RESEARCH COMMUNICATION

Cisplatin Induce Apotosis Via Upregulating Wrap53 in Osteosarcoma Cells U-2OS

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Abstract

Wrap531α, a newly identified natural antisense transcript of p53, can regulate p53 expression upon DNA damage. We sought to investigate changes in Wrap53 and p53 levels in an osteosarcoma cell line (U-2OS) exposed to cisplatin and to study apoptosis before and after knockdown of Wrap53. Our RT-PCR analysis showed a dose-dependent 3 to 40-fold increase in Wrap53 mRNA transcript levels in U-2OS exposed to 5 to 20 μM cisplatin. An approximate 2-fold increase was also observed in transcript levels of p53 mRNA. Furthermore, transient knockdown of Wrap53 by siRNAs in U-2OS cells treated with 10 μM cisplatin reduced p53 mRNA transcript levels by up to 50% of those of controls. Immunoblotting analysis showed that in U-2OS cells treated with siRNA against exon 4 of the Wrap53 gene, the protein level of p53 was also markedly reduced. Our findings suggest that cisplatin upregulates the expression of p53 in osteosarcoma cells by upregulating the transcript levels of Wrap53. Finally, measurement of apoptotic cell death by flow cytometry showed that knockdown of Wrap53 reduced apotosis in U-2OS cells induced by cisplatin.

Keywords: p53 - cisplatin - osteosarcoma - siRNAs - apotosis

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Introduction

Osteosarcoma is the second most common primary malignant bone tumor after multiple myeloma. The disease occurs predominantly in children, adolescents and young adults with a peak incidence during the second decade of life (Young and Miller, 1975).Currently, neoadjuvant chemotherapy followed by definitive resection with subsequent adjuvant chemotherapy is the accepted approach to the treatment of localized osteosarcomas. Chemotherapy can eradicate the micrometastatic disease that is believed to be present in the majority of patients with clinically resectable cancer and contributes to an improved outcome of osteosarcoma patients.

Cisplatin and oxaliplatin are widely used DNA cross-linking agents in the treatment of human cancer and has been used as part of the therapeutic regimen for osteosarcoma(Meyers et al., 2005). Cisplatin, a coordination complex that consists of Pt (II) bound to two ammine groups and two chloride ions, forms bivalent adducts with nucleophilic sites on purines in DNA, yielding predominantly DNA intrastrand crosslinks between adjacent purines. Lesions produced by cisplatin and other platinum-based compounds are widely known to activate the G1 and G2 cell cycle checkpoints. It has also been demonstrate that cisplatin can cause apoptosis in a p53-dependent manner (Caelles et al., 1994; Wagner et al., 1994; Fajac et al., 1996; Kanata et al., 2000). However, the mechanism where by p53 is activated in cells treated with cisplatin has not been fully defined.

WD40 encoding RNA antisense to p53 (Wrap53), a natural p53 antisense transcript that interacts with the 5'-untranslated region (UTR) of p53, is located on chromosome 17p13 and overlaps the p53 gene in a head-to-head fashion. Wrap53 also encodes a protein that is homologous to the WD40 protein family, while this protein have no obvoous regulation on p53 (Mahmoudi et al., 2009). Wrap53 regulates endogenous p53 mRNA levels and induces the expression of p53 protein upon DNA damage. Wrap53 have seventeen splice variants and only Wrap531α herein is called Wrap53 and has conclusive regulatory function on p53. Antisense transcription of Wrap53α has been proved to play a critical role in the activation of p53. When Wrap53α was upregulated, the function of p53 function was enhanced (Mahmoudi et al., 2009). Cisplatin is a powerful DNA crosslinking agent and induces predominantly DNA intrastrand crosslinks, and then causes apotosis of cells. Though the control of apoptosis by p53 have been well studied, will the activation of p53 have not been all clearly. As to chemotherapy, p53 has been shown upregulated upon DNA damages induced by cisplatin, its mechanism of activation of p53 in osteosarcoma cells exposed to cisplatin has remained undefined, and wrap53 whether

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play a key role in programmed cell death induced by cisplatin are not clearly. We hypothesized here that cisplatin may upregulate p53 expression in osteosarcoma cells exposed to cisplatin via upregulating Wrap53, and then cause apotosis. In the current study, we sought to investigate the changes in Wrap53 and p53 levels, and study the apotosis in an osteosarcoma cell line U-2OS cells exposed to cisplatin. We present here the first direct evidence that cisplatin upregulated the expression of p53 and cause apotosis in osteosarcoma cells by upregulating the transcript levels of Wrap53.

