Table 1 Results of completed phase III trials of molecularly targeted therapies in HCC

Drug	Main target	Design (trial)	TTP/PFS (months), HR, 95% CI	OS (months), HR, 95% CI
First-line advanced HCC				
Sorafenib	RAF, VEGFR, PDGFR, c-KIT	Sorafenib vs placebo (SHARP)	4.9 vs 4.1; $P = 0.77$; HR, 0.58; 95% CI, 0.45–0.74	10.7 vs 7.9; $P < 0.001$; HR, 0.69; 95% CI, 0.55–0.87
		Sorafenib vs placebo (Asia–Pacific)	2.8 vs 1.4; $P < 0.001$; HR, 0.57; 95% CI, 0.42–0.79	6.5 vs 4.2; $P = 0.014$; HR, 0.68; 95% CI, 0.50–0.93
Sunitinib	VEGFR, PDGFR, KIT, RET, Flt-3	Sunitinib vs sorafenib (SUN 1170)	4.1 vs 3.8; $P = 0.169$; HR, 1.13; 95% CI, 0.98–1.31	7.9 vs 10.2; $P = 0.0019$; HR, 1.30; 95% CI, 1.13–1.50
Brivanib	FGFR, VEGFR	Brivanib vs sorafenib (BRISK-FL)	4.2 vs 4.1; $P = 0.853$; HR, 1.01; 95% CI, 0.88–1.16	9.5 vs 9.9; $P = 0.373$; HR, 1.06; 95% CI, 0.93–1.22
Linifanib	VEGFR, PDGFR	Linifanib vs sorafenib (0100953)	5.4 vs 4.0; $P = 0.001$; HR, 0.76; 95% CI, 0.64–0.90	9.1 vs 9.8; P = NS; HR, 1.05; 95% CI, 0.90–1.22
Erlotinib	EGFR, HER-1	Erlotinib + sorafenib vs placebo + sorafenib (SEARCH)	3.2 vs 4.0; $P = 0.91$; HR, 1.13; 95% CI, 0.94–1.36	9.5 vs 8.5; $P = 0.2$; HR, 0.92; 95% CI, 0.78–1.1
Second-line advanced HCC				
Brivanib	FGFR, VEGFR	Brivanib vs placebo (BRISK-PS)	4.2 vs 2.7; $P < 0.001$ HR, 0.56; 95% CI, 0.42–0.78	9.4 vs 8.2; $P = 0.331$; HR, 0.89; 95% CI, 0.69–1.15
Everolimus	mTOR	Everolimus vs placebo (EVOLVE-1)	3.0 vs 2.6; HR, 0.93; 95% CI, 0.75–1.15	7.6 vs 7.3; $P = 0.68$; HR, 1.27; 95% CI, 0.86–1.27
Ramucirumab	VEGFR	Ramucirumab vs placebo (REACH)	2.8 vs 2.1; $P < 0.001$; HR, 0.63; 95% CI, 0.52–0.75†	9.2 vs 7.6; $P = 0.14$; HR, 0.87; 95% CI, 0.72–1.05

†Progression-free survival.

CI, confidence interval; FGFR, fibroblast growth factor receptor; HCC, hepatocellular carcinoma; HER-1, human epidermal growth factor receptor-1; HR, hazard ratio; mTOR, mammalian target of rapamycin; NS, not significant; OS, overall survival; PDGFR, platelet-derived growth factor receptor; PFS, progression-free survival; RET, rearranged during transfection, Flt-3, Fms-like tyrosine receptor kinase-3; TTP, time to progression; VEGFR, vascular endothelial growth factor receptor.

months, respectively ($P = 0.169$); however, median OS for sunitinib and sorafenib was 7.9 and 10.2 months (HR, 1.30; 95% CI, 1.13–1.50; $P = 0.0019$), respectively. The decision was based on a higher incidence of significant toxicities (including grade 3/4 thrombocytopenia [30%], neutropenia [25%] and hemorrhagic events [12%]) in the sunitinib arm and the futility of showing either superiority or non-inferiority in OS when compared with sorafenib. This trial was stopped prematurely after inferior outcomes were noted in the sunitinib arm.

Brivanib

Brivanib is a dual inhibitor of VEGFR and FGFR, both of which are implicated in the pathogenesis of HCC.[31] Two randomized phase III clinical trials were conducted to assess the use of brivanib in the first-line (BRISK-FL) and second-line (BRISK-PS) settings. BRISK-FL was a head-to-head randomized phase III clinical trial comparing brivanib with sorafenib as the first-line therapy in patients with unresectable HCC. Among the enrolled patients, the proportion of patients with Child–Pugh liver function class A and B disease was 92% and 8%, respectively, while that with BCLC stage B and C disease was 22% and 78%, respectively. The brivanib arm failed to achieve a non-inferior median OS, with 9.5 months for brivanib and 9.9 months for sorafenib (HR, 1.06; 95% CI, 0.93–1.22; $P = 0.373$). There was also no difference in TTP between brivanib and sorafenib (4.2 vs 4.1 months; HR, 1.01; 95% CI, 0.88–1.16; $P = 0.853$).[31] The study did not meet its primary OS objective based upon a non-inferiority statistical design. In the second-line setting, BRISK-PS compared brivanib with placebo in patients who were refractory or intolerant to first-line treatment with sorafenib. Although TTP was significantly longer in the brivanib arm than with placebo (4.2 vs 2.7 months; HR, 0.56; 95% CI, 0.42–0.78; $P < 0.001$), the primary end-point of the study was not met, with a median OS for brivanib and placebo of 9.4 and 8.2 months, respectively (HR, 0.89; 95% CI, 0.69–1.15; $P = 0.331$).[32] The most common grade 3/4 adverse events (AE) were hypertension (19%), hyponatremia (18%), fatigue (15%) and decreased appetite (12%).

Linifanib

Linifanib is an oral tyrosine kinase inhibitor (TKI) with selective activity against VEGFR and PDGFR. Linifanib was compared with sorafenib as first-line therapy in a non-inferiority phase III trial.[33] Enrolled patients were those with a histological and cytological diagnosis of unresectable HCC and Child–Pugh liver function class A. TTP with linifanib was significantly improved when compared with sorafenib (5.4 vs 4.0 months; HR, 0.76; 95% CI, 0.64–0.90; $P = 0.001$). However, median OS was 9.1 months with linifanib and 9.8 months with sorafenib (HR, 1.05; 95% CI, 0.90–1.22). Linifanib was less well tolerated than sorafenib, with significantly increased discontinuations and dose reductions/interruptions because of AE.

Erlotinib

Erlotinib is an orally active, potent selective inhibitor of the EGFR/human epidermal growth factor receptor-1-related tyrosine kinase enzyme. In the phase III SEARCH trial, advanced HCC patients were randomized to sorafenib plus either erlotinib or placebo.[34] Inclusion criteria were a histological and cytological diagnosis of unresectable HCC and Child–Pugh liver function class A. Median OS was 9.5 months with sorafenib plus erlotinib and 8.5 months with sorafenib (HR, 0.92; 95% CI, 0.78–1.1; $P = 0.2$). This result failed the prespecified boundaries for non-inferiority. TTP was 3.2 months with sorafenib plus erlotinib and 4.0 months with sorafenib (HR, 1.13; 95% CI, 0.94–1.36; $P = 0.91$).

Everolimus

The mTOR inhibitor, everolimus, has demonstrated antitumor activity in several malignancies. A phase III study comparing everolimus with placebo (EVOLVE-1) in patients who have failed or become intolerant to sorafenib has recently been completed. All patients had Child–Pugh liver function class A, and the proportion of patients with BCLC stage B and C disease was 14% and 86%, respectively. There were no significant difference in TTP between everolimus (3.0 months) and placebo (2.6 months) (HR, 0.93; 95% CI, 0.75–1.15). Furthermore, no significant difference in OS was seen between everolimus (7.6 months) and placebo (7.3 months) (HR, 1.05; 95% CI, 0.86–1.27; $P = 0.68$). The most common grade 3/4 AE for everolimus were anemia (7.8%), asthenia (7.8%) and decreased appetite (6.1%). No patients experienced hepatitis C viral flare. The EVOLVE-1 study failed to reach its primary end-point of extending OS with everolimus.[35]

Ramucirumab

Ramucirumab is a recombinant humanized antibody that specifically targets the extracellular domain of VEGFR-2. A phase II study of 42 patients with advanced HCC and primarily well-preserved liver function showed that first-line ramucirumab monotherapy produced a disease control rate of 69%. The median progression-free survival (PFS) was 4.0 months and

median OS was 12.0 months, respectively. Grade 3/4 toxicities included gastrointestinal bleeding (7%), hypertension (12%) and fatigue (10%). These findings prompted the initiation of the phase III RCT (REACH) comparing ramucirumab versus placebo in patients who failed or were intolerant to sorafenib (NCT01140347).[36] Eligible patients had advanced HCC, stage BCLC C or B disease that was refractory or not amenable to locoregional therapy, and Child–Pugh liver function class A. However, according to the preliminary results released at European Society for Medical Oncology Congress in 2014, ramucirumab failed to demonstrate superiority in terms of OS when compared with placebo. The OS HR was 0.866 (95% CI, 0.717–1.046; $P = 0.1391$); median OS was 9.2 months for ramucirumab versus 7.6 months for placebo. Median PFS with ramucirumab and placebo was 2.8 and 2.1 months, respectively (HR, 0.63, 95% CI, 0.52–0.75; $P < 0.0001$).[37]

ONGOING PHASE III CLINICAL TRIALS

IN ADDITION TO the antiangiogenic multi-targeted TKI, there is a growing number of biologics that target different molecular pathways, such as c-MET. Some of these treatments act on elements of intracellular signaling pathways. A number of agents have shown promising preliminary data for HCC. We also comment on ongoing phase III pivotal trials (Table 2). The inclusion criterion of all four phase III studies was Child–Pugh liver function class A disease.

