이학석사 학위논문

소세포폐암 유래 exosomal miRNAs을 활용한 진단 및 예후 예측

Clinical application of exosomal miRNAs in small cell lung cancer

울산대학교 대학원
의 과 학 과
박 효 정

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이 논문을 이학석사학위 논문으로 제출함

2022 년 2 월

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Abstract

Small cell lung cancer (SCLC), an aggressive lung tumor with a poor prognosis, has a problem that there are many somatic cell mutations, causes metastatic diseases, and is difficult to diagnose early. Cancer-derived exosomal miRNAs are considered as stable and non-invasive biomarkers for diagnosis and prognosis. Therefore, early detection of SCLC by using exosomal miRNAs can be critical to improving clinical outcomes. We investigated the miRNA of exosomes secreted from serum in 5 normal patients and 5 SCLC patients using microarray-based expression profiling. In the result of miRNA array, we found out highly expressed 15 miRNAs that are validated in large cohort consist of 76 SCLC patients. In comparison to the levels in 50 healthy volunteers, miR-3656, miR-3124-5p, 200b-3p, miR-6515, miR-9-5p, miR-3126-3p are highly expressed in SCLC serum samples. Moreover, among them, exosomal miR-3124-5p, miR-6515-5p and miR-9-5p indicate high expression of both limited disease (LD) and extended disease (ED) in SCLC although there was no exosomal miRNA with a significant difference in overall survival. In addition, functional enrichment analyses using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) showed that Ras signaling pathway and RNA polymerase II transcription involved in miRNA mechanisms. These results suggest that highly expressed exosomal miRNAs in patient serum can be utilized as potential diagnostic biomarkers.

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Introduction

Lung cancer is one of the most common cancer globally and poor diagnosis regardless gender [1, 2]. Lung cancer is classified non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). SCLC accounts for broadly 15% of all lung cancer [3-5]. Usually, SCLC is first located bronchial tube in the center of chest, generally having tiny, circular shape and scattered. The tumor tends to growing aggressively and disseminates fast. Also, SCLC can spread by a large number of circulating tumor cells (CTCs) in the body-fluids or via lymphatic system all over the body [6, 7]. For this reasons, 80–85% of patients represent with extensive disease (ED), called terminal cancer in SCLC, at diagnosis. Commonly, because of these histological properties, over 70% of patients spread to other organs at diagnosis such as liver, bone, brain and lung [8].

The usual treatment of SCLC is chemotherapy or radiation sometimes combine with both, rarely surgical resection [9, 10]. The therapies are highly responsive at first, but there is a tendency to recur within a year resulting in poor survival [9]. Among patients with SCLC, 5-year relative survival rates are about 30% for limited disease and below 10% for extensive disease. Thus, early diagnosis of SCLC is a critical to improve the therapeutic efficacy.

Microvesicles (MVs), also known as microparticles, are small, membrane-enclosed sacs that are thought to be shed from the surface of healthy or damaged cells under conditions such as cell activation, growth, and apoptosis [11, 12]. Such membrane-bound vesicular structures contain significant amounts of biologically active proteins, lipids, and nucleic acids acquired from their parental cells, which they can transport to other cells [13]. Growing evidence suggests that MVs are commonly found in blood, urine, saliva, tears, and many other body fluids [14-17]. Many studies have suggested possible roles for MVs as indicators in the diagnosis, prognosis, and surveillance of a variety of health conditions. Tumor cells may release more than one type of membrane-bound vesicle, each with unique morphological traits and functions. Those membrane-bound vesicles can affect many stages of tumor progression, including the development of the tumor microenvironment [18], angiogenesis [19, 20], the evasion of immune surveillance [21-23], invasion and metastasis [20, 25, 26], and the acquisition of aggressive phenotypes [27] and multi-drug resistance [28].

This study evaluated whether any exosomal miRNA can play as biomarker for detection of early SCLC. We purified the circulating exosomes from serum of normal and SCLC patients, and analysed the exosomal miRNAs by using microarray-based expression profiling. Furthermore, this miRNA was validated in large cohort, and evaluated the possibility for diagnosis of SCLC.

Material and Methods

Patient and clinical samples collection

A total of 76 samples of human SCLCs diagnosed with limited-stage and extensive stage were obtained from Asan Medical Center Institute, after approval by the local Institutional Review Board (2015-1323, 2018-0462), which waived the requirement for informed consent due to their retrospective nature.

Cell culture

cell culture Cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). We performed experiment using NSCLC (Blue: H1975, H1299, Calu-1), SCLC (RED: H209, H82, H69), Normal lung cell lines (Black: MRC5, Beas2B). All cell lines were cultured in RPMI 1640 (Invitrogen, Carsbad, CA, USA) medium that containing 10% fetal bovine serum (FBS) with 50 μl/ml Gentamicin and maintain in a humidified incubator at 37 C with 5% CO2.

Plasma exosome isolation

Patient's serum was centrifuged (300g for 10 min, 2000g for 10 min, 10,000g for 30 min and 10,000g for 30 min at 4 °C) to remove cell and other debris. And then, centrifuged serum was ultracentrifuged at 110,000g for 70 min. The pellet was washed with PBS, ultracentrifuged 110,000g for 70 min. Isolated exosomes were quantified using a standard

protein assay (Bio-Rad Laboratories, Hercules, CA). The pellet was resuspended in PBS and stored at −80 °C.

Electron microscopy

Purified exosomes were fixed in 2% (vol/vol) paraformaldehyde for 5 min at room temperature. After fixation, 10 μg of exosomal suspensions were applied to formvar/carbon-coated grids (200 mesh) for 3 min and stained with 2% uranyl acetate. After excess uranyl acetate was removed with filter paper, the grids were examined by transmission electron microscopy (TEM, Hitachi H7600, Japan) at 80 kV.

Nanoparticle tracking analysis

Exosome sizes and concentrations were analyzed using the NanoSight NS300 system (Malvern Instruments Ltd, Malvern, UK), which allows tracking of the Brownian motion of nanoparticles in a liquid suspension on a particle-by-particle basis. Data were then analyzed using nanoparticle tracking analysis software (NTA version 2.3 build 0017). Samples were diluted 10- to 100-fold in PBS to observe the reduced number of particles in the field of view to below 100 per frame, and readings were taken in triplicate over a 60 s period at 10 frames per second at room temperature.

Western blot assay

Cell and exosomes were lysated by EBC lysis buffer (50- mM Tris–HCl [pH 8.0], 120-mM NaCl, 1% Triton X-100, 1-mM EDTA, 1-mM EGTA, 0.3- mM phenylmethylsulfonylfluoride,

0.2-mM sodium orthovanadate, 0.5% NP-40, and 5-U/mL aprotinin). Protein was separated on SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Membranes were blocked with 5% skim milk for 1 h and then leaved the membrane with antibody overnight at 4 °C. Using anti-HSP70 (BD610607, 1:4000, BD Biosciences, San Diego, CA), anti-HSP90 (BD610418, 1:2000, BD Biosciences), anti-CD9 (ab92726, 1:1000, Abcam, Cambridge, UK), anti-calnexin (ab22595, 1:1000, Abcam), and anti-β-actin (SC47778,1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) for blotting. Proteins were detected with a chemiluminescence Western blotting kit (Amersham Biosciences), in accordance with the manufacturer's directions.

RNA extraction from exosomes and quantitative RT-PCR

RNA was extracted using the RNeasy Miniprep kit (Qiagen) and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturers' protocols. Quantitative real-time RT-PCR analysis was performed with an ABI 7900 Real-Time PCR System using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA), following the manufacturer's instruction. Let-7a-5p was used as a loading control. The primers used are listed in Table 1.

