Inhibition of Autophagy Enhances Anticancer Effects of Atorvastatin in Digestive Malignancies

Pei-Ming Yang¹, Yuan-Ling Liu¹, Yi-Chu Lin¹, Chia-Tung Shun², Ming-Shiang Wu³, and Ching-Chow Chen⁴

Abstract

Preclinical and clinical studies have shown that statins, the 3-hydroxy-3-methylglutaryl–CoA reductase inhibitors with cholesterol-lowering properties, exhibited anticancer effects. However, the underlying mechanisms remain ill defined. In this study, we showed that atorvastatin could inhibit the growth of hepatocellular carcinoma (HCC) and colorectal carcinoma (CRC) cells via induction of apoptosis. Atorvastatin also induced autophagy that is a physiologic process involved in the turnover of intracellular organelles. Atorvastatin-induced autophagy was found to be inhibited by AMP-activated protein kinase (AMPK) small interfering RNA. Examination of HCC patients showed the positive correlation between AMPK activity and autophagic marker (beclin-1). Atorvastatin-induced AMPK activation could induce p21 expression, which was also positively correlated with beclin-1 expression in CRC patients. AMPK/p21 signaling caused endoplasmic reticulum (ER) stress response leading to the induction of autophagy. Inhibition of autophagy by an autophagic inhibitor bafilomycin A1 or genetic knockout of autophagy-related gene 5 enhanced atorvastatin-induced cytotoxicity and apoptosis. In summary, activation of AMPK by atorvastatin enhances p21 expression and ER stress response, leading to autophagy, which promotes survival of cancer cells. Combinations of atorvastatin with bafilomycin A1 provide a novel and promising strategy to improve the treatment of digestive malignancies.

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Introduction

Statins, the 3-hydroxy-3-methylglutaryl (HMG)–CoA reductase inhibitors, are among the most widely prescribed drugs to lower cholesterol and prevent cardiovascular diseases (1, 2). By depleting precursors of the mevalonate pathway and its various downstream products with essential roles in cell cycle progression, cellular signaling, and membrane integrity, statins may affect various cellular effects (1, 2). Accordingly, some observational studies screen the effect of statins on the incidence of various cancers as part of the controlled trials on cardiovascular outcomes. Among them, a consistent association of decreased risk is found for hepatocellular carcinoma (HCC) and colorectal carcinoma (CRC; refs. 3–6). Furthermore, experimental data have unveiled a beneficial effect of statins on these two important malignancies. Of special interest is that the combination of statins with other anticancer drugs can enhance in vitro and in vivo antitumor efficacy of HCC, which is characterized by chemoresistance (7). Collectively, statins may play a dual crucial role as a lipid-lowering drug for the prevention of cardiovascular diseases and as an anticancer agent to prevent certain cancers.

Despite supporting epidemiologic evidence as well as experimental data, controversy exists about whether statins can indeed prevent cancer. There are epidemiologic studies showing the contrary: that statin use could lead to increased risk of cancer (8, 9). Several likely explanations might account for the disparity. These include insufficient control for potential confounders associated with exposure and outcome, inadequate power to detect cancer outcomes, and short follow-ups (10). Given the increasing prevalence of statin use and the controversial observational reports of its effect on cancers, further laboratory investigation should be conducted to better define the mechanisms by which statins show anticancer or cancer-promoting effects.

Autophagy is a physiologic process involved in the turnover of proteins or intracellular organelles and serves as a temporary survival mechanism during starvation, wherein self-digestion becomes an alternative energy source (11). Defective autophagy has been linked to a number of human pathologies, including cancer (12). It is noteworthy that the anticancer effect of statins could act through the mevalonate-independent pathway. Specifically, our group has found that statins induced p21
expression through inhibition of histone deacetylase (HDAC) activity and dissociation of HDAC1/2 from the promoter (13). To further address the biological mechanisms underlying the anticancer effect of statins, we used HCC and CRC as models. In the present study, we showed that atorvastatin inhibited tumor growth via induction of apoptosis in vitro and in vivo. Atorvastatin also induced autophagy, which was dependent on AMP-activated protein kinase (AMPK) activation, then led to p21 expression, and resulted in endoplasmic reticulum (ER) stress response. Inhibition of autophagy enhanced atorvastatin-induced cytotoxicity and apoptosis. Our results provide the molecular clues that the combination of autophagic inhibitors and statins may enhance efficacy of digestive malignances therapy.

