Liver kinase B1/adenosine monophosphate-activated protein kinase signaling axis induces p21/WAF1 expression in a p53-dependent manner

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Abstract. Liver kinase B1 (LKB1) encodes a serine/threonine kinase and functions as a tumor suppressor. LKB1 loss-of-function somatic mutations are frequently observed in sporadic types of cancer, particularly in lung cancer. Ectopic LKB1 induces growth arrest by upregulating p21/cyclin dependent kinase inhibitor 1A (WAF1) in LKB1 deficient cervical and melanoma cancer cell lines. However, the underlying molecular mechanism remains to be elucidated. The present study built upon previous observations by confirming that the ectopic expression level of LKB1 significantly reduced colony formation of LKB1-deficient lung cancer cells. Mechanistically, the present study demonstrated that LKB1 overexpression significantly induced p21/WAF1 expression in a kinase-dependent manner. Conversely, LKB1 stable knockdown resulted in a decrease in p21/WAF1 expression level in colon cancer cells. In addition, it was revealed that pharmacological activation of adenosine monophosphate protein kinase (AMPK) by 2-deoxyglucose significantly increased the p21/WAF1 expression level, suggesting that AMPK acts downstream of LKB1 to induce p21/WAF1 expression. Furthermore, the present study demonstrated that functional p53 was required for p21/WAF1 induction by LKB1. Phosphorylation of p53-Ser^{15} was increased by ectopic LKB1 or AMPK activation. Taken together, these results suggested that LKB1 acts via its substrate, AMPK, to upregulate p21/WAF1 expression in a p53-dependent manner. Therefore, the present study identified an important signaling axis, providing novel molecular insights into the tumor suppressor role of LKB1.

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Introduction

Liver kinase B1 (LKB1), also known as serine/threonine kinase 11, is a well-established tumor suppressor. Germline mutations in LKB1 are associated with Peutz-Jeghers syndrome, a disorder characterized by benign hamartomas of the gastrointestinal tract (1) and is predisposed to certain types of cancer (2). LKB1 somatic mutations are frequently identified in sporadic types of cancer, particularly in lung cancer (20-30%) (3,4), ranking it as the third most frequently mutated gene in lung adenocarcinoma following p53 and K-Ras (5). As a serine/threonine protein kinase, LKB1 acts as a master upstream kinase of a group of adenosine monophosphate-activated protein kinases (AMPKs), and is involved in a wide range of cellular functions (6,7).

AMPK, one of the major substrates of LKB1, is an energy sensor and metabolic switch. AMPK is activated by LKB1 under energy stresses, including adenosine triphosphate (ATP) depletion induced by glycolysis inhibitors. 2-deoxyglucose (2-DG) is a well-characterized glycolysis inhibitor (8). 2-DG is converted by hexokinase to 2-DG-P, which cannot be further metabolized, thus is trapped inside the cell and inhibits hexokinase. The inhibition of glycolysis by 2-DG treatment induces a decrease in intracellular ATP concentration and an increase in intracellular AMP concentration. The increased AMP expression level binds to AMPK and alters its conformation, resulting in AMPK activation by LKB1 via phosphorylation of AMPK at Thr172 (9).

As a tumor suppressor, LKB1 was previously demonstrated to arrest cell cycle and inhibit cancer cell growth by inducing p21/cyclin dependent kinase inhibitor 1A (WAF1) (10-13). However, the molecular mechanism underlying p21/WAF1 induction by LKB1 is not well understood. Firstly, it remains an open question whether LKB1-mediated p21/WAF1 induction is specific to certain cancer cell lines or whether it is a general characteristic. In addition, the substrate molecule downstream of LKB1 mediating p21/WAF1 induction has not yet been identified. Finally, there have been contradicting findings on whether functional p53, the key transcription factor of p21/WAF1 (14), is required for p21/WAF1 induction by LKB1. The present study built upon previous studies (10-12) by

confirming that ectopic LKB1-induced p21/WAF1 expression occurs in lung and colon cancer cells. Mechanistically, it was revealed that p53 was required for p21/WAF1 upregulation by LKB1. In addition, the results of the present study suggested that AMPK may act downstream of LKB1 to increase p21/WAF1 expression, possibly by phosphorylating p53-Ser^{15}. Taken together, the results demonstrated that LKB1 acts via its substrate AMPK to induce p21/WAF1 expression in a p53-dependent manner. Therefore, the results of the present study have shed new light on the molecular mechanism underlying the tumor suppressor role of LKB1.

