Table 6. Mitogenic and survival signal regulators, integrins, transcription factors and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
ERBB2	–	R, NC	S	CDDP, PTX	Yes (lung, DOX)	10, 22, 186–191
EGFR	–	R	–	DOX	No (lung, CDDP, DOX, PTX)	10, 22, 112, 192
KRAS2	–	R*	–	CDDP	–	193
HRAS	–	R*, NC	–	Ara-C, DOX, PTX	No (lung, DOX)	10, 193–197
RAF1	–	R	–	DOX	–	198
AKT1	–	NC, R	S	CDDP, DOX, PTX	–	199–201
AKT2	–	R	S	CDDP	–	200, 202
ITGB1	–	–	S	ETP, PTX	–	203, 204
JUN	–	R	–	CDDP	No (lung, DOX)	10, 205
FOS	U	R	S	CDDP	No (lung, DOX)	10, 206–208
MYC	NC, U	S, R	R, S, NC	CDDP, DOX	No (lung, DOX)	10, 209–216
NFKB1	U	–	S	5-FU, DOX, ETP	–	217–222

Alterations in drug-induced resistance cells (DIRC): NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs)): NC, no change; R, resistant; S, sensitive. Drugs: Ara-C, 1-beta-D-arabinofuranosylcytosine; CDDP, cisplatin; DOX, doxorubicin; ETP, etoposide; PTX, paclitaxel; 5-FU, 5-fluorouracil.
*Up-regulated with mutated K-ras gene.

Table 7. Apoptosis regulators and in vitro evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
TP53	–	S, R*	R, S	CDDP, DOX	Yes (brain)	223–229
					Yes (NCI-panel)	230
					No (breast, DOX)	231
					No (breast, DOX, PTX)	232
					No (lung, PTX)	22
MDM2	–	S, R	S	CDDP, DOX, PTX	–	169, 233–238
TP73	–	–	R	CDDP, ETP	–	239, 240
BCL2	U, D	R	–	CDDP, CPT, DOX	Yes (breast, DOX)	164, 198, 231, 241–244
					Yes (lung, PTX)	22
					No (breast, DOX)	232
BCL2L1	NC	R	S	CDDP, PTX	–	243–251
MCL1	–	–	S	DTIC	–	252
BAX	NC	S	R	CDDP, ETP, 5-FU	No (breast, DOX)	231, 244, 253–260
					No (lung, PTX)	22
BIRC4	–	NC	S	PTX	–	261, 262
BIRC5	–	R	S	CDDP, ETP	–	263–265
TNFRSF6	NC	–	S	CDDP	Yes (lung, DOX)	10, 242
CASP3	–	S	–	CDDP, DOX, ETP	No (lung, DOX)	10, 266–268
CASP8	–	–	R	CDDP	–	261
HSPB1	C	R	S	DOX	–	52, 269–273

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): NC, no change; R, resistant; S, sensitive. Drugs: CDDP, cisplatin; CPT, irinotecan; DOX, doxorubicin; DTIC, dacarbazine; ETP, etoposide; PTX, paclitaxel; 5-FU, 5-fluorouracil.
*Resistant in mutant *TP53* over-expressed cells.

Table 8. Gene categories and association with in vitro chemosensitivity

Category	No. of genes	Total no. of studies	No. of studies showing association (%)
Transporter	15	13	7 (54)
Drug target	8	5	3 (69)
Target associated protein	7	0	0 (0)
Intracellular detoxifier	7	6	6 (100)
DNA repair	10	3	2 (67)
DNA damage recognition protein	2	0	0 (0)
Cell cycle	6	5	3 (60)
Mitogenic signal	5	3	1 (33)
Survival signal	2	0	0 (0)
Transcription factor	4	3	0 (0)
Cell adhesion-mediated drug resistance protein	1	0	0 (0)
Apoptosis	13	12	5 (42)
Total	80	50	22 (44)

but not selected in the current study, because they have never caught the scientific eye for some reasons. Thus, the results of this study may be significantly influenced by publication bias. Nonetheless, we do believe that these genes have been selected reasonably carefully, and that they may be helpful for establishing a clinical predictive chemosensitivity test.

While the association between alterations of the 80 genes and the chemosensitivity of various cell lines was evaluated in 50 studies, significant association was observed in only 22 (44%) (Table 8). The cellular functions of a gene vary among cell types and experimental conditions. The evaluation of the gene functions, however, was conducted under only limited cellular contexts in these studies, as expected. Thus, for example, the conditions of a gene transfection experiment may differ from those of an experiment to evaluate the chemosensitivity for many cell lines. The gene functions may not necessarily be examined under all possible conditions, but the evaluation must be conducted under conditions similar to those in the clinical setting in order to develop clinical chemosensitivity testing using these genes.

The other possibility for the poor correlation to *in vitro* chemosensitivity may be that more than one gene alterations are involved in the chemosensitivity of tumors. This may be discussed from the standpoint of the signal transduction pathway and from the cellular standpoint. From the standpoint of the signal transduction pathway, more than one gene may be involved in the reaction to a cytotoxic agent. One of the best examples is cooperation of *TP53* with another

member of the p53 family, p73 (*TP73*), in the response to both DNA damage and chemosensitivity (3,4). From the cellular standpoint, several pathways may work additively, antagonistically, or complementally in determining the chemosensitivity of the cell. This can be understood well from the context of induction and inhibition of apoptosis being controlled by pro-apoptotic and anti-apoptotic pathways. Thus, it would be important to study several pathways at the same time, or to evaluate the net effect of the involvement of various pathways.

Complex factors influencing the cellular chemosensitivity may be operative on a tumor *in vivo*, in such a way that the tumor may exhibit highly heterogeneous gene alterations; that the tumor cells may interact with various host cells, including immune cells, fibroblasts and vascular endothelial cells; and that the differences in the distance between each tumor cell and blood vessels may affect the exposure level of tumor cells to a drug. No systematic approach has been developed to include this complex interplay of factors in the study of cellular chemosensitivity, although studies on cell adhesion-mediated drug resistance may be partly helpful.

Among the six genes for which the association was shown in two or more *in vitro* studies, four encode classical drug resistance proteins which are known to inhibit the drug–target interaction. These proteins are relatively specific for the drug as well as the cell type; e.g. *TYMS* is critical for 5-fluorouracil sensitivity. Thus, *TYMS* is a good candidate for chemosensitivity testing in patients with colorectal cancer who are treated with 5-fluorouracil (Table 2). *MVP* is involved in the transport of doxorubicin, therefore, it would be of interest to examine the association between the expression of *MVP* and the drug response in patients with breast cancer; the association of *MVP* with chemosensitivity has been evaluated only for brain tumor and lung cancer cell lines, to date (Table 1). However, the remaining two of the six genes, *TP53* and *BCL2*, are associated with apoptosis, and therefore may be relatively cell-type specific. Since all the three *in vitro* studies using breast cancer cell lines failed to show any associations between alterations of these genes and the chemosensitivity, the association should be evaluated in other tumor types in the clinical setting (Table 7).