**Materials and Methods**

**Materials**

Lovastatin was from Lotus Pharmaceutical Co. (Taiwan). Atorvastatin, pravastatin, and simvastatin were from Synpac Kingdom Pharmaceutical Co (Taiwan). Fluvoxinate was a gift from Novartis (Switzerland). All statins were dissolved in DMSO (Sigma Chemical Co.). Bafilomycin A1 was from Calbiochem. 4-Phenylbutyric acid was from Sigma-Aldrich. Antibodies specific for phosphorylated AMPK, AMPK, phosphorylated eukaryotic translation initiation factor α (eIF2α), and LC3B were from Cell Signaling. Antibodies specific for eIF2α, p21, caspase-3, and poly (ADP-ribose) polymerase (PARP) were from Santa Cruz Biotechnology. The EGFP-LC3 plasmid was kindly offered by Dr. Wei-Bon Huang (National Taiwan University, Taiwan). The human wild-type (wt) p21 plasmid and its mutation of Ser to Ala on nuclear localization sequence (NLSA) mutation were kindly donated by Dr. Neus Agell (University of Barcelona, Spain).

**Cell culture**

The human HCC cell lines Hep3B, HepG2, and Huh7 were kindly provided by Dr. Ann-Lii Cheng (Department of Internal Medicine, National Taiwan University Hospital, Taiwan) in 2008 to 2009. HCT116 wt and p53−/− cells were gifts from Dr. M.W. Van Dyke (M.D. Anderson Cancer Center, Houston, TX) in 2008. HCT116 p21−/− cells were kindly provided by Dr. Yan-Hwa Wu Lee (Department of Biotechnology, National Taiwan University, Taiwan). The human wild-type (wt) p21 plasmid and its mutation of Ser to Ala on nuclear localization sequence (NLSA) mutation were kindly donated by Dr. Neus Agell (University of Barcelona, Spain).

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**Transient transfection**

The plasmids (EGFP-LC3, EGFP-p21, and EGFP-p21-NLSA) or small interfering RNA (siRNA; AMPK and PERK) were transiently transfected into cells with Lipofectamine 2000 transfection reagent according to the manufacturer’s instructions (Invitrogen). After 24 hours, the cells were treated with atorvastatin for 24 hours and subjected to fluorescent analysis or Western blotting assay.

**Transmission electron microscopy**

Cell were fixed in a solution containing 8% paraformaldehyde, 5% glutaraldehyde, 1% tannic acid, and 30 mmol/L sodium cacodylate for 1 hour. The fixed cells were suspended in a buffered solution containing 1% osmic acid for 1 hour, followed by dehydration in a graded ethanol series, washing with acetone, and embedding into EPO epoxy resin. Ultrathin sections (60–80 nm) were prepared on an ultramicrotome and double stained with uranyl acetate and lead citrate. All sections were examined and photographed with a Philips EM300 transmission electron microscope.

**Immunofluorescence**

Huh7 or HCT116 cells were transfected with EGFP-LC3, EGFP-p21, or EGFP-p21-NLSA plasmids, followed by atorvastatin treatment. The cells were then rapidly washed with PBS and fixed at room temperature for 15 minutes with 3.7% paraformaldehyde. After being washed with PBS twice, the cells were blocked with 1% bovine serum albumin in TTBS and then mounted. The fluorescence of enhanced green fluorescence protein (EGFP) was observed under a fluorescence microscope.

**Immunoblotting**

Following treatment with atorvastatin, total cell lysates were prepared and subjected to SDS-PAGE. Western blot was done as described previously (14).

**Flow cytometry**

Cell cycle and apoptosis were determined by flow cytometry using a propidium iodide (PI) stain buffer or Annexin V-FITC/PI double staining and analyzed on a BD FACSCalibur cytometer with CellQuest software.

