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Bioinformatics analyses combined microarray identify the desregulated MicroRNAs in lung cancer

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Abstract. – BACKGROUND: MicroRNAs (miRNAs) play an important role in the regulation of cell growth, differentiation, apoptosis, and carcinogenesis. Deregulated miRNAs are found in blood cells of cancer patients recently.

AIM: This study aims to screen the differentially expressed miRNAs (DE-miRNAs) which could discriminate lung cancers from non-cancerous lung tissues as well as molecular signatures that differ in tumor histology.

MATERIALS AND METHODS: miRNA expression profiles of GSE17681 was downloaded from Gene Expression Omnibus database. Three test methods were used to identify DE-miRNAs between lung cancer tissue and healthy controls. Target genes of DE-miRNAs were retrieved from three databases and mapped to KEGG to investigate their roles in lung cancer. Further, a protein-protein interaction (PPI) network was constructed used STRING and Cytoscape.

RESULTS: A total of 17 DE-miRNAs were identified. Among them, hsa-miR-339-5p draw specific attention. Pathway analysis revealed that target genes of RASSF1 and KRAS play roles as oncogene or tumor suppressor gene in the progression of lung cancer. Besides, Target genes of RASSF1 and ERBB4 formed a module in the PPI network. Functional analysis suggested biological process of response to hypoxia was significantly enriched.

CONCLUSIONS: hsa-miR-339-5p play important role in the regulation of lung cancer and it may be potential to be used as biomarker to predict lung cancer progression.

Key Words:
MicroRNA, Differential expression analysis, Target genes, Pathway analysis.

Abbreviations

miRNA = microRNA.
DE-miRNAs = differentially expressed miRNAs.
PPI = protein-protein interaction
STRING = Search tool for the retrieval of interacting genes
RASSF1 = Ras association (RalGDS/AF-6) domain family member 1.
KRAS = V-ki-ras2 Kirsten rat sarcoma viral oncogene homolog.
ERBB = Receptor tyrosine-protein kinase
GEO = Gene Expression Omnibus
GO = Gene Ontology
DAVID = Database for Annotation, Visualization and Integrated Discovery.
KEGG = Kyoto Encyclopedia of Genes and Genomes.
NET = neuroendocrine tumor

Introduction

Lung cancer is the most common cause of cancer-related death in both men and women worldwide[1]. The five-year survival rate is the lowest of all cancer types[2]. The lung cancer is so difficult to be discovered that most of patients are at late stage when diagnosed[3]. Therefore, it is necessary for us to find new biomarkers to detect this disease in early stage.

MicroRNAs (miRNAs) are a type of small non-coding single-stranded RNAs with length of 20-25 nt typically[4]. Due to their function as regulator of gene expression, they play key roles in physiological and pathological process[5,6]. MiRNA expression is deregulated in cancer by a variety of mechanisms including amplification, deletion, mutation, and epigenetic silencing. Several studies have now shown that miRNAs are involved in the initiation and progression of cancer[7].

The miRNAs could be used to as a valuable tool in cancer diagnosis. Pioneering studies using miRNA microarray analysis[8] identified statistically unique profiles, which could easily discriminate cancers from noncancerous tissues. Indeed, miRNA expression profiles were more informative than traditional mRNA profiling. Thus, the profile of only 200 miRNAs was sufficient to classify poorly differentiated tumors in a recent study[9], with greater accuracy than a profile of 16,000 mRNAs. Another study[10] has achieved almost perfect accuracy in classifying the tissue origin of 400 tumor samples from 22 different tumor

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tissues and metastases. These findings demonstrate the effectiveness of miRNAs as biomarkers for tracing the tissue of origin of cancers of unknown primary origin, a major clinical problem[11].

The aim of the present study is to identify the miRNAs which may play important regulatory role in the progression of lung cancer and analyze their functions in the progression. Using three test methods (wilcox-test, *t*-test and exact-test) to deal with the raw data, we got the credible data of differentially expressed miRNAs (DE-miRNAs). Then, we drew an interaction network through STRING and Cytoscape. Our findings support the idea that miRNA expression was desregulated in blood cell of cancer patients compared with that of healthy individuals. Furthermore, we provide evidence that miRNA patterns can be used to detect human cancers from blood cells.

