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Original Article

Identification and characterization of biomarker MicroRNAs by high through-put sequencing in bone metastasis

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Abstract: Poor prognosis of patients with non-small cell lung cancer (NSCLC) indicates that metastases occur often before diagnosis of the primary tumor. To identify differentially expressed miRNAs that could function as specific biomarkers of bone metastasis in NSCLC. MiRNA high-throughput and mRNA sequence data were downloaded from GEO (Genome Expression Omnibus)dataset. Differentially expressed miRNAs between NSCLC bone metastasis samples and normal specimen were screened out. Hierarchical clustering and principal component analysis (PCA) were performed on these miRNAs and then target genes were predicted by Targetscan. Biological processes and network of putative and validated targets of miRNAs were analyzed by bioinformatics on GO annotation and KEGG pathway. Regulating network was constructed with differentially expressed miRNAs and the target genes. A total of 664 differentially expressed miRNAs and 1406 differentially expressed mRNAs were found between normal samples and bone metastasis specimens of NSCLC. Six interested miRNAs, including up-regulated miR365, miR-10b, miR-129-3P and down-regulated miRNA-671-5p, miR-141 and miR-25, were identified as predictors of bone metastasis in patients with NSCLC. In GO and KEGG analysis, the most enriched terms in both up- and down-regulated miRNAs were cytoplasmic membrane. As for the KEGG annotation of the target genes of interested miRNAs, pathway in cancer, cytokine-cytokine receptor interaction and Jak-STAT signaling pathway were found to be the most effective ones. Our results demonstrates that miR365, miR-10b, miR-129-3p, miRNA-671-5p, miR-141 and miR-25 could serve as prognostic and predictive markers for survival of bone metastasis, suggesting a potential application in improvement of prognostic tools and treatments.

Keywords: Bone metastasis, miRNA, prognostic marker, interaction

Introduction

Non-small cell lung cancer (NSCLC) is a leading cause of deaths related to cancer. More than half a million new cases of lung cancer are diagnosed every year all over the world and about 80% to 85% of tall cancer cases are of the type of non-small cell histological type in the US [1]. What's more, 30% to 40% NSCLC patients were prone to have bone metastasis [2]. Metastatic cancer is the main cause of mortality in patients with solid tumors [3]. Recently, many molecular genetic studies have been conducted in order to investigate genes and gene products that stimulate the metastatic process [4, 5]. However, due to the heterogeneity of the metastatic process and the focal nature of oncogene or suppressor gene alterations, the role of these genes in the onset of metastases and the diagnostic and prognostic value of such gene alterations are still limited [6].

MicroRNA (miRNAs), a growing class of small single-stranded noncoding RNAs found in diverse organisms, regulate gene expression at post-transcriptional level by regulating the expression of target genes [7]. Early studies showed that many miRNAs target mRNAs were involved in processes aberrant in tumorigenesis, such as proliferation, survival and differentiation. And what's more, evidence had shown that miRNAs played pivotal roles in the develop-

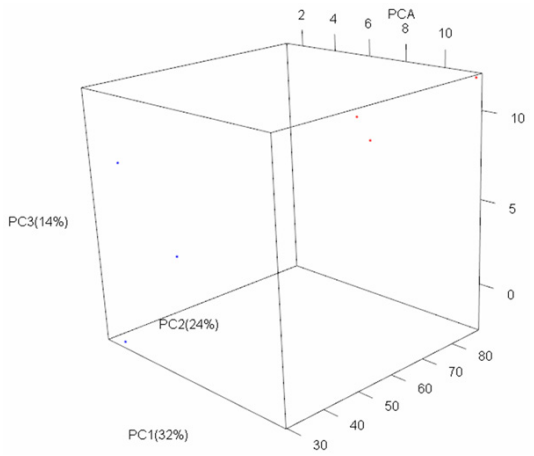

Figure 1. PCA of expression values of miRNA. The horizontal axis represents that the first component accounts for 33% while the vertical axis represents 21%. The red dots represent samples with bone metastasis in non-small-cell lung cancer patients while blue dots represent the normal ones.

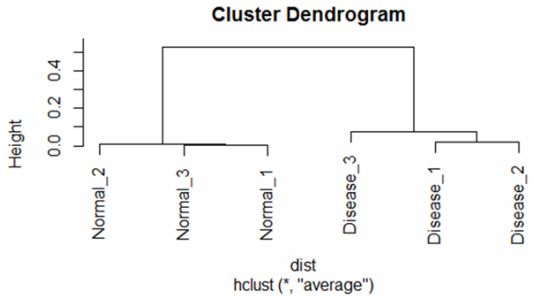

Figure 2. Cluster analysis of miRNA expression value. The vertical axis represents the height, revealing the differences among samples. The cluster tree suggests that samples with the similar expression value will be gathered.

ment and progression of human malignances. Previous studies have suggested that miRNA profiling can be used for prognostication in lung cancer [8-10].

