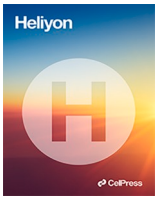
Heliyon 10 (2024) e30833

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Novel cancer-fighting role of ticagrelor inhibits GTSE1-induced EMT by regulating PI3K/Akt/NF-κB signaling pathway in malignant glioma

Enzhou Lu [a,b,1], Boxian Zhao [a,b,1], Chao Yuan [a,b], Yanchao Liang [a,b], Xiaoxiong Wang [a,b,**], Guang Yang [a,b,*]

[a] *Department of Neurosurgery, The First Affiliated Hospital of Harbin Medical University, China*
[b] *Heilongjiang Province Neuroscience Institute, Harbin, China*

ARTICLE INFO

Keywords:
Ticagrelor
GTSE1
Epithelial-mesenchymal transition
PI3K/Akt/NF-κB
Glioma

ABSTRACT

Background: Glioma is the most common malignant brain tumor of the central nervous system. Despite of the improvement of therapeutic strategy, the prognosis of malignant glioma patients underwent by STUPP strategy is still unexpected. Previous studies have suggested that ticagrelor exerted chemotherapeutic effects by inhibition of epithelial-mesenchymal transition (EMT) in various diseases including tumors. However, whether ticagrelor can exhibit the antitumor efficiency in glioma by affecting the EMT process is still unclear. In this study, we investigated the cancer-fighting role of ticagrelor and demonstrated its chemotherapeutic mechanism in glioma.
Materials and methods: The MTT assay was performed to detect the cytotoxicity of ticagrelor in glioma cells. We evaluated the expression of Ki67 in glioma cells by immunofluorescence assay after ticagrelor treatment. We conducted wound healing assay and transwell assay to determine the effects of ticagrelor on the migration and invasion of glioma cells. RNA-seq analysis was conducted to examine potential target genes and alternative signaling pathways for ticagrelor treatment. The expression levels of key EMT -related proteins were examined by Western blot experiment.
Results: Ticagrelor inhibited the proliferation, migration and invasion of glioma cells with a favorable toxicity profile in vitro. Ticagrelor downregulated the expression of GTSE1 in glioma cells. RNA-seq analysis explored that GTSE1 acted as the potential target gene for ticagrelor treatment. Upregulation of GTSE1 antagonized the inhibitory effect of ticagrelor on the invasion of glioma and EMT progression by regulation of PI3K/Akt/NF-κB signaling pathway. And ticagrelor also exhibited the similar chemotherapeutic effect of glioma in vivo.
Conclusions: Ticagrelor as a potential chemotherapeutic option induced the inhibition of the GTSE1-induced EMT progression by regulation of PI3K/AKT/NF-κB signaling pathway.

* Corresponding author. Department of Neurosurgery, The First Affiliated Hospital of Harbin Medical University; Heilongjiang Province Neuroscience Institute, No.23 Youzheng Street, Nangang District, Harbin, 150001, China.
** Corresponding author. Department of Neurosurgery, The First Affiliated Hospital of Harbin Medical University; Heilongjiang Province Neuroscience Institute, No.23 Youzheng Street, Nangang District, Harbin, 150001, China.
E-mail addresses: steven_xiaoxiong@yeah.net (X. Wang), yangguang@hrbmu.edu.cn (G. Yang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.heliyon.2024.e30833
Received 9 February 2024; Received in revised form 21 April 2024; Accepted 6 May 2024
Available online 7 May 2024

1. Introduction

Glioma accounts for approximately 80 % of all malignant primary brain tumors, and the incidence rate has increased annually from 5.9/100,000 in 1973 to 6.61/100,000 in 2016 [1,2]. The WHO grade 1 glioma grows slowly and the clinical course of WHO grade 2 and 3 glioma was moderate, whereas WHO grade 4 glioma progressed most rapidly (median survival from 14.5 to 16.6 months) [3,4]. In 2016, WHO firstly introduced the molecular alterations such as ATRX gene mutations, IDH mutations, and combined 1p/19q deletions into specific criteria for glioma classification [5]. And these changes in fifth WHO classification of CNS tumors added more biologically and molecularly defined entities with characterized natural histories, as well as introducing new types and subtypes [6].

Drug therapy remains an important part of the STUPP therapeutic strategy for glioma. Currently, chemotherapeutic agents such as temozolomide, bevacizumab, and lomustine are clinically used for the glioma treatment [7]. Most patients have poor 5-year overall survival despite of the standard STUPP care, surgical resection followed by temozolomide combined with radiotherapy [8]. Therefore, it is necessary to explore and develop new therapeutic drugs for glioma.

Ticagrelor is an antiplatelet agent that reversibly binds to the P2Y12 receptor on ADP [9]. It was approved by the FDA in 2011 for the treatment of acute coronary syndromes [10]. Several studies have reported that ticagrelor exerts antitumor effects in tumors such as breast, pancreatic, and ovarian cancer [11–13]. The epithelial-mesenchymal transition (EMT) enables epithelial-derived malignancies to acquire an invasive phenotype characterized by cadherin regulation, overexpression of specific transcription factors, and metabolic alterations [14,15]. In glioma, EMT confers an extremely strong migration and invasion characteristics, and the emergence of this phenomenon often limits the maximum extent of surgical resection and promotes treatment resistance, ultimately leading to malignant tumor progression and recurrence [16,17]. We speculate that ticagrelor may exert its antitumor effects in glioma by

