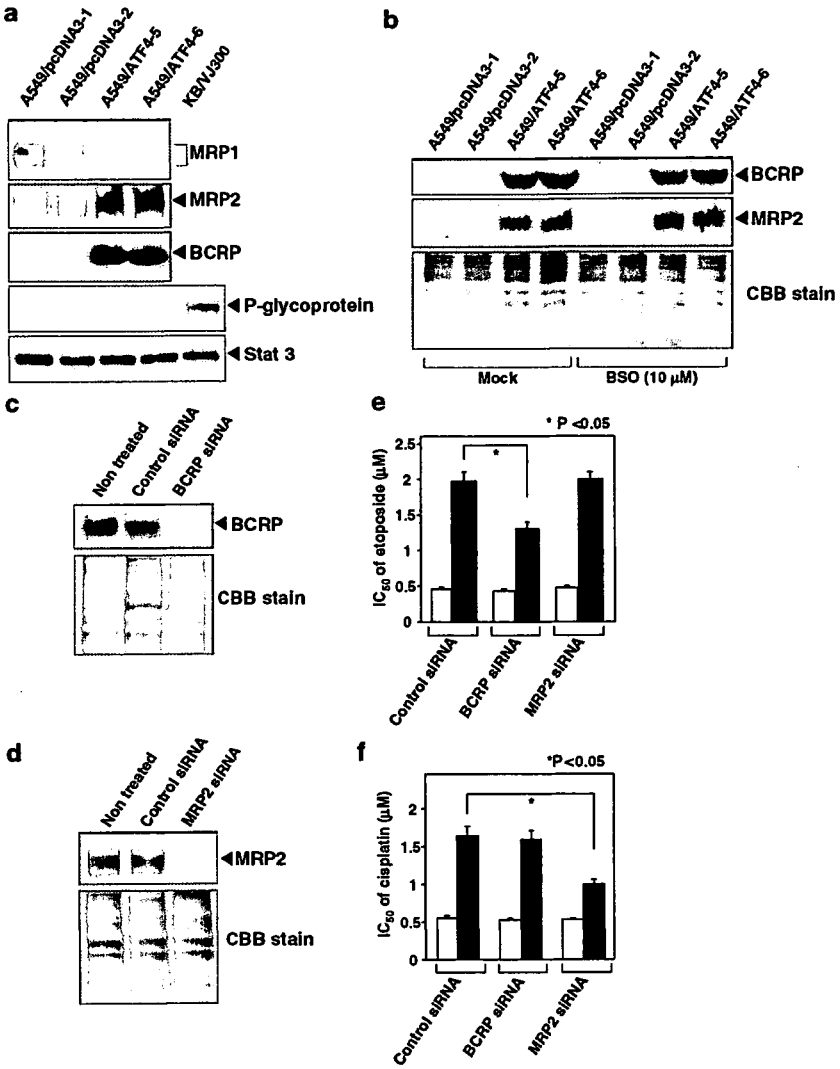

Figure 7 Cellular expressions of ABC transporter families in ATF4-overexpressing cells (A549/ATF4-5 and A549/ATF4-6) and control cells (A549/pcDNA3-1 and A549/pcDNA3-2) (**a**) Whole-cell extracts (100 μg) from the indicated cell lines were subjected to SDS–PAGE, and Western blotting analysis was performed 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 = ± 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.

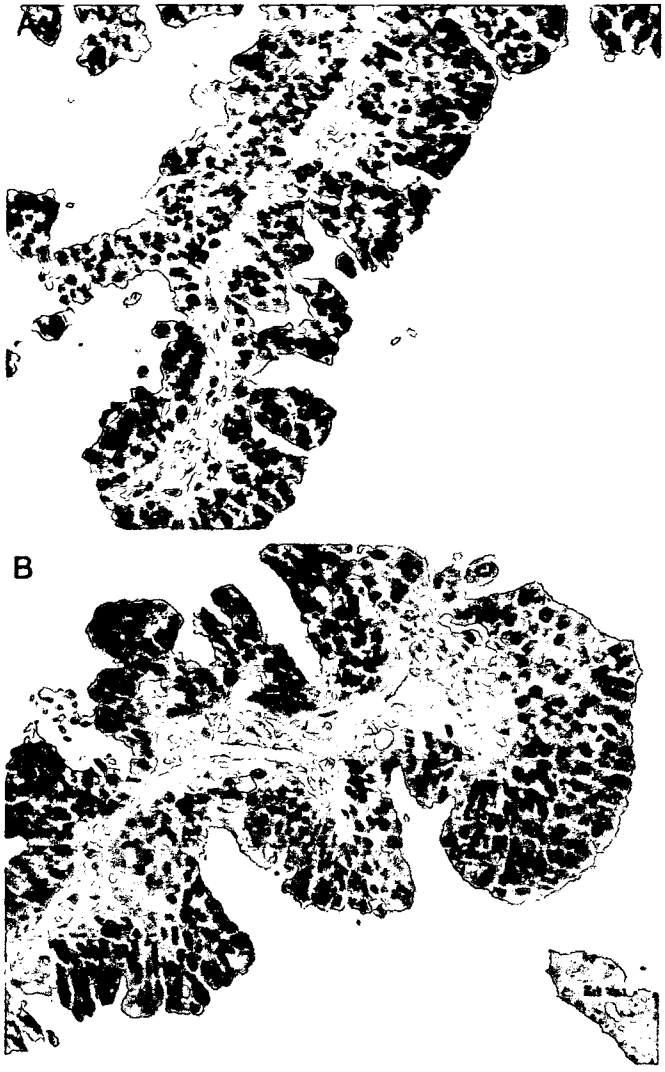

Fig. 1. Grade 2 and stage IIc serous cystadenocarcinoma of a 51-year-old woman. (A) Both nuclear and cytoplasmic expression of Y-box-binding protein-1 were observed in the tumor cells. (B) Strong and diffuse phosphorylated Akt expression was also evident in both the cytoplasm and nuclei. (C) Lung resistance-related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

