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Parthenolide induces gallbladder cancer cell apoptosis via MAPK signalling

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Objective: Parthenolide (PTL) has a wide range of clinical applications owing to its anti-inflammatory and antitumor effects. To date, the antitumor effect of PTL on gallbladder cancer (GBC) remains largely unknown. Therefore, we aimed to investigate the biological effects of PTL on GBC.

Methods: The cellular viability and proliferation of GBC-SD and NOZ cell lines after treatment with different concentrations of PTL were analyzed using the Cell Counting Kit-8 (CCK8)assay and colony formation assay. Apoptosis analysis was performed using flow cytometry. Hoechst staining was performed. RNA sequencing (RNA-seq) was performed to identify PTL-related genes and signalling pathways. Furthermore, we confirmed the involvement of these signalling pathways by qRT-PCR and western blotting. For the in-vivo experiments, a xenograft model was used to evaluate the effects of PTL on the proliferation of NOZ cells.

Results: PTL significantly inhibited GBC cell growth *in vitro* and induced apoptosis in the GBC-SD and NOZ cell lines in a dose-dependent manner. RNA sequencing data showed that the immune response and mitogen-activated protein kinase (MAPK) signalling pathways are closely associated with PTL-induced gallbladder cancer cell apoptosis. PTL upregulated BAX, cleaved PARP-1, cleaved caspase-3, cleaved caspase-9, P53 and decreased the expression of BCL-2, phosphorylated ERK, and phosphorylated MEK *in vitro*. Tumour volume and weight were also suppressed by PTL *in vivo*. Moreover, the effects of PTL on GBC cells might be mediated by the MAPK pathway.

Conclusion: PTL significantly inhibits gallbladder cancer cell proliferation and induces apoptosis through the MAPK pathway, which is a potential molecular reagent for treating GBC. However, further exploration is needed to verify the antitumor effects of PTL and its intracellular signalling mechanism.

Keywords: apoptosis, gallbladder cancer, parthenolide

Introduction

Gallbladder cancer (GBC), a malignant tumour that originates from gallbladder epithelial cells, is the most common invasive malignant tumour of the biliary tract system[1]. The incidence and mortality rates of GBC have been increasing worldwide. Moreover, the incidence of gallbladder cancer in China is much higher than that in Western countries[2]. However, chronic inflammation, gallbladder polyps, and pancreaticobiliary malfunction are risk factors for GBC[3]. Surgical resection is considered the best treatment option for GBC, but the average survival of patients after surgical resection is still less than 1 year. Due to the characteristics of asymptomatic onset, high degree of malignancy, rapid development, and early invasiveness and metastasis, more than 80% of patients with GBC are diagnosed at an advanced stage[4]. Despite improvements in early diagnosis and treatment, GBC still has poor prognosis[5]. Therefore, new therapeutic approaches related to molecular-targeted therapies may be effective for GBC treatment.

HIGHLIGHTS

- To reveal the biological effects and investigate possible mechanism of parthenolide (PTL) on gallbladder cancer cell, providing possible therapeutic target in cholangiocarcinoma.
- Our in-vivo and in-vitro studies suggested that PTL significantly inhibit gallbladder cancer cell proliferation and induces apoptosis through the mitogen-activated protein kinase (MAPK) pathway, which is a potential molecular reagent for gallbladder cancer.
- RNA sequencing data showed that the immune response pathway and MAPK signalling pathways was closely associated with PTL-induced gallbladder cancer cell apoptosis. In addition to the MAPK signalling pathway, immunoregulatory capacity of PTL may also be involved in PTL-induced cancer cell apoptosis, which needs to be further investigated
- These findings may aid for the development of possible therapeutic target in cholangiocarcinoma.

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Parthenolide (PTL), a natural sesquiterpene lactone, is extracted from the medicinal plant fever-few (*Tanacetum parthenium*). PTL has been shown to exert many pharmacological effects, including therapeutic effects on fever, headache, and arthritis, as well as antitumor, antimicrobial, anti-inflammatory, and antioxidant effects[6]. Previous studies have shown that PTL exerts anticancer effects by inhibiting proliferation and inducing apoptosis in various cancer cell lines[7,8]. Studies have shown that PTL can modulate several essential signalling pathways, including nuclear factor kappa B (NF-κB), Signal transducer and activator of transcription (STAT), and reactive oxygen species (ROS), thereby mediating its biological effects[9–11]. PTL, which has antitumor and anti-inflammatory properties, is a biologically active compound with great potential for the treatment of GBC. However, the mechanism by which PTL exerts antitumor effects in GBC remains unclear.

In the present study, we evaluated the biological effects and underlying mechanisms of PTL on gallbladder cancer. Our data showed that PTL exerted a good inhibitory effect and induced apoptosis in GBC cells via the mitogen-activated protein kinase (MAPK) signalling pathway, highlighting the potential applications of PTL in GBC therapy.

Materials and methods

Ethical approval

All animals were performed in accordance with the relevant guidelines and regulations, and approval by Animal Experiment Center of Xinjiang Medical University (ethical committee approval no. IACUC-20220301-06). The animals presented a healthy status and male mice were used for all experiments. The present study followed international, national and/or institutional guidelines for animal treatment and complied with relevant legislation; This work is fully compliant with the ARRIVE criteria, which provided in supplementary section[12].

Drugs and cell culture

Parthenolide was purchased from Santa Cruz Biotechnology. The molecular structure of PTL is shown in Fig. 1. The GBD-SD and NOZ gallbladder cancer cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences. GBC-SD cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS) (cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.), and 1% penicillin/streptomycin. NOZ cells were cultured in William's medium supplemented with 10% foetal bovine serum (FBS) (cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.), and 1% penicillin/streptomycin. Cells were grown at 37°C in a humidified incubator with 5% CO2.

Cell viability assay

Cell viability was evaluated using the CCK8 assay following the manufacturer's instructions. GBC-SD and NOZ cells were seeded in 96-well plates at a density of 3000 cells/well and cultured for 24 h. Various concentrations of PTL (0, 10, 20, 30, 60, and 90 μM) were then added. The cells were then incubated for 24 and 48 h. After each treatment, the CCK8 solution (10 μl) was added to each well and incubated for 2 h in the dark. The absorbance of each well was measured at 450 nm wavelength using a microplate reader (Bio-Tek). Three independent experiments with two replicates were conducted The IC50 values were determined using GraphPad Prism 5.

