Academic Editor

Dear Prof. Dr., Academic Editor in Chief, ***CANCERS***,

This is to submit our revised manuscript entitled "***Aberrant activation of hsa-miR-181d/STAT3 and hsa-miR-181d/5A ratios mediate the anticancer effect of Garcinol in STAT3/5A-addicted GBM***" for consideration of publication in the "***CANCERS***" journal.

Please be informed that this is a revised submission of our manuscript (**[Cancers] Manuscript ID: cancers-628385 R2**). We are thankful for your kind encouragement regarding to our manuscript. Herewith we are sending our revised manuscript including the improvements which were highlighted in red color in the manuscript following in accordance with the comments given by the reviewer.

Lastly, we would like to thank you once again for providing us the opportunity to improve our manuscript. We hope that these revisions are adequate, and that the manuscript is now acceptable for publication in the ***CANCERS.***

Sincerely,

Prof. Chien-Min Lin, MD., PhD.

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Answers to the comments:

Point-by-point responses to reviewer's comments:

We would like to thank the reviewer for the thorough reading of our manuscript as well as their valuable comments. We have followed their comments closely and feel that they have further improved the readability and appeal of our work, as well as strengthened the manuscript. Below are our point-by-point responses.

Q1: Reviewer #1: The Annexin V/7AAD staining represented in figure 2E is not complete. The dot plots were not so nice and the labeling of the axes is missing. Second the authors represent only double positive cells while usually the representation show both AnnV+/7AAD- and AnnV+/7AAD+ to differentiate apoptosis and late apoptosis/necrosis. Finally the authors used zVAD only in untreated cells. However the interest to use it is to check whether garcinol-induced cell death is mediated by caspases or not, so the authors have to use it in combination with garcinol.

A1: We are grateful for the reviewer's insightful comment. As requested by the reviewer, we have now provided more representation data of updated figure 2E for the ensured that the Annexin V/7-AAD staining, ensuring that the quality of the dot plots are improved, and that the axes labels are included. We have also indicated data for the early and late apoptosis. In addition, we have also added data for Z-VAD-fmk combined with Garcinol 'to check whether garcinol-induced cell death is mediated by caspases or not'. **Please see the updated figure 2 E.**

Please kindly see our revised Results section, Lines 354-391.

3.2. Garcinol significantly inhibits GBM cell viability and oncogenicity through induction of STAT3/5A signaling and enhanced apoptosis

Against the background of recent work demonstrating that garcinol inhibits CSC-like phenotype of human non-small cell lung carcinoma by suppressing the Wnt/β-catenin/STAT3 signaling axis (Huang et al., 2018), we investigated the probable STAT signaling-mediated anti-GBM effect of garcinol (**Figure 2A**). Firstly, to provide some mechanistic insight, we demonstrated that treatment of U87MG or GBM8401 cells with 2.5

μM or 5 μM garcinol significantly downregulated the expression of p-STAT3, p-STAT5, p-ERK, and p-AKT (**Figure 2B**), Synchronous with the observed inhibition of STAT3, STAT5 and AKT signaling, garcinol significantly suppressed the viability of GBM4801 and U87MG cells, with 10 μM eliciting 51% or 25% reduced viability of U87MG or GBM8401 cells, respectively, and 40 μM eliciting 94.7% reduction of U87MG and GBM8401 cell viability, indicating a dose-dependent GBM cell killing effect (**Figure 2C**), and this reduced viability was associated with markedly enhanced Bax/Bcl-xL apoptotic ratio, as 2.5 μM induced a 1.67-fold ($p < 0.05$) or 2.7-fold ($p < 0.05$) increase in U87MG or GBM8401 apoptotic ratio, while 5 μM increased the apoptotic ratio by 2.83-fold ($p < 0.001$) or 2.92-fold ($p < 0.001$) in the U87MG or GBM8401 cells, respectively (**Figure 2D**). In addition, using the Phycoerythrin (PE)-conjugated Annexin V/7- Amino-Actinomycin (7-AAD) staining, we demonstrated that compared to the cell death in the untreated control (U87MG: 0.379%, GBM8401: 0.208%) or 20 mM pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK)-treated negative control (U87MG: 0.157%, GBM8401: 0.071%), treatment with 2.5 μM garcinol enhanced cell death (U87MG: 18.76%, GBM8401: 17.92%), while 2.5 μM or 5 μM garcinol in the presence of 20 μM Z-VAD-FMK elicited 5.46% or 10.96% apoptosis of the GBM8401 cells, respectively, and 7.97% or 14.11% apoptosis of the U87MG cells (**Figure 2E**), indicating that the Garcinol-induced cell death was apoptotic and caspase-dependent. Since the highly invasive GBM spreads fast to surrounding brain tissue, thus, contributing to its documented lethality (Omuro & DeAngelis, 2013), we sought to understand if and how garcinol affects this invasive trait. We demonstrated that treatment with 2.5 μM or 5 μM dose-dependently suppressed the migration of the U87MG (~59%, $p < 0.01$ or 81%, $p < 0.001$, respectively) and GBM8401 (~48%, $p < 0.01$ or 76%, $p < 0.001$, respectively) cells at the 24 h time-point (**Figure 2F**). Similarly, 2.5 μM or 5 μM garcinol induced a 60% ($p < 0.01$) or ~80% ($p < 0.001$) reduction of U87MG invasive capacity, respectively, and 39% ($p < 0.01$) or 60% ($p < 0.001$) reduction in number of invaded GBM8401 cells (**Figure 2G**). Furthermore, in parallel assays to confirm the anticancer role of garcinol, consistent with earlier results, we demonstrated that treatment with 2.5 μM or 5 μM garcinol, significantly suppressed the expression of N-cadherin, vimentin and slug proteins, while conversely upregulating the expression of E-cadherin protein (**Figure 2H**), thus indicating that garcinol attenuates epithelial-to-mesenchymal transition (EMT) and the metastatic phenotype of GBM cells. Together, these

data suggest that garcinol significantly inhibits GBM cell viability and oncogenicity through induction of STAT3/5A and associated signaling with enhanced apoptosis.

Please also kindy see our updated Figure 2 and its legend, Lines 393-410.

