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Berberine inhibits human colon cancer cell migration via AMP-activated protein kinase-mediated downregulation of integrin β1 signaling

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ABSTRACT

Colon cancer is associated with a poor prognosis, motivating strategies to prevent its development. An encouraging preventative strategy is the use of nutraceuticals; however, scientific verification of therapeutic functions and mechanisms of biological activity are necessary for the acceptance of dietary supplements in cancer treatment. Berberine is a benzylisoquinoline alkaloid extracted from many kinds of medicinal plants that has been extensively used as a Chinese traditional medicine. Recently, berberine has been reported to possess antitumoral activities. Among the various cellular targets of berberine is AMP-activated protein kinase (AMPK), which regulates tumor progression and metastasis. However, the specific role of berberine-induced AMPK activation and its effects on the metastatic potential of colon cancer remain largely unknown. The present study investigated berberine-induced activation of AMPK and its effects on colon cancer cell migration. Berberine decreased the migration of SW480 and HCT116 cells. We found that berberine activated AMPK in human colon cancer cell lines. Notably, berberine-induced activation of AMPK reduced the integrin β1 protein levels and decreased the phosphorylation of integrin β1 signaling targets. Knockdown of AMPKα1 subunits using small interfering RNA significantly attenuated berberine-induced downregulation of integrin β1 and inhibition of tumor cell migration. Collectively, our results suggest that berberine-induced AMPK activation inhibits the metastatic potential of colon cancer cells by decreasing integrin β1 protein levels and downstream signaling.

1. Introduction

Metastasis is the last stage in the progression of cancer. A variety of molecules contribute to this complex, multistep process in which cancer cells detach from the primary tumor, migrate into blood vessels, and disseminate throughout the body, ultimately seeding in distant organs [1]. The natural history of colorectal carcinoma (CRC) predicts that many patients will experience metastasis to secondary organs and fail to respond to curative therapies. It has been reported that one-third of colon carcinoma patients developed hepatic metastasis [2–4]. Therefore, strategies for preventing CRC progression and metastasis have gained considerable research attention in recent years.

Berberine, a botanical alkaloid found in a number of clinically important medicinal plants, possesses diverse pharmacological effects and appears to exhibit relatively low toxicity toward normal human cells [5]. By contrast, berberine has demonstrated cytotoxic effects on most cancer cell types, highlighting the promise of berberine as a potential multispectrum anticancer therapeutic agent. In addition to its antineoplastic activity, novel therapeutic targets of berberine have been extensively investigated [5,6]. It has been recently reported that berberine strongly induces AMP-activated protein kinase (AMPK) activity by decreasing glucose levels and promoting reactive oxygen species (ROS) production [7].

A disturbed energy balance underlies a number of human metabolic diseases, including obesity, diabetes, and cancer. AMPK, which is activated by a decrease in the ATP/ADP ratio that may accompany such metabolic abnormalities, inhibits ATP-consuming metabolic pathways and facilitates activation of energy-producing pathways. AMPK is heterotrimer containing a catalytic subunit (α) and two regulatory subunits (β and γ) that essentially functions as an energy sensor. AMPK is phosphorylated by liver kinase B1 (LKB1) and calmodulin-dependent protein kinase kinase β (CaMKKβ). Tuberous sclerosis complex 2 (TSC2) and p53, which function as tumor suppressors, are also involved in the AMPK signaling network and thus contribute to the connections linking metabolism and cancer development [8,9]. Recent studies have indicated that AMPK controls metastasis of cancer cells [10–12]. However, the

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relationship between AMPK activity and metastasis is unclear, and whether treatment with therapeutic AMPK activators is an effective option for altering the metastatic potential of colon cancer cells remains a matter of controversy.

Sites of metastatic lesions in secondary organs are determined by the adhesion of tumor cells to endothelial cells in the blood vessel and the underlying extracellular matrix (ECM). Important to these interactions is the role played by integrin-mediated adhesion of malignant cells to ECM components [13]. Integrins are adhesion receptors composed of heterodimerically associated α and β subunits. Integrins transmit signals across the plasma membrane via the tyrosine kinases Src and focal adhesion kinase (FAK) and the CRK-associated tyrosine kinase substrate p130Cas, and thereby regulate cell adhesion, migration, invasion, proliferation, and differentiation [14]. We recently found that integrin β1 plays a significant role in regulating migration of human colon cancer [15]. Whereas, previous studies have demonstrated the involvement of integrin β1 in regulating metastasis, none has investigated berberine-induced activation of AMPK or its effect on integrin β1-mediated metastasis of human colon cancer.

