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# MicroRNA Signature Predicts Survival and Relapse in Lung Cancer

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## SUMMARY

**We investigated whether microRNA expression profiles can predict clinical outcome of NSCLC patients. Using real-time RT-PCR, we obtained microRNA expressions in 112 NSCLC patients, which were divided into the training and testing sets. Using Cox regression and risk-score analysis, we identified a five-microRNA signature for the prediction of treatment outcome of NSCLC in the training set. This microRNA signature was validated by the testing set and an independent cohort. Patients with high-risk scores in their microRNA signatures had poor overall and disease-free survivals compared to the low-risk-score patients. This microRNA signature is an independent predictor of the cancer relapse and survival of NSCLC patients.**

## INTRODUCTION

Lung cancer, predominantly non-small-cell lung cancer (NSCLC), is the most common cause of cancer deaths worldwide (Jemal et al., 2006). The relapse rate among patients with early-stage NSCLC is 40% within 5 years after potentially curative treatment (Miller, 2005). The current staging system for NSCLC is inadequate for predicting the outcome of treatment.

## SIGNIFICANCE

MicroRNAs are a class of small non-protein-coding RNAs that function in endogenous negative gene regulation and tumorigenesis. Lung cancer is the most common cause of cancer deaths worldwide. Current clinical-pathological staging methods are inadequate to predict treatment outcome for lung cancer. We identified a five-microRNA signature that can predict survival in lung cancer patients. This may have clinical implications in the molecular pathogenesis of cancer, development of targeted therapy, or selection of high-risk cancer patients for adjuvant chemotherapy.

Results of molecular research may improve the management of patients. Advances in genomics and proteomics have generated many candidate markers with potential clinical value (Ludwig and Weinstein, 2005). Gene expression profiling by microarray or real-time RT-PCR can be useful in the classification or prognosis of various types of cancer, including lung cancer (Chen et al., 2007; Endoh et al., 2004; Potti et al., 2006). With the advent of miRNA expression profiles, significant efforts have been made to correlate miRNA expressions with tumor prognosis (Calin and Croce, 2006a, 2006b; Cummins and Velculescu, 2006; Esquela-Kerscher and Slack, 2006; Garzon et al., 2006; Gregory and Shiekhattar, 2005). Since one microRNA can regulate hundreds of downstream genes, the information gained from miRNA profiling may be complementary to that from the expression profiling of protein-coding genes. Recent reports even suggest that the expression profiling of microRNAs may be a more accurate method of classifying cancer subtype than using the expression profiles of protein-coding genes (Calin and Croce, 2006b; Volinia et al., 2006).

MicroRNAs are a class of small non-protein-coding RNAs that can act as endogenous RNA interference (Hammond, 2006). MicroRNAs can posttranscriptionally regulate the expression of hundreds of their target genes, thereby controlling a wide range of biological functions such as cellular proliferation, differentiation, and apoptosis (Calin and Croce, 2006b). Recent evidence indicates that microRNAs may function as tumor suppressors or oncogenes, and alteration in microRNA expression may play a critical role in tumorigenesis and cancer progression (Calin and Croce, 2006a; Esquela-Kerscher and Slack, 2006).

Our understanding of microRNA expression patterns as potential biomarkers for diagnosis, prognosis, personalized therapy, and disease management is just starting to emerge. Several microRNAs were reported to be associated with the clinical outcome of chronic lymphocytic leukemia (Calin et al., 2005), lung adenocarcinoma (Takamizawa et al., 2004; Yanaihara et al., 2006), and breast (Iorio et al., 2005) and pancreas (Bloomston et al., 2007; Roldo et al., 2006) cancers. However, whether a microRNA signature can predict clinical outcome of NSCLC, including major histological or stage subgroups of NSCLC, is not known.

To investigate this problem, we conducted an extensive microRNA profiling study on a cohort of 112 NSCLC patients from a hospital in central Taiwan. By the splitting-sample approach, a five-microRNA signature was obtained from 56 patients for survival prediction and was validated on the other 56 patients. An independent cohort of 62 patients from a different hospital, located in Northern Taiwan, was used to reconfirm the effectiveness of this signature.

## RESULTS

### Detection of the Five-MicroRNA Signature from the Training Set

The 112 specimens were randomly assigned to a training set (n = 56) or a testing set (n = 56). Only the training data set is used for detection of the five-microRNA signature. We first applied the Cox proportional hazard regression to each of the 157 microRNAs for finding profiles that were correlated with the true overall survival times (which can only be inferred statistically because of heavy censoring). We identified five microRNAs that are significantly associated with patient survival. We then used these five significant microRNAs to construct a signature by the risk score method. We found that two microRNAs (hsa-miR-221 and hsa-let-7a) were protective, and the other three microRNAs (hsa-miR-137, hsa-miR-372, and hsa-miR-182*) were risky (see Table S1 available online). A risk-score formula (see Experimental Procedures) was obtained for patient survival prediction.

### Five-MicroRNA Signature and Patient Survival in the Training Set

We used the risk-score formula and calculated the five-microRNA signature risk scores for all patients in the training set. We ranked patients in the training set according to their risk scores and divided them into a high-risk group or low-risk group using the median risk score as the cutoff point. Table 1 gives the clinical characteristics of the 56 patients in the training set. Patients with high-risk five-microRNA signature had shorter median overall survival than patients with low-risk microRNA signature (20 months versus not reached, $p < 0.001$) (Figure 1A, left panel). Patients with high-risk five-microRNA signature had shorter median relapse-free survival than patients with low-risk microRNA signature (10 months versus not reached, $p = 0.002$) (Figure 1A, right panel).

