A MicroRNA Cluster at 14q32 Drives Aggressive Lung Adenocarcinoma

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Abstract

Purpose: To determine whether different subtypes of lung adenocarcinoma (AC) have distinct microRNA (miRNA) expression profiles, and to identify miRNAs associated with aggressive subgroups of resected lung AC.

Experimental Design: miRNA expression profile analysis was performed in 91 resected lung AC and 10 matched nonmalignant lung tissues using a PCR-based array. An independent cohort of 60 lung ACs was used for validating by quantitative PCR the top 3 prognostic miRNAs. Gene-expression data from 51 miRNA profiled tumors was used for determining transcript-specific miRNA correlations and gene-enrichment pathway analysis.

Results: Unsupervised hierarchical clustering of 356 miRNAs identified 3 major clusters of lung AC correlated with stage ($P = 0.023$), tumor differentiation ($P < 0.003$), and IASLC histologic subtype of lung AC ($P < 0.005$). Patients classified in cluster 3 had worse survival as compared with the other clusters. Eleven of 22 miRNAs associated with poor survival were encoded in a large miRNA cluster at 14q32. The top 3 prognostic 14q32 miRNAs (miR-411, miR-370, and miR-376a) were validated in an independent cohort of 60 lung AC. A significant association with cell migration and cell adhesion was found by integrating gene-expression data with miR-411, miR-370, and miR-376a expression. miR-411 knockdown significantly reduced cell migration in lung AC cell lines and this miRNA was overexpressed in tumors from patients who relapsed systemically.

Conclusions: Different morphologic subtypes of lung AC have distinct miRNA expression profiles, and 3 miRNAs encoded at 14q32 (miR-411, miR-370, and miR-376a) were associated with poor survival after lung AC resection. Clin Cancer Res; 20(12); 3107–17. ©2014 AACR.

Introduction

Lung cancer is the leading cause of cancer-related deaths for both sexes in industrialized countries (1, 2). Adenocarcinoma (AC) is the most common histologic subtype, accounting for about 40% of lung cancer diagnoses and 65,000 deaths each year in the United States. Lung AC is a heterogeneous disease and includes tumors with remarkably diverse clinical, pathologic, and molecular features. A new multidisciplinary lung AC classification has been recommended based on histopathology as well as clinical, radiological, and molecular features (3). According to the predominant histologic pattern, lung AC can be further classified in differentiated (comprising lepidic, invasive mucinous AC, acinar, papillary, and micropapillary) and undifferentiated/solid subtypes.

During the last decade, significant advances in understanding the critical molecular mechanisms and the tumor heterogeneity in lung AC have provided clinically relevant biomarkers that stratify patients according to their outcome. In addition, these biomarkers have contributed to the development of novel therapeutic strategies by identifying new targets as well as predictive markers for specific drugs (4). Analyses using mRNA genomic profiling from large cohorts of lung ACs have also provided significant information complementing histologic evaluation (5, 6).

MicroRNAs (miRNA) are short noncoding RNAs involved in many developmental processes that can negatively regulate gene expression by base pairing to a complementary sequence in the 3’ untranslated region of a target mRNA, leading to translational repression. In human cancer, miRNAs play a pathogenic role in the disease process by acting as oncogenes or tumor suppressor genes (7, 8). Because each
miRNAs associated with aggressive subtypes of lung adenocarcinoma may be actionable targets in the future.

**Material and Methods**

**Clinical samples**

We used 151 frozen primary tumors and 10 nonmalignant lung samples matched to the associated tumor from patients with lung AC who underwent resection at the University of Michigan Health System from 1991 to 2007. Informed consent was obtained for each subject and clinical investigations were conducted after approval by the Institutional Review Board. Tumor specimens were immediately frozen following resection and stored at −80°C. Regions containing a minimum of 70% tumor cellularity were used for RNA isolation. Tumor grade assessment as well as histopathological analysis of sections adjacent to regions used for RNA isolation was performed according the IASLC/ATS/ERS International Multidisciplinary Classification of Lung Adenocarcinoma (3) by 2 independent investigators. None of the patients included in this study received preoperative radiation or chemotherapy. Clinical data were retrospectively collected from the medical records and all cases were staged according to the revised seventh tumor–node–metastasis classification criteria (20). The median follow-up time was 8.12 years among the patients that remained alive. Primary tumors were randomly assigned to 2 independent sets: training and validation set, consisting of 91 and 60 samples, respectively. Patient characteristics are provided in Supplementary Table S1.

