J Biosci (2019) 44:96
DOI: 10.1007/s12038-019-9904-4
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Calycosin down-regulates c-Met to suppress development of glioblastomas

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MS received 15 October 2018; accepted 9 June 2019; published online 7 August 2019

The antitumor effect of calycosin has been widely studied, but the targets of calycosin against glioblastomas are still unclear. In this study we focused on revealing c-Met as a potential target of calycosin suppressing glioblastomas. In this study, suppressed-cell proliferation and cell invasion together with induced-cell apoptosis appeared in calycosin-treated U251 and U87 cells. Under treatment of calycosin, the mRNA expression levels of Dtk, c-Met, Lyn and PYK2 were observed in U87 cells. Meanwhile a western blot assay showed that c-Met together with matrix metalloproteinases-9 (MMP9) and phosphorylation of the serine/threonine kinase AKT (p-AKT) was significantly down-regulated by calycosin. Furthermore, overexpressed c-Met in U87 enhanced the expression level of MMP9 and p-AKT and also improved cell invasion. Additionally, the expression levels of c-Met, MMP9 and p-AKT were inhibited by calycosin in c-Met overexpressed cells. However, an AKT inhibitor (LY294002) only effected on MMP9 and p-AKT, not on c-Met. These data collectively indicated that calycosin possibility targeting on c-Met and exert an anti-tumor role via MMP9 and AKT.

Keywords. AKT; calycosin; c-Met; glioblastoma; MMP9

1. Introduction

Glioma is the most common tumor in human brains. There are five classifications in glioma based on the degree of developments and about 75% of them are at grade III and IV which are considered as the highly risky grade (Silvia *et al.* 2014). Glioblastoma is a type of malignant glioma with only 14.6 surviving months on average after diagnosis (Ellingson *et al.* 2012). Furthermore, the traditional treatments are still the primary therapy but with a poor curative effect and prognosis (Ganipineni *et al.* 2018). Therefore, a better treatment is in great need.

Calycosin is a main bioactive compound extracted from *radix astragali* which is a common herbal medicine commonly used in traditional Chinese medicine (Gao *et al.* 2014). Early studies showed that calycosin has various pharmacological properties like anti-inflammatory, antioxidant and neuroprotective effects (Guo *et al.* 2012; Gao *et al.* 2014). Recently several studies indicated that calycosin had a promising antitumor effect in various tumor cells (Qiu *et al.* 2014; Chen *et al.* 2015; Tian *et al.* 2017). Although it has been proved that calycosin showed anticancer effects against glioblastoma, the direct binding target of calycosin is still unclear (Nie *et al.* 2016).

The receptor tyrosine kinase (RTK) family is consists of a series of cell-surface receptors and play important roles in regulating various cellular processes (Lemmon and Schlessinger 2010). A gene-chip analysis in our preliminary experiment showed that calycosin inhibited the expression level of RTKs (Dtk, c-Met, Lyn and PYK2). Among them, c-Met is well known as a receptor of the hepatocyte growth factor (HGF) and regulates morphogenesis in embryonal cells (Bladt *et al.* 1995). Recently c-Met was reported to be related to regulating cancer development in various tissues (Sennino *et al.* 2012; Han *et al.* 2014) and a series of tremendous progress have been made in the development of targeted agents against c-Met treating various cancers (Granito *et al.* 2015; Park *et al.* 2017).

In this study we investigated that whether calycosin could regulate the development of glioblastoma through c-Met. Our results showed that calycosin could suppress the proliferation and invasion in two glioblastoma cell lines U251 and U87. Additionally, we also explored the downstream molecule system of c-Met which was related to regulating invasion in glioblastoma cells. Therefore we detected the expression levels of matrix metalloproteinases (MMP9), AKT and P-AKT which are strongly associated with cell invasion in various cancer cells in U251 and U87 cells after treated with calycosin. Our results showed that calycosin could suppress the expression level of c-Met and the expression levels of MMP9 and P-AKT were also down-regulated followed by the inhibition of c-Met.

These findings suggest that c-Met may be a potential target for calycosin against glioblastomas.

2. Materials and methods

2.1 *Cell culture*

HEK293T, U251 and U81 cells used in the this research were bought from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). All these cells were cultured in Dulbecco's minimum Eagle's medium (DMEM) at 37°C and 5% CO_2 were added. At the same time, 10% fetal calf serum (FCS) and 1% antibiotic (penicillin/streptomycin) were supplemented in DMEM.

