


# Serum SALL4 as a predictive biomarker for the prognosis of patients with hepatocellular carcinoma who underwent nonsurgical treatment

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## Abstract

To investigate the role of serum spalt like transcription factor 4 (SALL4) in the hepatocellular carcinoma (HCC) patients with nonsurgical treatment. Serum samples were collected from 101 patients with HCC without surgical treatment, then the SALL4 was detected by enzyme linked immunosorbent assay. According to subsequent treatment, patients were divided into 2 groups, best supportive care (BSC) (58 cases) and nonsurgical anticancer treatment (NSAT) (48 cases). Kaplan–Meier survival analysis and Cox multivariate regression analysis were applied to evaluate the relationship between SALL4 and survival time of 2 groups. In BSC group, there was no significant difference of the survival time between 2 groups (SALL4 < 800 ng/mL or SALL4 ≥ 800 ng/mL) ( $P = .339$ ). In NSAT group, the survival time of patients with low SALL4 concentration was significantly longer than patients with high SALL4 concentration ( $P = .005$ ). SALL4 have no predictive effect in BSC patients with HCC. But for patients received NSAT, low SALL4 concentration in serum may indicate longer survival.

**Abbreviations:** AFP = alpha-fetoprotein, BCLC = Barcelona Clinic Liver Cancer, BSC = best supportive care, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, NSAT = nonsurgical anticancer treatment, OS = overall survival, SALL4 = spalt like transcription factor 4, TACE = transcatheter arterial chemoembolization.

**Keywords:** biomarkers, hepatocellular carcinoma, prognosis, serum, spalt like transcription factor 4

## 1. Introduction

Hepatocellular carcinoma (HCC), as the most frequent pathological type of primary liver cancer, has been considered as the sixth most commonly cancer worldwide and fourth in China.<sup>[1,2]</sup> Annually, the new incidence and mortality of HCC in China account for over 50% of the world.<sup>[3]</sup> Surgery is a kind of standard treatment for patient with Barcelona Clinic Liver Cancer (BCLC) stage A. Nonsurgical anticancer treatment (NSAT), such as transcatheter arterial chemoembolization (TACE) and targeted therapy (sorafenib or lenvatinib) are used for patients with stage B or C.<sup>[4]</sup>

There were plenty of studies on the diagnostic, prognostic and predictive biomarkers for HCC. Alpha-fetoprotein (AFP), micro ribonucleic acid, long noncoding ribonucleic acid and circulating tumor cells have been confirmed as effective biomarkers in the early detection of cancer and surveillance of recurrence after surgery.<sup>[5–7]</sup> However, the investigation on prognostic and predictive biomarker was not satisfactory. AFP remains the most common used biomarker in the past half century. Elevated AFP

serum levels are related with poor prognosis of patients underwent NSAT.<sup>[7]</sup> But AFP is not only affected by HCC, but also by the liver cirrhosis caused by hepatitis B virus (HBV).<sup>[7]</sup> Its use may not be suitable for patients with HCC combined with HBV and cirrhosis, which is common in China.

The spalt like transcription factor 4 (SALL4), a zinc finger transcriptional activator, have important developmental roles during organogenesis.<sup>[8]</sup> At first, SALL4 was found to be related to the occurrence and development of hematological malignancies.<sup>[9,10]</sup> Subsequently, SALL4 expression has been reported in various solid malignancies. The overexpression of SALL4 gene was found to cause a series overexpression of genes related to cell proliferation and metastasis in HCC.<sup>[11]</sup> Patients who had SALL4-immunopositive surgical specimens, had a high probability of recurrence after operation and a short survival time.<sup>[12,13]</sup> In addition to histological immunopositivity, patients with high serum SALL4 level also showed a poor prognosis.<sup>[14]</sup> Furthermore, the expression status of SALL4 in surgical specimens were not correlation with liver function or staging.<sup>[12]</sup> Therefore, it has been considered that SALL4 could

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predict the therapeutic effect on HCC. However, the evidence of this conclusion is mainly from the data of patients after surgery. For those who are treated by NSAT, no clinical data have been found. With aim of identifying the relationship between serum SALL4 level and survival time of patients with HCC treated by NSAT, a previous study with a limited sample size conducted in 2017, showed that the survival time of low serum SALL4 level patients were longer than that of high level, which consistent with the results from surgical patients. However, these results were not observed in patients without anticancer treatment.<sup>[15]</sup>

Therefore, the current study was conducted to further evaluate the association between SALL4 and the prognosis of patients underwent nonsurgical treatment.

