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MicroRNA expression profiling of lung adenocarcinoma in Xuanwei, China

A preliminary study

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Abstract

MicroRNAs (miRNAs) have been proved to be related to the development and progression of lung cancer. However, the expression signatures of miRNAs in lung adenocarcinoma in Xuanwei are not yet clear. The current study aimed to identify the potential miRNA profiles in lung adenocarcinoma in Xuanwei by microarray.

The miRNA profiles in 24 lung adenocarcinoma and paired non-tumor tissues in Xuanwei were ascertained by using the Exiqon miRCURY LNA microRNA Array (v.18.0). The results of the microarrays were further verified by quantitative real-time polymerase chain reaction (qRT-PCR) detection. Bioinformatics analysis was used to carry out the functional annotations of differentially expressed miRNAs.

One hundred fifty five differentially expressed (≥2-fold change) miRNAs were identified (65 upregulated and 90 downregulated). QRT-PCR was used to validate the top 4 most upregulated and downregulated miRNAs, and the results were generally consisted with microarray. Furthermore, the differentially expressed miRNAs were significantly enriched in numerous common pathways that were bound up with cancer. The pathways included focal adhesion and signaling pathways, such as cyclic guanosine monophosphate -protein kinase G (cGMP-PKG) signaling pathways, mitogen-activated protein kinase (MAPK) signaling pathway, and Hippo signaling pathway, etc.

Our study identified the potential miRNA profiles in lung adenocarcinoma in Xuanwei by microarray. These miRNAs might be used as biomarkers for diagnosis and/or prognosis for lung cancer in Xuanwei and therefore warrant further investigation. Further study is needed to reveal the potential role of these miRNAs in the carcinogenesis of XuanWei Lung Cancer (XWLC).

Abbreviations: FC = fold change, qRT-PCR = quantitative real-time polymerase chain reaction, XWLC = XuanWei Lung Cancer.

Keywords: microarray analysis, MicroRNAs, non-small-cell lung carcinoma

1. Introduction

Xuanwei is a small City, locating in the eastern Yunnan province of China, which had the highest morbidity and mortality of lung cancer in China, even in the world.[1,2] XuanWei Lung Cancer (XWLC) also had some remarkable features, which were that the non-smoking women had rather high morbidity and mortality of lung cancer and the major pathological type was adenocarcinoma.[3] "Smoky coal," which is different from other types of coal, served as the main cooking and warming material and caused indoor air pollution that is may be the major reason to lung cancer in women. When this coal burns, it releases some carcinogen, such as particulate matter, polycyclic aromatic hydrocarbons (PAHs), and crystalline quartz etc.[4–6] In the late 1980s, for the sake of decreasing the incidence rate of XuanWei Lung Cancer, Chinese government had carried out a project called as "Stove Improvement," but the result was inconsistent with expectation.[2] All these evidences suggested the mechanisms of XuanWei Lung Cancer development have not been well understood; further investigation should be performed.

MicroRNAs (miRNA) are short, single-stranded, non-coding RNA of 19–25nt, which can bring about mRNA degradation and/or the inhibition of translation by monitoring the post-translational gene expression.[7] Recent studies have found aberrant miRNA may cause abnormal gene expression and tumorigenesis. A feature of many types of cancers is the changes in expression of miRNAs, which have been indicated to promote

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the development, invasion, and metastasis of tumors by many different ways.[8,9]

As the air pollution exposure in Xuanwei was complex and different from other air pollution, the miRNAs change in XWLC may be unique. Adenocarcinoma is the main pathological type of lung cancer in Xuanwei, so lung adenocarcinoma could represent the major feature of XWLC. There were few researches focus on miRNA in lung adenocarcinoma in Xuanwei. One previous study indicated miR-195 may act as potential tumor suppressor genes by inhibits non-small cell lung cancer cell proliferation, migration and invasion by targeting MYB in Xuanwei lung cancer cohort.[10] Another study provided evidence for the involvement of miR-29b in XWLC lymphatic metastasis and suggested that miR-29b may serve as a new biomarker for diagnosis of XWLC or therapeutic target.[11] However, there is no systemic study on the miRNA profiles of lung adenocarcinoma in Xuanwei. In the future, we hope our research will be useful to develop diagnostic tool for detecting and prognosis in lung cancer, especially for those lung adenocarcinoma patients in Xuanwei. Here, we use high-throughput technology to identify the miRNAs profile in lung adenocarcinoma in Xuanwei.

