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--Research Paper--

Suppression of p53R2 gene expression with specific siRNA sensitizes HepG2 cells to doxorubicin

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Abstract

Introduction: p53R2 is a p53-inducible protein that contributes to DNA repair by providing dNTPs in response to DNA damage. The roles of p53R2 in cancer cells and malignancies still remain controversial. Herein, we examined the effects of p53R2 silencing on HepG2 human hepatocellular carcinoma (HHC) cell line (wild-type p53) viability, apoptosis and cell cycle arrest in the presence and absence of doxorubicin. **Methods:** Cell transfection was performed using a liposomal approach. Gene silencing was determined by quantitative real-time PCR and western blot analysis. To evaluate the cell growth rate after transfection, trypan blue dye exclusion assay was employed. The cytotoxicity of the doxorubicin and p53R2 siRNA as single agents or in combination against HepG2 cell was analyzed by MTT assay and the drug combination effects was evaluated by calculating the combination index. The effects of treatments on different stages of cell cycle were analyzed by flow cytometry using propidium iodide (PI) and induction of apoptosis was assessed using DNA-histone ELISA. **Results:** We found that silencing of p53R2 alone had a strong effect on growth inhibition and spontaneous apoptosis in HepG2 cells. p53R2 siRNA synergistically enhanced the cytotoxic effect of doxorubicin. Furthermore, when used in combination with doxorubicin (0.4 μM), a significant increase in the rate of apoptosis was observed ($P<0.05$). Moreover, cell cycle at S and G2/M phases progressed at a lower rate after p53R2 combination treatment compared with doxorubicin mono-therapy. **Conclusion:** These findings suggest that siRNA-mediated silencing of p53R2 has great potential as a therapeutic tool and adjuvant in chemotherapy.

Key words: p53R2; siRNA, Doxorubicin, Chemotherapy, HepG2.

1. Introduction

p53R2 is a p53-inducible peptide, the substitute and homolog of R2 subunit of Ribonucleotide Reductase (RR) enzyme complex which is a rate limiting machinery in synthesis of dNTPs. RR is a tetramer composed of two dissimilar homodimers including hRRM1 (R1) and hRRM2 (R2) (1, 2). R1 and R2 are expressed in a cell cycle-dependent manner and their maximal level is during the S-phase. While R2 gene is regulated by cell cycle-associated transcription factors such as nuclear factor Y (NF-Y) and E2F, p53R2 is regulated by p53 in response to various genotoxic stresses, such as irradiation and exposure to chemotherapeutic agents such as doxorubicin (3-5). Upon DNA damage, p53 induces cell-cycle arrest in the G1 and G2 phases and enhances the expression and nuclear accumulation of p53R2, with subsequent induction of RR activity (6). Interestingly, p53 and p53R2 also interact at the protein level. In quiescent cells, p53 binds to p53R2 and R2 but not to R1. UV irradiation triggers the release of p53R2 and R2 from p53 (1).

In proliferating cells the levels of R1 is almost constant because of its long half-life and always in excess of R2 (7). In early G1-phase, R2 undergoes degradation by cadherin 1/anaphase promoting complex (Cdh1/APC) which binds to KEN box of R2 (8). The absence of a KEN box in p53R2 protects it from degradation in G1-phase. Therefore during G1, p53R2 associates with R1 instead of R2 to provide dNTPs for DNA repair in G1 which is the most important phase for DNA repair (8-10). Therefore, there are two independent pathways in providing dNTPs: i) R2 in S-phase and ii) p53R2 for DNA repair in cells arrested in G1 or G2-phase**.**

In addition to synthesis of dNTPs, p53R2 plays some other important roles involved in cell survival. The maximal levels of p53R2 have been detected in G1/S transition. Furthermore, through up-regulation of p21 and down-regulation of cyclin D, p53R2 causes cell cycle arrest in G1, thus providing both time and dNTPs for repair of damaged DNA. Based on the mentioned

facts, the blockage of p53-induced DNA repair could hypothetically help to enhance the efficacy of DNA-damaging chemotherapeutics. Moreover, p53R2 plays an essential role in mitochondrial integrity and mitochondrial DNA synthesis (2). This is confirmed by the fact that mutations in p53R2 are linked to severe depletion of mitochondrial DNA in both human and mouse cell lines (11, 12). Furthermore, p53R2 eliminates reactive oxygen species (ROS) and thereby protects mitochondrial membrane from oxidative stress-induced damage (13). p53R2 also, down-regulates MAPK/ERK signaling through direct interaction with MEK2 (14). Since MAPK/ERK signaling regulates numerous cellular processes including cell cycle progression and cell survival (15-17), the inhibition of this pathway by p53R2 can be yet another anticancer mechanism for p53R2. However, deregulated expression of p53R2 has been noted in various human cancers and p53R2 over-expression is associated with cancer progression and resistance to therapy (18).

