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High expression of Anxa2 and Stat3 promote progression of hepatocellular carcinoma and predict poor prognosis

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ABSTRACT

Aim: To elucidate whether the interaction between Anxa2 and Stat3 could promote the progression of hepatocellular carcinoma (HCC) and that high co-expression of Anxa2 and Stat3 could predict poor prognosis in HCC patients.

Methods: We investigated Anxa2 and Stat3 expression using Western blot analysis in 4 HCC and adjacent nontumor tissues and using immunohistochemistry in 100 patients' paraffin sections. Then we assessed the expression of Stat3, Anxa2 and co-expression of Stat3 and Anxa2 with relevant clinical pathological parameters and their prognostic value in HCC patients. The recurrence and overall survival rates were estimated using the Kaplan-Meier method and compared with the log-rank test. The prognostic analysis was carried out with univariate and multivariate Cox regressions models.

Results: The incidence of high Stat3 expression in HCC tissues (35%) was significantly higher than that in non-HCC tissues (8%) ($P < 0.001$). The same result was observed in Anxa2 ($P < 0.001$). Also, the overexpression of Stat3 or Anxa2 showed a significant relationship with the recurrence of the 100 HCC patients ($P = 0.012$; $P = 0.003$). Additionally, tumor size > 3 cm in diameter, multiple tumor number, and the presence of microvascular tumor thrombus were also significantly associated with recurrence in 100 patients. Then, all enrolled patients were divided into four groups according to IHC score of Stat3 and Anxa2, and the results indicated a significant difference in recurrence time between the subgroups ($P < 0.001$). What's more, co-highexpression of Stat3 and Anxa2 was related to the presence of microvascular tumor thrombus ($P = 0.003$) and poor tumor differentiation ($P < 0.001$), but not relevant with other clinical features (All $P > 0.05$).

Conclusion: The expression of Stat3, Anxa2, or co-high-expression of the two proteins was associated with HCC recurrence and survival.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common primary malignancies and the third leading cause of cancer-related deaths worldwide [1]. The high mortality and poor prognosis of HCC are mainly due to the difficulty of early detection since there are no explicit serum biomarkers except alpha-fetoprotein (AFP) [2,3]. Additionally, detection of serum AFP is limited in sensitivity and specificity particularly in early diagnosis of small HCCs [4]. The current treatment of HCC includes surgery, radiotherapy and chemotherapy [5]. However, these treatments are not ideal for advanced and metastatic HCC patients. In recent decades, targeted therapy against malignant tumors has become a novel and effective therapeutic tool in comprehensive cancer treatment and has been widely used in clinical patients [6]. Various new targeted drugs have been used to treat patients, such as rituximab for NHL patients who are CD20 positive and EGFR-TKIs (epidermal growth factor receptor-tyrosine kinase inhibitors) for NSCLC (non-small cell lung cancer) patients [7,8]. Nevertheless, several relevant studies

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Table 1
Clinical characteristics of hepatocellular carcinoma patients.

Characteristics	No. (%)	Values
Number of patients	100(100)	
Male/female ratio	82:18	
Median age, years (range)		53(35–74)
Tumor size (≤3 cm vs. > 3 cm)	45:55	
Tumor number (single vs. multiple)	82:18	
Microvascular tumor thrombus (absent vs. present)	75:25	
Liver cirrhosis (yes vs. no)	78:22	
AFP (≤400 ng/ml vs. > 400 ng/ml)	67:33	
Envelope invasion (yes vs. no)	44:56	
Tumor differentiation (well-differentiated vs. moderate vs. poor)	23:48:29	
HBV DNA level (< 4 log_{10} copies/mL vs. ≥ 4 log_{10} copies/mL)	71:29	
Median baseline biochemistry and hematology		
Albumin, g/L		35.5(21.3–50.6)
Total bilirubin, μmol/L		18.4(6.6–32.8)
Prothrombin time, s		13.4(10.2–15.6)
Okuda stage (I vs. II)	93:7	
Child-Tuecotte-pugh grade (A vs. B)	93:7	
Anxa2 (low vs. high)	59:41	
Stat3 (low vs. high)	65:35	
Recurrence vs. not	59:41	
Median time of recurrence (months)		41(4–69)
Median time of survival (months)		47(4–73)

AFP: serum alpha-fetoprotein; HBV DNA: Hepatitis B virus desoxyribonucleic acid; Anxa2: Annexin A2; Stat3: Signal transducer and activator of transcription 3.

Table 2
Clinical details of the selected four patients.

Clinical details	patient A	patient B	patient C	patient D
Sex	Male	Male	Female	Female
Age	53	47	64	59
Tumor size (cm)	6*5	3.5*3.4	4.5*3	2*1.5
Tumor number	1	1	2	1
Microvascular tumor thrombus	present	present	present	absent
Liver cirrhosis	absent	present	present	absent
AFP (ng/ml)	5.91	4.55	2456	14.76
Envelope invasion	yes	no	yes	no
Tumor differentiation	poor	high	poor	high
HBV DNA level	< 1*10^3	< 1*10^3	< 1*10^3	< 1*10^3
Albumin,g/L	34.7	33	44	38.7
Total bilirubin,μmol/L	19.4	16.8	27.4	11
Prothrombin time,s	13.4	14.6	14	13.2
Okuda stage	I	I	I	I
Child-Tuecotte-pugh grade	A	A	A	A

Table 3
Expression of Stat3/Anxa2 in cancerous and surrounding tissues.

