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Overexpressed Nuclear BAG-1 in Human Hepatocellular Carcinoma is Associated with Poor Prognosis and Resistance to Doxorubicin

Wenkai Ni,[1] Buyou Chen,[2] Guoxiong Zhou,[1] Cuihua Lu,[1] Mingbing Xiao,[1] Chengqi Guan,[1] Yixing Zhang,[3] Song He,[3] Aiguo Shen,[4] and Runzhou Ni[1]*

[1]*Department of Gastroenterology, Affiliated Hospital of Nantong University, Nantong, 226001, P.R., China*
[2]*Department of Radiochemotherapy, Affiliated Hospital of Nantong University, Nantong, 226001, P.R., China*
[3]*Department of Pathology, Affiliated Cancer Hospital of Nantong University, Medical College of Nantong University, Nantong, 226001, P.R., China*
[4]*Department of Immunology, Medical College, Nantong University, Nantong, 226001, P.R., China*

ABSTRACT

Bcl-2-associated athanogene-1 (BAG-1) is a multifunctional anti-apoptotic protein which regulates an array of cellular processes, including apoptosis, signaling, proliferation, transcription, and cell motility and has been reported to be over-expressed in a number of human malignancies. To investigate the possible involvement of BAG-1 in tumorigenesis of hepatocellular carcinoma (HCC), we performed Western blot analysis in eight paired samples of HCC and adjacent peritumoral tissues and immunohistochemistry in 65 paraffin sections of HCC, which both showed an enhanced expression of nuclear BAG-1 isoform in HCC tissues. Statistical analysis confirmed that overexpression of nuclear BAG-1 in HCC tissues was significantly associated with histological grading ($P < 0.001$), poor prognosis ($P = 0.004$), and was found to be an independent prognostic indicator for HCC ($P = 0.023$). We also noted that BAG-1 was overexpressed in four HCC cell lines compared with a normal hepatocyte cell line, and BAG-1 overexpression increased resistance of HCC cells to doxorubicin, a common chemotherapeutic agent for HCC. Furthermore, we observed that knock down of BAG-1 with siRNA in HepG2 cells increased the chemosensitivity of cells, a process mediated through inhibition of doxorubicin-triggered NF-κB activation; and knock down of BAG-1 suppressed proliferation and cell cycle transition of HepG2 cells. In consequence, our results for the first time indicated that BAG-1 was dysregulated in HCC and suppression of BAG-1 expression which resulted in inhibiting of NF-κB signaling might be developed into a new strategy in HCC therapy. J. Cell. Biochem. 114: 2120–2130, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: BCL-2-ASSOCIATED ATHANOGENE-1 (BAG-1); HEPATOCELLULAR CARCINOMA (HCC); PROGNOSIS; NUCLEAR FACTOR-κB (NF-κB); CHEMORESISTANCE

Human hepatocellular carcinoma (HCC), accounting for 90% of primary liver cancer, presents a globally significant challenge as it is the sixth most common cancer and third common cause of cancer-related mortality worldwide [Lu et al., 2009]. Hepatic resection is a potentially curative therapy for HCC, but the prognosis of postoperative HCC patients remains poor because of high recurrence and metastasis rates. Transarterial chemoembolization (TACE) and systemic therapy by doxorubicin (a common chemotherapeutic agent used to treat HCC) alone or combined with other chemotherapeutic drugs are widely accepted for the management of advanced-stage HCC, achieving an improvement in overall survival duration varying between 6.8 and 8.6 months [Patt et al., 1999; Llovet et al., 2002;

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*Correspondence to: Runzhou Ni, Affiliated Hospital of Nantong University, Nantong, 226001, Jiangsu Province, China. E-mail: nirz@163.com

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Llovet and Bruix, 2003]. The difficulty of developing effective chemotherapy is partly caused by inherent chemoresistantance of HCC, which is related to multidrug-resistant gene expression. However, the underlying molecule mechanisms are not fully clear.

BAG-1 (Bcl-2-associated athanogene-1) is a multifunctional anti-apoptotic protein which modulates growth control pathways important for both normal and malignant cells, including apoptosis, signaling, proliferation, transcription, and cell motility [Townsend et al., 2003]. Four isoforms of BAG-1, translated from the same mRNA, are found in humans: BAG-1L/p50, BAG-1M/p46, BAG-1S/p36, and BAG-1/p29. Different BAG-1 isoforms have distinct subcellular localizations within the cell. BAG-1L, containing a nuclear localization signal, is generally restricted to the nucleus; BAG-1S or BAG-1/p29 is predominantly a soluble cytoplasmic protein; and BAG-1M partitions itself between the nucleus and cytoplasm [Brimmell et al., 1999]. BAG-1 is upregulated in a wide rage of cancers, including breast, cervical, and colon cancers [Zapata et al., 1998; Yang et al., 1999; Clemo et al., 2008]. Intriguingly, the expression and prognostic role of specific BAG-1 isoforms vary among different tumor types. For example, overexpression of nuclear BAG-1 in colorectal cancer is associated with a risk of metastasis and a short overall survival [Kikuchi et al., 2002], whereas elevated expression of cytosolic BAG-1 in breast cancer predicts a longer overall survival and a distant metastasis-free survival [Turner et al., 2001].

Previous studies mainly focus on the role of BAG-1 acting as an anti-apoptotic protein through several complicated mechanisms. The major findings are: (1) BAG-1 has an ability to bind to BCL-2 for inhibiting apoptosis triggered by a variety of apoptotic agents [Takayama et al., 1995]. (2) BAG-1 modulates nuclear hormone receptors (NHR)-mediated regulation of cell proliferation and survival, which is an important component in cancer development. For example, BAG-1 may bind to oestrogen receptors and enhances its ability to mediate cell proliferation and survival responses to oestrogens in hormone dependent breast cancer [Cutress et al., 2001]. (3) BAG-1 interacts with vitamin D receptor (VDR) and leads to inhibition of vitamin D-induced apoptosis in cancer cells [Witcher et al., 2001].

It has been recently established that BAG-1 potentiates the transcriptional activity of nuclear factor (NF)-κB that plays a critical role in resistance to chemotherapy [Weldon et al., 2001; Clemo et al., 2008]. The NF-κB gene family comprises structurally related transcription factors such as p50 (NF-κB1), p65 (RelA), c-Rel, p52, and RelB, all of which have a conserved N-terminal Rel homology domain (RHD) that contains the DNA-binding and dimerization regions. Only p65, RelB, and c-Rel, however, contain potent transactivation domains within sequences C-terminal to the RHD [Nogueira et al., 2011]. In resting cells, NF-κB is sequestered in the cytosol by its inhibitor IκB protein in an inactive state. Upon stimulation with some agents, such as tumor necrosis factor (TNF-α), interleukin-1, or bacterial lipopolysaccharide, NF-κB is activated by the release from IκB to allow transactivation of target genes [Nogueira et al., 2011].

