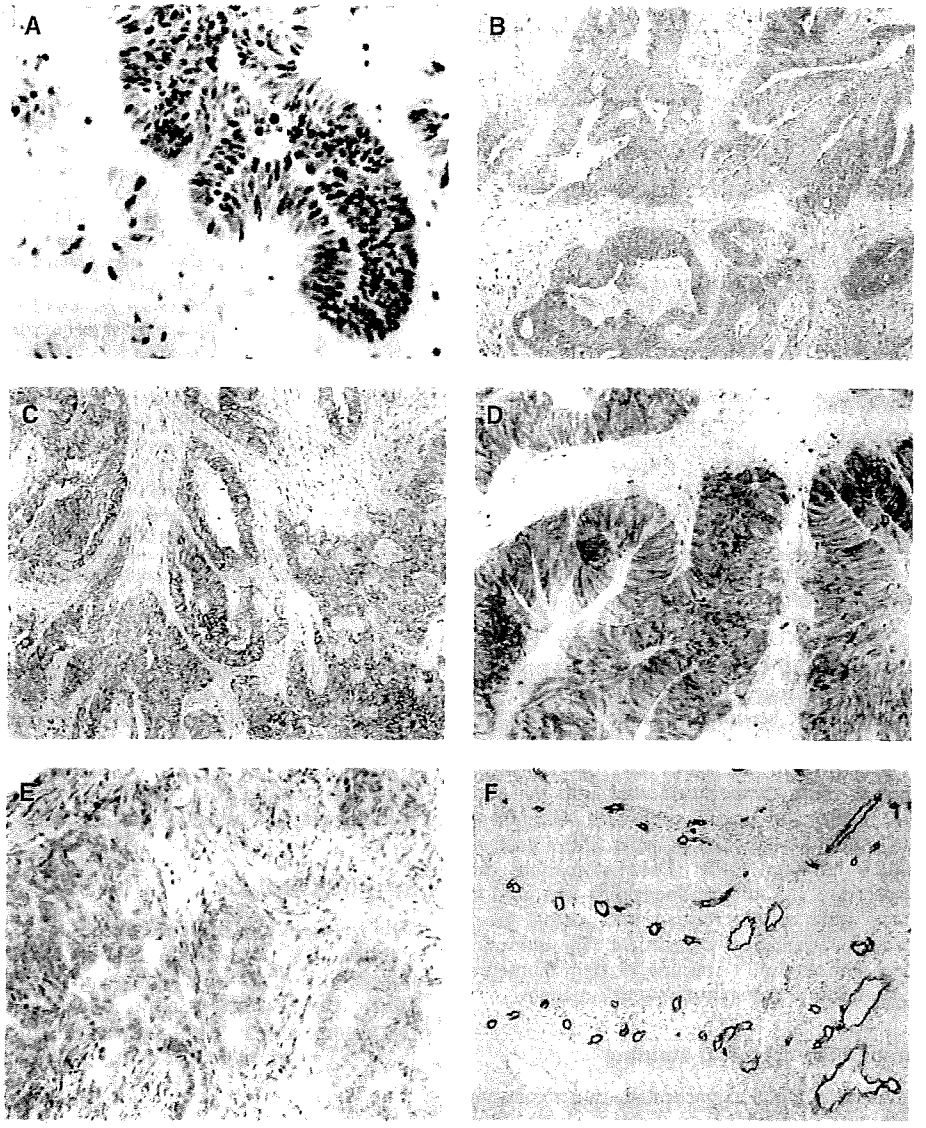

Figure 1 Immunohistochemical staining of pre-treatment rectal biopsy specimens from locally advanced rectal cancers. (**A**) Ki67 immunoreactivity, (**B**) Bax immunoreactivity, (**C**) Grp78 immunoreactivity, (**D**) TS immunoreactivity, (**E**) DPD immunoreactivity, and (**F**) CD34 immunoreactivity. Note Ki67 immunoreactivity confined to the tumour cell nuclei, Bax, and Grp78 to the tumour cell cytoplasm, TS and DPD to the tumour cell nucleus and cytoplasm, and CD34 to the endothelium of intratumoural microvessels.

pathological CR and greater than 95% pathological response groups achieve a significantly improved overall survival and recurrence-free survival when compared with less than 95% pathological response groups (Ruo *et al*, 2002; Guillem *et al*, 2005). Therefore, we divided the cases into two groups: Dworak grades 1 and 2, and grades 3 and 4 (Gavioli *et al*, 2005). The latter were considered as responders to CRT. A high Ki67, Bax score, and TS score and a low Grp78 score were well correlated with response. On the other hand, there were no associations with the other immunohistochemical factors, as well as clinicopathological factors (Table 3).

Multiple logistic regression analysis

Multiple logistic regression analysis was performed with a stepwise method (Tanaka *et al*, 1999). Independent variables were the data for Ki67 LI, Bax score, TS score, and Grp78 score, and dependent variables were no-response (0; Dworak regression grades 1 and 2) or response (1; Dworak regression grades 3 and 4). Other immunohistochemical markers and clinicopathological factors were not used. By the logistic regression analysis, we detected the Ki67 LI, Bax score, and TS score as independent factors (Table 4). The Bax score (odds ratio 18.1) had the strongest influence. The logistic regression formula was as follows:

$$\log_e (p/1-p) = -24 + 0.15 \times [\mathrm{Ki}_{67}\ \mathrm{LI}] + 2.90 \times [\mathrm{Bax\ score}] + 0.60 \times [\mathrm{TS\ score}].$$

Receiver-operating characteristic curve

A receiver-operating characteristic curve was generated by plotting the true-positive rate (sensitivity) on the *y* axis and the

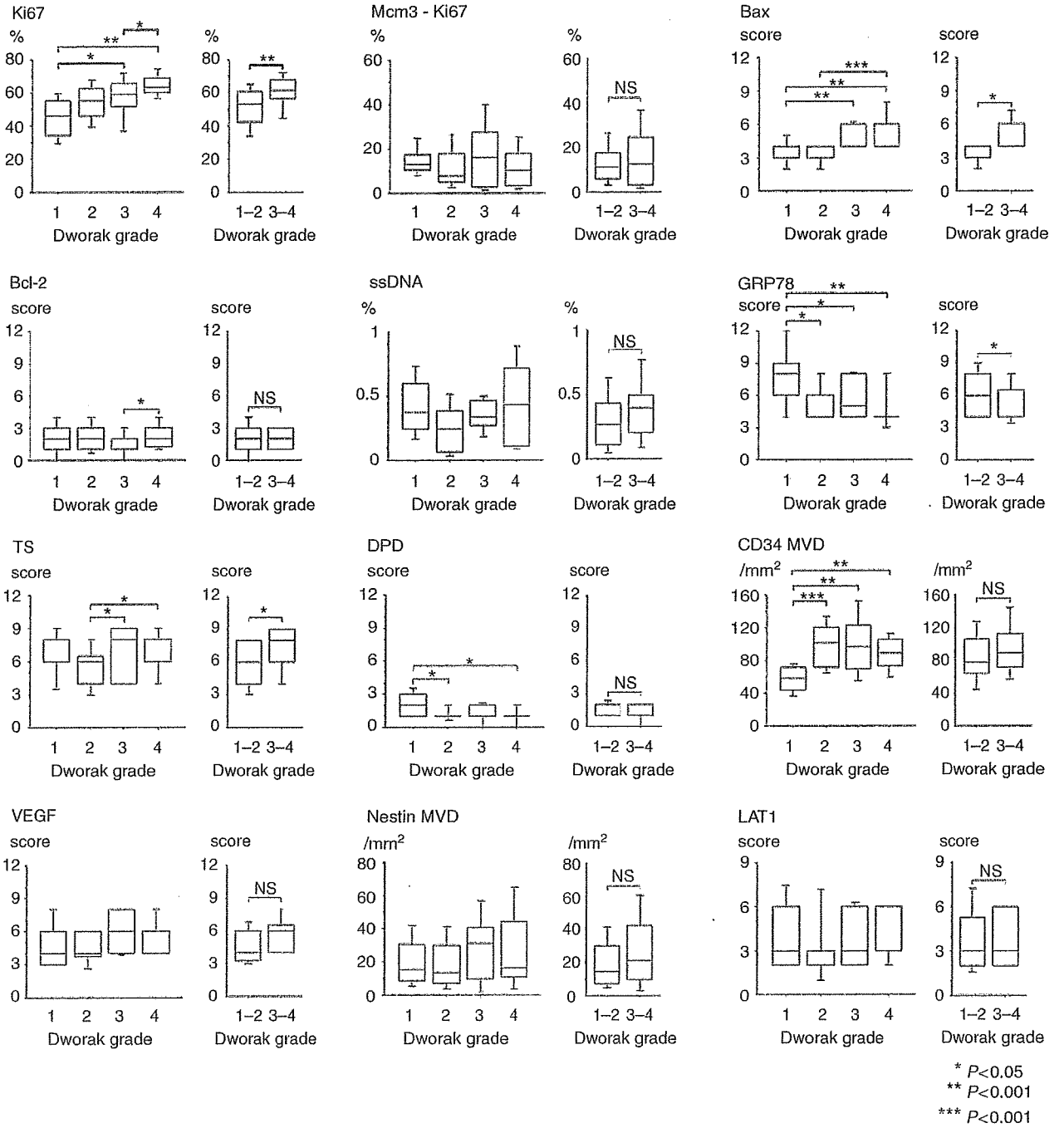

Figure 2 Ki67, Bax, Grp78, TS, DPD, and CD34 (MVD) were significantly related to chemoradiosensitivity ($P<0.05$). High Ki67 LI, Bax score, TS score, and low Grp78 were significantly correlated with tumour regression when responders were defined as having Dworak regression grades 3 and 4.

false-positive rate (1−specificity) on the *x* axis (Figure 3) (Tanaka *et al*, 1999).

Although the *P*-value at the point closest to the left upper corner on the curve is generally considered to represent the best balance of both sensitivity and specificity in distinguishing between response and no-response, we determined four points of *P* as the cut-off values (0.90, 0.50, 0.40, and 0.20) to construct practical criteria for the five categories 'responder', 'probable responder', 'unknown', 'probable non-responder', and 'non-responder' (Table 5). The points of $P=0.90$ and 0.20 meant the points of specificity 100% and sensitivity 100%, respectively. The point of $P=0.50$ meant the point at which the specificity was maximum and the sensitivity was more than 80%. The point of $P=0.40$ meant the point at which the specificity for prediction of non-responder was maximum and the sensitivity more than 80%.