### Validation of the Five-MicroRNA Signature for Survival Prediction by the Testing Set

We used the same risk score formula obtained from the training set and calculated the five-microRNA signature risk score for each of the 56 patients in the testing set. We then classify them into the high-risk group or low-risk group using the same cutoff point as in the training set. Table 1 gives the clinical characteristics of the 56 patients in the testing set. We carried out the same survival comparison procedures as in the training set. Similar to the findings from the training set, patients with high-risk microRNA signature had shorter median overall survival than patients with low-risk microRNA signature (25 months versus not reached, $p = 0.008$) (Figure 1B, left panel). Likewise, patients with high-risk microRNA signature had shorter median relapse-free survival than patients with low-risk microRNA signature (14 months versus not reached, $p = 0.003$) (Figure 1B, right panel).

We also showed the distribution of tumor microRNA expression, patient risk scores, and the survival status of 112 patients (combination of the training and testing sets) (Figure 2). Tumors with high risk scores tend to express risky microRNAs, whereas tumors with low risk scores tend to express protective microRNAs. Patients with high risk scores had more deaths than low-risk-score patients. Similar results were found in both the training set (Figure S1) and the testing set (Figure S2).

The entire microRNA data set is available in the Supplemental Data.

### Revalidation of the Five-MicroRNA Signature for Survival Prediction by an Independent Cohort

To reconfirm our microRNA signature in an independent cohort, we used 62 NSCLC patients from a different hospital, located in northern Taiwan. Table 1 gives the clinical characteristics of the 62 patients in the independent cohort. Patients were classified as high-risk or low-risk groups based on their microRNA

**Table 1. Clinical Characteristics of NSCLC Patients According to High- or Low-Risk MicroRNA Signature in the Training Set, the Testing Set, and an Independent Cohort**

| Characteristic | Patients with High-Risk MicroRNA Signature (%) | Patients with Low-Risk MicroRNA Signature (%) | p Value |
|---|---|---|---|
| Training data set (n = 56) | n = 28 | n = 28 | |
| Age (mean ± SD) | 65.7 ± 10.3 | 67.3 ± 9.7 | 0.549[a] |
| Gender | | | |
| Male | 21 (75) | 24 (86) | 0.503[b] |
| Female | 7 (25) | 4 (14) | |
| Stage | | | |
| I | 5 (18) | 16 (57) | 0.008[b] |
| II | 8 (28) | 6 (22) | |
| III | 15 (54) | 6 (21) | |
| Cell type | | | |
| Adenocarcinoma | 15 (54) | 10 (36) | 0.353[b] |
| Squamous cell carcinoma | 10 (36) | 15 (54) | |
| Others | 3 (10) | 3 (10) | |
| Testing data set (n = 56) | n = 26 | n = 30 | |
| Age (mean ± SD) | 66.5 ± 13.6 | 64.3 ± 14.7 | 0.57[a] |
| Gender | | | |
| Male | 22 (85) | 21 (70) | 0.224[b] |
| Female | 4 (15) | 9 (30) | |
| Stage | | | |
| I | 11 (42) | 15 (50) | 0.340[b] |
| II | 5 (19) | 9 (30) | |
| III | 10 (39) | 6 (20) | |
| Cell type | | | |
| Adenocarcinoma | 17 (65) | 13 (43) | 0.179[b] |
| Squamous cell carcinoma | 9 (35) | 16 (53) | |
| Others | 0 (0) | 1 (4) | |
| Independent cohort (n = 62) | n = 40 | n = 22 | |
| Age (mean ± SD) | 62.9 ± 10.3 | 64.1 ± 9.1 | 0.634[a] |
| Gender | | | |
| Male | 32 (80) | 10 (45) | 0.010[b] |
| Female | 8 (20) | 12 (55) | |
| Stage | | | |
| I | 15 (37) | 13 (59) | 0.152[b] |
| II | 6 (15) | 4 (18) | |
| III | 19 (48) | 5 (23) | |
| Cell type | | | |
| Adenocarcinoma | 21 (52) | 9 (41) | 0.563[b] |
| Squamous cell carcinoma | 13 (33) | 8 (36) | |
| Others | 6 (15) | 5 (23) | |

[a] Student's t test.
[b] Fisher's exact test.

signature risk scores. We found that patients with high-risk microRNA signature had shorter median overall survival than patients with low-risk microRNA signature (40 months versus not reached, p = 0.007) (Figure 1C, left panel). Likewise, patients with high-risk microRNA signature had shorter median relapse-free survival than patients with low-risk microRNA signature (20 months versus 48 months, p = 0.037) (Figure 1C, right panel).

