

## AMPK mediates curcumin-induced cell death in CaOV3 ovarian cancer cells

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**Abstract.** AMP-activated protein kinase (AMPK), an evolutionarily conserved serine/threonine protein kinase, serves as an energy sensor in all eukaryotic cells. Recent findings suggest that AMPK activation strongly suppresses cell proliferation and induces cell apoptosis in a variety of cancer cells. Our study demonstrated that chemopreventive agent curcumin strongly activates AMPK in a p38-dependent manner in CaOV3 ovarian cancer cells. Pretreatment of cells with compound C (AMPK inhibitor) and SB203580 (p38 inhibitor) attenuates curcumin-induced cell death. We also observed that curcumin induces p53 phosphorylation (Ser 15) and both compound C and SB203580 pretreatment inhibit p53 phosphorylation. Collectively, our data suggest that AMPK is a new molecular target of curcumin and AMPK activation partially contributes to the cytotoxic effect of curcumin in ovarian cancer cells.

### Introduction

Curcumin, a major yellow pigment and active component of turmeric, is widely used as dietary spice and herb medicine in Indian subcontinent, south Asia and Japan. Endowed with numerous beneficial pharmacological actions, curcumin brings new therapy strategy to various disorders. Research

over the last few decades has shown that curcumin is a potent anti-inflammatory agent with strong therapeutic potential against a variety of cancers. Curcumin has been shown to suppress transformation (1), proliferation (2) and metastasis and angiogenesis (3) of tumors. These effects are mediated through its regulation of various transcription factors, growth factors, inflammatory cytokines, protein kinases and other enzymes (4). Recently, we found that curcumin attenuates EGF-induced AQP3 upregulation and cell migration in ovarian cancer cells (5). Yet no published study has considered AMPK as a molecular target of curcumin while berberine, an active component of *Coptidis rhizoma* with similar characteristics and pharmacological actions, is capable of activating AMPK in adipocytes (6).

AMPK-activated kinase (AMPK) has emerged as a key kinase controlling many cellular processes, particularly pathways involved in cellular energy status. AMPK is activated during metabolic stress, resulting in not only activating numerous energy producing metabolic pathways, but also inhibiting energy consuming pathways (7). In case of depletion of intracellular energy, intracellular ATP level drops and AMP level rises, leading to AMPK allosteric activation by redundant AMP. Also AMPK activation is regulated by several signal molecules known as AMPK kinase such as LKB1 (8) and CaM-dependent protein kinase kinase (9). So far, plenty of stimuli including leptin (10), metformin (11) and berberine (6) have been reported to induce AMPK activation.

Once activated, AMPK phosphorylates a number of distinct substrates including several rate-limiting enzymes involved in fatty acid and glucose metabolism, resulting in less ATP consuming and more ATP generation. Besides metabolic enzymes, p38 (12) and p53 (13) are reported as direct substrates of AMPK, indicating a complex biological effects of AMPK activation. Curcumin also induces p38 and p53 activation in ovarian cancer cells (14), thus the question was raised whether AMPK is involved in p38 and p53 activation in the presence of curcumin in CaOV3 ovarian cancer cells.

Previous research supports the view that AMPK activation during ischemia is cardioprotective (15,16) while inhibition of AMPK seems neuroprotective during ischemic brain injury

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(17,18). AMPK activation strongly suppresses cell proliferation in non-malignant cells (19) as well as in tumor cells (20,21). The AMPK signaling contains a number of tumor suppressor genes including LKB1 (8), p53 (13,22), TSC1 and TSC2 (23), and overcomes growth factor signaling from a variety of stimuli (24). These observations suggest that AMPK activation is a logical therapeutic target for diseases rooted in cellular proliferation, especially cancer. In fact, numerous published papers have demonstrated that AMPK activation by various stimuli induces cell cycle arrest and apoptosis in a variety of cancer cells (12,20,21).

