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ScienceDirect
Chinese Journal of Natural Medicines 2022, **20**(4): 290-300
doi: 10.1016/S1875-5364(22)60166-3
Chinese Journal of Natural Medicines

•Research article•

EGCG and ECG induce apoptosis and decrease autophagy *via* the AMPK/mTOR and PI3K/AKT/mTOR pathway in human melanoma cells

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Available online 20 Apr., 2022

[ABSTRACT] Catechins have been proven to exert antitumor effects in different kinds of cancers. However, the underlying mechanisms have not been completely clarified yet. This study aimed to assess the effects and mechanisms of (−)-epigallocatechin-3-gallate (EGCG) and (−)-epicatechin-3-gallate (ECG) on human melanoma skin A375 cells. Results showed that EGCG and ECG inhibited the proliferation of A375 cells and ECG showed better inhibitory effect. Flow cytometry analysis had shown that EGCG and ECG induced apoptosis and led to cell cycle arrest. EGCG and ECG decreased Bcl-2 expression and upregulated Caspase-3 protein level, indicating the development of apoptosis. Furthermore, EGCG and ECG could decreased mitochondrial membrane potential of A375 cells. In addition, the expression of Beclin-1, LC3 and Sirt3 were downregulated at protein levels, which known to be associated with autophagy. After autophagy was increased by rapamycin, the apoptotic trend was not change, indicating that apoptosis and autophagy are independent. Mechanistically, EGCG and ECG treatments decreased phosphorylated-AMPK (p-AMPK) and increased the ratios of p-PI3K, p-AKT and p-mTOR in melanoma cells. Conclusively, EGCG and ECG induced apoptosis *via* mitochondrial signaling pathway, downregulated autophagy through modulating the AMPK/mTOR and PI3K/AKT/mTOR signaling pathway. It indicated that EGCG and ECG may be utilized in human melanoma treatment.

[KEY WORDS] EGCG; ECG; Apoptosis; Autophagy; PI3K/AKT/mTOR
[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2022)04-0290-11

Introduction

Melanoma has become a difficult public health problem due to its high aggressiveness and lethality [1]. It is reported that the incidence rates and mortality rates of melanoma are increasing every year [2]. Despite recent breakthroughs in the treatment, the clinical outcomes of existing treatments remain unsatisfactory. Therefore, it is essential to find the new treatment of melanoma to become an important medical need [1, 3, 4].

Apoptosis and Autophagy are two different ways to regulate programmed cell death [5]. Apoptosis, as one of the terminal pathways of cell death, participates in morphogenesis during embryonic progression and eliminates pre burning or harmful cells to maintain tissue steady state [6]. Autophagy is a traditionally self-feeding process that digests its cellular contents through lysosomal mediation, thus providing energy and basis for cancer cell survival under stress conditions. Autophagy inhibition is considered to be an effective strategy to make cancer cells sensitive to current therapies for advanced melanoma [7]. A study showed that Amlexanox suppressed human melanoma by the inhibition of autophagy [8]. AMPK is a key mediator for the initial process of autophagy [9]. Autophagy could be triggered *via* several signaling pathways. AMPK activation is one of the upstream activators of autophagy signaling [1]. AMPK not only directly phosphorylates and activates ULK1 to induce autophagy, but also indirectly activates ULK1 by inhibiting mTORC1 [10]. The PI3K/AKT/mTOR pathway, as a key regulator of autophagy, is involved in the initiation and promotion of a range of pathological disorders including various cancers [11]. It has been reported that Artesunate inhibits chondrocyte proliferation and accelerates cell apoptosis and autophagy *via* suppres-

[Received on] 11-Oct.-2021
[Research funding] This work was supported by the National Natural Science Foundation of China (No. 82004027) and the Fundamental Research Funds for the Central Universities (JUSRP11961).
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These authors have no conflict of interest to declare.

sion of the PI3K/AKT/mTOR signaling pathway [12]. A recent study demonstrated that Neogambogic acid could inhibit the proliferation of melanoma B16 cells and induce their apoptosis *via* the PI3K/Akt/mTOR signaling pathway [13].

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Materials and Methods

Materials

Catechins (EGCG, ECG, EGC, EC) with the purity of more than 98% were purchased from Nanjing Plant Origin Biological. Catechins were dissolved (DMSO) at 50 mmol·L^{-1} as a stock solution and were stored at −20 °C. Arbutin with the purity of 98% was purchased from Bidepharm. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Sigma. Antibodies against AMPK, p-AMPK, Bcl-2, Caspase-3, LC3 and *β*-actin were purchased from Beyotime Biotechnology. Antibody against Beclin-1 was purchased from Proteintech and Sirt3 was purchased from Cell Signaling Technology.

Cell culture

Human melanoma A375 cells were friendly obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences and identified by STR authentication. The cells used were in logarithmic growth phase and were tested for mycoplasma regularly. A375 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C under 5% CO_2.

Cell proliferation assay

Effects of catechins on cell proliferation were measured by using cell counting kit-8 (CCK-8, Meilun MA0218). Arbutin was used as a positive control at the same time. The control group was only treated with DMEM. A375 cells were seeded in 96-well plates with a density of 1 × 10^4 cells per well. After 24 h incubation, A375 cells were exposed to various concentrations of EGCG, ECG, EGC, EC (3.125, 6.25, 12.5, 25, 50, 100 μg·mL^{-1}) and Arbutin (0.5, 1, 2, 4, 8, 16 mg·mL^{-1}) for 24 h, 48 h and 72 h. Then 10 μL CCK-8 was added and incubated for 2 h. The absorbance was read at 450 nm using a microplate reader (TECAN Infinite M200pro Switzerland). Each concentration was analyzed in triplicate.

Apoptosis Measured by Annexin V-FITC/PI staining

To further quantify apoptosis, Annexin V-FITC Apoptosis Detection Kit was used to assess cell apoptosis according to the supplier's instructions. After treatment with EGCG, ECG (75, 100, 125 μg·mL^{-1}) and Arbutin (10, 13, 16 mg·mL^{-1}) for 48 h, cells and cell culture medium were collected and cells were gently resuspended with the Annexin V-FITC binding solution. Annexin V-FITC and PI staining solution were added, then incubated for 20 min in the dark at room temperature, and detected by flow cytometry on the computer.

