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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.
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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. Down-regulation 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. Knock-down of ATF4 expression lead to downregulation of glutathione metabolism. Our findings indicate an important contribution of both Clock and ATF4 to chemosensitivity.

Results

Overexpression of Clock in cisplatin-resistant cells

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

ATF4 is a direct target of Clock

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

Cellular expression of Clock correlates with cisplatin sensitivity

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

Multidrug resistance in ATF4-overexpressing cell lines

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

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

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

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

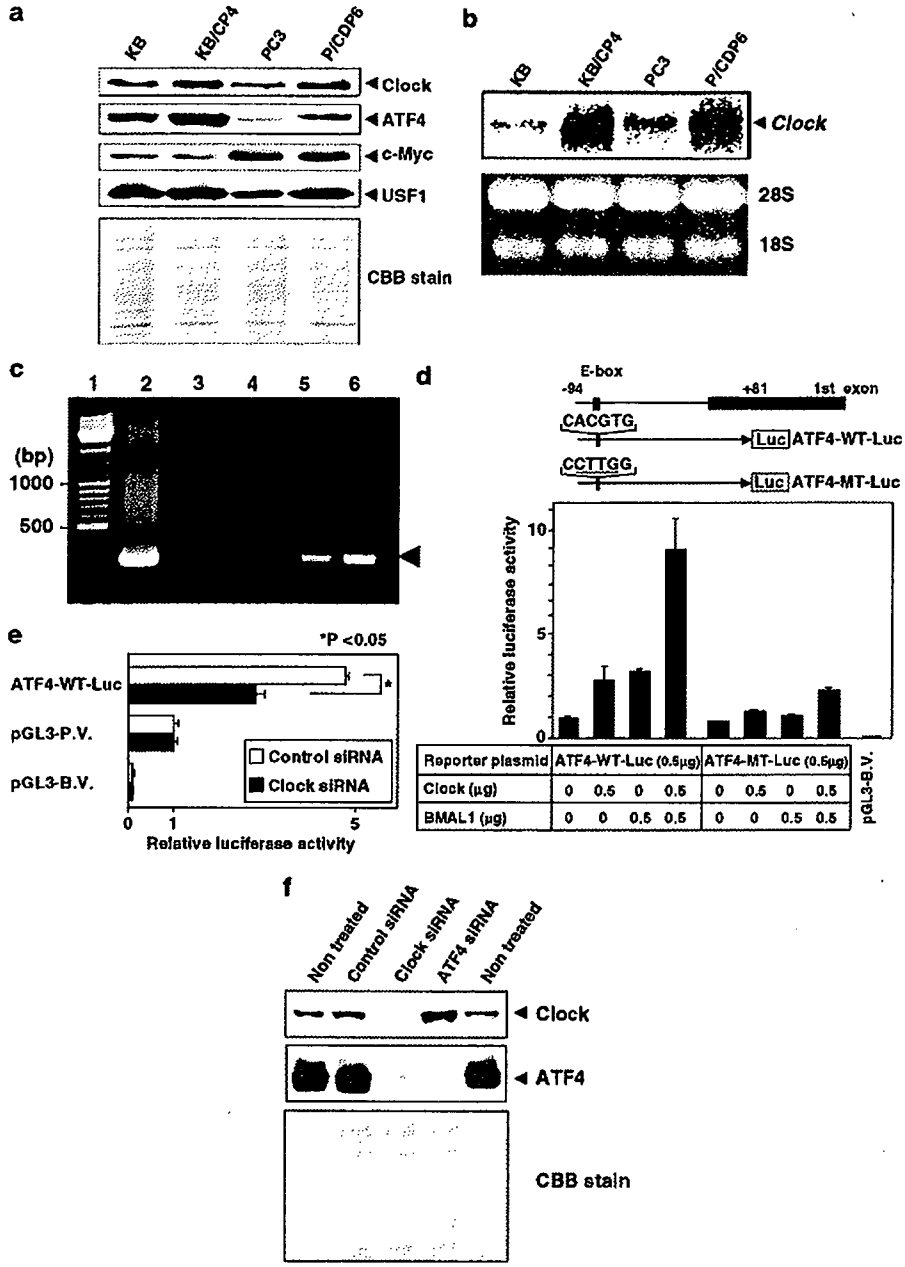

Figure 1 Clock regulates *ATF4* gene expression through binding to the E-box located in the promoter region. (a) Whole-cell extracts (75 μg for Clock, and 50 μg for c-Myc and USF1) and nuclear extracts (100 μg for ATF4) were subjected to SDS–PAGE, and Western blotting analysis was performed with the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

