Tivantinib Hampers the Proliferation of Glioblastoma Cells via PI3K/Akt/Mammalian Target of Rapamycin (mTOR) Signaling

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background:
Glioblastoma, the most common and malignant glial tumor, often has poor prognosis. Tivantinib has shown its potential in treating c-Met-high carcinoma. No studies have explored whether tivantinib inhibits the development of glioblastoma.

Material/Methods:
The correlation between c-Met expression and clinicopathological characteristics of glioblastoma was investigated. U251 and T98MG glioblastoma cells treated with tivantinib, PI3K inhibitor (LY294002), PI3K activator (740 Y-P), and/or mammalian target of rapamycin (mTOR) inhibitor were subjected to MTT assay or colony formation assay to evaluate cell proliferation. The expression of mTOR signaling and caspase-3 in tivantinib-treated glioblastoma cells was differentially measured by western blotting.

Results:
In a group of Chinese patients, expression of c-Met was elevated with the size of glioblastoma, but not with the other clinicopathological characteristics, including gender, age, grade, IDH status, 1p/19q status, and Ki67 status. High dose of tivantinib (1 µmol/L) obviously repressed the proliferation and colony formation of U251 and T98MG glioblastoma cells, but low dose (0.1 µmol/L) of tivantinib failed to retard cell proliferation. Tivantinib blocked PI3K/Akt/mTOR signaling but did not change the expression of cleaved caspase-3. PI3K activator 740 Y-P (20 µmol/L) significantly rescued tivantinib-induced decrease of cell proliferation. Tivantinib (1 µmol/L) in combination with PI3K inhibitor LY294002 (0.5 µmol/L) and mTOR inhibitor rapamycin (0.1 nmol/L) largely inhibited the proliferation of glioblastoma cells.

Conclusions:
c-MET inhibitor tivantinib blocks PIKE/Akt/mTOR signaling and hampers the proliferation of glioblastoma cells, which endows the drug a therapeutic effect.

MeSH Keywords:
Cell Proliferation • Glioblastoma • TOR Serine-Threonine Kinases

Full-text PDF:
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Background

Glioblastoma, the most common and malignant glial tumor, has a poor prognosis [1]. Treatment options include surgery, radiation therapy, chemotherapy, tumor treating fields therapy, and targeted drug therapy [2]. However, few effective drugs and/or therapeutic regimens are available. The discovery of novel drugs is a requirement for the prevention and cure of glioblastoma.

Tivantinib (ARQ197) antagonizes c-Met with antineoplastic activity [3]. Tivantinib binds to c-Met protein and disrupts c-Met signal transduction pathways, leading to the death of tumor cells overexpressing c-Met protein [4]. c-Met protein is translated from the proto-oncogene *c-Met* [5]. c-Met is highly expressed or mutated in proliferating tumor cells [6]. Unfortunately, the hepatocellular carcinoma (HCC) candidate tivantinib (ARQ197) showed no antitumor ability in the phase III MET-high, advanced HCC (METIV-HCC) trial [7]. Even so, tivantinib has shown therapeutic potential against c-Met-high carcinoma, including non-small-cell lung cancer, breast cancer, germ cell tumor, alveolar soft part sarcoma, metastatic gastric cancer, mesothelioma, papillary renal cell cancer, oral squamous cell carcinoma, myeloma, neuroendocrine tumor, prostate cancer, acute myeloid leukemia, colorectal cancer, and cholangiocarcinoma [8–21].

Anti-c-Met antibody (one-armed 5D5, OA-5D5) is capable of reducing cell proliferation and microvessel density and is capable of inducing apoptosis of glioblastoma in a nude mouse [22]. However, chemical drug targeting c-Met has not been reported in the treatment of glioblastoma. Here, we investigated the correlation between c-Met expression and clinicopathological characteristics in glioblastoma, the effect of tivantinib on glioblastoma cell proliferation, and the underlying mechanism.

