

# miRNA expression profiling of lung adenocarcinomas: correlation with mutational status

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**MicroRNA (miRNA) expression is deregulated in lung cancer, and some miRNAs are associated with poor prognosis and survival. In this study, we investigated the miRNA expression in lung adenocarcinomas with different oncogenic mutations, including *EGFR*-positive, *KRAS*-positive and *EGFR/KRAS*-negative tumors. The expression of 319 miRNAs was evaluated by Exiqon/Luminex microarray, and expression of individual miRNAs was validated by individual RT-PCR assays (Applied Biosystems). Overall, miRNA expression was similar among three mutationally different groups with most upregulated miRNAs being miR-20a, miR-328, miR-34c and miR-18b and most downregulated miRNAs being miR-32, miR-137 and miR-342. Four miRNAs (miR-155, miR-25, miR-495 and miR-7g) were expressed differently among these tumors. miR-155 was upregulated only in *EGFR/KRAS*-negative group, miR-25 was upregulated only in *EGFR*-positive group and miR-495 was upregulated only in *KRAS*-positive adenocarcinomas. In opposite, let-7g was downregulated in all three groups, with more significant downregulation in *EGFR/KRAS*-negative adenocarcinomas. Principal component analysis (PCA) revealed significant correlation between miRNA expression patterns and somatic mutations. In this study, we demonstrated that despite the similarity in miRNA expression among lung adenocarcinomas with different somatic mutations, some miRNAs showed unique expression patterns, which were in strong correlation with the mutation type, suggesting different carcinogenic pathway for these tumors. These miRNAs can be further explored for their diagnostic and prognostic use.**  
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Lung cancer is the leading cause of cancer deaths in both men and women in the United States.<sup>1</sup> Over the past two decades, adenocarcinoma has replaced squamous cell carcinoma as the most common subtype of non-small cell lung cancer (NSCLC) in the United States. There is a large amount of data published in literature regarding genetic alterations in lung carcinogenesis.<sup>2</sup> Alterations in the *p53* and *RB/p16* pathways are common in all subtypes of lung carcinomas. Somatic mutations in certain tyrosine kinases have emerged as central ‘drivers’ in lung adenocarcinoma carcinogenesis. Activating mutations of *EGFR* have been reported in adenocarcinomas responsive to *EGFR* tyrosine kinase

inhibitors (TKIs).<sup>3–5</sup> Other mutually exclusive mutations and negative predictors to *EGFR*-TKI, such as *KRAS* and *BRAF* mutations, represent alternative mechanisms of activating the MAP kinase pathway in lung adenocarcinomas.<sup>6,7</sup>

Previously unknown markers, such as noncoding RNA gene products, may be important contributors to lung cancer development as well. MicroRNA (miRNA) is a group of endogenous, small, noncoding RNA that can modulate protein expression by regulating translational efficiency or cleavage of target. They regulate the expression of known tumor suppressor genes and oncogenes. miRNA genes are frequently located at fragile sites, regions of loss of heterozygosity, minimal regions of amplifications or common chromosomal breakpoint regions. They are expressed in a tissue-specific manner, and has been shown that the pattern of expression accurately define the specific cancer types.<sup>8–14</sup>

Prognostic and diagnostic significance of miRNA expression has been reported in several malignancies,

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including chronic lymphocytic leukemia, breast, pancreas, colon and lung carcinomas.<sup>15–19</sup> Let-7 was the first miRNA reported to be aberrantly expressed in lung carcinomas.<sup>20–22</sup> It has been found that let-7 miRNA family controls the activity of *RAS* genes and function as tumor suppressor gene.<sup>23</sup> Lung tumors with reduced levels of let-7 had significantly increased levels of RAS protein and by some reports poor clinical outcome. In addition, induced overexpression of let-7g in human non-small cell lung tumors lead to significant growth reduction and reduced tumor burden.<sup>24</sup> It was also hypothesized that alterations in let-7 represent an early event in lung adenocarcinoma carcinogenesis. Another miRNAs that correlated with poor survival in lung carcinomas were miR-155, miR-21 and miR-106a.<sup>19</sup> Upregulation of miR-155 alone was found as independent negative prognostic factor in lung adenocarcinomas.<sup>19</sup> In addition, the expression of miRNA cluster miR-17-92 is increased in lung cancer, particularly in small cell carcinoma.<sup>25</sup> The predicted targets include *PTEN*, *RB2* and *c-myc*, the latest which is frequently amplified/overexpressed in small cell lung carcinoma.<sup>26</sup> A different profiles of miRNA expression was observed in lung carcinomas from never-smokers vs smokers.<sup>27</sup> Recent studies demonstrated that EGFR may be a functional target of miRNAs.<sup>28,29</sup> MiRNA-128, which is frequently deleted in lung cancers, directly downregulated *EGFR*, but did not correlate with *EGFR* mutation and prognosis. Overall, all these studies suggest that miRNAs can be used as diagnostic and prognostic markers of lung cancer.

