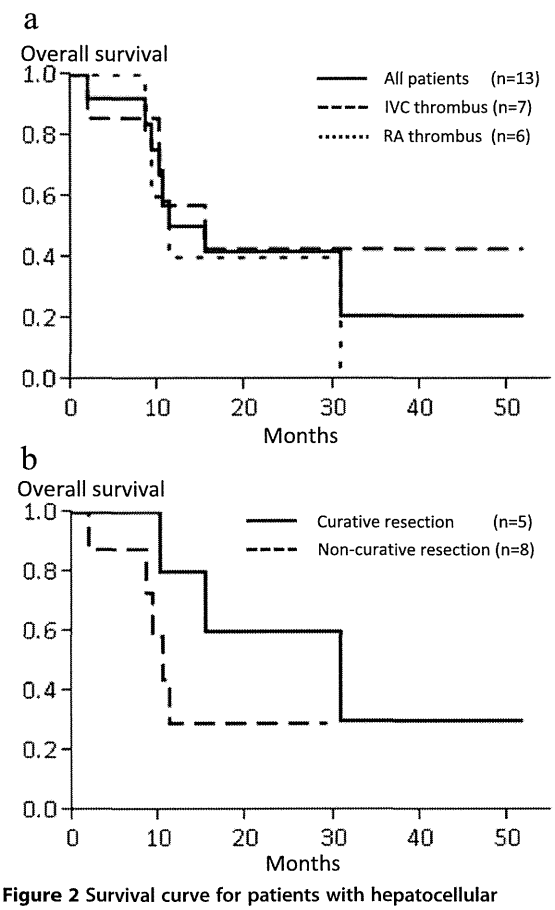

Figure 2 Survival curve for patients with hepatocellular carcinoma (HCC) and inferior vena cava (IVC) and right atrium (RA) thrombi. (a) Overall survival curve for all patients (n = 13), patients with IVC thrombus, and patients with RA thrombus. $P = 0.6056$ by log-rank test, IVC thrombus versus RA thrombus. **(b)** Overall survival curve for patients who underwent curative surgical resection and noncurative resection. $P = 0.2103$ by log-rank test, curative resection versus noncurative resection.

IVC or RA thrombectomy, because the degree of bleeding is a major predictive factor for operative morbidity and mortality [21]. In this study, hepatic parenchymal transection was routinely performed prior to thrombectomy using the Pringle maneuver. IVC occlusion at the suprahepatic portion with bulky tumor thrombi evokes Budd-Chiari syndrome and massive hepatic congestion [28]. In this study, we observed hepatic congestion in patients with outflow obstruction of spared hepatic veins by a massive tumor thrombus. Furthermore, occlusion of two of three major hepatic veins by venous invasion induced hepatic congestion, even though the spared hepatic vein was not obstructed. We used extracorporeal bypass from the PV and IVC to SVC to decompress the liver parenchyma and decrease bleeding during hepatic transection in two patients with an RA thrombus [29]. We performed IVC thrombectomy under a favorable field with good bleeding control by HVE. The duration of HVE, which could trigger hemodynamic deterioration, was short enough. Although CPB was mandatory for RA thrombectomy and was accompanied with a larger amount of blood loss and a higher rate of blood transfusion, patients with RA thrombi required minimal ICU stays and shorter postoperative hospitalization. These procedures contributed to a low incidence of postoperative non-serious complications that were medically manageable. Furthermore, we did not observe any operative mortality in this study. Therefore, hepatectomy with IVC or RA thrombectomy, although technically challenging, can be performed safely with appropriate inflow vascular control for patients with good hepatic reserve. Because almost all thrombi had capsules and did not adhere to the wall of the IVC or RA, they were simply removed by thrombectomy without wall resection. Although some authors indicate the efficacy of IVC resection, the benefits are controversial [30]. We experienced two cases of intra-IVC recurrence after surgery (Table 5); therefore, the management of such tumor thrombi should be reconsidered.

The prognosis of HCC patients with IVC or RA tumor thrombi is extremely poor. Earlier observations revealed a median survival duration after diagnosis of 1 to 5 months for untreated patients [4,15,31]. Although there is no consensus on the therapeutic options for HCC with IVC or RA thrombi, nonsurgical treatments such as TACE, as well as radiotherapy and chemotherapy, have been attempted. Previous reports concerning the therapeutic benefits of TACE with or without radiotherapy revealed insufficient results, with a median survival duration of 9.2 months (range, 4.2 to 18.4 months) [4,8,11-13,32]. Currently, the outcome of systemic chemotherapy for HCC has been disappointing, although sorafenib, which is the only effective agent against HCC, demonstrated a slightly better prognosis of 10.7 months in patients with unresectable HCC [33]. Some case reports have suggested the efficacy of surgical resection, and, recently, Wang *et al.* reported the significant superiority of a surgical approach to HCC with IVC or RA thrombi, with a median survival duration of 19 months, compared with TACE with or without chemoradiotherapy or no treatment [4,6,9,16, 17,20,26,27,34]. This study included 56 patients, of whom 25 underwent surgery, although 7 had an RA thrombus and only 3 underwent surgical resection for the same [4]. Therefore, the therapeutic benefit of surgical resection for HCC with an RA thrombus remains unclear. In the present study, the median overall survival duration of patients with an IVC or RA thrombus was comparable at 15.3 months and 11.2 months, respectively (Figure 2a). This finding indicates the equivalent therapeutic efficacy of surgical resection for RA or IVC thrombi. These results for patient survival are slightly worse than those of the

previous study [4] because our study included patients who underwent non-curative resection. The median survival duration of patients who underwent curative resection was 30.8 months, which is longer than that in the previous report [4] (Figure 2b). This result also surpasses nonsurgical treatment with sorafenib, which resulted in median survival duration of 8.1 months in patients with macrovascular invasion [35].