Material and Methods

Clinical cases

Twenty-nine glioblastoma cases were recruited from Jinan Fourth People's Hospital and Linyi Central Hospital from 2015 to 2019. Glioblastoma was diagnosed according to World Health Organization (WHO) 2007 and 2016 histopathologic criteria [23]. All high-density glioblastoma tissues were histopathologically confirmed by 2 pathologists. The study was approved by the Clinical Research Ethics Committee, Jinan Fourth People's Hospital (no. LL-20140005). Written informed consent was obtained from each participant.

Cell culture

U251 and T98MG glioblastoma cells were stored in our laboratory and cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, MA, USA) containing 10% fetal bovine serum (FBS) with 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA) in a 5% CO_2 incubator at 37°C.

MTT assay

Cell proliferation of U251 and T98MG glioblastoma cells were evaluated by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) assay. Then, 100 μL of cell suspension (5000 cells/well) was dispensed in a 96-well plate. The cells were treated with indicated concentrations of tivantinib (Selleck), 0.1 nmol/L rapamycin (Selleck), 0.5 μmol/L LY294002 (Selleck) or 20 μmol/L 740 Y-P (APExBIO) and cultured for 24 hours. MTT solution (5 mg/mL, 20 μL) was injected into each well of the plate. The plates were incubated for 4 hours in an incubator. After the medium and MTT were taken away, 150 mL dimethyl sulfoxide (DMSO) was supplemented into each well. The absorbance at 490 nm was measured using a microplate reader (Bio-Tek, Elx800, USA).

Colony formation assay

U251 and T98MG glioblastoma cells (5000 cells/well) were seeded into a 6-well plate. The cells were allowed to grow in a humidified incubator (37°C, 5% CO_2) for 2 weeks. Crystal violet (0.1%) was added into a plate to visualize formed colonies after removal of the medium. The pictures were taken by a commercial household camera (Canon, Japan).

Western blotting

Western blotting was used to analyze protein expression according to standard procedures [24]. After various treatments, glioblastoma cells were lysed with iced RIPA buffer [25]. The isolated proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) thereby transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Blocked with 5% non-fat dried milk, the membranes were subsequently incubated with appropriate primary and secondary antibodies. The primary antibodies used in this study included anti-caspase-3 (Proteintech, Wuhan, China), anti-S6 (Cell Signaling Technology), anti-p-S6 (Cell Signaling Technology), anti-Akt (Cell Signaling Technology), anti-p-Akt (Ser 473, Cell Signaling Technology), anti-p-S6K1 (T389, Abcam), and anti-Actin (Cell Signaling Technology).

Statistical analysis

Data from at least 3 independent experiments were analyzed using the SPSS (Version 22, Chicago, IL, USA). All data were expressed as mean±standard deviation (SD) and analyzed with one-way ANOVA followed by Bonferroni's test or chi-square test. *P* values of less than 0.05 were considered as statistically significant.

Table 1. Associations between c-Met expression and clinicopathological characteristics in glioblastoma.

Characteristics	n	c-Met*		OR (95%Cl)	P value**
		low	high		
Tumor size					
≤2 cm	14	11	3	10.083 (1.816–55.998)	0.006
>2 cm	15	4	11		
Gender					
Male	16	8	8	0.857 (0.198–3.713)	0.839
Female	13	7	6		
Age					
≤40	15	7	8	0.656 (0.151–2.843)	0.579
>40	14	8	6		
Grade					
I–II	20	11	9	1.528 (0.314–7.437)	0.605
III–IV	9	4	5		
IDH status					
Wildtype	23	10	13	0.154 (0.015–1.534)	0.087
Mutant	6	5	1		
1p/19q status					
Wildtype	25	13	12	1.083 (0.131–8.946)	0.942
Mutant	4	2	2		
Ki67 status					
Negative	16	9	7	1.5 (0.344–6.532)	0.595
Positive	13	6	7		

* The c-Met expression levels were divided at a cutoff point of 50%; ** P value for χ^2 test.

