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NUSAP1, a novel stemness-related protein, promotes early recurrence of hepatocellular carcinoma

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Abstract

Early recurrence (within 2 years after resection) is the primary cause of poor outcomes among hepatocellular carcinoma (HCC) patients, and liver cancer stem cells are the main contributors to postsurgical HCC recurrence. Nucleolar and spindle-associated protein 1 (NUSAP1) has been reported to be involved in tumor progression. We investigated the function and clinical value of NUSAP1 in early recurrence of HCC. Data from public datasets and our cohort were used to assess the association between NUSAP1 expression and early HCC recurrence. Gain- and loss-of-function experiments were carried out in vivo and in vitro. The predictive effect of NUSAP1 on early HCC recurrence was further evaluated by a validation cohort. We found that elevated NUSAP1 expression in HCC specimens was correlated with poor outcome, especially in cases with postoperative early recurrence. Functional studies indicated that NUSAP1 significantly promotes HCC progression. A postsurgical recurrence murine model further revealed that upregulated NUSAP1 dramatically increased the likelihood of HCC early recurrence. RNA sequencing data revealed that the gene sets of cancer stemness and the signal transducer and activator of transcription 3 (STAT3) pathway were enriched by NUSAP1 overexpression. Mechanistically, NUSAP1 enhanced cancer stemness through stimulating STAT3 nuclear translocation and activation through receptor of activated protein C kinase 1 (RACK1). In a validation cohort with 112 HCC patients, NUSAP1 effectively predicted HCC early recurrence. Our results indicated that NUSAP1 promotes early recurrence of HCC by sustaining cancer stemness and could serve as a valuable predictive indicator for postsurgical intervention in HCC patients.

KEYWORDS
hepatocellular carcinoma, early recurrence, nucleolar and spindle associated protein 1, STAT3 activation

1 | INTRODUCTION

Hepatocellular carcinoma is one of the most prevalent and aggressive types of cancers worldwide.[1] Currently, surgical resection of the liver is the first-line option for HCC patients to achieve long-term survival.[2] However, due to the high risk of early HCC recurrence (within 2 years after surgical resection), the survival rate for HCC patients remains poor.[3] Notably, commonly used tumor markers, such as serum AFP, cannot effectively predict early HCC recurrence.[4] Therefore, discovering more powerful predictive biomarkers for early HCC recurrence is vital in improving treatment efficacy among patients with advanced HCC.

Nucleolar and spindle-associated protein 1, an important mitotic regulator, plays a critical role in binding microtubules, mitotic processes, spindle formation, and stability.[5] Aberrant NUSAP1 expression is markedly correlated with poor prognosis across many types of cancers, including HCC.[6–8] Previous studies indicate the upregulation of NUSAP1 promotes the progression and invasion of astrocytoma and prostate cancer,[9,10] and that NUSAP1 plays an important role in cancer progression and aggressiveness. Recently, increasing evidence has identified *NUSAP1* as a stemness-related gene[11,12] that could impair immune infiltration and affect tumor occurrence in combination with other stemness-related genes, such as *MCM6*, *RACGAP1*, and *RRM2*.[12] Cancer stem cells, defined as cells with cancer stemness characteristics, play a vital role in tumor progression, metastasis, and recurrence. In addition, studies have reported that LCSCs are responsible for the early recurrence of HCC.[13] It has also been reported that the upregulation of NUSAP1 could promote metastasis of cervical cancer by enhancing CSC properties and EMT progression.[14] However, whether NUSAP1 contributes to early HCC recurrence by enhancing cancer stemness has not been systematically investigated, and clarification of the molecular mechanism needs further study.

In the present study, we examined whether NUSAP1 has any predictive value for the clinical outcomes of HCC patients who undergo curative resection, with an emphasis on the risk of postoperative recurrence. Furthermore, we established an orthotopic HCC implantation mouse model and a postsurgical recurrence mouse model to investigate the contribution of NUSAP1 to postsurgical HCC recurrence. Ultimately, we identified NUSAP1 as a novel mediator that links the RACK1/STAT3 pathway to the promotion of early HCC recurrence.

2 | MATERIALS AND METHODS

2.1 | Patients

A tissue microarray including 90 patients was purchased from OUTDO Biotech. Two hundred and sixteen patients receiving hepatic curative R0 resection were collected at the Affiliated Tumor Hospital of Guangzhou Medical University from 2006 to 2010. Another independent validation cohort including 139 patients receiving hepatic curative resection were collected in the First Affiliated Hospital of Jinan University from 2013 to 2019. Patients were excluded if they had clinical or histological evidence of portal vein thrombosis or extrahepatic metastases before or during operation. Patients followed up for less than 24 months were also excluded. Finally, 112 patients were enrolled. All experiments involving human tissues were approved by the research and ethics committee of the First Affiliated Hospital of Jinan University.

2.2 | Animal studies

For the subcutaneous xenograft model, 5×10^6 cells resuspended in 200 μl PBS (Cat# 16D13830; BOSTER) were injected into the right flank of nude mice (total of 16 mice were randomly allocated to two group). The tumor volume was repeatedly measured and recorded every 3 days; the xenografts were removed, fixed, weighed, and photographed 4 weeks following implantation.

For the lung metastasis model, 1×10^6 cells resuspended in 50 μl PBS (Cat# 16D13830; BOSTER) were slowly injected into the tail vein of nude mice (total of 16 mice were randomly allocated to two groups). Six weeks later, lungs were resected and metastatic nodules counted.

For the orthotopic HCC cancer mouse model, 1×10^6 cells in 25 μl PBS/Matrigel solution (1:1, Cat# 356234; Corning) were injected into the subcapsular region of the liver (total of 16 mice were randomly allocated to two groups). Twenty-one days after HCC orthotopic implantation, the mice were killed, and their livers were dissected and examined for tumor numbers and size.

For the postsurgical HCC recurrence mouse model, 1×10^6 cells in 25 μl PBS/Matrigel solution (1:1, Cat# 356234; Corning) were injected into the subcapsular region of the liver (total of 24 mice were randomly allocated to two groups). Ten days after implantation, the left lobe of the liver where tumors had been implanted was resected if tumors developed in the lobe and no intrahepatic metastasis occurred. Fourteen days after hepatectomy, the mice were killed, and their livers were dissected and examined for tumor recurrence.

All 4–6-week-old male BALB/c nude mice were purchased from the Guangdong Medical Lab Animal Center. All mice were maintained in specific pathogen-free conditions. Assignment of the mice into different groups was random. All animal studies were approved by the Animal Care and Use Committee at Jinan University and carried out according to established guidelines.

2.3 | Cell cultures

Hepatocellular carcinoma cell lines HepG2, Hep3B, MHCC-97h, and Huh7 were obtained from Sun Yat-sen University Cancer Center. All cells were maintained in high-glucose DMEM (glucose 4.5 g/L, Cat# 21885025; Gibco) with 10% FBS (Cat# FND500, ExCell), 100 U/ml penicillin, and 100 μg/ml streptomycin (Cat# 15140122; Gibco) in a humidified incubator at 37°C and 5% CO_2. The identity of all

cell lines was confirmed by genetic profiling using short tandem repeat loci. A specific inhibitor of STAT3, stattic (Stattic-10 mM/1 ml; Selleck) was used to inhibit STAT3 in vitro. The stattic was dissolved in DMSO (Cat# 317275; Sigma-Aldrich) and further diluted to the required concentration. In this study, the cells were treated with 10 μM stattic or DMSO for 24 h, then the applicable experiments were carried out.

2.4 | Co-immunoprecipitation

Overexpressed Flag- or HA-labeled cell protein extracts were isolated using Pierce IP Lysis Buffer (#87787; Thermo Fisher Scientific). Cell lysates were then incubated with 1 μg specific Ab and treated with Dynabeads magnetic beads (10004D; Invitrogen) at 4°C overnight. The magnetic bead-Ab-Ag complex was then washed three times with IP buffer, eluted with loading buffer, heated for 10 min at 70°C, and resolved by SDS-PAGE, followed by MS or immunoblotting.

2.5 | Affinity capture and MS

Huh7 cells overexpressed with Flag-NUSAP1, or vector control, were lysed and centrifuged and were then separately applied to an anti-Flag Ab affinity column. After washes, bound proteins were eluted from the column using Flag peptide and were subjected to SDS-PAGE. Gels were stained with Coomassie Blue, and the electrophoresis lanes were dissected separately and subjected to MS analysis (Beijing Genomics Institute).

