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Expression of TBX3 in Hepatocellular Carcinoma and Its Clinical Implication

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Data Collection B
Statistical Analysis C
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Manuscript Preparation E
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Background: Hepatocellular carcinoma (HCC) is the fifth most common malignancy in China, and China's annual number of new cases accounts for about 45% of the world total. This research was aimed to study the expression of TBX3 protein in HCC and exploring its clinical significance.

Material/Methods: We collected tumor tissues and adjacent non-tumoral tissues of 174 patients with HCC undergoing surgical resection. The expression of TBX3 protein in different tissues and cell lines *in vitro* (LO2, HHL-5, MHC97-L, MHC97-H) was detected by immunohistochemistry or Western blotting, and the relationship between TBX3 expression and clinical data of patients with HCC was analyzed.

Results: The expression of TBX3 protein in HCC was significantly correlated with histological grade, tumor size, cancer cell metastasis, hepatitis B surface antigen, and the expression of Ki-67 in tumor tissues ($P<0.05$), and it was positively correlated with serum AFP level ($r=0.766$, $P<0.05$). The expression of TBX3 increased with increased histological grade in HCC ($P<0.05$). Cox regression analysis showed that the expression of TBX3 protein in HCC was an independent risk factor for prognosis (OR=0.524, 95% CI=0.283-0.964). The 5-year survival rate of patients with HCC that highly expressed TBX3 protein was 20.83%, which was significantly lower than the 40.20% rate in patients with low expression ($P<0.05$).

Conclusions: The expression of TBX3 in HCC patients undergoing surgical resection is high, and its expression increases with the degree of tumor differentiation. It is related to the metastasis of tumor cells and is positively correlated with the serum level of AFP and may affect the survival time of HCC patients undergoing surgical resection.

MeSH Keywords: Liver Neoplasms • Lymphatic Metastasis • Prognosis

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Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China. It is the fifth most common malignant tumor after lung cancer, gastric cancer, esophageal cancer, and breast cancer. HCC is associated with high mortality. China's annual number of new cases accounts for about 45% of the world´s total HCC cases, and China has the world's highest incidence of this malignancy. In China, HCC is the leading cause of death due to malignant tumors in rural areas and the second leading cause in urban areas. China is now become the country with the highest incidence of HCC [1–3]. According to data released by the National Cancer Center, although the 1-year survival rate after radical resection in China increased from 39.3% to 87.0%, the 5-year survival rate after surgery is still only 15–40% [2,3]. Tumor metastasis and lack of effective targeted therapies are the main causes of poor prognosis in patients with HCC [4].

TBX3 protein is encoded by the *Tbx3* gene, which is an important member of the TBX (T-box) gene family and plays an important role in tumorigenesis and maintenance of tissue homeostasis [5,6]. Under normal physiological conditions, the end-product of *Tbx3* gene exists only in the embryo and participates in the regulation of embryonic development, but the abnormal expression of *Tbx3* gene can lead to many diseases, especially malignant tumors. It has been found that TBX3 protein is overexpressed in many malignant tumors such as breast cancer [7,8], prostate cancer [9], bladder cancer [10], and melanoma [11], and is closely related to tumor cell proliferation, apoptosis, invasion, and metastasis [12–14]. However, there is a lack of research on the expression of TBX3 protein in HCC. In the present study, the expression of TBX3 protein in HCC tissues and adjacent non-tumoral liver was detected by immunohistochemistry and Western blotting, and its clinical significance was analyzed. Our results may lay a theoretical foundation for studying the relationship between TBX3 protein expression and the occurrence and development of HCC.

