

IL-12 induces autophagy in human breast cancer cells through AMPK and the PI3K/Akt pathway

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Abstract. Interleukin-12 (IL-12) serves an important role in immune responses and antitumor activity. The study of the association between autophagy and cancer cells remains controversial. The present study aimed to investigate the effect of IL-12 on autophagy in the human breast cancer cell lines MDA-MB-231 and MCF-7, and the possible molecular mechanism. Breast cancer cells were treated with different concentrations of recombinant IL-12. The expression of the autophagy-associated protein microtubule-associated protein light chain 3 (LC3) was determined using western blot analysis, fluorescein isothiocyanate-labeled LC3 was detected using fluorescence microscopy and autophagosomes were examined using transmission electron microscopy. Alterations in the phosphatidylinositol 3-kinase/Rac- α serine/threonine protein kinase (PI3K/Akt) and 5'-AMP-activated protein kinase subunit β -1 (AMPK) pathways, in addition to pathway-associated proteins, were detected using western blotting, following treatment with IL-12 and pretreatment with the PI3K/Akt activator insulin-like growth factor or the AMPK inhibitor compound C. It was observed that IL-12 was able to upregulate the expression of the autophagy-associated protein LC3 in a concentration- and time-dependent manner, and induce the formation of autophagosomes in the two cell lines, and that the above effects involved the inhibition of the PI3K/Akt signaling pathway and the activation of the AMPK signaling pathway.

Introduction

Breast cancer is the leading cause of cancer mortality in women worldwide (1). Approximately one-half of breast cancer cases

and 60% of cancer mortalities occur in economically developing countries (2). Autophagy is a self-digestion mechanism for degrading damaged organelles and misfolded proteins in lysosomal compartments (3). Under metabolic stress, autophagy maintains a balance between synthesis, degradation and the subsequent recycling of macromolecules and organelles, in order to promote survival. Autophagy has been hypothesized to serve tumor suppressive and tumor promoting functions (4,5). These paradoxical effects may be due to alterations in the role of autophagy in cancer depending on the tumor types and the stage of tumorigenesis (6,7). Previous research into breast cancer has demonstrated that autophagy activation may suppress tumorigenesis. Liang *et al* (8) reported that the growth of MCF-7 breast cancer cells was suppressed by overexpression of beclin 1, an autophagy marker gene. Qu *et al* (9) and Yue *et al* (10) observed that 40-75% of human breast cancer, ovarian carcinoma and prostate cancer cells were beclin 1-deficient. Kondo and Kondo (11) reported that beclin 1 protein acted as a tumor suppressor by inhibiting cell proliferation and tumorigenesis *in vitro* and *in vivo*. It was additionally reported that autophagy-dependent cell death was induced by numerous anti-cancer drugs, including tamoxifen, rapamycin, arsenic trioxide and histone deacetylase inhibitors. Therefore, activating the autophagy pathway may be an effective way to inhibit breast cancer tumorigenesis.

The antitumor effects of interleukin (IL)-12 have been demonstrated in previous studies (12). IL-12 suppresses tumor growth by activating a number of types of immunity-associated cells, including T cells, macrophages and dendritic cells (13,14). IL-12 has been demonstrated to inhibit the growth of tumors by reducing nutrition and oxygen provision, through suppression of the growth of blood vessels (15). Tumor-associated cytokines secreted by cancer cells are activated or inhibited by IL-12. A previous study demonstrated that IL-12 was able to induce monocytic tumor cell polarization to macrophage-like cells through the up-regulation of the expression and phosphorylation of macrophage colony-stimulating factor 1 receptor (16). However, the mechanism underlying the antitumor effects of IL-12 remains to be elucidated. Additionally, the effects on tumor cell autophagy induced by IL-12, and the underlying mechanism, have not been reported.

In the present study, the induction of autophagy by IL-12 was investigated in human breast cancer cells, in addition to the possible signaling pathways involved, with the aim of

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providing novel research into the mechanism of IL-12-mediated antitumor effects.

