research article

Metformin enhanced *in vitro* radiosensitivity associates with G2/M cell cycle arrest and elevated adenosine-5'-monophosphate-activated protein kinase levels in glioblastoma

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Background. It is hypothesized that metabolism plays a strong role in cancer cell regulation. We have recently demonstrated improved progression-free survival in patients with glioblastoma who received metformin as an antidiabetic substance during chemoradiation. Although metformin is well-established in clinical use the influence of metformin in glioblastoma is far from being understood especially in combination with other treatment modalities such as radiation and temozolomide.

Materials and methods. In this study, we examined the influence of metformin in combinations with radiation and temozolomide on cell survival (clonogenic survival), cell cycle (routine flow cytometric analysis, FACScan), and phosphorylated Adenosine-5'-monophosphate-activated protein kinase (AMPK) (Phopho-AMPKalpha1 - ELISA) levels in glioblastoma cell lines LN18 and LN229.

Results. Metformin and temozolomide enhanced the effectiveness of photon irradiation in glioblastoma cells. Cell toxicity was more pronounced in O^6-methylguanine DNA methyltransferase (MGMT) promoter non-methylated LN18 cells. Induction of a G2/M phase cell cycle block through metformin and combined treatments was observed up to 72 h. These findings were associated with elevated levels of activated AMPK levels in LN229 cells but not in LN18 cells after irradiation, metformin, and temozolomide treatment.

Conclusions. Radiosensitizing effects of metformin on glioblastoma cells treated with irradiation and temozolomide *in vitro* coincided with G2/M arrest and changes in pAMPK levels.

Key words: metformin; glycolysis; metabolism; gliomas; proliferation; cell cycle

Introduction

The relationship of tumorigenesis, and tumor cell metabolism by an increased glycolysis was first described in the early 20th century by Otto Warburg.[1,2] Multiple modifications in cancer cell metabolism have subsequently been detected, but the influence on alterations in signaling path-

ways, cell growth, and therapy response is not yet understood. Nonetheless, tumor cell metabolism represents an intriguing potential target in the multidisciplinary treatment of cancer. The addition of metabolically active substances, particularly those with limited toxicity, with chemotherapy and radiation is an area of active research. In this context, the antidiabetic medication metformin is of particular interest as it demonstrated prolonged progression-free survival in a retrospective study in diabetic patients with glioblastoma (GBM).[3] The primary glucoregulatory effects of metformin are predominantly explained through reduced hepatic glucose production and increased glucose uptake in the periphery.[4] These effects lead to decreased mitochondrial-dependent ATP production and cell proliferation, increased glycolytic ATP production, induction of cell cycle arrest, autophagy, and apoptotic processes through activation of adenosine-5′-monophosphate-activated protein kinase (AMPK)[5] and inhibition of the mTOR (mammalian target of rapamycin) pathway in glioblastoma cells.[6,7]

AMPK is a serine/threonine kinase that functions as a cellular energy sensor. AMPK is an obligate heterotrimer, consisting of one catalytic subunit (α) and two regulatory subunits (β and γ).[8] Under cellular stress conditions, AMPK is activated by increased AMP-to-ATP ratios to promote catabolism and inhibit anabolism.[8] High cellular activated AMPK levels, particularly by phosphorylation (pAMPK), seems to be associated with tumor cell growth and cell survival.[9,10] Furthermore, increased AMPK phosphorylation has been observed in cells following radiation-induced DNA damage in several studies.[7,11,12] The activation of AMPK is hypothesized to regulate irradiation-induced metabolism changes and might be a key determinant of cell survival after exposure to ionizing radiation.[7] The AMPK pathway is therefore a potential objective for targeted therapies. Although the concomitant application of metformin with temozolomide (TMZ) and this effect is not well understood. In this study, we investigated the effects of metformin effect in combination with current standard of care therapy in glioblastoma cell lines.

Materials and methods

Cell lines and culture conditions

Two representative human GBM cell lines (ATCC; Manassas, VA, USA) were utilized in this study. LN18 is a GBM cell line with a mutant tumor suppressor protein 53 (p53mut) and an un-methylated O^6-methylguanine DNA methyltransferase (MGMT) promoter. LN229 is a GBM cell line with both mutant and wild type p53 (p53 mut/WT) and a methylated MGMT promoter. Both cell lines were cultured in Dulbeccos's modified Eagle medium (Biochrom, Berlin, Germany) supplemented with 10% Fetal Calf Serum (FCS) superior (Biochrom AG) and 1% penicillin/streptomycin (Gibco, Darmstadt, Germany). Cultures were maintained in exponential monolayer at incubator conditions with 37°C, 5% CO_2, and 95% humidity.

