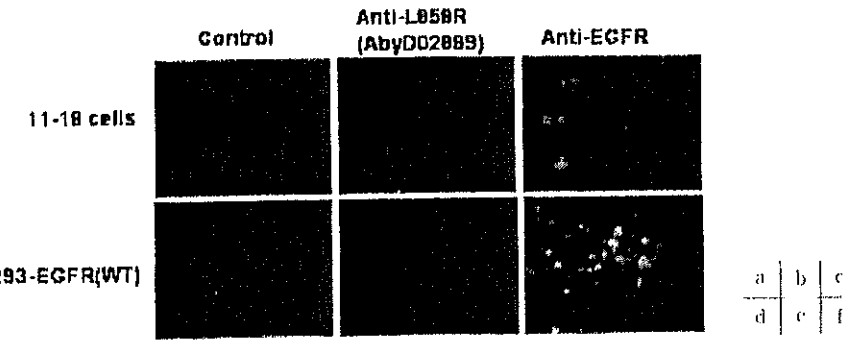

Fig. 5 Specificity of AbyD02889 against EGFR (L858R) protein intrinsically expressed in the 11-18 cells. The 11-18 cells and HEK293-expressing EGFR (WT)-myc (293-EGFR(WT)) were ectopically plated on a PLL-coated 24-well plate at 5,000 cells/well. After fixation, permialization, and blocking, the cells were probed with the AbyD02889 (b and e) and anti-EGFR antibodies (Cell Signaling) (c and f) followed by probing with the FITC-conjugated anti-His antibody as a second antibody. The signal was examined with fluorescence microscopy. The fluorescence views of 11-18 cells and 293-EGFR(WT) cells are shown in the upper (a, b, and c) and lower panels (g, h, and i), respectively.

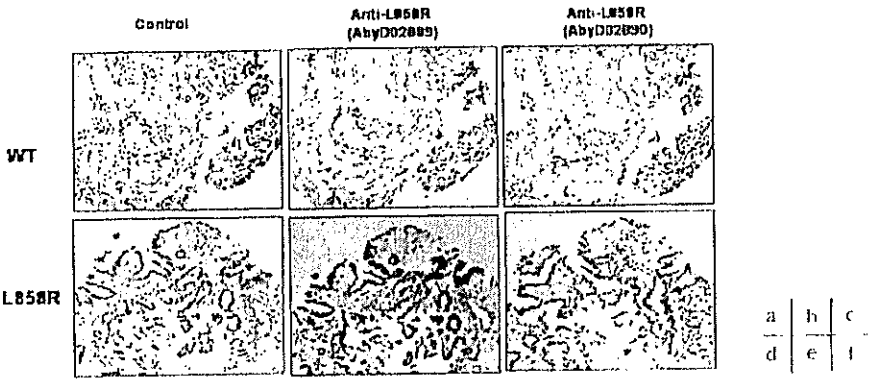

Fig. 6 Immunohistochemical analysis of two clinical specimens of non-small cell lung cancer whose EGFR status had been confirmed by the direct sequencing method.
The upper and lower three panels show DAB staining by AbyD02889 and AbyD02890 for tissue sections of EGFR (WT)- and EGFR (L858R)-expressing NSCLC, respectively.

cells transfected with the wild-type EGFR (293-EGFR (WT)) were negative for the antibody. On the contrary, an anti-EGFR antibody (from Cell Signaling) detected both the 11-18 and 293-EGFR (WT) cells (Figure 5, panels c and f). These results showed that the mutant EGFR protein expressed in human cells could be detected with specificity by AbyD0889.

Immunohistochemical evaluation of AbyD02889 and AbyD02890

Finally, we evaluated the immunohistochemical assay for two clinical specimens of NSCLC whose EGFR status had been confirmed by direct sequencing. The upper sections of an NSCLC tumor with no EGFR mutation were negative for both AbyD02889 and AbyD02890 (Figure 6, panels b and c) while the lower sections with the L858R mutation were positive for AbyD02889 but negative for AbyD02890, suggesting that AbyD02889 could specifically detect the EGFR (L858R)-expressing cells even in a paraffin embedded section and might be useful for the immunohistochemical examination of EGFR mutations.

Discussion

We developed an antibody specifically recognizing a mutant EGFR (L858R) protein by screening a HuCAL phage library. We found that this specific antibody (AbyD02889) could specifically detect the mutant protein and was available for ELISA, immunoblotting, immunocytochemistry, and immunohistochemistry. Here, we

would like to discuss the feasibility of the use of this antibody in the cancer research field and in the clinical situation

Recent cancer research has shown trends toward the discovery of a molecular target which is necessary to maintain tumor survival or growth.[14] This may possibly lead to the development of a specific inhibitor for molecular targets characteristic of various tumor species, which may become one promising therapeutic strategy for cancer. Typical cases of this strategy are the currently successful results of tyrosine kinase inhibitors such as Iressa, tarceva, and glivec.[15-19] Gefitinib has been reported to be effective especially against NSCLC patients with E746 A750del in exon 19 or L858R in exon 21, which account for approximately 90% of the NSCLC-associated EGFR mutations.[8,6] In other words, the development of Iressa has made a revolutionary contribution to lung cancer treatment. The EGFR mutation in Japanese NSCLC patients has been reported to be relatively high in frequency, although there are differences among the various human racial types.[20,21]

Under these circumstances, it is important to know how to select the group of patients in whom the benefit of Iressa may be maximized. Based on this view, we have developed the Scorpion ARMS method, a highly-sensitive PCR-based detection method of gene mutation, and have reported its reliability and feasibility for clinical use.[9,10] In addition to the high sensitivity, this method has another great advantage that contamination of the wild-type EGFR gene derived from normal tissue surrounding the tumor does not interfere with its sensitivity or specificity. However, because there were a few false negative or positive cases,[9] it is still impossible to say that this method is perfect. For example, when we examine the genome DNA containing a lower amount of the mutant EGFR gene than the lower detectable limit (this is considered to be caused by the situation that only a small number of tumor cells having EGFR mutation exist in the tissue sample), the Scorpion-ARMS technique may possibly fail to detect this mutation. Although this is considered as one limitation of the ARMS technology, we still have to make a greater effort to improve the precise detection of the mutation. One strategy is to detect the protein of the mutant EGFR using a mutant-specific antibody, which would enable us to improve the detectability through the use of the antibody and the Scorpion-ARMS method in combination. From this point, we stress the significance of the mutant EGFR (L858R)-specific antibody and would like to discuss its feasibility.

This antibody enables us to examine the mutation using one tumor cell, which is a great advantage in addition to its contribution to improvement of the detectability of the mutation. Actually, Figures 4 and 5 show that only the cells expressing mutant EGFR (L858R) protein were stained with the mutant-specific antibody. One tumor cell is the smallest tumor sample necessary for examination. Using the mutant-specific antibody we have developed, it is therefore possible to detect a single cell mutation, meaning that we can diagnose the EGFR mutation in one tumor cell derived from the smallest clinical sample with a less invasive approach. This antibody offers the potential to make a large contribution to the clinical evaluation of the EGFR mutation. Furthermore, this antibody shows promising importance when considering tumor oncogenesis and progression from the aspect of the research field. At present, it remains controversial whether a hit on the EGFR gene causes the development of cancer or if one heteropopulation of cancer cells in the tumor acquires the EGFR mutation. This discussion is considered to be related largely to the clinical responsiveness of cancer to Iressa, and its prognosis. According to the latter hypothesis of oncogenesis, the tumor cells with wild-type EGFR, to which Iressa may be less effective, will survive and grow even while Iressa responds well to the cells harboring the EGFR mutation. There we will find that the mutation-positive and negative tumor cells co-localize in one tumor mass using our EGFR (L858R)-specific antibody. Finally, we would like to say that our EGFR (L858R)-specific antibody will be a useful tool to obtain more important information on NSCLC in the clinical situation or even in the research field as well. Our current research strategy is focused on developing a specific antibody against deletional EGFR mutants.

