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Gambogic acid induces EGFR degradation and Akt/mTORC1 inhibition through AMPK dependent-LRIG1 upregulation in cultured U87 glioma cells

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common malignant tumor in adults' central nervous system (CNS). The development of novel anti-cancer agents for GBM is urgent. In the current study, we found that gambogic acid induced growth inhibition and apoptosis in cultured U87 glioma cells, which was associated with Akt/mTORC1 (mTOR complex 1) signaling in-activation. To restore Akt activation by introducing a constitutively active (CA) Akt attenuated gambogic acid-induced cytotoxicity against U87 cells. For mechanism study, we found that gambogic acid induced LRIG1 (leucine-rich repeat and Ig-like domain-containing-1) upregulation, which was responsible for EGFR (epidermal growth factor receptor) degradation and its downstream Akt/mTORC1 inhibition. Further, we provided evidence to support that AMPK (AMP-activated protein kinase) activation mediated gambogic acid-induced LRIG1 upregulation, U87 cell apoptosis and growth inhibition, while AMPK inhibition by shRNA or compound C reduced gambogic acid-induced EGFR/Akt inhibition and cytotoxicity in U87 cells. We here proposed novel signaling mechanism mediating gambogic acid-induced cytotoxic effects in glioma cells.

1. Introduction

Glioblastoma multiforme (GBM) is the most common malignant tumor in adults' central nervous system (CNS) [1–3]. Glioma cells show a high proliferation rate and diffusely infiltrate adjacent brain tissues, making complete surgical removal virtually impossible [1–3]. The current standard therapy of malignant glioma is postoperative radiation and/or temozolomide (TMZ). Currently, the mean survival time of patients diagnosed with malignant glioma is only around 14 months [4,5]. Consequently, development of novel anti-GMB agents is very important and urgent [1].

As the natural product isolated from gamboges, gambogic acid (C38H44O8) is a brownish dry resin exuded from the Garcinia hanburryi tree in Southeast Asia [6,7]. Many recent studies have shown the significant anti-cancer ability of gambogic acid. Gambogic acid inhibited the *in vivo* and/or *in vitro* growth of hepatocarcinoma, gastric carcinoma, lung carcinoma, breast cancer cells [8–10] and glioma cells [11,12]. The underlying mechanisms of gambogic acid's potent anti-tumor activity are, however, not fully understood. Although recent studies have shown that gambogic acid induces cancer cell G_2-M phase arrest, p53-dependent apoptosis and represses telomerase reverse transcriptase [8,13,14].

In malignant glioma, epidermal growth factor receptor (EGFR) over-activation and/or over-expression promotes glioma cell progression. Activated EGFR activates its downstream signaling pathways i.e. phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) complex 1 (mTORC1) cascade to promote glioma cell survival, metastasis and proliferation [15,16]. Meanwhile, activated EGFR also undergoes self-degradation [17]. Different groups have identified multiple endogenous EGFR negative regulators [18–20] such as leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) [19,21]. LRIG1 forms a complex with EGFR to cause EGFR degradation [22]. In malignant glioma cells, LRIG1 upregulation suppresses cell growth by attenuating EGFR expression [19]. These studies raised the possibility that LRIG1 might act as a endogenous suppressor of glioma [20,23].

In the current study we found that gambogic acid induces EGFR degradation and Akt/mTORC1 in-activation through AMP-activated protein kinase (AMPK) dependent-LRIG1 upregulation in cultured

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U87 glioma cells, these signaling events are responsible for U87 cell growth inhibition and apoptosis by gambogic acid.

2. Material and methods

2.1. Chemical and reagents

RAD001(Everolimus), MK-2206, Compound C, PD 153035, and AICAR (5-amino-1-β-Dffff-ribofuranosyl-imidazole-4-carboxamide) were obtained from Calbiochem (Darmstadt, Germany), Anti-EGFR, LRIG1, Akt, AMPK, acetyl-CoA carboxylase (ACC), S6K antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Mouse monoclonal antibody against tubulin was purchased from Sigma (Sigma, Shanghai, China), other antibodies used in this study were purchased from Cell Signaling Tech (Denver, MA).

2.2. Cell culture

U87 glioblastoma cells were maintained in RPMI 1640 (Sigma, St. Louis, MO), supplemented with a 10% FBS (Sigma), penicillin/streptomycin (1:100; Sigma) and 4 mM L-glutamine (Sigma), in a CO_2 incubator at 37 °C.

