Lupeol induces apoptosis and inhibits invasion in gallbladder carcinoma GBC-SD cells by suppression of EGFR/MMP-9 signaling pathway

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Abstract The cytostatic drug from fruits and other plant derived products have acted as a chemotherapeutic agent used in treatment of a wide variety of cancers. Lupeol, a dietary triterpene, present in many fruits and medicinal plants, has been shown to possess many pharmacological properties including anti-cancer effect in both in vitro and in vivo assay system. However, the cancer proliferative and invasive inhibitory effects and molecular mechanisms on gallbladder carcinoma GBC-SD cells have not been studied. In the present study, GBC-SD cells were treated by lupeol and subjected to methyl thiazolyl tetrazolium analysis, Hoechst 33342 staining, annexin V propidium iodide double-staining, transwell chamber assay and Western blot analysis. In addition, GBC-SD xenograft tumors were established in male nude BALB/c mice, and lupeol was intravenously administered to evaluate the anti-cancer capacity in vivo. Our results showed that lupeol inhibited the proliferation, migration, invasion and induced apoptosis of

GBC-SD cells in a dose-dependent manner in vitro. Furthermore, the expression of p-EGFR, p-AKT and MMP-9 levels were significantly down-regulated. These protein interactions may play a pivotal role in the regulation of apoptosis and invasion. More importantly, our in vivo studies showed that administration of lupeol decreased tumor growth in a dose-dependent manner. Immunohistochemistry analysis demonstrated the down-regulation of p-EGFR and MMP-9 in tumor tissues following lupeol treatment, consistent with the in vitro results. Taken together, our findings indicated that lupeol can induce apoptotic cell death and inhibit the migration as well as invasion of GBC-SD cells. The mechanism may be associated with the suppression of EGFR/MMP-9 signaling. These results might offer a therapeutic potential advantage for human gallbladder carcinoma chemoprevention or chemotherapy.

Keywords Lupeol · Gallbladder carcinoma · Apoptosis · Invasion · EGFR · MMP-9

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Introduction

Gallbladder carcinoma (GBC), most frequently diagnosed incidentally after a cholecystectomy for symptomatic diseases such as gallstones, is the fifth most common malignant tumor of the digestive system and one of the most common malignant neoplasm of the

biliary tract (Reid et al. 2007). It is reported that GBC is relatively common in Chile, Bolivia, and Mexico and over the past decades, the incidence rate of GBC has increased in China (Jia et al. 2011). The only potentially curative therapy for GBC is surgical resection. Unfortunately, most patients with this type of cancer present with advanced and unresectable disease—only 10–30 % of patients can be considered for surgery on presentation (Taner et al. 2004). Additionally, chemotherapy and radiotherapy have limited efficacy on GBC, which was evidenced by low response rates and no demonstrated survival benefit (Liu et al. 2013b). The prognosis of advanced GBC is extremely poor, and the 5-year survival rate is only about 5 % (Washiro et al. 2008). Therefore, novel agents are urgently needed for the treatment of advanced GBC. Nowadays, to discover and develop novel natural compounds that have therapeutic selectivity or that can preferentially kill cancer cells without significant toxicity to normal cells is an important tendency for cancer therapy (Yu et al. 2009).

Lupeol [Lup-20(29)-en-3b-ol] (Fig. 1), a dietary triterpene, found in several medicinal plants and in various fruits such as olive, mango, strawberry, grapes, figs and vegetables, is used for treatment of numbers of ailments worldwide (Imam et al. 2007; Saleem 2009; You et al. 2003). Lupeol has attracted much attention because of its low toxicity and wide pharmacological effects. Extensive research over the past decades have revealed various important pharmacological activities of lupeol under *in vitro* and *in vivo* conditions, including anti-inflammation, anti-arthritis, anti-heart diseases, anti-obesity, anti-hepatic toxicity and anti-renal toxicity (Saleem 2009; Khan et al. 2008; Chaturvedi et al. 2008; Ardiansyah et al. 2012). Recently, lupeol has been extensively studied, for their cancer chemopreventive potential against various cancers, for instance prostate cancer (Saleem et al. 2006), pancreatic cancer (Murtaza et al. 2009), skin cancer (Saleem et al. 2004), hepatocellular carcinoma (Liu et al. 2013a), epidermoid carcinoma (Prasad et al. 2009) and melanoma (Tarapore et al. 2010). However, there has no research reported about lupeol's effect and mechanism on GBC cells.

