2.4. Adhesion assay

Ninety-six well flat bottom plates (Corning Incorporated, Corning, New York) were coated with 4 mg/ml of hyaluronate or 10 mg/ml BSA in PBS overnight at 4 °C. Following procedures were performed as previously described [7].

2.5. In vitro cell proliferation

Cells were harvested from plates with 0.05% EDTA in PBS, suspended in SITA medium in single suspension and counted. Two thousands cells were added to the 96-well microtiter plate in triplicate, and allowed to grow at 37 °C with 5% CO_2 for 1–3 days. At the indicated time, the cell number was assessed with the Cell Counting Kit-8™ (Wako, Japan) according to the manufacturer's instruction.

2.6. Immunocytochemical staining for the single stranded DNA and TUNEL

To identify apoptotic cells, antibody specific for the single stranded DNA (DAKO Code number: A4506, Kyoto, Japan) was used to identify the cells exhibiting DNA fragmentation. In brief, 2×10^5 H322/CD44s cells were incubated with SITA medium on 9 cm dish coated with HA (4 mg/ml) or BSA (10 mg/ml) at 37 °C for 72 h. The cells were washed with PBS and adjusted to 5×10^3 cells/ml, and fixed on slides with the cytospin method. The slides were fixed with 4% paraformaldehyde, washed with PBS and incubated for 30 min in 10% normal goat serum. Then the anti-single stranded DNA polyclonal antibody was applied at a dilution of 1:400 and incubated overnight at 4 °C. Specific binding was detected with avidine–biotin peroxidase complex formation using the biotin conjugated goat anti-rabbit IgG (Vectastatin ABC kit, Vector, Burlingame, CA) and diaminobendizine (DAB) (Sigma, St. Louis, MI) as the substrate. Staining was absent when isotype-matched immunoglobulin was used as the control.

With other cytospun slides, Transferase-mediated dUTP nick end labeling (TUNEL) staining for apoptosis was also performed with an in situ Cell Death Detection kit (Roche, Indianapolis, IN) according to the manufacturer's instruction. Each experiment conducted with TUNEL reaction mixture without terminal transferase served as the negative control. The fluorescent images were obtained using an epifluorescence microscope.

2.7. In vitro chemosensitivity assay

Cells (1.0×10^4) cultured in 1% FCS/SITA medium were seeded to 96-well microtiter plates coated with HA (4 mg/ml) or BSA (10 mg/ml). Various concentration of chemotherapeutic agents including platinum agents (cis-platinum (II) diammine dichloride; CDDP, LKT Laboratories, MN), gemcitabine (GEM) provided by Eli Lilly (Indianapolis, IN) and vinorelbine (VNB) donated by Kyowa Hakko (Tokyo, Japan) in the absense or presense of 50 μM of (*E*)-3[[[3-[2-(7-chloro-2-qinolinyl)ethenyl]phenyl]-[[3-dimethylamino]-3-oxopropyl]thio]methyl]thio]-propanoic acid, MK571 (Calbiochem, San Diego, CA) were added to each well 1 h after seeding. After 72 h incubation, 10 μl of Cell Counting Kit-8 was added to each well. Four hours later, the optical density was measured at 450 nm with a microplate reader. Results are expressed as the percentage of cell viability (absorbance of exposed cells at 450 nm/absorbance of cells in SITA medium at 450 nm in the absence of platinum agents). Experiments were performed in triplicate in quadruplicate plates.

2.8. Immuno-fluorescence

Cells (1.0×10^6), seeded to 10 cm dish coated with HA or BSA, were cultured in 1% FCS/SITA medium. Incubation was allowed to proceed for 72 h at 37 °C in the absence or presence of 50 μM of MK571. The adherent cells were then detached from plates with 0.05% EDTA in PBS and pellet was fixed with 70% ethanol for 10 min at −20 °C. After permeabilizing in 1X FACS Permeabilizing Solution 2 (Becton and Dickinson, San Jose, CA)/dH_2O, cells were incubated for 5 min in PBS containing 0.5% BSA. Then, the cells (5×10^5) were incubated with mAb M2 I-4 (1 μg/ml) in 1% FCS/PBS at 4 °C for 30 min. Antibody binding was detected with fluorescent-labeled anti-mouse mAb (Sigma Chemical Co.). Fluorescence was analyzed with a FACScan™ (Becton–Dickson Co., Mountain View, CA).

2.9. Statistics

Statistical analysis was performed by analysis of variance. The Kruskal–Wallis method was used to compare the proliferation rate with H322/Mock, H322/CD44s, and H322/TR. All data are presented as means ± SD Differences between means were considered statistically significant at $p < 0.05$. Statview version 5.0 (Abacus corporation) was used for all analyses.

3. Results

3.1. Reintroduction of CD44s to a human non-small cell lung cancer cell line

To determine the role of interaction between HA and its receptor, CD44s, we reintroduced the CD44s cDNA back to the NSCLC cell line, H322, which originally lacks the CD44s isoform with electroporation. We also established the H322/TR and H322/Mock transfectants by reintroducing the CD44s gene encoding for a protein

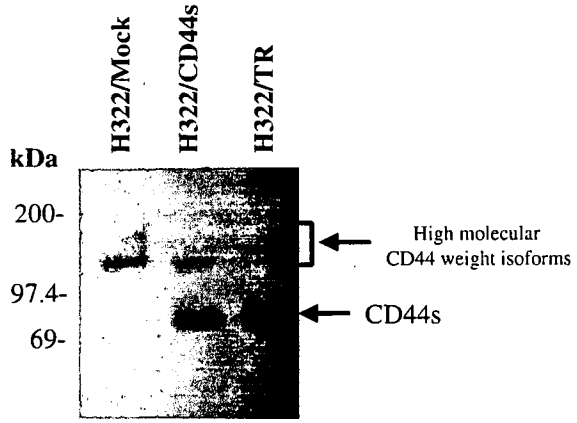

Fig. 1. Establishment of H322 transfected with an empty vector (H322/Mock), CD44s full-length gene (H322/CD44s), and CD44s gene whose cytoplasmic sequence was deleted (H322/TR). Expression of CD44s on H322 CD44s transfectants (H322/CD44s cells), control transfectants (H322/Mock) and CD44s genes whose cytoplasmic sequence is deleted (H322/TR) was assessed with Western blot. The upper arrow indicates endogenous high molecular weight CD44 isoforms. The lower arrow indicates transfected CD44s. Molecular weight markers are indicated on the left.

whose cytoplasmic region was deleted and control expression vector, respectively. G418-resistant clones expressing cell surface CD44s were identified with both Western blot (Fig. 1) and FACS analysis (data not shown). Several clones for CD44s transfectant designated H322/CD44s, H322/TR and H322/Mock were obtained.

