

## Original Article

# The role of AMP-activated protein kinase in quercetin-induced apoptosis of HL-60 cells

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**Our previous studies have shown that quercetin inhibits Cox-2 and Bcl-2 expressions, and induces human leukemia HL-60 cell apoptosis. In order to investigate the role of AMP-activated protein kinase (AMPK) on quercetin-induced apoptosis of HL-60 cells, we used flow cytometry to detect cell apoptosis. The expressions of LKB1, phosphorylated AMPK (p-AMPK), and Cox-2 protein were detected in HL-60 cells and normal peripheral blood mononuclear cells (PBMCs) by western blot. The expressions of LKB1, p-AMPK, and Cox-2 were detected in HL-60 cells after culture with quercetin. The expressions of p-AMPK were detected in HL-60 cells after culture with AMPK inhibitor Compound C. Then, the expressions of LKB1, p-AMPK, and Cox-2 were detected in HL-60 cells after culture with quercetin alone or quercetin + Compound C. It was found that there was no significant difference in LKB1 between PBMCs and HL-60, p-AMPK in PBMCs was higher than that in HL-60, while Cox-2 was lower. After culture of HL-60 with quercetin, p-AMPK was increased, Cox-2 was decreased, but LKB1 remained unchanged. After culture of HL-60 with Compound C, p-AMPK was decreased. There was no significant difference in LKB1 between the quercetin-alone and the quercetin + Compound C groups. p-AMPK decreased more significantly, while Cox-2 increased more significantly in the quercetin + Compound C groups than those in the quercetin-alone groups. Taken together, these findings suggested that quercetin activates AMPK expression in HL-60 cells independent of LKB1 activation, inhibits Cox-2 expression by activating AMPK, and further regulates the Bcl-2-dependent pathways of apoptosis to exert its anti-leukemia effect.**

**Keywords** leukemia; quercetin; AMP-activated protein kinase; cyclooxygenase-2

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## Introduction

The focus of research on anti-tumor drugs in recent years has been transferred from cytotoxic agents to target-specific new drugs for the purpose of improving safety, selectivity, and drug resistance. Drugs from natural plants that possess extensive mechanisms of action and low toxicity have become the hot point of research. Quercetin (3,3',4',5,7-pentahydroxyflavone) is a flavonoid compound widely existing in nature. Recent studies [1–5] have reported that quercetin can inhibit the growth of various malignant tumor cells including leukemia. Our previous study [1] found that quercetin could inhibit Cox-2 expression, reduce Bcl-2 expression, and induce Bax expression and the apoptosis of HL-60 cells by initiating a caspase-3 cascade reaction. However, further research is still needed to explore the upstream signaling molecule for Cox-2.

Tumorigenesis is closely associated with the energy metabolism of tumor cells. AMP-activated protein kinase (AMPK) participates in the development and progression of tumors by regulating cellular energy [6,7]. Activation of AMPK can inhibit the proliferation of tumor cells and induce their apoptosis. Cox-2 may be a site of action downstream of AMPK [8]. LKB1 is a generally existing anti-oncogene and its mechanism of action includes promoting apoptosis through release of cytochrome C (CYC) and inhibition of tumor angiogenesis by reducing the generation of vascular endothelial growth factor [9–11]. Studies have demonstrated that LKB1 is a protein kinase upstream of AMPK, able to phosphorylate and activate AMPK [11,12]. It was found that quercetin could induce apoptosis by activating the expression of AMPK in some solid tumor cell lines [6]. However, there is no study reporting the effect of AMPK in acute leukemia. The aim of the present study was to further explore the signal transduction pathway of leukemia cell apoptosis induced by quercetin, and to find the relationship between quercetin and AMPK, the effect of AMPK in leukemia, and the therapeutic target specific to AMPK.

## Materials and Methods

### Main reagents

Main reagents used in this study were quercetin (Sigma Chemical, St. Louis, USA); rabbit anti-human LKB1 IgG monoclonal antibody, rabbit anti-human phosphorylated AMPK (p-AMPK) IgG monoclonal antibody, and rabbit anti-human  $\beta$ -actin IgG monoclonal antibody (Cell Signaling Technology, Boston, USA); mouse anti-human Cox-2 IgG monoclonal antibody (Cayman Chemical, Ann Arbor, USA); and Compound C (Merck, Darmstadt, Germany).

