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Table 1. Clinicopathologic characteristics on surgically resected primary lung ADC

	Test cohort ($n = 40$)	Validation cohort ($n = 63$)	*P*
Age, y			0.20
Mean (range)	59 (47–67)	59 (32–72)	
Gender number (%)			
Male	23 (58)	28 (44)	0.23
Female	17 (42)	35 (56)	
Smoking (pack year) number (%)			
Never smoker	16 (40)	33 (52)	0.09
<20	3 (8)	10 (16)	
≥20	21 (52)	20 (32)	
TP53 genotype number (%)			0.49
Arg/Arg	20 (50)	24 (38)	
Arg/Pro	16 (40)	33 (52)	
Pro/Pro	4 (10)	6 (10)	
Driver oncogene mutation number (%)			
Negative	14 (35)	28 (44)	0.34
Positive	26 (65)	35 (56)	
EGFR mutation	19	28	
KRAS mutation	3	7	
HER2 mutation	3	0	
BRAF mutation	1	0	
Driver oncogene fusion number (%)			
Negative	36 (90)	NE	
Positive	4 (10)	NE	
ALK fusion	2	NE	
RET fusion	0	NE	
ROS1 fusion	2	NE	
TNM stage at initial diagnosis number (%)			0.83
IA	6 (15)	5 (8)	
IB	5 (13)	12 (19)	
IIA	4 (10)	10 (16)	
IIB	2 (5)	2 (3)	
IIIA	18 (45)	26 (41)	
IIIB	2 (5)	0	
IV	3 (8)	8 (13)	
TNM stage at recurrence number (%)			0.32
IA	0	0	
IB	0	0	
IIA	0	0	
IIB	0	0	
IIIA	0	0	
IIIB	2 (5)	3 (5)	
IV	38 (95)	60 (95)	
Recurrent portion			1
Local/regional	2 (5)	3 (5)	
Metastasis	38 (95)	60 (95)	
M1a	22	28	
M1b	16	32	
Platinum-based regimens number (%)			0.44
Platinum + paclitaxel	30 (75)	42 (67)	
Platinum + gemcitabine	9 (23)	14 (22)	

(Continued on the following page)

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Table 1. Clinicopathologic characteristics on surgically resected primary lung ADC (Cont'd)

	Test cohort ($n = 40$)	Validation cohort ($n = 63$)	P
Platinum + docetaxel	0	4 (6)	
Platinum + pemetrexed	0	2 (3)	
Platinum + vinorelbine	1 (2)	1 (2)	
Tumor response number (%)			
Responder	16 (40)	18 (29)	0.31
CR	1	0	
PR	15	18	
Nonresponder	24 (60)	45 (71)	
SD	21	35	
PD	3	10	

NOTE: The P value from the Fisher's exact test.

Abbreviations: NE, not examined; Platinum, cisplatin or carboplatin.

were subjected to a two-step screening procedure to identify miRNAs whose expression is associated with responses to platinum-based doublet chemotherapy (Fig. 1B).

Differential expression of miRNAs between responders and nonresponders

First, we used microarray analysis to identify miRNAs in test cohort samples that were differentially expressed in LADC tissues from responders and nonresponders to platinum-based doublet chemotherapy. Fifty-nine miRNAs were identified ($P < 0.05$; Welch t test; Supplementary Table S2). Of these, 28 were upregulated in responders and 31 were downregulated.

Next, to identify the limited number of miRNAs that can be used to deduce responsiveness in the clinic, we searched for miRNAs showing highly differential expression between responders and nonresponders. Twelve miRNAs (miR135a*, miR196b, miR1181, miR31, miR31*, miR1290, miR598, miR1, miR144*, miR628-5p, miR449a, and miR34b) showing a >5-fold change in expression were identified as potential candidates and investigated further (Fig. 2A; Supplementary Table S2). The expression of these 12 miRNAs was reanalyzed by qRT-PCR using the same RNA samples used in the microarray experiments. A good agreement between the qRT-PCR (ΔC_t value) and microarray ($\log_2$ signal) data (as indicated by the Pearson correlation coefficient) was observed for 10 of the 12 miRNAs (excluding miR1181 and miR598; Supplementary Fig. S1). qRT-PCR identified three miRNAs (miR1290, miR196b, and miR135a*) as differentially expressed in responders and nonresponders ($P < 0.001$, $P < 0.001$, and $P < 0.008$, respectively; Fig. 2B; Supplementary Table S3). The fold changes observed in the qRT-PCR experiment were lower than those observed in the microarray experiment. This may be due to the higher sensitivity of the former. Several samples that gave no significant signal (calculated as zero) in the microarray experiment yielded ΔC_t values in the qRT-PCR experiment (Supplementary Fig. S1), leading to a reduction in the fold change values (Supplementary Table S3).

