Original Paper

Erinacine Facilitates the Opening of the Mitochondrial Permeability Transition Pore Through the Inhibition of the PI3K/Akt/GSK-3 β Signaling Pathway in Human Hepatocellular Carcinoma

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Key Words

Erinacine • Hepatocellular carcinoma • Mitochondrial permeability transition pore • PI3K/Akt/GSK-3 β signaling pathway • LY294002

Abstract

Background/Aims: Erinacine, which is extracted from the medicinal mushroom *Hericium erinaceus*, is known to play anticancer roles in human cancers. The following study aims to investigate the role of erinacine in the opening of the mitochondrial permeability transition pore (MPTP) in hepatocellular carcinoma (HCC) through the PI3K/Akt/GSK-3 β signaling pathway and highlight the applicability of erinacine in HCC treatments. **Methods:** HCC and paired cancerous tissues were obtained from 85 HCC patients who've undergone surgical resection. Immunohistochemistry was adopted to detect positive expression of PI3K, Akt, and GSK-3 β . Treatment of HepG-2 with LY294002 (an inhibitor of the PI3K/Akt/GSK-3 β signaling pathway) and different concentration of erinacine was performed to determine the involvement of LY294002 in erinacine action. The expressions of PI3K, Akt, GSK-3 β , CyclinD1, E-cadherin, β -catenin, Bcl-2, E-cadherin, Bax, and caspase-9 were determined by RT-qPCR and western blot analysis. Cell viability, colony formation rate, migration, invasion, cycle, and apoptosis were detected by MTT, colony formation, wound healing assay, Transwell assay, and flow cytometry, respectively. The size and weight of xenograft tumors were observed in nude mice. Mitochondrial membrane potential in HepG-2 was determined using laser scanning confocal microscopy following JC-1 staining. Mitochondrial Ca²⁺ indicator Rhod-2, AM was used to detect the changes of mitochondrial Ca²⁺, while western blot analysis was employed to detect the presence levels of cytochrome C (cyt-C). **Results:** The results

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revealed that PI3K, Akt, and GSK-3 β were up-regulated in HCC tissues. Erinacine or LY294002 led to a decrease in mitochondrial membrane potential, increase in intracellular mitochondrial Ca²⁺, and the release of cyt-C in mitochondria. In addition, Erinacine was found to decrease the mitochondrial membrane potential, expression of PI3K, Akt, GSK-3 β , CyclinD1, Vimentin, β -catenin, and Bcl-2, cell proliferation, colony formation ability, migration, invasion, and xenograft tumor size, while E-cadherin, Bax, and caspase-9 expression, and cell apoptosis were elevated in a dose-dependent manner. Erinacine also stimulated the effects of LY294002 on the HCC. Following the addition of 500 μ M Erinacine and MPTP opening inhibitor CsA, we found that the mitochondrial membrane potential level increased, while mitochondrial Ca²⁺ and Cyt-C decreased from the mitochondria. **Conclusion:** The results from the study demonstrated that erinacine induced MPTP opening, facilitates the release of cyt-C, and inhibited cell proliferation, migration, and invasion, while it promotes apoptosis by inhibiting the PI3K/Akt/GSK-3 β signaling pathway, preventing the progression of HCC.

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Introduction

Liver cancer is known to be one of the most common malignancies with high morbidity and mortality, affecting large portions of the population worldwide [1]. The national population-based cancer registration data showed that the incidence rate of liver cancer is increasing in China, with higher susceptibility among the elderly population [2]. Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma account for majority of the primary liver cancer cases [3]. Chronic hepatitis B virus, hepatitis C virus, exposure to dietary aflatoxin, alcohol-induced cirrhosis, smoking, obesity, and diabetes have been identified as the risk factors for HCC [4-6]. Since the fact that the aforementioned risk factors are controllable, the incidence and mortality of HCC could be lowered through lifestyle modifications and hepatitis immunization [7]. Treatment for HCC is a multimodal and multidisciplinary task, with surgical resection and liver transplantation known to be the only potentially curative regimen [8]. In recent years, there have been findings highlighting mushroom polysaccharide, a traditional Chinese medical treatment, as a potential chemopreventive agent in the prevention of metastasis in HCC [9].

Erinacine is isolated from *Hericium erinaceus*, a genus of edible mushrooms in the *Hericiaceae* family consisting of polysaccharides which have anticancer properties and is involved in the immunomodulatory activity against human HCC [10, 11]. *Hericium erinaceus* polysaccharide is a traditional Chinese medicine [12], and beneficial nutrient native to China and other Asian countries, which has previously been employed for treating cancers, lymphatic disease and geriatric disorders [13]. Although the involvement of erinacine in HCC has been identified, very little is known on the molecular mechanisms by which erinacine suppresses human HCC cell growth. Genomic study has suggested that the PI3K/Akt signaling pathway is one of the most common signaling pathways that is most likely to be dysregulated in several kinds of cancer, thereby playing a critical role in carcinogenesis [14]. Abnormal activation of the PI3K/Akt pathway was observed in various types of human malignancies including HCC, and its activation is associated with HCC progression [15]. The polysaccharides present in *Ganoderma lucidum* (an edible mushroom) has been demonstrated to process anti-metastatic/-invasive activities through the modulation of the PI3K/Akt pathway [17]. Mitochondria plays a role in the regulation of the apoptotic and necrotic cell death, while the opening of the mitochondrial permeability transition pore (MPTP) has been identified to be a strategic regulator of cell death, but the presence of MPTP in tumor tissues has not been evidently found [18, 19]. MPTP is the principal mechanism in acetaminophen-induced or chronic ethanol-mediated liver injury [20, 21]. It has been reported that the PI3K/Akt pathway is associated with the MPTP opening participating in the cardiomyocyte apoptosis involved in oxidative stress [22]. The present study focuses on the effects of erinacine on MPTP opening in HCC via the PI3K/Akt/GSK-3 β signaling pathway and highlights the ability of erinacine to impede the progression of HCC.

Materials and Methods

Ethical statement

This study has been approved by the Ethics Committee of the Affiliated Hospital of North China University of Science and Technology. A written informed consent was obtained from all patients prior to the study and animal experiments were conducted in accordance with the Welfare Ethics of Experimental Animal.