In this study, we investigated berberine-induced activation of AMPK and its effects on colon cancer cell migration. Berberine decreased the migration of SW480 and HCT116 cells. This agent activated AMPK in human CRC cell lines. Berberine reduced the migration of colon cancer cells through a decrease in integrin β1 protein stability and a reduction in phospho-Src, FAK, and p130Cas. Importantly, depletion of the AMPKα1 subunit using small interfering RNA significantly attenuated the berberine-induced inhibition of tumor cell migration. Our results suggest that berberine-induced AMPK activation inhibits migration-promoting signaling by decreasing integrin β1 protein levels and downstream signaling, thereby reducing the metastatic potential of colon cancer cells.

2. Materials and methods

2.1. Material

Berberine and *N*-acetyl-cysteine (NAC) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Antibodies against AMPKα1, integrin β1, p130Cas, and phospho-FAK^{Y397} were from BD Biosciences (San Jose, CA, USA). Antibodies against total and phosphorylated (phospho) ACC^{S79}, phospho-p130Cas^{Y410}, Src, phospho-Src^{Y416} and 5-aminoimidazole-4-carboxaminde-1-β-D-ribofuranoside (AICAR) were from Cell Signaling Technology Inc. (Danvers, MA, USA). Antibodies against FAK and α-tubulin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). A predesigned siRNAs specific for AMPKα1 and integrin β1 were purchased from Bioneer (Seoul, Republic of Korea).

2.2. Cell culture and transfection

SW480, SW620, HT-29, and HCT116 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). SW480 and SW620 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and HT-29 and HCT116 cells were grown in McCoy's 5a medium; both media were supplemented with heat-inactivated 10% fetal bovine serum and antibiotics and all cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cells were transfected with an AMPK expression plasmid or si-AMPKα1 using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA).

2.3. Cell migration assay

Cell migration was assayed using Transwell chambers containing filters with a pore size of 8.0 μm (Corning Costar, Cambridge, MA, USA). Cells (1×10^5) were added into the upper chamber and incubated for 24 h at 37 °C. Twenty-four hours after plating, the cells on the lower surface were stained with Diff-Quick solution (Baxter Scientific, Deerfield, IL, USA). Images of migrating cells were captured in randomly three selected fields using a phase-contrast microscope, cell numbers were counted, and expressed as relative fold.

2.4. Cell viability assay

A Vi-CELL XR Cell Viability Analyzer (Beckman Coulter) cell counter, which performs an automated trypan blue exclusion assay, was used to measure cell viability. The instrument collects 100 images of cells to compute viability.

2.5. Protein preparation and immunoblot analysis

The cells were lysed with lysis buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Protein samples were denatured and resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then transferred onto a nitrocellulose membrane and blocked by 5% nonfat dry milk in TBST (Tris-buffered saline with 0.1% Tween-20). After blocking, the membrane was incubated with the primary antibody at 4 °C overnight and then with the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were visualized using enhanced chemiluminescence reagents.

2.6. Measurement of reactive oxygen species (ROS) generation

The cells were incubated with 10 μM 2′, 7′-dichlorodihydrofluorescein diacetate (DCF-DA; Molecular Probes) dye for 30 min. The adherent cells were trypsinized and collected. The intensity of DCF-DA fluorescence was determined using a FACSCanto II flow cytometer (BD Biosciences).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed using Omniscript transcriptase (Qiagen, Hilden, Germany). PCR amplifications were performed using the following primer pairs: integrin β1, sense 5′-AAT GAA GGG CGT GTT GGT AG-3′ and antisense 5′-CTG CCA GTG TAG TTG GGG TT-3′; GAPDH, sense 5′-TGC TGA GTA TGT CGT GGA GTC TA-3′ and antisense 5′-AGT GGG AGT TGC TGT TGA AGT CG-3′. PCR thermocycling conditions were 95 °C for 5 min followed by 30 cycles of 94 °C for 45 s, 60 °C for 2 min, and 72 °C for 2 min. The amplified products were visualized on ethidium bromide-stained 1% agarose gels.

2.8. Statistical analysis

Data are expressed as means ± standard deviations (SDs) of at least three experiments. Statistical significance was determined using Student's *t*-test for comparisons between two means at *p*-values less than 0.01 (∗) or 0.05 (∗∗).

