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Metformin enhances anti-cancer effects of cisplatin in meningioma through AMPK-mTOR signaling pathways

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Cisplatin is currently used to treat inoperable recurrent meningiomas, but its side effects and drug resistance limit its use. Metformin has recently been identified as a chemosensitizing agent. However, the combined treatment of cisplatin and metformin in high-grade meningiomas has not been reported. Herein, our findings demonstrate metformin significantly enhanced cisplatin-induced inhibition of proliferation in meningioma cells, which was associated with the induction of G0/G1 cell cycle arrest. Additionally, metformin activated adenosine monophosphate activated protein kinase (AMPK) and repressed the mammalian target of rapamycin (mTOR) signaling pathways via an AMPK-dependent mechanism. Furthermore, our xenograft murine model confirmed that metformin enhanced cisplatin's anti-cancer effect by upregulation of AMPK and downregulation of mTOR signaling pathways. In addition, in 63 patients with atypical meningiomas, the activation of AMPK was significantly associated with tumor recurrence and short disease-free survival (DFS). These results demonstrate metformin enhanced the anti-cancer effect of cisplatin in meningioma *in vitro* and *in vivo*, an effect mediated through the activation of AMPK and repression of mTOR signaling pathways. Our study suggests the combined treatment of metformin and cisplatin is an effective and safe treatment for high-grade meningiomas.

INTRODUCTION

Meningiomas, deriving from the arachnoid cells, are the most common benign primary tumors in the CNS, with an annual incidence of approximately 5 per 100,000 individuals.[1] The 2016 World Health Organization (WHO) classification for CNS tumors stratifies meningiomas into three grades.[2] Grade 1, accounting for more than 80% of meningiomas, are essentially benign and curable by surgical resection alone. In contrast, high-grade meningiomas (II [atypical] and III [anaplastic/malignant]) are less common but are characterized by their aggressive progression and often require combinatorial treatments, including radiation therapy and systemic chemotherapy.[3,4] Despite added therapeutic measures, high-grade meningiomas commonly recur after surgery and radiotherapy, which accounts for low (26%) 6-month progression-free survival estimates.[5] Presently, few pharmacological agents have demonstrated therapeutic efficacy against high-grade meningiomas in clinical studies, underscoring the need to identify new chemotherapeutic agents and treatment strategies.[6,7]

Cisplatin, one of the most potent and widely used anticancer agents, is a platinum-containing drug used as a first-line chemotherapy against many epithelial malignancies.[8] Cisplatin exerts anticancer activity via multiple mechanisms, but its most recognized mechanism involves induction of DNA damage by interacting with purine bases on DNA followed by activation of several signal transduction pathways that result in tumor cell apoptosis.[9] Indeed, preliminary *in vitro* studies demonstrated meningioma cell line sensitivity to cisplatin, and it is currently used to treat patients with inoperable recurrent meningiomas.[10,11] Despite cisplatin's positive therapeutic effects, its systemic side effects and drug resistance limit its use against meningiomas.[12] Considering other tumor types have demonstrated improved therapeutic outcomes with combinatorial chemotherapies involving cisplatin, investigation into combinatorial cisplatin therapies against meningioma is warranted.

Metformin is a widely used drug for reducing hyperglycemia in patients with type 2 diabetes mellitus (T2DM). Epidemiologic studies have suggested that metformin reduces the risk of cancers in patients with DM, including lung, prostate, colon, breast, and

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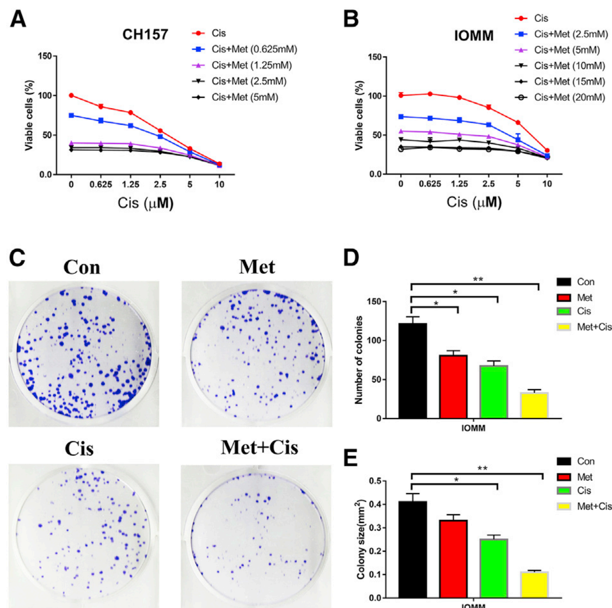


Figure 1. Metformin enhanced cisplatin-induced anti-proliferation and colony formation in meningioma cells

Cell viability was assessed by CCK8 assay. (A) CH157 cells were treated with metformin (0, 0.625, 1.25, 2.5, or 5 mM) and cisplatin (0, 0.625, 1.25, 2.5, 5, or 10 μM) for 48 h. (B) IOMM cells were treated with cisplatin (0, 0.625, 1.25, 2.5, 5, or 10 μM) and metformin (0, 2.5, 5, 10, 15, or 20 mM) for 48 h. (C) Cells were treated with metformin (1 mM) or cisplatin (1 μM) or the combination for 24 h and subsequently cultured in drug-free DMEM for 10 days. (D) The colony numbers and (E) colony sizes were both significantly suppressed in the Met, Cis, and Met+Cis groups, when compared with the control group. *$p < 0.05$; **$p < 0.01$.

pancreatic cancers.[13–16] Several preclinical studies have demonstrated that metformin inhibits the *in vitro* and *in vivo* cell growth of various cancer cell lines.[17,18] Furthermore, metformin has also been identified as an effective chemosensitizer when combined with other anticancer agents, such as cisplatin, in the treatment of various cancer types.[19–21] However, despite the aforementioned anticancer properties, the efficacy of the combined treatment of cisplatin and metformin against high-grade meningioma has never been reported.

Metformin's anticancer effect has been previously attributed to the activation of adenosine monophosphate activated protein kinase (AMPK) and inhibition of the mammalian target of rapamycin (mTOR) signaling pathways.[18,22–24] AMPK is an important energy-sensing enzyme involved in the maintenance of cellular energy homeostasis and plays a central role in reprogramming cellular metabolism pathways that favor tumor progression.[25,26] AMPK activation is normally mediated by an increase in the cellular AMP/ATP ratio but can also be activated by metformin. Various molecules and signaling pathways have been identified to be regulated by activated AMPK.[27] Notably, activated AMPK can directly phosphorylate and activate tuberous sclerosis complex 2 (TSC2), leading to repression of mTOR signaling pathways.[24,27]

The mTOR (including mTOR complex 1 and mTOR complex 2) pathway is essential for tumor cell growth, proliferation, and survival.[28] The mTORC1 consists of mTOR, regulatory-associated protein of mTOR (Raptor), and mammalian lethal with SEC13 protein 8 (MLST8), PRAS40, and DEPTOR.[28] The mTORC1 mediates phosphorylation of p70-S6 kinase 1 (S6K) and the eukaryotic initiation factor 4E binding protein 1 (4EBP1), which stimulate mRNA translation and ultimately cell growth and proliferation.[29] The mTORC2, which consists of mTOR, rapamycin-insensitive companion of mTOR (RICTOR), and mammalian stress-activated protein kinase interacting protein 1 (mSIN1), also regulates cellular proliferation and metabolism. mTORC2 phosphorylates the serine/threonine protein kinase (AKT) at serine residue S473 as well as serine residue S450.[30] However, the underlying role of AMPK-mTOR signaling pathways in meningioma has not been clearly elucidated.

