

Preclinical evidence of STAT3 inhibitor, Pacritinib, overcomes temozolomide resistance via down-regulating miR-21-enriched exosomes from M2 glioblastoma-associated macrophages

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Abstract

Background: Tumor microenvironment (TME) plays a crucial role in virtually every aspect of tumorigenesis of glioblastoma multiforme (GBM). The dysfunctional TME promotes drug resistance, disease recurrence and distant metastasis. Recent evidence indicates that exosomes released by stromal cells within TME may promote oncogenic phenotypes via transferring signaling molecules such as cytokines, proteins and microRNAs.

Results: In this study, clinical GBM samples were collected and analyzed. We found that GBM-associated macrophages (GAMs) secreted exosomes which were enriched with oncomiR-21. Co-culture of GAMs (and GAM derived exosomes) and GBM cell lines resulted in the increased GBM cells' resistance against temozolomide (TMZ) by upregulating pro-survival gene, PDCD4 and stemness markers Sox2, STAT3, Nestin and miR-21-5p and increased M2 cytokines, IL-6 and TGF- β 1 secreted by GBM cells, promoting the M2 polarization of GAMs. Subsequently, pacritinib treatment suppressed GBM tumorigenesis and stemness; more importantly, pacritinib-treated GBM cells showed markedly reduced ability to secrete M2 cytokines and reduced miR-21 enriched exosomes secreted by GAMs. Pacritinib-mediated effects were accompanied by a reduction of oncomiR miR-21-5p, by which tumor suppressor PDCD4 was targeted. We subsequently established a patient-derived xenograft models where mice bore patient GBM and GAMs. The treatment of pacritinib, and the combination of pacritinib/TMZ appeared to significantly reduce tumorigenesis of GBM/GAM PDX mice, overcome TMZ-resistance, and M2 polarization of GAMs.

Conclusion: In summation, we showed that potential of pacritinib alone or in combination with TMZ for suppressing GBM tumorigenesis via modulating STAT3/miR-21/PDCD4 signaling. Further investigations are warranted for adopting pacritinib for the treatment of TMZ-resistant GBM in the clinical settings.

Keywords: Tumor microenvironment (TME), glioblastoma multiforme (GBM), GBM-associated macrophages (GAMs), exosomes, oncomiR-21, STAT3 inhibitor.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive brain tumor of glial origin, with a poor median survival of 14 months[1]. One of the reasons for its malignancy and challenging for therapeutics development lies in the heterogeneous nature at the cellular and molecular levels. It is now generally recognized that GBM is composed of a subpopulation of glioma stem cells (GSCs), capable of tumor initiation, progression self-renewal upon treatments, and other cells within the tumor microenvironment (TME). TME contains cancerous cells surrounded with parenchymal cells, including endothelial/vascular cells, microglia, immune cells[2]. One of the major cell types from the GBM TME is glioma associated macrophages (GAMs). GAMs have been shown to contribute to the progression of GBM. For instance, the presence of M2 GAMs promoted the growth and metastasis of GBM cells [2,3].

More importantly, emerging evidence indicates the dynamic intercellular communications within the GBM TME via secretions of cytokines, chemicals, signaling molecules. Among these, secreted exosomes represents a class of small bilayered particles (ranging 50–150 nm in diameter) that have been extensively explored for their roles in GBM tumorigenesis for the past few years [4]. Recent studies have shed lights on the diverse functions of exosomes involved in GBM tumorigenesis. For instance, exosomes released from human GBM cell lines contain various types of heat shock proteins and TGF- β 1 proposed to exert immune suppressive roles in GBM [5]. In addition, serum-derived exosomes from patients of GBM and CSF-derived exosomes, were shown to contain high level of miR-221, serving as a potential GBM biomarker [6]. A recent study demonstrated that microglia also communicate and affect the function of glioma via the release of exosomes [7]. These findings suggest that there is a potential area for therapeutics development via the interrupting the intracellular communications between GBM cells and its TME, by means of exosomes. However, the role of exosomes derived from M2 GAMs has not fully appreciated.

In this study, we first demonstrated that when human GBM cell lines co-cultured with clinically isolated GAMs, a significantly enhanced ability of colony formation and tumor sphere generation, in association with increased expression of Sox2, STAT3, IL-6 and Nestin while decreased in GFP. Subsequently, exosomes released into the culture medium of GAMs were isolated and co-cultured with GBM cell lines. A similar increased tumorigenic property was observed in addition to the increased resistance against temozolomide (TMZ). More importantly, miR-21, an oncomiR, was identified as the most abundant microRNA species in the exosomes released from the GAMs. We then provided evidence for the positive association between the level of miR-21 and GBM malignancy; exogenously increased miR-21 in GBM cells increased their ability to polarize GAMs towards M2 phenotype and the reduction of miR-21 reversed these properties. In addition, we showed that miR-21-mediated oncogenic properties were associated with its targeting/inhibitory function on PDCD4 (a tumor suppressor). An increased miR-21 level in the GBM cells led to their increased ability to polarize GAMs towards M2 phenotype by the increased secretion of M2 cytokine, IL-6 and TGF- β 1.

Subsequently, we examined the feasibility of applying pacritinib, an inhibitor of STAT3-associated pathway, as an anti-GBM agent. We showed that pacritinib treatment significantly reduced the cell viability, colony/tumor sphere formation in association with reduced level of STAT3, Sox2, PDCD4 and miR-21, as well as reduced ability to generate M2 GAMs. Notably, pacritinib treated GAMs released less amount of miR-21

enriched exosomes. Finally, we demonstrated the preclinical support for using pacritinib to overcome TMZ-resistance using PDX model.