Doxorubicin belongs to the anthracycline class of anticancer agents derived from Streptomyces bacteria. Doxorubicin is extensively used in treatment of solid tumors and hematologic malignancies, for example breast, ovarian and lung carcinoma, transitional bladder cell cancer, neuroblastoma, Wilms' tumor, soft tissue sarcomas, osteosarcoma, acute lymphocytic–lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), non-Hodgkin's lymphoma and Hodgkin's disease (19, 20). Although an exact mechanism of action for doxorubicin is yet to be revealed, several mechanisms have been proposed. For example, it has been suggested that doxorubicin intercalates into DNA and leads to production of free radicals. Alternatively, doxorubicin can inhibit nucleic acid synthesis, and suppress topoisomerase II at clinically-achievable concentrations resulting in DNA breaks (20-22). Furthermore, doxorubicin-induced accumulation of ceramide is required for separation and subsequently activation of N-terminal of CREB3L1 as transcription factor for transcription of numerous genes involved in inhibition of

cell proliferation such as p21 (23). However, p21 expression cannot suppress cell proliferation *per se*. CREB3L1 may therefore function as a master regulator of cell proliferation.

With regard to the existing overlap in downstream effectors and mechanisms of p53R2 siRNA and doxorubicin; such as affection of p21 expression, induction of DNA damage, inhibition of nucleic acid synthesis and subsequently effect on DNA replication/repair, it seemed highly possible that the combined treatment of hematologic cancers using p53R2 siRNA and doxorubicin, could synergistically enhance the cytotoxicity of doxorubicin in HepG2 cell line. This hypothesis has been put to test in this study.

2. Materials and Methods

2.1. Cell culture. Human hepatocellular carcinoma (HHC) cell line (wild-type p53) was acquired from Pasteur Institute of Iran (Tehran, Iran). Cells were maintained in RPMI-1640 medium supplemented with 10% FBS, and penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

2.2. siRNA Transfection and Treatment. The p53R2 specific siRNA and negative control (NC) siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Briefly, 1×10^5 cells/ml were seeded per well in 6-well cell culture plates with antibiotic-free RPMI-1640 medium supplemented with 10% FBS, then incubated at 37°C in a CO_2 incubator for 1 day. Then, cells were transfected with p53R2 siRNA or NC siRNA (at a final concentration of 100 nM) using a transfection reagent. Cells were incubated in the transfection medium for 6 h and then replaced with normal cell culture medium. The cells treated with only the transfection reagent were considered as a blank control. The suppression of p53R2 was measured by

quantitative real-time PCR (qRT-PCR) and Western blotting for 12, 24 and 48 h. For combination treatments, cells were incubated with doxorubicin 24 h after siRNA transfection.

2.3. qRT-PCR. Total cellular RNA was prepared from transfected cells using AccuZolTM reagent (Bioneer, Daejeon, Korea) as described by the manufacturer. cDNA was synthesized from 1 μg of RNA using AccuPower® RT PreMix (Bioneer, Daejeon, Korea) primed with Oligo-dT_{18} primers under conditions of 70˚C for 15 min, 42˚C for 60 min, and 95˚C for 5 min. The Rotor- GeneTM 6000 system (Corbett Life Science, Mortlake, NSW, Australia) was utilized for performing all amplification reactions. cDNAs were diluted 1:4 in nuclease-free distilled water and 5 μl of diluted cDNA was added to 20 μl of PCR mixture containing SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan) and 0.2 μmol/L of each primer. Primer sequences were for p53R2 forward, 5'-CCTTGCGATGGATAGCAGATAGA-3' and reverse, 5'-GCCAGAATATAGCAGCAAAAGATC-3'; and for β–actin forward, 5'-TCCCTGGAGAAGAGCTACG-3' and reverse, 5'-GTAGTTTCGTGGATGCCACA-3'. PCR conditions were 95^{o}C for 5 min followed by 30 cycles at 94^{o}C for 45 sec, 60^{o}C for 45 sec and 72^{o}C for 30 sec. Relative gene expression of p53R2 was calculated with the $2^{-(\Delta\Delta Ct)}$ method (24), using β-actin as the endogenous expression standard.

2.4. Western blot analysis. Cells were washed twice times with cold PBS and re-suspended in lysis buffer with freshly added inhibitors (1% SDS, 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 1mM EDTA, pH 8 and 150 nM NaCl). Cell lysates were centrifuged at 12500×g for 20 min at 4˚C and supernatants were collected. Soluble proteins were then quantified using a Bradford analysis (Bio-Rad Laboratories). Proteins are normally separated based on their molecular weight in SDS-PAGE electrophoresis. In our experiment, we used 15% gel, and also 5x sample loading buffer containing 10% SDS, 0.5% bromophenol blue, 60 mM of Tris-HCL(PH 6.8), 50%

glycerol, and 14.4 mM of 2-mercaptoethanol, was added to each tube and subsequently boiled for 5 min. For blots, 100 μg of protein samples was loaded in each well. The sample proteins were separated on a 15% polyacrylamide gel and transferred to nitrocellulose membranes (Thermo) for 48 min at 110 V, using transfer buffer (15.6 Mm of Tris-base, 120 Mm of glycine, and 10% methanol, pH=8.4). The blots were incubated in 3% skim milk in PBS-T (0.05% Tween-20 at room temperature for 2-3 hrs on a rotating platform), and subsequently using monoclonal antibodies against p53R2 and polycolonal anti β-actin (Abcam; Cambridge, UK) that described previously.

