Original Article
Tob1 enhances radiosensitivity of hepatocellular carcinoma via inhibiting DNA double-strand break repair

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Abstract: Hepatocellular carcinoma (HCC) is the most common type of liver cancer. Tob1 has been proved to be a tumor suppressor protein. The aim of the present study was to evaluate the effect of Tob1 on radiosensitivity of HepG2 cells. Protein and mRNA levels of Tob1 were detected by Western blotting and RT-PCR, respectively. The cells were irradiated with different doses of X-rays. Cell survival was detected using clonogenic assay. Cell proliferation was measured by BrdU assay. Cell apoptosis were detected by flow cytometry. γ-H2AX and Rad 51 foci were analyzed by fluorescence microscope. The DNA-PK activity was measured by radioimmunoassay. The protein and mRNA levels of Tob1 were significantly decreased by 79% and 68% in HepG2 cells compared with HL-7702 cells. Overexpression of Tob1 inhibited the cell proliferation and clonogenic growth, and increased cell apoptosis. The γ-H2AX and Rad51 foci were significantly increased in the HepG2/Tob1 cells pretreated with 4 Gy of X-ray. Tob1 also inhibited the DNA-PK activity and phosphorylation of DNA-PKcs and Akt. In addition, the combined effect of Tob1 and LY294002 was also similar to the single effect of Tob1 or LY294002. The results showed that Tob1 enhances radiosensitivity of HepG2 cells via inhibiting DNA double-strand break repair. The PI3K/Akt signaling pathway was involved in the effect of Tob1 on the radiosensitivity of HepG2 cells.

Keywords: Hepatocellular carcinoma, Tob1, radiosensitivity, radiotherapy, DNA double-strand break repair

Introduction

Hepatocellular carcinoma (HCC), the most common type of liver cancer, is the fifth common cause of cancer related death worldwide [1]. Since HCC is commonly locally advanced and multifocal, the treatment of HCC is still a challenge. Currently, surgery and radiotherapy are two common therapies for the treatment of HCC, however, the five year survival rate is only 30% [2]. A major of patients with HCC are not suitable for liver resection or transplantation because of tumor size, distant metastasis, or other parenchymal disease [3]. For those patients, a recommended therapy is radiotherapy. However, due to the radioresistance of the cancer cells, the efficacy of radiotherapy was limited [4]. Therefore, a better understanding of the molecular mechanism involved in the radioresistance of HCC cancer cells will contribute to improve the efficacy of radiotherapy.

Transducer of ErbB-2.1 (Tob1) is a member of the B cell translocation gene (BTG)/Tob anti-

proliferative protein family [5]. Accumulated evidences proved that Tob1 is a tumor suppressor protein, and it is low-expressed or lost in a variety of cancers including hepatocellular carcinomas, lung cancer, breast, and pancreas [6-8]. Tob1 has been proved to modulate the radiosensitivity of lung cancer cells and breast cancer cells [9, 10]. The above findings suggest that Tob1 may be a novel molecular target of irradiation. However, the role of Tob1 on radiosensitivity of HCC cells is still unknown.

DNA double strand breaks (DSBs) is considered as the most crucial cellular damage caused by ionizing radiation (IR) [11]. The efficiency of DSBs repair is critical for cell survival during IR. It has been reported that there is a close relationship between cellular radiosensitivity and DSBs repair capacity [12, 13]. Thus the relationship between Tob1 and DSBs repair needs investigation.

The present study evaluated the effect of Tob1 on radiosensitivity of HepG2 cells and the role

of DSBs in the effect. The results showed that Tob1 enhances radiosensitivity of HepG2 cells via inhibiting DNA double-strand break repair. The PI3K/Akt signaling pathway was involved in the effect of Tob1 on the radiosensitivity of HepG2 cells.

Materials and methods

Cell culture and treatment

Human hepatoma cell line, HepG2, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human normal liver cell line, HL-7702, was obtained from the Cell Bank of the Chinese Academic of Science (Beijing, China). Cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated in a humidified atmosphere (37°C, 5% CO_2). For irradiation treatment, the cells were irradiated using 6-MeV X-ray linear accelerator (Siemens KD2, Germany) with 0, 2, 4, 6, or 8 Gy of X-rays.