Group	n	Stat3		*P* value	Anxa2		*P* value
		high	Low/neg.		high	Low/neg.	
HCC	100	35	65	< 0.001	41	59	< 0.001
No-HCC	100	8	92		12	88	

HCC: hepatocellular carcinoma; No-HCC: adjacent nontumor tissues; neg: negative.

have reported the use of these targeted drugs in the clinical treatment of HCC. Therefore, there is an urgent need to identify new biomarkers and possible targets for the early diagnosis and treatment of HCC patients.

Signal transducer and activator of transcription 3 (STAT3) signaling and Annexin A2 (Anxa2) participate in the oncogenesis and progression of numerous malignant tumors [9,10]. Stat3 exists in the cytoplasm while activated Stat3 (phosphorylated Stat3) can translocate into the nucleus and play a critical role in cell proliferation, metastasis, angiogenesis, host immune evasion and drug resistance [11–13]. Additionally, Anxa2 which is a member of annexin family consisting of up

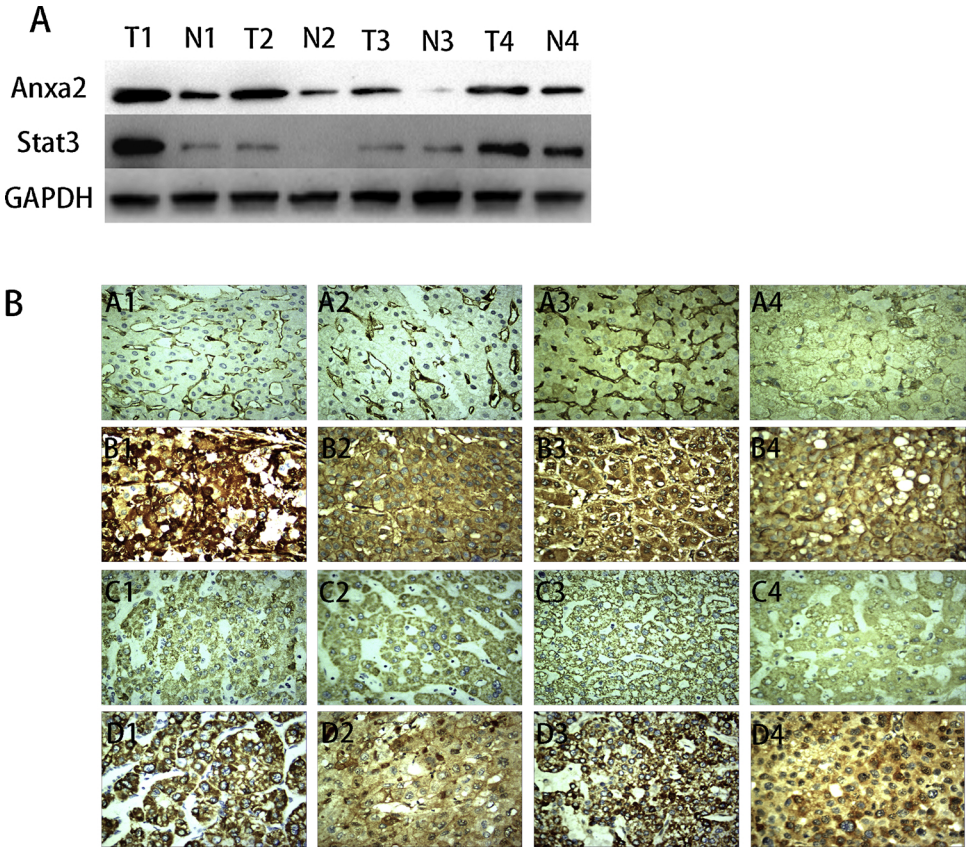

Fig. 1. The Western blot and immunohistochemical results of these four patients were as follows. (A) Anxa2 and Stat3 protein expressions in HCC and PCL tissues. A representative western blot image indicating Anxa2 and Stat3 protein expression in four paired samples of HCC and PCL tissues. GAPDH served as a control for protein loading and integrity. T1: the HCC tissues in patient A; N1: the adjacent nontumor tissues in patient A; T2: the HCC tissues in patient B; N2: the adjacent nontumor tissues in patient B; T3: the HCC tissues in patient C; N3: the adjacent nontumor tissues in patient C; T4: the HCC tissues in patient D; N4: the adjacent nontumor tissues in patient D. (B) the immunohistochemical results of these 4 patients were also shown in Fig. 1. A1-A4: Anxa2 expression of the four patients' adjacent nontumor tissues. B1-B4: Anxa2 expression of the four patients' HCC tissues. C1-C4: Stat3 expression of the four patients' adjacent nontumor tissues. D1-D4: Stat3 expression of the four patients' HCC tissues.