2.6 | Sphere formation assay

Hepatocellular carcinoma cells were plated in 96-well ultra-low attachment culture dishes (Cat# 3474; Corning) at 200 cells per well and incubated in DMEM/F12 medium (Cat# C11330500BT; Gibco) supplemented with 20 ng/ml basic fibroblast growth factor (Cat# HG10014-NH; Sino Biological) and 20 ng/ml epidermal growth factor (Cat# HG10325-M; Sino Biological), 2% B27 supplement (Cat# 17504–044; Thermo Fisher Scientific), for 7 days. The number of spheroids was counted, and representative views were imaged using a microscope (Cat# 135706; Nikon Corporation).

2.7 | Statistical analysis

Student's t-test or the nonparametric Mann–Whitney U-test was carried out to compare the values between the involved comparison groups. Survival curves were constructed using the Kaplan–Meier method and analyzed by the log–rank test. Univariate and multivariate analyses were undertaken using a Cox proportional hazards regression model. The χ^2-test was used to evaluate the association of NUSAP1 with categorical variables. Data were expressed as the mean ± SD; p values less than 0.05 were considered statistically significant. Statistical analyses were undertaken using SPSS software (version 23.0; IBM).

3 | RESULTS

3.1 | NUSAP1 is upregulated in HCC and correlates with poor outcomes

Four public biomedical databases were used to evaluate the expression and clinical value of NUSAP1 among HCC patients. We found that NUSAP1 expression was significantly upregulated in HCC samples at both the mRNA and protein levels ($p < 0.001$; Figures 1A,B and S1A). Consistent with these findings, we observed increased NUSAP1 expression in HCC on a tissue microarray of 90 paired human HCC specimens and non-neoplastic liver tissues. ($p < 0.001$; Figure 1C). Subsequently, we established our own exploratory discovery cohort with 216 HCC cases to investigate NUSAP1 protein expression and its clinical value (baseline demographic data are provided in Table S1). Our data indicated that the level of nuclear-localized NUSAP1 was higher in HCC tissues compared with adjacent nontumor tissues ($p < 0.001$; Figure 1D). In addition, data for three independent murine models from public datasets showed that NUSAP1 expression was markedly upregulated in liver tumors (Figure S1B).

Moreover, similar to the data in three public biomedical databases (Figures 1E and S2), we found that HCC patients with high NUSAP1 expression had poorer 5-year overall survival ($p = 0.002$) and a higher 5-year cumulative recurrence rate ($p = 0.004$; Figure 1F) in our exploratory discovery cohort.

3.2 | Upregulation of NUSAP1 contributes to postsurgical early recurrence in HCC patients

Our cohort showed that NUSAP1 expression was strongly correlated with poor tumor differentiation ($p = 0.005$), abnormal AFP levels ($p = 0.033$), the presence of tumor thrombus ($p = 0.001$), and early recurrence ($p < 0.001$), suggesting NUSAP1 plays an aggressive role in HCC invasion and metastasis (Table 1). Early recurrence within 2 years after hepatectomy is the main obstacle for long-term survival of HCC patients, even among early-stage patients who receive a radical resection.[15,16] In our discovery cohort ($n = 216$), we found that the incidence of early recurrence was higher in HCCs with elevated NUSAP1 expression than in those with relatively low NUSAP1 expression (84.09% vs. 58.33%, $p < 0.001$; Figure 2A, left panel). In addition, regardless of tumor stage, NUSAP1 expression was markedly upregulated when early recurrence occurred ($p < 0.001$; Figure 2A, right panel).
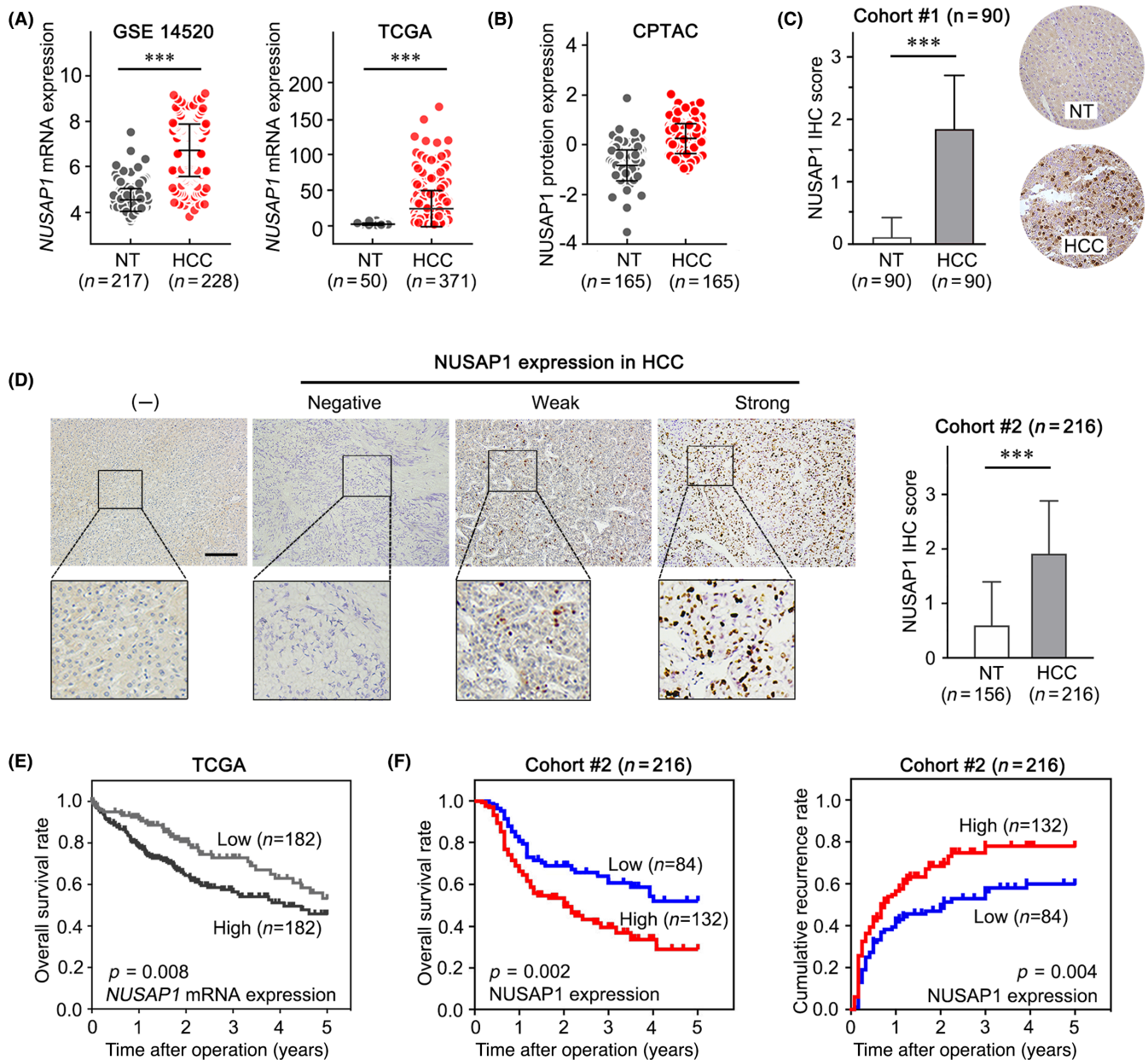
FIGURE 1 Nucleolar and spindle associated protein 1 (NUSAP1) is upregulated in hepatocellular carcinoma (HCC) and correlates with poor outcomes. (A,B) Relative mRNA and protein levels of NUSAP1 expression among HCC tissues and nontumor liver tissues (NT) (data from three datasets: GSE14520, The Cancer Genome Atlas [TCGA], and Clinical Proteomic Tumor Analysis Consortium [CPTAC]). (C) Representative images and quantification of NUSAP1 expression in HCC tissue microarray ($n = 90$) and (D) exploratory discovery cohort (cohort #2, $n = 216$); scale bar, 100 μm. (E) Kaplan–Meier curves for overall survival and cumulative recurrence rate according to NUSAP1 expression in HCC samples from the TCGA dataset and (F) cohort #2. Data are presented as mean ± SD. ***$p < 0.001$.

