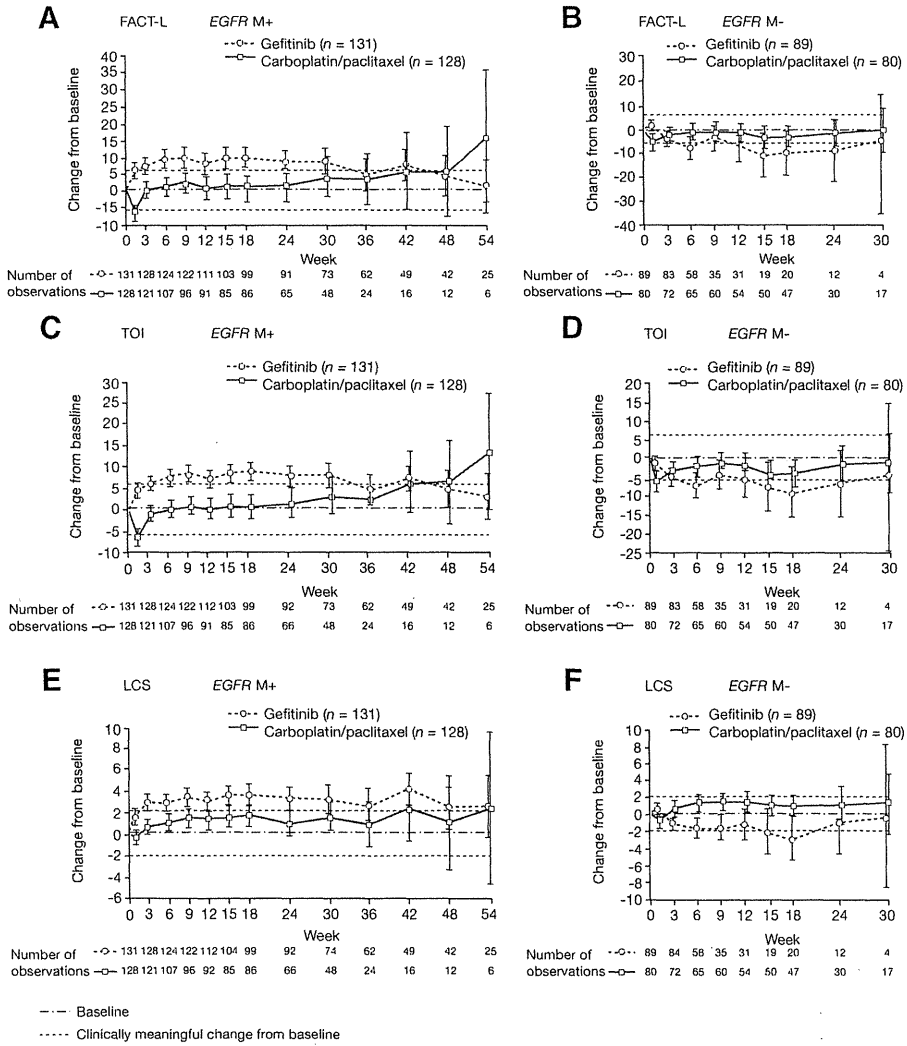

FIGURE 4. Mean change from baseline in HRQoL and symptoms for (*A*) FACT-L in patients with *EGFR* mutation-positive tumors, (*B*) FACT-L in patients with *EGFR* mutation-negative tumors, (*C*) TOI in patients with *EGFR* mutation-positive tumors, (*D*) TOI in patients with *EGFR* mutation-negative tumors, (*E*) LCS in patients with *EGFR* mutation-positive tumors, and (*F*) LCS in patients with *EGFR* mutation-negative tumors (EFQ population). Posthoc calculations. Error bars are 95% CI of the mean. Data after week 54 (*EGFR* M+) and week 30 (*EGFR* M−) not presented as <20 patients remain. HRQoL, health-related quality-of-life; FACT-L, Functional Assessment of Cancer Therapy-Lung; *EGFR*, epidermal growth factor receptor; TOI, Trial Outcome Index; LCS, Lung Cancer Subscale; EFQ, evaluable-for-quality-of-life; M+, mutation-positive; *n*, number of patients at baseline; M−, mutation-negative; CI, confidence interval.

TABLE 2. Time-to-Improvement in HRQoL and Symptoms in Patients with *EGFR* Mutation-Positive Tumors Treated with Gefitinib Who Improved (EFQ Population)

	Total FACT-L (*n* = 131)	TOI (*n* = 131)	Symptoms by LCS (*n* = 131)
Improved, *n* (%)	92 (70.2)	92 (70.2)	99 (75.6)
Median time-to-improvement, days	8	11	8
Range of time-to-improvement, days	6–209	6–85	6–255

HRQoL, health-related quality of life; *EGFR*, epidermal growth factor receptor; EFQ, evaluable-for-quality-of-life; FACT-L, Functional Assessment of Cancer Therapy-Lung; TOI, Trial Outcome index; LCS, Lung Cancer Subscale; *n*, number of patients.

with NSCLC. Patients with *EGFR* mutation-positive tumors reported a time-to-improvement of 8 days (FACT-L and LCS) and 11 days (TOI) with gefitinib, suggesting a rapid improvement in both HRQoL and symptoms. When the rapid improvement in HRQoL and symptoms are considered in conjunction with the significantly prolonged PFS and higher ORR seen in this subgroup,[7] gefitinib can be considered an important first-line treatment option in this molecularly defined population. Conversely, the lesser amount of improvement in HRQoL and symptoms, and significantly shorter PFS and lower ORR reported in patients with *EGFR* mutation-negative tumors treated with gefitinib versus carboplatin/paclitaxel,[7] highlight the importance of a chemotherapy-based regimen in this subpopulation. Additionally, impairment in HRQoL caused by adverse events, and immediacy and evidence of benefit, are known to impact on patient adherence to oral anticancer therapy.[22] The ability of gefitinib to rapidly improve HRQoL and symptoms during treatment, together with the significantly longer survival without CTC grade 3 or 4 toxicity with gefitinib reported in IPASS, could be important factors in maintaining a patient's adherence to therapy.

To summarize the HRQoL and symptom improvement data from IPASS, patients with *EGFR* mutation-positive tumors had greater improvement in HRQoL and symptoms

when treated with gefitinib compared with carboplatin/paclitaxel. Conversely, patients with *EGFR* mutation-negative tumors benefited most from carboplatin/paclitaxel treatment, further highlighting the importance of personalized NSCLC treatment based on the tumor molecular characteristics. IRESSA is a trademark of the AstraZeneca group of companies.

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Serum Heparan Sulfate Concentration is Correlated with the Failure of Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Treatment in Patients with Lung Adenocarcinoma

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Introduction: The epidermal growth factor receptor (*EGFR*) mutation status is a validated biomarker for the stratification of EGFR-tyrosine kinase inhibitor (EGFR-TKIs) treatment in patients with non-small cell lung cancer (NSCLC); however, its use is limited in patients with wild-type EGFR, and new biomarkers are needed. We hypothesized that the serum concentration of heparan sulfate (HS), which activates oncogenic growth factor receptor signaling through EGFR and non-EGFR signaling pathways, may be a novel glycobiological biomarker for EGFR-TKIs treatment in NSCLC.

Methods: The pretreatment serum HS concentrations were determined using enzyme-linked immunosorbent assay in 83 patients with stage IV non-small cell lung adenocarcinoma who received EGFR-TKIs treatment. The relationship between the serum HS concentrations and patient characteristics, tumor response, progression-free survival (PFS), and overall survival (OS) were analyzed.

Results: Patient sex, performance status, smoking history, and *EGFR* mutation status were associated with tumor response. The serum HS concentrations were significantly higher among patients with progressive disease than among those without progressive disease ($p = 0.003$). Furthermore, the serum HS concentrations were strongly associated with a poor PFS and OS in a univariate Cox analysis ($p = 0.0022$ and $p = 0.0003$, respectively). A stratified multivariate Cox model according to the *EGFR* mutation status showed that higher HS concentrations were significantly associated with a shorter PFS and OS ($p = 0.0012$ and $p = 0.0003$).

Conclusion: We concluded that a high-serum HS concentration was strongly related to a poor treatment outcome of EGFR-TKIs and may be a promising noninvasive and repeatable glycobiological biomarker in cancer treatment.

Key Words: Heparan sulfate, Non-small cell lung cancer, EGFR-tyrosine kinase inhibitors.

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Heparan sulfate proteoglycans (HSPGs) are composed of a core protein and one or more heparan sulfate (HS) glycosaminoglycan (GAG) chains. Many studies have demonstrated the importance of these molecules in development and normal physiology including metabolism, transport, information transfer, support, and regulation at the systemic level and the cellular level.[1] Heparin and HS consist of repeating disaccharide units that comprised a hexuronic acid and a D-glucosamine linked to each other and to other disaccharides by 1A4 linkages.[2] The HS component sugars (*N*-acetylgalactosamine and β-D-glucuronic acid/α-L-iduronic acid) and patterns of sulfating modifications create an extraordinarily large potential for structural diversity.[2] The structural diversity of HS is considered to be important because HS can bind and interact with a wide variety of proteins including thrombin, fibroblast growth factors (FGFs) 1 and 2, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), interleukin-8, MIP-1β, P-selectin, laminin, and fibronectin.[3] Such interactions are thought to mediate the enhancement of growth factor/receptor signaling activity, promote tumor growth, regulate differentiation, induce angiogenesis, modulate host immune cell responses to tumor cells, and promote metastasis in cancer cells.[3] Among the biological activities of HS, a large body of structural data

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has demonstrated that HS enhances FGFs/FGF receptor signaling by acting as a template that bridges FGF and the FGF receptor.[4] A structure-based proposal for an HS sequence able to bind FGF and FGFR showed that the interaction between HS and FGFs or FGFRs seemed to be determined by a specific sequence of 5-10 saccharides with sulfating modifications.[5] Other growth factor/receptor interactions may follow a similar binding and activation process. Thus, HS and HSPG expression may enhance the activity of oncogenic growth factor receptor signaling in cancer cells.

Meanwhile, selective epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) block EGFR signal transduction pathways implicated in the proliferation and survival of cancer cells[6–8] and have exhibited clinical activity against non-small cell lung carcinoma (NSCLC[9–11]). Several clinical and molecular biomarkers of EGFR-TKI treatment have been identified such as gender, smoking status, NSCLC histology, East Asian ethnicity, and an active *EGFR* mutation status that confers constitutively active tyrosine kinase activity and a hyperresponsiveness to gefitinib among patients with NSCLC.[12,13] These mutations are observed mostly in either point mutations in exons 18 (G719A/C) and 21 (L858R and L861Q) or in-frame deletions in exon 19 located at position 745.[14] Two recent phase III trials targeting adenocarcinoma in patients with NSCLC with *EGFR* mutations have demonstrated that the gefitinib group had a significantly longer progression-free survival (PFS) than the platinum-doublet therapy group.[15,16] These data indicated that the *EGFR* mutation status is a powerful predictor of the tumor response to EGFR-TKIs.