Table 1

The clinicopathological characteristics of the patients with lung adenocarcinoma in Xuanwei area.

Characteristics	Microarray analysis (n = 24)	qPCR validation (n = 30)
Age, y	55 ± 9.85	58.2 ± 11.33
Sex (M/F)	14/10	17/13
Smoking status		
Yes	5	8
No	19	22
Tumor		
T1	8	10
T2	13	15
T3	3	5
Lymphatic metastasis		
N0	19	22
N1	5	8
TNM stage		
I	10	13
II	8	10
III	4	5
IV	2	2

2. Material and methods

2.1. Sample collections

Fifty four pathologically confirmed lung adenocarcinoma patients who underwent surgical resection at the Yunnan Cancer Center from January 2015 to August 2016 were selected in our study. All these patients were from Xuanwei region and lived there for >3 generations. Any adjuvant treatments were not given to all patients enrolled in the study before surgery. Lung adenocarcinoma tissues and corresponding non-tumor tissues (>5 cm away from carcinoma tissues) were collected after surgery and immersed in RNAlater solution (Qiagen, Hilden, Germany) overnight, then kept in −80 °C fridge for next steps, such as RNA extraction. According to the 7th edition of the AJCC (American Joint Committee on Cancer) TNM staging system, we evaluated the tumor stage. All patients enrolled in our study subscribed the written informed consents before surgery, and the ethics committee of Yunnan Cancer Center approved the study. Table 1 contains the basic characteristics of the patients included in our study.

2.2. RNA extraction

According to manufacturer's protocols, we extracted the total RNA from tissues by using TRIzol Reagent (Invitrogen life technologies, Carlsbad, CA) and RNasey Mini Kit (Qiagen, Hilden, Germany). Electrophoresis on a denaturing agarose gel was used to estimate the integrity of the isolated RNA. A NanoDrop ND-1000 spectrophotometer (Thermo, Waltham, MA) was used to evaluate the quantity and purity of isolated RNA. Only samples with the purity of 1.8 to 2.1 (A260/A280) were deemed acceptable.

2.3. miRNAs expression analysis by microarray

Twenty four paired lung adenocarcinoma tissues and corresponding non-tumor tissues were prepared for microarray analysis. After quality control, miRCURYTM Array Power Labeling kit (Cat #208032-A, Exiqon, Vedbaek, Denmark) was applied for RNA labeling according to manufacturer's protocol. After the labeling procedure was finished, the Exiqon miRCURY LNA microRNA Array (v.18.0), which comprised >3100 capture probes, was applied to make Hy3-labeled samples to be hybridized on the 7th generation according to manufacturer's guidelines. And the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA) was used to scan the slides.

Then scanned images were imputed into the GenePix Pro 6.0 software (Axon) for information analysis. If the intensity in specimen is ≥30, which is the average value of the replicated miRNAs, the miRNAs were picked out to count the normalization factor. Through the median normalization, we completed to normalize the expressed data. After normalization, if expressed miRNAs got fold changes (≥2) and *P*-values (<.05), it showed the 2 groups were identified to be different significantly. Lastly, volcano plot figures and hierarchical clustering were used to demonstrate the significantly different expressed miRNAs.

2.4. qRT-PCR

We selected 4 top upregulated and 4 top downregulated miRNAs for quantitative real-time polymerase chain reaction (qRT-PCR) validation. According to the manufacturer's guidelines of miRcute Plus miRNA First-Strand cDNA Synthesis Kit (Tiangen Biotech, Beijing, China), total RNA was reverse transcribed to cDNA by using a T100 Thermal Cycler (Bio-Rad, California). Thirty paired lung adenocarcinoma tissues and corresponding non-tumor tissues were used for qRT-PCR validation in a ABI 7900HT Fast Real Time PCR System (Applied Biosystems, USA) by using miRcute Plus miRNA quantitative real-time polymerase chain reaction (qPCR) Detection Kit (Tiangen Biotech, Beijing, China).

