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p73 and MDM2 confer the resistance of epidermoid carcinoma to cisplatin by blocking p53

Syunji Hayashi [a,b], Toshinori Ozaki [a], Kaori Yoshida [a], Mitsuchika Hosoda [a], Satoru Todo [b], Shin-ichi Akiyama [c], Akira Nakagawara [a,*]

[a] *Division of Biochemistry, Chiba Cancer Center Research Institute, Chuoh-ku, Chiba 260-8717, Japan*
[b] *Department of General Surgery, Hokkaido University School of Medicine, Kita-ku, Sapporo 060-8638, Japan*
[c] *Department of Molecular Oncology, Graduate School of Medical and Dental Science, Kagoshima University, Kagoshima 890-8520, Japan*

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Abstract

p73 responds to DNA damage and exerts its pro-apoptotic function. However, p73 might contribute to the development of drug-resistance in certain tumor cells. In this study, we found that p73 and MDM2 correlate with cisplatin-resistant phenotype of human epidermoid carcinoma-derived cells. p73 and MDM2 were kept at low levels in the cisplatin-sensitive KB-3-1 cells, whereas p53 was induced to be phosphorylated at Ser-15 in response to cisplatin. In contrast, p73 and MDM2 were expressed at higher levels, and cisplatin-mediated p53 phosphorylation was undetectable in the cisplatin-resistant KCP-4 cells. Enforced expression of p73 in KB-3-1 cells caused an accumulation of unphosphorylated form of p53 and MDM2, and conferred the cisplatin resistance. Collectively, our results suggest that a loss of the cisplatin sensitivity is at least in part due to a lack of cisplatin-induced p53 phosphorylation, and p73 might cooperate with MDM2 to be involved in this process.

Keywords: Cisplatin; KB-3-1; MDM2; p53; p73

p53 plays a pivotal role in a variety of biological processes including cell cycle arrest and DNA damage-induced apoptosis. p53 is a sequence-specific nuclear transcription factor, and has an ability to transcriptionally activate its downstream target genes such as $p21^{WAF1}$, *MDM2*, *Bax*, and *PUMA*, whose products trigger cell cycle arrest and/or apoptosis [1,2]. The biological activities of p53 are tightly linked to its sequence-specific transactivation function, and more than 90% of p53 mutations found in human tumor tissues are located within its highly conserved DNA-binding domain. In response to genotoxic stresses, p53 is phosphorylated at multiple sites including Ser-15 and Ser-20, and thereby the latent form of p53 is converted to the active form for its function. DNA damage-induced phosphorylation of p53 increases its half-life and nuclear concentration by preventing its interaction with MDM2, which acts as an E3 ubiquitin ligase for p53 and directly mediates the ubiquitination-dependent proteolytic degradation of p53 [3–5].

There are several potential molecular mechanisms responsible for acquisition of drug resistance, including reduced drug uptake, increased drug inactivation and accelerated DNA damage repair [6,7]. These mechanisms, individually or collectively, reduce the apoptotic response of tumor cells following drug exposure and thus contribute to the development of drug resistance. Alternatively, it has been shown that p53 status is one of the critical determinants of cellular sensitivity to DNA-damaging agents [8,9]. Alterations in p53 caused by mutations result in a poor response to DNA-damaging agents. *p53* mutations found in tumor tissues lead to a significant accumulation of mutant forms of p53, because mutant forms of p53 lack

* Corresponding author. Fax: +81 43 265 4459.
E-mail address: akiranak@chiba-cc.jp (A. Nakagawara).

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an ability to transactivate the expression of *MDM2* [10]. Wild-type p53 functions as a tetramer, and mutant forms of p53 have an ability to hetero-oligomerize with wild-type p53. As a result of this heteromeric association, mutant forms of p53 inhibit the transactivation as well as pro-apoptotic function of wild-type p53. Thus, the defects in the p53-mediated apoptotic pathway play an important role in tumorigenesis and also render tumor cells resistant to anti-cancer drug.

p73 shares remarkable structural and functional similarities with p53 [11]. Contrary to the initial prediction based on the *in vitro* studies, mutations of *p73* in a wide variety of human tumors appear to be rare [12], and p73-deficient mice displayed a specific neuronal disorder as well as a defective immunological response but showed no increased susceptibility to spontaneous tumorigenesis [13]. In addition, p73 is expressed as multiple functionally distinct isoforms that differ at their NH_2- and COOH-termini, arising from alternative promoter utilization and differential mRNA splicing, respectively [14,15]. Among them, ΔNp73, which lacks an NH_2-terminal transactivation domain, has an oncogenic potential [16] and exhibits dominant-negative behavior toward wild-type p73 as well as p53 [17]. Intriguingly, *ΔNp73* itself is transactivated by p73, thus forming an autoregulatory feedback loop for inhibiting p73 function [18,19].