We next used linear discriminant analysis to examine these three miRNAs for their potential to discriminate responders from nonresponders. For this, continuous expression values for a single miRNA or a combination of two or three miRNAs, that is, ΔC_t values obtained by qRT-PCR, were included as variables in the analysis. ROC curves were plotted to examine both sensitivity and specificity. The results showed that a combination of all three miRNAs provided the best discrimination, with an AUC of 0.893 (Fig. 2C). This combination of three miRNAs is, henceforth, referred to as the "three-miRNA signature."

Ability of the three-miRNA signature to predict responses to chemotherapy

To validate the findings in the test cohort, we next examined the expression of the three-miRNA signature in the validation cohort (Supplementary Fig. S2 and Supplementary Table S3). We then performed a linear discriminant analysis to evaluate the potential of the three-miRNA signature as a biomarker. The mean expression levels of the three miRNAs were higher in responders than in nonresponders in the validation cohort, although the difference in the expression of miR135a* did not reach a statistical significance. However, a combination of all three miRNAs again was better able to distinguish responders from nonresponders (AUC = 0.837) than a single miRNA or a combination of two miRNAs (Supplementary Fig. S3).

PCA and support vector machine (SVM) analysis showed a predictive response of 37.5% for the test cohort containing 40% responders and a predictive response of 31.7% for the validation cohort containing 28.6% responders. The signature predicts responders and nonresponders in the test and validation cohorts with an accuracy of 82.5% and 77.8%, respectively (Fig. 3). The sensitivity, specificity, and positive and negative predictive values were similar for both cohorts (Supplementary Table S4). There was no significant difference in the clinical characteristics between true responders/

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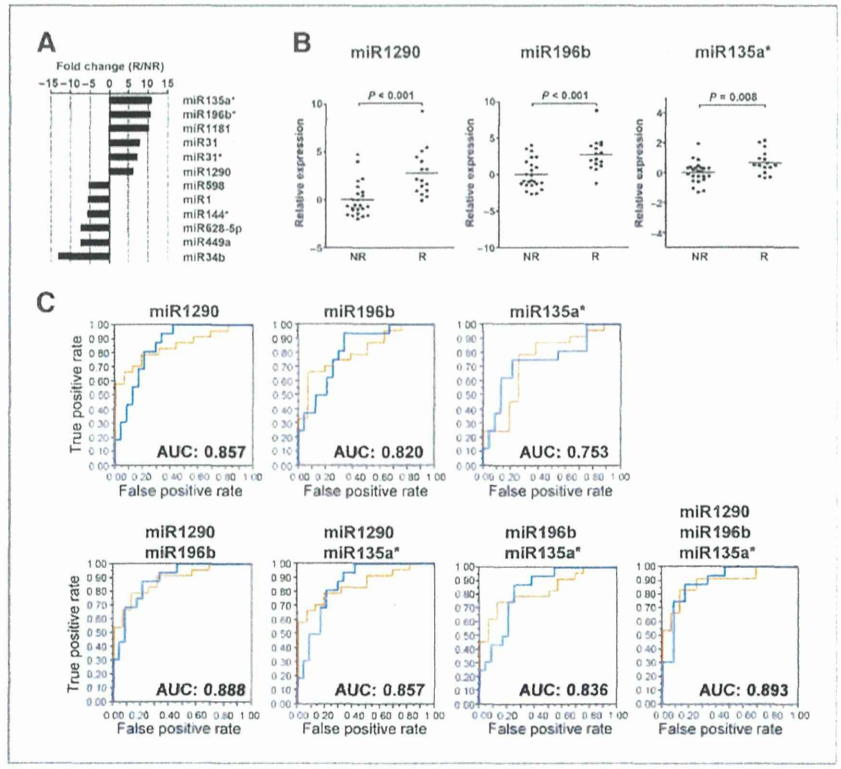

