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The p21$^{cip1/waf1}$ cyclin-dependent kinase inhibitor enhances the cytotoxic effect of cisplatin in human ovarian carcinoma cells

H. Lincet[a], L. Poulain[a], J.S. Remy[b], E. Deslandes[a], F. Duigou[c], P. Gauduchon[a], C. Staedel[a,*]

[a]*INSERM CJF 96-03 and UPRES-EA 1772, Laboratoire de Cancérologie Experimentale, Centre Régional de Lutte Contre le Cancer François Baclesse, route de Lion-sur-mer, 14076 Caen Cedex 05, France*

[b]*Laboratoire de Chimie Génétique, CNRS UMR 7514, Faculté de Pharmacie de Strasbourg, 74 route du Rhin, 67401 Illkirch, France*

[c]*Laboratoire d'Anatomie Pathologique, Centre François Baclesse, route de Lion-sur-mer, 14076 Caen Cedex 05, France*

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Abstract

The seriousness of ovarian cancer, which is related to the observed link between recurrency and cell cycle control defect, prompted us to explore the effect of ectopic expression of the cdk inhibitor p21$^{cip1/waf1}$ on ovarian carcinoma chemosensitivity. The transfection of p21$^{cip1/waf1}$ cDNA into SKOV3 and OVCAR3 cells led to reduction of tumor cell growth, enhanced susceptibility to cisplatin-induced apoptosis, and abolition of recurrency after cisplatin exposure. p21$^{cip1/waf1}$ gene transfer allowed a marked reduction of the cisplatin concentration needed to erradicate the tumor cell population. These results suggest exploring the possible use of p21$^{cip1/waf1}$ as an adjunctive to conventional chemotherapy.

Keywords: Ovarian carcinoma; cdk-inhibitor p21^{cip1}; Cisplatin; Chemoresistance; Apoptosis

1. Introduction

Chemoresistance is a major concern in cancer chemotherapy. Ovarian carcinoma is an example of cancer in which intrinsic and acquired resistance are clinically apparent. Aggressive treatment of the patients with platinum-based combination chemotherapeutic regimens produces a high primary rate of complete clinical complete response to the drug. However a large proportion of patients relapse, become refractory to the initial drugs used, and eventually die of their disease within 5 years. Several mechanisms of resistance to platinum compounds have been identified, including decreased intracellular drug accumulation, enhanced detoxification mechanisms, DNA repair, and tolerance toward platinum adducts [1]. Since most chemotherapeutic agents exert their cytotoxic effect on tumor cells by inducing apoptotic cell death [2], defects in the apoptosis program have also been suggested to be responsible for chemoresistance in malignant cells [1,3]. Having established an in vitro cellular model of chemoresistance in human ovarian carcinoma cells by mimicking a clinical protocol of cisplatin treatment, we found that the acquisition of chemoresistance was not directly linked to a defect in apoptosis induction, but rather to a defect in cell cycle control favoring the

* Corresponding author. Tel.: +33-2-3145-5070; fax: +33-2-3145-5053.

E-mail address: c.staedel@baclesse.fr (C. Staedel).

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proliferation of a few recurrent cells that had acquired a chemoresistant phenotype [4].

The most common genetic alteration in human cancer, including ovarian cancer, involves the *p53* tumor suppressor gene [5,6]. This results in defective control of cell cycle arrest and cell death following exposure to DNA-damaging agents [7], and thereby in genomic instability. *p53* encodes a transcriptional factor for a set of genes involved in the regulation of cell cycle progression, DNA repair, and apoptosis. One of the targets for p53-dependent transcriptional activation is the cyclin-dependent kinase (cdk) inhibitor $p21^{cip1/waf1}$, a negative regulator of cell cycle transitions following DNA damage [8–10]. p21 is also inducible in a p53-independent manner [11,12]. The cell cycle inhibitory effects of p21 may be attributed to its ability to bind cdks as well as the proliferating cell nuclear antigen (PCNA) [13,14], resulting in inhibition of progression from the G1 to the S phase, of DNA replication, and of progression through G2 phase [15]. In normal cells, p21, cyclin, cdk, and PCNA can form a tetrameric complex in which the binding of more than one p21 molecule is needed to inhibit the cdk activity.

Gene transfer of p21 inhibits tumor cell growth in vitro and in vivo, as reported for melanoma cells [16], breast carcinoma cells [17,18], lung cancer cells [19], colorectal carcinoma cells [20], and oncogene-transformed fibroblasts [21]. Having uniformly demonstrated the negative effect of p21 on malignant cell proliferation, all these data stress the potential of p21 to serve as a tumor suppressor for therapeutic purposes. However, inhibition of cell growth may not be sufficient to eradicate efficiently a tumor and should be associated with enhanced tumor cell death. The effect of p21 upregulation on apoptosis induction is controversial. p21 failed to induce apoptosis in normal and tumor cells of lung and mammary epithelial origin [17], whereas it induced the formation of giant cells that ultimately succumbed to apoptotic death in breast carcinoma [18] and colorectal carcinoma [22]. Other studies have also reported that p21 appears to protect from apoptosis [23–26], and p21-deficient cells are more sensitive to killing by irradiation than their p21-expressing counterparts [27].

Based upon data on the growth inhibition efficiency of p21 on a variety of malignant cell types, the aim of the present work was to determine the potential effect of p21 on ovarian carcinoma cell growth in vitro, alone or in combination with cisplatin.

2. Materials and methods

2.1. Cell lines

The human ovarian adenocarcinoma cell lines SKOV3, OAW42, and OVCAR3 were grown as monolayers at 37°C in a 5% CO_2 atmosphere. SKOV3 was grown in Mac Coy's medium supplemented with 10% fetal calf serum (Gibco BRL, Cergy-Pontoise, France) and 2 mM L-glutamine; OAW42 in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 2 μg/ml bovine insulin (Gibco BRL); and OVCAR3 in RPMI 1640 supplemented with 20% fetal calf serum, 2 mM L-glutamine, 2.5g/l D-glucose, 1 mM sodium pyruvate, and 10 μg/ml bovine insulin. The medium was replaced every 2 days. The cell lines were subcultured weekly, through detachment with tryspin/EDTA (Gibco BRL), at a plating density of 10^5 per 25 cm^2 flask for SKOV3 and OAW42, and 5×10^5 cells per flask for OVCAR3.