Table 1. Primer sequences

Primer name	Primer sequences
let-7a-5p (Forward)	5’-TGAGGTAGTAGGTTGTATAGTT-3’
Poly(T) adaptor	5’-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN -3’

adaptor reverse	5’-GCGAGCACAGAATTAATACGAC -3’
hsa-miR-6746-3p	5’- cagccgccgcctgtctccacag -3’
hsa-miR-3688-5p	5’- agtggcaaagtctttccatat -3’
hsa-miR-5706	5’- ttctggataacatgctgaagct -3’
hsa-miR-3919	5’- gcagagaacaaaggactcagt -3’
hsa-miR-6803-3p	5’- tccctcgccttctcaccctcag -3’
hsa-miR-4726-3p	5’- acccaggttccctctggccgca -3’
hsa-miR-3690	5’- acctggacccagcgtagacaaag -3’
hsa-miR-299-5p	5’- tggtttaccgtcccacatacat -3’
hsa-miR-548l	5’- aaaagtatttgcgggttttgtc -3’
hsa-miR-6818-3p	5’- ttgtctcttgttcctcacacag -3’
hsa-miR-130b-3p	5’-cagtgcaatgatgaaagggcat -3’
hsa-miR-3126-3p	5’-catctggcatccgtcacacaga -3’
hsa-miR-892b	5’-cactggctcctttctgggtaga -3’
hsa-miR-4742-3p	5’-tctgtattctcctttgcctgcag -3’
hsa-miR-450b-5p	5’-ttttgcaatatgttcctgaata -3’
hsa-miR-9-5p	5’-tctttggttatctagctgtatga -3’
hsa-miR-372-5p	5’-cctcaaatgtggagcactattct -3’
hsa-miR-664a-3p	5’-tattcatttatccccagcctaca -3’
hsa-miR-1185-1-3p	5’-atatacaggggggagactcttat -3’
hsa-miR-1254	5’-agcctggaagctggagcctgcagt -3’
hsa-miR-487b-5p	5’-gtggttatccctgtcctgttcg -3’
hsa-miR-16-5p	5’-tagcagcacgtaaatattggcg -3’
hsa-miR-152-5p	5’-aggttctgtgatacactccgact -3’
hsa-miR-2052	5’-tgttttgataacagtaatgt -3’
hsa-miR-3613-3p	5’-acaaaaaaaaaagcccaacccttc -3’
hsa-miR-4497	5’-ctccgggacggctgggc -3’
hsa-miR-5587-3p	5’-gccccgggcagtgtgatcatc -3’
hsa-miR-4695-3p	5’-tgatctcaccgctgcctccttc -3’

hsa-miR-606	5’-aaactactgaaaatcaaagat -3’
hsa-miR-3656	5’-ggcgggtgcgggggtgg -3’
hsa-miR-3124-5p	5’-ttcgcgggcgaaggcaaagtc -3’
hsa-miR-200b-3p	5’-taatactgcctggtaatgatga -3’
hsa-miR-3916	5’-aagaggaagaaatggctggttctcag -3’
hsa-miR-4496	5’-gaggaaactgaagctgagaggg -3’
hsa-miR-5010-3p	5’-ttttgtgtctcccattccccag -3’
hsa-miR-6515-5p	5’-ttggagggtgtggaagacatc -3’
hsa-miR-3684	5’-ttagacctagtacacgtcctt -3’
hsa-miR-144-3p	5’-tacagtatagatgatgtact -3’
hsa-miR-8054	5’-gaaagtacagatcggatgggt -3’

Small RNA sequencing libraries preparation

Extracted total RNA samples were analyzed with a Eukaryote Total RNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, United States) to confirm sufficient yield, quality, and size of RNA. Exosome RNA sequencing libraries were then constructed using the NEB Next Multiplex Small RNA Library Prep Kit for Illumina (BioLabs, New England) according to the manufacturer’s instructions. The raw data was pre-processed using BBDuk to remove Illumina adapters and reads shorter than 20 bp. Mapping was conducted using Bowtie2 to reference genome calculation read count using Bedtools. Quantile normalization and DEG master file production was carried out by using EdgeR and ExDEGA.

Data analysis and statistics

Vesiclepedia (Version 3.1, 2017) and Exocarta (July 2015) were downloaded to map previously reported EV proteins. KEGG and Gene ontology (GO) analyses were performed with Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8, using all the proteins identified by the whole-exosome lysate proteomics experiment as background. Data are presented as the means ± standard deviations. P-values were determined using unpaired or paired t-tests between groups (GraphPad Prism software, GraphPad Software Inc., San Diego, CA, USA).

Result

1. Characterization of exosomes from plasma of SCLC patients.

To search the expression of exosomal miRNAs in SCLC, we collected 5 healthy controls and 5 early SCLC patients-derived blood plasma and isolated exosomes using ultracentrifugation. Isolated exosomes analyzed its size and morphology through the transmission electron microscope (TEM) (Fig. 1A). To support the accuracy of its size, we also used a NanoSight NS300. As shown Fig. 1B, NanoSight analysis measured all of patient sample mode sizes ranged from 98.9 -182.7nm is the most. (Fig. 1B; Control, 108.0 ± 4.5 nm; SCLC, 105.0 ± 1.8 nm). Exosomes are heterogeneous in size, but the most numerous exosomes were less than 200 nm (Fig. 1C). Moreover, western blotting showed that exosome lysates were highly enriched for TSG101, HSP90, CD63, CD81, known markers for isolated exosome (Fig. 1D).

