

# Effect of Celecoxib in Combination with X-ray on the Apoptosis of Human Cholangiocarcinoma Cell Line QBC939 in Vitro

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**ABSTRACT Objective:** To investigate the effect of celecoxib in combination with x-ray on the apoptosis of human cholangiocarcinoma cell line QBC939 in vitro. **Methods:** The QBC939 cells were cultured in RMP1640 supplemented with 10% FBS and treated with celecoxib in combination with x-ray. Cell Counting Kit-8 (CCK-8) assay was used to investigate the inhibition ratio and the IC<sub>50</sub>. Cells were divided into five groups: control group, x-ray group, celecoxib group, x-ray after the celecoxib group and x-ray before celecoxib group. Apoptosis of each group were determined by flow cytometry. Expression of survivin was determined by western blot. **Results:** The apoptosis ratio of the combination treatment groups enhanced apparently. The efficacy of the x-ray after celecoxib group was more significant than the x-ray before celecoxib group ( $P < 0.05$ ). Western blot showed that survivin expression was down-regulated obviously after combination treatment. **Conclusion:** Celecoxib enhanced x-ray's effect of apoptosis-inducing. Celecoxib had the radiosensitivity-enhancing effect on the cholangiocarcinoma QBC939 cells in vitro, one of the possible mechanisms may be that it reduced the expression or accelerating the degradation of survivin.

**Key words:** Cholangiocarcinoma ; Celecoxib ; X-ray ; Survivin; Apoptosis

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## Introduction

Cholangiocarcinoma is the primary malignant neoplasm of the bile duct with high mortality in recent years. The special anatomy position makes its early diagnosis difficult. Only less than 20% of cholangiocarcinomas are potentially respectable at time of diagnosis<sup>[1]</sup>. Because of its special biological behavior, the sensitivity of cholangiocarcinoma to chemotherapy and radiotherapy remains rather low<sup>[2]</sup>. Single-drug chemotherapy and adjuvant radiotherapy also have been proven in vain for prolonging life span and reducing the recurrence rate after resection<sup>[3]</sup>.

Survivin, also as known as baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), is encoded by the BIRC5 gene. As a member of the inhibitor of apoptosis (IAP) family, survivin protein had down-regulation effect on apoptosis through inhibiting caspase activation. It interacts with tubulin of the mitotic spindle and plays an important role in mitosis<sup>[4]</sup>. Recent studies shown that destruction of survivin could promote apoptosis and inhibit tumour growth<sup>[5]</sup>. The survivin protein is expressed highly in most human tumours and fetal tissue, such as human cholangiocarcinoma cell line QBC939<sup>[6]</sup>, but is completely absent in terminally differentiated cells<sup>[7]</sup>. Survivin has been reported to play an important role in inhibiting the effect of radiotherapy by suppressing caspase-9 and caspase-3 activities, which are key enzymes in the apoptosis signaling pathway<sup>[8]</sup>. This makes survivin an ideal target for cancer therapy as its selectively cancer cells targeting characteristic.

Cyclooxygenase-2 (COX-2) is an inducible enzyme controlling the synthesis of lipid inflammatory mediator prostaglandins (PGs). Recent researches found that the expression of COX-2 increased in cholangiocarcinoma cells<sup>[9]</sup>, which suggested a potential role of COX-2-mediated PG pathway in biliary carcinogenesis. Celecoxib as one of the selective COX-2 inhibitors, has depressant effect on mammary cancer and gastrointestinal cancer such as colon cancer cells. Bai XM reported that prostaglandin E2 upregulate survivin expression via the EP1 receptor in hepatocellular carcinoma cells<sup>[9]</sup>. These achievements suggested that celecoxib could promote the apoptosis of some tumor cells and may disturb the expression of survivin. This study was to detect the celecoxib's effect on the apoptosis of cholangiocarcinoma and investigate the effects of celecoxib in combination with x-ray on the apoptosis of QBC939, in the hope of seeking a new approach to improve the treatment of human cholangiocarcinoma.