2.2. Drug treatment

Cisplatin (CDDP, *cis*-diamino-dichloro-platinum (II)) was obtained in the form of Cisplatyl® from Roger-Bellon (France). Exponentially growing cells were exposed to CDDP for 2 h at 37°C in serum-free medium. After exposure to the drug, the cells were rinsed and incubated in the culture medium.

2.3. Construction of expression vector of $p21^{cip1/waf1}$

A 560 bp fragment of the $p21^{cip1/waf1}$ cDNA, which contained the entire coding region, was obtained by RT-PCR from total RNA of OAW42 cells. In this cell line expressing wt *p53,*the *cip1/waf1*gene can be induced by serum deprivation for 6 h. The primers were determined from the published sequence (GenBank U03106): the forward primer was 5′-TATGAATTCATTCGCCGAGGCACCGAGGC-A-3′ (including *Eco*RI site at 5′), and the reverse primer 5′-ATTGCGGCCGCTTCCAGGACTGCAG-GCTTCC-3′ (including *Not*I site at 5′). One microgram of total RNA, extracted by RNAeasy kit

(Qiagen, Courtaboeuf, France), was reverse-transcribed with 200 units Superscript II reverse transcriptase (Gibco BRL), in first strand reaction conditions recommended by the manufacturer. The PCR reaction contained 3 mM $MgCl_2$, 250 μM dNTP, 1 μM of each primer and 5 units Taq polymerase (Eurobio, Paris, France) and was run for 25 cycles of successive heating at 94°C for 30 s, 55°C for 30 s and 72°C for 2 min within an Amplitron® II Thermolyne thermal cycler (Bioblock, Illkirch, France). The unique 560 bp amplified fragment was cleaved with *Eco*RI and *Not*I, and cloned into the pcDNA3.1 vector (In Vitrogen), under the control of the cytomegalovirus (CMV) promoter. Using the ThermoSequenase sequencing kit (Amersham, Paris, France) and the $5'$-^{33}P-labelled T7 promoter, the sequence of the p21 cDNA insert was identical to the p21 published sequence. Moreover, using an in vitro transcription-translation assay (Promega, Lyon, France), a unique band of 21 kDa was detected by Western blot with pcDNA-p21, while no band was detectable with the empty vector.

2.4. Transient cell transfection

Either DPPES (di-palmitoyl-phosphatidylethanolamine-spermine) or L-PEI (linear polyethylenimine 22KDa) were used to transfect the expression vector. DNA-lipopolyamine complexes or DNA-PEI were obtained as described in [28] and [29], respectively. Exponentially growing cells were incubated for 3 h in the presence of the complexes in serum-free medium and post-incubated in growth medium without rinsing. Optimal conditions of transfection, assessed with the Green Fluorescent-Protein expression vector pEGFP (Clontech, Palo-Alto, CA), were 1 μg DNA/4 nmol DPPES/10^5 cells or 1 μg/1.5 nmol L-PEI/10^5 cells: this was a compromise between the highest transfection efficiency without toxicity. Control cells were transfected either with the empty pcDNA vector or with the pEGFP vector.

2.5. Tet-Off cell line

In order to establish the relationship between p21 level and cell response, we established an inducible p21-expression system, using the Tet-Off System (Clontech Laboratories), in which the p21 gene can be induced after removal of Doxycycline (Dox). SKOV3 cells were transfected by the pTet-Off regulator plasmid, using DPPES. Stable pTet-Off transfectants were selected by 200 μg/ml of the neomycin analog G418 (Calbiochem). G418-resistant clones with low background and high Dox-dependent induction were screened after transient transfection of the reporter plasmid pTRE-d2EGFP. These clones were referred to as SKOV3-Tet-Off and were grown in Mac Coy's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 200 μg/ml G418. SKOV3-Tet-Off cells were transiently transfected with the pTRE-p21 plasmid, in which the p21 cDNA fragment was cloned in *Eco*R1 and *Xba*I sites, under the control of the tetracycline response element (TRE). p21 was repressed by 100 ng/ml Dox.

2.6. Assessment of drug cytotoxicity

The drug cytotoxic effect was assessed by two means: cell enumeration combined with the Trypan Blue exclusion assay, which analyses total cell death, and enumeration of apoptotic cells, revealed by their typical condensed and fragmented nuclear morphology visible after staining with DAPI (diamino-2-phenyl-indol, Boehringer Mannheim) or by TUNEL procedure, as described previously [4].

2.7. DNA flow cytometry

Cells were prepared as previously described [4] and samples were analyzed using an EPICS Profil I flow cytometer (Coulter, Paris, France). EPICS Profile Software (Coulter) was run for data acquisition and MultiCycleAV (Phoenix Flow System) for DNA ploidy and delineation of different cell cycle compartments.

2.8. Immunodetection of $p21^{cip1}$ in cultured cells

Cells grown on glass coverslips and fixed with 3% paraformaldehyde were processed for p21 immunodetection as previously described [4], except that the anti-human p21 was OP64 (Oncogene Research, Meudon, France) at a 100-fold dilution. Controls, consisting of cells incubated in the absence of the primary antibody, were totally negative.

2.9. Western immunoblotting

For Western blots, the conditions of cell lysis, SDS-PAGE, transfer onto Immobilon-P membranes (Milli-

pore), and incubation with the antibodies anti-p53 (DO7, Dako) at a 1/5000 dilution or anti-p21 (Oncogene Research, OP64) at a 250-fold dilution have been previously described [4]. Immunoreactivity was detected by enhanced chemoluminescence (Amersham).

3. Results

3.1. Cisplatin effects on growth inhibition and apoptosis

CDDP exerts its cytotoxic effect by inducing apoptosis [2]. In both SKOV3 and OAW42 cell lines, the number of viable cells decreased as a function of drug concentration (Fig. 1a). The drug concentrations inhibiting cell growth by 50 and 100% after 2 days were 5 and 20 μg/ml, respectively, for OAW42 cells, and 10 and 100 μg/ml CDDP, respectively, for SKOV3 cells. SKOV3 cells appeared, therefore, to be more resistant than OA4W2 cells to CDDP. The decrease of viable cell number was related to the commitment to apoptosis of the cells exposed to the drug (Fig. 1b): at concentrations higher than 5 μg/ml CDDP, the proportion of apoptotic cells was higher for OAW42 than for SKOV3. Using non-lethal CDDP concentrations, we followed OAW42 and SKOV3 cell growth for several weeks after a pulse exposure to the drug. Whereas cisplatin-induced apoptosis progressed in the cell population over several days, cell proliferation in both cell lines resumed (Fig. 1c) after a period of time. Despite the presence of 20% apoptotic cells during the 2 days following exposure to 5 μg/ml CDDP, the number of SKOV3 viable cells remained constant and a quite normal growth pattern resumed after less than a week. At the same drug concentration, OAW42 cell number dramatically decreased during 7 days and the proliferation slowly resumed thereafter, until recovery to a normally proliferating cell population after 6–8 weeks. A similar pattern of recovery was also observed in SKOV3 cells in only 2 weeks after exposure to a 4-fold higher concentration of CDDP (20 μg/ml).

