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Overexpression of HIPK2 circumvents the blockade of apoptosis in chemoresistant ovarian cancer cells

Rosa Puca [a], Lavinia Nardinocchi [a], Giuseppa Pistritto [b], Gabriella D'Orazi [a,c,*]

[a] Department of Experimental Oncology, Molecular Oncogenesis Laboratory, Regina Elena Cancer Institute, 00158 Rome, Italy
[b] Department of Neuroscience, University "Tor Vergata", 00133 Rome, Italy
[c] Department of Oncology and Neurosciences, University "G. d'Annunzio", 66013 Chieti, Italy

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Abstract

Objective. Chemoresistance, due to inhibition of apoptotic response, is the major reason for the failure of anticancer therapies. HIPK2 regulates p53-apoptotic function via serine-46 (Ser46) phosphorylation and activation of p53 is a key determinant in ovarian cancer cell death. In this study we determined whether HIPK2 overexpression restored apoptotic response in chemoresistant cancer cells.

Methods. Using cisplatin chemosensitive (2008) and chemoresistant (2008C13) ovarian cancer cell lines we compared drug-induced activation of the HIPK2/p53Ser46 apoptotic pathway. The levels of HIPK2, Ser46 phosphorylation, and PARP cleavage were detected by Western blotting. The p53Ser46 apoptotic commitment was evaluated by luciferase assay using the Ser46 specific AIP1 target gene promoter. The apoptotic pathway was detected by caspase-3, -8, and -9 activities.

Results. HIPK2 was expressed differently in sensitive versus chemoresistant cells in response to different chemotherapeutic drugs (i.e., cisplatin and adriamycin), though the p53Ser46 apoptotic pathway was not defective in chemoresistant 2008C13 cells. Thus, 2008C13 cells were resistant to cisplatin but sensitive to adriamycin-induced apoptosis through activation of the HIPK2/p53Ser46 pathway. HIPK2 knock-down inhibited the adriamycin-induced apoptosis in 2008C13 cells. Exogenous HIPK2 triggered apoptosis in chemoresistant cells, associated with induction of p53Ser46-target gene AIP1.

Conclusions. HIPK2 is an important regulator of p53 activity in response to a chemotherapeutic drug. These results suggest that different drug-activated pathways may regulate HIPK2 and that HIPK2/p53Ser46 deregulation is involved in chemoresistance. Exogenous HIPK2 might represent a novel therapeutic approach to circumvent inhibition of apoptosis in treatment of chemoresistant ovarian cancers with wtp53.
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Keywords: HIPK2; p53; Ovarian cancer; Chemotherapeutic drugs; Apoptosis; Caspase activity

Introduction

Ovarian cancer is the primary cause of death from gynecological malignancies. Although cisplatin (CDDP) has been used successfully to treat several types of cancers, including ovarian cancer, both intrinsic and acquired resistance to cisplatin occurs frequently to severely limit treatment success [1]. However, molecular mechanisms that underlie chemoresistance are largely unknown. As for many anticancer drugs, CDDP exerts its antitumoral effects by inducing apoptosis; therefore functional cell death machinery is necessary to allow successful treatment.

The mechanisms of chemoresistance appear to be multifactorial and include among others, inhibition of apoptosis and dysfunction of p53 oncosuppressor [2,3]. P53 is a key regulator of apoptosis through induction of both the intrinsic and extrinsic apoptotic pathways [4]. P53 is activated by cellular and genotoxic stresses through a large number of posttranscriptional modifications such as phosphorylation, acetylation, methylation, and ubiquitylation [reviewed in ref. 5,6], meaning that a very large number of proteins can regulate upstream p53 for an efficient oncosuppressor function. Importantly, p53 regulatory proteins are themselves in turn regulated by other interacting or

* Corresponding author. Fax: +39 06 52662505.
E-mail address: gdorazi@unich.it (G. D'Orazi).

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modification proteins induced differently from various processes (such as DNA damage) [7]. Among the multiple proteins that regulate p53 activity in response to genotoxic stress is the homeodomain interacting protein kinase 2 (HIPK2) that induces a p53 apoptotic pathway. HIPK2 is a serine–threonine kinase that belongs to a family of transcriptional co-repressors [8]. HIPK2 is activated in response to various types of DNA-damaging agents, including ultraviolet (UV) and ionizing irradiation (IR), cisplatin, adriamycin (ADR) and roscovitine chemotherapeutic drugs [9–14]. HIPK2 phosphorylates p53 at Ser46 for specific activation of proapoptotic target genes, including p53AIP1, PIG3, Bax, Noxa, and KILLER/DR5 [9–11,15] and contributes to the regulation of p53-induced apoptosis. Recent work elucidated some mechanisms regulating HIPK2 function during the initiation and execution phases of apoptosis [16]. After activating p53 HIPK2 is subsequently cleaved by p53-dependent caspase activation and thus the HIPK2 cleaved form shows a higher capacity to phosphorylate p53Ser46 before disappearing at the end of the apoptotic phase [16]. This regulatory pathway allows a tight and highly integrated control of p53-apoptotic activity [17]. On the other hand, the signalling pathways and the molecules involved in HIPK2 activation in response to different stimuli are very poorly understood. This consideration led us to examine the role of HIPK2 in response to chemotherapeutic drugs in a cisplatin-sensitive 2008 ovarian cancer cell line and in its resistant counterpart 2008C13 and whether HIPK2 overexpression could circumvent the inhibition of apoptosis induced by chemoresistance.