3. Results

3.1. Berberine inhibits human colon cancer cell migration

Berberine (Fig. 1A) has been strongly suggested to possess antimetastatic activity [5]. To determine whether berberine inhibits

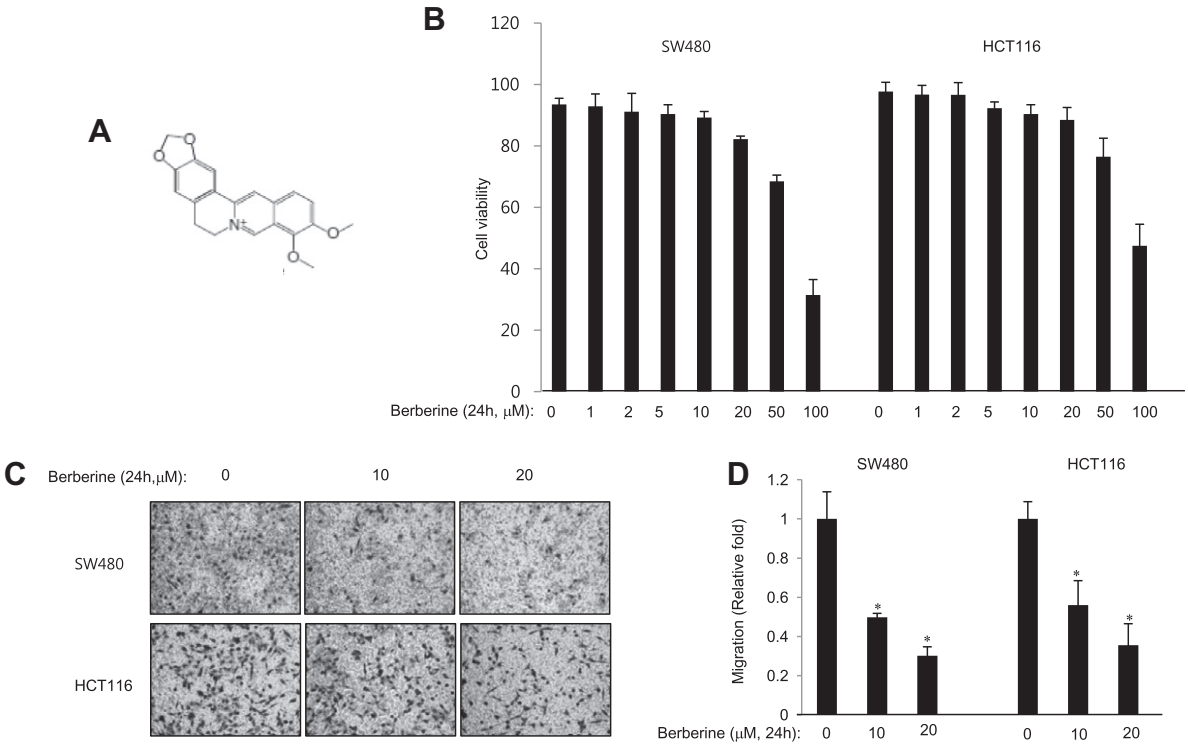

Fig. 1. Effect of berberine on colon cancer cell migration. (A) Structure of berberine. (B) Concentration-dependent effects of berberine on cell viability were determined on SW480 and HCT116 human cells using a Vi-CELL XR Cell Viability Analyzer. (C) SW480 and HCT116 cells were incubated with the indicated concentration of berberine for 24 h, and then cell migration was analyzed using a Transwell migration assay. Images of migrating cells were captured in randomly three selected fields using a phase-contrast microscope. (D) Migrating cells were counted and the results were expressed as the mean number of migratory cells ± SD per microscopic field for three determinations in triplicate.

migration of human CRC cells, we tested its effects on the migration of SW480 and HCT116 cells. Before examining migration, we performed a cell viability analysis to determine the lowest concentration of berberine that did not induce cell death. Berberine reduced cell viability in a concentration-dependent manner, effectively inhibiting proliferation at concentrations greater than 50 μM (Fig. 1B). We found that both 10 and 20 μM berberine, concentrations that inhibited cell proliferation by less than 20% after a 24 h treatment (Fig. 1B), reduced the migration of SW480 and HCT116 cells (Fig. 1C). The numbers of migrating cells per microscopic field are summarized in Fig. 1D.

