sensitivity to anticancer agents. Furthermore, ATF4-overexpressing cells showed multidrug resistance and marked elevation of intracellular glutathione. Knock-down of ATF4 expression lead to downregulation of glutathione metabolism. Our findings indicate an important contribution of both Clock and ATF4 to chemosensitivity.

Results

Overexpression of Clock in cisplatin-resistant cells

We have shown previously that the transcription factor ATF4 is overexpressed in cisplatin-resistant cell lines (Tanabe *et al.*, 2003). As an E-box is located in the core promoter region of *ATF4*, we examined the expression levels of the E-box-binding proteins c-Myc, upstream stimulatory factor 1 (USF1), and Clock. Western blotting analysis revealed that the Clock protein was overexpressed in cisplatin-resistant cell lines (Figure 1a). No significant alteration of c-Myc and USF1 expression was observed between parental and cisplatin-resistant cells. As the Clock/BMAL1 complex regulates the expression of circadian genes (Gekakis *et al.*, 1998), we analysed the BMAL1 expression. However, the BMAL1 expression was not upregulated in cisplatin-resistant cells (data not shown). Northern blotting analysis revealed that Clock messenger RNA (mRNA) was also overexpressed in cisplatin-resistant cells (Figure 1b), suggesting that Clock might be involved in the transcriptional regulation of *ATF4* by binding to its promoter E-box.

ATF4 is a direct target of Clock

To test whether the *ATF4* promoter is a direct target of Clock, we carried out chromatin immunoprecipitation (ChIP) assays using specific primer pairs for the *ATF4* promoter region and an anti-Clock antibody. As shown in Figure 1c, this analysis revealed that Clock bound specifically to the E-box region of the *ATF4* promoter. A luciferase reporter gene assay showed that both Clock and BMAL1 co-transfection transactivated the *ATF4* promoter, and that this transactivation was dependent on an intact E-box, as reporter gene expression was reduced following transfection of a mutated E-box (Figure 1d). We also verified the relationship between ATF4 expression and Clock using siRNAs. Inactivation of Clock by siRNA was shown to suppress the promoter activity of *ATF4* gene (Figure 1e) as well as cellular expression level of ATF4 in PC3 cells (Figure 1f).

Cellular expression of Clock correlates with cisplatin sensitivity

To explore whether Clock overexpression is involved in cisplatin resistance, we examined the correlation between Clock expression and cisplatin sensitivity in 11 lung cancer cell lines (Figure 2a). Clock expression significantly correlated with cisplatin sensitivity (Figure 2b) and with ATF4 expression (Figure 2c) in these cell lines, but c-myc expression did not (data not shown). To confirm these findings by an alternative approach, we used the siRNA strategy. Downregulation of the cellular expression of the Clock protein conferred cisplatin and etoposide, but not 5-fluorouracil (5-FU), sensitivity to A549 cells (Figure 2d). Similar results were also obtained when ATF4 expression was downregulated. Clock expression did not correlate with the cellular sensitivity of etoposide, doxorubicin and vincristine at all (data not shown). We next investigated whether downregulation of ATF4 expression overcomes cisplatin resistance in cisplatin-resistant cell line P/CDP6. As shown in Figure 2e, downregulation of ATF4 expression partially overcomes cisplatin resistance, because the IC_{50} value of cisplatin in PC3 cells is about 0.7 μM (data not shown).

Multidrug resistance in ATF4-overexpressing cell lines

In addition to our two previously established ATF4-overexpressing cell lines (Tanabe *et al.*, 2003), we derived two new cell lines that overexpressed ATF4 (A549/ATF4-5 and A549/ATF4-6) at levels 10–20-fold higher than vector-alone transfectants (A549/pcDNA-1 and A549/pcDNA-2) (Figure 4). The ATF4-overexpressing cell lines showed increased resistance to cisplatin, doxorubicin, etoposide, SN-38, and vincristine, but not to 5-FU (Table 1). To our knowledge, this is the first transcription factor that can induce multidrug-resistant phenotypes.

Intracellular glutathione level and drug resistance-related gene expression in ATF4-overexpressing cells

It has been reported that $ATF4^{-/-}$ cells demonstrate impaired glutathione biosynthesis (Harding *et al.*, 2003), whereas an increased level of glutathione has been shown to be involved in drug resistance (Lai *et al.*, 1989; Tew, 1994). Therefore, intracellular glutathione levels were evaluated in ATF4-overexpressing cell lines, and were found to be approximately 12.7-fold higher than in control cells (Figure 3a). This increase was abolished when cells were treated with the γ-glutamylcysteine synthetase inhibitor: buthionine-sulfoximine (BSO) (10 μM). To examine whether Clock and ATF4 are involved in glutathione biosynthesis, A549 cells were transfected with Clock-directed, ATF4-directed, or control siRNA oligomers. Downregulation of both Clock and ATF4 was found to reproducibly suppress intracellular glutathione levels to 75–80% of the control levels (Figure 3b).

It has been shown that resistant cells against cisplatin often upregulate both glutamate-cysteine ligase catalytic subunit (GCLC) and glutathione S-transferase π (GSTπ) (Saburi *et al.*, 1989; Yao *et al.*, 1995). On the other hand, the resistant cells against topoisomerase-targeted drugs often downregulate DNA topoisomerase (Takano *et al.*, 1992). We, therefore, examined drug resistance-related gene expressions in ATF4-overexpressing cells (Figure 4). Although ATF4-overexpressing cells were resistant to etoposide and SN-38, the expressions of DNA topoisomerase I and IIα were not downregulated. As we expected, the expressions of

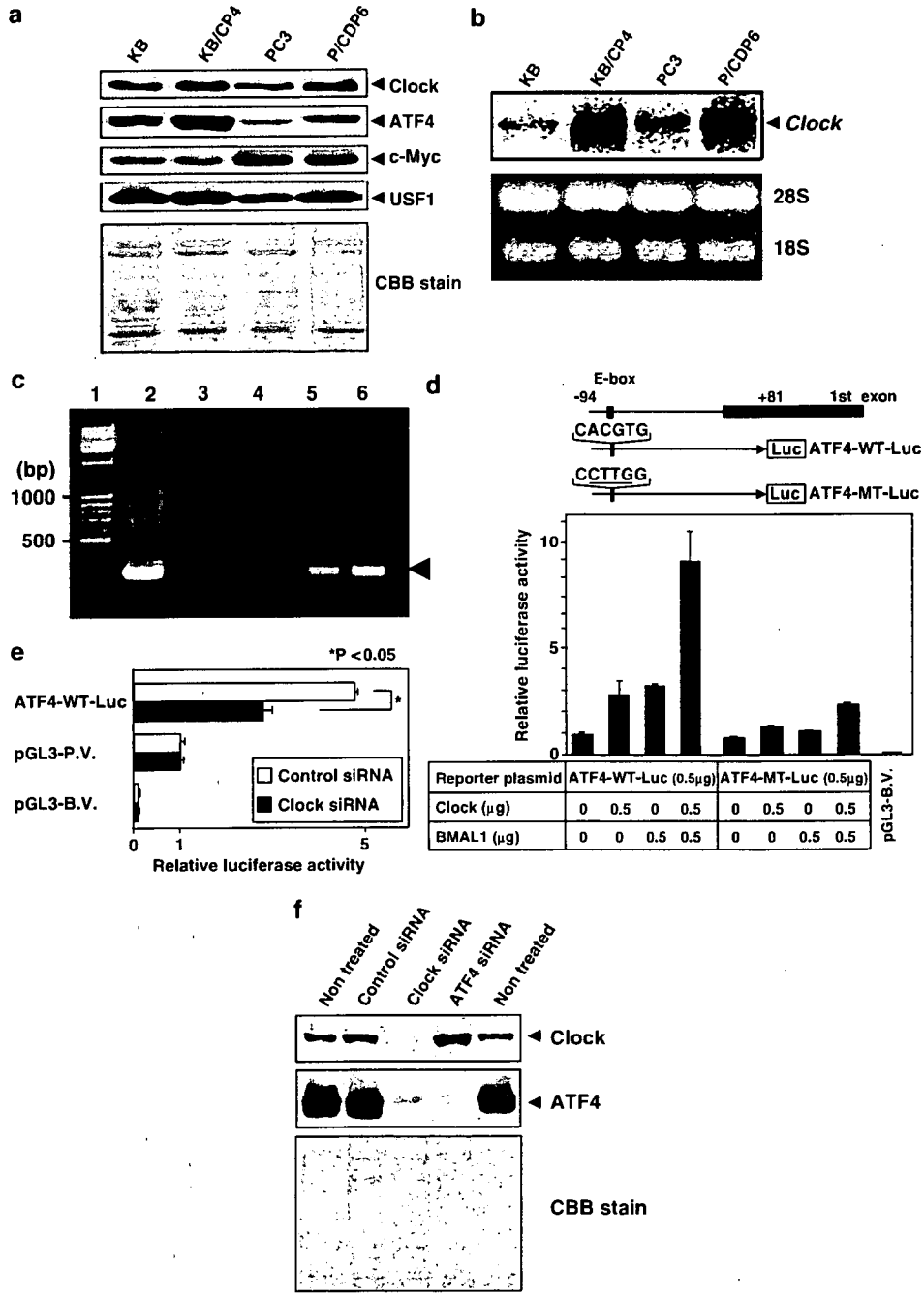

Figure 1 Clock regulates *ATF4* gene expression through binding to the E-box located in the promoter region. (**a**) Whole-cell extracts (75 μg for Clock, and 50 μg for c-Myc and USF1) and nuclear extracts (100 μg for ATF4) were subjected to SDS–PAGE, and Western blotting analysis was performed with the indicated antibodies. Gel staining with Coomassie Brilliant Blue (CBB) is also shown. (**b**) Total RNA (20 μg) prepared from the indicated cells was separated on a 1% formaldehyde-agarose gel and transferred to a Hybond N^+ membrane. Northern blotting analysis was performed with a Clock cDNA probe. Gel staining with ethidium bromide is also shown. (**c**) A ChIP assay of the PC3 cells was performed with antibodies against Clock or goat IgG. Immunoprecipitated DNAs (anti-goat IgG in lanes 3 and 4, and anti-Clock IgG in lanes 5 and 6) were amplified by PCR using specific primer pairs for the *ATF4* promoter region. The templates used for PCR were as follows: 1 μl (lane 2) of genomic DNA from cell lysate, and 1 μl (lanes 3 and 5) and 3 μl (lanes 4 and 6) of immunoprecipitated DNAs. Lane 1 contains a DNA size marker. The arrowhead indicates amplified partial *ATF4* promoters (278 bp). (**d**) Indicated amounts of Clock and/or BMAL1 expression plasmids were transiently co-transfected with ATF4-WT-Luc or ATF4-MT-Luc into MCF7 cells. The schematic representation of ATF4-WT-Luc and ATF4-MT-Luc is shown above. pGL3-B.V. indicates pGL3 basic vector. The results are normalized to β-galactosidase activity and are representative of at least three independent experiments. Bars = $\pm$s.d. (**e**) MCF7 cells were transfected with 50 nM control or Clock siRNAs. The following day, they were transfected with the indicated reporter plasmids. The results were normalized to β-galactosidase activity and pGL3 promoter vector (Promega). All values are the mean of at least three independent experiments. pGL3-PV and pGL3-BV indicate pGL3 promoter vector and pGL3 basic vector, respectively. Bars = $\pm$s.d. (**f**) Indicated siRNAs were transfected into PC3 cells. Whole-cell extracts (75 μg) for Clock and nuclear extracts (100 μg) for ATF4 were subjected to SDS–PAGE, and Western blotting analysis was performed.

