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ORIGINAL ARTICLE

Influence of Photodynamic Therapy on Apoptosis and Invasion of Human Cholangiocarcinoma QBC939 Cell Line

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Key words: photodynamic therapy; cholangiocarcinoma; apoptosis; invasion

Objective To investigate the effect of photodynamic therapy (PDT) mediated by hematoporphyrin derivative (HPD) on apoptosis and invasion of cholangiocarcinoma QBC939 cell lines.

Methods *In vitro* cultured cholangiocarcinoma QBC939 cell line was exposed to 2, 4, 6, 8, 10, 12, and 14 $\mu\text{g/ml}$ HPD with 5, 10, and 15 J/cm^2 light intensity, respectively. The optical density at 450 nm of the QBC939 cells was measured by CCK8 assay and its growth inhibition ratio was calculated. Flow cytometry and transwell migration assay were applied to detect cell apoptosis and invasion respectively. RT-PCR and immunocytochemistry analyses were used to detect expressions of vascular endothelial growth factor-C (VEGF-C), cyclooxygenase-2 (COX-2), and proliferating cell nuclear antigen (PCNA). Enzyme-linked immunosorbent assay (ELISA) was carried out to examine the secretion of VEGF-C and COX-2 in QBC939 cells.

Results Exposure to HPD-PDT can significantly suppress the growth of QBC939 cells (all $P < 0.05$). HPD-PDT can promote apoptosis of QBC939 cells at the early stage. When the concentration of HPD was 2 $\mu\text{g/ml}$ and light irradiation was 5 J/cm^2 , HPD-PDT had no obvious inhibitory effect on QBC939 cell growth, but can obviously inhibit cell invasion, and significant difference was observed between the HPD-PDT and control groups ($P < 0.01$). The HPD-PDT can reduce the mRNA and protein expressions of VEGF-C, COX-2, and PCNA, and decrease the secretion of VEGF-C and COX-2 in QBC939 cells.

Conclusion PDT could promote apoptosis and inhibit growth and invasion of cholangiocarcinoma cells QBC939 *in vitro*.

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CHOLANGIOCARCINOMA is a kind of malignant tumor that originates from biliary epithelial cells. The surgical resection rate is low because it could easily invade surrounding vessels, nerve,

lymph nodes, and liver tissues. Moreover, it is not sensitive to conventional radiation and chemotherapy.¹ The 5-year survival rate is lower than 5%.^{2, 3} Palliative biliary drainage could improve the patient's physical condition and life quality to some extent,⁴ but could not obviously improve prognosis and prolong survival time.⁵

Photodynamic therapy (PDT) mediated by hematoporphyrin

derivative (HPD) is an adjuvant treatment strategy, which used clinically to eliminate malignant tumor. PDT has been shown to be effective, minimally toxic and invasive, easy and simple to operate, and repeatable.

It has been reported that PDT can inhibit proliferation and invasion, as well as induce apoptosis of tumor cells. However, the specific molecular mechanisms involved in the process are not clear.^{6, 7} It has been confirmed that inhibiting cyclooxygenase-2 (COX-2) expression of QBC939 cells can inhibit cell proliferation and promote apoptosis. In addition, inhibiting COX-2 expression can influence vascular endothelial growth factor-C (VEGF-C) expression so that to develop the antineoplastic properties.⁸ Proliferating cell nuclear antigen (PCNA) binds to DNA polymerase protein to regulate DNA replication, which is widely used to monitor the proliferation activity of cancer cell. The increased PCNA expressions in the liver cancer and digestive tumors are related with its proliferation, infiltration, and metastasis of tumor cells.^{9, 10} Therefore, inhibiting PCNA expression might reduce the proliferation of tumor cells, and induce the apoptosis. As a primary lymphatic stimulating factor, VEGF-C can combine with its specific receptor—VEGF receptor 3 (VEGFR-3), and mediate the lymphatic metastasis of malignant tumor cells.¹¹ In this study, we investigated the potential effects of PDT on QBC939 cells.

MATERIALS AND METHODS

Cell culture

Human cholangiocarcinoma QBC939 cell line was bought from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). QBC939 cells were cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal calf serum (Hyclone, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 95% air and 5% CO₂ at 37°C. Cells in the logarithmic phase were used in the experiment.