Cell cycle analysis

After treatment with EGCG, ECG (75, 100, 125 μg·mL^{-1}) and Arbutin (10, 13, 16 mg·mL^{-1}) for 48 h, cells and cell culture medium were collected, centrifuged at 1000 r·min^{-1} for 5 min, then added to cold 70% ethanol overnight at 4 °C. The fixed cells were washed with cold PBS and centrifuged at 1000 r·min^{-1} for 5 min. PI staining was prepared according to the instructions, and then added to the cell samples, incubated at 37 °C in the dark for 30 min. On-line detection and analysis by flow cytometer.

Western blot

After treatment with EGCG, ECG (12.5, 25, 50 μg·mL^{-1}) and Arbutin (4, 8, 16 mg·mL^{-1}) for 48 h, A375 cells were harvested and washed with cold PBS. The proteins were lysed in RIPA buffer and kept on ice for at least 30 min and the total protein in the supernatant was obtained. The concentration of protein was measured by the BCA protein assay kit. Then equal amounts of protein for each sample were separated by SDS-PAGE gels and transferred to the PVDF membranes. Later, the membrane was sealed with 5% BSA (Albumin from bovine serum) in the TBST for 1 h, incubated with a primary antibody overnight at 4 °C. The next day membranes were incubated with the second antibody at room temperature for 1 h. The imaging was performed with a high sensitivity ECL chemiluminescence kit. The total gray level of each band is quantified by Image J software.

Mitochondrial membrane potential assay

A375 cells were seeded on glass bottom dishes with a density of 2 × 10^4 cells. After treatment with EGCG, ECG (12.5, 25, 50 μg·mL^{-1}) and Arbutin (4, 8, 16 mg·mL^{-1}) for 48 h, JC-1 staining working solution was added, incubated

for 20 min in the incubator, and at the end of the incubation, washed twice with JC-1 staining buffer and examined and photographed under a laser confocal microscope.

Statistical analysis

Data were analyzed using GraphPad presented as mean ± standard deviation of three independent experiments, *t*-test was performed to compare the individual treatment to the normal, and comparisons between different treatments were analyzed by one-way analysis of variance with a significance level of $P < 0.05$.

Results

Antiproliferation activity in A375 cells

Effects of the catechin (EGCG, ECG, EGC, EC) on A375 cell proliferation were determined by CCK8 assay, Arbutin was used as a positive control. The results suggested that EGCG and ECG inhibited A375 cell proliferation in a dose-dependent manner, while EGC and EC did not show obvious inhibitory effects. From the concentration of 4 mg·mL^{-1}, the positive drug Arbutin showed an inhibitory effect (Fig. 1). Considering the cytotoxicity, EGCG, ECG (12.5, 25, 50 μg·mL^{-1}) and Arbutin (4, 8, 16 mg·mL^{-1}) were used in the following experiments.

EGCG and ECG induce cell apoptosis in A375 cells

To explore the effects of EGCG and ECG on cell apoptosis, the apoptotic rates of A375 cells were detected by flow cytometry. Flow cytometry results showed that EGCG and ECG promoted cells apoptosis in a concentration-dependent

