BAMBI overexpression together with β-sitosterol ameliorates NSCLC via inhibiting autophagy and inactivating TGF-β/Smad2/3 pathway

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Received September 12, 2016; Accepted February 20, 2017

DOI: 10.3892/or.2017.5508

Abstract. Non-small cell lung cancer (NSCLC) has the highest mortality rate among all solid tumors with a poor prognosis. The BMP and activin receptor membrane bound inhibitor (BAMBI) has been identified as a hallmark of NSCLC and β-sitosterol possesses antitumor potentiality. This study explores the effect of BAMBI overexpression and β-sitosterol in the context of NSCLC. The results revealed that the transfection of pcDNA-BAMBI and β-sitosterol treatment significantly reduced the levels of autophagy markers light chain 3 (LC3) II and Beclin 1, whereas the levels of LC3 I and p62 were promoted. The reduced punctate accumulations of GFP-LC3 were detected in pcDNA-BAMBI and β-sitosterol groups, especially in pcDNA-BAMBI + β-sitosterol group. BAMBI overexpression and β-sitosterol induced G0/G1 cell cycle arrest and inhibted cell proliferation in A549 cells. In addition, the levels of transforming growth factor-β (TGF-β)/p-Smad2/3/c-Myc pathway proteins were decreased. The TGF-β overexpression further confirmed that BAMBI overexpression and β-sitosterol treatment suppressed autohagy and viability of A549 cells was through TGF-β/Smad2/3/c-Myc pathway. Finally, the tumor growth was suppressed in NSCLC xenografts, and the inhibitory effect was stronger under treatment of pcDNA-BAMBI together with β-sitosterol. These results indicate that BAMBI overexpression and β-sitosterol may serve as novel targets for the treatment of NSCLC.

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Abbreviations: NSCLC, non-small cell lung cancer; BAMBI, BMP and activin receptor membrane bound inhibitor; LC3, light chain 3; TGF-β, transforming growth factor-β

Key words: non-small cell lung cancer, BAMBI, β-sitosterol, autophagy, TGF-β

Introduction

Lung cancer is the leading cause of deaths with the most rapidly increasing incidence worldwide. Non-small cell lung cancer (NSCLC) has the highest mortality rate among all solid tumors with a poor prognosis (1). More than half of lung carcinomas are detected in a progressed or already metastasized state with a 5-year survival, for lacking of characteristic early symptoms (2). The general therapy treating the majority of patients is chemotherapy, which often induces resistance (3). It is necessary to develop novel therapeutic approaches to better understand the lung cancer progression.

Autophagy is a fundamental cellular homeostatic process that cells use to degrade and recycle cellular proteins (4). This process can be induced in response to either intracellular or extracellular factors, such as hypoxia, low cellular energy state and organelle damage (5). The microtubule-associated protein 1 light chain 3 (LC3), functions as a structural component in the formation of autophagosomes. The conversion of the cytosolic form of LC3 (LC3 I) to lipidated form (LC3 II) indicates autophagosome formation (6). Beclin 1, is required for the initiation and in the process of autophagosome formation (7). p62 acts as a receptor or adaptor for autophagic degradation of ubiquitinated proteins, the upregulation of p62 is commonly detected in human tumors and contributes directly to tumorigenesis (8). Thus, LC3, Beclin 1 and p62 were considered as autophagy markers in many studies (9,10).

Transforming growth factor-β (TGF-β) has a crucial role in homeostasis, fibrosis angiogenesis, carcinogenesis and differentiation of the cell. A report indicated that TGF-β reduced cell apoptosis via induction of autophagy (11). TGF-β also induces the transcriptional activation of several autophagy-related genes, including BECLIN 1, ATG5, ATG7 and death-associated protein kinase (DAPK) (12,13). Thus hinting that the inhibitor of TGF-β may control cell autophagy and proliferation. BMP and activin receptor membrane bound inhibitor (BAMBI) gene is evolutionally conserved in vertebrates (14). BAMBI was described as a modulator, with a putative function as a dominant negative, non-signaling, competitive pseudo-receptor for members of the TGF-β receptor type 1 (TβR1) family (14). Apart from being suggested as a competitive receptor antagonist for the TGF-β receptor family, BAMBI

was indicated as a negative regulator for the subsequent Smad pathway (15), indicating BAMBI may antagonize autophagy through downregulating TGF-β/Smad pathway.

β-Sitosterol is a natural product isolated from traditional Chinese herbs, including *Trifolium repens*, *Houttuynia cordata* and *Lasia spinosa* (16). β-Sitosterol has been applied in treating many diseases because of the anti-inflammatory, anti-proliferative and anticancer effects (17,18). However, the effect of BAMBI together with β-sitosterol on cell autophagy and proliferation in NSCLC is rarely reported.

In this study, we aimed to explore the effect of BAMBI overexpression and β-sitosterol in the context of NSCLC. We found that BAMBI overexpression and β-sitosterol suppressed the autophagy flux of A549 cells. Besides, BAMBI overexpression and β-sitosterol restrained tumor growth *in vitro* and *in vivo* through inactivating TGF-β/Smad2/3 pathway. Taken together, our results suggest BAMBI overexpression together with β-sitosterol treatment may provide novel insight into the mechanism and treatment of NSCLC.

Materials and methods

Cell culture. Human NSCLC A549, NCI-H1975 and H1299 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultivated in modified Eagle's medium (MEM), supplemented with 20% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were incubated at 37˚C in a humidified 21% O_2, 5% CO_2 atmosphere.

Constructs and transfection. Human BAMBI expression plasmid was constructed by inserting BAMBI cDNA purchased from Beyotime (Shanghai, China) into the pcDNA3-EGFP (Invitrogen, Shanghai, China) expressing vector. The plasmid was amplified with Top 10 bacteria (Invitrogen), extracted, and purified by Plasmid Midi kit (Qiagen, Shanghai, China). For transfection, the three NSCLC cells (A549, NCI-H1975 and H1299) were cultured in antibiotic- and serum-free α-MEM medium (at 50-60% confluence). BAMBI plasmid (2 μg/well) or the vector (2 μg/well) was transfected into NSCLC cells with Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instructions. After transfection for 48 h, BAMBI expression in transfected cells was examined by western blotting.