In the present study, we first demonstrated that metformin enhanced the anti-cancer effect of cisplatin against meningioma cells *in vitro* and *in vivo*. Subsequently, we evaluated the role of the metformin-activated AMPK-mTOR pathway in chemosensitizing the effect of cisplatin against meningioma. Last, we investigated the correlation of AMPK activation to disease-free survival (DFS) in patients with atypical meningiomas.

RESULTS

Metformin enhanced cisplatin-induced anti-proliferation *in vitro*

To evaluate whether metformin could enhance the cisplatin-induced anti-proliferative effect in meningioma cells *in vitro*, we first evaluated metformin's anti-meningioma effect alone. Metformin treatment significantly inhibited meningioma cell growth in a concentration-dependent manner (Figures S1A and S1B). Specifically, when incubated with metformin for 48 h, the 50% growth-inhibitory concentration (IC_{50}) values for CH157 and IOMM were 0.646 ± 0.544 mM and 4.94 ± 0.363 mM, respectively. Further, when co-treated with cisplatin, metformin significantly enhanced cisplatin-induced inhibition of proliferation in both CH157 and IOMM cells (Figures 1A and 1B). These data suggest that metformin chemosensitizes meningioma cells to cisplatin.

We subsequently confirmed metformin's chemosensitizing effect using a colony formation assay. IOMM cells were chosen, as CH157 cells could not form cell colonies. Treatment with metformin or cisplatin alone significantly decreased the colony numbers and colony sizes compared to the control group (Figures 1C–1E) ($p < 0.05$). Notably, the combination treatment further enhanced the suppression of colony formation in meningioma cells (Figures 1C–1E) ($p < 0.05$).

Metformin induced G0/G1 phase cell cycle arrest but could not enhance cisplatin-induced apoptosis in meningioma cells

To investigate how metformin influences meningioma cell growth, we analyzed tumor cell apoptosis and cell cycle. The pro-apoptotic effects of metformin were measured by flow cytometric analysis of annexin

V- fluorescein isothiocyanate/propidium iodide (FITC/PI) staining. As shown in Figures 2A and 2B, treatment with metformin alone did not increase meningioma cell apoptosis when compared to the control group ($p > 0.05$); moreover, when used in combination with cisplatin, metformin did not enhance cisplatin-induced apoptosis in CH157 and IOMM cells ($p > 0.05$).

Further, we analyzed cell cycle using flow cytometric analysis after the treatment of metformin and/or cisplatin (Figures 2C and 2D). Results showed that cisplatin had no significant effect on modulating the cell cycle in meningioma cells. In contrast, metformin alone or in combination with cisplatin significantly induced cell cycle arrest in the G0/G1 phase ($p < 0.05$).

Last, we assessed the levels of the main cell cycle regulatory proteins in meningioma cells following treatment with metformin and/or cisplatin (Figure 2E). Western blot analysis showed that metformin significantly reduced cyclin D1 expression and increased P27 expression but had no effect on the expression levels of cleaved caspase-3 and PARP. In contrast, cisplatin had no effect on the expression levels of cyclin D1 or P27 but did significantly increase the expression levels of cleaved caspase-3 and PARP. Taken together, these results indicated that metformin induced G0/G1 phase cell cycle arrest but did not enhance cisplatin-induced apoptosis in meningioma cells.

Metformin enhanced cisplatin-induced activation of AMPK and repression of both the mTORC1 and mTORC2 signaling pathways in meningioma cells

To evaluate whether metformin mediated the chemosensitizing effect through AMPK-mTOR signaling pathways, western blot was performed in meningioma cells treated with metformin and/or cisplatin (Figure 3), and the results demonstrated that phosphorylated AMPK (P-AMPK) expression was upregulated following treatment with metformin or cisplatin alone for 6 h and 24 h in meningioma cells. Notably, co-treatment with metformin and cisplatin significantly enhanced the activation of AMPK compared to treatment with metformin or cisplatin alone. Further investigation of mTORC1 and mTORC2 signaling pathways demonstrated that metformin or cisplatin alone downregulated P-mTOR at Ser2448 (a marker of mTORC1 activation) and Ser2481 (a marker of mTORC2 activation). Compared to single treatment, cells treated with metformin and cisplatin demonstrated a significantly enhanced suppression of mTORC1 and mTORC2 pathways, as evidenced by the repression of their downstream proteins P-S6K, P-4EBP1, and P-AKT at Ser473. Collectively, these results demonstrate that metformin chemosensitized the effect of cisplatin on meningioma cells, an effect that is mediated through the AMPK-mTORC1/2 signaling pathways.

Metformin inhibited meningioma cell growth in an AMPK-dependent manner

To confirm that AMPK activation is responsible for inhibiting the growth of meningioma cells, we used an AMPK inhibitor, compound C, and an AMPK-specific small interfering RNA (siRNA) to block AMPK expression in meningioma cells. We first assessed the cytotoxic effect of compound C in CH157 and IOMM cell lines (Figure S2). After pre-treatment with compound C, metformin-induced inhibition of meningioma cell growth was attenuated (Figures 4A and 4B). Similarly, knockdown of AMPK with siRNA abrogated metformin-induced inhibition of meningioma cell growth (Figures 4C and 4D) and further attenuated the suppression of mTORC1/2 signaling pathways (Figure 4E). Together, these experimental results confirmed that metformin inhibited meningioma cell growth by repressing mTORC1/2 signaling pathways in an AMPK-dependent manner.

Metformin enhanced anti-cancer effect of cisplatin in a mice xenograft meningioma model

To determine whether metformin enhanced the anti-cancer effect of cisplatin *in vivo*, IOMM meningioma cells were injected subcutaneously in nude/c mice to establish a meningioma xenograft tumor model. Once tumors reached 75 mm^3 (Figure 5A), mice were randomized into the following 4 treatment groups: control, Met, Cis, and Met+Cis groups. Mice were treated with saline (100 μL) or metformin (200 mg/kg), daily, or cisplatin (1.5 mg/kg) every other day. After treatment for 24 days, mice in the metformin or cisplatin treatment groups demonstrated significantly reduced tumor volumes when compared to mice in the control group. (Figures 5B–5D, $p < 0.05$). Of note, mice in the combination treatment group demonstrated significantly reduced tumor volumes and reduction in excised tumor weights when compared to mice in the other treatment groups (Figures 5B–5D). Collectively, these results suggest treatment with combination metformin and cisplatin synergize to inhibit the *in vivo* growth of meningioma cells.

Metformin inhibited meningioma cell proliferation through AMPK-mTOR signaling pathways *in vivo*

We performed H&E staining and immunohistochemical staining (Figure S3) to investigate the effect of metformin in inhibiting meningioma tumor growth in mice. H&E staining revealed more evidence of tumor necrosis and hyperplastic blood vessels in tumors extracted from the control group versus those extracted from the other three treatment groups. Immunohistochemical staining revealed that there were fewer Ki-67-positive tumor cells in mice treated with metformin alone and in mice treated with Met+Cis when compared with control and Cis treatment groups. Assessment of tumor cell apoptosis revealed that there were more apoptotic (TUNEL-positive) cells in the Cis and Met+Cis treatment groups when compared to control or Met treatment groups. Additionally, Met and Met+Cis treatment groups demonstrated significantly upregulated P-AMPK expression and significantly downregulated expressions of P-S6K, P-4EBP1, and P-AKT. Taken together, these results revealed that metformin enhanced the anti-cancer effect of cisplatin by inhibiting meningioma cell proliferation, an effect modulated by the AMPK-mTORC1/2 signaling pathways.

