

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 \pm 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 4h. 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 \pm 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: $\times 40$): lower right panel. BrdU, bromodeoxyuridine; HE, hematoxylin and eosin.

administration, HSF1^{-/-} 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- κ B 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 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- κ B pathway represents one of the most important antiapoptotic signals. In addition, BAG3 is also reported to control proteasomal degradation of IKK γ , the regulatory subunit (also called NF- κ B essential modulator) of the IKK complex, and

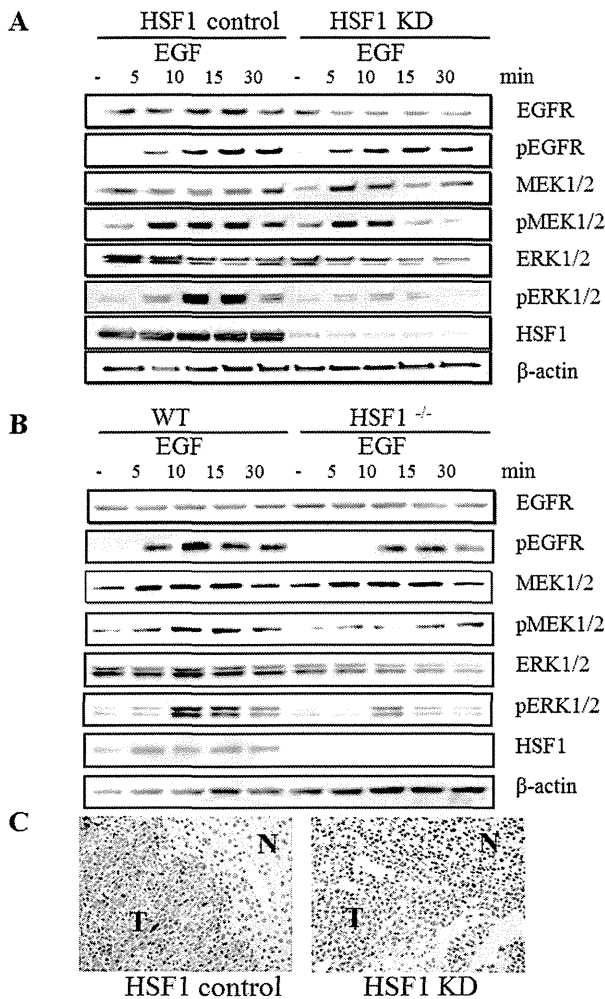


Fig. 2. EGF-mediated MEK/ERK activation is impaired in HSF1 KD cells and HSF1^{-/-} hepatocytes. (A) HSF1 control and KD cells were treated with EGF (10 ng/ml), lysed at the indicated times, gel separated and immunoblotted with antibodies against indicated proteins. (B) HSF1 WT and HSF1^{-/-} hepatocytes 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: $\times 40$). N, non-cancerous liver; T, tumor.

NF- κ B activity (33). Regarding the NF- κ B pathway, NF- κ B activation by TNF- α was decreased in HSF1 KD cells compared with the control cells (Figure 4A). In contrast, without any treatment, basal NF- κ B 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- κ B-regulated genes. We also performed NF- κ B 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- κ B activation and found that phosphorylated I κ B (p-I κ B), a marker of NF- κ B activation, was significantly decreased in HSF1^{-/-} hepatocytes and HSF1 KD cells compared with their controls. As expected, IKK γ protein levels were dramatically reduced in HSF1^{-/-} hepatocytes and HSF1 KD cells compared with their controls (Figure 4A and B). To investigate whether decreased IKK γ protein was degraded via proteasome, we used the proteasomal inhibitor, MG-132, and

found that protein levels of IKK γ 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 IKK γ 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 IKK γ protein necessary for NF- κ B 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- κ B-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- κ B-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