## 2. Methods

### 2.1. Study subjects

The patients diagnosed with HCC without surgery treatment were included. The inclusion criteria were: patients were unsuitable for surgery or refused surgery; patients  $\geq 18$  years old; the liver function was class A or B assessed by Child-Pugh classification and performance status score were between 0 and 2 according to Eastern Cooperative Oncology Group criteria; extrahepatic metastases were not found. Exclusion criteria included: insufficient blood test data for the judgement of liver function; neither computerized tomography nor magnetic resonance imaging scan found out the size and amount of the tumor nodules; patients cannot complete at least once follow up in first 3 months after enrollment.

The protocol was approved by the medical ethics committee and was conducted in accordance with the principles of the Declaration of Helsinki. Before the sample collection, written consent for blood sample and related medical information donation for research purposes was obtained from all patient.

### 2.2. Diagnostic criteria

Histological diagnosis was made according to World Health Organization criteria. Clinical diagnosis of HCC was made according to diagnosis standard of China.<sup>[16]</sup> Postoperative recurrent were confirmed by intravenous contrast-enhanced computerized tomography or magnetic resonance imaging.

### 2.3. Serum preparation

Blood samples were collected in fasting condition before any therapeutic procedures. After 1 hour standing at 4°C, specimens were centrifuged at 3000  $\times$  g for 5 minutes. Then serum was aliquoted to EP tube and stored at -80°C until analysis.

### 2.4. Enzyme linked immunosorbent assay

Serum samples were removed from -80°C refrigerator and dissolved completely at room temperature. Specimen processing followed instructions of the test kit (Human SALL4 Enzyme linked immunosorbent assay Kit, Shanghai Enzyme-linked Biotechnology Co. Ltd) and 450nm optical density values were obtained by enzyme-labeling measuring instrument (muLTISKAN FC, Thermo Fisher Scientific Inc). The actual SALL4 concentration of every serum sample could be calculated according to standard curve.

### 2.5. Clinical data collection and follow-up

Patients would be follow-up once a month in the first 3 months, then per quarter until lost or death. The overall survival (OS) time was identified the date from enrollment to final follow-up or death.

### 2.6. Statistical analysis

All analyses were performed with SPSS 22.0 (International Business Machines Corporation, Armonk, NY). The normal distribution of measurement data was evaluated by Kolmogorov-Smirnov test. For the comparison of normal distribution data, student's *t* test was used, while for the comparison of nonnormal distribution data, the Mann-Whitney U rank sum test in the nonparametric test was used. Counting data was compared using  $\chi^2$  test. Cox regression was used for multivariate analysis. The Kaplan-Meier method was used for univariate analysis. The age, Eastern Cooperative Oncology Group performance status, vascular invasion, Child-Pugh class, AFP, SALL4 were evaluated in multivariate analysis and univariate analysis. The difference of survival time of different types of patients was analyzed by the Log-Rank test.  $P < .05$  indicates the difference is statistically significant.

## 3. Results

### 3.1. Basic characteristics

One hundred and one patients were recruited in this study between April 1, 2017 and August 30, 2019. According to subsequent treatment, 101 patients were divided into 2 groups, best supportive care (BSC) and NSAT. Based on our initial research, each group is divided into high SALL4 group (SALL4-H) and low SALL4 (SALL4-L) group according the threshold 800 ng/mL. Last follow-up date was August 31, 2020. Median follow-up time is 8 months. Fifty-six patients were dead during the follow up (Table 1).

### 3.2. Analysis of prognostic factors

The median OS of BSC and NSAT were 3.9 months and 9.5 months, respectively ( $P = .008$ ). Such significant difference put the focus of attention on NSAT group, while the other group serviced only as a reference. Multivariate survival analysis found different distribution of prognostic factors in 2 groups (Table 2). In the BSC group, score of performance status was found to be statistically significant by multivariate analysis, while AFP was found by univariate analysis. In the NSAT group, Child-Pugh grade have statistical significance in multivariate analysis ( $P = .018$ ), but not in univariate analysis ( $P = .160$ ).