### Culture of human acute myeloid leukemia cell lines

HL-60 and HL-60/A cell strains were purchased from Tianjin Institute of Hematology of the Chinese Academy of Medical Sciences (Tianjin, China). After resuscitation of HL-60 and HL-60/A cells, they were seeded in RPMI 1640 containing 10% fetal bovine serum and 3% glutamine, cultured in a 37°C 5% CO<sub>2</sub> incubator, and passaged with medium changed at 2- or 3-day intervals. The cells in the logarithmic growth phase were used for the subsequent experiments.

### Isolation of peripheral blood mononuclear cells from normal subjects

Peripheral blood mononuclear cells (PBMCs) were collected from the healthy subjects who underwent routine physical examinations. Whole venous blood was drawn from the normal subjects aseptically, treated with ethylenediaminetetraacetic acid anti-coagulation, diluted with 2-fold volume of phosphate-buffered saline (PBS; pH 7.2), and mixed well. The cell suspension was added with caution to the lymphocyte separation liquid equal in volume to the blood, and centrifuged horizontally at 500 g at room temperature for 20 min. The PBMCs at the junction of the plasma layer and the lymphocyte separation liquid were sucked out, added with the equal amount of PBS, mixed well, and centrifuged at 500 g for 10 min. After discarding the supernatant, the cells were washed twice to remove the residual lymphocyte separation liquid.

### Cell apoptosis by flow cytometry

After treatment with various concentrations (0, 25, 50, and 100  $\mu$ M) of quercetin for 48 h, the cells were washed twice with PBS and diluted to a final concentration of  $1 \times 10^6$  cells/ml, and then incubated with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 30 min in the dark. The cell apoptosis was determined by flow cytometry (Becton-Dickinson, Temse, Belgium). Early stage of cell apoptosis was defined as Annexin V-FITC<sup>+</sup>PI<sup>-</sup> and late stage as Annexin V-FITC<sup>+</sup>PI<sup>+</sup>.

### Detection of LKB1, p-AMPK, Cox-2, and $\beta$ -actin protein expressions in HL-60 and HL-60/A cells, and PBMCs by western blotting

Total protein was extracted and the concentration was measured. Protein sample was loaded onto plate wells (50  $\mu$ g per well), treated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% skimmed milk at 37°C for 1 h and then incubated with the primary antibody (LKB1, 1 : 1000; p-AMPK, 1 : 1000; Cox-2, 1 : 1000;  $\beta$ -actin, 1 : 1000) on the rocking bed overnight at 4°C. After three washes with TBST, the PVDF membranes were placed in the blocking solution containing 1 : 10,000 horseradish peroxidase-labeled goat anti-mouse IgG monoclonal antibody (Cox-2) or goat anti-rabbit IgG monoclonal antibody (LKB1, p-AMPK and  $\beta$ -actin), shaken gently at room temperature for 1 h. After wash with TBST, membranes were detected with DBA kit (Pulilai, Beijing, China). The images were analyzed using the gel analysis software (Quantity one, Bio-Rad, USA).

### The effect of quercetin on LKB1, p-AMPK, and Cox-2 protein expressions in HL-60 cells

After co-culture with different concentrations (0, 25, 50, and 100  $\mu$ M) of quercetin for 48 h, HL-60 cells were harvested to detect the protein expressions of LKB1, p-AMPK, and Cox-2 by western blotting.

### The effect of AMPK inhibitor Compound C on p-AMPK protein expression in HL-60 cells

The p-AMPK protein expression in HL-60 cells was detected 48 h after co-culture of HL-60 cells with different concentrations (0, 5, 10, 25, 50, 75, and 100  $\mu$ M) of Compound C.

