

Bag3 promotes resistance to apoptosis through Bcl-2 family members in non-small cell lung cancer

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Abstract. In non-small cell lung cancer (NSCLC) certain molecular characteristics, which are related to molecular alterations have been investigated. These are responsible for both the initiation and maintenance of the malignancy in lung cancer. The aim of this study was to evaluate the influence of Bag3 (Bcl-2 associated athanogene 3) in the regulation of apoptosis on NSCLC. Bag3 and Hsp70 expression were examined by immunohistochemistry to confirm their potential roles in the prevalence of NSCLC. We also established human normal bronchial epithelial cells and HOP-62 cell line as the model to analyze cell apoptosis and the expression of Hsp70, Bcl-X_L and Bcl-2, which were affected by Bag3. In this study, we found that Bag3 and Hsp70 are highly expressed in few tissues and cell lines of NSCLC. Bag3 inhibits apoptosis in human normal bronchial epithelial cell lines and sustain the survival of NSCLC cells. Bag3, Hsp70, Bcl-X_L and Bcl-2 are up-regulated in NSCLC cell lines. At the same time, the silencing of Bag3 results in diminishing protein levels of Bcl-X_L and Bcl-2. The results of immunoprecipitation identified that Bag3 could interact with Hsp70, Bcl-X_L and Bcl-2 NSCLC cells directly or indirectly. We conclude that NSCLC cells were protected from apoptosis through increasing Bag3 expression and consequently promoted the expression of Bcl-X_L and Bcl-2.

Introduction

Lung cancer has the highest mortality rate in the world (1,2). It is a malignancy arising from different histology types of

respiratory epithelium cells (3). Among them, non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases (4). The subtypes of NSCLC are classified into adenocarcinoma, squamous cell carcinoma and large cell carcinoma (5,6). Adenocarcinoma of lung usually develops in the outer area of the lung and often presents as metastatic disease. It can occur in both smokers and non-smokers (7-9). Although treatment options, such as surgery and chemotherapy, have improved, the overall survival of lung adenocarcinoma remains at the original level (10). Lung adenocarcinoma presents specific genomic changes that might be responsible for both the initiation and maintenance of the malignancy (11-13). These investigations highlight the new mechanisms with vital molecules to develop more effective prognosis and treatments.

Bag3 (Bcl-2 associated athanogene 3), also known as CAIR-1 or Bis, is a 74-kDa cytoplasmatic protein. It is a member of Bag protein family and shares with the other members an evolutionarily conserved domain, that binds the ATPase domain of Hsp70 (14,15). Several studies showed that, in human primary lymphoid, myeloblastic leukemias and other neoplastic cell types, Bag3 expression sustains cell survival and underlies resistance to therapy, through down-modulation of apoptosis (16,17). On the contrary, down-modulation of Bag3 primary samples of B-cell chronic lymphocytic leukemia and acute lymphoblastic leukemia results in a dramatic increase of basal as well as drug-induced apoptosis (16,18). In addition, Bag3 expression is induced in different normal cell types in response to cell stressors, such as oxidants, high temperature, heavy metals and HIV-1 infection (17,19-21).

In this study, we explored the expression and apoptosis-associated proteins of Bag3 in human normal bronchial epithelial cells, NSCLC cell lines and tissues. Our research found that Bag3 is expressed in some cell lines and tissues of NSCLC. Moreover, we show that Bag3 can interact with anti-apoptotic Bcl-2 proteins to sustain tumor cells to resist apoptosis progression.

