

Evidence for the Involvement of COX-2/VEGF and PTEN/PI3K/AKT Pathway the Mechanism of Oroxin B Treated Liver Cancer

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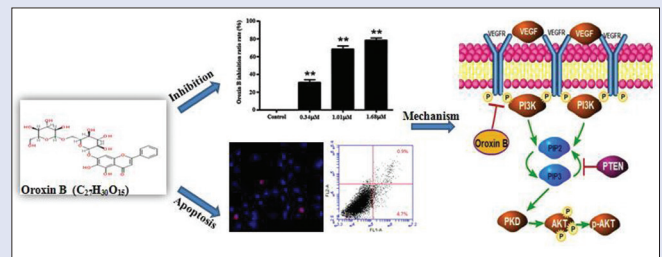
ABSTRACT

Background: Oroxin B (OB) is one of flavonoids isolated from traditional Chinese herbal medicine *Oroxylum indicum* (L.) Vent. Recent studies suggest that flavonoids have obvious anti-liver tumors effect, but the precise molecular mechanism is still unclear. **Objective:** The current study was performed to investigate the antitumor effects of OB on human hepatoma cell line SMMC-772 and explore the part of molecular mechanisms in this process. **Materials and Methods:** MTT method, terminal deoxynucleotidyl transferase dUTP nick end labeling assay and flow cytometry were utilized to detect the inhibition of proliferation and the apoptosis after treating OB in of SMMC-7721 cells. The mRNA and proteins expressions of COX-2, vascular endothelial growth factor (VEGF), phosphatidylinositol-3-kinase (PI3K), p-AKT, and PTEN were measured by a real-time polymerase chain reaction and Western Blot method. **Results:** The results showed that OB inhibited proliferation of SMMC-7721 cell in a dose-dependent manner, and induced its apoptosis. Moreover, OB unregulated PTEN and downregulated COX-2, VEGF, p-AKT, and PI3K. **Conclusion:** Our results demonstrated that OB significantly inhibits proliferation and induce apoptosis, which may be strongly associated with the inhibiting COX-2/VEGF and PTEN/PI3K/AKT pathway signaling pathway in SMMC-7721 cells, OB potentially be used as a novel therapeutic agent for liver cancer.

Key words: Anti-liver cancer, COX-2/vascular endothelial growth factor, Oroxin B, PTEN/phosphatidylinositol-3-kinase/AKT, SMMC-7721 cells

SUMMARY

- OB (Oroxin B) is one of the effective flavonoid components of traditional Chinese medicine *O. indicum* (L.)
- OB can inhibit the proliferation and promoted apoptosis of the human hepatoma cell line SMMC 7721
- OB plays a role of antitumor effect may to regulate COX 2/VEGF and PTEN/PI3K/AKT pathways directly or indirectly.



Abbreviations used: OB: Oroxin B; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; COX-2: cyclooxygenase-2; PI3K: phosphatidylinositol 3 kinase; PTEN: Phosphatase and tensin homolog deleted on chromosome ten; VEGF: Vascular endothelial growth factor; RT-PCR: Reverse transcription polymerase chain reaction; DAPI: Diamidino 2 phenylindole; PBS: Phosphate buffer saline; FITC: Fluorescein isothiocyanate; PI: Propidium Iodide; RIPA: Radio immunoprecipitation assay lysis buffer; PMSF: Phenylmethanesulfonyl fluoride; PAGE: Polyacrylamide gel electrophoresis.

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INTRODUCTION

Liver cancer is the common malignant tumor, cause of cancer deaths due to its high incidence and the lack of effective treatments.^[1] Now the clinical use of chemotherapy drugs has a lot of side effects for patients, such as liver and kidney toxicity, immunosuppressive effect, and drug resistance.^[2] For treating liver cancer, it is significant to look for effective and low toxic drugs, investigate the molecular mechanisms of liver cancer pathogenesis, and develop beneficial therapeutic treatments.