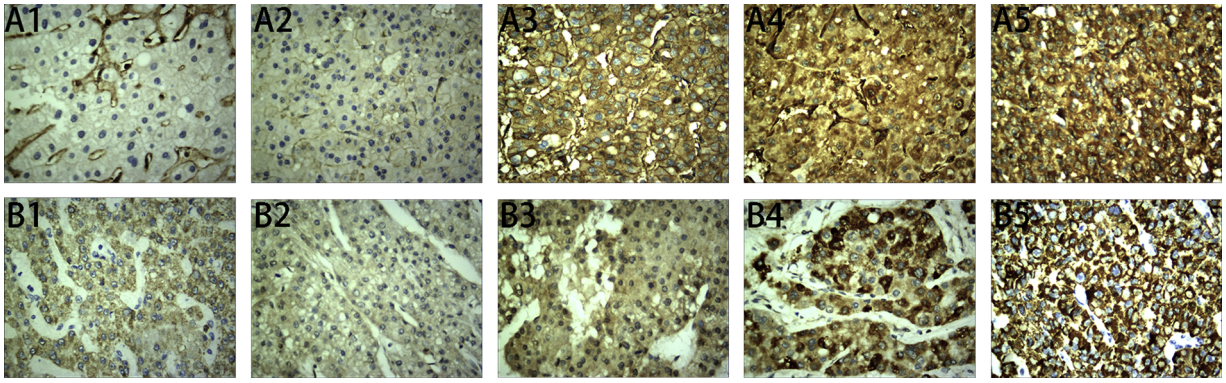

Fig. 2. Analysis of hepatic Anxa2 and Stat3 expression and its cellular distribution by immunohistochemistry: A1 and B1: Low or no Anxa2 and Stat3 expression in the para-cancerous tissues. A2-A5: The brown staining of Anxa2 gradually increases in cancerous tissues according to the expression score. B2-B5: The brown staining of Stat3 gradually increases in cancerous tissues according to the expression score. (original magnification, x400). We assess the A4/A5 and B4/B5 as high expression while the others as low expression.

Table 4
Anxa2 and stat3 expression status in relation to patient characteristics.

Characteristics	Anxa2 expression		P value	Expression level of Anxa2 and Stat3		P value
	low	high		Others	Double-positive	
Age(years)	59	41	0.018*	79	21	0.080
Sex						
Male	47	35	0.599	64	18	0.757
Female	12	6		15	3	
Tumor size						
≤3 cm	33	12	0.014*	39	6	0.138
> 3 cm	26	29		40	15	
Tumor number						
Single	49	33	0.795	67	15	0.200
Multiple	10	8		12	6	
Microvascular tumor thrombus						
Absent	48	27	0.101	65	10	0.003**
Present	11	14		14	11	
Liver cirrhosis						
No	15	7	0.462	18	4	0.485
Yes	44	34		61	17	
Serum AFP						
≤400 ng/ml	41	26	0.666	53	14	1.000
> 400 ng/ml	18	15		26	7	
Envelope invasion						
No	24	20	0.539	34	10	0.806
Yes	35	21		45	11	
Tumor differentiation						
Well or moderate	50	21	0.001**	65	6	< 0.001**
poor	9	20		14	15	
HBV DNA level						
< 4 log_{10} copies/mL	38	32	0.185	53	17	0.288
≥4 log_{10} copies/mL	21	9		26	4	
Albumin, g/L	59	41	0.913	79	21	0.325
Total bilirubin,μmol/L	59	41	0.967	79	21	0.726
Prothrombin time,s	59	41	0.928	79	21	0.892
Okuda stage						
I	53	40	0.235	72	21	0.340
II	6	1		7	0	
Child-Tuecotte-pugh grade						
A	55	38	1.000	73	20	1.000
B	4	3		6	1	

AFP: serum alpha-fetoprotein; HBV DNA: Hepatitis B virus desoxyribonucleic acid.
* $P < 0.05$ was considered statistically significant.
** $P < 0.01$ was considered statistically significant.

to 160 unique annexin proteins, also plays a pivotal role in carcinogenesis and cancer progression [14]. Anxa2 can bind to STAT3 and promote epithelial to mesenchymal transition which is a key step in cancer metastasis [15]. Phosphorylation of Anxa2 in Tyr23 site can activate Stat3 in sequence and promote proliferation and invasion in breast cancer [16].

In the present study, we hypothesize that Anxa2 can interact with Stat3 and promote the progression of hepatocellular carcinoma and that high co-expression of Stat3 and Anxa2 could cause poor prognosis in HCC patients. Therefore, we investigated the expression levels of Anxa2 and Stat3 in HCC and their paired paraneoplastic tissues. Furthermore, we assessed the expression of Stat3, Anxa2 and co-expression of Stat3 and Anxa2 with relevant clinical pathological parameters and their prognostic value in HCC patients.

Table 5
Factors Identified on Univariate and Multivariate Cox Regression Analysis That Influenced Cumulative HCC Recurrence in Patients.

Factors	P value	Hazard ratio	95% CI
Sex(male vs. female)	0.440	0.745	0.353–1.571
Age(years)	0.562	0.992	0.967–1.019
Tumor size(≤3 cm vs. > 3 cm)	0.028*	1.817	1.065–3.099
Tumor number(single vs multiple)	0.035*	1.952	1.047–3.640
Microvascular tumor thrombus(absent vs present)	0.008**	2.206	1.233–3.949
Liver cirrhosis(yes vs. no)	0.317	1.384	0.733–2.651
AFP(≤400 ng/ml vs. > 400 ng/ml)	0.781	1.081	0.625–1.867
Envelope invasion(yes vs no)	0.083	0.635	0.381–1.061
Tumor differentiation(well-differentiated、moderate vs. poor)	0.323	1.383	0.727–2.632
HBV DNA level(< 4 $\log_{10}$ copies/mL vs. ≥ 4 $\log_{10}$ copies/mL)	0.054	1.690	0.992–2.881
Albumin,g/L	0.551	0.983	0.930–1.039
Total bilirubin,μmol/L	0.434	1.017	0.975–1.060
Prothrombin time,s	0.625	0.941	0.738–1.200
Okuda stage(I vs II)	0.584	1.267	0.544–2.951
Child-Tuecotte-pugh grade(A vs B)	0.270	1.677	0.669–4.203
Anxa2(low vs high)	0.003**	2.266	1.317–2.898
Stat3(low vs high)	0.012*	2.073	1.171–3.670
Multivariate analysis			
Microvascular tumor thrombus(absent vs present)	0.011*	2.172	1.197–3.940
Anxa2(low vs high)	0.005**	2.192	1.261–3.811
Stat3(low vs high)	0.006**	2.287	1.272–4.113

AFP: serum alpha-fetoprotein; HBV DNA: Hepatitis B virus desoxyribonucleic acid; Anxa2: Annexin A2; Stat3: Signal transducer and activator of transcription 3; CI: confidence interval.
* P < 0.05 was considered statistically significant.
** P < 0.01 was considered statistically significant.