**RNA isolation and miRNA profiling**

We profiled 91 lung AC and 10 nonmalignant lung samples using TaqMan OpenArray Human microRNA panel (Applied Biosystems), which includes 754 miRNAs plus 3 controls (U6, RNU44, and RNU48). Details on the RNA extraction, quality control procedures, array preparation, and data normalization are provided in the Supplementary Material.

**Validation of miRNA expression by quantitative real-time PCR**

Quantitative real-time PCR (qRT-PCR) was performed using TaqMan microRNA assays (Applied Biosystems) to determine the expression values of 3 miRNAs (miR-411, miR-370, and miR-376a) in an independent cohort of 60 resected lung AC. Details about the qRT-PCR preparation and data normalization are provided in the Supplementary Material. A patient’s risk score was calculated as the sum of the expression levels of the 3 prognostic miRNAs in the test set, weighted by the corresponding regression coefficients (β) derived from the Cox regression analysis in the training set, as previously reported (21). The risk score was used to classify patients into high- or low-risk groups, with a high risk score indicating poorer survival. The distribution of risk scores was similar in both sets (Supplementary Fig. S1). In the test set, the median of the risk score was used as the cutoff value.

**Lung AC cell lines, transfection, and trans-well migration assay**

Two lung AC cells endogenously expressing high miR-411 (SK-LU-1 and NCI-H2228) were purchased from ATCC (Manassas) and were authenticated by genotyping using the Identifier Plus Kit (Applied Biosystems). These cells were transfected with miRCURY LNA microRNA power inhibitors (Exiqon) either with nontarget control A or antisense against miR-411 using Lipofectamine RNAiMax (Invitrogen), and their migration ability was tested by using Boyden chambers (8-μm pore size; BD Biosciences).

**Integration of miRNA profile with other genomic data**

SNP array data from 216 lung ACs were used for calculating the copy number of regions encoding selected miRNAs (22). Available Affymetrix U133A gene expression microarray data from 51 miRNA profiled lung AC tumors were used from a previous study (5). The original gene sets of embryonic stem cell (ESC), Myc targets, and Notch pathway were obtained from previous publications (23), and average expression of each gene set was calculated for each tumor. Using a 5% FDR, the correlation between

**Translational Relevance**

MicroRNAs (miRNA) are small noncoding RNAs involved in posttranscriptional regulation of gene expression. Lung cancer miRNA expression profiles have identified not only miRNAs differentially expressed among the distinct histologic subtypes of lung cancer, but also miRNAs that predict prognosis in early-stage non–small cell lung cancer. In this study, we found miRNAs differentially expressed among distinct morphologic subtypes of lung adenocarcinoma, which may have potential diagnostic utility in the future. In addition, we identified 3 miRNAs encoded at 14q32 region (miR-411, miR-370, and miR-376a) whose expression was associated with poor survival, and these miRNAs were validated as independent prognostic markers in patients with early-stage lung adenocarcinoma. These miRNAs associated with aggressive subtypes of lung adenocarcinoma may be actionable targets in the future.
specific miRNAs and mRNA expression was determined using 2 approaches: independently of whether they can be targeted by these specific miRNAs to capture genes that may be indirectly regulated and restricting the analysis to the predicted conserved targets downloaded from TargetScan v.6.2 and the Miranda and miRWalk websites (24–26). To assess biologic processes associated with selected miRNAs, a gene ontology (GO) enrichment analysis was performed based on significantly correlated genes using DAVID bioinformatics website (27).