Recently, we have shown that the serum concentrations of heparin binding growth factors including heparin-binding EGF-like growth factor (HB-EGF), HGF, and VEGF are closely related to the treatment response of EGFR-TKIs in patients with NSCLC.[17] Our results have demonstrated that the serum concentrations of these growth factors were strongly related to the outcome of EGFR-TKIs treatment and suggest that these levels could be used to refine the selection of patients expected to respond to EGFR-TKIs treatment. On the basis of these findings, we speculated that the serum concentration of HS, which activates oncogenic growth factor receptor signaling through EGFR and non-EGFR signaling pathways, may be a novel glycobiological biomarker for EGFR-TKIs treatment in NSCLC. Identifying such a marker would contribute to the further individualization of treatment for NSCLC. In this report, we retrospectively studied the pretreatment serum HS concentrations in patients with stage IV non-small cell lung adenocarcinoma who underwent treatment with EGFR-TKIs.

PATIENTS AND METHODS

Patients

Pretreatment serum samples from histologically confirmed adenocarcinoma and patients with stage IV NSCLC (n = 93) were evaluated in this study. Six patients were excluded because their tumor response was not evaluated. Three additional patients were excluded because a complete clinical data set was not available, and a sufficient serum sample was not available for one patient. Thus, 83 patients were included in the final analysis. All the patients had been treated with EGFR-TKIs (gefitinib, n = 78; erlotinib, n = 5) at one of three centers (Kanazawa University, Japan; Cancer Institute Hospital, Japan; and Tokyo Medical University, Japan). The tumor response was evaluated every 2 to 3 months using computerized tomography according to the Response Evaluation Criteria in Solid Tumors; the response was then classified as a complete response, a partial response (PR), stable disease (SD), or progressive disease (PD). Clinicopathological features including age, sex, Eastern Cooperative Oncology Group performance status (PS), TNM stage, smoking status and *EGFR* mutation status were recorded. To detect active *EGFR* mutations, direct sequencing of a tumor sample was performed in 37 patients; 18 of these samples were found to harbor an *EGFR* mutation, whereas the remaining 19 samples exhibited wild-type EGFR. The mutation status of the other 46 patients was not evaluated. The median follow-up period was 8.2 months. This study was approved by the institutional review boards of all the centers involved in the study.

Preparation of Serum Samples

Blood samples were collected before the initiation of EGFR-TKI treatment. The separated serum was stocked at −80°C until use.

Serum HS Concentrations

Serum HS concentrations were determined using a human heparan sulfate enzyme-linked immunosorbent assay (ELISA) Kit (Code. No. 280564; Seikagaku Biobusiness, Tokyo, Japan). This sandwich-type ELISA kit is composed of two specific monoclonal antibodies recognizing the disaccharide units of HS. It specifically detects HS but does not crossreact with heparin, hyaluronic acid, chondroitin sulfate (CS), or keratin sulfate. In brief, a 50 μl aliquot of serum was treated with 5 μl of actinase E at a concentration of 20 mg/ml at 37°C for 20 hours; the reaction was stopped by heating at 100°C for 5 minutes. The sample was then centrifuged at 10,000 rpm for 10 minutes, and the supernatant (20 μl) was used for the analysis. The sample was diluted with 40 μl of the reaction buffer. Then, 20 μl of the samples were measured in duplicate according to the manufacturer's instructions. The absorbance of the samples at 450 nm and 630 nm was measured using VERSAmax (Japan Molecular Devices, Tokyo, Japan). The average was used for the subsequent analyses.

Statistical Analysis

The primary objective was to investigate novel markers correlated with treatment efficacy independently of EGFR status. If a molecule was very strongly associated with survival after adjustments for the EGFR status and important prognostic factors, then that molecule was deemed as warranting further prospective study to determine whether it was a predictive factor, a prognostic factor, or both. The distributions of the clinical factors were compared between patients with PD and those without PD using the Fisher's exact test. In terms of the analysis for survival time (PFS and overall survival [OS]), clinical factors including age, sex, Eastern Cooperative Oncology Group PS, and smoking status were

examined using the Cox proportional hazards model. After selecting the important clinical variables, we considered these variables fixedly in a Cox proportional hazards model and then determined whether the molecule was associated with survival independent of the important clinical variables at a two-sided significance level of 0.05. Log-transformed values were used for the molecule in the Cox models. The proportional hazards assumption was assessed graphically and using an individual time-dependent component for each covariate. In the multivariate Cox models, the EGFR status (wild type/mutant/unknown) was treated as a stratified variable. We applied the above analyses to all the cases, to the cases in which the EGFR status was evaluated, and to the cases with wild-type EGFR to check the robustness of the conclusions. The survival curves for PFS and OS were estimated using the Kaplan-Meier method. All the statistical analyses were performed using SAS for Windows (version 9.1.3).

RESULTS

Patient Characteristics and Tumor Response

The patient characteristics are listed in Table 1. All 83 patients were of Asian ethnicity and had been treated with EGFR-TKIs (gefitinib, $n = 79$; erlotinib, $n = 4$). Sixty-six (80%) and four (5%) patients had previously received chemotherapy and radiotherapy, respectively. Nineteen patients had wild-type EGFR, 18 had active mutations (exon 19, $n = 13$ and exon 21, $n = 5$), and 46 had an unknown status because their samples had been collected before the identification of this biomarker.[12,13] Regarding the response to EGFR-TKIs treatment, a PR was observed in 34 (41%) patients, SD was observed in 20 (24%) patients, and PD was observed in 29 (35%) patients; none of the patients exhibited a complete response. Significant differences in the tumor response were observed for patients characteristics such as a sex ($p = 0.0002$), PS ($p = 0.04$), smoking history ($p = 0.003$), and EGFR status ($p = 0.00001$). These findings were consistent with those of many previous reports.

TABLE 1. Patient Characteristics, Serum Concentration of Heparan Sulfate, and Response to EGFR-TKIs

		Response		
	Total ($n = 83$) (%)	PR + SD ($n = 54$)	PD ($n = 29$)	p
Age (yr)				0.65
≤65	43 (52)	27	16	
>65	40 (48)	27	13	
Sex				0.0002
Male	46 (55)	22	24	
Female	37 (45)	32	5	
PS				0.04
0–1	60 (72)	43	17	
2–4	23 (28)	11	12	
Smoking				0.003
Yes	51 (61)	27	24	
No	32 (39)	27	5	
CTx				0.27
Yes	66 (80)	41	25	
No	17 (20)	13	4	
RTx				0.08
Yes	4 (5)	1	3	
No	79 (95)	53	26	
EGFR status				0.00001[a]
Wild	19 (23)	6	13	
Mutant	18 (22)	18	0	
Unknown	46 (55)	30	16	—
HS				
Range	3.3–85.8	3.3–51.0	3.8–85.8	
Mean ± SD	14.9 ± 16.5	10.9 ± 9.8	22.2 ± 23.1	0.003

p values are calculated using the t test for serum concentration of heparan sulfate and the Fisher's exact test for other variables.

[a] Comparison between wild type and mutant.

HS, serum concentration of heparan sulfate (μg/ml); PR, partial response; SD, stable disease; PD, progressive disease; PS, performance status; EGFR-TKIs, epidermal growth factor receptor tyrosine kinase inhibitors; CTx, prior chemotherapy; RTx, prior radiotherapy; —, not done.

Serum Concentrations of HS and Tumor Response

The serum concentration of HS ranged from 3.3 to 85.8 μg/ml in all the patients (Table 1) and were over 20 μg/ml in 13 patients, indicating the presence of large individual differences in serum HS concentration. The serum HS concentration is shown for the tumor response groups in Figure 1. Of note, the serum HS concentration was significantly higher among patients with PD (22.2 ± 23.1 μg/ml) than among those without PD (10.9 ± 9.8 μg/ml, $p = 0.003$). The sensitivity and specificity of HS for discriminating PD from PR + SD were determined using the optimal cutoff value (13.5 μg /ml) obtained from a receiver operating characteristic (ROC) curve according to a previous report.[17] The sensitivity and specificity of HS for discriminating PD from PR + SD were 0.448 and 0.851, respectively.

Univariate Analysis of Clinical Molecular Factors for PFS and OS

The median PFS and OS were 4.1 and 10.2 months, respectively. Among the clinical factors that were examined, a male sex, a positive smoking history, and a poor PS were significantly related with a poor PFS and OS (Table 2). A higher serum HS concentration was significantly associated with a shorter PFS (HR, 3.61; $p = 0.0022$) and OS (HR, 5.57; $p = 0.0003$; Table 2). Thus, similar to the results for tumor response, a high serum HS concentration was closely associated with a poor EGFR-TKIs treatment outcome.

Figures 2*A*, *B* shows the Kaplan-Meier estimates for PFS and OS with respect to the concentrations of serum HS. All the patients were divided into two groups according to the cutoff value (13.5 μg /ml) described earlier. The curves indicated that the high serum HS group had a significantly poorer treatment outcome with respect to both PFS (median, 47 versus 161 days; $p = 0.002$) and OS (median, 105 versus 406 days; $p = 0.0002$).

Multivariate Analysis of Clinical Molecular Factors for PFS and OS

As the EGFR status of half the patients in this study was unknown, we used a stratified multivariate Cox analysis

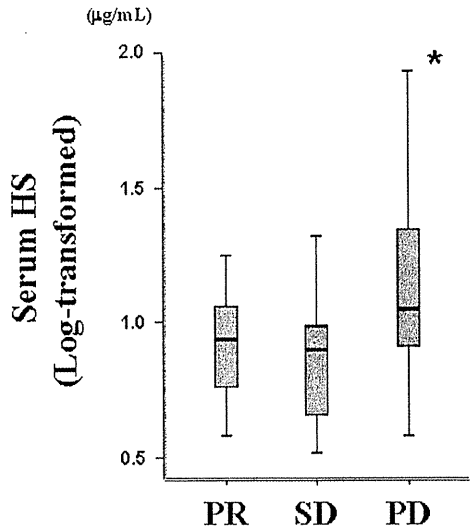

FIGURE 1. Box-whisker plots of serum HS concentration in patients with a partial response (PR, $n = 34$), stable disease (SD, $n = 20$), and progressive disease (PD, $n = 29$). HS, serum concentration of heparan sulfate (μg/ml). *Progressive disease (PD) versus PR + SD, $p < 0.05$.

TABLE 2. Univariate Analysis of Clinical and Molecular Factors for Progression-Free Survival and Overall Survival

	PFS			OS		
	Log Rank,	Cox		Log Rank,	Cox	
	p	HR	*p*	*p*	HR	*p*
Age (yr)						
>65 vs. ≤65	0.678	0.91	0.6791	0.978	0.99	0.9777
Sex						
Male vs. female	0.002	2.08	0.0026	<.0001	3.06	0.0001
Smoke						
Yes vs. no	0.024	1.74	0.0257	0.011	1.99	0.013
PS						
2–4 vs. 0–1	0.075	1.56	0.079	0.002	2.31	0.0021
HS						
Continuous	n.d.	3.61	0.0022	n.d.	5.57	0.0003

Univariate analyses of factors for progression-free survival (PFS) and overall survival (OS) were for all the patients. Log-transformed values were used for all the molecules.