2.2 *Cell counting kit-8 (CCK-8) assay*

A CCK-8 assay was used to analyze the cell proliferation. In brief, every 5×10^3 cells were seeded onto one well of 96-well plate and cultured in a cell incubator (Thermo Forma 3111) with 5% CO_2 at 37°C overnight. Followed by treating with a bunch of different concentrations (0, 25, 50, 100, 200, 400 and 800 μM) of calycosin (Sigma, B9938-10MG), the cell proliferation rate was analyzed by a spectrophotometer (Perlong, DNM-9602) after being treated for 0, 24, 48 and 72 h.

2.3 *Cell apoptosis assay*

U251 and U87 cells were cultured with calycosin at 0, 100, 200 and 400 μM for 48 h separately. After this about $5–10 \times 10^4$ cells were collected from each well. Following the treatment with sufficient Annexin V-FITC (Beyotime, C1063) and propidium iodide (Beyotime, C1063), a FACS instrument (BD, Accuri C6) was used to analyze the cell apoptosis.

2.4 *Transwell analysis*

The cells treated with calycosin or LY294002 (Merck, CAS 154447-36-6) were cultured in DMEM without serum for 24 h, and then seeded about 5×10^4 cells into the upper chamber precoated with matrigel followed by adding 0.7 mL RPMI 1640 Medium (Gibco, 22400089) with 10% FCS into the lower chamber and then the cells were cultured at 37°C with 5% CO_2. After 24 h, the cells still on the upper surface were gently removed. The invaded cells were fixed by 4% formaldehyde solution (Sinopharm, Shanghai, China) for 10 min followed by dyeing with 0.5% crystal violet (Solarbio, C8470) for another 30 min. After being washed with PBS three times, the cells were counted under a microscope (Caikang, Shanghai, China) at ×200 magnification.

2.5 *Western blot assay*

The cell samples cultured with calycosin or LY294002 were washed three times with cold PBS and then lysed in Radioimmunoprecipitation assay lysis buffer. The supernatant containing extracted protein was collected, after 10 min centrifugation at 12,000 rpm under 4°C. A bicinchoninic acid protein assay kit (Thermo, PICPI23223) was used to determine the concentrations of these protein samples. About 25 μg proteins from each sample were separated by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel followed by transferring them onto a nitrocellulose (NC) membrane (Millipore, HATF00010). 5% skim milk was used to block the NC membrane with target proteins and then primary antibodies (MMP9, Abcam, Ab38898; C-met, Abcam, Ab51067; AKT, CST, #4685; P-AKT, CST, #4060; GAPDH, CST, #5174) were added. The appropriate secondary horseradish peroxidase-conjugated antibodies (HRP-labeled Goat Anti-Rabbit IgG, Beyotime, A0208; HRP-labeled Donkey Anti-Goat IgG, Beyotime, A0181 and HRP-labeled Goat Anti-Mouse IgG, Beyotime, A0216) were added to the NC membranes and then washed with Tris buffer with 1% Tween (TBST) three times. The target bands were visualized with enhanced chemiluminescence reagents (Millipore, WBKLS0100) and then the NC membranes with target bands were imaged in the Tanon-5200 system (Tanon) and then analyzed with Image J software.

2.6 *Real-time polymerase chain reaction (PCR) analysis*

The total RNA from cell samples was extracted by using Trizol reagent (Invitrogen, 1596-026) according to manufacturer's instruction. Equal amounts of RNA were used to reverse transcribed into complementary DNA (cDNA) with a RevertAid First Strand cDNA Synthesis Kit (Fermentas, K1622). The real-time PCR was conducted with a SYBR Green PCR kit (Thermo, K0223) on a ABI-7300 instrument (ABI). GAPDH was served as an internal control. The primer information is shown as follows: DTK, 5′ TGTGGC TCACGGTAGAAGG 3′ and 5′ GCTTGAAGGTGAACAGTGGC 3′; c-Met, 5′ TACCACTCCTTCCCTG 3′ and 5′ CATTTCCCTTAATTTCC 3′; Lyn, 5′ CCCTATGATGGCATCCAC 3′ and 5′ CTTTCTGCGTCCTTCCTG 3′; PYK2, 5′ AGATTCCCGACGAAACCC 3′ and 5′ GACAC CTTCATAGACCTCCC 3′ and GAPDH, 5′ AATCCCATCACCATCTTC 3′ and 5′ AGGCTGTTGTCATACTTC 3′.