2.5. Functional group analysis

Gene ontology (GO) analysis that supplies 3 structured networks of defined terms for describing the attributes of gene product is based on Gene Ontology (www.geneontology.org). The significance of GO term enrichment in the target genes of differentially expressed mRNAs is indicated with *P* value ($P < .05$ is considered statistically significant). The pathways for the target genes of

differentially expressed mRNAs derived from the latest KEGG (Kyoto Encyclopedia of Genes and Genomes) database are also been analyzed. The results of analysis enables us to identify the biological pathways and the target genes of differentially expressed mRNAs are enriched ($P < .05$ and FDR < 0.1 were considered statistically significant).

3. Results

3.1. Overview of miRNA profiles

By means of microarray analysis, we distinguished the differentially expressed miRNAs in lung adenocarcinoma relative to non-tumor tissues. The results showed 65 upregulated miRNAs and 90 downregulated miRNAs, according to $P < .05$ and FC (fold change) ≥2-fold. Table 2 presented 20 top upregulated and 20 top downregulated miRNAs, and Hsa-miR-135b-3p and hsa-miR-144-5p were the most upregulated and downregulated miRNA, respectively. On the basis of expression level, the samples were divided into different group through hierarchical clustering analysis (Fig. 1).

3.2. qRT-PCR verification of microarray results

To identify the expression levels of miRNAs of the microarray, top 4 most upregulated miRNAs (hsa-miR-135b-3p, hsa-miR-485-5p, hsa-miR-135b-5p, hsa-miR-9-5p), and downregulated miRNAs (hsa-miR-144-5p, hsa-miR-486-5p, hsa-miR-30a-3p, and hsa-miR-144-3p) were selected for qPCR validation. The results of qPCR were in good agreement with the results of microarray except the difference of hsa-miR-30a-3p between cancer and normal tissue did not reach a significant level ($P = .088$) (Fig. 2).

3.3. Prediction of the target genes

For the sake of further understanding the functions of the differentially expressed miRNAs, we found out the target genes of these miRNAs (65 upregulated and 90 downregulated miRNAs) by using 3 databases (miRanda, miRBase, and TargetScan), then the target genes included in all 3 databases was selected. As a result, 1167 and 904 target genes were predicted by upregulated and downregulated miRNAs, respectively (Fig. 3).

3.4. GO analysis and KEGG pathway analysis

Gene ontology analysis (GOs) was used to predict the target genes and found that the biological processes, cellular components and molecular functions were the gene product enrichments. Results showed the highest enriched GOs targeted by upregulated miRNAs were biological process (system development), cellular component (intracellular organelle), and molecular function (protein binding). While, the highest enriched GOs targeted by downregulated miRNAs were biological process (negative regulation of cellular process), cellular component (intracellular), and molecular function (protein binding) (Fig. 4).

Ten pathways corresponded to upregulated miRNAs were identified through KEGG pathway analysis. The most enriched cancer related pathway were the cGMP-PKG signaling pathway, MAPK signaling pathway, Focal adhesion, Transcriptional misregulation in cancer, and so on. One pathway corresponded to downregulated miRNAs were identified including cAMP signaling pathway (Table 3).

4. Discussion

Previous studies have suggested the mutation type in certain genes in XWLC might be different from other type of lung cancer. Epidermal growth factor receptor (EGFR) might be one of the examples. Our previous work suggested the mutation rate of G719X in exon 18 was higher than that of the general population, while the incidence of 19 deletions and L858R mutations was lower than that of the general population.[12] Our founding is similar to the study by Hosgood et al[13] and Chen

Table 2

Top 20 significantly upregulated and downregulated miRNAs ($P < .05$, FDR < 0.1).

