AMPK Promotes p53 Acetylation via Phosphorylation and Inactivation of SIRT1 in Liver Cancer Cells

Chi-Wai Lee, Leo Lap-Yan Wong, Edith Yuk-Ting Tse, Heong-Fai Liu, Veronica Yee-Law Leong, Joyce Man-Fong Lee, D. Grahame Hardie, Irene Oi-Lin Ng, and Yick-Pang Ching

Abstract

AMP-activated protein kinase (AMPK), a biologic sensor for cellular energy status, has been shown to act upstream and downstream of known tumor suppressors. However, whether AMPK itself plays a tumor suppressor role in cancer remains unclear. Here, we found that the α2 catalytic subunit isoform of AMPK is significantly downregulated in hepatocellular carcinoma (HCC). Clinicopathologic analysis revealed that underexpression of AMPK-α2 was statistically associated with an undifferentiated cellular phenotype and poor patient prognosis. Loss of AMPK-α2 in HCC cells rendered them more tumorigenic than control cells both in vitro and in vivo. Mechanistically, ectopic expression of AMPK enhanced the acetylation and stability of p53 in HCC cells. The p53 deacetylase, SIRT1, was phosphorylated and inactivated by AMPK at Thr344, promoting p53 acetylation and apoptosis of HCC cells. Taken together, our findings suggest that underexpression of AMPK is frequently observed in HCC, and that inactivation of AMPK promotes hepatocarcinogenesis by destabilizing p53 in a SIRT1-dependent manner.

Introduction

AMP-activated protein kinase (AMPK), which functions as a cellular energy sensor, occurs as heterotrimers composed of α, β, and γ subunits. The α-subunit serves as the catalytic subunit, whereas the β- and γ-subunits serve regulatory functions (1, 2). As its name implies, AMPK is activated by increases in the cellular AMP:ATP and ADP:ATP ratio caused by conditions of metabolic stress, including hypoxia and nutrition depletion. Recently, AMPK has been implicated in carcinogenesis because of the finding that a well-known tumor suppressor, LKB1, is an upstream activating kinase for AMPK (3, 4).

Furthermore, activation of AMPK was shown to induce a p53-dependent G1 cell-cycle arrest, suggesting that AMPK possesses tumor suppressor activity (5).

SIRT1 is a NAD-dependent protein deacetylase, originally discovered in yeast, involved in gene silencing, telomere regulation, and extension of lifespan following caloric restriction (6). It is predominantly localized in the nucleus, and deacetylates both histone and nonhistone proteins, such as p53, FOXOs, and Ku70 (7). SIRT1 deacetylates p53 and inhibits p53-mediated cell death following DNA damage, and SIRT1-deficient mice exhibit p53 hyperacetylation following DNA damage (8). All of these observations suggest that SIRT1 is a negative regulator of p53.

In the present study, we explored the role of AMPK in the pathogenesis of hepatocellular carcinoma (HCC). Using quantitative real-time PCR (qRT-PCR), we found that the AMPK-α2 subunit was frequently underexpressed in human HCCs. Functional analysis showed that stable knockdown of AMPK-α2 in HCC cells increased their tumorigenicity. Given loss of AMPK induces HCCs, we observed that AMPK enhanced acetylation of p53 through phosphorylation and inhibition of SIRT1 in HCC cells, resulting in promotion of apoptosis. Thus, our study provides new evidence for the tumor suppressor role of AMPK, and suggests that activation of AMPK may present a potential therapeutic target in HCC.

Materials and Methods

Cell culture and transfection

The human hepatoma cell lines, HepG2 and PLC/PRF/5, were purchased from the American Type Culture Collection. Human HCC cell line SMMC-7721 was a gift from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Beijing, China). Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) high-glucose medium (Life Technologies) supplemented with 10% heat-inactivated FBS (JRH Biosciences) and 1 mmol/L sodium pyruvate. Cells were transfected with DNA constructs using Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocol. For establishment of stable clones, PLC/PRF/5 cells were transfected with shAMPK-α2 expression constructs, and were selected with the antibiotic G418 (0.8 mg/mL) 48 hours after transfection.
AMPK kinase assay

Kinase assays were conducted as described (9). The GST-AMPK catalytic domain was incubated with His-SIRT1 in the AMPK kinase buffer (50 mmol/L HEPES, 0.02% Brij35, pH 7.0) in the presence of 10 μCi [γ-32P] ATP. Proteins were resolved by SDS-PAGE and radioactive signal visualized by autoradiography.