Recent studies have linked NF-κB signaling activation to the tumor development and metastasis [Staudt, 2010]. In an animal model of HCC, NF-κB inhibition in later stages of tumor development results in apoptosis of transformed hepatocytes and failure in progressing toward HCC [Pikarsky et al., 2004]. NF-κB enhances proliferation of cancer cells by increasing the expression of cyclin-D1 [Guttridge et al., 1999]. Another important role of NF-κB signaling in tumorigenesis appears to mediate chemoresistance probably owing to the ability of NF-κB to activate a number of anti-apoptotic genes, such as Bcl-xL, A1, c-FLIP, c-IAP1, c-IAP2, x-IAP, TRAF1, and TRAF2 [Arlt and Schafer, 2002; Karin and Lin, 2002].

Although BAG-1 has been extensively examined in a wide range of cancers, the issue of whether BAG-1 is dysregulated in tumorigenesis of HCC remains unclear, and the role of BAG-1 as a predictive marker in HCC is also not well studied. Therefore, in this study, we aimed to investigate BAG-1 expression in HCC and to explore the relationship of BAG-1 expression with HCC prognosis. Considering that BAG-1 increases the transcriptional activity of NF-κB and impacts apoptosis in some cancer cells, we further tested whether suppression of BAG-1 expression could increase chemosensitivity of HCC cells through NF-κB signaling and retard HCC progression.

MATERIALS AND METHODS

PATIENTS AND TISSUE SPECIMENS

The paired samples of HCC and adjacent peritumoral tissues were obtained from 65 patients, who had undergone hepatic surgical resection without preoperative systemic chemotherapy at the Affiliated Hospital of Nantong University between May 2002 and January 2005. Our study was approved by the research ethics committee of the institute, and written informed consent was obtained from all patients. The main clinical and pathologic variables of patients are listed in Table I. Immediately after surgical removal, the tissue samples were fixed in formalin and embedded in paraffin to undergo the following procedures.

CELL LINE AND CELL CULTURE

Four HCC cell lines, including SMMC-7721, HepG2, Bel-7404, and Huh7, and a normal human hepatocyte cell line, LO_2 cells, were obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 atmosphere at 37°C.

WESTERN BLOT ANALYSIS

For Western blot analysis, tissue or cell proteins were extracted using standard protocols as described previously [Fei et al., 2009]. The primary antibodies used included anti-BAG-1 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-BCL-2 (1:500, Santa Cruz Biotechnology), anti-cleaved-caspase 3 (1:500, Santa Cruz Biotechnology), anti-PCNA (1:1,000, Santa Cruz Biotechnology), and anti-β-actin (1:1,000 Sigma Chemical Co.).

IMMUNOHISTOCHEMISTRY

Tissue sections (4 μm thick) were placed on 3-amino propyltriethoxy silane (APES)-pretreated slides, deparaffinized, rehydrated through graded alcohol, and quenched in 3% hydrogen peroxide. Antigen retrieval was performed by microwave heating at high power (750 W)

TABLE I. Nuclear BAG-1 Expression and Clinicopathological Parameters in 65 HCC Specimens Based on Immunohistochemistry

Parameters	Total	Nuclear BAG-1		P-value[a]
		Low (final score 0–2; n = 30)	High (final score 3–7; n = 35)	
Gender				
Male	49	22	27	0.722
Female	16	8	8	
Age (years)				
≤45	20	8	12	0.507
>45	45	22	23	
Histological grade				
Well	18	16	2	
Mod	27	10	17	0.000
Poor	20	4	16	
Tumor size (cm)				
≤5	43	20	23	0.936
>5	22	10	12	
Metastasis				
Negative	52	24	28	1.000
Positive	13	6	7	
HBsAg				
(−)	12	6	6	0.767
(+)	53	24	29	
Cirrhosis				
Negative	31	17	14	0.180
Positive	34	13	21	
AFP (ng/ml)				
≤50	32	16	16	0.540
>50	33	14	19	

[a]The P-value was calculated by chi-squared test or Fisher's exact test. $P < 0.05$ was considered significant.

in 10 mM sodium citrate buffer (pH 6.0) for three cycles of 5 min each. After rinse in phosphate-buffered saline (PBS, pH 7.2), the sections were blocked with donkey serum for 1 h at room temperature, followed by incubation with rabbit anti-BAG-1 antibody (1:100, Santa Cruz Biotechnology) overnight at 4°C. Negative control slides were incubated in parallel using a nonspecific immunoglobulin IgG (Sigma, St. Louis, MO) at the same concentration as the primary antibody. All slides were processed using a peroxidase–antiperoxidase method (Dako, Hamburg, Germany). Diaminobenzidine was used as the final chromogen, and Gill's hematoxylin was used for counterstaining [Fei et al., 2009].

IMMUNOHISTOCHEMISTRY EVALUATION

All immunostained sections were evaluated independently by two observers in a blinded manner without knowledge of clinical and pathological variables of patients. For nuclear BAG-1 assessment, the nuclear staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). Extent of nuclear staining was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%) according to the percentage of the positive staining nucleus in relation to the whole cancer nucleus. The sum of the intensity and extent score was considered as the final staining score (0–7) for nuclear BAG-1. A final staining score of ≥3 were considered as high [Xiao et al., 2011]. For all tissue samples, staining was repeated twice to avoid possible technical errors, and similar data were obtained in these samples. The evaluation procedures performed by two observers needed to reach a consensus.

TRANSFECTION WITH siRNA

The sequences of BAG-1 siRNA used for transfection were: #1 5′-GGGAAAAUCUCUGAAGGAAtt-3′ [Clemo et al., 2008], and #2 5′-AGAACAGUCCACAGGAAGAtt-3′ [Maier et al., 2010] which were both specific to all BAG-1 isoforms, and sequence of negative control siRNA was 5′-UUCUCCGAACGUGUCACGUTT-3′. They were synthesized by Genepharma Company (Shanghai, China). Cell transfection was performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

REPORTER GENE ASSAY

Cells were aliquoted into 24-well plates and transfected with BAG-1 siRNA or control RNA for 24 h, and then treated with 2 μM BAY-117082 (a NF-κB inhibitor, Santa Cruz Biotechnology) or 0.1% dimethylsulfoxide (DMSO, a vehicle control) for 1 day. Two days after transfection, each well was co-transfected with pNF-κB-luciferase reporter constructs (0.8 μg/well, Beyotime Biotechnology, Haimen, China) and the renilla construct pRL-TK (0.04 μg/well, Promega, Madison, WI) in the presence of lipofectamine 2000. One day later, cells were treated with or without 30 μM doxorubicin for 3 h. The luciferase activity was measured using a dual luciferase assay system (Promega) and normalized to Renilla luciferase activity.

