Table II. *Therapeutic regimens*

		No. of patients
SCLC	cisplatin + etoposide	6
	cisplatin + etoposide + TRT	2
	cisplatin + irinotecan	4
	cisplatin + irinotecan + etoposide	2
	carboplatin + etoposide	3
	cisplatin + TRT	1
NSCLC	cisplatin + gemcitabine	7
	cisplatin + vinorelbine	3
	cisplatin + vinorelbine + TRT	2
	cisplatin + vindesine + TRT	3
	cisplatin + irinotecan	1
	cisplatin +TRT	2
	carboplatin + etoposide	1
	carboplatin + paclitaxel	1
	nedaplatin + irinotecan	6
	paclitaxel + irinotecan	3

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; TRT, thoracic radiation therapy.

expression, ubiquitin, liver glyceraldehydes 3-phosphate dehydrogenase, 23-kDa highly basic protein, 60S ribosomal protein L13A and 40S ribosomal protein S9. When we analyzed the data for correlations between gene expression levels and tumor response to chemotherapy, nine genes showed the a significantly different expression in responders to chemotherapy compared with non-responders to chemotherapy (Table III, $p<0.01$). Stepwised multivariate regression analysis revealed that allogenic inflammatory factor, HLA-DR antigen associated invariant subunit and MHC class II HLA-DR-beta precursor were independent chemo-resistant factors (Table IV, $p<0.0001$). Expression levels of the each of these three genes were significantly elevated in non-responders compared with responders, and thus these three independent genes were defined as resistant genes. Furthermore, the expression levels of one or more independent resistant genes were elevated compared to the mean expression level of control genes expression in ten out of 29 responders and 14 out of 18 non-responders, respectively (Table V, $p=0.0039$).

When we analyzed the differences in independent resistant gene expression levels between patients with SCLC and NSCLC, the expression levels of one or more independent resistant genes were elevated compared with the mean expression level of control genes in five out of 18 SCLC patients and 19 of the 29 NSCLC patients, respectively (Table V, $p=0.012$).

DISCUSSION

We examined cancer-related gene expressions in lung cancer samples obtained before chemotherapy using cDNA microarray screening, and analyzed the relationship between gene expression levels and clinical outcome after chemotherapy. We identified three specific genes whose expression levels were correlated with the response of the tumor to chemotherapy. These three resistant genes identified as

Table III. *Genes closely associated with sensitivity in chemotherapy for lung cancer.*

		Expression of each genes compared to control					
		Responder			*Non-responder*		
Description	Symbol	n	mean	SD	n	mean	SD	—
allograft inflammatory factor 1 (AIF1); ionized calcium-binding adapter molecule 1	U19713	18	1.67	21.13	29	-10.17	7.59	0.0084
lymphocyte antigen	M81141	18	11.39	26.37	29	-4.76	13.85	0.0085
hepatocyte growth factor-like protein; macrophage-stimulating protein (MSP)	M74178	18	15.17	20.48	29	3.55	8.36	0.0092
HLA-DPB1 precursor; HLA class II histocompability antigen SB beta chain	K01615; M83664;	18	3.17	16.99	27	-7.56	8.67	0.0078
IgG receptor FC large subunit P51 precursor (FCRN); neonatal FC receptor; IgG FC fragment receptor transporter alpha chain	U12255	8	28.50	31.89	17	4.47	11.02	0.0096
HLA-DR antigen-associated invariant subunit	X00497	18	130.44	190.18	28	-8.25	57.39	0.0007
MHC class II HLA-DR-beta (DR2-DQW1/DR4 DQW3) precursor	M20430	15	25.60	31.81	25	-11.08	12.96	<0.0001
HLA class II histocompatibility antigen alpha chain precursor	K01171	18	-3.67	194.09	29	-161.86	65.00	0.0002
vimentin (VIM)	X56134; M14144	18	15.72	252.30	29	-150.83	126.75	0.0043

Four housekeeping genes were used as controls for gene expression.

Table IV. *Stepwized multivariate regression analysis on chemotherapeutic response.*

Description	coefficient	SE
allograft inflammatory factor 1	-0.014	0.002
HLA-DR antigen-associated invariant subunit	-0.001	0.0003
MHC class II HLA-DR-beta (DR2-DQW1/DR4 DQW3) precursor	-0.010	0.002

Coefficient; responder 1, non-responder 0

Table V. *Correlation between resistance gene expression and objective response to chemotherapy.*

		Cases of elevated gene expression of independent resistant genes			*p*
		1 to 3 genes	0 gene	Total	
Pathology	SCLC	5	13	18	0.012
	NSCLC	19	10	29	
Response to chemotherapy	Responder	10	19	29	0.0039
	Non-responder	14	4	18	

Elevated gene expression was defined as higher expression than mean of expressions of 4 house keeping genes. SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer.

predictive markers of chemotherapy response in the present study had a role in host immunity. Anticancer drugs cause rapid and persistent depletion of lymphocytes, possibly by the direct induction of apoptosis in mature T and B cells. *In vivo* chemotherapy induces a significant increase in lymphocyte apoptosis *ex vivo*. Chemotherapy-induced lymphocyte depletion involves distinct mechanisms of apoptosis induction, such as direct mitochondrial and caspase-dependent pathways in resting lymphocytes and p53-dependent pathways in cycling lymphocytes (13). Furthermore, we have previously shown that responders to chemotherapy demonstrate greater gene-specific damage in MNC compared with non-responders (3). These data support the observation that most of the genes identified as resistant genes in the present study were involved in host immunity. The expression level of each resistant gene in our study was elevated in non-responders, and was expected to oppose apoptosis induction by anticancer drugs *in vivo*. Upon reference to other studies, it was confirmed that three genes involved in host immunity had some influence on a patient's response to treatment.

Some researchers have described an activation of these immunity-related genes with chemotherapy, such as interferon (IFN) treatment. One investigation demonstrated that IFN-treated renal cell carcinoma (RCC) cells induced HLA-DR expression. A significant correlation was found between the expression of an MHC antigen-associated invariant chain and the degree of lymphocyte infiltration (14). A previous genetic analysis study has demonstrated that MHC class II genes influence the outcome of chronic C hepatitis treatment with IFN (15). The findings of this study are not applicable to the treatment of cancer with chemotherapy; some human cancers such as melanomas and RCC are also treated with IFN, and the gene may be implicated in the mechanism of chemosensitivity of cancer cells. Keratinocyte-bound HLA-DR antigens were observed after treatment with IFN in melanomas and RCC (16). The allograft inflammatory factor-1, which is encoded within the HLA class III genomic region, is a modulator of the immune response during macrophage activation (17,18). From these data, it may be suggested that host immune response is closely related to tumor depression by anticancer drugs.

Using computational analysis, in the study reported here, we selected genes likely to be associated with chemo-resistance, and were able to distinguish SCLC from NSCLC according to the different biological natures of the genes. The expression levels of resistant genes were elevated in about two thirds of NSCLCs, but not in most of the SCLCs in the present study. The data indicated that SCLC is highly sensitive to chemotherapy, while NSCLC is only moderately sensitive.

We need to undertake prospective evaluations to determine whether the selected genes in this study are truly important and potentially useful for predicting chemoresistance. It is also necessary to determine whether administration of drugs will result in changes to the expression levels of the resistant genes we identified, and if any such changes are related to tumor response. If the expression level of a gene changes with treatment, that gene will be the new target of

cancer chemotherapy. In this study we measured the expression levels of genes in patients treated with platinum-based chemotherapy. Recently, patients with NSCLC have been treated with non-platinum chemotherapy. It is thus also necessary that the expression levels of our resistant genes can be used to predict clinical outcome with non-platinum chemotherapy. Accumulation of these data could eventually lead to the prescription of "personalized chemotherapy" with effective anticancer drugs.

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REFERENCES

1. Shinkai T, Eguchi K, Sasaki Y, Tamura T, Ohe Y, Kojima A, Oshita F, Miya T, Okamoto H, Iemura K, Saijo N. A prognostic-factor risk index in advanced non-small-cell lung cancer treated with cisplatin-containing combination chemotherapy. *Cancer Chemother. Pharmacol* **30**:1-6, 1992.
2. Oshita F, Arioka H, Heike Y, Shiraishi J, Saijo N. Correlation of gene-specific damage with cisplatin between human adenocarcinoma cells and peripheral blood mononuclear cells analyzed by polymerase chain reaction-stop assay. *Jpn J Cancer Res* **86**:233-238, 1995.
3. Oshita F, Yamamoto N, Fukuda M, Ohe Y, Tamura T, Eguchi K, Shinkai T, Saijo N. Correlation of therapeutic outcome in non-small cell lung cancer and DNA damage assayed by polymerase chain reaction in leukocytes damaged in vitro. *Cancer Res* **55**:2334-2337, 1995.
4. Oshita F, Yamada K, Nomura I, Noda K. Gene-specific damage produced by topoisomerase inhibitors in human lung cancer cells and peripheral mononuclear cells as assayed by polymarase chain reaction-stop assay. *Anticancer Res* **18**:3389-3394, 1998.
5. Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M, Pergamenschikov A, Lee JC, Lashkari D, Shalon D, Myers TG, Weinstein JN, Botstein D, Brown PO. Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* **24**:227-235, 2000.
6. Scherf U, Ross DT, Waltham M, Smith LH, Lee JK, Tanabe L, Kohn KW, Reinhold WC, Myers TG, Andrews DT, Scudiero DA, Eisen MB, Sausville EA, Pommier Y, Botstein D, Brown PO, Weinstein JN. A gene expression database for the molecular pharmacology of cancer. *Nat Genet* **24**:236-244, 2000.
7. Staunton JE, Slonim DK, Coller HA, Tamayo P, Angelo MJ, Park J, Scherf U, Lee JK, Reinhold WO, Weinstein JN, Mesirov JP, Lander ES, Golub TR. Chemosensitivity prediction by transcriptional profiling. *Proc Natl Acad Sci USA* **98**:10787-10792, 2001.
8. Dan S, Tsunoda T, Kitahara O, Yanagawa R, Zembutsu H, Katagiri T, Yamazaki K, Nakamura Y, Yamori T. An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines. *Cancer Res* **62**:1139-1147, 2002.
9. Zembutsu H, Ohnishi Y, Tsunoda T, Furukawa Y, Katagiri T, Ueyama Y, Tamaoki N, Nomura T, Kitahara O, Yanagawa R, Hirata K, Nakamura Y. Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. *Cancer Res* **62**:518-527, 2002.
10. Kikuchi T, Daigo Y, Katagiri T, Tsunoda T, Okada K, Kakiuchi S, Zembutsu H, Furukawa Y, Kawamura M, Kobayashi K, Imai K, Nakamura Y. Expression profiles of non-small cell lung cancers on cDNA microarrays: identification of genes for prediction of lymph-node metastasis and sensitivity to anti-cancer drugs. *Oncogene* **22**:2192-2205, 2003.
11. WHO Hand Book for Reporting Results of Cancer Treatment. WHO Offset Publication No. 48. Geneva, Switzerland: World Health Organization, 1979.
12. Chenchik A , Zhu YY, Diatchenko L, Li R, Hill J, Siebert PD. Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In:Gene cloning and analysis by RT-PCR. Siebert PD and Larrick JW (ed.) Bio Techniques Book, MA, USA, 305-319, 1998.
13. Stahnke K, Fulda S, Friesen C, Strauss G, Debatin KM. Activation of apoptosis pathways in peripheral blood lymphocytes by in vivo chemotherapy. **Blood 98**:3066-3073, 2001.
14. Saito T, Kimura M, Kawasaki T, Sato S, Tomita Y. MHC class II antigen-associated invariant chain on renal cell cancer may contribute to the anti-tumor immune response of the host. *Cancer Lett* **109**:15-2, 1996.
15. Dincer D, Besisik F, Oguz F, Sever MS, Kaymakoglu S, Cakaloglu Y, Demir K, Turkoglu S, Carin M, Okten A. Genes of major histocompatibility complex class II influence chronic C hepatitis treatment with interferon in hemodialysis patients. *Int J Artif Organs* **24**:212-214, 2001.
16. Volc-Platzer B, Steiner A, Radaszkiewicz T, Wolff K. Recombinant gamma interferon and in vivo induction of HLA-DR antigens. *Br J Dermatol* **119**:155-160, 1988.
17. Utans U, Quist WC, McManus BM, Wilson JE, Arceci RJ, Wallace AF, Russell ME. Allograft inflammatory factory-1. A cytokine-responsive macrophage molecule expressed in transplanted human hearts. *Transplantation* **61**:1387–1392, 1996.
18. Utans U, Arceci RJ, Yamashita Y, Russell ME. Cloning and characterization of allograft inflammatory factor-1: a novel macrophage factor identified in rat cardiac allografts with chronic rejection. *J Clin Invest* **95**:2954-2962, 1995.