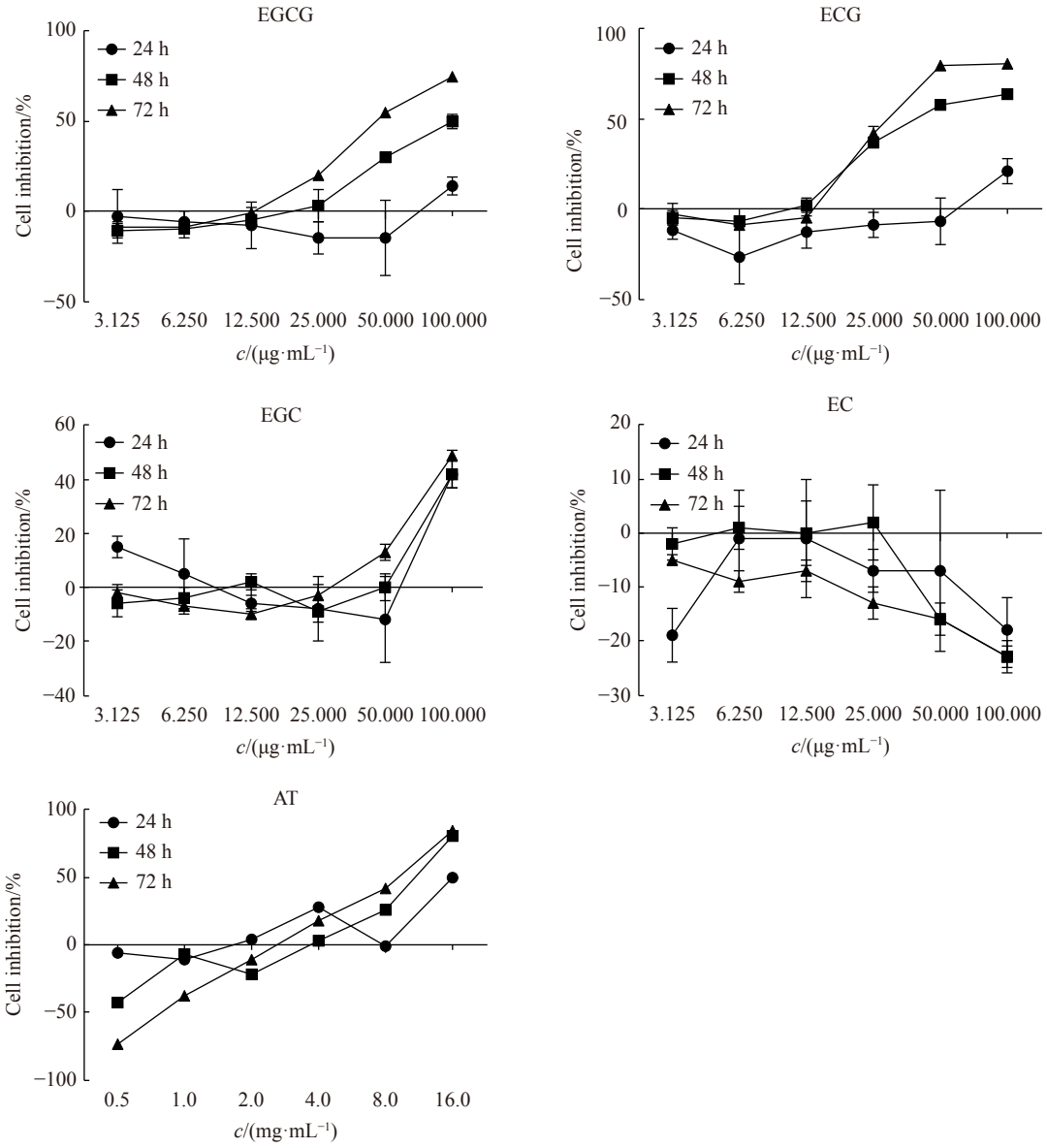

Fig. 1 The inhibition of cell proliferation was detected by CCK-8 assay. A375 cells were treated with increasing concentrations of catechins (3.125, 6.25, 12.5, 25, 50, 100 μg·mL^{-1}) and Arbutin (0.5, 1, 2, 4, 8, 16 mg·mL^{-1}) for 24 h, 48 h and 72 h, respectively. AT, Arbutin

manner. The apoptotic rates were 37.6%, 59.20%, 79.9% for EGCG and 44.50%, 43.80%, 34.5% for ECG, respectively. EGCG exerted a better effect than ECG (Fig. 2).

EGCG and ECG induced cell cycle arrest in A375 cells

In this experiment, cell cycle analysis was performed by flow cytometry using PI staining. The result showed that EGCG, ECG and Arbutin could induce cell cycle arrest at G0/G1 phase in A375 cells. Meanwhile, ECG could also significantly block A375 cells in G2/M phase (Fig. 3).

EGCG and ECG promoting apoptosis in A375 cells

Furthermore, the apoptosis-related proteins were investigated by western blotting. The results indicated that EGCG and ECG reduced the expression of Bcl-2 in a concentration-dependent manner and EGCG downregulated Bcl-2 expression by 47% at a concentration of 50 μg·mL^{-1}. On the contrary, the expression level of Caspase-3 was upregulated by 27% by ECG at a concentration of 25 μg·mL^{-1} (Fig. 4).

EGCG and ECG decreased mitochondrial membrane potential

The change of JC-1 from red fluorescence to green fluorescence can also be used as an early indicator of apoptosis. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is an inhibitor of the mitochondrial electron transport chain, as shown in Fig. 5, compared with the control group, CCCP (10 μmol·L^{-1}) changed the fluorescence from red to green, indicated the decrease of mitochondrial membrane potential. In the treatment groups, EGCG, ECG and Arbutin significantly reduced mitochondrial membrane potential in A375 cells.

EGCG and ECG decreased autophagy-related indicators at protein levels in A375 cells

LC3 and Beclin-1 are autophagy related indicators, and their upregulation indicates autophagy activation. As shown in Fig. 6B, EGCG and ECG reduced Beclin-1 expression in a concentration-dependent manner. The expression level of LC3-II/ LC3-I was reduced obviously by EGCG and ECG in A375 cells (Fig. 6C). Sirt3 overexpression promote autophagy. Sirt3 expression was also significantly decreased by EGCG and ECG (Fig. 6D). These results revealed that the treatment of EGCG and ECG could decrease autophagy in A375 cells.