Upregulated miRNAs				Downregulated miRNAs			
miRNA	Fold change	*P* value	FDR	miRNA	Fold change	*P* value	FDR
hsa-miR-135b-3p	8.0007942	.0006959	0.0056219	hsa-miR-144-5p	0.0764973	.0115454	0.0436158
hsa-miR-485-5p	5.7646738	.0177208	0.0602508	hsa-miR-486–5p	0.1861492	6.607E–06	0.0001934
hsa-miR-135b-5p	5.6953045	.0059377	0.0269368	hsa-miR-30a-3p	0.2076893	6.248E–08	9.408E–06
hsa-miR-9–5p	4.8773565	.0265475	0.0810238	hsa-miR-144-3p	0.2088273	4.014E–06	0.0001426
hsa-miR-369-3p	4.3489718	.0331098	0.095068	hsa-miR-1-3p	0.2116755	7.967E–05	0.0011717
hsa-miR-4732-5p	4.1500094	.0248131	0.0769205	hsa-miR-218-5p	0.2179132	4.907E–08	8.62E–06
hsa-miR-301b-3p	3.8361571	.0063486	0.0283534	hsa-miR-551b-3p	0.2474182	8.069E–05	0.0011812
hsa-miR-134-5p	3.5468529	1.886E–05	0.0004263	hsa-miR-126-5p	0.2593944	3.464E–07	2.434E–05
hsa-miR-224-5p	3.2389712	.0228252	0.0724631	hsa-miR-223-3p	0.2766898	8.832E–05	0.0012303
hsa-miR-501-5p	3.1688483	.0223758	0.0713947	hsa-miR-3665	0.2770479	1.842E–07	1.734E–05
hsa-miR-708-3p	2.8656563	.0009682	0.007087	hsa-miR-20b-5p	0.2811925	.0009354	0.0069104
hsa-miR-3913-3p	2.8502806	.0024444	0.0136076	hsa-miR-126–3p	0.2862527	4.129E–06	0.0001451
hsa-miR-764	2.7468242	.0011602	0.0080981	hsa-miR-15b-3p	0.2874967	.0125728	0.046826
hsa-miR-4676-5p	2.7460479	8.398E–05	0.0012049	hsa-miR-30a-5p	0.2884915	1.338E–07	1.549E–05
hsa-miR-210-3p	2.7421208	.0020588	0.0120854	hsa-miR-20a-5p	0.289276	.0002852	0.0028632
hsa-miR-548l	2.7283936	.0193407	0.0642387	hsa-miR-3195	0.2933574	8.097E–07	4.492E–05
hsa-miR-548t-5p	2.719158	.0022558	0.0128753	hsa-miR-99a-3p	0.2964608	2.229E–08	7.306E–06
hsa-miR-96-5p	2.6332503	.0126074	0.0468444	hsa-miR-4462	0.3001976	4.716E–08	8.62E–06
hsa-miR-4539	2.6100567	2.767E–05	0.0005593	hsa-miR-139-5p	0.3007603	4.326E–09	2.736E–06
hsa-miR-938	2.2220253	.0110123	0.0420213	hsa-miR-598-3p	0.4300214	.0000000	0.0000000