2. Materials and methods

2.1. Liver specimens

A total of 100 consecutive HCC patients pathologically confirmed after hepatectomy were recruited in our study between January 2010 and September 2011 at the Affiliated Hospital of Nantong University, Jiangsu Province, China. Four paired HCC tissues with detailed clinical data were frozen in liquid nitrogen immediately after surgery and stored at −80 °C. All of the recruited patients were in accordance with the following inclusion criteria: pathologically diagnosed as primary HCC after surgery without previous treatment; clinical information and related data parameters were available. The exclusion criteria were as follows: HCC patients accompanied with other carcinomas or died in hospital due to other reasons including postoperative hepatic failure; lack of patients' data. All patients previously received no other oncological surgery, chemotherapy, radiotherapy or any other anticancer therapy. This study was approved by the Ethics Committee with permission from the Affiliated Hospital of Nantong University. All patients signed written informed consent at the time of admission, with which the blood, tissue and related clinical data were authorized for scientific purposes.

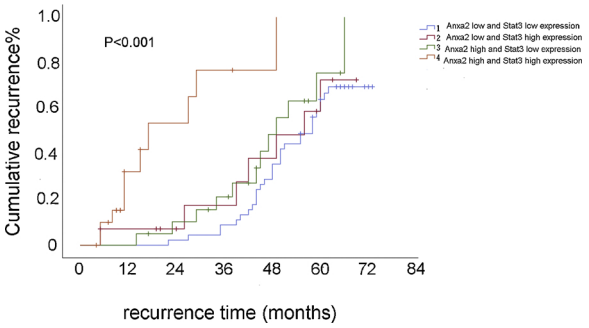

Fig. 4. Recurrence analysis of Anxa2 and Stat3 co-expression. The patients were divided into four groups (1, 2, 3 and 4 in the figure) depend on the expression of Stat3 and Anxa2. The patients in four subgroups had a different cumulative recurrence (P < 0.001) and the patients simultaneously with high Anxa2 and Stat3 expression (P4 subgroup) tend to have a high recurrence rate.

2.2. Clinical parameters of patients

During the follow-up study, 100 patients corresponded to the above criteria were analyzed as follows. Of those patients with HCC, liver biopsy specimens and their related clinical parameters were obtained. As shown in Table 1, the patients include 82 males and 18 females, between 35 and 74 years old (median 53.52). We considered the expression of Anxa2 and Stat3, the survival time, the differentiation, tumor grade, tumor size and spread, liver cirrhosis, HBV infection, periportal embolus, α-fetoprotein (AFP) levels, Okuda and TNM stage of these patients. The histological grade of tumors was delimited as well, moderately, and poorly differentiated according to the Edmondson grading system [17]. The tumor-node-metastasis (TNM) stages were defined according to 2010 AJCC staging system for HCC [18]. Tumor staging was 59 cases at I and II and 41 cases at III and IV based on the TNM classification. The Okuda and Child stage were both designated according to the current clinical application [19]. We chose serum AFP level < 400 or ≥400 ng/ml to group HCC patients based on domestic diagnostic criteria for liver cancer in China, 1999 [20]. The clinical and pathological parameters were collected once the patients were admitted to the hospital for HCC diagnosis before the operation. All of the patients were followed up regularly by telephone per 3 months. The follow-up time was delimited from the day of the surgery to the day of either death or the last follow-up. All patients were followed-up, and we recorded the recrudescence time, if applicable, and

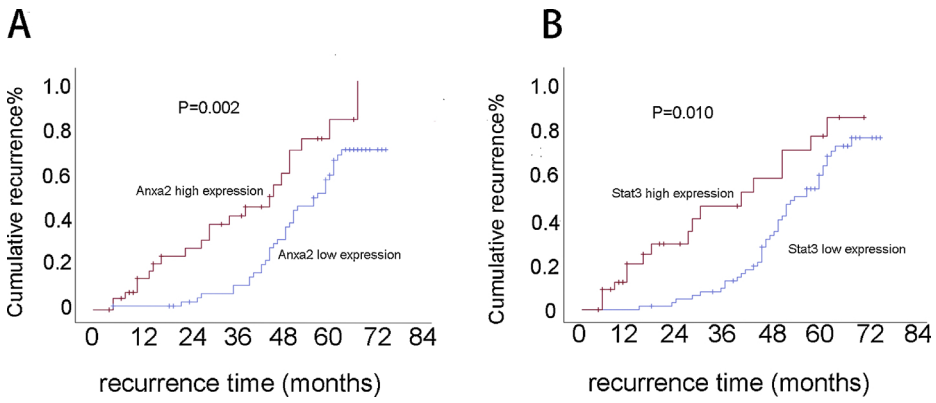

Fig. 3. Cumulative HCC recurrence related to the expression of Anxa2/Stat3 in the resected tumors (P = 0.002/P = 0.010, log-rank test).