The goals of this study were to determine whether lupeol could inhibit the growth and invasion, and induce apoptosis in GBC-SD cells and to clarify the related mechanisms, which may offer a promising new approach in the effective treatment of GBC.

Fig. 1 The chemical structure of lupeol

Materials and methods

Reagents and antibodies

Lupeol was purchased from Sigma-Aldrich (St. Louis, MO, USA) and a stock solution of lupeol (30 mmol/l) was prepared by resuspension in warm alcohol and dilution in DMSO at 1:1 ratio. The final Lupeol concentrations used for the different experiments were prepared by diluting the stock solution with complete cell culture medium. Materials used included 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Company St. Louis, MO, USA), annexin V–fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (MultiSciences Biotech, Shanghai, China), Hoechst-33342 staining assay kit (Molecular Probes, Beyotime Institute of Biotechnology, Shanghai, China), Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) antibodies (Kangchen Bio-tech, Shanghai, China), phospho-Akt (Thr308), total-Akt, phospho-EGFR, total-EGFR, matrix metalloproteinase 9 (MMP-9), horseradish peroxidase-conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG antibodies (Cell Signaling Technology, Danvers, MA, USA).

Cell lines and culture

GBC-SD cells were purchased from the Shanghai Cell Institute Country Cell Bank. The cells were maintained in high-glucose DMEM (Gibco, Gaithersburg, MD, USA) supplemented with 10 % fetal bovine serum (Gibco), 100 µg/ml streptomycin and 100 units/ml penicillin (Hyclone, Logan, UT, USA), and incubated in a humidified atmosphere with 5 % CO₂ at 37 °C. The cells were kept in an exponential growth phase during experiments.

Cell viability assay

The effect of lupeol on the viability of GBC-SD cells was determined by MTT assay. Briefly, cells were trypsinized and plated into 96-well plates at a density of 5×10^3 cells per well. The cells were cultured overnight and then replenished with fresh medium containing various concentrations (0–120 μM) of lupeol for 24, 36, and 48 h. Thereafter, 20 μl MTT (5 mg/ml) was added. Four hours later, 150 μl DMSO was added to each well to dissolve the resulting formazan crystals. The absorbance values at 492 nm were measured by micro-enzyme-linked immunosorbent assay plate reader. The effect of lupeol on growth inhibition was assessed as percent cell proliferation inhibition, where vehicle-treated cells were taken as 0 % inhibition.

Hoechst 33342 staining

Hoechst staining was employed to evaluate the apoptosis of GBC-SD cells treated with lupeol. Briefly, the cells were exposed to lupeol for 36 h and then stained with Hoechst 33342 (5–10 $\mu\text{g/ml}$) for 10 min. After being washed with PBS, they were observed using an inverted fluorescence microscope. Live cell nuclei showed dispersion and uniform fluorescence, while dead cells were not stained with Hoechst. Following apoptosis, the nuclei underwent significant morphological changes, and blue fluorescent-stained compact particulates could be seen in the nucleus or cytoplasm.

Annexin V–FITC/PI staining

Annexin V–FITC/PI double staining was employed to quantify the apoptotic rate of GBC-SD cells treated with lupeol. Briefly, cells were seeded into six-well plates at 2×10^5 cells/well and exposed to lupeol (15, 30 and 60 μM) for 36 h. The cells were then stained using annexin V–FITC/PI double fluorescence apoptosis detection kit following the manufacturer's instruction. After incubation for 15 min at room temperature in the dark, the cells were analyzed with flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) within 1 h after the staining.