Figure 2. Garcinol significantly inhibits GBM cell viability and oncogenicity through induction of STAT3/5A signaling and enhanced apoptosis. (A) Chemical structure of garcinol with molecular formula $C_{38}H_{50}O_6$ and molecular weight 602.80 g/mol. (B) Representative western blot photo-images of the effect of 2.5 μM – 5 μM on the expression of p-STAT3, STAT3, p-STAT5, STAT5, p-ERK, ERK, p-AKT, and AKT proteins in GBM8401 or U87MG cells. (C) Graphical representation of the effect of 2.5 μM – 40 μM on the viability of GBM8401 or U87MG cells. (D) Representative western-blot photo-images showing the effect of 2.5 μM – 5 μM on the expression of Bax and Bcl-xL proteins in GBM8401 or U87MG cells. (E) Flow-cytometry data (*upper*) and graphical representation (*lower*) showing the effect of Garcinol, alone or in presence of Z-VAD-FMK, on U87MG or GBM8401 cells co-stained with PE-conjugated Annexin V and 7- AAD, compared with untreated control or Z-VAD-FMK -treated negative control groups. Annexin V-stained Q4 cells are early apoptotic cells, whereas Q2 cells are late stage apoptotic (necrotic) cells. Apoptosis (%), sum of Q4+Q2; CTL, vehicle-treated; Neg CTL, pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK). Representative photo-images (*upper*) and graphical representation (*lower*) of the effect of 2.5 μM or 5 μM on the (F) migration and (G) invasion of GBM8401 or U87MG cells. (H) Representative western-blot photo-images showing the effect of 2.5 μM – 5 μM on the expression of E-cadherin, N-cadherin, vimentin and slug proteins in GBM8401 or U87MG cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$; GAPDH is loading control.

Q1: Reviewer #2: I am satisfied that the authors have addressed all my comments made regarding the original manuscript.

A1: We thank the reviewer for the time taken to review our work, the critical assessment of our findings and the final satisfactory predisposition towards our revised work. The reviewer's helpful suggestions has helped us improve the acceptability and appeal of our work.

Q1: Reviewer #3: Most of the critics raised during the first revision was answered.

A1: We thank the reviewer for the time taken to review our work, the assessment of our findings and the helpful suggestions given to help us improve the acceptability and appeal of our work.

Q2: Reviewer #3: In particular the diagram of the Figure 6 should be modified: hsa-miR-181 does not directly block STAT3 phosphorylation (because this is not the role of miRNAs). Some intermediate molecule (maybe a protein Kinase) is the direct target of hsa-miR-181. Then lacking of this "Kinase" avoids STAP3 phosphorylation.

A2: We sincerely thank the reviewer for this insightful comments. Making use of the reviewer's suggestion, we have modified the graphical abstract **in updated Figure 7. Please kindly see our updated Figure 7 and its legend, Lines 675-678.**

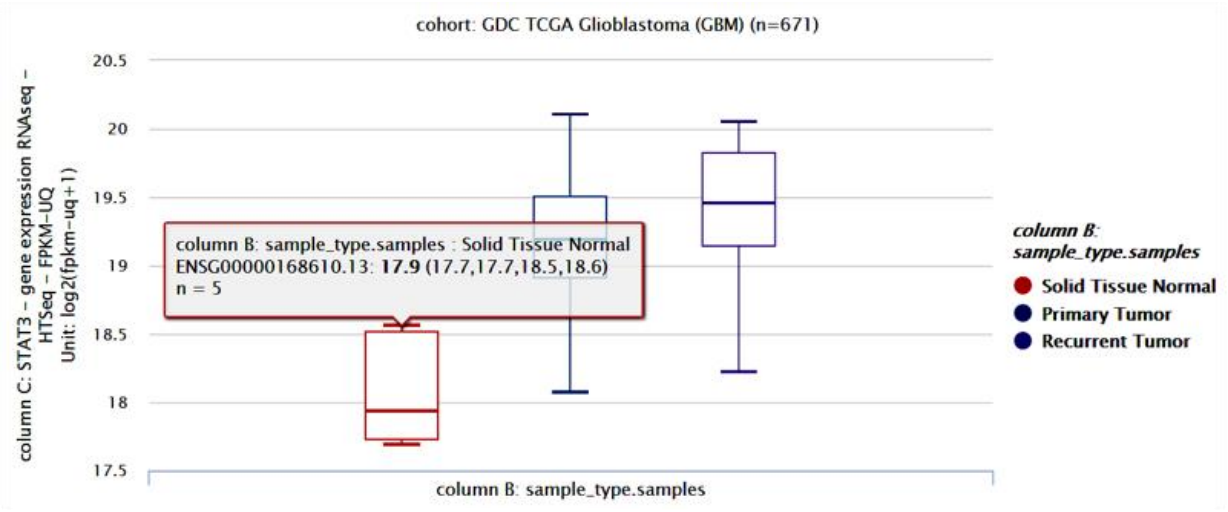
Figure 7. Graphical abstract showing how activation of STAT3/5A in GBM is inversely correlated with suppressed hsa-miR-181d expression, and that JAK2-mediated garcinol-induced upregulation of hsa-miR-181d/STAT3 and hsa-miR-181d/5A ratios underlies the anti-GBM-SCs effect of garcinol in STAT3/5A-addicted glioblastoma.

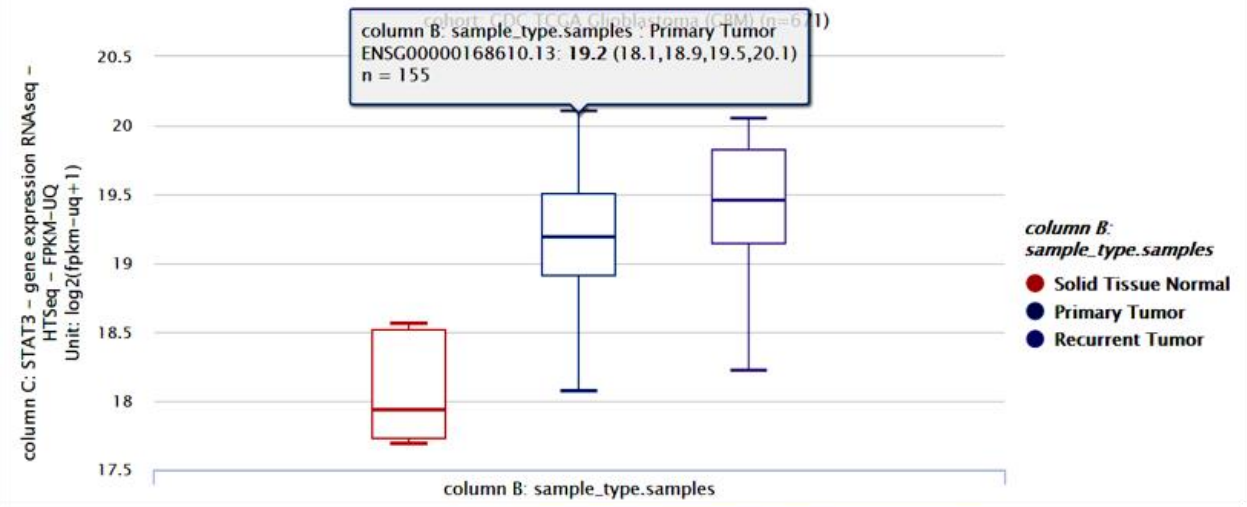
Q1: Reviewer #4: The authors have made significantly effort to address my questions and comments. The manuscript they have re-submitted is significantly improved.