Materials and Methods

Microarray data

The miRNA expression profile of GSE 17681 was downloaded from the Gene Expression Omnibus (GEO) database, which was deposited by Keller et al[12]. we got the expression data of miRNA: GSE17681, whose test purposes were peripheral blood expression of lung cancer's samples and normal cells' samples. The expression profiles of 866 miRNAs in 17 blood samples of patients with lung cancer and in 19 blood samples of healthy controls were available. We downloaded the original CEL files and the platform probe annotation information file for the next step of bioinformatics analysis.

Identification of differentially expressed miRNAs (DE-miRNAs)

The raw data were transformed into identifiable expression measures, and then the missing part of the data was filled[13]. Robust multiarray average (RMA) was used to perform background correction and quartile data normalization with defaulted parameters in R affy package[14].

Three tests: *t* test, Wilcox test and Fisher exact test were used to identify DE-miRNAs between lung cancer patients and healthy controls, respectively. The *p*-value adjusted by the Benjamin and Hochberg (BH) method of 0.05 was used as the cut-off criterion. Then we selected the miRNAs which could be captured by these three methods as the final result.

Predicting target genes of DE-miRNAs

This analysis used the three most popular databases: TargetScan[15], miRanda[16] and PicTar[17] to predict target genes of DE-miRNAs. To reduce the false positive results, the genes predicted by at least two of these three databases were selected as DE-miRNA targets for subsequent analysis.

Network analysis and functional annotation

The STRING (Search Tool for the Retrieval of Interacting Genes)[18] database provides both experimental and predicted interaction information. Version 9.0 of STRING covers more than 1100 completely sequenced organisms. To identify the interactive relationships among target genes or other genes, we mapped the target genes of DE-miRNAs to STRING. Then, Cytoscape software[19] was used to visualize these relationships and mined modules.

The DAVID (Database for Annotation, Visualization and Integrated Discovery) provides a comprehensive set of functional annotation tools for investigators to understand biological meaning behind large list of genes[20]. In this study, we used the DAVID to annotate the function of genes in module. We selected the GO terms with adjusted *p*-value less than 0.05 and count larger than 2.

Pathway analysis

The target genes of DE-miRNA were further put into the KEGG database to identify the enriched pathways in lung cancer based on hypergeometric distribution, The count number lager than 2 and adjusted p-value less than 0.05 were chosen as cut-off criterion.

Results

Identification of DE-miRNAs

Due to various reasons, such as efficiency of RNA extraction, reverse transcription, label incorporation, exposure, and spot detection[21], the original microarray data maybe systematically biased. Therefore, we performed data preprocessing first. From Figure 1, we could find that the fluctuations after normalization were less significantly than that before normalization.

To obtain a result with high confidence, we analyzed the miRNA expression data by three statistical tests, and then the overlapping miRNAs from these three tests were selected for further analysis (Figure 2). At an adjusted *p*-value of 0.05, 17 miRNAs showed significant differential expression (Table I).