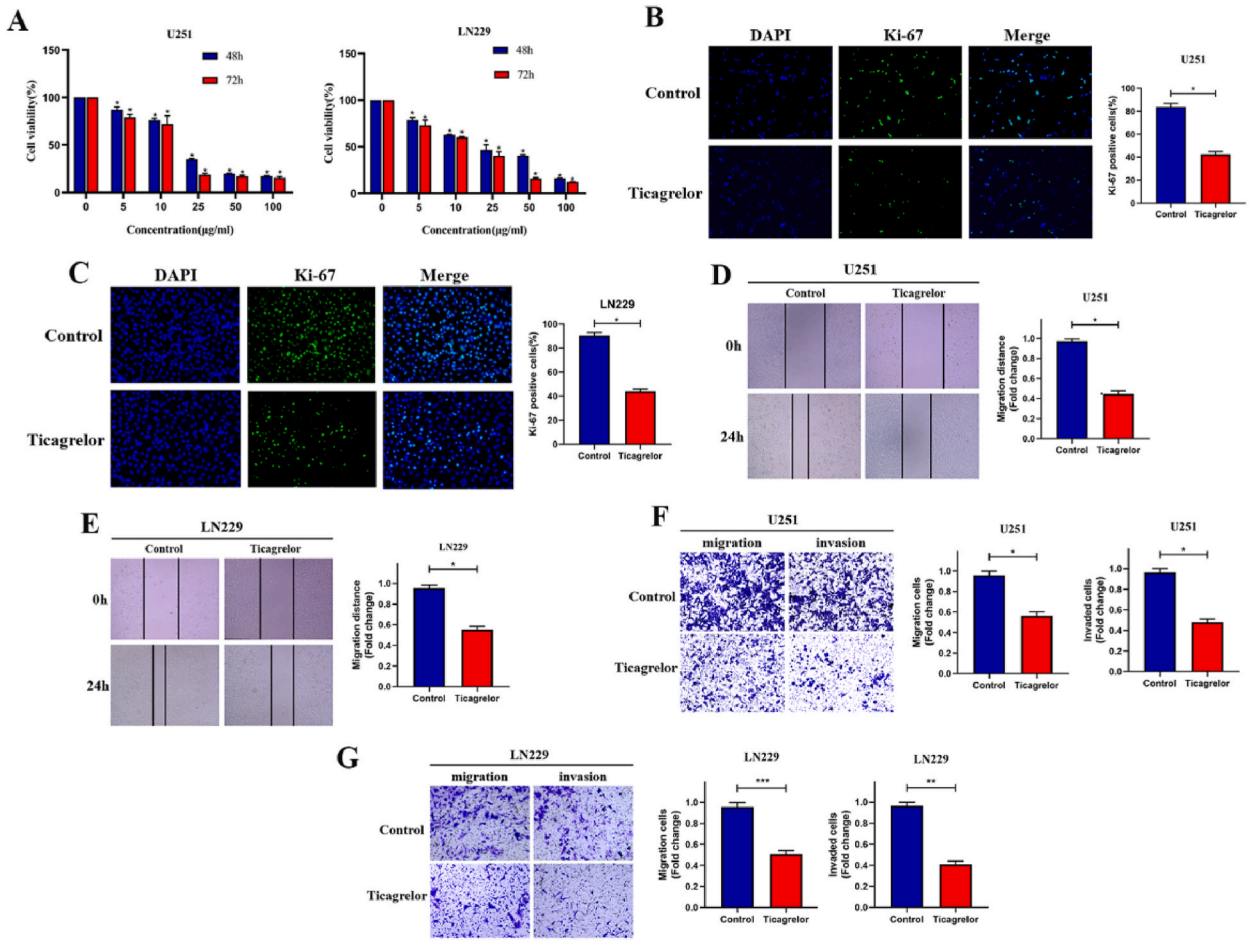


Fig. 1. Ticagrelor inhibited the proliferation, migration and invasion of glioma cells. A The viability of U251 and LN229 glioma cells treated with ticagrelor for 48 and 72 h was measured by MTT assay. **B–C** Expression of Ki67 was detected by immunofluorescence in U251 and LN229 glioma cells treated with ticagrelor for 48 h. **D-E** The migration ability of U251 and LN229 glioma cells was measured by wound healing assay after 24 h of ticagrelor treatment. **F-G** The migration and invasion ability of U251 and LN229 glioma cells were detected by transwell assay after 48h treatment with ticagrelor. Error bars show mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001. The experiments were repeated 3 times independently.

modulating the EMT process, but the molecular mechanism involved in this effect still need to be further investigated. The purpose of this study was to investigate the regulatory effects of ticagrelor on the EMT process in glioma, and to explore new potential evidence for the antitumor effect of ticagrelor.

2. Results

2.1. Ticagrelor inhibited the proliferation, migration and invasion of glioma

In order to investigate the effect of ticagrelor on the proliferation of glioma cells, MTT assay was performed to detect the effect of ticagrelor on the viability of human glioma cell lines U251 and LN229 at different treatment times (48 h and 72 h) and concentrations (0, 5, 10, 25, 50, 100 μg/ml). MTT assay results showed that ticagrelor inhibited the growth of human glioma cell lines U251 and LN229 in vitro in a time-dependent and concentration-dependent manner (Fig. 1A). According to the results of MTT assay, IC_{50} of ticagrelor treated U251 and LN229 human glioma cells for 48 h in vitro were 19.32 μg/ml and 20.25 μg/ml, respectively. The expression of Ki-67 was detected by immunofluorescence assay, which showed that ticagrelor significantly inhibited Ki-67 expression on U251 and LN229 cells compared with the control group (Fig. 1B and C). The quantification results of Ki-67 expression also showed that compared with the control group, the proportion of Ki-67 positive cells was significantly reduced after ticagrelor treatment ($p < 0.05$). These results indicate that ticagrelor can inhibit the proliferation of human glioma cell lines U251 and LN229 in vitro.

Next, we further investigated the effect of ticagrelor on glioma cell migration by wound healing assay and transwell assay. The results of wound healing assay showed that U251 glioma cells migrated significantly shorter distances from the edge to the midline of the wound after ticagrelor treatment compared with the control group after 24 h of wound healing assay (Fig. 1D) similar to the results in LN229 glioma cells (Fig. 1E). The results of the transwell assay indicated that the number of migrating U251 and LN229 glioma cells in the ticagrelor treatment group was significantly lower than that in the control group after 48 h (Fig. 1F and G). In addition, transwell assay was performed to investigate the effect of ticagrelor on glioma cell invasion. The results of the transwell assay indicated that after 48 h, the number of invasive U251 and LN229 glioma cells in the ticagrelor treatment group was significantly lower than that in the control group (Fig. 1F and G). These results indicate that ticagrelor can inhibit the migration and invasion of glioma cells in vitro.

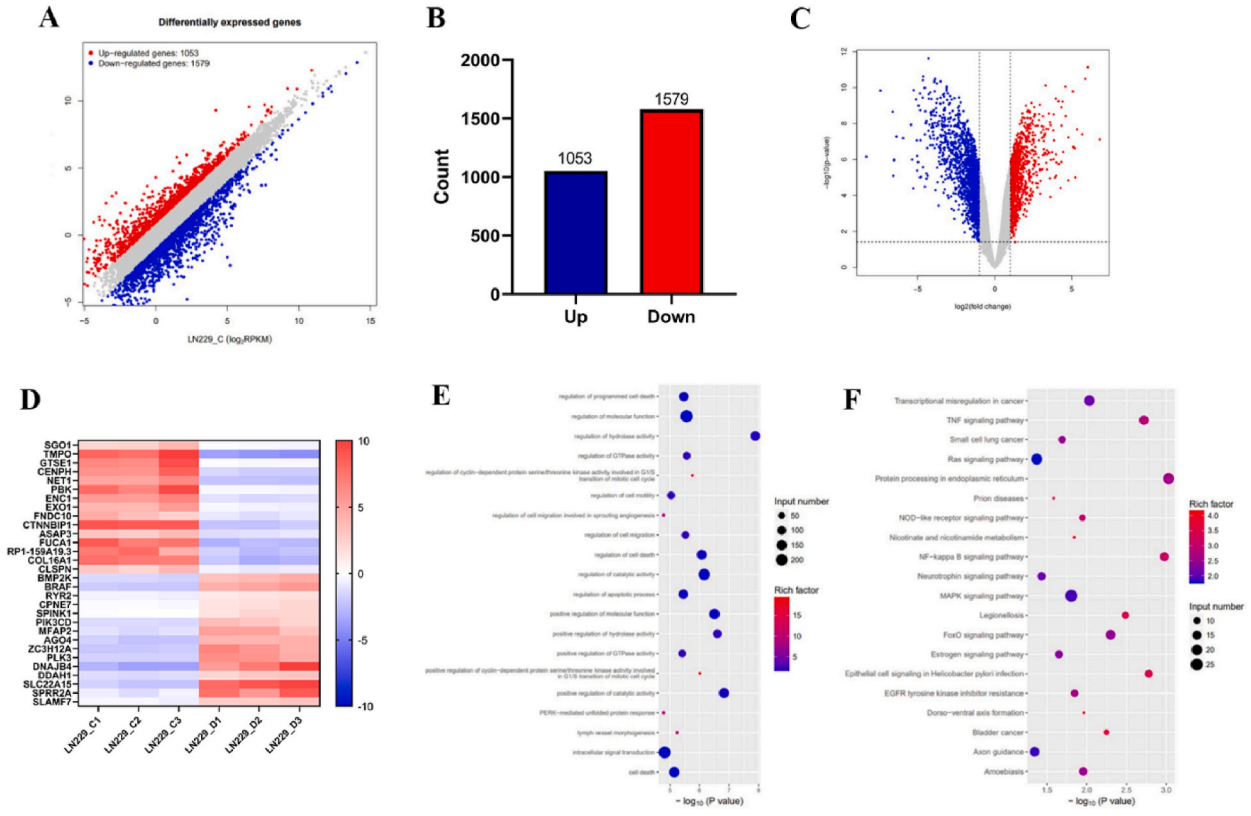


Fig. 2. GTSE1 is a therapeutic target of ticagrelor. A-B RNA-seq assay screened out differentially expressed genes. **C** Volcano plot of differentially expressed genes with differential expression ratios greater than value of fold change 1. **D** Heat map showed selected differentially expressed genes following ticagrelor treatment. **E** GO analysis showed enriched biological functions of differential genes. **F** KEGG analysis showed differential gene-enriched signaling pathways.

2.2. GTSE1 is a potential therapeutic target of ticagrelor

In order to identify the potential genes and signaling pathways involved in ticagrelor regulation, we performed RNA-seq assay on LN229 human glioma cell lines treated with ticagrelor and control group to explore the differential gene expression profiles and differential signaling pathways after ticagrelor treatment. The RNA-seq assay results showed that 2632 differentially expressed genes (1053 differentially expressed genes up-regulated and 1579 differentially expressed genes down-regulated) were present in the ticagrelor-treated group compared with the control group (Fig. 2A and B).

Next, the GO analysis and KEGG analysis were performed for differentially expressed genes. The results of GO analysis showed that genes involved in cell motility and cell migration function were significantly enriched in ticagrelor-treated groups among the 836 gene functions analyzed by GO significant enrichment (Fig. 2E). KEGG analysis showed that 16 molecular signaling pathways, including NF-kappa B and TNF signaling pathways, were significantly enriched in ticagrelor treated group compared with control group (Fig. 2F). In addition, both volcano maps and heat maps of differentially expressed genes showed that GTSE1 was significantly down-regulated in ticagrelor-treated LN229 glioma cells (Fig. 2C and D). Lin et al. found that GTSE1, as an oncogene, participates in the EMT process of tumors [18]. Therefore, we suppose that ticagrelor inhibits EMT process in glioma cells mainly by reducing the expression of the oncogene GTSE1 as the potential target gene.