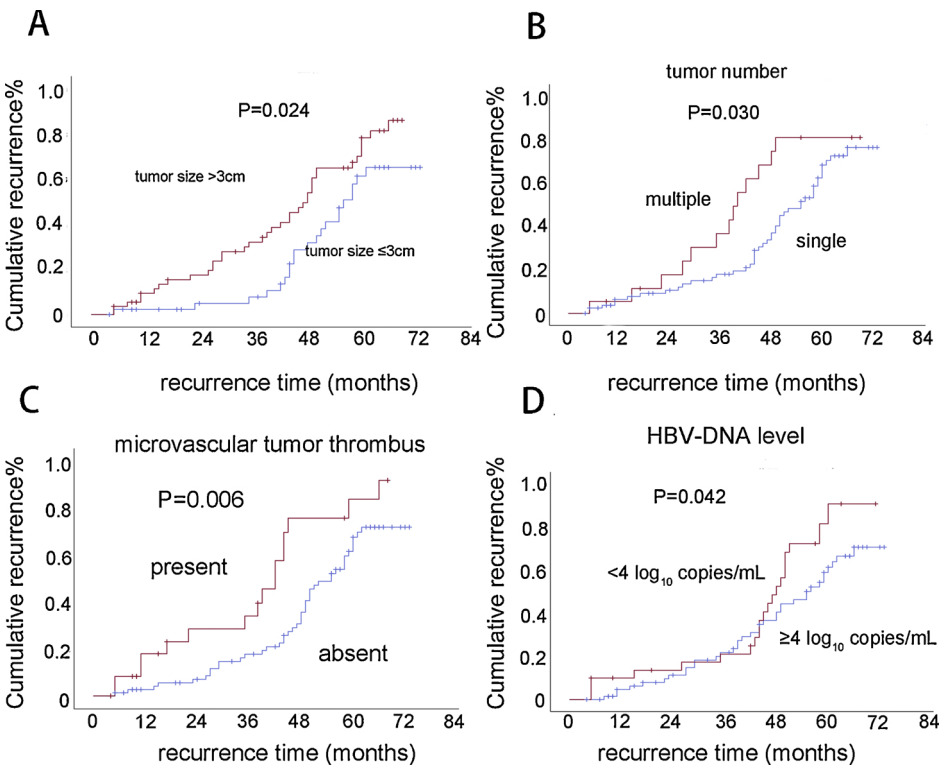

Fig. 5. Cumulative HCC recurrence related to other clinical pathological parameters in the resected tumor. (A) Cumulative HCC recurrence related to the tumor size in the resected tumors (P = 0.024, log-rank test). (B) Cumulative HCC recurrence related to tumor number in the resected tumor (P = 0.030). (C) Cumulative HCC recurrence related to the presence of microvascular tumor thrombus in the resected tumor (P = 0.006). (D) Cumulative HCC recurrence related to HBV-DNA level in the resected tumor (P = 0.042).

Table 6
Factors Identified on Univariate and Multivariate Cox Regression Analysis That Influenced HCC Overall survival time in Patients.

Factors	P value	Hazard ratio	95% CI
Sex (male vs. female)	0.573	1.164	0.687–1.971
Age (years)	0.751	0.997	0.978–1.016
Tumor size (≤3 cm vs. > 3 cm)	0.170	1.324	0.887–1.975
Tumor number (single vs multiple)	0.122	1.506	0.896–2.530
Microvascular tumor thrombus (absent vs present)	0.003**	2.033	1.279–3.230
Liver cirrhosis (yes vs. no)	0.322	1.271	0.790–2.064
AFP (≤400 ng/ml vs. > 400 ng/ml)	0.503	1.156	0.756–1.768
Envelope invasion(yes vs no)	0.443	0.855	0.574–1.275
Tumor differentiation(well-differentiated、moderate vs. poor)	0.011*	1.774	1.140–2.761
HBV DNA level (< 4 log_{10} copies/mL vs. ≥ 4 log_{10} copies/mL)	0.854	0.960	0.624–1.478
Albumin, g/L	0.073	0.958	0.913–1.004
Total bilirubin, μmol/L	0.348	1.016	0.982–1.052
Prothrombin time, s	0.443	0.931	0.777–1.117
Okuda stage (I vs II)	0.536	0.783	0.361–1.698
Child-Tuecotte-pugh grade (A vs B)	0.397	0.698	0.303–1.605
Anxa2(low vs high)	< 0.001**	2.330	1.547–3.509
Stat3(low vs high)	< 0.001**	3.009	1.956–4.630
Multivariate analysis			
Microvascular tumor thrombus (absent vs present)	0.006**	1.952	1.206–3.159
Anxa2(low vs high)	< 0.001**	2.137	1.398–3.267
Stat3(low vs high)	< 0.001**	2.021	1.272–4.918

AFP: serum alpha-fetoprotein; HBV DNA: Hepatitis B virus desoxyribonucleic acid; Anxa2: Annexin A2; Stat3: Signal transducer and activator of transcription 3; CI: confidence interval.
* P < 0.05 was considered statistically significant.
** P < 0.01 was considered statistically significant.

death. The last follow-up time was December 2017.

2.3. Diagnosis of HCC

All of the patients were diagnosed with HCC according to relevant inspection, including abdominal ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and/or biochemical results (AFP serology and liver function enzymes). All tissues were diagnosed accurately by postoperative pathologic examination based on hematoxylin and eosin (HE) staining. The diagnostic criteria were according to Chinese National Collaborative Cancer Research Group [17].

