Clinics of Oncology

Research Article

ISSN: 2640-1037 |Volume 6

Combined Analysis of Micro RNA and Proteomic Profiles and Interactions in Patients with Primary Lung Adenocarcinoma and Lung Adenocarcinoma Brain Metastases

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Received: 02 Mar 2022
Accepted: 15 Mar 2022
Published: 21 Mar 2022
J Short Name: COO

Citation:

Tang C, Liang J, Zhang S, Combined Analysis of Micro RNA and Proteomic Profiles and Interactions in Patients with Primary Lung Adenocarcinoma and Lung Adenocarcinoma Brain Metastases. Clin Onco. 2022; 6(3): 1-10

Keywords:

Brain metastases; Lung adenocarcinoma; microRNAs, Proteomics

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1. Abstract

1.1. Objective: Brain metastasis of lung adenocarcinoma is the main cause of death in such patients. There is an urgent need to explore highly sensitive and specific biomarkers for the early detection and treatment of lung adenocarcinoma brain metastases.

1.2. Materials and Methods: We carried out an analysis of miRNAs expression profiles and protein spectrums of non-metastatic primary Lung Adenocarcinoma (LP) and patients with Brain Metastases (BM) to better explore the molecular basis of BM. The potential roles and interactions of differentially expressed miRNAs and proteins were classified by gene ontology enrichment, Kyoto Encyclopedia of Genes and Genomes pathway analyses. The interaction between the differentially expressed miRNAs and proteins was analyzed by R soft and shown by Cytoscape.

1.3. Results: Compared with LP patients, 16 miRNAs were upregulated and 4 miRNAs were downregulated in the tissue of BM patients, 53 proteins were upregulated and 35 proteins were downregulated meanwhile. Enrichment pathway analysis revealed that NF-κB signaling pathway and primary immunodeficiency pathway played critical roles in the occurrence and development of brain metastasis in lung adenocarcinoma.

1.4. Conclusion: This study broadened the understanding of the regulatory network of microRNA and proteomic expressions and provided clues for the pathogenitic molecular mechanism of brain metastases in lung adenocarcinoma at the miRNA and protein levels.

2. Introduction

Lung cancer is characterized by a high incidence of brain metastases, with about 40% of patients developing brain metastases

during their disease course [1]. The prognosis of Non-Small Cell Lung Cancer (NSCLC) patients with brain metastasis is very poor, which is the main cause of death. The post-brain metastasis survival time of these patients is only 1-2 months if untreated [2]. The efficacy remains unsatisfactory even though the treatments for brain matastasis include surgery, radiotherapy, chemotherapy and immunotherapy nowadays. Therefore, it is an urgent task that further study of biological and molecular mechanism underlying BM in NSCLC needs to be done to identify new treatment targets.

MicroRNAs have been revealed as essential part of the un coding genome, playing crucial roles through a complicated regulation in all the most important processes and in different species [3]. Early studies have shown that microRNA expression profiling was associated with tumor development, progression and response to therapy, which suggests that their possible use as diagnostic, prognostic and predictive biomarkers [4]. Several miRNAs are known to play key roles in the development of NSCLC [5]. NSCLC metastasis could be inhibited through manipulation of some miRNAs in preclinical studies [6, 7]. Increasingly evidence indicate that microRNAs are target keys involved in metastasis, which could act as biomarkers for combating brain metastasis [8, 9].

Proteomic analysis is currently considered to be a powerful tool for global evaluation of protein expression which has been widely applied in fields of cancer research. Quantitative protein expression profiling is a crucial part of proteomics, which requires methods that are able to provide accurate and reproducible differential expression values for proteins in two or more biological samples efficiently [10].

Such profilings have been used for screening metastasis-associated proteins in recent studies [11, 12]. In this study, we analyzed the pathogenesis of Lung Adenocarcinoma(LAC) brain metastasis by detecting the differences in miRNA expression and proteome expression in primary lesion of LAC and brain metastasis tissue samples, with a view to finding biomarkers of LAC brain metastasis and researching for potential therapeutic targets.

3. Materials and Methods

3.1. Samples

This study was reviewed and approved by the Medical Ethics Committee of Peking University International Hospital and informed consent forms were signed by all patients. All experiments were carried out in accordance with relevant guidelines and regulations or Declaration of helsinki. From September 2018 to October 2019, Participants with histologically confirmed LAC treated in our hospital were recruited. The inclusion criteria were as follows:

1) patients diagnosed as pulmonary adenocarcinoma or LAC brain metastasis by surgery;

2) Eastern Cooperative Oncology Group performance status (ECOG) was 0–2;

3) function of the bone marrow, heart, liver, kidney and other organs was not abnormal;

4) survival period longer than 3 months;

5) patients agreed with complete ctDNA tests for tissue samples;

6) informed consent form was signed voluntarily by participants.

