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Original research paper

Ampelopsin induces MDA-MB-231 cell cycle arrest through cyclin B1-mediated PI3K/AKT/mTOR pathway *in vitro* and *in vivo*

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ABSTRACT

Breast cancer is one of the most common malignant tumors in women and it is the most frequently diagnosed cancer in the world. Ampelopsin (AMP) is a purified component from the root of *Ampelopsis grossedentata*. It is reported that AMP could significantly inhibit the proliferation of breast cancer cells. However, the antitumor mechanism against breast cancer has not yet been fully elucidated. The purpose of this work was to study the role of AMP against breast cancer MDA-MB-231 cells and to further investigate the underlying mechanism. PI3K/AKT/mTOR plays a very important role in tumor cell growth and proliferation and we hypothesize that AMP may inhibit this pathway. In the present work, the results showed that AMP could significantly inhibit the growth of breast cancer MDA-MB-231 cells *in vitro* and *in vivo*. In addition, treatment with AMP decreased the levels of PI3K, AKT and mTOR, as well as cyclin B1 expression, followed by p53/p21 pathway activation to arrest the cell cycle at G2/M. Moreover, it demonstrated a positive association between cyclin B1 and PI3K/AKT/mTOR levels. Importantly, this pathway was found to be regulated by cyclin B1 in MDA-MB-231 cells treated with AMP. Also, it was observed that cyclin B1 overexpression attenuated cell apoptosis and weakened the inhibitory effects of AMP on cell proliferation. Together, AMP could inhibit breast cancer MDA-MB-231 cell proliferation *in vitro* and *in vivo*, due to cell cycle arrest at G2/M by inactivating PI3K/AKT/mTOR pathway regulated by cyclin B1.

Keywords: PI3K/AKT/mTOR pathway, cyclin B1, breast cancer, cell cycle arrest, ampelopsin

Cancer occurs due to the uncontrolled growth and spread of abnormal cells, which is mainly related to hormones, immune conditions and oncogene disorders (1). Breast cancer is one of the most common malignant tumors in women in the world. Nowadays, this kind

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of cancer has surpassed lung cancer as the most frequently diagnosed cancer worldwide, with an estimated 2.3 million new cases, and causes large numbers of deaths among women every year (2). Recently, a variety of drugs have been developed to treat breast cancer, but due to the emergence of drug resistance, most of the chemotherapy drugs for breast cancer have no obvious therapeutic effect (3). Therefore, novel chemotherapies that overcome drug resistance and improve patient outcomes are urgently needed. Also, finding more effective alternative drugs is of great value in reducing the mortality rate associated with breast cancer.

Plant-derived natural products occupy a very important position in the area of cancer chemotherapy. The use of natural plant-derived compounds as a potential cancer preventive and/or therapeutic agents has become a fascinating strategy (4). The identification and research of active ingredients in plant-derived natural products play an important role in clinical evaluation and application. At present, many ingredients purified from herbs have been widely used in the treatment of breast cancer and other cancers (5). For example, paclitaxel is a natural chemotherapeutic drug derived from the bark of the Pacific yew, which has been widely used in the treatment of breast cancer (6). Therefore, exploring new therapeutic drugs from natural sources has potential value for the treatment of breast cancer. Ampelopsin (AMP) (Fig. 1) is derived from the tender stem and leaves of the plant species, which is one of the most common flavonoids (7). It is a major bioactive constituent of *Ampelopsis grossedentata*, which has been reported to exert antitumor effects including breast cancer (8–9). Furthermore, it was reported that AMP is a useful chemotherapeutic agent in the treatment of human breast cancer (MCF-7) cell line (10). However, the antitumor mechanism remains to be further studied.

Cell cycle arrest plays a very important role in the treatment of targeted cancer cells. Studies have shown that many cell cycle proteins are overexpressed or overactive in human cancers (11). Cyclin B1 is a cell cycle protein, which is involved in checkpoint control, promotion of the G2/M phase transition and acceleration of the cell cycle (12), and it is particularly critical for the maintenance of the mitotic state (13). In the process of normal cells turning into the cancerous state, cyclin B1 is overexpressed causing uncontrolled abnormal cell growth (14). It was reported that cyclin B1 knockdown prevents mitotic entry (15), also indicating that cyclin B1 is an essential regulator of mitosis. Previous studies have demonstrated that cyclin B1 is increased in breast cancer (16). Furthermore, uncontrolled expression of cyclin B1 is significantly associated with malignant transformation and abnormal proliferation of tumor cells (17). So, the elevated level of cyclin B1 indicates more aggressive cancer and a poor prognosis.

The PI3K/AKT/mTOR pathway is demonstrated to function in a serial biological activity by transmitting signal transduction events in response to extracellular stimuli (18). By docking with AKT to the cellular membrane, activated PI3K directly activates downstream

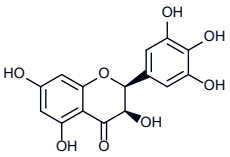

Fig. 1. The chemical structure of AMP ($C_{15}H_{12}O_8$; M_r 320.25).