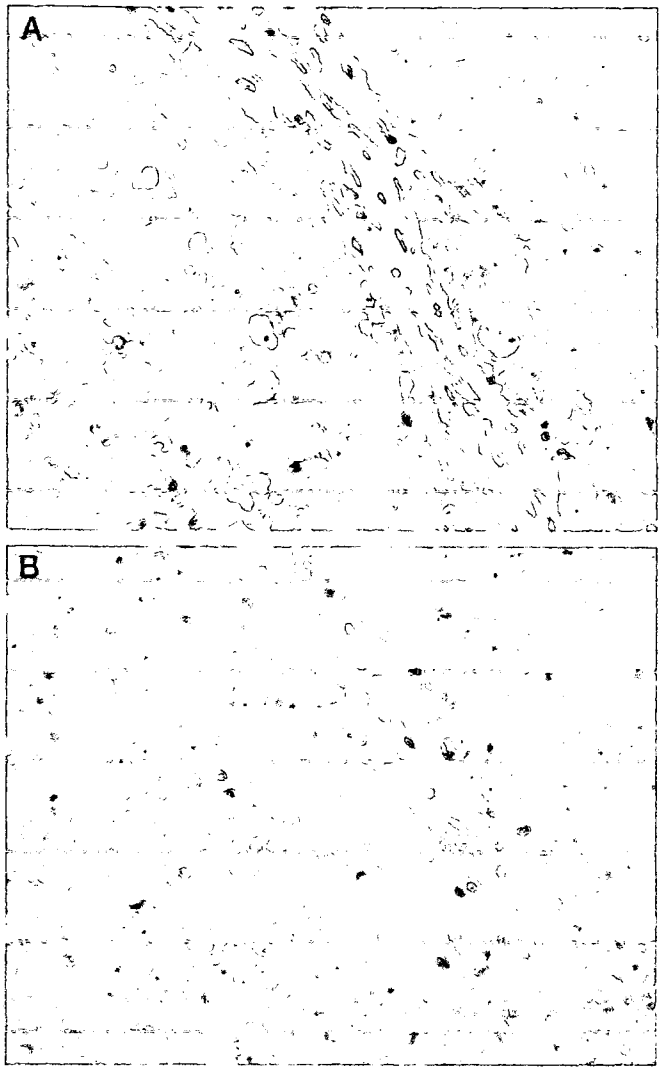

Fig. 2. Grade 3 and stage IIIc serous adenocarcinoma of a 66-year-old woman. Y-box-binding protein-1 expression was observed only in the cytoplasm (A), whereas immunoreactivity for phosphorylated Akt was recognized very faintly in a few tumor cells and was interpreted as negative (B). The patient currently 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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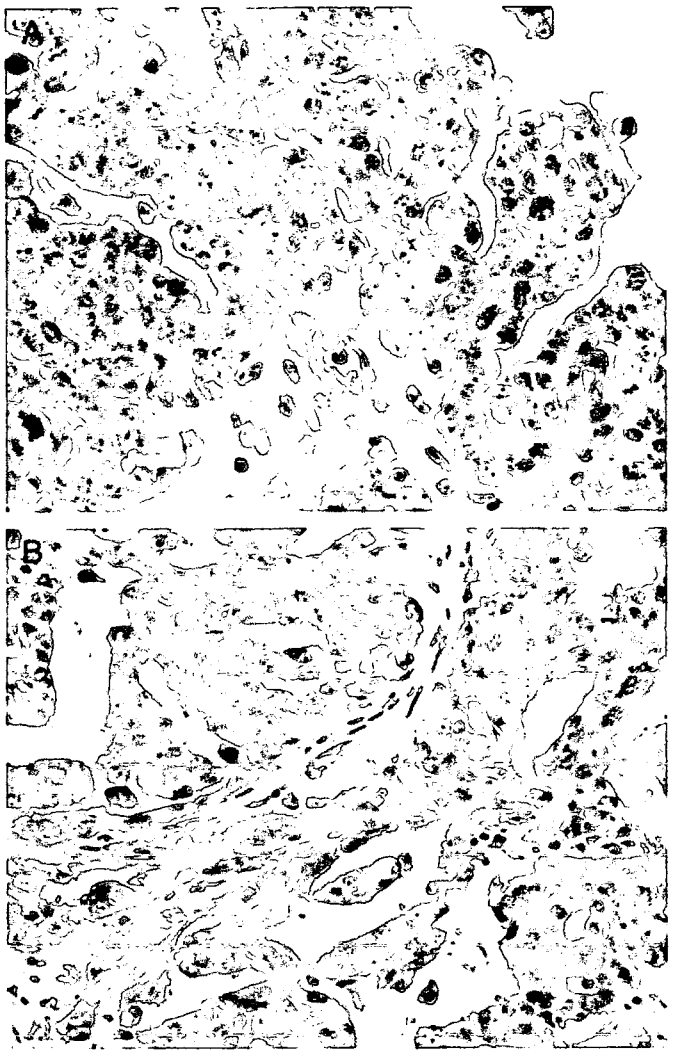

Fig. 3. Grade 3 and stage IIIc serous adenocarcinoma of a 76-year-old woman. P-glycoprotein was expressed in this case as a diffuse membranous staining pattern (A). CXCR4 expression was diffusely visible in the cytoplasm as well as in a few nuclei (B). Y-box-binding protein-1 nuclear expression was also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

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.

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).

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 1 *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

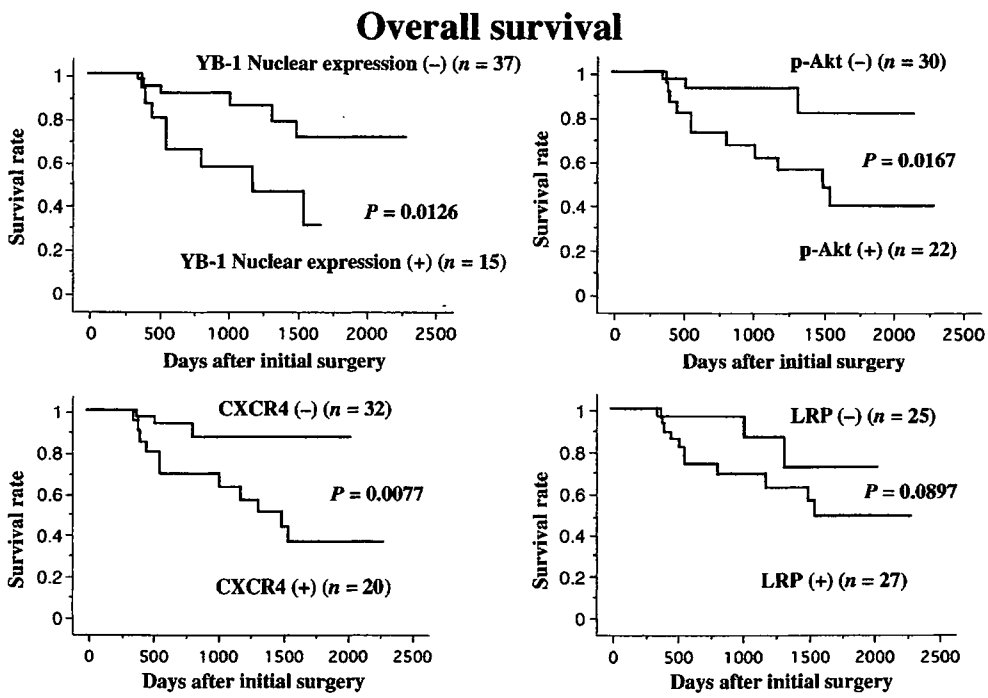

Fig. 4. Overall survival according to immunohistochemical expression in 52 patients with ovarian carcinoma. Y-box-binding protein-1 nuclear expression, and phosphorylated Akt and CXCR4 expression have a significant predictive value for survival.