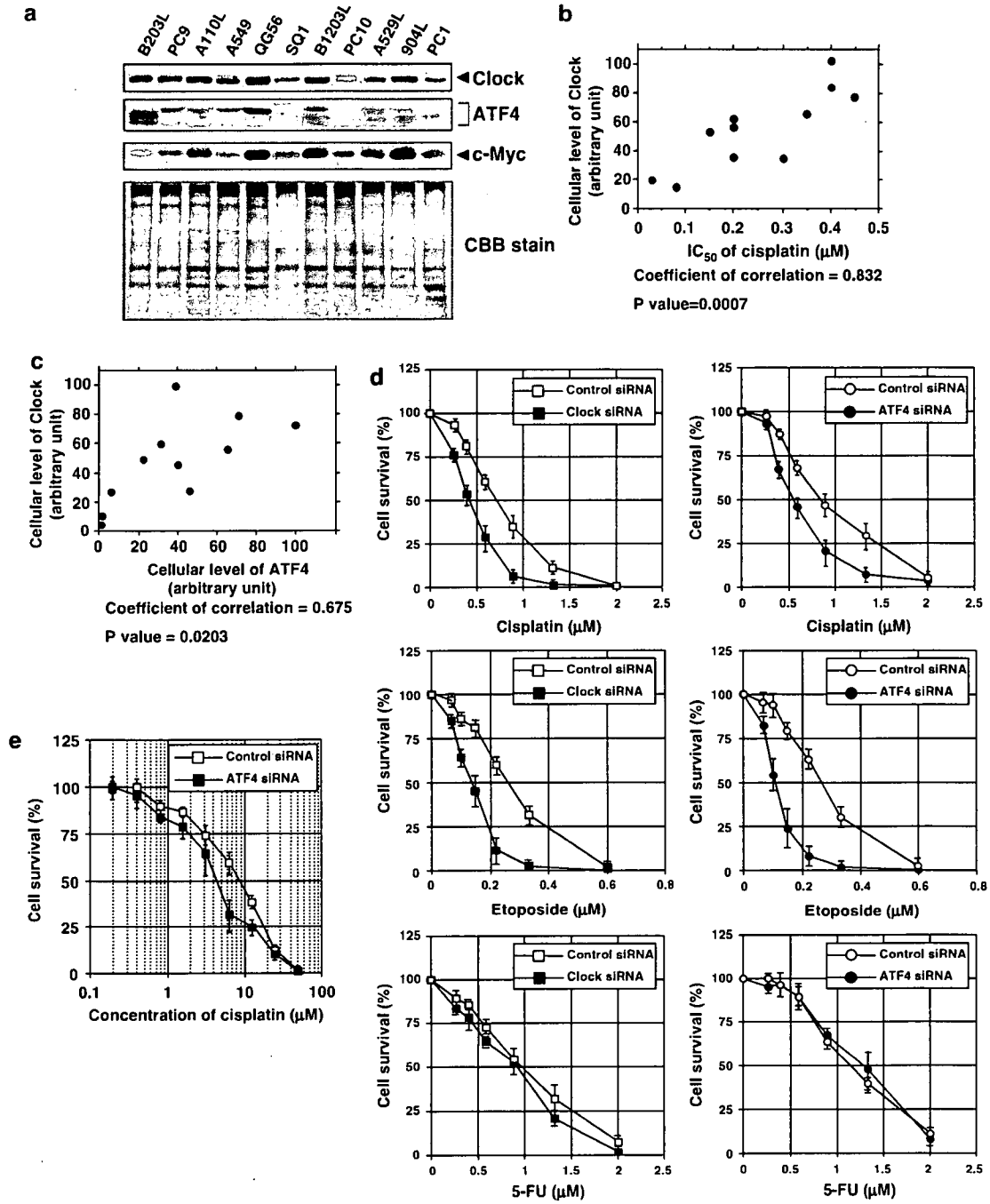

Figure 2 Clock expression correlates with cisplatin resistance and ATF4 expression. (**a**) Western blotting analysis was performed with 100 μg nuclear extracts for ATF4 expression and 75 μg whole-cell extracts for Clock and c-Myc expressions in 11 human lung cancer cell lines. Gel staining with CBB is also shown. (**b**) and (**c**) Expression levels of Clock and ATF4 (whole band) were determined by NIH imaging using Figure 2a, and were normalized by each CBB stain. The maximum expression levels of Clock or ATF4 were set to 100, and the IC_{50} of each cell line was calculated from the concentration–response curves for cisplatin. (**d**) Downregulation of either Clock or ATF4 confers sensitivity of A549 cells to cisplatin and etoposide. Cells were transfected with the indicated siRNAs, and exposed to various concentrations of cisplatin, etoposide, and 5-FU for 7 days. The colony number in the absence of drugs corresponded to 100%. All values are the mean of at least three independent experiments. Bars = $\pm$s.d. (**e**) P/CDP6 cells were transfected with 50 nM control or Clock siRNAs. The following day, various concentrations of cisplatin were treated. After 72 h, cell survival was analysed with a WST-8 assay. Cell survival in the absence of cisplatin corresponded to 100%. All values are the mean of at least three independent experiments. Bars = $\pm$s.d.

GCLC and GSTπ were upregulated in ATF4-overexpressing cells. Drug resistance is also modulated by the expression of both anti-apoptotic and apoptotic molecules. We then examined the expression of several molecules involved in apoptosis such as Bcl-2, Bcl-X_L, Bax and BAK. However, we could not detect the significant alteration between drug-resistant cells and ATF4-overexpressing cells (data not shown). To explore

Table 1 Drug sensitivity (half-maximal inhibitory concentration [IC_{50}]) and relative resistance of ATF4-overexpressing cell lines

	Cell line		
Drug	*A549/pcDNA3*[a]	*A549/ATF4*[b]	*Relative resistance*[c]
5-FU (μM)	1.38 (±0.04)	1.35 (±0.21)	1.0
Cisplatin (μM)	0.67 (±0.04)	2.11 (±0.02)	3.1
Doxorubicin (μM)	0.03 (±0.01)	0.14 (±0.04)	3.6
Etoposide (μM)	0.39 (±0.01)	2.02 (±0.64)	5.2
SN-38 (nM)	5.25 (±1.06)	19.0 (±1.41)	3.6
Vincristine (nM)	2.75 (±0.78)	6.10 (±1.56)	2.2

[a]Control cell lines A549/pcDNA3-1 and A549/pcDNA3-2. [b]ATF4-overexpressing cell lines A549/ATF4-5 and A549/ATF4-6. [c]IC50 ratio of ATF4-overexpressing cell lines to control cell lines. The cell viability after drug exposure was analysed with a WST assay. In the absence of drugs, the viability was 100%. The IC_{50} of each cell line was calculated from the concentration–response curves. All values indicate the mean±standard deviation (s.d.). 5-FU, 5-fluorouracil; SN-38, 7-ethyl-10-hydroxycamptothecin.

a potential role for glutathione, we tested BSO for its ability to reverse drug resistance in ATF4-overexpressing cells, and found that cellular sensitivity of cisplatin and etoposide was almost completely reversed by addition of BSO (Figure 5a and b). We also examined the expression of drug resistance-related genes after BSO treatment. However, no significant alteration of gene expression was observed (data not shown).

Microarray analysis of ATF4-regulated genes

Because the available information regarding the transcriptional regulation by ATF4 was limited, we used microarray technology to enable the simultaneous analysis of large numbers of genes. To confirm further transcriptional changes by the ATF4 siRNA, oligonucleotide microarray study was carried out in A549 cells treated with or without ATF4 siRNA (0.3 nM of ATF4 siRNA downregulated the cellular expression of ATF4 to 50%). Data analysis identified 121 genes, which were downregulated more than 2.5-fold and only eight genes which were upregulated (Supplementary Information). Among downregulated genes, only glutathione peroxidase 2 (GPX2) gene was identified in relation to glutathione metabolism. Then, we analysed the subset of genes for glutathione metabolism. As shown in Figure 6a, the genes for glutathione metabolism were generally downregulated by ATF4 knockdown including GCLC, glutamate-cysteine ligase modifier subunit (GCLM), γ-glutamyltransferase 1 (GGT1), γ-glutamyltransferase 2 (GGT2), glutamic pyruvate transaminase 2 (GPT2), GPX2, glutathione S-transferase M4 (GSTM4) and microsomal glutathione S-transferase 2 (MGST2). The ATF4-binding site was found in the proximal promoter region of these eight genes (data not shown). It was reported that GCLC was a key enzyme to determine the cellular glutathione levels and often involved in drug resistance (Tipnis *et al*., 1999). To evaluate the microarray study, we carried out Western blotting analysis and revealed that the GCLC expression was downregulated by the ATF4 siRNA (Figure 6b). As

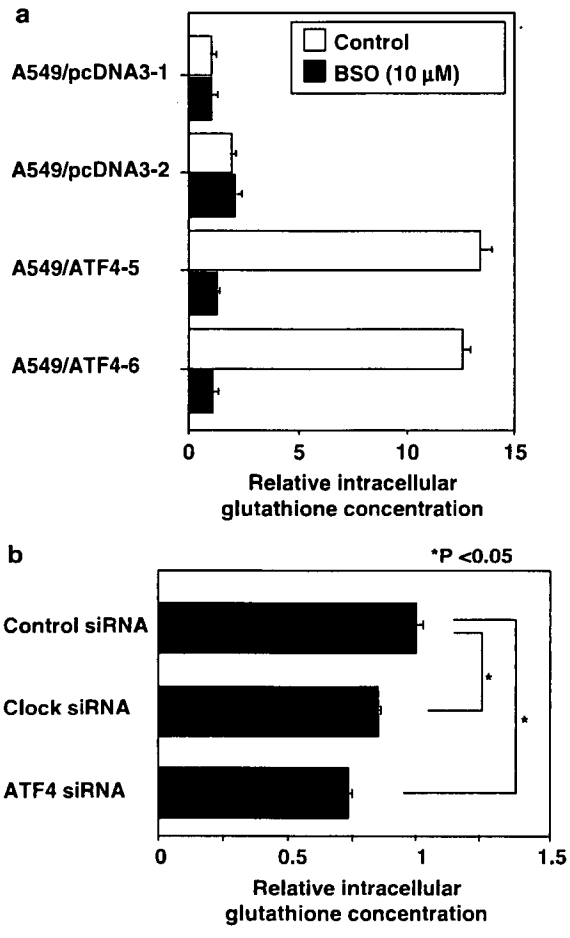

Figure 3 Intracellular glutathione levels are evaluated in ATF4-overexpressing cells and downregulated by Clock or ATF4 siRNAs. (**a**) Intracellular glutathione levels of ATF4-overexpressing cell lines (A549/ATF4-5, 6) and control cell lines (A549/pcDNA3-1, 2) with or without 10 μM BSO treatment for 72 h were measured. Each glutathione concentration indicates a relative level to A549/pcDNA3-1. (**b**) A549 cells were transfected with Clock or ATF4 siRNAs, and intracellular glutathione levels were measured. Each glutathione concentration indicates a relative level to control siRNA. All values are the mean of at least three independent experiments. Bars = ±s.d.

shown in Figure 6c, the expressions of GCLC and GSTπ were significantly upregulated in cisplatin-resistant cells. These data were comparable with our microarray analysis. However, the GCLC expression was not downregulated by the Clock siRNA (data not shown).

The expression of the ATP-binding cassette transporter family

As shown in Table 1, the ATF4-overexpressing cell lines showed multidrug-resistant phenotypes. It has been reported that intracellular glutathione could support the drug efflux by ATP-binding cassette (ABC) transporters (Renes *et al*., 2000). Thus, we next examined the expression of major ABC transporters such as multidrug resistance protein 1 (MRP1/*ABCC1*), multidrug resistance protein 2 (MRP2/*ABCC2*), breast cancer-resistance protein (BCRP/*ABCG2*) and

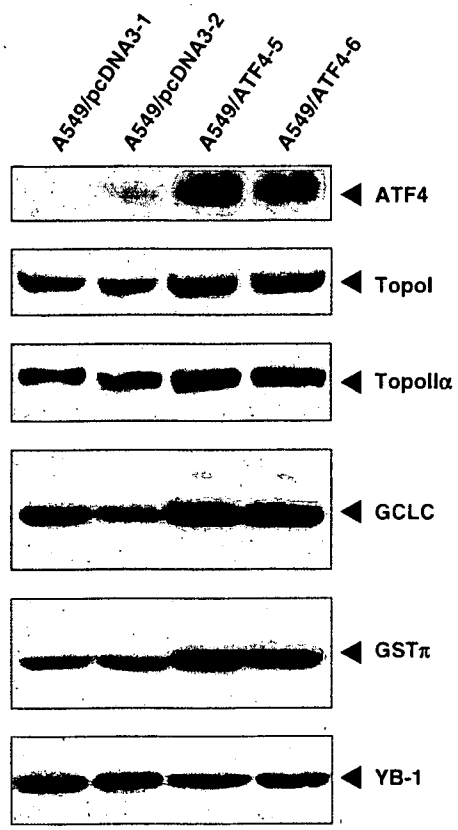

Figure 4 Cellular expressions of drug resistance-related genes in ATF4-overexpressing cells (A549/ATF4-5 and A549/ATF4-6) and control cells (A549/pcDNA3-1 and A549/pcDNA3-2). Whole-cell extracts (75 μg) for GCLC, GSTπ, YB-1 and nuclear extracts (100 μg) for ATF4, TopoI, TopoIIα from ATF4-overexpressing cells were subjected to SDS–PAGE, and Western blotting analysis was performed with the indicated antibodies.

