Original Article

Radioactive ^{125}I Seed Inhibits Cell Migration and Invasion and Promotes Apoptosis by Inactivating the VEGFR2 Signaling Pathway in Cholangiocarcinoma

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Abstract

Objectives: To investigate the potential mechanisms of ^{125}I seed implantation therapeutic treatment on inactivating the VEGFR2/PI3K/AKT pathway in cholangiocarcinoma.

Methods: The human cholangiocarcinoma cell lines HCCC-9810 and HuCCT1 were purchased for in vitro studies. The BALB/c nude mice were obtained for in vivo studies. The proliferation of cells was detected by CCK-8, colony formation, and BrdU staining. The migration and invasion of cells were determined by wound healing assay and Transwell assay, respectively. Hematoxylin and eosin staining was utilized for histological evaluation. Protein expression was determined by western blotting and immunohistochemistry.

Results: Compared with the control group, .6 mCi group and .8 mCi group inhibited cholangiocarcinoma cells proliferation, invasion, migration, and promoted apoptosis, the protein expression of p-VEGFR2, VEGFR2, PI3K, p-AKT/AKT, cyclin B1, cyclin A, CDK1, and Bcl-2 was decreased. Similar results were obtained from in vitro experiments. However, when VEGF is overexpressed, the inhibitory effect of .8 mCi was partially significantly reversed on cholangiocarcinoma cells. The in vivo studies further confirmed the inhibitory effects of .6 mCi group and .8 mCi group on cholangiocarcinoma.

Conclusion: ^{125}I seed irradiation could inhibit cholangiocarcinoma cells proliferation, migration, and invasion and promote apoptosis through inactivation of the VEGFR2/PI3K/AKT signaling pathway.

Keywords

radioactive ^{125}I seed, cholangiocarcinoma, invasion, apoptosis, VEGFR2

Introduction

The incidence and mortality rate of cholangiocarcinoma was gradually increased during the past 40 years.[1] Early cholangiocarcinoma is mostly asymptomatic, resulting in the high percentage of diagnosis in the advanced stage and compromised therapeutic methods.[2] Surgical resection, served as the main potential treatment for patients with cholangiocarcinoma, might be led to high rates of recurrence or metastasis (>70%).[3] Moreover, platinum with gemcitabine treatment only provided patients with limited

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survival benefit.[4] Therefore, effective treatments were needed to be investigated for improving the prognostic of patients with cholangiocarcinoma.

Recently, ^{125}I seed implantation has become an effective method for tumor treatment, whose main anti-tumor mechanism is to promote apoptosis and inhibit proliferation of cancer cells.[5] Clinical studies reported that ^{125}I seeds implantation was effective in cholangiocarcinoma, whereas its specific mechanism has not been fully investigated.[6] A recent study indicated that anlotinib had the ability to inhibit the proliferation and invasion of tumor cells in intrahepatic cholangiocarcinoma through downregulating the phosphorylation of VEGFR2 and inactivating of PI3K/AKT signaling, which further promoted apoptosis *via* cell-cycle arrestment.[3] ^{125}I seeds could also inhibit the growth, migration, and invasion of cancer cells by inactivating VEGF-A/ERK signaling in nasopharyngeal carcinoma.[7] Moreover, lobaplatin could enhance the effects of ^{125}I seeds on promoting apoptosis and inhibiting proliferation of cancer cells through suppressing the AKT/mTOR pathway in non-small cell lung cancer.[8] However, the molecular mechanism of ^{125}I seed implantation in the treatment of cholangiocarcinoma has not been comprehensively elucidated.

In the present study, we hypothesized that ^{125}I seed implantation could inhibit proliferation, migration, and invasion of cancer cell and promote cell apoptosis in cholangiocarcinoma through inactivating the VEGFR2/PI3K/AKT pathway.

Methods

Cell Culture

The human cholangiocarcinoma cell lines HCCC-9810 and HuCCT1 were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China for in vitro experiments. The cells were grown in an RPMI-1640 medium (Invitrogen, Waltham, MA, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/mL) (Invitrogen, Waltham, MA, USA) in a 37°C humidified incubator with 5% CO_2.

The ^{125}I seeds (4.5 mm long, .8 mm diameter) and the related implantation instrument were obtained from Junan Pharmaceutical Technology Company, Ningbo, China, and the experiments were performed according to the manufacturer's instructions. Totally two kinds of seeds with different apparent radio activities were included: .6 mCi/seed and .8 mCi/seed. The average energy of ^{125}I was ranged from 27.2 to 34.6 keV, and the half-life of ^{125}I was around 59.5 days. The apparent activity of the seeds was tested for experimental confirmation.[9] Cells were then divided into 4 groups: the control group, the sham group with sham seed treatment, the .6 mCi group with .6 mCi/seed irradiation treatment, and the .8 mCi group with .8 mCi/seed irradiation treatment.

For the construction of overexpression models of VEGF, the lentivirus expression system was prepared by Hanbio Biotechnology, Shanghai, China. The vectors were transfected into 293T cells using Lipo2000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocols. The HCCC-9810 and HuCCT1 cells were transduced with lentivirus at a multiplicity of infection of 100. The successfully transfected cells were screened for the subsequent experiments.[10]

Animal Model

A total of 24 male BALB/c nude mice (4–5 weeks old) were purchased from Taconic Biosciences, Leverkusen, Germany. The mice were housed under environmentally controlled conditions (50 ± 10% humidity, 18–23°C) with 12-h light/dark cycle and free access to food and water. Patient-derived xenograft (PDX) models were constructed according to the previous studies.[11,12] The ^{125}I seeds were obtained as described above. When the tumor volume reached around 200 mm^3, the mice were randomly divided into 4 groups (n = 6 in each group): the control group, the sham group with sham seed implantation, the .6 mCi group with .6 mCi/seed implantation, and the .8 mCi group with .8 mCi/seed implantation. After 3 weeks of intervention, the mice were sacrificed using dislocation, and the tumor tissues were harvested for the subsequent experiments. All experiments were approved by the Research Ethical Committee of Zhejiang Cancer Hospital.