**The effect of quercetin plus Compound C on LKB1, p-AMPK, and Cox-2 protein expressions in HL-60 cells**  
LKB1 and Cox-2 expressions were detected after inhibition of Compound C on quercetin-activated AMPK to determine the effect of AMPK on apoptosis of the quercetin-induced leukemia cells. The experiment was conducted in five groups: the control group (without addition of quercetin), the 50  $\mu$ M quercetin group, the 50  $\mu$ M quercetin + 25  $\mu$ M Compound C group, the 100  $\mu$ M quercetin group, and the 100  $\mu$ M quercetin + 25  $\mu$ M Compound C group. After 48 h co-culture with HL-60 cells, the expression levels of LKB1, p-AMPK, and Cox-2 protein were detected by western blotting.

### Statistical analysis

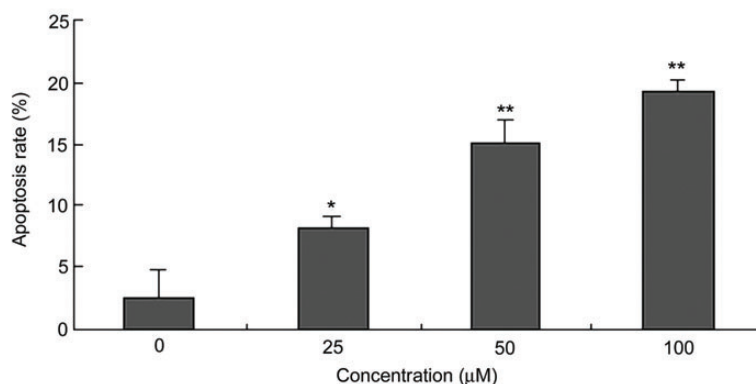
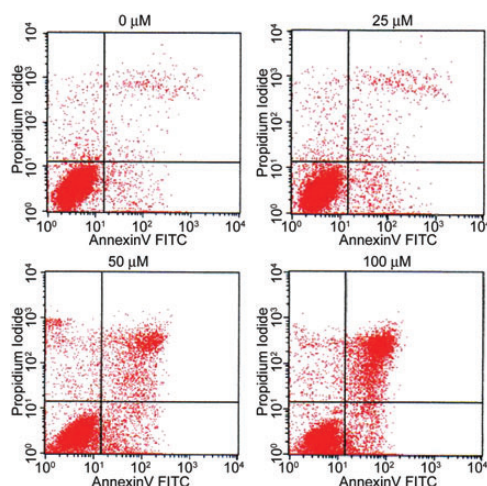
Measurement data are expressed as mean  $\pm$  standard deviation. Comparison of the mean values of two independent specimens was conducted using Student's *t*-test. Comparison

of the mean values between the multiple groups was conducted using single factor analysis of variance. Correlations between variants were analyzed using Pearson correlation analysis. All data were treated using SPSS 16.0 statistical software.  $P < 0.05$  was considered statistically significant.

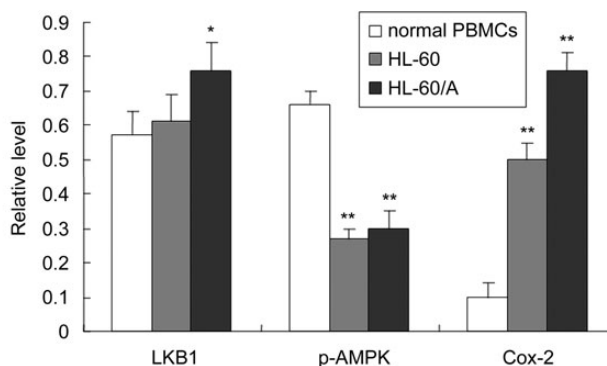
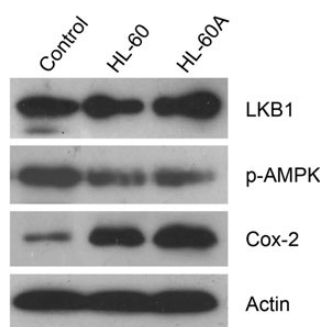
## Results

### Quercetin induced HL-60 cell apoptosis

The cells were treated with quercetin for 48 h and the apoptosis rate was then calculated by flow cytometry. The average of apoptosis rate at early stage was 2.4%, 8.2%, 15.06%, and 19.29% when treated with quercetin at 0, 25, 50, and 100  $\mu\text{M}$ , respectively (Fig. 1). The  $P$  value was  $< 0.05$  between the control group (0  $\mu\text{M}$ ) and the group treated with 25  $\mu\text{M}$  quercetin, and was  $< 0.01$  between the control group and the other two treatment groups (50 and 100  $\mu\text{M}$ ), suggesting that quercetin significantly suppressed cell growth by inducing apoptosis.



