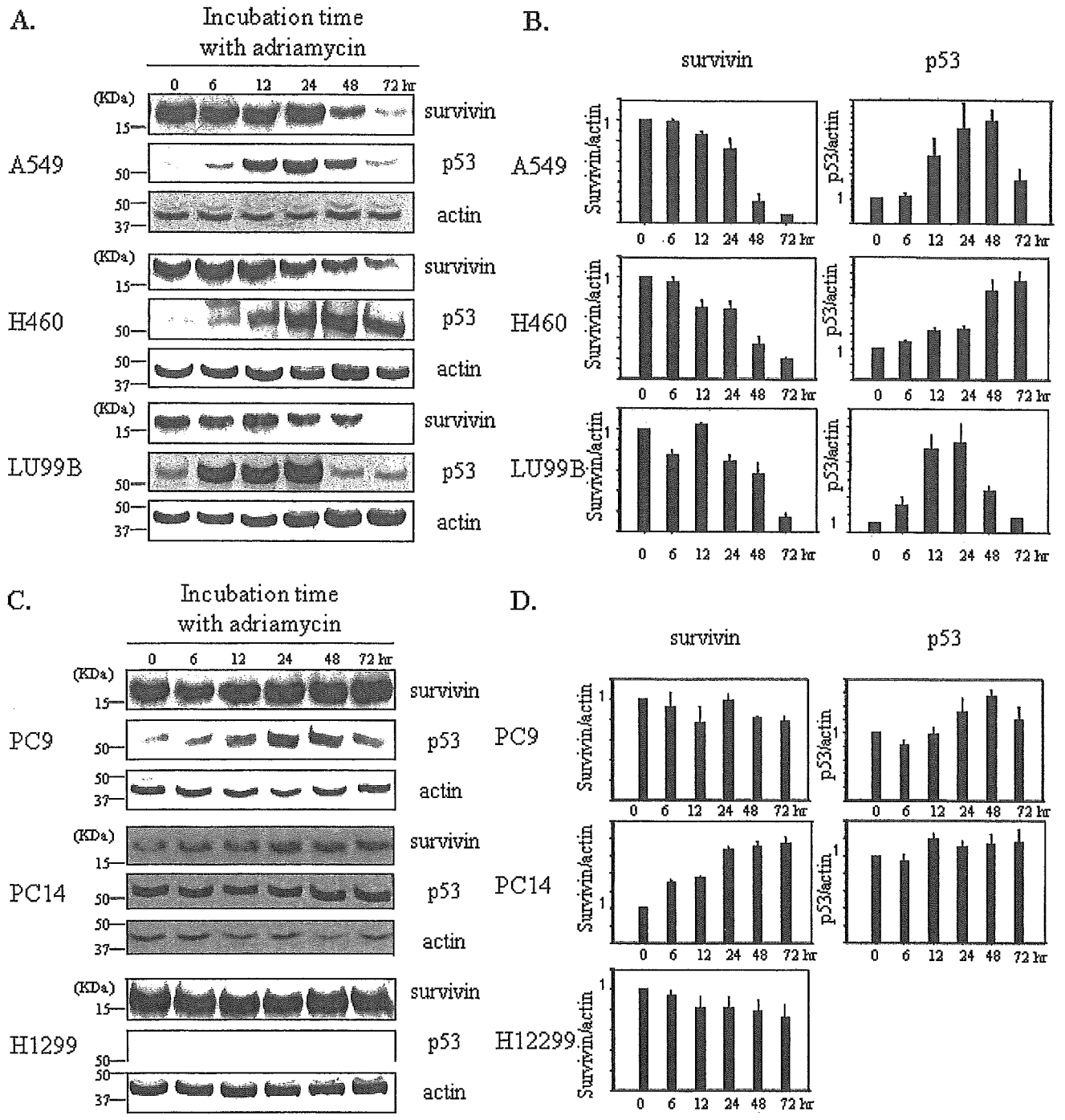


FIGURE 2 – Expression of survivin and p53 protein in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. (*a*) Western-blotting analysis for expression of survivin and p53 in cell lines possessing wild-type p53, including A549, NCI H460 and LU99B. Each of the cell lines was incubated with adriamycin at the $IC_{50}$ dose for the indicated time. Actin served as a control. Treatment, harvest and analysis were repeated 3 times. (*c*) Western-blotting analysis for expression of survivin and p53 in PC9 and PC14, possessing mutated p53, and in NCI H1299, possessing deleted p53. Each of the cell lines was incubated with adriamycin at the $IC_{50}$ dose for the indicated time. Actin served as a control. Treatment, harvest and analysis were repeated 3 times. (*b,d*) Protein expression levels were presented as the mean±SD.

mately $1 \times 10^5$ stained cells were analyzed by flow cytometry in a Becton Dickinson FACS calibur.[28]

*siRNA transfection*

The siRNA duplexes for survivin and p53 were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2′-ACE protection chemistry. The siRNA targeting survivin corresponded to the coding region 206–404 relative to the start codon (GenBank NM001168). The siRNA targeting p53 corresponded to the coding region 775–793. BLAST searches of the human genome database were carried out to ensure the sequences would not target other gene transcripts. Cells in the exponential phase of growth were

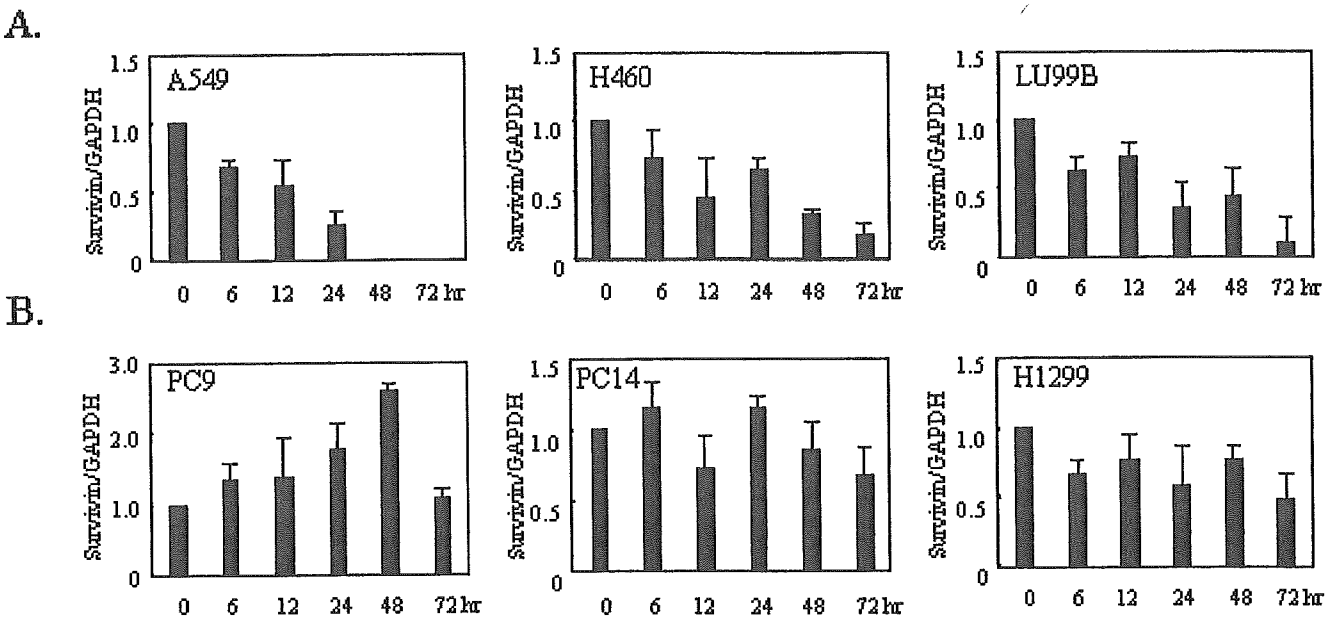


FIGURE 3 – Expression of survivin mRNA in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. Each of the cell lines with wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin at the $IC_{50}$ dose for the indicated time and analyzed by real-time PCR, as described in Material and methods. All data were normalized relative to the concentration of mRNA for the housekeeping gene GAPDH, and are presented as the mean ± SD for at least 3 independent experiments.

plated in 12-well tissue culture plate at $4 \times 10^4$ cells/well, grown for 24 hr and then transfected with 300 nM siRNA using oligofectamine and OPTI-MEM. Serum media (Invitrogen Life Technologies, Inc., Carlsbad, CA) were reduced according to the manufacturer's protocol. Gene silencing was examined with Western blotting 24–72 hr after transfection. Control cells were treated with siRNA duplex targeting scramble (Dharmacon). These studies were repeated 3 times and the data was presented as mean ± SE.

*TUNEL assay*

Cells were fixed in 4% paraformaldehyde (pH 7.4) and then stained and analyzed for apoptosis using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany). Fixed cells were permeabilized using a mixture containing 0.1% sodium citrate and 0.1% TritonX100 and incubated with TUNEL reaction mixture containing terminal deoxynucleotidyltransferase and fluorescein-dUTP at 37°C for 60 min. Flow cytometric analysis using a FACS calibur was done to quantitate apoptosis.[29]

*Cell viability analysis*

Cells treated with adriamycin or transfected with siRNA duplex were washed with medium once and PBS twice, after staining with trypan blue.

*Statistical analysis*

All data are presented as mean ± SD or mean ± SE, and statistical analysis was done by Student's 2-tailed *t*-test (Stat View, SAS Institute, Inc.). Differences at $p < 0.05$ were considered significant.

## Results

*Survivin mRNA expression in lung cancer cell lines*

The level of expression of survivin mRNA in the 22 human lung cancer cell lines was analyzed by TaqMan real-time PCR (Fig. 1). Normalization was performed using GAPDH as an internal control. Harvest and analysis of each cell line was repeated at least 3 times, and the mean and standard deviation for each cell lines is shown. All lung cancer cell lines expressed survivin mRNA, although the expression level varied. Among the 22 cell lines, the p53 status of 17 has been reported. The mean survivin expression of cells with wild-type p53, except for SBC3/ADM, tended to be less than that of cells with mutated or deleted p53 ($p = 0.0192$). Moreover SBC3/ADM, which is 8 times more adriamycin-resistant than SBC3 in terms of $IC_{50}$, expressed about 3 times more survivin mRNA than did SBC3.

*Decrease of survivin expression after adriamycin exposure is dependent on functional p53 accumulation*

To examine the p53 regulation of survivin expression, we monitored the expression of survivin protein in cells treated continuously with adriamycin at the $IC_{50}$ dose by Western blotting (Fig. 2). Harvest, treatment and analysis of each cell line were repeated 3 times. The p53 phenotype of cell lines A549, NCI H460 and Lu99B has been reported previously as wild-type p53; PC9, PC14 and NCI H1299 possess mutant or deleted p53. In the cells with wild-type p53 (A549, H460 and Lu99B), p53 expression was induced 6 hr after adriamycin exposure and reached a peak level by 24 hr or later. Survivin protein expression was repressed for 72 hr after p53 accumulation (Fig. 2*a*). On the other hand, expression of survivin protein in cells with mutated or deleted p53 (PC9, PC14 and H1299) was not significantly decreased, and in fact appeared to be strongly increased in PC14 (Fig. 2*b*). Additionally, we analyzed survivin mRNA modification after adriamycin exposure using real-time PCR (Fig. 3). As was observed for the protein, the level of survivin mRNA showed a temporal decrease in all cell lines (A549, H460 and LU99B) containing wild-type p53. Repression of survivin mRNA in these cell lines started with accumulation of p53 during the first 6 hr (Fig. 3*a*). In contrast, in cell lines with mutated or deleted p53 (PC9, PC14 and H1299), survivin mRNA did not decrease throughout the period of adriamycin exposure. Furthermore, in cell line PC9, the level of survivin mRNA tended to increase (Fig. 3*b*).

*Dependence of altered cell cycle distribution on p53 phenotype following exposure to adriamycin*

In each of the cell lines treated with adriamycin, the cell cycle distribution was analyzed by flow cytometry (Fig. 4). It was found that the cell cycle distribution varied markedly depending on the p53 phenotype. That is, following exposure to adriamycin cells possessing wild-type p53 tended to show arrest in G1/S phase, whereas cells with mutated or deleted p53 became arrested in G2 phase. In cells containing wild-type p53, the G2/M peak tended to decline along with repression of survivin protein after 24 hr of adriamycin exposure, and the proportion of apoptotic cells (sub-G1) increased. On the other hand, in cells with mutated or deleted p53, the decline in the G2 peak was delayed in comparison with wild cells possessing wild-type p53, and only a small proportion of the cells became apoptotic after 24 hr of expression to adriamycin (Fig. 4).

