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Interferon-α enhances sensitivity of human osteosarcoma U2OS cells to doxorubicin by p53-dependent apoptosis[1]

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Key words

Interferon-α; doxorubicin; apoptosis; p53; osteosarcoma

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Abstract

Aim: To determine whether interferon-α (IFNα) can enhance doxorubicin sensitivity in osteosarcoma cells and its molecular mechanism. **Methods:** Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis was studied using Flow cytometry analysis, Hoechst33258 staining, DNA fragmentation assay, as well as the activation of caspase-3 and poly (ADP-ribose) polymerase. Protein expression was detected by Western blotting. The dependence of p53 was determined using p53-siRNA transfection. **Results:** IFNα increased doxorubicin-induced cytotoxicity to a much greater degree through apoptosis in human osteosarcoma p53-wild U2OS cells, but not p53-mutant MG63 cells. IFNα markedly upregulated p53, Bax, Mdm2, and p21, downregulated Bcl-2, and activated caspase-3 and PARP cleavage in response to doxorubicin in U2OS cells. Moreover, the siRNA-mediated silencing of p53 significantly reduced the IFNα/doxorubicin combination-induced cytotoxicity and PARP cleavage. **Conclusion:** IFNα enhances the sensitivity of human osteosarcoma U2OS cells to doxorubicin by p53-dependent apoptosis. The proper combination with IFNα and conventional chemotherapeutic agents may be a rational strategy for improving the treatment of osteosarcoma with functional p53.

Introduction

Osteosarcoma is the most common primary malignant tumor of bone[1]. The current treatment is the combination of surgery and neoadjuvant chemotherapy using multidrugs, including methotrexate, doxorubicin, cisplatin and cyclophosphamide[2]. Although chemotherapy significantly increases patient survival, there is no effective treatment for metastatic osteosarcoma. The frequent acquisition of drug-resistant phenotypes and unwanted side-effects are often associated with chemotherapy and remain a serious problem[3]. Therefore, novel therapeutic strategies to increase chemosensitivity need to be developed.

Interferonα (IFN)-α, which belongs to the type I IFN, is a multifunctional cytokine exerting immunomodulatory, antiviral, and anticancer effects[4-6]. It interacts with the IFN-α/β receptor on the cell surface to induce the activation of JAK/STAT1 (Janus activated kinase/signal transducers and activators of transcription 1) pathway to regulate the transcription of the genes controlling antiproliferative activities[6].

p53 is a famous tumor suppressor gene which is commonly destroyed by mutation or deletion in malignant tumors including osteosarcoma[7]. It controls cell cycle arrest and apoptosis induced by chemotherapeutic agents including doxorubicin, by activating Bax, p21, PUMA (p53 Upregulated Modulator of Apoptosis) and Noxa, which are target genes of p53[8]. Recently, p53 was found to be correlated with Type I IFN-induced apoptosis in human cancer cells[9,10]. Thus we hypothesize that IFNá may cooperate with chemotherapeutic drugs to enhance antitumor effects by modulating p53-dependent apoptosis. To test this

hypothesis, we utilized the p53-wild and p53-mutant osteosarcoma cells to examine whether IFNα increases doxorubicin sensitivity to determine its molecular mechanism. We report that IFNα enhances doxorubicin sensitivity in osteosarcoma p53-wild U2OS, but not p53-mutant MG63 cells and define for the first time a p53-dependent apoptosis as the molecular mechanism. This work also supports the view that the proper combination of IFNα and conventional chemotherapeutic agents may be a rational strategy for improving the treatment of osteosarcoma with functional p53.

Materials and methods

Cell culture The human osteosarcoma U2OS cells[11] containing wild p53 and MG63 cells[12] containing mutant p53 were maintained in DMEM (Gibco BRL, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ºC in a 5% CO_2 humidified atmosphere.

Drugs and reagents Doxorubicin, Hoechst 33258 and MTT were obtained from Sigma (St Louis, MO, USA). Human recombinant IFNα2a were purchased from Peprotech (Rocky Hill, NJ, USA).