PROTEASOME INHIBITION ASSAY

HepG2 cells were initially treated with or without 1 μM doxorubicin. At 40 h after the treatment, MG132 (20 μM) and 0.1% DMSO (control) were added to the medium for another 8 h, and whole-cell lysates were prepared and subjected to Western blot analysis as the above mentioned.

CELL VIABILITY ASSAY

To evaluate sensitivity of different HCC cell lines to doxorubicin, the cells were seeded in 96-well plates at the density of 5×10^3/well to allow 24-h incubation, followed by treatment with doxorubicin at different concentrations ranged from 8.5×10^{-3} to 34 μM (5×10^{-3}

to 20 μg/ml) for 48 h. To detect the effect of suppressed BAG-1 expression on the cell viability of HepG2 cells under the stimulation of doxorubicin, the cells were pretreated as above described and transfected with BAG-1 siRNA or control siRNA for 24 h, and then incubated with 2 μM BAY-117082 for 24 h, followed by treatment with 1 μM doxorubicin for 48 h. To verify the role of BAG-1 expression on proliferation of HepG2 cells, the cells were transfected with control siRNA or BAG-1 siRNA followed by adding the Cell Counting Kit reagent at time points as 0, 1, 2, and 3 days. The cell viability was assessed by using the Cell Counting Kit (Dojindo, Kumamoto, Japan). The absorbance was recorded at 450 nm with an Immuno Mini NJ-2300 plate reader (NJ InterMed, Tokyo, Japan).

IMMUNOCYTOCHEMISTRY

HepG2 cells grown on coverslips were transfected with BAG-1 siRNA or control RNA for 48 h, and treated with a graded series of doxorubicin (0, 5, 10, 20, or 30 μM) for 3 h. Afterwards, the cells were fixed with 4% formaldehyde for 30 min, treated with 0.1% Triton X-100/PBS for 5 min, and incubated with PBS containing 3% normal goat serum for 1 h. Then the cells were incubated with primary antibody anti-NF-κB p65 (1:100, Santa Cruz Biotechnology) overnight at 48°C. After rinse, the cells were further reacted with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody, and 4′, 6-diamidino-2-phenylin-dole (DAPI) was used for DNA staining, followed by observation under a confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

TUNEL ASSAY

HepG2 cells were initially seeded in 24-well plates on coverslips for 24 h incubation, and then transfected with BAG-1siRNA or control RNA for 1 day, followed by 24 h treatment with or without 2 μM BAY-117082. After stimulation of 1 μM doxorubicin for 48 h, the cells were subjected to TUNEL staining by using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) in combination with 4′, 6-diamidino-2-phenylindole (DAPI) staining. Apoptotic cells with characteristic nuclear fragmentation were counted in at least 300 cells in randomly chosen fields. The data were expressed as a percentage of apoptotic cells to the total cells.

CELL CYCLE ANALYSIS

Cells were trypsinized, fixed in 70% ethanol for 1 h at 48°C and then incubated with 1 mg/ml RNase A for 30 min at 37°C. Subsequently, cells were stained with propidium iodide (50 mg/ml PI; Becton Dickinson, San Jose, CA) in PBS, 0.5% Tween-20, and analyzed using a Becton Dickinson flow cytometer BD FACScan (San Jose, CA) and Cell Quest acquisition and analysis programs.

STATISTICAL ANALYSIS

For correlation analysis of nuclear BAG-1 expression and clinicopathological variables, chi-square test or Fisher′s exact test was performed in 2 × 2 tables. Survival curves were calculated using the Kaplan–Meier method, and compared by standard log-rank tests. Multivariate analysis was performed using Cox′s proportional hazards model. All other data were analyzed with Student′s *t*-test. The Stat View 5.0 software package was used, and *P* values of less than 0.05 were considered statistically significant.

RESULTS

EXPRESSION OF BAG-1 PROTEIN IN HCC AND ADJACENT PERITUMORAL TISSUES

Western blot analysis was performed to compare the differential expression of BAG-1 in eight paired samples of HCC and adjacent peritumoral tissues (Fig. 1A,B). Interestingly, the difference in BAG-1 expression between HCC and peritumoral tissues varied among the isoforms of BAG-1. The observations are summarized as follows: (1) BAG-1L staining was positive in 6/8 (75%) of HCC tissues but only in 2/8 (25%) of peritumoral tissues, and BAG-1L was high expressed in six HCC tissues (patient 1, 3, 4, 5, 7, and 8) compared to the peritumoral tissues in all the eight paired HCC tissues. (2) BAG-1M staining was positive in 3/8 (37.5%) of HCC tissues and in 1/8 (12.5%) of peritumoral tissues. All the three BAG-1M positive staining HCC tissues (patient 1, 3, and 4) showed higher expression than the matched peritumoral tissues. (3) BAG-1S staining was positive in 7/8 (87.5%) of either HCC or peritumoral tissues, but no significant difference in BAG-1S expression was observed between paired HCC and peritumoral tissues. Only four HCC tissues (patient 1, 3, 4, and 8) showed noticeable higher expression compared to the peritumoral tissues whereas the remaining showed lower or similar expression. (4) BAG-1/p29 expression was not detected in any of eight tumoral or peritumoral tissues. However, following studies in HCC cell lines showed marked BAG-1/p29 expression which might be accounted for tissue specificity and small sample size. Collectively, these observations indicated that BAG-1L was overexpressed in HCC tissues as compared to matched peritumoral tissues, and dysregulation of BAG-1 might be involved in the progression of HCC.

CORRELATION OF NUCLEAR BAG-1 EXPRESSION WITH CLINICOPATHOLOGICAL FEATURES IN HCC

Considering that BAG-1L is over expressed in HCC tissues and mainly expressed in the cell nucleus, we further investigated expression of nuclear BAG-1 in HCC tissues using immunohistochemical staining. Even though we did not find the antibody specific to BAG-1L and used antibody for all isoforms, the immunohistochemistry results demonstrated that nuclear BAG-1 (mainly BAG-1L) was null or very low expressed in peritumoral tissues (Fig. 2A,B), but significantly highly expressed in HCC tissues (Fig. 2C,D).

To explore the statistical relationship between nuclear BAG-1 expression and clinicopathological variables, HCC tissue samples were divided into two groups based on immunostaining score: nuclear BAG-1 expression was considered as high with the final score ranging from 3 to 7, and considered as low with the final score ranging from 0 to 2. As shown in Table I, a strong correlation was observed between nuclear BAG-1 expression and the histologic grade ($P < 0.001$); however, nuclear BAG-1 expression did not significantly correlate with the gender, age, metastasis, tumor size, HBsAg, liver cirrhosis, or AFP level.

