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World J Gastroenterol 2004;10(21):3103-3106
World Journal of Gastroenterology
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• LIVER CANCER •

Transfection of p27 ^{kip1} enhances radiosensitivity induced by ^{60}Co γ-irradiation in hepatocellular carcinoma HepG$_2$ cell line

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Received: 2004-03-27 **Accepted:** 2004-04-13

Abstract

AIM: To study the cell cycle alterations of human hepatoma cell line HepG$_2$ *in vitro* after ^{60}Co γ-irradiation and further to examine the mechanisms underlying the enhancement of radiosensitivity to γ-irradiation in HepG$_2$ transiently transfected with wild type p27^{kip1}.

METHODS: The proliferation of HepG$_2$ cells was evaluated with MTT assay, and the cell cycle profile and apoptosis were assessed by cell morphology, DNA fragmentation analysis and flow cytometry. HepG$_2$ cells were transfected with p27^{kip1} wild type by using Lipofectamine (LF2000), and the expression and subcellular localization of p27^{kip1} in HepG$_2$ were detected by immunocytochemistry.

RESULTS: ^{60}Co γ-irradiation inhibited the growth of HepG$_2$ cells in a dose-dependent manner. Apoptosis of HepG$_2$ cells was induced 48 h after γ ray exposure. Furthermore research was carried out to induce exogenous expression of p27^{kip1} in HepG$_2$. The expression of p27^{kip1} induced G_0/G_1 phase arrest in HepG$_2$ cells. The overexpression of p27^{kip1} enhanced ^{60}Co γ-irradiation-induced radiosensitivity in HepG$_2$ cells.

CONCLUSION: Overexpression of p27^{kip1} is a rational approach to improve conventional radiotherapy outcomes, which may be a possible strategy for human hepatoma therapy.

Guan XX, Chen LB, Ding GX, De W, Zhang AH. Transfection of p27 ^{kip1} enhances radiosensitivity induced by ^{60}Co γ-irradiation in hepatocellular carcinoma HepG$_2$ cell line. *World J Gastroenterol* 2004; 10(21): 3103-3106
http://www.wjgnet.com/1007-9327/10/3103.asp

INTRODUCTION

Hepatocellular carcinoma (HCC) is a relatively common malignancy, ranking fifth in frequency on a worldwide basis and causing more than one million deaths annually[1,2] and there has been a progressive increase in the number of hepatoma cases over the past two decades[3]. Unfortunately, most of the cases of hepatoma are not curable because extensive resection is not possible. Though many approaches, such as transarterial chemoembolization (TACE), percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), radiotherapy and liver transplantation have been developed to treat it, and the effective and survival rates are increased, a large number of patients would die from recurrence and metastasis[4-8]. It is well known that improving the overall therapeutic effects of liver cancer depends on the combined therapies. The purpose of combined interventional therapies for HCC is to increase their therapeutic efficiencies and to reduce the side effects and complications.

Radiotherapy presents another interesting option for the treatment of HCC amidst the wide array of non-surgical modalities available[11] and experimental and clinical studies have been reported that gene therapy is one of the more promising approaches for patients with advanced liver tumours[9-12].

It is unknown about the combination of radiotherapy and gene therapy, especially the relationship between p27^{kip1} and radiosensitivity, although p27^{kip1} is a target for cancer therapeutics[13]. p27^{kip1} is a key molecule in cell cycle control because of their specific and periodic expression during cell cycle progression. Knowledge of the function of cell cycle checkpoints in tumour cells may be important to develop treatment strategies for human cancers. Recent studies indicate that mutations in the p27^{kip1} gene have not been seen in many tumors including human hepatoma[14]. Down-regulation and mislocalization expression of p27^{kip1} have recently been found to be associated with a poor prognosis in patients with hepatoma[15]. However, the role of p27^{kip1} expression and gamma irradiation-induced apoptosis in human hepatoma cells has not been examined previously.

