



Targeting PLA2G16, a lipid metabolism gene, by Ginsenoside Compound K to suppress the malignant progression of colorectal cancer

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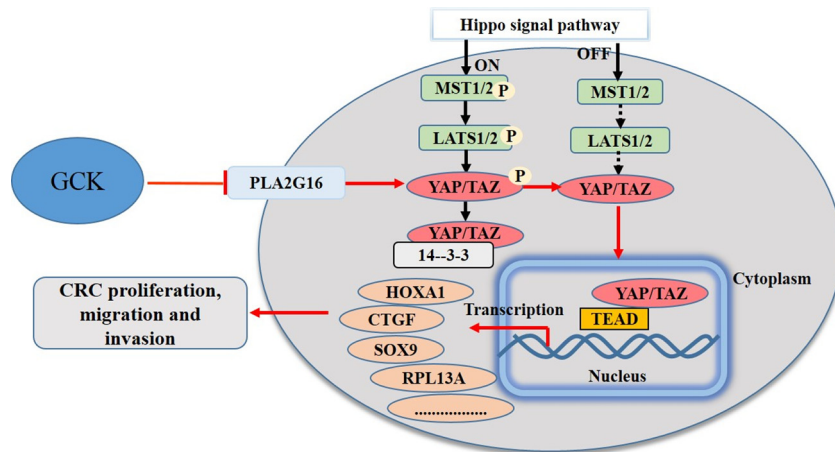
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HIGHLIGHTS

- PLA2G16 is up-regulated in CRC, and high expression of PLA2G16 is associated with the advanced stages.
- PLA2G16 promotes the malignant progression of CRC through the Hippo signaling pathway.
- GCK exerts its anti-CRC effects by inhibiting the protein expression of PLA2G16.
- Provide a new insights towards the development of effective therapeutic strategies for CRC treatment by targeting PLA2G16.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 7 March 2021

Revised 16 April 2021

Accepted 9 June 2021

Available online 12 June 2021

Keywords:

Colorectal cancer

PLA2G16

Ginsenoside Compound K

ABSTRACT

Introduction: Colorectal cancer (CRC) is a common malignant tumor with a high global incidence, metastasis rate and low cure rate. Changes in lipid metabolism-related genes can affect the occurrence and development of CRC, and may be a potential therapeutic target for CRC. Therefore, starting from lipid metabolism-related genes to find natural medicines for tumor treatment may become a new direction in CRC research.

Objectives: This study aimed to investigate the effect of PLA2G16, a key gene involved in lipid metabolism, on the biological function of CRC, and whether the anti-CRC effect of GCK is related to PLA2G16.

Methods: To explore the role of PLA2G16 in CRC *in vitro* and *in vivo*, we performed cell proliferation, migration, invasion and nude mice tumorigenesis assays. As for the mechanism, we designed RNA-seq

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Lipid metabolism gene
Lipidomics
Hippo signaling pathway

analysis and verified by western blotting and immunofluorescence experiments. Subsequently, we found the anti-CRC effect of GCK is related to PLA2G16 through western blotting and rescue experiments.

Results: We showed that PLA2G16 was significantly higher in CRC tissues than the adjacent normal appearing tissues, and high PLA2G16 expression correlates with unfavorable prognosis of CRC patients. Further, PLA2G16 promoted the malignant progression of CRC by inhibiting the Hippo signaling pathway determined by RNA-seq analysis, and GCK exerted anti-CRC effects by inhibiting the protein expression of PLA2G16 *in vitro* and *in vivo*.

Conclusion: Our results suggested that PLA2G16 promote the malignant progression of CRC by inhibiting the Hippo signaling pathway and the anti-CRC effect of GCK is through inhibiting the protein expression of PLA2G16.

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Introduction

Colorectal cancer (CRC) is a gastrointestinal malignancy with a high global incidence rate and degree of metastasis as well as a low survival rate [1]. It was reported that the incidence and mortality rates of CRC in the 20- to 30- year-old group is expected to increase by 90% and 124.2%, respectively, by the year 2030 [2]. In China, according to the 2018 cancer statistics, CRC ranks fourth in incidence and fifth in mortality among men, while it ranks third in incidence and fourth in mortality among women [3]. In addition, it's reported that high-fat diet may drive the progression of CRC, leading to increased mortality [4]. Abnormal lipid metabolism is one of the hallmark features of cancer cells. Lipids or lipid metabolites provide energy for the growth and metastasis of cancer cells [5,6]. Changes in the lipid or lipid metabolism-related genes could affect the occurrence and development of CRC, and may act as potential biomarkers for early detection of CRC [7,8]. Therefore, the key genes related to lipid metabolism might be a potential target for CRC treatment. Further elucidation of the underlying mechanisms could have important clinical significance.

Lipid or lipid metabolites promote the proliferation and invasion of cancer cells by aiding in the synthesis of biofilm synthesis and the production of cholesterol lipids [9,10]. Abnormal lipid metabolism is observed in CRC patients [11]. Free fatty acids (FFA) in adipocytes can provide energy for the growth of cancer cells, and promote proliferation and invasion [6]. Lipid metabolism is regulated by the enzyme, adipocyte-specific phospholipase A2 (PLA2G16), also known as AdPLA, which is a rate-limiting enzyme in the process of FFA biosynthesis, and is specifically and highly expressed in adipocytes. Its mRNA expression is 1,000-100,000 times higher than that of other enzymes in the PLA2 family [12]. PLA2G16 plays an important role in adipocyte lipolysis and obesity formation. Jaworski's research team found that PLA2G16 null mice exhibited a significant reduction in adipose tissue and triglyceride and they also showed that PLA2G16 worked against obesity caused by leptin deficiency or high-fat diet *via* the PGE₂/EP3/cAMP pathway [12,13]. Meanwhile, studies showed that PLA2G16 plays an oncogenic role in several cancers. It's revealed that high expression of PLA2G16 may be an independent prognostic factor for the poor overall survival of patients with osteosarcoma [14]. Li found that PLA2G16 promoted metastasis and drug resistance through the MAPK pathway in osteosarcoma [15]. Moreover, PLA2G16 increased the growth of non-small cell lung (NSCL) cancer by activating the mitotic pathway [16]. In addition, microarray-based gene expression profiling analysis of tumor revealed an increased expression of PLA2G16 in NSCL cancer, colon cancer, rectal cancer, and gastric cancer, suggesting that PLA2G16 might play an oncogenic role in these tumors [16]. However, the specific role of PLA2G16 in CRC and the underlying mechanisms have not been clarified.

At present, the clinical treatment of CRC mainly includes surgery, chemotherapy, and radiotherapy [17,18]. However, surgical

intervention is only suitable for patients who are diagnosed at an early stage. The efficacy of chemotherapy is affected by resistance to chemotherapy and serious adverse reactions [19,20]. In recent years, it has become important to search for lead compounds that are not only effective but also have fewer side effects, this led to an increased interest in natural products [21]. Ginsenoside Compound K (GCK) is a natural diol-type ginsenoside Rb1, Rb2, and Rc metabolites in the human intestine [22]. It has various pharmacological activities, such as cardiovascular protection along with anti-inflammatory and neuroprotective effects as well as anti-tumor activity in several tumors [23–26]. Clinical randomized double-blind experiments have shown that GCK is safe and well-tolerated in healthy subjects [27]. Additionally, in adipocytes (3T3-L1 cells), GCK has been shown to inhibit lipid deposition, the expression of lipid genes, leptin and PPAR γ , accumulation of triglycerides, and lipid droplet synthesis by activating the lipid metabolism-related genes such as PPAR α and MAPK. Thus, it has been used to treat diseases related to lipid accumulation [28,29]. In addition, GCK could reduce the expression of PGE2 catalyzed by PLA2G16 [30,31]. Therefore, we speculated that the anti-CRC effect of GCK is might be related to PLA2G16.

In our study, we demonstrated that PLA2G16 was up-regulated in CRC tissues, which was associated with the poor prognosis in the patients of CRC. Mechanistically, we found that PLA2G16 promotes CRC cells proliferation, migration and invasion through inhibiting the Hippo pathway. Meanwhile, we found that GCK could inhibit the proliferation, migration and invasion of CRC cells by decreasing the protein expression of PLA2G16 *in vitro* and *in vivo*. These discoveries will provide a new target and aid in the development of effective therapeutic strategies for CRC treatment.

