Research Article

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14-3-3η is a novel growth-promoting and angiogenic factor in hepatocellular carcinoma

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Background & Aims: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. The continued search for novel therapeutic strategies for HCC is urgently required. In this study, we aimed to investigate the functions and clinical significance of 14-3-3η protein in HCC.

Methods: Expressions of genes and proteins were determined by quantitative reverse transcription polymerase chain reaction, Western blot, and immunohistochemistry. Their functions were assessed by endothelial cell recruitment, tube formation, wound healing, flow cytometry, immunostaining, immunoprecipitation, and xenograft assay. A tissue microarray followed by univariate and multivariate analyses was performed to indicate the clinical significance.

Results: In HCC specimens, overexpression of 14-3-3η was observed not only in tumors but also in intratumoral vessels. In HCC and vascular endothelial cells, 14-3-3η stimulated proliferation and angiogenesis, but attenuated the functions of sorafenib. Briefly, 14-3-3η facilitated the phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2). Then, by binding to the phosphorylated-ERK1/2 (p-ERK1/2), formed a functional positive feed-back loop. A xenograft model showed that, blockage of either 14-3-3η or ERK1/2 inhibited the tumor growth. Finally, tissue microarray analyses showed that overexpression of 14-3-3η, either in tumors or intratumoral vessels, contributed to the poor survival.

Conclusions: The 14-3-3η-ERK1/2 feedback loop played a characteristic growth-promoting role in HCC, not only in tumors but also in intratumoral vessels. Further, 14-3-3η could be a potential therapeutic target for HCC and a biomarker for predicting sorafenib treatment response.

Lay summary: Here we found that, 14-3-3η protein exhibited a characteristic growth-promoting effect in both tumor and intratumoral vessels of hepatocellular carcinoma by interacting with ERK1/2 signaling.

Keywords: Hepatocellular carcinoma; 14-3-3η; Extracellular signal-regulated kinase1/2; Tumor growth; Angiogenesis.

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Abbreviations: Cxcl1, C-X-C motif-ligand 1; ECs, endothelial cells; EMT, epithelial to mesenchymal transition; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FRA-1, fos related antigen 1; NK-κB, nuclear factor kappa B; PCNA, proliferating cell nuclear antigen; TGF-β, transforming growth factor beta; TUNEL, transferase-mediated deoxyuridine triphosphate-biotin nick end labeling; VEGF, vascular endothelial growth factor; Zeb-1, zinc finger E-box-binding protein-1.

Introduction

Hepatocellular carcinoma is one of the most common solid tumors and the second leading cause of cancer-related mortality worldwide [1,2]. Current standard curative practices for treatment of HCC are the liver resection or liver transplantation, however, the long-term outcome after these therapies is less than satisfactory because of the high post-surgical recurrence [3]. Consequently, the continued search for novel therapeutic strategies for HCC is urgently needed.

The 14-3-3 proteins are a family of approximate 28 to 33 kDa acidic polypeptides; there are seven mammalian isoforms of the 14-3-3 protein (α/β, γ, σ, ε, ζ, η, and θ/τ), which regulate multiple cellular functions via interactions with intracellular proteins by phosphoserine and phosphothreonine bindings [4,5]. Studies indicate that 14-3-3 proteins can regulate cancer cell proliferation, survival, migration/invasion, and function as potential therapeutic targets [6,7]. Up to date, five members of 14-3-3 family proteins (α/β, γ, σ, ε, and ζ) have been identified to be involved in the HCC progression, including tumor growth, metastasis, and resistance to sorafenib [8–13], however, the functions and clinical significances of 14-3-3θ/τ and η in HCC are still largely uninvestigated.

Cancer

Here we identified that overexpression of 14-3-3η was observed both in HCC tumors and intratumoral vessels, and that, overexpression of 14-3-3η prognosticated poor overall/recurrence-free survival. In the context of the molecular mechanisms involved, 14-3-3η and phosphorylated extracellular signal-regulated kinase (p-ERK)1/2 formed a functional positive feed-back loop, which enhanced cellular proliferative and angiogenic abilities, but attenuated the functions of sorafenib. Collectively, our findings have provided the underlying mechanisms of 14-3-3η in the growth and angiogenesis of HCC, and implicated 14-3-3η as a potential prognostic biomarker and therapeutic target for HCC.

Materials and methods

Patients and tissue microarray

This study was approved by Medical Ethics Committee of Nanjing Medical University, and the participants' written informed consents were obtained from each patient for the study of tissue excised from surgical specimens. A cohort of 256 Chinese HCC patients was enrolled in this study, the clinic-pathologic data was listed in Supplementary Table 1. To validate the clinical significance of 14-3-3η in HCC, we performed a tissue microarray constructed by Shanghai Zhuoli Biotechnology Co., Ltd (Zhuoli Biotechnology Co, Shanghai, China). In each case, 1–2 μm thick sections from paraffin tissue blocks were cut, dewaxed, pretreated and transferred to glass slides used with an adhesive tape transfer system, in order to carry out ultraviolet cross linkage. All reactions were performed on an automated staining device. The quantitation of immunostaining for 14-3-3η was completed by two independent researchers who were blinded regarding patient details. The immunostaining score of 14-3-3η in tumor parenchyma was semi-quantified by Quick-score (Q-score) based on intensity and heterogeneity [13]. The positive rates were scored as 0 point (0%), 1 point (1–25%), 2 points (26–50%), 3 points (51–75%), and 4 points (76–100%). The score of the staining intensity was presented as 0 point (none), 1 point (low), 2 points (medium), and 3 points (high). The Q-score was the sum of heterogeneity and intensity. The expression was defined as positive/high when the combination scores were $\geqslant$2/4. The immunostaining in intratumoral vessels was merely scored intensity (none, low, medium, and high to 0, 1, 2, 3) and the expression was defined low when the score $\leqslant$1.

Cell culture and transfection

HepG2, HuH7, and human umbilical vein endothelial cell line (HUVECs), were obtained from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The MHCC97H cells were obtained from the Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China). These cells were identified by China Center for Type Culture Collection (Wuhan, China). Cells were maintained in a 37 °C humidified incubator with 5% CO_2. HepG2, HuH7, and MHCC97H cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies/Gibco, Grand Island, NY), while HUVECs were cultured in extracellular matrix (ECM) medium (Invitrogen, Carlsbad, USA). The mediums were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 100 μg/ml heparin, and 30 μg/ml ECs growth supplement (for HUVECs, Sigma-Aldrich, MO, USA). Phenol red was added into the medium to reflect the pH. A mycoplasma stain assay kit (Beyotime Co. Ltd, Haimeng, China) was used for mycoplasma testing. For cell transfection, the pcDNA-3.1-14-3-3η-FLAG plasmid that overexpressed both 14-3-3η and FLAG was created by inserting the coding sequences of 14-3-3η (YWHAH, 741 bp) into pcDNA3.1 plasmid, followed by adding a FLAG-tag at its N-terminal (Generay Biotech Co. Ltd, Shanghai, China). The commercial specific 14-3-3η small interfering (si)RNA was purchased from Santa Cruz Biotechnology (http://datasheets.scbt.com/sc-43581.pdf). Cells were transiently transfected using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol. Briefly, cells were seeded in 6-well plates at a density of 1×10^5 per well. After 24 h, these cells were transfected with 5 ng/ml vector-Con or 14-3-3η-FLAG, or 20 nM si-Con or si-14-3-3η for 12 h. After transfection, such cells were cultured in fresh medium supplemented with 10% FBS for another 24 h before being used for other experiments.