Colony formation assay

GBD-SD and NOZ cells were seeded in 6-well plates and treated with PTL (0, 5, 15, and 25 μM and 0, 15, 30, and 45 μM)) for 15 days. Then, the cells were fixed with 10% formalin and stained with 0.1% crystal violet (Sigma–Aldrich). After washing, the plates were dried and colonies (with more than 50 cells) were observed under a microscope.

Apoptosis analysis by flow cytometry

GBC-SD and NOZ cells were seeded in 6-well plates and treated with PTL (0, 5, 15, and 25 μM and 0, 15, 30, and 45 μM) for 48 h. An annexin V/PI staining assay was used to evaluate apoptotic GBC-SD and NOZ cells. Briefly, after trypsinization and centrifugation at 200*g* for 5 min, the cells were mixed with 1× Annexin V binding buffer and then incubated with 5 μl Annexin V and PI at 37°C for 30 min in the dark. After washing twice with PBS, cell apoptosis was measured using flow cytometry. Flow cytometry data were analyzed with FlowJo 7.6 (Treestar Inc.).

Hoechst 33342 staining

GBC-SD and NOZ cells were treated with PTL (0, 5, 15, and 25 μM and 0, 15, 30, and 45 μM)) for 48 h. After the cells were fixed with 1 ml methanol/acetic acid (3:1) and stained with 5 μg/ml Hoechst 33342, a fluorescence microscope was used to analyze the cell morphological changes.

Western blot analysis

Tissues were homogenized in RIPA lysis buffer (cat. no. P0013B; Beyotime Biotechnology) and incubated on ice for 30 min. After centrifugation at 12 000 rpm for 15 min, the supernatant was used for western blotting. Western blotting was performed as previously described[8]. The blots were incubated overnight with primary antibodies against the following target proteins: Bax (1:1000, ABclonal, China), Bcl-2 (1:1000, ABclonal, China), cleaved PARP-1(1:2000, ABclonal, China), cleaved caspase-3 (1:1000, ABclonal, China), cleaved caspase-9 (1:1000, ABclonal, China), P53 (1:1000, Cell Signalling Technology), ERK (1:1000, ABclonal, China), p-ERK (1:1000, ABclonal, China), MEK (1:1000, ABclonal, China), p-MEK (1:1000, ABclonal, China), and GAPDH (1:1000, ABclonal, China). Western blotting was performed using enhanced chemiluminescence reagent (EpiZyme) and visualized using an electrophoretic gel imaging system (Tanon).

RNA sequencing

GBC-SD cells were treated with 15 μM PTL for 48 h. RNA sequencing was performed by the Shanghai Yueda Technology Co., Ltd. Each sample was analyzed in triplicate. Three independent biological replicates were used for each experiment. Relying on the genomic information of known species or complete transcriptome information, the sequenced reads can be compared to the reference genome in order to annotate and quantify genes or transcripts. After comparing reads to the reference genome, we annotated and quantified the expression using StringTie[13]. StringTie applies network flow algorithms and optional de-novo assembly to assemble complex datasets into