Lenvatinib

Lenvatinib is an oral multi-tyrosine kinase inhibitor that targets VEGFR-1–3, FGFR-1–3, RET, mast/stem cell growth factor receptor kit and PDGFR.[38] A phase I/II trial of lenvatinib in patients with advanced HCC and Child–Pugh score A liver function status showed a median OS of 18.7 months (95% CI, 12.8–25.1) and a median TTP of 7.4 months (95% CI, 5.5–9.4). Based on these results, a phase III trial was designed to compare the safety and efficacy of lenvatinib versus sorafenib in patients with unresectable or advanced HCC and Child–Pugh A liver status (NCT01761266).[39] Subjects were categorized as stage B (not applicable for transarterial chemoembolization [TACE]) or stage C based on the BCLC staging system.

Regorafenib

Regorafenib is a multikinase inhibitor that targets kinases involved in angiogenesis (e.g. VEGFR-1–3), oncogenesis (e.g. c-kit, RET and BRAF) and the tumor microenvironment (e.g. PDGFR and FGFR).[40] Regorafenib (160 mg/day) was tested in an uncontrolled phase II study in patients with advanced HCC after failure of prior sorafenib therapy (RESORCE).[41] Median TTP was 4.3 months and median OS was 13.8 months. The most common grade 3/4 AE included fatigue (17%), hand–foot skin reaction (14%) and diarrhea (6%). Based on this data, a phase III RCT in the second-line setting is under development (NCT01774344). Inclusion criteria were BCLC stage B or C disease, and failure to receive prior treatment with sorafenib.

Tivantinib

Tivantinib is a selective inhibitor of c-MET.[42] In a randomized phase II trial comparing the use of tivantinib

Table 2 List of ongoing phase III trials of novel targeted therapy for HCC

Drug	Main target	Design (trial)	Status	NCT number
1st line				
Lenvatinib	VEGFR, PDGFR, FGFR, RET, SCFR	Lenvatinib vs sorafenib (E7080)	Recruiting	NCT01761266
2nd line				
Regorafenib	VEGFR, PDGFR, BRAF, FGFR, KIT, RET	Regorafenib vs placebo (RESORCE)	Recruiting	NCT01774344
Tivantinib	c-MET	Tivantinib vs placebo in subjects with c-MET overexpressing (JET-HCC)	Recruiting	NCT01755767
Cabozantinib	c-MET, VEGFR, RET	Cabozantinib vs placebo (CELESTIAL)	Recruiting	NCT01908426

c-MET, c-mesenchymal-epithelial transition factor-1; FGFR, fibroblast growth factor receptor; HCC, hepatocellular carcinoma; PDGFR, platelet-derived growth factor receptor; RET, rearranged during transfection; SCFR, stem cell growth factor receptor kit; VEGFR, vascular endothelial growth factor receptor.

Table 3 Results of completed phase III trials of molecularly targeted therapy in combination with TACE for HCC

Drug	Main target	Design	TTP (months, HR, 95% CI)	OS (months)
Sorafenib	RAF, VEGFR, PDGFR, c-KIT	TACE + sorafenib vs TACE + placebo	5.4 vs 3.7; $P = 0.252$; HR, 0.87; 95% CI, 0.70–1.09	29.7 vs NE; $P = 0.790$; HR, 1.06; 95% CI, 0.69–1.64
Brivanib	FGFR, VEGFR	TACE + brivanib vs TACE + placebo	12.0 vs 10.9; $P = 0.62$; HR, 0.94; 95% CI, 0.72–1.22	26.4 vs 26.1; $P = 0.53$; HR, 0.90; 95% CI, 0.66–1.23
Orantinib	VEGFR, PDGFR, FGFR	TACE + orantinib vs TACE + placebo	†	†

†Full data have not yet been reported at November 2014.
CI, confidence interval; HCC, hepatocellular carcinoma; FGFR, fibroblast growth factor receptor; HR, hazard ratio; NE, not estimable due to immaturity of data; OS, overall survival; PDGFR, platelet-derived growth factor receptor; TTP, time to progression; VEGFR, vascular endothelial growth factor receptor.

versus placebo as second-line treatment, the overall analysis showed a marginal but significant improvement in TTP in tivantinib over placebo (1.6 vs 1.4 months; HR, 0.64; 95% CI, 0.43–0.94; $P = 0.04$). A preplanned analysis of patients whose tumors demonstrated overexpression of MET by immunohistochemistry revealed a more notable improvement in TTP, with 2.7 months in the MET-high tivantinib subset versus 1.4 months in the MET-high placebo subset (HR, 0.43; 95% CI, 0.19–0.97; $P = 0.03$). Median OS was 7.2 months for patients with MET-high tumors who received tivantinib versus 3.8 months for MET-high patients who received placebo (HR, 0.38, 95% CI, 0.18–0.81; $P = 0.01$).[43] The most common grade 3/4 AE in the tivantinib group were neutropenia and anemia; severe neutropenia rates were higher prior to mandated dose reduction. Currently, a phase III study is underway to compare tivantinib versus placebo in subjects with c-MET-overexpressing HCC who have failed one prior systemic therapy (NCT01755767).

Cabozantinib

Cabozantinib, a multikinase inhibitor that inhibits MET, VEGFR-2 and RET, was studied in a phase II trial of HCC patients who had received at most one prior systemic therapy.[44] Impressive efficacy was observed; the PFS was 4.4 months while the median OS was 15.1 months in the cabozantinib arm.[45] A phase III clinical trial testing the efficacy of cabozantinib in the second-line setting is planned (NCT01908426).

Combination therapy

With regard to molecularly targeted agents combined with other treatments, surgical resection and local ablation are curative therapies for BCLC stage A, whereas TACE is used for the management of patients of BCLC stage B. Hepatic arterial infusion chemotherapy (HAIC) is used for the management of patients of BCLC stage B to C. In this article, we focused mainly BCLC stage B to C. Tables 3 and 4 summarizes data regarding the use of molecularly targeted agents combined with TACE or HAIC.

The high rate of HCC recurrence after TACE may be due to its enhancement of angiogenesis and upregulation of VEGF and PDGFR expression, resulting in the formation of rich vascular beds in residual tumors.[46] Administration of an antiangiogenic agents with TACE may block angiogenesis and may therefore lengthen time to recurrence and improve survival.

A phase III study of sorafenib in combination with TACE versus TACE alone performed in Japan and Korea likewise did not demonstrate any benefit with the combination (TTP; sorafenib vs placebo [5.4 vs 3.7 months, HR, 0.87; 95% CI, 0.70–1.09; $P = 0.252$]; OS sorafenib vs placebo; 29.7 months vs not estimable due to immaturity of data [HR, 1.06; 95% CI, 0.69–1.64;

Table 4 List of ongoing phase III trials of therapy in combination with TACE or HAIC for HCC

Drug	Design (trial)	Status	NCT number
Sorafenib	TACE + sorafenib vs TACE + placebo	Recruiting	NCT01004978
Sorafenib	TACE + sorafenib vs TACE + placebo	Recruiting	NCT01324076
Sunitinib	TACE + sunitinib vs TACE + placebo	Recruiting	NCT01164202
Sorafenib	HAIC + sorafenib vs sorafenib	Recruiting	NCT01214343

HAIC, hepatic arterial infusion chemotherapy; HCC, hepatocellular carcinoma; TACE, transcatheter arterial chemoembolization.

$P = 0.79$]).[47] Two other phase III, randomized, placebo-controlled trials evaluating the efficacy of sorafenib in combination with conventional TACE are ongoing (NCT01004978 and NCT01324076).

Other phase III RCT exploring the combinations of TACE and orantinib (ORIENTAL trial, NCT01465464) and brivanib (BRISK-TA trial) have been completed, and sunitinib (TURNE trial, NCT01164202) are ongoing.

In the BRISK-TA trial, although brivanib improved time to radiographic progression (brivanib vs placebo; 8.4 vs 4.9 months; HR, 0.61; 95% CI, 0.48–0.77; $P < 0.0001$), brivanib did not improve TTP (brivanib vs placebo; 12.0 vs 10.9 months; HR, 0.94; 95% CI, 0.72–1.22; $P = 0.62$) or OS (brivanib vs placebo; 26.4 vs 26.1 months; HR, 0.90; 95% CI, 0.66–1.23; $P = 0.53$).[48]

Orantinib is an oral small molecule inhibitor of VEGFR, PDGFR and FGFR.[49] A recent press release announced that a phase III trial comparing TACE plus orantinib versus TACE plus placebo did not meet the primary end-point, but the full dataset has not yet been reported.

