



Alantolactone Suppresses YAP Signaling and Stemness Properties in Glioblastoma Cells

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ABSTRACT

Glioblastoma (GBM) remains one of the most common and lethal primary brain tumors due to its high malignancy and heterogeneity. Despite recent advances in medical technology and concerted effort to improve therapeutic methods, the prognosis of GBM still remains very poor. The poor prognosis is associated with cancer stem cells (CSCs) which hold the potential of self-renewal, and differentiation into pathogenomic heterogeneous tumor formation leading to therapeutic resistance. Therefore, targeting CSCs and their associated signaling pathways is crucial for effective treatment of GBM. In this study, we showed that alantolactone (ALT) hold the capacity to suppress stem-cell-like phenotype (SCLP) of glioblastoma cells. ALT inhibited the growth, reduced mammosphere formation and down-regulated the expression of CSCs markers such as CD133, OCT4, SOX2, and NANOG in U87 and U251 glioblastoma cells in a dose-dependent manner. Further mechanistic study showed that ALT inhibits glioma SCLP and tumor growth through repression of EGFR/MOB1/LATS1/YAP signaling pathway. These findings were further validated using *in vivo* xenograft mouse model. Taken together, the data provides convincing evidence of ALT-mediated inhibition of glioma SCLP and tumor growth via repression of EGFR/MOB1/LATS1/YAP pathway and suggests ALT as a promising therapeutic drug candidate, and YAP as a potential therapeutic target for glioblastoma treatment.

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Authors' Contribution

BY performed experimental work and wrote initial draft. MMZ and XC provided assistance during experimental work. YL and TM designed study and evaluated data. MK evaluated the data and edited manuscript

Key words

Glioblastoma, Stemness, YAP signaling, Alantolactone

INTRODUCTION

Glioblastoma (GBM) is considered one of the most common and lethal primary brain tumors. Even with gold standard treatment, the median survival of GBM remains approximately 13-16 months (Wang *et al.*, 2018; Luo *et al.*, 2021). The exact mechanism of GBM has not yet been fully explored, and it is significant to study the driving factors of glioma origin for accurate diagnosis and treatment. Although, GBM is very complicated, multifactorial and extremely heterogeneous cancer, considerable research evidence has shown that unlike bulk tumor population, glioma CSCs are relatively few in number and insensitive to chemotherapeutic agents, thus play critical role in malignant relaps, and resistance to gold

standard therapies (Tang *et al.*, 2021). Cancer stem cells (CSCs) are possessed with the capabilities for self-renewal and differentiation. They are blessed with extreme potential to divide asymmetrically to progenitor cells and also to new stem cells, leading to formation of heterogeneous tumor tissues which are phenotypically more diverse than the bulk tumor (Bao *et al.*, 2006). The aforesaid findings suggest that glioma CSCs are prime drug target and targeting stemness of glioma CSCs, and signaling pathways implicated in stemness of glioma CSCs could offer an unprecedented therapeutic opportunity for GBM.

The stemness properties of glioma CSCs are regulated by multiple signaling pathways including the Hippo signaling pathway (Hao *et al.*, 2014). The Hippo pathway

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List of abbreviations

GBM, Glioblastoma; CSCs, Cancer stem cells; SCLP, Stem-cell-like phenotype, ALT, Alantolactone; OCT4, Octamer-binding transcription factor 4; SOX2, Sex determining region Y-box-2; EGFR, Epidermal growth factor receptor; MST1/2, mammalian ste20 like 1 and 2; LATS1/2, large tumor suppressor land 2; YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; TCGA, The cancer genome atlas program; DMSO, Dimethyl sulfoxide; ECM, Extracellular matrix; DMEM, Dulbecco's Modified Eagle Medium; EGF, Epidermal growth factor; FGF, Fibroblast growth factor; BSA, Bovine serum albumin; PBS, Phosphate buffer saline; TEAD, Transcriptional enhanced associated domain.

is an evolutionarily conserved pathway among different organisms that controls organ size and tissue development. The Hippo pathway consists of a kinase signaling cascade which is composed of MST1/2 and LATS1/2 which control the nuclear translocation of YAP/TAZ. The Hippo pathway is activated by a myriad of intrinsic and extrinsic signals such as cell-cell contact, stress signals, stiffness of ECM and cell polarity. Upon activation, MST1/2 kinase phosphorylate and activate LATS1/2 kinase which in turn phosphorylate YAP/TAZ resulting in cytoplasmic retention or proteasomal degradation of YAP/TAZ. On the other hand, when Hippo pathway is switched off, unphosphorylated YAP/TAZ translocate into nucleus and regulates the expression of various genes involved in tumorigenesis and stemness properties of CSCs (Bae and Luo, 2018; Masliantsev *et al.*, 2021). It is well established now that Hippo pathways is frequently dysregulated in multiple human cancers including GBM leading to the unrestrained activity of its downstream target YAP/TAZ which is implicated in cell proliferation, tumor growth, stemness properties of CSCs and drug resistance (Pobbati and Hong, 2020). EGFR is most frequently amplified in GBM and considered as major driving force in development of GBM (Jureczek *et al.*, 2020). About 57.4% of patients with primary GBM have detectable EGFR gene amplification, resulting in high levels of EGFR protein contributing to tumorigenesis and progression (Kharbanda *et al.*, 2020). Moreover, EGFR activation has been shown to activate and translocate YAP/TAZ into nucleus (Huh *et al.*, 2019; Ando *et al.*, 2021).

Alantolactone (ALT) is a potent bioactive sesquiterpene lactone compound and has been extensively studied for its anticancer activity and anticancer mechanism in multiple human cancer cells (Babaei *et al.*, 2021) including lung cancer (Maryam *et al.*, 2017), liver cancer (Khan *et al.*, 2013), and GBM (Khan *et al.*, 2012) using *in vitro* and *in vivo* models. However, the effect of ALT on glioma CSCs and Hippo signaling pathway remains unexplored. In this study, we have investigated the effect and possible mechanism of ALT on Hippo pathway and stemness properties of glioma CSCs.

MATERIALS AND METHODS

Chemicals and reagents

ALT (purity >98%) was obtained from Meilunbio (Dalian, China). Antibodies against Phospho-MST1/MST2(80093-1-RR, Oct4 (60242-1-Ig), Nanog (14295-1-AP), CD133 (66666-1-Ig), and β -actin (81115-1-RR) were obtained from Proteintech (Wuhan, China) while EGFR (R22778), p-EGFR (R24173) were purchased from Zen-bioscience (Chengdu, China). All other antibodies were

obtained from CST.