**Figure 1. Apoptosis induced by quercetin in HL-60 cells** The cells were treated with quercetin at the concentrations of 0, 25, 50, and 100  $\mu\text{M}$ , respectively, for 48 h. The apoptosis rate was calculated by flow cytometry. Cell apoptosis at early stage was defined in Annexin V-FITC<sup>+</sup>PI<sup>-</sup>. \* $P < 0.05$  and \*\* $P < 0.01$  compared with the control.



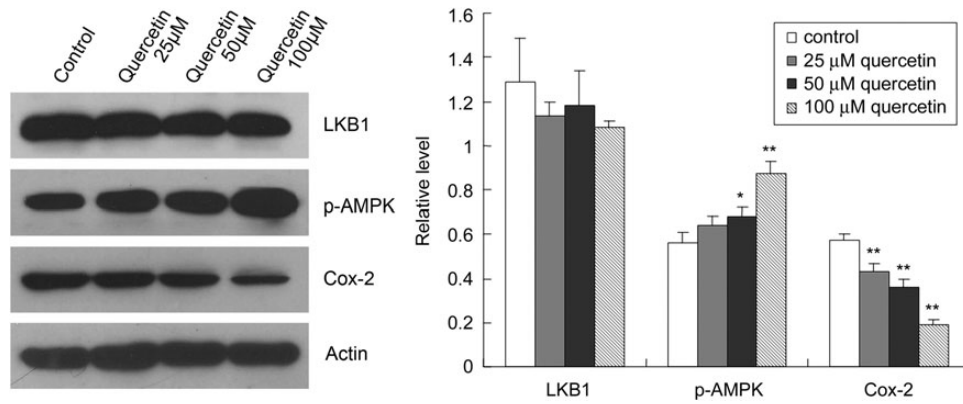
**Figure 2. LKB1, p-AMPK, and Cox-2 protein expression levels in normal PBMCs, HL-60, and HL-60A cells** L: Western blot protein expression profiles. R: Statistical charts (\* $P < 0.05$  compared with the control group; \*\* $P < 0.01$  compared with the control group).

### LKB1, p-AMPK, and Cox-2 protein expression levels in normal PBMCs, HL-60, and HL-60/A cells

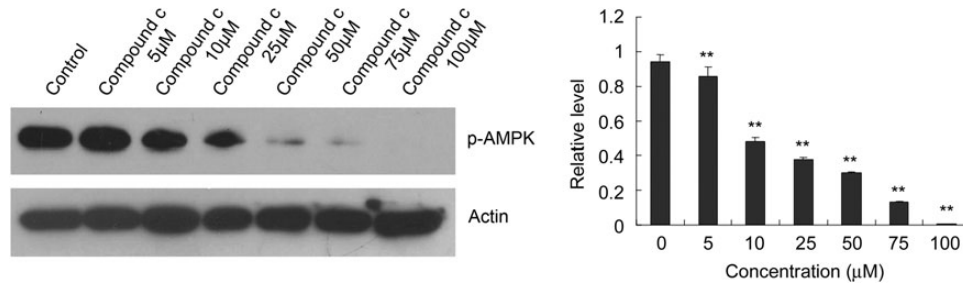
There was no significant difference in the LKB1 protein expression between the normal PBMCs and HL-60 cells ( $P = 0.61$ ), while the LKB1 expression in HL-60/A cells was significantly higher than that in normal PBMCs ( $P < 0.05$ ). The p-AMPK expression in the normal PBMCs was significantly higher than that in the leukemia HL-60 and HL-60/A cells ( $P < 0.01$ ). The Cox-2 expression in the normal PBMCs was relatively low, while it was significantly elevated in HL-60 and HL-60/A cells ( $P < 0.01$ ). The Cox-2 expression level in the HL-60/A cells was 1.52 fold as high as that in the HL-60 cells (Fig. 2).