In vitro deacetylase activity assay

GST-p53 was acetylated in the presence of purified p300 protein (Enzo Life Science) and acetyl-CoA in acetylation buffer [50 mmol/L HEPES pH 7.9, 1 mmol/L EDTA, 50 μg/mL bovine serum albumin (BSA)]. The acetylated p53 was subjected to SIRT1 deacetylation in deacetylation buffer (50 mmol/L Tris-HCl, pH 8.0, 137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl2, and 1 mg/mL BSA), supplemented with 10 mmol/L NAD+ (Sigma). Proteins were resolved by SDS-PAGE, and the level of p53 acetylation was visualized by probing with anti-acetyl-p53(K382) antibody.

Luciferase reporter assay

Luciferase reporter assay was conducted with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instruction. Cells were transfected with various SIRT1 mutants and a p53-luciferase reporter plasmid. Data were calculated by normalizing luminescence of Firefly luciferase to that of Renilla luciferase.

Annexin V apoptosis detection assay

Annexin V labeling was conducted with BD Pharmingen Annexin V:PE Apoptosis Detection Kit I according to the manufacturer’s protocol. Cells were transfected with GFP-tagged SIRT1 mutants and fluorescein isothiocyanate-positive cells were gated for Annexin V/7-AAD labeling.

Results

Underexpression of AMPK-α2 transcripts in HCC

To address the significance of AMPK in human HCCs, we examined the mRNA expression levels of the AMPK catalytic subunit isoform, AMPK-α2, in HCCs using (q)RT-PCR. We were interested in AMPK-α2 because the allelic region of AMPK-α2, that is chromosome region 1p31, has previously been reported to be deleted in HCC (10, 11). Our results indicate that AMPK-α2 was frequently underexpressed in HCCs (mean decrease 47.6%, 20 of 42 cases; P < 0.001) as compared with their corresponding nontumorous livers (Fig. 1A). The reduced expression of AMPK-α2 protein was confirmed in some of the HCC samples by Western blotting (Supplementary Fig. S1). In line with this observation, at least 5 out of 9 HCC cell lines examined (Fig. 1B), including SMMC-7721, Hep3B, HepG2, CL48, and Huh-7 cells, had low expression of AMPK-α2 detected by Western blotting suggesting that downregulation of AMPK is a frequent event in HCC tissues and cell lines. To understand the significance of underexpression of AMPK-α2 in human HCC, we conducted statistical analysis of the association of clinicopathologic
features with underexpression of AMPK-α2. This showed that underexpression of AMPK-α2 (T/NT < 0.5) was significantly correlated with a poorer tumor differentiation classified according to Edmonson grading (Table 1) and a poorer disease-free survival rate (Fig. 1C), indicating that underexpression of AMPK-α2 was associated with a poor prognosis for patients with HCC.

Table 1. Clinicopathologic correlation of AMPKζ2 expression in human HCC

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*According to Edmonson grading.

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AMPK inhibited HCC cell growth

To explore whether AMPK is involved in HCC cell growth, we treated HCC cell lines with various AMPK activators, such as resveratrol, metformin, and phenformin, and conducted cell proliferation and colony formation assays. As shown in Fig. 2A and B, incubation of these AMPK activators with PLC/PRF/5 and HepG2 (Supplementary Fig. S2) cells suppressed cell proliferation and colony formation in a dose-dependent manner. To rule out off-target effects of these pharmacologic activators, DNA encoding AMPK-α2 was also transfected into HepG2 cells and colony formation assays conducted. As expected, HepG2 cells expressing AMPK-α2 formed far fewer colonies than cells transfected with the empty vector or an unrelated control gene (chloramphenicol acetyl transferase, CAT), indicating that forced expression of AMPK-α2 suppressed the growth of HepG2 cells (Fig. 2C). To further characterize the tumor suppressor effects of AMPK-α2, we endogenous α2 in PLC/PRF/5 cells (which have a high AMPK-α2 expression level) was specifically knocked down using short-hairpin RNA (shRNA). Two stable clones (shAMPK#1 and shAMPK#2) were isolated and the knockdown of AMPK-α2 confirmed by immunoblotting. Consistently, the proliferation rates of these stable knockdown clones were significantly lower than those of the vector control, and were highest for shAMPK#2 cells, which have a greater degree of knockdown of expression than shAMPK#1 cells. To assess anchorage-independent growth and in vivo tumor growth of the stable clones, we conducted assays of growth of cells in soft agar and xenografts in nude mice. Our data show that the 2 stable knockdown clones formed significantly more, and larger, colonies than the vector control cells in the soft agar growth assay (Fig. 2E) and that tumors generated from the stable knockdown clone (shAMPK#1) exhibited a significant enhancement in the onset of tumor formation, faster growth rates, and higher weights than those from the vector control cells (Fig. 2F) suggesting that loss of expression of AMPK-α2 promoted tumor growth in vivo.