Material and Methods

Study objects and clinical specimens

A total of 174 pairs of HCC tissues and adjacent non-tumoral liver tissues (>3 cm away from the tumor) were collected from January 2010 to December 2012 in the Affiliated Hospital of Inner Mongolia Medical University. Of these 174 HCC patients undergoing surgical resection, 135 were men and 39 were women; 90 were histological grade I–II and 84 were grade III–IV cases; the age range was 34–72 years old with a median age of 54; and 139 patients received preoperative Epirubicin combined with cisplatin, or sorafenib and other adjuvant therapies. Clinical data, including tumor size and number are shown in Table 1. All clinical specimens were approximately the size of a soybean and were immediately preserved in liquid nitrogen, while the rest of the tissues were fixed by formalin and made into paraffin sections for use. Patients were followed up every month by phone or on-site visits or when they returned to the hospital for a check-up after surgery or after discharge. Follow-up ended when patients died or had completed 5 years of follow-up. Exclusion criteria were: Patients with incomplete information such as age, sex, disease history or tumor information, postoperative follow-up loss or unknown cause of death, with other malignancies or cannot be identified as HCC, death due to other sudden-onset diseases (such as cardiovascular and cerebrovascular diseases), poor physical condition or poor postoperative mental status affecting prognosis of patients, and pregnant or breast-feeding patients. In addition, all patients participating in this study were informed of the details of this study. The informed consent form was signed by all patients or their family members and all participation was voluntary. The study was approved by the Ethics Committee of the Affiliated Hospital of Inner Mongolia Medical University.

Immunohistochemistry

TBX3 protein expression was detected by immunohistochemical staining according to the instructions of the VECTASTAIN® Elite® ABC Kit (Vector Laboratories, USA). First, the paraffin sections were heated at 60°C for 2 h, dewaxed and hydrated sequentially with xylene and ethanol, washed with PBS and double-distilled water, and stained after nuclear antigen retrieval. Secondly, we used TBX3 antibody (1: 300, Abcam, UK) or Ki-67 antibody (1: 300, Abcam, UK) as primary antibody (PBS instead of primary antibody as negative control) and incubated cells overnight at 4 C. Goat Anti-Rabbit IgG H and L (HRP) (Abcam, UK) was incubated as secondary antibody at 37°C for 2 h. Finally, 5 fields per slice were photographed and scored [15]: TBX3 protein localized in cytoplasm and Ki67 protein was localized in the nucleus. The cells in the slices were stained yellow (1 point), brown (2 points), and tan (3 points) for positive staining, while the cells that were not stained were scored as negative (0 point). In each field of view, the number of positive cells was scored as: ≤25%=0 points, 25–75%=1 point, and ≥75%=2 points. Results of the staining score multiplied by the percentage score of positive cells was the immunohistochemical score. In this study, TBX3 immunohistochemical score ≥4 was regarded as high expression.

Western blot

Tissue sample and cell lines (LO2, HHL-5, MHC97-L, MHC97-H) total protein was extracted using the Total Protein Extraction Kit (Beyotime Biotechnology, China) according to the instructions. The BCA Protein Concentration Determination kit (Beyotime

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Table 1. Relationship between TBX3 protein expression and clinical data on HCC.

Items	Cases	TBX3		χ^2	P
		Low expression	High expression		
Gender					
Female	39	26	13	1.342	0.274
Male	135	76	59		
Age (year)					
<45	59	30	29	2.224	0.136
≥45	115	72	43		
Serum AFP (ng/ml)					
<50	69	47	22	4.250	0.039
≥50	105	55	50		
Histology classification					
I–II	90	64	26	11.990	0.001
III–IV	84	38	46		
Tumor size (cm)					
<45	74	63	11	37.317	0.000
≥45	100	39	61		
Cirrhosis					
Yes	137	82	55	0.404	0.525
No	37	20	17		
Cancer metastasis					
Yes	150	78	72	19.652	0.000
No	24	24	0		
Tumor number					
Single	117	65	52	1.383	0.240
Multiple	57	37	20		
HBsAg					
Yes	135	86	49	6.415	0.011
No	39	16	23		
Microvascular invasion					
Yes	53	32	21	0.097	0.756
No	121	70	51		
Ki-67					
Low expression	63	27	36	10.117	0.001
High expression	111	75	36		