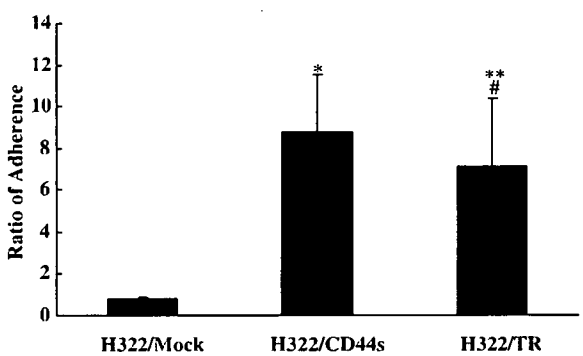

Fig. 2. Adhesion assay. In vitro cell adhesion assay of H322/Mock cells, H322/CD44s cells, or H322/TR cells to BSA or HA. Cells were allowed to adhere to BSA (10 mg/ml) or HA (4 mg/ml) at 4 °C for 1 h. The ratio of adherence (percent specific adhesion to HA/percent specific adhesion to BSA) of H322/CD44s and H322/TR was significantly greater than that of H322/Mock. $^{*}p = 0.0057$ vs. H322/Mock, $^{**}p = 0.5307$ vs. H322/CD44s, $^{\#}p = 0.045$ vs. H322/Mock. Data are presented as the means ± SD in triplicates.

3.2. Adhesion assay

To evaluate the effect of CD44s transfected to H322 cells on HA binding, in vitro cell adhesion assay was performed using H322/CD44s, H322/TR and H322/Mock cells. Cells were investigated for adhesion to either HA or BSA. The ratio of adherence (percent specific adhesion to HA/percent specific adhesion to BSA) of H322/CD44s and H322/TR was significantly greater than that of the H322/Mock (Fig. 2).

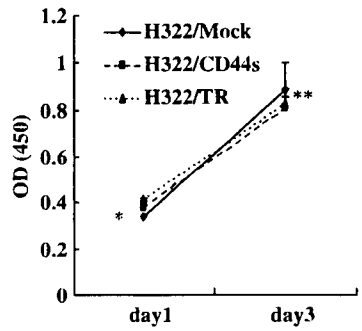

Fig. 3. In vitro cell proliferation assay. Two thousand H322/Mock, H322/CD44s and H322/TR cells were added to the 96-well microtiter plate in triplicate, and allowed to grow for 1–3 days. Cell number was assessed with the cell counting kit-8™. There was no difference in growth speed among the three clones. $^{*}p = 0.2521$, $^{**}p = 0.5611$ among three clones.

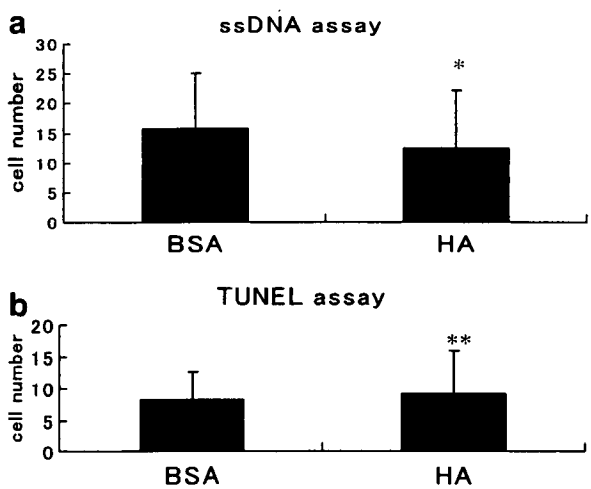

Fig. 4. Immunocytochemical staining for single stranded DNA and TUNEL. Immunocytochemistry for single stranded DNA (ssDNA) (a) and immunofluorescence analysis for Transferase-mediated dUTP Nick-End-Labeling (TUNEL) staining (b) of H322/CD44s cells cultured on HA or BSA coated dish. The number of ssDNA positive cells cultured on HA coated plate was similar to that of the BSA coated dish with both methods. The number of ssDNA-positive cells or TUNEL-positive cells in five fields of cytospin was counted at 400× and presented as means ± SD. $^{*}p = 0.6069$ vs BSA. $^{**}p = 0.7843$ vs BSA.