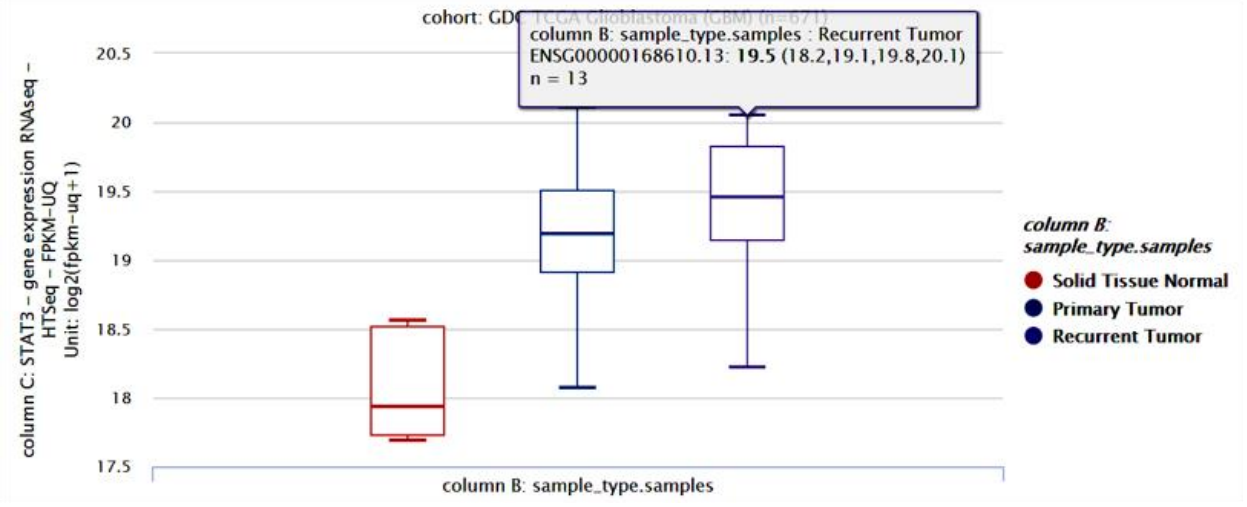
A1: We sincerely thank the reviewer for the time taken to review our work, for the encouraging words, and the important suggestions given to help us improve the quality of our work.

Q2: Reviewer #4: The GDC-TCGA glioblastoma (GBM) cohort indeed contains 173 samples- however, the annotation of this cohort mentions only 6 recurrent tumor samples and not 13 as mentioned in the text- how valid are the statistics in these conditions? (Photo evidence? - xena)

A2: We thank the reviewer for this comments, however, we humbly believe the reviewer is mistaken, as we have double checked our data and do confirm its correctness. We therefore provide a photo-evidence to support our claim. **Please kindly see the original screenshot from University of California Santa Cruz Xena Functional Genomics Explorer below.**


cohort: GDC TCGA Glioblastoma (GBM) (n=671)
column C: STAT3 – gene expression RNAseq – HTSeq – FPKM-UQ
Unit: log2(fpkm-uq+1)
20.5
20
19.5
19
18.5
18
17.5
column B: sample_type.samples : Solid Tissue Normal
ENSG00000168610.13: 17.9 (17.7,17.7,18.5,18.6)
n = 5
column B: sample_type.samples
column B: sample_type.samples
Solid Tissue Normal
Primary Tumor
Recurrent Tumor


column C: STAT3 – gene expression RNAseq – HTSeq – FPKM-UQ
Unit: log2(fpkm-uq+1)
20.5
20
19.5
19
18.5
18
17.5
column B: sample_type.samples : Primary Tumor
ENSG00000168610.13: 19.2 (18.1,18.9,19.5,20.1)
n = 155
column B: sample_type.samples
column B: sample_type.samples
Solid Tissue Normal
Primary Tumor
Recurrent Tumor


column C: STAT3 – gene expression RNAseq – HTSeq – FPKM-UQ
Unit: log2(fpkm-uq+1)
20.5
20
19.5
19
18.5
18
17.5
column B: sample_type.samples : Recurrent Tumor
ENSG00000168610.13: 19.5 (18.2,19.1,19.8,20.1)
n = 13
column B: sample_type.samples
column B: sample_type.samples
Solid Tissue Normal
Primary Tumor
Recurrent Tumor

Q3: Reviewer #4: The authors have added some in vivo data performed on ex situ xenografts (sub-cutaneous U87 xenografts in the flanks of SCID mice), but have not performed any in situ xenografts and survival analyses, which would be required to get an idea of the potential development of resistance mechanisms to their drug.

A1: We sincerely appreciate the reviewer's comment. We do agree with the reviewer that an orthotopic transplant of GBM cells would have been an ideal experimental design. We also humbly provide corroborating evidence of the validity and clinical-relevance of the method used in this current study, i.e. sub-cutaneous "flank model".

[1] William D, et al. Optimized creation of glioblastoma patient derived xenografts for use in preclinical studies. J. Transl. Med 2017; 15 (1):27. doi: 10.1186/s12967-017-1128-5.

[2] Husain SR, et al. Complete regression of established human glioblastoma tumor xenograft by interleukin-4 toxin therapy. Cancer Res. 1998; 58:3649-3653.

[3] Carlson BL, et al. Establishment, maintenance and in vitro and in vivo applications of primary human glioblastoma multformis (GBM) xenograft models for translational biology studies and drug discovery. Curr Protoc Pharmacol. 2011; 52(14):1-14. doi: 10.1002/0471141755.ph1416s52.

[4] Palanichamy K, et al. Clinically relevant brain tumor model and device development for experimental therapeutics. J. Analyt. Oncol. 2015; 4(1):5-12.

Please see the updated figure 5C of the effect of garcinol on the survival of mice GBM xenograft models.

Please kindly see our revised Results section, Lines 506-527.