We undertook this study to investigate whether AMPK is involved in curcumin-induced cell death in ovarian cancer cells and the cell signaling pathways associated with AMPK activation. We report for the first time that curcumin induces AMPK activation in ovarian cancer cells and p38 and p53 are involved in AMPK signaling. Our study further suggests that AMPK may be a new molecular target of curcumin.

## Materials and methods

**Chemicals and reagents.** SB203580 and compound C were purchased from Calbiochem (San Diego, CA). Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti- $\beta$ -actin was obtained from Sigma (St. Louis, MO). Phospho-AMPK (Thr172), phospho-p38 (Thr180/Tyr182), phospho-LKB1 (Ser428), phospho-ACC (Ser79), phospho-p53 (Ser 15) and cleaved caspase-3 (Asp175) were from Cell Signaling Technology (Beverly, MA).

**Cell culture.** Human ovarian cancer cells (CaOV3 cells) were maintained in DMEM medium (Sigma) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (1:100, Sigma) and 4 mM L-glutamine, in a CO<sub>2</sub> incubator at 37°C. For Western blotting, cells were reseeded in 6-well plates at a density of 0.2x10<sup>6</sup> cells/ml with fresh complete culture medium.

**Western blot analysis.** As described previously (25-28), cultured cells with and without treatments were washed with cold PBS and harvested by scraping into 150  $\mu$ l of RIPA buffer with protease inhibitor. Proteins (20-40  $\mu$ g) were separated by SDS-PAGE and transfer onto PVDF membrane (Millipore, Bedford, MA). After blocking with 10% milk in TBS, membranes were incubated with specific antibodies in dilution buffer (2% BSA in TBS) overnight at 4°C followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG at appropriate dilutions and room temperature for 1 h. Antibody binding was detected using ECL detection system from Amersham Biosciences (Piscataway, NJ) following manufacturer's instructions and visualized by fluorography with Hyperfilm.

**Cell viability assay (MTT dye assay).** Cell viability was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) method. Briefly, cells were collected and seeded in 96-well plates at a density of 2x10<sup>5</sup> cells/cm<sup>2</sup>. After incubation for 24 h, cells were exposed

to fresh medium containing reagents at 37°C. After incubation for certain period, 20  $\mu$ l of MTT tetrazolium (Sigma) salt dissolved in Hank's balanced salt solution at a concentration of 5 mg/ml was added to each well and incubated in CO<sub>2</sub> incubator for 4 h. Finally, the medium was aspirated from each well and 150  $\mu$ l of DMSO (Sigma) was added to dissolve formazan crystals and the absorbance of each well was obtained using a Dynatech MR5000 plate reader at a test wavelength of 490 nm with a reference wavelength of 630 nm.

**Statistical analysis.** The values in the figures are expressed as the means  $\pm$  standard error (SE). The figures in this study are representatives of more than three different experiments. Statistical analysis of the data between the control and treated groups was performed by a Student's t-test. Values of P<0.05 were considered as statistically significant.

## Results

**Curcumin induces AMPK activation in CaOV3 ovarian cancer cells.** First we examined whether curcumin induces AMPK activation in human ovarian cancer cells. Cells were treated with 50  $\mu$ M curcumin for different time (15, 30, 60 and 120 min) or curcumin at different concentration (10, 25, 50  $\mu$ M) for 60 min, cell lysate was then analyzed for phospho-AMPK by Western blotting as described above. As shown in Fig. 1A, curcumin induced AMPK activation in a time- and concentration-dependent manner. AMPK activation was observed within 15 min and peaked at 120 min. Twenty-five and 50  $\mu$ M but not 10  $\mu$ M of curcumin significantly induced AMPK activation (Fig. 1B and C). As LKB1 and acetyl-CoA carboxylases (ACC) are the most important upstream kinase and downstream substrate of AMPK respectively, we measured phospho-LKB1 and phospho-ACC. As shown in Fig. 1F, phospho-LKB1 level increased rapidly and peaked within 15 min, and then decreased after curcumin treatment (Fig. 1F and G). Curcumin stimulated ACC phosphorylation as early as 15 min and ACC activity remained elevated for 120 min (Fig. 1F and H).