Recent studies in thyroid cancer demonstrated correlation between miRNA expression profiles and specific oncogenic mutations, but only one report indicated correlation between the presence of *EGFR* mutations and miRNA profile in lung adenocarcinomas.<sup>30</sup> Oncogenic mutations represent a significant event in lung adenocarcinoma development and therapeutic target. In this study, we determined and compared miRNA expression profiles in stage I surgically resected lung adenocarcinomas with different oncogenic mutations. The results may suggest a potential therapeutic strategy targeting specific miRNAs in combination with *EGFR*-TKI or by itself.

## Materials and methods

### Lung Tissue Samples

Snap-frozen tissue samples from surgically resected lung specimens were obtained from the University of Pittsburgh Health Sciences Tissue Bank. Thirty-four non-neoplastic and neoplastic lung samples were analyzed, including 17 primary stage matched lung adenocarcinomas (five *EGFR* mutated (*EGFR* +), six *KRAS* mutated (*KRAS* +) and six wild types (wt)).

### RNA Isolation

Total RNA was extracted from surgical specimens using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as previously described.<sup>30,31</sup> RNA yield and purity were determined using the NanoDrop 1000 spectrophotometer (ThermoScientific, Wilmington, DE, USA). RNA integrity was assessed for 1  $\mu$ l of sample using the Agilent 6000 NANO kit for the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol.

### miRNA Expression Microarray

Quantitation of expression of 319 mature miRNA was performed using FlexmiR human microRNA pool, version 8 (Exiqon, Vedbaek, Denmark) and analyzed on Luminex 200 (Austin, TX, USA) according to the manufacturer's instructions. In detail, 2.5  $\mu$ g total RNA was labeled with biotin using the FlexmiR MicroRNA Labeling Kit (Luminex) and subsequently hybridized to beads coated with locked nucleic acid probes complementary to mature miRNA sequences. The system calibration was performed using the xMAP calibration control reagents (Luminex). A blank control set of beads was used to normalize the background value for every individual miRNA. The five synthetic controls and four small nucleolar RNA, snoRNA, normalization controls were used to adjust mean fluorescence intensities between samples and between runs using the average correction factor for these controls, as recommended by the manufacturer. Finally, the miRNA expression in tumor samples was quantitated relatively to normal lung tissue using Luminex IS software v.2.3 (Luminex).

### miRNA Expression by Real-Time RT-PCR

The measurement of the expression levels of individual microRNAs was performed using miRNA sequence-specific primers (Applied Biosystems, Foster City, CA, USA) by the real-time RT-PCR-based detection methodology. Briefly, 10 ng of total RNA was reverse transcribed using High-Capacity cDNA Archive kit (Applied Biosystems) followed by amplification on an ABI 7500 Real-Time PCR System (Applied Biosystems). Small nucleolar RNA RNU44 was used as endogenous control. miRNA expression levels were calculated by relative quantitation using the ABI 7500 Real-Time PCR SDS 1.2 software (Applied Biosystems), and the fold expression changes were determined by  $2^{-\Delta\Delta C_t}$  method.<sup>30</sup> The data were presented as the fold change of miRNA expression in lung tumors relatively to normal lung tissues after normalization to an endogenous control (RNU44).

### Statistical Analysis

Principal component analysis (PCA) was conducted for an unsupervised assessment of the relationship

between mutation type and the miRNA expression. The Kruskal–Wallis test was also applied to assess the difference in the expression levels of miRNA among mutations. Statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA). A significance level is set at 0.05 and all *P*-values reported are two-sided.

### Search for Predicted Target Genes

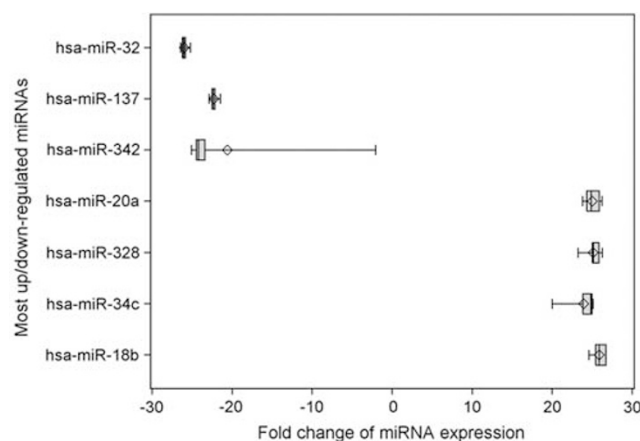
Putative miRNA target genes were identified using miRBase (<http://microrna.sanger.ac.uk>), TargetScan (<http://genes.mit.edu/targetscan>) (Lewis BP 2003 Cell 115:787–798)<sup>32</sup> and PicTar (<http://pictar.bio.nyu.edu>) target prediction programs.<sup>33</sup>

