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ORIGINAL ARTICLE

High expression of WSB1 is associated with poor prognosis in hepatocellular carcinoma and affects epithelial-mesenchymal transition

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Summary

Purpose: *To explore the expression of WD-40 repeat and SOCS box containing 1 (WSB1) and its clinical significance in hepatocellular carcinoma (HCC).*

Methods: *Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression level of WSB1 in clinical tissues. Survival curves were platted using the Kaplan-Meier method and log rank test was used to compare survival between groups. The influencing factors for the long-term survival were analysed using Cox univariate and multivariate analysis. In in vitro study, Western blot was used to evaluate the effects of WSB1 on epithelial-mesenchymal transition (EMT) of tumor cells.*

Results: *The expression of WSB1 was much higher in cancer tissues than that in para-normal tissues, and the WSB1 expression was correlated with tumor differentiation, lymph node metastasis and clinical stage. In addition, HCC patients with higher WSB1 expression had significantly poorer progression-free survival (PFS) and overall survival (OS). Both univariate analysis and multivariate analyses indicated that high expression of WSB1 was an independent predictor of poor prognosis in HCC. Further in in vitro study, downregulation of WSB1 in HCC cell line could reduce the expression of epithelial phenotype protein (E-cadherin) while increase the expression of mesenchymal phenotype protein (N-cadherin and Vimentin).*

Conclusions: *WSB1 might contribute into the development of HCC and serve as a clinical biomarker and a therapeutic target for HCC patients.*

Key words: *hepatocellular carcinoma, WD-40 repeat and SOCS box containing 1, progression-free survival, overall survival, epithelial-mesenchymal transition.*

Introduction

Primary liver cancer is one of the ten major malignant tumors in the world, with morbidity and mortality ranking fifth and third, respectively [1, 2]. Its histopathological types include hepatocellular carcinoma (HCC), cholangiocellular carcinoma and combined hepatocellular-cholangiocellular carcinoma, dominated by HCC [3]. Due to the insidious onset and atypical symptoms in the early stage, 80-90% of HCC patients had lost the opportunity of curative resection when diagnosed [4]. In terms of the treatment of HCC, radical resection is also one of the efficacious methods, but the postoperative recurrence rate remains high. In addition, HCC is insensitive to both chemotherapy and radiotherapy. Therefore, exploring the target genes and molecular pathways related to the occurrence and development of HCC is of high significance to ameliorate the prognosis and lower the mortality of HCC patients.

Tumor microenvironment refers to the environment where the tumor cells are generated and grown. The tumor cells and the microenvironment

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are interdependent, and they interact with each other, jointly promoting the malignant progression of tumor. Hypoxia is an unavoidable environmental condition during the development of solid tumors, and it has been confirmed in clinical and animal models that the hypoxic environment indeed exists in 90% of solid tumors. Moreover, the oxygen pressure of the solid tumors is lower than 15 mmHg in the great majority of patients, far lower than that in normal tissues surrounding these tumors (about 50 mmHg) [5]. Hypoxia-inducible factor-1α (HIF-1α), a transcription regulator in human body and mammals are extremely unstable under normal oxygen partial pressure and prone to degradation through the ubiquitin-proteasome pathway. Under the hypoxic environment, HIF-1α can accumulate stably and bind to HIF-1β to form heterodimers, which can facilitate the transcription process of downstream genes by acting on the hypoxia response elements of target genes [6]. When the tumor cells are adapting to the hypoxic environment, HIF-1α regulates various relevant signaling pathways and plays particularly important roles in tumor cell proliferation, neovascularization, insensitivity of tumor to radiotherapy and chemotherapy as well as malignant invasion [7-9].