Although the biological functions of most miRNAs are not yet fully understood, their role in the regulation of cellular differentiation, proliferation, apoptosis and gene regulation, cancer development has drawn much attention in clinical management [11, 12]. Differentially expressed miRNAs in normal tissues and cancers contribute to cancer development and progression [13]. However, the specific role of miRNAs in the metastatic process is still unknown. Therefore, screening specific miRNAs that are differentially expressed in bone metastasis of NSCLC will provide a better understanding of the mechanism for the occurrence of the disease. However, in the selection of miRNA markers for bone metastasis in NSCLC prognosis, the applying of following aspects, such as small dataset, explanatory variables, single miRNA analysis, pre-selection of miRNAs and use of approaches, finally lead to a variety set of different miRNA markers.

The main purpose of this study is to identify specific miRNA markers that are closely associated with progression of bone metastasis in patients with NSCLC by analyzing significantly altered miRNAs in a large dataset. Another goal is to investigate the availability and rationality of interactions of interested miRNAs as prognostic and predictive indictors for clinical outcome of bone metastasis in NSCLC patients. In this study, we found that six miRNAs, miR-365, miR-10b, miR-129-3p, miR-671-5p miR141, miR-25 and three miRNA-interactions could function as prognostic and predictive markers of bone metastasis in patients with NSCLC.

Materials and methods

Data source and data preprocessing

Microarray data of miRNA and mRNA were downloaded from Genome expression omnibus (GEO) dataset, with the accession number of GSE10096 and GSE47056. High-throughput sequencing of miRNA and mRNA sequencing data were downloaded, and nineteen samples were included, including miRNAs samples (three normal miRNA samples and three samples from bone metastasis of NSCLC), and mRNA samples (four normal mRNA and nine samples from bone metastasis of NSCLC patients). The data platform was Affymetrix Human Gene 1.0 ST Array. Data with low quality and batch difference was eliminated.

Screening of differentially expressed miRNAs and mRNAs

SAMR package [14] in R language was used to screen the differentially expressed miRNAs between the normal bile duct tissue and bone samples with non-small-cell lung cancer. FC=2 and FDR <0.05 were used as the cut-off criterion. Cluster analysis was performed on these differentially expressed miRNAs to ensure whether the difference between normal sam-

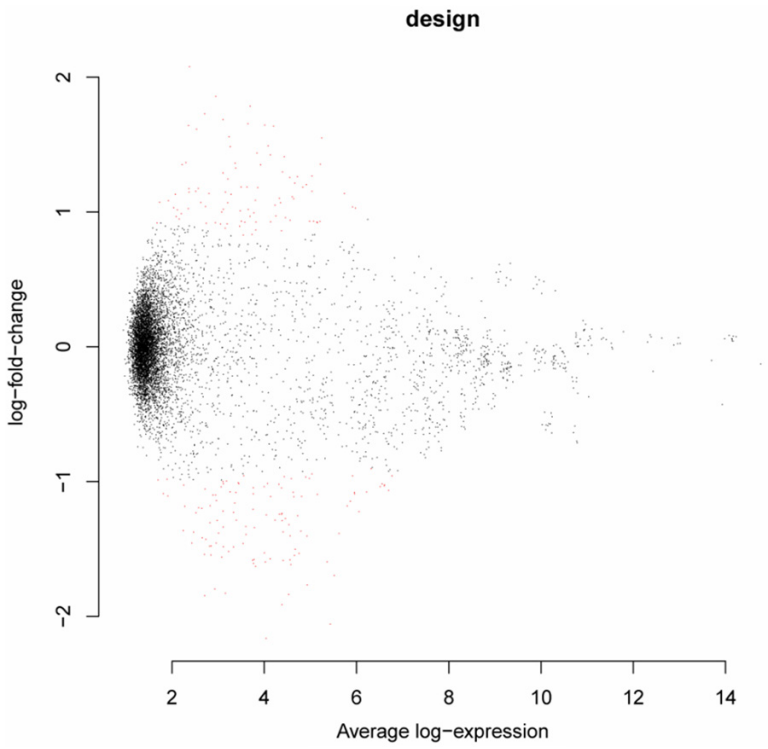

Figure 3. Differentially expressed analysis of miRNA. The relation between log-expression and miRNA. The black dots represent the no differentially expressed miRNAs while the red dots represent the differentially expressed miRNAs. The red dots above the figure stand for the miRNAs with the increased expression values while the below ones represent those with the decreased expression values.

ples and bone metastasis specimens of non-small-cell lung cancer was significant. Cluster heatmap was drawn out afterwards. PCA (Principal Component Analysis) [15] was used to justify whether normal bile duct tissues can clearly differentiate from those bone tissues with non-small-cell lung cancer. Meanwhile, differentially expressed mRNAs were screened out in the same way.