**MTT assay**

The cell viability after atorvastatin treatment was measured using MTT assay (Sigma). Cells were plated in triplicate in 96-well plates and treated with increasing concentrations of atorvastatin. After 48 hours of incubation, 0.5 mg/mL of MTT was added to each well for an additional 4 hours. The blue MTT formazan precipitate was then dissolved in 100 μL of DMSO. The absorbance at 550 nm was measured on a multiwell plate reader. Cell viability was expressed as a percentage of control.
Animal xenograft model

Female BALB/c nude mice (4–6 weeks old) were injected with 2 × 10^6 Huh7 cells (suspended in 0.1 mL PBS and mixed with 0.1 mL Matrigel) in the rear left flank. After 2 weeks, animals were divided into two groups and p.o. received either control vehicle (soy oil) or atorvastatin (50 mg/kg/d) for 32 days. Tumor volume was measured twice per week with calipers and calculated using the formula, \[ V = \frac{a^2b}{2} \], wherein \( a \) is the length and \( b \) is the width of the tumor.

Histopathologic evaluation

Formalin-fixed colonic tissues from mice were embedded in paraffin by routine procedures. H&E-stained, 4-μm sections of major organs were evaluated microscopically by a pathologist. The expression of relevant biomarkers in representative sections of tumor tissues was detected by immunohistochemical staining using specific primary antibodies.

Statistical analysis

Data were analyzed using Student’s t test. \( P \) values of <0.05 were considered significant. The correlation between phosphorylated AMPK or p21 and beclin-1 expression in normal and tumor tissues was evaluated by the Spearman’s rank correlation coefficients. The calculated formula was \( r_s = 1 - \frac{6\Sigma d_i^2}{n(n^2 - 1)} \), wherein \( d_i \) was the difference in the ranks given to the paired samples and \( n \) was the paired sample numbers.

Results

Atorvastatin exhibited anticancer effects in vitro and in vivo

To investigate the anticancer potential of statins, HCC cells (Huh7), and CRC cells (HCT116) were treated with atorvastatin, and cell proliferation was examined by MTT assay. As shown in Fig. 1A, atorvastatin inhibited cell proliferation was examined by MTT assay. As shown in Fig. 1A, atorvastatin inhibited cell growth of both cells. To further characterize atorvastatin-induced cytotoxicity, apoptotic cell death was assessed by Annexin V/PI double staining and sub-G1 analysis. Increase of apoptosis in early stage (Annexin V positive/PI negative) and late stage (Annexin V positive/PI positive) was detected in atorvastatin-treated Huh7 cells (Fig. 1B and Supplementary Fig. S1). Similarly, atorvastatin induced apoptosis in HCT116 cells, as indicated by an increase in sub-G1 phase (Fig. 1B). Apoptotic induction by atorvastatin in both cells was further confirmed by the time- and dose-dependent decrease of procaspase-3 and the cleavage of PARP (Fig. 1C). These results suggested that atorvastatin might exhibit anticancer effects through the induction of apoptosis.

To investigate whether atorvastatin inhibited tumor growth in vivo, the nude mice bearing Huh7 tumor xenografts were given p.o. atorvastatin (50 mg/kg/d) or vehicle for 32 days. Tumor growth was significantly reduced by atorvastatin, and liver metastasis was not observed (Fig. 1D). Intriguingly, the transmission electron microscopic analysis of excised tumor tissues showed the double-membrane and autophagosome-like structures in atorvastatin-treated tumor cells (Fig. 1E), suggesting that the cells underwent autophagy. Western blot analysis indicated that atorvastatin simultaneously induced apoptosis and autophagy, which were respectively characterized by PARP cleavage and beclin-1 (Atg6) expression (Fig. 1F).