MTT assays The logarithmically growing U2OS and MG63 cells were seeded in 96-well plate at a density of 5×10^3 cells/well. After overnight growth, the cells were treated with IFNα and doxorubicin, alone or in combination. After the indicated time courses, 10 μL of 5 mg/mL MTT was added into each well followed by incubation for an additional 4 h. The supernatants were removed and 200 μL DMSO was added. After the crystals had dissolved, absorbance at 450 nm was measured in the microplate reader.

Flow cytometry analysis The cells were collected, washed twice with ice-cold phosphate-buffered saline (PBS), resuspended in cold PBS, and fixed with 70% ethanol. After fixation overnight and subsequent rehydration in PBS for 30 min at 4 ºC, the samples were stained for 30 min in the dark with 50 μg/mL propidium iodide (Sigma, USA) containing 125 units/mL protease-free RNase, both diluted in PBS in a flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Morphological analysis of apoptosis The cells were collected by centrifugation at 1000×g for 5 min, washed twice with PBS, and stained with 10 μg/mL Hoechst 33258 (Sigma, USA) for 15 min, followed by examination using a Olympus fluorescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan).

DNA fragmentation assay The cells (3×10^6) were collected and washed once with PBS. DNA was extracted using DNAZol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and electrophoresed on 2% agarose gel containing 0.5 μg/mL ethidium bromide. The gel was photographed with UV illumination.

Western blot analysis The cells were collected and lysed with the lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 25 mmol/L NaF, fresh 100 μmol/L Na_3VO_4 and l mmol/L dithiothreitol]. An equal amount of protein determined by Bradford assay was resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Grenzacherstrasse, Basel, Switzerland). The blots were incubated with primary antibody overnight at 4 ºC, followed with 3 washes in TBST [20 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 0.1% Tween-20] for 5 min, and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Signals were detected using enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposed to X-ray film (Kodak, Shanghai, China). All the antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RT-PCR Total RNA was extracted with TRIZol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. For the RT reaction, 2 μg RNA was combined with 0.5 μg oligo $(dT)_{15}$ (15 μL total volume). The mixture was incubated at 70 ºC for 5 min and placed on ice. Then 5 μL 5× M-MLV (moloney murine leukemia virus) reaction buffer, 1.25 μL 4× dNTP (10 mmol/L), 1 μL M-MLV (Promega, Madison, WI, USA; 200 U/μL), 0.625 μL RNaseOUT (40 U/μL) was added (25 μL total volume). The tube was incubated at 42 ºC for 60 min and then at 75 ºC for 10 min for termination. The PCR reaction was performed in the presence of *Taq* DNA polymerase, dNTP mix, and PCR buffer primers (all from Invitrogen, USA). After denaturation at 94 ºC for 2 min, the samples underwent 30 cycles of amplification (1 min at 94 ºC, 1 min at 58 ºC for p53 or 55 ºC for β-actin, and 1 min at 72 ºC) with a 10 min extension at 72 ºC following the last cycle. The sense and antisense primers were: 5'-CAG CCA AGT CTG TGA CTT GCA CGT AC-3' and 5'-CTA TGT CGA AAA GTG TTT CTG TCA TC-3' for p53 and 5'-ACT ACC TCA TGA AGA TCC TC-3' and 5'-CTA AAG ATT GCG TGG CGA GG-3' for β-actin. Products were electrophoresed on 1.5% agarose gels containing 0.5 μg/mL ethidium bromide.

Small interfering RNA transfection The U2OS cells were plated onto 6-well plates at a density of 3×10^5 cells/well with growth medium without antibiotics. After overnight incubation, transfection was performed at a confluency of 50% by using Opti-MEM media (Invitrogen, Carlsbad, CA, USA), Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA),

and specific or non-specific siRNA for p53 according to the manufacturer's recommendations. Six hours later, the medium was replaced with growth medium without antibiotics. After transfection for 20 h, the cells were trypsinized and sub-seeded onto 96 well plates. After incubation for another 4 h, the cells were treated as described for the MTT assay. The p53 siRNA duplexes were synthesized by Genepharma (Shanghai, China). The mRNA sequence to be targeted by p53-siRNA was 5'-CTA CTT CCT GAA AAC AAC G-3'.