## 1 Materials and methods

### 1.1 Main reagents and materials

The human cholangiocarcinoma cell line QBC939 was purchased from Shanghai Institute of Cell Library. Celecoxib was purchased from Debao Biotechnology Co.Ltd (Nanjing, China). Cell Counting Kit-8 (CCK-8), Annexin V-FITC Apoptosis Detection Kit, RIPA Lysis Buffer and Enhanced BCA protein Assay Kit were purchased from Beyotime Institute of Biotechnology (China). Antibodies against survivin and  $\beta$ -actin were from Santa-Cruz

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Biotechnology Inc.(Santa Cruz, CA, USA).The Enhanced Chemiluminescence (ECL) Detection System was purchased from Millipore(MA,USA). RIMP1640 medium and fetal bovine serum (FBS) were purchased from Hyclone (USA).

### 1.2 Cell culture

The human cholangiocarcinoma cell line QBC939 was cultured in RIMP1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin and 100  $\mu$ g/mL streptomycin at 37  $^{\circ}$ C in a humidified incubator containing 5% CO<sub>2</sub>. The culture medium was changed everyday. Cells were passaged with 0.25% trypsin when the density reached 70-80%. Logarithmic phase cells were collected to adopt in the following experiments.

### 1.3 Cell groups and therapies induction

For the cell proliferation assay, logarithmic phase cells were seeded in 96-well microtitre plates with a concentration of 5000 cells/well, 100 $\mu$ l per well, and incubated at 37  $^{\circ}$ C for 24 h. Thereafter, for the groups of celecoxib, the culture medium was replaced with fetal bovine serum-free medium with different concentrations (25  $\mu$ mol/L, 50  $\mu$ mol/L, 75  $\mu$ mol/L, 100  $\mu$ mol/L respectively) of celecoxib. Medium without celecoxib served as control. Each assay was repeated in six simultaneous holes. For the groups of x-ray, the medium was replaced with fresh fetal bovine serum medium, and then the cells were treated with a series of doses(2 Gy, 4 Gy, 6 Gy, 8 Gy ,10Gy respectively). The cells without the treatment of x-ray served as control. Each assay was repeated in six simultaneous holes too.

### 1.4 CCK-8 cell proliferation assay

Following the steps described above, all the cells were incubated for another 48 h at 37  $^{\circ}$ C. Then, 10  $\mu$ l of CCK-8 solution was added to each well and incubated for 2h, and then the optical absorbance (A) of each well was read in a spectrophotometer at a wavelength of 450 nm. The quantity of viable cells was used to reflect the cell growth inhibition ratio using the following formula: Growth inhibition ratio(%)=(1-optimum A of experimental group/optimum A of control group)  $\times$  100%.

### 1.5 Determination of apoptosis rate through flow cytometry

Human cholangiocarcinoma cells QBC939 were divided into five groups: control group, x-ray group (R), celecoxib group(C), x-ray after celecoxib group (C+R) and x-ray before celecoxib group (R+C). All the cells were incubated in 25 cm<sup>2</sup> tissue culture flasks with a concentration of 2 $\times$  10<sup>5</sup>/ml, 3ml per flask. Group C was added celecoxib with the concentration of 75  $\mu$ mol/L and incubated for 48h. Group R was treated by x-ray radiation with the dose of 6Gy and incubated for 48h; Group C+R was added celecoxib with the concentration of 75  $\mu$ mol/L for 24 h, thereafter, the cells were treated by x-ray radiation with the dose of 6Gy and incubated for another 24 h. Group R+C was treated by the x-ray ra-

diation for the first 24 h, and then celecoxib was added for another 24 h. Cells without any treatment served as control. After 48 h, cells were collected with 0.25% trypsin and stained with Annexin V-FITC and PI double staining kit. According to the manufacture's instructions, the cells were immediately analyzed by flow cytometer (Epics XL·MCL,USA). At least three independent experiments were conducted.

