumor invasion (P=0.012), lymph node metastasis (P<0.001) and TNM stage (P=0.002). By contrast, HBXIP expression displayed no association with age, sex, smoking status, tumor location, or tumor size.

Relationship between HBXIP expression and prognosis in ESCC patients

To evaluate the prognostic value of HBXIP, the OS and DFS curves were plotted with the Kaplan-Meier and compared using the log-rank

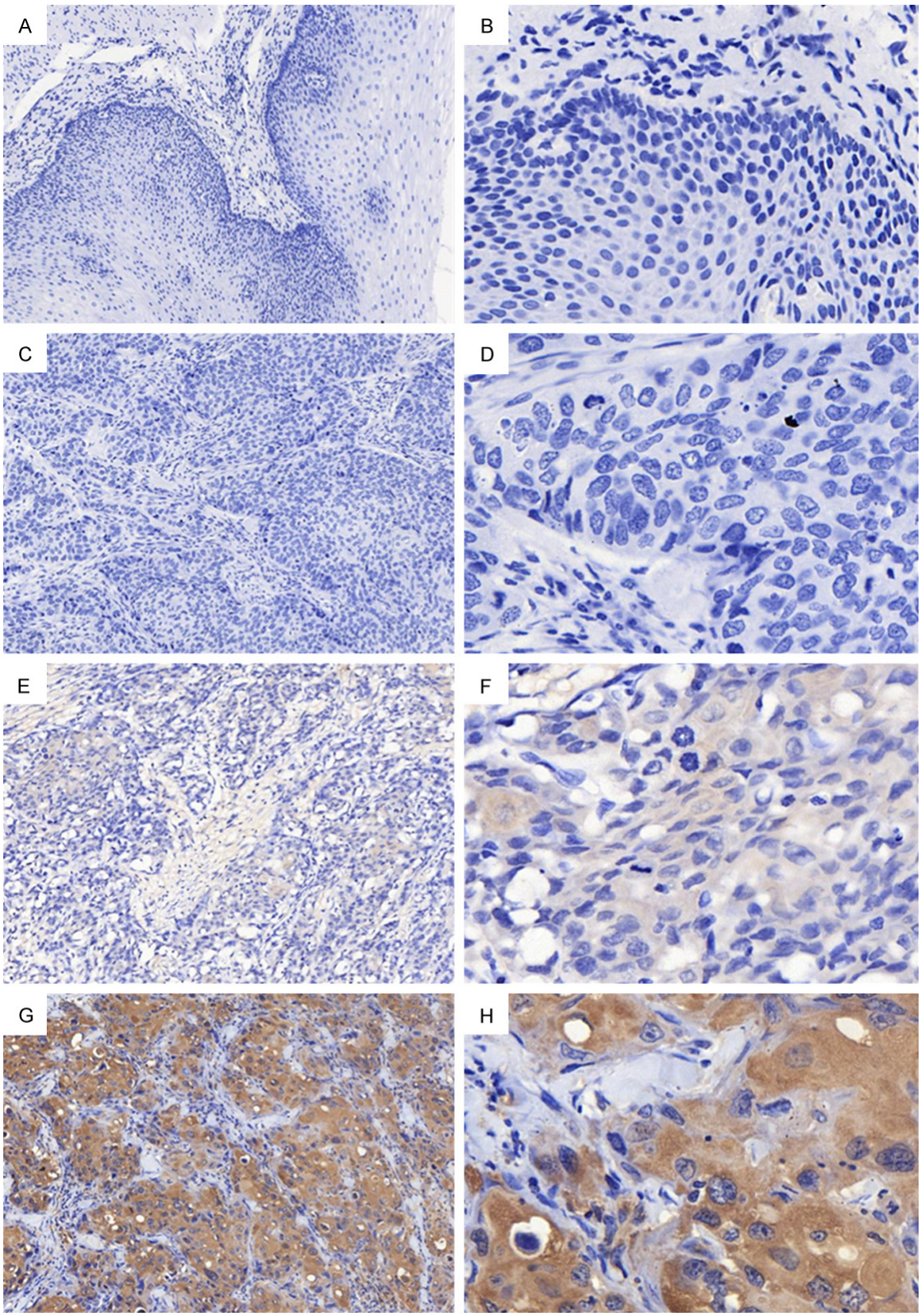

Figure 2. Expression of HBXIP was detected by immunohistochemistry in esophageal squamous cell carcinoma. The samples were stained with an anti- HBXIP