PROGNOSTIC SIGNIFICANCE OF NUCLEAR BAG-1 EXPRESSION

The correlation between nuclear BAG-1 expression level and patients′ survival was analyzed by using Kaplan–Meier analysis. Survival information was available in the cohort of all patients at the endpoint. Kaplan–Meier survival curves showed that HCC patients with high

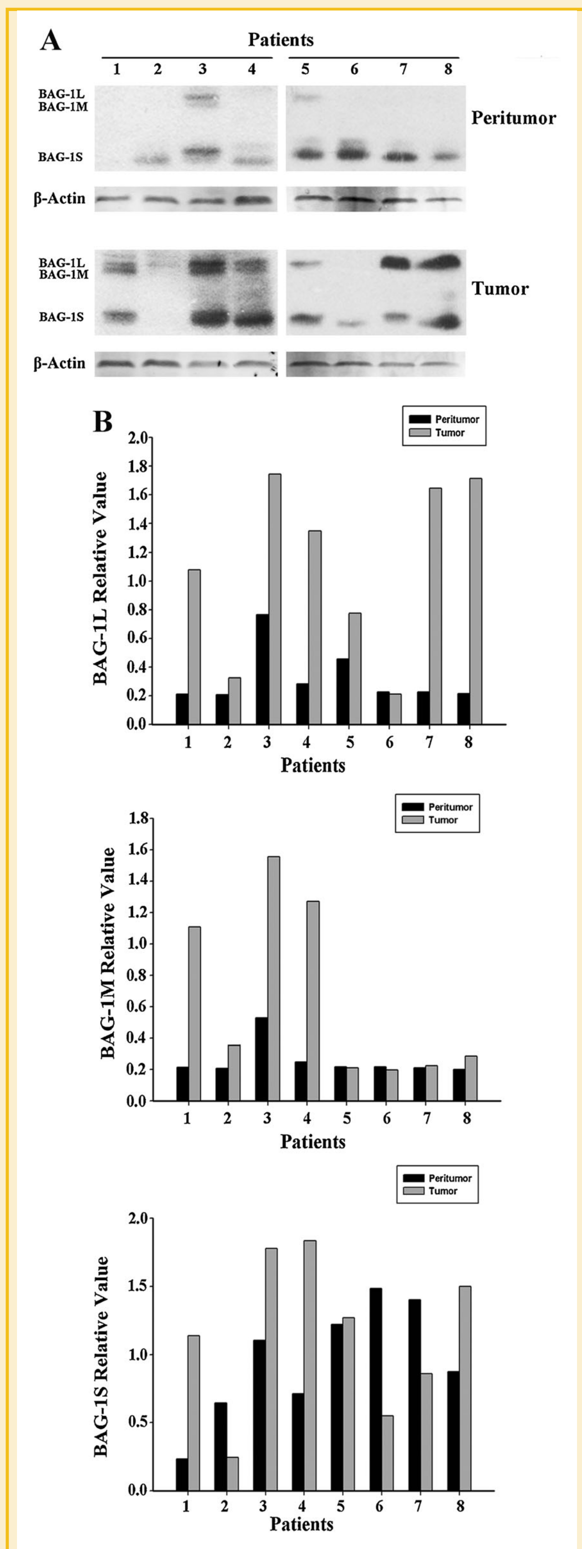

Fig. 1. The expression of four BAG-1 isoforms in eight paired samples of HCC and adjacent peritumor tissues, as measured by Western blot analysis. A: The paired tumoral and peritumoral tissues of patient 1, 2, 3, 4 or 5, 6, 7, 8 were applied respectively in the same gel. β-actin was served as a loading control. B: The semiquantitative analysis for BAG-1 isoforms expression was normalized by relative β-actin level and representative quantification graphs were shown.

nuclear BAG-1 expression were significantly associated with poor overall survival ($P = 0.004$; Fig. 3). Univariate analysis for overall survival using log-rank test identified the histological differentiation ($P < 0.001$) and nuclear BAG-1 expression ($P = 0.004$) as significant prognostic predictors (Table II). To assess whether nuclear BAG-1 expression was an independent prognostic factor or derived from other variables, multivariate analysis based upon the Cox's proportional hazards model was performed, and the result showed that nuclear BAG-1 expression ($P = 0.023$), as well as the histological grade ($P = 0.013$), was an independent prognostic indicators for HCC patients (Table III).

CYTOTOXICITY OF DOXORUBICIN IN HCC CELL LINES AND DOXORUBICIN EFFECTS ON EXPRESSION OF APOPTOSIS-RELATED PROTEINS

Four available HCC cell lines (SMMC-7721, HepG2, Bel-7404, and Huh-7) were assessed for basal abundances of BAG-1 to compare with normal hepatocyte cell line LO_2. Western blot analysis showed that expression of endogenous BAG-1L and BAG-1M was higher expressed in all of the four HCC cell lines than in LO_2 cells while the predominant isoform BAG-1S was only higher expressed in SMMC-7721 cells. Interestingly, BAG-1/p29 which could not be detected in eight paired HCC and peritumoral tissues was clearly shown in all of the five cell lines and was higher expressed in SMMC-7721 and HepG2 cells. These results demonstrated that BAG-1 was high expressed in HCC cells while BAG-1L and BAG-1M were the main isoforms (Fig. 4A).

The cytotoxic effect of doxorubicin was examined in 3 HCC cell lines, including SMMC-7721, HepG2, and Bel-7404. We noted that SMMC-7721 and HepG2 which both showed high level of BAG-1 expression seemed to be more resistant to higher concentrations of doxorubicin than Bel-7404 which showed a lower BAG-1 expression (Fig. 4B). The issue whether BAG-1 expression was involved in the proliferation of various cell lines, however, is to be further investigated.

We further determined expressions of BAG-1 and other apoptosis-related proteins after doxorubicin treatment of HCC cell lines. Pretreatment with 1 μM doxorubicin for 48 h led to decrease in expression of BAG-1 or BCL-2, but increase in expression of cleaved-caspase3 in each of three HCC cell lines (Fig. 4C). Given that all BAG-1 isoforms contain a ubiquitin-like domain (ULD), and BAG-1 is a target for ubiquitin conjugation [Sourisseau et al., 2001], we hypothesized that down regulation of BAG-1 by doxorubicin might be through a ubiquitin-proteasome pathway. Contrary to our expectation, however, addition of MG132, a proteasome inhibitor, failed to reverse the decreased expression of BAG-1 in HepG2 cells (Fig. 4D), suggesting that inhibition of BAG-1 was not modulated by ubiquitin-proteasome dependent degradation.