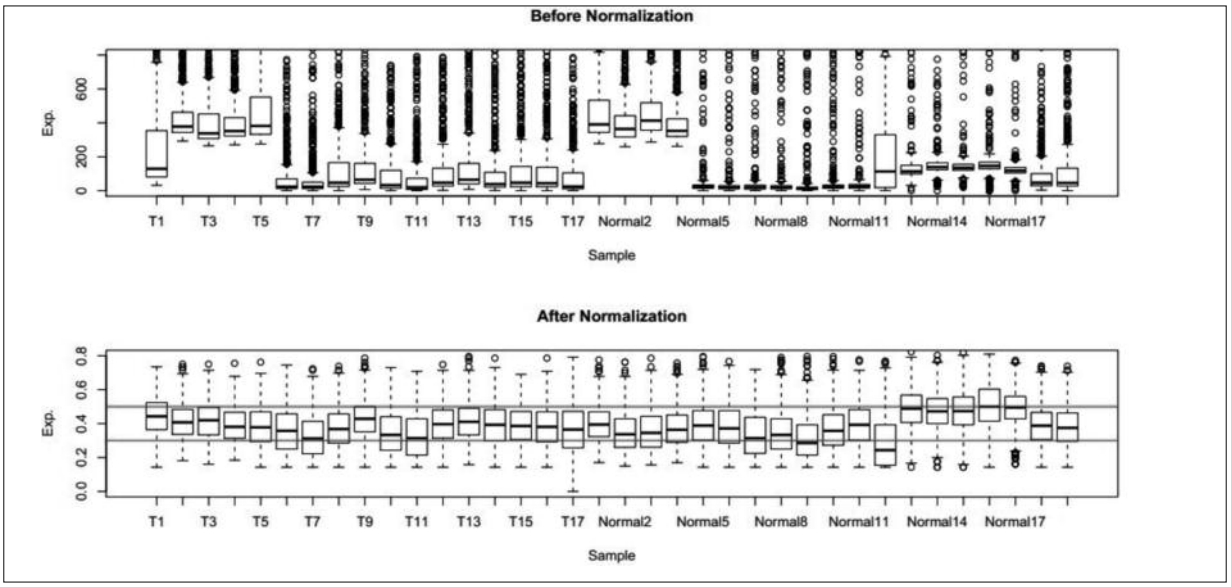

Figure 1. Boxplot shows the difference between before and after normalization. After normalization, the expression was distributed between 0.3 and 0.5.

Target genes prediction

Because miRNAs play roles in post-transcriptional regression by targeting mRNAs, we retrieved the putative target genes of DE-miRNAs from three databases and selected the target genes retrieved by at least two databases. We found target genes of six DE-miRNAs were associated with lung cancer, including hsa-miR-339-5p, hsa-miR-423-3p, hsa-miR-19a, hsa-miR-151-3p, hsa-miR-324-3p and hsa-miR-29b (Table II). Among them, the hsa-miR-339-5p had the largest number of target genes that related with lung cancer.

Pathway analysis

The target genes of DE-miRNAs were performed pathway enrichment using KEGG pathways to find the pathway of lung cancer. Figure 3 shows the distribution of target genes in the pathway of non-small cell lung cancer. The genes plus red were target genes. We could see that the predicted target genes: RASSF1 and KRAS play key roles as oncogene or tumor suppressor gene in the progression of lung cancer (Figure 3).

Interaction network construction and module analysis

We mapped the target genes of all DE-miRNAs to STRING database and screened the significant interactions with confidence score larger than 0.8. Then an protein-protein interaction (PPI) network was constructed by Cytoscape (Figure 4A).

The PPI network may aid in understanding the molecular mechanism of lung cancer, however, it contains so many nodes and interactions, which is hard to draw the useful information for us. Therefore, we mined the modules in the PPI network using Cytoscape (Figure 4B). In this module, the target genes of DE-miRNAs, RASSF1 and ERBB4 were involved. Functional analysis revealed that the genes in this module could enriched into 41 functional GO terms (the top 10 significant terms are showed in Table III). Among these functional nodes, the most significant GO category is response to hypoxia (FDR = 2.00E-02). The target genes of RASSF1 and ERBB4 were involved in GO categories of negative regulation of biological process,

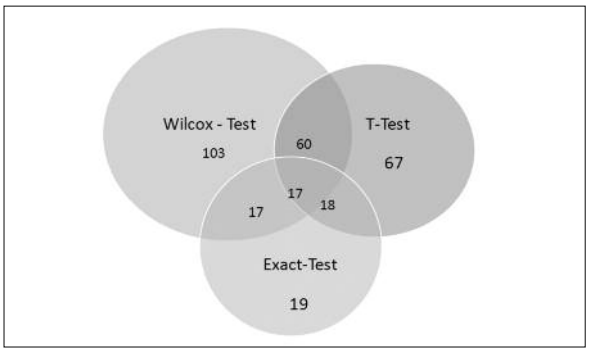

Figure 2. VENN figure shows the number of differentially expressed miRNAs identified by the three test methods. The intersection of the 3 parts was selected for further analysis.

Table I. The differentially expressed miRNAs from the three tests.