HR, hazard ratio; n.d. not done; HS, serum concentration of heparan sulfate (μg/ml); PS, performance status.

that included the EGFR status as a stratification factor[18] (Table 3). First, sex and PS remained statistically significant at a level of 0.05 in a multivariate model after backward selection. The smoking status was no longer significant ($p = 0.46$ and $p = 0.40$ for PFS and OS, respectively) because it was highly correlated with sex ($p < 0.0001$, Fisher's exact test). Thus, we used sex and PS as fixed factors in the Cox model. The serum HS concentration was significantly correlated with poor treatment outcomes for PFS (HR = 3.98, $p = 0.0012$) and OS (HR = 5.42, $p = 0.0003$) in the final model; a high concentration of HS was correlated with a shorter PFS and OS independently of the EGFR status, sex, and PS (Table 3). In the final model, no interaction was shown between the EGFR status and the serum HS concentration ($p > 0.20$ for both PFS and OS). The results presented in Table 3 were also stable in analyses of subsets of patients with a known EGFR status as well as patients with wild-type EGFR (data not shown). Regarding *EGFR* mutations and the HS concentration, we verified the results in additional experiments using an independent set of 48 serum samples from patients whose tumor EGFR status was known. The results showed that a high serum HS level was reproducibly associated with a poor PFS during EGFR-TKI treatment, although the p value was not significant ($p = 0.087$, Supplementary Figure 1A, http://links.lww.com/JTO/A109). The EGFR status did not seem to be associated with the serum HS concentrations in the 48 additional samples ($p = 0.48$, Supplementary Figure 1B, http://links.lww.com/JTO/A109).

Taken together, these observations suggested that a high serum HS concentration was significantly associated with the failure of EGFR-TKIs treatment and may be a novel glycobiological biomarker.

DISCUSSION

The major GAG in the blood is CS, and other serum GAGs include HS, keratin sulfate, and hyaluronan.[19] Many methods are now available to measure the concentration of serum/plasma GAGs; these methods include cellulose acetate membrane electrophoresis, paper, affinity, and gas chromatography, capillary electrophoretic analysis, and HPLC. Nevertheless, no standardized methods exist for serum/plasma GAG isolation and quantification.[19] Our approach using a sandwich ELISA was easy to perform, quantitative, and reproducible. The C.V. value was below 10% in intraplate, intrakit, and intraday analyses (data of Seikagaku-kogyo). Regarding individual differences, the Alcian blue dot blot method showed that the GAG concentration of plasma from hospitalized patients exhibited a variation of plasma GAGs of 0.1 to 17.6 μg/ml,[20] and our result for the HS concentration was 3.3 to 85.8 μg/ml. Identifying the cause of these individual differences will require further study. Recent studies have shown that the pleural fluid/serum GAG ratio may be useful for the simultaneous differentiation of exudates from transudates and of malignant exudates from benign exudates.[21] In ovarian cancer, the serum CS level may be useful as a discriminator between benign ovarian disorders and malignant ovarian diseases.[22]

Accumulating evidence has demonstrated that HSPG has oncogenic roles in cancer cells. Perlecan is a potent inducer of bFGF-mediated neovascularization in vivo.[23] In addition, several studies have shown that large deposits of perlecan were observed in the tumor stroma and blood vessel walls in liver tumor and invasive breast cancer in clinical specimens.[24,25] The strong reactivity for perlecan in tumoral stromal vessels suggests a role for these HSPGs in tumoral angiogenesis, and the angiogenic effect is considered to interact with various proangiogenic ligands.[26] On the other hand, high expression levels of shed/soluble syndecans-1 are found in the serum of patients with myeloma and lung cancer,

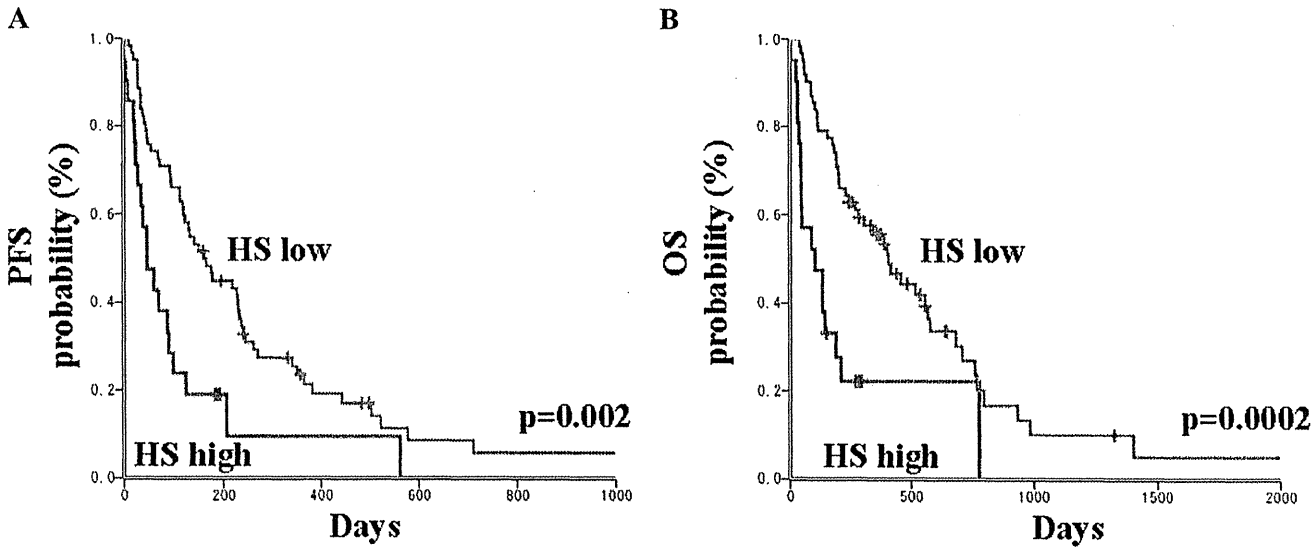

FIGURE 2. Kaplan-Meier curves for progression-free survival (PFS) and OS according to serum concentrations of heparan sulfate (HS). Optimal cutoff point (13.5 μg/ml) of serum HS was determined from a receiver operating characteristic (ROC) curve to discriminate progressive disease (PD) or without PD. Kaplan-Meier curves for PFS (*A*) and OS (*B*) are shown. HS high, patients with a serum HS concentration >13.5 μg/ml. HS low, serum HS concentration <13.5 μg/ml.

TABLE 3. Final Multivariate Model for Progression-Free Survival and Overall Survival

	PFS		OS	
	HR	*p*	HR	*p*
Sex				
(male vs. female)	1.82	0.0231	2.43	0.0031
PS				
(2–4 vs. 0–1)	1.09	0.7610	1.95	0.0235
HS[a]	3.98	0.0012	5.42	0.0003

Sex and PS were fixed in the model. Molecular markers were then selected using the backward selection procedure with a removal probability of 0.05. In all the steps, a Cox model stratified according to the EGFR status (wild type/mutant/unknown) was applied.

[a] Log-transformed values are used for HS.

PFS, progression-free survival; OS, overall survival; HR, hazard ratio; EGFR, epidermal growth factor receptor; HS, serum concentration of heparan sulfate (μg/ml); PS, performance status.

and these high expression levels were predictors of a poor prognosis.[27,28] Compared with the normal form of syndecans-1, the shed form of syndecans-1 gains oncogenic functions leading to hyperinvasiveness and the increased tumor growth of myeloma tumors in vivo.[29] Thus, shed HSPGs remain highly biologically active and can regulate cell growth and metastasis in cancer.[30] In line with this observation, our findings that a high HS expression level was correlated with a poor clinical outcome may be associated with the expression of the shed/soluble form of HSPGs. Regarding the correlation between the HS expression levels in tumor and serum samples, we examined the HS expression levels in 10 independent pairs of serum and surgical samples. Representative results of the immunostaining for tumor HS expression are shown in Supplementary Figure 2 (http://links.lww.com/JTO/A110). An anti-HS antibody was used in this experiment with or without heparitinase I digestion. Heparitinase I digestion completely abolished the staining (left panel). Under such conditions, HS was strongly expressed on the membranes of lung cancer cells (lower right panel). Furthermore, HS expression in the tumor tissues was relatively weak in all five cases in the group with a low serum HS level (lower panel, Supplementary Figure 3, http://links.lww.com/JTO/A111), whereas strong HS expression in the tumor tissues was observed in three of the five cases in the group with a high serum HS level (upper panel, Supplementary Figure 3). These results suggested that the tumor and serum HS expression levels may be positively correlated.

The activation of EGFR signaling occurs as a result of mutations affecting the adenosine triphosphate-binding cleft of EGFR, and EGFR mutants exhibit constitutive tyrosine kinase activity independently of any ligand. We found that the serum HS concentration was significantly higher among patients with PD and was strongly associated with a poor PFS and OS in EGFR-TKIs-treated patients. No difference in the serum HS concentration was observed between the PR and SD groups, but a difference was seen between the PD and non-PD groups (Figure 1). Therefore, the serum HS concentration may be involved in drug resistance but not in sensitivity. Regarding resistance to EGFR-TKIs treatment, the amplification of met proto-oncogene (MET) causes gefitinib resistance by driving the v-erb-b2 avian erythroblastic leukemia oncogene homolog 3 (ERBB3)-dependent activation of phosphoinositide-3-kinase, and previous authors have proposed that MET amplification may promote drug resistance in other ERBB-driven cancers.[31] Yano et al.[32] showed that HGF-mediated MET activation is involved in gefitinib resistance in lung adenocarcinoma with EGFR-activating mutations. A recent study has clearly demonstrated that HGF accelerates the devel-

opment of MET amplification both in vitro and in vivo, mediating the EGFR kinase inhibitor resistance caused by either MET amplification or autocrine HGF production.[33] These studies indicate that the activation of HGF-MET signaling confers resistance to EGFR-TKIs. Our previous study also showed that a high concentration of serum HGF is a predictive biomarker for EGFR-TKIs treatment.[17] Therefore, the activation of HGF-MET signaling in lung cancer cells is considered to be a cause of drug resistance to EGFR-TKIs. In addition, combined with data on the serum HGF, VEGF, HB-EGF, and PDGF-BB levels from a previous study,[17] the correlation coefficient between the serum HS and the HGF, VEGF, HB-EGF, and PDGF-BB levels were 0.45, 0.46, −0.03, and −0.13, respectively. These results indicated that the expression pattern of the serum HS level was weakly similar to those of the HGF and VEGF levels but was not correlated with the HB-EGF or PDGF-BB levels.