2.3. CCK-8 cell viability assay

Cell viability was measured by Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) assay according to manufacturer's protocol as reported [24]. The OD value of group received the indicated treatment was normalized to OD value of vehicle control group.

2.4. Clonogenicity assay

U87 cells (5×10^3) were suspended in 1 ml of RPMI containing 1% agar (Sigma, St. Louis, MO), 10% FBS and with indicated treatments or vehicle controls. The cell suspension was then added on top of a pre-solidified 1% agar in a 100 mm culture dish. The medium was replaced every 2 days. After 8 days of incubation, the number of colonies was manually counted in each group.

2.5. Quantification of apoptosis by enzyme-linked immunosorbent assay (ELISA)

The Cell Apoptosis ELISA Detection Kit, purchased from Roche (Palo Alto, CA), was used to quantify U87 cell apoptosis according to the manufacturer's protocol as reported early [25].

2.6. Caspase-3 activity assay

Cytosolic proteins of U87 cell were extracted in hypotonic cell lysis buffer (25 mm HEPES, pH 7.2, 5 mm MgCl2, 5 mm EDTA, 5 mm dithiothreitol, 0.05% phenylmethylsulfonyl fluoride). A total of 20 μg of cytosolic extracts was added to caspase assay buffer (312.5 mm HEPES, pH 7.5, 31.25% sucrose, 0.3125% CHAPS) with benzyloxycarbonyl-DEVD-7-amido-4-(trifluoromethyl)coumarin as substrates (Calbiochem, Darmstadt, Germany). Release of 7-amido-4-(trifluoromethyl)coumarin (AFC) was detected, after 1 h of incubation at 37 °C with a fluorescence reader (BD), set to an excitation value of 355 nm and emission value of 525 nm. The results were expressed as relative fluorescence units/μg of protein, and the number in the treatment group was normalized to the number of control group.

2.7. Protein isolation, western blot and quantification

The cells were washed with ice-cold phosphate buffered saline (PBS) and then lysed using lysis buffer (pH, 7.4) containing 1% NP-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 150 mmol/L sodium chloride and 10 mM Tris–HCl. The lysates were separated on 10% SDS–polycrylamide gel, and after electro-blotting onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA), the membranes were blocked with 10% milk in PBS plus Tween-20 (0.5%), incubated overnight at 4 °C with the primary antibody, and then incubated with HRP-conjugated anti-rabbit/mouse IgG. Detection was performed by Supersingnal West Pico Enhanced Chemiluminescent (ECL) Substrate. The blot intensity was quantified by ImageJ (Free download from NIH website). The intensity of each phosphorylated blot was normalized to the intensity of non-phosphorylated control blot. The number was expressed as fold change vs. Vehicle control group, Vehicle was expressed as "1.00".

2.8. RNA interference (RNAi)

SiRNA duplexes against LRIG1 were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Lipofectamine™ 2000 was applied to transfect RNAi (100 nM/well) into U87 cells according to manufacturer's protocol [26]. Same amount of scramble siRNA (Santa Cruz Biotech, Santa Cruz, CA) was transfected into control cells. After 48 h, the LRIG1 expression in transfected cells was examined by western blots to confirm knockdown efficiency.

2.9. AMPKα shRNA and stable cell selection

The lentiviral particles containing AMPKα1/2 short hairpin RNA (shRNA) (Santa Cruz Biotech, Santa Cruz, CA) or scramble shRNA control (Santa Cruz Biotech) [27] were added to U87 cells for 24 h, cell culture medium was then replaced by fresh medium and cells were cultured for additional 24 h. Stable clones expressing target shRNA were selected by puromycin (0.5 μg/ml). Cell culture medium was replaced with fresh puromycin-containing medium every 24 h, until resistant colonies can be identified. The expression level of AMPKα1/2 was always detected by western blots in the resistant colonies.

2.10. Constitutively active Akt (CA-Akt) transfection and stable cell selection

2 μg/well of plasmid encoding a constitutively active Akt (CA-Akt, a gift from Dr. Zhi-gang Bi [28]) was transfected to U87 cells through Lipofectamine PLUSTM protocol as described [28]. 12 h after transfection, cells were switched back to culture medium (RMPI + 10% FBS) and cultured for additional 48 h. Stable clones expressing target shRNA were selected by puromycin (0.5 μg/ml). Cell culture medium was replaced with fresh puromycin-containing medium every 24 h, until resistant colonies can be identified.