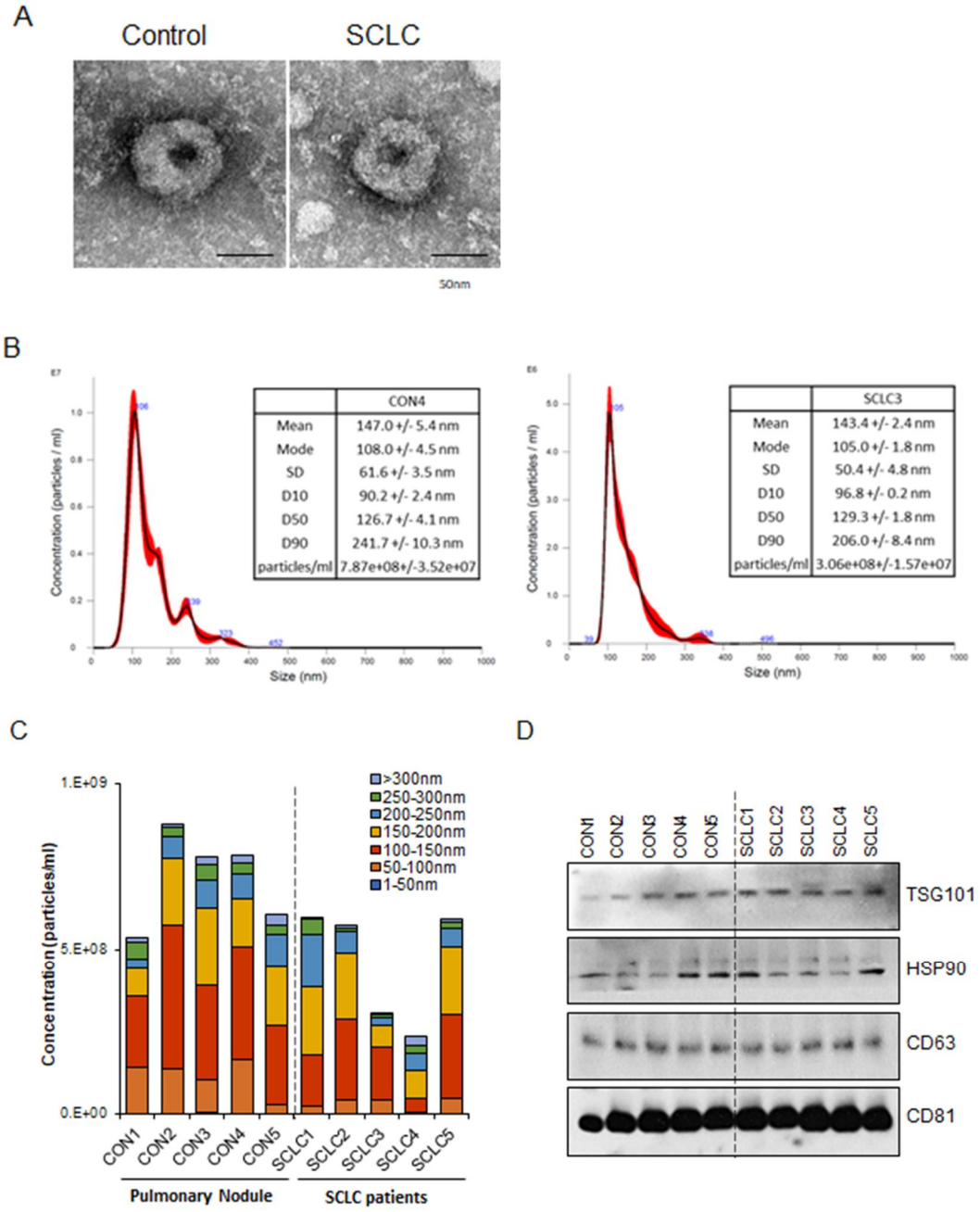


Figure 1. Isolation and characterization of exosome in serum. Exosomes were isolated from SCLC patients (n=5) and control group (n=5). (A) Transmission electron microscopy view of exosome derived from plasma. (B) Nanoparticle tracking analysis of isolated exosome. (C) Particle distribution of exosome. (D) Western blot of exosomal marker TSG101, HSP90, CD63, CD81 in exosome lysate.

2. Identification of exosomal RNA and Comparison of miRNA property between SCLC and normal group.

Most of the miRNAs are located inside the cell, but a large proportion of these circulates outside in body-fluid. Exosomal RNAs was isolated using QIAGEN kit as described in materials and methods. RNA quality control was conducted to confirm the possibility of miRNA sequencing analysis. miRNA is a small, non-coding RNA and 20-25nt in length. By showing the graph reached peak point at around 20 nt, we identified that miRNAs were enriched in exosome. (Fig. 2).

To evaluate similarities and differences in exosomal miRNA profile of two groups on a world scale, we conducted principal component analysis (PCA). All exosomal miRNAs of normal group (Blue circle) were similar to patterns, while SCLC patients (orange circle) dispersed in all direction. This is shown to reflect property of cancer cell including heterogeneity (Fig. 3A).

From the miRNA sequencing results, we identified 1255 miRNAs in normal, 1236 miRNAs in SCLC and 856 commonly expressed among them. In total, around 1200 miRNAs detected in serum at each group and averagely 600 miRNA expressed each patient, there were no significant differences on the number of miRNA between the two groups (Fig. 3B). The Venn diagram also showed the number of all differentially expressed miRNAs cross-referenced it on publicly available exosome databases including Vesiclepedia and Exocarta (Fig. 3D). Among 1500 miRNAs in our sample, 1369 miRNAs in Exocarta, 962 miRNAs in Vesiclepedia are reported. (Fig. 3C) This analysis showed that more than 90% of our detections have been previously presented in extracellular function, and we also identified another 135 miRNAs that were not previously reported to exist in exosomes.

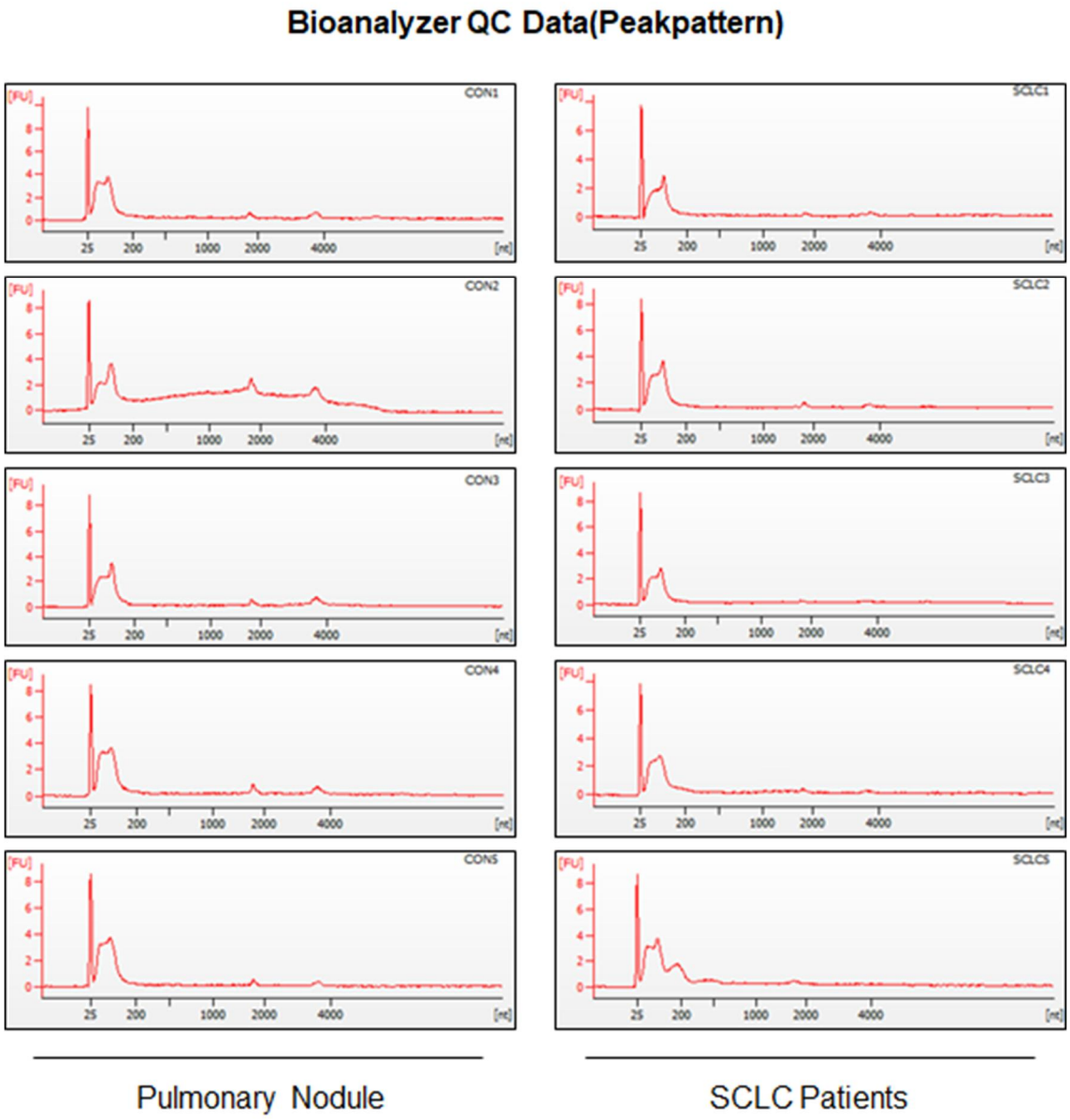


Figure 2. Identification of exosomal miRNA profile through BioAnalyzer.

Isolated exosomal RNAs are conducted RNA Quality control to confirm the possibility of miRNA sequencing analysis. The presentative images showed RNA profiles in SCLC patients-derived exosome (n=5) and control group exosome (n=5).