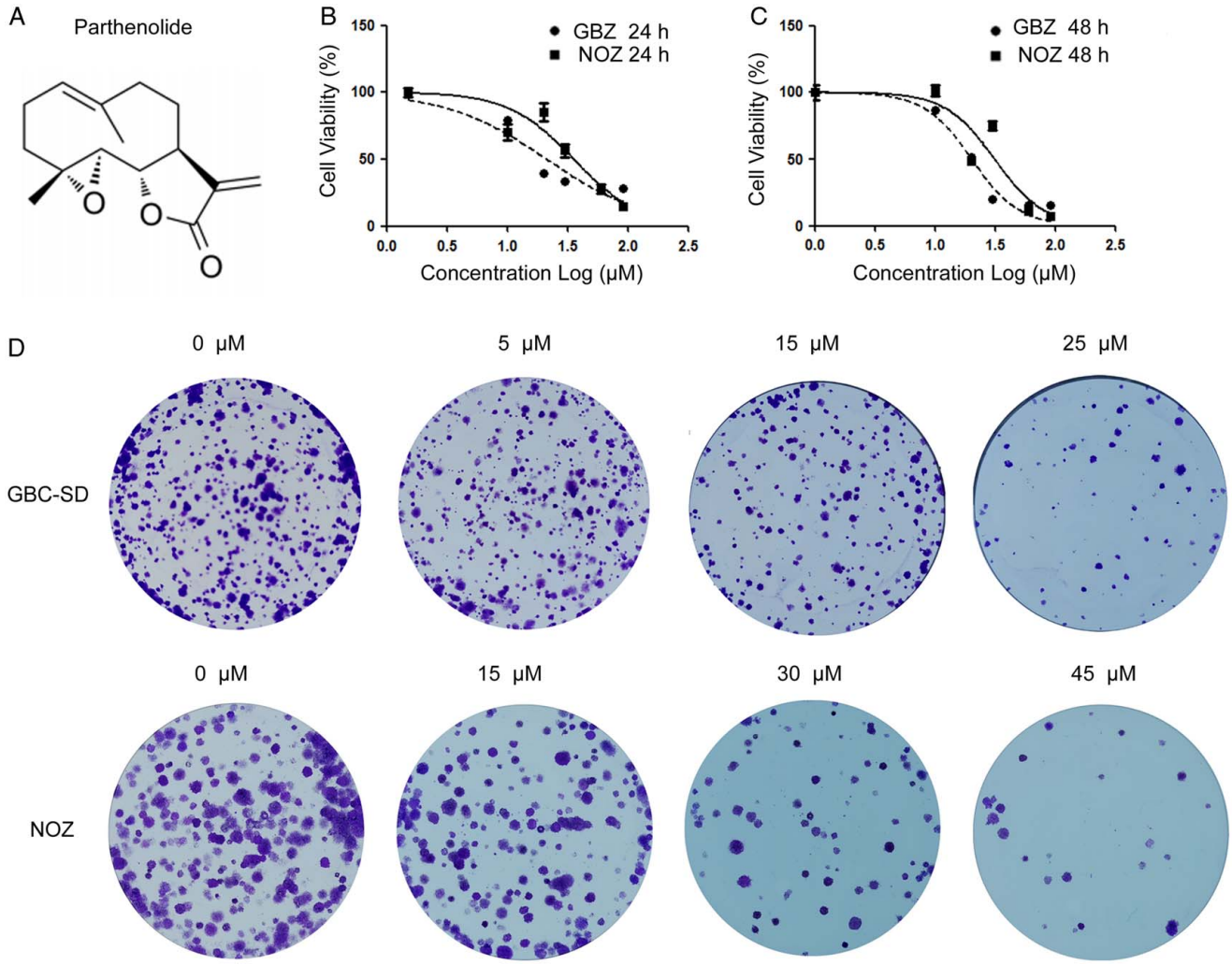

Figure 1. PTL inhibits proliferation and colony formation of GBC-SD and NOZ cells *in vitro*. (A) Chemical structure of PTL. (B, C) GBC-SD and NOZ cells treated with PTL in different concentration for 24 and 48 h. Cell viability measured by CCK8 assays. ($n = 3$). (D) PTL suppressed the colony formation abilities of GBC-SD and NOZ cells. Cells were treated with PTL and then cultured in fresh medium for 14 days to form colonies. ($n = 3$). CCK8, Cell Counting Kit-8; GBC, gallbladder cancer; PTL, parthenolide.

transcripts. And further calculate the FPKM values of genes and transcripts. DESeq2 was used to compare the differences between the two groups and obtain significant P values, pad just values, and fold changes; In this analysis, two groups will be compared based on the pad just values(< 0.05) and fold changes(≥ 2 or ≤ 0.5).RNA-Seq analysis identified a total of 1559 genes that were significantly changed after PTL treatment. Based on the differentially expressed genes, clustering analysis was performed on the genes and samples. it can be found that gene clusters clustered into a group have similar expression patterns and may have similar functions in the sample. Then, GO, KEGG, and other related analyses were conducted based on the DAVID database. (https://david.ncifcrf.gov). The supplementary data contain the full RNA-seq dataset.

RNA extraction and qRT-PCR

Total RNA was extracted from cell samples using a TRIzol RNA isolation kit (Invitrogen), and cDNA was generated following the manufacturer's instructions (Vazyme Biotech). The expression of the selected genes was determined using the LightCycler 480 System (Roche) and SYBR Green chemistry (Vazyme Biotech). Calculations were performed using the comparative computed tomography method (ΔΔCt method). Fold induction was adjusted for GAPDH levels. A primer list is as follows : GAPDH:

F: CATGAGAAGTATGACAACAGCCT; R: AGTCCTTCCACGATACCAAAGT

MAPK15: F: AGAAGCCGTCCAATGTGCTC; R: CAAGGGTGTATCGGTGCGA

MMP9: F: GGGACGCAGACATCGTCATC; R: TCGTCATCGTCGAAATGGGC

TP53: F: ACAGCTTTGAGGTGCGTGTTT; R: CCCTTTCTTGCGGAGATTCTCT

MKI67: F: GCCTGCTCGACCCTACAGA; R: GCTTGTCAACTGCGGTTGC

In-vivo tumour xenograft study

Pathogen-free BALB/C nude mice ($n = 5$) (aged 4–5 weeks; weight, 20 g; male) were obtained. The National Research Council Guide