Drug treatment and irradiation

We performed clonogenic assays to evaluate treatment effects on cell survival. Low passage cells were plated in T25 flasks (Becton, Dickinson, Heidelberg, Germany) with 5 ml medium as described previously.[13] TMZ was obtained by Schering-Plough (Kenilworth, NJ, USA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Deisenhofen, Germany) at concentrations < 0.5%. Cell samples were incubated with 10 μM or 50 μM TMZ for 4 hours prior to irradiation. 1,1-Dimethylbiguanide hydrochloride (metformin; 97%) was provided by Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and applied in concentrations of 1 mM or 20 mM for 24 hours prior to irradiation. Immediately prior to irradiation, all samples were rinsed twice with phosphate buffered saline and fresh medium was added. Adherent cells were irradiated using a 6MV photon linear accelerator (XRAD 320 Precision X-ray Inc., N.Bradford, USA) with single photon doses up to 6 Gy. Cells were fixed with 70% ethanol and stained with methylene blue. Colonies of > 50 were counted. Experiments were repeated in triplicate at least three times.

Cell cycle analyses

To evaluate cell cycle distributions, cells were harvested, washed and fixed in ice-cold 70% ethanol after 24, 48 or 72 hours and stained with propidium iodide (Sigma-Aldrich). 10.000 events were counted for each experimental setup with routine flow cytometric analysis (FACScan, Becton-Dickinson, Heidelberg, Germany). Histograms were created and analyzed using ModFit software (Verity Software House, Topsham, ME, USA). Each experiment was repeated at least three times on different days for validation.

Phopho-AMPKα1 - ELISA

ELISA for pAMPK levels in LN229 and LN18 cell lysates was performed by commercially available DuoSet ELISA development kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instruction using goat anti-human AMPKα1 (T183) as primary antibody and biotinylated rabbit anti-human phospho-AMPKα1 (T183) as secondary antibody. Recombinant human phospo-AMPKα1 (T183) was utilized as standard. The results were presented as relative values to the control value, which was set as 1. Data analysis was carried out using the Infinite® F50/Robotic ELISA plate reader (absorbance at 450 nm, correction wavelength at 570 nm) and Magellan for F50 software (Tecan Group Ltd, Crailsheim, Germany). Measurements were repeated at least three times on three different days.

Statistical analysis

Clonogenic survival was calculated from the measured plating efficiencies. With the data of the combined treatment approaches survival curves were generated with the linear quadratic (LQ) - model as described earlier.[14] Sigma Plot's (Systat Software GmbH, Erkrath, Germany) non-linear least-squares regression option was used to fit the calculated survival curves. Clonogenic survival was calculated using the sensitizer enhancement ratio (SER), comparing the radiation dose at 20% cell survival, due to the high efficiency of TMZ and metformin on cellular killing.

$$SER = \frac{radiation\ dose\ without\ sensitizer}{radiation\ dose\ with\ sensitizer}$$

The Student's two-sided t-test was used for comparison of cell survival curves, differences in cell cycle analysis distribution and pAMPK levels. Data are shown as mean values ± standard deviation. Statistical significance was set at $p < 0.05$.

Results

Metformin enhances the effectiveness of irradiation in glioblastoma cells

Metformin sensitivity of glioblastoma cell lines LN18 and LN229 was investigated by clonogenic survival assays. Increasing concentrations of metformin (1 mM and 20 mM) and TMZ (10 μM and 50 μM) were chosen for all baseline experiments. Representative concentrations (20 mM metformin and 50 μM TMZ) were chosen for subsequent combined experiments. Clonogenic survival of the MGMT promoter methylated LN229 cell line was significantly reduced after treatment with TMZ compared to the untreated control and LN18 cell lines. Combined treatment approaches with irra-