The purpose of this study was to investigate the function of p27^{kip1} and ^{60}Co γ-irradiation in the HepG$_2$ cell cycle progress and apoptosis, and then to examine the molecular mechanisms of radiosensitivity induced by p27^{kip1} and gamma irradiation in human hepatoma cells, focusing on the possibility that it might act, at least in part, by increasing the expression of p27^{kip1} in human hepatoma cells. This study may help us to understand radiosensitivity and develop a new treatment strategy.

MATERIALS AND METHODS

Reagents

RPMI-1640 medium and Lipofectamine (LF2000) were purchased from GIBCO. Anti-Flag (M2) was purchased from Sigma. FITC conjugated-IgG was purchased from Santa Cruz Biotechnology. The plasmid containing p27^{kip1} was kindly provided by Dr. Keiichi Nakayama at Kyushu University, Japan.

Cells and treatment with gamma-irradiation radiation treatment

Human hepatoma cell line, HepG$_2$, was routinely maintained in RPMI-1640 medium supplemented with 100 mL/L heat-inactivated fetal bovine serum at the 100 mL/L concentration and incubated in 100 U/mL penicillin-streptomycin in 50 mL/L CO_2 in air at 37 ℃ with 50 mL/L CO_2. Hepatoma cells were plated into 6-well culture plates (5×10^4 cells/well) and exposed to 0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 Gy of γ-irradiation from a telecobalt therapy source at a dose rate of 5.0 cGy/minute.

Transient transfection of p27^{Kip1}

Transfection of the cells with wild type p27^{kip1} cDNA constructs was performed using Lipofectamine (LF2000) reagent according to the manufacturer's instructions. Briefly, cells were plated in a 6-well plate at a density of 5×10^4 /well and incubated overnight in RPMI-1640 supplemented with 100 mL/L FCS. The cDNA constructs encoding p27^{kip1} were diluted in RPMI-1640 (100 μL) and then mixed with the transfection solution for 15 min. After washed twice with phosphate-buffered saline (PBS) to remove serum, the cells were incubated with the transfection mixture at 37 ℃ for 4 h and then refreshed with RPMI-1640 medium.

Proliferation assays

Cell proliferation was measured by MTT assay. After $HepG_2$ cells were treated with different dose gamma-irradation for indicated time and dose, then 10 μL MTT (5 mg/mL) was added to each well and incubated for an additional 4 h, and then the liquid in the wells was evaporated. To dissolve the formazam, 200 μL of DMSO was added. Control wells were treated with 1 g/L DMSO alone. The absorbance was detected in the microplate reader 550 model at 570 nm wavelength. Growth inhibition was equal to (1-absorbance of the treated wells)/(absorbance of the control wells) ×100%.

Immunohistochemistry

Immunohistochemical staining was performed to determine the expression of Flag-P27^{kip1} fusion protein. $HepG_2$ cells transiently expressing p27^{kip1} were grown on a glass coverslip in a 6-well plate for 24 h after transfetion. The cells on the coverslips were fixed in 20 g/L paraformaldehye at room temperature for 5 min on the next day of transfection, and sequentially incubated for 60 min with anti-mouse Flag (M2) and then FITC-conjugated second antibody. The slides were lightly counterstained with Hoechst 33258, washed with water and then mounted. Finally coverslips were mounted with other glass slides, the cells were examined by immunofluorescence microscopy at the excitation/emission wavelengths of 488 nm and 520 nm alternatively.

Flow cytometry analysis

$HepG_2$ cells were plated onto a 6-well plate (5×10^4 cells/well) in RPMI-1640 containing 100 mL/L FBS and grown overnight to allow cell attachment. They were then treated with irradiation, harvested, fixed with 700 mL/L ethanol, centrifuged, resuspended in 400 μL of PBS, and 2 mg/mL RNase was added to avoid double-stranded RNA staining, then stained with 400 μL of 0.1 mg/mL propidium iodide (PI). The cell suspension was filtered through a 60-μm Specrta/Mesh nylon filter. Samples of 20 000 cells were then analyzed for DNA histograms and cell cycle phase distributions by flow cytometer using a FACSCalibur instrument (Becton Dickinson), and the data were analyzed by a CELLQuest computer program.