3.2. Berberine-induced ROS generation is involved in AMPK activation

We next investigated the relationship between berberine-induced inhibition of colon cell migration and AMPK activation, which has previously been linked with cancer cell metastatic potential. We first demonstrated that the AMPKα1 catalytic subunit is expressed at the protein level in SW480, HCT116, HT-29, and SW620 cells (Fig. 2A). To test the functional expression of AMPK, we examined the phosphorylation level of acetyl CoA carboxylase (ACC), a target of AMPK known to be a reliable indicator of AMPK activity [7]. Berberine strongly activated AMPK in human colorectal cell lines (Fig. 2B), as evidenced by the enhanced levels of phosphorylated ACC. AMPK activation was increased by 20 μM berberine treatment within 8 h and was sustained for up to 24 h (Fig. 2C). To elucidate the mechanisms of berberine-induced AMPK activation, we assessed the levels of ROS, known to be a potent activator of AMPK [8]. Consistent with previous reports, we found that berberine increased ROS generation in HCT116 cells (Fig. 2D). We confirmed that NAC, an ROS scavenger, blocked berberine-induced ROS generation (Fig. 2D) and AMPK activation (Fig. 2E), as reflected in a decreased level of phospho-ACC. These results indicate that ROS is a mediator of berberine-induced AMPK activation in human colon cancer cells.

3.3. AMPK acts through a reduction in integrin β1 levels and downstream signaling to play a critical role in berberine-induced inhibition of human colon cancer

Integrin β1 plays a significant role in tumor metastasis. It binds to the ECM and initiates adhesion by recruiting cytoplasmic proteins, such as Src, FAK, and p130Cas [16]. Previously, we reported that knockdown of integrin β1 abrogated the adhesion and migration of SW480 colon cancer cells [15]. Therefore, we examined whether the inhibitory effect of berberine on the migration of colon cancer cells involved integrin function. We found that integrin β1 levels were significantly decreased by berberine treatment in SW480 and HCT116 cells (Fig. 3A). In addition, berberine treatment for 24 h resulted in a significant reduction in the phosphorylated forms of Src, FAK, and p130Cas (Fig. 3A). To investigate whether the berberine-induced decrease in integrin β1 is caused by inhibition of transcription or post-translational regulation, we determined integrin β1 mRNA using RT-PCR. Integrin β1 protein levels were reduced substantially in cells treated with berberine, but integrin β1 mRNA levels were not changed (Fig. 3A). Therefore, the reduction in integrin β1 levels induced by berberine treatment likely reflects post-translational regulation. To test the effect of berberine on integrin β1 protein stability, we measured protein degradation in cells treated with the protein synthesis inhibitor cycloheximide. These assays showed that integrin β1 stability was decreased by berberine treatment (Fig. 3B). To examine the relevance of berberine-induced AMPK activation in the control of integrin β1, we treated cells with the AMPK activator, AICAR [17],