Migration and invasion assays

Cell migration and invasion were evaluated using transwell chamber assay (Millipore, Billerica, MA, USA) according to the manufacturer's instruction. For invasion assay, totally 4×10^5 cells were seeded on an 8- μm pore size transwell insert coated with extracellular matrix (ECM) (1:6) (BD Biosciences, China), while cell migration assay did not coat with ECM. After incubated for 36 h, the cells adherent to the upper surface of the filter were removed using a cotton applicator, then the cells on the underlying surface of the membrane were fixed and stained with crystal violet, and the values obtained were calculated by averaging the total numbers of cells from triplicate determinations.

Western blot analysis

GBC-SD cells were plated in 6-well plates (as described above) and after being treated with lupeol for 36 h, cells were washed twice in ice-cold PBS and lysed on the culture dishes using RIPA lysis buffer (1 % NP-40, 0.1 % SDS, 0.5 % sodium deoxycholate, 150 mmol/l NaCl and 10 mmol/l Tris–HCl) containing 1/100 phenylmethanesulfonyl fluoride solution (Beyotime Institute of Biotechnology, Shanghai, China). The total protein concentration was determined using the bicinchoninic acid (Beyotime Institute of Biotechnology) method and 40 μg of each sample was separated by SDS-PAGE (8, 10, or 12 %) and transferred to polyvinylidene difluoride membrane (Beyotime Institute of Biotechnology). Non-specific binding sites were blocked by incubating with TBST containing 5 % (w/v) non-fat dried milk for 2 h at room temperature. The membranes were incubated with primary antibodies overnight at 4 °C and then with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. After each incubation period, the membranes were washed three times with TBST. Signals were visualized by ECL chemiluminescence. Relative levels of total proteins were determined by western blot analysis using the antibodies specified in the Reagents section. Equal protein loading was assessed by the expression of GAPDH. The bands were semi-quantified using Image J software.

In vivo efficacy of lupeol

Male BALB/c nude mice at 4 weeks of age (Shanghai SLAC Laboratory Animal Center of Chinese

Academy of Sciences, Shanghai, China) were maintained throughout in specific pathogen-free (SPF) environment. Exponentially growing GBC-SD cells (4×10^6) were suspended in 150 μ l PBS and subcutaneously injected into the right axillary fossa of each nude mouse. On day 5, twenty-four nude mice whose tumors were similar in size (4–6 mm in diameter) were chosen and equal numbers were assigned to four groups ($n = 6$ per group). Then, the treatment groups were injected intravenously at two doses of lupeol (30 and 60 mg/kg) on alternative days, respectively. Positive and negative control group animals were given 5-Fluorouracil (manufactured by Jiangsu Tongtai Pharmaceuticals Company, Rufu, China) 20 mg/kg and normal saline, respectively. All mice were sacrificed on day 31 after these drugs had been administered ten times and tumors were dissected and weighed. The tumor volume was calculated using the formula " $0.5 \times a \times b^2$ ", in which a and b represent the maximal and minimal diameters. The tumor inhibition rate was calculated using the formula " $(\text{tumor weight of the control group} - \text{tumor weight of the treatment group}) / \text{tumor weight of the control group} \times 100 \%$ ". The animal studies were approved by the Xuzhou medical college, and the principles of laboratory animal care were followed in all animal experiments.

Then formaldehyde-fixed, paraffin-embedded tissue blocks were prepared from xenograft tissue and cut into serial sections (3 μ m) for examining the expression of p-EGFR, MMP-9 and PCNA (mouse anti-PCNA antibody: GeneTex Inc., Irvine, CA, USA) by immunohistochemistry with the streptavidin-peroxidase (S-P) kit (Fuzhou Maier Biotechnology Development Co., Fuzhou, China). Each slice was enumerated under five-fold of medium magnification (400 $\times$) to determine the proportion of positive cells.