2.5. Cell proliferation assay. The antiproliferative effect of p53R2 silencing was assessed by trypan blue exclusion assay. In brief, cells (5×10^4 cells/well) were transfected with p53R2 siRNA and NC siRNA in 24-well cell culture plates for 5 days. At various time points, cells were harvested and then stained with 0.4% trypan blue (Merck KGaA, Darmstadt, Germany) for 2 min. The Number (N) of viable cells (unstained) was counted using a hematocytometer and an inverted microscope (Nikon Instrument Inc., Melville, NY, USA) and averaged over 3 independent experiments. The percentage of viability was determined using the following formula: Cell viability (%) = (N Treatment/N Control) × 100.

2.6. Cytotoxicity assay. The cytotoxicity was monitored using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, cells ($1x10^4$) were seeded in 96-well plates into eight groups: Dox, p53R2 siRNA, NC siRNA, p53R2 siRNA+Dox, NC siRNA+Dox, siRNA blank control, Dox blank control and combination blank control. After an incubation period of 24 h, 50 μl of MTT (2 mg/ml PBS) was added to each well and incubated for an additional 3 h. The medium was aspirated off and 200 μl of DMSO was added. Absorbance (A) was measured at 570 nm using an ELISA plate reader

(Awareness Technology, Palm City, FL, USA). Survival rate (SR) was calculated as the following formula: SR (%) = (A Treatment/A Control) ×100%. IC50 values (concentration that produced 50% cytotoxicity) were calculated by drawing the plots of cytotoxicity index (% CI=(1−(ODtreated/ODcontrol))×100) versus different concentrations of chemotherapeutic, and calculating the slop in Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

The coefficient of drug interaction (CDI) is calculated as below:

$$CI = \frac{D_1}{D_1 x} + \frac{D_2}{D_2 x} + \alpha\left(\frac{D_1 \times D_2}{D_1 x \times D_2 x}\right)$$

The cytotoxicity of NCsiRNA /Dox and p53R2 siRNA /Dox combinations was calculated by the formula, D1x is dose of drug 1 alone; D1 is dose of drug in combination with drug 2; D2x is dose of drug 2 alone; D2 is dose of drug 2 in combination with drug 1 ; and α=0 for mutually exclusive or 1 for mutually nonexclusive modes of drug action.

2.7. Cell cycle analysis. Cell cycle distribution was evaluated using propidium iodide (PI) staining followed by flow cytometry. Cells were washed with PBS, fixed in 70 % ethanol at 20˚C overnight. Cells were then centrifuged, washed in PBS, re-suspended in 500 µl PBS buffer solution containing RNaseA (10 µg/ml; Roche) and then incubated at room temprature for 30 min. Following RNaseA treatment, PI was added (10 µg/ml; Boehringer Mannheim Corp.) and incubated for another 20 min, protected from light. Analysis of cell cycle was done with FACS Caliber flow cytometer (BD Biosciences Clontech, Palo Alto, CA). The percentages of cells in the various phases of the cell cycle were evaluated on 50,000 cells with doublet discrimination.

2.8. Apoptosis assay. Apoptosis was measured using ELISA cell death detection kit (Roche Diagnostics GmbH, Germany) that quantify mono- and oligonucleosomes released into the cytoplasm of apoptotic cells. Briefly, the cells were lysed and centrifuged at 250×g for 10 min. 20 μl of the supernatants and 80 μl of immunoreagent containing antihistone-biotin and anti-DNA-peroxidase were then transfered to each well of a streptavidin-coated plate and the plate was incubated for 2 hrs in 25ºC. After washing with incubation buffer, 100 μl of 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) solution was added to each well. Finally, the reaction was stopped with stop solution and absorbance was determined with an ELISA plate reader at 405 nm (with a reference wavelength of 490 nm).

2.9. Statistical analysis. Data were presented as the mean±standard deviation (SD). Analysis of variance (ANOVA) followed by Bonferroni's test was used to determine the significant differences between groups. $p<0.05$ was considered significant. All statistical analyses were performed using Graph-Pad Prism software Version 6.

3. Results

3.1. siRNA effectively down-regulates p53R2 mRNA and protein levels in HepG2 cells. Firstly, the silencing effect of siRNA on p53R2 expression levels of mRNA and protein in HepG2 cells were investigated using qRT-PCR and Western blot. As shown in Fig. 1 and 2, p53R2 siRNA was markedly reduced the p53R2 mRNA and protein levels as compared to the control in a time-dependent manner ($p<0.05$). At 12, 24 and 48 h after the transfection, the relative p53R2 mRNA expression levels reduced to 39%, 15%, 12% of the blank control, respectively (Fig. 1) and the relative p53R2 protein expression levels reduced to 42%, 20% and

17%, respectively (Fig. 2). The NC siRNA did not affect the p53R2 expression levels compared to the blank control group.