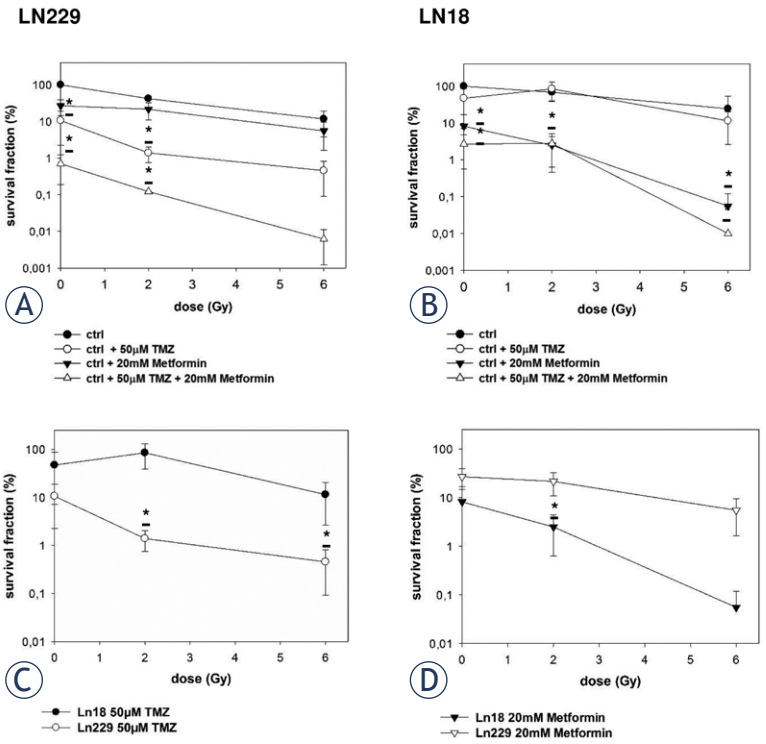

FIGURE 1. (A,B) Clonogenic survival assays of LN229 and LN18 glioblastoma cell lines after treatment with photon irradiation. **(C,D)** Clonogenic survival assays after comparing LN229 and LN18 cell lines after combined treatment with TMZ **(C)** or metformin **(D)**. Error bars represent standard deviations. * shows a statistical significance (P <0.05). In LN229 enhanced cell kill was observed by TMZ and in LN18 cells lines metformin treatment resulted in increased cell toxicity compared to the control, as well as the combination of both agents itself. Combined bimodal and trimodal treatment results in superior cell toxicity.

TABLE 1. Sensitizer enhancement ratio (SER 20%) for LN229 and LN18 cells after treatment with metformin, temozolomide (TMZ) and combined treatment with 2Gy irradiation

SER (20% survival) / 2 Gy		
Cell line	LN18	LN229
50 μM TMZ	1.11	2.15
1mM Metformin	0.67	0.74
20 mM Metformin	3.13	0.61
50 μM TMZ + 20mM Metformin	2.57	2.69

SER values (ranging from 0.67–3.13) depending on cell line, chemotherapeutic agent and dose

TABLE 2. Cell cycle distribution into G1, S, and G2/M phase of LN18 and LN229 cells after various treatments. Measurements were performed after 72h. * shows a statistical significance (P <0.05) of the treatment compared to the control

Ln18 24h 72h	G1 (%) ± std.dev.	S (%) ± std.dev.	G2/M (%) ± std.dev.
Ctrl	74.5 ± 8	5.6 ± 0	19.9 ± 1
50µM TMZ	66.0 ± 8	9.5 ± 1	24.6 ± 3
1mM Metformin	71.0 ± 5	6.3 ± 1	22.7 ± 0
20mM Metformin	46.9 ± 3	9.3 ± 2	**43.8 ± 2***
50µM TMZ + 1mM Metformin	64.5 ± 0	8.6 ± 4	26.9 ± 4
50µM TMZ + 20mM Metformin	41.2 ± 0	19.4 ± 7	39.4 ± 2
2Gy	71.7 ± 1	7.5 ± 1	20.8 ± 4
6Gy	56.2 ± 5	8.0 ± 1	35.7 ± 1
2Gy + 50µM TMZ	62.0 ± 5	6.6 ± 5	31.4 ± 4
2Gy + 1mM Metformin	68.2 ± 7	10.3 ± 1	21.5 ± 2
2 Gy + 20mM Metformin	41.3 ± 0	16.1 ± 1	**42.6 ± 4***
2Gy + 50µM TMZ + 1mM Metformin	60.1 ± 7	8.6 ± 1	31.3 ± 0
2Gy + 50µM TMZ + 20mM Metformin	33.5 ± 2	20.0 ± 3	**46.5 ± 4***
6Gy + 50µM TMZ	47.1 ± 7	11.6 ± 3	**41.3 ± 3***
6Gy + 1mM Metformin	51.3 ± 2	10.4 ± 0	38.2 ± 3
6Gy + 20mM Metformin	36.1 ± 3	14.2 ± 0	**49.7 ± 3***
6Gy + 50µM TMZ + 1mM Metformin	46.3 ± 4	10.7 ± 1	**42.9 ± 2***
6Gy + 50µM TMZ + 20mM Metformin	27.9 ± 1	14.1 ± 3	**58.0 ± 2***