DNA methylation and histone deacetylation are 2 major epigenetic mechanisms that lead to gene silencing in cancer. To investigate whether underexpression of AMPK-α2 is due to epigenetic regulation, the HCC cell line HepG2, which shows no detectable AMPK-α2 mRNA or protein, was treated with the DNA methylation inhibitor, 5-aza-2’-dCytosine (5-Aza), or the histone deacetylase inhibitor, trichostatin A (TSA). As shown in Fig. 1D (i), treatment with TSA, but not 5-Aza, significantly induced the reexpression of AMPK-α2 transcripts, indicating that the α2 promoter was repressed by histone acetylation. To further confirm the role of histone modification on AMPK-α2 expression, we conducted chromatin immunoprecipitation assays using anti-acetylated histone antibody. HepG2 cells pretreated with TSA showed a large increase in DNA fragments containing the AMPK-α2 promoter after immunoprecipitation using anti-acetylated histone antibody, as compared with unrelated antibody (anti-myc) control (Fig. 1D (ii)). Collectively, these data suggested that an alteration of histone acetylation might occur at the promoter region of AMPK-α2, leading to its underexpression in human HCCs.

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AMPK promoted the acetylation of p53

As the pharmacologic AMPK activator 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) has been reported to regulate the cell cycle through the stabilization of p53 (5), we wondered whether AMPK might regulate p53 to modulate hepatocarcinogenesis. To explore the potential role of AMPK on posttranslational modifications of p53, HepG2 cells were cotransfected with increasing doses of a plasmid-expressing GFP fused to an activated AMPK-α2 mutant (T172D) and the phosphorylation of Ser20 and acetylation of Lys382 on p53 was analyzed. Interestingly, overexpression of AMPK-α2 significantly enhanced both Ser20 phosphorylation and acetylation.

Figure 2. Effect of AMPK-α2 on HCC tumorigenicity. A, PLC/PRF/5 cells were cultured in medium containing either dimethyl sulfoxide (DMSO; 1 μL/mL) as control, or resveratrol, phenformin, or metformin. The cell number was determined for 6 consecutive days and results are mean ± SD. B, HepG2 cells were cultured in medium containing DMSO, resveratrol, phenformin, or metformin for 2 weeks for assays of colony formation. Results are mean ± SD. C, HepG2 cells were transfected with constructs expressing wild-type AMPK-α2, vector, or an unrelated gene (CAT) as a control for colony formation assays. Error bars, mean ± SD. *, P < 0.05 compared with vector control by t test. D, two stable shAMPK-α2-expressing PLC/PRF/5 cells [shAMPK#1 (−−) and #2 (−−)] and vector control (−−) were used to carry out cell proliferation assays. The curve shows the proliferation rate of these cell clones. E, shAMPK#1 and #2 stable clones were used for assays of growth in soft-agar. Representative pictures of the colonies are shown and the bar chart indicates quantification (mean ± SD). F, the shAMPK#1 and vector control cells were subcutaneously injected into nude mice, and tumors were allowed to grow for 4 weeks. The tumor size and weight (n = 5 per group, error bars, mean ± SD) are presented, and a representative picture of tumors with shAMPK and vector control is shown. *, P < 0.01; **, P < 0.001 (Student t test) compared with vector.
of p53 in a dose-dependent manner (Fig. 3A). Previous studies have suggested that AMPK regulates p53 by promoting the phosphorylation at the Ser15 site. Thus, in vitro kinase assays were conducted to evaluate whether AMPK directly phosphorylated p53. A glutathione S-transferase (GST fusion) of the catalytic domain of AMPK-α2 [amino acids (a.a.) 1–312, GST-AMPK-CA] was used to phosphorylate wild-type p53 and mutant versions in which Ser15 or Ser20 was mutated to alanine (S15A and S20A mutants). As shown in Fig. 3B, AMPK directly phosphorylated wild-type, S15A and S20A mutants of p53 to a similar extent suggesting that while p53 is a substrate of AMPK, Ser15 and Ser20 may not be direct phosphorylation sites for AMPK.