3.2. Silencing of p53R2 inhibits the proliferation of HepG2 cells. To investigate whether down-regulation of p53R2 could inhibit the growth of HepG2 cells, the cells were transfected with p53R2 siRNA and NC siRNA and cell viability was then examined every 24 h for 5 days by trypan blue assay. The cell growth curve showed p53R2 siRNA significantly reduced cell viability over a period of 5 days ($p<0.05$; Fig. 3). After 24 h transfection of p53R2 siRNA, the cell viability decreased to 69.72 % and dropped to 54.66 % at the end of the experiment (day 5). However, No statistical differences in cell viability were found between the NC siRNA and the blank control group ($p>0.05$; Fig. 3).

3.3. p53R2 siRNA synergistically enhances the cytotoxic effect of doxorubicin in HepG2 cells. To analyze whether reduced p53R2 expression could enhance the sensitivity of HepG2 cells to doxorubicin, a combination treatment with p53R2 and doxorubicin was examined. As shown in Fig. 4, mono-treatment with doxorubicin induced cytotoxicity in a dose-dependent manner. We found that p53R2 siRNA by itself had a significant cytotoxic effect on HepG2 cells after 24 h ($p<0.05$) and lowered the cell survival rate to 77.50 %, relative to the blank control. Furthermore, its combination with doxorubicin for 24 h significantly enhanced cell killing capacity of doxorubicin ($p<0.05$) and the IC50 value of doxorubicin in HepG2 cells drastically decreased from 0.43 μM to 0.27 μM. The CDI values were also less than 0.9 in all concentrations of doxorubicin, which indicates the significant synergistic effects in combination therapy (Fig. 4).

3.4. p53R2 siRNA increase doxorubicin-induced cell cycle arrest in HepG2 cells. To assess whether down-regulation of p53R2 could regulate cell cycle distribution, p53R2 silencing and doxorubicin treatment on HepG2 cells was investigated at 24 h. As shown in Fig. 5, the percent of cells in G2/M phases after doxorubicin treatment elevated to 35%. p53R2 siRNA alone had no significant effects in the cell cycle arrest. Pre-treatment with p53R2 siRNA affected a 20% net increase in G2/M phases compared to blank controls after doxorubicin treatment. Therefore, the responses of HepG2 cells to combinational treatment caused a distinct cell cycle arrest compared to doxorubicin single treatment. These results propose that the increase in cytotoxic effects of p53R2 siRNA+doxorubicin on HepG2 cells may be exerted through the induction of other cellular processes, including apoptosis.

3.5. Suppression of p53R2 increases *doxorubicin*-induced apoptosis in HepG2 cells. To determine whether the observed synergistic cytotoxic effects between p53R2 siRNA and doxorubicin treatment were related to the increase in the extent of apoptosis, the apoptotic effects of p53R2 silencing and/or doxorubicin were investigated using an ELISA-based cell death detection system. As shown in Fig. 6, treatment of HepG2 cells with p53R2 siRNA or doxorubicin alone significantly increased apoptosis by 3.9 and 13.70 fold respectively, compared to the blank control. Furthermore p53R2 siRNA+doxorubicin further enhanced apoptosis to 26.14 fold ($p<0.05$). NC siRNA alone or in combination with doxorubicin showed no distinct changes in the extents of apoptosis relative to control or doxorubicin alone, respectively ($p>0.05$). These data indicate that the chemosensitization effect of p53R2 silencing is partially due to the enhancement of apoptosis.

4. Discussion

Doxorubicin administration is associated with some severe side effects such as myelosuppression, acute nausea and vomiting, alopecia and cardio-toxicity related to cumulative dose (25-27). On the other hand, chemoresistance and tumor relapse decrease the efficacy of doxorubicin (28). RNA interference (RNAi) technology is viewed as a promising tool for cancer therapy. siRNAs have been shown to synergistically enhance the cytotoxicity of anticancer agents (29-32). Therefore, in the present study we investigated the effect of doxorubicin and p53R2 siRNA combination therapy to enhance the efficacy of doxorubicin in inhibition of HepG2 cell cycle progression and cell proliferation. Not only such a combination might be more effective in treatment of cancer, also it can reduce the doxorubicin dose required for the treatment and as such, reduce its associated side effects in the clinic.