P-glycoprotein. Interestingly, both expressions of MRP2 and BCRP were significantly upregulated in the ATF4-overexpressing cells, but MRP1 was not (Figure 7a). We could not detect the P-glycoprotein in these cells at all. However, the ATF4-binding site was not in the promoter region of both *MRP2* and *BCRP* genes, suggesting that both genes were not direct targets of ATF4. We confirmed the expression levels of ABC transporters were not reduced when cells were treated with BSO (Figure 7b). We next investigated whether downregulation of BCRP or MRP2 expressions overcome etoposide or cisplatin resistance in ATF4-overexpressing cells. We prepared the specific siRNAs for both BCRP and MRP2 (Figure 7c and d). As shown in Figure 7e, we found that downregulation of BCRP significantly decreased the IC_{50} value of etoposide. On the other hand, downregulation of MRP2 significantly decreased the IC_{50} value of cisplatin (Figure 7f).

Discussion

We have previously shown that the transcription factor ATF4 can be induced by cisplatin, and that overexpression of ATF4 confers cisplatin resistance to cells (Tanabe *et al.*, 2003). We have also shown that ATF4 expression is a possible predictor for sensitivity to cisplatin (Tanabe *et al.*, 2003; Kohno *et al.*, 2005). The current analysis explored the molecular mechanism of ATF4 expression and drug resistance.

Both expression and function of ATF4 have been reported to be regulated by the post-transcriptional pathways (Blais *et al.*, 2004). Phosphorylation of the α subunit of translation initiation factor (eIF2α) promotes translation of ATF4 and ATF4 phosphorylated by RSK2 increases transactivation ability (Yang *et al.*, 2004). We initially examined the cellular expression levels of eIF2α kinase PERK, which was activated by endoplasmic reticulum stress, in cisplatin-resistant cells. However, we could not find the significant difference of PERK expression in cisplatin-resistant cells (data not shown). Moreover, mRNA level of ATF4 was increased in cisplatin-resistant cells (Tanabe *et al.*, 2003) then, we investigated the transcriptional regulation of *ATF4*. The core promoter region of *ATF4* contains an E-box, so we analysed the cellular expression levels of E-box-binding proteins. Among these proteins, only Clock was overexpressed in cisplatin-resistant cell lines that were independently established (Figure 1a and b). It has been shown that the extent of phosphorylation can determine the cellular localization and stability of Clock proteins (Lee *et al.*, 2001). However, Clock mRNA is also overexpressed in cisplatin-resistant cells. This indicates that cellular Clock might be involved in the transcriptional regulation in these cell lines. We confirmed the role of Clock in the regulation of *ATF4* gene expression by three independent approaches: ChIP with an anti-Clock antibody, E-box-dependent promoter activity in reporter gene assays, and the downregulation of Clock using a siRNA strategy (Figure 1c–f). We also demonstrated the positive correlation of Clock expression with sensitivity to cisplatin and ATF4 expression (Figure 2b and c). To investigate more clearly whether cellular expression of Clock and ATF4 contribute to chemosensitivity, siRNA oligomers were used to knockdown the expression of these transcription factors. Transfection of both Clock and ATF4 siRNAs in A549 cell led to sensitization to cisplatin and etoposide, but not to 5-FU (Figure 2d).

It has been shown that the *Per2*-mutant mouse demonstrates increased sensitivity to γ-radiation (Fu *et al.*, 2002). As the *Per2* gene is regulated by Clock, it is possible that the expression of this protein might be involved in DNA damage-induced apoptosis. The analysis of the expression profile showed that several DNA damage-inducible genes such as members of the growth-arrest and DNA damage (*GADD*) family that block cell-cycle progression (Liebermann and Hoffman, 2002) and cyclin genes were controlled by circadian regulators (Fu *et al.*, 2002). Taken together, these results indicate that the cell cycle-regulating mechanism in cisplatin-resistant cells is regulated by Clock.

The second aim of our current work was to investigate the molecular mechanisms of drug resistance regulated by the Clock and ATF4 transcription system. Although

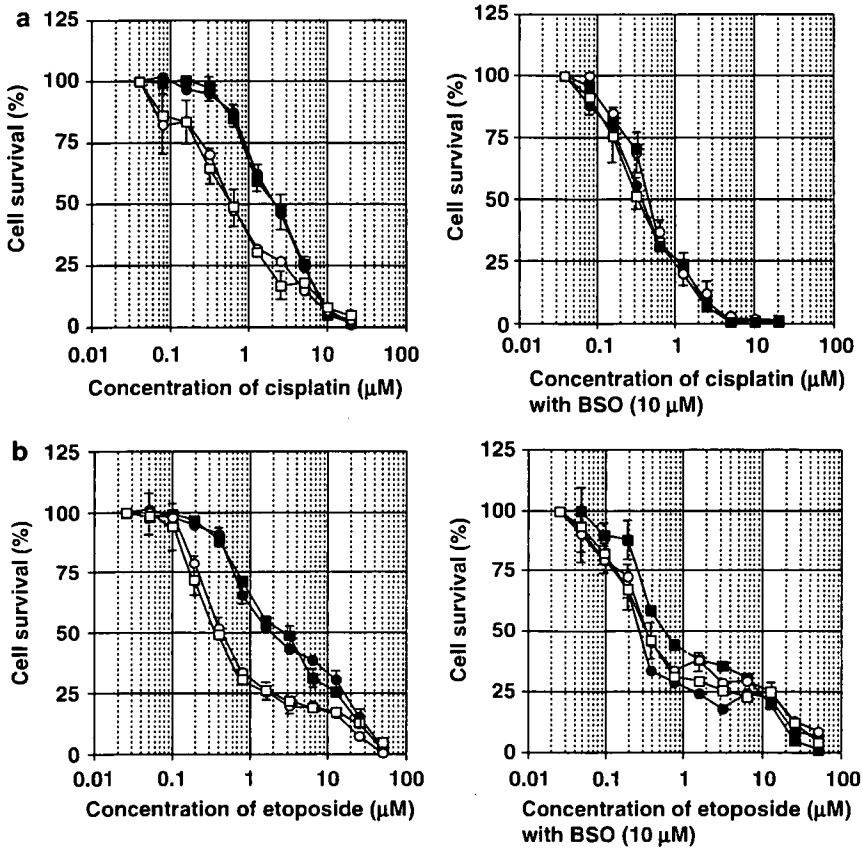

Figure 5 BSO overcomes the drug resistance of cisplatin and etoposide in ATF4-overexpressing cells. ATF4-overexpressing cells (A549/ATF4-5, closed square; A549/ATF4-6, closed circle) and control cells (A549/pcDNA3-1, open square; A549/pcDNA3-2, open circle) were pretreated with or without 10 μM BSO for 24 h, and exposed to various concentrations of cisplatin (**a**) and etoposide (**b**), with or without 10 μM BSO. After 72 h, cell survival was analysed with a WST-8 assay. Cell survival in the absence of drugs corresponded to 100%. All values are the mean of at least three independent experiments. Bars = $\pm$s.d.

we were unable to establish Clock-overexpressing cells, we successfully derived two ATF4-overexpressing cell lines. These cells were resistant to various anticancer agents, such as cisplatin, etoposide, doxorubicin, SN-38, and vincristine, but not to 5-FU suggesting that ATF4 contributes to the multidrug resistance of human cancer cell lines.

Another important finding was the elevation of intracellular glutathione levels in these cell lines. This was consistent with the report that the ATF4$^{-/-}$ cells showed impaired glutathione biosynthesis (Harding *et al.*, 2003). Elevated glutathione clearly contributes to drug resistance, because the depletion of glutathione by BSO was able to reverse the resistance in ATF4-overexpressing cell lines. Western blotting analysis showed that GCLC and GSTπ expressions were upregulated in ATF4-overexpressing cells. Microarray data also revealed that genes for glutathione metabolism were generally downregulated in ATF4 siRNA-treated cells, suggesting that glutathione metabolism may be a key role involved in drug sensitivity. It has been reported that BSO overcomes Bcl-2-mediated drug resistance and hypothesized that BSO could possess an unique activity via mitochondria-independent pathway (Yoshida *et al.*, 2006). However, there are no significant changes in the expression of apoptosis-related genes (data not shown). DNA-binding activity of Clock and BMAL1 is regulated by the redox state of NAD cofactors (Rutter *et al.*, 2001). It would be interesting to examine the possible involvement of glutathione in regulating Clock/BMAL1 and ATF4 transcriptional activity. The oxidation–reduction status of the cell is an important regulator of various metabolic functions, and glutathione is one of the main compounds involved in reducing oxidative stresses (Dickinson and Forman, 2002). Furthermore, glutathione S-transferases (GSTs) are ubiquitous enzymes that play an important role in drug resistance by conjugating drugs to glutathione. The genes for biosynthesis of the antioxidant glutathione were regulated by Clock and ATF4 transcription system (Figures 3b and 6b). Thus, the cellular protection against oxidative stresses and hepatic function for drug metabolism could be regulated by the circadian rhythm, with the involvement of the transcription factor, Clock. Our findings will provide some clue that may be helpful to understand the oxidative stress-associated disorders including cancer and systemic chronotherapy.

Membrane transporters of the ABC superfamily function as a pump, and can lead to resistance against multiple anticancer agents (Gottesman *et al.*, 2002;

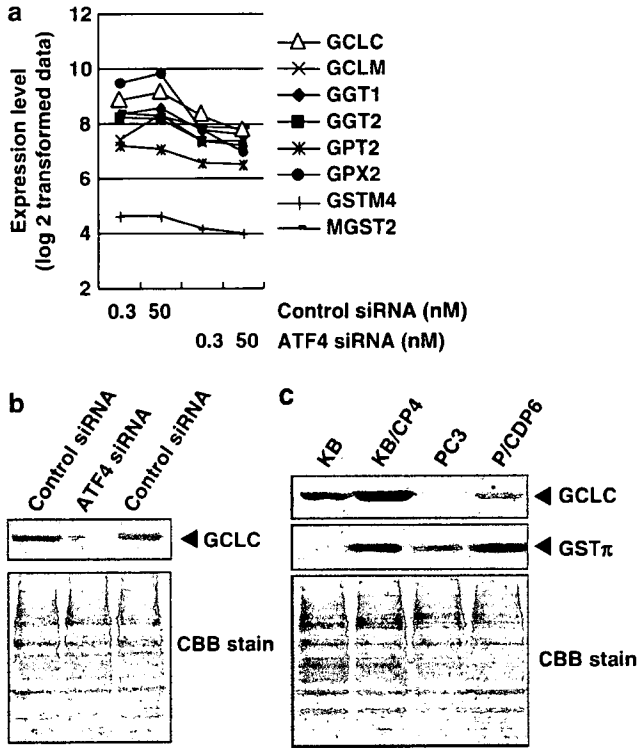

Figure 6 The genes for glutathione metabolism are regulated by ATF4. (a) The graph shows gene expression changes of glutathione metabolism subset mediated by ATF4 siRNA. The data were obtained from normalized and log 2-transformed microarray expression signal intensities. The samples were collected from A549 cells transfected with ATF4 siRNA (50 or 0.3 nM) and control siRNA (50 or 0.3 nM) in duplicate. Eight GeneChips were used for analysis and duplicated GeneChip data was averaged for each of the four conditions. The subset of genes was further selected if fold change marked > 1.5 between averaged ATF4 siRNA and control siRNA samples. (**b**) Whole-cell lysates (50 μg) from A549 cells transfected with indicated siRNAs were subjected to SDS–PAGE, and Western blotting analysis was performed with anti-GCLC antibody. Gel staining with CBB is also shown. (c) Western blotting analysis with whole-cell lysates (50 μg) from cisplatin sensitive/resistant cells was performed with anti-GCLC and anti-GSTπ antibodies.

Szakacs *et al*., 2006). Among these transporters, P-glycoprotein and the MRP families have been extensively studied (Annereau *et al*., 2004). MRP2-overexpressing cells show cross-resistance to anticancer agents such as cisplatin, doxorubicin and epirubicin (Cui *et al*., 1999). BCRP can transport diverse anticancer agents, including etoposide, doxorubicin and SN-38 (Deeley *et al*., 2006; Krishnamurthy and Schuetz, 2006). It has been reported that both MRP2 and GCLC are coordinately expressed in acquired drug-resistance cell lines (Ishikawa *et al*., 1996; Kuo *et al*., 1998). Although the molecular mechanism of this is unknown, a significant correlation was found between the glutathione content and drug resistance (Fojo and Bates, 2003). Interestingly, in our studies, both MRP2/*ABCC2* and BCRP/*ABCG2* were upregulated in ATF4-overexpressing cells. These two ABC transporters partially contribute to drug resistance in ATF4-overexpressing cell lines. Downregulation of BCRP partially reverse etoposide resistance but not cisplatin resistance. On the other hand, downregulation of MRP2 reverse cisplatin resistance but not etoposide resistance. These data are consistent with the previous reports that cisplatin is one of the substrates of MRP2 (Cui *et al*., 1999), and etoposide is one of the substrates of BCRP (Deeley *et al*., 2006; Krishnamurthy and Schuetz, 2006). Microarray analysis showed that BCRP was downregulated by the ATF4 knockdown (data not shown). High level of intracellular glutathione might be involved in the function of transcription factors, which regulate the expression of these genes. However, the regulatory mechanism of ABC transporter expression in ATF4-overexpressing cells remains unclear.