Materials and methods

Cell culture. Specimens of human bronchial epithelial cells were obtained from the autopsied lungs of patients without respiratory disorders. The study was approved by the Ethics

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Committee of our university and the investigation conformed to the principles outlined in the Declaration of Helsinki. Tissues were dissected and rinsed 5 times in Ca^{2+} and Mg^{2+} free HBSS and incubated overnight at 4°C in 0.1% protease solution in Ham's F12 medium containing penicillin (100 U/ml), streptomycin (100 U/ml) and fungizone (1 $\mu\text{g}/\text{ml}$). Cells were detached by a gentle jet of 20% FCS in Ham's F12 medium. The suspension was centrifuged at 1,100 rpm for 8 min, and the cell pellet was resuspended in F12/DMEM medium containing 5% heat-inactivated FCS, penicillin (10 U/ml) and streptomycin (100 U/ml). The medium was changed each day for five days to F12/10X medium and changed on alternate days thereafter. The purity of the cultures and identity of the cells were confirmed by light microscopy and immunocytochemical staining using specific monoclonal antibodies directed against cytokeratin.

BEAS-2B cell line was purchased from ATCC (USA). The cells were cultured in Lechner and LaVeck media (LHC-9) containing retinoic acid (33 nM) and epinephrine (2.75 μM). Culture flasks and multi-well plates were coated with LHC-basal media containing BSA (100 $\mu\text{g}/\text{ml}$), collagen (30 $\mu\text{g}/\text{ml}$) and fibronectin (10 $\mu\text{g}/\text{ml}$) for at least 4 h at 37°C . The cells were maintained in 75- cm^2 flasks at 37°C and 5% CO_2 . Media were replaced every second day, and cells were passaged when $>85\%$ confluent by washing with Ca^{2+} and Mg^{2+} free PBS and digested with 0.05% trypsin.

HOP-62, EKVX and A549 cells were maintained by Department of Thoracic Surgery, Fourth Military Medical University. Cells were cultured in DMEM medium with 10% FCS including penicillin/streptomycin.

Plasmid and siRNA construction and transfection. The Bag3 expression plasmid was obtained by cloning Bag3 full-length cDNA from HOP-62 cell line in the expression vector pcDNA3.1. Lipofectamine (Invitrogen, USA) was used for cell transfection with plasmid.

siRNA transfections were performed using 0.2 nM of Stealth siRNA (Invitrogen, USA) per 10^7 cells (control and Bag3 sense sequence, 5'-AAGGUUCAGACCAUCUUGGAA-3') and Lipofectamine (Invitrogen) according to the manufacturer's protocol. Bag3 siRNA was transfected in Opti-MEM for 4 h. Cells were allowed 24 h to adhere before performing the experiment.

Immunohistochemistry examination. The expression of Bag3 and Hsp70 protein in NSCLC tissues was detected via immunohistochemistry according to the manufacturer's instructions. Tissues were incubated with primary antibodies against Bag3 and Hsp70 (Santa Cruz Biotechnology, Inc., CA, USA) followed by horseradish peroxidase conjugated secondary antibodies. The results were observed with an Olympus microscope and imaging system (BX51; Olympus, Tokyo, Japan). Image analysis was done with the Scion image analyzer (Scion Corp., Frederick, MD, USA).

Assessment of apoptosis. The cells were washed with PBS and stained with Annexin V/PI (BD Pharmingen) for 30 min at room temperature, separately. Cells were then processed and analyzed using a Becton-Dickinson FACScan cytofluorometer (Mansfield, USA) with the use of CellQuest software (Becton-