AMPK suppressed SIRT1 deacetylase activity on p53

To investigate how AMPK promotes the acetylation of p53, the potential regulation of the p53 deacetylase, SIRT1, by AMPK was examined (12). HepG2 cells were cotransfected with Myc-p53 and Flag-SIRT1 constructs, followed by activating AMPK using the selective AMPK activator, A769662. Coexpression of SIRT1 with p53 dramatically reduced the level of acetylated p53; however, treatment with A769662 partially reversed the effect of SIRT1 on p53 acetylation, in a dose-dependent manner (Fig. 3C). To further confirm that AMPK blocks the SIRT1-mediated deacetylation of p53, a p53-dependent luciferase reporter assay was conducted by cotransfecting SIRT1 with increasing amounts of constructs expressing the activated form of AMPK-α2 (T172D). This showed that ectopic expression of SIRT1 suppressed p53 transcriptional activity, but also that expression of activated AMPKα2 inhibited the suppressive effect of SIRT1 on p53 transcriptional activity (Fig. 3D). Taken together, these data support the hypothesis that AMPK suppresses the deacetylase activity of SIRT1 to promote p53 transcriptional activity.

AMPK directly binds to the deacetylase domain of SIRT1

To elucidate the mechanism by which AMPK inhibits SIRT1, we examined the interaction of these 2 proteins using a coimmunoprecipitation assay. As shown in Fig. 4A, GFP-AMPKα2, but not GFP alone, coprecipitated with Flag-SIRT1 in HEK293T cells cotransfected with DNA encoding GFP-AMPK-α2 and Flag-SIRT1. In addition, the interaction of
AMPK-α2 and SIRT1 was further confirmed using a GST-affinity pull-down assay, in which His-SIRT1 was pulled down with GST-AMPK-CA (Fig. 4B) suggesting that these 2 proteins interact in intact cells. Using confocal immunofluorescence staining, colocalization of SIRT1 and AMPK-α2 was observed in the nuclei of HepG2 cells (Fig. 4C). To map the region of SIRT1 that interacts with the AMPK-α2 kinase domain, 3 truncation mutants, that is, M1 (1–235), M2 (236–525), and M3 (526–747) of SIRT1 were generated. Using the GST affinity pull-down assay, we showed that only the mutant M2, but not M1 and M3, bound to GST-AMPK-CA (Fig. 4D), indicating that the kinase domain of AMPK-α2 directly interacts with the deacetylase domain of SIRT1.

AMPK phosphorylated SIRT1 at Thr344

Because AMPK is a serine/threonine kinase, we next tested whether AMPK phosphorylates SIRT1 directly. In vitro kinase assays were conducted using a purified GST fusion with the activated AMPK-α2 catalytic domain (a.a.1–312 T172D, GST-AMPK-CAD) and full-length His-SIRT1. The result showed that GST-AMPK-CAD phosphorylated SIRT1, but not another member of the sirtuin family, SIRT2 (Fig. 5A). As a positive control, we showed that GST-AMPK-CAD also phosphorylated a known substrate (13), the catalytic domain of 3-hydroxy-3-methyl-glutaryl coA reductase (HMG-CoA reductase; Fig. 5A). Moreover, the region of SIRT1 phosphorylated by AMPK was mapped to the M2 truncation mutant of SIRT1 (Fig. 5A). To further delineate the AMPK phosphorylation site on SIRT1, the amino acid sequence of the catalytic domain of SIRT1 was compared with other known AMPK consensus phosphorylation sequences revealing that the catalytic domain of SIRT1 contains a threonine residue (Thr344) surrounded by a sequence, which has a close similarity to a consensus AMPK recognition site (refs. 13–15; although with aspartate at the P+4 position rather than the preferred hydrophobic residue; Fig. 5B). To confirm phosphorylation of SIRT1 at this threonine, Thr344 was mutated to alanine and the mutant used in a cell-free kinase assay. While both the GST-AMPK-CAD and heterotrimeric forms of AMPK robustly phosphorylated the M2 mutant of SIRT1, this phosphorylation was completely abolished with a T344A substitution in M2 (Fig. 5C). Consistently, a significant reduction of SIRT1 phosphorylation by GST-AMPK-CAD was also observed in a full-length SIRT1 carrying the T344A substitution (Fig. 5D). To test whether this phosphorylation also occurs in intact cells, HEK293T cells were transfected with DNAs encoding wild-type or a T344A mutant of Flag-SIRT1, and treated with A769662. After immunoprecipitation of Flag-SIRT1, threonine phosphorylation was determined. Treatment with A769662 significantly increased AMPK activity and enhanced the threonine phosphorylation of wild-type SIRT1, but any effect with the T344A mutant was not statistically significant (Fig. 5E), implying that AMPK activation induces the phosphorylation of SIRT1 primarily at Thr344.