Prognostic Impact of Survivin, Cyclin D1, Integrin β1, and VEGF in Patients With Small Adenocarcinoma of Stage I Lung Cancer

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Abstract: The purpose of this study was to investigate the impact of survivin, cyclin D1, integrin β1, and vascular endothelial growth factor (VEGF) in tumor on survival of patients with small adenocarcinoma of the lung. Seventy-two patients with pathologic stage I resected tumors <2 cm in diameter were entered into the study. Each patient underwent curative surgical resection for lung cancer between July 1992 and November 1999. The resected tumors were subjected to immunostaining for each gene. Thirty-five, 26, 6, and 16 patients had tumors with >10% survivin-, >20% cyclin D1-, >10% integrin β1-, and >10% VEGF-positive cells, respectively. When the survival of 72 patients was compared according to each gene expression, the overall survival of patients with positive expression of survivin, cyclin D1, and integrin β1 was significantly worse than that of individuals whose tumors had negative expression of each gene. By multivariate analysis controlling for each gene expression, no gene expression was an independent marker of poor prognosis, however, the overall survival of the complex gene expression (2 or more gene-positive) group (n = 35) was significantly worse than that of 0 or 1 gene-positive group (n = 37; log-rank test, $P = 0.0011$; Wilcoxon test, $P = 0.0011$). When the association between survival and pathologic factors, including lymphatic invasion, venous invasion, type of bronchioalveolar carcinoma, and complex gene positive expression was analyzed, only complex gene-positive expression was found to be a significant independent factor (hazard ratio = 0.085, $P = 0.0299$). It can be concluded that multiple increased expression of oncogene is a poor prognostic factor in patients with small adenocarcinoma of the lung.

Key Words: oncogene, lung cancer, prognostic factor

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Non–small-cell lung cancer (NSCLC) is the leading cause of cancer death in Japan. To improve the prognosis of lung cancer patients, attempts have been made to develop tests that will facilitate the early diagnosis and treatment of lung cancer and thereby decrease the mortality from this disease. Many chest roentgenogram-negative lung cancers can be detected on chest computed tomography scans, but a significant number of patients with early stage disease show aggressive tumors. Although locoregional control of NSCLC can be achieved by surgery, more than 70% of relapses in patients with stage I disease occur at distant sites.[1] Thus, most patients with NSCLC must have systemic disease, even at the earliest stage. Recent efforts at improving the management and outcome of patients with this disease have been directed at neoadjuvant and adjuvant chemotherapy to reduce the high systemic relapse sites.

New therapeutic strategies for NSCLC are required a better understanding of the cell biology of early stage NSCLC. Several molecular markers have been evaluated in association with established histologic and clinical prognostic parameters of early stage NSCLC,[2–7] and it is suspected that tumor invasion and metastasis involve complex alterations of gene expression that may be selective for specific cancer types. However, none is currently being used in treatment decision making.

Initiated cancer cells at early stage disease are considered to acquire other gene alterations in addition to early genetic alteration, and progress to locally advanced or metastatic tumors. Many genetic alterations, which relates to cell proliferation, apoptosis, vascularization, and tumor invasion, were reported as prognostic factors in resected NSCLC. However, there is no study showing which gene alterations mostly influence tumor progression and metastasis in the early stage of NSCLC. Clarification of the gene alterations that influence tumor progression from early to advanced stage in NSCLC is required when considering new therapeutic strategies for resectable NSCLC.

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Retrospective analysis of the predictive factors associated with the response and survival benefit of gefitinib in patients with advanced non-small-cell lung cancer

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KEYWORDS
Gefitinib;
Non-small-cell lung cancer;
Prognostic factor;
Smoking index;
Female;
Performance status (PS);
Retrospective analysis

Summary

Background: The purpose of the study was to identify the potential predictive features associated with the response and survival benefit of gefitinib administration. We have retrospectively reviewed data of all patients who received a single regimen of gefitinib in our institution from August 1998 until July 2003.

Methods: Overall 101 patients with non-small-cell lung cancer (NSCLC) who have received a single use of gefitinib were analyzed. Potential factors associated with the response of gefitinib included smoking index, gender, histology, performance status (PS), number of pre-treatments, age and stage. Univariate analysis was performed for these strata by Fisher's exact test and multivariate analysis was then performed using the logistic regression model.

Results: The overall response rate was 19.8%. Univariate analysis revealed that significant predictive factors were associated with the response for 'adenocarcinoma', 'female', 'good PS' (0–1) and 'non-smoker' categories. Multivariate analysis limited the predictive factors associated with the response for 'female' ($P = 0.0032$), 'good PS' ($P < 0.02$) and 'non-smoker' ($P = 0.0417$). In survival analyses, 'female' ($P < 0.005$), 'good PS' ($P < 0.0001$), and a low level of the smoking index ($P < 0.05$) indicated significantly prolonged survival. Response and survival data in elderly patients were equivalent to those in younger patients. Adverse events (AEs) were generally mild and were almost always skin reactions and diarrhea. Interstitial lung disease (ILD) occurred in 4% of the group under observation.

Conclusions: Gefitinib provided clinical benefit for the following factors 'female', 'good PS' and 'non-smoker'. A low smoking index is reported as a novel predictive prognostic factor following a single regimen of gefitinib.

Abbreviations: NSCLC, non-small-cell lung cancer; EGFR, epidermal growth factor receptor; IDEAL-1, Iressa dose evaluated advanced lung cancer-1; PS, performance status; NCI-CTC, National Cancer Institute-Common Toxicity Criteria; INTACT-1, Iressa NSCLC trial assessing combination treatment-1; INTACT-2, Iressa NSCLC trial assessing combination treatment-2

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TABLE 1. Characteristics of Gene Expression and Overall Survival*

			P Value	
		No. of Patients	Log-rank	Wilcoxon
Survivin	Negative	37	0.014	0.014
	Positive	35		
Cyclin D1	Negative	26	0.049	0.052
	Positive	46		
Integrin β1	Negative	66	0.021	0.01
	Positive	6		
VEGF	Negative	46	0.68	0.65
	Positive	26		
Combination	0–1	37	0.0011	0.0011
	2–4	35		

*Combination; survivin, cyclin D1, integrin β1, and VEGF were included.

Thirty-seven patients had tumor with positive expression of 0 or 1 gene and 35 patients had tumor with positive expression of 2 to 4 genes.

In an attempt to better understand tumor progression in NSCLC, expression of survivin, cyclin D1, integrin β1, and vascular endothelial growth factor (VEGF), which have different mechanisms in tumor progression, were investigated prognosis in adenocarcinoma <2 cm in diameter of stage I in the present study.

PATIENTS AND METHODS

Patients with lung adenocarcinoma <2 cm in diameter of pathologic stage I, resected between January 1992 and December 1999, were enrolled in the present study.

The tumor specimens obtained by resection were subjected to immunostaining for survivin, cyclin D1, integrin β1, and VEGF. Formalin-fixed, paraffin-embedded, 5-im-thick tumor sections were mounted on charged glass slides, deparaffinized and rehydrated in a graded alcohol series. Immunohistochemical staining was performed using an automated processor. Details of immunostaining were shown in previous reports.[8–11] Each factors immunostaining levels were classified as positive (>10% of cells stained for survivin, integrin β1, and VEGF, and >20% of cells stained for cyclin D1) or negative (≤10% of cells stained for survivin, integrin β1, and VEGF, and ≤20% of cells stained for cyclin D1).

Two pathologists examined the staining patterns of each factor independently, and recorded the percentage of positive cells in each specimen. At least 20 high-power fields were chosen randomly and 2000 cells were counted. The ratio of each gene-positive cell was calculated by dividing the number of positive cells by the total number of cells, and was expressed as a percentage.

Kaplan-Meier survival curves were constructed and analyzed for statistical significance by means of the log-rank and generalized Wilcoxon tests. The influence of each variable on survival was examined by the Cox proportional hazards model in multivariate regression analyses. Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Seventy-two patients with resected tumors <2 cm in diameter of pathologic stage I were entered into the study. There were 29 males and 43 females, with a median age of 64 years (range 26–83 years). Each patient underwent curative surgical resection for lung cancer between July 1992 and November 1999. The resected tumors were subjected to immunostaining for each gene. Thirty-five, 26, 6, and 16 patients had tumors with >10% survivin-, >20% cyclin D1-, >10% integrin β1-, and >10% VEGF-positive cells, respectively.

When the survival of 72 patients was compared according to each gene expression, the overall survival of patients with positive expression of survivin, cyclin D1, and integrin β1 was significantly worse than that of individuals whose tumors had negative expression of each gene (Table 1). We analyzed how many of the 4 genes expressed positively in each resected tumor, 9, 28, 24, and 11 patients had tumors with positive expression of 0, 1, 2, and 3 genes, respectively. There were no patients with tumor expressed every 4 genes.