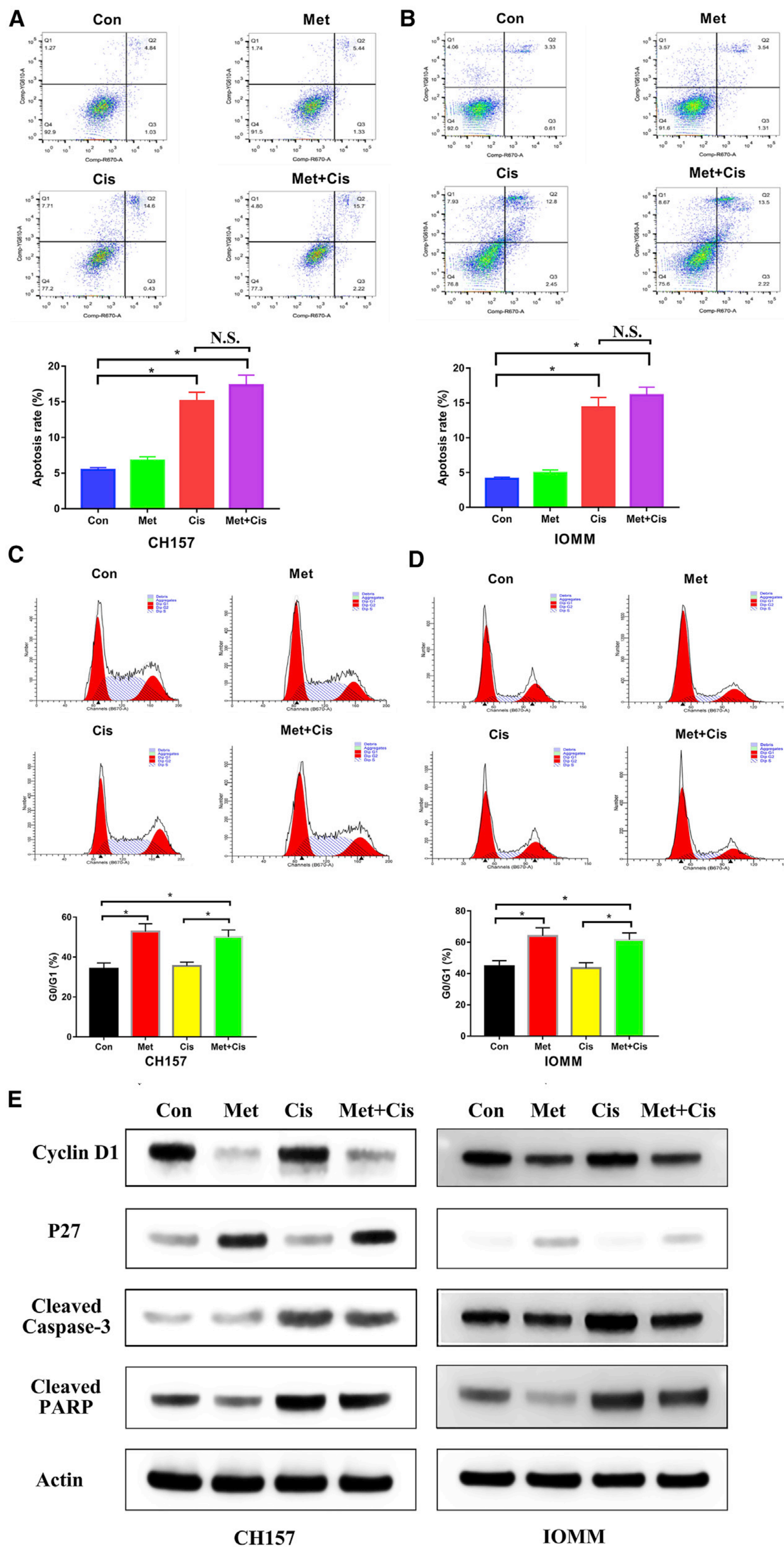
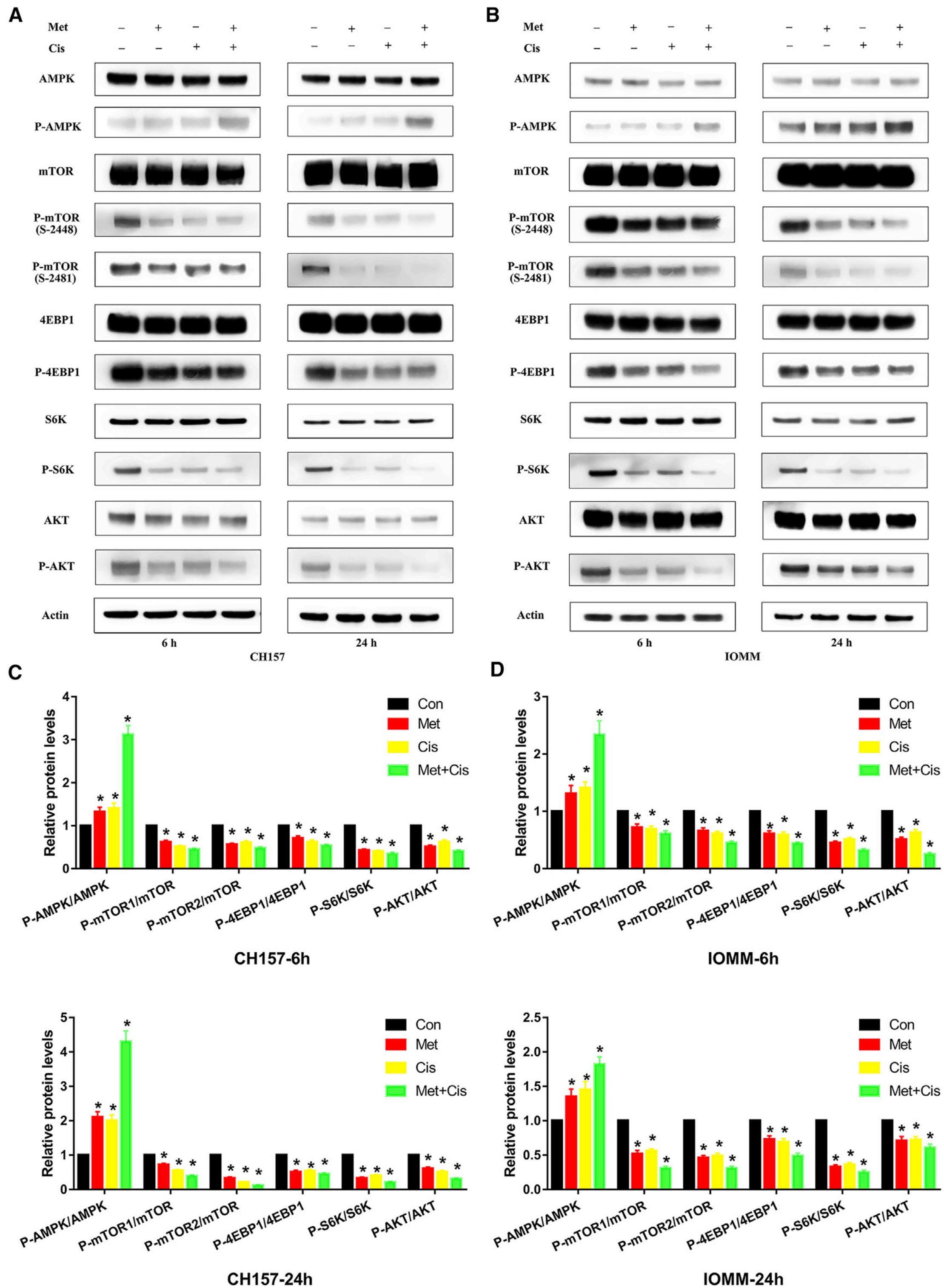