2.11. Statistical analysis

The data presented were mean ± standard error (SE). Statistical differences were analyzed by one-way ANOVA followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 15). Values of $p < 0.01$ were considered statistically significant.

3. Results

3.1. Gambogic acid induces growth inhibition and apoptosis in cultured U87 glioma cells

To test the potential anti-glioma effect of gambogic acid, we administrated gambogic acid to cultured U87 glioma cells. Cell counting (Fig. 1A), CCK-8 cell viability assay (Fig. 1B) and "clonogenicity" survival assay (Fig. 1C) were utilized to test U87 cell proliferation. Meanwhile, histone-DNA ELISA (Fig. 1D) and caspase-3 activity assay (E) were performed to examine U87 cell apoptosis. Results in Fig. 1 clearly demonstrated that gambogic acid dose-dependently induced growth inhibition and apoptosis in U87 glioma cells (Fig. 1). These results confirmed the cytotoxic effects of gambogic acid against U87 cells, as shown in Fig. 1F.

3.2. Gambogic acid inhibits Akt/mTORC1 signaling in cultured U87 glioma cells

Activation of Akt/mTOR signaling is critical for glioma cell proliferation and survival [29]. By using western blots testing indicated phosphorylated proteins, we found that gambogic acid significantly suppressed Akt/mTOR activation in cultured U87 cells (Fig. 2A and B). Note that mTOR complex 1 (mTORC1) activation was reflected by phosphorylation of S6 K (Thr 389), S6 (Ser 235/236) and 4E-BP1 (Ser 65) (Fig. 2B), while Akt activation was reflected by phosphorylation of Akt (Ser 473 and Thr 308) (Fig. 2A). Importantly, we found that EGFR inhibitor PD 153035 (PD) almost blocked the above mentioned signalings in U87 cells (Fig. 2A and B). These results confirmed that gambogic acid inhibits Akt/mTORC1 activation in cultured U87 glioma cells. An constitutively-active Akt (CA-Akt) was introduced to U87 cells to restore Akt activation in gambogic acid-treated U87 cells. Results in Fig. 2C showed that CA-Akt restored U87 cell viability after gambogic acid treatment. On the other hand, MK-2206, a novel Akt inhibitor [30], and RAD001, the mTORC1 inhibitor [31], inhibited U87 cell growth (Fig. 2D), more importantly, combination of the two resulted a further reduction of cell viability (Fig. 2D). These results together suggest that gambogic acid inhibits Akt/mTORC1 activation in U87 cells, which might be responsible for growth inhibition and/or cell apoptosis.

3.3. LRIG1 induction by gambogic acid promotes EGFR degradation, growth inhibition and apoptosis in cultured U87 glioma cells

The above results showed that both gambogic acid and EGFR inhibitor PD 153035 supressed Akt/mTORC1 signaling, we then tested the potential role of gambogic acid on EGFR expression in U87 cells. Western blot results in Fig. 3A showed gambogic acid inhibited EGFR expression and Akt activation while up-regulating its negative regulator LRIG1. Meanwhile, inhibition of LRIG1 using target RNAi restored EGFR expression and Akt activation in gambogic acid-treated U87 cells (Fig. 3A). Further, LRIG1 RNAi also suppressed gambogic acid-induced apoptosis (Fig. 3C) and viability loss (Fig. 3B). Interestingly, EGFR inhibitor PD 153035 also promoted U87 cell apoptosis and viability loss, and LRIG1 RNAi had no effects on PD 153035 (Fig. 3B and C). These data suggest that gambogic acid induces EGFR degradation and Akt inhibition through LRIG1 upregulation in U87 cells.