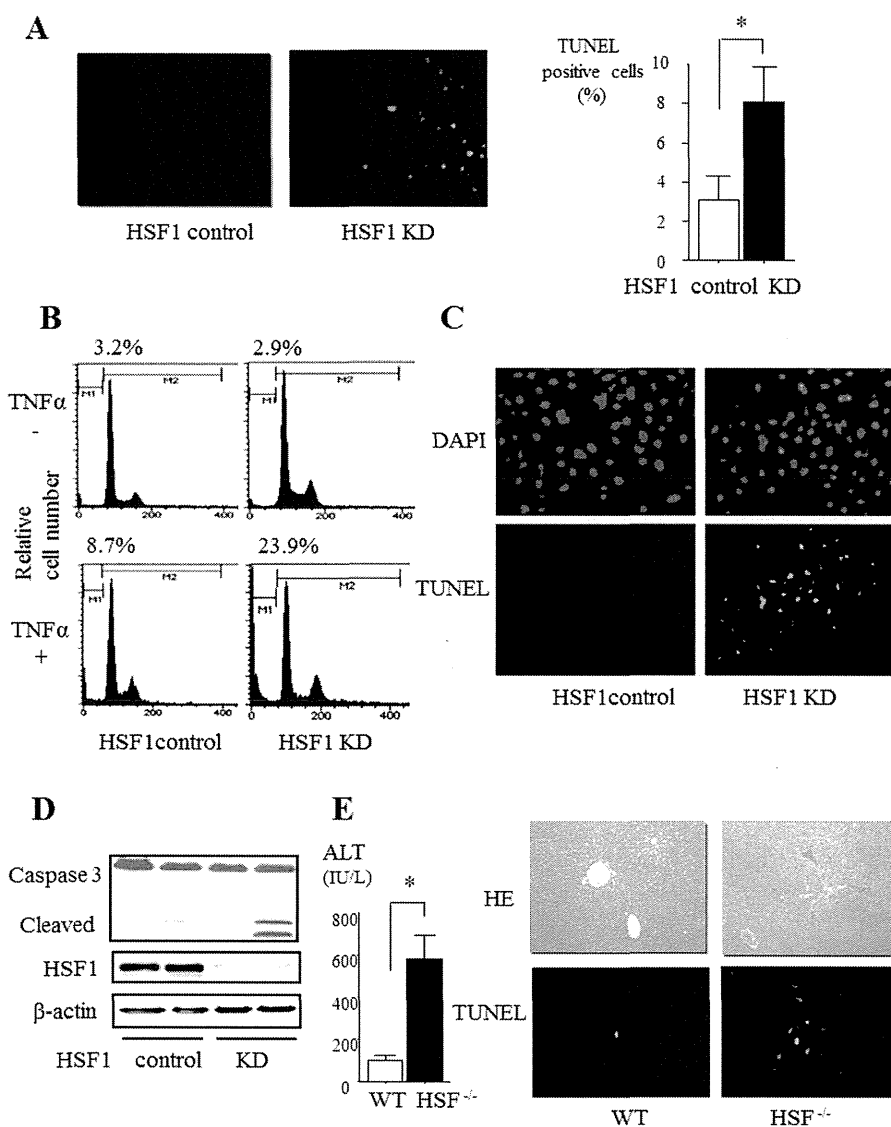


Fig. 3. Antiapoptotic effect of HSF1 in HCC cells and hepatocytes. (A) TUNEL staining was performed in tumors of HSF1 control and HSF1 KD cells from orthotopic implanted mice (left panel). TUNEL-positive cells were counted in tumors of HSF1 control and HSF1 KD cells. $*P < 0.05$. Bars: SEM (right panel). (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-G₁ 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 μ g/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 μ g/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- κ B signaling pathway. Although, in a previous study, heat shock blocked the degradation of I κ B (36) and nuclear translocation of NF- κ B, the recent literature has reported that the presence of constitutively active HSF1 does not block TNF- α -induced activation of the NF- κ B pathway or expression of a set of NF- κ B-dependent genes (37). The current study established HSF1 KD cells and showed that HSF1 was necessary for TNF- α -induced NF- κ B activation. We analyzed the function of BAG3 as a candidate for the molecule connecting HSF1 with NF- κ B activation. BAG3 has reportedly been characterized by the