Animals and xenografts

This study was approved by Nanjing Medical University Institutional Animal Care and Use Committee, and animals were treated humanely and with regard for alleviation of suffering. Briefly, the BALB/c nude mice were obtained from SLRC laboratory animal center (Shanghai, China), and kept in a specific pathogen-free and temperature-controlled environment (20–22 °C) with a 12 h light dark cycle and with free access to drinking water and chow. For xenograft study, 2×10^6 cells in 100 μl matrigel were injected subcutaneously into the right armpit of the mice (5 mice per group) for 3 weeks. To determine the effects of 14-3-3η or ERK1/2 on the *in vivo* growth of HCC, we performed the intratumoral injection assay. Briefly, 100 μl of siRNA (si-Con or si-14-3-3η, 100 nM) or 100 μl of ERK1/2 inhibitor, U0126 (20 μM, Beyotime) were intratumoral injections every 3 days. Tumors were measured every 3 days and their volumes were calculated using the formula: $V = \frac{1}{2}(\text{width}^2 \times \text{length})$. After 21 days, the mice were sacrificed, and tumor tissues were removed for further investigation.

Please see the Supplementary materials and methods section for additional procedures.

Results

Identification of 14-3-3η as a characteristic cancer-promoting factor in HCC

We initially evaluated the expression of 14-3-3η in HCC patients. As shown in Fig. 1A, compared with adjacent non-tumor liver tissues, a considerable elevation of *14-3-3η* mRNA was observed in HCC tissues (Fig. 1A). Moreover, overexpression of 14-3-3η protein was also demonstrated in HCC tissues by immunohistochemistry (IHC) (Fig. 1B; Supplementary Fig. 1). Interestingly, in addition to the well-defined location in tumor cells, 14-3-3η showed a continuously capillary endothelial staining, which exhibited a different characteristics compared to the other six family members (Supplementary Fig. 2). As HCC is a highly vascularized tumor [14], we hypothesized that the higher expression of 14-3-3η might be associated with more aggressive types of tumors. To confirm this hypothesis, we then divided these HCC specimens into two groups ("14-3-3η low" *vs.* "14-3-3η high", Supplementary Fig. 3). As shown in Fig. 1C, the transverse diameters of tumors in 14-3-3η high group were significantly larger than those in 14-3-3η low group. Moreover, compared with well differentiated HCC tissues, overexpression of 14-3-3η was observed in poorly differentiated HCC tissues (Fig. 1D, E; Supplementary Fig. 4). A stronger staining for 14-3-3η was also observed with increasing TNM Classification of Malignant Tumors stage (Fig. 1F, G, and Supplementary Fig. 5). Collectively, these results suggested that 14-3-3η had a characteristic role in leading HCC progression at least in part through promoting the growth and neovascularization both in carcinoma and vascular ECs.

Effects of 14-3-3η on the proliferative, anti-apoptotic, and angiogenic abilities in HCC cells

We analyzed the expression of 14-3-3η in a panel of well-established human HCC cell lines [15]. As shown in Supplementary Fig. 6, the expressions of 14-3-3η were increased in six HCC cell lines compared to the non-transformed hepatic cell line, L02. Interestingly, in HepG2 cells (relative lower expression of 14-3-3η), forced expression of 14-3-3η shortened the doubling time and promoted the cell cycle transition (Fig. 2A and B). Moreover, these cells exhibited increased expressions of proliferating

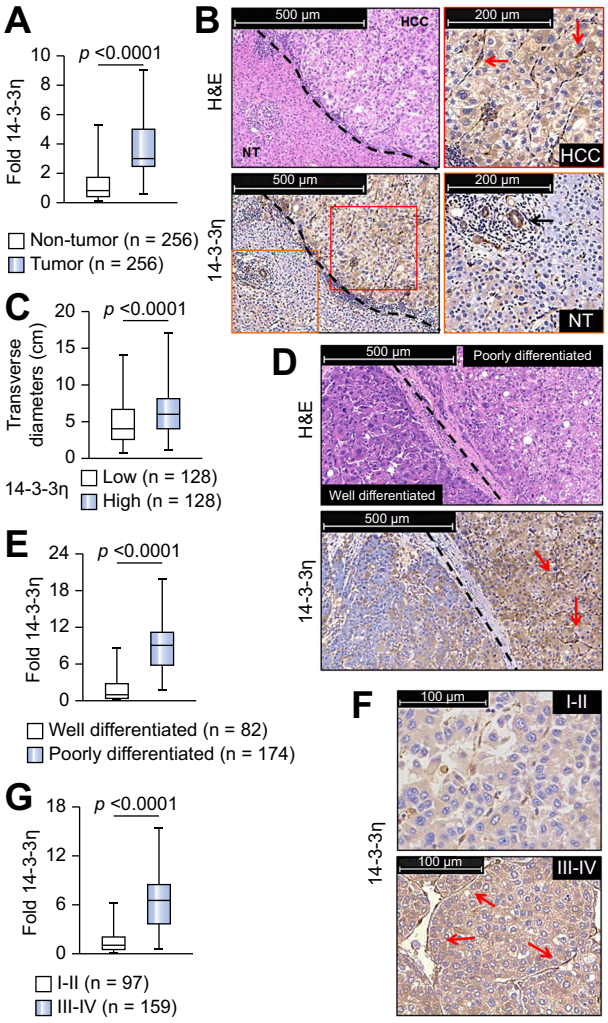


Fig. 1. Identification of 14-3-3η as a characteristic cancer-promoting factor in HCC. (A) qRT-PCR analyses in triplicate and (B) IHC staining of 14-3-3η in HCC and adjacent tissues (NT). (C) The transverse diameters of tumors in 14-3-3η high and low groups. (D) IHC staining and (E) qRT-PCR analyses in triplicate of the 14-3-3η in HCC with different differentiated degrees. (F) IHC staining and (G) qRT-PCR analyses in triplicate of the 14-3-3η in HCC tissues with different TNM stage. (This figure appears in colour on the web.)

cell nuclear antigen (PCNA), cyclinD1, and survivin (Supplementary Fig. 7A). In contrast, knockdown of 14-3-3η in HuH7 and MHCC97H cells (relative higher expressions of 14-3-3η) showed the opposite effects and enhanced the apoptosis induced by doxorubicin (Fig. 2A and C; Supplementary Fig. 7B–C, Supplementary Fig. 8). Angiogenesis is a prerequisite for cancer growth, during which, cancer cells secrete several proinflammatory and proangiogenic chemokines, such as C-X-C motif-ligand (Cxcl)1, Cxcl2, vascular endothelial growth factor (VEGF)-A, and VEGF-C, promoting the proliferation and migration in vascular endothelial cells [16,17]. Here, overexpression of 14-3-3η in HepG2 cells elevated the expressions of *Cxcl1*, *Cxcl2*, *VEGF-A*, and *VEGF-C* mRNAs; conversely, knockdown of 14-3-3η in HuH7 and MHCC97H cells showed the opposite effects (Supplementary Fig. 9). Moreover, the 14-3-3η-overexpressed HepG2 cells recruited more HUVECs in comparison with vector control group (Fig. 2D). Further, compared with conditioned medium collected from si-Con-transfected HuH7 or MHCC97H cells, tube formation was reduced dramatically in HUVECs grown in conditioned medium collected from si-14-3-3η-transfected cells (Fig. 2E; Supplementary Fig. 10). Finally, the *in vivo* HuH7 xenografts assay showed that knockdown of 14-3-3η inhibited the tumor growth and the expressions of Ki67 and CD34 (Fig. 2F and G), but increased the apoptosis (as determined by TUNEL staining, Fig. 2H). Collectively, these results indicated that 14-3-3η could improve the proliferative and angiogenic abilities in HCC cells.