2.4. Western blot analysis

Western blot analysis was performed as normally described [21]. Briefly, the tissues were treated with pyrolysis, homogenized and centrifuged. The supernatant was removed and stored at −80 °C. For the experiment, loading buffer was added to the supernatant, which was then boiled for 15 min and centrifuged 10–15 minutes. SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis) was performed using the Laemmli method [22]. Western blot analysis was performed using a mouse monoclonal antibody to Anxa2 at a 1:1000 dilution (Santa Cruz Biotechnology, INC, sc-28385), and a mouse monoclonal antibody to Stat3 at a 1:500 dilution (Cell Signaling Technology, Orders-877-616-CELL). The membranes were incubated with specific primary antibodies overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibodies. After incubation with secondary antibodies, the reactive bands were identified using a commercially available Enhanced Chemiluminescence Kit (Amersham Bioscience).

2.5. Immunohistochemistry

IHC staining was processed as previously described [21]. First, the TMA slides were cut into 4-μm sections, deparaffinized and rehydrated through different graded alcohols. Second, the sections were quenched in H2O2 for 15 min and then boiled in antigen retrieval solution

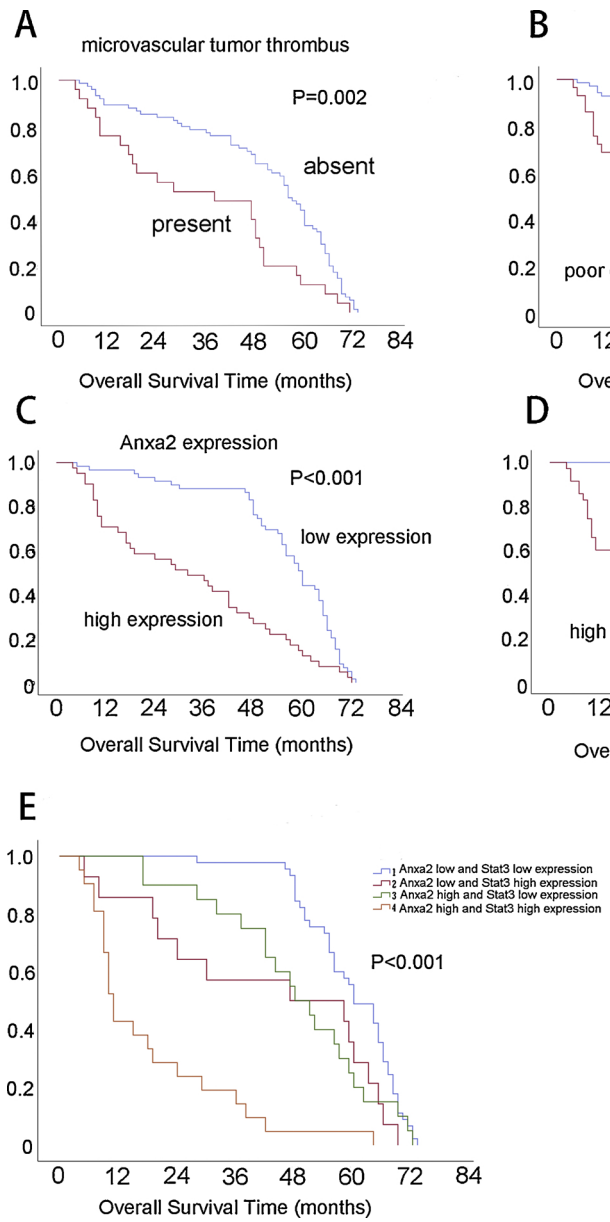

Fig. 6. Overall survival curves of 100 hepatocellular carcinoma patients. Kaplan-Meier analysis showed that these factors below had a different overall survival. (A) The presence of microvascular tumor thrombus tend to have little survival time (P = 0.002). (B) The poor differentiated patients tend to have little survival time (P = 0.007). (C) (D) The expression of Anxa2/Stat3 showed differences in the survival time (P < 0.001/P < 0.001). (E) The patients were divided into four groups (1, 2, 3 and 4 in the figure) depend on the expression of Stat3 and Anxa2. The patients in four subgroups had a different overall survival time (P < 0.001) and the patients simultaneously with high Anxa2 and Stat3 expression (P4 subgroup) tend to cause poor survival.

(sodium citrate, pH 6.0) under pressure for 30 min. After, the tissues were incubated at 4 °C overnight with a mouse primary anti-Stat3 antibody diluted 1:500 and rabbit primary anti-Anxa2 antibody diluted 1:1000. After washing, the sections were incubated with horseradish peroxidase conjugated goat anti-mouse IgG for 15 min at a 1:2500 dilution and washed again with PBS. The slides were processed using the peroxidase–anti-peroxidase method (Dako Deutschland GmbH, Hamburg, Germany). The sections were further counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and cover-slipped. PBS was used instead of the primary antibody for the negative control reactions.

2.6. Immunohistochemical evaluation

Anxa2 and Stat3 expression levels were assessed by the percentage of positive cells and the intensity of positive staining. The percentages for the two proteins were scored as follows: 0 (0%), 1 (1%–33%), 2 (34%–66%), and 3 (67%–100%). The intensity of positive staining was also scored as follows: 0 (negative), 1 (borderline), 2 (weak), 3 (moderate) and 4 (strong). The sum of the percentage and staining score was defined as the overall IHC score. According to the above criterion, we divided the tissues with Anxa2 or Stat3 expression into two groups: low with 0–3 scores and high with 4–7 scores.