Modern research has shown that the antitumor action of medicine might be attributed to inducing cell apoptosis, inhibiting tumor angiogenesis, adjusting the cell signal transduction, regulating the body's immune function, etc.^[3,4] Moreover, numbers of targets and pathways have been proposed to taking part in the antitumor mechanisms, such as COX-2/vascular endothelial growth factor (VEGF) and PTEN/phosphatidylinositol 3-kinase (PI3K)/AKT pathway.^[5-7] COX-2/VEGF pathway is an important pathway related to tumor angiogenesis. COX-2, which is highly induced in response to cell

apoptosis, tumor invasiveness, and angiogenesis, is a rate-limiting enzyme and have been found overexpression in varieties of cancers.^[8,9] VEGF as a pro-angiogenic factor is increased in many tumor types is associating with tumor vascularity and tumor invasiveness. Recently, research in cancer cells suggests that overexpression of COX-2, which play roles in regulating angiogenesis by modulating the production of many angiogenic factors, including VEGF, is along with growing tumor.^[10,11] In this study, the expression of VEGF and COX-2 was investigated after

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OB was treated with liver cancer SMMC-7721 cells. The antitumor mechanism was illustrated.

PI3K/AKT signaling pathways is an important metabolic pathway concerning to apoptosis *in vivo*. PI3K, which is frequently over-activated in human cancers, including hepatoma cancer, is a fundamental enzyme involved in cell growth and proliferation.^[12,13] The signaling is naturally antagonized by the tumor suppressor gene PTEN, PTEN which is a phosphatase that terminates the signaling of PI3K/AKT pathway by dephosphorylating PIP3, PTEN inhibited PI3K and AKT abnormal activation, and stop all downstream regulated by AKT signaling, thus inhibiting tumor cell proliferation and promote tumor cell apoptosis, and play a role for the treatment of liver tumors.^[14-20] In our research, OB effect on liver cancer cell apoptosis and its mechanisms were studied by flow cytometry instrument, reverse transcription polymerase chain reaction (RT-PCR) and Western blot.

Oroxin B (OB, Baicalein7-*O*- β -gentiobioside, C₂₇H₃₀O₁₅, MW 594.52 Da) is one of the flavonoids isolated from traditional Chinese herbal medicine *Oroxylum indicum* (L.) Vent. Flavones have been approved to be effective constituents in *O. indicum* (L.) Vent.^[21,22] Recent studies suggest that flavonoids possess antitumor, anti-inflammatory activities, etc.^[23,24] Although the current research shows that flavones have obvious anti liver tumors effect, the precise molecular mechanism is still unclarified. Accordingly, systematic study on the anti-liver cancer effect and molecular mechanisms of OB are particularly important for the development of new therapeutic strategies. In this research, the anti-liver tumors effects of OB using *in vitro* (human liver cancer SMMC-7721 cells) models were explored. Our results demonstrated that OB possessed obvious inhibitory effect and induced cell apoptosis on liver cancer cells *in vitro* through upregulation of PTEN, downregulation of COX-2, VEGF, PI3K, and p-AKT. This experiment was based on related genes and proteins to interpret the mechanism of antitumor effect on SMMC-7721 cells after treated with OB. Proving that OB possessed treatment of liver cancer by taking part in multiple metabolic pathways and targets directly or indirectly, such as PI3K-Akt signaling pathway, and COX-2/VEGF signaling pathway.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line SMMC-7721 was supplied by the Shanghai Cell Bank, Cell Bank, Chinese Academy of Science (Shanghai, China). This cell line was culture in RPMI 1640 medium (Gibco, GrandIsland, USA) supplemented with 10% fetal bovine serum (Gibco, GrandIsland, USA) and 1% penicillin/streptomycin (Gibco, GrandIsland, USA) at 37°C in a humidified incubator containing 5% CO₂.

MTT assay

Cell viability was determined through MTT assays. Briefly, cells were plated in 96-well plates at a density of 1 × 10⁴ per well. After overnight culture, different concentrations of OB (0.34, 1.01, 1.68 μM) were added to the wells and cells were incubated for 48 h. After drug treatment, the culture medium was replaced with MTT (Sigma St. Louis, MO, USA) fresh serum-free medium (final concentration, 2.5 mg/ml), followed by a 4 h incubation. In addition, 150 μl of dimethyl sulfoxide (Sigma St. Louis, MO, USA) was added to each well. Absorbance was measured with a Spectra Max Plus microplate reader (Molecular Devices, CA, USA) at a wavelength of 492 nm.