2.3. Overexpression of GTSE1 is associated with poor clinical prognosis in glioma

To investigate clinical correlation between GTSE1 expression and prognosis of glioma patients, we evaluated the relative expression of GTSE1 in gliomas in the GEPIA bioinformatics database. We found that GTSE1 expression was higher in 518 low grade glioma (LGG) samples than that in 207 non-tumor brain tissue samples similar in 163 high grade glioma (HGG) samples. In addition, the level of GTSE1 was higher in HGG than in LGG (Fig. 3A). Based on these results of the database, we next validated them by qRT-PCR and Western blot in the collected clinical glioma samples. Our results showed that GTSE1 expression was higher in glioma tissues, which was also higher in HGG (Fig. 3B and C). Immunohistochemical staining supported the similar results in Fig. 3D. High-grade

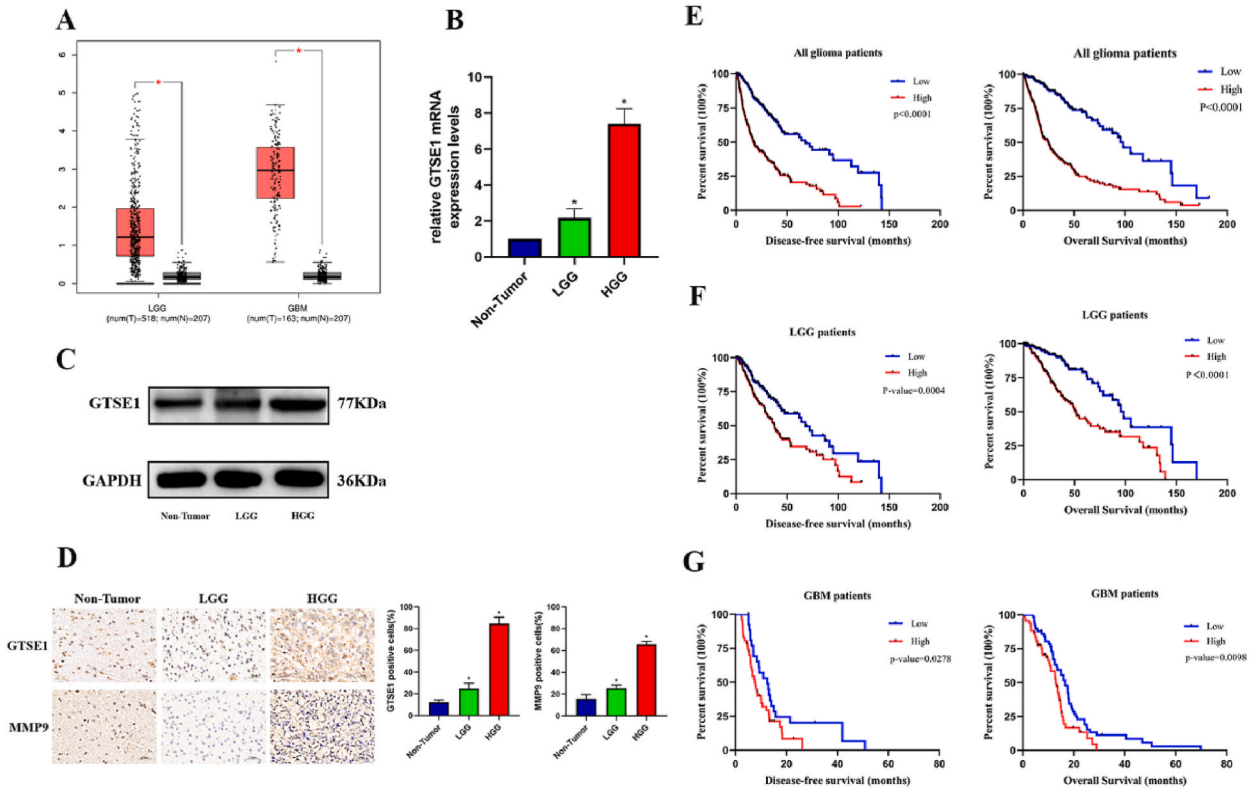


Fig. 3. Clinical data show that GTSE1 is highly expressed in gliomas and is associated with poor clinical prognosis. **A** The GEPIA database was used to analyze the expression level of GTSE1 in glioma. **B** The expression of GTSE1 mRNA in non-tumor and glioma tissues was analyzed by qRT-PCR. **C** The expression of GTSE1 in non-tumor and glioma tissues was analyzed by Western blot. **D** Representative images of immunohistochemical staining results for GTSE1 and MMP9 in different grades of glioma and non-tumor brain tissue. **E** Kaplan–Meier survival analysis of GTSE1 for all glioma patients (670 glioma tissue samples). **F** Kaplan–Meier survival analysis of GTSE1 for LGG glioma patients (516 LGG tissue samples). **G** Kaplan–Meier survival analysis of GTSE1 for GBM patients (154 GBM tissue samples). Error bars show mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. The experiments were repeated 3 times independently.

glioma tends to be highly aggressive, which is high risk for its poor prognosis [19]. And to this characteristic, the EMT process plays an important role, in which matrix metalloproteinase 9 (MMP9) is one of the key molecules. Immunohistochemical staining results suggested that MMP9 expression was higher in glioma tissues and similar in HGG (Fig. 3D). Meanwhile, we investigated the relationship between GTSE1 expression and the prognosis of glioma patients. Our results showed that the expression of GTSE1 was negatively correlated with the prognosis of glioma patients (Fig. 3E and F). These results indicated that highly expressed GTSE1 was correlated with tumor malignancy, which might be used as a prognostic biomarker for glioma patients.

2.4. Up-regulation of GTSE1 promotes EMT in glioma

Emerging evidence suggested that EMT was involved in tumor cell migration and invasion. Therefore, we determined the expression of E-cadherin, N-cadherin and vimentin, the key molecules in the EMT process. We assessed the changes of these key molecules by upregulating the expression of GTSE1 in glioma cells, and then explored the underlying mechanism. After GTSE1 overexpression plasmid constructed, we verified the efficiency of the plasmid by qRT-PCR and Western blot in vitro. The results showed that the relative level of GTSE1 in U251 glioma cells in the overexpression plasmid group was higher than that in the empty plasmid group (Fig. 4A). Similarly, the Western blot results showed that compared with the empty plasmid group, the trend of GTSE1 expression in U251 glioma cells transfected with GTSE1 overexpression plasmid group was consistent with that of GTSE1 RNA level (Fig. 4C). LN229 glioma cells were also treated in the same way, and the same trend was observed by qRT-PCR and Western blot (Fig. 4B and D).

Next, we investigated whether GTSE1 upregulation after ticagrelor treatment could promote the EMT process in glioma cells. After 48 h of ticagrelor treatment, U251 glioma cells were divided into two groups: one group was transfected with empty plasmid, and the other group was transfected with GTSE1 overexpression plasmid. Meanwhile, the expression of E-cadherin in U251 glioma cells