**p38 mediates curcumin-induced AMPK activation.** As p38 is an important downstream signal of AMPK in most cases (12) and curcumin also activates p38 in human ovarian cancer cells (14), then we tested whether p38 is activated under our experimental condition and whether p38 is involved in AMPK signaling in the presence of curcumin. The results showed that p38 phosphorylation increased as early as 15 min and then decreased at 120 min after 50  $\mu$ M of curcumin treatment (Fig. 1A, D and E). Furthermore, our data showed that p38 mediates AMPK activation, since pretreatment with SB203580 (selective p38 inhibitor) blocked curcumin-induced AMPK phosphorylation (Fig. 2A and B) while compound C (selective AMPK inhibitor) did not affect p38 phosphorylation (Fig. 2A and C).

**Both AMPK and p38 inhibitors alleviates curcumin-induced cell death.** To investigate the anti-tumor effect of curcumin in CaOV3 cells, MTT dye assay was applied. The results showed that 10, 25 and 50  $\mu$ M of curcumin incubation for 24 h decreased the cell viability by 12, 37 and 62%,

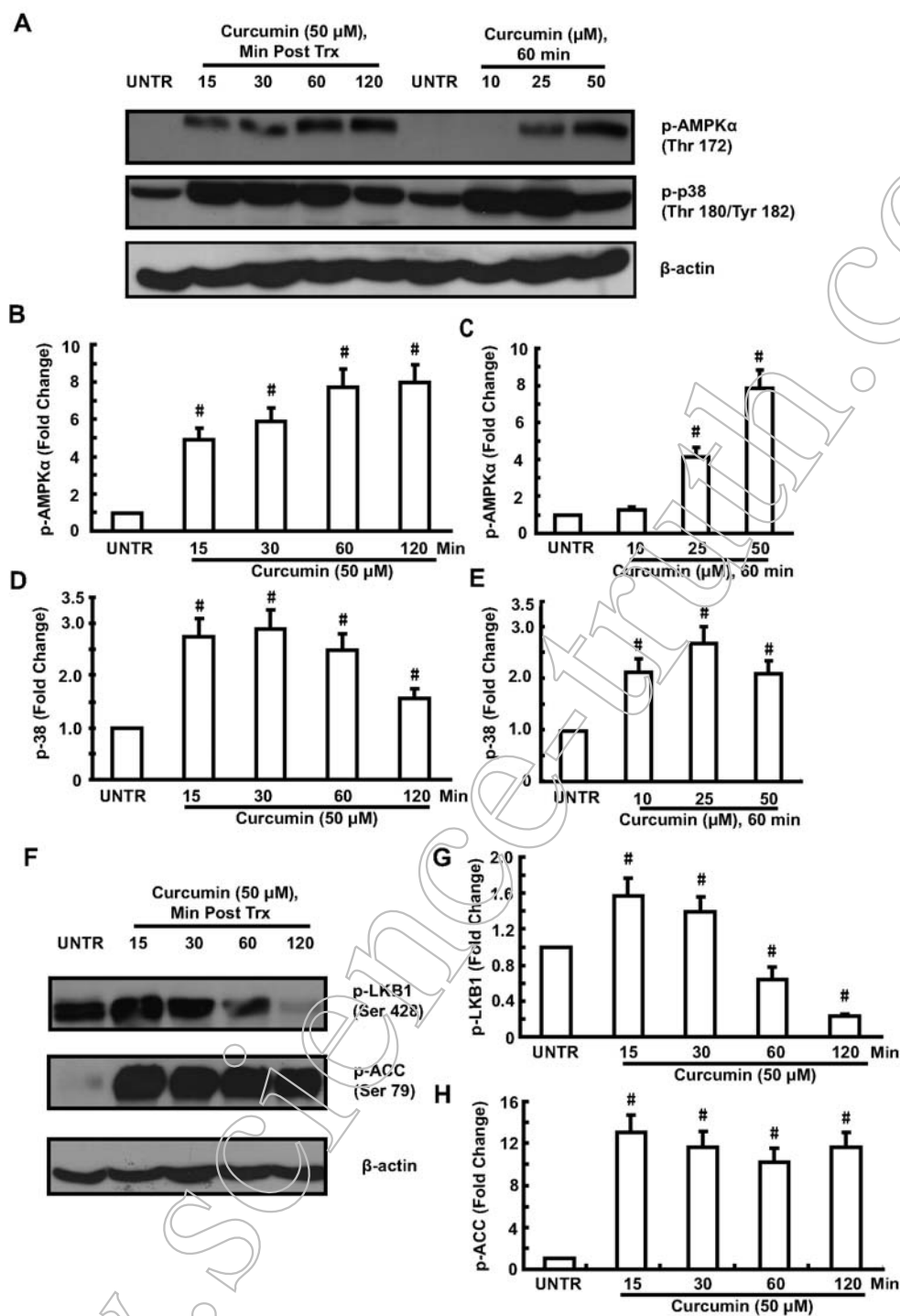


Figure 1. Curcumin induces AMPK and p38 activation in CaOV3 cells. CaOV3 cells were treated with 50  $\mu$ M curcumin for different time-points (15, 30, 60 and 120 min) or treated with curcumin at different