Statistical analysis

Significance analysis of microarrays (SAM) algorithms for paired and unpaired samples were used for identifying differentially expressed miRNAs among tumor and nonmalignant samples using 5,000 permutations as previously described (28). DIANA-miRPath software version 2.0 was used for pathway enrichment for miRNAs discriminating lung AC and nonmalignant samples (29). To identify miRNA expression patterns, an unsupervised hierarchical centroid linkage cluster analysis was performed using Cluster v3.0 (30) after mean-centering miRNAs and arrays and heat maps were visualized using the TreeView software (31). Pearson’s and ANOVA tests were used to determine the correlation between the clusters and the clinicopathologic variables. ANOVA tests were used for comparing the mean expression of ESC, Myc, and Notch gene sets among the miRNA clusters. A multivariate regression analysis adjusted by tumor grade was performed for miRNAs significantly up- or downregulated in each specific histologic subtype for identifying miRNAs differentially expressed in solid, lepidic, and invasive mucinous AC subtypes. Spearman correlation coefficients and a linear regression analysis, adjusted by gender, were performed to test the association between tobacco consumption (measured in pack year) and miRNA expression. Survival curves were plotted using the Kaplan–Meier method, and survival differences were assessed by the log-rank test using the median of each individual miRNA as a cutoff. Univariate or multivariate Cox proportional hazards were calculated considering individual miRNA as a continuous variable. Multivariate analysis was adjusted by age, gender, and stage. To identify miRNAs associated with metastatic recurrence, the expression of miRNAs from patients with lung AC who developed metastasis within 5 years of follow-up was compared with recurrence-free patients at 5 years.

Results

Identification of differentially expressed miRNAs in lung AC versus nonmalignant lung

A total of 78 miRNAs were differentially expressed in lung AC as compared with nonmalignant lung tissue by paired class-comparison analysis at an FDR of 0.65% (SAM plot is shown in Supplementary Fig. S2). Thirty-seven were found to be significantly upregulated and 41 downregulated in the tumor tissues (Fig. 1). Using TCGA miRNAseq data from 126 lung AC versus 78 nonmalignant lung samples, 31 of 78 miRNAs were validated as differentially expressed by class-
comparison analysis in this independent cohort at an FDR <1% (Supplementary Table S2).

The top 10 deregulated miRNAs discriminating lung AC from nonmalignant lung included miR-21, miR-210, miR-183-3p, miR-135b, miR-182 (upregulated) and miR-144, miR-126, miR30a-3p, miR-195, and miR-145-3p (downregulated). Interestingly, pathway enrichment analysis based on the predicted gene targets for the top 10 discriminating miRNAs yielded pathways relevant in lung cancer biology such as p53, PI3K-Akt, MAPK, TGF-β, WNT, MAPK, and ErbB signaling pathways, as well as regulation of transcription and posttranslational processes such as ubiquitin-mediated proteolysis (Supplementary Fig. S3).

Hierarchical clustering yielded three clusters of lung AC significantly associated with clinicopathologic features and outcome

Unsupervised hierarchical clustering analysis based upon 356 expressed miRNAs yielded three major clusters of lung AC, with all nonmalignant lung samples clustering tightly together within cluster 1 (Fig. 2A). When nonmalignant samples were not included in the analysis, we observed that tumors classified in cluster 3 still clustered together and most tumors overexpressed miRNAs encoded at 14q32 (Supplementary Fig. S4). Using unsupervised clustering analysis and the most variable miRNAs expressed in a large cohort of lung ACs from TCGA, a subset of tumors overexpressing 14q32 miRNAs was clearly identified (Supplementary Fig. S5).
The correlation of these 3 clusters with the clinicopathologic features was determined, and detailed information is provided in Table 1. A significant association between cluster and stage ($P = 0.023$) as well as differentiation ($P = 0.003$) was found. Cluster 1 included more well-differentiated tumors (70%) and fewer stage II to III patients (17% and 6%, respectively), whereas clusters 2 and 3 contained more poorly differentiated tumors (48% and 45%, respectively) and more stage II to III patients. As an ESC profile correlates with poorly differentiated lung AC (23), we analyzed using microarray gene expression data on 51 lung AC the expression of the ESC, Myc, and Notch gene sets according to the 3 miRNA clusters. Remarkably, cluster 3 had significantly higher expression of Notch ($P = 0.035$) and Myc target genes ($P = 0.032$) as compared with clusters 1 and 2 (Supplementary Fig. S6). On the other hand, clusters 2 and 3 overexpressed ESC signature genes ($P < 0.001$) as compared with cluster 1.