## Multivariate Regression Analysis Shows that the MicroRNA Signature Is Independent from Stage or Histology

The multivariate Cox proportional hazard regression analysis and stepwise variable selection were used to evaluate independent prognostic factors associated with patient survivals in this independent cohort of 62 NSCLC patients. The microRNA signature,

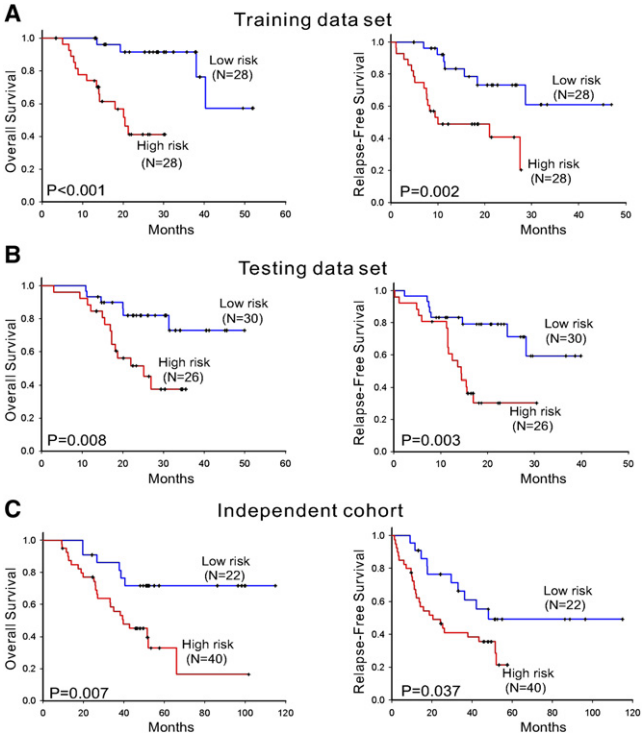


**Figure 1. Kaplan-Meier Estimates of Overall Survival and Relapse-Free Survival of NSCLC Patients According to the MicroRNA Signature**

(A) Fifty-six patients in the training data set.
(B) Fifty-six patients in the testing data set.
(C) Sixty-two patients in the independent cohort.

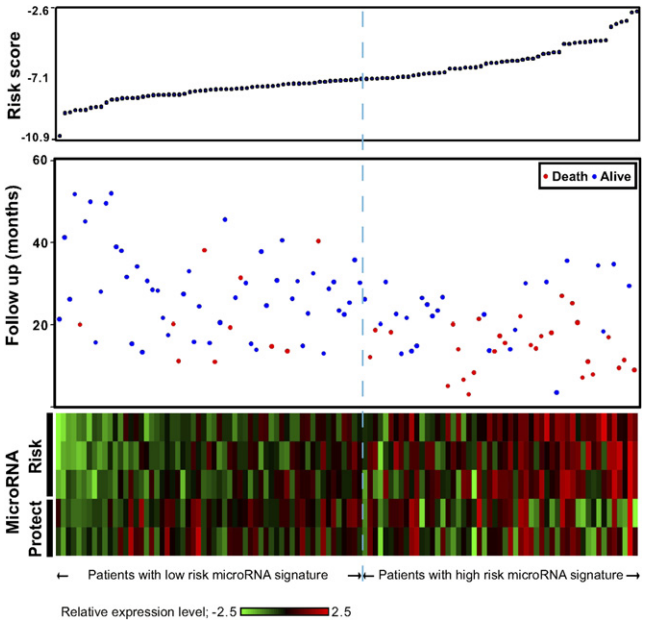


**Figure 2. MicroRNA Risk-Score Analysis of 112 NSCLC Patients**

(Upper panel) MicroRNA risk-score distribution. (Middle panel) Patients' survival status. (Bottom panel) Color-gram of microRNA expression profiles of NSCLC patients; rows represent high-risk and protective microRNAs, and columns represent patients. The blue dotted line represents the median microRNA signature cutoff dividing patients into low-risk and high-risk groups.

age, sex, stage, and histology were used as covariates. Multivariate regression analysis showed that the microRNA signature (HR = 2.81, p = 0.026) and stage (HR = 2.35, p = 0.022) are independent prognostic factors associated with overall survival or disease-free survival of NSCLC patients and that the prognostic ability of the microRNA signature is independent from stage or histology (Table 2). Similar results were also found in the training and the testing sets (see Table S2).

## MicroRNA Signature Can Predict Patient Survivals within Cancer Stages and Histological Subgroups of NSCLC Patients

In order to investigate whether this microRNA signature can distinguish high-risk versus low-risk groups of patients within each stage stratum (NSCLC stage I, II, or III) and to predict their survivals, we used the combined samples of the testing set and the independent cohort for this analysis. From the survival curves shown in Figure 3, we found that the high-risk survival curve lies below the low-risk curve in all three stages.

We conducted a log-rank test for each stage. Unlike the situation in the beginning, the two risk groups are introduced and compared (Figure 1); the one-sided log-rank test makes better sense here. The high-risk group defined before stratification is expected to have a shorter survival time even after stratification, and the low-risk group is expected to have a longer survival time after stratification. We conducted the one-sided log-rank test because the purpose of the test is not only to tell if the two survival curves for the high-risk group and the low-risk group are separable, but also to tell if the separation is in the correct direction—namely, low-risk-signature patients have longer survival and high-risk-signature patients have shorter survival (Figures 3A–3C).

We found that the microRNA signature is significantly associated with relapse-free survival of patients with stage I disease (p = 0.033). For the overall survival, the p value is 0.057, slightly over the 5% level (Figure 3A). For stage II disease, the results are marginal (p = 0.148, p = 0.095). This may be because the sample size is too small (only 24 patients) to draw any firm conclusions (Figure 3B). For stage III disease, the microRNA signature is again significantly associated with the overall survival and relapse-free survival of NSCLC patients (p = 0.0095, p = 0.044) (Figure 3C).