In this study, we have used chemosensitive-2008 and chemoresistant-2008C13 ovarian cancer cell lines as an *in vitro* model. We found that the HIPK2 apoptotic function appeared to be differently regulated in response to different chemotherapeutic drugs (i.e., cisplatin and adriamycin) in sensitive versus chemoresistant cells, although the p53Ser46 apoptotic pathway was not defective in chemoresistant-2008C13 cells. These results suggest that different drug-activated pathways might regulate HIPK2 and that HIPK2/p53Ser46 deregulation was involved in chemoresistance. Finally, HIPK2 overexpression circumvented inhibition of apoptosis in chemoresistant cells. Thus, exogenous HIPK2 might represent a novel therapeutic approach to induce apoptosis in chemoresistant ovarian cancers with wtp53.

Materials and methods

Cell lines and treatments

Cisplatin sensitive human ovarian carcinoma cell line 2008 and its resistant variant 2008C13, both carrying endogenous wtp53 (kindly provided by Dr. S.B. Howell, University of San Diego, La Jolla, CA, USA) were maintained in RPMI-1640 (GIBCO-BRL, Life Technology, Grand Island, NY) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO-BRL) plus glutamine and antibiotics, in a humidified atmosphere with 5% CO_2 at 37 °C. The 2008 cell line, established from a patient with serous cystadenocarcinoma of the ovary, and its resistant subclone 2008C13, derived from 2008 cells by *in vitro* exposure to CDDP, have been previously characterized by Howell et al. [18] and Chaney et al. [19]. Unless otherwise specified, 5 µg/ml CDDP and 2 µg/ml ADR were added to the cells in culture medium at 24 h before harvest. The 5 µg/ml dose of CDDP and the 2 µg/ml dose of ADR were chosen because of their ability to induce HIPK2 activation, p53Ser46 phosphorylation, and apoptosis, as previously shown [11,20].

Western immunoblotting

Cell were washed in ice-cold phosphate-buffered saline (PBS), collected by trypsinization, rinsed with PBS, and lysed for 20 min on ice in lysis buffer. For total cell extracts, cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.5; 1 mM ethylene diamine tetraacetic acid — EDTA; 150 mM NaCl; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate — SDS; 1% Nonidet P-40) or in high-salt lysis buffer (for HIPK2 detection) (50 mM Tris–HCl pH 7.5; 300 mM NaCl; 5 mM EDTA pH 8; 1% NP-40) plus a mix of protease inhibitors (Sigma Chemical Company, St. Louise, MO), sonicated and spun at top speed for 15 min. For subcellular fractionations, cells were lysed in hypotonic buffer (10 mM HEPES, pH 7.9; 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid — EGTA) plus NP-40 to a final concentration of 0.5%. After 15 min in ice cells were spun at top speed to collect the cytoplasmic fraction (supernatant). The remaining pellet was washed with hypotonic buffer, resuspended in lysis buffer, sonicated and spun at top speed for 20 min to remove debris and collect the nuclear fraction (supernatant). Equal amounts of total cell lysates were mixed with Laemmli sample buffer, resolved on 9–12% SDS-polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene fluoride membrane (PVDF, Millipore, Bedford, MA, USA). After transfer, membranes were stained with ponceau stain (Sigma) to verify uniform loading and transfer. Western immunoblot was performed by incubating with primary antibodies followed by anti-immunoglobulin–G-horseradish peroxidase antibody (BioRad Laboratories, Inc., Hercules, CA, USA).

The antibodies used were: anti-p53 (DO1) mouse monoclonal (Santa Cruz Biotechnology), anti-p53Ser46 rabbit polyclonal (Cell Signaling Technology, Danvers, MA, USA), anti-PARP mouse monoclonal (BD Pharmingen, San Diego, CA, USA), anti-HIPK2 rabbit polyclonal (kindly provided by M.L. Schmitz, Justus-Liebig-University, Giessen, Germany), and anti-tubulin mouse monoclonal (Immunological Sciences). Immunoreactivity was detected by the enhanced chemiluminescence (ECL) reaction kit (Amersham Corp., Arlington Heights, IL) in accordance with the manufacturer's instructions.

Transfection and plasmids

Transient transfection was carried out using the *N*,*N*-bis-(2-hydroxyethyl)-2-amino-ethanesulphonic acid-buffered saline (BBS) version of the calcium phosphate procedure [21]. The amount of plasmid DNA in each sample was equalized by supplementing with empty plasmid. The comparable transfection efficiency between 2008 and 2008C13 cells was assayed by the use of a fluorescent microscope after co-transfecting with a green fluorescent protein (GFP) tagged vector. The expression vectors used in this study were: wild-type HIPK2-Flag and its kinase defective K221R-Flag mutant [9]; pCAG3.1wtp53, p53S46A (nonphosphorylatable Ser46) (kindly provided by Dr. E. Appella, NIH, Bethesda, MD, USA) [9], and p53S46D mutants (phosphorylation mimic) (kindly provided by Dr. L. Mayo, Case Western Reserve University, Cleveland, Ohio, USA); dominant-negative mutants specific for caspase-8 (DN-caspase-8) and caspase-9 (DN-caspase-9) (kindly provided by Dr. Valerie Castle, University of Michigan, MI, USA); and pSUPER vectors carrying HIPK2 or aspecific RNA-interfering sequences [11].

Viability assay

Exponentially proliferating cells were transfected with HIPK2, kinase defective K221R mutant and Flag-empty expression vectors. Soon after transfection, cells were trypsinized and re-plated in duplicate for assessment of cell death at daily intervals by direct counting with a hemocytometer. Both floating and adherent cells were collected and cell viability was determined by trypan blue exclusion. The percentage of viable cells, i.e. blue/total cells, was determined by scoring 100 cells per field for three times. Unless specified numbers are means of three different experiments.

