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ALLN hinders HCT116 tumor growth through Bax-dependent apoptosis

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ABSTRACT

Continual high expression of cysteine proteases calpain I and II have been implicated in tumorigenicity; conversely, N-acetyl-leu-leunorleucinal (ALLN), which inhibits calpain I and II, should also influence tumor growth and carcinogenesis. To explore the role of ALLN against colon cancer and in promoting apoptosis, we used colon cancer HCT116 cell lines, p53 or Bax-deficient HCT116 cell lines. Cell viability and tumor growth decreased in a concentration-dependent manner when treated with 0–26 μM ALLN. Treatment with ALLN induced apoptosis in HCT116 cell; however, flow cytometry showed that apoptosis significantly decreased in Bax-deficient HCT116 cell lines, but not in p53-deficient HCT116 cell lines. In addition, the ALLN-induced apoptosis response was through Bax translocation from cytosol to mitochondria. In this study we showed intraperitoneally injected ALLN to inhibit colon tumor formation in nude mice, and found ALLN to inhibit tumor growth in colon cancer cells, mainly through apoptosis that depends on translocation of Bax to a mitochondrial endogenous pathway; this implies a molecular mechanism for ALLN against human colon cancer. These results suggest that ALLN could become a novel agent for prevention of colon cancer.

1. Introduction

Cancer is a major public health problem in the modern world. In the United States, one in four deaths is due to cancer [1]. Colorectal cancer is the third leading cause of cancer death in each sex and second overall in men and women combined [2]. Although cancer generally reflects growth deregulation at a cellular level, such deregulated cells will normally undergo apoptosis to protect the organism; inactivation of the apoptotic pathway can thus lead to cancer [3], and often, to drug resistance in cancers; such inactivation is sometimes due to mutation of the *Bax* gene, which is an important regulator of the mitochondrial apoptosis pathway [4–7].

Apoptosis is also called programmed cell death. The Bcl-2 family plays an important role in mitochondria pathway-mediated apoptosis. Bax, as one of the Bcl-2 family's pro-apoptotic members, is normally located in cytoplasm, but will, under certain stresses, translocate to the mitochondria and oligomerize, resulting in release of cytochrome C from mitochondria which activates apoptotic protease activating factor-1 and Caspase 9, and forming apoptosomes, which induce apoptosis. Bax deficiency renders cancer cells resistant to several anticancer drugs that act through the mitochondria [8,9].

Calpains are a conserved family of cysteine proteinases, which catalyze their specific substrates in a calcium-dependent manner [10]. Calpains, especially calpain I and II, participate in many physiological processes, including cell signaling, apoptosis and cell-cycle regulation; their increased expression is associated with tumorigenicity, and they are implicated in processes critical to cancer development and progression. As high calpain levels can influence cancer therapy, possibly by affecting apoptosis, they could be targets for anti-cancer therapies [11]. N-acetyl-leu-leunorleucinal (ALLN), also called calpain inhibitor 1, inhibits calpain I and II, and reportedly activates p53-dependent apoptosis [12]. However, the mechanism and specific function of ALLN in apoptosis are yet unclear.

Our investigation showed that ALLN greatly inhibits colon cancer cell tumor growth both *in vivo* and *in vitro*. ALLN was seen to promote apoptosis in HCT116 cell line by inducing Bax translocation from cytoplasm to mitochondria [13]. Whereas ALLN-activated apoptosis and Bax translocation was significantly hindered in isogenic *Bax*-deficient human colon cancer HCT116 cell line, ALLN-induced Bax translocation and apoptosis was rescued through reintroduction of Bax in these Bax-deficient cells. All these

Abbreviations: ALLN, N-acetyl-leu-leunorleucinal; CCK-8, Cell Counting Kit-8; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.

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results imply that ALLN could become the basis of a therapeutic approach for cancers with functioning Bax proteins.

2. Materials and methods

2.1. Ethics statement

All studies on human cells were approved by the Committee on the Use of Human Subjects in Research at Wuhan University. All of the animal studies were conducted in accordance with the Guidelines of the China Animal Welfare Legislation, as proved by the Committee on Ethics in the Care and Use of Laboratory Animals of Wuhan University (Permit Number: 11030h). All efforts were made to minimize suffering.

