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Iodine-125-induced cholangiocarcinoma cell death is enhanced by inhibition of endoplasmic reticulum stress-mediated protective autophagy

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Cholangiocarcinoma (CCA) is the second most common primary liver malignancy, however, it is difficult to diagnose and treat, and only a few patients with CCA are suitable for surgery. Iodine-125 (I-125) is an effective treatment for cancer, but the molecular mechanisms underlying the effects of I-125 differ among different cancers. This study aimed to explore the effects of I-125 on CCA cell activity and determine the possible mechanisms of action of I-125 in this type of cancer. CCA cell proliferation, cycling, apoptosis, autophagy, and endoplasmic reticulum (ER) stress were determined after irradiation of CCA cells with I-125 seeds. The effects of I-125 on autophagy and ER stress in three CCA cell lines were evaluated using western blotting, while the effects of I-125 on apoptosis and autophagy in QBC939 cells treated with si-Beclin1 or si-PERK, respectively, were assessed using flow cytometry. I-125 suppressed cell viability and induced cell cycle G2/M-phase arrest in three CCA cell lines (QBC939, TFK-1, HuCCT1). I-125 induced apoptosis, autophagy, and ER stress by altering the expression levels of some related proteins in each of the three CCA cell lines. Furthermore, autophagy inhibition (treatment with si-Beclin1) increased expression of apoptosis-related proteins (cleaved-PARP and cleaved-caspase-3, Bax/Bcl2) in QBC939 cells irradiated with I-125 seeds, while ER stress inhibition (with si-PERK) suppressed the expression of autophagy-related proteins (LC3-I, LC3-II, p62). Therefore, I-125 induces ER stress, thereby activating protective autophagy in CCA cells through the PERK signaling pathway. Combined inhibition of ER stress and autophagy signaling may increase the killing effect of I-125 on cancer cells and serve as a new auxiliary method in I-125 radiotherapy.

Key words: cholangiocarcinoma (CCA); autophagy; iodine-125 (I-125); endoplasmic reticulum (ER) stress

Cholangiocarcinoma (CCA) is the second most common primary liver malignancy [1, 2]. According to its initial location, CCA can be divided into two categories, intrahepatic (ICC) and extrahepatic (ECC). CCA is difficult to diagnose and cure, the 5-year survival rate is approximately 5%, and the patients usually die within 6–12 months after diagnosis due to biliary sepsis and liver failure. In Asia, CCA is a relatively common disease with a poor prognosis. Furthermore, CCA is difficult to diagnose and has been associated with high mortality rates in Europe and North America in recent decades [1, 3]. Surgery, radiation therapy, and chemotherapy are used for treating CCA. Surgery is a potential curative treatment option but is not suitable for most patients because of recurrence or metastasis following resection [1].

CCA is less sensitive to chemotherapy compared with other gastrointestinal tumors, such as colon cancer. This may be due to typical characteristics of CCA, such as high levels of fibrosis and profound genetic heterogeneity, leading to drug resistance [2]. Additionally, the role of neoadjuvant and adjuvant therapies in improving the overall prognosis of patients with CCA is limited, but radiotherapy is reported to prolong survival in some cases [4]. Iodine-125 (I-125) radioactive particles (brachytherapy) are recommended as a treatment for localized tumor control and it is a safe and effective method against chemo-refractory and radio-refractory carcinomas [5]. In recent years, I-125 radiation therapy has played a crucial role in the treatment of many types of cancer. I-125 seeds, as implanted brachytherapy radiation particles, can reduce disease lesions and minimize the injury region [6]; for example, these seeds have been applied in early-onset colorectal cancer and for lower urinary tract symptoms and localized prostate cancer [7]. I-125 seed irradiation is also effective against gastric cancer and acts by increasing

the expression of apoptosis and cell cycle-related genes [8]. Hence, the use of I-125 seeds is an important adjuvant anti-cancer therapy for patients with short-term recurrence and is also a promising new treatment for gastric cancer [9–11]. In CCA, I-125 seeds combined with percutaneous transhepatic biliary stenting significantly improved the liver function of patients with an advanced ECC form of the disease and prolonged survival time [12]. However, the functional mechanisms underlying the effects of I-125 in CCA have yet to be elucidated.

Autophagy is an intracellular dynamic metabolic process, and its cytoplasmic target molecules are transferred to lysosomes for degradation and recycling. Beclin 1, p62, and LC3 are commonly used as markers of autophagy flux [13]. Studies have suggested autophagy plays an important role in the energy balance of cancer cells and may regulate the survival mechanism of cancer cells as a tumor promoter or tumor suppressor, and its intervention has become an effective strategy for cancer treatment [14, 15]. Moreover, autophagy induction or autophagy inhibition has been used for the treatment of CCA in clinical studies, indicating that autophagy is an effective target for CCA therapy [16, 17]. However, the molecular basis of the relationship between autophagy and I-125 seed radiation in CCA has not yet been further studied, to the best of our knowledge. And therefore, the present study aimed to explore the potential role of I-125 against CCA and its possible molecular mechanisms of action.

Materials and methods

Cell culture. Three CCA cell lines were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences. Two of the cell lines, QBC939 (poorly differentiated) and TFK-1 (highly differentiated), were derived from an ECC tumor, while the HuCCT1 cell line (moderately differentiated) was derived from an ICC tumor. All CCA cells were cultured under adherent conditions (Roswell Park Memorial Institute [RPMI]-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37 °C in a 5% CO_2 incubator.

I-125 seed irradiation *in vitro* model. The I-125 seed irradiation model was obtained from Shanghai Xinke Pharmaceutical Co., Ltd. (Shanghai, China). The seeds (diameter, 0.8 mm; length, 4.4 mm) had a 59-day half-life, with 15.1 MBq of surface activity, and average energy of 27.1-35.3 Kev. The *in vitro* I-125 seed irradiation model was used as described previously, nine seeds were confined within a 50 mm polystyrene disc, which was chosen for its approximate tissue equivalence and its very high resistance to radiation damage. Among them, one I-125 seed was confined to the center within a 6 mm diameter hole, and eight I-125 seeds were evenly spaced within recesses around the circumference of a 30 mm diameter × 1 mm thick polystyrene disc. CCA cell lines (5×10^6/cells) were seeded into each culture dish, and the discoid radioactive source was designed to provide a relatively homogeneous dose distribution at the surface of the dish. The activity of single 125I seeds was 0.8 mCi, the corresponding initial dose rate in model cells was 2.77 cGy/h, and the exposure time for delivering cumulative radiation doses of 2–3 Gy is 72.2–108.3 h. Therefore, we set the I-125 seed irradiation group to be continuously exposed to the radiation source for 96 h, while the control group was not exposed to I-125.