Hematoxylin and Eosin (H&E) Staining

According to the H&E staining kit (C0105S, Boyetime, China) manufacturer's instructions, tumor tissues were fixed in 4% paraformaldehyde for 24 h. After paraffin imbedding, the tissues were sliced into 4 μm-thick sections. The sections were dehydrated with gradient ethanol, and then stained with hematoxylin for 5 min. After being differentiated in 1% hydrochloric acid alcohol for 2 s, the sections were then incubated in ammonia water, followed by the staining with eosin. Ultimately, the sections were dehydrated, cleared, mounted with neutral resin, and observed under a light microscope (Olympus, Japan).

BrdU Staining

When confluence of cells reached 90%, the culture medium was removed, and cells were washed thrice with PBS. Cell proliferation was measured using the BrdU cell proliferation kits (6813S, Cell Signaling, USA) in accordance with the manufacturer's instructions. The cells were stained with DAPI for nuclear counterstaining, and immunofluorescent photographs were captured under an inverted microscope (Olympus, Tokyo, Japan). Cell counts were performed on randomly selected areas at ×200 magnification. The proliferation was evaluated by the number of BrdU-positive cells against the total cell number.

Immunohistochemistry

The expression of KI67 was detected by Immunohistochemistry. The sections were isolated from tumor tissues and then treated with conventional deparaffinization, rehydration, and blocking of endogenous peroxidase activity for 15 min. Sections were pretreated for the purpose of antigen retrieval by microwaving, and then washed with PBS. Sections were incubated for 2 h at room temperature with anti-KI67 (ab279653, Abcam, USA), after which sections were rinsed with PBS, developed with DAB, counterstained with hematoxylin, cleared with xylene, and observed under a light microscope. A mean percentage of positive tumor cells was determined in at least five areas at × 400 and assigned to one of five of categories: (a) 0. < 5%; (b) 1. 5–25%; (c) 2. 25–50%; (d) 3. 50–75%; and (e) 4. > 75%. According to cell staining intensity score: (a) cell no color, 0 points; (b) straw colored, 1 point; (c) brownish-yellow, 2 points; and (d) tan, 3 points. Divided into 4 levels based on cell staining intensity score: (a) negative, 0-1points; (b) weak positive, 2-3 points; (c) positive, 4-5 points; (d) string positive, 6-7 points.

TUNEL Staining

Tumor specimens were subjected to a TUNEL assay using the In Situ Cell Death Detection kit (Roche, Basel, Switzerland), according to manufacturer's instructions for detecting apoptosis. As noted above, the fixed tissues were incubated with 100 μL Proteinase K for 30 min at 37°C. Slides were rinsed twice with PBS. TUNEL reaction mixture was added to the sample at 37°C and incubated for 60 min. Converter-POD solution was added to the sample at 37°C and incubated for 30 min. The results were analyzed under an Olympus BX51TPHD-J11 microscope (Tokyo, Japan). The apoptotic rate was determined as the percentage of TUNEL positive cells to overall tumor cells. The analysis software (Image Pro Plus, Media Cybernetics, USA) was used for image and data acquisition and analysis.

Transwell Assay

Transwell assays were performed using 24-well transwell permeable supports with Matrigel coating (Corning, New York, USA). Briefly, cell suspensions were obtained 24 hours after irradiation. Then, 100 μl containing 10^6cells in serum-free RPMI 1640 media were added to the upper chamber and 500 μl RPMI 1640 media with 10% FBS was added to the lower chamber. Cells were incubated for 48 hours at 37°C, and the membrane was stained with crystal violet to calculate the average number of migrated cells. To investigate the effect of VEGF-A on migration, the growth factor was added (20 ng/mL) prior to irradiation, and cells were harvested 24 hours later for transwell assays.

Wound Healing Assay

Cells were cultured with FBS-free tumor supernatant in 6-well plates and were incubated until they reached 90% confluence. A scratch was created across the center of the cell layer using a sterile 100-μl pipette tip. After 24 h, photographs were taken under the microscope, and cell migration distance was calculated using Image J software.

Colony Formation

For the colony formation assay, cells were placed into 6-well plates at a density of 1000 cells/well and incubated under different treatment. After staining with .1% crystal violet for 30 min, the colonies were visualized and quantified.

Western Blotting

Tumor tissues were ground in liquid nitrogen and treated with RIPA for 15~30 min. Then the tissues or cells were ultrasonicated at 5 s × 4 times and centrifuged at 4°C and 10 000×g for 15 min. The supernatant was stored at −20°C. The protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membrane. After block by 5% skim milk for 2 h, the membrane was incubated in primary antibody (1:1000) at 4°C overnight. Next, the membrane was further incubated in secondary antibody (1:1000) at room temperature for 30 min. After washing with phosphate-buffered saline/Tween, the membrane was mixed with a chemiluminescent agent for 1 min and developed. Protein image processing system software and Quantity One software were adopted for scanning and calculation. Antibody: anti-p-VEGFR2 (ab38473, Abcam, USA); anti-VEGFR2 (ab11939, Abcam, USA); anti-PI3K (12402S, Cell Signaling, USA); anti-p-AKT (4060S, Cell Signaling, USA); anti-AKT(9272S, Cell Signaling, USA); anti-Cyclin B1 (ab181593, Abcam, USA); anti-Cyclin A(ab185619, Abcam, USA); anti-CDK1(ab32094, Abcam, USA); anti-Bax (ab32053, Abcam, USA); anti-Bcl-2(ab182858, Abcam, USA); and anti-GADPH(ab9485, Abcam, USA).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 7. The results were presented as mean ± standard deviation values. Student's *t* tests were used for comparisons between two groups. One way ANOVA analysis was performed for comparisons among multiple groups. *P*-values <.05 were considered to indicate statistically significant results. All triplicate results were quantifications of independent experiments.