A phase III study of sorafenib plus low-dose cisplatin/fluorouracil HAIC versus sorafenib in patients with advanced HCC is ongoing (NCT01214343).

Biomarkers

Studies have investigated whether several biomarker can predict the response to sorafenib. Tissue markers, such as FGF3/FGF4,[50] αB-crystallin,[51] c-Jun N-terminal kinase,[52] VEGF-A[53] and pERK,[54] serum marker and angiogenesis-related cytokine have been reported.[55] Conventional tumor markers for the diagnosis of HCC, namely, des-γ-carboxyprothrombin and α-fetoprotein, have been reported to show contrasting behavior after administration of sorafenib.[56–60] However, no definitive biomarker for sorafenib has been identified. Lovelt *et al.* reported that no biomarker was significantly associated with the response to sorafenib within the SHARP study, which was the largest study of sorafenib.[61] The difficulty in identifying a specific biomarker in sorafenib therapy for HCC may be due to the presence of multiple molecular targets.

FUTURE DIRECTIONS

NINE PHASE III clinical trials (i.e. SHARP, Asia–Pacific, SUN 1170, BRISK-FL, 0100953, SEARCH, BRISK-PS, EVOLVE-1, REACH) of patients with advanced HCC have been completed, and four phase III clinical trials (i.e. E7080, RESORCE, JET-HCC, CELESTIAL) are ongoing. No targeted agent or regimens other than sorafenib significantly improve OS in patients with advanced HCC, according to phase III trials in the first- or second-line setting. Three phase III clinical trials did not demonstrate any benefit with combination therapy.

Potential reasons for negative results include heterogeneous patient population and the lack of understanding of critical drivers of tumor progression/dissemination. Other reasons include liver toxicity, flaws in trial design or marginal antitumoral efficacy of the agents. When dissecting the results of recent trials,[30–34] we can speculate that the main shortcomings for sunitinib are liver toxicity and issues with trial design.[30] Other shortcomings include lack of efficacy for erlotinib,[34] toxicity for linifanib[33] and lack of efficacy and issues with trial design for brivanib.[31,32]

Hepatocellular carcinoma is a heterogeneous disease, both in regard to its clinical manifestations with underlying liver disease, and its complex pathogenesis involving aberrant signaling in several molecular pathways. Advances in targeted therapy for HCC require a better understanding of various molecular events driving the progression of HCC as well as identification of biomarkers to predict treatment response to targeted agents. Due to the complexity of the mechanisms involved in progression of HCC, the establishment of personalized therapy will require the identification of tissue biomarkers in HCC.

Regarding patient selection, recommendations emphasized the need for standardization of inclusion criteria based on stage, such as the BCLC classification. It is evident that the population of patients with unresectable HCC consists of a highly heterogeneous group of patients with a wide spectrum of survival, ranging from a few months to longer than 2 years.[62,63] Therefore, it is difficult to precisely estimate the survival of patients during the design of clinical trials that encompass a heterogeneous population. As a result, the staging system is suboptimal in identifying a homogeneous group of patients in terms of prognosis and disease behavior.

In summary, success in the development of targeted agents for HCC relies on concerted efforts of testing of novel agents in clinical trials, advancement of knowledge of the molecular events of HCC, discovery of biomarkers to guide personalized treatment and improvements in patient selection.

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Heat shock factor 1 accelerates hepatocellular carcinoma development by activating nuclear factor-κB/mitogen-activated protein kinase

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Heat shock factor 1 (HSF1), a major transactivator of stress responses, has been implicated in carcinogenesis in various organs. However, little is known about the biological functions of HSF1 in the development of hepatocellular carcinoma (HCC). To clarify the functional role of HSF1 in HCC, we established HSF1-knockdown (HSF1 KD) KYN2 HCC cells by stably expressing either small hairpin RNA (shRNA) against HSF1 (i.e. HSF1 KD) or control shRNA (HSF1 control). Tumorigenicity was significantly reduced in orthotopic mice with HSF1 KD cells compared with those with HSF1 control cells. Reduced tumorigenesis in HSF1 KD cells appeared attributable to increased apoptosis and decreased proliferation. Tumor necrosis factor-α-induced apoptosis was increased in HSF1 KD cells and HSF1$^{-/-}$ mouse hepatocytes compared with controls. Decreased expression of IκB kinase γ, a positive regulator of nuclear factor-κB, was also observed in HSF1 KD cells and HSF1$^{-/-}$ mouse hepatocytes. Furthermore, expression of bcl-2-associated athanogene domain 3 (BAG3) was dramatically reduced in HSF1 KD cells and HSF1$^{-/-}$ mouse hepatocytes. We also found that epidermal growth factor-stimulated mitogen-activated protein kinase signaling was impaired in HSF1 KD cells. Clinicopathological analysis demonstrated frequent overexpression of HSF1 in human HCCs. Significant correlations between HSF1 and BAG3 protein levels and prognosis were also observed. In summary, these results identify a mechanistic link between HSF1 and liver tumorigenesis and may provide as a potential molecular target for the development of anti-HCC therapies.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and the third leading cause of cancer death worldwide (1). Despite marked advances in diagnostic and therapeutic techniques, prognosis remains unsatisfactory for HCC patients (2,3). An understanding of HCC carcinogenesis at the molecular level is thus urgently needed in order to identify novel molecular targets for the development of more effective therapies.

Heat shock factor 1 (HSF1) is the main regulator of the heat shock response, which is involved in protecting cells and organisms from heat, ischemia, inflammation, oxidative stress and other noxious conditions (4,5). Under various forms of physiological stress, HSF1 drives the production of heat shock proteins (HSPs), such as HSP27, HSP70 and HSP90, which act as protein chaperones (5,6). The functions of HSF1 are not limited to increasing the expression of chaperones; HSF1 also modulates the expression of hundreds of genes other than chaperones that are critical for survival under an array of potentially lethal stressors (6–8). As a result, HSF1 influences fundamental cellular processes such as cell cycle control, protein translation, glucose metabolism and proliferation (7–12). In human tumors, constitutive expression of Hsp27, Hsp70 and Hsp90 at high levels predicts poor prognosis and resistance to therapy (13–15). These effects are often attributable to HSF1-dependent mechanisms (16). Thus, as a master regulator of cellular processes, the roles of HSF1 in carcinogenesis and tumor progression are now emerging. Several recent investigations using mouse models have suggested that HSF1 is involved in carcinogenesis (9,17). In clinical samples, HSF1 is often constitutively expressed at high levels in a variety of tumors, including breast cancer (7,18), pancreatic cancer (19), prostate carcinoma (20) and oral squamous cell carcinoma (21).

Hepatocarcinogenesis is a multistep process, in the majority of cases slowly developing within a well-defined etiology of viral infection and chronic alcohol abuse, leading to the chronic hepatitis and cirrhosis that are regarded as preneoplastic stages (22). A great number of factors, receptors and downstream elements of signaling cascades regulate proliferation and apoptosis. Dysregulation of the balance between cell proliferation and apoptosis thus plays a critical role in hepatocarcinogenesis (23,24). Two of the major pathways of cell proliferation and apoptosis are nuclear factor kappa B (NF-κB) signaling and mitogen-activated protein kinase (MAPK) signaling. NF-κB transcription factors are critical regulators of genes involved in inflammation and the suppression of apoptosis. NF-κB has been shown to be instrumental for tumor promotion in colitis-associated cancer and inflammation-associated liver cancer (25,26). Activation of the extracellular signal-regulated kinase (ERK)/MAPK pathway regulates many important cellular processes, such as proliferation, differentiation, angiogenesis, survival and cell adhesion (27). Importantly, the ERK/MAPK pathway is constitutively activated in HCC (28).

The present study investigated the biological influences of HSF1 in HCC cell proliferation and apoptosis involving the NF-κB and MAPK signal pathways. We found that HSF1 deficiency significantly diminished NF-κB and MAPK activation in primary hepatocytes and HCC cells, so HSF1 deficiency inhibited the development of HCC. Furthermore, clinicopathological analysis demonstrated a significant correlation between HSF1 protein level and prognosis. Our results suggest HSF1 as a promising molecular target for the development of anti-HCC therapeutics.

Abbreviations: BAG3, bcl-2-associated athanogene domain 3; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; HCC, hepatocellular carcinoma; HSF1, heat shock factor 1; HSF1 KD, HSF1 knockdown; HSP, heat shock protein; IKKγ, IκB kinase gamma; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; mRNA, messenger RNA; NF-κB, nuclear factor kappa B; PCNA, proliferating cell nuclear antigen; SCID, severe combined immune-deficient mice; shRNA, small hairpin RNA; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; WT, wild type.

Materials and methods

Cell cultures and reagents

Human HCC cell lines HepG2, PLC/PRF/5, HLE and HLF were obtained from the American Type Culture Collection. Huh7 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Japan). KIM-1 and KYN2 were kindly provided by Dr Hirohisa Yano (Department of Pathology, Kurume University, Kurume, Japan). Li7 was kindly provided by Dr Yae Kanai (Division of Molecular Pathology, National Cancer Center Research Institute,

Tokyo, Japan). HepG2, PLC/PRF/5, Huh7, HLE and HLF cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. KIM-1 and KYN2 was maintained in RPMI medium containing 10% fetal bovine serum.