The recently developed cDNA microarray technique allows analysis of the mRNA expression of more than 20 000 genes at once, and as many as 100–400 genes have been statistically shown as potentail chemosensitivity-related genes in various studies (5–7). The 80 genes in the current study were selected theoretically based on their known functions, and their contribution to *in vitro* chemosensitivity was shown in the experiments. Thus, it would be of interest to evaluate the expression profiles of these genes by cDNA microarray analysis, even if the difference in expression between sensitive and resistant cell lines does not reach statistical significance.

In conclusion, 80 *in vitro* chemosensitivity associated genes were identified from a review of the literature, which

may be considered to be future candidates for clinical predictive chemosensitivity testing.

Acknowledgments

Conflict of interest statement

None declared.

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Detection of Epidermal Growth Factor Receptor Mutation in Transbronchial Needle Aspirates of Non-Small Cell Lung Cancer*

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Background: Somatic mutations of epidermal growth factor receptor (EGFR) are closely associated with an objective response to *EGFR* tyrosine kinase inhibitors. However, it is difficult to obtain sufficient tumor samples from patients with non-small cell lung cancer (NSCLC), so these diagnoses are often made using cytology procedures alone. The aim of this study was to detect *EGFR* mutations in transbronchial needle aspiration (TBNA) samples using both direct sequencing and a highly sensitive assay (Scorpions Amplified Refractory Mutation System; DxS; Manchester, UK) [ARMS], and to compare the sensitivity of these methods.
Methods: We enrolled 94 patients (63 men and 31 women) with NSCLC in this study. Cytologic diagnoses were adenocarcinoma (n = 58), squamous cell carcinoma (n = 24), and other types of NSCLC (n = 12). We extracted DNA from the TBNA samples, and *EGFR* mutations were analyzed using both direct sequencing (exons 19 and 21) and the Scorpions ARMS method (E746 A750del and L858R).
Results: Mutations were detected in 31 patients (33%; 14 women and 17 men). Of these, 23 patients had adenocarcinoma, 4 had squamous cell carcinoma, and 4 had other types of NSCLC. Direct sequencing detected 13 mutations (14%) in 13 patients (E746-A750del, n = 6; L858R, n = 7), and the Scorpions ARMS method detected 27 mutations (29%) in 27 patients (E746 A750del, n = 16; L858R, n = 11 patients).
Conclusions: Both methods detected *EGFR* mutations in TBNA samples, but Scorpions ARMS is more sensitive than direct sequencing.   (*CHEST 2007; 131:1628–1634*)

Key words: epidermal growth factor receptor; epidermal growth factor receptor mutation; epidermal growth factor receptor tyrosine kinase inhibitor; Scorpions Amplified Refractory Mutation System; transbronchial needle aspiration

Abbreviations: ARMS = Amplified Refractory Mutation System; Ct = cycle threshold; EGFR = epidermal growth factor receptor; NSCLC = non-small cell lung cancer; PCR = polymerase chain reaction; TBLB = transbronchial lung biopsy; TBNA = transbronchial needle aspiration

L ung cancer is among the most common malignancies worldwide and one of the few types of cancer with an increasing incidence. Advanced non-small cell lung cancer (NSCLC) is treated with a combination of chemotherapy and radiotherapy, but

the outcome remains poor. Gefitinib and erlotinib are inhibitors of the tyrosine kinase activity of epi-

dermal growth factor receptor (EGFR), and have recently been used to treat advanced NSCLC.[1] These agents are dramatically effective in some

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patients yet completely ineffective in others. The response rate to gefitinib is high among individuals with an Asian background.[2]

In May and June of 2004, two independent groups reported an association between somatic EGFR mutations and a dramatic clinical response to gefitinib, respectively.[3,4] Thereafter, EGFR mutations were extensively investigated.[5–17] The mutations consist of small, in-frame deletions or substitutions clustered around the adenosine triphosphate-binding site in exons 18, 19, and 21 of the EGFR gene, and approximately 90% of patients with EGFR mutations have one of two major mutations. One is a 15-base pair nucleotide in-frame deletion (E746 A750del) in exon 19, and the other is a point mutation involving the replacement of leucine with arginine at codon 858 (L858R) in exon 21.[18] The above studies included genetic analyses of surgical tissues or biopsy specimens. However, to obtain sufficient amounts of tumor samples from inoperable NSCLC patients is often difficult. Some studies[19,20] of patients with advanced NSCLC have found a correlation between clinical manifestations and EGFR mutation status obtained from small tumor samples, such as those obtained using standard transbronchial lung biopsy (TBLB). All of the above studies are limited by the fact that the rate of usable samples obtained from enrolled patients is very low. Therefore, a method is required to detect mutant EGFR, especially the two major mutations, using samples other than surgical tissues from NSCLC patients. We addressed this problem using a sensitive technique for actual tumor sampling, and a highly sensitive assay for detecting EGFR mutations.

Pulmonary lesions are most often clinically diagnosed using flexible bronchoscopy. Common bronchoscopic sampling techniques used for pulmonary lesions are transbronchial needle aspiration (TBNA) and TBLB. One report has indicated that TBNA is superior to TBLB in diagnosing pulmonary lesions: Gasparini et al[21] found that the diagnostic sensitivity of these techniques is 50.0% for TBLB, 70.1% for TBNA, and 76.0% for TBLB and TBNA together. We thus presumed that TBNA is a highly sensitive means of tumor sampling, and that DNA obtained from such specimens might provide useful information about the mutation status of the EGFR gene.

We postulated that Scorpions Amplified Refractory Mutation System (ARMS) [DxS; Manchester, UK] technology would enhance the sensitivity of detecting EGFR mutations. Scorpion primers are used with a fluorescence-based method that specifically detects polymerase chain reaction (PCR) products.[22] A "scorpion" consists of a specific probe sequence held in a hairpin loop configuration by complementary stem sequences on the 5′ and 3′ ends of the probe. A scorpion can be combined with ARMS to enable the detection of single-base mutations.[22,23] The ARMS method is used for allele discrimination, and additional mismatches have been introduced near the 3′ termini of the primers to enhance specificity. The ARMS method is superior to both direct sequencing and the WAVE method (Transgenomic; Omaha, NE) for detecting EGFR mutations.[24] Here, we aimed to detect major EGFR mutations in TBNA specimens and to verify the sensitivity of these methods for detecting EGFR mutations.