2.7 *C-Met overexpression in U87 cells*

The full-length c-Met sequence was cloned into a lentiviral plasmid pLVX-puro (Clontech, USA). The constructed lentiviral vector or control vector was co-transfected into

HEK293T cells with helper plasmids. The supernatant containing target lentivirus was collected after 48 h and then infected U81 cells. The c-Met expression level was evaluated by the real-time PCR and western blot.

2.8 *Statistical analysis*

All experiments were performed at least three times and the data were analyzed by GraphPad Prism software Version 7.0 and mean ± standard deviation was shown. One-way or two-way analysis of variance (ANOVA) was performed to determine the statistical significance. Statistically significant was marked when $P<0.05$.

3. Results

3.1 *Calycosin suppressed cell proliferation in a dose-dependent way*

To study the inhibitory effect of calycosin on glioblastoma cell proliferation, we treated U251 and U87 cells with a bunch of different concentrations (0, 25, 50, 100, 200, 400 and 800 μM) of calycosin. As shown in figure 1, calycosin inhibited cell proliferation only at higher concentration (≥50 μM). When the concentration was 25 μM, calycosin had a promotion on cell proliferation in both U251 and U87 cells. So we chose 0, 100, 200 and 400 μM concentrations of calycosin to continue our following experiments.

3.2 *Calycosin inhibited induced cell apoptosis and cell invasion in U251 and U87*

We examined the change of cell apoptosis and cell invasion related to calycosin treatment to investigate the anticancer activity of calycosin in glioblastomas. The results from the CCK8 assay (figure 2A and B) showed that when the concentration of calycosin was above or equal to 100 μM, there was a significant inhibition of cell apoptosis in both U251 and U87 cells compared with the untreated groups. In addition, the results from a transwell assay showed that calycosin could inhibit cell invasion but only at higher concentrations. As shown in figure 2A and B, when the calycosin concentration was at 100 μM, there was no difference in U251 ($P>0.05$) or no extremely significant ($0.01<P<0.05$) in U87 cells on cell invasion compared with untreated groups.

3.3 *Calycosin inhibited the expression of RTKs in U87*

To investigate that RTKs were related to the anti-glioma effect of calycosin, 71 human RTKs had been detected with Human Receptor Tyrosine Kinase Phosphorylation Antibody Array 1 Kit (RayBiotech, USA, Norcross GA) in our pre-experiment. The expression levels of Dtk, c-Met, Lyn and PYK2 were down-regulated after being treated with calycosin.

Furthermore, the mRNA expression levels of Dtk, c-Met, Lyn and PYK2 were detected by the real-time PCR. In this study, we first analyzed the effect of calycosin on the expression of c-Met. As shown in figure 3A, low concentration of calycosin showed no significant effect on c-Met. However, the mRNA expression level of c-Met was suppressed, when the concentration of calycosin was higher than 100 μM. Based on this result, we further analyzed the mRNA expression levels of Dtk, Lyn and PYK2 in U87 cells which were treated with calycosin (0, 100, 200 and 400 μM). As shown in figure 3B, calycosin could inhibit the expression levels of Dtk, Lyn and PYK2 in U87 cells compared with untreated groups.

3.4 *C-Met acted as a potential target of calycosin in glioblastoma via MMP9 and AKT pathways*

To further analyze the downstream molecular mechanism of c-Met as a specific target of calycosin, we measured the expression levels of c-Met, MMP9, AKT and p-AKT in U251 and U87 cells after treating with 0, 100, 200 and 400 μM calycosin by western blot. As illustrated in