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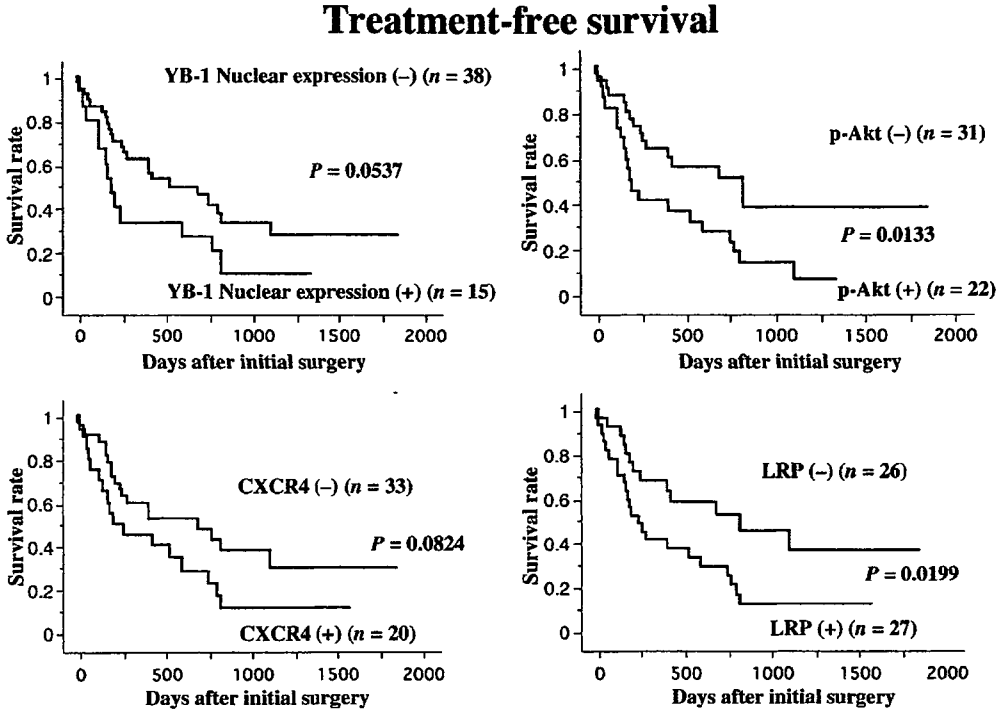

Fig. 5. Treatment-free survival. Cases with Y-box-binding protein-1 nuclear expression tended to show poor prognosis. Cases with phosphorylated Akt and lung resistance-related vault protein expression showed adverse prognosis, whereas CXCR4 expression had no impact on prognosis.

demonstrated no association between LRP expression and clinical outcome.[26] 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.

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LUNG CANCER

SHORT COMMUNICATION

Sequential occurrence of non-small cell and small cell lung cancer with the same *EGFR* mutation

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EGFR mutation;
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Summary We report a case of small cell lung cancer (SCLC) developing after prolonged treatment (more than 2 years) for primary adenocarcinoma of the lung, and we show that both the SCLC and non-small cell lung cancer (NSCLC) tissues obtained from the same site share the same deletion in exon 19 of *EGFR*. This case suggests that the activating *EGFR* mutations may confer the pathogenesis of a subset of SCLC.

1. Introduction

The identification of somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) in patients with NSCLC and the association of such mutations with the clinical response to EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib have had a substantial impact on the treatment of this disease [1,2]. To date, however, only a few *EGFR* mutations have been detected in other solid tumors including SCLC.

2. Case report

A 46-year-old Japanese woman with no smoking history was diagnosed in July 2003 with stage IIIB adenocarcinoma (acinar type) of the lung, with a primary tumor in the left lower lobe and pleural disseminations. A computed tomography (CT) scan showing the tumor (arrow) and hematoxylin–eosin (HE) staining of a tumor biopsy specimen are shown (Fig. 1A). The patient received first-line treatment with cisplatin and vinorelbine and showed a brief partial response. She

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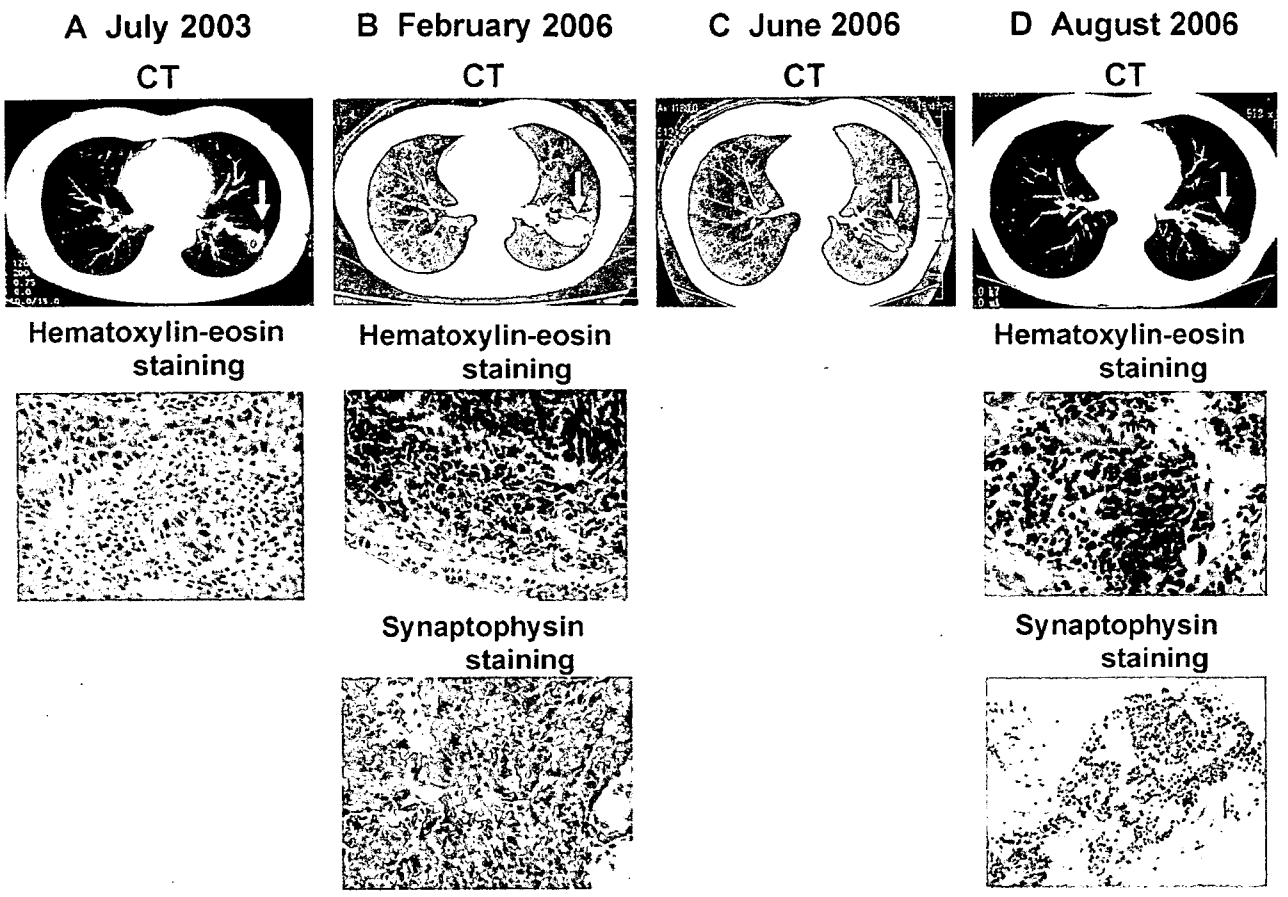