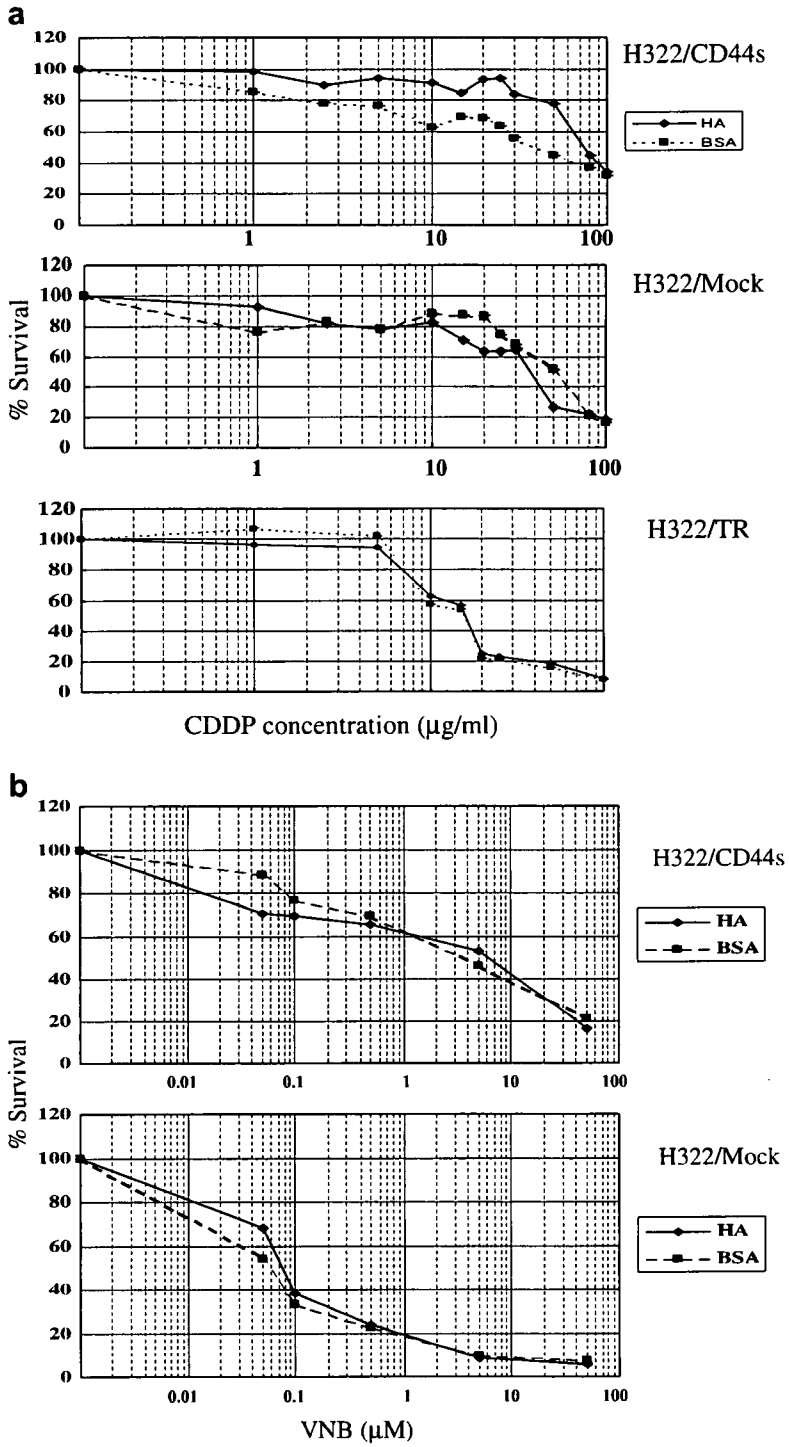

Fig. 5. In vitro chemosensitivity assay. Both H322/CD44s and H322/Mock cells were seeded to the 96-well flat bottom plates coated with HA (4 mg/ml) or BSA (10 mg/ml) and cultured for 72 h in the absence or the presence of various concentration of cisplatin (CDDP; a), vinorelbine (VNB; b), gemcitabine (GEM; c). The in vitro chemosensitivity of each cell line was evaluated by determining the number of surviving cells with the Cell-Counting kit-8. H322/TR cells were also examined in vitro chemosensitivity for CDDP. H322/CD44s cells cultured on HA were more resistant to CDDP than the cells cultured on BSA, while H322/Mock or H322/TR cells cultured on HA did not demonstrate resistance to CDDP (a). Interestingly, there were no resistance revealed for H322/CD44s cells cultured on HA to GEM or VNB compared with the cells cultured on BSA (b and c). As expected, H322/Mock cells cultured on HA did not reveal resistance against GEM or VNB (b and c).

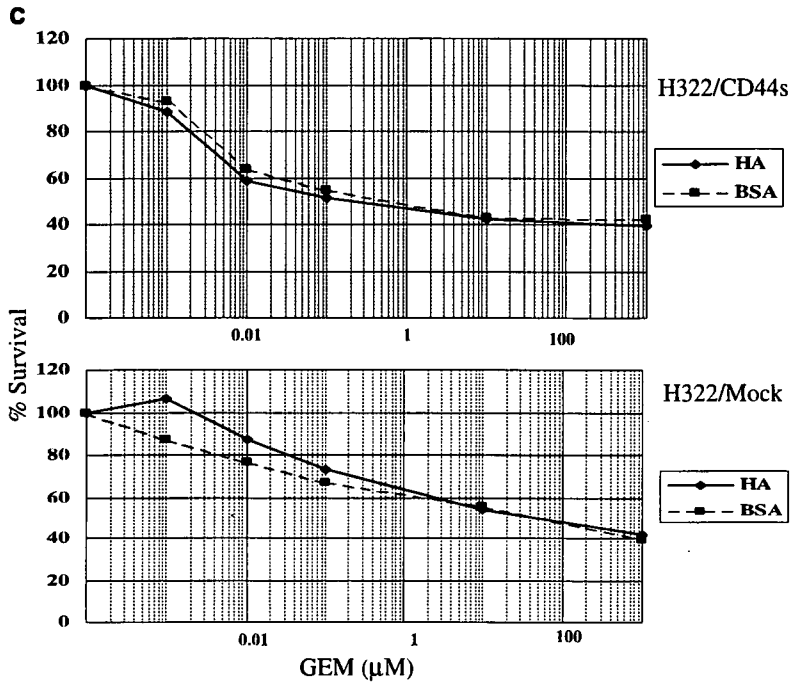

Fig 5. *(continued)*

3.3. The effect of reintroduction of CD44s on in vitro cell growth

To determine whether reintroduction of CD44s to NSCLC cells influences cell growth, the H322/Mock, H322/CD44s, and H322/TR cells were investigated in vitro. As expected reintroduction of CD44s or CD44TR to H322 NSCLC cell line did not influence in vitro cell growth rates (Fig. 3).

3.4. Examination for DNA fragmentation

To investigate whether the binding to HA or BSA influence apoptosis of H322/CD44s cells, we performed both single stranded DNA and TUNEL staining. The number of single stranded DNA positive cells cultured on BSA coated plate was not statistically different in comparison to that of the HA coated plate (Fig. 4a). In the same way, TUNEL positive cells observed in the wells pre-coated with BSA were similar to those of the wells pre-coated with HA (Fig. 4b). These results suggest that the binding of H322/CD44s cells to HA did not influence cell viability.

3.5. In vitro chemosensitivity assay

To evaluate the cytotoxicity of anti-cancer agents, H322 transfectants were exposed continuously to various concentration of CDDP, GEM, VNB for 72 h on either BSA or HA. H322/CD44s cells cultured on HA were more resistant to CDDP than the cells cultured on BSA, while neither H322/Mock cells nor H322/TR cells cultured on HA demonstrated resistance to CDDP (Fig. 5a). At the point of IC 50, H322/CD44s cells cultured on HA was twice more resistant to CDDP than the H322/Mock cells cultured on HA. Interestingly, H322/CD44s cells cultured on HA did not acquire chemoresistance to GEM or VNB compared to the cells cultured on BSA (Fig. 5b and c). H322/Mock cells cultured on HA did not demonstrate resistance to GEM or VNB (Fig. 5b and c). We also tested other clones of H322/CD44s, H322/Mock, and H322/TR. As expected, all clones revealed similar results (data not shown). These data indicate that the interaction between HA and CD44s receptor reduces chemosensitivity against CDDP in H322 cells.

3.6. Induction of MRP2 expression on H322/CD44s stable transfectants cultured on HA

To determine, the mechanism of acquiring chemoresistance, we investigated MRP2 expression on cell surface of H322 transfectants. Strong expression of MRP2 was induced on H322/CD44s cells by culturing on HA, but not on BSA (Fig. 6). In contrast, neither H322/Mock cells nor H322/TR cells cultured on HA induced upregulation of MRP2 expression compared to cells cultured on BSA. Additionally, we also examined whether MRP1 expression