Phosphorylation of SIRT1 at Thr344 inhibited its activity towards p53

To understand the effect of AMPK phosphorylation on the activity of SIRT1, potentially phospho-mimetic (T344E) and nonphosphorylatable (T344A) mutants of SIRT1 were generated for in vitro deacetylation assays. As expected, both the wild-type and the T344A mutant of SIRT1 readily deacetylated...
acetyl-p53 in the presence of NAD$^+$; in contrast, the T344E mutant of SIRT1 failed to deacetylate acetyl-p53 (Fig. 6A). Furthermore, while overexpression of wild-type and the T344A mutant of SIRT1 in HepG2 cells markedly reduced the acetyl-p53 level upon etoposide treatment and coexpression of p300, the T344E mutant failed to reduce the acetylation level of p53, similar to results with the catalytically inactive H363Y mutant of SIRT1 (Fig. 6B). Comparable etoposide treatment results were also observed in Hep3B cells ectopically expressing Myc-p53 (Supplementary Fig. S4A). We examined the subcellular localization of these SIRT1 mutants and observed that the wild-type and all of the phosphorylation mutants localized in the nucleus (Supplementary Fig. S3) suggesting that this phosphorylation site had no effect on SIRT1 localization.

**Figure 5.** AMPK phosphorylated SIRT1 at Thr344. A, full-length His-SIRT1 or GST-SIRT2 or truncations (M1–M3) of His-SIRT1 and GST-AMPKα2-CA were used to carry out in vitro kinase assays. The expressed catalytic domain of HMG-CoA reductase (HMGR), was used as a positive control. The autoradiograph shows phosphorylation and the Coomassie blue–stained gel shows total protein. B, amino acid alignment of SIRT1 with other known AMPK substrates. The phosphorylation site is underlined. C, the wild-type or T344A mutant of the SIRT1 M2 fragment was phosphorylated using GST-AMPKα2-CA or a trimeric form (α2β1γ1) of AMPK in in vitro kinase assays. An autoradiograph is shown; HMGR was used as a positive control. D, similar to C, but the wild-type or T344A mutant of full-length SIRT1 were incubated with GST-AMPKα2-CA. E, HEK293T cells were transfected with the Flag-tagged wild-type or T344A mutant of SIRT1 and treated with 100 μmol/L A769662 for 4 hours. SIRT1 proteins were immunoprecipitated with anti-Flag antibody and probed with phosphothreonine (p-Thr) antibody. The A769662-treated lysates were probed with the indicated antibodies. The bar chart shows the intensity of the anti-pThr signal relative to anti-Flag signal.
Figure 6. Phosphorylation at Thr344 inactivates SIRT1 deacetylase activity. A, preacetylated p53 was incubated with wild-type or mutants of His-SIRT1 in the presence or absence of NAD⁺, and the level of p53 acetylation was detected by acetylation-specific anti-p53 antibody. B, HepG2 cells were transfected with DNAs encoding wild-type or mutant Flag-SIRT1 and cotreated with 100 μmol/L etoposide overnight. The level of p53 acetylation was detected and quantified. Results are mean ± SD; *, P < 0.05. C, His-tagged mutants of SIRT1 were pulled down with GST, GST-p53, or GST-acetyl-p53. WT, wild type; TA, T344A; TE, T344E. D, 0.3 or 0.9 mg of DNA-encoding SIRT1 mutants was transfected into HepG2 cells together with a p53-luciferase reporter and luciferase activity measured. Results are mean ± SD; *, P < 0.05; **, P < 0.01. E, HepG2 cells were transfected with DNAs encoding wild-type or mutant SIRT1 and treated with 100 μmol/L etoposide and 0.5 μM TSA for 6 hours. The transcript level of p21CIP and Bax were measured using qRT-PCR. Results are mean ± SD; *, P < 0.001. F, SMMC-7721 cells were transfected with DNAs encoding GFP-SIRT1 and treated with 100 μmol/L etoposide for 24 hours. Cells were stained with Annexin V-PE and 7-AAD. Only GFP-positive cells were gated for analysis. G, HepG2 cells were transfected with DNAs encoding SIRT1 for colony formation assays. Results are mean ± SD; *, P < 0.01. H, diagram summarizes the role of AMPK in stabilizing p53. P, phosphate; Ac, acetyl.