Transactivation assay

Cells were plated in 60 mm dishes and transiently transfected with the luciferase reporter driven by the p53-dependent promoter AIP1 (kindly provided by H. Arakawa, National Cancer Center, Tokyo, Japan), using the BBS-version

of the calcium phosphate procedure and either treated with CDDP and ADR for 24 h or co-transfected with HIPK2 and K221R expression vectors. The amount of plasmid DNA was equalized in each sample by supplementing with empty vector. Thirty-six hours after transfection luciferase activity was assayed. Transfection efficiency was normalized with the use of a co-transfected CMVβ-galactosidase (β-gal) plasmid. Luciferase activity was assayed on whole-cell extract and the luciferase values were normalized to β-gal activity and protein content. At least three independent experiments were performed in duplicate.

Caspase activity

For caspase activity both adherent and floating cells were collected by centrifugation at 1100 rpm for 10 min. After washing with PBS, the cell pellets were lysed in ice-cold lysis buffer provided by the caspase assay kit (Biovision, Mountain View, CA, USA), according to the manufacturer's instructions. Total cell lysates were centrifuged at top speed for 20 min at 4 °C. The resulting supernatants were analysed for protein concentration using the Lowry method for normalization of assay results on a protein basis. The caspase fluorometric enzymatic activity assay was carried out following the manufacturer's instructions. The fluorogenic substrates were DEVD-amino-4-trifluromethyl coumarin (AFC) (caspase-3), IETD-AFC (caspase-8), and LEHD-AFC (caspase-9) (Biovision, Mountain View, CA, USA). At least three independent experiments were performed.

RNA extraction and reverse transcriptase-PCR (RT-PCR)

Equal number of cells was harvested in TRIzol Reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions. The first strand cDNA was synthesized using the Moloney murine leukemia virus reverse transcriptase enzyme according to the manufacturer's instructions (Applied). Semi-quantitative Reverse-Transcriptase PCR was carried out with HOT-MASTER Taq enzyme (Eppendorf) using 2 µl cDNA reaction and HIPK2 specific oligonucleotides [11] under conditions of linear amplification. PCR was performed in duplicate in two different sets of cDNA. PCR products were run on a 2% agarose gel and visualized with ethidium bromide. The housekeeping GAPDH mRNA, used as internal standard, was amplified from the same cDNA reaction mixture.

Results

Deregulation of HIPK2/p53Ser46 expression in cisplatin-resistant cells

HIPK2 takes a significant part in mediating apoptotic response, including that induced by p53Ser46 activation following cisplatin treatment [11]. To investigate the role of HIPK2 in chemoresistance we first analysed gene expression in cisplatin-sensitive 2008 and its resistant counterpart 2008C13 cells and found similar HIPK2 mRNA expression levels (Fig. 1A). Next, HIPK2 protein levels were analysed in 2008 and 2008C13 cells in the presence or absence of CDDP treatment as it is known that the levels of HIPK2 protein increase after DNA damage such as UV irradiation, cisplatin, and ADR treatments [9–11,14]. As shown in Fig. 1B, the administration of different doses of CDDP to 2008 and 2008C13 cells resulted in comparable induction of the endogenous HIPK2 protein levels; however, the 5 µg/ml dose of CDDP induced upregulation of HIPK2 only in resistant 2008C13 cells while the sensitive 2008 cells showed downregulation of HIPK2 that correlated with cleavage of the apoptotic marker PARP. (Fig. 1B). CDDP induced apoptosis in a concentration-dependent manner in chemosensitive 2008 cells but had no effect in its resistant counterpart 2008C13, as previously reported [22]. In agreement with the induction of apoptosis, the 5 µg/ml dose of CDDP induced phosphorylation of p53 at Ser46 only in 2008 cells compared to the resistant 2008C13 cells (Fig. 1C). These findings are in agreement with the proposed regulatory loop between p53 and HIPK2 in response to an apoptotic dose of a drug that is: activation of HIPK2 activates p53Ser46 which in turn induces caspase-mediated degradation of HIPK2 allowing for the rapid amplification of the apoptotic response [16]. Therefore,

