

Full-length article

Antisense oligonucleotide targeting p53 increased apoptosis of MCF-7 cells induced by ionizing radiation¹Li-cheng DAI², Xiang WANG, Xing YAO, Li-shan MIN, Fu-chu QIAN, Jian-fang HE*Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, Huzhou 313000, China***Key words**

antisense oligodeoxyribonucleotides; MCF-7; p53 genes; radiation tolerance; cell cycle

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Abstract

Aim: To investigate the effect of antisense compounds (AS) targeting human p53 mRNA on radiosensitivity of MCF-7 cells. **Methods:** Western blotting and RT-PCR were used to analyze the protein content and mRNA level. Additionally, cell proliferation, cell cycle and cell apoptosis were all analyzed in irradiated or sham-irradiated cells. **Results:** Among the five antisense compounds (AS), AS3 was identified to efficiently inhibit p53 mRNA level and protein content. Interestingly, AS3 transfer has little effect on cell proliferation in DU-145 cells (mutant p53) after ionizing radiation (IR). In contrast, a marked increase of cell apoptosis and growth inhibition were observed in MCF-7 cells (wild-type p53), suggesting that AS3 can increase radiosensitivity of MCF-7 cells. Additionally, it was also observed that the transfection of AS3 decreased the fraction of G₁ phase cells, and increased the proportion of S phase cells compared to untreated cells 24 h after IR in MCF-7 cell lines. **Conclusion:** AS3 transfection increases MCF-7 cell apoptosis induced by 5 Gy-radiation, and this mechanism may be closely associated with abrogation of G₁ phase arrest.

Introduction

Ionizing radiation (IR) is widely used in the therapy of solid tumours, with more than half of all cancer patients receiving radiation therapy (RT) during the course of treatment. After surgery, RT is now proved to be the most powerful medical treatment in the fight against cancer, especially for localized disease that has not spread. Rational and effective use of its killing power depends on understanding IR-mediated responses at the molecular, cellular and tissue levels. It is known that irradiation is used to treat virtually all types of solid malignancies, but to varying degrees of success. That is, some tumors are highly responsive to low doses of radiation (eg, lymphomas, seminomas), and other tumors are typically very radioresistant and tend to progress even after high radiation doses (eg, melanoma, glioblastoma)^[1]. The reasons for such radioresistance in those tumors are multiple and varied.

Nowadays, the main focus of radiation-related studies has switched to the analysis of molecular mechanisms of the IR-induced cellular response. With the hope that increased

understanding would facilitate the development of new strategies for overcoming the radioresistance. Accumulated evidence demonstrate that p53 is an important molecule for determining cell radiosensitivity^[2]. It is reported that the degree of radiation-induced apoptosis has been shown to correlate with the p53 wild-type (wt) status^[3]. Furthermore, apoptosis is induced when wt-p53 is transfected into certain cell lines lacking p53^[4,5]. It is also known that several downstream genes of p53, such as p21, cyclin G, and GADD45, whose expression products function as regulators of diverse aspects of cell growth, is rapidly upregulated after ionizing radiation-induced DNA damage^[6-8]. The induction of the p21 protein after ionizing radiation mediates p53-dependent G₁ arrest via its inhibitory effects on cyclin-dependent kinase (CDKs) required for S-phase entry^[9]. Recently, it is believed that cell cycle phase is an important factor determining cell radiosensitivity^[10,11]. Proper cell cycle arrest may allow cells the time to repair DNA damage, which is thought to be associated with cell survival in response to radiation. This suggests that p53 can not only induce cell apoptosis, but also plays a role in protecting cells against apoptosis by regulat-

ing the progression through the cell cycle when cells respond to irradiation.

Because of the complexity of p53 function, a problem arises as to whether wt p53 functions as a cell apoptotic activator or not when responding to radiation. There are many published reports of investigations of the relationship between p53 mutations and radiosensitivity *in vitro* and in animals^[12-16]; however, the role of p53 in determining sensitivity to radiotherapy is still controversial. Böhnke *et al*^[17] showed that p53 wt cells (MCF-7, MCF-BB, LNCaP, *etc*) were on average 1.3-times more radiosensitive than mutant cells (DU-145, RT112, and SCC4451), suggesting that wt p53 may mainly serve as a pro-apoptotic gene to increase radiosensitivity. However, this result was obtained mainly through statistical analysis, and might be limited to reflect the truth. Further study is needed on this.