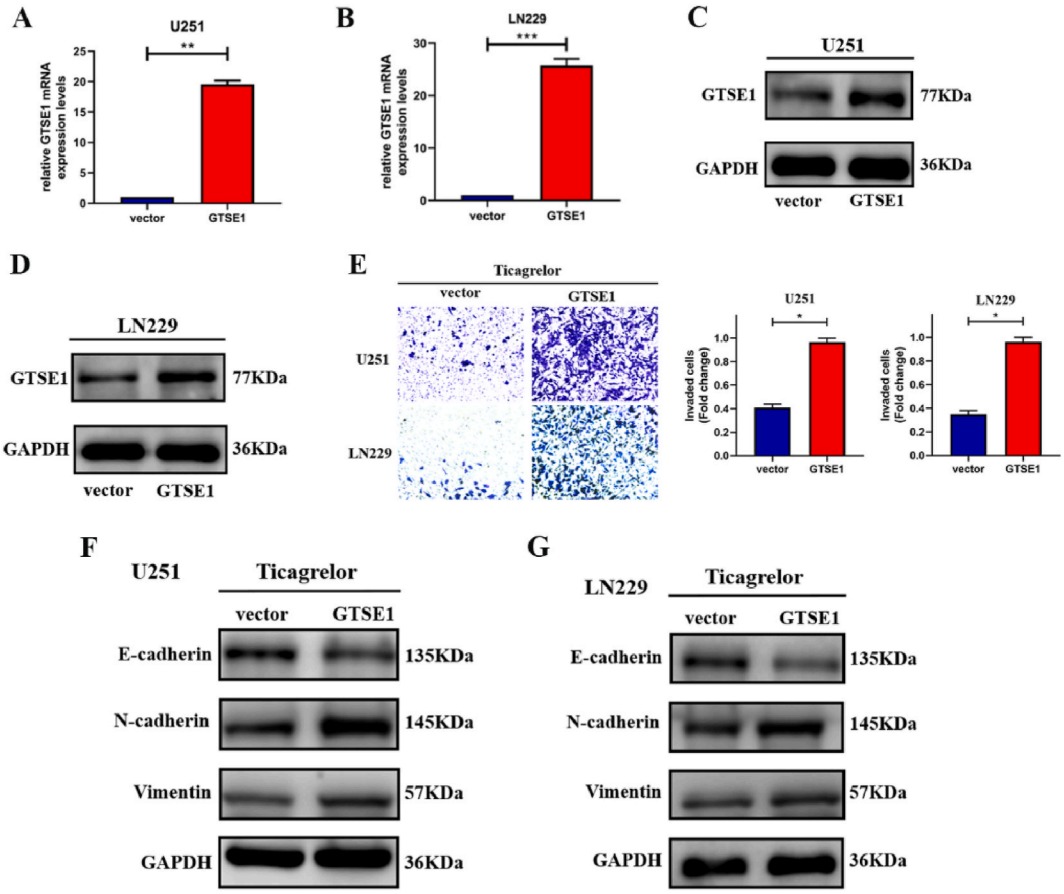


Fig. 4. Up-regulation of GTSE1 expression was able to promote glioma invasion and EMT under ticagrelor treatment. A-B QRT-PCR assay demonstrated relative expression levels of GTSE1 in GTSE1-overexpressing U251 and LN229 glioma cells. **C-D** Western blot demonstrated increased GTSE1 expression in U251 and LN229 glioma cells overexpressed with GTSE1. **E** Ticagrelor treatment of U251 and LN229 glioma cells for 48 h, up-regulation of GTSE1 expression can promote the invasion of U251 and LN229 glioma cells. **F-G** Western blot assays were performed to detect the expression levels of key molecular proteins during EMT. Error bars show mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001. The experiments were repeated 3 times independently.

transfected with GTSE1 overexpression plasmid group decreased, while the expression of N-cadherin and vimentin increased (Fig. 4F), similar in LN229 glioma cells (Fig. 4G).

We performed Transwell assay to verify whether up-regulation of GTSE1 expression could promote glioma cell invasion. After 48 h of ticagrelor treatment, U251 glioma cells were divided into two groups: one group was transfected with empty plasmid, and the other group was transfected with GTSE1 overexpression plasmid. The results suggested that the number of U251 glioma cells that invaded in the overexpression plasmid group was higher than that in the empty plasmid group after 48 h (Fig. 4E). In LN229 glioma cells, the number of invasive LN229 glioma cells in the GTSE1 overexpression plasmid group was also significantly higher than that in the empty plasmid group (Fig. 4E). These results suggested that ticagrelor inhibited glioma cell invasion by regulating the levels of key molecules during EMT by downregulation of GTSE1.

2.5. Inhibition of PI3K-Akt/NF-κB attenuates GTSE1-induced glioma invasion and EMT

Previous studies have shown that PI3K-Akt/NF-κB signaling pathway plays an important role in tumor proliferation and invasion. To further investigate the effect of PI3K-Akt/NF-κB signaling pathway on the efficacy of ticagrelor in the treatment of glioma, we treated U251 glioma cells with ticagrelor for 48 h into four groups with empty plasmid, GTSE1 overexpression plasmid, empty plasmid combined with PI-103 and GTSE1 overexpression plasmid combined with PI-103, respectively. The Western blot assay showed that

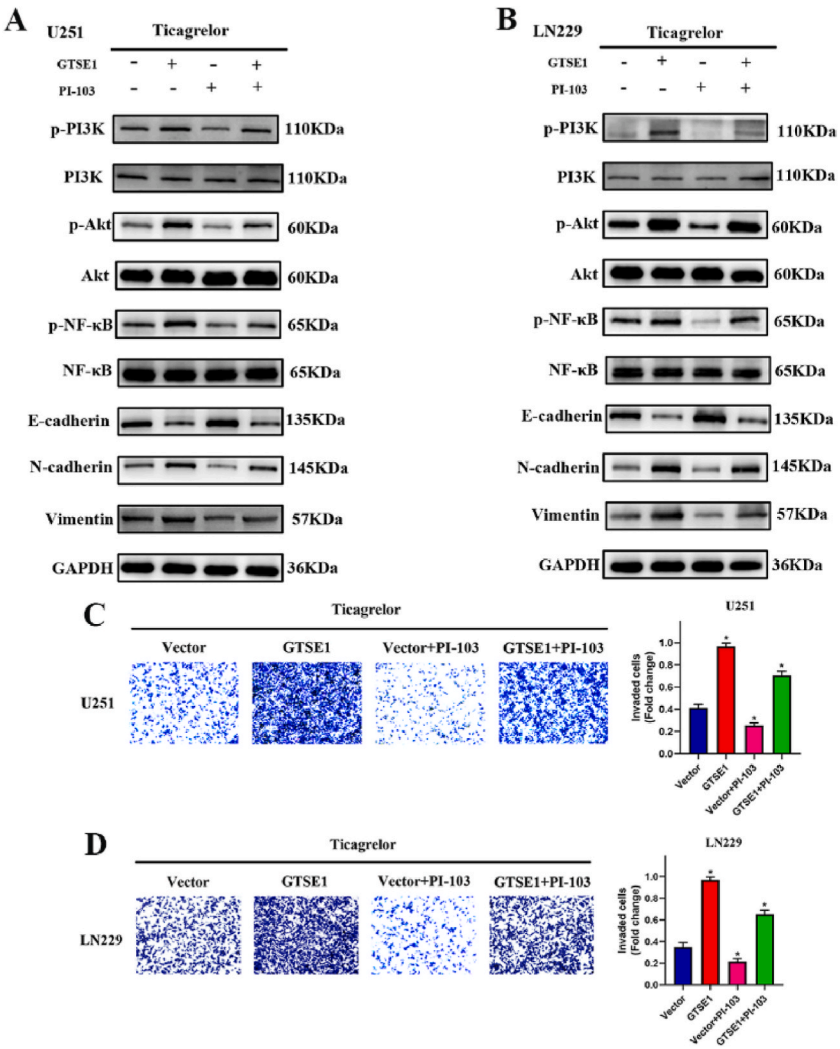


Fig. 5. Inhibition of PI3K-Akt/NF-κB attenuates GTSE1-induced glioma invasion and EMT under ticagrelor treatment. A-B Western blot assays showed the expression levels of key molecular proteins during EMT and PI3K-Akt/NF-κB signaling pathway. **C-D** Ticagrelor treatment of U251 and LN229 glioma cells for 48 h, inhibition of PI3K-Akt/NF-κB attenuates GTSE1-induced invasion of U251 and LN229 glioma cells. The experiments were repeated 3 times independently. Error bars show mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. The experiments were repeated 3 times independently.

compared with the GTSE1 overexpression plasmid group, the expression of E-cadherin in U251 glioma cells transfected with GTSE1 overexpression plasmid combined with PI-103 treatment group was increased, while the expression of N-cadherin and vimentin was decreased (Fig. 5A). Compared with the empty plasmid group, the expression of E-cadherin was increased, while the expression of N-cadherin and vimentin was decreased in U251 glioma cells transfected with the empty plasmid combined with PI-103 treatment group (Fig. 5A). Compared with the GTSE1 overexpression plasmid group, the expression of p-PI3K, *p*-Akt and p–NF–κB in U251 glioma cells transfected with GTSE1 overexpression plasmid combined with PI-103 were decreased (Fig. 5A). Compared with the empty plasmid group, the expression of p-PI3K, *p*-Akt and p–NF–κB in U251 glioma cells transfected with empty plasmid combined with PI-103 were decreased (Fig. 5A). LN229 glioma cells also received the similar treatment and results (Fig. 5B). The results of transwell assay showed that the number of invading U251 glioma cells in the GTSE1 overexpression plasmid group was more than that in the GTSE1 overexpression plasmid combined with PI-103 treatment group (Fig. 5C). The number of U251 glioma cells that invaded in the empty plasmid group was more than that in the empty plasmid combined with PI-103 treatment group (Fig. 5C). In LN229 glioma cells, more LN229 glioma cells developed invasion in the GTSE1 overexpression plasmid group than in the overexpression plasmid combined with PI-103 treatment group (Fig. 5D). The number of LN229 glioma cells that invaded in the empty plasmid group was more than that in the empty plasmid combined with PI-103 treatment group (Fig. 5D).