Materials and Methods

Sample collection and cell culture

Tumor sample and stromal GAMs were collected from our department of neurosurgery, Taipei Medical University-Shuang Ho Hospital, under the strict adherence of IRB (approval number: IRB: N201801070) where the patients were fully informed and written consent form was signed prior to the operation. The pathological examination was performed by the department of pathology and verified all cases met the criteria of GBM. Samples (tumor samples and stromal cells) were isolated and cultured according previously established protocols [8,9]. Human GBM cell lines U87MG, LN18 were obtained from American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS. Neurospheres from both cell lines and clinical samples were generated using sphere forming medium containing growth factors supplemented DMEM-F12 1:1 medium, as previously described [10]. For co-culture experiments, a previously established protocol was followed with minor modifications [11]. In brief, U87MG and LN18 (2×10^5 cells) were seeded in a transwell insert (0.4µm pore size) with GAMs (2.5×10^5 cells) seeded in the lower chamber of a 6-well system. Cells were cultured in DMEM medium as described above. Cells were maintained for 48h and harvested for further analyses. In the case of exosome co-culture, GBM cells were cultured in serum-free DMEM in the presence of exosomes for 48h and harvested.

Transfection

In order to explore the functional roles of miR-21 in GBM cells, the up-regulation or down-regulation of miR-21 was achieved using mimic and inhibitor respectively. MiR-21-5p mimic (HMI0372, Sigma, China) and inhibitor (HSTUD0371, Sigma, China) were transfected into GBM cells according using Lipofectamine 2000 reagent (Invitrogen, USA) according to the vendor's instructions. The change in the expression of miR-21-5p was then determined by real-time PCR 48h post transfection in both GBM cell lines. hsa-miR-21-5p primers (MPH02337, Abm, USA) were purchased and used for qPCR experiments.

Exosome Isolation

Glioma-associated macrophages (GAMs) were cultured in serum-free medium for 48 hours (with and without pacritinib treatment, 0.5 µM) before exosome isolation. Culture medium collected and a standard procedure was performed accordingly [12]. In short, we carried out a serial centrifugation procedure (500 g for 10 min, 1200 g for 20 min, and 10,000 g for 30 min) followed by filtration with a 0.22-µm pore syringe, and a spin at 100,000 g for 60 min. The pellet collected was washed in PBS three times before another ultracentrifugation at 100,000 g for 60 min. The exosomes were used for further analyses. A small portion of the pellet was processed for transmission electron microscopic examination. In brief, purified exosomes were fixed with 1% glutaraldehyde (1 h, room temperature), washed and followed by 1% reduced osmium tetroxide fixation (1 h). The sample was washed, stained with 0.3% thiocarbohydrazide and fixed again in OsO₄. Finally, the sample was embedded into Epon. Ultrathin sections were placed on formvar-coated grids. EM analysis was performed as previously described [13] using anti-CD63 antibody.

miRNA PCR array analysis

Total RNA (200 ng) isolated from exosomes derived from GAMs was transcribed to cDNA using miScript II RT kit (Qiagen, USA) using protocol provided by the vendor. The miRNA PCR array (Qiagen, China) was used for profiling by the instructions provided.

Real-time PCR

Total RNAs were extracted, purified and reverse-transcribed using RNeasy kit (Qiagen, China) and OneStep RT-PCR Kit (Qiagen, China). Real time PCR (RT-PCR) was performed using an I-Cycler IQ Multicolor RT-PCR Detection System (Bio-Rad) with SsoFast Eva Green Supermix (Bio-Rad). All experimental Ct values were normalized against the Ct value of internal control, GAPDH. Relative abundance was determined by $2^{-\Delta\Delta Ct}$ and expressed as fold changes. Primer sequences were listed in **Supplementary Table 1**.

SDS-PAGE and western blotting

A standard SDS-PAGE and western blotting was carried out according previously established protocols [14]. Primary antibodies used in this study were all purchased from AbCam (Shanghai, China) unless otherwise specified. Anti-STAT3 (ab119352, 1:1500); anti-IL-6 (ab6672, 1:500); anti-Sox2 (ab93689, 1:800); anti-Nestin (ab105389, 1:800); anti-CD9 (ab92726, 1:400); anti-CD63 (ab217345, 1:400); anti-CD81 (ab79559, 1:400); anti-actin (ab179467, 1:2000); anti-tubulin (ab6046, 1:1000).

In vivo patient-derived xenograft model

A tumor sample from a GBM patient with TMZ resistance was used to establish PDX mouse model for in vivo evaluation according to previously established protocol [15]. In brief, NOD/SCID mice were anaesthetized (10mg/kg, ketamine/xylazine, and buprenorphine, 0.05 mg/kg, before and after injection). GBM cells (5×10^5 cells) were stereotactically injected into the right striata of the mice. One-week post-injection, mice were randomly divided into vehicle, pacritinib (100 mg/kg, 5 times/week), TMZ (30 mg/kg, 5 times/week) group, or the combination of pacritinib (100 mg/kg) and TMZ (30 mg/kg) group. Both drugs were administered via oral gavage. Mice were humanely sacrificed by sodium pentobarbital at the end of experiments. The presence of tumor and size were determined in the mice via necropsy and cranial dissection. Tumor samples were harvested for further analyzes. The tumor size (average area) was determined from the cross-sections of tumor samples. Image J software was used for calculating the tumor size. The animal study protocol was approved by the Animal Care and User Committee at Taipei Medical University (Affidavit of Approval of Animal Use Protocol # Taipei Medical University- LAC-2017-0512).

Statistical analysis

The miRNA expression levels from the array experiments was analyzed by SDS software version 2.2.2 (Applied Biosystems). The delat Ct values were calculated against U6 internal control. Heatmap of differentially expressed miRNAs were analyzed by R software. Other data was analyzed using Student's t-tests for the determination of statistical significance among different groups. P-values (represented by asterisks) where *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Results

M2-polarization of Glioma associated macrophages (GAMs) promotes GBM tumorigenesis

Initially, we co-cultured clinically isolated GAMs with human GBM cell lines, U87MG and LN18 and showed that increased colony (Fig. 1A) and neurosphere (Fig. 1B) forming abilities. Consistently, qPCR analysis demonstrated that the presence of GAMs was associated with an increased mRNA level of stemness markers (Fig. 1C). The results from western blots were consistent where the expression of Sox2, Wnt, STAT3 and Nestin were elevated while GFAP was decreased in the presence of GAMs (Fig. 1D).