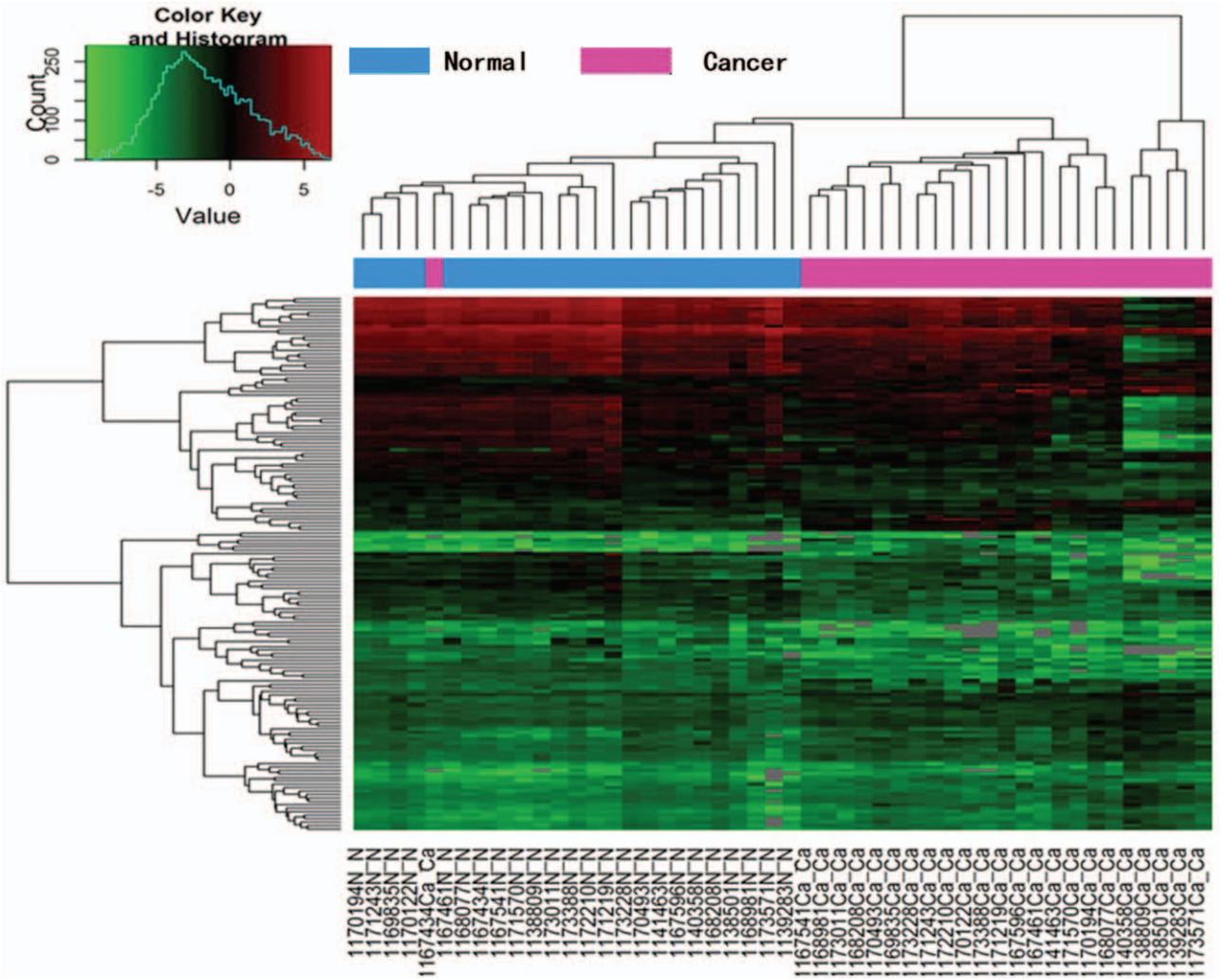

Figure 1. Hierarchical clustering analysis of differential expressed microRNAs in lung adenocarcinoma tissue and adjacent noncancerous tissue.

et al[14] KRAS and TP53 mutation, which spectra in nonsmokers in Xuanwei were different from the other smokers, were as similar as the EGFR mutation.[15] These results suggest the mutations of EGFR, TP53, and KRAS genes may reflect a exposure to particular environment. Nevertheless, which component, particulate matter, PAHs, crystalline quartz, or their interactions, may play a leading role? What are the underlying mechanisms? Look forward to further research.

As a result, the etiology of XuanWei Lung Cancer (XWLC) is unique and complex. Both environmental, genetic, epigenetic factors, and their interaction should be taken into consideration. In current study, we only focus on miRNA, which was a closely related environmental factor and took a prominent part in tumorigenesis and progression in lung cancer.[16] Microarray technologies unable us compressively analyze the potential miRNAs related to lung cancer in Xuanwei.

Our analysis have identified 155 miRNAs (FC ≥2) might be related to carcinogenesis in XWLC. Some cancer-related pathways have been enriched by pathway analysis of potential target predicted by these miRNAs, such as cGMP-PKG signaling pathway, MAPK signaling pathway, focal adhesion, suggesting these miRNAs would play an important part in XWLC. In certain previous studies, some miRNAs identified to be upregulated/downregulated by our study have been proved dysregulated in great deal of tumors, such as hsa-miR-9-5p,[17,18] miR-485-5p,[19] hsa-miR-135b-5p,[20] hsa-miR-144-5p,[21] hsa-miR-486-5p,[22] hsa-miR-144-3p[23] and hsa-miR-218-5p,[24] etc. However, some have been seldom reported, such as hsa-miR-369-3p, hsa-miR-325, hsa-miR-4732-5p, hsa-miR-30a-3p, hsa-miR-1-3p, hsa-miR-551b-3p, and hsa-miR-3665. More attentions should be paid in these seldom reported miRNAs to found new biomarkers and elucidated new mechanism in XWLC.