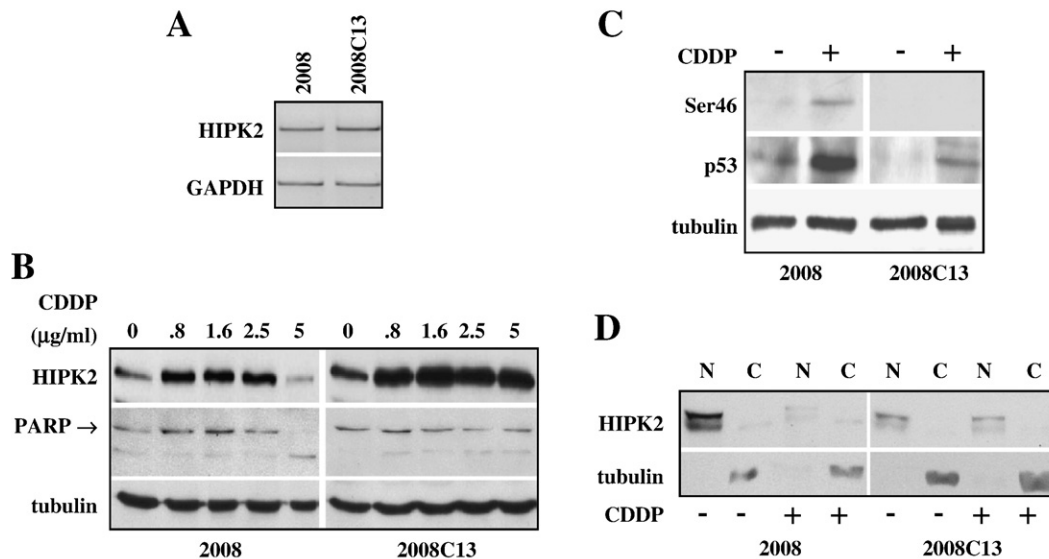

Fig. 1. HIPK2/p53Ser46 expression in cisplatin-sensitive 2008 and cisplatin-resistant 2008C13 cells. (A) The results show that HIPK2 mRNA was equally expressed in 2008 and 2008C13 cells. GAPDH was amplified as internal control. (B) The expression of HIPK2 and PARP cleavage as a result of CDDP (0.8, 1.6, 2.5, and 5 µg/ml) treatment. Cells were harvested 24 h after treatment and the expression of HIPK2 and PARP cleavage was determined by Western blotting with whole-cell lysates. Anti-tubulin was used as protein loading control. (C) 2008 and 2008C13 cells treated with 5 µg/ml of CDDP for 24 h were harvested and the expression of p53 and p53Ser46 phosphorylation was assessed by Western blotting with whole-cell lysates. Anti-tubulin was used as protein loading control (D) HIPK2 subcellular localization. 2008 and 2008C13 cells treated with 5 µg/ml of CDDP for 24 h were subjected to nuclear and cytoplasmic extraction and expression of HIPK2 was assessed by Western blotting. Anti-tubulin was used as a cytoplasmic marker. The results show representative bands from at least two independent experiments.

among of the mechanisms that contribute to the inhibition of p53 apoptotic function in 2008C13 chemoresistant cells might be the lack of proper HIPK2 activation with impairment of p53Ser46-induced apoptosis.

To test whether the lack of HIPK2 function in 2008C13 cells following CDDP treatment might depend on its cytoplasmic relocalization that impairs p53 phosphorylation, as previously shown [23], analysis of nuclear/cytoplasmic cell extracts was performed. As shown in Fig. 1D, HIPK2 protein was mainly localized in the nucleus of both 2008 and 2008C13 cells; moreover, it was downregulated only in sensitive 2008 cells following apoptotic dose of CDDP treatment, in agreement with the results described above. Altogether, these data suggest that the impairment of HIPK2/p53Ser46 function in resistant 2008C13 cells, following an apoptotic dose of CDDP treatment, does not depend on HIPK2 cytoplasmic relocalization.