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Gefitinib Efficacy Associated with Multiple Expression of HER Family in Non-small Cell Lung Cancer

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Abstract. *The aim of this study was to compare the relationship between HER family expression and clinical response to gefitinib. Patients and Methods: Tissues from thirty-one non-small cell lung cancer (NSCLC) patients treated with a monotherapy of gefitinib were analyzed. Expressions of HER family in 31 tumors were examined by immunohistochemistry. Results: The total expressions were 21 for EGFR (68%), 24 for HER2 (77%), 17 for HER3 (55%) and 4 for HER4 (13%). Fourteen out of 31 (45%) demonstrated triple expression of EGFR and HER2, as well as HER3 or HER4. A significantly better response rate (RR) and time to progression (TTP) were observed for the group with the triple expression than for the other groups (RR 50 vs. 11%; p<0.05, median TTP 4.29 vs. 1.2 months; p<0.05). Conclusion: Mutliple expression of the HER family might be related with the clinical response to gefitinib and EGFR mutation status.*

The epidermal growth factor receptor (EGFR/HER1) is a promising target for anticancer therapy. Gefitinib (ZD 1839, Iressa; AstraZeneca, London, UK) is an orally active, selective EGFR-tyrosine kinase inhibitor (1), which showed promise in a recent clinical trial of non-small cell cancer (NSCLC) cases, in terms of rapid symptom improvements (2-7) and clinically meaningful benefit in some patients (5, 7). Thus, the selection of individuals who may demonstrate a response to gefitinib is important. The degree of EGFR expression seems not to directly determine the response, although gefitinib is considered to be a targeted therapy by virtue of its selective inhibition of EGFR tyrosine kinase (8-10). Recent reports show that specific missense and deletion mutations in the tyrosine kinase domain of the EGFR gene (11-13) are associated with EGFR tyrosine kinase inhibitor sensitivity. Although these EGFR mutations can account for almost all objective responses to tyrosine kinase inhibitors, the clinical benefit observed with these drugs and the survival benefit cannot be explained only by the presence of mutations.

The HER family includes the following four distinct receptors: EGFR, HER2 (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4) and recent preclinical studies indicated that gefitinib causes reduced phosphorylation levels of not only EGFR, but also of HER2 and HER3 (14), inducing the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers (15). Based on these data, the co-expression profile of HER family receptors (especially the additional expression of HER2 and HER3) was hypothesized to play an important role in determining the efficacy of gefitinib in NSCLC cases. The relationship between the co-expression status of HER family members and gefitinib efficacy was evaluated with regard to response rate (RR), time to progression (TTP) and overall survival (OS).

Patients and Methods

Between September 2002 and January 2004, 31 advanced or recurrent NSCLC patients from whom tumor tissues were available, were treated with 250 mg of gefitinib monotherapy until disease progression at our institution. The medical records, pathology slides and imaging studies of these patients were retrospectively reviewed. The study was conducted after obtaining approval of the appropriate ethical review boards following recommendations of the Declaration of Helsinki for biomedical research involving human subjects.

For all patients, archival paraffin blocks of transbrochial lung biopsy (TBLB) specimens taken at the time of initial diagnosis (n=14) or tumor tissue specimens obtained by surgical resection (n=17) were sectioned for staining with antibodies against EGFR, HER2, HER3 and HER4 using an EGFR pharmDx kit (DAKO), Herceptest (DAKO), anti-HER3 (Chemicon) and anti-HER4 (Chemicon), respectively, with the Autostainer (DAKO).

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Key Words: NSCLC, gefitinib, HER2, tyrosine kinase inhibitor, molecular target agent, EGFR mutation.

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The results of the immunostaining were reviewed by an experienced pathologist (Y.I.). For HER2 staining, only moderate to strong cell membrane-specific immunostaining was considered positive, whereas cytoplamic staining was also taken into account for the other receptors.

Genetic analysis of the *EGFR* gene was performed on the 17 frozen tumor specimens obtained by surgical resection. Genomic DNA was extracted from 1-2 mm^3 tumor specimens using REDExtract-N-AmpTM Tissue PCR Kit (Sigma) and the DNA was purified with a QIAmpDNA blood mini kit.

Genetic analysis of the *EGFR* gene was performed by PCR amplification of exons 18, 19 and 21. The following primers, specifically designed for this study, were used for PCR amplification: exon 18 (forward, 5'-AGGTGACCCTTGTCTCTGTGTTCT-3'; reverse, 3'-CACCSGACCATGAGAGGCCCTGCG-5'), exon 19 (forward, 5'-GA TCACTGGGCAGCATGTGGCACC-3'; reverse, 3'- TGGACCCCCAC ACAGCAAAGCAAAGCAGA-5'), exon 21 (forward, 5'-TTCCCATG ATGATCTGTC-3'; reverse, 3'-ATGCTGGCTGACCTAAA -5'). PCR was performed in a total volume of 20 μL, containing reaction buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 100 nmol/L each primer, 0.5 units AmpliTaq (Biosystems) and 4 μL genomic DNA. Thermal cycling conditions included 3 min at 94°C, followed by 35 cycles of 94°C for 20 sec, 68°C for 40 sec and 72°C for 3 min.

After completion of the PCR reaction, the products were denatured (5 min at 90°C), immediately cooled on ice and loaded onto a nondenaturing polyacrylamide gel. The concentration of acrylamide was 1%-14% gradient. Electrophoresis was carried out for 3 h at 0°C at 72V/cm. The shifted bands were removed from the gel, and the recovered DNA was amplified in duplicate and subjected to bidirectional dye-terminator sequencing using the same primers used for amplification. Sequencing fragments were detected by capillary electrophoresis. SSCP and sequencing were performed by Hitachi Hitechnology Co. (Tokyo, Japan).

All 31 patients were evaluated for responses to gefitinib using WHO criteria (16). TTP and OS were measured from the date of initial gefitinib treatment to the date of disease progression and to the date of death or last follow-up examination, respectively, and were estimated using the Kaplan-Meier method (17).

Since recent clinical studies suggested that females with no smoking history and an adenocarcinoma were positive predictors for gefitinib responses (5, 7, 18), these were evaluated as potential prognostic factors for gefitinib sensitivity. Age, gender, performance status, histology type, number of prior chemotherapy, prior platinum or docetaxel use, smoking history and HER family-expression were analyzed using the Fisher's exact test and the Students *t*-test. Differences in TTP and OS between the two groups were tested using the log-rank test. All statistical analyses were performed using SPSS Version 8 statistical software (SPSS, Inc., IL, USA).

Results

Patients characterization. Of the 31 patients, eleven (35%) were females and 74% of the patients had adenocarcinomas. Eleven patients (35%) had never smoked (Table I). The median age was 62 years (range, 51 to 77 years). Eight patients (25%) had no prior chemotherapy and the remainder had received platinum-based chemotherapy (Table I).

Immunohistochmical staining of HER family in tumors. Total positive staining included 21 for EGFR (68%), 24 for HER2

Table I. *Patient characteristics.*

Variable	No. of patients	%
Portial response		
yes	9	29
no	22	71
Gender		
male	20	65
female	11	35
Age, years		
median	62	
range	51 - 77	
Smoking history		
never	11	35
former/current	20	65
Histology		
adenocarcinoma	23	74
non-adenocarcinoma	8	25
Stage		
III	5	16
IV	12	39
recurrence after surgery	14	45
Performance status		
0.1	18	58
>2	13	42
No. of prior chemotherapy regimens		
0	8	25
1	14	45
2	7	23
3	2	6
Prior platinum		
yes	23	74
no	8	26
Prior docetaxel		
yes	7	23
no	24	77

Table II. *EGFR/HER2/HER3/HER4 expression status.*

Status	No. of patients	%
EGFR		
negative	10	32
positive	21	68
HER2		
negative	7	23
positive	24	77
HER3		
negative	14	45
positive	17	55
HER4		
negative	27	87
positive	4	13
Co-expesion		
no expression of EGFR	10	32
mono-expression of EGFR	4	13
double-expression of EGFR/HER2	3	10
triple-expression of EGFR/ HER2/HER3 or HER4 +	14	45

(77%), 17 for HER3 (55%) and 4 for HER4 (13%) (Table II). Fourteen out of 31 (45%) samples demonstrated triple expression of HER family members (13 were positive for EGFR/HER2/HER3 and 1 for EGFR/HER2/HER4). Three (10%) double-expressed EGFR /HER2, 4 (13%) mono-expressed EGFR and 10 (32%) exhibited no expression of EGFR (Table II).