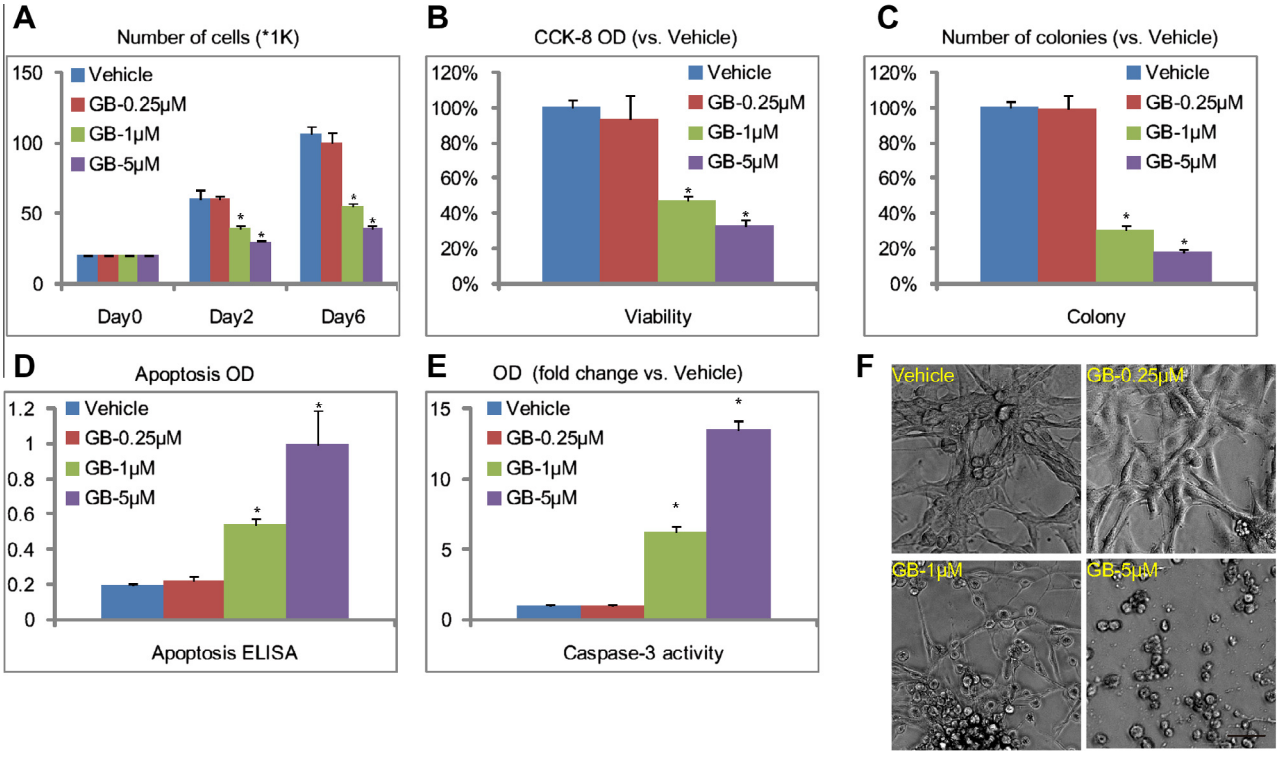

Fig. 1. Gambogic acid induces growth inhibition and apoptosis in cultured U87 glioma cells. *In vitro* cultured U87 glioma cells were treated with vehicle (0.1% DMSO) or indicated concentration of gambogic acid (GB, 0.25, 1 and 5 μM), cells were further cultured for 2 and 6 days, cell number was counted and recorded (A), cell viability was analyzed using CCK-8 assay 48 h after indicated treatment (B), number of large colonies was also manually counted 8 days after treatment, (C) histone-DNA ELISA was performed 36 h after drug administration (D), while caspase-3 activity assay was performed 24 h after treatment (E). Cell morphology after 4 days of drug treatment was also shown in (F). Values in this figure were expressed as mean ± SE, experiments were repeated three times and similar results were obtained. $^{*}p < 0.01$ vs. vehicle control group. Bar = 25 μm.