CCK8 assay

QBC939 cells were seeded in 96-well plates at a density of 6×10^3 per well, and continued to culture for 24 hours. After supernatant was discharged, 0, 2, 4, 6, 8, 10, 12, and 14 $\mu\text{g/ml}$ HPD (Huading Modern Biological Pharmaceutical Co., Ltd., Chongqing, China) was added respectively, 3 wells in each concentration group. After 24 hours of incubation, QBC939 cells were exposed to red light (630 nm) at the different dose rate of 0, 5, 10, and 15 J/cm² (Diode laser Treatment Apparatus, Gibco) respectively at room temperature. The medium was replaced with serum-free RPMI-1640 medium thereafter, and CCK8 assay was

carried out after 48 hours. Optical density (OD) value at 450 nm was measured using a spectrophotometer (UV-3600, Gibco). The cell growth inhibition ratio was calculated as follows: $(\text{OD}_{\text{control}} - \text{OD}_{\text{HPD-PDT}}) / \text{OD}_{\text{control}} \times 100\%$. CCK8 kit was purchased from Dojindo (Kumamoto, Japan).

Flow cytometry

QBC939 cells were seeded in 6-well plates at a density of approximately 2×10^5 cells/well, and cultured for 12 hours. After cells were serum-starved for 24 hours, they were randomly divided into the PDT only (5 J/cm²), HPD only, HPD-PDT, and control groups, 3 wells in each group. Cells in the HPD only group were incubated with RPMI-1640 medium supplemented with 10% fetal calf serum and 8 $\mu\text{g/ml}$ HPD for 24 hours. The apoptosis of suspension cells was determined by Annexin V/Propidium Iodide double staining and flow cytometry on a flow cytometer (Cell Lab Quanta SC, Gibco) according to manufacturer's instruction. Apoptosis rate = number of apoptotic cells/number of total cells $\times 100\%$. Apoptosis Detection Kit was purchased from Invitrogen (USA).

Transwell migration assay

QBC939 cells were seeded in the upper layer of transwell at a density of approximately 4×10^4 cells/well. They were randomly divided into the PDT only, HPD only, HPD-PDT, and control groups with 2 wells in each group. The final concentration of HPD for the HPD-PDT and HPD only groups was 2 $\mu\text{g/ml}$. Cells in the control group and PDT only group were cultured with the same volume of serum-free RPMI-1640. After incubated in dark place for 24 hours, cells in the HPD-PDT and PDT only groups were exposed to 5 J/cm² light intensity for 79 seconds. When the medium in the upper layer was changed to fresh serum-free RPMI-1640, 600 μl culture medium containing 10% fetal calf serum was added in the lower layer of transwell. Following an incubation period for 36 hours in dark place, the migratory cells were stained with 0.1% crystal violet, and quantitated under microscope. Afterwards, decolorization of crystal violet was carried out with 33% acetic acid, after 10 minutes OD value at 630 nm was detected under spectrophotometer UV-3600. Transwell Model was obtained from Dow Corning Co. (USA) and Matrigel from BD Co. (USA).

RT-PCR assay

QBC939 cells were seeded in 6-well plates and were cultured at a density of approximately 1.5×10^5 cells/well. They were randomly divided into the HPD-PDT and control groups with 6 wells per group. Total RNA was extracted and

reversely transcribed into cDNA. VEGF-C, COX-2, PCNA, and hGAPDH primers (Table 1) were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). PCR was performed in a 20 μ l of reaction system, including cDNA 1 μ l, forward primer (0.5 μ mol/L) 1 μ l, reverse primer (0.5 μ mol/L) 1 μ l, Premix Taq (TaKaRa Bio Inc., Dalian, China) 11.5 μ l, and distilled water 5.5 μ l. VEGF-C reaction was conducted by initial denaturation at 95°C for 5 minutes, and followed by denaturation at 98°C for 10 seconds, annealing at 61.7°C for 30 seconds, extension at 72°C for 30 seconds in a total of 35 cycles, and an extension step at 72°C for 10 minutes. COX-2 reaction was carried out by initial denaturation at 95°C for 5 minutes, 33 cycles of denaturation at 98°C for 10 seconds, annealing at 55.8°C for 30 seconds, extension at 72°C for 30 seconds, and an extension at 72°C for 10 minutes. PCNA reaction was performed by initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 98°C for 10 seconds, annealing at 63.9°C for 30 seconds, extension at 72°C for 30 seconds, and an extension at 72°C for 10 minutes. The amplification product was electrophoresed on 2% sepharose gel and in 0.5×TAE buffer solution at 110 V for 30 minutes. Transcript values were normalized to GAPDH.