Study subjects

A total of 85 HCC tissue samples were obtained from HCC patients who had undergone surgical resection and had received no radiotherapy, chemotherapy, or other anti-tumor treatments prior to surgery in Hepatobiliary Surgery department of the Affiliated Hospital of North China University of Science and Technology. The corresponding para-cancerous tissue samples were selected as normal controls. The study subjects included 60 males and 25 females (between the ages of 30 and 74 years) with the mean age of (51.37 ± 9.3) years and 49 patients over the age of 50 years. The HCC samples were classified into the well-differentiated (26 cases), moderately differentiated (37 cases), and poorly differentiated (22 cases) groups based on their histopathological findings. According to the tumor-node-metastasis (TNM) staging classification by the International Union against Cancer (UICC), there were 6 cases of stage I, 15 cases of stage II, 28 cases of stage III, and 36 cases of stage IV. Based on the Cancer of the Liver Program (CLIP) scoring system, 7 cases were scored as point 0, 17 cases as point 1, 13 cases as point 2, and 48 cases as point ≥ 3 . Among these cases, there was lymphatic metastasis present in 10 patients. The fresh tissue samples were then frozen in liquid nitrogen in order to extract the total RNA. The control group was composed of HCC specimens without bleeding necrosis.

Immunohistochemistry

After drying in an incubator at 60°C overnight, the paraffin-embedded sections were dewaxed with xylene, dehydrated with gradient alcohol (100%, 95%, 80%, 5 min each), washed with tap water for 5 min, and then immersed in phosphate-buffered solution (PBS) for 3×3 min. The sections were then rinsed in 0.3% H_2O_2 -methanol to block endogenous peroxidase activity. After being washed with PBS 3 times, the sections were incubated with 1% goat serum (36119ES03, Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China) at room temperature for 30 min and incubated at 4°C overnight with rabbit anti-human PI3K (1: 1000 dilution, ab67189, Abcam Inc., Cambridge, MA, UK), rabbit anti-human Akt (1: 500, ab8805), and mouse anti-human GSK-3 β (1: 4000, ab93923, Abcam Inc., Cambridge, MA, UK), which were purchased from Abcam Inc. (Cambridge, MA, UK) with PBS considered as the negative control. Next, a horseradish peroxidase [HRP, 0343-10000U, Imunbio (Beijing) Biotechnology Co., Ltd., Beijing, China]-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (1:1000, ab6807, Abcam Inc., Cambridge, MA, UK) was added for a 30-minute incubation at room temperature. The same was developed by diaminobenzidine (DAB, P0203, Beyotime Institute of Biotechnology, Shanghai, China) until brown with the development process controlled under a microscope in a dark environment. Afterwards, the samples were washed with running water for 5 min, counterstained with hematoxylin for 1 min, differentiated for 5 s with 1% HCl/ethanol, and washed in running water. Consequently, the samples were mounted in neutral resin, and observed under an optical microscope, with the images obtained. Positive cells were then identified based on a brownish yellow appearance of the cytoplasmic membrane. Finally, five fields of vision in each section were selected. The average absorbance of five fields of vision was calculated using Image-Pro Plus Version 7.0.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The TaKaRa (TaKaRa, Dalian, Liaoning, China) was employed to extract the total RNA from tissues and cells, after which the concentration and purity of the total RNA were determined. RT-qPCR was performed in accordance with the manufacturer's instructions of RNA reverse transcription kit (K1621, Fermentas, MD, USA). The reaction conditions were as follows: at 70°C for 5 min, ice bath for 3 min, reverse transcription at 37°C for 60 min, and reverse transcriptase enzyme inactivation at 95°C for 10 min. The cDNA was then preserved at -20°C. Primer sequences (Table 1) of PI3K, Akt, GSK-3 β , CyclinD1, Vimentin, β -catenin, Bcl-2, E-cadherin, Bax, and caspase-9 were synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). Fluorescence quantitative PCR kit (TaKaRa, Dalian, Liaoning, China) was used to determine relative mRNA expression of genes. The reaction system was as follows: 5.3 μ L of $2 \times$ Taq MasterMix, 1 μ L of forward primer (5 μ M), 1 μ L of reverse primer (5 μ M), 1 μ L of cDNA template, and 11.7 μ L of RNase-free H_2O . The reaction condition was shown as follows: pre-denaturation at 95°C for 5 min, and 35 cycles of denaturation at 95°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 45 s. Real-time fluorescence PCR was performed

with an ABI7500 instrument (Applied Biosystems, Inc., Carlsbad, CA, USA), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The $2^{-\Delta\Delta Ct}$ formula was used to calculate the relative mRNA expression of the target gene. The experiment was repeated three times.

Western blot analysis

Total protein was extracted from tissues. Protein concentration was determined according to the instructions on the bicinchoninic acid (BCA) protein kit (23225, Pierce, Rockford, IL, USA) and adjusted to a concentration of 1 $\mu\text{g}/\mu\text{L}$. The pre-treated protein was added to the sampling wells (20 μg for each well) for protein isolation on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, P1200, Solarbio Science & Technology Co., Ltd., Shanghai, China). Firstly, the electrophoresis was performed at 8 V/cm. Following the addition of the protein into the separation gel, the voltage for electrophoresis was changed to 15 V/cm. The electrophoresis was terminated when the protein was close to the bottom of the separation gel. The protein samples were transferred onto the polyvinylidene fluoride (PVDF) membranes (HVLPO4700, Millipore, Bedford, MA, USA) through semi-dry transfer method and stained with ponceau S (P0012, Solarbio, Beijing, China). The membranes were washed with Tris-buffered saline Tween-20 (TBST), blocked at room temperature with 5% skimmed milk for 2 h, and then washed by TBST 3 times. The protein samples were incubated in a 4°C refrigerator overnight with rabbit anti-human p-PI3K antibody (1 : 1000, ab86714), monoclonal antibody against rabbit phosphorylated PI3K (p-PI3K) (1 : 1000, ab152073), rabbit anti-human Akt antibody (1 : 1800, PAB1310), rabbit anti-human p-Akt (1 : 500, ab32167), mouse anti-human GSK-3 β antibody (1 : 500, ab93926), rabbit anti-human p-GSK-3 β antibody (1 : 500, ab32230), rabbit anti-human Bcl-2 antibody (1 : 1000, ab32124), rabbit anti-human Bax antibody (1 : 1000, ab32503), rabbit anti-human CyclinD1 antibody (1 : 10000, ab134175), rabbit anti-human E-cadherin antibody (1 : 5000, ab32572), rabbit anti-human Vimentin antibody (1 : 100, ab8978), rabbit anti-human caspase-9 antibody (1 : 1000, ab32539), and mouse anti-human GAPDH antibody (1 : 500, ab8245) (all from Abcam Inc. (Cambridge, MA, UK) with the exception of Akt antibody from Abnova, Shanghai, China and p-GSK-3 β antibody from Shenzhen Otwo Biotech Inc. Shenzhen, Guangdong, China), after which the samples were washed by TBST 3 times (10 min per wash). Next, the samples were incubated with an HRP-conjugated anti-rabbit IgG antibody (1 : 2000, ab6721, Abcam Inc. (Cambridge, MA, UK)) and goat anti-mouse IgG antibody (1 : 1000, ab6789, Abcam, USA) at room temperature for 2 h, and rinsed in TBST for 3 times. DAB was applied for coloring and Gel Doc XR imager system (Bio-Rad Laboratories, Inc. CA, USA) for imaging. The gray value ratio of target protein to that of the internal reference GAPDH was regarded as the relative protein expression. These procedures were also performed during cell experiment.