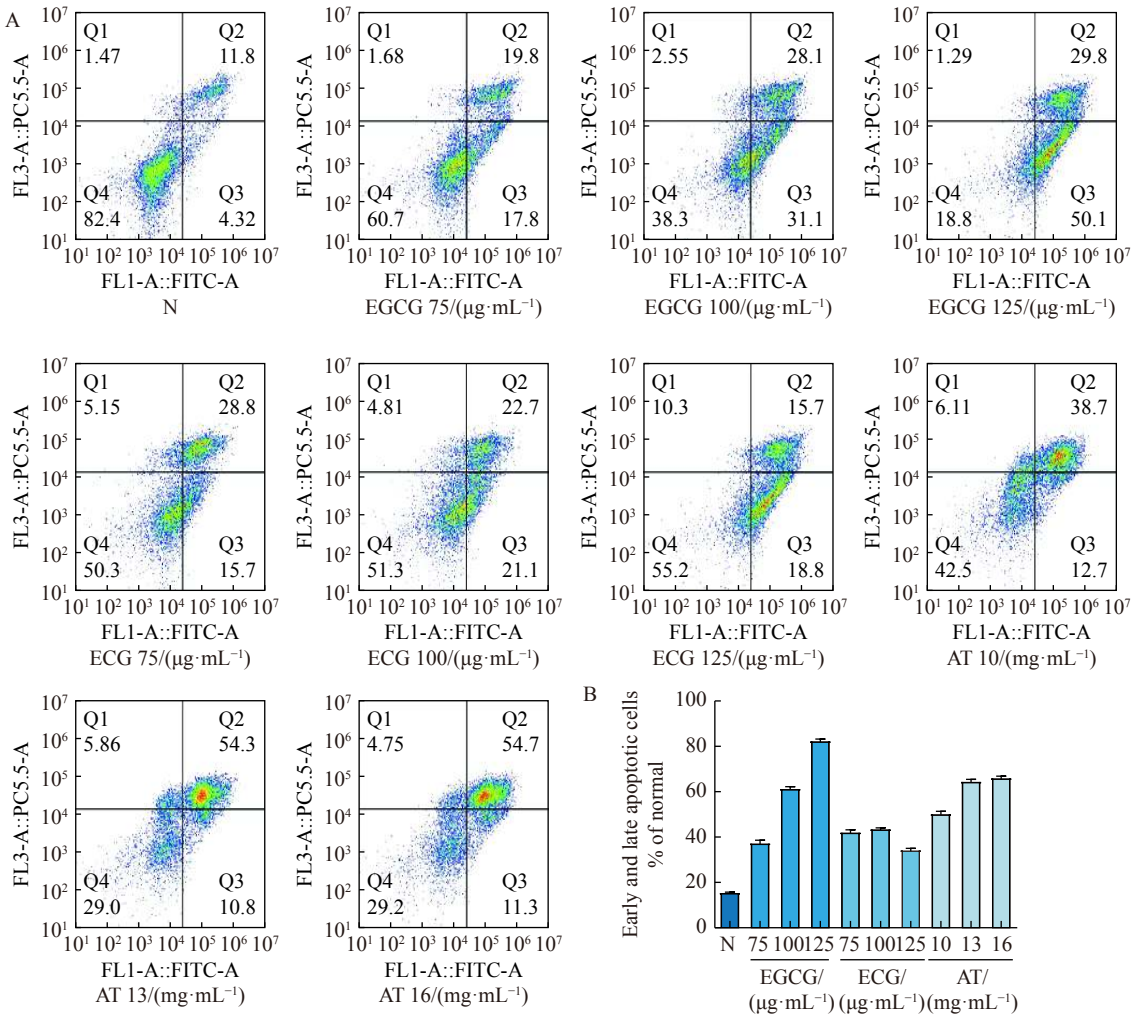

Fig. 2 EGCG and ECG induce cell apoptosis in A375 cells. A: After treated with EGCG and ECG, the apoptotic rates of A375 cells were detected by flow cytometry. B: Quantitative analysis of the apoptotic rates of EGCG and ECG-treated A375 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the control A375cells. The values were presented as mean ± SD ($n = 3$)

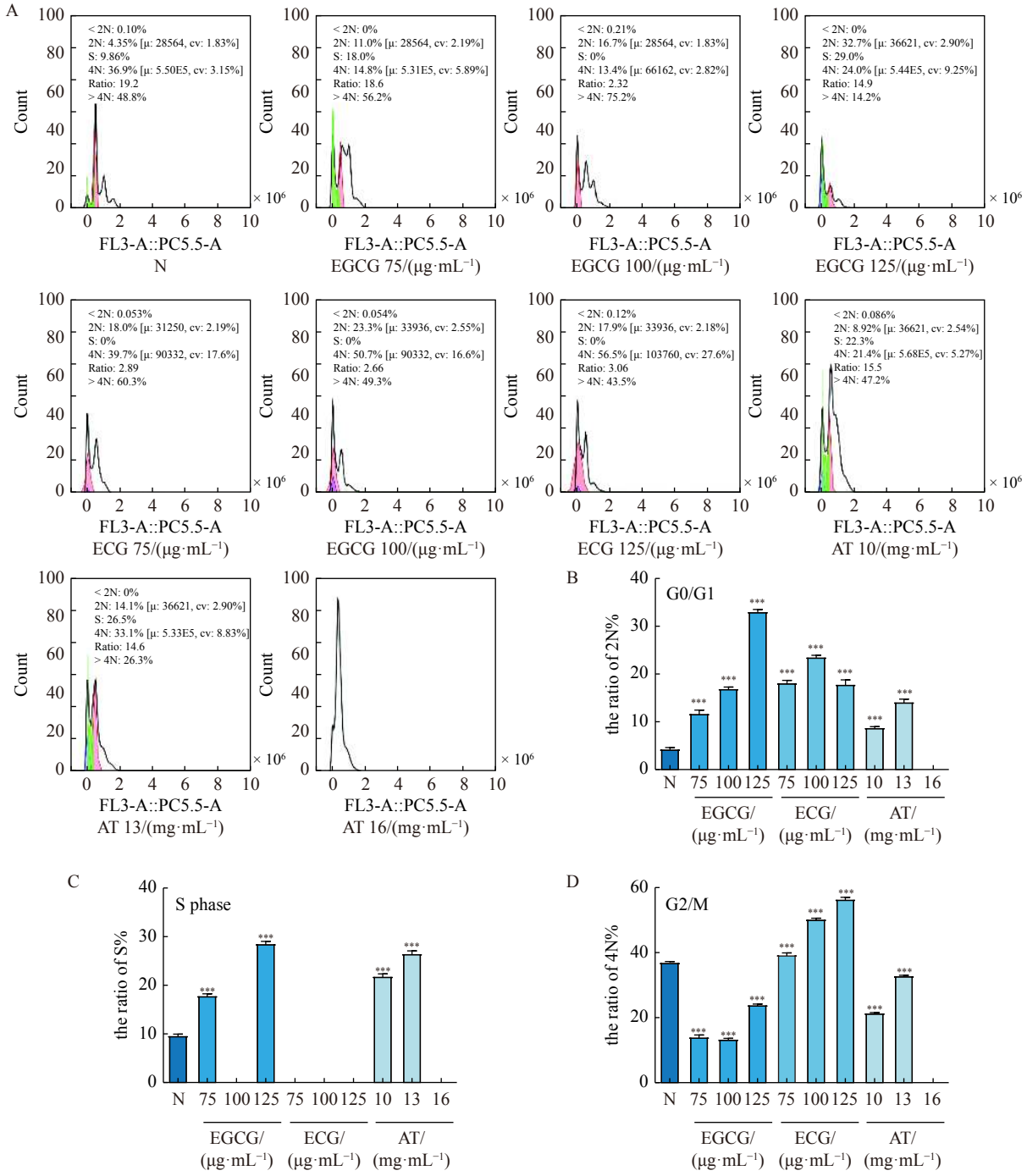

Fig. 3 EGCG and ECG induce cell cycle arrest in A375 cells. A: The effects of EGCG and ECG on A375 cell cycle distribution were measured by flow cytometry. (B, C, D): Statistical analysis of the cell cycle distribution of EGCG and ECG-treated A375 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the control A375cells. The values were presented as mean ± SD ($n = 3$)

EGCG and ECG decreased autophagy in A375 cells

EGCG and ECG decreased autophagy by downregulating the AMPK pathway

In order to explore the potential mechanism of EGCG and ECG in autophagy, western blotting was used to detect the AMPK pathway. AMPK is a negative regulator of autophagy. The results showed that EGCG could reduce the expression level of p-AMPK in a concentration-dependent manner (Fig. 7A and B). However, ECG exerted a better effect on reducing p-AMPK expression than EGCG. These results indicated that EGCG and ECG could decrease autophagy by downregulated the AMPK pathway.