Clinical response to therapy. The response to treatment was evaluated in all 31 patients. Nine partial responses (PR; 29%) were observed. The results of univariate analysis of the significance of potential prognostic factors for gefitinib sensitivity using Fisher's exact test and a *t*-test for age are provided in Table III. An objective response was observed in 6 out of 11 females and in 3 out of 20 males ($p<0.05$), 6 out of 11 non-smokers and 3 of 20 current or former smokers ($p<0.05$), 8 out of 23 adenocarcinoma and 1 out of 8 non-adenocarcinoma ($p=0.37$) cases (Table III).

No correlation was found between the EGFR-expression status and gefitinib efficacy. However, there was a significant difference in the gefitinib response between the group with triple expression of EGFR/HER2 /HER3 or HER4 and the remainder (50% *vs.* 11%, $p=0.043$) (Table III). There was also a significant difference in TTP between these groups (TTP; 4.3 *vs.* 1.2 months, $p=0.0449$, Figure 1A). Median OS time of the group with triple expression was longer time than that of the others remainder, but was not significant (15.3 *vs.* 6.7 months, $p=0.099$, Figure 1B).

EGFR mutations. The genomic status of the TK domain of the *EGFR* gene was evaluated in 17 frozen primary NSCLC tumor specimens. Exons 18, 19 and 21 were subjected to mutational analysis. PCR amplification followed by SSCP analysis was used since SSCP analysis is more sensitive than direct sequencing (19). A total of 6 of the shifted bands were found and were directly sequenced. Four mutations were located in exon 19, 1 in exon 21, and 1 in exon 18. Of the 6 mutations identified, 4 were in frame deletions in exon 19, and 2 were aminoacidic substitutions in exons 21 and 18. The deletions "E746-A750 del", "L747-S752" and "L747-S752 del, P753S" in exon 19, the leucine to arginine mutation (L858R) in exon 21 and "E709D, T710del" in exon 18 were found.

EGFR mutations were more frequently found in adenocarcinomas than non-adenocarcinomas (6 out of 12 adenocarcinomas and 0 out of 5 non-adenocarcinoma;). There were no obvious differences in *EGFR* mutation status with gender or smoking history (3/6 females and 3/11 males; 3/5 non-smokers and 3/12 current or former smokers). The clinical responses to gefitinb in 6 cases with *EGFR* mutations were 3 partial response (PR) and 3 stable disease. No PR was observed in the 11 cases without *EGFR* mutations.

In addition, all 6 cases with *EGFR* mutations showed triple expression of EGFR/HER2/HER3 or HER4 and no *EGFR* mutation was detected in non-triple expression cases; $p=0.035$.

Table III. *Univariate analysis of features associated with sensitivity to genitinib.*

Variable	Response (n=9)	No response (n=22)	*P*
	No. of patients	No. of patients	
Gender			
male	3	17	0.037
female	6	5	
Age, years			
median	62	63	0.97
range	53 - 74	51 - 77	
Histology			
adenocarcinoma	8	15	0.37
non-adenocarcinoma	1	7	
No. of prior chemotherapy regimens			
0	1	7	0.37
>1	8	15	
Prior platinum			
yes	8	15	0.38
no	1	7	
Prior docetaxel			
yes	1	6	0.64
no	8	16	
Performance status			
0,1	5	13	0.99
>2	4	9	
Smoking history			
never	6	5	0.037
former/current	3	17	
HER family expression status			
EGFR			
negative	1	9	
positive	8	13	0.21
HER2			
negative	1	6	0.64
positive	8	16	
HER3			
negative	3	11	0.46
positive	6	11	
HER4			
negative	7	20	0.56
positive	2	2	
Co-expression of HER family			
triple expression of EGFR/ HER2 /HER3 or HER4+	7	7	0.043
Other	2	15	

Discussion

We studied the correlation between HER family expression status and sensitivity to gefitinib monotherapy in patients with advanced NSCLC and we showed that sensitivity to gefitinib is related to the triple expression of EGFR, HER2 and HER3 or HER4.

Although inhibition of EGFR tyrosine kinase is considered an essential mode of action of gefitinib, previous studies

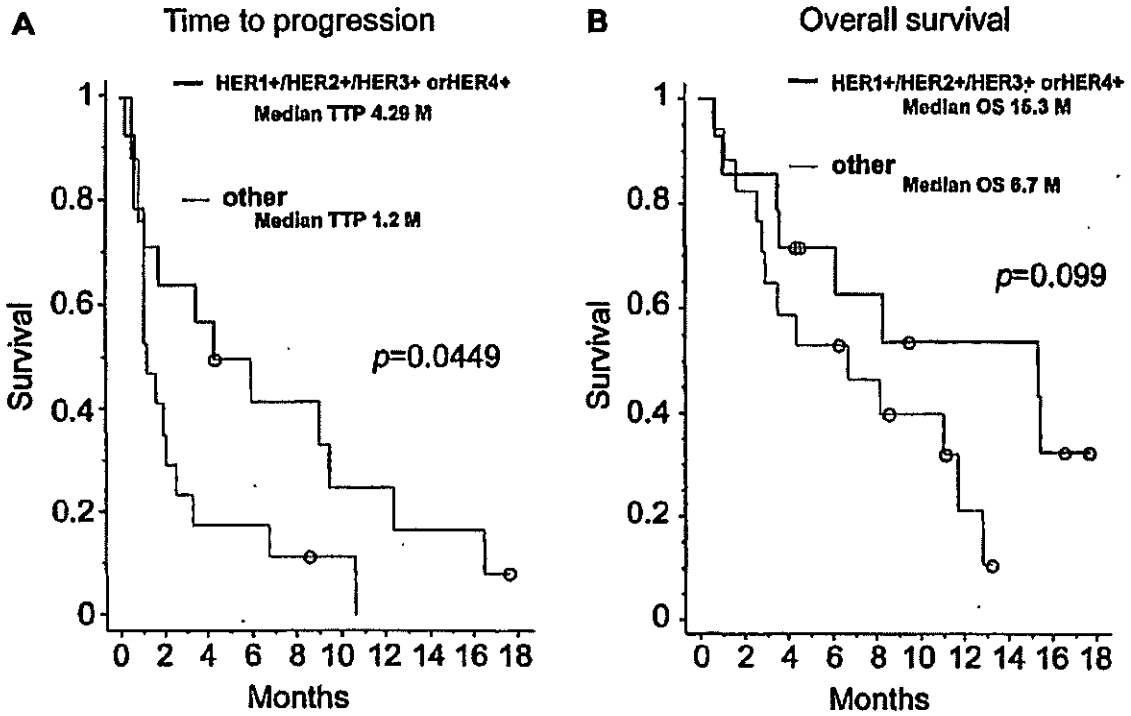

Figure 1. *(A) Time to progression (TTP) and triple expression of HER family (EGFR1+/HER2+/HER3+ or /HER4+); (B) Overall survival (OS) and triple expression of HER family (EGFR+/HER2+/HER3+ or /HER4+).*

indicated that expression of EGFR does not itself determine sensitivity to gefitinib therapy (8-10, 14, 20, 21). We also found no correlation between the EGFR-expression status and gefitinib efficacy. However, there was a significant difference in the gefitinib response between the group with triple expression of EGFR/HER2/HER3 or HER4 and the remainder (50% *vs.* 11%, p=0.0439). There was also significant difference in TTP between these groups (4.3 *vs.* 1.2 months, respectively; p=0.0449). The results are, thus, in line with those of several recent studies which indicated that all of the HER family members are targeted by gefitinib. Preclinical studies indicate that heterodimer formation is a factor impacting on sensitivity. Gefitinib causes reduced basal phosphorylation of EGFR/HER2, EGFR/HER3 and HER2/HER3 and this might correlate with the antitumor activity of this agent (14, 15, 22).