### The effect of quercetin on LKB1, p-AMPK, and Cox-2 protein expressions in HL-60 cells

There was no significant difference in LKB1 expression before and after quercetin treatment ( $P > 0.05$ ). The p-AMPK protein expression level was increased, and the Cox-2 protein



**Figure 3. The effect of quercetin on LKB1, p-AMPK, and Cox-2 protein expressions in HL-60 cells** The protein expression level was detected by western blotting 48 h after culture with 0, 25, 50, and 100 µM quercetin. L: Western blot protein expression profiles of LKB1, p-AMPK, and Cox-2. R: Statistical charts (\* $P < 0.05$ , compared with the control group; \*\* $P < 0.01$  compared with the control group).



**Figure 4. The effect of Compound C on p-AMPK protein expression in HL-60 cells** HL-60 cells were cultured with different concentrations of Compound C (0, 5, 10, 25, 50, 75, and 100 µM) for 48 h to observe the activation of AMPK. L: Western blot protein expression profiles of p-AMPK. R: Statistical chart (\*\* $P < 0.01$  compared with the control group).

expression level was decreased. The higher the quercetin concentration, the more pronounced was the change in p-AMPK and Cox-2 expressions. There was no significant difference in p-AMPK expression between the 25 µM quercetin group and the control group. The p-AMPK expression level in the 50 µM quercetin group was 1.29 fold as high as that in the control group ( $P < 0.05$ ). The p-AMPK expression level in the 100 µM quercetin group was 1.55 fold as high as that in the control group ( $P < 0.01$ ). The Cox-2 expression levels in the experiment groups were all significantly lower than that in the control group ( $P < 0.01$ ) (Fig. 3).

**The effect of AMPK inhibitor Compound C on p-AMPK protein expression in HL-60 cells**

Compound C inhibited AMPK activation in a dose-dependent manner ( $P < 0.01$ ). At 25 µM Compound C, the p-AMPK expression decreased to 40.42% of the control group. With the increase of the Compound C concentration, the p-AMPK expression decreased correspondingly. When the Compound C concentration increased to 100 µM, the p-AMPK expression was at a very low level. Because 25 µM Compound C

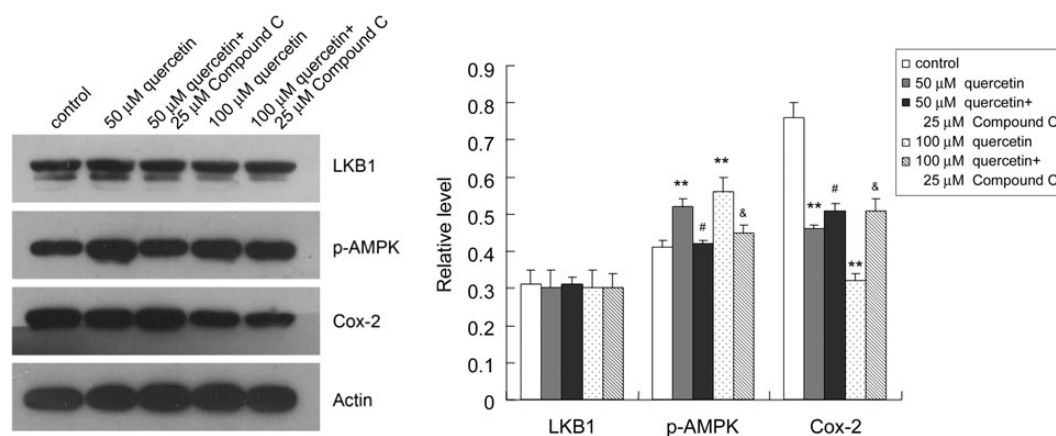
was able to inhibit AMPK activation, this concentration was used for the subsequent experiments (Fig. 4).