Transfection

The pcDNA3.0-Tob1 plasmid was constructed by inserting the cDNA fragment retro-transcribed from the full-length of Tob1 mRNA. HepG2 cells were transfected with Tob1-overexpression plasmid (pcDNA3.0-Tob1) or empty plasmid (pcDNA3.0) using Lipofectamine™ 2000 (Invitrogen). Stable expression clones were selected by G418 (800 µg/mL, Sigma, MO, USA). For some experiments, cells were treated in the presence or absence of LY294002 (100 ng/mL, Sigma), which is a specific inhibitor of the PI3K/Akt signaling pathway.

RT-PCR

Total RNA of HepG2 and HL-7702 cells were extracted using Trizol reagent (Invitrogen) according to the manufacture's instructions. And then the total RNA was reverse transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, IN, USA). Real-time PCR was carried out using SYBR Green Gene Expression Assay Kit (Qiagen, Valencia, CA, USA). The primers of Tob1 and GAPDH were synthesized by Sangon (Shanghai, China). The PCR primers were as follows: TOB1 sense, 5'-CTC GAC CGG ACA TTT GTC TCT AG-3' and anti-sense-5'-TAG CTC GCA CCA CGC TAA AGC GC-3'; GAPDH sense, 5'-CTG CTA ACA TGG TGT TCT ACA TCC-3' and antisense, 5'-CTG TCC TGG TCC TAA AGC AG-3'. Relative quantification was calculated as fold changes according to the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Total cell lysate of HepG2 and HL-7702 cells was assessed by western blotting as previously described [14]. Briefly, 40 µg of proteins were separated by SDS-PAGE, and then transferred to a nitrocellulose membrane (Amersham Pharmacia, Germany). The membranes were blocked with 5% skimmed milk and incubated with the antibodies against Tob1, catalytic subunit of DNA dependent protein kinase (DNA-PKcs), p-DNA-PKcs (T2609), Akt, p-Akt (S473) or β-actin (Santa Cruz Biotech, Santa Cruz, CA, USA, 1:1000 dilution), and then incubated with secondary antibody. Finally, the blots were scanned using Bio-Rad ChemiDoc MP Imaging System.

Cell proliferation assay

Cell proliferation of HepG2 cells was measured by BrdU cell proliferation assay kit (Millipore, Billerica, MA, USA) according to manufacturer's instructions. Briefly, HepG2 cells (2×10^3 cells/well) were seeded in a 96-well plate and irradiated with 4 Gy of X-rays. After incubating for 48 h, BrdU was added to the medium and incubated at 37°C for 4 h. After removing the medium, the cells were fixed by fixing solution followed by incubation with detection antibody solution. After adding the HRP-conjugated secondary antibody solution, substrate solution was added. Finally, the plates were detected at 450 nm by a micro-plate reader (Bio-Rad, Hercules, CA).

Clonogenic assay

Cell survival of HepG2 cells was measured using clonogenic assay. HepG2 cells were seeded into 6-well plates and were exposed to different doses of IR on the following day. The culture medium was replaced every two days. After 14 days, cells were fixed with 4% methanol and were stained with crystal violet. Colonies containing more than 50 cells were counted. The plating efficiency and surviving fraction for each treatment were calculated based on the survival of non-irradiated cells.
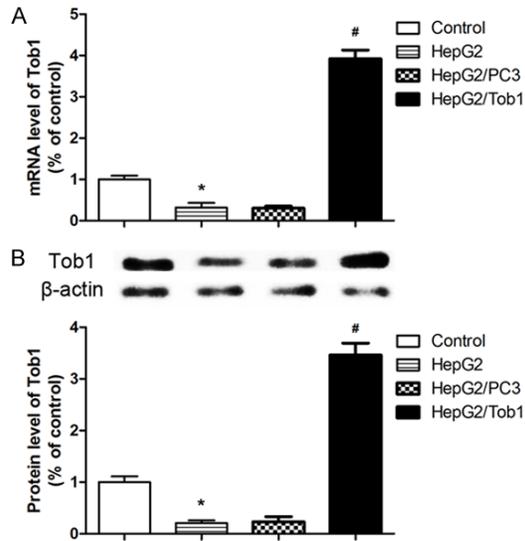
Figure 1. Expression of Tob1 in HepG2 and HL-7702 cells. The mRNA and protein levels of Tob1 in HepG2 and HL-7702 cells were detected by RT-PCR and western blotting, respectively. A. mRNA level of Tob1 in HepG2 and HL-7702 cells; B. Protein level of Tob1 in HepG2 and HL-7702 cells. *$P < 0.05$ vs. control group, #$P < 0.05$ vs. HepG2 or HepG2/PC3 group. Data are represented as mean ± SD of three dependent experiments. Control group: HL-7702 cells; HepG2: Normal HepG2 cells; HepG2/PC3: HepG2 cells transfected with pcDNA3.0 empty plasmid; HepG2/Tob1: HepG2 cells transfected with pcDNA3.0-Tob1.