AKT, which subsequently triggers signalling pathways (19–20). As one of the complicated signalling pathways in cells, PI3K/AKT/mTOR is regulated by the stimulation of nutrients, hormones and growth factors, and it has also been confirmed that this pathway plays a very important role in tumor cell growth and proliferation (21). The activation of the PI3K/AKT/mTOR pathway leads to severe interference with cell growth and survival control, and ultimately results in competitive growth advantage, metastatic ability, angiogenesis, and treatment resistance (22). For cell cycle progression and cell growth, several targets of the PI3K/AKT/mTOR pathway are involved in cell cycle regulation (23). Also, more and more data show that PI3K/AKT/mTOR inhibitors have strong inhibitory activity in the treatment of breast cancer (24).

p53 protein can induce both cell cycle arrest and cell death and the regulation of cell fate decision has been the focus of numerous studies. Cell cycle arrest driven by p53 requires the transcription of p21, which is a cyclin-dependent kinase inhibitor (25). The p53/p21 pathway is involved in the cell apoptosis progression and plays an important role in the cell cycle arrest of the G2/M phase (26).

Thus, we hypothesize that the potential for treatment with AMP against breast cancer may be accomplished by induced cell growth inhibition and cell cycle arrest through PI3K/AKT/mTOR signal pathway, which may be associated with cyclin B1. In this study, we aimed to investigate whether AMP has antitumor effects on breast cancer and whether decreased cyclin B1 levels and PI3K/AKT/mTOR pathway, as well as p53/p21 pathway are required for AMP-induced cell growth inhibition.

EXPERIMENTAL

Reagents, cell lines and antibodies

AMP (purity ≥ 98 %), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT, purity ≥ 98 %) and all other chemicals (analytical grade) in this study were purchased from Sigma-Aldrich (USA). AMP in a properly sealed glass vial can be stored in the refrigerator (4 °C), and dissolved in DMSO, which concentrations in the cell culture experiments did not exceed 0.1 %. Then the solution could be stored at –20 °C. The human MDA-MB-231 breast cancer cell line was purchased from China Center for Type Culture Collection (CCTCC) (China). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10 % (*V*/*V*) fetal bovine serum (FBS) (HyClone, USA) and 1 % penicillin-streptomycin solution (Biological Industries, Israel). The cells were cultured in a humidified incubator in an atmosphere with 5 % CO_2 at 37 °C. Anti-cyclin B1, anti-p53, anti-p21, anti-PI3K, anti-AKT, anti-mTOR, anti-β-actin antibodies, and IgG against rabbit with HRP-linked secondary antibodies were from Boster (China).

MTT assay

The cells were seeded into 96-well cell culture plates at a density of 1.5×10^3 cells/well in a fresh medium and incubated overnight. After that, they were treated with different concentrations of AMP (0, 10, 25 and 50 μmol L^{-1}). Then the cells were maintained for 0, 6, 12, 24 and 48 hours. After that, the supernatant was removed followed by loading with 100 μL MTT solution (1 mg mL^{-1}) for each well. The cells were incubated for 4 hours and then added 100 μL DMSO. Cell proliferation was determined by shaking for 10 minutes in the

dark to solubilize the formazan completely. Absorbance at 490 nm was measured using an automatic microplate reader (MQX200, Bio-Rad, USA). The data were obtained from three independent experiments of six replicates. The survival rate is calculated as: the survival rate = (The absorbance of treated groups/The absorbance of the control group) × 100 %.

Clonogenic assay of cells in vitro

MDA-MB-231 cells were incubated in triplicate in a 6-well culture plate containing 2 mL of fresh complete medium with 1,000 cells per well. After 24 hours, cultures were replaced with a fresh medium with 10 % FBS or the same medium containing AMP (10, 25, 50 μmol L^{-1}), and then the cells were cultured in a humidified atmosphere of 5 % CO_2 and 95 % air at a temperature of 37 °C. After further incubation for 14 days, the cells were washed with cold PBS three times followed by fixing for an hour with a solution containing 0.5 % (*m*/*V*) crystal violet in methanol. Then the plate was rinsed with tap water 3 times to remove excess dye. ImageJ software could be used to count colonies only when a single clone contains more than 100 cells. Clonogenic assays were repeated at least three times as independent experiments, each with internal duplicate cultures.

Tumor induction in nude mice

40 female BALB/c nude mice aged 4–5 weeks were from Shanghai Laboratory Animal Center. They were fed in an SPF environment that had regular ultraviolet radiation. MDA-MB-231 cells (0.5×10^6) were inoculated subcutaneously into the right flank of each nude mouse. After implantation for a week, tumors had grown to a diameter between 0.4 and 0.6 cm with a model success rate of 100 %. They were divided randomly into four groups with 10 mice in each group: the untreated group, AMP treatment groups including 20, 50 and 100 mg kg^{-1} b.w. The mice in the control group were fed the vehicle alone (normal saline). All mice were intragastrically (*i.g.*) orally treated twice per week for 4 weeks. During the experiment, the tumor volume was monitored every week and calculated using the following formula: tumor volume = $(a^2 \times b)/2$, in which a is the shortest diameter and b is the longest diameter of the tumor. All measurements were performed in a coded, blinded fashion. In addition, peripheral blood (0.1 mL) from these mice was obtained by tail incision every two days and kept at –80 °C for further ELISA analysis. The levels of cyclin B1, PI3K, AKT and mTOR in peripheral blood were quantitated using ELISA analysis following the manufacturer's instructions. All commercially available ELISA kits were purchased from Boster.

At the end of the study, the mice were euthanized and tumor weight was also measured. All animal procedures were approved by the Committee on Animal Experimentation of the First Affiliated Hospital of Xiamen University (Reference Number: XMU-AEA-20200636) and the procedures complied with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985).