Figure 2. Metformin induced G0/G1 phase cell cycle arrest but could not enhance cisplatin-induced apoptosis in meningioma cells

Meningioma cells were treated with metformin (5 mM) or cisplatin (4 μM) alone or the combination for 6 h (for cell cycle experiments) or 24 h (for apoptosis experiment). (A and B) Representative results showed the distribution of apoptosis in CH157 (A) and IOMM (B) cells by flow cytometric analysis of annexin V-FITC/PE staining after treatment. Histograms show the percentage of apoptotic cells following treatments. (C and D) Representative results demonstrate the distribution and percentage of CH157 (C) and IOMM (D) cells in G0/G1, S, and G2 phases. Histograms show the percentage of G0/G1, S, and G2 phases following treatments. (E) Western blots show the expression of cell cycle regulatory proteins cyclin D1 and P27, as well as apoptotic proteins cleaved caspase-3 and cleaved PARP following treatments. *$p < 0.05$; N.S., not significant.



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Metformin did not increase cisplatin-induced systemic side effects *in vivo*

To assess the physiologic impact of aforementioned treatments, we monitored the fluctuations in animal body weight throughout the course of the study. Significant weight loss was not observed in mice in the Met, Cis, or Met+Cis treatment groups when compared to mice in the control group (Figure S4A; $p > 0.05$). Furthermore, blood plasma of mice from each treatment group was analyzed for glucose levels and markers of kidney function (creatine and serum urea nitrogen). Similarly, no significant difference was observed in glucose levels (Figure S4B; $p > 0.05$) or kidney function (Figure S5; $p > 0.05$) among the four treatment groups.

Metformin attenuated cisplatin-induced neurotoxicity *in vitro*

Considering that neurotoxicity is one of the most severe side effects of cisplatin treatment, two types of neuronal cells (PC12 cells and primary cultured rat cortical neuron [PCN] cells) were used to assess the effect of metformin in modulating cisplatin-induced neurotoxicity. Cisplatin's neurotoxic effect was first confirmed by incubating PC12 cells with different concentrations of cisplatin for 48 h. Indeed, cisplatin-incubated PC12 cells demonstrated a significant dose-dependent decrease in cell proliferation (Figure S6A; $p < 0.05$). PC12 cells were also incubated with different concentrations of metformin for 48 h to assess metformin's neurotoxic effect. In contrast to cisplatin-incubated cells, PC12 cells incubated with metformin did not reveal a significant difference in cell proliferation compared to non-treated PC12 cells (Figure S6B; $p > 0.05$). Last, PC12 cells were incubated with cisplatin and metformin (at different concentrations) to assess the neurotoxic effect of the combined treatments. PC12 cells incubated with cisplatin and metformin demonstrated no significant increase in cell proliferation when compared with PC12 cells incubated with cisplatin or metformin alone (Figures S6A and S6B; $p > 0.05$). These results revealed that metformin did not increase the neurotoxic effect of cisplatin in PC12 cells.

Neurotoxic effects of treatments were similarly assessed in PCN cells. PCN cells incubated with 0.1 μM cisplatin for 24 h demonstrated a significant inhibition of axonal and dendritic growth when compared with non-treated PCN cells (Figure S7). In contrast, PCN cells treated with 2 μM metformin demonstrated no significant difference in neurite outgrowth when compared to non-treated PCN cells (Figure S7). Interestingly, PCN cells pretreated with 2 μM metformin (2 h) and subsequently incubated with cisplatin (24 h) demonstrated a reduction in the cisplatin-induced inhibition of axonal and dendritic growth (Figure S7). This result suggests that metformin attenuated the cisplatin-induced neurotoxicity in PCN cells.

Low P-AMPK expression was associated with tumor recurrence and short DFS in atypical meningiomas

The cytoplasmic expression of P-AMPK was investigated in meningiomas of different grades (Figures 6A–6E) and with variable intensity of staining (IS) and the area of staining positivity (ASP) (Table S1). Of the 63 atypical meningiomas assessed, 46 (73.0%) demonstrated positive P-AMPK immunostaining. IS was homogeneous throughout each section. Intensity distribution (ID) scores ranged from 1–12, with a median value of 6. The median ID score (6) was defined as the cut-off point to distinguish between low and high immune expression of P-AMPK. A total of 34 (54.0%) meningiomas demonstrated a low P-AMPK immune expression. The remaining 29 (46.0%) meningiomas demonstrated a high P-AMPK immune expression. Low P-AMPK expression was significantly associated with development of tumor recurrences and short DFS (Table 1; $p < 0.05$). No significant correlations were found between P-AMPK immune expression and the other clinicopathological variables, including age, sex, tumor location, or associated Simpson grade (Table 1; $p > 0.05$). A dot-plot distribution was created to illustrate the relationship between P-AMPK expression (ID scores) and patients' DFS (Figure 7A). Further, Kaplan-Meier curves demonstrated that low P-AMPK expression was significantly associated with shorter DFS in patients with atypical meningiomas (Figure 7B).

DISCUSSION

Metformin has been recently identified as a chemosensitizing agent when combined with chemotherapeutic drugs (such as cisplatin, gemcitabine, and rapamycin) in the treatment of various cancers.[19–21,31,32] Metformin's chemosensitizing effect was believed to be mediated via the activation of AMPK pathways, which are associated with cellular metabolism and proliferation.[25,26]

Present data on AMPK's effect on either promoting or preventing tumor progression is mixed. Several recent studies have suggested that loss of AMPK activity could activate various oncogenic pathways and promote tumor progression. These include studies by Chen et al.[33] and Zheng et al.,[34] which demonstrated that loss of AMPK activation or low expression of P-AMPK correlated with aggressive clinicopathologic features and poor prognosis in pancreatic cancer and hepatocellular carcinoma. However, competing data exist demonstrating that increased AMPK activity may play an antitumorigenic role. In a cohort of patients with non-small cell lung cancer, for example, William et al.[35] reported that high P-AMPK expression levels were associated with increased patient survival. Similarly, our investigation revealed that increased expression of P-AMPK was associated with longer survival in patients with atypical meningiomas.

Figure 3. Metformin enhanced cisplatin-induced activation of AMPK and repression of both the mTORC1 and mTORC2 signaling pathways in meningioma cells

(A and B) CH157 (A) and IOMM (B) cells were treated with metformin (5 mM) or cisplatin (4 μM) alone or in combination for 6 h and 24 h. Western blots show the expression of AMPK-mTORC1/2-mediated signaling pathway proteins. (C and D) The relative expressions of target proteins, normalized with β-actin as loading control, was calculated as ratio to the control group. *$p < 0.05$ versus control.