MATERIALS AND METHODS

Patients

We studied patients with NSCLC diagnosed using specimens obtained by TBLB and/or TBNA. Tumors in saline solution were not collected from enlarged lymph nodes only. After obtaining written informed consent from the patients to participate in all study protocols approved by the Institutional Review Board of the Cancer Institute Hospital, tumor tissues, tumors in saline solution obtained using TBNA, and clinical data were collected. We recorded age at diagnosis, gender, cytologic diagnosis of NSCLC, clinical stage, and smoking status. Cytologic diagnoses were based on the World Health Organization pathology classification. Clinicopathologic staging was determined according to the International Union Against Cancer TNM classification of malignant tumors. Nonsmokers were defined as those who had smoked < 100 cigarettes in their lifetime. We obtained detailed information about smoking history, including age at first cigarette, packs per day, and number of smoking and smoke-free years (after quitting). Patients were categorized as follows: never smoked (< 100 lifetime cigarettes), former smokers (quit ≥ 1 year ago), or current smokers (quit < 1 year ago).

TBNA Sampling

Four experienced operators performed standard flexible bronchoscopy (Olympus P260F; Olympus; Tokyo, Japan) using 21-gauge cytology needles and aspirated for 10 s in the standard fashion.[25] Paired samples consisted of two aspirates that were obtained in immediate succession in an identical manner, with the needle insertion points ideally 1 mm apart. At least four aspirates (two pairs) were obtained from each site. For cytologic analysis, the aspirate was immediately placed onto a glass slide, covered with a second slide, and the slides were drawn apart under continuous gentle pressure. The smear was spray-fixed using ethanol, processed routinely and visualized by Papanicolaou staining. The second aspirate was mixed into 2 mL of saline solution and stored at − 80°C until DNA extraction.

DNA Extraction

Samples obtained by TBNA in saline solution were digested with proteinase K, and then DNA was extracted with phenol-

chloroform and precipitated with ethanol. Precipitated DNA was eluted in 50 μL of sterile, double-distilled water. The concentration and purity of the extracted DNA were determined by spectrophotometry and then the DNA was stored at −20°C.

PCR Amplification and Direct Sequencing

Genomic PCR was performed in 25-μL volumes using 50 ng of template DNA, 0.75 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer; Roche Molecular Systems; Branchburg, NJ), 2.5 μL of PCR buffer (Perkin-Elmer), 0.8 μmol/L deoxynucleotide triphosphate (Perkin-Elmer), 0.5 μmol/L of each primer, and various concentrations of MgCl₂, depending on the polymorphic marker. Exons 19 and 21 were amplified by nested PCR. Primer sequences were obtained as described by Lynch et al.[3] Initial PCR analyses proceeded in a volume of 25 μL as follows: 35 cycles of denaturation at 94°C for 45 s, primer annealing at 58°C for 30 s, and elongation at 72°C for 30 s. A final extension proceeded at 72°C for 10 min. Nested PCR was performed using 20 cycles under the same conditions as the initial PCR. The bands of PCR products were visualized using a 2100 bioanalyzer and the DNA 500 Labchip kit (Agilent Technologies; Palo Alto, CA). Each sample was sequenced in duplicate in both forward and reverse directions using the BigDye Terminator kit (Applied Biosystems; Foster City, CA) and an ABI prism 310 (Applied Biosystems) according to manufacturer instructions. The sequences were then compared with the GenBank-archived human sequence for EGFR (accession number AY588246).

Scorpions ARMS for the Detection of E746 A750del and L858R

We used the EGFR Scorpions kit, which combines two technologies, namely ARMS and Scorpions, to detect mutations in real-time PCR reactions. All reactions proceeded in 25-μL volumes using 1 μL of template DNA, 7.5 μL of reaction buffer mix, 0.6 mL of primer mix, and 0.1 mL of Taq polymerase. Real-time PCR was performed using a SmartCycler II (Cepheid; Sunnyvale, CA) under the following conditions: initial denaturation at 95°C for 10 min, 50 cycles of 95°C for 30 s, and 62°C for 60 s with fluorescence reading (set to FAM, which allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. Data were analyzed using Cepheid SmartCycler software (Version 1.2b). The cycle threshold (Ct) was defined as the cycle at the highest peak of the second derivative curve that represented the point of maximum curvature of the growth curve. Both Ct and maximum fluorescence were used for interpretation of the results. Positive results were defined as Ct ≤ 45 and maximum fluorescence intensity ≥ 30. When only the curve that indicated the wild-type increased, the sample was considered wild-type with respect to EGFR. When both wild- and mutant-type curves increased, the sample was considered mutant-type with respect to EGFR. These analyses were performed in duplicate for each sample.

Statistical Analysis

The rates of EGFR mutation between the two groups were compared using χ^2 or Fisher exact tests. The latter test was applied to five or fewer observations in a group. We used logistic regression models to further explore observed differences and to identify baseline factors that might independently predict an EGFR mutation. Probability values of < 0.05 were defined as being statistically significant. All statistical tests were two sided.

RESULTS

Patient Characteristics

Ninety-four patients were enrolled in this study (63 men and 31 women; median age, 66 years) [Table 1]. Among these, 58 patients had adenocarcinoma, 24 patients had squamous cell carcinoma, 5 patients had large cell carcinoma, 2 patients had other classifications of NSCLC, and 5 patients had unclassified NSCLC. Disease in 70 patients was diagnosed from both TBNA and TBLB samples, disease in 23 patients was diagnosed using only TBNA samples, and disease in 1 patient was diagnosed using TBLB samples alone (Table 2). The DNA from TBNA samples in all 93 patients was extracted at a median concentration of 8.7 ng/μL (range, 0.1 to 39.0 ng/μL).

Detection of EGFR Mutations Using Direct Sequencing

We performed direct sequencing in all patients. We could determine EGFR mutation status using direct sequencing in samples from 83 patients. We could not evaluate the mutation status of the other 10 patients because we did not obtain sufficient PCR products; bands were undetectable for these 10 patients. In 13 of the 83 patients (15.7%), EGFR mutations were detected using direct sequencing. All 13 were heterozygous. E746 A750del was detected in five patients, E746 A752del insA was detected in

Table 1—*Patient Characteristics*

Characteristics	No.	EGFR Mutation, No. (%)
Patients	94	31 (33.0)
Gender		
Male	63	17 (27.0)
Female	31	14 (45.1)
Age, yr		
Mean	67	
Range	26–86	
Stage		
I	44	11 (25.0)
II	3	0 (0)
III	28	13 (46.4)
IV	15	6 (40.0)
Recurrence after surgery	4	1 (25.0)
Cytologic diagnosis		
Adenocarcinoma	58	23 (39.7)
Squamous cell carcinoma	24	4 (16.7)
Large cell carcinoma	5	0 (0)
Other	2	1 (50.0)
Unclassified	5	3 (60.0)
Smoking history		
Current	26	7 (26.9)
Former	34	10 (29.4)
Never	34	14 (41.2)

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Table 2—*Diagnostic Yield of Different Bronchoscopic Sampling Techniques**

TBLB	TBNA	
	Positive	Negative
Positive	70 (74.4)	1 (1.1)
Negative	23 (24.5)	

*Data are presented as No. (%).

one patient, and L858R was detected in seven patients. E746 A750 deletion and L858R substitution mutations were frequent (12 of 13 patients with detectable *EGFR* mutations; 92.3%). Figure 1 shows the results of direct sequencing in a patient with E746 A750del (patient 50; Fig 1, *top*, A), and a patient with L858R (patient 70; Fig 1, *bottom*, B). None of the patients had more than one mutation.