MiRNA	t-test	wilcox-test	exact-test	logFC
hsa-let-7e	0.017128	0.019364631	0.048314	-1.1692
hsa-miR-18a*	0.000715	0.001227822	0.014645	1.400385
hsa-miR-19a	0.001217	0.000975057	0.020934	1.322033
hsa-miR-324-3p	0.000211	0.00161427	0.044037	1.147487
hsa-miR-339-5p	3.71E-05	0.000125347	0.014757	1.400421
hsa-miR-361-5p	0.001429	0.00170178	0.024794	1.283974
hsa-miR-423-3p	0.000662	0.001170831	0.016045	1.380185
hsa-miR-93*	0.000144	0.000182898	0.005093	1.617376
hsa-miR-98	0.015294	0.002608095	0.008583	-1.57124
hsa-miR-126	0.077809	0.027500805	0.042787	-1.20017
hsa-miR-140-3p	0.003054	0.029932099	0.09577	0.943873
hsa-miR-22	0.00688	0.007377646	0.156253	0.80017
hsa-miR-423-5p	0.037698	0.038326302	0.278657	0.607389
hsa-miR-604	0.005902	0.004333637	0.114741	0.894142
hsa-miR-574-5p	0.096829	0.049428098	0.356751	0.51585
hsa-miR-675	0.129957	0.044937283	0.22132	0.68868
hsa-miR-939	0.096335	0.032540417	0.104941	0.919219

negative regulation of cellular process, regulation of cellular process, and regulation of biological process.

Discussion

In this study, we found 17 DE-miRNAs that may play important regulatory role in the progression of lung cancer. By retrieving the target genes of the DE-miRNAs from three widely-used databases, we found target genes of six DE-miRNAs were associated with lung cancer. Through PPI network construction and module analysis, target genes of RASSF1 and ERBB4 were formed a module in the progression of lung cancer. Functional analysis showed the module was most related with oxygen response.

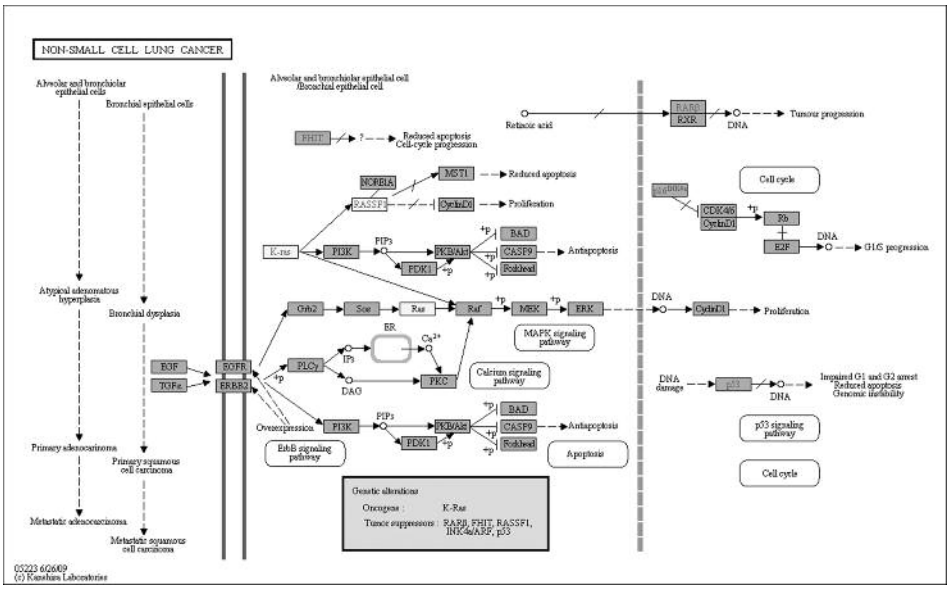

Figure 3. The KEGG pathway map of non-small cell lung cancer. The genes marked gray represent target genes of differentially expressed miRNAs.

Table II. The target genes of differentially expressed MiRNA predicted by three databases.