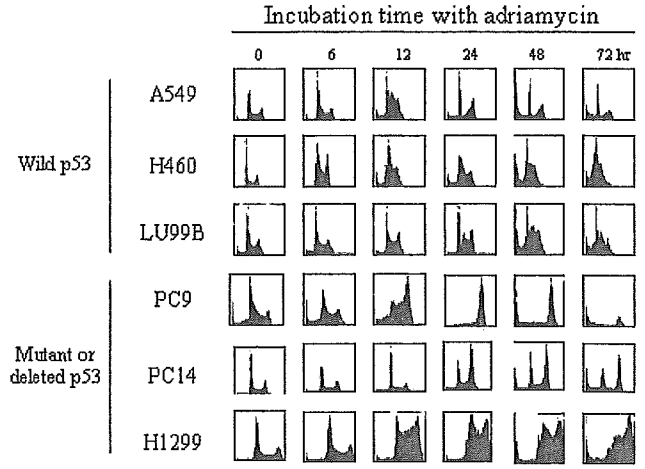


FIGURE 4 – Cell cycle analysis of lung cancer cell lines with different p53 phenotypes after exposure to adriamycin. Each of the cell lines possessing wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin at the $IC_{50}$ dose for the indicated time and analyzed by flow cytometry as described in Material and methods.

*Inhibition of p53 using siRNA duplex, and resulting change in survivin expression*

We examined whether wild-type p53 functionally regulates survivin, using the novel siRNA technique, which specifically inhibits p53. The siRNA duplex was designed to target coding region 775–793 after the start codon of p53. A549, a lung cancer cell line possessing wild-type p53, was transfected with siRNA duplex targeting p53, or scramble as a control, and the resulting levels of survivin expression were determined by Western blotting (Fig. 5*a*). All siRNA molecules have some intrinsic effect on treated cells. We compared cells treated with scrambled siRNA and cells treated with distilled water about p53 and survivin expression. In a result, there is not a significant difference between these. The siRNA duplex targeting p53 reduced p53 protein expression to 54% of the control level within 48 hr (Fig. 5*b*), and this was accompanied by an increase of survivin protein by as much as 2 times the control level (Fig. 5*c*).

*Inhibition of survivin expression by siRNA duplex inhibits cell proliferation and induces cell death*

To evaluate the biological effect of survivin inhibition in lung cancer cell lines, transfection with siRNA duplex was performed. Cell line PC9, with mutated p53, was transfected with siRNA duplex targeting survivin or with that targeting scramble as a con-

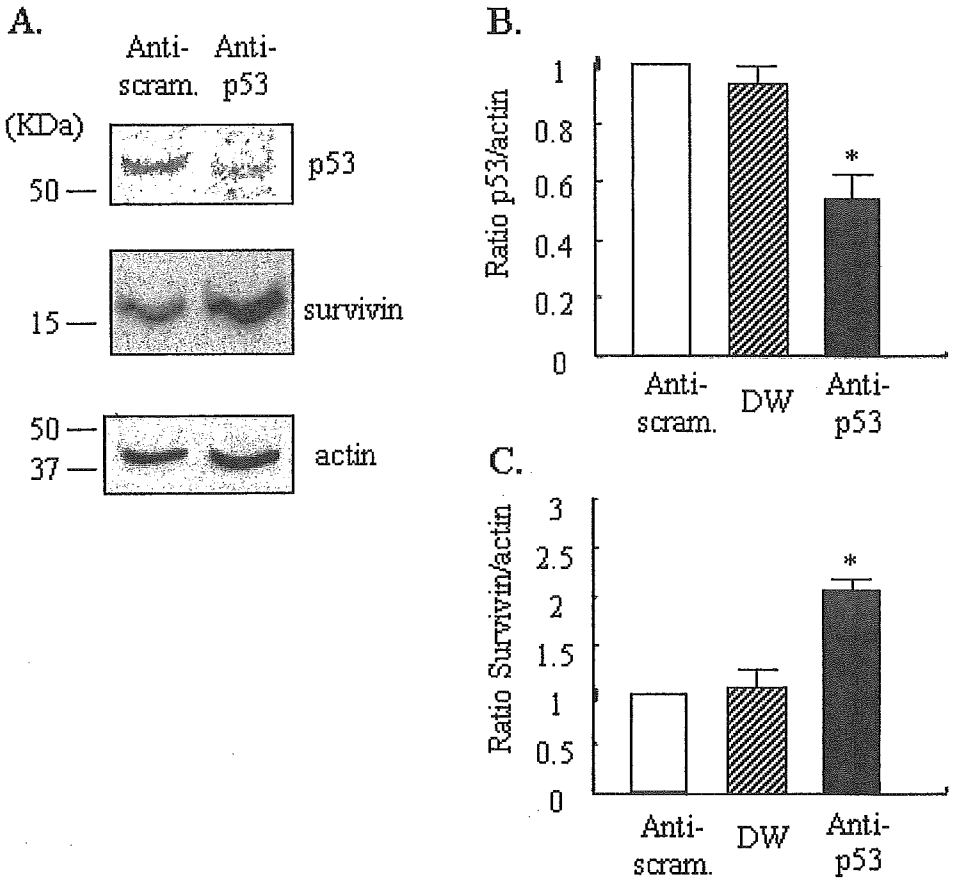


FIGURE 5 – (*a*) Increasing survivin expression in A549 lung cancer cells possessing wild-type p53 as a result of p53 inhibition by siRNA duplex. A549 cells were treated with siRNA duplex targeting p53, scramble or distilled water and then 48 hr later, cell lysates were prepared from the siRNA-treated cells. (*a*) Expressions of p53, survivin and actin were analyzed by Western blotting. (*b*) The expression of p53 protein was analyzed densitometrically using a ChemiImager AlphaImager (ASTEC Co., Japan) and corrected relative to actin. (*c*) The expression of survivin protein was analyzed densitometrically using a ChemiImager AlphaImager and corrected relative to actin. All data are presented as the mean ± SD for at least 3 independent experiments. Statistical analysis was performed by Student's 2-tailed *t*-test. $*p < 0.05$ *vs.* cells treated with siRNA duplex targeting scramble.

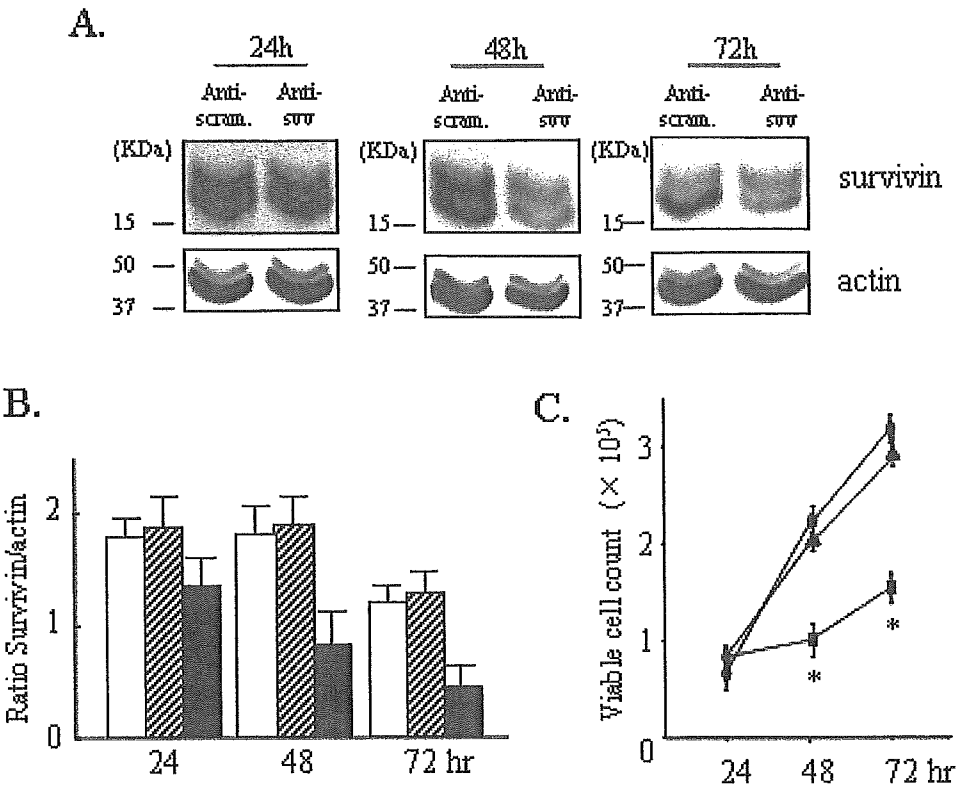


FIGURE 6 – Effects of siRNA targeting survivin on proliferation of PC9 lung cancer cells. PC9 cells were treated with siRNA duplex targeting surviving, scramble or distilled water. At the indicated time, the cells were harvested and assayed using the following procedure. (*a*) Expression of survivin and actin was analyzed by Western blotting, and actin was used as a control. (*b*) The expression of survivin protein was analyzed densitometrically using a ChemiImager AlphaImager, and corrected relative to actin. (*c*) Effect of siRNA targeting survivin (closed square), scramble (closed circle) or distilled water (closed triangle) on proliferation of PC9 cells. Cell proliferation was measured by counting the viable cells using trypan blue staining. All data are presented as the mean±S.E. for at least 3 independent experiments. Statistical analysis was performed by Student's 2-tailed t test. $*p < 0.05$ versus cells treated with siRNA duplex targeting scramble.

trol. Scrambled siRNA did not have unspecific effect on survivin expression compared to distilled water in each point. It was found that expression of survivin protein was significantly repressed after transfection with anti-survivin, compared to the control (Fig. 6*a*,*b*). The level of survivin protein was reduced to 62% of the control within 48 hr and to 45% within 72 hr. We then counted the number of viable cells after siRNA transfection. As shown in Figure 6*c*, the repression of survivin had a direct effect on cell proliferation. At 48 hr post-siRNA, survivin repression significantly reduced the viable cell count to 45% of the scrambled siRNA treated cells ($p < 0.05$) and 47% of the control level at 72 hr ($p < 0.05$). Viable cell count of the scrambled siRNA treated cells was not different from distilled water treated cells in each point. In addition, apoptosis was induced to a greater extent by survivin repression, which is measured by the TUNEL assay (data not shown).

*Sensitization of lung cancer cell lines to adriamycin by siRNA targeting survivin*

Based on the fact that cell lines with mutated or deleted p53 stably expressed survivin after exposure to adriamycin, we investigated the impact of survivin inhibition on adriamycin sensitivity in cells with mutated p53. Cell line PC9 possessing mutated p53 was transiently transfected with siRNA duplex targeting survivin, or with that targeting scramble as a control, for 48 hr. After the transfection, which significantly inhibited survivin expression, the medium was replaced and adriamycin at the $IC_{50}$ dose, or water, was added. Adriamycin exposure was continued for 48 hr, and the cells were then harvested separately for Western blotting, viable cell assay, TUNEL assay and procaspase 3 assay. It was found that siRNA inhibited the expression of survivin by 57% at the start of adriamycin exposure and that survivin inhibition was weakened to 20% by 48 hr (data not shown). In terms of cell proliferation, anti-survivin siRNA duplex alone, adriamycin alone or a combination of both was significantly more repressive than anti-scramble siRNA followed by water, as a control ($*p < 0.05$, Fig. 7). That is, 48 hr after exposure to adriamycin or water, anti-survivin siRNA alone inhibited cell growth to 55% of the control, adriamycin alone reduced cell growth to 39%, and a combination of the 2r reduced cell growth to 21% of the control. Within 12 hr after exposure to adriamycin or water, exposure to anti-survivin siRNA or adriamycin alone did not significantly inhibit cell proliferation compared to the control; however the combination of the 2 significantly repressed cell proliferation to 44% of the control ($*p < 0.05$), and we compared anti-scrambled siRNA with distilled water followed by adriamycin or not. As a result, the scrambled siRNA effect on cell proliferation was small.

*Induction of apoptosis in lung cancer cells by siRNA targeting survivin, and resulting sensitization to adriamycin*

Additionally, we performed a TUNEL assay to evaluate apoptosis (Fig. 8). Cells were transfected with anti-scramble, anti-survivin siRNA duplex or distilled water for 48 hr and harvested for the assay 24 hr after exposure to adriamycin or water. Cells treated with water after anti-scramble were 5.1% TUNEL-positive, whereas cells treated with anti-survivin siRNA alone or adriamycin alone were 24.1% and 18.8% TUNEL-positive, respectively. Anti-survivin siRNA duplex induced significantly more apoptosis than that seen in the control ($*p = 0.0298$). Finally, the combination of anti-survivin siRNA duplex and adriamycin exposure resulted in 51.2% TUNEL-positivity, which was a significantly more potent effect than each of the other treatments ($**p < 0.05$). Intrinsic effect of scrambled siRNA on apoptosis was small, compared to cells treated with scrambled siRNA and cells treated with distilled water.