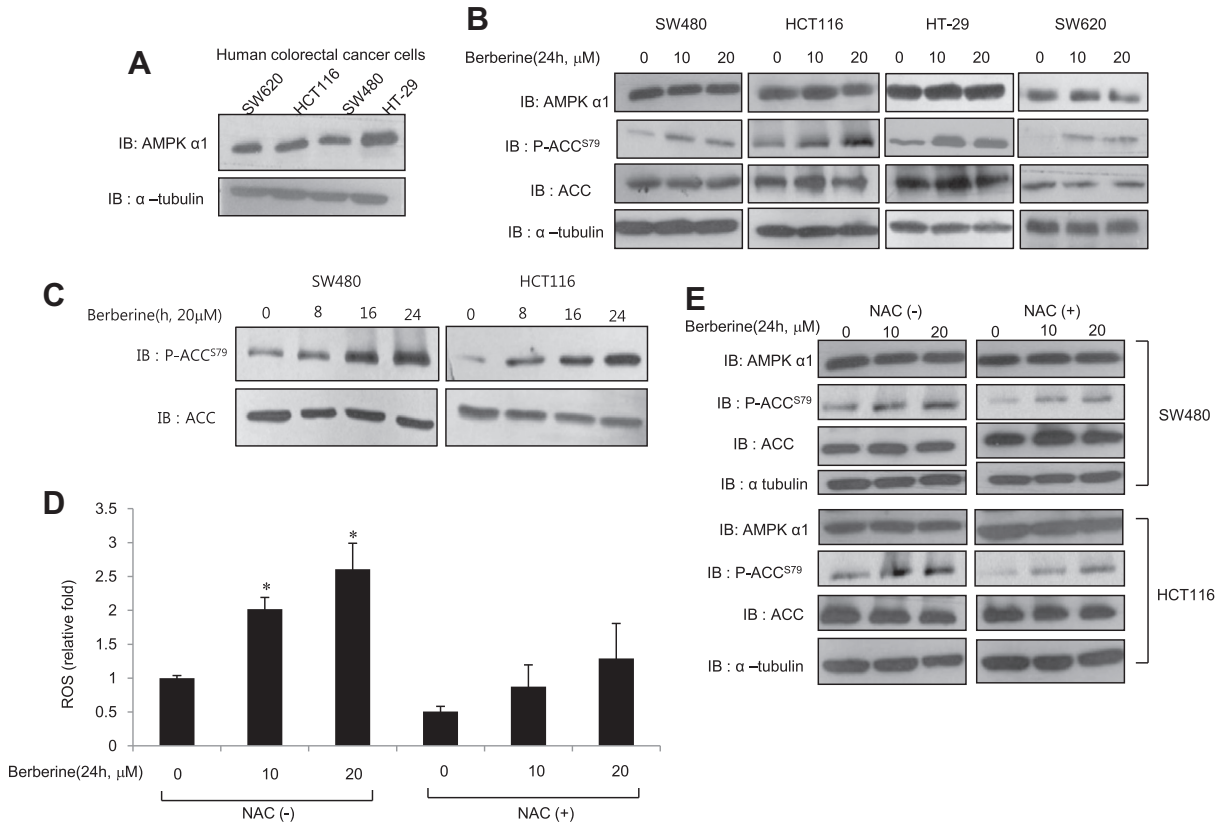

Fig. 2. Berberine activates AMPK in human colon cancer cells via ROS generation. (A) Expression of the AMPKα1 catalytic subunit in SW480, SW620, HCT116, and HT-29 cells. (B) SW480 and HCT116 cells were exposed to the indicated concentration of berberine for 24 h. Thereafter, cell extracts were prepared and analyzed by immunoblotting using anti-phospho-ACC^{S79}, anti-ACC, anti-AMPKα1, and anti-α-tubulin antibodies. (C) SW480 and HCT116 cells were incubated with 20 μM berberine for different periods of time. After treatment, cell lysates were analyzed by immunoblotting using anti-phospho-ACC^{S79} and anti-ACC antibodies. (D) After incubation with berberine in the presence or absence of NAC (5 mM) for 1 h, ROS levels in HCT116 cells were determined using the fluorescent dye, DCF-DA. The data are expressed as means ± SD for three determinations in triplicate. (E) After exposure of SW480 and HCT116 cells to the indicated concentration of berberine for 24 h in the presence or absence of 5 mM NAC, the levels of phospho-ACC, ACC, AMPKα1, and α-tubulin were determined by immunoblot analysis.

or overexpressed a Myc-tagged, constitutively active form of AMPK (AMPK-CA) [18–20] and determined integrin β1 levels. As shown in Fig. 3C, integrin β1 and the phosphorylated forms of Src and FAK were decreased by AICAR treatment or overexpression of constitutively active AMPK, assessed by an increase in the level of phospho-ACC. Next, we examined whether depletion of AMPK affected integrin β1 function. Notably, siRNA-mediated knockdown of the AMPKα1 catalytic subunit significantly increased the levels of integrin β1 protein and the phosphorylated forms of Src, FAK, and p130Cas (Fig. 3D). To rule out the possibility that integrin β1 acts upstream of AMPK, we knocked downed integrin β1 protein with si-integrin β1 and then examined the level of AMPKα1 and the phosphorylated form of ACC. As shown in Fig. 3E, there was no change in the phosphorylation status of ACC in response to depletion of integrin β1. Taken together with the siRNA results, these data demonstrate that berberine induces a decrease in the levels of integrin β1 and phosphorylated Src, FAK and p130Cas via an AMPK-dependent mechanism.

3.4. Knockdown of the AMPKα1-catalytic subunit attenuates the berberine-induced inhibition of colon cancer cell migration

Given that AMPK may negatively regulate integrin β1 signaling, we tested whether the berberine-induced reduction in cell migration was dependent on AMPK activity. To this end, we transfected SW480 and HCT116 cells with si-AMPKα1 prior to treatment with 20 μM berberine for 24 h. Compared with its effects on cell migration in parental cells ($p < 0.01$), berberine inhibition of migration was incomplete in AMPKα1-deficient cells ($p < 0.05$; Fig. 4A and B). Consistent with the diminished effects of berberine on migration, integrin β1 levels were reduced to a lesser extent by berberine in AMPKα1-knockdown cells (Fig. 4C). Taken together, these findings indicate that berberine-induced inhibition of colon cancer migration involves down regulation of integrin β1 signaling by AMPK.

4. Discussion

Once the CRC cells metastasize to the liver or lung, most anticancer therapies fail and overall survival is poor. Notably, more than 50% of deaths due to CRC are related to metastasis [2–4]. Therefore, the development of more effective strategies and identification of new agents and novel therapeutic targets for the prevention of metastasis is essential. A considerable research effort has been devoted to identifying naturally occurring therapeutic agents that are able to destroy metastatic cancer cells. The value of berberine as a pharmacological agent in the treatment of a variety of human diseases in Asian countries has been well documented [5], and accumulating evidence indicates that treatment with berberine contributes to the inhibition of cancer metastasis [21]. However, research on the anticancer effects of berberine currently remains preclinical and there are no available data on the effects of berberine on the metastasis of human CRC.