2.2. Cell lines and plasmid

Human colorectal carcinoma HCT116 cells, RKO cells and human colorectal adenocarcinoma SW480 cells were obtained from the American Type Culture Collection. Human hepatoma cells (HepG2) were kindly provided by Dr. Deyin Guo (Wuhan University). HCT116/p53$^{-/-}$ cells, HCT116/Bax$^{-/-}$ cells were kindly provided by Dr. Bert Vogelstein (The Johns Hopkins University). The pEGFP-C3-Bax and pEGFP-C3-Bax (C62/126S) expression vectors were a gift from Quan Cheng (Institute of Zoology, Chinese Academy of Sciences).

2.3. Cell culture

HCT116, RKO, SW480, HCT116/*p53*$^{-/-}$, HCT116/*Bax*$^{-/-}$ cells were maintained in McCoy's 5A Medium (AppliChem, A1324, 9050) with 10% (v/v) fetal bovine serum (FBS; HyClone) and 100 U penicillin–streptomycin (GIBCO) at 37 °C in a 5% CO_2 incubator. HepG2 cells were maintained in RPM1640 Medium (HyClone) with 10% (v/v) FBS (HyClone) and 100 U penicillin–streptomycin (GIBCO) at 37 °C in a 5% CO_2 incubator.

2.4. Medicine and antibodies

ALLN (208719) was purchased from CalBioTech. Caspase inhibitor VI (219007) was obtained from Merck. Rabbit monoclonal antibodies against Bax (#5023) and Caspase 3 (#9665), and rabbit polyclonal antibodies against Caspase 9 (#9502), and cleaved PARP (#9541), were obtained from Cell Signaling Technology (Beverly, MA). Anti-mouse monoclonal antibody P53 (sc-126) and anti-goat polyclonal antibody HSP60 (sc-1052) came from Santa Cruz Biotechnology. Anti-mouse monoclonal β-actin antibody (AC004) came from ABClonal. Anti-mouse monoclonal β-Tubulin antibody (AbM59005-37B-PU) came from BPI. Horseradish peroxidase-conjugated secondary antibody came from Jackson ImmunoResearch.

2.5. Transient and stable transfection

Transient transfections used Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions (Life Technologies, Inc.). Transfection reagents and DNA were mixed in Opti-MEM (Invitrogen); the complex was added to cells grown to 40–80% confluence and cultured for about 4 h, when the medium was replaced by fresh medium. For *Bax-EGFP* stable transfections, 0.5 mg/ml G418 added to the medium 48 h after transient transfection and cells selected 2 weeks later. Stable cells were maintained in 0.25 mg/ml G418 medium.

2.6. Colony-formation and soft agar assay

Colony-formation and soft agar assays were used to detect viability and tumorigenicity of HCT116 cells in the presence of ALLN [24]. Briefly, HCT116 cells (3×10^3) were exposed to ALLN (0–26 μM) for 24 h; medium and drugs were then replaced with fresh medium. About 10 days later, HCT116 cells colonies were stained with crystal violet and photographed. For the soft agar assay, 2 ml 0.7% lower agar-McCoy's 5A-calpain inhibitor 1 mix (0–26 μM) was plated onto each well of 6-well plates. 1 ml HCT116 cells (1×10^4) were mixed with 1 ml 0.7% agar-McCoy's 5A-calpain inhibitor 1 (0–26 μM) mix and added to curdled lower agar; 2 ml McCoy'5A medium was added to upper agar, and the plates were incubated at 37 °C in 5% CO_2 for about 3 weeks. Finally, the numbers of clones were scored and photographed.

2.7. Cell viability assay

Cells viability was assayed using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) following manufacturer's protocol. All HCT116 cell types (1×10^4) were seeded into each well of 96-well plate, cultured to 80% density and treated with different doses of ALLN for 24 h. Medium was then replaced by 100 μl fresh McCoy's 5A complete medium with 10% CCK-8 reagent and incubated for 1 h. Absorbance was measured at 450 nm using a microplate reader (BioTek, ELx800, USA). Results are shown as death percentages.

2.8. Apoptosis assay

All HCT116 cell types (1×10^4) were plated in 6-well plates and cultured to 80% density. After treatment with 26 μM ALLN for 24 h, all cells were collected, washed twice with phosphate-buffered saline (PBS), and resuspended in binding buffer at a concentration of 1×10^6 cells/ml. Cells were stained with PI and Annexin V for 15 min at room temperature. These samples were analyzed by flow cytometry (Beckman Coulter, Fullerton, CA).