Moreover, HCC patients with high NUSAP1 expression were likely to experience early recurrence in both HCCs and their subgroup with early-stage (BCLC-0/A) patients (both $p < 0.005$; Figure 2B). The NUSAP1 expression status and prognostic clinicopathologic parameters identified by univariate analysis ($p < 0.10$) were entered into a multivariate model and we found that NUSAP1 upregulation was an independent risk factor for early recurrence both in HCCs and their subgroup of BCLC-0/A stage ($p < 0.005$; Figure 2C), suggesting that upregulation of NUSAP1 could play a pivotal role in the early recurrence of HCC patients regardless of tumor stage.

3.3 | NUSAP1 promotes HCC progression and postsurgical recurrence in multiple murine models

Postsurgical HCC early recurrence is closely associated with the aggressive tumor phenotype.[15,17] Therefore, we sought to investigate the role of NUSAP1 in HCC growth and metastasis. First, HCC cell lines with NUSAP1 overexpression or knockdown were generated and we found that overexpression of NUSAP1 markedly enhanced cell proliferation, invasion, and migration ability in vitro, whereas the knockdown of NUSAP1 reversed such effects (Figures S3 and S4).

TABLE 1 Correlation of NUSAP1 expression with clinicopathologic parameters in 216 patients with hepatocellular carcinoma

Variable	n	NUSAP1 Low, n (%)	High, n (%)	x^2-test	p value
Gender					
Female	29	12 (54.5)	10 (45.5)	2.527	0.112
Male	194	72 (37.1)	122 (62.9)		
Age, years					
≤50	92	43 (46.7)	49 (53.3)	1.194	0.275
>50	83	32 (38.6)	51 (61.4)		
HBsAg					
Negative	25	8 (32.0)	17 (68.0)	0.505	0.477
Positive	188	74 (39.4)	114 (60.6)		
HCV-Ab					
Negative	128	46 (35.9)	82 (64.1)	0.202	0.653
Positive	4	1 (25.0)	3 (75.0)		
AFP, ng/ml					
≤400	108	44 (40.7)	64 (59.3)	0.418	0.576
>400	107	39 (36.4)	68 (63.6)		
GGT, U/L					
≤50	44	21 (47.7)	23 (52.3)	0.513	0.474
>50	130	54 (41.5)	76 (58.5)		
ALT, U/L					
≤50	141	57 (40.4)	84 (59.6)	0.239	0.625
>50	73	27 (37.0)	46 (63.0)		
AST, U/L					
≤40	88	32 (36.4)	56 (63.6)	0.523	0.470
>40	126	52 (41.3)	74 (58.7)		
Cirrhosis					
No	94	39 (41.5)	55 (58.5)	0.747	0.491
Yes	122	45 (36.9)	77 (63.1)		
Child–Pugh grade					
A	200	79 (39.5)	121 (60.5)	0.079	0.779
B	14	5 (35.7)	9 (64.3)		
Tumor size, cm					
≤5	99	44 (44.4)	55 (55.6)	2.227	0.136
>5	116	40 (34.5)	76 (65.5)		
Tumor number					
Single	165	64 (38.8)	101 (61.2)	0.003	0.956
Multiple	51	20 (39.2)	31 (60.8)		
Tumor thrombus					
No	181	79 (43.6)	102 (56.4)	10.638	**0.001**
Yes	35	5 (14.3)	30 (85.7)		
Tumor capsule					
No	143	59 (41.3)	84 (58.7)	0.573	0.449
Incomplete/complete	33	16 (48.5)	17 (51.5)		
Tumor differentiation					
I–II	140	65 (46.4)	75 (53.6)	7.837	**0.005**
III–IV	28	5 (17.9)	23 (82.1)		

(Continues)

TABLE 1 (Continued)

Variable	n	NUSAP1 Low, n (%)	High, n (%)	x^2-test	p value
Early recurrence[a]					
No	56	35 (62.5)	21 (37.5)	17.734	**<0.001**
Yes	160	49 (30.6)	111 (69.4)		

Abbreviations: AFP, alpha fetoprotein; ALT, alanine transaminase; AST, aspartate transaminase; GGT, gamma-glutamyl transferase; HBsAg, hepatitis B surface antigen; HCV-Ab, hepatitis C antibody.

Bold values ($p < 0.05$) are statistically significant.

[a]Tumor detected within 2 years after surgical operation.

Next, to further determine the function of NUSAP1 in HCC tumor progression and metastasis in vivo, we established three murine models: a subcutaneous implantation model, lung metastasis model, and orthotopic implantation model. Both subcutaneous tumor growth and lung metastatic nodules were remarkably suppressed by NUSAP1 knockdown but enhanced by NUSAP1 overexpression (Figures 3A,B and S5A,C). In addition, in orthotopic implantation models, NUSAP1 overexpression significantly promotes tumor invasive growth (tumor diameter: 0.849 ± 0.139 cm vs. 1.660 ± 0.530 cm; $p < 0.001$) and intrahepatic metastasis (intrahepatic metastasis rate: 60% vs. 100%; Figure 3C), while NUSAP1 knockdown caused the opposite effect (Figure S5D).

Subsequently, we investigated the function of NUSAP1 in early recurrence in a murine hepatectomy model for postsurgical HCC recurrence with MHCC-97h, an HCC cell line with high metastatic potential (Figure 3D). On day 10, when all mice developed tumors on the left lobe of liver and no intrahepatic metastasis occurred, we resected the left liver lobe (Figure 3E, left panels) and evaluated HCC recurrence on day 14 after radical tumor resection.[18,19] We found that overexpression of NUSAP1 slightly promoted tumor growth compared to controls on day 10 after HCC orthotopic implantation ($p = 0.031$; Figure 3F). All mice (12/12) with NUSAP1-overexpressing tumors experienced postsurgical recurrence after hepatectomy, whereas a fraction of the control mice (8/12) relapsed (Figure 3G). Moreover, a higher level of Ki-67 expression was observed in NUSAP1-overexpressing tumors (Figures 3E, right panels, and S5B). Collectively, our results indicated that upregulation of NUSAP1 enhances tumor progression and further promotes postsurgical recurrence.

## 3.4 | NUSAP1 promotes CSC properties to facilitate early HCC recurrence

To address the gene profile regulated by NUSAP1, we undertook RNA sequencing in Huh7 cells (Scramble vs. shNUSAP1). Approximately 18,089 differentially expressed genes were detected, in which stemness-related genes, the main contributors to HCC recurrence,[20] were markedly downregulated (Figures 4A and S6A). NUSAP1 was significantly upregulated in the self-renewing spheroids compared to the attached cells and further increased during serial passages of

cell spheroids (all $p < 0.001$; Figure 4B). Furthermore, the expression of LCSC markers (CD24, CD133, and EpCAM) and stemness-related transcription factors (Nanog, Bmil, SOX2, and OCT-4) increased when NUSAP1 was overexpressed (Figure 4C). Moreover, NUSAP1 mRNA expression was strongly correlated with EpCAM and stemness-related transcription factors in the TCGA dataset (Figures 4D and S6B). Flow cytometry analysis revealed that the number of LCSCs (CD133$^+$EpCAM$^+$ cells) significantly increased after NUSAP1 overexpression ($p < 0.001$; Figure 4E), while the number of LCSCs significantly decreased after NUSAP1 knockdown (Figure S6C). Importantly, spheroid formation was enhanced almost 4-fold in NUSAP1-overexpressing cells compared to controls (Figures 4F and S6D). Furthermore, NUSAP1 was positively correlated with EpCAM and Nanog in primary HCCs of patients showing early recurrence after surgery and the postsurgical recurrence mouse model (Figures 4G and S6E,F).

Taken together, these results suggested that NUSAP1 was enriched in LCSCs, which facilitated the cancer stemness properties of HCC cells.