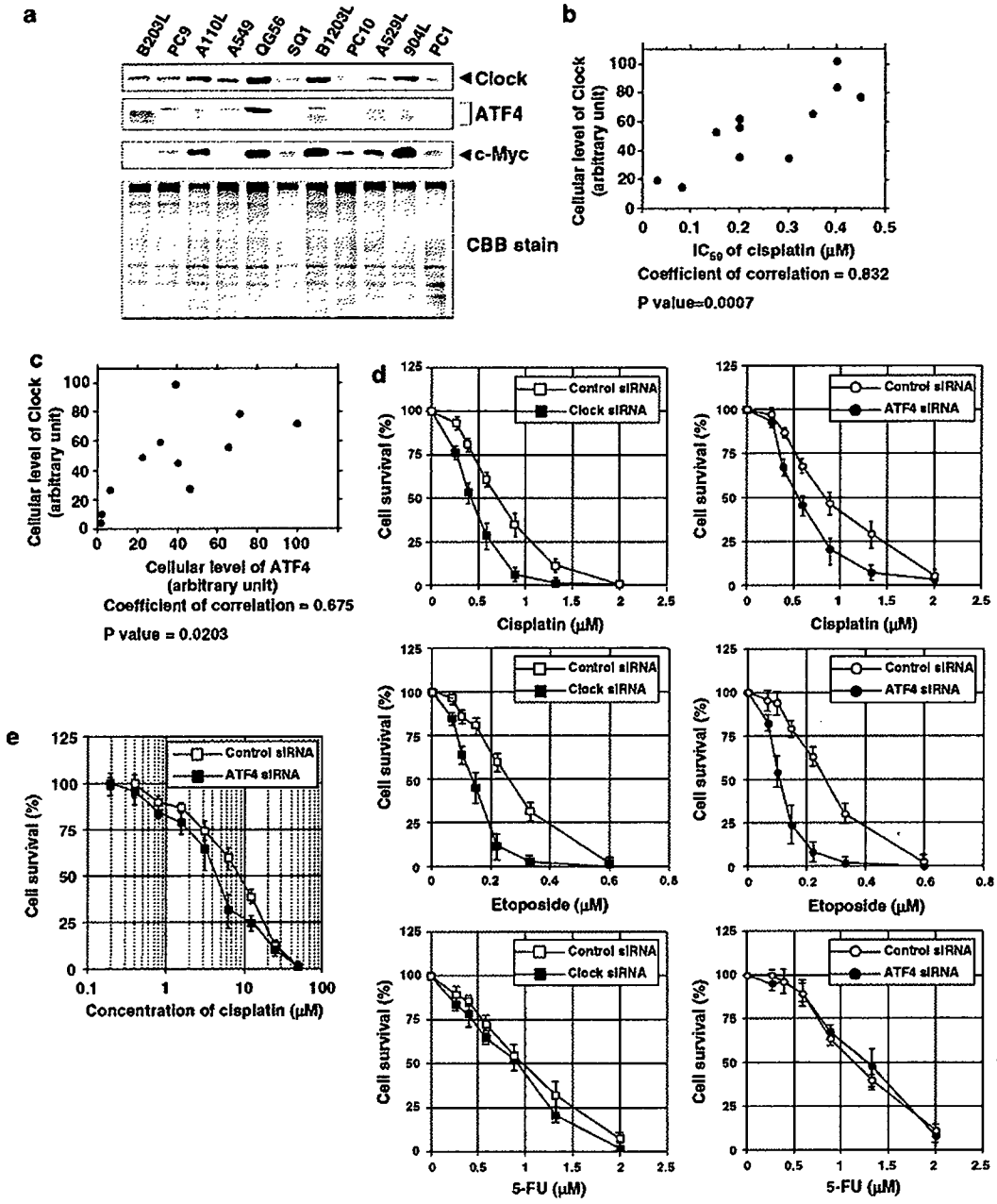

Figure 2 Clock expression correlates with cisplatin resistance and ATF4 expression. (a) Western blotting analysis was performed with 100 μg nuclear extracts for ATF4 expression and 75 μg whole-cell extracts for Clock and c-Myc expressions in 11 human lung cancer cell lines. Gel staining with CBB is also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

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

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

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

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

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

Microarray analysis of ATF4-regulated genes

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

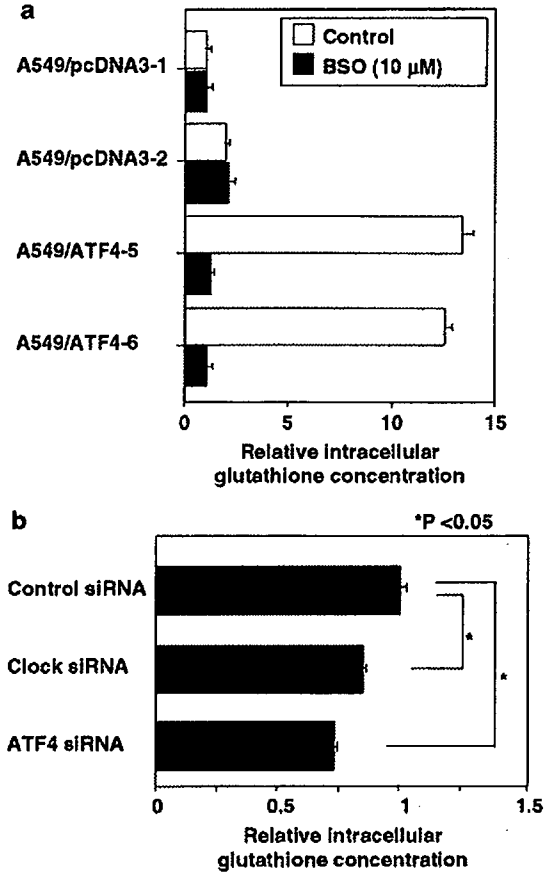

Figure 3 Intracellular glutathione levels are evaluated in ATF4-overexpressing cells and downregulated by Clock or ATF4 siRNAs. (a) Intracellular glutathione levels of ATF4-overexpressing cell lines (A549/ATF4-5, 6) and control cell lines (A549/pcDNA3-1, 2) with or without 10 μM BSO treatment for 72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

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

The expression of the ATP-binding cassette transporter family

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

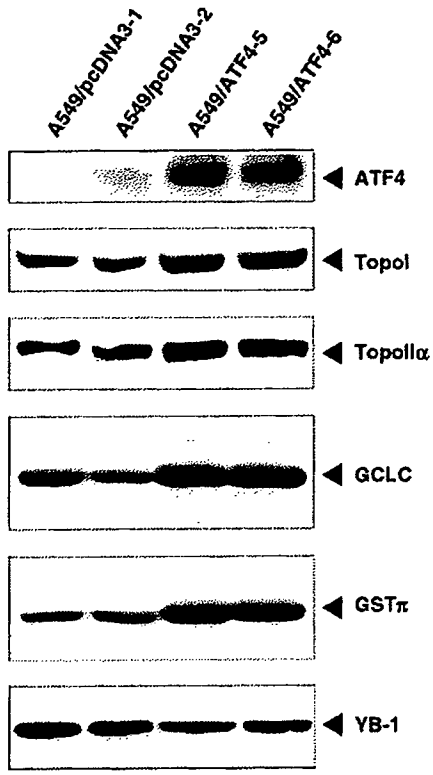

Figure 4 Cellular expressions of drug resistance-related genes in ATF4-overexpressing cells (A549/ATF4-5 and A549/ATF4-6) and control cells (A549/pcDNA3-1 and A549/pcDNA3-2). Whole-cell extracts (75 μg) for GCLC, GSTπ, YB-1 and nuclear extracts (100 μg) for ATF4, TopoI, TopoIIα from ATF4-overexpressing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