SALL4 and AFP showed significant prognostic value in multivariate and univariate analysis in NSAT group (Table 2). In NSAT group, the *P* value for AFP was 0.005 and 0.001 in multivariate and univariate analysis respectively, while is was 0.001 and 0.005 for SALL4. It should be emphasized that when the threshold of AFP was adjusted from 400 to 5000  $\mu$ g/mL, the results of univariate analysis of AFP in the BSC, the univariate analysis and multivariate analysis in NSAT are consistent with foregoing conclusions about AFP. However, it was not found in the process of adjusting SALL4 threshold. In other words, SALL4 has a threshold of 800 ng/mL, while AFP does not.

### 3.3. The survival of the different groups

The survival curves of high AFP ( $\geq 400$  ng/mL) patients and low AFP ( $< 400$  ng/mL) patients in BSC group and NSAT group were significantly different, while the difference of survival curves of SALL4 could only be found in NSAT group (Fig. 1). In NSAT group, median OS of SALL4-H group and SALL4-L group were 5.3 and 12.7 m, respectively ( $P = .005$ ). In BSC group, median OS of SALL4-H group and SALL4-L group were 4.8 and 3.0 m, respectively ( $P = .339$ ).

### 3.4. Relationship between clinical characteristics and SALL4

To exclude interference from therapeutic factors, we analyzed the data of 42 newly diagnosed patients in this study. The

**Table 1**  
**Clinical characteristics.**

	BSC (n = 53)			NSAT (n = 48)			P
	SALL4-L	SALL4-H	P	SALL4-L	SALL4-H	P	
n	26	27		23	25		
age	60.58 ± 11.83	55.74 ± 13.48	.172	61.96 ± 13.80	57.44 ± 11.57	.213	.555
Sex							.302
Male	24	26	.610	21	21	.668	
Female	2	1		2	4		
AFP							.111
≥400 ng/mL	9	9	1.000	8	16	.082	
<400 ng/mL	17	18		15	9		
Child-Pugh class							.005*
A	10	9	.779	13	18	.367	
B	16	18		10	7		
ECOG performance status							.011*
0	5	4	.166	7	4	.468	
1	3	9		10	12		
2	18	14		6	9		
BCLC stage							.092
A	5	1	.088	3	1	.506	
B	0	2		4	4		
C	21	24		16	20		
Hepatitis virus infection							.565
HBV	21	27	.023*	19	24	.286	
HCV	0	0		1	0		
None	5	0		3	1		
Vascular invasion							.308
Yes	11	11	1.000	6	9	.542	
No	15	16		17	16		
Previous therapy							.179
Surgery	4	3	.601	1	1	.758	
TACE and/or ablation	9	13		14	13		
Targeted therapy	0	0		0	1		
None	13	11		8	10		
Subsequent treatment	/	/					
TACE alone or combined with other treatments	/	/		22	20	.221	
Targeted treatment	/	/		1	3		
Immunotherapy	/	/		0	2		

\* Significant difference.

AFP = Alpha-fetoprotein, BCLC = Barcelona Clinic Liver Cancer, BSC = best supportive care, ECOG = Eastern Cooperative Oncology Group, HBV = hepatitis B virus, HCV = hepatitis C virus, NSAT = nonsurgical anticancer treatment, SALL4 = spalt like transcription factor 4, TACE = transcatheter arterial chemoembolization.