Cell grouping

Human HCC HepG2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in an incubator with 5% CO₂ at 37°C with Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL penicillin/streptomycin solution. When the cell density reached 70%, the culture medium was discarded. The cells were then washed with PBS twice, digested in 0.25% trypsin (GIBCO-BRL, Gaithersburg, MD, USA), triturated in DMEM supplemented with 10% FBS to single cell suspension, and then passed. Cells in logarithmic growth phase were collected for the following experiments and incubated at 37°C with 5% CO₂/95% air mixture.

HepG-2 cells were treated and grouped into the control, the 100 μM erinacine, the 300 μM erinacine, the 500 μM erinacine, the LY294002 (as an inhibitor of the PI3K/Akt/GSK3 β signaling pathway), and the LY294002 + 500 μM erinacine groups. Cells in logarithmic growth phase were seeded in a 6-well plate (2

Table 1. Primer sequences of related genes for reverse transcription quantitative polymerase chain reaction. Note: PI3K, phosphoinositide 3-kinase; GSK-3 β , glycogen synthase kinase-3 β ; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Gene	Primer sequence (5' - 3')
PI3K	Forward: TGGCCTTAGCTCTTAGCCAAAG Reverse: ATTGGAACACGGCCTTTGACA
Akt	Forward: CAACTTCTCTGTGACAGTC Reverse: TGGTTGTAGAAGGACACGCA
GSK-3 β	Forward: GGAACCTCCAACAAGCCTCA Reverse: TTCGGGGTCCGACACCCCT
Bcl-2	Forward: GTGAACTGGGCTGATTC Reverse: GGAGAAATCACTGAGGCC
Bax	Forward: CCCGAGAGCTCTTCTGACG Reverse: CCAGCCGATCTGGTCTCT
Vimentin	Forward: GACAACTCTCTGGCAGCTCTT Reverse: TCCTCCCTCTGTCAGGTTCTT
E-cadherin	Forward: CACATCAGGCTCAGAAATGAA Reverse: CACCTTCCTCCATCCTGTTT
CyclinD1	Forward: ATGCTGCTGCTTAAGATGA Reverse: AATCTTCTGAGCTTGTTT
Caspase-9	Forward: CAGATGGAGTTGGACATGGC Reverse: CAGAGAGGATGTGGATACCTCC
GAPDH	Forward: CAGCCAGATGCTGTCCCAT Reverse: CCAAGGTCTCGATGTACCAGGAA
PI3K	Forward: ACAGCCTCAAGATCATCAGCAA Reverse: ATGGCATGGACTGTGGTCATG

$\times 10^5$ cells per well). The cells in the above group were then added with 500 μM culture medium, 100 μM erinacine, 300 μM erinacine, 500 μM erinacine, 1 μM LY294002, and 1 μM LY294002 + 500 μM erinacine according to the grouping, twenty-four hours after the adherence of the cells to the wall.

Confocal microscopic analysis for mitochondrial membrane potential

JC-1 was clustered in mitochondria in a potential-dependent manner. The membrane potential of normal mitochondria is relatively high, and JC-1 multimeric aggregates in the mitochondrial matrix emitted red fluorescence. JC-1 in the abnormal mitochondria could not accumulate in the mitochondrial matrix due to a decrease or loss of membrane potential and thus, emitted green fluorescence. A total of $\times 10^4$ cells were resuspended in 1 mL culture medium, added with 1 mL JC-1 dye (40706ES60, Shanghai Yeasen Biotechnology Co., Ltd, Shanghai, China), mixed, and cultured, with the avoidance of light at 37°C for 20 min. The redundant staining solution was washed off with JC-1 staining buffer. The suspension was centrifuged at 1000 rpm for 3 min, followed by the removal of the supernatant. The cells were then added with 500 μL JC-1 dye, resuspended, observed under an Olympus FV100 confocal microscope (Olympus, Tokyo, Japan), and imaging was performed. Five fields of vision were randomly selected. The fluorescence intensity was calculated using Image-Pro Plus Version 6.0. An increase in the ratio of red/green fluorescence intensity indicated a relatively high mitochondrial membrane potential.

Detection of mitochondrial Ca^{2+}

Cells were loaded with Rhod2, AM (5 μM) for 40 min at 37°C in order to measure changes in mitochondrial Ca^{2+} . Afterwards, the cells were then washed and resuspended in calcium-free buffer solution. A laser scanning confocal microscope (Olympus fluo FV10i, Olympus, Tokyo, Japan) was applied to observe the changes in mitochondrial Ca^{2+} . The mitochondrial mediated influx of Ca^{2+} was measured by means of Image Pro Plus 6.0 [23].

Expression of cytochrome-C (cyt-C) in mitochondria and cytoplasm

The isolation of the mitochondrial and cytosolic protein was conducted by a commercial mitochondria isolation kit (Applygen Technologies Inc., Beijing, China). The protein levels of cyt-C in mitochondrial and cytosolic fraction were measured using BCA Protein Assay (Pierce and Warriner, Warriner, Nanjing, China). In addition, cyt-C expression in mitochondrion and cytoplasm was examined with the use of western blot analysis [24].