Screening of target genes and functional enrichment analysis

Target gene data of differentially expressed miRNA were extracted out from Mirtarbase [16] dataset. Then, GO (Gene Ontology) [17] functional annotation and KEGG (Kyoto Encyclopedia of Genes and Genomes) [18] pathway analysis were performed by DAVID (Database for Annotation, Visualization, and Integrated Discovery). The enriched *p* value and the corrected *p* value after multiple test (Benjamini rectification) were obtained by DAVID [19, 20] analysis. Potential targets of miRNA were predicted by Targetscan software, and then GO and KEGG functional analysis were performed on these genes in order to obtain the potential therapy targets [21].

Construction and analysisofmiRNA-mRNA interaction network

Network of target genes and protein was constructed by Cytoscape and then the topology character was analyzed by Network Analyzer, plug-in of Cytoscape [22]. Cytoscape is an open source software project and used to integrate the network with expression profiles, phenotypes, and other molecular state visually [22]. Clusterone, another plug-in of Cytoscape, was used to module functions in the network. *P*-value =1.0e-0.5 was used as the threshold and modules ranging the top three was selected out to carry the functional analysis. Correlation coefficient of miRNA-mRNA interactions was calculated out and the network structure was observed by constructing NP network. Function of each cluster tree was obtained by analyzing the cluster tree with different network structures.

Results

Data source

A total of 20023 miRNA expression values were identified from the six sample dataset (three normal samples and three bone tissue from NSCLC samples). The difference between normal samples and bone tissue from non-small-cell lung cancer specimens were figured out by PCA and cluster analysis (**Figures 1** and **2**). As can be clearly seen from the figure, bone metastasis samples from non-small cell lung cancer were clustered on the top-right area while the normal samples were on the below-right area, suggesting the significant difference among the samples.

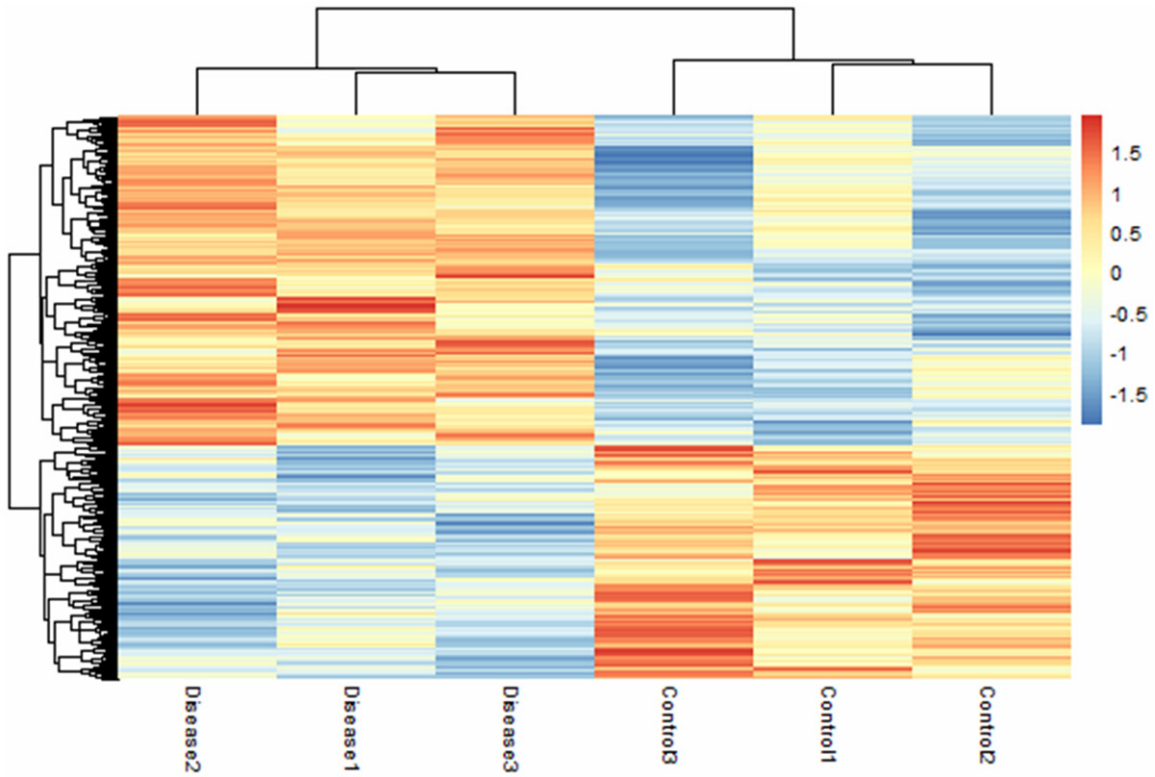

Figure 4. Cluster analysis of differentially expressed miRNA. The horizontal axis below represents sample names. The vertical axis on the right stands for the names of miRNA, while the left ones represent the cluster of miRNA. The red color stands for the up-regulation of miRNA while the blue color represents the down-regulation of miRNA. Two clusters of samples are included, one is the normal bile duct tissue while the other is from the bone metastasis of non-small-cell lung cancer patients. MiRNAs can also be divided into two kinds, one is the down-regulated miRNAs in bone metastasis tissue of non-small-cell lung cancer and the other is the up-regulated miRNAs.