Cell culture

The HCM3, U87 and U251 human glioma cell lines were obtained from the Laboratory of Pharmacy at Dalian, Medical University. HCM3, U87 and U251 cells were maintained in DMEM. All culture media were supplemented with 10% fetal bovine serum (FBS), U87/shYAP cells were obtained from lentiviral infection (Shumi, China). Overexpression was obtained from the recombinant lentiviral vector (Genechem, China), and the empty vector was used as a control.

Mammosphere formation assay

U87 (5×10^4) and U251 (5×10^4) cells were seeded in 6-well culture plates (Corning, USA) and treated with ALT. After drug treatment, cells were left to grow in serum-free DMEM containing 20 ng/ml EGF, (PeproTech, USA), 20 ng/ml basic FGF, (PeproTech), and B27 (Gibco) for 14 days. After two weeks, tumorspheres were examined and counted under a light microscope. Spheroid cells were collected for western blotting (Qin *et al.*, 2018).

Real-time PCR

RNA was extracted using an RNA extraction kit (YeaSEN, China) and reverse-transcribed with a high-capacity cDNA conversion kit (Vazyme, China). The expression of genes was estimated using SYBR Green master mix (YeaSEN, China).

YAP Forward: 5'-CCTGATGGATGGGAACAAGC-3'
Reverse: 5'-GCACTCTGACTGATTCTCTGG-3'.
 β -actin Forward: 5'-CCTTCCGTGTTCCCTACCCC-3'
Reverse: 5'-GCCCAAGATGCCCTTCAGT-3'.
 $\Delta\Delta$ CT equation was employed to calculate data and is expressed as multiples of change relative to a control sample.

Western blotting

After extraction and quantification, proteins were separated, transferred to membrane and subjected to immunoblotting as described by us previously (Maryam *et al.*, 2017).

Immunofluorescence

U87-spheroid cells were seeded into the L-PLL coated cover glass (Diamond, China) for 4 h followed by treatment with ALT (20 μ M) for 96 h. The cover glass was fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100/PBS and blocked with 5% BSA/PBS. The cover glass was incubated with anti-YAP antibody (#14074CST) and anti-CD133 antibody (66666-1-Ig, Protein Tech) overnight at 4 $^{\circ}$ C. Alexa Flour 568 and Flour 488 conjugated secondary antibody (CST, USA)

was added at room temperature for 1h. Nuclei was counter stained with mounting medium with DAPI (Abcam, USA). Laser-scanning confocal or Thunder fluorescence microscope (Leica, Germany) was used to take images.

Bioinformatic analyses

Genetic data from glioma samples were extracted from TCGA. The results of the analysis were used for graphing by GraphPad Prism software. Information on the three glioma subtypes (grade II, grade III, grade IV) was classified according to WHO classification. According to the WHO classification, each point in the dot plot represents an individual sample, which was assembled using glioma data. In an independent cohort study performing overall survival of glioma patients, glioma data downloaded from the TCGA website were divided into two groups; high expression and low expression groups. The data were divided into two groups based on median YAP expression. Survival curves were plotted using the Kaplan-Meier method, and comparative analysis of data between groups was performed using the Log-Rank test. GraphPad Prism 8.0 software was used for graphing. Glioma data were downloaded from the TCGA website to divide the cases into a high expression YAP group and a low expression YAP group.

Intracranial mouse model

The intracranial glioma mouse model was established by transfecting U87MG luciferase cells (3×10^5) and stereotactically implanting into the brains of nude mice (Qinglongshan Animal Breeding Center (Nanjing, China)). The nude mice used in this study were 4 weeks old. After 1 week, the mice were segregated into 2 groups randomly ($n = 10$). ALT was dissolved in DMSO and a total of 100 μ L ALT (20mg/kg b.w.) was injected to animal via intraperitoneal injection every two days for 2 weeks. The control group was injected with an equal volume of vehicle for 2 weeks. The intracranial tumor growth was detected by bioluminescence imaging on 21st days. Kaplan-Meier survival curves were plotted to determine the survival time and weight. Tumor tissues from brain were harvested at 21st days and subjected to H and E staining and immunohistochemistry following the established protocols.

Statistical analysis

Graphpad Prism was used to analyze data (mean \pm SD). Student's t-test and One-way ANOVA was applied on data to compare means between groups and within groups. Significance was marked as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

RESULTS

YAP is highly expressed in GBM cells and glioma stem cells

To study the differential expression and role of YAP in tumorigenesis, we studied the glioma cancer database of TCGA. A careful analysis of the TCGA database revealed that glioma cells with YAP transcripts ($n = 689$) exhibits a significantly higher expression level than normal samples ($n = 1157$) ($P < 0.0001$) (Fig. 1A). Using WHO expression profiling, YAP expression was also tested in the four grades. First, we found that G3 and G4 have the high expression levels of YAP ($p < 0.0001$) (Fig. 1A). We also evaluated the role of YAP as an oncogenic biomarker for overall survival of patients suffering from glioma cancer. Based on differential YAP expression, the clinical data from the TCGA database were classified into two groups. The findings showed a strong correlation between cancer expressing higher level of YAP and mortality ($P < 0.05$) (Fig. 1A). To validate the overexpression of YAP gene in glioma cancer, U87 and U251 glioma cells and human Microglia Clone 3 cells (HMC3) were used to examine YAP protein and mRNA expression by immunoblotting and q-PCR (Fig. 1B, C). We found that YAP expresses more in sphere cells than adhesion cells at both mRNA and protein levels (Fig. 1D, E). Moreover, the higher expression of YAP in glioma CSCs was further confirmed by immunofluorescence staining (Fig. 1F). The data suggest that YAP is highly expressed in glioma stem cells than glioma cells.

ALT suppresses stemness properties in glioma

First, we examined the effect of ALT on U87 and U251 cell viability by CCK8 assay. The cells incubated with ALT for 48 h exhibited significantly retarded cell proliferation in a dose-dependent fashion (Fig. 2B). Next, we evaluated the activity of ALT on mammosphere formation. To test the effect of ALT on U87- and U251-sphere formation, we treated the cells with ALT for 96 h, and found that ALT dose-dependently reduced the formation of glioma mammosphere (Fig. 2C) indicating that ALT has inhibitory effect on glioma CSCs. Moreover, Western blot data showed that expressions of glioma stem cells marker CD133 was downregulated after treatment with ALT in both U87 and U251 cells (Fig. 2D). These data suggest that ALT has suppressive effect on stemness properties of glioma cancer cells.