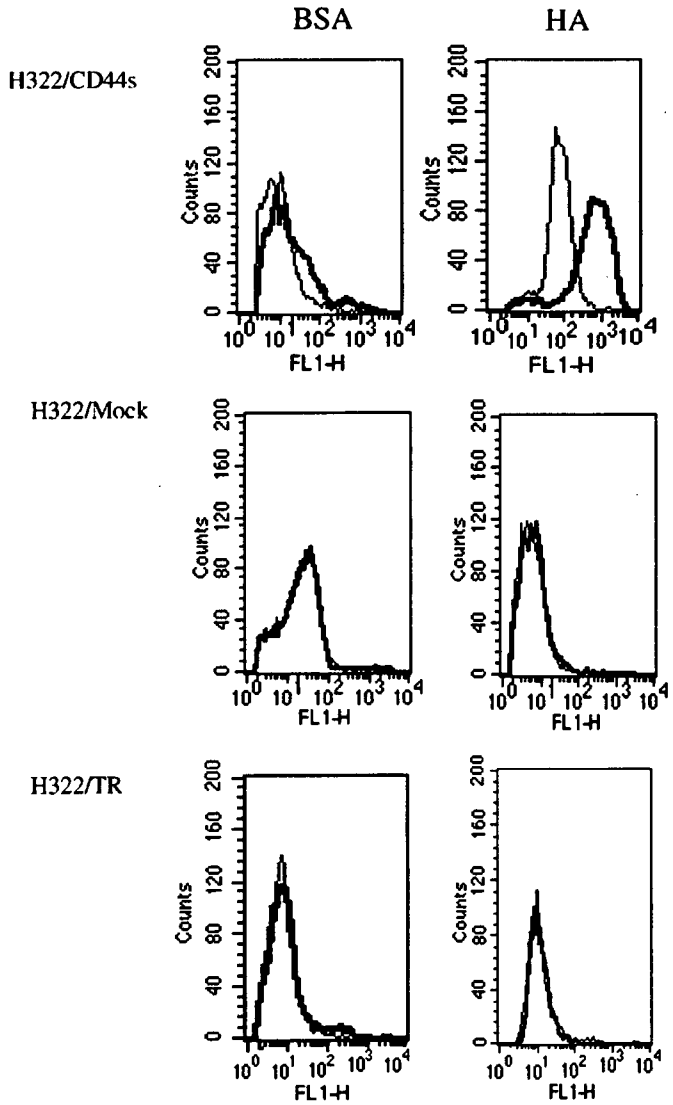

Fig. 6. Expression of MRP2 on H322/CD44s transfectants, H322 Mock, or H322/TR transfectants cultured on either BSA or HA. To determine MRP2 expression on H322/CD44s, cells were incubated with mAb M2 I-4 and analyzed with a FACScan™. Note that cell surface MRP2 expression was induced in H322/CD44s cultured on HA, but not on BSA. In contrast, neither H322/Mock nor H322/TR cells cultured on HA demonstrated induced upregulation of MRP2 expression compared to cells cultured on BSA. Bold lines indicate MRP2 expression, while normal lines indicate background immunofluorescence.

on H322/CD44s cells cultured on HA is induced or not. H322/CD44 cells natively express MRP1, however no induction was observed (data not shown).

3.7. Abrogation of induced MRP2 expression on H322/CD44s transfectants cultured on HA by MRP2 inhibitor, MK571

To determine whether the upregulation of MRP2 expression on H322/CD44s cells cultured on HA is involved in the mechanism of acquiring chemoresistance to CDDP, we also performed chemosensitivity assay and cytometric analysis in the absence or the presence of MRP2 inhibitor, MK571. As expected, significant reversal of CDDP resistance was achieved (Fig. 7a and induced MRP2 expression was diminished (Fig. 7b) at final MK 571 concentration of 50 μM. These results suggest that the upregulation of MRP2 expression on H322/CD44s cells cultured on HA is involved in the mechanism of acquiring chemoresistance to CDDP.

4. Discussion

According to past reports, tumor cells may actively reorganize their microenvironment to increase cell adhesion and drug resistance [12–15].

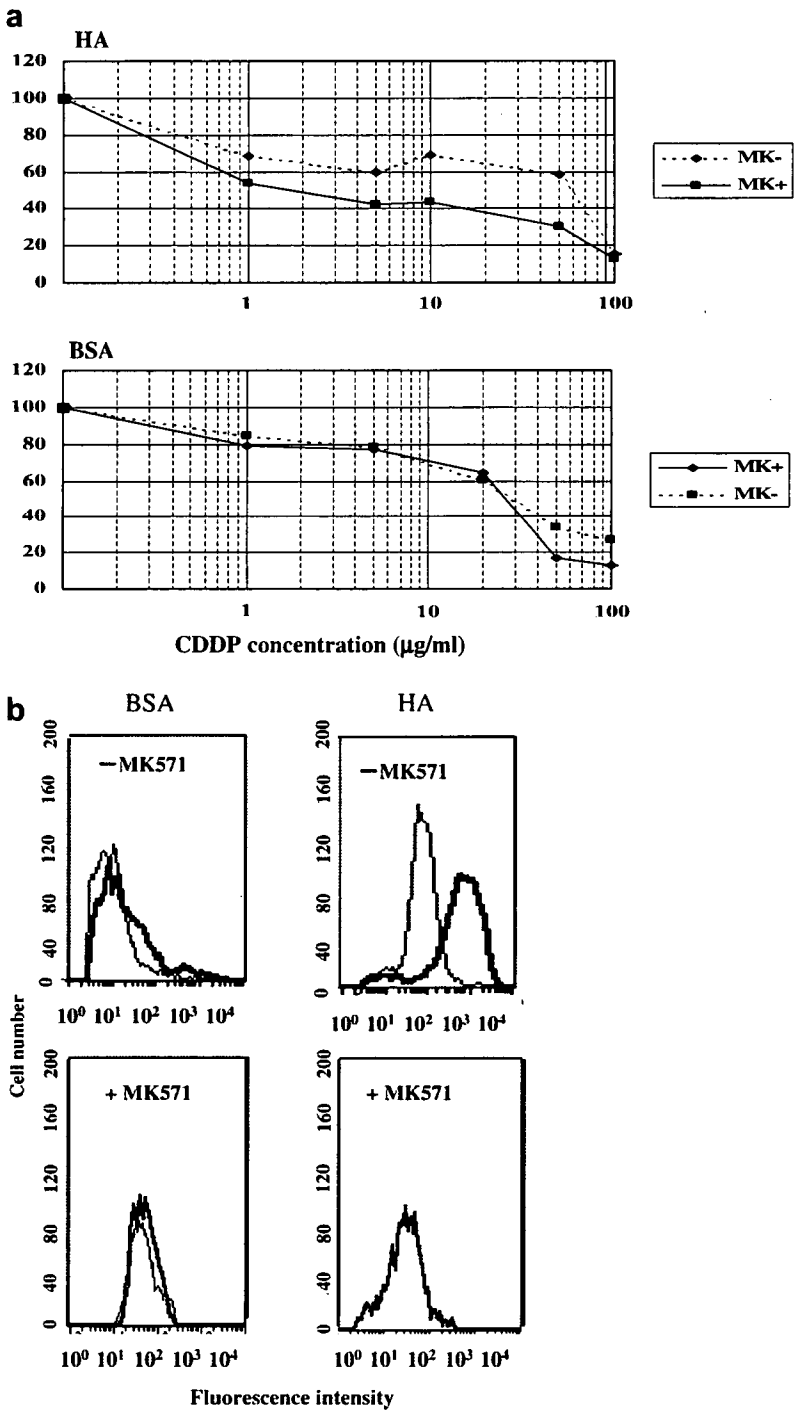

Fig. 7. Abrogation of induced MRP2 expression on H322/CD44s transfectants cultured on HA reverse CDDP resistance. (a) H322/CD44s cells were seeded to the 96-well flat bottom plates coated with HA (4 mg/ml) or BSA (10 mg/ml) and cultured for 72 h with various concentration of CDDP in the absence or presence of 50 μM of MRP2 inhibitor, MK571. As expected, significant reversal of CDDP resistance of H322/CD44s cells cultured on plates coated with HA was achieved in the presence of MK571. (b) MRP2 expression was analysed with FACScan™ in H322/CD44s cells cultured on HA or BSA in the absence or the presence of MK571. MRP2 expression was completely abolished in the presence of MK571 in H322/CD44s cells cultured on HA, while it was not affected by MK571 on BSA. Bold lines indicate MRP2 expression, while normal lines indicate background immunofluorescence.