Mutation Analysis Using the Scorpions ARMS Method

We performed Scorpions ARMS in all patients. We could analyze *EGFR* mutation status of 91 patients using the *EGFR* Scorpions kit. Because curves corresponding to neither the wild-type nor the mutant-type were detectable in two patients, we could not determine their *EGFR* mutation status. NSCLC was diagnosed in another patient with

TBLB alone. Curves corresponded to *EGFR* mutations in 27 patients, indicated the E746 A750del in exon 19 in 16 patients, and indicated L858R in exon 21 in 11 patients (Fig 2).

Comparison of the Two Methods for Detecting the Two Major Mutations

EGFR mutations were detected in 31 patients (Table 3). Both methods together could determine mutation status in 9 patients, whereas either Scorpions ARMS or direct sequencing could do so in 18 patients and 4 patients, respectively. The *EGFR* mutations were more frequently detected by the Scorpions ARMS method than by direct sequencing (Table 4).

EGFR Mutation Status and Clinical Manifestations

The frequency of *EGFR* mutations was higher in patients with adenocarcinomas (23 of 58 patients, 39.7%; vs 8 of 36 patients, 22.2% in nonadenocarcinomas), women (14 of 31 patients, 45.2%; vs 17 of 62 patients, 27.4% in males), and nonsmokers (14 of 34 patients, 41.2%; vs 17 of 59 patients, 28.8% of current or former smokers), although the differences were not statistically significant. The *EGFR* status detected by direct sequencing alone was not statistically correlated with cytologic diagnosis, gender, or response to gefitinib (data not shown).
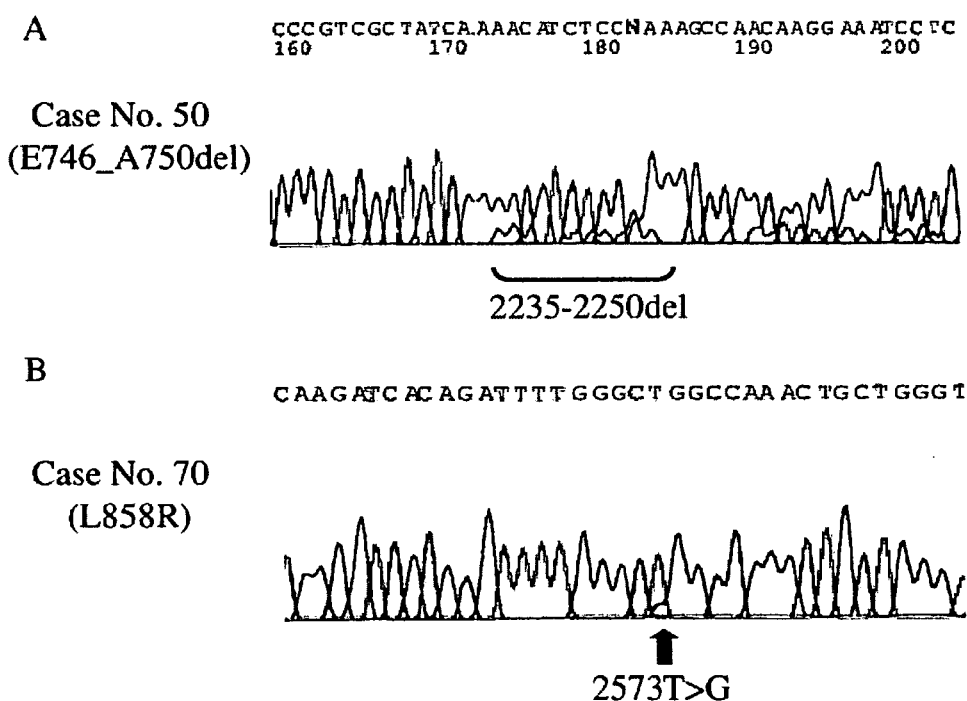
A

CCC GT CGC T A7CA.AAAC AT CT CCNA AAGCC AAC AAGG AA A7CC 7C
160   170   180   190   200

Case No. 50
(E746_A750del)

2235-2250del

B

C AAG A7C AC AG AT T T T G GGCT GGCCAA AC 7G CT GGG T

Case No. 70
(L858R)

2573T>G

FIGURE 1. Wave figures generated by direct sequencing. *Top*, A: E746 A750 del in exon 19. *Bottom*, B: L858R in exon 21. All mutations were confirmed bidirectionally with forward and reverse sequencing.
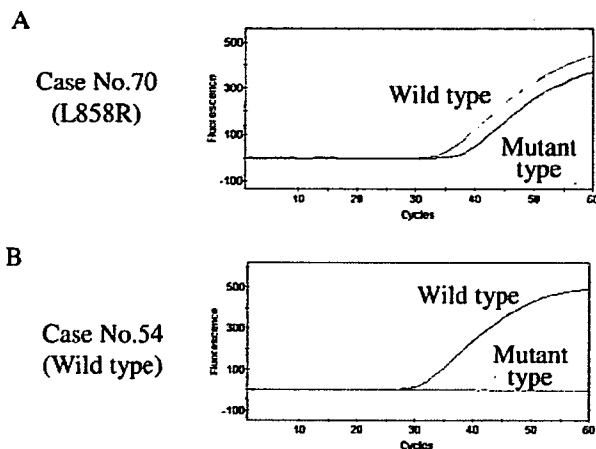
A

Case No.70
(L858R)

B

Case No.54
(Wild type)

FIGURE 2. Curves for exon 21 using the Scorpions ARMS method. Top, A: L858R. Bottom, B: wild-type. Top, A: Curves for both wild-type and mutant-type have increased, so this sample was considered mutant-type with respect to EGFR. Bottom, B: Only one curve indicating the presence of wild-type has increased, so the sample was considered wild-type with respect to EGFR.

Correlation With Responsiveness to Tyrosine Kinase Inhibitors

Only two patients received gefitinib, one of whom was a 63-year-old woman with a cytologic diagnosis of adenocarcinoma who had never smoked (patient 70). She had partially responded to gefitinib administered from September 2005 to August 2006. Her mutation status according to both direct sequencing and the Scorpions ARMS methods was L858R (Table 3). The other patient was a 69-year-old woman with a cytologic diagnosis of adenocarcinoma who had also never smoked (patient 94). Her condition had stabilized in response to gefitinib that had been administered from August 2005 to October 2005. We determined her mutation status as wild-type in exons 19 and 21.