ALT suppresses stem-cell-like properties of glioma stem cells by suppressing YAP expression

Since ALT inhibited mammosphere formation and YAP is highly expressed in glioma mammosphere, we were

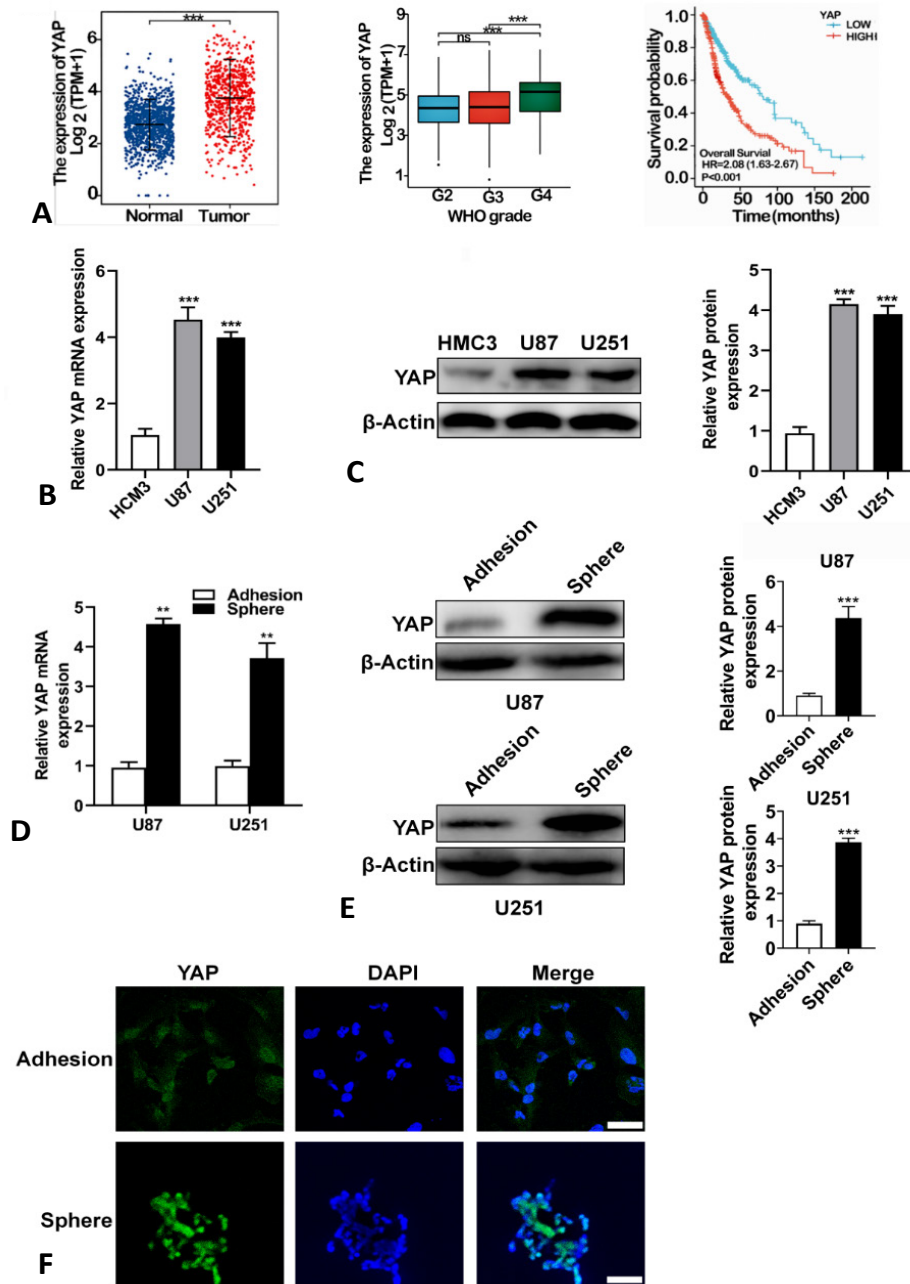


Fig. 1. YAP is highly expressed in glioma cells and glioma stem cells. (A) Higher expression of YAP in gliomas ($n = 689$) than in normal brain tissue ($n = 1157$) was obtained from the TCGA database ($***P < 0.0001$). Median and interquartile ranges are indicated by black lines. According to WHO grading criteria, ($n = 635$, G2=224, G3=243, G4=168). Medians with interquartile spacing are indicated by black lines. Gliomas ($n = 697$ patients) were evaluated with YAP mRNA levels and the results were correlated with an overall survival of 18 years. The red line indicates patients with high YAP transcripts ($n = 349$) and the blue line indicates patients with low YAP transcripts ($n = 348$). P values were analyzed by Kaplan-Meier analysis using the GraphPad prism. (B) YAP expression levels were detected in Human Microglia Clone (HCM3) and human glioma cancer cells (U87 and U251) by qRT-PCR. $*P < 0.05$, $**P < 0.01$. (C) YAP expression levels were detected in Human Microglia Clone (HCM3) and human glioma cancer cells (U87 and U251) by Western blotting $*P < 0.05$, $**P < 0.01$. (D) mRNA expression levels of YAP in spheroid cells and adherent cells. (E) Protein expression of YAP in spheroid cells and adherent cells. (F) Immunofluorescence staining of YAP in spheroid cells and adherent cells in U87 cells (scale bar = $50\mu\text{m}$).