3.5 | NUSAP1 stimulated the activation of STAT3 by interacting with RACK1

To elucidate the mechanism underlying the stimulative role of NUSAP1 in HCC recurrence, we analyzed our RNA sequencing data and found significant enrichment of the STAT3 activation pathway among several stemness-associated pathways (Figures 4A and 5A). These data were consistent with the Gene Set Enrichment Analysis (Figure S7A,B). Signal transducer and activator of transcription 3 has been widely accepted as a key oncogenic factor and plays an essential role in the development of HCC.[21–23] In the present study, our results revealed that NUSAP1 did not influence total STAT3 protein levels, but markedly increased the phosphorylation levels of STAT3 (Y705) in HCC cells (Figure 5B). Subsequently, Co-IP was carried out by transfecting Flag-NUSAP1 plasmids. We found that NUSAP1 and STAT3 could potentially bind to each other (Figure S7C), and this was further validated by immunoprecipitation and immunofluorescence (Figures 5C,D and S7D). Consistently, the expression of STAT3 downstream target genes was increased in NUSAP1-overexpressing cells; however, there was a negative correlation between the primary
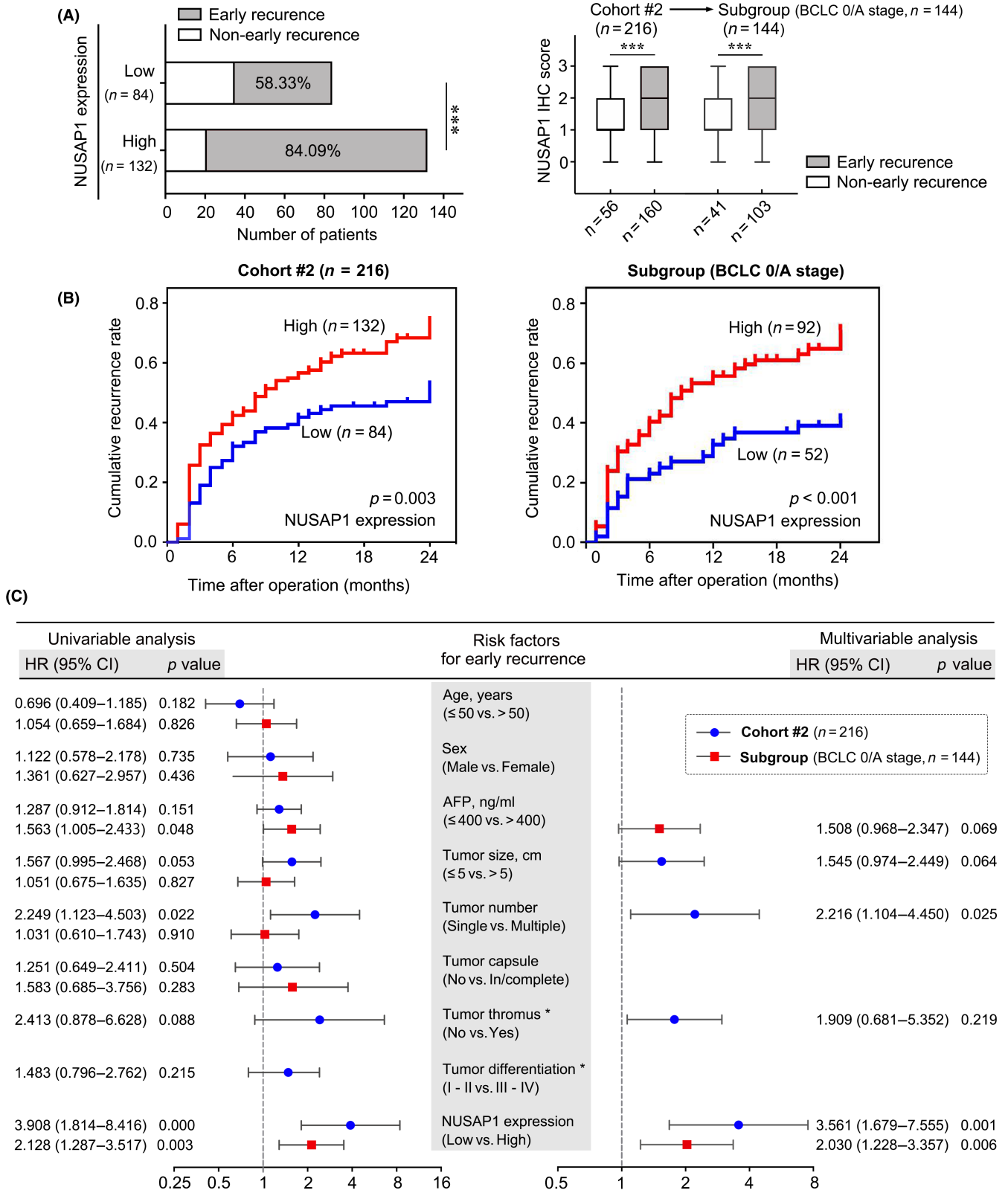
FIGURE 2 Upregulation of nucleolar and spindle associated protein 1 (NUSAP1) contributes to postsurgical early recurrence in hepatocellular carcinoma patients. (A) NUSAP1 expression was related to early recurrence in cohort #2 (left panel). Quantification immunohistochemistry (IHC) score of NUSAP1 expression and early recurrence in cohort #2 (n = 216) and subgroup with Barcelona Clinic Liver Cancer (BCLC) stage 0/A (n = 144) (right panel). (B) Cumulative rates of early recurrence in cohort #2 (n = 216) and their subgroup (n = 144). (C) Univariate and multivariate analyses of potential factors associated with early recurrence for cohort #2 (n = 216) and their subgroup (n = 144). ***p < 0.001. AFP, alpha-fetoprotein; HR, hazard ratio.
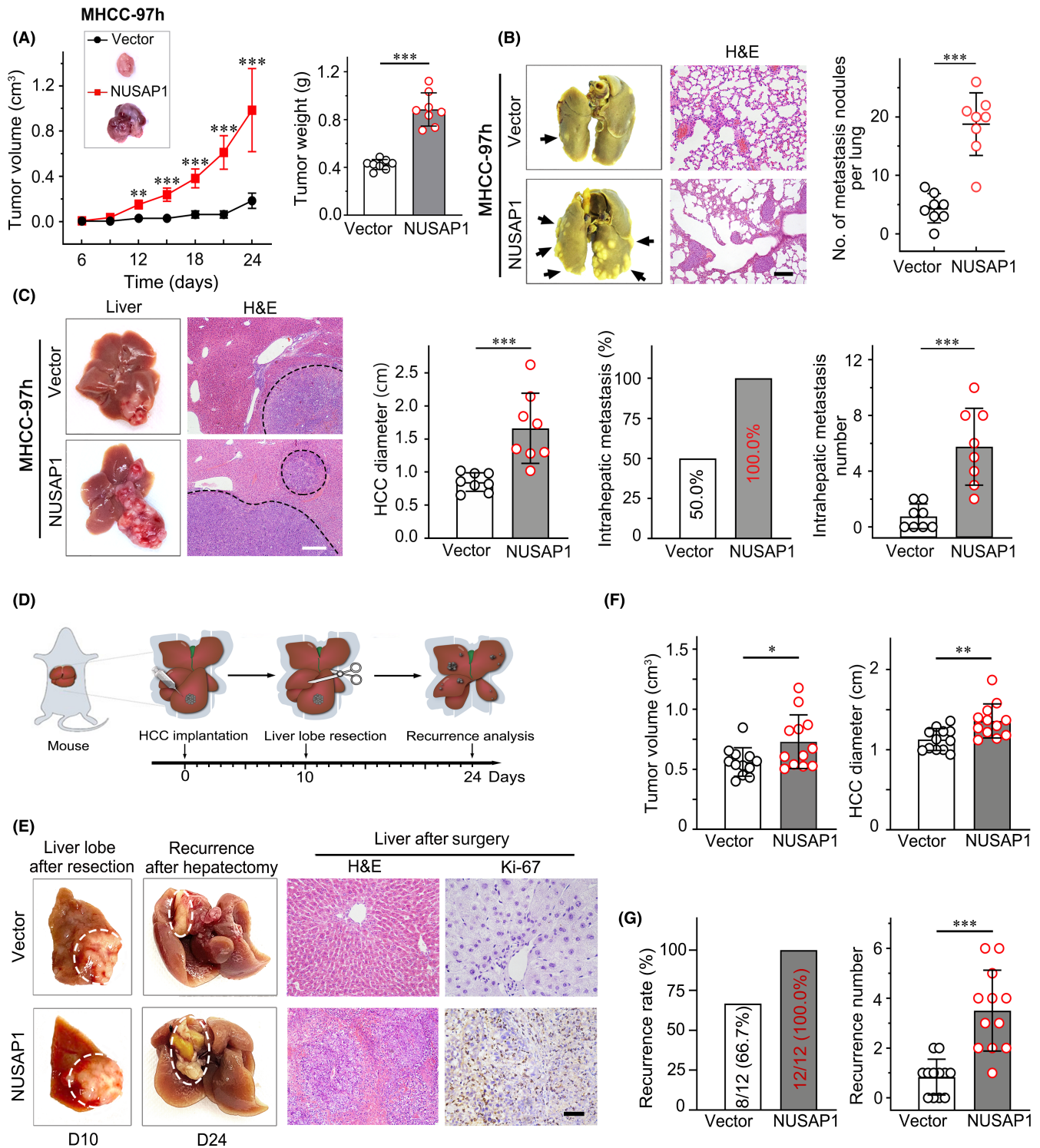
FIGURE 3 Nucleolar and spindle associated protein 1 (NUSAP1) promotes hepatocellular carcinoma (HCC) progression and postsurgical recurrence in multiple murine models. (A) NUSAP1-overexpressing and NUSAP1-vector MHCC-97h cells were subcutaneously injected into BALB/c nude mice for observation of tumor growth. $n = 8$, each group. (B) Representative lung pictures, H&E staining images, and quantification of lung metastasis numbers of the indicated groups. Scale bar, 100 μm. $n = 8$, each group. (C) Representative liver pictures and H&E staining images of the indicated group are shown in the left panel. Scale bar, 100 μm. Quantification of the orthotopic implantation tumor diameter, intrahepatic metastasis rate, and intrahepatic metastasis number of the indicated mice. $n = 8$, each group. (D) Schematic of the postsurgical recurrence model established in mice with orthotopic implantation. (E) Representative example of orthotopic implantation of tumor on day 10 and recurrence on day 14 after hepatectomy (left panel). $n = 12$ for each group. Representative H&E staining and immunohistochemical staining for Ki-67 in HCC recurrence tissues between the indicated groups (right panel). Scale bars, 100 μm. (F) Quantification of tumor volume and diameter of the indicated group on day 10 before hepatectomy. (G) Quantification of the recurrence rate and number of indicated groups at day 14 after hepatectomy. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
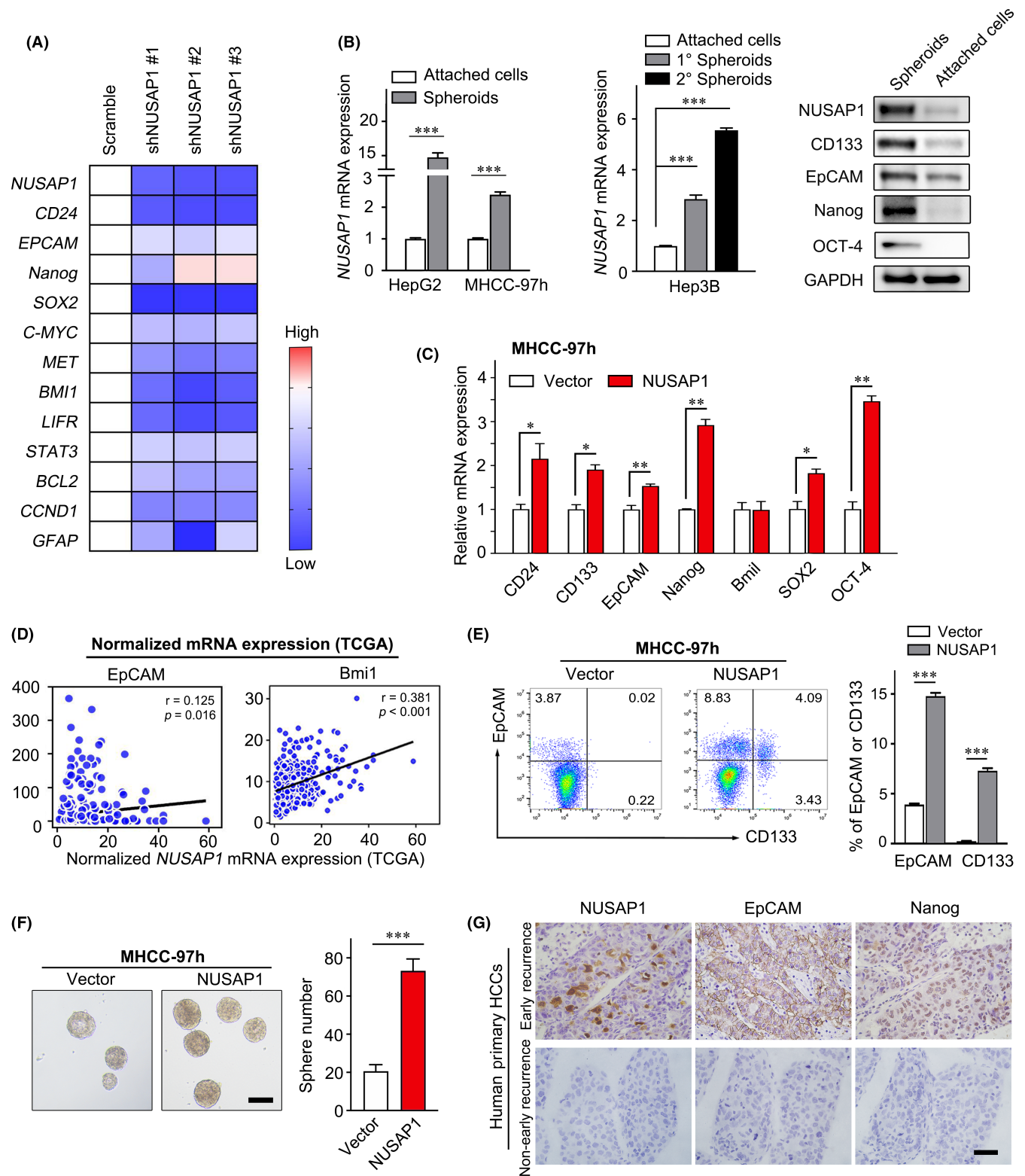
FIGURE 4 Nucleolar and spindle associated protein 1 (NUSAP1) promotes cancer stem cell properties to facilitate early recurrence of hepatocellular carcinoma (HCC). (A) Main stemness-related markers and signal transducer and activator of transcription 3 (STAT3) signaling pathways indicated by RNA sequencing. (B) NUSAP1 expression in hepatoma spheroids and serial passages of HCC cell spheroids was examined by real-time PCR (left and middle). Expression of NUSAP1, CD133, epithelial cellular adhesion molecule (EpCAM), Nanog, OCT-4, and GAPDH was detected by western blotting (right). (C) Expression of liver cancer stem cell markers and stemness-related transcription factors in NUSAP1-overexpressing cells was examined by real-time PCR. (D) Correlation between NUSAP1 mRNA expression and stemness-related transcription factors (EpCAM, Bmi1) in The Cancer Genome Atlas (TCGA) dataset. (E) Flow cytometry analysis of CD133^{+} or EpCAM^{+} population cells. (F) Representative images of spheroids of MHCC-97h. The number of each population was counted and compared. Scale bars, 100 μm. (G) Representative immunohistochemistry of NUSAP1, EpCAM, and Nanog in clinical HCC tissues showing early recurrence after surgery compared with non-early recurrence after surgery (cohort #2). Scale bars, 100 μm. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