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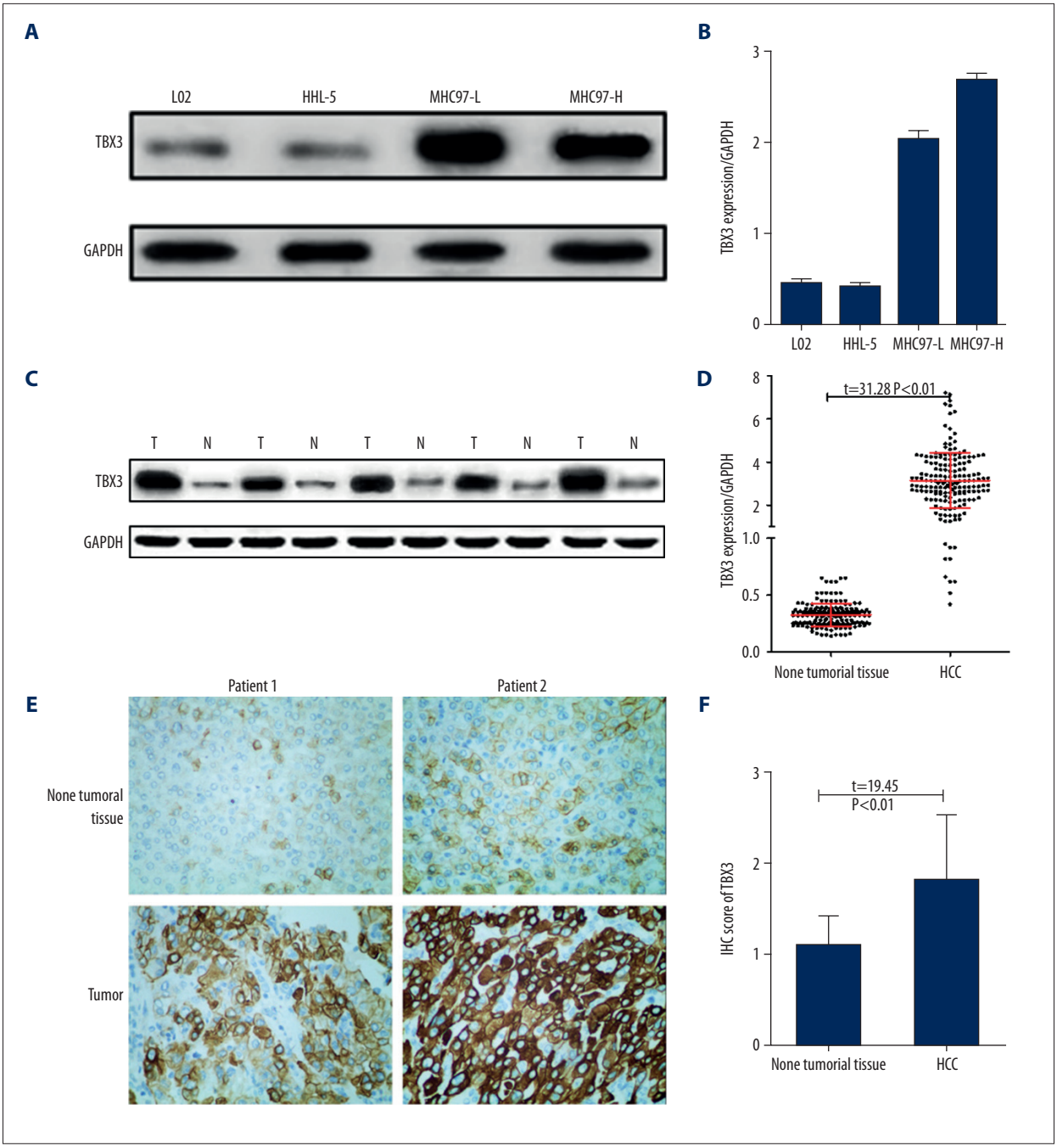

Figure 1. TBX3 protein is highly expressed in human HCC and HCC cells. (**A**) Western blot of TBX3 in human hepatocytes LO2 and HHL-5, HCC cells MHC97-L and MHC97-H; (**B**) Relative expression of TBX3 in human hepatocytes LO2 and HHL-5, human HCC cells MHC97-L, and MHC97-H; (**C**) Western blot of TBX3 in human HCC and the adjacent non-tumoral tissues. T: HCC tissues, N: non-tumoral tissues; (**D**) Relative expression of TBX3 in human HCC and adjacent non-tumoral tissues; (**E**) Immunohistochemical results of TBX3 in human HCC and the adjacent non-tumoral tissues (200×); (**F**) IHC score of TBX3 in human HCC and the adjacent non-tumoral tissues.

Biotechnology, China) was used to determine the total protein concentration of tissue/cell total protein. We loaded 75 ug total protein on SDS-PAGE electrophoresis (120 V, 1.5 h) and then transferred it to a PVDF membrane (Amersham Biosciences, UK) in transfer buffer (400 mA, 1.5 h). The membrane was fixed in methanol and washed with TBST buffer and blocked, then incubated overnight with primary antibody at 4°C with TBX antibody and GAPDH antibody (Abcam, UK), followed by washing

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with TBST buffer again and incubating with secondary antibody (Goat Anti-Rabbit IgG H & L (HRP)) for 1 h at room temperature and developed using the ECL kit after washing with TBST buffer. Protein expression level was analyzed using Image J software with gray scale value of the target protein/GAPDH protein to represent the relative expression of the target protein.