Statistical analysis

All experiments presented here derived from at least three independent experiments. All data are expressed as mean $\pm$ standard deviation (SD) and analyzed by the SPSS 17.0 software. One-way ANOVA followed by the appropriate post hoc test (Bonferroni) was used to establish whether significant differences existed among groups. The P value <0.05 was considered statistically significant.

Fig. 2 Lupeol inhibits the proliferation of GBC-SD in vitro. GBC-SD cells were incubated in the absence or presence of lupeol at different concentrations, and harvested at different time points. The proliferation of GBC-SD cells was assessed by the MTT method to calculate the proliferation inhibition rate (%). Dose- and time-dependent inhibition of GBC-SD cells growth could be observed ($P < 0.05$, ANOVA analysis). Each experiment was conducted in triplicate

Results

Lupeol inhibited the growth and proliferation of GBC-SD cells

The proliferation inhibition effect of lupeol on GBC-SD cells was determined with MTT assay. Within definite dose and time, we found lupeol showed inhibitory effects on the growth and proliferation of GBC-SD cells in dose- and time-dependence manners (Fig. 2). The results showed that at the concentration of 0–60 μ M of lupeol, the inhibition rate sharply increased. However, higher concentration of lupeol had a saturated inhibitory effect. During the following experiment at 36 h, lupeol showed a significantly higher inhibiting effect than that at 24 h. In contrast, there was no significant difference in cell inhibition rate among prolonged treatment for 48 h. The IC₅₀ for lupeol were estimated to be 101, 44 and 46 μ M after treatment for 24, 36, 48 h, respectively. Based on these observations, we selected a dose of 15–60 μ M and a period of 36 h post-lupeol treatment for further mechanism studies.

Fig. 3 Apoptotic nuclear morphology changes induced by lupeol (15, 30, and 60 μM) treatment for 36 h, were observed by Hoechst 33342 staining in GBC-SD cell lines

Lupeol induced apoptosis in GBC-SD cells dose-dependently

The fastest way to detect apoptosis is the observation of variations in nucleus morphology of incubated cells using Hoechst staining. In the present study, morphological changes in the apoptotic cells were revealed by the Hoechst 33342 staining (Fig. 3). In the untreated GBC-SD cells, the nuclei were stained weakly homogeneously blue, whereas in the group treated with lupeol, the cells exhibited strong blue fluorescence, revealing the typical apoptosis characteristics. And cell nuclei appeared to be highly condensed and crescent-shaped. This was confirmed by flow cytometry assay. The apoptotic rates of the cells were 27.79 ± 2.04 , 39.59 ± 2.95 , 60.41 ± 2.66 %, respectively, after treatment with 15, 30, 60 μM lupeol, which were all higher than that of GBC-SD cells cultured under normal conditions (10.50 ± 1.7 %; $P < 0.01$; Fig. 4). The 60 μM lupeol group was the highest in all experiments. These data suggested that lupeol induced apoptosis in GBC-SD cells dose-dependently.

Lupeol inhibited the migration and invasion of GBC-SD cells

The migration and invasion exert important effects on the complicated process of metastasis of cancer cells. Therefore, we examined the effects of lupeol on the migration and invasion in GBC-SD cells (Fig. 5). Our results indicated that lupeol at the concentrations of 2.5–7.5 μM significantly reduced the rate of GBC-SD cell migration and invasion when compared with the control group after the cells were treated for 36 h ($P < 0.01$). In addition, the inhibitory rates of each treatment group differed significantly ($P < 0.01$). Furthermore, lupeol at the concentrations did not

significantly reduce the viability of GBC-SD cells after the cells were treated for 36 h. These results suggested that the inhibition of GBC-SD cell migration and invasion by lupeol was not the result from a reduction of cell viability. These observations indicated that lupeol plays an important role in the regulation of the invasive and metastatic capacity of GBC-SD cells.