Materials and methods

Materials. Mouse monoclonal antibody against LKB1 was purchased from Abcam (Cambridge, UK). Antibodies against p21/WAF1, phosphorylated (p)-p53-Ser^{15} and p-AMPK-Thr 172 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti-GAPDH antibody was purchased from Santa Cruz Biotechonology, Inc. (Dallas, TX, USA). 2-DG was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The pEGFP-C2, pEGFP-LKB1 and pEGFP-LKB1-K78M plasmids were provided by Professor Wei Zhou (The Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA, USA). The lentiviral LKB1 short hairpin RNA (shRNA) construct and a negative control construct, which was created in the same vector system (pLKO.1), were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Lentiviral helper plasmids, pCMV-dR8.2 dvpr and pCMV-VSV-G, were obtained from Addgene, Inc. (Cambridge, MA, USA).

Cell lines and cell culture. A549 and H460 lung cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The p53 wild-type HCT116 and the isogenic HCT116 p53$^{-/-}$ colon cell lines were provided by Professor Wei Zhou (The Winship Cancer Institute, Emory University School of Medicine). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific Inc.) at 37˚C in a humidified atmosphere with 5% CO_2.

Western blotting. Following treatment, cells were lysed on ice for 20 min using the radioimmunoprecipitation assay lysis and extraction buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.). The protein concentration of each cell sample was determined using the bicinchoninic acid protein assay reagent (Thermo Fisher Scientific, Inc.). A total of 20 μg protein/lane was denatured in sodium dodecyl sulfate sample buffer (cat. no. 9173; Takara Biotechnology Co., Ltd., Dalian, China) and separated using SDS-PAGE (10% gel). Subsequently, proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Followed blocking with 5% skimmed milk for 1.5 h at room temperature, the membranes were incubated with the recommended dilutions of primary antibodies against LKB1 (cat. no. ab-15095; dilution, 1:1,000; Santa Cruz Biotechnology, Inc.), p21/WAF1 (cat. no. 2947; dilution, 1:1,000; Cell Signaling Technology, Inc.), p-AMPK (cat. no. 8208; dilution, 1:1,000; Cell Signaling Technology, Inc.), p-p53 Ser^{15} (cat. no. 9284; dilution, 1:1,000; Cell Signaling Technology, Inc.), p53 (cat. no. 9282; dilution, 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (cat. no. sc-47724; dilution, 1:3,000; Santa Cruz Biotechnology, Inc.) at 4˚C overnight and rinsed twice with PBS. The membranes were subsequently incubated with the following secondary antibodies: Horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG; cat. no. ZB2301; dilution, 1:10,000; OriGene Technologies, Inc.) or the HRP-conjugated anti-mouse IgG antibody (cat. no. ZB2305; dilution, 1:10,000; OriGene Technologies, Inc.) at room temperature for 2 h. Peroxidase-labeled bands were visualized using an enhanced chemiluminescence detection reagent (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Results were analyzed using Image software (version 1.5; National Institutes of Health, Bethesda, MD, USA) to compare the relative target protein expression.

Colony formation assay. A549 cells were plated at a density of 2x10^5 cells/well in six-well plates overnight at 37˚C in a humidified incubator containing 5% CO_2. The following day, cells were transfected with plasmids encoding wild-type LKB1 or mutant LKB1-K78M (kinase-deficient) in duplicate with Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 2 μg plasmid and 6 μl Lipofectamine 2000 were added to each well. Cells were selected using G418 (2 mg/ml) 72 h after transfection for 2 weeks at 37˚C in a humidified incubator containing 5% CO_2. The medium was replaced every 4 days. Finally, the cells were fixed with 10% trichloroacetic acid solution for 20 min and stained with 0.5% crystal violet for 30 min at room temperature. The stained cells were washed and air-dried, then observed under a light microscope (x40, magnification). Colony numbers were assessed visually and colonies containing >50 normal-appearing cells were counted. Statistical differences in colony numbers between the wild-type LKB1 and LKB1-K78M plasmid-transfected cells were evaluated using the two-tailed Student's t-test.

LKB1 stable knockdown using lentiviral shRNA. Lentivirus stocks were prepared according to the manufacturer's protocol, as previously described (15). Briefly, 1.5x10^6 293T cells were plated in 10-cm dishes. Cells were co-transfected with shRNA constructs (3 μg) together with pCMV-dR8.2 dvpr (3 μg) and pCMV-VSV-G (0.3 μg) helper constructs. Following 2 days of incubation at 37˚C in a humidified atmosphere containing 5% CO_2, viral stocks were harvested from the culture medium, which was filtered to remove non-adherent 293T cells. In order to select the colon cancer cells that were stably expressing shRNA constructs, HCT116 cells were plated at sub-confluent densities, and infected with a cocktail of 1 ml virus-containing RPMI medium, 3 ml RPMI-medium (no antibiotics, heat inactivated serum) and 8 μg/ml polybrene at 37˚C in a humidified atmosphere with 5% CO_2. Selection with 2 μg/ml puromycin was initiated 48 h after lentivirus infection. Following ~4 weeks of selection, monolayers of stably infected pooled clones were harvested for use and cryopreserved.