activators of STAT3, interleukin-6, and NUSAP1 (Figure S7E). Moreover, a significantly positive correlation between NUSAP1 and p-STAT3 (Y705) was observed in 112 human HCC samples ($p < 0.001$; Figure 5E). Additionally, NUSAP1-induced STAT3 activation (Figure S7F) and self-renewal capacity (Figure 5F) was effectively reversed by stattic, a specific inhibitor of STAT3.

Next, we analyzed protein identification to determine the mechanism of how NUSAP1 regulates STAT3 activation by MS. These data revealed that NUSAP1 and RACK1 were potential binding partners (Figure S7C). It has been reported that RACK1 can act as a specific mediator during the recruitment of STAT3 for activation, and promoted self-renewal of LCSCs and maintained murine embryonic stem cell function.[24,25] Co-immunoprecipitation of Flag-NUSAP1 and HA-RACK1 both in HEK-293T and HCC (HepG2 and Huh7) cells was carried out to confirm the potential interaction between NUSAP1 and RACK1 (Figure 6A,B). Furthermore, the phosphorylation level of STAT3 (Y705) and STAT3 nuclear translocation were significantly inhibited in NUSAP1-overexpressing cells treated with specific siRACK1 (Figure 6C,D). Moreover, flow cytometry analysis of the stem cell (EpCAM$^+$ or CD133$^+$) population showed that RACK1 could reverse tumor stemness induced by NUSAP1 (Figure 6E). In addition, in the subcutaneous implantation model, NUSAP1 overexpression significantly promoted tumor xenograft volumes and weights, while stattic-treated mice could rescue the in vivo phenotypes caused by NUSAP1 overexpression ($p < 0.001$; Figure 6F).