p53R2 has a dual role in cell proliferation and survival(33). p53R2 was suggested to mediate both cell cycle progression and subsequently exacerbate the malignancy, and cell cycle suppression, hence inhibition of cancer(34-42). Cancer can exploit p53R2 against different cytotoxic agents such as chemo/radio therapy. For example, Okumura et al. reported that siRNA silencing of p53R2 increased radiosensitivity in esophageal squamous cell carcinoma(43). It was shown that silencing p53R2 expression sensitizes both colorectal cancer HCT116-p53−/− and HCT116-p53+/+ cells to DNA damage agent, Adriamycin, through increase in apoptotic death(44). These findings indicate that targeting p53R2 could enhance the effectiveness of ionizing radiation or DNA-damaging chemotherapy and might amplify the efficacy of anticancer drugs such as doxorubicin.

p53R2 siRNA could successfully suppress the p53R2 mRNA and protein p53R2 expression in HepG2 cells in a time-dependent manner (Figure 1 and 2). Results of the trypan blue assay

indicated that the down-regulation of p53R2 significantly decreased the viability of cells compared with the blank control group during a 5-day period. These results show that p53R2 may play a critical role in the survival and growth of HepG2 cells. The MTT assay further demonstrated that pretreatment with p53R2 siRNA synergistically enhanced the cytotoxicity of doxorubicin in HepG2 cells, and the doxorubicin IC_{50} was substantially reduced from 0.43 μM to 0.27 μM. Therefore, not only down-regulation of p53R2 sensitized HepG2 cells to doxorubicin, but it also reduced the required doxorubicin dose, which can have great clinical implications with regard to the manifested side effects. p53R2 Based on the above may play an important role in cancer cell viability, and as such, p53R2 silencing can be considered as an adjuvant therapy along with different anticancer drugs to reduce the effective dose and enhance the efficacy of these drugs.

Doxorubicin disrupts DNA replication and repair by inhibition of topoisomerase II(45, 46). This results in induction of single and double strand breaks in DNA (47, 48). DNA is synthesized during S phase in the cell cycle, in which two exact copies of the chromosomes are produced. On the other hand, DNA repair is mostly done in G1 and at the second step in G2 phase (49). Therefore, doxorubicin is supposed to increase the number of cells in S and/or G1 phases by inhibition of DNA replication and repair. We also noted an increase in the number of cells in S and G2/M phases upon treatment with doxorubicin.

In addition, p53R2 has been shown to be regulated by p53 in response to various genotoxic stresses such as irradiation and exposure to chemotherapeutic agents such as doxorubicin (2, 9, 12). Following DNA damage, p53 induces cell-cycle arrest in the G1 and G2 phases(50, 51), and enhances the expression and nuclear accumulation of p53R2, with subsequent induction of RR activity, to produce dNTPs for DNA repair. Considering the role of p53R2 in dNTPs synthesis

during G1 and G2 phases, p53R2 siRNA is expected to increase the number of cells in G1 and/or G2 phases; however, the results of present study did not confirm this hypothesis. This is probably due to the fact that p53R2 has a more prominent role in providing dNTPs for cells under DNA stress than quiescent cells (7), such as those treated with siRNA alone.

In spite of the synergism in arresting cell cycle, doxorubicin and p53R2 siRNA have opposite effects on p21 expression. While doxorubicin induces p21 expression through ceramide synthesis (22, 23, 52), p53R2 siRNA decreases p21 through inhibition of p53R2 (53). P21 play an important role in G/S transition which is the most important checkpoint in cell cycle. However, due to the induction of DNA damage by both doxorubicin and p53R2 siRNA and subsequent induction of p53 -as the strongest p21 inducer (3), the increased expression of p53 compensates for the suppressed p53R2. However, a previous study has shown that a combination of doxorubicin and p53R2 siRNA can increase p21 in the mRNA level, but decrease p21 in the protein level in LNCa prostate cancer cell line (53).

The combination of doxorubicin and p53R2 siRNA increases the number of cells in the S phase of the cell cycle more effectively than when doxorubicin is used alone. Furthermore, the combination of doxorubicin with p53R2 siRNA can arrest cell cycle and subsequently suppress cell proliferation and growth.

The current study showed that p53R2 siRNA can induce apoptosis in HepG2 cells. It was also shown that the combination of doxorubicin and p53R2 siRNA increases the rate of apoptosis. This can get back to the synergism of these two treatment modalities in induction of DNA damage and subsequent execution of the intrinsic pathway of apoptosis. Previous study showed that an increase in activity of caspase-3 as an apoptotic enzyme following single p53R2 siRNA or combination therapy with doxorubicin(52). This may be linked to the role of p53R2 siRNA in

prevention of DNA repair especially with doxorubicin. Damaged DNA brings about p53 up-regulation and subsequently arrests cell cycle or finally induces apoptosis in case of intense DNA damage. On the other hand, caspase-3 up-regulation might be happening due to the direct effect of p53R2 in prevention of caspase-3 activation. Anyway, p53R2 siRNA augments the effect of doxorubicin in induction of apoptosis in HepG2 cancer cells.

In summary, we showed that p53R2 siRNA can enhance the cytotoxicity of doxorubicin in HepG2 cells especially through induction of apoptosis. Moreover combination therapy with doxorubicin decreased the dose required to kill cancer cells and can potentially decrease the side effects of doxorubicin in the clinic. Therefore, p53R2 siRNA with doxorubicin can be considered as a potential combination therapy strategy in treatment of the HCC.