In this study, the serum HS concentration was identified as another candidate biomarker for treatment resistance, and this finding may provide novel glycobiological insight into drug resistance to EGFR-TKIs. The results suggest that a high serum HS concentration may be related to the activation of non-EGFR signaling, such as HGF, FGF, and VEGF signaling in cancer cells. We plan to conduct a prospective study to validate the ability of the serum HS concentration to predict the response to EGFR-TKIs treatment.

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Laboratory-Clinic Interface

Critical comments for roles of biomarkers in the diagnosis and treatment of cancer

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ABSTRACT

A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic/pharmacodynamic responses to a therapeutic intervention". Various assays, including immunohistochemistry, gene constitution such as amplification, mutation, and rearrangement, gene and protein expression analysis such as single gene or protein expression, exhaustive analysis and gene or protein signature and single nucleotide polymorphism have been used to identify biomarkers in recent years. No therapeutic effects have yet been predicted based on the results of such exhaustive gene analysis because of low reproducibility although some correlate with the prognosis of patients. Biomarkers such as HER2 for breast cancer or EGFR mutation for lung cancer and KRAS mutation in colon cancer have contributed to identify a patient population that might show a good and bad treatment response, respectively. On the other hand, other biomarkers such as bcr-abl, c-kit gene mutation and CD20 expression, which are positive for CML, GIST and B cell lymphoma, respectively, have crucial biological significance but have not necessarily been used for practical clinical screening since pathological diagnosis coincide with finding of biomarkers. Hence, much work remains to be done in many areas of biomarker research.

Introduction

Most cancer patients treated with chemotherapy will suffer severe toxicity, because response rates to a single therapy with anticancer drug are much lower than that to therapy for other diseases and also effective dose levels of anticancer drugs are often close to or overlap the toxic dose level. Thus, it is important to identify a patient population that is likely to be responsive to treatment with anticancer drugs. To address this challenge, various biomarkers have recently been studied. In addition, molecular-targeted agents have been extensively developed by many pharmaceutical companies and some of these agents are currently available in clinical practice. One group of molecular-targeted agents exert their antitumor activity by modification of a tumor cell-specific target. Development of biomarkers is necessary for predicting the effects of these agents on the relevant targets. The goal of the development of biomarkers will be to design ways to predict efficacy of molecular-targeted agents including response rate, progression-free survival (PFS) and overall survival (OS). If biomarkers allow us to select a patient population that might show a good treatment response, they are believed to be beneficial to both patients and physicians. Furthermore, biomarkers are expected to provide valuable information for developing new drugs, thereby reducing development costs and duration as well as the number of patients enrolled in clinical studies while no reliable biomarkers have yet been identified for tumor-environment-specific molecular targeted agents such as antiangiogenic drugs. Recently, biomarker research has become complicated because of the emergence of molecular targeted agents with multiple targets. In this review, I will attempt to discuss current trends and the clinical significance of biomarker research.

Definition of biomarkers

A biomarker has been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic/pharmacodynamic responses to a therapeutic intervention".[1,2] In routine clinical oncology practice, patients' characteristics and findings such as performance status (PS), disease stage, histological type, X-ray, MRI, CT, scintigram and other laboratory examinations have been assessed as biomarkers. With recent progress in molecular biological research, various advanced technologies, including pharmacogenomics, such as transcriptomics, proteomics, metabolomics and molecular imaging, have been introduced in clinical settings to analyze factors regulating both the effects and the adverse events of treatments. Furthermore, the following attempts have been made: understanding the effects of the cancer and the drug actions on DNA, RNA, proteins and their metabolites, and assessment of the significance of surrogate endpoint biomarkers as alternatives for true clinical endpoints. The main purpose of using anticancer drugs

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is to increase total survival time and the complete remission rate. Under these circumstances, it is important for clinicians to identify a biomarker that correlates directly with these parameters. In other words, clinicians need to determine whether the effect on a biomarker correlates directly with the effect on a true endpoint.

Necessity of biomarkers and rationale for biomarker research

In general, response rates to anticancer drugs are much lower than those to drugs for other diseases. Even with limits on the approved products, the rate of anticancer drug responsiveness is as low as approximately 20% according to RECIST criteria. These criteria are not strict for evaluating the antitumor activity, because, in particular, a partial response is defined as at least a 30% decrease in tumor size sustained for at least 4 weeks with one direction measurement.[3]

Success rates in anticancer drug development remain low or have gradually declined in recent years. Moreover, the price of newly approved anticancer drugs has been exorbitant due to increases in their development costs. If a patient population that is expected to show a good response to an anticancer drug can be selected by a reliable biomarker, these problems will be resolved. For instance, it is known that in trastuzumab-based therapy, HER2-positive patients had a good response rate of 50%; while those without screening for HER-2 had a poor response rate of only 10%.[4]

Positioning of biomarkers (diagnostic, prognostic and effect predictive markers)

Biomarkers basically include tumor markers (including diagnostic function tests) and are a characteristic that is evaluated using pharmacogenomic methods reflecting PK/PD reaction when administering anticancer drugs. Biomarkers can be divided into the following three types: (1) diagnostic markers (such as tumor markers), (2) prognostic markers and (3) predictive markers. Special care should be taken to determine whether a certain biomarker is a prognostic or predictive marker. When a biomarker correlates with anti-tumor effects including response rate, progression free survival, time to progression and overall survival, it will be regarded as a surrogate endpoint of clinical effect. Such a correlation between an identified biomarker and clinical effect supports molecular targeted agents exerting an anti-tumor effect via molecular target modulation. This approach is thought to be translational studies in United States. Application to biomarker research in anticancer drug development based on pharmacogenetics can generally be divided into three steps: target identification in the drug discovery process, elucidation of the action mechanism and identification of the biomarker. In the process of this research, pre-clinical studies focus on elucidating the in vitro/vivo mechanisms of action and pharmacogenomic reasons for the toxicities of drugs. While clinical studies aim to elucidate the in vivo mode of action and to develop and validate biomarkers. Diligent and intensive research activities are required and validation of biomarker analysis methods is the most important aspect on these processes.

Conditions of biomarkers

A new biomarker will become beneficial to patients, when newer and more important information becomes available as compared with the old prognostic and/or predictive markers used in current clinical practice. A new technology, regardless of its cost-saving and user-friendly properties, will be meaningless unless there is true clinical significance.

Practical questions that clinicians commonly face are as follows: (1) can data from molecular biomarkers, genomics or proteomics provide more correct information than those from prognostic and/or predictive markers routinely used in clinical practice? (2) Can the gene panel or molecular signature be an independent prognostic and/or predictive marker rather than a surrogate marker for a factor previously used? and (3) why do results from gene prediction differ among researchers?

It is well known that substantial efforts in biomarker validation are required to use a new biomarker in clinical practice. In such biomarker validation research, a prospective study design using high quality tumor samples from patients, who are enrolled in a controlled clinical study, should be adopted.[5,6] A sufficient sample size is also necessary to assess the specificity, sensitivity and predictive values (positive and negative) before developing a hypothesis and the endpoints introduced for biomarker validation.[7] However, high quality tumor samples are not consistently collected from all patients enrolled, suggesting that data from tumor samples of some cases may not reflect data that would be obtained from the entire patient population. Patients treated with adjuvant chemotherapy after surgery would be the best subjects for biomarker validation, because great amount of tumor samples can be collected from all such patients.

Henceforth, the clinical significance of new biomarkers should be evaluated by comparing the predictability between molecular biomarkers and clinical prognostic factors, therapeutic gain factors and predictive factors, such as age, sex, clinical stage and type of tissue, which are routinely used in clinical settings, in such a patient population.

Classification of biomarkers

In light of the clinical significance, biomarkers could be classified into the following three groups[8] (Table 1). First, biomarkers in Group 1 are known as valid markers which are well known to correlate with clinical response. This biomarker group includes expressions of human epidermal growth factor receptor 2 (HER2) expression for trastuzumab (Herceptin)[9,10] epidermal growth factor receptor (EGFR) expression[11] and K-RAS mutation[12,13] for cetuximab (Erbitux), EGFR mutation for epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI).[14,15] These biomarkers are thought to be essential for deciding indication of anticancer drugs and now used to optimize patient selection when administering Herceptin, Erbitux EGFR-TK I in clinical settings. When using EGFR-TKI, previous data suggest that patients with EGFR mutations have high response rates of 70–80% with extremely high predictability. While approximately 10% of patients without this mutation also show a good response.[16–18] Recent IPASS trial, however showed that response rate of EGFR-wild type patient was only 1% if EGFR mutation was analysed by highly sensitive Scolpion Arms method.[19,20] In addition patient with EGFR mutation showed an amazingly longer progression free survival when treated by

Table 1
Classification of biomarkers.

Known Valid Biomarkers: Test required
– Accepted by scientific community at large to predict clinical outcome
Her2(Herceptin), EGFR mt(EGFR-TKI), EGFR, KRASmt(Erbitux),
BRCA1&2 mt,def. PARP-inhibitor,
EML4-ALK(ALK-inhibitor),Mamma Print, Oncotype Dx (Chemotherapy)
Probable Valid Biomarkers: Test recommended
– Appears to have predictive value but not yet replicated or widely accepted
UGT1A1*28,*6, (irinotecan), Cytidine deaminase*3(gemcitabine)
Exploratory Biomarkers: (Valid, non Valid) Information only
– Supported by initial identification data
Genomic & Proteomic predictors
(Single gene:ERCC1,RRM1,MSH2, TS,
Exhaustive Analysis : Gene/Protein Signatures)

EGFR-TK I compared with standard chemotherapy.[21] Thus, there are following advantages to tests for these biomarkers in clinical practice: (1) high predictability of cases with high response rates and (2) optimizing patient selection when administrating EGFR-TKI in a high risk group. Second, biomarkers in Group 2 are probable valid biomarkers, which can probably predict good clinical response or adverse events. This group includes UGT1A1*28 or *6 for irinotecan.[22,23] These marker tests should be conducted before using these drugs to avoid severe adverse events. The biomarker test for UGT1A1*28, however, may not be reliable in clinical use, because it is based on data that only three of six patients homozygous for this genotype had neutropenia of grade 4.[24,25] Another problem is that it's frequency is very low. Finally, biomarkers in Group 3 are still in the process of evaluation, and some have not been validated as yet. This group includes ERCC1 and MSH-2 for platinum-based drugs[26,27], RRM1 for gemcitabine[28], and thymidylate synthetase (TS) for pemetrexed and fluorinated pyrimidines.[29] Also, majorities of expression profiles of genes and proteins signature have not been validated.[31] Other biomarkers such as CD20 in B-cell lymphoma, bcr-abl in chronic myeloid leukemia (CML)or c-kit in gastro intestinal stromal tumors (GIST) are not always essential for optimizing patient selection when administering rituximab or imatinib because expression of markers and the presence of its gene mutation in tumor cell coincide with results of cytopathological diagnosis. These biomarkers however, will play an important role in identifying the second mutation with resistance and finding a compound for a new molecular target with a mutation in addition to a crucial role to understand biology of each disease.[33]

Prognostic versus predictive factors

Prognostic factors are defined as patient- and tumor-side factors that provide information about the natural histories of diseases, such as survival time after surgery, with no relationship to treatment. Predictive factors represent patient- and tumor-side factors that allow clinicians to assess clinical effects of chemotherapy and molecular targeted agents on response rate and survival time. For example, if survival times of patients without treatment, whose performance status (PS) is 0/1 or 2/3, are 6 and 3 months, respectively, PS will be a prognostic factor. If the survival times of patients, with PS of 0/1 or 2/3, increase from 6 to 8 months and from 3 to 4 months with treatment, respectively, both hazard ratios (HRs) will be 0.75. In this case PS would not be a predictive factor for clinical effect. Recent studies suggested that gene signature and proteomics may become prognostic factors for lung cancer, breast cancer and colon cancer[34,35], although there is no evidence showing either to be a predictive factor for clinical effects.