Recently, it has been shown that p73 or p63 (another member of p53 family) is required for the p53-dependent apoptosis in response to DNA damage [20]. On the other hand, enforced expression of p73 reduced the transcriptional activity of p53 by inhibiting its DNA-binding ability [21], and is also associated with the resistance to DNA-damaging agents in ovarian cancer cells that carry wild-type *p53* [22]. In the present study, we have found that overexpression of p73 as well as MDM2 correlates quite well with the cisplatin-resistant phenotype of human epidermoid carcinoma cells, and the cisplatin-resistant phenotype might be due to a lack of the induction of p53 phosphorylation at Ser-15 in response to cisplatin.

Materials and methods

Cell culture and transfection. Human epidermoid carcinoma KB-3-1, a cisplatin-resistant subline KCP-4, and a cisplatin-sensitive KCP-4R derived from KCP-4 were cultured in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and penicillin (100 IU/ml)/streptomycin (100 μg/ml). Human breast carcinoma cell lines MCF-7 and MDA-MB-231 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS and antibiotic mixture. Human pancreas carcinoma cell lines JHP1, MIAPaCa, Panc-1, SW1990, and AsPC-1; human non-small cell lung carcinoma cell lines A549 and H1299; human colon carcinoma cell lines COLO320, SW480 were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS and antibiotic mixture. Cells were maintained in an incubator at 37 °C with humidified 5% CO_2 and 95% air. For transient transfection, cells were seeded in 6-well plates at a density of 5×10^4/ml. The day after plating, transfection was performed using the calcium phosphate/DNA precipitation procedure.

Semi-quantitative reverse transcription-PCR analysis. Total RNA was extracted from the indicated cells in the presence or absence of cisplatin by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The first strand cDNA was generated from 1 μg of total RNA using random primers and a SuperScript II reverse transcriptase (Invitrogen) at 42 °C for 1 h. When the reaction was complete, the cDNA was used for PCR amplification with rTaq DNA polymerase (Takara). Primer sequences were designed as follows: forward, 5′-ATTTGATGCTGTCCC CGGACGATATTGAAC-3′, and reverse, 5′-ACCCTTTTTGGACTTC AGGTGGCTGGAGTG-3′ for *p53*; forward, 5′-CCGGGAGAACTTT GAGATCC-3′, and reverse, 5′-ATCTTCAGGGCCCCCAGGTC-3′ for *p73*; forward, 5′-CGCCTACCATGCTGTACGTCA-3′, and reverse, 5′-G TGCTGGACTGCTGGAAAGT-3′ for *ΔNp73*; forward, 5′-GTCCCAG AGCACACAGACAA-3′, and reverse, 5′-GAGGAGCCGTTCTGAAT CTG-3′ for *p63*; forward, 5′-CTGGAAAACAATGCCCAGAC-3′, and reverse, 5′-GGGTGATGGAGAGAGAGCAT-3′ for *ΔNp63*; forward, 5′-ATGAAATTCACCCCCTTTCC-3′, and reverse, 5′-CCCTAGGCTG TGCTCACTTC-3′ for *p21*^{WAF1}; forward, 5′-ACTTGAGCCGAGGAG TTCAA-3′, and reverse, 5′-TTGCTCTGTCACCTGGACTG-3′ for *MDM2*; forward, 5′-ACTTGAGCCGAGGAGTTCAA-3′, and reverse, 5′-TCCCGGCAAAAACAAATAAG-3′ for *GADD45*; forward, 5′-TTT GCTTCAGGGTTTCATCC-3′, and reverse, 5′-CAGTTGAAGTTGCC GTCAGA-3′ for *Bax*; forward, 5′-AGGTGGACCTGTTTCGTGAC-3′, and reverse, 5′-ACCCTGTGATCCACCAGAAG-3′ for *MRP1*; forward, 5′-TGCTTCCTGGGGATAATCAG-3′, and reverse, 5′-CACGGATAA CTGGCAAACCT-3′ for *MRP2*; forward, 5′-GGCGTCTATGCTGC TTTAGG-3′, and reverse, 5′-CCTTGGAGAAGCAGTTCAGG-3′ for *MRP3*; forward, 5′-TGCTCT GGAGTGTGCATTTC-3′, and reverse, 5′-AAGCCGAGTACGGAC TCTCA-3′ for *ATP7B*; forward, 5′-TGGG ACGAAGAAAAGGAATG-3′, and reverse, 5′-GATCAGGCAGGTT AGCAAGC-3′ for *MLH1*; forward, 5′-GCCATTTTGGAGAAAGGA CA-3′, and reverse 5′-CTCACATGGCACAAAACACC-3′ for *MSH2*; forward, 5′-CTCCCACTGCTGCCTTAGTC-3′, and reverse, 5′-CTTGC AAGCAATGGTGAAGA-3′ for *XPA*; forward, 5′-GCGGCAGAGAT TCTTGGTAG-3′, and reverse 5′-GGCCCCAGACATAGAACTCA-3′ for *XPB*; forward, 5′-CCATGAGGACACACACAAGG-3′, and reverse 5′-ACAACCACCCTCCAAGACAG-3′ for *XPC*; forward, 5′-AGCCAC GTTCCCCTATGTG-3′, and reverse, 5′-ACAGCTTCCTTTCAGCCA AA-3′ for *XPE*; forward, 5′-TGCGTGAATTTCGAAGTGAG-3′, and reverse, 5′-TGGAGATGCACTGGCTGTAG-3′ for *XPF*; forward, 5′-C AGACACAGCTCCGAATTGA-3′, and reverse, 5′-TTCTGGGTTTTT CGTTTTGC-3′ for *XPG*; forward, 5′- ACCTGACCTGCCGTCTAGA A-3′, and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′ for *GAPDH*. *GAPDH* was used as a control for each PCR for semi-quantitative purposes. PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and visualized by UV-induced fluorescence.