Lupeol suppressed the activation of EGFR and MMP-9 in GBC-SD cells

The EGFR family plays an important role in cell apoptosis and migration. Therefore, to determine whether EGFR family members were involved in the lupeol-induced GBC-SD cells apoptosis and migration, the analysis measured EGFR, p-EGFR, AKT and p-AKT at the protein level (Fig. 6a). The results revealed that the expression level of EGFR and AKT in the lupeol treatment groups were not significantly different from that in the control group while the expression level of p-EGFR and p-AKT were significantly decreased compared with the control group ($P < 0.01$). Another important factor in cell invasion is MMP-9, an endopeptidase of the large MMPs family. In our analysis, the expression of MMP-9 treated with different concentrations of lupeol also decreased in a dose-dependent manner (Fig. 6b). These results suggested that lupeol inhibited the activation of EGFR and its downstream AKT signaling molecules, and suppressed the activity of MMP-9.

Lupeol inhibited the tumor growth in vivo

We further studied the effects of lupeol on tumor cell growth in vivo. The xenograft model was established in BALB/c nude mice following subcutaneous

Fig. 4 Apoptosis was induced in GBC-SD cells by lupeol. **a** Apoptosis analysis of GBC-SD cells induced by different concentrations of lupeol (0, 15, 30 and 60 μM) for 36 h using flow cytometry with annexin V–FITC/propidium iodide (PI) binding assay. **b** Flow cytometric analysis of GBC-SD cells stained with annexin V–FITC/PI after treatment with 0–60 μM lupeol. Data are presented as mean ± standard deviation of three independent experiments, and each experiment was carried out in triplicate (**P* < 0.01 versus control group)

Fig. 5 Effect of lupeol on GBC-SD cell migration and invasion. After incubation with lupeol for 36 h, cells were stained with *crystal violet* and the number of cells invading the lower side of the filter was measured as metastatic and invasive activity (original magnification ×100). **a** Microphotographs of

metastatic and invasive GBC-SD cells. **b** The number of metastatic and invasive cells. **P* < 0.01 versus control group. The data shown are representative of three independent experiments. (Color figure online)

Fig. 6 Effect of lupeol on protein expression of EGFR family and MMP-9. **a** Expression of EGFR, p-EGFR, AKT, p-AKT and MMP-9 was analysed by western blot assay. GAPDH was used as the sample loading control. For one experiment, three assays were carried out but only one set of gels is shown. In GBC-SD cells treated with various lupeol concentrations for 36 h, the expression of EGFR and AKT did not show significant changes

while p-EGFR, p-AKT and MMP-9 expression declined remarkably compared to the control group. **b** The expression of p-EGFR, p-AKT and MMP-9 protein levels in every group of GBC-SD cells was analyzed by densitometry normalized with the corresponding total AKT and ERK and GAPDH density with the ratio of p-AKT/AKT, p-ERK/ERK and MMP-9/GAPDH. Considered as $P < 0.01$ versus the control group

Fig. 7 Lupeol inhibits the growth of GBC-SD cells in vivo. **a** GBC-SD cells were subcutaneously injected into mouse right axillary fossa to establish xenograft models and all mice were divided into four groups ($n = 6$ per group): control (sterile physiological saline), 5-Fu (20 mg/kg), and lupeol (30 and

60 mg/kg) treatment groups. Lupeol significantly reduced the tumor weight in vivo. **b** Tumor weight and volume in the each group. Considered as $*P < 0.01$ versus control group; $^{#}P < 0.01$ versus lupeol 60 mg/kg group