We additionally assessed procaspase-3 expressed in cells exposed to adriamycin after treatment with anti-scramble, anti-survivin siRNA duplex or distilled water (Fig. 9). It has already been reported that survivin potentially inhibits caspase-3 acti-

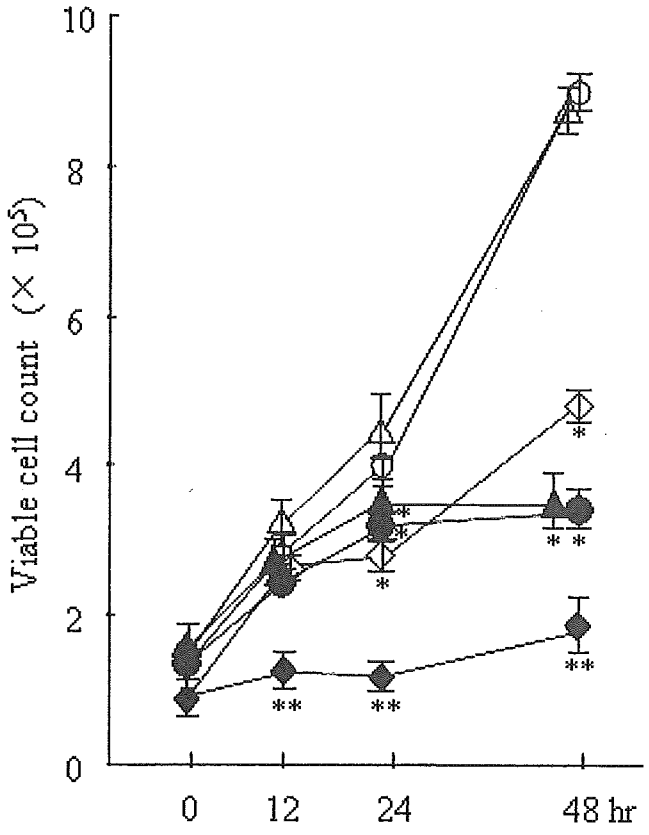


FIGURE 7 – Effects of siRNA duplex targeting of survivin on proliferation of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin or water after 48 hr transfection with siRNA duplex targeting surviving, scramble or distilled water. Open triangle:water after distilled water; open circle: water after transfection with siRNA duplex targeting scramble; open diamond: water after transfection with siRNA duplex targeting survivin; closed triangle: adriamycin after distilled water; closed circle: adriamycin after transfection with siRNA duplex targeting scramble; closed diamond: adriamycin after transfection with siRNA duplex targeting survivin. The data are presented as the mean±S.E. from 3 independent experiments. Statistical analysis was performed by Student's 2-tailed *t*-test. $*p < 0.05$ *vs.* cells treated with water after transfection with siRNA duplex targeting scramble. $**p < 0.05$ vs. other treatments.

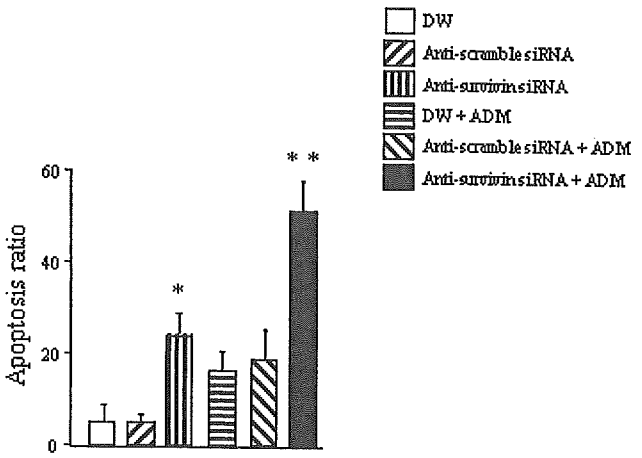


FIGURE 8 – Effects of siRNA targeting survivin on apoptosis of PC9 lung cancer cells treated with adriamycin, evaluated by TUNEL assay. PC9 cells were exposed to adriamycin or water for 24 hr after 48 hr transfection with duplex siRNA targeting surviving, scramble or distilled water. The data are presented as the mean±S.E. for 3 independent experiments. Statistical analysis was performed by Student's 2-tailed *t*-test, $*p<0.05$ *vs.* cells treated with anti-scrambled siRNA. $**p<0.05$ *vs.* cells treated with each of the other treatments.

vation and inhibits apoptosis. The procaspase-3 level in the cells exposed to adriamycin and treated with anti-survivin siRNA decreased to 50% of the level in cells exposed to adriamycin followed by treatment with anti-scramble siRNA duplex. We treated distilled water to replace anti-scramble siRNA, and there is small effect on pro-caspase3 expression in anti-scrambled siRNA.

## Discussion

Survivin mRNA is expressed to various degrees in all of the 22 lung cancer cell lines used in our study. It has been reported that survivin mRNA is detectable in 85.5% of NSCLC tissue samples and that its expression level is correlated with poor prognosis.[3] The mean survivin expression in 6 cell lines with wild-type p53, except for SBC3/ADM, tended to be low in comparison with the mean expression in 10 cell lines possessing mutant p53 ($p = 0.019$). There is no relationship between survivin expression and histology or origin of carcinoma (Table I). It has been reported that survivin expression is associated with accumulation of mutant p53 in gastric cancer and pancreatic carcinoma, assayed by immunohistochemical staining.[30–31] These data suggest that p53 might regulate survivin expression. In addition, after exposure to adriamycin, survivin expression show a transcriptional decrease following accumulation of wild-type p53. Adriamycin is generally classified as a topoisomerase II inhibitor that induces DNA double-strand breaks. The cellular response to DNA damage, which includes nuclear accumulation of p53, has been studied extensively using adriamycin. Thus, we used adriamycin in this study. In our study, p53 inhibition by siRNA duplex resulted in downregulation of survivin expression. The dependence of survivin repression on functional p53 has been investigated previously in a number of different cell models and cancer cell lines.[14,15] Although it is generally accepted that p53 activates a number of genes through direct interaction with their promoter DNA, the mechanism whereby p53 regulates survivin expression is still unclear.[8] One possibility is that p53 might directly bind to the promoter of survivin and repress survivin transcription. In fact, a p53-binding motif is reported to exist within the promoter of survivin.[14,15] In contrast, Mirza *et al.*[15] suggested that a p53-binding motif was not required for transcriptional repression of survivin. They suggested that chromatin deacetylation in the survivin promoter could contribute to p53-dependent repression of survivin gene expression. It is also possible that p53 might increase the level of another transcriptional regulator (*e.g.*, p21) and indirectly downregulate survivin elsewhere downstream.[11] In our study, both survivin and p53 expressions were low in 2 cell lines with wild-type p53 treated with adriamycin for 72 hr (Fig. 2*a*). It may be explained by indirect survivin regulation by another transcriptional factor. Z. Wang *et al.*[32] previously showed that survivin post-translationally increased Mdm2 protein, and subsequently ubiquitination of p53, by blocking caspases that could cleave Mdm2 protein. We showed that p53 functionally repressed survivin expression. In our study, there is a possibility that survivin repression followed by adriamycin exposure might affect p53 accumulation in wild-type p53 cell lines. Survivin expression increased after adriamycin treatment in PC14 possessing mutant p53. Wall NR *et al.*[33] also showed survivin protein increase in MCF7 following adriamycin treatment, and they suggested that survivin was phosphorylated by cdc2 and very little degraded by an ubiquitination-dependent mechanism.

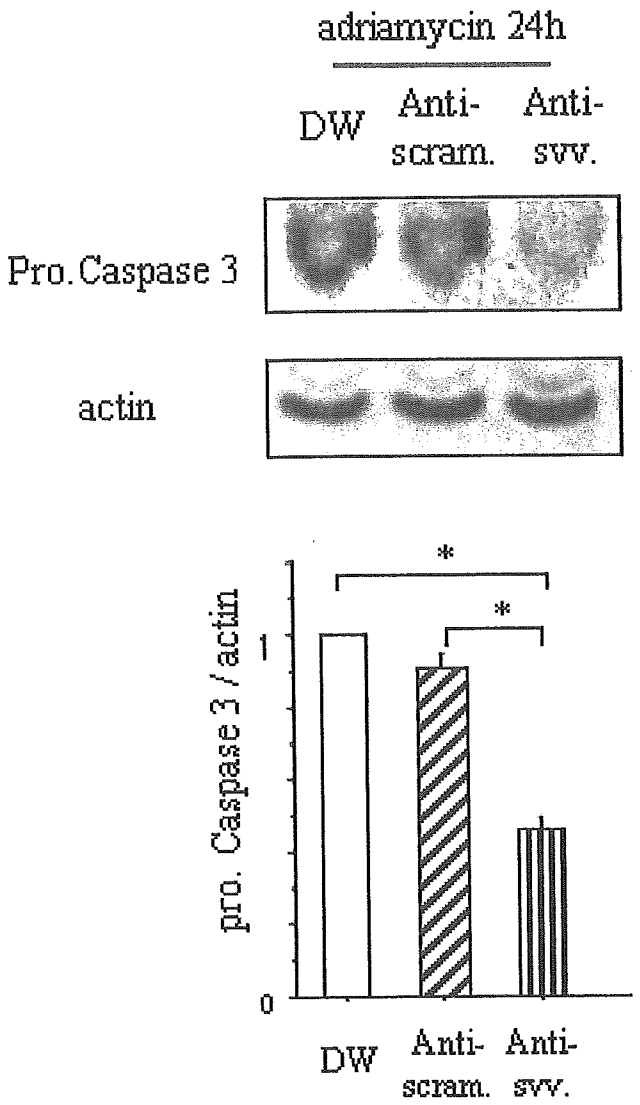


FIGURE 9 – Effects of siRNA targeting survivin on pro-caspase3 expression of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin for 24 hr after 48 hr transfection with duplex siRNA targeting survivin, scramble or distilled water, and each sample was analyzed by Western blotting. The data are presented as the mean ± S.E. for the 3 independent experiments. A representative blot is shown. Statistical analysis was performed by Student's 2-tailed *t*-test, $*p < 0.05$ *vs.* cells treated with other agents.

TABLE I – HISTOLOGY AND ORIGIN OF EACH CELL LINE[1]

| Cell Line | Histology | Origin |
|---|---|---|
| LU99 | La | Prim. |
| A549 | Ad | Prim. |
| EBC1 | Sq | Prim. |
| MA-46 | Sq | Effu. |
| RERF-LC-KJ | Ad | Prim. |
| OBALK1 | La | Effu. |
| Lu99B | La | Effu. |
| PC9 | Ad | Prim. |
| SBC3 | Sm | Prim. |
| NCI-H292 | Muc | Prim. |
| LK-2 | Sq | Prim. |
| LU65 | La | Prim. |
| NCI-H358 | Ad | Prim. |
| PC14 | Ad | Prim. |
| Sq1 | Sq | Prim. |
| NCI-H226 | Metho | Effu. |
| NCI-H460 | La | Effu. |
| NCI-H522 | Ad | Prim. |
| Lu 135 | Sm | Prim. |
| NCI-H1299 | La | Lym. |
| NCI-H69 | Sm | Prim. |

[1]Ad: adenocarcinoma, Sq: squamous cell carcinoma, La: large cell carcinoma, Sm: small cell carcinoma, Metho: mesothelioma, Muc.: mucoepidermoid carcinoma, Prim.: primary, Lym.: lymph node, Effu.: effusion.

Investigation of cell cycle distribution after exposure to adriamycin has shown that cells possessing wild-type p53 tend to become arrested in G1 phase. In these cell lines, transcriptional p21 activation generally leads to G1 arrest. Additionally, we found G2/M phase repression and apoptosis progression accompanying repression of survivin protein. It has been reported previously that transfection with survivin anti-sense or dominant negative survivin gene resulted in accumulation of apoptotic cells and concomitant loss of G2/M phase cells.[34,35] Li *et al.*[7] showed that cells transfected with a mutant survivin gene or survivin anti-sense appeared to show increased caspase3 activity when synchronized in G2/M phase but not in G1/S phase. We therefore analyzed the cell cycle distribution of cell lines possessing mutated or deleted p53. In contrast to cells with wild-type p53, these cells became arrested in G2/M phase. Thus, survivin retention in cells possessing mutant p53 might make them able to resist apoptosis at the G2/M checkpoint.

One critical point of our study was to investigate differences in the proliferation of cancer cells following survivin repression, with the expectation that survivin inhibition itself would have a potent anti-proliferation effect. In cells possessing mutated or deleted p53, survivin was stably expressed even after adriamycin exposure and cell cycle arrest at the G2/M phase, indicating an anti-apoptotic effect. Survivin inhibition by siRNA downstream of p53 induced cell apoptosis and enhanced the anti-proliferative effect. Survivin associates with microtubules of the mitotic spindle at the beginning of mitosis, and disruption of survivin-microtubule interactions increases caspase-3 activity.[7] In order to inhibit survivin specifically, we used siRNA. This efficiently repressed survivin expression and inhibited cell proliferation in the absence of any cytotoxic stimulus. It has been reported that antisense targeting of survivin induces apoptosis in lung cancer cells. Using TUNEL assay, we also confirmed that anti-survivin siRNA duplex induced apoptosis.

Finally, survivin inhibition was found to sensitize PC9 to an anti-cancer agent. Exposure to Adriamycin after repression of survivin by siRNA significantly inhibited cell proliferation compared to cells exposed to either adriamycin alone or anti-survivin siRNA alone. Data obtained by the TUNEL assay confirmed that the difference in cell proliferation was based on apoptosis. *In vitro* binding experiments have indicated that survivin specifically binds to caspase-3 and -7, but not to caspase-8.[6] We also identified repression of procaspase-3 (which means activation of caspase-3) in cells exposed to adriamycin after treatment with anti-survivin siRNA. Activation of caspase-3 by inhibition of survivin may thus promote sensitivity to adriamycin. In our study, the expression of survivin mRNA in SBC3/ADM cells was greater than that in the parental SBC cells (Fig. 1*b*), indicating that survivin expression is related to cell resistance to adriamycin. We identified survivin inhibition by siRNA in cells with mutated p53 sensitized to adriamycin. Combining transfection with a mutant survivin gene with exposure to adriamycin did not enhance apoptosis in HeLa cells and MCF-7 cells, which have wild-type p53, compared to a mutant survivin gene transfection alone or adriamycin alone.[36] The combined effect of the two against apoptosis may be dependent on the character of each cell type, including p53 status or the compound targeting survivin. Additional studies will be needed to

determine the combined effect of survivin inhibition and other drugs on other cell lines.