Antisense compounds are useful tools for biological research and can reduce gene expression by efficient annealing of complementary sequences to the target mRNA^[18, 19]. The ability to suppress the expression of individual genes with a high degree of specificity could distinguish antisense therapy from other modes of treatment. Therefore, the aim of our study is to identify antisense compounds targeting p53 and clarify its effect on radiosensitivity in MCF-7 cells.

Materials and methods

Cell line and culture DU-145 and MCF-7 cells were obtained from Chinese Academy of Medical Science (Beijing, China). Cells were cultured in DMEM medium (Invitrogen, San Diego, CA, USA) containing 10% fetal bovine serum (GIBCO BRL, Grand Island, NY, USA), 100 kU/L penicillin and 100 mg/L streptomycin. All cultures were incubated at 37 °C in a 5% carbon dioxide (CO₂) atmosphere.

Radiation Cells after transfection for 24 h were irradiated with 5 Gy IR by using a ⁶⁰Co irradiator at a dose-rate of about 2.5 Gy/min for 2 min.

Antisense oligodeoxynucleotides Antisense sequences used in these experiments were designed by a computational neural network mode and mfold server^[20,21]. BLAST confirmed they were specific for the human p53 mRNA. 20 mer antisense ODNs were synthesized to about 99% (MGW Biotech, Ebersberg, Germany). p53 antisense oligodeoxynucleotides (AS) sequences corresponding to different regions of human p53 mRNA were as follows:

AS1: 5'-ACGCTC CCA GCC CGA ACG CA-3' (bases 169 to 188)

AS2: 5'-TGG CGC GGA CGC GGG TGC CG-3' (bases 710 to 729)

AS3: 5'-CAC CAC CAC ACT ATG TCG AA-3' (bases 886 to 905)

AS4: 5'-TCC CCA TCC TCC TCC CCA CA-3' (bases 1615 to 1634)

AS5: 5'-AAA GTT TTA TTG TAA AAT AA-3' (bases 2603 to 2622).

Lip-mediated transfection of antisense oligodeoxynucleotides Cells were plated in 6-well plates at a density of 1×10^5 cells per well. Transfections were carried out after plating for 24 h when cells reached a confluence of 50%–80%. Lipofectamine reagent (Invitrogen) was used for transfection in this experiment, and performed according to the manufacturer. Antisense ODNs concentrations were selected as 0.2 μmol/L, 0.4 μmol/L, and 0.8 μmol/L respectively. After transfection (incubation for 6 h at 37 °C), cells were washed with PBS and incubated in fresh culture medium until additional analyses in this experiment.

Cell viability The effects of AS on cellular viability were determined using MTS assay. Briefly, 3×10^3 cells were seeded in 96-well microtiter plate and allowed to attach overnight. Cells were then transfected with different antisense ODNs concentrations (0.2 μmol/L, 0.4 μmol/L, and 0.8 μmol/L). After transfection for 24 h, samples were treated with 5 Gy IR. At post-irradiation 24 h, 20 μL of MTS (Sigma, St Louis, MO) was added to each well. Then the 96-well microtiter plate was incubated at 37 °C for 2 h, and 490 nm absorbance value was determined using a MR 600 Microplate reader (Wallac 1420 Multilabel counter). Each assay was performed in quadruplicate. Cell survival rate (%) is calculated as: $[1 - (A_{\text{control}} - A_{\text{sample}}) / (A_{\text{control}} - A_{\text{blank}})] \times 100$