Statistical analysis

The data were statistically analyzed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA) and data were calculated as (mean ± standard deviation). The *t* test was used to compare differences between the 2 groups, and one-way ANOVA was used to compare the differences between groups (Duncan method for the post-test). Pearson method was used for analysis of the correlation between continuous variables, Kaplan-Meier curve and log rank test were used to analyze the survival data, and the Cox regression model was used to analyze factors that affect the survival of patients with HCC. $P<0.05$ indicates a significant difference.

Results

TBX3 protein is highly expressed in human HCC and HCC cells

The relative expression levels of TBX3 protein in human hepatocytes LO2 and HHL-5, human HCC cells MHC97-L and MHC97-H, and 174 pairs of HCC tissues and adjacent non-tumoral tissues were determined by Western blotting. The results showed that the expression of TBX3 in human HCC cells was significantly higher than that in normal hepatocytes ($P<0.05$), as shown in Figure 1A, 1B. The relative expression level of TBX3 in human HCC was (3.15±1.29), which was significantly higher than that in the adjacent non-tumoral tissues (0.33±0.10) ($P<0.05$), as shown in Figure 1C, 1D.

Immunohistochemistry was used to detect the expression of TBX3 protein in 174 human HCC tissues and adjacent non-tumoral tissues. The results showed that there were 72 cases with high expression of TBX3 protein (immunohistochemical score ≥4) and 102 cases with low expression. The immunohistochemistry score was (3.57±1.46), which was significantly higher than that in normal tissues (2.16±0.67), as shown in Figure 1E, 1F.

The relationship between TBX3 protein expression and clinical data of patients with HCC

According to the immunohistochemical detection of TBX3 in HCC, 174 patients with HCC were divided into a TBX3 low-expression group (102 cases) and a high-expression group (72 cases) to analyze the relationship between expression of TBX3 protein and clinical data of patients with primary hepatic cancer. The results showed that the expression of TBX3 protein had no correlation with sex, age, cirrhosis, tumor number, or microvascular invasion in patients with primary hepatic cancer ($P>0.05$). There was a significant correlation between histological grade, tumor size, metastasis of cancer cells, and HBsAg and Ki-67 expression in tumor tissues ($P<0.05$), and a positive correlation with serum AFP levels in patients with HCC ($r=0.766$, $P<0.05$), as shown in Table 2 and Figure 2.

The relationship between the expression of TBX3 and the histological grade of HCC

The histological grade of 174 patients with HCC was grade I in 44 cases, grade II in 46 cases, grade III in 62 cases, and grade IV in 22 cases. The results of Western blot analysis showed that the expression of TBX3 protein in grade I, grade II, grade III and grade IV HCC were 1.66 ± 0.66, 2.74±0.24, 3.72±0.54, and 5.30±1.07, respectively, as shown in Figure 3A, 3B. Immunohistochemistry showed that TBX3 protein immunohistochemical scores were 2.33±0.47 points, 2.77±0.73 points, 4.29±1.22 points, and 5.23±0.70 points in grade I, grade II, grade III, and grade IV tumors, respectively (Figure 3C, 3D).

TBX3 protein expression and HCC metastasis

In 174 cases of HCC, there were 150 cases with HCC cell metastasis and 24 cases without HCC cell metastasis. The relative expression of TBX3 in tumor tissue was 3.46±1.08, significantly higher than that without distal metastasis (1.18±0.43) ($P<0.01$), as shown in Figure 4A, 4B. The immunohistochemical score of TBX3 in patients with metastasis of HCC was 3.73±1.50, significantly higher than that of patients without metastasis (2.58±0.50), as shown in Figure 4C, 4D.

The effect of TBX3 protein expression on the prognosis of patients with HCC

Cox regression model analysis of 174 patients with HCC survival factors analysis showed that the expression of TBX3 protein in HCC is an independent risk factor for survival of HCC (OR=0.524, 95% CI=0.283–0.964) (Tables 2, 3). In addition, 174 patients with HCC were followed up for 5 years after the operation. The results showed that the 5-year overall survival rate of patients with high expression of TBX3 protein was 40.20%, while that of patients with low expression of TBX3 was only 20.83%, as shown in Figure 5.