Atorvastatin induced autophagy in vitro

Because atorvastatin induced autophagy in vivo, whether this event occurred in vitro was further investigated. Huh7 and HCT116 cells were treated with atorvastatin, and morphologic changes were examined by transmission electron microscopy. Compared with cells in the basal state, atorvastatin-treated Huh7 and HCT116 cells exhibited obvious autophagic vacuoles in the cytoplasm (Fig. 2A). To confirm this, a green fluorescent EGFP-LC3 plasmid was transfected into Huh7 and HCT116 cells. Microtubule-associated LC3 protein is used as a biomarker of autophagy. This protein normally exhibits diffused cytosolic distribution but is processed and localized to autophagosomes during autophagy (15). As shown in Fig. 2B, diffused distribution of EGFP-LC3 in the basal state was observed in the whole cells. After treatment with atorvastatin, punctate patterns of EGFP-LC3 representing autophagic vacuoles were formed in the cytoplasm. This phenomenon was confirmed by immunoblot analysis to detect LC3 conversion from the cytosolic 16-kDa LC3-I to the autophagosome-associated 18-kDa LC3-II. Because LC3-II tends to be more sensitive than LC3-I in immunoblotting, single comparison of LC3-I and LC3-II for ratio determinations might not be appropriate (16). Therefore, we compared the amount of LC3-II between samples. The accumulation of LC3-II was induced by atorvastatin in Huh7, Hep3B, HepG2, and HCT116 cells (Fig. 2C). Moreover, atorvastatin-induced LC3-II accumulation was blocked in Atg5 knockout MEF cells (Fig. 2D). These results suggested that atorvastatin induced autophagy of cancer cells in vitro.

To examine whether inhibition of HMG-CoA reductase was involved in the atorvastatin-induced autophagy, Huh7 and HCT116 cells were given with mevalonate. Replenishment of mevalonate did not affect autophagosome-induced LC3-II accumulation (Fig. 2E). In addition, various statins including lovastatin, fluvastatin, pravastatin, and simvastatin were able to induce LC3-II accumulation (Fig. 2F). These results suggested that statin-induced autophagy was independent of HMG-CoA reductase activity.

Inhibition of autophagy potentiated the anticancer effects of atorvastatin

To investigate the role of autophagy in the anticancer effects of atorvastatin, wt and Atg5 knockout (Atg5−/−) MEF cells were treated with atorvastatin, and then cell viability was analyzed. As show in Fig. 3A, atorvastatin induced growth inhibition in wt MEF cells, and inhibition was enhanced in Atg5−/− cells. This event correlated with the increase of procaspase-3 and PARP cleavage in Atg5−/− cells (Fig. 3B). An inhibitor of the vacuolar type H^+ ATPase, bafilomycin A1, which blocks the fusion of autophagosome and lysosome, enhanced atorvastatin-induced cytotoxicity in Huh7 cells (Fig. 3C). Cotreatment of bafilomycin A1 with atorvastatin also enhanced the procaspase-3 and PARP
Figure 1. A, MTT cell viability in Huh7 and HCT116 cells treated with 50 μmol/L atorvastatin (Atorva). B, Annexin V-FITC/PI double staining or sub-G₁ analysis in cells treated with 50 μmol/L atorvastatin for 5 d (Huh7) or 2 d (HCT116). C, the cleavages of procaspase-3 and PARP in atorvastatin-treated Huh7 (top, 50 μmol/L; bottom, 2 d) or HCT116 (bottom, 1 d). D, tumor outgrowth in Huh7 xenograft mice given 50 mg/kg/d atorvastatin p.o. Top photograph, xenograft mice on the 32nd day; second photograph, dissected tumors from the first graph; third photograph, histologic analysis of tumor xenografts; fourth photograph, no lungs or liver metastasis in xenograft mice. E, electron microscopic features of xenograft tumors on the 32nd day. N, nucleus. Arrowhead, autophagosomes. F, Western blot of PARP and beclin-1 in the excised tumors.
cleavage (Fig. 3D). Similarly, atorvastatin-induced apoptosis, caspase-3 activation, and PARP cleavage in HCT116 cells were augmented in the presence of bafilomycin A1 (Fig. 3E and F). To examine the potentiation of bafilomycin A1 on the in vivo anticancer effect of atorvastatin, the nude mice bearing Huh7 tumor xenografts were given atorvastatin p.o. (20 mg/kg/d) and were s.c. injected with bafilomycin A1 (1 mg/kg/d). As shown in Supplementary Fig. S2, enhancement of bafilomycin A1 on in vivo anticancer effect of atorvastatin was observed. Therefore, atorvastatin-induced autophagy might be an adaptive response that promoted cell survival.