Microarray analysis is a powerful tool to identify the target genes for transcription factors. Classification according to the function suggests that ATF4 mainly mediate the cellular physiological process and metabolism (Supplementary Information). Identification of the ATF4-binding site in the 5′ upstream from these genes and functional analysis of the promoter activity are now in progress.

In conclusion, we describe here a novel mechanism of multidrug resistance. Two transcription factors, Clock and ATF4, were unequivocally demonstrated to cause multidrug resistance in human cancer cell lines. Clock has been identified as a protein with regulating function of circadian rhythmicity, which is primary through actions at suprachiasmatic nucleus and the supraoptic nucleus (Moore, 1997). The systemic circadian rhythm is known to be important for the clinical treatment of cancer patients (Canaple *et al*., 2003; Gorbacheva *et al*., 2005), and our results imply that cellular rhythm can modulate cellular sensitivity to anticancer agents. Further study is required to prove the fundamental issue how cellular rhythm at a single cell level contributes to systemic chemotherapy. Regulation of Clock gene expression and Clock-targeted genes in cancer cells will be an important question to address in future work. Further, elucidation of the molecular network regulating transcription factor genes in multidrug-resistant cells should improve the understanding of genomic responses against anticancer agents and drug resistance.

Materials and methods

Cell culture

Human epidermoid cancer KB cells and human prostate cancer PC3 cells were cultured in Eagle's minimal essential medium. Human breast cancer MCF7 cells were cultured in Dulbecco's modified Eagle medium. These mediums were purchased from Nissui Seiyaku (Tokyo, Japan) and contained 10% fetal bovine serum. The cisplatin-resistant KB/CP4 and P/CDP6 cells were derived from KB and PC3 cells as described previously (Murakami *et al*., 2001) and found to be 23–63-fold more resistant to cisplatin than their parental cells (Fujii *et al*., 1994). Vincristine-resistant KB/VJ300 cell derived from KB was generated as described previously (Kusaba *et al*., 1999). Eleven lung cancer cell lines and newly generated

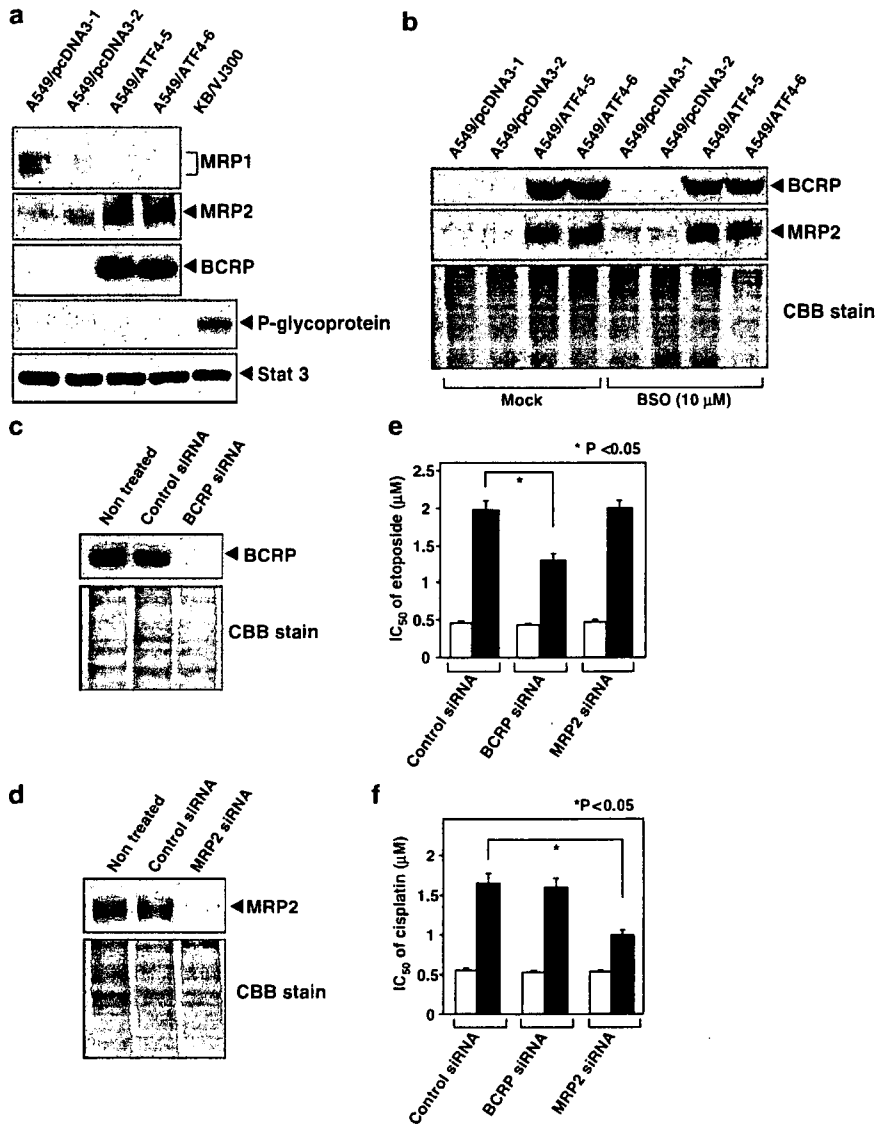

Figure 7 Cellular expressions of ABC transporter families in ATF4-overexpressing cells (A549/ATF4-5 and A549/ATF4-6) and control cells (A549/pcDNA3-1 and A549/pcDNA3-2) (**a**) Whole-cell extracts (100 μg) from the indicated cell lines were subjected to SDS–PAGE, and Western blotting analysis was performed with the indicated antibodies. (**b**) ATF4-overexpressing cells and control cells were treated with or without 10 μM BSO for 96 h. Whole-cell extracts (100 μg) were subjected to SDS–PAGE, and Western blotting analysis was performed with the indicated antibodies. Gel staining with CBB is also shown. (**c**) and (**d**) ATF4-overexpressing cells (A549/ATF4) were transfected with the indicated siRNAs (50 nM). Whole-cell extracts (100 μg) were subjected to SDS–PAGE, and Western blotting analysis was performed with the indicated antibodies. Gel staining with Coomassie Brilliant Blue (CBB) is also shown. (**e**) and (**f**) Both control cells (A549/pcDNA3) and ATF4-overexpressing cells (A549/ATF4) were transfected with the indicated siRNAs (50 nM) and exposed to various concentrations of cisplatin or etoposide for 72 h. The IC_{50} values were determined by WST-8 assay. Open column and closed column indicate control cells (A549/pcDNA3) and ATF4-overexpressing cells (A549/ATF4), respectively. All values are the mean of at least three independent experiments. Bars = $\pm$s.d.

ATF4-overexpressing cell lines were described previously (Tanabe *et al*., 2003). Cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

Antibodies and drugs

Antibodies against c-Myc (sc-764), Clock (sc-6927), USF1 (sc-8983), ATF4 (sc-200), TopoI (sc-5342), TopoIIα (sc-5346), GCLC (γGCSc, sc-22755), Stat3 (sc-482) and donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-BCRP (MAB4146) and MRP2 (MAB4150) antibodies were purchased from Chemicon (Temecula, CA, USA). Anti-MRP1 and anti-P-glycoprotein antibodies were purchased from MONOSAN (Netherlands) and Fujirebio Diagnostic (Malvern, PA, USA), respectively. Anti-YB-1 (Ohga *et al*., 1996) and anti-GSTπ (Saburi *et al*., 1989) antibodies were prepared as described previously. Cisplatin, vincristine, 5-FU), etoposide, BSO) were purchased from Sigma (St Louis, MO, USA). Doxorubicin was purchased from Kyowa Hakko Kogyo Co. Ltd, (Tokyo, Japan). 7-Ethyl-10-hydroxycamptothecin (SN-38) was kindly gifted by Yakult Co. Ltd. (Tokyo, Japan).

Plasmid construction
To obtain the full-length complementary DNA (cDNAs) of Clock and BMAL1, polymerase chain reaction (PCR) was carried out on a SuperScript cDNA library (Invitrogen Life Technologies, CA, USA) using the following primer pairs (single underlining indicates the start codons): ATGTTGTT TACCGTAAGCTGTAG and CTACTGTGGTTGAACCT TGGAAG for Clock; and ATGGCAGACCAGAGAATG GAC and TTACAGCGGCCATGGCAAGTC for BMAL1. These PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). To construct mammalian expression plasmids, the *Not*I Clock cDNA fragment and the *Eco*RI BMAL1 cDNA fragment were ligated into the pcDNA3 vector (Invitrogen). The core promoter and the partial first exon (−94 to +81) of *ATF4* were amplified by PCR using the placenta DNA and the following primer pairs: AGATCTGAGACGGTCACGTGGTCGCGGC and AAG CTTGGCCGTGGACCCTGAGGGC. PCR was also performed to obtain the E-box-mutant promoter of the *ATF4* using the following primer pairs: AGATCTGAGACGGTC CTTGGGTCGCGGC and AAGCTTGGCCGTGGACCCT GAGGGC. Single and double underlining indicate the wild-type and mutated E-box, respectively. These PCR products were cloned and ligated into the *Bgl*II-*Hind*III site of the pGL3-basic vector (Promega). ATF4-WT-Luc and ATF4-MT-Luc plasmids contain a wild-type and mutated E-box, respectively.

Northern blotting analysis
Northern blotting analysis was performed as described previously (Uramoto *et al*., 2002). RNA samples (20 μg/lane) were separated on a 1% formaldehyde–agarose gel and transferred to a Hybond N^+ membrane (Amersham Biosciences, Piscataway, NJ, USA) with 10 × SSC. After prehybridization and hybridization with radiolabeled cDNA fragment of Clock, signal intensities were quantified using a bio-imaging analyzer (BAS2000, Fuji Film, Co. Ltd, Tokyo, Japan).

Western blotting analysis
Whole-cell lysates and nuclear extract were prepared as described previously (Uramoto *et al*., 2002). The indicated amounts of whole-cell lysate or nuclear extract were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblotting analysis was performed with an appropriate dilution of the antibodies, and the membrane was developed using a chemiluminescence protocol (Amersham Biosciences).

Chromatin immunoprecipitation assay
The ChIP assay was performed as described previously (Uramoto *et al*., 2002). Soluble chromatin from 1×10^6 cells was incubated with 2 μg anti-Clock antibody or anti-goat immunoglobulin G (IgG). The purified DNA was dissolved with 20 μl dH_2O. The DNA (1 or 3 μl) was then used for PCR analysis with the following primer pairs for the *ATF4* promoter region (−241−+38): GACTCTGATCATAGAAGC CTAG forward primer and GCAGAGAAAACTACAT CTGTGG reverse primer. The PCR products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Transient transfection and luciferase assay
Transient transfection and a luciferase assay were performed as described previously (Uramoto *et al*., 2002). The indicated amounts of the ATF4 reporter plasmid and the expression plasmid were co-transfected using Superfect reagent (Qiagen, Hilden, Germany). After transfection, the cells were cultured for 48 h. Luciferase activity was detected by a Picagene kit (Toyoinki, Tokyo, Japan), and the light intensity was measured with a luminometer (Luminescencer JNII RAB-2300; ATTO, Japan). All of the cells were co-transfected with pCH110 as a control for transfection efficiency. The results shown are normalized to β-galactosidase activity and are representative of at least three independent experiments.