EFFECTS OF BAG-1 EXPRESSION ON NF-κB ACTIVITY IN HepG2 CELLS

Although a decreased expression of BAG-1 has been shown to significantly suppress NF-κB transcriptional activity in colorectal carcinoma cells [Clemo et al., 2008] and Hela cells [Maier et al., 2010], it remains unclear whether such a regulation by BAG-1 exists also in HCC cell lines. To clarify this question, BAG-1 was knocked down in HepG2 cells by using BAG-1 siRNA (Fig. 5A), and NF-κB luciferase

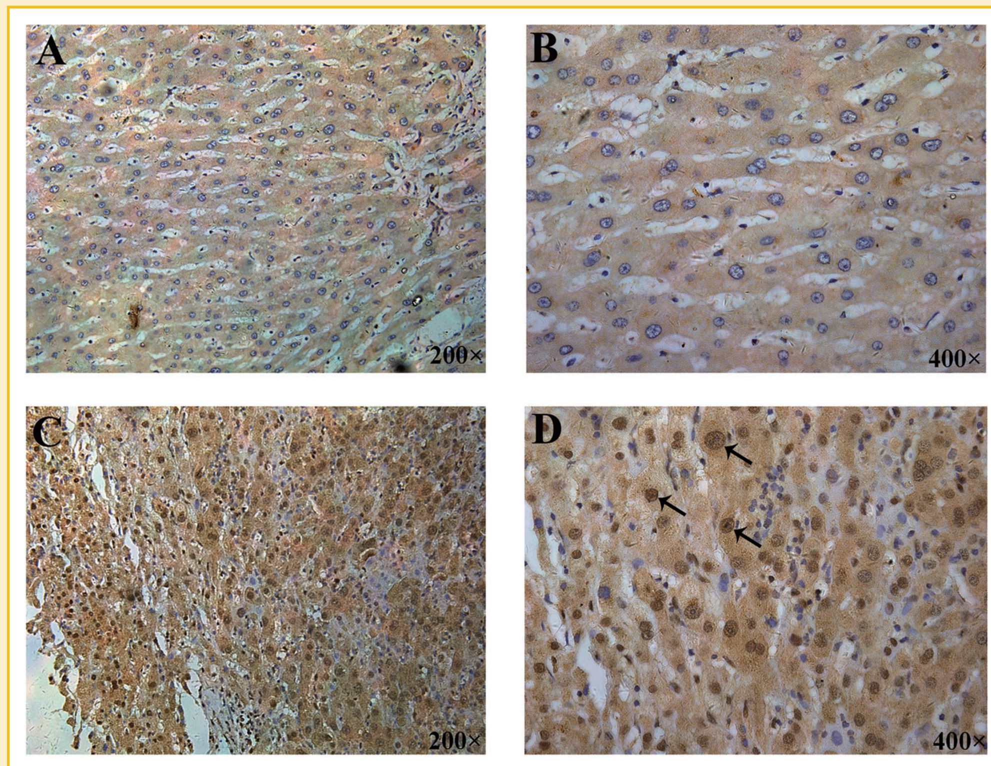

Fig. 2. Immunohistochemical analysis of BAG-1 in HCC and adjacent peritumor tissues. The tissue sections were immunostained with antibodies against BAG-1 and counterstained with hematoxylin. Negative staining of nuclear BAG-1 was shown in adjacent peritumor tissue (A,B). Strong staining of nuclear BAG-1 was detected in HCC tissues (C,D). Magnification, 200× (A,C) and 400× (B,D).

reporter assay was used to test the effect of BAG-1 knockdown on NF-κB transcriptional activity. We noted that NF-κB activity was moderately inhibited in the BAG-1 siRNA transfected cells despite in a quiescent state with a low basal level (Fig. 5B). Moreover, expression of BCL-2 was also decreased after transfection with BAG-1 siRNA. Since NF-κB can be activated by a number of chemotherapeutic agents [Das and White, 1997], we further determined whether NF-κB could be activated in HCC cell lines by doxorubicin which is the most common chemotherapeutic agent used for HCC [Yoo et al., 2010]. As predicted, after treatment with doxorubicin NF-κB transcriptional

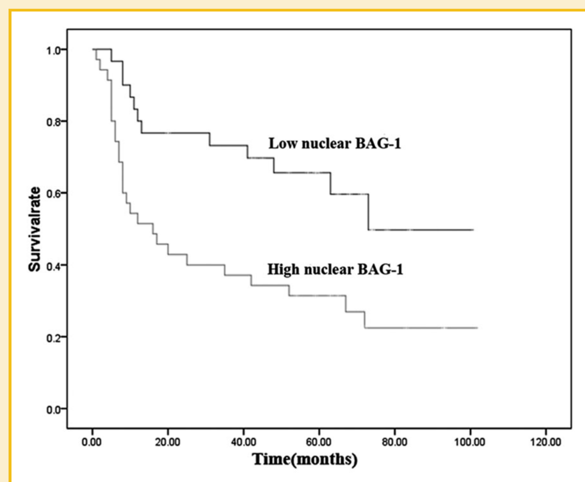

Fig. 3. Kaplan–Meier survival curves for low nuclear BAG-1 expression versus high expression in 65 HCC patients showed a highly significant separation ($P = 0.004$, log-rank test).

TABLE II. Univariate Analysis of Prognostic Variables

	Univariate[a]	
Parameters	χ^2 value	*P*-value
Gender		
Male/female	0.004	0.952
Age (years)		
≤45/>45	0.023	0.879
Histological grade		
Well/Mod and poor	19.293	0.000
Tumor size (cm)		
≤5/>5	0.719	0.396
Metastasis		
Negative/positive	0.263	0.608
HBsAg		
(−)/(+)	1.345	0.246
Cirrhosis		
Negative/positive	0.452	0.502
AFP (ng/ml)		
≤50/>50	0.680	0.410
Nuclear BAG-1		
Negative/positive	8.475	0.004

[a]Statistical analyses were performed by log-rank test. $P < 0.05$ was considered significant.

TABLE III. Contribution of Various Potential Prognostic Factors to Survival by Cox Regression Analysis in 65 HCC Specimens

	Hazard ratio	95% Confidence interval	*P*-value[a]
Gender	0.736	0.314–1.724	0.481
Age (years)	1.422	0.664–3.046	0.365
Histological grade	2.016	1.157–3.512	0.013
Tumor size (cm)	1.584	0.722–3.474	0.251
Metastasis	0.740	0.283–1.935	0.539
HBsAg	1.087	0.368–3.213	0.880
Cirrhosis	1.388	0.702–2.744	0.346
AFP (ng/ml)	1.228	0.577–2.611	0.594
Nuclear BAG-1	8.593	1.338–55.170	0.023

[a]Statistical analyses were performed by the Cox regression model. $P < 0.05$ was considered significant.

activity was notably increased, and then suppressed by depletion of BAG-1 or addition of BAY-117082, a NF-κB inhibitor (Fig. 5B). The immunofluorescence assay provided consistent results and showed that NF-κB P65 subunit translocated to the nucleus of HepG2 cells which implied activation of NF-κB in a doxorubicin dose dependent manner while remained restricted mainly in the cytoplasm when transfected with BAG-1 siRNA (Fig. 5C). It should be noted that low concentration of doxorubicin (1 μM) for 48 h stimulation caused massive apoptosis in BAG-1 knocked down cells. As a result, we chose the high concentration as high as 30 μM for a shortly time stimulation (3 h) to see the alteration of NF-κB activity. These observations implied that doxorubicin caused activation of NF-κB in a dose dependent manner and, in turn, knock down of BAG-1 caused inhibition of the doxorubicin triggered NF-κB activation.

