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Research paper

# Two lung development-related microRNAs, miR-134 and miR-187, are differentially expressed in lung tumors

F. Mirzadeh Azad [a], P. Naeli [a], M. Malakootian [b], A. Baradaran [c], M. Tavallaei [d], M. Ghanei [e], S.J. Mowla [a,*]

[a] Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[b] Rajaie Cardiovascular Medical and Research Center, Iran University of Medical Sciences, Tehran, Iran
[c] Department of Pathology, Alzahra Hospital, Isfahan University of Medical Sciences, Isfahan, Iran
[d] Genetic Research Center, Baqiatallah University of Medical Sciences, Tehran, Iran
[e] Chemical Injuries Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

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ABSTRACT

*Introduction:* MicroRNAs (miRNAs) are involved in various cellular events needed for embryonic development and tumorigenesis. As some of the development-specific gene expression patterns could be observed in cancers, we speculated that the expression pattern of lung development-specific miRNAs miR-134 and miR-187 might be altered in lung tumor samples. Lung cancer is the first cause of cancer related deaths worldwide, mostly due to its late diagnosis. Therefore, finding a reliable diagnostic tumor marker, based on molecular profile of tumorigenesis, would be critical in lowering lung cancer mortality.
*Methods:* We employed a real-time RT-PCR approach to evaluate the expression alteration of two lung development-related miRNAs in lung tumor tissues. The suitability of miRs expression alterations as lung tumor biomarkers was tested by receiver operating characteristic (ROC) curve analysis. The effect of miR-187 overexpression on a lung carcinoma cell cycle was assessed using flow cytometry analysis.
*Results:* Our data revealed a significant upregulation (7.8 times, $p < 0.02$) of miR-134 in lung tumors. However, its expression level failed to discriminate different tumor types and grades of malignancies from each other. Moreover, the ROC curves analysis did not give it a good score as a reliable biomarker (AUC = 0.522, P = 0.729). In contrast, miR-187 showed a significant down-regulation (P = 0.008) in lung tumors. Similarly, its expression level failed to differentiate different tumor types or grades of malignancies. Nevertheless, ROC curve analysis gave it an AUC score of 0.669 (P = 0.012), which suggests its suitability as a potential biomarker for lung cancer. Furthermore, ectopic expression of miR-187 in A549 cells caused a cell cycle arrest in G1 phase (P = 0.013).
*Conclusion:* Altogether, our data demonstrated an altered expression of two development-related miRNAs namely miR-134 and miR-187 in lung tumors for the first time. Moreover we have shown that miR-134 and miR-187 expression alternation were in accordance with their approved regulatory roles, therefore these miRNAs could serve as new biomarkers with potential usefulness in lung cancer diagnosis and treatments. In addition, miR-187 expression in tumor cells could perturb cell cycle which supported its possible role as tumor suppressor.


## 1. Introduction

Lung cancer is the leading cause of cancer-related deaths around the world (Herbst et al., 2008). Nearly 85% of all lung tumor cases are non-small cell carcinomas (NSCC). In Iran, it is among the five most frequent cancer types and its incidence is growing mostly due to the environmental pollutions and life style changes (Hosseini et al., 2009). The main reason for high mortality rate of lung cancer is its late diagnosis. In fact, 75% of all lung carcinomas are diagnosed at late stages, when tumors have already become invasive and refractor to treatments (Hoffman et al., 2000; Herbst et al., 2008; Ettinger et al., 2012; Zhang et al., 2013). Therefore, finding reliable lung cancer biomarkers with high sensitivity and specificity is of great importance (Brambilla et al., 2003).

MicroRNAs (miRNAs) are a group of small (18–22 nucleotides) non-coding RNAs that post-transcriptionally regulate gene expression. By binding to their target mRNAs, they either repress translation or degrade their targets (Bartel, 2004; Ketting, 2011). Regulating more than 60% of human protein-coding genes, miRNAs profoundly control different cellular pathways and biological events (Friedman et al., 2009). Two important cellular events that are under tight control of these small regulators are embryonic development and oncogenesis (Lin et al., 2010).

Several previous investigations have emphasized on significant similarities between lung tumorigenesis and lung embryonic development.

*Abbreviations:* miRNA, microRNA; RT-PCR, reverse transcriptase polymerase chain reaction; ROC curve, receiver operating characteristic curve; AUC, area under curve; NSCC, non-small cell carcinomas; rRNA, ribosomal RNA.

* Corresponding author.
*E-mail address:* sjmowla@modares.ac.ir (S.J. Mowla).

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In fact, some processes such as cell growth, division, and differentiation recruit the same genetic machinery in both states. In other words, genes which have strategic roles in developmental events, most likely have a part in tumorigenesis as well (Bonner et al., 2004; Liu and Kohane, 2009; Whitsett et al., 2011). MiRNAs that are down-regulated during embryonic lung development are proved to function as tumor suppressor, while those with elevated level in developing lung are mostly considered as oncomiRs (Mendell, 2008; Liu and Kohane, 2009).

First indications of miRNAs orchestrating lung development came from studies on conditional knockout mice for Dicer in which affected epithelial cells were unable to succeed epithelial branching (Harris et al., 2006). Furthermore, analyzing miRNAs expression pattern in different stages of lung development exhibited a unique profile for each stage (Williams et al., 2007; Bhaskaran et al., 2009). These miRNAs are responsible for fine-tuning expression of growth and transcription factors or act as molecular switches for cellular events (Brennecke et al., 2003; Tay et al., 2008; Croce, 2009; Shenouda and Alahari, 2009; Liu et al., 2010).