2.9. Mitochondrial isolation

HCT116 cells were treated with ALLN (0.13 μM) for 0 h or 24 h. Isolation of mitochondrial and cytosol proteins was performed using the Mitochondria/cytosol Fractionation Kit (Beyotime Inst. Biotech, Peking, PR China). Shortly, cells were resuspended in the Mitochondria isolation buffer and homogenized on ice with a 2-ml glass homogenizer. Centrifuge cell lysises 1000*g* at 4 °C for 10 min and collected supernatant. Then, Spin down 14,000*g* for 15 min at 4 °C, and the resulting supernatant is the cytosolic fraction, the pellet represents the mitochondrial fraction.

2.10. GFP-Bax translocation assay

GFP-Bax stable expressing HCT116 Bax$^{-/-}$ cells were transfected with 2 μg Mito Red. After 24 h transfection, cells were exposed to ALLN (0 or 26 μM) for 12 h. After fixed with 4% paraformaldehyde, the cells were washed with PBS and observed with an Olympus FluoView FV1000 confocal laser scanning microscope to (Olympus, Japan) equipped with objective lenses (40×, 60/1.4× oil) and operated with the Olympus FluoView software. The distribution pattern of both GFP-Bax and Mito Red were imaged simultaneously. Bax redistribution was assessed by the matching fluorescence of GFP-Bax and Red CMXRos emission.

2.11. Immunoblotting analysis

Immunoblotting analysis was performed as described previously. Briefly, cells were cultured on 60-mm dishes to 80% confluence, and then were treated with ALLN (0–26 μM) for periods of 0–24 h. The cells were scraped and centrifuged at 1000 rpm for 5 min and washed once with ice-cold PBS. Finally, the cells were lysed by SDS-sample buffer and boiled 10 min. Protein concentration was determined by BCA protein assay kit (Thermo). The 30 μg samples were loaded on SDS–PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane, and the membranes blocked with TBST (PBS, 0.1% Tween 20) containing 5% dry fat milk, probed with primary antibodies overnight at 4 °C followed by an HRP-conjugated secondary antibody for 1 h at room temperature; members were then developed with the Immobilon Western Chemiluminescent HRP Substrate kit (Merck Millipore).

2.12. Xenografts

Female athymic nude mice were bought from Peking HFKBio. When mice were 6 weeks old, HCT116 cells were collected and washed with PBS twice; finally 5×10^6 HCT116 cells were resuspended in 0.2 ml PBS and inoculated into the flanks of 10 mice. About 10 days after being inoculated, mice were divided into two groups: the control group was intraperitoneally injected with vehicle containing a saline and ethanol mixture, and the treatment group was intraperitoneally injected with 200 μl saline containing 10 mg/kg ALLN. Tumor volumes were measured every 3 days; volume was calculated by formula ([length × width^2] 0.5×). Tumors were removed and weighed 26 days after injection.

3. Results

3.1. ALLN reduced growth of colon cancer HCT116 cells and tumorigenicity in vivo

We aimed to understand the effects of ALLN on tumor growth in human colon cancer HCT116 cells. As shown in Fig. 1, HCT116 cells were exposed to 0–26 μM ALLN for 24 h and then cultured for 10 days. Viability of HCT116 cells significantly decreased with ALLN treatment (Fig. 1A). Soft agar assays were used to test tumor formation *in vitro*; after about 2 weeks, anchorage-independent tumor growth was greatly inhibited in cells incubated with ALLN (Fig. 1B). These two experiments demonstrated that ALLN has inhibitory effects on human colon cancer HCT116 cell growth *in vitro*. We also examined the inhibition role of ALLN on HCT116 cell tumorigenicity *in vivo*, by injecting HCT116 cells into the flanks of nude mice. About 10 days after injection, 10 mice were randomly divided into two groups, a vehicle control group and the ALLN treatment group. The vehicle control mice were intraperitoneally injected with vehicle containing saline and ethanol mixture, while the treatment mice were intraperitoneally injected with ALLN. Injections were carried out once a day for 15 days. As shown in Fig. 1C, ALLN did not affect body weights of the mice. However, tumor weight (Fig. 1D) and volume (Fig. 1E) of treated mice were significantly less at day 26 compared to control mice. Before treat-