In conclusion, siRNA targeting survivin could be of potential value for increasing the sensitivity of cancer cells to anti-cancer drugs, especially drug-resistant cells that possess mutated p53.

**Acknowledgements**

We thank E. Hatashita, Y. Yamada, T. Wada and M. Nagasaka for experimental assistance. This investigation was selected for a Scholar-in-Training Award at the 95th Annual Meeting of the AACR.

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ANTICANCER RESEARCH *26*: 777-782 (2006)

# Phase II Study of Weekly Paclitaxel for Relapsed and Refractory Small Cell Lung Cancer

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**Abstract.** *The purpose of this study was to evaluate the efficacy and toxicity of single-agent paclitaxel given weekly to patients with relapsed and refractory small cell lung cancer (SCLC). Patients were treated with 80 mg/m$^2$ paclitaxel administered weekly for 1 h for 6 weeks in an 8-week cycle. Twenty-two patients were enrolled, 21 of whom were eligible. The patient characteristics included: 20 males, 1 female; median age 66 years (range 48 - 75); performance status 0/1 in 19 and 2 in 5 patients. Grade 3/4 leukopenia and neutropenia occurred in 47.5% and 64%, respectively. Other grade 3/4 toxicities included infection, skin rash, neuropathy and pulmonary toxicity. There were 5 partial responses in 3 out of the 11 sensitive cases and 2 out of the 10 refractory cases, respectively. Paclitaxel, administered as a weekly infusion at a dose of 80 mg/m$^2$, was effective in treating relapsed and refractory SCLC.*

More than 95% of patients with small cell lung cancer (SCLC), who are initially treated with paclitaxel 80 mg/m$^2$, present a relapse and their response to a second-line therapy is poor. The responses obtained are usually brief, and the median survival is generally less than 4 months (1). Nevertheless, second-line chemotherapy may provide a significant palliation of symptoms and does result in a prolongation of survival in many patients.

The activity of paclitaxel as a single agent has been investigated in both previously-untreated and -treated SCLC patients. Two phase II trials were conducted to investigate its efficacy as a first-line treatment for SCLC. In a trial conducted by the Eastern Cooperative Oncology Group (ECOG), Ettinger *et al.* administered 250 mg/m$^2$ paclitaxel as a 24-h infusion to 36 patients (2), among whom 11 partial responses were observed. Kirschling *et al.* obtained a similar response rate, 41%, in a group of 37 patients on an identical paclitaxel dose-schedule (3). The results of a phase II study in previously treated patients were reported by Smit *et al.* (4). All 24 patients in that trial developed progressive disease within 3 months of receiving at least one previous chemotherapy regimen. Seven patients (29%) had a partial response to 175 mg/m$^2$ paclitaxel as a 3-h infusion. These data show that paclitaxel exhibits single-agent efficacy in SCLC comparable to that of the best agents. The results of Smit *et al.'s* study in patients with refractory SCLC are particularly impressive, since most response rates reported with single-agent or combination regimens in this population have been less than 15%. However, life-threatening toxicity occurred in 4 of these patients, 2 of whom experienced hematological toxicity.

Recent reports of the activity and tolerability of weekly doses of paclitaxel have generated a great deal of clinical interest. Weekly paclitaxel therapy has generally been quite well tolerated, causing minimal toxicity and no apparent cumulative myelosuppression. Substantial evidence from clinical trials indicates that weekly paclitaxel is effective and generally well tolerated as both a first- and second-line treatment for advanced NSCLC. A phase I/II trial by Koumakis *et al.* in a second-line setting tested weekly paclitaxel infused for the first 6 weeks of each 8-week cycle, and demonstrated that a paclitaxel dose escalation from 60 mg/m$^2$ to 90 mg/m$^2$ was tolerated (5).

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*Key Words:* Paclitaxel, small cell lung cancer.

Fennelly *et al.* reported a recommended dose of 80 mg/m$^2$ administered weekly for 6 weeks of an 8-week cycle in patients with recurrent ovarian cancer (6).

Based on this evidence, a phase II trial of 80 mg/m$^2$ weekly paclitaxel as a 1-h infusion for 6 consecutive weeks followed by 2 weeks without treatment (8-week cycle) was conducted in patients with relapsed SCLC. The objective of this study was to evaluate the efficacy and safety of weekly paclitaxel in patients with relapsed and refractory SCLC. The primary end-point was the response rate, while the secondary end-points were the toxicity profile and survival rate.

## Patients and Methods

*Patient selection.* Patients who met all of the following criteria were considered eligible: a) histological or cytological proof of SCLC with no response to prior chemotherapy or progression after chemotherapy, b) measurable disease, c) most recent cytotoxic treatment less than 4 weeks before entry, d) ECOG performance status 0-2, e) age ≤75 years, f) adequate bone marrow function (leukocyte count ≥4,000/μl, hemoglobin level ≥9.0 g/dl and platelet count ≥100,000/μl), hepatic function (transaminases ≤2.5 times the upper limit of normal, bilirubin level ≤1.5 mg/dl), and renal function (creatinine ≤1.5 times upper limit of normal) and g) arterial oxygen partial pressure ≥60 torr. Excluded patients were those with any active concomitant malignancy, symptomatic brain metastases, a past history of drug allergy reactions, complication by interstitial pneumonia, treatment with non-steroidal anti-inflammatory drugs or steroids or other serious complications such as uncontrolled angina pectoris, myocardial infarction within 3 months, heart failure, uncontrolled diabetes mellitus or hypertension, massive pleural effusion or ascites or serious active infection. All patients gave written informed consent and our institutional review board for human experimentation approved the protocol.

*Treatment schedule.* Paclitaxel was infused intravenously (*i.v.*). over a 1-h period at a dose of 80 mg/m$^2$ each week for 6 consecutive weeks followed by a 2-week break. This 8-week period comprised one treatment cycle. Premedication consisted of 20 mg dexamethasone, 50 mg ranitidine and 50 mg diphenhydramine given *i.v.* 30 min prior to paclitaxel.

If the leukocyte count fell below 2,000/μl or the neutrophil count fell below 1,000/μl, recombinant granulocyte colony-stimulating factor ( rhG-CSF ) at a daily dose of 2 μg/kg was administered until the leukocyte count recovered to ≥10,000/μl, except on the days of paclitaxel administration. The toxicity assessment was based on the National Cancer Institute – Common Toxicity Criteria version 2.0. If grade 3 leukopenia, grade 4 neutropenia, grade 2 neuropathy or other grade 3 non-hematological toxicities occurred, the dose of paclitaxel in subsequent cycles was reduced by 10 mg/m$^2$ from the planned dose. Paclitaxel was not administered if the leukocyte count was <2,000/μl, the platelet count was <5,000/μl, or if there was grade 3 nausea/vomiting, infection with a fever of more than 38°C, or other grade 2 non-hematological toxicities except alopecia. The treatment was discontinued if there was disease progression, grade 3 neuropathy, other grade 4 non-hematological toxicities or a 2 consecutive weeks without paclitaxel administration.

*Evaluation of response and survival.* The tumor response was classified according to the WHO criteria (7). A complete response (CR) was defined as the total disappearance of all measurable and assessable disease for at least 4 weeks. Partial response (PR) was defined as a ≥50% decrease in the sum of the products of the 2 largest perpendicular diameters of all measurable tumors lasting for at least 4 weeks without the appearance of any new lesions. No change (NC) was defined as a decrease of <50% or an increase of <25% in tumor lesions for at least 4 weeks with no new lesions. Progressive disease (PD) was defined as the development of new lesions or an increase of 25% in the sum of the products of the 2 largest perpendicular diameters of all measurable tumors. The overall survival was measured from the time of study entry until death.

*Statistical methods.* The median probability of survival was estimated by the method of Kaplan and Meier (8). This study was designed as a phase II study, with the response rate as the main end-point. According to the Simons minimax design, with a sample size of 20 our study had a 90% power to accept the hypothesis that the true response rate was greater than 25%, while a 10% significance sufficed for rejection of the hypothesis that the true response rate was less than 5% (9).

## Results

*Patient characteristics.* Between December 1999 and February 2002, a total of 22 patients were enrolled in the study, 1 of whom was deemed ineligible due to age (>75 years), leaving a total of 21 patients assessable for toxicity, response and survival. The main demographic characteristics of the cohort are summarized in Table I. The patient cohort consisted of 1 female and 20 males with a median age of 66 years (range, 48 to 75). Four patients exhibited limited disease and 19 exhibited extensive disease at the start of treatment. The majority of the patients had received no prior surgical treatment, while 67% had received prior radiation therapy. All patients had been treated with some form of cisplatin- or carboplatin-based combination chemotherapy regimen. Eighteen patients had received prior etoposide-containing chemotherapy and 10 prior irinotecan-containing chemotherapy. The median number of previous chemotherapy regimens administered was 1 (range, 1 to 2). Among the 10 patients who proved refractory to chemotherapy, 5 had NC or PD on first- or second-line treatment, 2 had PR but experienced disease progression during treatment and 3 had a relapse within a 90-day treatment-free interval after completing their treatments.

*Toxicity.* The toxicity of the regimen is summarized in Table II. Neutropenia was the main toxicity, with 6 out of the 21 patients experiencing grade 4 neutropenia during the entire study. Grade 3 anemia was observed in 2 patients. One patient experienced grade 4 anemia, secondary to digestive tract bleeding. Thrombocytopenia remained infrequent throughout the study. No cases of grade 3 or 4 thrombocytopenia were observed and there was no evidence of cumulative hematological toxicity.

Table I. *Baseline characteristics of all patients.*

| Baseline characteristics | | No. of patients |
|---|---|---|
| Sex | Male / Female | 20 / 1 |
| Age (years) | Median (Range) | 66 (48-75) |
| ECOG PS | 0/1/2 | 5 /12 /4 |
| Disease extent | LD/ ED | 4 / 17 |
| Previous treatment | Chemotherapy only | 4 |
| | Chemotherapy + radiotherapy | 14 |
| | Chemotherapy + others | 3 |
| Previous chemotherapy | Platinum + etopoisde +/- others | 18 |
| | Including irinotecan HCl | 10 |
| | Others | 1 |
| No. of previous chemotherapy regimens | 1 / 2 / 3 | 16 / 4 / 1 |
| Response to prior chemotherapy | CR / PR / NC / PD / NE | 2 / 13 / 5 / 0 / 1 |

No.: number
PS: performance status, LD: limited disease, ED: extensive disease.

Table II. *Toxicity of treatment for all cycles.*

| Toxicity | No. of patients with event by grade | | | | |
|---|---|---|---|---|---|
| | G0 | G1 | G2 | G3 | G4 |
| Nausea | 12 | 7 | 2 | 0 | 0 |
| Vomiting | 19 | 1 | 1 | 0 | 0 |
| Diarrhea | 17 | 3 | 1 | 0 | 0 |
| Constipation | 10 | 5 | 6 | 0 | 0 |
| Mucositis | 21 | 0 | 0 | 0 | 0 |
| Gastric ulcer | 20 | 0 | 1 | 0 | 0 |
| Fever | 16 | 3 | 2 | 0 | 0 |
| Fatigue | 13 | 0 | 8 | 0 | 0 |
| Skin rash | 20 | 0 | 0 | 1 | 0 |
| Infection | 18 | 0 | 0 | 3 | 0 |
| Neuropathy | 9 | 9 | 1 | 2 | 0 |
| Myalgia | 16 | 4 | 1 | 0 | 0 |
| Dyspnea | 17 | 0 | 1 | 2 | 1 |
| Hemoglobin | 1 | 9 | 9 | 1 | 1 |
| WBC count | 2 | 1 | 8 | 8 | 2 |
| Neutrophil count | 0 | 5 | 2 | 8 | 6 |
| Platelet count | 16 | 5 | 0 | 0 | 0 |
| GOT | 12 | 7 | 2 | 0 | 0 |
| GPT | 16 | 4 | 1 | 0 | 0 |
| Total bilirubin | 19 | 1 | 1 | 0 | 0 |

Other grade 3 and 4 toxicities included infection, skin rash, neuropathy and pulmonary toxicity. Grade 1 or 2 neuropathy was seen in 10 patients, and greater than grade 2 was observed in 2 individuals. No hypersensitivity reactions were encountered. Grade 3 or 4 pulmonary toxicity was reported in 3 patients and was characterized by dyspnea. Life-threatening complications of grade 4 infection and grade 4 dyspnea were encountered in 1 patient, who experienced febrile neutropenia and respiratory failure secondary to pneumonia after the third weekly dose. He was treated with antibiotics and supportive measures, but the respiratory distress worsened and he died on day 41. One of 2 grade 3 pulmonary toxicities was pneumonitis, probably induced by paclitaxel, but was resolved by steroid therapy.