ERCC1 is known to be a nucleotide excision repair (NER) enzyme associated with the repair of DNA damage caused by platinum-based drugs.[36] In lung cancer field, considerable research on ERCC1 has been conducted in recent years.[37,38] Simon reported that ERCC1 expression is a prognostic factor of survival in patients with resected non-small cell lung cancer (NSCLC) and that those with resected NSCLC with high ERCC1 expression show better survival than patients with low ERCC1 expression.[37] Lord et al. demonstrated response rate and prognosis to be better in IV-stage NSCLC patients with low ERCC1 expression than in those with high expression after cisplatin plus gemcitabine chemotherapy.[38] They also concluded that ERCC1 is a predictive factor for tumor response to platinum-based chemotherapy. This finding was supported by results of the IALT study, which demonstrated a correlation between ERCC1 protein expression and adjuvant chemotherapy response. This analysis indicated significant effect of adjuvant chemotherapy in patients with ERCC1-negative tumors; however, the survival time of patients with ERCC1-positive tumors treated by chemotherapy tended to be shorter.[26] In a comparison between patients without adjuvant chemotherapy, survival was longer in the ERCC1-positive than in ERCC1-negative group.[26] On the basis of these results, that ERCC1 is a tumor cell biomarker has been established in western countries. Caution, however, is necessary when interpreting the results of these subgroup analyses. In such subgroup data, biomarkers can be interpreted as both prognostic and predictive factors. If a specified subgroup shows response to chemotherapy, subgroup interaction is considered to be present in the statistical sense of the term. For example, if the patient subgroup with ERCC1-negative tumors shows response to adjuvant chemotherapy while a subgroup with ERCC1-positive tumors does not show tumor response[39], ERCC1 is interpreted as a predictive factor. This is statistically called "treatment with baseline covariate interaction".

These interactions include quantitative and qualitative interactions. The former interactions originally had the same trend but different intensities. Thus, each treatment group shows a tumor response without notable problems. For interactions between response with some drug and marker A, the subgroup with marker A has a higher response rate while another subgroup without marker A also shows a response to the agent although the response rate is low. On the other hand, the latter interactions may differ in mechanism between subgroups. Careful attention is required when such a mechanism is observed. Examples include interaction concerning pemetrexed for subgroups with squamous cell carcinoma and non-squamous cell carcinoma[40], interaction concerning platinum-based chemotherapy for ERCC1-positive and negative subgroups[38], and interaction in cetuximab therapy for KRAS mutation positive and negative subgroups in colon cancer.[41,42] It must be determined whether or not these interactions are statistically significant by testing differences in two hazard ratios. However, this test power will not be sufficiently robust for the following reasons: (1) there are two sources of variation and (2) sample sizes are small in subgroups. Thus, tumor responses with treatment in each subgroup evaluated are likely to be obtained by chance. Data including ERCC1, KRAS-mutation and histological type should be evaluated keeping this mind. A recent prospective randomized trial (COIN trial) in patients with KRAS type could not demonstrate the survival benefit of cetuximab if combined with FOLFOX regimen. (Press release)

RRM1 is associated with nucleoside metabolism and is a molecular target of gemcitabine. It has been reported that this RRM1 is a positive prognostic factor for early-stage lung cancer. In advanced-stage lung cancer patients with high RRM1 expression, the tumor response to gemcitabine + cisplatin combination is low, thereby showing that RRM1 is a predictive factor for advanced lung cancer.[37,38] A relationship between these factors including the recent results of MSH2 is similar to that with ERCC1.

A report demonstrated that the KRAS mutation is a predictive factor for increased survival with adjuvant chemotherapy. In spite of the relationship between the KRAS mutation and responses to cetuximab in colon cancer[41,42], KRAS mutation was identified not as a predictive factor of cetuximab treatment in lung cancer.[43] Recent topic is the effect of PARP inhibitor in BRCA1&2 mutant/deficient population. It has been demonstrated that PARP inhibitors inhibit repair of damaged DNA. PARP inhibitors has showed activity in those populations of breast and ovarian cancer by itself. PARP inhibitor has also been identified to be active against triple negative breast cancer by the combination-with carboplatin and gemcitabine, both of which are DNA damaging agents.[44–46]

Molecular targeted agent development and biomarkers

To establish molecular targeted therapies, the following are required: (1) validated molecular target; (2) assay that can deter-

mine expression and activity of target and signaling pathway; (3) expression in targeted tumors; (4) potent and specific inhibitor with high pharmacological activity; and (5) proved suppression targeting human tumors. It is more essential that a target with over-expression or a mutation should be present in tumor cells, or that a target is associated with cell proliferation, cell death or metastatic capacity and target suppression inhibits cell proliferation or promotion. Biomarkers play a role in monitoring such targets; however, various challenges remain, including tumor proliferation, the clinical significance of target related to survival, the reliability of assay methods for the target (sensitivity, specificity and precision), tissue specificity in targeted expression, heterogeneity, availability of tissue (sample) and clinical significance. In recent years (2001–2006), 67% of FDA approved anticancer drugs have been molecular targeted agents while only 33% were cytotoxics. Among anticancer drugs approved between 2000 and 2005, 37% have a filed application of pharmacogenetic data showing a rapid increase in the proportion of molecular targeted agents.

To use molecular targeted agents more effectively, clinicians should consider not only pharmacokinetics (PK) and pharmacodynamics (PD) but also pharmacogenomics (PGx) which lies in the background of PK/PD. In PK analysis, whether or not a clearance saturation or protein binding present when C_{max} or trough value reaches to effective blood concentration should be considered. In PD analysis, general observations include side effects or tumor reduction. In addition, elimination of target molecular and expression changes in downstream molecules can be assessed by PGx methods.

PGx markers are also used to determine whether or not a target molecule is expressed and to estimate the intensity of tumor response to anticancer drugs. Thus, it is essential to evaluate proof-of-principles (POP), for setting an optimal dosage and its schedule. For anticancer drugs, in particular, PGx analysis must be performed in cancer cells themselves, even if excellent PK analysis results are obtained. This is because such responses to drugs differ between tumors due to their features.

Application of biomarkers to non-clinical studies can provide the following advantages: (1) identifying targets for drugs; (2) clarifying the action mechanisms of drugs; and (3) predicting possible toxicity in humans. At this time, there are very few reliable methods (biomarkers) to identify responders, and biomarker development is needed in the future. The advantages in clinical studies include: (1) identifying responders and non-responders; (2) designing a clinical study for only responders; and (3) excluding patients with possible serious adverse events. Although application to routine clinical practice has not yet been achieved, identification of a biomarker that can predict serious a pulmonary fibrosis caused by EGFR-TKI, such as gefitinib or erlotinib, may lead to the prevention of side effects of the drug. In addition, the advantages of developing promising biomarkers are: (1) facilitating patient entry into clinical studies and decreasing the development period; (2) decreased sample size and development cost; (3) simplifying GO/NO GO decision; and (4) decreasing new drug approval period. The disadvantage of developing biomarkers include: (1) complicated clinical study plan; (2) segmentalizing the market; and (3) costly study for application of biomarkers.

Design for clinical studies with individualized therapeutics using biomarkers

Order-made or tailor-made therapy, i.e. individualized therapeutics based on pharmacogenomic information, became popular several years ago. However, only a few individualized therapies have been applied in clinical practice and there are no comprehensive data from genes and proteins useful for optimizing patient selection. In this section, several clinical study designs are discussed for evaluating tailor-made therapies that can optimize patient selection by using biomarkers.[47,48]

All comer's design

The all comer's design has been adopted by most of clinical studies using biomarkers. This design should be selected, when a biomarker has not been established or validated. In the study design, the relationship between presence/absence or higher/lower levels of biomarkers and the response rates or survival times are analyzed retrospectively, although positive or negative marker status could not be used to randomize patients even if biomarker levels are determined in all the patients enrolled. It is difficult to conclude that optimizing patient selection on the basis of study results will lead to increased responses to treatment.

Marker + design

The marker + design has been applied to clinical studies of Herceptin for breast cancer[10], standard chemotherapy versus imatinib for CML[31] and rituximab for B cell lymphoma.[30] In a study for B cell lymphoma, this design is not intended to optimize patient selection, because the pathologic disease entity is almost always CD20-positive. Biomarkers used in this study design must be established and validated. In the IPASS trial using gefitinib, clinical characteristics are used as biomarkers to optimize patient selection.[19] The WJOG and the North East Japan Gefitinib Study Group conducted a randomized controlled study with selecting patients with EGFR mutations.[21] When both biomarker positive and negative patients are randomized into treatment and controlled groups, interactions between the biomarker and response to treatment can be evaluated.

Marker strategy design

The marker strategy design is an important approach that can determine whether or not individualized therapeutics based on a biomarker has clinical significance. Generally, patients are allocated into two groups: the A group is treated with standard chemotherapy without biomarker measurement and the B group with biomarker measurement includes B1 (marker-positive) and B2 (marker-negative) subgroups which are treated with new agents and standard chemotherapy, respectively. In this study design, when survival time is significantly longer in the B (B1 + B2) group than in the A group, the significance of individualized therapeutics can be confirmed. Rosell et al. conducted a clinical study for the clinical significance of platinum-based chemotherapy based on the ERCC1 expression level, using this marker strategy design. In this study, the control group received CDDP + DTX. In the selection group, however, patients with low ERCC1 mRNA and patients with high ERCC1 mRNA received CDDP + DTX and DTX + GEM (excluding CDDP), respectively. The results showed patients who were selected based on their ERCC1 mRNA level to show a higher response rate, although survival times were similar in the two groups.[49] In conclusion, the selection of patients based on their ERCC1 expressions did not provide a good prognosis with chemotherapy for advanced-stage lung cancer, although response rate was significantly better than control group.