Results

c-Met was positively correlated with tumor size of glioblastoma

To investigate whether c-Met mediated the development of glioblastoma, we retrospectively looked at the clinicopathological characteristics and c-Met expression in 29 Chinese glioblastoma cases. High expression of c-Met was positively associated with the tumor size of glioblastoma (odds ratio [OR]: 10.083, 95% confidence interval [Cl]: 1.816–55.998, P=0.006, Table 1). However, c-Met expression showed no indications with the other clinicopathological characteristics, including gender, age, grade, IDH status, 1p/19q status, and Ki67 status (Table 1). Thus, these results indicated that c-Met might mediate the growth of glioblastoma cells.

Tivantinib repressed the growth of glioblastoma cells

We treated U251 and T98MG glioblastoma cells with 0, 0.1, 1, or 10 μmol/L tivantinib, a selective human c-Met receptor tyrosine kinase inhibitor, then subjected to MTT assay and colony formation assay. High dose of tivantinib (1 μmol/L and 10 μmol/L) largely inhibited the proliferation of glioblastoma cells, but low dose (0.1 μmol/L) of tivantinib failed to retard cell proliferation (Figure 1A, 1B). We also found that 1 μmol/L tivantinib completely inhibited the colony formation of glioblastoma cells (Figure 1C).

Next, we examined whether tivantinib had a stimulative effect on the apoptosis of glioblastoma cells. Western blotting revealed that 1 μmol/L tivantinib did not change the expression of cleaved caspase-3 (Figure 2A, 2B), suggesting its invalid role on caspase-dependent apoptosis of glioblastoma cells.
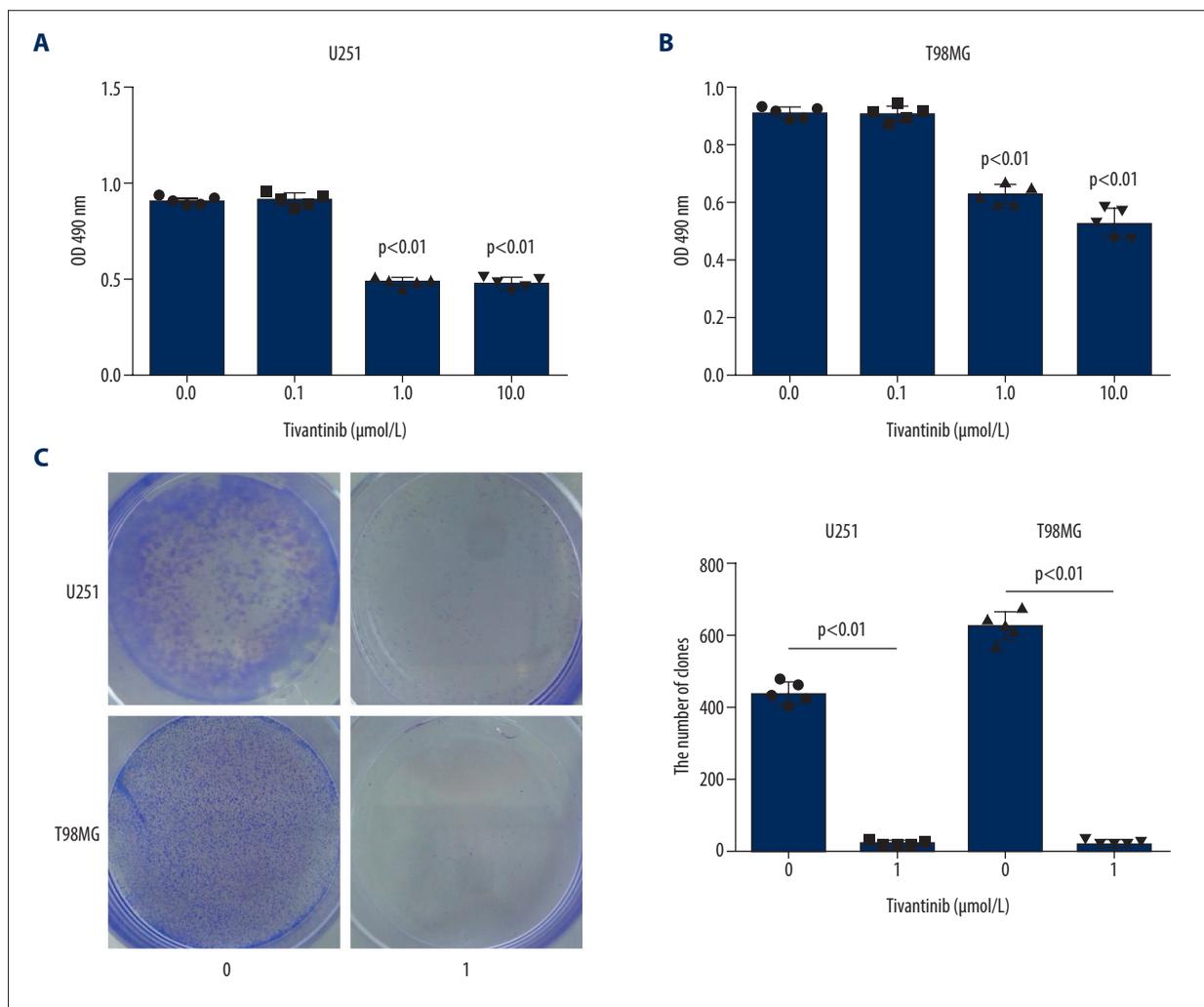
Figure 1. Tivantinib inhibits the proliferation of glioblastoma cells. (**A, B**) U251 and T98MG glioblastoma cells were incubated with tivantinib and subjected to MTT assay. 1 µmol/L and 10 µmol/L tivantinib significantly inhibited the proliferation of U251 and T98MG cells. (**C**) U251 and T98MG glioblastoma cells were incubated with 1 µmol/L tivantinib and subjected to colony formation assay. Tivantinib significantly decreased the number of the colonies of U251 and T98MG cells.