3.5. Garcinol inhibits tumor growth in GBM mice models through inversely correlated STAT3/5A and hsa-miR-181d expressions

Having shown that treatment with garcinol suppresses the cancer stem cell-like phenotype of U87MG and GBM8401 cells in vitro, to determine the probable suppressive effect of garcinol on the formation and growth of tumor, in vivo, we generated NOD/SCID mice xenograft models derived by inoculation with 1×10^6 U87MG cells subcutaneously in the hind-flank. Mice were randomly placed into control or garcinol treatment group. We demonstrated that treatment with 1 mg/kg garcinol significantly reduced the size of tumors

formed in the treated mice, compared to the untreated control group (U87MG: ~7.1-fold smaller, $p < 0.001$ by week 4) (Figure**s 5A**), without adversely affecting the mice body weight (**Figure 5B**). We also observed that mice treated with garcinol showed 100% survival as compared to 60% in the control counterparts, over the 4-week treatment period. (**Figure 5C**). In subsequent experiments using protein lysates derived from the tumors extracted from the untreated and garcinol-treated mice, we demonstrated that compared to the untreated control group, STAT3, pSTAT3, STAT5A, p-STAT5A, Ki-67, and Bcl-xL protein expression levels were concomitantly suppressed, while Bax expression was significantly enhanced (**Figure 5D**). Moreover, for tumors extracted from the 1 mg/kg garcinol-treated U87MG tumor-bearing mice, compared to the untreated control group, STAT3, or STAT5A mRNA expression were suppressed by 4-fold ($p < 0.01$) or 3.87-fold ($p < 0.01$), while miR-181d expression was enhanced by 3.52-fold ($p < 0.01$) in the U87MG mice treated with 1 mg/kg garcinol (**Figure 5E**). These findings indicate that garcinol inhibits tumorigenicity and growth of GBM by abrogating STAT3/5A signaling, and upregulating hsa-miR-181d, with concomitant suppression of Ki-67 proliferation index and enhancement of Bax/Bcl-xL apoptotic ratio, *in vivo*.

Please kindly see our updated Figure 5 and its legend, Lines 529-538.

Figure 5. Garcinol inhibits tumor growth in GBM mice models through inversely correlated STAT3/5A and hsa-miR-181d expressions. Representative image and graph showing the effect of garcinol on the (A) tumor volume and (B) body weight of U87MG-tumor-bearing mice. p-values were determined by 2-way ANOVA. (C) Kaplan-Meier Survival curve. Mice treated with garcinol showed 100% survival as compared to 60% in the control counterparts, over the 4-week treatment period. (D) Representative images and histograms of the differential expression of STAT3, pSTAT3, STAT5A, pSTAT5A, Ki-67, Bax, and Bcl-xL proteins level in tumors extracted from mice bearing U87MG cell-derived tumors, treated with or without garcinol. (E) Histograms showing the effect of garcinol treatment on STAT3, STAT5A and miR-181d expression levels in U87MG-tumor-bearing mice. *p<0.05, **p<0.01, ***p<0.001; GAPDH is loading control.

Q4: Reviewer #4: Furthermore, the data remain limited to cell lines (of which U87 remains controversial) and have not been confirmed in primary cultures of GBM; As such, the results remain of very limited relevance for the clinic.

A2: We thank the reviewer for this important comment. As suggested by the reviewer, we have now included results from our primary GBM culture studies data in our revised manuscript. **Pleas see the updated figure 6.**

Please kindly see our revised Results section, Lines 539-566.

3.6. Garcinol, akin to Stattic, a selective inhibitor of STAT3/5A activation, inhibits the metastatic and cancer stem cell-like phenotypes of primary GBM culture cells.

Sequel to our data demonstrating that garcinol suppresses the cancer stem cell-like phenotype of U87MG and GBM8401 cells in vitro, and that garcinol also suppresses the formation and growth of tumor in mice GBM models by upregulating hsa-miR-181d expression and inhibiting STAT3/5A activation, we further investigated if indeed these findings could be replicated in GBM primary culture cells. Comparative analyses of the anti-GBM therapeutic effects of garcinol and stattic, a selective inhibitor of STAT3/5A activation and dimerization, revealed that akin to the 25% - 98% reduction in cell viability of the primary GBM culture cells by 2.5 – 40 μM static, equimolar garcinol treatment dose-dependently elicited a 13% - 96.8% reduced viability of the primary GBM culture cells (μM). We also observed that concurrent with reduced cell viability, 2.5 and 5 μM garcinol or stattic induced significant downregulation of p-STAT3 (garcinol: 40% and 85%, $p < 0.01$; stattic: 56% and 89%, $p < 0.01$) and p-STAT5A (garcinol: 35% and 83%, $p < 0.01$; stattic: 48% and 74%, $p < 0.01$) in the primary GBM culture cells **(Figure 6B**). Furthermore, while treatment with 2.5 and 5 μM stattic attenuated the migration of the primary culture cells by 49.3% ($p < 0.05$) and 88.9% ($p<0.001$), 2.5 and 5 μM garcinol elicited 48% ($p<0.05$) and 87.5% ($p<0.001$) (**Figure 6C**). Similarly, suppression of cell invasion was demonstrated, with 2.5 and 5 μM stattic causing 64% ($p<0.01$) and 91% ($p<0.001$) reduction in migration, respectively, and equimolar garcinol induced 51% ($p < 0.01$) and 84% ($p<0.001$), respectively (**Figure 6D**). We also showed that comparable to stattic, 2.5 and 5 μM garcinol reduced the number of colonies formed by 50.7% ($p<0.01$) and 91% ($p<0.001$), respectively (**Figure 6E**). Moreover, akin to stattic, 5 μM garcinol

reduced the number of primary GBM culture tumorspheres formed by 91.7% ($p<0.001$) and the tumorsphere sizes by ~90% ($p<0.001$) (**Figure 6F**). These findings not only validate the replicability of garcinol anti-GBM effect in vitro, in vivo and ex vivo using primary GBM culture cells, and enhance clinical or translational relevance, but also demonstrate, at least in part, that akin to stattic, garcinol inhibits the metastatic and stemness phenotypes of primary GBM culture cells by inhibiting the activation of STAT3/5A, which is consistent with results in Figures 4 and 5 showing that garcinol inhibits metastasis, cancer stemness and tumor growth through enhanced hsa-miR-181d/STAT3 or hsa-miR-181d/STAT5A.

Also see our newly included Figure 6 and its legend, Lines 568-579.

Figure 6. Garcinol, akin to Stattic, a selective inhibitor of STAT3/5A activation, inhibits the metastatic and cancer stem cell-like phenotypes of GBM primary culture cells. (A) Graphical representation of the effect of 2.5 μM – 40 μM on the viability of GBM primary culture cells. (B) Representative western-blot photo-images comparing the effect of stattic and garcinol on the expression level of p-STAT3, STAT3, p-STAT5, and STAT5 proteins in primary culture cells. (C) Representative images (*left*) and histograms (*right*) comparing the effect of 2.5 and 5 μM stattic or garcinol on the migration of U87MG cells over 24 h, as determined by wound-healing assay. Representative images (*upper*) and histograms (*lower*) comparing the effect of 2.5 and 5 M stattic or garcinol on the (D) invasion and (E) colony formation capacity of GBM primary culture cells. (F) Representative photo-images (*left*) and histograms comparing the effect of 5 μM stattic and garcinol on the number and size of tumorspheres formed by the GBM primary culture cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$; GAPDH is loading control.