Recombinant adenovirus and infection. Adenovirus-p73α and adenovirus-LacZ were generated as described previously [18]. For adenovirus gene transfer, KB-3-1 cells were incubated with the indicated multiplicity of infection of adenovirus constructs at 37 °C for 48 h.

Western blot analysis. Cells were extracted directly with the lysis buffer containing 25 mM Tris–HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100, 1 mM PMSF, and protease inhibitor mixture (Sigma). Total protein concentrations were determined using the Bradford protein assay according to the instructions of the vendor (Bio-Rad). Equal amounts of protein (50–100 μg) were boiled for 5 min in an SDS sample buffer consisting of 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol, and 0.01% bromophenol blue, subjected to 10% SDS–PAGE under reducing conditions, and then electro-transferred onto Immobilon-P membranes (Millipore) using a semi-dry transfer apparatus at room temperature for 1 h. Nonspecific antigen–antibody binding on the membranes was blocked by incubating in TBS-T [50 mM Tris–Cl (pH 7.6), 100 mM NaCl, and 0.1% Tween 20] containing 5% non-fat dried milk at room temperature for 1 h. The membranes were then incubated for 1 h with antibodies against p73 (Ab-4; NeoMarkers), p53 (DO-1; Oncogene Research Products), MDM2 (SMP14; Santa Cruz Biotechnologies), p21^{WAF1} (H-164; Santa Cruz Biotechnologies), p53 phosphorylated at Ser-15 (Cell Signaling), p53 phosphorylated at Ser-20 (Cell Signaling), p53 phosphorylated at Ser-392 (Cell Signaling), ΔNp73 (18), or actin (20–33, Sigma) in TBS-T, followed by an incubation with the appropriate sec-

ondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The membranes were washed extensively with TBS-T and protein bands were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Cell survival assays. Cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay. In brief, KB-3-1 cells were seeded at a density of 5×10^3 cells/96-well microtiter plates with 100 μl of complete medium and allowed to attach in a 37 °C incubator. The next day, the medium was changed and cells were exposed to cisplatin at a final concentration as indicated for 48 h. For the MTT assay, 10 μl MTT solution was added to each well for 3 h at 37 °C. The absorbance readings for each well were carried out at 570 nm using the microplate reader (Model 450, Bio-Rad).

Results

Expression of p53 family genes in various human cancerous cell lines

We examined the expression levels of *p53* family genes including *p53*, *p73*, *ΔNp73*, *p63*, and *ΔNp63* in various human cell lines derived from cancers of multiple origins. Total RNA was purified from the indicated cell lines and subjected to the semi-quantitative RT-PCR analysis. The levels of *GAPDH* mRNA were comparable among these cell lines. Under our experimental conditions, the expression of *p53* was undetectable in *p53*-deficient AsPC-1 and H1299 cells, whereas *p53* was readily detectable in the remaining cell lines (Fig. 1). Relatively higher levels of *p63* expression were observed in MIAPaCa, Panc-1, A549, and MB-231 cells. The expression of *ΔNp63* was detected in the majority of pancreas carcinoma cell lines, but not in the other tumor-derived cell lines except MCF-7 cells. Additionally, *p73* was expressed at higher levels in KCP-4, MIAPaCa, Panc-1, H1299, MCF-7, and SW480 cells, whereas *ΔNp73* was undetectable in all of the cell lines that we examined. It is worth noting that, in a sharp contrast to the cisplatin-resistant human epidermoid carcinoma KCP-4 cells, *p73* was undetectable or remained extremely low level in the cisplatin-sensitive parental KB-3-1 or incomplete revertant KCP-4R cells, respectively. These results showed that the elevated level of *p73* expression is closely associated with the acquisition of cisplatin resistance in human epidermoid carcinoma KB cell lines, and also suggested that p73 could play an important role in the development of resistance to cisplatin in certain cell types. We focused subsequent studies on KB cell lines.