2.6. *Ticagrelor inhibits the growth of glioma in vivo*

To investigate the efficacy of ticagrelor therapy in vivo, we established an intracranial xenograft model of U251 glioma cell line in nude mice. The tumor-bearing nude mice were randomly divided into control group and drug treatment group. We found that the body weight loss showed no significant difference between the control group and the drug treatment group (Fig. 6A). Ticagrelor inhibited intracranial tumor growth compared with the control group (Fig. 6B). In addition, HE and immunohistochemical staining were performed on intracranial transplanted tumor tissues. Compared with the control group, the expression of GTSE1, vimentin, *p*-Akt, and p–NF–κB was decreased and the expression of E-cadherin was increased in the ticagrelor treatment group (Fig. 6C). The results of immunohistochemical staining were consistent with previous results of in vitro. More importantly, the survival time of nude mice in the ticagrelor treatment group was longer than that in the control group (Fig. 6D). Overall, these results support that ticagrelor can exert an anti-glioma effect in vivo.

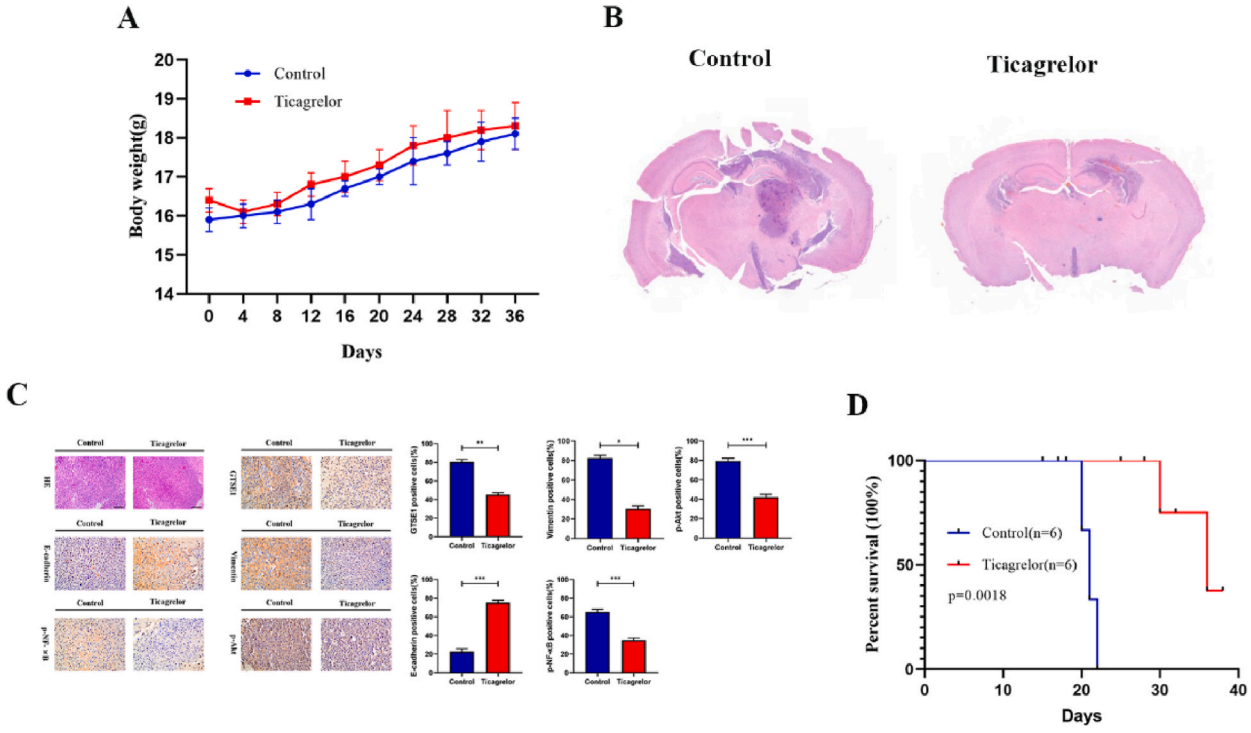


Fig. 6. Ticagrelor inhibits the growth of glioma in vivo. A Body weight change of tumor-bearing mice. **B** The tumor size of intracranial tumor mouse model. Scale bar represents 5000 μm. **C** HE and immunohistochemical staining results of GTSE1, E-cadherin, Vimentin, p–NF–κB and *p*-Akt. **D** The survival time of tumor-bearing mice. Error bars show mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001. The experiments were repeated 3 times independently.

3. Discussion

Cancer constitutes an enormous burden on society following with the continuous development of economy and human living environment [20]. Despite the emergence of many novel treatment modalities in tumor treatment, drug therapy still plays an important role in tumor treatment. Owing to breakthrough advances in the field of new drug development, such as metastatic melanoma and lung cancer, the prognosis of patients with these cancers is currently greatly improved compared with before20. Exploring new uses of existing drugs is a valuable strategy compared to developing a new drug. Glioma is the most common intracranial malignant tumor. Current drugs include temozolomide, lomustine and bevacizumab. However, the emergence of drug side effects and drug resistance leads to unsatisfactory compliance and treatment effect. Therefore, exploring new uses of existing drugs can provide solutions for the development of novel therapeutic strategy for glioma patients.

GTSE1 as an essential factor for cell mitosis in G2 and S phases is mainly located in the cytoplasm and is associated with cytoplasmic tubulin and microtubule activity during mitosis is located in microtubules [21]. It has been confirmed that the protein GTSE1 is mainly located in the cytoplasm and is associated with cytoplasmic tubulin and microtubule activity during mitosis [22]. Previous studies have suggested that GTSE1 is a key regulator of chromosome motility and spindle integrity during mitosis [23,24]. In a variety of tumors, overexpression of GTSE1 is involved in various malignant biological behaviors. Guo et al. found that in hepatocelleular carcinoma, GTSE1 could promote the malignant proliferation and metastasis of hepatocelleular carcinoma to reducing the 5-FU sensitivity [25]. Lin et al. found that GTSE1 activates the Akt pathway in a distinct p53 mutation-dependent manner and promotes proliferation, migration, and invasion of breast cancer cells [18]. Vinod et al. found that GTSE1 confers resistance to cisplatin in gastric cancer cells by inhibiting the transduction of p53 apoptotic signals in gastric cancer cells [26]. In this study, we found that ticagrelor could significantly inhibit the proliferation, migration and invasion of glioma cells in vitro by MTT assay, Ki67 immunofluorescence staining, wound healing assay and transwell assay. We performed RNA-seq analysis of ticagrelor-treated samples to further screen out the target gene of ticagrelor - GTSE1. Our findings demonstrate that ticagrelor inhibits the malignant biological behavior of glioma cells by decreasing GTSE1 expression, which reveals a critical role of GTSE1 in ticagrelor therapy.

The concept of EMT was first proposed in 1968 [27]. EMT is involved in a variety of pathological and physiological processes in vivo, tissue regeneration and embryonic development, and also regulates tumor stemness, tumorigenesis and malignant progression, tumor cell migration and invasion, tumor metastasis and therapeutic resistance [28,29]. EMT refers to the transition of epithelial cells from cell polarity and adhesion to mesenchymal cells with an enhanced invasive phenotype, which is also important in tumor progression and metastasis [30]. Malignant tumor progression usually consists of three steps, namely invasion, dissemination and metastasis, which are closely related to the classical EMT process [31]. When EMT is activated, E-cadherin expression decreases. In addition, the increase of N-cadherin and vimentin is also a key marker of the EMT process during tumor progression [32]. At present, EMT has been shown to occur in a variety of tumors, such as breast cancer, colorectal cancer and esophageal squamous cell carcinoma [33,34]. In gliomas, this highly invasive ability conferred by EMT leads to surgical inability to completely remove the tumor and contributes to the development of treatment resistance, ultimately leading to tumor recurrence [35]. In this study, we found that up-regulation of GTSE1 expression in glioma cells resulted in decreased expression of E-cadherin, while N-cadherin and vimentin expression were increased after glioma cells were treated with ticagrelor, indicating that EMT was activated. Transwell assays also showed that upregulation of GTSE1 enhanced the invasive ability of glioma cells treated with ticagrelor.