**Figure 2.** Atorvastatin induced autophagy in vitro. A, electron microscopic ultrastructures of Huh7 and HCT116 cells treated with 50 μmol/L atorvastatin for 24 h. N, nucleus. Arrowhead, autophagosomes. B, fluorescence microscopy analysis of EGFP-LC3-transfected Huh7 and HCT116 cells treated with 50 μmol/L atorvastatin for 24 h. C to F, Western blot of LC3 expression in Huh7 and HCT116 cells treated with 10, 30, and 50 μmol/L atorvastatin for 24 h (C), in MEF wt and Atg5−/− cells treated with 50 μmol/L atorvastatin for 24 h (D), in Huh7 and HCT116 cells treated with 100 μmol/L mevalonate and 50 μmol/L atorvastatin for 24 h (E), and in Huh7 and HCT116 cells treated with 50 μmol/L statins (atorvastatin, fluvastatin, simvastatin, lovastatin, pravastatin) for 24 h.

**Atorvastatin induced autophagy through AMPK activation**

AMPK is a serine/threonine kinase that regulates energy balance and serves as an energy sensor in all eukaryotic cells (17). Once activated, AMPK promotes energy production and limits energy utilization to ensure cellular survival (18). AMPK activation has been reported to induce autophagy through phosphorylating and activating the TSC1/TSC2 complex that negatively regulates mammalian target of rapamycin complex (19). We found that atorvastatin induced AMPK activation in Huh7 and HCT116 cells (Fig. 4A). To ascertain whether AMPK was required for atorvastatin-induced autophagy, Huh7 and
HCT116 cells were transfected with AMPK siRNA. Western blot analysis showed that atorvastatin-induced LC3-II accumulation was inhibited when AMPK expression was knocked down (Fig. 4B), showing that atorvastatin induced autophagy through activation of AMPK. Furthermore, the expression of beclin-1 and AMPK activity was examined in the HCC patients. Examination of 11 patients showed that the expression of beclin-1 and phosphorylated AMPK was elevated in tumor tissues compared with the adjacent normal tissues. Activated AMPK was positively correlated with the beclin-1 expression in patients (Fig. 4C; Supplementary Fig. S3). Therefore, targeting AMPK-mediated autophagy would be an effective strategy to enhance the efficacy of therapy for HCC.

**Atorvastatin induced autophagy through cytoplasmic p21**

The tumor suppressor protein p53 acts as a transcription factor that transactivates proapoptotic and cell cycle arresting genes (20). p53 can also transactivate an autophagy-inducing gene, DRAM, which encodes a lysosomal protein (21). However, cytoplasmic p53 exerts autophagy-inhibitory function, and its degradation is actually required for the induction of autophagy (22). The p53 downstream gene p21 is a universal inhibitor of cyclin-dependent kinases (CDK) belonging to the CIP/KIP family of CDK inhibitors (23). Accumulating evidence indicates that p21 has other functions, for example, inducing apoptosis or enhancing the apoptotic response to chemotherapeutic agents (24–26). We previously found that statins induced p53-independent p21 expression (13); therefore, the roles of p53 and p21 in atorvastatin-induced autophagy were investigated using HCT116 wt, p53−/−, and p21−/− cells. Atorvastatin-induced LC3-II accumulation or autophagosome formation was seen in HCT116 wt and p53−/−, but not in p21−/− cells (Fig. 5A and B), indicating the independence of p53 and dependence on p21 in atorvastatin-induced autophagy. Although atorvastatin induced both cytoplasmic and nuclear p21 expression,
the LC3-II was predominantly located in the cytoplasm (Fig. 5C), implying that cytoplasmic p21 might play a more important role in autophagic induction. To clarify this, wt and NLSA (mutation of Ser to Ala on nuclear localization sequence) p21 were transfected into HCT116 p21−/− cells, and their distributions were examined by Western blot and immunofluorescence analysis. wt p21 was found in both nucleus and cytoplasm, whereas NLSA p21 was predominantly detected in the cytoplasm (Fig. 5D). Overexpression of wt or NLSA p21 both increased LC3-II accumulation (Fig. 5E), suggesting that cytoplasmic p21 was sufficient to induce autophagy. To examine the effect of p21 on atorvastatin-induced apoptosis, HCT116 p21−/− cells, reconstituted with wt or NLSA p21, were treated with atorvastatin. PARP cleavage induced by atorvastatin was reduced by NLSA p21 (Fig. 5F). Furthermore, the expression of p21 and beclin-1 was also examined in normal and tumor colon tissues. Examination of 30 CRC patients showed that the expressions of p21 and beclin-1 were positively correlated (Fig. 5G; Supplementary Fig. S4).