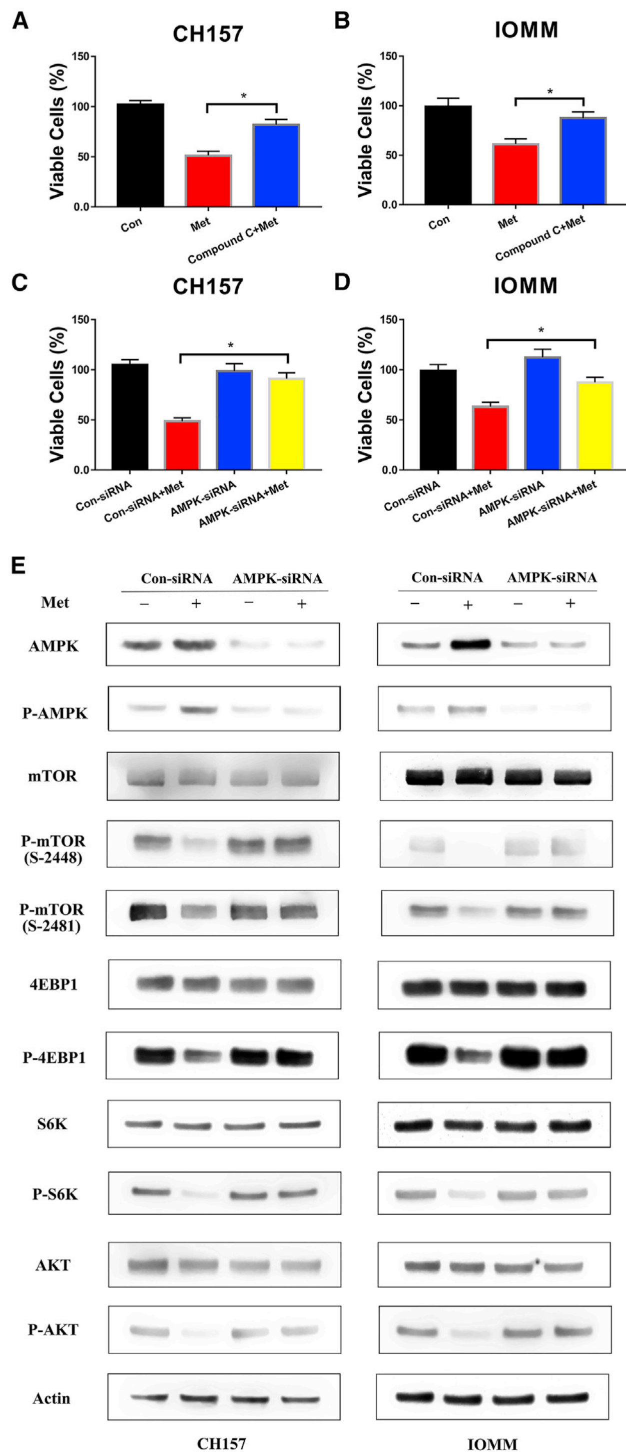

Figure 4. Metformin inhibited meningioma cell growth in an AMPK-dependent manner

Cell viability was assessed by CCK8 assay. (A and B) Compound C (1 μM), AMPK inhibitor, rescued metformin (5 mM)-induced cell growth inhibition in CH157 (A) and IOMM (B) cells. (C and D) In addition, siRNA-mediated knockdown of AMPK in CH157 (C) and IOMM (D) cells attenuated metformin (5 mM)-induced cell growth inhibition. (E) Western blots show that siRNA-mediated knockdown of AMPK in meningioma cells failed to repress phosphorylation of the mTORC1/2 signaling pathways.

Considering previous clinicopathologic findings and previous reports demonstrating that metformin could downregulate AMPK pathways, we assessed if metformin could chemosensitize the effect of cisplatin, a well-established drug used against high-grade meningiomas. Our *in vitro* work demonstrates that metformin activates AMPK signaling, which decreases mTORC1/2 pathways that are known to prevent tumorigenesis.[35] Moreover, when assessed in *in vivo* models, we find that the combination of cisplatin and metformin significantly reduced meningioma tumor growth. In light of metformin's chemosensitizing effect on cisplatin, we investigated if combination treatment produced adverse side effects on treated animals. Our results suggest that combination treatment has no impact on body weight or kidney function. Importantly, our study also found that metformin may have a neuroprotective effect against cisplatin-induced neurotoxicity. These results suggest that the pharmacological suppression of the AMPK pathway with metformin holds promising therapeutic potential in the treatment of high-grade meningiomas.

Accumulating evidence suggests metformin alone, or in combination with other anticancer drugs, exerts an anti-cancer effect in many cancer types.[19,31,32,36] Numerous cellular and molecular mechanisms have been attributed to metformin's anti-cancer effect. Specifically, these mechanisms can be classified as AMPK- and mTORC1-independent or AMPK- and mTORC1-dependent pathways.[37] The AMPK- and mTORC1-independent mechanism has been shown to decrease glucose and insulin blood levels and decrease the production of biosynthetic precursors generated by the tricarboxylic acid (TCA) cycle.[37] In contrast, the AMPK-dependent mechanism of metformin is mediated through the direct inhibitory phosphorylation of mTORC1 subunits, inhibition of lipid synthesis and nuclear factor-κB (NF-κB) pathway, and increased protein acetylation.[37] The mTORC1-dependent mechanisms are due to metformin-mediated and AMPK-independent inhibition of mTORC1.[37] In addition to effects at the cellular and molecular level, metformin has systemic effects that include inhibition of tumor development by reducing insulin/insulin-like growth factor (IGF)-1 signaling, reducing pro-inflammatory cytokine levels, reducing expression of cell adhesion molecules, suppressing the Warburg effect, and releasing of lactate by tumors.[38,39] In the present study, we first demonstrated that metformin promoted the activation of AMPK and repression of mTORC1/2, as well as their downstream proteins, by western blot *in vitro*; further, the upregulation of P-AMPK expression and downregulation of mTORC1/2 downstream proteins were confirmed by immunohistochemical staining *in vivo*. Both pharmacologic and siRNA knockdown of AMPK were found to abrogate metformin-induced cell growth inhibition and further attenuated the repression of mTORC1/2 signaling pathway, confirming that metformin inhibited meningioma cell growth via an AMPK-dependent mechanism. In addition, the results of western blot and