Conclusion

The clinical significance of biomarkers has been recognized among clinicians. Considerable research on biomarkers has been conducted in recent years; however established findings are as

yet limited. Limitations include validation of methods, the feasibility of obtaining tumor samples and low sensitivity. For examples, samples could not always be collected from all patients, samples for determination were not consistently of adequate volume, and the detection methods were not available at all facilities participating in the study. The most important issue is whether or not a biomarker is validated; if not, various data obtained in clinical studies will be minimally useful in clinical practice. In order to improve the results of cancer treatment, reliable biomarkers fully validated in relation to specific drugs must be established.

Conflict of interest

None declared.

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Epidermal Growth Factor Receptor Mutation Status in Circulating Free DNA in Serum

From IPASS, a Phase III Study of Gefitinib or Carboplatin/Paclitaxel in Non-small Cell Lung Cancer

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Introduction: In IPASS (IRESSA Pan-Asia Study), clinically selected patients with pulmonary adenocarcinoma received first-line gefitinib or carboplatin/paclitaxel. This preplanned, exploratory analysis was conducted to increase understanding of the use of surrogate samples, such as serum, versus tumor biopsy samples for determining *EGFR* mutation status in the Japanese cohort (n = 233).

Methods: *EGFR* mutations were assessed using tumor tissue-derived DNA (n = 91) and circulating free (cf) DNA from pretreatment serum samples (n = 194).

Results: Fewer patients were *EGFR* mutation positive when assessed using pretreatment cfDNA (23.7%) versus tumor tissue-derived DNA (61.5%). cfDNA results identified no false positives but a high rate of false negatives (56.9%). There was a significant interaction between cfDNA *EGFR* mutation status and treatment for progression-free survival (PFS) (p = 0.045). PFS was significantly longer and objective response rate (ORR) higher with gefitinib than carboplatin/paclitaxel in the cfDNA *EGFR* mutation-positive subgroup (PFS: hazard ratio [HR], 0.29; 95% confidence interval [CI], 0.14–0.60; $p < 0.001$; ORR: odds ratio [OR], 1.71; 95% CI, 0.48–6.09; 75.0% versus 63.6%; p = 0.40). There was a slight numerical advantage in PFS and ORR for gefitinib over carboplatin/paclitaxel in the cfDNA *EGFR* mutation-negative subgroup, likely due to the high rate of false negatives within this subgroup.

Conclusions: These results merit further investigation to determine whether alternative sources of tumor DNA, such as cfDNA in serum, could be used for determining *EGFR* mutation status in future; currently, where a sample is available, analysis of tumor material is recommended.

Key Words: EGFR, Mutation, Gefitinib, NSCLC, Serum.

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The epidermal growth factor receptor (EGFR) superfamily has been implicated in the regulation of tumor cell biology and, as such, has emerged as a therapeutic target.[1] In 2004, mutations in the *EGFR* were reported to be associated with sensitivity to EGFR tyrosine kinase inhibitors (EGFR-TKIs).[2–4] The presence of such mutations in tumor tissue is associated with a number of clinical factors including Asian origin, female sex, adenocarcinoma histology, and a never-smoking history, and these factors have additionally been correlated with response to gefitinib (IRESSA, AstraZeneca, Macclesfield, UK), an EGFR-TKI.[5]

The IRESSA Pan-Asia Study (IPASS) compared gefitinib with carboplatin/paclitaxel as first-line treatment in 1217 never-smokers/light ex-smokers with advanced adenocarcinoma of the lung in East Asia.[6] Subgroup analysis of patients with *EGFR* mutations (n = 261) detected in DNA derived from tumor tissue samples demonstrated significantly longer progression-free survival (PFS) with gefitinib versus carboplatin/paclitaxel (hazard ratio [HR], 0.48; 95% confidence interval [CI], 0.36–0.64; $p < 0.001$).[6] In the *EGFR* mutation-negative (M−) subgroup (n = 176), PFS was significantly longer with carboplatin/paclitaxel versus gefitinib (HR, 2.85; 95% CI, 2.05–3.98; $p < 0.001$). Objective response rates (ORR) were 71.2% versus 47.3% ($p < 0.001$) and 1.1% versus 23.5% ($p = 0.001$) with gefitinib versus carboplatin/paclitaxel in *EGFR* M+ and M− patients, respectively.

The difficulties of collecting sufficient tumor tissue for biomarker analyses have stimulated interest in analyses using surrogate samples, such as serum and plasma samples, which frequently contain circulating free (cf) DNA derived from tumor tissues. Previous studies in relatively few patients had detected *EGFR* mutations in cfDNA in serum or plasma samples and suggested that using such methodology to predict response to gefitinib was worthy of further evaluation.[7–12] However, most of these studies were retrospective.

Herein, we report the evaluation of *EGFR* mutations in cfDNA from serum samples of patients in the IPASS study recruited in Japan. This preplanned, exploratory analysis was conducted to increase the understanding of the use of surrogate samples, such as serum, versus tumor biopsy samples for determining *EGFR* mutation status.

MATERIALS AND METHODS

Study Design and Patients

Full details of the IPASS study design (ClinicalTrials.gov identifier NCT00322452) have been published previously.[6] Planned objectives of this substudy of IPASS were evaluations of efficacy between the gefitinib and carboplatin/paclitaxel treatment groups by cfDNA *EGFR* mutation status from pretreatment serum samples and evaluation of the concordance between *EGFR* mutation status in pretreatment cfDNA versus tumor. Comparison of *EGFR* mutation status in pretreatment versus postprogression serum samples was also performed; however, not all patients with a pretreatment sample had a postprogression sample, which limited the comparison. In addition, comparisons with postprogression serum and pretreatment pleural effusion samples are reported in Supplemental Digital Content 1 (Methods http://links.lww.com/JTO/A152). Preplanned analysis of the Japanese subset of the IPASS population was performed to meet Japanese regulatory requirements.

All patients provided written informed consent. Provision of samples for biomarker research was optional and involved separate consent procedures for tumor and serum sampling. An independent ethics committee at each participating institution approved the study protocol. The study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation Guidelines for Good Clinical Practice, applicable regulatory requirements, and AstraZeneca's policy on bioethics.

Biomarker Analyses

Sample collection and DNA extraction are described in Supplemental Digital Content 1 (Methods http://links.lww.com/JTO/A152). *EGFR* mutations were detected using the DxS *EGFR* Mutation Test Kit for Research Use Only (DxS, Manchester, UK), which combines Amplification Refractory Mutation System (ARMS) (allele-specific polymerase chain reaction [PCR]) with the Scorpions real-time PCR technology.[13,14] Modified run conditions and cutoffs (delta Ct values [dCt]) used to define M+ samples for cfDNA derived from serum and pleural effusion samples were as follows: 50 cycles of PCR were carried out and the dCt for exon 19 deletions was 12, L858R was 14, and T790M was 8 (for tumor DNA, 40 cycles of PCR were carried out and the dCt cutoffs were 9, 11, and 8, respectively). In analyses of tumor DNA, all 29 mutations detected by the kit were assayed (19 deletions in exon 19, L858R, T790M, L861Q, G719X [S, A, or C], S768I, and 3 insertions in exon 20); whereas for serum and pleural effusion samples, the 21 most common mutations (19 deletions in exon 19, L858R, and T790M) were assayed (to make the best use of limited cfDNA yield). Samples were tested in duplicate, and only if both replicates were positive for at least one of the mutations was the sample defined as M+. Patients without a tumor sample evaluable for mutation analysis and samples which were not successfully analyzed were classified as *EGFR* mutation unknown. Biomarker samples were assayed blinded to clinical outcome and randomized treatment.

Statistical Analyses

Serum samples were collected for patients recruited in Japan and who consented to this optional analysis. Analyses of efficacy end points comparing treatment groups in the Japanese subset (intent-to-treat [ITT] population) were assessed as described previously for the overall IPASS population.[6] However, for the analyses in the cfDNA M+ and M− subgroups, the prespecified covariates of World Health Organization (WHO) performance status (PS), smoking history, and sex could not be included as covariates because of the small number of patients who had a WHO PS 2, were ex-smokers, or were males; therefore, models without covariates were used. Because of the lack of power to detect treatment differences, the result of the Japanese subset should be interpreted with caution, taking into account the associated variability and overlap in plausible range of effects (CIs). Analyses comparing treatment groups were performed for PFS (by Cox proportional hazards model) and ORR (by logistic regression model) in subgroups defined by cfDNA *EGFR* mutation status. A test for interaction between cfDNA *EGFR* mutation status (M+ or M−) and treatment was used to assess whether the PFS treatment effect was statistically different between subgroups.

Comparison of pretreatment cfDNA versus tumor *EGFR* mutations was based on the 21 mutations analyzed for cfDNA using patients with known mutation status (M+ or M−) in both samples. The sensitivity, specificity, positive

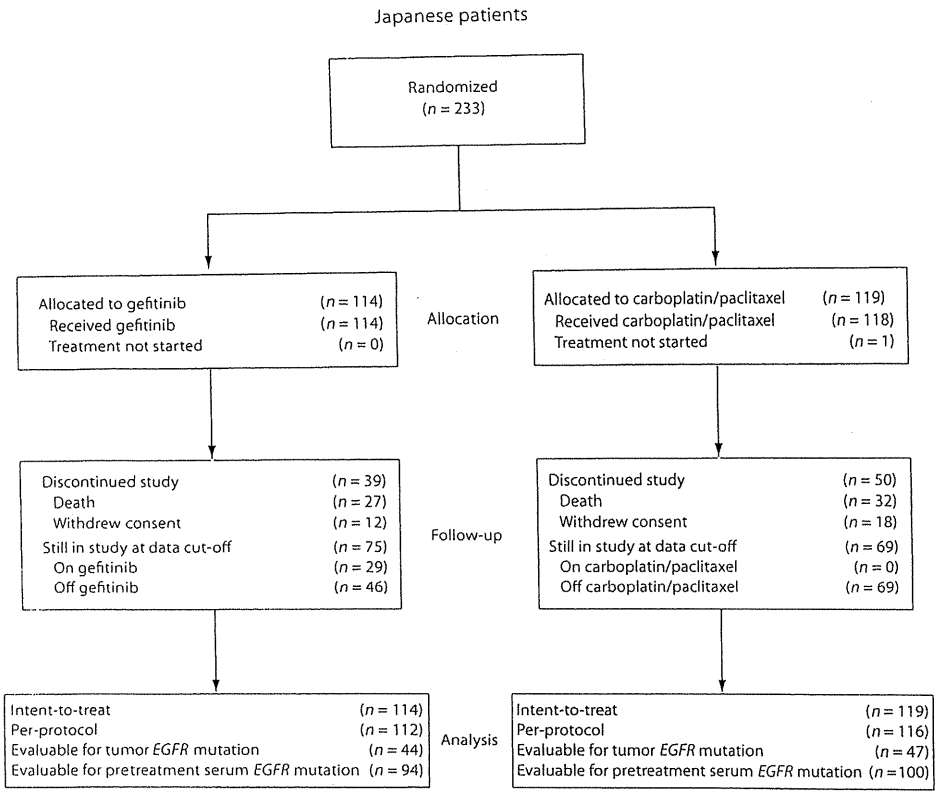

FIGURE 1. CONSORT diagram representing patient disposition (including number of patients with tumor tissue or serum evaluable for *EGFR* mutation status). *EGFR*, epidermal growth factor receptor.

and negative predictive values and their exact 95% CIs, and the kappa coefficient and 95% CI, for *EGFR* mutation status in serum samples, were evaluated assuming that the *EGFR* mutation status in tumor tissue was a true reflection of tumor biology. The proportion of concordance between cfDNA and tumor was calculated on a similar basis by excluding patients judged as unknown using either cfDNA or tumor samples.