subcellular localization. On the other hand, previous structural studies have shown that amino acid residues 344–348 of SIRT1 are highly conserved among different species and are involved in the binding of acetyl-p53 (16). Thus, we hypothesized that the phosphorylation at Thr344 may abolish the interaction between SIRT1 and acetyl-p53. GST-affinity pull-down assays were conducted using GST-p53 or preacetylated GST-p53 to precipitate the wild-type, T344A, or T344E mutants of SIRT1. As shown in Fig. 6C, both wild-type and T344A SIRT1, but not the T344E mutant, bound strongly to acetylated p53, implying that the phosphorylation of SIRT1 at Thr344 abrogates the binding of SIRT1 to acetylated p53. Thus, our results suggest that phosphorylation of T344 leads to a loss of SIRT1 deacetylase activity towards p53 by attenuating the affinity of SIRT1 for its substrate p53.

Phosphorylation of SIRT1 at Thr344 inhibited suppression of the transcriptional activity of p53

Because the phosphorylation of SIRT1 at T344 position affects the affinity of substrate binding, the effect of this phosphorylation on p53 transcriptional activity was assessed using p53 luciferase reporter assays. While wild-type and T344A mutant SIRT1 was found to significantly suppress p53 transcriptional activity in a dose-dependent manner; this suppression was abolished with the phosphomimetic T344E mutant and the catalytic inactive H363Y mutant of SIRT1 (Fig. 6D). Similar results were also observed in another HCC cell line, SMMC-7721 (Supplementary Fig. S4B), suggesting that phosphorylation at Thr344 causes SIRT1 to lose its ability to suppress p53 activity. This was further supported by the observation that the T344E mutant had lost its ability to suppress the transcription of target genes downstream of p53, including p21WAF1 and Bax, upon etoposide treatment (Fig. 6E).

Thr344 phosphorylation of SIRT1 promoted apoptosis of HCC cells

Because phosphorylation of SIRT1 at Thr344 enhances the expression of p53 target genes, we analyzed whether the effect on SIRT1 phosphorylation affects apoptosis of HCC cells. SMMC-7721 cells were transfected with GFP-tagged SIRT1 wild-type or mutant constructs, and apoptosis was assessed using fluorescence-activated cell sorting analysis. GFP-positive cells were gated and counted for the apoptotic cells that were Annexin V+/−/− dual positive. As shown in Fig. 6F, transfection with wild-type or T344A mutant SIRT1 significantly reduced etoposide-induced apoptosis compared with the GFP control, whereas transfection with the T344E mutant failed to rescue the etoposide-induced apoptosis, showing that the T344E substitution prevented the antiapoptotic effect of SIRT1. We next examined the effect of the T344E mutant on HCC cell growth using a colony formation assay. Forced expression of H363Y and T344E mutants, but not the wild-type and T344A mutant, of SIRT1 significantly reduced the number of colonies formed when compared with a vector control in HepG2 cells (Fig. 6G), indicating that T344E mutant suppressed HCC cell growth. Taken together, these results show that phosphorylation of Thr344 abolishes the antiapoptotic activity of SIRT1 (Fig. 6H).