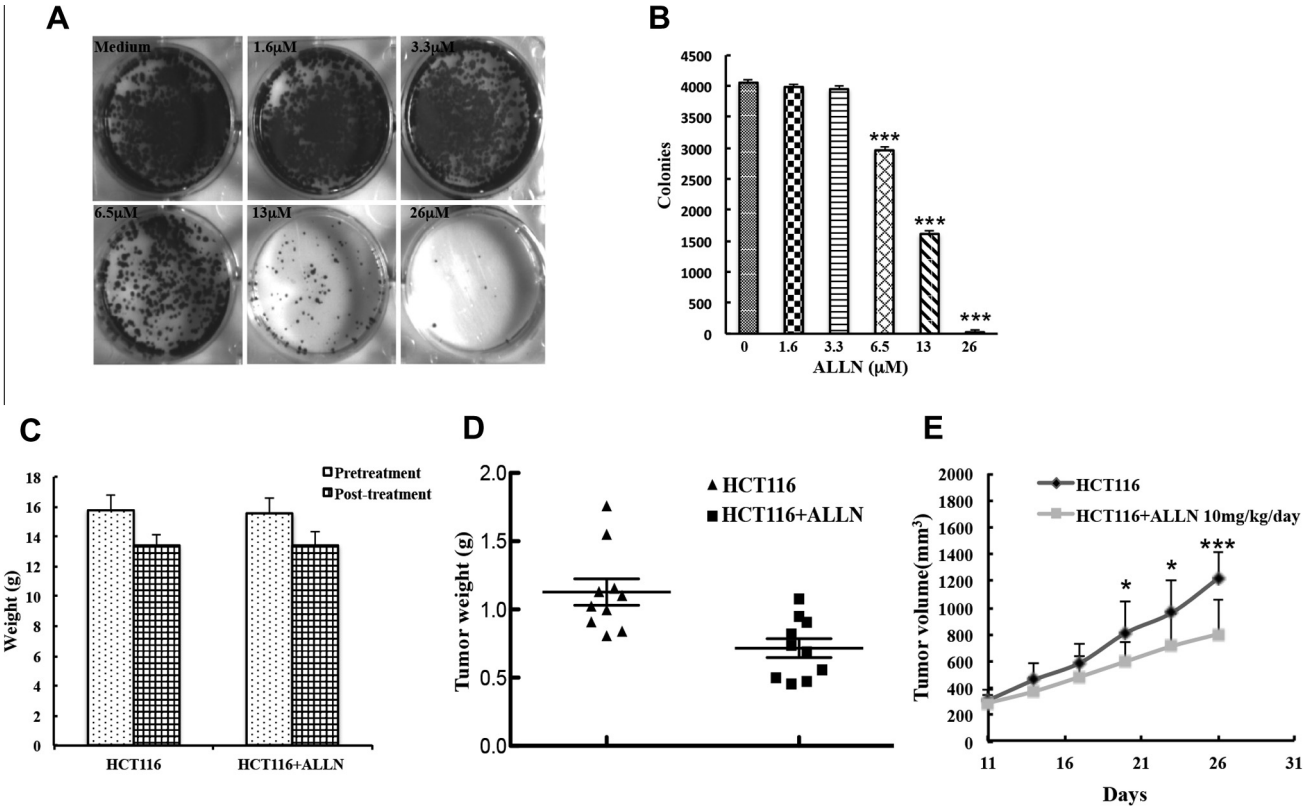

Fig. 1. ALLN represses growth of colon cancer HCT116 cells and tumorigenicity in nude mice. (A) HCT116 cells (3×10^3) were exposed to ALLN (0–26 μM) for 24 h; drugs were then removed and culture continued for about 10 days. HCT116 cell colonies were stained with crystal violet and photographed. (B) HCT116 cells (1×10^4) were exposed to ALLN (0–26 μM) in 0.35% upper agar and 0.7% lower agar for 14 days. (C) Average body weights of nude mice pre- and post-treatment, with and without ALLN. (D and E) Xenograft experiments were carried out by injecting HCT116 cells (5×10^6) into the flanks of 6-week-old nude mice to form tumors. Mice were then randomly divided into two groups: the vehicle control group and the ALLN treatment group. Vehicle control mice were intraperitoneally injected with vehicle (saline and ethanol mixture); treatment mice were intraperitoneally injected with 10 mg/kg/day ALLN. Injections were carried out once a day for 15 days. Tumor volumes were recorded every 3 days. Numbers of colonies are expressed as means ± S.E. from three assays. $^*P < 0.05$, $^{***}P < 0.001$ compared to controls.