Fig. 1 Chest CT scan: (A) before treatment and HE staining of a tumor biopsy specimen; (B) before second lung biopsy and HE and synaptophysin stainings of a tumor biopsy specimen; (C) after four cycles of cisplatin and irrinotecan; (D) before third lung biopsy and HE and synaptophysin stainings of a tumor biopsy specimen.

subsequently underwent combination chemotherapy with gemcitabine and paclitaxel, manifesting a minor response on radiographic examination. In September 2004, the mass in the left lower lobe had progressed and treatment with gefitinib (250 mg daily) was initiated. After 10 months of treatment with gefitinib alone and transient disease stabilization, a repeat evaluation in July 2005 showed progression of the primary lung tumor. Gefitinib was discontinued, and the patient was enrolled in a phase I clinical trial of new agents. The primary tumor showed no evidence of regression on radiological examination. A magnetic resonance imaging (MRI) scan in December 2005 revealed multiple brain metastases in both hemispheres, which were accompanied by symptoms including headache, nausea, and visual disturbances. After surgical resection of the largest tumor in the right parietal lobe, the patient was exposed to 10 fractions of 3 Gy whole-brain radiotherapy. Her symptoms improved markedly, and MRI scans after radiotherapy revealed almost complete regression of the brain metastases. Histological examination of the resected brain tumor revealed a synaptophysin-positive small cell cancer. The patient provided informed consent to repeated lung biopsies for histological examination. A biopsy specimen of the progressive mass in the left lower lobe in February 2006 revealed SCLC by HE staining and was positive for synaptophysin by immunohistochemical analysis (Fig. 1B). A second lung biopsy specimen was microdissected for extraction of genomic DNA and analysis of *EGFR* mutations. A heterozygous in-frame 15-bp deletion in exon 19 of *EGFR* was detected with the use of the amplification refractory mutation system (ARMS); the genomic DNA of the patient was thus subjected to amplification by the polymerase chain reaction with primers specific for the wild-type (Fig. 2A, left panel) or mutant (Fig. 2A, right panel) versions of exon 19. The deletion was confirmed to be delE746–A750 by nucleotide sequencing. On the basis of the histological diagnosis of SCLC, the patient was treated with four cycles of cisplatin and irrinotecan, and she achieved a partial response (Fig. 1C). A repeat chest CT evaluation in August 2006 showed progression of the primary lung tumor (Fig. 1D). A new lung biopsy specimen revealed nests of adenocarcinoma cells forming small tubular structures, the same subtype of the adenocarcinoma at initial diagnosis on July 2003, and was negative for synaptophysin staining (Fig. 1D). In addition, ARMS analysis of the adenocarcinoma specimen detected the same in-frame 15-bp deletion in exon 19 of *EGFR* that had been identified in the previous SCLC specimen (Fig. 2B).

3. Discussion

EGFR mutations are more frequent in women, Asians, individuals with adenocarcinoma, or those who have never

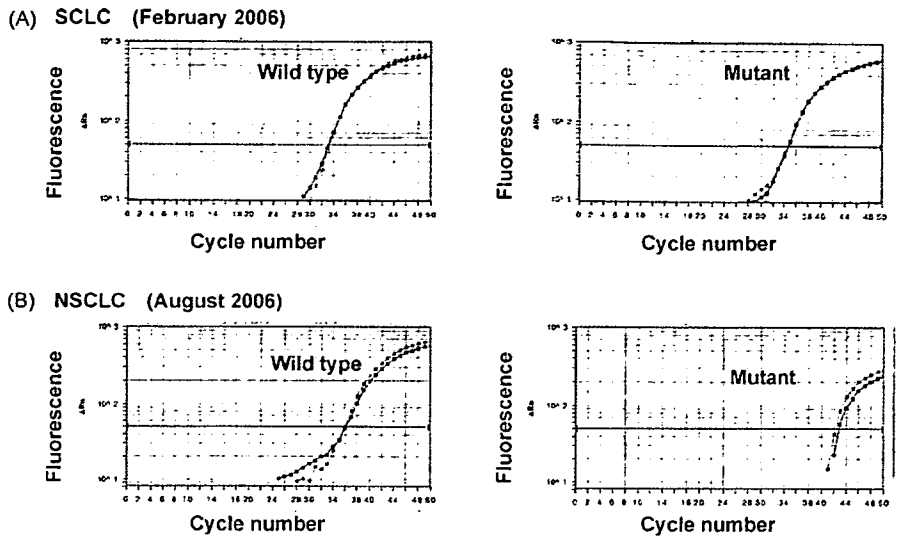

Fig. 2 Results of ARMS analysis of (A) the SCLC. Ascending curves, performed in duplicate (green and red), indicate that wild type (left panel) and deletion mutation in exon 19 (right panel) were detected; (B) the adenocarcinoma. Ascending curves, performed in duplicate (green and red), indicate that wild type (left panel) and deletion mutation in exon 19 (right panel) were detected.