Fig. 8 Lupeol down-regulates p-EGFR, MMP-9 and PCNA in GBC-SD xenografts. **a** H&E staining analyses of the pathological features of the tumors from the four groups (original magnification $\times 400$). **b–d** The expression of p-EGFR, MMP-9 and PCNA in xenograft tumors was analyzed by immunohistochemistry (original magnification $\times 400$). **e–g** Expression of

p-EGFR, MMP-9 and PCNA was quantified in percentages of positive cells within five medium-power fields under microscope and shown in histograms. Considered as $*P < 0.05$ versus control group; $**P < 0.01$ versus control group

transplantation of GBC-SD cells. As shown in Fig. 7, the weight and volume of transplanted tumors were significantly inhibited after administration of 30 and 60 mg/kg lupeol compared with the negative control group ($P < 0.01$), and the inhibitory rates were 33.75 and 56.91%, respectively. In addition, the inhibition levels of tumor growth between 30 mg/kg and 60 mg/kg lupeol treated groups have statistically significance ($P < 0.01$). The 20 mg/kg 5-Fu treated mice had the smallest tumor weight among these four groups.

Further the subcutaneous tumor tissues were examined by H&E staining. The tumor tissue from control mice showed disorganized arrangement and a high cell density of GBC cells. The ratios of nucleus to cytoplasm were increased and nuclei showed clear

signs of heteromorphism. In contrast, in tumor tissues from mice treated with lupeol or 5-Fu, the ratio of nucleus to cytoplasm was reduced and the nuclei were polygonal and lightly stained. The GBC cells were loosely arranged, and there were marked signs of widespread tumor destruction: coagulation, apoptotic and necrotic cells were observed (Fig. 8a). In addition, the expression of p-EGFR, MMP-9 and PCNA in lupeol-treated tumors was examined by immunohistochemistry. As shown in Fig. 8b–g, the mean areas that stained positive for p-EGFR, MMP-9 and PCNA were all down-regulated compared with the negative control group ($P < 0.01$). This result also confirmed the western blot analysis of p-EGFR and MMP-9 expressions (shown in Fig. 6).

Discussion

To the best of our knowledge, this is the first study to reveal the ability of the natural compound lupeol to induce apoptosis and inhibit migration and invasion in GBC-SD cells. Lupeol has been shown to exhibit various pharmacological activities under *in vitro* and *in vivo* conditions. In recent years, studies have shown that lupeol can inhibit the growth and induce apoptosis of a variety of tumor cells. For example, Saleem et al. (2005, 2009) found that lupeol could induce apoptotic death of human pancreatic adenocarcinoma cells via inhibition of Ras signaling pathway, as well as inhibit proliferation of human prostate cancer cells by targeting beta-catenin signaling. Here, we have shown the biochemical and molecular mechanisms of apoptosis induction and invasion inhibition by lupeol in GBC-SD cells.

Cell apoptosis is an autonomous cell death process, which can be induced by a variety of physical and chemical factors and drugs (Liang et al. 2012). The induction of apoptosis has been described as a standard and best strategy in anticancer therapy (Kelly and Strasser 2011; Yang et al. 2014). Cancer metastasis, the main causes of morbidity and mortality in millions of patients with cancer, depends on the invasion and migration of cancer cells (Zhang et al. 2009). During the complicated process of metastasis, the invasion of cancer cells plays a critical role, which is the final stage of tumor progression (Yilmaz et al. 2007). The migration of cancer cells is one of the important steps during the invasion. Clearly, an agent which could efficiently inhibit the proliferation and invasion of cancer cells would be a helpful candidate to suppress cancer progression and metastasis and thus could reduce mortality. Therefore, we examined whether lupeol could induce apoptosis and inhibit migration and invasion in GBC-SD cells in this study. Results from MTT, Hoechst 33342 staining, and annexin V-FITC/PI staining suggested that lupeol inhibited the proliferation and induced apoptosis of GBC-SD cells in a dose-dependent manner in the range of 15–60 μ M. In addition, transwell chamber assay revealed low concentrations of lupeol could inhibit the migration and invasion of GBC-SD cells in a dose-dependent manner, and the inhibition of migration and invasion is not the result from the reduction of cell viability.