EGCG and ECG decreased autophagy by upregulating the PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR pathway has attracted extensive attention as the modulators of autophagy [11]. In order to ex-

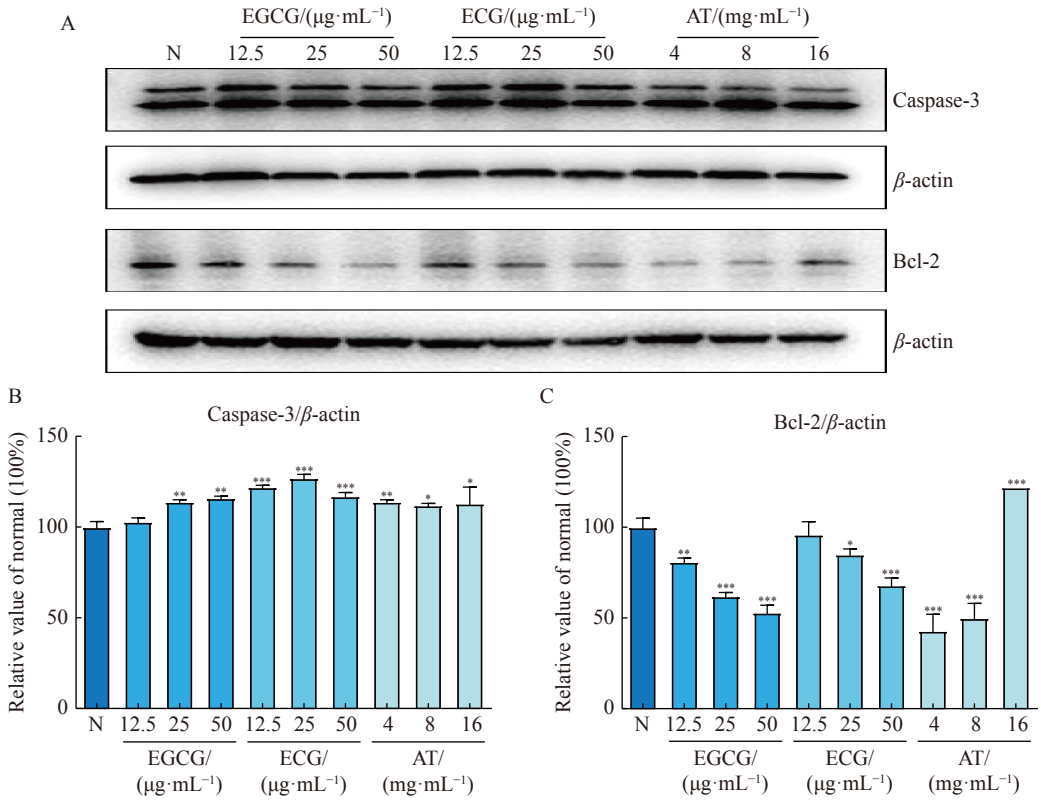

Fig. 4 Effects of EGCG and ECG on apoptosis in A375 cells. A: The apoptosis-related protein expression levels of Caspase-3 and Bcl-2 in EGCG and ECG-treated A375 cells were detected by western blotting. (B, C): Quantitative analysis of the protein expression levels of Caspase-3 and Bcl-2 in A375 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the control A375cells. The values were presented as mean ± SD ($n = 3$)

plore the role of the PI3K/AKT/mTOR pathway in autophagy, the expressions of the PI3K, AKT and mTOR was detected by western blotting. As shown in Fig. 7C, D and E, EGCG and ECG upregulated the levels of p-PI3K, p-AKT and p-mTOR expression in A375 cells. Differently, ECG showed better results than EGCG in elevating the level of the PI3K, AKT and mTOR phosphorylation. These results suggested that EGCG and ECG might decrease autophagy through upregulated the PI3K/AKT/mTOR pathway.

Autophagy and apoptosis are independent of each other

The above results showed that EGCG and ECG induced apoptosis and decreased autophagy in A375 cells. Rapamycin, an autophagy inducer that targets mTOR, was used to investigate the relationship between apoptosis and autophagy. As shown in Fig. 8, LC3 expression was significantly increased with EGCG, ECG and rapamycin co-treatment compared with those after treatment with EGCG and ECG alone. Compared with EGCG and ECG treatment alone, rapamycin and EGCG, ECG co-treatment did not change the trend of apoptosis. It indicated that autophagy and apoptosis are independent of each other.

Discussion

Melanoma is the most common cause of skin malignancy and results from malignant transformation of melanocytes. The effects of treatments of melanoma are limited [20]. Some natural extracts, including tea polyphenols, paclitaxel, quercetin and paclitaxel, have been shown to activate or inhibit certain signaling pathways and inhibit the proliferation of cancer cells [21]. EGCG, the main constituent of green tea catechins, is nontoxic, effective cancer preventives for humans [22]. Although there have many studies reported that the effects of catechins in varies cancers, the mechanisms of EGCG and ECG inhibiting melanoma were unclear. Therefore, in this study, we investigated the effects and mechanisms of EGCG and ECG on A375 cells. The result investigated that EGCG and ECG could inhibit the proliferation of A375 cells and the mechanism might be due to over stimulation of apoptosis accompanied by down-regulating autophagy.

Apoptosis plays an important role in the development of melanoma [23]. In this study, flow cytometry experiment showed that EGCG and ECG induced the apoptosis of A375 cells. Previous studies have shown that the expression of Bcl-2 and Caspase-3 are the key factor to initiate apoptosis through the mitochondrial pathway [24]. Bcl-2 family proteins play an important role in regulating cell death [25]. Caspase-3 is a key molecule in the "executioner stage" of apoptosis and plays a key role in the proliferation of peripheral cells [26]. In