### KRAS and EGFR Mutational Analysis

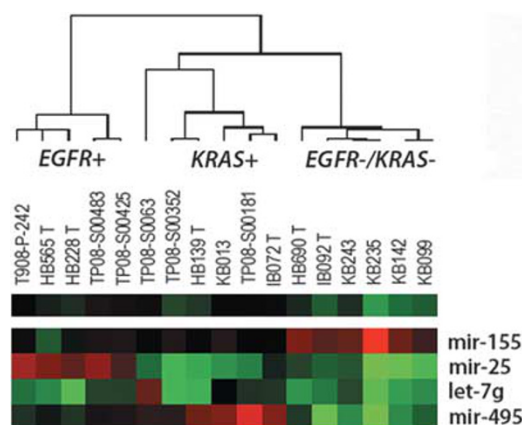
Tumor targets were manually microdissected from the 4- $\mu$  unstained formalin-fixed paraffin-embedded histological sections. DNA was isolated from each target using the DNeasy tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For the detection of mutations, DNA was amplified with primers flanking exon 2 of the *KRAS* gene (forward primer 5'-GGTGAGTTTGTATT AAAAGGTAATATGCA-3' and reverse primer 5'-TCCTG CACCAGTAATATGCA-3'), exon 19 of the *EGFR* gene (forward primer 5'-CCCAGCAATATCAGCCTT AGGTG-3' and reverse primer 5'-CCACTAGAGCTA GAAAGGGAAAGAC-3') and exon 21 of the *EGFR* gene (forward primer 5'-CCTCACAGCAGGGTCTTC TC-3' and reverse primer 5'-CCTGGTGTGTCAGGAAA ATGCT-3'). Then, PCR products were sequenced in both sense and antisense directions using the BigDye Terminator v3.1 cycle sequencing kit on ABI 3130 (Applied Biosystems), according to the manufacturer's instructions. The sequences were analyzed using Mutation Surveyor software (SoftGenetics, LLC., State College, PA, USA). Each case was classified as positive or negative for the *KRAS* and *EGFR* mutation based on the sequencing results.

## Results

Initially, six lung adenocarcinomas (two positive for *EGFR* mutation, two positive for *KRAS* mutation and two *EGFR*- and *KRAS*-negative tumors) and corresponding normal tissue samples were analyzed by microarray analysis for expression levels of 319 human mature miRNAs. Overall, miRNA expression was similar among three mutationally different groups, confirming the same histopathologic type of these tumors. Several miRNAs (ie, miR-20a, miR-328, miR-34c and miR-18b) were consistently upregulated with more than 20-fold difference as compared with normal lung tissue (Figure 1). Similarly, miR-32, miR-137 and miR-342 downregulated at more than 20-fold as compared with



**Figure 1** Box plot analysis of the most upregulated and downregulated miRNAs in lung adenocarcinomas.



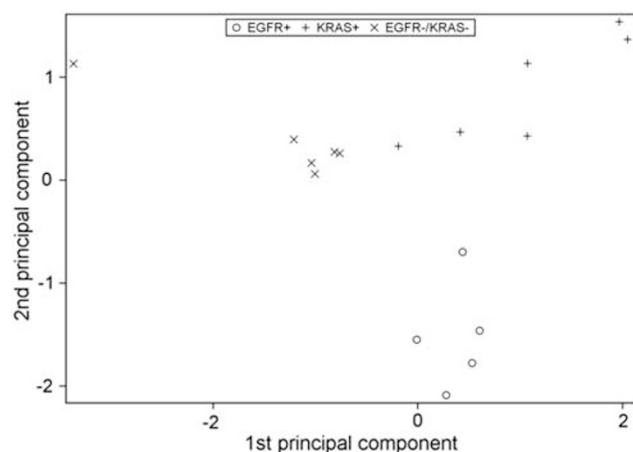
**Figure 2** Cluster dendrogram of miRNA expression in lung adenocarcinoma with different oncogenic mutations.

corresponding normal lung tissue (Figure 1). Only one case of adenocarcinoma positive for *EGFR* mutation did not demonstrate downregulation of miR-342 at such level.