3.2. Effect of p21 expression on cell growth

In the CDDP-sensitive OAW42 cells, CDDP induced the accumulation of p53, and in parallel, that of $p21^{cip1}$ (Fig. 2a). SKOV3 cells are defective in p53 [30]; p21 was basically undetectable and was not induced after exposure to CDDP. Both the unde-

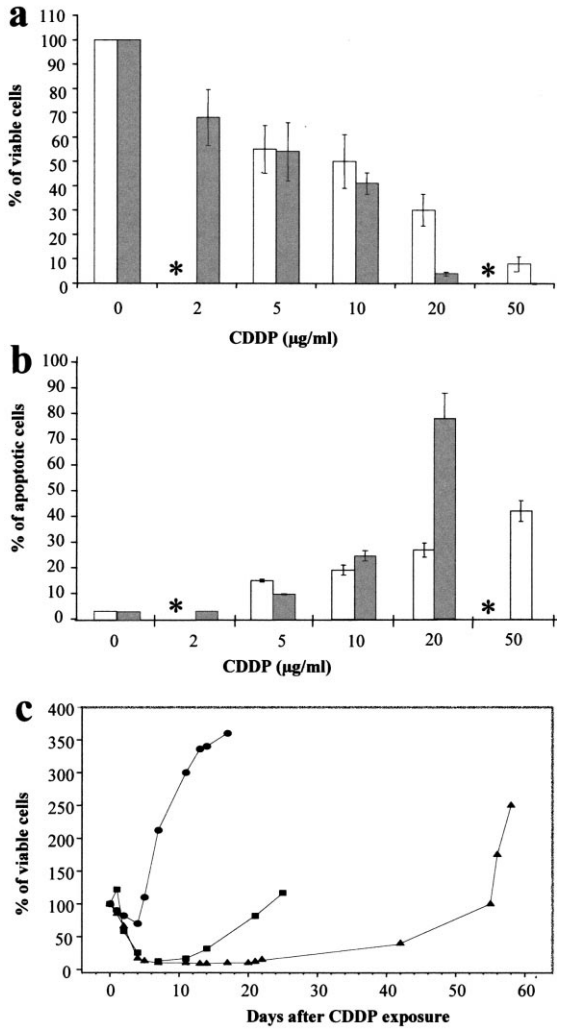

Fig. 1. Cisplatin effect on (a) the percent of viable SKOV3 (open bars) and OAW42 (gray bars) cells remaining 2 days after a 2 h pulse exposure to the drug, and (b), in parallel, apoptosis induction determined by nuclear morphology after DAPI staining. The values in (a) and (b) represents the mean ± SEM in a representative experiment out of 4 for SKOV3 and 2 for OAW42. (*, not done). In (c), cell growth was determined at long term after a 2 h pulse exposure to CDDP: 5 μg/ml in SKOV3 cells (dots), 20 μg/ml in SKOV3 cells (squares) and 5 μg/ml in OAW42 (triangles). The results are expressed as the percent of viable cells compared with the number of cells at the day of treatment. A typical experiment out of three is shown.

tectable level of p21 and the high dose of drug necessary to totally kill these cancer cells in vitro prompted us to assess the effects of ectopic expression of p21 on SKOV3 cell growth. One day after the transient transfection with a cDNA containing the entire translated sequence of the human *waf1/cip1* gene, the expected 560 bp band was present (Fig. 2b), indicating that the transfected cDNA has been transcribed. The appearance of a p21 protein in the transiently transfected SKOV3 cells was shown by Western blot and immunostaining (Fig. 2c,d, left). Accumulation of p21 protein was induced in pTRE-p21-transfected SKOV3-Tet-Off cells, in an inverse correlation with the concentration of Dox (Fig. 2c,d, right). Immunostaining of p21 revealed that the protein accumulated in the nucleus at a very heterogeneous level. About 40% of the cells were positive.

The effect of p21 expression on cell growth was evaluated. Two days after transient transfection, the growth rate was null; it slowly increased thereafter, remaining at a level of 50% lower than that of controls (Fig. 3a). The efficiency of growth inhibition declined after 5 days, due to the transient modality of transfection leading to the progressive loss of p21 expression after day 3 (data not shown) and to the partial transfection of the cell population. DNA flow cytometry depicted in Fig. 3b shows a noticeable reduction of the number of cells in S phase in p21-transfected cells as compared with untransfected cells, indicating that p21 expression arrested the cells in G1 and G2. The relationship between growth inhibition and the p21 level was demonstrated in SKOV3-Tet-Off cells transfected with pTRE-p21, in which the growth rate was proportional to the level of p21 repression by Dox. As before, in the presence of p21 expression (0 or 0.1 ng/ml Dox), cell growth was efficiently inhibited during the 4 days following tranfection. Overexpression of p21 either after transient transfection or induction after Dox removal did not affect cell morphology (data not shown).