RNA extraction and quantitative polymerase chain reaction (qPCR). Total RNA was isolated from HCT116-pLKO.1 and HCT116-LKB1 isogenic colon cancer cell lines using TRIzol®

reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The first-strand cDNA was prepared using the PrimeScript First strand cDNA Synthesis kit (Takara Bio, Inc.), according to the manufacturer's protocol, using 1 μg total RNA. All qPCR reactions were performed in a 20 μl mixture containing 1X SYBR Green supermix (Takara Bio, Inc.), 0.2 μmol/l of each primer and 2 μl cDNA template. Primers for LKB1 were as follows: Forward, 5'-CTGGGGTCACCCTCTACAAC-3'; and reverse, 5'-ACTCAAGCATCCCTTTCAGC-3'. Primers for GAPDH were as follows: Forward, 5'-GGAGTCAACGGATTTGGTCG-3'; and reverse, 5'-CTTGATTTTGGAGGGATCTCG-3'. qPCR was performed using the Applied Biosystems 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following cycling conditions: (Step 1) 95˚C for 30 sec; (step 2) 40 cycles of 95˚C for 5 sec; 60˚C for 34 sec; the melting curve stage was followed. The relative expression level of LKB1 was normalized to GAPDH. Relative mRNA concentrations were determined using the $2^{-\Delta\Delta Cq}$ method (16).

RNA interference. The LKB1 small interfering RNA (siRNA) sequence was as follows: Sense, 5'-CCAACGUGAAGAAGGAAAUTT-3' and antisense, 5'-AUUUCCUUCACGUUGGTT-3'. The control siRNA sequences was as follows: Sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. All siRNAs were synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China). The HCT116 and HCT116p53-/- cancer cell lines were used for RNA interference. Cells were plated at a density of $2x10^5$ cells/well in 6-well plates overnight at 37˚C in a humidified incubator containing 5% CO_2. Cells were subsequently transfected with 100 nmol/l of LKB1 siRNA or negative control siRNA using the Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific Inc.), according to the manufacturer's protocol. For every 105 cells, 0.5 μg LKB1 siRNA and control siRNA were diluted and mixed with 3 μl transfection reagent. After incubation for 30 min at room temperature, the transfection mixture was added to the cells. Following 6 h of incubation at 37˚C in a humidified incubator containing 5% CO_2, the RPMI-1640 medium was replaced with serum-enriched 1640-RPMI medium (RPMI-1640 medium supplemented with 10% fetal bovine serum) and the cells were cultured for an additional 48 h at 37˚C in a humidified incubator containing 5% CO_2. Subsequently, the transfected cells were collected.

Statistical analysis. SPSS software (version 17; IBM Corporation, NY, USA) was used for statistical analysis of the results. The majority of the results are representative ≥3 independent experiments and are presented as the mean ± standard deviation of triplicate samples. Error bars represent standard deviations between experiments. One-way analysis of variance and Student's t-test (≤2 groups) were used to determine statistical significance. Fisher's least significant difference post-hoc test was used to compare the differences between ≥3 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Restoring LKB1 activity in lung cancer cells induces growth suppression. To investigate whether the kinase activity of LKB1 is required for growth inhibition in lung cancer cells, LKB1 mutant A549 cells were transfected with plasmids encoding wild-type LKB1 or mutant LKB1-K78M (kinase-deficient). Subsequently, these transfections were subjected to G418 (2 mg/ml) selection for 14 days. As presented in Fig. 1A and B, ectopic expression of wild-type LKB1 resulted in a significantly reduced number of colonies compared with those transfected with LKB1-K78M. In order to evaluate the transfection efficiency of LKB1, the protein expression level of LKB1 was evaluated using western blotting. As presented in Fig. 1C, the expression level of wild-type LKB1 and the mutant were similar, suggesting equal transfection efficiencies. This result suggests that the kinase activity of LKB1 is required for the suppression of lung cancer cell proliferation.