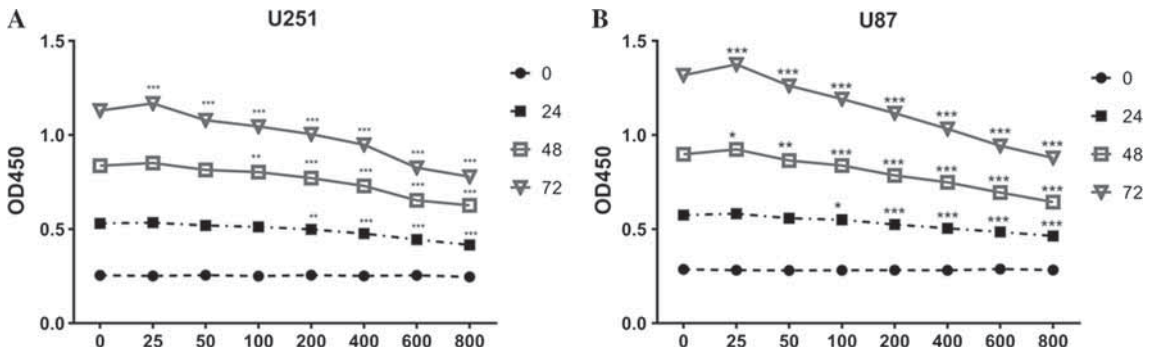

Figure 1. Calycosin suppressed cell proliferation in glioblastomas. Cell proliferations of U251 (**A**) and U87 (**B**) were analyzed by the CCK8 assay at 0, 24, 48 and 72 h after treated with different concentrations of calycosin (0, 25, 50, 100, 200, 400 and 800 μM). *$P<0.05$ *vs* control group (0 μM), **$P<0.01$ *vs* control group (0 μM), ***$P<0.001$ *vs* control group (0 μM).