**Atorvastatin induced autophagy through p21-mediated ER stress response**

ER is an organelle in eukaryotic cells playing an important role as a sensor for cellular stress by detecting the changes in cell homeostasis and by responding to different signal pathways (27). The hallmark of the ER stress response is the activation of eIF2α protein kinase (PERK) to phosphorylate eIF2α and then induce expression of a transcription factor CCAAT/enhancer binding homologous protein (CHOP/GADD153; ref. 28). To examine whether atorvastatin-induced autophagy was associated with ER stress response, phosphorylation of eIF2α was examined. Atorvastatin induced phosphorylation of eIF2α in Huh7 and HCT116 cells (Fig, 6A and B). A chemical chaperone 4-phenylbutyrate, which is an ER stress inhibitor, inhibited atorvastatin-induced phosphorylation of eIF2α (Fig. 6B). Moreover, knockdown of PERK expression inhibited atorvastatin-induced phosphorylation of eIF2α (Fig. 6C). In addition, atorvastatin-induced phosphorylation of eIF2α and expression of GADD153 were not seen in HCT116 p21−/− cells (Fig. 6E), suggesting that...
p21 acts upstream of ER stress. Indeed, overexpression of wt and NLSA p21 to HCT116 wt and p21<sup>+/−</sup> cells increased the phosphorylation of eIF2α and the expression of GADD153 (Fig. 6F). Therefore, atorvastatin induced autophagy through AMPK activation, which led to p21 expression, and then caused ER stress response.

**Discussion**

In epidemiologic observations, statins use was associated with the decreased risk of HCC and CRC, but the underlying mechanisms remained ill defined. Given the chemoresistant nature of HCC and high prevalence of CRC, we thus
investigated how statins can have anticancer effects for these two fatal malignancies. Our results indicated that atorvastatin exhibited antitumor activities in cancer cells of HCC (Huh7) and CRC (HCT116) through induction of apoptosis. Furthermore, autophagy was induced in both cells, as well as in atorvastatin-induced Huh7 xenograft tumors. Intriguingly, inhibition of autophagy increased cellular sensitivity to atorvastatin in vitro and in vivo. Taken together, our results not only elucidated the detailed mechanisms of anticancer effects of statins but also provided novel insights that combination of autophagic inhibitors and statins might be an attractive and promising strategy for the management of HCC and CRC.

Previous studies have reported that either blockage of autophagy or induction of autophagy could lead to tumor growth (12). The effect of manipulated autophagy seems to vary with intrinsic properties of the tumor and with the nature of combined therapy. Our finding that statins induced autophagy might provide a plausible explanation for the varying relationships of statins and cancer in different tissues (10). In this study, genetic inhibition of AMPK was found to reduce atorvastatin-induced autophagy, and examination of HCC patients showed the positive correlation between the expression of beclin-1 and phosphorylated AMPK in tumor tissues. This finding as well as in vitro and in vivo hepatoma models indicated that autophagy might promote survival of liver cancer cells. This was further supported by the result that inhibition of autophagy by bafilomycin A1 indeed enhanced atorvastatin-induced apoptosis and anticancer effect in vivo. In this aspect, our observations were consistent with the studies using different cancer cells and agents. One study revealed that inhibition of AMPK can overcome drug resistance and sensitize human prostate cancer DU145 cells to Fas-induced apoptosis (29). The chemotherapeutic agent cisplatin activates AMPK in various cancer cells, and inhibition of AMPK results in a marked augmentation of cisplatin-induced apoptosis (30). Because atorvastatin induced autophagy through AMPK activation, combination with AMPK or autophagy inhibitors would enhance the therapeutic effect on cancer therapy.