### 1.6 Detection of the survivin expression by Western Blot

Cells were scraped from the culture flasks and lysed for 30min on ice in chilled RIPA Lysis Buffer. Then, total protein was extracted by centrifugation at 12000g for 10min at 4  $^{\circ}$ C. The supernatant was collected and measured by Enhanced BCA protein Assay Kit. Each group's protein was processed as previously reported<sup>[9]</sup>. Incubated the membranes with rabbit survivin polyclonal antibody(1:1000 dilution) at room temperature for 3 h. After three washes with TBST, the membranes were incubated with goat anti-rabbit IgG (1:6000 dilutions) at room temperature for 1.5 h. Then, the membranes were washed with TBST and TBS three times respectively. The immunoreactive proteins were detected using Enhanced Chemiluminescence's (ECL) Detection System.  $\beta$ -actin was used as an internal control. Densitometry analysis was repeated for three times.

### 1.7 Statistical analysis

All the data are expressed as means  $\pm$  S. Multiple group comparison was analyzed using ANOVA test, and LSD method was used to compare each two tests. For each variable at least three independent experiments were carried out. All analyses were performed using SPSS version 17.0, and differences were considered statistically significant at P<0.05.

## 2 Results

### 2.1 Effects of celecoxib and x-ray on the growth of QBC939

After treatment of celecoxib with different concentrations (25  $\mu$ mol/L, 50  $\mu$ mol/L, 75 $\mu$ mol/L, 100  $\mu$ mol/L) for 48h, the inhibition ratio were 19.90%, 27.52%, 50.37%, 82.49%, respectively. The half maximal (50%) inhibitory concentration (IC<sub>50</sub>) was 74.7  $\mu$ mol/L. While after the treatment of x-ray (2 Gy, 4 Gy, 6 Gy, 8 Gy, 10 Gy), the inhibition ratio were 15.31%, 26.52%, 47.67%, 65.71%, 78.93%, respectively. The IC<sub>50</sub> was 6.34Gy. We determined IC<sub>50</sub> of 75  $\mu$ mol/L and 6 Gy to adopt in the flow cytometry and western blot.

### 2.2 Apoptosis promotion effect of celecoxib

The apoptosis rate in control group and the group C or R was significant difference (P<0.05; Table 1). The apoptosis rate after the celecoxib in combination with x-ray enhanced apparently, as show in Table 1 (P<0.05), Fig 1. These data indicated that the effect of combination celecoxib with radiotherapy may be enhanced, and celecoxib showed as a radiosensitizer.

Table 1 Assay of FCM and western blotting to the effect of combination celecoxib with x-ray

Group	Apoptosis(FCMassay,%)	Density(survivin/ $\beta$ -actin)
Control	3.267 $\pm$ 0.569	0.361 $\pm$ 0.053
C	8.267 $\pm$ 0.702	0.648 $\pm$ 0.051
R	11.233 $\pm$ 1.050	1.238 $\pm$ 0.070
C+R	22.133 $\pm$ 1.258	1.953 $\pm$ 0.083
R+C	15.733 $\pm$ 1.504	1.536 $\pm$ 0.062
P Value	<0.05	<0.05

Note: The apoptosis ratios of FCM assay and density of western blotting are expressed as mean  $\pm$  S. Any two tests of data comparison have obvious statistical significance( $P < 0.05$ )