Taken together, our data indicated that NUSAP1 promoted STAT3 activation through RACK1 to facilitate stem cell-like properties in HCC.

3.6 | Validation cohort: NUSAP1 effectively predicts early recurrence of HCC

Alpha-fetoprotein, the most widely used biomarker for HCC diagnosis, has also been applied as a predictive biomarker of HCC recurrence.[4] Here, we compared the predictive effect on HCC recurrence between NUSAP1 and AFP in our discovery cohort. Notably, NUSAP1 showed a similar sensitivity but much better specificity on prediction of HCC recurrence than AFP (AUC 0.816, sensitivity 70%, and specificity 94.12% vs. AUC 0.680, sensitivity 72.96%, and specificity 64.71%; $p = 0.087$). In addition, combined scores of AFP and NUSAP1 (AUC 0.822, sensitivity 68.55%, and specificity 94.12%) showed a similar sensitivity and specificity on prediction of HCC early recurrence than NUSAP1 ($p = 0.465$) (Figure 7A). Moreover, NUSAP1 also showed promising predictive potential for AFP-negative HCC (both *AFP* < 400 ng/ml and AFP < 20 ng/ml) (Figure S8A). Next, an independent validation cohort study of 112 participants was undertaken to confirm the role of NUSAP1 in early recurrence (the patient enrollment flowchart is shown in Figure 7B, and the baseline demographic data are provided in Table S1). In this validation cohort, we found that NUSAP1 expression was strongly correlated with tumor size ($p = 0.002$), abnormal AFP levels ($p = 0.007$), the presence of tumor thrombus ($p = 0.001$), poorer BCLC stage, as well as

early recurrence ($p = 0.001$) (Figures 7C and S8B,C). Subsequently, patients were divided into two groups based on the presence or absence of early recurrence; patients with high NUSAP1 expression was more prone to early recurrence (Figure 7D). The multivariate model indicated that NUSAP1 upregulation and tumor differentiation were independent risk factors for early recurrence ($p = 0.002$ and 0.019, respectively; Table 2). Moreover, Kaplan–Meier survival analysis showed that NUSAP1 could effectively predict early recurrence, even within 1 year after radical hepatectomy (both $p < 0.001$; Figure 7E). Furthermore, we undertook a subgroup analysis according to serum AFP levels,[26,27] and we found that NUSAP1 could effectively predict postsurgical HCC recurrence in patients with low AFP level (serum AFP levels <400 ng/ml; $p < 0.05$; Figure 7F).

Collectively, these data suggested that NUSAP1 might serve as a sensitive predictor for early recurrence of HCC, even in these AFP-negative patients.

4 | DISCUSSION

Early recurrence (within 2 years after hepatectomy) is the primary cause of poor outcomes among HCC patients who underwent radical hepatectomy, and identification of predictive biomarkers for early recurrence is an unmet need. In the current study, we found that NUSAP1 facilitated cancer stemness by activating STAT3 signaling, thus promoting HCC postsurgical recurrence and metastasis. Moreover, we showed that NUSAP1 could serve as a potential biomarker for prediction of HCC early recurrence, especially in AFP-negative patients.