Results

^{125}I Seed Irradiation Inhibits Cholangiocarcinoma Cells Proliferation

Cell proliferation was determined to evaluate the effects of ^{125}I seed irradiation on the progression of cholangiocarcinoma.

As presented in Figure 1A, the CCK8 assay revealed that the application of ^{125}I seed irradiation significantly decreased the proliferation of both HCCC-9810 cells and HuCCT1 cells in a dose-dependent manner. Consistently, the BrdU staining results in Figure 1B and colony formation data in Figure 1C also indicated that the proliferation of HCCC-9810 cells and HuCCT1 cells was suppressed by ^{125}I seed irradiation in a dose-dependent manner.

^{125}I Seed Irradiation Inhibits Cholangiocarcinoma Cells Invasion and Migration and Promotes Apoptosis

The invasion of cholangiocarcinoma cells was detected using Transwell assay. As shown in Figure 2A, the invasion ability of HCCC-9810 cells and HuCCT1 cells was dramatically decreased after ^{125}I seed irradiation, while .8 mCi had the strongest inhibitory effect compared with .6 mCi. Wound healing assay (Figure 2B) revealed that ^{125}I seed irradiation inhibited cell migration ability in a dose-dependent manner. TUNEL assay showed that implantation of ^{125}I seed promoted cell apoptosis (Figure 2C).

^{125}I Seed Irradiation Inhibited the VEGFR2/PI3K/AKT Signaling Pathway

Western blotting analysis (Figure 3) suggested that ^{125}I seed irradiation downregulated the phosphorylation level of VEGFR2 and AKT, indicating that the VEGFR2/PI3K/AKT signaling pathway was inhibited. Moreover, the protein levels of cell cycle–related proteins that included CDK1, cyclin A, and cyclin B1 were also decreased after implantation of ^{125}I seed, which revealed that the cell cycle was arrested. The apoptosis-related proteins bcl-2 was downregulated and bax was upregulated when

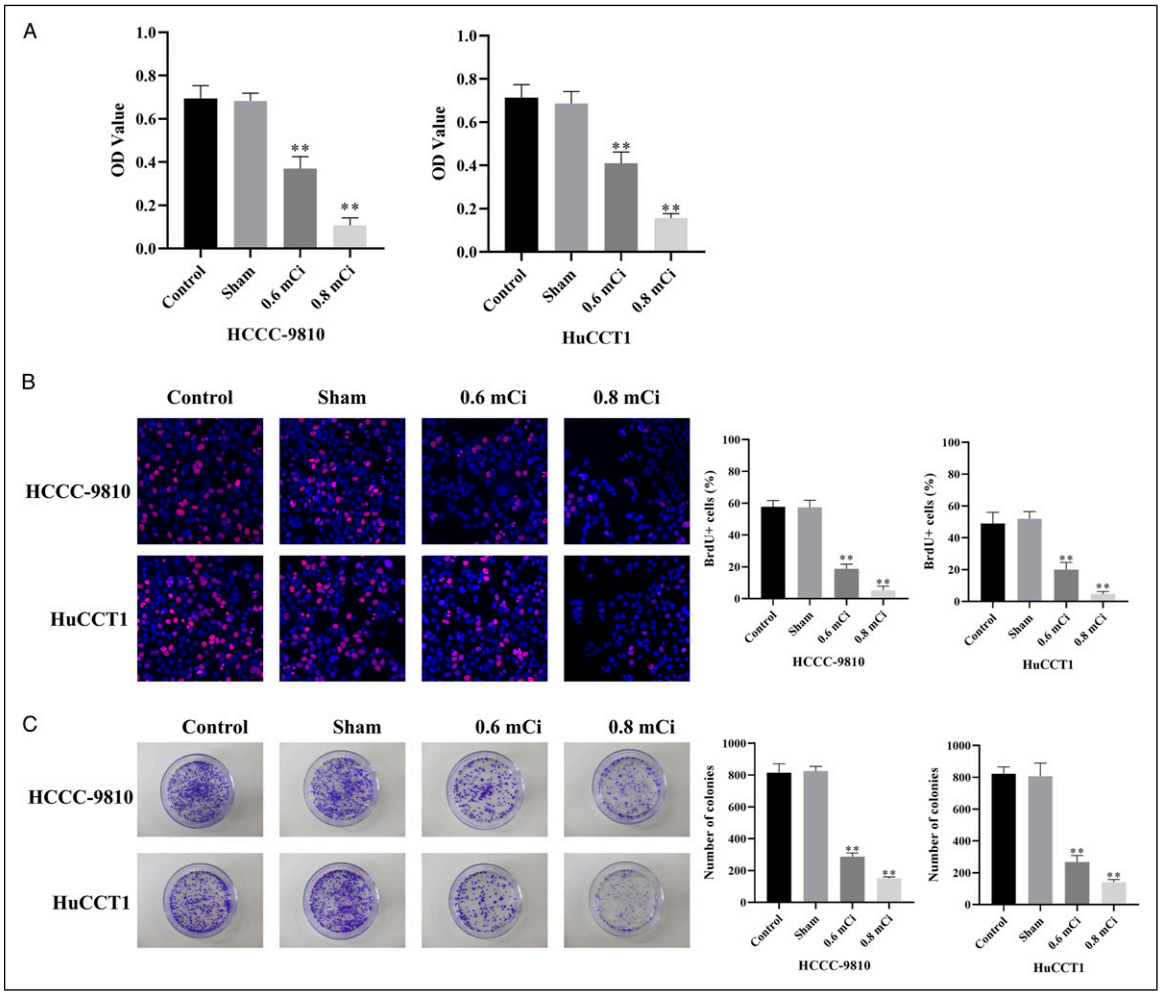

Figure 1. Effects of ^{125}I seed irradiation on the proliferation of HCCC-9810 cells and HuCCT1 cells. (A) The cell proliferation detected by CCK-8 assay. (B) The BrdU staining results, scale bar = 100 μm. (C) The results of colony formation analysis. *$P < .05$ vs control group, **$P < .01$ vs control group.