Here, as part of an effort to establish the potential clinical relevance of berberine, we investigated the anti-metastatic activity of

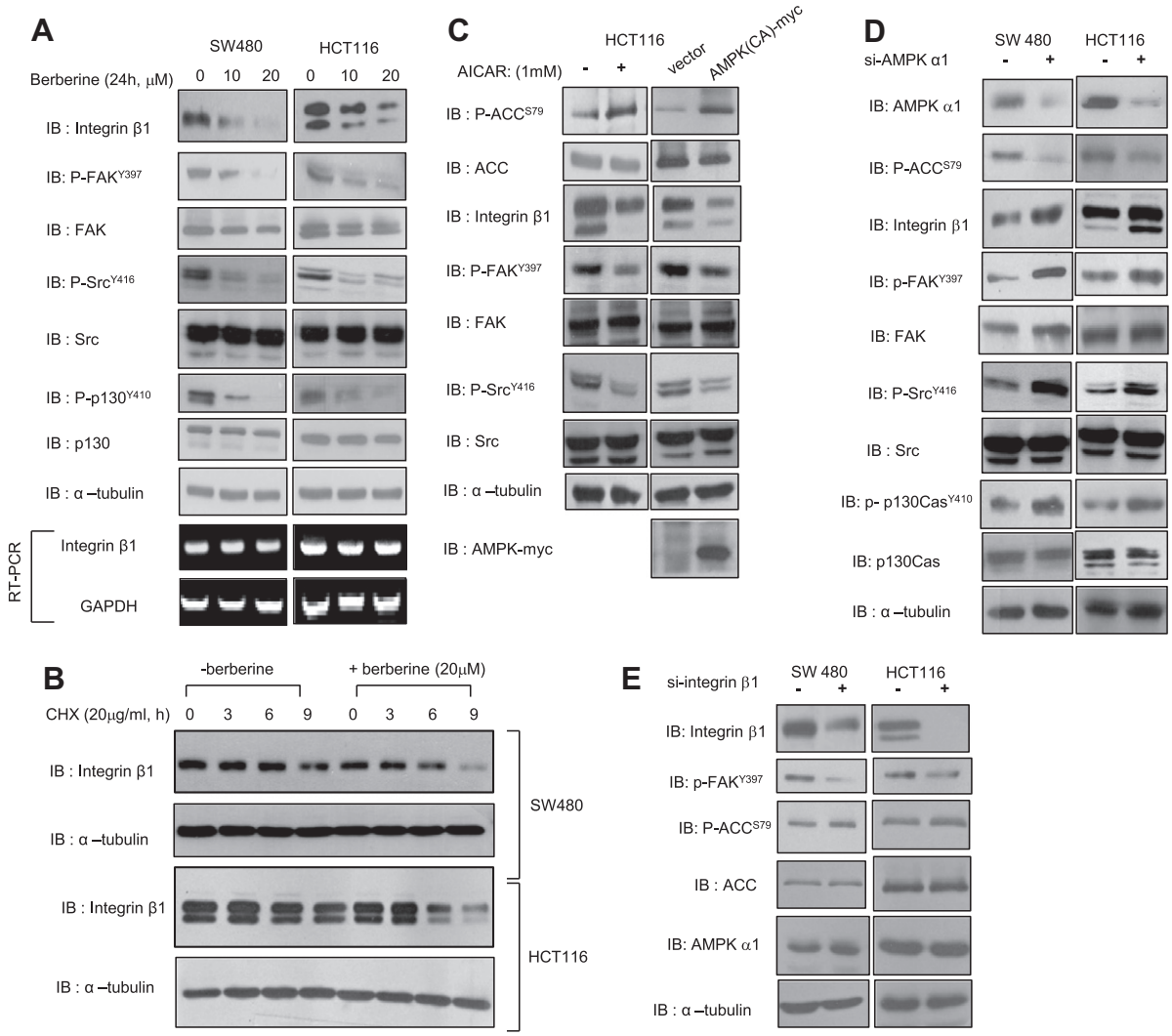

Fig. 3. Berberine decreases the levels of integrin β1 protein through AMPK activation. (A) After treatment of SW480 and HCT116 cells with berberine for 24 h, levels of integrin β1, phospho-Src^{Y416}, Src, phospho-FAK^{Y397}, FAK, phospho-p130Cas^{Y410}, p130Cas, and α-tubulin were determined by immunoblot analysis. Integrin β1 RNA level were analyzed by RT-PCR. (B) SW480 and HCT116 cells were treated with 100 μg/ml of cycloheximide with or without 20 μM berberine. Cell lysates were harvested at the indicated time points, and integrin β1 protein levels were determined by immunoblotting. (C) HCT116 cells were incubated with 1 mM AICAR for 24 h or were transfected with a Myc-tagged, constitutively active AMPK expression plasmid (AMPK-CA). Phospho-ACC^{S79}, ACC, phospho-FAK^{Y397}, FAK, and integrin β1 levels were determined by immunoblot analysis. (D) Expression of the AMPKα1 catalytic subunit was knocked down in SW480 and HCT116 cells by transfecting with si-AMPKα1. Twenty-four hours after transfection, AMPKα1, phospho-ACC^{S79}, phospho-Src^{Y416}, integrin β1, phospho-FAK^{Y397}, p130Cas^{Y410}, and α-tubulin protein levels were determined by immunoblotting. (E) After siRNA-mediated knockdown of integrin β1, AMPKα1, phospho-ACC^{S79}, ACC, integrin β1, phospho-FAK^{Y397}, and α-tubulin levels were determined by immunoblot analysis.

berberine on human CRC cells, focusing on AMPK activation and its effect on integrin signaling. We found that berberine (Fig. 1A) reduced the migration of SW480 and HCT116 cells (Fig. 1C and D). Berberine clearly activated AMPK in SW480, HCT116, SW620, and HT29 cells (Fig. 2B and C) via ROS production (Fig. 2D and E). Importantly, we demonstrated that berberine down