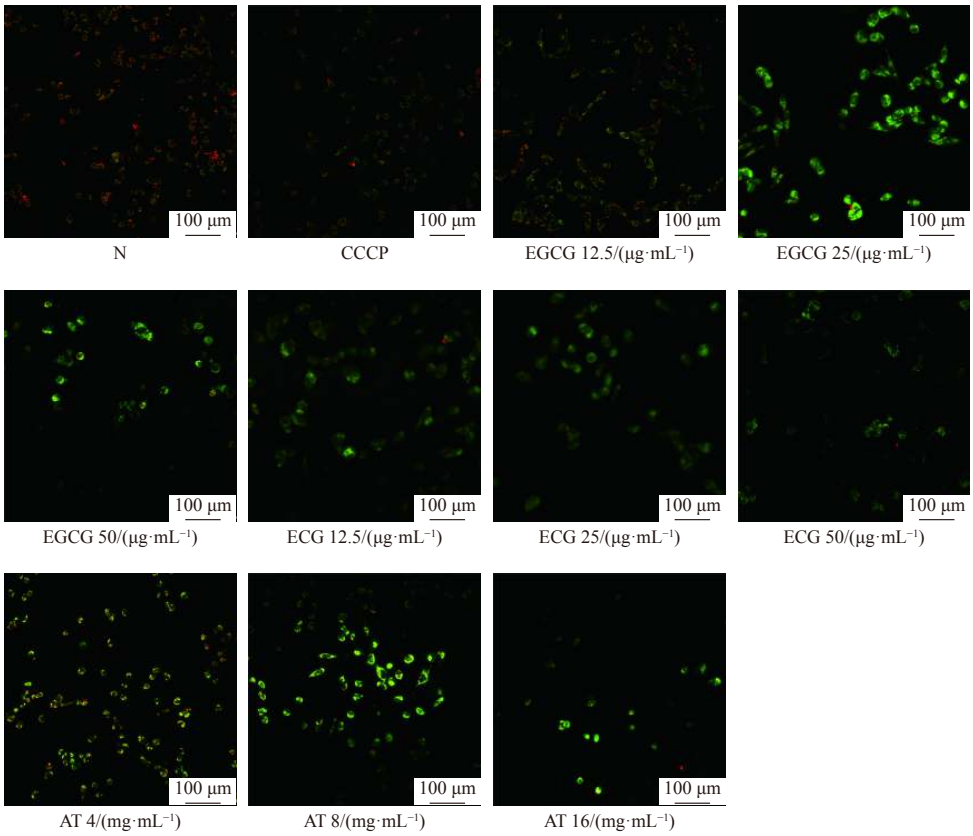

Fig. 5 The detection of mitochondrial membrane potential by confocal laser scanning (10 ×). EGCG, ECG and Arbutin could decrease mitochondrial membrane potential in a concentration dependent manner. The change of mitochondrial membrane potential could be detected by the transition of JC-1 from red fluorescence to green fluorescence. Laser: red fluorescence: 561 nm; green fluorescence: 488 nm

the key medium of programmed cell death, Caspase-3, as a frequently activated death protease, is essential for certain processes related to cell breakdown and apoptotic body formation [27]. Our results suggested that EGCG and ECG could significantly increase Caspase-3 expression and decrease Bcl-2 protein level, and the effect of EGCG was better than ECG. On the other side, EGCG and ECG could decrease mitochondrial membrane potential in a concentration dependent manner. For the intrinsic apoptosis signaling pathway, the loss of mitochondrial membrane potential, which could be detected by JC-1 in living cells, is an early event [28]. Intrinsic apoptosis signaling pathway is regulated by the Bcl-2 family of proteins [29]. It suggested that EGCG and ECG could inhibit the proliferation of A375 cells through apoptosis pathway *via* the mitochondrial signaling pathway.

Cellular autophagy is a dynamic process and plays an environment-dependent role in tumor development [30]. Autophagy has a dual role in different stages of cancer treatment, it not only protect cells, but also promote cell death [31]. However, the mechanism of autophagy inducing or inhibiting cell proliferation still needs more research. We assessed autophagy-related indicators from protein levels by western blotting assays. Our results have shown that EGCG and ECG could decrease the expression levels of Beclin-1 and LC3 at protein levels. LC3 and Beclin-1 are two markers of autophagy [32]. LC3 transformed from cytosolic soluble form LC3-I to liposoluble autophagy related form LC3-II is considered as a marker of autophagy. It should be pointed out that the conversion from LC3-I to LC3-II is a dynamic process. It has been reported that the deletion of Beclin-1 blocks autophagy at an early stage, leading to typical I-cell death [32, 33]. Beclin-1 plays a central role in autophagy and accumulates when cells are under pressure [34]. More and more evidence suggested that Beclin-1 may be involved in other processes that help to inhibit tumor potential [35]. The relative binding levels of Bcl-2/Beclin-1 were downregulated by EGCG while ECG did not exert effect on the relative binding levels of Bcl-2/Beclin-1 (Supplementary information). Downregulation of Beclin-1 and LC3 suggest that EGCG and ECG could downregulate autophagy in A375 cells.

Sirt3 played an important role in the alleviation of many diseases through regulating apoptosis and autophagy [36]. However, the role of Sirt3 in cancer seems to be complex; there is evidence that Sirt3 has both tumor promoter and tumor suppressor effects [37]. Previous studies had shown that Sirt3 was overexpressed in human melanoma cells and tis-