Apoptosis assay

HepG2 cells were harvestes and assessed for cell apoptosis using an Annexin V-PI Apoptosis Detection Kit (Abcam, Cambridge, UK) according to the manufacture's recommendations. Briefly, HepG2 cells were resuspended in 500 μL of binding buffer. Then the cells were stained with Annexin V-FITC and propidium iodide (PI). Cell apoptosis was analyzed by a FACScan flow cytometer.

γ-H2AX analysis

Cells were seeded on a cover slip and cultured for 48 h. Then the cells were irradiated with 4 Gy of X-rays for another 24 h, and were fixed in 4% paraformaldehyde for 30 min. Cells were permeabilized with 10% Triton X-100 in PBS for 15 min, and blocked with 5% skimmed milk for 1 h at 37°C. Then the cells were incubated with γ-H2AX or Rad 51 primary antibody (Santa Cruz) at 4°C overnight, and then incubated with secondary antibody conjugated with Alexa Fluor

555 or conjugated with Alexa Fluor 488 (Abcam, MA, USA) for 1 h at 37°C. The slides were washed for three times with PBST and were mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA). γ-H2AX and Rad 51 foci were visualized using a fluorescence microscope.

Detection of DNA-PK activity

The detection of DNA-PK activity was performed using a SignaTect DNA-PK assay system (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. Briefly, the crude cell extract was prepared, and was mixed with DNA-PK activation buffer, reaction buffer, a DNA-PK biotinylated p53-derived peptide substrate and 0.5 μCi (γ-^{32}P) ATP. The mixture was then incubated at 30°C for 5 min. Termination buffer was added to the mixture, and 10 μl of mixture was spotted onto a SAM^2 capture membrane. Finally, the membrane was washed with 2 mol/L NaCl, dried, and incorporated ^{32}P-phosphorylated substrate. Then the membrane was measured by scintillation counting.

Statistical analysis

All data were expressed as means ± SD. Each experiment was performed three times. Statistical analysis was performed with GraphPad Prism 6 software (GraphPad Prism Software Inc., San Diego, CA, USA). The differences were evaluated by one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Tob1 expression was decreased in HepG2 cells

It was reported that Tob1 is low-expressed or lost in a variety of cancers [5]. The expression level of Tob1 in HepG2 cells and HL-7702 cells was detected by western blotting and RT-PCR. As shown in **Figure 1**, the protein and mRNA levels of Tob1 were significantly decreased by 79% and 68% in HepG2 cells compared with HL-7702 cells. To study the effect of Tob1 on the radiosensitivity of HepG2 cells, pcDNA3.0 or pcDNA3.0-Tob1 were transfected into HepG2 cells. The results showed that the protein and mRNA levels of Tob1 in pcDNA3.0-Tob1 transfected HepG2 cells (HepG2/Tob1) were in-
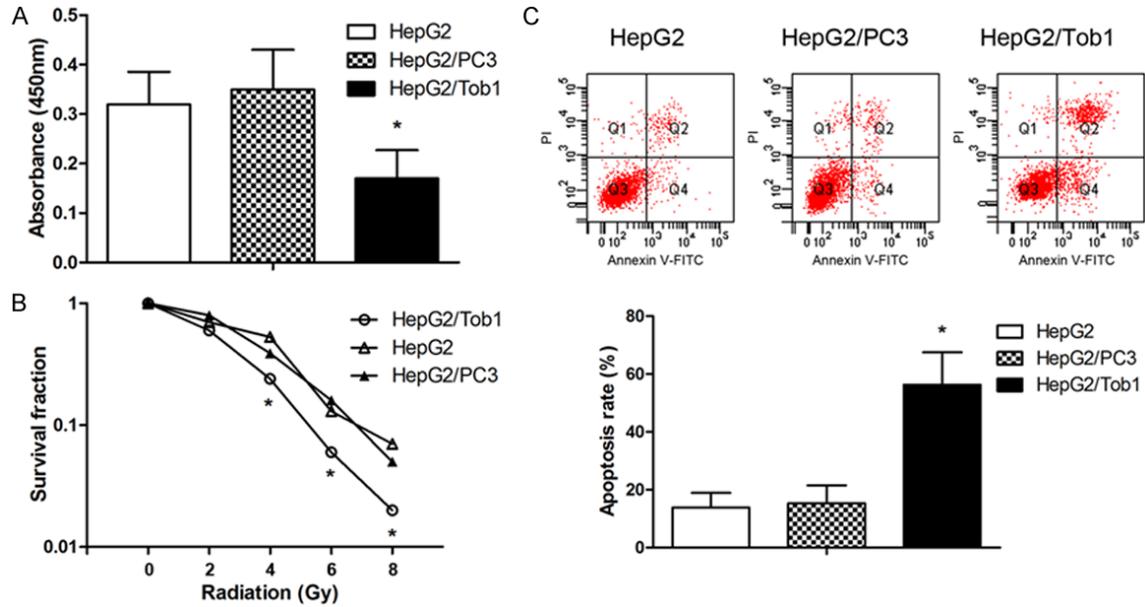
Figure 2. Tob1 increased the radiosensitivity of HepG2 cells. A. The cells were irradiated with 4 Gy of X-rays. Cell proliferation was detected by BrdU assay; B. The cells were irradiated with 0, 2, 4, 6, or 8 Gy of X-rays. Clonogenic cell survival assay was performed; C. The cells were irradiated with 4 Gy of X-rays. Cell apoptosis were detected by flow cytometry. *P < 0.05 vs. HepG2 or HepG2/PC3 group. Data are represented as mean ± SD of three dependent experiments.