All patients who underwent curative surgical resection experienced local recurrence or distant metastasis in the early postoperative phase, despite the fact that almost all patients received adjuvant chemotherapy (Table 5). It has been recognized that the poor prognosis of HCC with tumor thrombi in the IVC or RA is strongly related to a high incidence of postoperative recurrence at a relatively early stage, even after curative surgery. In this study, most patients who underwent curative resection developed postoperative lung metastases and intrahepatic recurrence at an equal rate. Preoperative or intraoperative dissemination of tumor cells to the lung can contribute to postoperative metastatic recurrence. To prevent potential intraoperative dissemination by intraoperative handling, some authors indicated the benefit of separated thrombectomy before hepatic transection [36]. However, it could be technically difficult to remove a thrombus en-bloc without up-front hepatic transection; therefore, further improvements in surgical techniques are required. To date, there is no clear modality established for preventing HCC recurrence [4,16,37]. The efficacy of preoperative radiotherapy is indicated for PV tumor thrombi [38]; however, the benefit for IVC or RA thrombi is unclear and there remains a risk of thrombi dislodgment during radiation. In this study, locally recurrent tumors were controlled by TACE or RFA and distant metastatic tumors were treated by chemotherapy with or without radiotherapy or surgical excision if resectable. These vigorous repetitive treatments contribute to improvement in survival, even after recurrence.

Surgical resection is commonly contraindicated for patients with unresectable metastatic tumors because incomplete resection is a crucial factor for poor prognosis [6]. However, hepatic lesions, but not distant metastasis, are the major factors influencing poor prognosis for death in the early postoperative phase [39]. On the basis of the fact that the survival duration of patients with IVC or RA thrombi is extremely short with nonsurgical treatment, distant metastasis itself should not be considered a contraindication for surgery. In this study, eight patients underwent noncurative surgery, and the median survival duration of 10.5 months was relatively better than that for patients who underwent nonsurgical treatment or no treatment in previous studies, including patients who received sorafenib, which is the only highly evidenced agent for advanced HCC treatment and results in median survival duration of 8.9 months in patients with extrahepatic metastases [4,8,11-13,15,30-32,35]. Recent reports indicate the efficacy of aggressive treatment for HCC metastases, including those to the lung, adrenal gland, and lymph nodes, by surgery or radiotherapy [40-42]. In this study, a patient with lung metastases at primary surgery underwent resection of the metastases and survived for 29.3 months. These findings indicate that reductive surgical resection can be justified in patients with IVC or RA thrombi accompanied by distant metastases or unresectable intrahepatic metastases. Control of the life-threatening progression of intrahepatic HCC and prevention of unexpected death by pulmonary embolism would give these patients a chance to undergo multidisciplinary treatments for improving survival. Therefore, if intrahepatic HCC and IVC or RA thrombi can be totally or partially resected, surgical resection may be beneficial.

The limitations of this study include its retrospective design, its single-center design, the small sample size, and patient heterogeneity. Because IVC and RA thrombi associated with HCC are rare, a multicenter prospective study with a large patients sample is necessary to definitively establish the benefits of surgical management.

Conclusions

In conclusion, surgical resection of HCC with IVC or RA thrombosis can be performed safely with appropriate inflow vascular control in patients with good hepatic reserve. We suggest that aggressive surgical resection may be more beneficial than existing therapeutic modalities; however, early recurrence and treatment of recurrent or metastatic tumors remain unresolved issues. Further studies on adjuvant therapies and establishment of therapeutic strategies for recurrent and metastatic tumors are important challenges to improve survival.

Abbreviations

Af: Atrial fibrillation; ARF: Acute renal failure; HBV: Hepatitis B virus; CDDP: Cisplatin; CPB: Cardiopulmonary bypass; CT: Computed tomography; 5-FU: 5-fluorouracil; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HVE: Hepatic vascular exclusion; ICG R_{15}: Indocyanine green retention rate at 15 minutes after injection; IRHV: Inferior right hepatic vein; IVC: Inferior vena cava; LHV: Left hepatic vein; MHV: Middle hepatic vein; MRI: Magnetic resonance imaging; PV: Portal vein; RA: Right atrium; RAdV: Right adrenal vein; RFA: Radio frequency ablation; RHV: Right hepatic vein; S-1: Tegafur gimeracil oteracil potassium; SVC: Superior vena cava; TACE: Transarterial chemoembolization; UFT: Tegafur uracil.

Competing interests

The authors have no conflicts of interest to declare.

Authors' contributions

KW and TK designed the research, KW, TK, HY, TK, HK, YT, KN, TS, ST, and AT contributed to acquisition of data, and KW and TK analyzed and interpreted data. All authors read and approved the final manuscript.