antibody. A, B. Negative HBXIP staining in nontumorous esophageal epithelial tissues. C, D. Negative HBXIP staining in tumor tissues. E, F. Low HBXIP staining in tumor tissues. G, H. High HBXIP staining in tumor tissues. A, C, E and G. Magnifications × 100; B, D, F and H. Magnifications × 400.

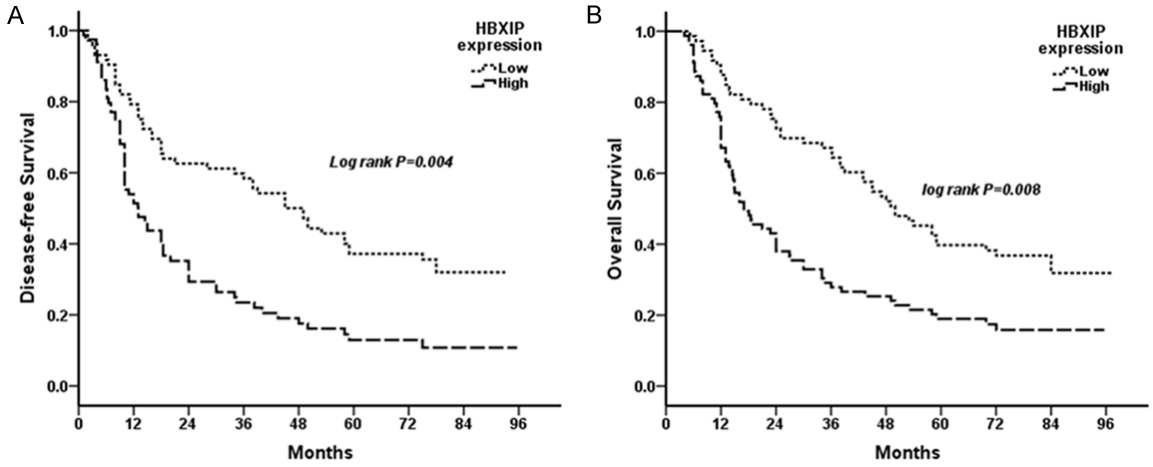

Figure 3. Kaplan-Meier survival curve and log-rank test revealed DFS and OS of all ESCC patients. A. DFS curves of all ESCC patients with high or low HBXIP expression (*P*=0.004). B. OS curves of all ESCC patients with high or low HBXIP expression (*P*=0.008).