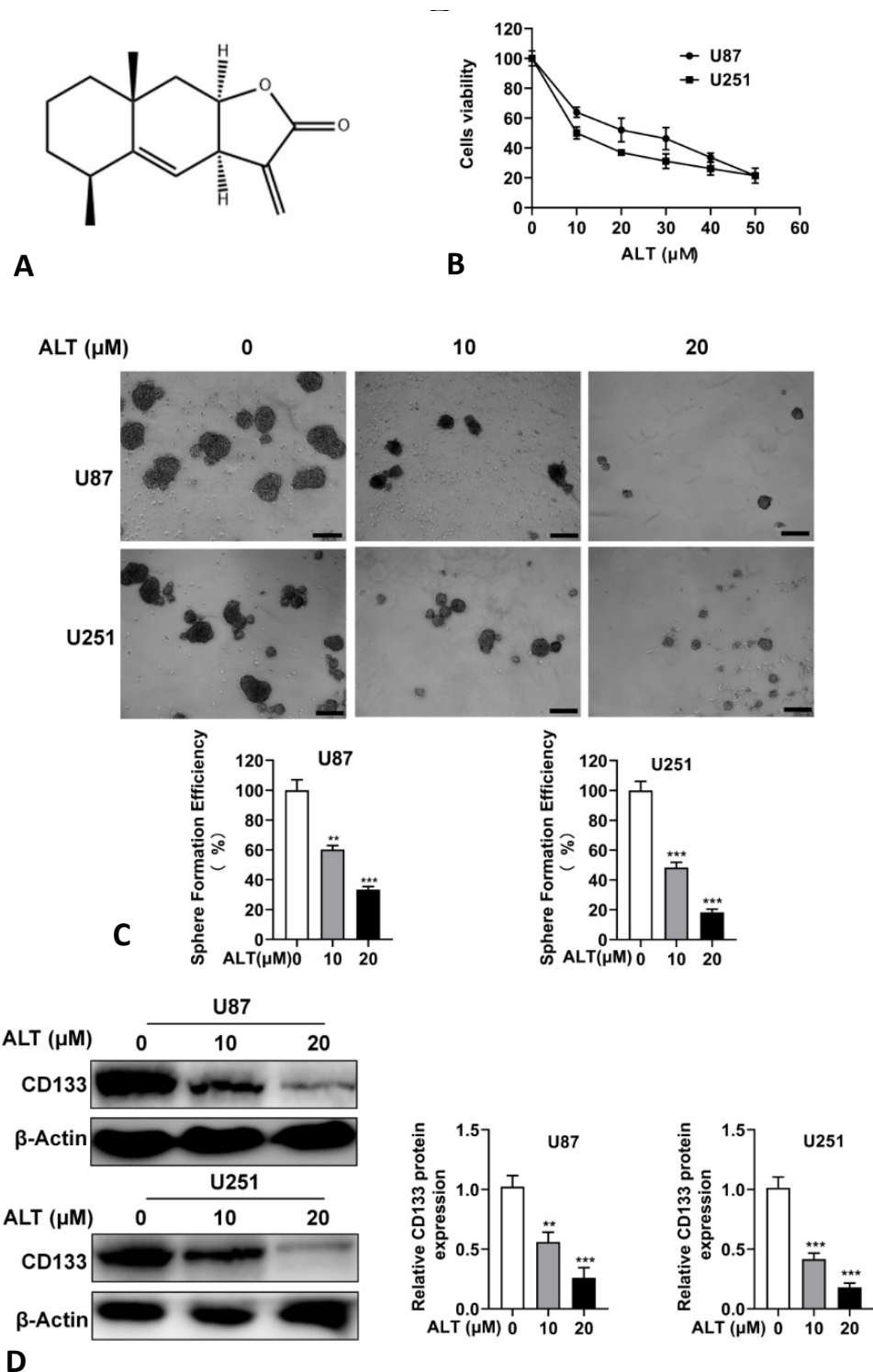


Fig. 2. ALT suppresses stemness properties of glioma. (A) Chemical structure of ALT. (B) U87 and U251 cells were treated with ALT for the 48 h and CCK8 assay was performed. (C) The cells were treated with ALT for 96 h and mammosphere formation was measured. Pictures were taken under a reverse microscope (scale bar = 200 μm). (D) U87- and U251-spheroid cells were treated with ALT for 96 h, and protein expressions level of glioma stem cells marker gene CD133 was analyzed by Western blotting.