Sensitivity and specificity

A *P*-value for each case was calculated with three immunohistochemical markers examined in 60 sets of biopsy specimens. Using

the calculated *P*-value, we classified the 60 patients into one of the above five categories with criteria distinguishing between responder and non-responder. Sensitivities and specificities of the criteria are shown in Table 5.

DISCUSSION

In this study, we sought clinicopathological factors and immunohistochemical markers that might contribute to prediction of chemoradiation effects on locally advanced rectal cancer. Our conclusion is that it is possible to predict a responder to preoperative CRT, with 82.8% sensitivity and 83.9% specificity, using the value calculated with the three elements of the Ki67 LI, the Bax score, and the TS score in biopsy specimens before CRT. In fact, high expression of Ki67, Bax, and TS was positively correlated with therapeutic effects.

The first factor, high proliferative activity with Ki67 as the marker, was earlier found to correlate with PCNA immunostaining, and mitotic counts after radiation of rectal cancer (Willett *et al*, 1995). Later, beneficial effects of radiotherapy for patients with various carcinoma with high Ki67 LIs were reported (Nakano *et al*, 1997; Raybaud-Diogene *et al*, 1997). However, in other reports, no relation was noted between Ki67 values in biopsy specimens before radiation and response rate in rectal cancers (Suzuki *et al*, 2004; Debucquoy *et al*, 2008). Suzuki *et al* (2004) performed preoperative radiotherapy only. Debucquoy *et al* (2008) combined preoperative radiotherapy and/or 5-FU/LV. Because we adopted CRT for all patients, the response may be more influenced by chemotherapy than radiation.

The second factor, Bax expression, was also reported by Chang *et al* (2005) to correlate well with chemoradiation therapeutic effects, and the authors considered that apoptosis may be important in rectal carcinoma response to CRT. Similarly, Bax overexpression has been found to correlate with anticancer drug sensitivity in a variety of human cancers, through enhanced induction of apoptosis (Krajewski *et al*, 1995; Guo *et al*, 2000; Teranishi *et al*, 2007). However, Gosens *et al* (2008) did not find any link between Bax expression and rectal cancer regression for neoadjuvant chemoradiation. They evaluated the regression grading system described by Rödel *et al* (2005): (1) no regression or <25% of tumour mass, (2) 25 to >50% tumour regression, and

Table 3 Clinicopathological characteristics of the patients separated by Dworak grades 1, 2 *vs* 3, 4

	Dworak grade 3, 4 (responder) ($n=29$)	Dworak grade 1, 2 (non-responder) ($n=31$)	
Age (year) (mean ± s.d.)	63.5 ± 11.4	63.5 ± 9.8	$P=0.11$
Sex			
Male	21	24	
Female	8	7	$P=0.65$
Tumor size (mm) (mean ± s.d.)	46.7 ± 14.4	48.0 ± 19.7	$P=0.98$
Histological type (biopsy)			
Well	17	20	
mod/por	12	11	$P=0.64$
CEA (mg/100 ml) (mean ± s.d.)	8.5 ± 12.7	9.4 ± 8.5	$P=0.23$
CA19-9 (ng/ml) (mean ± s.d.)	17 ± 25	22 ± 29	$P=0.054$

Well, well-differentiated adenocarcinoma; mod/por, moderately to poorly differentiated adenocarcinoma; s.d., standard deviation.

Table 4 Results of multiple logistic regression analysis

	Regression coefficient	*P*-value	Odds ratio	95% CI
Variable				
Ki67 LI	0.15	0.002	1.17	1.06–1.29
Bax score	2.90	0.001	18.1	3.11–105.7
TS score	0.60	0.019	1.83	1.11–3.03
Constant	−24.47	<0.001		

LI, labelling index; CI, confidence interval.

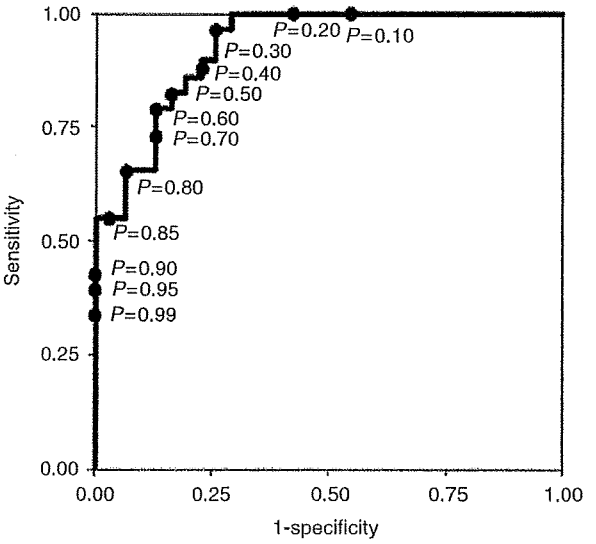

Figure 3 Receiver-operating characteristic curve with the logistic regression model. The area under the curve is 0.928 (95% confidence interval; 0.867–0.988).

Table 5 Criteria for Dworak grades 1, 2 *vs* 3, 4, and their validities tested among the 60 patients

			Pathological response		Validity			
			DG 3, 4	DG1, 2	DG 3, 4		DG 1, 2	
Category	**Definition (*P*)**	**Definition (Π)**	**Responder ($n=29$)**	**Non-responder ($n=31$)**	**Se**	**Sp**	**Se**	**Sp**
Responder	$0.90 \leqslant P$	$2.20 \leqslant \Pi$	13	0	44.8	100		
Probable responder	$0.50 \leqslant P < 0.90$	$0 \leqslant \Pi < 2.20$	11	5	82.8	83.9		
Unknown	$0.40 \leqslant P < 0.50$	$-0.41 < \Pi < 0$	1	1				
Probable no-responder	$0.20 < P \leqslant 0.40$	$-1.39 < \Pi \leqslant -0.41$	4	7			80.6	86.2
No-responder	$P \leqslant 0.20$	$\Pi \leqslant -1.39$	0	18			58.1	100

P, probability; DG, Dworak grade; Se, sensitivity; Sp, specificity; $\Pi = \log_e (P/1-P)$.

(3) complete regression. In addition, Bax immunohistochemical values were only intensity of cytoplasmic staining 0–3. Differences in grading systems and immunohistochemical expression scoring could clearly influence the results.

Rau *et al* (2003) immunohistochemically investigated the expression of p53, Bax, p21, Ki67, hMSH2 in pre- and post-therapeutic rectal carcinoma with preoperative radiotherapy. Only low p21 expression in tumour samples was significant in no-response to neoadjuvant chemoradiation. They reported no relation with Bax expression but classified responders as CR or partial response, histopathologically defined with resected post-therapeutic rectum, again differing from our definition as Dworak grades 3 or 4.

The third factor, TS, is important in pyrimidine nucleotide synthesis and represents an important chemotherapeutic target for 5-FU chemotherapy. Immunohistochemically, high TS expression in pre-treatment locally advanced rectal cancer biopsies was earlier shown to be predictive of a higher pathological response in the fluorouracil/oxaliplatin-base chemotherapy (Negri *et al*, 2008). A trend toward a direct correlation between the level of TS expression and response of 5-FU/LV treatment in patients with metastatic colon cancer has been noted (Johnston *et al*, 2003). Similar results have also been reported by Edler *et al* (2002) and Kornmann *et al* (2003).

However, low TS expression was a predictor of response to 5-FU chemotherapy for colorectal cancer metastases (Aschele *et al*, 1999) and advanced colorectal cancer (Cascinu *et al*, 1999). Aschele *et al* (1999) used a regimen of schedule-specific biochemical modulation of 5-FU plus methotrexate, and Cascinu *et al* (1999) applied 5-FU/LV. In both studies, cases with metastases and/or recurrence were included, and TS expression was evaluated as intensity 0 (undetectable staining) to 4 (very high intensity of staining), and then intensity levels 0–2 were considered as low, and 3 and 4 as high expression. We examined both cytoplasmic TS expression intensity and percentage of positive cells, as well as the Bax value. In another study, by Liersch *et al* (2006), TS expression was examined in surgically resected rectal cancer. In the reports, high TS expression correlated with cancer relapse. The clinical meaning of evaluation of TS expression needs further clarification.

The multiple logistic regression analysis revealed Ki67 LI, Bax score, and TS score to be independent factors, with a sensitivity and specificity for prediction of responder cases of 82.8 and 83.9%, respectively. Although the logarithm model is difficult to calculate for daily use, it can be easily converted to a linear model. It is sufficient for users to know the values of $\log_e (P/1-P)$ at the point of criteria. Practically, users can directly substitute the Ki67 LI, Bax score, and TS score into the formula:

$$\prod = \log_e(p/1-p) = -24 + 0.15\times[\text{Ki67 LI}] + 2.90 \times[\text{Bax score}] + 0.60\times[\text{TS score}].$$

If this value Π ($\log_e (P/1-P)$) is larger than 0.00, it indicates a responder case. If it is smaller than -0.41, it indicates a non-responder case (Table 5).

At present, CRT with subsequent surgical resection is performed without selection of cases. However, with our approach, likely responder cases can be chosen before therapy. In the future, our multivariate model should be revised using new factors to improve the sensitivity and specificity. The treatment strategy for locally advanced rectal cancer should be further developed toward so-called tailor-made therapy including such evaluation before preoperative therapy and/or surgical resection.

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Molecular Diagnostics

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ORIGINAL REPORT

Relapse-Related Molecular Signature in Lung Adenocarcinomas Identifies Patients With Dismal Prognosis

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The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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ABSTRACT

Purpose
In order to aid the development of patient-tailored therapeutics, we attempted to identify a relapse-related signature that allows selection of a group of adenocarcinoma patients with a high probability of relapse.

Patients and Methods
Whole-genome expression profiles were analyzed in 117 lung adenocarcinoma samples using microarrays consisting of 41,000 probes. A weighted voting classifier for identifying patients with a relapse-related signature was constructed with an approach that allowed no information leakage during each training step, using 10-fold cross-validation and 100 random partitioning procedures.