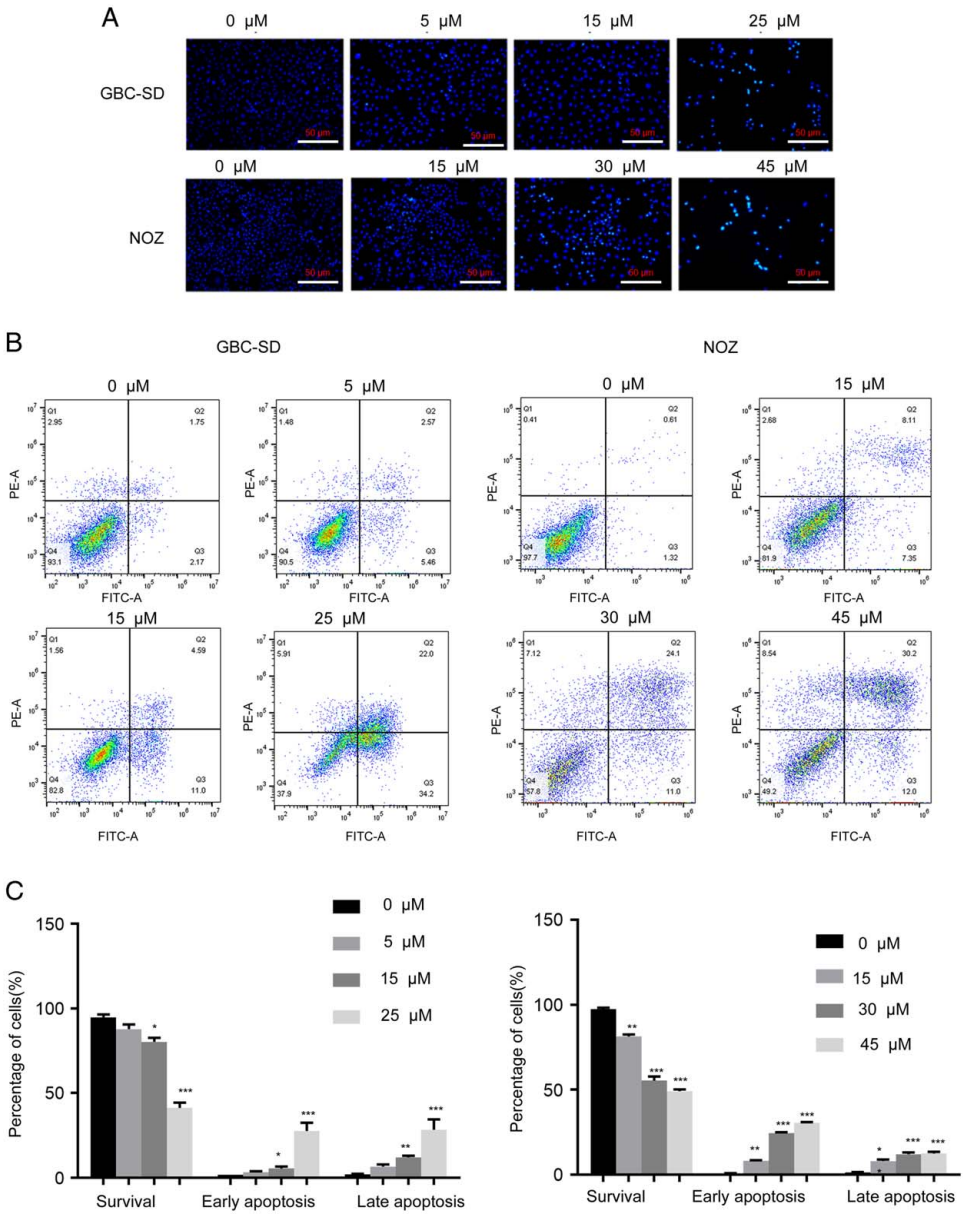

Figure 2. PTL induces apoptosis of GBC-SD and NOZ cells. (A) GBC-SD and NOZ cells were treated different concentration with PTL, and performed with Hoechst 33342 staining and visualized by fluorescence microscopy (scale bar, 50 μm). (B–D) GBC-SD and NOZ cells were analyzed by flow cytometry with annexin V–FITC/propidium iodide (PI) staining after using different concentration with PTL. (C–E) Quantified data of Annexin V/PI staining assay. The apoptosis percentage increased dramatically after PTL exposure of GBC-SD and NOZ cells. Annexin V versus PI plots from the gated cells show the populations corresponding to viable (annexin V −/PI −), necrotic (annexin V −/PI +), early apoptotic (annexin V + /PI −), and late apoptotic (annexin V + /PI +) cells. The data are presented as the mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.01$ versus control. GBC, gallbladder cancer; PTL, parthenolide.

for the Care and Use of Laboratory Animals was followed, and ethical approval was obtained from the Ethics Committee. The mice were housed in a room with a 12:12 h light-dark cycle, temperature of 24°C, and humidity of 65%. In addition, mice were provided with free access to food and water. NOZ cells were subcutaneously injected into the right flank of mice (10^6 cells/mouse). Twenty-four hours later, the mice were randomly divided into three groups (control, 5 mg/kg PTL, and 15 mg/kg PTL). The mice received PTL at the appropriate dose (0, 5, or 15 mg/kg) every three days for up to 28 days. On day 29, the animals were sacrificed and their tumours were dissected and weighed.

Hematoxylin and eosin (H&E) staining and immunohistochemical (IHC)

Tissues were fixed in 10% buffered formalin and embedded in paraffin at room temperature. Sections (5 μm thick) were cut for H&E staining and IHC evaluation. HE staining was performed following the instructions of the Eosin Y Stain Solution (Beijing Solarbio Science & technology (G1100). For IHC, the tissue sections were immunostained with primary antibodies against Ki67 (1:1000; ABclonal, China) overnight at 4°C. The following day, the sections were incubated with a goat anti-rabbit IgG secondary antibody at 37°C for 30 min. The sections were then