Immunocytochemistry assay

QBC939 cells were seeded into 6-well plates and cultured at a density of approximately 1.5×10^5 cells/well. Then 4% formalin-fixed cells were incubated with the primary antibody (1:200 in dilution) for 1 hour at 37°C. The rabbit anti-human VEGF-C antibody, COX-2 antibody, and PCNA antibody were purchased from Abcam Co. (MA, USA). A horseradish peroxidase-labeled goat anti-rabbit IgG polyclonal was used as the secondary antibody. The DAB kit (Beijing Boisynthesis Biotechnology Co. Ltd., China) was used for colour development and improved hematoxylin (CWBIO Co., China) was used as counterstain.

MIAS-1000 (Beijing BEONY Science and Technology Co. Ltd., China) was used for determining the number of positive cells. The positive cells showed yellow particles in their cytoplasm and membrane. A sample with <5% positive cells was scored as –, that with positive cells accounting for more than 5% but less than 25% of the total was scored as +, that with more than 25% but less than 50% positive cells was scored as ++, that with more than 50% but less than 75% positive cells was scored as +++, and that with more than 75% positive cells was scored as ++++.

ELISA

QBC939 cells were seeded in 6-well plates and were cultured at a density of approximately 1.5×10^5 cells/well. They were randomly divided into the HPD-PDT group (8 μ g/ml+5 J/cm²), PDT only group (5 J/cm²), HPD only group (8 μ g/ml), and control group with 3 wells/group. After the sample was centrifuged at 716-1610 $\times g$ for 20 minutes in the sterile tube, the culture supernatant containing VEGF-C and COX-2 was collected. The assayed supernatants were incubated with anti-VEGF-C or anti-COX-2 antibodies coated plate. The concentration of VEGF-C and COX-2 in the sample was measured at 450 nm using a spectrophotometer. ELISA kits were from Qingdao Effort Biotechnology Co., Ltd (Shandong, China).

Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences, version 17.0 (SPSS, Chicago, IL, USA) software. The measurement data were expressed as means $\pm$ standard deviation. The means of multiple groups were compared using a one-way analysis of variance (ANOVA), and the means between two groups was analyzed using least significant difference (LSD)-*t* test. *P*<0.05 was considered statistically significance.

Table 1. Primer sequences for polymerase chain reaction

Genes	Primers	Primer sequences	Fragment length (bp)
VEGF-C	Forward	5'-TCAAGGCCCCAAACCAGTAACAAT-3'	228
	Reverse	5'-CCAGCATCCGAGGAAAACATAAAA-3'	
COX-2	Forward	5'-AATGGGGTGATGAGCAGTTGTTC-3'	202
	Reverse	5'-GGATGCCAGTGATAGAGGGTGTTA-3'	
PCNA	Forward	5'-AAGCACCAAACCAGGAGAAAGT-3'	364
	Reverse	5'-ACCGTTGAAGAGAGTGGAGTG-3'	
hGAPDH	Forward	5'-AACAGCCTCAAGATCATCAGCAA-3'	146
	Reverse	5'-GACTGTGGTCATGAGTCCTTCCA-3'	

VEGF-C: vascular endothelial growth factor-C; COX-2: cyclooxygenase-2; PCNA: proliferating cell nuclear antigen; hGAPDH: human glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

HPD-PDT can depress the growth of QBC939 cells

As shown in Fig. 1, compared with the control group (0 $\mu\text{g/ml}$ +0 J/cm^2), 4 $\mu\text{g/ml}$ HPD combined with 5 J/cm^2 light irradiation can significantly inhibit the growth of QBC939 cells (approximately 26%, $P<0.05$). When HPD concentration was raised to 8 $\mu\text{g/ml}$ and light irradiation dose to 5 J/cm^2 , inhibition rate was increased to about 80%. Thereafter, increasing the quantity of HPD-PDT exposure did not significantly increase the severity of growth inhibition. As a result, we chose 8 $\mu\text{g/ml}$ of HPD and 5 J/cm^2 of light irradiation for the rest of this study.