3.3. Increased cytotoxic effect of CDDP in p21-expressing cells

To study if p21 expression would affect the cell sensitivity to CDDP, we exposed p21-transfected

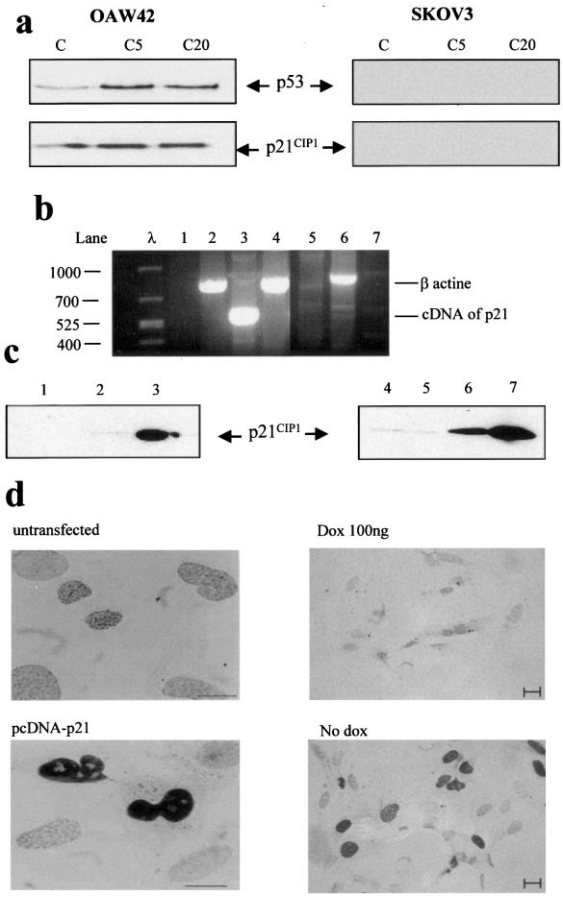

Fig. 2. (a) Modification of p53 and $p21^{cip1}$ expression in OAW42 and SKOV3 cells, 6 h after a pulse exposure to 5 (C5) or 20 μg/ml (C20) CDDP, revealed by Western blot. (b) Expression of $p21^{cip1}$ mRNA, revealed by RT-PCR, as described in Section 2.3, of untransfected SKOV3 cells (lane 1), after transfection with a plasmid construct encoding $p21^{cip1}$ (p21-pcDNA, 2 μg) (lane 3) or a control plasmid (pcDNA3.1, 2 μg) (lane 5). RT-PCR of β-actin mRNA was used as positive control in untransfected (lane 2), p21-pcDNA-transfected (lane 4) and pcDNA3.1-transfected (lane 6) cells. In lane 7, p21 cDNA was amplified without reverse transcriptase reaction, to assess the absence of genomic DNA in the RNA preparations. The base pair ladder at left was given by the Euroladder M (Eurobio). (c) Western blot of p21 in SKOV3 cells, 1 day after transfection: untransfected cells (lane 1), cells transfected with 2 μg pcDNA3.1 (lane 2), and cells transfected with 2 μg p21-pcDNA (lane 3). Western blot of p21 in SKOV3-Tet-Off cells, 1 day after transfection with 2μg pTRE-p21 plasmid, in the presence of Dox at 100 ng/ml (lane 4), 10 ng/ml (lane 5), 0.1 ng/ml (lane 6) or without Dox (lane 7). (d) Immunocytochemistry revealed by a streptavidin–biotin–peroxidase system of untransfected and p21-pcDNA-transfected SKOV3 cells (left), and of pTRE-p21-transfected SKOV3-Tet-Off, with 100 ng/ml Dox or without Dox. Bars: 10 μm

SKOV3 cells to different concentrations of the drug. Combination of p21 gene transfert and CDDP decreased the number of surviving cell, compared

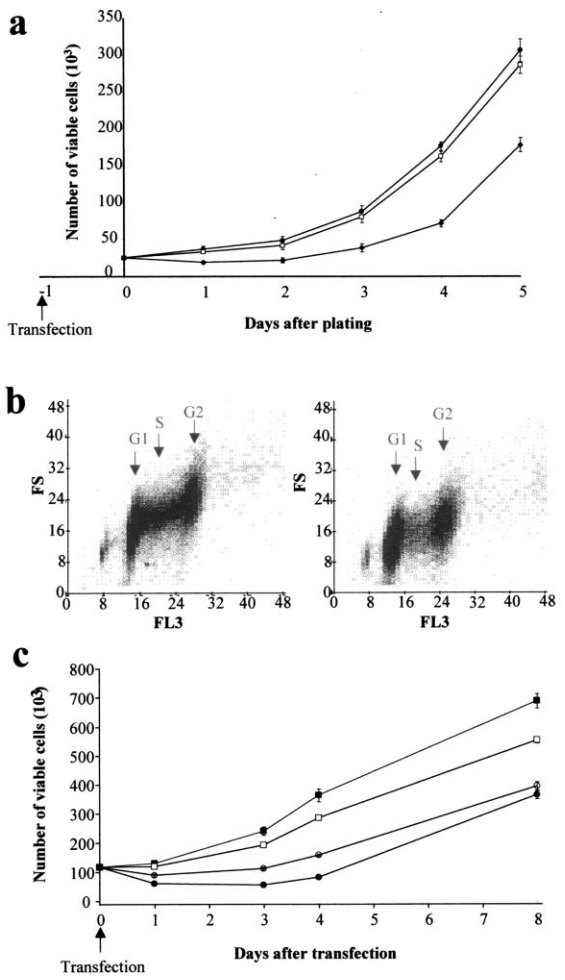

Fig. 3. Effect of $p21^{cip1}$ on cell growth. In (a), SKOV3 cells were transfected with 2 μg p21-pcDNA (black diamonds), or with the irrelevant pEGFP plasmid (open squares) at 70% confluency, and plated the next day at 25×10^3 cells per flask. The curve in black dots represent untransfected cells plated in the same conditions. Cell growth was determined by cell numeration on duplicate flasks, excluding Trypan Blue-positive cells. In (b), DNA flow cytometry, expressed as forward scatter (FS) in function of propidium iodide fluorescence (FL3), was determined on untransfected (left) and p21-transfected cells (right), at day 3 after transfection. The phases of the cell cycle are indicated by an arrow. In (c), SKOV3-Tet-Off cells were transfected with pTRE-p21 at day 0 and incubated in the presence of decreasing concentration of Dox: 100 ng/ml (black squares), 10 ng/ml (open squares), 0.1 ng/ml (open dots) or without Dox (back dots). Cell growth was evaluated as in (a).