**Table 2**  
**Multivariate and univariate analysis of risk factors.**

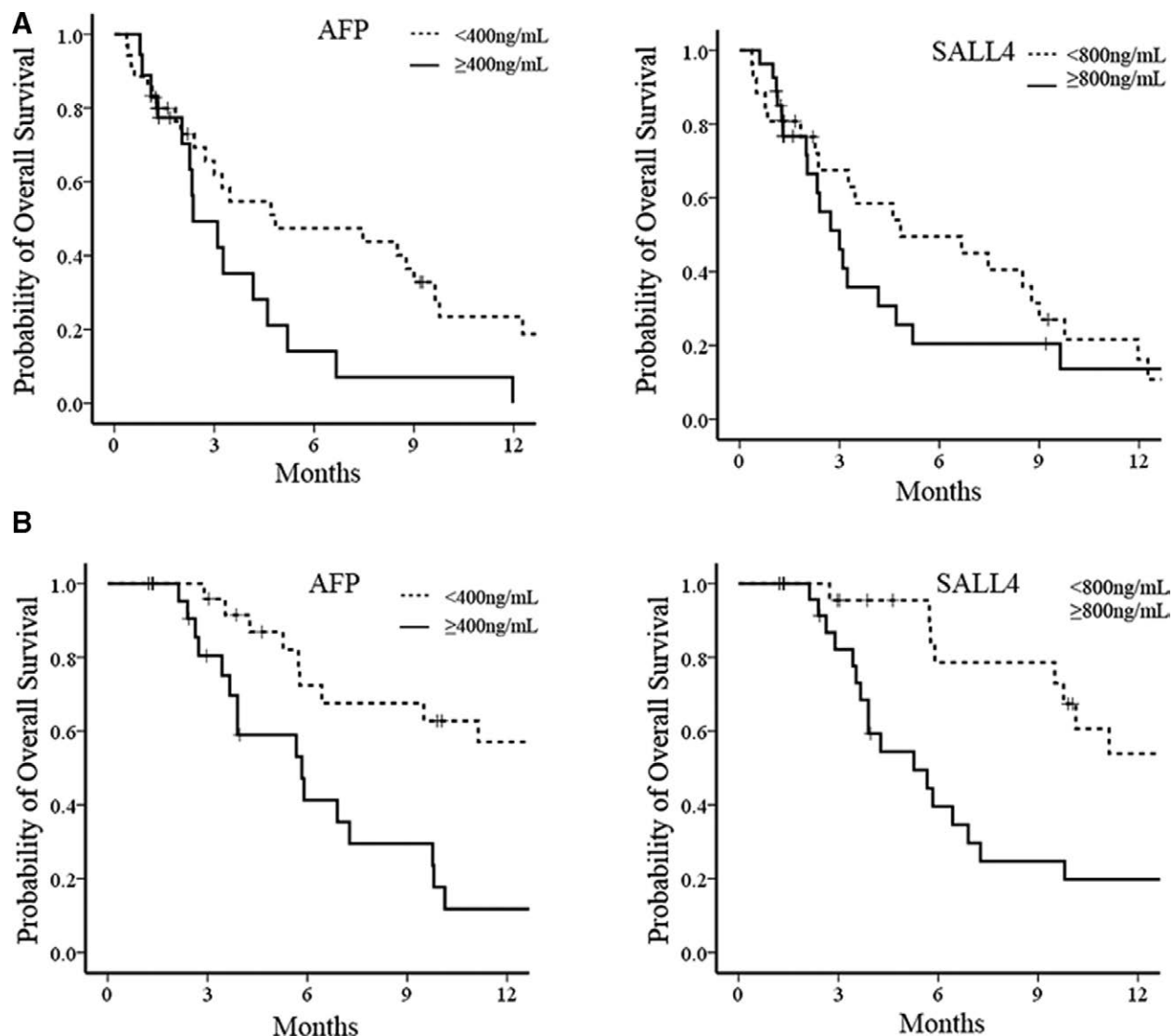
	BSC			NSAT		
	n	P		n	P	
		Multi*	Uni†		Multi*	Uni†
Age		.851	.433		.155	.668
<65	38			29		
≥65	15			19		
ECOG performance status		.022‡	.056		.747	.283
0	9			11		
1	12			22		
2	32			15		
Vascular invasion		.669	.212		.761	.285
Yes	22			15		
No	31			33		
Child-Pugh class		.996	.114		.018‡	.160
A	20			31		
B	33			17		
AFP		.157	.034‡		.005‡	.001‡
<400 ng/mL	35			24		
≥400 ng/mL	18			24		
SALL4		.135	.339		.001‡	.005‡
<800 ng/mL	26			23		
≥800 ng/mL	27			25		

\*Multi: multivariate analysis.

†Uni: univariate analysis.

‡Statically significant.

AFP = Alpha-fetoprotein, BSC = best supportive care, ECOG = Eastern Cooperative Oncology Group, NSAT = nonsurgical anticancer treatment, SALL4 = spalt like transcription factor 4.



**Figure 1.** Survival curves of patients in 2 groups (A, BSC; B, NSAT). The survival curves of high AFP ( $\geq 400$  ng/mL) patients and low AFP ( $< 400$  ng/mL) patients in BSC group and NSAT group are significantly different, while the difference of SALL4 can only be found in NSAT. AFP = alpha-fetoprotein, BSC = best supportive care, NSAT = nonsurgical anticancer treatment, SALL4 = spalt like transcription factor 4.

clinical characteristics, including age, BCLC stages, Child-Pugh grade, HBV infection, cirrhosis and vascular invasion were not related to SALL4 level.