Discussion

Tbx3 gene is located at human chromosome 12q24.21; it consists of 12 895 bases and it encodes 2 protein subtypes of TBX3 and TBX3+2α. Both protein subtypes contain a carboxyl

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Table 2. Cox regression model for univariate analysis of survival results of 174 patients with HCC.

Varieties	n	95%CI	OR	P
Sex Female/Male	39/135	1.312–4.406	2.294	0.004
Age (years) <45/≥45	59/115	0.681–2.995	1.428	0.346
AFP (ng/ml) <50/≥50	69/105	0.254–1.023	0.523	0.001
Histology classification I–II/III–IV	90/84	0.812–2.756	1.594	0.023
Tumor size (cm) <4.5/≥4.5	74/100	0.765–2.064	1.268	0.352
Cirrhosis Yes/No	137/37	1.175–5.624	3.521	0.021
Cancer metastasis Yes/No	150/24	2.050–6.257	4.022	<0.001
Tumor number Single/multiple	117/57	0.785–3.546	2.254	0.131
HBsAg Yes/No	135/39	2.451–9.254	3.248	<0.001
Microvascular invasion Yes/No	53/121	1.032–4.781	2.364	<0.001
Ki67 Low expression/high expression	63/111	1.652–4.415	9.124	<0.001
TBX3 Low expression/high expression	102/72	1.222–4.658	2.459	0.011

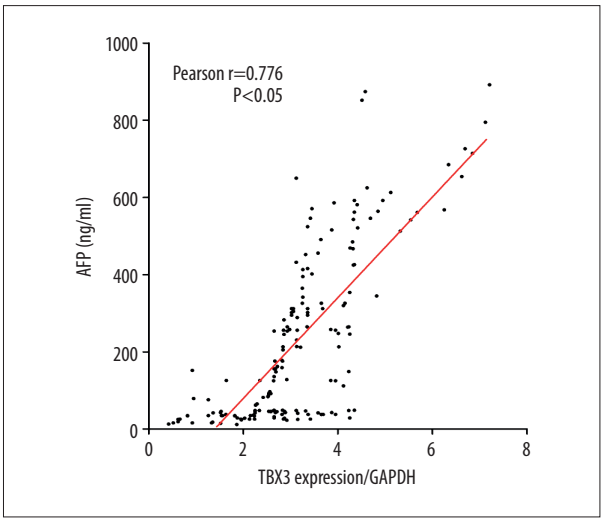

Figure 2. Expression of TBX3 protein in HCC was positively correlated with serum AFP level.

terminal, an amino terminal, and a T region. Carboxyl termini amino acids 567–623 are their functional domains. TBX3 protein is the main functional subtype of *Tbx3* gene. Under normal physiological conditions, TBX3 protein, a transcriptional repressor, plays an important role in all stages of embryonic development [6,16]. Once its expression is dysregulated or the gene is mutated, it can cause a variety of diseases [17,18]. A number of studies [7,8,10,11] have shown that TBX3 is overexpressed in various malignant tumors and is considered as an oncogene.

The present study found that TBX3 protein expression in HCC tissue was significantly higher than in normal tissue, and in human HCC cultured cells lines its expression was significantly