*Response to treatment and survival.* The responses to therapy are shown in Table III according to whether the patient had primary refractory disease or primary sensitive cancer that subsequently relapsed. Although 1 out of the 21 patients was not assessable for response, having died during the first cycle, a ≥50% decrease in the sum of the products of the 2 largest perpendicular diameters of the tumor was achieved in this patient. Five of the 22 patients had a PR, but no CRs were observed and the overall response rate was 23.8% (95% confidence interval, 5.59 to 42.03). When only evaluable patients were included in the analysis, however, the response rate improved to 25% (95% confidence interval, 6.02 to 43.98). Two PRs (20%) occurred in refractory cases and 3 PRs (27%) were achieved in sensitive cases. Four patients showed no change, and 1 exhibited disease progression. The survival analysis was performed in January 2003, by which point 10 patients had died and 2 were still alive. The median survival time (MST) was 5.8 months and the 1-year survival rate was 13.4% (Figure 1).

Table III. *Response data.*

| | | No. of patients | | | | | Response rate (%) |
|---|---|---|---|---|---|---|---|
| | | CR | PR | NC | PD | NE | |
| Total | 21 | 0 | 5 | 4 | 11 | 1 | 23.8 |
| Sensitive | 11 | 0 | 3 | 3 | 5 | 0 | 27.3 |
| Refractory | 10 | 0 | 2 | 1 | 6 | 1 | 20.0 |

CI = confidence interval; CR = complete response; NE = not evaluable; PD = progressive disease; PR = partial response; NC = no change.

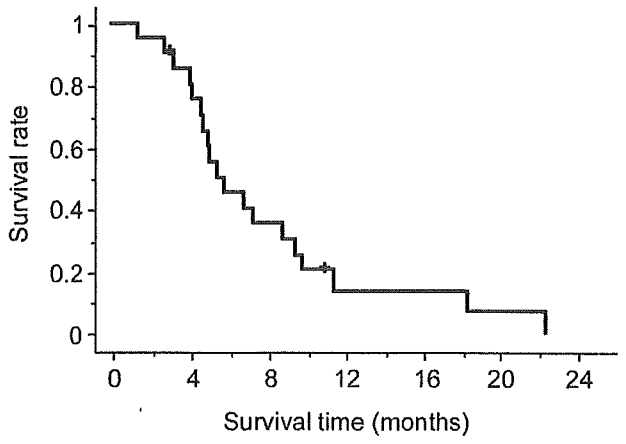


Figure 1. *Overall survival.*

## Discussion

Since the outlook for SCLC patients who receive second-line therapy is poor, several new drugs, such as paclitaxel, docetaxel, gemcitabine, vinorelbine, topotecan and irinotecan, are currently under investigation. The new chemotherapy agents that have been most extensively evaluated in SCLC are the topoisomerase I inhibitors, including topotecan and irinotecan. Von Pawel *et al.* conducted a phase III study comparing single-agent topotecan with cyclophosphamide, doxorubicin and vincristine (CAV) in patients with progression at least 60 days after initial therapy and reported response rates of 24.3% for topotecan and 18.3% for CAV with a median survival time (MST) of 25.0 and 24.7 weeks, respectively, and found that topotecan was at least as effective as CAV in the treatment of patients with recurrent SCLC (10). Two studies of irinotecan in patients with refractory SCLC have been reported in Japan and the response rates in both studies were high, *i.e.*, 50% in 16 patients, and 47% in 15 patients, respectively (11, 12). We therefore consider that topoisomerase I inhibitors, such as topotecan and irinotecan, are key drugs in the second-line treatment of SCLC. However, the number of SCLC patients treated with an irinotecan-containing regimen as first-line chemotherapy has increased in Japan since, in a randomized phase III trial in Japan (13), a combination of irinotecan and cisplatin was shown to yield better survival than the standard etoposide and cisplatin regimen in patients with untreated extensive SCLC. Therefore, the search for effective drugs, other than topoisomerase I inhibitors, for previously treated SCLC, especially refractory SCLC, must be continued.

Single-agent paclitaxel, at a dose of 175 mg/m$^2$ as a 3-h infusion every 3 weeks in patients with previously treated SCLC, produced a response rate of 29% and an MST of 100 days (4). The results of our phase II study demonstrated that weekly paclitaxel at a dose of 80 mg/m$^2$ yielded a similar response rate of 23.8% and a much better MST of 5.8 months than that of paclitaxel given every 3 weeks. Because the antiproliferative activity of paclitaxel is cell-specific, prolonging patient exposure to a low dose of the drug beyond a threshold concentration is ultimately more efficacious than a short-term exposure to higher drug concentrations, a hypothesis supported by *in vitro* experiments with a variety of cell lines and suggested by the results of clinical studies. As clinical experience with paclitaxel treatment of various types of tumors has progressed, so has the use of weekly regimens at lower doses administered as 1-h infusions, as opposed to standard higher doses delivered once every 3 weeks as 3-h infusions.

A response rate of more than 10% is considered evidence of drug efficacy in previously-treated SCLC patients (14). Before newer drugs, such as topoisomerase I inhibitors, taxane, gemcitabine and vinorelbine were introduced, salvage chemotherapy did not usually prolong survival in SCLC and MSTs after relapse were 2.5 – 3.9 months (1). Single-agent phase II trials of gemcitabine, docetaxel and vinorelbine in patients with relapsed or refractory SCLC have been reported. Smyth *et al.* (15), using a 100 mg/m$^2$ dose of docetaxel, obtained a response rate of 25% in 28 assessable patients who had received prior chemotherapy. A trial of gemcitabine in 46 previously-treated patients yielded an 11.9% response rate (16) and vinorelbine provided response rates of 12% and 16% in second-line patients with sensitive disease (17,18). Thus, the MST of 5.8 months and response rate of 23.8% in this study compare favorably with those of published single-agent trials in relapsed or refractory SCLC.

The toxicity profile noted in this trial was predictable based on the toxicity profile previously described in weekly paclitaxel trials, neutropenia being the major toxic effect. All side-effects, except fatal neutropenic pneumonia in 1 case, were manageble. Grade 3 or 4 neutropenia occurred in 14 of the patients in our study but was immediately alleviated by treatment with G-CSF. Grade 3 or 4 anemia occurred in 1 patient, but there was no grade 3 or 4 thrombocytopenia in our study. The incidence of grade 3/4 myelosuppression was considered tolerable. There were 3 cases of grade 3 or 4 pulmonary toxicity, 2 of which occurred due to bacterial infection. This regimen required a dose of 20 mg of dexamethasone weekly as premedication. We believe that this occurrence of bacterial pneumonia might be related to the use of steroids.

Testing new drugs in previously-treated patients has the clear advantages of determining the degree of non-cross resistance with other drugs. Its greatest disadvantage is the risk of a considerable dose reduction (especially of myelotoxic drugs) to avoid extensive hematological side-

effects, perhaps resulting in doses that are too low to fairly evaluate the drug. Since a weekly administration of paclitaxel causes only mild myelosuppression and as there may be no cross resistance with platinum, etoposide, irinotecan, or topotecan, which are usually used to treat SCLC, we find this regimen suitable for previously-treated SCLC.

In summary, the weekly paclitaxel regimen is moderately effective in SCLC patients who have received prior chemotherapy. Based on the statistical design of this study, the 5 PR observed suggest that weekly paclitaxel warrants further evaluation in this patient population. Additional investigations will serve to clarify the role of this agent, either alone or in combination with other agents. Combining paclitaxel with other agents with proven non-cross resistance such as irinotecan, topotecan, or gemcitabine or new target-based agents is the next step needed to evaluate second-line situations, especially in patients with resistant disease.

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*Received September 20, 2005*
*Accepted November 10, 2005*

# Cisplatin Resistance and Transcription Factors

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**Abstract:** Cisplatin is one of the most potent and widely used anti-cancer agents in the treatment of various solid tumors. However, the development of resistance to cisplatin is a major obstacle in clinical treatment. Several mechanisms are thought to be involved in cisplatin resistance, including decreased intracellular drug accumulation, increased levels of cellular thiols, increased nucleotide excision-repair activity and decreased mismatch-repair activity. In general, the molecules responsible for each mechanism are upregulated in cisplatin-resistant cells; this indicates that the transcription factors activated in response to cisplatin might play crucial roles in drug resistance. It is known that the tumor-suppressor proteins p53 and p73, and the oncoprotein c-Myc, which function as transcription factors, influence cellular sensitivity to cisplatin. So far, we have identified several transcription factors involved in cisplatin resistance, including Y-box binding protein-1 (YB-1), CCAAT-binding transcription factor 2 (CTF2), activating transcription factor 4 (ATF4), zinc-finger factor 143 (ZNF143) and mitochondrial transcription factor A (mtTFA). Two of these—YB-1 and ZNF143—lack the high-mobility group (HMG) domain and can bind preferentially to cisplatin-modified DNA in addition to HMG domain proteins or DNA repair proteins, indicating that these transcription factors may also participate in DNA repair. In this review, we summarize the mechanisms of cisplatin resistance and focus on transcription factors involved in the genomic response to cisplatin.

**Key Words:** ATF4, cisplatin, c-Myc, CTF2, mtTFA, p53/p73, YB-1, ZNF143.

## INTRODUCTION

*cis*-diamminedichloroplatinum (II) (cisplatin) plays a crucial role in the treatment of many solid tumors. The mechanisms of cisplatin-induced cytotoxic activity are not completely understood; however, the therapeutic effect of cisplatin is believed to result from the formation of covalent adducts with DNA [1, 2]. Cisplatin has been shown to cause the formation of intrastrand cross-links between adjacent purines in genomic DNA. The major cisplatin cross-links are intrastrand 1, 2-d(GpG) and d(ApG); DNA damage signals then induce apoptosis in various solid tumor cells [1, 2]. Cisplatin treatment induces not only DNA damaging stress, but also oxidative and endoplasmic reticulum (ER) stresses [3, 4]. This, along with the other available evidence, demonstrates the highly complex nature of cellular sensitivity to cisplatin. Of the induced genomic responses, anti-apoptotic defenses are activated simultaneously with apoptotic signaling [5]. The major limitation to clinical treatment is the development of cisplatin resistance by tumors through these mechanisms, which include efflux and detoxification of cisplatin, and DNA repair. Other genes that are differentially expressed in association with acquired cisplatin resistance have been identified, including cytochrome oxidase I, ribosomal protein S28, elongation factor 1α, α-enolase, stathmin and HSP70 [6]. Understanding the molecular basis of cisplatin-induced genomic responses in cisplatin resistance is therefore important for determining clinical strategies.

Many genes have been identified that affect cancer cells during programmed cell death following various genotoxic stresses. The activation of the typical tumor-suppressor proteins p53 and p73 can result in cell-cycle arrest, DNA repair or apoptosis [7, 8]. Loss of p53 function confers resistance to cisplatin in various human cancer cell lines [9], whereas overexpression of p73 is associated with cisplatin resistance [10]. Recently, it has been shown that codon 72 polymorphic variants of p53 display altered mitochondrial translocation and apoptotic potential [11]. Furthermore, mutations in the *p53* gene have been widely detected in various human cancer cells, indicating that p53 might be critical in determining drug sensitivity [12]. However, it is not clear how many transcription factors play significant roles in cisplatin-induced stress responses and drug sensitivity. We believe that transcription factors for genes involved in cisplatin resistance are often activated by DNA damage; therefore, identification and characterization of cisplatin-induced transcription factors might provide a shortcut to deciphering cisplatin sensitivity and resistance in clinical treatment.

In this article, we describe the main mechanisms of cisplatin-induced apoptosis and cisplatin resistance, and discuss the transcription factors involved in resistance to

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1568-0118/05 $50.00+.00 

cisplatin: p53/p73, c-Myc, YB-1, CTF2, ATF4, ZNF143 and mtTFA. Additionally, we refer to the potential responses of some other transcription factors, including octamer transcription factor Oct1 and the zinc-finger protein Sp1, to anti-cancer agents.

## CISPLATIN-INDUCED APOPTOSIS

DNA is the primary target of cisplatin in cancer cells and one of the major cytotoxicities of cisplatin is thought to be caused by the formation of cisplatin–DNA adducts. Cisplatin binds preferentially to the N7 atom of guanine residues, especially in regions of two or more consecutive guanines. Thus, the major cisplatin cross-links are intrastrand 1, 2-d(GpG) and d(ApG), whereas the minor cross-links include intrastrand 1, 3-d(GpNpG), as shown in Fig. (1) [1, 2]. Intrastrand 1, 2-d(GpG) and d(ApG) provide the strongest basis for cisplatin-induced cytotoxicity. Cisplatin is hydrolyzed and equilibrium is maintained between cisplatin (the Cl-Cl species; $(NH_3)_2PtCl_2$), the charged species (the $H_2O$-Cl species; $[(NH_3)_2PtCl(H_2O)]^+$), and the neutral species (the OH-Cl species; $(NH_3)_2PtCl(OH)$) in physiological conditions of intracellular pH and chloride concentration. Charged species under low $Cl^-$ and/or low pH conditions, such as the $H_2O$-Cl and $H_2O$-$H_2O$ species, are more reactive than the Cl-Cl species because of their nucleophilic properties (Fig. (2)). Thus, intracellular $Cl^-$ and pH levels could modulate the cytotoxicity of cisplatin [13].