#### 4. Discussion

Intermediate and advanced stages (BCLC stage B and C) are usually found at the time of diagnosis due to the absence of symptoms in early stages of HCC, which means most patients can only receive NSAT.<sup>[17]</sup> Our current study showed in NSAT group, the survival time of patients with low SALL4 concentration was longer than patients with high SALL4 concentration.

AFP as an indicator of tumor burden has been used as a prognostic factor, but not for predicting the response to treatment. In addition, its concentration is interfered by hepatitis infection and cirrhosis. Furthermore, a small percentage of patients with HCC had normal levels of AFP.<sup>[18]</sup> Although 400 ng/mL is commonly used as threshold, no robust data exist has defined its cut-off value at treatment modification.<sup>[17]</sup> Previous study also found the adjustable range of AFP threshold could be very large to produce statistically significant differences of survival curves,<sup>[15]</sup> which was in accordance with our results. Yong et al<sup>[12]</sup> found

the expression status of SALL4 in surgical specimens was not correlated with liver function or staging, while we found the similar association with SALL4 in serum. In addition, SALL4 was not associated with HBV infection, cirrhosis and vascular invasion in this study. It seems to be an independent factor. Our study indicated that low SALL4 level in serum predicted longer survival in patients with NSAT, while it was not found in patients with BSC. It could be considered that AFP concentration was a result of HCC and may only be used as a prognostic reference, but SALL4 may be 1 of the causes of heterogeneity of HCC, thus it might be a potential predictive factor for HCC treatment.

Down-regulation of SALL4 can decrease tumorigenicity and reversing the aggressive progenitor-like phenotype.<sup>[12]</sup> Downstream targets of SALL4 involve vascular endothelial growth factor A (VEGFA) which promotes vascular regeneration.<sup>[19]</sup> The mechanism of TACE and targeted agents for HCC is mainly through anti-angiogenic effects.<sup>[20–22]</sup> It may explain the result that patients with high SALL4 expression have a poorer response to treatment. However, it is not reasonable to explain why there is no difference in the survival for BSC patients, as patients with high SALL4 expression should have



a worse prognosis theoretically. Gene expression pattern analysis showed high-SALL4 HCC clustered with hepatic progenitor cells.<sup>[12]</sup> SALL4 plays an essential role in the regulation network of stemness state, which maintains cell renewal and pluripotent capacities.<sup>[11,23–25]</sup> SALL4 was found to directly connect with the epigenetic modulator nucleosome remodeling and deacetylase complex, thereby possible affecting the histone modification associated with stemness and SALL4 was reported to play a role in controlling histone deacetylase activity and 8 contributing to the maintenance of HCC with stem cell features.<sup>[11]</sup> In other words, SALL4 could be considered as an indicator of the stemness of tumor cell. This tumor stemness may be activated under certain external pressure. Therefore, we hypothesize that the function of SALL4 was activated by the stimulation of treatment to maintain tumor cell regeneration, while in the untreated tumor was not. In addition to TACE and targeted therapies, immunotherapy had made encouraging breakthroughs in the treatment of HCC.<sup>[26]</sup> SALL4 was a transcription repressor of the phosphatase and tensin homologue protein (PTEN), while the dysfunction or mutation of PTEN will lead to resistance to immunotherapy, suggesting that SALL4 may also be a predictive marker for the effect of immunotherapy.<sup>[27]</sup> Therefore, SALL4 is worthy for further study and the development of relative targeted drugs to improve the therapeutic effect.

Our study firstly evaluated the relationship between serum SALL4 level and the prognosis of HCC patients underwent nonsurgical treatment. There were also some limitations in this study. The sample size of this study was relatively small. The patients were included from 1 center. Further study with multi-center and large sample size was still needed.

In conclusion, SALL4 has no predictive effective in HCC patients with best supportive care. But for the patients received NSAT, low SALL4 may indicate longer survival. This may be attributed to that SALL4 represents the tumor stemness of HCC. Our results showed that SALL4 was be a reliable biomarker on the prognosis of HCC, which might help to support the further improvement of treatment on HCC.

## Author contributions

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**Writing – review & editing:** Zhixian Chen, Huifeng Wu, Simin Jiang, Xingli Liu, Meihua Luo, Yawei Yuan.

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