**Table 2. Multivariate Cox Regression* Analysis of the MicroRNA Signature and Survivals in an Independent Cohort**

| Variable | Hazard Ratio | 95% CI | p Value |
|---|---|---|---|
| Overall survival | | | |
| MicroRNA expression signature | 2.81 | 1.13–7.01 | 0.026 |
| Stage | 2.35 | 1.13–4.89 | 0.022 |
| Relapse-free survival | | | |
| MicroRNA expression signature | 2.39 | 1.12–5.10 | 0.024 |
| Stage | 2.76 | 1.43–5.34 | 0.003 |
| Age | 0.93 | 0.90–0.97 | <0.001 |

*Variables were selected through the stepwise selection method.

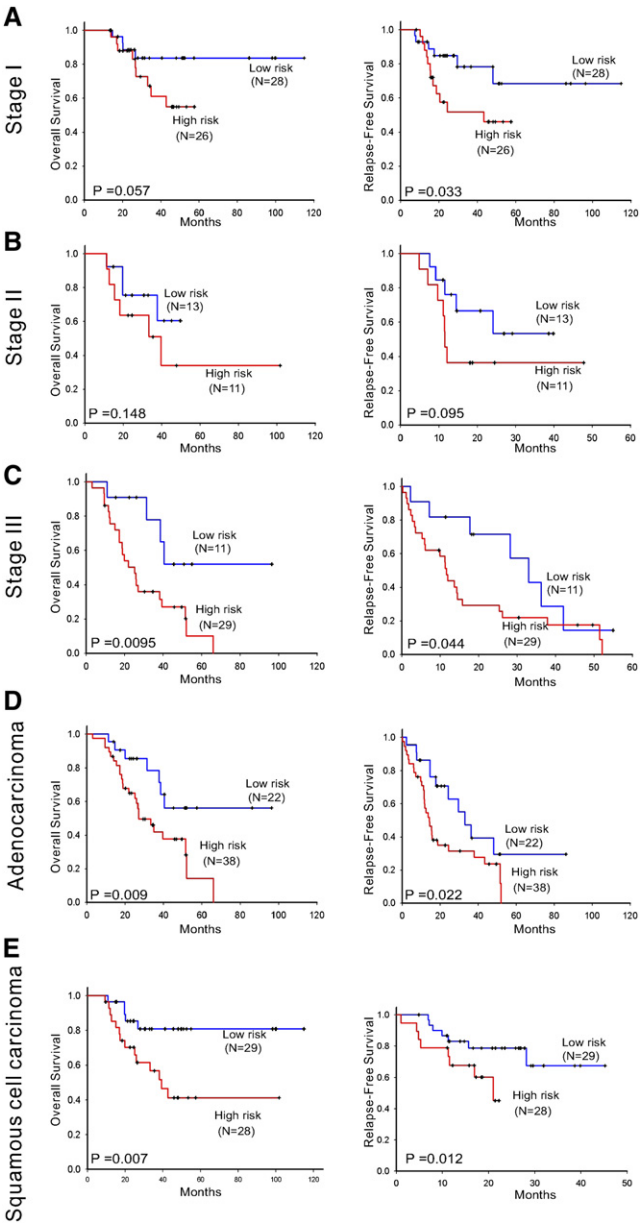


**Figure 3. Kaplan-Meier Estimates of Overall Survival and Relapse-Free Survival According to the MicroRNA Signature in Subgroups of NSCLC Patients in the Combination of the Testing and Independent Data Sets**

(A) Stage I disease (n = 54).
(B) Stage II disease (n = 24).
(C) Stage III disease (n = 40).
(D) Adenocarcinoma patients (n = 60).
(E) Squamous cell carcinoma patients (n = 57).

To reach the overall conclusion that the survival prediction of microRNA signature is independent from stage, we conducted the overall chi-square test that combines the three log-rank tests together. The p value is 0.028 for the overall survival and 0.046 for the relapse-free survival (Table S3).

Next, we stratified the NSCLC patients by the histological subtype of adenocarcinoma or squamous cell carcinoma. The microRNA signature can predict patient survival within each lung cancer histology subtype (Figures 3D and 3E). The overall chi-square test that combines the two log-rank tests together gives p values of 0.003 and 0.010 for overall survival and relapse-free survival, respectively (Table S3).

## All Five MicroRNAs Are Required in the Signature

To confirm that a set of five microRNAs is essential for this microRNA signature, we constructed five competing four-microRNA signatures by deleting one microRNA in turn from the set. We then repeated the survival analysis for each of these ''five-minus-one'' microRNA signatures and compared the results with the original five-microRNA signature using the log-rank analysis. The results showed that, unlike the five-microRNA signature, none of the ''five-minus-one'' microRNA signatures was consistently correlated with overall survival and relapse-free survival in the training data set, the testing data set, or the independent cohort of NSCLC patients (Table S4).

## Expression of MicroRNAs Alters Invasiveness of Lung Cancer Cells

Two components of this five-microRNA signature, hsa-let-7a and hsa-miR-372, can play opposite roles in tumorigenesis through regulation of cell proliferation (Johnson et al., 2005; Lee and Dutta, 2007; Mayr et al., 2007; Takamizawa et al., 2004; Voorhoeve et al., 2006). In addition to cell growth, cell invasiveness is another key determinant of malignancy. However, the association of microRNAs with cancer cell invasive ability is unknown. To investigate the role of the five microRNAs in regulating the invasiveness of cancer cells, we transfected each precursor of high-risk microRNAs (hsa-miR-137, hsa-miR-182*, and hsa-miR-372) into a low-invasive lung cancer cell line (CL1-0) and each precursor of protective microRNAs (hsa-miR-221 and hsa-let-7a) into a highly invasive lung cancer cell line (CL1-5). The invasiveness of the transfectants was measured. We found that the invasion ability of cancer cells to penetrate Matrigel membrane was significantly increased by the three high-risk microRNAs (hsa-miR-137, hsa-miR-182*, and hsa-miR-372) and decreased by one protective microRNA (hsa-miR-221) compared to control. Only hsa-let-7a did not affect the invasiveness of lung cancer cells (Figure 4A).