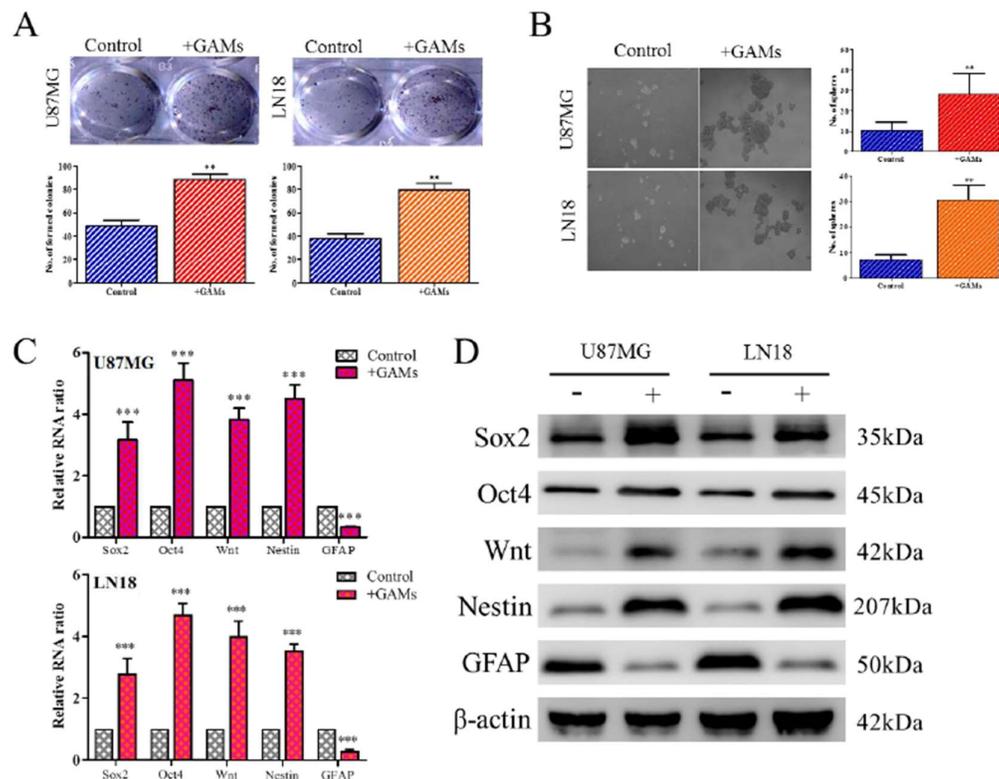


Figure 1 M2 GAMs promotes GBM tumorigenesis. GBM cells U87MG and LN18 co-cultured with M2 GAMs showed a significantly increased colony forming ability (A) and tumor sphere generating ability (B) as compared to their parental controls. Comparative real-time PCR (C) and western blots (D) showed that M2 GAM co-cultured GBM cells expressed a significantly higher level of stemness markers, Sox2, STAT3, Wnt, and Nestin while GFAP was reduced. ** $p < 0.01$; *** $p < 0.001$.

Exosome enriched with miR-21 from GAMs promotes tumorigenic properties

To further investigate the underlying tumorigenesis by isolating and characterizing exosomes secreted into the culture medium by GAMs. First, we used different markers for exosomes, CD9, CD63 and CD81, to verify the identity of the exosomes isolated from the GAMs (Fig. 2A). Next, we showed that incubation of GAM-derived exosomes significantly increased TMZ resistance in both U87MG and LN18 cells (Fig. 2B). For example, the estimated IC50 value for U87MG increased approximately 4-fold after the incubation with GAM-derived exosomes while even more significantly in the LN18 cells post exosome treatment. This increased TMZ resistance was accompanied by the increased colony forming (Fig. 2C) and tumor sphere forming (Fig. 2D) abilities. We

then screened a small cohort of microRNAs in two batches of GAM-derived exosomes and found that miR-21 appeared to be the most abundant microRNA (Fig. 2E). As shown in the heatmap, miR-21 level appeared to be the most enriched in the exosomes collected from two samples of GAMs.