Cappuzzo *et al.* reported no correlation between co-expression of EGFR and HER2 in NSCLC patients and the results of treatment with gefitinib with regard to RR, TTP and OS (8). However, these authors did not evaluate the expressions of HER3 and HER4, and HER3 positive rate which is relatively high (>50%) in this study. This might be critical for sensitivity to gefitinib. In addition, the RR in our study was relatively high compared with their value (29% *vs.* 15.9%, respectively) and an ethnic difference may account for this difference.

Sensitivity to gefitinib appears to be greatly influenced by the presence of activating mutations within the kinase domains (11-13) and the mutations were more frequent in tumors from Japanese and other East Asian patients (23-25). *EGFR* gene mutations in exons 18, 19, 21 were analyzed by SSCP in this study and a comparable frequency of *EGFR* mutations was detected by the SSCP methods in a previous report (6 out of 17 cases; 35%). *EGFR* mutations were detected in cases with triple expression of EGFR/HER2 /HER3 or HER4, but no mutation was found in cases without triple expression of HER family receptors. Although these *EGFR* mutations can account for almost all objective responses obtained with gefitinib, the clinical benefits, such as long stable disease, cannot be explained only by the presence of mutations.

Recently, Hirata *et al.* reported that NSCLC cells trasfected with the *HER2* gene (LK2/HER2) were approximately 5-fold more sensitive to gefitinib than LK2/mock cells and cell survival and death were dependent on HER2/HER3 signaling. However, the sensitivity was about 20-fold lower in the LK2/HER2 cells than in the PC9 cells, which harbor in-frame deletion mutation of *EGFR* (E746-A750) in exon 19 (22). These results support our findings that multiple expressions of HER family members may contribute to gefitinib efficacy and multiple expressions of HER family members may play more important roles in cases without *EGFR* mutations than in case with mutations of this gene.

This small pilot study is not enough to conclude that triple expression of HER family members is strong predictive factor for response to gefitinb. Further large-scale prospective trials are necessary to confirm these results.

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INVITED REVIEW SERIES: LUNG CANCER

Clinical aspects of epidermal growth factor receptor inhibitors: Benefit and risk

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Clinical aspects of epidermal growth factor receptor inhibitors: Benefit and risk
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Abstract: Gefitinib and erlotinib are small molecules that selectively inhibit epidermal growth factor receptor (EGFR) tyrosine kinase activity. Developmental studies of either drug have failed to show synergistic effects when combined with cytotoxic drugs as the first line treatment in patients with advanced non-small cell lung cancer, but erlotinib has shown survival prolongation when compared with best supportive care in patients with recurrence. Female gender, adenocarcinoma histology and lack of smoking history are considered to be clinical factors predicting response. Being positive for EGFR mutations in exons 18–24 in cancer cells has a strong correlation with response. On the other hand, preceding idiopathic pulmonary fibrosis, male gender and history of smoking appear to be risk factors for EGFR tyrosine kinase inhibitor-induced interstitial lung disease in the Japanese population. Reports on these factors predicting response or risk for interstitial lung disease have attracted great interest in the relation between cancer genetics and drugs, as well as the relation between ethnicity and genetics. In clinical practice, EGFR tyrosine kinase inhibitor should be prescribed with careful consideration and it is essential to assess benefit and risk of the drug.

Key words: interstitial lung disease, lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death, with 1.18 million deaths worldwide.[1] Incidence and mortality rates are increasing because the disease is very much influenced by past exposure to tobacco smoking.[1] Eighty-five per cent of tumours are non-small cell lung cancer (NSCLC), the others, small cell lung cancers. More than half of NSCLC patients are diagnosed at an advanced stage at which mainly systemic chemotherapy is recommended.

For patients with previously untreated advanced NSCLC, combination chemotherapy with cisplatin or carboplatin and third generation agents, such as docetaxel, gemcitabine, irinotecan, paclitaxel or vinorelbine, have yielded a response in 30–40% of the patients, 7–12 months of median survival times (MST) and 30–40% achieved 1-year survival.[2–5] When these tumours become refractory to the first-line chemotherapy, docetaxel[6,7] and pemetrexed[8] are reportedly effective cytotoxic agents as second-line treatments, with response rates of about 10%, an 8-months MST and 30% achieving 1-year survival with symptom palliation.

In addition to these cytotoxic agents, two molecular targeting agents have been approved for the treatment of advanced NSCLC, gefitinib and erlotinib. Both agents are orally active epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), small molecules belonging to the quinazolinamine class, which inhibit EGFR tyrosine kinase activity by competing for the ATP-binding domain, thereby inhibiting cellular proliferation, angiogenesis and consequently reducing tumour invasion and metastasis (Fig. 1).

Gefitinib was a first molecular targeting agent approved for lung cancer in the world. Because of ever higher response rates for recurrent disease, gefitinib was made public based on to the results of phase II trials. To date, it has, however, failed to prolong survival in NSCLC patients, and the problem of interstitial lung disease (ILD), especially in Japan, has also been recognized. In this review, the current status of

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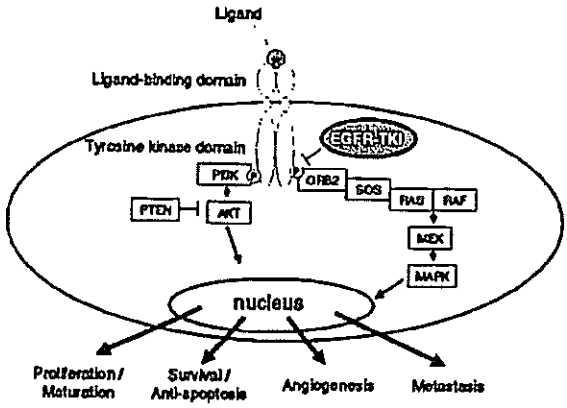

Figure 1 Epidermal growth factor receptor (EGFR) signal transduction, its biological consequences and EGFR tyrosine kinase inhibition (TKI).

the gefitinib and erlotinib will be discussed especially in terms of benefits and risks in clinical practice.

DEVELOPMENT

Two EGFR-TKIs, gefitinib and erlotinib, have been developed using similar procedures. Both drugs were well tolerated and had higher response rates in single agent phase I and phase II studies, but failed to indicate synergistic effects in phase III studies with combination of cytotoxic agents. Results of single agent phase III studies for patients with recurrent or resistant disease differed between the two drugs. Although erlotinib apparently prolonged survival, gefitinib did not.

Phase I trials

Four phase I trials of gefitinib were performed in 252 patients with a variety of solid tumours, including NSCLC, head and neck cancer and colorectal cancer.[9–12] Major adverse events were rash and diarrhoea. These events were generally mild and tolerable at doses not exceeding 600 mg/day and 700 mg/day came to be the maximal tolerated dose (MTD). Because doses of 150–800 mg/day were associated to tumour responses, 250 and 500 mg/day were selected for subsequent phase II trials. In the case of erlotinib, phase I trials identified a dose of 150 mg/day for further clinical development. Adverse events were similar to those of gefitinib and the incidence and severity of the adverse events generally increased as the dose increased.

Second-line phase II trials

Among single-agent phase II studies, one erlotinib and two gefitinib studies were conducted in patients with NSCLC. Two large scale multicentre phase II trials of gefitinib were performed; Iressa™ dose evaluation in advanced lung cancer (IDEAL)-1 in Japan, Europe and Australia,[13] and IDEAL-2 in North America.[14] Eligibility criteria of the IDEAL-1 included having failed only one prior platinum-containing regimen, whereas the IDEAL-2 criteria were to have failed a platinum-containing regimen and docetaxel. Patients were randomized to gefitinib 250 or 500 mg/day. In the IDEAL trials, response rates ranged from 9 to 19% and severe toxicities were relatively uncommon. Treatment related toxicities, that is, diarrhoea, rash, acne, dry skin, nausea and vomiting, were slightly more severe and more frequent on 500 mg/day than on 250 mg/day. Because no additional response was observed with 500 mg/day, gefitinib at a dose of 250 mg/day was approved in Japan and the USA for treatment of advanced NSCLC.