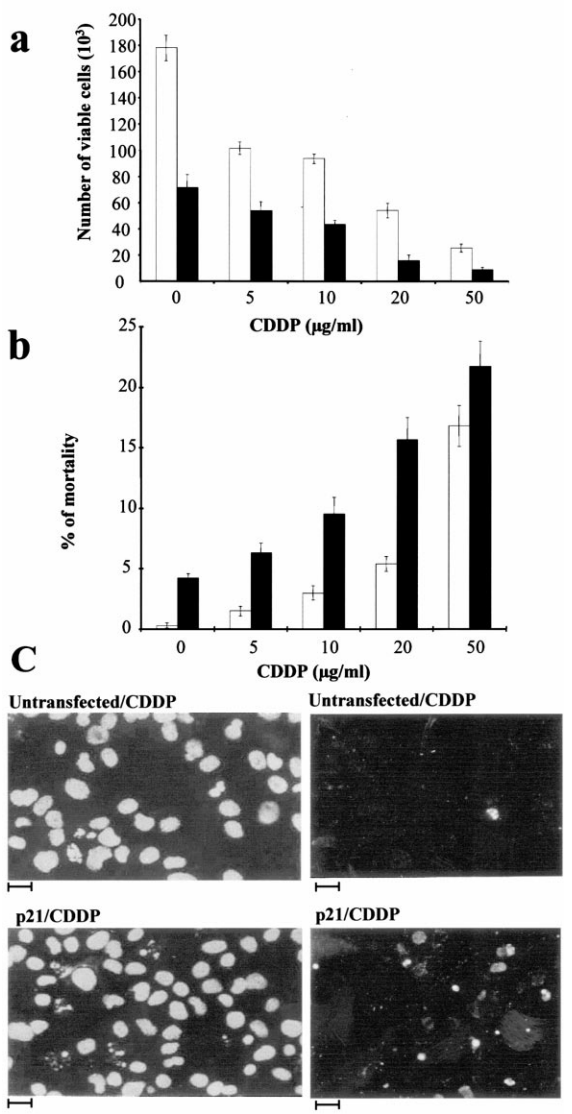

Fig. 4. Cytotoxic effect of p21 in combination with CDDP in SKOV3 cells. Parental SKOV3 cells (open bars) or 2 μg p21-pcDNA-transfected SKOV3 (black bars) were exposed for 2 h to increasing concentrations of CDDP, the day following transfection. Cell viability (a) was determined by Trypan Blue exclusion, 2 days after drug treatment. Cell mortality (b) represents the proportion of Trypan Blue-positive cells relative to the total number of cells. The results are expressed as the mean ± SEM of duplicates, and correspond to a representative experiment out of four. (c) Morphological assessment of apoptosis, by DAPI staining (left) or by in situ TUNEL procedure (right), as described in Section 2.6, the day following a 2 h pulse exposure to 20 μg/ml CDDP. Apoptotic cells display a fragmented nucleus, with condensed chromatine, visible as well after DAPI staining than after TUNEL reaction.

with CDDP alone (Fig. 4a). For example, the number of residual cells after the association p21-20 μg/ml CDDP was two times lower than that of cells surviving 50 μg/ml CDDP without tranfection, indicating that p21 decreased by at least a factor two the concentration of CDDP necessary to achieve the same reduction of tumor cells. The p21-CDDP combination actually enhanced cell death (Fig. 4b). In the absence of CDDP, p21 gene transfert gave rise to a low proportion (4%) of dead cells; in the presence of CDDP, the proportion of dead cells was enhanced in p21-transfected cells as compared with parental cells. Apoptosis took part of this cell death, since an enhanced number of cells with characteristic nuclear fragmentation and positive TUNEL reaction were visible as soon as one day after CDDP treatment (Fig. 4c).

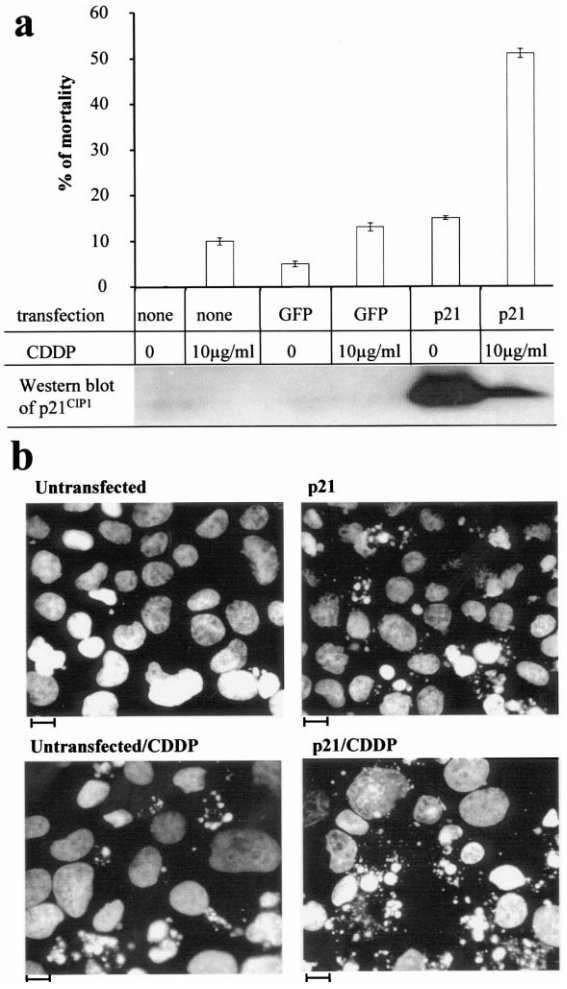

Fig. 5. Cytotoxic effect of p21 in combination with CDDP in OVCAR3 cells. OVCAR3 cells were transfected either with pEGFP (2 μg) or with p21-pcDNA (2 μg), using PEI as described in Section 2.4. The next day, they were exposed for 2 h to 10 μg/ml CDDP. In (a) cell mortality was quantified 2 days after drug treatment by the proportion of Trypan Blue-positive cells, relative to the total number of cells. Western blot of p21 was used to qualitatively verify p21 expression before and after transfection with p21-pcDNA. In particular, the decreased p21 level after the combination p21/CDDP was only due to the decreased number of cells used for the Western blot. In (b), OVCAR3 cells were stained with DAPI to detect apoptotic cells two days after drug treatment. Bars: 20 μm

The enhanced cytotoxic effect of CDDP after p21 transfection was verified in another p21-negative cell line, OVCAR3. In this cell line also, the reestablishment of p21 expression markedly enhanced the cytotoxic effect of CDDP (Fig. 5a). This was confirmed by the increased proportion of apoptotic cells and amount of cell debris after the combination p21/CDDP, compared with CDDP alone, as depicted in Fig. 5b.