Genetic inhibition of AMPK was found to reduce atorvastatin-induced p21 expression, indicating that AMPK acts

![Figure 6. AMPK/p21/ER stress signaling was involved in atorvastatin-induced autophagy.](image)
upstream of p21 expression. Activation of AMPK by the widely used AMP-mimetic 5-aminimidazole-4-carboxamide ribonucleoside (AICAR) also showed induction of p21 expression (31). The mechanism for AICAR-induced p21 expression involves accumulation of p53 through phosphorylation of Ser15 (31). However, our previous studies showed that atorvastatin-induced p21 expression was independent of p53 because the p21 promoter activity was not affected by the deletion of p53 (13). Moreover, statins were shown to induce p21 expression through inhibition of HDAC activity (13). Therefore, AMPK might regulate p21 expression through modulation of HDAC activity. The posttranslational modification of HDAC is crucial for its enzyme activity and localization. For example, phosphorylation of HDAC1 at Ser421 and Ser423 by protein kinase CK2 regulates its deacetylase activity and capacity to form protein complexes (32–34). Aligning the consensus motif of AMPK phosphorylation sites (X-I-X-R-X-S-X-X-V; X, any amino acid; I, isoleucin; R, arginine; S, serine; V, valine; ref. 35), Ser42 of HDAC1 and Ser50 of HDAC2 are found to be the putative AMPK phosphorylation sites. In support of this notion, we found that AMPK was activated and translocated to the nucleus on statin stimulation and AMPK can phosphorylate pure HDAC2 protein and GST-HDAC2 in vitro (Supplementary Fig. S5). It is possible that phosphorylation of HDAC2 by AMPK leads to inhibition of HDAC activity, which is crucial for statin-induced p21 expression.

p53 exerts an ambiguous function in the regulation of autophagy. Within the nucleus, p53 acts as an autophagy-inducing transcription factor. p53 in the cytoplasm exerts a tonic autophagy-inhibitory function (36). We found that atorvastatin induced LC3-II accumulation in HCT116 p53−/− cells, indicating the independence of p53 in atorvastatin-induced autophagy. Interestingly, three lines of evidence showed that the p53 downstream gene p21 promotes autophagy. First, atorvastatin-induced autophagy was abolished in p21−/− cells. Second, ectopic expression of wt or NLSA p21 induced autophagy in HCT116 wt and p21−/− cells. Third, examination of patients with CRC showed positive correlation of p21 and beclin-1 expression. Although autophagy is activated by nutrient starvation (37), we showed a novel induction pathway indicating that accumulation of p21 in the cytoplasm leads to autophagy in nutrient-rich conditions. Inhibition of cell growth by p21 is strongly correlated with its nuclear localization (23, 38). However, recent lines of evidence have shown that p21 can also localize in the cytoplasm and plays a protecting role against apoptosis (39, 40). Both nuclear p21 and cytosolic p21 were induced by atorvastatin. The localization of p21 is important for the regulation of autophagy. Because autophagy occurs in the cytoplasm, only cytoplasmic p21 is expected to play a role in this event. This was shown by the result that overexpression of p21 with mutation of Ser to Ala in nuclear localization sequence still induced autophagy. Therefore, accumulation and redistribution of p21 in the cytoplasm might be important for atorvastatin-induced autophagy. Akt has been reported to phosphorylate p21 at Thr145 and retained it in the cytoplasm, leading to the loss of cell cycle inhibition (40). Instead of cell cycle arrest, p21 in the cytoplasm induces an apoptosis-resistant phenotype. Cytoplasmic p21 can form a complex with the apoptosis signal-regulating kinase 1 and inhibits c-Jun NH2 terminal kinase–induced apoptosis (39). It can also enter mitochondria and interacts with procaspase-3 to inhibit its activation (41). Therefore, cytoplasmic p21 itself might act as an inhibitor of apoptosis. We further showed that p21-induced autophagy depends on ER stress response. A recent report has shown that overexpression of p21 or p27 enhanced acid–induced phosphorylation of PERK and eIF2α and increased the numbers of autophagic vesicles in primary hepatocytes (42). These results strengthen our findings that cytoplasmic p21 increases ER stress and autophagy and plays an important role beyond cell cycle regulation.

To our knowledge, this is the first report showing that activation of AMPK by atorvastatin induced cytoplasmic p21-dependent ER stress, which regulates autophagy. The atorvastatin-induced autophagy leads to cell survival in HCC and CRC cancer cells. The combination of autophagic inhibitors enhanced cytotoxicity and apoptosis. Pharmacologic targeting of autophagy provides a promising strategy for the management of HCC and CRC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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