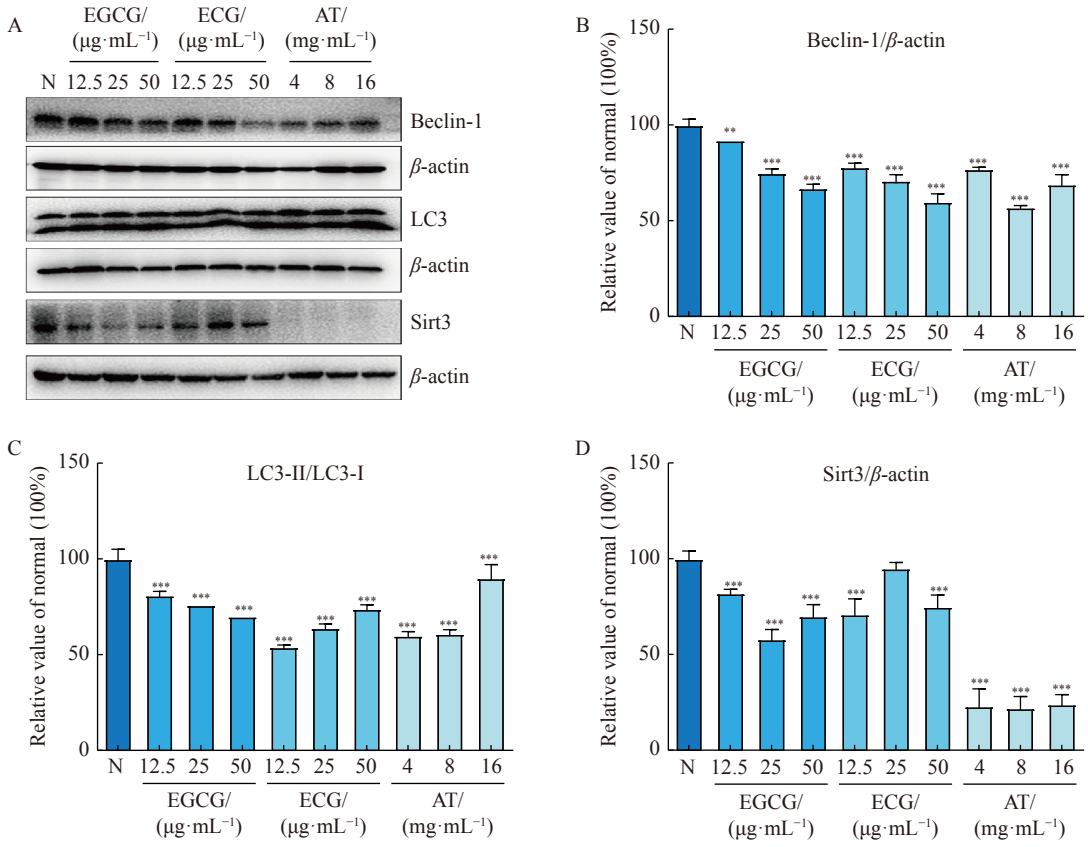

Fig. 6 EGCG and ECG decreased autophagy in A375 cells. A: The autophagy-related protein expression levels of Beclin-1, LC3 and Sirt3 in EGCG and ECG-treated A375 cells were detected by western blotting. (B, C, D): Quantitative analysis of the protein expression levels of Beclin-1, LC3 and Sirt3 in A375 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the control A375 cells. The values were presented as mean ± SD ($n = 3$)

sues, and the inhibition of Sirt3 produced a significant anti-proliferative response in human melanoma cells [38]. A novel role of Sirt3 in regulating phosphorylation of AMPK to promote autophagy has been reported, it has positive regulation on phosphorylation of AMPK. AMPK is a positive regulator of autophagy [39, 40]. In this study, we found that EGCG and ECG downregulated the expression of Sirt3, but because of its complexity, the molecular mechanism is the subject of future investigation.

Furthermore, to elucidate the mechanism of autophagy involved in the inhibition of human melanoma, we confirmed whether EGCG and ECG affect AMPK and PI3K/AKT/mTOR pathway by western blotting. AMPK is a key molecule in the stress response that is involved in a variety of cell physiological functions, including autophagy of cancer cells and the catabolic pathway of energy production. These functions are closely related to the cell cycle, autophagy and apoptosis [41]. In this study, the phosphorylation level of AMPK was decreased by EGCG and ECG, and ECG showed an obvious effect on decreasing the expression of p-AMPK than EGCG. Besides, the expression level of Sirt3 was consistent with the trend of p-AMPK. Therefore, it indicated that EGCG and ECG decreased autophagy via downregulating the AMPK pathway in A375 cells.

Autophagy is negatively regulated by the PI3K/AKT/mTOR pathway [42]. It has been proven that PI3K/AKT/mTOR-mediated autophagy played vital roles in the occurrence, development, and drug resistance of cancers [11]. The PI3K/AKT/mTOR pathway is one of the most common dysregulated signaling pathways in melanoma survival and progression [43]. More importantly, previous studies have proved that the PI3K/AKT/mTOR signaling pathway could regulate cell proliferation, apoptosis and autophagy, which are key functions of melanoma [44, 45]. In our research, EGCG and ECG could increase the expression levels of p-PI3K, p-AKT and p-mTOR, and ECG exerted a better result. It indicated that EGCG and ECG downregulated autophagy *via* activating the PI3K/AKT/mTOR signaling pathway in A375 cells.

We investigated the relationship between apoptosis and autophagy. Apoptosis is the main mechanism of programmed cell death that maintains the balance of death and survival in mammals. Autophagy can also promote death and survival, depending on the intensity of specific stimulation and different cell types. Although there is cross-talk between the pathways leading to apoptosis and autophagy, the stimulating factors and underlying mechanisms are still un-