Differentially expressed analysis of miRNA

A total of 664 differentially expressed miRNAs were found out between normal samples and bone metastasis of non-small-cell lung cancer specimens by R package, including 387 up-regulated miRNAs and 277 down-regulated ones, accounting for 58.3% and 41.7%, respectively. Hereinto, miR365, miR-10b, miR-129-3P were identified significantly different among up-regulated miRNAs; while miRNA-671-5p, miR-141 and miR-25 were the significant ones identified from the down-regulated miRNAs (**Figures 3** and **4**).

Functional analysis of target genes of miRNA

4366 target genes of down-regulated miRNAs were predicted while 3708 target genes of up-regulated miRNAs were found out (**Figures 5** and **6**). GO and KEGG pathway analysis were performed by DAVID on these target genes (P=0.05). There were 11 GO terms and 5 KEGG pathways enriched in target genes of down-regulated miRNAs. Hereinto, ion-binding was found to be the most obvious in GO term and graft-versus-host disease was the most enriched KEGG pathway. Meanwhile, there were 13 GO terms and 4 KEGG pathways enriched in up-regulated miRNAs. Cation binding and Jak-STAT signaling pathway were the most significant terms in GO annotation and KEGG annotation, respectively. The most enriched terms in up- and down-regulated miRNAs were both related with cytoplasmic membrane, in response to the exchange of material, energy and information with the outside environment, which indicted its association with the occurrence of the disease.

Differentially expressed mRNAs analysis

There were 1406 differentially expressed mRNAs between the normal samples and bone metastasis samples from non-small-cell lung cancer by R package, including 625 up-regulat-