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References

1. Okuda K: **Hepatocellular carcinoma: clinicopathological aspects.** *J Gastroenterol Hepatol* 1997, **12**:S314–S318.
2. Kojiro M, Nakahara H, Sugihara S, Murakami T, Nakashima T, Kawasaki H: **Hepatocellular carcinoma with intra-atrial tumor growth. A clinicopathologic study of 18 autopsy cases.** *Arch Pathol Lab Med* 1984, **108**:989–992.
3. Tse HF, Lau CP, Lau YK, Lai CL: **Transesophageal echocardiography in the detection of inferior vena cava and cardiac metastasis in hepatocellular carcinoma.** *Clin Cardiol* 1996, **19**:211–213.
4. Wang Y, Yuan L, Ge RL, Sun Y, Wei G: **Survival benefit of surgical treatment for hepatocellular carcinoma with inferior vena cava/right atrium tumor thrombus: results of a retrospective cohort study.** *Ann Surg Oncol* 2013, **20**:914–922.
5. Chun YH, Ahn SH, Park JY, Kim-do Y, Han KH, Chon CY, Byun SJ, Kim SU: **Clinical characteristics and treatment outcomes of hepatocellular carcinoma with inferior vena cava/heart invasion.** *Anticancer Res* 2011, **31**:4641–4646.
6. Le Treut YP, Hardwigsen J, Ananian P, Saisse J, Gregoire E, Richa H, Campan P: **Resection of hepatocellular carcinoma with tumor thrombus in the major vasculature. A European case–control series.** *J Gastrointest Surg* 2006, **10**:855–862.
7. Sun JH, Zhang YL, Nie CH, Chen LM, He JD, Wang WL, Zheng SS: **Long-term survival after chemoembolization of metastatic right atrial tumor thrombus as a presenting feature of hepatocellular carcinoma: a case study.** *Oncol Lett* 2012, **3**:975–977.
8. Hou JZ, Zeng ZC, Zhang JY, Fan J, Zhou J, Zeng MS: **Influence of tumor thrombus location on the outcome of external-beam radiation therapy in advanced hepatocellular carcinoma with macrovascular invasion.** *Int J Radiat Oncol Biol Phys* 2012, **84**:362–368.
9. Jung H, Lee KU, Shin WY, Ahn H: **Treatment outcomes of surgical resection for hepatocellular carcinoma with inferior vena cava invasion and/or thrombosis.** *Hepatogastroenterology* 2011, **58**:1694–1699.
10. Matsuda M, Shiba S, Asakawa M, Kono H, Fujii H: **Complete remission of multiple recurrent hepatocellular carcinomas by oral administration of enteric-coated tegafur/uracil in a patient with huge hepatocellular carcinoma extending to the inferior vena cava after hepatic resection: analysis of mRNA expression of fluoropyrimidine metabolism enzymes in the primary tumor.** *Int J Clin Oncol* 2009, **14**:245–248.
11. Chern MC, Chuang VP, Cheng T, Lin ZH, Lin YM: **Transcatheter arterial chemoembolization for advanced hepatocellular carcinoma with inferior vena cava and right atrial tumors.** *Cardiovasc Intervent Radiol* 2008, **31**:735–744.
12. Cheng HY, Wang XY, Zhao GL, Chen D: **Imaging findings and transcatheter arterial chemoembolization of hepatic malignancy with right atrial embolus in 46 patients.** *World J Gastroenterol* 2008, **14**:3563–3568.
13. Sugiyama S, Beppu T, Ishiko T, Takahashi M, Masuda T, Hirata T, Imai K, Hayashi H, Takamori H, Kanemitsu K, Hirota M, Murakami R, Baba Y, Oya N, Yamashita Y, Baba H: **Efficacy of radiotherapy for PV and IVC tumor thrombosis in unresectable HCC.** *Hepatogastroenterology* 2007, **54**:1779–1782.
14. Zeng ZC, Fan J, Tang ZY, Zhou J, Qin LX, Wang JH, Sun HC, Wang BL, Zhang JY, Jiang GL, Wang YQ: **A comparison of treatment combinations with and without radiotherapy for hepatocellular carcinoma with portal vein and/or inferior vena cava tumor thrombus.** *Int J Radiat Oncol Biol Phys* 2005, **61**:432–443.
15. Chang JY, Ka WS, Chao TY, Liu TW, Chuang TR, Chen LT: **Hepatocellular carcinoma with intra-atrial tumor thrombi. A report of three cases responsive to thalidomide treatment and literature review.** *Oncology* 2004, **67**:320–326.
16. Fukuda S, Okuda K, Imamura M, Imamura I, Eriguchi N, Aoyagi S: **Surgical resection combined with chemotherapy for advanced hepatocellular carcinoma with tumor thrombus: report of 19 cases.** *Surgery* 2002, **131**:300–310.
17. Tanaka A, Morimoto T, Ozaki N, Ikai I, Yamamoto Y, Tsunekawa S, Kitai T, Yamaoka Y: **Extension of surgical indication for advanced hepatocellular carcinoma: is it possible to prolong life span or improve quality of life?** *Hepatogastroenterology* 1996, **43**:1172–1181.
18. Papp E, Keszthelyi Z, Kalmar NK, Papp L, Weninger C, Tornoczky T, Kalman E, Toth K, Habon T: **Pulmonary embolization as primary manifestation of hepatocellular carcinoma with intracardiac penetration: a case report.** *World J Gastroenterol* 2005, **11**:2357–2359.
19. Rajasekharan C, Ganga V: **A rare cause for acute cor pulmonale.** *Case Rep Gastroenterol* 2011, **5**:330–335.
20. Sung AD, Cheng S, Moslehi J, Scully EP, Prior JM, Loscalzo J: **Hepatocellular carcinoma with intracavitary cardiac involvement: a case report and review of the literature.** *Am J Cardiol* 2008, **102**:643–645.
21. Kamiyama T, Nakanishi K, Yokoo H, Kamachi H, Tahara M, Yamashita K, Taniguchi M, Shimamura T, Matsushita M, Todo S: **Perioperative management of hepatic resection toward zero mortality and morbidity: analysis of 793 consecutive cases in a single institution.** *J Am Coll Surg* 2010, **211**:443–449.
22. Strasberg SM: **Nomenclature of hepatic anatomy and resections: a review of the Brisbane 2000 system.** *J Hepatobiliary Pancreat Surg* 2005, **12**:351–355.
23. Lee IJ, Chung JW, Kim HC, Yin YH, So YH, Jeon UB, Jae HJ, Cho BH, Park JH: **Extrahepatic collateral artery supply to the tumor thrombi of hepatocellular carcinoma invading inferior vena cava: the prevalence and determinant factors.** *J Vasc Interv Radiol* 2009, **20**:22–29.
24. Llovet JM, Bustamante J, Castells A, Vilana R, Ayuso Mdel C, Sala M, Bru C, Rodes J, Bruix J: **Natural history of untreated nonsurgical hepatocellular carcinoma: rationale for the design and evaluation of therapeutic trials.** *Hepatology* 1999, **29**:62–67.
25. Shivathirthan N, Shimoda M, Kosuge T, Kato M, Kijima H, Sawada T, Kubota K: **Recurrent hepatocellular carcinoma with tumor thrombus in right atrium - report of a successful liver resection with tumor thrombectomy using total hepatic vascular exclusion without concomitant cardiopulmonary bypass.** *Hepatogastroenterology* 2012, **59**:872–874.
26. Nonami T, Nakao A, Harada A, Kaneko T, Kurokawa T, Takagi H: **Hepatic resection for hepatocellular carcinoma with a tumor thrombus extending to inferior vena cava.** *Hepatogastroenterology* 1997, **44**:798–802.
27. Togo S, Shimada H, Tanaka K, Masui H, Fujii S, Endo I, Sekido H: **Management of malignant tumor with intracaval extension by selective clamping of IVC.** *Hepatogastroenterology* 1996, **43**:1165–1171.
28. Okuda K, Kage M, Shrestha SM: **Proposal of a new nomenclature for Budd-Chiari syndrome: hepatic vein thrombosis versus thrombosis of the inferior vena cava at its hepatic portion.** *Hepatology* 1998, **28**:1191–1198.
29. Fortner JG, Kallum BO, Kim DK: **Surgical management of hepatic vein occlusion by tumor: Budd-Chiari syndrome.** *Arch Surg* 1977, **112**:727–728.
30. Matsuda H, Sadamori H, Shinoura S, Umeda Y, Yoshida R, Satoh D, Utsumi M, Onishi T, Yagi T: **Aggressive combined resection of hepatic inferior vena cava, with replacement by a ringed expanded polytetrafluoroethylene graft, in living-donor liver transplantation for hepatocellular carcinoma beyond the Milan criteria.** *J Hepatobiliary Pancreat Sci* 2010, **17**:719–724.
31. Florman S, Weaver M, Primeaux P, Killackey M, Sierra R, Gomez S, Haque S, Regenstein F, Balart L: **Aggressive resection of hepatocellular carcinoma with right atrial involvement.** *Am Surg* 2009, **75**:1104–1108.
32. Zeng ZC, Fan J, Tang ZY, Zhou J, Wang JH, Wang BL, Guo W: **Prognostic factors for patients with hepatocellular carcinoma with macroscopic portal vein or inferior vena cava tumor thrombi receiving external-beam radiation therapy.** *Cancer Sci* 2008, **99**:2510–2517.
33. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Haussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J: **Sorafenib in advanced hepatocellular carcinoma.** *N Engl J Med* 2008, **359**:378–390.
34. Yogita S, Tashiro S, Harada M, Kitagawa T, Kato I: **Hepatocellular carcinoma with extension into the right atrium: report of a successful liver resection by hepatic vascular exclusion using cardiopulmonary bypass.** *J Med Invest* 2000, **47**:155–160.
35. Bruix J, Raoul JL, Sherman M, Mazzaferro V, Bolondi L, Craxi A, Galle PR, Santoro A, Beaugrand M, Sangiovanni A, Porta C, Gerken G, Marrero JA, Nadel A, Shan M, Moscovici M, Voliotis D, Llovet JM: **Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial.** *J Hepatol* 2012, **57**:821–829.
36. Shirabe K, Shimada M, Tsujita E, Maehara S, Yamashita Y, Rikimaru T, Tanaka S, Sugimachi K: **Thrombectomy before hepatic resection for hepatocellular carcinoma with a tumor thrombus extending to the inferior vena cava.** *Int Surg* 2001, **86**:141–143.