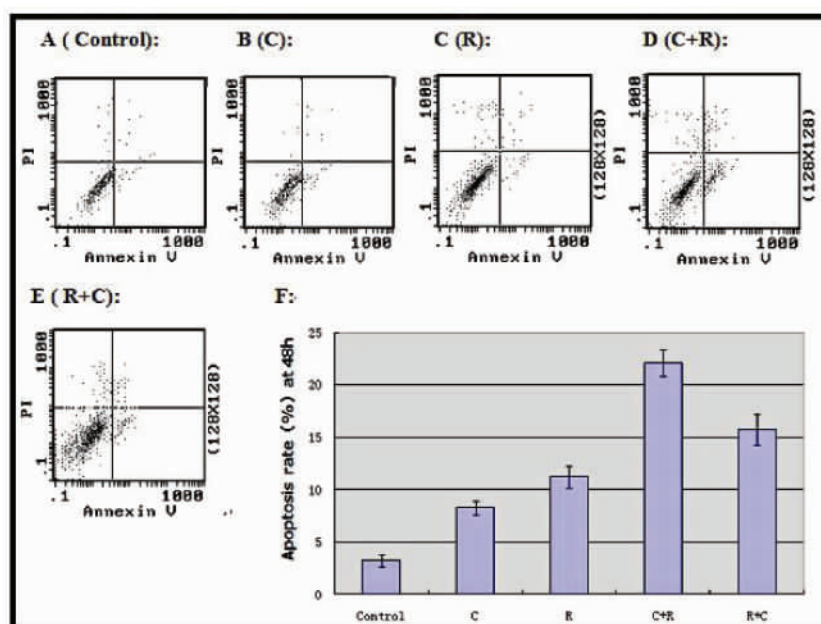


Fig.1 Flow cytometry analysis apoptosis in QBC939 cells:A-E Assessment of apoptosis using flow cytometry with Annexin V-FITC/PI staining, showing the dot-plot graph;F: Apoptosis ratio of each group at 48 h. Lane 1: untreated cells; Lane 2: cells treated with 75 $\mu$ mol/L; Lane 3: cells treated with 6Gy x-ray; Lane 4: cells treated with celecoxib for the first 24h, and then added the x-ray radiation for the other 24h; Lane 5: cells treated with x-ray for the first 24h, and then added the celecoxib for the other 24h. The apoptosis ratios are expressed as mean  $\pm$  S,  $p < 0.05$ .

### 2.3 The expression of survivin

Western blot analysis was performed to determine the survivin protein expression in different treatment groups. The densitometry for whole cell protein samples was determined by normalizing the integrated survivin band intensity to the integrated  $\beta$ -actin band intensity in the same sample (Fig.2). The result showed that survivin protein expression was down-regulated obviously by celecoxib combined with x-ray. The efficacy of the x-ray after the celecoxib group was better than the x-ray before celecoxib group( $P < 0.05$ ; Table 1).

## 3 Discussion

Cholangiocarcinoma is a malignant neoplasm originating from epithelium. It often occurs in patients suffering long-standing cholangitis, injury and reparative biliary epithelial cell proliferation. Nonresectable biliary tract cancer is associated with a poor

prognosis due to wide resistance to chemotherapeutic agents and radiotherapy. Radiotherapy and systemic chemotherapy have been evaluated in unresectable cholangiocarcinoma, but failed to show satisfactory results [10-13]. Recent studies had reported the inhibitory effects of celecoxib on various malignancies, while its effect and synergistic effect with radiotherapy on cholangiocarcinoma were scarcely reported.

Radiation therapy in cancer therapy directly or indirectly damages DNA and induces apoptosis. Defects in the apoptotic machinery can lead to radiation resistance. The radiation could activate the endogenous signaling pathways including the activation of caspase8 and finally caspase3 to induce apoptosis of cancer cells. Inhibition of apoptosis confers a survival advantage on cell containing genetic alterations and promotes the acquisition of further mutations to increase neoplastic progression [14]. Apoptosis is programmed cell death, it provides a vital protective mechanism a