Ln229 24h 72h	G1 (%)	S (%)	G2/M (%)
Ctrl	86.0 ± 2	3.6 ± 2	10.4 ± 2
50µM TMZ	37.4 ± 3	8.1 ± 2	**54.6 ± 5***
1mM Metformin	87.4 ± 3	3.3 ± 1	9.3 ± 0
20mM Metformin	63.9 ± 10	14.9 ± 1	21.2 ± 9
50µM TMZ + 1mM Metformin	38.9 ± 0	6.1 ± 2	**55.0 ± 4***
50µM TMZ + 20mM Metformin	25.2 ± 1	25.4 ± 2	**49.4 ± 2***
2Gy	83.5 ± 5	3.3 ± 2	13.2 ± 5
6Gy	67.1 ± 2	6.2 ± 1	26.7 ± 1
2Gy + 50µM TMZ	44.4 ± 1	6.9 ± 1	**48.7 ± 4***
2Gy + 1mM Metformin	82.3 ± 1	3.5 ± 1	14.2 ± 2
2 Gy + 20mM Metformin	55.6 ± 2	11.8 ± 2	**32.6 ± 1***
2Gy + 50µM TMZ + 1mM Metformin	46.0 ± 1	7.9 ± 1	**46.1 ± 0***
2Gy + 50µM TMZ + 20mM Metformin	32.3 ± 3	28.9 ± 7	**38.8 ± 5***
6Gy + 50µM TMZ	41.6 ± 2	8.9 ± 3	49.5 ± 3
6Gy + 1mM Metformin	55.5 ± 11	8.8 ± 4	35.7 ± 23
6Gy + 20mM Metformin	56.5 ± 18	7.8 ± 6	35.7 ± 16
6Gy + 50µM TMZ + 1mM Metformin	41.9 ± 2	5.9 ± 2	**52.2 ± 2***
6Gy + 50µM TMZ + 20mM Metformin	18.4 ± 1	16.2 ± 3	**65.4 ± 3***

diation, TMZ and metformin showed additive cell toxicity compared to the control ($P < 0.05$, Student's t-test) (Figure 1A,B and Table 1). Furthermore, clonogenic survival after metformin exposure was reduced in LN18 cells when compared to the untreated control and to LN229 cells ($P < 0.05$) (Figure 1C,D and Table 1). Additionally, additive cell toxicity could be reached in LN229 cells adding ionizing irradiation (2 Gy and 6 Gy) to TMZ and Metformin, whereas additional effects with ionizing irradiation could only be reached for metformin in LN18 cells.

Metformin induces a G2/M phase block in combination with irradiation

Cell cycle assessment of glioblastoma cell lines was carried out using FACS analyses. Exposure to metformin (20 mM) resulted in accumulation at G2/M phases after 72 hours to a higher degree in LN18 cells (G2 phase cells: ctrl vs 20mM metformin: 19.9% *vs* 43.8%). However, the results did not reach statistical significance. Combined treatment approaches with irradiation and metformin resulted in a more pronounced G2/M block after 72 hours ($P < 0.05$, Student's t-test) (Table 2). Accumulations in G2/M phases after 72 hours were even more marked when using higher radiation doses (6 Gy) (Table 2) and trimodal approaches with irradiation, 50 µM TMZ, and 20 mM metformin ($P < 0.05$, Student's t-test) (Table 2). Analysis of sub-G1 populations indicating apoptosis did not show any measurable results.

Irradiation, TMZ and metformin enhance activated AMPK levels in glioblastoma cells

Phosyphorylated serine/threonine kinase AMPK levels induced by irradiation, TMZ, and metformin exposure were investigated as a potential mechanism for the described metformin sensitivity of glioblastoma cell lines. Increased pAMPK levels were demonstrated in LN229 cells after treatment with the following regimens: 2 Gy + 50 µM TMZ, 2 Gy + 20 mM metformin, 2 Gy + 50 µM TMZ + 20 mM metformin, 6 Gy, 6 Gy + 50 µM TMZ, 6 Gy + 20 mM metformin, and 6 Gy + 50 µM TMZ + 20 mM metformin ($P<0.05$, Student's t-test) (Figure 2A). All other treatment approaches showed a trend towards higher pAMPK levels in LN229 cells ($P<0.1$, Student's t-test). Higher radiation doses were associated with increased pAMPK levels ($P<0.05$, Student's t-test) (Figure 2A). Interestingly, LN229

demonstrated a twofold higher level of pAMPK after treatment with 6 Gy compared to the untreated control ($P<0.05$, Student's t-test). pAMPK measurements obtained from LN18 cell lines did not show a significant increase after each treatment combination (Figure 2B).