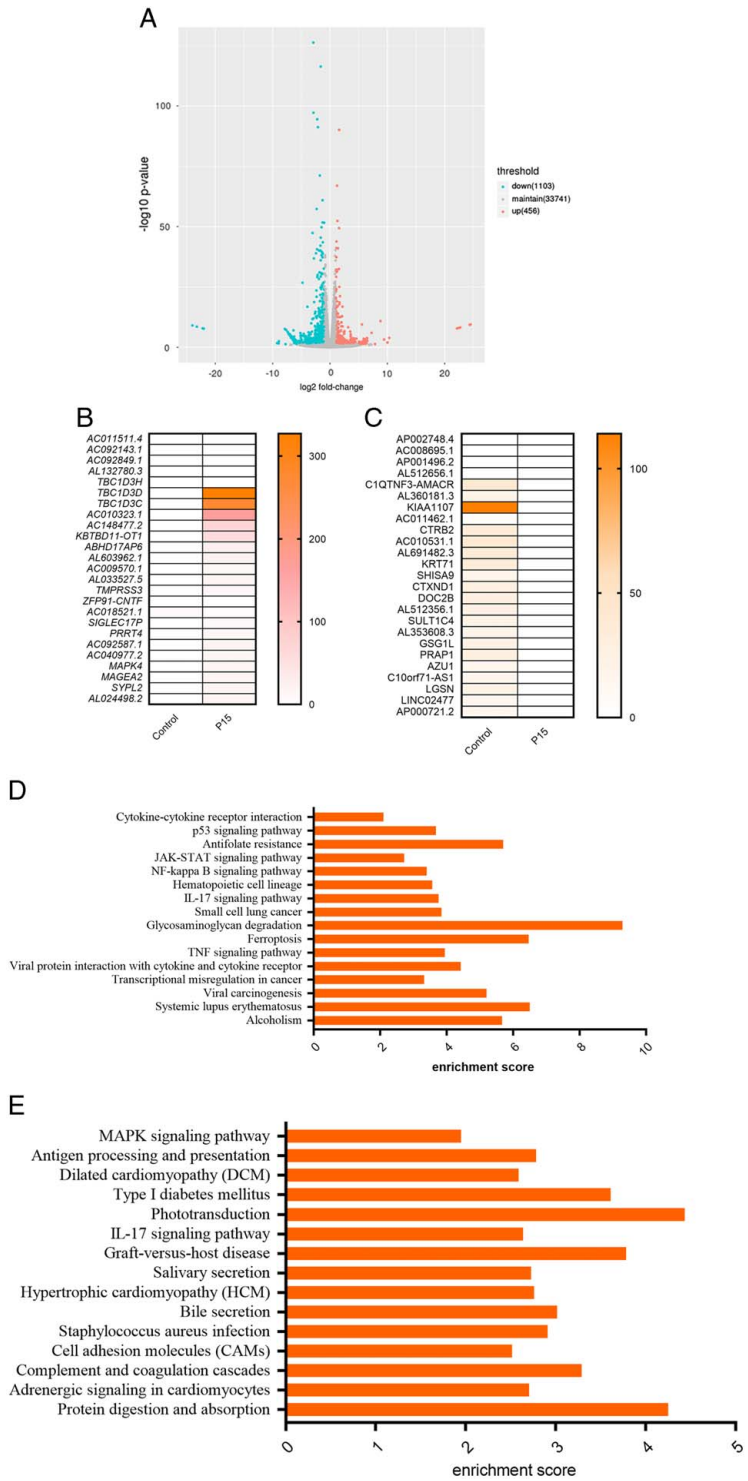

Figure 3. Analysis of RNA-seq data. (A) Analysis of differentially expressed genes using a volcano plot. (B, C) The heatmap of the top 25 genes with the most significant upregulation (B) and the top 25 genes with the most significant downregulation (C) (D, E) Quantified data of KEGG pathways using DAVID database. Significantly upregulated gene enriched KEGG pathway (D) and significantly downregulated gene enriched KEGG pathway (E).

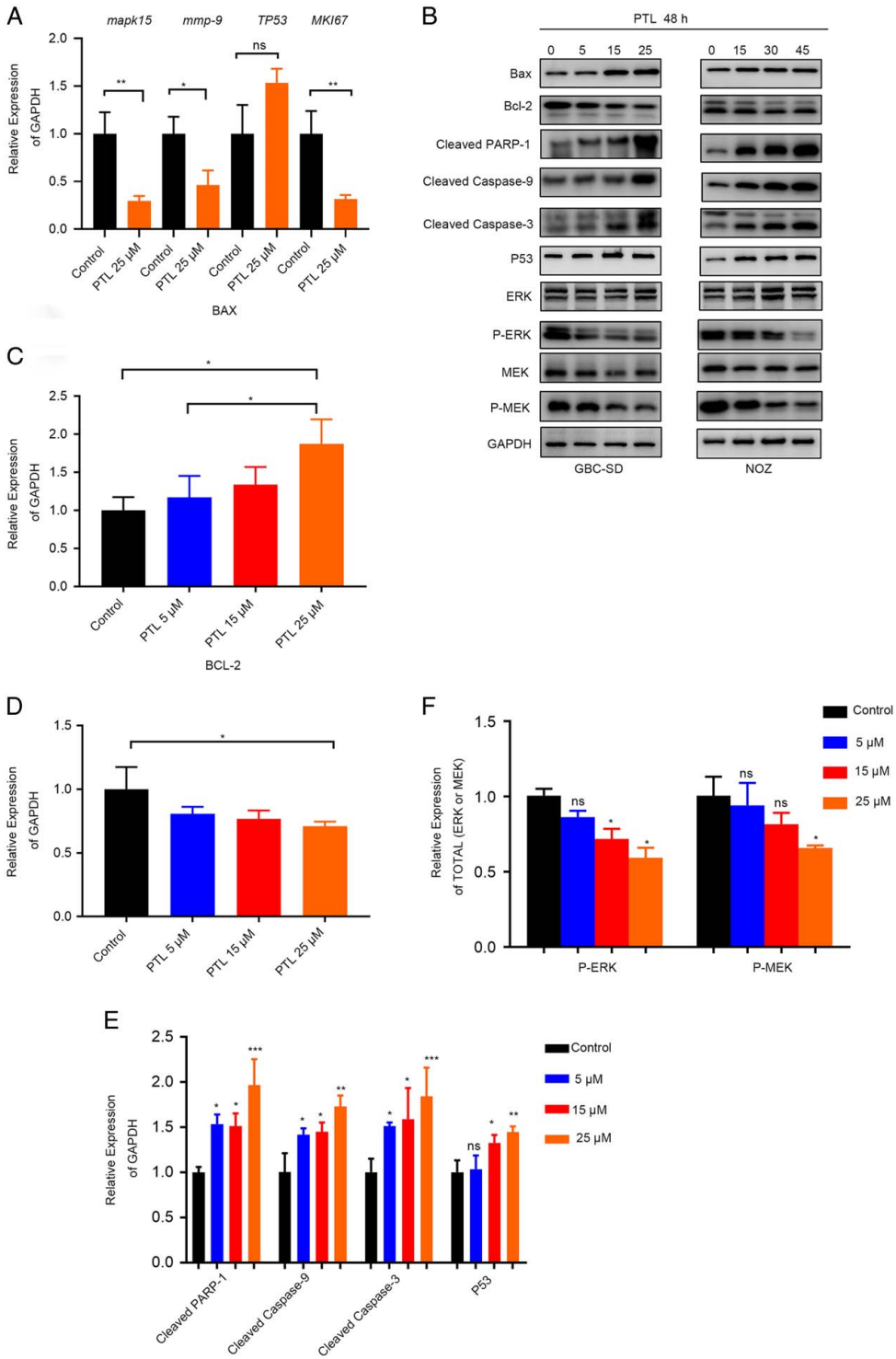

Figure 4. MAPK signalling pathway was related to the PTL-induced GBC cells apoptosis. (A) The mRNA expression of pathway-related genes in GBC-SD cells treated by PTL. The mRNA expression of genes was examined after treatment of 25 μM PTL for 48 h. (B) Western blot analysis of apoptosis and ERK-MEK pathway-related proteins expression after using different concentration with PTL of GBC-SD and NOZ cells. GAPDH was used as a loading control ($n = 3$). (C–F) Quantified data of apoptosis and ERK-MEK pathway-related proteins expression after using different concentration with PTL of GBC-SD cells $n = 3$. (***) $P < 0.001$, (**) $P < 0.01$ and (*) $P < 0.05$ compared with the control group. GBC, gallbladder cancer; MAPK, mitogen-activated protein kinase; PTL, parthenolide.