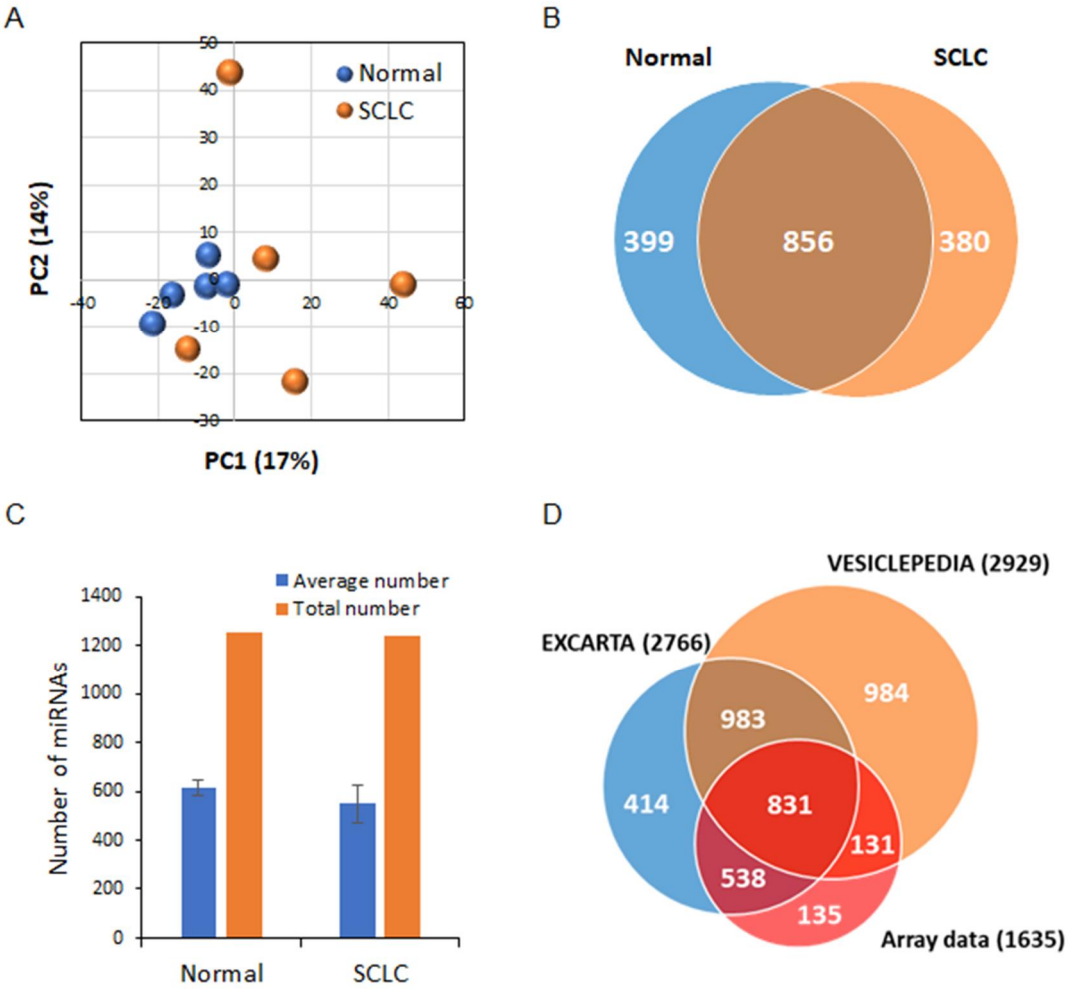


Figure 3. Comparison of expressed miRNAs.

(A) Principal component analysis (PCA). (B) Venn diagram of miRNA between SCLC patients (n=5) and control group (n=5). (C) Graph of Quantitative data about the number of miRNAs. (D) Identification of representing miRNA in analyzed array data compared with noted in the Excarta and Vesiclepedia.

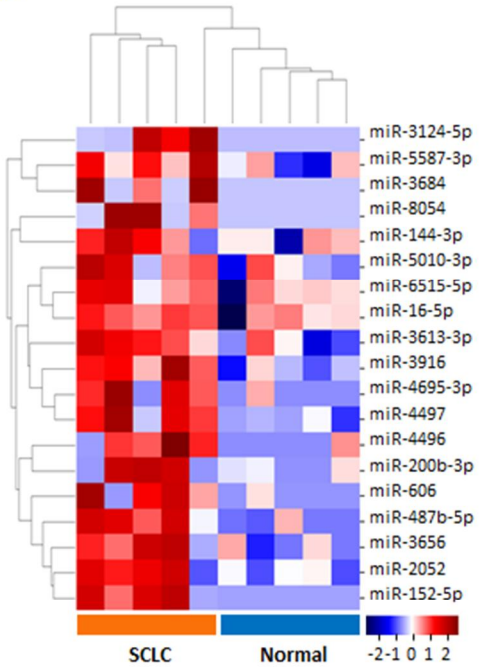
3. Identification and validation of SCLC-specific exosomal miRNAs.

We examined the enrichment of miRNAs within each group through miRNA profiling (Fig. 4A). These results revealed SCLC-specific miRNAs that are showing high expression, and we could pick out list of top 20 miRNAs over two-fold up- and p-value. To verify these array data, quantitative real-time RT-PCR (qRT-PCR) was performed and 10 miRNAs were selected to match the array results (Fig. 4B, C).

In addition, we also made a heatmap based on expression level. This was analyzed based on fold change and p-value as well (Fig. 5A). As a result, we could select 5 miRNAs through the qRT-PCR (Fig. 5B, C). From the miRNA array results, we could choice 15 miRNA to verify in a large cohort.

Before investigation in a large cohort, qRT-PCR was conducted *in vitro* to know how to express lung cancer cell lines in each miRNA. The experiments were performed using lung cancer cell lines, MRC5 (lung fibroblast), BEAS2B (lung epithelial virus transformed), H69, H82, H209 (SCLC carcinoma), Calu-1, H1299, H1975 (NSCLC lung adenocarcinoma). SCLC carcinoma cell lines were dominant all of miRNAs, so that it is demonstrated that SCLC cell lines much higher those miRNAs among lung cancer cell lines. Also, lung cancer cell derived-exosomal miRNAs showed identical tendency, too (Fig, 6).

A



B

	miRNA array		QRT-PCR	
P-value ≤ 0.05	SCLC/CON		SCLC/CON	
Gene symbol	FC	p-value	FC	p-value
hsa-miR-487b-5p	4.16	0.005	**3.32**	**0.013**
hsa-miR-16-5p	2.80	0.006	1.45	0.181
hsa-miR-152-5p	3.32	0.007	1.86	0.078
hsa-miR-2052	2.51	0.008	ND	ND
hsa-miR-3613-3p	2.31	0.008	**5.39**	**0.008**
hsa-miR-4497	5.24	0.016	**3.23**	**0.074**
hsa-miR-5587-3p	2.04	0.018	**3.65**	**0.009**
hsa-miR-4695-3p	2.66	0.022	1.75	0.013
hsa-miR-606	4.64	0.023	ND	ND
hsa-miR-3656	6.29	0.025	**4.16**	**0.049**
hsa-miR-3124-5p	2.20	0.025	**4.33**	**0.042**
hsa-miR-200b-3p	7.88	0.025	**2.85**	**0.040**
hsa-miR-3916	7.48	0.028	1.55	0.248
hsa-miR-4496	2.17	0.037	**3.78**	**0.017**
hsa-miR-5010-3p	3.02	0.038	1.73	0.156
hsa-miR-6515-5p	2.43	0.041	**2.77**	**0.036**
hsa-miR-3684	3.23	0.046	2.15	0.132
hsa-miR-144-3p	2.23	0.047	ND	ND
hsa-miR-8054	4.60	0.049	**4.55**	**0.035**