The kinetics of cell viability after a pulse exposure to CDDP was assessed in pTRE-p21 transfected SKOV3-Tet-Off in relation to the level of p21 expression (Fig. 6). Two days after CDDP treatment, cell growth was stopped at any level of p21 expression. When p21 was repressed by Dox, cell growth resumed at a rate inversely correlated to the level of p21. In particular, pTRE-p21 transfected SKOV3-Tet-Off incubated with 100 ng/ml Dox behaved like parental SKOV3 cells in the presence of low concentrations of the drug (Fig. 1c). When p21 was induced after Dox omission, the number of residual cells after CDDP treatment dramatically decreased during several days.

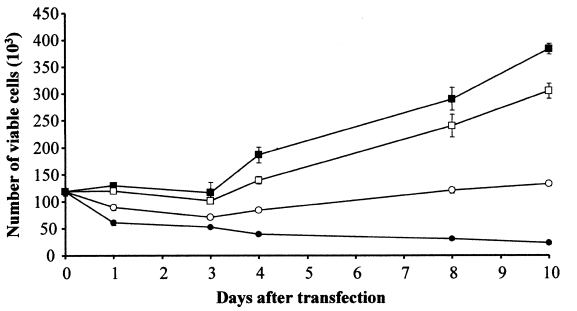

Fig. 6. Kinetics of cell viability of 2 μg pTRE-p21 transfected SKOV3-Tet-Off cells, after a 2 h exposure to 5 μg/ml CDDP at day 1, and in the presence of Dox at 100 ng/ml (black squares), 10 ng/ml (open squares), 0.1 ng/ml (open dots) or in the absence of Dox (back dots). Cell growth was determined as in Fig. 3a.

Cell survival to CDDP was examined after 2 weeks. Parental SKOV3 cells exposed for 2 h to 20 μg/ml CDDP resumed cell growth before 2 weeks, as described in Fig. 1c, in the form of small colonies, whereas in p21-transfected cells exposed to the same dose of drug, no colonies were visible (Fig. 7a). In pTRE-p21-transfected SKOV3-Tet-Off cells, recurrent cell growth could be inhibited either by 20 μg/ml CDDP or by the combination of p21 induction after Dox omission and only 5 μg/ml CDDP (Fig. 7b).

4. Discussion

The seriousness of ovarian cancer, which is basically related to the acquisition of chemoresistance after conventional therapy, combined with extensive data unanimously indicating the tumor suppressor effect of $p21^{cip1}$, prompted us to explore the effect of ectopic expression of p21 in ovarian carcinoma cells. In the present report, we show that *waf1/cip1*gene transfert enhanced the cytotoxic effect of CDDP and hindered the proliferation of recurrent ovarian carcinoma cells.

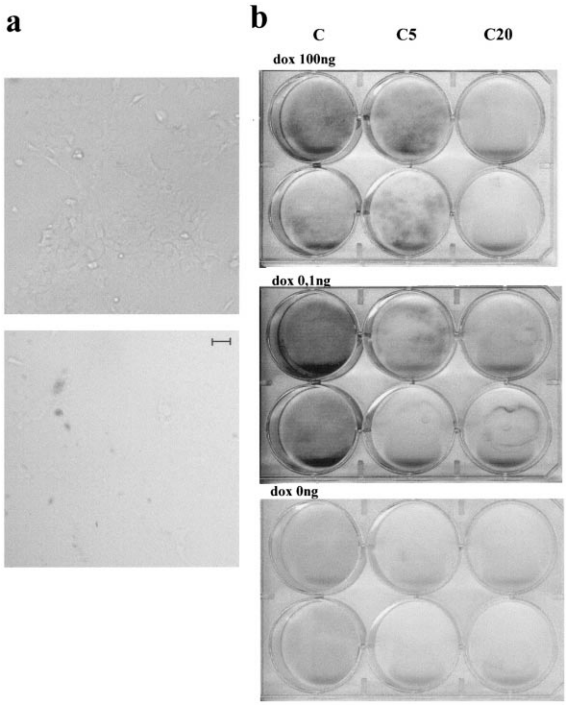

Fig. 7. Recurrent cell growth after CDDP treatment. (a) Phase contrast microphotography of SKOV3 cell culture 14 days after a pulse exposure to 20 μg/ml CDDP (upper photography), or after the combination p21-20 μg/mlCDDP (lower photography). Bars: 100 μm. (b) Photography of 2 μg pTRE-p21 transfected SKOV3-Tet-Off cell plates, colored by Giemsa, 15 days after a pulse exposure to 5 (C5) or 20 (C20) μg/ml CDDP or without CDDP (C).

p21 is a universal and potent cdk inhibitor, in part under the control of the tumor suppressor gene *p53* product [8]. Whereas *p53* status is well established in ovarian cancer, with 50% of the tumors expressing a mutated form [6], mutations in the *waf1/cip1* gene were not found in ovarian carcinomas [31] or in tumors in general [32]. Heterogeneous expression of p21 was described among ovarian cancer cell lines and in vivo tumor specimens, but was unrelated to *p53* status or to the tumor cell proliferation rate [33]. p21 mRNA or protein was undetectable in SKOV3 and in OVCAR3 cells; this could be related to the defective *p53*gene in these cell lines [30].

p21 cDNA transient transfection in SKOV3 and p21 induction in SKOV3-Tet-Off cells led to p21 accumulation in the nucleus of a high proportion of cells. In consequence, cell growth was reduced in correlation with the amount of expressed p21: this is in accordance with a large body of data [8,16–19]. Accumulation of cells in G1 and G2 phase after p21 expression is consistent with its dual inhibitory role of cdks and PCNA [13–15]. However, p21 alone was insufficient to maintain totally growth inhibition of SKOV3 cells (Fig. 3) and OVCAR3 cells (data not shown), in contrast to melanoma cells [16], breast carcinoma cells [18] or lung cancer cells [19]. In these tumors, p21 not only restricted cell cycle in G1/G0 phase, but also altered cell morphology [18,19] and differentiation [16]. No morphological changes were observed in any p21-transfected ovarian carcinoma cell lines as compared with the parental cell line. The ineffectiveness of p21 to suppress totally cell growth may be due to an insufficient proportion of transfected cells and/or to a low level of expression. Indeed, since p21 inhibits a variety of cyclin/Cdk complexes in a stoechiometric fashion, insufficient expression may allow an active residual pool of Cdk2, Cdk4, Cdk6, or Cdc2. This could not be resolved by increasing the amount of DNA or transfection agent, because of undesirable toxicity, which

was also observed with unrelated genes such as that coding for GFP. Moreover, some cell lines, including SKOV3, present additional genetic defects conferring to a growth advantage, such as loss of $p16^{ink4a}$ [34] and amplification of the oncogene *c-erbB2* [35]. Correction of only one of these defects is likely to be insufficient to inhibit totally tumor cell growth.