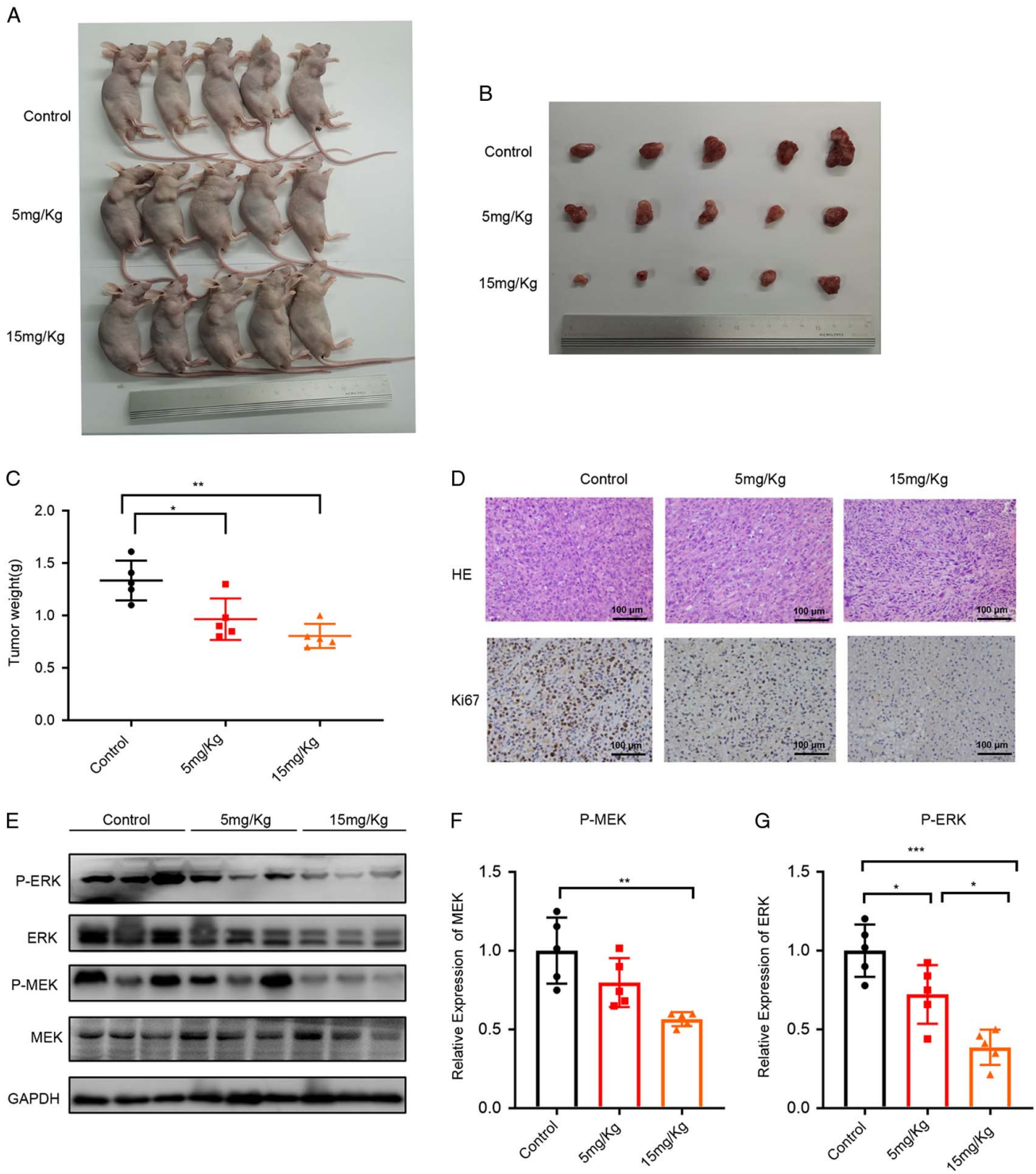

Figure 5. PTL suppresses the growth of xenograft GBC tumours in nude mice. (A–C) GBC-SD cells injected to the flank of the nude mice and tumour xenografts were established. Mice were treated with 0.1 ml vehicle (PBS) or PTL (5 or 15 mg/kg) i.p. every day for up to 4 weeks. The tumour volumes were measured and quantified. Data represent the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. (D) Representative images of H&E and Ki67 staining of tumour sections collected from different groups at 30 days (scale bar = 100 μm) and quantified data of the percentage of KI67 positive cells. (E–G) Western blot analysis and quantification of p-ERK, ERK, p-MEK, and MEK in tumours. GAPDH served as a loading control ($n = 5$). GBC, gallbladder cancer; H&E, hematoxylin and eosin; PTL, parthenolide.

stained with 3,3′-diaminobenzidine and counterstained with hematoxylin. For each sample, a light microscope (Olympus Corp.) was used to capture the images. Three optical fields areas were selected for each sample using a light microscope (Olympus Corporation).

Statistical analysis

The results are presented as mean ± standard deviation. Comparisons between groups were made using Student's *t*-test. All statistical analyses were performed using SPSS (version 22; IBM Corp.) and GraphPad Prism (version 5; GraphPad Software, Inc.), and *P* less than *0.05*.

Results

PTL inhibits the proliferation of GBC-SD and NOZ cells in vitro

To investigate the effect of PTL on gallbladder cancer cells, we analyzed the proliferation of GBC-SD and NOZ gallbladder cell lines using a CCK8 assay. CCK8 assay showed that PTL inhibited the growth of GBC-SD and NOZ cells in a dose-dependent manner at 24 and 48 h. The IC50 values in GBC-SD cells at 24 and 48 h were 15.6 and 17.55 μM, respectively. The IC50 values in NOZ cells at 24 and 48 h were ~31.54 and 28.75 μM, respectively (Fig. 1B-C). Therefore, in our follow-up experiment, we selected 5, 15, and 25 μM as the concentration gradient for treating GBC-SD cells and 15, 30, and 45 μM as the concentration gradient for treating NOZ cells to complete the experiment. Compared to normal cells, tumour cells can be expanded from single cells and allow the formation of cell colonies *in vitro*. We investigated whether PTL could affect the proliferation of GBC-SD and NOZ gallbladder cancer cells *in vitro*. The results showed that after PTL treatment, the number of clones and the size of a single clone of gallbladder cancer cells significantly decreased, and these values were significantly different from those of the control group (Fig. 1D). Our data indicate that PTL treatment significantly inhibited the proliferation of gallbladder cancer cells *in vitro*.