Antibodies and chemicals

The antibodies used included: anti-HSF1, ERK1/2, phosphor-ERK1/2, MAPK kinase (MEK), phospho-MEK, phosphor- efficiently activated epidermal growth factor receptor (EGFR), cyclin D1, cdc2, CDK4, phospho-IκBα, IκB kinase gamma (IKKγ), IKKβ, caspase-3 and Bcl-X_L (Cell Signaling Biotechnology, Danvers, MA); anti-HSP90, HSP72, β-actin and proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-EGFR (Millipore, Billerica, MA); anti-HSP70/HSP72 (Enzo Life science, NY); and anti-BAG3 (Abcam, Cambridge, UK).

Biochemical and immunohistochemical analyses

Protein lysates were prepared from tissues and cultured cells, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto Immobilon membranes (Millipore) and analyzed by immunoblotting. Total cellular RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), then cDNA was synthesized using SuperScript II (Invitrogen), and expression of specific messenger RNAs (mRNAs) was quantified using real-time PCR and normalized against glyceraldehyde-3-phosphate dehydrogenase mRNA expression. Details of real-time PCR conditions and primer sequences are available in Supplementary Materials and methods, available at *Carcinogenesis* Online. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections using immunoperoxidase methods, as described previously (15). For array analysis, we used the Human WG-6 BeadChip-kit (Illumina, San Diego, CA) in accordance with the instructions from the manufacturer (details are given in Supplementary Materials and methods, available at *Carcinogenesis* Online).

Establishment of HSF1-knockdown cells

A HSF1 small hairpin RNA (shRNA) plasmid and negative control plasmid were purchased from SABiosciences (QIAGEN, Valencia, CA). The shRNA sequences targeting HSF1 were from position 5′-CAGGTTGTTCATAGTCAGAAT-3′ as in the nucleotide sequence of HSF1. As a negative control, a shRNA was designed with the sequence 5′-GGAATCTCATTCGATGCATAC-3′. Transfection was achieved using Oligofectamine reagent (Invitrogen) according to the instructions from the manufacturer. To establish stable knockdown cell lines, shRNA plasmids were transfected into KYN2 cells and cultured in the presence of puromycin (Sigma–Aldrich, St Louis, MO).

Cell proliferation and bromodeoxyuridine assay

Cell proliferation in response to HSF1 silencing was determined by trypan blue exclusion assay. DNA synthesis was determined by bromodeoxyuridine assay according to the instructions from the manufacturer (Roche Diagnostics, Basel, Switzerland). The result was expressed as a percentage of the maximum absorbance at 450 nm, based on three independent experiments. Cells were counted using a Coulter Counter (Beckman Coulter, Pasadena, CA).

Apoptosis assay

Assessment of apoptosis was performed by measuring the intensity of the sub-G_1 peak. For the sub-G_1 peak, HSF1 control KYN2 cells or HSF1-knockdown (HSF1 KD) KYN2 cells were tumor necrosis factor alpha (TNF-α) treatment for 24 h. Cells were treated with propidium iodide and then the sub-G_1 peak was analyzed with a fluorescence-activated cell sorting (FACS) flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed in accordance with the manufacturer's instructions (ApopTag kit; Intergen, Burlington, MA).

Animals

HSF1-deficient ($HSF^{-/-}$) mice have been described previously (29). C57BL/6 wild-type (WT) mice were purchased from CLEA Japan (Tokyo, Japan) for use in the experiments, with primary hepatocytes isolated using a collagenase perfusion method as described in a previous report (26). For orthotopic implantation, C.B-17/Icr-scid/scidJcl [severe combined immune-deficient mice (SCID)] mice were obtained from CLEA Japan. All mice were maintained in filter-topped cages on autoclaved food and water at the University of Hokkaido and the Institute for Adult Diseases, Asahi Life Foundation, according to National Institutes of Health (NIH) guidelines. All experimental protocols were approved by the ethics committee for animal experimentation at Hokkaido University and Asahi Life Foundation. Orthotopic implantation of KYN2 cells and KYN2 transfectants were performed as described previously (30). Briefly, mice were inoculated orthotopically with 5×10^6 HSF1 control ($n = 12$) and HSF1 KD ($n = 12$) cells in 100 μl of phosphate-buffered saline, injected into the liver. Mice were killed 6 weeks after inoculation and autopsies were performed immediately. In the lipopolysaccharide (LPS)/D-galactosamine (GalN)-induced liver injury model, mice were injected intraperitoneally with LPS (20 lg/kg; Sigma) and GalN (1000 mg/kg; Wako, Osaka, Japan) (24).

Patients and tissue samples

For immunohistochemical analysis, a total of 226 adult patients with HCC who underwent curative resection between 1997 and 2006 at Hokkaido University Hospital were enrolled in this study. A preoperative clinical diagnosis of HCC was required to meet the diagnostic criteria of the American Association for the Study of Liver Diseases. Briefly, inclusion criteria were as follows: (i) distinctive pathological diagnosis, (ii) no preoperative anticancer treatment or distant metastases, (iii) curative liver resection (exclusion of extrahepatic tumor spread/metastasis) and (iv) complete clinicopathological and follow-up data. The study protocols were approved by the institutional review board and performed in compliance with the Helsinki of Declaration. Written informed consent was obtained from as many of the patients who were alive as possible (deceased cases were approved for use without written informed consent). Histological diagnosis was made according to World Health Organization criteria. The main clinicopathological features are presented in Table I. During follow-up, clinical evaluations and biochemical tests were performed every 1–3 months. Patients underwent triphasic computed tomography of the liver every 2–3 months.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Significant differences were detected using non-parametric testing. Correlations between protein expression and clinicopathological features of the specimens were assessed, and the resulting data were analyzed using the χ^2 test and Fisher's exact test. Cumulative survival rate was calculated from the first date of treatment using the Kaplan–Meier life-table method. Differences were evaluated by log-rank testing. Independent factors for survival were assessed with the Cox proportional hazard regression model. Differences between the two groups were analyzed using the log-rank test. Statistical analyses were performed using Stat View software (version 5.0; SAS Institute, Cary, NC). Values of $P < 0.05$ were considered significant.

Results

Effect of HSF1 on tumor growth

We first investigated expression of HSF1 in cultured HCC cell lines. HSF1 expression was detected in all eight HCC cell lines analyzed. KYN2 cells showed significantly higher expression of HSF1 than other cell lines (Figure 1A). To further elucidate the functional role of HSF1 in HCC, we established HSF1 KD KYN2 cells by expressing the shRNA against HSF1 or control shRNA. To evaluate the effects of HSF1 on cell growth, we measured cell numbers at several time points and found that the growth of HSF1 KD cells was significantly inhibited compared with control cells (HSF1 control) (Figure 1B). Cell cycle regulators including PCNA, cyclin D1, cdc2 and CDK4 were suppressed in HSF1 KD cells compared with HSF1 control cells (Figure 1C). These results indicate that HSF1 enhances HCC cell growth. Concordantly, HSF1 KD reduced DNA synthesis as measured by bromodeoxyuridine incorporation (Figure 1D).

To evaluate the effects of HSF1 on HCC *in vivo*, orthotopic xenografts were established by HSF1 control and HSF1 KD KYN2 cells in nude mice. Maximum primary tumor diameters and tumor volumes were significantly decreased in HSF1 KD xenografts compared with HSF1 control ones (Figure 1E), suggesting that HSF1 accelerated HCC tumor growth *in vivo*. We confirmed that the tumor of HSF1 KD cells showed significantly lower expression of HSF1 and PCNA than the tumor of HSF1 control cells (Figure 1E).

We performed gain-of-function experiments for HSF1 *in vitro*. No apparent changes in cell growth were seen with overexpression of HSF1 in HCC cell lines with low HSF1 expression (Supplementary Figure 1, available at *Carcinogenesis* Online), whereas cell growth was reduced in HSF1 KD experiments, as above. Based on these

Table I. HSF1, BAG3 expression and clinicopathological variables in HCC

Parameter	Total	HSF1		*P*	BAG3		*P*
		High	Low		High	Low	
		n = 115	*n* = 111		*n* = 112	*n* = 114	
		≥30	<30		≥25	<25	
Age (years)							
≥60	126	66	60	0.69	59	67	0.42
<60	100	49	51		53	47	
Sex							
Male	185	95	90	0.86	94	91	0.49
Female	41	20	21		18	23	
Etiology							
HBsAg(+)/HCV(−)	85	45	40	0.70	39	46	0.67
HBsAg(−)/HCV(+)	84	43	41		44	40	
HBsAg(+)/HCV(+)	6	4	2		2	4	
HBsAg(−)/HCV(−)	51	23	28		27	24	
Cirrhosis							
Presence	121	64	57	0.59	62	59	0.59
Absence	105	51	54		50	55	
Tumor size (cm)							
<5	149	67	82	0.017*	66	83	0.035*
≥5	77	48	29		46	31	
No. of tumor nodules							
Solitary	168	78	90	0.032*	79	89	0.22
Multiple (≥2)	58	37	21		33	25	
TNM stage							
I and II	139	62	77	0.017*	63	76	0.11
III and IV	87	53	34		49	38	
BCLC stage							
A	81	27	54	<0.001*	32	49	0.065
B	108	64	44		58	50	
C	37	24	13		22	15	
Differentiation							
Well	36	11	25	0.010*	10	26	0.014*
Moderate	143	74	69		75	68	
Poor	47	30	17		27	20	
Capsular formation							
Presence	184	95	89	0.73	91	93	1.0
Absence	42	20	22		21	21	
Vascular invasion							
Present	37	24	13	0.073	22	15	0.21
Absent	189	91	98		90	99	
Serum AFP level							
<20	117	53	64	0.086	52	65	0.14
≥20	109	62	47		60	49	

AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; HCV, hepatitis C virus; TNM, tumor node metastasis.
*Significant *P* value.

findings, we concluded that HSF1 expression is a necessary condition for cell growth, but it is not a sufficient condition. We, therefore, did not further investigate gain of function of HSF1.