Analysis of cell cycle and cell apoptosis

MDA-MB-231 cells for cell cycle analysis were cultured in DMEM complete medium and grown in a humidified atmosphere of 37 °C and 5 % CO_2. After treating the cells with AMP (0, 10, 25, 50 μmol L^{-1}) for 24 hours, the cells were washed with PBS three times, and then fixed completely using 70 % ethanol in PBS at 4 °C overnight. Following centrifuga-

tion with 200×g force for 5 minutes, the cells were washed three times with PBS for 5 minutes each time and then incubated with 100 mg mL^{-1} Rnase A and 40 μg mL^{-1} propidium iodide (PI) at room temperature in the darkness for half an hour. The cell cycle distribution was analyzed using a flow cytometer (Canto II, BD) and the analysis of the data was performed by ModFit LT 3.0 program. All cell cycle analysis data were shown as mean ± standard deviation (SD) of three independent experiments.

For cell apoptosis, MDA-MB-231 cells under indicated conditions were grown in Petri dishes (6-cm diameter) to 80 % confluence, and then treated with AMP (0 and 50 μmol L^{-1}) for 48 hours. Following that the cells were collected by trypsinization, and PBS was added to quickly wash the cells. The cells were then resuspended in 200 μL of binding buffer. Then, 1 mg mL^{-1} Annexin V-FITC (10 μL) and 2.5 mg mL^{-1} PI (10 μL) were added to the cell sample and further incubated in the dark at room temperature for 15 minutes. Finally, samples were then analyzed by flow cytometer (Canto II, BD) and the percentage of apoptotic cells was assessed based on the percentage of positive cells for Annexin V using a flow cytometer (Canto II, BD) and the analysis of the data was performed by ModFit LT 3.0 program. Experiments were performed at least three times.

The analysis of Western blot

Total protein from cells under different conditions was extracted using 50 μg mL^{-1} phenylmethylsulfonyl fluoride (PMSF) and RIPA lysis buffer. Cells were collected and the protein concentration was measured by bicinchoninic acid (BCA) protein assay kit (Beyotime, China). An equal amount of protein was separated by 10 % sodium dodecyl sulfate--polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). Then the membranes were blocked in a blocking buffer containing 5 % non-fat milk followed by overnight incubation with the primary antibodies in Tris-buffered saline at 4 °C. After that, the blot was incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for an hour at room temperature. Lastly, the bands were detected and the signal intensity was determined by ImageJ software. All Western blot assays were successfully repeated at least three times.

Cell transfection and plasmid construction

Cyclin B1 overexpression plasmid (pcDNA-cyclin B1) and an empty vector (pcDNA), considered as a negative control, were both designed and purchased from Shanghai GenePharma Ltd. Company (China). When cells reached 80–90 % confluence, Lipofectamine 2000 specification (Invitrogen, Thermo Fisher Scientific, Inc.) was used for cell transfection according to the manufacturer's protocols (The transfection mixture was added to cultured cells, which were in DMEM without FBS, and incubated at 37 °C). Cells were harvested 48 hours later, cells were collected and then Western blot analysis was performed to confirm the level of cyclin B1 overexpression. After that, transfected cells were used to further utilization of Western blot, flow cytometry, and MTT test. All procedures were performed on the basis of the above protocols.

Statistical analysis

Experimental data are shown as mean ± SD, and each experiment *in vitro* was performed in triplicate. SPSS 25.0 software (Chicago, IL) was used as the statistical software.

Multiple comparisons were analyzed using a one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. Pearson's correlation analysis was used to examine the relationship between cyclin B1 and PI3K, AKT, mTOR in peripheral blood, respectively. Statistical significance was set at $p < 0.05$. Correlation coefficients (R) were also made between certain parameters.

RESULTS AND DISCUSSION

AMP inhibits MDA-MB-231 cell viability in vitro

Tumor is a disease state characterized by a disorder of cell proliferation and uncontrolled cell growth is a key feature of carcinogenesis. So, inhibition of proliferation is an effective method of tumor treatment. Some anti-cancer agents cause cell proliferation disorders by inducing cell cycle arrest (27). MTT analysis was used to measure cell viability. Following treatment with different concentrations (0, 10, 25 and 50 μmol L^{-1}) of AMP for 0, 6, 12, 24 and 48 hours, cell growth activity was significantly suppressed in a dose- and time-dependent manner (Fig. 2a). It showed that AMP significantly inhibited the growth of MDA-MB-231 cells, and it reached the maximum level at a concentration of 50 μmol L^{-1} for 48 hours (Fig. 2a). Also, the results also showed that the cell viability decreased by 2.91 times after the cells were treated with different concentrations of AMP for 48 hours ($p < 0.05$, Fig. 2a). Besides, it could also be found from the results that 50 μmol L^{-1} AMP strongly inhibited cell viability after stimulation on MDA-MB-231 cells for different periods of time (6–48 hours) compared with the untreated control, from 34.40 to 53.13 % ($p < 0.05$, Fig. 2a). We next tested the effect of AMP on colony forming potential of MDA-MB-231 cells on 6-well cell culture plates in either the presence or absence of AMP for 14 days. Consistent with the results of the MTT test, the colony formation assay demonstrated that AMP effectively inhibited the proliferation of MDA-MB-231 cells. AMP decreased colony formation at concentrations as low as 10 μmol L^{-1} (Fig. 2b). Quantitative analysis revealed that the number of cell colonies was dose-dependently decreased by AMP (Fig. 2c, $p < 0.05$), with a 97.6 % inhibition at dosages as high as 50 μmol L^{-1} AMP in comparison with untreated control cells (Fig. 2d). All these data indicated that AMP suppressed the proliferation and colony formation of MDA-MB-231 cell *in vitro*. Moreover, the observation was also in line with the previous report from Li and coworkers (28). However, its exact anti-breast cancer mechanism needs to be further explored.