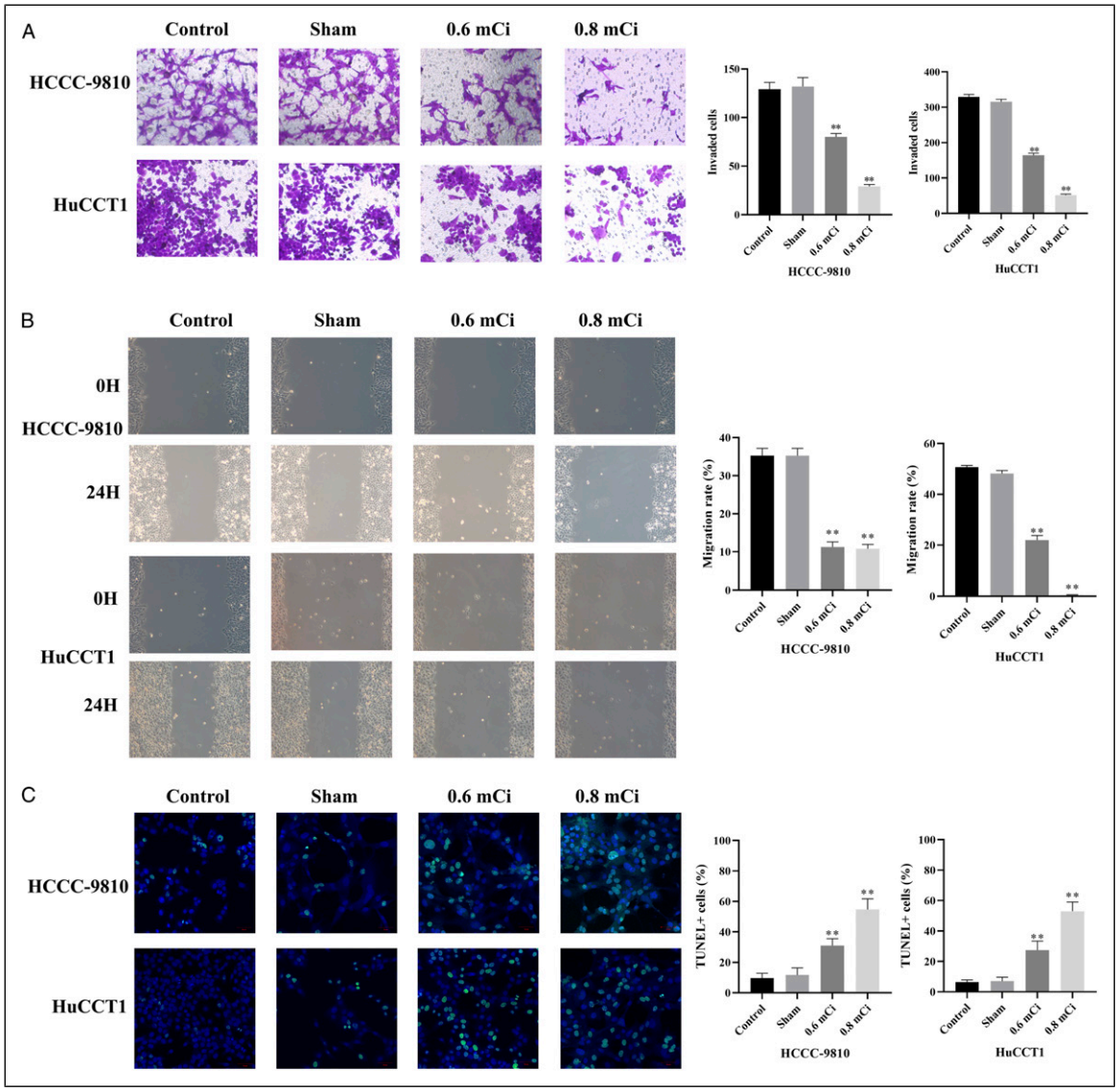

Figure 2. Effects of ^{125}I seed irradiation on the invasion, migration, and apoptosis of HCCC-9810 cells and HuCCT1 cells. (A) The migration of cells determined by Transwell assay. (B) The wound healing results revealed the cell migration ability. (C) The cell apoptosis measured by TUNEL staining, scale bar = 100 μm. *P < .05 vs control group, **P < .01 vs control group.

HCCC-9810 cells and HuCCT1 cells were treated with ^{125}I seed irradiation.

^{125}I Seed Irradiation Inhibits Cholangiocarcinoma Cells Proliferation, Migration, and Invasion and Promotes Apoptosis by Inactivating the VEGFR2/PI3K/AKT Signaling Pathway

For further investigating the molecular pathway of ^{125}I seed irradiation in cholangiocarcinoma cells, the expression of VEGF was overexpressed using siVEGFR2 in HCCC-9810 cells and HuCCT1 cells. As presented in Figure 4A, overexpression of VEGF could significantly reverse the inhibitory effects of ^{125}I seed irradiation on cell proliferation. Wound healing assay (Figure 4B) and Transwell analysis (Figure 4C) also demonstrated that the ^{125}I seed-suppressed migration and invasion ability could be reversed by overexpression of VEGF. Furthermore, the TUNEL staining showed that the apoptosis was promoted after ^{25}I seed irradiation (Figure 4D), whereas overexpression of VEGF had the reversed effects. Western blotting results (Figure 5) showed that overexpression of VEGF in ^{125}I seed irradiation group could upregulate the phosphorylation level of VEGFR2 and AKT, increase the protein levels of cell cycle–related proteins that included CDK1, cyclin A, and cyclin B1, and increase the expression of bcl-2 and inhibit the expression of bax. These data suggested that ^{25}I seed irradiation might exhibit its effects on inhibiting cholangiocarcinoma cells proliferation, migration, and invasion