Effects of 14-3-3η on the proliferative and migrated abilities in vascular ECs

Studies have indicated that, compared with HCC-derived vascular ECs, the immortalized HUVECs have almost the same proliferative and migrated abilities [18]. Here, overexpression of 14-3-3η shortened the doubling time in HUVECs, and these cells expressed increased levels of PCNA and survivin; however, knockdown of 14-3-3η showed the opposite effects (Fig. 3A; Supplementary Fig. 11). Furthermore, compared with si-Con-transfected HUVECs, the mobility and the tube formation ability of 14-3-3η-knockdown cells were significantly decreased (Fig. 3B-C). Similar results were observed in another vascular endothelial cell line, HMEC-1 (Supplementary Fig. 12). Collectively, these results indicated that 14-3-3η enhanced the proliferative and migrated abilities in vascular ECs.

Effects of 14-3-3η on the tumor growth and angiogenesis in an MHCC97H/HUVECs xenografts model

To mimic the crosstalk between HCC cells and ECs, and to further determined the effects of 14-3-3η on the *in vivo* tumor growth and angiogenesis, we conducted an MHCC97H/HUVECs xenografts model (Supplementary Fig. 13). Similar to Fig. 2F, knockdown of 14-3-3η also significantly inhibited the growth of this co-injected xenografts tumor (Fig. 3D). Moreover, IHC assays showed that 14-3-3η protein was mainly located in tumor cells, vascular ECs, as well as the vasculogenic mimicry established by MHCC97H cells. Further, compared with xenografts tumor tissues injected with con-siRNA, the 14-3-3η-siRNA-injected group showed decreased formation of intratumoral capillary tubes, and the lower expressions of PCNA and VEGF-A (Fig. 3E and F). Collectively, these results indicated that, via regulating the proliferative/angiogenic abilities and the crosstalk between carcinoma and vascular ECs, 14-3-3η stimulated the *in vivo* growth of HCC.

14-3-3η activated ERK1/2 by forming a functional complex with phosphorylated ERK1/2

Accumulating studies highlight an important role for ERK1/2 in the development of HCC [9,19]; moreover, several family members of 14-3-3 proteins (α/β, ε, and δ/ζ isoforms) promote the progression of HCC via the activation of ERK1/2 [20]. So we firstly determined the expressions/activations of 14-3-3η and ERK1/2 protein in HCC tissues and analyzed the relationship between these two molecules. Intriguingly, with the increased expression of 14-3-3η, there was more enhanced activation of ERK1/2 (as determined by the phosphorylation on Thr202/Tyr204, Supplementary Fig. 14). Next, we investigated the effects of 14-3-3η on the phosphorylation of ERK1/2. In both HCC and vascular ECs, overexpression of 14-3-3η enhanced the phosphorylation

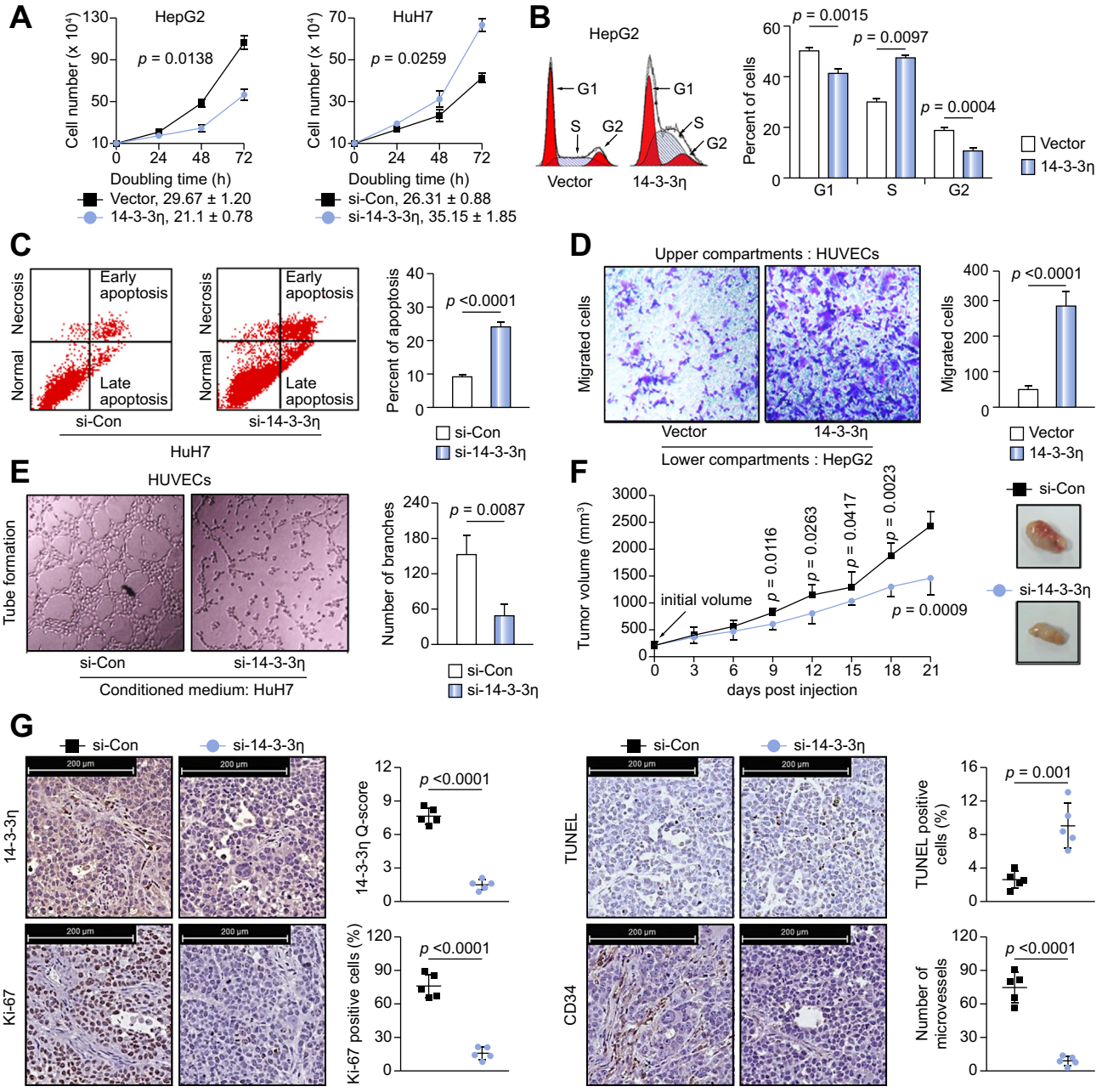


Fig. 2. Effects of 14-3-3η on the proliferative, anti-apoptotic, and angiogenic abilities in HCC cells. (A) Growth kinetics analyses in triplicate in HepG2 and HuH7 cells with different 14-3-3η levels. (B) Flow cytometry analysis in triplicate of cell cycle in vector- or 14-3-3η-transfected HepG2 cells. (C) Flow cytometry analysis in triplicate of apoptosis in si-Con- or si-14-3-3η-transfected HuH7 cells in the presence of doxorubicin. The percent of apoptosis was the sum of early apoptosis (%) and late apoptosis (%). (D) ECs recruitment assay analyses of the effects of 14-3-3η on HepG2-induced recruitment of HUVECs. (E) Capillary tube formation assay analyses of the effects of 14-3-3η on angiogenic ability in HuH7 cells. (F) The volumes of xenografts tumors injected with con-siRNA or 14-3-3η-siRNA. (G, left) IHC and TUNEL staining; (G, right) The Q-scores of 14-3-3η, the Ki67- or TUNEL-positive cells, and the number of microvessel. Note: Each point represented the mean of one xenografts tumor section calculating in 10 high-power fields; The Q-scores of 14-3-3η were the sum of Q-score (tumor) and Q-score (vessel); The number of microvessel was quantitated based on the CD34 staining. (This figure appears in colour on the web.)