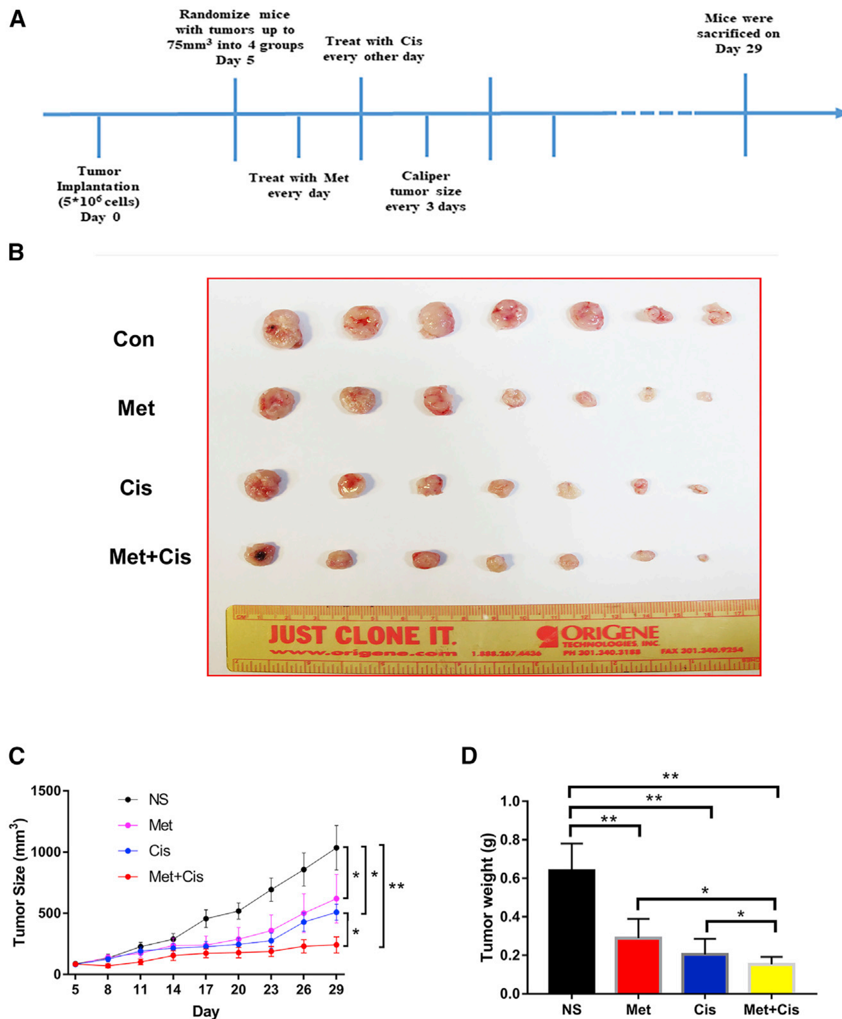

Figure 5. Metformin enhanced anti-cancer effect of cisplatin in a mice xenograft meningioma model

(A) Nude/c mice were treated with saline (100 μL) or metformin (200 mg/kg) per day or cisplatin (1.5 mg/kg) every other day or combination treatment after subcutaneous implantation of IOMM cells. (B) The sizes of excised tumors in each group are presented and compared. (C) The tumor volumes in each group were monitored every 3 days. (D) The excised tumor weights of each group are compared.

immunohistochemical staining also revealed that AMPK could be activated by cisplatin alone, which is consistent with previous studies.[40] Collectively, our *in vitro* and *in vivo* experiments demonstrate that metformin enhanced the cisplatin-induced activation of AMPK and strengthened its anti-cancer effect.

Consistent with several previous studies,[18] we found that metformin repressed both mTORC1 and mTORC2 signaling pathways, which exerts a more effective anti-cancer activity when compared to mTORC1 inhibition alone.[41] Specifically, inhibition of the mTORC1 pathway leads to decreased expressions of mTOR (Ser-2488), P-S6K, and P-4EBP1 proteins, resulting in the inhibition of mRNA translation and cell proliferation. Inhibition of the mTORC2 pathway decreases expression of the P-AKT (Ser473) protein, which further inhibits cell proliferation. Further, flow cytometric analysis revealed that this anti-proliferative effect was associated with G0/G1 cell cycle arrest, not apoptosis, and this finding was also consistent with several previous studies in which metformin alone could not induce apoptosis.[18,42,43] Whether or not metformin is capable of inducing apoptosis in tumor cells is an area of ongoing investigation, with some studies demonstrating that metformin can indeed promote apoptosis or autophagy in several tumor cell lines.[36,44,45] Based on our investigation, we believe metformin's antitumor effect may be multifactorial and may differ among different cell lines.

Our study confirmed that metformin enhanced the anti-cancer effect of cisplatin in a meningioma xenograft mouse model and demonstrated that a dose of 200 mg/kg per day was safe and effective for treating mice with meningiomas. The murine dose of metformin can be translated to the human equivalent dose by using the well-established Reagan-Shaw method.[46] According to the formula, the human equivalent dose (mg/kg) = animal dose (mg/kg) × animal Km/human Km (Km values are based on body surface area; Km for a 60 kg human adult is 37 and for a 20 g mouse is 3).[46] Thus, the human equivalent of the murine dose of 200 mg/kg in a mouse is 973 mg in an average-sized human (60 kg), while the standard human treatment dose of metformin is 1,000 to 2,500 mg (per day). Thus, the dose of metformin used in this murine study is one that falls within safe therapeutic range when translated for humans.

Aside from augmenting cisplatin's anticancer effect on meningioma cells, we found that combination treatment with metformin did not result in adverse side effects on body weight, kidney function, or glucose levels. It was reported that metformin reduced tubular cell death in cisplatin-induced acute kidney injury through AMPKα-regulated autophagy induction[47] or the AKT/mTORC2 pathway.[48] Mao-Ying et al.[49] recently reported that metformin protected against chemotherapy-induced peripheral neuropathy in a mouse model, and Cheki et al.[50] described that metformin attenuates cisplatin-induced genotoxicity and apoptosis in rat bone marrow cells. Furthermore, our assessment of secondary neurotoxic effects revealed that metformin, when delivered in combination with cisplatin, attenuates cisplatin-induced neurotoxicity *in vitro*. Although this neuro-protective mechanism needs further exploration, emerging data have shown that this effect was mediated mainly through the AMPK axis.

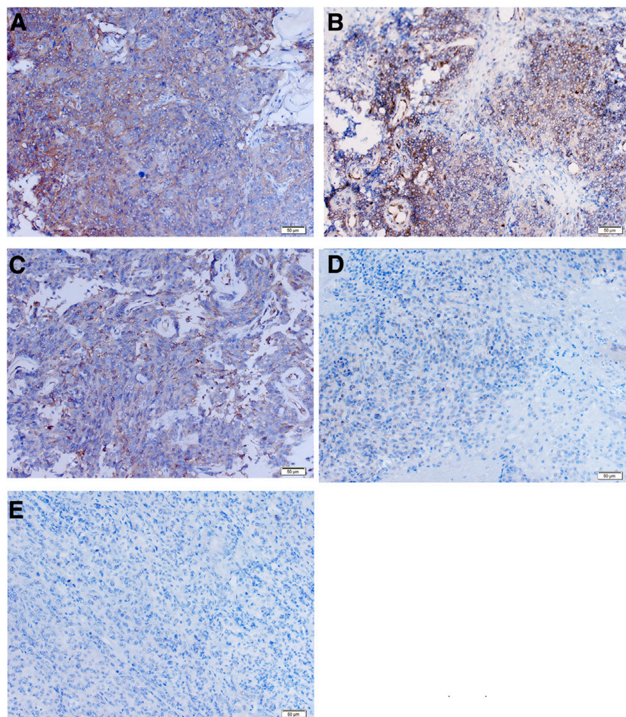

Figure 6. Immunohistochemical staining for P-AMPK in meningiomas of various grades showed low P-AMPK expression was associated short DFS in atypical meningiomas

Cytoplasmic P-AMPK immunohistochemical staining was investigated in meningiomas of different grades with variable IS and ASP. (A) Strong staining intensity patterns were demonstrated in benign meningiomas (grade I). (B–D) Strong (B), moderate (C), and weak (D) staining intensity patterns were indicated in atypical meningiomas (grade II). (E) Negative staining was seen in malignant meningiomas (grade III).

Table 1. Statistical correlation between clinicopathological features and p-AMPK immuno-expression in 63 atypical meningiomas

	p-AMPK immune expression		
	Low p-AMPK (ID score ≤ 6)	High p-AMPK (ID score > 6)	p value
Age (years)	58.29 ± 2.07	58.83 ± 2.36	0.865
Gender			
Male	14	16	0.268
Female	20	13	
Site			
Convexity	21	14	
Sagittal	4	2	0.299
Basal	9	13	
Simpson			
1	4	4	
2	15	14	0.880
3	15	11	
Recurrence			
No	17	23	0.016
Yes	17	6	
DFS (months)	23.44 ± 4.22	50.45 ± 4.84	< 0.001

ID, intensity distribution; DFS, disease-free survival.