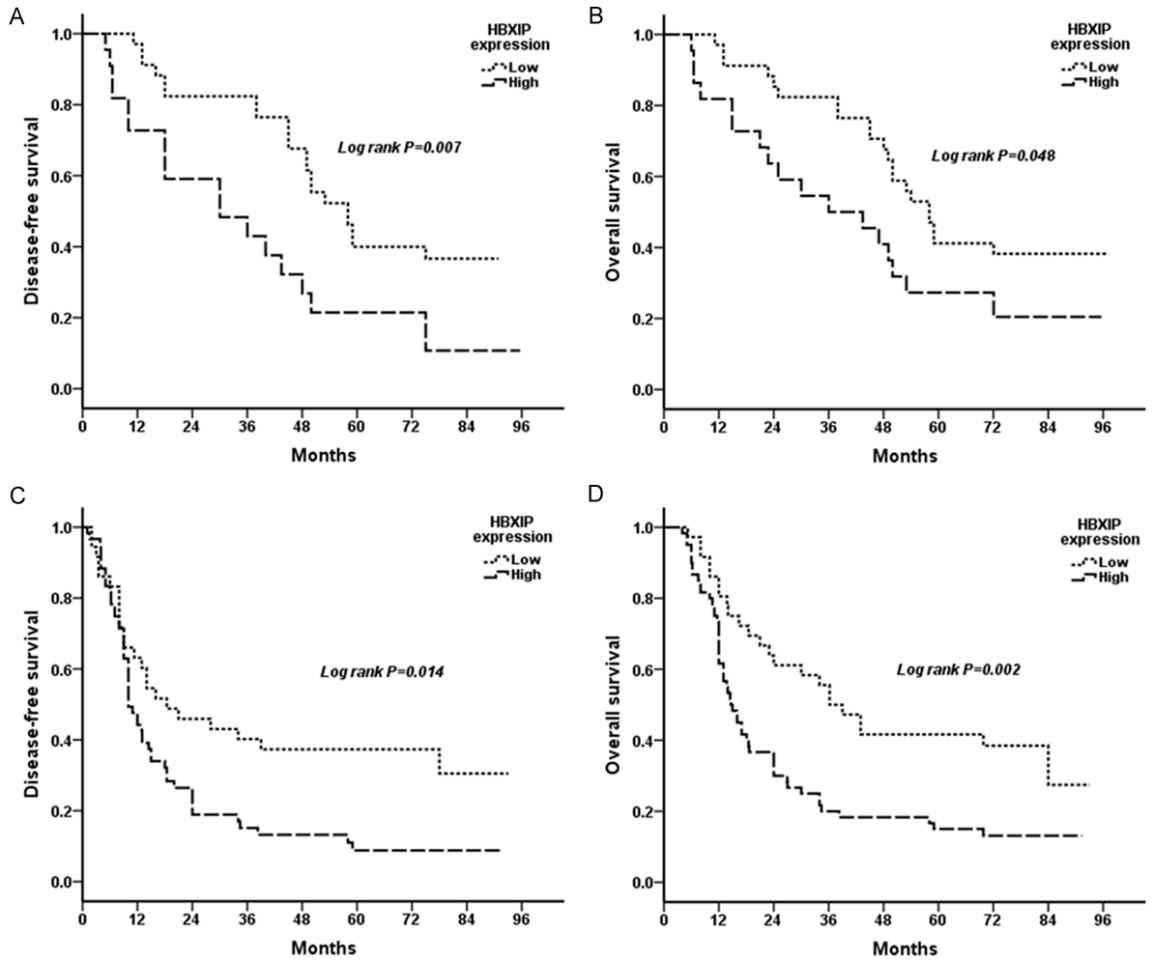

Figure 4. Kaplan-Meier survival curve and log-rank test revealed DFS and OS of ESCC patients in different TNM stage. A. DFS curves of TNM stage I+II ESCC patients with high or low HBXIP expression (*P*=0.007). B. OS curves of TNM stage I+II ESCC patients with high or low HBXIP expression (*P*=0.048). C. DFS curves of TNM stage III ESCC patients with high or low HBXIP expression (*P*=0.014). D. OS curves of TNM stage III ESCC patients with high or low HBXIP expression (*P*=0.002).