Materials and methods

Ethics statement

The experiments involving patient were approved by the Ethics Committee of the Institute of Clinical Pharmacology of Central South University (Registration no. CTXY-150001-2). Clinical research registration was approved by the Chinese Clinical Trial Registry (Registration no. ChiCTR-DCD-15006289). All patients signed an informed consent form. Also, the animal experiments were carried out at the Experimental Animal Center of Central South University and approved by the Experimental Animal Ethics Review Committee (No. 2019sydw0018).

Bioinformatics analysis

The expression profile of CRC samples and corresponding clinical information were downloaded from TCGA database (<https://cancergenome.nih.gov/>). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were conducted by KEGG

pathway database (<http://www.kegg.jp/>), which aimed to explore the potential gene involved in lipid metabolism. Survival was conducted by using Kaplan-Meier Plotter database (<https://km-plot.com/analysis/>).

Clinical samples

A total of 135 CRC patients who were admitted to the Xiangya Hospital, Central South University (Changsha, China) from 2014 to 2016 were enrolled in the study. Among the 135 pairs of CRC and adjacent normal appearing tissues, a total of 95 pairs of fresh tissues were used for RNA extraction, while the remaining 40 pairs of paraffin-embedded tissues were used for IHC staining. Adjacent normal tissues were located 3 cm away from the patient's CRC tissue.

Drugs

Ginsenoside Compound K (CAS no.39262-14-1) was purchased from Chengdu Chroma-Biotechnology Co., Ltd. (Chengdu, China). The HPLC purity of GCK was $\geq 98\%$. Capecitabine (CAS no.154361-50-9) was purchased from MedChemExpress (New Jersey, USA). The HPLC purity of Capecitabine was $\geq 99.97\%$. They were diluted by the corresponding solvents to be used in further experiments.

Immunohistochemical analysis

Paraffin-embedded sections from CRC patients were used for IHC staining, and was scored by two pathologists followed the criteria: negative = 0, weak = 1, moderate = 2, and strong = 3. And PLA2G16-positive cells was scored as follows: $\leq 5\%$ = 0, 5–25% = 1, 25–50% = 2, 50–75% = 3, and $>75\%$ = 4, then multiplying the two scores. The cut-off value was set to 4.5, according to the ROC curves.

Cell lines and cell culture

The human CRC cell lines (SW480, HT29, HCT116, and LOVO) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), cultured in RPMI-1640, McCoy's 5A and F12 basic medium with 10% FBS (v/v), respectively, and incubated at 37°C in a 5% CO₂ atmosphere.

siRNA and transfection of CRC cell

The targets sequences of si-PLA2G16 were GGAGTCATGTCT-CAAGAA (si-PLA2G16-001) and GCGAGCACTTTGTGAATGA (si-PLA2G16-002). The negative control and siRNAs were all synthesized by Ribobio (Guangzhou, China). Transient transfection was performed by using the lipofectamine[®] RNAiMAX reagent (Invitrogen, USA). Western blotting and RT-qPCR were used for evaluating the transfection efficiency. PLA2G16 cDNA was sub-cloned into pcDNA3.1 to construct plasmid, then perform sequencing analysis.

Cell proliferation and colony formation assays

A total of 3×10^3 CRC cells per well were cultured in 96-well plates. MTS was used to detect cell proliferation after corresponding treatment. 1×10^3 cells per well were cultured in 6-well plates for around 15 days to perform the colony formation assay, then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Images were captured, after PBS washing.

EdU cell proliferation assay

3×10^3 CRC cells per well were cultured in 96-well plates, and treated with different concentrations of GCK for 48 h. Then incubated with EdU (Cell-Light[™] Edu Apollo488 *in vitro* Flow Cytometry Kit, RiboBio biotechnology), according to the manufacturers protocol. Images were captured using a fluorescent inverted microscope (Nikon, Japan) and analyzed using the Image-J software (Bethesda, USA) to quantify the Edu⁺ cells. The percentage of Edu⁺ cells in each field of view was analyzed.

Wound-healing assay

1×10^6 CRC cells per well were seeded in 6-well plates until the cell density reached more than 90%, 100 μ L sterile tips were used to scratch the bottom to form a cell-free area and treated for 48 h. Finally, the area was monitored by using a photographic microscope (Leica, Germany). The scratched width was quantified using the Image-J software.

Cell migration and invasion assays

For the transwell assay, CRC cells were suspended in the culture medium with 1% FBS and cells were cultured in the upper chamber (pore size 8.0 μ m; Costar, USA), while the culture medium with 20% FBS was added to the lower chamber, then fixed with paraformaldehyde and stained with 0.1% crystal violet. 24-well transwell chamber was paved with 60 μ L of a mixture (Matrigel: corresponding medium = 1: 9) when performing the invasion assay, added the cells to the chamber after 3 h. All the remaining steps were similar to those of the migration assay.

RNA isolation and RT-qPCR analysis

Total mRNA was extracted using TRIzol reagent (Invitrogen, USA) and reverse transcribed into cDNA using the PrimeScript[™]RT reagent kit (Takara, Japan). RT-qPCR was performed using SYBR Premix DimerEraser kit (Takara, Japan) on the Roche LightCycler480 (Roche, Switzerland). The primers for RT-qPCR are shown in [Table S1](#).

Protein extraction and western blotting analysis

Protein extraction was carried out in ice-cold RIPA buffer with phosphatase and protease inhibitors. The protein concentrations were measured by the BCA protein assay kit (Beyotime, Haimen, China). PLA2G16 was detected using a polyclonal human PLA2G16 antibody (1 μ g/mL, AF6190, R&D Systems, Inc.). Antibodies to TAZ (1:1000, sc-293183) and MST1 (1:1000, sc-515051) were purchased from Santa Cruz. LATS1 (1:100, #3477), p-LATS1 (Thr1079) (1:1000, #8654), p-TAZ (Ser89) (1:1000, #59971), and YAP (1:1000, #14074) were provided by Cell Signaling Technology. Antibodies to E-cadherin (1:500, cat. No. WL00941), N-cadherin (1:500; cat. No.WL01047), MMP-2(1:500; cat. No.WL1579), MMP-9 (1:500; cat. No.WL01580), Snail (1:500; cat. No. WL01863), Vimentin (1:500; cat. No.WL01960) and ZEB1 (1:500; cat. No.WL01657) were purchased from Wanleibio. Other antibodies were obtained from Abcam, including p-MST1 (T183) (1:2000, ab76323), p-YAP (S127) (1:10000, ab76252) and GAPDH (1:10000, ab181603).

RNA-sequencing (RNA-seq) analysis

mRNA was collected after siRNA transfection in 6-well plates for 48 h. Two replicate samples of mRNA were extracted, one for testing the transfection efficiency and the other for transcriptome

sequencing. The NEBNext® UltraTM RNA Library Prep Kit from Illumina® (NEB, USA) was used as sequencing libraries.

Immunofluorescence

CRC cells were cultured on coverslips, washed 3 times after transfection with siRNAs and fixed in paraformaldehyde. After permeabilization with 0.2% Triton X-100 and block with 5% BSA, incubated with the primary antibody overnight at 4°C and secondary antibodies for 1 h at 37°C, then coverslips were mounted on glass slides with the anti-fade mounting medium containing DAPI.