37. Okada S: **How to manage hepatic vein tumour thrombus in hepatocellular carcinoma.** *J Gastroenterol Hepatol* 2000, **15**:346–348.
38. Kamiyama T, Nakanishi K, Yokoo H, Tahara M, Nakagawa T, Kamachi H, Taguchi H, Shirato H, Matsushita M, Todo S: **Efficacy of preoperative radiotherapy to portal vein tumor thrombus in the main trunk or first branch in patients with hepatocellular carcinoma.** *Int J Clin Oncol* 2007, **12**:363–368.
39. Kamiyama T, Nakanishi K, Yokoo H, Kamachi H, Tahara M, Kakisaka T, Tsuruga Y, Todo S, Taketomi A: **Analysis of the risk factors for early death due to disease recurrence or progression within 1 year after hepatectomy in patients with hepatocellular carcinoma.** *World J Surg Oncol* 2012, **10**:107.
40. Hwang S, Kim YH, Kim DK, Ahn CS, Moon DB, Kim KH, Ha TY, Song GW, Jung DH, Kim HR, Park GC, Namgoong JM, Yoon SY, Jung SW, Park SI, Lee SG: **Resection of pulmonary metastases from hepatocellular carcinoma following liver transplantation.** *World J Surg* 2012, **36**:1592–1602.
41. Chua TC, Morris DL: **Exploring the role of resection of extrahepatic metastases from hepatocellular carcinoma.** *Surg Oncol* 2011, **21**:95–101.
42. Jiang W, Zeng ZC, Zhang JY, Fan J, Zeng MS, Zhou J: **Palliative radiation therapy for pulmonary metastases from hepatocellular carcinoma.** *Clin Exp Metastasis* 2011, **29**:197–205.

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

Makoto Chuma*, Naoya Sakamoto, Akira Nakai[1], Shuhei Hige, Mitsuru Nakanishi, Mitsuteru Natsuizaka, Goki Suda, Takuya Sho, Kanako Hatanaka[2], Yoshihiro Matsuno[2], Hideki Yokoo[3], Toshiya Kamiyama[3], Akinobu Taketomi[3], Gen Fujii[4], Kosuke Tashiro[5], Yoko Hikiba[6], Mitsuaki Fujimoto[1], Masahiro Asaka and Shin Maeda[7]

Department of Gastroenterology and Hepatology, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan, [1]Department of Biochemistry and Molecular Biology, Yamaguchi University, Ube, Japan, [2]Department of Pathology and [3]Department of Gastroenterological Surgery I, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan, [4]Division of Cancer Prevention, National Cancer Center Research Institute, Tokyo, Japan, [5]Graduate School of Genetic Resources Technology, Kyushu University, Fukuoka, Japan, [6]Division of Gastroenterology, Institute for Adult Diseases, Asahi Life Foundation, Tokyo, Japan and [7]Department of Gastroenterology, Yokohama City University, Yokohama, Japan

*To whom correspondence should be addressed. Tel: +81 11-716-1611; Fax: +81 11-706-7867;
Email: mchuuma@med.hokudai.ac.jp