Knockdown analysis using siRNAs
The following double-stranded RNA 25 base pair oligonucleotides were generated from Stealth Select RNAi (Invitrogen): 5′-UAAAGUCUGUUGUUGUAUCAUGUGC-3′ and 5′-GCA CAUGAUACAACAACAGACUUUA-3′ for Clock; 5′-UU CAGUGAUAUCCACUUCACUGCCC-3′ and 5′-GGGCA GUGAAGUGGAUAUCACUGAA-3′ for ATF4; 5′-UAAU GAUGUCCAAGAAGAAGUCUGC-3′ and 5′-GCAGACU UCUUCUUGGACAUCAUUA-3′ for BCRP/*ABCG2*; 5′-CUAUAAAUAACCAUCAUCAAGGCUG-3′ and 5′-CAG CCUUGAUGAUGGUUAUUUAUAG-3′ for MRP2/*AB CC2*. siRNA transfections were performed according to the manufacturer's instructions (Invitrogen) with modification. Ten microliters of Lipofectamine 2000 (Invitrogen) was diluted in 250 μl Opti-MEM I medium (Invitrogen) and incubated for 5 min at room temperature. Next, 250 pmol of Clock, ATF4, and inverted control duplex Stealth RNA (Invitrogen) diluted in 250 μl Opti-MEM I were added gently and incubated for 20 min at room temperature. Oligomer–lipofectamine complexes and aliquots of 1×10^6 A549 cells in 500 μl culture medium were combined and incubated for 10 min at room temperature. Aliquots of 300 cells were used for a colony-formation assay as described below. The remaining cells were seeded in 100 mm dishes with 10 ml culture medium and harvested after 96 h culture for Western blotting analysis as described above.

Cytotoxicity analysis by colony formation and WST-8 assays
For the colony-formation assay, 300 cells transfected with siRNAs were seeded in 35 mm dishes with 2 ml culture medium. The following day, the cells were treated with the indicated concentrations of cisplatin, etoposide and 5-FU. Seven days post-transfection, the numbers of colonies were counted. For the water-soluble tetrazolium salt (WST)-8 assay, 1.5×10^3 ATF4-overexpressing cells or 4×10^3 P/CDP6 cells transfected with indicated amounts of siRNAs were seeded in 96-well plates. The following day, the indicated concentrations of the drugs were applied. After 72 h, the surviving cells were stained with TetraColor ONE (Seikagaku Corporation, Tokyo, Japan) for 90 min at 37°C according to the manufacturer's instructions. The absorbance was then measured at 450 nm. For the WST-8 assay with BSO, 10 μM BSO were treated when ATF4-overexpressing cells were seeded, and the following day the indicated concentrations of the drugs were applied.

Quantitative analysis of intracellular glutathione
Aliquots of 5×10^5 ATF4-transfected cells treated with or without 10 μM BSO for 72 h, and siRNA-transfected A549 cells were washed twice with phosphate-buffered saline. Then, 10 nmol/l HCl was added to the cells and they were rapidly freeze–thawed twice. Next, 5% sulfosalicylic acid was added and the cell lysates were centrifuged at 8000 *g* for 10 min at room temperature. The intracellular glutathione concentration of the supernatants was measured using the Total Glutathione

Quantification Kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions.

Oligonucleotide microarray study and microarray analysis

A microarray procedure was performed as described previously (Arao *et al.*, 2006). In brief, total RNA extracts were collected from A549 cells transfected with ATF4 siRNA (50 or 0.3 nM) or control siRNA (50 or 0.3 nM) in duplicate. Eight GeneChips (Affymetrix, Santa Clara, CA, USA) were used for analysis. The microarray analysis were performed using the BRB Array Tools software ver. 3.3.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) developed by Dr Richard Simon and Amy Peng. In brief, a log base 2 transformation was applied to the microarray raw data, and global normalization was used to median over entire array. Genes were excluded if percent of data missing or filtered out exceeds 20%. We analysed subset of genes including glutathione metabolism subset (based on KEGG PATHWAY Database, http://www.genome.jp/kegg/pathway.html). The selected genes were further selected if fold change marked > 1.5 between averaged ATF4 siRNA sample and control siRNA samples. Next, the 129 genes that fold change marked > 2.5 between averaged ATF4 siRNA sample and control siRNA samples from all normalized and filtered genes were listed and clustered in Supplementary Information.

Statistical analysis

Expression levels of Clock, ATF4, and c-Myc were assessed numerically with the NIH image program (NIH, Bethesda, MD, USA). The Pearson correlation was used for statistical analysis, and significance was set at the 5% level.

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Prognostic implications of the nuclear localization of Y-box-binding protein-1 and CXCR4 expression in ovarian cancer: Their correlation with activated Akt, LRP/MVP and P-glycoprotein expression

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The nuclear localization of Y-box-binding protein-1 (YB-1) is known to be a poor prognostic factor in several human malignancies, including ovarian carcinoma. Following on from our basic study dealing with microarray analyses of YB-1-associated gene expression in ovarian cancer cells, we examined whether nuclear localization of YB-1 is associated with the expression of CXCR4, a vault protein named lung resistance-related vault protein (LRP/MVP), phosphorylated Akt (p-Akt) or P-glycoprotein (P-gp) in human ovarian carcinoma. Fifty-three surgically resected ovarian carcinomas treated with paclitaxel and carboplatin were examined immunohistochemically for nuclear YB-1 expression and intrinsic expression of p-Akt, P-gp, LRP/MVP and CXCR4. Nuclear expression of YB-1 demonstrated significant correlation with p-Akt, P-gp and LRP expression, but no relationship with CXCR4 expression. By multivariate analysis, only YB-1 nuclear expression and CXCR4 expression were independent prognostic factors with regard to overall survival. These results indicate that YB-1 nuclear expression and CXCR4 expression are important prognostic factors in ovarian carcinoma. (*Cancer Sci* 2007; 98: 1020–1026)

Y-box-binding protein-1 (YB-1) has been identified as a transcription factor that binds to the promoter region of several genes involved in positive regulation of the cell cycle, such as proliferating cell nuclear antigen, DNA topoisomerase II α, and multidrug resistance 1 gene (*MDR1*) which is linked to classical multidrug resistant (*MDR*).[1–3] Nuclear expression of YB-1 has been reported to have a close relationship with MDR1/P-glycoprotein (P-gp) expression in several human malignancies.[4–7] Moreover, YB-1 has been reported to be a prognostic marker of breast cancer,[4] ovarian cancer,[6] lung cancer[7] and synovial sarcoma.[8] These clinicopathological studies consistently supported the notion that the absence or presence of YB-1 within the nucleus plays a critical role in the acquisition of malignant characteristics, including global drug resistance.

Sutherland *et al.* have also reported that YB-1 phosphorylation by Akt is required for the nuclear translocation of YB-1:[9] Akt is a signal transduction protein that plays an important role in inhibiting apoptosis, stimulating angiogenesis, and promoting tumor formation in a variety of human malignancies.[10] Taking these findings together, translocation of YB-1 into the nucleus would seem to be mediated through pleiotropic signaling pathways. Our recent study demonstrated that the nuclear translocation of YB-1 is in part stimulated through Akt activation, and also that YB-1 is involved in upregulation and downregulation of various genes including *P-gp*, lung resistance-related vault protein (*LRP/MVP*) and *CXCR4* in human ovarian cancer cells.[11]

The lung resistance-related vault protein (LRP) has been identified as the major vault protein (MVP), which is the major component of vaults, of subcellular particles that have been implicated in transmembrane transport processes.[12] YB-1 also has been reported to promote basal and 5-fluorouracil-induced expression of the *LRP/MVP* gene, the promoter of which contains the Y-box in human colon cancer.[13] Furthermore, the chemokine stroma-derived factor 1 (SDF-1)/CXCL12, and its receptor, CXCR4, have recently been shown to play an important role in metastasis of several kinds of carcinoma.[14,15] This SDF-1/CXCR4 pathway has also been implicated in the invasion and metastasis of ovarian cancer.[16,17] Our preliminary study demonstrated that a human ovarian cancer cell line treated with YB-1 knockdown by small interfering RNA showed downregulated expression of CXCR4, using oligonucleotide microarray analysis.[11]

In the present study, we focused on whether nuclear localization of YB-1 could be associated with the expression of these molecular targets, p-Akt, LRP/MVP, CXCR4 as well as P-gp in ovarian cancer patients, using immunohistochemical analysis. We also studied the various clinicopathological characteristics and the prognostic impact in ovarian carcinoma when patients were treated with a regimen containing both paclitaxel and carboplatin (CBDCA). The coupling of the nuclear localization of YB-1 with p-Akt and global drug resistance-related markers will be discussed with regard to its possible association with the therapeutic efficacy of paclitaxel and carboplatin.

Materials and Methods

Patients. Fifty-three patients with primary ovarian carcinoma who had undergone debulking surgery at Kyushu University Hospital between 1998 and 2004 were examined. Patients were staged according to the International Federation of Obstetrics and Gynecology classification.[18] All of the patients were subjected to chemotherapy using a regimen containing both taxanes (paclitaxel for 51 patients, 180 mg/m^2 body surface/day; docetaxel for two patients, 70 mg/m^2 body surface/day) and CBDCA. The doses of CBDCA were calculated using Calvert's formula.[19] The effect of chemotherapy was evaluated 3–4 weeks after each administration of chemotherapy by ultrasonography or computed tomography. After chemotherapy, all patients were followed up every 2 months

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for the first year, every 3 months for the next year, every 4 months for the next year, every 6 months for the next 2 years, and every year thereafter.

Clinical outcome was measured by treatment-free survival, defined as the interval from the date of the end of the treatment to the date of the diagnosis of progression (drug-free interval), as well as overall survival.

Primary tumors were classified according to a recent WHO classification[20] and were graded as grade 1, 2 or 3 according to Silverberg's proposal[21] using extensively sampled paraffin-embedded samples. We obtained written informed consent from all patients. For strict privacy protection, identifying information for all samples was removed before analysis.

Antibodies. The polyclonal antibody to YB-1 was prepared against a 15-amino acid synthetic peptide (residues 299–313) in the tail domain of the YB-1 protein.[22] The working dilution of anti-YB-1 polyclonal antibody was 1:100. Polyclonal anti-pAkt (Ser473) (diluted 1:100) was obtained from Cell Signaling Technology (Beverly, MA, USA). The monoclonal antibodies 12G5 (BD PharMingen, San Diego, CA, USA; diluted 1:100) for the detection of CXCR4, LRP56 (Nichirei, Tokyo, Japan; diluted 1:50 for LRP), and JSB-1 (Sanbio, Uden, the Netherlands; diluted 1:20) for P-gp were used. Tissue from a normal kidney served as a control for LRP56 and JSB-1, whereas primary breast cancer tissue with regional lymph node metastasis was used as a control for anti-YB-1, anti-pAKt and 12G5.

Immunohistochemistry. Surgically resected specimens prior to chemotherapy were fixed with 10% formalin and embedded in paraffin. Four-micrometer-thick sections on silane-coated slides were stained using the streptavidin–biotin–peroxidase method with a Histofine Sab-Po kit (Nichirei) according to the manufacturer's instructions. At least one representative section was examined in each tumor. After deparaffinization, rehydration and inhibition of endogenous peroxidase, sections were exposed to the primary antibodies at 4°C overnight. After incubation of the secondary antibody and streptavidin–biotin–peroxidase complex at room temperature, the sections were then incubated in 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted. For staining with all of the antibodies, sections were pretreated with microwave irradiation for the purpose of antigen retrieval.

Scoring of immunohistochemical results. The evaluation of immunohistochemical results was scored by two pathologists (Y. Oda and Y. Ohishi) without knowledge of the clinical data of the patients. YB-1 expression was evaluated as to whether its expression was localized in both the nucleus and the cytoplasm, or only in the cytoplasm.[6] For P-gp and LRP, when >10% of the tumor cells showed a positive reaction, either weakly or strongly, we judged the case to be positive in accordance with a previous study.[23] As for P-gp expression, only membranous staining was evaluated, whereas cytoplasmic granular staining pattern was estimated for LRP expression. A consensus judgment was adopted as to the proper immunohistochemical score of the tumors based on the strength of p-Akt and CXCR4 expression: 0, negative; 1+, weak staining; 2+, moderate staining; or 3+, strong staining. The distribution of positive cells was also recorded to portray the diffuse or focal nature of the positive cells: sporadic (positive cells <10%); focal (positive cells ≥11% but <50%); diffuse (positive cells ≥50%). Samples with immunohistochemical scores of 2+ and 3+ with focal to diffuse distributions were considered to be positive for p-Akt and CXCR4 antibodies.[24]

Statistics. Association between two dichotomous variables was evaluated by a two-sided Fisher's exact test. Differences in progression-free survival and overall survival were analyzed using log-rank statistics. Multivariate analysis was carried out with a Cox proportional hazards regression model. $P < 0.05$ was considered statistically significant.

Table 1. Clinical and pathological characteristics of 53 patients

Characteristic	*n*
Age (years)	
<56	26
≥56	27
Stage (FigO)	
I/II	7
III/IV	46
Grade	
I/II	37
III	16
Histology	
Endometrioid	4
Serous	49
Residual tumor (cm)	
<2	36
≥2	11
Unknown	6
Chemotherapy	
Paclitaxel/carboplatin	51
Docetaxel/carboplatin	2

Results

Patients. Clinical and pathological characteristics at diagnosis are summarized in Table 1.