HPD-PDT can induce the apoptosis of QBC939 cells

As illustrated in Fig. 2, the apoptosis rates of the control, HPD only, and PDT only groups were $33.4\%\pm 1.4\%$, $41.0\%\pm 1.6\%$, and $37.2\%\pm 2.1\%$, respectively. There were no significant differences between the three groups (all $P>0.05$). The apoptosis rate of the HPD-PDT was $74.6\%\pm 1.5\%$, which was significantly higher than that of the other three groups (all $P<0.05$).

HPD-PDT can arrest QBC939 cells migration

The number of QBC939 cells through Matrigel in the HPD-PDT

group (light irradiation dose 5 J/cm^2 , HPD 2 $\mu\text{g/ml}$) was 13.33 ± 1.16 , which was significantly less than that in the control (31.67 ± 2.52), PDT only (28.00 ± 2.65), and HPD only groups (27.67 ± 2.08), respectively (all $P<0.01$). There were no significant differences among the control, PDT only, and HPD only groups (Fig. 3). After acetic acid decolorization, the OD₆₃₀ values showed significant differences ($P<0.01$) between the HPD-PDT group (0.1207 ± 0.0085) and control group (0.2550 ± 0.0070), PDT only group ($0.2397\pm$

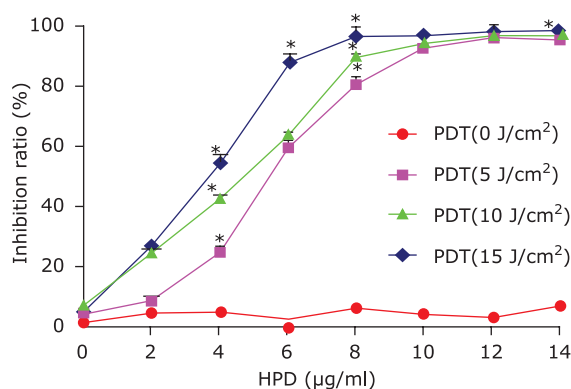


Figure 1. Analysis result of growth inhibition rate of QBC939 cells exposed to HPD-PDT ($n=3$).

HPD: hematoporphyrin; PDT: photodynamic therapy.

* $P<0.05$ compared with the control group (0 $\mu\text{g/ml}$ +0 J/cm^2).