MiR-134 and miR-187 were previously reported as lung development-related miRNAs, with different expression status in mature and developing lung in human and mice (Williams et al., 2007). MiR-187 is reported to be down-regulated in developing lung. Moreover, its ectopic expression in renal cell lines affects cellular proliferation rate (Zhao et al., 2013). MiR-134 is known as a modulator of cell growth, apoptosis, and migration and is upregulated in developing lung (Williams et al., 2007; Zhang et al., 2012). Considering the important role of miRNAs in cancers, several studies have investigated miR-134 and miR-187 expressions and functions in different tumors; however, their expression alterations in lung cancer are still unclear.

## 2. Materials and methods

### 2.1. Tissues samples

36 FFPE samples of lung tumor along with their adjacent apparently normal tissues were obtained from Al-zahra hospitals pathology archive (Isfahan, Iran). Clinical and pathological characteristics of obtained tissues were as shown in Table 1. Tissue blocks were examined by an expert pathologist and the representative tumor and non-tumor areas were punched off and sectioned into 10 μm sections by a microtome

### 2.2. RNA extraction

FFPE tissues were deparaffinized with xylene, followed by absolute ethanol wash. Then tissues were digested by proteinase K (Fermentas, UK) for 3 h at 56 °C. Total RNA was isolated using RiboEx reagent (GeneAll, South Korea), according to the manufacturer's instructions, and stored at −80 °C until further investigations.

**Table 1**
Clinico-pathological characteristics of the patients with lung cancer.

| Variables | | No. of individuals |
|---|---|---|
| All samples | | 36 |
| Gender | Male | 25 |
| | Female | 11 |
| Tumor types | Adenocarcinoma | 15 |
| | Large cell carcinoma | 9 |
| | Squamous cell carcinoma | 12 |
| Tumors differentiation grade | G 1 | 7 |
| | G 2 | 10 |
| | G 3 | 11 |
| | G 4 | 8 |

### 2.3. MicroRNA quantification

MiR-134, miR-187 and 5S rRNA, as an internal control, were reverse transcribed using PARSGENOME MiR-Amp kit (Parsgenome, Iran) following manufacturer's instruction. Briefly, 2 μg of total RNA sample was polyadenylated by poly (A) polymerase and reversely transcribed into cDNA using reverse transcriptase enzyme and adaptor primers. Real-time qPCR was done in 20-μl PCR reaction using HOT FIREPol Eva Green qPCR Mix (Solis BioDyne, Estonia) and specific primer mix (Parsgenome, Iran) via an ABI 7500 Instrument (Applied Biosystems, USA). After analyzing melt curves, PCR products were sequenced to validate the accuracy of amplification. Relative expressions of miRs to 5S rRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. All reactions were performed in duplicate.

### 2.4. Construction of miR-187 overexpressing vector

Genomic sequence of miR-187 precursor was amplified by Pfu polymerase (GeneAll, South Korea), using GGGTACCCATGCACAGCAAGTC GGATT as forward primer and GGGCCCTGTGTCGAGTCCCTC as reverse primer. The amplified sequence was directly cloned into pTracer™-SV40 vector (Invitrogen, USA) and transformed to DH5α competent cells (TaKaRa, Japan). Positive selection against zeocin™ (Life Technologies, USA) was used to identify recombinant colonies. The accuracy of the recombinant vectors was confirmed by direct DNA sequencing (Macrogen, South Korea).

### 2.5. Cell cycle analysis

The human lung adenocarcinoma (A549) cell line was obtained from Stem Cell Technology Company (Tehran, Iran). Cells were cultured at 37 °C with 5% humidified $CO_2$ in high glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) and 1% Penicillin/Streptomycin solution (Biowest, Canada). For transfection, A549 cells were plated overnight in 24-well plates ($7 \times 10^4$ cells per well). After complete adhesion, cells were transfected with 1 μg of miR-187 overexpressing vector using Lipofectamin 2000 (Invitrogen, USA), according to the manufacturer instruction. Transfection rate was checked by visualizing GFP signal and by performing real-time PCR against miR-187. To determine cell cycle distribution, cells were harvested 24 h post transfection using trypsin (Gibco, USA) treatment. After ethanol fixation, cells were stained by 60 mg/mL propidium iodide solution containing RNaseA (20 mg/mL) and analyzed by FACScan cell sorter (Partec Flow Cytometry, Deutschland). Flowing software (version 2.5) was used in order to determine cell cycle profile.

### 2.6. Statistical analysis

Statistical analyses were performed using GraphPadPrism 6 software. Student t-test, one way ANOVA and receiver operating characteristic (ROC) curve analysis were employed for examining the statistical significance of observed miRNAs expression differences, and also to test whether they have enough sensitivity and specificity to distinguish tumor from non-tumor states. All values were expressed as mean ± standard error (SE), and P values less than 0.05 were considered as statistically significant.

## 3. Results

### 3.1. MiR-134 is significantly upregulated in lung tumor tissues

A real-time quantitative RT-PCR approach was applied to investigate the expression pattern of miR-134 in different types of lung tumors (adenocarcinoma, large cell carcinoma, and squamous cell carcinoma). Using a poly-A tailing method, we amplified miR-134 mature form in