Preparation of cells for further experiments. Control and I-125 seed-irradiated cells were digested with trypsin (0.25%), washed with fresh serum-containing medium, washed with phosphate buffer saline (PBS), and then were centrifuged at 500×g for 3 min and the supernatant was discarded. Finally, cells were counted.

Cell viability assay. Irradiated and control CCA cells (QBC939, TFK-1, and HuCCT1) were collected. Cell viability was evaluated using a cell counting kit-8 (CCK-8) (Beyotime, China). Briefly, cells were seeded in 96-well plates (2×10^5/well) in 100 μl media; control wells containing only medium were prepared to measure background luminescence. CCK-8 solution (10 μl) was added to each well and the plate was incubated at 37 °C for 1–4 h in a 5% CO_2 incubator. Then the absorbance at 450 nm was measured by a microplate reader (Molecular Devices, Menlopark, CT, USA).

Cell-cycle analysis by flow cytometry, western blotting. Distribution of the CCA cell cycle in G0/G1, S, and G2/M phases was analyzed by flow cytometry analysis using Vybrant® DyeCycle™ Violet Stain (V35003, Thermo Fisher Scientific, Inc.). Expression levels of the cell cycle-related genes, Cyclin B1, CDC25C, and p21, were detected by western blotting. The primary antibodies used in western blotting were Cyclin B1 (AF6168, Affinity), CDC25C (4688T, Cell Signaling Technology (CST)), p21 (2947T, CST), β-Actin (AB0035, Abways).

Apoptosis analysis by flow cytometry, western blotting. An apoptosis detection kit (C1052, Beyotime) was used for quantitative analysis of the proportion of living and apoptotic cells. Irradiated and control CCA cells were treated with trypsin for 3 min at 37 °C, collected, washed with PBS, and rates of apoptosis were detected using Fluorescein Isothiocyanate (FITC)-Annexin V/Propidium Iodide (PI) staining (C1052, Beyotime) for flow cytometry analysis (BD Biosciences, BD Calibur, USA). Expression levels of the apoptosis-related molecules, BCL-2/BAX, cleaved caspase-3, and cleaved poly ADP-ribose polymerase (PARP) were detected by western blotting. The primary antibodies used in western blotting were BCL-2 (AF6139, Affinity), BAX (AF0120, Affinity), cleaved caspase-3 (9661S, CST), PARP (AF7023, Affinity), β-Actin (AB0035, Abways).

Autophagy analysis by western blotting, RT-qPCR. Plasmid pEGFP-LC3, expressing a green fluorescent protein (GFP)-human LC3 fusion protein, was purchased from Addgene (Cambridge, MA, USA). CCA cells (QBC939, TFK-1, and HuCCT1; 2×10^5/well) were seeded in 6-well

plates, incubated for 24 h, then transfected with 2 mg plasmid using Lipofectamine™ 3000 transfection reagent (Invitrogen, L3000015). GFP fusion proteins were observed under a confocal laser scanning microscope (Leica, TSC-SP5). The percentage of GFP-LC3-positive cells with GFP-LC3 punctuate dots was determined in three independent experiments. Cells with more than five GFP-LC3 punctuate dots were counted under blinded conditions. A minimum of 30 GFP-LC3-positive cells, assessed from at least six random fields per sample, were counted in triplicate per condition in each experiment [18]. Autophagy was evaluated by detecting the expression of GFP-LC3 under a scanning electron microscope. GFP-LC3 was considered to be at the same level as endogenous LC3. Expression levels of the autophagy-related molecules LC3 II/I, p62, and Beclin1 were detected by western blotting. The primary antibodies used in western blotting were LC3 II/I (2775S, CST), p62 (ab91526, Abcam), Beclin1 (AF5128, Abcam), β-Actin (AB0035, Abways). The expression of Beclin1 in QBC939 cell lines after transfection of siRNA-Beclin1 was detected by Real Time Quantitative PCR (RT-qPCR). The primer sequences were as follows: Beclin1, 5'-GGTTGCGGTTTTTCTGGGAC-3' (forward), 5'-ACGTGTCTCGCCTTTCTCAA-3' (reverse); β-Actin, 5'-CTCCATCCTGGCCTCGCTGT-3' (forward), 5'-GCTGTCACCTTCACCGTTCC-3' (reverse).

ER stress analysis by western blotting. Expression levels of the ER stress-related proteins BIP, PERK, eIF2α, ATF4, and CHOP were detected by western blotting. The primary antibodies used in western blotting were BIP (AF5366, Affinity), PERK (5683T, CST), p-PERK (DF7576, Affinity), eIF2α (4688T, CST), p-eIF2α (3398T, CST), ATF4 (ab184909, Abcam), CHOP (AF6277, Affinity), β-Actin (AB0035, Abways).

Statistical analysis. GraphPad Prism 8.0 software was used to process data. All data were presented as mean ± standard deviation (SD) and differences were assessed by Student's t-test or analysis of one-way variance (ANOVA). All experiments were independently measured three times. A p-value ≤0.05 was considered statistically significant.

Results

I-125 inhibited cell viability. Three CCA cell lines, QBC939, TFK-1, and HuCCT1, were evaluated after treatment with (2–3 Gy) or without (0 Gy, control group) I-125 seed irradiation. Cell viability was significantly decreased (QBC939, p=0.0015; TFK-1, p=0.0013; QBC939, p=0.0016) in each of the three CCA cell lines treated with I-125 seed irradiation compared with untreated controls (Figure 1A).