Results

IFNα enhances doxorubicin-induced cytotoxicity in p53-wild U2OS, but not p53-mutant MG63 cells We first determined whether IFNα could enhance doxorubicin-induced cytotoxicity using MTT assay. After treatments with IFNα, doxorubicin or both for 72 h, the proliferation of the U2OS cells was not found to be inhibited by IFNα and was slightly suppressed by doxorubicin. However, doxorubicin-induced cytotoxicity was significantly enhanced by IFNα (Figure 1). In contrast, such effects were not observed in p53-mutant osteosarcoma MG63 cells (Figure 1). The MTT results show that IFNα enhances doxorubicin-induced cytotoxicity in p53-wild U2OS, but not p53-mutant MG63 cells. The results indicate that p53 may contribute to this phenomenon.

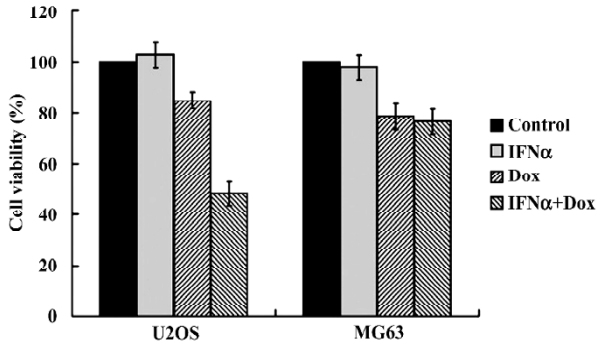

Figure 1. Effects of IFNα on doxorubicin-induced cytotoxicity in U2OS and MG63 cells. U2OS and MG63 cells were treated with IFNα (5000 U/mL) and doxorubicin (2 μmol/L), alone or in combination for 72 h. Cell viability was examined using MTT assay and shown as mean±SD. Experiment was repeated 3 times with similar results.

IFNα enhances doxorubicin-induced apoptosis in p53-wild U2OS, but not p53-mutant MG63 cells We next used flow cytometry analysis to evaluate the effect of IFNα on doxorubicin-induced apoptosis in U2OS and MG63 cells. The fraction of sub-G1 cells is a dependable way of determining cells undergoing apoptosis[13]. IFNα did not induce obvious apoptosis, but notably increased doxorubicin-induced apoptosis in p53-wild U2OS cells (Figure 2A), but not p53-mutant MG63 cells (Figure 2B). Moreover, Hoechst33258 staining, which stains nuclei to manifest apoptotic morphological change, revealed a higher level of nuclear condensation and fragmentation in the U2OS cells treated for 72 h with the IFNα/doxorubicin combination than either alone (Figure 3A). We next used agarose gel electrophoresis to further confirm apoptosis. The internucleosomal DNA fragmentation was observed in the U2OS cells treated with the IFNα/doxorubicin combination for 72 h, compared with either alone (Figure 3B). In contrast, such results were not obtained for the p53-mutant MG63 cells (data not shown). These results from different methods strongly indicated that IFNα enhanced doxorubicin-induced apoptosis in p53-wild U2OS, but not p53-mutant MG63 cells.