of ERK1/2; in contrast, knockdown of 14-3-3η showed the opposite effects (Fig. 4A). As 14-3-3 proteins are the well-known phosphothreonine-binding factors [20], we speculated that there might be a protein-protein binding between 14-3-3η and phosphorylated ERK1/2 (p-ERK1/2) in HCC. We therefore performed a Co-IP assay and found that, in 14-3-3η-transfected HepG2 and HUVECs cells, the 14-3-3η-FLAG was immunoprecipitated with the specific FLAG antibody, and its p-ERK1/2-binding status was confirmed with p-ERK1/2 antibody; in contrast, knockdown of 14-3-3η attenuated this binding effect (Fig. 4B and Supplementary Fig. 15). Then we performed an immunofluorescence staining assay to further confirm our findings. As shown in

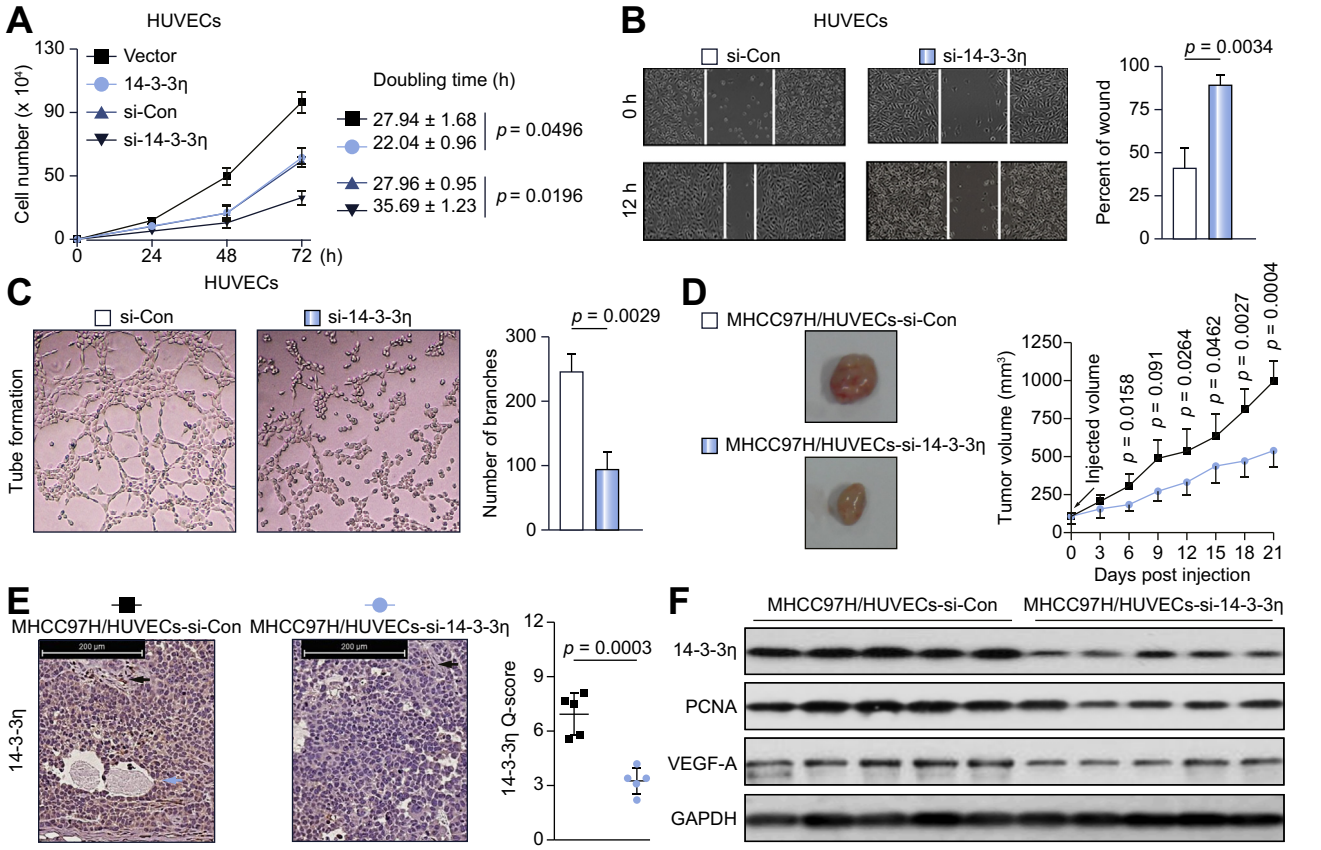


Fig. 3. Effects of 14-3-3η on the proliferative and migrated abilities in vascular ECs and in MHCC97H/HUVECs xenografts: (A) Growth kinetics analyses in triplicate in HUVECs with different 14-3-3η levels. (B) Wound healing assay analyses of the effects of 14-3-3η on the migrated ability in HUVECs. (C) Capillary tube formation assay analyses of the effects of 14-3-3η on the tube formation in HUVECs. (D) The volumes of xenografts tumors injected with con-siRNA or 14-3-3η-siRNA. (E) IHC staining of 14-3-3η, the IHC Q-scores of 14-3-3η were provided as described before in Fig. 2G. Note: the black arrow represents the vascular ECs, while the blue arrow represents the vasculogenic mimicry established by MHCC97H cells. (F) Western blot analyses of the 14-3-3η, PCNA or VEGF-A in con-siRNA- or 14-3-3η-siRNA-treated MHCC97H/HUVECs xenografts tissues. (This figure appears in colour on the web.)

Fig. 4C, the 14-3-3η and p-ERK1/2 were well co-located in the cytosol and partly co-located in the nuclear; furthermore, overexpression of 14-3-3η enhanced the fluorescence intensity of p-ERK1/2. Studies indicate that the translocation of activated ERK1/2 into nuclei can upregulate its well-established substrate, FRA1, in a series of human cancer cell lines, including HCC [9,21]. Here, the up or downregulation of 14-3-3η expression in HCC and ECs resulted in a corresponding increase or decrease in the expression of FRA1, respectively (Fig. 4A). Collectively, these results indicate that, in HCC and ECs, 14-3-3η stimulates the phosphorylation of ERK1/2, and then, by forming a functional complex with p-ERK1/2, 14-3-3η enhances the activity of ERK1/2.

Inhibition of ERK1/2 attenuated the 14-3-3η expression and HCC growth

As 14-3-3η could form a functional complex with p-ERK1/2, we further hypothesized that the activation of ERK1/2 influenced the 14-3-3η expression. Here, in MHCC97H and HUVECs, inhibition of ERK1/2 attenuated the 14-3-3η mRNA and protein level (Fig. 4D and Supplementary Fig. 16). Then we used the MHCC97H/HUVECs xenografts model to further determine the effects of ERK1/2 on the *in vivo* 14-3-3η expression and HCC growth. Similarly to Fig. 3E, blockage of ERK1/2 significantly inhibited the tumor growth (Fig. 4E) and the expressions of 14-3-3η, PCNA and VEGF-A (Fig. 4F and G). Based on our aforementioned findings, we considered that the ERK1/2 cascade might be a potential up-stream regulator, which enhanced the expression of 14-3-3η in HCC and ECs.