A randomized phase II study was also performed with erlotinib in patients with previously treated advanced NSCLC.[15] The study, a comparison with best supportive care, revealed 150 mg/day of the drug to produce a 12.3% of objective response rate in patients with previously treated advanced NSCLC. MST was 8.4 months and the 1-year survival rate was 40% with no grade 4 toxicity.

First-line combination phase III trials

In a preclinical study, EGFR-TKI showed an additive effect on antitumour activity with no toxicity increase when combined with cytotoxic agents.[16–18] On the basis of these data, four randomized trials were conducted with gefitinib; Iressa™ NSCLC trial assessing combination treatment (INTACT)-1, 2,[19,20] and with erlotinib; TALENT[21] and TRIBUTE,[22] in chemotherapy-naïve patients with advanced NSCLC to compare chemotherapy plus EGFR-TKIs to chemotherapy alone.

In the INTACT-1 and TALENT trials, the chemotherapy regimen consisted of cisplatin and gemcitabine. In the INTACT-2 and TRIBUTE trials, the chemotherapy regimen was carboplatin and paclitaxel. Unfortunately, none of those studies showed any definitive benefit of adding an EGFR-TKI to standard chemotherapy in patients with NSCLC. These trials failed to support the concept of synergistic preclinical studies and to show additive or synergistic effects when combined with platinum-based chemotherapy as a first-line treatment for NSCLC.

Second-line phase III trials

To investigate the survival benefit of EGFR-TKIs as single agents, two large scale placebo controlled phase III trials were conducted as second- or third-line treatment for the patients with NSCLC. Iressa™ survival evaluation in lung cancer (ISEL) trial was planned to compare gefitinib with a placebo with 1692 patients.[23] Although the results of the study showed a response in the gefitinib group, there was no survival prolongation effect with gefitinib. MST

was 5.6 months in the gefitinib arm and 5.1 months in the placebo arm. On the other hand, the BR.21 trial investigated erlotinib as compared with a placebo.[24] Results of the trial included 731 patients showing a 9% of response rate in the erlotinib arm and less than 1% in the placebo arm. In terms of survival, 2-month prolongation of MST was achieved in the erlotinib arm, 6.7 months compared with 4.7 months in the placebo arm. Based on these data, erlotinib was approved in the USA and European countries.

Although study results were similar in phase I and phase II trials, only erlotinib, not gefitinib, produced a survival benefit compared with the placebo. The reason for the difference may be explained partly by the administered dose of gefitinib possibly being lower. In a phase I trial of gefitinib, a dose of 250 mg/day was less toxic and as effective as a dose of 500 mg/day but the MTD in the trial was 700 mg/day. On the other hand, a dose of 150 mg of erlotinib is nearly the MTD in a phase I trial. Another explanation is an issue of the difference in the response to previous treatment between participants in two trials. In terms of the best response, 18% of the patients in the gefitinib group had responded and 45% had progressed in the ISEL trial. By contrast, 38% of the patients in the erlotinib group had responded and 28% had progressed in BR.21 trial.

EGFR-TKI-INDUCED INTERSTITIAL LUNG DISEASE

Incidence

Although toxicities, like myelosuppression and vomiting, were not dose limiting for patients receiving gefitinib, a proportion of Japanese patients experienced ILD.[25–27] This type of adverse event has also been reported from Korea[28] and Taiwan.[29] In a large scale surveillance by the West Japan Thoracic Oncology Group (WJTOG), among 1976 patients, 70 patients (3.5%) were identified as having ILD after a panel review of 102 patients who were suspected by their physicians, and 31 patients (1.6%) who had died due to the event.[30] Another large scale post marketing surveillance conducted in Japan, an analysis involving 3322 patients, found that the incidence of ILD was 5.8%, and mortality due to ILD 2.3%.[31] Other smaller but detailed studies reported similar ratios of 5.4% and 4.5% for incidence, 3.6% and 2.4% for mortality.[32,33] Report of the incidence of ILD in first-line single agent treatment is limited. In a phase II study of chemo-naïve patients with advanced NSCLC, 4 of 37 patients (11%) died due to severe ILD.[34] In this study, only a CXR was required to exclude preceding interstitial pneumonia or pulmonary fibrosis.

A Korean report indicated two of 65 patients (3%) to have ILD.[28] Another study in Taiwan, of patients with brain metastases reported four of 76 patients (6%) to have experienced non-lethal ILD.[29] Regional differences seem to exist because relatively higher ratios of pulmonary involvement have only been reported in East Asian countries. The incidence of ILD is reportedly only 1.0–1.1% in the USA or Europe.[19,20,35,36] The ISEL study conducted in both Europe and Asia was the only study in which a difference in incidence between ethnicities could be compared directly. East and South-east Asian patients tend to suffer more ILD in 3–4% of the patients although the frequency of ILD in all population was 1%. However, there were no differences in incidence between patients receiving gefitinib versus a placebo.[20]

Risk factor

Several Japanese studies have reported risk factors for ILD. In the WJTOG surveillance, the presence of idiopathic pulmonary fibrosis (IPF), male gender and history of smoking were independent predictive factors for developing ILD.[30] In other studies, multivariate analysis revealed that preceding IPF, poor performance status (PS), smoking history, prior history of irradiation or chemotherapy were independent risk factors for ILD.[31–33,37] In these studies, the most striking factor was pre-existing IPF with a higher odds ratio. Factors associated with a poor prognosis have been analysed. These included a short interval from initiation of gefitinib treatment to the onset of ILD, an acute interstitial pneumonia (AIP) pattern on CT, the presence of preceding IPF, male gender and a poor PS.[30–33,37] (Table 1)

Diagnosis and patterns

Most patients with EGFR-TKI-induced ILD experienced symptoms such as coughing, increasing

Table 1 Factors associated to EGFR-TKI-induced interstitial lung disease

	Risk factors for ILD	Factors associated to poor prognosis after ILD
Definitive	Preceding IPF	
Possible	Male gender	AIP patterns
	History of cigarette smoking	Preceding IPF
	Poor PS	Early onset of ILD
	Prior chemotherapy	Male gender
	Japanese/East Asian ethnicity	Squamous cell carcinoma

AIP, acute interstitial pneumonia; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; PS, performance status.

Table 2 Predictive markers for response to EGFR-TKI

	Clinical	Molecular
Definitive	Female gender adenocarcinoma Lack of smoking history Japanese/East Asian	Positive EGFR mutation
Possible	Better PS No preceding IPF Skin eruption as adverse event	Increased EGFR gene copy number Positive p-Akt expression Negative K-ras mutation

EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; IPF, idiopathic pulmonary fibrosis; PS, performance status.

dyspnoea and fever. It is difficult to diagnose ILD and differentiate it from other respiratory conditions that produce similar symptoms, such as infections, and cancer progression. According to the surveillance, suspicion of ILD by the attending physician was refuted by an expert panel consisting of pulmonologists and radiologists in 15.7–31.4% of patients.[30,31,34,37] These findings indicate that a diagnosis of ILD might not always have been correct in other reports.

As well as other pulmonary infiltrative disease, it must be emphasized that accurate diagnosis before the start of the treatment is necessary. When any sign or symptom or CXR abnormality appears, CT, especially high-resolution CT (HRCT), is recommended to diagnose interstitial shadows on the CXR. Screening for respiratory tract infection is also essential, including culture and polymerase chain reaction examination for pneumocystis carinii and aspergillus, for example. Transbronchial biopsy or BAL may contribute to making a correct diagnosis, and may be the key to the mechanism of EGFR-TKI-induced ILD.