DISCUSSION

We demonstrated the feasibility of detecting EGFR mutations in DNA from TBNA samples from NSCLC patients. Furthermore, we showed that the diagnostic sensitivity of TBNA in our patients was higher than that of TBLB, which agreed with reported findings. The volume of DNA extracted from TBNA samples was measurable by spectrophotometry using our methods and was sufficient to analyze EGFR mutation status. Therefore, TBNA samples are apparently suited to such analysis. The mutation rate in this study was lower (33.3%) than that found by other studies of Japanese NSCLC patients.[11,12] However, in line with previous results, we detected

EGFR mutations at a higher frequency in women, adenocarcinoma patients, and nonsmokers.[6,9] We did not find a relationship between EGFR mutation status and response to EGFR tyrosine kinase inhibitors such as gefitinib. Only two patients had already received gefitinib at the time the study was implemented, and the others were to receive gefitinib as a second-line (or later) treatment. The relationship between EGFR mutation status and response to gefitinib will be determined in the near future.

The results of this study suggest that the Scorpions ARMS method is more sensitive than direct sequencing for detecting the two major EGFR mutations. Direct sequencing is currently the routine method of detecting EGFR mutations in tumor samples, and a standard method for detecting EGFR mutations in tumor specimens other than surgical tissues has been established. Our results indicated that the EGFR Scorpions Kit is superior to direct sequencing for detecting EGFR mutations, especially the major deletion mutations in exon 19 and L858R. We previously showed that EGFR mutation status in serum DNA detected using the Scorpions ARMS method is a useful predictive marker of the response to gefitinib. That study showed that Scorpions ARMS is more sensitive than direct sequencing for detecting EGFR mutations in a mixture of normal and mutant DNA.[26] We inferred from these results that the differences in the determined mutation status for the 18 patients who tested positive using Scorpions ARMS and negative using direct sequencing are due to the density of tumor cells in the sample. However, the reason for the differences in the determined mutation status for those patients who tested negative using Scorpions ARMS and positive using direct sequencing remains obscure. The two methods detected different mutations in the same patient (patient 58), indicating that the primer for the deletion mutation of exon 19 can detect not only E746   A750del but also E746   S752del insA in the Scorpions ARMS method. The differences were frequent in patients with L858R in exon 21 (21.4% of patients with L858R, 5.9% of patients with other mutations). The sensitivity of Scorpions ARMS for detecting L858R was approximately equivalent to that for the detection of E746   A750del in our previous study. Some reports[19,27] have indicated that the presence of EGFR gene amplification is more predictive of responses than EGFR mutation. However, this does not alter the fact that an EGFR mutation is one predictor of response. To detect EGFR gene amplification from cytology samples is complicated by the difficulty of defining fluorescent in situ hybridization. Because there were few cancer cells in cytology samples, and these samples did not yield interpretable signals (data not shown).

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Table 3—EGFR Mutation Status and Characteristics of Patients With Mutations*

Patient No.	Cytologic Diagnosis	Gender	Age, yr	Smoking History	Mutation Status	
					Direct Sequencing	Scorpions ARMS
50	Ad	Male	63	Former	E746_A750del	E746_A750del
54	Ad	Male	49	Former	E746_A750del	E746_A750del
58	Ad	Male	57	Former	E746_S752del insA	E746_A750del
87	Ad	Female	75	Never	E746_A750del	E746_A750del
91	Ad	Female	69	Never	E746_A750del	E746_A750del
47	Ad	Male	74	Former	E746_A750del	Wild-type
12	NS	Male	86	Former	Wild-type	E746_A750del
22	Ad	Male	67	Current	Wild-type	E746_A750del
28	Ad	Female	56	Current	Wild-type	E746_A750del
40	Ad	Male	52	Current	Wild-type	E746_A750del
43	Sq	Male	70	Former	Wild-type	E746_A750del
44	Ad	Female	72	Never	Wild-type	E746_A750del
49	Ad	Male	73	Former	Wild-type	E746_A750del
67	Ad	Male	76	Former	Wild-type	E746_A750del
77	NS	Male	62	Current	Wild-type	E746_A750del
79	Ad	Female	66	Never	Wild-type	E746_A750del
92	NS	Male	68	Current	Wild-type	E746_A750del
4	Ad	Female	55	Never	L858R	L858R
70	Ad	Female	63	Never	L858R	L858R
82	Ad	Male	50	Current	L858R	L858R
89	Ad	Female	55	Never	L858R	L858R
56	Sq	Male	55	Former	L858R	Wild-type
61	Ad	Female	71	Never	L858R	Wild-type
62	Sq	Female	73	Never	L858R	Wild-type
6	Ot	Male	26	Never	Wild-type	L858R
10	Ad	Female	73	Never	Wild-type	L858R
15	Ad	Female	73	Never	Wild-type	L858R
17	Ad	Male	65	Current	Wild-type	L858R
23	Sq	Male	77	Former	Wild-type	L858R
32	Ad	Female	69	Never	Wild-type	L858R
74	Ad	Female	75	Never	Wild-type	L858R

*Ad = adenocarcinoma; Sq = squamous cell carcinoma; NS = unclassified non-small cell carcinoma; Ot = other classification of non-small cell carcinoma.

Some investigators have tried to improve the sensitivity of detecting EGFR mutations. The novel peptide nucleic acid-locked nucleic acid PCR clamp method[28] and the mutant-enriched PCR assay[29] are both rapid and sensitive. Although the minimum detectable mutation volumes were not evaluated in these studies, the sensitivity of these methods seems to be comparable to that of Scorpions ARMS and thus sufficient for clinical use. Since the Scorpions ARMS method is simple and very fast, it might be suitable for mutation screening. However, one limitation of the EGFR Scorpions kit is that it can detect

Table 4—EGFR Mutation Analysis of Different Genetic Assays*

Variables	E746-A750del	L858R	Total
Direct sequencing	6 (6.5)	7 (7.5)	13 (14.0)
Scorpions ARMS	16 (17.2)	11 (11.8)	27 (29.0)
Total	17 (18.3)	14 (15.0)	31 (33.3)

*Data are presented as No. (%).

only mutations targeted by the designed Scorpions primers. Not all EGFR mutations are found at the two targeted sites, as some are clustered around the adenosine triphosphate-binding site in exons 18, 19, and 21.[3–6,9,10] Minor variations of deletional mutations in exon 19, such as E747 P753del insS and L747 T751del, and point mutations other than L858R cannot be detected using Scorpions ARMS. Although approximately 90% of NSCLC-associated EGFR mutations comprise the two major EGFR mutations,[18] others might be missed using Scorpions ARMS. Moreover, a secondary mutation, a substitution of methionine for threonine at position 790, leads to gefitinib resistance in NSCLC patients with EGFR mutations that are responsive to gefitinib.[30,31] These mutation states may also be critical factors for gefitinib therapy. Scorpions primers need to be designed to detect these mutations, and further study using these primers is required. In conclusion, both direct sequencing and Scorpions ARMS can detect EGFR mutations in DNA extracted from

TBNA samples obtained from NSCLC patients, but the latter method is more sensitive.