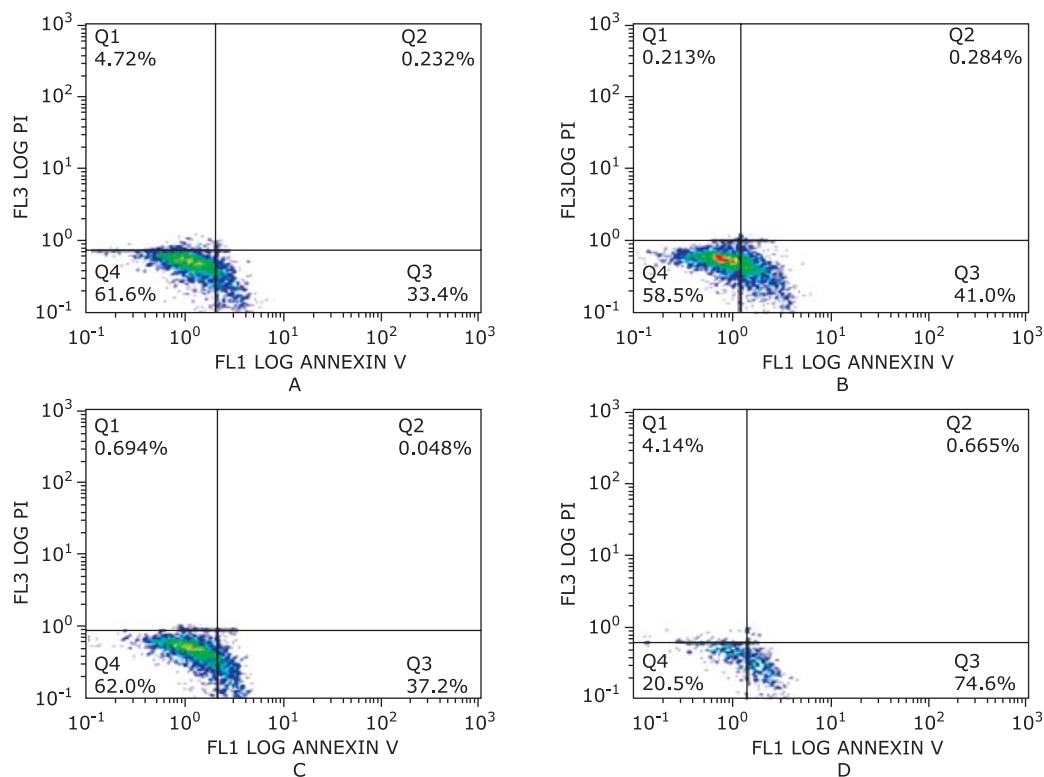


Figure 2. Analysis results of apoptosis rate of QBC939 cells exposed to HPD and/or PDT detected by flow cytometry.

PI: propidium iodide.

A. control group; B. HPD only group (8 $\mu\text{g/ml}$); C. PDT only group (5 J/cm^2); D. HPD-PDT group (8 $\mu\text{g/ml}$ +5 J/cm^2).

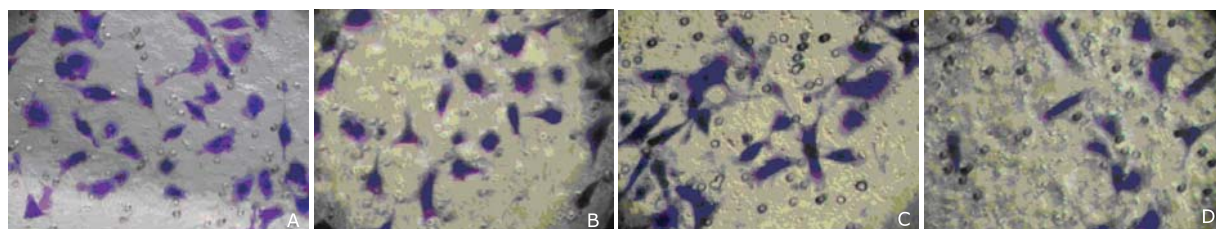


Figure 3. QBC939 cells that have migrated through Matrigel were stained with crystal violet in Transwell migration assay. x400
A. control group; B. HPD only group (2 µg/ml); C. PDT only group (5 J/cm²); D. HPD-PDT group (2 µg/ml+5 J/cm²).

0.0143), HPD only group (0.2373 ± 0.0167) respectively. There were no significant differences among the control group, HPD only group, and PDT only group (all $P > 0.05$).

HPD-PDT can decrease VEGF-C, COX-2, and PCNA expression levels in QBC939 cells

Fig. 4 indicated that relative mRNA levels of VEGF-C, COX-2, and PCNA in QBC939 cells of the control group were 0.84 ± 0.02 , 0.98 ± 0.01 , and 0.95 ± 0.08 , respectively. After treated with 8 µg/ml HPD and 5 J/cm² light irradiation, relative mRNA levels of VEGF-C, COX-2, and PCNA significantly dropped to 0.46 ± 0.02 , 0.51 ± 0.08 , and 0.68 ± 0.06 , respectively. The difference between photodynamic group and control group was statistically significant (all $P <$

0.05). When cells were treated with 2 µg/ml HPD and 5 J/cm² light irradiation, the relative mRNA level of VEGF-C was significantly decreased to 0.54 ± 0.05 ($P < 0.05$).

Fig. 5 showed that proportions of VEGF-C positive cells of the HPD-PDT, PDT only, HPD only, and control groups were $10.5\% \pm 1.1\%$ (+), $70.6\% \pm 1.7\%$ (+++), $26.5\% \pm 2.2\%$ (++), and $72.5\% \pm 2.3\%$ (+++), respectively, those of COX-2 positive cells were $3.3\% \pm 0.5\%$ (-), $40.9\% \pm 0.8\%$ (++), $46.5\% \pm 2.2\%$ (++), and $53.7\% \pm 1.2\%$ (+++), and those of PCNA positive cells were $4.5\% \pm 1.4\%$ (-), $30.5\% \pm 1.6\%$ (++), $47.7\% \pm 1.2\%$ (++), and $52.5\% \pm 1.3\%$ (+++). The differences between the HPD-PDT group and control group were statistically significant (all $P < 0.05$).