I-125 seeds induced cell-cycle arrest in the G2/M phase. The flow cytometric cell-cycle assay demonstrated that irradiation with I-125 seeds decreased the percentage of CCA cells in the G0/G1 phase of the cell cycle (QBC939, p=0.0306; TFK-1, p=0.0414; HuCCT1, p=0.0377), but increased the percentage of cells in the G2/M phase and induced a delay in the G2/M phase in cells (QBC939, p=0.0307; TFK-1, p=0.0391; HuCCT1, p=0.0149) (Figures 1B, 1C). Furthermore, expression levels of the cell cycle-related proteins,

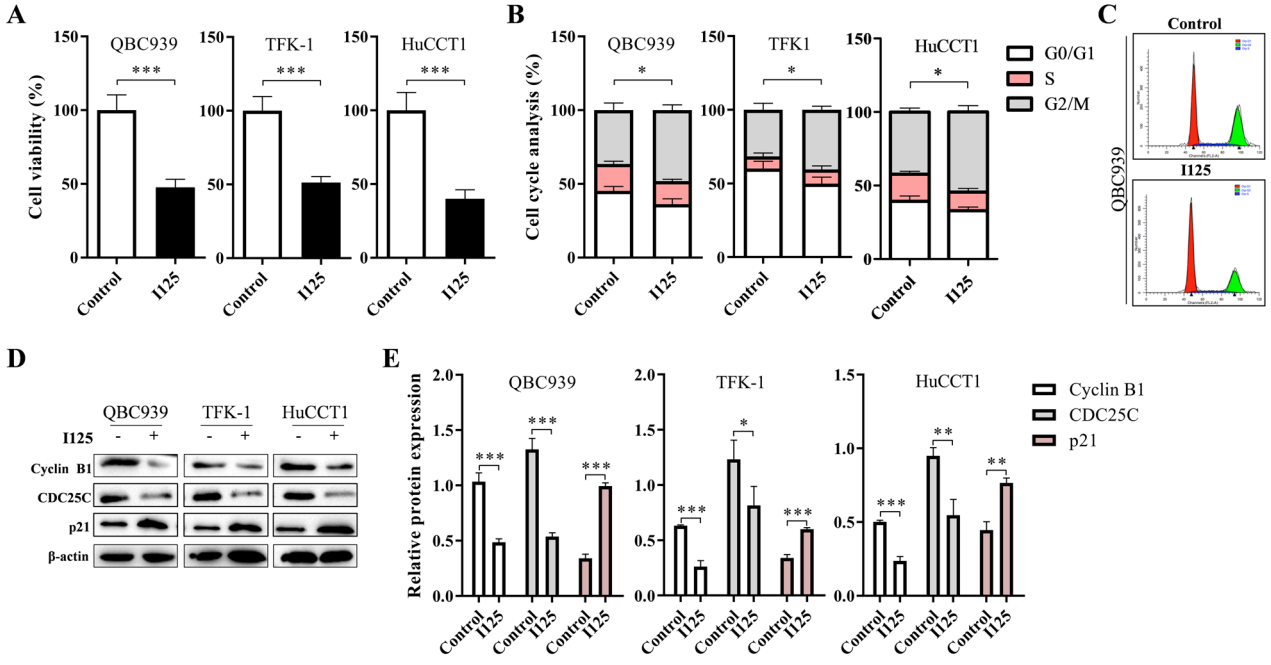

Figure 1. I-125 reduced cell viability and induced cell cycle G2/M arrest in CCA cell lines (QBC939, TFK-1, HuCCT1). Effects of I-125 treatment on: A) cell viability; B, C) cell cycle status; D, E) expression of cell cycle-related proteins. *p<0.05, **p<0.01, and ***p<0.001

Cyclin B1 (QBC939, p=0.0004; TFK-1, p=0.0003; HuCCT1, p=0.0001) and CDC25C (QBC939, p=0.0002; TFK-1, p=0.0416; HuCCT1, p=0.0046), in each of the three CCA cell lines treated with I-125 seed irradiation were significantly lower than those of the untreated group (Figures 1D, 1E). In contrast, an expression level of the cyclin-dependent kinase inhibitor p21 (QBC939, p<0.0001; TFK-1, p=0.0002; HuCCT1, p=0.0012) was significantly increased relative to the untreated group (Figures 1D, 1E).

I-125 seeds induced apoptosis *in vitro*. Apoptosis rates of the three CCA cell lines, examined using FITC-Annexin V-PI staining and a cell apoptosis detection kit, were significantly increased by I-125 seed irradiation compared with the untreated control group (QBC939, p=0.0021; TFK-1, p=0.0153; HuCCT1, p=0.0028) (Figures 2A, 2B). Furthermore, treatment with I-125 seeds reduced protein expression of the apoptosis inhibitor BCL-2 and increased expression of BAX, a pro-apoptosis protein (Figure 2C). In addition, levels of cleaved-PARP (QBC939, p=0.0006; TFK-1, p=0.0003; HuCCT1, p<0.0001) and cleaved-caspase-3 (QBC939, p=0.0001; TFK-1, p=0.0005; HuCCT1, p<0.0001) were higher in the I-125 seed-irradiation group than in the control group, as well as the ratio of Bax/Bcl2 (QBC939, p=0.0003; TFK-1, p<0.0001; HuCCT1, p<0.0001) (Figures 2C, 2D), confirming that I-125 seed irradiation leads to apoptosis of CCA cells through activation of caspase-3 and cleaved-PARP.

I-125 seeds induced autophagy. The percentage of GFP-LC3B-positive cells, as an indicator of autophagosome formation, was evaluated in QBC939, TFK-1, and HuCCT1 cell lines after treatment with I-125 seed irradiation. The percentages of GFP-LC3B-positive cells in all three CCA cells lines treated with I-125 seed irradiation was significantly higher than those of the control groups (QBC939, p<0.0001; TFK-1, p=0.0004; HuCCT1, p=0.0002) (Figures 3A, 3B). Autophagy-related proteins, LC3-I, LC3-II, and Beclin1, were expressed at high levels in all three CCA cell lines after irradiation with I-125 seeds. However, the autophagy-related protein, p62, was inhibited during autophagy activation in the treated groups of cells (QBC939, p<0.0001; TFK-1, p<0.0001; HuCCT1, p<0.0001) (Figures 3C, 3D). Western blotting and quantitative real-time PCR analysis revealed significantly increased expression of LC3-II/I (QBC939, p=0.0007; TFK-1, p<0.0001; HuCCT1, p<0.0001) and Beclin1 (QBC939, p<0.0001; TFK-1, p=0.0065; HuCCT1, p=0.0042) in all three CCA cell lines treated with I-125 seed irradiation (Figures 3C, 3D).