Ramamurthy et al.[51] demonstrated that the energy-sensing AMPK pathway regulated neuronal structure in distinct regions of developing neurons at multiple stage of development (not only during axon outgrowth but also during dendrite growth and arborization). Houshmand et al.[52] suggested that metformin-induced AMPK activation could stimulate remyelination through induction of neurotrophic factors, downregulation of Nogo A, and recruitment of Olig2+ precursor cells. Tao et al.[53] revealed AMPK mediated activity-dependent axon branching by recruiting mitochondria to axons. In addition, Zhu et al.[54] uncovered that AMPK interacted with Down syndrome cell adhesion molecule (DSCAM) and played an important role in netrin-1-induced neurite outgrowth. Therefore, metformin might have distinct effects in cancer cells and in normal cells, as its activation could enhance anti-cancer effects but also ameliorate cisplatin-induced toxicities. Indeed, further studies are needed to investigate the chemosensitizing effects of metformin on cisplatin and also further elucidate its protective effect on cisplatin-induced neurotoxicity.

In conclusion, our study demonstrated that metformin enhanced the anti-cancer effect of cisplatin in meningioma *in vitro* and *in vivo*. Metformin's chemosensitizing effect was associated with the activation of AMPK and the dual repression of mTORC1 and mTORC2 signaling pathways, leading to G0/G1 cell cycle arrest. Furthermore, in patients with atypical meningiomas, the low expression of P-AMPK (secondary to AMPK activation) was significantly associated with tumor recurrence and shorter DFS. Combination treatment with metformin and cisplatin did not increase cisplatin-induced systemic side effects in a meningioma xenograft mice model and attenuated cisplatin-induced neurotoxicity *in vitro*. Collectively, our investigation found that metformin, when combined with cisplatin, is an effective and safe chemosensitizing drug for the treatment of high-grade meningioma.

MATERIALS AND METHODS

Cell lines and cultures

The human immortal meningioma cell lines (CH157-MN, IOMM-Lee) and PC-12 (a rat pheochromocytoma) cell line were purchased from American Type Culture Collection (ATCC, USA). All three cell lines were maintained in complete medium, specifically Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, Invitrogen) and supplemented with L-glutamine, 1 mM sodium pyruvate (PAA), and 1% penicillin/streptomycin (Invitrogen) at 37°C and 5% CO_2.

PCN cells

Cultured cortical cells were prepared from the cerebral cortices of 1-day-old Sprague-Dawley rats. After the brain was dissected, the blood

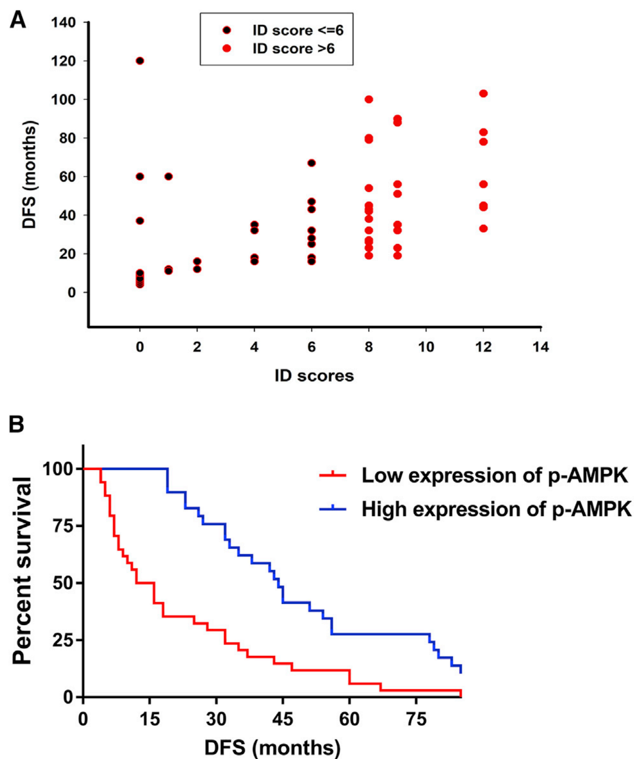

Figure 7. Low P-AMPK expression was associated with poor prognosis in patients with atypical meningiomas

(A) A dot-plot distribution was created to investigate the relationship between P-AMPK expression levels (ID scores) and DFS of the 63 patients with atypical meningiomas. (B) Kaplan-Meier curves demonstrate that low P-AMPK expression level is significantly associated with short DFS.

vessels and meninges were removed under a microscope. Cortices were placed in ice-cold DMEM and minced. The tissue chunks were incubated with papain solution (100 U/mL papain, 0.5 μM EDTA, 0.2 mg/mL cysteine, 1.5 mM $CaCl_2$, DNase I) at 37°C for 20 min to dissociate the cells. The reactions were terminated by adding heat-inactivated horse serum. After the cell suspension was centrifuged at 200 g, the pellet was re-suspended in DMEM supplemented with 10% horse serum. Cells were plated onto poly-D-lysine-coated Petri dishes and incubated at 37°C in a humidified incubator with 5% CO_2. Two hours after plating, the medium was replaced with neurobasal containing B27, 25 μM glutamine, and 0.5 mM glutamine. On the 4th day *in vitro*, the medium was changed and replaced with neurobasal/B27 without glutamate. The PCN cells were grown for another 10 days to permit the growth of axons and dendrites. Morphological changes were conducted using a phase-contrast inverse microscope (EVOS Cell Imaging Systems, Life Technologies, USA).

Assessment of AMPK expression in patients with atypical meningiomas

Sixty-three atypical meningiomas were diagnosed according to WHO criteria in Renji Hospital, Shanghai Jiaotong University School of Medicine from January 2008 to January 2018. All enrolled patients provided written informed consent, and the study protocol was approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiaotong University. Clinical characteristics, including age, sex, tumor site (basal, convexity, and sagittal), extent of tumor resection, development of recurrences, and DFS were available for all cases.

All 63 study patients with malignant meningiomas underwent surgical resection to remove their tumors. Excised meningiomas were subdivided based on their Simpson's grade: grade 1 (complete excision, including dura and bone), grade 2 (complete excision plus apparently reliable coagulation of dura attachments), and grade 3 meningiomas (complete excision, but insufficient dura coagulation or bone excision)

Reagents

Metformin (Calbiochem, Sigma-Aldrich, USA) was dissolved in phosphate-buffered saline (PBS) as a stock solution of 1 M. Cisplatin (Sigma-Aldrich, USA) was also dissolved in PBS as a stock solution of 2.5 mg/mL (8.33 mM). AMPK inhibitor Compound C (Calbiochem, Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 1 M. AMPKα 1/2-siRNA and scramble siRNA were purchased from Santa Cruz Biotechnology.

Primary antibodies against Cyclin D1, p27, cleaved Caspase-3, cleaved PARP (Asp214), and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA).

Primary antibodies for specific detection of AMPK, P-AMPK (Thr172), mTOR, P-mTOR (Ser2481), P-mTOR (Ser2448), S6K, P-S6K (Thr389), 4EBP1, P-4EBP1 (Thr37/46), AKT, and P-AKT (Ser473) were purchased from Cell Signaling Technology (Danvers, MA, USA).