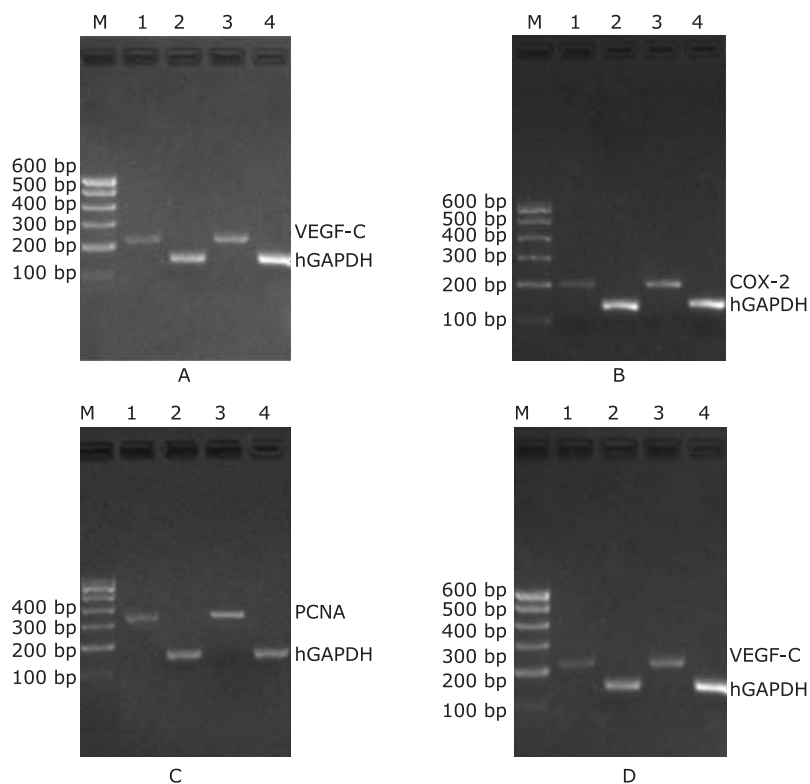


Figure 4. RT-PCR results of expression levels of VEGF-C (A, D), COX-2 (B), and PCNA (C) in QBC939 cells.

Cells in the HPD-PDT group (lanes 1 and 2) were cultured with 8 (A) and 2 (D) µg/ml HPD respectively for 24 hours, and followed by exposure to 5 J/cm² light intensity. And QBC939 cells in the control group (lanes 3 and 4) were cultured with the same volume of serum-free RPMI-1640.