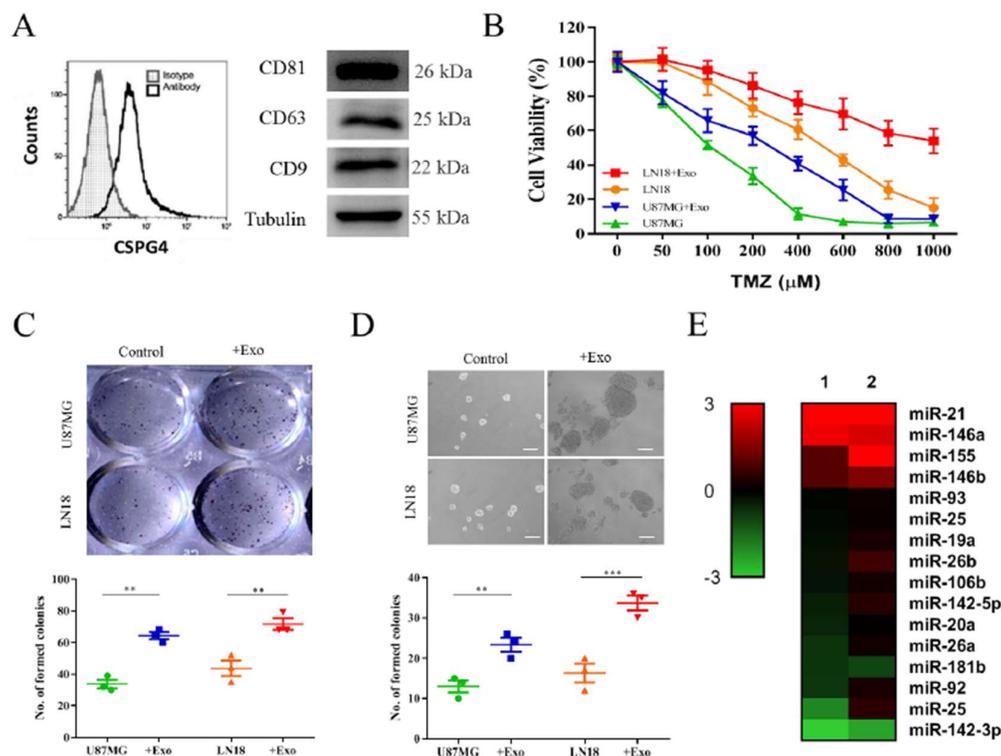


Figure 2 GAM secreted miR-21 enriched exosomes promoted GBM tumorigenesis. (A) Representative transmission electronic micrograph of exosomes isolated from clinical GAMs (left); western blot validation of exosomes isolated from GAM culture medium showed the expression of CD9, CD63 and CD81. (B) Increased TMZ resistance in U87MG and LN18 cells co-cultured with exosomes (+exo). Enhanced colony forming ability (C) and tumor sphere generating ability (D) in the presence GAM-derived exosomes. (E) MicroRNA profiling analyses showed that exosomes (two samples) isolated from M2 GAMs contained a high level of miR-21. ** $p < 0.01$; *** $p < 0.001$.

MiR-21 is associated with GBM tumorigenic properties

Next, we examined the effects of miR-21 on GBM cells by gene silencing and overexpression techniques. First, we demonstrated that an increased miR-21 level in both U87MG and LN18 cells post co-cultured with GAM-derived exosomes (Fig. 3A). We then transfected GBM (post-incubation with GAM exosomes) with either mimic or inhibitor molecules of miR-21-5p. We found that the stemness markers Sox2, STAT3, Wnt and Nestin were all significantly increased when mimic miR-21-5p was added to both cells while the opposite occurred after the level of miR-21-5p was inhibited (Fig. 3B). In support, the results from western blots agreed with the real-time PCR results (Fig. 3C) where an increased level of miR-21-5p led to the increased expression of Sox2, STAT3, Nestin and Wnt and a decreased level of GFAP. More importantly, tumorigenic properties such as colony formation and tumor sphere formation were also positively correlated with the level of miR-21-5p. For instance, an increased miR-21-5p level by mimic molecules led to the increased number of colonies (Fig. 3D) and neurospheres (Fig. 3E) generated and the opposite was true with the decreased level of

miR-21-5p with the inhibitor treatment. Furthermore, miR-21-5p mimic transfection made both U87MG and LN18 cells more resistant against TMZ whereas miR-21-5p inhibitor reversed the resistance (Fig. 3F).

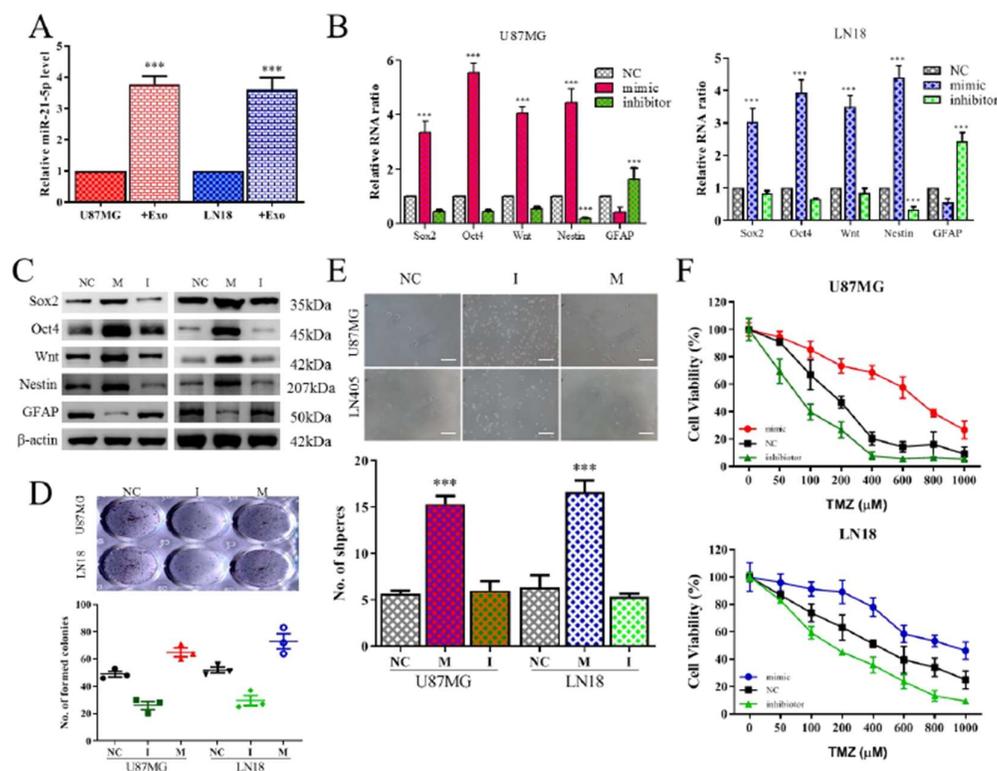


Figure 3 GAM-derived exosomes promoted GBM tumorigenesis via miR-21-5p. (A) U87MG and LN18 cells incubated with GAM-derived exosomes showed a significantly increased level of miR-21-5p. GBM tumorigenesis was associated with miR-21-5p. Increased miR-21-5p level (by mimic molecules) in GBM cells showed an increased mRNA level of Sox2, STAT3, Wnt and Nestin while decreased GFAP while a decreased in miR-21-5p (inhibitor) led to the opposite phenomenon (B, C). Incubation with GAM-derived exosomes increased colony forming ability (D) and tumor sphere generating ability (E) in both U87MG and LN18 cells. (F) U87MG and LN18 cells transfected miR-21-5p mimic molecules (increased miR-21-5p level) resulted in a significantly increased TMZ resistance while reduced miR-21-5p with decreased TMZ resistance. ** $p < 0.01$; *** $p < 0.001$.