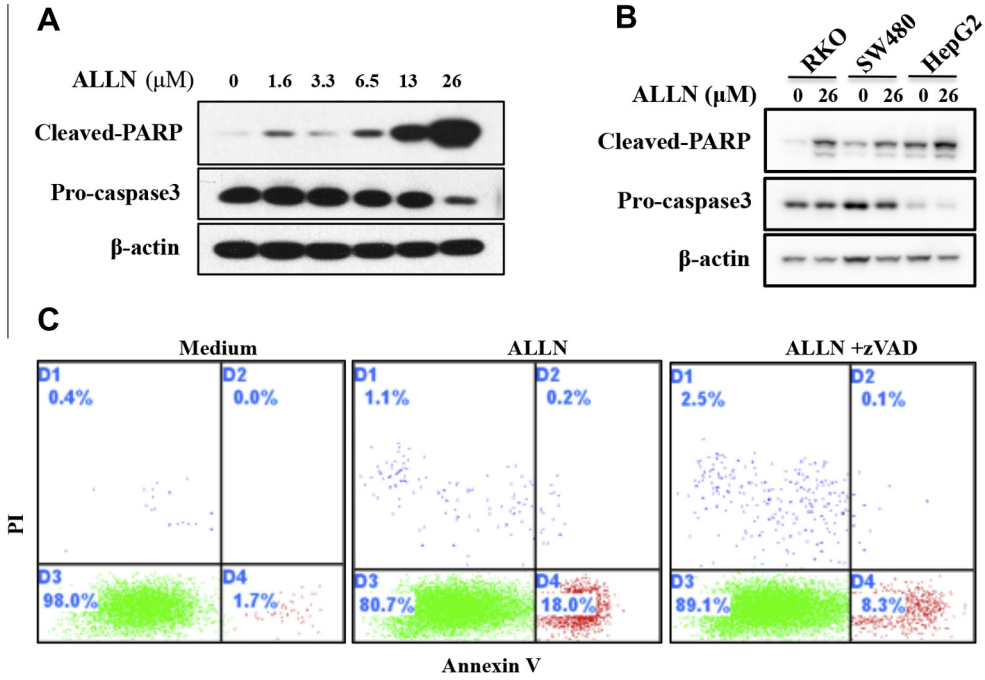

Fig. 2. ALLN induces apoptosis in colon cancer cells. (A) Pro-caspase-3 and cleaved PARP were detected by Western blot 24 h after treating HCT116 cells with various concentrations of ALLN. (B) Pro-caspase-3 and cleaved PARP were detected by Western blot 24 h after treating RKO, SW480 and HepG2 cells with 26 μM ALLN (C) Annexin-V and PI stained cells were detected by flow cytometry.