Discussion

Standard of care multidisciplinary management of GBM entails surgical resection followed by radiotherapy with concomitant and adjuvant TMZ, resulting in overall survival rates of approximately one year. Given this dismal prognosis, the need to improve the efficacy of chemoradiation for these common primary brain tumors is urgent. Recently, we demonstrated improved progression-free survival rates in diabetic patients receiving metformin[3], a biguanide that is commonly used and well tolerated in patients with type II diabetes. Accordingly, combined approaches targeting cell metabolism became attractive. Metformin is known to exhibit anticancer effects via LKB1/AMPK/mTOR/S6K1 pathway blockade[15,16], inhibition of tumor growth[17,18] and induction of autophagy and apoptosis[19,20] in various cancer cell lines. Accordingly, integration of approaches targeting cell metabolism into standard therapy is an attractive area of investigation. In the present study, we examined the interaction of metformin in combination with photon irradiation and the alkylating agent TMZ. Furthermore, we demonstrated that metformin has antitumoral effects and increases sensitivity to ionizing radiation, which was particularly pronounced in a non-MGMT methylated cell line (LN18).

Of note, both the LN18 and LN229 cell lines express wildtype for PTEN, which is associated with increased sensitivity to metformin.[6] This susceptibility may be explained by opposing PI3K signaling, thus leading to a down-regulated AKT survival pathway and decreased glucose consumption.[6] Therefore, the higher metformin sensitivity for LN18 cannot solely be explained by the more effective deactivation of AKT in these cells. The authors believe the MGMT promoter methylation in metformin sensitive LN18 cells is rather a coincident than the cause of this finding. In fact, glioma cells are known to have a high intrinsic radiation sensitivity caused by several intrinsic factors such as high efficient radiation damage repair, a high ratio of hypoxic cell fraction and rapid repopulation following irradiation.[21] The intrinsic sensitivity of

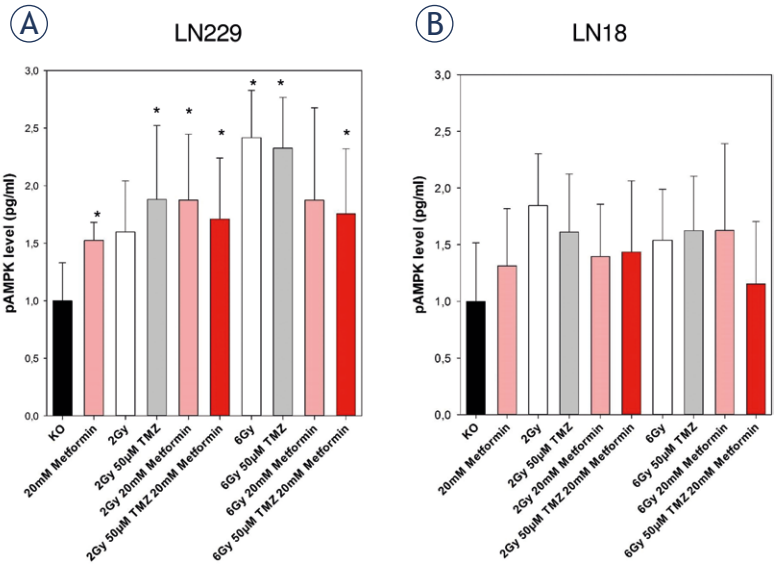

FIGURE 2. (A) In LN229 pAMPK measurements were significantly increased after treatment with Metformin, 2 Gy and temozolomide and 6 Gy combined with Metformin compared to the control if not otherwise specified (* $p<0.05$). **(B)** No significant increase of pAMPK levels were observed in cell line LN18. Error bars represent standard deviation.