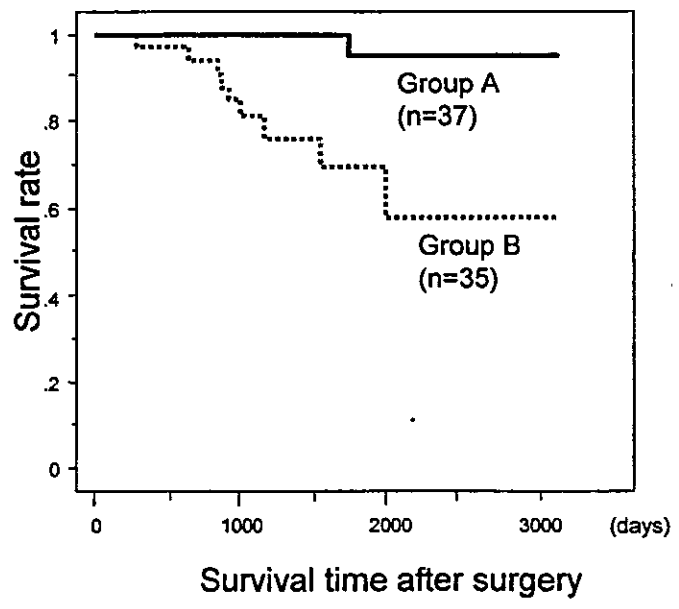

FIGURE 1. Survival curves according to gene immunostaining, constructed using the Kaplan-Meier method. Survival after surgery of patients with 2 or more positive expression of genes in tumor was worse than that of those with 0 or 1-positive expression of gene in tumor (log-rank $P = 0.0011$, Wilcoxon $P = 0.0011$).

TABLE 2. Multivariate Regression Analysis of Variables in Predicting Overall Survival

Variable	Assigned Score	Hazards Ratio	95% CI	*P* Value
Lymphatic invasion		0.488	0.094–2.549	0.400
Negative	0			
Positive	1			
Vessel invasion		0.493	0.098–2.473	0.390
Negative	0			
Positive	1			
Bronchioalveolar carcinoma		1.062	0.102–11.053	0.960
yes	0			
no	1			
Combination of gene expression		0.087	0.009–0.801	0.031
Positive 0–1	0			
Positive 2–4	1			

By multivariate analysis controlling for each gene expression, no gene expression was an independent marker of poor prognosis. When we examined whether the number of positive gene expression in the tumors influence the prognosis, the overall survival of complex gene expression (2 or more gene-positive) group (n = 35) was significantly worse than that of the 0 or 1 gene-positive group (n = 37; log-rank test, $P = 0.0011$; Wilcoxon test, $P = 0.0011$, Fig. 1 and Table 1). When the association between survival and pathologic factors, including lymphatic invasion, venous invasion, type of bronchioalveolar carcinoma, and complex gene expression was analyzed, only complex gene expression was found to be a significant independent factor (hazard ratio = 0.085, $P = 0.0299$, Table 2). It can be concluded that multiple but not single increased expression oncogene is a poor prognostic factor in patients with small adenocarcinoma of the lung.

DISCUSSION

Changes in gene expression are at the basis of many crucial physiological and pathologic processes. Tumorigenesis involves a loss of balance between regulators of cell proliferation and apoptosis. A previous study showed positive expression of survivin was a poor prognostic factor in small adenocarcinomas <2 cm in diameter.[11] However, the present study showed that not only survivin but also cyclin D1 and integrin β1 were poor prognostic factors. The present study demonstrated that 49%, 64%, 8%, and 22% of resected tumors <2 cm in diameter of pathologic stage I showed positive expression of survivin, cyclin D1, integrin β1, and VEGF, respectively. Only 9 patients (12.5%) had no expression of every 4 genes in resected small adenocarcinoma but many others had single or multiple gene expression in this study. This fact may explain that small adenocarcinoma <2 cm in diameter of pathologic stage I is in a transition from early to advanced stage. After all, multiple regression analysis demonstrated that no gene expression was an independent marker of poor prognosis, but complex gene expression show poor prognosis in small adenocarcinoma of the lung.

Lung cancer has a high potential of distant metastasis, and induction therapy followed by surgery and/or radiotherapy has become standard therapy for stage III disease.[12] Several gene expression analyzed in the present study is an important predictive factor for recurrence after curative resection in early stage lung cancer. The information obtained by this analysis is a powerful prognostic discriminator for patients with stage I disease and may be useful for decisions concerning which patients should and should not receive systemic treatment in addition to surgical resection. Furthermore, new strategies may be also considered with reference to multiple oncogene expression to improve treatment of locally advanced NSCLC. Targeted chemotherapy against positive expressed gene, such as using monoclonal antibodies, may be an ideal approach to treating multiple oncogene expressed tumors. When adjuvant chemotherapy after surgical resection is considered, not only single target therapy but also multitarget therapy such as combination of antiapoptotic, anticell cycle, antiadhesion and others, should be required, because multiple gene expressions in resected tumor is a poor prognostic factor presented in this study.

Lung cancer appears as small nodules in the peripheral part of the lung, and pathologic or cytologic diagnosis is essential. Patients suspected of having lung cancer often undergo fiberscopic examination, with a tumor biopsy examination or a cytologic approach. When a lesion is inaccessible to bronchoscopic biopsy, or when the biopsy specimen is nondiagnostic, a diagnosis of cancer may be possible by cytologic examination of bronchoalveolar lavage fluid (BALF). In a previous report, we demonstrated that detection of the K-*ras* mutation in BALF cells, by PCR-PIREMA, aids the diagnosis of lung cancer in patients with small pulmonary lesions with negative cytologic findings.[13] BALF from patients with small adenocarcinoma may contain survivin, cyclinD1, integrin β1, and VEGF, and it is possible that the gene expressions can be detected as a diagnostic marker.

In conclusion, multiple but not single oncogene expressions in tumor cells is a poor prognostic factor in patients with small adenocarcinoma of the lung. Detection of the gene expressions appears to be not only a useful diagnostic marker but also a potential new target for anticancer therapy for early stage NSCLC.

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REFERENCES

1. Martini N, Ginsberg R. Treatment of stage I and II disease, in Aisner J, Arriagada R, Green M, et al, eds. *Comprehensive Textbook of Thoracic Oncology*. Baltimore: Williams & Wilkins; 1996:339–350.
2. Tomizawa Y, Nakajima T, Kohno T, et al. Clinicopathological significance of Fhit protein expression in stage I non-small cell lung carcinoma. *Cancer Res*. 1998;58:5478–5483.
3. Ohta Y, Tomita Y, Oda M, et al. Tumor angiogenesis and recurrence in stage I non-small cell lung cancer. *Ann Thorac Surg*. 1999;68:1034–1038.
4. Herbst RS, Yano S, Kuniyasu H, et al. Differential expression of E-cadherin and type IV collagenase genes predicts outcome in patients with stage I non-small cell lung carcinoma. *Clin Cancer Res*. 2000;6: 790–797.
5. Khuri FR, Lotan R, Kemp BL, et al. Retinoic acid receptor-β as a prognostic indicator in stage I non-small-cell lung cancer. *J Clin Oncol*. 2000;18:2798–2804.
6. Hashimoto T, Tokuchi Y, Hayashi M, et al. p53 null mutations undetected by immunohistochemical staining predict a poor outcome with early-stage non-small cell lung carcinomas. *Cancer Res*. 1999;59:5572–5577.
7. Nelson HH, Christiani DC, Mark EJ, et al. Implications and prognostic value of K-ras mutation for early-stage lung cancer in women. *J Natl Cancer Inst*. 1999;91:2032–2038.
8. Betticher DC, Heighway J, Hasleton PS, et al. Prognostic significance of CCND (cyclin D1) overexpression in primary resected non-small-cell lung cancer. *Br J Cancer*. 1996;73:294–300.
9. Ito H, Oshita F, Kameda Y, et al. Expression of vascular endothelial growth factor and basic fibroblast growth factor in small adenocarcinoma. *Oncol Rep*. 2002;9:119–123.
10. Oshita F, Kameda Y, Ikehara M, et al. Increased expression of integrin β1 is a poor prognostic factor in small-cell lung cancer. *Anticancer Res*. 2002;22:1065–1070.
11. Ikehara M, Oshita F, Kameda Y, et al. Expression of survivin correlated with vessel invasion is a poor prognostic factor in small adenocarcinoma of the lung. *Oncol Rep*. 2002;9:835–838.
12. Van Zandwijk N, Smit EF, Krame GWP, et al. Gemcitabine and cisplatin as induction regimen for patients with biopsy-proven stage IIIA N2 non-small-cell lung cancer: A phase II study of the European Organization for Research and Treatment of Cancer Lung Cancer Cooperative Group (EORTC 08955). *J Clin Oncol*. 2000;18:2658–2664.
13. Oshita F, Nomura I, Yamada K, et al. Detection of K-ras mutation of bronchoalveolar lavage fluid cells aids the diagnosis of lung cancer in small pulmonary lesions. *Clin Cancer Res*. 1999;5:617–620.

1. Introduction

Patients with advanced non-small-cell lung cancer (NSCLC) have a poor prognosis with 1–5% 5-year survival rates [1]. A recent meta-analysis demonstrated that platinum-based combination chemotherapy is currently considered to be the most effective treatment for advanced NSCLC, and these have improved the median survival time (MST) by 2 months and caused a 10% increase in 1-year survival rates [2]. As platinum-based chemotherapy improves survival and quality of life in advanced NSCLC patients, most patients will receive second line chemotherapy. With recurrence or progression, docetaxel has been approved as a second line chemotherapy treatment due to demonstrated survival benefit compared with best supportive care (BSC) or vinorelbine/ifosfamide [3,4]. Currently, there is no proven effective chemotherapy for patients previously treated with platinum-based and docetaxel therapies.

The epidermal growth factor receptor (EGFR) is a promising target for anticancer therapy because many types of cancer cells express or overexpress EGFR (including NSCLC, renal cell carcinoma and breast cancer) [5,6]. EGFR overexpression has been reported as a poor prognostic factor in many types of human solid tumors including NSCLC in several studies [7–9]. Currently, monoclonal antibodies that bind to the extracellular domain of EGFR and intracellular tyrosine kinase inhibitors have been developed [10,11]. Gefitinib is an orally active, selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways implicated in the proliferation, angiogenesis, invasion, metastasis and survival of cancer cells [12,13]. Several phase I trials demonstrated safety and tolerability of gefitinib in pretreated patients with solid tumors, in which trials an 11% response rate was seen in 100 patients with heavily pretreated advanced NSCLC [14]. On the other hand, in Japan, a phase I trial demonstrated five responders out of a total of 31 patients who all had adenocarcinoma of the lung [12]. To confirm anti-tumour activity and the safety profile of gefitinib, an international phase II study (IDEAL-1) and United States trial (IDEAL-2) were conducted as a second or third line treatment in patients with advanced NSCLC [15,16]. Patients enrolled in these studies were randomized into two different doses, 250 and 500 mg/day. These trials demonstrated that toxicity was mild and showed an encouraging response rate with an RR of 18.4 and 11.8% of patients in the 250 mg arm, respectively, and an improvement in disease related symptoms and quality of life were observed. The IDEAL-1 study has also confirmed that there were statistically significant differences in efficacy for 'adenocarcinoma' and 'female' using multivariate analysis. Two large randomized phase III studies [17,18], which are standard chemotherapy (cisplatin/gemcitabine or carboplatin/paclitaxel) with or without gefitinib, failed to demonstrate a survival benefit for advanced NSCLC patients as a first line chemotherapy. Although the results of the phase III studies were negative, gefitinib is still considered a promising molecular targeted agent as a new generation treatment in patients with advanced NSCLC. Information on the clinical prognostic factors following a single regimen of gefitinib should be helpful in finding which patients are likely to receive benefit, and in the development of a future treatment. Although the previous phase II trial (IDEAL) showed that several predictive factors were associated with the response to gefitinib, the population was essentially biased towards the young, with good performance status (PS) and conserved, good organ functions.