INCREASED SENSITIVITY OF HepG2 CELLS TO DOXORUBICIN BY KNOCK DOWN OF BAG-1 VIA NF-κB SIGNALING

The above results revealed that doxorubicin could activate NF-κB signaling, which was strikingly repressed by BAG-1 suppression in HepG2 cells. In other cases, activation of NF-κB has been reported to inhibit apoptosis and mediate chemoresistance through different mechanisms. Hence, it was reasonable to hypothesize that knock

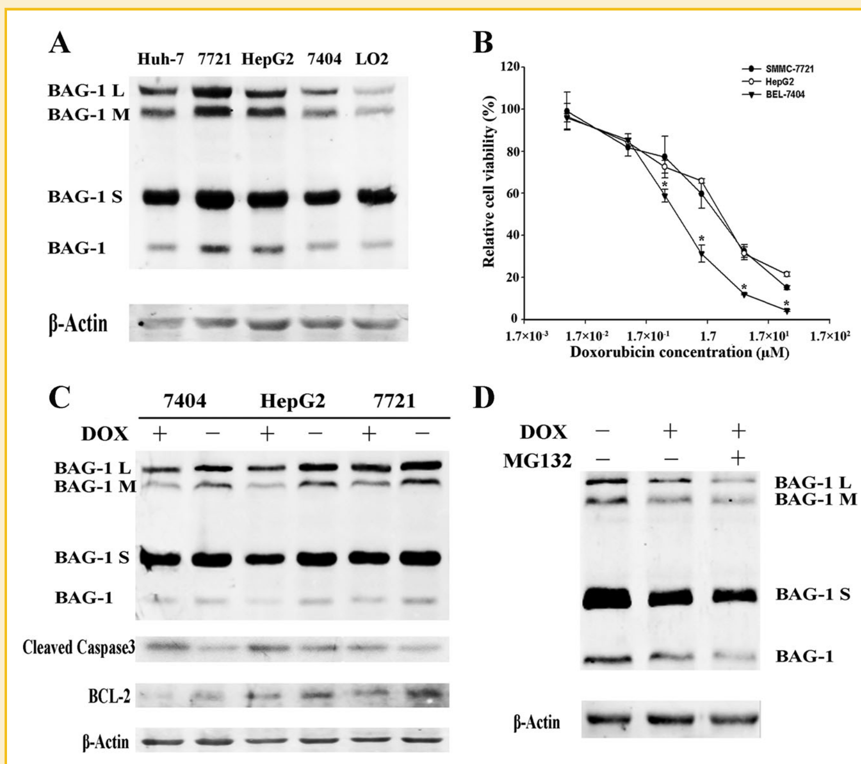

Fig. 4. Expression of BAG-1 and cytotoxicity in different HCC cell lines upon treatment with doxorubicin. A: Expression of BAG-1 in four HCC cell lines (Huh-7, SMMC7721, HepG2, and Bel7404) and normal hepatocytes (LO_2). B: Cytotoxicity assay for HCC cell lines treated with doxorubicin (DOX) at concentrations ranging from 8.5×10^{-3} to 34 μM (5×10^{-3} to 20 μg/ml). Relative cell viability was measured by a Cell Counting Kit at 48 h after doxorubicin (DOX) treatment. $^*P < 0.05$ versus SMMC7721 or HepG2. C: Three HCC cell lines were treated with or without 1 μM doxorubicin (DOX) for 48 h and then subjected to Western blot analysis for detecting expressions of BAG-1, BCL-2, and cleaved-caspase-3, respectively. D: Proteasome inhibition assay was applied to HepG2 cells which had been treated with doxorubicin (DOX) and MG132 (a proteasome inhibitor) or DMSO (control), and whole-cell lysates were then prepared for Western blot analysis. β-Actin served as a loading control in (A), (C), and (D).

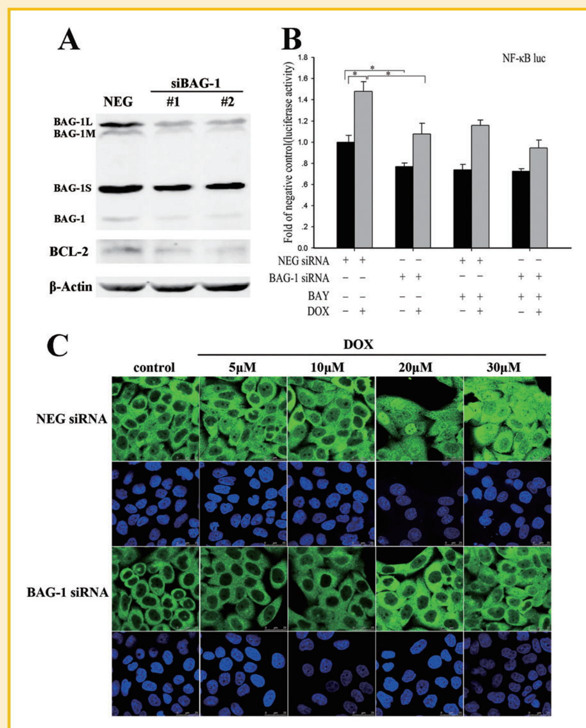

Fig. 5. Effects of BAG-1 expression on NF-κB activity in HepG2 cells. A: HepG2 cells were transfected with BAG-1 siRNA#1, BAG-1 siRNA#2 and negative control (NEG) siRNA, respectively, the efficiency of siRNA-mediated knock down of BAG-1 and subsequently suppression of BCL-2 were evidenced by Western blot analysis at 48 h after transfection. B: Basal levels and doxorubicin (DOX, 30 μM)-induced NF-κB activity, shown as the average fold (±SD) of negative control, were inhibited by BAG-1 siRNA#1 transfection or 2 μM BAY-117082 (BAY) treatment in HepG2 cells. The data were obtained from a representative measurement (in triplicates). $^*P < 0.05$. C: HepG2 cells were transfected with negative control (NEG) siRNA or BAG-1 siRNA#1 for 48 h, and then treated with doxorubicin (DOX) at indicated concentrations for 3 h, NF-κB p65 antibody was used to determine the cellular localization of NF-κB subunits by immunocytochemistry.