smoked [3–5]. However, EGFR expression has been shown to be low or undetectable in SCLC, and screening of SCLC for *EGFR* mutations has yielded negative results [5]. We previously described the first case of SCLC with a deletion in exon 19 of *EGFR* in a nonsmoking Japanese woman [6]. Another case of SCLC with an 18-bp deletion in exon 19 of *EGFR* in a nonsmoking woman was also recently reported [7]. All reported cases of SCLC with *EGFR* mutations, including the present case, have thus been in women who have never smoked, even though SCLC occurs almost exclusively in smokers. Furthermore, all three of these SCLC cases were initially diagnosed as adenocarcinoma. In the present case, SCLC developed after prolonged treatment (>2 years) for primary adenocarcinoma, and both SCLC and NSCLC (adenocarcinoma) tissues obtained from the same site shared the same *EGFR* mutation. Small cell carcinoma of the prostate, which shares histological similarities with SCLC, has been shown to arise during the course of treatment for prostatic adenocarcinoma, suggesting that prostatic small cell carcinoma may originate from multipotent stem cells of the prostate that have the ability to differentiate into either epithelial or neuroendocrine type carcinoma [8–10]. It remains unclear whether the primary tumor of the present patient originally had a minor SCLC component or whether SCLC arose from transdifferentiation of the adenocarcinoma. Our finding that SCLC and NSCLC developed at the same site in the lung and shared the same somatic *EGFR* mutation suggests, however, that different types of lung cancer may arise from a common stem cell with multiple potential pathways of differentiation.

Conflict of interest

We, all authors, indicate no potential conflicts of interest.

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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.

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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

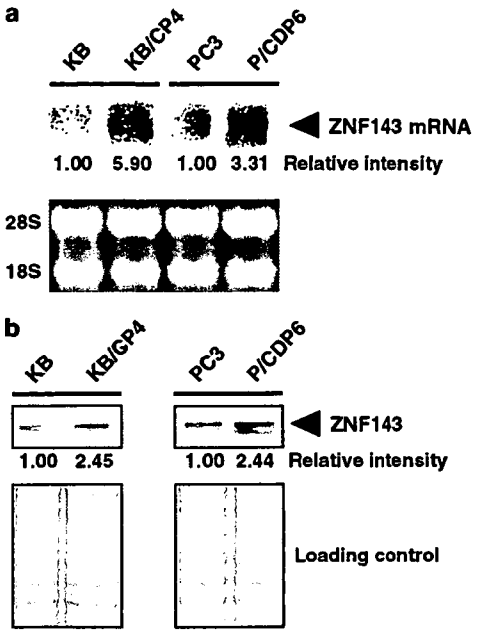


Figure 1 Expression of ZNF143 in cancer cell lines. (a) Northern blotting analysis of ZNF143 mRNA. Total RNA (20 μg/lane) of KB and PC3 cells and their cisplatin-resistant KB/CP4 and P/CDP6 were loaded. ZNF143 mRNA (2.6 kb) was detected by Northern blotting analysis. Relative intensity was shown. Gel 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.

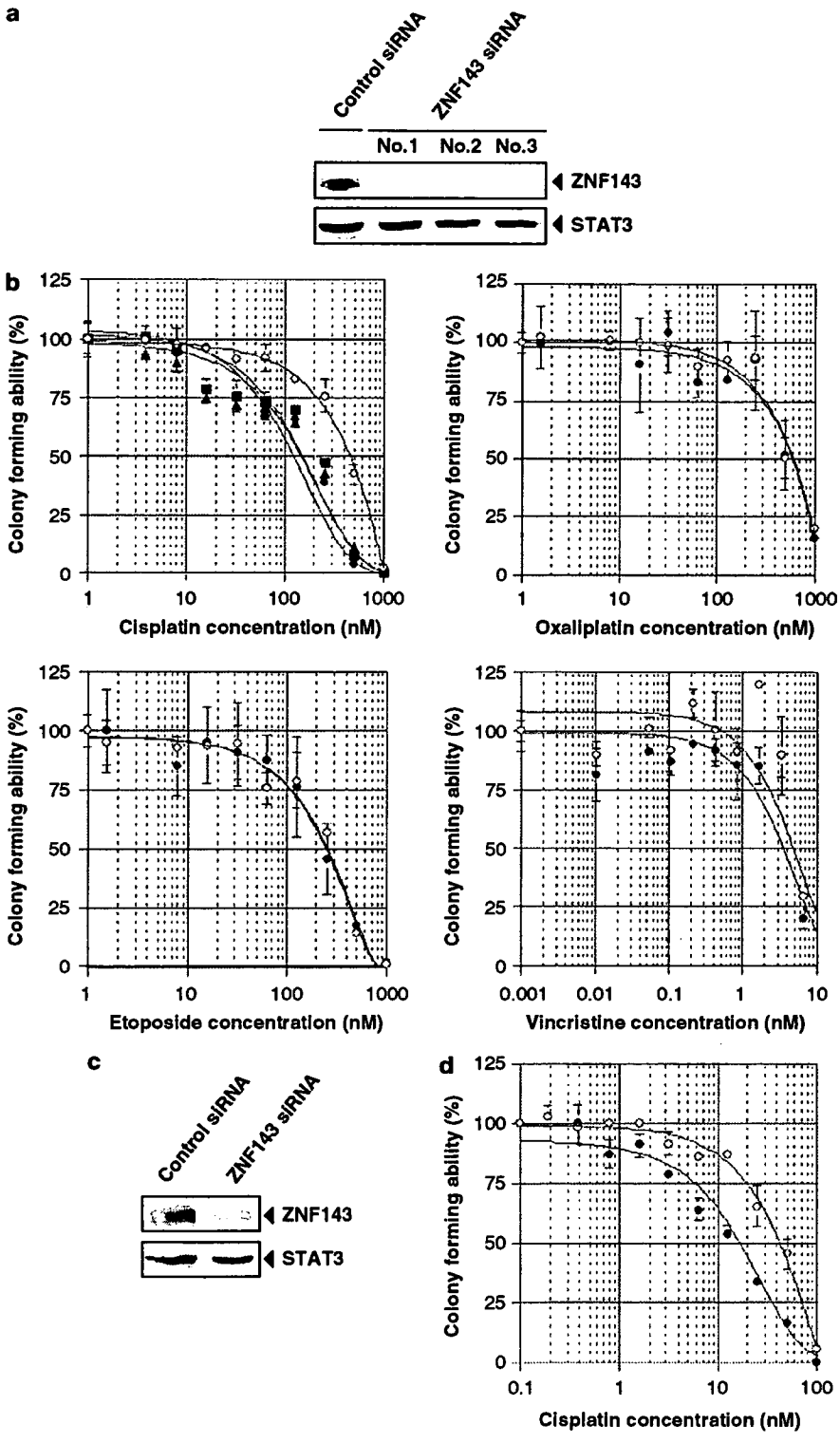
Potential ZNF143 target genes for DNA repair pathways