The exclusion criteria included patients with multiple primary carcinomas, severe systemic diseases, active infections, and immune diseases.

All participants were divided into the Lung Primary (LP) group (n=5, named as L1, L2, L3, L4, and L5) and the Brain Metastasis (BM) group (n=5, named as B1, B2, B3, B4, and B5).Tissues from the primary tumor sites in LP group, and from the brain metastasis tumor sites in BM group were sampled respectively, which were placed into 10% neutral buffered formalin solution within 5 minutes after excision, fixed at room temperature (8–72 h) and then processed routinely into a paraffin embedded tissue block (FFPE). All samples were stored at 4℃, until we had compiled the cohort and were ready to begin the study. Then, the FFPE blocks were sectioned to prepare an H&E stained slide and unstained sections (5μm thick) on glass slides for RNA extraction. The location of tumor tissue was determined under microscope on H&E stained slide and sections of adjacent layers were taken.

3.2. RNA Extraction and Sequencing

Total RNA was isolated from the Formalin-Fixed Paraffin-Embedded (FFPE) tumors using the Trizol reagent (Invitrogen, USA). The purity and integrity of RNA were detected by using an Agilent 2100 Bioanalyzer system (Agilent, USA). RNA samples with RIN ≥8 and 28S/18S ≥1 were considered. The cDNA library of all samples was sequenced using the Illumina HiSeq 2000 sequencing platform (Illumina, USA).

3.3. Liquid Chromatography Tandem Mass Spectrometry

The Liquid Mass System(LMS) includes an Easy nLC1000 (Thermo Fisher) coupled ultra-high resolution mass spectrometer Orbitrap Fusion Lumos (Thermo Fisher) with a Thermo Fisher electrospray source. Each injection is sent to a preset column (Acclaim PepMap C18, 100 μm x 2 cm, Thermo Scientific) for adsorption at a flow rate of 3 L/min. The sample is then sent to the analyzer column (Acclaim PepMap C18, 75 μm x 15 cm, Thermo Scientific) for separation.

Orbitrap Fusion Lumos was set to OT-OT mode. For a full first-level scan, the AGC Target was set to 5E5, with a scanning range of 350-1550 m/z, a resolution of 120,000 and a maximum injection time of 50 ms. For the second scan, the cycle time was set to 3.5s, the peptides with a charge of 2-7 were selected for fragmentation, and the collision energy was 32%. Maxquant (1.6.2.10) was used for data analysis. Mass tolerance was set at 20ppm and FDR at 1%.

3.4. Data Quality Control

The raw reads obtained included low quality and other problematic reads, such as those with missing insert tags, oversized inserts, poly(A) tags, and small tags. Data cleaning was therefore performed on the FASTQ file to obtain the final clean reads. Data cleaning was performed as follows: 1. Reads in which more than 50% of bases had a Qphred less than or equal to 5 were discarded; Reads in which N accounted for more than 10% were discarded. (N indicates that base information was indeterminable.); Reads with 5' primer contamination were discarded; Reads lacking a 3' primer or insert tag were discarded; The 3' primer sequence was trimmed; Reads with poly(A), poly(T), poly(G), or poly(C) tails were discarded.

3.5. Prediction of Novel miRNA

The small RNA reads were mapped to the genome using Bowtie so as to analyze their expression and distribution on the genome. The above reads on the reference sequence were compared with the specified sequence in the miRBase to obtain details of the sRNAs on each sample match, including the secondary structure of the matched miRNAs, the sequence of miRNAs in each sample, length, the number of occurrences and other information. Then we used mirdeep2 to predict novel miRNA [13].

3.6. GO and KEGG Enrichment Analysis of Target Gene of Differentially Expressed Mirnas and Proteins

Gene Ontology (GO) enrichment analysis of target gene of differentially expressed miRNAs was implemented by the topGO R package, in which gene length bias was corrected. GO terms with corrected P value less than 0.05 were considered significantly enriched by differential expressed genes.

KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (http://www.genome.jp/kegg/). We used cluster Profiler R package to test the statistical enrichment of differential expression genes in KEGG pathways.

3.7. Statistical Analysis

Statistical differences were analysed using SPSS (version 20.0, IBM SPSS Statistics) by independent-samples t test. Multiple comparisons were carried out using one-way analysis of variance. P values below .05 was defined as the significance. To control for multiple testing the FDR was estimated [14]. To assess the relevance, a linear model was applied for continuous covariates and the ANOVA test for categorical covariates. All statistical analysis was performed using R software v4.0.3.