C

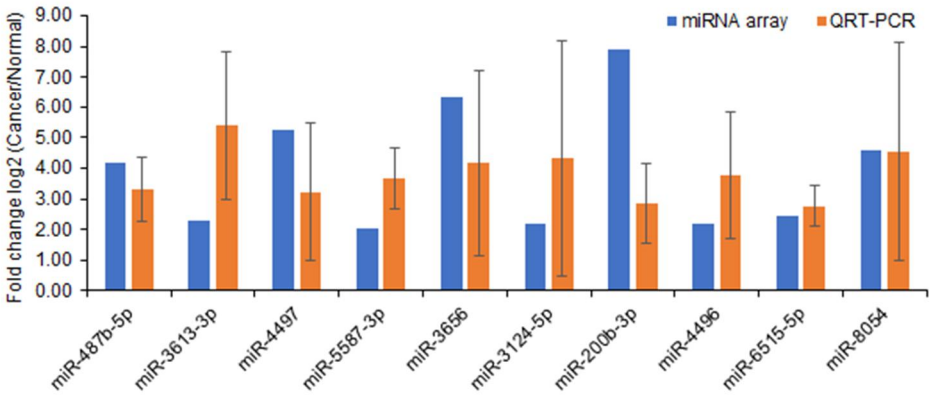
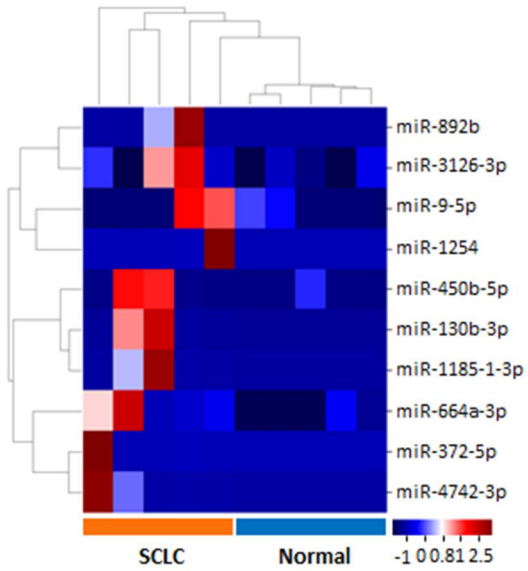


Figure 4. miRNA microarry analysis and qRT-PCR validation base on the p-value.

(A) Heat map of expressing miRNA based on online database. (B) Validation of miRNA array data in patients (n=5) and normal (n=5) group. Determination of the top 10 rank of miRNA based on two-fold up and p-value. (C) Quantitative real-time RT-PCR analysis to verify sample array data. All data represent the mean ± standard deviation. *$P < 0.05$, **$P < 0.005$, ***$P <$ 0.0005 compared to the control group.

A



B

	miRNA array		QRT-PCR	
Expression Top 10	SCLC/CON		SCLC/CON	
Gene symbol	FC	p-value	FC	p-value
hsa-miR-130b-3p	64.02	0.133	3.45	0.093
hsa-miR-3126-3p	42.58	0.119	3.90	0.050
hsa-miR-892b	36.35	0.164	3.98	0.072
hsa-miR-4742-3p	35.49	0.168	2.98	0.064
hsa-miR-450b-5p	34.23	0.073	ND	ND
hsa-miR-9-5p	29.09	0.089	6.25	0.019
hsa-miR-372-5p	26.64	0.173	6.47	0.016
hsa-miR-664a-3p	26.49	0.135	5.81	0.021
hsa-miR-1185-1-3p	26.12	0.161	7.49	0.005
hsa-miR-1254	26.03	0.173	2.65	0.080

C

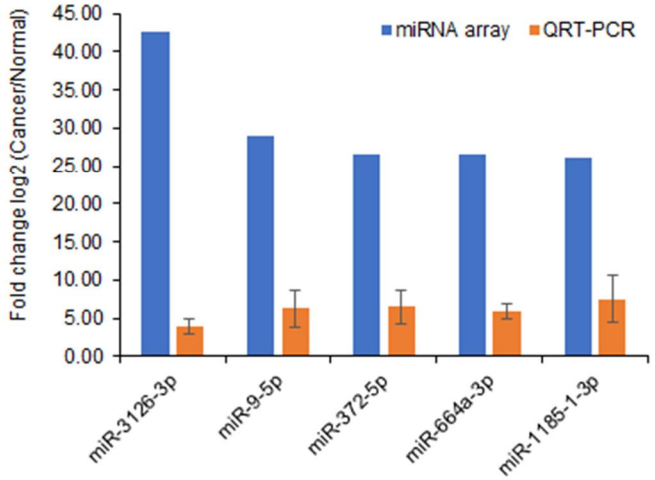


Figure 5. Identification of SCLC patients (n=5) specific miRNAs by miRNA microarray analysis and qRT-PCR validation based on the expression level.

(A) Heat map of expressing miRNA based on online database. (B) Validation of miRNA array data in patients (n=5) and normal (n=5) group. Determination of the top 5 rank of miRNA based on expression level. (C) Quantitative real-time RT-PCR analysis to verify sample array data. All data represent the mean ± standard deviation. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ compared to the control group.

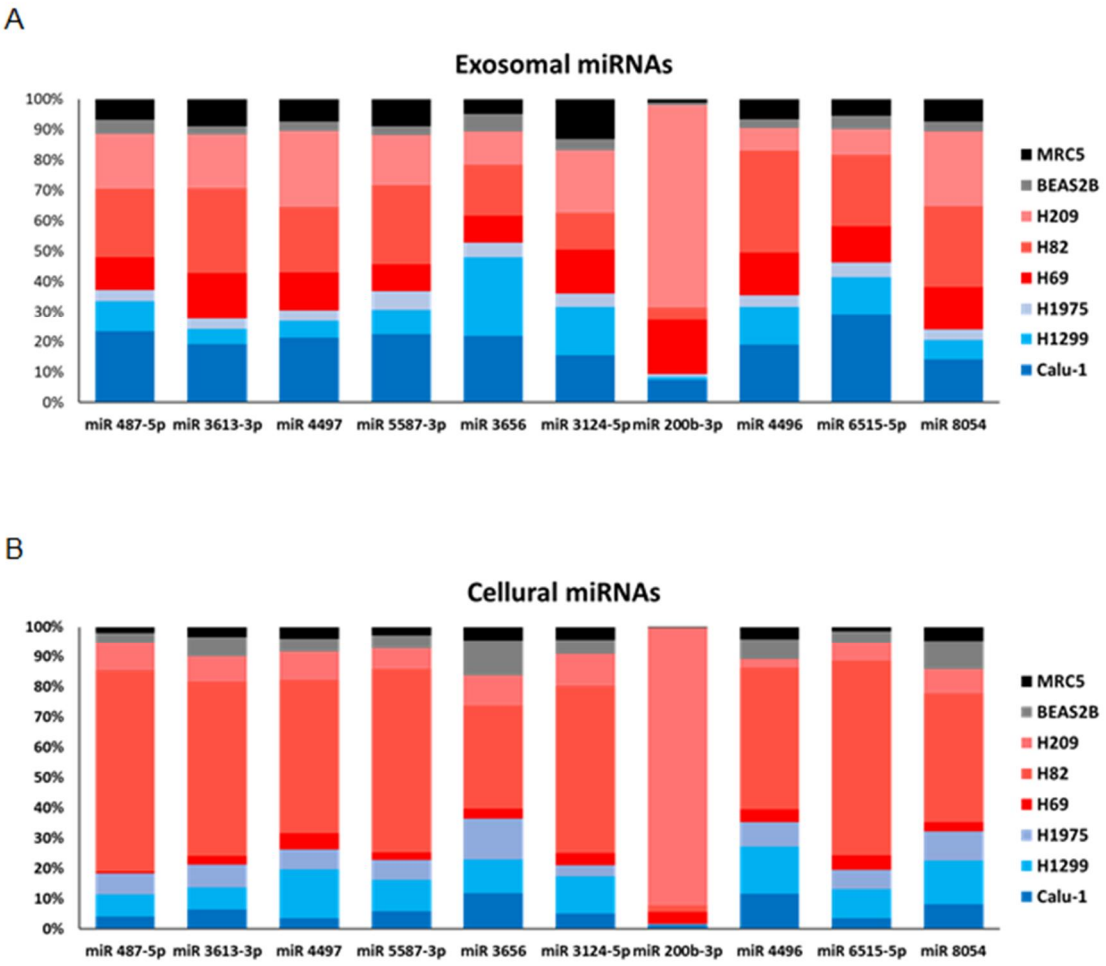


Figure 6. Comparison of miRNA expression in the cell line.