Cisplatin-induced Ser-15 phosphorylation of p53 is impaired in the cisplatin-resistant KCP-4 cells

As described previously [23,24], the cisplatin-resistant KCP-4 cells were isolated by culturing their parental KB-3-1 cells in the presence of cisplatin after EMS mutagenesis. The cisplatin-sensitive incomplete revertant KCP-4R cells were derived from KCP-4 cells. Consistent with the previous results [23,24], KCP-4 cells were highly resistant to cisplatin as compared with the parental KB-3-1 cells (Fig. 2A). Under our experimental conditions, KCP-4R cells exhibited about 3-fold higher resistance to cisplatin than KB-3-1 cells. To examine whether the differential cytotoxic response of these cells to cisplatin could correlate with the differential expression of *p73* in more detail, cells were exposed to cisplatin at a final concentration of 3 or 90 μM, and the expression levels of *p73*, *p53*, and their direct transcriptional target genes including *MDM2*, $p21^{WAF1}$, and *Bax* were analyzed by RT-PCR at various time points. At 3 μM of cisplatin, KB-3-1 and KCP-4R cells underwent apoptosis, whereas KCP-4 cells did not (Fig. 2A). At 90 μM cisplatin, KCP-4 cells showed an apoptotic response to cisplatin (data not shown). As shown

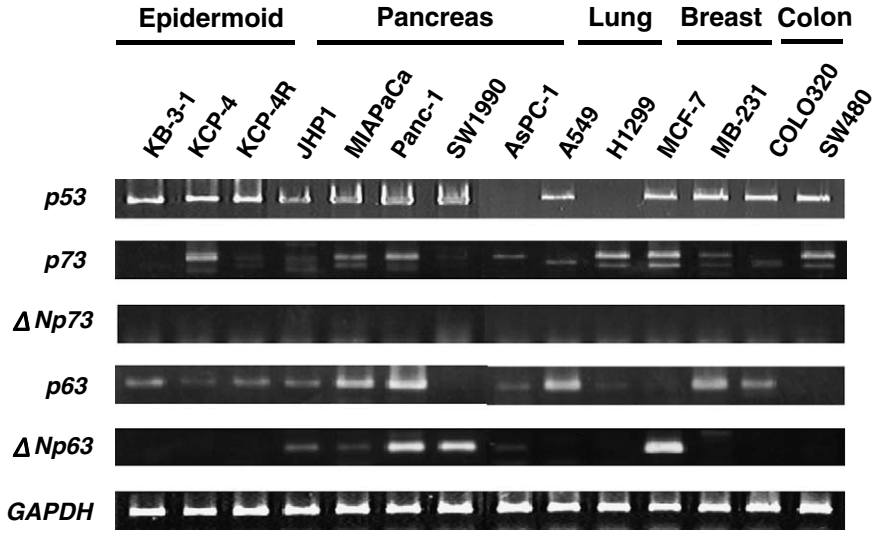

Fig. 1. The expression of *p53* family genes in various human cancerous cell lines. Total RNA prepared from the indicated cell lines was subjected to semi-quantitative reverse transcription-PCR to examine the expression levels of *p53*, *p73*, *ΔNp73*, *p63* and *ΔNp63*. The PCR-amplified products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. RNA samples were also amplified using *GAPDH* gene primers as an internal control.

