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Original Article

FOXS1 is a prognostic factor for hepatocellular carcinoma

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Abstract: Forkhead box (FOX) proteins control divergent and even opposing cell fate decision by regulating gene networks involved in cell cycle progression, proliferation and differentiation. However, the relation between FOXS1, a member of FOX proteins and neoplasms remains poorly defined. Therefore, this study aimed to examine the expression of FOXS1 in hepatocellular carcinoma (HCC) and explore the relation of FOXS1 expression with clinical features and prognosis of HCC. mRNA expression of FOXS1 was detected in 29 fresh frozen tissues of HCC patients. Immunohistochemistry was performed to detect the expression of FOXS1 in tumor samples from 90 HCC patients. The correlations between FOXS1 expression and clinicopathological factors and prognosis were evaluated. We found that FOXS1 expression was lower at both mRNA and protein levels in most HCC tissues than in normal liver tissues. FOXS1 expression was significantly correlated with tumor size, AJCC stage, and tumor differentiation. Moreover, decreased expression of FOXS1 was an important factor for predicting the prognosis of HCC patients. In conclusion, FOXS1 is a significant prognostic factor for HCC patients.

Keywords: Forkhead box, FOXS1, prognosis, hepatocellular carcinoma

Introduction

Forkhead box (FOX) proteins constitute an extended family of transcriptional regulators that are grouped based on the presence of an evolutionary conserved 'fork-head' or 'winged-helix' DNA-binding domain (DBD) [1]. FOX proteins control divergent and even opposing cell fate decision by regulating gene networks that are involved in cell cycle progression, proliferation and differentiation, as well as metabolism, senescence, survival and apoptosis [2]. Other than the conserved DBD domain, the members of FOX proteins vary greatly in sequence and function, acting as transcriptional activators or repressors to regulate developmental processes such as morphogenesis and differentiation [3].

A new forkhead family transcription factor FOXS1 has been identified to be localized on chromosome 20 [4, 5]. FOXS1 was also called FKHL18 and it has many similarities to the mouse gene Fkh3 [6, 7]. A more extensive sequence analysis showed that the mouse orthologue of human gene FKHL18 was indeed Fkh3 [8]. Recently, the nomenclature committee has approved FOXS1 as the new name for human and mouse gene FKHL18 and Fkh3. FOXS1 has been established as an early sensory neuronal marker [9]. While other members of FOX proteins such as FOXM1, FOXOs and FOXPs play important role in many aspects of neoplasms, the relation between FOXS1 and neoplasms remains poorly defined [10-13]. Therefore, this study aimed to examine the expression of FOXS1 in hepatocellular carcinoma (HCC) and explore the relation of FOXS1 expression with clinical features and prognosis of HCC. We found that FOXS1 expression was low in HCC tissues compared to peritumor tissues and its level could indicate the prognosis of HCC patients.

Materials and methods

Ethics statement

The study protocols followed the Declaration of Helsinki and were approved by Ethics

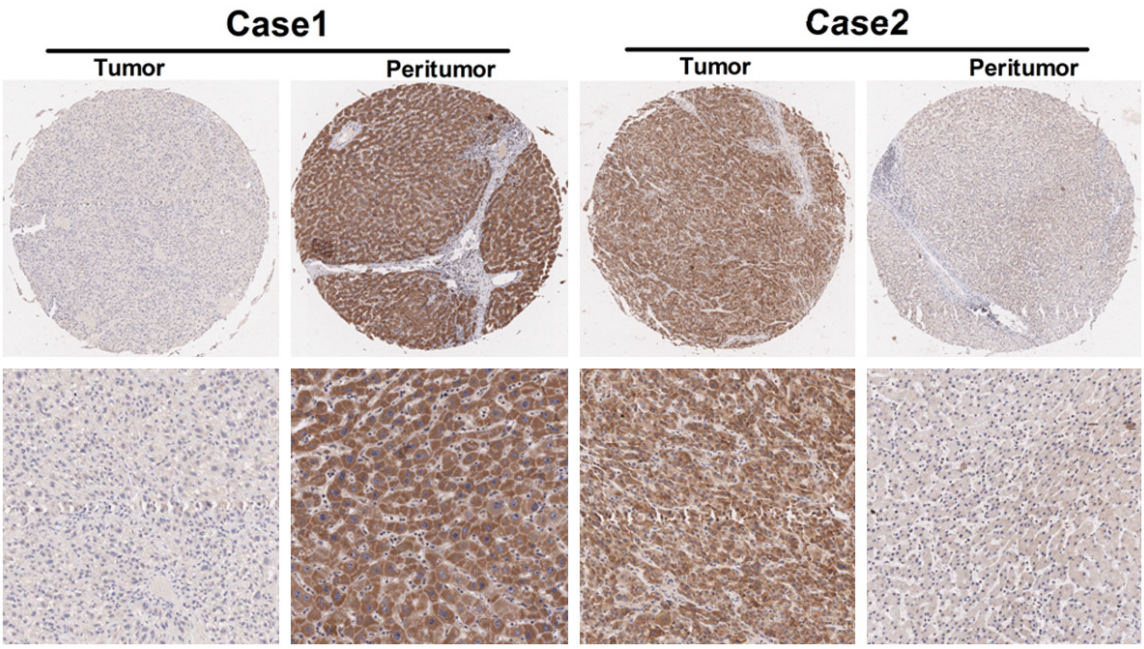

Figure 1. FOXS1 protein expression in tumor and peritumor tissues of HCC patients. In case 1, FOXS1 expression was lower in HCC than in normal tissues. In case 2, FOXS1 expression was higher in HCC than in normal tissues (magnification ×400).