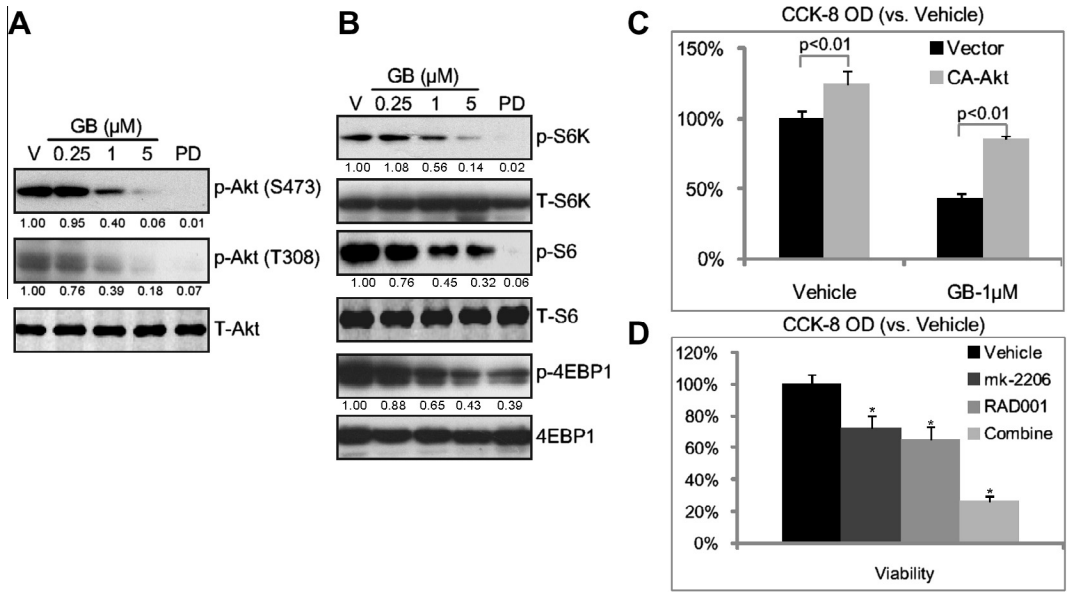

Fig. 2. Gambogic acid inhibits Akt/mTORC1 signaling in cultured U87 glioma cells U87 glioma cells were treated with indicated concentration of gambogic acid (GB, 0.25, 1 and 5 μM) or EGFR inhibitor PD 153035 (10 μM) for 12 h, phospho- and nonphospho levels of Akt (Ser 473, Thr 308), S6 K (Thr 389), S6 (Ser 235/236) and 4E-BP1 (Ser 65) were tested by western blot (A and B). The intensity of indicated blot was quantified as described (A and B). Vector- or constitutively active Akt- (CA-Akt) transfected stable U87 cells were treated with 1 μM of gambogic acid (GB) for 48 h, cell viability was analyzed by MTT assay (C). U87 glioma cells were treated with Akt inhibitor MK-2206 (1 μM), mTORC1 inhibitor RAD001 (1 μM) or a combination of both, cell viability was analyzed by CCK-8 assay (D). Values in this figure were expressed as mean ± SE, experiments were repeated three times and similar results were obtained. $^{*}p < 0.01$ vs. vehicle control group.

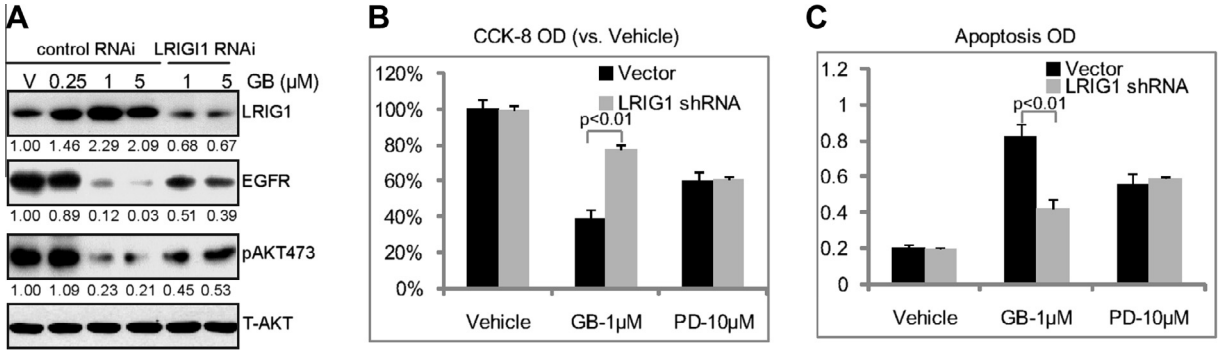

Fig. 3. LRIG1 induction by gambogic acid causes EGFR degradation, growth inhibition and apoptosis in cultured U87 glioma cells. Scramble and LRIG1 siRNA transfected U87 cells were treated with vehicle or indicated concentration of gambogic acid (GB), after 24 h, expression of LRIG1, EGFR, p-Akt (Ser 473) and total-Akt were tested by western blot (A). Above cells were also treated gambogic acid (1 μM) or EGFR inhibitor PD 153035 (PD, 1 μM), cell viability and apoptosis were analyzed by CCK-8 assay (B) and histone-DNA ELISA assay (C), respectively. Values in this figure were expressed as mean ± SE, experiments were repeated three times and similar results were obtained.