concentration (10, 25, 50  $\mu$ M) for 60 min, phospho-AMPK (Thr 172) and phospho-p38 (Thr 180/Tyr 182) were detected by Western blotting (A). AMPK and p38 activation were quantified (B-E). Phospho-LKB1 and phospho-ACC were detected at 15, 30, 60 and 120 min post 50  $\mu$ M curcumin treatment (F). Phospho-LKB1 and phospho-ACC were quantified (G and H). \* $P$ <0.05 vs. untreated group. Data are presented as the mean  $\pm$  SEM for three independent experiments.

respectively ( $P$ <0.05) (Fig. 3A). Also, 25  $\mu$ M of curcumin incubation for 12, 24, 48 and 72 h decreased the cell viability to 88, 58, 27 and 9%, respectively ( $P$ <0.05) (Fig. 3B). The result indicated that curcumin induces CaOV3 cell death in a concentration- and time-dependent manner. Then we tested whether the cytotoxic effect of curcumin is mediated, at least partially, by AMPK activation. As shown in Fig. 3C, treat-

ment of the cells with 50  $\mu$ M curcumin for 24 h decreased the cell viability to 38% while pretreatment with compound C and SB203580 restored the cell viability to 70 and 62%, respectively. To confirm the result, cleaved caspase-3 was also analyzed. The results showed that curcumin strongly induced caspase-3 cleavage at 18 h, which was attenuated by compound C and SB203580 pretreatment (Fig. 3D and E).