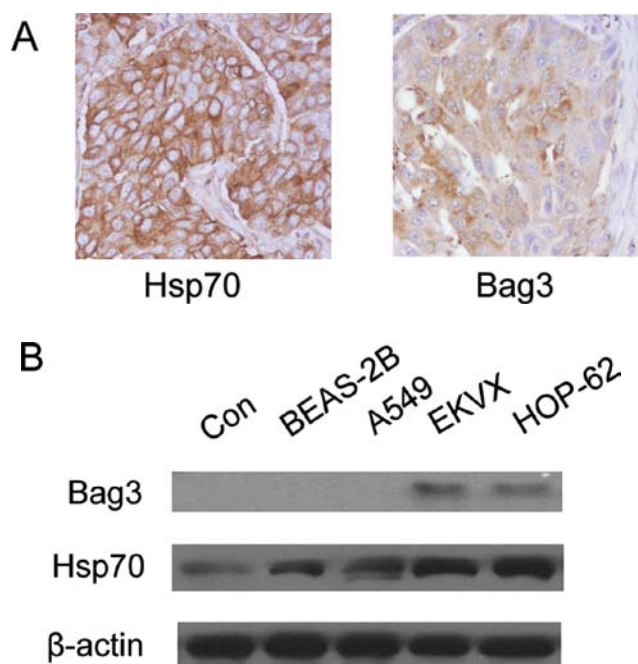


Figure 1. The expression of Bag3 and Hsp70 were detected by immunohistochemistry in NSCLC tissues. (A) Bag3 and Hsp70 were expressed in the cytoplasm in NSCLC cells. (B) Western blotting was performed to show the expression of Bag3 and Hsp70 in human normal bronchial epithelial cells (marked as 'Con'), BEAS-2B, A549, EKVX and HOP-62.

Dickinson, USA). Cells were considered to be apoptotic if they were either Annexin V⁺/PI⁻ (early apoptotic) or Annexin V⁺/PI⁺ (late apoptotic).

Quantitative real-time RT-PCR. Total RNA was extracted using TRIzol Reagent (Invitrogen) and digested with DNase (Invitrogen). A quantitative RT-PCR assay was performed using the LightCycler 480 SYBR-Green I Master (Roche Diagnostics, GmbH) with a Roche 480 LightCycler. Real-time reactions were performed on a Bio-Rad iCycler. Real-time PCR primers include the following: Bag3 (upper, 5'-CCTTGGACTGAAGGG GTTTT-3'; lower, 5'-ATGGCTCCTTGCATAGCTCATA-3'); Hsp70 (upper, 5'-CACTGTACCAGGGGATTATGTTT-3'; lower, 5'-ATGTTAGTACACAGTGCCAAGACG-3'); Bcl-X_L (upper, 5'-ATGGCAGCAGTGAAGCAAGC-3'; lower, 5'-ACGATGCGACCCAGTTTACTC-3'); Bcl-2 (upper, 5'-GTCGCTACCGTCGTGACTT-3'; lower, 5'-CAGCCTCCGTTATCCTGGA-3'); and β-actin (upper, 5'-GCTCTTTTCCAGCCTTCCTT-3'; lower, 5'-TGATCCACATCTGCTGGAAG-3'). Each sample was run in triplicate. Primer specificity was confirmed by melting curve analysis and electrophoresis of PCR on a 1.5% agarose gel to confirm the size of products.

Co-immunoprecipitation and Western blotting. Proteins (300 μg) was used for immunoprecipitation assays using 3 μg of anti-Bag3 monoclonal antibody (Santa Cruz Biotechnology, Inc.) and incubated at 4°C overnight on a tube rotator. Protein A-Sepharose, 25 ml, was then added, and the immuno-complexes were precipitated and washed five times with

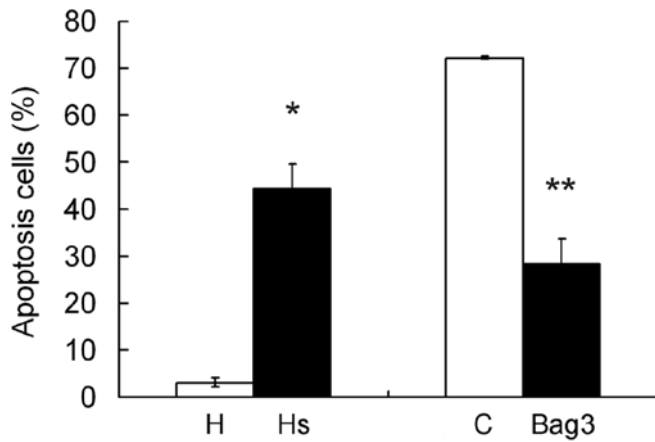


Figure 2. The regulation of Bag3 in apoptosis of NSCLC. Bag3 gene silencing sensitized HOP-62 cells to apoptosis. Bag3 overexpression promoted anti-apoptosis in human normal bronchial epithelial cells. *Bag3 interfere group vs. original HOP-62 group, $P<0.05$; **Bag3 overexpression group vs. original human normal bronchial epithelial cell group, $P<0.05$.