Nucleolar and spindle associated protein 1 is a microtubule and chromatin-associated protein, which regulates cell proliferation by promoting microtubule aggregation, spindle assembly, chromosome segregation, and cytokinesis.[5,28] As a mitosis-related protein, NUSAP1 is involved in diverse physiological and pathological processes, including embryogenesis and carcinogenesis.[29,30] Previous studies have highlighted the malignant role of NUSAP1 in several types of cancers, including gastric cancer, prostate cancer, non-small-cell lung cancer, as well as HCC.[16,31–35] It has been reported that NUSAP1 is upregulated in HCC tissues and predicts poor prognosis.[8,36,37] However, very few studies have focused on illustrating the role of NUSAP1 in HCC postsurgical early recurrence. In the present study, we found that patients with high NUSAP1 expression were more likely to experience recurrence within 2 years after surgical resection. Moreover, multivariate analysis indicated that NUSAP1 was an independent risk factor for early HCC recurrence. Furthermore, our data showed that NUSAP1 overexpression promotes intrahepatic and pulmonary metastasis in a postsurgical recurrence mouse model, which could partially explain the predictive effect of NUSAP1 on early HCC recurrence. Of note, although HCC metastasis includes intrahepatic metastasis and multicentric carcinogenesis, it is difficult to distinguish these two types of metastasis. Based on our data, we speculated that NUSAP1 might promote intrahepatic metastasis but not multicentric carcinogenesis.
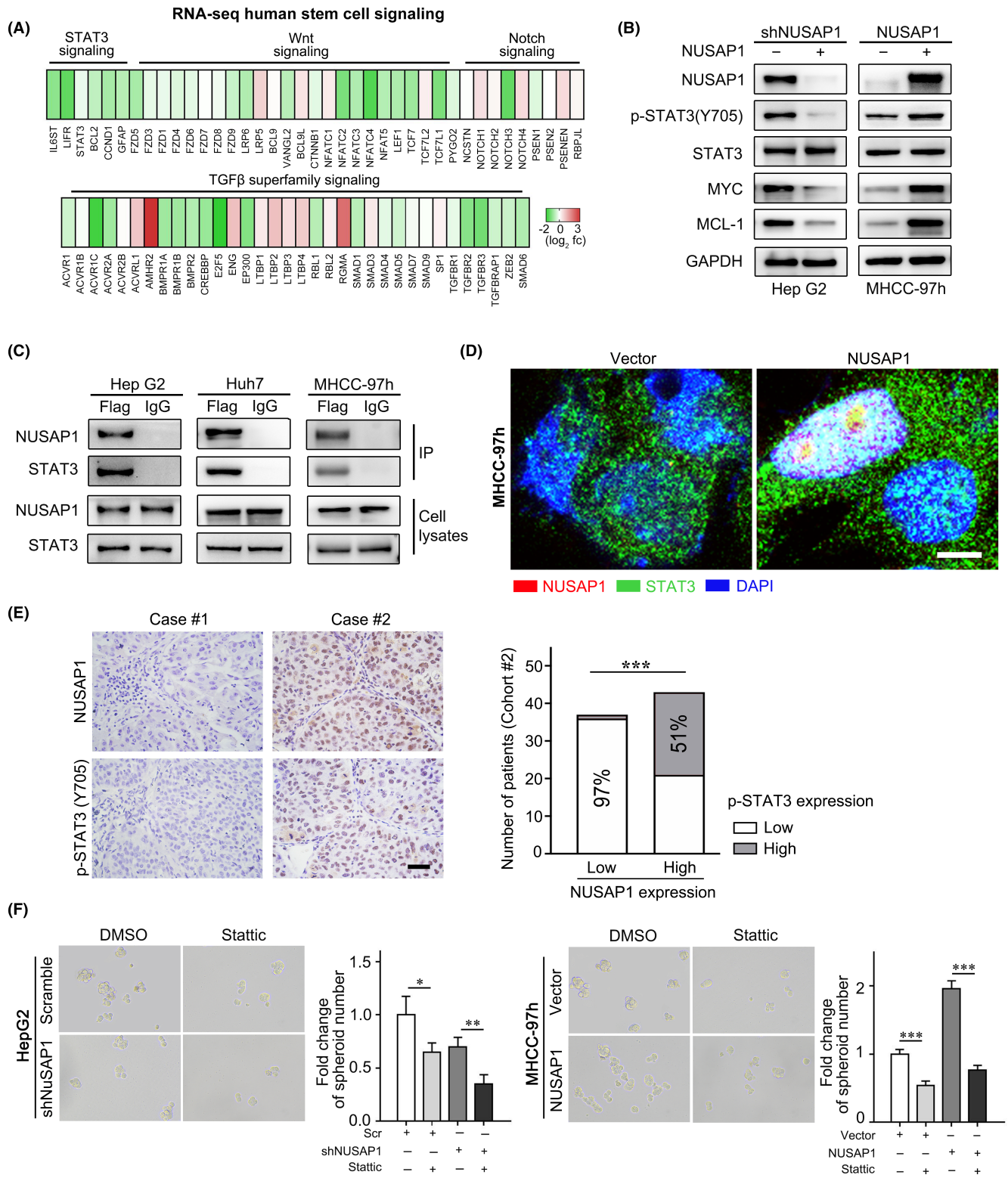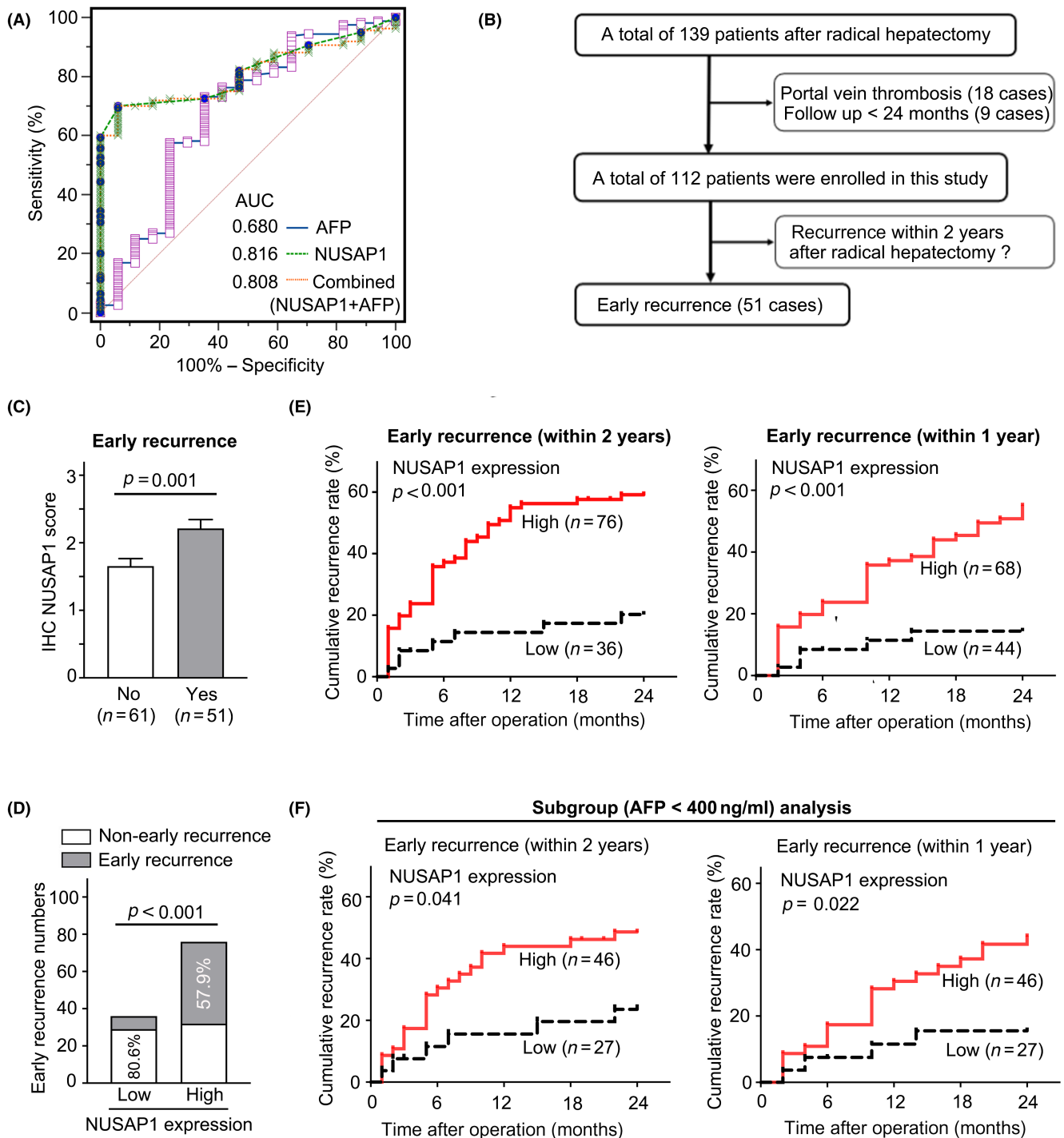
FIGURE 5 Nucleolar and spindle associated protein 1 (NUSAP1) interacts with signal transducer and activator of transcription 3 (STAT3) and activates its transcriptional activity. (A) Indicated main stemness-associated signaling pathways were analyzed. RNA-seq, RNA sequencing; TGFβ, transforming growth factor-β. (B) Phosphorylation of STAT3 (Y705), MYC, and MCl-1 was determined by western blot analysis. (C) Interaction between endogenous NUSAP1 and STAT3 protein was analyzed by co-immunoprecipitation. (D) Representative immunofluorescence images of STAT3 nuclear translocation in MHCC-97h cells. Scale bars, 25 μm. (E) Representative immunohistochemistry images of NUSAP1 and p-STAT3 in human hepatocellular carcinoma samples. Correlation between NUSAP1 and p-STAT3 was detected. Scale bars, 100 μm. (F) Colony-forming capacity of NUSAP1-knockdown or NUSAP1-overexpressing cells was analyzed in the presence of stattic (100 μm) medium for 2 weeks. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
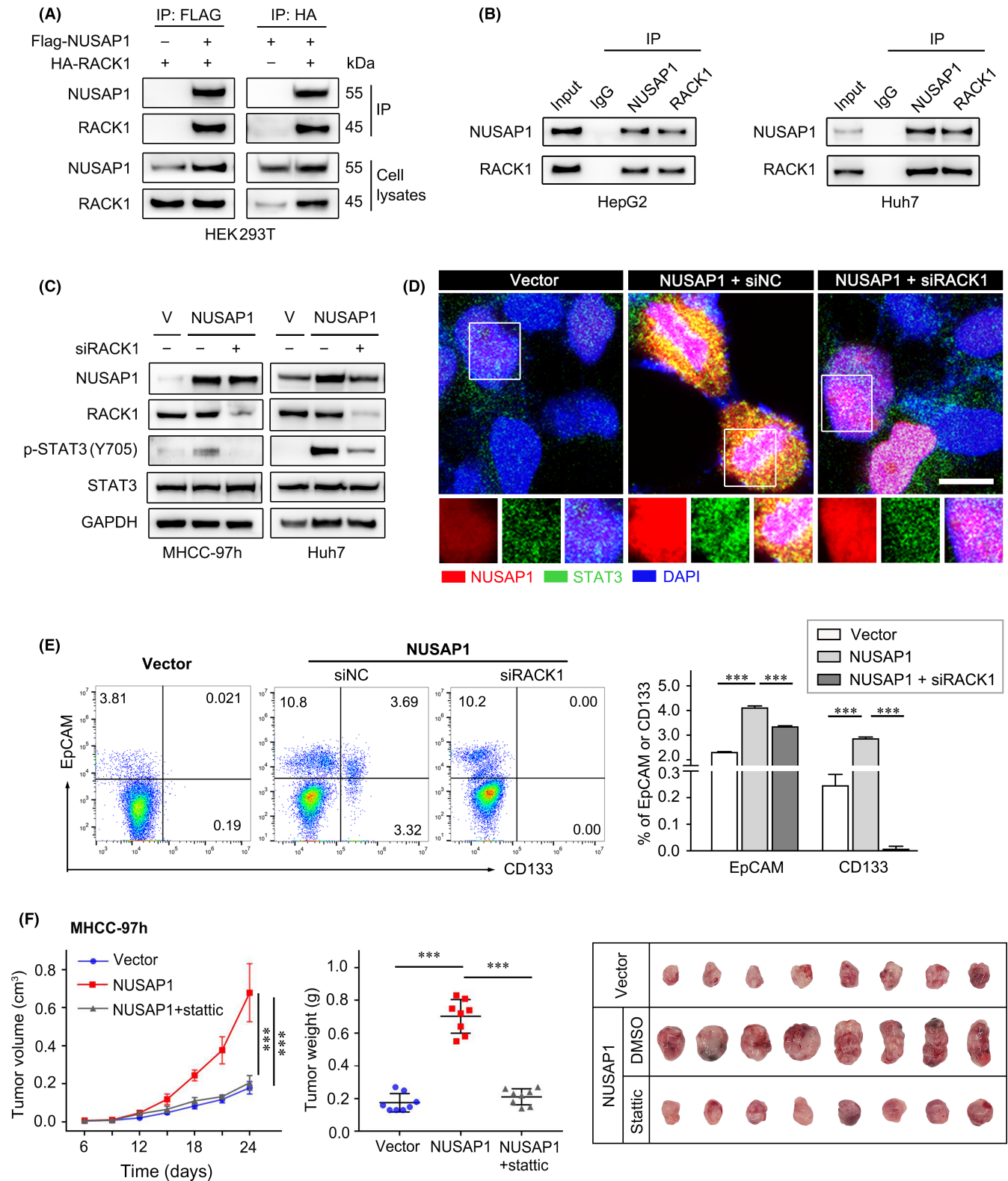
FIGURE 6 Nucleolar and spindle associated protein 1 (NUSAP1) interacts with signal transducer and activator of transcription 3 (STAT3) and activates its transcriptional activity. (A) Co-immunoprecipitation (IP) of Flag-NUSAP1 and HA-RACK1 in HEK-293T cells and (B) HepG2 and Huh7 cells was carried out. (C) Expression of phosphorylated STAT3 (p-STAT3; Y705) and STAT3 in NUSAP1-overexpressing cells treated with siRACK1 was detected by western blotting. (D) STAT3 nuclear translocation in hepatocellular carcinoma cells was analyzed by immunofluorescence, and representative images are shown. Scale bars, 25 μm. (E) Flow cytometry analysis of the CD133^{+} or epithelial cellular adhesion molecule (EpCAM)[+] population in NUSAP1-overexpressing cells treated with siRACK1. Representative results from three independent experiments are shown. (F) Tumor xenograft volumes and weights from different groups were compared. $n = 8$ for each group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