Lipidomics

CRC cells were seeded in 6-well plate for 24 h, then were subjected to GCK treatment followed by lipid extraction using dichloromethane. Samples were processed using LC-MS/MS for detection. ACQUITY UPLC BEH C₈ (1.7 μm, 2.1 × 100 mm) was used for sample separation. The mobile phases A and B for negative ion mode were acetonitrile: methanol: ddH₂O (1:1:1, v/v/v) and isopropanol (including 5 mM ammonium acetate. For positive ion mode, the mobile phase A and B were acetonitrile: ddH₂O (1:1, v/v) and isopropanol: acetonitrile (1:1, v/v) (including 10 mM ammonium formate). The gradient elution conditions were as follows: for negative ion mode: 0–0.10 min, 20% B; 0.10–1.50 min, 20% B; 1.50–2.50 min, 40% B; 2.50–4.00 min, 60% B; 4.00–12.00 min, 77% B; 12.00–12.50 min, 98% B; 12.50–14.00 min, 98% B; 14.00–14.10 min, 20% B; 14.10–18.00 min, stop; For the positive ion mode: 0–0.10 min, 70% B; 0.10–1.50 min, 70% B; 1.50–12.00 min, 85% B; 12.00–12.50 min, 98% B; 12.50–14.00 min, 98% B; 14.00–14.10 min, 70% B; 14.10–18.00 min, stop. The flow rate was 0.26 μL/min, injection volume was 1.0 μL and the column temperature was 45 °C. For the negative ion mode, the MS conditions were as follows: CUR was 35 psi, CAS was 9 psi, IS was –4500.0 V, TEM was 375.0 °C, GS1 was 50.0 psi and GS2 was 60.0 psi. For the positive ion mode, CUR was 35.0 psi, CAD was 9 psi, IS was 5500.0 V, TEM was 375.0 °C, GS1 was 50.0 psi and GS2 was 60.0 psi.

Nude mouse experiment

BALB/c female nude mice (6–8 weeks, weight 18 ± 1 g), purchased from the Hunan Slake Jingda Experimental Animal Co., Ltd, and were acclimatized for a week before performing the sub-

sequent experiments. 6 × 10⁷ CRC cells were inoculated into the right forelimbs. Then randomly divided into groups and given the corresponding treatment when the tumors were grew to 100 mm³. Mice were sacrificed after 4 weeks of continuous intra-gastric administration with GCK (50, 100 or 200 mg/kg) and a course of Capecitabine (359 mg/kg, 5 days/week for 3 weeks and stop for 1 week), weight and tumor volume were recorded every three days ($V = (L \times W^2)/2$).

Statistical analysis

All data are represented as mean ± sd and the difference between more than two groups was investigated by one-way ANOVA. The difference of PLA2G16 expression between tumor and matched adjacent normal tissues were analyzed by using paired student's *t*-test, the survival curves and statistical difference in survival were calculated by the Kaplan-Meier and log-rank test, respectively. Using SPSS 18.0 for statistical analysis and *P* < 0.05 was considered statistically significant.

Results

Bioinformatics analysis revealed that PLA2G16 plays an important role in the progression of CRC

From the data in the KEGG and TCGA databases, a series of genes related to lipid metabolism are differentially expressed in CRC tissues and adjacent normal tissues, such as ACACA, ACACB, GPD2, and PLA2G16. Among those, the expression of PLA2G16 was found to be the most significant (Fig. 1A). Analysis of data from the Kaplan-Meier plotter database showed that PLA2G16 mRNA expression level is related to the relapse free survival probability of CRC patients, the relapse free survival probability of the low expression of PLA2G16 group is significantly longer than that of the high expression of PLA2G16 group (Fig. 1B). Those bioinformatics analysis results revealed that PLA2G16 could be an oncogene in CRC.

PLA2G16 is up-regulated in CRC, and high expression of PLA2G16 is associated with the advanced stages

To investigate the role of PLA2G16 in CRC, we evaluated the expression of PLA2G16 in CRC tissue samples. We found that the

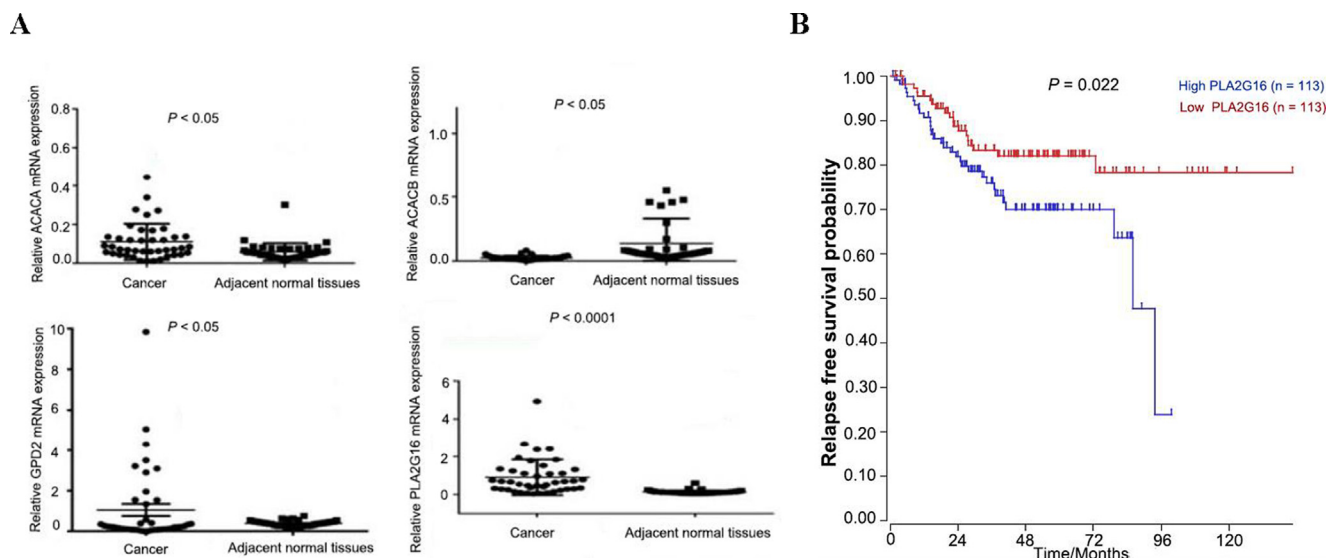


Fig. 1. Bioinformatics analysis of the effect of PLA2G16 in CRC. (A) The mRNA expression of genes related to lipid metabolism selected from the TCGA and KEGG databases in CRC and adjacent normal appearing tissues. (B) Relapse free survival probability according to PLA2G16 mRNA expression.