In order to confirm that the four microRNAs can alter the invasive ability of cancer cells, we constructed microRNA expression vectors that can produce these microRNAs driven by the CMV promoter. The increased expression level of mature hsa-miR-137, hsa-miR-182*, hsa-miR-372, and hsa-miR-221 was quantified by real-time RT-PCR (Figure 4B). We confirmed that ectogenic expression of hsa-miR-137, hsa-miR-182*, and hsa-miR-372 promoted invasiveness of lung cancer cell lines as compared with parent cell control and mock negative control. We also confirmed that hsa-miR-221 inhibited cell invasion activity (Figure 4C).

## DISCUSSION

In this study, we identified a five-microRNA signature (hsa-let-7a, hsa-miR-221, hsa-miR-137, hsa-miR-372, and hsa-miR-182*) that is associated with survival and cancer relapse in NSCLC patients. We confirmed these findings in a testing set and an independent cohort of NSCLC patients. Patients with

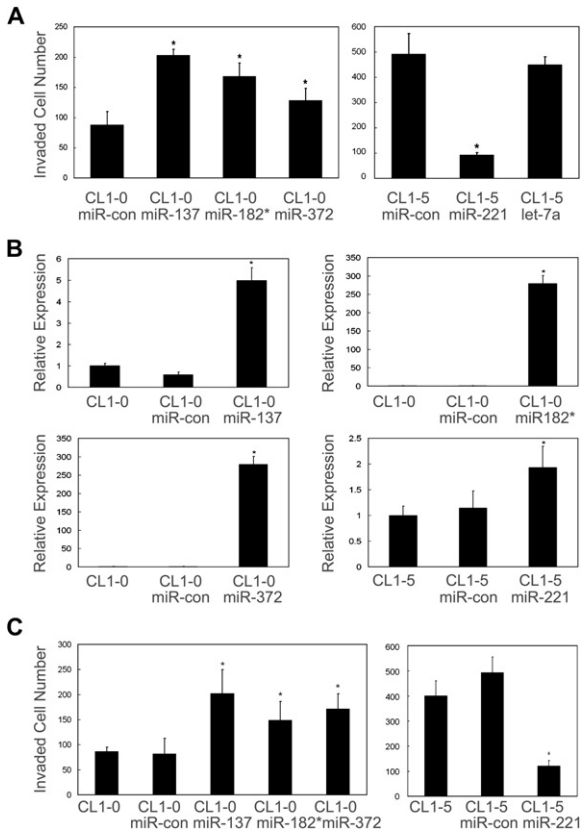


**Figure 4. MicroRNA Expression Alters the Invasiveness of Lung Cancer Cells In Vitro**

(A) The effect of microRNAs on cancer cell invasiveness. The low invasive lung cancer cell line (CL1-0) was transfected with high-risk microRNA precursors (hsa-miR-137, hsa-miR-182*, or hsa-miR-372), and the high invasive cell line (CL1-5) was transfected with protective microRNA precursors (hsa-miR-221 and hsa-let-7a). Twenty-four hours after transfection, cells were seeded onto Transwell culture inserts and cultured for 18 hr. The invasive ability of transfected cells was measured by modified Boyden chamber assay. The number of invaded cells in cells transfected with negative control 1 precursor miRNA acts as microRNA control (miR-con), and relative invasiveness of each microRNA was normalized to miR-con. Each type of cell was assayed in quaternary.

(B) Ectogenic microRNA expression quantified by real-time RT-PCR. Quantification of microRNA expression utilized total RNA isolated from cells transiently transfected with microRNA expression vector. The expression of microRNA was normalized to U6 RNA expression, and the microRNA transfectants were normalized to parental cell control (CL1-0 or CL1-5). Each type of cells was assayed in triplicate.

(C) The effect of microRNAs on invasiveness evaluated by using ectogenic microRNA expression vectors. Invasion assay was performed as mentioned in (A). The number of invaded cells in cells transfected with p*Silencer*4.1-CMV puro negative control acts as microRNA control (miR-con). Each type of cells was assayed in quaternary.

All data were processed by two-sided Student's t test and presented as mean ± SD. * $p < 0.05$.

a high-risk score of this five-microRNA signature in their tumor specimens had increased cancer relapse and shortened survival, even after stratifying patients by stage or histology (adenocarcinoma or squamous cell carcinoma) subgroups. These results suggest that microRNAs may play an important role in the molecular pathogenesis, clinical cancer progression, and prognosis of NSCLC.

MicroRNAs are small noncoding RNAs that can function as endogenous negative gene regulators, act as tumor suppressors or oncogenes, and play important roles in cancer progression (Calin and Croce, 2006b). Gene expression profiling using microarray or RT-PCR can be useful in the classification or prediction of prognosis of NSCLC (Endoh et al., 2004; Potti et al., 2006). Since each microRNA may regulate hundreds of downstream genes, the information gained from microRNA profiling may be complementary to the microarray gene expression profiling of protein-coding genes. Lu et al. reported that microRNA profiles are more effective in cancer classification than mRNA profiles containing over 16,000 genes (Lu et al., 2005). Our knowledge about the relationship between microRNA expression and clinical outcome of patients is just emerging.