STAT3 and PDCD4 were target of miR-21-5p

Subsequently, we examined the potential target(s) for miR-21-5p using bioinformatics tools and we identified STAT3, a well-known oncogene and PDCD4, an established tumor suppressor, as the top-ranking candidates from all three algorithms used (PITA, miRmap and miRanda). A potential site of interaction between miR-21-5p and Stat3, PDCD4 were identified in the 3'UTR (upper panel, Fig. 4A); more importantly, based on TCGA database, a strong negative correlation between the expression level of PDCD4 and miR-21-5p was established within a cohort of GBM patients (N=525, lower panel, Fig. 4B). We then demonstrated that increased miR-21-5p by mimic molecules in both U87MG and LN18 cells supported the negative correlation between the expression of miR-21-5p and PDCD4. Conversely, a decrease level of miR-21-5p by inhibitor molecules restored the expression of PDCD4 (Fig. 4C). We then co-cultured miR-21-5p silenced GBM cells with GAMs and observed a significantly reduced M2 signature, CD68⁺/CD206⁺ (Fig. 4D). More importantly, the M2 cytokines,

VEGF, TGF- β 1 and IL-6 released by GAMs co-cultured with miR-21-5p silenced U87MG cells were significantly reduced and restored partially after co-cultured with miR-21-5p silenced U87MG were transfected with mimic of miR-21-5p (**Fig. 4E**).

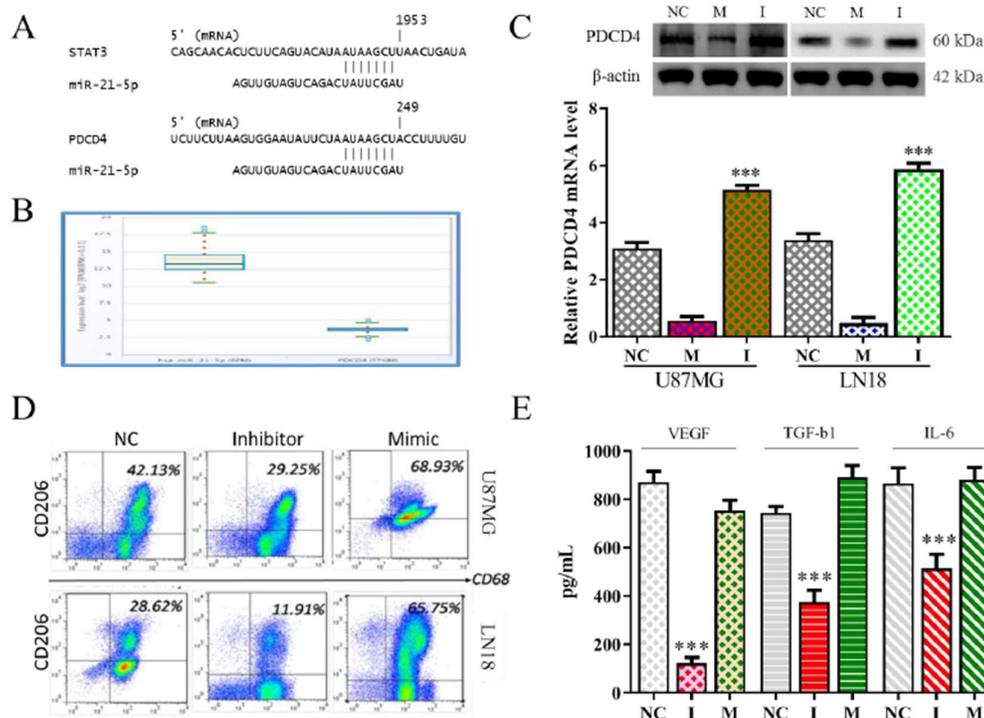


Figure 4. MiR-21-5p targets STAT3 and PDCD4. (A) Bioinformatics tool shows miR-21-5p binding to the 3'UTR of STAT3 and PDCD4 (upper panel). (B) A negative correlation between the expression of miR-21-5p and PDCD4 in GBM database (N=525, TCGA). (C) An increased miR-21-5p level (by mimic molecule, lane M), led to the significantly reduced PDCD4 expression in both U87MG and LN18 cells; the reverse was true with the inhibitor of miR-21-5p. (D) Flow cytometry analysis showed a significantly reduced CD206⁺/CD68⁺ population in GAMs co-cultured with miR-21-5p silenced U87MG and LN18 cells; the reverse was observed in miR-21-5p mimic transfected co-culture experiments. (E) The inhibitor of miR-21-5p resulted in the reduction of VEGF, TGF- β 1 and IL-6 secreted by the U87MG cells into the culture medium. **P<0.01; ***P<0.001.

Pacritinib suppresses GBM tumorigenesis and M2 polarization of GAMs

An elevated STAT3 signaling has been attributed to the malignancy of GBM and the generation of glioma stem cells [16]. In addition, increased STAT3 signaling is associated with the increased miR-21 level in the promotion of tumorigenesis [17,18]. Based on this premises, we examined a clinical STAT3 inhibitor, pacritinib for its potential GBM inhibitory effects. We found that the pacritinib treatment significantly suppressed the cell viability of both U87MG and LN18 cells at low IC₅₀ values, 0.5 and 1.7 μ M respectively (**Fig. 5A**). Subsequently, we showed that pacritinib-treated U87MG and LN18 cells contained a significantly lower ability to generate M2-polarized GAMs (**Fig. 5B**) as reflected by the reduced CD206 (M2 marker) and increased TNF- α (M1 marker). In addition, the addition of pacritinib prominently suppressed the colony formation (**Fig. 5C**) and tumor sphere generation (**Fig. 5D**). Furthermore, pacritinib treatment led to a decreased expression of Sox2, PDCD4 and STAT3; more importantly the level of miR-21-5p in both GBM cell lines was

suppressed as well (**Fig. 5E**). Notably, pacritinib treatment led to a significantly reduced exosome release and corresponding level of miR-21-5p from GAMs (**Fig. 5F**).