14-3-3η attenuated the repressive effect of sorafenib on ERK1/2 and cell viability in HCC and ECs

Studies indicate that by inhibiting the ERK1/2 cascade and blocking the receptor tyrosine kinases, sorafenib functions as the only approve systemic therapy for HCC [9,22]. As 14-3-3η and p-ERK1/2 formed a functional positive feed-back loop, we further tried to assess whether the sorafenib-inhibited ERK1/2 phosphorylation and cell viability could be antagonized by 14-3-3η. As shown in Fig. 5A–C, after the treatment with 10 μM sorafenib, ERK1/2 phosphorylation and cell viability were obviously decreased in vector-transfected HepG2 and HUVECs cells, but only slightly decreased in the 14-3-3η-transfected cells. In contrast, knockdown of 14-3-3η in HuH7 and HMEC-1 cells showed the opposite effects. To further determine the relationship

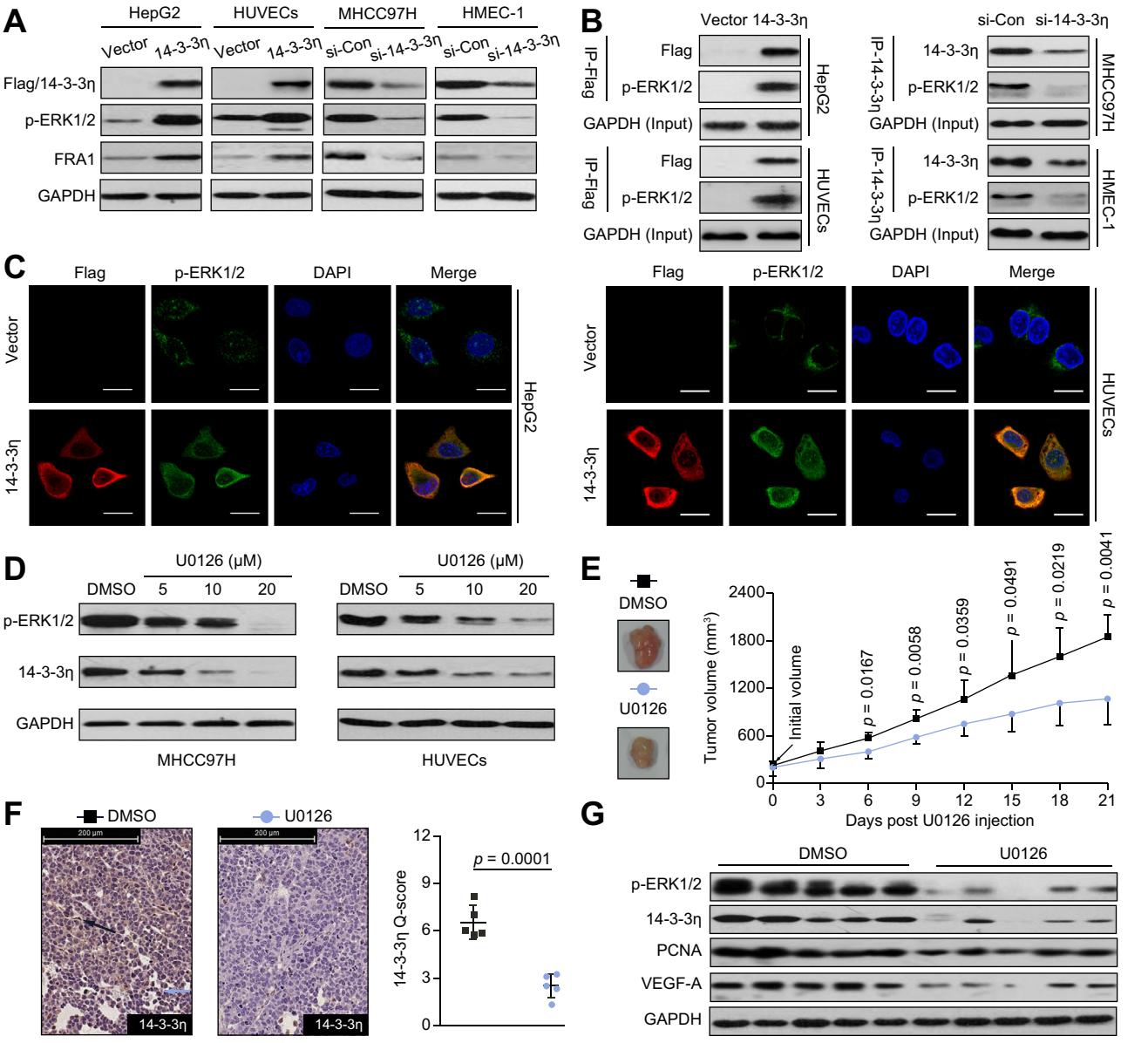
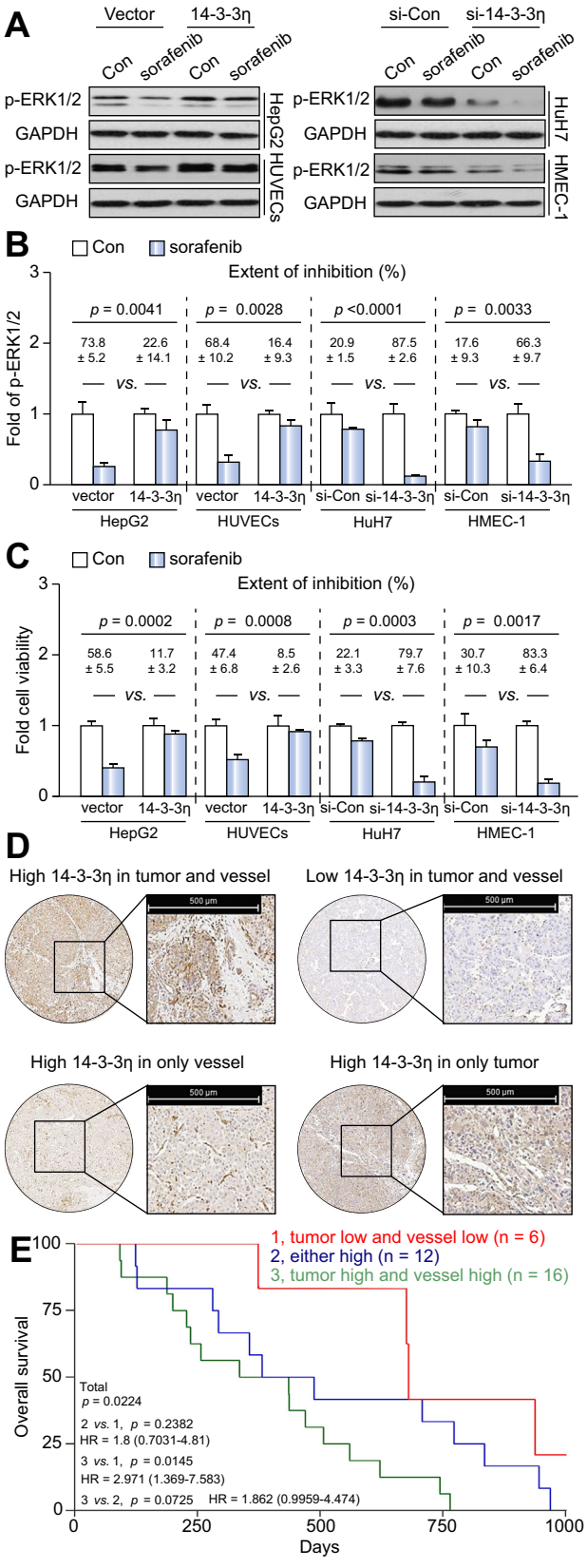