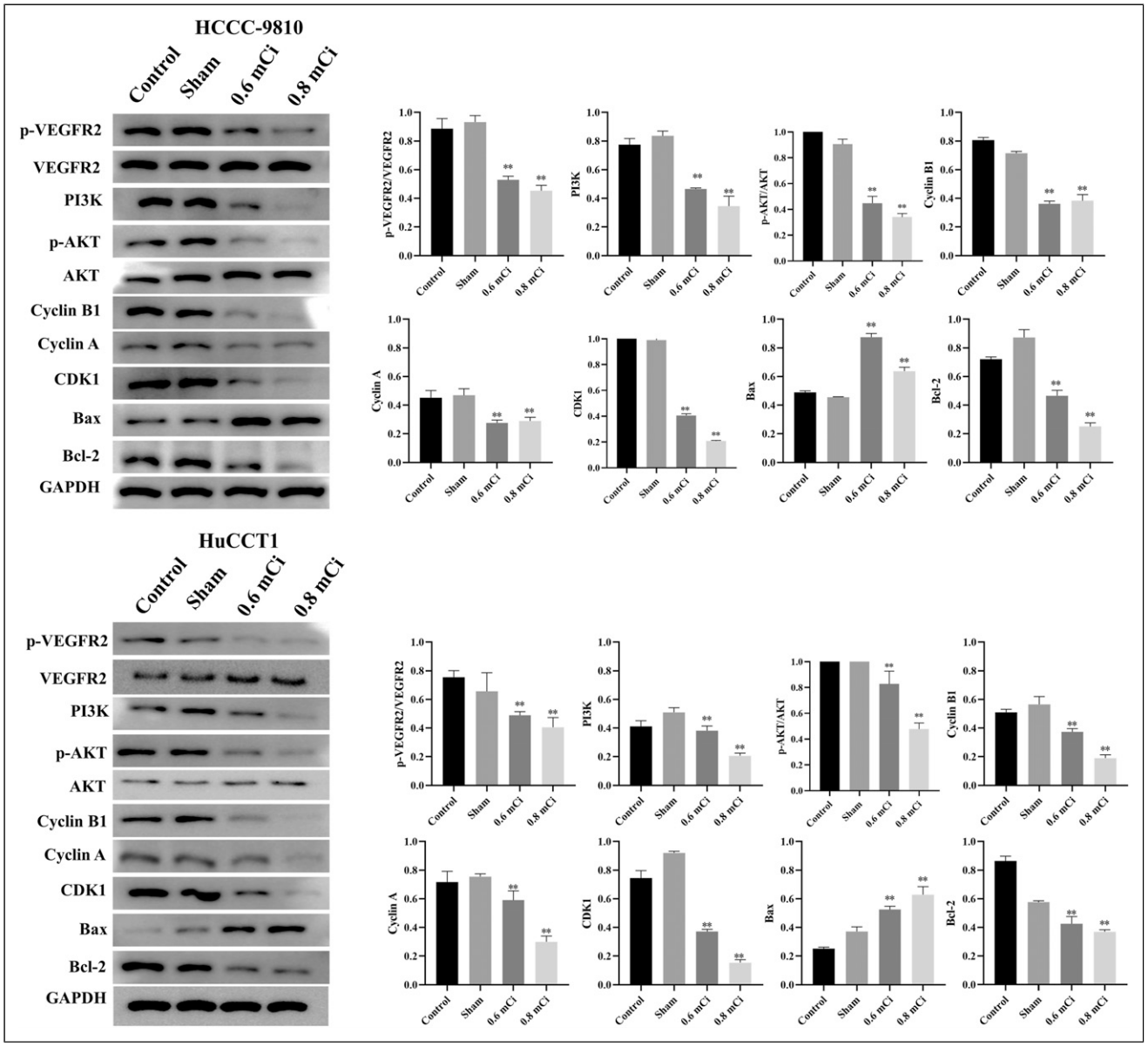

Figure 3. Western blotting analysis demonstrated that ^{125}I seed irradiation inhibited the protein expression of the VEGFR2/PI3K/AKT signaling pathway, cell cycle–related proteins and increased apoptosis-related proteins. The relative protein expression was calculated via normalization to GAPDH. *P < .05 vs control group, **P < .01 vs control group. #P < .05 vs oe-VEGF group. ##P < .01 vs oe-VEGF group.

and promoting apoptosis through the VEGFR2/PI3K/AKT signaling pathway.

^{125}I Irradiation Reduced Xenograft Tumor Growth in Vivo

The animal experiments were conducted for the verification of effects of ^{125}I seed irradiation in vivo. The tumor growth was significantly inhibited by ^{125}I seed irradiation in a dose-dependent manner (Figure 6A). Consistently, immunohistochemistry analysis revealed that the expression of KI67 was dramatically decreased by ^{125}I seed irradiation (Figure 6B). H&E staining demonstrated that the histological conditions were improved when mice were treated with ^{125}I seed (Figure 6C). Moreover, the apoptosis in mice tumor tissues was significantly promoted after implantation of ^{25}I seed (Figure 6D). The phosphorylation level of VEGFR2 and AKT was downregulated, the protein levels of cell cycle–related proteins that included CDK1, cyclin A, and cyclin B1 were decreased, the apoptosis-related proteins bcl-2 was down-regulated and bax was upregulated in mice tumor tissues after ^{125}I seed irradiation treatment (Figure 6E).