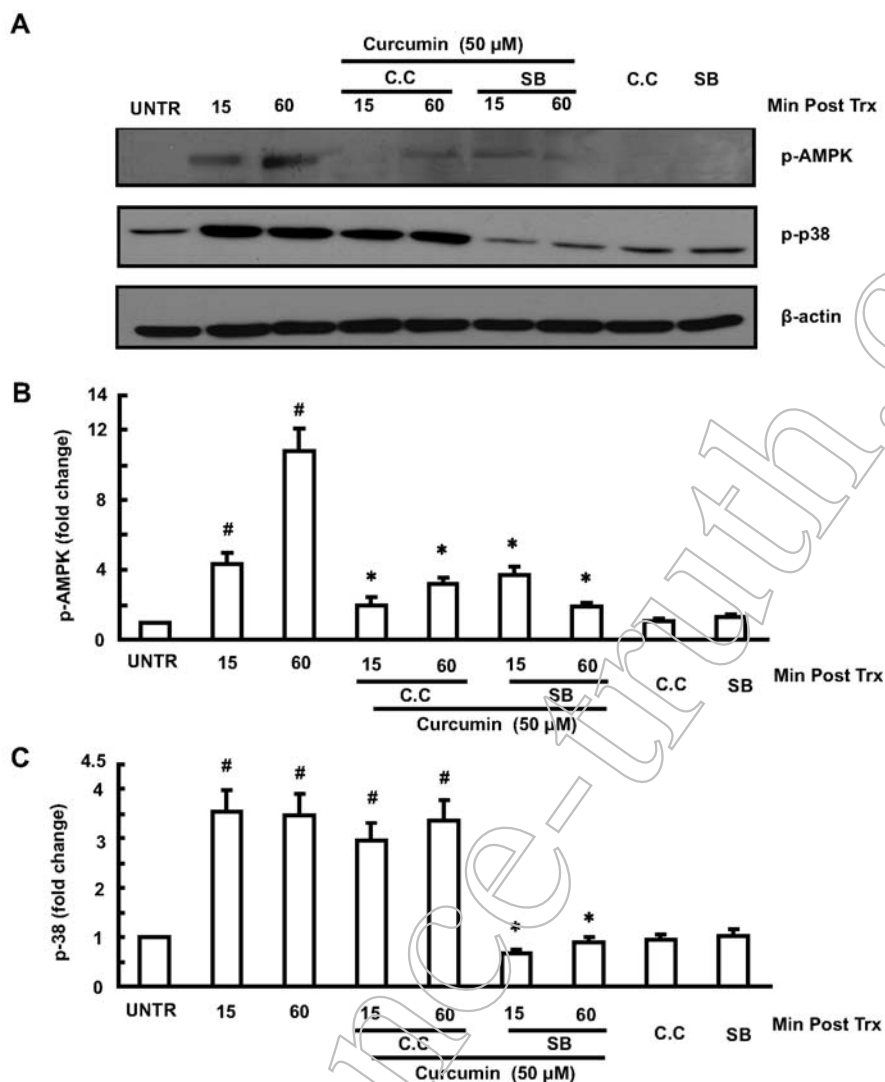


Figure 2. p38 mediates curcumin-induced AMPK activation. Cells were pretreated with 10  $\mu$ M SB203580 (p38 inhibitor) and 10  $\mu$ M compound C (AMPK inhibitor) 1 h before 50  $\mu$ M curcumin incubation. Cells were collected 15, 30 and 60 min after curcumin incubation and phospho-AMPK and phospho-p38 were detected. SB203580 (10  $\mu$ M) blocked curcumin induced AMPK activation whereas 10  $\mu$ M compound C had no effect on curcumin-induced p38 phosphorylation (A). AMPK and p38 activation were quantified (B and C). # $P$ <0.05 vs. untreated group. \* $P$ <0.05 vs. same time-point of curcumin treated group. Data are presented as the mean  $\pm$  SEM for three independent experiments. C.C, compound C; SB, SB203580.

*Both AMPK and p38 inhibitors attenuate curcumin-induced phosphorylation of p53.* Previous studies indicate that AMPK is a key regulator of p53 phosphorylation (13,22) and curcumin also induces p53 phosphorylation (29). The p53 tumor suppressor is associated with cell cycle arrest and apoptosis. Then we tested whether AMPK is a regulator of p53 phosphorylation after curcumin-treatment. As expected, compound C and SB203580 attenuated curcumin-induced p53 phosphorylation (Ser 15), indicating that AMPK and its upstream signal p38 regulate p53 phosphorylation (Fig. 4). This result suggests that AMPK may exert its pro-apoptotic effect through p53 activation.