3.4. AMPK activation mediates gambogic acid- induced LRIG1 upregulation, apoptosis and growth inhibition in cultured U87 glioma cells

We then studied the molecular mechanisms of gambogic acid-induced LRIG1 induction by focusing on AMPK signaling. Western blot results in Fig. 4A showed a clear AMPK activation in gambogic acid-treated U87 cells. Compound C, the AMPK inhibitor, and shRNA-mediated AMPK knockdown reduced gambogic acid-induced AMPK activation (Fig. 4A) and LRIG1 expression (Fig. 4B). Reversely, AMPK activator AICAR promoted LRIG1 expression in U87 cells (Fig. 4C). Importantly, as shown in Fig. 4D and E, AMPK inhibition by compound C and shRNA suppressed gambogic acid-induced U87 cell apoptosis (Fig. 4D) and viability loss (Fig. 4E). These data together suggest that AMPK activation mediates gambogic acid-induced LRIG1 upregulation and cytotoxicity in U87 cells (Fig. 4F).

4. Discussion

In the current study, we found that gambogic acid induces apoptosis and growth inhibition in cultured U87 glioma cells, which is associated with inactivation of of Akt/mTORC1 signaling. Restoring Akt activation by introducing a CA-Akt inhibited gambogic acid-mediated cytotoxic effects against U87 cells. Gambogic acid induces LRIG1 upregulation to cause EGFR degradation and its downstream Akt/mTORC1 inhibition. Further, AMPK activation is required for gambogic acid-induced LRIG1 upregulation and its cytotoxicity in U87 cells.

Although several studies have implicated the function of LRIG1 in the inhibition of tumorigenesis in glioma, whether gambogic acid-induced anti-glioma effects are also associated with LRIG1 is not known. Further the signaling mechanism regulating its expression remains obscure. Recent studies have shown that multiple anti-cancer drugs including vincristine [32,33], taxol [34,35],