In this study, to find factors associated with an objective response and survival benefit of gefitinib, we retrospectively analysed patients who received a single regimen of gefitinib at our institute.

2. Methods

All patients with stage IIIB or IV NSCLC, who received a single regimen of gefitinib from August 1998 until July 2003 at the Kinki University School of Medicine, Osaka, were retrospectively reviewed. We evaluated patients who participated in clinical trials (phase I trial, phase II trial; IDEAL-1), or phase II trial for investigating surrogate gene therapy, and in 53 patients who were administered the drug after marketing (including elderly or poor performance status patients). Patients who received gefitinib as part of a compassionate use program were excluded. All patients were checked for age, gender, histology, Eastern Cooperative Oncology Group (ECOG), PS, stage, pre-treatment regimen, number of prior regimen, and smoking status before treatment of gefitinib. Smoking status was evaluated by the Brinkmann index; number of cigarettes per day multiplied by number of years. We analyzed the response, overall survival rate and the adverse effects of gefitinib, and investigated predictive factors associated with response and prognosis. The response was assessed using physical examination, biochemical profile, chest X-ray, chest computed tomography (CT), head CT or magnetic resonance imaging (MRI) scan, abdominal echo-graphic or abdominal CT scan, bone scinti-graph, bronchoscope, and was evaluated according to the response eval-

uation criteria in solid tumor (RECIST) [19]. The severity of all the adverse events (AEs) that related to gefitinib administration was assessed by the NCPCTC (version 2.0) grading system. The predictive factors associated with the response that were analyzed in this study were age, gender, PS, histology, stage, number of prior regimen and smoking status. Variables were tested for any possible relationship with the response to gefitinib, at first by univariate analysis, and subsequently by the application of a multivariate model. Response rates were compared between strata using Fisher's exact test. Logistic regression models were used to explore observed differences and identify baseline factors that may independently predict for response rates. The survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Patient profiles

From August 1998 until July 2003 at our institute, a total of 105 patients, who were already cytologically or histologically diagnosed as NSCLC, were treated by a single regimen of gefitinib. Patients received gefitinib until disease progression or intolerable toxicity. Of these, 101 patients were evaluated as suitable for analysis; four patients were excluded from analysis because they received gefitinib as part of a compassionate use program. As shown in Table 1, the 101 patients included: 2 patients who received gefitinib at a once daily dose of 50 mg; single patients who each received 100, 225, 400, 525 and 700 mg, respectively; 89 patients who received 250 mg; and 5 patients who received 500 mg. In the phase I trial, we used an intermittent administration schedule with 14 days continuous dosing followed by 14 days off.

Table 1 Patient characteristics

	Number of patient ($N = 101$)
Phase I	7
50 mg	2
100 mg	1
225 mg	1
400 mg	1
525 mg	1
700 mg	1
Phase II (IDEAL-1)	11
250 mg	6
500 mg	5
Phase II (gene expression) (250 mg)	30
Post marketing (250 mg)	53

Table 2 Patient characteristics ($N = 101$)

	Number of patients
Age (year)	
Median (range)	62 (31–84)
<69	74
≥70	27
Gender	
Male	64
Female	37
Performance status	
0	15
1	62
2	17
3	7
Tumor histology	
Adenocarcinoma	81
Squamous	18
Large-cell	2
Stage	
III	18
IV	83
Previous treatment	
No treatment	5
Failed 1 previous chemotherapy regimens	53
Failed 2 previous chemotherapy regimens	34
Failed 3 previous chemotherapy regimens	9
Smoking (smoker:never-smoker)	55:46
Index[a] 0:1–999:1000	46:32:23

[a] Index: number of cigarettes per day multiplied by number of years.

Patient characteristics are shown in Table 2. The median age was 62 years (ranging from 31–84) and 74 patients (73.3%) were less than 69 years old. 63.4% of the patients were male, 76.2% had performance status (ECOG) 0–1, 80.2% had adenocarcinoma of which 83.2% had stage IV disease. Fifty-three patients had received one prior regimen, 43 had more than two prior regimens and only five had previously been untreated. 54.5% of them were smokers, and the non-smokers were almost all female. This study included patients

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Topoisomerase I Inhibitors in Small-Cell Lung Cancer

The Japanese Experience

ABSTRACT

Among patients with lung cancer, approximately 15% have small-cell lung cancer (SCLC). The clinical characteristics of SCLC tend to be more aggressive, but also more sensitive to chemotherapy and radiation therapy than those of non-SCLC. Irinotecan (Camptosar) is a derivative of camptothecin, an inhibitor of the nuclear enzyme topoisomerase I. Irinotecan has been shown to exhibit excellent antitumor activity against SCLC in monotherapy regimens and in combination with cisplatin. A phase III trial comparing irinotecan and cisplatin (IP) with etoposide and cisplatin (EP) in patients with previously untreated extensive-stage SCLC (ED-SCLC) was conducted. Patients in the IP arm responded significantly better than patients in the EP arm. In the IP arm, the response rate was 84%, and median overall survival was 12.8 months. A phase II trial of irinotecan, cisplatin, and etoposide (IPE) administered weekly (arm A) or every 4 weeks (arm B) for ED-SCLC (JCOG 9902-DI) was also performed. In arm B, the response rate was 77% and the median overall survival was 12.9 months. A randomized trial comparing IP with IPE administered every 3 weeks in patients with previously untreated ED-SCLC is presently being performed in Japan.

An estimated 75,000 new cases of lung cancer were diagnosed in Japan in 2002. Approximately 15% of these cases were diagnosed as small-cell lung cancer (SCLC), which is strongly associated with tobacco use, as is non–small-cell lung cancer (NSCLC). The clinical characteristics of SCLC tend to be more aggressive, but also more sensitive to chemotherapy and radiation therapy than those of NSCLC. Small-cell lung cancer is usually staged as either limited disease (LD) or extensive disease (ED).[1]

Platinum-based chemotherapy remains the mainstay of treatment regimens for ED-SCLC. In a meta-analysis of 19 randomized trials comparing a cisplatin-based regimen with a non–cisplatin-based regimen, patients randomized to a regimen containing cisplatin had a significantly higher probability of response and survival, with no significant increase in toxicity.[2] Berghmans et al presented a detailed analysis of the roles of etoposide and cisplatin in the treatment of SCLC.[3] Between 1980 and 1998, 36 eligible trials were performed. These trials concluded that the use of cisplatin and/or etoposide offered a significant survival advantage to patients with SCLC.

Irinotecan (Camptosar) has been semisynthesized as a water-soluble derivative of camptothecin, an inhibitor of nuclear enzyme topoisomerase I, in an attempt to reduce its toxicity and to improve its therapeutic efficacy.[4-8] In a phase II trial of irinotecan for SCLC, the response rate was 47%.[9,10] In preclinical studies, irinotecan and cisplatin exhibited synergistic activities. Their toxicity pro-

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10. Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R, Talpaz M. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med* 2001; **344**: 1038–42.
11. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2002; **344**: 1031–7.
12. Slamon DJ, Leyland-Jones B, Shak S *et al.* Use of chemotherapy plus a monoclonal antibody against Her2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2004; **344**: 783–92.
13. Coiffier B, Lepage E, Briere J *et al.* CHOP chemotherapy plus rituximub compared with CHOP alone in elderly patients with diffuse B-cell lymphoma. *N Engl J Med* 2002; **346**: 235–42.
14. Bevacizumab (anti VEGFAb) prolongs survival in first line CRC. *ASCO* 2003; abstract.
15. Shepherd FA, Giaccone G, Seymour L, Debruyne C, Bezjak A, Hirsh V, Smylie M, Rubin S, Martins H, Lamont A, Krzakowski M, Sadura A, Zee B. Prospective, randomized, double-blind, placebo-controlled trial of marimastat after response to firstline chemotherapy in patients with small-cell lung cancer: a trial of the National Cancer Center Institute of Canada-clinical trials group and European Organization for Research and Treatment of Cancer. *J Clin Oncol* 2002; **20**: 4434–9.
16. Gatzemeier U, Groth G, Hirsh V *et al.* Gemcitabine/cisplatin alone and with trastuzumab (Herceptin) in patients with non-small cell lung cancer overexpressing HER2: results of a randomised phase II study. *Proc Am Soc Clin Oncol* 2002; **21**: 297a (abstr 1185).
17. Johnson BE, Fischer T, Fischer B, Dunlop D, Rischin D, Silberman S, Kowalski MO, Sayles D, Dimitrijevic S, Fletcher C, Hornick J, Salgia R, Le Chevalier T. Phase II study of imatinib in patients with small cell lung cancer. *Clin Cancer Res* 2003; **9**: 5880–7.
18. Naruse I, Fukumoto H, Saijo N, Nishio K. Enhanced anti-tumor effect of trastuzumab in combination with cisplatin. *Jpn J Cancer Res* 2002; **93**: 574–81.
19. Lynch TJ, Raju R, Lind M, Riviere A, Gatzemeier U, Drorr A, Holmlund J, Yuen A, Sikic B. Randomized phase III trial of chemotherapy and antisense oligonucleotide LY900003 (ISIS 3521) in patients with advanced NSCLC: initial report. *Proc Am Soc Clin Oncol* 2003; **22**: 623 (abstr 2504).
20. Rubin C, Kosloff M, Edelman MJ, Hoffman PC, Szeto L, Vokes EE. Phase I study of G3139 (oblimersen sodium), carboplatin, and etoposide in previously untreated extensive stage small cell lung cancer (SCLC). *Proc Am Soc Clin Oncol* 2003; **22**: 631 (abstr 2538).
21. Herbst RS, Giaccone G, Schiller JH, Natale RB, Miller V, Manegold C, Scagliotti G, Rosell R, Oliff I, Reeves JA, Wolf MK, Krebs AD, Averbuch SD, Ochs JS, Grous J, Fandi A, Johnson DH. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 2. *J Clin Oncol* 2004; **22**: 785–94.
22. Giaccone G, Herbst RS, Manegold C, Scagliotti G, Rosell R, Miller V, Natale RB, Schiller JH, von Pawel J, Pluzanska A, Gatzemeier U, Grous J, Ochs JS, Averbuch SD, Wolf MK, Rennie P, Fandi A, Johnson DH. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 1. *J Clin Oncol* 2004; **22**: 777–84.
23. Saijo N. Translational study in cancer research. *Intern Med* 2003; **41**: 770–3.
24. Saijo N, Nishio K, Tamura T. Translational and clinical studies of target-based cancer chemotherapy. *Int J Clin Oncol* 2003; **8**: 187–92.
25. Paez JM, Janne PA, Lee JC, Tracy S, Geulich H, Gabriel S, Heman P, Key FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Seller WR, Johnson BE, Meyerson M. EGFR mutation in lung cancer: correlation with clinical response to Gefitinib therapy. *Science* 1099314, April 30, 2004.
26. Lynch T, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brain BS, Brannigan W, Harris PL, Haselat SM, Supko JG, Haluska FG, Haselat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA. *N Engl J Med* 2004; **350**: 2129–39.