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

involves in these gene expressions, we performed EMSA and chromatin immunoprecipitation (ChIP) assay. As shown in Figure 6b, GST–ZNF143 could recognize the putative ZNF143 binding site located in both gene promoters. These signals disappeared after the addition of unlabeled oligonucleotides in the reaction mixture (data not shown). Before the ChIP assay, we established the stable transfectants that expressed 3 × Flag-tagged ZNF143, because no adequate anti-ZNF143 antibody was available for the immunoprecipitation. We



confirmed the ectopic expression of 3 × Flag-ZNF143 by Western blotting (Figure 6c). Stable transfectant expressed slightly larger ZNF143 than endogenous protein in the molecular weight due to the additional tag peptides. The ChIP assay showed that substantial enrichment of the region spanning the ZNF143 binding site in the promoter regions of *Rad51* and *FEN-1* was observed when cells expressing 3 × Flag-ZNF143 were used (Figure 6d, lane 5), but not when cells transfected with vector alone were used (Figure 6d, lane 2). No

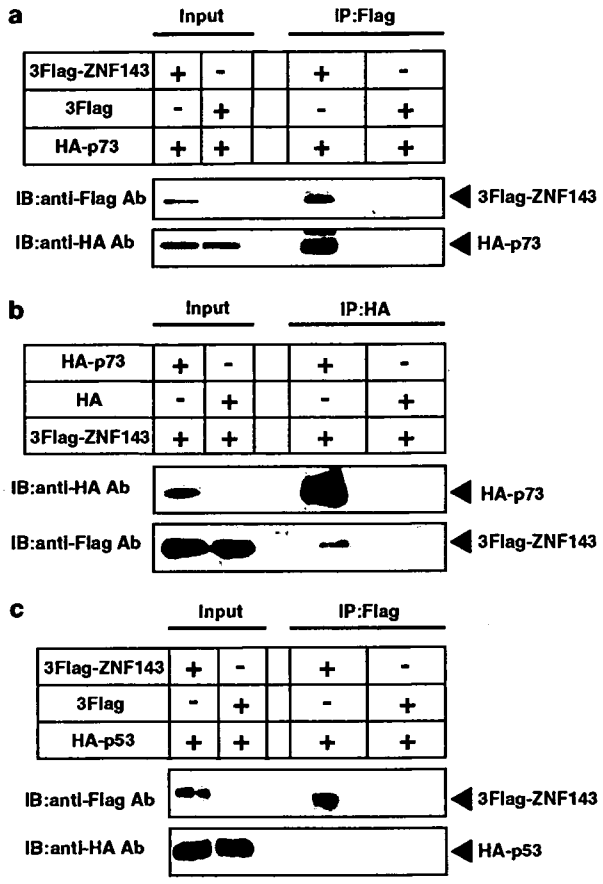


Figure 3 Interaction of p73 with ZNF143. (a) Whole-cell lysates (300 μg) prepared from PC3 cells co-transfected with HA and 3 × Flag expression plasmids were immunoprecipitated with anti-Flag (M2) antibody. The resulting immunocomplexes and whole-cell lysates (50 μg) were subjected to SDS–PAGE. Transferred membrane was blotted with either anti-Flag or anti-HA antibodies. (b) A reciprocal immunoprecipitation assay and Western blotting were performed. (c) p53 expression plasmid was transfected instead of p73 expression plasmid, and immunoprecipitation assay and Western blotting were performed.

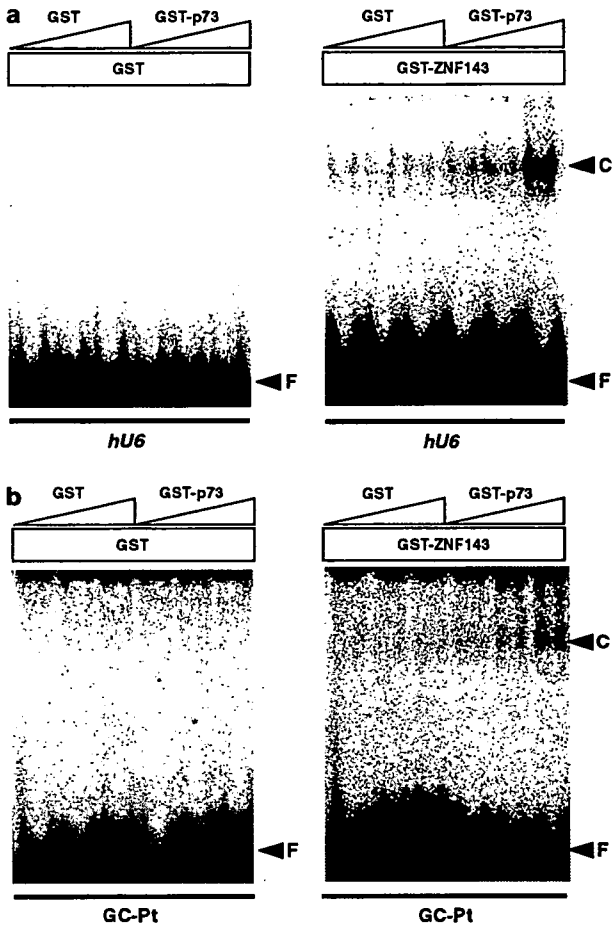


Figure 4 Stimulation of ZNF143 binding to DNA by p73. (a) Enhancement of ZNF143 binding to its binding site of *U6 RNA* promoter by p73. Purified GST or GST-p73 (50, 250 and 500 ng) were mixed with GST (100 ng) or purified GST-ZNF143 (100 ng), and incubated with ^{32}P-labeled *U6 RNA* oligonucleotides containing ZNF143 binding site. The reaction mixtures were resolved by electrophoresis on a 4% polyacrylamide gel with 0.5 × TBE buffer. The gel was dried and analysed by a bio-imaging analyzer (FLA2000). (b) Enhancement of ZNF143 binding to cisplatin-modified DNA by p73. Purified GST-p73 and GST-ZNF143 were incubated with ^{32}P-labeled cisplatin-modified DNA (GC-Pt), and EMSA was performed by the same method as described in (a).