4. Results

4.1. Data Quality Control

Age and gender of the patients are described in (Table 1), and all patients had no smoking history. RNAs were extracted from FFPE tumors of each sample, and were carried out quality detection according to the previous experimental design. The results are shown in (Table 2). The proportion of quality score ≥20 (Q20) was higher than 98%, and the proportion of quality score ≥30 (Q30) was higher than 94%. The single base error rate was 0.001.

Table 1: Characteristics of the patients

Sample	Gender	Age	Smoking history
BM1	Male	63	No
BM2	Male	75	No
BM3	Female	82	No
BM4	Female	52	No
BM5	Female	48	No
LP1	Female	70	No
LP2	Male	66	No
LP3	Female	60	No
LP4	Male	62	No
LP5	Female	57	No

Table 2: Results of data quality control

Sample	Raw Reads	Bases	GC (%)	Q20	Q30	Avg. Quality
BM1	3839266	0.192(GB)	53	98.63%	95.68%	35.83
BM2	3735819	0.187(GB)	53	98.60%	95.61%	35.82
BM3	4123090	0.206(GB)	53	98.58%	95.50%	35.8
BM4	3340001	0.167(GB)	53	98.48%	95.38%	35.77
BM5	4210563	0.211(GB)	54	98.16%	94.84%	35.66
LP1	3679273	0.184(GB)	52	98.54%	95.32%	35.77
LP2	3834373	0.192(GB)	52	98.64%	95.60%	35.82
LP3	5482979	0.274(GB)	53	98.74%	95.82%	35.86
LP4	3604748	0.180(GB)	52	98.73%	95.88%	35.87
LP5	7561029	0.378(GB)	52	98.37%	95.49%	35.77

4.2. Identification of Differentially Expressed Mirnas and Proteins

Bioinformatics analysis was performed to screen out differentially expressed miRNAs and proteins between the LP and BM groups. Volcano plots provided an overview of the differential expression of miRNAs and proteins, as shown in (Figure 1). Each point in the graph represents a miRNA or protein. The abscissa represents the logarithmic fold change value of the multiple of difference of miRNA or protein expression between the two groups. The ordinate represents the negative logarithmic P value of miRNA or protein expression change.

An absolute Fold Change cut-off value of 2 and $P < .05$ were utilized as the criteria to confirm significantly downregulated and upregulated miRNAs and proteins (FDR <0.05). Red points rep-

resent upregulated miRNAs or proteins in BM vs LP, while green points represent downregulated ones.

Heatmaps (Figures 2,3) show the significant changes of miRNA and protein expression between LP and BM(FDR <0.05). The figure shows the bidirectional clustering of differentially expressed miRNAs or proteins and groups. The depth of colour represents the level of expression; orange represents high and purple represents low levels of miRNA and protein expression (Figure 2,3). The above results suggested that 16 miRNAs, such as miR-31-3p, miR-654-5p, miR-1185-1-3p, miR-487a-5p, as well as 53 proteins, including VIM, RAB2A, RPLs, KRTs proteins were highly expressed in BM patients, while miR-219a-5p, miR-1269a, miR-615-3p, miR-196a-5p, 4 miRNAs and AHSG, HBD, SERPINC1, COL6As, totally 35 proteins were low expressed significantly, which may be related to the pathogenesis of brain metastasis.

The interaction network of differentially expressed miRNAs and proteins was mapped using Cytoscape (Figure 4). The network graph is composed of node and edge. Nodes represented genes and proteins, and edges represented interaction relationships between genes. The black nodes represented miRNAs, proteins with high expression were represented by green nodes, while those with low expression are represented by blue nodes. The lines represented the correlation and interaction between genes and proteins.

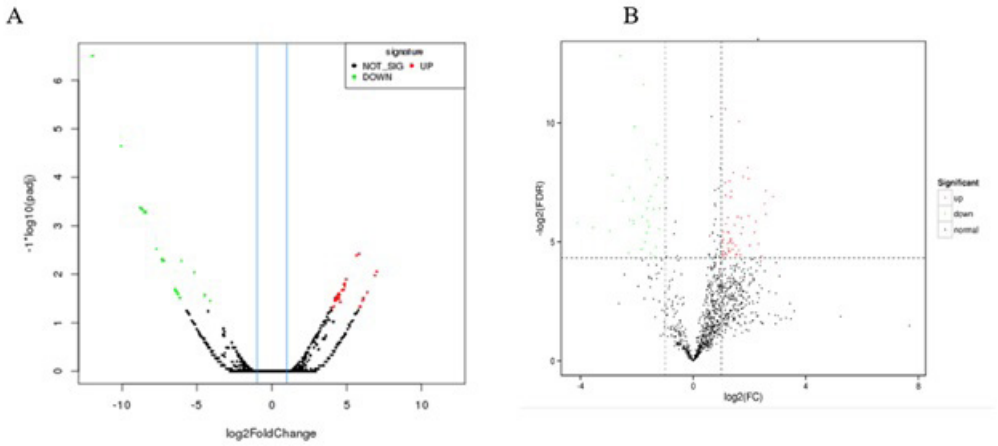

Figure 1: Volcano plots of differentially expressed microRNAs (A) and proteins (B)