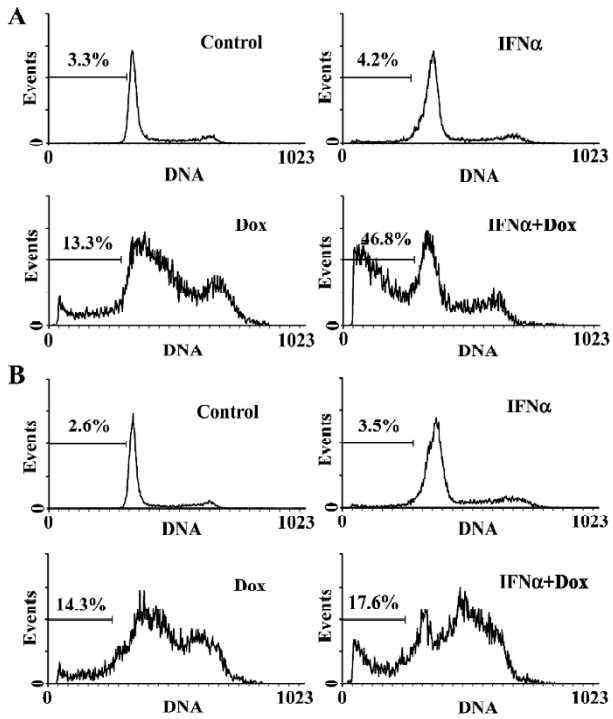

Figure 2. Effect of IFNα on doxorubicin-induced apoptosis in U2OS and MG63 cells by flow cytometry analysis. U2OS cells (A) and MG63 cells (B) were treated with IFNα (5000 U/mL) and doxorubicin (2 μmol/L) alone or in combination for 72 h. Apoptotic cells were examined by flow cytometry analysis. Experiment was repeated 3 times with similar results.

Caspase-3 is a key executor of apoptotic cell death signals by selectively cleaving proteins, such as poly (ADP-ribose) polymerase (PARP). Both caspase-3 activation and

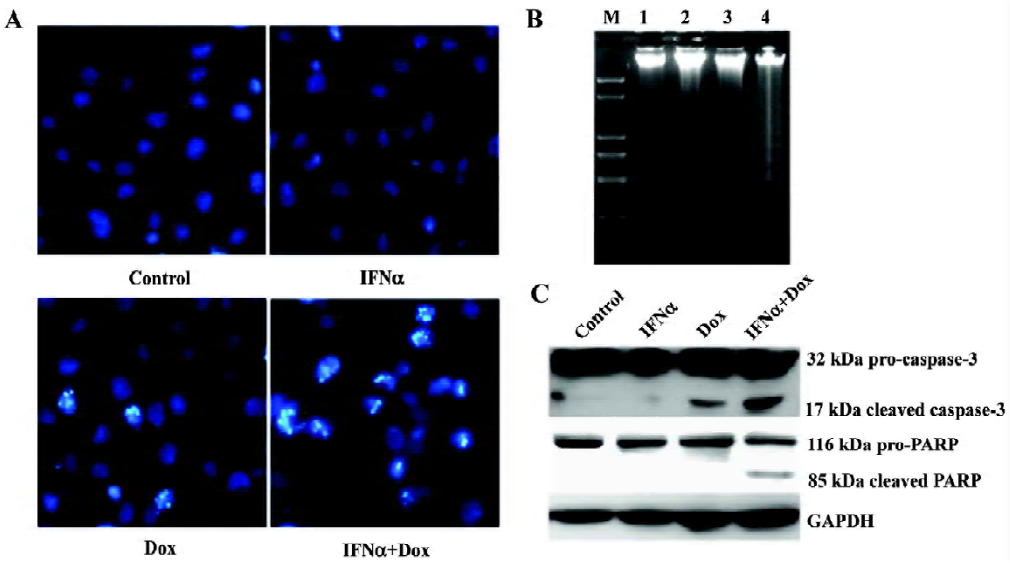

Figure 3. IFNα enhances doxorubicin-induced apoptosis in U2OS cells. U2OS cells were treated with IFNα (5000 U/mL) and doxorubicin (2 μmol/L) alone or in combination for 72 h. (A) apoptotic cells were determined by Hoechst 33258 staining of nuclei as a result of chromatin condensation and nuclear fragmentation. (B) DNA fragmentation assay was examined to confirm the ladder change representing apoptosis. Lane M: DNA molecular marker; lane 1: control cells; lane 2: IFNα-treated cells; lane 3: doxorubicin-treated cells; lane 4: IFNα/doxorubicin combination-treated cells. (C) IFNα increases caspase-3 activation and PARP cleavage in U2OS cells by Western blotting. Proteins were resolved by SDS-PAGE and blotted with antibodies for caspase-3 and PARP. GAPDH was used as a loading control.