In fact, we often experience that the response to chemotherapeutic agents is variable among the metastasized organs in the clinical setting. These findings suggest that the tumor surrounding microenvironment may play a pivotal role in regulating chemosensitivity to anticancer drugs. For instance, it has been revealed that leukemic cells and small cell lung cancer cells attain chemoresistance by interacting integrin with fibronectin on stroma cells [4,16]. However, to the best of our knowledge, there have been no studies revealing that HA regulates NSCLC cell resistance to anticancer drugs. In our study, we demonstrated that interaction between HA and CD44s is involved in chemoresistance of NSCLC to CDDP. The signal induced by CD44s binding to HA appears to play a pivotal role in the acquisition of chemoresistance by H322/CD44s cells since the binding to HA of H322/TR cells whose intracellular domain of CD44s is deleted did not acquire chemoresistance to CDDP.

One of the principle chemotherapeutic agents for the treatment of advanced NSCLC patients is CDDP. Its accumulation can be modulated by ouabine, osmolarity, pH, cyclic adenosine monophosphate (cAMP) and amphotericin B [17–20]. Decreased accumulation of CDDP is reported in NSCLC cell lines resistant to CDDP [3]. One of the ATP-binding cassette transporters, cMOAT2/MRP3, which is 45% identical to MRP2/cMOAT, actively transports anticancer drugs including CDDP out of the cells in an ATP dependent manner [21], and MRP2 is considered to play a principle role in CDDP resistance [22]. Moreover, there are various other pathways considered in the acquisition of chemoresistance against CDDP. Nakagawa et al. reported that low gluthatione S transferase (GST) mRNA levels in small cell lung cancer cell lines correlated inversely with high sensitivity to CDDP and suggested that GST may play an important role in intracellular inactivation of CDDP [23,24]. Metallothioneins are also reported as modulators of cellular sensitivity to electrophilic anticancer agents including CDDP [25]. High mobility group (HMG) 1, which recognizes CDDP-modified DNA, have been identified as possible DNA repair-proteins, and that the HMG1 cDNA transfectant demonstrated resistance to CDDP [26]. Recently, excision repair cross-complementation group 1 (ERCC1) protein is also involved in the sensitivity of NSCLC cells to CDDP [27]. In this study, we could not comprehensively determine how much these mechanisms contribute to CD44s-HA mediated chemoresistance to CDDP. However, MRP2 upregulation appears to be involved in CDDP chemoresistance in this study, because MRP2 inhibitor, MK571, reverses CDDP resistance by completely diminishing MRP2 expression.

Even though we could not exclude the contribution of other mechanisms in the acquisition of chemoresistance, the determination of the amount of HA and CD44s expression on NSCLC specimens appears to provide valuable information regarding chemoresistance to CDDP in NSCLC. Moreover, HA–CD44s signaling upregulates matrix metalloproteinase-2 secretion in a human lung carcinoma cell line [28] and melanoma cell line [29]. In addition to chemoresitance mediated by HA–CD44s interaction, these findings provide supporting evidence to explain the previous report that increased HA and/or CD44 expression has correlated with unfavorable outcomes in patients with lung adenocarcinoma [30].

In this study, we revealed that the interaction of HA and CD44s upregulates MRP2, resulting in the acquisition of CDDP chemoresistance in NSCLC in vitro. Perturbation of this HA–CD44s-mediated signaling pathway may be a promising target to overcome CDDP resistance in NSCLC in the future.

Conflict of interest statement

None of the authors have financial relationship with a commercial entity that has an interest in the content of this manuscript.

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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

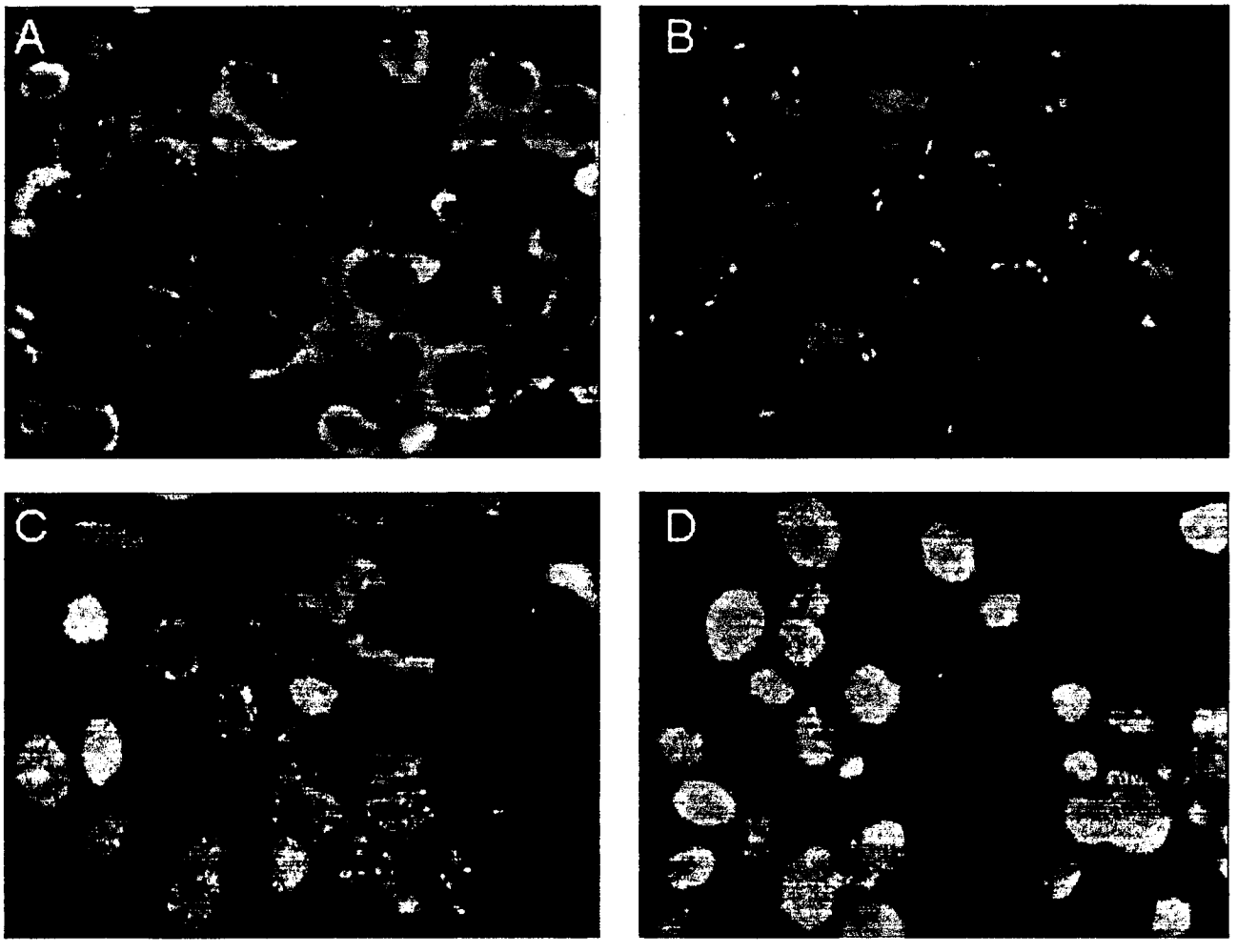

FIGURE 1. Fluorescence in situ hybridization of nonsmall cell lung cancer cells. The chromosome 7 centromere (CEP7) signals are green, and the epidermal growth factor receptor gene (*EGFR*) signals are red. A, disomy; B, low trisomy; C, high polysomy; D, high gene copy number.