Materials and Methods

Cells and drug treatments

The human osteosarcoma cell line U-2OS was obtained from ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37 ºC. U-2OS cells (4×10^5/well, 6-cm dish) were cultured and treated with cisplatin (Sigma, St Louis, MO) at 5, 10 and 20 μM for 36 h. In addition, U-2OS cells were treated with 10μM for 6, 12, 24 and 36 h.

Measurement of Cell viability by MTT

Cell viability was measured by the modified MTT assay described previously (Van et al., 2002; Peng et al., 2010; Ze et al., 2010). Briefly, cells were seeded in 96-well plates in DMEM culture medium. After incubation of 24 hr for U-2OS cells solid cancer cells, experimental media containing either excipient control or were added to appropriate wells. Six concentrations of each cisplatin for 36-hr treatment were used to determine the vitro IC50 growth inhibitory values of cisplatin in cancer cells, and the concentration ranges are indicated in table 1. After incubation, 10μl of MTT (MTT, Sigma Chemical Co., St Louis, MO, USA) solution (5 mg/ml in ddH2O) was added to each well. The plates were then incubated for 4 hr at 37 °C. Intracellular formazan crystals were dissolved by addition of 100μl of isopropanol-HCI-SDS solution to each well. After an overnight incubation at 37 °C, the optical density of the samples was determined at 492 nm. Rate of inhibition was calculated by using the equation: Rate of inhibition = (Ac)At)/Ac · 100, where At and Ac represent the absorbance in treated and control cultures, respectively. The IC50 values were calculated by the SPSS 13.0 software (SPSS Inc, Chicago, IL, USA) using Probit analysis.

Real-time PCR

Total cellular RNA was extracted with the TRIZOL reagents (Invitrogen, Carlsbad, CA) and reverse-transcribed using the PrimeScriptTM RT Reagent Kit by the manufacturer's instructions (TaKaRa, Kusatsu, Japan). The sequences for the primers used in real-time PCR were as follows:

Wrap53,5'-TGGCAC AAAGC TGGA CAGT-3'(sense) and 5'-GC TGGGTC CTGGT CTGAAG-3'(antisense); p53, 5'-CGTC AGGG AGCAG GTAG-3'(sense) and 5'-GCTC GACGCT AGGAT CTGAC-3' (antisense); GAPDH 5'-GGAC CTGACCT GCCGT CTAG-3'(sense) and 5'-TAGC CCAGG ATGCC CTTGAG-3' (antisense).The sequence of the probe for Wrap531α was 5'-CAACC GTTAGCT CCGGA CTGCTG-3' and the primers for p53 were located in exon 1 and exon 2 (Hs00153340_m1) (assay ID). Quantitative real-time PCR was carried out using the Applied Biosystems 7300 using transcript-specific TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's recommendations.

Western blotting studies

A modified method as previously described was used (Dorsey et al., 2000; Liu et al., 2009). Cells were rinsed with ice-cold phosphate buffered saline (PBS) and lyzed with the lysis buffer containing 20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EGTA, 1 mM Na2EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na3VO4, and 1g/mL leupeptin. The cell lysates were then sonicated for 30 s. Cell debris was removed by centrifugation at 12,000 g for 10 min. The cell lysates were resolved by 12% SDS–PAGE electrophoresis and transferred to the PVDF membrane (Bio-Rad, Hercules, CA). The blots were incubated in 5% PBS for 5 min, and then the PVDF membranes were blocked with 5% nonfat dry milk in Tris borate saline containing 1% Tween 20 (TBST) for 1 h and incubated overnight with anti-p53 antibody at 4 ºC with gentle shaking. The blots were extensively washed with TBST. Then, the blots were incubated with horseradish peroxidase-conjugated secondary antibody in TBST for 1h at room temperature. The blots were then washed, and the signals were visualized by chemiluminescence and quantified and analyzed with Quantity-One (Bio-Rad).

Transfection with small interfering RNA (siRNA)

U-2OS cells were grown in 6 well plates and when the cells became 30 to 50% confluent, they were transfected simultaneously with three small interfering RNAs (siRNAs) against exon 1, 2 and 4 of the Wrap53 gene at a concentration of 5 nM for each using Lipofectamine™ RNAiMAX by the manufacturer's protocol (Invitrogen). The siRNA sequences for Wrap53 (exon 1) were 5'-CAG UCGC CAUGACAAGUAATT-3'(sense) and 5'-UUACU UGUCAU GGCGACUGTT-3'(antisense); for Wrap53 (exon2), 5'-GAGCC UUUCUGAAG AAGAATT-3'(sense) and 5'-UUCU UCUUC AGAA AGGCU CTT-3'(antisense); for Wrap53 (exon 4), 5'-GUU CCUGCAU CUUGA CCAATT-3'(sense) and 5'-UU GGUCA AGAUGC AGGAACTT-3'(antisense). A scrambled RNA sequence was used as control: 5'-AAAC GTGAC ACGTTCG GAGAA-3'(sense) and 5'-AATT CTCCG AACGT GTCACGT-3'(antisense). Transfected cells were treated with appropriate concentrations of cisplatin when they were 70% confluent.