Materials and methods

Cell lines and cell culture. The MDA-MB-231 and MCF-7 human breast cancer cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Beit-Haemek, Israel), 100 U/ml penicillin and 100 μ g/ml streptomycin (both Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 5% CO₂. Human breast cancer cells (1 \times 10⁶) were cultured in 6-well culture dishes at 37°C. When the cells had grown to 60% confluence, they were treated with recombinant (r) IL-12 (PeproTech, Inc., Rocky Hill, NJ, USA) at a concentration of 5 ng/ml for 1, 2, 3, 6, 12 or 24 h, or for 12 h at concentrations of 0.1, 0.5, 1, 5, 10 or 20 ng/ml at 37°C. In addition, breast cancer cells were pretreated with 100 ng/ml insulin-like growth factor 1 (IGF-1; R&D Systems, Inc., Minneapolis, MN, USA) or 10 μ mol/ml BML-275 (Enzo Life Sciences, Inc., Farmingdale, NY, USA) for 2 h at 37°C prior to treatment with 5 ng/ml rIL-12. The positive group was starved using 1% FBS (Biological Industries) at 37°C for 3 h.

Western blot analysis. MDA-MB-231 and MCF-7 cells were harvested, rinsed 3 times with pre-cooled PBS and lysed in 100 μ l radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 1 μ l NaF (500 mM), 1 μ l Na₃VO₄ (100 mM) and 1 μ l protease inhibitor phenylmethylsulfonyl fluoride (100 mM) for 30 min on ice, followed by centrifuging for 30 min at 15,000 \times g and 4°C. The protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of total protein (50 μ g/lane) were electrophoresed on a 12% SDS-PAGE gel, followed by transfer to polyvinylidene fluoride (0.22/0.45 μ m) membranes using a Trans-Blot system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated overnight at 4°C with specific primary antibodies as follows: Rabbit anti-microtubule-associated protein light chain 3 (LC3) I and II (cat no. NB600-1384; 1:1,000; NOVUS Biologicals, LLC, Littleton, CO, USA); rabbit anti- β -actin (cat no. 9587; 1:2,000; ImmunoWay Biotechnology Co., Plano, TX, USA); rabbit anti-phosphatidylinositol 3-kinase (PI3K)/Rac- α serine/threonine protein kinase (Akt; cat no. 4691) and phosphorylated (p)-PI3K/p-Akt (cat no. 4060; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA); rabbit anti-5'-AMP-activated protein kinase subunit β -1 (AMPK; cat no. 2795) and p-AMPK (cat no. 2535; 1:1,000; Cell Signaling Technology, Inc.); and rabbit anti-serine/threonine protein kinase mTOR (mTOR; cat no. 2972) and p-mTOR (cat no. 5536; 1:1,000; Cell Signaling Technology, Inc.). Following washing in TBS with Tween-20, the membranes were incubated at 37°C for 2 h with horseradish peroxidase-conjugated secondary antibodies (cat no. ZB-2301; 1:1,000; ZSGB-Bio, Beijing, China). Following further washing, the immunoreactive bands were

visualized using an enhanced chemiluminescence reagent (cat no. WBKLS0500; EMD Millipore, Billerica, MA, USA). The optical density of each blot was measured, and the relative expression of target proteins was calculated and normalized to the housekeeping protein actin, using Quantity One software version 4.5.2 (Bio-Rad Laboratories, Inc.).