Strikingly, these miRNA clusters were highly correlated with the predominant histologic pattern according to the IASCL/ATS/ERS classification ($P = 0.005$). Cluster 1 included fewer acinar and solid tumors, whereas nearly all tumors categorized as lepidic or invasive mucinous ACs were included in this cluster. Conversely, clusters 2 and 3 were enriched for acinar and solid tumors and included fewer lepidic and invasive mucinous ACs. These results suggest that miRNA profiles might recapitulate to some extent the morphologic classification of lung AC. To identify individual miRNAs associated with predominant histologic subtypes, a logistic regression adjusted by tumor grade was performed, and 19 miRNAs were independently associated with a predominant solid pattern within the tumor sample.

### Table 1. Correlation between clinicopathologic characteristics and clusters defined by unsupervised hierarchical clustering analysis

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cluster 1 ($n = 24$)</th>
<th>Cluster 2 ($n = 33$)</th>
<th>Cluster 3 ($n = 34$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>65.9 ($\pm$9.9)</td>
<td>62.5 ($\pm$10.4)</td>
<td>68.1 ($\pm$8.9)</td>
<td>0.066</td>
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<tr>
<td>Sex, $n$ (%)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10 (25)</td>
<td>11 (27.5)</td>
<td>19 (47.5)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>14 (28)</td>
<td>22 (43)</td>
<td>15 (29)</td>
<td>0.172</td>
</tr>
<tr>
<td>Smoking, $n$ (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smokers</td>
<td>5 (50)</td>
<td>4 (40)</td>
<td>1 (10)</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>19 (24)</td>
<td>28 (35)</td>
<td>33 (41)</td>
<td>0.097</td>
</tr>
<tr>
<td>Stage, $n$ (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>19 (39)</td>
<td>17 (35)</td>
<td>13 (26)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>4 (17)</td>
<td>10 (42)</td>
<td>10 (42)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1 (6)</td>
<td>6 (33)</td>
<td>11 (61)</td>
<td>0.023</td>
</tr>
<tr>
<td>Histological subtype, $n$ (%)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinar predominant</td>
<td>10 (19)</td>
<td>23 (43)</td>
<td>20 (38)</td>
<td></td>
</tr>
<tr>
<td>Lepidic predominant</td>
<td>6 (26)</td>
<td>1 (14)</td>
<td>0 (0)</td>
<td></td>
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<tr>
<td>Papillary predominant</td>
<td>3 (13.0)</td>
<td>1 (4.7)</td>
<td>4 (18)</td>
<td></td>
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<tr>
<td>Invasive mucinous AC</td>
<td>3 (18.0)</td>
<td>1 (4.7)</td>
<td>1 (4.7)</td>
<td></td>
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<tr>
<td>Solid predominant</td>
<td>2 (11.0)</td>
<td>7 (39.0)</td>
<td>9 (50.0)</td>
<td>0.005</td>
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<tr>
<td>Differentiation, $n$ (%)</td>
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<td></td>
</tr>
<tr>
<td>Well</td>
<td>7 (70)</td>
<td>1 (10)</td>
<td>2 (20)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>15 (29)</td>
<td>18 (54.5)</td>
<td>19 (58)</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>2 (7)</td>
<td>14 (48)</td>
<td>12 (40)</td>
<td>0.003</td>
</tr>
<tr>
<td>Life status, $n$ (%)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dead</td>
<td>9 (19)</td>
<td>14 (44.5)</td>
<td>25 (52)</td>
<td>0.008</td>
</tr>
<tr>
<td>Alive</td>
<td>15 (33)</td>
<td>19 (44)</td>
<td>9 (21)</td>
<td></td>
</tr>
<tr>
<td>Recurrence, $n$ (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>9 (21)</td>
<td>11 (26)</td>
<td>23 (53)</td>
<td>0.006</td>
</tr>
<tr>
<td>No</td>
<td>15 (34)</td>
<td>20 (45.5)</td>
<td>9 (20.5)</td>
<td></td>
</tr>
<tr>
<td>Metastasis, $n$ (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5 (15)</td>
<td>9 (27)</td>
<td>19 (58)</td>
<td>0.006</td>
</tr>
<tr>
<td>No</td>
<td>19 (35)</td>
<td>22 (41)</td>
<td>13 (24)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Histologic type based upon the IASLC/ATS/ERS Classification Of Lung Adenocarcinoma.
and 30 were associated with lepidic pattern or mucinous invasive AC (Supplementary Table S3).