Impaired EGF-mediated MEK/ERK activation in HSF1 KD cells and HSF1$^{-/-}$ hepatocytes

Activation of the MEK/ERK pathway regulates many important cellular processes in carcinogenesis. To further elucidate the function of HSF1 on tumor growth, we investigated the cascade of MAPK. In WT hepatocytes and HSF1 control cells, EGF, a potent activator of MAPK, efficiently activated EGFR, MEK1/2 and ERK1/2 (Figure 2A). In contrast, activation of EGFR, MEK1/2 or ERK1/2 was significantly decreased in HSF-knockout mice (HSF$^{-/-}$) hepatocytes and HSF1 KD cells (Figure 2A and B). Regarding protein levels of EGFR, MEK1/2 and ERK1/2, EGFR protein levels were significantly decreased in HSF1$^{-/-}$ hepatocytes and HSF1 KD compared with controls, whereas other proteins were unchanged (Figure 2A and B). This result was consistent with the previous report (31). Immunohistochemical staining revealed that HSF1 control tumor showed strong phosphorylated ERK1/2 levels, whereas almost no ERK1/2 activation was observed in HSF1 KD tumors (Figure 2C).

Role of HSF1 in TNF-α-induced apoptosis

Since tumor growth inhibition is caused mainly by increased cell death and decreased cellular proliferation, we compared numbers of apoptotic cell deaths in HSF1 control and HSF KD xenografts using the TUNEL assay. Significantly more apoptotic tumor cells were found in HSF1 KD tumors than in HSF1 control tumors (Figure 3A). Next, we examined whether HSF1 was involved in apoptosis *in vitro*. FACS analysis showed very few apoptotic cells in HSF KD or HSF control in the absence of any stimuli. In contrast, treatment with TNF-α, a potent inducer of apoptosis, caused more extensive apoptotic cell death in HSF1 KD cells (23.9%) than in HSF control cells (8.7%) (Figure 3B). Furthermore, we also confirmed increased TNF-α-induced apoptosis in HSF KD cells as determined by TUNEL assay and caspase-3 activation (Figure 3C and D). To examine whether HSF1 is required for TNF-α-induced liver apoptosis *in vivo*, we used an LPS/GalN liver injury model that depends on TNF-α-mediated apoptosis (32). At 7 h LPS/GalN

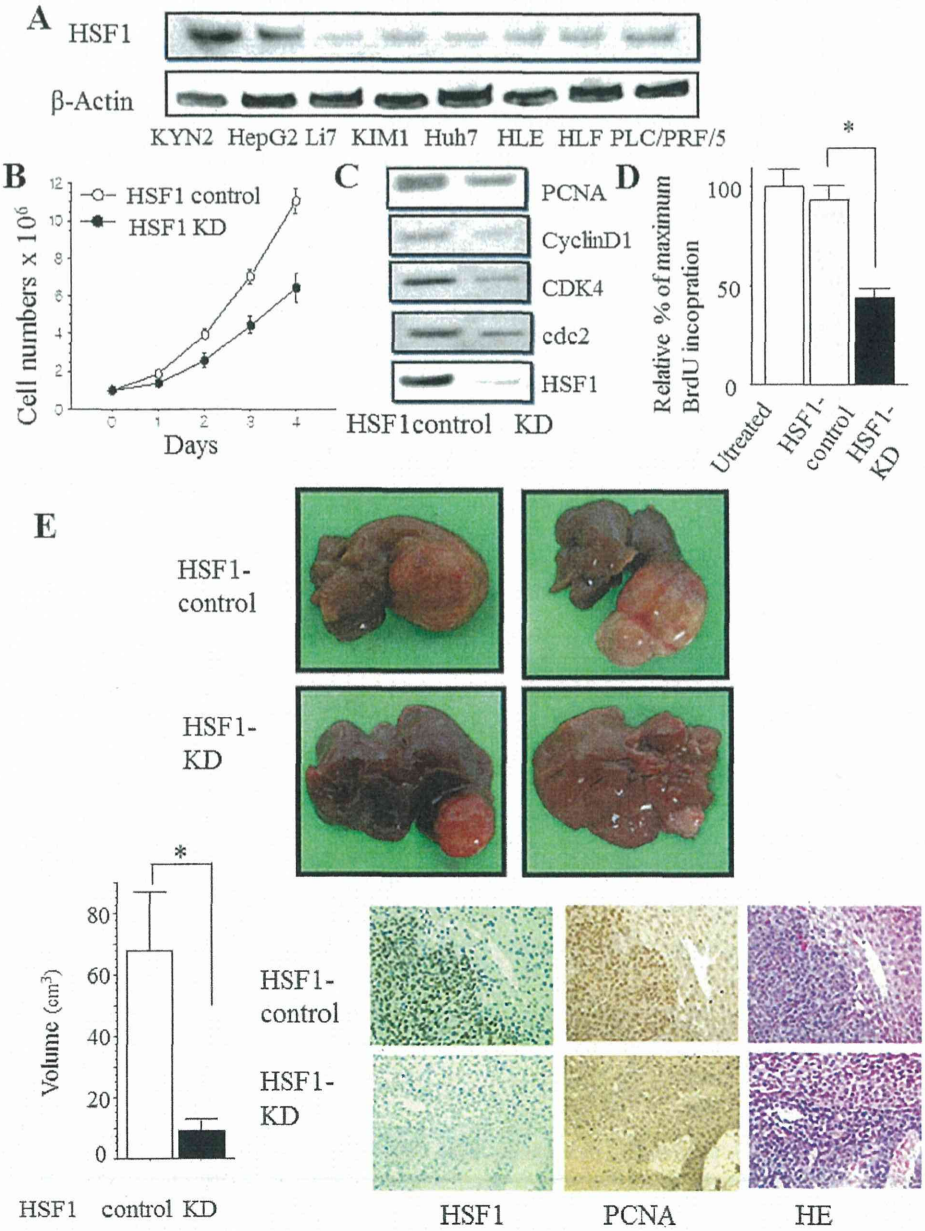

Fig. 1. Role of HSF1 in HCC growth. (**A**) Expression of HSF1 in the eight indicated HCC cell lines was determined by western blot analysis, using β-actin as a control. (**B**) Cell growth of HSF1 control KYN2 cells and HSF1 KD KYN2 cells was measured by counting the number of cells. One representative experiment from three experiments is shown. Data are plotted as mean ± SEM. (**C**) Expression of cell-cycle-related protein in HSF1 control KYN2 cells and HSF1 KD KYN2 cells, as determined by western blot analysis. (**D**) Cells were pulsed with BrdU (10 mmol/l) for 4 h. Optical density values are expressed as a percentage relative to the group expressing control. $^*P < 0.05$. Bars: SEM. (**E**) Growth appearance of HSF1 KD and HSF1 control cells in SCID mice after orthotopic implantation (upper panel). Orthotopic tumor volume was measured. Data are expressed as mean ± SEM (HSF1 control, $n = 12$; HSF1 KD, $n = 12$). $^*P < 0.05$. Bars: SEM (lower left panel). HE and immunohistochemical staining for HSF1 and PCNA (original magnification: ×40): lower right panel. BrdU, bromodeoxyuridine; HE, hematoxylin and eosin.

administration, $HSF^{-/-}$ exhibited marked alanine aminotransferase elevation (Figure 3E), severe histological liver damage and hepatocyte apoptosis compared with WT mice (Figure 3E). This was also in accordance with the notable depression of HSF1 inducing apoptosis *in vitro*.