Committees of the Second Affiliated Hospital, Kunming Medicine University. All patients provided informed consent.

Subjects

HCC tissues and matched peritumor tissues were collected from 90 patients with HCC who were hospitalized at the Second Affiliated Hospital of Kunming Medical University between 2012 and 2014. Patient medical records were reviewed to obtain clinical data about the age, gender, tumor size, AFP, HBsAg, vascular invasion, TNM stage (AJCC), tumor differentiation, and death or time of last follow-up. Patient survival was calculated from the day of surgery until death in months.

Immunohistochemistry

Immunohistochemistry was performed on HCC tissues and matched peritumor tissues by routine procedures with the incubation with primary antibody against FOXS1 (1:250; Sigma) overnight at 4°C. For negative control non-immune mouse immunoglobulin G was used instead of primary antibody. Sections were then incubated with HRP conjugated secondary antibody at room temperature for 30 min, and were visualized using DAB as chromogen for 5-10 min. Sections were scored semi-quantitatively as follows: (negative, -), 0% immunoreactive cells; (weak positive, +), ≤5% immunoreactive cells; (intermediate positive, ++), 5-50% immunoreactive cells; (high positive, +++), ≥50 immunoreactive cells. Cases with negative and weak positive were considered low expression and those with intermediate and high positive were considered high expression.

Quantitative real-time PCR

Total RNA was extracted from 29 paired tumor and peritumor tissues from HCC patients and cDNA was synthesized using RT kit (Bio-Rad, Hercules, CA, USA). PCR was performed on Applied Biosystems 7500 Fast Real-Time PCR System (ABI, Foster City, CA, USA) with the following primers: FOXS1 sense 5'-GAACTCTCGAAGGACCCAGC-3' and reverse 5'-TAAATCCCAAGAGGCCCTGC-3'; β-actin sense 5'-TGTCCACCTTCCAGCAGATG-3' and reverse 5'-TGTCACCTTCACCGTTCCAG-3'.

Statistical analysis

The data were analyzed using SPSS version 17.0. χ^2 test was used to evaluate any potential association between FOXS1 expression and

Table 1. Correlation of FOXS1 expression and clinicopathological characteristics of HCC patients

Variable	Low	High	*P* value
In general	55	35	
Gender			
Male	46	32	0.545
Female	9	3	
Age (years)			
≤50	18	9	0.491
>50	37	26	
Tumor size (cm)			
≤5	33	8	0.001
>5	22	27	
AFP (ng/ml)			
≤400	28	19	0.566
>400	27	16	
HBsAg			
Positive	48	32	0.488
Negative	7	3	
Anti-HCV			
Positive	4	1	0.584
Negative	51	34	
Vascular invasion			
Yes	14	7	0.576
No	41	28	
AJCC stage			
I-II	30	12	0.038
III-IV	25	23	
Tumor differentiation			
I-II	38	16	0.026
III-IV	17	19	
Liver cirrhosis			
Yes	30	19	0.547
No	25	16	

clinicopathologic parameters. Overall survival was calculated with the Kaplan-Meier method and the statistical difference between survival curves was determined with the log-rank test. P<0.05 indicated significant difference.

Results

FOXS1 expression in HCC tissue samples

Immunohistochemistry was performed to detect the expression of FOXS1 in tumor tissues from 90 HCC patients. The expression of FOXS1 was generally localized in the cytoplasm of HCC cells (**Figure 1**). Based on relative expression levels of FOXS1 in tumor and peritumor tissues, 90 patients with HCC were divided into two subgroups. As shown in **Table 1**, 55 patients (low-expression group) showed low expression level of FOXS1 in carcinoma compared with adjacent peritumor liver tissues, and 35 patients (high-expression group) showed high expression level of FOXS1.

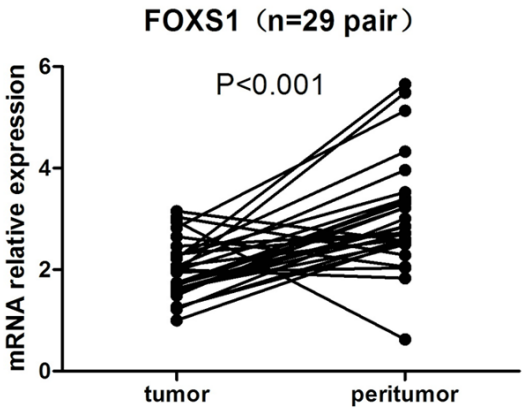

Figure 2. The mRNA level of FOXS1 in HCC tissue samples. mRNA level of FOXS1 was lower in tumor tissues compared to peritumor tissues (P=0.001).