Results
We identified a relapse-related molecular signature represented by 82 probes (RRS-82) through genome-wide expression profiling analysis of a training set of 60 patients. The robustness of RRS-82 in the selection of patients with a high probability of relapse was then validated with a completely blinded test set of 27 adenocarcinoma patients, showing a clear association of high risk RRS-82 with very poor patient prognosis regardless of disease stage. The discriminatory power of RRS-82 was further validated using an additional independent cohort of 30 stage I patients who underwent surgery at a distinct period of time as well as with the Duke data set on a different platform. Furthermore, completely separate training and validation procedures using another data set recently reported by the Director's Challenge Consortium also successfully confirmed the predictive power of the genes comprising RRS-82.

Conclusion
RRS-82 may be useful for identifying adenocarcinoma patients at very high risk for relapse, even those with cancer in the early stage.

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INTRODUCTION

Lung cancer remains the leading cause of cancer death in industrialized countries, including Japan and the United States.[1,2] Adenocarcinomas, which account for more than 50% of non–small-cell lung cancer (NSCLC) cases, are the most frequent type of NSCLC with a heterogeneous nature in various aspects, including clinicopathologic and molecular features, and are showing an increasing trend.[3] The TNM clinical staging system has become the standard for predicting prognoses, however, the best hope for cure relies on surgical resection, which is considered as standard treatment for operable adenocarcinoma patients.[4] Nevertheless, 30% to 35% of surgically treated stage I patients eventually face relapse after the initial surgery, indicating the existence of a subgroup of patients clinically diagnosed as having early-stage disease, who actually have residual cancer cells undetectable by currently available imaging techniques used for staging.[4]

Although a number of prognostic biomarkers, such as altered expressions of oncogenes, and tumor suppressor genes have been proposed, the TNM staging system remains the standard method for predicting patient prognosis, indicating that such prediction may require information derived from the expression status of multiple genes and molecules. At the same time, the advent of microarray technology and completion of the genome project has made it possible to carry out genome-wide profiling of gene expressions.[5] These developments have provided an opportunity for establishing patient-tailored therapeutic strategies, leading to the identification of gene-expression profiles that are associated with the prognosis of individuals with lung cancer.[6-12] However, few prognostic prediction

classifiers have been validated with a sufficient number of independent cases.[12]

In this study, we report successful identification of a relapse-related molecular signature in adenocarcinomas through analysis of genome-wide expression profiles using a training set of 60 patients with lung adenocarcinomas. General applicability of the resultant classifier was successfully validated in a blind test set of 27 cases with stage I to III disease as well as with another independent cohort of 30 stage I patients. Moreover, additional validation using two data sets on a different platform further confirmed the predictive power of the genes comprising the relapse-related molecular signature.

PATIENTS AND METHODS

Patient Samples

Eighty seven lung adenocarcinoma samples from patients who underwent potential curative resection between December 1995 and August 1999 were collected at Aichi Cancer Center, Nagoya, Japan (herein referred to as data set I; online-only Appendix Table A1). An additional independent cohort of 30 adenocarcinoma samples from patients with pathologic stage (pStage) I disease were also collected at Aichi Cancer Center between February 2002 and December 2004 (herein referred to as data set II; Appendix Table A1). None of the 117 patients received adjuvant chemotherapy. General schedule of follow-up examinations was chest x-ray (every month for the first 3 months, and 3 months interval thereafter) and chest and abdominal computed tomography (CT; every year) until 5 years after surgery. Additional examinations, such as CT, bone scan, and brain magnetic resonance imaging, were also considered, if any signs of possible relapse were suspected. The median follow-up periods for patients alive at the last follow-up examination in data set I and data set II were 90 months (range, 64 to 108 months) and 64 months (range, 55 to 75 months), respectively. All tumor specimens were collected under approval from the institutional review boards of Aichi Cancer Center and Nagoya University with written informed consent from each patient.

Acquisition of Expression Profiles and Analysis of EGFR, p53, and K-ras Mutations

Double-stranded cDNA was synthesized from 500 ng of total RNA using Moloney murine leukemia virus-reverse transcriptase (Agilent Technologies, Palo Alto, CA) and poly dT primer incorporating the T7 promoter. Cy5-sample cRNA and Cy3-common reference cRNA were generated and hybridized to a Whole Human Genome oligo DNA microarray kit (G4112F, Agilent Technologies) with 41,000 distinct probes, which was scanned using an Agilent DNA microarray scanner (G2505B, Agilent Technologies), basically as described previously.[13] The mutation status of *EGFR*, *p53*, and *K-ras* was previously reported in the same set of patients.[13] All the microarray data and the pathologic and clinical data used for this study are available at Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/; accession number GSE13213). Cross-platform validation was carried out using the Duke[11] and Director's Challenge Consortium[12] data sets as detailed in the online-onlyAppendix.

Biostatistical and Bioinformatic Analyses

To identify a relapse-related signature using signals that were expressed above the background in at least 90% of samples, we used a weighted voting algorithm, in which each weight value was calculated as the signal-to-noise ratio, basically according to the detailed method that we described previously.[14] Kaplan-Meier survival curves and Cox proportional hazards model analyses (Stata, version 7.0; Stata Corp, College Station, TX) were used to analyze the relationships of the resultant relapse-related signature with overall and relapse-free survival. All statistical tests were two sided. The CLUSTER[15] program was used for average linkage hierarchical clustering of both genes and cases, and the TREEVIEW[15] program was used for display (http://rana.lbl.gov/EisenSoftware.htm).

Identification of Relapse-Related Signature

A schematic diagram of our strategy for constructing and validating a relapse-related signature in surgically treated lung adenocarcinoma patients is shown in Figure 1, which was formed with the intention of blocking any information leakage between the training and validation data sets. First, we divided expression profile data obtained from 87 patients into 60 training and 27 validation data sets, the latter of which was completely set aside during training. In order to identify a generic signature with clear associations with relapse in the training set of patients with lung adenocarcinomas, we selected 28 favorable samples (alive > 5 years after surgery without any evidence of relapse) and 21 fatal samples (dead in 5 years after initial surgery with evidence of relapse). The remaining 11 patients in the training set were excluded from analysis of a possible relapse-related signature, because of ambiguity related to the aggressiveness of their tumors,

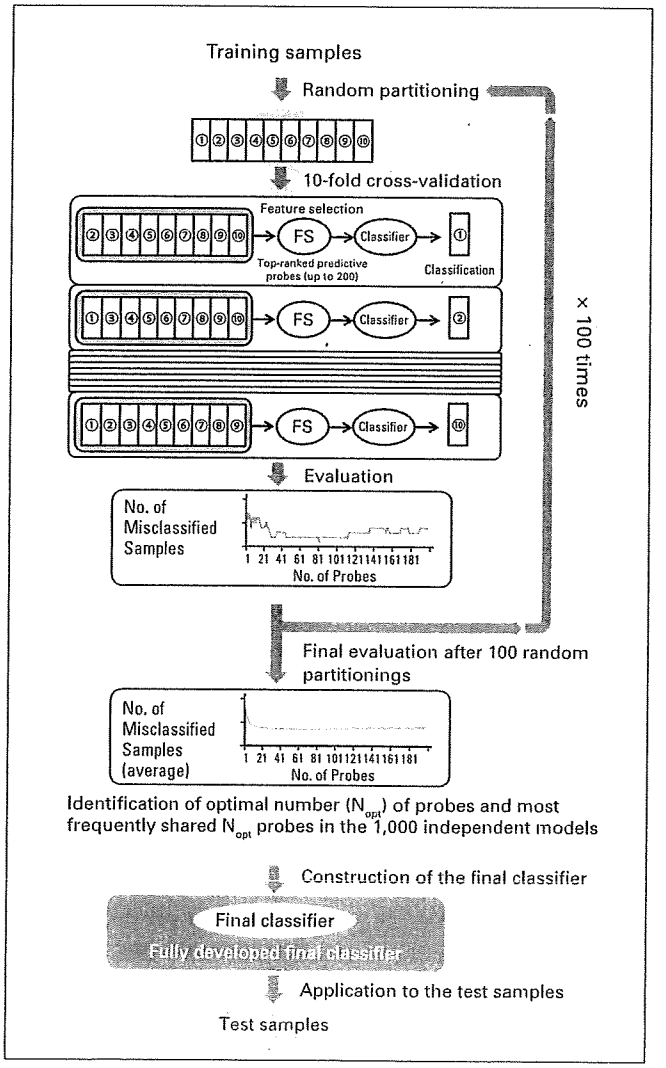

Fig 1. Schematic diagram of our training-validation stratergy for identifying replapse-related signature using 10-fold cross-validation procedures with 100 random partitions of the training data set.

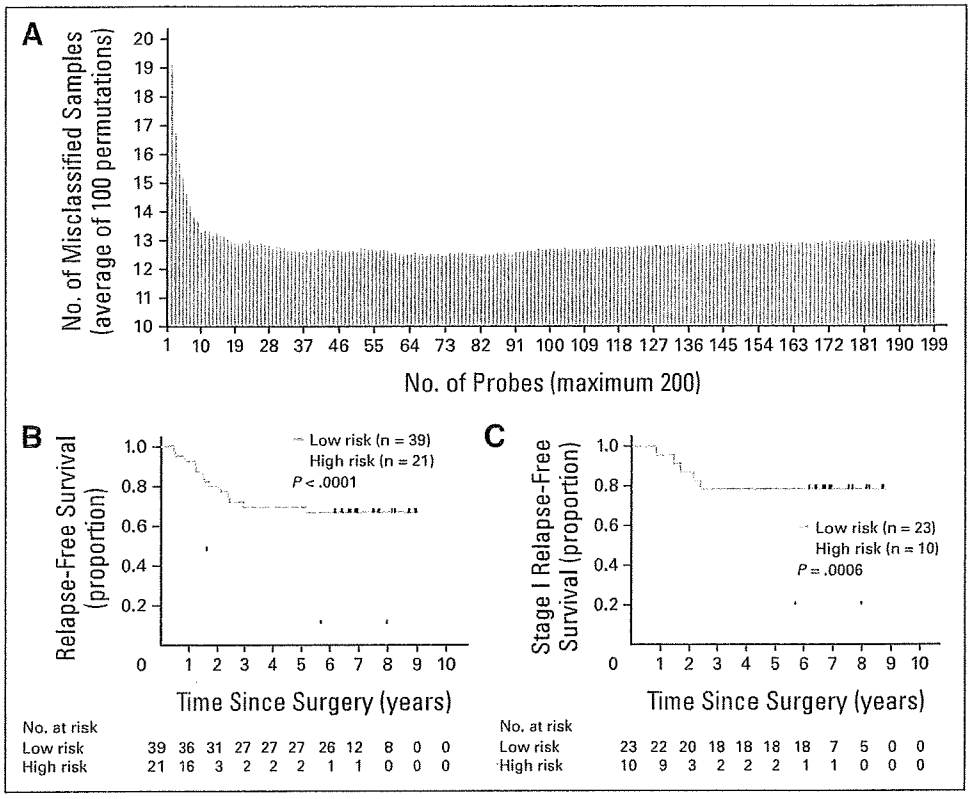

Fig 2. Results of the training procedure for identifying a relapse-related signature. (A) Results of our search for the optimum number of probes for defining a relapse-related signature. Kaplan-Meier survival curves were used to estimate survival in the training cohort. Relapse-free survival curves for patients in (B) all stages and (C) stage I.

which were five who survived for more than 5 years with some signs of relapse during follow-up, five who died of cancer after surviving for more than 5 years, and one who died within 5 years without evidence of relapse.