Some reports have tried to classify radiological patterns and clinical course.[31,33,35,38] There seem to be four patterns. About a half of the ILD patients showed non-specific ground-glass attenuation on CT or HRCT without lung volume loss on CXR.[33,35,38] This group and two other small groups, including cryptogenic organizing pneumonia-like pattern and acute eosinophilic pneumonia pattern, seemed to correlate with a fair prognosis or better response to steroid therapy.[38] The remaining one third of the patients showed AIP-like pattern with extensive bilateral ground-glass attenuation or airspace consolidation with traction bronchiectasis on CT and lung volume loss on CXR. The prognosis of AIP-like pattern patients were very poor with 75–100% dying early.[33,38] In some patients, the histopathology at autopsy revealed diffuse alveolar damage.[25,31] These findings may support the EGFR-TKI-induced ILD hypothesis that EGFR inhibitor suppresses lung injury repair and results in irreversible alveolar damage.

PROGNOSTIC FACTORS

According to the trials and surveillance of EGFR-TKIs used as first-line treatments or for refractory cases, as single agents or combined with other therapies, several factors have been proposed to predict response, long-term prognosis, or risk for ILD.

In summary, female gender, adenocarcinoma, lack of smoking history, being Japanese or another East Asian ethnic group are considered to be clinical factors predicting response. In addition, better PS and lack of preceding pulmonary fibrosis and skin rash while taking the drug could be predictive factors.[13,14,30,32,39] (Table 2)

Based on molecular analysis, being positive for mutations in EGFR exons 18–24, which encode the kinase domain of the protein, strongly predict response to EGFR-TKI, especially in the Asian population.[40–46] Other molecular factors include an increased EGFR gene copy number,[47–49] p-Akt expression[28,50] and lack of K-ras mutation,[51,52] but are still controversial. To clarify whether being positive for EGFR mutations correlates with better survival, some prospective studies are now underway.

In addition to these molecular factors associated with primary response or resistance, a second mutation T790M in exon 20 is reported in acquired resistance to EGFR-TKI.[53,54]

A numbers of research reports and practical experience from Asian countries support the favourable 'benefit to risk' balance of treating NSCLC patients. However, these results still lack survival advantages, and there is the problem of ILD, which appears to limit the use in patients with advanced NSCLC in taking EGFR-TKIs, even as second- or third-line treatments. It is necessary to assess benefit and risk individually before prescribing the drug and to give the patients adequate information to make an informed decision. Even in East Asian countries, gefitinib should be used only in clinical trials or for well-assessed patients.

SUMMARY

It remains unclear why gefitinib appears to produce a greater response in Asian patients than in patients from the rest of the world. Now, several genetic studies are starting to provide clarification of the mechanism underlying the differences in response and adverse events between ethnicities.

The benefits of using EGFR-TKI as the first-line treatment for NSCLC also remain unclear. There are

no results confirming the superiority of EGFR-TKI even showing clinical or genetic prediction of a better response, because these trials failed to show better survival over standard combination regimens including platinum agents.

We continue to await the results of research which will demonstrate clinical benefits in terms of survival even in selected patients, and which may help us to identify patients who are most likely to benefit from treatment with EGFR-TKI. Such results would tell us when and to whom we should prescribe the best drug to treat NSCLC.

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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

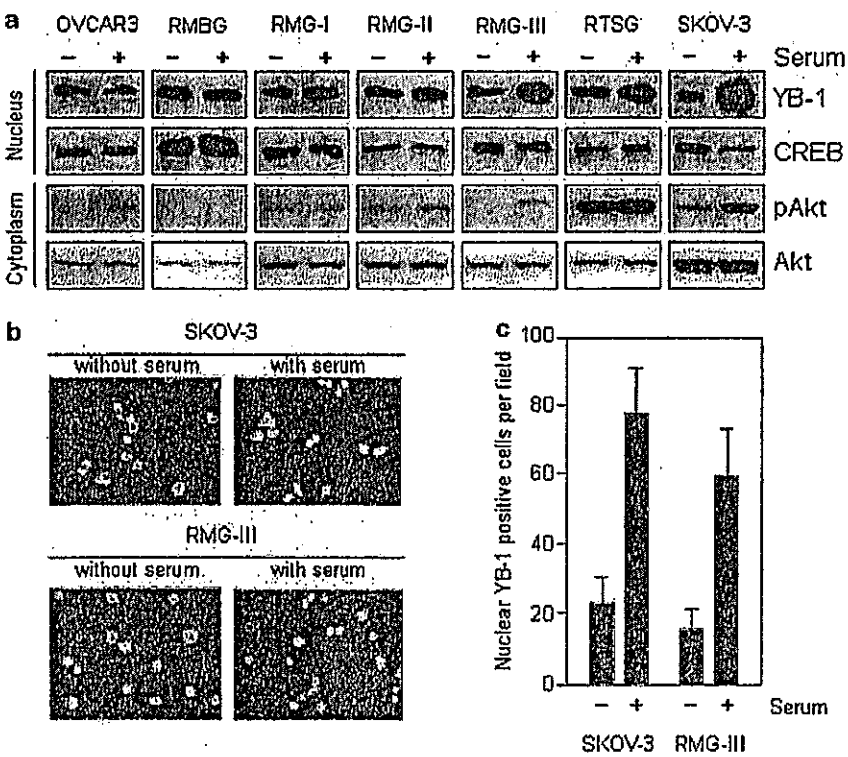

Figure 1 Levels of Akt phosphorylation and nuclear localization of YB-1 in ovarian cancer cell lines with or without serum stimulation. (a) Cytoplasmic and nuclear extracts were prepared 1 h after 10% serum stimulation. Anti-YB-1 and anti-CREB immunoblots were performed on nuclear extracts, and anti-pAkt 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.

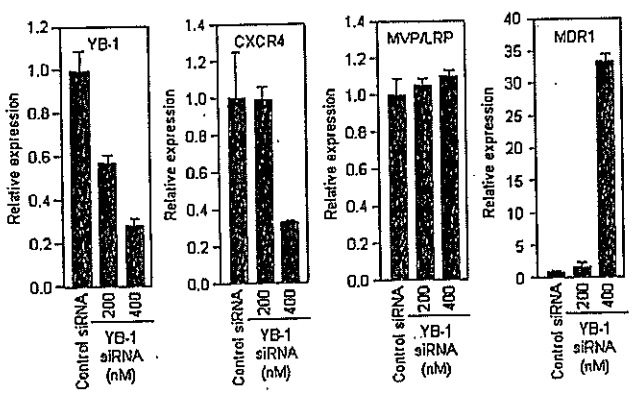

Figure 2 Effect of YB-1 knock down on expression of MDR1, MVP/LRP and CXCR4. SKOV-3 cells were treated with YB-1 siRNA for 48 h and then total RNA was prepared. QRT-PCR was performed for MDR1, MVP/LRP, CXCR4, YB-1 and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\text{GAPDH})-C_t(\text{target}))}$ 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,

Table 1 List of genes differentially expressed in YB-1 siRNA-transfected SKOV-3 cells