As a member of the SOCS box family and a core element of ubiquitin-ligase complex, WD-40 repeat and SOCS box containing 1 (WSB1) exerts critical effects in mediating the degradation of substrate proteins through the ubiquitin-proteasome pathway [10]. Currently, there are few studies on the biological functions of WSB1, but it has been reported that WSB1 could promote the ubiquitination and degradation of DIO2 to stimulate the activation of thyroid hormones [11]. Besides, WSB1 is also able to repress the cell apoptosis mediated by p35 and CtBP by facilitating the ubiquitination and degradation of HIPK2 [12]. Although research on the role of WSB1 protein in the occurrence and development of tumors is in its infancy yet, studies have demonstrated that WSB1 is highly expressed in a variety of tumors [13-15]. According to a study on HCC, WSB-1 serves as a target gene of HIF-1α to mediate the hypoxia-induced drug resistance of HCC cells [16]. However, the above reports only focus on the expression in clinical samples, without deep investigation in the molecular mechanism.

Our study aimed to explore the expression of WSB1 and its clinical significance in HCC, and hoped to contribute to clarifying the biological mechanism of the tumor as well as to provide valuable targets for corresponding diagnosis and treatment.

Methods

Tissue specimens collected

A total of 80 pairs of tissue specimens were collected. Specifically, the HCC tissues and para-carcinoma tissues (more than 5 cm away from the carcinoma tissues) were surgically resected in the Department of Hepatobiliary Surgery. All the tissue specimens were confirmed as HCC by postoperative pathological examination. The personal information and detailed clinical data of patients, including patient's gender, age, AFP levels, HBsAg levels, tumor size, tumor number, lymph node metastasis and clinical stage were collected intact. The patients received no anti-tumor therapy before operation, and the patients and their families were communicated adequately and signed the informed consent of the experiments. This study was approved by the Ethics Committee of Zibo Central Hospital.

Cell culture and transfection

The human HCC SMMC-7721 cells and normal liver HL-7702 cells were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA). All the cell lines were placed in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 mg/mL penicillin and 100 mg/mL streptomycin and cultured in an incubator with 5% CO_2 at 37°C. The cells were transfected according to the instructions of the Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) when the cell density reached 50-60%. Groups were divided in si-NC group (SMMC-7721 cells transfected by si-NC) and si-WSB1 group (SMMC-7721 cells transfected by si-WSB1).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total ribonucleic acid (RNA) was extracted from the tissues according to the instructions of RNA extraction kit (Invitrogen, Carlsbad, CA, USA). Then the first-strand complementary deoxyribonucleic acid (cDNA) was syn-

Table 1. Primer's sequence

Gene		*Primer sequence*
GAPDH	Forward primer	5'-GTCATCCATGACAACTTTGG-3'
	Reverse primer	5'-GAGCTTGACAAAGTGGTCGT-3'
WSB1	Forward primer	5'-CGTACTATAGGTGAACTTTTAGCTCCT-3'
	Reverse primer	5'-CCAAAGGAAAACTGCTTTACTGG-3'

thesized with the total RNA as the template. After that, the cDNA was utilized as the template to detect WSB1 expression via ABI 7500 real-time polymerase chain reaction (PCR) instrument (Applied Biosystems, Foster City, CA, USA). PCR procedures: 95°C for 5 s and 60°C for 34 s for 40 cycles. Finally, WSB1 expression was analyzed with glyceraldheyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The sequence is shown in Table 1.

Western blot analysis

The tissues were homogenized on ice using radioimmunoprecipitation assay (RIPA) cell lysate (Beyotime, Shanghai, China), so as to extract the total protein. After quantification by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China), the protein was loaded (50 μg/well) for electrophoresis and then transferred onto a membrane. After that, the membrane was sealed in 5% skim milk powder at room temperature for 1.5 h and then washed with Tris-buffered saline-Tween 20 (TBST) for 3 min. Subsequently, antibodies were diluted in accordance with the instructions and added for incubation at 4°C overnight, followed by membrane washing for 5 min×3 times. Next, corresponding secondary antibodies were added and incubated at room temperature for 1.5 h, and the membrane was washed with TBST for 3 times (10 min/time), followed by image development via electrochemiluminescence (ECL). Finally, the relative expression of each index was analyzed with GAPDH as the internal reference.