Immunofluorescence. MDA-MB-231 and MCF-7 breast cancer cells (2 \times 10⁵ cells) were grown on slides (8 \times 8 mm) in 24-well plates. When the cells had reached 60% confluence, they were treated with IL-12 (5 ng/ml) for 24 h, followed by washing 3 times in pre-cooled PBS. The cells were fixed in 4% paraformaldehyde for 20 min at room temperature, penetrated with 0.1% Triton X-100 (diluted in PBS) for 15 min and blocked with 10% goat serum (Sangon Biotech Co., Ltd., Shanghai, China) for 30 min at room temperature. The cells were incubated overnight at 4°C with rabbit anti-LC3 primary antibody (1:200 diluted in 10% goat serum). Following rinsing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (cat no. ZF-0314; 1:200 dilution in 10% goat serum; ZSGB-Bio) away from light at room temperature for 2 h. Following further washing, the cells were stained with 10% DAPI (diluted in PBS) for 5 min at room temperature, washed 3 times and sealed with 70% glycerol, prior to visualization using a fluorescence microscope (Nikon Corporation, Tokyo, Japan). Images were captured of DAPI and FITC in the same field of view.

Transmission electron microscopy (TEM). MDA-MB-231 and MCF-7 cells were grown on culture dishes and treated with rIL-12 (5 ng/ml) for 12 h, prior to washing 3 times in cold PBS (4°C). The cells were digested with 0.1% trypsin and collected by centrifugation at 1,500 \times g and 4°C for 10 min. The supernatant was removed. Glutaraldehyde fixative (2.5%, 1.5 ml) was added along the side of a disposable micro-centrifuge tube for treatment at 4°C for >2 h. Following rinsing, dehydration, saturation and embedding with epoxy resin at 35°C for >12 h, the cell samples (60 nm) were examined using a Hitachi-7500 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan).

Statistical analysis. Each experiment was performed \geq 3 times. Experimental data were analyzed using one-way analysis of variance and least significant difference tests. SPSS software (version 19.0; IBM Corp., Armonk, NY, USA) was used to perform the statistical analyses. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

IL-12 enhances the expression of LC3 protein in human breast cancer cells. In order to assess the effects of rIL-12 on human breast cancer cell autophagy, MDA-MB-231 and MCF-7 breast cancer cells were treated with 5 ng/ml rIL-12 for 1, 2, 3, 6, 12 or 24 h, or with 0.1, 0.5, 1, 5, 10 or 20 ng/ml rIL-12 for 12 h, to determine the time- and dose-effect associations. In addition, the expression of LC3 induced by 5 ng/ml rIL-12 was detected using immunofluorescence. MDA-MB-231 and MCF-7 breast cancer cells were treated by serum starvation for 3 h to act as positive controls. Western