Autophagy inhibition enhanced I-125-induced apoptosis. Beclin1-targeting small interfering RNA (siRNA) (si-Beclin1) suppressed expression levels of the autophagy-

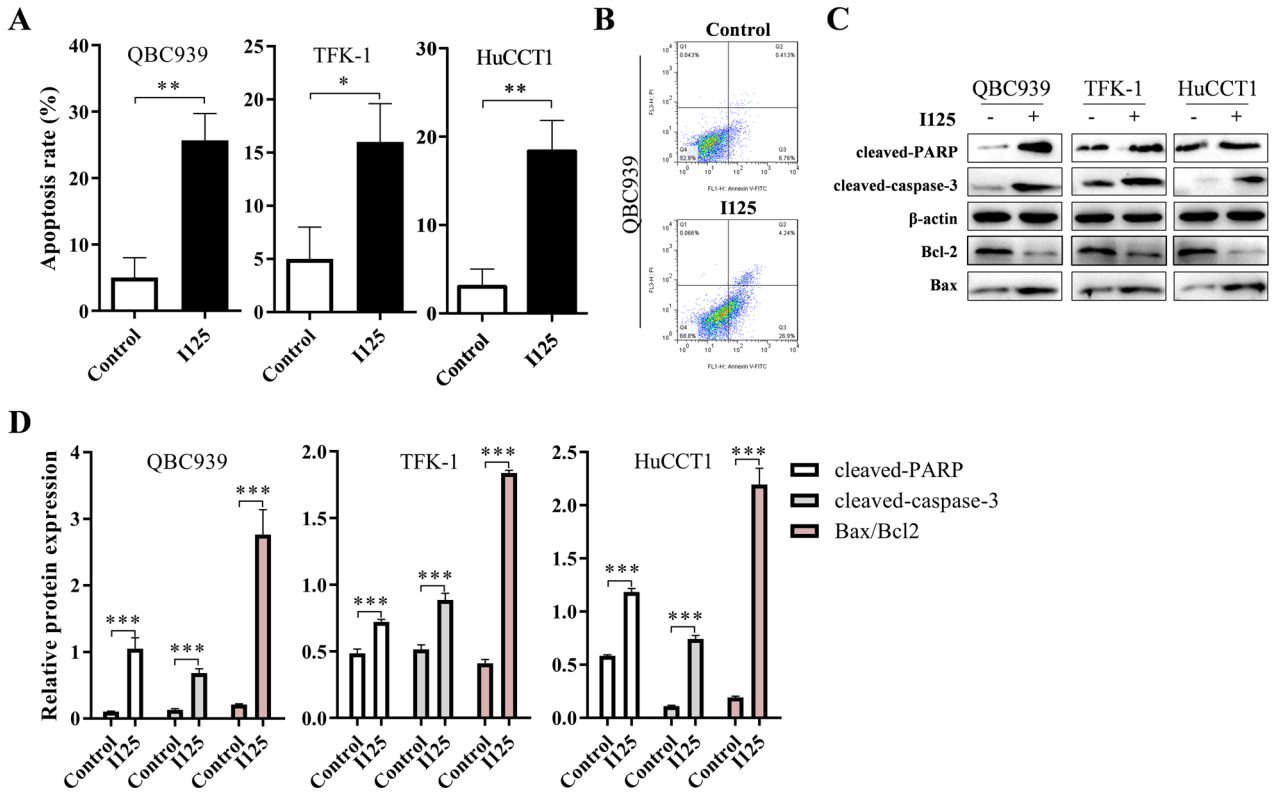

Figure 2. I-125 promoted increased cell death and apoptosis in CCA cell lines (QBC939, TFK-1, HuCCT1). A, B) Detection of apoptosis after I-125 treatment. C, D) Expression of apoptosis-related proteins following I-125 treatment. *p<0.05, **p<0.01, and ***p<0.001