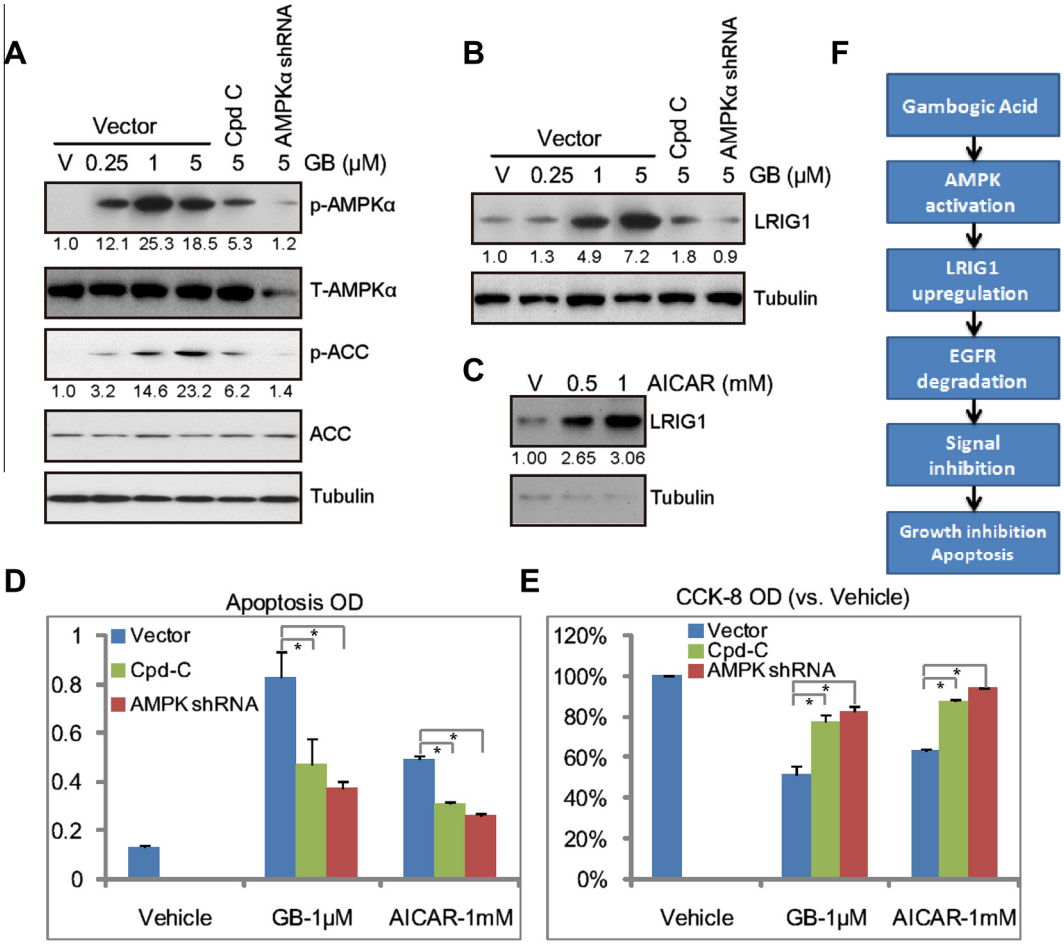

Fig. 4. AMPK activation mediates gambogic acid- induced LRIG1 upregulation, apoptosis and growth inhibition in cultured U87 glioma cells. Vector- or AMPKα shRNA – transfected stable U87 cells were treated with vehicle (0.1% DMSO) or indicated concentration of gambogic acid (GB), after 6 h, phospho- and total- AMPKα (Thr 172), ACC (Ser 79) and total-tubulin were tested by western blots (A); After 24 h, LRIG1 and tubulin were also tested (B). U87 cells were treated with vehicle (0.1% DMSO) or indicated concentration of AICAR (0.5 and 1 mM) for 24 h, LRIG1 and tubulin were tested by western blot (C). The effect of compound C (Cpd C, 10 μM) or AMPK shRNA on gambogic acid (GB, 1 μM) or AICAR (1 mM)-induced apoptosis ELISA (D) and viability loss (E) were tested, respectively. (F) The proposed signaling pathways involved in this study, gambogic acid activates AMPK to promote LRIG1 upregulation, the latter promotes EGFR degradation and Akt/mTORC1 inhibition, eventually causing U87 cell growth inhibition and apoptosis. Values in this figure were expressed as mean ± SE, experiments were repeated three times and similar results were obtained. $^{*}p < 0.01$.

temozolomide [36] and doxorubicin [37,38] activate AMPK-dependent cell apoptosis pathways. Importantly, the study by Zhao et al., confirmed AMPK can be activated by gambogic acid [39]. Here we provided evidence to support that AMPK activation is important for gambogic acid-induced LRIG1 upregulation, Akt/mTOR inhibition, and cytotoxicity in U87 cells. We proposed new signaling pathways mediating gambogic acid-induced cytotoxic effects and new downstream target of AMPK to achieve its anti-tumorigenesis ability. More evidence is, however, needed to explore how AMPK activation promotes LRIG1 expression. Also same experiments need to be repeated in other cancer cell lines.

In consistent with other studies, our inhibitors data suggested that Akt/mTORC1 activation is required for glioma cell proliferation and survival. Studies in other cancer cell lines have confirmed that gambogic acid suppresses Akt/mTOR activation [40,41], however, the underlying mechanism is not well-studied. Here we proposed that LRIG1-dependent EGFR degradation might be responsible for Akt/mTORC1 inhibition by gambogic acid. The fact that LRIG1 knockdown by RNAi restored Akt activation and cell growth in gambogic acid-treated U87 cells further supported our hypothesis. Future studies will be focusing on the detailed link between LRIG1 expression, EGFR degradation and Akt/mTOR inhibition in glioma cells after gambogic acid treatment. Further, the significance of these signaling changes observed *in vitro* needed to be explored in *in vivo* models.

In conclusion, we found that AMPK-dependent LRIG1 upregulation mediates gambogic acid-induced cytotoxic effects in U87 glioma cells, probably through promotes EGFR degradation and Akt/mTORC1 inhibition.

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