Also kindly see our revised Materials and Methods section, Lines 150-165.

2.3. Cell lines and Primary Culture Cell Culture

The human U-87 MG (ATCC® HTB-14™) (ATCC, Manassas, VA) and GBM8401 GBM cell lines used in the study were purchased from (Bioresource Collection Research Center, Hsinchu, Taiwan). The cell lines were cultured in Gibco DMEM (Cat. No. 11965175, Thermo Fisher Scientific, Inc. Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Life Technologies, Carlsbad, CA,

USA) and incubated in 5% humidified CO_2 incubator at 37oC. The cells were sub-cultured when they reached 80-90% confluency and the media changed every 48-72h. Patient-derived CD133+ GBM spheres was kindly provided by our collaborator Dr. Alexander T.H. Wu at Taipei Medical University. In brief, the patient-derived GBM cells were first sorted using established flow cytometric method. Once CD133+ cells were sorted, they were expanded in advanced DMEM/F12 (Gibco) mixed with Neurobasal TM-A medium (Gibco) (1:1) supplemented with B-27 (1×), FGF (20 ng/mL) and EGF (20 ng/mL); culturing under these conditions maintained CD133+ cell population and stemness (as well as TMZ-resistant), the tumor-initiating ability was demonstrated in vivo as described previously (Wei et al., 2016).

The reference has been added in the line 838-841.

Wei, L., Su, Y. K., Lin, C. M., Chao, T. Y., Huang, S. P., Huynh, T. T., Jan, H. J., Whang-Peng J., Chiou J. F., Wu A. T., Hsiao M. (2016) Preclinical investigation of ibrutinib, a Bruton's kinase tyrosine (Btk) inhibitor, in suppressing glioma tumorigenesis and stem cell phenotypes. Oncotarget. Oct 25;7(43):69961-69975.

Q5: Reviewer #4: The rationale to look at miR181-d as the link between garcinol, STAT3/5 phosphorylation remains obscure – the authors do not explain why they have looked at this miR and how garcinol is supposed to alter the expression of this miR. Theseare major shortcomings of this work.

A4: We thank the reviewer for this comment. We have tried to address the reviewer's lack of clarity on the rationale for the study of garcinol's effect on miR-181d, and STAT3/5 activation in our revised work. **Please kindly see our revised Introduction section, Lines 94-132.**

Over the last 2 decades, there has been increase in the documentation of the critical roles of microRNA (miRNAs/miRs) in the malignantization and progression of many human malignancies, including GBM; by their ability to hamper or facilitate cancer initiation, miRNAs have emerged as therapeutically-relevant actionable biomolecules for anticancer therapy (Bhaskaran et al., 2019; Floyd & Purow, 2014). One such miRNA family is miRNA-181, with varied expression of its isoforms (miRNA-181a/b/c/d) being

touted as independent predictors of clinical outcome in patients with different cancer types (Pop-Bica et al., 2018). A notable feature of treatment-resistant GBM is the enzyme O^6-methylguanin-DNA methyltransferase (MGMT), a DNA-repair protein that removes alkyl group from the O^6 position of alkyl groups, and consequently diminish the curative effects of chemotherapeutic agents (Hegi et al., 2005), conversely, the silencing of MGMT through promoter methylation is associated with prolonged OS and disease-free survival (DFS) in patients with GBM (Bell et al., 2018). Interestingly, recent studies show that MGMT activity is inversely correlated with the expression of microRNA-181d/miRNA-181d; as higher expression of miRNA-181d is positively correlated with improved OS in patients with GBM (Zhang et al., 2012). There is also accruing evidence that the prognostic implications of altered miRNA expression, is connected to their roles in modulation of stemness signaling such as Notch, Hedgehog, and JAK/STAT3 (Floyd & Purow, 2014; F. Yang et al., 2017), and the consequent acquisition of stem cell-like traits by GBM cells; Thus, the present study's rationale for exploring the actionability of miRNA-181d in the context of STAT signaling in GBM.

Due to the accruing adverse reactions to most chemotherapeutic agents, phytochemicals and nutraceuticals have garnered interest as possible safe alternatives or adjuvants in cancer treatment (Saadat & Gupta, 2012). Building on previous works by our team demonstrating that Garcinol inhibits CSCs-like phenotype by suppressing the Wnt/β-catenin/STAT3 signaling axis in human non-small cell lung carcinoma (Huang et al., 2018), we now examined the probable effect of Garcinol on GBM stem cells (GBM-SCs), and the implication of same for sensitivity to conventional chemotherapy and better prognosis. Garcinol, a major bioactive constituent of the fruit *Garcinia indica*, has widely documented antioxidant and anticancer effects, and is chemically similar in structure to the well-known curcumin (Ashad et al., 2017; Saadat & Gupta, 2012). In fact, Hong et al. (Hong et al., 2007) demonstrated that garcinol significantly inhibited the growth of the colon cancer cells, and importantly, provided evidence that unlike conventional chemotherapeutics, garcinol preferentially targets cancerous cells; thus inhibiting cancer growth without adversely affecting the neighboring 'normal' non-cancerous cells (Hong et al., 2007). In breast cancer, garcinol was shown to inhibit STAT3-NF-kB signaling, resulting in reduced invasiveness, *in vitro*, and significantly attenuated tumor growth in NOD-SCID mice (Ahmad et al., 2012, 2010); thus, building a case for the preclinical investigation of the probable anti-GBM effect of garcinol in this stance. This present study,

for the first time, to the best of our knowledge, investigated and documents the effect of garcinol on GBM, consistent with current theme-relevant knowledge, especially in the context of the therapeutic effects of garcinol alone or in synergism with conventional anticancer treatment modalities on GBM-SCs through the mediation of STAT3/5A signaling and miRNA-181d.

Also kindly see our revised discussion and conclusion section, Lines 617-673.

In the context of the GBM SCs-phenotypes including enhanced proliferation, oncogenicity, therapy resistance, recurrence and poor prognosis, we further demonstrated that garcinol significantly inhibited GBM8401, U87MG, and GBM primary culture cell viability in a dose-dependent manner, as well as suppresses the cell invasive and migratory potentials, thus, demonstrating the anti-proliferative and anti-metastatic efficacy of garcinol in GBM. This is consistent with documented robust growth-inhibitory effects of garcinol demonstrated against colon cancer and immortalized intestinal cells (Hong et al., 2007). Since STAT3 and STAT5A are implicated in the maintenance of the stem cell-like characteristics of GBM, we examined garcinol's inhibitory effect on the GBM-SCs profile. Interestingly, low dose garcinol ($\leq$ 5 μM) deregulated STAT3/5A signaling with repressed AKT and ERK crosstalk, and this was sufficient to significantly impeded GBM cell migration, invasion, clonogenicity, and tumorsphere formation, with associated increase in apoptotic index and nuclear expression of SOX2 and OCT4. Our findings are corroborated by recent evidence that STAT3 and STAT5 are constitutively activated in malignant cells, and that their persistent activation facilitates cancer development and progression by altering downstream gene expression through epigenetic modification, EMT induction, oncogenic modification of the tumor microenvironment, and enhancing of CSCs self-renewal and differentiation (Yuan et al., 2015), as well as evidence implicating high ERK1/2 activity in the acquisition and maintenance of SOX2-expressing Glioma stem cells (Kwon et al., 2017).