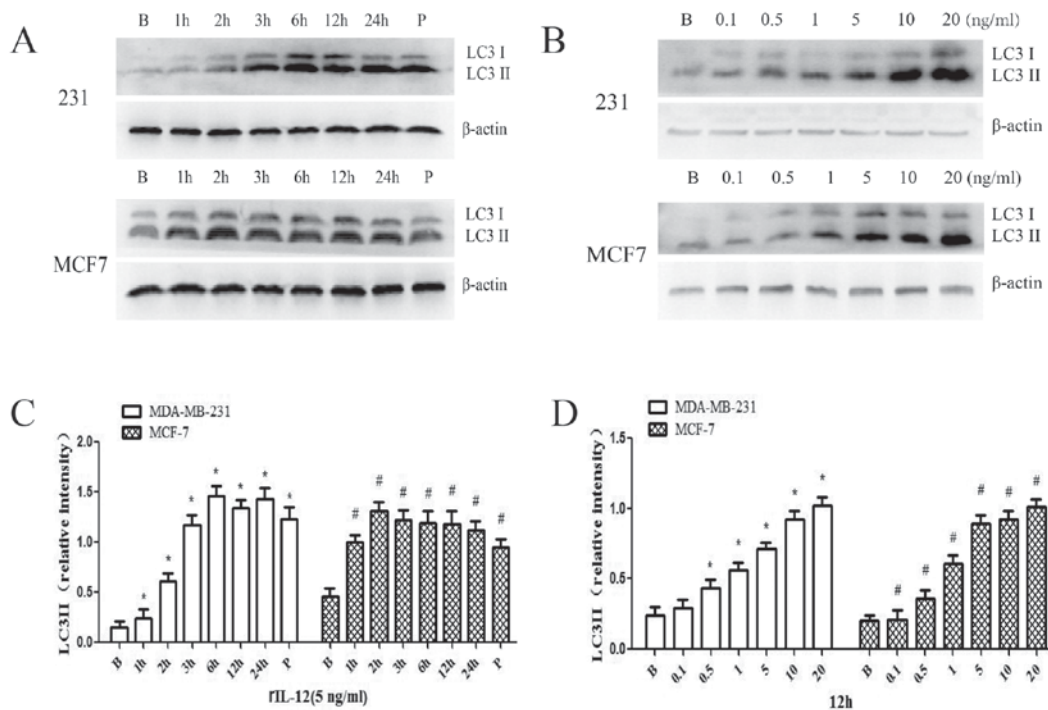


Figure 1. Western blot analysis of LC3 expression in breast cancer cells induced by IL-12. (A) The breast cancer cells were treated with rIL-12 (5 ng/ml) for 1, 2, 3, 6, 12 and 24 h. The cells in the P group were starved for 3 h. The whole-cell lysates were analyzed using western blotting. The results demonstrated that IL-12 increased the expression of LC3 in a time-dependent manner, particularly the expression of LC3II. (B) The breast cancer cells were treated with 0.1, 0.5, 1, 5, 10 and 20 ng/ml rIL-12 for 12 h. The whole-cell lysates were analyzed using western blotting. The results demonstrated that IL-12 upregulated the expression of LC3II in a dose-dependent manner. (C) Quantification of the results of the western blotting from cells treated for varying lengths of time. (D) Quantification of the results of the western blotting from cells treated with varying concentrations. *P<0.05 vs. MDA-MB-231 B group. #P<0.05 vs. MCF-7 B group. B, negative control; P, positive control; rIL-12, recombinant interleukin-12; LC3, microtubule-associated protein light chain 3.

blot analysis revealed that the expression of the autophagy marker protein LC3 in breast cancer cells was enhanced by treatment with IL-12, particularly the expression of LC3II. It was observed that IL-12 induced the expression of LC3II in a concentration- and time-dependent manner (Fig. 1). Consistently, the immunofluorescence results indicated that IL-12 upregulated the protein expression of LC3. LC3 protein stained with FITC was observed to be aggregated around the cell nucleus, and the stimulation effect of 5 ng/ml rIL-12 on LC3II protein expression increased compared with the positive control (Fig. 2).

IL-12 induces the formation of autophagosomes. TEM is the gold-standard method of analyzing autophagosome formation. MDA-MB-231 and MCF-7 breast cancer cells were treated with 5 ng/ml rIL-12 for 12 h. Following digestion with trypsin and preparation of the samples, cells were observed using TEM to view the micro-morphological characteristics of the autophagosome. The autophagosome is a foam-like structure with a double or multilayer membrane. In the later stages of autophagy, autophagosomes appear to be vesicular with inclusions. In the present study, autophagosomes were observed in the IL-12 group (Fig. 3). The results of the present study indicated that IL-12 is able to induce autophagosome formation in breast cancer cells.

IL-12 induces the expression of LC3 protein in breast cancer cells by inhibiting the Akt pathway and activating the AMPK pathway. In order to study alterations in the expression of

LC3 proteins induced by IL-12, the cells were divided into a number of groups. In the IL-12+IGF-1/BML-275 groups, MDA-MB-231 and MCF-7 breast cancer cells were pretreated with 100 ng/ml IGF-1, a PI3K/Akt activator, or 10 μ mol/ml BML-275, an AMPK inhibitor, for 2 h. A total of 5 ng/ml rIL-12 was added for a further 4 h. The IL-12 groups were treated with 5 ng/ml rIL-12 only for 4 h.