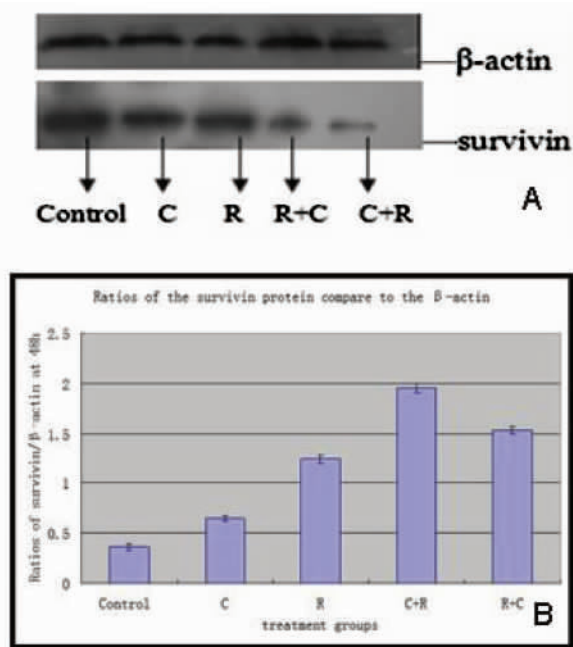


Fig.2 Western blot analysis showing the levels of survivin :A: Western blot show the expression of surviving in each group ;B: Ratios of the survivin protein compare to the  $\beta$ -actin by different treatments. The ratios are expressed as mean  $\pm$  S,  $p < 0.05$ .

against the genesis of neoplasia by removing cells with DNA damage. There are mainly two groups of proteins participate in apoptosis: pro-apoptotic and anti-apoptotic protein. We have found that tumor cells over-express anti-apoptosis proteins, such as inhibitors of apoptosis proteins (IAPs), protect cells from apoptosis [16]. Survivin is the smallest protein in the IAP family and is over-expressed in various human cancers. It suppress key enzymes' activities, such as caspase-9 and caspase-3, in the apoptosis signaling pathway [15]. Survivin inhibit the effect of radiotherapy through blocking the caspase pathway.

In our experiment, human cholangiocarcinoma cells QBC939 were divided into five groups as described in the method. After being treated with interference factor for a certain time, apoptosis of each group were determined by flowcytometry. Data indicated that the effects of combining celecoxib with radiotherapy may be enhanced, and celecoxib showed as a radiosensitizer.

As all known, celecoxib is a kind of non-steroidal anti-inflammatory drugs (NSAIDs). It's reported that NSAIDs could potentiate the lethal effect of radiation on murine tumor cells and conversely protect irradiated immune cells in vitro and in vivo at doses that have been shown to inhibit prostaglandin production. NSAIDs are known to interfere with the ability of the cyclooxygenase-1 and -2 (COX-1 and COX-2) to convert arachidonic acid to prostaglandins. It has been established that COX-2 inhibitors synergize with radiotherapy in several animal tumor models [17]. Kazuhiro reported that celecoxib induced apoptosis in HeLa cells at least partially due to reduction of the expression of survivin, by

the suppression of transcriptional activity partially through the -75/-66 bp region relative to the initiating codon in the survivin promoter in HeLa cells [18]. Li X found that the detection of Survivin and COX-2 expression appear to be correlated in gastric cancer [19]. As it has been reported that over-expression of survivin in cancer overcomes the apoptotic checkpoint and favors the aberrant progression of transformed cells through mitosis [20], the ability of celecoxib to decrease survivin expression may be responsible for the chemopreventive effects of this drug. Therefore, celecoxib was used as a drug aiming at reduce the expression of survivin to sensitise the effect of radiotherapy. Results support our anticipation. the survivin protein level were determined by western blot and find that it was down-regulated significantly in C+R group. Result indicated that celecoxib might reduce survivin mRNA expression or accelerated the degradation of survivin protein in cholangiocarcinoma cells.