PARP cleavage are hallmarks of apoptosis. We next used Western blotting for their detection them. Caspase-3 and PARP were cleaved to yield 17 and 85 kDa fragments in response to the IFNα/doxorubicin combination in the U2OS cells, respectively (Figure 3C).

IFNα activates p53 as a transcription factor in response to doxorubicin in osteosarcoma U2OS cells To determine whether IFNα affects the doxorubicin-induced activation of the p53 pathway, Western blotting was applied to examine the expression of p53 and well-known transcriptional target genes of p53, such as Bax, Bcl-2, Mdm2, and p21. The p53 protein level was unaltered by IFNα, but was enhanced by doxorubicin and further augmented by the IFNα/doxorubicin combination. The expression of pro-apoptotic Bax and p21 was further increased by the IFNα/doxorubicin combination than either alone. The Mdm2 expression level was also increased. Adversely, the expression of anti-apoptotic Bcl-2 was decreased by the IFNα/doxorubicin combination compared with either alone (Figure 4A). In contrast, such results were not observed in the MG63 cells (Figure 4A). In addition, RT-PCR showed that the p53 mRNA level was increased greatly by doxorubicin, but was not further augmented by the IFNα/doxorubicin combination in the U2OS cells (Figure 4B), suggesting that the combination-induced p53 protein upregulation was mediated in a post-transcrip-

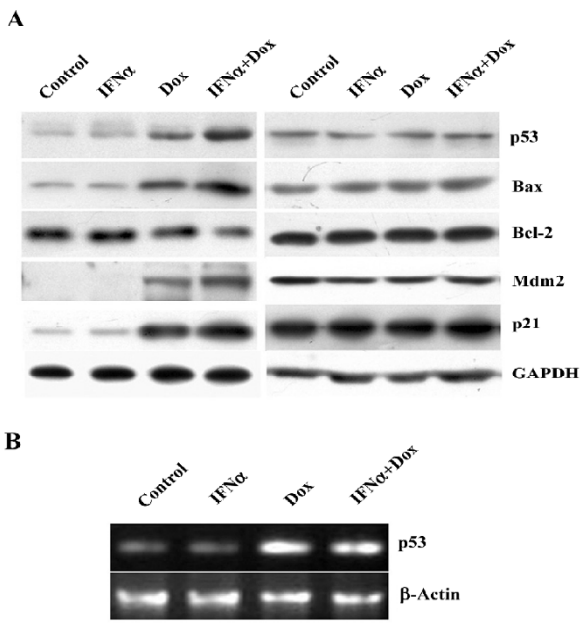

Figure 4. Effect of IFNα on the expression of p53, Bax, Bcl-2, Mdm2, and p21 in response to doxorubicin in osteosarcoma U2OS and MG63 cells. (A) cells were treated with IFNα and doxorubicin as indicated for 48 h. Expression levels of the indicated proteins were detected in the U2OS and MG63 cells by Western blotting. (B) mRNA level of p53 was examined at 48 h post-treatment in the U2OS cells using RT-PCR.

tional manner.

p53 silencing mediated by small interfering RNA in U2OS cells results in the decrease of cytotoxicity and apoptosis induced by the IFNα/doxorubicin combination To study the presence of the IFNα/β receptor in U2OS and MG63 cells, Western blotting was used to detect its expression. The IFNα/β receptor was found to be expressed equally in both cell lines (Figure 5A). To examine whether p53 was required for this enhanced apoptosis, we next used p53-specific siRNA transfection to suppress p53 expression in the U2OS cells. p53 siRNA effectively inhibited p53 protein expression in the U2OS cells treated with the IFNα/doxorubicin combination (Figure 5B). This p53 silencing significantly reduced the combination-induced cell death (Figure 5C). Furthermore, as key markers of apoptosis, caspase-3 activation and PARP cleavage were also completely suppressed in U2OS cells where p53 expression had been suppressed, compared with the nonspecific control (Figue 5D). These results show that the enhanced apoptosis induced by the IFNα/doxorubicin combination was p53-dependent.