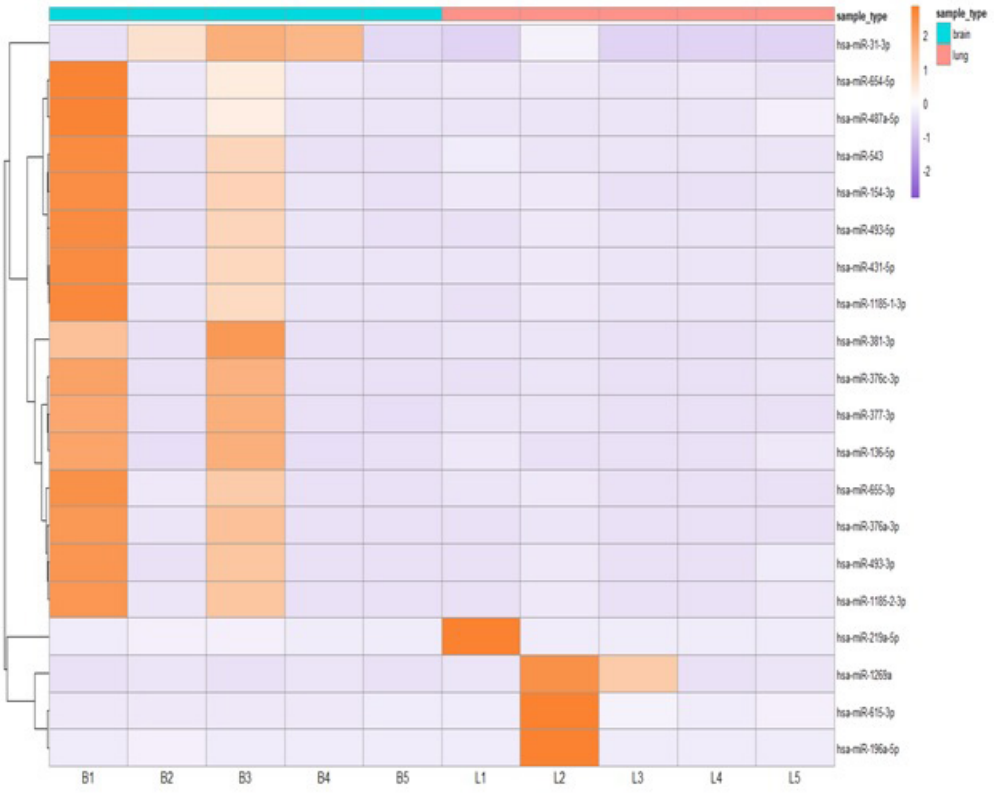

Figure 2: Heatmap of differentially expressed miRNAs

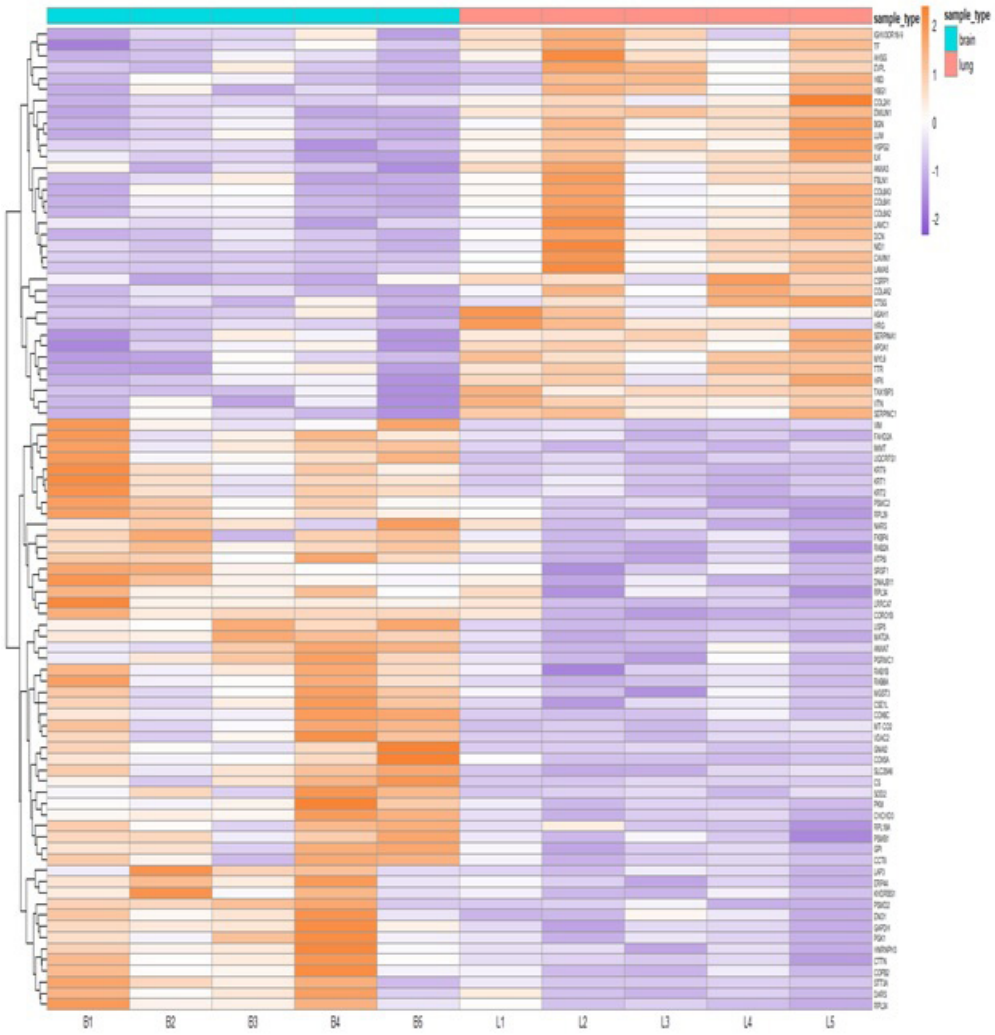

Figure 3: Heatmap of differentially expressed proteins

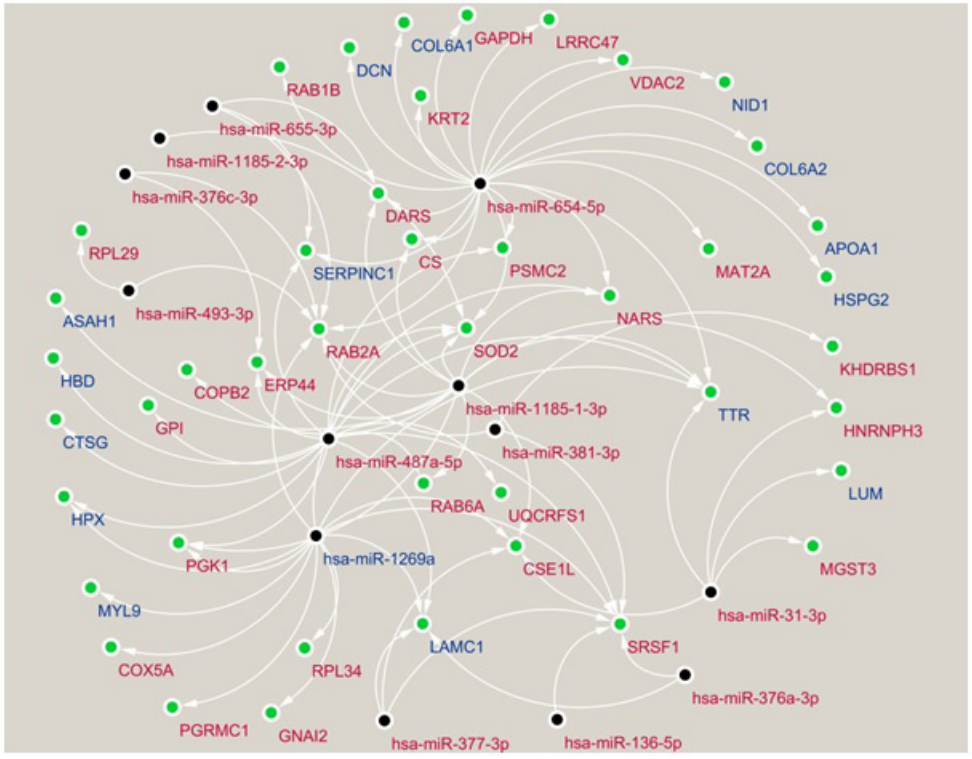

Figure 4: The interaction network of differentially expressed miRNAs and proteins

4.3. Enrichment Analysis

According to the relative level of expression between the two groups, the differentially expressed miRNAs and proteins can be classified as upregulated group and downregulated group. The results of GO enrichment analysis with differentially expressed genes and proteins are shown in (Figure 5 and Figure 6) respectively. The main information of enrichment results areis shown in the figures, and other information can be found in tables in the original document of analysis results.