β-Sitosterol treatment. For the treatment of β-sitosterol, $2x10^4$ A549 cells transfected with or without pcDNA-BAMBI were added to 96-well plates. Then, the RPMI-1640 medium was added to make the volume in each well up to 200 μl. After culturing for 24 h, 10 mg/ml β-sitosterol extracts were added to each well, respectively. Control group was added with equal volume of 0.01 mol/l phosphate-buffered saline (PBS). After incubation at 37˚C and 5% CO_2 for another 24 h, cells were collected for the following experiments.

Western blotting. NSCLC cells were lysed in lysis buffer (Beyotime) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF). The protein concentration was determined using the BCA protein assay (Tiangen Biotech Co., Ltd., Beijing, China). Twenty micrograms of protein in each sample was separated by 12% SDS-PAGE and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) for immunoblotting. The following primary antibodies were used: anti-BAMBI (1:500, ab203070), anti-LC3 (1:500, ab48394), anti-Beclin 1 (1:500, ab55878), anti-p62 (1:200, ab56416), anti-Smad2/3 (1:1,000, ab202445), anti-p-Smad2/3 (1:1,000, ab63399), anti-c-Myc (1:10,000, ab32072), anti-TGF-β (1:500, ab66043), anti-caspase-3 (1:5,000, ab32351), anti-caspase-8 (1:1,000, ab32397), anti-caspase-9 (1:2,000, ab202068), anti-mTOR (1:2,000, ab32028) and anti-GAPDH (1:2,500, ab9485) (all from Abcam, Cambridge, MA, USA), which was used as the internal reference. After incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), proteins were detected using a ChemiDoc XRS imaging system and Quantity One analysis software (Bio-Rad Laboratories, Inc., San Francisco, CA, USA).

Autophagy measurement using GFP-LC3. The autophagy measurement was conducted according to a previous study (19). Briefly, A549 cells were transfected with a GFP-LC3 expression plasmid (Sigma-Aldrich, St. Louis, MO, USA) incorporated into the lentiviral vector using Lipofectamine 2000 reagent. After selection with puromycin, expression of fluorescence was confirmed by microscopic evaluation before irradiation. Cells were then observed for the fluorescence of GFP-LC3 under a fluorescence microscope.

Autophagic flux measurement. A549 cells were treated with or without 20 μM chloroquine for 1 h prior to β-sitosterol treatment and BAMBI transfection. The recovered cell lysates were used to detect the accumulation of LC3 II, autophagic flux were detected by immunoblotting according to a previous study (20), briefly, cells were rinsed with PBS and lysed with an lysis buffer. The proteins were then separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies against LC3 and GAPDH and probed with an HRP-labeled secondary antibody and detected using an ECL reagent. Protein expression level was measured by a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc.).

Flow cytometry analysis of the cell cycle. A549 cells at $1x10^6$ cells/well were cultured in 6-well plates and transfected with or without pcDNA-BAMBI for 48 h. The cells were then treated with or withour 10 mg/ml β-sitosterol extracts, respectively. Cells were harvested and fixed in 70% ice-cold ethanol for 24 h, followed by staining with propidium iodide (PI). The different cell cycle phases were analyzed with the FACSCalibur instrument using CellQuest software (Becton-Dickinson, Mountain View, CA, USA).

Detection of apoptosis. For the apoptosis assay, A549 cells with β-sitosterol treatment and BAMBI transfection were washed with PBS and stained with PI and Annexin V-FITC (V13241; Invitrogen Life Technologies) on ice for 10-15 min. The stained cells were analyzed using a FACScan flow cytometer (Becton-Dickinson) and the number of apoptotic cells

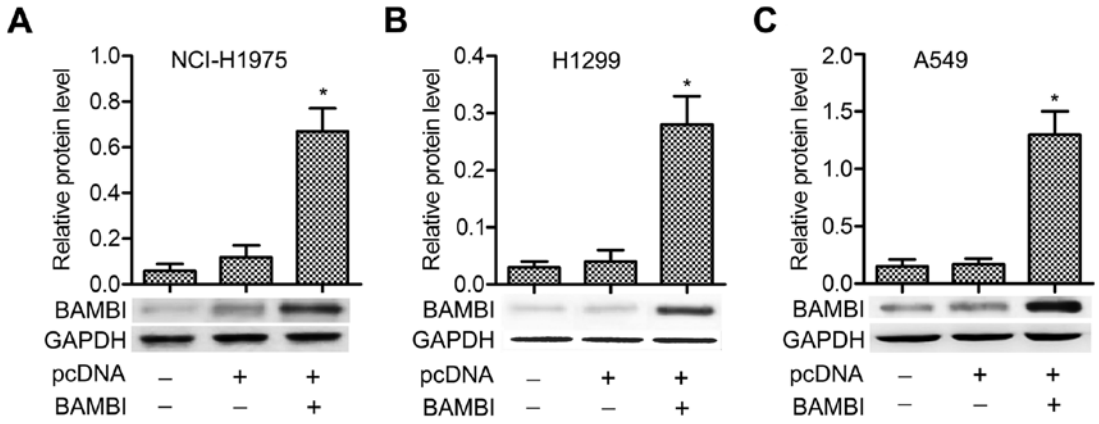

Figure 1. BAMBI is overexpressed by transfecting the plasmid of pcDNA-BAMBI into NSCLC cells. The plasmid of pcDNA-BAMBI was constructed and transfected into NSCLC cells. The expression of BAMBI in (A) NCI-H1975, (B) H1299 and (C) A549 cells was measured by western blotting. BAMBI, BMP and activin receptor membrane bound inhibitor; NSCLC, non-small cell lung cancer. *P<0.05.

was quantified using FlowJo software (Tree Star Inc., Ashland, OR, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cell viability of A549 cells was assessed using MTT assay. Shortly afterwards, cells were transfected according to the above description and were seeded in 96-well plates at $6x10^3$ cells/well. The surviving fractions were determined at 0, 24, 48, 72, 96 and 120 h. For the detection the cytotoxicity of autophagy inhibitors on A549 cells, cells were pretreated with or without 100 nM bafilomycin A1 for 1 h prior to β-sitosterol treatment and BAMBI transfection. The surviving fractions were determined after 24 h. Thereafter, the old medium was discarded and fresh medium containing MTT (5 mg/ml MTT in PBS; Sangon Biotech Co., Ltd., Shanghai, China) was added and incubated for an additional 4 h. Then, cell proliferation was measured with a spectrophotometer (Bio-Rad Laboratories, Inc.) at 470 nm. Each experiment was performed in triplicate.