Many studies have confirmed that PI3K-AKT/NF-κB signaling pathway plays an important role in the occurrence and development of glioma. It has been reported that the expression of PI3K is significantly associated with the WHO grade and poor prognosis of glioma [36]. Activation of AKT can promote tumor metastasis [37]. The expression of AKT is positively correlated with the malignant degree of glioma, and inhibition of AKT can effectively inhibit the proliferation and invasion of glioma cells [38]. NF-κB is an important transcription factor in cancer, which is closely related to the occurrence and development of cancer [39,40]. A large number of studies have demonstrated that NF-κB is highly expressed in various solid tumors such as pancreatic cancer [41], breast cancer [42], hematological tumors and other tumor cells, and it can inhibit the apoptotic of tumor cells by inducing and up-regulating anti-apoptotic factors, while the abnormal expression of NF-κB is related to the degree of tumor differentiation and tumor category. In glioblastoma, EGFR can activate NF-κB [43] by linking PI3K-AKT signaling. In this study, we showed that inhibition of PI3K-AKT/NF-κB signaling pathway weakened GTSE1-induced glioma invasion and EMT under ticagrelor treatment.

Malignant glioma has a poor prognosis, and the limitation of treatment methods is the main reason why we performed this experiment, while ticagrelor is widely used in the treatment of acute coronary syndrome (ACS) and other diseases, and it should be found to have certain anti-cancer potential in subsequent follow-up studies, which is the main reason why we explore its anti-glioma. On the basis of this conjecture we performed phenotypic experiments, completed sequencing to find a new target, GTSE1, and explored its molecular mechanism of inhibiting EMT. However, the application of ticagrelor has a certain anticoagulant effect, which needs attention in the therapeutic effect of anti-glioma, and blood routine and coagulation items need to be monitored in the future treatment. However, its exact efficacy needs to be verified by further single-center or multicenter clinical trials, and whether it can enhance the effect of existing STUPP treatment also needs to be deeply explored. As a representative drug of P2Y12 inhibitors in clinical application, ticagrelor, has potential anti-glioma effects, then whether other inhibitors of P2Y12 have related anti-glioma effects also needs to be studied in depth. At the same time, studies have reported that hydroclopidogrel, as a representative of commonly used drugs to improve circulation, has different sensitivities of its receptors of action in Asian populations, so whether ticagrelor has the same situation needs to be verified by large-scale epidemiological data.

In conclusion, our study reveals a new potential mechanism for ticagrelor in the treatment of glioma. We found that ticagrelor inhibited proliferation and invasion of glioma cells in vivo and in vitro. Further mechanistic studies have shown that ticagrelor blocked the EMT process in glioma by inhibiting GTSE1 expression, a potential target of ticagrelor, which affected the PI3K-AKT/NF-κB

signaling pathway (Fig. 7). This study has some limitations. The in vivo model used in this study may not fully capture the complexity of human glioma. In future studies, clinically relevant models constructed using patient-derived xenografts would be beneficial. Additionally, exploring the potential combinatorial effects of ticagrelor with existing glioma treatments could offer valuable insights into developing more effective therapeutic strategies. Meanwhile, the safety of ticagrelor as a drug that has been widely used in clinical practice has been verified. Ticagrelor, as a circulatory drug, has a risk of bleeding in tumor inhibition. Monitoring blood routine and coagulation items may have a certain early warning effect for its clinical application. This study provides a novel theoretical basis and strategy for the clinical application of ticagrelor in the glioma treatment.

4. Materials and methods

4.1. Cell culture and chemical reagents

Human glioblastoma cell lines (U251, LN229 and HUVEC) were taken from China Infrastructure of Cell Line Resource (National Science and Technology Infrastructure,

NSTI). Dulbecco's modified Eagle's medium (DMEM; D6429, Sigma, USA) with 10 % fetal bovine serum (FBS; 16000-044, Gibco, USA) was used to culture glioblastoma cells in a 5 % CO_2 incubator at 37 °C. Ticagrelor (Cat#HY-10064, USA) and PI-103 (Cat#HY-10115, USA) were purchased from MedChemExpress (MCE, USA).

4.2. Clinical tissue samples collection

From January 2021 to January 2022, non-tumor brain tissues (N = 6), low-grade glioma (LGG; N = 6), and high-grade glioma (HGG; N = 6) were collected from surgical resection at the Department of Neurosurgery, First Affiliated Hospital of Harbin Medical University. This research was approved by the Ethics Committee of First Affiliated hospital of Harbin Medical University and performed in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent.

4.3. MTT assay

The glioma cell suspension (200 μl) of were seeded in each well of a 96-well plate at a density of 5000 cells per well. After the cells were treated with different experimental conditions, 10 μl of MTT solution (5 mg/ml) was added to each well, and then the cells were cultured in a cell incubator for 4 h. Then, the culture supernatant from each well was carefully aspirated, followed by the addition of 150 μl of DMSO to each well. Shake on a horizontal shaker for 10 min. Cell viability was evaluated by recording absorbance values of each well at a wavelength of 490 nm using a BioTek ELx800 (USA) microplate reader.

4.4. Immunofluorescence staining

After 10^5 glioma cells were treated with ticagrelor for 48 h, we fixed them with 1 ml of 4 % paraformaldehyde for 20 min. After glioma cells were permeabilize in 1 ml of Triton X-100 for 10 min, we blocked them with 5 % BSA solution for 30 min. Cells were incubated overnight at 4 °C in the Ki-67 primary antibody followed by 2 h in secondary antibody. Cells were incubated for 5 min in DAPI solution in the dark to stain nuclei and examined by the fluorescence microscope.

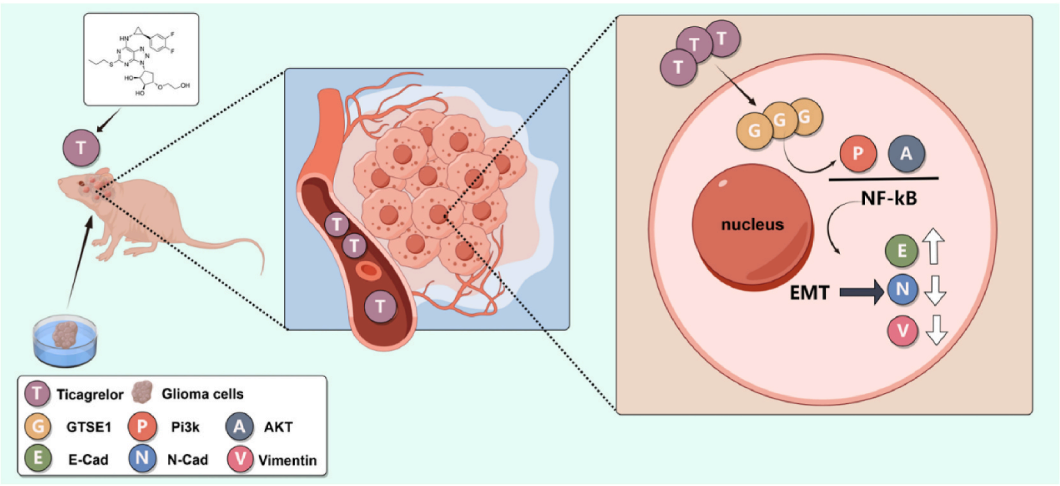


Fig. 7. Schematic diagram of the molecular mechanism of ticagrelor in the treatment of glioma.

4.5. Wound healing assay

Cells are inoculated at the appropriate density onto a six-well plate. Make a straight line scratch in the center of the plate with a pipette. The plates were gently rinsed with PBS buffer and cultured in a serum-free medium. After 0, 24, and 48 h, a microscope was used to observe and photograph the width of the scratch, which was subsequently measured by ImageJ v1.8.0.

4.6. Transwell assay

The 1×10^5 cells/well were inoculated in a 100 μL serum-free culture medium added into the upper transwell (Corning, CORNING, NY, USA). In the lower chamber of a 24-well plate, 600 μL of medium containing 10 % FBS was added. After 48 h, cells were fixed with 4 % paraformaldehyde for 30 min and stained by crystal violet (0.1 %). An inverted microscope was used to observe and record the number of cells migrating into the lower chamber.