The ordinate is the classification of GO for miRNAs, while the abscissa represents the number of enriched target genes. Differentially expressed miRNAs were enriched in signalling pathways such as binding, cell part, cell, organelle, intracellular organelle and protein binding. For proteins, the abscissa is the classification of GO; on the right is the number of target proteins, and on the left is the percentage of the number of target proteins. Differentially expressed proteins were enriched in signalling pathways such as cellular process, cell, cell part, organelle and binding. Both enrichment pathway analysis with differentially expressed miRNAs and proteins revealed that cell, cell part, organelle and binding signaling pathways play important roles in the development of LAC BM.

KOBAS3.0 was used to analyze the enrichment functional areas of the top 10 significantly differentially expressed miRNAs target genes, shown in (Figure7), which was demonstrated that the differentially expressed miRNAs were mainly enriched in extracellular vesicles, extracellular space, extracellular organelles functional areas.

Bubble map is a graphical representation of KEGG. KEGG Pathway analysis was performed on the differentially expressed miRNAs and proteins, the results of KEGG enrichment analysis were shown in (Figure8 and Figure9). KEGG enrichment was measured by Gene Ratio, p value and number of genes enriched in this pathway, respectively. Gene Ratio refers to the Ratio between the number of differentially enriched genes and the total number of differentially enriched genes in this pathway. The higher Gene Ratio, the greater the degree of enrichment; The value range of p value is between [0,1], and the closer it is to zero, the more significant the enrichment. The top 30 pathways with the most significant enrichment of miRNAs and top 20 pathways of proteins were showed in the item diagram. The ordinate is the description of the corresponding pathways, while the abscissa is the enrichment factor, the bubble size is the number of different genes or proteins, and the bubble color is the P value of enrichment significance. Both

enrichment pathway analysis with differentially expressed miRNAs and proteins revealed that NF-κB signaling pathway and primary immunodeficiency pathway play important roles in the development of lung adenocarcinoma BM.

Figure 5: Gene ontology enrichment analysis of differentialy expressed miRNAs

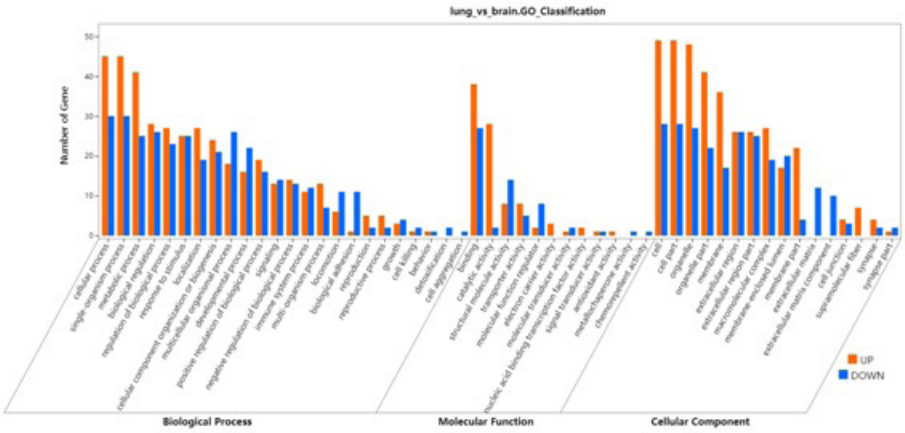

Figure 6: Gene ontology enrichment analysis of differentially expressed proteins

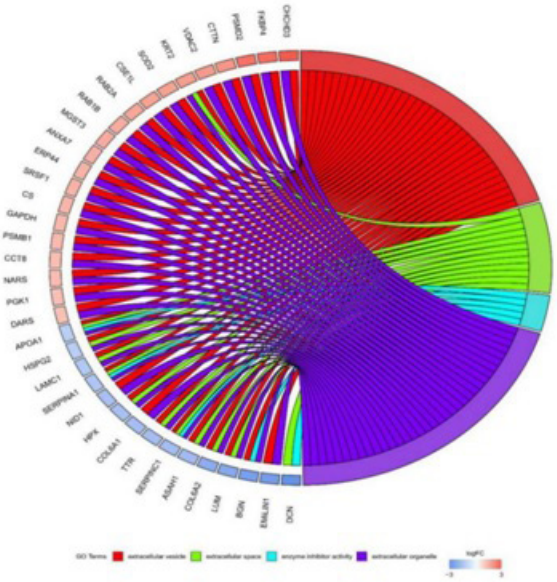

Figure 7: Gene ontology enrichment analysis of top 10 significantly differentially expressed miRNAs target genes