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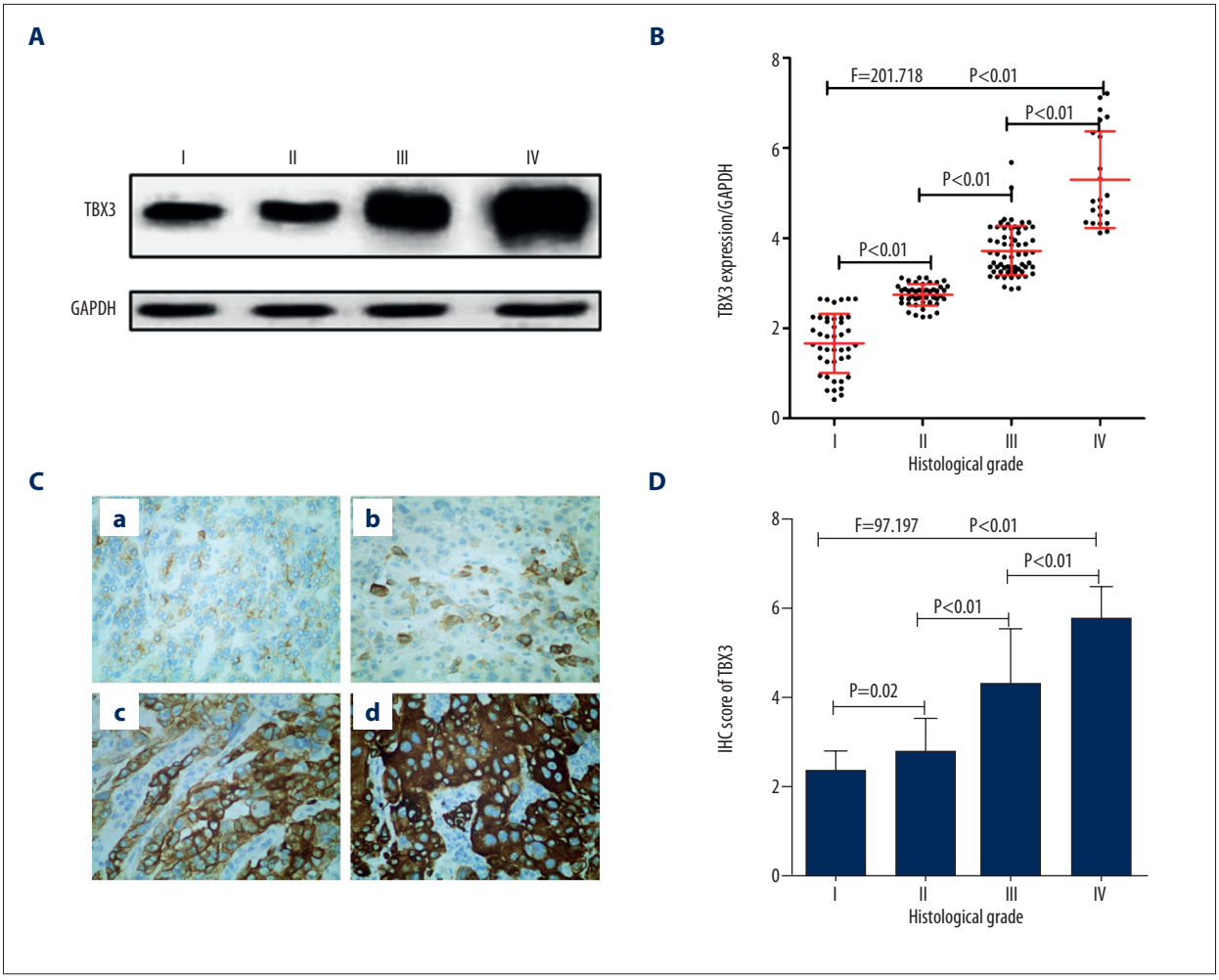

Figure 3. TBX3 protein expression in HCC tissues with different histological grade. (**A**) Western blot of TBX3 in in grade I, grade II, grade III, and grade IV; (**B**) Relative expression of TBX3 in in grade I, grade II, grade III, and grade IV; (**C**) **a**, Immunohistochemical results of TBX3 in grade I; **b**, grade II; **c**, Immunohistochemical results of TBX3 in grade III; **d**, Immunohistochemical results of TBX3 in grade IV (200×); (**D**) IHC score of TBX3 in grade I, grade II, grade III, and grade IV.

higher than that expressed in hepatic parenchyma. In the process of human liver development, *Tbx3* gene is expressed in the hepatic lobule. When its expression is missing, the liver becomes smaller, resulting in the reduction of hepatocytes and the increase of cholangiocytes *in vitro*. However, the molecular mechanism of its regulation is still not clear [19]. Suzuki et al. [20] concluded that Tbx3 gene expression was inhibited by inhibiting the expression of p19ARF protein, thereby enhancing hepatic progenitor cell proliferation and hepatocyte differentiation and inhibiting cholangiocyte differentiation. More importantly, the high expression of TBX3 protein in the liver may be associated with liver failure, chronic hepatitis and cirrhosis, and other liver diseases [21]. These data suggest that high expression of TBX3 in HCC tissues is related to the occurrence and development of HCC.