Cell apoptosis

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the

manufacturer's instructions. The cells were treated with OB for 12 h, then 1% paraformaldehyde in PBS fixed 30 min, 0.2% Triton X-100 in PBS permeabilized for 5 min, rinsed with PBS, and incubated with PE buffer (10 mM Tris-HCl, pH 7.5, 80% ethanol) at 37°C for 1 h. Then, they were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA) at 37°C for 5 min. Finally, fluorescence was recorded on an inverted fluorescence microscope (Nikon Eclipse Ti). The flow cytometer was used FITC/PI double-staining method, each group cells including treated cells (cells were treated with OB for 48 h) were collected and rinsed twice with cold PBS, mixed with 500 μl of 1 × binding buffer (1 × 10⁶/ml), 5 μl Annexin V-FITC, 5 μl propidium iodide, and incubated in the dark for 15 min and finally sent to the BD Accuri C6 flow cytometer to analyze the cell apoptosis.

RNA isolation and real-time quantitative polymerase chain reaction analysis

Total RNA of each group cells was extracted using TRIzol (Ambion, Texa, USA) according to the manufacturer's recommendations. Total RNA was reverse-transcribed using the TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China). Quantitative real-time PCR was performed using TransStart Top Green qPCR SuperMix (TransGen Biotech, China) with Piko Thermal Cycler 96-well system (Piko, Hawaii State, USA). Each sample was analyzed in triplicate. Relative levels of mRNA expression were normalized for

Table 1: First-strand cDNA synthesis system

Reagent	Volme
Random primer (N9) (0.1 μg/μl)	1 μl
2×TS reaction mix	10 μl
TransScript RT/RI enzyme mix	1 μl
Total RNA	50 ng to 5 μg/5-500 ng
RNase Free dH ₂ O	3 μl
Total	20 μl

Table 2: First-strand cDNA synthesis conditions

Reaction temperature (°C)	Reaction time (min)
25	10
42	30
85	5

Table 3: Reverse transcription polymerase chain reaction synthesis system

Reagent	Volme
cDNA	2 μl
Forward primer (10 μM)	1 μl
Reverse primer (10 μM)	1 μl
2×EasyTaq PCR SuperMix	12.5 μl
RNase Free dH ₂ O	3.5 μl
Total	20 μl

PCR: Polymerase chain reaction

Table 4: Reverse transcription polymerase chain reaction synthesis conditions

Reaction temperature (°C)	Reaction time	Cyc
94	5 min	1
94	10 s	30
58	10 s	
72	15 s	
72	5 min	1
4	∞	

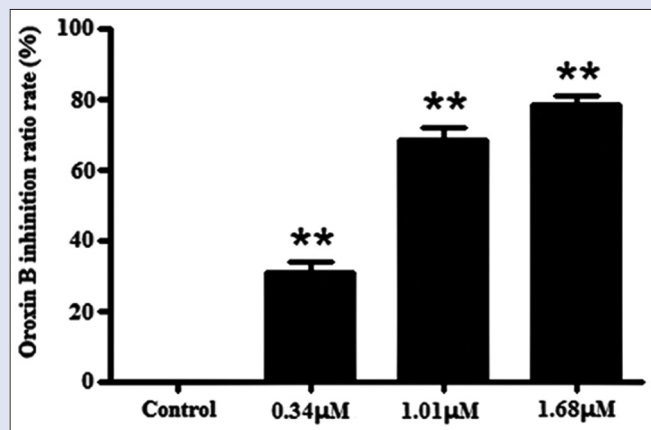


Figure 1: Oroxin B inhibits the proliferation in the human hepatoma cell line SMMC-7721 ($n=3$). X-axis is the inhibition ratio of SMMC-7721 cells, and y-axis is the different concentrations of Oroxin B (0.34, 1.01, and 1.68 μM), ** $P < 0.01$ versus control group

β -actin mRNA expression and calculated according to the formula $2^{-(\Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{control}})}$. Primer sequences for the genes: h-PTEN F: 5'-CCCAGTCAGAGGCGCTATG-3'; R: 5'-GGCAGACCACAAACTGAGGATT-3'; h-COX2 F: 5'-AGCAGGCAGATGAAATACCAGTCT-3'; R: 5'-ATACAGCTCCA CAGCATCGATGT-3'; h-pAKT F: 5'-GGAGGAGATCACTGGCAA-3'; R: 5'-CATGGTCAGTGTGCCTCTCA-3'; h-PI3K F: 5'-AACGAGA ACGTGT GCCATTTG-3'; R: 5'-AGAGATTGGCATG CTGTCGAA-3'; h-VEGF F: 5'-AGGAGTACCCTGATGAGATCGAGTA-3'; R: 5'-TGGTGAGGTTTG ATCCGCATA; β -actin F: 5'-CACCCGCGAGTACAACCTTC-3'; R: 5'-CCCA TACCACCATCACACC-3' (Invitrogen, USA). All primers final concentration is 100 $\mu\text{M/l}$. The reaction system and reaction time are shown in Tables 1-4.