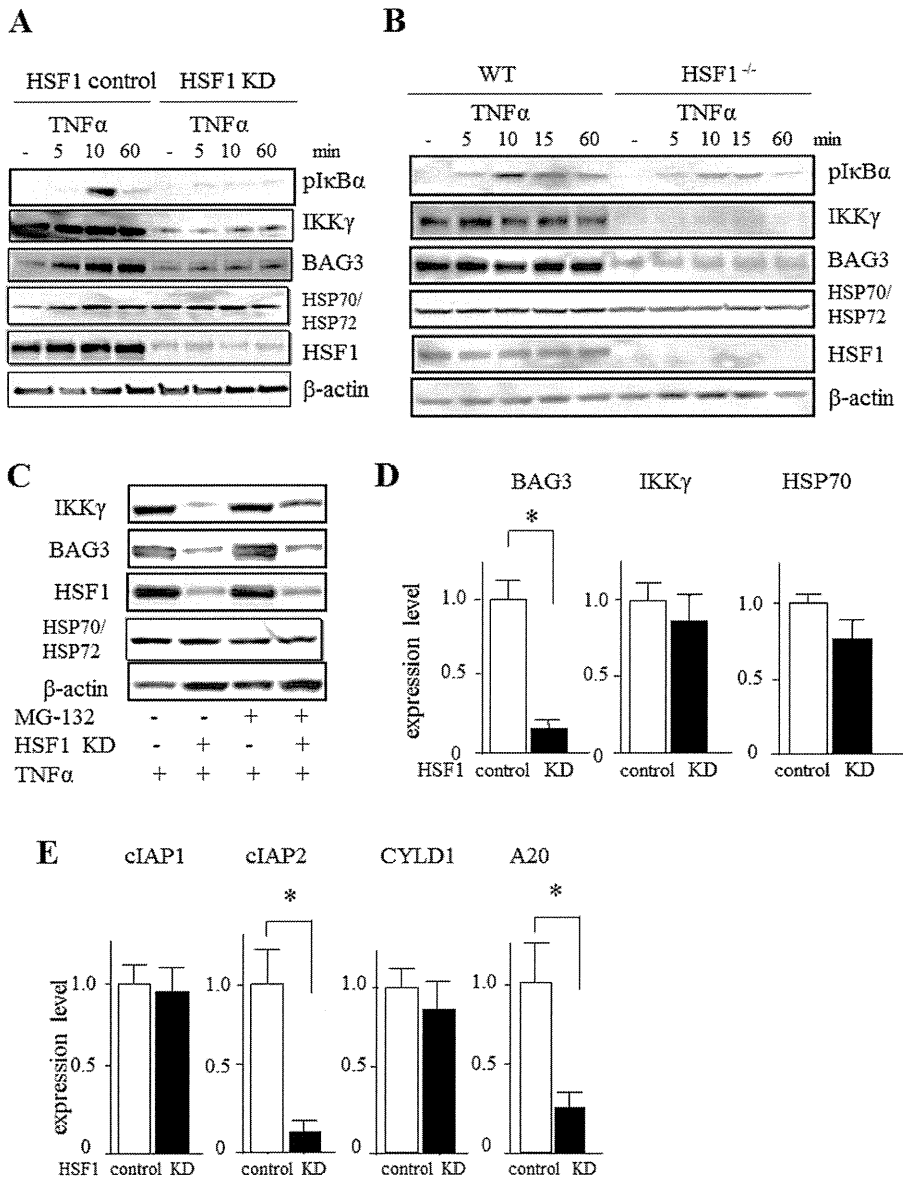


Fig. 4. HSF1 is involved in TNF- α -mediated NF- κ B activation. (A) HSF1 control and KD cells were treated with TNF- α (30 ng/ml), lysed at the indicated times, gel separated and immunoblotted with antibodies against the indicated proteins. (B) HSF1 WT and HSF1^{-/-} hepatocytes treated with TNF- α (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, IKK γ and HSP70 in HSF1 control and KD cells determined by real-time PCR. Data are expressed as mean \pm 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 \pm 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 IKK γ levels, increasing tumor cell apoptosis and inhibiting tumor growth (33). Based on these considerations, we investigated whether attenuating HSF1 would enhance IKK γ protein expression, and data with MG-132 show that proteasomal degradation of IKK γ 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 IKK γ in this setting.