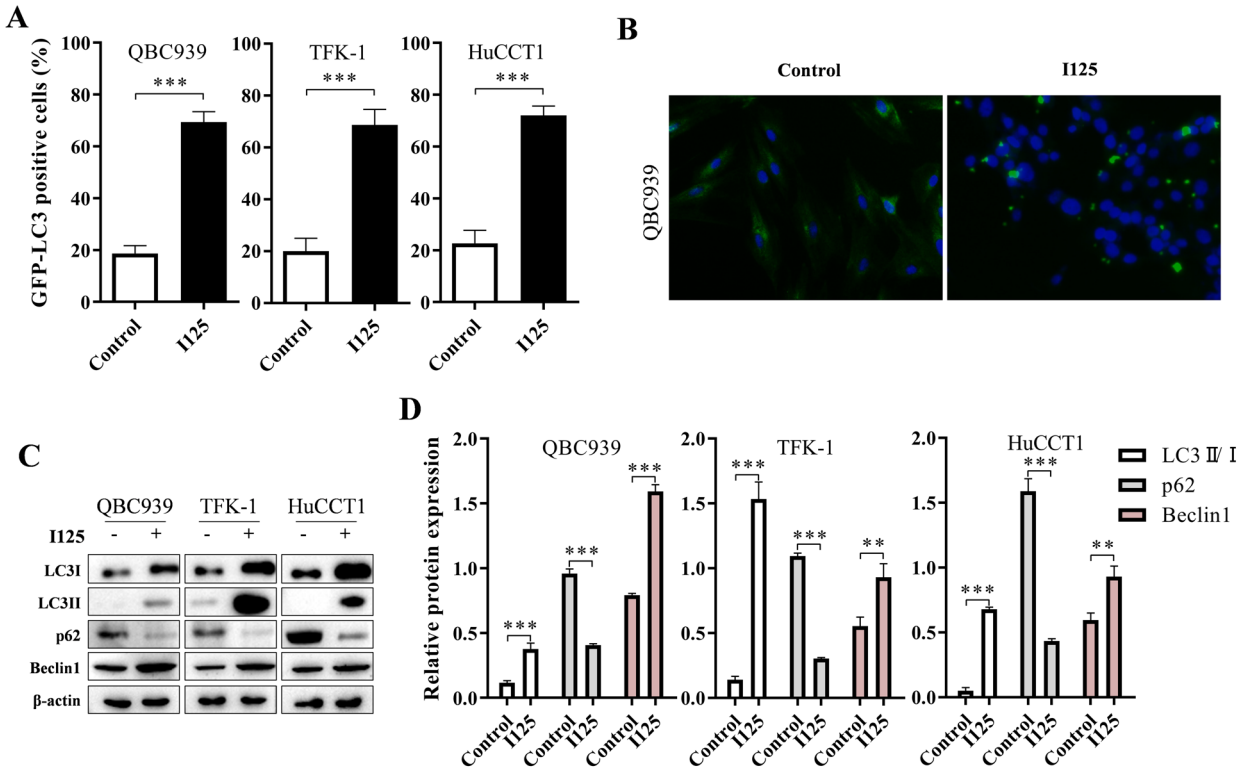

Figure 3. I-125 induced autophagy in the human CCA cell line, QBC939. A, B) Determination of autophagosome formation. C, D) Expression of autophagy-related proteins. **p<0.01 and ***p<0.001

related protein Beclin1 in QBC939 cells (p=0.0008, Figure 4A), and led to enhanced I-125-induced apoptosis (p=0.0428, Figure 4B). In addition, si-Beclin1 treatment induced high expression of the apoptosis-related proteins cleaved-PARP (p=0.0007) and cleaved-caspase-3 (p<0.0001), low expression of BCL-2 (an inhibitor of apoptosis), high expression of BAX (a pro-apoptosis protein), and a high BAX/BCL-2 ratio (p=0.0024) in QBC939 cells treated with I-125 seed irradiation compared with the I-125 group (Figures 4C, 4D).

I-125 seeds induced endoplasmic reticulum (ER) stress. The expression of the ER stress-related proteins including binding immunoglobulin protein (BIP) (QBC939, p=0.0003; TFK-1, p<0.0001; HuCCT1, p<0.0001), phosphorylation of PRKR-like endoplasmic reticulum kinase (PERK) and eukaryotic initiation factor 2 alpha (eIF2α) was also enhanced, and the ratio of pPERK/PERK (QBC939, p=0.0003; TFK-1, p=0.0003; HuCCT1, p=0.0007) and peIF2α/eIF2α (QBC939, p=0.007; TFK-1, p=0.0003; HuCCT1, p=0.0344) was higher in the three CCA cell lines treated with I-125 seed irradiation relative to the control group (Figures 5A, 5B). Furthermore, activating transcription factor 4 (ATF4) (QBC939, p=0.0003; TFK-1, p<0.0001; HuCCT1, p<0.0001), and C/EBP homologous protein (CHOP) (QBC939, p<0.0001; TFK-1, p<0.0001; HuCCT1, p=0.0018) were increased in all three CCA cell lines treated with I-125 seed irradiation when compared with untreated controls (Figures 5A, 5C).

ER stress inhibition decreased I-125-induced autophagy. Western blotting and quantitative real-time PCR analysis revealed that siRNA targeting PERK (si-PERK) suppressed expression of the autophagy-related proteins PERK and LC3-II/LC3-I, but increased p62 gene expression in QBC939 cells treated with I-125 seed irradiation compared with the I-125 group (p=0.0002; p=0.0035; p=0.0191) (Figures 6A, 6B). Treatment with si-PERK also suppressed the GFP-LC3-positive cells treated with I-125 seed irradiation (p=0.0008, Figure 6C). The apoptosis rate induced by I-125 seed irradiation significantly increased in QBC939 cells after treatment with si-PERK compared with the I-125 group (p=0.0387, Figure 6D).

Discussion

The current study investigated the potential mechanism of the anticancer effect of I-125 seed irradiation on CCA cells (Supplementary Figure S1). The results showed that I-125 seed irradiation reduced the viability and induced apoptosis in three human CCA cell lines, as well as promoted