Fig. 4. Involvement of 14-3-3η-ERK1/2 feed-back loop in the growth and angiogenesis of HCC: (A) Western blot analyses of the expressions of 14-3-3η, p-ERK1/2, and FRA1 in HCC and ECs with different 14-3-3η levels. (B) Co-immunoprecipitation and (C) immunostaining analyses of the relationship between 14-3-3η and p-ERK1/2 in HCC and ECs. (D) Western blot analyses of the expressions of 14-3-3η and p-ERK1/2 in HCC and ECs treated by U0126. (E) The volumes of xenografts tumors injected with dimethyl sulfoxide (DMSO) or U0126. (F) IHC staining of 14-3-3η, the IHC Q-scores of 14-3-3η and the representation of black/blue arrows were as described above in Fig. 3E. (G) Western blot analyses of the 14-3-3η, p-ERK1/2, PCNA, and VEGF-A in DMSO- or U0126-treated xenografts tissues. (This figure appears in colour on the web.)

between 14-3-3η expression and sorafenib response, another retrospective data from 34 advanced recurrent HCC patients receiving combined sorafenib treatment and transarterial chemoembolization therapy were analyzed (the clinic-pathologic data was listed in Supplementary Table 2). We divided these tissue samples into "tumor high and vessel high (both high)", "tumor high or vessel high (either high)", and "tumor low and vessel low (both low)" groups (Fig. 5D). Kaplan-Meier survival analysis showed that the overall survival of the "14-3-3η both high" group was much lower than that of "14-3-3η both low" group (Fig. 5E). Collectively, these data indicated that a high level of 14-3-3η could lead to a sorafenib resistance in HCC and ECs, and that 14-3-3η might be a potential biomarker for predicting sorafenib treatment response in advanced recurrent HCC patients.

The clinical significance of 14-3-3η in HCC

As a characteristic cancer-promoting factor, 14-3-3η promoted the *in vivo* growth and neovascularization in both carcinoma and vascular endothelial cells. So we finally validated the clinical significance of 14-3-3η in HCC. The cohort of 256 HCC patients was divided into "14-3-3η both high", "14-3-3η either high", and "14-3-3η both low" groups (Fig. 6A) as described in



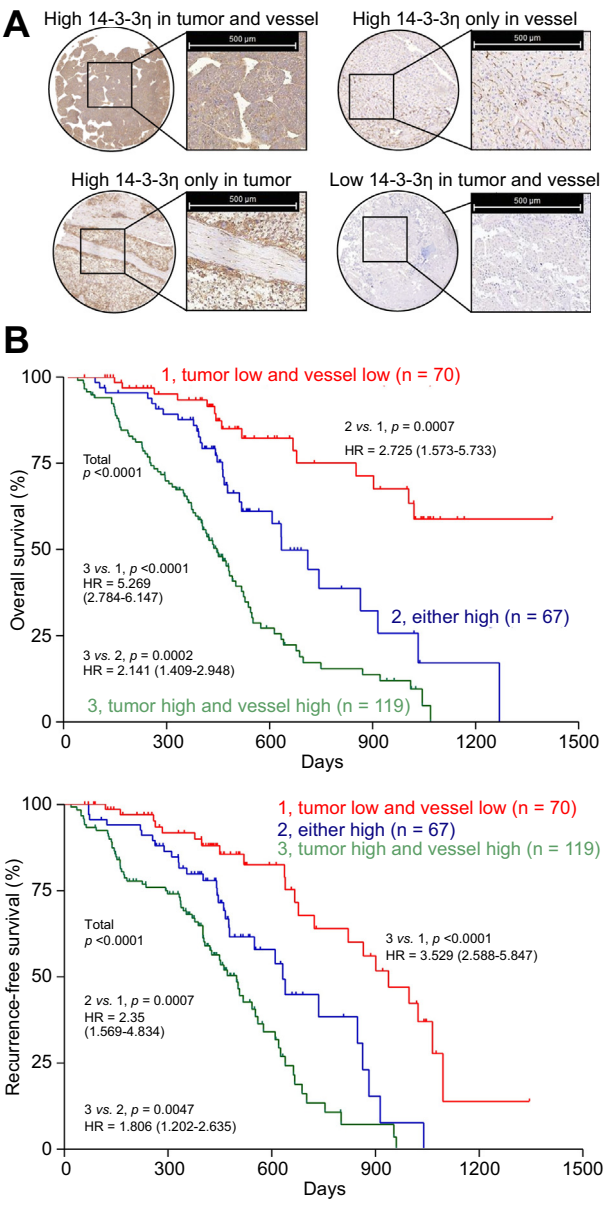


Fig. 6. The clinical significance of 14-3-3η in HCC: (A) Tissue microarray and IHC analyses of the 14-3-3η in 256 HCC patients' tissue samples. (Note: these specimens were collected from surgery when the patients were first diagnosed with HCC). We then divided these specimens into 3 groups as described above. (B) Kaplan-Meier analyses of the prognostic significances of 14-3-3η in these patients. (This figure appears in colour on the web.)

Fig. 5. Effects of 14-3-3η on sorafenib in HCC and ECs: (A) Western blots and (B) densitometric analyses in triplicate of the p-ERK1/2 expression in sorafenib-treated HCC and ECs. (C) Cell viability analyses in triplicate by CCK-8 solution. (D) Tissue microarray and IHC analyses of the 14-3-3η in 34 advanced recurrent HCC patients' tissue samples. (Note: these specimens were collected from surgery when the patients were first diagnosed with HCC. When the recurrence occurred, they received combined sorafenib treatment and transarterial chemoembolization therapy). These specimens were then divided into "tumor high and vessel high (both high)", "tumor high or vessel high (either high)", and "tumor low and vessel low (both low)" groups according to the Q-scores. (E) Kaplan-Meier analyses of the prognostic significances of 14-3-3η in these patients. (This figure appears in colour on the web.)

Cancer

Table 1. Univariate and multivariate analyses of factors associated with overall survival and recurrence-free survival of HCC patients (n = 256).

Clinical variables	Overall survival		Recurrence-free survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Univariate analysis				
Age (≤50 *vs.* >50)	1.599 (0.8486 to 3.038)	0.1474	2.029 (1.123 to 3.826)	0.0206
Gender (male *vs.* female)	1.486 (0.6792 to 3.652)	0.2930	1.114 (0.4821 to 2.606)	0.7999
HBsAg (negative *vs.* positive)	0.5104 (0.2030 to 1.721)	0.3402	0.4184 (0.2004 to 1.415)	0.2104
Hepatic fibrosis (S1/S2 *vs.* S3/S4)	0.8189 (0.4293 to 1.560)	0.5472	0.6867 (0.3770 to 1.250)	0.2247
Serum AFP (≤20 ng/ml *vs.* >20 ng/ml)	0.7323 (0.3621 to 1.537)	0.43	0.6693 (0.3514 to 1.334)	0.2739
Tumor size (≤5 cm *vs.* >5 cm)	0.3996 (0.2168 to 0.7759)	0.0063	0.3653 (0.1975 to 0.6604)	0.0011
Multinodular tumor (no *vs.* yes)	0.551 (0.2385 to 1.050)	0.0695	0.6612 (0.3123 to 1.261)	0.1956
Tumor differentiation [well (Edmondson grade I/II) *vs.* poorly (III/IV)]	0.7129 (0.4223 to 1.567)	0.461	0.7414 (0.5944 to 1.4893)	0.3072
Vascular invasion (no *vs.* yes)	0.3876 (0.1965 to 0.7199)	0.0033	0.443 (0.2192 to 0.7622)	0.0056
TNM stage (I/II *vs.* III/IV)	0.3534 (0.1872 to 0.6924)	0.0028	0.3954 (0.2259 to 0.7189)	0.0024
14-3-3η				
Both high *vs.* both low	5.269 (2.784 to 6.147)	<0.0001	3.529 (2.588 to 5.847)	<0.0001
Either high *vs.* both low	2.725 (1.573 to 5.733)	0.0007	2.35 (1.569 to 4.834)	0.0007
Multivariate analysis				
Tumor size (≤5 cm *vs.* >5 cm)	0.532 (0.261 to 0.984)	0.042	0.395 (0.202 to 0.775)	0.007
Vascular invasion (no *vs.* yes)	0.44 (0.223 to 0.87)	0.018	0.457 (0.241 to 0.865)	0.016
14-3-3η				
Both high *vs.* both low	4.799 (2.095 to 9.529)	0.0037	2.717 (1.23 to 3.143)	0.005
Either high *vs.* both low	3.38 (1.202 to 8.150)	0.026	1.966 (1.28 to 5.747)	0.009