**The effect of quercetin plus Compound C on LKB1, p-AMPK, and Cox-2 protein expressions in HL-60 cells**

Compared with the control group, there was no significant difference in the LKB1 expression in HL-60 cells in either the quercetin-alone groups or the quercetin + Compound C groups ( $P > 0.05$ ). Compared with the quercetin-alone groups, p-AMPK in the quercetin + Compound C groups was decreased ( $P < 0.05$ ). Compared with the 50 µM quercetin group, the p-AMPK expression was decreased by 19% in the 50 µM quercetin + 25 µM Compound C group. Compared with the 100 µM quercetin group, the p-AMPK expression was decreased by 20% in the 100 µM quercetin + 25 µM Compound C group. Compared with the quercetin-alone groups, the Cox-2 expression was increased in the quercetin + Compound C groups ( $P < 0.05$ ). The Cox-2 protein expression in the 50 µM quercetin + 25 µM Compound C group was 1.11 fold as high as that in the 50 µM quercetin-alone group. Cox-2 protein expression in the 100 µM quercetin + 25 µM

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**Figure 5.** The effect of quercetin plus Compound C on LKB1, p-AMPK, and Cox-2 protein expressions in HL-60 cells After 48 h treatment of HL-60 cells in different groups (control, 50  $\mu$ M quercetin, 50  $\mu$ M quercetin + 25  $\mu$ M Compound C, 100  $\mu$ M quercetin, and 100  $\mu$ M quercetin + 25  $\mu$ M Compound C), cells were harvested to detect LKB1, p-AMPK, and Cox-2 protein expression levels by western blot. L: Western blot protein expression profiles. R: Statistical chart (\*\* $P < 0.01$  compared with the control; # $P < 0.05$  compared with the 50  $\mu$ M quercetin-alone group; & $P < 0.05$  compared with the 100  $\mu$ M quercetin-alone group).

Compound C group was 1.59 fold as high as that in the 100  $\mu$ M quercetin-alone group (Fig. 5).

## Discussion

Studies [1–4] have shown that quercetin could inhibit various tumor cells including leukemia cells, mainly through regulating cell growth, proliferation, and apoptosis signaling pathways such as mitogen-activated protein kinases and phosphatidylinositol-3-kinase/protein kinase B, and the key target points are nuclear factor- $\kappa$ B and Cox-2. Our previous study [1] showed that quercetin could inhibit Cox-2 expression, reduce Bcl-2 expression, and induce Bax expression and the apoptosis of HL-60 cells by initiating caspase-3 cascade reaction. Based on these findings, we made a further research to explore the mechanism of leukemia cell apoptosis induced by quercetin.

AMPK is the regulator of energy metabolism of cells and is believed to be closely associated with tumorigenesis. AMPK activation can inhibit the proliferation of various tumor cells [7]. Metformin is a potent activator of AMPK. It was found in recent years that metformin could be used to prevent tumor occurrences [13], probably due to AMPK activation. Green *et al.* [12] found that metformin had an obvious action against myeloid leukemia by reducing the growth of leukemia cells in nude mice via affecting the LKB1/AMPK pathway. They also pointed out that the treatment targeting at the LKB1/AMPK signaling pathway may be a promising strategy for the treatment of leukemia. Sengupta *et al.* [14] found that the AMPK activator AICAR could inhibit various acute lymphocyte leukemia cell strains and induce their apoptosis by activating AMPK, and arrest the cell cycle at G1 phase. LKB1 is an anti-oncogene and highly expressed in the PBMCs of normal people. LKB1 gene mutation in a variety of solid tumors could lead to LKB1 inactivation and non-expression or

low-expression of LKB1 [10,15,16]. However, the state of LKB1 and AMPK in leukemia cells remains unclear. It was found in this study that LKB1 protein expression in leukemia HL-60 and HL-60/A cell strains was not decreased, suggesting that LKB1 gene does not undergo mutation in HL-60 and HL-60/A cells. p-AMPK is an activated state of AMPK, a state that can exert its function. For this reason, we detected p-AMPK in the experiments. The result showed that the p-AMPK expression in HL-60 and HL-60/A cells was significantly lower than that in the normal PBMCs. Previous studies [6,7] have demonstrated that AMPK is closely associated with tumor development and progression. The abnormally low expression of p-AMPK in HL-60 cells suggests that the treatment targeting at AMPK may help cure acute leukemia.