Unigene	*Accession*	*Symbol*	*Description*	*Mean fold change*
Hs.489033	NM_000927	ABCB1	MDR1, ATP-binding cassette, sub-family B (MDR/TAP), member 1	2.46
Hs.369762	AB077208	TYMS	Thymidylate synthetase	1.71
Hs.198363	NM_018518	MCM10	MCM10 minichromosome maintenance deficient 10	1.70
Hs.405958	U77949	CDC6	CDC6 cell division cycle 6 homolog (*S. cerevisiae*)	1.66
Hs.442658	AB011446	AURKB	Aurora kinase B	1.65
Hs.516484	NM_005978	S100A2	S100 calcium-binding protein A2	1.48
Hs.23960	NM_031966	CCNB1	Cyclin B1	1.40
Hs.460184	AA604621	MCM4	MCM4 minichromosome maintenance deficient 4 (*S. cerevisiae*)	1.40
Hs.438720	AF279900	MCM7	MCM7 minichromosome maintenance deficient 7 (*S. cerevisiae*)	1.36
Hs.433168	NM_002960	S100A3	S100 calcium binding protein A3	1.33
Hs.115474	NM_002915	RFC3	Replication factor C (activator 1) 3, 38 kDa	1.28
Hs.122908	NM_030928	CDT1	DNA replication factor	1.28
Hs.329989	NM_005030	PLK1	Polo-like kinase 1 (*Drosophila*)	1.21
Hs.334562	NM_001786	CDC2	Cell division cycle 2, G1 to S and G2 to M	1.21
Hs.74034	NM_001753	CAV1	Caveolin 1, caveolae protein, 22 kDa	1.19
Hs.477481	NM_004526	MCM2	MCM2 minichromosome maintenance deficient 2, mitotin	1.16
Hs.284244	M27968	FGF2	Fibroblast growth factor 2 (basic)	1.10
Hs.179565	NM_002388	MCM3	MCM3 minichromosome maintenance deficient 3 (*S. cerevisiae*)	1.08
Hs.194698	NM_004701	CCNB2	Cyclin B2	1.04
Hs.506989	BC001866	RFC5	Replication factor C (activator 1) 5, 36.5 kDa	1.02
Hs.171596	NM_004431	EPHA2	EPH receptor A2	1.01
Hs.194143	NM_007294	BRCA1	Breast cancer 1, early onset	0.75
Hs.156346	NM_001067	TOP2A	Topoisomerase (DNA) II alpha 170 kDa	0.64
Hs.473163	NM_001719	BMP7	Bone morphogenetic protein 7 (osteogenic protein 1)	0.54
Hs.391464	NM_004996	ABCC1	MRP-1, ATP-binding cassette, sub-family C (CFTR/MRP), member 1	0.20
Hs.256301	NM_199249	MGC13170	Multidrug resistance-related protein	0.15
Hs.513488	NM_017458	MVP	Major vault protein	−0.05
Hs.482526	NM_014886	TINP1	TGF beta-inducible nuclear protein 1	−0.23
Hs.525557	NM_000295	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member	−1.01
Hs.500466	BG403361	PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	−1.05
Hs.25292	NM_002229	JUNB	Jun B proto-oncogene	−1.06
Hs.132225	AI934473	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	−1.16
Hs.83169	NM_002421	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	−1.22
Hs.508999	NM_002742	PRKCM	Protein kinase C, mu	−1.29
Hs.326035	NM_001964	EGR1	Early growth response 1	−1.29
Hs.2256	NM_002423	MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	−1.32
Hs.197922	NM_018584	CaMKIINalpha	Calcium/calmodulin-dependent protein kinase II	−1.36
Hs.132966	AA005141	MET	Met proto-oncogene (hepatocyte growth factor receptor)	−1.39
Hs.208124	NM_000125	ESR1	Estrogen receptor 1	−1.50
Hs.73793	M27281	VEGF	Vascular endothelial growth factor	−1.53
Hs.381167	AW512196	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	−1.70
Hs.413111	NM_002661	PLCG2	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	−1.75
Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	−1.92
Hs.472793	AI631895	SGK2	Serum/glucocorticoid regulated kinase 2	−2.04
Hs.372914	NM_006096	NDRG1	*N-myc* downstream regulated gene 1	−2.34
Hs.421986	NM_001008540	CXCR4	Chemokine (C-X-C motif) receptor 4	−2.64

High-density oligonucleotide array was performed on 400 nM YB-1 siRNA-treated SKOV-3 cells and mock-treated cells. siRNA duplexes were transfected using LipofectAMINE2000 with Opti-MEM mediums. At 48 h after siRNA transfection, total RNA was prepared, and subjected to double-stranded cDNA synthesis and *in vitro* transcription. The labeled cRNA was applied to the oligonucleotide microarray.

respectively, and silencing effects of siRNA were analysed by immunoblotting (Figure 6a). In Akt siRNA almost completely silenced both Akt1 and Akt2, and siRNA for ILK, the upstream kinase for Akt, silenced ILK on protein level. Treatment with Akt siRNA and ILK siRNA resulted in a marked decrease in both pAkt expression and nuclear accumulation of YB-1 (Figure 6a). As both Akt and ILK siRNA blocked the nuclear translocation of YB-1, we examined their effects on expression of YB-1-regulated genes (Figure 6b). Treatment with Akt and ILK siRNA downregulated the expression of *CXCR4* gene, and upregulated the expression of *MDR1* gene. By contrast there appeared no marked effect on the expression of *MVP/LRP* and *YB-1* genes when treated with both siRNAs (Figure 6b).

Effect of LY294002 treatment on Akt phosphorylation and YB-1 nuclear localization in SKOV-3 xenograft

To further investigate the involvement of Akt in tumoural YB-1 nuclear localization, an *in vivo* xenograft

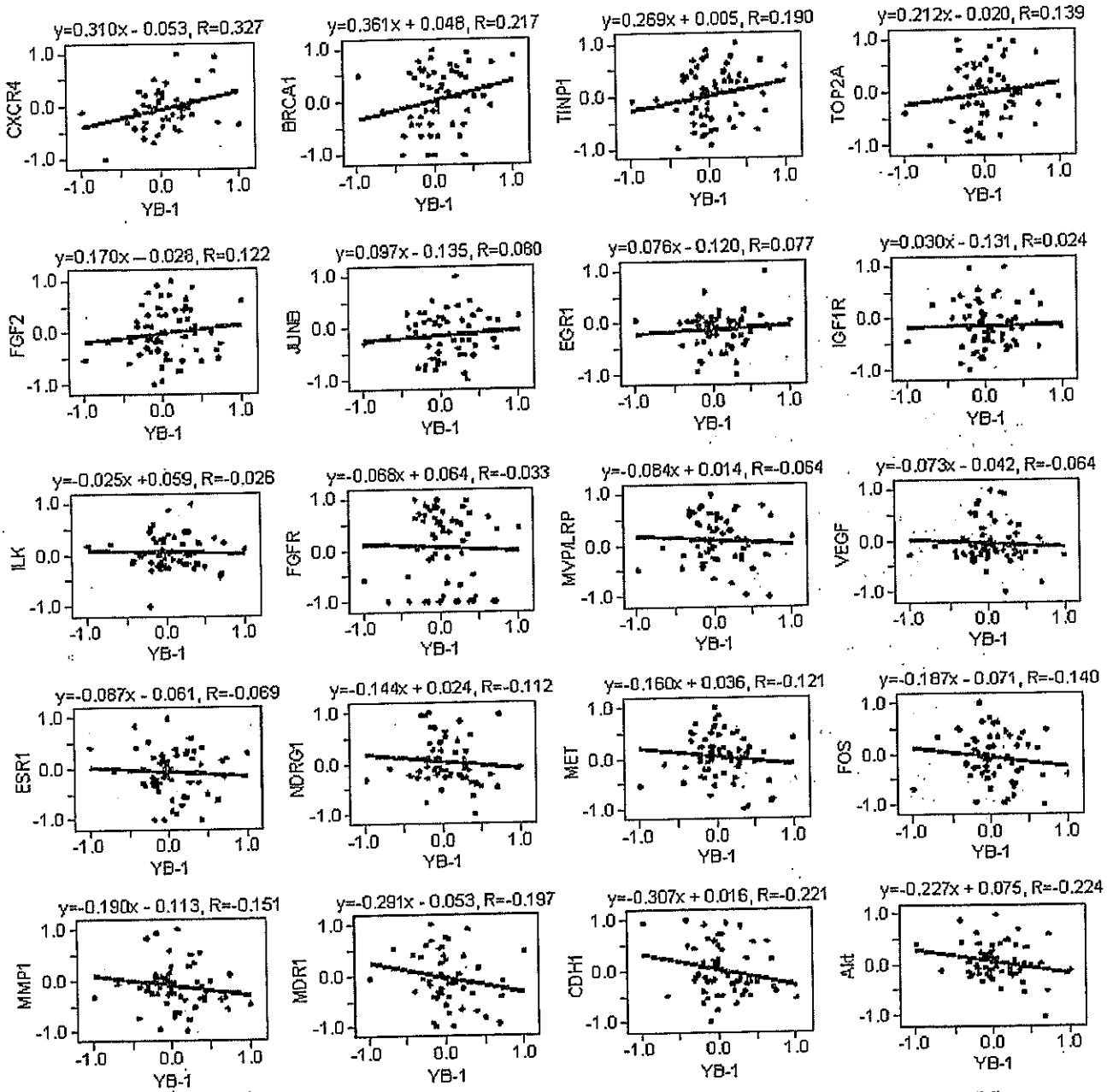

Figure 3 Correlation analysis of gene expression in NCI-60 screen. Gene expression data for the 60 human tumor cell lines were obtained from the Developmental Therapeutics Program (http://www.dtp.nci.nih.gov/), expressed as log of the mRNA levels in cell line/mRNA levels in reference pool in the NCI screen. Pearson correlation coefficients were calculated for each gene–gene pair.