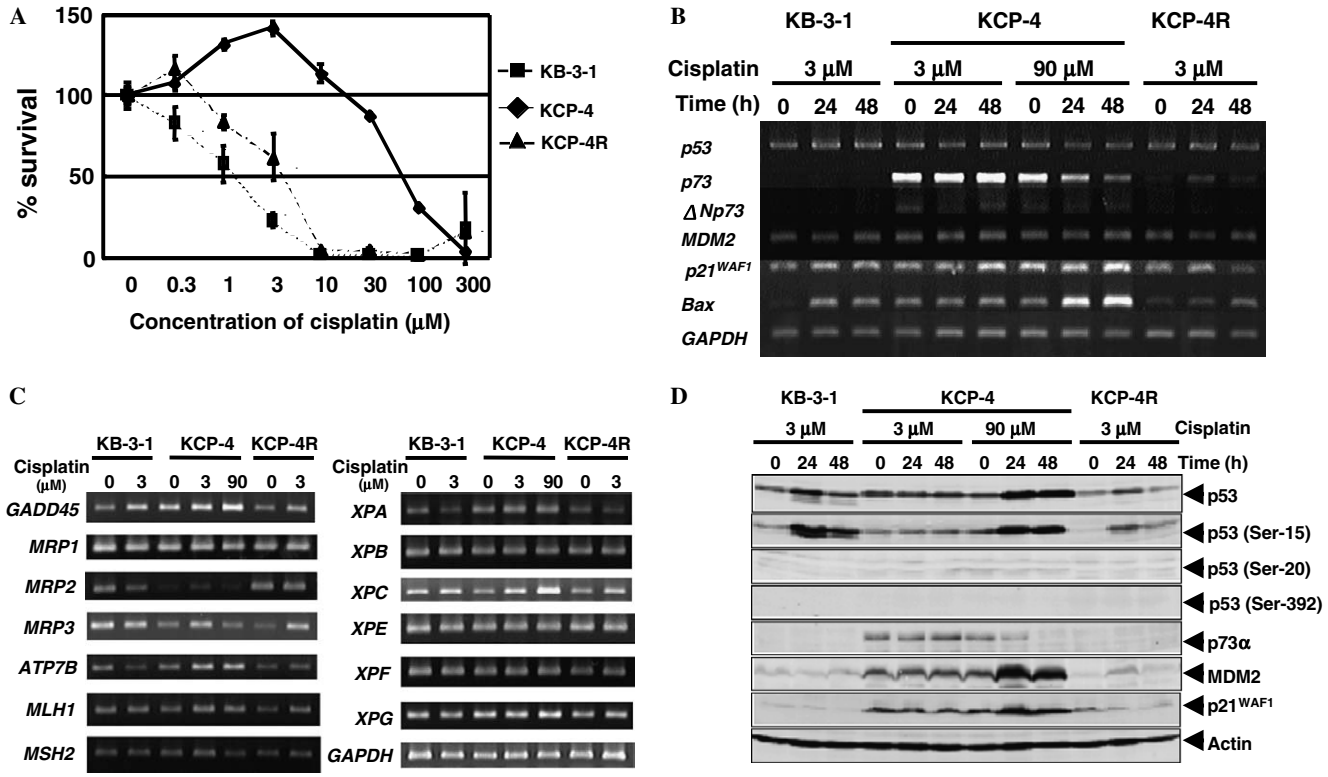

Fig. 2. *p73* expression is up-regulated in the cisplatin-resistant KCP-4 cells. (A) Concentration-response curves of KB-3-1, KCP-4, and KCP-4R cells to cisplatin. Cells were seeded at a density of 5×10^3 cells/96-well microtiter plates, incubated in the absence of cisplatin for 24 h, and subsequently exposed to various concentrations of cisplatin for 48 h. Cell viability was determined by MTT assay as described in Materials and methods. Data are presented as mean values ± the standard deviation (SD) of three independent experiments. (B) RT-PCR analysis. KB-3-1, KCP-4, and KCP-4R cells were left untreated or exposed to cisplatin at a final concentration of 3 or 90 μM. At the indicated time periods after the treatment with cisplatin, total RNA was purified and subjected to RT-PCR to examine the expression levels of *p53*, *p73*, *ΔNp73*, *MDM2*, $p21^{WAF1}$, and *Bax*. PCR with *GAPDH* primers is shown as an internal control. (C) RT-PCR analysis of the multi-drug resistance-associated genes, the copper-transporting ATPase gene, DNA-damage recognition protein genes, and DNA repair-related genes. KB-3-1, KCP-4, and KCP-4R cells were treated with the indicated concentrations of cisplatin for 48 h, and total RNA was subjected to RT-PCR. (D) Immunoblot analysis. KB-3-1, KCP-4, and KCP-4R cells were treated with cisplatin (3 or 90 μM) for 0, 24, and 48 h, followed by immunoblotting with the indicated antibodies. Anti-actin immunoblot was used as a loading control.

in Fig. 2B, the expression levels of *p53*, $p21^{WAF1}$ and *MDM2* remained almost constant in KB-3-1, KCP-4, and KCP-4R cells regardless of cisplatin treatment. On the other hand, during the cisplatin-induced apoptosis, the expression level of *Bax* was significantly increased in KB-3-1 and KCP-4 cells. Similarly, *Bax* was also induced in KCP-4R cells in response to cisplatin but to a lesser degree as compared with KB-3-1 and KCP-4 cells. Of note, *p73* was maintained at higher levels in KCP-4 cells exposed to cisplatin at 3 μM, however, *p73* was markedly down-regulated in KCP-4 cells in response to 90 μM of cisplatin. At 3 μM cisplatin, *p73* was undetectable in KB-3-1 cells, whereas the expression level of *p73* was slightly increased in KCP-4R cells in response to cisplatin. Next, we examined the expression levels of the multi-drug resistance-associated (MRP) genes (*MRP1*, *MRP2*, and *MRP3*), the copper-transporting P-type ATPase gene (*ATP7B*), DNA damage recognition protein genes (*MLH1* and *MSH2*), and the DNA repair-related genes (*GADD45*, *XPA*, *XPB*, *XPC*, *XPE*, *XPF*, and *XPG*) in response to cisplatin. As shown in Fig. 2C, there was no significant correlation between the expression levels of these genes and the cisplatin resistance.