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

Discussion

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

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

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

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

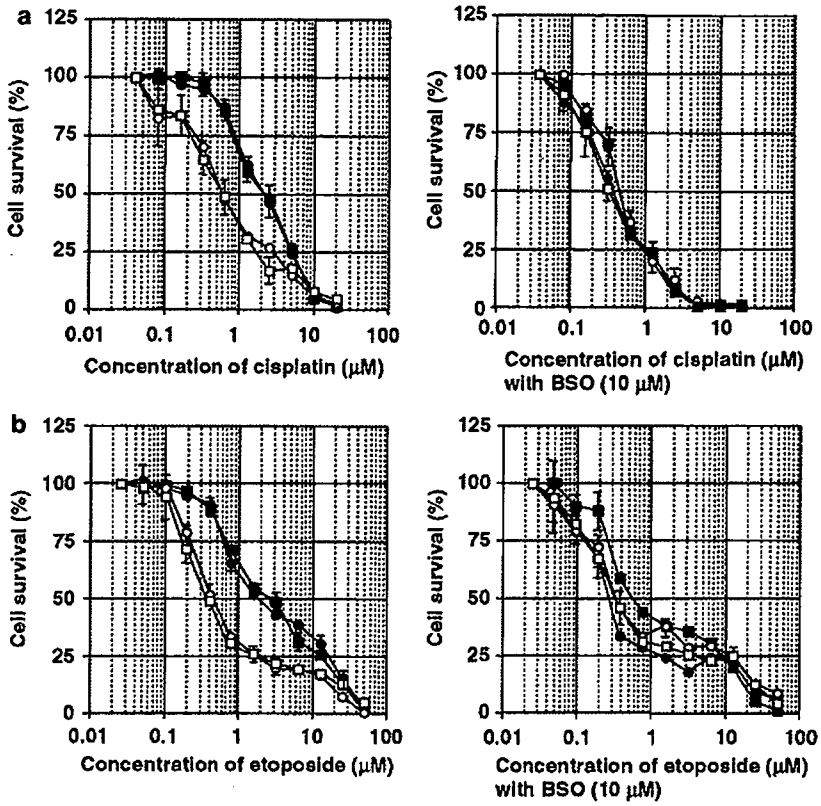

Figure 5 BSO overcomes the drug resistance of cisplatin and etoposide in ATF4-overexpressing cells. ATF4-overexpressing cells (A549/ATF4-5, closed square; A549/ATF4-6, closed circle) and control cells (A549/pcDNA3-1, open square; A549/pcDNA3-2, open circle) were pretreated with or without 10 μM BSO for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

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

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

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

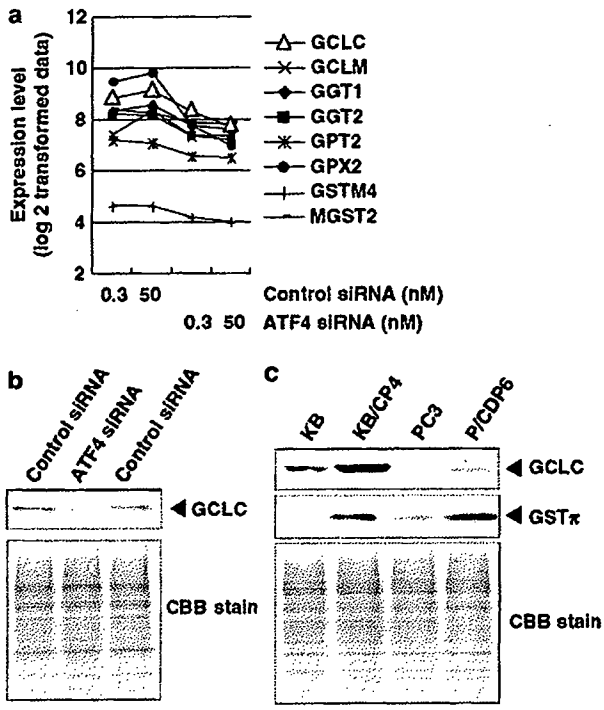

Figure 6 The genes for glutathione metabolism are regulated by ATF4. (a) The graph shows gene expression changes of glutathione metabolism subset mediated by ATF4 siRNA. The data were obtained from normalized and log 2-transformed microarray expression signal intensities. The samples were collected from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

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

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

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

Materials and methods

Cell culture

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

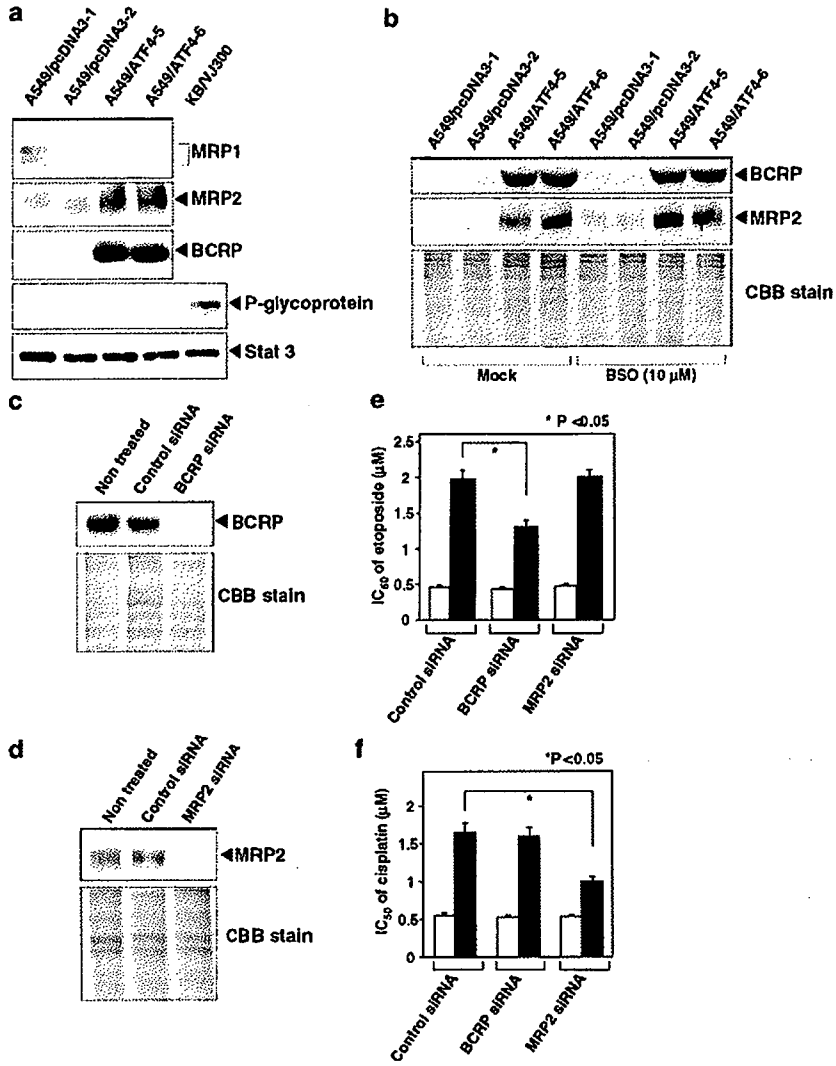

Figure 7 Cellular expressions of ABC transporter families in ATF4-overexpressing cells (A549/ATF4-5 and A549/ATF4-6) and control cells (A549/pcDNA3-1 and A549/pcDNA3-2) (a) Whole-cell extracts (100 μg) from the indicated cell lines were subjected to SDS–PAGE, and Western blotting analysis was performed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