NF- κ B activation is a master regulatory step in antiapoptosis. Several mechanisms have been reported regarding this antiapoptotic effect of NF- κ B activation (41). NF- κ B 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- κ B 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- κ B activation, whereas apoptosis-related genes such

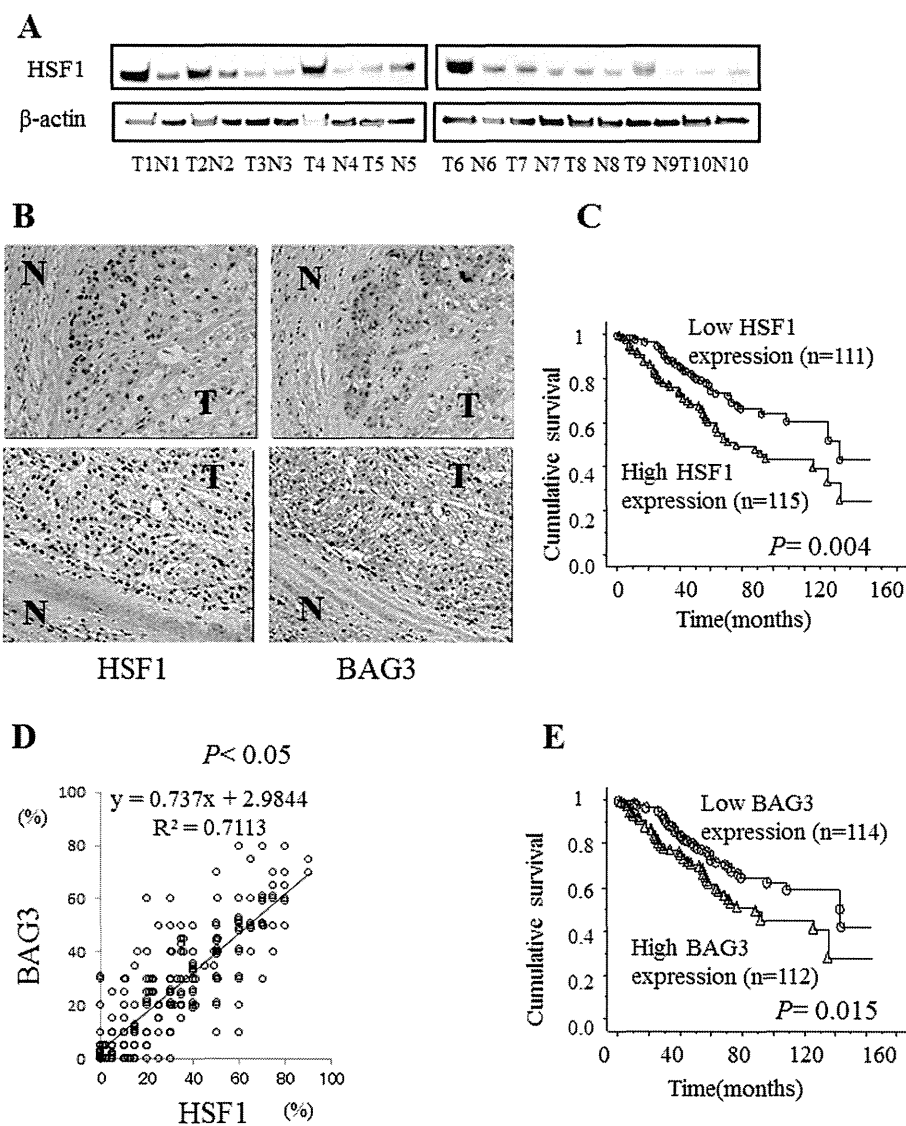


Fig. 5. Overexpression of HSF1 protein in human HCCs and pathological relationship between HSF1 and BAG3 in HCC samples. (A) HSF1 protein expression was determined in paired samples of human non-neoplastic liver and HCC by western blot, using β -actin as a control. N, non-cancerous liver; T, tumor. (B) 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- κ B activation, were apparently unaffected. Whether gene expression regulated by NF- κ B 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 IKK γ 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 IKK γ , 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- κ B 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 1 and Figures 1 and 2 can be found at <http://carcin.oxfordjournals.org/>

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