Figure 2 Downregulation by ZNF143 siRNA transfection and drug sensitivity. (a) Downregulation of ZNF143 expression by three kinds of ZNF143 siRNAs (No. 1, No. 2 and No. 3). Control siRNA (50 pmol) or ZNF143 siRNA were transfected into PC3 cells and whole-cell lysates (50 μg) were subjected to SDS–PAGE. Transferred membrane was blotted with anti-ZNF143 and anti-STAT3 antibodies. (b) Treatment of ZNF143-siRNA sensitized cisplatin. PC3 cells were treated with 50 pmol ZNF143-siRNAs (No. 1; closed circles, No. 2; closed triangle and No. 3; closed square) or 50 pmol control-siRNA (open circles) for 24 h, and exposed to various concentrations of cisplatin, oxaliplatin, etoposide and vincristine for 7 days. The colony number in the absence of drug corresponded to 100%. All values were the mean of least three independent experiments with ± s.d. (c) Downregulation of ZNF143 expression in cisplatin-resistant cells by ZNF143 siRNA. Control siRNA (100 pmol) or ZNF143 siRNA (No. 1) were transfected into P/CDP6 cells and whole-cell lysates (50 μg) were subjected to SDS–PAGE. Transferred membrane was blotted with anti-ZNF143 and anti-STAT3 antibodies. (d) Treatment of ZNF143-siRNA partially reversed cisplatin resistance. P/CDP6 cells were treated with 100 pmol ZNF143 siRNA No. 1 (closed circles) or control siRNA (open circles) for 24 h, and exposed to various concentrations of cisplatin for 7 days. The colony number in the absence of drug corresponded to 100%. All values were the mean of least three independent experiments with ± s.d.

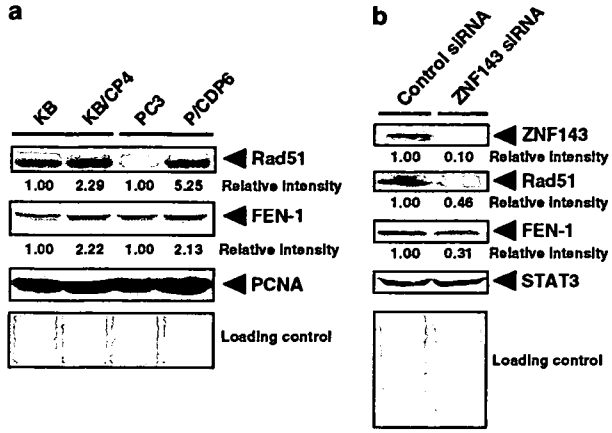


Figure 5 Expression of Rad51 and FEN-1 in cancer cell lines. (a) Expression of Rad51 and FEN-1 protein in KB and PC3 cells and their cisplatin-resistant KB/CP4 and P/CDP6. Nuclear extracts (50 μg) were subjected by SDS–PAGE. Transferred membrane was blotted with anti-Rad51, anti-FEN-1 or anti-PCNA antibodies. Relative intensity was shown at the bottom of each panel. Gel staining with CBB was also shown (lower panel). (b) Down-regulation of ZNF143 expression reduces cellular level of Rad51 and FEN-1. PC3 cells were treated with 50 pmol ZNF143-siRNA or control-siRNA for 72 h, and whole-cell lysates (100 μg) were subjected by SDS–PAGE, and Western blotting with anti-ZNF143, anti-Rad51, anti-FEN-1 and anti-STAT3 antibodies was performed. Relative intensity was shown at the bottom of each panel. Gel staining was also shown.

promoter enrichment was observed when unrelated *peroxiredoxin 4* (*PRDX4*) gene promoter was assayed.

We next performed ChIP assay and reporter assay with transient transfection to gain greater insight into the transcriptional regulation of two DNA repair genes. PC3 cells were co-transfected with the reporter plasmid driven by the promoter of *Rad51* or *FEN-1* genes with p73 expression plasmid. These promoter regions contain ZNF143 binding site. p73 activated both promoter activities (Figure 7a).

Next, we determined whether p73 expression induced by cisplatin treatment enhances the ZNF143 binding to the promoter of these DNA repair genes. We have previously shown that ZNF143 expression was also induced by cisplatin treatment as well as p73 (Ishiguchi *et al.*, 2004; Uramoto *et al.*, 2002). Thus, we employed the stable transfectant for ChIP assay to avoid the effect of the enhanced expression of endogenous ZNF143 by cisplatin treatment on the binding to the promoters. We assessed the effect of cisplatin treatment on p73 expression in PC3 cells. p73 expression by cisplatin treatment was substantially increased relative to untreated control cells (data not shown).

ChIP assay demonstrated that the promoter sequence of both DNA repair genes was concentrated in the immunocomplexes prepared after cisplatin treatment (Figure 7b, lanes 5 and 6). Collectively, these results show that the expression of two DNA repair genes is mediated, at least in part, by ZNF143 binding stimulated by p73 expression after cisplatin treatment.

Discussion

We have previously reported that ZNF143 is induced by cisplatin treatment and that it binds preferentially to cisplatin-modified DNA (Ishiguchi *et al.*, 2004), suggesting that it plays an important role in cisplatin resistance. In the present study, we found that ZNF143 interacts with p73 and is directly involved in cisplatin sensitivity through the regulation of DNA repair gene expression.

ZNF143 is overexpressed at both mRNA and protein levels in cisplatin-resistant cells (Figure 1). Interestingly, an increase in ZNF143 protein was observed when the total nuclear fraction of cisplatin-resistant cells was analysed (Figure 1b), but not when nuclear protein eluted with salt buffer was loaded (data not shown). This indicates that ZNF143 binds tightly to cisplatin-modified chromatin and could not be eluted easily under low salt condition.

Functional analysis of ZNF143 provides considerable insight into the epigenetics of cisplatin-resistance and might be of use in revealing targets for overcoming drug resistance. ZNF143 depletion using siRNA confers cell sensitivity 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). It is noteworthy that ZNF143 does not affect cellular sensitivity to oxaliplatin, which is a third-generation platinum drug that has shown a lack of cross-resistance with cisplatin (Raymond *et al.*, 2002). We previously demonstrated that etoposide can induce ZNF143 expression (Ishiguchi *et al.*, 2004), but the current results suggest that upregulation of ZNF143 by etoposide treatment is not directly involved in etoposide sensitivity. ZNF143 might, however, be involved specifically in DNA repair following DNA damage by cisplatin.