4.7. RNA-sequencing (RNA-seq) and TCGA data analysis

Total RNA was isolated from those treated cells and untreated cells as well. Three biological replicates were mixed and provided for library construction and sequencing (RiboBio. Ltd). Briefly, libraries were constructed from polyadenylated RNAs and sequenced with an Illumina HiSeq 4000 on an SR-50 run aiming for 30 million reads per sample. Reads were aligned to the mm10 mouse transcriptome using TopHat. Significant genes were defined by the p value and false discovery rate of the cutoff of 0.05 and fold changes $\geq$1.2, and differential gene expression was subsequently analyzed using the DAVID bioinformatics platform and determined using Cuffdiff. Next, the GO analysis and KEGG analysis were performed for differentially expressed genes. The TCGA data used in this study were downloaded from https://cancergenome.nih.gov.

4.8. Western blotting

The cells were washed with cold PBS and then lysed with IP buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5 % NP-40, and 1 mM DTT), plus 1 × protease and phosphatase inhibitors (Roch). After centrifuge, soluble protein concentrations were determined using the BCA kit (Pierce). Proteins were applied to SDS-PAGE and transferred to nitrocellulose membranes, followed with primary antibodies incubation overnight at 4 °C, and HRP-conjugated secondary antibody (Cell Signaling Technology) for 2 h at room temperature. Chemiluminescence was determined using the Super Signal West Dura detection system (Thermo Scientific). Proteins were visualized and quantitated by the Bio-Rad ChemiDoc XRS system.

4.9. Immunohistochemical staining

The samples were embedded with paraffin and sliced into thick sections. Then sample sections were immunostained for GTSE1, MMP9, E-cadherin, Vimentin, p–NF–κB and *p*-Akt primary antibodies at 4 °C overnight and secondary antibodies at 37 °C for 30 min. The target protein in the tissue was observed under the microscope.

4.10. RNA extraction and quantitative real-time PCR

Total RNA of collected tissues were extracted with TRIzol RNA Isolation Reagents (Thermo-Invitrogen) and reversetranscribed with HiScript II Q RT SuperMix for qPCR (Vazyme). Real-time PCR was performed with FastStart Universal SYBR Green Master (Roche) and with different primer sets on QuantStudio 7 Flex Real Real-Time PCR System (Applied Biosystems). The primers used for real-time PCR are shown in Supplementary Data 1. The 2–ΔΔCt method was used to calculate relative expression changes.

4.11. Intracranial xenograft mouse model

The BALB/C nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). For orthotopic transplantation, the mice were divided into control and experimental groups. Serum-free DMEM (5 μl) containing 2×10^6 cells was injected into the mouse brain. The injection location was 2.5 mm to the right of the midline and 0.5 mm behind the coronal suture at a depth of 3.5 mm. All animal studies were approved by the Ethics Committee of First Affiliated Hospital of Harbin Medical University and performed in accordance with the principles of the Declaration of Helsinki.

4.12. Statistical analysis

Statistical analyses comparing data between groups were performed using Student's t-test or one-way ANOVA (Prism software version 7.0). $P < 0.05$, <0.01, <0.001 were denoted in graphs by different asterisks.

Ethical approval

This research was approved by the Ethics Committee of First Affiliated Hospital of Harbin Medical University (2022063) and

performed in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Enzhou Lu: Writing – original draft, Conceptualization. **Boxian Zhao:** Data curation. **Chao Yuan:** Validation, Formal analysis. **Yanchao Liang:** Visualization. **Xiaoxiong Wang:** Writing – review & editing, Supervision, Resources, Project administration. **Guang Yang:** Methodology, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments and funding

This work was supported by Distinguished Young Foundations of the First Affiliated Hospital of Harbin Medical University (HYD2020JQ0014 to GY), the National Natural Science Foundation of China, China (81902554 to XXW), Postdoctoral Science Foundation of Heilongjiang Province (LBHZ19190 to XXW), Young Medical Talent Foundation of the Frist Affiliated Hospital of Harbin Medical University (HYD2020YQ0018) and Innovative scientific Research Fund of Harbin Medical University(2021-KYYWF-0222).

References

[1] P.Y. Wen, D.A. Reardon, Neuro-oncology in 2015: progress in glioma diagnosis, classification and treatment, Nat. Rev. Neurol. 12 (2016) 69–70, https://doi.org/10.1038/nrneurol.2015.242.
[2] J.S. Barnholtz-Sloan, Q.T. Ostrom, D. Cote, Epidemiology of brain tumors, Neurol. Clin. 36 (2018) 395–419, https://doi.org/10.1016/j.ncl.2018.04.001.
[3] R. Stupp, M.E. Hegi, W.P. Mason, M.J. van den Bent, M.J. Taphoorn, R.C. Janzer, S.K. Ludwin, A. Allgeier, B. Fisher, K. Belanger, et al., Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial, Lancet Oncol. 10 (2009) 459–466, https://doi.org/10.1016/S1470-2045(09)70025-7.
[4] M.R. Gilbert, M. Wang, K.D. Aldape, R. Stupp, M.E. Hegi, K.A. Jaeckle, T.S. Armstrong, J.S. Wefel, M. Won, D.T. Blumenthal, et al., Dose-dense temozolomide for newly diagnosed glioblastoma: a randomized phase III clinical trial, J. Clin. Oncol. 31 (2013) 4085–4091, https://doi.org/10.1200/JCO.2013.49.6968.
[5] D.N. Louis, A. Perry, G. Reifenberger, A. von Deimling, D. Figarella-Branger, W.K. Cavenee, H. Ohgaki, O.D. Wiestler, P. Kleihues, D.W. Ellison, The 2016 world health organization classification of tumors of the central nervous system: a summary, Acta Neuropathol. 131 (2016) 803–820, https://doi.org/10.1007/s00401-016-1545-1.
[6] C. Horbinski, T. Berger, R.J. Packer, P.Y. Wen, Clinical implications of the 2021 edition of the WHO classification of central nervous system tumours, Nat. Rev. Neurol. 18 (2022) 515–529, https://doi.org/10.1038/s41582-022-00679-w.
[7] Q.T. Ostrom, H. Gittleman, J. Fulop, M. Liu, R. Blanda, C. Kromer, Y. Wolinsky, C. Kruchko, J.S. Barnholtz-Sloan, CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2008-2012, Neuro Oncol. 17 (Suppl 4) (2015) iv1–iv62, https://doi.org/10.1093/neuonc/nov189.
[8] P.G. Morris, A.B. Lassman, Medical oncology: optimizing chemotherapy and radiotherapy for anaplastic glioma, Nat. Rev. Clin. Oncol. 7 (2010) 428–430, https://doi.org/10.1038/nrclinonc.2010.98.
[9] M. Cattaneo, New P2Y(12) inhibitors, Circulation 121 (2010) 171–179, https://doi.org/10.1161/CIRCULATIONAHA.109.853069.
[10] A. Mitrugno, O.J.T. McCarty, Ticagrelor breaks up the tumor-platelet party, Blood 130 (2017) 1177–1178, https://doi.org/10.1182/blood-2017-07-795898.
[11] O. Elaskalani, A. Domenichini, N.B. Abdol Razak, E.D. D, M. Falasca, P. Metharom, Antiplatelet drug ticagrelor enhances chemotherapeutic efficacy by targeting the novel P2Y12-AKT pathway in pancreatic cancer cells, Cancers 12 (2020), https://doi.org/10.3390/cancers12010250.
[12] A.J. Gareau, C. Brien, S. Gebremeskel, R.S. Liwski, B. Johnston, M. Bezuhly, Ticagrelor inhibits platelet-tumor cell interactions and metastasis in human and murine breast cancer, Clin. Exp. Metastasis 35 (2018) 25–35, https://doi.org/10.1007/s10585-018-9874-1.
[13] M.S. Cho, K. Noh, M. Haemmerle, D. Li, H. Park, Q. Hu, T. Hisamatsu, T. Mitamura, S.L.C. Mak, S. Kunapuli, et al., Role of ADP receptors on platelets in the growth of ovarian cancer, Blood 130 (2017) 1235–1242, https://doi.org/10.1182/blood-2017-02-769893.
[14] I. Pastushenko, C. Blanpain, EMT transition states during tumor progression and metastasis, Trends Cell Biol. 29 (2019) 212–226, https://doi.org/10.1016/j.tcb.2018.12.001.
[15] I. Georgakopoulos-Soares, D.V. Chartoumpekis, V. Kyriazopoulou, A. Zaravinos, EMT factors and metabolic pathways in cancer, Front. Oncol. 10 (2020) 499, https://doi.org/10.3389/fonc.2020.00499.
[16] I. Paw, R.C. Carpenter, K. Watabe, W. Debinski, H.W. Lo, Mechanisms regulating glioma invasion, Cancer Lett. 362 (2015) 1–7, https://doi.org/10.1016/j.canlet.2015.03.015.
[17] Q. Xie, S. Mittal, M.E. Berens, Targeting adaptive glioblastoma: an overview of proliferation and invasion, Neuro Oncol. 16 (2014) 1575–1584, https://doi.org/10.1093/neuonc/nou147.
[18] F. Lin, Y.J. Xie, X.K. Zhang, T.J. Huang, H.F. Xu, Y. Mei, H. Liang, H. Hu, S.T. Lin, F.F. Luo, et al., GTSE1 is involved in breast cancer progression in p53 mutation-dependent manner, J. Exp. Clin. Cancer Res. 38 (2019) 152, https://doi.org/10.1186/s13046-019-1157-4.
[19] A. Giese, R. Bjerkvig, M.E. Berens, M. Westphal, Cost of migration: invasion of malignant gliomas and implications for treatment, J. Clin. Oncol. 21 (2003) 1624–1636, https://doi.org/10.1200/JCO.2003.05.063.
[20] R.L. Siegel, K.D. Miller, H.E. Fuchs, A. Jemal, Cancer statistics, 2022, CA Cancer J Clin 72 (2022) 7–33, https://doi.org/10.3322/caac.21708.
[21] X.S. Liu, H. Li, B. Song, X. Liu, Polo-like kinase 1 phosphorylation of G2 and S-phase-expressed 1 protein is essential for p53 inactivation during G2 checkpoint recovery, EMBO Rep. 11 (2010) 626–632, https://doi.org/10.1038/embor.2010.90.
[22] S. Jiang, Z. Yang, S. Di, W. Hu, Z. Ma, F. Chen, Y. Yang, Novel role of forkhead box O 4 transcription factor in cancer: bringing out the good or the bad, Semin. Cancer Biol. 50 (2018) 1–12, https://doi.org/10.1016/j.semcancer.2018.04.007.
[23] M. Monte, R. Benetti, G. Buscemi, P. Sandy, G. Del Sal, C. Schneider, The cell cycle-regulated protein human GTSE-1 controls DNA damage-induced apoptosis by affecting p53 function, J. Biol. Chem. 278 (2003) 30356–30364, https://doi.org/10.1074/jbc.M302902200.