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

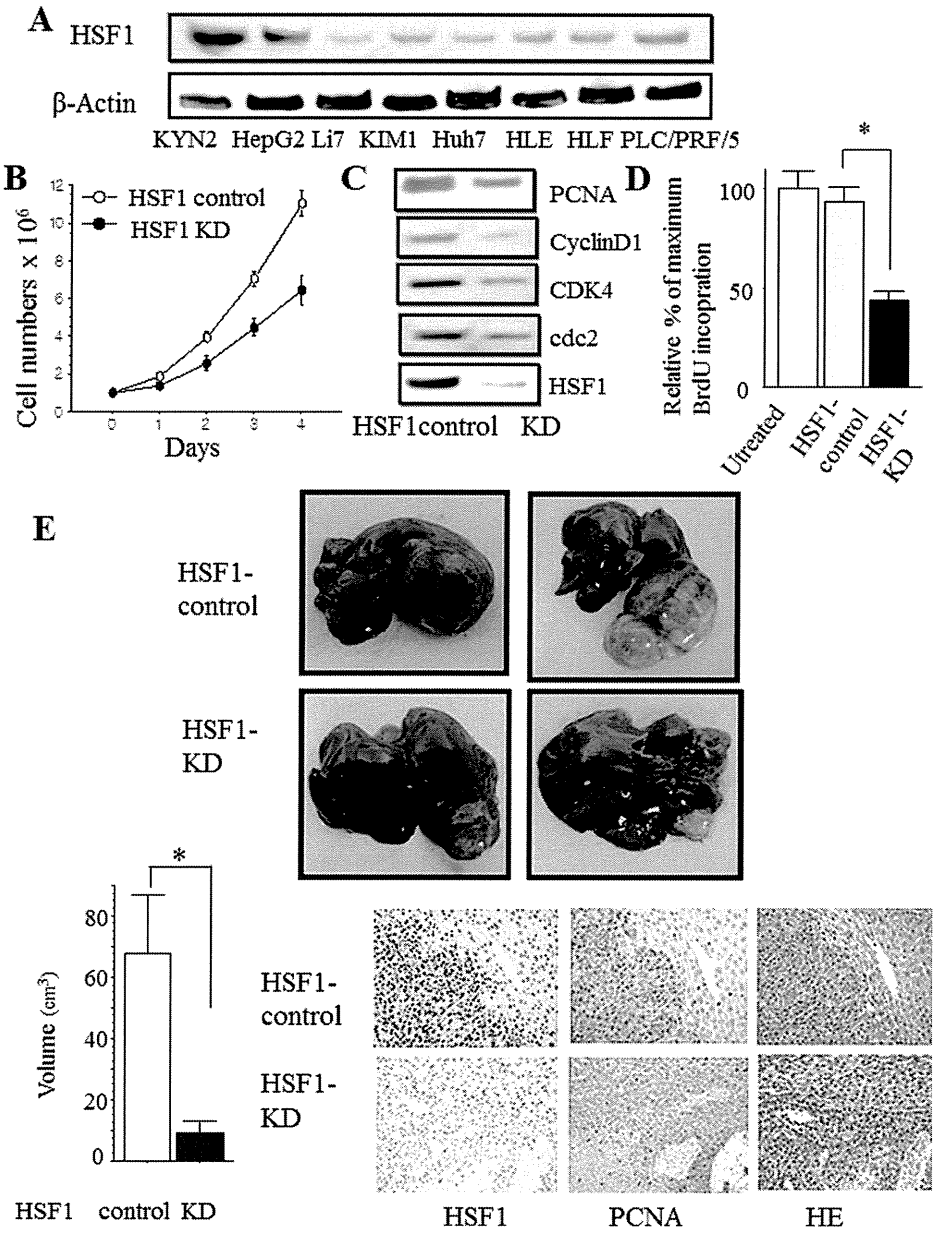

Fig. 1. Role of HSF1 in HCC growth. (**A**) Expression of HSF1 in the eight indicated HCC cell lines was determined by western blot analysis, using β-actin as a control. (**B**) Cell growth of HSF1 control KYN2 cells and HSF1 KD KYN2 cells was measured by counting the number of cells. One representative 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

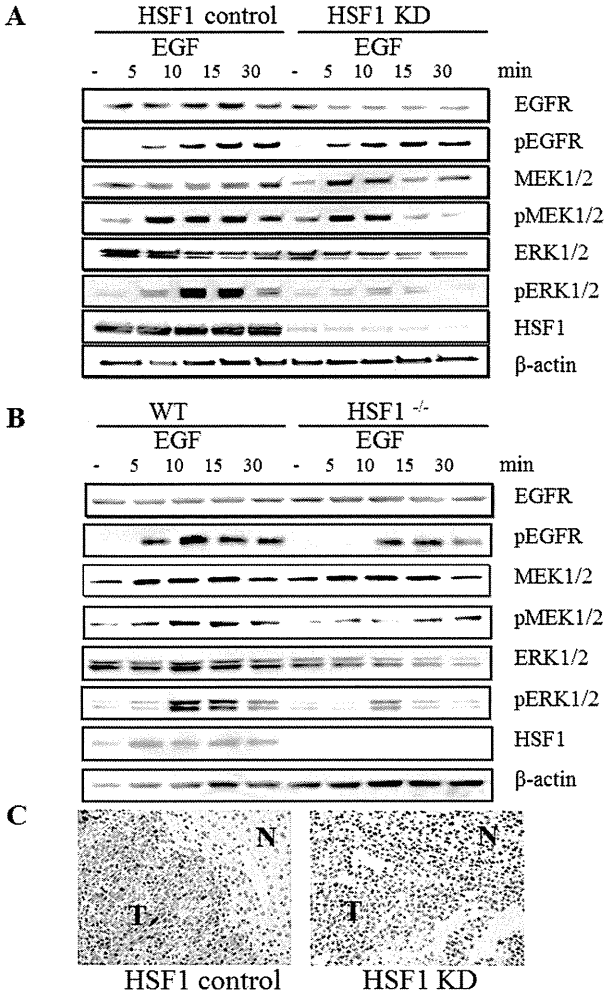

Fig. 2. EGF-mediated MEK/ERK activation is impaired in HSF1 KD cells and HSF1$^{-/-}$ hepatocytes. (**A**) HSF1 control and KD cells were treated with EGF (10 ng/ml), lysed at the indicated times, gel separated and immunoblotted with antibodies against indicated proteins. (**B**) HSF1 WT and HSF$^{-/-}$ 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