Cisplatin can induce two major distinct apoptotic pathways via various stress signalings: the first is p53-dependent mitochondrial apoptosis, which begins with translocation of the p53-induced Bax from the cytosol to the mitochondria, followed by cytochrome *c* release and activation of caspase-9 and -3 [14]; the second is the Fas/Fas ligand-dependent caspase-8-induced apoptotic cascade [15].

Mitogen-activated protein kinase (MAPK) signaling pathways, including the extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) pathways, play important roles in cellular responses to stress conditions, including various anti-cancer agents [16]. The ERK signaling pathway is involved in the regulation of cell growth, differentiation, proliferation and survival. By contrast, the p38 and JNK signaling pathways are stress dependent and have apoptotic regulatory functions. Cisplatin-induced activation of ERK signaling could contribute to resistance to cisplatin [17]; conversely, induction of the JNK/p38 signaling pathway in response to cisplatin induced apoptosis via Fas ligand induction in ovarian cancer cells [15]. However, Wang *et al.* have shown that ERK activation plays an important role in the cisplatin-induced apoptosis of Hela cells [3]. These results suggest that such differential effects of MAPK signals in response to drug-induction could reflect cell-type specificity. The functions of the JNK signaling pathway in apoptosis induced by cisplatin also remain unclear, as does ERK signaling [15, 18]. Further investigation is necessary to probe the exact cisplatin-induced mechanisms of these signaling pathways.

Initially, DNA damage signals induced by cisplatin can activate so-called sensor kinases. It was reported that cisplatin induces the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway to mediate p21 expression, suggesting that it might be involved in cell-cycle regulation; however, inhibition of the PI3K/AKT pathway had no influence on sensitivity to cisplatin [19]. However, it was recently reported that AKT phosphorylates the X-linked inhibitor of apoptosis protein (XIAP) and is involved in cisplatin resistance [20]. Moreover, cisplatin could phosphorylate p53 at serine 15 and induce p53 downstream genes via activation of ataxia telangiectasia mutated and Rad3-related protein (ATR) kinase [21]. ATR signaling has also

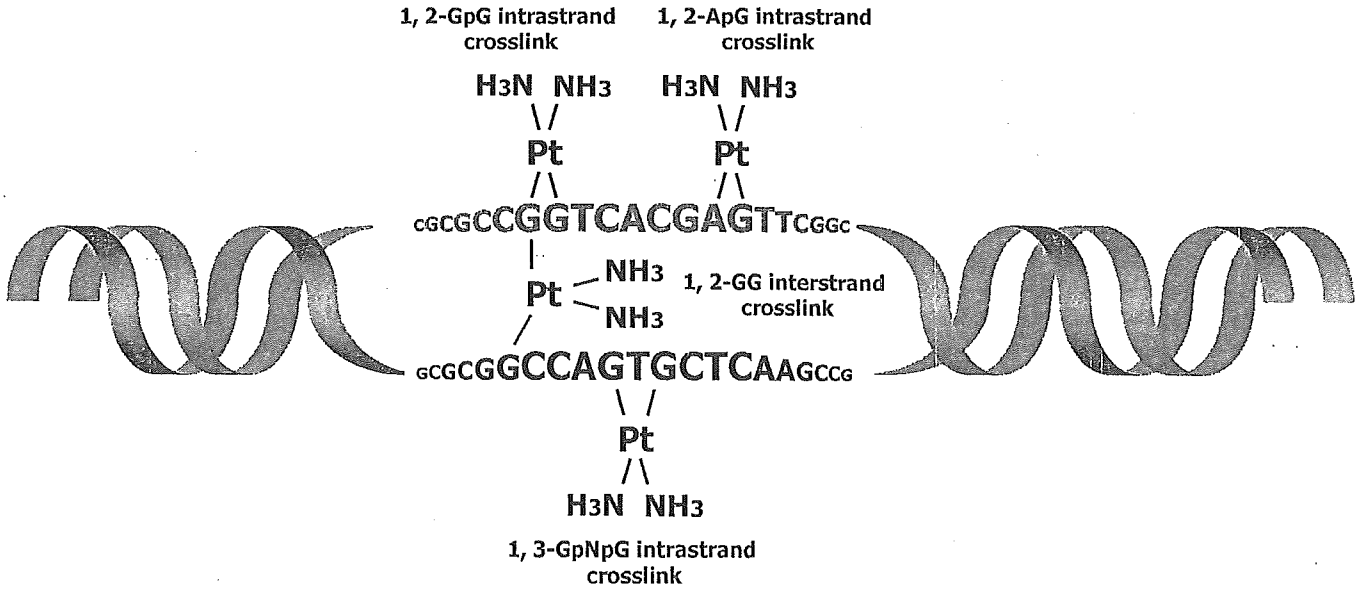


**Fig. (1). Schematic diagram of cisplatin-DNA adducts.**

Intrastrand 1, 2-d(GpG) and d(ApG) are the major cisplatin cross-links (85-90% of total lesions), whereas the minor cross-links is intrastrand 1, 3-d(GpNpG). The major lesions provide the strongest basis for cisplatin-induced cytotoxicity.

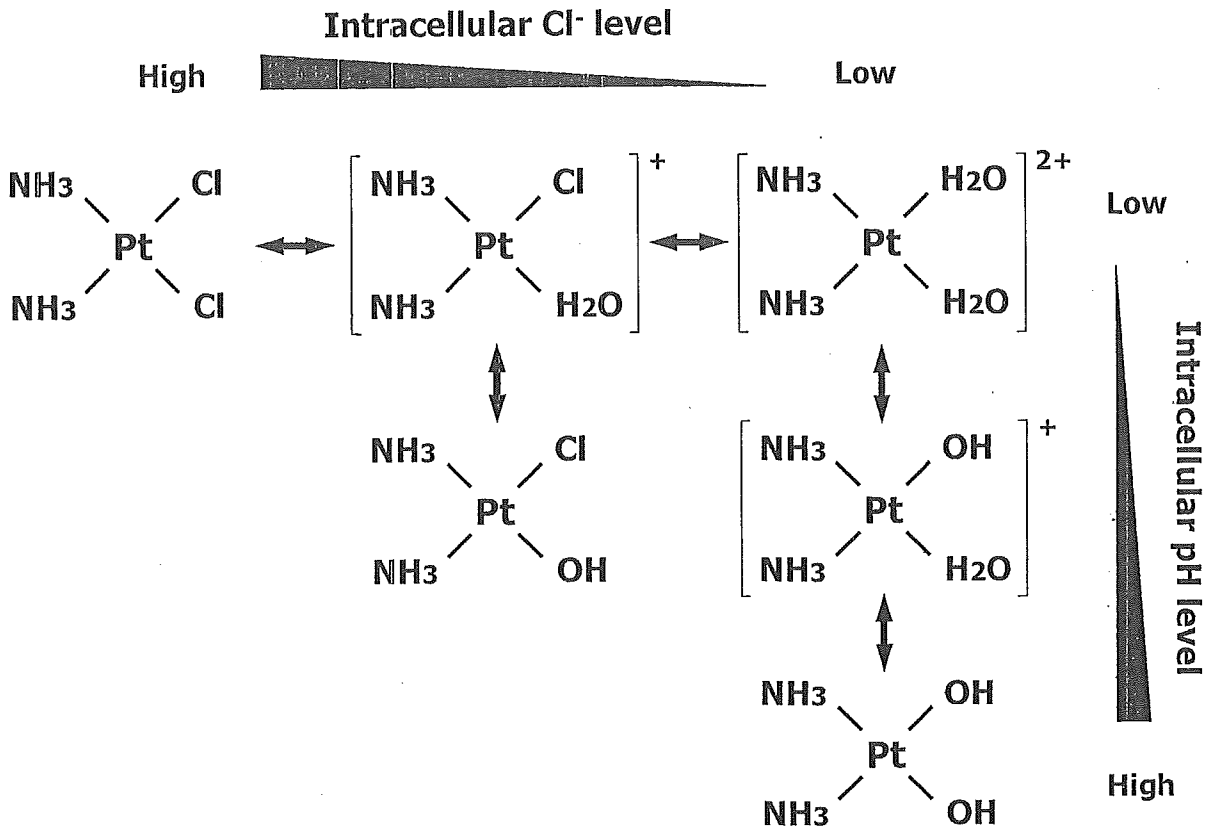


**Fig. (2). Formation of intracellular cisplatin.**

The equilibrium of cisplatin is affected by physiological conditions of intracellular pH and chloride concentration. The charged species under low $Cl^-$ and/or acidic conditions (the $H_2O$-Cl, $H_2O$-OH and $H_2O$-$H_2O$ species) are the most active forms.

been linked to the MAPK signaling cascade [22]. Another kinase, the c-Abl tyrosine kinase, is also activated by cisplatin. This kinase phosphorylates p73 and induces apoptosis [23]. The c-Abl pathway is also associated with the JNK signaling pathway, which is a member of the MAPK family [24]. The evidence therefore suggests that DNA damage signals might undergo crosstalk with each other.

Protein phosphatase is also involved in the cisplatin-induced signaling pathway through regulating the cellular phosphorylation state. Nuclear Src homology 2 domain-containing tyrosine phosphatase (SHP-2) was constitutively associated with c-abl and its phosphatase activity was significantly enhanced in response to DNA damage. It was reported that cells lacking SHP-2 showed markedly decreased apoptosis in response to DNA-damaging agents, such as cisplatin and γ-irradiation [25]. Furthermore, cisplatin has been shown to interact with the tumor-suppressor phosphatase and tensin homolog (PTEN), which plays an important role in cell growth and apoptosis [26]. The enzymatic activity of protein tyrosine phosphatases (PTPs) containing PTEN is often regulated by a redox system, including thioredoxin-1 [27, 28]. Apoptosis signal-regulating kinase 1 (ASK1) is a MAPK kinase kinase that activates p38 and JNK cascades, and is activated in response to oxidative stress [29]. Protein phosphatase 5 interacts with ASK1 and inhibits its activity [30]. These results provide evidence that protein phosphatase is an important modulator of apoptosis through cisplatin-induced DNA damage and oxidative stress, and that it contributes to drug sensitivity.

Reactive oxygen species (ROS) are produced upon various stress stimulations—including ultraviolet (UV) irradiation and cytotoxic agents such as cisplatin—and are closely involved in stress-induced apoptosis. ROS production can enhance sensitivity to cisplatin through activation of the JNK or p38 pathways [31], or through Fas aggregation [32]. Furthermore, it has been recently reported that cisplatin could induce apoptosis in the absence of DNA damage, through ER stress [4]. Cisplatin induced the activation of the calcium-dependent protease calpain, which activated caspase-3 and ER-specific caspase-12 in cytoplasts [4]. These data suggest that the ER might be the non-nuclear target of cisplatin.

Cisplatin-induced apoptotic pathways are complicated, as cisplatin might cause different stresses, such as DNA damaging, oxidative and ER stresses. Various cisplatin-induced stress signals can activate each pathway through specific transcription factors that act as the ultimate drug targets. Cell death or survival in response to cisplatin might be dependent on the relative intensity of, and the crosstalk between, these signal pathways. Fig. (3) shows a schematic summary of cisplatin-induced cellular signaling involved in cell death and survival. Further studies will lead to a better

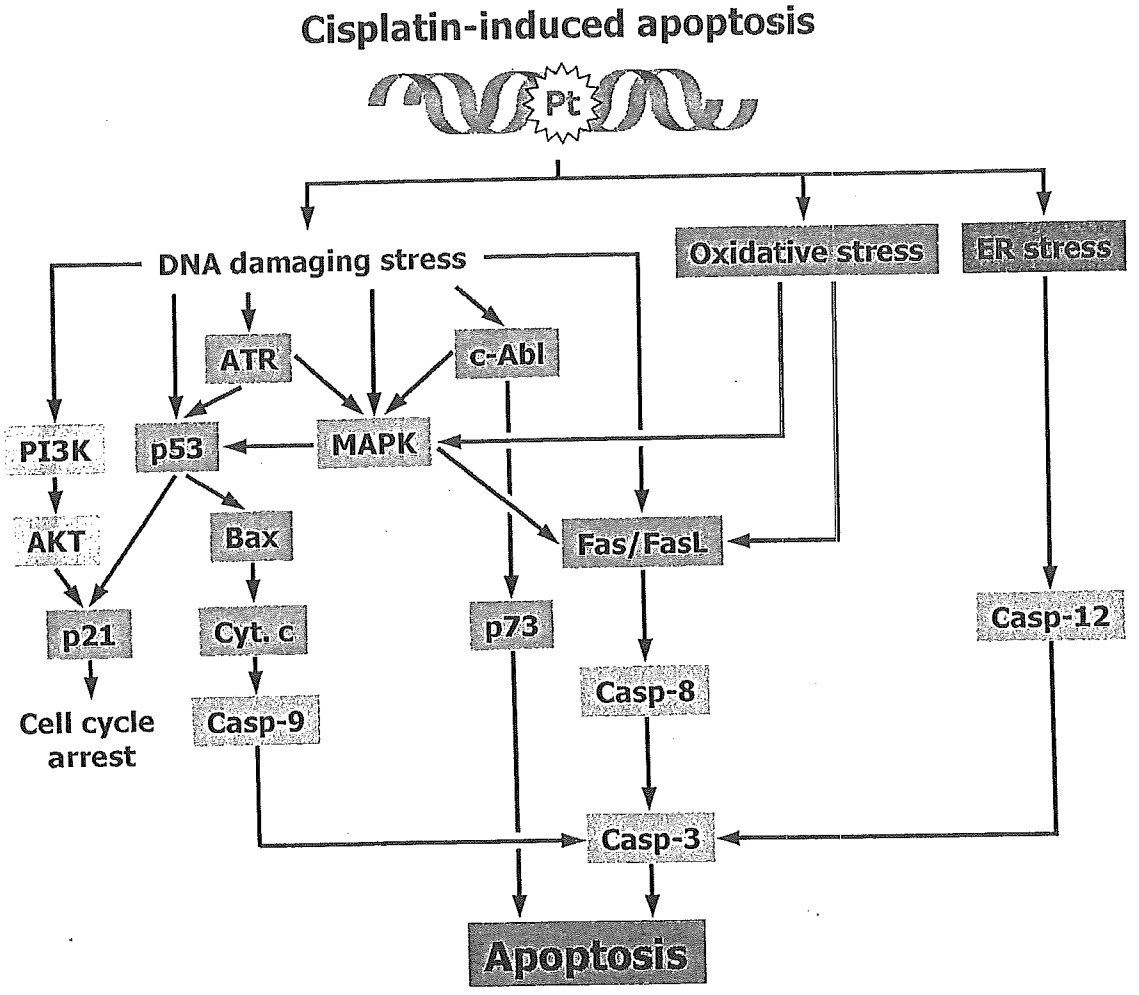


**Fig. (3). Schematic summary of cisplatin-induced damaging signals.**

Cisplatin induces different damaging signals, such as DNA damaging, oxidative and ER damaging stresses. These stresses can activate each pathway through specific phosphorylation cascades, which include transcription factors as the ultimate targets. The fate of cancer cells in cisplatin treatment is determined by the relative intensity of, and the crosstalk between, these signaling pathways.

understanding of the mechanisms involved in cisplatin-induced apoptosis.