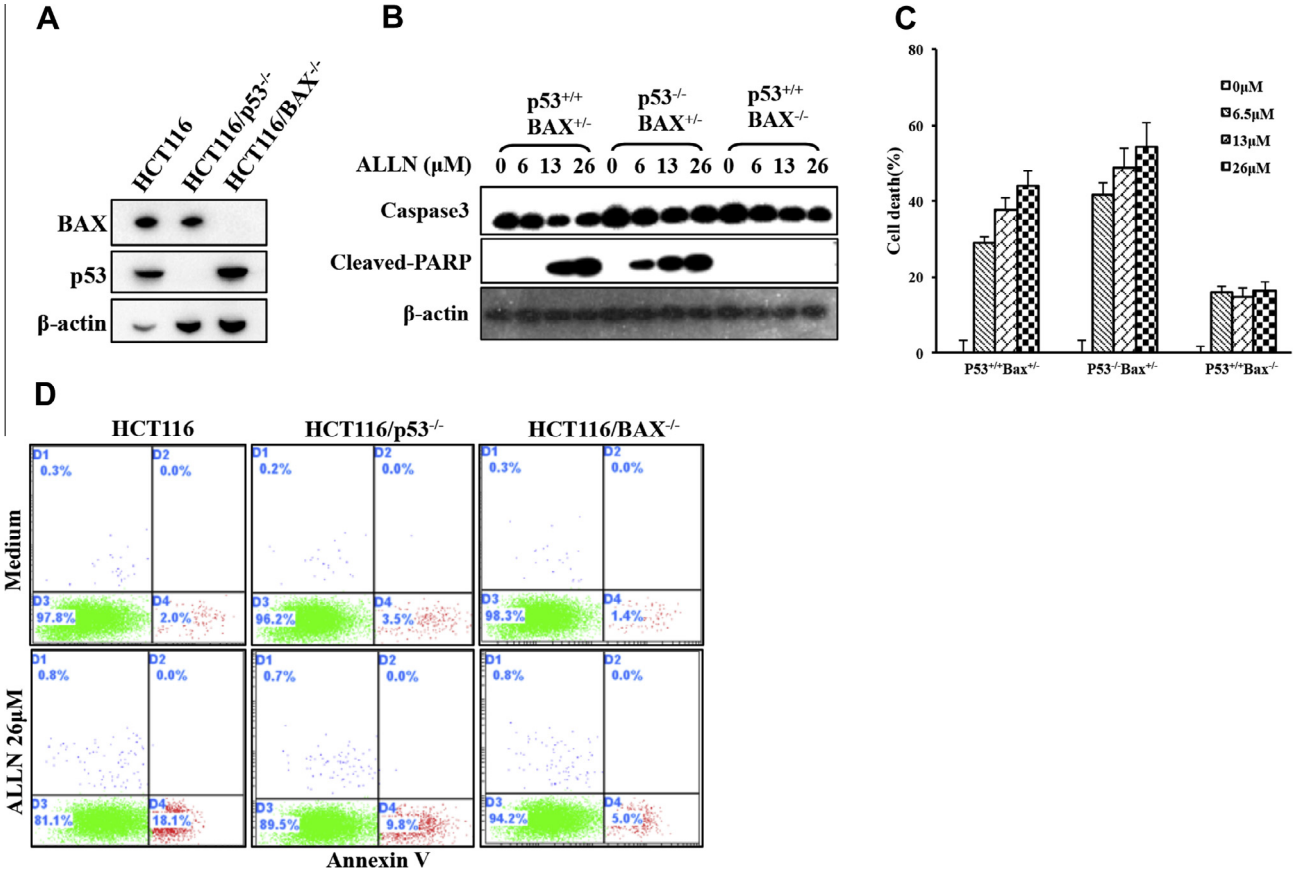

Fig. 3. ALLN-induced apoptosis by a Bax-dependent mechanism. (A) HCT116 (wild type), HCT116-*p53*$^{-/-}$ and HCT116-*Bax*$^{-/-}$ cells protein levels were confirmed by western blot with indicated antibodies. (B) Protein levels of apoptosis markers in HCT116, HCT116-*p53*$^{-/-}$ and HCT116-*Bax*$^{-/-}$ cells were detected by western blot. (C and D) HCT116, HCT116-*p53*$^{-/-}$ and HCT116-*Bax*$^{-/-}$ cells were treated with 0–26 μM ALLN; cell death and apoptosis were assayed by CCK-8 24 h later (C); Annexin-V PI staining flow cytometry assay (D).