However, several miRNAs were differentially expressed between the mutational groups by microarray analysis. To validate these results, we evaluated expression of each individual miRNA by real-time RT-PCR analysis on six primary studied tumors and on additional 12 adenocarcinoma samples (three *EGFR*-positive, five *KRAS*-positive and four *EGFR* and *KRAS*-negative tumors). The confirmed dysregulated miRNAs were miR-155, miR-25, miR-495 and miR-7g (Figure 2). Expression of miR-155 and let-7g was of particular interest, as it has been shown that the expression of these miRNAs correlate with a poor prognosis in lung cancer. MiR-155 was upregulated more than two-fold in wt adenocarcinomas (*EGFR/KRAS* negative) and let-7g was downregulated in all three tumor groups, however, more significantly in wt adenocarcinomas as compared with corresponding normal

**Table 1** Differentially expressed miRNAs between EGFR-positive, KRAS-positive and EGFR/KRAS-negative lung adenocarcinomas

miRNA/folds	EGFR+	KRAS+	EGFR-/KRAS-	P-value
miR-155	1.03	1.14	2.62	<0.0005
miR-25	2.37	0.44	0.38	0.0016
miR-495	0.97	2.43	0.37	<0.0001
Let-7g	0.47	0.85	0.38	0.24

**Figure 3** Principle component analysis (PCA) for the assessment relationship between mutation groups and miRNA expression levels.

lung tissue (Table 1). Our study also identified some novel miRNA not previously reported in lung carcinoma: miR-25, which was upregulated more than twofold only in EGFR-positive tumors, and miR-495, which was upregulated more than twofold only in KRAS-positive tumors, as compared with corresponding normal tissue (Table 1). The noted difference in miRNA expression pointed toward the possibility of correlation of miRNA expression with the mutational profile of tumors. To explore this possibility, the unsupervised hierarchical clustering analysis of miRNA expression in the tumors positive for mutations was performed. It demonstrated that EGFR-positive, KRAS-positive and EGFR/KRAS-negative adenocarcinomas formed individual clusters (Figure 2). In addition, PCA was used for an unsupervised assessment of the relationship between mutation type and expression levels of the miR-25, miR155, miR-495 and let-7g. It revealed a strong relationship between miRNA expression and mutational status (Figure 3). Similar results were obtained using the Kruskal–Wallis test, which demonstrated significant difference in expression for miR-25, miR-495 and miR-155 ( $P=0.0016$  to  $P<0.0001$ ), whereas the expression of let-7g was not significantly different ( $P=0.24$ ) (Table 1).

We analyzed the predicted target genes for miR-25, miR-495, miR-155 and let-7g (Table 2). The analysis was performed using three publicly available algorithms to predict human miRNA gene

**Table 2** Putative target genes of miRNAs dysregulated in lung adenocarcinomas with different oncogenic mutations

Dysregulated miRNA	miRNA chromosomal location	Predicted gene name	Gene description
miR-25	7q22.1	MAP2K4 MALAT1 RAB23	Mitogen-activated protein kinase kinase 4 Metastasis-associated lung adenocarcinoma transcript 1 Member RAS oncogene family
miR-495	14q32.31	CADM1 (TSLC1) TCF4	Tumor suppressor lung cancer 1 Transcription factor 4
miR-155	21q21.3	AKAP10 TCF4	<i>Homo sapiens</i> A kinase anchor protein 10 Transcription factor 4
let-7g	3p21.1	NRAS HMGA2	Neuroblastoma RAS viral (v-ras) oncogene homolog High mobility group AT-hook 2

targets, ie, miRBase, TARGETSCAN and PICTAR. To reduce number of false positives, we listed only putative target genes predicted by at least two of the programs and which were previously implicated in lung carcinogenesis. Table 2 shows target genes putatively regulated by these miRNAs.

## Discussion

Lung adenocarcinoma represents a very heterogeneous group of tumor with a large spectrum of morphology, genetics, clinical behavior and variable responses to therapies. Discovery of prognostic and predictive value of EGFR and KRAS somatic mutations in lung adenocarcinomas has changed our understanding of lung carcinogenesis and our perception of lung cancer as a disease. In addition, discovery of differences in miRNA expression between different tumor types provided an insight into lung carcinogenesis and hope for improvement in lung cancer diagnosis, prognosis and potentially new treatment. In this study, we demonstrated that lung adenocarcinomas with different oncogenic mutations have distinct miRNA profiles. As expected, there were many overlapping miRNAs that were upregulated and downregulated regardless of the presence or absence of EGFR and KRAS mutations. This finding correlates with known tissue specificity of miRNA and confirms that all studied tumors were of the same histopathologic type. However, overexpression of miR-25 in EGFR-positive, miR-495 in KRAS-positive and miR-155 in EGFR/KRAS-negative adenocarcinomas may be attributed to the difference in their carcinogenic pathways.