## MECHANISMS OF CISPLATIN RESISTANCE

The development of cisplatin resistance by tumor cells is a major clinical limitation in cancer chemotherapy. This resistance might arise due to changes in the biochemical pharmacology of cisplatin. Cisplatin resistance is induced through various mechanisms, including the reduction of cisplatin accumulation inside cancer cells [5]. One of the several possible efflux pumps for cisplatin is the multidrug resistance-associated protein 2 (MRP2; also designated cMOAT). MRP2 is a member of the MRP gene family and these ABC membrane proteins have been connected with the efflux of various drugs [33]. A recent study has shown that expression of MRP2 coincides with resistance to cisplatin [34] and Koike *et al.* have demonstrated that cisplatin sensitivity is increased by antisense MRP2 constructs [35]. These data give an insight into the relationship between MRP2 expression and drug resistance. Moreover, the copper transporters ATP7A and ATP7B have been shown to be involved in cisplatin efflux [36], and have potential as clinical markers in ovarian cancer specimens [37, 38]. However, the P-glycoprotein, which is a membrane channel encoded by the multidrug resistance 1 (*MDR1*) gene, has been reported not to participate in cisplatin resistance [39].

In another mechanism of resistance, increased activity of intracellular pathways of thiol production—including glutathione (GSH), metallothionein and thioredoxin—can contribute to the detoxification of cisplatin [5]. A small fraction of the intracellular cisplatin can bind to genomic DNA. However, a major fraction, about 60% of the intracellular cisplatin, is conjugated with GSH [40]. GSH is one of the most abundant SH-containing molecules, which can interact with cisplatin through the catalytic action of glutathione *S*-transferase π (GSTπ). GS-platinum complexes, which show inactivated cytotoxicity, are discharged from cancer cells via the glutathione conjugate export pump (GS-X pump) [1, 2]. GSTπ and γ-glutamylcysteine synthetase (γ-GCS), which is the enzyme involved in GSH biosynthesis,

were also shown to be associated with cisplatin resistance [41, 42]. Metallothionein is rich in thiol-containing cysteine and is presumed to function in the detoxification of heavy metals such as cadmium. Overexpression of metallothionein has been observed in cisplatin-resistant cell lines [43]. Thioredoxin, which is another intracellular thiol, is a redox-active protein induced by various stresses and secreted from cells. Cellular levels of thioredoxin, thioredoxin reductase and glutaredoxin are associated with cisplatin resistance, as are GSH and metallothionein [5, 44]. The glutathione adducts, which are GS-platinum complexes, inhibit the thioredoxin and glutaredoxin systems; thus, these results are consistent with the correlation between increased thioredoxin and cisplatin resistance [45]. Moreover, it has been recently reported that thioredoxin, acting as a downstream effector of Smad7; inhibitor of transforming growth factor (TGF)-β1 signaling, could suppress cisplatin-induced apoptosis in pancreatic cancer [46]. Reduced thioredoxin is also an inhibitor of ASK1 [29], and peroxiredoxin that is dependent upon thioredoxin activity might protect cancer cells from apoptosis caused by cisplatin-induced oxidative stress [47].

The cytotoxicity of cisplatin is ascribed to the formation of cisplatin-DNA adducts, and to the induction of DNA damage signals and apoptosis. The following damage-recognition proteins have been identified: HMG domain proteins (high-mobility group 1 and 2 (HMG1/2), mtTFA and hUBF); transcription factors lacking an HMG domain (TATA-binding protein (TBP), YB-1 and ZNF143); nucleotide excision repair (NER) proteins (XPE, XPA); and mismatch repair (MMR) proteins (hMutSα and hMSH2) (Table (**1**) [48-57]). These proteins can recruit repair complexes to damaged regions or shield them by inhibiting DNA damage signals.

The NER system is a vital pathway in the removal of cisplatin-DNA adducts and in the repair of DNA damage. First, damage-recognition proteins, such as XPA, detect cisplatin-DNA adducts. Then, XPG and ERCC1/XPF complexes make 3′ and 5′ incisions, respectively. Cisplatin-induced DNA damage regions are excised and these gaps are repaired in a proliferating cell nuclear antigen (PCNA)-dependent manner [2]. Cellular defects in the NER system have resulted in hypersensitivity to cisplatin [1, 2, 5] and NER related proteins, such as ERCC1 and XPA, have been overexpressed in cisplatin-resistant ovarian cancers [58, 59]. Recently, it has been shown that transcription-coupled NER is more closely related to cisplatin resistance than global genomic NER [60]. ERCC1 was shown to physically interact with a MMR protein, MSH2; these proteins might act cooperatively in cisplatin resistance [61]. The MMR system consists of various proteins, including MSH2, MSH3, MSH6 and MLH1. A defective MMR pathway in cisplatin resistance is associated with microsatellite instability [62] and these repair proteins were also demonstrated to contribute to drug resistance [63]. These data represent NER and MMR pathways as critical mechanisms in cisplatin resistance. The Fanconi anemia-BRCA1 pathway also regulates cisplatin sensitivity [64, 65]. BRCA1 colocalizes at DNA damage lesions and interacts with various DNA repair proteins residing within a large DNA repair protein complex known as the BRCA1-associated genome-surveillance complex; this indicates that BRCA1 is a critical component of multiple repair pathways [66]. However, there is no evidence that BRCA1 directly binds to cisplatin-modified DNA. A recent report from Wang and Lippard has demonstrated that cisplatin treatment could induce the phosphorylation of histone H3 at serine 10, mediated by the p38 signaling pathway and acetylation of histone H4 [67]. These chromatin modifications are thought to be involved in drug resistance, because they increase the accessibility of DNA for transcription factors and DNA repair proteins.

In general, tumor cells upregulate glycolysis and can grow in a severe microenvironment with hypoxia and/or acidosis; therefore, pH regulators are upregulated in highly proliferative cancer cells to avoid intracellular acidification [68, 69]. We have previously shown that subunit genes of one of the pH regulators, vacuolar $H^+$-ATPase (V-ATPase), are induced by cisplatin treatment and are overexpressed in cisplatin-resistant cell lines [70]. Intracellular pH was markedly higher in cisplatin-resistant cell lines than in sensitive parental cell lines. Furthermore, DNA-binding activity of cisplatin was markedly increased in acidic conditions [13, 70]. The DNA topoisomerase II inhibitor TAS-103, which can induce intracellular acidosis [71], also enhanced expression of the V-ATPase subunit genes [72]. In addition, we found that combining the V-ATPase inhibitor bafilomycin A1 with cisplatin or TAS-103 could enhance drug-induced apoptosis in cancer cells [70, 72]. Thus, elevated expression of pH regulators, such as V-ATPase subunit genes, contributes to the avoidance of apoptosis induced by intracellular acidosis and/or the drug cytotoxicity of cisplatin and TAS-103.

## TRANSCRIPTION FACTORS INVOLVED IN CISPLATIN RESISTANCE

Resistance to cisplatin is orchestrated via several mechanisms (as described above). These might be regulated

**Table 1. Cisplatin-Induced Damage-Recognition Proteins**

| | Protein | References |
|---|---|---|
| Transcription factors possessing an HMG domain | **mtTFA***, UBF | 49, 50 |
| Transcription factors lacking an HMG domain | TBP, **YB-1***, **ZNF143*** | 51-53 |
| HMG domain proteins | HMG1/2, SRY, SSRP1 | 48, 142, 143 |
| Repair proteins | XPE, XPA, MutSα, MSH2 | 54-57 |
| Chromatin protein | Histone H1 | 144 |

* indicates transcription factors focused on in this article.

by various transcription factors, which are often activated in response to cisplatin treatment. We now realize that molecular mechanisms of DNA damage signaling and cisplatin resistance are much more complex than was previously predicted. Transcription factors participate not only in gene expression, but also in DNA repair and apoptosis at the end of all signal transduction and stress-induced pathways. Various classified molecules mutually interact and function in nuclei; these interaction profiles might be altered by DNA damage, indicating that analysis of protein-interaction profiles is critical for future post-genomic research in cancer treatment. Fig. (4) illustrates DNA damage signaling and the ways in which this pathway might be associated with drug resistance, DNA repair and apoptosis. Cisplatin-induced transcription factors are closely involved in cisplatin resistance, and investigation of their mechanisms of action might allow us to overcome drug resistance. Furthermore, these transcription factors might be promising potential targets for clinical cancer treatment. Table (2) shows a summary of transcription factors and interacting molecules involved in cisplatin resistance.

### p53/p73

The p53 tumor-suppressor gene family proteins p53 and p73 are central to the cellular response to DNA damage. These proteins accumulate in nuclei after DNA damage and control cell proliferation [73, 74]. Cisplatin treatment can stabilize p53 through ATR- and MAPK-induced phosphorylation of p53 at serine 15; this treatment also induces p53 downstream genes [21, 75]. Several genes transcriptionally controlled by p53 have been identified, including the CDK inhibitor *p21/Waf1/Cip1* gene, the growth arrest and DNA damage-inducible *GADD45* gene and the pro-apoptotic *bax* gene [73]. Another significant role of p53 is its possible involvement in DNA repair. p53 preferentially associates