Fig. 5D. Kaplan-Meier survival analysis also showed that HCC patients in "14-3-3η both high" group had the worst overall survival and recurrence-free survival than those in "14-3-3η both low" group (Fig. 6B). Furthermore, multivariate analysis identified that, 14-3-3η was an independent predictor for postoperative recurrence and overall survival (Table 1).

Discussion

It has been well known that HCC is a highly vascularized tumor, and that the cancer-related angiogenesis has been proposed as the major hallmark of malignancy in HCC [14,23]. So, the molecular mechanisms underlying the regulation of angiogenesis have been extensively studied to discover both new therapeutic targets and predictive markers. In our present study, we identified a novel characteristic HCC-promoting factor, 14-3-3η, which facilitated the growth and angiogenesis in both tumor and ECs. On one hand, overexpression of 14-3-3η in HCC cells not only enhanced the proliferation but also stimulated these cells autosecreted chemotactic factors (Cxcl1, Cxcl2, VEGF-A, and VEGF-C), which in turn recruited the vascular endothelial cells to form intratumoral capillaries. On the other hand, the 14-3-3η-mediated proliferation and tube formation in vascular ECs could play a critical role in providing oxygen and nutrients, which is necessary for tumor growth, intra-hepatic and extra-hepatic metastasis and, post-surgical recurrence [14,16,24]. So these two processes synergistically promoted the tumor progression, with the core factor, 14-3-3η. Furthermore, our tissue microarray analysis showed that either high level of 14-3-3η in tumors or high level of 14-3-3η in intratumoral vessels predicts the poor overall/recurrence-free survival, and that the combined analysis of 14-3-3η in tumor and vessels showed the strongest prognostic value. Collectively, these data suggested that 14-3-3η could be a competent prognostic marker for HCC.

Several studies have shown that, isoform-specific expressions of 14-3-3 family members are associated with various biological behaviors of HCC [20]. For example, increased expressions of 14-3-3α/β and γ are associated with cancer progression, extrahepatic metastasis, and worse survival rates [11,12]. In addition, as a potent epithelial to mesenchymal transition (EMT) inducer, 14-3-3ε promotes HCC growth and metastasis via elevating the expressions/activations of snail, zinc finger E-box-binding protein-1 (Zeb-1), focal adhesion kinase (FAK), and nuclear factor kappa B (NF-κB) [25–27]. Moreover, overexpression of 14-3-3σ facilitates the HCC cell proliferation and migration via up-regulating the β-catenin/heat shock factor-1alpha (HSF-1α)/HSP70 signal pathway [8,28]. Further, 14-3-3ζ has been considered as a potential tumor-associated antigen in immunodiagnosis of HCC [29]; meanwhile, as an important HCC promoter, it facilitates the EMT, autocrine transforming growth factor beta (TGF-β), metastasis and vessel invasion, and resistance to sorafenib via interacting with AXL and/or αB-Crystallin [9,10]. Collectively, these findings indicate that, selective 14-3-3 isoforms contribute to HCC progression by regulating distinct targets and signal pathways. Particularly, in these studies, only the well-defined cytoplasmic (HCC cells) immunostaining for 14-3-3α/β, γ, σ, ε, and ζ are found via IHC in tissue specimens. In our present study, 14-3-3η also showed a continuously intratumoral capillary endothelial staining in HCC patients and xenografts model. In addition, 14-3-3η effectively antagonized the sorafenib-inhibited ERK1/2 phosphorylation and cell viability in both HCC and vascular ECs. Furthermore, HCC patients in "14-3-3η both

high" group had the worst survival than those in "14-3-3η both low" group. So we considered that 14-3-3η might be a promising biomarker for predicting sorafenib response, and that 14-3-3η could be a competent therapeutic target for HCC, especially for anti-angiogenesis therapy.

The activation of RAS/RAF/MEK/ERK1/2 signal pathway plays a key role in the progression of HCC by promoting the tumor growth and angiogenesis. On one hand, a progressive and aberrant ERK1/2 activation, in the presence or absence of RAS mutations, promotes the HCC progression and causes a pronounced increase in patients with poor prognosis [30,31]. On the other hand, inhibition of ERK1/2 attenuated the viability of vascular endothelial cells effectively [32]. In HCC, several proinflammatory/proangiogenic chemokines, like interleukin (IL)-6, and VEGF, can activate ERK1/2 [33,34]. In our present study, overexpression of 14-3-3η elevated the expressions of Cxcl1, Cxcl2, VEGF-A, VEGF-C, and p-ERK1/2; on the contrary, knockdown of 14-3-3η showed the opposed effects. These results suggested that, the 14-3-3η-mediated phosphorylation of ERK1/2 might be attributed to the 14-3-3η-stimulated auto-secretion of proinflammatory/proangiogenic chemokines. It has been shown that 14-3-3β can bind to and maintain the activity of RAF-1, which in turn enhances the RAF/MEK/ERK1/2 signaling [35]. Here we found that, by binding to the phosphorylated ERK1/2 (p-ERK1/2), 14-3-3η and p-ERK1/2 formed a functional positive feed-back loop. Further, inhibition of either 14-3-3η or ERK1/2 broke the feed-back loop and decreased the tumor growth. So our study expanded our understanding in the activation of ERK1/2 by 14-3-3 family in HCC.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

The conception and design: Yuan Li and Jianping Zhang. Performed experiments: Jian Shen, Fei Jiang, Qinqiang Liu, Lijun Chen, Liang Ju, Ming Lu, Fei Zhou, Chi Zhang, Xiagang Luo, and Xiaojun Yang. Collation and analysis of HCC samples: Ye Yang, Fuxing Pu, Guangming Huang, Qinqiang Liu, Chengyu Jiao, and Xiangcheng Li. Wrote the manuscript: Jian Shen, Ye Yang, Yuan Li and Jianping Zhang.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2016.05.017.