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

Antibodies and drugs

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

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

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

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

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

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

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

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

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

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

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

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

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Comparative Analysis of Epidermal Growth Factor Receptor Mutations and Gene Amplification as Predictors of Gefitinib Efficacy in Japanese Patients With Nonsmall Cell Lung Cancer

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BACKGROUND. Because the investigation of epidermal growth factor receptor gene (*EGFR*) status as a predictor of gefitinib efficacy in Japanese patients has shown promise, the authors evaluated *EGFR* mutations and gene amplification in biopsy specimens from Japanese patients with nonsmall cell lung cancer (NSCLC) who received treatment with gefitinib to analyze the correlation between *EGFR* gene status and clinical outcome.

METHODS. Fifty-nine patients were enrolled in this study. *EGFR* gene amplification was evaluated by fluorescence in situ hybridization (FISH), and *EGFR* mutations in exons 18, 19, and 21 were analyzed by polymerase chain reaction and direct sequencing.

RESULTS. *EGFR* mutations were detected in 17 patients (28.8%). FISH-positive results were observed in 26 patients (48.1%). The response rate was significantly higher in the patients with *EGFR* mutations than in the patients without mutations (58.8% vs 14.3%; $P = .0005$). No significant difference in the response rate was observed between FISH-positive patients and FISH-negative patients (31.8% vs 21.4%; $P = .4339$). *EGFR* mutation was correlated with both a longer time to progression (TTP) (7.3 months vs 1.8 months; $P = .0030$) and longer overall survival (OS) (18.9 months vs 6.4 months; $P = .0092$). No significant differences in TTP or OS were observed between FISH-positive patients and FISH-negative patients. The results from a multivariate analysis indicated that *EGFR* mutations maintained a significant association with longer TTP and longer OS.

CONCLUSIONS. The results of this study suggested that *EGFR* mutations may serve as predictors of response and survival and that the role of *EGFR* gene amplification is not a predictor of gefitinib efficacy in Japanese patients with NSCLC. *Cancer* 2007;109:1836–44. © *2007 American Cancer Society.*

KEYWORDS: gefitinib, epidermal growth factor receptor gene, mutations, gene amplification, fluorescence in situ hybridization, Japanese.

Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that belongs to the ErbB family and has been implicated in cell proliferation and survival. The *EGFR* gene (*EGFR*) frequently is overexpressed in many solid tumors, including nonsmall cell lung cancer (NSCLC).[1] Gefitinib (Iressa; Astra Zeneca) is a targeted agent that inhibits the tyrosine kinase activity of EGFR by competitively blocking the adenosine triphosphate binding site.[2] Two Phase II trials (THE Iressa Dose Evaluation in Advanced Lung Cancer [IDEAL] 1 and IDEAL 2 studies) confirmed that gefitinib was active in from 10% to

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20% of patients with NSCLC who had failed on standard therapy,[3,4] and both trials revealed significant variability in response to gefitinib. Objective responses to gefitinib frequently are observed in women, never-smokers, and patients with adenocarcinoma histology,[3,4] and molecular predictors of response to gefitinib have been investigated. Protein expression of EGFR and its downstream molecules have been studied widely immunohistochemically, but the results have not been consistent.[5–8]

In 2004, 3 groups reported that tumors with somatic mutations in the kinase domain of *EGFR* were associated with gefitinib sensitivity.[9–11] Several retrospective studies have revealed that *EGFR* mutations are observed more frequently in women, never-smokers, patients with adenocarcinoma histology, and East-Asian patients[12–17] and that mutations are associated with objective response to gefitinib and a prolonged time to progression (TTP). A survival benefit of gefitinib treatment has been observed in many East-Asian patients with *EGFR* mutations, but no such benefit has been reported in Caucasian patients with *EGFR* mutations.[7,12,13,15,18,19]

Cappuzzo et al. investigated *EGFR* gene amplification by fluorescence in situ hybridization (FISH) in patients with NSCLC who received treatment with gefitinib, and their results indicated that *EGFR* gene amplification was associated with an objective response to gefitinib, a longer TTP, and a longer overall survival (OS).[14,18] Those authors also demonstrated that *EGFR* gene amplification assessed by FISH was a more reliable biomarker for predicting a survival benefit of gefitinib therapy in Caucasian patients with NSCLC than *EGFR* mutation status. However, it is unclear whether *EGFR* gene amplification assessed by FISH is an effective predictor of gefitinib efficacy in Japanese patients with NSCLC, who frequently have *EGFR* mutations and in whom the mutations seem to be reliable predictors of gefitinib efficacy. In the current study, we evaluated the *EGFR* mutation status and gene amplification in biopsy specimens from Japanese patients with advanced NSCLC to assess their predictive value in regard to the efficacy of gefitinib in this population.

MATERIALS AND METHODS

Patients

This was a retrospective study and was conducted at 5 institutions: Kanazawa University Hospital, Ishikawa Prefectural Central Hospital, Kouseiren Takaoka Hospital, Houju Kinen Hospital, and Kanazawa Municipal Hospital. Gefitinib therapy in the enrolled patients was started between July 2002 and October 2005. The patients were chosen for this study based on the following eligibility criteria: histologically confirmed NSCLC treated with gefitinib (250 mg per day), the ability to obtain tumor samples from each institution after receiving approval from the institution's review board and the patient's written informed consent, and the ability to obtain complete clinical information and physical examination records, including information on Eastern Cooperative Oncology Group performance status (PS).[20]

Patients who received treatment with gefitinib were evaluated for response every 4 weeks according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.[21] Tumor response was assessed by computed tomography (CT), and a confirmatory evaluation was repeated after 4 weeks in patients who had a complete response (CR), a partial response (PR), or stable disease (SD). TTP was calculated from the date gefitinib therapy was started to the date of disease progression or the date of last contact. OS was calculated from the date gefitinib therapy was started to the date of death or the most recent date on which the patient was known to be alive. Some patients in this study had been enrolled in our previous study, which was a Phase II trial that evaluated the clinical benefit of gefitinib in chemotherapy-naive patients with NSCLC.[22] The investigators who examined the biopsy specimens were blinded to the clinical outcome.