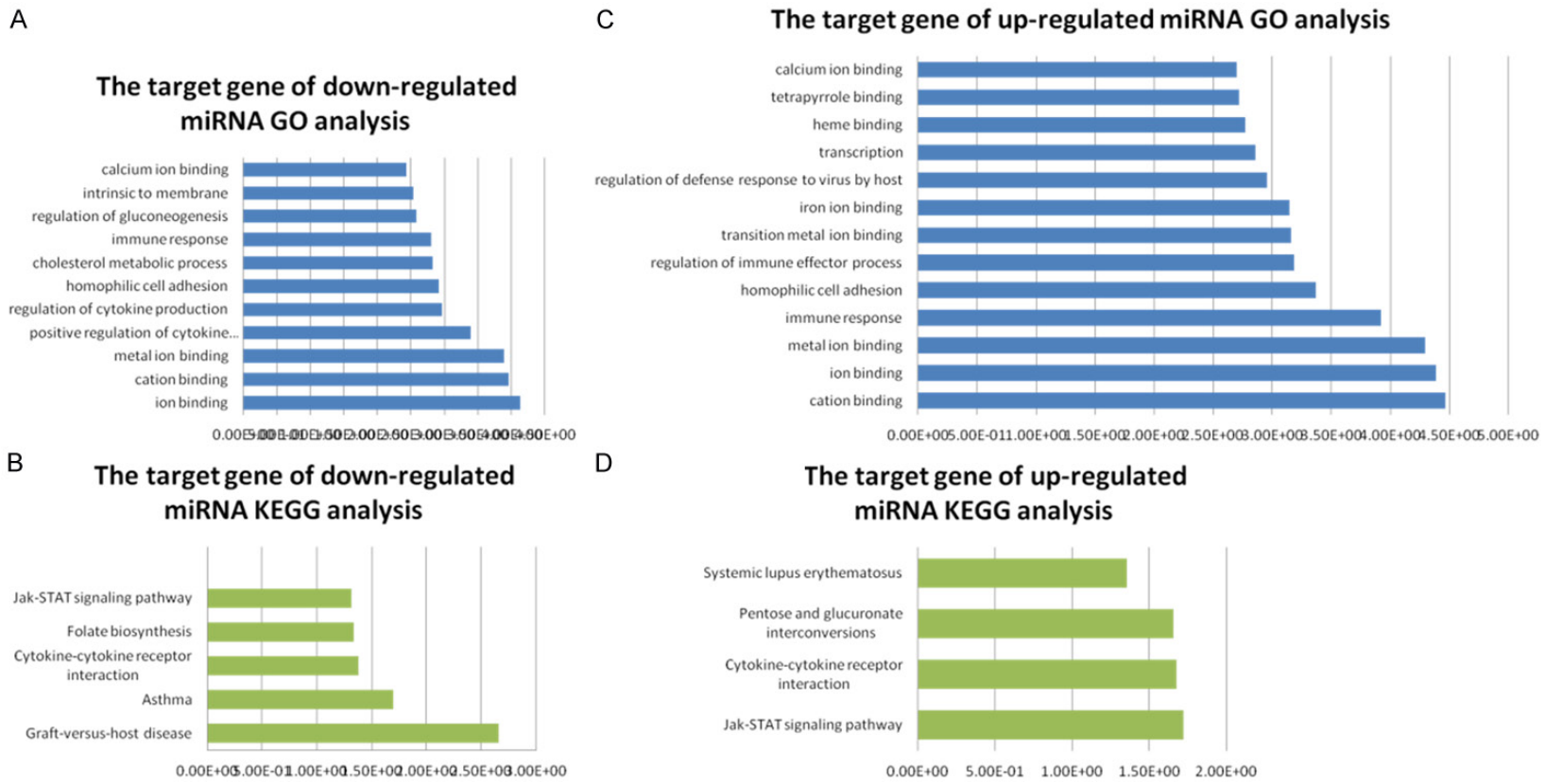

Figure 5. Go and KEGG analysis of target genes of differentially expressed miRNA. A, B. Are the GO analysis of target genes of up-regulated miRNA. C, D. Are the KEGG analysis of target genes. The horizontal axis represents the significant degree while the vertical axis stands for the function annotation.

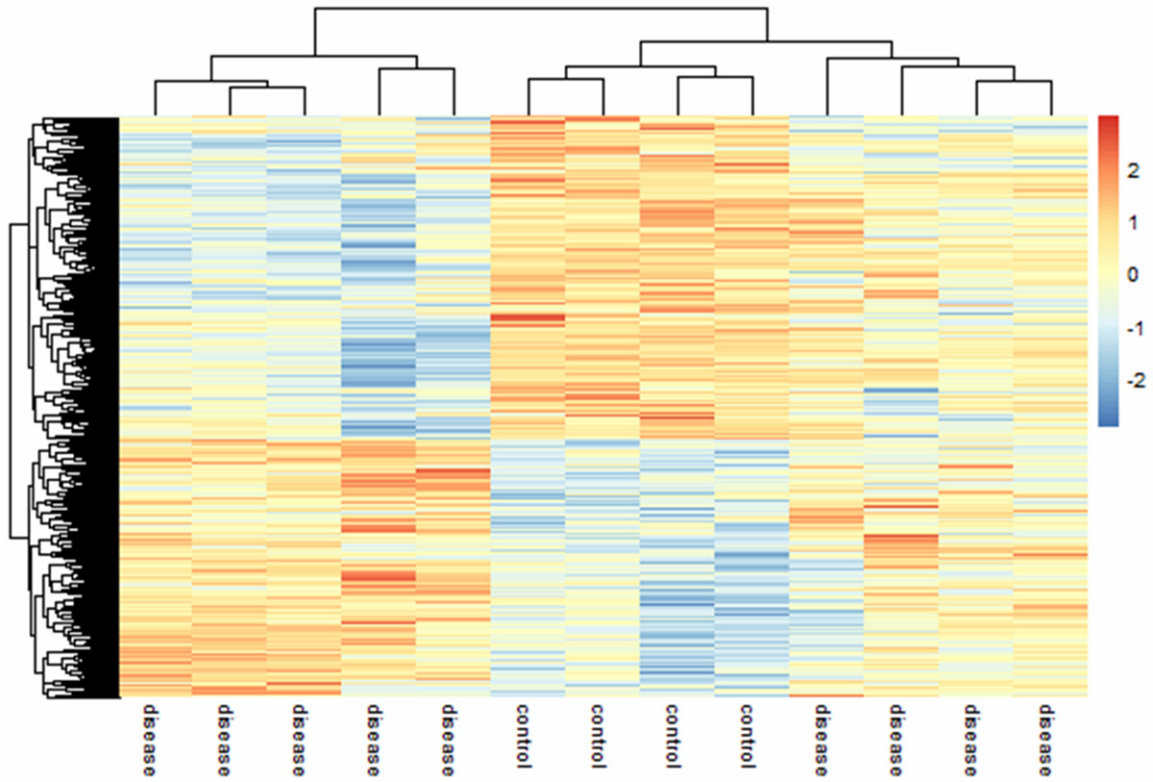

Figure 6. Differentially expressed mRNA analysis. The horizontal axis below is the sample names. The vertical axis on the right is the mRNA names while the left ones are the cluster situation of miRNAs. The red color represents the up-regulated miRNAs while the blue represents the down-regulated mRNA. There are two kinds of clusters, one is the normal tissue and the other is the bone metastasis of non-small-cell lung cancer. Expression of miRNAs can also be divided into two clusters, one is the down-regulated mRNA in bone metastasis of non-small-cell lung cancer and the other is the up-regulated mRNA.

ed mRNA and 781 down-regulated mRNA, accounting for 44.5% and 55.5%, respectively.