Measurement of apoptotic cell death by flow cytometry

Apoptosis was determined on the single cell level by measuring the DNA content of individual cells by flow cytometry as described (Friedrich et al., 2001; Philipp

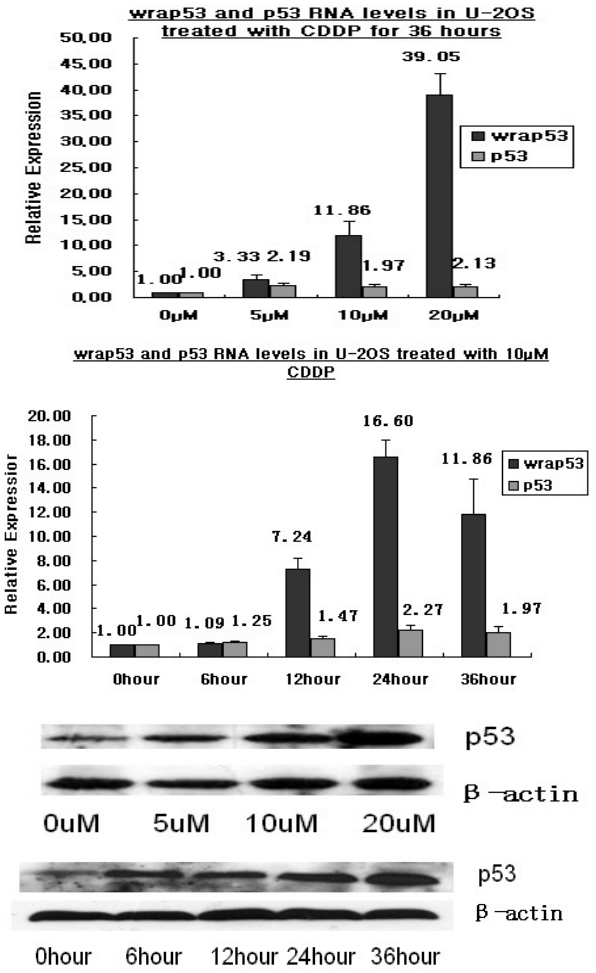

Figure 1. Measurement of Cell Viability by MTT in U-2OS Cells Treated with Cisplatin. Error bars represent the standard error of four independent experiments

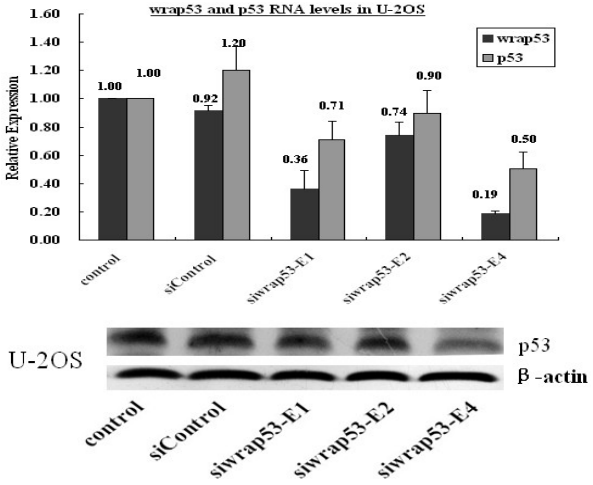

Figure 2. Wrap53 and p53 mRNA Levels in U-2OS Cells Treated with Cisplatin (CDDP) for 36 h (A).50.0 CDDP caused a dose-dependent increase in Wrap53 and p53 mRNA levels; Wrap53 and p53 RNA levels at different time points with 10 μM CDDP by quantitative real-time PCR (B); Immunoblotting assays showed that CDDP also caused an25.0 increase in the level of p53 protein