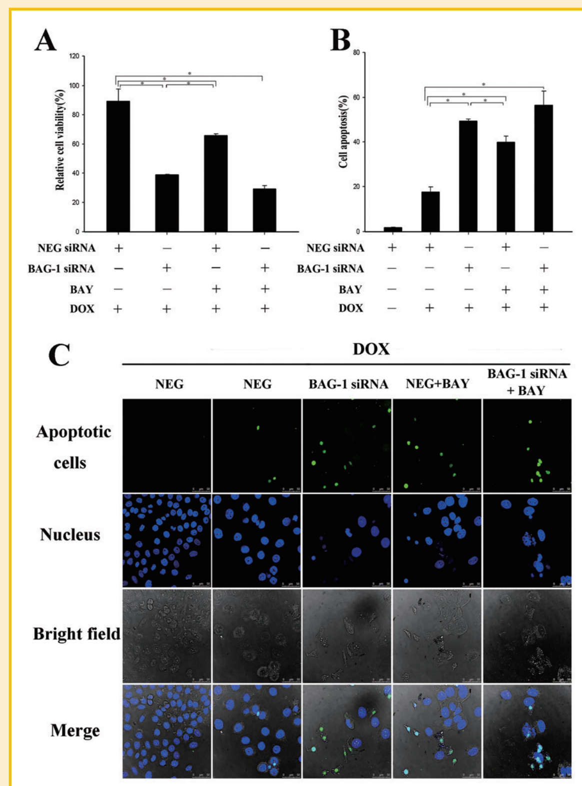

Fig. 6. Increased sensitivity of HepG2 cells to doxorubicin-induced apoptosis through BAG-1 knocking down via NF-κB signaling pathway. A: After transfection with BAG-1 siRNA#1 and/or treatment with 2 μM BAY-117082 (BAY) in the presence of 1 μM doxorubicin (DOX) for 48 h, the cell viability of HepG2 cells was assessed by Cell Counting Kit. The cell viability is expressed as the percentage of viable cells to total cells. Data are presented as mean ± SD of three independent experiments. B,C: Cells were pretreated as described above, cell apoptosis was evaluated by TUNEL assay in 24-well plate and was calculated as percentage of apoptotic cells in relation to all cells counted in four randomly chosen fields. $^*P < 0.05$ (A,B).

down of BAG-1 may sensitize HepG2 cells to doxorubicin-induced apoptosis through inhibiting doxorubicin-triggered NF-κB activation. To test this hypothesis, BAG-1 siRNA or control siRNA transfected HepG2 cells were respectively treated with 1 μM doxorubicin combined with or without 2 μM BAY-117082, and 48 h later the cell viability was assessed. Compared with control cells, either BAG-1 knocked-down cells or BAY-117082 treated cells exhibited a significant decrease in the cell viability of HepG2 cells (Fig. 6A). In other words, we observed that knock down of BAG-1 could reduce resistance to doxorubicin-induced apoptosis. Moreover, TUNEL assay yielded a consistent result that either knocking down of BAG-1 or inhibition of NF-κB by BAY-117082 enhanced doxorubicin-induced apoptosis (Fig. 6B,C). It was worthy to mention that BAG-1 knocked-down cells seemed to acquire more sensitivity to doxorubicin-induced apoptosis than BAY-117082 treated cells. This result might be because suppression of BAG-1 could also decrease expressions of BCL-2 (Fig. 5B) and even other anti-apoptotic genes. Another important point should be raised was that no significant increase of apoptosis was detected in cells with combined treatment of BAY and BAG-1 knocking down compared to the cells with simply BAG-1 knocking down, which further confirmed that BAG-1 inhibited apoptosis in HCC cells through potentiating NF-κB activity.

THE ROLE OF BAG-1 ON PROLIFERATION OF HepG2 CELLS

Apart from anti-apoptotic function, BAG-1 has proven to contribute to cell proliferation and survival [Townsend et al., 2005; Elliott and Ginzburg, 2009], but there has been an opposite view proposed for HaCaT cells [Hinitt et al., 2010]. In this study, HepG2 cells were serum-deprived for 72 h, causing most cells to arrest in G1 phase [Chen et al., 2012] and reentering the cell cycle upon serum refeeding. As expected, expression of BAG-1 in HepG2 cells was increased as early as 4 h after serum refeeding and reached the highest level 24 h after serum refeeding. To confirm the efficiency of the cell model, proliferating cell nuclear antigen (PCNA) was examined (Fig. 7A). Furthermore, we measured the cell viability for BAG-1 knocked-down cells. It was found that cells transfected with BAG-1 siRNA had a significantly reduced cell viability at each indicated time points (Fig. 7C). Since NF-κB promotes cell cycle progression and stimulate

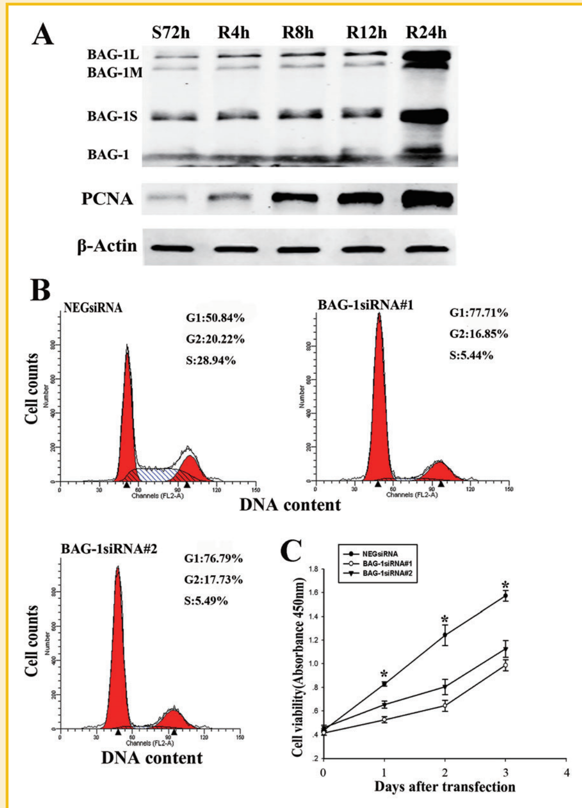

Fig. 7. The effects of BAG-1 expression on proliferation of HCC Cells. A: HepG2 cells were subjected to serum starvation (S) for 72 h, and then serum refeeding (R) for indicated times (4, 8, 12, and 24 h). Afterwards, cell lysates were prepared and analyzed by Western blot using antibodies against BAG-1, PCNA. β-Actin served as a loading control. B: HepG2 cells were transfected with NEG siRNA, BAG-1 siRNA#1, BAG-1 siRNA#2, respectively. Forty-eight hours after transfection, cells were collected, fixed in 70% alcohol, and incubated in PBS containing 10 mg/ml of RNase A at 37°Cfor 30 min, after incubation, the cells were stained with 5 mg/ml PI. Cells were then subjected to cell-cycle analysis. C: Cells (5×10^3 cells/well) were seeded in 96-well plate followed by transfection of BAG-1 siRNA#1, BAG-1 siRNA#2, or NEG siRNA respectively, and the cell viability was measured at indicated time points by Cell Counting Kit. Data are presented as mean ± SD of three independent experiments. $^*P < 0.05$.

cell growth by activating cyclin-D1 expression [Dolcet et al., 2005], we investigated whether the suppression of BAG-1 which significantly reduced NF-κB activity could inhibit cell cycle and proliferation of HepG2 cells. The cell cycle analysis results showed a decreased cell population in the S-phase (from 28.94% to 5.44% or 5.49%) and an increased cell population in the G1-phase (from 50.84% to 77.71% or 76.79%) with transfection of BAG-1siRNA compared with transfection of NEGsiRNA in HepG2 cells (Fig. 7B). These data suggested that BAG-1 played a key role in regulating cell cycle progression and cell proliferation in HepG2 cells.