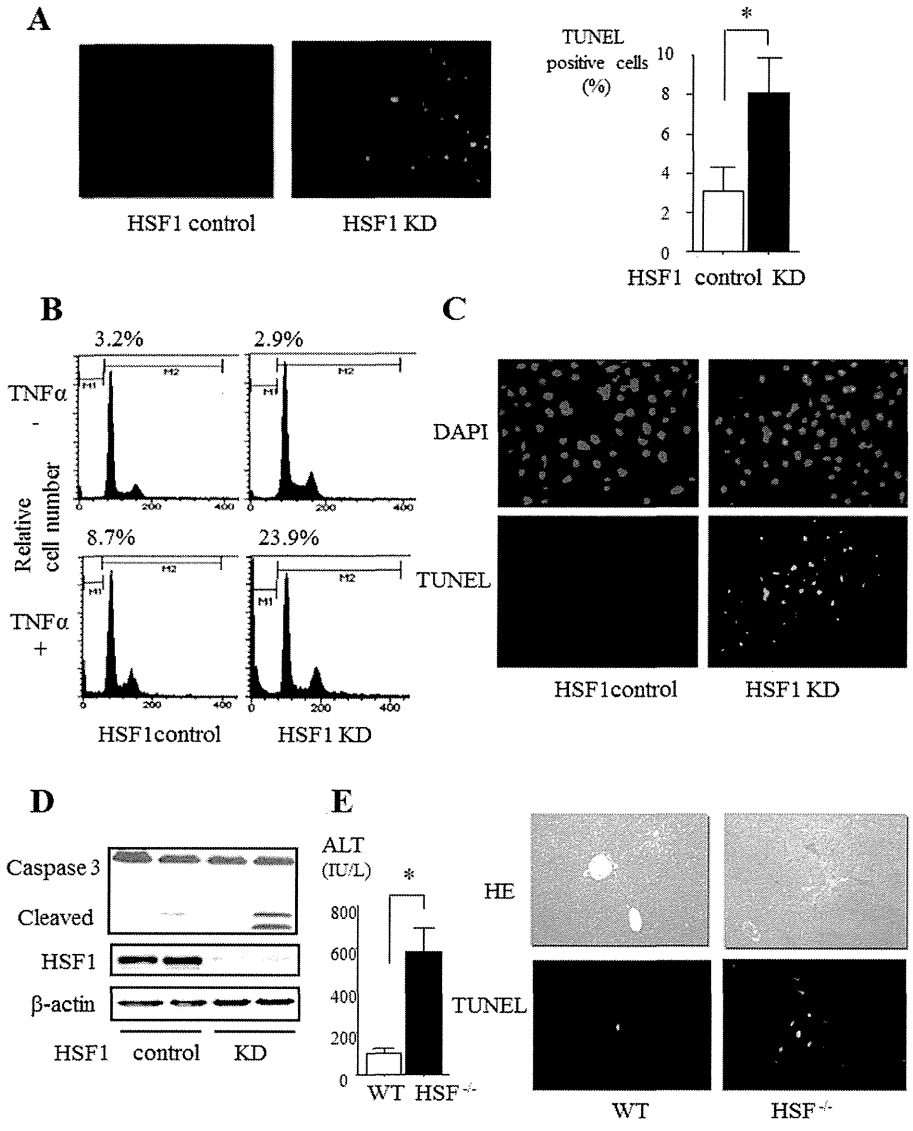

Fig. 3. Antiapoptotic effect of HSF1 in HCC cells and hepatocytes. (**A**) TUNEL staining was performed in tumors of HSF1 control and HSF1 KD cells from orthotopic implanted mice (left panel). TUNEL-positive cells were counted in tumors of HSF1 control and HSF1 KD cells. $*P < 0.05$. Bars: SEM (right 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