The median age of the patients was 58 years (range, 36–77 years). Four tumors were considered to be stage I, three stage II, 29 stage III, and 17 stage IV. Six tumors showed histological grade I, 31 grade II, and 16 grade III. Histologically, 49 tumors were serous adenocarcinoma and four were endometrioid adenocarcinoma. As for overall survival, follow-up data were available for 52 of the 53 patients. The median treatment-free survival of all 53 patients was 307 days (range, 2–1854 days), whereas the median survival was 858 days (range, 138–2292 days). The median treatment-free follow-up of those patients who are currently progression free is 783 days (range, 30–1854 days).

Immunohistochemistry. The results of the immunohistochemical analyses are summarized in Table 2. Of the 53 tumors, 15 (28.3%) showed intense YB-1 expression in the nucleus but weak expression in the cytoplasm of the tumor cells (Fig. 1A). These cases

Table 2. Correlation between nuclear expression of Y-box-binding protein-1 (YB-1) and phosphorylated Akt (p-Akt), P-glycoprotein (P-gp), lung resistance-related vault protein (LRP) or CXCR4 expression

Protein	Nuclear expression of YB-1		*P*-value
	+	–	
p-Akt			
+	12	10	0.0005*
–	3	28	
P-gp			
+	4	1	0.0191*
–	11	37	
LRP			
+	12	15	0.0084*
–	3	23	
CXCR4			
+	7	13	0.2963
–	8	25	

*Statistically significant.

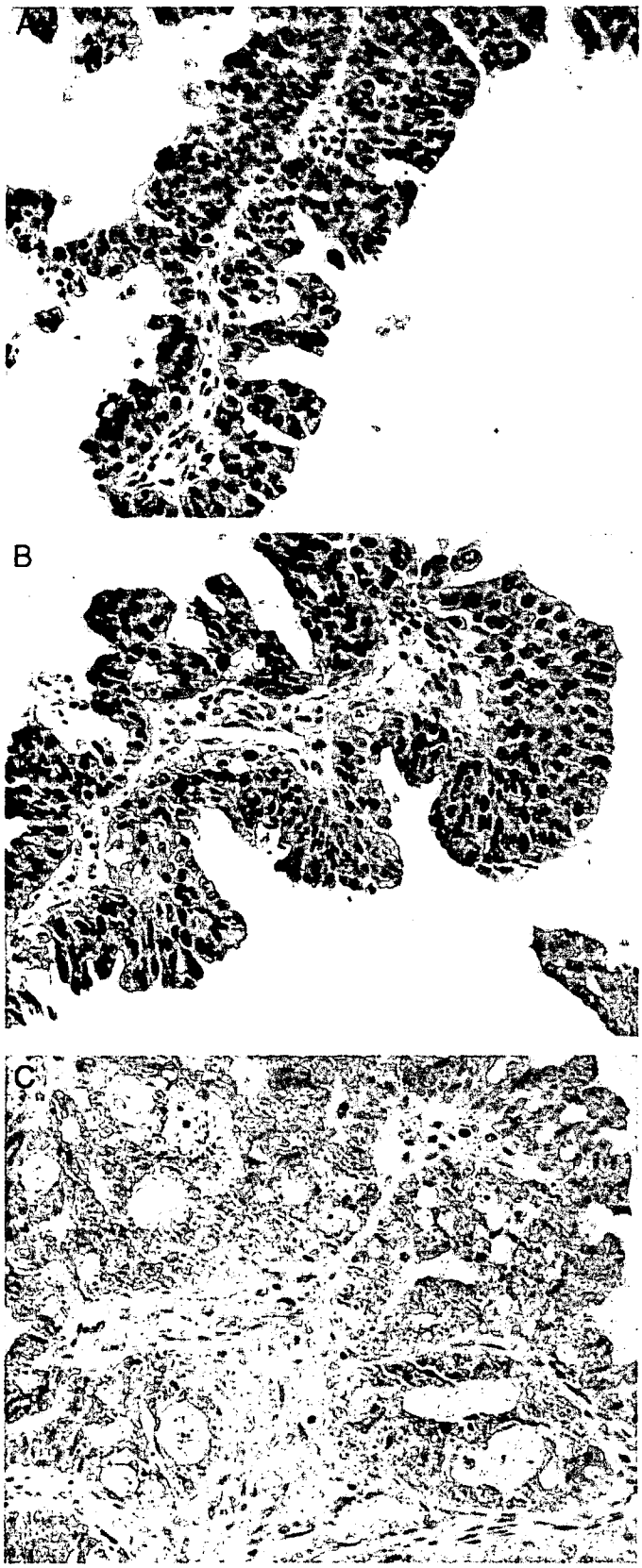

Fig. 1. Grade 2 and stage IIc serous cystadenocarcinoma of a 51-year-old woman. (A) Both nuclear and cytoplasmic expression of Y-box-binding protein-1 were observed in the tumor cells. (B) Strong and diffuse phosphorylated Akt expression was also evident in both the cytoplasm and nuclei. (C) Lung resistance-related vault protein was expressed as a granular cytoplasmic staining pattern. The patient died of disease 51 months after initial surgery.

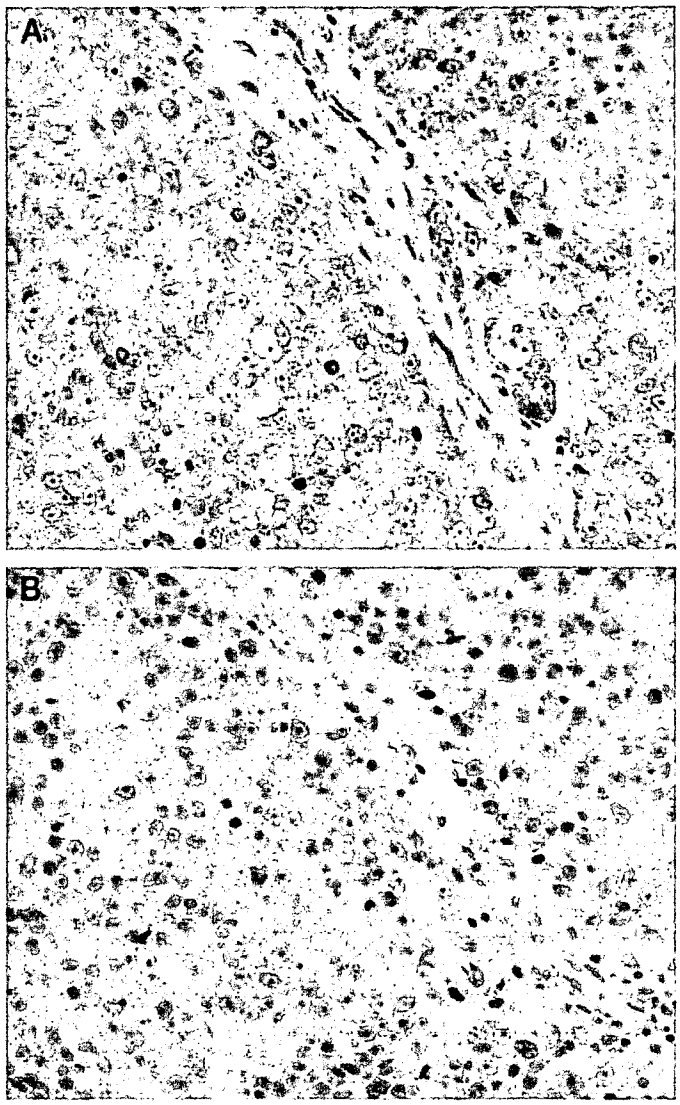

Fig. 2. Grade 3 and stage IIIc serous adenocarcinoma of a 66-year-old woman. Y-box-binding protein-1 expression was observed only in the cytoplasm (A), whereas immunoreactivity for phosphorylated Akt was recognized very faintly in a few tumor cells and was interpreted as negative (B). The patient currently shows no evidence of disease 22 months after surgery.

were interpreted as nuclear expression of YB-1-positive cases. The remaining 38 tumors (71.7%) revealed YB-1 expression only in the cytoplasm, and were interpreted as nuclear expression of YB-1-negative cases. Positive immunostaining for p-Akt was found in 22 tumors (41.5%) with it being predominantly cytoplasmic staining. In 5 of these 22 tumors, immunoreactivity was also recognized in the nucleus (Fig. 1B). Of the 15 tumors in which YB-1 expression was observed in the nucleus, 12 (80%) showed positive immunoreaction for p-Akt, and there was a significant correlation between the nuclear expression of YB-1 and p-Akt expression ($P = 0.0005$) (Fig. 1A,B,2). The membranous expression of P-gp was detected in only five tumors (9.4%) (Fig. 3A). A statistical significance was found between P-gp and YB-1 nuclear expression ($P = 0.0191$). LRP immunostaining was positive in 27 (50.9%) tumors with a granular cytoplasmic staining pattern. There was a significant correlation between LRP expression and YB-1 nuclear expression ($P = 0.0084$) (Fig. 1A,C). Positive immunoreactivity for CXCR4 was

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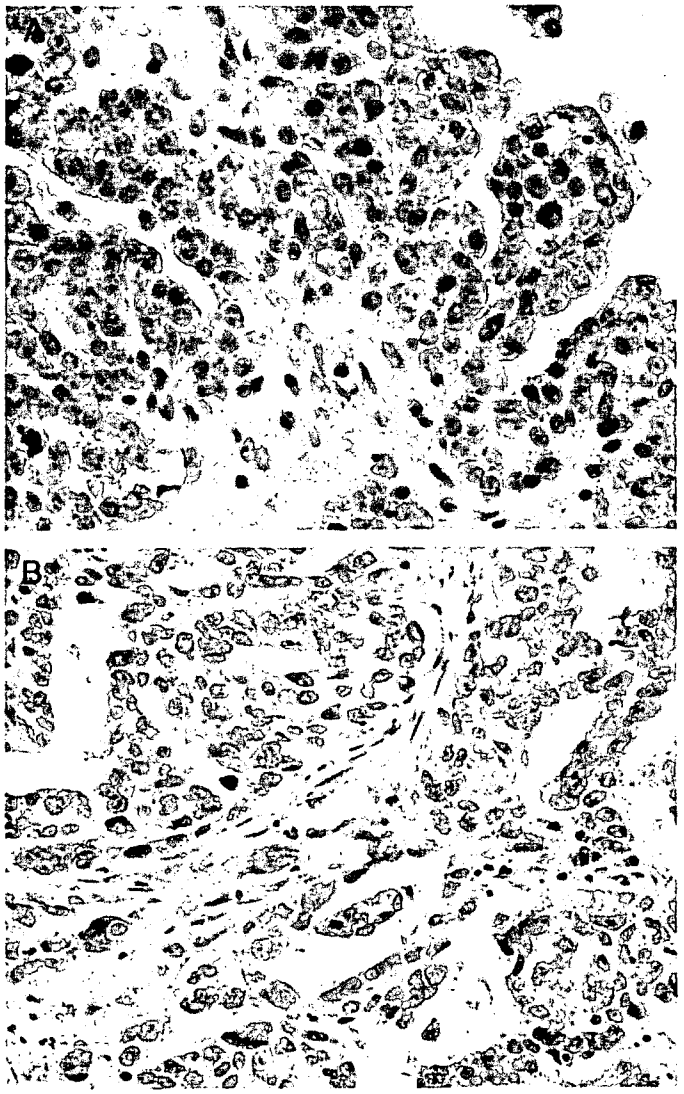

Fig. 3. Grade 3 and stage IIIc serous adenocarcinoma of a 76-year-old woman. P-glycoprotein was expressed in this case as a diffuse membranous staining pattern (A). CXCR4 expression was diffusely visible in the cytoplasm as well as in a few nuclei (B). Y-box-binding protein-1 nuclear expression was also recognized in this case and the patient died of disease 18 months after surgery.

observed in 20 tumors (37.7%) (Fig. 3B); however, it showed no significant relationship with YB-1 nuclear expression.

p-Akt expression was also related to P-gp ($P = 0.0092$), LRP ($P < 0.0001$) and CXCR4 ($P = 0.0078$) expression. Moreover, a significant correlation was found between LRP immunostaining and P-gp ($P = 0.0281$) or CXCR4 ($P = 0.0001$) expression. As for the correlation between clinicopathological parameters and immunohistochemical results, LRP expression was significantly correlated with an age higher than 56 years ($P = 0.0363$). No association was found between any other clinicopathological characteristics and immunostaining for YB-1, p-Akt, P-gp, LRP or CXCR4.