Table 2. Univariate analysis of DFS and OS for all ESCC patients

	DFS			OS		
	95% CI	HR	*P* value	95% CI	HR	*P* value
Age (>65, ≤65)	1.076-2.265	1.561	0.019	1.084-2.276	1.571	0.017
Sex (male, female)	0.595-1.431	0.923	0.719	0.585-1.407	0.907	0.664
Smoking status (no, yes)	0.471-1.096	0.719	0.125	0.469-1.090	0.715	0.119
Tumor location (upper, middle, lower)	0.960-2.266	1.475	0.076	0.967-2.282	1.486	0.071
Tumor size (≥4 cm, <4 cm)	1.127-2.376	1.636	0.010	1.140-2.400	1.654	0.008
Histological grade (G1, G2, G3)	0.964-1.859	1.339	0.082	0.932-1.764	1.282	0.127
Depth of tumor invasion (T1-T2, T3-T4)	1.213-2.963	1.896	0.005	1.252-3.057	1.956	0.003
Lymph node metastasis (negative, positive)	1.557-3.313	2.271	<0.001	1.379-2.915	2.005	<0.001
HBXIP (low, high)	1.480-3.171	2.166	<0.001	1.430-3.040	2.085	<0.001

DFS: disease-free survival; OS: overall survival; CI: confidence interval; HR: hazard ratio; G1 was defined as well-differentiated, G2 was defined as moderately-differentiated and G3 was defined as poorly-differentiated.

Table 3. Multivariate analysis of DFS and OS for all ESCC patients

	DFS			OS		
	95% CI	HR	*P* value	95% CI	HR	*P* value
Age (>65, ≤65)	0.890-1.926	1.309	0.171	0.896-1.934	1.316	0.162
Tumor size (≥4 cm, <4 cm)	0.957-2.120	1.424	0.082	0.940-2.079	1.398	0.098
Depth of tumor invasion (T1-T2, T3-T4)	0.675-1.852	1.118	0.665	0.803-2.126	1.306	0.283
Lymph node metastasis (negative, positive)	1.233-2.747	1.841	0.003	1.104-2.393	1.625	0.014
HBXIP (low, high)	1.203-2.685	1.797	0.004	1.152-2.544	1.712	0.008

DFS: disease-free survival; OS: overall survival; CI: confidence interval; HR: hazard ratio; G1 was defined as well-differentiated, G2 was defined as moderately-differentiated and G3 was defined as poorly-differentiated.

test. The 5-year DFS rate and the median survival time in the high HBXIP expression group were significantly lower than those in the low HBXIP expression group (12.9% vs 37.2% [12.9 vs 45.0 months], *P*=0.004; **Figure 3A**). The 5-year OS rate was 19.7% for the high HBXIP expression group and 39.7% for the low HBXIP expression group, and the median survival time was 17.0 and 50.0, respectively (*P*=0.008; **Figure 3B**). When the analysis was stratified by TNM stage (I+II, III), we found that DFS and OS were better in the low HBXIP expression group compared with the high HBXIP expression group for the TNM stage I+II, and III subgroups (stage I+II: *P*=0.007 for DFS, *P*=0.048 for OS, **Figure 4A**, **4B**; stage III: *P*=0.014 for DFS, *P*=0.002 for OS, **Figure 4C**, **4D**).

As shown in **Table 2**, a univariate analysis revealed that age (*P*=0.019 and *P*=0.017), tumor size (*P*=0.010 and *P*=0.008), depth of tumor invasion (*P*=0.005 and *P*=0.003), lymph node metastasis (*P*<0.001 and *P*<0.001), and HBXIP expression (*P*<0.001 and *P*<0.001) were significantly associated with DFS and OS, respectively. To assess independent prognostic factors, those that were significant (*P*<0.05) based on the above univariate analysis were entered in a multivariate analysis (**Table 3**). Our results demonstrated that HBXIP expression remained an independent prognostic factor for DFS (hazard ratio [HR]=1.797, 95% confidence interval [CI]=1.203-2.685, *P*=0.004) and OS (HR=1.712, 95% CI=1.152-2.544, *P*=0.008).

Discussion

In this study, we assessed HBXIP expression and its value in the prediction of DFS and OS in ESCC patients. The correlation between HBXIP expression and the clinicopathological characteristics of ESCC patients was also investigated. To the best of our knowledge, this is the first research to investigate the prognostic value of HBXIP in ESCC patients.