2.7. Statistical analysis

The correlation between Stat3 and Anxa2 expression level and clinical parameter features, including gender, tumor size, tumor number, microvascular tumor thrombus, liver cirrhosis, AFP level, envelope invasion, tumor differentiation, HBV DNA level, Okuda stage, and Child-Turcotte-Pugh grade, were evaluated by chi-square test. Additionally, Student's t tests or Fisher exact tests were performed for continuous variables, such as age, albumin, total bilirubin, and prothrombin time. The multivariate Cox proportional hazards regression model was used to assess the potential risk factors contributing to late recurrence and 95% confidence intervals (CI) of hazard ratios (HR) were calculated. Survival and one minus survival curves were calculated using the Kaplan–Meier method, and differences were compared using the log-rank test. Statistical significance was defined as a P value of less than 0.05. Statistical analysis was performed using SPSS v. 17.0

(SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Patient characteristics

A total of 100 HCC patients were enrolled in this study. During the follow-up period, survival and recurrence information was available for all patients. The patients' relevant original clinical data were allowed to obtain, and immunohistochemistry results were divided into two groups (high or low expression). Of these, the cumulative recurrence rates and overall survival rates at 1, 2, 3, 4, and 5 years after surgery were 16%, 26%, 35%, 48%, and 60% and 86%, 77%, 71%, 56%, and 31%. The clinical parameter characteristics of the patients are shown in Table 1.

3.2. High expression of Stat3 and Anxa2 in HCC

To investigate whether Stat3 and Anxa2 might promote the development and progression of HCC corporately, we analyzed their expression in 4 paired of HCC and adjacent nontumor tissues. Western blot analysis revealed that both expression of Stat3 and Anxa2 were significantly higher in HCC tissues compared with adjacent nontumor tissues (Fig. 1A), which was consistent with other similar basic research [14,23–25]. Moreover, the immunohistochemical results of these 4 patients were attached at the same time (Fig. 1B) and the clinical details of the 4 patients were shown in Table 2. Stat3 and Anxa2 were differently expressed in 100 HCC and adjacent nontumor tissue slices. According to the above separation, we chose the expression score of 4 (≥ 4 vs. < 4) as the cut-off point for further recurrence and survival analysis. The incidence of high Stat3 expression in HCC tissues (35%) was significantly higher than that in non-HCC tissues (8%) ($P < 0.001$). The same result was found for Anxa2 ($P < 0.001$). The results are shown in Table 3.

To further confirm the different expression of Anxa2 and Stat3 in different clinical staging of HCC tissues, we chose the 100 HCC tissue slices for further analysis. The relationship between Anxa2/Stat3 expression and clinical staging of HCC is shown in Fig. 2. According to the clinical staging criteria, there were 20 cases at staging I (20%), 39 at II (39%), 36 at III (36%), and 5 at IV (5%) among 100 cancerous tissues. The incidences of high Anxa2 expression in HCC tissues were 20% (4 of 20) at I staging, 35.9% (14 of 39) at II staging, 52.8% (19 of 36) at III staging, and 80% (4 of 5) at IV staging, respectively. Besides, the incidences of high Stat3 expression in HCC tissues were 15% (3 of 20) at I staging, 33.3% (13 of 39) at II staging, 44.4% (16 of 36) at III staging, and 60% (3 of 5) at IV staging. Both Anxa2 and Stat3 expression were gradually increased in different clinical stages and that the advanced stage was mostly very strong staining.

3.3. Relationship between Stat3/Anxa2 expression and clinical features of HCC

To illustrate the biological significance, we analyzed the correlations between the expression of Stat3 and Anxa2 and their clinical pathologic parameters. As shown in Table 4, high Anxa2 expression in HCC tissues was associated with a high probability of age ($P = 0.018$), tumor size ($P = 0.014$), and tumor differentiation ($P = 0.001$) and was not related to any other clinical pathological features (all P values > 0.05), such as gender, tumor number, microvascular tumor thrombus, liver cirrhosis, serum AFP, envelope invasion, HBV DNA level, albumin, total bilirubin, prothrombin time, Okuda stage and Child-Turcotte-Pugh grade. Additionally, high Stat3 expression in HCC tissues was associated with a high probability of tumor differentiation ($P < 0.001$) and was not related to the others mentioned above. Then, we combined the expression of Stat3 and Anxa2 to analyze their relationships with the clinical parameters, and we found that a high expression of Stat3 and Anxa2 was related to microvascular tumor thrombus ($P = 0.003$) and tumor differentiation ($P < 0.001$) and was not related to other clinical features (All $P > 0.05$). The results are shown in Table 4.

3.4. Prognostic significance of Stat3/Anxa2 expression for recurrence

All of the factors mentioned in Table 1 were used in a univariate and multivariate analysis. As expected, overexpression of Stat3/Anxa2 showed a significant relationship with the recurrence of HCC patients ($P_{Stat3} = 0.012$; $P_{Anxa2} = 0.003$). Moreover, as shown in Table 5, tumor size > 3 cm in diameter ($P = 0.028$), tumor number > 1 single tumor node ($P = 0.035$), and the absence of microvascular tumor thrombus ($P = 0.008$) were also significantly associated with recurrence in patients. To further assess the value of the expression of Stat3 and Anxa2 in the prognosis of HCC, we subsumed these single factors related to recurrence into a new multivariate analysis. The results in Table 5 showed that only the absence of microvascular tumor thrombus ($P = 0.011$), Stat3 ($P = 0.006$), and Anxa2 ($P = 0.005$) were identified as predictive factors for poor HCC recurrence.