Furthermore, we selected 29 fresh frozen tissues of HCC patients and detected the mRNA level of FOXS1 in these samples. Real-time PCR analysis showed that among the 29 HCC patients, mRNA level of FOXS1 was significantly lower in tumor tissues than in peritumor tissues (P=0.001, **Figure 2**).

Association between FOXS1 expression and clinicopathological features of HCC patients

The correlations between the expression level of FOXS1 and patient characteristics such as the gender, age, tumor size, tumor differentiation, TNM stage were investigated. FOXS1 protein expression was significantly correlated with tumor size, AJCC stage, tumor differentiation (P=0.001, 0.038 and 0.026, respectively) (**Table 1**). However, there was no association between FOXS1 expression and the age, gender, AFP, vascular invasion, and liver cirrhosis.

Association between FOXS1 and the prognosis of HCC patients

Finally we performed survival analysis. Patients in FOXS1 low expression group had worse overall survival than FOXS1 high expression group (**Figure 3**). The 3-year and 5-year overall sur-

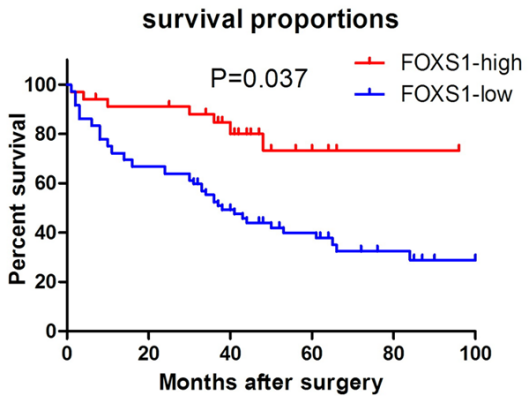

Figure 3. Kaplan-Meier curve analysis of overall survival of HCC patients. The low expression of FOXS1 predicted poor prognosis in HCC patients (P=0.037).

Table 2. Relationship between FOXS1 expression and survival of HCC patients

Survival rate	FOXS1 expression		*P* value
	Low	High	
1-year overall survival (%)	75.0±7.4	91.0±5.9	0.037
3-year overall survival (%)	55.5±7.9	83.0±6.8	
5-year overall survival (%)	37.5±3.6	72.2±7.4	

vival rate were much worse for FOXS1-low than FOXS1-high expression group. The 5-year overall survival rate for low expression group and high expression group was 37.5% and 72.2% (P=0.037), respectively (**Table 2**). FOXS1 low expression in HCC predicts a poor prognosis.

Discussion

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide comprising diverse and histologically distinct hepatic neoplasms. HCC is among the most lethal liver cancers, representing 70-90% of primary liver cancers [14, 15]. Tumorigenesis of HCC is a multistep process of oncogenic activation and tumor suppressor inactivation [16]. However, our understanding of genetic alterations underlying the initiation and development of HCC is still limited.

Forkhead proteins are characterized by a conserved DBD domain, known as the FH (forkhead) domain or WHD (winged-helix domain) [17, 18]. The unique DBD domain is composed of about 100 amino acids which are evolutionarily conserved and essential for DNA recognition [19]. Several three-dimensional structures of DBD, including FoxA3, Foxd3, FOXO4, FOXC2, and FOXK1, have been determined using x-ray crystallography or NMR spectroscopy [20-25]. The members of Fox family are known to perform important regulatory function in cell proliferation, transformation, differentiation, and longevity [26-29]. Many members of FOX family such as FOXM1, FOXOs and FOXPs have been implicated in breast cancer, lung cancer, ovarian cancer, liver cancer, lymphoma [30-32]. FOXS1, as a new member of FOX family, was established as an early sensory neuronal marker [9], but the relation between FOXS1 and neoplasms remains unknown.

In this study, we measured mRNA level of FOXS1 in fresh frozen HCC and peritumor tissues of 29 HCC patients. The expression of FOXS1 was decreased in tumor tissues compared to peritumor tissues. Furthermore, immunohistochemical staining showed that the expression of FOXS1 was weak in HCC tissues compared to peritumor tissues. Correlation analysis showed that low expression of FOXS1 was correlated with poor prognosis and short survival of HCC patients. These data indicate that loss of FOXS1 may promote the development of HCC.

During early embryonic development, FOXS1 expression is present in all sensory nervous systems regardless of cellular origin, but is not detected in neural crest-derived cell types [9]. Thus FOXS1 may play important function in development and differentiation. According to clinical pathologic features, we supposed that the downregulation of FOXS1 in HCC tissues may promote the proliferation of HCC cells. Further studies are needed to explore the role of FOXS1 in HCC.

In conclusion, for the first time we reported the status of FOXS1 expression in HCC. Decreased levels of FOXS1 mRNA and protein were observed in the majority of HCC tumors compared to non-cancerous peritumor tissues. Moreover, low expression of FOXS1 is correlated with poor prognosis of HCC patients. Thus FOXS1 is a promising prognostic factor for HCC.

Disclosure of conflict of interest

None.

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