radioimmunoprecipitation assay buffer. For Western blot analyses, proteins obtained from immunoprecipitations or 30 μ g of total protein were run on 10% (wt/vol) SDS/PAGE gels and transferred to nitrocellulose. Nitrocellulose blots were blocked and incubated with antibodies against Bag3, Hsp70, Bcl- X_L , Bcl-2 and β -actin (Santa Cruz Biotechnology, Inc.) followed by horseradish peroxidase conjugated secondary antibodies. Detection was performed by using enhanced chemiluminescence (ECL) (Pierce, USA).

Image analysis and statistical processing. All experimental data are reported as the mean \pm SE. Variance was analyzed with the SPSS statistical software package (version 10.0; SPSS, Chicago, IL, USA), with multiple comparisons verified with the least significant difference t-test. Statistical significance was set at $p<0.05$.

Results

Bag3 and Hsp70 were highly expressed in cytoplasm of some NSCLC tissues and cell lines. To describe the expression of Bag3 and Hsp70 in cells and tissues of NSCLC, we identified Bag3 and Hsp70 protein expression by immunohistochemistry in cells and tissues from different types and grades of NSCLC (Fig. 1A). Human normal bronchial epithelial cells and BEAS-2B cell line were detected as normal control. In this experiment, we observed that Bag3 and Hsp70 proteins were expressed in HOP-62 and EKVX cell lines and highly in the cytoplasm in 27.3% cases of NSCLC (6 in 22 cases). We detected weak Hsp70 and no Bag3 expression in human normal bronchial epithelial cells and BEAS-2B cell line (Fig. 1B).

Bag3 inhibits the apoptosis in human normal bronchial epithelial cell lines in vitro. We investigated the effects of Bag3 silencing in apoptosis of NSCLC cell lines and the overexpression of protein in human normal bronchial epithelial cell lines *in vitro*. Our results showed that down-regulation of Bag3 in HOP-62 cell line by a specific siRNA resulted in