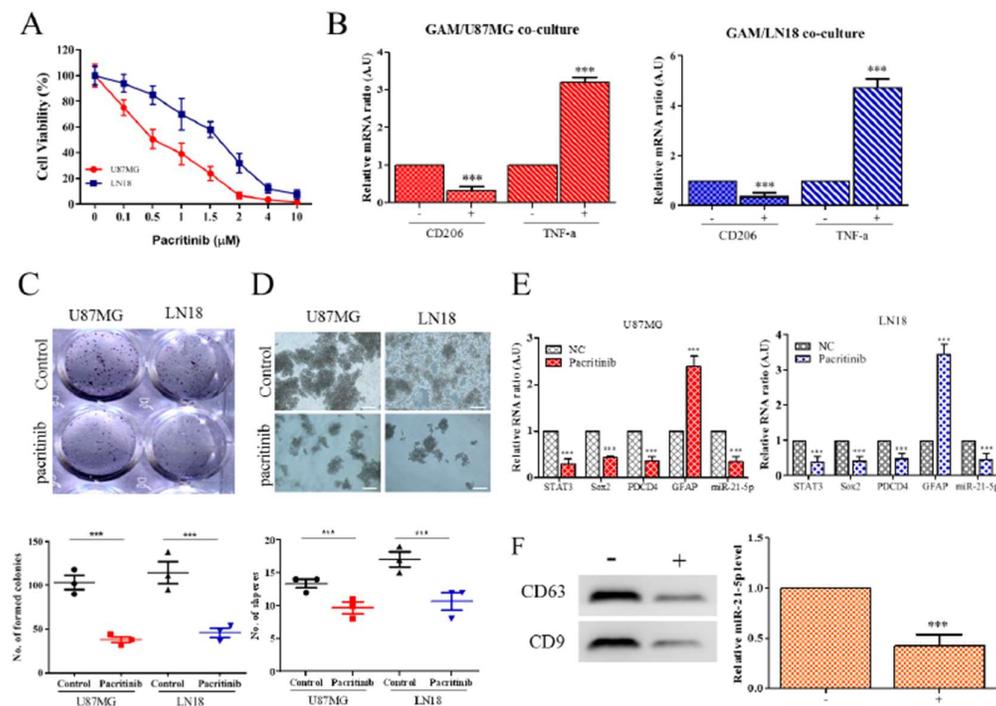


Figure 5 Pacritinib treatment suppresses GBM tumorigenesis and GSC properties (A) Pacritinib treatment significantly suppressed both U87MG and LN18 cells (approximate IC50 values 0.5 μM and 1.5 μM respectively). (B) Pacritinib treatment significantly reduced GBM cells' ability to induce M2 GAMs. CD206 mRNA in GAMs was significantly reduced while TNF-α was increased. Pacritinib treatment significantly reduced the colony formation (C) and tumor sphere generation (D) in both U87MG and LN18 cells. (E) Pacritinib treatment led to the significantly reduced mRNA level of STAT3, Sox2, PDCD4 and miR-21-5p while increased GFAP in both U87MG and LN18 cells. (F) GAMs treated with pacritinib resulted in the decreased release of exosomes. Western blot of exosomes collected from GAMs showed a significantly lower abundance of exosomes (CD63 and CD9, markers of exosomes). The exosomes collected showed a significantly lower miR-21-5p level. ***P<0.001.

In vivo evaluation of pacritinib

Finally, we evaluated the potential of using pacritinib as a treatment for GBM using preclinical mouse model bearing TMZ-resistant LN18 cells (co-cultured with exosomes isolated from GAMs). Representative brain slices showed that single treatment of pacritinib suppressed the tumorigenesis of TMZ-resistant LN18 cells as compared to TMZ single treatment and vehicle control (**Fig. 6A**). Notably, there was no significant difference in tumor size between vehicle control and TMZ single treatment group (**Fig. 6A**) while the combination of pacritinib and TMZ appeared to produce the most significant inhibitory effect on tumor progression (right panel, **Fig. 6A**). In support, tumor samples harvested from the combination of pacritinib and TMZ showed the lowest level of STAT3, Sox2, PDCD4, and miR-21-5p while increased level of GFAP (**Fig. 6B**). Microglial cells isolated from the single pacritinib treatment and the combination of pacritinib and TMZ group also demonstrated a significantly reduced CD206 mRNA level and increased TNF-α level (**Fig. 6C**).