Tissue Preparation

The tumor specimens were fixed in formalin and embedded in paraffin wax. Serial sections that contained representative malignant cells were deparaffinized in xylene washes and dehydrated in 100% ethanol. No specimens were microdissected.

EGFR Mutations

DNA was extracted from 5 serial, 10-μm-thick sections by using the QIAamp DNA Mini kit (Qiagen, Tokyo, Japan) according to the protocol described in the manufacturer's instructions. The DNA obtained was eluted in 50 μL of buffer AE, and the concentration and purity of the extracted DNA were assessed by spectrophotometry. The extracted DNA was stored at −20°C until it was used. *EGFR* mutations were detected by polymerase chain reaction (PCR)-based direct sequencing of exons 18, 19, and 21. PCR amplification was performed in 100 ng of genomic DNA with the TaKaRa Ex Taq Hot Start Version kit (TaKaRa, Tokyo, Japan). The following primers were used: exon 18, 5′-CCTTGTCTCTGTGTTCTTGT-3′ (forward) and 5′-CTGCGGCCCAGCCCAGAGGC-3′ (reverse); exon 19, 5′-CATGTGGCACCATCTCACA-3′ (forward) and 5′-CCA-

CACAGCAAAGCAGAAAC-3′ (reverse); and exon 21, 5′-CAGGGTCTTCTCTGTTTCAG-3′ (forward) and 5′-TAAAGCCACC TCCTTACTTT-3′ (reverse). DNA was amplified for 35 cycles at 95°C for 30 seconds, at 61°C for 30 seconds, and at 72°C for 60 seconds followed by 7 minutes of extension at 72°C. Sequencing was performed with a 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan), and the results were analyzed by using Sequencer 3.11 software (Applied Biosystems, Foster City, Calif) to compare variations. The sequences were compared with the GenBank sequence for human *EGFR* (accession number AF288738).

EGFR Amplification

Each slide was deparaffinized in xylene washes and dehydrated in 100% ethanol. After incubation in 0.2 N HCL at room temperature for 20 minutes and in a pretreatment reagent (NaSCN; Vysis, Tokyo, Japan) at 80°C for 30 minutes, the slides were digested with a proteinase reagent at 37°C for 10 to 60 minutes. Slides were refixed in 10% neutral buffered formalin at room temperature for 10 minutes and rinsed twice in 2 × standard saline citrate (SSC), pH 7.3, at room temperature for 5 minutes each. The slides were incubated in 70% formamide/2 × SSC, pH 7.0 to 8.0, at 72°C for 5 minutes to allow the chromosomes to denature and were then dehydrated in a series of increasing concentrations of ethanol solutions (70%, 85%, and 100%). The *EGFR*/chromosome 7 centromere (*CEP7*) probe set (Vysis) was applied to an area that was selected based on the presence of tumor foci on each slide, and the hybridization area was covered with a cover glass and sealed with rubber cement. The slides were incubated in a humidified chamber at 37°C for 20 to 24 hours to allow hybridization to occur. A posthybridization wash was performed in 2 × SSC/0.3% NP-40 at 73°C for 2 minutes and then in 2 × SSC at room temperature for 5 minutes. Finally, 4′,6-diamidino-2-phenylindole was applied to the target area, which then was covered with a coverslip.

The patients were classified into the following 6 categories according to *EGFR* gene copy numbers per cell and frequency of tumor cells with a specific copy number of the *EGFR* gene and *CEP7*, as described[18] by Cappuzzo et al.: disomy (≤2 copies in >90% of cells), low trisomy (≤2 copies in ≥40% of cells, 3 copies in 10–40% of cells, ≥4 copies in <10% of cells), high trisomy (≤2 copies in >40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells), low polysomy (≥4 copies in 10–40% of cells), high polysomy (≥4 copies in ≥40% of cells), and high gene copy number (defined as the presence of tight *EGFR* gene clusters and a ratio of *EGFR* gene to chromosome of ≥2 copies or ≥15 copies of *EGFR* per cell in ≥10% of cells) (Fig. 1A-D). FISH analysis was performed independently by 2 investigators who were blinded to the patients' clinical characteristics and all other molecular variables. Patients with high polysomy or high gene copy numbers were classified as FISH-positive, and the remaining groups (with disomy, low trisomy, high trisomy, or low polysomy) were classified as FISH-negative.

Statistical Analysis

Associations between response to gefitinib and other variables, including sex, histology, smoking history, *EGFR* mutations, and *EGFR* gene amplification, were analyzed for independence by using the chi-square test. A logistic regression model was used to identify which independent factors had a joint significant influence on the rate of objective response to gefitinib. TTP and OS were calculated by using the Kaplan-Meier method, and differences between patient groups according to *EGFR* mutations or FISH analysis were compared by using the log-rank test. A Cox proportional-hazards model was used for the multivariate analysis of survival. A P value of .05 was regarded as statistically significant, unless stated otherwise, and all comparisons were 2-sided. StatView 5.0 statistical software was used to perform all analyses.

RESULTS

Patient Characteristics

The characteristics of the patients are shown in Table 1. Tumor samples were collected from 59 patients. We were able to obtain complete clinical information on 101 patients with NSCLC who received treatment with gefitinib at the 5 institutions. It was impossible to obtain tumor samples from 37 of 101 patients; because, in 22 patients, the diagnosis was made cytologically, and the 15 other patients already had been diagnosed at other hospitals before their admission to 1 of our institutions. Response to gefitinib was not evaluated in 5 patients, because they did not have lesions that could be evaluated.