Western blotting analysis

Protein extracts were isolated from each group cells in using RIPA protein lysis buffer containing 1mM PMSF. Total protein was separated by 10% SDS-PAGE, transferred with polyvinylidene difluoridemembrane, blocked in 5% BSA (Solarbio, Beijing, China) and probed with appropriate primary

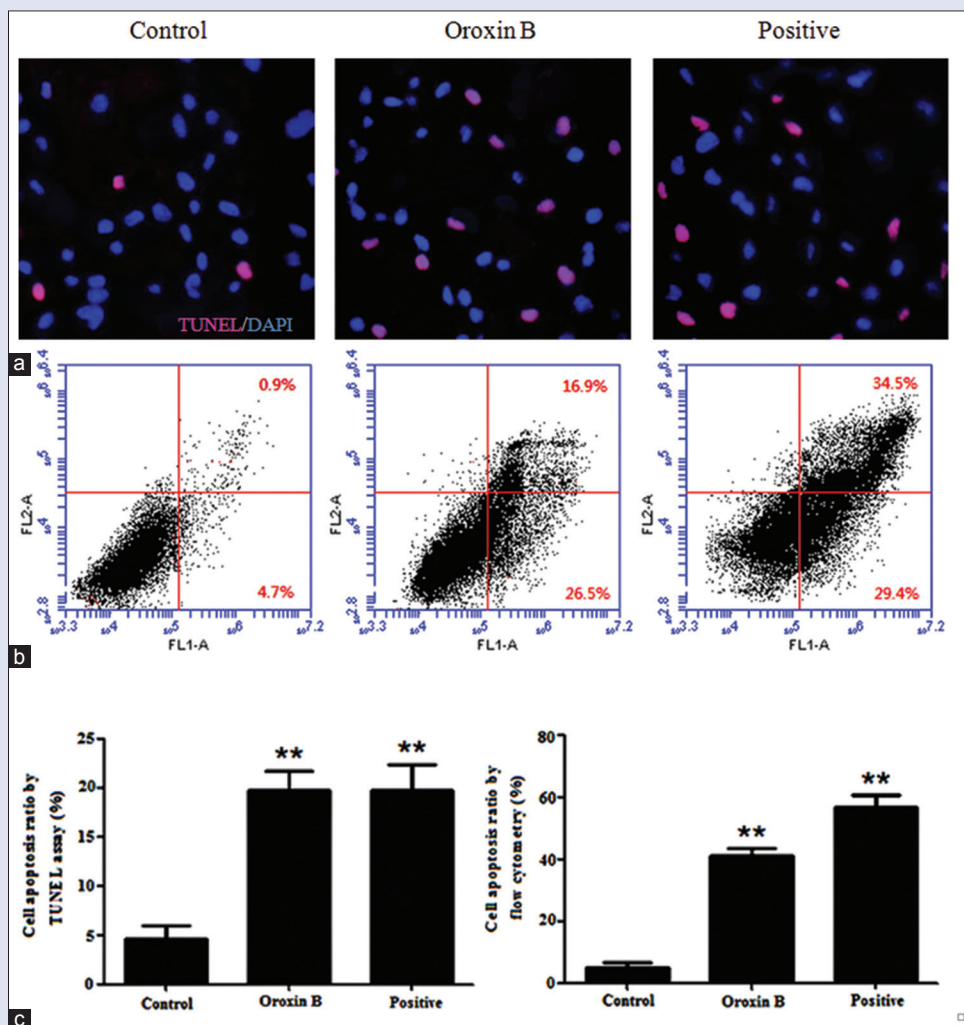


Figure 2: The effect of Oroxin B on cell apoptosis. (a) Cell apoptosis profiles by terminal deoxynucleotidyl transferase dUTP nick end labeling assay of SMMC-7721 cells treated with Oroxin B (1.68 μM) and Cisplatin (positive control group) for 12 h. (b) cell apoptosis profiles of SMMC-772 cells, treated with Oroxin B (1.68 μM) and Cisplatin for 48 h, were subjected to Annexin-V-FITC/PI binding and analyzed by flow cytometry. (c) Statistical analysis results of cell apoptosis ratio by terminal deoxynucleotidyl transferase dUTP nick end labeling assay. (d) Statistical analysis results of cell apoptosis ratio by flow cytometry. ** $P < 0.01$ versus control group

antibodies against the target proteins, VEGF antibody was purchased from Abcam (Santa Cruz, USA). PTEN, PI3 Kinase p85, p-AKT, COX-2, and β -actin antibodies were purchased from Cell Signaling (Beverly, MA, USA). These were followed by incubation with Goat Anti-Rabbit immunoglobulin G (H + L) (Protein tech, USA), and antigen-antibody complexes were visualized using the chemiluminescent ECL (TransGen Biotech, China) detection system.