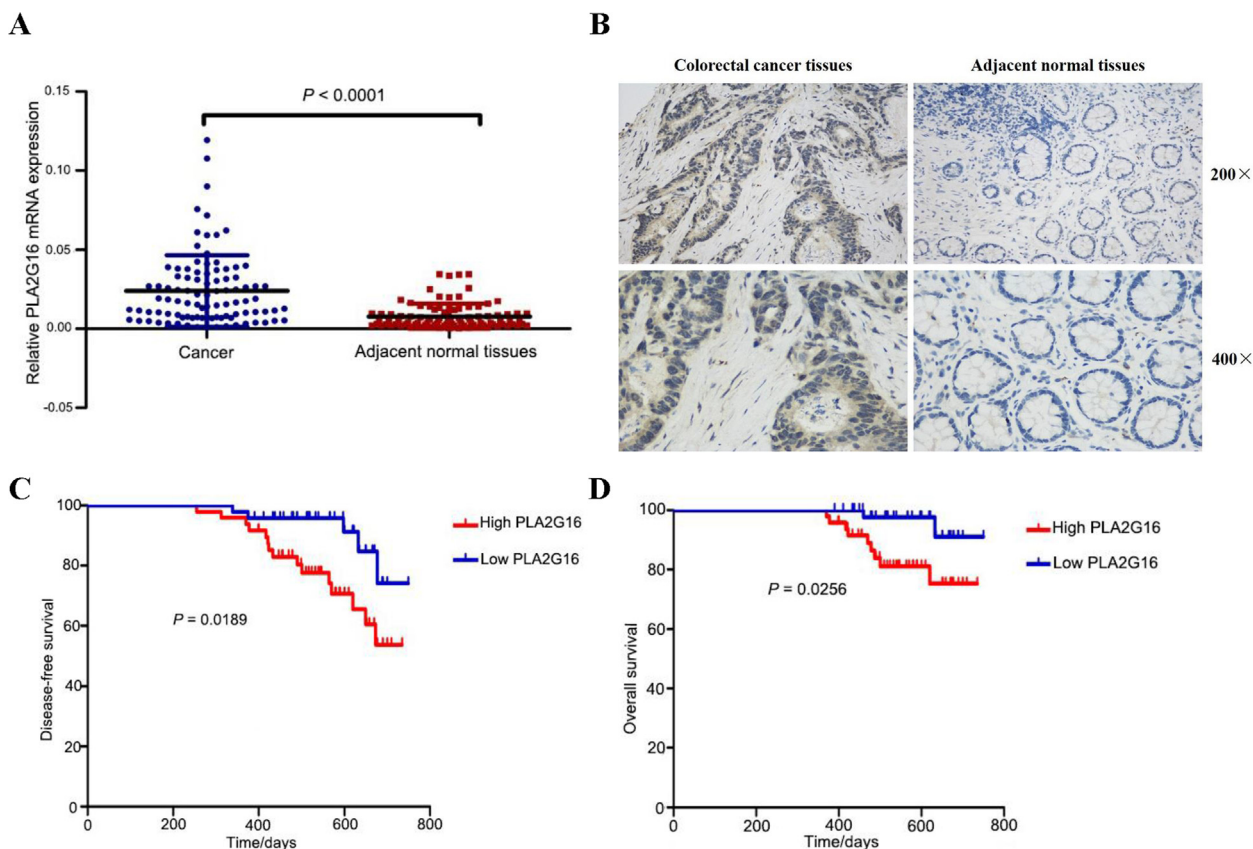


Fig. 2. Clinical significance of PLA2G16 in CRC patients. (A) Relative mRNA expression of PLA2G16 in 95 paired CRC and adjacent normal appearing tissues ($P < 0.0001$). (B) Representative images of IHC staining of PLA2G16 in 40 paired specimens of CRC and adjacent normal appearing tissues. (C) Kaplan-Meier analysis of disease-free survival of the 95 CRC patients according to PLA2G16 mRNA expression. (D) Kaplan-Meier analysis of overall survival of the 95 CRC patients according to PLA2G16 mRNA expression.

mRNA expression of PLA2G16 was significantly increased in the CRC tissues compared to the matched normal appearing tissues (Fig. 2A). IHC staining revealed that the PLA2G16 was mainly expressed in the cytoplasm, and was markedly up-regulated in CRC tissues than that in the adjacent normal appearing tissues (Fig. 2B) (Table 1). Furthermore, this study was performed using samples from 95 CRC patients with complete clinical information of age, sex, tumor location, and staging as well as physiological and biochemical indexes. Significantly, the level of PLA2G16 mRNA expression was related to clinical pathologic stages, and high expression of PLA2G16 was correlated with abnormal FFA biosynthesis (Table S2A). Moreover, the PLA2G16 mRNA expression was found to be negatively correlated with disease-free survival and overall survival (Fig. 2C-D) (Table S2B). These findings suggest that PLA2G16 is up-regulated in CRC and is related to the malignant progression of CRC.

PLA2G16 knockdown inhibits CRC cells proliferation, migration, and invasion in vitro and in vivo

To explore the specific role of PLA2G16 in CRC, RT-qPCR and western blotting were employed to detect the mRNA and protein

expression of PLA2G16 in different CRC cells lines (HT29, SW480, HCT116, and Caco2) (Fig. 3A-B). HT29 and SW480 cell lines were used for subsequent studies due to the high protein expression of PLA2G16. The interference efficiency of PLA2G16 in HT29 and SW480 cells were validated by RT-qPCR and western blotting (Fig. 3C-D). Then, MTS and colony formation assays were used to evaluated the role of PLA2G16 on CRC cell proliferation. The results showed that PLA2G16-silencing significantly suppressed cell proliferation and reduced the number of colonies (Fig. 3E). In addition, PLA2G16-silencing could also inhibit the migration and invasion of CRC cells compared to the control group (Fig. 3F-G). It is known that, epithelial mesenchymal transition (EMT) is related to cell migration and invasion, hence, western blotting was employed to detect the EMT pathway related proteins. It was found that PLA2G16-silencing increased the protein expression of E-cadherin, and decreased the protein expression of N-cadherin and Vimentin, thereby these results suggest that PLA2G16-silencing might reverse the EMT process (Fig. 3H).

To further investigate the effects of PLA2G16 on CRC growth *in vivo*, a xenograft nude mouse model was established. HT29 cells with a stable PLA2G16 knockdown were injected subcutaneously in BALB/c nude mice (6–8 weeks of age), and formed significantly

Table 1
The protein expression of PLA2G16 in 40 paired CRC and adjacent normal tissues.

Variable	No. of patients	No. of patients		P
		No/Weak expression	Moderate/Strong expression	
Colon adenocarcinomas	40	24(60)	16(40)	0.01
Adjacent normal tissues	40	35(87.5)	5(12.5)	

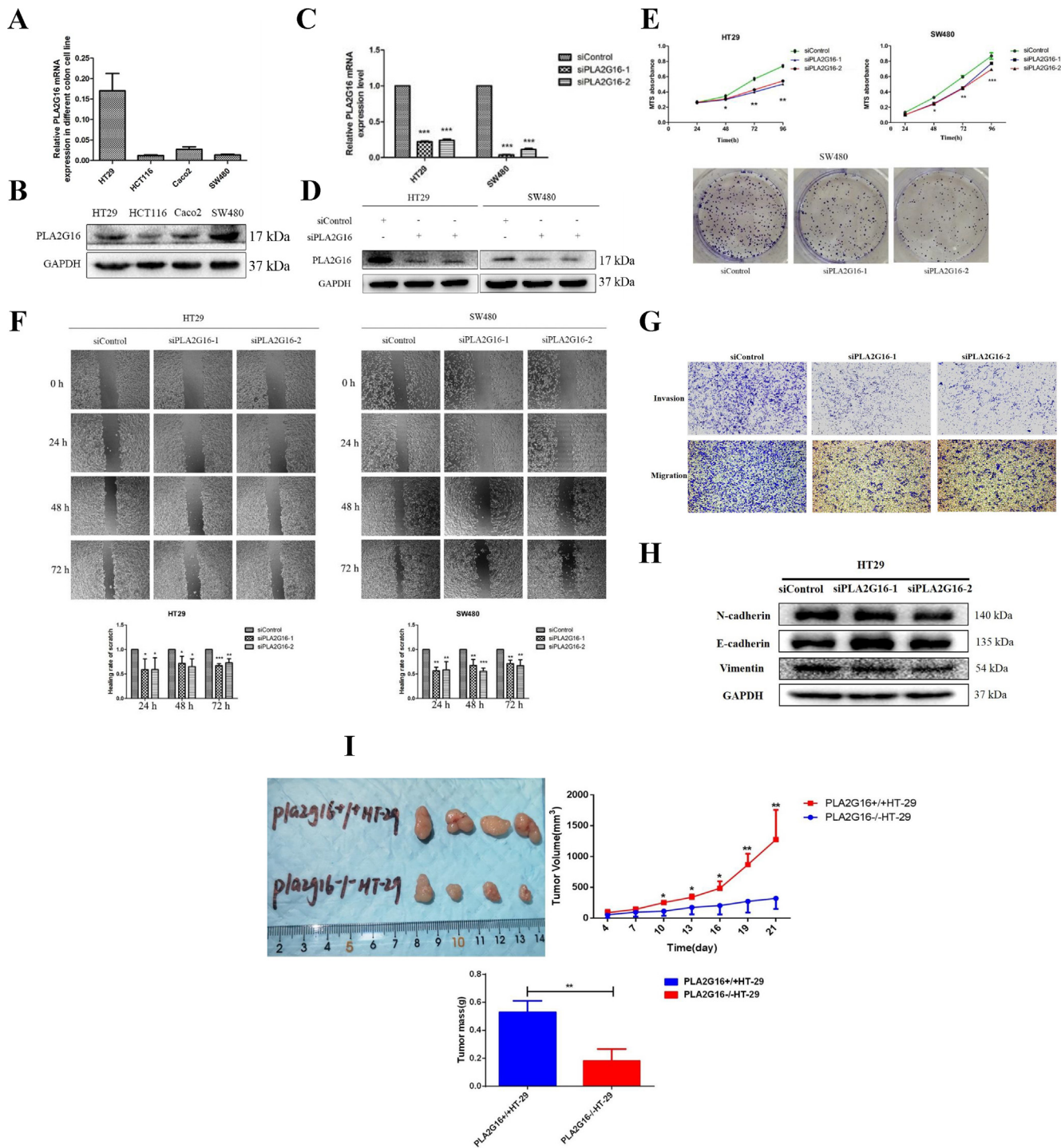


Fig. 3. PLA2G16 promotes CRC cells' proliferation, migration, and invasion *in vitro* and *in vivo*. (A–B) The mRNA and protein expression of PLA2G16 in difference CRC cell lines. (C–D) Levels of PLA2G16 mRNA and protein were detected by RT-qPCR and western blotting after PLA2G16-silencing in HT29 and SW480 cell lines. (E) PLA2G16-silencing inhibited the proliferation of CRC cells by MTS and colony formation assays. (F) PLA2G16-silencing inhibited the migration of CRC cells by wound healing assay, the magnification was 50×. (G) PLA2G16-silencing inhibited the invasion and migration of CRC cells, the magnification was 100×. (H) PLA2G16-silencing decreased the protein expression of N-cadherin and Vimentin, and increased the protein expression of E-cadherin. (I) Knockdown of PLA2G16 decreased the growth and weights of xenograft.

smaller tumors compared to those in the control group (Fig. 3I). Taken together, our results suggest that PLA2G16 knockdown inhibits CRC cell growth *in vivo*.