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Original Research

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ORIGINAL ARTICLE

Clock and ATF4 transcription system regulates drug resistance in human cancer cell lines

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The mechanisms underlying cellular drug resistance have been extensively studied, but little is known about its regulation. We have previously reported that activating transcription factor 4 (ATF4) is upregulated in cisplatin-resistant cells and plays a role in cisplatin resistance. Here, we find out a novel relationship between the circadian transcription factor Clock and drug resistance. Clock drives the periodical expression of many genes that regulate hormone release, cell division, sleep-awake cycle and tumor growth. We demonstrate that *ATF4* is a direct target of Clock, and that Clock is overexpressed in cisplatin-resistant cells. Furthermore, Clock expression significantly correlates with cisplatin sensitivity, and that the downregulation of either Clock or ATF4 confers sensitivity of A549 cells to cisplatin and etoposide. Notably, ATF4-overexpressing cells show multidrug resistance and marked elevation of intracellular glutathione. The microarray study reveals that genes for glutathione metabolism are generally downregulated by the knockdown of ATF4 expression. These results suggest that the Clock and ATF4 transcription system might play an important role in multidrug resistance through glutathione-dependent redox system, and also indicate that physiological potentials of Clock-controlled redox system might be important to better understand the oxidative stress-associated disorders including cancer and systemic chronotherapy.
Oncogene (2007) **26,** 4749–4760; doi:10.1038/sj.onc.1210289; published online 12 February 2007

Keywords: Clock; ATF4; multidrug resistance; glutathione; chronotherapy

Introduction

Cisplatin is a potent anticancer agent that is used in the treatment of various solid tumors, but the development of resistance is a major obstacle in a clinical setting (Wang and Lippard, 2005). Several mechanisms are involved in the acquisition of cisplatin resistance, including decreased drug accumulations (Komatsu et al., 2000; Nakayama et al., 2002), increased levels of cellular glutathione (Lai et al., 1989; Tew, 1994), and increased DNA-repair activity (Chaney and Sancar, 1996; Husain et al., 1998). We have been interested in the transcription factors activated in response to cisplatin, which might play a crucial role in cisplatin resistance (Kohno et al., 2005; Torigoe et al., 2005). We believe that the transcription factors of genes involved in cisplatin resistance are often overexpressed or activated in cisplatin-resistant cells.

Activating transcription factor 4 (ATF4) is a member of the cyclic adenosine monophosphate responsive element-binding (CREB) protein family, and is involved in multiple intracellular stress pathways (Rutkowski and Kaufman, 2003). ATF4 is ubiquitously expressed in human cancer cells, and is essential for normal cellular proliferation (Fawcett et al., 1999), especially the high-level proliferation required during fetal liver hematopoiesis (Masuoka and Townes, 2002). ATF4-null cells also show impaired glutathione biosynthesis (Harding et al., 2003). We have shown previously that ATF4 is upregulated in cisplatin-resistant cell lines and is involved in cisplatin resistance (Tanabe et al., 2003).

We herein investigate the molecular regulation of *ATF4* gene expression and drug resistance. Interestingly, a database search revealed an E-box in the core promoter region of *ATF4*, and we show that the essential circadian regulator Clock binds to this E-box and is overexpressed in cisplatin-resistant cells. It has been reported previously that Clock/BMAL1 heterodimers activate transcription from E-box elements (Gekakis et al., 1998); therefore, *ATF4* is thought to be regulated by circadian transcription factors. Downregulation of either Clock or ATF4 using small interfering RNAs (siRNAs) was shown to confer cell