It was observed that the expression of p-PI3K/Akt was inhibited by IL-12, and p-mTOR was additionally down-regulated. LC3 protein expression, particularly LC3II, was upregulated in the IL-12 group compared with the negative control group. The expression levels of p-PI3K/Akt and p-mTOR recovered when the cells were treated with IGF-1 to activate the PI3K/Akt pathway, and the expression of LC3II protein was inhibited (Fig. 4A). In addition, it was observed that the expression of p-AMPK was activated by IL-12 and p-mTOR was simultaneously downregulated. As the p-AMPK pathway was inhibited by BML-275, the ability of IL-12 to induce the expression of LC3II in the BML-275-treated cells was decreased (Fig. 4B).

Consistent results were observed in the immunofluorescence assay. The fluorescence intensities of LC3 proteins were increased by IL-12. However, this effect was reversed when the cells were pretreated with IGF-1 or BML-275 (Fig. 4C and D). The results of the present study demonstrated that IL-12 upregulated the expression of LC3 proteins and promoted the autophagy of MDA-MB-231 and MCF-7 breast cancer cells, due to inhibition of the PI3K/Akt signaling pathway and activation of the AMPK signaling pathway.

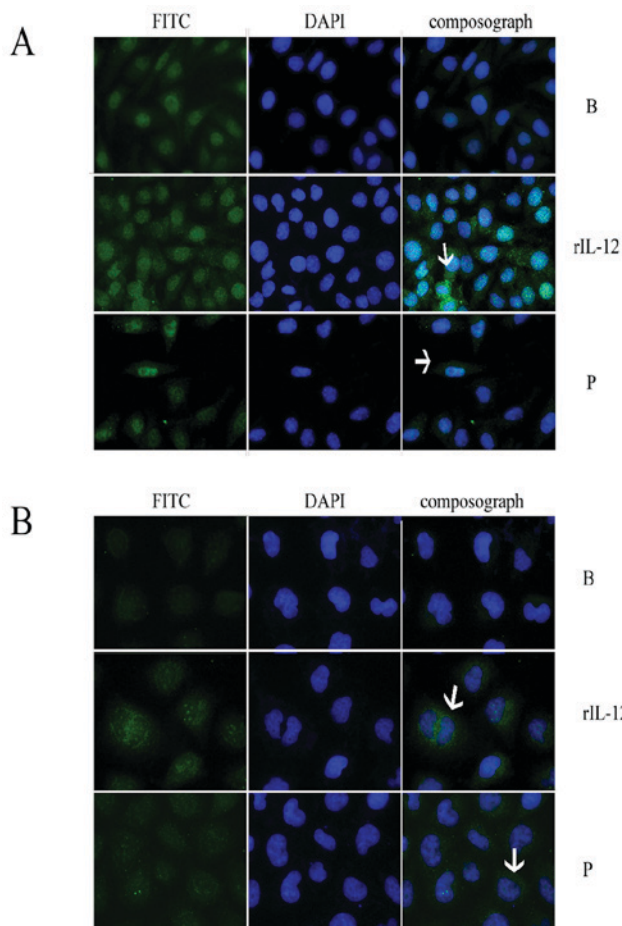


Figure 2. Immunofluorescence analysis of LC3 expression in breast cancer cells treated with IL-12. Untreated breast cancer cells acted as the B group. The cells in group P were starved for 3 h. The breast cancer cells were treated with 5 ng/ml rIL-12 for 6, 12 and 24 h. The cells were observed using a fluorescence microscope. The results indicated that IL-12 upregulated the expression of LC3. White arrows indicate aggregation around the cell nucleus. The results at 12 h are presented. (A) MDA-MB-231 cells. (B) MCF-7 cells. B, negative control; P, positive control; rIL-12, recombinant interleukin-12; LC3, microtubule-associated protein light chain; FITC, fluorescein isothiocyanate.