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	.0001
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*
Exon 21	5	3	60.0	
Wild type	42	6	14.3	
EGFR gene amplification†				
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.

* *P* value for comparison of mutations in exons 19 and 21.

† *EGFR* gene amplification could not be evaluated in 5 patients because of the small size of the tissue specimen.

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	.0885
Nonadenocarcinoma	3/15	20		4/14	28.6	
Smoking status						
Never	10/21	47.6	.0177	11/17	64.7	.0988
Current/former	7/38	18.4		15/37	40.5	

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

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%), 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

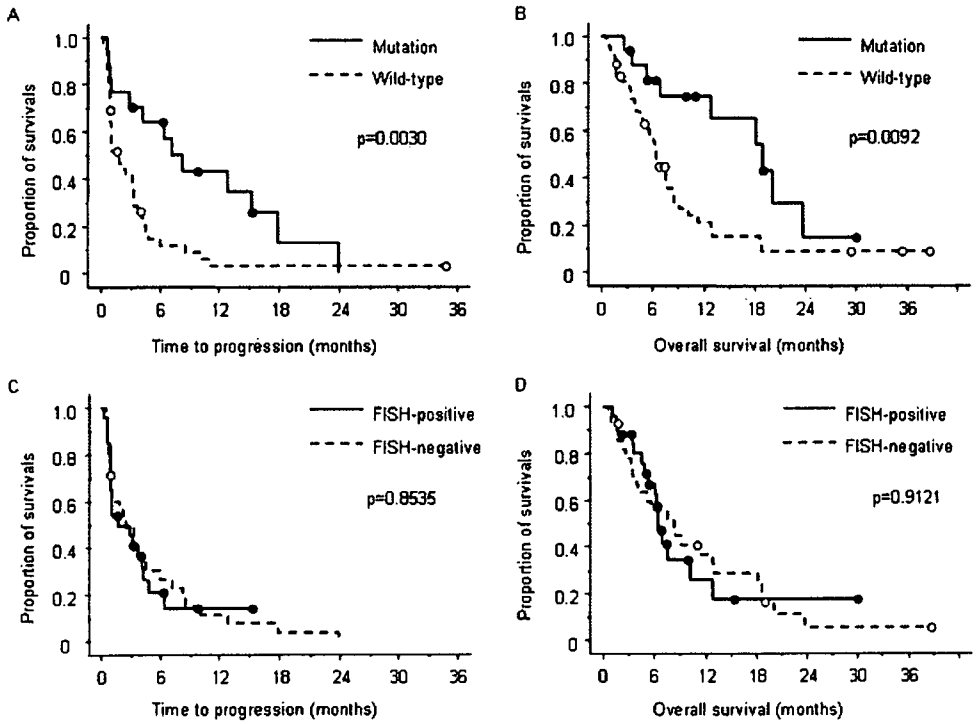

FIGURE 2. Kaplan-Meier estimates of time-to-progression and overall survival grouped by epidermal growth factor gene (*EGFR*) mutation (A,B) and by *EGFR* amplification (C,D).The solid and open dots represent censored patients. FISH indicates fluorescence in situ hybridization.

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

not associated with prolonged survival. Previous studies have shown an approximately 3 times higher incidence of *EGFR* mutations in East Asians than in Caucasians.[7,9–12,16,17,19,21,28–30] FISH-positive results do not appear to contribute significantly to the response to gefitinib or to survival in populations with high percentages of *EGFR* mutations.

The incidence of L858R in our study seemed low compared with the incidence of exon 19 deletion. Previous studies have demonstrated that the incidence of deletion mutations in exon 19 is almost the same as the incidence of point mutations in exon 21.[7,9–12,16,17,19,21,28–30] Because the direct sequencing method usually was used to detect *EGFR* mutations, it is unlikely that the low frequency of the L858R mutation was caused by assay-related, false-negative findings. Our results of the incidence of L858R mutation and exon 19 deletion mutations may also produce some distortion with regard to the analysis of gene copy numbers because the number of patients with high gene copy numbers has been observed to be higher in those with deletion mutations in exon 19 than with point mutations in exon 21. Further analyses in much larger groups of patients will be necessary to clarify the frequency of the 2 most common mutations.

Takano et al. demonstrated an association between increased *EGFR* copy numbers measured by quantitative PCR (qPCR) and both higher a response rate and longer TTP.[15] Dziadziuszko et al. reported that EGFR messenger RNA (mRNA) expression in tumor samples measured by qPCR was a predictive biomarker for response to gefitinib and longer progression-free survival. Those investigators also demonstrated that *EGFR* mRNA expression measured by qPCR was correlated significantly with FISH-positive results.[31] It is possible that qPCR may enable us to make a more reliable distinction between specific and nonspecific amplification of the *EGFR* gene.[13] We plan to compare *EGFR* gene copy numbers in corresponding samples measured with qPCR to confirm our results. We classified all patients into a FISH-positive group and a FISH-negative group according to the scoring system published by Cappuzzo et al. *EGFR* gene copy numbers also may vary according to ethnicity, similar to the differences in *EGFR* mutation frequency. The definition of FISH-positive results may need to be modified to use it as a predictor of gefitinib efficacy in Japanese patients with NSCLC.

In conclusion, the results of the current study suggest that the presence of *EGFR* mutations detected in biopsy specimens is an independent and significant predictor of response to gefitinib and survival in Japanese patients with advanced NSCLC. However, the role of *EGFR* gene amplification was not identified as a predictor of gefitinib efficacy in Japanese patients. Precise measurements are needed, and the validity of the classification must be confirmed in a prospective study.

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ORIGINAL ARTICLE

Akt-dependent nuclear localization of Y-box-binding protein 1 in acquisition of malignant characteristics by human ovarian cancer cells

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Y-box-binding protein 1 (YB-1), which is a member of the DNA-binding protein family containing a cold-shock domain, has pleiotropic functions in response to various environmental stimuli. As we previously showed that YB-1 is a global marker of multidrug resistance in ovarian cancer and other tumor types. To identify YB-1-regulated genes in ovarian cancers, we investigated the expression profile of YB-1 small-interfering RNA (siRNA)-transfected ovarian cancer cells using a high-density oligonucleotide array. YB-1 knockdown by siRNA upregulated 344 genes, including *MDR1*, *thymidylate synthetase*, *S100 calcium binding protein* and *cyclin B*, and downregulated 534 genes, including *CXCR4*, *N-myc downstream regulated gene 1*, *E-cadherin* and *phospholipase C*. Exogenous serum addition stimulated YB-1 translocation from the cytoplasm to the nucleus, and treatment with Akt inhibitors as well as Akt siRNA and integrin-linked kinase (ILK) siRNA specifically blocked YB-1 nuclear localization. Inhibition of Akt activation downregulated *CXCR4* and upregulated *MDR1* (*ABCB1*) gene expression. Administration of Akt inhibitor resulted in decrease in nuclear YB-1-positive cancer cells in a xenograft animal model. Akt activation thus regulates the nuclear translocation of YB-1, affecting the expression of drug-resistance genes and other genes associated with the malignant characteristics in ovarian cancer cells. Therefore, the Akt pathway could be a novel target of disrupting the nuclear translocation of YB-1 that has important implications for further development of therapeutic strategy against ovarian cancers.