TGF-β overexpression. The TGF-β overexpression was achieved by PCR amplification using TGF-β cDNA as a template, and the hNUDC expressing vector was constructed by inserting the TGF-β cDNA into pcDNA3.1 vector. The recombinant plasmid was transfected into $3x10^6$ A549 cells with or without pcDNA-BAMBI transfection and β-sitosterol treatment using a nucleofector instrument. Forty-eight hours later, subsequent experiments were performed on the cells. The experiment was replicated thrice for data calculations.

NSCLC xenografts. Twenty NOD/SCID mice (Jackson Laboratory, Bar Harbor, ME, USA) (male; body weight, 20-22 g; age, 8-weeks) were purchased from the Institute of Zoology, Chinese Academy of Medical Sciences (Shanghai, China). A549 cells ($5x10^6$) were injected subcutaneously into ten NOD/SCID mice, another ten mice were injected with equal number of A549 cells transfected with pcDNA-BAMBI. Half of the mice with or without pcDNA-BAMBI transfection were then injected with β-sitosterol (1 mg/kg body weight) every other day. The tumor volumes were measured daily after the injection, and all the rats were assigned to euthanasia at the end of measurements on day 25. All animal experiments were performed according to current prescribed guidelines and under a protocol approved by the Institutional Animal Care and Use Committee.

Statistical analysis. All results are presented as mean ± SD from a minimum of three replicates. Differences between groups were evaluated by SPSS version 15.0 statistical software (SPSS, Inc., Chicago, IL, USA) by one-way analysis of variance (ANOVA) followed by Bonferroni's test. Differences were considered statistically significant at P<0.05.

Results

BAMBI is overexpressed by transfection of the plasmid of pcDNA-BAMBI into NSCLC cells. The pcDNA-BAMBI plasmid was transfected into three NSCLC cell lines (NCI-H1975, H1299 and A549). No significant difference of BAMBI level was detected between control and pcDNA groups, but the expression of BAMBI was strongly promoted in pcDNA-BAMBI group compared with pcDNA group, especially in A549 cells (P<0.05) (Fig. 1A-C). The results indicated that BAMBI was overexpressed successfully by inserting its plasmid into NSCLC cells.

BAMBI overexpression and β-sitosterol suppress autophagy of A549 cells. We next explored whether BAMBI overexpression played a functional role in autophagy. A549 cells were chosen for the following experiments. β-Sitosterol was added alone or with pcDNA-BAMBI transfection. Western blotting indicated that LC3 II and Beclin 1 expression levels declined significantly in pcDNA-BAMBI and β-sitosterol groups compared with control group (P<0.05). In comparison with β-sitosterol function alone, the levels of LC3 II and Beclin 1 were particularly decreased when adding β-sitosterol in pcDNA-BAMBI group (P<0.05). Conversely, the levels of LC3 I and autophagy substrate p62 were increased significantly under the treatment of pcDNA-BAMBI and β-sitosterol (P<0.05). The expression of LC3 I and p62 were strongly promoted under treatment of pcDNA-BAMBI together with β-sitosterol (P<0.05) (Fig. 2A-C). In addition, immunofluorescent assay