PLA2G16 promotes the malignant progression of CRC through the Hippo signaling pathway

To investigate the molecular mechanism, we conducted RNA-seq analyses. The results showed that a total of 105 and 71 genes

to be significantly down- and up-regulated, respectively, after transfection with siPLA2G16-01, while a total of 11 and 57 genes were significantly down- and up-regulated respectively, after transfection with siPLA2G16-02, respectively. The standard of significant difference was adjusted to $P < 0.05$ (Fig. 4A). A total of 12 differential genes were obtained from the intersection of the two sets of differential genes (Fig. 4B). The results were validated by RT-qPCR, and were found to be consistent with the results of RNA-seq (Fig. 4C). And the results of KEGG pathway enrichment

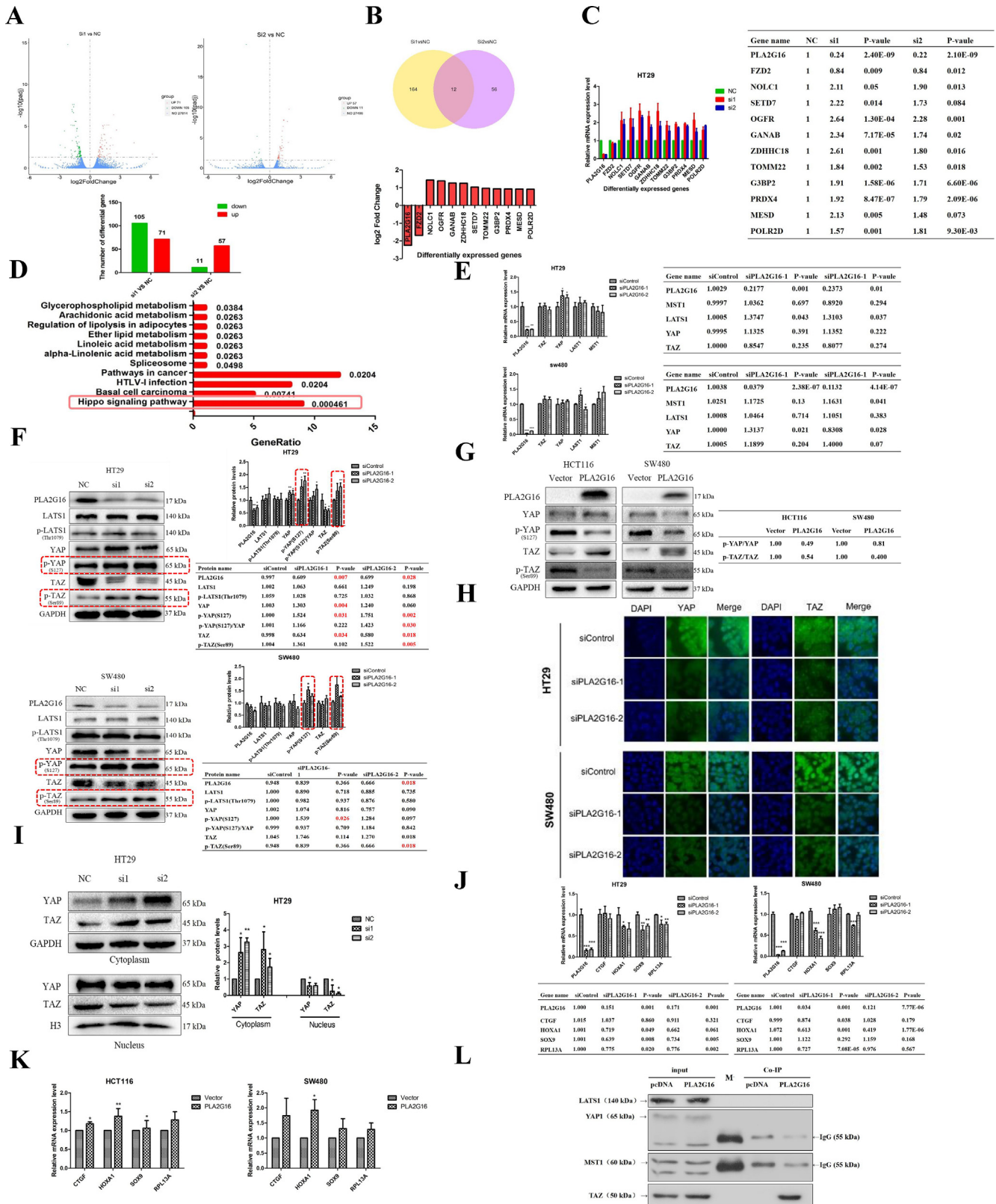


Fig. 4. PLA2G16 promotes the malignant progression of CRC through the Hippo signaling pathway. (A) Differentially expressed gene in PLA2G16-siRNA-1 and PLA2G16-siRNA-2 compared to Ctrl-siRNA. (B) The same differentially expressed gene in PLA2G16-siRNA-01 and PLA2G16-siRNA-02. (C) RT-qPCR to validate the results of same genes in PLA2G16-siRNA-01 and PLA2G16-siRNA-02 by RNA-seq. (D) KEGG pathway enrichment analysis of PLA2G16-regulated pathway. (E) The mRNA levels of YAP, TAZ, LATS1, and MST1 were measured by RT-qPCR after HT29 and SW480 cells were transfected with PLA2G16-siRNA. (F) The protein levels of LATS1, p-LATS1, YAP, p-YAP, TAZ, and p-TAZ upon PLA2G16 knockdown in HT29 and SW480 cells. (G) The protein expression of YAP, p-YAP, TAZ, and p-TAZ after PLA2G16-overexpression. (H) Immunofluorescence assay to detect the protein expression of YAP and TAZ in HT29 and SW480 cells, the magnification was 600 ×. (I) The expressions of YAP and TAZ in the cytoplasmic and nucleus. (J-K) RT-qPCR analysis of the mRNA expression of the downstream genes of the Hippo signaling pathway after PLA2G16 knockdown or PLA2G16-overexpression. (L) Co-IP was used to study how PLA2G16 regulated the Hippo signaling pathways.

analysis showed the differentially expressed genes to be enriched in the Hippo signaling pathway (Fig. 4D). It is already known that the Hippo signaling pathway is related to cell proliferation, apoptosis, and tumor genesis, hence, dysregulations of this pathway can lead to uncontrolled cell growth and malignant transformation [32,33]. Next, we explored whether PLA2G16 enhances CRC cells proliferation, migration, and invasion via the Hippo signaling pathway. RT-qPCR analysis showed that the mRNA expressions of YAP, TAZ, LATS1, and MST1 did not change significantly after PLA2G16-silencing (Fig. 4E). Western blotting analysis showed that PLA2G16-silencing significantly increased the protein expressions of p-YAP and p-TAZ, and decreased the protein expressions of YAP and TAZ (Fig. 4F). Conversely, opposite results were obtained on overexpressing PLA2G16 (Fig. 4G). Immunofluorescence assay indicated that the protein expressions of YAP and TAZ were down-regulated after PLA2G16 silencing, and they were mainly expressed in the cytoplasm (Fig. 4H). To further verify the effect of PLA2G16 on the Hippo pathway, we examined the expressions of YAP and TAZ in the cytoplasmic and nucleus. The results confirmed that the expressions of YAP and TAZ increased in the cytoplasm and decreased in the nucleus (Fig. 4I). After entering the nucleus, YAP and TAZ bind to the transcription factor TEAD, which causes the transcription of the downstream target genes, hence, we next examined the mRNA expressions of the downstream target genes, such as HOXA1, SOX9, and RPL13A. The result showed that the mRNA expressions of these target genes were decreased after PLA2G16 silencing (Fig. 4J). Conversely, the mRNA expression of these target genes were increased after PLA2G16 overexpression (Fig. 4K). Next, we sought to investigate the mechanism that PLA2G16 regulates the Hippo signaling pathways. Using co-immunoprecipitation (Co-IP), we confirmed that PLA2G16 interacted with TAZ (Fig. 4L). Taken together, our results indicated that PLA2G16 regulates the malignant progression of CRC by promoting PLA2G16-TAZ interactions to inhibit the Hippo signaling pathway.