Discussion

Our in vitro and in vivo experiments have shown that: ^{125}I seed irradiation could inhibit cholangiocarcinoma cells

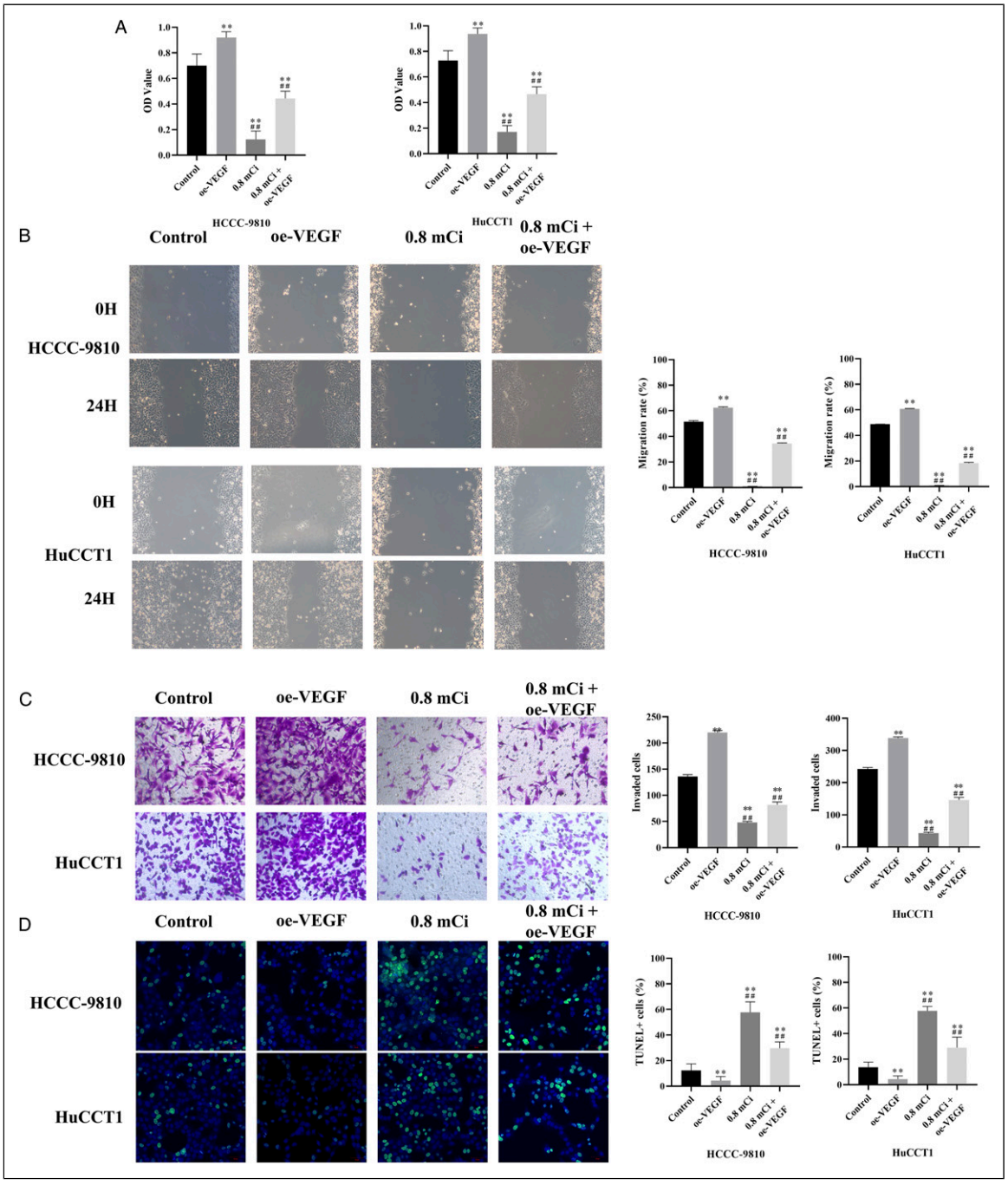

Figure 4. Activating the VEGFR2/PI3K/AKT signaling pathway reversed the effects of ^{125}I seed irradiation. (A) The cell proliferation detected by CCK-8 assay. (B) The wound healing results revealed the cell migration ability. (C) The migration of cells determined by Transwell assay. (D) The cell apoptosis measured by TUNEL staining, scale bar = 100 μm. *P < .05 vs control group, **P < .01 vs control group.

proliferation, migration, and invasion and promote apoptosis through inactivation of the VEGFR2/PI3K/AKT signaling pathway.

In the past few decades, major progress has been made in surgery, radiotherapy, and chemotherapy, however, the survival rate of patients with advanced cancers has not been significantly improved.[13] Recently, the inhibitory effects of ^{125}I seed on tumor growth have been investigated, and ^{125}I seed irradiation has gradually been regarded as an alternative therapeutic method for advanced cancers.[14] Wang et al. compared the effects of ^{125}I seeds radiation and 60Co γ-ray radiation on non–small-cell lung cancer cells, they found that the tumor growth was remarkably inhibited after ^{125}I seeds radiation and the apoptosis-related proteins that included