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Introduction

The Y-box-binding protein 1 (YB-1), which is a DNA/RNA-binding protein also known as dbpB, regulates transcription, translation, DNA damage repair and other biological processes in both the nucleus and cytoplasm (Matsumoto and Wolffe, 1998; Kohno *et al.*, 2003). In the cytoplasm, YB-1 regulates mRNA stability and translational regulation (Evdokimova *et al.*, 2001; Ashizuka *et al.*, 2002; Fukuda *et al.*, 2004), while in the nucleus, it plays a pivotal role in transcriptional regulation through specific recognition of the Y-box promoter element (Ladomery and Sommerville, 1995; Kohno *et al.*, 2003). Interaction of YB-1 with its cognate Y-box-binding site (inverted CCAAT box) is promoted by cytotoxic stimuli, including actinomycin D, cisplatin, etoposide, ultraviolet (UV) and heat shock, leading to the activation of a representative ABC transporter *MDR1/ABCB1* and DNA topoisomerase IIα genes (Asakuno *et al.*, 1994; Furukawa *et al.*, 1998; Ohga *et al.*, 1998). YB-1 also selectively interacts with damaged DNA or RNA, and protects from cytotoxic effects following cellular exposure to cisplatin, mitomycin C, UV and oxygen radicals (Ohga *et al.*, 1996; Ise *et al.*, 1999).

Royer and co-workers were the first to report that nuclear localization of YB-1 is associated with intrinsic *MDR1* expression in human primary breast cancer (Bargou *et al.*, 1997). Immunostaining analysis of various human cancers also supported this result, and showed that nuclear expression of activated YB-1 was closely associated with the acquisition of P-glycoprotein-mediated multidrug resistance (Kuwano *et al.*, 2004). YB-1 has also been shown to induce basal and 5-fluorouracil-induced expression of the major vault protein (*MVP/LRP*) gene, the promoter of which contains a Y-box (Stein *et al.*, 2005). In human malignancies, vault proteins are involved in acquiring drug resistance (Mossink *et al.*, 2003). Taken together, these findings suggest that nuclear localization of YB-1 might play a key role in the acquisition of global drug resistance through transcriptional activation of relevant genes and the repair of damaged DNA (Kuwano *et al.*, 2004).

The nuclear localization of YB-1 is required for transcription and DNA repair in response to various environmental stimuli, such as adenovirus infection (Holm *et al.*, 2002), DNA-damaging agents, UV irradiation, hyperthermia (Stein *et al.*, 2001) and serum stimulation (En-Nia *et al.*, 2005). However, as a nucleocytoplasmic shuttling protein, it is important to understand which signalling molecules are involved in the translocation of YB-1 into the nucleus. Koike *et al.* (1997) first reported the possible role of protein kinase C in YB-1 nuclear translocation in cancer cells exposed to UV irradiation, and highlighted the importance of the YB-1 C-terminal region in cytoplasmic retention. Other studies have suggested the involvement of additional molecules: thrombin-mediated YB-1 nuclear translocation was shown to be inhibited by protein tyrosine phosphatase inhibitor in endothelial cells (Stenina *et al.*, 2000), while Dooley *et al.* (2006) demonstrated the involvement of Jak1 in YB-1 nuclear translocation. Sutherland *et al.* (2005) recently reported that phosphorylation of YB-1 by Akt at serine 102 in the cold-shock domain is required for YB-1 nuclear translocation in cancer cells. Another mechanism for nuclear translocation of YB-1 was shown to be promoted by various cytotoxic anticancer agents, which trigger the proteolytic cleavage by the 20S proteasome of the YB-1 C-terminal fragment containing the cytoplasmic retention signal (Sorokin *et al.*, 2005). In our present study, we have provided evidence that Akt activation is one of the mechanisms for nuclear translocalization of YB-1, and also that YB-1 regulates expression of various cell growth and malignant progression-related genes as well as global drug resistance-related genes including *MDR1*.

Results

Suppression of YB-1 leads to an enhancement of MDR-1 expression and decrease of CXCR-4 expression

We previously reported that YB-1 was expressed in the nucleus in almost 30% of serous ovarian cancers, and that YB-1 nuclear-positive patients had a poor prognosis (Kamura *et al.*, 1999). As nuclear translocation of YB-1 is highly susceptible to environmental stimuli, we first examined whether the stress-inducing exogenous addition of serum could stimulate nuclear translocation of YB-1 in seven serum-deprived human ovarian cancer cell lines. Among the seven cell lines, nuclear YB-1 translocation was stimulated more than twofold in two: RMG-III and SKOV-3 (Figure 1a). In these two lines, serum incubation markedly enhanced Akt phosphorylation and increased translocation of YB-1 into the

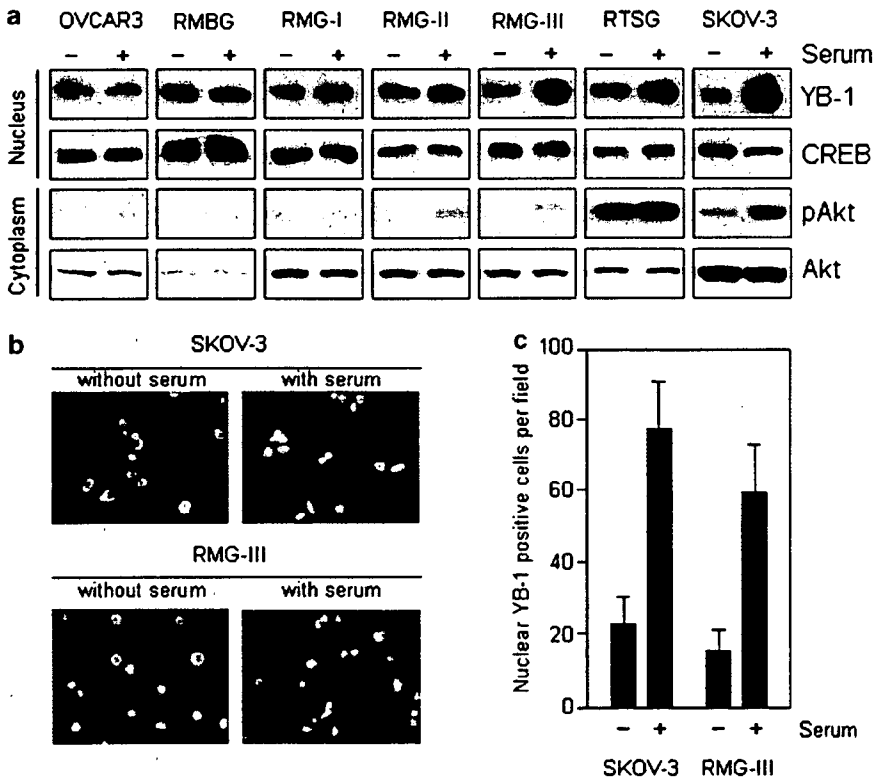

Figure 1 Levels of Akt phosphorylation and nuclear localization of YB-1 in ovarian cancer cell lines with or without serum stimulation. (a) Cytoplasmic and nuclear extracts were prepared 1 h after 10% serum stimulation. Anti-YB-1 and anti-CREB immunoblots were performed on nuclear extracts, and anti-pAkt and anti-Akt immunoblots were performed with cytoplasmic extracts. CREB and Akt are shown as a loading control. (b) Immunofluorescent staining of YB-1 in ovarian cancer cells. Cells stimulated with or without serum for 1 h were fixed and permeabilized, incubated at 4°C with the primary YB-1 antibody, then with the Alexa Flour 546-labelled secondary antibody. (c) Quantitative analysis of YB-1 nuclear localization as shown in Figure 1b. Data are mean of three independent experiments; bars ±s.d.