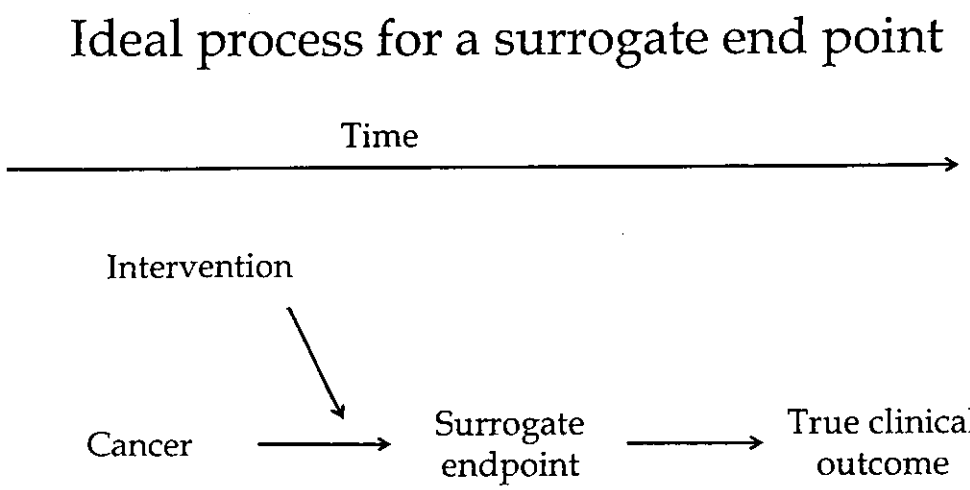

Fig. 2. Ideal process for a surrogate end point. A surrogate end point should be evaluated and obtained before the data of true clinical outcome. It should be a predictor for true clinical outcome.

onstrate that the target effect actually results in a survival benefit. However, many surrogate end points have failed. The reasons can be summarized as follows. The surrogate is not in any of the causal pathways of the disease. There are several causal pathways of the disease, but molecular-target-based drugs affect only the pathway mediated through the surrogate. The surrogate is insensitive to the effect or is not in the pathway of the intervention's effect. The intervention has mechanisms of action that are independent of the disease process. Requirements for target-based therapy are a validated molecular target, reliable assay to measure expression and activity of the target or pathway, difference between target expression in heterogeneous tumor cells, potent and specific inhibitor with good pharmacological properties, and demonstrable target inhibition in human tumors. Valid targets should be expressed, overexpressed, or mutated in tumor tissues. The target should be essential to cell proliferation, cell death, or metastatic ability, and inhibition of the target should result in inhibition of tumor growth and spread. Simple correlates do not make a surrogate. Surrogates can be divided into surrogate-effect end points and surrogate-benefit end points. Surrogate-effect end points involve various problems, such as reliability of the target in tumors, the reliability of the assay method, the tissue specificity of target expression, the extent of heterogeneity, the accessibility of the tissue, and validation against a clinical benchmark. Surrogates for epidermal growth factor receptor (EGFR) inhibition and for anti-angiogenesis are shown in Table 8. Surrogate-benefit end points are objective tumor response, changes in a tumor marker, and changes in tumor metabolism on a positoron emission tomography (PET) scan, and they require validation by linkage to a definitive clinical end point in a prospective trial.

Table 8.

Surrogates for EGFR inhibition
1) Rash: skin biopsy
2) EGFR amount: phosphorylation state
3) MAP kinase activation
4) AKT activation
5) Induction of $p27^{kip1}$
6) Cell proliferation index
Surrogates for anti-angiogenesis
1) Tumor microvessel density
2) Tumor blood flow (MRI)
3) Tumor metabolism (PET)
4) Tumor apoptosis
5) Circulating endothelial cell apoptosis
6) Circulating VEGF and VEGFR levels

EGFR, epidermal growth factor; MRI, magnetic resonance imaging; PET, positron emission tomography; VEGF, vascular endthelial growth factor.

6. How can positive data be obtained from phase III trials?

Standard approval of a molecular-target-based drug requires demonstration of a clinical benefit and improvement of the ultimate outcome, including improvement of survival, relief of symptoms, or a delay in the onset of symptoms. Appropriate preclinical and early clinical trials are essential to obtain positive results in phase III trials. Scientific decision-making is required. Enrichment of the responsive population based on clinical information and translational research is important. Of course, good and feasible clinical trial designs should be adopted. Urgent requirements include the development of a validated test to define the target population and more effective molecular-target-based drugs to reduce sample size.

Recently a Dana-Farber group reported the identification of EGFR mutations in subset of human lung adenocarcinomas and the association between EGFR mutation and gefitinib sensitivity.

Screening for such mutations in lung cancers may enrich patients who will have a response to gefitinib.[25,26]

1. Saijo N. The molecular biology and signal transduction of epidermal growth factor receptor. *New Dev Lung Cancer* 2003; **1**: 3–6.
2. Sekine I, Saijo N. Growth-stimulting pathways in lung cancer: implications for targets of therapy. *Clin Lung Cancer* 2001; **2**: 299–306.
3. Saijo N, Tamura T, Yamamoto N, Nishio K. New strategies for cancer therapy in 21st century. *Cancer Chemother Pharmacol* 2001; **48**: S102–6.
4. Schilsky RL. Endpoints in cancer clinical trials and the drug approval process. *Clin Cancer Res* 2002; **8**: 935–8.
5. Stadler WM, Ratain MJ. Development of target based antineoplastic agents. *Invest New Drugs* 2000; **18**: 7–16.
6. Fox E, Curt GA, Balis FM. Clinical trial design for target-based therapy. *Oncologist* 2002; **7**: 401–9.
7. Gardner SN, Ferandes M. Cytostatic anticancer drug development. *J Exp Ther Oncol* 2004; **4**: 9–18.
8. Saijo N, Tamura T, Nishio K. Problems in the development of target-based drugs. *Cancer Chemother Pharmacol* 2000; **46**: S43–5.
9. Saijo N, Tamura T, Nishio K. Strategy for the development of novel anticancer drugs. *Cancer Chemother Pharmacol* 2003; **52**: S97–101.

prior chemotherapy, but the results showed the same efficacy as for docetaxel alone. Randomized controlled trials comparing paclitaxel and carboplatin with or without Ly900003 have shown no difference in antitumor effect or median survival, and the frequency of grade (Gr) 3/4 thrombocytopenia was significantly higher in the Ly900003-containing regimen.[19] At least four problems can be pointed out in this example. The molecular target of Ly900003 is not essential for tumor growth, invasion, or metastasis, the antitumor activity of Ly900003 is inadequate, the evaluation of the results of the combination phase I/II trial is incorrect, and the target populations of the phase I/II and III trials were different. A trial of oblimersen sodium combined with standard combination chemotherapy consisting of etoposide+carboplatin was conducted in patients with extensive small-cell lung cancer.[20] The appropriate dose of oblimersen was determined to be 7 mg/kg with etoposide 80 mg/m^2 and carboplatin area under the curve (AUC)–5. The response rate was 86% (12/14), and the median survival time was 12.5 months. No Bcl2 suppression was observed in peripheral blood mononuclear cells. Although the data are not interesting, and the results of proof-of-principle study were negative, Cancer and Leukemia Group B (CALGB) initiated a phase III randomized controlled trial to evaluate the efficacy of oblimersen sodium against small-cell lung cancer.

The clinical study design and sample size are decided based on expected differences in antitumor effect between regimens, the feasibility of the study, and the baseline clinical treatment effect of the control regimen. Possible reasons for the well-known negative results of the Iressa NSCLC Trial Assessing Combination Treatment (INTACT) trial are low response rate to gefitinib, absence of survival benefit of gefitinib, crossover use of gefitinib in the control group, and small sample size.[21, 22] Table 7 shows the sample sizes required for phase III trials. Postulating 1-year survival prolongation in responders, no overall survival, and 1-year median survival prolongation in non-responders, and no crossover of treatment regimens, it would be necessary to accrue 7095×2 patients if the response rate to the molecular target-based drug were 10%. An astronomical number of patients would be needed to obtain positive data (Table 7).

5. Role of surrogate end points

Surrogate endpoints are measurements or signs that are used as substitutes for clinically meaningful end points that directly measure how a patient survives and functions.[23, 24] Changes in a surrogate end point in response to therapy should reflect changes in clinical endpoint. Fig. 2 shows the ideal process for a surrogate end point. Measurement of surrogate effects has been considered essential in the clinical evaluation of molecular-target-based drugs, because it seems very important to dem-

Table 6. Reasons for poor preclinical prectictability of combined effects

1) Molecular target of each drug undetermined
2) *In vitro* problems
A. Concentration, incubation time, timing, protein binding
B. Target tumor
C. Effect on normal cells
D. End point of combined effect (evaluation method)
3) *In vivo* problems
A. Dose, timing
B. Target tumor
C. Species specificity (metabolism, protein binding)
D. Endpoint of combined effect

Table 7. Sample size for phase III studies

RR (%)	MST (months)	2-Year survival (%)	#pts.
0	12.0	12.2	—
10	12.7	16.0	7095×2
20	13.6	19.8	1670×2
30	14.3	23.5	709×2
50	16.4	31.1	232×2

α=0.05, β=0.20.

- 1-Year survival prolongation in responders.
- No survival prolongation in non-responders.
- MST: 1 year in non-responders.
- No crossover.

Ishizuka: personal communication. RR, response rate; MST, median survival time.