3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cells were seeded in a 96-well plate with a density of 5×10^3 cells/well, cultured in a CO_2 incubator at 37°C, and added with 10 μL MTT solution (Sigma-Aldrich, St. Louis, MO, USA) at the 24th, 48th, and 72th h. Following a 4-hour incubation, the culture medium was removed and each well was reacted with 100 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich Corp., St. Louis, MO, USA) with the avoidance of light, followed by 15 min of gentle shaking on a shaking table. The optical density (OD) value of each well was measured at 570 nm using a microplate reader (BioTek, Winooski, VT, USA). The following formula was then used to measure the rate of cell inhibition: Cell inhibition rate = $(\text{OD}_{\text{blank control group}} - \text{OD}_{\text{experiment group}}) / \text{OD}_{\text{blank control group}}$. The above procedure was repeated three times.

Cloning formation

Luria-Bertani medium plate (D0110, Nobleryder, Beijing, China) was prepared. The cells were digested, centrifuged, washed, and re-suspended in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD, USA). Next, the cells were seeded in culture medium and cultured in an incubator with 5% CO_2 at 37°C for 2 weeks. After the culture medium was removed, the cells were washed with PBS 3 times, fixed in 4% paraformaldehyde at 4°C for 20 min, and stained with crystal violet dye for 1 h. The staining solution was removed and washed. The cells were then air-dried and the number of clones including more than 50 cells was counted under a microscope. The experiment was repeated three times.

Wound healing assay

After 48-hour culture, the cells were seeded in a 6-well plate with a density of 1×10^5 cells/well. When cell confluence was 90%, cell monolayers were wounded by scratching using a sterile 200- μL pipette tip. There were 4 scratches of identical strength in each well. The cells were then rinsed 3 times with PBS to remove debris. Each well was added with 2 mL culture medium containing 10% serum and incubated in an incubator. Images of the wounds were acquired at the very beginning and the 24th h under an inverted microscope. Five fields of vision were randomly selected from each sample in order to count the cells and measure the scratch width. The healing rate was calculated using the formula: healing rate = $[(\text{scratch width}_{0\text{h}} - \text{scratch width}_{24\text{h}}) / \text{scratch width}_{0\text{h}}] \times 100\%$. The above experiments were repeated three times.

Transwell assay

The Matrigel (354230, Shanghai Qcbio Science & Technologies Co. Ltd., Shanghai, China) was dissolved at 4°C overnight. After being diluted with serum-free RPMI 1640 medium (Matrigel : RPMI 1640 medium = 1 : 3), the Matrigel (30 µL) was added to the upper chamber three times (15 µL, 7.5 µL, 7.5 µL) at 10-minute intervals. The diluted Matrigel was evenly spread out to cover all pores. After 48-hour culture, the HepG2 cells were collected to make cell suspension, and seeded in the upper chamber of the Transwell chamber (Corning, NY, USA). The basolateral chamber with a 24-well plate was added with 0.5 mL RPMI 1640 medium containing 10% FBS. After culturing at 37°C with 5% CO₂ for 24 h, the cells that failed to move through the Matrigel on the upper chamber were removed. Subsequently, the Transwell chambers were fixed in 95% ethanol for 15 – 20 min, stained with methyl violet for 10 min, and rinsed by water. Cell invasion was observed with an inverted fluorescence microscope and the images were obtained. Five fields of vision were then randomly selected from each sample to count the number of cells moving through the Matrigel as an index of cell invasion ability. The experiment was repeated three times.

Flow cytometry

After being cultured for 48 h, the culture medium was removed. The cells were washed with PBS, detached by 0.25% trypsin, and centrifuged at 1000 rpm for 5 min, after which the supernatant was removed. Then, the cells were fixed overnight with cold 70% ethanol at 4°C, washed with PBS, centrifuged at 1000 rpm for 5 min, and incubated with 10 µL RNAase at 37°C for 1 h. Next, 1 µL propidium iodide (PI; 40710ES03, Shanghai Qianchen Biological Technology Co. Ltd., Shanghai, China) was used in order to stain the culture for 30-minute with the avoidance of light. Cell cycle was analyzed by an excitation wavelength of 488 nm using a flow cytometer (FACS Calibur; Becton-Dickinson, San Jose, CA, USA). The experiment was repeated for 3 times.

Following culturing for 48-h, the cells were digested by ethylenediaminetetraacetic acid (EDTA)-free trypsin, and centrifuged at 1000 rpm for 5 min at 4°C. Following the removal of the supernatant, the cells were rinsed in cold PBS, followed by a centrifugation at 1000 rpm for 5 min. The supernatant was discarded afterwards. Cell apoptosis was detected using the Annexin V-FITC/PI Apoptosis Detection kit (CA1020, Solarbio, Beijing, China). The cells were washed with binding buffer, and resuspended in the mixture of Annexin-V-FITC and binding buffer (1:40), mixed, and incubated. Following 30-minute culturing at room temperature, the cells were added with the PI and binding buffer (1:40), mixed, and cultured at room temperature for 15 min. Cell apoptosis was then detected using the FACS Calibur (Becton-Dickinson, USA). The experiment was repeated for 3 times.

Xenograft tumors in nude mice

Total 48 BALB/c nude mice (age: 6 weeks; weight: 20 ± 5 g) purchased from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, Guangdong, China) were grouped randomly into the control, the 100 µM erinacine, the 300 µM erinacine, the 500 µM erinacine, the LY294002, and the LY294002 + 500 µM erinacine groups, with 8 mice in each group. They were fed under constant temperature in the condition in a sterile environment. Before tumor inoculation, the mice were observed for about one week. Following treatment, the cells were collected, centrifuged at 1000 rpm for 5 min, rinsed in PBS twice, and mixed with PBS diluted Matrigel (PBS : Matrigel = 2: 1). After anesthesia, 5 × 10⁶ HCC cells were inoculated in the nude mice *via* subcutaneous injection. The length and width of tumor were recorded every five days using vernier caliper to calculate the tumor volume using the following formula: Volume = (Length × Width²) / 2. On the day after inoculation, the nude mice were sacrificed through neck dislocation and the tumors were collected to determine the weight.