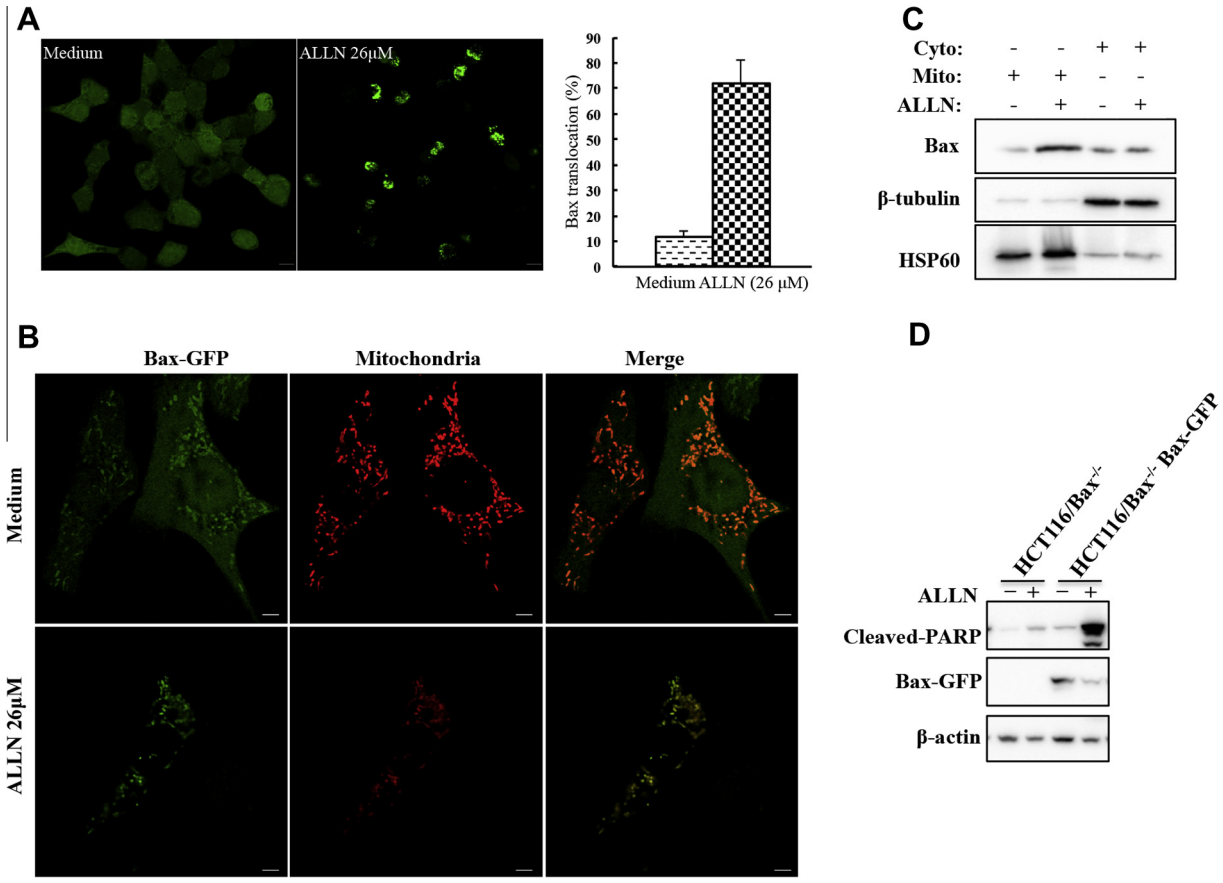

Fig. 4. ALLN-induced Bax translocation from cytoplasm to mitochondria. (A) Bax–EGFP was stably overexpressed in *Bax*$^{-/-}$ HCT116 cell. These Bax–EGFP cells were treated with 26 μM ALLN; 24 h later Bax-EGFP location was observed under fluorescence microscope and compared to untreated Bax–EGFP control cells. (B) A vector that expressed RFP mitochondria marker protein transfected into Bax–EGFP cells, which were then fixed and observed by fluorescence microscope. (C) HCT116 cells growth in 10-cm dishes were treated with 13 μM ALLN; 24 h later, mitochondria and cytosolic were isolated. Bax was detected by western blot. HSP60 and β-tubulin was used as loading controls. (D) ALLN induced apoptosis is repressed in *Bax*$^{-/-}$ HCT116 cells, but rescued by reintroduction of the *Bax* gene.

ment, tumor volume was almost the same between the two groups of mice. These *in vitro* and *in vivo* experiments show ALLN can inhibit growth of colon cancer HCT116 cells.

3.2. ALLN induced apoptosis in HCT116 and other colon cancer cell lines

To understand the molecular mechanism of ALLN's anti-cancer activity, we studied ALLN-induced apoptotic activity in colon cancer cell lines. HCT116 cells were exposed to 0–26 μM ALLN for 24 h. Pro-caspase 3 and cleaved PARP-two apoptosis markers-were then detected by western blot, which showed decreased pro-caspase 3 and increased cleaved PARP as ALLN doses increased (Fig. 2A). Other colon cancer cell lines and the hepatocarcinoma line HepG2 also underwent apoptosis after being treated with 26 μM ALLN for 24 h (Fig. 2B). Incubation by HCT116 cells with 26 μM ALLN for 24 h resulted in >18% of the HCT116 cells undergoing apoptosis, which was greatly prevented by adding the pan-caspase inhibitor Z-VAD-fmk (Fig. 2C).

3.3. ALLN induced Bax-dependent apoptosis in HCT116 cells

Bax is a pro-apoptosis Bcl-2 family member. Its translocation from cytosol to mitochondria can result in increased activated caspase 9, which induces cell apoptosis [14]. P53, as a tumor suppressor protein, participates in maintenance of genomic stability and cell apoptosis [15]. Our result showed that colon cancer cells and hepatocarcinoma HepG2 cells undergo apoptosis when treated with 26 μM ALLN for 24 h. To see whether p53 or Bax participate in ALLN-induced apoptosis, we analyzed apoptotic effects after ALLN treatment in HCT116 cells, HCT116/*p53*$^{-/-}$ cells and HCT116/*Bax*$^{-/-}$ cells. Deficient Bax and p53 in HCT116 cell lines were verified with Bax- and p53-specific antibodies (Fig. 3A). HCT116 cells were treated with 26 μM ALLN for 24 h; significant cell death was detected in the HCT116 cells and HCT116/*p53*$^{-/-}$ cells, but was inhibited in HCT116/*Bax*$^{-/-}$ cells (Fig. 3B). Decreased pro-caspase-3 and cleaved PARP were seen in ALLN-treated HCT116 cells and HCT116/*p53*$^{-/-}$ cells, but pro-caspase-3 remained almost steady and cleaved PARP was greatly prevented in HCT116/*Bax*$^{-/-}$ cells (Fig. 3C). Annexin V and PI staining further confirmed these results (Fig. 3D). Bax translocation from cytosol to mitochondria plays an important role in apoptotic initiation. The *Bax-EGFP* stable cell line, which was constructed by adding G418 drugs to medium, allowed us to follow the location of Bax. Control cells showed diffuse average distribution of green fluorescence using the fluorescence microscope, whereas almost 80% of the ALLN-treated cells showed points of fluorescence (Fig. 4A). To see whether these Bax-EGFP points co-localized to the mitochondria, a red fluorescence mitochondria marker protein was transfected into *Bax-EGFP* calls. We found that the red fluorescence points coincide with EGFP points (Fig. 4B). Western blot showed Bax protein levels in the mitochondria to be higher than those in the cytosol (Fig. 4C). At the same time, inhibition of ALLN-induced PARP protein cleavage in HCT116/*Bax*$^{-/-}$ cells was reversed by reintroducing the *Bax* gene into HCT116/*Bax*$^{-/-}$ cells (Fig. 4D). These data demonstrate that Bax is required for ALLN-induced apoptosis.