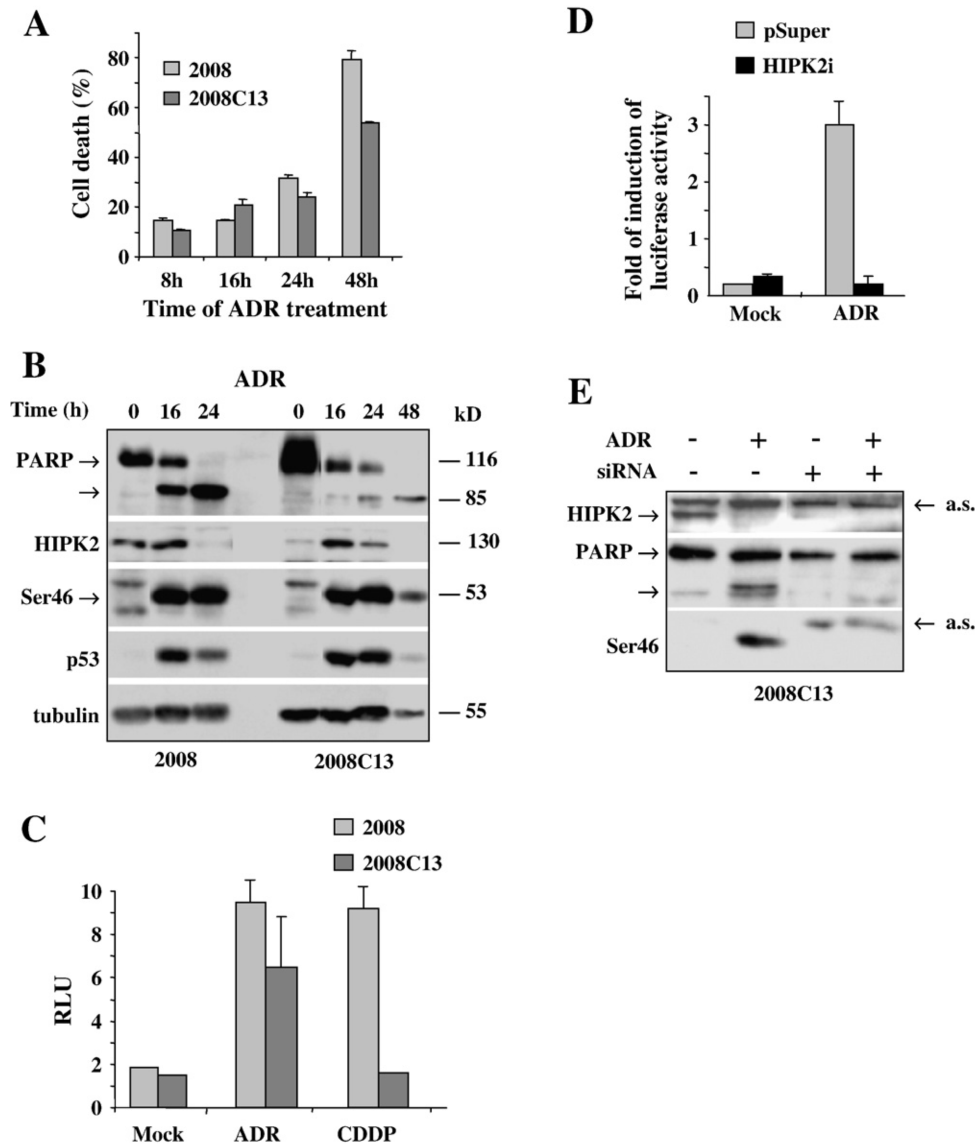

Fig. 2. P53Ser46 activity is not impaired in 2008C13 chemoresistant cells. (A) Time-course analysis of ADR treatment (2 μg/ml) on cell viability in 2008 and 2008C13 cells. Trypan blue exclusion assay was performed to quantify cell viability at 8, 16, 24 and 48 h after treatment. The results are the mean of two independent experiments performed in triplicate. Standard deviation is indicated. (B) 2008 and 2008C13 cells were treated with 2 μg/ml ADR for 16, 24, and 48 h, harvested and the expression of HIPK2, p53, p53Ser46, and PARP cleavage was determined by Western blotting with whole-cell lysates. Anti-tubulin was used as protein loading control. The protein molecular weights are indicated. (C) 2008 and 2008C13 cells were transfected with AIP1-luc reporter and treated with CDDP (5 μg/ml) and ADR (2 μg/ml) for 24 h. Relative Luciferase Activity (RLU) normalized to β-gal is shown. The shown data represent the mean ± SD from three independent experiments performed in duplicate. (D) 2008C13 cells were transfected with pSUPER or pSUPER-HIPK2-interfering vectors and 48 h later transfected with AIP1-luc reporter and treated with ADR (2 μg/ml) for 24 h. Luciferase activity normalized to β-gal is shown. The result is shown as fold of induction of relative luciferase activity. Data are representative of three independent experiments performed in duplicate. Standard deviation from the mean is indicated. (E) 2008C13 cells were transiently transfected with siRNA to knock-down HIPK2 and treated with ADR (2 μg/ml for 24 h). The expression of HIPK2, p53Ser46 phosphorylation, and PARP cleavage was assessed by Western blotting with whole-cell lysates. Arrows indicate aspecific signals (a.s.).

The p53Ser46 pathway is not compromised in 2008C13 cisplatin-resistant cells

To evaluate whether 2008C13 cells were resistant to other chemotherapeutic drugs, cells were treated with ADR that has been shown to induce Ser46 phosphorylation and apoptosis [20] and to accumulate HIPK2 at protein level [14]. Moreover, we have recently shown that ADR can induce apoptosis in 2008 cells and that HIPK2 knock-down inhibits this effect [15]. As shown in Fig. 2A, ADR treatment induced cell death in 2008 cells and in a similar extent in 2008C13 cells. Immunoblot analysis of 2008 and 2008C13 cell extracts treated with ADR for 16, 24, and 48 h showed cleavage of the apoptotic marker PARP from its 116 kDa to 85 kDa fragment in both cell lines, although the 2008C13 cells showed a slightly slower kinetics; it also showed p53Ser46 phosphorylation in both cell lines, and HIPK2 downregulation concomitant to PARP cleavage (Fig. 2B). These findings suggest that the 2008C13 cells were sensitive to ADR with induction of HIPK2/p53Ser46 apoptotic pathway.

The involvement of p53 function in drug-induced apoptosis was then evaluated by luciferase assay using the AIP1 promoter that is a specific target of p53Ser46 phosphorylation [20]. To this end 2008 and 2008C13 cells were transfected with the AIP1-luc vector and treated with CDDP and ADR. As shown in Fig. 2C, the reporter activity of the AIP1 promoter was induced in 2008 cells after treatment with both drugs while it was induced in 2008C13 cells only following ADR treatment. To investigate the role of HIPK2 in p53-induced AIP1 activation, a similar luciferase assay was performed in 2008C13 cells depleted of endogenous HIPK2 by transient transfection with pSUPER vectors carrying HIPK2, or aspecific RNA-interfering sequences. A shown in Fig. 2D, the ADR-induced reporter activity of the AIP1 promoter was inhibited by HIPK2 knock-down; in agreement, PARP cleavage and Ser46 phosphorylation were not detected in 2008C13 cells depleted of HIPK2 and treated with