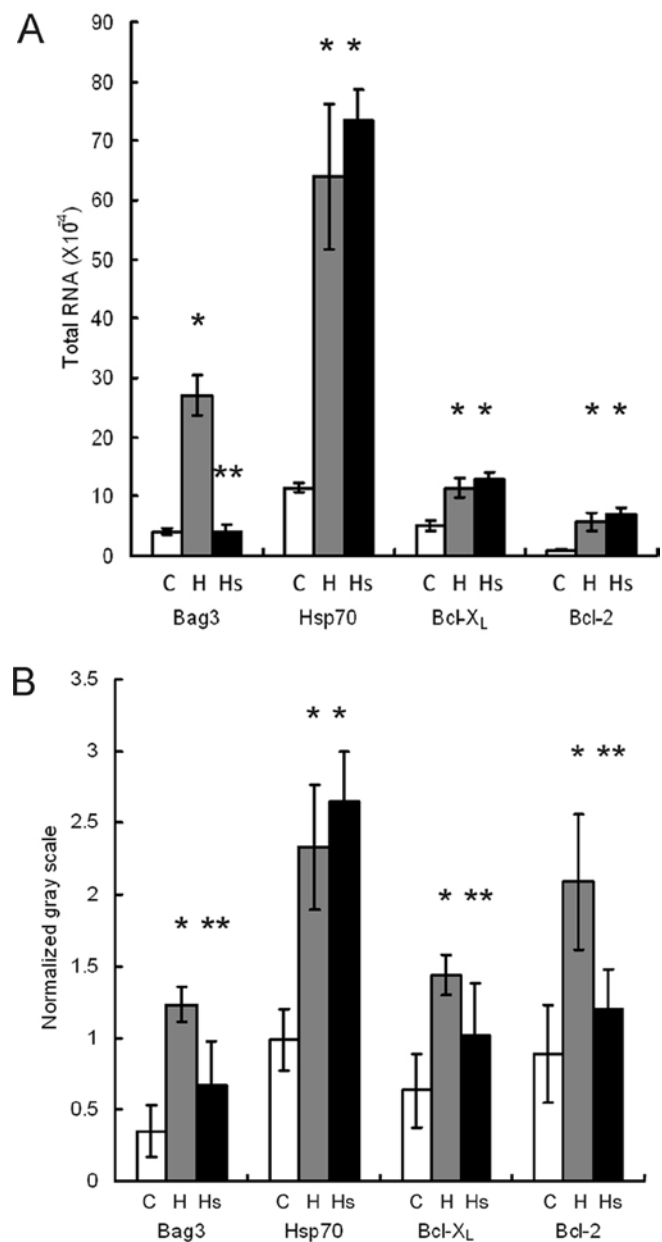


Figure 3. Bag3 gene silencing has no effect on Hsp70, Bcl- X_L and Bcl-2 on transcription level, neither on the protein expression level (except Hsp70) in NSCLC cells. (A) RT-PCR analysis of Bag3 and other mRNA levels. (B) Western blot analysis of their protein level. *H or Hs group vs. control group, $P<0.05$; **Hs group vs. H group, $P<0.05$. (C) Control (human normal bronchial epithelial cells); H, HOP-62; Hs, HOP-62 with Bag3 silencing.

significantly increasing cell apoptosis ($P<0.05$, Fig. 2). It is consistent that overexpression of Bag3 in human normal bronchial epithelial cells protected cells from apoptosis ($P<0.05$, Fig. 2). The results confirmed the anti-apoptotic property of Bag3 in NSCLC *in vitro*.

Bag3, Hsp70, Bcl- X_L and Bcl-2 are up-regulated in NSCLC cell line in vitro. According to previous results, Bag3 inhibits apoptosis in human normal bronchial epithelial cell lines, while the silencing of Bag3 increased the apoptosis in NSCLC cell line. Thus the expression of Bag3 and apoptosis-associated proteins were explored in HOP-62 cell line by RT-PCR and

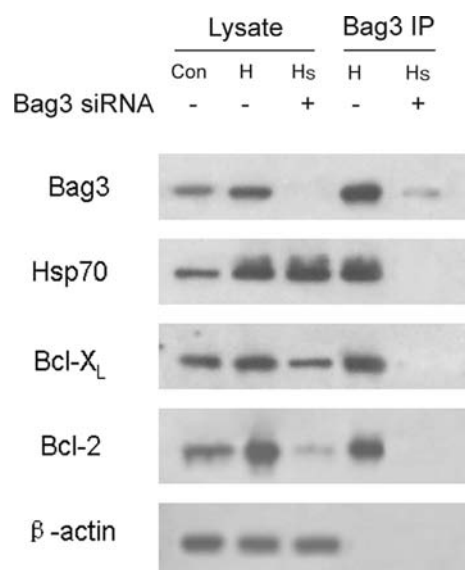


Figure 4. Interactions and regulations among Bag3 and other proteins. Whole lysates from H and Hs group cells were immunoprecipitated with the anti-Bag3 antibody IMG-5676 (Imgenex Corp., CA) and analyzed by immunoblotting with anti-Bag3 antibody P-17, anti-Hsp70, anti-Bcl-X_L, anti-Bcl-2 and anti-β-actin (Santa Cruz Biotechnology, Inc.).

Western blot analysis. The results showed that the production of Bag3, Hsp70, Bcl-X_L and Bcl-2 in HOP-62 cell line was more than that in human normal bronchial epithelial cell lines on gene transcription and protein translation level ($P < 0.05$, Fig. 3).