miRNA	Diseases	Target Gene	Sources TargetScan	miRanda	PicTar
hsa-miR-339-5p	NSCLC	RASSF1	√	√	√
	lung cancer	ARHGEF11	√	√	
	lung cancer	CAMKK1	√	√	
	lung cancer	CDKN1A	√	√	
	lung cancer	ERBB4	√	√	
	lung cancer	MMP2	√	√	
	breast cancer, colorectal cancer, lung cancer, stomach cancer	MYLK2	√	√	
	breast cancer, colorectal cancer, leukemia, liver cancer, lung cancer, stomach cancer	PTPRT	√	√	
	lung cancer	VEGFA	√	√	
	bladder cancer, leukemia myeloid, lung cancer	CYP1A1	√		
hsa-miR-423-3p	lung cancer	VEGFA	√	√	
	bladder cancer, leukemia myeloid, lung cancer	NQO1	√		
	lung cancer	NQO1	√		
hsa-miR-19a	bladder cancer, leukemia, lung cancer, upper aerodigestive tract cancer	KRAS	√		√
	lung cancer	KRAS	√		√
	lung cancer	ERBB4	√		√
	lung cancer	IGFBP3	√		√
	breast cancer, cervical cancer, colorectal cancer esophageal cancer, lung cancer, stomach cancer	CASP8	√		
hsa-miR-151-3p	bladder cancer, leukemia, lung cancer, upper aerodigestive tract cancer	TP53	√		
hsa-miR-324-3p	lung cancer	MMP2	√		√
hsa-miR-29b	breast cancer, colorectal cancer, leukemia liver cancer, lung cancer, stomach cancer	PTPRT	√	√	
	breast cancer, colorectal cancer, lung cancer, stomach cancer	MYLK2	√	√	
	lung cancer	CAMKK1	√	√	
	lung cancer	LEP	√	√	
	lung cancer	DNMT3B	√		√

The expression of 6 DE-miRNAs, including hsa-miR-339-5p which we screened could be used as biomarkers to monitor cancer progression and early diagnosis.

The pathogenesis of cancer and its associated genes has been the object of medical workers, and lung cancer due to its low survival rate and got more attention[22]. Recently reports on lung cancer-related miRNA are more and more. such as MiR-7 and miR-133 have been specified as an important regulator in the lung cancer procession[23,24]. In this study, hsa-miR-339-5p had the largest number of target genes that related with lung cancer. Ueda suggested that Dicer is responsible for the generation of the mature miR-339, which suppress intercellular cell adhesion molecule-1 expression on tumor cells, thereby down-regulating the susceptibility of tumor cells to cytotoxic T-lymphocytes-mediated cytolysis[25]. Besides, Wu et al showed that miR-339-5p could significantly

Table III. GO analysis of target genes (The top 10, FDR< 0.05).

GO-ID	FDR	Description	Genes in test set
1666	2.00E-02	Response to hypoxia	EPAS1|EPO|ARNT
48519	2.00E-02	Negative regulation of biological process	COL18A1|SERPINF1|VHL|RASSF1|TIMP2|GSTP1|EPO|ARNT
42127	3.45E-02	Regulation of cell proliferation	COL18A1|VHL|TIMP2|EPO|ARNT
43193	3.45E-02	Positive regulation of gene-specific transcription	EPO|ARNT
48523	3.45E-02	Negative regulation of cellular process	COL18A1|VHL|RASSF1|TIMP2|GSTP1|EPO|ARNT
50793	3.45E-02	Regulation of developmental process	SERPINF1|VHL|TIMP2|TIMP3|GSTP1|ARNT
51244	3.91E-02	Regulation of cellular process	COL18A1|NRG3|ERBB4|EPAS1|VHL|ITGB3|TIMP2|TIMP3|AHR|ARNT|SERPINF1|RASSF1|ITCH|SHC2|NRG2|GSTP1|EPO
32583	3.91E-02	Regulation of gene-specific transcription	EPO|ARNT
50791	3.91E-02	Regulation of biological process	COL18A1|NRG3|ERBB4|EPAS1|VHL|ITGB3|TIMP2|TIMP3|AHR|ARNT|SERPINF1|RASSF1|ITCH|SHC2|NRG2|GSTP1|EPO
6950	3.91E-02	Response to stress	EPAS1|VHL|ITCH|ITGB3|AHR|EPO|ARNT