Of the 41,000 probes in the entire genome microarray, 23,828 passed the initial filtering criteria for selecting informative probes, and were then ranked according to a signal-to-noise metric and used to identify a relapse-related signature that could best distinguish patients who died with relapse from those cured by surgery. The learning errors for each model, to which increasing numbers of the predictive probes were applied, were calculated using 10-fold cross-validation and repeated with new randomly partitioned data sets 100 times. Thus, 1,000 independent sets consisting of up to 200 predictive probes each were selected for constructing a relapse-related signature-based classifier. As a result, 82 predictive probes were found to yield the fewest numbers of learning errors (Fig 2A), and the group of 82 probes most frequently shared among each of the 1,000 independent sets of 82 predictive probes was identified as a relapse-related signature (hereafter referred to as RRS-82; online-only Appendix Table A2). RRS-82 was able to distinguish patients with a very poor prognosis when all stages or only stage I were considered (Figs 2B and 2C for relapse-free survival and online-only Appendix Fig. A1 for overall survival). There were no associations of RRS-82 with the presence of *EGFR*, *K-ras*, or *p53* gene mutations, none of which showed any prognostic significance (Appendix Fig. A2).

Validation of RRS-82 in the Test Cohort of Data Set I

To evaluate the robustness of RRS-82, we analyzed its discriminatory power using a completely blinded data set of 27 adenocarcinomas. Results with the validation data set indicated that RRS-82 could distinguish between patients with high and low risks of recurrence and death. Relapse-free survival was significantly different between the two groups ($P = .0003$; Fig 3A), and the proportions of relapse-free patients in the high- and low-risk groups were 38% and 78%, respectively, after 2 years. In the high-risk group, the overall survival rate after surgical resection was also significantly lower than that in the low-risk group ($P = .026$; Fig 3B). It was of note that all stage I patients, who were predicted as high-risk based on RRS-82, experienced relapse within 5 years, and died during the follow-up period (Figure 3C for relapse-free survival; $P = .0008$; Fig 3D for overall survival; $P = .043$; both by log-rank test). Interestingly, Kaplan-Meier curves for both relapse-free and overall survival showed tendencies to have modest associations with pathologic disease stage ($P = .15$ for relapse-free survival and $P = .18$ for overall survival) among patients in the low-risk group but not in patients with high-risk RRS-82 (online-only Appendix Fig. A3). The presence of a high risk signature of RRS-82 was not associated with site of relapse (online-only Appendix Table A3).

Further Validation of RRS-82 With an Additional Independent Cohort of pStage I Patients

Further validation of the predictive power of RRS-82 in early-stage patients was conducted using another completely independent cohort of 30 stage I adenocarcinomas in patients who underwent surgery during a different period of time (data set II). RRS-82 was again shown capable of predicting which stage I patients were at extreme high risk (Figs 4A and 4B). In the combined validation cohort

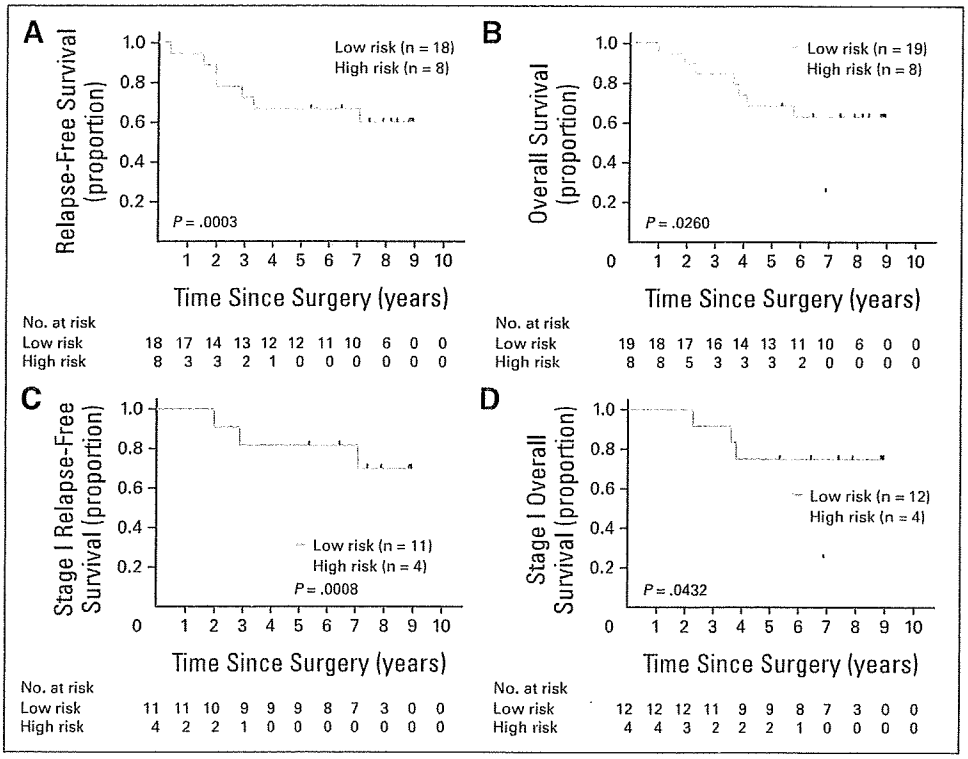

Fig 3. Validation of the RRS-82 signature with the use of completely blinded data set of 27 patients. Relapse-free survival curves for (A) all stages, (C) stage I. Overall survival curves for (B) all stages and (D) stage I.

consisting of 46 stage I cases (16 and 30 from datasets I and II, respectively), Kaplan-Meier survival curves based on RRS-82–based predictions were markedly different, showing relapse-free survival in 74% and 10% of patients with low- and high-risk signatures, respectively ($P < .0001$; Fig 4C). Overall survival was also significantly worse in the high-risk group as compared with the low-risk group ($P = .002$; Fig 4D). Data for patients in all stages are shown in online-only Appendix Figure A4. Multivariate Cox regression analysis of the combined validation data sets, in which the results of RRS-82–based predictions were considered as one of the variables, revealed that RRS-82 was highly predictive and independent of disease stage for both relapse-free survival ($P < .001$) and overall survival ($P = .005$; Table 1).

Confirmation of Predictive Capability of RRS-82 Using Two Additional Data Sets With a Different Platform

The robustness of RRS-82 for predicting survival of patients with lung adenocarcinomas was further validated using a completely independent Duke University data set of 39 lung adenocarcinomas. We conducted an unsupervised hierarchical clustering based on the expression profiles of the 46 genes, which corresponded to those constituting RRS-82 (Appendix Table A4). Thirty-nine adenocarcinomas were clearly clustered into two distinct subsets (Fig 5A), with significantly different postoperative survival results shown ($P = .028$; Fig 5B). The vast majority of genes corresponding to those related to relapse in RRS-82 showed a higher expression in patients in cluster 2, who had a poor prognosis, supporting the general applicability of RRS-82 for lung adenocarcinomas.

We further confirmed the predictive capability of the gene set constituting RRS-82 with a different approach by utilizing recently reported large training-testing, multisite data sets (Fig 5C). Using the University of Michigan data set consisting of 75 alive and 102 dead patients, we calculated each weighted value for 31 genes, which corresponded to the gene set constituting RRS-82, as the signal-to-noise ratio and then applied it to the 104 Memorial Sloan-Kettering samples, all of which had valuable information regarding relapse. The resultant RRS-82-based classifier built on the University of Michigan data set was able to predict patients at high risk in the Memorial Sloan-Kettering validation data set (Fig 5D). Taken together, these results demonstrated the predictive power of the gene set constituting RRS-82 for identifying patients at high risk for disease recurrence. Since the 31 genes in the set were selected based only on the presence of corresponding genes between the two distinct platforms, our findings suggest that potential future development of an optimally downsized classifier with sufficient predictive power based on RRS-82 is possible.

DISCUSSION

In this study, we identified a molecular signature, termed RRS-82, which was significantly associated with relapse and death in patients with adenocarcinomas of the lung. Based on the RRS-82 signature, we were able to construct a prognosis prediction classifier, which may ultimately aid in patient-tailored selection for therapeutic strategies. The robustness of the RRS-82 signature was successfully validated through application in four attempts with two independent Nagoya data sets as well as with the Duke and Director's Challenge Consortium data sets. Notably, the RRS-82–based classifier clearly distinguished patients with very poor prognosis from those with favorable outcome, including the duration of relapse-free survival, even in stage