Network analysis of differentially expressed miRNA and target genes

Topology calculation of the network was conducted by the properties of node degree distribution, the shortest path distribution, the closeness centrality, and the topology degree (**Figure 7**). Codes degree distribution of protein-protein interaction (PPI) network of these miRNAs was in the form of power rate and had the structure of small-world, revealed by the character of average shortest path and the larger average accumulation character. The top three miRNAs with the largest degree of nodes were selected out for further functional analysis. As for the down-regulated miRNAs, there were 264 target genes of has-miR-141, 206 target genes of miR-20 and 206 target genes of miR-183. Meanwhile, among the up-regulated miRNAs, there were 204 target genes of has-miR-365, 132 target genes of has-miR-576, 78 target genes of has-miR-184 (**Figure 8**). Then, KEGG annotation was performed on these target genes. Three pathways, pathway in cancer, cytokine-cytokine receptor interaction and Jak-STAT signaling pathway were found to be the most effective pathways (**Table 1**).

Discussion

Non-small-cell lung cancer is the leading causes of all cancer-related death worldwide. And it accounts for 80% to 85% of all lung cancer in the US [1]. Metastasis was recognized as a late event in the natural history of epithelial tumors and micrometastases occur often before diagnosis of the primary tumor by the poor prognosis of patients with lung cancer [23]. Therefore, the discovery of new biomarkers of bone metastasis in non-small cell lung cancer could be of much benefit in diagnostic and prognostic use.

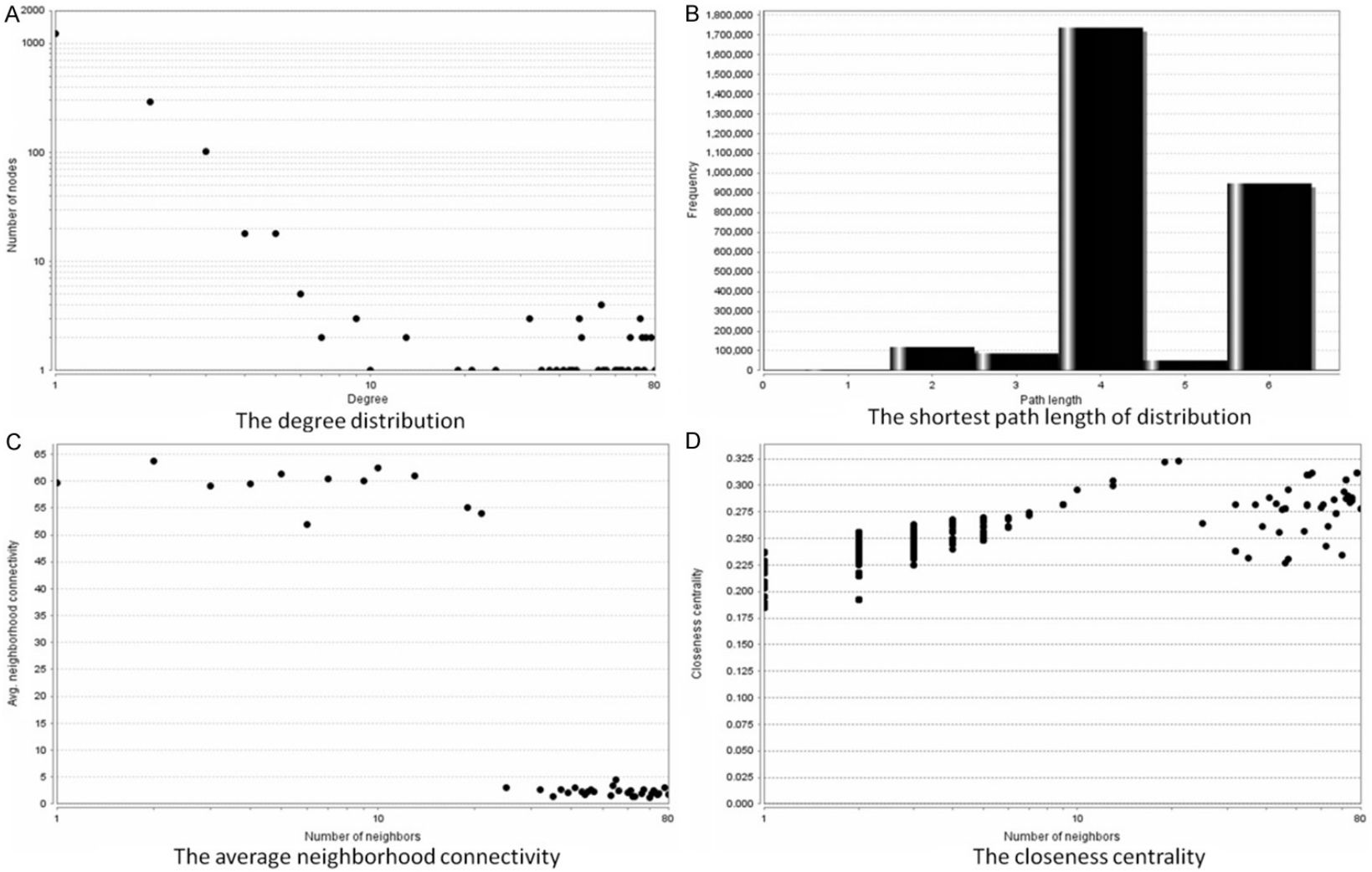

Figure 7. The topological structure analysis of network. A. Is the node degree distribution. B. Is the shortest length distirbution. C. Is the closeness centrality. D. Is the topological degree.