Figure 2. Selection of the three miRNAs whose expression was associated with responses to platinum-based doublet chemotherapy upon LADC recurrence. A, twelve miRNAs differentially expressed in responders (R) and nonresponders (NR) to platinum-based doublet chemotherapy in the test cohort. The diagram depicts miRNAs showing a >5-fold change in expression and with a *P* value of <0.05. The fold change is represented by the ratio of R to NR derived from the microarray data. B, expression of miRNAs in NR and R in the test cohort, as measured by qRT-PCR. Dot plots, miRNA relative threshold cycle values. Expression was normalized to that of RNU66. Threshold cycle values relative to the mean value in NR are shown on a $\log_2$ scale. Horizontal bars, the mean expression value; *P* values (Mann–Whitney test) are indicated. C, ROC analysis was performed for miR1290, miR196b, and miR135a' in the test cohort. The AUC value is shown. The blue line, the results for responders and the orange line represents the results for NR.

nonresponders and predicted responders/nonresponders (Supplementary Table S1). Taken together, these results show that the predictive ability of the three-miRNA signature was confirmed in the independent validation cohort, and that the signature is still predictive even if archive FFPE tissues are used for analysis. Specificity and negative predictive values greater than sensitivity and positive predictive values suggest that the three-miRNA signature predicts nonresponders better than responders (Supplementary Table S4).

Combining the three-miRNA signature with the TP53-Arg72Pro polymorphism genotype

We previously showed in the same study population (i.e., the 640 cases shown in Fig. 1A) that the Arg72Pro polymorphism in the *TP53* gene in noncancerous (germline) DNA is associated with responses to platinum-based doublet chemotherapy: The response rate is higher in those harboring the TP53-72Pro polymorphism (35). Therefore, we combined the three-miRNA signature with the *TP53*-Arg72Pro genotype data to ascertain whether the predictive power of the miRNA signature was enhanced. We dichotomized the study cohorts into two subgroups (patients with the TP53-72Pro allele and those without) and examined the predictive accuracy of the three-miRNA signature. We found that the predictive accuracy marginally improved in both the test (85.0%) and validation (82.5%) cohorts (Fig. 1B).

The three-miRNA signature predicts responses to chemotherapy irrespective of driver oncogene aberrations and clinical characteristics

Driver oncogene aberrations in LADC are a critical factor that determines the therapeutic strategy for each patient. In addition, such aberrations are associated with clinical characteristics, as represented by the predominance of *EGFR* mutation in females and never-smokers. Therefore, we next addressed whether the ability of the three-miRNA signature to predict responses to chemotherapy was affected by driver oncogene alterations or clinical characteristics. The three-miRNA signature failed to predict responses in 21 of 103 cases (21%). The test and validation cohorts contained seven (one with an *EGFR* mutation, one with a *HER2* mutation, and five aberration-negative cases) and 14 (six with *EGFR* mutations, one with a *KRAS* mutation, and seven aberration-negative cases) nonpredicted cases, respectively (Fig. 4). The nonprediction rate was 15% (7/47 cases) for patients harboring the *EGFR* mutation, 10% (1/10) for those harboring the *KRAS* mutation, 33% (1/3) for those harboring the *HER2* mutation, and 32% (12/38) for aberration-negative cases; therefore, there was no significant correlation between driver gene status and nonprediction. Similarly, there was no significant association between clinical factors and nonprediction; thus, the three-miRNA signature may be a useful biomarker for predicting the responses of patients with LADC to chemotherapy,

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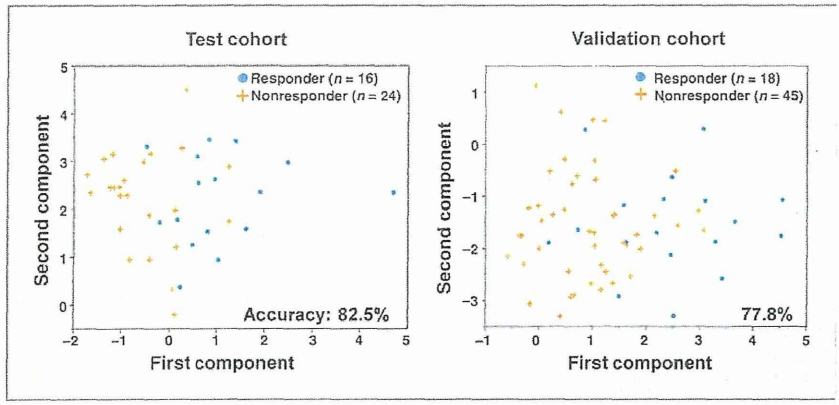

Figure 3. PCA. A PCA–SVM strategy using three miRNAs (miR1290, miR196b, and miR135a*) was used to construct a classifier, which could distinguish responders from nonresponders. The blue dots represent the responders and the orange crosses represent the nonresponders in the test (left) and validation cohorts (right). The classifier had a predictive accuracy of 82.5% for the test cohort and an accuracy of 77.8% for the validation cohort.

irrespective of driver oncogene aberrations and clinical characteristics.