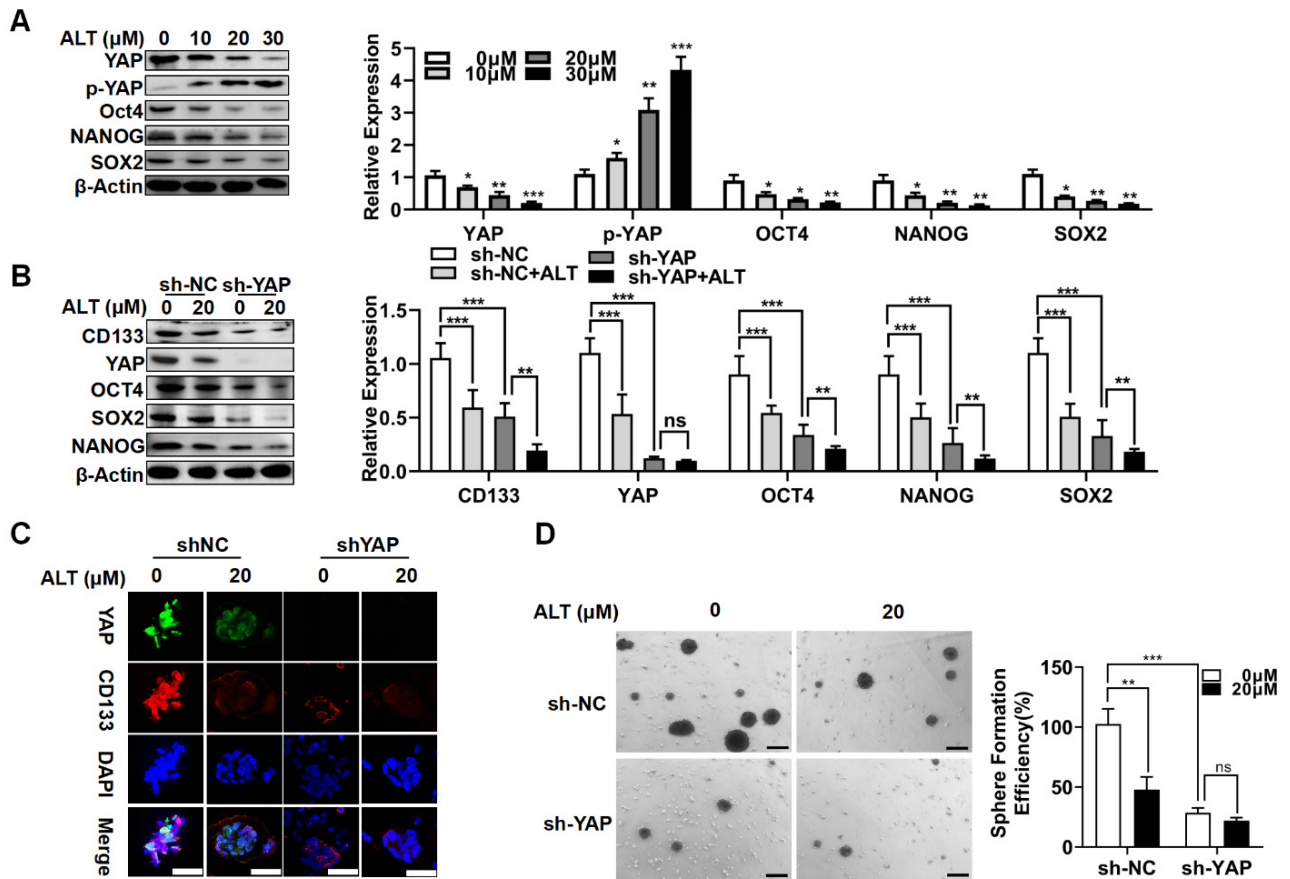


Fig. 3. ALT suppresses stem-cell-like properties of glioma CSCs via suppressing YAP expression. (A) Western blot was used to examine the expression of stem cell markers (SOX2, OCT4, NANOG) and YAP, p-YAP in U87-spheroid cells treated with ALT for 96 h. (B) U87-spheroid-shScramble cells and U87-spheroid-shYAP cells were treated with ALT for 96 h, and expression of CD133, YAP, OCT4, SOX2 and NANOG was measured by Western blotting. (C) U87-spheroid-shScramble cells and U87-spheroid-shYAP cells were treated with ALT (20 μM) for 96 h, and expression of CD133 and YAP was observed by immunofluorescence (scale bar = 50 μm). (D) Comparison of mammosphere formation between U87-shScramble or U87-shYAP cells treated with ALT for 96 h. (Scale bar = 200 μm).

interested to know whether ALT could inhibit YAP expression. For this, we measured the expression of YAP in mammosphere treated with ALT. The data showed that ALT dose-dependently inhibited the expression of YAP (Fig. 3A). We further measured the expression of various glioma CSCs markers including OCT4, SOX2 and NANOG in mammospheres treated with ALT. ALT significantly inhibited the expression of all these markers associated with stemness properties of glioma (Fig. 3A). In order to figure out the functional role of YAP in glioma stemness, we knocked down YAP in U87-spheroid cells and measured the expression glioma CSCs markers (CD133, OCT4, SOX2 and NANOG) by Western blot. YAP knockdown by shRNA significantly inhibited the expression of CD133, OCT4, SOX2 and NANOG and

addition of ALT further reduced the expression of all these markers (Fig. 3B). The expression of CD133 was also evaluated by immunofluorescence in YAP knockdown cells and data was in line with Western blot data (Fig. 3C). Moreover, knockdown of YAP significantly inhibited the formation of spheroids and additional treatment with ALT further inhibited the formation of spheroids (Fig. 3D).

In order to validate the above finding, we overexpressed YAP and measured the expression of CD133, OCT4, SOX2 and NANOG. The data demonstrated that overexpression of YAP significantly increased the expression of OCT4, SOX2, NANOG and CD133 (Fig. 4A, B). Addition of ALT remarkably suppressed YAP-induced expression of OCT4, SOX2, NANOG and CD133 (Fig. 4A, B). Furthermore, overexpression of YAP significantly promoted spheroid

formation, as well as their size and volume in U87 control cells, and addition of ALT restrained spheroid formation, as well as their size and volume (Fig. 4C). Taken together, the above data showed that YAP is a key factor affecting tumor stem cells and ALT affects glioma stem cell properties by inhibiting the expression of YAP.

ALT suppresses YAP expression via EGFR inhibition

A large body of research evidence has shown that the Hippo-YAP pathway plays a vital role in glioma CSCs (Hiemer *et al.*, 2014; Lau *et al.*, 2014; Bartucci *et al.*, 2015). Therefore, we sought to investigate if ALT could interfere with Hippo-YAP signaling pathway. Our immunoblotting data showed that ALT suppressed YAP expression and phosphorylation of EGFR, an upstream modulator of YAP signaling in a concentration-dependent way, in U87 stem cells (Fig. 5A). Taking into account that YAP is the downstream target of Hippo signaling cascade, we next evaluated the possible effect of ALT on Hippo signaling cascade molecules. LATS1 and MOB1 are upstream regulators of YAP, and phosphorylation of YAP by LATS1 promotes the cytoplasmic retention or proteasomal degradation of YAP. Therefore, we measured the expression and phosphorylation of YAP upstream regulators. Our findings showed that ALT treatment

dose-dependently induced phosphorylation of LATS1 and YAP and dephosphorylated MOB1 (Fig. 5A). To further corroborate the finding that ALT suppresses YAP signaling via EGFR inhibition, we used “erlotinib” a clinical inhibitor of EGFR as a single agent as well as in combination with ALT and measured the expression and phosphorylation of EGFR and its downstream targets. The data showed that erlotinib significantly reduced the phosphorylation of EGFR and MOB1 and increased the phosphorylation of LATS1 and YAP (Fig. 5B). The changes in phosphorylation status of aforesaid proteins were further augmented when cells were treated with a combination of erlotinib and ALT (Fig. 5B). These set of data clearly indicate that ALT suppresses YAP signaling via EGFR inhibition. We also measured the expression of YAP downstream targets (OCT4, SOX2, NANOG) in cells treated with erlotinib alone or in combination with ALT and found that both drugs inhibited the expression of OCT4, SOX2, and NANOG as a single agent however; erlotinib in combination with ALT synergistically inhibited the expression of OCT4, SOX2, and NANOG (Fig. 5C). Collective data from above set of experiments clearly demonstrated that ALT suppresses YAP signaling and stemness properties of glioma cells via EGFR inhibition.