Discussion

As a downstream target of the tumor suppressor LKB1, the role of AMPK in cancer is being extensively researched. The LKB1-AMPK axis has been shown to be important in the suppression of the mTOR signaling pathway, which regulates cell proliferation and is hyperactivated in the majority of solid tumors (17). However, the activation of AMPK in LKB1-null non–small cell lung cancer cells can also induce apoptosis, suggesting that alternative pathways can be involved in the tumor suppressor effects of AMPK, independent of LKB1 (18). In this study, we explored the involvement of AMPK in HCC development. Our results indicate that AMPK is a potential tumor suppressor in human HCCs based on 3 lines of evidence. First, AMPK-α2 transcripts, encoding one of the catalytic subunit isoforms of AMPK, were significantly downregulated in HCCs compared with corresponding nontumorous liver tissue (Fig. 1A) and underexpression of AMPK-α2 correlated with a poorer prognosis for the patients (Fig. 1C and Table 1). Second, treatment with pharmacologic activators of AMPK led to a dose-dependent inhibition of cell proliferation (Fig. 2A) and colony formation (Fig. 2B) with HCC cells indicating that activation of AMPK inhibits HCC cell growth. Third, using HCC cell lines with stable knockdown of AMPK-α2, we showed that loss of AMPK-α2 enhanced the tumorigenicity of HCC cells addressed by colony formation (Fig. 2C), cell proliferation (Fig. 2D), growth in soft agar (Fig. 2E), or xenografts in nude mice (Fig. 2F). Thus, these data strongly support the notion that AMPK-α2 is a tumor suppressor in HCC. As to how AMPK-α2 expression is downregulated in HCCs, we speculated that AMPK-α2 expression may be repressed by promoter methylation, because a CpG island was found in the promoter region of AMPK-α2 when analysed using the software EMBOSS CpgPlot. Surprisingly, however, our results indicate that treatment with a histone deacetylase inhibitor, TSA, but not 5-Aza, restored the expression of AMPK-α2 in HCC cell lines expressing low levels of endogenous AMPK-α2 (Fig. 1C and D). This suggests that the AMPK-α2 promoter was suppressed by histone acetylation rather than DNA methylation in HCC cells.

Activation of AMPK by AICAR has been shown to induce cell-cycle arrest through the activation of the p53–p21 pathway (5, 19). Furthermore, activation of AMPK is associated with the phosphorylation of p53 at Ser15 and Ser20, which is coupled to the acetylation of p53 at Lys382 (19, 20). Consistent with these reports, we showed that overexpression of AMPK promoted Ser20 phosphorylation and Lys382 acetylation of p53 (Fig. 3A). However, our data suggested that AMPK did not directly phosphorylate the Ser15 or Ser20 sites on p53 (Fig. 3B). One possibility to explain this apparent discrepancy is that our investigation was carried out using a direct in vitro kinase assays, whereas previous studies were conducted in transfected cells; in the intact cells these sites on p53 might be phosphorylated by another kinase downstream of AMPK. It is worth noting that the sequences around Ser15 or Ser20 do not...
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The previously identified consensus recognition motifs for AMPK (14, 15).

Previous reports have shown that p53 is acetylated by p300/CPB at Lys382 (21) and is deacetylated by histone deacetylases (22). In this study, we showed that AMPK promoted the acetylation of p53 by inactivating the class III histone deacetylase SIRT1 by phosphorylation. We showed that AMPK directly phosphorylated SIRT1 at a novel site, Thr344, in cell-free assays and in intact cells, and that activation of SIRT1 in SIRT1-transfected cells inhibited the SIRT1-mediated deacetylation of p53 (Fig. 3C). To understand the mechanism by which AMPK regulates SIRT1, we explored the potential effects of AMPK-α2 on the localization and phosphorylation of SIRT1, because these mechanisms have previously been shown to play a role in modulating the activity of SIRT1 (23–25). First, interactions between AMPK-α2 and SIRT1 were clearly shown using protein interaction assays in cell-free systems and in intact cells (Fig. 4A and B) and the 2 proteins colocalized in the cell nucleus (Fig. 4C). Second, the interaction was mapped to the respective catalytic domains (Fig. 4D). Following these observations, we hypothesized that AMPK might regulate the activity of SIRT1 through direct phosphorylation. Using a cell-free kinase assay, we showed that AMPK robustly phosphorylates SIRT1 (Fig. 5A) and the site of phosphorylation was mapped to Thr344, which lies within the catalytic domain of SIRT1. Although it has been reported previously that there was no direct phosphorylation of SIRT1 by AMPK (26), we clearly observed a direct phosphorylation using various forms of purified AMPK in cell-free assays, as well as in intact cells (Fig. 5E). One explanation for this discrepancy may be the different methodologies used. Nevertheless, our findings strongly support the view that SIRT1 is a direct target for AMPK.