Table 2. WSB1 expression and clinical features of patients with HCC

Features		*WSB1 expression level*		*p*
		high	*low*	
	No.80	*40*	*40*	
Gender				0.647
Male	49	23	26	
Female	31	17	14	
Age (years)				0.364
< 60	33	19	14	
≥ 60	47	21	26	
AFP (g/L)				0.815
< 20	28	13	15	
≥ 20	52	27	25	
HBsAg				0.113
Positive	46	27	19	
Negative	34	13	21	
Tumor size (cm)				0.162
< 5	29	11	18	
≥ 5	51	29	22	
Tumor number				0.091
Solitary	55	24	31	
Multiple	25	16	9	
Tumor differentiation				0.001
Well	32	9	23	
Moderate	31	15	16	
Poor	17	16	1	
Lymph node metastasis				0.001
Absence	46	13	33	
Presence	34	27	7	
Cirrhosis				0.180
Absence	42	24	18	
Presence	38	16	22	
Clinical stage				0.001
I + II	37	10	27	
III + IV	43	30	13	

Patient follow-up

The follow-up was conducted by phone calls or outpatient visit to record the survival of patients. The deadline for follow-up was 2018. The total survival period was from the date of onset to the date of the final follow-up or death, in months.

Statistics

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for the processing of result data, and measurement data were expressed as mean ± standard deviation (x±s) or median. Paired t-test was used to compare the expression level of WSB1 in primary HCC tissues and normal adjacent tissues. X^2 test was adopted to analyze the associations of the expression of WSB1 in HCC tissues with the clinicopathological features of patients. Overall survival (OS) and progression-free survival (PFS) of the patients was evaluated with Kaplan-Meier survival analysis and the intergroup differences were analyzed by Log-rank test. Cox proportional hazard regression models were respectively chosen for single factor analysis and multiple factor analysis of survival analysis. P<0.05 suggested that the difference was statistically significant.

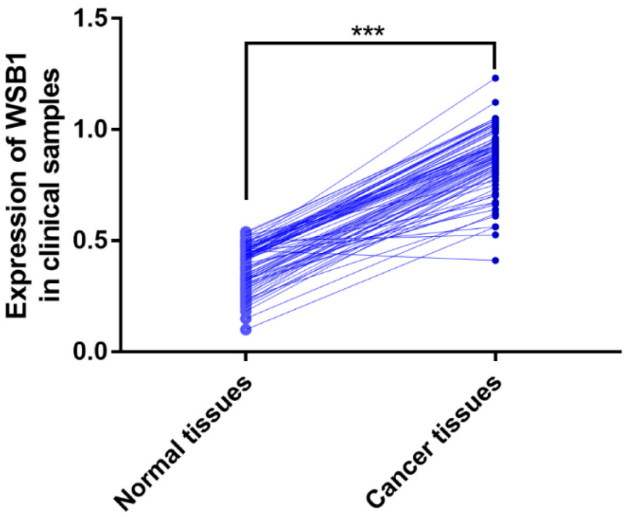

Figure 1. The expression level of WSB1 was measured in cancer tissues and para-cancer tissues by qRT-PCR. The results showed that the expression of WSB1 in HCC tissues was much higher than in normal tissues (***p<0.001).

Results

The expression of WSB1 in HCC tissues.

The expression of WSB1 in 80 pairs of organizations (cancer tissues and para-normal tissues) was detected as mentioned above. The results showed that the expression of WSB1 in HCC tissues was much higher than that of normal tissues, with statistically significant difference (p<0.001) (Figure 1).

The HCC samples were divided into WSB1-high-expression group (n=40) and WSB1-low-expression group (n=40) based on the median expression level of WSB1 from qRT-PCR result. The relationship between WSB1 expression and clinicopathological features of patients was further analyzed, and it was found that there was statistical difference between WSB1 expression and tumor differentiation, lymph node metastasis and clinical stage (Table 2).

Effect of WSB1 on the prognosis of patients with HCC

The correlation between WSB1 expression and survival time of patients was estimated using the Kaplan-Meier method. The results revealed that the patients with high expression of WSB1 had worse PFS and OS by comparing with WSB1-low-expressed patients (p<0.05; Figure 2), indicating that high expression of WSB1 in HCC patients predicted poor prognosis.