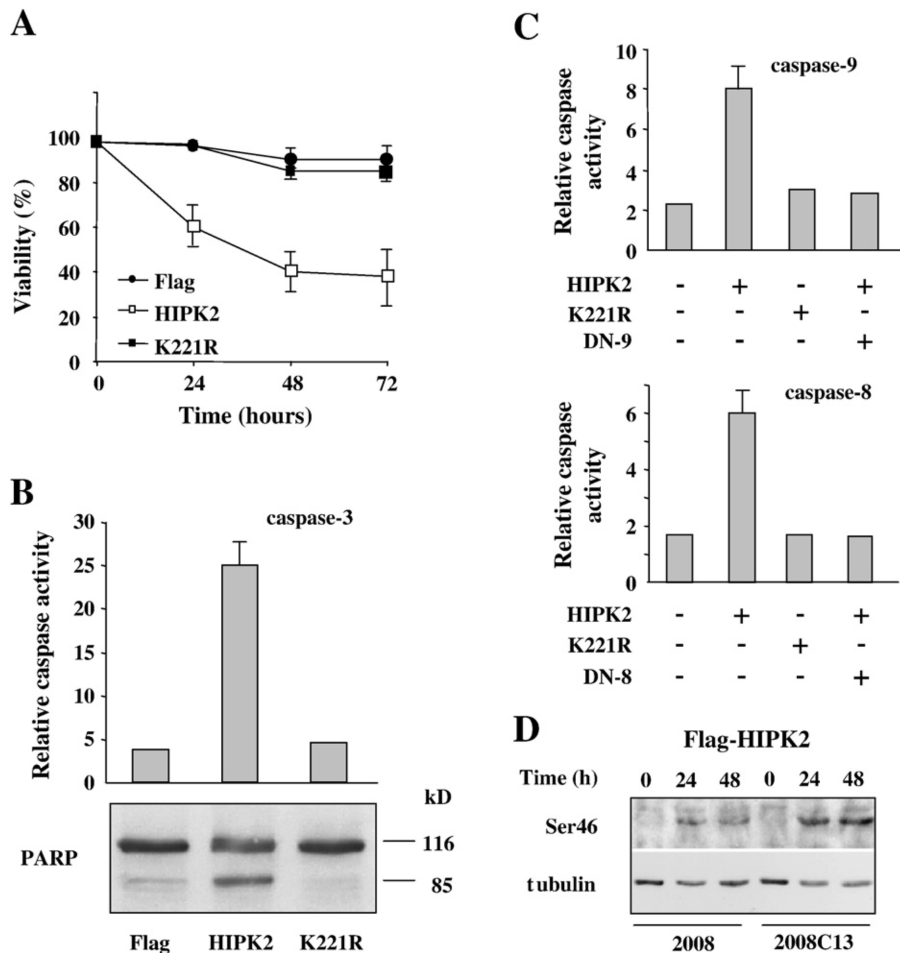

Fig. 3. HIPK2 overexpression induces apoptosis in 2008C13 chemoresistant cells. (A) 2008C13 cells were transfected with HIPK2, K221R, and Flag-empty expression vectors and cell viability was measured by trypan blue exclusion 24, 48, and 72 h post-transfection. The results shown are representative of two independent experiments performed in triplicate. Standard deviation is indicated. (B) The expression of caspases-3 and PARP cleavage as result of HIPK2 overexpression. 2008C13 cells were transfected as in (A) and 24 h later the caspase-3 activity was detected by fluorogenic assay (upper panel) and the expression of PARP cleavage was assessed by Western blotting of whole-cell lysates (lower panel). The uncleaved (116 kDa) and cleaved (85 kDa) forms of PARP are indicated. (C) 2008C13 cells were co-transfected with HIPK2 and dominant-negative expression vectors for caspases-8 (DN-8) and -9 (DN-8). Caspase-8 and -9 activities were detected by fluorogenic assay 24 h post-transfection. Data are representative of three independent experiments. Standard deviation is indicated. (D) The p53Ser46 phosphorylation after HIPK2 overexpression. 2008 and 2008C13 cells were transfected with HIPK2 and the expression of Ser46 phosphorylation was assessed 24 and 48 h after transfection by Western blotting with whole-cell lysates. Anti-tubulin was used for loading control. The results show representative bands from two independent experiments.

ADR (Fig. 2E). Similar results were obtained in 2008 cells (not shown), as previously reported [15]. These experimental results reveal that the p53Ser46 apoptotic pathway is not defective in 2008C13 cells and that HIPK2 takes part in this regulation following ADR treatment; they also suggest that HIPK2/p53Ser46 deregulation is involved in chemoresistance.

HIPK2 overexpression induces p53Ser46 apoptotic pathway in chemoresistant 2008C13 cells

To investigate the possibility to evade inhibition of apoptosis and circumvent chemoresistance, exogenous HIPK2 was overexpressed in 2008C13 cells. HIPK2 overexpression reduced cell viability, compared to Flag-empty or K221R mutant expression vectors (Fig. 3A). Similar results were obtained previously in 2008 cells [15]. Induction of apoptosis was evident after overexpression of HIPK2 but not of K221R mutant, as assessed by caspases-3 activity, a downstream effector of caspases-8 and -9 (Fig. 3B, upper panel), and PARP cleavage (Fig. 3B, lower panel). The caspases' involvement was then evaluated by fluorogenic assays of 2008C13 cells co-transfected with HIPK2 and K221R, along with specific caspase-8 and caspase-9 dominant-negative (DN) expression vectors. As shown in Fig. 3C, specific induction of both caspase-8 and -9 activities was reached only after HIPK2 overexpression, and inhibited by specific dominant-negative mutants co-expressed with HIPK2.

The involvement of p53 in HIPK2-mediated apoptosis was evaluated by Western blot analysis and by luciferase assay. As shown in Fig. 3D, p53Ser46 phosphorylation was comparably induced in both 2008 and 2008C13 cells after HIPK2 overexpression. Luciferase assay showed that AIP1 promoter was induced in 2008C13 cells only after HIPK2 overexpression, compared to K221R mutant (Fig. 4A). Finally, a luciferase assay was performed in 2008C13 cells transiently transfected with AIP1-luc reporter and wtp53, or Ser46A (nonphosphorylatable Ser46) and Ser46D (phosphorylation mimic) mutants. As shown in Fig. 4B, the AIP1 promoter reporter activity was induced in a bigger extent by Ser46D compared to wtp53 while the Ser46A mutant did not induce AIP1 reporter activity. Altogether, these results show that exogenous HIPK2 exerts p53Ser46-induced apoptosis with involvement of both caspase-8 and -9 pathways, no matter whether the cells are drug resistant or not.

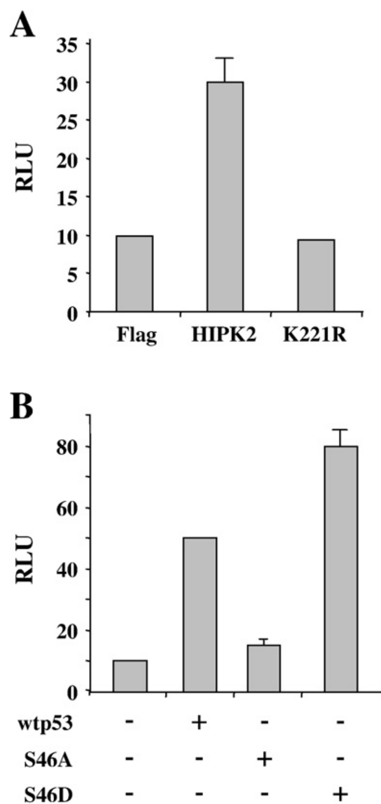

Fig. 4. HIPK2 overexpression induces p53Ser46-dependent AIP1 promoter. (A) 2008C13 cells were co-transfected with AIP1-luc reporter and HIPK2 or K221R kinase defective mutant. Relative Luciferase Activity (RLU) normalized to β-gal is shown. The shown data represent the mean ± SD from three independent experiments performed in duplicate. (B) 2008C13 cells were co-transfected with AIP1-luc reporter and wtp53, S46A, or S46D mutants. Relative Luciferase Activity (RLU) normalized to β-gal activity is shown. Data are representative of three independent experiments performed in duplicate. Standard deviation from the mean is indicated.