Statistics analysis

Statistical analysis was performed using *t*-test or one-way ANOVA with GraphPad Prism 5. The *P* values were considered statistically significant at *P* < 0.05. All data are means \pm standard deviation for at least three separate experiments.

RESULTS

Oroxin B inhibits the proliferation in the human hepatoma cell line SMMC-7721

To investigate the effect of OB on the proliferation of human hepatoma cell line SMMC-7721, we set up different concentrations of OB, 0.34, 1.01, and 1.68 μ M then continued to incubate for 48 h. The results showed that each concentration has obvious proliferation inhibition on hepatoma cancer SMMC-7721 cells *in vitro*, and the inhibition rate increased with the increase of concentration, it indicated that OB can

inhibited the proliferation of SMMC-7721 cells in a dose-dependent manner [Figure 1].

Oroxin B induces apoptosis in the human hepatoma cell line SMMC-7721

To identify the effect of OB on apoptosis in SMMC-7721 cells, Annexin V-FITC/PI double fluorescence staining method and TUNEL assay were used. The results of the TUNEL assay, the proportion of apoptotic cells was calculated as the percentage of red cells relative to the total DAPI-positive cells. The statistical analysis showed that the rate of apoptosis in OB group was $21.55\% \pm 2.58\%$, (*P* < 0.01 compared with the Control group, [Figure 2c]). The rate of apoptosis in positive group was $25.13\% \pm 2.74\%$, (*P* < 0.01 compared with the Control group, [Figure 2c]), but no significant difference was found between OB and positive groups. In the flow cytometry analysis, the statistical analysis showed that the rate of apoptosis in OB group was $43.03\% \pm 3.07\%$, (*P* < 0.01 compared with the Control group, [Figure 2c]), the statistical analysis showed that the rate of apoptosis in positive group was $58.87\% \pm 4.86\%$, (*P* < 0.01 compared with the Control group, [Figure 2c]), and the difference between two groups was that OB can induce early stage of apoptosis clearly, and positive group can induce late apoptosis, and difference from the control group was obvious [Figure 2b].