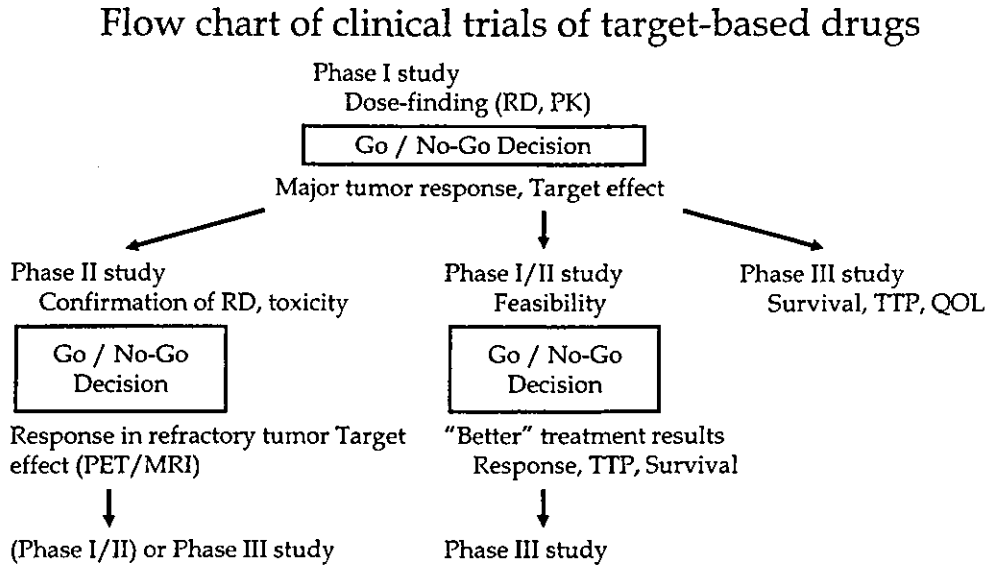

Fig. 1. Flow chart of clinical trials of target-based drugs. Target effects are measured for correlation with effect, dose-finding, and monitoring. RD, recommended dose; PK, pharmacokinetics; TTP, time to progression; QOL, quality of life; PET, positron emission tomography; MRI, magnetic resonance imaging.

Table 1. Classification of target-based therapies according to molecular targets

1) Inhibitors of growth factors/receptors & signal transduction
Anti-EGFR Ab, Anti-Her-2/Neu Ab, EGFR-TKI, c-Kit receptor TKI, Bcr-abl-TKI, Farnesyltransferase inhibitors, PKC inhibitors
2) Inhibitors of the cell cycle
Cyclin-dependent kinase (CDK) inhibitor
3) Inhibitors of metastasis & angiogenesis
Anti-VEGF Ab, VEGF inhibitor, MMP inhibitor, Thalidomide, Angiostatin, Endostatin

Table 2. Requirements of paradigm shifts for new anticancer drug development

1) Concept and screening		
	Paradigm shift	
Seek and destroy	→	Target and control
Random screening against tumors	→	Target-based screening against tumor-specific molecules
2) PK and surrogate end point		
	Paradigm shift	
?	→	Target effect
	No paradigm shift	
PK	→	PK
Tumor shrinkage	→	Tumor shrinkage
Toxicity	→	Toxicity
3) Primary end point		
	No paradigm shift	
Survival	→	Survival

Table 3. Results of phase III trials of specific-target-based drugs

Agent	Tumor	Combination	Results
ZD1839	NSCLC	Y	Negative
		N	Too early
OSI774	NSCLC	Y	Negative
		N	Positive
STI571	CML	N	Positive
	GIST	N	Positive
Trastsuzumab	Breast	Y	Positive
	NSCLC	Y	Negative
Rituximab	NHL	Y	Positive
Affinitac	NSCLC	Y	Negative

NSCLC, non-small cell lung cancer; CML, chronic myelocytic leukemia; GIST, gastrointestinal stromal tumor; NHL, non-Hodgkin's lymphoma; Y, yes (+); N, no (–).

explain the negative results for matrix metalloprotease inhibitors[15] and small molecules against vascular endothelial growth factor (VEGF) tyrosine kinase.

Another hypothesis is that the target tumor contains no, or only a low level of the molecular target. The results of the randomized trial of trastsuzumab for the treatment of non-small cell lung cancer (NSCLC) were completely negative,[16] but expression of Her-2 is very low in NSCLC. This explanation is also valid for the results of the trial of imatinib against small cell lung cancer, which expresess only low levels of c-kit.[17] Enrichment of the target population with the molecular target is essential to obtain positive results. These are likely to be the reasons why the molecular-target-based drugs did not exert adequate antitumor activity.

The majority of molecular-target-based drugs tested in the clinical trials have been evaluated in combination with cytotoxic drugs and other modalities. There are many problems with predictability in preclinical models, especially in regard to

Table 4. Phase II* and phase III trials of non-specific or tumor-environment-specific target-based drugs**

Agent	Tumor	Combination	Results
Marimastat	SCLC	N	Negative**
	Pancreas	N	Negative**
	Stomach	N	Negative**
	NSCLC	Y	Negative**
Prinomastat	NSCLC	Y	Negative**
Tanomastat	SCLC	Y	Negative**
SU5416	Colon	Y	Negative**
	Breast	Y	Negative**
Avastin	Colon	Y	Positive**
	Renal	N	Positive*

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; Y, yes (+); N, no (–).

Table 5. Why have so many phase III trials been negative?

1. Poor predictability of preclinical models, especially of combined effects
2. Target tumor contains no, or a low level of the molecular target.
Enrichment of the target population is inadequate.
3. The "molecular target" is not essential for the growth, invasion, or metastasis of the tumor.
4. Molecular-target-based drugs have inadequate antitumor activity.
5. Clinical decision-making for moving from phase I/II to phase III studies is inappropriate.
6. Clinical study design is inappropriate.
Small sample size
Comparison of 3 drugs vs. 2 drugs

combined effects.[18] The purpose of combination therapy is to increase the spectrum and intensity of the anticancer effect. The criteria for the selection of drugs for use in combinations are: 1) each drug must be independently active against the target tumor, 2) the toxicities of the drugs must not overlap, 3) each drug can be used at its most appropriate dose and schedule, 4) the mode of action of each drug is different, and 5) no cumulative toxicity is observed and the regimen can be administered repeatedly. If an active drug is combined with another active drug, a synergistic or additive effect is expected. The majority of molecular-target-based drugs are used as sensitizing drugs, and a synergistic effect is expected based on biochemical or molecular biological interaction. The purposes of preclinical evaluation of combinations are prediction of synergism of effect and toxicity, demonstration of biochemical modulation, provision of a rationale for clinical combination to the physician/IRB/patient. The conditions required for preclinical studies of combination chemotherapy are demonstration of a synergistic/additive antitumor effect *in vitro*, no increase in *in vitro* toxicity against normal cells, molecular proof-of-principle study for synergism, demonstration of a synergistic/additive antitumor effect *in vivo*, and no increase in *in vivo* toxicity. There are many problems in preclinical prediction of combined effects of anticancer drugs, and the results of preclinical prediction of combined effects have been very poor. Most preclinical data for combination chemotherapy have been obtained after the clinical evaluation. In other words, the purpose of preclinical evaluation is confirmation of a combined effect observed clinically. The reasons for the poor preclinical predictability of combined effects are shown in Table 6.

Decision-making to proceed from phase I/II to phase III studies is quite difficult. For example, a combination of docetaxel and Ly900003 has been tried in NSCLC patients after

Review Article

What are the reasons for negative phase III trials of molecular-target-based drugs?

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The results of molecular-biological studies of cancer are changing the way we diagnose and treat cancer. Target-based drug discovery selects agents for development based on their mechanisms of action. The interaction between target-based drugs and their targets can be described by classical drug-receptor theory. Clinical trials have demonstrated that some effective target-based drugs induce apoptosis, even though they are considered to be cytostatic. Numerous phase III trials of target-based drugs have been conducted. Although some have yielded strongly positive results, the majority of the results have been negative. This article seeks to clarify the value of molecular-target-based therapy and to discuss the reasons for negative results in phase III trials. The importance of proof-of-principle studies is stressed throughout preclinical and clinical trials of molecular-target-based drugs. (Cancer Sci 2004; 95: 772–776)

In an attempt to modulate specific molecular targets in tumor cells and the tumor environment, the emphasis in anticancer drug discovery has shifted from an empirical approach characterized by random screening of a variety of natural and synthetic compounds by means of cell-based cytotoxicity assays to more rational and mechanistic molecular screening.[1–5]

The concept of molecular-target-based therapy is not new, because modern therapy of breast cancer and prostate cancer is in reality target-based therapy. What is new is that we now recognize that tumor cells contain many targets, and drugs specifically directed at such targets have been introduced clinically.[6–9]

1. Classification and characteristics of molecular-target-based drugs

The molecular-target-based drugs that are currently available can be classified according to mechanism of action into inhibitors of growth factors/receptors and signal transduction, inhibitors of the cell cycle, and inhibitors of metastasis and angiogenesis, and they can further be classified according to site of action into two groups: tumor-specific and tumor-environment-specific. Based on the formulation of the drug products, they can be classified into small molecules and macromolecules (Table 1).

Compared with the process of discovery of empirical drugs, that of molecular-target-based drugs is target-based, and their mechanism of action is the basis for selection. Many researchers consider the pharmacological effect of target-based drugs to be cytostatic/reversible, as opposed to the cytotoxic/irreversible effect of cytotoxic drugs; however, all recently approved antitumor molecular-target-based drugs cause tumor shrinkage except for avastin. Since the effects of molecular-target-based drugs are more selective, the drugs are expected to be less toxic. The clinical data suggest that molecular-target-based drugs have different spectra of adverse events. They are expected to be more effective when given continuously at tolerable doses.[6–8]

2. End points and strategies of clinical trials

It is generally said that the paradigms for the development of molecular-target-based drugs should be shifted from empirical to more scientific. The concept of drugs has shifted from "seek and destroy" to "target and control," and screening has shifted from "random screening against tumors" to "target-based screening against tumor-specific molecules". It is extremely important to evaluate the effect of drugs on their targets pharmacodynamically, although no proof-of-principle studies are required for cytotoxic drugs. The paradigms for other pharmacodynamic effects, such as tumor shrinkage and toxicities, as well as pharmacokinetics have not changed. The primary end point of clinical trials, "survival," is the same as for evaluation of cytotoxic drugs. The major end points for ordinary approval of oncology drugs are survival and response rate (Table 2).

Only one drug, trastsuzumab, has been approved based on data showing an increase in time-to-progression. However, trastsuzumab itself causes tumor shrinkage and a survival benefit when combined with other anticancer drugs. The initial clinical study for cytotoxic drugs is a phase I study. Phase II and I/II studies follow, and the final conclusive study is a phase III study. Phase II studies are sometimes skipped for molecular-target-based drugs, and the phase III study sometimes follows the phase I study (Fig. 1).

3. Clinical trials of molecular-target-based drugs

Table 3 shows the results of clinical trials of specific-target-based drugs.