A borderline association was also found between cluster and patient age \((P = 0.066)\) and between cluster and smoking history \((P = 0.097)\). Clusters 2 and 3 contained more heavy smokers than cluster 1. We determined the association between miRNA expression and the tobacco pack-year consumed, and 13 miRNAs were significantly associated with patient’s smoking intensity: miR-210 was positively correlated with pack-year, whereas miR-342, miR-151, miR-501-3p, miR-29b, miR-30d, miR-497, miR-222, miR-505, miR-34b, miR-500, and miR-99a-3p were inversely correlated with pack-year consumed.

Furthermore, we found that the clusters were associated with clinical outcome, and patients classified in cluster 3 had a significantly worse disease-free survival (DFS) and OS \((\text{log-rank test}, P = 0.002\text{ and }0.001, \text{respectively, Fig. 2B and C})\) as compared with other patients.

**Prognostic miRNAs and validation of the top 3 prognostic miRNAs in an independent cohort of lung AC**

The statistically significant clinicopathologic covariates in the multivariate Cox model for OS were used in the multivariate analysis examining the prognostic value of individual miRNAs (Supplementary Table S4). The univariate and multivariate analysis (adjusted by age, gender, and stage) for OS generated 46 and 40 significant miRNAs respectively, including 22 overlapped in both analyses (Supplementary Table S5). Eleven of these 22 miRNAs were structurally associated by their genomic location on the long arm of chromosome 14 \((14q32)\). Interestingly, this region encodes one of the larger miRNA clusters in humans and is organized into 2 miRNA segments \((14q32.2 \text{ and } 14q32.31)\).

We selected 3 miRNAs, which were significantly associated with survival in the univariate and multivariate analysis (Supplementary Fig. S7), that are encoded at 14q32.2 \((\text{miR-370})\) and 14q32.31 \((\text{miR-411 and miR-376a})\) for validating their prognostic value in an independent cohort of 60 lung AC by qRT-PCR. Patients were classified as high risk or low risk according to their median risk score value. In this test set, patients with a high risk score had a shorter median DFS \((25.3\text{ months}; 95\% \text{ confidence interval, CI }, 19.0–31.6)\) as compared with low-risk patients \((\text{median not reached, log-rank } P = 0.002, \text{Fig. 3A})\). The estimated DFS rates at 24 months for high- and low-risk patients were 53.3\% ± 9.1\% and 80.0\% ± 7.3\%, respectively. Similarly, high-risk patients had a significantly shorter median OS \((46.3; 95\% \text{ CI }, 29.7–62.9)\) as compared with low-risk patients \((\text{median not reached, log-rank } P = 0.005, \text{Fig. 3B})\). In the multivariate Cox model (Supplementary Table S6), a high risk score remained as an independently prognostic marker for DFS \((HR = 3.32; 95\% \text{ CI }, 1.61–6.88; P = 0.001)\) as well as for OS \((HR = 3.37; 95\% \text{ CI }, 1.42–8.00; P = 0.006)\). The prognostic value of these 3 miRNAs encoded at the 14q32 \((\text{miR-411, miR-370, and miR-376a})\) was therefore validated in an independent set of resected lung AC.