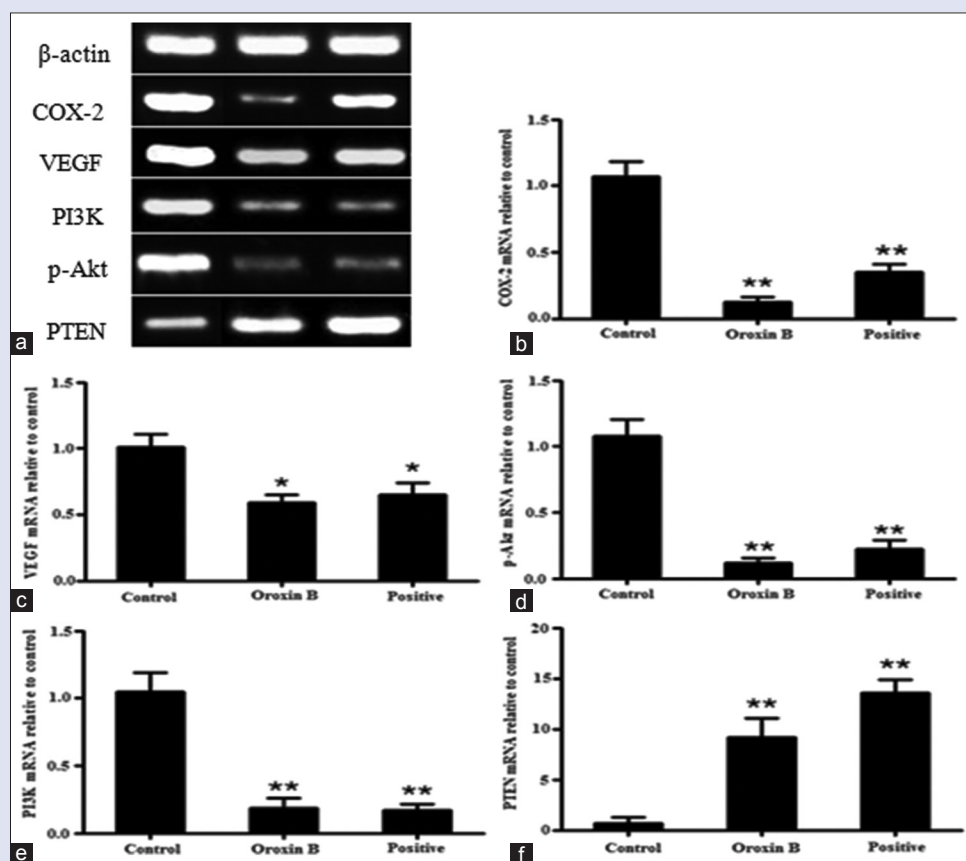


Figure 3: The effect of OB on COX-2, VEGF, p-Akt, PI3K, and PTEN mRNA expression in SMMC-7721 cells. mRNA expression levels of COX-2, VEGF, PI3K, p-Akt, and PTEN in SMMC-7721 cells following OB treatment for 48 h. The mRNA levels of mRNAs were detected by real-time PCR and measured using Piko Real Software with β -actin as an internal reference. All data, repeated by three independent experiments, are presented as mean \pm standard deviation. **P* < 0.05 and ***P* < 0.01 versus control group. (a) The expression of COX-2, VEGF, PI3K, p-Akt, and PTEN mRNA in SMMC-7721 cells. (b) Expression of COX-2 in each group. (c) Expression of VEGF in each group. (d) Expression of p-AKT in each group. (e) Expression of PI3K in each group. (f) Expression of PTEN in each group. VEGF: Vascular endothelial growth factor; PI3K: Phosphatidylinositol-3-kinase; OB: Oroxin B

Effect of Oroxin B on COX-2, vascular endothelial growth factor, p-Akt, phosphatidylinositol 3-kinase, PTEN mRNA, and protein expression in SMMC-7721 cells

To explore the OB's influence on the COX-2/VEGF and PI3K/AKT signaling pathway, RT-PCR method was used to study the effects on the expression of COX-2, VEGF, PI3K, p-AKT, and PTEN mRNA in SMMC-7721 cells when treated with OB. We observed that COX-2, VEGF, PI3K, and p-AKT mRNA high expression in SMMC-7721 cells, but the levels were greatly decreased when treated with OB [Figure 3]. We also found that the expression of PTEN mRNA a was increased compared with control group (***P* < 0.01) after being treated by OB. Western blot analyses were further confirmed that whether lower levels of COX-2, VEGF, p-AKT, PI3K protein and higher levels of PTEN were observed in the OB-treated cells. As shown in the Figure 4, OB downregulated COX-2, VEGF, PI3K, and p-AKT protein's expression obviously when compared with Control group **P* < 0.05 [Figure 4b-e],

the expression of PTEN protein is significantly higher in OB-treated cells when compared to the control group (***P* < 0.01) [Figure 4f]. Western blot results consistent with the results of RT-PCR.

DISCUSSION

OB, monomer composition, is a flavonoid compound originated from traditional Chinese medicine *O. indicum* (L.). Recent studies have suggested that flavonoids in *O. indicum* (L.) are beneficial to treat liver cancer,^[25] but the mechanisms of their antitumor activity remain incompletely understood. In this research, OB treatment possessed obvious inhibition of the proliferation in the human liver cancer SMMC-7721 cells, and the inhibition was enhanced with the increasing dose. The result of TUNEL assay showed that, OB-induced cell apoptosis in the human liver cancer SMMC-7721 cells [Figure 2]. We selected cisplatin as positive control which is a conventional chemotherapy drug used in clinical lines when defended against tumors, especially lung and hepatoma tumor. The flow cytometry results showed that cisplatin could obviously induce late apoptosis and OB-induced early apoptosis