assay was performed. Administration of LY294002 (i.p.) to mice carrying SKOV-3 cell tumors inhibited the phosphorylation of Akt (Figure 7a and b). Akt phosphorylation and YB-1 nuclear localization were also evaluated by immunohistochemical analysis. Tumors in the LY294002-treated group displayed a lower level of pAkt staining (3.3 ± 0.5) than those in the control group, where the mean number of nuclear YB-1-positive cells was 24.7 ± 3.4 (Figure 7c and d). Taken together, these results suggest that nuclear localization of YB-1 in ovarian cancer cells is closely associated with Akt phosphorylation activity *in vitro* and *in vivo*.

Discussion

The nuclear localization of YB-1 is essential process for YB-1-driven transcription of various genes and DNA repair in cancer cells in response to various environmental stimuli. One should understand which signalling pathway specifically controls the translocation of YB-1 from cytoplasm into nucleus. Our previous study has demonstrated that PKC activates the nuclear localization of YB-1 in cancer cells treated with UV irradiation or cisplatin, and also that the C-terminal region of YB-1 was important for its cytoplasmic

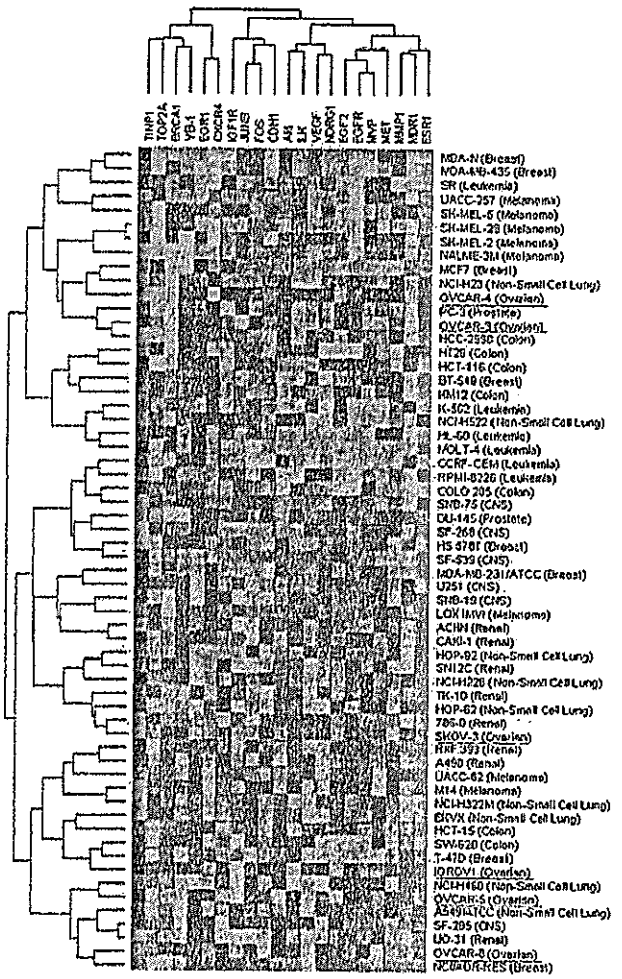

Figure 4 Hierarchical clustering of gene expression in NCI-60 screen. Hierarchical clustering can be used to group cell lines and genes in term of their patterns of gene expression. To obtain cluster trees for genes that showed distinct expression patterns across the 60 cell lines, we used the program 'Cluster' and 'Tree View' (http://rana.lbl.gov/) with average linkage clustering and a correlation metric.

retention (Koike *et al.*, 1997). Sutherland *et al.* (2005) have presented more definitive mechanism at molecular basis that phosphorylation of serine 102 at cold-shock domain of YB-1 by Akt is essential for the nuclear YB-1 localization in breast cancer cells, and also that ILK phosphorylate its downstream Akt, resulting in activation of YB-1 and it nuclear localization. Consistent with this study, our present study also demonstrated that Akt as well as ILK played a critical role in the nuclear YB-1 localization and YB-1-driven-transcriptional control of various genes including *CXCR4* and *MDR1* in human ovarian cancer cells.

In our present study, we examined whether expression of two multidrug resistance relevant genes, *MVP/LRP* and *MDR1/ABCB1*, was affected by knockdown of YB-1. Stein *et al.* (2005) have reported that the *MVP/LRP* gene is transcriptionally activated by YB-1 in response to cytotoxic anticancer agents including doxorubicin and 5-fluorouracil: *MVP/LRP* is an essential vault protein involving acquirement of multidrug resistance. However, in ovarian cancer cells, there was no causative association between the two genes when assayed by microarray and QRT-PCR. YB-1 might not regulate *MVP/LRP* expression in ovarian cancer cells used in our present study. In contrast, in human breast cancer cells, treatment with YB-1 siRNA markedly upregulated *MVP/LRP* expression (Shimoyama T, Nishio K, Basaki Y, Ono M and Kuwano M, unpublished data), suggesting that YB-1-induced regulation of *MVP/LRP* gene expression depends upon cancer cell types and/or types of stimuli. In contrast, knockdown or nuclear translocation inhibition of YB-1 upregulated expression of another drug resistance *MDR1* gene in ovarian cancer cells. Various environmental stimuli often upregulated *MDR1* gene in various human cancer cells through pleiotropic transcriptional regulations (Kuwano *et al.*, 2004). Our present study further presented a novel regulation of YB-1-induced negative control of *MDR1* gene in ovarian cancer cells, and further study should be required to understand its underlying mechanism at molecular basis.

In our present study, we first observed that the knockdown of YB-1, ILK and Akt as well as an Akt inhibitor all downregulated expression of *CXCR4* gene. Consistent with recent study by Sutherland *et al.* (2005), ILK-Akt activation could be responsible for the nuclear localization of YB-1, resulting in enhanced expression of *CXCR4* gene. The 2.6 Kb 5′-flanking region located upstream of the *CXCR4* gene contains a TATA box and the transcription start site characteristic of a functional promoter (Caruz *et al.*, 1998) and this region also contained putative consensus Y-box-binding site (inverted CCAAT box) form −685 to −681. However, it remains unknown whether ILK-Akt-induced activation of YB-1 is directly involved in the upregulation of *CXCR4* gene.

CXCL12 (SDF-1α) is a specific ligand of CXCR4. CXCL12 induced a dose dependent proliferation of human ovarian cancer cells through its specific interaction with CXCR4 (Porcile *et al.*, 2005). This CXCR4 activation by CXCL12 further stimulated EGF receptor phosphorylation and its downstream kinases, ERK1/2, Akt and c-Src that might link several signallings of cell proliferation in ovarian cancer cells (Porcile *et al.*, 2005). On the other hand, VEGF, a potent angiogenic factor, induced upregulation of *CXCR4* gene expression in vascular endothelial cells, and expression of both VEGF and CXCL12 was very high in ascites of patients with advanced ovarian cancers (Kryczek *et al.*, 2005). The cross-talk of CXCL12/CXCR4 with EGF/EGF receptor and/or VEGF/VEGF receptor might thus provide important signallings for both cell proliferation and angiogenesis in ovarian cancers.

CXCL12/CXCR4 pathway is also expected to be clinically involved in acquirement of malignant characteristics of human ovarian cancers. Of 14 chemokine receptors, only CXCR4 protein was found to be expressed in ovarian cancer cell lines and in ascites from patients with ovarian cancers (Scotton *et al.*, 2001). The CXCL12/CXCR4 pathway has been implicated in