Comparison of the validated *P*-value top 10 miRNAs expressed in NSCLC (Blue: H1975, H1299, Calu-1), SCLC (RED: H209, H82, H69), Normal lung cell lines (Black: MRC5, Beas2B). (A) Exosomal miRNA (B) Cellular miRNA expression levels.

4. Clinical implications of exosomal miRNAs in SCLC patients.

To validate the expression of SCLC-specific exosomal miRNAs in the serum of healthy and SCLC patients, we extended our studies in a larger cohort, comprising 76 SCLC patients and 50 healthy volunteers. In previous studies, we found that Let-7a-5p was maintained at the same concentration within exosomes of the healthy and NSCLC patients serum compared to other miRNAs, including RNU6B and RNU48 [29]. For this reason, we used let-7a-5p as an endogenous control to evaluate the expression of exosomal miRNAs.

To confirm prognosis-related miRNAs, the candidate 15 miRNAs were determined by using qRT-PCR to validate the possibility of prognositic biomarker. Among 15 miRNAs, 6 miRNAs showed high expression levels in SCLC patients (miR-3656; $p = 0.0010$, miR-3124-5p; $p = 0.0004$, 200b-3p; $p = 0.0045$, miR-6515; $p = 0.0011$, miR-9-5p; $p = 0.0002$, miR-3126-3p; $p = 0.0217$).

The association between the 6 exosomal miRNAs and survival rate was analyzed in patients in the large cohort. We divided people into two groups, with expression level high and low. However, all of the 6 exosomal miRNAs were no significant difference in overall survival outcomes of patients with SCLC. Taken together, although there was no exosomal miRNA with a significant difference in overall survival, compared with the control group, the exosomal miR-3124-5p, miR-6515-5p and miR-9-5p, which were significantly increased in both LD and ED, were identified.

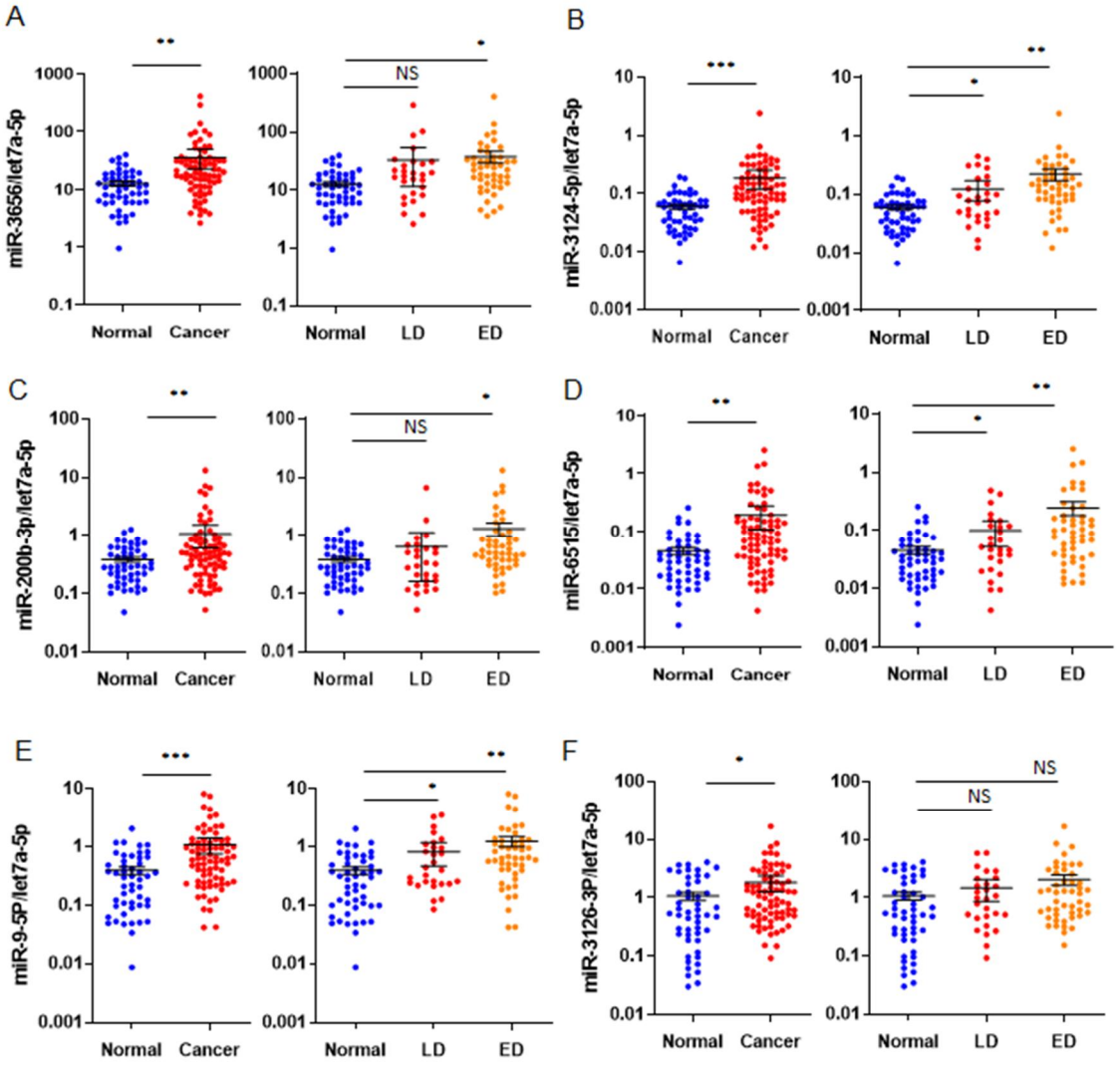


Figure 7. The expression levels of miRNAs in patients serum-derived exosome.

Exosomal miRNAs were determined by qRT-PCR assay as plots in SCLC patients (n=76) and normal (n=50) at different pathological stages. (A) miR-3656 (B) miR-3124-5p (C) 200b-3p (D) miR-6515 (E) miR-9-5p (F) miR-3126-3p $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.