PTL induces the apoptosis of GBC-SD and NOZ cells

Hoechst 33342 staining was performed to evaluate the effect of PTL on the apoptotic morphology of GBC cells. PTL-treated cells showed obvious nuclear shrinkage, and some cells exhibited nuclear fragmentation and formed multiple spherical particles (Fig. 2A). Additionally, after treatment with PTL for 48 h, the early apoptosis rate of GBC cells significantly increased. The late apoptosis rate also significantly increased with increasing PTL concentration. When the PTL concentration was 25 μM, the early apoptotic GBC-SD cells increased from 2.18 ± 0.25% to 28.4 ± 4.98% and that of late apoptotic cells proportion of GBC-SD cells were increased from 1.78 ± 0.22% to 27.67.20 ± 4.03%, (Fig. 2B, C). When the concentration of PTL was 45 μM, the early apoptosis rate of NOZ cells increased from 1.39 ± 0.71% to 12 ± 3.16% and the late apoptosis rate of NOZ cells increased from 0.73 ± 0.11% to 30.34 ± 2.16% (Fig. 2D, E). The rate of GBC cell apoptosis increased with PTL concentration, and tumour cells also exhibited apoptosis-related nuclear morphology, confirming that PTL has antitumor properties with great potential in the treatment of GBC. Taken together, our results indicate that PTL significantly induced GBC cell apoptosis *in vitro*.

MAPK signalling pathway is related to PTL-induced GBC cell apoptosis

RNA sequencing (RNA-seq) was performed to determine the footprint of PTL-related apoptosis. GBC-SD cells were treated with or without 15 μM PTL (p15 and control groups, respectively). Three independent biological replicates were used for each experiment. The DAVID database was used to analyze functional annotation clustering. RNA-Seq analysis identified a total of 1559 genes that were significantly changed after PTL treatment (Fig. 3A, log2[fold change] ≥ 2; adjusted $P < 0.05$) (Fig. 3A). The heatmap shows the top 25 genes with the most significant upregulation (Fig. 3B) and the top 25 genes with the most significant downregulation (Fig. 3C). KEGG pathway analysis revealed that many immune-related genes and pathways were included, supporting the role of PTL in regulating immune response (Fig. 3D-E). In addition, our results showed that P53 signalling, MAPK signalling, and IL-17 signalling pathway-related genes were significantly different between the two groups (Fig. 3D-E). These data suggest that PTL controls the induction of a broad spectrum of immune responses and P53, MAPK signalling-related genes, affecting cancer cell apoptosis. After GBC-SD and NOZ gallbladder cancer cells were treated with PTL for the indicated times, the expression of apoptosis-related genes and proteins was measured. As shown in Fig. 4, PTL treatment suppressed the mRNA expression of mapk15, mmp-9, and ki67 and increased the expression of P53(Fig. 4A). Furthermore, we performed western blotting on NOZ and GBC-SD cells. Western blot analysis suggested that the expression of BAX, Cleaved PARP-1, Cleaved Caspase-3, Cleaved Caspase-9, P53 was significantly upregulated, and the expression of BCL-2, phosphorylated ERK, and phosphorylated MEK was significantly downregulated after PTL treatment *in vitro* (Fig. 4B-F). Phosphorylation levels of ERK and MEK were remarkably decreased after PTL treatment, indicating that PTL-induced apoptosis may be correlated with the MAPK signalling pathway.

PTL inhibits GBC cell proliferation in vivo

To further verify the antitumor effect of PTL *in vivo*, GBC-SD cells were subcutaneously injected into nude mice. After 24 h, nude mice were randomly divided into three groups, of which two groups were intraperitoneally injected with different concentrations of PTL (5 and 15 mg/kg). The solvent control group was established and injected every 3 days for a total of 30 days. The size and weight of the tumours are shown in the Fig. 5A. One month after intraperitoneal injection, the tumour weights of the control group and the 5 and 15 mg/kg PTL groups were 1.36 ± 0.37 g, 0.97 ± 0.17 g, and 0.81 ± 0.11 g, respectively. Compared with the control group, PTL significantly decreased the tumour weight of nude mice and showed an obvious dose-dependent effect (Fig. 5B-C). HE staining indicated that the model was successfully established. Immunohistochemical staining of ki67 also performed. PTL significantly decreased ki67 expression *in vivo*, which confirmed our results (Fig. 5D). PTL also decreased the levels of phosphorylated ERK and MEK *in vivo* (Fig. 5E-G). These data suggest that PTL can significantly inhibit tumour growth *in vivo*.

Discussion

GBC is one of the most common malignant tumours of the biliary tract. A previous study showed that surgical resection is considered the main method for GBC treatment; however, GBC still has a poor prognosis[14]. The exploration of new methods of GBC therapy is an interesting topic. Natural Chinese medicine has been recognized as an alternative approach to gallbladder cancer therapy[15]. Our results also showed that PTL, isolated from traditional Chinese medicine, inhibited GBC cells and exerted antitumor effects.