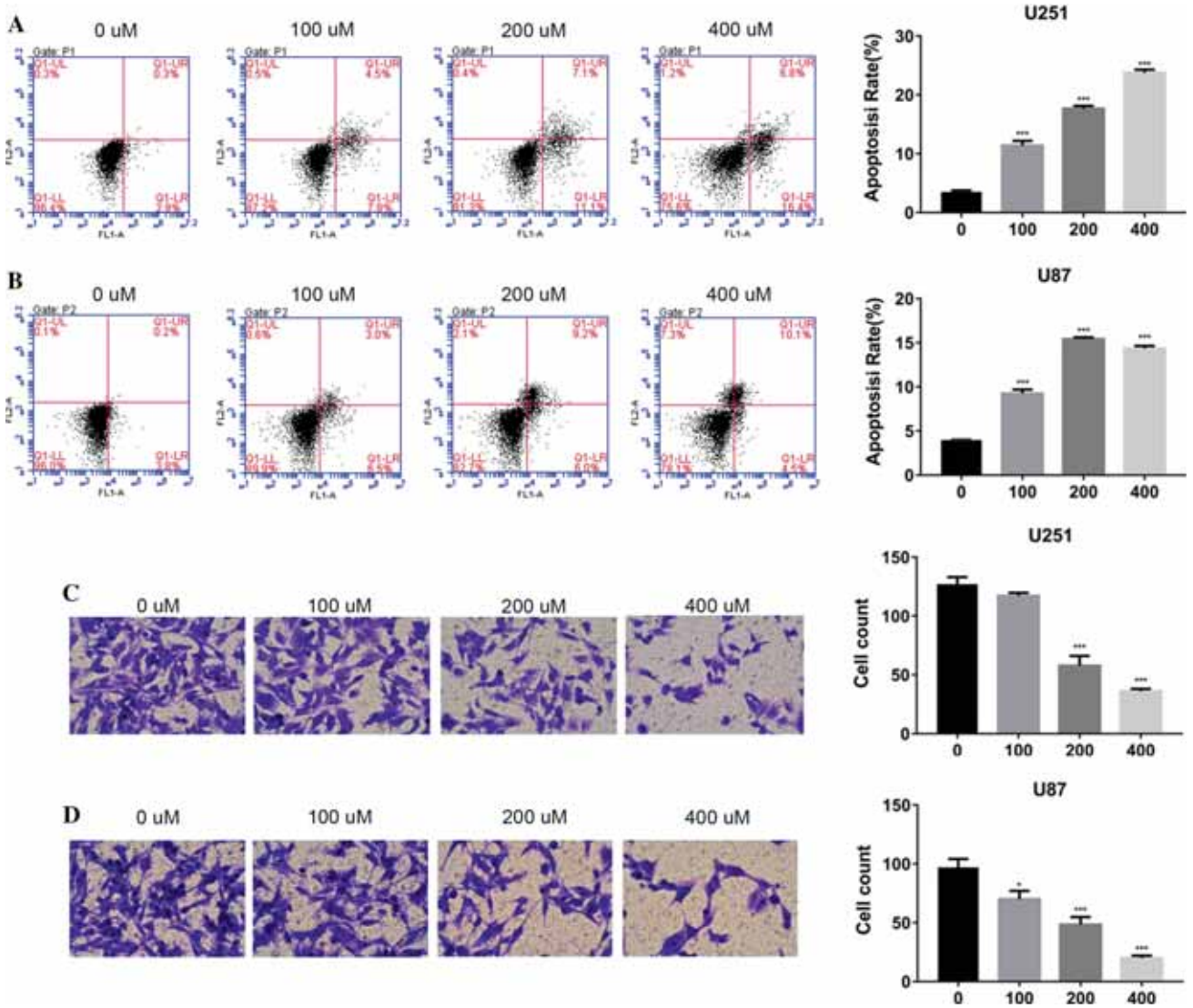

Figure 2. Calycosin suppressed cell invasion and induced cell apoptosis in glioblastomas. Cell apoptosis was analyzed by a flow cytometer in U521 (**A**) and U87 (**B**) after treated with calycosin at 0, 100, 200 and 400 μM. The invasion capacities of U521 (**C**) and U81 (**D**) were detected by the transwell assay after treated with calycosin at 0, 100, 200 and 400 μM. *P<0.05 *vs* control group (0 μM). ***P<0.001 *vs* control group (0 μM).

figure 3C and D, the protein expression level of c-Met was inhibited by calycosin in both U251 and U87 cells. Followed by the down-regulated c-Met, the expression levels of MMP9 and p-AKT were also reduced. These results indicated that the MMP9 and p-AKT were crucial downstream molecules of c-Met as a target of calycosin.

3.5 *Overexpression of c-Met suppressed the antitumor function of calycosin in U87*

To further evaluate the connection among calycosin, c-Met, MMP9 and AKT, we overexpressed c-Met in U87 cells. As shown in figure 4A and B, compared with control and vector groups, the expression level of c-Met was significantly improved in both mRNA and protein levels.

Afterwards, we used c-Met overexpressed and vector control cells to continue our further experiments. The results from the transwell assay (figure 5A) showed that overexpressing c-Met in U87 could improve cell invasion and those improvements were suppressed by the treatment of 200 μM calycosin or 10 μM LY294002, an inhibitor of AKT. At the same time the results from western blot (figure 5B) showed that the expression levels of MMP9 and p-AKT were up-regulated by c-Met overexpression compared with vector groups and these up-regulations were inhibited by calycosin or LY294002 significantly. Calycosin could also suppress the expression level of c-Met in both vector control and c-Met overexpressed cells. However, no significant inhibitory effect on c-Met expression was shown with the treatment of LY294002.