4. Discussion

We investigate the anti-cancer effect of ALLN on human colon cancer HCT116 cell growth *in vitro,* and found its molecular basis to be associated with apoptosis by Bax-dependent means; ALLN's significant effect on colon cancer tumorigenicity in nude mice may also be due to apoptosis. Additionally, we found that ALLN-induced apoptosis could be controlled by a caspase inhibitor. Use of HCT116/*Bax*$^{-/-}$ and HCT116/*p53*$^{-/-}$ cell lines demonstrated that apoptosis induced by ALLN is greatly Bax-dependent and slightly p53-dependent. Co-localization of Bax in the mitochondria at the presence of ALLN shows that Bax is required for ALLN-induced apoptosis; reintroduction of Bax into *Bax*$^{-/-}$ cells rescued the ALLN-induced apoptosis response.

Apoptosis is a normal process during cell development and aging. Inappropriate apoptosis is a factor in many diseases, including colon cancer. Induction of apoptosis by anti-cancer agents may be mediated by multiple mechanisms, for instance, a platinum anticancer drug cisplatin could interact with nucleic acid and induce replication arrest, transcription inhibition, cell-cycle arrest, DNA repair and apoptosis [16]. Apoptosis in initiated through two alternative pathways: extrinsic and intrinsic. Mitochondria play a significant role in the intrinsic way and regulate drug-induced apoptosis. The intrinsic pathway is regulated by members of Bcl-2 family, which includes the anti-apoptotic Bcl-2 and Bcl-XL proteins and the pro-apoptotic Bax and Bid proteins [17]. Bax ordinarily locates in the cytoplasm, but under stress, translocates to the mitochondria, resulting in apoptosis. To identify the role of the Bax in ALLN-induced apoptosis, we investigated apoptosis induction in wild-type HCT116 and *Bax*$^{-/-}$ cells with or without ALLN treatment. Increased cleaved-PARP was observed in HCT116 cells, but there was no change in *Bax*$^{-/-}$ cells. Our results suggest that ALLN-induced apoptosis depended on Bax. Bax translocation from cytosol to mitochondria was also detected in the *Bax-EGFP* stable cell line treated with ALLN. Results obtained from our experiments suggested that ALLN-induced apoptosis was Bax-dependent.

Calpains are pivotal proteins in various pathways, and are important regulators of carcinogenesis [18,19]. As ALLN-a major inhibitor of calpain 1-has been shown to stabilize p53 protein levels, found that calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines, using a recombinant adenovirus that expresses p53 in *p53*-mutation tumor cell lines [20]. ALLN reportedly induces apoptosis in human promyelocytic leukemia HL-60 cells [21], but counterintuitively, it blocks dexamethasone-induced apoptosis in thymocytes and cycloheximide-induced apoptosis in metamyelocytes [22]. Our results, which show ALLN-induced apoptosis greatly depends on Bax rather than p53, vary from previous Atencio studies in using *p53*-null and *Bax*-null HCT116 cells [23].

In summary, our findings indicate that ALLN decreased HCT116 cell tumorigenicity, *in vitro* and *in vivo*, by activating the HCT116 cells' apoptotic response, through a Bax-dependent and p53-independent pathway. This suggests that ALLN could be the basis of a therapeutic approach to tumors that lack *Bax* mutations.

Acknowledgments

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