GCK inhibits the proliferation, migration, and invasion of CRC in vitro and in vivo

As the main active ingredient of ginsenosides in the human body, GCK has various pharmacological effects, but its anti-CRC effect and its molecular mechanisms have not been reported. To investigate the effect of GCK on the proliferation of CRC cells, MTS, colony formation, and EdU assays were performed, which demonstrated that GCK decreased the proliferation of CRC cells in a dose- and time-dependent manners (Fig. 5A-D). Migration and invasion abilities are key factors for cancer progression [34]. Therefore, the wound healing and transwell assays were used to detect the effects of GCK on the migration and invasion of CRC cells, and the results showed that GCK suppressed the migration and invasion of CRC cells in a dose-dependent manner (Fig. 5E-F). Western blotting analysis showed that the protein expressions of Snail1, ZEB1, MMP-9 and Vimentin were decreased, while the protein expression of E-cadherin was increased in a dose-dependent manner (Fig. 5G). Taken together, these results demonstrated that GCK inhibits the proliferation, migration, and invasion of CRC cells *in vitro*.

To further investigate the effects of GCK on CRC growth *in vivo*, stable CRC cells were inoculated into the right forelimbs of female nude mice at 6–8 weeks of age. Then, they were randomly divided into groups and administered with GCK (50, 100, or 200 mg/kg) by gavage for four weeks and a course of Capecitabine (359 mg/kg, 5 days/week for 3 weeks and stop for 1 week). Compared to the control group, the GCK group showed a significant reduction in tumor size and weight (Fig. 5H). Moreover, H&E staining was performed to observe the histological morphology of the tumor. The

result showed that the degree of tumor cell necrosis increased, compared to that in the control group (Fig. 5I). IHC staining results showed that the positive expression of Ki-67 in tumor tissues decreased with the increased doses of GCK (Fig. 5J). These findings indicate that GCK has an anti-proliferation effect *in vivo*. Western blotting analysis showed that GCK also increases the protein expression of E-cadherin and reduces the protein expressions of N-cadherin, ZEB1, MMP-9, MMP-2, and Vimentin (Fig. 5K). To a certain extent, this indicates that GCK can inhibit CRC cell migration and invasion *in vivo*.

GCK corrects the abnormal lipid metabolism and inhibits the protein expression of PLA2G16 in vitro and in vivo

Abnormal lipid metabolism is found in patients of CRC. An abnormal increase in lipid metabolism, especially an abnormal increase in FFA, may be related to cancer cell metastasis. Therefore, the inhibition of CRC cell proliferation, migration, and invasion by GCK may be related to the correction of abnormal lipid metabolism. Hence, a lipidomic analysis of the CRC cells was carried out after the GCK treatment. The normalized data was imported into the SIMCA-P version 14.1 to create an OPLS-DA model. The result showed that the control group (0.1% DMSO) and GCK group are completely separated in the direction of the [t1] component with the explained parameters $R^2X = 0.911$ and $R^2Y = 0.98$ and the predictive parameter $Q^2 = 0.925$ (Fig. 6A), and permutation test of corrects the OPLS-DA model indicated that the model was not overfitting the data (Fig. 6B). These results showed that GCK could reverse the abnormal lipid metabolism in CRC. Furthermore, we analyzed the lipids with significant changes by GCK. The overall lipid levels with significant changes (fold change > 2 and $P < 0.05$) are all presented in the volcano plots, scatter diagram and cluster analysis map (Fig. 6C-E). We found that GCK could significantly reverse the abnormal metabolism of some lipids, including FFA.

PLA2G16 is the rate-limiting enzyme in the biosynthesis process of FFA. We found that PLA2G16 could promote the proliferation, migration, and invasion of CRC by inhibiting the Hippo signaling pathway (Fig. 4). Thus, we speculated that the anti-CRC effect of GCK may be related to the inhibition of PLA2G16. Hence, we used western blotting to detect the effect of GCK on PLA2G16. The results indicated that GCK could inhibit the protein expression of PLA2G16 in a dose-dependent manner *in vitro* and *in vivo* (Fig. 6F).

Overexpression of PLA2G16 attenuates the anticancer effect of GCK

To further study whether the expression of PLA2G16 affects the anti-CRC effect of GCK, we overexpressed PLA2G16 in CRC cells, which was confirmed by RT-qPCR and western blotting. Both the mRNA and protein expression analysis showed that PLA2G16 were overexpressed (Fig. 7A). Then the PLA2G16-overexpressed CRC cell lines were subjected to the treatment of different concentrations of GCK and tested using MTS and colony formation assays. The results indicated that PLA2G16 overexpression attenuated GCK's ability to suppress cell proliferation (Fig. 7B-C), migration and invasion (Fig. 7D-F). These results indicate that GCK might inhibit the proliferation, migration, and invasion of CRC cells by decreasing the protein expression of PLA2G16.

Discussion

Changes in lipid metabolism enzymes and related pathways can cause a variety of diseases, including metabolic, immunity, and central nervous system diseases as well as cancer. This indicates