Statistical analysis

SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was used for data analysis. The measurement data was presented as mean ± standard deviation. Comparison between two groups was performed by *t*-test, and the comparisons among multiple groups were assessed by one-way analysis of variance. Enumeration data are expressed as percentage and analyzed by *chi*-square tests. *p* < 0.05 was considered to be statistically significant value.

Results

High positive expression rate of PI3K, Akt, and GSK-3β in HCC

Based on the results from immunohistochemistry, the positive expression rate of PI3K, Akt, and GSK-3β in the para-cancerous tissues was (15.69 ± 1.91) %, (14.23 ± 1.66) %, (25.66 ± 2.51) %, and (63.29 ± 6.88) %, (55.41 ± 5.50) %, (58.21 ± 6.11) % in the HCC tissues (*p*

$p < 0.05$) (Fig. 1). The PI3K- or Akt-positive cells in the HCC tissues appeared to have yellow-brown granules in their cytoplasm, while the GSK-3 β -positive cells presented with diffused and strong yellow-brown staining in cytoplasm. Compared with paracancerous tissues, HCC tissues exhibited increased positive expression rate of PI3K, Akt, and GSK-3 β ($p < 0.05$). The results indicated that the positive expression rate of PI3K, Akt, and GSK-3 β in the HCC is higher than that in the paracancerous tissues.

High expression of PI3K, Akt, and GSK-3 β in the HCC is associated with differentiation degree, TNM stage, CLIP score, and lymphatic metastasis

RT-qPCR and Western blot analysis were employed in order to determine the expression of PI3K, Akt, and GSK-3 β in the HCC and paracancerous tissues. The mRNA expression of PI3K, Akt, and GSK-3 β in the HCC tissues were elevated when compared with paracancerous tissues (all $p < 0.05$) (Fig. 2A). In comparison with the paracancerous tissues, the HCC tissues showed increased levels in protein expressions of PI3K, Akt, GSK-3 β , p-PI3K, p-Akt, and p-GSK-3 β (all $p < 0.05$) (Fig. 2B&C). Based on the analysis of clinical pathological data of the HCC patients, we found that the mRNA expressions of PI3K, Akt, and GSK-3 β were associated with differentiation degree, TNM stage, CLIP score, and lymphatic metastasis of HCC (all $p < 0.05$), while there was no correlation to the sex and age of the HCC patients (both $p > 0.05$) (Table 2). These results verified that there was a correlation between high expression of PI3K, Akt, and GSK-3 β and differentiation degree, TNM stage, CLIP score, and lymphatic metastasis in HCC.

Fig. 1. High positive expression rate of PI3K, Akt, and GSK-3 β in the HCC tissues ($\times 200$). Note: A, the immunohistochemistry images for the positive expression of PI3K, Akt, and GSK-3 β ; B, the average absorbance of PI3K, Akt, and GSK-3 β , $p < 0.05$ vs. paracancerous tissues; HCC, hepatocellular carcinoma; PI3K, phosphoinositide 3-kinase; GSK-3 β , glycogen synthase kinase-3 β .

Fig. 2. High mRNA and protein expression levels of PI3K, Akt, and GSK-3 β in the HCC tissues. Note: A, the mRNA expression of PI3K, Akt, and GSK-3 β in the HCC and paracancerous tissue; B, protein bands of PI3K, Akt, and GSK-3 β observed after Western blot analysis; C, the protein expressions of PI3K, Akt, and GSK-3 β in the HCC and paracancerous tissue; *, $p < 0.05$, vs. paracancerous tissues; HCC, hepatocellular carcinoma; PI3K, phosphoinositide 3-kinase; GSK-3 β , glycogen synthase kinase-3 β ; p-, phosphorylated.

Erinacine regulates the expression level of the PI3K/Akt/GSK-3 β signaling pathway to relieve the HCC

The results of RT-qPCR and western blot analysis are as shown in Fig. 3. Compared with the control group, the mRNA and protein expressions of PI3K, Akt, GSK-3 β , CyclinD1, Vimentin, β -catenin, Bcl-2 and the phosphorylation levels of p-PI3K, p-Akt, p-GSK-3 β were decreased in the remaining five groups, but the mRNA and protein expressions of E-cadherin, Bax, and caspase-9 were increased (all $p < 0.05$). These changes were dose-dependent on the amount of erinacine; higher dose could lead to an increase or decrease, respectively. In contrast to the 500 μ M erinacine group, there was a decrease in the mRNA and protein expression of PI3K, Akt, GSK-3 β , CyclinD1, Vimentin, β -catenin, Bcl-2 and the phosphorylation levels of p-PI3K, p-Akt, p-GSK-3 β and an increase in the mRNA and protein expression of E-cadherin, Bax, and caspase-9 in the LY294002 and LY294002 + 500 μ M erinacine groups (all $p < 0.05$). The LY294002 + 500 μ M erinacine group presented with lowered levels in mRNA and protein expression of PI3K, Akt, GSK-3 β , CyclinD1, Vimentin, β -catenin, Bcl-2 and the phosphorylation levels of p-PI3K, p-Akt, p-GSK-3 β , and increased levels in mRNA and protein expression of E-cadherin, Bax, and caspase-9 in comparison with the LY294002 group (all $p < 0.05$). The aforementioned results suggested that LY294002 and erinacine inhibited the PI3K/Akt/GSK-3 β signaling pathway to relieve HCC, and the effect of erinacine occurred in a dose-dependent manner.

HepG2 cell proliferation is decreased following treatment with erinacine or LY294002
The relative inhibitive inhibition rate of HepG2 cells in each group is shown in Fig. 4. The groups treated with erinacine or LY294002 presented with increased rate in the inhibition of HepG2 cells compared with the control group ($p < 0.05$). When compared with the 500 μ M erinacine group, LY294002 and LY294002 + 500 μ M erinacine groups showed an increase in the inhibition rate of HepG2 cells ($p < 0.05$). The LY294002 + 500 μ M erinacine group indicated a higher inhibition rate of HepG2 cells than the LY294002 group ($p < 0.05$). The above results indicated that the proliferation of HepG2 cells was decreased following treatment with erinacine or LY294002, and erinacine took effect in a dose-dependent manner.