Co-immunoprecipitation assay showed that the tumor suppressor gene product p73 interacts with ZNF143. We previously reported that p53 interacts with high mobility group box 1 (HMGB1) and stimulates the binding of HMGB1 to cisplatin-modified DNA (Imamura *et al.*, 2001). We therefore investigated whether p73 plays a similar role and found, using EMSA, that p73 enhances the cisplatin-modified DNA binding of ZNF143 (Figure 4b). We could not detect a p73 supershift, suggesting that although p73 stimulates ZNF143 binding to cisplatin-modified DNA, it cannot interact stably during electrophoresis. p73 overexpression is associated with resistance to DNA-damaging agents (Vikhanskaya *et al.*, 2001), so both ZNF143 and p73 might be cooperatively involved in cisplatin resistance. p73 also enhances the ZNF143 binding to its binding site located in the promoter region of human *U6 RNA* gene. As both p73 and ZNF143 expression are induced by DNA damage signal, p73 might function cooperatively to activate the ZNF143 target gene expression. Little is known about potential ZNF143 target genes for DNA repair pathways. Among several DNA repair pathways, it has been extensively studied that excision repair cross-complementation group 1 (ERCC1) has the critical role in nucleotide excision repair pathway and high ERCC1

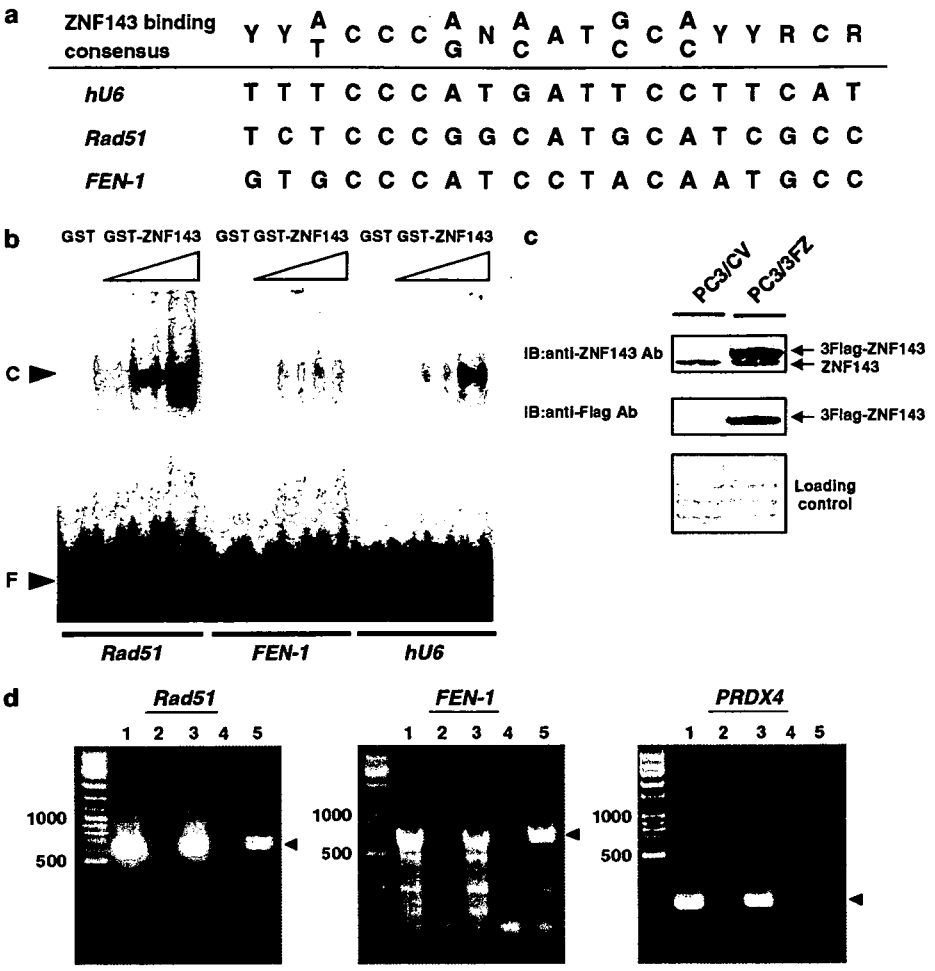


Figure 6 ZNF143 binds to ZNF143 binding sites of DNA repair genes promoter *in vitro* and *in vivo*. (a) Schematic representation of ZNF143 binding sites. ZNF143 binding sites in human *U6 RNA* promoter (*hU6*), human *Rad51* promoter and human *FEN-1* promoter were compared with ZNF143 binding consensus motif (Schaub *et al*., 1997). (b) GST-ZNF143 binding to ZNF143 binding site of both *Rad51* and *FEN-1* gene promoters *in vitro*. Purified GST (500 ng) or GST-ZNF143 (50, 250 and 500 ng) were incubated with ^{32}P-labeled oligonucleotides containing ZNF143 binding sites. The reaction mixtures were resolved by electrophoresis and analysed by a bio-imaging analyzer. (c) Cloning of stable transfectants. Whole-cell lysates (50 μg) of stable transfectant PC3/control vector (PC3/CV) and PC3/3 × Flag-ZNF143 (PC3/3FZ) were subjected to SDS–PAGE. Transferred membrane was blotted with anti-ZNF143 (upper panel) and anti-Flag (middle panel) antibodies. Gel staining with CBB was also shown (lower panel). (d) ZNF143 binding to the promoter *in vivo*. ChIP assay of the PC3/control vector (lanes 1 and 2) and PC3/3 × Flag-ZNF143 (lanes 3–5) was performed with antibodies against Flag (M2) or mouse IgG. Immunoprecipitated DNAs (anti-Flag (M2) in lanes 2 and 5, and anti-mouse IgG in lane 4) and pre-immunoprecipitated DNA (lanes 1 and 3) were amplified by PCR using specific primer pairs for the *Rad51*, *FEN-1* and *PRDX4* promoter regions. Amplification products (682 bp for *Rad51*, 844 bp for *FEN-1* and 157 bp for *PRDX4*) were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The arrowhead indicates amplified PCR fragment containing the promoter region of gene.

expression is associated with cisplatin resistance (Altaha *et al*., 2004). Both BRCA1 and Rad51 have been shown to be involved in recombinational repair and also associated with cisplatin resistance (Bhattacharyya *et al*., 2000; Spiro and McMurray, 2003). The FEN-1 is a 5′ endonuclease and has been implicated in various DNA repair processes (Lieber, 1997). Based on these reports, we searched putative ZNF143 binding site in the promoter region of these DNA repair genes and found that putative ZNF143 binding sites are located in the core promoter region of these genes. We confirmed the role of ZNF143 in the regulation of both *Rad51* and *FEN-1* gene expression by three independent approaches: siRNA strategy, EMSA and ChIP assay as shown in Figures 5b, 6b and d, respectively. In addition, p73 transactivated the promoter activities of two DNA repair genes (Figure 7a) and cisplatin treatment resulted in the enhanced binding of ZNF143 to these promoters (Figure 7b). Thus, p73 interacts with ZNF143 and modulates its function, and therefore has the potential to broadly regulate the DNA repair gene expression.

We also carried out a search of the sequence database to identify the distribution of the ZNF143 binding sites of all DNA repair genes. Surprisingly, we found that a