Discussion

Here, we performed miRNA expression profiling of patients who initially underwent surgical resection for primary LADC and were then treated with platinum-based doublet chemotherapy upon recurrence. We identified a three-miRNA signature (miR1290, miR196b, and miR135a*) that predicts whether patients with recurring LADC respond to and, therefore, will benefit from platinum-based doublet chemotherapy. Even patients with LADC harboring druggable oncogene aberrations (that are resistant to treatment with TKIs) may be treated with platinum-based doublet chemotherapy; therefore, platinum-based doublet chemotherapy is a major therapeutic strategy for almost all patients with LADC (39–41). Personalized therapy, in which a drug with the greatest chance of eliciting a response (i.e., tumor shrinkage) is chosen specifically for each patient, is the first critical step toward improved prognosis for patients with LADC with advanced and recurrent disease; indeed, clinical trials examining the effect of new drugs on NSCLC have set improved response rates as their primary endpoint (10); thus, response to treatment according to the RECIST criteria rather than survival was the outcome measure selected for the present study. The three-miRNA signature will facilitate personalized therapy for LADC and will include platinum-based doublet therapy as an option.

Here, we examined the three-miRNA signature of primary tumors to predict the responsiveness of recurrent tumors. It is noteworthy that the biologic characteristics of recurrent tumors are not the same as those of primary tumors due to tumor cell heterogeneity in the primary lesions and the accumulation of additional genetic/epigenetic changes during progression. Thus, at present our findings are applicable to the treatment of recurrent tumors for which corresponding primary tumor tissue samples are available. The finding that the three-miRNA signature is predictive in archived primary tumor tissues is an advantage; patients are spared the additional burden of further tissue sampling for genetic analysis. However, it is also worth analyzing recurrent tumors and inoperable advanced tumors to find out whether the three-miRNA signature is applicable to patients for whom archived surgical tissues are not available. The finding that the signature can be identified in archived FFPE tissues is also an advantage and will facilitate translation to the clinic.

We also examined the combination of the three-miRNA signature with the TP53-Arg72Pro polymorphism genotype to see whether this provided greater predictive accuracy, as blood cells used for genotyping polymorphisms are easily obtained from patients. However, the improvement was only marginal. Therefore, more polymorphisms associated with responses to platinum-based doublet therapy must be identified if we are to achieve any marked improvement over the three-miRNA signature alone. In addition, we used a fold change >5 as a criterion for identifying candidate miRNAs that are differentially expressed between responders and nonresponders. Using less stringent or other statistical criteria may lead to the identification of more miRNAs that are useful for prediction.

The three-miRNA signature predicted responses irrespective of the presence of driver oncogene aberrations. This is important when we consider that LADCs that have acquired resistance to specific TKIs are treated with platinum-based doublet chemotherapy. However, unfortunately, the present study cohort did not include samples from patients with EGFR- or ALK-positive LADC that received TKIs before platinum-based doublet chemotherapy. Such cases should be examined to address this issue.

This study has several limitations. First, it was retrospective in nature, so the ability of the three-miRNA signature to predict responses needs to be further validated using more samples. Here, different types of tumor tissue for the test and validation sets (bulk frozen and microdissected FFPE tissues, respectively), which contained patients that had undergone several different chemotherapeutic regimens, were subjected to analysis; therefore, we may have over- or underestimated predictive value. Thus, further studies that use a larger number of samples obtained according to a defined experimental procedure and take factors such as previous treatment regimen, disease stage, and PS into account are required. In addition, prospective studies, for