creased by 3.25- and 3.60-fold, respectively (**Figure 1**).

Tob1 increased the radiosensitivity of HepG2 cells

To assess the effect of Tob1 on the radiosensitivity of HepG2 cells, BrdU assay was carried out to evaluate cell proliferation. The results showed that Tob1 overexpression inhibited cell proliferation of HepG2 cells (**Figure 2A**). The results of clonogenic assay indicated that overexpression of Tob1 significantly reduced the clonogenic growth of HepG2 cells (**Figure 2B**). In addition, cell apoptosis of HepG2 cells after irradiation was also measured, and the percentage of apoptosis in HepG2/Tob1 cells was significantly increased by 42.5%, compared to the normal HepG2 cells (**Figure 2C**).

Tob1 enhanced the DNA-DSBs of irradiated HepG2 cells

Rad51 and γ-H2AX are considered as key markers of DNA-DSBs [15]. The γ-H2AX and Rad51 foci were detected in the present study. As shown in **Figure 3A**, the γ-H2AX and Rad51 foci were slightly increased in the HepG2 cells pretreated with 4 Gy X-ray irradiation. While the γ-H2AX and Rad51 foci were significantly

increased HepG2/Tob1 cells, which was consistent with the expression of γ-H2AX and Rad51 (**Figure 3A** and **3B**), data suggested that Tob1 enhanced the DNA-DSBs of irradiated HepG2 cells.

DNA dependent protein kinase (DNA-PK) is a key enzyme during DNA repair [16]. The result of **Figure 3C** proved that DNA-PK activity was significantly increased by irradiation, while Tob1 significantly inhibited the activity of DNA-PK. It has been confirmed that the phosphorylation of DNA-PKcs plays important roles in regulating DNA-PK activity. And we find that Tob1 inhibited the phosphorylation of DNA-PKcs (T2609) 1.3 fold (**Figure 3D**). The result of **Figure 3D** showed that Tob1 also inhibited the Akt (S473) phosphorylation, indicating that the PI3K/Akt signaling pathway was involved in the effect of Tob1.

Tob1 enhanced radiosensitivity of HepG2 cells and inhibited DNA double-strand break repair via inhibiting the PI3K/Akt signaling pathway