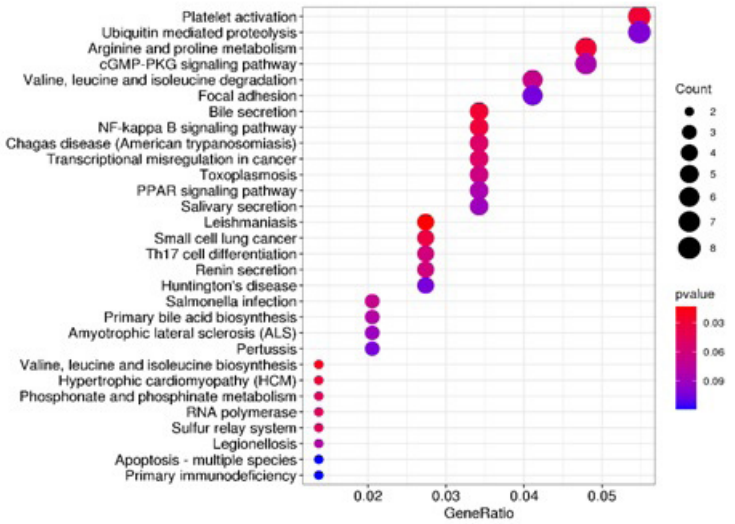

Figure 8: The bubble map of Kyoto Encyclopedia of Genes and Genomes enrichment analysis of differentialy expressed miRNAs

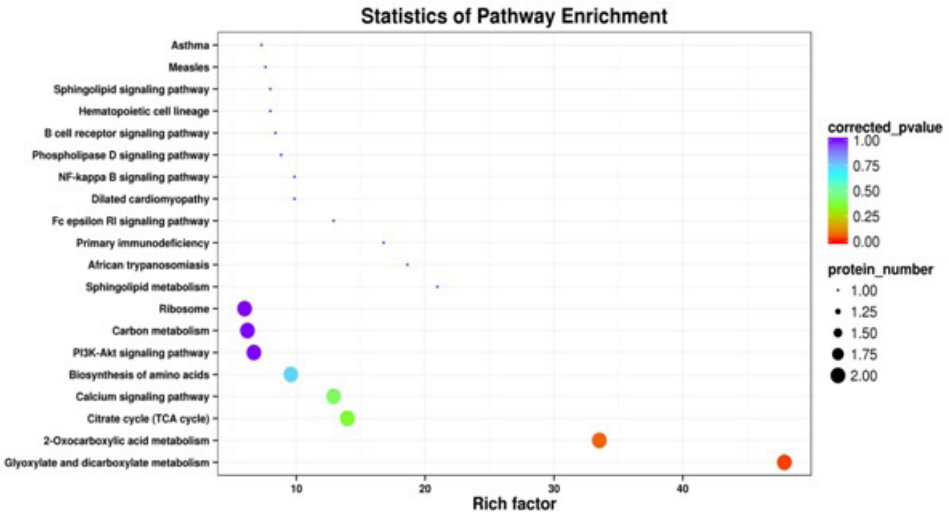

Figure 9: The bubble map of Kyoto Encyclopedia of Genes and Genomes enrichment analysis of differentialy expressed proteins

5. Discussion

In this study, it was found that compared with LP group, 16 miRNAs and 53 proteins were highly expressed while 4 miRNAs and 35 proteins were significantly low expressed in BM group, which may be related to the pathogenesis of LAC brain metastasis.

We found that Tissue Factor (TF) was low expressed in BM group, which could mean that TF can inhibit brain metastasis of lung adenocarcinoma patients. Previous research has reported that metastasis had a predilection to organs with low levels of TF in the microcirculation, which enables tumor cell retention [15]. SERPINA1 (Serpin Family A Member 1) was significantly different expressed in BM group and LP group in current study and other study [16]. Some studies have reported that Epithelial-Mesenchymal Transition (EMT) proteins and matrix metalloproteinases (MMPs) play important roles in brain metastasis by regulating the Blood-Brain Barrier (BBB) [17], high expression of Inner Membrane Mitochondrial Protein (IMMT) was significantly correlated with advanced disease stage and poor prognosis of LAC patients [18], the NARS levels were positively associated with LAC lymph node metastasis [19], which were consistent with our study. We also found that proteins like KRTs, COL6As, RAB2A, AHSG were associated to pathogenesis of LAC brain metastasis. However, the sample size of this study is limited, and more samples is needed in the future for further confirmation.