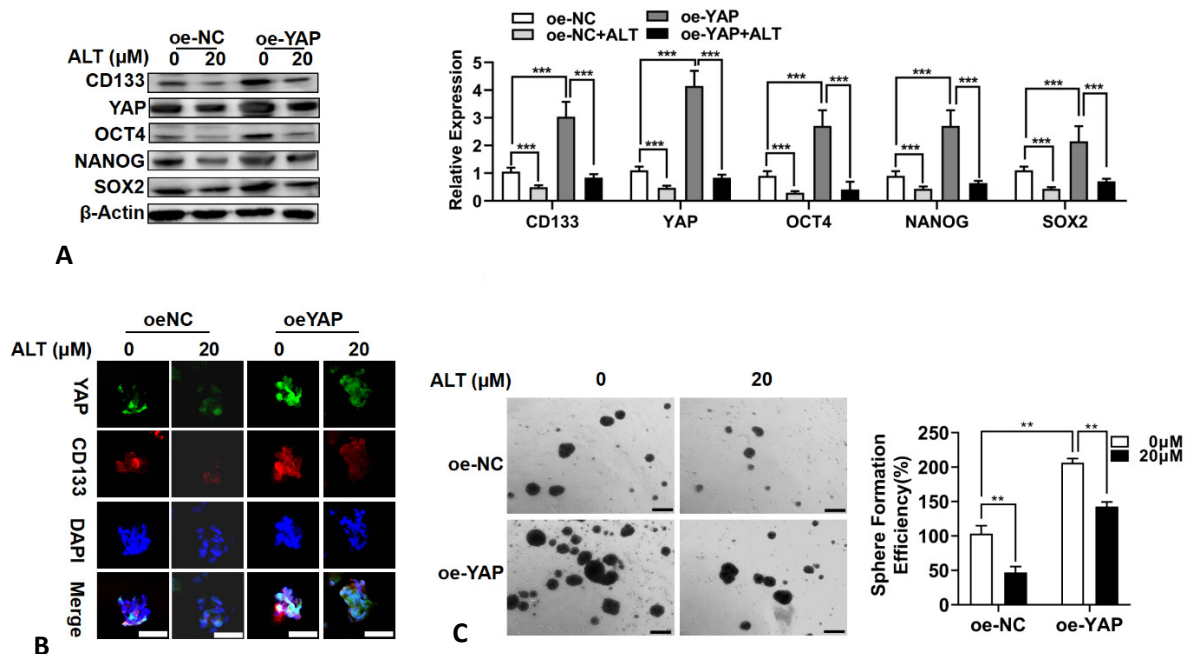


Fig. 4. ALT suppresses stem-cell-like properties of glioma stem cells via suppressing YAP expression. (A) U87 spheroid-oeScramble cells and U87 spheroid-oeYAP cells were treated with ALT for 96 h and expression of CD133, YAP, OCT4, SOX2 and NANOG was measured by Western blotting. (B) Immunofluorescence was used to detect the expression of CD133 and YAP in U87spheroid-oeScramble cells and U87spheroid-oeYAP cells (scale bar = 50 μm). (C) Comparison of mammosphere formation between U87-oeScramble or U87-oeYAP cells were treated with ALT for 96 h. (Scale bar = 200 μm).

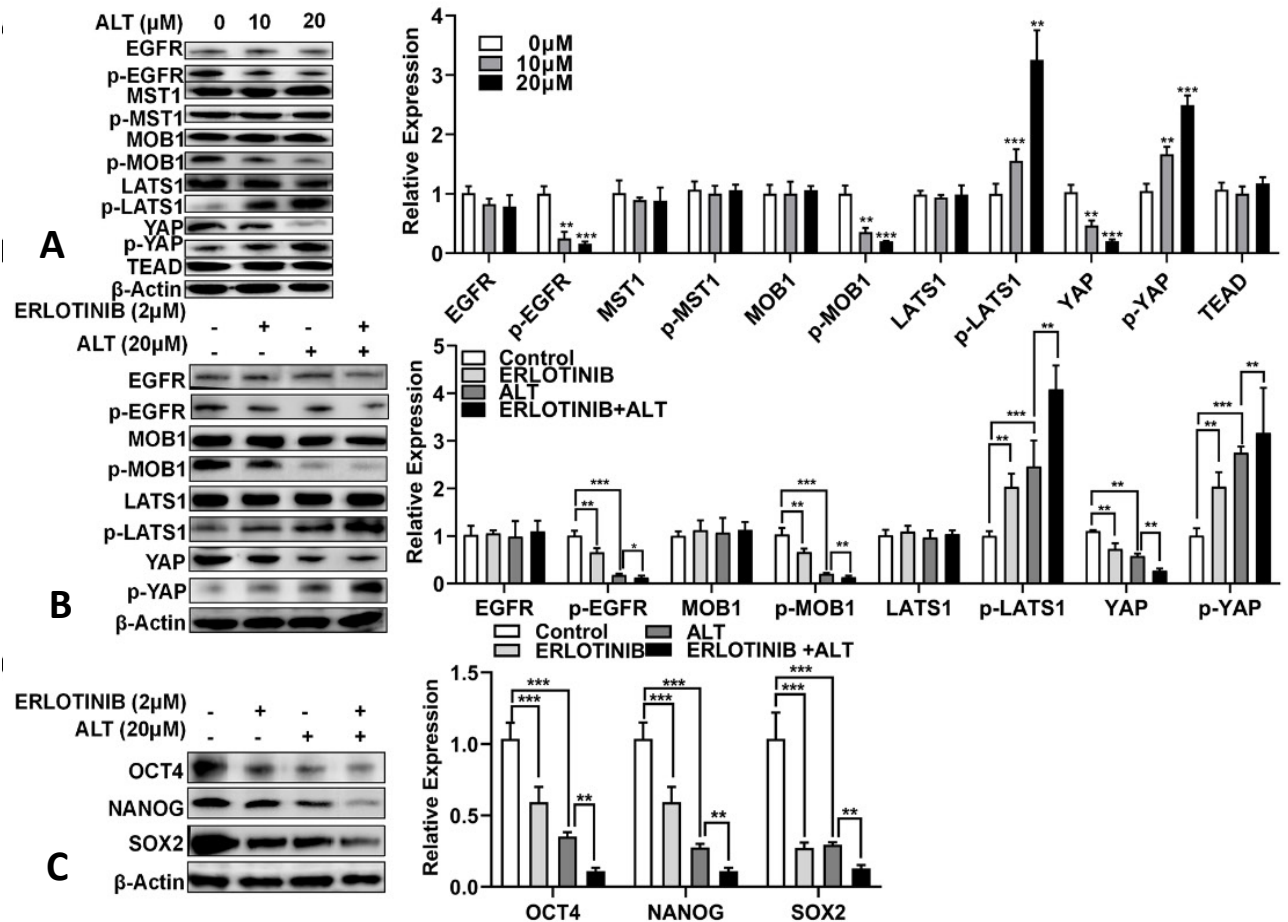


Fig. 5. ALT Suppresses YAP expression via EGFR inhibition. (A) Western blot was performed to detect the effect of ALT on the expression and phosphorylation level of various members of Hippo pathway in U87-spheroid cells. (B) Protein expression and phosphorylation level of various members of Hippo pathway in U87-spheroid cells treated with erlotinib or ALT as single agent or combination of both for 96 h was examined by Western blotting. (C) Western blot was used to examine the effect of erlotinib or ALT on the expression of OCT4, SOX2 and NANOG in U87-spheroid cells.