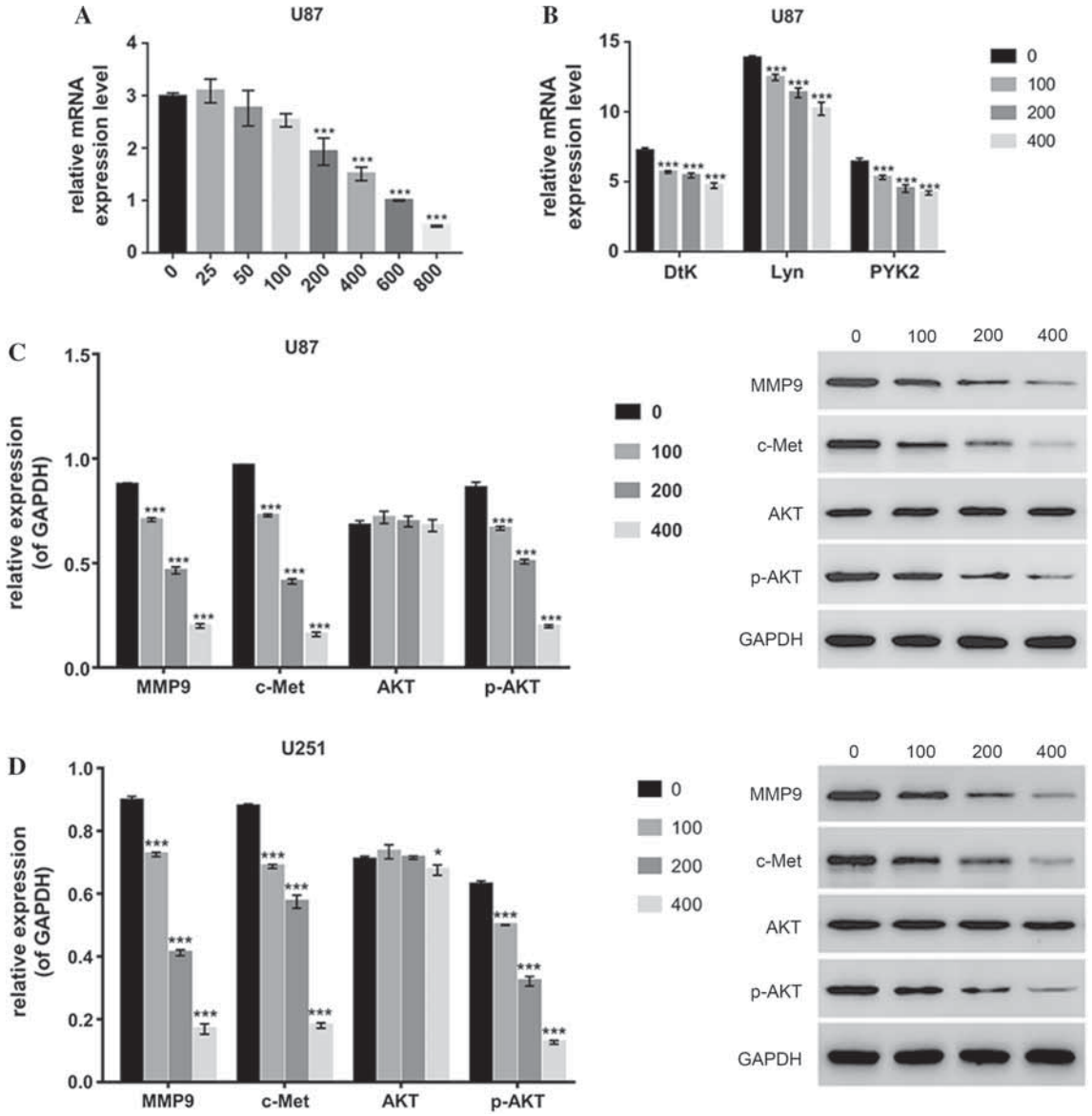

Figure 3. Down-regulation of RTKs by calycosin induces inhibition of MMP9 and p-AKT. (**A**) U87 cells were treated with different concentrations of calycosin (0, 25, 50, 100, 200 and 400 μM) and then the mRNA expression of c-Met were analyzed by the real-time PCR. (**B**) The mRNA expression levels of Dtk, Lyn and PYK2 in U97 cells treated with 0, 100, 200 and 400 μM calycosin were analyzed by the real-time PCR. The protein expression of c-Met, MMP9, AKT and p-AKT in U251 (**B**) and U87 (**C**) was analyzed by western blot after treated with different concentrations of calycosin (0, 100, 200 and 400 μM). **P*<0.05 *vs* control group (0 μM), ****P*<0.001 *vs* control group (0 μM).

4. Discussion

Glioblastoma is one of the most fatal tumors in brain with high invasion and proliferation rates as well as a character to escape from chemotherapy and radiation (Stupp *et al.* 2006). Therefore, more effective therapies are urgent need. The functions of calycosin have been studied for several years and among them the antitumor property is most attractive. Our research showed that calycosin could inhibit the development of glioblastoma by regulating cell proliferation, apoptosis and invasion at relatively high concentrations. However, the underlying mechanism of how calycosin suppress glioblastoma development is still unclear.

RTKs are reported to regulate a series of vital physiological activities in human, such as cell growth and survival. However, abnormal activation of RTKs has been found in a variety of cancers, therefore RTKs are also considered to regulate the occurrence and development of cancers (Regad 2015). The results from this research showed that calycosin suppressed the expression levels of Dtk, c-Met, Lyn and PYK2 which are family members of RTKs. As one of the most researched members of RTKs, c-Met is a specific receptor of the HGF, which is reported to involve in the recurrence of glioblastoma