RT-PCR After transfection with AS for 48 h, total RNA was extracted using TRIzol (Invitrogen) from MCF-7 cells as the manufacturer's instruction (Qiagen, Chatsworth, CA, USA). RNA was obtained from both untransfected MCF-7 cells and cells transfected with 0.4 μmol/L AS. RT-PCR was carried out by using 2 μg of RNA for the RT reaction (as per the manufacturer's instruction, Qiagen). p53 and Bax primers toward human p53 and Bax gene were then used in the PCR to measure p53 RNA levels (5'-GAATCTCCGCAA-GAAAGG-3', forward; 5'-TGGGCATCCTTGAGTTCC-3', reverse) and Bax RNA level (5'-GTTGTCGCCCTTTTCTACTT-3', forward; 5'-TCAGCCCATCTTCTTCCA-3', reverse), respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse) were used on the same RT reaction as a quantitative control for PCR. Briefly, first strand cDNA was synthesized using a Oligo (dT)₁₅ primer at 42 °C for 2 h. PCR reaction for p53 and Bax were performed following 30 cycles of denaturing at 95 °C for

30 s, annealing at 55 °C, and 50 °C for 30 s, respectively, and extending at 72 °C for 30 s.

Western-blot analysis Cells were lysed in RIPA buffer [10 mmol/L Tris-HCl (pH 7.4), 1% deoxycholate, 1% NP 40, 150 mmol/L NaCl, 0.1% SDS, 0.2 mmol/L phenylmethyl sulfonyl fluoride, 1 µg/mL aprotinin and 1 µg/mL leupeptin] for 30 min on ice. The lysates were centrifuged at 15 000×g for 15 min to remove debris. Proteins sample (30 µg) were separated by 12% SDS-PAGE gel and transferred onto PVDF membranes (Hybond-polyvinylidene difluoride membranes, Amersham Biosciences). p53 and Bax protein was identified using anti-p53 or anti-Bax primary and peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Finally, the reactive band was visualized by an ECL-plus Detection Kit (Amersham Biosciences) and scanned by Gel Doc 1000 (Bio-Rad).

Cell cycle and apoptosis analysis Cell cycle assays were performed as described previously^[22]. Briefly, cells were harvested at 24 h after IR, and fixed with 70% ethanol at -20 °C overnight. The fixed cells were washed twice again with PBS, and stained with 50 µg/mL propidium iodide (PI) in the presence of RNase A. The stained cells were analyzed for DNA content by fluorescence-activated cell sorting (FACS) in a FACScan (SOBR model, Becton Dickinson Instrument, San José, CA, USA). Cell cycle fractions were quantified with CellQuest (Becton Dickinson). For cell apoptosis analysis, MCF-7 cells were examined by annexin V-propidium iodide staining in conjunction with FACS analysis. Briefly, MCF-7 were plated and either mock-treated or irradiated with 5 Gy IR. After 24 h, both untreated and irradiated MCF-7 was incubated with annexin V-fluorescein isothiocyanate (FITC, BAOSAI Biosea Biochnology, Beijing, China) following the manufacturer's specifications. Binding of FITC-conjugated annexin V and propidium iodide (PI) was analyzed by flow cytometry.

Statistics Data were expressed as mean ± SD. Statistical analysis was carried out using Student's *t*-tests (two-tailed). *P*<0.05 indicates statistical significance.

Results

Effect of antisense ODNs on p53 mRNA level We firstly obtained five antisense oligonucleotides (AS) targeting p53 using AO predict tool based on a neural network. To evaluate expression of p53 mRNA in MCF-7 cells, RT-PCR was performed on extracted mRNA aliquots from cells. Of the five AS synthesized, only AS3 markedly downregulated p53 mRNA level in MCF-7 cells after transfection with the aid of Lipofectamine reagent (Figure 1). Therefore, AS3 was used

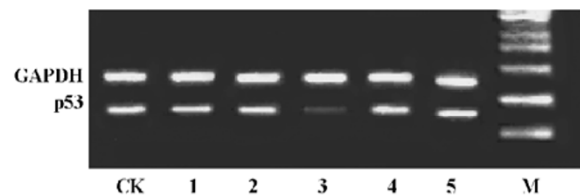


Figure 1. RT-PCR analysis of p53 mRNA level in MCF-7 cells transfected with 0.4 µmol/L antisense ODNs (antisense-1, antisense-2, antisense-3, antisense-4, and antisense-5). Cells transfected only with lipofectamine™ reagent were used as CK. GAPDH was used on the same RT reaction as a quantitative control for PCR.

to analyze the effect of AS targeting p53 on radiosensitivity in cells in the following experiments.