The inhibitory effects of AMP on the tumor growth in nude mice

We next determined whether AMP would have a negative effect on the tumorigenicity of breast cancer cells *in vivo*. MDA-MB-231 cells were injected into the flanks of nude mice and then the inhibitory effects of AMP on the growth of tumors in nude mice were observed. The tumor volume was measured in nude mice weekly for four weeks. As shown in Fig. 3a, compared with the control group, the tumor volumes of the AMP-treated groups were significantly decreased at the end of 28 days, with 20, 50, 100 mg kg^{-1} b.w. AMP being 592.16 ± 50.12, 376.09 ± 43.08 and 169.12 ± 32.03 mm^3, respectively, and also it showed a dose- and time-dependent manner ($p < 0.05$). In addition, the final tumor weights of the AMP--treated groups were 463.83 ± 59.01, 289.29 ± 41.65, and 125.81 ± 30.48 mg, respectively, which were significantly dose-dependently reduced than that of the control group (Fig. 3b,

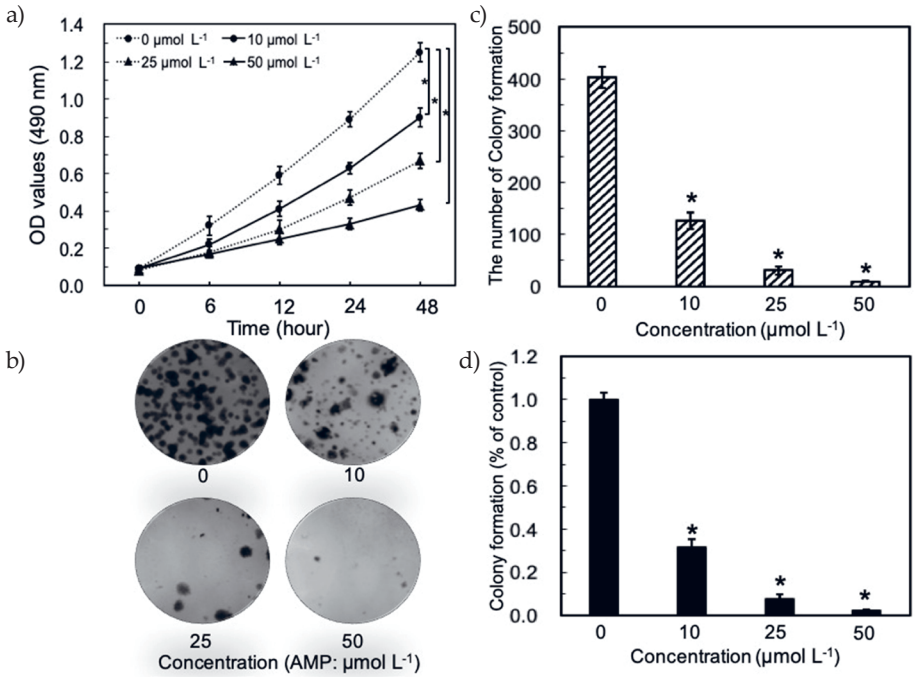

Fig. 2. The inhibitory effect of AMP on cell growth in MDA-MB-231 cells. a) Cell proliferation inhibition activity of AMP was evaluated by the MTT test. The cells were treated with different doses of AMP (0, 10, 25 and 50 μmol L^{-1}) for different times (0, 6, 12, 24 and 48 hours). Values represent the mean ± SD (3 independent experiments, * $p < 0.05$); b) AMP inhibited MDA-MB-231 cell colony formation *in vitro*. MDA-MB-231 cells were treated with different concentrations of AMP (0, 10, 25 and 50 μmol L^{-1}), and colony formation was observed after two weeks of incubation. Shown are the representative results from three independent experiments; c) the quantitative data are expressed as the mean ± SD (3 independent experiments, * $p < 0.05$); d) results show the percentage of clone formation compared with the control group, in which MDA-MB-231 cell was treated with 0.1 % DMSO (3 independent experiments, *$p < 0.05$).

$p < 0.05$). Together, these data showed that AMP potentially inhibited *in vivo* growth of human breast cancer cell line MDA-MB-231. Interestingly, it was also demonstrated that AMP could lead to growth inhibition and shrinkage of breast cancer MCF-7 tumors in nude mice (10).

AMP induces cell arrest at G2/M in the MDA-MB-231 cell line

Since uncontrolled cell growth is a key feature of carcinogenesis (29), inhibition of proliferation is an effective method of tumor treatment. The endless cell cycle progression is the main cause of proliferation and tumor transformation (30). Some anti-cancer agents cause cell proliferation disorders by inducing cell cycle arrest (31). Therefore, different concentrations of AMP (0, 10, 25 and 50 μmol L^{-1}) were next used to treat MDA-MB-231 cells and then the cell cycle distribution was observed to further study the anti-prolifera-