References

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[1] Dhanasekaran R, Venkatesh SK, Torbenson MS, Roberts LR. Clinical implications of basic research in hepatocellular carcinoma. J Hepatol 2016;64:736–745.
[2] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359–E386.
[3] European Association for the Study of the LiverEuropean Organisation for Research and Treatment of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol 2012;56:908–943.
[4] Morrison DK. The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. Trends Cell Biol 2009;19:16–23.
[5] **Yang X**, **Lee WH**, Sobott F, Papagrigoriou E, Robinson CV, Grossmann JG, et al. Structural basis for protein-protein interactions in the 14-3-3 protein family. Proc Natl Acad Sci U S A 2006;103:17237–17242.
[6] Zhao J, Meyerkord CL, Du Y, Khuri FR, Fu H. 14-3-3 proteins as potential therapeutic targets. Semin Cell Dev Biol 2011;22:705–712.
[7] Freeman AK, Morrison DK. 14-3-3 Proteins: diverse functions in cell proliferation and cancer progression. Semin Cell Dev Biol 2011;22:681–687.
[8] **Liu CC**, **Jan YJ**, **Ko BS**, Wu YM, Liang SM, Chen SC, et al. 14-3-3sigma induces heat shock protein 70 expression in hepatocellular carcinoma. BMC Cancer 2014;14:425.
[9] Huang XY, Ke AW, Shi GM, Zhang X, Zhang C, Shi YH, et al. AlphaB-crystallin complexes with 14-3-3zeta to induce epithelial-mesenchymal transition and resistance to sorafenib in hepatocellular carcinoma. Hepatology 2013;57:2235–2247.
[10] Reichl P, Dengler M, van Zijl F, Huber H, Fuhrlinger G, Reichel C, et al. Axl activates autocrine transforming growth factor-beta signaling in hepatocellular carcinoma. Hepatology 2015;61:930–941.
[11] **Liu TA**, **Jan YJ**, **Ko BS**, Chen SC, Liang SM, Hung YL, et al. Increased expression of 14-3-3beta promotes tumor progression and predicts extrahepatic metastasis and worse survival in hepatocellular carcinoma. Am J Pathol 2011;179:2698–2708.
[12] Ko BS, Lai IR, Chang TC, Liu TA, Chen SC, Wang J, et al. Involvement of 14-3-3gamma overexpression in extrahepatic metastasis of hepatocellular carcinoma. Hum Pathol 2011;42:129–135.
[13] **Liu TA**, **Jan YJ**, **Ko BS**, **Wu YJ**, Lu YJ, Liang SM, et al. Regulation of aldo-keto-reductase family 1 B10 by 14-3-3epsilon and their prognostic impact of hepatocellular carcinoma. Oncotarget 2015;6:38967–38982.
[14] Yang X, Zhang XF, Lu X, Jia HL, Liang L, Dong QZ, et al. MicroRNA-26a suppresses angiogenesis in human hepatocellular carcinoma by targeting hepatocyte growth factor-cMet pathway. Hepatology 2014;59:1874–1885.
[15] **Yang ZF**, **Ho DW**, **Ng MN**, **Lau CK**, Yu WC, Ngai P, et al. Significance of CD90+ cancer stem cells in human liver cancer. Cancer Cell 2008;13:153–166.
[16] Wang R, Zhao N, Li S, Fang JH, Chen MX, Yang J, et al. MicroRNA-195 suppresses angiogenesis and metastasis of hepatocellular carcinoma by inhibiting the expression of VEGF, VAV2, and CDC42. Hepatology 2013;58:642–653.
[17] **Cao Z**, **Fu B**, **Deng B**, Zeng Y, Wan X, Qu L. Overexpression of Chemokine (C-X-C) ligand 1 (CXCL1) associated with tumor progression and poor prognosis in hepatocellular carcinoma. Cancer Cell Int 2014;14:86.
[18] **Xiong YQ**, **Sun HC**, Zhang W, Zhu XD, Zhuang PY, Zhang JB, et al. Human hepatocellular carcinoma tumor-derived endothelial cells manifest increased angiogenesis capability and drug resistance compared with normal endothelial cells. Clin Cancer Res 2009;15:4838–4846.
[19] **Schmitz KJ**, **Wohlschlaeger J**, Lang H, Sotiropoulos GC, Malago M, Steveling K, et al. Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis C virus infection. J Hepatol 2008;48:83–90.
[20] **Wu YJ**, **Jan YJ**, **Ko BS**, Liang SM, Liou JY. Involvement of 14-3-3 Proteins in Regulating Tumor Progression of Hepatocellular Carcinoma. Cancers 2015;7:1022–1036.

[21] Basbous J, Chalbos D, Hipskind R, Jariel-Encontre I, Piechaczyk M. Ubiquitin-independent proteasomal degradation of Fra-1 is antagonized by Erk1/2 pathway-mediated phosphorylation of a unique C-terminal destabilizer. Mol Cell Biol 2007;27:3936–3950.
[22] Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 2006;66:11851–11858.
[23] **Welti J**, **Loges S**, Dimmeler S, Carmeliet P. Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. J Clin Invest 2013;123:3190–3200.
[24] Shih TC, Tien YJ, Wen CJ, Yeh TS, Yu MC, Huang CH, et al. MicroRNA-214 downregulation contributes to tumor angiogenesis by inducing secretion of the hepatoma-derived growth factor in human hepatoma. J Hepatol 2012;57:584–591.
[25] **Liu TA**, **Jan YJ**, **Ko BS**, Liang SM, Chen SC, Wang J, et al. 14-3-3epsilon overexpression contributes to epithelial-mesenchymal transition of hepatocellular carcinoma. PLoS One 2013;8 e57968.
[26] Ko BS, Chang TC, Hsu C, Chen YC, Shen TL, Chen SC, et al. Overexpression of 14-3-3epsilon predicts tumour metastasis and poor survival in hepatocellular carcinoma. Histopathology 2011;58:705–711.
[27] **Ko BS**, **Jan YJ**, Chang TC, Liang SM, Chen SC, Liu TA, et al. Upregulation of focal adhesion kinase by 14-3-3epsilon via NFkappaB activation in hepatocellular carcinoma. Anti-Cancer Agents Med Chem 2013;13:555–562.
[28] **Zhang Y**, Li Y, Lin C, Ding J, Liao G, Tang B. Aberrant upregulation of 14-3-3sigma and EZH2 expression serves as an inferior prognostic biomarker for hepatocellular carcinoma. PLoS One 2014;9 e107251.
[29] Liu M, Liu X, Ren P, Li J, Chai Y, Zheng SJ, et al. A cancer-related protein 14-3-3zeta is a potential tumor-associated antigen in immunodiagnosis of hepatocellular carcinoma. Tumour Biol 2014;35:4247–4256.
[30] Ito Y, Sasaki Y, Horimoto M, Wada S, Tanaka Y, Kasahara A, et al. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. Hepatology 1998;27:951–958.
[31] Mehdizadeh A, Somi MH, Darabi M, Jabbarpour-Bonyadi M. Extracellular signal-regulated kinase 1 and 2 in cancer therapy: a focus on hepatocellular carcinoma. Mol Biol Rep 2016;43:107–116.
[32] Ou DL, Shen YC, Liang JD, Liou JY, Yu SL, Fan HH, et al. Induction of Bim expression contributes to the antitumor synergy between sorafenib and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor CI-1040 in hepatocellular carcinoma. Clin Cancer Res 2009;15:5820–5828.
[33] Baek JH, Jang JE, Kang CM, Chung HY, Kim ND, Kim KW. Hypoxia-induced VEGF enhances tumor survivability via suppression of serum deprivation-induced apoptosis. Oncogene 2000;19:4621–4631.
[34] **Liu F**, **Zhang W**, Yang F, Feng T, Zhou M, Yu Y, et al. Interleukin-6-stimulated progranulin expression contributes to the malignancy of hepatocellular carcinoma cells by activating mTOR signaling. Sci Rep 2016;6:21260.
[35] Fantl WJ, Muslin AJ, Kikuchi A, Martin JA, MacNicol AM, Gross RW, et al. Activation of Raf-1 by 14-3-3 proteins. Nature 1994;371:612–614.