Effects of AS3 on p53 protein expression In order to analyze the effect of AS3 on p53 protein content in cells, AS3 were transfected into MCF-7 and DU-145 cells before irradiation. At 24 h after transfection of AS3, both MCF-7 and DU-145 cells were treated with 5-Gy irradiation. The total proteins were extracted at 24 h after irradiation. In this experiment, it was observed that AS transfer led to a remarkable decline of p53 protein level in both MCF-7 and DU-145 cells at 24 h after irradiation (Figure 2).

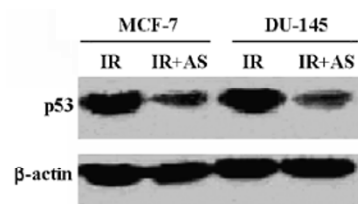


Figure 2. Western blots analysis of p53 protein expression in MCF-7 and DU-145 cells transfected with 0.4 µmol/L AS3 or no antisense ODNs. Cells were irradiated with 0 Gy (-IR) or 5 Gy, and proteins were extracted at 24 h after radiation.

Effects of AS3 on cell viability after IR For examination of effect of AS3 targeted to p53 on the radiosensitivity in DU-145 and MCF-7 cells, AS3 was added to both cells at final concentrations of 0.2, 0.4, and 0.8 µmol/L before irradiation or at 6 h after irradiation. From Figure 3, it was clear to observe that transfection of AS3 before irradiation had little effect on cell survival in DU-145 cells at 24 h after IR. However, survival rate of MCF-7 cells decreased significantly at 24 h, and its inhibitory effects exhibited a dose-dependent manner, suggesting that transfection of AS3 before radiation can increase MCF-7 cells radiosensitivity to certain extent (Figure 3). To examine whether AS3 transfer after IR still increased radiosensitivity of MCF-7, we added the AS3 to

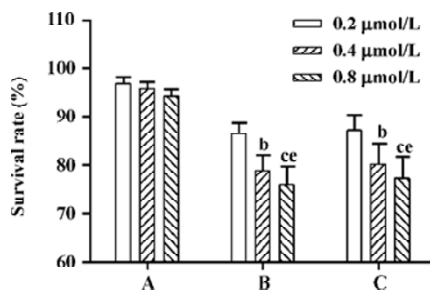


Figure 3. Effect of AS3 at different concentrations on proliferation of cells treated with 5 Gy radiation. Results are expressed as percentage of antisense-untreated controls. Data are means±SD of three independent measurements. ^b*P*<0.05, ^c*P*<0.01 vs antisense 0.2 μmol/L; ^e*P*<0.05 vs antisense 0.4 μmol/L. A, B: cell proliferation was measured at 24 h after IR in DU-145 and MCF-7 cells transfected with 0.2, 0.4, 0.8 μmol/L AS3 before irradiation, respectively. C: cell proliferation was measured at 30 h after irradiation in MCF-7 cells which transfected with 0.2, 0.4, 0.8 μmol/L AS3 at 6 h after IR.

MCF-7 cells at 6 h after treatment with 5-Gy. Interestingly, transfection of AS3 after irradiation also increased MCF-7 cells radiosensitivity in this experiment.

Effects of AS3 on cell cycle distribution and cell apoptosis

Flow cytometry was then used to quantify changes in the cell apoptosis at 24 h after IR. As shown in Figure 4, it was clearly observed that IR induced slight apoptosis in MCF-7 cells. It is noted that transfection of AS3 induced marked apoptosis in MCF-7 cells. To examine the effect of AS3 on cell-cycle progression, MCF-7 with wt p53 was transfected with AS3. At 24 h after IR, cells were fixed at -20°C overnight,

and then fixed cells were stained with 50 μg/mL propidium iodide (PI) in the presence of RNase and analyzed cell cycle progression. Cell-cycle redistribution was shown in Table 1 and Figure 5. Transfection of AS3 decreased the fraction of G₀-G₁ phase cell from 60.38%±1.07% to 45.94%±1.53%, suggesting that AS3 abrogated G₁ phase arrest to certain extent by inhibition of p53 expression (Table 1). In comparison, S phase cells increased from 20.42%±2.33% to 28.82%±1.68%. It is interesting to observe that transfection of AS3 increased G₂ phase cells from 19.19%±1.15% to 25.24%±2.46%.