LKB1 upregulates p21/WAF1 expression in lung and colon cancer cells. p21/WAF1 serves an essential role in arresting cell cycle and inducing growth suppression; therefore, the present study suggested that p21/WAF1 may be functionally important for the tumor suppressive effects of LKB1 in lung cancer cells. To evaluate whether LKB1 regulates p21/WAF1 expression in NSCLC cells, LKB1 mutant A549 and H460 cells were used. According to our previous study, H460 and A549 cells harbor a nonsense mutation at codon 37 of LKB1, thus LKB1 protein is not expressed in these cells lines (15). The cells were transfected with plasmids encoding wild-type LKB1, LKB1 K78M or vector, and the protein expression level of p21/WAF1 was determined through western blotting. As presented in Fig. 2A, compared with the control group, ectopic LKB1 significantly increased p21/WAF1 protein expression level in both cell lines. Conversely, kinase deficient LKB1-K78M did not have a significant effect on p21/WAF1 expression level. These results suggest that the kinase activity of LKB1 is required to upregulate p21/WAF1 expression in lung cancer cells.

In order to validate this observation in other types of cancer cells, the LKB1 wild-type colon cancer cell line HCT116 was used. Using the lentivirus system, isogenic HCT116-pLKO.1 and HCT 116-LKB1 shRNA cell lines were established. As presented in Fig. 2B and C, compared with the control group, LKB1 mRNA and protein expression levels were significantly suppressed in HCT 116 cells stably expressing LKB1 shRNA. Additionally, it was revealed that LKB1 knockdown significantly decreased the expression level of p21/WAF1 (Fig. 2C), confirming that LKB1 positively regulated p21/WAF1 expression level in colon cancer cells.

AMPK acts downstream of LKB1 to regulate p21/WAF1 expression level. It has been reported that deficiency of AMPK-α1, a canonical substrate of LKB1 as described above, reduces p21/WAF1 expression level in mouse embryonic fibroblasts (17). Thus, the present study suggested that LKB1 acts indirectly to induce p21/WAF1 expression via its substrate AMPK. To investigate this hypothesis, 2-DG, a glycolysis inhibitor and AMPK activator, was used. HCT116 cells were treated with 25 mM 2-DG for 24 h, the expression levels of p-AMPK Thr172 and p21/WAF1 were evaluated using western blotting. As presented in Fig. 3A, 2-DG treatment induced AMPK phosphorylation and significantly enhanced p21/WAF1 expression, suggesting that AMPK activation serves a role in p21/WAF1 induction.

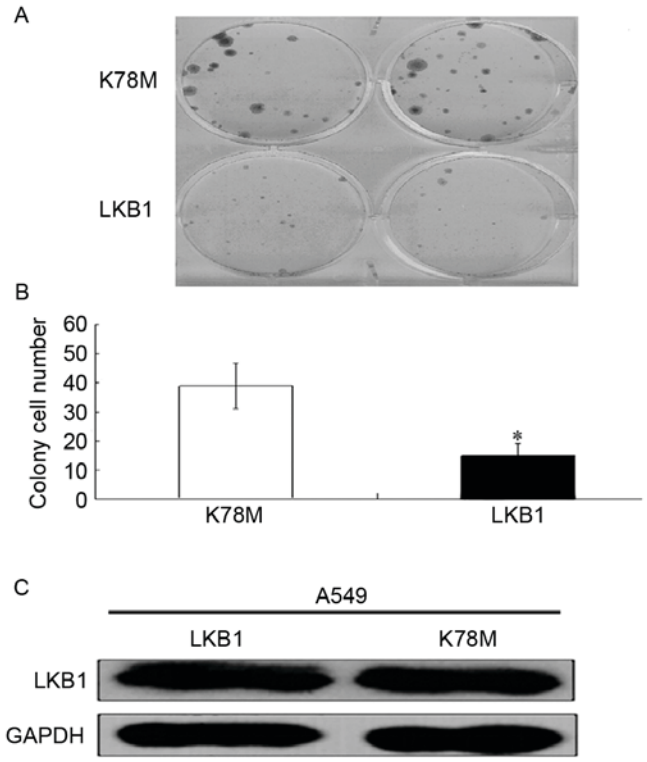

Figure 1. Restoring LKB1 activity in lung cancer cells induces growth suppression. (A) A549 cells were transfected with plasmids encoding wild-type LKB1 or mutant LKB1-K78M (kinase-deficient) in duplicate, selected with G418 (2 mg/ml) 72 h after transfection for 2 weeks and subsequently colony formation assays were performed. Colony numbers were assessed visually and colonies containing >50 normal-appearing cells were counted. (B) Experiments were performed as in (A), and colony numbers were evaluated using the two-tailed Student's t-test. Results are presented as the mean ± standard error of the mean from three independent experiments. *P<0.05 vs. the control. (C) Experiments were performed as in (A) and LKB1 protein expression level was determined through western blotting with GAPDH as the loading control. Results are presented as the mean ± standard deviation. LKB1, liver kinase B1.