## Discussion

Ovarian cancer is the most deadly cancer of the female reproductive system. Its high death rate is partly due to the lack of early detection and screening. Despite good initial

responses to chemotherapy, side effects and drug resistance are the main disadvantages of current chemotherapeutics. There is a great demand for drugs with low toxicity and high efficiency. Curcumin is one such agent, derived from turmeric, used for thousands of years in the Orient as a healing agent for various illnesses. Curcumin has been shown to suppress transformation, proliferation and metastasis of tumors. In phase I clinical studies, curcumin with doses up to 3600-8000 mg daily for 4 months did not result in discernible toxicities except mild nausea and diarrhea (30), indicating its high pharmacological safety.

Carcinogenesis is a multistep process in which several biochemical pathways and hundreds of molecules are deregulated. These include the growth factors and their receptors, cytokines, enzymes and gene regulating apoptosis and proliferation. Curcumin has been shown to target several of these molecules involved in carcinogenesis, including transcription factors such as NF- $\kappa$ B, STAT, AP-1 and Nrf-2,



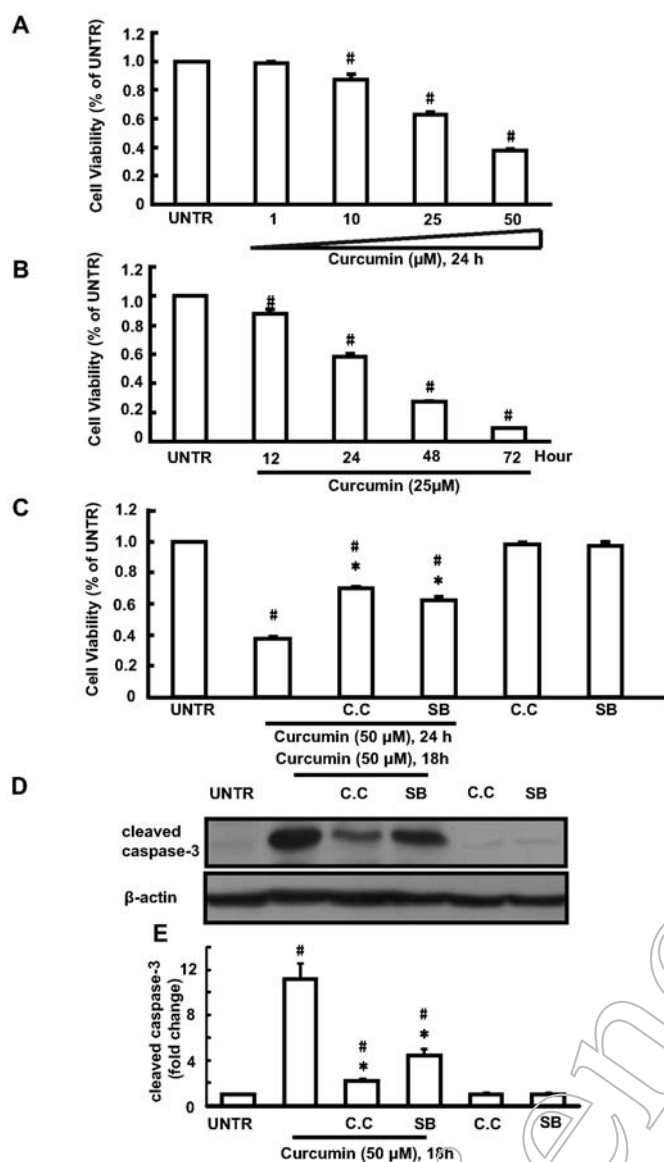


Figure 3. Compound C and SB203580 attenuate curcumin-induced cell death. Curcumin induced CaOV3 ovarian cancer cell death in a time- (A) and concentration-dependent manner (B). SB203580 (10  $\mu$ M) and 10  $\mu$ M compound C pretreatment alleviated cell death induced by 50  $\mu$ M curcumin incubation (C). Cell viability was determined by MTT assay. Cells were pretreated with 10  $\mu$ M SB203580 and 10  $\mu$ M compound C for 1 h followed by 50  $\mu$ M curcumin treatment. Cells were collected at 18 h after curcumin treatment. Cleaved caspase-3 was detected (D) and quantified (E). <sup>#</sup>P<0.05 vs. untreated group. \*P<0.05 vs. same time-point of curcumin treated group. Data are presented as the mean  $\pm$  SEM for three independent experiments.