Table 1. Cell cycle distribution after IR for 24 h. *n*=3. ^b*P*<0.05 vs control group (cells transfected only with lipo, IR+Lipo)

MCF-7 cells	G ₀ -G ₁	G ₂ /M	S
-IR	57.01±3.63	7.81±1.68	35.19±4.51
IR+Lipo	60.38±1.07	19.19±1.15	20.42±2.33
IR+AS3	45.94±1.53 ^b	25.24±2.46 ^b	28.82±1.68 ^b

Effects of AS3 transfer on Bax mRNA level It is known that p53 protein is a positive regulator of the proapoptotic gene Bax transcription. However, in this experiment, inhibition of p53 expression had little effect on Bax mRNA level and protein content measured in MCF-7 cells at 24 h after IR (Figure 6). This result suggests that the increased apoptosis after transfection with AS3 in MCF-7 cells may not be associated the Bax gene expression.

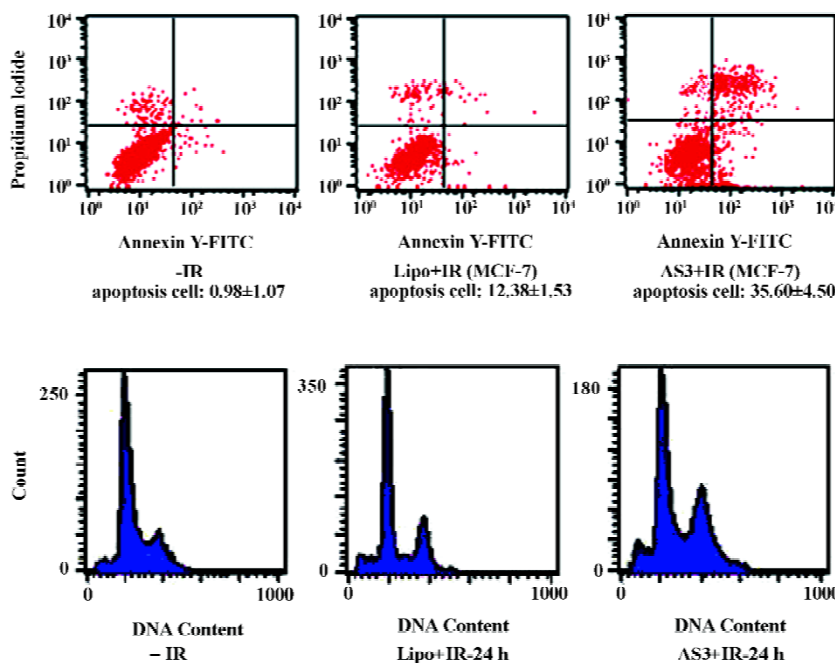


Figure 4. Effect of AS3 on cell apoptosis in MCF-7 cells. Cells were transfected with 0.4 μmol/L AS3 before irradiation, and then were treated with 5-Gy IR. Cell apoptosis were measured at 24 h after IR. Cells transfected with lipofectamine alone used as control (CK).

Figure 5. Effect of AS3 on cell cycle distribution. Cells were transfected with 0.4 μmol/L AS3 before irradiation, and then were treated with 5-Gy IR. Cell cycles were measured at 24 h after IR. Cells transfected with lipofectamine alone used as control (CK).