sensitivity to anticancer agents. Furthermore, ATF4-overexpressing cells showed multidrug resistance and marked elevation of intracellular glutathione. Knockdown 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
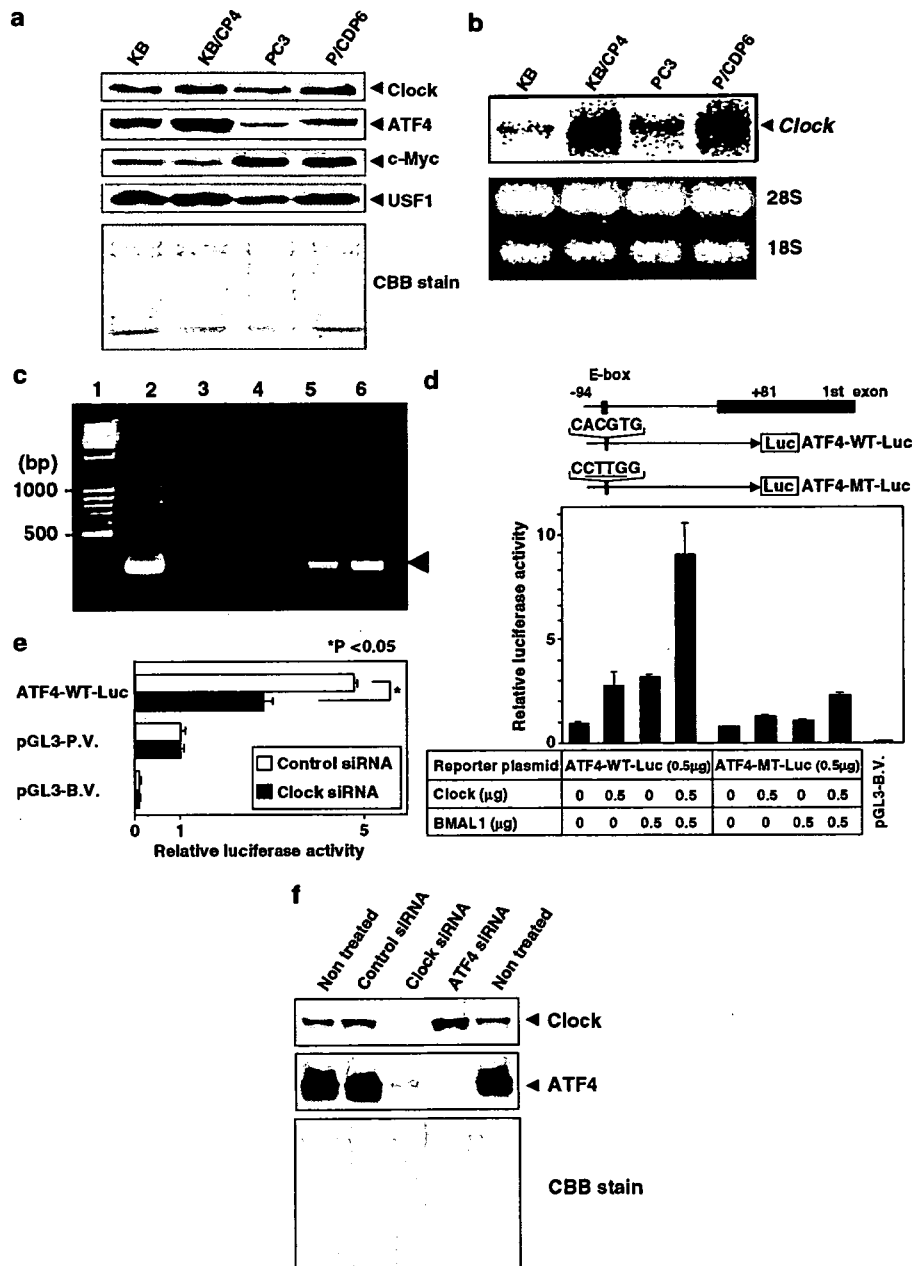
Figure 1 Clock regulates *ATF4* gene expression through binding to the E-box located in the promoter region. (a) Whole-cell extracts (75 μg for Clock, and 50 μg for c-Myc and USF1) and nuclear extracts (100 μg for ATF4) were subjected to SDS–PAGE, and Western blotting analysis was performed with the 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 = ± 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 = ± 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.
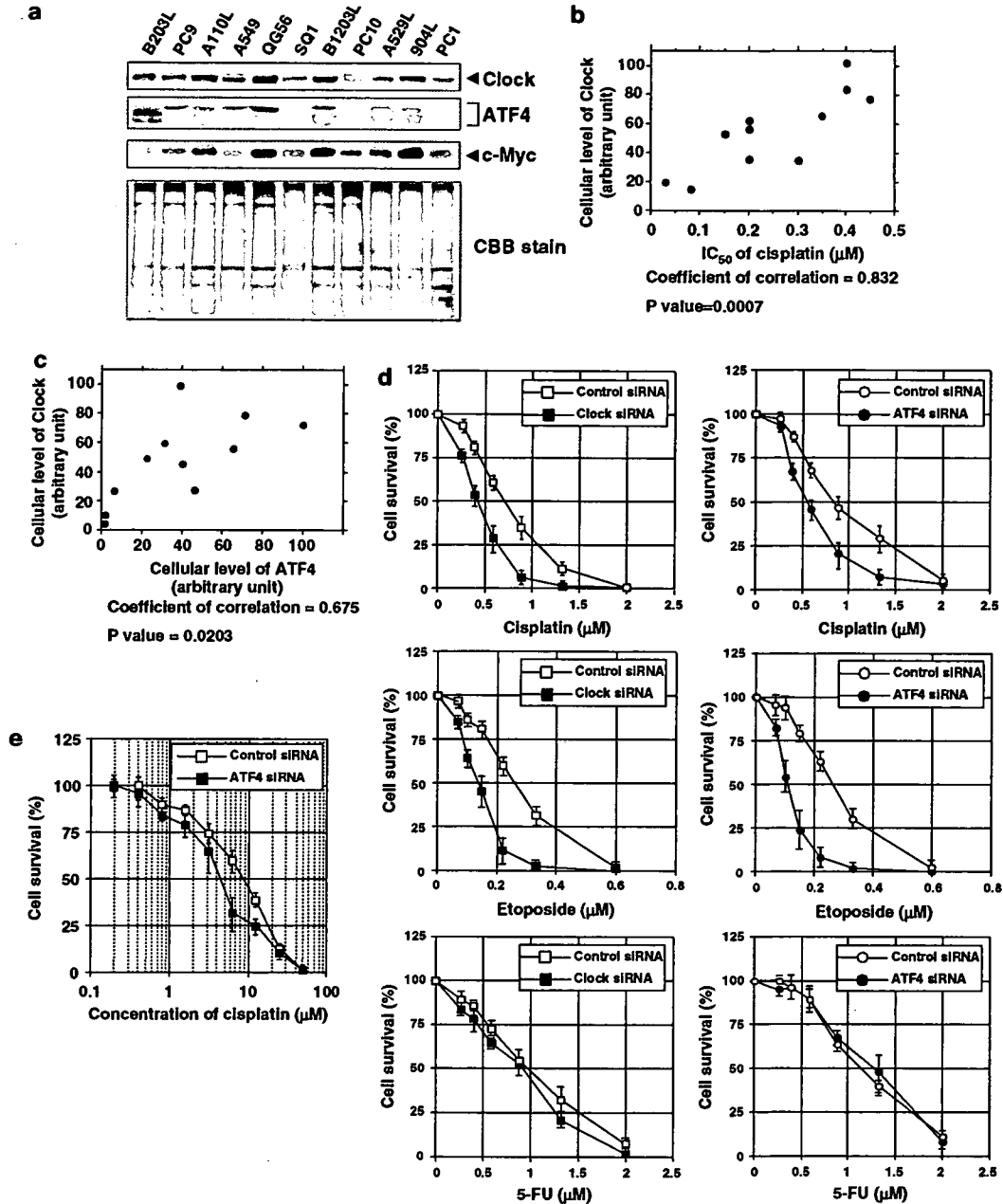
Figure 2 Clock expression correlates with cisplatin resistance and ATF4 expression. (a) Western blotting analysis was performed with 100 μg nuclear extracts for ATF4 expression and 75 μg whole-cell extracts for Clock and c-Myc expressions in 11 human lung cancer cell lines. Gel staining with CBB is also 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 IC₅₀ 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 = ± 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 = ± s.d.