This study also demonstrated for the first time to the best of our knowledge, that garcinol-induced suppression of STAT3 and STAT5A is associated with significant upregulation of hsa-miR-181d expression, in vitro, ex vivo and in vivo; interestingly we also showed direct interaction between hsa-miR-181d and STAT3 or STAT5A protein. We posit that upon treatment with garcinol, miR-181d canonically represses the

activation/phosphorylation of STAT3/5A in a JAK2-mediated manner, or as also documented herein, miR-181d non-canonically binds directly to the coding region of STAT3 or STAT5A mRNA, eliciting STAT3/5A degradation, and consequently impair activation of STAT3 or STAT5A in the GBM cells. This demonstrated tumor suppressor role of hsa-miR-181d is consistent with findings showing that overexpression of miR-181d significantly suppressed esophageal squamous cell carcinoma (ESCC) by downregulating Derlin-1, inhibiting cancerous cell proliferation, migration and cell cycle progression *in vitro*, as well as inhibiting tumorigenicity *in vivo* (Li D et al., 2016), as well as in glioma samples and cell lines, where ectopic expression of miR-181d suppressed proliferation and induced cell cycle arrest and apoptosis by targeting K-ras and Bcl-2 (Wang et al., 2012). This demonstrated garcinol-modulated miR-181d/STAT3/5A signaling axis is of therapeutic relevance, considering that well documented role of the JAK-STAT signaling pathway, and more particularly its molecular effectors namely STAT3 and STAT5 which act as a point of convergence for several signaling pathways in cancerous cells and oncogenic processes (Luo and Balko, 2019). It is worth mentioning however, that while we cannot fully explain how garcinol induced hsa-miR-181d inhibited the activation of STAT3 and STAT5, our data finds some corroboration in increasingly documented role of miRs in the (de)activation of the JAK-STAT signaling (Zhuang G et al., 2012, Lam et al, 2013, Liu X et al., 2018), and of particular interest is miR-204 which similarly had very insignificant effect on total STAT3 expression, but impaired STAT3 phosphorylation, consequently inducing cancerous cell apoptosis and suppressed cell proliferation, migration in vitro and tumor growth in vivo (Liu X et al., 2018). Moreover, recent report that the Kaposi's sarcoma-associated herpes virus (KSHV) miRNAs impair the activation/phosphorylation of STAT3/5, and inhibit the activation of STAT3-dependent reporter upon IL6-treatment, also lend credence to our finding that hsa-miR-181d may bind directly with STAT3/5A and impair activation of the later (Ramlingam and Ziegelbauer, 2017).

5. Conclusion

Taken together, as depicted in our graphical abstract in **Figure 7**, the present study provides evidence that the constitutive activation of STAT3/5A in GBM is inversely correlated with suppressed hsa-miR-181d expression, and that JAK2-mediated garcinol-induced upregulation of hsa-miR-181d/STAT3 and hsa-miR-181d/5A ratios underlies the

anti-GBM-SCs effect of garcinol in STAT3/5A-addicted GBM. These findings are of translational relevance as they highlight the therapeutic efficacy of a relatively novel small molecule inhibitor of STAT3/5A in the highly invasive and often therapy resistant GBM. In addition, findings documented in the present pilot study form a basis for further large cohort exploration of the preclinical feasibility and subsequent clinical applicability of garcinol-modulated hsa-miR-181d/STAT ratio as a therapeutic strategy in GBM.

The reference has been added in the line 815-816.

Ramalingam, D., & Ziegelbauer, J. M. (2017). Viral microRNAs Target a Gene Network, Inhibit STAT Activation, and Suppress Interferon Responses. Scientific reports, 7, 40813. doi:10.1038/srep40813

Q6: Reviewer #4: The result section toitle "4. Garcinol increases the expression of hsa-miR181d, which has inhibitory effects on STAT3 and STAT5" is absolutely not supported by the results: mir181d affects the PHOSPHORYLATION of STAT3 &5, not their expression- it also alters the expression of JAK2, ERK and Akt, which aall alter the phosphorylation of these STAT transcription factors- In view of these results, the authors should look at the interaction of mir181d with the genes of these kinases rather than between this miR and STAT genes.

A5: We thank the reviewer for this insightful comment. As suggested by the reviewer, we have now modified the sub-title in question. We have also incorporated the reviewer's suggestion on the likely interaction between mir181d and the JAK2 kinase in our revised manuscript. **Please kindly see our revised Results section, Lines 447-491.**

3.4. Garcinol increases the expression of hsa-miR181d, which has inhibitory effects on STAT3 and STAT5 activation

Having established that garcinol impairs STAT3 and STAT5A activation, we probed for likely modulators and/or mediators of the interaction between garcinol and the STAT proteins. Hsa-miR-181d shown in Figure 4A, has been implicated in the worse OS of patients with GBM (Zhang et al., 2012). Consistent with this, using the Schrodinger PyMOL 2.3 molecular docking and visualization software (http://pymol.org) we demonstrated that hsa-miR-181d interacts with and binds directly to STAT3 (docking score = -254.49, ligand root mean square deviation (RMSD) = 195.10 Å) or STAT5A (docking