Following the identification of the AMPK phosphorylation site on SIRT1, the effect of SIRT1 phosphorylation by AMPK was studied through the generation of nonphosphorylatable (T344A) and potentially phosphomimetic mutants (T344E). Thr344 phosphorylation of SIRT1 did not affect its localization, but had a large effect on the deacetylase activity of SIRT1, both in cell-free assays and in intact cells. Our results show that the phosphomimetic mutant (T344E) had very little p53 deacetylase activity, comparable with that observed with the dominant-negative H363Y mutant of SIRT1 (Fig. 6A). Furthermore, the T344E mutant was incapable of deacetylating p53 on treatment of HepG2 cells with etoposide, indicating that phosphorylation at Thr344 inactivates the deacetylase activity of SIRT1 against p53 (Fig. 6B). It has been reported that amino acid residues Thr344 to Asp348 of SIRT1 are highly conserved among different species and are important for the deacetylase activity (27, 28). Moreover, Ile347 of SIRT1, which is in close proximity to Thr344, interacts with the acetyl group of acetylated p53 (16), suggesting that phosphorylation of Thr344 might directly interfere with SIRT1 substrate binding. Indeed, we showed that phosphorylation of Thr344 interrupts the interaction of SIRT1 with acetylated p53 as shown by a GST affinity pull-down assay (Fig. 6C). Although, our data strongly indicates that Thr344 phosphorylation blocks SIRT1 substrate binding, the possibility exists that this phosphorylation also affects its binding with the cofactor NAD⁺, as this region of the protein also has extensive interactions with NAD⁺ (27, 29).

It has been well characterized that the acetylation status of p53 plays an important role in its transcriptional activity (21, 30). Consistently, we showed that the T344E substitution silenced the ability of SIRT1 to suppress p53 transcriptional activity in HCC cells, and promoted the transcription of the p53 target genes, p21<sup>cip1</sup> and Bax (Fig. 6F). Because p21<sup>cip1</sup> and Bax are important proteins involved in induction of apoptosis (31, 32), we next showed that ectopic expression of the wild-type or phosphorylation-defective T344A mutant of SIRT1, but not the phosphomimetic T344E mutant, promoted etoposide-induced apoptosis and inhibited cell proliferation in HepG2 cells (Fig. 6F and G). Thus, the ability of AMPK to phosphorylate and inactivate SIRT1, and the subsequent activation of the p53-dependent apoptotic pathway, suggested a possible mechanism for AMPK to inhibit tumor growth in HCC. Taken together, these data are in line with the hypothesis that AMPK mediates its tumor suppressive effect in HCCs by promoting p53 acetylation.

Although 2 studies on cultured myotubes have shown a positive influence of AMPK on SIRT1 in studies of metabolism (26, 33), we have clearly shown a negative regulation of SIRT1 by AMPK in the HCC cancer model. Although there are parallels between the roles of AMPK and SIRT1 in metabolic regulation, their roles in cancer are distinct. One explanation for these apparently opposing findings is that liver cell function was examined in different contexts in the 2 studies. The LKB1-AMPK pathway is implicated in p53-dependent senescence (5), whereas SIRT1 promotes cellular senescence through the inactivation of p53 (34). Our finding that AMPK inhibits the activity of SIRT1 and reverses the suppressive effect of SIRT1 on p53 supports the idea that these 2 proteins have opposing effect on p53 regulation. As we found that AMPK is frequently downregulated in HCC, our model provided an explanation on how the dysregulation of the AMPK pathway would promote tumor growth through the SIRT1-p53 pathway.

Disclosure of Potential Conflicts of Interest
LOL. Ng is Loke Yew Professor in Pathology. No potential conflicts of interest were disclosed by the other authors.

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