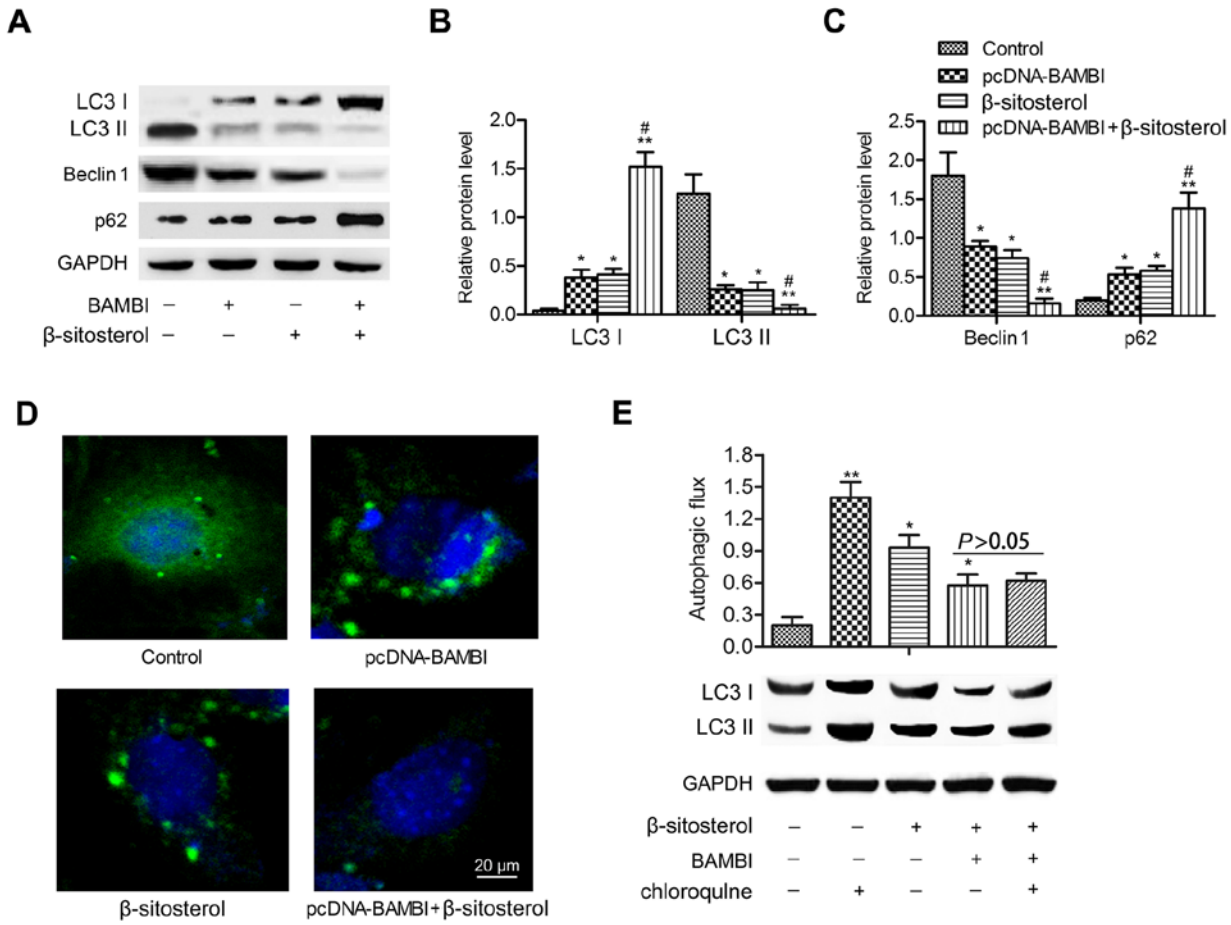

Figure 2. BAMBI overexpression and β-sitosterol suppress autophagy of A549 cells. A549 cells were divided into four groups: control group, A549 cells without any treatment; pcDNA-BAMBI group, cells transfected with pcDNA-BAMBI plamid; β-sitosterol group, cells were incubated in 10 mg/ml β-sitosterol for 24 h; pcDNA-BAMBI + β-sitosterol group, cells were incubated in 10 mg/ml β-sitosterol for another 24 h after pcDNA-BAMBI transfection for 24 h. (A) The levels of autophagy markers LC3 I, LC3 II, Beclin 1 and p62 were detected by western blotting. (B) The relative protein expression of LC3 I and LC3 II. (C) Beclin 1 and p62 was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Data are represented as the mean ± SD of three experiments. (D) GFP-LC3 staining was examined by immunofluorescence, scale bar, 20 μm. (E) Autophagic flux was detected by immunoblotting. A549 cells were treated with or without 20 μM chloroquine for 1 h prior to β-sitosterol treatment and BAMBI transfection. *P<0.05, **P<0.01 vs. control group; $^{\#}$P<0.05 vs. β-sitosterol group. BAMBI, BMP and activin receptor membrane bound inhibitor; LC3, light chain 3.

displayed reduced punctate accumulations of GFP-LC3 in pcDNA-BAMBI and β-sitosterol groups, especially in pcDNA-BAMBI + β-sitosterol group compared with control group (Fig. 2D). In addition, lipidated LC3 II degradation were used to monitor autophagic flux as the LC3 II is degraded by autolysosome. Thus, LC3 II in cells with or without chloroquine was used to examine the effects of BAMBI overexpression and β-sitosterol on autophagic flux. Compared to the control group, the level of LC3 II was higher in A549 cells with BAMBI overexpression and β-sitosterol treatment (P<0.05). However, no significant difference was observed in cells pretreated with or without chloroquine and treated with β-sitosterol with BAMBI transfection (P>0.05) (Fig. 2E). These results suggested that BAMBI overexpression and β-sitosterol suppressed autophagy of A549 cells.

BAMBI overexpression and β-sitosterol inhibit cell progression and proliferation of A549 cells. Flow cytometric analysis exhibited that G0/G1 phase arrest in A549 cells was observed in pcDNA-BAMBI and β-sitosterol groups. Besides, a large accumulation of G0/G1 phase was measured in pcDNA-BAMBI + β-sitosterol group (Fig. 3A and B). These results suggested an inactivation of G0/G1 progression in A549 cells following incubation with pcDNA-BAMBI and β-sitosterol. Besides, cell apoptosis assay indicated that BAMBI transfection strengthened the apoptosis-inducing effect of β-sitosterol in A549 cells. The number of apoptotic cells was increased in pcDNA-BAMBI + β-sitosterol group compared with β-sitosterol group (P<0.05) (Fig. 3C and D). Accordingly, the expression level of caspase-3, -8 and -9 was increased, while the level of mTOR was decreased in cells treated with β-sitosterol together with BAMBI overexpression compared with β-sitosterol group (P<0.05) (Fig. 3E). Further, the cell proliferation assay was performed in A549 cells. The transfection of pcDNA-BAMBI and β-sitosterol treatment strongly decreased cell growth compared with control group (P<0.01). The inhibitory effect was stronger in pcDNA-BAMBI + β-sitosterol group compared β-sitosterol group (P<0.05) (Fig. 3F). The cytotoxicity of autophagy inhibitor bafilomycin A1 on A549 cells was measured, the results displayed that pcDNA-BAMBI and β-sitosterol decreased the viability of A549 cells, the adding of bafilomycin A1 enhanced this effect (Fig. 3G). These findings suggest that BAMBI overexpression and β-sitosterol inhibited the viabilityof A549 cells.