TNF, cyclooxygenase-2, cyclin D1 and protein kinases such as EGFR and Mitogen-Activated protein kinases (4). Moreover, recently we found that curcumin is also targeting AQP3 in CaOV3 cells (5). Exploration of curcumin's molecular targets helps to comprehend its pharmacological effects and finally provides experimental data to guide its clinical application.

In the present study, we found that AMPK is another molecular target of curcumin in CaOV3 ovarian cancer cells. AMPK kinase LKB1 and AMPK substrate ACC are phosphorylated simultaneously by curcumin treatment, which further confirmed AMPK activation. As reported, p38

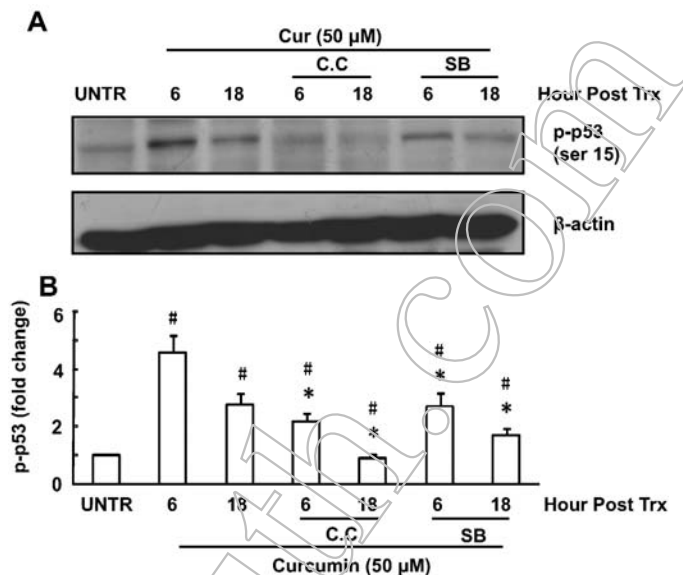


Figure 4. Compound C and SB203580 attenuate curcumin-induced p53 phosphorylation. Cells were pretreated with 10  $\mu$ M SB203580 and 10  $\mu$ M compound C for 1 h followed by 50  $\mu$ M curcumin treatment. Cells were collected at 6 and 18 h after curcumin treatment. Phospho-p53 (Ser 15) was detected (A) and quantified (B). <sup>#</sup>P<0.05 vs. untreated group. \*P<0.05 vs. same time-point of curcumin treated group. Data are presented as the mean  $\pm$  SEM for three independent experiments.

activation was also observed. Pretreatment with SB203580, a selective p38 inhibitor, almost abolished AMPK activation by curcumin whereas compound C, a selective AMPK inhibitor had no effect on p38 activation, indicating that p38 lies upstream of AMPK. Also, our data showed that curcumin induces apoptosis in CaOV3 cells and pretreatment with SB203580 and compound C attenuates curcumin induced cell death. These results indicate that AMPK and its upstream signal p38 contribute to the cytotoxic effect of curcumin in cancer cells. Curcumin-induced p53 phosphorylation was also weakened by SB203580 and compound C pretreatment which may partially explain why p38 and AMPK activation by curcumin is pro-apoptotic in cancer cells. Collectively, our findings suggest that AMPK is a neo molecular target of curcumin and its activation is associated with the cytotoxic effect of curcumin in ovarian cancer cells.