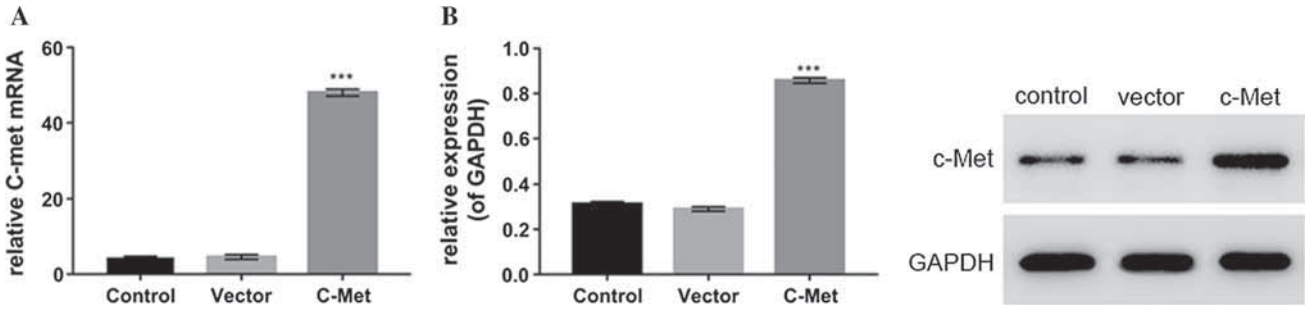

Figure 4. Overexpression of c-Met in U87 cells. Efficiency of c-Met overexpression on mRNA and protein levels was analyzed respectively by the real-time PCR (**A**) and western blot (**B**) in U87 cells. ***P<0.001 *vs* control. (Control: U87 cells without treatment; vector: U87 cells were infected with lentivirus expressing control vectors; c-Met: U87 cells were infected with lentivirus expressing c-Met).