To confirm the differential expression of p73 at protein level, whole cell lysates prepared from KB-3-1, KCP-4, and KCP-4R cells exposed to cisplatin (3 or 90 μM) for the indicated time periods were subjected to immunoblot analysis. Consistent with the RT-PCR analysis, p73α was maintained at higher levels in KCP-4 cells treated with cisplatin at 3 μM, however, its expression level was strongly reduced in KCP-4 cells exposed to cisplatin at 90 μM in a time-dependent manner (Fig. 2D). After treatment with 90 μM cisplatin, KCP-4 cells underwent apoptosis. On the other hand, p73α was undetectable at protein level in the cisplatin-sensitive KB-3-1 and KCP-4R cells. Thus, there exists a clear correlation between the degree of cisplatin sensitivity and the decrease in the expression level of p73α in KCP-4 cells. The steady-state expression level of p53 was much lower in KB-3-1 and KCP-4R cells than in KCP-4 cells. Cisplatin treatment resulted in a remarkable accumulation of p53 in KB-3-1 and KCP-4R cells. In KCP-4 cells, 3 μM cisplatin had no effect on p53, however, p53 was significantly induced in the presence of 90 μM cisplatin. The cisplatin-mediated accumulation of p53 was associated with its remarkable induction of phosphorylation at Ser-15, whereas the cisplatin-dependent

phosphorylation of p53 at Ser-20 or Ser-392 was undetectable. Intriguingly, MDM2 as well as $p21^{WAF1}$ was maintained at higher levels in KCP-4 cells as compared with KB-3-1 and KCP-4R cells, and their amounts were increased in response to 90 μM cisplatin. Since 90 μM cisplatin had no detectable effect on *MDM2* and $p21^{WAF1}$ transcripts (Fig. 2B), their up-regulation might be due to their increased protein stability. According to the expression study, it is likely that the accumulation of p53 phosphorylated at Ser-15 is required for the cisplatin-mediated apoptosis in KB cells, and the cisplatin-resistant phenotype might be caused at least in part by a defect in the induction of active p53 even in the presence of cisplatin.

Ectopic expression of p73 inhibits cisplatin-mediated induction of p53 phosphorylation at Ser-15 and increases resistance to cisplatin

As described above, we found that, in the cisplatin-resistant KCP-4 cells, the steady-state expression level of p73α correlates quite well with those of p53 as well as MDM2. Recently, it has been reported that p73α has an ability to stabilize p53 at protein level independent of its transcriptional activity [25]. To determine whether p73 could contribute to the development of resistance to cisplatin, the cisplatin-sensitive KB-3-1 cells were infected with the recombinant adenovirus for LacZ or p73α. As shown in Fig. 3A, ectopic expression of p73α was associated with a strong accumulation of p53 and MDM2, but phosphorylation at Ser-15 of p53 was undetectable. Under our experimental conditions, mRNA levels of *p53* and *MDM2* remained unchanged even in the presence of exogenous p73α (data not shown). Twenty-four hours after infection, cells were left untreated or exposed to the indicated concentrations of cisplatin for another 48 h, and their cisplatin sensitivity was examined by cell survival assay. As shown in Fig. 3B, KB-3-1 cells expressing exogenous p73α displayed an increased resistance to cisplatin, with a 1.5- to 3-fold higher IC_{50} as compared with those of cells expressing LacZ. Intriguingly, cisplatin-mediated induction of p53 phosphorylation at Ser-15 was inhibited by exogenous p73α (Fig. 3C).

To further investigate the role of p73 in the regulation of the cisplatin resistance, we have constructed an expression plasmid for small interfering RNA (siRNA) targeting p73. KCP-4 cells were transfected with the empty plasmid or with the expression plasmid for p73 siRNA. Twenty-four hours after transfection, cells were left untreated or treated with cisplatin at a final concentration of 5 μM for the indicated time periods, and then cell survival assay was performed. As shown in Fig. 4A, RT-PCR analysis demonstrated that a significant reduction of the endogenous *p73*