score = -234.19, ligand RMSD = 143.04 Å), complementing earlier prediction that hsa-miR-181d binds with STAT3 with a mirSVR or PhastCons score of -0.26 or 0.69, respectively, while it binds with STAT5A with a mirSVR or PhastCons score of -0.21 or 0.49, respectively (**Figure 4A**). Concomitantly, as shown in Figure 4A, results of our nucleotide complementarity analysis indicate that the 5'-UTR (untranslated region) of hsa-miR-181d binds to the 3' UTR of JAK2 with a mirSVR or PhastCons score of -0.79 or 0.61, respectively. Where the mirSVR shows the likelihood of hsa-miR-181d down-regulating the target mRNA STAT3 and STAT5A based on the sequence and structure features in the miRNA/mRNA predicted target sites. Moreover, the PhastCons score shows the likelihood that the predicted miRNA/mRNA binding nucleotides are conserved. In concordance, our qRT-PCR analysis of 5 μM garcinol-treated U87MG and GBM8401 cells showed that garcinol significantly induced higher expression of miR-181d in the U87MG (2.7-fold, $p < 0.01$) and GBM8401 (2.1-fold, $p < 0.01$) cells (**Figure 4B**). Furthermore, having implicated STAT3/5A in enhanced migration and invasiveness of U87MG or GBM8401 cells, we examined the probable effect of hsa-miR-181d on these metastatic phenotypes of GBM cells. Using the scratch wound-healing assay, we demonstrated that compared to the untreated control or syn-mir-treated cells, treatment with mir-181d inhibitor significantly enhanced the ability of the U87MG cells to migrate (4.64-fold, $p<0.01$), while treatment with the mir-181d-mimic elicited marked attenuation of migration (3.80-fold, $p<0.01$) (**Figure 4C**), which is reminiscent of suppressed migration induced by 5 M garcinol (4.17-fold, $p<0.01$) earlier. Similarly, while treatment with mir-181d inhibitor significantly enhanced the invasiveness of the U87MG cells (4.63-fold, $p<0.01$), treatment with the mir-181d-mimic elicited profound suppression of invasion (4.15-fold, $p<0.01$) (**Figures 4D**), and this was akin to the effect of 5 μM garcinol (4.22-fold, $p<0.01$). To confirm a direct relationship between the STAT proteins and miR-181d expression, western blot analysis was done comparing samples exposed to mir-181d inhibitor, mir-181d-mimic, or mir-181d inhibitor/garcinol combination. The results showed that mir-181d inhibitor significantly enhanced the expression of p-STAT3, p-STAT5, N-cadherin and vimentin proteins, but suppressed E-cadherin protein expression compared to the control group, while for the mir-181d-mimic-treated cells, the p-STAT3, p-STAT5, N-cadherin, and vimentin protein expression levels were significantly lower, but E-cadherin was upregulated; For cells incubated with mir-181d inhibitor and 5 μM garcinol concomitantly, p-STAT3 and p-STAT5 protein expression levels were markedly higher than in the mir-

181d-mimic group but lower than the mir-181d-inhibitor group (**Figure 4E**). Concomitantly, we observed that compared with the control group, syn-mir-treated cells, or even mir-181d inhibitor-treated cells, treatment with mir-181d-mimic markedly suppressed the expression of JAK2 protein (2.22-fold, $p<0.01$), akin to the effect elicited by treatment with concurrently with mir-181d inhibitor and 5 μM garcinol (2.94-fold, $p<0.01$), which is consistent with the results above and suggestive of a miR-181d-mediated JAK2-modulated phosphorylation of STAT3/5A. These results indicate that garcinol can activate mir-181d activity which suppresses JAK2-modulated STAT3/5A activation.

Also kindly see our revised Discussion section, Lines 635-662.

This study also demonstrated for the first time to the best of our knowledge, that garcinol-induced suppression of STAT3 and STAT5A is associated with significant upregulation of hsa-miR-181d expression, in vitro, ex vivo and in vivo; interestingly we also showed direct interaction between hsa-miR-181d and STAT3 or STAT5A protein. We posit that upon treatment with garcinol, miR-181d canonically represses the activation/phosphorylation of STAT3/5A in a JAK2-mediated manner, or as also documented herein, miR-181d non-canonically binds directly to the coding region of STAT3 or STAT5A mRNA, eliciting STAT3/5A degradation, and consequently impair activation of STAT3 or STAT5A in the GBM cells. This demonstrated tumor suppressor role of hsa-miR-181d is consistent with findings showing that overexpression of miR-181d significantly suppressed esophageal squamous cell carcinoma (ESCC) by downregulating Derlin-1, inhibiting cancerous cell proliferation, migration and cell cycle progression *in vitro*, as well as inhibiting tumorigenicity *in vivo* (Li D et al., 2016), as well as in glioma samples and cell lines, where ectopic expression of miR-181d suppressed proliferation and induced cell cycle arrest and apoptosis by targeting K-ras and Bcl-2 (Wang et al., 2012). This demonstrated garcinol-modulated miR-181d/STAT3/5A signaling axis is of therapeutic relevance, considering that well documented role of the JAK-STAT signaling pathway, and more particularly its molecular effectors namely STAT3 and STAT5 which act as a point of convergence for several signaling pathways in cancerous cells and oncogenic processes (Luo and Balko, 2019). It is worth mentioning however, that while we cannot fully explain how garcinol induced hsa-miR-181d inhibited the activation of STAT3 and STAT5, our data finds some corroboration in increasingly documented role of miRs in the

(de)activation of the JAK-STAT signaling (Zhuang G et al., 2012, Lam et al, 2013, Liu X et al., 2018), and of particular interest is miR-204 which similarly had very insignificant effect on total STAT3 expression, but impaired STAT3 phosphorylation, consequently inducing cancerous cell apoptosis and suppressed cell proliferation, migration in vitro and tumor growth in vivo (Liu X et al., 2018). Moreover, recent report that the Kaposi's sarcoma-associated herpes virus (KSHV) miRNAs impair the activation/phosphorylation of STAT3/5, and inhibit the activation of STAT3-dependent reporter upon IL6-treatment, also lend credence to our finding that hsa-miR-181d may bind directly with STAT3/5A and impair activation of the later (Ramlingam and Ziegelbauer, 2017).

Also kindly see our revised Conclusion section, Lines 663-673.

5. Conclusion

Taken together, as depicted in our graphical abstract in **Figure 7**, the present study provides evidence that the constitutive activation of STAT3/5A in GBM is inversely correlated with suppressed hsa-miR-181d expression, and that JAK2-mediated garcinol-induced upregulation of hsa-miR-181d/STAT3 and hsa-miR-181d/5A ratios underlies the anti-GBM-SCs effect of garcinol in STAT3/5A-addicted GBM. These findings are of translational relevance as they highlight the therapeutic efficacy of a relatively novel small molecule inhibitor of STAT3/5A in the highly invasive and often therapy resistant GBM.

Q7: Reviewer #4: In light of 4., the statement in the discussion that "We posit that upon treatment with garcinol, miR-181d binds directly to the coding region of STAT3 or STAT5A mRNA, eliciting STAT3/5A degradation, and consequently reduce the expression and/or activation of STAT3 or STAT5A in the GBM cells. " is dfinitely not supported by the results.