As shown in **Figure 4**, the effects of Tob1 and LY294002 (an inhibitor of PI3K/Akt) on cell apoptosis, DNA-DSBs and DNA-PK activity were similar. However, the combined effect of Tob1 and LY294002 was also similar to the single effect of Tob1 or LY294002. The results indi-
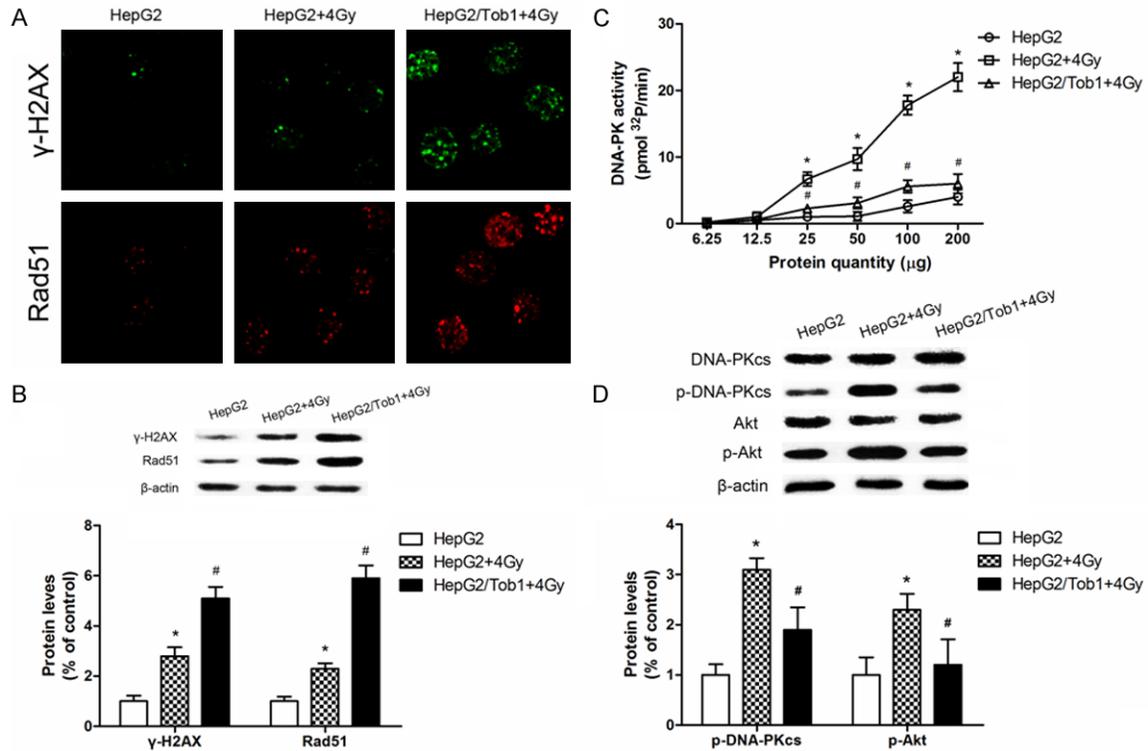
Figure 3. Tob1 enhanced the DNA-DSBs of irradiated HepG2 cells. A. γ-H2AX and Rad 51 foci; B. Expression of γ-H2AX and Rad 51 were detected by western blotting; C. DNA-PK activity; D. Phosphorylation of DNA-PKcs (T2609) and Akt (S473) were detected by western blotting. *P < 0.05 vs. HepG2 group, #P < 0.05 vs. HepG2+4 Gy group. Data are represented as mean ± SD of three dependent experiments.

cated that Tob1 enhanced radiosensitivity of HepG2 cells and inhibited DNA double-strand break repair via inhibiting the PI3K/Akt signaling pathway.

Discussion

Tob1, a well-known tumor suppressor, is decreased in many cancer cells [17]. The relationship between Tob1 and cell radiosensitivity is controversial [9, 18]. The discrepancy indicates that the role of Tob1 on cell radiosensitivity is different in different cancer cells. It has been demonstrated that mice lacking *Tob* are prone to spontaneous formation of liver tumor [19]. To further investigate the role of Tob1 in HCC, the expression of Tob1 in HepG2 cells was detected in the present study. The results showed that Tob1 was decreased in HepG2 cells, compared to the normal liver cells (**Figure 1**). We also find that Tob1 enhanced the radiosenditivity of HepG2 cells (**Figure 2**).

Since DSBs repair is closely related with the tumor radioresistance, we evaluated the effect of Tob1 on DSBs repair. H2AX is a histone variant which can be induced to γ-H2AX by DSBs [15, 20]. Rad51 is marker protein that plays a major role in homologous recombination of DSBs repair [20]. In the present study, γ-H2AX and Rad51 were selected to be the marker proteins of DSBs. The results proved that overexpression of Tob1 increased the γ-H2AX and Rad51 foci, indicating that Tob1 promoted the process of DSBs.