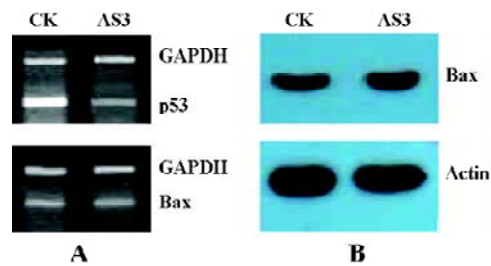


Figure 6. A: RT-PCR analysis of p53 and Bax mRNA level in MCF-7 cells transfected with 0.4 $\mu\text{mol/L}$ AS3. Cells transfected only with lipofectamine reagent were used as control (CK). GAPDH was used on the same RT reaction as a control for PCR. B: Effect of 0.4 $\mu\text{mol/L}$ AS3 transfer on Bax protein content in MCF-7 cells after IR. Actin was used as a control in this experiment.

Discussion

In this study, we firstly designed antisense sequences using a neural network. Furthermore, in order to improve the predicted effective antisense ODNs, the p53 mRNA secondary structure was also predicted according to Zuker^[21]. Based on the two results, we obtained several candidate sequences. Among those, AS3 was identified to successfully suppress p53 mRNA and protein level in MCF-7 and DU-145 cells (Figure 1 and 2). The results indicated that computational prediction is a suitable method to find putative target sites.

To investigate the effect of AS3 on cell radiosensitivity, we selected MCF-7 cells with wt p53 and DU-145 cells with mutant p53. It is believed that loss of p53 protein function caused by mutation or deletion results in ability of anti-apoptosis in cell^[17,23,24]. However, in this experiment, down-regulation of wt p53 using antisense compounds leads to increase of apoptosis in MCF-7 cells after irradiation (Figure 4). It is well known that loss of p53 function decreases the apoptotic response in several cell types after irradiation programmed for proliferation-independent early-interphase apoptosis^[25,26]. However, in most cell types of solid tumors, early apoptosis after irradiation before the release from G₂ block is not a general phenomenon in all mammalian cells. A prerequisite for radiation-induced late apoptosis is cell-cycle progression beyond the radiation-induced G₂ phase block. The apoptosis in irradiated cells in our experiment occurred mainly after 24 h, which belongs to IR-induced late apoptosis. This kind of apoptosis may be closely related to cell-cycle progression beyond the radiation-induced cell-cycle arrest.

Presently, it is generally believed that cell phase is crucial for determining cell radiosensitivity. The cell-cycle checkpoints pathway has been of interest to the field of irradiation biology because it is related to the radiosensitivity of cancer.

Such checkpoint mechanisms allow the cell time to repair the DNA damage before cell-cycle progression is resumed. In this experiment, the fraction of G₁ phase cells decreased significantly in MCF-7 transfected with AS3 at 24 h after IR (Table 1). Several studies indicated that increasing radiosensitivity by transfection of antisense oligonucleotide targeting p21 is associated with G₁ cell arrest block^[27–29]. Therefore, abrogation of G₁ cell arrest through inhibition of p53 gene expression might be an important cause for the increase of radiosensitivity in MCF-7 cells. In fact, Sak *et al*^[30] reported that antisense oligonucleotide targeting p53 transfer increased A549 (wt p53) apoptosis induced by IR, and decreased the fraction of G₁ and G₂ phase cells. However, in our experiment, we did not observe AS3 transfer decreased G₂/M phase cell at 24 h after IR. In contrast, a slight increase of G₂/M phase cells was observed in MCF-7 cells. Interestingly, AS3 transfection after IR also increased MCF-7 cells radiosensitivity. In addition to regulation of cell-cycle checkpoints that arrest cells, giving them time to repair damage, p53 also activates genes that are in some way more directly involved in DNA repair responses^[31]. However, it is believed that IR only induced p53-dependent DNA repair not but p53-dependent apoptosis under low dose radiation^[32]. We therefore speculate that the increased radiosensitivity by AS3 transfer after IR may be associated with down-regulation of DNA repair related genes expression.

In conclusion, antisense ODNs targeting against p53 transfer inhibit p53 gene expression efficiently. The inhibition of p53 abrogated G₁ phase arrest to a certain extent, and increased MCF-7 cell radiosensitivity. We speculated the increase of radiosensitivity might be associated with abrogation of cell arrest. Moreover, the developed efficient antisense ODNs may be used as tools for continuative investigations with regard to the role of p53 in tumor events.

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