Conflict of interest

The authors declare no conflict of interest.

The limitations of experimental design and future works. With regard to the relationship between p53R2 and p21 to more investigate the molecular mechanism of p53R2-targeting siRNA in treatment of the HepG2 cells, it is better to focus on the translocation ability of p53R2 into the nucleus, p21 expression level, p21 translocation to cytoplasm and the impairment of DNA repair activity by p53R2 silencing in HepG2 cells.

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Figure Legends

Fig. 1 Down-regulation of p53R2 mRNA expression levels by siRNA in HepG2 cells. Relative p53R2 mRNA expression level was quantified by qRT-PCR in HepG2 cells 12, 24 and 48

h after transfection. p53R2 siRNA significantly decreased the level of p53R2 mRNA all times. Data are shown as mean±SD (n=3); *p<0.05 versus blank control

Fig. 2 p53R2 protein expression level in HepG2 cells transfected with p53R2 siRNA. (A) Representative western blot showing the levels of p53R2 and β-actin proteins in HepG2 cells 12, 24, 48 h after exposure to siRNAs. (B) Relative p53R2 expression level of each sample was calculated using densitometry and normalized to the respective β-actin. Data are shown as mean±SD (n=3); *p<0.05 versus blank control

Fig. 3 Growth curve of HepG2 cells transfected with NC siRNA or p53R2 siRNA. Cell viability was determined by trypan blue staining in each day. Data are shown as the mean ± SD (n=3); *p<0.05 versus blank control

Fig. 4 Effects of P53R2 siRNA and doxorubicin on HepG2 cell survival 24 h after treatment with doxorubicin. The combination was treated with doxorubicin 24 h after transfection. The coefficient of drug interaction (CDI) was calculated to analyze the interaction effects between doxorubicin and P53R2 siRNA. Data are shown as the mean ± SD (n=4); *p<0.05 versus doxorubicin.

Fig. 5 Effect of p53R2 siRNA on the cell cycle arrest on HepG2 Cells. After silencing (24 h), doxorubicin was added and the cell cycle distribution was examined 24 h later using propidium iodide staining. (A) The flow cytometric histogram and the cell counts in each of the phases of the cell cycle. (B) The numerical representation of the

cells counts in each of the phases of the cell cycle. Results are of three independent experiments and are represented as the mean ± SD (n =3).

Fig. 6 Effect of p53R2 suppression on doxorubicin-induced apoptosis. Cells were exposed to p53R2 siRNA and doxorubicin, alone or in combination, as described in methods section. At 24 h after treatment, apoptosis was evaluated by ELISA cell death assay. Results are expressed as mean±SD (n=3); *p<0.05 compared to blank control; #p<0.05