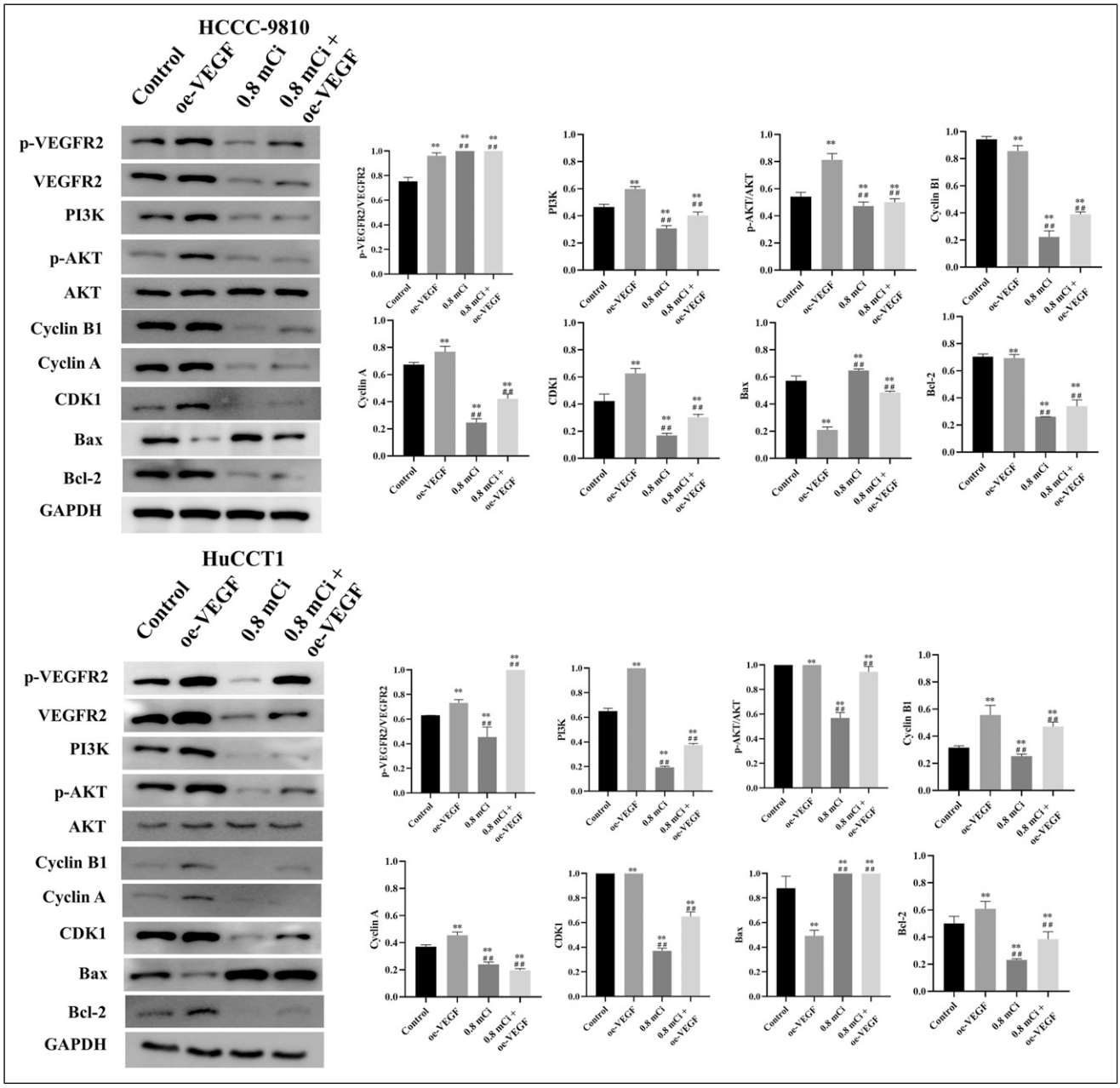

Figure 5. Figure 5. **Western blotting demonstrated the effects of activating the VEGFR2/PI3K/AKT signaling pathway on the expression of cell cycle–related proteins and apoptosis-related proteins.** The relative protein expression was calculated via normalization to GAPDH. *$P < .05$ vs control group, **$P < .01$ vs control group.

caspase-3 and bax were upregulated.[15] Zhang et al. reported that ^{125}I radioactive seeds brachytherapy might increase the efficacy of therapeutic treatment and reduce the mortality rate compared with chemotherapy, improving the overall survival of patients with advanced non–small-cell lung cancer.[16]

With respect to the clinical treatment of cholangiocarcinoma, the second most common hepatic malignancy, novel silicone-coated ^{125}I seeds for extrahepatic methods as well as novel brachytherapy drainage tube loaded with double ^{125}I strands were investigated and applicated.[17,18] In the present study, we similarly found that the ^{125}I seed irradiation could significantly suppress cholangiocarcinoma cells proliferation, migration, and invasion. Moreover, caspase-3 expression and apoptosis index were reported to be increased in hepatocellular carcinoma after ^{125}I interstitial brachytherapy.[19] Wang et al. also showed that ^{125}I seed radiation induced damage of DNA, caused cell cycle arresting in G2/M stage, and contributed to reactive oxygen species-mediated apoptosis and autophagy in human esophageal squamous cell carcinoma cells.[20] Consistently, we found that