Cell proliferation and migration is involving several growth factors, for binding to receptors on cell surface,

stimulating downstream signaling pathways, and resulting in cytoskeletal reorganization and stimulation of motility machinery of the cell (Wang et al. 2011). To elucidate the molecular mechanisms how lupeol induces apoptosis and suppresses the migration and invasion of GBC-SD cells, we investigated several related proteins changes in lupeol-treated and non-treated GBC-SD cells. The epidermal growth factor receptor (EGFR), which is overexpressed in a variety of malignancies, plays a key role in essential cellular functions including tumor cell proliferation, apoptosis, differentiation, metastasis and tumor-induced angiogenesis (Marshall 2006; Tomas et al. 2014; Mlcochova et al. 2013). This makes it an attractive target in cancer therapy, and its inhibition a strategy for augmentation of the efficacy of chemotherapy and radiotherapy (Chi et al. 2013). EGFR is one of the receptor tyrosine kinases which can autophosphorylate upon ligand binding, and activates three major downstream signaling pathways, Ras-mitogen-activated protein kinase (Ras-MAPK), phosphatidylinositol 3-kinase-protein kinase B (PI3K/Akt) and signal transducers and activators of transcription (STAT) (Ayyappa et al. 2013; Mitsudomi and Yatabe 2010). Interestingly, over the past years, studies have also shown that the PI3K/Akt pathway is critically involved in the control of tumor cell growth, survival, progression, invasiveness, and metastasis formation (Martini et al. 2013; Wachsherger et al. 2014). Blockage of the PI3K/Akt signaling pathway results in programmed cell death and growth inhibition of tumor cells (Liu et al. 2013a). In this study, we found that after treatment with lupeol, the expression of phosphorylation of EGFR and PI3K/Akt in GBC-SD cells was significantly decreased compared with the control group ($P < 0.01$). The results indicated that the anti-proliferation, anti-migration and anti-invasion effects of lupeol are likely to be associated with the inhibition of EGFR signaling.

It is reported that EGFR signaling can contribute to tumor cell invasion through up-regulating the expression of MMPs (Mitsudomi and Yatabe 2010). Qiu et al. (2004) showed EGF-stimulated MMP-9 expression and activity through PI3K and MAPK signal pathway. Tian et al. (2007) also found the induction of MMP-9 via EGF-induced p-ERK activation, and blocking of EGFR signal pathway could dramatically down-regulated MMP-9 expression. Since MMPs play an important role in promoting angiogenesis and tumor metastasis, agents inhibiting that expression

may prove useful in treating such diseases. An enhanced expression of MMP-9 was shown to be associated with the migration and invasion of tumors (Hlobilkova et al. 2009; Park et al. 2010). Therefore, to further investigate the mechanism by which lupeol reduced GBC-SD cell migration and invasion, we explored MMP-9 expression after lupeol treatment. Here, our data showed that lupeol suppressed the expression of MMP-9 protein levels in GBC-SD cells in a dose-dependent manner. These evidences and results support the view that EGFR signal could regulate the expression and activity of MMP-9.

In addition, in our *in vivo* study, we found that lupeol (30, 60 mg/kg) and 5-Fu significantly inhibited the tumors weight and volume during the treatment period. H&E staining analyses of the tumors from mice treated with lupeol or 5-Fu revealed morphological features characteristic of apoptotic cells. Furthermore, immunohistochemical analysis confirmed the downregulation of p-EGFR, MMP-9 and PCNA following treatment with lupeol or 5-Fu *in vivo*, which were consistent with our findings *in vitro*.

In conclusion, the present investigation confirms the anti-proliferative and anti-invasive effects of lupeol in GBC-SD cells. More interestingly, our study provided first evidence that lupeol inhibited EGFR signal-induced apoptosis, migration and invasion of GBC-SD cells via blocking of EGFR/MMP-9 signaling. The dramatic effects of lupeol in GBC-SD cells indicated that lupeol could be recognized to be a useful candidate as chemotherapeutic agent against GBC.

Conflict of interest The authors declare no conflict of interest.

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