Fig. 1

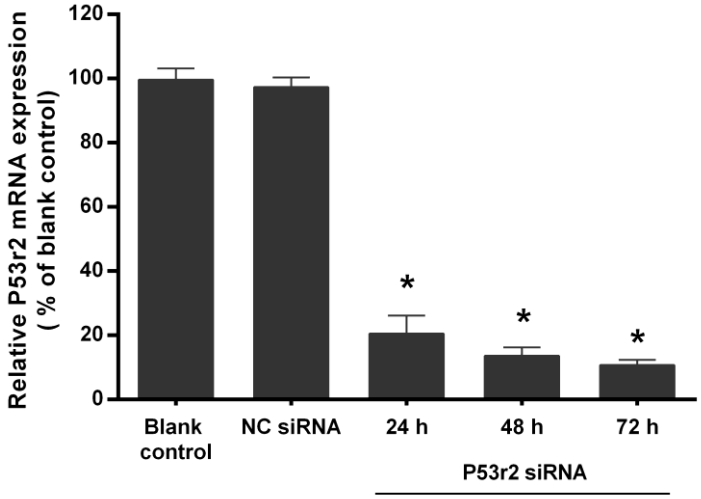


Fig. 2

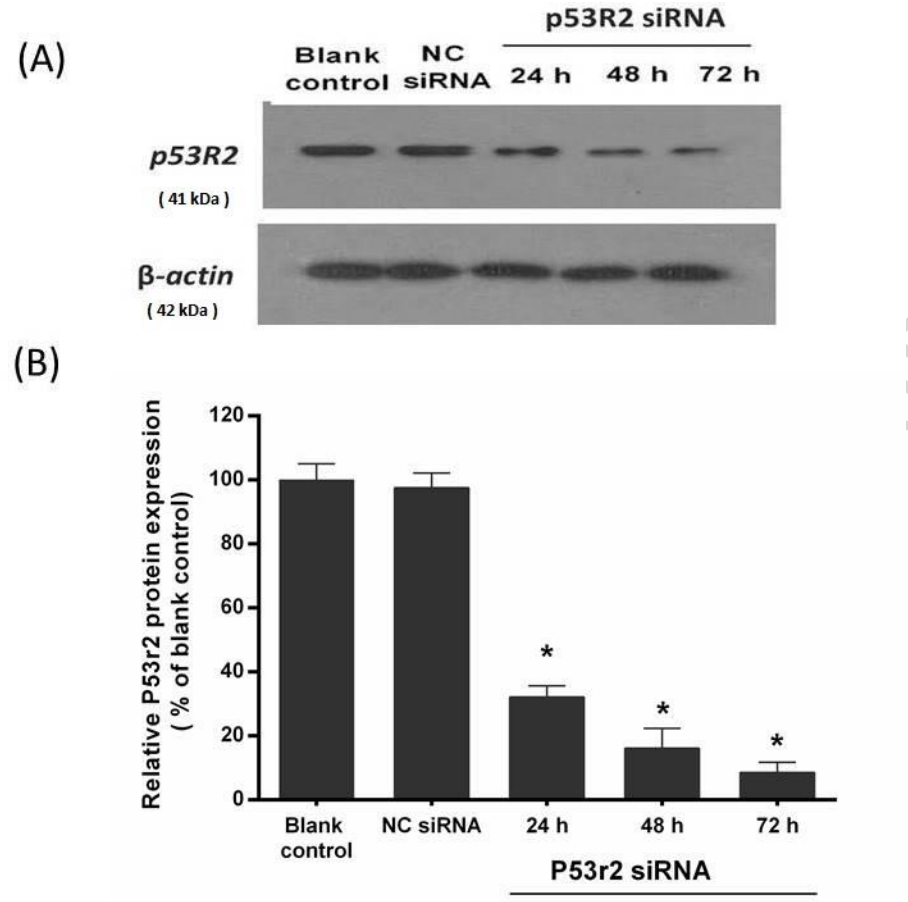


Fig. 3

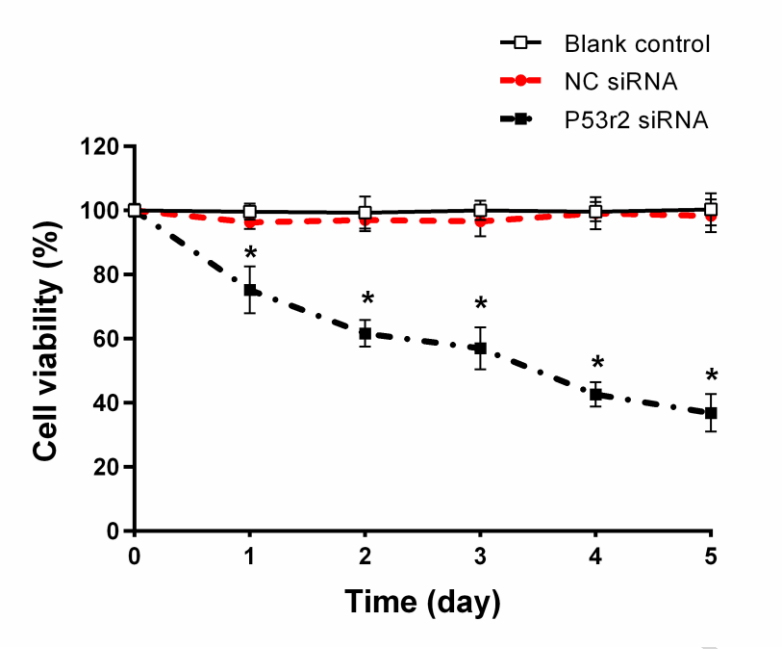


Fig. 4

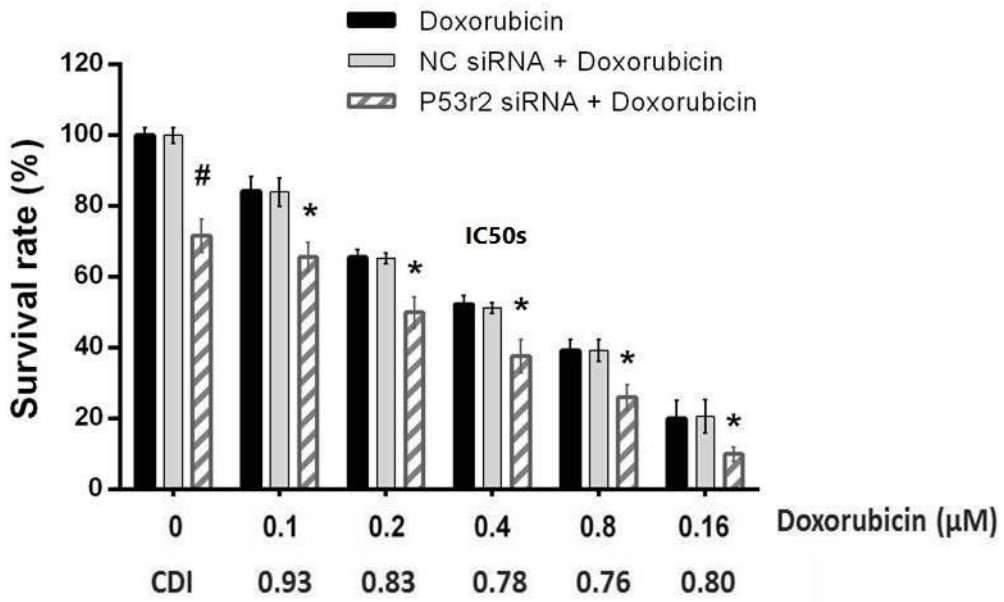


Fig. 5

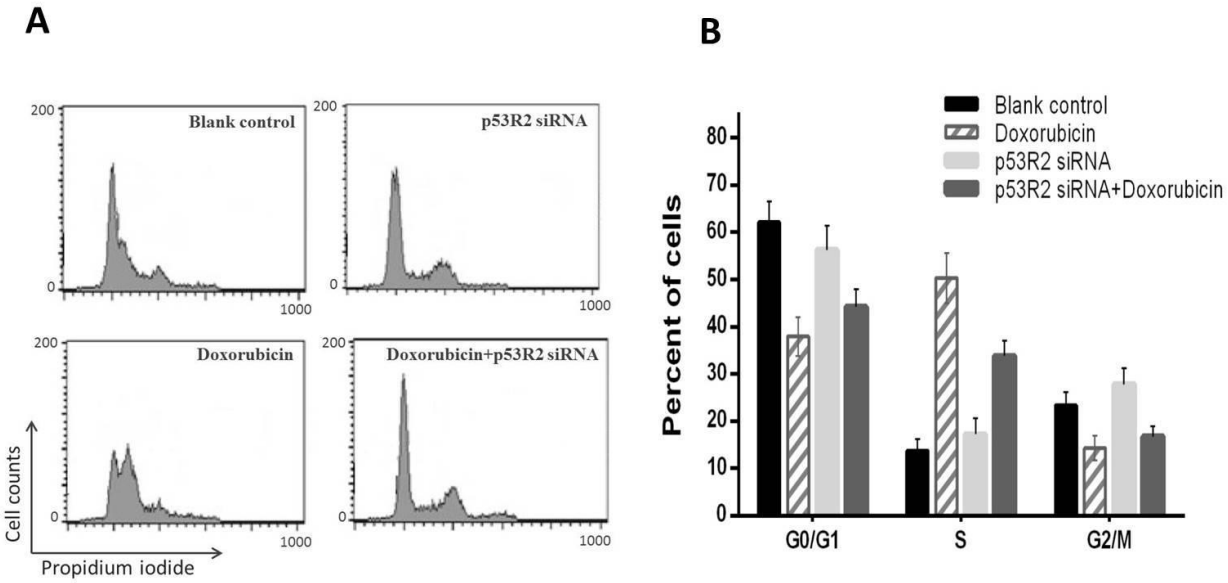
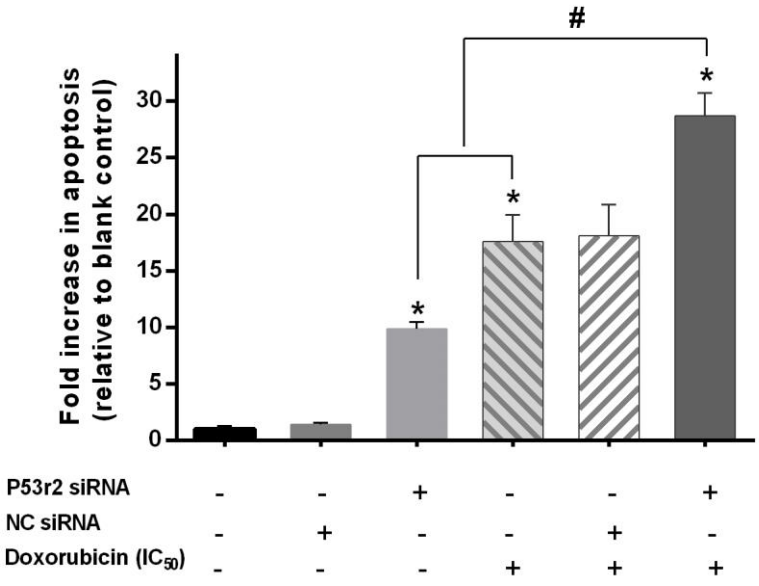


Fig. 6



Research highlights

p53R2 is a p53-inducible protein and provides dNTPs for DNA repair before the formation of cancer cells. After the formation of malignancy and their increasing demands dNTPs, p53R2 provides dNTPs required for cancer cell progression. siRNA-mediated silencing of p53R2 in HepG2 cells can effectively induce apoptosis and may be a potent adjuvant in chemotherapy.

Abrreviations:

dNTP , Deoxynucleotide

HHC, Hepatocellular carcinoma

PI, Propidium iodide

qRT-PCR, quantitative real-time PCR