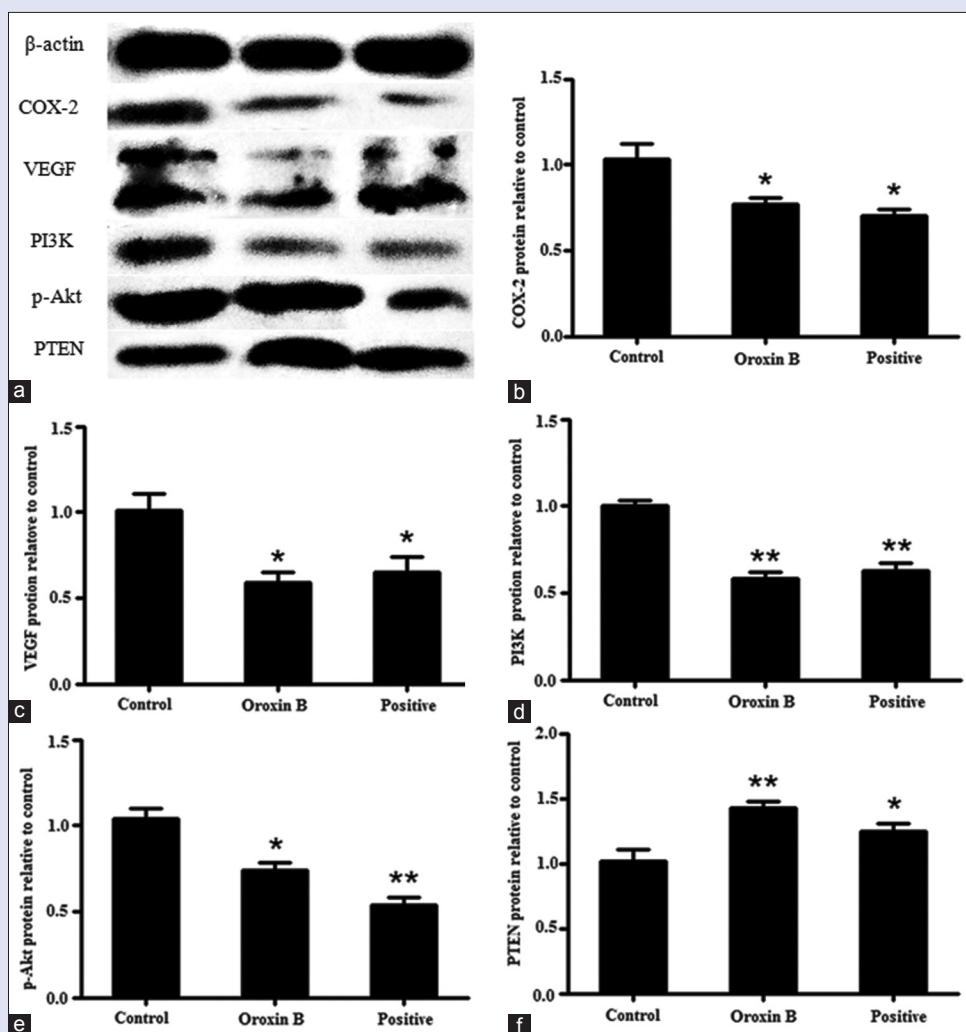


Figure 4: The effect of OB on COX-2, VEGF, p-Akt, PI3K, PTEN protein expression in SMMC-7721 cells. Protein expression levels of COX-2, VEGF, PI3K, p-Akt, and PTEN in SMMC-7721 cells following OB treatment for 48 h. The protein levels were detected by Western blotting analyses. All data, repeated by three-independent experiments, are presented as mean ± standard deviation. **P* < 0.05 and ***P* < 0.01 versus control group. (a) Western blotting analyses of COX-2, VEGF, PI3K, p-Akt, and PTEN protein expression in SMMC-7721 cells. (b) COX-2 protein expression in cells. (c) VEGF protein expression in cells. (d) PI3K protein expression in cells. (e) p-Akt protein expression in cells. (f) PTEN protein expression in cells. VEGF: Vascular endothelial growth factor; PI3K: Phosphatidylinositol 3-kinase; OB: Oroxin B

of SMMC-7721 cells [Figures 3 and 4]. Compared with cisplatin, OB had a good curative effect, could induce early apoptosis rather than late apoptosis, which laid the foundation for us to study on related targets. Therefore, OB as an antitumor drug had great value for further development.

For investigating the potential mechanism of OB antitumor effects, we evaluated the regulating effect of OB on COX-2, VEGF, PI3K, AKT, and PTEN expression in the human liver cancer SMMC-7721 cells. Recent research suggests that highly expressed COX-2 and VEGF indicates a poor prognosis, and overexpression of COX-2 in the growth and diffusion of tumor is triggered by its regulation of several angiogenic factors, such as VEGF,^[26,27] which is widely accepted as a primary inducer of angiogenesis.