p21-induced cell growth inhibition was accompanied by a slight but reproducible rise of the number of apoptotic cells, suggesting that p21-transfected cells became more susceptible to apoptosis. Examining the effect of p21 on the response to cisplatin, we found that the cytotoxic effect of the drug was enhanced, as demonstrated by the increased rate of cell death, the early induction of apoptosis, and the decreased concentration of drug needed to achieve the same cytotoxic effect than in control cells. Whereas p21-transfection was efficient in growth inhibition only for 4–5 days, p21-transfection in combination with CDDP allowed the erradication of the ovarian tumor cell population, with a dose of drug much lower than in parental cells. This suggests that p21 indeed rendered ovarian tumor cells more sensitive to the cytotoxic effect of the drug and did not protect them from cell death. This contrasts with data supporting the protective role of p21 from apoptosis in other cancer cells [24–27], which could be linked to its ability to enhance DNA repair [36,37]. The discrepancy in the kinetics of p21-induced growth inhibition, which lasted 4–5 days, and p21-induced cell death after CDDP treatment, which lasted at least 2 weeks, raises the possibility of independent p21-induced pathways responsible for enhanced drug cytotoxicity and p21-induced cell growth regulation. p21 gene transfer could have affected some parameters determining chemosensitivity, such as drug uptake and metabolism, DNA repair, and the expression of apoptosis regulators and effectors. These parameters are worth exploring to understand the mechanism by which this cell cycle inhibitor also sensitized ovarian tumor cells to a DNA damaging drug.

Recent studies have suggested that gene transfer could be used to make conventional chemotherapy more effective [38,39] or to deliver two co-operating genes for the induction of apoptosis and tumor growth inhibition [40]. Reduction of tumor cell growth, increased sensitivity to apoptotic stimuli, and abolition of recurrency shown in this report suggest exploring the possible use of p21 as an adjunctive to either conventional chemotherapy.

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References

[1] N. Auersperg, M.I. Edelson, S.C. Mok, S.W. Johnson, T.C. Hamilton, The biology of ovarian cancer, Semin. Oncol. 25 (1998) 281–304.

[2] J.A. Hickman, Apoptosis induced by anticancer drugs, Cancer Metastasis Rev. 11 (1992) 121–139.

[3] E. Segal-Bendirdjian, A. Jacquemin-Sablon, Cisplatin resistance in a murine leukemia cell line is associated with a defective apoptotic process, Exp. Cell Res. 218 (1995) 201–212.

[4] L. Poulain, H. Lincet, F. Duigou, E. Deslandes, F. Sichel, P. Gauduchon, C. Staedel, Acquisition of chemoresistance in a human ovarian carcinoma cell is linked to a defect in cell cycle control, Int. J. Cancer 78 (1998) 454–463.

[5] M. Hollstein, D. Sidransky, B. Vogelstein, C.C. Harris, *p53* mutation in human cancers, Science 253 (1991) 49–53.

[6] B.J. Milner, L.A. Allan, D.M. Eccles, H.C. Kitchener, R.C.F. Leonard, K.F. Kelly, D.E. Parkin, N.E. Haites, *p53* mutation is a common genetic event in ovarian carcinoma, Cancer Res. 53 (1993) 2128–2132.

[7] S.E. Morgan, M.B. Kastan, *P53* and ATM: cell cycle, cell death and cancer, in: G.F. Vande Woude, G. Klein (Eds.), Advances in Cancer Research, Vol. 71, Academic Press, San Diego, CA, 1997, pp. 1–19.

[8] W.S. El-Deiry, J.W. Harper, P.M. O'Connor, V.E. Velculescu, C.E. Canman, J. Jackman, J.A. Pietenpol, M. Burrell, D.E. Hill, Y.S. Wang, K.G. Wiman, W.E. Mercer, M.B. Kastan, K.W. Kohn, J.J. Elledge, K.W. Kinzler, B. Vogelstein, *WAF1/CIP1* is induced in p53-mediated G1 arrest and apoptosis, Cancer Res. 54 (1994) 1169–1174.

[9] V. Dulic, W.K. Kaufmann, S.J. Wilson, T.D. Tlsty, E. Lees, J.W. Harper, S.J. Elledge, S.I. Reed, p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest, Cell 76 (1994) 1013–1024.

[10] T. Waldman, K.W. Kinzler, B. Vogelstein, p21 is necessary for the p53-mediated G1 arrest in human cancer cells, Cancer Res. 55 (1995) 5187–5190.

[11] A. Elbendary, A. Berchuck, P. Davis, L. Havrileski, R.C. Bast, J.D. Iglehard, J. Marks, Transforming growth factor beta 1 induces *Cip1/Waf1* independent of the p53 pathway in ovarian cancer cells, Cell Growth Differ. 3 (1994) 1301–1307.

[12] Y.X. Zeng, W. El-Deiry, Regulation of p21 WAF1/CIP1 expression by p53-independent pathways, Oncogene 12 (1996) 1557–1564.

[13] J.W. Harper, S.J. Elledge, K. Keyomarsi, B. Dynlacht, I.H. Tsai, P. Zhang, S. Dobrowolski, C. Bai, I. Connel-Crowley, E. Swindell, M.P. Fox, N. Wei, Inhibition of cyclin-dependent kinases by p21, Mol. Biol. Cell. 6 (1995) 387–400.

[14] S. Waga, G.J. Hannon, D. Beach, B. Stillman, The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA, Nature (Lond.) 369 (1994) 574–578.

[15] C. Cayrol, M. Knibiehler, B. Ducommun, p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells, Oncogene 16 (1998) 311–320.

[16] Z.Y. Yang, N.D. Perkins, T. Ohno, E.G. Nabel, G.J. Nabel, The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity in vivo, Nat. Med. 10 (1995) 1052–1056.