et al., 2002). Briefly, cells were seeded at a density of 5×10^5cells in 75 cm^2 flasks, cultured over night and treated with 10M cisplatin for 36 hours or infected with siRNAs targeted wrap53's 4exon for 32 hours and then treated with 10M cisplatin for 36 hours under serum free conditions, cells were trypsinized and collected by centrifugation at 300×g for 5 min, washed once with PBS at 4 °C and fixed in PBS/2% (v/v) formaldehyde for 30 min on ice. Thereafter, cells were incubated in 70% ethanol/ PBS (v/v) for 20 min on ice, pelleted and resuspended in PBS containing 40μg/ml DNase-free RNase A. After incubation for 30 min at 37
°C, cells were pelleted again and resuspended in PBS containing 50 mg/ml propidum iodide. Cellular DNA content was measured with a logarithmic amplification in the FL-2 channel of a FACScan flow cytometer equipped with the CELL-Quest software. Data are given in percent hypoploidy, which reflects the percentage of apoptotic cells with fragmented genomic DNA.

Results

Cisplatin upregulated the expression of Wrap53 and p53 in U-2OS cells when they were treated with 0 to 20μM cisplatin for 36 h. Our RT-PCR analysis showed a dose-dependent increase in Wrap53 mRNA transcript levels with an approximate 3-fold increase in Wrap53 mRNA transcript levels at 5 μM cisplatin and an approximate 40-fold increase in the transcript levels of Wrap53 mRNA at 20 μM cisplatin (Figure 1A). We further examined whether cisplatin affected the Wrap53 mRNA transcript levels in a time-dependent manner. Measurement of Cell viability by MTT determined that the vitro IC50 growth inhibitory values of cisplatin in U-2OS cells was approximately 10μM (Figure 3). So We treated U-2OS cells with 10 μM cisplatin over the course of 36 h and examined the Wrap53 mRNA transcript levels by RT-PCR. Our results showed that cisplatin indeed exerted a time-dependent effect on Wrap53 mRNA transcript levels. Ten μM cisplatin at 6 h caused no apparent change in Wrap53 mRNA transcript levels and at 12 h there was an approximate 7-fold increase in Wrap53 mRNA transcript levels, which peaked at 24 h post treatment with a 16-fold increase in Wrap53 mRNA transcript levels (Figure 1B). Cisplatin at 5 to 20 μM also caused an approximate 2-fold increase in the transcript levels of p53 mRNA ($P<0.05$ compared with controls) and, unlike Wrap53 mRNA, the increase in the transcript levels of p53 mRNA was not dose-dependent. Furthermore, cisplatin also exerted a time-dependent effect on p53 mRNA transcript levels with an approximate 1 to 2-fold increase in the transcript levels. Immunoblotting assays further revealed that the increase in p53 mRNA transcript levels in U-2OS cells treated with 5 to 20μM cisplatin was also accompanied by a corresponding increase in p53 protein levels (Figure 1C and 1D).

Wrap53 suppression aborted cisplatin-induced increase in p53 levels. We further investigated the effect of Wrap53 on p53 expression by transiently knocking down the expression of Wrap53 by siRNAs in U-2OS cells treated with 10 μM cisplatin. Our RT-PCR assays showed that siRNA against exon 1, 2 or 4 of the Wrap53 gene reduced the transcript levels of Wrap53 mRNA to approximately 20% to 80% of those of controls (Figure 2A). There was a concomitant decrease of p53 mRNA transcript levels to 50% to 90% of those of controls Immunoblotting analysis

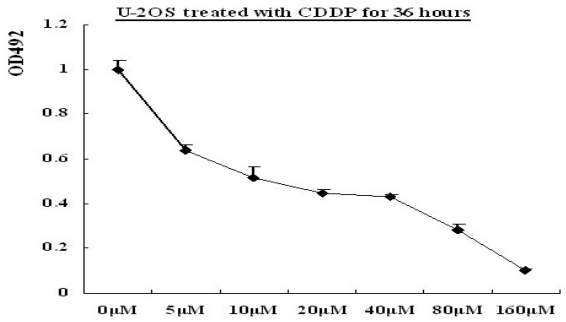

Figure 3. Depletion of Wrap53 in U-2OS Cells by siRNA Decreased p53 RNA and Protein Levels. U-2OS cells were transfected with siRNA against Wrap53 and scrambled siRNA control, and the mRNA levels of Wrap53 and p53 were determined by RT-PCR (A). The protein levels were analyzed by Western blotting assays (B)