regulated integrin β1 protein, leading to a reduction in the phosphorylated forms of Src, FAK and p130-downstream elements in the integrin β1 signaling pathway (Fig. 3A). This decrease in integrin β1 protein was not accompanied by a change in integrin β1 mRNA levels (Fig. 3A and B), suggesting a post-translational mechanism. To examine the link between AMPK activity and integrin β1 levels, we silenced AMPKα1 using siRNA and examined integrin β1 protein levels and Src, FAK, and p130Cas phosphorylation. Knockdown of the AMPKα1 catalytic subunit increased the levels of integrin β1 and the phosphorylated forms Src, FAK, and p130Cas (Fig. 3D). Conversely, activation of AMPK by treatment with AICAR, which functions as an AMP analogue, or transfection with the constitutively active AMPK form (AMPK-CA), decreased integrin β1 levels (Fig. 3C). Finally, knocking down AMPKα1 attenuated the berberine-induced reduction in SW480 and HCT116 cell migration (Fig. 4). Taken together, these results suggest that berberine acts through AMPK activation to reduce integrin β1 protein stability, thereby downregulating integrin β1 signaling through Src, FAK, and p130Cas and reducing cell migration.

The metastatic potential of tumors depends on integrin complexes, which function as cellular "feet". Integrins promote migration of cells on the surrounding ECM, and the signals initiated by integrin binding to ECM proteins are necessary for the maintenance of cell survival. Focal adhesion sites contain integrins and complexes of signaling elements such as Src, FAK, p130Cas, MAP kinases, small GTPases, and phosphoinositide 3-kinase [16]. Previous reports have shown that integrin signaling is essential for cell adhesion, migration, and chemo- or radioresistance [13,22]. Our current findings support this, demonstrating that downregulation of integrin β1 protein levels and downstream signaling through