Several studies reported that quercetin could activate the expression of AMPK. Lee and Park [17] reported that quercetin could activate AMPK of breast cancer MCF-7 cells and induce tumor cell apoptosis by affecting the Akt pathway. Kim *et al.* [18] found that quercetin could induce AMPK activation and apoptosis of colon cancer HT-29 cells in a p53-dependent manner. Ahn *et al.* [19] found that quercetin could induce apoptosis of fat cells by activating AMPK. Lee *et al.* [8] reported that quercetin could induce the apoptosis of breast cancer and colon cancer cells via the regulatory effect of the AMPK/Cox-2 pathway. However, whether quercetin could activate the expression of AMPK in HL-60 cells has not been reported in the literature. We therefore detected the changes in p-AMPK expression before and after quercetin intervention and confirmed that quercetin was also able to activate AMPK in HL-60 cells. After 48 h culture with quercetin, no significant change in LKB1 expression was observed in HL-60 cells. We postulated that the activating effect of quercetin on AMPK of HL-60 cells is not dependent on LKB1. Zhang *et al.* [20] also reported the

similar phenomenon, saying that galangin activated AMPK and inhibited the proliferation of hepatocellular carcinoma cells in an LKB1-independent way. In our study, we tested the total LKB1 protein level but not the phosphorylation status of LKB1. Although some studies suggested that phosphorylation of LKB1 is necessary to allow it to phosphorylate and activate AMPK and other downstream kinases, others have suggested that LKB1 is constitutively active and is not rate-limiting for the activation of AMPK [21,22]. In our future study, we will explore LKB1's effect on quercetin-induced apoptosis using siRNA to down-regulate LKB1 expression.

Our previous study [1] has demonstrated that Cox-2 is the key target point in the apoptosis of quercetin-induced human leukemia HL-60 cells. Lee *et al.* [8] reported that quercetin induced tumor cell apoptosis via the AMPK/Cox-2 pathway in breast cancer MCF-7 cells and colon cancer HT-29 cells. Quercetin activated AMPK by stimulating the generation of reactive oxygen species. Park *et al.* [23] found that catechin, another plant flavonoid, could induce the apoptosis of colon cancer cells by activating the AMPK/Cox-2 pathway in a low-dose H<sub>2</sub>O<sub>2</sub> condition. Hwang *et al.* [24] showed that genistein could inhibit the proliferation of tumor cells by activating AMPK and further affecting Cox-2. Quercetin expresses its anti-tumor function by a complicated mechanism. AMPK/Cox-2 pathway may be one part of the complicated mechanism. Reports demonstrated that quercetin could induce cancer cell apoptosis through the p53 pathway [25]. Suppressing the expression of heat-shock protein in cancer cells was also one of the pathways for quercetin to induce cell apoptosis [26]. It was found in our study that when quercetin-activated AMPK was inhibited by Compound C, the expression of Cox-2 that was originally inhibited by quercetin began to increase, suggesting that AMPK is an upper stream kinase of Cox-2; quercetin-mediated AMPK activation of HL-60 cells inhibited the expression of Cox-2, thus inducing the apoptosis of HL-60 cells. The treatment targeting at AMPK, a key regulator of energy metabolism of tumor cells, may be a promising strategy for acute leukemia.

In summary, abnormally low expression of p-AMPK and abnormally high expression of Cox-2 were observed in human acute leukemia HL-60 and HL-60/A cells. Quercetin could activate AMPK and further inhibit the expression of Cox-2 independent of LKB1 activation. Based on this finding and the results of previous studies [1], we postulate that quercetin inhibits the expression of anti-apoptosis gene Bcl-2 and increases the expression of pro-apoptotic gene Bax by activating AMPK and further inhibiting Cox-2 expression, and eventually initiates the caspase-3-dependent cascade reaction to induce the apoptosis of HL-60 cells. AMPK and Cox-2 may become potential targets for the treatment of acute leukemia.

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