nucleus, as shown by immunofluorescense analysis (Figure 1b and c).

Although YB-1 is known to regulate the expression of several genes at the transcriptional level, the complete network of genes associated with YB-1 has not been elucidated. We therefore, explored the expression profile of YB-1 siRNA-treated SKOV-3 cells and mock-treated SKOV-3 cells using a high-density oligonucleotide microarray. We transfectd YB-1 siRNA into SKOV-3 cells at a concentration of 200 and 400 nM. Transfection of 200 nM YB-1 siRNA decreased expression of YB-1 mRNA by only 45%, whereas 400 nM YB-1 siRNA decreased by 70% (Figure 2). Of the 54675 RNA transcripts and variants in the microarray, we identified 344 genes that were increased more than twofold and 534 genes that were decreased 0.5-fold or less in both 200 and 400 nM YB-1 siRNA-transfected cells (Supplementary Table S1). Upregulated genes were classified into 'cell cycle' ($P<0.0001$), 'cytoskeleton organization and biogenesis' ($P=0.0003$), 'cell growth and/or maintenance' ($P=0.0005$), and GO SLIMS Biological Process' ($P=0.0013$). Downregulated genes were classified into 'catalytic activity' ($P=0.0007$) and 'transferase' ($P=0.0010$). We selected 46 genes that we expected to be associated with drug resistance, cell growth, cancer malignant progression and cell signalling (Table 1), and chose three of these for further study: *MDR1*, *MVP/LRP* and chemokine (C-X-C motif) receptor 4 (*CXCR4*).

We used quantitative real-time PCR (QRT-PCR) to confirm whether expression of these three genes was modulated in YB-1 siRNA-transfected cells. Expression of *CXCR4* decreased by 67%, whereas expression of *MVP/LRP* was unaffected by the siRNA (Figure 2). *MDR1* expression was increased approximately 30-fold in 400 nM YB-1 siRNA-transfected cells compared with control siRNA-transfected cells. The results of QRT-PCR are broadly consistent with those of the microarray analysis.

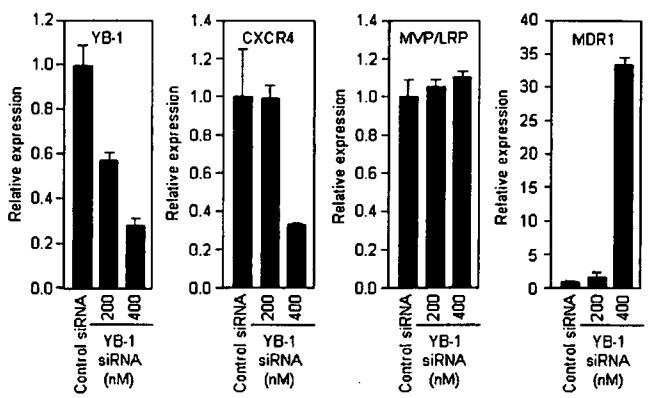

Figure 2 Effect of YB-1 knock down on expression of MDR1, MVP/LRP and CXCR4. SKOV-3 cells were treated with YB-1 siRNA for 48 h and then total RNA was prepared. QRT-PCR was performed for MDR1, MVP/LRP, CXCR4, YB-1 and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\mathrm{GAPDH}) - C_t(\mathrm{target}))}$ which reflected target gene expression normalized to GAPDH levels. Data were mean of three independent experiments; bars ± s.d.

Pearson correlation and hierarchical cluster analysis of selected NCI-60 genes

We next examined a database containing the expression profile of the National Cancer Institute (NCI)-60 panel from the Developmental Therapeutics Program (http://www.dtp. nci.nih.gov/), shown as a log of mRNA expression level in the NCI screen. When the Pearson correlation coefficients were calculated, YB-1 was negatively correlated with *MDR1* expression, positively correlated with *CXCR4* expression and showed little correlation with *MVP/LRP* (Figure 3). Moreover, the hierarchical dendrogram of gene expression revealed that *YB-1* and *CXCR4* belong to the same cluster, whereas *MDR1* and *MVP/LRP* are clustered in a separate group (Figure 4). Together, these NCI-60 panels suggest that cellular levels of YB-1 negatively modulate expression of *MDR1* and positively regulate expression of *CXCR4*. In this cluster analysis, six ovarian cancer cell lines including SKOV-3 showed various correlation coefficiencies with YB-1 expression. Our oligonucleotide array analysis was performed only with SKOV-3, and correlation coefficiencies among ovarian cancer cell lines would depend upon which cell line was analysed.

Akt activity is prerequisite for nuclear translocation of YB-1and transcriptional regulation by YB-1

Phosphorylation of YB-1 by Akt is a necessary requirement for its translocation from the cytoplasm into the nucleus (Sutherland *et al.*, 2005). We therefore investigated the effect of two inhibitors of Akt activation (LY294002 and 1L-6-hydroxymethyl-*chiro*-inositol 2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate) on serum-stimulated SKOV-3 cells. Both Akt inhibitors markedly blocked the nuclear accumulation of YB-1, whereas treatment with inhibitors of MEK (U0126), p38MAPK (SB203580) and JNK (SP600125) had no effect on nuclear translocation (Figure 5a). In addition, phosphorylation of Akt was inhibited by LY294002 and octadecylcarbonate, but not by U0126, SB203580 and SP600125. Immunofluorescence analysis with a YB-1 antibody also demonstrated the predominant accumulation of YB-1 in the cytoplasm when treated with LY294002 and octadecylcarbonate (Figure 5b and c). As Akt inhibitors blocked the nuclear translocation of YB-1, we examined whether they could also affect expression of YB-1-regulated genes. *CXCR4* expression was found to be downregulated in a dose-dependent manner following treatment with the Akt inhibitors when determined by QRT-PCR analysis (Figure 5d). Treatment with Akt inhibitors upregulated the expression of *MDR1*, but not *MVP/LRP*.

SKOV-3 cells expressed high level of Akt1 protein, very low level of Akt2 protein, and no Akt3 protein when assayed by immunoblotting analysis (Figure 6a). We introduced siRNA targeting Akt or ILK into SKOV-3 cells at a concentration of 100 and 10 nM,