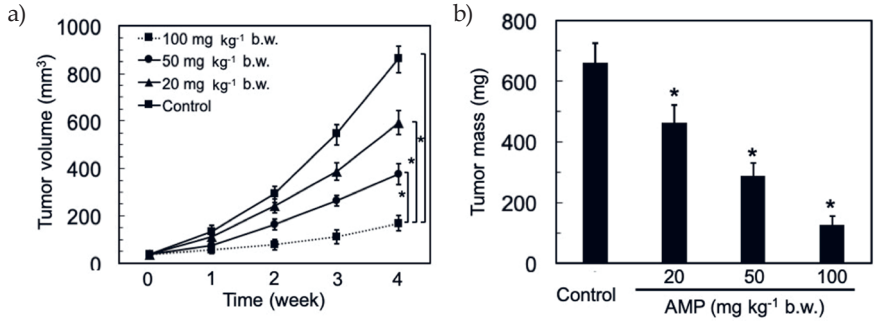

Fig. 3. AMP inhibits tumor growth *in vivo*. Either vehicle control or AMP (20, 50 and 100 mg kg^{-1} b.w., intraperitoneally) were administered twice per week to female BALB/c nude mice bearing MDA-MB-231 breast cancer xenografts for 4 consecutive weeks. Tumor growth curves were recorded every week. a) tumor volume (mm^3) was calculated every week according to the formula: ($a^2 \times b$)/2, in which *a* and *b* are the shortest and longest diameters of the tumor, respectively; b) at the end of the experiment, the mice were euthanized and the tumor weights were measured. All data are expressed as the mean ± SD (n = 10, *p < 0.05).

tive activity of AMP. The results of DNA content analysis showed that AMP caused a dose-dependent increase in G2/M phase cells, while the ratio of cells in the G0/G1 phase and S phase reduced with increasing concentration (Figs. 4a,b). When the cells were stimulated by 50 μmol L^{-1} AMP, the percentage of MDA-MB-231 in the G0/G1 phase and S phase was decreased to 2.11 and 16.01 %, respectively (Fig. 4b). As shown in Fig. 4c, in response to different concentrations of AMP, the percentage of cells in G2/M phase was 31.84 ± 3.10, 56.54 ± 4.09 and 81.87 ± 5.12 % in 10, 25 and 50 μmol L^{-1} AMP groups, which was increased compared to control group, respectively (p < 0.05). The results showed that AMP induced the MDA-MB-231 cell cycle arrest at G2/M. Therefore, these data demonstrated that the inhibition of breast cancer cell viability by AMP stimulation may be associated with a dose-dependent shift of cell distribution into the G2/M phase, which means that it causes cell cycle arrest at G2/M and then inhibits cell proliferation.

Cyclin B1 is related to PI3K/AKT/mTOR pathway in AMP-induced inhibition of cell growth

The PI3K/AKT/mTOR signalling pathway is an important regulator of cell growth, proliferation and cell cycle progression (32–33). In the subsequent paper of this work, we will further investigate whether the activity of PI3K was affected in MDA-MB-231 cells treated with AMP. Interestingly, it was observed that the level of PI3K was greatly and dose-dependently decreased (Fig. 5a, p < 0.05). PI3K promotes the activation of AKT, and increased AKT is thought to be important in fully activating AKT (34–35). So AKT activity was also further tested. Similar to PI3K in the present study, the levels of AKT were strikingly lower in AMP-treated groups than that of the control (Fig. 5a, left), and the difference was also statistically significant (Fig. 5a, right, p < 0.05). AKT has been suggested to be an activator of mTOR, which regulates signaling networks to control cell growth in response to cell stress (36). To determine the effect of PI3K/AKT inaction induced by AMP on mTOR, the expression of mTOR was examined. Our results demonstrated that the level of mTOR

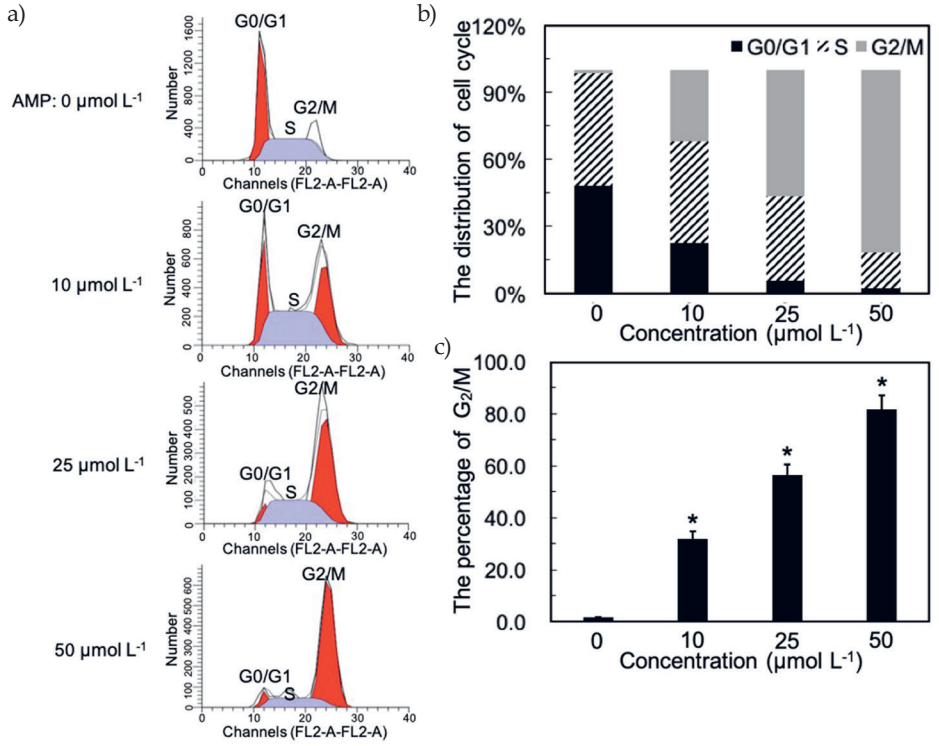

Fig. 4. Cell cycle arrest at G2/M is observed following treatment with different concentrations of AMP (10, 25 and 50 μmol L^{-1}). a) Flow cytometry was used to determine the DNA content of MDA-MB-231 cells treated with AMP for 24 hours at 37 °C. The experiment was repeated three times, and representative results are presented; b) cell cycle distribution in MDA-MB-231 cells, showing the percentage of cells in the G0/G1, S and G2/M phases of the cell cycle; c) the percentage of cells in the G2/M phase. Values represent the mean ± SD (3 independent experiments, * $p < 0.05$).