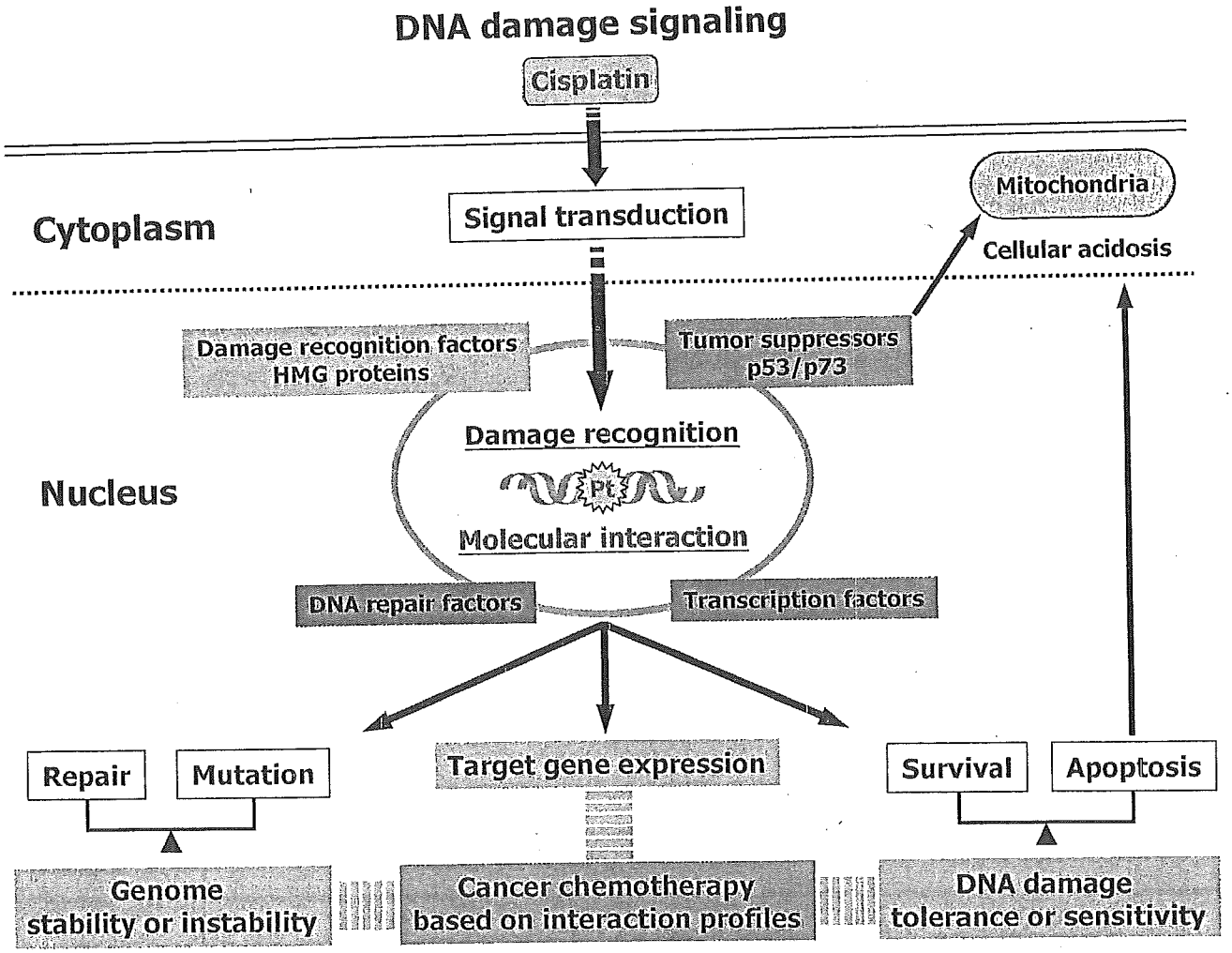


**Fig. (4). Flow diagram illustrating the cellular effects of cisplatin.**

Cisplatin treatment can activate various classified molecules, including DNA damage-recognition factors, DNA repair factors and transcription factors involved in cisplatin resistance via cisplatin-induced signal transductions. These factors mutually interact and function in nuclei, and interaction profiles might be altered by DNA damage. Thus, these molecular interactions are closely involved in genome stability, DNA damage tolerance and damage-induced apoptosis. The analysis of protein-interaction profiles is critical for future post-genomic research in cancer chemotherapy.

Table 2. Transcription Factors Involved in Cisplatin Sensitivity

| Transcription factor | Target gene | Interacting protein involved in drug resistance |
|---|---|---|
| p53/p73 | *p21, GADD45, Bax* | c-Myc [83], YB-1 [85], CTF2 [104], TBP [73], mtTFA [127], HMG1 [105], XPB [76], XPD [76] |
| Myc/Max | *Myc/Max, YB-1* | p53/p73 [83] |
| **YB-1*** | *MDR1, cyclin A/B1, TopoIIα, Fas, PTP1B, GPX1* | p53 [85], PCNA [52], MSH2 [92], Ku80 [92], DNA polymerase δ [92], WRN protein [92] |
| CTF2 | *HMG1* | p53/p73 [104] |
| ATF4 | *ZNF143, CHOP* | Nrf1/2 [110, 111] |
| **ZNF143*** | *mtTFA, MRP S11* | unknown |
| **mtTFA*** | | p53 [127] |
| Oct1 | *ATP6L (V-ATPase c subunit), TrxR* | Sp1 [145], HMG2 [136] |
| Sp1 | *ATP6L (V-ATPase c subunit), DNA-PKcs, VEGF, TrxR* | p53 [137], Oct1 [145], c-Jun [137], NF-κB [146], TBP [146], p38 [147], BRCA1 [137] |

* indicates specific recognition of cisplatin-modified DNA
Bracketed numbers indicate references.

with damaged DNA and interacts with DNA repair proteins, such as XPB and XPD [76]. Additionally, wild-type p53, but not mutant p53, has been demonstrated to exert intrinsic 3'→5' exonuclease activity [77]. Cancer cells carrying loss-of-function mutants of p53 are also less sensitive to anti-cancer agents [12].

p73 possesses structural and functional similarities to p53, and is able to activate p53-responsive genes and induce apoptosis. p73 was initially cloned from neuroblastoma cell lines and is involved in the development of the central nervous system [74]. The c-Abl tyrosine kinase can activate p73 by phosphorylating the p73 protein and induce apoptosis in response to DNA damage [23]. However, it has recently been reported that p73α overexpression is associated with resistance to DNA damaging agents [10]. These findings indicate that the major roles of p73 remain unclear and might be different from those of p53 in tumor cells. Further investigation is necessary to understand the functional differences between p53 and p73 in drug resistance.

### c-Myc

The oncoprotein c-Myc is a transcription factor that binds to E-box and transactivates various genes. c-Myc has been shown to function in many cellular processes, including cell proliferation, differentiation and transformation [78]. However, c-Myc is also able to induce apoptosis under certain conditions, such as deprivation of survival factors or treatment with anti-cancer agents [79, 80]. The mechanisms of c-Myc-induced cell growth and apoptosis remain unclear.

A recent report has shown that c-Myc downregulation is involved in cisplatin sensitivity in human melanoma cells [81]. Low expression of c-Myc or c-Myc downregulation by antisense oligonucleotides resulted in increased susceptibility to cisplatin, via glutathione depletion [82]. We have previously demonstrated that p73 interacts with c-Myc to regulate gene expression *via* E-box binding [83]. Furthermore, p73 stimulated the interaction of Max; the dimerization partner of the Myc oncoprotein with c-Myc and promoted binding of the c-Myc/Max complex to its target DNA. Our findings might help explain the complicated functions of c-Myc in drug resistance of cancer cells.

### Y-Box Binding Protein-1 (YB-1)

YB-1 is the most highly evolutionarily conserved nucleic-acid-binding protein and is a member of the cold-shock domain (CSD) protein superfamily. It functions in various biological processes, including transcriptional regulation, translational regulation, DNA repair, drug resistance and cell proliferation [84, 85]. YB-1 is a transcription factor, which was first identified by its ability to bind to the inverted CCAAT box (Y-box) of the MHC class II promoter. YB-1 has also been shown to regulate the expression of various genes through a Y-box in promoter regions, including *MDR1*, *cyclin A/B1*, *DNA topoisomerase IIα*, *Fas* and Protein tyrosine phosphatases 1B (*PTP1B*) [85-87].

YB-1 comprises three domains: a variable N-terminal domain; a highly conserved nucleic-acid-binding domain (the CSD); and a C-terminal basic and acidic amino-acid cluster domain (called a B/A repeat) [84, 85]. The N-terminal domain is thought to be a *trans*-activation domain and the CSD has an affinity for double-stranded DNA *in vitro*. The C-terminal region functions as either a nucleic acid-binding domain or a protein-protein interaction domain; the C-terminal domain also has a strong affinity for single-stranded DNA/RNA *in vitro* and is involved in dimerization [88]. YB-1 has pleiotropic functions, which are conferred through molecular interactions with a diverse range of proteins. It regulates human gene expression via interactions with transcription factors, including p53, p65, AP2, CTCF and Smad3 [85, 89]. YB-1 also interacts with the RNA-binding proteins IRP2 and hnRNPK to regulate mRNA translation and splicing, respectively [90, 91]. An examination of its physical partners might help to elucidate the integrated functions of YB-1.

YB-1 might be one of the components necessary for DNA repair. We previously reported that YB-1 preferentially binds to cisplatin-modified DNA, similarly to HMG domain

proteins [52]. This is the first evidence that a sequence-specific transcription factor can recognize cisplatin-modified DNA. YB-1 interacts with PCNA, which is necessary for nucleotide-excision repairs [52], in addition to DNA repair proteins such as MSH2, DNA polymerase δ, Ku80 and WRN protein [92]. YB-1 also possesses 3′→5′ exonuclease and endonucleolytic activities [88, 92], and is thus thought to be involved in base-excision repair. Furthermore, YB-1 preferentially binds to RNA containing 8-oxoguanine, which suggests that it might be able to detect damaged RNA molecules [93].

YB-1 is mainly localized in the cytoplasm. When cells are challenged with anti-cancer agents, hyperthermia or UV irradiation, YB-1 is immediately translocated from the cytoplasm to the nucleus [85, 94]. We have demonstrated that promoter activity of the *MDR1* gene increases in response to various environmental stresses in a Y-box-dependent manner [95]. We have also shown that YB-1 is overexpressed in human cancer cell lines, which are resistant to cisplatin, and that cisplatin sensitivity is increased by antisense YB-1 constructs [96]. Moreover, the disruption of one allele of the *YB-1* gene increased sensitivity to cisplatin and mitomycin C in mouse embryonic stem cells [97]. Additionally, increased YB-1 expression in clinical specimens has been reported to be significantly correlated with tumor progression and poor prognosis in lung cancer, ovarian cancer, prostate cancer and synovial sarcoma [85, 98, 99]. Interestingly, it has been shown that YB-1 can recognize the selenocysteine insertion-sequence element in glutathione peroxidase (*GPX1*) gene transcripts, suggesting that increased YB-1 might enable the effective translation of selenoproteins under cisplatin-induced oxidative stress [100]. These data indicate that YB-1 might have the capacity to protect the genome from DNA damaging agents in cancer cells, might play an important role in drug resistance and, thus, might be a new molecular target in cancer treatment.

### CCAAT-Binding Transcription Factor 2 (CTF2)

The CCAAT-binding transcription factor/nuclear factor I (CTF/NF-I) family of ubiquitous transcription factors was initially discovered as part of an adenovirus-DNA replication complex. CTF/NF-I group proteins recognize the sequence TTGGC($N_5$)GCCAA and are involved in the transcriptional regulation of various genes [101, 102]. CTF2 is one of four different splice variants of the CTF/NF-I protein. We have previously determined that CTF2 is overexpressed in cisplatin-resistant cells, and that overexpression of this transcription factor might be responsible for the trans-activation of the *HMG1* gene [103]. p53 and p73 physically interact with CTF2 and reciprocally regulate *HMG1* gene expression; p73α upregulates the activity of the *HMG1* gene promoter and enhances the DNA binding activity of CTF2, although p53 does not [104].

HMG1 (also designated HMGB1) and HMG2—the nonhistone chromosomal proteins—are ubiquitously expressed in higher eukaryotes, function as class II transcription factors and preferentially bind to cisplatin-modified DNA [48]. Furthermore, physical interaction of HMG1 with p53 enhances binding of cisplatin-modified DNA [105]. HMG1 and HMG2 have been implicated in cisplatin resistance [106, 107]. CTF2 might thus be a potential target in overcoming cisplatin resistance, because it could regulate *HMG1* gene expression in cancer cells. However, Wei *et al.* demonstrated recently that *HMG1* knockout of mouse embryonic fibroblasts has no effect on cisplatin sensitivity [108]. Further studies are needed to establish the mechanisms involving CTF2 and HMG1/2 in cisplatin resistance.

### Activating Transcription Factor 4 (ATF4)

ATF4 is a member of the ATF/cyclic AMP-responsive element-binding (CREB) family of transcription factors, and is widely expressed in a variety of tissues and tumor cell lines. It has been reported previously that ATF4 forms a homodimer *in vitro* and binds to the consensus ATF/CRE site TGACGTCA [109]. Various stress-inducible genes, including DNA repair genes, contain an ATF/CRE site in their promoter regions. ATF4 interacts with nuclear-factor erythroid 1 (Nrf1)- and Nrf2-related factors, which are recruited to the antioxidant-response element and regulate the expression of genes encoding enzymes with antioxidant or detoxification functions [110, 111]. Moreover, ATF4-null cells show impaired expression of genes involved in glutathione biosynthesis and resistance to oxidative stress [112]. Additionally, ATF4 participates in ER stress-induced gene expression and transactivates ER stress response-related protein; CHOP (a CCAAT/enhancer-binding protein (C/EBP) family protein) in response to amino-acid starvation [113]. Thus, these data might provide insights into the relation between ATF4 expression and cisplatin resistance. Transcription profiling by cDNA arrays that are inducible by genetic-suppressor elements has demonstrated that ATF4 is upregulated in drug-resistant cells [114]. ATF2, which is another member of the ATF/cAMP-responsive element binding family, is phosphorylated via JNK activation following cisplatin treatment. Phosphorylated ATF2 plays a critical role in drug resistance by promoting p53-independent DNA repair [115].

We have previously shown that ATF4 is a cisplatin-induced gene and is overexpressed in cisplatin-resistant cell lines [116]. ATF4 expression in human lung cancer cell lines correlated significantly with cisplatin sensitivity, and two stable transfectant ATF4-overexpressing derivatives of human lung cancer A549 cells were less sensitive to cisplatin than the parental cells. This is the first demonstration that ATF4 closely correlates with resistance to cisplatin. Cellular levels of ATF4 expression might aid the prediction of cisplatin efficacy; however, further study of the expression of ATF4 target genes is necessary to clarify the relationship between ATF4 expression and cisplatin resistance.

### Zinc-Finger Factor 143 (ZNF143)

ZNF143 is a zinc-finger transcription factor and is the human homologue of selenocysteine tRNA gene-transcription activating factor (Staf), which was identified originally in the frog. Staf is involved in transcriptional regulation of snRNA type and mRNA promoters transcribed either by RNA polymerase II or III [117]. Two human Staf homologues, ZNF143 and ZNF76, were recently isolated; these are 84 and 64% identical to *Xenopus* Staf, respectively [118]. ZNF143 is required for transcriptional activation of