HSF1 is involved in TNF-α-mediated NF-κB activation

Regarding the association between HSF1 and antiapoptosis, expression of bcl-2-associated athanogene domain 3 (BAG3) was reportedly reduced in HSF1 KD cells compared with control cells (7,11). In addition, microarray array analysis showed that BAG3 was dramatically downregulated in HSF1 KD cells compared with HSF1 control cells (Supplementary Table I, available at *Carcinogenesis* Online). Immunoblot analysis showed that BAG3 protein expression was reduced in $HSF1^{-/-}$ hepatocytes and HSF1 KD cells relative to the respective controls (Figure 4A and B). Meanwhile, activation of IKK and NF-κB pathway represents one of the most important antiapoptotic signals. In addition, BAG3 is also reported to control proteasomal degradation of IKKγ, the regulatory subunit (also called NF-κB essential modulator) of the IKK complex, and

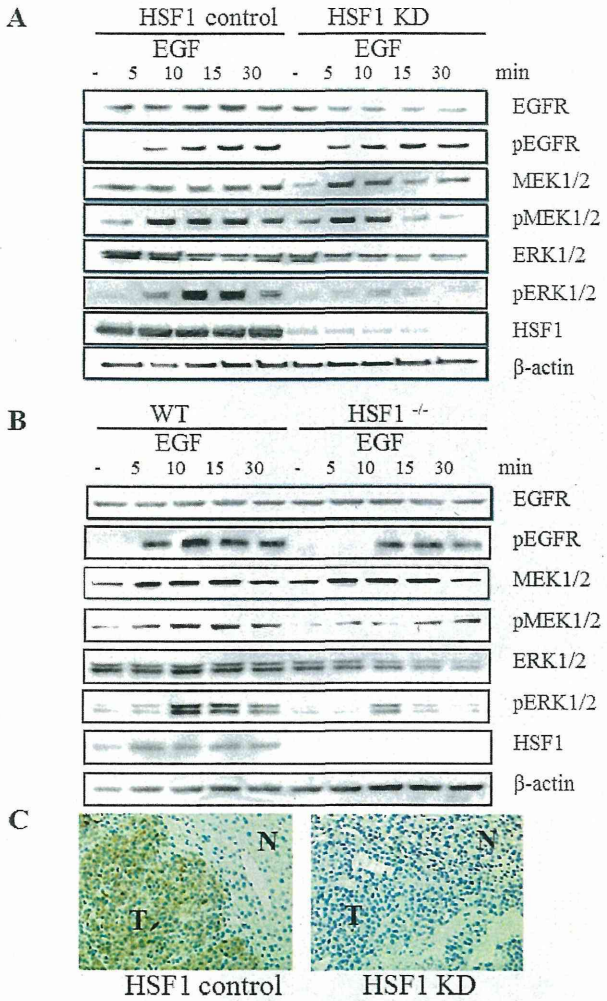

Fig. 2. EGF-mediated MEK/ERK activation is impaired in HSF1 KD cells and HSF1$^{-/-}$ hepatocytes. (**A**) HSF1 control and KD cells were treated with EGF (10 ng/ml), lysed at the indicated times, gel separated and immunoblotted with antibodies against indicated proteins. (**B**) HSF1 WT and HSF$^{-/-}$ hepatocytes were treated with TNF-α (30 ng/ml), lysed in indicated times, gel separated and immunoblotted with antibodies against indicated proteins. (**C**) Representative phosphorylated ERK (p-ERK) staining of orthotopic tumors of HSF1 control and KD cells (original magnification: ×40). N, non-cancerous liver; T, tumor.

NF-κB activity (33). Regarding the NF-κB pathway, NF-κB activation by TNF-α was decreased in HSF1 KD cells compared with the control cells (Figure 4A). In contrast, without any treatment, basal NF-κB activity was very weak and no differences were apparent between HSF1 control cells and HSF1 KD cells (Figure 4A). Consistent with this, microarray analysis showed no apparent differences in the expression of typical NF-κB-regulated genes. We also performed NF-κB pathway analysis and found that the pathway was not overrepresented by the microarray results (Supplementary Figure 2, available at *Carcinogenesis* Online). Next, we investigated whether HSF1 is involved in TNF-α-mediated NF-κB activation and found that phosphorylated Iκk-B (p-IκB), a marker of NF-κB activation, was significantly decreased in HSF$^{-/-}$ hepatocytes and HSF1 KD cells compared with their controls. As expected, IKKγ protein levels were dramatically reduced in HSF1$^{-/-}$ hepatocytes and HSF1 KD cells compared with their controls (Figure 4A and B). To investigate whether decreased IKKγ protein was degraded via proteasome, we used the proteasomal inhibitor, MG-132, and found that protein levels of IKKγ in HSF1 KD cells recovered with the inhibitor, whereas protein expression of BAG3 was unchanged (Figure 4C). Although mRNA levels of BAG3 were significantly downregulated in HSF1 KD cells compared with HSF1 control cells, mRNA levels of IKKγ were not changed (Figure 4D). HSP70 mRNA and protein levels were similar between HSF1 control and HSF1 KD cells (Figure 4A–D). These results suggest that HSF1 positively regulated BAG3 expression, which stabilized the IKKγ protein necessary for NF-κB activation. Immunohistochemical staining revealed that downregulation of HSF1 dramatically reduced BAG3 levels in HSF1 KD xenografts compared with the HSF1 control xenografts.

We performed real-time PCR analysis of the putative NF-κB-regulated antiapoptotic genes. The levels of A20, cellular inhibitor of apoptosis 2 (c-IAP2) RNA expression were decreased in HSF1 KD cells by TNF-α-mediated compared with HSF1 control cells, whereas cylindromatosis, cIAP1 were unchanged (Figure 4E). These results suggest that HSF1 plays an important role in tumor growth via MAPK-mediated cellular proliferation and NF-κB-mediated antiapoptosis.

HSF1 and BAG3 were frequently overexpressed in human HCCs

To analyze the involvement of HSF1 in HCCs, we examined expression levels of HSF1 in human primary HCCs. Immunoblot analysis showed that levels of HSF1 in HCC tissues were significantly higher than in non-cancerous liver tissues in 5 of 10 samples (50%) (Figure 5A). We tested 226 samples from tumor tissues of patients with HCCs by immunohistochemistry. The median percentage of positive cells was 30% (range: 0–90.0%) and we divided patients into two groups of high expressers and low expressers based on the percentage of HSF1-positive cells using a cutoff level of 30%, representing the median value of HSF1. We found that 50.9% (115/226) of tumor samples showed high HSF1 expression. Typical examples of high HSF1 expression samples are shown in Figure 5B. The characteristics of patients in this analysis are shown in Table I. Significant differences were apparent between high and low HSF1 expression groups in terms of tumor size ($P = 0.017$), tumor node metastasis stage ($P = 0.017$), Barcelona Clinic Liver Cancer stage ($P < 0.001$), number of tumor nodules ($P = 0.032$) and histological grade ($P = 0.010$) (Table I), but no significant correlations were observed between HSF1 expression and other clinicopathological variables such as etiology or cirrhosis (Table I). Furthermore, patients with tumors showing HSF1 overexpression displayed significantly shorter overall survival (median: 75.2 months) compared with patients whose tumors showed HSF1 low expression (median: 136.0 months; $P = 0.004$, log-rank test) (Figure 5C). These findings suggest that overexpression of HSF1 was frequently observed in human HCCs, particularly in tumors exhibiting aggressive features.

To explore the pathological relationship between HSF1 and BAG3 in HCC samples, we performed immunohistochemical analysis for BAG3 in 226 HCC samples, which were also analyzed for HSF1 immunohistochemistry. The median percentage of positive cells was 25% (range: 0–85.0%) and we divided them into two groups—high expressers and low expressers—based on the percentage of BAG3-positive cells using a cutoff level of 25%, representing the median value of BAG3. Representative examples of immunohistochemical reactivity for BAG3 are shown in Figure 5B. Expressions of BAG3 protein were significantly increased in HCC specimens, whereas no or only low BAG3 expression was seen in adjacent non-cancerous tissue. BAG3 expression correlated significantly with histological grade ($P = 0.014$), and tumor size ($P = 0.035$), but no significant correlations were observed between BAG3 expression and other clinicopathological variables (Table I). Furthermore, a positive correlation between expressions of HSF1 and BAG3 was found in HCC ($P < 0.05$; Figure 5D) and patients with tumors showing BAG3 overexpression displayed significantly shorter overall survival (median: 84.0 months) compared with those patients whose tumors showed BAG3 low expression (median: 134.2 months; $P = 0.015$, log-rank test) (Figure 5E). Multivariate Cox regression