The presence of hsa-let-7a in this microRNA signature in this NSCLC study is consistent with two recent reports that reduced hsa-let-7a expression is associated with shortened survival of lung adenocarcinoma patients (Takamizawa et al., 2004; Yanaihara et al., 2006). On the other hand, the microRNA (hsa-miR-155) reported to be associated with survival in lung adenocarcinoma patients by Yanaihara et al. was not included in our microRNA signature (Yanaihara et al., 2006). In protein-coding gene expression profiling, the genes associated with patient survival can vary substantially from study to study, with few genes being consistently reported in different lung cancer studies (Beer et al., 2002; Potti et al., 2006). For the microRNA profiling, it remains uncertain whether the results between different studies may be equally inconsistent or not.

The identification of a microRNA signature that can predict survival of patients with squamous cell carcinoma or adenocarcinoma is an important finding. It suggests that microRNAs may have an important role in the enhancement of cancer progression for a broad spectrum of NSCLC, thus indicating a wide clinical applicability.

The current clinical-pathological staging method has limited success in predicting patient survival. For patients with identical clinical-pathological characteristics or the same stage of lung cancer, great uncertainties remain regarding how some patients will be cured while other patients will have cancer recurrence, metastasis, or death after surgical resection. Applying our five-microRNA signature to the combined samples of the testing set and the independent cohort, in all stages, we found a clear separation between the low- and the high-risk curves in the time range that is clinically most relevant. For instance, at stage III, consider the relapse-free survival time at 36 months. Our data show that a survival probability of 36 months is 0.43 for the low-risk microRNA signature group and is 0.22 for the high-risk signature. The results of chi-square testing showed that the five-microRNA signature is independent of stage or histology types (Table S3). When the subgroup stratified analysis was performed to test the independence of the signature, we found a pattern of persistently small p values. Separately, because of the small sample sizes, two of them (overall and relapse-free survivals of patients with stage II) are only marginally significant. However, combining all tests together, the overall p values do reach statistical significance.

We showed that the five-microRNA signature can distinguish high-risk versus low-risk patients within stage subgroups. This finding may potentially enable doctors to identify and select

high-risk patients for effective adjuvant therapy in addition to standard surgery in order to improve the treatment outcome of NSCLC. Cisplatin-based adjuvant chemotherapy was recently reported to be effective for improvement of survival of NSCLC patients after surgical resection (The International Adjuvant Lung Cancer Trial Collaborative Group, 2004). The report that gene expression (ERCC1) can predict NSCLC patients' response to cisplatin-based adjuvant chemotherapy was also recently reported (Olaussen et al., 2006).

Because there are still possibilities of false positives in selection of the five-miRNAs for the prediction of clinical outcome, we further validated our findings using an independent cohort of lung cancer patients. The validity of a gene signature as a predictor of clinical outcome lies in the internal validation (the training set and the testing set), the external validation (in an independent cohort), and validity across different subgroups (cancer stages and cell types) of patients (Simon et al., 2003). This five-microRNA signature may have satisfied these criteria. Our finding that this five-microRNA signature can predict high-risk and low-risk patients within stage subgroups of NSCLC patients is an important finding that may add supportive evidence that microRNAs may play an important prognostic role in NSCLC patients. In fact, the results of predictor power generated from microRNA or mRNA signatures had been reported in the literature (Beer et al., 2002; Bloomston et al., 2007; Calin et al., 2005; Chen et al., 2007; Endoh et al., 2004; Lossos et al., 2004; Potti et al., 2006; Yanaihara et al., 2006). They showed good performance in survival prediction. Our five-microRNA signature not only predicted overall survival and predicted relapse-free survival well across three data sets (the training, testing, and independent sets) but also showed good predictive power in overall survival and relapse-free survival using a real-time RT-PCR assay.

Determination of microRNA signature in patients using real-time RT-PCR and assessing a small number of microRNAs as in this study may be a clinically applicable procedure. This is because it gives accurate and reproducible RNA quantification results from small amount of bronchoscopy sampling or paraffin-embedded specimens (Bast and Hortobagyi, 2004; Ramaswamy, 2004). In contrast, the reproducibility of hybridization-based microarray technologies is still questionable, and frozen fresh tissues are usually needed in the assay (Jiang et al., 2005; Lu et al., 2005; Tang et al., 2006).

The five microRNAs identified in this study that can predict clinical outcome of NSCLC may generate potential molecular targets for the development of anticancer therapy (Czech, 2006). hsa-let-7a is a protective microRNA that suppresses RAS and other transcription factors, and hsa-let-7a expression is associated with prolonged survival in NSCLC patients (Grosshans et al., 2005; Johnson et al., 2005; Takamizawa et al., 2004). In addition, recently evidence demonstrated that hsa-let-7a inhibits cell proliferation and anchorage-independent growth through repression of the HMGA2 oncogene (Lee and Dutta, 2007; Mayr et al., 2007). hsa-miR-221 is another protective microRNA that inhibits erythroleukemic cell growth and angiogenesis via kit receptor downmodulation, suggesting a possible role for miR-221 at least as a modulator of the formation of vessels in tumor microenvironment (Felli et al., 2005; Poliseno et al., 2006). However, a recent report suggested that hsa-miR-221 directly targets tumor suppressor p27Kip1 to cause the acceleration of cell cycle in prostate cancer cells (Galardi et al., 2007). It is well known that one microRNA can regulate many targets. Therefore, it may be possible that the same microRNA may play opposite roles in cancer progression, both as a tumor suppressor in certain cancers and as an oncogene in others.