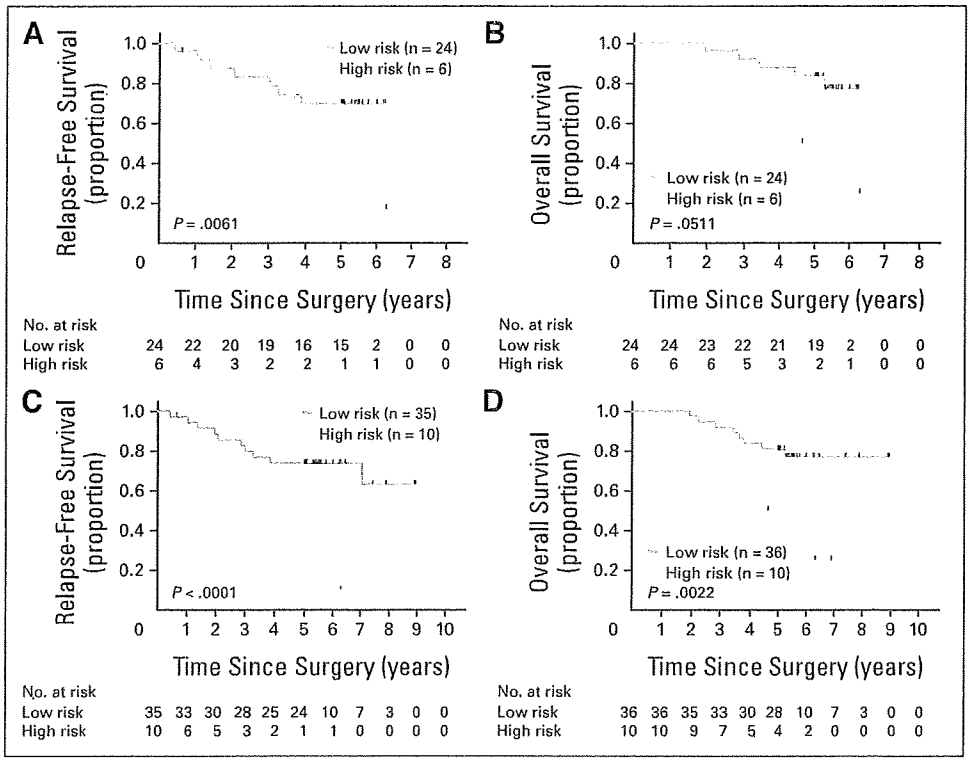

Fig 4. Independent validation of the RRS-82 signature using an additional independent cohort of 30 patients with stage I disease. (A) Relapse-free survival curves and (B) overall survival curves. Kaplan-Meier survival curves were used to estimate (C) relapse-free survival and (D) overall survival in the 46 stage I patients from data sets I and II.

I cases. These findings suggest that patients with the high-risk RRS-82 signature, who are overlooked using current diagnostic procedures for staging because of the inability of detection, are likely to have minimal residual disease. We previously reported that a 25-peak proteomic signature could also identify patients with very unfavorable outcome after surgery with curative intent at the protein level,[14] similarly to the present RRS-82 signature. Taken together, these findings support the notion that patients with very poor prognosis are certainly predictable even in stage I cases and that inclusion of molecular signature-based prognosis predictions, which take molecular and biologic characteristics manifested as signatures into consideration, may improve our capabilities for evaluating each patient with the ultimate aim of better therapeutic options.

Several studies have presented evidence supporting a model in which the propensity to metastasize reflects the predominant genetic/epigenetic state of a primary tumor, rather than the emergence of rare cells with a metastatic phenotype.[16-18] In this regard, it is interesting that disease stage at surgery appeared to have a modest tendency to affect patient outcome only in patients with a low-risk RRS-82 signature and not in those with a high-risk signature. A similar tendency was consistently observed in our previous proteomic analysis using matrix-assisted laser desorption/ionisation time of flight mass spectrometry, in which a 25-peak-based prediction model was constructed.[14] These findings therefore suggest a potential difference in biologic aggressiveness between the groups with high- and low-risk RRS-82 signatures.

Table 1. Univariate and Multivariate Cox Regression Analysis for the Combined Test Cohort (n = 57)

Variable	Unfavorable/Favorable	Univariate			Multivariate		
		Hazard Ratio	95% CI	P	Hazard Ratio	95% CI	P
Relapse-free survival (n = 56)*							
Age	> 61/≤ 61	0.68	0.32 to 1.47	.331	0.91	0.41 to 2.02	.817
Sex	Male/female	1.46	0.68 to 3.10	.329	1.19	0.54 to 2.60	.668
Stage	II-III/I	2.41	1.05 to 5.54	.038	2.00	0.84 to 4.72	.115
RRS-82	High risk/low risk	5.48	2.50 to 12.0	< .001	4.92	2.17 to 11.2	< .001
Overall survival (n = 57)							
Age	> 61/≤ 61	1.00	0.44 to 2.32	.991	1.21	0.50 to 2.91	.668
Sex	Male/female	1.61	0.70 to 3.74	.265	1.33	0.55 to 3.19	.526
Stage	II-III/I	2.56	1.04 to 6.32	.041	2.15	0.84 to 5.47	.106
RRS-82	High risk/low risk	3.68	1.58 to 8.56	.003	3.60	1.48 to 8.77	.005

*Information of relapse was not available in a single case.

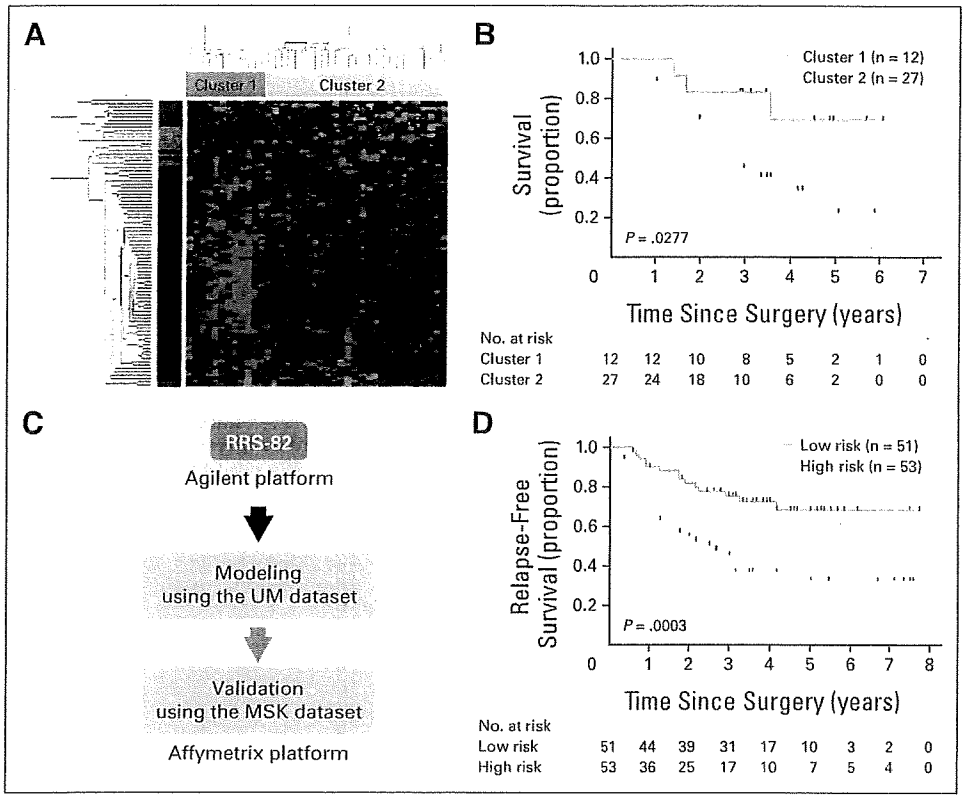

Fig 5. Results of (A) unsupervised hierarchical clustering analysis and (B) Kaplan-Meier survival curves for clusters I and II of the Duke data set. Schematic diagram showing further verification of RRS-82 constituents using another completely (C) independent training-validation data sets of 177 University of Michigan (UM) patients and 104 Memorial Sloan Kettering (MSK) patients, and (D) relapse-free survival curves for MSK patients.

The highly predictive nature of our RRS-82 signature, especially in terms of risk of relapse, may have been accomplished by our strategy used in the identification process, which paid special attention to relapse-free duration in a training cohort with high quality follow-up data. In fact, relapse within 5 years after surgery was observed in 80% and 90% of the patients with a high-risk RRS-82 signature in the training and combined validation cohorts with stage I disease, respectively. Although relapse-free survival data were not available for the 50 gene–based prediction classifier presented by the Michigan group[8] or the "A method" model by the Director's Challenge Consortium,[12] fatal outcome within 5 years after surgery was observed in approximately 55% and 60%, respectively, of those patients. In addition, the high-risk Duke metagene signature composed of nine metagene groups corresponding to 133 probes[11] was reported to correctly predicted relapse in 69% and 79% in their American College of Surgeons Oncology Group (stages I and II) and Cancer and Leukemia Group B (stages I to III) validation cohorts, respectively. Interestingly, the constituents of the RRS-82 signature do not have a significant overlap with other predictive signatures thus far reported by us and others.[8,9,11,12,19-23] Such variability among studies is commonly observed in molecular signatures for class prediction, and we suspect that it may reflect the use of different platforms for expression profiling and/or existence of distinct genes with similar predictive information, because of the presence of similarly coregulated genes that do not necessarily have similar biologic and/or biochemical properties.[24] For example, *PSMD12*, *FIP1L1*, and *UBE2V2*, included in RRS-82, are a part of the cluster six-gene set reported by the Director's Challenge Consortium, while *SMARCE1* in RRS-82 is included in the cluster 10-gene set. Additional analyses using the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) and Gene Ontology (http://www.geneontology.org/) databases identified only a few common pathways and networks containing predictive gene sets in such studies (examples shown in the Data Supplement). However, those results may not be surprising, since all of these studies including our own were not aimed at identifying functionally relevant gene sets or pathways associated with differences in clinical behavior such as relapse after surgery.

A number of negative results have been reported in regard to the benefits of adjuvant chemotherapy in patients with early-stage lung cancers,[25-28] although we believe that those do not preclude the potential clinical importance of molecular signature–based classification. Instead, such classification will likely add additional important information for patient-tailored evaluation of the nature of those diseases, considering that the current staging procedures, which rely on the measurement of disease spread by imaging techniques with insufficient power for detecting minute residual tumors, may be causing stage-migration of actual advanced cases into false early stages.

In conclusion, we succeeded in identifying a relapse-related molecular signature for use with patients diagnosed with adenocarcinomas, which was able to select those at extremely high risk for relapse, even in early-stage patients. In the field of breast cancer, a molecular signature–based prediction of surgically treated patients has been approved by the US Food and Drug Administration, and development of a similar useful means is urgent for lung cancer, which claims the highest number of lives each year. A future confirmatory study and clinical trial for patient-tailored adjuvant therapy with stratification according to the RRS-82 molecular signature are therefore warranted

to evaluate whether such selection may ultimately improve patient prognosis after surgery for this deadly cancer.