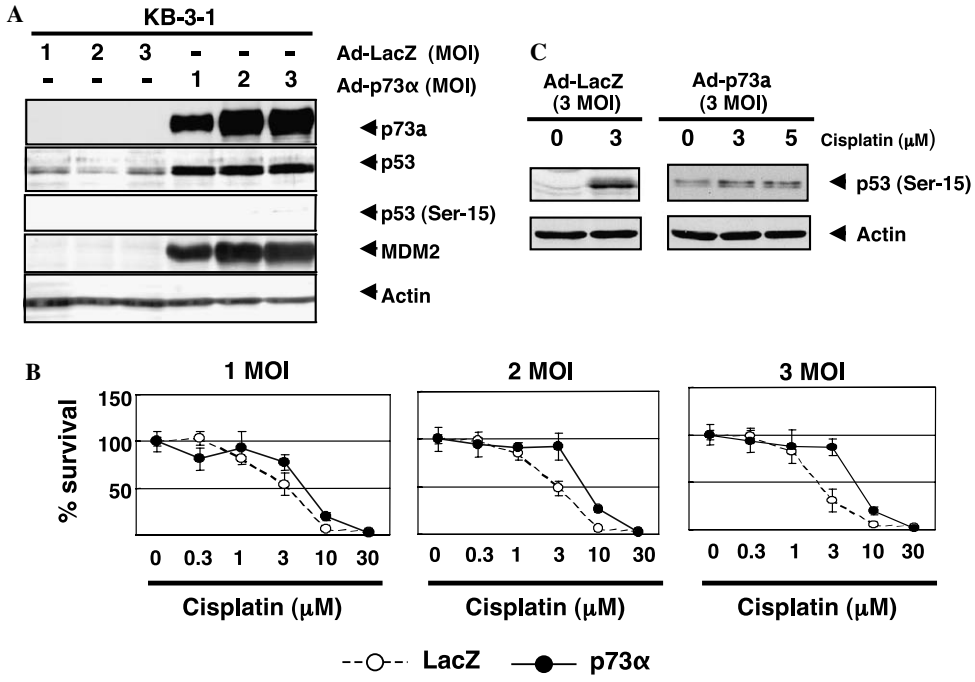

Fig. 3. Enforced expression of p73 increases resistance to cisplatin in KB-3-1 cells. (A) Immunoblot analysis. KB-3-1 cells were infected with the recombinant adenovirus encoding LacZ or p73α (MOI = 1, 2 or 3) for 72 h. Whole cell lysates were prepared from KB-3-1 cells infected with the recombinant adenovirus, and analyzed for the expression of p73α, p53, p53 phosphorylated at Ser-15 and MDM2 by immunoblotting. Anti-actin immunoblot was used as a loading control. (B) Cell survival assay. KB-3-1 cells (5×10^3 cells/well) were infected with the recombinant adenovirus for LacZ or p73α (MOI = 1, 2 or 3). Twenty-four hours after infection, cells were left untreated or treated with the indicated concentrations of cisplatin for 48 h, and then their viability was determined by MTT assay. The graphs indicate relative viability based on the percent viable cells compared to the control infection (adenovirus-LacZ). (C) Immunoblotting. KB-3-1 cells infected with the adenovirus encoding LacZ or p73α (MOI = 3) were exposed to the indicated concentrations of cisplatin for 24 h, and whole cell lysates were analyzed for the amounts of p53 phosphorylated at Ser-15. Anti-actin immunoblot was used as a loading control.

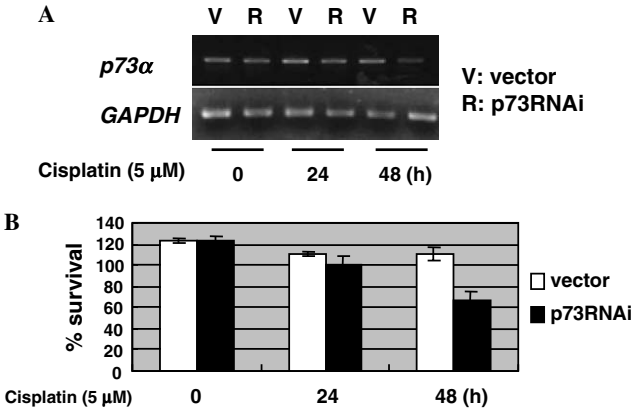

Fig. 4. p73 siRNA treatment increases cisplatin sensitivity of KCP-4 cells. (A) Down-regulation of *p73α* in KCP-4 cells transfected with p73 siRNA plasmid. The empty plasmid or p73 siRNA plasmid was introduced into KCP-4 cells. Twenty-four hours after transfection, cells were exposed to cisplatin at a final concentration of 5 μM. At the indicated time periods after the treatment with cisplatin, total RNA was purified and subjected to RT-PCR to examine the expression level of *p73α*. Amplification of *GAPDH* was used as an internal control. (B) Cell survival assay. KCP-4 cells were transfected with the empty plasmid or p73 siRNA plasmid. Twenty-four hours after transfection, cells were treated with cisplatin (5 μM) for the indicated times, and then their viability was determined by MTT assay.

mRNA was detected in cells transfected with the expression plasmid for p73 siRNA, whereas no detectable effect on the expression levels of *p73* was observed in the control cells transfected with the empty plasmid. Under our experimental conditions, the control KCP-4 cells were resistant to cisplatin at a final concentration of 5 μM (Fig. 4B). In contrast, the down-regulation of the endogenous *p73* levels in KCP-4 cells resulted in an increased cell killing by cisplatin. Thus, it is likely that p73 might contribute to the cisplatin-resistant phenotype of KB cells at least in part through the inhibition of the cisplatin-mediated induction of p53 phosphorylation at Ser-15.