AMPK-siRNA transfection

CH157 and IOMM cells were transfected with siRNA targeting the AMPK-α1 and α2 subunits (Santa Cruz, CA, USA) or scrambled siRNA (Santa Cruz, CA, USA) as a control using the Lipofectamine 2000 (RNAiMAX) Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection for 6 h, the culture medium was replaced with DMEM, followed by further studies.

Cell viability assay

Cell viability was assessed with Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plates (5×10^3/well for CH157 and PC12, and 3×10^3/well for IOMM) and incubated with or without metformin and/or cisplatin at the indicated concentrations at 37°C for 48 h. Subsequently, cells were incubated for an additional 1 h with 10 μL of CCK-8 at 37°C. Absorbance values were determined at a wavelength of 450 nm by spectrophotometric measurements (BioTek Instruments, Winooski, VT, USA)

Cell apoptosis assay

Cell apoptosis was measured using an Annexin V-FITC/PI Apoptosis kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, cells (CH157 and IOMM) were seeded at 1×10^6 cells per well in 6-well plates and incubated with or without metformin and/or cisplatin for 24 h. Cells were then harvested and washed with PBS buffer and re-suspended in 100 μL binding buffer. Annexin V-FITC (5 μL) was then added, and the cell suspension was incubated in the dark for 5 min before incubation for another 15 min in the dark in the presence of 5 μL PI. Fluorescence intensity was measured by flow cytometry (Guava Technologies, Hayward, CA, USA).

Cell cycle assay

Cells (CH157 and IOMM) were first seeded at 1×10^6 cells per well in 6-well plates overnight and incubated with or without metformin and/or cisplatin for 6 h. Cells were then harvested and permeabilized overnight with pre-cooled 75% ethanol at 4°C. Further, cells were treated with 1 mg/mL RNase A for 30 min at 37°C and stained with 50 μg/mL PI in the dark for 15 min. Finally, cells were analyzed by flow cytometry (Guava Technologies, Hayward, CA, USA).

Colony forming assay

500 viable IOMM cells were seeded per well in 6-well plates and maintained in DMEM overnight. CH157 cells were not used in this experiment, as they cannot form cell colonies. IOMM cells were then treated with metformin, cisplatin, or the combination of both for 24 h. Further, the medium was refreshed and maintained for another 10 days. The cultured cells were rinsed, fixed, and stained with 0.5% crystal violet containing 10% methanol for 20 min. Finally, colony numbers and sizes were counted and analyzed.

Preparation of whole cell extract

Cells were seeded onto 6-well plates at a density of 5×10^5 cells per well. After treatment, cells were washed with cold PBS and lysed with RIPA cell lysis buffer (Pierce RIPA Buffer, Thermo Scientific, USA) containing phosphatase and protease inhibitors at 4°C for 15 min. Cell lysates were then transferred into a microtube. The supernatant of cell lysates was collected by centrifugation, and the protein concentration was determined by Bradford dye binding method (Bio-Rad, Hercules, CA, USA).

Western blot analysis

After quantification, protein extracts were separated on 4%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were then blocked with 5% non-fat dried milk in Tris-buffered saline-Tween 20 (TBS-T, 20 mM Tris [pH 7.6], 137 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. The membranes were then washed and incubated with the appropriate primary antibody overnight at 4°C. The next day, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody in TBS-T at room temperature for 1 h. The immuno-complexes were visualized using KwikQuant Imager system (Kindle Biosciences, Greenwich, CT, USA).

Murine xenograft tumor model

A murine xenograft meningioma model was established to evaluate the anti-tumor efficiency of metformin in combination with cisplatin *in vivo*. Female BALB/c nude mice (National Institute of Cancer Animal Production Program, Frederick, MD, USA) aged 8 weeks were used for all *in vivo* studies. Mice were injected subcutaneously into the right flank with 5×10^6 IOMM cells suspended in 100 μL PBS buffer. After approximately 5 days, when tumor volumes reached a size of 75 mm^3, 28 mice were randomly assigned into four groups: Control, Met, Cis, and Met+Cis groups.

Control group mice were treated with 100 μL normal saline (NS) (intraperitoneal [i.p.] injection). Treatment with metformin (200 mg/kg, dissolved in 100 μL NS, i.p.) was given every day, while treatment with cisplatin (1.5 mg/kg, dissolved in 100 μL NS, i.p.) was administrated every other day. The mice were monitored for body weight and tumor volume every 3 days. Tumor volume was calculated as $0.5 \times$ length $\times$ width.[2]

After 24 days of treatment, blood from mice in all 4 treatment groups was extracted through the eyelid venous plexus, and the blood plasma was isolated and analyzed for kidney function (creatinine and urea nitrogen) and glucose levels. After study, mice were sacrificed by cervical dislocation and tumors were dissected and frozen in liquid nitrogen or fixed in formalin. All animal studies were conducted in accordance with the principles and procedures outline in the National Institutes of Health Guide for the Care and Use of Animals and approved by the Animal Care and Use Committee of the National Institutes of Health.

H&E staining

Consecutive tissue sections (thickness, 5 μm) of paraffin-embedded brain and tumor specimens were prepared. After staining with hematoxylin for 5 min and rinsed with running water for 5 min, tissue sections were soaked in hydrochloric acid solutions for 5 s, rinsed with running water for another 10 min, and then immersed in ammonia for 5 s. Tissue sections were then rinsed with running water for 10 min, stained with eosin solution for 30 s, rinsed with running water, and briefly immersed in distilled water. Last, the sections were rapidly dehydrated in graded ethanol (80%, 95%, and 100%), cleared in Xylene, and mounted with neutral gum.

TUNEL assay

Tumor cell apoptosis was evaluated on tumor specimen sections using the Promega TUNEL staining kit (Penzberg, Germany) following the manufacturer's instructions. The TUNEL-positive cells were counted in five randomized areas per section and expressed as the number of positively stained cells per square millimeter.

Immunohistochemistry

Immunohistochemical staining was performed on paraffin-embedded sections (thickness, 5 μm) of the mouse xenograft tumors. Antibodies against P-AMPKα (1:100), P-S6K (1:100), P-4EBP1 (1:1000), P-AKT (1:100), and Ki-67 (1:500) were used to determine protein expression. Expression levels were blindly scored by two independent individuals using an Olympus CX31 microscope.

For P-AMPK immunohistochemical staining of atypical meningiomas, we considered the IS and the ASP. IS was scored as: 1 (weak), 2 (moderate), or 3 (strong). ASP represented the percentage of positive cells, and it was scored as follows: 1 (5%–25% positive cells), 2 (26%–50% positive cells), 3 (51%–75% positive cells), or 4 (>75% positive cells). Cases with less than 5% positive cells were considered to be negative for P-AMPK. For each meningioma analyzed, we calculated an ID score by multiplying IS and ASP. The median ID score in the cohort was used to define low (ID score below cutoff, ≤ 6) and high (ID score above cut-off, >6) P-AMPK immunohistochemical expression.

Statistical analysis

Statistical analysis was performed on results from at least two independent experimental replicates. Data were presented as means ± SD. The two-sided Student's t test was applied to determine statistical significance between groups. Ordinary one-way ANOVA test was used for comparison between more than two groups. Survival curves were generated using the Kaplan-Meier estimate and compared using the log-rank test. All the experiments were performed in triplicate. A p value less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.11.004.

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AUTHOR CONTRIBUTIONS

Y.L. and Z.Z. designed the experiments, analyzed data, and wrote the article. L.G. and J.C. performed the main experiments. H.W., R.M., and S.Z. assisted with the experiments. R.M. and X.Z. assisted in analyzing the data and revised the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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