Univariate and multivariate analysis of WSB1 expression and HCC clinicopathological data

Univariate and multivariate Cox regressions analyses were used to analyze pathological parameter of HCC. Similar to the tumor number, the results revealed that tumor differentiation, lymph node metastasis, cirrhosis and clinical stage, expression of WSB1 was independent prognosis risk factor of patients with HCC (p<0.05). These findings suggested that WSB1 might play an important role in the progression of HCC (Table 3).

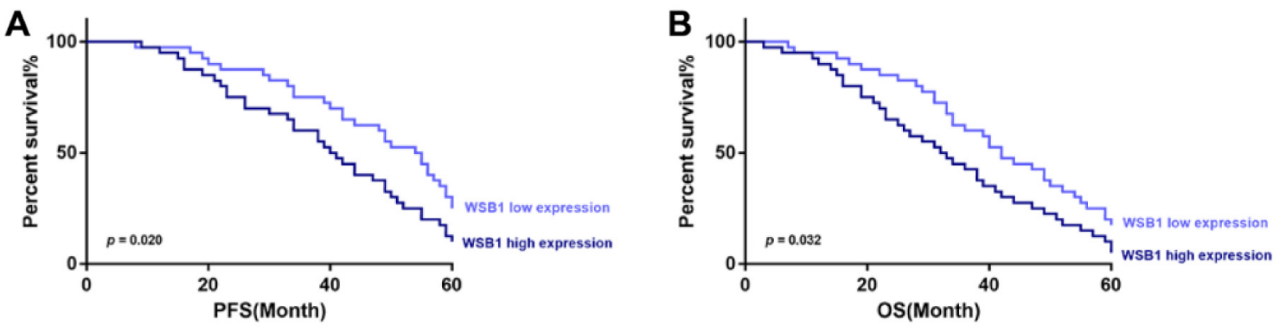

Figure 2. The relationship of WSB1 expression with progression-free survival (A) and overall survival (B) of HCC patients.

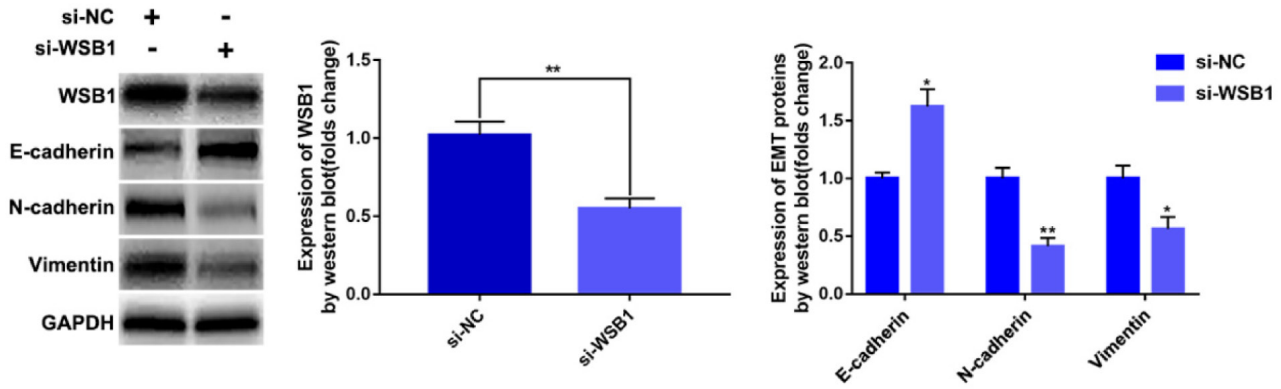

Figure 3. The protein expression of WSB1 and EMT-marker detected by Western blot (*p<0.05, **p<0.01).