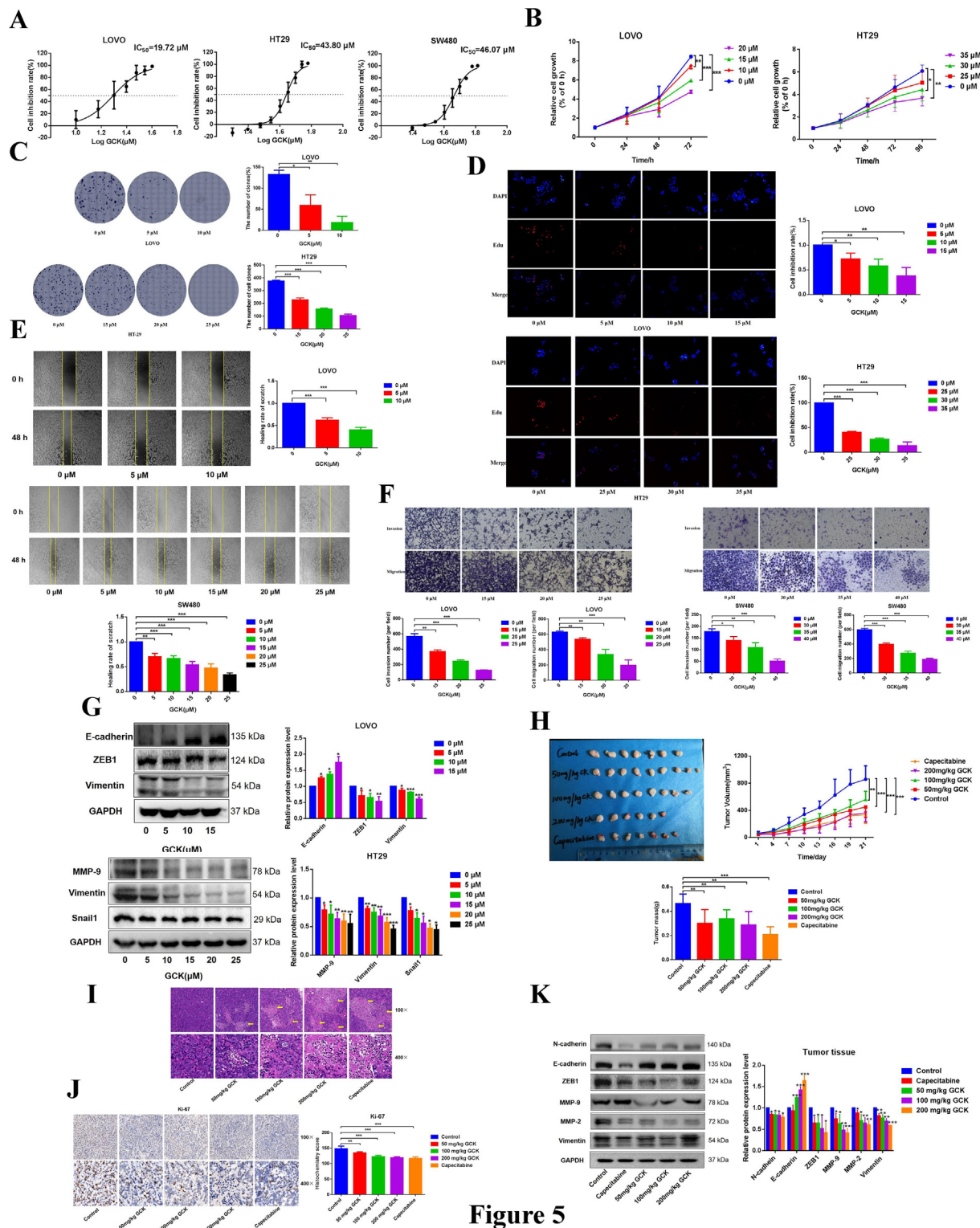


Figure 5

Fig. 5. GSK inhibits CRC cells' proliferation, migration and invasion *in vitro* and *in vivo*. (A) IC_{50} of GSK in different CRC cells. (B–D) GSK inhibited the proliferation of CRC cells by MTS, colony formation and Edu assays (the magnification was 100 \times). (E–F) GSK inhibited the migration and invasion of CRC cell by wound healing (the magnification was 50 \times) and transwell assays (the magnification was 100 \times). (G) GSK increased the protein expression of E-cadherin, and decreased the protein expressions of ZEB1, Vimentin, Snail1 and MMP-9 *in vitro*. (H) GSK inhibited the growth of xenograft tumors. (I) H&E staining to detect tumor necrosis. (J) IHC detected the expression of Ki-67 in tumor tissues. (K) GSK increased the protein expression of E-cadherin, and decreased the protein expressions of N-cadherin, ZEB1, MMP-9, MMP-2 and Vimentin *in vivo*.

that lipids show an important role in maintaining the balance in the intracellular environment and sustaining normal cellular function [35,36]. PLA2G16, an adipocyte-specific phospholipase, can regulate adipocyte lipid metabolism. It generates FFA by hydrolyzing the membrane phospholipid *sn*-2, and FFA can provide energy

for the growth and invasion of cancer cells [6]. This indicated that PLA2G16 could provide a steady stream of energy for cancer cells and promote tumor progression. Our research results showed that PLA2G16 is highly expressed in CRC tissues compared with the adjacent normal tissues. High PLA2G16 expression was associated

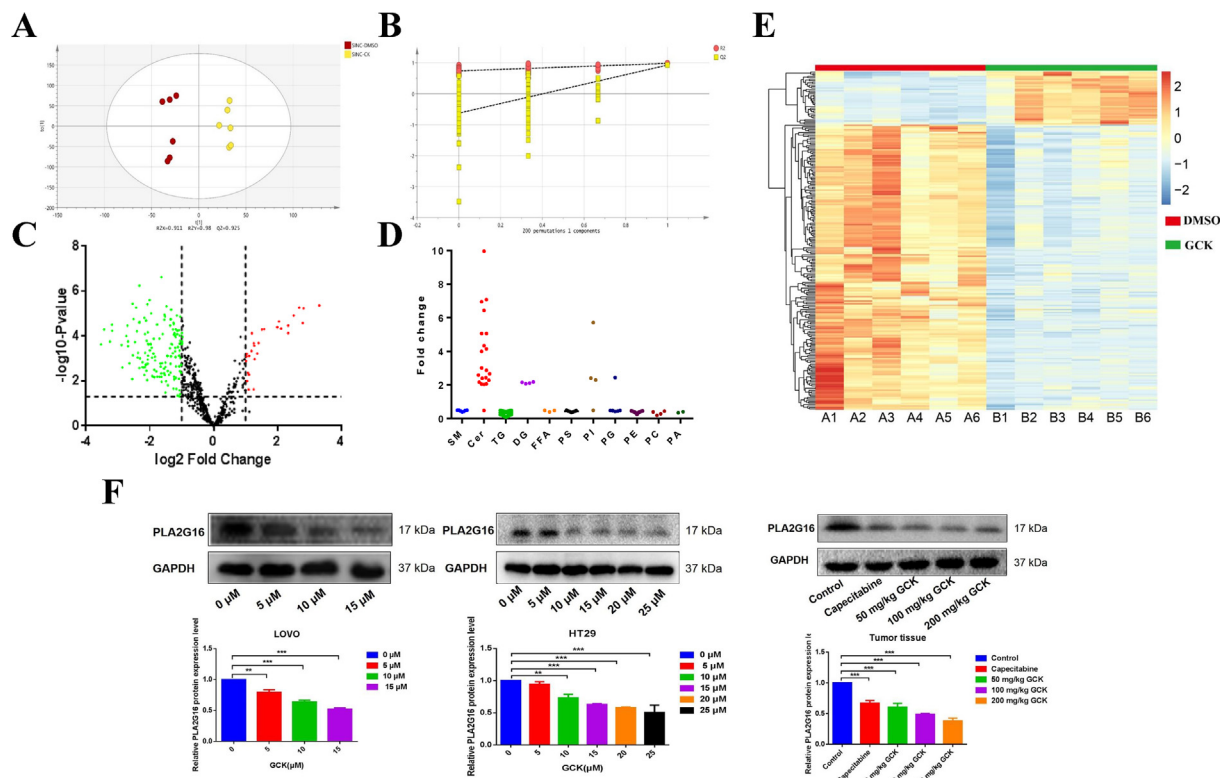


Fig. 6. Effect of GCK on abnormal lipid metabolism in CRC cells. (A) The effect of control group and GCK group on CRC cell lipid metabolism was clearly separated on OPLS-DA model [R^2X (cum) = 0.911, R^2Y (cum) = 0.98, Q^2 (cum) = 0.925]. (B) Permutation test of the OPLS-DA model. The lipid metabolites in CRC cells by non-targeted lipidomics were presented in volcano plot (C), scatter plot (D) and cluster analysis diagram (E). (F) GCK decreased the protein expression of PLA2G16 *in vitro* and *in vivo*.