Discussion

As described previously [23], cisplatin was actively effluxed from the cisplatin-resistant KCP-4 cells but not from the cisplatin-sensitive KB-3-1 cells, however, there was no clear correlation between the degree of cisplatin resistance and the decreased accumulation of cisplatin. In addition, Komatsu et al. reported that overexpression of the copper-transporting P-type ATPase (ATP7B) renders KB-3-1 cells resistant to cisplatin, whereas the expression level of endogenous ATP7B in KCP-4 cells is almost identical to that in KB-3-1 as well as the revertant KCP-4R cells [26], suggesting that there could exist another unknown molecular mechanism(s) behind the acquisition of cisplatin resistance in human epidermoid KB carcinoma cells.

Our cell-based study demonstrated that p73 and MDM2 are significantly associated with the cisplatin-resistant phenotype of KCP-4 cells. Vikhanskaya et al. described that the enforced expression of p73 results in the resistance to DNA-damaging agents through the up-regulation of a variety of DNA repair-related genes [21]. Under our experimental cell systems, however, there was no clear correlation between the expression levels of *p73* and DNA repair-related genes. MDM2 overexpression has been shown to induce the expression of multi-drug resistance (MDR) P-glycoprotein, and thereby conferring resistance to DNA-damaging agents [27]. As described previously [23,28], P-glycoprotein was undetectable in KB-3-1 as well as KCP-4 cells, suggesting that p73-mediated up-regulation of the DNA repair-related genes and MDM2-dependent induction of P-glycoprotein might not be responsible for the acquisition of cisplatin resistance of KB cells.

According to our present results, p53 was highly phosphorylated at Ser-15 in KB-3-1 and KCP-4R cells exposed to cisplatin. Of particular interest was a lack of the induction of p53 phosphorylation at Ser-15 in KCP-4 cells in response to cisplatin. Rodicker and Putzer described that a loss of p53 pro-apoptotic activity in pancreatic cancer cells might be due to the lack of p53 phosphorylation at Ser-46 [29]. At a higher concentration of cisplatin (90 μM), KCP-4 cells underwent apoptosis in association with a significant down-regulation of p73 as well as a remarkable induction of p53 phosphorylation at Ser-15. Accumulating evidence suggests that the stress-induced phosphorylation of p53 at Ser-15 causes its stabilization as well as increase in its sequence-specific DNA-binding activity [1,2]. It has been shown that cisplatin preferentially activates ATR and thereby enhancing p53 function [30,31]. Although it remains unclear which is the primary p53 Ser-15 kinase that actually phosphorylates p53 in response to cisplatin, our present results strongly suggest that p53 phosphorylation at Ser-15 is required, at least in part, for the cisplatin-mediated apoptosis in KB cells.

Adenovirus-mediated overexpression of p73α in KB-3-1 cells resulted in a remarkable accumulation of MDM2 at protein level and a decrease in cisplatin-sensitivity. Of note, cisplatin-mediated induction of p53 phosphorylation at Ser-15 was inhibited significantly by exogenous p73α in KB-3-1 cells. These results strongly indicate that p73 and/or MDM2 might be involved in an acquisition of resistance to cisplatin in KB cells. Mounting evidence suggests that MDM2 binds to the NH_2-terminal transactivation domain of p53 and thereby inhibiting p53 phosphorylation at Ser-15 [1,2]. Thus, it is likely that p73-mediated stabilization of MDM2 could block the cisplatin-induced phosphorylation of p53 at Ser-15 in KCP-4 cells. In support of this notion, Wang et al. described that MDM2 is stabilized in the presence of p73 in human lung carcinoma-derived H1299 cells [32]. Additionally, Yu et al. showed that siRNA-mediated knockdown of MDM2 increases the cisplatin sensitivity in colorectal adenocarcinoma cells [33]. In another report, overexpression of p73 attenuates the transcriptional activity of p53 by sequestrating p53 from its DNA-binding site through competitive binding with p73 [20]. Since the precise molecular mechanism(s)

behind the p73-dependent stabilization of MDM2 is unclear, further studies are needed to elucidate the functional role of p73 and MDM2 in the acquisition of cisplatin resistance.

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