Discussion

Autophagy is a Greek term coined by Christian DeDuve to mean 'self-digesting' (17). The process of autophagy may be divided into three parts. The first phase begins with the activation of mTOR. mTOR is an atypical serine/threonine protein kinase and is associated with cell growth. The second phase is the regulation of autophagy related gene (Atg), which is regulated by interactions between proteins or signal transduction molecules. The third phase is the late stage of autophagy regulation, and is associated with autophagosome maturation and lysosome-phagosome fusion (18). Autophagy was first associated with human cancer due to the identification of beclin 1, an autophagy associated gene, as a haploid-insufficient tumor suppressor. Beclin 1 has been mapped to a tumor susceptibility locus on human chromosome 17q21, which is monoallelically deleted in 40-75% of ovarian, breast and prostate cancer, suggesting that it may act as a tumor suppressor (8,19).

At present, autophagy research was primarily based on the direct observation of autophagosomes, or the indirect detection

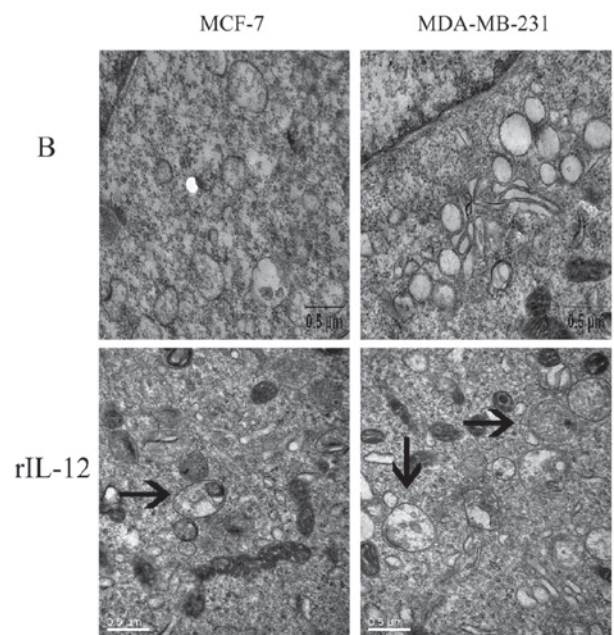


Figure 3. Transmission electron microscopy analysis of autophagosomes in breast cancer cells treated with IL-12. The untreated breast cancer cells acted as the B group. The rIL-12 groups of breast cancer cells were treated with 5 ng/ml rIL-12 for 12. The results demonstrated that IL-12 promoted autophagosome formation. The black arrows indicate the autophagosomes, which exhibit double membranes or inclusions. Magnification, x30,000. B, negative control; IL-12, recombinant interleukin-12.

of surface markers or associated proteins. Comprehensive evaluation of the function of autophagy and the control of autophagic pathways in cell behavior is required. Electron microscopy remains one of the most accurate methods for the detection of autophagy and the quantification of autophagosome accumulation. Autophagosomes exhibit a foam-like structure with a double or multilayer membrane, and contain numerous types of undigested organelles. Following fusion with lysosomes, autophagosomes assume the form of a monolayer with undigested organelles (20). An alternative method of studying autophagy is based on LC3 protein expression. During the production of autophagosomes, cytoplasmic LC3I proteins are converted to LC3II proteins in the membrane of autophagosome. Alterations in autophagy may be investigated indirectly by assessing LC3 protein expression (21,22). In the present study, it was demonstrated that IL-12 is able to upregulate cellular autophagy in MDA-MB-231 and MCF-7 breast cancer cells, using TEM and LC3 protein expression analysis. The alteration in LC3 protein expression was detected using western blotting and immunofluorescence.