**Potential mechanisms of regulation of the miRNAs encoded in the 14q32 region**

The 14q32 region is an imprinted locus depicted in Fig. 4A, which contains multiple paternally imprinted genes \((\text{DLK1, DIO3, and RTL1})\) and maternally imprinted non-coding RNAs \((\text{MEG3, MEG8, and asRTL1})\; \text{ref. 32})\). The 14q32 miRNAs are only expressed from the maternal allele and are organized into 2 clusters: the 14q32.2 cluster, which contains 10 miRNAs that overlap with the gene antisense \(\text{RTL1} \text{asRTL1}\) and the 14q32.31, which encodes more than 40 miRNAs. These 2 miRNA segments are separated by a C/D small nucleolar RNA \(\text{snRNA}\) segment, which overlaps with the noncoding RNA \(\text{MEG8}\). As multiple miRNAs encoded at 14q32 were associated with outcome, it is likely that these miRNAs share a common mechanism of regulation. Several reasons might explain why only specific 14q32 miRNAs were associated with prognosis such as the...
MicroRNAs at 14q32 Are Prognostic in Lung Adenocarcinoma

Figure 4. Regulation of 14q32 region. A, genomic map of 14q32 miRNA clusters. The paternally imprinted genes (DLK1, RTL1, and DIO3) are depicted in black and the maternally imprinted noncoding RNAs (MEG3, anti-RTL1, and MEG8) are depicted by diagonal lines. The miRNAs at 14q32 region are organized in 2 segments separated by a C/D snoRNA cluster. The intergenic differentially methylated region (IG-DMR) is methylated in the paternal chromosome. Bold miRNAs were associated with poor outcome in the univariate and multivariate analysis. B, median copy-number value was calculated using SNP 250K data available from 216 patients with lung AC for the region 100, 445, 178–100, 582, 998. Dashed lines, criteria for considering the samples amplified (≥3) or deleted (≤1). C, scatter plot showing the significant correlation among the mean of 14q32 miRNAs expression and MEG3 expression (r = 0.42; n = 51; P = 0.003).

diversity of target genes regulated by individual miRNAs, differences in the transcript processing or maturation, and the fact that 14q32 miRNAs are not all consistently expressed in lung AC. Correspondingly, we found that not all the expressed 14q32 miRNAs were significantly correlated among them (Supplementary Fig. S8).

To examine whether structural chromosomal changes are associated with the expression of the miRNAs encoded at the 14q32 region, we used dense SNP 250K data from 216 lung ACs [22]. The median copy-number value was calculated for the 14q32 region, including both miRNA clusters, and only 1 patient (0.5%) harbored a deletion, whereas 2 patients (0.9%) had amplification at this region (Fig. 4B). These results suggest that copy number is not the main mechanism influencing expression of the miRNAs encoded at this chromosomal region. Next, we determined the correlation of 14q32 miRNAs with other genes encoded at 14q32 region using miRNA expression data available from the same 51 lung ACs included in this study. The mean of 24 expressed miRNAs located at 14q32 was calculated and correlated to the expression values of 25 probes corresponding to 18 transcripts (coding and noncoding) flanking the 14q32 miRNAs. The mean expression of 14q32 miRNAs was significantly correlated to DLK1 and MEG3 expression (P = 0.041 and 0.003, respectively; Fig. 4C). We hypothesize that 14q32 miRNAs are epigenetically regulated, because genes and miRNAs encoded within this imprinted locus might be regulated by DNA methylation of two differentially methylated regions located upstream of the transcription initiation site of MEG3 [32–35].
Genes correlated with miR-370, miR-411, and miR-376a expression and GO analysis

We determined the correlation between the expression of the top 3 prognostic miRNAs (miR-370, miR-411, and miR-376a) and mRNA expression of 22,823 probes in a subset of 51 lung AC, which had both miRNA and mRNA expression data. Using a 5% FDR, we detected a significant correlation (P < 0.05) among 3,621 unique miRNA–mRNA pairs. A gene set enrichment analysis was performed based upon genes significantly correlated with each of the 3 miRNA gene sets, focusing on GO gene set collections. The top 12 GO terms for these 3 miRNAs are provided in Supplementary Table S7. Interestingly, all 3 miRNAs were significantly associated with regulation of cell migration or cell motion (P < 0.001), whereas miR-370 and miR-411 target genes were also associated with cell adhesion (P ≤ 0.001). In addition, the predicted genes targeted by miR-370, miR-411, and miR-376a were downloaded from TargetScan v6.2, Miranda, and miRWalk to determine whether the miR-correlated genes are potential direct miRNA targets. When the gene enrichment analysis was restricted to genes that are predicted targets and significantly correlated with these miRNAs (959 miRNA–mRNA pairs), the top terms were involved in the regulation of cell migration and motility as well.