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EGFR and HER2 Genomic Gain in Recurrent Non-small Cell Lung Cancer After Surgery

Impact on Outcome to Treatment with Gefitinib and Association with EGFR and KRAS Mutations in a Japanese Cohort

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Background: Sensitivity to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) and frequency of activation mutations in EGFR is lower in Caucasian than Asian non small-cell lung cancer (NSCLC) patients. Increased EGFR gene copy numbers evaluated by fluorescence in situ hybridization (FISH) has been reported as predictor of clinical benefit from EGFR-TKIs in Caucasian NSCLC patients. This study was carried out to verify whether EGFR FISH had similar performance in Japanese patients.

Methods: A cohort of 44 Japanese patients with recurrent NSCLC after surgery was treated with gefitinib 250 mg daily. The cohort included 48% females and 52% never-smokers; 73% had prior chemotherapy and 57% had stage III-IV at the time of surgery. Adenocarcinoma was the most common histology (86%). FISH was performed using the EGFR/Chromosome Enumeration Probe 7 and PathVysion DNA probes (Abbott Molecular). Specimens were classified as FISH positive when showing gene amplification or high polysomy (≥4 copies of the gene in ≥40% of tumor cells). Tumor response to gefitinib was assessed by RECIST for 33 patients with measurable diseases.

Results: Twenty-nine tumors (66%) were EGFR FISH+ and 23 (53%) were HER2 FISH+. Overall response rate was 52%, representing 65% of EGFR FISH+ patients and 29% of EGFR FISH− patients ($p = 0.0777$). Survival was not impacted by the EGFR FISH ($p = 0.9395$) or the HER2 FISH ($p = 0.0671$) status. EGFR FISH+ was significantly associated with HER2 FISH+ ($p = 0.015$) and presence of EGFR mutation ($p = 0.0060$). EGFR mutation significantly correlated with response ($p < 0.0001$) and survival after gefitinib ($p = 0.0204$). EGFR and HER2 FISH status were not associated with KRAS mutation.

Conclusion: Frequency of EGFR FISH+ status was higher and its predictive power for TKI sensitivity was lower in this Japanese cohort than in Western NSCLC cohorts. These findings support differences in the mechanisms of EGFR pathway activation in NSCLC between Asian and Caucasian populations. Confirmation of these results in larger cohorts is warranted.

Key Words: FISH, EGFR, HER2, KRAS, Biomarkers, NSCLC, Tyrosine inhibitors.

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Tumor dependence on specific molecular pathways may identify the best target for therapy exploration. Activation of the epidermal growth factor receptor (EGFR)-related signaling pathways drives numerous cancer-promoting processes, such as cell proliferation, apoptosis inhibition, angiogenesis, cell adhesion, and motility and invasion, and also controls development of drug resistance.[1] Therefore, anti-EGFR approaches (antibodies directed against the extracellular domain and small inhibitors of the tyrosine kinase activity) have been one of the most successful examples of molecular target therapy in human solid neoplasias, mainly in

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non small-cell lung cancer (NSCLC), head and neck, pancreatic and colorectal carcinomas.[2]

Targeted therapies are expected to be effective when the targeted molecule is a major player in the tumor cellular processes, which usually does not occur in all patients with any specific solid tumor. Strategies for patient selection for targeted therapy are almost universally considered to be necessary but are not fully implemented, even for anti-EGFR therapies. In NSCLC, causally associated with EGFR activation are mutations in the adenosine triphosphate-binding site of the tyrosine kinase domain that sustain abnormal response to the ligand,[3,4] activate multiple signaling transduction pathways[5,6] and selectively activate AKT and signal transducers and activators of transcription signaling.[6,7] Increased gene copy numbers is also a well known mechanism for activation of EGFR-related pathways in gliomas,[8] breast,[9] colon,[10] head and neck cancers,[11]and NSCLC.[12]

In NSCLC, at least three molecular markers have been consistently associated with sensitivity or resistance to EGFR-TKIs (tyrosine kinase inhibitors): mutations and amplification/overrepresentation of the EGFR gene[3–5,12–14] and mutation in the KRAS genes.[15–18] The impact on survival has been extensively investigated for activating EGFR mutations,[19] and less for the EGFR gene copy numbers[12,14,20,21] or for the KRAS mutations[16,22] and results among studies have not been totally concordant. Distinct technologies have been used to identify mutations and genomic gain and part of the discrepancies among results from different studies may due to technical factors. However, other factors such as smoking status, gender, and ethnicity have been demonstrated to impact sensitivity to EGFR-TKIs. Patients of Eastern Asian origin have significantly better clinical outcome to EGFR-TKIs than western populations[23,24] but reasons for these differences are not completely understood. The most important factor so far accounting for this finding is that the Asian NSCLC patients including Japan, have high incidence of activating EGFR mutations.[4,25]

This study aimed to verify the role of EGFR genomic gain as a marker for sensitivity to gefinitib in a Japanese cohort using fluorescence in situ hybridization (FISH), a technology proved to be successful for prediction of outcome to EGFR TKIs in some Caucasian NSCLC populations. In addition, the study aimed to compare EGFR genomic gain with two other gefitinib-related markers, activating mutations in EGFR and resistant mutations in KRAS, which were previously investigated in this cohort.[13]

PATIENTS AND METHODS

Description of Patient Population and Definition of Effectiveness of Gefitinib Treatment

From a population of NSCLC patients who underwent surgery between 1999 and 2003 in the Aichi Cancer Center Hospital in Nagoya, Japan, 75 had recurrent disease and were treated with 250 mg/daily of gefitinib for recurrent disease. From those, response to treatment could not be evaluated in 6 cases, tumor material was not available in 24 cases, and FISH analyses failed in 4 cases. Thus, the current study reports on 44 patients, all of whom provided consent for the study.

Tumor materials obtained at initial tumor resection for these 44 NSCLC cases had been previously investigated for EGFR and KRAS mutations.[13,16] Tumor response to gefitinib treatment was evaluated for 33 patients eliminating 11 patients who did not have measurable diseases. Tumor response was judged according to the RECIST, without requirement of confirmation of tumor response no less than 4 weeks apart. The length of gefitinib therapy was used as a surrogate for disease free survival and overall survival was calculated form the start of gefitinib administration to death from any cause or the most recent date on which the patient was known to be alive.

EGFR and HER2 Fluorescence In Situ Hybridization Assays

Formalin-fixed, paraffin-embedded tumor blocks were sectioned at 4 μm and submitted to dual-color FISH assays using the Locus Specific Indicator EGFR SpectrumOrange/CEP 7 SpectrumGreen and the PathVysion DNA Probe Kit (HER2 SpectrumOrange/CEP 17 SpectrumGreen Vysis/Abbott Molecular) following protocol previously described.[12] Briefly, slides were deparaffinized in CitriSolv (Fisher Scientific) and washed in 100% ethanol for 5 minutes. The slides were then sequentially incubated in 2× SSC (saline sodium citrate) at 75°C for 13 to 18 minutes, digested in 0.25 mg/ml Proteinase K/2× SSC at 45°C for 14 to 18 minutes, washed in 2× SSC for 5 minutes and dehydrated in ethanol series. Probes were applied according to the manufacturer instructions to the selected hybridization areas, which were covered and sealed. DNA denaturation was performed in dry oven for 15 minutes at 80°C and hybridization was allowed to occur for 20 hours at 37°C in a humidified chamber. Posthybridization washes were performed consecutively in 2× SSC/0.3% NP-40 at 72°C and 2× SSC for 2 minutes each. Following dehydration in ethanol, chromatin was counterstained with 4' = 6-diamidino-2-phenylindole (DAPI) (0.3 μg/ml in antifade Vectashield mounting medium, Vector Laboratories). Analysis was performed on epifluorescence microscopes using single interference filters sets for green, red (Texas red) and blue (DAPI) as well as dual (red/green) and triple (blue, red, green) band pass filters. For documentation, images were acquired using charged-coupled device camera with Z-stacking and merged using dedicated software (CytoVision, Applied Imaging).

At least 50 tumor nuclei were analyzed in tumor areas selected using the correspondent HE stained slide as a guide. Scoring system followed previous publications.[12] According to the frequency of tumor cells with specific number of copies of the gene and the CEP targets, the tumors were initially classified into six FISH categories (disomy, low and high trisomy, low and polysomy, and gene amplification) and finally grouped into two strata: (a) FISH negative including disomy to low polysomy tumors, which basically have ≥4 copies of the gene in <40% of cells; and (b) FISH positive including tumors with high polysomy (≥4 copies in ≥40% of cells) and gene amplification (defined by a ratio gene/chromosome per cell ≥2, presence of small or nonenumerable clusters of the gene signal or ≥15 copies of the gene signal in ≥10% of the analyzed cells).

TABLE 1. Population Characteristics

Variable	Categories	Statistics
Age (years)	Median	60.9 × 10.3
	Range	38–79
Gender	Male	23 (52.3%)
	Female	21 (47.7%)
Smoking	Never	23 (52.3%)
	Ever	21 (47.7%)
Histology	Adenocarcinoma	38 (86.4%)
	Nonadenocarcinoma (SqC, LC)[a]	6 (13.6%)
Differentiation	Poor	10 (26.3%)
	Moderate	22 (57.9%)
	Well	6 (15.8%)
	Not determined	6
Stage	Early (I–II)	19 (43.2%)
	Advanced (III–IV)	25 (56.8%)
Prior chemotherapy	Yes	12 (27.3%)
	No	32 (72.7%)
Survival after surgery (days)	Median	2081
	Range	250–2655
Tumor response (RECIST)	Yes	17 (52%)
	No	16 (48%)
Disease free interval (days)	Median	375
	Range	99–1818
Survival after gefitinib (days)	Median	562
	Range	69–724
Death	Dead	15 (34.1%)
	Alive	29 (65.9%)

[a] SqC, Squamous cell carcinoma; LC, Large cell carcinoma.

Statistical Analysis

For comparisons of proportions, the Pearson's χ^2 test or the Fisher's exact test was used. Nonparametric Wilcoxon rank sum test or Kruskal-Wallis test was used to compare the difference in continuous variables. The Kaplan-Meier method was used to estimate the probability of survival as a function of time, and survival differences between groups were analyzed by the log-rank test. The two-sided significance level was set at $p < 0.05$. All analyses were performed using SAS version 9.1 (SAS Institute Inc, Cary, NC) software.

RESULTS

Clinical and demographical characteristics are summarized in Table 1. The patients were evenly split between males and females, never or ever smokers and with early or advanced stage disease. Adenocarcinoma histology and poorly or moderately differentiated histologic grade were prevalent. Most patients had not received prior chemotherapy. Median disease free interval after surgery was 375 days, median survival after gefitinib treatment was 562 days, and 66% of patients were alive at the time of last follow up.