was reduced in a dose-dependent manner after MDA-MB-231 cells in response to AMP (Fig. 5a, $p < 0.05$), showing the same trend of change as the PI3K/AKT pathway. Surprisingly and interestingly, the above results were in line with the data *in vivo*, suggesting that the levels of PI3K, AKT and mTOR were significantly decreased after AMP treatment compared with the control (Fig. 5b, $p < 0.05$). The p53/p21 pathway is involved in the cell apoptosis progression and plays an important role in the cell cycle arrest of the G2/M phase in response to stress (37). The results also suggested that AMP was able to activate the p53/p21 pathway in a dose-dependent manner (Fig. 5a, $p < 0.05$). Collectively, the data stated above implied that the inactivation of the PI3K/AKT/mTOR pathway was involved in AMP-induced inhibition of cell growth, which was at least partly due to p53/p21 pathway activation. However, how the PI3K/AKT/mTOR pathway is regulated in the antitumor action of AMP against breast cancer is still an urgent problem to be solved (10).

Cell proliferation is restrained through the control of the cell cycle and cyclin B1 is the key component of the cell cycle machinery (38–39). In the current study, it was observed

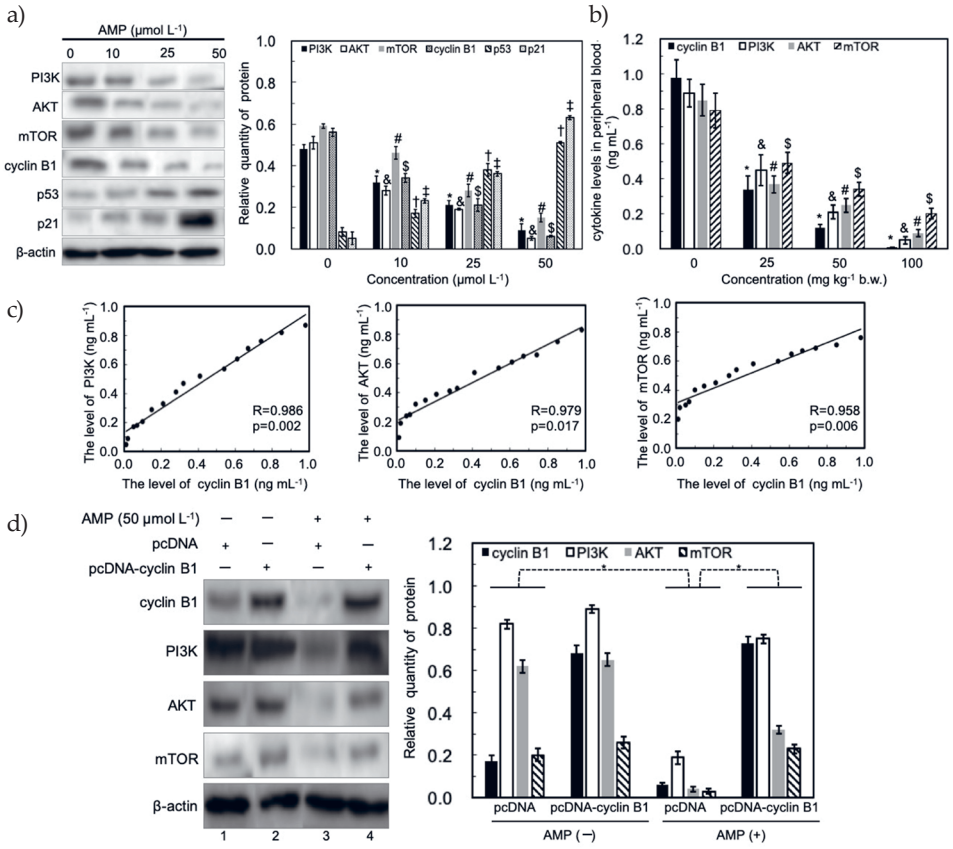

Fig. 5. Cyclin B1 is in a positive correlation with the level of PI3K/AKT/mTOR pathway in response to AMP treatment *in vitro* and *in vivo*. a) MDA-MB-231 cells were cultured in the presence of AMP (0, 10, 25 and 50 μmol L^{-1}) for 48 hours, and then the whole-cell lysate was extracted by 50 μg mL^{-1} PMSF and RIPA lysis buffer. Protein expressions were analyzed by Western blot and β-actin was used as a loading control. The quantitative results (normalized to **β**-actin levels) are shown as the mean ± SD (3 independent experiments, *, &, #, $, †, ‡ $p < 0.05$ compared with vehicle-treated controls using one-way ANOVA followed by a post hoc Tukey's test); b) the cytokine levels including cyclin B1, PI3K, AKT and mTOR in peripheral blood were analyzed at the end of the study by ELISA analysis, described in the Materials and Methods section ($n = 10$, *, &, #, $, $p < 0.05$ compared with vehicle-treated controls by one-way ANOVA followed by a post hoc Tukey's test); c) levels of cyclin B1, PI3K, AKT, and mTOR *in vivo*. Pearson's correlation analysis was performed to analyze the correlation between the level of cyclin B1 and PI3K, AKT, and mTOR from ELISA analysis in the serum of mice, respectively; d) cyclin B1 overexpression leads to activation of PI3K/AKT/mTOR pathway in AMP-treated MDA-MB-231 cells. pcDNA-cyclin B1-transfected and pcDNA-transfected MDA-MB-231 cells were treated with 50 **μ**mol L^{-1} AMP or 0.1 % DMSO for 48 hours, respectively. After treatment, cell lysates were prepared, resolved by SDS-PAGE and subjected to immunoblotting analysis of cyclin B1, PI3K, AKT, mTOR and β-actin. The results were normalized to the level of expression of β-actin and the results of three independent experiments were shown (mean ± SD, 3 independent experiments, * $p < 0.05$).