Randomized trials have shown positive results for imatinib, trastsuzumab, and rituximab.[10, 11–13] Imatinib was used as a single agent, whereas trastsuzumab and rituximab were evaluated as combination therapy. Table 4 shows the results of clinical trials of non-specific or tumor-environment-specific target-based drugs, which include antiangiogenic and/or antimetastatic drugs. The results of all of the phase III clinical trials were negative, except for the trial of avastin for colon cancer.[14] Even the results of the phase III trial of avastin for breast cancer were completely negative. Generally speaking, antibodies and some small molecules have shown promise. Signal transduction modulators that act upstream of a growth signal have been found to show a survival benefit. All of the compounds approved for commercial sale caused tumor shrinkage when given alone.

4. Reasons for negative phase III trials

Several hypotheses have been proposed to explain the negative results of phase III trials (Table 5).

One is that the molecular target was not essential for growth, invasion, or metastasis of the tumor, and this hypothesis may

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5. Sethi T, Rintoul RC, Moore SM, et al. Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance *in vivo*. *Nature Med* 1999;5:662–8.
6. Boudreau N, Sympson CJ, Werb Z, et al. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* 1995;267:891–3.
7. Brown JM, Wouters BG. Apoptosis, p53 and tumor cell sensitivity to anticancer agents. *Cancer Res* 1999;59:1391–9.
8. Tada M, Matsumoto R, Iggo RD, et al. Selective sensitivity to radiation of cerebral glioblastomas harboring p53 mutations. *Cancer Res* 1998; 58:1793–7.
9. Zamble DB, Jacks T, Lippard SJ. p53-dependent and -independent responses to cisplatin in mouse testicular teratocarcinoma cells. *Proc Natl Acad Sci U S A* 1998;95:6163–8.
10. Fan S, Chang JK, Smith ML, et al. Cells lacking CIP1/WAF1 genes exhibit preferential sensitivity to cisplatin and nitrogen mustard. *Oncogene* 1997;14:2127–36.
11. World Health Organization. *WHO hand book for reporting results of cancer treatment. WHO Offset Publication 1979, No. 48.* Geneva, Switzerland: World Health Organization, 1979.
12. Lai SL, Goldstein LJ, Gottesman MM, et al. MDR1 gene expression in lung cancer. *J Natl Cancer Inst* 1989;81:1144–50.
13. Tamura M, Gu J, Tran H, et al. PTEN gene and integrin signaling in cancer. *J Natl Cancer Inst* 1999;91:1820–8.
14. Miyamoto S, Akiyama SK, Yamada KM. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science* 1995;267:883–5.
15. Frisch SM, Vuori K, Ruoslahti E, et al. Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* 1996;134:793–9.
16. Suzuki J, Kaziro Y, Koide H. Synergistic action of R-Ras and IGF-1 on Bcl-xL expression and caspase-3 inhibition in BaF3 cells: R-Ras and IGF-1 control distinct anti-apoptotic kinase pathways. *FEBS Lett* 1998; 437:112–6.
17. Khwaja A, Rodriguez-Viciana P, Wennstrom S, et al. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J* 1997;16: 2783–93.
18. Miyamot S, Teramoto H, Coso OA, et al. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J Cell Biol* 1995; 131:791–805.
19. Guan JL, Shalloway D. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature* 1992;358:690–2.
20. Kornberg L, Earp HS, Parsons JT, et al. Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J Biol Chem* 1992;267:23439–42.
21. Ilic D, Almeida EA, Schlaepfer DD, et al. Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J Cell Biol* 1998;143:547–60.
22. Anthoney DA, McIlwrath AJ, Gallagher WM, et al. Microsatellite instability, apoptosis, and loss of p53 function in drug-resistant tumor cells. *Cancer Res* 1996;56:1374–81.
23. Perego P, Giarola M, Righetti SC, et al. Association between cisplatin resistance and mutation of *p53* gene and reduced bax expression in ovarian carcinoma cell systems. *Cancer Res* 1996;56:556–62.
24. Gallagher WM, Cairney M, Schott B, et al. Identification of p53 genetic suppressor elements which confer resistance to cisplatin. *Oncogene* 1997;14:185–93.
25. Gannon JV, Greaves R, Iggo R, et al. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J* 1990;9:1595–602.
26. Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. *Nature* 1991;351:453–6.

TABLE 2. Multivariate Regression Analysis of Variables in Predicting Overall Survival

Variable	Assigned Score	Hazards Ratio	95% Confidence Interval	*P* Value
Gender		0.622	0.251–1.541	0.305
Male	0			
Female	1			
Age (y)		0.869	0.527–1.431	0.58
≦65	0			
>65	1			
PS (ECOG)		0.710	0.397–1.268	0.247
0 or 1	0			
2	1			
Clinical stage		0.590	0.346–1.006	0.0524
LD	0			
ED	1			
Integrin β1/p53*		0.394	0.233–0.666	0.0005
Negative	0			
Positive	1			

*Positive was defined as patients with tumor specimens of high expression of either integrin β1 or p53, and negative as others. ECOG, Eastern Cooperative Oncology Group; PS, performance status.

inary evidence that adhesion to ECM proteins is essential in SCLC cell resistance to chemotherapy.[5] Cancer cells bound to the ECM may escape chemotherapy-induced cell death and then, with subsequent genetic damage, drug-resistant clones are selected. This is a model to explain not only SCLC behavior in vivo but also why a partial response and local recurrence of SCLC are often seen after chemotherapy. One report has indicated that resistance to chemotherapy induced by integrin β1-mediated adhesion to ECM is due to an increase in the level of PTK activity.[5] However, it is not known how integrin-stimulated PTK activation suppresses the early phase of apoptosis in SCLC cells. R-Ras and insulinlike growth factor-1, which activate phosphatidylinositol-3 kinase (PI3 K), cooperatively inhibit caspase-3 activation, preventing apoptosis of BaF3 cells.[16] Activation of PI3 K by integrins protects epithelial cells from detachment-induced apoptosis.[17] Thus, integrin-stimulated PI3 K activation may impinge on the nuclear response to DNA-damaging agents.

Activation of intracellular signals includes tyrosine phosphorylation of focal adhesion kinase (FAK), which binds to the integrin β1 cytoplasmic domain and is one of the molecules that coclusters with β1 integrins aggregated by noninhibitory antiintegrin antibodies.[18–20] FAK is also reported to suppress a p53-dependent pathway activated by protein kinase C and cytosolic phospholipase A2, and it inhibits apoptosis under serum-starved conditions.[21] From these data, integrin β1 and p53 are considered to be part of the same signal pathway that induces cells in apoptosis. In support of a role for p53 in the cytotoxic mechanism of cisplatin, several studies of ovarian carcinoma cell lines have demonstrated that disruption of p53 function conferred drug resistance.[22–24] Our data from this study show that the high expression of p53 in SCLC was associated with a poor prognosis. We did not confirm whether the high expression of p53 was wild or mutant type, although most are considered to be mutant type, since p53 protein accumulation in tumor cells correlates well with mutations of the p53 gene due to a prolonged half-life of mutated p53 protein[25,26] and high expression of mutated p53 is considered to reflect resistance to chemotherapy.

SCLC is not a candidate for surgical resection, and only a small specimen obtained by transbronchial biopsy was available to investigate the genetic characteristics. It is questionable whether the small tissue sample available truly reflects the genetic characteristics of the total tumor in heterogeneous tumor tissues, although because SCLC is relatively homogeneous we feel justified in using small specimens obtained by transbronchial biopsy to analyze the genetic characteristics of the tumor. High p53 expression in SCLC cells is associated with a poor prognosis, although in this study p53 expression was determined in only 50% of the biopsy specimens because the nucleus of SCLC cells was easily crushed during the biopsy procedure, whereas the ECM was less badly damaged. Therefore, if we investigate the genetic characteristics in small transbronchial biopsy specimen of tumor obtained before chemotherapy, not only p53 but also integrin β1 should be examined for analysis of SCLC prognosis, in spite of the fact that integrin β1 expression in tumor cells appeared to be less closely related to SCLC prognosis than p53 expression.

Both p53 and integrin β1 expression are more closely related to SCLC prognosis than is the clinical stage of the disease. Because each gene is related to cell apoptosis after treatment with anticancer drugs, identification of the apoptotic pathway mediating integrin β1- and p53-dependent survival signals may provide new therapeutic strategies to improve the response of SCLC to chemotherapy.

REFERENCES

1. Noda K, Nishiwaki Y, Kawahara M, et al. Irinothecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med* 2002;346:85–91.
2. Smith PJ, Soues S, Gottlieb T, et al. Etoposide-induced cell cycle delay and arrest-dependent modulation of DNA topoisomerase II in small-cell lung cancer cells. *Br J Cancer* 1994;70:914–21.
3. Aas T, Borresen AL, Geisler S, et al. Specific p53 mutations are associated with *de novo* resistance to doxorubicin in breast cancer patients. *Nature Med* 1996;2:811–4.
4. Tallet T, Chilvers ER, Hannah S, et al. Inhibition of neuropeptide-stimulated tyrosine phosphorylation and tyrosine kinase activity stimulates apoptosis in small cell lung cancer cells. *Cancer Res* 1996;56:4255–63.

showed tumor response (77% and 89%, respectively). Only 46 patients could be evaluated for p53 immunostaining. The response rate to chemotherapy in patients with high expression of p53 was similar to that in patients with low expression of p53 (82% vs. 84%, respectively).

When the survival of 80 patients evaluable for integrin β1 or p53 was compared after stratification according to clinical stage, the overall survival of patients with high expression of integrin β1 (n = 22) was significantly worse than that of individuals whose tumors had low expression of integrin β1 (n = 54; log-rank test, $p = 0.046$; Wilcoxon test, $p = 0.043$). The overall survival of patients with high expression of p53 (n = 27) was also significantly worse than that of patients with low expression of p53 (n = 19; log-rank test, $p = 0.0067$; Wilcoxon test, $p = 0.033$). When other prognostic factors were considered, no significant difference in survival was observed according to gender, age, or PS except for clinical stage (Table 1).

Survival curves were constructed for group B of patients with high expression of either integrin β1 or p53 (n = 42) and group A of other patients without high expression of integrin β1 and p53 (n = 38; Fig. 1). The overall survival of group B was significantly worse than that of group A (log-rank test, $p = 0.0003$; Wilcoxon test, $p = 0.0026$). When patients were stratified according to clinical stage (LD and ED), the survival of group B was also significantly worse (log-rank test, $p = 0.0007$; Wilcoxon test, $p = 0.0034$). The association between survival and prognostic factors, including gender, age, PS, clinical stage, and integrin β1/p53 expression was examined by the Cox proportional hazards model (Table 2). Only integrin β1/p53 expression was found to be a significant independent factor (hazard ratio = 0.394, $p = 0.0005$).