Survival analysis. The results of overall survival analysis and treatment-free (drug-free) survival analysis are summarized in Tables 3 and 4, respectively. As for overall survival, immunohistochemical YB-1 nuclear expression ($P = 0.0126$), p-Akt expression ($P = 0.0167$) and CXCR4 expression ($P = 0.0077$) were adverse prognostic factors, using univariate analysis (Table 3; Fig. 4). No clinicopathological parameters demonstrated a predictive value for overall survival. By multivariate analysis including clinicopathological and immunohistochemical parameters, only YB-1 nuclear expression ($P = 0.0216$) and CXCR4 expression ($P = 0.0316$) were found to be independent prognostic factors with regard to overall survival (Table 3).

Table 3. Overall survival in 52 cases of ovarian carcinoma

Variable	*n*	*P*-value in survival analysis		HR (95% CI)
		Univariate	Multivariate	
Clinicopathological				
Age (years)				
<56	26	0.8903	0.5488	1
≥56	26			1.582 (0.36–6.98)
Stage				
I/II	7	0.1577	0.2087	1
III/IV	45			4.064 (0.46–36.19)
Grade				
I/II	36	0.7422	0.6	1
III	16			1.553 (0.3–8.06)
Residual tumor (cm)				
<2	35	0.82	0.2039	1
≥2	11			2.714 (0.58–12.67)
Immunohistochemical				
YB-1 nuclear expression				
–	37	0.0126*	0.0216*	1
+	15			6.014 (1.3–27.81)
P-gp				
–	47	0.8995	0.6383	1
+	5			0.619 (0.08–4.57)
p-Akt				
–	30	0.0167*	0.5195	1
+	22			1.866 (0.28–12.46)
CXCR4				
–	32	0.0077*	0.0316*	1
+	20			9.007 (1.21–66.88)
LRP				
–	25	0.0897	0.458	1
+	27			0.44 (0.05–3.85)

*Statistically significant. CI, confidence interval; HR, hazard ratio; p-Akt, phosphorylated Akt; P-gp, P-glycoprotein; LRP, lung resistance-related vault protein; YB-1, Y-box-binding protein-1.

As for treatment-free survival, high-stage tumors ($P = 0.0102$) and cases with p-Akt expression ($P = 0.0133$) and LRP expression ($P = 0.0199$) showed adverse prognosis, whereas CXCR4 expression had no impact on prognosis by univariate analysis (Table 4; Fig. 5). Although the cases with YB-1 nuclear expression tended to have worse prognosis, the difference was not statistically significant ($P = 0.0537$; Fig. 5). By multivariate analysis, tumor stage ($P = 0.0428$) and CXCR4 expression ($P = 0.0373$) were poor prognostic factors for treatment-free survival (Table 4).

Discussion

Nuclear expression of YB-1 is reported to be associated with poor prognosis in malignant solid tumors.[7,8] As for ovarian cancer, Kamura *et al.* first demonstrated the prognostic value of YB-1 nuclear expression on disease-free survival in a group of advanced (stage III) serous adenocarcinoma patients who had been treated with cisplatin, epirubicin and cyclophosphamide.[6] In contrast, Huang *et al.* could detect no significant difference in overall survival between patients with YB-1 nuclear expression and those without such expression among patients with epithelial ovarian cancers that consisted of several histological subtypes.[25] These studies help us to further understand why the nuclear localization of YB-1 is associated with poor prognosis in patients

Table 4. Treatment-free survival in 53 cases of ovarian carcinoma

Variable	*n*	*P*-value in survival analysis		HR (95% CI)
		Univariate	Multivariate	
Clinicopathological				
Age (years)				
<56	26	0.7085	0.3508	1
≥56	27			1.536 (0.62–3.79)
Stage				
I/II	7	0.0102*	0.0428*	1
III/IV	46			4.869 (1.05–22.51)
Grade				
I/II	37	0.237	0.2335	1
III	16			0.577 (0.23–1.43)
Residual tumor (cm)				
<2	36	0.8	0.4657	1
≥2	11			1.424 (0.55–3.68)
Immunohistochemical				
YB-1 nuclear expression				
–	38	0.0537	0.6326	1
+	15			1.236 (0.52–2.95)
P-gp				
–	48	0.1768	0.1859	1
+	5			2.415 (0.65–8.92)
p-Akt				
–	31	0.0133*	0.7813	1
+	22			1.149 (0.43–3.07)
CXCR4				
–	33	0.0824	0.0373*	1
+	20			3.102 (1.07–9.00)
LRP				
–	26	0.0199*	0.7685	1
+	27			0.844 (0.27–2.61)

*Statistically significant. CI, confidence interval; HR, hazard ratio; p-Akt, phosphorylated Akt; P-gp, P-glycoprotein; LRP, lung resistance-related vault protein; YB-1, Y-box-binding protein-1.

with various malignancies, including ovarian cancers. In the current study, all of the ovarian cancer patients were treated with taxanes and carboplatin and YB-1 nuclear expression was found to be a poor prognostic marker with regard to overall survival by univariate analysis. As for treatment-free survival, the patients with YB-1 nuclear expression tended to show worse prognosis compared with the patients without YB-1 nuclear expression. Moreover, multivariate analysis revealed that the nuclear expression of YB-1 was an independent adverse prognostic marker with regard to overall survival.

We then asked ourselves how YB-1 could affect the prognosis of patients with ovarian cancer and other malignancies. One representative ATP-binding cassette superfamily protein, P-gp, is often overexpressed in various types of human tumors including ovarian cancer, breast cancer, osteosarcoma and synovial sarcoma.[4–6,8] YB-1 has been identified as a transcription factor that binds to the Y-box of the *MDR1* promoter.[22] Some investigators have shown the prognostic value of intrinsic P-gp expression in ovarian carcinoma,[26] whereas others have failed to demonstrate its predictive value for survival.[23,25] In the current study, we could detect P-gp expression in only 9.4% of the examined cases. Although a statistically significant correlation between P-gp expression and nuclear YB-1 expression was observed, P-gp expression did not affect the patient's prognosis because of the small number of P-gp-positive cases. Further studies with an increased number of patients with P-gp-positive ovarian cancer are required to clarify the notion that the close association of YB-1 with P-gp could play a clinically significant role in the acquisition of drug resistance in ovarian cancer when patients are treated with paclitaxel and cisplatin.

Recently, Stein *et al.* showed an increased expression of endogenous LRP protein by transduction of YB-1 cDNA I *in vivo*, and a strong coexpression of LRP and YB-1 in human colon cancer specimens.[13] The prognostic value of LRP expression in ovarian carcinoma is also controversial. LRP has been shown to be a predictor of poor response to chemotherapy and prognosis in ovarian cancer patients,[23] whereas other authors have

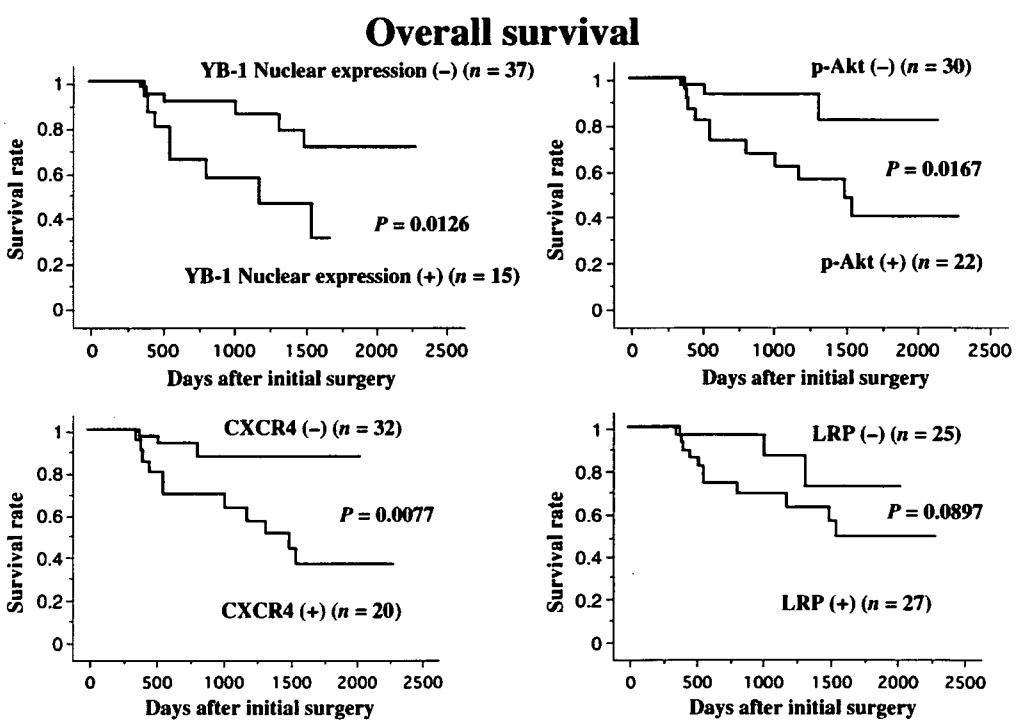

Fig. 4. Overall survival according to immunohistochemical expression in 52 patients with ovarian carcinoma. Y-box-binding protein-1 nuclear expression, and phosphorylated Akt and CXCR4 expression have a significant predictive value for survival.

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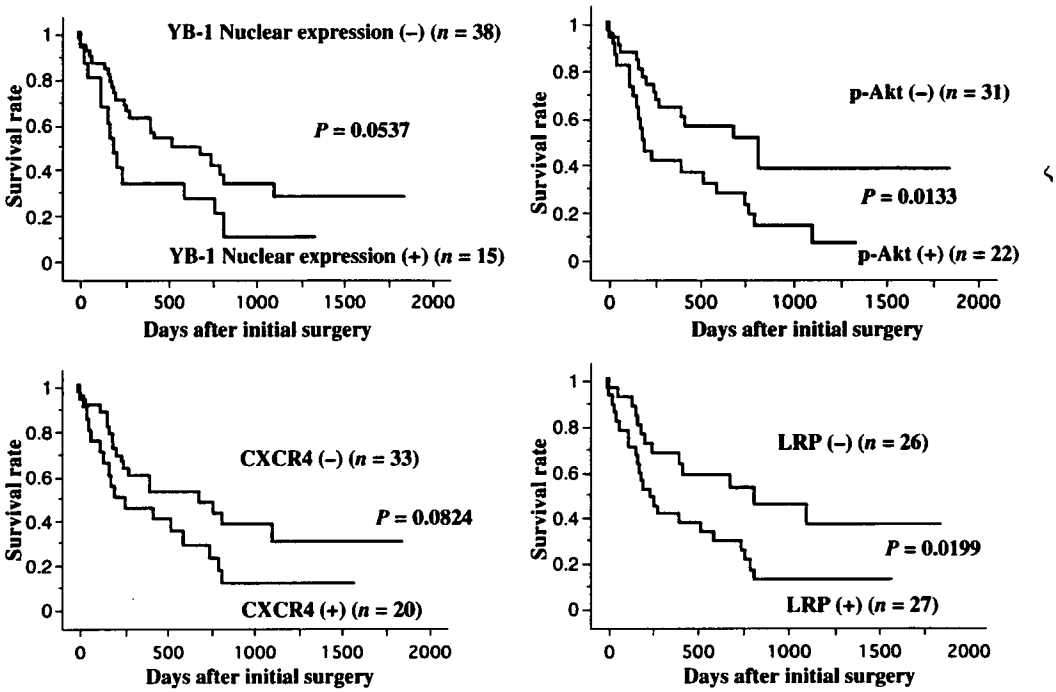

Fig. 5. Treatment-free survival. Cases with Y-box-binding protein-1 nuclear expression tended to show poor prognosis. Cases with phosphorylated Akt and lung resistance-related vault protein expression showed adverse prognosis, whereas CXCR4 expression had no impact on prognosis.

demonstrated no association between LRP expression and clinical outcome.[20] In contrast, the present study demonstrated a close correlation between YB-1 nuclear expression and LRP expression in ovarian carcinoma, as has also been reported in colon cancer. Moreover, LRP expression in untreated ovarian carcinoma was an unfavorable prognostic factor with regard to treatment-free survival. This YB-1-LRP/MVP network may also play a role in global drug resistance in ovarian cancer treated with chemotherapy.

In the present study, we demonstrated a very high association of nuclear localization of YB-1 with p-Akt for the first time. Activated Akt (p-Akt) is known to be predictive of poor clinical outcome in breast cancer,[27] prostate cancer[28] and non-small cell lung cancer.[29] One author failed to demonstrate a significant correlation between p-Akt expression and prognosis,[30] whereas another author suggested the possibility that PTEN and Akt, as well as pathways involving other genes, might play a role in ovarian carcinogenesis.[31] Recently, Sutherland *et al.* have shown that phosphorylation of YB-1 by Akt is required for its translocation into the nucleus from the cytoplasm, and they concluded that YB-1 is a new Akt substrate and disruption of this specific site inhibits tumor cell growth in breast cancer cells.[9] In the current study, p-Akt expression was observed in 22 out of 53 (41.5%) cases and it had a significant correlation with poor prognosis with regard to both overall survival and treatment-free survival, using univariate analysis. Moreover, there was a close relationship between p-Akt expression and YB-1 nuclear expression.