ALT inhibits the growth of glioma xenografts

Previously, it has been reported that ALT can cross the blood-brain barrier (Khan *et al.*, 2012). Therefore, we measured the growth inhibitory effects of ALT in GBM xenograft. Modified U87 luciferase cells were implanted in nude mice. After a week of normal feeding, ALT was injected intraperitoneally as described above. The survival rate of animals in treatment group was longer than those controls (Fig. 6A). The tumor sizes were significantly reduced in ALT group compared to control group as evident from bioluminescence imaging data (Fig. 6B). H and E staining of tumors revealed that tumor collected from treatment groups exhibits a smaller size and more defined borders as compared to control group (Fig. 6C). Our immunohistochemistry analysis revealed low expression of p-EGFR and high expression of p-YAP in

ALT group compared to control group (Fig. 6D). The data is in line with findings of *in vitro* studies.

DISCUSSION

Researchers are working to identify and develop new targets or therapeutic strategies for effective treatment of glioblastoma. Glioma CSCs are thought to be the root cause of glioma onset and progression. While most existing CSC studies have focused on stemness signaling regulation, we demonstrated that downregulation of YAP in glioma CSCs can inhibit the self-renewal capacity. Therefore, inhibition of YAP may be a potential therapeutic target for glioma. Since YAP is a key factor in the Hippo signaling pathway, exploring novel drugs that hold the capacity to inhibit the Hippo signaling pathway can be sought to suppress YAP

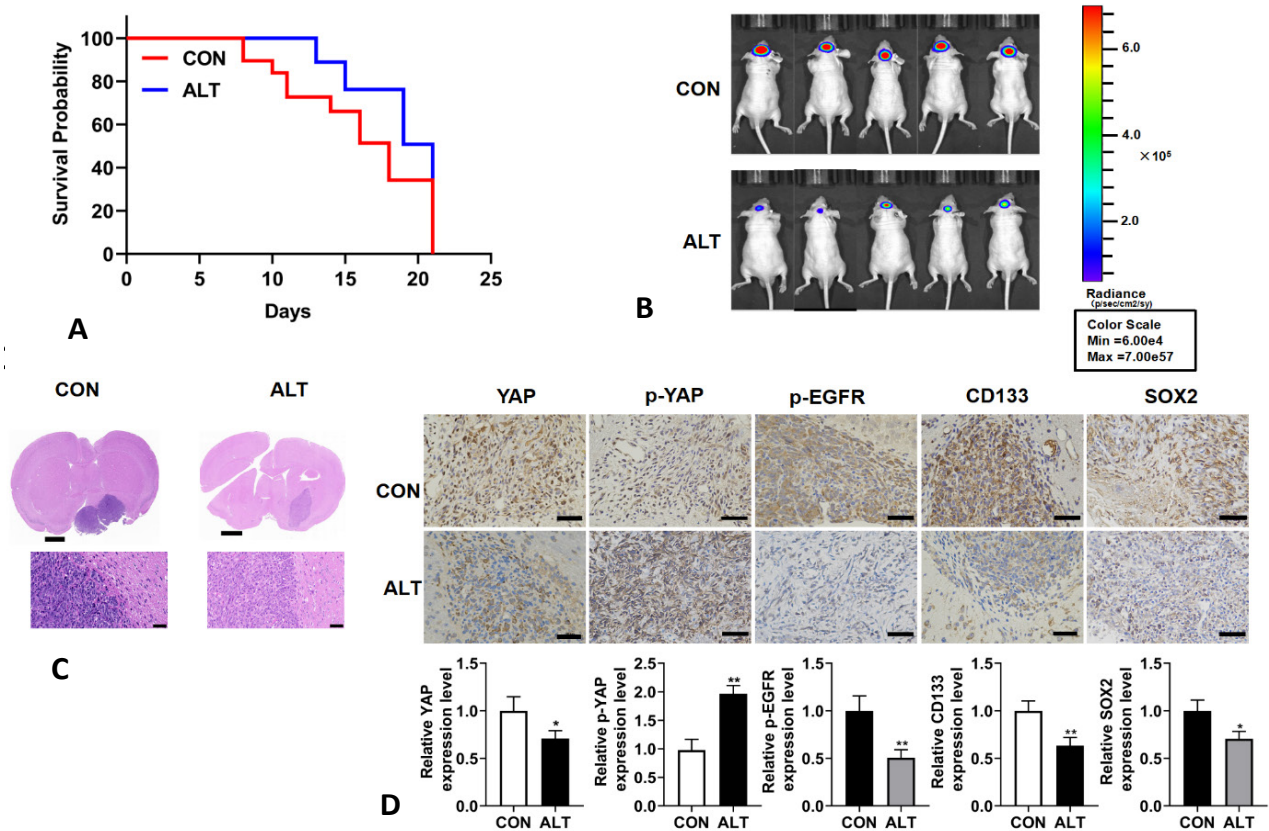


Fig. 6. ALT inhibits the growth of glioma xenografts. (A) Survival analysis for animals treated with 0.9% saline or ALT (20mg/kg) ($P < 0.01$ by log-rank test; n 0.9% saline = 12, n ALT = 12). (B) Bioluminescence imaging showed the tumor size. (C) H and E staining of sections from mouse brains with U87 xenografts treated with 0.9% saline or ALT (20mg/kg) at 21 days. (D) IHC for YAP, p-YAP, p-EGFR, CD133 and SOX2 in sections from indicated xenografts (scale bar = 100 μ m).

expression and provide ideas for clinical treatment. In the present study, we found that ALT could inhibit YAP expression and consequently inhibit glioma CSCs self-renewal. YAP may become a diagnostic and prognostic biomarker for glioma in future patients. Interestingly, YAP overexpression has also been implicated in the pathogenesis of oncogenic transformation and tumorigenesis in hepatocellular and colorectal cancers (Cheung *et al.*, 2020; Zhu *et al.*, 2020). Recent studies have shown that YAP, a specific key factor for maintaining self-renewal and maintenance of phenotype in CSCs, is inactivated by phosphorylation in the cytoplasm, or degraded upon binding to 14-3-3 protein, while unphosphorylated YAP enters the nucleus where it binds with TEAD and transcriptionally regulates downstream target genes associated with stemness properties of cancer cells (Kharbanda *et al.*, 2020). In a previous study, Ando *et al.* (2021) has shown that the EGFR suppresses the Hippo pathway by promoting the tyrosine phosphorylation of MOB1, that leads to YAP activation and translocation into

nucleus (Ando *et al.*, 2021). In this study, we demonstrated that ALT inhibits the stem cell-like properties of glioma cells and tumor growth by suppressing YAP signaling via EGFR inhibition through EGFR/MOB1/LATS1/YAP signaling cascade. However, the molecular mechanism involved in ALT mediated inhibition of EGFR remains unexplored in this study and needs to be further investigated.