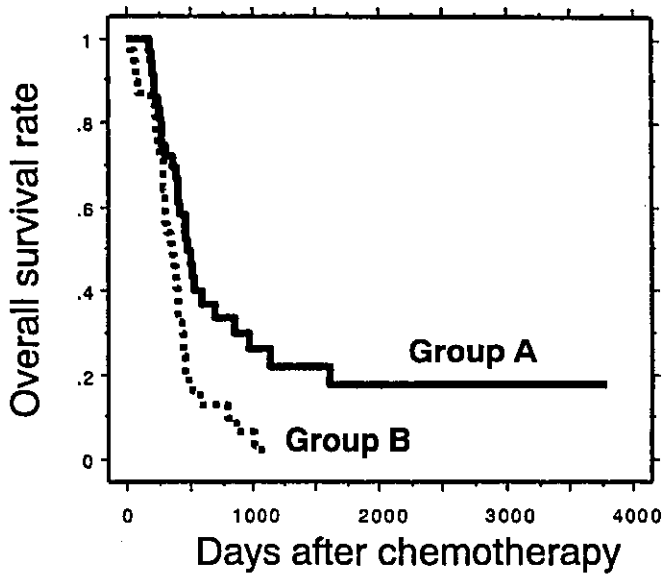

FIGURE 1. Kaplan-Meier survival curves according to integrin β1 and p53 immunostaining. Survival after chemotherapy of patients with high expression of either integrin β1 or p53 (n = 42; group B) was worse than that of other patients without high expression of integrin β1 and p53 (n = 38; group A; log-rank test, $p = 0.0003$; Wilcoxon test, $p = 0.0026$).

TABLE 1. Characteristics of Patients and Overall Survival

	No. of Patients	*P* Value	
		Log-Rank	Wilcoxon
Gender			
Male	74	0.36	0.27
Female	6		
Age			
≦65	42	0.7	0.9
>65	38		
PS (ECOG)			
0	6	0.36	0.27
1	52		
2	22		
Clinical stage			
LD	30	0.012	0.028
ED	50		
Integrin β1			
High	22	0.046	0.043
Low	54		
p53			
High	27	0.0067	0.033
Low	19		

ECOG, Eastern Cooperative Oncology Group; ED, extensive disease; LD, limited disease; PS, performance status.

Analysis was performed by stratification of clinical stage between LD and ED except for analysis of clinical stage.

DISCUSSION

Overexpression of the multidrug resistance gene *MDR1* is not common in SCLC, indicating that, unlike in other tumors, this is not an important mechanism for drug resistance in these tumors.[12] During the past few years, it has become clear that integrins are involved not only in cell adhesion but also in signal transduction in both normal cells and tumor cells. Integrins can directly activate many intracellular signaling events after stimulation by ECM proteins or by antibodies that bind to specific sites of integrins.[13] Both receptor clustering and ligand occupancy are critical for the activation of intracellular integrin-mediated responses.[14]

Many mammalian cell types are dependent on adhesion to the ECM for their continued survival. A variety of normal cell types undergo apoptosis when they lose attachment to an appropriate ECM.[15] In vitro and in vivo data provide prelim-

to confer sensitivity to drugs whose toxicity is modulated by nucleotide excision repair, such as nitrogen mustard and cisplatin.[9,10]

From these data, chemosensitivity-related genes such as integrin β1 and p53 are suggested to relate to cancer prognosis. Most SCLC respond to chemotherapy, to determine the relationship between integrin β1 and p53 expression in SCLC and clinical resistance to treatment, the expression of these genes in tumors and patients' survival were investigated.

PATIENTS AND METHODS

Patients

Between January 1989 and June 1999, patients with histologically proven SCLC, who had received a full dose of chemotherapy, were entered in this study. Patients who had not received a full dose of chemotherapy because of poor performance status (PS) on the Eastern Cooperative Oncology Group (ECOG) scale were excluded. The tumor responses were evaluated according to the World Health Organization criteria.[11]

Immunohistochemistry

Transbronchial biopsy specimens of tumors obtained before chemotherapy were subjected to immunostaining for integrin β1 and p53. Formalin-fixed, paraffin-embedded, 5-μm-thick tumor sections were mounted on charged glass slides, deparaffinized, and rehydrated in a graded alcohol series. Immunohistochemical staining was performed using an automated processor. The slides were immersed in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity and the sections were then immersed in 10 mmol/l citrate buffer (pH 6.0) for 1 hour at 94°C, and nonspecific staining was blocked with immunoglobulins from normal rabbit serum for 10 minutes at room temperature. Excess serum was removed and the sections were incubated for 1 hour at 4°C with a mouse antiintegrin β1 monoclonal antibody (Oncogene Research Products, U.S.A.) or mouse anti-p53 monoclonal antibody (Novocastra, UK) diluted 1:100 with phosphate-buffered saline (PBS), followed by incubation for 10 min at room temperature with the secondary antibody, biotinylated antimouse immunoglobulin (Nichirei Corp., Japan), for 10 min at room temperature. The slides were again washed in PBS and incubated with streptavidin peroxidase complex (Nichirei Corp., Japan) for 10 min at room temperature. The slides were then incubated with 3,3-diaminobenzidine as the substrate for 5 min to visualize positively immunostained cells. Finally, the slides were counterstained with hematoxylin and coverslips were applied. The identical reaction times used permitted consistent reproducibility, thus allowing accurate comparison of all samples.

Scoring of integrin β1 and p53 immunostaining

Two pathologists examined the staining patterns of integrin β1 and p53 independently, and recorded the percentage of positive cells in each specimen. At least 20 high-power fields were chosen randomly and 2,000 cells were counted. The ratio of integrin β1- and p53-positive cells was calculated by dividing the number of positive cells by the total number of cells, and was expressed as a percentage. The integrin β1 immunostaining levels were classified as high (>25% of the cells were stained) or low (≤25% of the cells were stained), and p53 immunostaining levels were classified as high (>50% of the cells were stained) or low (≤50% of the cells were stained).

Statistical Analysis

Kaplan-Meier survival curves were constructed and analyzed for statistical significance by means of the log-rank and generalized Wilcoxon tests. The influence of each variable on survival was examined by the Cox proportional hazards model in multivariate regression analyses. Differences at $p < 0.05$ were considered to be statistically significant.

RESULTS

From February 1989 to July 1999, 104 patients received an initial course of chemotherapy for SCLC. There were 91 males and 13 females with a median age of 65 years (range 40–85 years). The ECOG PS was 0 in 7 patients, 1 in 69, and 2 in 28. The clinical stages of the tumors were limited disease (LD) in 43 patients and ED in 61.

Transbronchial biopsy specimens were subjected to integrin β1 and p53 immunostaining. Twenty-eight and 58 patients could not be evaluated for integrin β1 and p53, respectively, because the tissue samples had been crushed during the biopsy procedure. Fifty-four and 22 patients had tumors with less than or equal to 25% and more than 25% integrin β1-positive cells, respectively, whereas 19 and 27 patients had tumors with less than or equal to 50% and more than 50% p53-positive cells, respectively.

Each patient received a full dose of combination chemotherapy after confirmation of SCLC. Fourteen of 45 patients treated with PE and 1 of 12 patients treated with carboplatin plus etoposide received concurrent thoracic radiotherapy. Among 80 patients with biopsy specimens evaluated for integrin β1 or p53, 11 patients achieved a complete response and 56 patients achieved a partial response. In three patients there was disease progression, and the remaining 10 patients showed no change. The overall response rate to chemotherapy was 84%. When the relationship between gene expression and tumor response to chemotherapy was considered, 17 of 22 patients with high expression of integrin β1, and 48 of 54 patients with low expression of integrin β1

High Expression of Integrin β1 and p53 is a Greater Poor Prognostic Factor Than Clinical Stage in Small-Cell Lung Cancer

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Abstract: The purpose of this study was to investigate the possible association between expression of integrin β1 and p53 and survival in patients with small-cell lung cancer (SCLC). One hundred four patients with SCLC who had received an initial course of a full-dose of combination chemotherapy between February 1989 and July 1999 were entered in the study. Transbronchial biopsy specimens of tumors obtained before chemotherapy were subjected to immunostaining for integrin β1 and p53. Twenty-eight and 58 patients could not be evaluated for integrin β1 and p53 immunostaining, respectively, because the tissue samples had been crushed during the biopsy. Fifty-four patients had tumors with less than or equal to 25% integrin β1-positive cells and 22 patients had tumors with more than 25% integrin β1-positive cells, whereas 19 and 27 patients had tumors with less than or equal to 50% and more than 50% p53-positive cells, respectively. By comparison, the overall survival of patients with high expression of integrin β1 and p53 were significantly worse than those of individuals whose tumors had low expression (log-rank test, $p = 0.046$ and $p = 0.0067$, respectively). Moreover, the overall survival of patients with high expression of either integrin β1 or p53 (n = 42) was significantly worse than that of other patients without high expression of integrin β1 and p53 (n = 38; log-rank test, $p = 0.0003$; Wilcoxon test, $p = 0.0026$). The association between survival and prognostic factors, including gender, age, performance status, clinical stage, and integrin β1/p53 expression was examined by the Cox proportional hazards model; only integrin β1/p53 expression was found to be a significant independent factor (hazard ratio = 0.394, $p = 0.0005$). In conclusion, the high expression of integrin β1 and p53 in tumor cells is a greater poor prognostic factor than clinical stage in patients with SCLC.

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Small-cell lung cancer (SCLC) is one of the most chemosensitive solid tumors, and treatment with combination chemotherapy is known to improve survival. Recently, the Japanese Clinical Oncology Group (JCOG) showed that cisplatin plus irinotecan improved the survival of patients with extensive disease (ED)-SCLC when compared with cisplatin plus etoposide (PE) in a phase III study.[1] However, despite the high response rates and prolonged survival, relapse occurred in the majority of these patients. New therapeutic strategies for SCLC are therefore urgently needed, and these will most likely result from a better understanding of the cell biology of SCLC.

Some forms of chemotherapy exert their cytotoxic effects by inducing apoptosis.[2,3] The regulation of apoptosis in tumor cells is poorly understood; however, the level of protein tyrosine kinase (PTK) activity may determine whether SCLC cells survive and proliferate or die as a result of apoptosis.[4] A recent study showed that SCLC was surrounded by an extensive stroma of extracellular matrix (ECM) at both primary and metastatic sites.[5] Adhesion of SCLC cells to the ECM confers resistance to chemotherapeutic agents as a result of integrin β1-stimulated tyrosine kinase activation, which suppresses chemotherapy-induced apoptosis.[5]

Mutations of p53 in tumors are also suspected to induce resistance to cancer chemotherapy.[6] One response to genotoxic stress involves the p53 tumor suppressor gene product.[7,8] This p53 accumulates after DNA damage and controls cellular proliferation predominantly through its activity as a transcription factor. The expression of downstream genes contributes to tumor suppression either by activating cell arrest, possibly to give the cell time to repair the damage and avoid genetic instability. Mutations in p53 have been shown