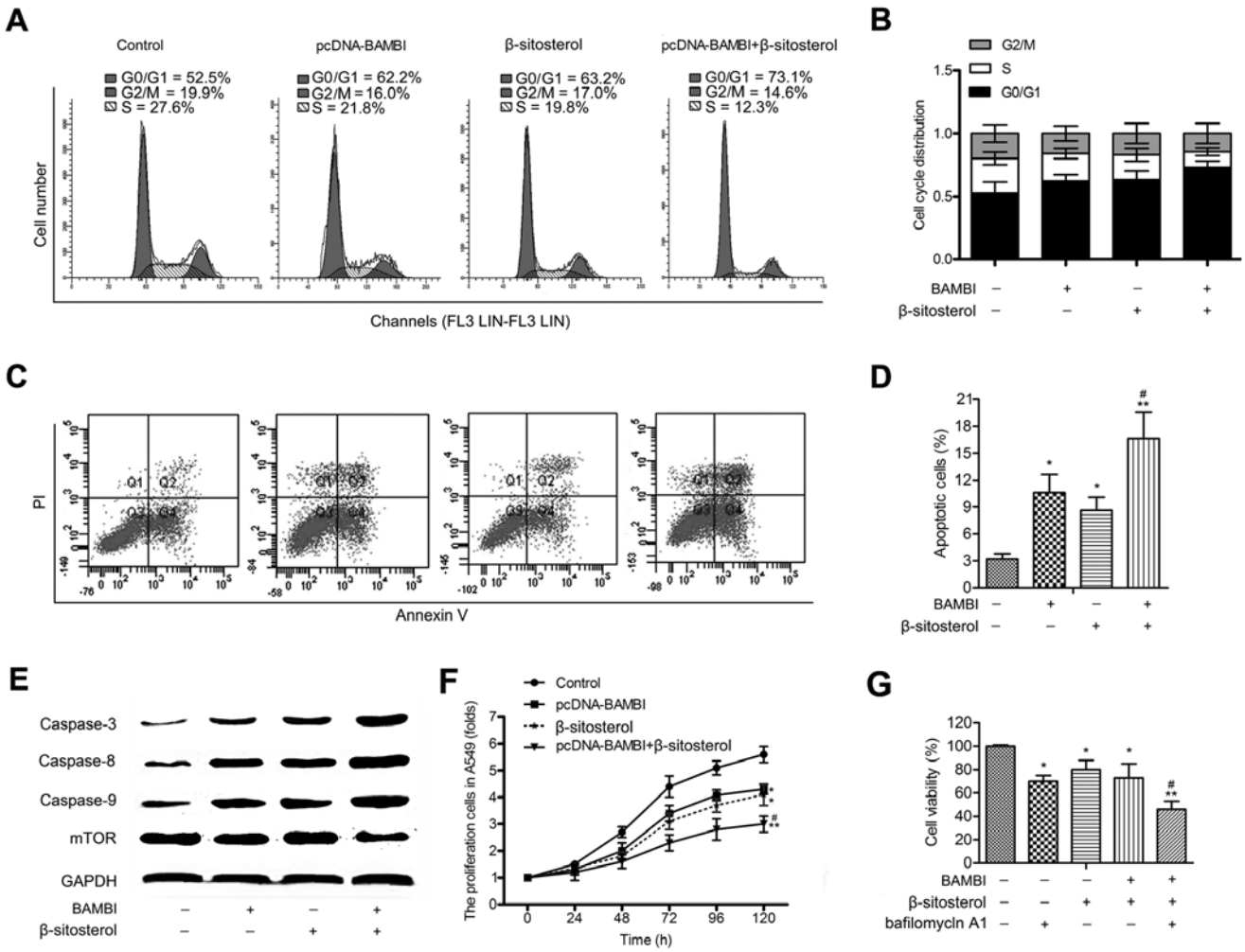

Figure 3. BAMBI overexpression and β-sitosterol inhibit the progression and proliferation of A549 cells. (A) The cell cycle analysis of A549 cells was determined by flow cytometric analysis. (B) The distributions of G0/G1, S and G2/M phases were evaluated in A549 cells treated with or without pcDNA-BAMBI and/or β-sitosterol. (C) Cell apoptosis was determined by flow cytometric. (D) Histogram represents apoptotic cells in each group. (E) The expression of caspase-3, -8 and -9 and mTOR was measured by western blotting. (F) The proliferation of A549 cells was determined at various time-points (0, 24, 48, 72, 96 and 120 h) by MTT assays. (G) MTT assays were also applied to detect the cytotoxicity of autophagy inhibitors bafilomycin A1 on A549 cells, cells were pretreated with or without 100 nM bafilomycin A1 for 1 h prior to β-sitosterol treatment and BAMBI transfection. The surviving fractions were determined after 24 h. All experiments were repeated thrice with three replicates. *P<0.05, **P<0.01 vs. control group; $^{\#}$P<0.05 vs. β-sitosterol group. BAMBI, BMP and activin receptor membrane bound inhibitor.

The TGF-β/Smad2/3 pathway is restrained by BAMBI overexpression and β-sitosterol. BAMBI is a member of TGF-β family, we further explored the effect of pcDNA-BAMBI and β-sitosterol on the expression of TGF-β pathway proteins. The results indicated that the level of Smad2/3 exhibited no significant difference, while the expression of TGF-β, p-Smad2/3 and c-Myc were decreased by pcDNA-BAMBI and β-sitosterol compared with control group (P<0.05). Moreover, the protein expression was strongly suppressed under the treatment of pcDNA-BAMBI together with β-sitosterol (Fig. 4A-E). These results suggested that BAMBI overexpression and β-sitosterol restrained the TGF-β/Smad2/3/c-Myc pathway.

The inhibitory effect of BAMBI and β-sitosterol on autophagy and viability of A549 cells is through TGF-β/Smad2/3 pathway. To confirm the TGF-β pathway was involved in the inhibition effect of BAMBI and β-sitosterol on the viability of A549. Firstly, the plasmid of pcDNA-TGF-β was constructed and transfected into A549 cells. Western blotting revealed that the level of TGF-β, p-Smad2/3 and c-Myc was promoted in pcDNA-TGF-β group in comparison with control group (P<0.01). The downregulation of their expression was partially counteracted when cells in pcDNA-BAMBI + β-sitosterol group were transfected with pcDNA-TGF-β (P<0.05) (Fig. 4F-I).

We measured the levels of autophagy markers, the results displayed that A549 cells transfected pcDNA-TGF-β exhibited enhanced expression of LC3 II and Beclin 1 with decreased LC3 I and p62 levels compared with control group (P<0.05). The expression of LC3 II and Beclin 1 was strongly restrained in pcDNA-BAMBI + β-sitosterol group in comparison with control group (P<0.05), and their levels were increased adding pcDNA-TGF-β in pcDNA-BAMBI + β-sitosterol group (P<0.05). The opposite behavior was observed in the expression of LC3 I and p62 (Fig. 5A and B). In addition, a large accumulation of GFP-LC3 was measured by pcDNA-TGF-β transfection. The GFP-LC3 II punctate accumulation was significantly reduced in pcDNA-BAMBI + β-sitosterol, but increased