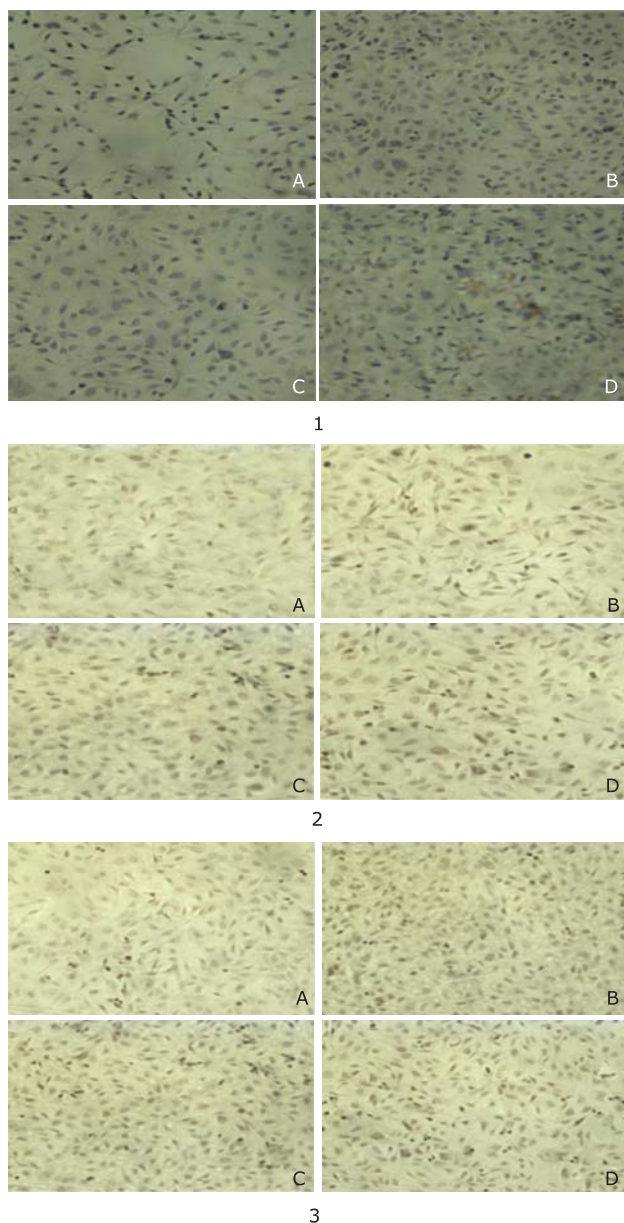


Figure 5. Images of VEGF-C (1), COX-2 (2), and PCNA (3) staining in QBC939 cells section ($n=5$). DAB $\times 200$. A. HPD-PDT group (8 $\mu\text{g/ml}+5 \text{ J/cm}^2$); B. PDT only group (5 J/cm^2); C. HPD only group (8 $\mu\text{g/ml}$); D. control group.

HPD-PDT can inhibit QBC939 cells to secrete VEGF-C and COX-2

VEGF-C concentrations in the supernatant of the HPD-PDT, HPD only, PDT only, and control groups were 1.47 ± 0.04 , 1.58 ± 0.03 , 1.57 ± 0.05 , and $1.65 \pm 0.03 \text{ ng/ml}$, respectively, and COX-2 concentrations were 32.57 ± 1.02 , 34.66 ± 0.50 , 34.75 ± 0.60 , and $35.27 \pm 0.54 \text{ ng/ml}$, respectively. Compared with the control group, the secretion of both VEGF-C and COX-2 by QBC939 cells were significantly decreased after photodynamic therapy (all $P < 0.05$).

DISCUSSION

PDT is a noninvasive or minimally invasive technique used clinically in tumor auxiliary treatment in recent years, which destroys the targeted tissue and cells by photochemical reaction without generating heat and thermal damage. In the local treatment of cancers, an appropriate wavelength of the light was applied locally to excite the photosensitizer that has been selectively taken up and accumulated in tumor tissue to produce reactive oxygen species, which can trigger a series of cellular response.¹²⁻¹⁴ The mechanisms by which PDT inhibits tumor growth *in vivo* include: regulating signal transduction and inducing tumor cell apoptosis and necrosis, and autophagic cell death; destroying the microvessels of tumors; stimulating the immunoreaction and inflammatory response, etc.^{15, 16}

PDT can significantly inhibit the growth of tumor cells *in vitro*.¹⁷ The underlying mechanism of inhibiting the cellular growth of cholangiocarcinoma remains unclear. The results of this study showed that PDT can inhibit growth of cholangiocarcinoma QBC939 cells *in vitro*. The different HPD-PDT protocols had different effects: When PDT reached 5 J/cm^2 and HPD was more than $8 \mu\text{g/ml}$, QBC939 cell growth can be significantly inhibited with a growth inhibition rate of 80% or above; at the same time, the early apoptosis of QBC939 cells can be significantly increased with the early apoptosis rate of 74.6%. We also applied transwell chamber (Matrigel migration assay) to find the fact that *in vitro* invasive capacity of QBC939 cells was greatly decreased after being treated with $2 \mu\text{g/ml}$ HPD and a light energy density of 5 J/cm^2 . In the meantime, we found PDT can induce apoptosis and inhibit invasion of QBC939 cells.