In total, 59 tumor samples, 46 from primary tumors (29 obtained by transbronchial lung biopsy, 3 obtained by percutaneous lung biopsy, and 14 obtained at surgery) and 13 samples from lung tumor metastases to other sites (5 in lymph nodes, 5 in the brain, 1 in the small bowel, 1 in bone, and 1 in muscle) were obtained. All resections were performed for histologic diagnosis or palliative therapy. Twenty-four of 59 patients (40.7%) were women, and 21 patients (35.0%) were never-smokers. According to histologic type, there were 44 adenocarcinomas (73.3%; including 2 that contained bronchioloalveolar carcinoma

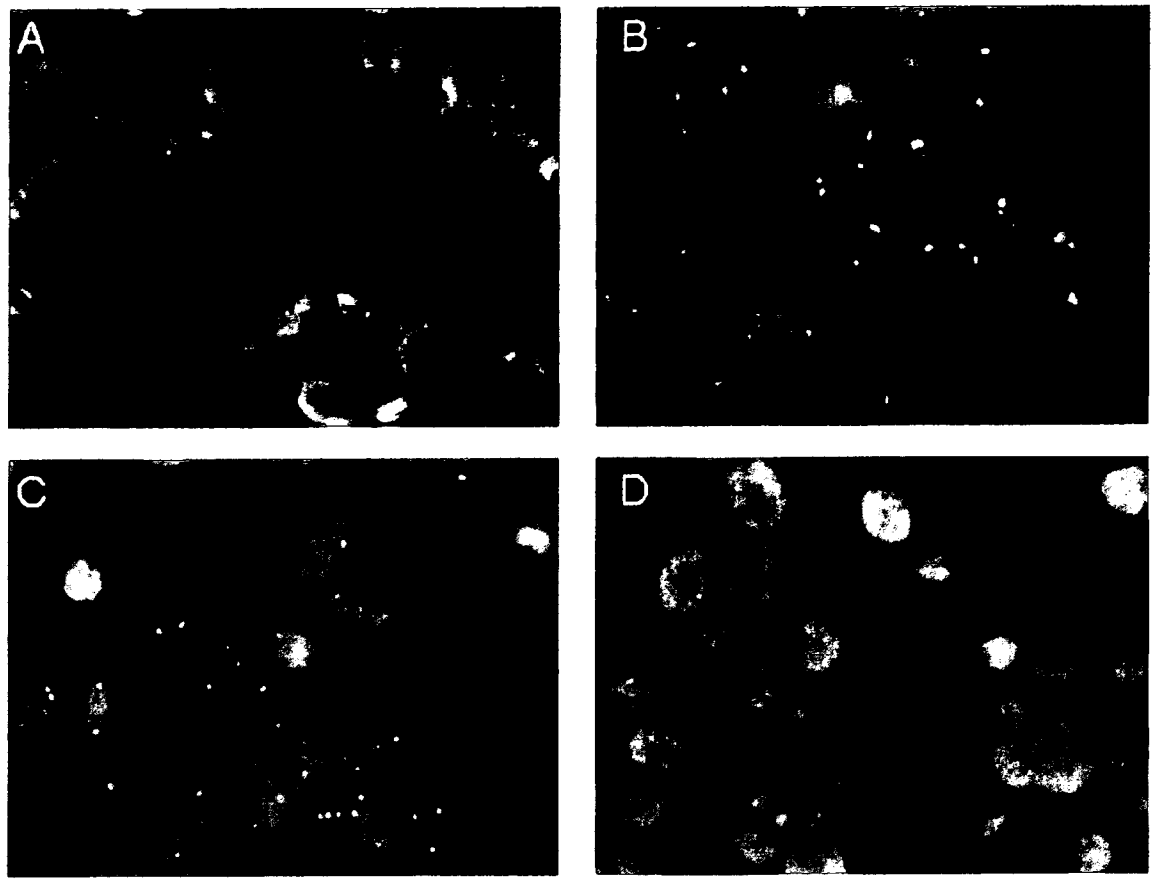

FIGURE 1. Fluorescence in situ hybridization of nonsmall cell lung cancer cells. The chromosome 7 centromere (CEP7) signals are green, and the epidermal growth factor receptor gene (*EGFR*) signals are red. A, disomy; B, low trisomy; C, high polysomy; D, high gene copy number.

components), 11 squamous cell carcinomas, 3 large cell carcinomas, and 1 adenosquamous cell carcinoma. Thirty-six patients (61.0%) had a good PS (0 or 1). Fifty-four patients (91.5%) had stage IV disease at the start of gefitinib therapy.

Twenty-five patients had been treated with chemotherapy, and 34 patients were chemotherapy-naive. A PR was achieved in 16 patients (27.1%), 20 patients had SD (33.9%), and 23 patients had progressive disease (PD) (39.0%). The overall response rate was 27.1% (95% confidence interval, 15.8–38.5%), and the response rate was significantly higher among women, patients with adenocarcinoma, and never-smokers (Table 1).

Fifteen patients were alive at the time of the analysis, and 5 of those patients still were receiving gefitinib therapy without tumor progression. The median follow-up was 9.9 months, the median TTP was 3.1 months (range, 0.2–34.8 months), and the median OS was 7.6 months (range, 0.7–38.8 months). The median duration of gefitinib therapy was 3.5 months. The TTP and OS were significantly longer among women (TTP: 4.3 months vs 1.1 months; $P = .0084$; OS: 18.2 months vs 6.4 months; $P = .0022$), among patients with adenocarcinoma (TTP: 3.7 months vs 1.1 months; $P = .0024$; OS: 10.2 months vs 6.1 months; $P = .0025$), and among never-smokers (TTP: 6.5 months vs 3.3 months; $P = .0013$; OS: 18.2 months vs 6.1 months; $P = .0003$). There were no differences in TTP or OS between patients who received gefitinib as first-line therapy and patients who received gefitinib as second-line therapy (TTP, 3.3 months vs 2.6 months, respectively; $P = .3287$; OS: 8.2 months vs 6.2 months, respectively; $P = .2689$).

EGFR Mutations

EGFR mutations of exons 18, 19, and 21 were analyzed in every patient were detected in 17 patients (28.8%).

TABLE 1
Patient Characteristics and Objective Response

Variable	No. of patients	No. of responders	Response rate, %	P
All patients	59	16	27.1	
Sex				
Women	24	11	45.8	.0074
Men	35	5	14.3	
Histology				
Adenocarcinoma	44	15	34.1	.0391
Nonadenocarcinoma	15	1	6.7	
Smoking history				
Never	21	12	57.1	.0081
Current/former	38	4	10.5	
PS				
0–1	36	9	25.0	.6470
2–3	23	7	30.4	
Stage				
III	5	1	20.0	.7082
IV	54	15	27.8	
Prior chemotherapy				
Present	25	7	28.0	.8961
Absent	34	9	26.5	
EGFR mutation				
Mutation	17	10	58.8	.0005
Exon 18	0	0	-	
Exon 19	12	7	58.3	.9493[b]
Exon 21	5	3	60.0	
Wild type	42	6	14.3	
EGFR gene amplification[c]				
FISH positive	26	8	30.8	.4339
FISH negative	28	6	21.4	

PS indicates performance status; *EGFR*, epidermal growth factor receptor; FISH, fluorescence in situ hybridization.
[b] *P* value for comparison of mutations in exons 19 and 21.
[c] *EGFR* gene amplification could not be evaluated in 5 patients because of the small size of the tissue specimen.