Table 3. Univariate and multivariate analyses of postoperative prognosis in patients with HCC

Features	*Univariate analysis*		*Multivariate analysis*	
	Hazard ratio/CI (95%)	*p*	*Hazard ratio/CI (95%)*	*p*
Gender	0.883/0.623-1.464	0.733		
Age	1.273/0.700-1.404	0.458		
AFP level	1.637/0.937-2.585	0.077		
HBsAg level	1.480/0.894-2.265	0.133		
Tumor size	1.690/1.089-2.801	0.054		
Tumor number	1.821/1.274-3.116	0.040	1.774/1.093-2.884	0.048
Tumor differentiation	2.223/1.348-3.772	0.031	2.036/1.193-3.552	0.042
Lymph node metastasis	3.455/1.920-4.861	0.014	3.108/1.672-4.506	0.024
Cirrhosis	3.487/2.003-4.929	0.014	3.114/1.830-4.524	0.023
TNM stage	4.155/2.873-5.677	0.003	3.872/2.469-5.017	0.010
WSB1 expression level	1.994/1.370-3.752	0.037	1.780/1.281-3.472	0.048

Effect of WSB1 on the cell epithelial-mesenchymal transition (EMT)

In order to demonstrate the effect of WSB1 on EMT of HCC cells, EMT-related markers were detected after interfering intracellular WSB1 expression levels. As shown in Figure 3, the expression of mesenchymal phenotype protein (N-cadherin and Vimentin) was suppressed while the expression of epithelial phenotype protein (E-cadherin) resulted in recovery in HCC cells with low WSB1 expression, indicating the EMT was partially inhibited after si-WSB1 intervention.

Discussion

HCC, the most common primary malignant tumor in the liver, accounts for 70-85% of the total cases of liver cancer worldwide. The incidence rate of HCC has tripled over the past two decades, with 5-year survival rate of the patients still low [17]. Hence, an urgent need exists to seek for reliable diagnostic markers and effective therapeutic targets. Hypoxic microenvironment could accelerate the malignant progression of tumors by promoting the chemotherapy resistance, metastasis enhancement and metabolic disorders of the tumors. The role of WSB1 in tumor progression as a HIF protein and its biological functions were worth studying [18]. The analysis of histopathological sections from HCC patients revealed that WSB1 had a relatively high expression level in carcinoma tissues. Furthermore, the expression level of WSB1 and the clinical features of HCC patients were analyzed and the results suggested the WSB1 expression was correlated with tumor differentiation, lymph node metastasis and clinical stage, indicating that WSB1 might be involved in regulating the malignant progression of HCC.

EMT is referred to the process in which epithelial cells transform into mesenchymal cells under specific physiological or pathological conditions [19]. As it has been confirmed to play vital roles in tumor metastasis in recent years, EMT occurs in the initial stage of tumor metastasis, which will lead to loss of expression of linker molecules in epithelial cells such as E-cadherin, Claudin and Oc-

cludin, thereby destroying the cell polarity. Meanwhile, EMT could increase the expression of some lytic enzymes (including matrix metalloproteinases) that participate in the degradation of extracellular matrix and basement membrane and damage the histologic barrier against tumor cell invasion, thus facilitating the isolation and detachment of tumor cells from the primary tumor and triggering metastasis [20]. In the case of HCC, cancer promoters induce EMT of tumor cells by activating the upstream Notch [21] and Wnt [22] signaling pathways of EMT, or enhance EMT by modulating the activity of EMT-related transcription factors such as ZEB2 [23] and Snail [24]. This evidence shows that EMT was a crucial link of HCC metastasis. Therefore, how to restrain the incidence of EMT has also become an important task of studies on the mechanism of HCC metastasis. According to our *in vitro* experiments, EMT of HCC cells was reversed after silencing WSB1 expression, indicating that WSB1 was involved in the regulation of EMT process.

The limitation of this study was that only the expression of WSB1 in HCC tissues and the relevant clinical significance were preliminarily discussed. The biological functions and specific mechanisms of WSB1 in HCC invasion and migration require further exploration.

Conclusions

In conclusion, WSB1 might contribute to the development of HCC and serve as a clinical biomarker and a therapeutic target for HCC patients.

Conflict of interests

The authors declare no conflict of interests.

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