miR-411 expression is associated with metastatic relapse and EMT genes

As regulation of genes involved in cell motility and cell adhesion can contribute to the acquisition of a more metastatic phenotype, we examined which miRNAs were differentially expressed in tumors from patients who developed distant relapse as compared with metastasis-free patients during the follow-up. Strikingly, miR-411 was identified among the 7 miRNAs differentially expressed in tumors from patients with metastatic disease progression (Fig. 5A). Accordingly, patients with higher miR-411 expression had a significantly shorter median metastasis-free survival as compared with patients expressing low levels of miR-411 in both the training and in the validation set (Fig. 5B and C). Next, we determined the functional impact of miR-411 knockdown on migration by transfecting 2 lung AC cell lines (SK-LU-1 and NCI-H2228), which endogenously overexpress miR-411. A significant reduction in cell migration was observed in SK-LU-1 and NCI-H2228 cells transfected with an anti–miR-411 as compared with non-target (Fig. 5D). Interestingly, a significant number of genes overexpressed during epithelial–mesenchymal transition (EMT; ref. 36) were found positively correlated with miR-411 expression, such as ZEB1, SNAI1, SNAI2, MMP2, COL5A2, or SPARC (Fig. 5E, Supplementary Table S8).

Discussion

miRNA expression is deregulated in human cancers, including lung cancer, and contributes to cancer initiation and progression by targeting genes involved in biologic processes that are hallmarks of cancer such as sustained proliferative signaling, resisting cell death, activating cell migration and invasion, inducing angiogenesis, and avoiding immune destruction or deregulating cell energetics (37). The investigation of miRNAs associated with a more aggressive behavior in lung cancer may provide prognostic and disease monitoring markers and potentially define novel therapeutic targets.

In this study, we identified 78 miRNAs differentially expressed in lung AC comparing with nonmalignant lung tissue. Interestingly, 7 of these deregulated miRNAs were found differentially expressed in lung squamous cell carcinoma (SCC) versus nonmalignant lung in our previous miRNA study (11): miR-210, miR-200c, miR-200a, miR-106b, miR-182, and miR-183 were upregulated, whereas let-7e was downregulated. Previous studies demonstrated that miRNA profiles can discriminate the main histologic subtypes of lung cancer, small cell lung cancer, and NSCLC as well as between lung AC and SCC (14, 16, 38). However, it is unknown whether different morphologic and molecular subtypes of lung AC have specific miRNA expression profiles. We identified 3 major clusters of lung AC by unsupervised hierarchical clustering analysis based on the expression of 356 miRNAs. These clusters were significantly associated not only with tumor differentiation grade and disease stage, but also with the predominant morphologic subtype of lung AC according to the IASLC/ATS/ERS Classification. Cluster 1 was significantly enriched for lepidic and mucinous invasive tumors, whereas cluster 3 contained more solid lung AC. These results suggest that miRNA expression profiles may recapitulate to some extent the significant heterogeneity of lung AC, and specific miRNAs are dysregulated in distinct morphologic types of lung AC. The morphologic subtypes are indicative of biologic behavior and therefore, miRNA expression profiles, which reflect the morphologic subtypes of lung AC, may be potential surrogate markers of the underlying lung cancer and have potential clinical utility as diagnostic tools in the future. Further prospective studies will be needed for validating these findings. Of note, differential miRNA expression profiles have been reported to be reflective of distinct histologic subtypes of breast or ovarian tumors as well (39, 40).

When we examined the effect of tobacco smoking on miRNA expression, we found several miRNAs were significantly correlated with the amount of tobacco consumed. miR-210, which was highly expressed in heavy smokers, has been associated with hypoxia in lung cancer and positively regulates HIF-1α (41). Certain miRNAs negatively correlated with smoking status (such as miR-342-3p and miR-34b) may act as tumor suppressor miRNAs, and their expression may be regulated by epigenetic mechanisms (42).