Is there any connection between COX-2 inhibitors and survivin expression? Some studies had been done on this issue. Bai XM reported that Prostaglandin E2 could up-regulate expression of survivin via the EP1 receptor in hepatocellular carcinoma cells by activating the EGFR/PI3K pathway [9]. Thomas W also reported the synergy between celecoxib and radiotherapy resulted from inhibition of cyclooxygenase-2-derived Prostaglandin E2 [17]. This study speculated that celecoxib may increase the tumor cells' radiosensitivity through down-regulating survivin expression by inhibition of Prostaglandin E2.

From the results of cell proliferation assay and flow cytometric analysis, it can be seen that x-ray after the celecoxib group get higher apoptosis rate than the x-ray before celecoxib group. This may indicate that celecoxib as a radiosensitizer could enhance the effect of radiotherapy. However, the mechanism of this phenomenon by which agents interact is unknown. Zhu AX had described some novel targeted biologic agents including celecoxib and bevacizumab being explored in combination with standard chemotherapy and radiation therapy [21]. They speculated that the mechanism may be associated with the reduction of Akt phosphorylation, expression of bcl-2, and expression of survivin, as well as the inhibition of caspase3 activation [22]. There is a need to determine the further mechanisms of why x-ray after celecoxib group is better than x-ray before celecoxib group.

However, studies showed that celecoxib still inhibited caspase-3 activity significantly even with adequate survivin [23]. This result suggested that the induction of apoptosis by celecoxib is not dependent on one manner. Indeed, Kim et al. reported that celecoxib accelerated cytochrome C release from mitochondria via increasing the expression of pro-apoptotic protein Bak in HeLa cells [24]. Moreover, other scholars have reported that celecoxib affects the Wnt/b-catenin signaling pathway [8,25]. Thus, anti-carcinogenic



effects of celecoxib can be induced by several different mechanisms. The mechanisms between celecoxib and radiotherapy on the cholangiocarcinoma cells and is there any relationship between celecoxib and survivin need further investigation.

This study found that the effect of celecoxib in combination with radiotherapy was more significant than that of radiotherapy alone on the proliferation inhibition and apoptosis of human cholangiocarcinoma cell line QBC939 in vitro. The usage of celecoxib increased the radiosensitivity of cholangiocarcinoma cells and therefore enhanced its effect of apoptosis-inducing and proliferation inhibition. One of the mechanisms may be that celecoxib reduces expression or accelerates the degradation of survivin. This study indicated that the combination of celecoxib and radiotherapy may promote the treatment of cholangiocarcinoma. More studies should be done to confirm the mechanisms and examine its clinical curative effect of the combination treatment on cholangiocarcinoma.

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# Celecoxib 联合 X 线对胆管癌细胞株 QBC939 凋亡的影响

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**摘要** 目的：研究塞来昔布联合 X 线在体外环境下对人胆管癌细胞株 QBC939 凋亡的影响并对其机制进行初步探讨。方法：CCK-8 检测出不同浓度的塞来昔布及不同剂量的 X 线对 QBC939 细胞株的增值抑制率，确定塞来昔布组的 IC<sub>50</sub> 及 X 线组的 IC<sub>50</sub>。将 QBC939 细胞株分为 5 组：对照组(control)、X 线组(R)、塞来昔布组(C)、塞来昔布再加 X 线组(C+R)、X 线再加塞来昔布组(R+C)。用流式细胞仪检测各组的凋亡率，western blot 检测凋亡相关基因 survivin 蛋白的表达。结果：联合使用塞来昔布和放疗组的凋亡率有明显的增加，从 8.268%、11.233%到 15.733%、22.133% (P<0.05)。western 结果显示联合组 survivin 蛋白的表达也明显低于对照组 (P<0.05)。先用塞来昔布再加放疗的效果要优于先用放疗后用塞来昔布的效果。结论：塞来昔布联合 X 线对 QBC939 细胞株有凋亡增敏作用，作用可能机制之一是通过降低或下调凋亡相关基因 survivin 蛋白的表达。

**关键词** 胆管癌；塞来昔布；X 线；Survivin；凋亡

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