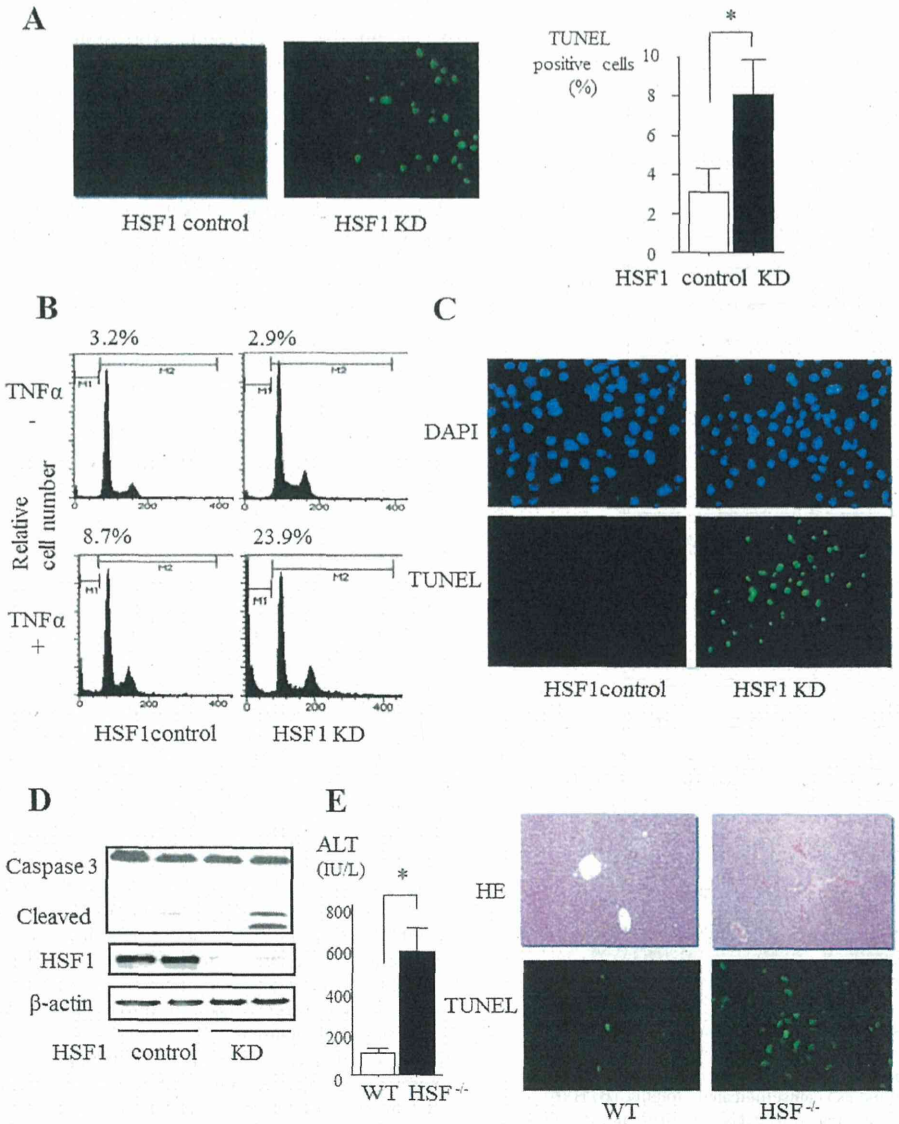

Fig. 3. Antiapoptotic effect of HSF1 in HCC cells and hepatocytes. (**A**) TUNEL staining was performed in tumors of HSF1 control and HSF1 KD cells from orthotopic implanted mice (left panel). TUNEL-positive cells were counted in tumors of HSF1 control and HSF1 KD cells. *$P < 0.05$. Bars: SEM (right panel). (**B**) Apoptotic cells were evaluated by FACS at 24 h after incubation with TNF-α (30 ng/ml). Values indicate percentages of cells with sub-G_1 DNA content. Representative data are shown from three independent experiments. (**C**) TUNEL staining was performed in HSF1 control and KD cells after incubation with TNF-α. (**D**) Protein expressions of caspase 3, HSF1 and β-actin in TNF-α-treated HSF1 control and KD cells were determined by western blot analysis. (**E**) Serum ALT levels 7 h after injection of WT and HSF1$^{-/-}$ mice with LPS (5 μg/kg) and GalN (500 mg/kg). *$P < 0.05$, compared with WT mice (left panel). HE and TUNEL stainings were performed in sections of livers obtained 7 h after injecting LPS (5 μg/kg) and GalN (500 mg/kg) into WT and HSF1$^{-/-}$ mice (right panel). ALT, alanine aminotransferase; DAPI, 4′,6-diamidino-2-phenylindole; HE, hematoxylin and eosin.

analysis identified high HSF1 expression (hazard ratio: 2.07; $P = 0.04$) as an independent prognostic factor for overall survival (Table II).

Discussion

As a master regulator of the heat shock response, HSF1 enhances organism survival and longevity in the face of environmental challenges. However, HSF1 can also act to the detriment of organisms by supporting malignant transformation (34). As reported previously, loss of HSF1 negatively impacts tumorigenesis driven by p53 or Ras mutations (8,16). Since HSF1 does not act as a classic oncogene, the increased resistance to proteotoxic stress induced by HSF1 was suggested to support tumor initiation and growth by enabling cells to accommodate the genetic alterations that accumulate during malignancy (35). However, the specific mechanisms by which HSF1 may support the growth of tumors are not well understood. Here, we have demonstrated that HSF1 has detrimental effects on liver tumor growth. We also proposed that the antiapoptotic effect of HSF1 may play a role in HCC tumor growth.

To clarify the mechanisms underlying this effect, we investigated associations between HSF1 and the NF-κB signaling pathway. Although, in a previous study, heat shock blocked the degradation of IκB (36) and nuclear translocation of NF-κB, the recent literature has reported that the presence of constitutively active HSF1 does not block TNF-α-induced activation of the NF-κB pathway or expression of a set of NF-κB-dependent genes (37). The current study established HSF1 KD cells and showed that HSF1 was necessary for TNF-α-induced NF-κB activation. We analyzed the function of BAG3 as a candidate for the molecule connecting HSF1 with NF-κB activation. BAG3 has reportedly been characterized by the

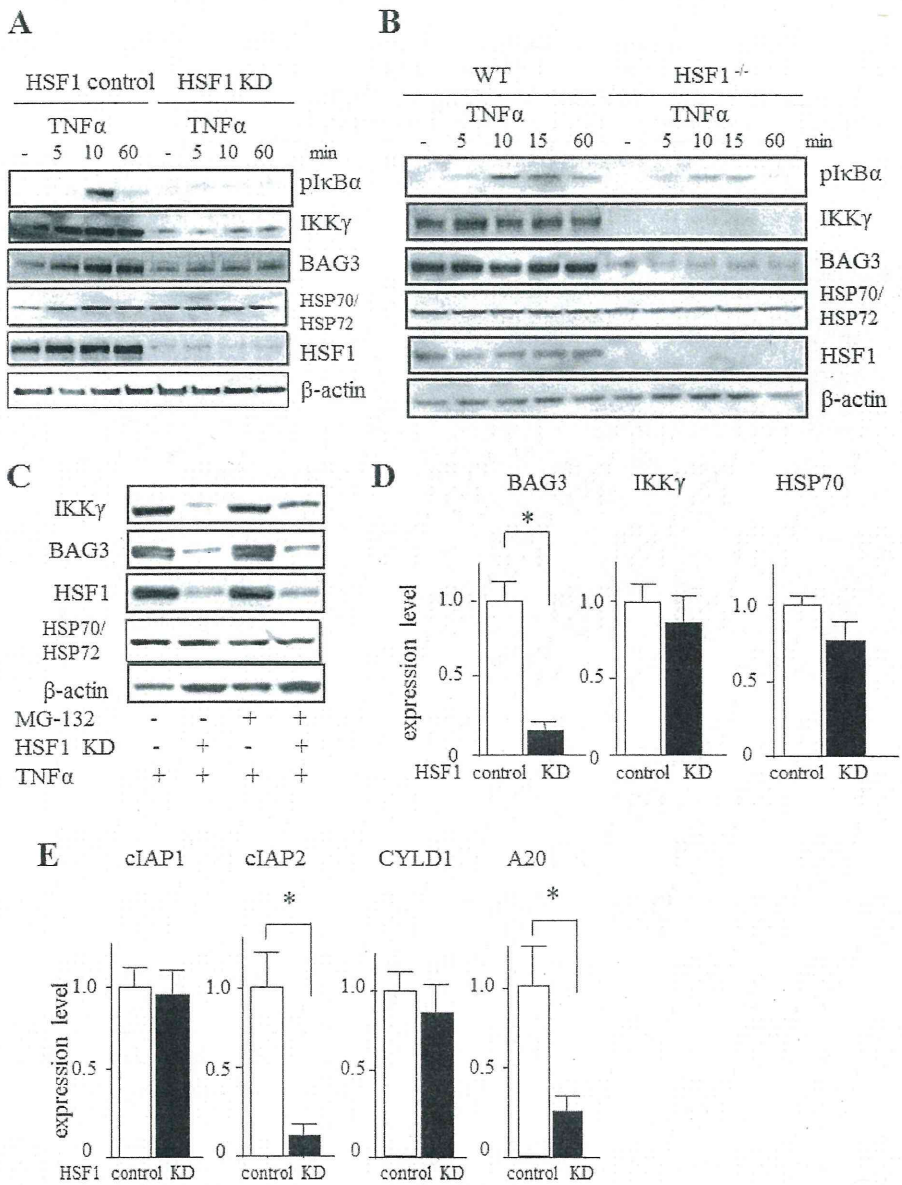

Fig. 4. HSF1 is involved in TNF-α-mediated NF-κB activation. (**A**) HSF1 control and KD cells were treated with TNF-α (30 ng/ml), lysed at the indicated times, gel separated and immunoblotted with antibodies against the indicated proteins. (**B**) HSF1 WT and HSF$^{-/-}$ hepatocytes treated with TNF-α (30 ng/ml), lysed at the indicated times, gel separated and immunoblotted with antibodies against the indicated proteins. (**C**) HSF1 control and KD cells were treated with TNF-α (30 ng/ml) with or without MG-132, lysed at 24 h, gel separated and immunoblotted with antibodies against indicated proteins. (**D**) Relative mRNA levels for BAG3, IKKγ and HSP70 in HSF1 control and KD cells determined by real-time PCR. Data are expressed as mean ± SEM ($n = 4$ per group). *$P < 0.05$. Bars: SEM. (**E**) Relative mRNA levels for antiapoptosis-related gene in HSF1 control and KD cells as determined by real-time PCR. Data are expressed as mean ± SEM ($n = 4$ per group). *$P < 0.05$. Bars: SEM. CYLD, cylindromatosis.

interaction with a variety of partners (Raf-1, steroid hormone receptors and HSP70) and is involved in regulating a number of cellular processes, particularly those associated with antiapoptosis (38). This molecule was expressed in response to stressful stimuli in a number of normal cell types and appears constitutively in a variety of tumors (33,39), and gene expression is regulated by HSF1 (40). In addition, knockdown of BAG3 protein decreased IKKγ levels, increasing tumor cell apoptosis and inhibiting tumor growth (33). Based on these considerations, we investigated whether attenuating HSF1 would enhance IKKγ protein expression, and data with MG-132 show that proteasomal degradation of IKKγ is enhanced in HSF1 KD cells. In addition, knowledge of the role BAG3 plays in preventing the proteasomal turnover of certain proteins suggests that the loss of BAG3 in HSF1 KD cells may be responsible for the enhanced turnover of IKKγ in this setting.