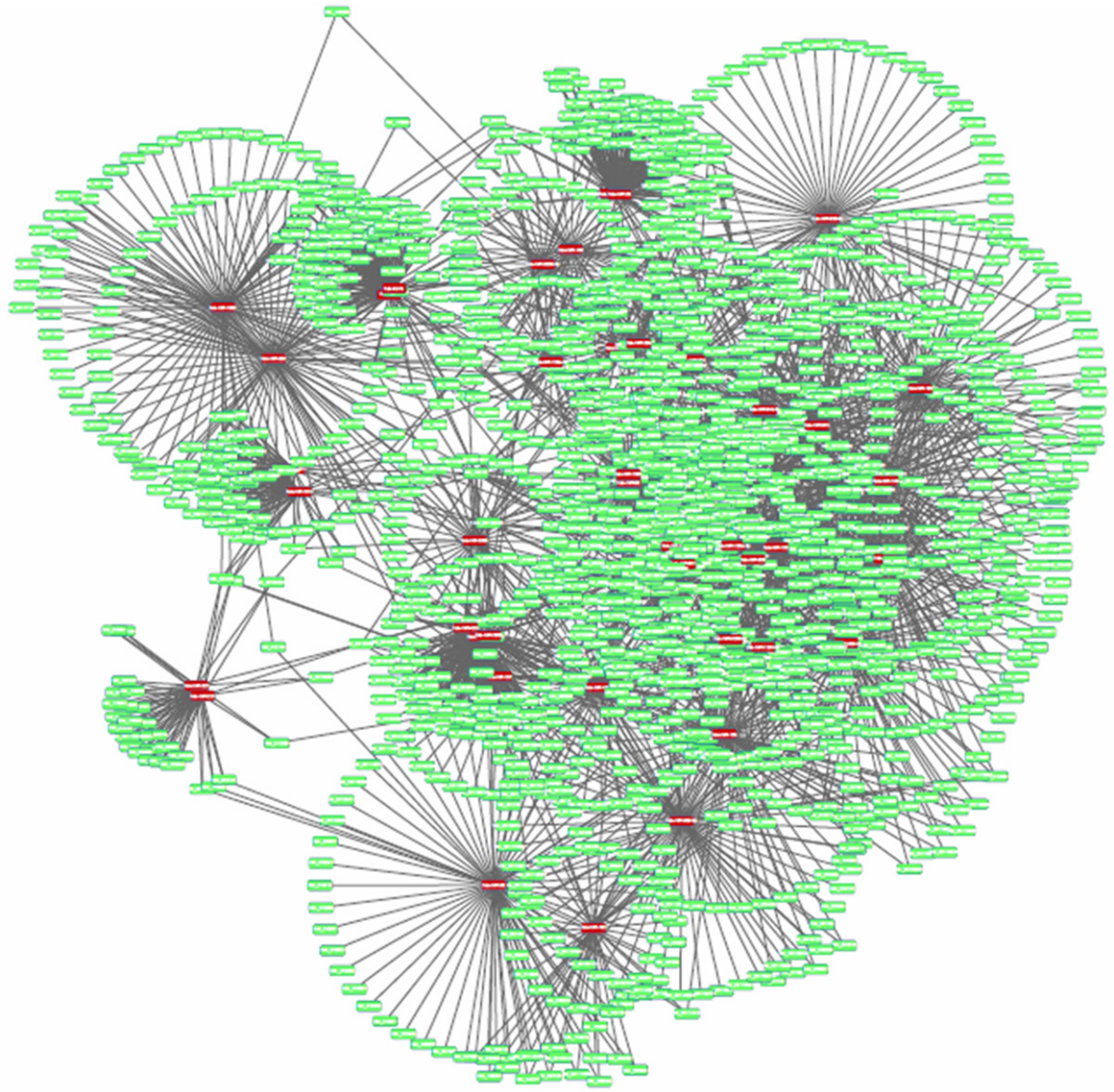

Figure 8. Network of the differentially expressed miRNA with the target genes. The red dots represent the miRNAs while the blue dots represent the target genes. As is shown in the figure, each miRNA has a dozen of target gens, and furthermore, more than one miRNAs can act on the same target gene.