HBXIP, a conserved 18 kDa protein, was originally identified for interacting with hepatitis B virus X proteins [4]. HBXIP sequences are well

conserved among mammalian species. In HepG2 cells, HBXIP overexpression suppressed hepatitis B virus replication, in addition to suppressing the transactivation phenotype of HBx [4]. Recently, people have been focusing on the study of biological functions of HBXIP on cancers and have confirmed that HBXIP is elevated in cancers and acts as an oncoprotein in controlling cell growth, proliferation, apoptosis, angiogenesis, and migration. Wang et al. reported that HBXIP up-regulates yes-associated protein via activation of transcription factor c-Myb to promote growth of liver cancer [8]. Zhang et al. revealed that HBXIP upregulates the platelet-derived growth factor beta polypeptide through activating transcription factor 1 Sp1 to promote breast cancer cells proliferation [9]. As a cofactor of surviving, HBXIP serves as a link between the cellular apoptosis machinery and a viral pathogen involved in hepatocellular carcinogenesis. In hepatocellular carcinoma, HBXIP overexpression enhanced HepG2 cell-induced endothelial cells proliferation, migration and angiogenesis [10]. HBXIP was able to stimulate the activity of Skp2 promoter via transcription factor Sp1 thus promoting the migration of ovarian cancer cells [11].

Firstly, we use qRT-PCR and western blot to detect HBXIP mRNA and protein expression in ESCC tissues and corresponding nontumorous tissues. Compared with its corresponding nontumorous tissues, our results showed that HBXIP mRNA and protein levels were significantly increased in ESCC tissues. These findings suggest that HBXIP may function as an oncogene in ESCC and play a role in ESCC tumorigenesis.

It has been revealed that elevated HBXIP expression was significantly associated with worse prognosis in malignancies including breast cancer, cervical cancer, and ovarian cancer [12-14]. To this regard, we assessed HBXIP expression in 152 ESCC patients who underwent radical surgery. IHC results indicated that high HBXIP expression was found in 52% of all studied cases. High HBXIP had a positive correlation with histological grade, depth of tumor invasion, lymph node metastasis and TNM stage. The results are in agreement with the findings of Wang et al. [14], showing that elevated HBXIP expression was associated with the occurrence of lymph node metastases, histological grade and clinical stage. Our results implicated that abnormal HBXIP expression might be involved with the progression of ESCC and might also be used as a marker to identify malignant phenotype of ESCC. The significant difference in DFS and OS for patients with high HBXIP expression and those with low HBXIP expression also supports the hypothesis that elevated HBXIP expression is associated with invasive and metastasis process in ESCC. In addition, we also separately evaluated the prognostic value of HBXIP expression in patients with TNM stage I+II and III, and our results revealed that the HBXIP expression level is significantly associated with DFS and OS in stage I+II and III. The results of survival analysis in TNM stage subgroup were consistent with the outcomes of the whole study population, indicating that our findings are reliable. Most importantly, multivariate analysis demonstrated that elevated HBXIP expression was an independent and unfavorable marker for DFS and OS in ESCC patients. Thus, HBXIP expression has the potential to predict ESCC prognosis.

Our study is the first to investigate the prognostic value of HBXIP in ESCC, however, several potential limitations must be recognized. First, the present study is a retrospective single-center study, we cannot fully exclude selection bias. Second, all enrolled ESCC patients are Chinese, and we do know if these findings are generalizable worldwide. In the future, further studies are needed to confirm our results. Basic studies are also needed to analyze the molecular mechanism of HBXIP involved in the tumorigenesis and progression of ESCC.

In conclusion, our study presents the first evidence that HBXIP mRNA and protein expression are higher in ESCC tissues than in the corresponding nontumorous tissues. Elevated HBXIP expression was associated with aggressive ESCC phenotype and was an independent and unfavorable prognostic marker for ESCC patients.

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Disclosure of conflict of interest

None.

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