GCLC and GSTπ were upregulated in ATF4-over-expressing 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 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
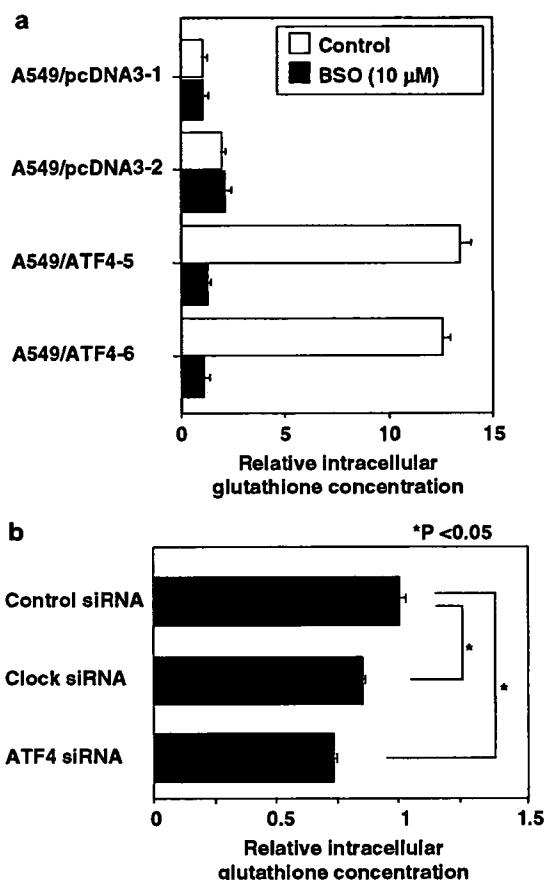
Drug	Cell line		
	A549/pcDNA3[a]	A549/ATF4[b]	Relative resistance[c]
5-FU (μM)	1.38 ($\pm$0.04)	1.35 ($\pm$0.21)	1.0
Cisplatin (μM)	0.67 ($\pm$0.04)	2.11 ($\pm$0.02)	3.1
Doxorubicin (μM)	0.03 ($\pm$0.01)	0.14 ($\pm$0.04)	3.6
Etoposide (μM)	0.39 ($\pm$0.01)	2.02 ($\pm$0.64)	5.2
SN-38 (nM)	5.25 ($\pm$1.06)	19.0 ($\pm$1.41)	3.6
Vincristine (nM)	2.75 ($\pm$0.78)	6.10 ($\pm$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$\pm$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 = $\pm$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
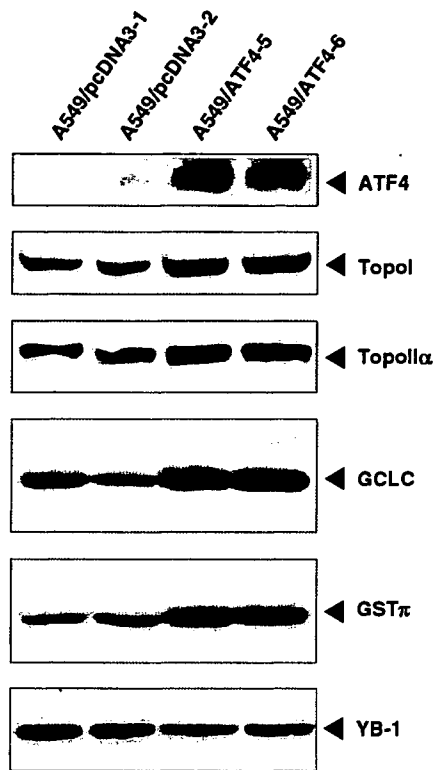
Figure 4 Cellular expressions of drug resistance-related genes in ATF4-overexpressing cells (A549/ATF4-5 and A549/ATF4-6) and control cells (A549/pcDNA3-1 and A549/pcDNA3-2). Whole-cell extracts (75 μg) for GCLC, GSTπ, YB-1 and nuclear extracts (100 μg) for ATF4, TopoI, TopoIIα from ATF4-overexpressing 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 over-

expression 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 over-expressed 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
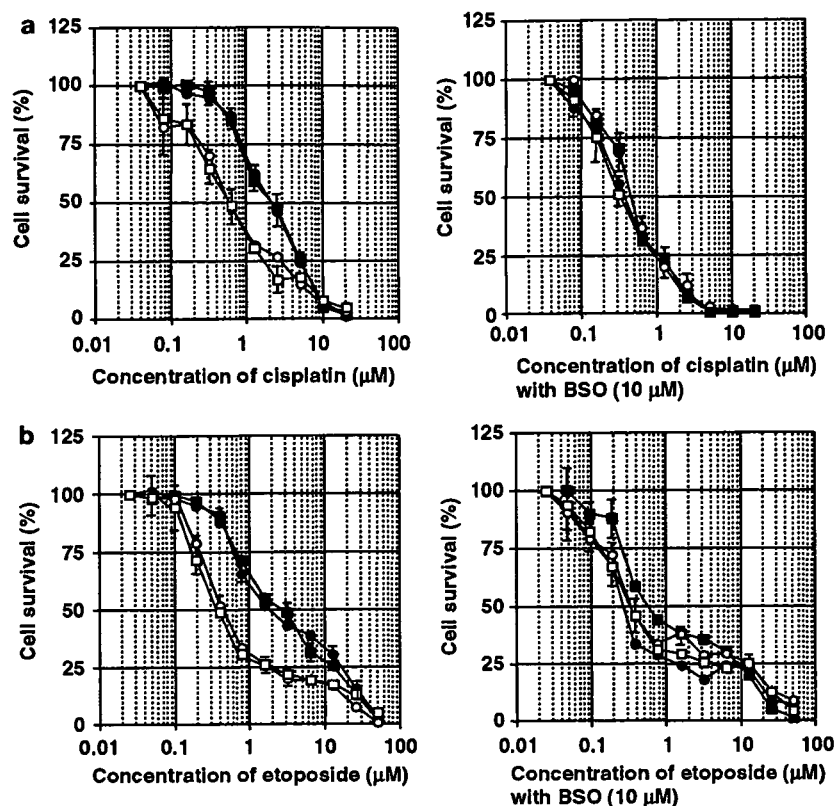
Figure 5 BSO overcomes the drug resistance of cisplatin and etoposide in ATF4-overexpressing cells. ATF4-overexpressing cells (A549/ATF4-5, closed square; A549/ATF4-6, closed circle) and control cells (A549/pcDNA3-1, open square; A549/pcDNA3-2, open circle) were pretreated with or without 10 μM BSO for 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 = ±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;
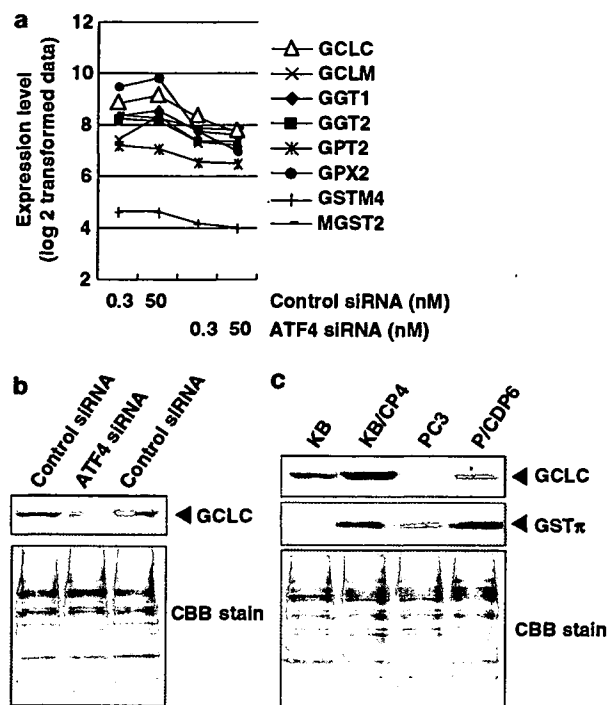
Figure 6 The genes for glutathione metabolism are regulated by ATF4. (a) The graph shows gene expression changes of glutathione metabolism subset mediated by ATF4 siRNA. The data were obtained from normalized and log 2-transformed microarray expression signal intensities. The samples were collected from 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