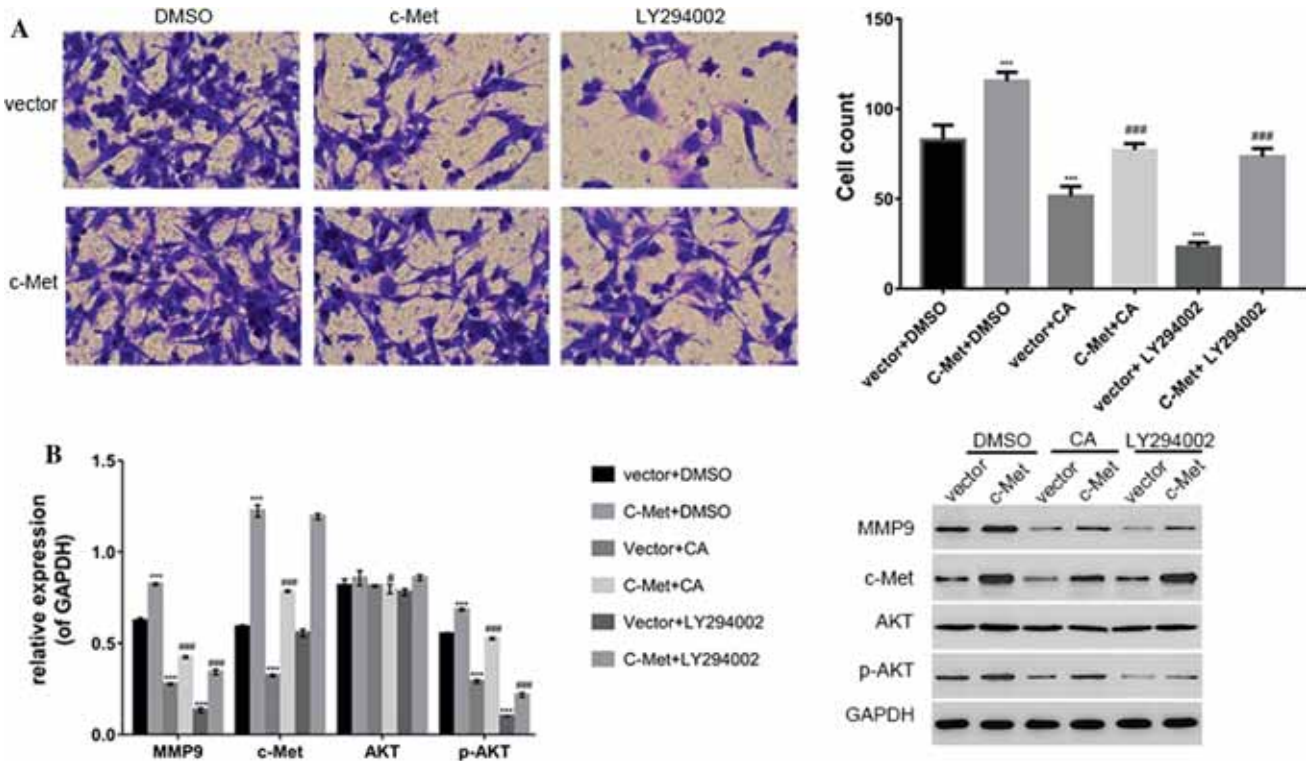

Figure 5. Overexpression of c-Met suppressed the antitumor function of calycosin via MMP9 and p-AKT. (**A**) The expression levels of c-Met, MMP9, AKT and p-AKT in vector control (vector) or c-Met overexpressed (c-Met) U87 cells were measured by western blot after treated by vehicle (DMSO), calycosin (CA) or LY294002, respectively. (**B**) The invasion capacity was analyzed by the transwell assay in vector control or c-Met overexpressed U87 cells after treated with vehicle (DMSO), calycosin (CA) or LY294002, respectively. ***P<0.001 *vs* vector+DMSO; $^{\#}P$<0.05 *vs* c-Met+DMSO, $^{\#\#\#}P$<0.001 *vs* c-Met+DMSO.

in patients (Liu *et al.* 2011). Furthermore, an anti-c-Met antibody, OA-5D5, has been reported to be involved in suppressing the growth of glioblastomas *in vivo* (Martens *et al.* 2006). Little is known about the effect of calycosin-inhibited c-Met on glioblastoma development, therefore we further investigated the change of some protein expression levels associated with tumor development to analyze whether they are related to the down-regulation of c-Met in U521 and U87 cells by calycosin treatment.

Epithelial–mesenchymal transition (EMT) was reported to play a crucial role in various malignant cancers and our previous research showed that EMT induced by MMPs was associated with glioblastoma development (Nie *et al.* 2016). MMP9 is a key member of the MMP family and plays a critical role in extracellular matrix degradation. Another protein closely related to oncogenesis is serine/threonine kinase AKT which is known as regulating various signaling pathways such as cell growth processes and metabolic activities (Bellacosa *et al.* 2004). Previous research studies showed that abnormal activation of AKT was related to non-small cell lung cancer and breast cancer (Brognard *et al.* 2001; Stemke-Hale *et al.* 2008) and calycosin could inhibit the phosphorylation of AKT to suppress tumor growth or enhance the efficiency of antitumor drugs (Chen *et al.* 2014;

Zhou *et al.* 2017). In this research the results from western blot showed that after treated with calycosin the expression levels of c-Met, MMP9 and p-AKT were obviously down-regulated in U251 and U87 cells. These results suggested that the anti-cancer activity of calycosin in glioblastoma was related to c-Met, MMP9 and p-AKT expression levels. However, the reaction order of these three proteins was still unclear. Previous studies showed that RTKs could active mTORC2 which further phosphorylated AKT (Manning and Cantley 2007). In addition, PI3K/AKT has been reported to be a downstream pathway of HGF/c-Met in regulating various cancers (Matsumura *et al.* 2013; Trovato *et al.* 2013). Therefore we speculated that the suppressed c-Met by calycosin may be the reason for the reduction of MMP9 and p-AKT expression levels. To prove this we overexpressed c-Met in U87 cells. Our further results from western blot showed that overexpressing c-Met in U87 indeed induced the expression of MMP9 and p-AKT significantly. Meanwhile, after being treated with the AKT inhibitor (LY294002), the expression level of c-Met was not influenced but the expression levels of MMP9 and p-AKT were significantly suppressed. These data indicated that c-Met was the upstream effector of AKT and followed by MMP9. At the same time, the results from western blot also showed that after being treated with calycosin, the expression levels of c-Met, MMP9 and p-AKT were suppressed concurrently. These results confirmed that c-Met is one of the major targets through which calycosin suppresses tumor cell growth. In addition, the results from the transwell assay showed that overexpressed c-Met promoted cell invasion in U87, while this increased invasion was inhibited by calycosin and LY294002 significantly. Together with the results from western blot, we concluded that calycosin targeted on c-Met and executed its anti-cancer programs via AKT and MMP9.

In summary, calycosin showed an antineoplastic activity on glioblastoma *in vitro* but only at relative high concentration. And the c-Met is an effective target for calycosin against glioblastoma followed by MMP9 and AKT pathways to execute anti-tumor efficacy.

Acknowledgements

Funding was provided by Social Development Project of Public Welfare Technology Research, from Science Technology Department of Zhejiang Province (Grant No. 2016C3220) and National Natural Science Foundation of China (Grant No. 81703752).

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Corresponding editor: Rita Mulherkar