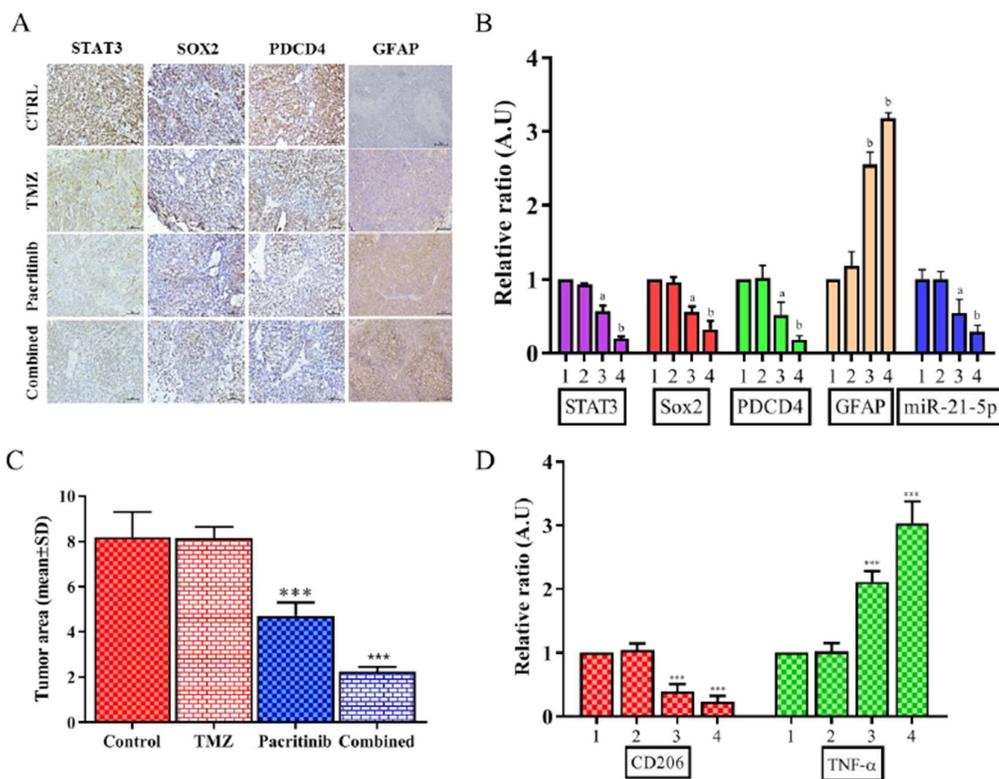


Figure 6 In vivo evaluation of pacritinib for treating GBM and reduction of M2 GAMs (A) TMZ-resistant LN18 bearing mice treated with pacritinib showed significantly reduced tumor size and the combination of pacritinib and TMZ led to the most significantly reduced tumor size. NS, statistically non-significant. (B) Comparative real-time PCR analyses showed that the reduced mRNA level of STAT3, Sox2, PDCD4, and miR-21-5p while increased GFAP in pacritinib group and pacritinib/TMZ combination. (C) M2 GAMs from tumor samples showed a significantly reduced CD206 (M2 marker) mRNA level (lane 3, pacritinib alone; lane 4, pacritinib/TMZ combination) while an increase in TNF- α (lane 3 and lane 4). ***P<0.001

Discussion

Despite advances in therapeutics development over the past decade, GBM remains challenging to treat due to its heterogeneity and malignant nature. The tumor microenvironment plays a crucial role in promoting GBM tumorigenesis. Glioma associated microglia (or GAMs) have been shown to be one of the key players of the GBM microenvironment. We first demonstrated that clinical samples of GAMs promoted GBM tumorigenesis. For instance, U87MG and LN18 GBM cells co-culture with clinical M2 GAMs showed an increased in colony-forming and tumor sphere generating abilities in association with increased stemness markers, Sox2, STAT3, Wnt and Nestin, in the GBM cells. Accumulating evidence has supported the observations where GAMs induced EMT in GBM cells and subsequently generated properties of glioma stem cells (GSCs)[19]. In addition, our observations were in agreement with previous studies where interactions between GBM and GAMs increased CD133+ GSCs and malignant phenotypes [20,21]. GAM-mediated GBM-promoting effects were through different communicating molecules such as M2 cytokines (IL-6, VEGF, TGF- β 1)[2]. Here, we showed that the presence of GAMs promoted GBM tumorigenesis and stemness not only via the cytokines but also through the aid of exosomes. More specifically, we found that GBM cells incubated with exosomes derived from GAMs exhibited enhanced ability in colony and tumor sphere formation; more importantly,

exosomes incubated GBM cells became more resistant against TMZ. Emerging evidence indicates the functional roles of exosomes in GBM tumorigenesis. A recent study showed that exosomes secreted from GBM cells promoted the oncogenic transformation of astrocytes in the tumor microenvironment [22]. This observation complements the results of our study where an intimate communication between the tumor microenvironment and tumor cells via the exchange of exosomes.

We performed an array analysis on the exosomes secreted by GAMs and found that the most abundant microRNA species was miR-21. Notably, a recent review points out that miR-21 plays a pivotal role in GBM pathogenesis where miR-21 functions through the modulation of insulin-like growth factor associated signaling pathway, RECK, and TIMP3 to promote GBM tumorigenesis [23]. Our results provided an added feature of miR-21 in GBM tumorigenesis where miR-21 was enriched in the exosomes secreted by GAMs. It is very plausible that GAM-derived miR-21-enriched exosomes were incorporated into GBM cells and executed its tumor-promoting functions. It has been well demonstrated that the transfer and uptake of exosomes between donor and recipient cells represents one of the major routes for intercellular communications in many diseases including cancer[24]. We provided support that an increased miR-21-5p in GBM cells by miR-21-5p mimic molecules, resulted in the similar tumorigenic and stemness properties in GBM cells co-cultured with GAM-derived exosomes; the reduction of miR-21-5p significantly reduced the tumorigenic properties in both GBM cell lines. Furthermore, GBM cells transfected with miR-21-5p inhibitor showed a significantly reduced ability to generate M2 GAMs, based on our co-culture experiments; this was attributed to the decreased secretion of M2 cytokines such as IL-6, VEGF by miR-21-5p silenced GBM cells while an increased secretion of TNF- α , M1 marker. More importantly, we provided evidence that miR-21-5p targets PDCD4, a tumor suppressor in both GBM cell lines. PDCD4 has been shown to be frequently suppressed in GBM cells and associated with poor prognosis[25,26]. In agreement, a previous study also demonstrated that PDCD4 was targeted by miR-21 in GBM [27].

According to our experimental results, miR-416a ranks as the second most abundant microRNA species in the GAM-secreted exosomes. It has been shown that miR-416a plays a key role in the progression of malignant melanoma via the activation of notch signaling [28]. The activation of notch has also been shown to be responsible for the generation of GSCs[29,30]. The fact that miR-21 and miR-416a, two powerful oncogenic microRNA molecules were enriched in the GAM exosomes further supports our notion that GAMs play a key contributing role in GBM malignancy and should be targeted in treatment design. As the roles of exosomal miR-416a in GBM tumorigenesis is currently under intense investigation in our laboratory.