DISCUSSION

HCC ranks among the most common causes of cancer-related death worldwide with approximately 700,000 deaths reported annually [Munoz et al., 2011]. Despite advances in diagnostic and therapeutic strategy, the prognosis for HCC patients remains unsatisfactory due to high recurrence and metastasis as well as chemoresistance. Although the importance of BAG-1 has been documented in a number of cancers, its involvement in HCC tumorigenesis is yet to be defined. In this study, Western blot analysis showed that BAG-1L mainly restricted to nucleus was significantly overexpressed in HCC tissues of patients, and immnohistochemisty further confirmed that expression of nuclear BAG-1 (mainly BAG-1L) was remarkably higher in HCC tissues than in adjacent peritumoral tissues. Although the expression pattern and prognostic significance of BAG-1 vary depending on the cancer type as described previously [Weldon et al., 2001; Kikuchi et al., 2002; Millar et al., 2009], our statistical analysis of 65 HCC patients confirmed that nuclear BAG-1 was associated with histological grade, and overexpression of nuclear BAG-1 had relevance to the poor outcome of HCC patients, thus highlighting the prognostic significance of BAG-1 in HCC.

BAG-1, as a multifunctional protein regulating diverse cellular processes related to cancer, has been shown to be overexpressed in drug-resistant cells [Ding et al., 2000] and mediate resistance to chemotherapy-induced apoptosis of cancer cells [Chen et al., 2002; Liu et al., 2009]. In this study, we demonstrated that BAG-1 was overexpressed in HCC cell lines compared to normal hepatocytes, and cells with the lower expression of BAG-1 seemed to be more susceptible to doxorubicin-induced apoptosis. This result suggested that BAG-1 might mediate anti-apoptotic effects and induce chemoresistance in HCC cell lines. To confirm this, we knocked down the endogenous BAG-1 protein using siRNA in HepG2 cells and the result showed that the expression of BCL-2 and transcriptional activity of NF-κB was concomitantly reduced which may clarify the anti-apoptotic effects of BAG-1.

Although BCL-2 is vital to prevent doxorubicin-induced apoptosis in HCC cells [Takahashi et al., 2003], the molecular mechanisms by which BAG-1 regulates BCL-2 have been fully illustrated that BAG-1 can enhance stability of BCL-2 protein and decrease its degradation. Therefore, we were concerned about the actions of BAG-1 as a NF-κB regulator. The crucial role of NF-κB has been documented in many types of cancer, including HCC [Pikarsky et al., 2004], and many genes involved in suppression of cell death, such as FLIP (an inhibitor of apoptosis), are known to be regulated by NF-κB [Deveraux et al., 1998; Kreuz et al., 2001]. For this reason, cancer cells with constitutive activation of NF-κB usually show increased resistance to chemotherapy. On the other hand, several cancer cells show enhanced activation of NF-κB upon exposure to radiation or chemotherapeutic drugs, which may be responsible for resistance of these cancer cells to certain treatments. Although regulation of NF-κB activity by BAG-1 has been observed in colorectal cancer cells and Hela cells, the underlying mechanisms are different between these two cell types. It has been proposed that BAG-1 interacts with p50–p50 NF-κB homodimers to form a complex required for regulating epidermal growth factor receptor (EGFR) and COX-2 (PTGS2) genes, and thereby acts as a co-regulator of an atypical NF-κB pathway [Southern et al., 2011]. Another report, however, claims that suppression of BAG-1 expression results in decrease in P65, P50 nuclear accumulation in response to phorbol ester [Maier et al., 2010]. These studies suggested BAG-1 may be able to modulate the function of NF-κB at more than one stage in the signaling pathway depending on the

cell types. The present study corresponded with the latter one that decreased expression of BAG-1 downregulated transcriptional activity of NF-κB and inhibited doxorubicin-induced NF-κB P65 subunit nuclear accumulation in HepG2 cells. Our data also showed that knock down of BAG-1 significantly enhanced doxorubicin-induced apoptosis possibly through inhibition of NF-κB activity, and BAG-1 promoted proliferation of HepG2 cells by facilitating cell cycle progression, in which NF-κB signaling might be involved because of the ability of NF-κB to regulate cyclin-D1 expression.

In addition, BAG-1 is expressed in four protein isoforms and each one may have special function. A study performed to investigate the relationship between BAG-1 isoforms in endometrial cancer revealed that BAG-1 nuclear isoforms appeared more frequently in grade 2 tumors than in grade 1 and 3 tumors, and the cytoplasmatic isoforms were expressed more strongly than the nuclear one. The other study proposed that distinct isoforms of BAG-1 have different anti-apoptotic functions in breast cancer cells, and that the BAG-1L isoform can potentiate the role of estrogen in ER-positive breast cancer. Although we have validated the role of BAG-1 on NF-κB activity and chemoresistance in HCC cell lines, the respective function of specific isoforms of BAG-1 in HCC remains to be elucidated. As stated previously, BAG-1L played an important role on sensitivity to chemotherapeutics in different cancer types [Chen et al., 2002; Liu et al., 2009]. The research performed with Hela cells also showed that BAG-1L can interact with IκBα which may act as an inhibitor protein on NF-κB activity, and reintroduction of BAG-1L to BAG-1 depleted cells partially restored NF-κB activation. Similarly, our studies noted that BAG-1L was significantly overexpressed in HCC tissues and cells by Western blotting. Therefore it is reasonable to propose that no matter how the other isoforms function, at least BAG-1L participates in the regulation of NF-κB and chemoresistance to doxorubicin in HCC cells.

To summarize, this study for the first time showed that nuclear BAG-1 (mainly BAG-1L) was significantly highly expressed in HCC, and this overexpression was correlated with the poor prognosis as well as histological grade, suggesting a prognostic value of BAG-1 in HCC. We further revealed that suppression of BAG-1 expression inhibited NF-κB activity and reduced doxorubicin-induced nuclear accumulation of NF-κB P65 subunit, thus enhancing the chemosensitivity of HCC cells. Moreover, we established that BAG-1 might have a potential ability to promote proliferation of HCC cells. Collectively, it is supposed that suppression of BAG-1 expression by inhibiting NF-κB signaling might be developed into a new strategy in HCC therapy.

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