NF-κB activation is a master regulatory step in antiapoptosis. Several mechanisms have been reported regarding this antiapoptotic effect of NF-κB activation (41). NF-κB exerts its prosurvival activity primarily through the induction of target genes, the products of which inhibit components of the apoptotic machinery. These include Bcl-X_L and c-IAP (41), which binds directly to and inhibits the effect of caspases. This study showed that inactivation of NF-κB promoted apoptotic effects against TNF-α in HSF1$^{-/-}$ hepatocytes and HSF1 KD HCC cells. Real-time PCR analyses indicated that expression levels of apoptosis-related genes such as A20 and c-IAP2 were decreased by inhibition of NF-κB activation, whereas apoptosis-related genes such

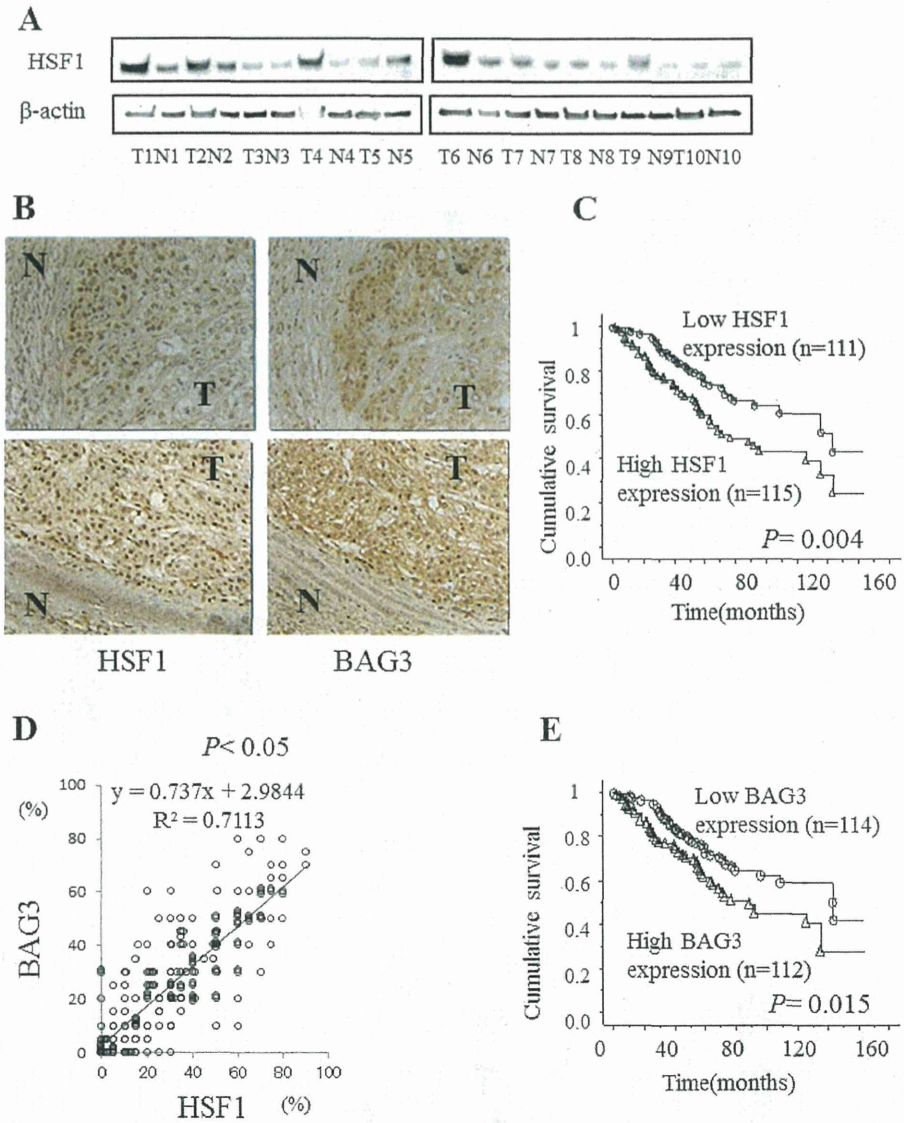

Fig. 5. Overexpression of HSF1 protein in human HCCs and pathological relationship between HSF1 and BAG3 in HCC samples. (**A**) HSF1 protein expression was determined in paired samples of human non-neoplastic liver and HCC by western blot, using β-actin as a control. N, non-cancerous liver; T, tumor. (**B**) Representative HSF1 and BAG3 staining of HCC and surrounding tissue. (**C**) Correlation of HSF1 overexpression with overall survival rates of patients. (**D**) Relationship between BAG3 and HSF1 expression in HCC. Scatterplot of BAG3 versus HSF1 with regression line displaying a correlation according to Spearman's correlation coefficient ($P < 0.01$). (**E**) Correlation of BAG3 overexpression with overall survival rates of patients.

as cIAP1 and cylindromatosis, which are known to be regulated by NF-κB activation, were apparently unaffected. Whether gene expression regulated by NF-κB activity differs between inducible and basal activation remains to be determined.

Regarding the relationship between HSF1 and HCC development, HSF1-deficient mice recently revealed dramatically reduced numbers and sizes of tumors compared with WT controls when tumors were induced by the chemical carcinogen, diethylnitrosamine. The same study suggested that the presence of extensive pathology associated with severe steatosis by diethylnitrosamine was prevented by HSF1 deletion and may be associated with reduced HCC development (42). On the other hand, ablation of IKKγ in liver parenchymal cells caused spontaneous development of HCC in mice, with tumor development preceded by steatohepatitis (43). Based on these observations, we assume that reductions in diethylnitrosamine-induced HCC development among HSF1-deficient mice may be associated with reduced expression of IKKγ, the reduction of which caused the steatosis.

BAG3 is a critical regulator of apoptosis in HSF1-deficient hepatocytes and HSF1 KD HCC cells. Moreover, the relationship between HSF1 and BAG3 has been shown not only in cell cultures and mouse models, but also in human HCC tissue samples; a correlation between HSF1 expression and BAG3 expression was found in HCC. Clinicopathological features and biological results provide a mechanistic link between HSF1 and HCC development via BAG3.

As for the ERK signal, a previous study demonstrated that impairment of JNK and ERK signaling in $HSF1^{-/-}$ MEF cells was caused in part by the reduced expression of EGFR (33). We showed a slight decrease in expression of EGFR among HSF1-deficient hepatocytes and HSF1 KD cells. On the other hand, the level of reduced activation of ERK, as a downstream molecule of EGFR, was larger than expected. However, the detailed mechanisms by which HSF1 regulates MAPK need further investigation.

In conclusion, we found that HSF1 deficiency significantly diminished NF-κB and MAPK activation in HCC hepatocytes and

Table II. Multivariate analysis with a Cox proportional hazards regression model

Characteristic	Univariate analysis	Multivariate analysis	Hazard ratio (95% CI)
Age (≥60 years)	0.22	0.15	
Gender (male)	0.92	0.53	
HCV status (positive)	0.28	0.82	
Cirrhosis (positive)	0.15	0.066	
Tumor size (≥50 mm)	<0.01*	0.011*	2.21 (1.18–4.12)
No. of tumor nodule (multiple)	<0.01*	<0.01*	2.67 (1.38–5.62)
Tumor differentiation (poor)	<0.01*	0.031*	2.34 (1.33–4.11)
Capsular formation (absence)	0.18	0.36	
Vascular invasion (presence)	0.062	0.10	
TNM stage (III + IV versus I + II)	<0.01*	0.020*	2.35 (1.14–4.82)
AFP (≥20 ng/ml)	0.18	0.36	
HSF1 expression (high)	0.018*	0.040*	2.07 (1.22–3.50)
BAG3 expression (high)	0.043*	0.056	

AFP, alpha-fetoprotein; CI, confidence interval; HCV, hepatitis C virus; TNM, tumor node metastasis.
*Significant *P* value.

HCC cells; accordingly, HSF1 deficiency inhibited the development of HCC. Furthermore, clinicopathological analysis demonstrated a significant correlation between HSF1 or BAG3 protein levels and prognosis. Our results demonstrate the importance of HSF1 in human HCCs and suggest inhibition of HSF1 as a novel strategy to target that subset of HCC patients in whom this protein is overexpressed.

Supplementary material

Supplementary Materials and methods, Table I and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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