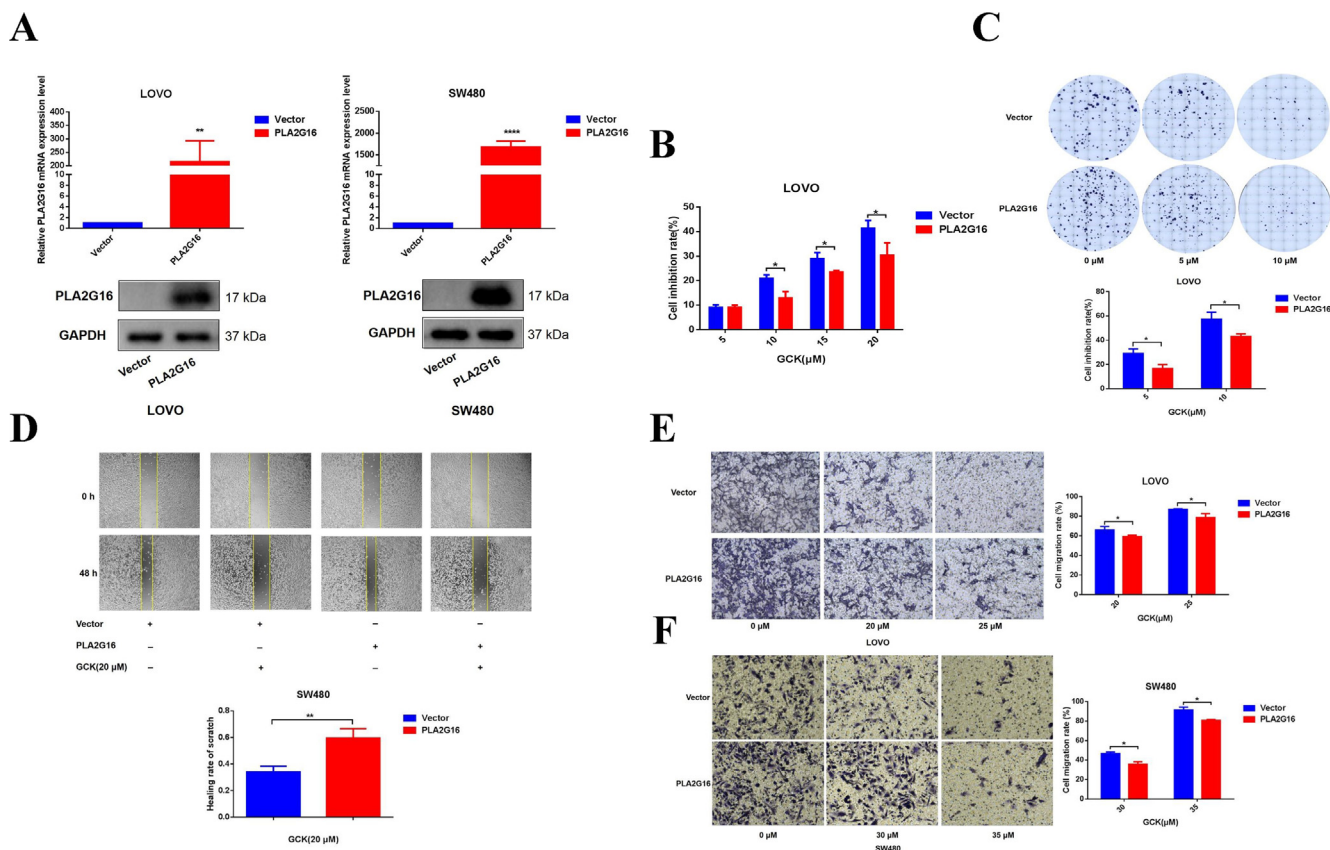


Fig. 7. GCK's ability to suppress CRC was attenuated by overexpressing the protein of PLA2G16. (A) PLA2G16 was successfully overexpressed in mRNA and protein levels. (B–C) GCK'S inhibition effect on the proliferation and colony formation of CRC cell was attenuated by overexpressing the protein of lipid metabolism gene PLA2G16. (D) GCK'S inhibition effect on the migration of CRC cells was attenuated by overexpressing the protein expression of PLA2G16, the magnification was 50 ×. (E–F) GCK'S inhibition effects of on migration and invasion were weakened after the protein expression of PLA2G16 was overexpressed in the same CRC cells, the magnification was 100 ×.

with poor prognosis of CRC patients. Therefore, we suspect that abnormal expression of PLA2G16 may act as a biomarker for CRC diagnosis and prognosis. Furthermore, we then explored the effect of PLA2G16 on the biological function of CRC cells *in vitro* and *in vivo*. The results showed that PLA2G16 not only promoted CRC cell growth, but also enhanced the migration and invasion ability of CRC cells. Mechanistically, we identified the differentially expressed genes in PLA2G16 knockdown cells *via* RNA-sequencing, and those genes were significantly enriched in the Hippo signaling pathway by KEGG enrichment analysis.

The core of the Hippo pathway consists of MST1 and LATS1 along with the two effectors, YAP and TAZ. When the pathway is dysregulated, YAP and TAZ are up-regulated and localized in the nucleus [33,37]. Studies have shown that YAP participates in the occurrence and development of CRC, and is highly expressed in CRC compared to matched adjacent normal tissues [38]. Increasing the protein expression of YAP could promote liver metastasis and tumor recurrence in CRC [39]. Our results indicated that PLA2G16-knockdown significantly increased the protein expressions of p-YAP and p-TAZ, while decreased YAP and TAZ nuclearization. After entering the nucleus, YAP and TAZ combine with transcription factors to promote the transcription of target genes related to proliferation and metastasis. In our study, we found that the expressions of these downstream target genes, especially HOAX1 and RPL13A were down-regulated after PLA2G16-knockdown. Conversely, PLA2G16-overexpressed led to the opposite phenomena. These results indicated that PLA2G16 mainly reduce the protein expression of p-YAP and p-TAZ to dysregulate the Hippo pathway, and promote the malignant progression of CRC. Together, we identified an oncogenic role of PLA2G16 as a novel negative regulator of the Hippo signaling by interacting with TAZ. These discoveries not only enhance our understanding of the Hippo pathway regulated by PLA2G16 in CRC, but also provide a new therapeutic target for CRC.

The current standard therapy for CRC involves surgical intervention along with adjuvant therapy, such as radiotherapy, chemotherapy, or chemoradiotherapy [17,18]. However, these strategies are ineffective and have serious side effects. In recent years, there has been a gradual shift in attention towards natural medicines due to the low toxicity and high efficiency [21]. GCK is a metabolite of the natural diol-type ginsenosides Rb1, Rb2, and Rc in the human intestinal tract and the data of clinical trials showed that GCK is safe for subjects [27]. It's reported that GCK has a variety of pharmacological effects, such as anti-tumor [40,41], anti-inflammatory [42], antiaging [43], neuroprotective [44] and antidiabetic [45]. Our research found that GCK has a good anti-CRC effect *in vitro* and *in vivo*. However, the results *in vivo* indicated that the anti-tumor effect of GCK is not in a dose-dependent manner, and the inhibitory effect only at a high concentrations (200 mg/kg of GCK). The possible reason may be that the concentration setting of GCK is inappropriate. Considering the side effects, the concentration of GCK did not continue to increase.

It is reported that abnormal lipid metabolism is a hallmark of tumors, and that adipocytes could provide energy for the rapidly growing tumor cells and promote metastasis. Our results indicated that GCK corrected the abnormal lipid metabolism in CRC cells, and regulated the biosynthesis of FFA through the lipid metabolism gene PLA2G16. And GCK can inhibit the protein expression of PLA2G16 in a dose-dependent manner both *in vitro* and *in vivo*, PLA2G16-overexpressed attenuated the anti-CRC effect of GCK. Furthermore, the results of molecular docking showed that GCK was well docked with PLA2G16 at four active sites including TYR21, VAL68, GLU638 and LYS61 (Supplementary Fig. 7), suggesting that the anti-CRC effect of GCK is related to PLA2G16.

The major limitation of our study was that lacking experiment to confirm that PLA2G16 promotes the proliferation, migration

and invasion of CRC through the Hippo signaling pathway. Furthermore, it is also important to design animal experiments to confirm the results *in vitro*. More efforts are warranted to answer those questions in future studies.

Conclusions

In conclusion, we found that PLA2G16 promote the malignant progression of CRC by inhibiting the Hippo signaling pathway, and GCK exerted its anti-CRC effects by inhibiting the protein expression of PLA2G16. Our study provides significant new insights towards the development of effective therapeutic strategies for CRC treatment by targeting PLA2G16.

Ethics statement

- (1) This study was approved by the Ethics Committee of the Institute of Clinical Pharmacology of Central South University (Registration no. CTXY-150001-2).
- (2) All animal experiments were approved by the Experimental Animal Ethics Review Committee (No. 2019sydw0018).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81974510), the National Development of Key Novel Drugs for Special Projects of China (Nos. 2018ZX09736007-004, 2017ZX09304014-02 and 2018ZX09736-07) and the Natural Science Foundation of Hunan Province (No. 2020JJ4124, 2020JJ9057). In addition, the authors appreciate Prof. Jianbo Xiao (University of Vigo-Ourense Campus), Dr. Yongchao Gao (The Xiangya Hospital of Central South University) and Dr. Hecun Zou (Chongqing Medical University), for their help.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2021.06.009>.

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