Since targeting microRNA for therapeutic purposes still remains challenging, miR-21-5p represents a potential therapeutic target. Thus, we evaluated the feasibility of using small-molecule agent which may indirectly increase miR-21 level to convey therapeutic functions in GBM. STAT3 signaling has been shown to be important in GBM tumorigenesis as well as linked to the expression of miR-21[17,31,32]. Based on these premises, we evaluated pacritinib, a recent FDA-approved inhibitor of STAT3/JAK2 signaling for treating myelofibrosis [33,34]. We found pacritinib treatment suppressed cell viability, colony/tumor sphere formation in association with decreased expression of STAT3, Sox2, PDCD4, and miR-21-5p while an increased GFAP expression. Equally important, GAMs co-cultured with pacritinib-treated

U87MG and LN18 GBM cells showed a significantly reduced M2 marker, CD206 and increased M1 marker, TNF- α , strongly suggesting pacritinib not only suppressed GBM tumorigenesis but also affected GAM polarization. This tumor inhibitory and tumor microenvironment normalizing effects of pacritinib could be attributed to the suppression of STAT3/JAK2 signaling. Our observations were supported by a recent report that the inhibition of JAK/STAT3 pathway resulted in the disrupted intercellular communications between microglia and GBM cells [35] and pronounced anti-GBM effects [36,37]. In addition, we found that pacritinib treatment was able to suppress the number of miR-21-enriched exosomes secreted by GAMs.

Finally, we provided support for combining pacritinib and TMZ using TMZ-resistant GBM PDX mouse model. Single treatment of pacritinib was sufficient to suppress GBM growth while the combination of pacritinib and TMZ exerted the most significant inhibitory effect. Several studies have demonstrated the benefit of using STAT3 inhibitor for overcoming TMZ resistance[38,39]. Notably, one report showed that STAT3 inhibitor treatment promoted the infiltration of tumoricidal lymphocytes [40]. Another study also lend support to our results where the sequential combination of STAT3 inhibition and TMZ led to the induction of GBM apoptosis with an increased level of miR-21[41].

Conclusion

In conclusion, as the schema abstract **Figure 7**, we have provided translational evidence that miR-21-enriched GAM-derived exosomes contributed to GBM malignancy via increasing stemness. The feasibility of using pacritinib to modulate STAT3/miR-21/PDCD4 signaling was demonstrated both in vitro and in vivo GBM models. Further investigation is warrant for conducting potential clinical trails for GMB patients experiencing TMZ resistance.

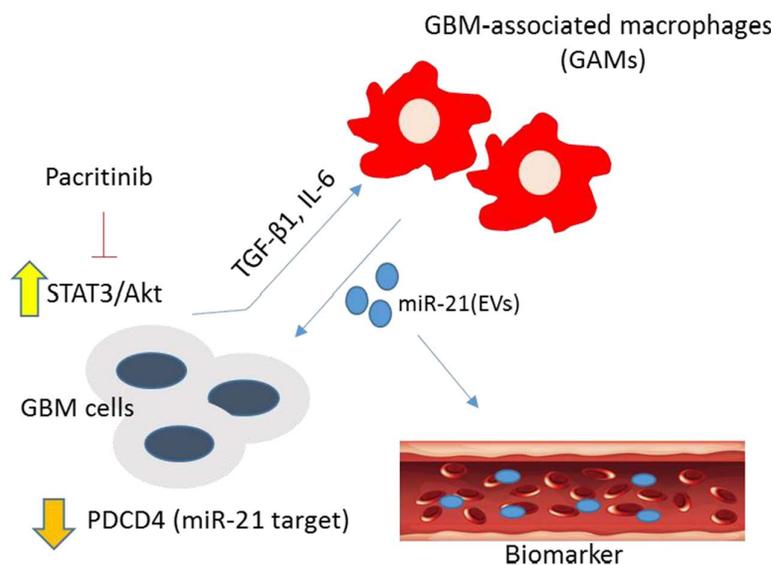


Figure 7 GBM-associated macrophages (GAMs) in the tumor microenvironment promotes the survival of GBM cells via miR-21 enriched EVs. Mir-21 targets and suppresses the expression of tumor suppressor PDCD4 in GBM cells, leading to the elevated STAT3/Akt signaling. In turn, GBM cells secrete inflammatory cytokines, TGF- β 1 and IL-6 and promotes M2 polarization. Pacritinib (STAT3 inhibitor) treatment suppresses GBM tumorigenesis by inhibiting STAT3 signaling and reducing M2 polarization of GAMs.

List of Abbreviations

M2-polarization of glioma associated microglia (M2-GAMs), negative controls (NC), Glioblastoma (GBM), long non-coding RNAs (lncRNAs), temozolomide sensitive (TMZ-S), resistant (TMZ-R), tumor microenvironment (TME),

Declarations

Authors' contribution

Conceived and designed the study: Hao-Yu Chuang, Yu-kai Su. Performed the experiments: Heng-Wei Liu, Chao-Hsuan Chen. Analyzed the data: Shao-Chih Chiu and Der-Yang Cho. Bioinformatics: Shinn-Zong Lin. Wrote the manuscript: Hao-Yu Chuang and Yu-kai Su. Provided reagents, materials, experimental infrastructure and administrative oversight: Yueh-Sheng Chen and Chien-Min Lin. All authors read and approved the final version of the manuscript.

Funding

This work was supported by the grants from Ministry of Science and Technology of grant to Dr. Chien-Min Lin (MOST 107-2314-b-038-056-MY3)

Ethics approval and consent to participate

Clinical samples were collected from Taipei Medical University (Taipei, Taiwan). All enrolled patients gave written informed consent for their tissues to be used for scientific research. The study was approved by the Institutional Review Board (IRB) of the Taipei Medical University (IRB: N201801070), consistent with the recommendations of the declaration of Helsinki for biomedical research (Taipei Medical University (Taipei, Taiwan) and followed standard institutional protocol for human research. Moreover, the animal study protocol was approved by the Animal Care and User Committee at Taipei Medical University (Taipei, Taiwan) (Affidavit of Approval of Animal Use Protocol # Taipei Medical University - LAC-2017-0512).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author in response to reasonable requests.

Conflict of interest

The authors declare that they have no potential financial competing interests that may in any way gain or lose financially from the publication of this manuscript at present or in the future. Additionally, no non-financial competing interests are involved in the manuscript.

Acknowledgments

The authors thank the laboratory assistant Mr. Alfred Fong (Department of Neurosurgery, Taipei Medical University Shunag-Ho Hospital) for his technical assistance.

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