In the first phase of autophagy regulation, the PI3K/Akt-mTOR and AMPK-mTOR signaling pathways serve important roles. The activation of autophagy is associated with the inhibition of PI3K/Akt-mTOR and mTOR, or the activation of AMPK (4). In previous studies, Zhang *et al* (23,24) and Danielsen *et al* (25) demonstrated that activation of Akt pathway was associated with the inhibition of cellular apoptosis and the promotion of cell proliferation. Guo *et al* (26) observed that transforming growth factor β 1 (TGF β 1) was overexpressed in various types of cancer. TGF β 1 overexpression in cancer cells may activate the Akt pathway and

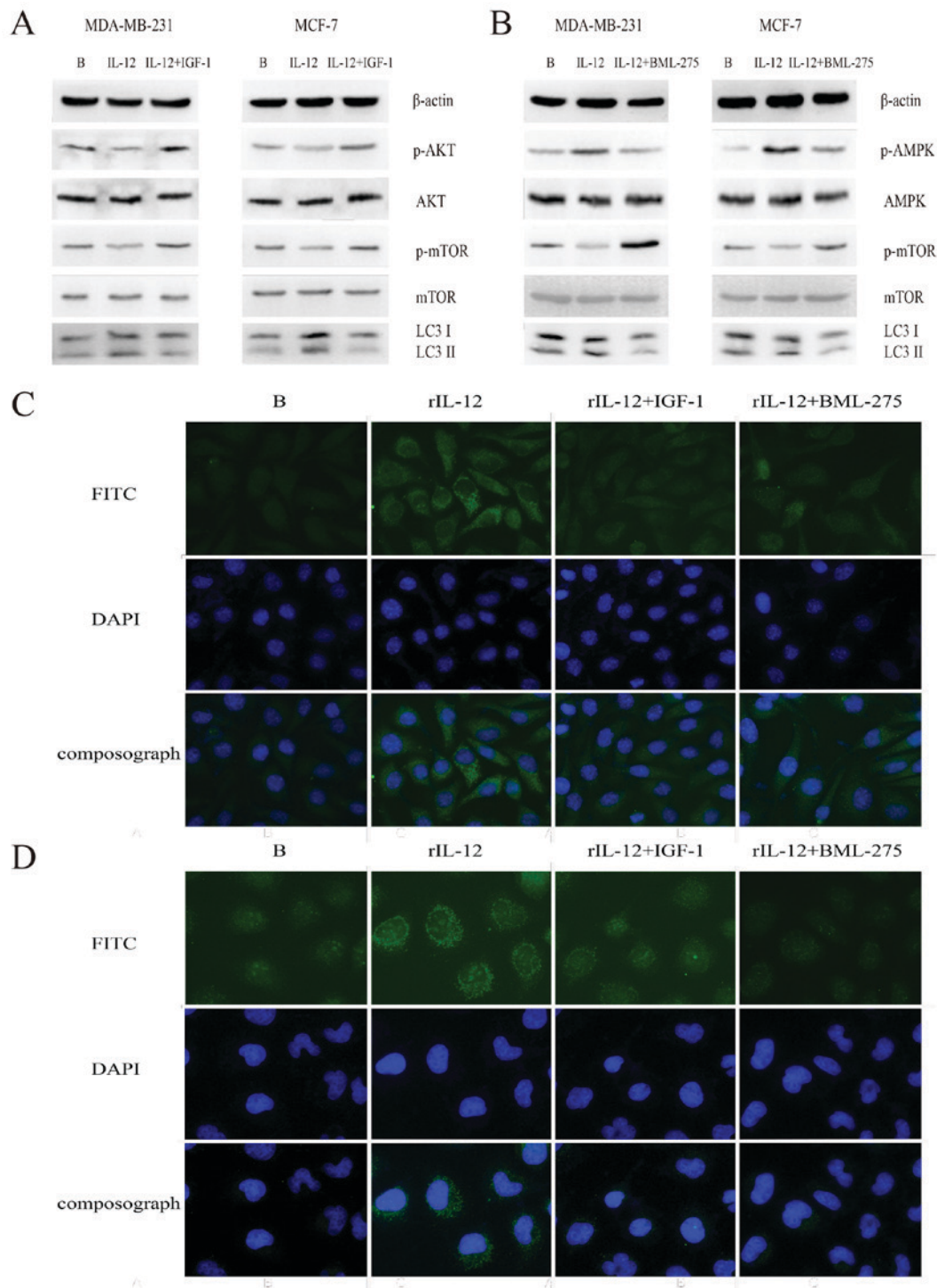


Figure 4. IL-12 induced the expression of LC3 in breast cancer cells by inhibiting the PI3K/Akt pathway and activating AMPK pathway. The breast cancer cell groups IL-12+IGF-1 and IL-12+BML-275 were pretreated with 100 ng/ml IGF-1 or 10 μ mol/ml BML-275, respectively, for 2 h. A total of 5 ng/ml rIL-12 was added for a further 4 h. The IL-12 groups were treated with 5 ng/ml rIL-12 for 4 h. β -actin, p-Akt/Akt, p-mTOR/mTOR, pAMPK/AMPK and LC3 proteins were detected using western blotting: (A) IL-12+IGF-1 groups and (B) IL-12+BML-275 groups. The expression and distribution of LC3 proteins was examined using fluorescence microscopy with FITC-labeled secondary antibodies in (C) MDA-MB-231 and (D) MCF-7. B, negative control; rIL-12, recombinant interleukin-12; LC3, microtubule-associated protein light chain; FITC, fluorescein isothiocyanate; PI3K, phosphatidylinositol 3-kinase; Akt, Rac- α serine/threonine protein kinase; AMPK, 5'-AMP-activated protein kinase subunit β -1; IGF-1, insulin-like growth factor 1; mTOR, serine/threonine protein kinase mTOR.

enhance cell proliferation and migration. The Akt signaling pathway is able to activate numerous downstream targets, including mTOR complex 1, which inhibits tumor formation. Previous studies have demonstrated that the inactivation of negative regulatory proteins upstream of mTOR occurs in