In the survival analysis, we identified a set of overexpressed miRNAs associated with poor outcome after lung AC resection, which were encoded in the long arm of chromosome 14 (14q32). Overexpression of 14q32 miRNAs has been also associated with poor outcome in other solid tumors (43, 44), but it has not been previously...
MicroRNAs at 14q32 Are Prognostic in Lung Adenocarcinoma

We compared the prognostic miRNAs identified in previous studies and a modest overlap was observed across all these studies (Supplementary Table S9). This minimal overlap might be influenced by tumor heterogeneity, differences in assay platforms and normalization methods, different tumor histology distribution, and patient selection. Large collaborative studies such as The Cancer Genome Atlas (TCGA) or the Strategic Partnering to Evaluate Cancer Signatures will be extremely valuable for testing the accuracy and reliability of miRNAs as prognostic markers.

The top 3 prognostic miRNAs encoded at 14q32 (miR-411, miR-376a, and miR-370) were selected for validation by qRT-PCR in an independent cohort of lung AC. This supervised miRNA signature seemed to be superior to the clusters identified by unsupervised clustering because it not only predicted poor outcome independently of other clinical variables, but it is also more practicable when analyzing clinical samples.

We were interested in understanding the mechanisms of regulation of 14q32 region which contains several prognostic miRNAs. Our analysis of 14q32 copy number using SNP 250K data from a large cohort of lung AC did not reveal a significant frequency of genomic events. Because miRNA expression may be transcriptionally linked to the expression of host genes (protein-coding and noncoding RNAs), we...
determined the correlation of miRNAs encoded at 14q32 with other genes located in this region. We found that expression of 14q32 miRNAs correlated with two genes (DLK1 and MEG3) located in an imprinted locus, which is regulated by DNA methylation and histone modifications. We hypothesize that 14q32 miRNAs may also be epigenetically regulated but further studies are needed to elucidate the mechanism that controls the expression of this miRNA cluster. miRNAs encoded at 14q32 region play an important role in stem cell pluripotency as well as lung development and differentiation. Indeed, they were overexpressed in the lungs of mice at the late embryonic stage or newborn mice and then decrease dramatically during the first weeks of life (45, 46). A recent study that performed RNAseq in \textit{KRASG12D} transgenic tumors found that miRNAs encoded at the \textit{Dlk1-Dio3} locus on mouse chromosome 12qF1 were consistently increased in tumors as compared with nonmalignant lung (47). Interestingly, these miRNAs were repressed when \textit{KRASG12D} cells were grown \textit{in vitro} and were reactivated when transplanted \textit{in vivo}.

To investigate the biologic processes related to specific 14q32 miRNAs, we determined which genes were correlated with specific miRNAs using gene expression data from 51 lung ACs. We recognize a limitation of using this approach as miRNAs may reduce protein expression without affecting mRNA stability. Nevertheless, this approach might be hypothesis generating for identifying cellular processes that are deregulated in primary tumors overexpressing certain miRNAs. We found that several gene families were consistently correlated with expression of these miRNAs, such as regulation of cell motility, which may confer a more aggressive phenotype. We also observed that migration of lung AC cells was reduced after knocking down miR-411. Furthermore, miR-411 was significantly overexpressed in tumors from patients who developed a distant recurrence and miR-411 expression correlated with genes involved in EMT. Indeed, it has been recently reported that the expression of various miRNAs encoded at 14q32 significantly increased after treating lung AC cells with TGF-\(\beta\), and knockdown of these miRNAs inhibited the EMT process (48).

In conclusion, miRNA expression profiles not only identified miRNAs differentially expressed in lung AC compared with nonmalignant lung, but also were correlated with tumor differentiation and morphologic subtype of lung AC, suggesting that miRNA profiles may subclassify lung AC. We identified specific miRNAs located at 14q32 independently associated with poor outcome after lung AC resection. We focused on miR-411, which was overexpressed in patients who develop metastasis after surgery and was correlated with genes involved in cell migration and EMT. These findings may have implications for the diagnosis of lung AC, and specific miRNAs might be relevant targets in the future using novel therapeutic approaches such as antisense oligonucleotides.

Disclosure of Potential Conflicts of Interest

R.M. Reddy reports receiving commercial research support from GlaxoSmithKine. No potential conflicts of interest were disclosed by the other authors.

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