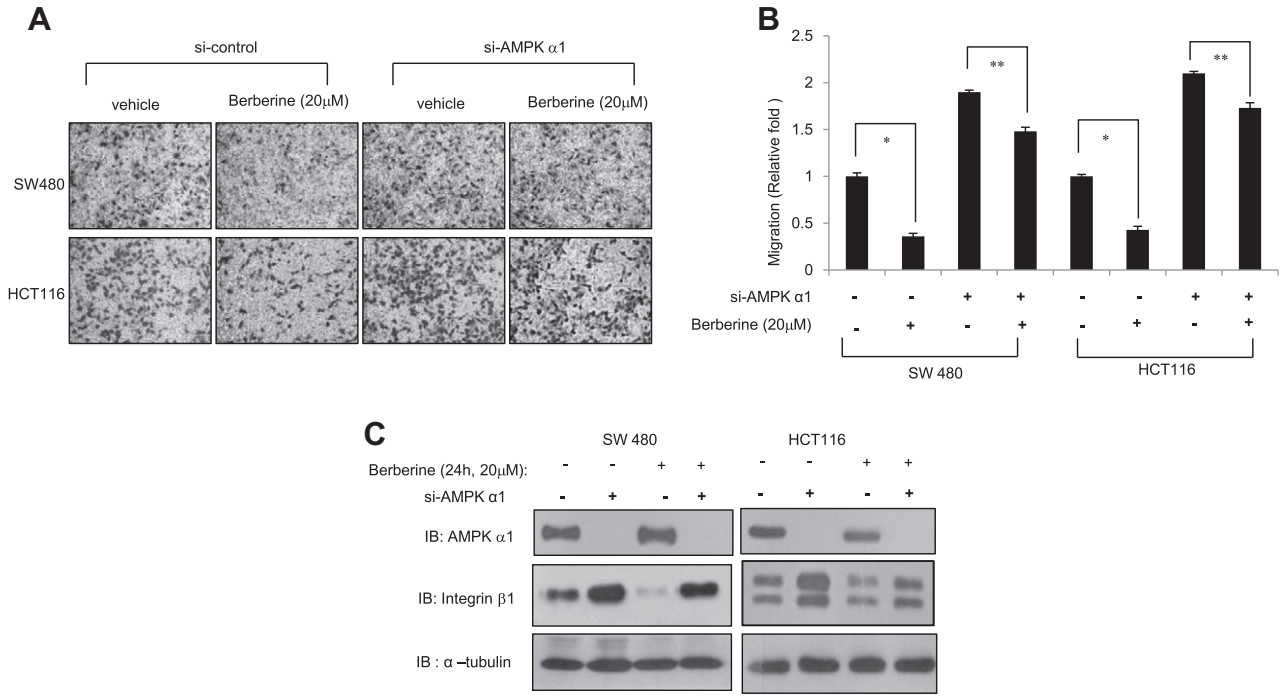

Fig. 4. Knockdown of the AMPKα1 catalytic subunit abrogates the berberine-induced inhibition of cell migration. SW480 and HCT116 cells were transfected with si-control or si-AMPKα1, and then treated with 20 μM berberine for 24 h. A fixed number of cells were added to the upper Transwell chamber. After 24 h, images of migratory cells that had moved to the lower surface of the membrane were captured using a phase-contrast microscope (A). Migrating cells were counted and the results were expressed as the mean number of migratory cells ± SD per microscopic field for three determinations in triplicate (B). Cells exposed to the same conditions were harvested and AMPKα1, integrin β1, and α-tubulin protein levels were determined by immunoblot analysis (C).

AMPK activation is a novel mechanism for the suppressive effect of berberine on the migration of colon cancer cells.

Our findings demonstrate that berberine strongly activated AMPK and this activation of energy sensor clearly downregulated the levels of integrin β1 and phosphorylated forms of the downstream targets Src, FAK and p130Cas, resulting in a decrease in the migration of CRC cells. Although metabolic pathways and cellular signaling have often been considered as separate entities, reciprocal regulation of energy metabolism and signal transduction pathways is a well-accepted paradigm [23]. One significant example of this is diabetes patients using metformin, who have lower risk of colon cancer development compared with non-metformin users [24]. This previous study strongly suggests that metformin, a potent AMPK activator, inhibits colon carcinogenesis. In addition, aspirin, a synthetic derivative of a plant product, was shown to reduce the development of CRC through direct activation of AMPK [25,26]. Clearly, identification of AMPK activators is a promising strategy for the development of novel therapeutic drugs for the treatment of both metabolic disorders and colon cancer.

As reported by others, post-translational modifications such as phosphorylation, acetylation, methylation, and glycosylation are highly linked to the metabolic status of cells. In particular, glycosylation has a crucial role at the interface of metabolism and signaling in cancer [23]. As shown previously by others, the stability of membrane glycoprotein integrin β1 is critically regulated by N-glycosylation [27]. Because AMPK acts as a nutrient sensor, it is possible that berberine-induced AMPK activation affects integrin β1 surface expression, trafficking and/or protein stability via energy-sensitive modulation of post-translational modifications such as N-glycosylation. We are currently investigating the effect of berberine on the glycosylation status of integrin β1 and other metastasis-related glycoproteins.

In conclusion, our investigation identified a critical role of AMPK in the attenuation of CRC cell metastatic potential by berberine, showing that berberine acts through activation of AMPK to inhibit integrin signaling and cell migration. Our findings provide insight into the mechanism underlying berberine actions against colon cancer cell migration, a previously neglected aspect of botanical product-based therapy, and suggest that berberine could be developed as a pharmacological agent for use in combination with other chemotherapy or anti-metastatic drugs in the prevention of metastatic human colon cancer.

Acknowledgments

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