Tivantinib blocked PI3K/Akt/mTOR signaling of glioblastoma cells

Given that mTOR is the key regulator of cell growth, we hypothesized that tivantinib inhibited cell proliferation via downregulating mTOR signaling in glioblastoma. Western blotting revealed that 1 µmol/L tivantinib significantly decreased the phosphorylation of S6K1 and S6, the downstream targets of mTOR complex, but not S6 expression (Figure 2A, 2B). We also found that 1 µmol/L tivantinib significantly downregulated the phosphorylation of Akt, a direct downstream target of PI3K, but not Akt expression (Figure 3A, 3B). These results indicated that tivantinib can block c-Met and PI3K/Akt/mTOR signaling in glioblastoma cells.

Tivantinib inhibited cell proliferation via PI3K/Akt/mTOR signaling

MTT assay revealed that 0.1 nmol/L rapamycin (a specific mTOR inhibitor) and 1 µmol/L tivantinib had similar suppressive effects on cell proliferation (Figure 4A, 4B). Next, we challenged glioblastoma cells with 20 µmol/L 740 Y-P (a PI3K activator), finding that 740 Y-P significantly rescued tivantinib-induced decrease of cell proliferation (Figure 4A, 4B). The combined treatment of tivantinib, 740 Y-P and rapamycin remarkably retarded cell proliferation (Figure 4A, 4B), suggesting that mTOR was the downstream target of PI3K/Akt. Thus, these results indicated that tivantinib inhibits cell proliferation via PI3K/Akt/mTOR signaling in glioblastoma.
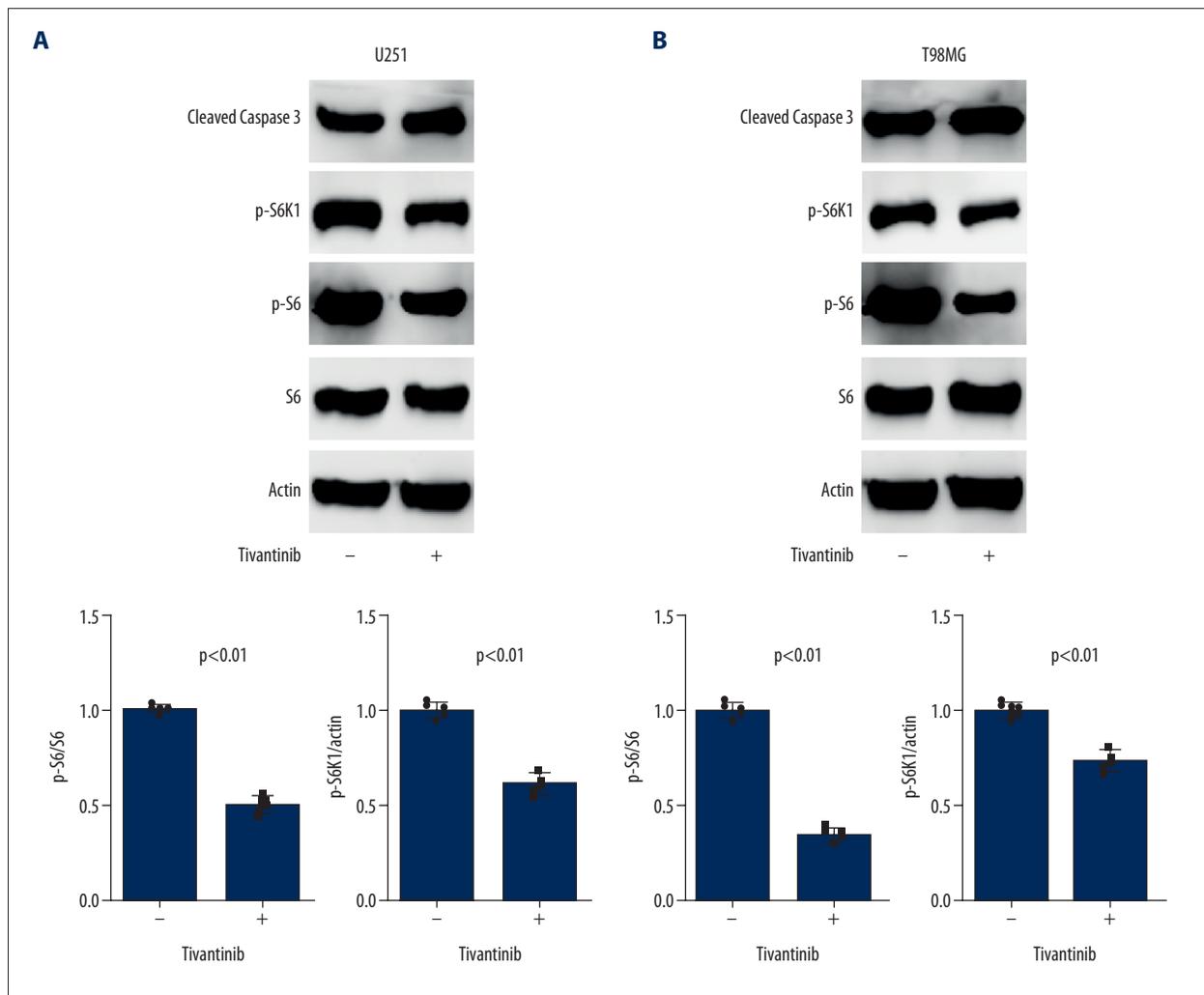
Figure 2. Tivantinib downregulates mTOR signaling, but not apoptotic signaling. U251 (**A**) and T98MG (**B**) glioblastoma cells were treated with 1 µmol/L tivantinib for 1 hour. Cellular lysates were subjected to western blotting. Tivantinib downregulated the phosphorylation of S6K1 (p-S6K1) and the phosphorylation of S6 (p-S6), 2 downstream targets of mTOR complex. Tivantinib did not change the expression of cleaved caspase-3. Actin was the internal control.