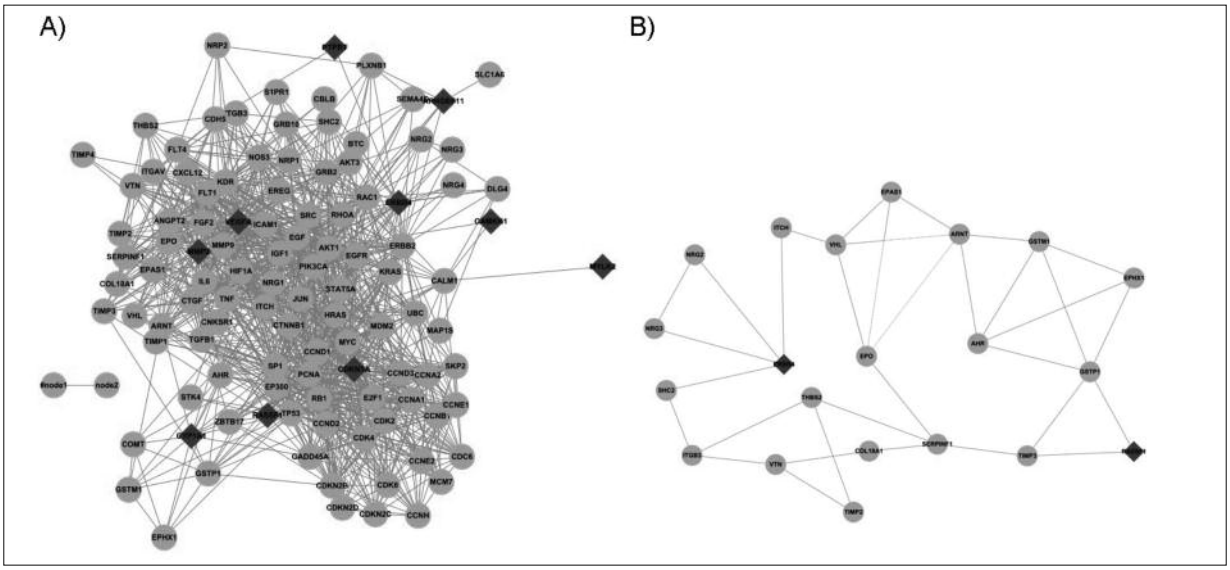

Figure 4. ***A,*** Protein-protein interaction network construction. ***B,*** The module identified from the protein-protein interaction network. The diamond nodes represent target genes of differentially expressed miRNAs. The round nodes represent their interactive genes predicted from STRING.

decrease tumor cell migration and invasion capacity, which associated with downregulation of BCL-6 expression in breast cancer cells[26]. Indeed, altered expression of miR-339-5p has been reported in several kinds of tumors, such as gastric cancer and colorectal cancer[27]. Our result is consistent with these previous studies that miR-339-5p play a role in regulation of lung cancer progression.

Target genes of RASSF1 and ERBB4 were formed a module in the PPI network constructed in lung cancer, suggesting the important roles of these two genes. RASSF1 (Ras association domain family 1) is located on chromosome 3p21.3[28,29] and encodes at least eight different transcripts (RASSF1 A-H) under alternative splicing modalities[30]. Loss or altered expression of this gene has been associated with the pathogenesis of a variety of cancers, most often include gene promoter methylation and loss of heterozygosity, suggesting the tumor suppressor function of this gene[31-33]. In addition, Pelosi et al[34] suggested that RASSF1 has dual function as tumor suppressor gene in all types of neuroendocrine

tumors (NET) for RASS1A/E isoform and as oncogene impairing patients' survival for RASSF1C isoform in high-grade NET.

ERBB 4 (Receptor tyrosine-protein kinase) is a member of the Tyr protein kinase family and the epidermal growth factor receptor subfamily. This gene has a significant role in the growth and survival of many types of human tumors[35]. Mutations in this gene have been associated with cancer[36]. Starr et al[37] provide evidence that ERBB-4 plays a significant role in human lung cancer and may serve as a molecular target for anticancer therapy.

Functional analysis showed that GO category of response to hypoxia was significant desregulated in lung cancer. Hypoxia is a common phenomenon in human tumors, with most tumors possessing lower oxygenation than their corresponding tissue of origin[38]. An aggressive phenotype has been associated with hypoxic tumors, encompassing both the well-studied resistance of poorly oxygenated cancers to radiotherapy and chemotherapy as well as a propensity for hypoxic tumors to exhibit increased potential for invasion, growth, and metastasis[39-42]. Our result suggests that hypoxia response play important role in the progression of lung cancer, which is consistent with previous studies.

Conclusions

We identified 6 DE-miRNAs which may play important regulatory roles in the progression of lung cancer. We suggested the expression profiles of these DE-miRNAs in peripheral blood cells have potential to be used as biomarkers to predict cancer progression. However, further study will be needed to verify the usefulness of these miRNAs as biomarkers before used in the clinic.

Competing Interest

None to declare.

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