The high expression of Stat3 and Anxa2 were significantly associated with high tumor recurrence probabilities (Stat3 hazard ratio (HR): 2.073, 95% confidence interval (CI): 1.171–3.670, $P = 0.012$; Anxa2 hazard ratio (HR): 2.266, 95% confidence interval (CI): 1.317–2.898, $P = 0.003$). The median recurrence time was 24 months for the high-expression Stat3 group, in contrast to 50 months for the low-expression group ($P = 0.003$). The data for Anxa2 were similar. Fig. 3 demonstrates that high expression of Stat3 or Anxa2 was associated with a significantly higher cumulative risk of tumor recurrence time ($P_{Stat3} = 0.010$; $P_{Anxa2} = 0.002$). Further, we analyzed the value of Stat3 and Anxa2 coexpression. The patients were separated into four groups using the IHC score of Stat3 and Anxa2, and the results indicated that the recurrence times ($P < 0.001$, Fig. 4) were significantly different in the subgroups.

Multivariate analysis demonstrated that microvascular tumor thrombus, Anxa2 expression, and Stat3 expression were three independent factors in HCC recurrence (Table 5). The figures have demonstrated that high expression of Stat3, Anxa2, and their coexpression was associated with a significantly higher cumulative risk of tumor recurrence time (Figs. 3 and 4). Additionally, we further analyzed whether other clinical parameters are associated with HCC recurrence time. Tumor size ($P = 0.024$), tumor number ($P = 0.030$), microvascular tumor thrombus ($P = 0.006$), and HBV DNA level ($P = 0.042$) were significantly associated with HCC recurrence time, which was slightly different from the univariate Cox regression analyses (Fig. 5).

3.5. Prognostic significance of Stat3/Anxa2 expression for overall survival

As shown in Table 6, microvascular tumor thrombus ($P = 0.003$), tumor differentiation ($P = 0.011$), Anxa2 expression ($P < 0.001$) and Stat3 expression ($P < 0.001$) were significantly associated with OS after surgery. However, in multivariate analysis, microvascular tumor thrombus ($P = 0.006$), Anxa2 expression ($P < 0.001$) and Stat3 expression ($P < 0.001$) influenced HCC OS, which was consistent with the recurrence time analysis (Table 5). In this retrospective cohort study, we found that OS was highly associated with recurrence time ($P < 0.001$), which confirmed the credibility of the clinical data. The Kaplan-Meier survival curves (Fig. 6) demonstrated that microvascular tumor thrombus ($P = 0.002$), tumor differentiation ($P = 0.007$), Stat3 expression ($P < 0.001$), Anxa2 expression ($P < 0.001$), and their coexpression ($P < 0.001$) were significantly associated with OS.

4. Discussion

HCC is one of the most common primary alimentary tract malignancies in the world [26]. Anxa2 is mostly localized in cell membrane or cytoplasm and involved in multiple cellular processes, especially in

cancer progression [14,27,28]. The expression of Anxa2 is not only correlated with tumor size and differentiation in HCC (Table 4) but also associated with development of other cancers, such as breast cancer, clear cell renal cell carcinoma, colorectal cancer, lung cancer, etc. [9] Interestingly, Stat3 is another key molecular in regulating HCC progression via persistent activation through phosphorylation to induce liver injury [26]. Direct interaction of Anxa2/Stat3 promotes proliferation and invasion of cancer cells while potential molecular mechanism that Tyr23 phosphorylation of Anxa2 activates Stat3 in sequence [14,26,29,30].

Recently, many studies concerning on new therapies for cancer treatment have attracted attention worldwide. Immunotherapy, a newly developed cancer therapy that concentrates on immunosuppressive markers, tumor targets or combinational approaches, has demonstrated that dampening the tumor immunosuppressive environment might be a newly effective approach for HCC treatment [31–33]. A relevant study did show that a Stat3-blocking whole-cell hepatoma vaccine could induce a cellular and humoral immune response against HCC. Additionally, several studies have shown that Stat3's interactions with other proteins, such as hexokinase 2, Jab1/Csn5, could contribute to cancer progression [10,34].

However, there are possible limitations influencing this study. Only a relatively small number of patients were included in the analysis, and the study design was retrospective. Additionally, these patients were limited by the resectable tumors' indication. Many clinical parameters were predictable, such as albumin, total bilirubin, prothrombin time, Okuda stage and Child-Turcotte-Pugh grade, which leads to one-sidedness in clinical data analysis. Most patients were included in stage I (93 of 100) or grade A (93 of 100), which may lead to the analysis results were not appropriate. We analyzed whether the coexpression of Stat3 and Anxa2 could promote the progression of HCC and cause poor prognosis in patients, but we did not investigate whether the integration occurs in the nucleus or cytoplasm, as well as whether the active form p-Anxa2 was present.

In conclusion, this is the first analysis to investigate whether the combination of Anxa2 and Stat3 can influence HCC recurrence and overall survival time. By collecting clinical data, we confirmed that the expression of Stat3, Anxa2, or co-high-expression of the two proteins was associated with HCC recurrence and survival. Additionally, we propose that our results may lead to further research on these pathways and cancer immunotherapy.

Conflict of interest

These authors declare that they have no conflict of interest.

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