RESULTS

Patients

In total, 233 patients from Japan were randomized to study treatment (19.1% of the overall IPASS population). Preplanned evaluations of efficacy, quality of life, and safety for the overall Japanese study population have been previously presented[15,16] and are summarized in Supplemental Digital Content 2 (Results http://links.lww.com/JTO/A153) and 3 (Figure http://links.lww.com/JTO/A154). The patient disposition for the Japanese subset of IPASS is shown in Figure 1.

EGFR Mutation Status

An evaluable DNA sample for *EGFR* mutation status derived from tumor tissue was available for 91 patients; of these, 56 (61.5%) patients were *EGFR* M+, with a lower proportion of *EGFR* M+ patients in the gefitinib group compared with the carboplatin/paclitaxel group (52.3% [23/44] versus 70.2% [33/47]) (Figure 2). A total of 194 patients provided a pretreatment serum sample for mutation analysis; all were evaluable. Of these, 46 (23.7%) patients were cfDNA *EGFR* M+ (25.5% [24/94] and 22.0% [22/100] in the gefitinib and carboplatin/paclitaxel groups, respectively) (Figure 2). Data from pretreatment pleural effusion (9 patients) and postprogression serum analyses (144 patients) are presented in Supplemental Digital Content 2 (Results http://links.lww.com/JTO/A153) and 4 (Table http://links.lww.com/JTO/A155).

Demographic and Baseline Characteristics of Patients with Known *EGFR* Mutation Status

Key demographic and baseline characteristics for patients with known (i.e., evaluable) cfDNA or tumor *EGFR* mutation status were generally consistent with the overall Japanese study population (Table 1).

Pretreatment cfDNA *EGFR* Mutation Status and Clinical Outcomes

The subset of patients with known cfDNA *EGFR* mutation status could be assumed to be representative of the overall Japanese study population (and therefore the overall study population) as shown by similar PFS and ORR results (Table 1).

A significant interaction between cfDNA *EGFR* mutation status and treatment was evident for PFS (interaction test $p = 0.045$). PFS was significantly longer with gefitinib than carboplatin/paclitaxel in the cfDNA *EGFR* M+ subgroup (HR, 0.29; 95% CI, 0.14–0.60; $p < 0.001$) (Figure 3*A*). In the cfDNA *EGFR* M− subgroup, there were no significant differences for PFS with gefitinib compared with carboplatin/paclitaxel (HR, 0.88; 95% CI, 0.61–1.28; $p = 0.50$) (Figure 3*B*). However, the HR was not constant over time. We

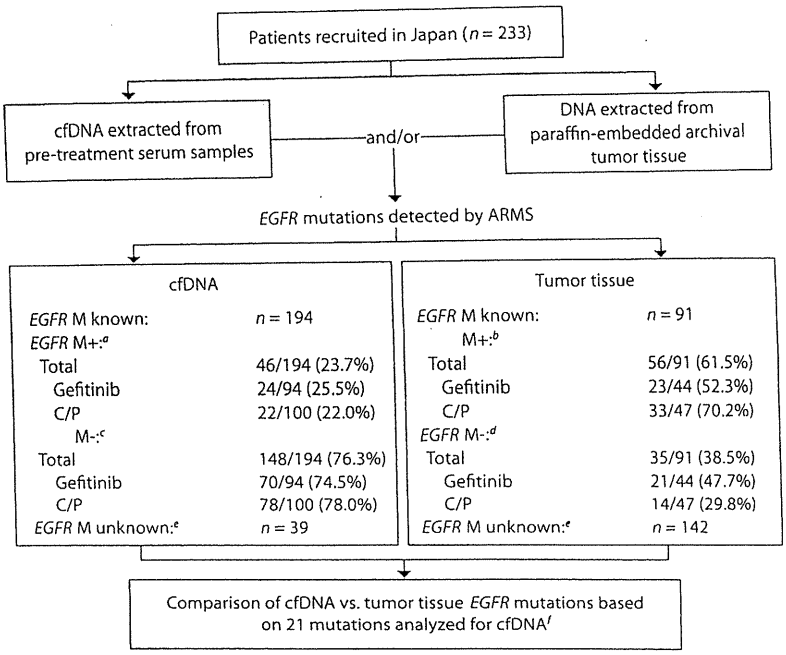

FIGURE 2. Flow and results of *EGFR* mutation analysis. [a]Sample positive for ≥1 of 21 mutations tested; detected 19 deletions in exon 19, L858R, and T790M. [b]Sample positive for ≥1 of 29 mutations tested; detected 19 deletions in exon 19, L858R, T790M, L861Q, G719S, G719A, G719C, S768I; 3 insertions in exon 20. [c]Sample negative for all 21 mutations tested. [d]Sample negative for all 29 mutations tested. [e]Unknown *EGFR* mutations: no sample available or failed analysis. [f]86 patients had known mutation status by both tumor tissue and cfDNA. C/P, carboplatin/paclitaxel; *EGFR*, epidermal growth factor receptor; M, mutation; M+, mutation-positive; M−, mutation-negative.

TABLE 1. Patient Demographics, Baseline Characteristics, and Efficacy (PFS and ORR) for Patients with Samples (cfDNA or Tumor) Evaluable for *EGFR* Mutation Status Compared with the Overall Japanese[a] Study Population (Japanese ITT Population)

	Evaluable for *EGFR* Mutation Status (cfDNA) (n = 194)[b]	Evaluable for *EGFR* Mutation Status (Tumor) (n = 91)[b]	Overall Japanese Study Population (n = 233)
Demography, n (%)			
Female	172 (88.7)	84 (92.3)	204 (87.6)
WHO PS 0/1	185 (95.4)	89 (97.8)	223 (95.7)
Never-smoker	177 (91.2)	83 (91.2)	212 (91.0)
Stage IIIB	66 (34.0)	27 (29.7)	73 (31.3)
Age <65 yr	97 (50.0)	45 (49.5)	121 (51.9)
Efficacy			
PFS HR[c] (95% CI)	0.68 (0.49–0.95)	1.08 (0.68–1.72)	0.69 (0.51–0.94)
ORR OR[d] (95% CI)	1.45 (0.80–2.61)	0.99 (0.41–2.40)[e]	1.34 (0.78–2.30)

[a] Refers to the country of recruitment and not necessarily to racial origin.
[b] Includes both mutation-positive and mutation-negative samples.
[c] HR <1 indicates a difference in favor of gefitinib.
[d] OR >1 indicates a greater chance of response on gefitinib.
[e] These results should be interpreted with caution as the logistic regression model did not converge.
cfDNA, circulating free DNA; CI, confidence interval; *EGFR*, epidermal growth factor receptor; HR, hazard ratio; ITT, intent-to-treat; OR, odds ratio; ORR, objective response rate; PFS, progression-free survival; PS, performance status; WHO, World Health Organization.

believe that this result was due to the high rate of false negative results as described later (i.e., this group included both tumor *EGFR* M+ and M− patients).

In the cfDNA M+ subgroup, ORR was not significantly different in the gefitinib group compared with carboplatin/paclitaxel treatment (75.0% [18/24] and 63.6% [14/22], respectively; odds ratio [OR], 1.71; 95% CI, 0.48–6.09; p = 0.40). In the cfDNA M− subgroup, there were no significant differences in ORR with gefitinib compared with carboplatin/paclitaxel (27.1% [19/70] and 21.8% [17/78], respectively; OR, 1.34; 95% CI, 0.63–2.84; p = 0.45) (Figure 4). Again, this subgroup included both tumor *EGFR* M+ and M− patients as described later.

The results for clinical outcome by *EGFR* mutation status (M+, M−) for the Japanese subset of patients with known tumor *EGFR* mutation status (n = 91) are included in Supplemental Digital Content 2 (Results http://links.lww.com/JTO/A153).

Comparison of *EGFR* Mutation Status in Pretreatment cfDNA and Tumor Tissue

A total of 108 patients had a known mutation result by cfDNA but not by tumor; 5 patients had a known mutation

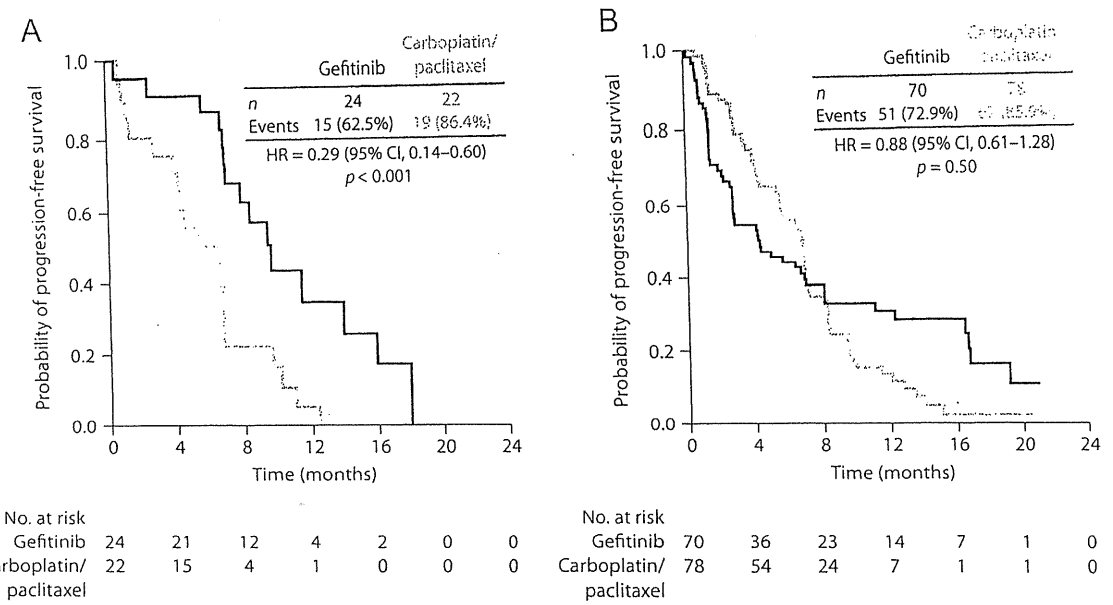

FIGURE 3. Kaplan-Meier curves of progression-free survival in cfDNA *EGFR* mutation-positive (*A*) and cfDNA *EGFR* mutation-negative (*B*) patients in the Japanese subset of IPASS. HR <1 indicates a difference in favor of gefitinib. CI, confidence interval; cfDNA, circulating free DNA; *EGFR*, epidermal growth factor receptor; HR, hazard ratio.