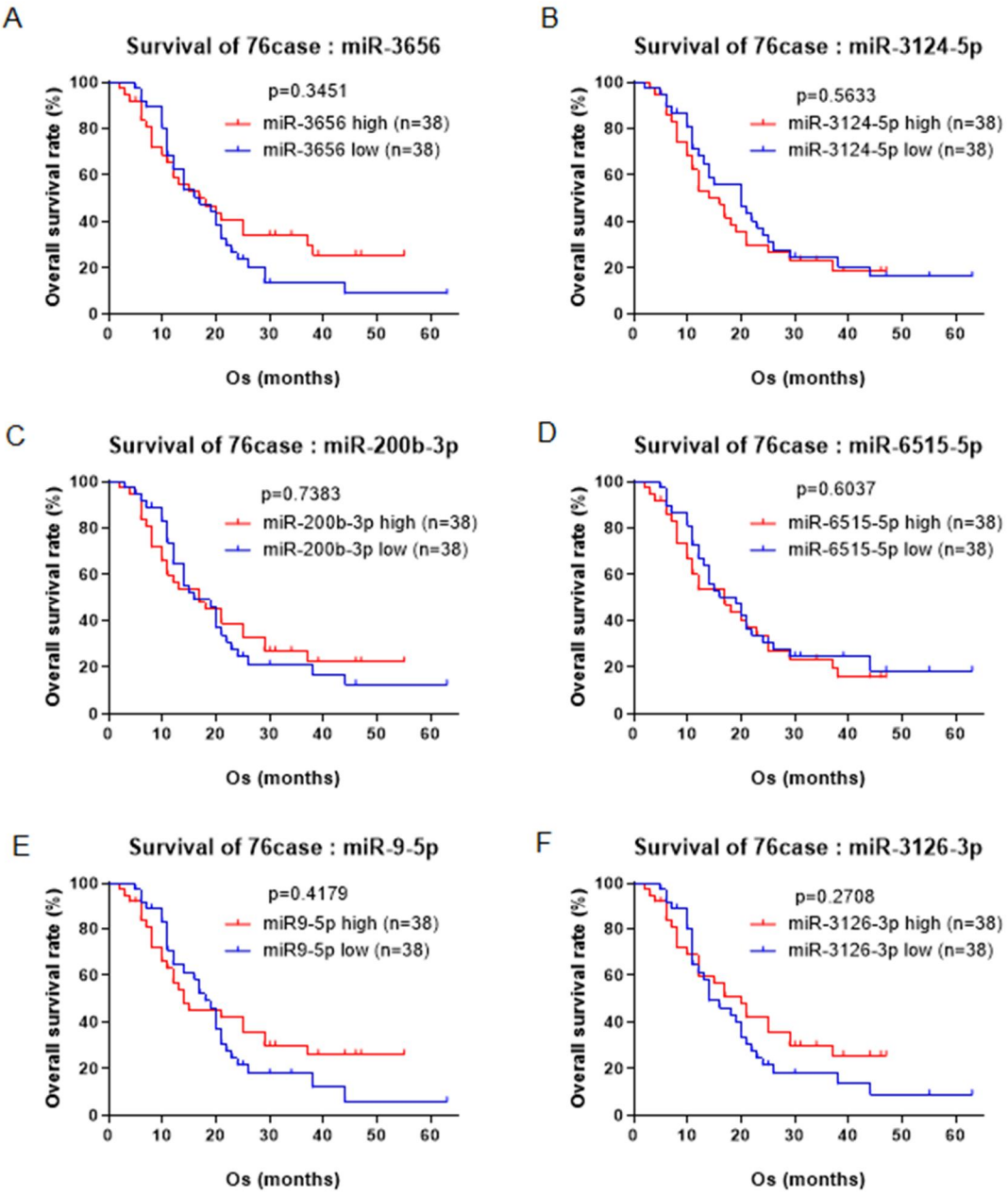


Figure 8. Kaplan–Meier survival curves for the overall survival (OS).

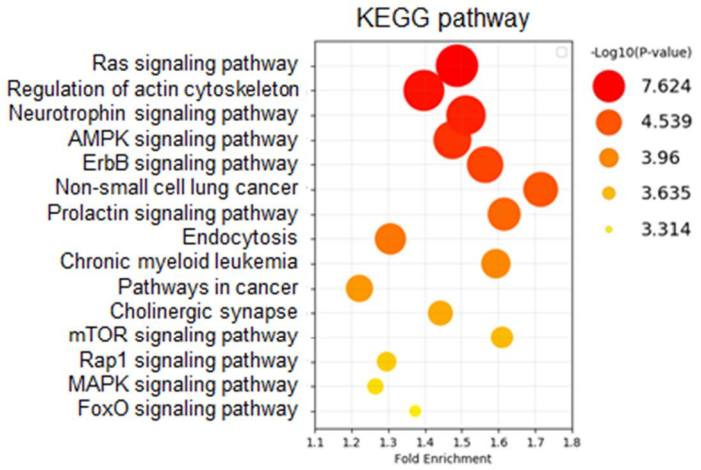
Kaplan–Meier survival curve stratified by high and low exosomal miRNA expression levels. (A) miR-3656 (B) miR-3124-5p (C) 200b-3p (D) miR-6515 (E) miR-9-5p (F) miR-3126-3p *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

5. Prediction of the role of SCLC-specific exosomal miRNAs.

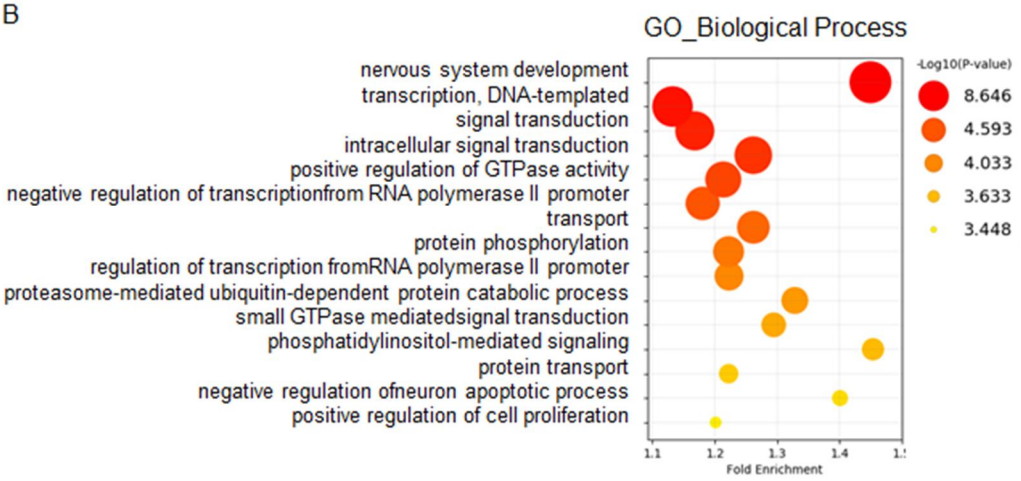
We performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) enrichment analyses to find out pathways and processes enriched in the genes regulated by miRNAs. These proteins were associated with key molecules directly or indirectly in signaling pathways such as the Ras signaling pathway, endocytosis, ErbB signaling pathway. Furthermore, these results indicated that miRNAs were involved in the processes of signal transduction, positive regulation of GTPase activity and regulation of transcription from RNA polymerase II promoter.

In short example matching between two analysis, activated Ras signaling pathway associates with ErbB signaling and transduces extracellular signals to diverse intracellular pathways, including AMPK, MAPK signaling pathway and so on [30-32]. In previous study, it is also shown that signal transduction pathways through Ras/ PKA activity also can directly regulation the elongation step of RNA polymerase II transcription targeting Spt5p elongation factor [33].