In many physiologic and pathologic processes, angiogenesis is the basis of chronic inflammation, ischemic disease and cancer.^[28] It is a multistep process occurring in preexisting blood vessels, including newly blood vessels migration, extracellular matrix remodeling, and endothelial cell proliferation and migration.^[29,30] The results of the present study, COX-2 and VEGF expression were decreased significantly after treated with OB [Figure 4], it suggested that OB could play a potential role for treating liver cancer through the regulation of COX-2/VEGF signaling pathway, cutting off the blood supply a neoplastic lesion could be potentially starved into remission, then indirectly play antitumor effect.

PI3K/AKT signaling participates in multiple signaling pathways which modulate cell proliferation, differentiation, migration, and apoptosis.^[31] PI3K is located at the cell membrane; when it is activated by a number of receptor tyrosine kinases (such as VEGFR), it initiates the signaling cascade through phosphorylation, leads PIP2 into PIP3. However, the role of PTEN is just the opposite.^[32,33] PTEN has been identified to participate in the PI3K/AKT apoptotic pathways and related to cellular apoptosis or cell proliferation. Therefore, PTEN, PI3K, and AKT have been demonstrated to be the targets for intervening liver cancer.^[34-38] Our study proved that OB could down regulate VEGF, PI3K, and p-AKT both in mRNA and protein level, meanwhile, it could upregulate the expression of PTEN mRNA and protein [Figure 4]. This study indicated that OB could decrease VEGF protein expression to reduce the activation

of PI3K, thus inactive the PI3K/AKT signaling pathway. It could also be concluded that PTEN served as a negative regulatory factor in the PI3K/AKT signaling pathway, thus inhibiting the abnormal activation of PI3K and AKT could contribute to cellular apoptosis thus play a role of antitumor [Figure 5]. The results suggested that OB was participated in PI3K/AKT signaling pathways, influenced the expression of VEGF, PTEN, PI3K, and p-AKT, thus induced the apoptosis and proliferation of cancer cell.

In conclusion, our findings revealed that OB effectively inhibited proliferation, and it also could induce early apoptosis of human liver cancer cells. COX-2, VEGF, PI3K, and p-AKT expression levels were found to be downregulated, while PTEN was upregulated after OB treatment. We could infer that OB inhibited cell proliferation and reduced angiogenesis partly through regulation of COX-2/VEGF pathways, and OB induced cancer cell apoptosis may relate to PTEN/PI3K/AKT pathways directly or indirectly. Thus, we can infer that OB plays a role of antitumor effect may to regulate these targets, and this paper laid the foundation of OB as antitumor targets drugs development and the research of antitumor mechanism. In addition, for developing nontoxic OB as a potential of liver cancer, clinical trials will be necessary.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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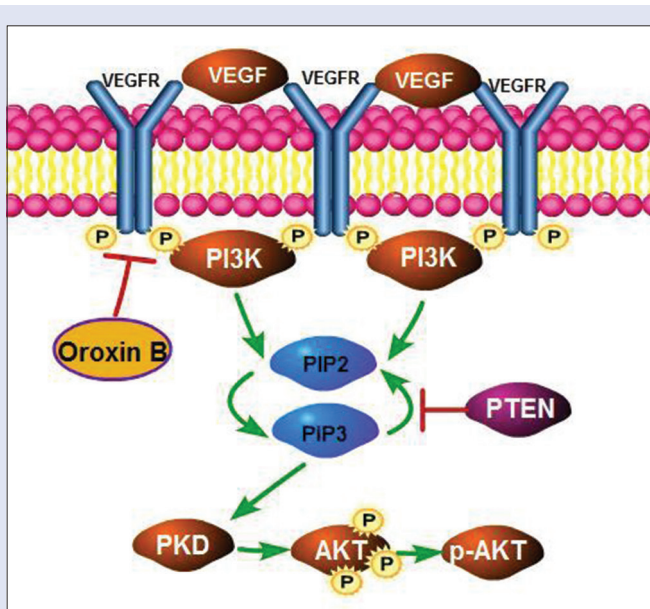


Figure 5: The relationship between VEGF and PI3K/Akt/PTEN signaling pathways. VEGF: Vascular endothelial growth factor; PI3K: Phosphatidylinositol 3-kinase

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