Further analysis showed that the expression of TBX3 was significantly associated with tumor histological grade, tumor size, metastasis of cancer cells, and HBsAg and Ki-67 expression in HCC (P<0.05). p19ARF is a tumor-suppressor gene that not only inhibits normal cells from cancerization, but also activates p19ARF to induce p53 expression through inhibition of p53-negative regulator MdM2 *in vitro*, which in turn leads to cell cycle arrest and apoptosis [22,23]. In the liver, TBX3 can inhibit the priming sequence of p19ARF in a specific way, thereby inhibiting the expression of p19ARF protein [20]. Therefore, the high expression of TBX3 protein in malignant cells can inhibit the expression of p19ARF protein to promote tumor cells to avoid aging and apoptosis. When the abnormal expression of TBX3 gene and their over-accumulation reach a certain critical value, the rapid proliferation of many cells reach the critical point, and it is likely to lead to malignant transformation of cells [24]. On the other hand, both a hepatic carcinoma cell

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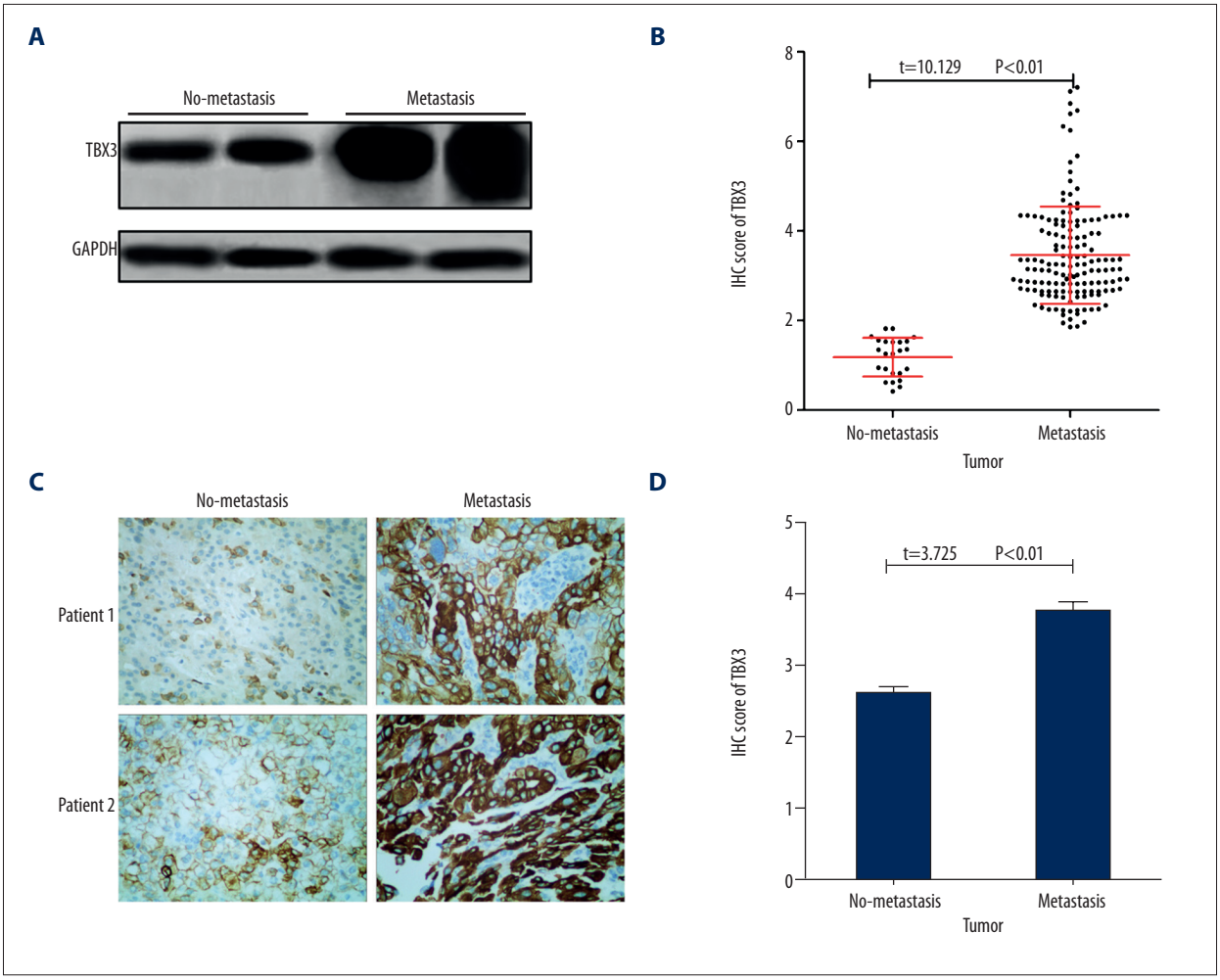