Regarding the potential role of the three risky microRNAs in this microRNA signature, hsa-miR-372 was reported to act as an oncogene in testicular germ cell tumors by downregulating the tumor suppressor LATS2 (Voorhoeve et al., 2006). To our best knowledge, the molecular mechanism of hsa-miR-137 and hsa-miR-182* in cancer biology has not been reported yet.

Two components of this five-microRNA signature, hsa-let-7a and hsa-miR-372, can play opposite roles in tumorigenesis through regulation of cell proliferation (Johnson et al., 2005; Lee and Dutta, 2007; Mayr et al., 2007; Takamizawa et al., 2004; Voorhoeve et al., 2006). In addition to cell growth, cell invasiveness is another key determinant of malignancy. However, the association of microRNAs with cancer cell invasive ability is unknown. We found that the risky microRNAs (hsa-miR-137, hsa-miR-182*, and hsa-miR-372) promote the invasive ability of lung cancer cells and the protective microRNA (hsa-miR-221) inhibits cancer cell invasiveness. A somewhat unexpected finding is that hsa-let-7a does not lower the invasiveness of the more invasive lung cancer cell line. This suggests that the cancer-protective effect of hsa-let-7a may come from suppression of cell proliferation (Johnson et al., 2005; Mayr et al., 2007; Takamizawa et al., 2004). Further investigation of the regulatory mechanism of these microRNAs and their interactions may increase our understanding of the molecular pathogenesis of NSCLC.

In conclusion, the five-microRNA signature can predict cancer recurrence and survival of NSCLC patients. This may have prognostic or therapeutic implications for the future management of NSCLC patients.

## EXPERIMENTAL PROCEDURES

### Patients and Tissue Specimens

We studied frozen specimens of lung cancer tissue from 112 consecutive patients who underwent surgical resection of NSCLC at the Taichung Veterans General Hospital between September 2000 and December 2003. The patients had not received adjuvant chemotherapy. We validated the microRNA signature using an independent cohort of 62 consecutive patients who underwent surgical resection of NSCLC at the National Taiwan University Hospital between February 1995 and December 2001.This study was approved by the Institutional Review Boards of the Hospitals. Written informed consent was obtained from all patients.

### MicroRNA Profiling

MicroRNA expression profiling was performed using ABI PRISM 7900 Real Time PCR System and TaqMan MicroRNA Assays Human Panel-Early Access Kit containing 157 mature human microRNAs (Applied Biosystems, Foster City, CA). The cDNA was made using TaqMan MicroRNA RT reagent and specific primers for each microRNA. The transcripts were amplified with reagent (TaqMan 2x Universal PCR Master mix). MicroRNA expression was quantified in relation to the expression of small nuclear U6 RNA. The U6 RNA is a common internal control for microRNA quantification assays (Jiang et al., 2005; Yanaihara et al., 2006). Only five microRNAs are measured in the independent cohort because we only need five microRNAs to produce the signature.

### Statistical Analysis

The 112 specimens were randomly assigned to a training data set (n = 56) or a testing data set (n = 56). The expression level of each microRNA was

assessed. Hazard ratios from univariate Cox regression analysis were used to identify which microRNAs were associated with death from recurrence of cancer or any cause. Protective microRNAs were defined as those with hazard ratio for death < 1. High-risk microRNAs were defined as those with hazard ratio for death > 1. In most clinical studies, heavy censor rates higher than 60% are typical. For example, the censoring rate for our study is 66%, and that for the study reported by Beer et al. (2002) is 72%. Censoring refers to the patients who may drop out or still are alive at the end of the study. If we divide the patients into long overall survival and short overall survival according to the survival time being longer than 24 months or not, we will end up with 24 patients in the long survival group and 16 patients in the short survival group in the training data set. The other patients cannot be placed in either group because we don't know their actual survival time. In fact, leaving censored patients out would introduce bias to the remaining uncensored samples, and it is difficult to make adjustment for such bias. The approach we use, Cox proportional hazard regression (Cox, 1972), is a standard method in biostatistics for dealing with survival data.

The permutation test procedure is described in the section "MicroRNA selection from the training set" of the Supplemental Experimental Procedures. We found 5 out of 157 microRNAs that are significantly associated with the patient survival by Cox proportional hazard regression in the training data set. To investigate the effectiveness of these five microRNAs as a microRNA-based gene signature for clinical outcome prediction, a mathematical formula for survival prediction was constructed, taking into account both the strength and the positive or negative association of each microRNA with survival. More specifically, we assigned each patient a risk score according to a linear combination of the expression level of the microRNAs, weighted by the regression coefficients derived from the aforementioned univariate Cox regression analyses (Lossos et al., 2004). From our five-microRNA signature, the risk score for each patient was calculated as follows:

Risk-score = (0.15 × expression level of hsa-miR-137) + (0.31 × expression level of hsa-miR-372) + (0.28 × expression level of hsa-miR-182*) + (−0.13 × expression level of hsa-miR-221) + (−0.14 × expression level of hsa-let-7a).

Patients having higher risk scores are expected to have poor survival outcomes.

We divided patients in the training data set into high-risk and low-risk groups using the median microRNA signature risk score as the cut-off point. The difference in patient characteristics between the high-risk and the low-risk groups was analyzed using Student's t test for continuous variables or using Fisher's exact test for categorical variables. The Kaplan-Meier method was used to estimate overall survival and relapse-free survival. Differences in survival between high-risk and the low-risk patients were analyzed using the two-sided log-rank test.