More interestingly, we found correlation with expression of let-7 and miR-155, two most studied miRNAs in lung cancer, and mutational status. Takamizawa *et al*<sup>22</sup> were first to report reduced expression of let-7 in lung carcinomas associated with shorter survival after potentially curative surgical resection. Yanaihara *et al*<sup>19</sup> also found reduced expression of let-7 family in lung adenocarcinomas. Only let-7a-2 correlated with prognosis by miRNA microarray analysis, but was not statistically significant in the independent set of adenocarcinomas analyzed by real-time RT-PCR analysis. None of these studies compared miRNA profiles with respect to *EGFR* or *KRAS* mutations. In study by Inamura *et al*,<sup>21</sup> let-7 failed to show prognostic significance in bronchioloalveolar (carcinoma *in-situ*) and invasive adenocarcinomas. However, mucinous bronchioloalveolar carcinoma showed significantly lower expression than non-mucinous bronchioloalveolar carcinoma. Authors suggested that aberrant let-7 expression most likely represents an early event in lung adenocarcinoma carcinogenesis. A subset of cases was also analyzed for *KRAS* and *EGFR* mutations, and no differences were found in let-7 expression between adenocarcinomas with or without *EGFR* or *KRAS* mutations. In our study, we found reduced expression of let-7g in all mutational groups with more significant downregulation in tumors negative for *EGFR/KRAS* mutations. In the same group, we found upregulation of miR-155, another miRNA associated with poor prognosis. Yanaihara *et al*<sup>19</sup> reported a negative prognostic impact of high miR-155 in patients with lung adenocarcinomas. This miRNA was also reported as a prognostic factor in chronic lymphocytic leukemia.<sup>15</sup> Therefore, on the basis of the reported finding and our results, it is possible to assume that *EGFR/KRAS*-negative tumors with upregulation of miR-155 and downregulation of let-7g may have the less favorable prognosis as compared with other mutational groups. Our study does not have any survival data, but it would be interesting to further explore prognostic significance of let-7 and miR-155 in stage I wt adenocarcinomas.

Abnormal expression of miR-25 noted in *EGFR*-mutated adenocarcinoma has never been reported in lung carcinoma. Recent analysis of miR-25 expression indicated its role in the regulation of airway smooth muscle cell phenotype by modulating the expression of inflammatory mediators such as *RANTES*, eotaxin and *TNF $\alpha$* , as well as genes involved in extracellular matrix turnover and contractile proteins, most notably myosin heavy chain.<sup>34</sup> Its role in lung cancer is uncertain, but possible role in interaction between malignant epithelial cells and extracellular matrix may be considered. In addition, we report *MALAT-1* as one of potential targets of miR-25. Upregulation of this gene was found to be associated with the development of distant metastases in early-stage non-small lung cancer.<sup>35,36</sup> In our study, miR-25 was upregu-

lated only in *EGFR*-positive group, however, prognostic significance of this finding in our study is uncertain.

Many miRNAs are known to function as oncogenes or tumor suppressors depending on the regulation of their target genes. We identified several interesting genes as predicted targets to differentially expressed miRNAs. A tumor suppressor gene, *CADM1* (originally referred as tumor suppressor lung cancer 1 (*TSLC1*) gene), was a predicted target for miR-495, which is found to be upregulated only in *KRAS*-positive adenocarcinomas. This gene is known to be inactivated in 44% of NSCLC, especially in tumors with invasion or metastasis.<sup>37</sup> The *TCF4* gene was identified as target gene for miR-155 and miR-495. This gene is a downstream target of the Wnt/ $\beta$ -catenin/TCF pathway, and has been implicated as a marker of metastatic activity in lung cancer.<sup>38</sup> *RAS* genes are known predicted targets for let-7.<sup>23</sup> Finally, the *HMGA2* gene is involved in modulation of DNA repair, and overexpression of *HMGA2* leads to the promotion of genome instability and tumorigenesis.<sup>39,40</sup>

In conclusion, our results indicate correlation between expression of several miRNAs and somatic mutations in lung adenocarcinomas. These miRNAs may have a role in cancer development and may be associated with more aggressive behavior of some tumor types. With the development of RNAi-based treatments, it might be crucial to learn about specific miRNAs expression and lung adenocarcinoma mutational profile, which may lead to the development of novel therapeutic approaches in lung carcinomas.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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