In this study, we found that YAP may be a predictor of poor prognosis in gliomas. Previous clinico-pathological analyses have shown that YAP is overexpressed in aggressive gliomas (Orr *et al.*, 2011). Therefore, YAP expression levels may serve as a specific oncogene for glioma progression to predict patient prognosis. We further assessed that CSCs are blessed with stem cell properties, and ability to self-renew and are distinguished by symmetric or asymmetric cell division. Self-renewal and progenitor cell production in the cell population of CSCs has been shown to be the underlying cause of the poor outcome of radiotherapy and chemotherapy.

Therefore, the elimination of these cells is a novel strategy for cancer therapy. However, limited markers are available for the identification of glioma stem cells. Although researchers have demonstrated that CD133 is a key biomarker of glioma CSC (Chinot *et al.*, 2014), our data suggest that YAP gene is significantly associated with CSC characteristics such as self-renewal and formation of progenitor cells. It has been reported that YAP is involved in regulating CSCs-related proteins such as SOX2 and OCT4 (Sun *et al.*, 2020; Vigneswaran *et al.*, 2021). In line with the previous reports, YAP knockdown by shRNA repressed the expression of OCT4, SOX2, and NANOG and inhibited mammosphere formation in glioma cells while overexpression of YAP significantly increased the expression of OCT4, SOX2, and NANOG and promoted mammosphere formation. Our findings suggest that YAP gene expression confers stemness and may act as a marker

of CSCs in key glioma cancers (Zanconato *et al.*, 2016; Yang *et al.*, 2019; Yan *et al.*, 2020).

CONCLUSION

In conclusion, our data showed that YAP can regulate the biological activities of CSCs via EGFR//MOB1/LATS1/YAP signaling. These findings not only confirm the glioma inhibitory effect of ALT as previously reported, but also reveal novel cellular targets of ALT associated with glioma stemness. Moreover, the therapeutic efficacy of ALT was also evaluated using *in vivo* mouse model study. A schematic model showing the cellular targets and underlying mechanism of ALT implicated in suppression of YAP signaling and stemness properties of glioma cells has been shown in Figure 7.

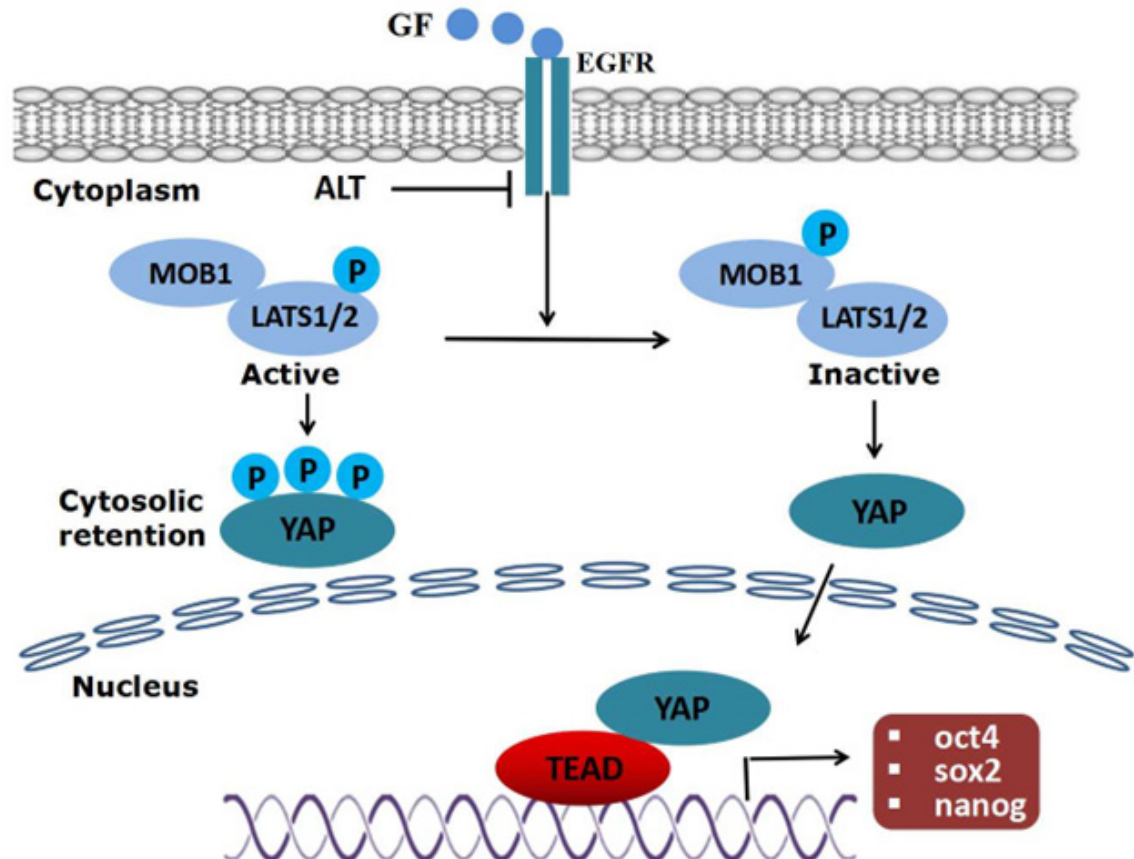


Fig. 7. A schematic diagram representing intervention of Alantolactone with EGFR-mediated YAP signaling pathway and stemness properties. In the absence of EGFR ligand (EGF), LATS1/2 is phosphorylated (active). Upon activation, LATS1/2 induces YAP phosphorylation which ultimately results in cytoplasmic retention or degradation of YAP. Upon ligation with growth factor, EGFR become activated leading to MOB1 tyrosine phosphorylation and LATS1/2 dephosphorylation (inactive). Inactivation of LATS1/2 results in translocation of YAP into nucleus where it interacts with TEAD transcriptional factor and induces the expression of various genes associated with stemness properties of cell.

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IRB approval

Animal studies were approved by the Committee on Animal Research and Ethics of Nanjing University of Chinese Medicine.

Statement of conflict of interest

The authors have declared no conflict of interest.

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