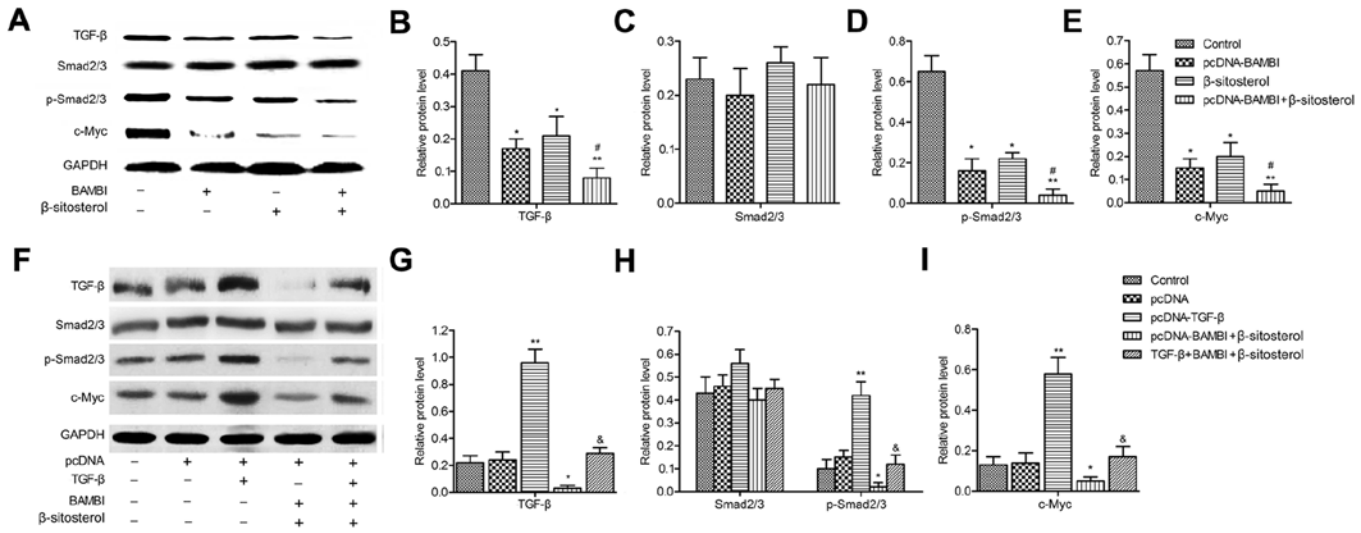

Figure 4. BAMBI overexpression and β-sitosterol inhibit the TGF-β/Smad2/3/c-Myc pathway. (A) The expressions of TGF-β, Smad2/3, p-Smad2/3 and c-Myc were detected by western blotting and quantified using Image-Pro Plus 6.0 software and normalized to GAPDH (B-E). TGF-β was then overexpressed by inserting the plasmid of pcDNA-TGF-β into A549 cells. Cells were divided into five groups: control, pcDNA, pcDNA-TGF-β, pcDNA-BAMBI + β-sitosterol and TGF-β + BAMBI + β-sitosterol groups, cells were co-transfected with pcDNA-TGF-β and pcDNA-BAMBI and incubated in 10 mg/ml β-sitosterol for 24 h. (F) The expressions of TGF-β, Smad2/3, p-Smad2/3 and c-Myc were detected by western blotting and quantified using Image-Pro Plus 6.0 software and normalized to GAPDH (G-I). Data are represented as the mean ± SD of three experiments. *P<0.05, **P<0.01 vs. control group; $^{\#}$P<0.05 vs. β-sitosterol group; $^{\&}$P<0.05 vs. pcDNA-BAMBI + β-sitosterol group. BAMBI, BMP and activin receptor membrane bound inhibitor.

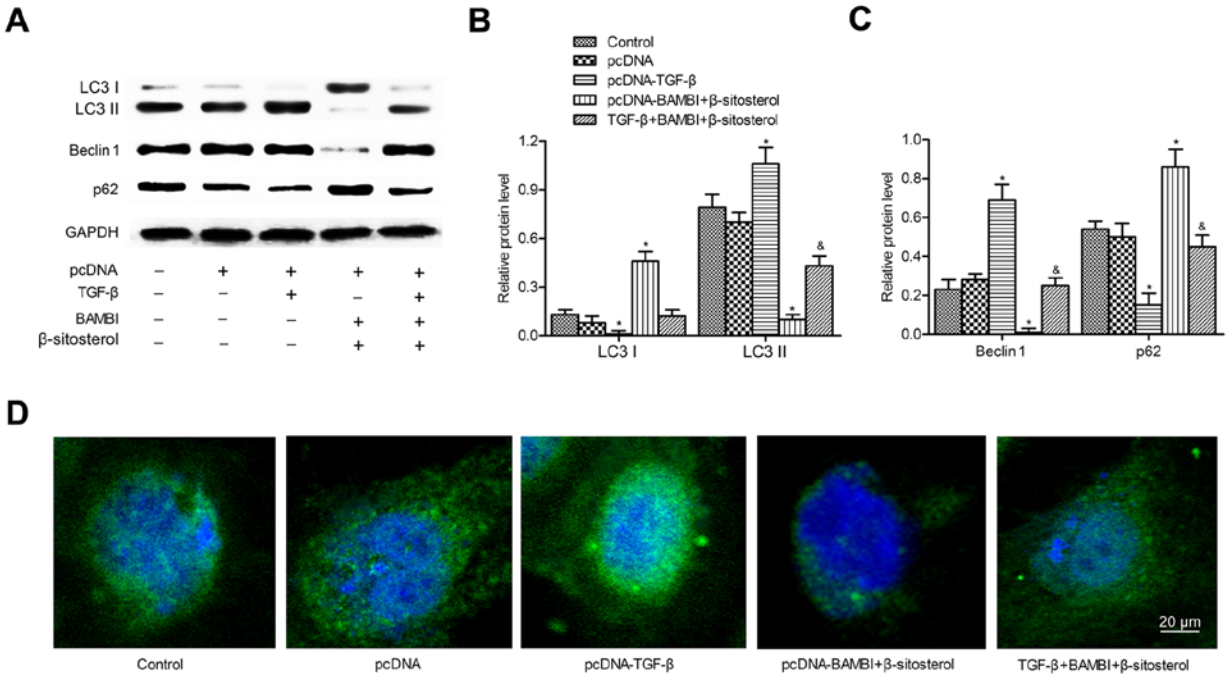

Figure 5. TGF-β overexpression partially counteracted the suppression effect on autophagy of A549 cells. (A) The levels of autophagy markers LC3 I, LC3 II, Beclin 1 and p62 were detected by western blotting. (B) The relative protein expression of LC3 I and LC3 II. (C) Beclin 1 and p62 was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Data are represented as the mean ± SD of three experiments. (D) GFP-LC3 staining was examined by immunofluorescence, scale bar, 20 μm. *P<0.05 vs. control group; $^{\&}$P<0.05 vs. pcDNA-BAMBI + β-sitosterol group. LC3, light chain 3; BAMBI, BMP and activin receptor membrane bound inhibitor.

adding pcDNA-TGF-β in pcDNA-BAMBI + β-sitosterol groups (Fig. 5C).

In accordance with these results, the G0/G1 phase and cell proliferation were accelerated in pcDNA-TGF-β group compared with control group. In addition, the cell cycle arrest and the inhibition of cell growth were partially offset when cells in pcDNA-BAMBI + β-sitosterol group were transfected with pcDNA-TGF-β (P<0.05) (Fig. 6A-C). These results indicated that the inhibitory effect of BAMBI overexpression and β-sitosterol on the viability of A549 is through the TGF-β/Smad2/3/c-Myc pathway.