Inhibition of Bag3 results in diminished levels of Bcl-X_L and Bcl-2 in NSCLC cell line. In this experiment, we investigated whether silencing Bag3 was responsible for the concomitant decreases in apoptosis-associated proteins in NSCLC cell line. Accordingly, siRNA was used to silence Bag3 and the effect on the expression of Hsp70, Bcl-X_L and Bcl-2 proteins were examined. Real-time PCR and Western blot analysis showed a significant reduction in Bag3 mRNA and protein levels in cells transfected with Bag3 specific siRNA. Silencing Bag3 did not affect on the gene transcription of Hsp70, Bcl-X_L and Bcl-2 ($P > 0.05$, Fig. 3A), but it could decrease levels of Bcl-X_L and Bcl-2 in protein translation ($P < 0.05$, Fig. 3B). The inhibition of Bag3 hardly changed the Hsp70. Bag3 was able to interact with Hsp70, Bcl-X_L and Bcl-2 in NSCLC cell line by immunoprecipitation.

It has been reported that Bag3 interacts with Hsp70, Bcl-X_L and Bcl-2 in different tumor cells to inhibit tumor apoptosis (22-24). Therefore, we hypothesized that in NSCLC cell line, Bag3 can bind to apoptosis-associated proteins to decrease the apoptosis of tumor cells (Fig. 4), coimmunoprecipitation experiments were performed in HOP-62 to confirm the interaction between Bag3 and Hsp70, Bcl-X_L and Bcl-2. These data imply the existence of a multiprotein complex between Bag3, Hsp70, Bcl-X_L and Bcl-2 in the NSCLC cell line.

Discussion

Although NSCLC has been categorized in histology, NSCLC tumors have heterogeneous histology within the same lesion

in many cases. Morphology remains the standard for clinical diagnosis and treatment. At the same time, significant effort has recently been dedicated to elucidate the molecular biology of NSCLC to help clinical research to acquire more useful information in the treatment of NSCLC (25-27).

In this study, we demonstrate for the first time that the expression of Bag3 and its effects on the anti-apoptotic proteins in NSCLC tissues and cell lines. The results of IHC indicated that the expression of Bag3 and Hsp70 are up-regulated in some cases of NSCLC. Furthermore, many reports showed that Bag3 expression attenuates apoptosis in a few cancer cells via stabilization of anti-apoptotic Bcl-2 proteins (28-32). We hypothesized that Bag3 could inhibit apoptosis of NSCLC cells by regulating the expression of Bcl-2 and other functional components.

In this study, we cultured human normal bronchial epithelial and NSCLC cell lines for exploring the potential mechanism of Bag3 as an anti-apoptotic in tumor progression. Our results showed that the regulation of Bag3 changed the effect of apoptosis of NSCLC cell line. We found that Bcl-X_L and Bcl-2 protein expression were significantly reduced, while their mRNA levels were not affected by silencing Bag3 (Figs. 3 and 4). It suggested that this effect of Bag3 is mediated at the level of protein by molecule interaction and degradation (Fig. 4). This style of protein interaction regulation by Bag3 could be effective in modulating different components in several pathways to cooperate on inhibition of apoptosis of tumor cells at the same time.

Our study suggests that Bag3 may be important in the survival and apoptosis in NSCLC. A previous report also showed that Bag3 can regulate the motility and adhesion of epithelial cancer cells (33). Bag3 can interact with different molecules to increase its effect on the progression and invasion in many cancers. Further studies are needed to explore the clinical characteristic of Bag3 in NSCLC. Because of the small number of NSCLC cases (22 cases), further exploring the relationship among the tissue morphology, survival and Bag3 expression was not possible. We will perform associated work to study this further.

In conclusion, we demonstrate that Bag3 expression is increased in some cases of NSCLC. It was able to inhibit apoptosis of NSCLC cells by interacting with several different apoptosis-associated proteins *in vitro*. Bag3 may be a good target for developing new methods to improve the survival in NSCLC.

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