EGFR FISH and mutation status in relation to demographics are summarized in Table 2. While EGFR mutation was associated with female gender, never-smoking status, and adenocarcinoma histology, none of these was related with EGFR-FISH status.

Distribution of patients through the FISH categories is illustrated in Figure 1*A* for the EGFR gene and Figure 1*B* for the HER2 gene. The majority of tumors (29 cases [66%]) were EGFR FISH positive, predominantly due to a large representation of tumors with high polysomy (23 cases, 52%, Figure 2*A*) rather than gene amplification (6 cases, 14%, Figure 2*B*). Also, a high number of tumors (23 cases, 53%) were positive for HER2 FISH, of which 21 cases (48%) were represented by high polysomy and only 2 cases (5%) by gene amplification (illustrated in Figure 2*C*). EGFR and HER2 patterns were significantly associated ($p = 0.015$): 19 cases (43%) of tumors were positive and 11cases (25%) were negative for both genes, while 14 cases (32%) had discordant patterns; EGFR FISH positives were more likely to be HER2 FISH positives (19/29 = 66%) than EGFR FISH negatives (4/15 = 27%).

Overall, the specimens with amplification of the EGFR or HER2 genes exhibited clusters of loosely associated signals (Figures 2*B*, *C*) indicating that the amplification occurred as homogenously staining regions. However, one specimen displayed EGFR gene amplification as numerous, diffuse signals mimicking the extrachromosomal double minutes (Figure 2*D*). Heterogeneity for both EGFR and HER FISH

TABLE 2. EGFR FISH and Mutation Status According to Demographics

		EGFR FISH			EGFR Mutation		
Variable	Categories	Positive	Negative	*p*	Positive	Negative	*p*
Age (years)	Median	61.0	62.0		61.0	61.0	
Gender	Male	15 (65%)	8	$p = 0.9193$	11 (48%)	12	$p = 0.0536$
	Female	14 (67%)	7		16 (76%)	5	
Smoking	Never	15 (67%)	8	$p = 0.9193$	18 (78%)	5	$p = 0.016$
	Ever	14 (65%)	7		9 (43%)	12	
Histology	Adenocarcinoma	25 (66%)	13	$p = 0.9664$	26 (68%)	12	$p = 0.0151$
	Nonadenocarcinoma (SqC, LC)[a]	4 (67%)	2		1 (17%)	5	

FISH, fluorescence in situ hybridization; EGFR, epidermal growth factor receptor.
[a] SqC, Squamous cell carcinoma; LC, Large cell carcinoma.

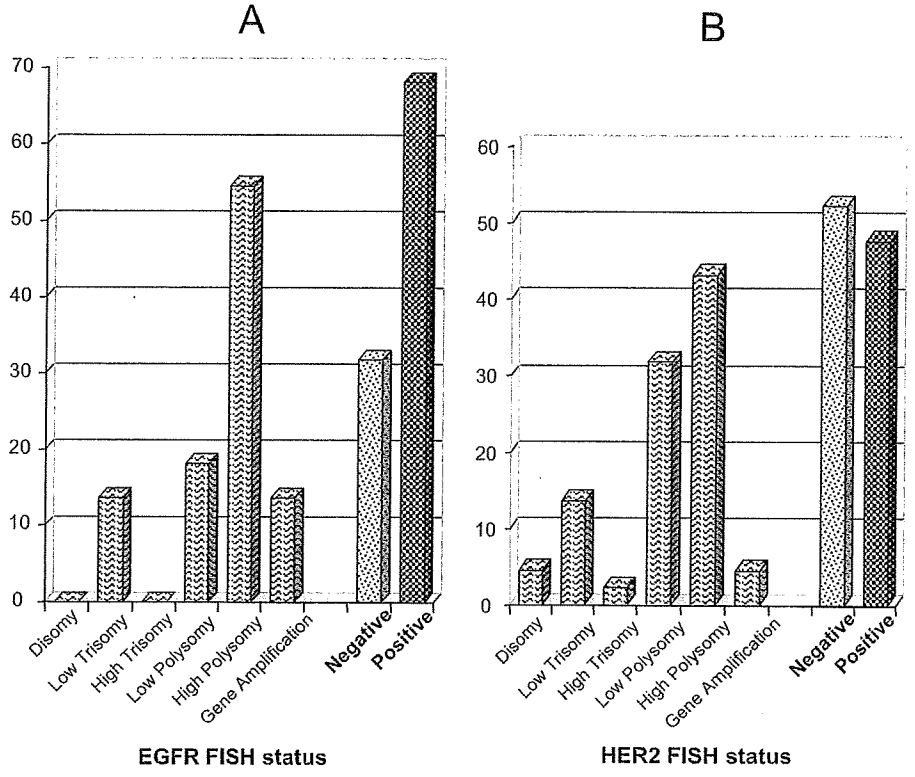

FIGURE 1. Frequencies of tumors with distinct categories for the epidermal growth factor receptor-fluorescence in situ hybridization (EGFR-FISH) (*A*) and the HER2 FISH (*B*) assays. Negative category includes disomy to low polysomy. Positive category includes high polysomy and gene amplification.

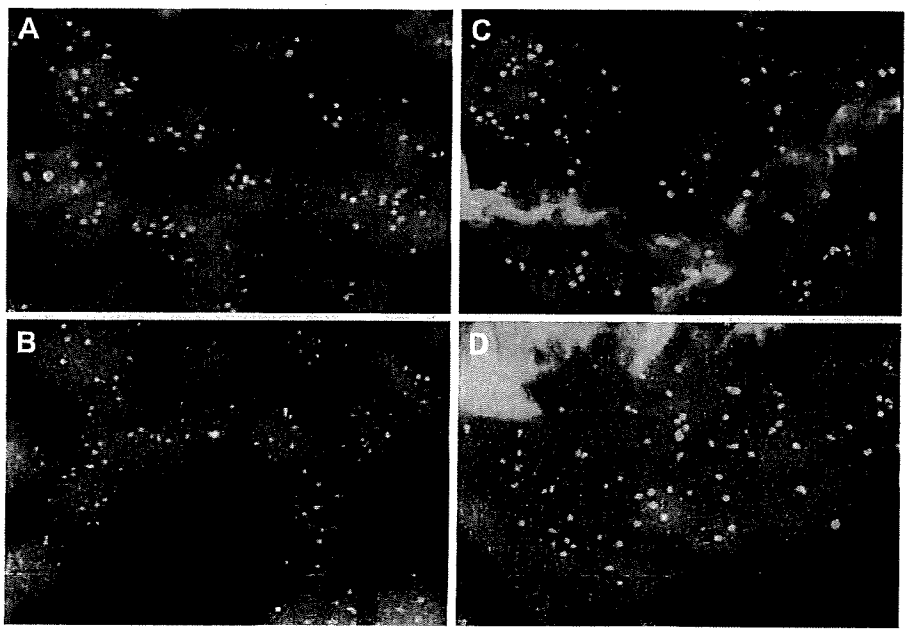

FIGURE 2. Hybridization of the non small-cell lung cancer (NSCLC) sections with the epidermal growth factor receptor EGFR/CEP7 (*A, B, D*) and the PathVysion probe set (*C*) showing EGFR high polysomy (*A*), EGFR clustered gene amplification (*B*), HER2 gene amplification (*C*) and EGFR amplification as double minutes (*D*).

patterns was common, with tumor foci showing nuclei with high copy numbers (including gene amplification) interspaced with nuclei with low copy numbers.

The association between FISH patterns and response to the gefitinib treatment for 33 patients with measurable diseases is shown in Table 3. Response to gefitinib was marginally higher in EGFR FISH positive (65%) than negative (29%) patients ($p = 0.0777$). Patients with EGFR gene amplification had a trend towards better benefit (response in 4 of 4 = 100%) than patients with high polysomy (response in 9 of 16 = 56%). HER2 FISH positive pattern trended no impact, including 47% of responders ($p =$

TABLE 3. Tumor Response in Relation to EGFR FISH, HER2 FISH, EGFR Mutation and KRAS Mutation Status

		Patients		Tumor response			
Molecular marker	**Categories**	***n***	**%**	**PR (%)**	**SD**	**PD**	***p***
EGFR	Positive (+)	20	61	13 (65%)	1	6	$p = 0.0777$
	Negative (−)	13	39	4 (29%)	0	9	
HER2	Positive (+)	17	52	8 (47%)	0	9	$p = 0.4426$
	Negative (−)	16	48	9 (56%)	1	6	
EGFR and HER2	+/+	13	39	8 (62%)	0	5	$p = 0.2451$
	+/−	7	21	5 (71%)	1	1	
	−/+	4	12	0 (0%)	0	4	p^a
	−/−	9	27	4 (44%)	0	5	
EGFR mutation	Positive (+)	20	61	17 (85%)	1	2	$p < 0.0001$
	Negative (−)	13	39	0 (0%)	0	13	
EGFR FISH and EGFR mutation	+/+	16	48	13 (81%)	1	2	$p = 0.0029$
	+/−	4	12	0 (0%)	0	4	
	−/+	4	12	4 (100%)	0	0	p^a
	−/−	9	27	0 (0%)	0	9	
KRAS mutation	Positive (+)	4	13	0 (0%)	0	4	$p = 0.0995$
	Negative (−)	26	87	14 (54%)	1	11	
EGFR FISH and KRAS mutation	+/+	0	0	0 (0%)	0	0	p^a
	+/−	17	57	10 (59%)	1	6	
	−/+	4	13	0 (0%)	0	4	p^a
	−/−	9	30	4 (44%)	0	5	

FISH, fluorescence in situ hybridization; EGFR, epidermal growth factor receptor; PR, partial response; PD, progressive disease.
[a] *p* value could not be calculated because of blank cells.