DNA-dependent protein kinase (DNA-PK) is a protein complex, which is essential for the DSBs repair [16]. DNA-PK is composed of DNA-PKcs and a double-stranded DNA end binding subunit (Ku70/Ku80) [16]. The phosphorylation of DNA-PKcs (T2609) is related to the DSB repair and cellular radiosensitivity. And we found that Tob1 inhibited the phosphorylation of DNA-PKcs (T2609), which indicated that Tob1 blocked the DSB repair. It has been reported that the PI3K/Akt pathway was involved in the regulation of DNA-PKcs [21]. And the inhibitor of Akt blocks the radiation induced DNA-PKcs phosphorylation, which is consistent
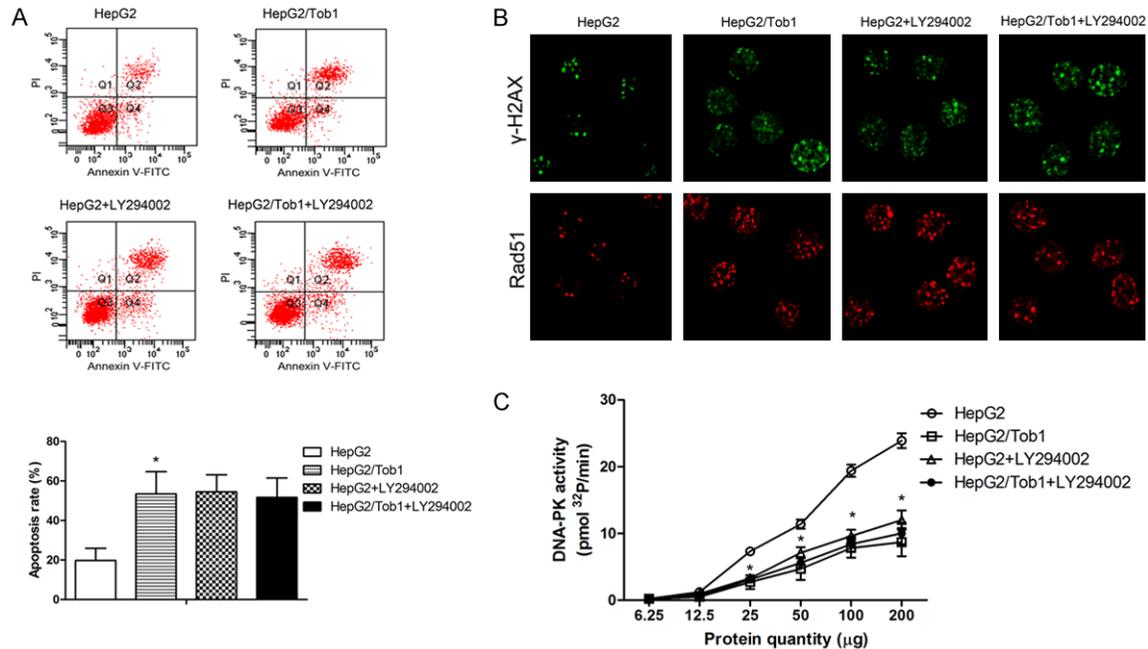
Figure 4. PI3K/Akt was involved in the effect of Tob1 on the IR-induced HepG2 cells. A. Cell apoptosis of HepG2 cells with different treatment; B. γ-H2AX and Rad 51 foci; C. DNA-PK activity. *P < 0.05 vs. HepG2 group. Data are represented as mean ± SD of three dependent experiments.

with our results. We also found that Tob1 also inhibited the phosphorylation of DNA-PKcs and Akt. In addition, the combined effect of Tob1 and LY294002 was also similar to the single effect of Tob1 or LY294002, but not stronger than that of Tob1 or LY294002. Thus, we concluded that Tob1 enhanced radiosensitivity of HepG2 cells and inhibited DNA double-strand break repair via inhibiting the PI3K/Akt signaling pathway. However, the further molecular mechanism and the *in vivo* effects need further investigation.

In summary, the present study evaluated the effect of Tob1 on the radiosensitivity of hepatocellular carcinoma. The results showed that Tob1 enhances radiosensitivity of HepG2 cells via inhibiting DNA double-strand break repair. The PI3K/Akt signaling pathway was involved in the effect of Tob1 on the radiosensitivity of HepG2 cells. These findings suggested that Tob1 might be a promising molecular in gene therapy for the treatment of hepatocellular carcinoma.

Acknowledgements

Disclosure of conflict of interest

None.

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