According to Simon et al. (2003), there are at least two ways of validation: internal validation (splitting sample or crossvalidation) versus external validation. The splitting-sample strategy is to separate patients into a training set and a testing set. First, the classifier genes are selected based on data from the training set and are validated with data from the testing cohort. There is no overlap between the two sets, which is an advantage over crossvalidation. The generalization ability of the statistical procedures from the training set to other data can be more faithfully examined.

Simon et al. suggested that the splitting-sample method appears to be a better choice than the repeated random partition method and crossvalidation if the sample size is not too small.

In internal validation, the training and the testing samples are taken from the same population. Because it is important to examine how well the detected biomarkers will perform in other populations, Simon et al. urged for an external validation in which additional independent samples should be taken. Conforming with their suggestions, the samples used in our external validation come from a different medical center. We validated the performance of our microRNA signature risk-score model using patients in the testing data set and the independent cohort of NSCLC.

We employed two statistical approaches to investigate whether the microRNA signature is an independent predictor of overall survival and disease-free survival in NSCLC patients, especially whether it is independent of stage.

The starting approach is multivariate Cox regression analysis. In the Cox regression, the stepwise selection method is performed to select the optimal combination of variables. The concept of multivariate analysis is that the estimated hazard ratio of our five-microRNA signature is adjusted by the effects of potential confounding variables (e.g., stage). On the other hand, if the confounders are controlled in the multivariate model, the effect of our microRNA signature is an independent prognostic factor. The multivariate Cox proportional hazard regression analysis and stepwise variable selection were used to evaluate the contribution of independent prognostic factors to patient survival. The microRNA signature risk score, age, sex, stage, and histology were used as covariates. All analyses were done with SAS version 9.1 software (SAS Institute Inc). Two-tailed tests and p values < 0.05 for significance were used.

The second approach is the use of stage stratification and histology stratification to avoid the confounding effect of stage and histology. We stratified patients by stage (NSCLC stage I, II, or III) and by histology (adenocarcinoma or squamous cell carcinoma) and then performed Kaplan-Meier estimates of overall survival and relapse-free survival of patients according to their high-risk or low-risk microRNA signature. Survivals were compared using the one-sided log-rank test. The one-sided test may be appropriate here because the hypothesis we wish to test is the one-sided hypothesis that the survival time is longer for patients with the low-risk microRNA signature than that for patients with the high-risk signature, not the two-sided hypothesis that the survival time is different between the low-risk signature and high-risk signature (Green et al., 2003; Koch and Gillings, 1988).

To seek statistical evidence for supporting the one-sided hypothesis that the high risk score of microRNA signature can predict poor survival in NSCLC patients, the combined samples of testing set and independent cohort were analyzed with the one-sided log-rank test (Flanigan et al., 2001; Seymour et al., 2007).

To test if the signature is independent from the stage, we use chi-square distribution of three degrees of freedom to find the p value for combining the three log-rank tests from stage stratification. The degree of freedom is two for combining the two log-rank tests from histology stratification (see Supplemental Experimental Procedures for details).

### Cell Culture and Transfection

The human lung adenocarcinoma cell lines CL1-0 and CL1-5 were established in a previous study in which the invasive competence of CL1-5 is higher than that of CL1-0 (Chen et al., 2001). Cells were cultured in DMEM medium (GIBCO, Grand Island, NY) with 10% fetal bovine serum.

All of the following partially double-stranded RNAs that mimic endogenous precursor miRNAs were purchased from Ambion (Ambion, Austin, TX): hsa-miR-137, hsa-miR-182*, hsa-miR-221, hsa-miR-372, hsa-let-7a, and negative control 1 precursor miRNAs. They were transfected into cells at final concentrations of 100 nM each using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Twenty-four hours after transfection, the invasiveness of cells was analyzed.

To transiently express microRNAs, paired oligonucleotides based on the precursor sequences of hsa-miR-137, hsa-miR-182*, hsa-miR-221, and hsa-miR-372 (http://microrna.sanger.ac.uk/) were cloned into the BamHI and HindIII sites of an expression vector p*Silencer*4.1-CMV puro (Ambion, Austin, TX). The p*Silencer*4.1-CMV puro Negative Control is a negative control plasmid encoding a hairpin siRNA whose sequence is not found in the human genome databases (Ambion, Austin, TX).

### Invasion Assay

Transwell culture inserts with their companion 24-well plates (Costar, Cambridge, MA) were used for the assessment of cell migration and extracellular matrix invasion as described previously with slight modification (Shridhar et al., 2004). Briefly, the culture inserts consist of an 8 mm pore-size polycarbonate filter upon which cells can be seeded and grown. The filters were coated with appropriate Matrigel (Becton Dickinson, Bedford, MA). Appropriate number of transfected cells ($1 \times 10^5$ for CL1-0 and $1 \times 10^4$ for CL1-5) were seeded onto the Matrigel and incubated for 18 hr. The filters coated with Matrigel were swabbed with a cotton swab, fixed with methanol, and then stained with Giemsa solution. The number of cells attached to the lower surface of the polycarbonate filter was totally counted at 200× magnification under a light microscope. Each type of cell was assayed in quaternary.

## Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, two supplemental figures, and four supplemental tables, as well as the entire microRNA data set, and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/1/48/DC1/.

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