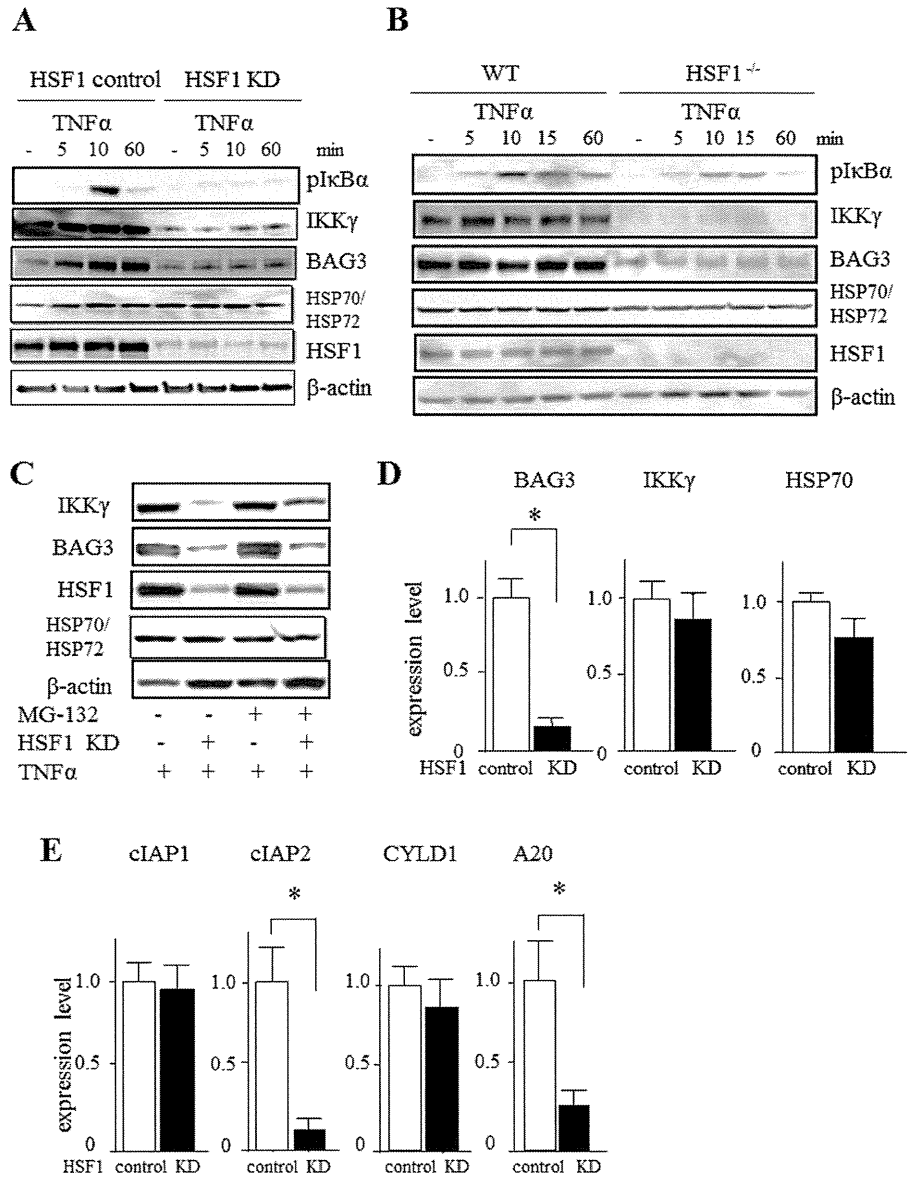

Fig. 4. HSF1 is involved in TNF-α-mediated NF-κB activation. (**A**) HSF1 control and KD cells were treated with TNF-α (30 ng/ml), lysed at the indicated times, gel separated and immunoblotted with antibodies against the indicated proteins. (**B**) HSF1 WT and HSF$^{-/-}$ hepatocytes treated with TNF-α (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

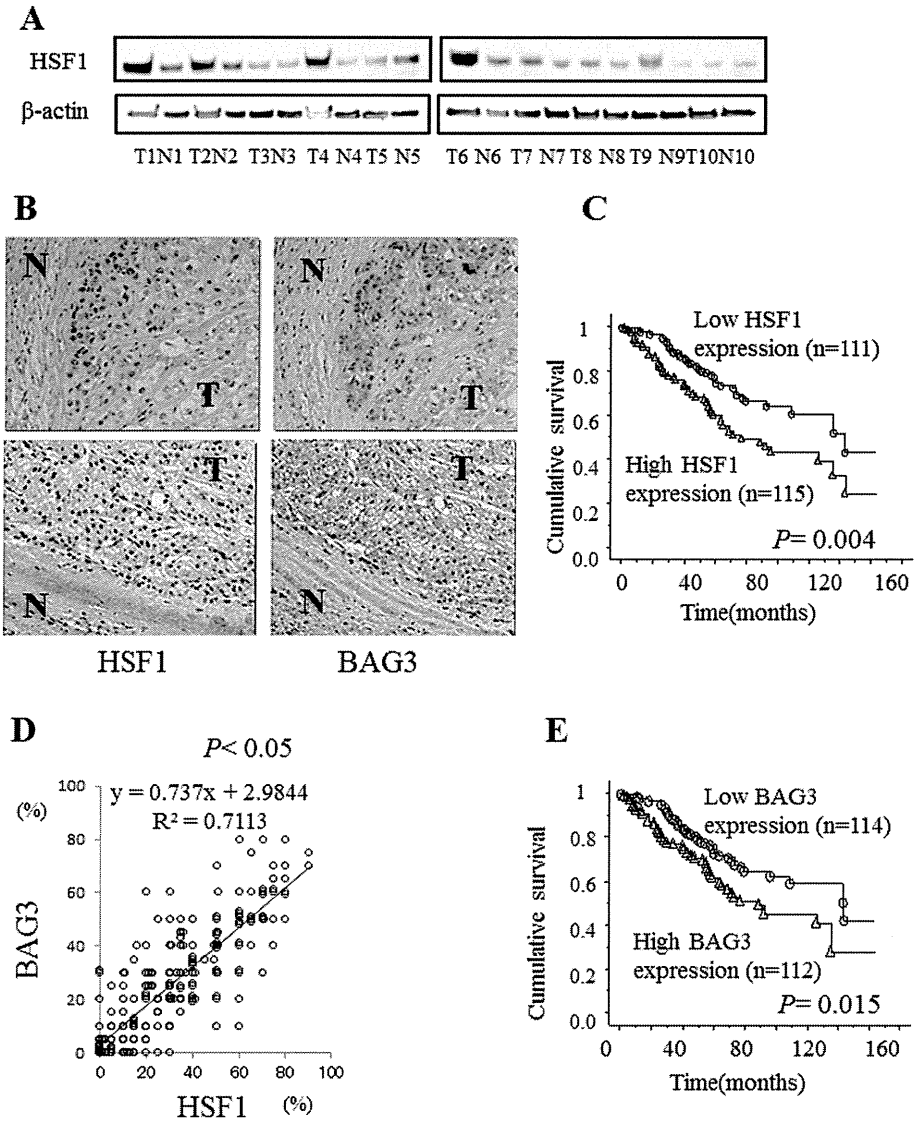

Fig. 5. Overexpression of HSF1 protein in human HCCs and pathological relationship between HSF1 and BAG3 in HCC samples. (**A**) HSF1 protein expression was determined in paired samples of human non-neoplastic liver and HCC by western blot, using β-actin as a control. N, non-cancerous liver; T, tumor. (**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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References