The mutations consisted of in-frame deletions in exon 19 (n = 11 patients), an in-frame deletion with point mutation in exon 19 (n = 1 patient), and point mutations in exon 21 (n = 5 patients). *EGFR* mutations were significantly more frequent among women (12 of 24 women; 50.0%; 5 of 35 men, 14.3%; $P = .0028$) and never-smokers (10 of 21 never-smokers; 47.6%; 7 of 38 smokers; 18.4%; $P = .0177$). Mutations frequently were detected in adenocarcinomas (14 of 44 tumors; 31.8%); they were detected in 2 squamous cell carcinomas and in 1 large cell carcinoma, but none of the differences according to histologic subtype were statistically significant (Table 2).

EGFR Amplification

EGFR gene amplification was assessed by FISH in 54 patients. The tissue specimens in the other 5 patients were small and inadequate for FISH analysis. Disomy for the *EGFR* gene was present in 5 patients (9.3%),

TABLE 2
Epidermal Growth Factor Receptor Gene Mutations, Gene Amplification, and Patient Characteristics

Variable	No. of patients with *EGFR* mutations	%	P	No. of FISH-positive patients	%	P
All patients	17/59	28.8		26/54	48.1	
Sex						
Women	12/24	50	.0029	11/21	52.4	.6195
Men	5/35	14.3		15/33	45.5	
Histology						
Adenocarcinoma	14/44	31.8	.3828	22/40	55	.0365
Nonadenocarcinoma	3/15	20		4/14	28.6	
Smoking status						
Never	10/21	47.6	.0177	11/17	64.7	.0388
Current/former	7/38	18.4		15/37	40.5	

EGFR indicates epidermal growth factor receptor gene; FISH, fluorescence in situ hybridization.

low trisomy was present in 5 patients (9.3%), high trisomy was present in 0 patients (0%), low polysomy was present in 18 patients (33.3%), high polysomy was present in 5 patients (9.3%), and high gene copy numbers were present in 21 patients (38.9%). Twenty-six patients (48.1%) were FISH-positive, and 28 patients (51.9%) were FISH-negative. FISH-positive results were observed frequently among patients with adenocarcinomas and among never-smokers, but the differences were not statistically significant. FISH status was not associated with sex (Table 2)., FISH-positive results were observed in 62.5% of patients with *EGFR* mutations (10 of 16 patients) and in 42.1% of patients without *EGFR* mutations (16 of 38 patients). The rate of FISH-positive results was not correlated with the presence of *EGFR* mutations ($P = .1708$). The clinical characteristics of the patients with *EGFR* mutations and their classification according to *EGFR* gene amplification in are summarized in Table 3.

EGFR Mutations and Gene Amplification As Predictors of Gefitinib Efficacy

The response rate among patients with *EGFR* mutations was significantly higher than the response rate among patients without mutations (58.8% vs 14.3%; $P = .0005$) (Table 1), but there was little difference in the response rate between FISH-positive patients and FISH-negative patients (30.8% vs 21.4%, respectively; $P = .4339$) (Table 1). A multivariate analysis of response to gefitinib that included the variables sex, histology, smoking status, and *EGFR* gene status revealed that *EGFR* mutation was the only factor that contributed significantly toward a response to gefitinib ($P = .0196$) (Table 4).

TABLE 3
Clinical and Molecular Features of Patients With Epidermal Growth Factor Receptor Gene Mutations

Patient	Sex	Histology	Smoking status	Response to gefitinib	Median OS, mo	Median TTP, mo	*EGFR* gene amplification	*EGFR* mutation
3	W	ADC	Never	PR	33.4	6.5	HGCN	L858R
8	M	ADC	Smoker	PR	23.7	23.9	D	L858R
9	W	ADC	Never	PR	18.2	17.9	LP	del E746-A750
15	W	ADC	Never	PR	12.9	4.3	HGCN	del E746-A750
18	M	ADC	Smoker	PR	18.8	18.3	HGCN	del E746-A750
28	W	ADC	Smoker	SD	14.4	14.4	LP	del L747-S752, E746I
30	W	ADC	Never	SD	13.3	13.3	HGCN	L858R
33	W	ADC	Never	PR	22.4	8.3	LT	del L747-S752
34	M	SCC	Smoker	PD	2.7	0.9	LT	del E746-A750
37	W	ADC	Never	SD	13.6	0.9	HGCN	L858R
38	M	LCC	Smoker	PD	5.4	1	HP	del E746-A750
39	W	ADC	Never	PR	20	12.9	LT	del E746-A750
42	W	ADC	Never	PR	9.7	7.7	HGCN	L858R
44	M	SCC	Smoker	SD	6.8	1.1	HGCN	del E746-A750
49	W	ADC	Never	PR	6.6	6.6	HP	del E746-A750
53	W	ADC	Smoker	SD	3.6	3	HGCN	del E746-A750
58	W	ADC	Never	PR	18.9	15.3	NE	del E746-A750

OS indicates overall survival; TTP, time to progression; *EGFR*, epidermal growth factor receptor gene; W, women; ADC, adenocarcinoma; PR, partial response; HGCN, high gene copy number; M, men; D, disomy; LP, low polysomy; SD, stable disease; SCC, squamous cell carcinoma; PD, progressive disease; LCC, large-cell carcinoma; HP, high polysomy; LT, low trisomy; NE, not evaluable.

TTP and OS were significantly longer in patients with *EGFR* mutations than in patients without *EGFR* mutations (TTP: 7.3 months vs 1.8 months; $P = .0030$) (Fig. 2A) (OS: 18.9 months vs 6.4 months; $P = .0092$) (Fig. 2B), but no significant difference in the median TTP or OS was observed between FISH-positive patients and FISH-negative patients (TTP: 1.8 months vs 2.6 months, respectively; $P = .8535$) (Fig. 2C) (OS: 6.4 months vs 8.2 months, respectively; $P = .9121$) (Fig. 2D). Five variables (sex, histology, smoking status, *EGFR* mutation, and *EGFR* FISH-positive results) were included in a Cox hazards model to define which variables were predictive of longer survival. The results showed that *EGFR* mutation was the only factor associated with both longer TTP and longer OS (Table 4). The median OS of patients with mutations in exon 21 was longer than of patients with exon 19 deletion mutations (23.0 months vs 18.2 months; $P = .0271$), but there was no difference in response rate or TTP between patients with mutations in exon 19 and patients with mutations in exon 21 (response rate: 60.0% vs 58.3%, respectively; $P = .9493$; TTP: 8.3 months vs 6.4 months, respectively; $P = .5158$). TTP and OS were significantly longer in the 10 responders with mutations than in the 6 responders without mutations (TTP: 15.3 months vs 3.3 months; $P = .0022$; OS: 20.0 months vs 7.7 months; $P = .0007$).