[17] D. Katayose, R. Wersto, K.H. Cowan, P. Seth, Effects of a recombinant adenovirus expressing WAF1/Cip1 on cell growth, cell cycle, and apoptosis, Cell Growth Differ. 6 (1995) 1207–1212.

[18] M.S. Sheikh, H. Rochefort, M. Garcia, Overexpression of p21WAF1/CIP1 induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines, Oncogene 11 (1995) 1899–1905.

[19] U.S. Joshi, Y.Q. Chen, G.P. Kalemkerian, M.R. Adil, M. Kraut, F.H. Sarkar, Inhibition of tumor cell growth by p21WAF1 adenoviral gene transfer in lung cancer, Cancer Gene Ther. 5 (1998) 183–191.

[20] Y.Q. Chen, S.C. Cipriano, J.M. Arenkiel, F.R. Miller, Tumor suppression by $p21^{WAF1}$, Cancer Res. 55 (1995) 4536–4539.

[21] P. Michieli, W. Il, M.V. Lorenzi, T. Miki, R. Zakut, D. Givol, J.H. Pierce, Inhibition of oncogene-mediated transformation by ectopic expression of $p21^{Waf1}$ in NIH-3T3 cells, Oncogene 12 (1996) 775–784.

[22] M.S. Sheikh, Y.Q. Chen, M.L. Smith, A.J. Fornace, Role of p21Waf1/Cip1/Sdi1 in cell death and DNA repair as studied using a tetracycline-inducible system in p53-deficient cells, Oncogene 14 (1997) 1875–1882.

[23] J. Wang, K. Walsh, Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation, Science 273 (1996) 359–361.

[24] M. Gorospe, X. Wang, K.Z. Guyton, M.J. Holbrook, Protective role of p21(Waf1/Cip1) against prostaglandin A2-mediated apoptosis in human colorectal carcinoma cells, Mol. Cell Biol. 16 (1996) 6654–6660.

[25] M. Gorospe, C. Cirielli, X. Wang, P. Seth, M.C. Capogrossi, N.J. Holbrook, p21 (Waf1/Cip1) protects against p53-mediated apoptosis of human melanoma cells, Oncogene 14 (1997) 929–935.

[26] K. Polyak, T. Waldman, T.C. He, K.W. Kinzler, B. Vogelstein, Genetic determinants of p53-induced apoptosis and growth arrest, Genes Dev. 10 (1996) 1945–1952.

[27] T. Waldman, C. Lengauer, K.W. Kinzler, B. Vogelstein, Uncoupling of S phase induced by anticancer agents in cells lacking p21, Nature 381 (1996) 713–7716.

[28] C. Staedel, J.S. Remy, X. Hua, T. Broker, L. Chow, J.P. Behr, High-efficiency transfection of primary human keratinocytes with positively charged lipopolyamine:DNA complexes, J. Invest. Dermatol. 102 (1994) 768–772.

[29] L. Poulain, C. Ziller, C.D. Muller, P. Erbacher, T. Bettinger, J.F. Rodier, J.P. Behr, Ovarian carcinoma cells are effectively transfected by polyethylenimine (PEI) derivatives, Cancer Gene Ther. 7 (2000) 644–652.

[30] Y. Yaginuma, H. Westphal, Abnormal structure and expression of the p53 gene in human ovarian carcinoma cell lines, Cancer Res. 52 (1992) 4196–4199.

[31] M. Wan, K.J. Cofer, L. Dubeau, *Waf1/Cip1* strucutral abnormalities do not contribute to cell cycle deregulation in ovarian cancer, Br. J. Cancer 7 (1996) 1398–1400.

[32] H. Wanatabe, K. Fukuchi, Y. Takagi, S. Tomoyasu, N. Tsurukoa, K. Gomi, Molecular analysis of the CIP1/WAF1 gene in diverse types of human tumors, Biochem. Biophys. Acta 1263 (1995) 275–280.

[33] N. Barboule, P. Mazars, V. Baldin, S. Vidal, S. Jozan, P. Martel, A. Valette, Expression of p21 WAF1/CIP1 is heterogeneous and unrelated to proliferation index in human ovarian carcinoma, Int. J. Cancer 63 (1995) 611–615.

[34] Y. Yaginuma, H. Hayashi, K. Kawai, T. Kurakane, Y. Saitoh, S. Kitamura, K. Sengohu, M. Ishikawa, Analysis of the Rb gene and cyclin-dependent kinase 4 inhibitor genes (p16INK4 and p15INK4B) in human ovarian carcinoma cell lines, Exp. Cell Res. 233 (1997) 233–239.

[35] J. Deshane, F. Loechel, R.M. Conry, G.P. Siegal, C.R. King, D.T. Curiel, Intracellular single-chain antibody directed against erbB-2 down regulates cell surface erbB-2 and exhibits a selective antiproliferative effect in erbB-2 overexpressing cell lines, Gene Ther. 1 (1994) 332–337.

[36] E.R. McDonald, G.S. Wu, T. Waldman, W.S. El-Deiry, Repair defect in p21WAF1/CIP1 $-/-$ human cancer cells, Cancer Res. 56 (1996) 2250–2255.

[37] S. Ruan, M.F. Okcu, J.P. Ren, P. Chiao, A. Andreef, V. Levin, W. Zhang, Overexpressed WAF1/Cip1 renders glioblastoma cells resistant to chemotherapy agents 1,3-bis(2-chloroethyl)-1-nitrosurea and cisplatin, Cancer Res. 58 (1998) 1538–1543.

[38] A. Inoue, K. Narumi, N. Matsubara, S. Sugawara, Y. Saijo, K. Satok, T. Nukiwa, Administration of wild-type p53 adenoviral vector synergistically enhances the cytotoxicity of anti-cancer drugs in human lung cancer cells irrespective of the status of p53 gene, Cancer Lett. 157 (1) (2000) 105–112.

[39] S. Osaki, Y. Nakanishi, K. Takayama, X.K. Pei, H. Ueno, N. Hara, Alteration of drug chemosensitivity caused by the adenovirus-mediated transfer of the wild-type p53 gene in human lung cancer cell, Cancer Gene Ther. 7 (2) (2000) 300–307.

[40] V. Sandig, B. Karsten, S. Herwig, J. Lukas, J. Bartek, M. Strauss, Adenovirally transferred $p16^{INK4/CDKN2}$ and p53 genes cooperate to induce apoptotic tumor cell death, Nat. Med. 3 (1997) 313–319.