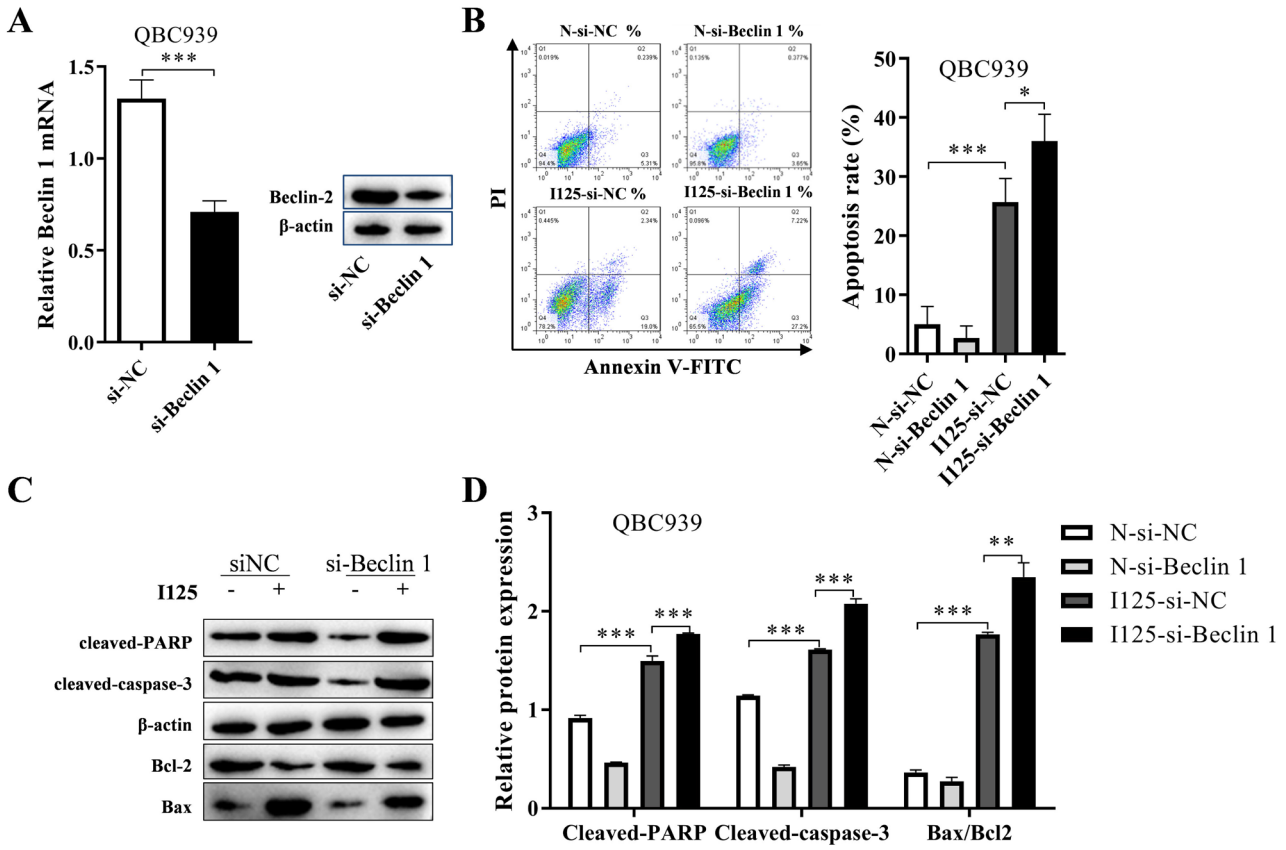

Figure 4. Autophagy inhibited I-125-induced apoptosis. A) The Beclin1 expression level in QBC939 cells after transfection with si-Beclin1. B) Expression of apoptosis-related proteins (cleaved-PARP, cleaved-caspase-3, BAX, BCL-2). C, D) mRNA and protein expression levels of apoptosis-related proteins. *p<0.05, **p<0.01, and ***p<0.001

the expressions of autophagy- and ER stress-related proteins. Furthermore, inhibition of ER stress and autophagy promoted I-125-induced apoptosis of QBC939 cells. These data explained the killing effect of I-125 seeds in CCA cells and clarified critical mechanisms underlying I-125-triggered ER stress, autophagy, and apoptosis in QBC939 cells.

There are numerous imbalances between tumor cell proliferation and death, which can lead to an unrestricted proliferation of cells. Consequently, apoptosis has emerged as a target for cancer therapeutics [19]. Cell apoptosis induction is also considered to be the main mode of anti-cancer effect of I-125 seed radiation, and cell cycle delay contributes to enhancing the cellular radiosensitivity and eventually induces cell death [20]. In the current study, an irradiation range of <2 cm from I-125 seeds containing 0.4–0.8 mCi of ionizing radiation source effectively attenuated the cell viability in each of the CCA cell lines, and led to the accumulation of cells in the G2/M phase of the cell cycle, and induced cell apoptosis through activation of PARP and caspase-3. Similarly, previous studies also demonstrated that the cancer-killing effect of I-125 seed radiation was dependent on inducing cell cycle arrest and apoptosis in gastric cancer [9], lung cancer [21], and pancreatic cancer cells [22]. In addition, a recent *in vitro* and *in vivo* study showed that I-125 seeds inhibited the proliferation and promoted apoptosis of CCA cells by increasing the expression of p-p38 MAPK and p-p53 [23]. These data at least partly supported our results that the molecular mechanism of I-125 seed irradiation involved inhibition of CCA cell viability, cell cycle progression, and promotion of cell apoptosis. In addition, due to the strong radiation sensitivity of G2/M cells [24], the I-125-induced G2/M arrest in this research may reinforce the killing impact of radiation on CCA cells. Further, a previous study has indicated that radiation sensitivity is negatively correlated with the degree of cell differentiation [25]. The present study also revealed similar results that I-125 radiation-induced apoptosis was strongest in poorly differentiated QBC939 and weakest in well differentiated TFK-1 cells.

However, the molecular mechanism underlying the effects of I-125 seeds differs among different cancers, and those underlying the improvements in CCA have yet to be elucidated. In our study, ER stress was assessed by the levels of ER-related markers including the enhanced phosphorylation of PERK and eIF2α, as well as expressions of BIP,