A7: We thank the reviewer for this comment. We have however rephrased the statement to address the reviewer's concern. **Please kindly see our revised Discussion section, Lines 635-662.**

This study also demonstrated for the first time to the best of our knowledge, that garcinol-induced suppression of STAT3 and STAT5A is associated with significant upregulation of hsa-miR-181d expression, in vitro, ex vivo and in vivo; interestingly we also showed direct

interaction between hsa-miR-181d and STAT3 or STAT5A protein. We posit that upon treatment with garcinol, miR-181d canonically represses the activation/phosphorylation of STAT3/5A in a JAK2-mediated manner, or as also documented herein, miR-181d non-canonically binds directly to the coding region of STAT3 or STAT5A mRNA, eliciting STAT3/5A degradation, and consequently impair activation of STAT3 or STAT5A in the GBM cells. This demonstrated tumor suppressor role of hsa-miR-181d is consistent with findings showing that overexpression of miR-181d significantly suppressed esophageal squamous cell carcinoma (ESCC) by downregulating Derlin-1, inhibiting cancerous cell proliferation, migration and cell cycle progression *in vitro*, as well as inhibiting tumorigenicity *in vivo* (Li D et al., 2016), as well as in glioma samples and cell lines, where ectopic expression of miR-181d suppressed proliferation and induced cell cycle arrest and apoptosis by targeting K-ras and Bcl-2 (Wang et al., 2012). This demonstrated garcinol-modulated miR-181d/STAT3/5A signaling axis is of therapeutic relevance, considering that well documented role of the JAK-STAT signaling pathway, and more particularly its molecular effectors namely STAT3 and STAT5 which act as a point of convergence for several signaling pathways in cancerous cells and oncogenic processes (Luo and Balko, 2019). It is worth mentioning however, that while we cannot fully explain how garcinol induced hsa-miR-181d inhibited the activation of STAT3 and STAT5, our data finds some corroboration in increasingly documented role of miRs in the (de)activation of the JAK-STAT signaling (Zhuang G et al., 2012, Lam et al, 2013, Liu X et al., 2018), and of particular interest is miR-204 which similarly had very insignificant effect on total STAT3 expression, but impaired STAT3 phosphorylation, consequently inducing cancerous cell apoptosis and suppressed cell proliferation, migration in vitro and tumor growth in vivo (Liu X et al., 2018). Moreover, recent report that the Kaposi's sarcoma-associated herpes virus (KSHV) miRNAs impair the activation/phosphorylation of STAT3/5, and inhibit the activation of STAT3-dependent reporter upon IL6-treatment, also lend credence to our finding that hsa-miR-181d may bind directly with STAT3/5A and impair activation of the later (Ramlingam and Ziegelbauer, 2017).

Q8: Reviewer #4: The title of the manuscript remains unsupported by the results and should be modified: "Aberrant Activation of Hsa-miR-181d/STAT3 and Hsa-miR-181d/STAT5A Ratios Mediate the Anticancer Effect of Garcinol in STAT3/5A-Addicted GBM" as The authors have not demonstrated any 'aberrant activation of Has-miR-181-

d/STAT3-5' in GBM; Such an 'activation' certainly does not explain the effect of Garcinol (at best, an inhibiton of the phosphorylation of these STAT proteins, possibly mediated by a down regulation of several kinases (JAK2, ERK, Akt).

A8: We thank the reviewer for this comment. To allay the reviewer's concern, we have now modified the title of the manuscript. **Please kindly see title page, Lines 1-4.**

Enhanced Hsa-miR-181d/p-STAT3 and Hsa-miR-181d/p-STAT5A Ratios Mediate the Anticancer Effect of Garcinol in STAT3/5A-addicted Glioblastoma

Q9: Reviewer #4: The authors fail to demonstrate tha GBM are 'addicted' to STAT3/5 activation (in particular, given their lack of survival experiments using xenografts to demonstrate the absence of resistance mechanisms.

A9: We thank the reviewer for this important comments. We have made included the 'survival experiments using xenografts to demonstrate the absence of resistance mechanisms' suggested by the reviewer.

Please see the updated figure 5C of the effect of garcinol on the survival of mice GBM xenograft models.

Please kindly see our revised Results section, Lines 506-527.

3.5. Garcinol inhibits tumor growth in GBM mice models through inversely correlated STAT3/5A and hsa-miR-181d expressions

Having shown that treatment with garcinol suppresses the cancer stem cell-like phenotype of U87MG and GBM8401 cells in vitro, to determine the probable suppressive effect of garcinol on the formation and growth of tumor, in vivo, we generated NOD/SCID mice xenograft models derived by inoculation with 1×10^6 U87MG cells subcutaneously in the hind-flank. Mice were randomly placed into control or garcinol treatment group. We demonstrated that treatment with 1 mg/kg garcinol significantly reduced the size of tumors formed in the treated mice, compared to the untreated control group (U87MG: ~7.1-fold smaller, $p < 0.001$ by week 4) (Figure**s 5A**), without adversely affecting the mice body weight (**Figure 5B**). We also observed that mice treated with garcinol showed 100% survival as compared to 60% in the control counterparts, over the 4-week treatment period.

(**Figure 5C**). In subsequent experiments using protein lysates derived from the tumors extracted from the untreated and garcinol-treated mice, we demonstrated that compared to the untreated control group, STAT3, pSTAT3, STAT5A, p-STAT5A, Ki-67, and Bcl-xL protein expression levels were concomitantly suppressed, while Bax expression was significantly enhanced (**Figure 5D**). Moreover, for tumors extracted from the 1 mg/kg garcinol-treated U87MG tumor-bearing mice, compared to the untreated control group, STAT3, or STAT5A mRNA expression were suppressed by 4-fold ($p < 0.01$) or 3.87-fold ($p < 0.01$), while miR-181d expression was enhanced by 3.52-fold ($p < 0.01$) in the U87MG mice treated with 1 mg/kg garcinol (**Figure 5E**). These findings indicate that garcinol inhibits tumorigenicity and growth of GBM by abrogating STAT3/5A signaling, and upregulating hsa-miR-181d, with concomitant suppression of Ki-67 proliferation index and enhancement of Bax/Bcl-xL apoptotic ratio, in vivo.

Please kindly see our updated Figure 5 and its legend, Lines 529-538.

Figure 5. Garcinol inhibits tumor growth in GBM mice models through inversely correlated STAT3/5A and hsa-miR-181d expressions. Representative image and graph showing the effect of garcinol on the (A) tumor volume and (B) body weight of U87MG-tumor-bearing mice. p-values were determined by 2-way ANOVA. (C) Kaplan-Meier Survival curve. Mice treated with garcinol showed 100% survival as compared to 60% in the control counterparts, over the 4-week treatment period. (D) Representative images and histograms of the differential expression of STAT3, pSTAT3, STAT5A, pSTAT5A, Ki-67, Bax, and Bcl-xL proteins level in tumors extracted from mice bearing U87MG cell-derived tumors, treated with or without garcinol. (E) Histograms showing the effect of garcinol treatment on STAT3, STAT5A and miR-181d expression levels in U87MG-tumor-bearing mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$; GAPDH is loading control.