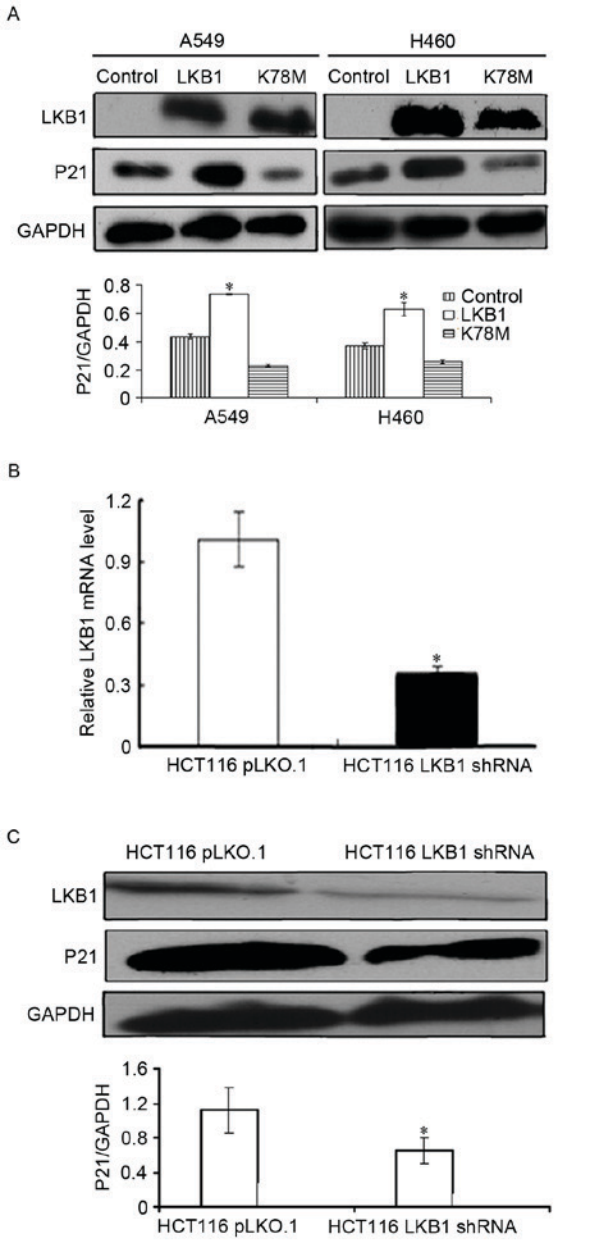

Figure 2. LKB1 upregulates p21/WAF1 expression level in lung and colon cancer cells. (A) LKB1 mutant A549 and H460 NSCLC cells were transfected with plasmids encoding wild-type LKB1, LKB1 K78M or vector, and LKB1 and p21/WAF1 protein expression level was examined using western blotting with GAPDH used as the loading control. (B) Isogenic colon cancer cell lines, HCT116-pLKO.1 and HCT116-LKB1 shRNA, were established using the lentivirus system. LKB1 mRNA expression level was determined using quantitative polymerase chain reaction normalized to GAPDH. (C) The cell lysates of isogenic HCT116-pLKO.1 and HCT116-LKB1 shRNA cells were collected, and the LKB1 and p21/WAF1 expression level was analyzed using western blotting. GAPDH served as a loading control. *P<0.05 vs. the control. Each blot is representative of three blots obtained from three independent experiments. Results are presented as the mean ± standard deviation. NSCLC, non-small cell lung cancer; LKB1, liver kinase B1; shRNA, short hairpin RNA; WAF1, cyclin dependent kinase inhibitor 1A.

2-DG also demonstrated an off-target effect, which is independent of LKB1/AMPK signaling. To confirm that 2-DG induced p21/WAF1 expression mediated by LKB1/AMPK signaling, the isogenic HCT116-pLKO.1 and HCT116-LKB1 shRNA cell lines were treated with 25 mM 2-DG for 24 h. As presented in Fig. 3B, 2-DG activated AMPK and significantly increased p21/WAF1 expression in HCT116-pLKO.1 cells. In contrast, in HCT116-LKB1 shRNA cells, 2-DG failed to induce AMPK activation, and LKB1 depletion diminished the 2-DG-inducing effects on p21/WAF1. In addition, it was revealed that the phosphorylation of p53-Ser^{15} was increased following treatment with 2-DG, which was significantly attenuated by LKB1 loss. These results suggest that AMPK acts downstream of LKB1 to induce p21/WAF1 expression.