Discussion

Apoptosis is the unique outcome that may lead to a successful cancer therapy. Indeed, the failure to die in response to several genotoxic and/or chemotherapeutic agents due to defects in one or more components of the apoptotic pathway, is a determinant of tumor cell resistance to antineoplastic treatments. Therefore, genetic restoration of the apoptotic pathway or introduction of proapoptotic molecules is an attractive approach for treating cancers.

Resistance to p53-induced apoptosis is an important phenotype of tumor cells expressing wtp53. We have previously shown that HIPK2 regulates p53 oncosuppressor activity through Ser46 phosphorylation and induction of apoptotic target genes in response to genotoxic stress including that induced by chemotherapeutic drugs [9,11,15]. In the present study, we have found that HIPK2 is differently expressed at protein level, in response to CDDP, in sensitive 2008 versus chemoresistant 2008C13 cells with impairment of downstream p53Ser46 phosphorylation. It has been reported that 2008C13 cells express a wild-type p53 that is necessary for the induction of apoptosis [22], however the presence of wild-type p53 does not necessarily ensure a chemosensitive phenotype because when p53 is not mutated, deregulated upstream molecules can inhibit its function [24–27]. In this regard, it has been recently found that HIPK2 cytoplasmic localization can inhibit p53 activation [23]. However we have found that HIPK2 was mainly nuclear in both 2008 and 2008C13 cells, suggesting that the impairment of HIPK2/p53Ser46 function in resistant 2008C13 cells following apoptotic dose of CDDP treatment, does not depend on HIPK2 cytoplasmic relocalization. HIPK2 inactivation might instead depend on deregulation of upstream molecules induced by different stimuli, as we have found that HIPK2-mediated p53Ser46 activation was induced in sensitive 2008 cells by both CDDP and ADR while it was induced in

2008C13 cells only by ADR. These findings suggest that the HIPK2/p53Ser46 complex was not defective in 2008C13 cells but rather it was deregulated by chemoresistance induced by CDDP. How HIPK2 regulation is involved in chemoresistance awaits further investigations. Thus, the existence of different signalling pathways and regulatory proteins that finally lead to HIPK2 activation in response to different signals is very poorly understood. In this regard, recent findings showed that IR-induced HIPK2 accumulation might be regulated by the ATM pathway, and the authors suggest the existence of an essential HIPK2 cofactor which is required to form an active p53Ser46 kinase complex [13]. Therefore, it is important to understand the upstream role of individual as well as combinatorial post-translational modifications or protein/protein interaction events in regulating HIPK2 response that may be altered in acquired chemoresistance to better understand the impact of HIPK2 on p53 activity.

As mentioned, HIPK2-induced apoptosis is mainly due to positive regulation of p53 function [9–13,15]. Here we have shown that overexpression of HIPK2 induced apoptosis in chemoresistant ovarian cancer cells with activation of p53Ser46 target gene AIP1, supporting the HIPK2 potential application in chemoresistant ovarian cancer therapy. It has been shown that p53 phosphorylation at Ser46 is a late event after DNA damage and is a necessary step for inducing irreversible apoptosis with activation of a specific target gene such as AIP1 [20,28]. It has been shown that a defect in Ser46 phosphorylation contributes to the acquisition of the p53 resistance in an oral squamous cell carcinoma cell line [29]. Therefore, the lack of p53Ser46 phosphorylation by deregulation of activating kinases may be responsible of inhibition of p53-induced apoptosis and development of chemoresistance. In this regard, we have previously found that silencing of endogenous HIPK2 reduces p53Ser46 phosphorylation and p53 apoptotic function in response to CDDP [11] and found here that HIPK2 knock-down reduced p53Ser46-dependent apoptosis in response to ADR, indicating that impaired HIPK2 function could contribute to the development of chemoresistance. Further work is required to determine the feasibility of using HIPK2 overexpression for treatment of human ovarian cancer with wtp53. However, recent studies have explored the possibility to treat tumors with p53Ser46 downstream mediators. Thus, if the p53 protein is insufficiently modified in cancer cells due to alterations of phosphorylation kinases a Ser46 mutant (Ser46-phenilalanine) or the downstream mediator AIP1 target gene offer the advantage of enhancing transcription of p53 target genes and induce apoptosis [30,31].