The chemokine-CXCL12 and its receptor, CXCR4, have recently been shown to play an important role in regulating the directional migration of breast cancer cells to sites of metastasis.[14] Scotton *et al.* found that of the 14 chemokines that they investigated, only CXCR4 was expressed in ovarian cancer cells.[16] They also described that CXCR4 may influence cell migration in the peritoneum, a major route for ovarian cancer spread, and accordingly, it could be a therapeutic target.[16] Although CXCR4 is a seven-domain membrane G-protein-coupled receptor, cytoplasmic CXCR4 expression has been described in many human cancers.[17,24] Engl *et al.* demonstrated distinct CXCR4 expression at the intercellular boundaries and strong intracellular accumulation, using confocal laser scanning microscopic analysis.[32] In the current study ovarian cancer cells mainly showed cytoplasmic CXCR4 staining, as previously reported.[17] Jiang *et al.* demonstrated that CXCR4 expression was one of the independent prognostic factors in clinical samples of ovarian cancer.[17] In our recent study we demonstrated the close correlation between CXCR4 and YB-1 expression *in vitro*;[11] however, we failed to reveal such a correlation in the current study. This discrepancy may be due to differences in materials (cell line and clinical tumor sample) and methods (quantitative reverse transcription–polymerase chain reaction and immunohistochemistry). Although no association was detected between YB-1 nuclear expression and CXCR4 expression, CXCR4 expression demonstrated a correlation with adverse prognosis with regard to overall survival, using univariate analysis. Moreover, by multivariate analysis, CXCR4 expression was found to be an independent poor prognostic factor with regard to both overall survival and treatment-free survival. Therefore, these results support the possibility that CXCR4 could be a new molecular therapeutic target in the treatment of ovarian cancer.

In conclusion, by using our basic information on the expression of which genes are closely coupled with YB-1, we were able to further examine whether YB-1 could be significantly associated with relevant genes such as *P-gp*, *p-Akt*, *LRP/MVP* and *CXCR4*. Nuclear localization of YB-1 was found to be closely associated with *P-gp*, *LRP/MVP* and *P-Akt*, but not with *CXCR4* in ovarian cancer. Nuclear YB-1 expression and CXCR4 expression may be independent global poor prognostic markers in ovarian cancer, and these two molecules could be novel candidates as therapeutic targets in patients with ovarian cancer.

Acknowledgments

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ORIGINAL ARTICLE

ZNF143 interacts with p73 and is involved in cisplatin resistance through the transcriptional regulation of DNA repair genes

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Zinc-finger protein 143 (ZNF143) is a human homolog of *Xenopus* transcriptional activator staf that is involved in selenocystyl tRNA transcription. We previously showed that ZNF143 expression is induced by treatment with DNA-damaging agents and that it preferentially binds to cisplatin-modified DNA. In this study, the potential function of ZNF143 was investigated. ZNF143 was overexpressed in cisplatin-resistant cells. ZNF143 knockdown in prostate cancer caused increased sensitivity for cisplatin, but not for oxaliplatin, etoposide and vincristine. We also showed that ZNF143 is associated with tumor suppressor gene product p73 but not with p53. p73 could stimulate the binding of ZNF143 to both ZNF143 binding site and cisplatin-modified DNA, and modulate the function of ZNF143. We provide a direct evidence that both Rad51 and flap endonuclease-1 are target genes of ZNF143 and overexpressed in cisplatin-resistant cells. Taken together, these experiments demonstrate that an interplay of ZNF143, p73 and ZNF143 target genes is involved in DNA repair gene expression and cisplatin resistance.
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Keywords: ZNF143; p73; Rad51; FEN-1; cisplatin; DNA repair

Introduction

Cisplatin is an important chemotherapy drug used in the treatment of many solid tumors (Zamble and Lippard, 1995; Cohen and Lippard, 2001). Its major limitation is the development of resistance (Torigoe *et al.*, 2005). The mechanisms of cisplatin resistance are not completely understood. Cisplatin resistance is influenced by many factors, which affect intracellular drug accumulation (Fujii *et al.*, 1994), levels of cellular thiols (Tew, 1994) and DNA repair activity (Chaney and Sancar, 1996). Drug-induced responses are mediated by transcription factors and include DNA damage signals that lead to the induction of apoptosis in tumor cells by cisplatin (Torigoe *et al.*, 2005; Kohno *et al.*, 2005). Loss of p53 function confers resistance in cancer cell lines (Keshelava *et al.*, 2001). Further, p73 overexpression is associated with cisplatin resistance (Vikhanskaya *et al.*, 2001). Thus, molecular links between transcription factors and drug resistance promises to provide the foundation for novel molecular targeted chemotherapy (Kohno *et al.*, 2005). We previously reported that transcription factor, Y-box binding protein 1 (YB-1), binds preferentially to cisplatin-modified DNA (Ise *et al.*, 1999) and YB-1 expression is upregulated in cisplatin resistance cells (Ohga *et al.*, 1996; Kohno *et al.*, 2003; Kuwano *et al.*, 2004).

We identified the cisplatin-inducible genes such as *activating transcription factor 4* (*ATF4*) (Tanabe *et al.*, 2003) and *Mitochondrial ribosomal protein S11* (*MRP S11*) (Ishiguchi *et al.*, 2004) using differential display (Murakami *et al.*, 2001). ATF4 is upregulated in cisplatin-resistant cells and its expression correlates with cisplatin resistance in lung cancer (Tanabe *et al.*, 2003). Analysis of the *MRP S11* promoter region gene revealed that the zinc-finger transcription factor zinc-finger protein 143 (ZNF143) is involved in the cisplatin induction. ZNF143 is a human homolog of *Xenopus* Staf (Myslinski *et al.*, 1998), and is involved in the transcriptional regulation of small nuclear RNA (snRNA) and snRNA-type genes by RNA polymerase II or III (Schaub *et al.*, 1997; Rincon *et al.*, 1998). It is induced by DNA-damaging agents and binds preferentially to cisplatin-modified DNA (Ishiguchi *et al.*, 2004). In this study, we show that ZNF143 is upregulated in cisplatin-resistant cells. p73 interacts with ZNF143 and promotes the binding of ZNF143 to both ZNF143 binding site and cisplatin-modified DNA. And we also show that ZNF143 plays an important role in the control of DNA repair gene expression.

Results

ZNF143 is upregulated in cisplatin-resistant cell lines

ZNF143 gene expression was shown to be increased in cisplatin-resistant cells in comparison with the parental

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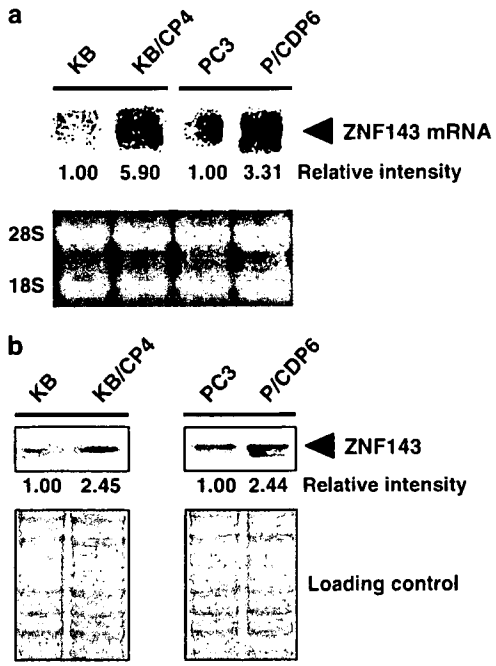

Figure 1 Expression of ZNF143 in cancer cell lines. (**a**) Northern blotting analysis of ZNF143 mRNA. Total RNA (20 μg/lane) of KB and PC3 cells and their cisplatin-resistant KB/CP4 and P/CDP6 were loaded. ZNF143 mRNA (2.6 kb) was detected by Northern blotting analysis. Relative intensity was shown. Gel staining is shown (lower panel). (**b**) Expression of ZNF143 protein in KB and PC3 cells and their cisplatin-resistant KB/CP4 and P/CDP6. Fifty micrograms of sonicated nuclear fractions were subjected by SDS–PAGE. Transferred membrane was blotted with anti-ZNF143 antibody. Relative intensity was also shown. Gel staining with Coomassie Brilliant Blue (CBB) was also shown (lower panel).

cells (Figure 1a). Western blotting analysis revealed that ZNF143 protein was overexpressed in cisplatin-resistant cells when sonicated nuclear fractions were loaded (Figure 1b). However, inverse result was obtained when eluted nuclear extracts using salt buffer were loaded (data not shown), suggesting that ZNF143 may be tightly bound to chromatin in cisplatin-resistant cells.

The effects of ZNF143 expression on cisplatin sensitivity
To determine whether ZNF143 protein plays a role in cisplatin sensitivity, we inhibited its expression using small interfering RNA (siRNA) knockdown. Western blotting analysis showed that three kinds of ZNF143 siRNAs specifically downregulated ZNF143 expression in comparison with control siRNA treatment (Figure 2a). The effect of ZNF143 inhibition on clonogenic survival was also assessed (Figure 2b). Downregulation of ZNF143 expression by three kinds of siRNAs rendered cell sensitive to cisplatin (Figure 2b, left upper panel). Specific inhibition of ZNF143 had significantly sensitized PC3 cells to cisplatin, but not to oxaliplatin, etoposide and vincristine (Figure 2b). Further, downregulation of ZNF143 could partially reverse the cisplatin resistance of P/CDP6 cells (Figure 2c and d).

Association of p73 with ZNF143
As damage to DNA increases the nuclear accumulation of tumor suppressor gene products, we next investigated the interaction of ZNF143 with p53 and p73. We employed transient transfection using both 3 × Flag-ZNF143 and hemagglutinin (HA)-p53 or HA-p73-expressing plasmids. The complexes immunoprecipitated with anti-Flag antibody contained HA-p73 (Figure 3a), but not HA-p53 (Figure 3c). And we also verified that the complex contained 3 × Flag-ZNF143 when HA-p73 was reciprocally immunoprecipitated using HA antibody (Figure 3b).

p73 stimulates the DNA binding of ZNF143
We previously reported that ZNF143 preferentially binds to cisplatin-modified DNA (Ishiguchi *et al*., 2004). To examine the effect of p73 on ZNF143 binding to oligonucleotide containing ZNF143 binding site of human *U6 RNA* promoter and cisplatin-modified DNA, we performed electrophoretic mobility shift assay (EMSA). Both glutathione-*S*-transferase (GST) and GST-p73 could not bind to both ZNF143 binding site (Figure 4a, left panel) and cisplatin-modified DNA (Figure 4b, left panel). The DNA binding of ZNF143 was significantly enhanced by GST-p73 in a dose-dependent manner, but not by control GST. However, p73 did not alter the electrophoretic mobility of the ZNF143 complex formed with DNA.

Potential ZNF143 target genes for DNA repair pathways
More than 150 genes for DNA repair pathways were identified and listed (Wood *et al*., 2005). A 19 bp consensus sequences for staf binding site was reported (Schaub *et al*., 1997; Rincon *et al*., 1998) and was used in a computer search of the human genome database. Initially, we surveyed and selected the putative staf binding sites, which show more than 70% homology in the promoter region containing 1000 bp upstream from the transcriptional start site. Among about 150 DNA repair genes, the putative binding sites were found in the promoter region of 78 genes. As C residues at position 4–6 and 13 are almost invariably conserved more than 95%, this criteria was considered to select the potential ZNF143 binding sites. Finally, we found that the 83 staf binding sites of 62 genes contained these conserved C residues (Supplementary Data). This suggests that ZNF143 functions as the pivotal factor to control gene expressions for DNA repair pathways.

DNA repair-associated gene expression regulated by ZNF143
We found that *Rad51* and *flap endonuclease-1* (*FEN-1*) had putative ZNF143 binding site in the core promoter region as shown in Figure 6a and Supplementary Data. We carried out Western blotting analysis. As shown in Figure 5a, both cellular Rad51 and FEN-1 proteins were upregulated in cisplatin-resistant cells. Reciprocally, cellular Rad51 and FEN-1 proteins were decreased when PC3 cells were treated with ZNF143 siRNA (Figure 5b). To determine whether ZNF143 directly