BAMBI overexpression and β-sitosterol suppress the proliferation of NSCLC in vivo. To further explore the

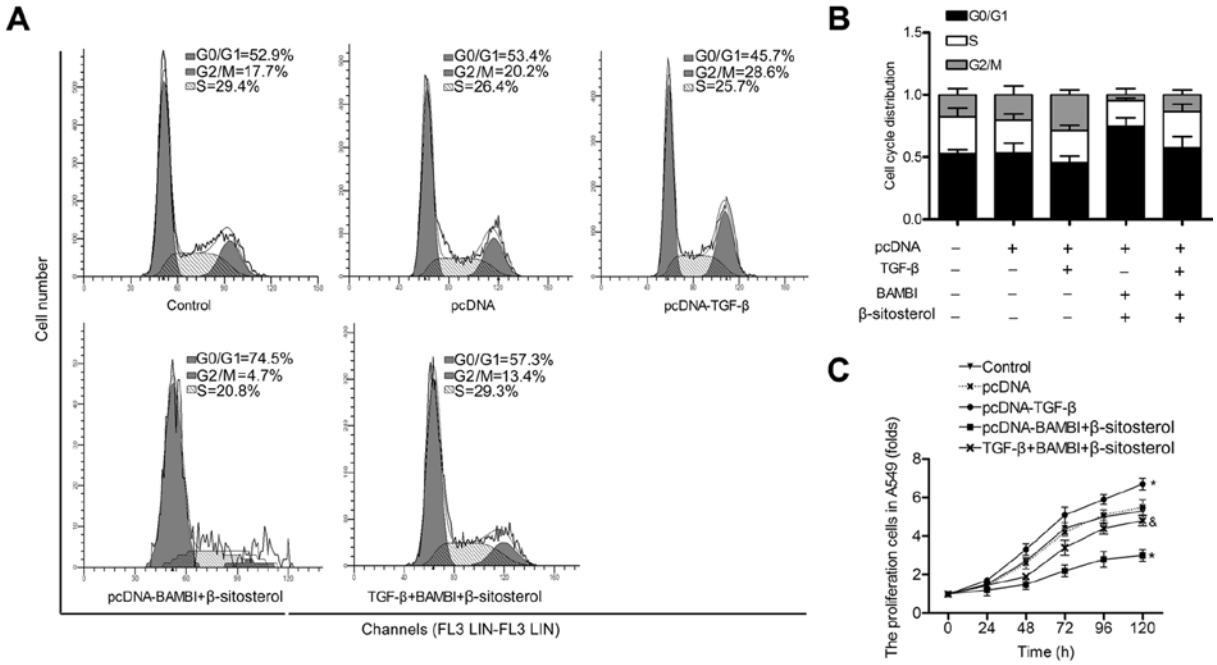

Figure 6. TGF-β overexpression partially offset the inhibitory effect on the viability of A549 cells. (A) The cell cycle analysis of A549 cells in five groups was determined by flow cytometric analysis. (B) The distribution of G0/G1, S and G2/M phases were evaluated in A549 cells. (C) The proliferation of A549 cells was determined at various time-points (0, 24, 48, 72, 96 and 120 h) by MTT assays. Data are represented as the mean ± SD of three experiments. *P<0.05 vs. control group; $^{\&}$P<0.05 vs. pcDNA-BAMBI + β-sitosterol group. BAMBI, BMP and activin receptor membrane bound inhibitor.

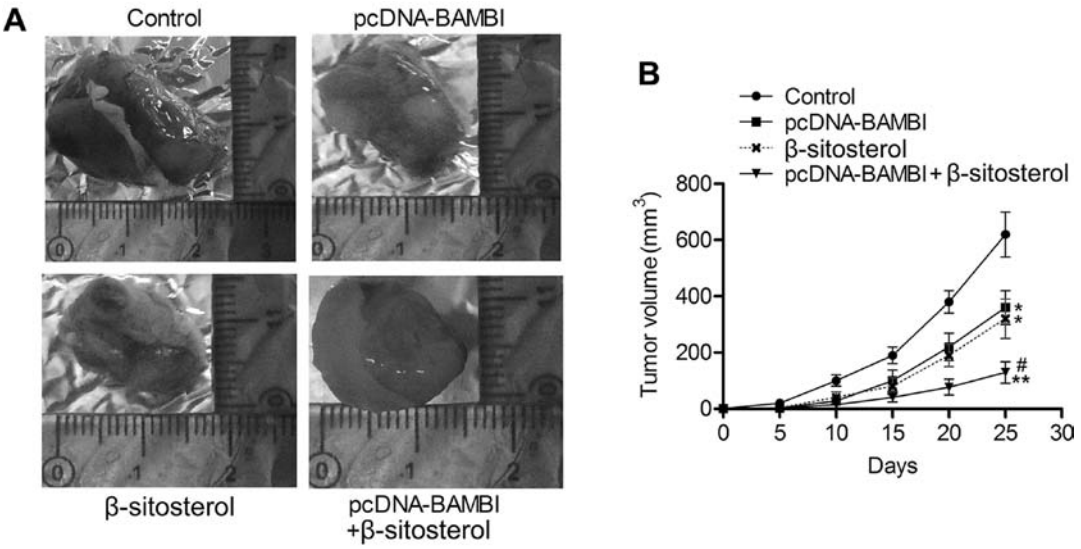

Figure 7. BAMBI overexpression and β-sitosterol suppress the proliferation of NSCLC *in vivo*. A549 cells ($5x10^6$) were injected subcutaneously into ten NOD/SCID mice, another ten mice were injected with equal number of A549 cells transfected with pcDNA-BAMBI. Half of the mice with or without pcDNA-BAMBI transfection were then injected with β-sitosterol (1 mg/kg body weight) every other day, representative images of NSCLC xenograft and tumor volumes are indicated (A and B). The experiments were repeated thrice with three replicates. *P<0.05, **P<0.01 vs. control group; $^{\#}$P<0.05 vs. β-sitosterol group; $^{\&}$P<0.05 vs. pcDNA-BAMBI + β-sitosterol group. BAMBI, BMP and activin receptor membrane bound inhibitor; NSCLC, non-small cell lung cancer.

tumor suppression effect of BAMBI overexpression and β-sitosterol, we assessed tumor growth of NSCLC xenografts under treatment of pcDNA-BAMBI and/or β-sitosterol. As shown in Fig. 7A and B, tumor growth was suppressed in pcDNA-BAMBI and β-sitosterol groups compared with control group (P<0.05), and the minimum tumor volume was detected in pcDNA-BAMBI + β-sitosterol group. The *in vivo* experiments were convincing that BAMBI overexpression and β-sitosterol suppressed the proliferation of NSCLC.