Discussion

IFNα is an approved treatment option for tumor therapy, however; biological activity remains elusive. For example, IFNα induces cell cycle arrest, triggers apoptosis, and increases chemotherapy-induced cytotoxicity in specific cancer cells[14-16]. Recently, IFNα was reported to directly suppress the growth of some osteosarcoma cells[17]. However, the role of IFNα in the chemosensitivity of human osteosarcoma is largely unknown. In the present work, we showed that IFNα alone unaltered cell growth but promoted doxorubicin-induced cytotoxicity pronouncedly in osteosarcoma U2OS cells containing wild p53. Furthermore, the enhanced cytotoxicity was demonstrated to be mediated by apoptosis using FACS, Hoechst 33258 staining, DNA fragmentation, caspase-3 activation and PARP cleavage. In contrast, such effects were not observed in the p53-mutant MG63 cells. Although IFNα can directly induce apoptosis in a large group of tumor cells, such an effect was not observed in the U2OS and MG63 cells. This is not unusual because these 2 cell lines are both null of ARF (Alternative Reading Frame) [18], which is required for IFNα-induced apoptosis in specific cell types[19].

Although neoadjuvant chemotherapy shows a promising efficacy in treating osteosarcoma, the resistance against chemotherapy and drug-induced side-effects remain serious problems[2]. The topoisomerase II inhibitor doxorubicin is

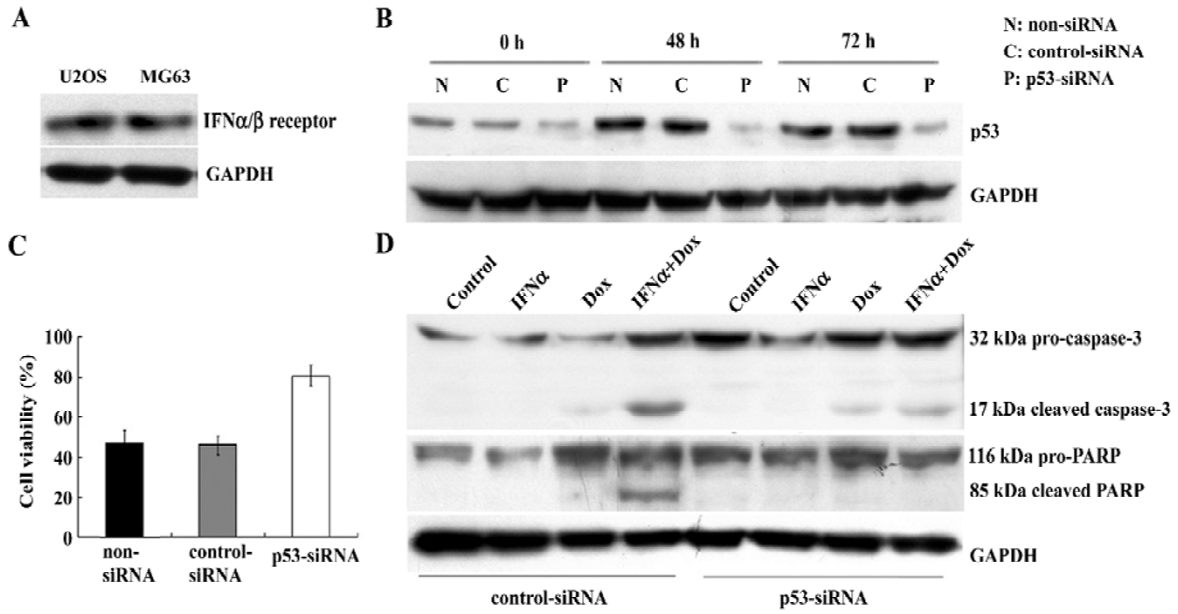

Figure 5. p53 silencing mediated by small interfering RNA in the U2OS cells results in a decrease of cytotoxicity and apoptosis induced by the IFNα/doxorubicin combination. (A) expression of the IFNα/β receptor in the U2OS and MG63 cells was detected by Western blotting. U2OS cells were transfected with p53 specific or non-specific siRNA duplexes and treated with the IFNα/doxorubicin combination. p53 protein level was examined by Western blotting (B). Cell viability was determined by MTT assay at 72 h post-treatment (C). Data is shown as mean±SD and is representative of 3 separate experiments. (D) U2OS cells were transfected with siRNA duplexes and treated as described for 72 h. Caspase-3 activation and PARP cleavage were detected by Western blotting.