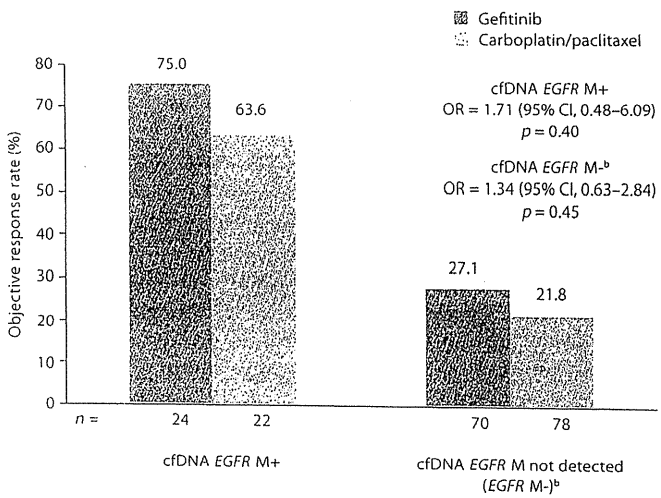

FIGURE 4. Objective response rates by treatment and by cfDNA (serum) *EGFR* mutation status (Japanese ITT population[a]). [a]Refers to the country of recruitment and not necessarily to racial origin. [b]There was a high rate of false-negative results, i.e., this group included both tumor *EGFR* M+ and M− patients. OR >1 implies a greater chance of response on gefitinib. OR, CI, and *p* values from logistic regression. cfDNA, circulating free DNA; CI, confidence interval; *EGFR*, epidermal growth factor receptor; ITT, intent-to-treat; M+, mutation-positive; M−, mutation-negative; OR, odds ratio.

result by tumor but not cfDNA (no serum sample provided); and 86 patients had a known mutation status by both tumor and cfDNA.

Of the 86 patients who had a known tumor and cfDNA mutation status, no false positives were identified (i.e., no samples were tumor M− but cfDNA M+). All 22 patients identified as cfDNA *EGFR* M+ were tumor *EGFR* M+, i.e., the positive predictive value was 100% (all samples that were cfDNA M+ were tumor M+) and the specificity was 100% (all samples that were tumor M− were cfDNA M−) (Table 2). However, the rate of false negatives was high: 29/51 (56.9%) of patients identified as tumor *EGFR* M+ were cfDNA *EGFR* M− (Table 2).

TABLE 2. Comparison of *EGFR* Mutation Status in cfDNA and Tumor Samples in 86 Patients with a Known *EGFR* Mutation Status Using Both Methods (Japanese[a] ITT Population)

	Mutation Status (Tumor Tissue), *n*		
	M+	M−	Total
Mutation status (cfDNA), *n*			
M+	22	0	22
M−	29	35	64
Total	51	35	86

Sensitivity = 43.1% (22 cfDNA M+ out of 51 tumor M+).[b]
Specificity = 100% (all 35 tumor M- were cfDNA M−).[b]
Positive predictive value = 100% (all 22 cfDNA M+ were tumor M+).[b]
Negative predictive value = 54.7% (35 tumor M- out of 64 cfDNA M−).[b]
Concordance = 66.3% (cfDNA and tumor results agreed in 57 of 86 cases).[b,c]

[a] Refers to the country of recruitment and not necessarily to racial origin.
[b] Those with a known *EGFR* mutation status using both methods.
[c] Kappa coefficient 0.38 (95% CI, 0.24–0.53).
cfDNA, circulating free DNA; CI, confidence interval; *EGFR*, epidermal growth factor receptor; ITT, intent-to-treat; M+, mutation positive; M−, mutation negative.

EGFR Mutation Types in Pretreatment cfDNA and Tumor Tissue

Of the patients classified as *EGFR* M+ at pretreatment by both tumor and cfDNA, all had the same mutation type in

TABLE 3. *EGFR* Mutations in Pretreatment cfDNA vs. Tumor Samples (Japanese[a] ITT Population)

	Tumor *EGFR* Mutation[b]						
cfDNA *EGFR* Mutation	Exon 19 Deletions Only	Exon 20 T790M Only	Exon 21 L858R Only	Exon 20 T790M and Exon 21 L858R	Negative	Unknown	Total
Exon 19 deletions only	11	0	0	0	0	15	26
Exon 20 T790M only	0	0	0	1	0	1	2
Exon 21 L858R only	0	0	10	0	0	8	18
Exon 20 T790M and exon 21 L858R	0	0	0	0	0	0	0
Negative	18	0	11	0	35	84	148
Unknown	2	1	0	0	2	34	39
Total	31	1	21	1	37	142	233

The categories are mutually exclusive. The categories "Exon 19 deletions and exon 20 T790M" and "Exon 19 deletions and exon 21 L858R" were 0 for both tumor and cfDNA and have been omitted from the table.

[a] Refers to the country of recruitment and not necessarily to racial origin.

[b] Mutations that were tested in tumor tissue samples but not serum included: exon 20 insertion, exon 21 L861Q, exon 18 G719X, and exon 20 S768I. Two patients with tumor samples had these mutations (1 with exon 20 insertion and 1 with exon 21 L861Q). These patients were excluded from the comparative analysis of mutation detection by sample type.

cfDNA, circulating free DNA; *EGFR*, epidermal growth factor receptor; ITT, intent-to-treat.

tumor and cfDNA except one patient who had exon 20 T790M and exon 21 L858R by tumor but exon 20 T790M only by cfDNA (Table 3).

DISCUSSION

The feasibility of using cfDNA to detect *EGFR* mutations was assessed in the Japanese subset of patients from the IPASS study. The proportion of patients identified as *EGFR* M+ was lower when assessed in cfDNA (23.7%) compared with tumor tissue (61.5%). Although cfDNA results identified no false positives, a high rate of false negatives (56.9%) was observed, with more than half of the tumor M+ patients not detected by cfDNA testing (of patients with evaluable mutation status from both cfDNA and tumor). Further research into appropriate methods and analysis needs to be performed before it could be accepted as an option in the diagnostic or screening setting. If larger patient series confirmed the absence of false-positive results and demonstrated an improvement or lowering of false-negative results, serum testing may prove useful for patients for whom tumor samples are not available.

Testing of biopsied tumor tissue remains the current recommended method for *EGFR* mutation analysis.[8] However, tumor tissue is often difficult to obtain, particularly from patients with advanced non-small cell lung cancer (NSCLC), and a lack of tumor cells in a given sample and subsequently failure on pathological examination can make *EGFR* mutation analysis very difficult. The increased recognition of the relevance of mutation testing to treatment selection may stimulate efforts to better obtain tissue for *EGFR* mutation testing in the future. In the meantime, detection of *EGFR* mutation status in cfDNA derived from serum/plasma may allow patients without diagnostic tumor material the opportunity to benefit from personalized treatment and also has a use in the clinical trial setting where tumor material is not always available.

Although minimally invasive, the use of serum as a nontumor surrogate sample may be limited by the amount of cfDNA available in the sample, meaning that some positive samples are not detected. In addition, some patients may not have cf tumor DNA as their tumors may not be releasing this material into the bloodstream, giving rise to false-negative results. Because of the limited yields of cfDNA obtained from serum, two changes (in addition to duplicate tests) were made to the *EGFR* mutation ARMS kit used to detect *EGFR* mutations in this study: an increase in the number of PCR cycles and an alteration of the cutoffs used to define M+ samples (dCt values). Further analysis is underway to investigate whether these conditions are the most appropriate and whether less stringent settings could result in more true positives (fewer false negatives) while retaining no false positives.

There have been several reports on the detection of cfDNA *EGFR* mutation status using different methods. A significant correlation between cfDNA *EGFR* mutation status and clinical response to gefitinib was found in two previous small studies that assessed cfDNA *EGFR* mutation status using the ARMS method of detection, a highly sensitive (1% sensitive) targeted technique to detect specific known *EGFR* mutations.[9,11] Other screening techniques detect all *EGFR* mutations, known and novel variants, by PCR amplification followed by sequencing, pyrosequencing, or melt analysis (10–30% sensitivity).[8] However, although these methods are widely used for *EGFR* mutation analysis of DNA derived from tumor tissue, not all of these methods have demonstrated utility for *EGFR* mutation analysis of cfDNA. In a small study that used DNA sequencing to detect *EGFR* mutations in serum, mutations were more frequently observed in patients experiencing partial response or stable disease compared with those whose disease progressed, although the difference did not reach statistical significance.[10] No statistically significant association between cfDNA *EGFR* mutation status and PFS by multivariate analysis (HR, 1.48; 95% CI, 0.93–2.36; $p = 0.09$) was found in the study by Rosell et al.[12] which assessed *EGFR* mutations by PCR-based methods in the presence of a protein nucleic acid (PNA) clamp in the cfDNA extracted from serum of 164 patients

treated with erlotinib. In another study that used denaturing high-performance liquid chromatography to analyze for mutations in exons 19 and 21 from matched plasma and tumor samples, patients with plasma *EGFR* mutations had significantly higher ORR and prolonged PFS.[7] The present study using ARMS demonstrated that the treatment effect for the Japanese cfDNA *EGFR* M+ subgroup followed the same pattern as the tumor *EGFR* M+ subgroup of the overall IPASS population (i.e., PFS HR significantly in favor of gefitinib and higher ORR with gefitinib versus carboplatin/paclitaxel).[6] There was a significant interaction between cfDNA *EGFR* mutation status and treatment for PFS.

Any variance in concordance rates for mutation results between pretreatment serum versus tumor tissue (66.3% in our study and between 58 and 93% in previously reported studies)[7,9–11] may be attributed to different methods of extraction, detection, run conditions, the size and yield of the DNA fragments, and the fact that cfDNA may not be present in the circulation of all patients with NSCLC. For example, targeted sequences amplified by ARMS are short, at 100–150 bp, leading to decreased assay failure rates (particularly from formalin-fixed paraffin-embedded material or fragments of cfDNA) compared with sequencing methods, which tend to involve the amplification of longer target sequences of 150–250 bp or above.[8,13,14,17,18]

In patients who were cfDNA *EGFR* M− in this study, no significant difference for PFS was seen with gefitinib compared with carboplatin/paclitaxel; however, the HR was not constant over time (as was observed for the overall Japanese study population). These results should be interpreted with caution as there was a high rate of false negatives, and this subgroup is likely to include tumor *EGFR* M+ and M− patients.

In conclusion, these results merit further investigation to determine whether alternative samples, including serum or plasma, may be considered for determining *EGFR* mutation status in future, particularly in cases where diagnostic tumor material is not available. Currently, analysis of tumor material is the recommended method for determining *EGFR* mutation status.

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