tumors (27,28). The inactivation of the phosphatase and tensin homolog gene (PTEN) is frequently observed in breast cancer, thyroid carcinoma and prostate cancer. The inactivation of PTEN is associated with the oncogenic activity of Akt and the activity of the mTOR signaling pathway. It is due to these

associations that rapamycin may be used to treat cancer (29). AMPK is a sensor of cellular energetic status expressed in all eukaryotes. Previous studies have indicated that AMPK activation inhibits cell proliferation in tumor cells, which require increased rates of protein synthesis and *de novo* fatty acid synthesis due to their rapid growth. The AMPK signaling pathway is associated with tumor growth and proliferation through regulation of the mTOR pathway. Therefore, AMPK is emerging as an important metabolic tumor suppressor and a promising target for cancer prevention and therapy (30,31).

In order to elucidate the mechanism of IL-12-induced autophagy in breast cancer cells, the involvement of the PI3K/Akt and AMPK signaling pathways was investigated. It was observed that the induction of autophagy-associated protein expression by IL-12 accompanied the decrease in p-AKT and increase in p-AMPK in breast cancer cells. However, following activation of the PI3K/Akt pathway by IGF-1, the ability of IL-12 to promote the expression of LC3 was inhibited, and the expression of mTOR was recovered in the IL-12 group. The AMPK pathway was inhibited by BML-275, and it was observed that the alteration of LC3 and mTOR expression was consistent compared with cells treated with the PI3K/Akt activator IGF-1. The results of the present study demonstrated that IL-12 is able to induce autophagy by inhibiting the PI3K/Akt signaling pathway and activating the AMPK signaling pathway.

In conclusion, the results of the present study demonstrated that IL-12 was able to induce autophagy in breast cancer cells in a time- and concentration-dependent manner, by inhibiting PI3K/Akt and activating the AMPK signaling pathway. The present study may offer a novel insight into the IL-12 anti-cancer mechanism.

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