Discussion

Chemotherapy is common in treatment of NSCLC, but the development of chemoresistance in NSCLC is a major obstacle in treating patients (21). A study indicated that human lung cancer tissues that experienced chemotherapy showed increase of autophagy (22). Thus, a strategy that is specific to anti-autophagy with tumor-suppressive effect would be beneficial in treating NSCLC. BAMBI is a type I TGF-β receptor

antagonist, the epigenetic silencing of BAMBI was identified as a hallmark of NSCLC (3). The methanol extract from β-sitosterol possesses antitumor potentiality. The mechanism of BAMBI and β-sitosterol on cell autophagy and growth was explored in this study.

The physiological function of BAMBI remains unclear, high expression of BAMBI has been detected in colorectal cancer (11) and ovarian cancer (12). On the contrary, BAMBI is epigenetically silenced in high-grade bladder cancer (14) and absent from breast cancer (15). In the context of lung diseases, BAMBI was almost completely absent from the lung cancer tissues compared with the tumor-free lung tissues. Besides, BAMBI downregulation drives the invasiveness of NSCLC (3), indicating that the upregulation of BAMBI could reduce the severity of NSCLC. In this study, BAMBI was overexpressed successfully by inserting its plasmid into three NSCLC cell lines (NCI-H1975, H1299 and A549). Recent studies revealed the paradoxical nature of autophagy in deciding cell-fate machinery. Autophagy induces cell death, suppresses inflammation and enhances genomic stability; on the contrary, autophagy also renders cells viable in stressful conditions and is considered a pro-survival mechanism (23,24). Studies indicated that TGF-β controls autophagic responses during angiogenesis, and fibrogenesis in many human cellular systems, such as atrial myofibroblasts (25), renal tubular epithelial (26) and endothelial cells (27). Research also indicated that the TGF-β-activated fibroblasts increased autophagy and greatly sustained the growth of breast cancer cells (28). These findings suggested that the inhibitor of TGF-β may prevent tumorigenesis by regulating autophagy. As the antagonist of TGF-β receptor, the transfection of pcDNA-BAMBI together with β-sitosterol treatment in this study significantly reduced the levels of LC3 II and Beclin 1 with decreased GFP-LC3 punctuate structures, whereas the levels of LC3 I and p62 were strongly promoted. Besides, no significant increase of autophagy flux was observed in cells pretreated with or without chloroquine and treated with β-sitosterol and BAMBI transfection. These results suggested that BAMBI overexpression and β-sitosterol suppressed autophagy in NSCLC cells.

Apart from the effect in cell autophagy, BAMBI gene suppression was indicated as one of the epigenetic events affecting the invasiveness or aggressiveness of bladder cancers (29) and NSCLC (3). BAMBI overexpression significantly increased the number of apoptotic cells in T24 line (29). Research also indicated that BAMBI was stimulated by fibroblast growth factor 18, which increased apoptosis in ovarian granulosa cells (30). These studies suggested a cell apoptosis-inducing role of BAMBI. As component in active fractions of plants, β-sitosterol has a protective effect against colon, prostate, stomach, ovarian and breast cancers via interfering with multiple cell signaling pathways, including cell cycle, apoptosis, proliferation, invasion, angiogenesis and carcinogenesis (31-33). Animal studies have shown that β-sitosterol, extracted from *Aristolochia mollissima* Hance, has an inhibitory effect on the proliferation of osteosarcoma HOS cells (34). β-Sitosterol has also been reported to affect cell cycle progression by inducing sub-G1 arrest in human colon cancer cells (HT-29) (35) and U266 multiple myeloma cells (36). Consistent with these studies, pcDNA-BAMBI and β-sitosterol applied in this study suppressed cell proliferation and induced accumulation of G0/G1 arrest and cell apoptosis in A549 cells. The inhibitory effect was stronger when cells were under pcDNA-BAMBI transfection plus β-sitosterol treatment. These findings revealed an anticancer effect of BAMBI overexpression and β-sitosterol in NSCLC cells.

The TGF-β signaling pathway is believed to contribute to carcinoma development by increasing cancer cell motility, invasiveness and metastasis (37). TGF-β has immunosuppressive properties and may enable cancer cells to evade the immune responses (38). Upon binding of the ligand to TGF-β receptors type 2 (TGF-βR2), TGF-βR1 is phosphorylated and recruits SMAD2 and SMAD3, migrates to the nucleus, and stimulates target gene expression (39). In lung cancer, high TGF-β serum levels is associated with a poor prognosis, lymph node metastasis, and tumor progression (40,41). In addition, TGF-β was suggested as an independent risk factor for the occurrence of pulmonary metastasis in NSCLC (42). A study also revealed that the presence of p-SMAD2 and 3 was significantly higher in the NSCLC cancer tissues compared with the tumor-free lung tissues (3). In this study, the level of TGF-β, p-Smad2/3 and c-Myc was decreased by pcDNA-BAMBI transfection and β-sitosterol treatment. The transfection of pcDNA-TGF-β further confirmed the inhibitory effect in this pathway. In addition, the inhibition of cell autophagy and cell survival were partially offset when cells in pcDNA-BAMBI + β-sitosterol group were transfected with pcDNA-TGF-β. These results confirmed that BAMBI overexpression and β-sitosterol suppression of cell autophagy and viability of NSCLC is through the TGF-β/Smad2/3/c-Myc pathway.

In vivo experiment showed that BAMBI transduction abolished protumor effects of an orthotopic breast cancer xenograft model (43) and modulated the effects of diabetes via inhibiting TGF-β signaling (44). Our study found that BAMBI overexpression and β-sitosterol suppressed tumor growth in NSCLC xenografts, revealing the antitumor effect of BAMBI overexpression and β-sitosterol.

In conclusion, this study explored pcDNA-BAMBI transfection and β-sitosterol treatment on autophagy and progression of NSCLC cells. We found that BAMBI overexpression and β-sitosterol suppressed cell autophagy, induced G0/G1 cell cycle arrest and inhibited cell proliferation *in vitro* and *in vivo* possibly through inactivating the TGF-β/Smad2/3/c-Myc pathway. Moreover, the inhibitory effect was stronger when pcDNA-BAMBI and β-sitosterol functioned together. These results indicate that BAMBI overexpression and β-sitosterol may serve as novel targets for the treatment of NSCLC.

Acknowledgements

The authors would like to thank the members of Internal Medicine, the Cancer Hospital of Linyi, for providing technical support and helpful discussions concerning the present study.

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