LKB1/AMPK requires p53 to regulate p21/WAF1. The phosphorylation of p53 Ser^{15} is important for the stabilization of p53 protein (18), thus the present study suggested that p53 may be involved in p21/WAF1 induction by LKB1/AMPK. To determine whether p53 is required for LKB1-mediated p21/WAF1 induction, the p53 wide-type HCT116 colon cell line and its isogenic clones with p53 depletion (HCT116 p53$^{-/-}$) were used. As presented in Fig. 4A, the protein expression level of p53 was almost undetectable in HCT116 p53$^{-/-}$ cells. These cells were then transiently transfected with control short interfering (si)RNA or LKB1 siRNA, and the expression

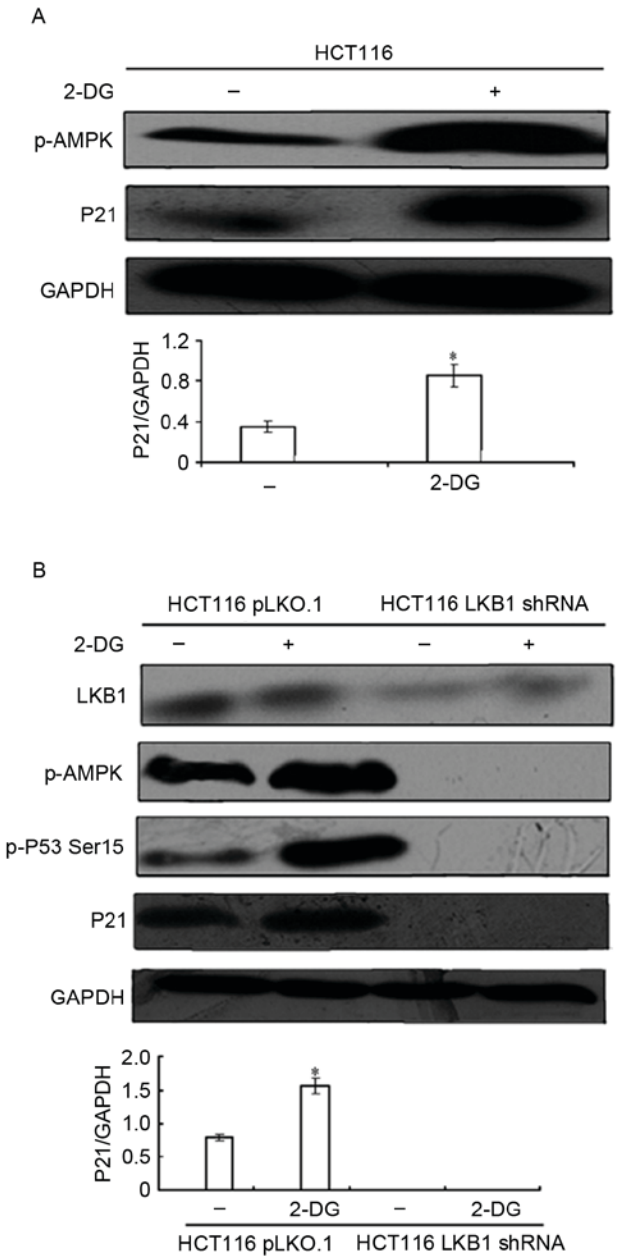

Figure 3. AMPK acts downstream of LKB1 to regulate p21/WAF1 expression level. (A) HCT116 cells were treated with 25 mM 2-DG for 24 h and western blotting was performed to evaluate p-AMPK Thr172 and p21/WAF1 level, with GAPDH as the loading control. (B) The isogenic HCT116 pLKO.1 and HCT116 LKB1 shRNA cell lines were treated with 25 mM 2-DG for 24 h, p-AMPK, p-p53Ser[15] and p21/WAF1 expression levels were detected by western blotting and GAPDH served as the loading control. *P<0.05 vs. the control. Each blot is representative of three blots obtained from three independent experiments. Results are presented as the mean ± standard deviation. AMPK, adenosine monophosphate protein kinase; LKB1, liver kinase B1; 2-DG, 2-deoxyglucose; p, phosphorylated; shRNA, short hairpin RNA; WAF1, cyclin dependent kinase inhibitor 1A.