PTL has been widely used for its anti-inflammatory and antioxidant properties and its biological effects in treating headaches, fever, and rheumatoid arthritis[16]. PTL has been studied for its inhibitory effect on NF-κB and its anticancer effects on many tumours, including breast cancer, prostate cancer, papillary thyroid carcinoma, and lung cancer[16]. A previous study indicated that PTL has minimal cytotoxicity against cancer stem cells in nasopharyngeal carcinoma[17]. Other studies have also shown that PTL inhibits the tumour-promoting effects of nicotine on lung cancer by inducing P53-dependent apoptosis and inhibiting VEGF expression[18]. Additionally, PTL can induce cell death in human osteosarcoma cells via ROS-mediated autophagy[19]. In a murine animal model, PTL inhibited the lung colonization of osteosarcoma cells[20]. PTL was found to inhibit hypoxia-inducible factor-1α (HIF-1α) through epithelial-mesenchymal transformation induced by signal transduction and hypoxia in colorectal cancer[21]. Cui *et al.*[22] found that PTL can activate autophagy and apoptosis by inhibiting the rapamycin/phosphatidylinositol 3 kinase/protein kinase B (mTOR/PI3K/AKT) signalling pathway, thereby inhibiting MDA T32 papillary thyroid cancer cells. Based on previous functional studies on PTL, we aimed to explore its biological effects on gallbladder cancer. The present study showed that PTL significantly inhibited GBC cell growth *in vivo* and *in vitro*. Our experimental evidence showed that PTL-induced apoptosis in GBC-SD and NOZ cell lines in a dose-dependent manner. To further investigate the mechanism underlying PTL-induced apoptosis in GBC cells, RNA sequencing was performed. Our RNA sequencing results indicate that this mechanism may be related to a variety of signalling pathways and genes, which may form a complex network. Consistent with previous research, our RNA-seq results also suggest that PTL may influence other signalling pathways, such as the IL-17, JAK-STAT, P53 and NF-KB signalling pathways[16], which are very hot topic in field of apoptosis and cancer. our results showed that PTL leads to tumour cell apoptosis, combined with RNA-seq results, it may affect the implementation of P53 cell apoptosis pathways. At the same time, we have also observed changes in MAPK and other pathways although MAPK is not the most significantly altered pathway. However, the final validation experiment results show that the ERK and MEK phosphorylation levels were remarkably decreased after PTL treatment in NOZ and GBC-SD cells. Nevertheless, MAPK pathway changes are more significant, Therefore, we focus on studying the relationship between PTL and MAPK pathways.as fa as we considered that the NF-kB and immune-related signalling pathway are related to the anti-inflammatory effects of PTL which reported in previous literature. In future research, we will focus on this area and conduct more specific research to reveal underlying mechanism. Indeed, our subsequent validation results also showed significant changes in the MAPK pathway after PTL treatment. Although other signalling pathways were also significantly enriched after PTL administration, to the best of our knowledge, apoptosis may be more closely related to the MAPK pathway. Therefore, our subsequent research focused on the MAPK signalling pathway. After examining the MAPK-related signalling pathway using qPCR and western blotting, encouraging results were obtained *in vivo* and *in vitro*.

The MAPK family plays an important role in regulating the proliferation, differentiation, transformation, apoptosis, and metabolism of malignant tumours[23,24]. MAPK signalling is also involved in the occurrence and development of a variety of cancers, indicating its important role in tumour biological processes such as cancer cell proliferation, apoptosis, and differentiation[25,26]. The extracellular regulated protein kinase (ERK1/2) family was the first classic MAPK signal transduction pathway to be discovered, and includes Ras, RAF, and MAPK. ERK1/2 comprises five subgroups: ERK1/2, ERK3/4, and ERK5. ERK1 and ERK2 are two highly homologous subclasses, and the first members of the MAPK family have been cloned. ERK1/ERK2 is closely related to cell proliferation and apoptosis[27]. Activated ERK1/2 translocate to the nucleus and activates transcription by phosphorylating p90rsk, MSK, and the transcription factors Elk-1 and STAT3, resulting in cell growth, proliferation and differentiation[28].

A previous study also showed that multiple signalling pathways are related to GBC progression[29]. The ERK/MEK pathway is one MAPK signalling pathway, and it is a modulatory pathway that regulates several fundamental cellular processes, including apoptosis[30]. Our Western blotting results demonstrated that upregulation of the apoptosis-related proteins Bax, cleaved caspase-3, caspase-9, and PARP-1, and downregulation of the anti-apoptotic proteins BCL-2 and p53 were also upregulated after PTL treatment. Our results showed that PTL significantly decreased the expression and phosphorylation of ERK/MEK proteins *in vivo* and *in vitro*, indicating that its antitumor effect may be mediated by the MAPK pathway in GBC.

In this study, traditional Chinese medicines PTL showed a strong growth inhibitory effect towards NOZ and GBC-SD cells and suppresses the growth of xenograft GBC tumours in nude mice. Previous studies have showed that PTL has therapeutic effects on skeletal diseases, primary and metastasis bone cancers, and inflammation-induced cytokine storm[22,31,32]. All this research strongly suggests that PTL offers an excellent opportunity to be an effective candidate drug for many diseases, including GBC , and further understanding the mechanistic insights into the role of PTL in a disease specific manner will not only provide many drug targets but also help us to develop therapeutic applications of PTL for autoimmune disease, cancer, and infection disease such as COVID-19. GBC is characterized high malignancy, lack of effective drugs and poor prognosis. Therefore, providing effective drugs and targets will be indispensable for GBC. In the future, we will study the immunoregulatory capacity of PTL and explore the potential interaction mechanism of its immune and anticancer functions. In addition, there are limitations regarding the clinical use of PTL, including clinical trial studies, and future studies needs to explore for these issues, which will enhance the clinical applications of PTL in various diseases.

In summary, this study is the first to demonstrate the potential anticancer effects of PTL in GBC. PTL inhibits proliferation and induces apoptosis of GBC cells. RNA sequencing and other experiments have demonstrated that MAPK signalling contributes to PTL-related GBC apoptosis. Therefore, PTL can be

considered as a new potential reagent for the treatment of GBC. However, further exploration is needed to verify the antitumor effects of PTL and its intracellular signalling mechanism.

Limitations

Our studies have focused on the efficacy of PTL in GBC with lack of reports on toxicity assessments and in-depth mechanistic insights. *in vivo*, sample size was small and experiment not being blinded. Thus, further studies will help to better understand the draggability of PTL.

Ethical approval

Ethics approval for patients was not required for this research. All animals were performed in accordance with the relevant guidelines and regulations, and approval by Animal Experiment Center of Xinjiang Medical University (ethical committee approval no. IACUC-20220301-06). The animals presented a healthy status and male mice were used for all experiments.

Consent

The present study followed international, national and/or institutional guidelines for humane animal treatment and complied with relevant legislation; The present study involved client-owned animals and demonstrated a high standard (best practice) of veterinary care and involved informed client consent.

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Author contribution

H.O., G.A. and A.A. conducted the experiments, Y.L. performed the statistical analysis, and S.D. wrote the manuscript.

Conflicts of interest disclosure

The authors declare no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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This research did not involve human subjects.

Guarantor

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Provenance and peer review

Not applicable.

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