The cloning capability of HepG2 cells in vitro is inhibited following treatment with erinacine or LY294002

Clone formation assay was employed to detect the cloning capability of HepG2 cells *in vitro* (Fig. 5). The groups treated with erinacine or LY294002 had decreased cloning capability of HepG2 cells compared with the control group ($p < 0.05$). When compared with the 500 μ M erinacine group, cloning capability of HepG2 cells was inhibited in the LY294002 and

Table 2. Expression levels of PI3K, Akt, and GSK-3 β in the HCC and paracancerous tissues is associated with differentiation degree, TNM stage, CLIP score, and lymphatic metastasis. Note: HCC, hepatocellular carcinoma; PI3K, phosphoinositide 3-kinase; GSK-3 β , glycogen synthase kinase-3 β ; TNM, tumor node-metastasis; UICC, International Union Against Cancer; CLIP, Cancer of the Liver Italian Program

Clinicopathological characteristics	PI3K	p	Akt	p	GSK-3 β	p
Age (year)		0.666		0.666		0.666
≤ 50	2.10 ± 0.21		2.18 ± 0.23		1.27 ± 0.14	
> 50	2.08 ± 0.21		2.16 ± 0.24		1.25 ± 0.14	
Sex		0.56		0.56		0.56
Male	2.08 ± 0.20		2.17 ± 0.23		1.24 ± 0.13	
Female	2.11 ± 0.25		2.18 ± 0.26		1.27 ± 0.17	
Differentiation		< 0.001		< 0.001		< 0.001
Well	1.87 ± 0.07		1.87 ± 0.07		1.09 ± 0.06	
Moderate	2.03 ± 0.10		2.18 ± 0.10		1.24 ± 0.04	
Poor	2.26 ± 0.19		2.37 ± 0.16		1.42 ± 0.09	
TNM stage		< 0.001		< 0.001		< 0.001
I	1.77 ± 0.05		1.76 ± 0.08		1.00 ± 0.05	
II	1.91 ± 0.05		1.99 ± 0.14		1.13 ± 0.07	
III	2.03 ± 0.05		2.10 ± 0.06		1.20 ± 0.03	
IV	2.26 ± 0.19		2.37 ± 0.16		1.36 ± 0.12	
CLIP score		< 0.001		< 0.001		< 0.001
0	1.78 ± 0.05		1.77 ± 0.08		1.01 ± 0.05	
1	1.90 ± 0.07		1.97 ± 0.09		1.12 ± 0.04	
2	2.04 ± 0.04		2.12 ± 0.06		1.21 ± 0.03	
≥ 3	2.28 ± 0.17		2.39 ± 0.14		1.37 ± 0.10	
Lymphatic metastasis		< 0.001		< 0.001		< 0.001
No	2.19 ± 0.17		2.29 ± 0.17		1.31 ± 0.11	
Yes	1.88 ± 0.08		1.92 ± 0.11		1.10 ± 0.06	

Fig. 3. Relative mRNA and protein expression levels of PI3K/Akt/GSK-3 β signaling pathway were inhibited following treatment with erinacine or LY294002. Note: A, the histogram of mRNA expression of related genes in HCC cells; B, protein bands observed after western blot; C, the histogram of protein expression in HCC cells; *, $p < 0.05$, vs. the control group; #, $p < 0.05$ vs. the 500 μM erinacine group; &, $p < 0.05$ vs. the LY294002 group; HCC, hepatocellular carcinoma; PI3K, phosphoinositide 3-kinase; GSK-3 β , glycogen synthase kinase-3 β ; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p-, phosphorylated; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

LY294002 + 500 μM erinacine groups (all $p < 0.05$). The LY294002 + 500 μM erinacine group presented with repressed cloning capability of HepG2 cells compared with the LY294002 group ($p < 0.05$). These results showed that the cloning capability of HepG2 cells was inhibited following treatment with erinacine or LY294002, and erinacine exerted effect in a dose-dependent manner.

The migration of HepG2 cells is suppressed following the treatment with erinacine or LY294002

After scratching, the cells migrated to the narrow part in the lower end of the scratches. As shown in Fig. 6, migration ability was stronger in all groups at 0 h, after culturing for 24 h. There was a decrease in migration rate of HepG2 cells in groups that had received erinacine or LY294002 treatment compared with the control group ($p < 0.05$). When compared with the 500 μM erinacine group, LY294002 and LY294002 + 500 μM erinacine groups presented with decreased migration

Fig. 4. Cell proliferation was following treatment with erinacine or LY294002. Note: MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; *, $p < 0.05$ vs. the control group; #, $p < 0.05$ vs. the 500 μM erinacine group; &, $p < 0.05$ vs. the LY294002 group.

rate of HepG2 cells ($p < 0.05$). The LY294002 + 500 μM erinacine group showed lowered levels in migration rate of HepG2 cells in contrast to the LY294002 group ($p < 0.05$). These results showed that the migration ability of HepG2 cells was inhibited following treatment with erinacine or LY294002, and erinacine took effect in a dose-dependent manner.

The invasion of HepG2 cells is decreased after the treatment with erinacine or LY294002

The result of Transwell assay is shown in Fig. 7. The groups treated with erinacine or LY294002 showed decreased invasion of HepG2 cells compared with the control group ($p < 0.05$). When compared with the 500 μM erinacine group, invasion of HepG2 cells was inhibited in the LY294002 and LY294002 + 500 μM erinacine groups ($p < 0.05$). The LY294002 + 500 μM erinacine group indicated repressive invasion of HepG2 cells compared with the LY294002 group ($p < 0.05$). These results provided evidence that the invasion ability of HepG2 cells was inhibited following treatment with erinacine, LY294002, and LY294002 + 500 μM erinacine in a dose-dependent manner.

Erinacine enhances the inhibition effect of LY294002 on triggering apoptosis and inhibiting cell cycle progression of HepG2 cells

Flow cytometry single staining was performed in order to detect cell cycle of HepG2 cells, as illustrated in Fig. 8A-B. Compared with the control groups, the remaining five groups showed increased cell number in the G1 phase and decreased number in the S phase, suggesting that the proliferation of HepG2 cells was inhibited ($p < 0.05$). When compared with the 500 μM erinacine group, LY294002 and LY294002 + 500 μM erinacine groups showed an increase in the number of cells in the G1 phase and a decrease in the S phase (both $p < 0.05$). The LY294002 + 500 μM erinacine group indicated increased cell number in the G1 phase and decreased number in the S phase in contrast to the LY294002 group ($p < 0.05$).