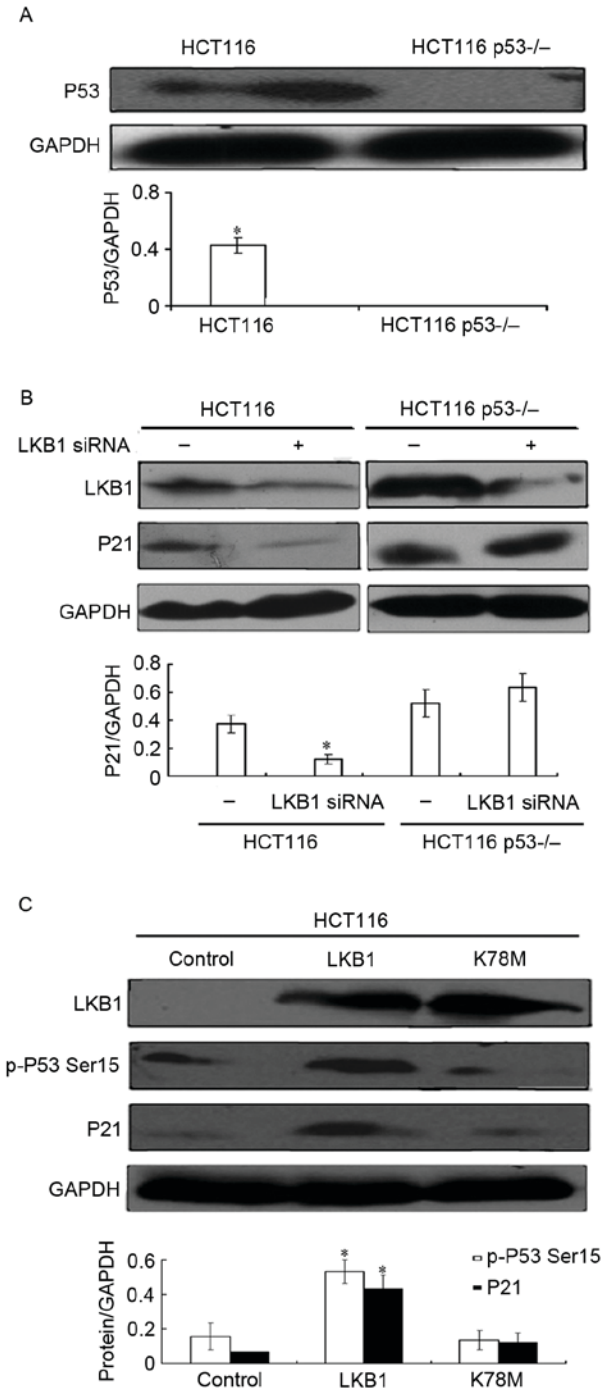

Figure 4. LKB1/AMPK requires p53 to regulate p21/WAF1. (A) P53 protein expression level in P53 wide-type HCT116 colon cell line and the isogenic HCT116 $p53^{-/-}$ cells. (B) P53 wild-type HCT116 and the isogenic HCT116 $p53^{-/-}$ cells were transiently transfected with control siRNA or LKB1 siRNA, and LKB1 and p21/WAF1 expression levels were analyzed using western blotting, with GAPDH used as a loading control. (C) HCT116 cells were transfected with plasmids encoding wild-type LKB1, LKB1 K78M or vector, and western blotting was performed to analyze p-p53 Ser[15] and p21/WAF1 expression levels. GAPDH serves as the loading control. *P<0.05 vs. the control. Each blot is representative of three blots obtained from three independent experiments. Results are presented as the mean ± standard deviation. siRNA, short interfering RNA; LKB1, liver kinase B1; AMPK, adenosine monophosphate protein kinase; WAF1, cyclin dependent kinase inhibitor 1A.

level of p21/WAF1 was analyzed by western blotting. As presented in Fig. 4B, it was revealed that LKB1 depletion by siRNA in p53 wide-type HCT116 cells induced a lower expression level of p21/WAF1. Conversely, in p53-absent HCT116 $p53^{-/-}$ cells, LKB1 transient knockdown did not significantly alter the p21/WAF1 expression level. Therefore, these results indicate that LKB1-mediated p21/WAF1 induction is p53-dependent.

To directly evaluate whether LKB1 was involved in p53-Ser[15] phosphorylation, HCT116 cells were transfected with plasmids encoding wild-type LKB1, LKB1 K78M or

vector, and western blot analysis was performed to analyze p-p53 Ser^{15} and p21/WAF1 expression levels. As presented in Fig. 4C, wild-type LKB1 significantly increased the expression levels of p-p53 Ser^{15} and p21/WAF1 compared with the control, whereas kinase-deficient LKB1 did not have any significant effects. These results suggest that LKB1 induces the phosphorylation of p53-Ser^{15} in a kinase-dependent manner, which may contribute to the upregulation of p21/WAF1 expression.