that compared with control, the level of cyclin B1 *in vitro* and *in vivo* was significantly decreased by the treatment of AMP when the concentration was as low as 10 or 25 mg kg^{-1} b.w. (Figs. 5a,b, $p < 0.05$). cyclin B1-CDK1 complex plays an important role in the G2/M cell cycle (40). In addition, activation of the PI3K/AKT pathway appears to be required for CDK1 expression levels during the G2/M transition of the cell cycle (41). Therefore, we were considering whether cyclin B1 was related to the PI3K/AKT/mTOR pathway in the process of cell arrest at G2/M induced by AMP treatment. The association between variables in peripheral blood of 100 mg kg^{-1} b.w. AMP-treated groups were determined using Pearson's correlation analysis and the data presented in Fig. 5c. Strikingly, cyclin B1 showed strong positive association with PI3K ($R = 0.986$, $p = 0.002$), AKT ($R = 0.979$, $p = 0.017$) and mTOR ($R = 0.958$, $p = 0.006$). Taken together, these results showed that cyclin B1 is in positive correlation with the regulation of the PI3K/AKT/mTOR pathway in response to AMP treatment.

PI3K/AKT/mTOR pathway is regulated by cyclin B1 in the cell arrest induced by AMP

Cyclin B1 is a central regulator of the transition from the G2 phase of the cell cycle to mitosis (42). Also, the role of cyclin B1-CDK1 complex in the G2/M cell cycle and the requirement of PI3K/AKT pathway for CDK1 expression during the G2/M of the cell cycle (43). On the basis of the above data, we were trying to directly identify whether cyclin B1 was the regulator of suppression of the PI3K/AKT/mTOR pathway induced by AMP. pcDNA-cyclin B1-transfected and pcDNA-transfected MDA-MB-231 cells were treated with 50 μmol L^{-1} AMP or 0.1 % DMSO. Then Western blot analysis was further employed 48 hours post-treatment using the whole-cell lysate. Notably, cyclin B1 was detected in pcDNA-cyclin B1-transfected cells, which were treated with 50 μmol L^{-1} AMP or not (lines 2 and 4). In addition, following pcDNA-transfected MDA-MB-231 cells were treated with 50 μmol L^{-1} AMP, the levels of PI3K, AKT and mTOR were decreased with cyclin B1 reduction, which was also supported by the results above in this study (line 3) (Fig. 5d). More importantly and interestingly, it was observed that the expression of PI3K, AKT and mTOR was increased in pcDNA-cyclin B1-transfected cells treated with 50 μmol L^{-1} AMP than that of in pcDNA-transfected cells as detected by immunoblot (lines 3 and 4, Fig. 5d, left). Also, the above differences were statistically significant ($p < 0.05$, Fig. 5d, right). Therefore, it was confirmed that suppression of the PI3K/AKT/mTOR pathway was regulated by inhibition of cyclin B1 activity induced by AMP in the MDA-MB-231 cell line.

Cyclin B1 overexpression inhibits cell apoptosis of MDA-MB-231 in response to AMP treatment

To further identify whether cyclin B1 was involved in the cytotoxic effects of AMP, pcDNA-transfected and pcDNA-cyclin B1-transfected MDA-MB-231 cells were seeded on 6-well plates and incubated for 48 hours. After that, either 50 μmol L^{-1} AMP or 0.1 % DMSO was used to stimulate cells, which were cultured for another two days. Firstly, flow cytometry was employed to determine whether cyclin B1 played an important role in MDA-MB-231 cell apoptosis induced by treatment of AMP. The resulting data demonstrated that cell apoptosis was greatly inhibited in the pcDNA-cyclin B1-transfected group (percentage of cell apoptosis: 40.0 ± 5.2 % than that of the pcDNA-transfected group (percentage of cell apoptosis: 93.8 ± 6.6 % when cells were treated with 50 μmol L^{-1} AMP (Fig. 6a), and the difference