Annexin-V/PI double staining was used for the detection of apoptosis of HepG2 cells, which is shown in Fig. 8C-D. The groups treated with erinacine or LY294002 revealed an increase in the apoptosis rate of HepG2 cells compared with the control group ($p < 0.05$). When compared with the 500 μM erinacine group, apoptosis rate of HepG2 cells was elevated

Fig. 5. The cloning capability of HepG2 cells. Note: A, images of the formation of HepG2 cells; B, relative cloning capability of HepG2 cells among 6 groups; *, $p < 0.05$ vs. the control group; #, $p < 0.05$ vs. the 500 μM erinacine group; &, $p < 0.05$ vs. the LY294002 group.

Fig. 6. Cell migration in each group ($\times 40$). Note: A, the cell migration images of HepG2 cells among six groups; B, relative migration ability of HepG2 cells detected by the wound healing assay; *, $p < 0.05$ vs. the control group; #, $p < 0.05$ vs. the 500 μM erinacine group; &, $p < 0.05$ vs. the LY294002 group.

Fig. 7. Inhibition of cell invasion among the six groups following treatment with erinacine or LY294002 ($\times 200$). Note: A, the cell invasion images of HepG2 cells among six groups; B, the relative invasion ability of HepG2 cells among six groups; *, $p < 0.05$ vs. the control group; #, $p < 0.05$ vs. the 500 μM erinacine group; &, $p < 0.05$ vs. the LY294002 group.

in the LY294002 and LY294002 + 500 μM erinacine groups ($p < 0.05$). The LY294002 + 500 μM erinacine group presented with increased apoptosis rate of HepG2 cells in contrast to the LY294002 group ($p < 0.05$). These results indicated that the treatment with erinacine or LY294002 triggers cell apoptosis, while it suppresses cell cycle progression of HepG2 cells.

The xenograft tumor volume and weight in nude mice were decreased following the treatment with erinacine or LY294002

As the Fig. 9 showed, the effect of erinacine on HCC growth was further confirmed by xenograft tumor experiments in male BALB/c mice. The volume and weight of tumor were decreased in the erinacine or LY294002 groups compared with the control groups ($p < 0.05$). In comparison to the 500 μM erinacine, the LY294002 and LY294002 + 500 μM erinacine groups showed decreased tumor volume and weight ($p < 0.05$). The LY294002 + 500 μM erinacine group revealed reduced tumor volume and weight in contrast to the LY294002 group ($p < 0.05$). These findings demonstrated that the volume and growth of the xenograft tumors of HCC were decreased following the treatment with erinacine or LY294002, and erinacine took effect in a dose-dependent manner.

Erinacine or LY294002 affects the mitochondrial membrane potential and the opening of MPTP in HepG2 cells

Mitochondrial membrane potential in HepG-2 was determined after JC-1 staining by laser scanning confocal microscopy. Compared with the control group, green fluorescence and subdued red fluorescence were enhanced in the remaining five groups (Fig. 10A-B), suggesting that the mitochondrial membrane potential was reduced. The mitochondrial membrane potential was gradually regulated by the concentration of erinacine. Based on intensity in the channels of green/red fluorescence, the LY294002 and the LY294002 + 500 μM erinacine groups showed a decrease in the mitochondrial membrane potential in contrast to the 500 μM erinacine group, and the mitochondrial membrane potential was reduced in the LY294002 + 500 μM erinacine group compared with the LY294002 group. The addition of 500 μM erinacine and MPTP opening inhibitor CsA resulted in a significant decrease in green fluorescence and markedly increased red fluorescence in the 500 μM erinacine + CsA group compared with the 500 μM erinacine group, indicating that mitochondrial membrane potential was increased.

The Ca^{2+} indicator Rhod2, AM was applied to detect mitochondrial Ca^{2+} concentration in HCC HepG2 cells. As shown in Fig. 10C-D, compared with the control group, the groups that received LY294002 or erinacine treatment had significantly increased mitochondrial Ca^{2+} concentration, and an increase in the dose of erinacine resulted in elevated mitochondrial Ca^{2+} concentration. The expression of cyt-C in mitochondria and cytoplasm was then further investigated and the results found that the expression of cyt-C in mitochondria gradually decreased and increased in the cytoplasm with increasing doses of erinacine compared with the control group (Fig. 10E), and the relative mitochondrial/cytoplasmic expression level gradually decreased (Fig. 10F). The same trend was observed when LY294002, the PI3K/Akt-GSK-3 β signaling pathway inhibitor, was added. In addition, when 500 μM erinacine was added, the difference was even more significant. In comparison with the 500 μM erinacine group, the 500 μM erinacine + CsA group had promoted cyt-C

Fig. 8. Apoptosis and the cell cycle among six groups. Note: A, the cell cycle images of HepG2 cells among six groups; B, proportion of HepG2 cells in different cell cycles among six groups; C, the cell apoptosis images of HepG2 cells among six groups; D, the apoptosis rate of HepG2 cells among six groups; *, $p < 0.05$ vs. the control group; #, $p < 0.05$ vs. the 500 μM erinacine group; &, $p < 0.05$ vs. the LY294002 group.

Fig. 9. The volume and weight of the xenograft tumor in mice with HCC. Note: A, the line chart of tumor volume among six groups; B, tumor weight among six groups; *, $p < 0.05$ vs. the control group; #, $p < 0.05$ vs. the 500 μM erinacine group; &, $p < 0.05$ vs. the LY294002 group; HCC, hepatocellular carcinoma.

expression in mitochondria while cyt-C expression was suppressed in the cytoplasm. These results demonstrated that erinacine increases the intracellular Ca^{2+} concentration, stimulates the opening of mitochondrial membrane permeability, reduces the mitochondrial transmembrane potential, and promotes the release of cyt-C by inhibiting the activation of PI3K/Akt-GSK-3 β signaling pathway; and the erinacine effect is dose-dependent.