Besides switching the cell status from anabolic metabolism to catabolic mechanism, AMPK is believed to possess other functions such as regulation of cell proliferation and apoptosis. AMPK activation strongly represses cell growth and induces cell apoptosis in a variety of cancer cells (21,22), suggesting that AMPK is a new target of cancer therapy. It is not surprising that AMPK activation is pro-apoptotic in cancer cells, because the AMPK signaling contains a number of tumor suppressor genes including LKB1 (8), p53 (13,22), TSC1 and TSC2 (23). In this study, we found that curcumin induces AMPK phosphorylation as well as its upstream signal LKB1 and downstream signal ACC.

Tumor suppressor LKB1 is a 50 kDa serine/threonine kinase that was originally identified as the product of gene mutated in the autosomal dominantly inherited Peutz-Jepfers syndrome. LKB1 tumor suppressor kinase, in complex with the pseudokinase STRAD and the scaffolding protein

MO25, phosphorylates and activates AMPK (8). Hence, it is likely that curcumin induces LKB1 activation serving as an AMPK kinase. Further experiments are necessary by using LKB1 deficient cell to confirm this notion.

ACC or acetyl-CoA carboxylases, a rate limiting enzyme in fatty acid synthesis, is phosphorylated and inactivated by AMPK. Series of studies showed that lipogenic enzymes are upregulated or hyper activated in cancer cells, inhibition of FAS or ACC limits cancer cell growth and survival (31,32). Thus, curcumin-induced ACC phosphorylation via AMPK activation may also contribute to the cytotoxicity of curcumin in ovarian cancer cells.

Most studies indicate that p38, a mitogen-activated protein kinase (MAPK) regulated by stress and cytokines, is another important downstream molecule of AMPK signaling. Activation of p38 is involved in inflammation, cell growth and differentiation as well as cell survival and apoptosis. As curcumin induces p38 activation in ovarian cancer cells, we tested the relationship between p38 and AMPK. In contrast to previous studies, we found that curcumin-induced AMPK activation is p38-dependent. In addition, p38 phosphorylation increased as early as 15 min but decreased at 120 min when AMPK phosphorylation was still on a rise (Fig. 1A). These data suggest that p38 activation is an early event in comparison to AMPK activation. This notion is supported by a recent report that AMPK is downstream of p38 MAPK, mediating the effects of adenosine on glucose utilization in hearts stressed by transient ischemia (33). Thus, it is conceivable that AMPK signaling may vary upon different stimuli in different cell types.

AMPK activation possesses multiple functions as discussed above. However, in this work, our main concern is whether this AMPK activation by curcumin is pro-apoptotic in cancer cells. As both AMPK and p38 inhibitors alleviate curcumin induced cell death in CaOV3 ovarian cancer cells, we propose that AMPK contributes to the cytotoxicity induced by curcumin in cancer cells. AMPK activation strongly suppresses cell proliferation and induces cell cycle arrest in a variety of cells. This may be achieved by regulation of p53 tumor suppressor. Several lines of evidence have indicated that AMPK positively regulates p53 phosphorylation (Ser 15) and causes accumulation of p53 protein as well as its downstream p21 and p27 (22). We found that AMPK inhibitor and p38 inhibitor attenuate but not abolish curcumin-induced upregulation of phospho-p53, suggesting that AMPK plays a certain role in curcumin-induced upregulation of p53 phosphorylation. Our data suggest that regulation of p53 phosphorylation by AMPK is important for the cytotoxic effect of curcumin. Besides p53, mTOR, the principal activator of cell growth and survival in response to growth factor, is reported to be inhibited by AMPK (23,24). Thus, whether curcumin inhibits mTOR through AMPK activation and exerts anti-tumor activity warrants further investigation.

In summary, our study demonstrated for the first time that AMPK is a new molecular target of curcumin and this AMPK activation partially accounts for the cytotoxicity of curcumin in ovarian cancer cells. Our data provide insights into understanding of the molecular mechanism of curcumin's effect on cancer cell death and may offer better clinical management of ovarian cancer.

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