TABLE 4. Time to Treatment Failure According to EGFRFISH, HER2 FISH, EGFR Mutation and KRAS Mutation Status

		Patients		TTF after Gefitinib (Days)	
Molecular marker	**Categories**	***n***	**%**	**Median**	***p***
EGFR	Positive (+)	29	66	169	0.722
	Negative (−)	15	34	97	
HER2	Positive (+)	23	53	121	0.1815
	Negative (−)	21	47	144	
EGFR and HER2	+/+	19	43	169	0.0179
	+/−	10	23	118	
	−/+	4	9	56	
	−/−	11	25	144	
EGFR mutation	Positive (+)	27	61	311	<0.0001
	Negative (−)	17	39	83	
EGFR FISH and EGFR mutation	+/+	22	50	182	<0.0001
	+/−	7	16	67	
	−/+	5	11	916	
	−/−	10	23	83	
KRAS mutation	Positive (+)	5	12	87	0.0248
	Negative (−)	36	88	146	
EGFR FISH and KRAS mutation	+/+	1	2	113	0.0767
	+/−	25	61	169	
	−/+	4	10	57	
	−/−	11	25	144	

FISH, fluorescence in situ hybridization; EGFR, epidermal growth factor receptor; TTF, time to treatment failure.

0.4426). Response rate was 62% of patients with EGFR and HER2 FISH positive tumors, in 45% of patients with EGFR or HER2 FISH positive tumors, and in 44% of patients EGFR and HER2 FISH negative tumors. Time to treatment failure (TTF) was not significantly associated with EGFR or HER2 FISH positivity (Table 4). Overall survival was not associated with patterns of EGFR FISH ($p = 0.93$) or HER2 FISH ($p = 0.69$), as shown in Figure 3*A*, *B*. EGFR FISH+ patients with high polysomy (score 5) and true gene amplification (score 6) did not differ regarding survival ($p = 0.6607$; Figure 3*C*).

Among these 44 NSCLC patients, 27 (61%) had activating mutations in the tyrosine kinase domain of the EGFR gene and, among 41 who were tested for KRAS mutations, 5

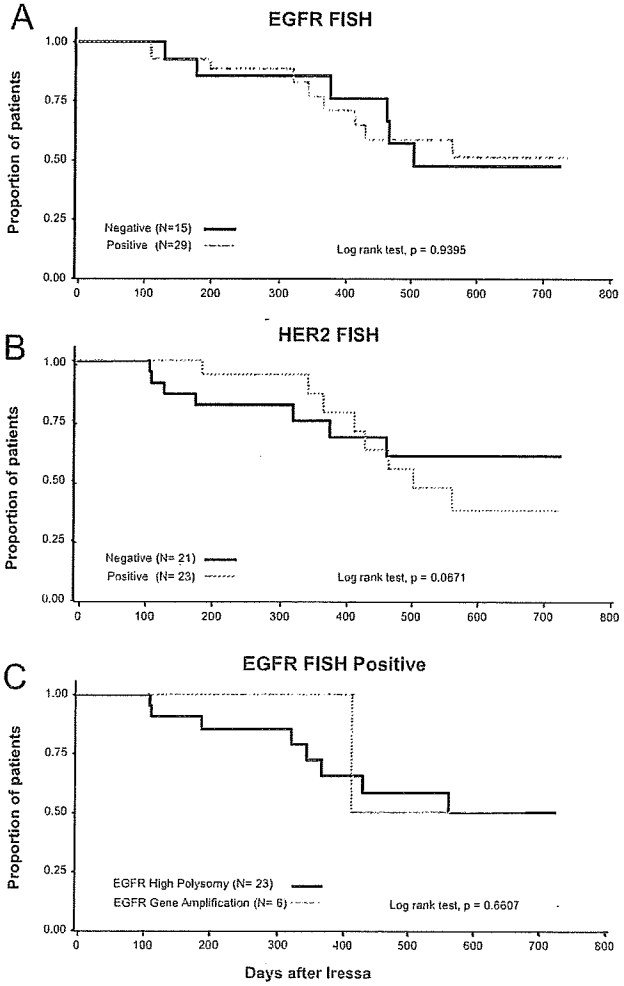

FIGURE 3. Effect on survival from the day of initiating gefitinib treatment in recurrent non small-cell lung cancer (NSCLC) after surgery by epidermal growth factor receptor fluorescence in situ hybridization (EGFR FISH) status (*A*), HER2 FISH status (*B*), and EGFR high polysomy and gene amplification (*C*).

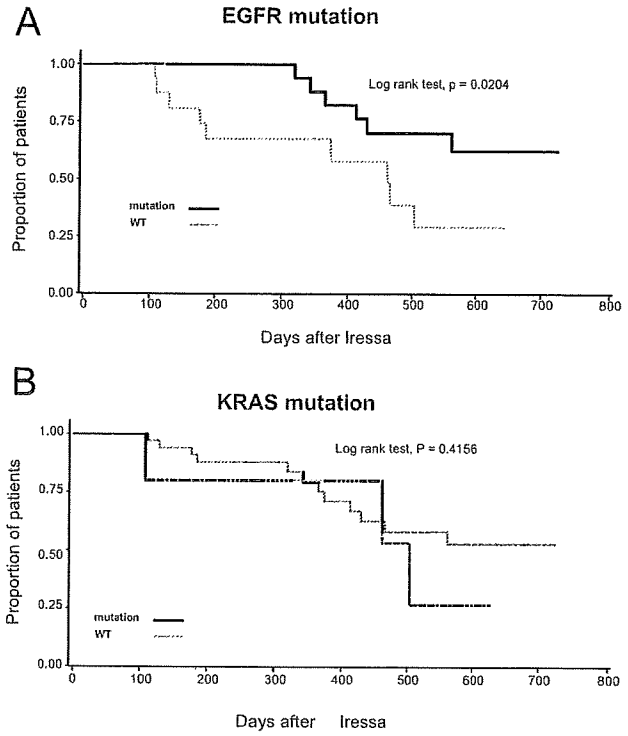

FIGURE 4. Effect on survival from the day of initiating gefitinib treatment in recurrent non small-cell lung cancer (NSCLC) after surgery by epidermal growth factor receptor (EGFR) activating mutation (*A*) and KRAS mutation (*B*) status.

(12%) had point mutations in codons 12 or 13. Table 3 also shows tumor response according to presence or absence of EGFR and KRAS mutations, both individually and in combination with EGFR FISH. EGFR mutation was significantly associated with tumor response ($p < 0.0001$) and prolonged TTF ($p < 0.0001$) or survival ($p = 0.02$; Figure 4*A* and Table 4). EGFR FISH positivity was significantly associated with presence of EGFR mutation ($p = 0.0060$). Patients with EGFR mutation were more likely to be EGFR FISH positive (22/27 = 81%) than patients with wild type EGFR (7/17 = 41%). EGFR mutations were present in all 6 tumors with EGFR gene amplification and in 16 out of 23 tumors with EGFR high polysomy (70%). Response rate was 81% of 16 cases positive for both EGFR FISH and mutation and all 4 EGFR FISH negative/EGFR mutation positive cases responded to gefitinib (Table 3).

Conversely, none of the 4 patients with KRAS mutation (none of whom were EGFR FISH positive) or of the 13 patients with EGFR wild type (4 of whom were EGFR FISH positive) benefited from gefitinib treatment. Presence of KRAS mutation was significantly associated with TTF ($p = 0.0248$) but not with lack of response ($p = 0.0995$) or overall survival ($p = 0.4156$, Figure 4*B*).

DISCUSSION

The EGFR FISH positive status had a borderline association to response of gefitinib treatment, but no impact on

survival in this cohort of Japanese NSCLC patients. These results do not support a predictive role of the established EGFR FISH assay to gefitinib sensitivity in Japanese NSCLC patients. This observation contrasts with previous findings in Caucasian NSCLC populations obtained by our group[12,20,21] and others,[14] that had identified EGFR genomic gain by FISH as a significant predictor of outcome to EGFR-TKIs. In the current study, EGFR mutation was highly predictive of both response and survival to gefitinib. Lack of predictive value of EGFR FISH or EGFR gene copy numbers as assessed by quantitative polymerase chain reaction have also been reported by Korean[17] and Japanese[26] groups. Therefore, there seems to be ethnic differences as to whether EGFR genomic gain is predictive for response or survival after geftinib treatment.

The clinical and demographical characteristics of this Japanese cohort were distinctive, including high proportion of female, never smokers, early stage disease, no prior chemotherapy, and adenocarcinomas. Unselected cohorts of Asian origin usually have higher frequency of females (40%[27]) and never smokers (40%[27]) than Caucasians (34% for females, 9% for never smokers according to Kobrinsky et al.[28]). In addition, this cohort had one of the highest reported frequencies of EGFR FISH+ tumors (68%) and EGFR mutations (61%). Taken only studies that evaluated gene copy numbers by FISH with identical or similar scoring criteria, the frequency of EGFR FISH+ tumors ranged from 44 to 48% in Asian patients[17,26,29] and from 32 to 45% in Caucasian NSCLCs.[14,21] EGFR activating mutations are well known to be more prevalent in Asian (40–50% of adenocarcinomas[27,30]) than Caucasian NSCLCs (10% of adenocarcinomas[25]). Altogether, these findings substantiate the interesting hypothesis that there are ethnicity-associated molecular peculiarities in NSCLC.

The two EGFR gene markers, activating mutation and genomic gain, were significantly correlated in this cohort. Association between EGFR gene amplification and activating mutations has been reported in NSCLC cell lines[31] and clinical specimens of Caucasian[12] and Asian origins.[17,32] Furthermore, the selective amplification of the mutant allele was verified in the cell lines H3255, H827, PC-9, KT-2, KT-4 and Ma-1,[31] as well as in Asian patients.[32] These findings support the hypothesis that there is a selection of cells carrying the amplification of the mutant allele in lung tumorigenesis. Interestingly, high EGFR copy numbers due to chromosomal aneusomy or structural rearrangements (high polysomy) were also associated with mutations in this cohort and in Caucasian NSCLC.[33]

Status of the HER2 gene in NSCLC has been poorly explored and discrepant results have been reported in association with outcome to EGFR-TKIs.[34] In this cohort, HER2 genomic gain showed up as a negative impact factor for survival after gefinitib treatment, in contrast to our previous results in an Italian cohort.[34] Conversely, none of the five KRAS mutant tumors showed treatment efficacy in this study, in agreement with previously findings that KRAS mutations are primary resistance factors to EGFR-TKIs.[18,35]

In summary, the study showed that the EGFR FISH scoring criteria proposed for stratification of NSCLC for therapy with EGFR-TKIs was not effective in Japanese patients as in Caucasian patients. Confirmation of these results in larger cohorts is warranted and investigation of factors that may underlie distinct molecular mechanisms of activation of the EGFR pathway in these populations should be investigated.

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