DNA fragmentation assay

The integrity of DNA was assessed by agarose gel electrophoresis. Cells (1×10^6) were centrifuged for 5 min at 3 000 r/min, washed once with PBS, and cell pellets were resuspended in 100 μL of lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L tetraacetic acid, 4 g/L SDS, 0.5 g/L proteinase K) and incubated for 8 h at 50 ℃, then 10 μL of 0.5 g/L RNase A was added. The samples were incubated for 1 h at 50 ℃ and heated to 70 ℃ for 5 min, then 100 μL of phenol: chloroform:isopropanol (25:24:1) was added. After centrifugation, the supernatants were transferred to new tubes, and twice volume ethanol (ice cold) was added. After centrifugation, the pellets were solubilized in TE buffer and loaded on 18 g/L agarose gel for electrophoresis. The gel was stained with ethidium bromide, and photographed with UV illumination.

Hoechst 33258-propidium iodide counterstaining

Apoptosis and death cells were identified by Hoechst 33258-PI counterstaining[16]. Briefly, cell pellets (1×10^9) were suspended by 100 μL PBS containing Hoechst33258 at the concentration of 1 μL/mL. The cells were incubated at 37 ℃ for 7 min, and then centrifuged. The cell pellets were resuspended in 100 μL staining solution containing PI at the concentration of 5 μg/mL. The stained cells were analyzed using a fluorescence microscope. Samples of 200 cells were then analysed.

Statistical analysis

Data were represented as mean±SD. Differences were evaluated by SPSS10.0 software. $P\leqslant0.05$ was considered statistically significant.

RESULTS

Growth inhibition of $HepG_2$ cells by ^{60}Co γ-irradiation

The irradiation of $HepG_2$ cells caused a dose-dependent cell growth inhibition, and a maximum inhibitory effect was observed at 6 Gy. MTT assay showed that irradiation had anti-proliferative effects on $HepG_2$ cells in a dose-dependant manner. At the dose of 0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 Gy, the inhibitory rate was 2.62±0.18%, 26.85±0.21%, 54.11±0.47%, 57.19±0.54%, 60.26±0.53% and 67.12±0.65% respectively (Figure 1). The cell growth inhibitory effect was due to apoptosis or necrosis induced by excessively high dose irradiation. The counterstaining of PI and Hoechst 33258 proved to be an excellent probe to distinguish apoptotic cells from necrotic cells. Live and apoptotic cells were only probed by Hoechst 33258 (blue in color) and exclusion of propidium iodide due to plasma membrane integrity. The morphological features of apoptotic cells were cell shrinkage, nuclear condensation and genomic fragmentation down to the size of individual nucleosome units. On the contrary, necrotic cells were probed by PI (red in colors) (Figure 2). The apoptotic rate reached the top at the dose of 4.0 Gy, when irradiated by 6.0 Gy, most of $HepG_2$ cells were in necrotic state (Figure 3).

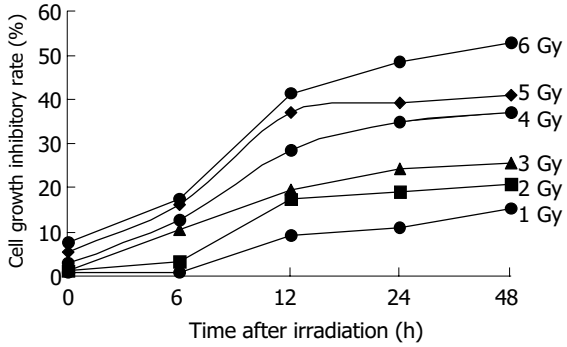

Figure 1 Effect of irradiation on proliferation of $HepG_2$ cells.

p27^{kip1} expression enhanced radiosensitivity induced by ^{60}Co γ-irradiation in $HepG_2$ cells

Overexpression of p27^{kip1} protein was observed in transfected cells (Figure 4). As a result, the proliferation of $HepG_2$ cells was greatly inhibited and cell cycle was arrested in G_1 phase after exogenous p27^{kip1} expression (Figure 5). Further results showed that overexpression of p27^{kip1} enhanced radiosensitivity in $HepG_2$ cells induced by ^{60}Co γ-irradiation, and 2.0 Gy irradiation induced maximum apoptotic rate by 40.6% in $HepG_2$ cells transfected with p27^{Kip1}, whereas $HepG_2$ cells transfected empty vector, only 4.0 Gy reached the maximum apoptotic rate of 35.3% (Figure 6). Apoptosis of $HepG_2$ cells indicated by flow cytometry and Hoechest33258 staining was then confirmed by DNA fragmentation, which was visualized as the characteristic oligonucleosome-sized fragmentation in ethidium bromide after DNA agarose gel electrophoresis (date not shown).