TABLE 4
Multivariable Analysis for Response to Gefitinib and Survival

Variable	OR for response	*P*	HR for TTP	*P*	HR for OS	*P*
Sex						
Women	0.515	.6274	1.677	.2777	1.549	.4322
Men	1		1		1	
Histology						
Adenocarcinoma	2.130	.5481	0.648	.2933	0.656	.2869
Nonadenocarcinoma	1		1		1	
Smoking status						
Never	12.765	.0676	0.443	.135	0.342	.1145
Current/former	1		1		1	
EGFR mutation						
Mutation	5.880	.0289	0.219	.0006	0.339	.0125
Wild type	1		1		1	
EGFR gene amplification						
FISH positive	0.773	.7488	1.723	.1136	1.678	.1696
FISH negative	1		1		1	

OR indicates odds ratio; HR, hazard ratio; TTP, time to progression; OS, overall survival; *EGFR*, indicates epidermal growth factor receptor gene; FISH, fluorescence in situ hybridization.

DISCUSSION

The results of this study showed that *EGFR* mutation was the only factor significantly associated with a better response to gefitinib, longer TTP, and longer OS in Japanese patients with NSCLC. A multivariate analysis revealed that *EGFR* mutation was a more reliable predictor of gefitinib efficacy than sex, histology, smoking status, or *EGFR* gene amplification. All biopsy specimens were examined successfully for *EGFR* mutations, and the results were comparable with those from previous studies in which surgical specimens were used,[12,17,19] even though the amount of tumor tissue available in the biopsy specimens

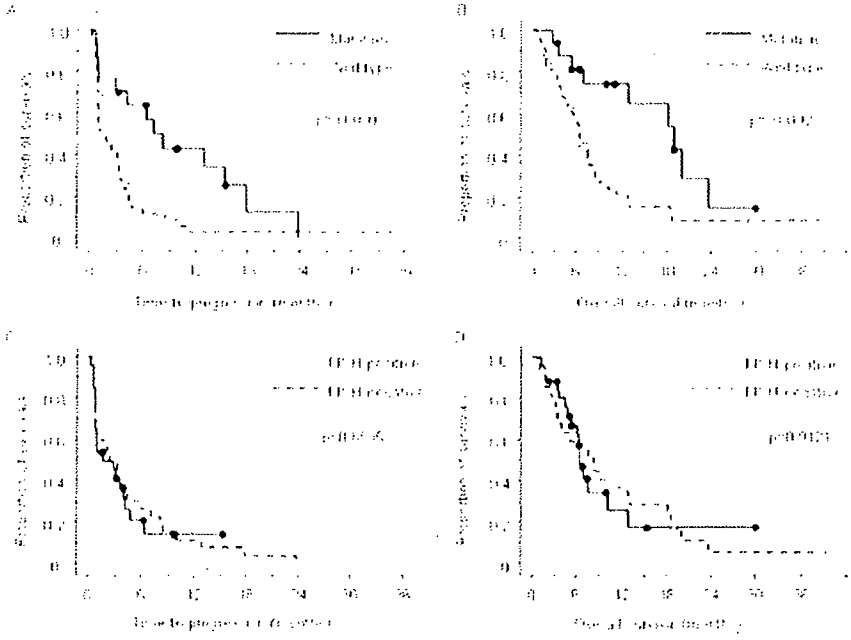

FIGURE 2. Kaplan-Meier estimates of time-to-progression and overall survival grouped by epidermal growth factor gene (*EGFR*) mutation (A,B) and by *EGFR* amplification (C,D).The solid and open dots represent censored patients. FISH indicates fluorescence in situ hybridization.

was limited. The results suggested that gefitinib efficacy in Japanese patients with NSCLC can be predicted by detecting *EGFR* mutations in biopsy specimens.

Several recent studies produced higher response rates in patients with mutations in exon 19 of *EGFR* than in patients with L858R in exon 21 and demonstrated that TTP and OS were longer in patients with the deletion than in the patients with L858R.[23,24] However, our current results showed no difference in response or TTP between patients with exon 19 mutations and patients with exon 21 mutations, and patients with the L858R point mutation in exon 21 had a longer median OS than patients with a point mutation in exon 19. We cannot explain why our results were not in line with the those obtained in previous studies.[23,24] Our study was not designed investigate the differences in gefitinib efficacy between patients with mutations in exon 19 and patients with mutations in exon 21, and the sample size of the study was too small to detect a statistically significant difference.

The small number of patients is a clear limitation of this study. A large-scale, single-arm study of Western NSCLC reported that both *EGFR* mutations and gene amplification were associated with response rate to gefitinib, longer TTP, and longer OS, and that study also demonstrated a significant correlation between FISH-positive results and the presence of *EGFR* mutations in paired specimens.[18] Results from samples in prospective, placebo-controlled, Phase III studies were published subsequently, and the usefulness of *EGFR* gene amplification as a predictor of gefitinib efficacy was validated in a Phase III, placebo-controlled study, the Iressa Survival Evaluation in Lung Cancer (ISEL) trial.[25] The results of that prospective study indicated that high *EGFR* gene copy numbers were a predictor of a better response to gefitinib and a survival benefit.[26] The results from the current study conflict with the results from the ISEL trial and do not demonstrate any association between gene amplification and the efficacy of gefitinib therapy.

One possible explanation for the discrepancies between findings from the studies described above and our own findings is the difference in *EGFR* gene status according to ethnicity. Han et al. investigated *EGFR* gene mutations, gene amplification, K-*ras* mutation, and Akt phosphorylation in tumor samples from East-Asian patients with NSCLC[27] and demonstrated that *EGFR* mutation was an independent predictor of response and survival in a multivariate analysis that included *EGFR* gene amplification. Those authors also demonstrated that FISH-positive results were associated with a better response rate the same as *EGFR* mutation in the univariate analysis but was