Tivantinib in combination with LY294002 and rapamycin largely inhibited cell proliferation

MTT assay revealed that 0.5 µmol/L LY294002 (a PI3K inhibitor) and 1 µmol/L tivantinib had similar suppressive effects on cell proliferation (Figure 5A, 5B). Next, the combined treatment of tivantinib, LY294002, and rapamycin remarkably retarded cell proliferation (Figure 5A, 5B), suggesting a novel pharmaceutical composition for inhibiting the proliferation of glioblastoma cells.

Discussion

Tivantinib (ARQ197), the first non-ATP-competitive c-Met inhibitor, does not act with Ron or inhibit EGFR, InsR, PDGFRα, or

FGFR1/4. Although tivantinib showed no antitumor effect in the phase III METIV-HCC trial [7], there have been numerous successful trials: non-small-cell lung cancer (randomized phase II study of erlotinib plus tivantinib versus erlotinib plus placebo, randomized phase III of double-blind study of tivantinib plus erlotinib versus placebo plus erlotinib), metastatic solid tumor (phase I dose-escalation study), relapsed or refractory germ cell tumor (phase 2 multicenter study), metastatic triple-negative breast cancer (phase II study), stage IIIB/IV nonsquamous non-small-cell lung cancer harboring wild-type epidermal growth factor receptor (randomized, double-blind, placebo-controlled, phase III trial of erlotinib with or without tivantinib), metastatic gastric cancer (phase II trial), metastatic colorectal cancer with wild-type KRAS (randomized, placebo-controlled, phase 1/2 study), and relapsed or relapsed/refractory multiple myeloma (phase II), metastatic colorectal cancer (phase II) [4,8,12,20,26–30]. However,
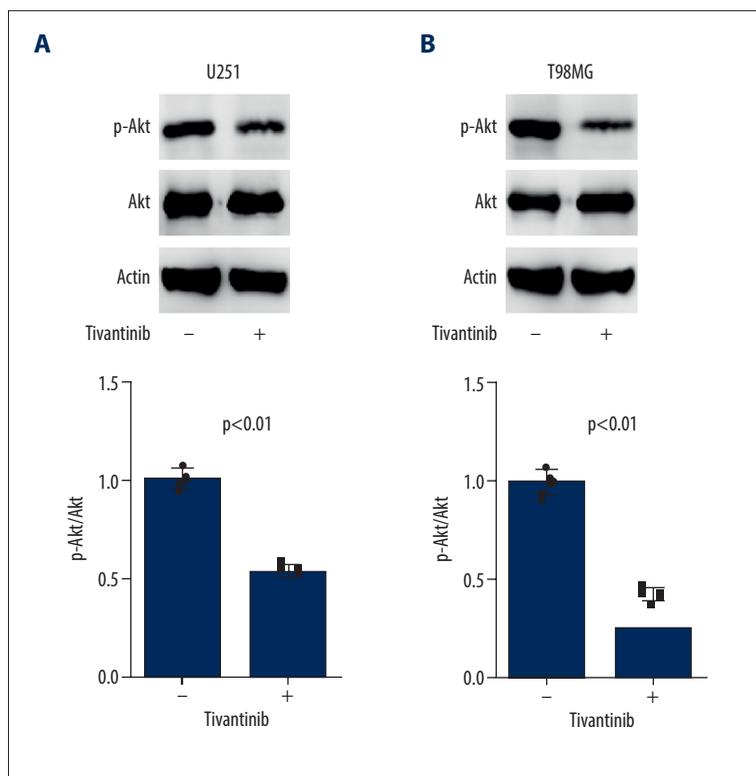
Figure 3. Tivantinib suppresses PI3K/Akt signaling. U251 (**A**) and T98MG (**B**) glioblastoma cells were treated with 1 μmol/L tivantinib for 1 hour. Cellular lysates were subjected to western blotting. Tivantinib suppressed the phosphorylation of Akt (p-Akt), a downstream target of PI3K. Actin was the internal control.
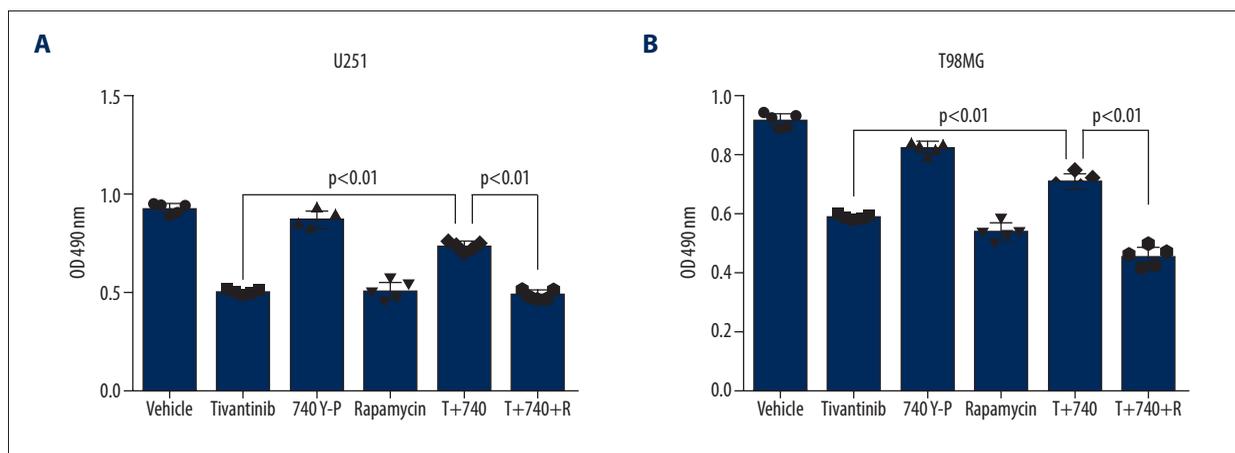