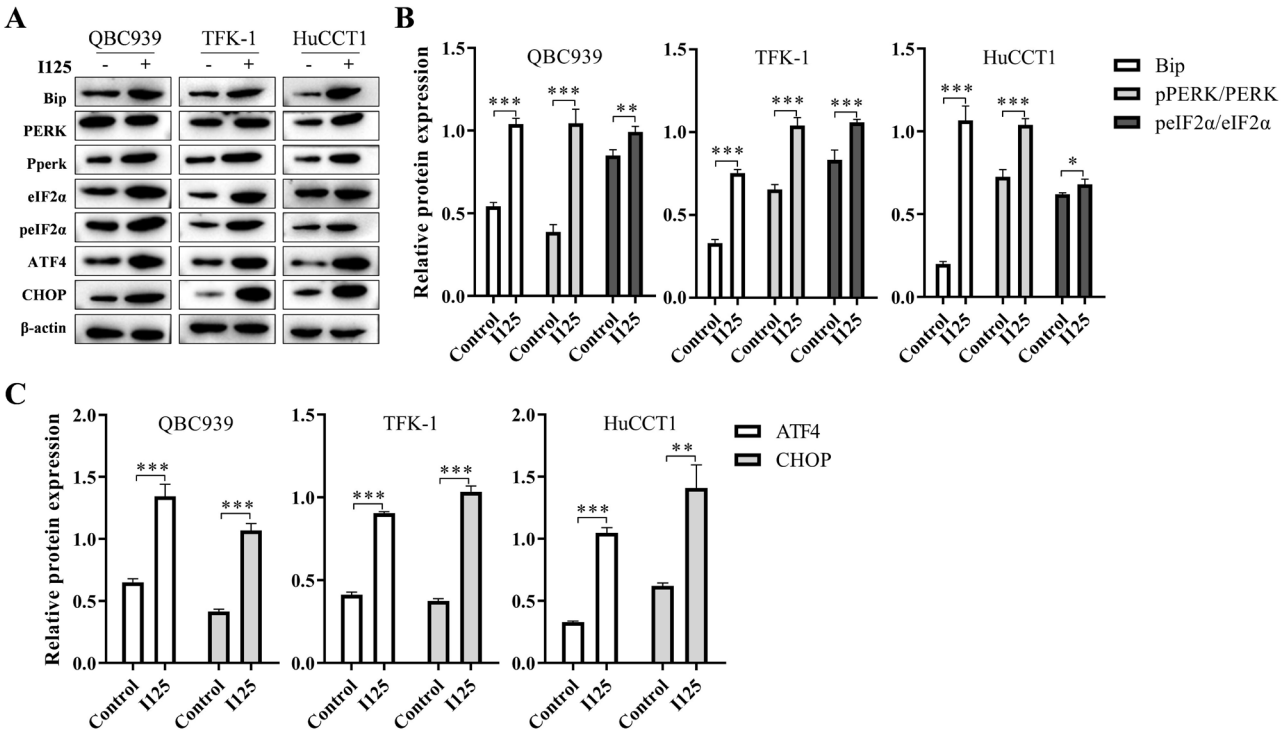

Figure 5. ER stress was induced by I-125 in QBC939, TFK-1 and HuCCT1 cell lines. A–C) Expression of ER stress-related proteins (BIP, pPERK, peIF2α, ATF4, CHOP) following I-125 treatment. *p<0.05, **p<0.01, and ***p<0.001

ATF4, and CHOP proteins, and autophagy was assessed by autophagy-related markers, for example, the induction of the increase of LC3-II/LC3-I ratio and the reduction of Beclin1 and p62. As expected, our data demonstrated that I-125 seed irradiation induced ER stress and autophagy in CCA cells. Further, our study revealed that I-125 seed radiation unexpectedly increased ER stress mediated pro-survival autophagy, and inhibition of ER stress enhanced the effects of autophagy inhibition on I-125-induced cell apoptosis. Recently, autophagy is thought to play a dual role in cancer progression, inhibiting the initial stages of tumor progression and promoting tumor malignancy when the tumor is well established [26, 27]. Autophagy can be activated by tumor suppressors at the initiation of cancer, thereby reducing ROS through mitochondrial damage and preventing tumor malignant progression [26, 28]. Increased LC3, Beclin1, and p62 in infiltrating cancer tissue may be associated with tumor suppression and resistance to oncogenic transformation of cholangiocytes during the early stages of CCA development. Instead, autophagy is also considered to be a protective strategy for tumor cells to evade various therapies and promote tumor survival under stress conditions [29, 30]. Previously, hypoxia-inducible factor-1 (HIF-1) alpha was positively correlated with Bcl-2 19-kDa interacting protein 3 (BNIP3), a pro-apoptotic molecule, and phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3), a component of beclin1-PI3K complex, and was associated with poor prognosis of CCA, suggesting a protective autophagy mechanism of CCA under hypoxia stress [31]. In addition, ER stress is an effective inducer of cancer cell autophagy. Studies have confirmed that ER stress can activate cancer-protective autophagy, and autophagy inhibition can enhance the anticancer effect [32, 33]. Further, radiation therapy has also been shown to induce protective autophagy, which is not intended, and inhibition of autophagy increases the radiosensitivity of cells [34–36]. These findings all confirmed our conclusion that the anti-cancer effect of I-125 is affected by ER stress-induced protective autophagy, and combined inhibition of ER stress and autophagy is an important factor in I-125-induced cell apoptosis, which suggested a potent auxiliary method to enhance the effect of I-125 radiation therapy.

Finally, our research has some limitations. First, the effect of I-125 on cell activity, circulation, autophagy, and ER stress was similar among QBC939, TFK-1, and HuCCT1 cells. ECC accounts for a large proportion (80–90%) of CCA, while ICC accounts for the remaining 10–20% [37]. QBC939 is a poorly differentiated human CCA cell line, which is typical of ECC. Thus, our mechanism study only included QBC939 cells due to time and funding limitations, and further studies on various cell lines of CCA are needed in the future. Secondly, this study only focused on the related mechanisms of I-125-