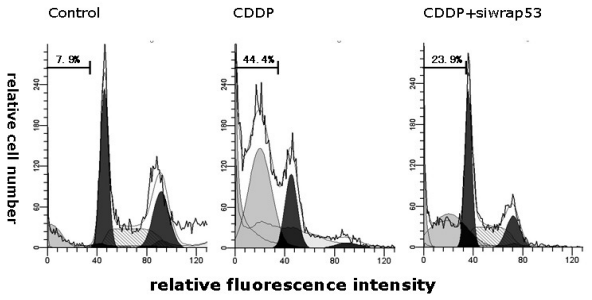

Figure 4. Measurement of apoptotic cell death by flow cytometry in U-2OS cells treated with cisplatin. Wrap53 played a key role in the apoptotic cell death induced by cisplatin in U-2OS cells. The rate of apotosis of U-2OS cells treated with 10 μM CDDP was 44.4%, while as knowdown wrap53 with siRNA taargeted exon4, the rate of apotosis in U-2OS cells induced by 10 μM CDDP was reduced to 23.9%

further showed that in U-2OS cells treated siRNA against exon 4 of the Wrap53 gene, the protein level of p53 was also markedly reduced (Figure 2B). These findings suggest that suppression of Wrap53 mRNA levels by siRNA aborted cisplatin-induced increase in the transcript levels.

Wrap53 suppression aborted cisplatin-induced apotosis in U-2OS cells treated with 10 μM cisplatin. We also studied U-2OS cells apotosis induced by 10 μM cisplatin for 36 h, the rate of apotosis was 44.4%, while when knowdown of Wrap53 mRNA levels by siRNA, this rate was reduced to 23.9% (Figure 4). These results above reveal that wrap53 may play a key role in the apotosis of osteosarcoma cells treated with cisplatin.

Discussion

Cisplatin induces apotosis in a variety of cell lines with predominantly DNA intrastrand crosslinks. While the exactm molecular biology mechanisms of apotosis induced by CDDP are vague, the most research of apotosis induced by CDDP in tumor cells was about p53 (Caelles et al., 1994; Wagner et al., 1994; Hirokazu and Katsunari, 2000). The protein encoded by TP53 controls multiple cellular functions including cell proliferation, DNA repair, senescence and apoptosis. p53 serves as a regulator of the apoptotic process that can modulate key control points in both the extrinsic and intrinsicpathways (Jordan and Scott, 2003). Tumor resistance to chemotherapeutic agents has also been reported to arise through failed p53 signaling (Gasco and Crook, 2003; Murray-Zmijewski et al., 2008). We have shown in this study that cisplatin upregulated the expression of p53, which is consistent with findings by other investigators. We further showed that cisplatin caused a dose-dependent increase in the transcript levels of Wrap53 mRNA. Additionally, suppression of Wrap53 by siRNAs aborted the cisplatin-induced upregulation in the protein and mRNA transcript levels of p53, and what's more apotosis induced by cisplatin was also reduced. This is consistent with the recent finding by others that Wrap53 is required for p53 induction upon DNA damage (Mahmoud et al., 2009), this also suggest that wrap53 play a key role in the p53-dependent apotosis induced by cisplatin in U-2OS osteosarcoma cells. Other investigators have shown that knowdown of Wrap53 causes a reduction in the protein and mRNA transcript levels of p53 not via blocking transcription but via posttranscriptional regulation (Mahmoudi et al., 2009). Although adjuvant chemotherapy results in significant improvement in patient survival, resistance against chemotherapy remains as a serious problem in the clinical treatment of osteosarcoma. Dysregulated p53 function is a major contributor to the development of multiple types of cancer and the p53 response to chemotherapeutic agents is a significant determinant of prognosis of a variety of cancer types. The p53 status also appears to determine cellular response to cisplatin in osteosarcoma (Fan and Bertino, 1999; Martelli et al., 2007). The control of p53 is complex with tight regulation at several levels, both posttranscriptional and posttranslational. The upregulation of p53 in response to cisplatin in osteosarcoma cells has not been fully elucidated. Our identification of Wrap53 as a novel regulator of p53 in the cellular response of osteosarcoma cells to cisplatin sheds further light into the regulatory mechanisms of p53 action in response to DNA damage and shows that the activities of p53 could be regulated by RNAs. Our study also opens the possibility of using regulatory RNAs such as Wrap53 to modulate cellular response to chemotherapeutic agents. However most of the osteosarcoma cell lines are p53 mutant (Diller et al., 1990), many investigator rebuilded p53 expression in these tumor cell lines to recover sensitivity to chemotherapeutics, from our studies recovery of p53 without wrap53 in p53 mutant cell lines cant't get a suitable p53 expression like normal. Future studies will explore whether upregulation or downregulation of Wrap53 in p53 wild and mutant cell lines could enhance or suppression p53 expression in vivo and modulate the viability of osteosarcoma cells both in vitro and in vivo.

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