[24] H. Lee, J. Palm, S.M. Grimes, H.P. Ji, The Cancer Genome Atlas Clinical Explorer: a web and mobile interface for identifying clinical-genomic driver associations, Genome Med. 7 (2015) 112, https://doi.org/10.1186/s13073-015-0226-3.
[25] L. Guo, S. Zhang, B. Zhang, W. Chen, X. Li, W. Zhang, C. Zhou, J. Zhang, N. Ren, Q. Ye, Silencing GTSE-1 expression inhibits proliferation and invasion of hepatocellular carcinoma cells, Cell Biol. Toxicol. 32 (2016) 263–274, https://doi.org/10.1007/s10565-016-9327-z.
[26] V.V. Subhash, S.H. Tan, W.L. Tan, M.S. Yeo, C. Xie, F.Y. Wong, Z.Y. Kiat, R. Lim, W.P. Yong, GTSE1 expression represses apoptotic signaling and confers cisplatin resistance in gastric cancer cells, BMC Cancer 15 (2015) 550, https://doi.org/10.1186/s12885-015-1550-0.
[27] R. Fleischmajer, Epithelial-mesenchymal interactions, Science 157 (1967) 1472–1482, https://doi.org/10.1126/science.157.3795.1472.
[28] M.A. Nieto, R.Y. Huang, R.A. Jackson, J.P. Thiery, Emt: 2016, Cell 166 (2016) 21–45, https://doi.org/10.1016/j.cell.2016.06.028.
[29] B. De Craene, G. Berx, Regulatory networks defining EMT during cancer initiation and progression, Nat. Rev. Cancer 13 (2013) 97–110, https://doi.org/10.1038/nrc3447.
[30] M. Zeisberg, E.G. Neilson, Biomarkers for epithelial-mesenchymal transitions, J. Clin. Invest. 119 (2009) 1429–1437, https://doi.org/10.1172/JCI36183.
[31] H. Hugo, M.L. Ackland, T. Blick, M.G. Lawrence, J.A. Clements, E.D. Williams, E.W. Thompson, Epithelial–mesenchymal and mesenchymal–epithelial transitions in carcinoma progression, J. Cell. Physiol. 213 (2007) 374–383, https://doi.org/10.1002/jcp.21223.
[32] B. Fan, B. Su, G. Song, X. Liu, Z. Yan, S. Wang, F. Hu, J. Yang, miR-363-3p induces EMT via the Wnt/beta-catenin pathway in glioma cells by targeting CELF2, J. Cell Mol. Med. 25 (2021) 10418–10429, https://doi.org/10.1111/jcmm.16970.
[33] X. Ye, T. Brabletz, Y. Kang, G.D. Longmore, M.A. Nieto, B.Z. Stanger, J. Yang, R.A. Weinberg, Upholding a role for EMT in breast cancer metastasis, Nature 547 (2017) E1–E3, https://doi.org/10.1038/nature22816.
[34] H. Li, M. Rokavec, L. Jiang, D. Horst, H. Hermeking, Antagonistic effects of p53 and HIF1A on microRNA-34a regulation of PPP1R11 and STAT3 and hypoxia-induced epithelial to mesenchymal transition in colorectal cancer cells, Gastroenterology 153 (2017) 505–520, https://doi.org/10.1053/j.gastro.2017.04.017.
[35] T. Eisemann, B. Costa, J. Strelau, M. Mittelbronn, P. Angel, H. Peterziel, An advanced glioma cell invasion assay based on organotypic brain slice cultures, BMC Cancer 18 (2018) 103, https://doi.org/10.1186/s12885-018-4007-4.
[36] A. Chakravarti, G. Zhai, Y. Suzuki, S. Sarkesh, P.M. Black, A. Muzikansky, J.S. Loeffler, The prognostic significance of phosphatidylinositol 3-kinase pathway activation in human gliomas, J. Clin. Oncol. 22 (2004) 1926–1933, https://doi.org/10.1200/JCO.2004.07.193.
[37] L. Huang, X.O. Zhang, E.J. Rozen, X. Sun, B. Sallis, O. Verdejo-Torres, K. Wigglesworth, D. Moon, T. Huang, J.P. Cavaretta, et al., PRMT5 activates AKT via methylation to promote tumor metastasis, Nat. Commun. 13 (2022) 3955, https://doi.org/10.1038/s41467-022-31645-1.
[38] D.D. Sarbassov, D.A. Guertin, S.M. Ali, D.M. Sabatini, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex, Science 307 (2005) 1098–1101, https://doi.org/10.1126/science.1106148.
[39] E. Pikarsky, R.M. Porat, I. Stein, R. Abramovitch, S. Amit, S. Kasem, E. Gutkovich-Pyest, S. Urieli-Shoval, E. Galun, Y. Ben-Neriah, NF-kappaB functions as a tumour promoter in inflammation-associated cancer, Nature 431 (2004) 461–466, https://doi.org/10.1038/nature02924.
[40] L. Huang, M. Ravi, X.O. Zhang, O. Verdejo-Torres, N.A.M. Shendy, M.A.M. Nezhady, S. Gopalan, G. Wang, A.D. Durbin, T.G. Fazzio, Q. Wu, PRMT5 orchestrates EGFR and AKT networks to activate NFkappaB and promote EMT, bioRxiv (2024), https://doi.org/10.1101/2024.01.03.574104.
[41] A. Liu, H. Chen, H. Tong, S. Ye, M. Qiu, Z. Wang, W. Tan, J. Liu, S. Lin, Emodin potentiates the antitumor effects of gemcitabine in pancreatic cancer cells via inhibition of nuclear factor-kappaB, Mol. Med. Rep. 4 (2011) 221–227, https://doi.org/10.3892/mmr.2011.414.
[42] H.S. Ko, H.J. Lee, S.H. Kim, E.O. Lee, Piceatannol suppresses breast cancer cell invasion through the inhibition of MMP-9: involvement of PI3K/AKT and NF-kappaB pathways, J. Agric. Food Chem. 60 (2012) 4083–4089, https://doi.org/10.1021/jf205171g.
[43] G.S. Kapoor, Y. Zhan, G.R. Johnson, D.M. O'Rourke, Distinct domains in the SHP-2 phosphatase differentially regulate epidermal growth factor receptor/NF-kappaB activation through Gab1 in glioblastoma cells, Mol. Cell Biol. 24 (2004) 823–836, https://doi.org/10.1128/MCB.24.2.823-836.2004.