A



B



C

KEGG pathway

Term	Count	P-Value
Ras signaling pathway	126	2.37E-08
Regulation of actin cytoskeleton	110	1.09E-05
Neurotrophin signaling pathway	68	2.89E-05
AMPK signaling pathway	68	8.21E-05
ErbB signaling pathway	51	1.10E-04
Non-small cell lung cancer	36	1.20E-04
Prolactin signaling pathway	43	1.59E-04
Endocytosis	113	2.32E-04
Chronic myeloid leukemia	43	2.45E-04
Pathways in cancer	180	4.55E-04
Cholinergic synapse	60	5.07E-04
mTOR signaling pathway	35	8.29E-04
Rap1 signaling pathway	102	9.13E-04
MAPK signaling pathway	120	9.72E-04
FoxO signaling pathway	69	0.001071

D

GO_Biological Process

Term	Count	P-Value
nervous system development	162	2.26E-09
transcription, DNA-templated	862	5.55E-07
signal transduction	528	1.99E-06
intracellular signal transduction	198	2.55E-05
positive regulation of GTPase activity	267	3.77E-05
negative regulation of transcription from RNA polymerase II promoter	331	6.92E-05
transport	171	9.26E-05
protein phosphorylation	217	1.25E-04
regulation of transcription from RNA polymerase II promoter	210	1.54E-04
proteasome-mediated ubiquitin-dependent protein catabolic process	105	2.33E-04
small GTPase mediated signal transduction	124	2.50E-04
phosphatidylinositol-mediated signaling	60	3.44E-04
protein transport	188	3.57E-04
negative regulation of neuron apoptotic process	72	3.64E-04
positive regulation of cell proliferation	218	0.000404

Figure 9. Representative of function of miRNAs to perform KEGG and GO analysis.

(A) Top gene sets enriched in EVs of the SCLC patients or normal EVs, by GSEA. RNAs in each subset of EVs are ranked by GSEA based on their differential expression level. (B) Gene ontology enrichment analysis of the EV RNAs identified using the DAVID database.

Discussion

One of the methods analyzing molecular characterization of patient's tumor is using solid tumor biopsy. Using of this method is really limited because of accuracy, cost, ongoing monitoring and reflecting cancer cell property, such as heterogeneity, invasiveness [34]. Compared to solid tumor biopsy, the use of liquid biopsy provides a minimally invasive method for early and longitudinal evaluation of disease-related prediction and prognosis information [35]. Liquid biopsy are performed using body fluids including sweat, blood, saliva, tears and urine. The body fluid has circulating tumor proteins, circulating tumor nucleic acids (ctDNA and ctRNA), CTCs, EVs, and TEPs copying the component of tumor. Among the components used in liquid biopsies, ctDNA and CTC have limitations for use in profiling due to low sensitivity and efficiency, and instability of ctRNA [36]. For these reasons, we used extracellular vesicles called exosomes for genetic profiling using liquid biopsies.

Exosomes are helpful vesicle to communicate between the cells, even cancer. Vesicles pass thorough plasma membrane of target cell having constantly interaction according to transmission of various biological signal [37, 38]. Then, cancer-derived exosomes are capable of tumor progression including invasion, angiogenesis and remodeling microenvironment [19, 39-41]. Among the exosomal components that effect target cells, it is demonstrated that exosomal miRNA can function in cancer and contribute tumor growth. [37, 42-44]. As miRNA have activity of dysfunction in most cancer, exosomal miRNAs are able to interrupt translation binding to target mRNA [45]. A number of miRNA are also known tumor-specific [46], miRNAs can use as disagnostic and prognostic biomarker.

Our data showed that miR-3124-5p, miR-6515-5p and miR-9-5p are highly expressed at both ED and LD stage. miR-3124-5p and miR-6515-5p were not much previously reported its function in exosomes. In contrast, miR-9-5p play a powerful role in tumor progression by regulating StarD3 in prostate cancer and also facilitates proliferation, migration and invasion by targeting ESR1 expression in hepatocellular carcinoma cell [47-49]. Exosomal miR-9-5p also already consider promising diagnostic biomarker in renal cancer [50].

Furthermore, validation data expected that highly expressed miRNAs in patients showed poor diagnosis, but there were no difference according to expression level of miRNA. It could be explained miRNA might have not dual-function in cancer as diagnostic and prognostic

biomarker. Additional study is needed such as ROC curve analysis to clarify the possibility as prognosis marker. To identify the functionality of the identified miRNAs, each predicted target was identified using a miRNA target matching program. In addition, functional enrichment analysis using GO and KEGG in the DAVID database showed that the Ras signal pathway and RNA polymerase II transcription were common and important signal pathway of target miRNAs.

Ras mutations correlates with poor survival of NSCLC patients, whereas these mutations less than 1% in SCLC tumor [51-52]. Although lack of activating mutations in SCLC, Ras activation effects SCLC tumor progression. Inhibiting Ras signaling with simvastatin, the 3-hydroxy-3methylglutaryl CoA (HMG–CoA) reductase inhibitor, disrupts proliferation and survival in SCLC cells. Also, gefitinib known as EGFR-TKI also shown inhibition of Ras-MAPK pathway in three SCLC cell lines [53]. Thus, it is demonstrated that targeting Ras pathway can provide indirect effect of SCLC prognosis.

We are not able to provide an answer to which of the six biomarkers will play a better role in early diagnosis of SCLC patients. This is because the patient samples we used cannot represent the entire SCLC, and a lot of biomarker research continue to provide new insights, especially in the accelerating exosome field. We will investigate effect of identified 6 miRNA on sensitivity and specificity in SCLC patients further confirm their functions through additional research.

Conclusion

miRNA signature for diagnosis and prognosis can be useful to improve clinical outcome. We investigated exosomal miRNAs profile secreted by SCLC patients serum. 6 miRNAs showed high expression levels in SCLC patients and only miR-3124-5p, miR-6515-5p and miR-9-5p represent high expression of LD stage. Ras signaling pathway involved in miRNA mechanisms and it showed that miRNA regulates signal transduction and RNA polymerase II transcription in miRNA analysis data. It suggested that exosomal 6 miRNAs from serum of SCLC patients might have potential as a biomarker.

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국문 요약

폐에서 발생하는 악성종양은 조직형에 따라 분리하게 되는데 전체 폐암의 20~30%를 차지하는 소세포폐암 (Small Cell Lung Cancer, SCLC)은 암의 조직학적 특성 때문에 대부분의 환자들이 제한기인 진행성 병기에 발견되는 특징이 있으며 재발률이 높고 항암제의 반응성이 떨어져 환자의 생존율이 좋지 않은 것으로 알려져 있다. 기존 폐암환자의 진단은 조기 진단의 정확성 면에서 한계가 있다. 따라서 비교적 환자에게 부담을 주지 않고 치료 성적을 높일 수 있는 조기 소세포폐암의 진단 방법은 오랫동안 필요성이 강조되어 왔다.

이번 연구에서는 SCLC 환자와 정상 대조군을 대상으로 혈액을 수집해 환자에서 증가한 miRNA 를 확인함으로써 진단 및 예후인자로써 유효성을 검증하였다. 첫번째로, 각 환자 검체에서 exosome 을 분리하고 이를 miRNA profile 을 통해 SCLC 특이적으로 증가된 miRNA 들을 확인할 수 있었다. 이는 확장된 임상 코호트에서 한 번 더 검증하였고 그 결과 최종적으로 소세포암의 초기 병기인 제한병기와 암이 진행된 상태인 확장병기 모두에서 miR-3124-5p, miR-6515-5p and miR-9-5p 인자들이 환자에서 증가되는 것을 검증하였다. 하지만 이는 환자 예후를 반영하지는 못했다. 온라인 데이터 베이스를 통해 해당 miRNA 를 분석한 결과 암의 증식에 영향을 미치는 Ras signaling, ErBb pathway 를 포함한 다양한 신호전달 기작에 관여하고 있음을 확인할 수 있었으며, 이는 비정상적인 기능을 하도록 세포내 신호와 RNA 중합효소 Ⅱ 전사과정에 기여함을 보여준다. 결론적으로, 이번 연구는 소세포폐암 특이적으로 증가하는 miRNA 가 존재하였으며, 이는 추후 연구에서 진단 및 예후인자로서 고려되어야 한다는 점을 시사한다.