Table 1. KEGG pathway analysis of modules ranging the top 3

	Term	Genes
Module 1	Pathway in cancer	NM_001079846, NM_000639, NM_000059, NM_053056, NM_005559, NM_005560, NM_001077493, NM_003466
Module 2	Cytokine-cytokine receptor interaction	NM_005114, NM_020346, NM_173678, NM_176823, NM_022970, NM_001135636, NM_019892, NM_201533, NM_201532, NM_001111125, NM_014043
Module 3	Jak-STAT signaling pathway	NM_020814, NM_012282, NM_015160, NM_001039396, NM_001042432, NM_014638, NM_003557, NM_001135636, NM_018209, NM_024591, NM_019619, NM_175609, NM_015075

Recent evidence indicates that the non-coding molecular RNA known as small microRNAs (miRNAs) can function as tumor suppressor or oncogenes [24]. Disregulation and mis-expression of miRNAs in bone metastasis of NSCLC could be beneficial for the molecular mechanism research of the disease in clinical utility. Differentially expressed genes screening and corresponding functional identification can get easier and more accurate by high-throughput

screening and then help us to make clear the molecular mechanism and diagnosis of the disease. Unfortunately, the study of high-throughput screening is rare due to expensive equipment and annotation probe [21]. In this study, we screened the differentially expressed miRNAs in the bone metastasis of non-small-cell lung cancer and explored their relationship with the occurrence with bone metastasis of NSCLC. As a result, a total of 799 differentially expressed miRNAs were identified out, among which six miRNAs, including up-regulated miR365, miR-10b, miR-129-3p and down-regulated miRNA-671-5p, miR-141 and miR-25, were figured as the most important network nodes, which may function as biomarkers of bone metastasis in non-small-cell lung cancer diagnosis.

Early reports of miRNAs in bone metastasis of non-small-cell lung cancer may help us better understand the mechanism. MiRNA 21 was identified up-regulated in the serum of patients with bone metastasis of non-small-cell lung cancer and there had been reports about its role in cell proliferation and apoptosis [11, 25]. Once it's inhibited, the proliferation of RBE cells was also prohibited and the speed of apoptosis increased. Let-7 family had also been proposed to function in tumor progression and reduced expression of let-7 family members were common in bone metastasis of non-small cell lung cancer [26]. In this study, let-7a was found to be down-regulated in the bone metastasis of NSCLC samples, corresponding with the early discoveries, confirming its close association with the disease. Besides, its target gene, NF-2, was found to be anti-oncogene. NF-2 exerted negative regulation on Stat-3, one of the signal transmitting and transportation activating factor, which happened to be one of the enriched GO terms we found in the biological function analysis.

One of the six differentially expressed miRNAs, miR365, was reported to regulate cellular proliferation and modulate cell growth phenotypes and can stimulate chondrocyte differentiation through targeting histone deacetylase 4. Mir-129-3p was also reported to regulate cell proliferation by down-regulating Cdk6 expression [27]. Hence, we speculated their up-regulation had essential predictive significance of the bone metastasis in NSCLC samples, the compensatory mechanism, perhaps. As for down-regulated miRNAs, miR-141 and miR-671-5p and miR25 had been reported to be associated with colon cancer with metastasis [13, 28] and their down-regulation in NSCLC may have pathogenic effect on bone metastasis, which needs more exploration to confirm its diagnostic value. Therefore, the exact mechanism of the regulation effect of these miRNAs needs more research.

The most enriched GO term we found in the study, STAT (signal transducers and activators of transcription) signaling pathway, was reported to be activated with subsequent suppression of apoptosis in early stage of bone metastasis of NSCLC [29]. Stat regulates a number of pathways that's important in tumorigenesis, including cell cycle progression, apoptosis, tumor angiogenesis, invasion and metastasis, and tumor cell evasion of the immune system [30, 31]. One critical role of Stat3 is to protect cells against apoptosis through the transcriptional level [32-34]. Results of certain researches were consistent with the role of target genes of the differentially expressed miRNA played in the disease [32-34]. By the way, in the functional analysis of the target genes of miRNA, we found that the occurrence of bone metastasis of NSCLC was mainly related to the ion-binding ability of proteins. For example, once the location of phosphorylation protein was affected, the disregulation of activation and inactivation of proteins would be triggered. What's worse, the process can also affect the regulating interaction of protein and DNA. Protein phospholyation was reported to be associated with various signaling pathways and plays essential roles in regulating many biological processes. Abnormal regulation of protein phosphorylation had been found in a serial of diseases, such as cancer, Alzheimer's disease, non-small-cell lung cancer, etc. The discovery of its association with bone metastasis in NSCLC samples hadn't been reported before, which made our discovery more meaningful [13, 27, 28]. What's more, in GO and KEGG analysis, the most enriched terms in both up- and down-regulated miRNAs were cytoplasmic membrane, which in charge of the exchange of material, energy and information with the outside environment, making our conclusions more reliable.

In conclusion, metastatic miRNA signature in NSCLC samples has never been evaluated. We consequently undertook to find a specific miRNA expression signature characteristic of

the metastatic phenotype of non-small-cell carcinoma. Our results suggest that six specific miRNAs, miR-365, miR-10b, miR-129-3p, miR-671-5p, miR-141 and miR-25, may be directly involved in bone metastasis of NSCLC samples and may represent a novel diagnostic tool in the characterization of bone metastasis of NSCLC cancer gene targets. What's more, phosphorylation and Stat signaling pathways were found to be closely associated with bone metastasis of non-small-cell lung cancer, providing new insights into the pathogenesis of bone metastasis of NSCLC and feasible suggestions for treatment strategies.

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Disclosure of conflict of interest

None.

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