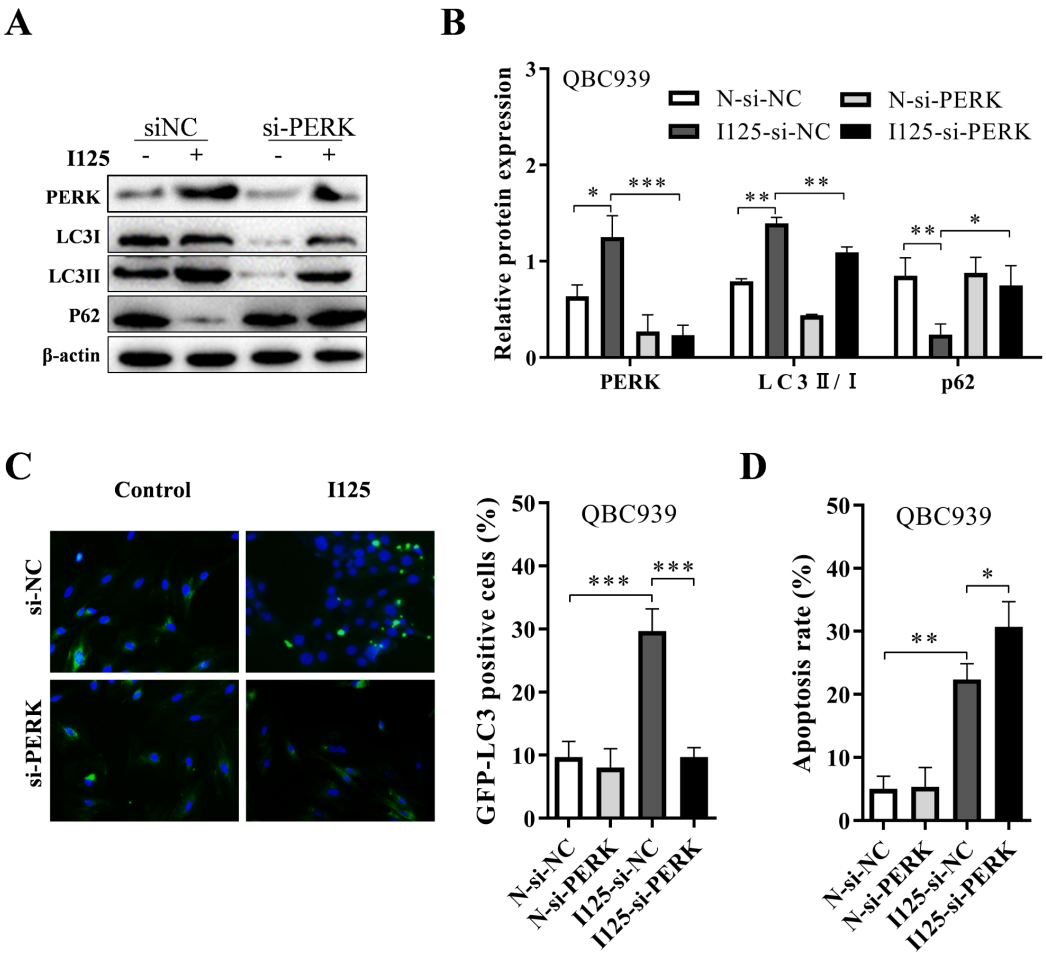

Figure 6. Inhibition of ER stress reduced autophagy. A, B) Expression of autophagy-related proteins after transfection of QBC939 cells with si-PERK. C) Determination of autophagosome formation. D) Rate of apoptosis. *p<0.05, **p<0.01, and ***p<0.001

induced cell apoptosis but did not explore the related mechanisms of cell viability and cell cycle due to time and funding limitations. Third, the significant role of I-125 seeds is to be applied in clinical work. However, radiation is often accompanied by some adverse effects, such as pneumonia, esophagitis, bleeding, fistula, skin injury, heart injury, and so on [38]. Thus, future studies will therefore focus on establishing xenograft models of CCA in mice to investigate whether I-125 seeds can increase the killing effect of I-125 based on combined inhibition of ER stress and autophagy.

In summary, during I-125 radiotherapy, ER stress and autophagy were induced and might cause some unwanted side effects that promote tumor survival, and inhibition of ER stress decreased I-125-induced autophagy in QBC939 cells, and inhibition of autophagy enhanced I-125-induced apoptosis in QBC939 cells. This indicates that expression levels of autophagy- and ER stress-related proteins are both predictors of the effects of I-125 in CCA. We thus conclude that the combination of inhibition of ER stress and autophagy can increase I-125-induced apoptosis in QBC939 cells, and that combination of autophagy inhibition and I-125 radiation may represent a novel therapy for CCA.

Supplementary information is available in the online version of the paper.

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Iodine-125-induced cholangiocarcinoma cell death is enhanced by inhibition of endoplasmic reticulum stress-mediated protective autophagy

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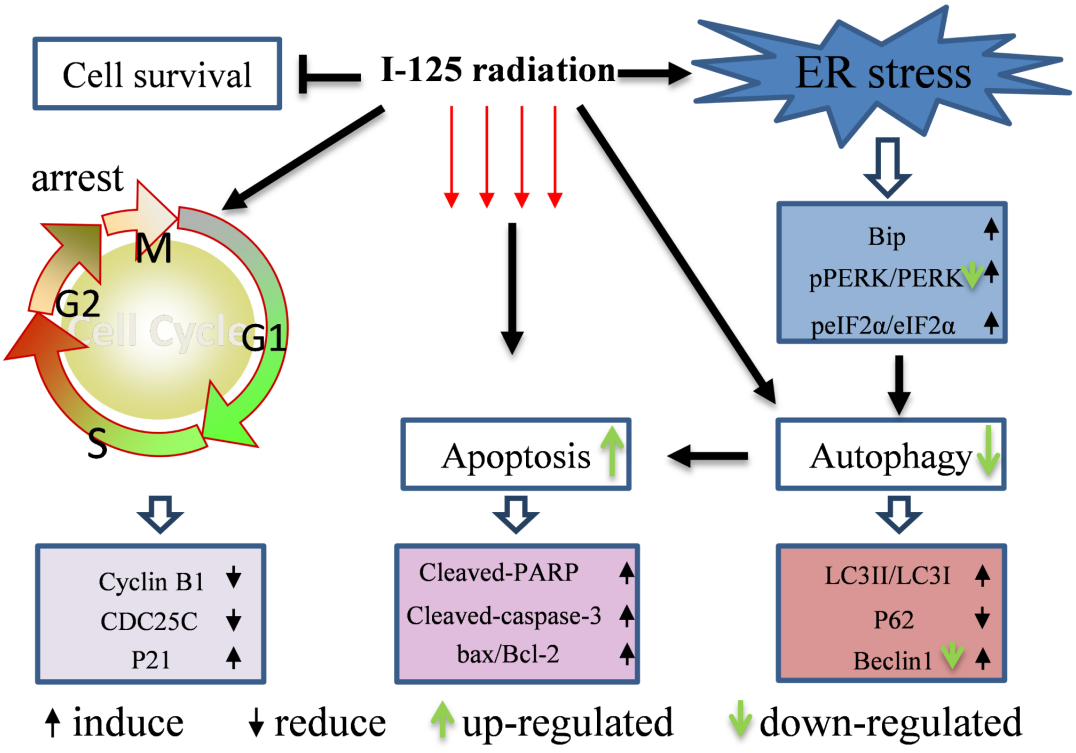
Supplementary Information



Supplementary Figure S1. Schematic diagram of the killing effect of I-125 on CCA cells. I-125 radiation suppresses cell survival and induces cell cycle G2/M-phase arrest, which is characterized with decreased Cyclin B1 and CDC25C, increased p21. I-125 radiation induces cell apoptosis, which is manifested by enhanced cleaved-PARP, cleaved-caspase-3 and the ratio of Bax/Bcl-2. I-125 radiation leads to cell autophagy, which was manifested by enhanced LC3II/LC3I and Beclin1, decreased p62. Further, si-PEAK (ER stress inhibition) promotes I-125 induced autophagy, and si-Beclin1 (autophagy inhibition) promoted I-125 induced apoptosis.