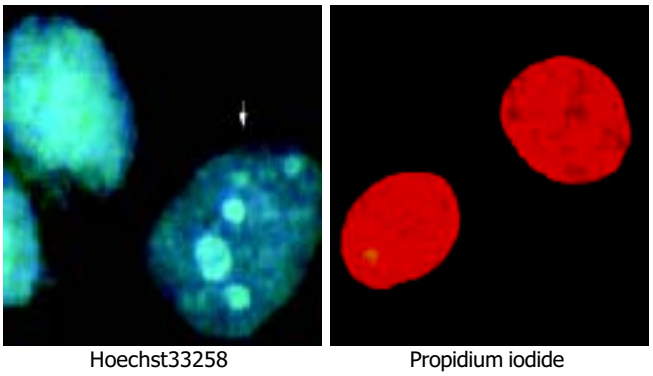

Figure 2 Counterstaining of Hoechst 33258 and propidium iodide to distinguish apoptotic from necrotic cells.

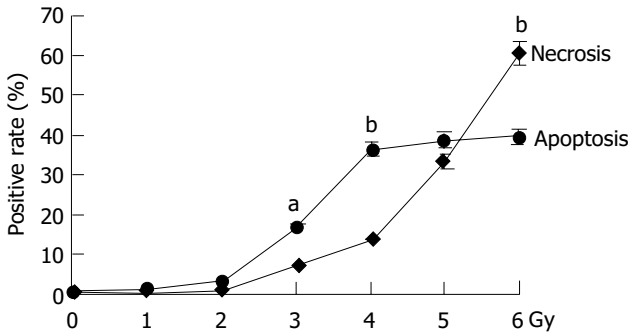

Figure 3 Comparsion of apoptosis and necrosis rate of HepG$_2$ treated by ^{60}Co γ-irradiation. $^aP<0.05$, $^bP<0.01$ *vs* necrosis.

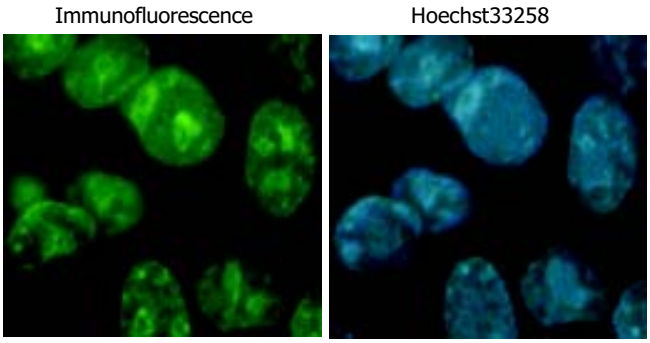

Figure 4 Subcellular localization and expression of p27^{kip1} in tranfected HepG$_2$ cells.

DISCUSSION

HCC remains one of the most difficult tumors to treat. Primary HCC is the second most common cancer and the leading cause of cancer deaths behind gastric cancer in China. Surgical resection has been accepted as the only curative therapy for primary liver cancer. Unfortunately, most patients are surgically unresectable, and are sometimes recommended to receive non-surgical therapies, including radiotherapy, radiofrequency hyperthermia, genetherapy or combination of the above methods. Radiation therapy has been commonly used in the treatment of unamenable human hepatoma. Unfortunately, the cause of this radiosensitization has not met expectations fully. New approches that may reduce side-effects and provide good quality of life are required. Thus, it is imperative to develop new and effective treatments, such as gene therapy, in order to treat this disease.