Figure 4. Tivantinib inhibits glioblastoma cell proliferation via PI3K/Akt/mTOR signaling. U251 (**A**) and T98MG (**B**) glioblastoma cells were treated with 1 μmol/L tivantinib, 0.1 nmol/L rapamycin, 20 μmol/L 740 Y-P, tivantinib plus 740 Y-P, or tivantinib plus 740 Y-P plus rapamycin. Cells were subjected to MTT assay. Cell proliferation was inhibited by tivantinib treatment, and rescued by the incubation of PI3K activator 740 Y-P. The mTOR inhibitor rapamycin blocked the PI3K/Akt signaling and inhibited cell proliferation.

the effect of tivantinib on glioblastoma has not been studied. For this study, from 2015 to 2019, we recruited 25 glioblastoma cases in Jinan Fourth People's Hospital and 35 patients in Linyi Central Hospital. Among them, 18 cases from Jinan Fourth People's Hospital and 15 patients from Linyi Central Hospital had undergone surgery after diagnosis. For this study, we demonstrated that the expression increased with glioblastoma size in Chinese patients. Moreover, we demonstrate that tivantinib

significantly inhibited the proliferation and colony formation of glioblastoma cells. Tivantinib directly blocked c-Met, significantly downregulated the phosphorylation of PI3K/Akt, and thus suppressed mTOR activity of glioblastoma cells.