VEGF-C, as the most important angiogenic factor that has been identified by far, is closely related with tumor angiogenesis and enables local lymphatic and capillary proliferation.¹⁸ High expression of VEGF-C has been observed in tumor tissues.¹⁹ "Skip" metastasis along perineural lymphatics is one of the important characteristics of cholangiocarcinoma.²⁰ As the main lymphangiogenic stimulating factor, VEGF-C can bind to its specific receptor VEGFR-3 and plays its specific role in promoting lymphangiogenesis and mediating lymph node metastasis in malignant tumor cells.²¹ High expression of COX-2 has been reported to be responsible for tumorigenesis and development of various tumors, the possible mechanism of which is currently considered to be relevant to promoting tumor cell proliferation, inhibiting apoptosis in tumor cells, and increasing malignant transformation of tumors.²² In addition, it has been verified that activation of COX-2 gene

in a tumor can up-regulate COX-2 protein expression, thus induce the expression of VEGF-C, and thereby promote angiogenesis in the tumor, providing a favorable micro-environment for tumor cell proliferation, invasion, and metastasis. On the contrary, inhibiting COX-2 can down-regulate the expression of the VEGF-C.¹⁹ PCNA, also known as cyclin, existing in the nucleus, is an auxiliary protein for DNA polymerase δ and ϵ . The synthesis and expression of PCNA are closely related to cell proliferation, considered as a major biological indicator of cell proliferation.^{23, 24} High expression of PCNA has been observed in many tumors—the more active the tumor cell proliferation is, the higher the PCNA expression can be.^{25, 26} PCNA can also be used to determine malignancy and prognosis of tumors.^{27, 28} It coordinates the synthesis of leading strands and lagging strands in DNA. The intracellular content of PCNA changes cyclically—PCNA exists in small amount at the G₀ phase, begins to increase during late G₁ phase, peaks at S phase, and declines significantly during G₂ and M phases, which plays an important role in DNA replication, cell proliferation, and regulation of cell cycle. The cyclical change of PCNA is relevant to the process of cell proliferation, and tumorigenesis is relevant to block of apoptosis and excessive proliferation.²⁹ PCNA would interact with DNA polymerase, which pushes the cells into S phase and leads to excessive proliferation and cancerization of cells.

The results of this study demonstrated that the relative gene and protein expression levels of VEGF-C, COX-2, and PCNA were significantly down-regulated, indicating that PDT might inhibit QBC939 cell growth by down-regulating VEGF-C, COX-2, and PCNA expressions and thereby inducing apoptosis of cholangiocarcinoma cells. It is known that apoptosis is induced *via* two routes including mitochondrial pathway and death receptor pathway.³⁰ Nuclear factor-kappa B (NF- κ B) signaling pathway, up-regulation of proapoptotic proteins, transcription factors and cytokines, and down-regulation of the expression of anti-apoptotic molecules and adhesion molecules are involved in the anti-tumor mechanisms of death receptor pathway.^{31, 32} A significant positive correlation was found between the expression of COX-2 and NF- κ Bp65. Based on the fact that photodynamic effect down-regulates COX-2 expression in this study, it is speculated that photodynamic effect can promote apoptosis by impacting NF- κ Bp65 pathway and thereby down-regulating COX-2 expression in cholangiocarcinoma cells. It has been demonstrated that down-regulation of PCNA expression in tumor cells can delay transition from G₁ to S phase and thus reduce the content of cells in S phase. Its specific mechanism is that since PCNA-P21 can form quaternary complexes with a

variety of cyclin dependent kinase (CDK)/cyclin,²⁵ CDK activity would be down-regulated when PCNA expression was suppressed. The down-regulation of CDK activity would affect the phosphorylation of retinoblastoma proteins and the release of transcription factors that bind to them, which results in the failure for cells to pass the G₁/S phase checkpoint, and thereby induces cell apoptosis and inhibits cell proliferation. It has proven PCNA to be an excellent indicator for the detection of apoptosis. PCNA expression is significantly down-regulated in the process of tumor cell apoptosis.³³ This study revealed that PDT inhibited PCNA expression, so we considered that PDT might induce QBC939 cells apoptosis by interfering with the cell cycle. In addition, PDT can suppress the *in vitro* invasion ability of QBC939 cells, the mechanism of which might be relevant to down-regulation of VEGF-C expression.

In summary, PDT can promote apoptosis and inhibit invasion of cholangiocarcinoma cells QBC939. The detailed signaling cascades and underlying molecular mechanisms remain unclear. The further study is needed to explore the mechanisms involved.

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