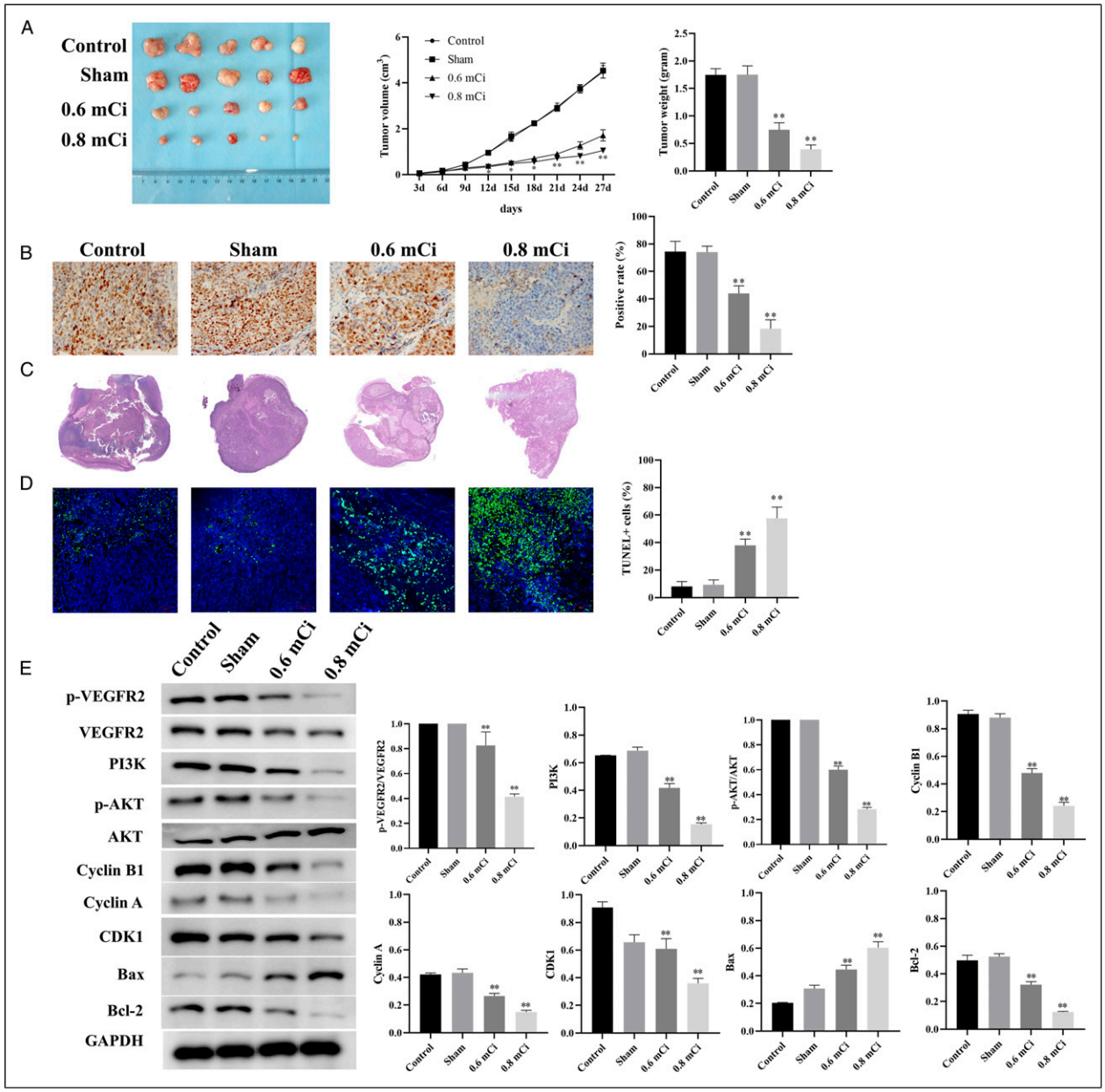

Figure 6. Effects of ^{125}I seed irradiation on the tumor growth and apoptosis in vivo. (A) The tumor volume detected after ^{125}I seed irradiation. (B) The expression of KI67 determined by immunohistochemistry analysis. (C) The histological conditions revealed by H&E staining. (D) The apoptosis cells measured by TUNEL staining, scale bar = 100 μm. (E) Protein expression detected by western blotting. The relative protein expression was calculated via normalization to GAPDH. *P < .05 vs control group, **P < .01 vs control group.

the apoptosis-related proteins were upregulated, and the cell apoptotic rate was increased after ^{125}I seed irradiation. We also showed that the cell cycle–related proteins including CDK1, cyclin A, and cyclin B1 were all downregulated by ^{125}I seed irradiation.

Several studies indicated that the VEGFR2/PI3K/AKT pathway was related with cell proliferation, cell cycle progression, and cell apoptosis.[21,22] Karar et al. revealed that hypoxia-induced hypoxia-inducible factor 1 increasing would lead to the upregulation of VEGF, which further activated PI3K and promoted the phosphorylation of AKT, finally leading to the promoted angiogenesis and cell migration.[23] Bromodomain, an emerge therapeutic targets for cancers, were reported to decrease VEGF receptor 2 phosphorylation, reduced PI3K/AKT pathway expression, and induced apoptosis of glioma stem cells.[24] Tian et al. demonstrated that radioactive ^{125}I seed had the ability to inhibit cell growth and migration of nasopharyngeal carcinoma through inducing DNA damage and inactivating expression of VEGFA.[7] In cholangiocarcinoma, we elucidated that the phosphorylation

level of VEGFR2 and AKT was downregulated by ^{125}I seed irradiation. To verify the role of VEGFR2, the overexpression model was constructed, and the data showed that overexpression of VEGF could significantly reverse the phenomenon observed after implantation of ^{125}I seed, revealing that ^{125}I seed irradiation might exhibit its cancer inhibitory effects through suppressing the VEGFR2/PI3K/AKT signaling pathway. Our study also has limitations, such as overexpressing VEGF without directly altering the expression of VEGFR2. In addition, animal experiments did not involve overexpression of VEGF. In subsequent experiments, we will improve the shortcomings.

In conclusion, the present study indicated that ^{125}I seed irradiation could inhibit cholangiocarcinoma cells proliferation, migration, and invasion and promote apoptosis through inactivation of the VEGFR2/PI3K/AKT signaling pathway. Therefore, ^{125}I seed irradiation might be served as an effective therapeutic method for the clinical treatment of cholangiocarcinoma. However, the comprehensive understanding of molecular pathway regulations of ^{125}I seed irradiation in cholangiocarcinoma cells needs further investigations using transcriptomes or proteomes. Clinical studies are also needed to verify and strengthen the clinical usage of ^{125}I seed irradiation.

Authors Contribution

GS contributed to the conception of the study, JL performed the experiment and contributed significantly to analysis and manuscript preparation, JZ and HY performed the data analyses and wrote the manuscript, BW and ZZ helped perform the analysis with constructive discussions.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

All procedures performed in studies involving animals were approved by the Research Ethical Committee of Zhejiang Cancer Hospital.

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Availability of Data and Materials

All data generated or analyzed during this study are available on request to the corresponding author. Sources of the material used in this study are presented in the manuscript.

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