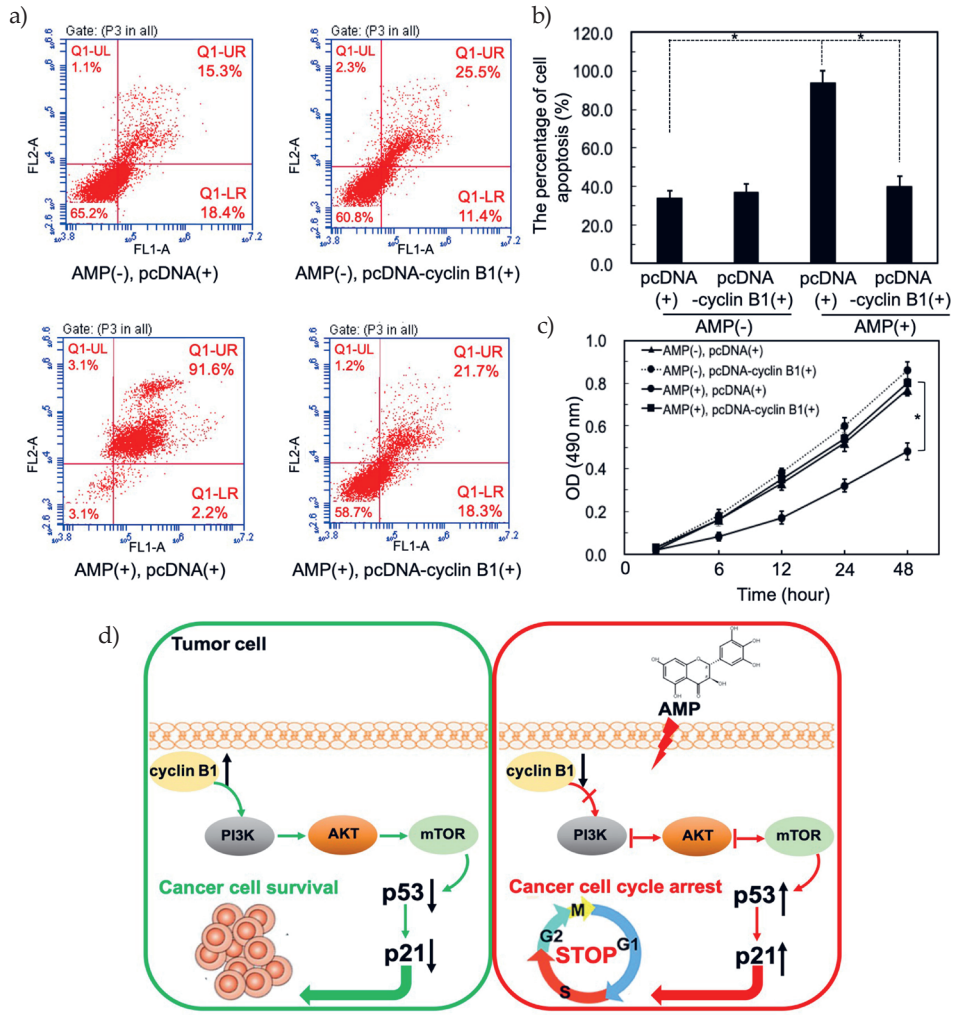

Fig. 6. Overexpression of cyclin B1 inhibits cell apoptosis and enhances cell proliferation in AMP-treated MDA-MB-231 cells. a) MDA-MB-231 cells were transfected with cyclin B1 targeted overexpression vector (pcDNA-cyclin B1) and blank vector plasmid (pcDNA), followed by treatment with 50 μmol L^{-1} AMP or 0.1 % DMSO. After another 48 hours of incubation, cell apoptosis was investigated using flow cytometry, and the representative plots are presented; b) the number of apoptotic cells was quantitatively analyzed, represented as the mean ± SD from at least three independent experiments, and *$p < 0.05$; c) MTT assay in MDA-MB-231 cells used to detect the growth changes following overexpression of cyclin B1 in combination with 0.1 % DMSO or 50 μmol L^{-1} AMP for different times (0, 6, 12, 24, 48 hours). Quantified results of three independent experiments are shown. * $p < 0.05$ vs. corresponding control; d) schematic representation of signal pathway involved in cell cycle arrest in the presence of AMP. Briefly, AMP downregulates the level of cyclin B1 expression to inactivate PI3K/AKT/mTOR pathway, thereby resulting in p53/p21 pathway activation and finally regulating cell proliferation.

was statically significant ($p < 0.05$, Fig. 6b). The data showed that overexpressed cyclin B1 could significantly attenuate this effect of cell apoptosis induced by AMP. Consistently, the overexpression of cyclin B1 eliminated AMP-induced suppression of cell proliferation ($p < 0.05$, Fig. 6c), showing that the OD value in the pcDNA-cyclin B1-transfected group was less than that in the pcDNA-transfected group. These findings confirmed that cyclin B1 was associated with the AMP-induced inhibition of cell growth, and promotion of cell apoptosis.

CONCLUSIONS

In this study, we found that the antitumor effect of AMP in breast cancer MDA-MB-231 cells was associated with inhibition of cell growth due to cell cycle arrest at G2/M. Furthermore, mechanistic studies showed that the inhibition of the PI3K/AKT/mTOR pathway regulated by cyclin B1, as well as the action of the p53/p21 pathway, was involved in the arrest of G2/M phase transition of MDA-MB-231 cells after AMP treatment (summarized in Fig. 6d). These findings will provide greater insights into the antitumor mechanism activation of AMP, thus, possibly contributing to the development of novel antitumor agent of breast cancer in future.

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Authors contributions. – All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. M.M., Q.Y., S.C. and W.C. designed the research and wrote the manuscript; M.M., Q.Y., Z.O., Q.Y., X.W., Y.H., Y.S., S.C. and W.C. conducted the experiments and performed the data analysis; S.C. and W.C. reviewed and edited the manuscript.

Availability of data and material. – The data and material will be made available upon request.

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