GBM cells is probably independent of the MGMT promotor methylation status. The mechanistic effects of these findings are beyond the scope of this manuscript and will be evaluated in future experiments. An inverse effect was shown for TMZ, where the MGMT promoter-methylated LN229 cells were more sensitive to TMZ compared to MGMT promoter non-methylated LN18 cells. This finding can be explained by the lack of the MGMT DNA-repair protein in LN229 cells which normally removes O6-MG-DNA and counteracts the antineoplastic effect of TMZ.[22]

We performed cell cycle analyses in order to further investigate the antiproliferative effects of metformin on glioblastoma cell lines. In PTEN wildtype cells, cytotoxic effects have been described starting at 48 hours.[6] However, in LN18 and LN229 cell lines, significant increase of G2/M block rates started delayed, chiefly 72 hours after irradiation. This effect was pronounced in combined treatment approaches with higher radiation doses and TMZ administration. These results indicate that the antiproliferative effects of metformin on glioblastoma cell lines might be mediated trough cell cycle arrest starting at 72 hours post-exposure. These results are in line with results from *in vitro* data of Yu *et al.* who observed G2/M cell cycle arrests after TMZ and metformin. A combined treatment showed synergistic effects.[23] In a former study G1 arrests were described after metformin exposure,

whereas mainly G2/M phase arrests were observed in the current study. This effect might be due to the two-fold metformin dose (10 nM vs 20 nM) with varying impact. Furthermore metformin effects were mainly observed after 72 hours but the observation period of Sesen *et al.* ended after 48 hours.[6] Nevertheless, molecular mechanisms of metformin are far from understood and further research to examine its role in tumor cell metabolism is necessary.

Previous studies have demonstrated that activation of AMPK leads to an inhibition of mTOR[6,15,24,25] and is essential for glioma proliferation by promoting cell cycle progression *in vitro* and *in vivo*.[26] These findings are supported by reports indicating a major AMPK phosphorylation and activation through the tumor suppressor LKB1.[27-29] Furthermore, AMPK has been associated with p53-dependent apoptosis through p53 phosphorylation[30], underlining the potential function of AMPK activation as an "energy checkpoint".[31] This proposed mechanism permits proliferation and cell growth in cells with intact AMPK signaling only in favorable metabolic cell conditions. Conversely, cancer cells with deficient AMPK signaling might be capable of receiving a metabolism-independent growth stimulus. In these cases, induction of AMPK activation could present a valuable therapeutic approach.[26] Although the role of AMPK as a metabolic sensor in homeostasis is well described, its function in cancer remains opaque. *In vitro* studies have shown highly efficient inhibition of tumor cell growth across multiple glioblastoma cell lines with several AMPK agonists, including 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and activated AMPK adenovirus.[32-35]

In this study, we demonstrated a dose-dependent increase of AMPK in LN229 glioblastoma cells following radiation in combination with metformin and TMZ. Although AMPK level changes did not show statistically significant changes in the LN18 cell line, the radiosensitizing effect of metformin was more pronounced in these cells. Another reason for the absent of significant findings could be attributed to a high standard deviation masking subtle changes (Figure 4B). The authors carefully performed pAMPK level measurements with at least n=3. Even though, future in-depth analyses may help to unmask subtle changes. Interestingly, the failure of LN18 to phosphorylate AMPK compared to LN 229 cells could be a reason for the enhanced radiosensitivity in the latter, since AMPK activation might be a keyregulator for glioma cell proliferation.[26] On the other hand, it is likely that metformin exhibits both AMPK dependent and AMPK-independent effects which are contingent on molecular tumor characteristics.[6,15]

Taken together, the present finding that activated AMPK levels are elevated after treatment with radiation, TMZ, and metformin contributes to the understanding of GBM metabolism following therapeutic intervention. However, more detailed knowledge of the antitumoral effects of metformin, the role of AMPK, and tumor cell biology is necessary to establish a novel multidisciplinary approach to glioblastoma therapy. We planned to perform mechanistic *in vitro* metformin experiments in the future based on the current baseline results. Additional challenges, including the ability of AMPK activating agents such as AICAR to cross the blood brain barrier more effective, are ongoing. Nonetheless, our results suggest that the development of an AMPK activating agent with high central nervous system bioavailabity may be a promising new therapeutic avenue in the treatment of this aggressive malignancy.

Conclusions

Together with our previously published clinical findings[3] and the well-established use of metformin in clinical practice, these data show that radiosensitizing effects of metformin on glioblastoma cells treated with irradiation and temozolomide *in vitro* coincided with G2/M arrest and changes in pAMPK levels.

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