Figure 4. TBX3 protein expression promotes HCC cell metastasis. (**A**) Western blot of TBX3 in tumor tissue without distal metastasis and with metastasis; (**B**) Relative expression of TBX3 in tumor tissue without distal metastasis and with metastasis; (**C**) Immunohistochemical results of TBX3 in tumor tissue without distal metastasis and with metastasis (200×); (**D**) IHC score of TBX3 in tumor tissue without distal metastasis and with metastasis.

Table 3. Cox regression model for multivariate analysis of survival results of 174 patients with HCC.

Items	95%CI	OR	P
Sex	1.123–4.352	2.152	0.031
AFP (ng/ml)	0.747–2.657	1.915	0.045
Cirrhosis	0.254–5.12	1.368	0.756
Histology classification	0.143–1.358	0.512	0.085
Cancer cell metastasis	1.152–10.364	3.784	0.027
Microvascular invasion	0.584–3.621	1.425	0.421
Ki-67	1.302–4.425	2.321	0.004
TBX3	0.283–0.964	0.524	0.036

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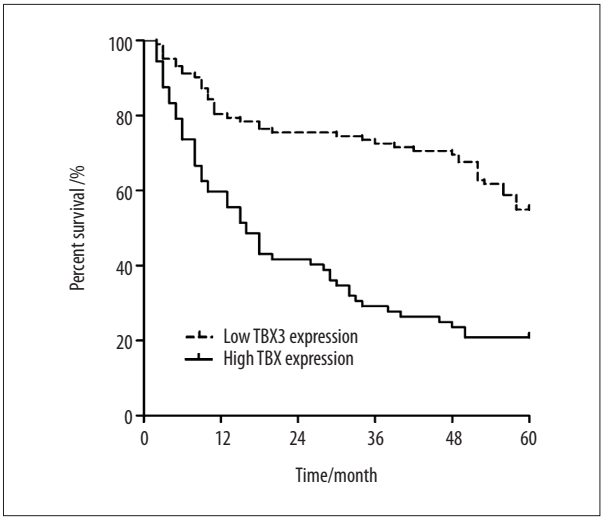

Figure 5. Effects of TBX3 protein expression on the prognosis of patients with HCC.

model [25] and a cell experiment [26] have found that TBX3 is a downstream target of the Wnt/β-catenin pathway and an important mediator of β-catenin function in HCC. Inhibition of TBX3 expression not only effectively inhibits the growth of xenografts in nude mice, but also can repress the level of β-catenin, thereby inhibiting activation of the Wnt/β-catenin signaling pathway and reducing the invasion and migration of HCC cells. Therefore, TBX3 may be a potential therapeutic target for primary hepatic carcinoma [27].

Our study also found that the expression of TBX3 protein in HCC was positively correlated with serum AFP level ($r=0.766$, $P<0.05$). Alpha fetoprotein (AFP) is derived from embryonic liver cells. Normal human serum AFP content is less than 20 ug/L. When HCC occurs, about 80% of hepatocytes in HCC patients will recover to synthesize AFP again. With the deterioration of HCC, the level of APF in the blood appears to sharply increase, and this is one of the main specific indicators of HCC in clinical diagnosis [28]. Highly-expressed AFP can promote the proliferation of HCC cells through activating the PI3K/Akt signaling pathway [29]. Chemotherapy can increase the sensitivity of HCC cells to chemotherapeutic drugs and reduce the growth of HCC cells by decreasing the AFP level in the serum of patients, which can also inhibit the proliferation, invasion, and migration of HCC cells [30]. The expression of TBX3 was positively correlated with serum AFP level, suggesting that TBX3 participates in the progress of HCC by regulating the synthesis of AFP in hepatocytes.

Limitation

We did not study all patients with HCC; we only studied patients with HCC that were submitted to hepatectomy/resection, which is a limitation of our study.

Conclusions

The expression of TBX3 in HCC is high and associated with tumor differentiation. It is associated with metastatic disease and AFP elevation, being a possible useful instrument for survival prediction in patients with resected HCC.

Conflict of interest

None.

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