1. El-Serag,H.B. (2012) Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology*, **142**, 1264–1273.e1.
2. Cheng,A.L. *et al.* (2009) Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol.*, **10**, 25–34.
3. Breuhahn,K. *et al.* (2011) Strategies for hepatocellular carcinoma therapy and diagnostics: lessons learned from high throughput and profiling approaches. *Hepatology*, **53**, 2112–2121.
4. Pirkkala,L. *et al.* (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J.*, **15**, 1118–1131.
5. Sorger,P.K. (1991) Heat shock factor and the heat shock response. *Cell*, **65**, 363–366.
6. Guertin,M.J. *et al.* (2010) Chromatin landscape dictates HSF binding to target DNA elements. *PLoS Genet.*, **6**, e1001114.
7. Mendillo,M.L. *et al.* (2012) HSF1 drives a transcriptional program distinct from heat shock to support highly malignant human cancers. *Cell*, **150**, 549–562.
8. Page,T.J. *et al.* (2006) Genome-wide analysis of human HSF1 signaling reveals a transcriptional program linked to cellular adaptation and survival. *Mol. Biosyst.*, **2**, 627–639.
9. Dai,C. *et al.* (2007) Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell*, **130**, 1005–1018.
10. Hayashida,N. *et al.* (2006) A novel HSF1-mediated death pathway that is suppressed by heat shock proteins. *EMBO J.*, **25**, 4773–4783.
11. Jacobs,A.T. *et al.* (2007) Heat shock factor 1 attenuates 4-hydroxynonenal-mediated apoptosis: critical role for heat shock protein 70 induction and stabilization of Bcl-XL. *J. Biol. Chem.*, **282**, 33412–33420.
12. Vydra,N. *et al.* (2006) Spermatocyte-specific expression of constitutively active heat shock factor 1 induces HSP70i-resistant apoptosis in male germ cells. *Cell Death Differ.*, **13**, 212–222.
13. Neckers,L. *et al.* (2012) Hsp90 molecular chaperone inhibitors: are we there yet? *Clin. Cancer Res.*, **18**, 64–76.
14. Khalil,A.A. *et al.* (2011) Heat shock proteins in oncology: diagnostic biomarkers or therapeutic targets? *Biochim. Biophys. Acta*, **1816**, 89–104.
15. Chuma,M. *et al.* (2003) Expression profiling in multistage hepatocarcinogenesis: identification of HSP70 as a molecular marker of early hepatocellular carcinoma. *Hepatology*, **37**, 198–207.
16. Cai,L. *et al.* (2003) The tumor-selective over-expression of the human Hsp70 gene is attributed to the aberrant controls at both initiation and elongation levels of transcription. *Cell Res.*, **13**, 93–109.
17. Min,J.N. *et al.* (2007) Selective suppression of lymphomas by functional loss of Hsf1 in a p53-deficient mouse model for spontaneous tumors. *Oncogene*, **26**, 5086–5097.
18. Santagata,S. *et al.* (2011) High levels of nuclear heat-shock factor 1 (HSF1) are associated with poor prognosis in breast cancer. *Proc. Natl Acad. Sci. USA*, **108**, 18378–18383.
19. Dudeja,V. *et al.* (2011) Prosurvival role of heat shock factor 1 in the pathogenesis of pancreatobiliary tumors. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **300**, G948–G955.
20. Hoang,A.T. *et al.* (2000) A novel association between the human heat shock transcription factor 1 (HSF1) and prostate adenocarcinoma. *Am. J. Pathol.*, **156**, 857–864.
21. Ishiwata,J. *et al.* (2012) State of heat shock factor 1 expression as a putative diagnostic marker for oral squamous cell carcinoma. *Int. J. Oncol.*, **40**, 47–52.
22. Kojiro,M. *et al.* (2009) Pathologic diagnosis of early hepatocellular carcinoma: a report of the international consensus group for hepatocellular neoplasia. *Hepatology*, **49**, 658–664.
23. Fabregat,I. *et al.* (2007) Survival and apoptosis: a dysregulated balance in liver cancer. *Liver Int.*, **27**, 155–162.
24. Nakagawa,H. *et al.* (2011) Apoptosis signal-regulating kinase 1 inhibits hepatocarcinogenesis by controlling the tumor-suppressing function of stress-activated mitogen-activated protein kinase. *Hepatology*, **54**, 185–195.
25. Sun,B. *et al.* (2008) NF-kappaB signaling, liver disease and hepatoprotective agents. *Oncogene*, **27**, 6228–6244.
26. Maeda,S. *et al.* (2005) IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell*, **121**, 977–990.
27. Beeram,M. *et al.* (2005) Raf: a strategic target for therapeutic development against cancer. *J. Clin. Oncol.*, **23**, 6771–6790.
28. Whittaker,S. *et al.* (2010) The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene*, **29**, 4989–5005.
29. Inouye,S. *et al.* (2003) Activation of heat shock genes is not necessary for protection by heat shock transcription factor 1 against cell death due to a single exposure to high temperatures. *Mol. Cell. Biol.*, **23**, 5882–5895.
30. Chuma,M. *et al.* (2004) Overexpression of cortactin is involved in motility and metastasis of hepatocellular carcinoma. *J. Hepatol.*, **41**, 629–636.
31. O'Callaghan-Sunol,C. *et al.* (2006) Heat shock transcription factor (HSF1) plays a critical role in cell migration via maintaining MAP kinase signaling. *Cell Cycle*, **5**, 1431–1437.

32. Nowak,M. *et al.* (2000) LPS-induced liver injury in D-galactosamine-sensitized mice requires secreted TNF-alpha and the TNF-p55 receptor. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **278**, R1202–R1209.
33. Ammirante,M. *et al.* (2010) IKK{gamma} protein is a target of BAG3 regulatory activity in human tumor growth. *Proc. Natl Acad. Sci. USA*, **107**, 7497–7502.
34. Meng,L. *et al.* (2010) Heat-shock transcription factor HSF1 has a critical role in human epidermal growth factor receptor-2-induced cellular transformation and tumorigenesis. *Oncogene*, **29**, 5204–5213.
35. Solimini,N.L. *et al.* (2007) Non-oncogene addiction and the stress phenotype of cancer cells. *Cell*, **130**, 986–988.
36. Malhotra,V. *et al.* (2002) Heat shock inhibits activation of NF-kappaB in the absence of heat shock factor-1. *Biochem. Biophys. Res. Commun.*, **291**, 453–457.
37. Janus,P. *et al.* (2011) NF-κB signaling pathway is inhibited by heat shock independently of active transcription factor HSF1 and increased levels of inducible heat shock proteins. *Genes Cells*, **16**, 1168–1175.
38. Rosati,A. *et al.* (2011) BAG3: a multifaceted protein that regulates major cell pathways. *Cell Death Dis.*, **2**, e141.
39. Homma,S. *et al.* (2006) BAG3 deficiency results in fulminant myopathy and early lethality. *Am. J. Pathol.*, **169**, 761–773.
40. Franceschelli,S. *et al.* (2008) Bag3 gene expression is regulated by heat shock factor 1. *J. Cell. Physiol.*, **215**, 575–577.
41. Luo,J.L. *et al.* (2005) IKK/NF-kappaB signaling: balancing life and death–a new approach to cancer therapy. *J. Clin. Invest.*, **115**, 2625–2632.
42. Jin,X. *et al.* (2011) Heat shock transcription factor 1 is a key determinant of HCC development by regulating hepatic steatosis and metabolic syndrome. *Cell Metab.*, **14**, 91–103.
43. Luedde,T. *et al.* (2007) Deletion of NEMO/IKKgamma in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. *Cancer Cell*, **11**, 119–132.

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