FIGURE 7 Validation cohort: nucleolar and spindle associated protein 1 (NUSAP1) effectively predicts the early recurrence of hepatocellular carcinoma (HCC). (A) Areas under the receiver operating characteristic (ROC) curves (AUC) for NUSAP1 and alpha-fetoprotein (AFP) were compared in cohort #2 ($n = 216$). Immunohistochemistry (IHC) score was used for ROC analysis. (B) Patient enrollment flowchart for our validation cohort (cohort #3, $n = 112$). (C) Quantification IHC score of NUSAP1 expression in early recurrence in validation cohort. (D) Association between NUSAP1 expression levels (IHC score) and early recurrence in validation cohort. (E,F) Cumulative rates of early recurrence in cohort #3 and their subgroup (AFP < 400 ng/ml, $n = 73$) of HCC patients within 1 and 2 years after resection, respectively. Data are presented as mean ± SD.

Serum AFP level is commonly recommended as a biomarker for monitoring postoperative HCC recurrence. However, the prognostic capacity of AFP is still unsatisfactory due to its low sensitivity, particularly in AFP-negative patients.[4,38] Among our exploratory

discovery cohort, we found that NUSAP1 is a better independent biomarker for HCC early recurrence than AFP, which was confirmed by an independent validation cohort study. Consistently, patients with higher NUSAP1 expression showed a higher early recurrence

TABLE 2 Univariate and multivariate analysis of factors associated with early recurrence in 112 hepatocellular carcinoma patients

Variable	Rate of recurrence within 2 years (%)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	p value	Hazard ratio (95% CI)	p value
Age, years (≤50 vs. >50)	45.0 vs. 47.2	1.052 (0.469–2.360)	0.902	NA	NA
Sex (female vs. male)	43.8 vs. 45.8	0.901 (0.406–2.000)	0.798	NA	NA
HBsAg (negative vs. positive)	52.9 vs. 43.5	0.796 (0.386–1.641)	0.536	NA	NA
AFP, ng/ml (≤400 vs. >400)	38.4 vs. 59.0	1.852 (1.066–3.220)	**0.029**	1.799 (0.870–3.722)	0.113
ALT, U/L (≤50 vs. >50)	42.1 vs. 52.8	1.360 (0.771–2.400)	0.288	NA	NA
AST, U/L (≤40 vs. >40)	36.4 vs. 58.7	1.892 (1.090–3.282)	**0.023**	1.158 (0.595–2.254)	0.667
Liver cirrhosis (no vs. yes)	47.5 vs. 44.4	0.805 (0.456–1.422)	0.455	NA	NA
Child–Pugh score (A vs. B)	41.8 vs. 76.9	2.684 (1.337–5.387)	**0.005**	1.902 (0.783–4.619)	0.155
Tumor size, cm (≤5 vs. >5)	33.3 vs. 58.2	2.215 (1.253–3.914)	**0.006**	1.482 (0.690–3.182)	0.313
Tumor number (single vs. multiple)	38.5 vs. 70.8	2.124 (1.184–3.812)	**0.012**	1.646 (0.609–4.445)	0.326
Tumor capsule (no vs. incomplete/complete)	37.5 vs. 45.6	1.225 (0.381–3.940)	0.733	NA	NA
Microvascular invasion (no vs. yes)	41.7 vs. 100	5.353 (2.202–13.011)	**0.006**	1.487 (0.584–3.785)	0.405
Tumor differentiation (I/II vs. III/IV)	30.6 vs. 58.0	2.219 (1.188–4.143)	**0.012**	2.338 (1.152–4.745)	**0.019**
Tumor stage, BCLC (0/A vs. B/C)	36.3 vs. 68.8	2.511 (1.438–4.385)	**0.001**	1.295 (0.405–4.135)	0.663
AFP, IHC (negative vs. positive)	41.9 vs. 45.5	1.225 (0.684–2.196)	0.495	NA	NA
NUSAP1 expression (low vs. high)	19.4 vs. 57.9	3.812 (1.713–8.483)	**0.003**	4.492 (1.727–11.685)	**0.002**

Abbreviations: AFP, alpha fetoprotein; ALT, alanine transaminase; AST, aspartate transaminase; BCLC, Barcelona Clinic Liver Cancer; HBsAg, hepatitis B surface antigen; IHC, immunohistochemistry; NA, not included in analysis.
Bold values ($p < 0.05$) are statistically significant.

rate. Importantly, NUSAP1 effectively predict HCC early recurrence for AFP-negative patients before resection. Although more extensive clinical studies are needed to further evaluate the clinical value of NUSAP1, the predictive effect of NUSAP1 on HCC early recurrence would be of great value for precise stratification of patients to determine optimal treatment and follow-up.

Cancer stemness is a key player in promoting HCC recurrence.[13,39,40] A study showed that NUSAP1 promotes metastasis of cervical cancer by enhancing CSC properties and EMT progression.[33] Herein, we explored whether NUSAP1 promotes HCC early recurrence by enhancement of cancer stemness. We found that numerous stemness-related markers, including EpCAM and CD133,[41] were markedly downregulated in HCC cells with NUSAP1 knockdown. Consistently, upregulation of NUSAP1 was detected in self-renewing spheroids with enhanced cancer stemness. Our data also showed that, compared with nonrecurrent HCC tissue, NUSAP1 expression was positively correlated with EpCAM and Nanog in early recurrent HCC tissues.

Studies showed that STAT3 plays a key role in regulating cancer stemness in many tumor types, including HCC.[42,43] Consistently, our data indicated that NUSAP1 promotes tumor stemness by activating STAT3 signaling, and inhibition of STAT3 rescues the prometastatic phenotypes caused by NUSAP1 overexpression. To further explore how NUSAP1 regulates STAT3 activation, IP-MS was carried out, and we found that NUSAP1 and RACK1 were potential binding partners. A study indicated that RACK1 serves as an adaptor for STAT3 activation in ovarian cancer cells.[24] In our study, phenotypic rescue experiments showed that NUSAP1-maintained tumor stemness and

STAT3 activation were reversed by knockdown of RACK1. These results suggest that NUSAP1 promotes STAT3 activation through RACK1 and facilitates stem cell-like properties in HCC.

In the present study, we found that NUSAP1 could serve as a clinical biomarker for predicting postsurgical HCC early recurrence, especially for AFP-negative patients. Assessment of NUSAP1 expression could help to identify patients with a high risk of postoperative early recurrence who might benefit from timely and active personalized adjuvant therapy after primary tumor resection.

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ETHICS STATEMENT

Approval of the research protocol by an institutional review board: All liver samples were obtained under protocols approved by the First Affiliated Hospital of Jinan University Office for Protection of Human Subjects.

Informed consent: Informed consent was obtained from all patients.

Registry and the registration no. of the study/trial: N/A.

Animal studies: Animal experiments were approved by the Animal Care and Use Committee at Jinan University and performed according to established guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.