Fig. 10. Erinacine prevents MPTP opening in HepG2 cells. A, fluorescence images of HepG2 cells after JC-1 staining observed by an Olympus FV10i confocal microscope (×400); B, fluorescence intensity by Image-Pro Plus Version 6.0; C, free Ca²⁺ concentration changes detected by mitochondrial Ca²⁺ indicator Rhod2, AM detection (×200); D, Ca²⁺ fluorescence intensity changes detected by Image-Pro Plus 6 software; E, mitochondria and intracytoplasmic cytochrome C expression detected by Western blot analysis; F, mitochondrial and intracytoplasmic cytochrome C expression ratio. *, p<0.05 vs. the control group; #, p<0.05 vs. the 500 μM erinacine group; &, p<0.05 vs. the LY294002 group; HCC, hepatocellular carcinoma; MPTP, mitochondrial permeability transition pore; cyt-C, cytochrome C.

Discussion

HCC is known to be the most common malignancy in the liver and is the leading cause of cancer-related death worldwide [25, 26]. The presence of cirrhosis and portal vein hypertension makes the treatment and management of HCC a very complex process [27]. The anticancer properties of several traditional Chinese medicine-derived compounds have previously been studied and have been identified as promising candidates in the suppression of HCC metastasis [28]. This study provided evidence that erinacine facilitated the opening of MPTP and induced the apoptosis of HCC HepG2 cells in a dose-dependent manner, in

addition to suppression of xenograft tumor growth in nude mice through the inhibition of PI3K/Akt-GSK-3 β signaling pathway activation. Our results indicated that erinacine may be used as anticancer drug for HCC in the future.

In this study, we demonstrated that the PI3K/Akt-GSK-3 β signaling pathway was activated in HCC tissues and cells, and that treatment with erinacine and LY294002 resulted in the inhibition of this pathway. A consistent study revealed that the extract of *Pleurotus pulmonarius* (an edible mushroom) inhibited the proliferation and invasion of HCC *in vitro* and *in vivo* through the suppression of the PI3K/Akt signaling pathway [29]. Moreover, the PI3K/Akt/mTOR pathway was found to be activated in HCC and enhance invasion and metastasis through the up-regulation of the metalloproteinase-9 matrix in HCC [30]. *Hericium erinaceus* has been demonstrated to play a role in chemotherapy resistance against HCC by facilitating intracellular doxorubicin accumulation through the inhibition of the NF- κ B activity [31]. Extracts of edible mushroom have indicated considerable cytotoxic, antitumor and antioxidant properties in HCC HepG2 and may be utilized as a potent anticancer agent against HCC [31]. Another study showed that the Akt/GSK-3 β pathway facilitates the lowering of the CD44 and TGF- β 1 migration in HCC cells [32]. We also demonstrated that the PI3K/Akt-GSK-3 β signaling pathway was associated with distinct clinicopathological characteristics in HCC such as differentiation degree, TNM stage, CLIN score and lymphatic metastasis.

The combination of erinacine and LY294002 showed a decrease in the expression of CyclinD1, Vimentin, β -catenin, Bcl-2 as well as an increase in the expression of E-cadherin, Bax, and caspase-9. A key finding in our study was that erinacine promoted the effect of LY294002 (a PI3K inhibitor) on reducing proliferation, cloning capability, migration, invasion and xenograft tumor growth of HepG2 cells, as well as enhanced apoptosis. The Bax and Bak are regarded as multidomain proapoptotic proteins and required for initiation of apoptosis at the mitochondria, while Bcl-2 and Bcl-xl as anti-apoptotic proteins play a functional role in the apoptotic stimuli triggered cyt-C effluence [33]. The downregulation of Bcl-2/BAX ratio is known to participate in the mechanism that PI3K/Akt pathway inhibited cell growth through DNA fragmentation and subG1 accumulation in HCC [34]. The inhibition of CyclinD1 and β -catenin, which were involved in cell invasion, apoptosis, and angiogenesis, has been proved to play a therapeutic role in HCC after treatment *via* PI3K pathway inactivation [35, 36]. The upregulation of Bax and the down-regulation of Vimentin, which occur as a result of reduced phosphorylation of Akt, have been reported to be involved in the PI3K/Akt pathway inactivation which inhibit cell migration, invasion and the epithelial-mesenchymal transition in HCC [37]. Previous studies reported that EMT of HCC was promoted through activation of the PI3K/Akt-GSK-3 β signaling pathway by CXCR2/CXCL5 axis, and this pathway may serve as a potential therapeutic target for HCC [38].

Lastly, we found that erinacine or LY294002 treatment leads to a decrease in the mitochondrial membrane potential in HepG2 cells. Mitochondria damage induced by the opening of MPTP may cause the disruption of cell function and eventually lead to cell death [39]. The opening of MPTP has been demonstrated to induce the release of apoptogenic factors, depolarization of the transmembrane potential ($\Delta\psi$) and loss of oxidative phosphorylation, ultimately resulting in the induction of the apoptotic process [40]. It has been demonstrated that the opening of MPTP attributes to the induction of apoptosis in HCC HepG2 cells, where the molecular mechanism involved decreased $\Delta\psi$ and the release of cyt-C [41, 42]. These findings were also consistent with our study and provided further confirmation for our results. Erinacine increases the intracellular Ca^{2+} concentration, stimulates the opening of mitochondrial membrane permeability, reduces the mitochondrial transmembrane potential, and promotes the release of cyt-C by inhibiting the activation of PI3K/Akt-GSK-3 β signaling pathway; and the erinacine effect is dose-dependent. Other researches have also identified that the varying Ca^{2+} concentration determines the cyt-C release [43, 44] and the Ca^{2+} concentration induces its release [45]. The MPTP opening takes part in reactive oxygen species induced by iron overload in liver damage [46]. Furthermore, previous researches have also proved that activation of mitochondrial apoptosis pathway was associated with MPTP [47]. Thus, we concluded that erinacine could alleviate HCC through the promotion of MPTP opening *via* the PI3K/Akt pathway.

In conclusion, this study provided evidence in support with the idea that erinacine facilitates the MPTP opening and inhibits cell invasion and migration in HCC in a dose-dependent manner through the inactivation of the PI3K/Akt/GSK-3 β signaling pathway. These findings suggest that erinacine could potentially be used as an anti-cancer drug for

HCC in the future and that the PI3K/Akt/GSK-3 β pathway is a key biomarker for further therapeutic studies. However, due to the limited sample size included, further study is required.

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Disclosure Statement

The authors have declared that no competing interests exist.

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