Rapamycin, a macrolide compound obtained from *Streptomyces hygroscopicus*, can specifically inhibit mTOR. Rapamycin functions as an immunosuppressor in organ allografting [31].
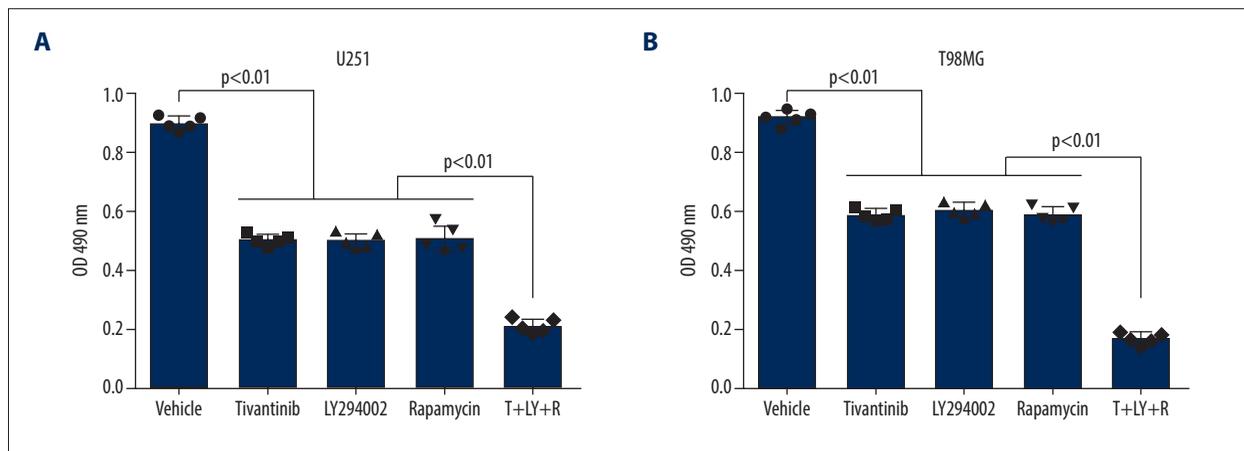
Figure 5. Tivantinib in combination with LY294002 and rapamycin largely inhibits the proliferation of glioblastoma cells. U251 (**A**) and T98MG (**B**) glioblastoma cells were treated with 1 µmol/L tivantinib, 0.5 µmol/L LY294002, 0.1 nmol/L rapamycin, or tivantinib plus LY294002 plus rapamycin. Cells were subjected to MTT assay. Cell proliferation was largely inhibited by the combination of tivantinib, LY294002, and rapamycin.

Hosoi et al. first reported rapamycin inhibited c-MYC signaling in human cancer cells [32]. Rapamycin blocks steroid driven transition of T47D breast cancer cells from G1 to S-phase [33]. Rapamycin prevents tumor growth and metastatic progression and prolongs the survival of mice inoculated with renal cancer cells or T24 human bladder cancer cells [34]. Everolimus (RAD001, Novartis), an oral rapamycin, acts in relapsed non-small-cell lung cancer [35,36]. Rapamycin enhances 5-aza-dC in suppressing cell growth and arresting cell cycle in human gastric cancer [37]. Rapamycin in combination with cisplatin inhibits cell growth and induces the apoptosis of endometrial cancer and pancreatic cancer [38,39]. Mendiburu-Elicabe et al. reported rapamycin attenuated cancer stem cells' proliferation and tumorigenic potential *in vitro* [40]. In this study, tivantinib blocked c-Met/PI3K/Akt/mTOR signaling of glioblastoma cells, indicating that combined tivantinib and rapamycin can suppress glioblastoma growth.

PI3K/Akt/mTOR signaling pathway is engaged in various cellular processes. LY294002 can strongly block the performance of PI3K [41,42]. Intracellular HMGB1 of glioblastoma cells is released and activates Akt and ERK signaling pathways, thus

promoting glioblastoma cell invasion in hypoxia [43]. We found that the combined treatment of tivantinib, LY294002, and rapamycin largely inhibited tumor cell proliferation, suggesting a novel pharmaceutical composition for suppressing cell proliferation in glioblastoma. Wang et al. reported that PTPN3 was an independent prognostic factor in glioblastoma [44]. Our data and the study of Cheng et al. [43] indicate that the expression of c-Met/Akt might be used to identify high-risk glioblastoma patients.

Conclusions

c-MET inhibitor tivantinib blocks PI3K/Akt/mTOR signaling and inhibits the proliferation of glioblastoma cells. Tivantinib in combination with PI3K inhibitor LY294002 and mTOR inhibitor rapamycin shows therapeutic effect for glioblastoma.

Conflicts of interest

None.

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