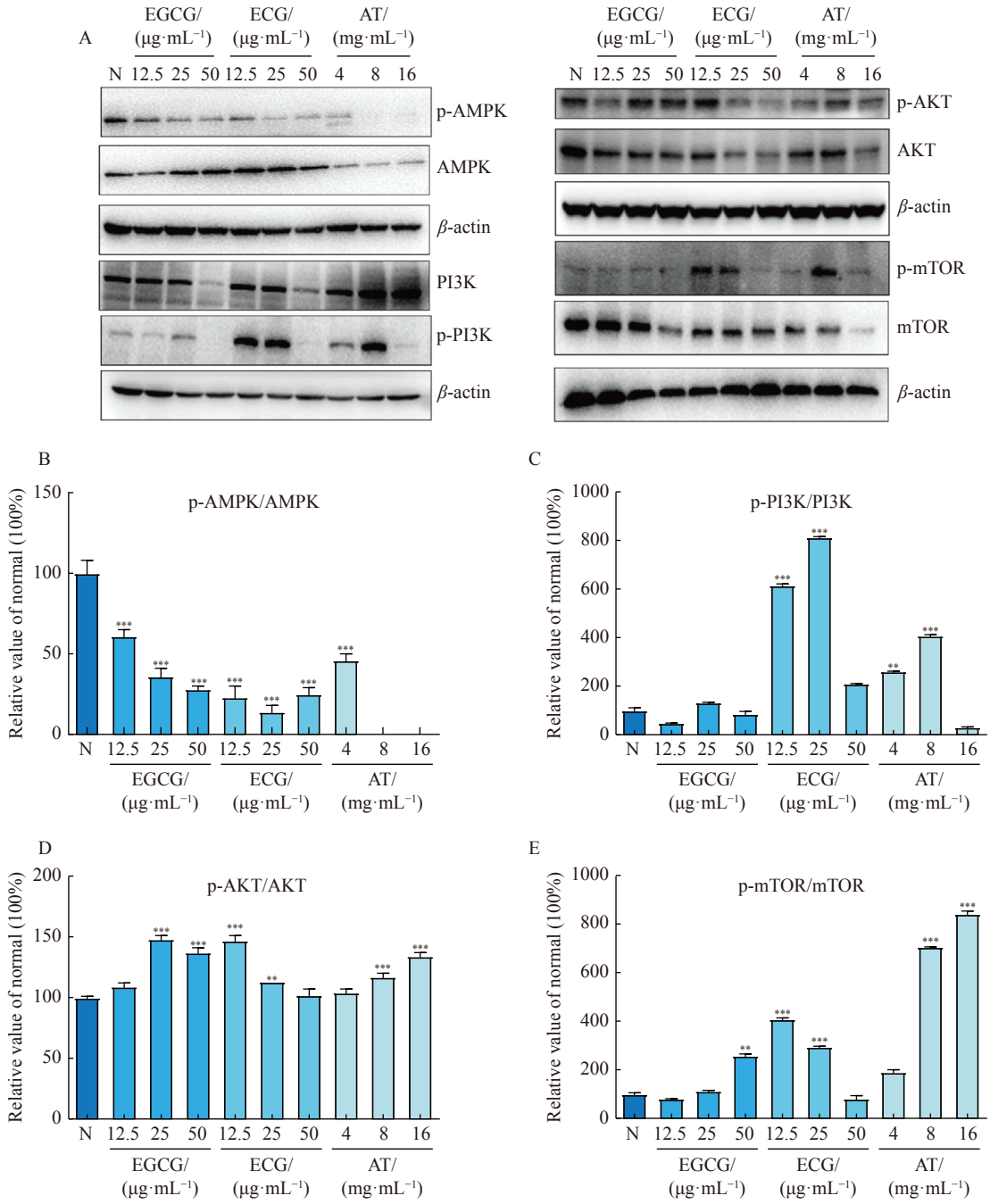

Fig. 7 EGCG and ECG decreased autophagy via the AMPK and PI3K/AKT/mTOR pathway in A375 cells. A: The autophagy-related protein expression levels of p-AMPK, AMPK, p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR in EGCG and ECG-treated A375 cells were detected by western blotting. (B, C, D, E): Quantitative analysis of the protein expression levels of p-AMPK, p-PI3K, p-AKT, p-mTOR in A375 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the control A375cells. The values were presented as mean ± SD ($n = 3$)

clear [46, 47]. Under some stimuli, they can appear simultaneously, while in others, they can appear independently. At the same time, autophagy can protect or promote apoptosis [48]. Our results showed that there is no change in the apoptotic trend upon activation of autophagy with the addition of rapamycin, indicating that apoptosis and autophagy are independent of each other.

Conclusion

In conclusion, EGCG and ECG have significant anticancer effects on A375 cells by inhibition of cell proliferation *via* induction of apoptosis and downregulation of autophagy through the AMPK/mTOR and PI3K/AKT/mTOR signaling pathway. These results indicated that EGCG and ECG may prove vital drugs and deserve further studies to investigate

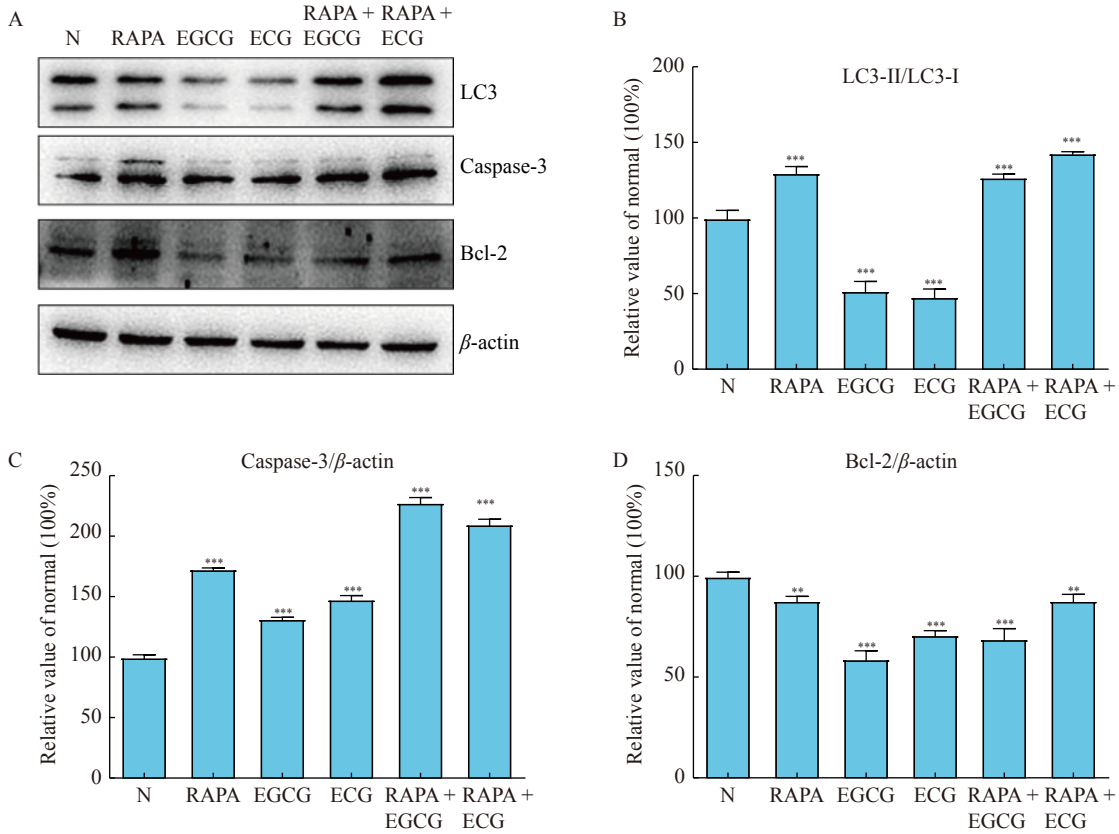

Fig. 8 Apoptosis and autophagy are independent of each other. A: A375 cells were treated with EGCG and ECG (50 μg·mL^{-1}) or combined with rapamycin (200 nmol·L^{-1}), protein expression levels of LC3, Caspase-3 and Bcl-2 were detected by western blotting. (B, C, D): Quantitative analysis of the protein expression levels of LC3, Caspase-3 and Bcl-2 in A375 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the control A375 cells. The values were presented as mean ± SD ($n = 3$). RAPA, rapamycin

whether they represent new agents for melanoma cancer therapy.

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Cite this article as: DU Bing-Xin, LIN Pei, LIN Jun. EGCG and ECG induce apoptosis and decrease autophagy *via* the AMPK/mTOR and PI3K/AKT/mTOR pathway in human melanoma cells [J]. *Chin J Nat Med*, 2022, **20**(4): 290-300.