Gene therapy is one of the more promising approaches for patients with advanced liver tumour. Experimental and clinical studies have reported that gene therapy and molecular prevention are becoming a part of patient management and

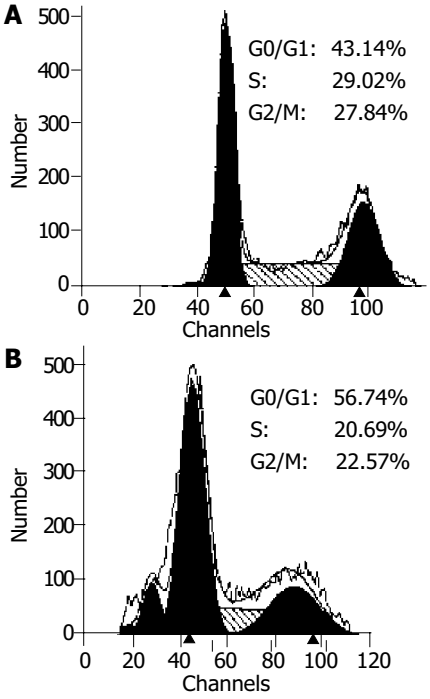

Figure 5 Flow cytometry analysis of cell cycle of nontransfected and transiently transfected HepG$_2$ cells. A: nontransfected HepG$_2$ cells. B: p27^{kip1} transiently transfected HepG$_2$ cells.

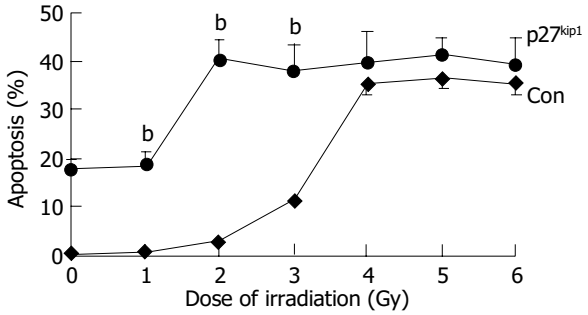

Figure 6 Apoptotic rates induced by γ-irradiation in nontransfected and transfected HepG$_2$ cells. $^bP<0.01$ *vs* control.

would eventually complement or in part replace the existing therapeutic and preventive strategies[17]. Recent researches have indicated that p27^{kip} is a new target for gene therapy and p27^{kip1} is a new suitable candidate for gene therapy[9,18]. The protein p27^{kip1} is an important factor that regulates cell cycle progression and apoptosis. Mutations in p27^{kip1} gene have not been seen in many tumors including human hepatoma. Down-regulation and mislocalized expression have recently been found to be associated with poor prognosis in patients with hepatoma. Therefore, regulation of p27^{kip1} activity is a new strategy for hepatoma therapy.

Pretreatment of hepatocellular carcinoma cells with overexpression of p27^{kip1} protein before irradiation enhanced the cell-killing effect of irradiation. Interaction with moderate doses of radiation caused a substantial increase in tumor cell killing. The beneficial effect of this interaction was further evidenced by the significant increase in the number of apoptic cells. The HepG$_2$ cells treated with p27^{kip1} and exposed to 6.0 Gy showed a maximum apoptosis percentage when compared to the other irradiation doses or p27^{kip1} transfection. Our results showed that overexpression of p27^{kip1} could enhance the radiosensitivity in HepG$_2$ cells. Overexpression of p27^{kip1} in HepG$_2$ cells cound sensitize cells to ionizing radiation. Recent advances have been made in the understanding of molecular

events following cell exposure to ionizing radiation. Our results suggest that $p27^{kip1}$ protein could be used to modulate radio-induced cellular responses.

However, the molecular machanism of $p27^{kip1}$ in radiosensitivity induced by ^{60}Co γ-irradiation is still unclear, and further studies are needed to identify the molecular mechanisms for the radiosensitizing activity, with emphasis on the study of $p27^{kip1}$ in cell cycle progress and radiosensitivity.

Our results here provide some experimental evidences that overexpression of $p27^{kip1}$ increases the radiosensitivity of gamma irradiation. It may help us understand radiosensitivity and develop strategies for liver cancer.

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Edited by Zhang JZ and Wang XL **Proofread by** Xu FM