Discussion

Numerous previous studies provide evidence that LKB1 serves an essential role as a tumor suppressor. Tiainen *et al* (10) demonstrated that LKB1 induced cell cycle arrest and inhibited cell growth by upregulating the p21/WAF1 expression level in LKB1 deficient cervical (HeLa), S3, and melanoma (G361) cancer cell lines. However, it remains unclear whether LKB1 regulates p21/WAF1 expression in other types of cancer cells. The results of the present study confirmed and extended previous observations in lung cancer and colon cancer cells (10-12). Firstly, the present study demonstrated that ectopic LKB1 increased p21/WAF1 expression level in LKB1 mutant NSCLC cells in a kinase-dependent manner. Furthermore, by establishing an isogenic LKB1 stable knockdown colon cell line, it was revealed that LKB1 depletion significantly reduced p21/WAF1 protein expression level. Human LKB1 is a nuclear and cytoplasmic protein. There have been contradicting studies investigating the effect of LKB1 cellular localization on p21/WAF1 expression levels (11-13). Future investigations are required to identify which part of LKB1, localized in the cytoplasm or in the nucleus, serves a more significant role in p21/WAF1 induction.

AMPK, one of the key substrates of LKB1, is considered as the 'cellular fuel gauge' in sensing and modulating metabolic processes (9). AMPK is a heterotrimeric enzyme composed of two regulatory β and γ subunits and a catalytic α subunit (α1 and α2) (19). Xu *et al* (17) demonstrated that AMPKα1 deficiency induced p21/WAF1 reduction in mouse embryonic fibroblasts. Consistent with their findings, the present study revealed that pharmacological activation of AMPK by 2-DG significantly increased the p21/WAF1 expression level, and depletion of LKB1/AMPK impaired the ability of 2-DG to induce p21/WAF1. Thus, the present study identified AMPK as a potential downstream molecule of LKB1 involved in the mediation of p21/WAF1 induction. We previously revealed that 2-DG treatment potently inhibits the proliferation of LKB1 wild-type lung cancer cells (8). Given the importance of p21/WAF1 in growth arrest, it has been suggested that 2-DG-mediated p21/WAF1 induction may contribute to the inhibitory effect of the compound. Upregulation of p21/WAF1 by LKB1/AMPK may represent the mechanism underlying growth arrest when cancer cells are exposed to energy stresses with ATP depletion.

Contradicting findings have been reported regarding the role of transcription factor p53 in p21/WAF1 induction mediated by LKB1. Tiainen *et al* (11) and Zeng *et al* (12) reported that LKB1 required p53 to induce p21/WAF1 expression. In contrast, a previous study by Setogawa *et al* (20) demonstrated that LKB1 has the potential to induce p21/WAF1 expression in collaboration with LIM domain only 4, GATA binding protein 6 and LIM domain binding, 1 in the p53-deficient HelaS3 cell line (20), suggesting a p53-independent mechanism. The present study confirmed that p53 was required for LKB1-mediated p21/WAF1 induction, as p53 depletion in colon cancer cells were able to inhibit p21/WAF1 regulation by LKB1. Furthermore, the present study revealed that the phosphorylation of endogenous p53-Ser^{15} was increased by LKB1 overexpression or AMPK activation, suggesting that p53-Ser^{15} phosphorylation, a modification essential for p53 stabilization, may be involved in p21/WAF1 upregulation. The results of the present study are consistent with previous reports indicating that AMPK induces phosphorylation of p53-Ser15 in hepatoma HepG2 cells (21), mouse embryonic fibroblasts (22), human aortic smooth muscle cells and rabbit aortic strips (23). Other kinases have been demonstrated to phosphorylate p53 at Ser^{15}, including ATM and ATR serine/threonine kinases, as they also target p53 at this site (24). Thus, some phosphorylation does occur, despite the fact that the results of the present study demonstrated that LKB1 depletion induced a significant reduction in the expression levels of p-p53. Further investigations are required to confirm that LKB1/AMPK-mediated p53-Ser^{15} contributes to p21/WAF1 induction.

In conclusion, the results of the present study demonstrated that in lung and colon cancer cells, LKB1 acts via AMPK to induce p21/WAF1 expression in a p53-dependent manner. Therefore, the present study provides novel molecular insights into the tumor suppressor gene, LKB1.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DZ was responsible for study conception; QM, PX, LS designed and performed the research; QM, PX, LS, and JW analyzed the data; PX, LS and DZ wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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