In conclusion, the data presented here indicate that HIPK2/p53Ser46 can be deregulated in chemoresistance, suggesting that various stress stimuli activate distinct signalling pathways leading to HIPK2 regulation that eventually activates p53 apoptotic response. Our results also suggest that the use of exogenous HIPK2 might be able to circumvent inhibition of apoptosis in chemoresistant tumors that harbour wtp53. Collectively, our findings might have important contributions for cancer treatment. HIPK2 inhibition reduces the efficacy of chemotherapeutic agents and inhibits p53Ser46 apoptotic pathway therefore HIPK2 might be a potential target for gene therapy of chemoresistant ovarian cancer with wtp53.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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References

[1] Borst P, Jonkers J, Rottenberg S. What makes tumors multidrug resistant? Cell Cycle 2007;6:2782–7.
[2] Fraser M, Leung BM, Yan X, Dan HC, Cheng JQ, Tsang BK. p53 is a determinant of X-linked inhibitor of apoptosis protein/Akt-mediated chemoresistance in human ovarian cancer cells. Cancer Res 2003;63:7081–8.
[3] Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003;22:7265–79.
[4] Haupt S, Berger M, Goldberg Z, Haupt Y. Apoptosis—the p53 network. J Cell Sci 2003;116:4077–85.
[5] Xu Y. Regulation of p53 responses by post-translational modifications. Cell Death Differ 2003;10:400–3.
[6] Brooks CL, Gu W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin Cell Biol 2003;2:164–71.
[7] Giaccia AJ, Kastan MB. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev 1998;12:2973–83.
[8] Kim YH, Choi CY, Lee S, Conti MA, Kim Y. Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. J Biol Chem 1998;273:875–9.
[9] D'Orazi G, Cecchinelli B, Bruno T, Manni I, Higashimoto Y, Saito S, et al. Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser46 and mediates apoptosis. Nat Cell Biol 2002;4:11–9.
[10] Hofmann TG, Moller A, Sirma H, Zentgraf H, Taya Y, Droge W, et al. Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. Nat Cell Biol 2002;4:1–10.
[11] Di Stefano V, Rinaldo C, Sacchi A, Soddu S, D'Orazi G. Homeodomain-interacting protein kinase-2 activity and p53 phosphorylation are critical events for cisplatin-mediated apoptosis. Exp Cell Res 2004;293:311–20.
[12] Wesierska-Gadek J, Schmitz ML, Ranftler C. Roscovitine-activated HIP2 kinase induces phosphorylation of wtp53 at Ser-46 in human MCF-7 breast cancer cells. J Cell Biochem 2007;100:865–74.
[13] Dauth I, Kruger J, Hofmann TG. Homeodomain-interacting protein kinase 2 is the ionizing radiation-activated p53 serine 46 kinase and is regulated by ATM. Cancer Res 2007;67:2274–9.
[14] Choi DW, Seo Y-M, Kim E-A, Sung KS, Ahn JW, Park S-J, et al. Ubiquitination and degradation of homeodomain-interacting protein kinase 2 (HIPK2) by WD40-repeat/SOCS box protein WSB-1. J Biol Chem 2007, doi:10.1074/jbc.M708873200.
[15] Pistritto G, Puca R, Nardinocchi L, Sacchi A, D'Orazi G. HIPK2-induced p53Ser46 phosphorylation activates the KILLER/DR5-mediated caspase-8-extrinsic apoptotic pathway. Cell Death Differ 2007, doi:10.1038/sj.cdd.4402186.
[16] Gresko E, Roscic A, Vichalkovski S, del Sal G, Schmitz ML. Autoregulatory control of the p53 response by caspases-mediated processing of HIPK2. EMBO J 2006;25:1883–94.
[17] Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. Oncogene 2005;24:2899–908.
[18] Andrews PA, Velury S, Mann SC, Howell SB. *cis*-Diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. Cancer Res 1998;48:68–73.

[19] Delmastro DA, Li J, Vaisman A, Solle M, Chaney SG. DNA damage inducible-gene expression following platinum treatment in human ovarian carcinoma cell lines. Cancer Chemother Pharmacol 1997;39:245–53.
[20] Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, et al. P53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 2000;102:849–62.
[21] Chen C, Okayama H. High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 1987;7:2754–6.
[22] Yan X, Fraser M, Qiu Q, Tsang BK. Over-expression of PTEN sensitizes human ovarian cancer cells to cisplatin-induced apoptosis in a p53-dependent manner. Gynecol Oncol 2006;102:348–55.
[23] Pierantoni GM, Rinaldo C, Mottolese M, Di Benedetto A, Esposito F, Soddu S, Fusco A. High-mobility group A1 inhibits p53 by cytoplasmic relocalization of its proapoptotic activator HIPK2. J Clin Invest 2007;117:693–702.
[24] Hollstein M, Sidransky D, Vogelstein B, Harris CC. P53 mutations in human cancers. Science 1991;253:49–53.
[25] Greenblatt MS, Bennet WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994;54:4855–78.
[26] Hollstein M, Hergenhahn M, Yang Q, Bartsch H, Wang ZQ, Hainaut P. New approaches to understanding p53 gene tumor mutation spectra. Mutat Res 1999;432:199–209.
[27] Vousden KH, Prives C. P53 and prognosis: new insights and further complexity. Cell 2005;120:7–10.
[28] Mayo LD, Rok Seo Y, Jackson MW, Smith ML, Rivera Guzman JR, Koegaonkar CK, et al. Phosphorylation of human p53 at serine 46 determines promoter selection and whether apoptosis is attenuated or amplified. J Biol Chem 2005;280:25953–9.
[29] Ichwan SJA, Yamada S, Sumreijkanchanakij P, Ibrahim-Auerkari E, Eto K, Ikeda MA. Defect in serine 46 phosphorylation of p53 contributes to acquisition of p53 resistance in oral squamous cell carcinoma cells. Oncogene 2006;25:1216–24.
[30] Yoshida K, Monden M, Nakamura Y, Arakawa H. Adenovirus-mediated p53AIP1 gene transfer as a new strategy for treatment of p53-resistant tumors. Cancer Sci 2004;95:91–7.
[31] Nakamura Y, Futamura M, Kamino H, Yoshida K, Nakamura Y, Arakawa H. Identification of p53-46F as a super p53 with an enhanced ability to induce p53-dependent apoptosis. Cancer Sci 2006;97:633–41.