MiR-31-3p are related to the occurrence and development of various malignant tumors such as colorectal cancer, as well as lymph node metastasis and poor prognosis of lung adenocarcinoma have reported in previous studies [20]. In current study we also demonstrated that miR-31-3p significantly increased in BM group, suggesting that miR-31-3p may promote the occurrence and development of brain metastasis in lung adenocarcinoma patients. MiR-493-5p is related to the pathogenesis of various malignant tumors such as liver cancer, colorectal cancer and osteosarcoma, which is also related to the prognosis of patients with NSCLC. It can affect the malignant behavior of NSCLC cells by regulating the expression of integrin β1 (ITGB1) [21]. We found that miR-493-5p can promote lung adenocarcinoma brain metastasis as well. These findings indicate the possibility of miRNAs as biomarkers for the occurrence, progression, prognosis, as well as potential therapeutic targets of lung cancer BMs. Our study also found the differential expression of these miRNAs in the two groups, which is in line with previous studies, suggesting these miRNAs are correlated with the pathogenesis of brain metastasis in lung adenocarcinoma. In addition, this study also found the differential expression of miR-487a-5p, miR-654 −5p, miR-1185-1-3p, miR-376c-3p and miR-196a-5p between BM and LP groups for the first time, which may be associated with the brain metastasis of lung adenocarcinoma. Previous studies have reported that miR-654-5p can promote the progression of colon cancer, breast cancer, and oral squamous cell carcinoma, which is also associated with paclitaxel resistance of ovarian cancer. We found that miR-654-3p may promote the progression of lung adenicarcinoma brain metastasis MiR-1185-1-3p can be used for the early diagnosis of bladder cancer. We reported the association of miR-1185-1-3p with brain metastasis in lung adenocarcinoma patients for the first time, no studies have focused on the relationship between these miRNAs and lung cancer or brain metastasis before.

We found that the pathogenesis of brain metastasis in LAC was related to NF-κB signaling pathway, which is in line with previous studies [22]. It was shown that primary immunodeficiency signaling pathway also plays important role in the occurrence and development of brain metastasis of LAC, which is a newly discovered mechanism in this study. NF-κB is a key signaling pathway in the development and metastasis of various inflammatory and malignant tumors, and has also been found to be associated with brain metastasis of breast cancer and lung cancer [22]. In addition, previous studies have found that there are multiple mechanisms involved in the occurrence and progression of lung cancer brain

metastases: The highly open calcium-activated potassium (KCA) channels in lung cancer brain metastases and their microvascular endothelial cells can be used as targets for Blood-Brain Tumor Barrier (BTB) and permeability regulation [23]. Vascular endothelial growth factor C plays a role in the development and progression of lung cancer brain metastasis by enhancing the affinity of tumor cells to specific organs and promoting the movement of tumor cells to lymphatic vessels [24, 25]. The interaction between neurotransmitter receptors and neurons can promote the metastasis of lung cancer cells to the brain [25]. The abnormal expression of tumor suppressor genes, matrix metalloproteinases, carcinoembryonic antigen and other related proteins are also associated with LAC brain metastasis.

The samples of primary and metastatic lesions from the same patient which can exclude the interference of expression differences caused by histological differences in brain tissues and lung tissues were not obtained for the present study. Relevant samples will be collected in the future for further studies. In addition, a number of studies have explored the expression of cerebrospinal fluid miRNAs and proteins in central nervous system malignant tumors, as well as their diagnostic and application value. It has been found that miRNAs differentially expressed in cerebrospinal fluid can distinguish glioblastoma from metastatic brain malignancies and can be used to monitor changes in disease condition and response to treatment [26]. Due to the difficulty in obtaining BMs tissue specimens, subsequent attempts can be made to explore the feasibility of using CSF specimens of BMs and non-BMs to detect the differentially expressed non-coding RNA and proteins.

However, a larger cohort will be required to examine and validate the datas due to the limitation of the small size sample and cohort selection in the present study.

In conclusion, RNA sequencing and proteomics analysis provided a list of interesting altered transcriptomes in LAC patients with brain metastasis. The findings of this study contribute to the elucidation of the molecular mechanisms of LAC brain metastasis and contribute to an increased understanding of the complex pathogenetic mechanisms underlying brain metastasis, which will ultimately lead to novel treatment strategies in the future.

6. Acknowledgements

This study was supported by the Beijing CSCO Oncology Research Foundation(No: Y-HS2019/2-056, Y-HR2018-315)

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