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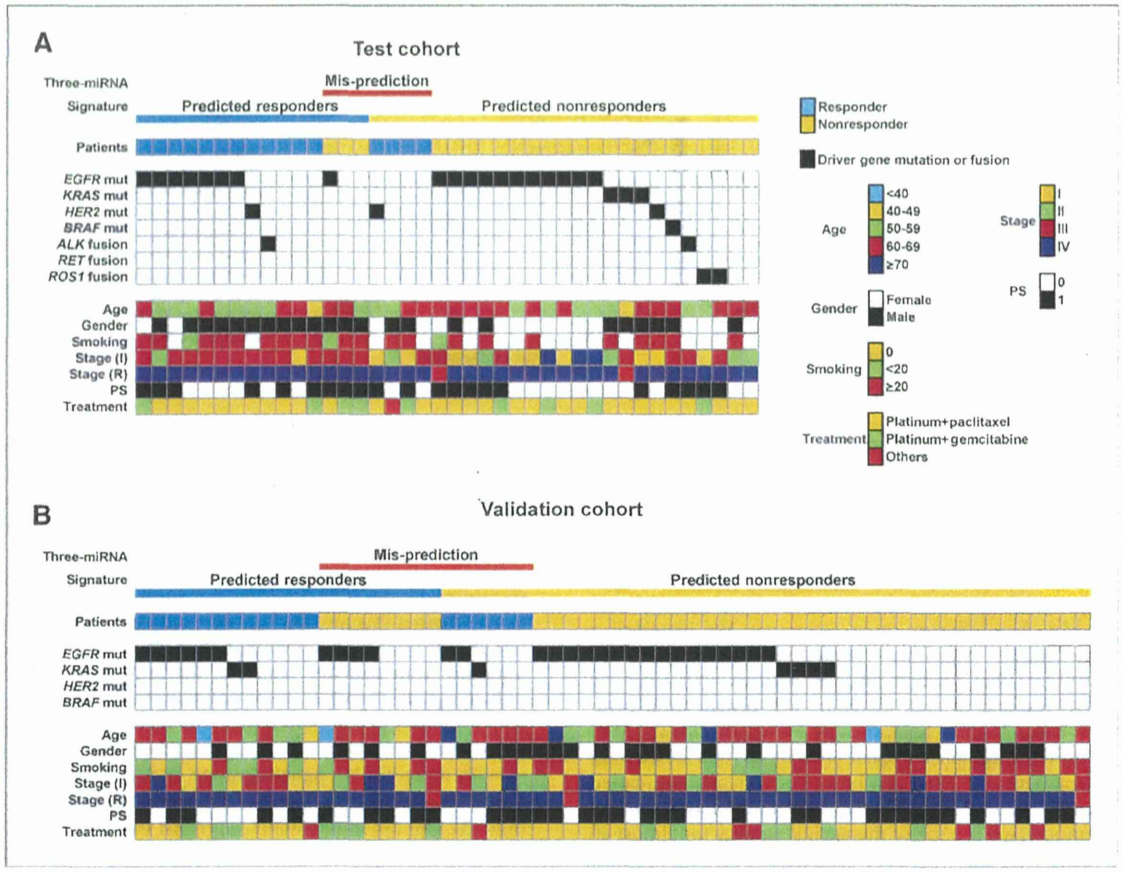

Figure 4. Response prediction by the three-miRNA signature according to clinicopathologic factors in the test (A) and validation cohorts (B). Driver gene mutations and clinical features are shown: patients (blue, responder; orange, nonresponder); driver gene (black, *EGFR*, *KRAS*, *HER2*, *BRAF* mutation- or *ALK*, *RET*, or *ROS1* fusion-positive; white, negative); age (blue, <40; orange, 40–49; green, 50–59; red, 60–69; and navy blue, ≥70); gender (white, female; black, male); smoking (orange, pack years = 0; green, <20; and red, ≥20); tumor stage at initial diagnosis (I) and at recurrence (R; orange, I; green, II; red, III; and navy blue, VI); PS (white, 0; black, 1); treatment (orange, platinum + paclitaxel; green, platinum + gemcitabine; and red, other).

example, studies using samples from patients treated with a single therapeutic regimen, and the analysis of primary and recurrent tumors and inoperable advanced tumors, should be conducted to confirm the utility of the three-microRNA signature. Second, although the three-miRNA signature was significantly associated with response to chemotherapy, differences in progression-free survival were only suggestive (Supplementary Fig. S4). We chose the response as the primary endpoint of efficacy to identify subgroups for which chemotherapy does work (35). However, treatment is continued after the failure of platinum-based doublet chemotherapy; therefore, clinical response alone would not be enough to improve the outcome. Third, the functional relevance of miR1290, miR196b, and miR135a* to the chemosensitivity of LADC remains unclear. Interestingly, a recent study shows that the expression of miR196b is upregulated in patients with rectal adenocarcinoma that respond to neoadjuvant chemoradiotherapy (capecitabine or 5-fluorouracil), which supports the findings of the present study (42). However, preliminary experiments examining the exogenous expression of the three miRNAs in LADC cell lines did not show increased sensitivity to a platinum agent, cisplatin (CDDP). Therefore, the direct or indirect effects of miRNAs on chemosensitivity should be further investigated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Study supervision: J. Yokota, T. Kohno

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