an antitumor drug widely used in treating human osteosarcoma[1]. Topoisomerase II is a nuclear enzyme that functions during both DNA replication and transcription[2]. Doxorubicin is able to induce DNA damage and leads to cell cycle arrest or apoptosis by activating p53[2]. As a tumor suppressor gene which is often disrupted in human malignancies including osteosarcoma[8,20], the p53 gene product is also involved in type I IFN-induced apoptosis[9,10]. Based on these findings and our result that the enhanced apoptosis occurred in the p53-wild U2OS, but not p53-mutant cells, we hypothesize that p53 may contribute to the IFNα/doxorubicin combination-induced apoptosis. Our results showed that the enhanced apoptosis in response to IFNα/doxorubicin combination in the U2OS cells was associated with an accumulation of the p53 protein, which confirmed our hypothesis.

The Mdm2-p53 feedback loop is the main mechanism in the regulation of the p53 level[21]. Mdm2, another transcriptional target of p53, inhibits p53 by directly binding to it to antagonize its activity and enhance its degradation. Adversely, when the nuclear p53 level is elevated, it activates the transcription of the Mdm2 gene[21]. After treatment with the combination, Mdm2 expression was consistent with p53 upregulation. This is possibly because p53 upregulation induced Mdm2 expression according to this negative feedback loop. However, the p53 mRNA level did not further increase following the IFNα/doxorubicin combination, suggesting that p53 upregulation is mediated in a post-transcriptional manner. However, the exact mechanism is not clear.

Bax and Bcl-2, members of the Bcl-2 family, exert pro-apoptotic or anti-apoptotic functions respectively to regulate p53-dependent apoptosis[22]. The Bcl-2 protein is able to repress a number of apoptotic death programs[23]. The 21 kDa protein partner Bax, which overexpresses to counter the death repressor activity of Bcl-2, can enhance apoptosis. The ratio of Bcl-2 to Bax determines survival or death following an apoptotic stimulus[23-24] In this study, the combination subsequently increased Bax and decreased Bcl-2 expression resulting from p53 upregulation. The wild-type p53 gene is a negative regulator of cell growth by the transcriptional activation of p21 which plays a crucial role in controlling DNA repair, cell differentiation, and apoptosis in response to p53 activation[25]. Furthermore, the expression of p21 was also further increased by the combination. These events may induce the mitochondrial permeability transition, which can release cytochrome c and culminate in apoptotic cell death[22].

Caspase-3 is a key executor of the apoptotic machinery[26]. Once activated by apoptotic signals, caspase-3 is proteolytically cleaved to active its substrates, such as PARP, resulting in the activation of the DNA fragmentation of apoptosis[27]. Our study showed that IFNα markedly promoted caspase-3 activation and PARP cleavage in doxorubicin-treated U2OS cells, suggesting that a caspase-3 activation pathway was involved.

Although we proved that p53 activation is involved in enhanced apoptosis induced by the IFNα/doxorubicin combination, whether it is required for this effect remains unclear. We show here that siRNA-mediated p53 knockdown markedly decreased the apoptotic response to the IFNα/doxorubicin combination, as determined by the decrease of caspase-3 activation and PARP cleavage, indicating that p53 function is required for the IFNα/doxorubicin combination-induced cytotoxicity and apoptosis.

In conclusion, we demonstrated that IFNα enhanced the sensitivity of human osteosarcoma U2OS cells to doxorubicin by apoptosis, and defined a p53-dependent pathway as the underlying mechanism. The combination of IFNα and standard chemotherapeutic agents may help to achieve enhanced chemosensitivity and reduce the side-effects in treating osteosarcoma with functional p53.

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