Other studies also investigated the genetic and epigenetic profile in lung cancer in Xuanwei, such as somatic mutation spectrum,[25] DNA copy number alterations (CNAs),[26] differentially expressed genes (DEGs),[27] DNA methylation,[28] and long non-coding RNAs (lncRNAs).[29] The miRNAs profile have not been investigated yet, here, we offered the whole genome expression profiling of miRNA in XWLC. In the near future, further work is needed to identify their potential functions, even the molecular mechanisms.

Our study may have such advantages. Firstly, our sample size used in microarray analysis was relatively larger, large sample size may yield stronger static power that made our result more

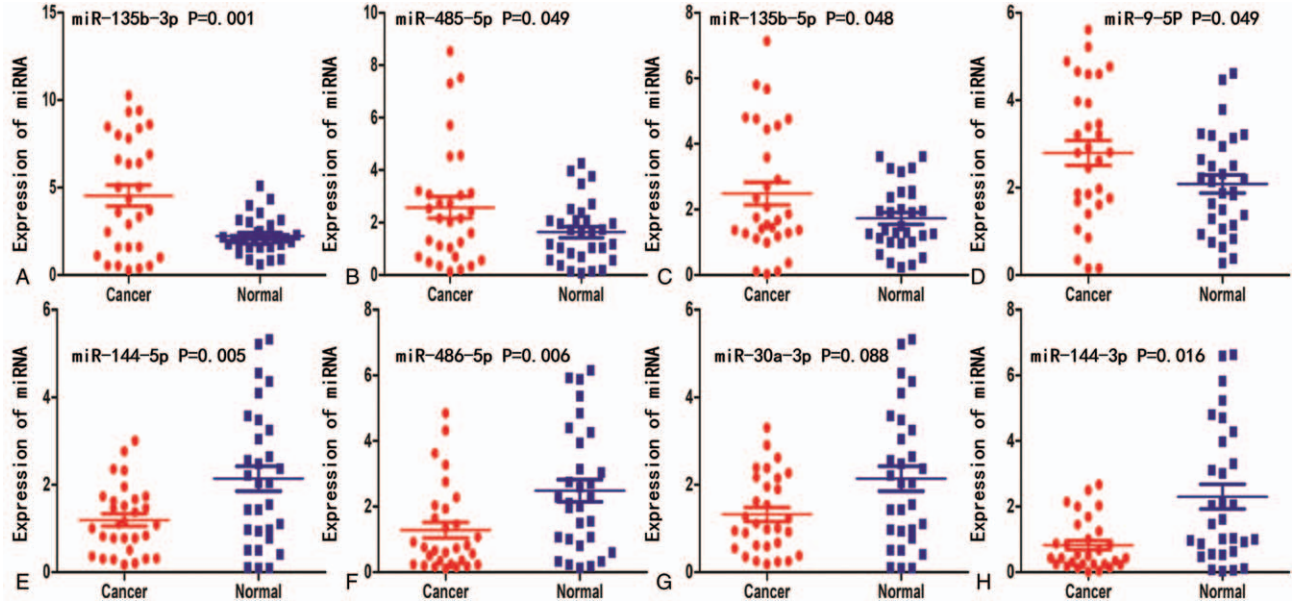

Figure 2. Quantitative reverse transcription-polymerase chain reaction validation of 4 upregulated miRNAs (miR-135b-3p, miR-485-5p, miR-135b-5p, and miR-9–5p) and 4 downregulated miRNAs (miR-144-5p, miR-486-5p, miR-30a-3p, and miR-144-3p).

robust. Secondly, our sample might have good representative, as the adenocarcinoma was the major type of Xuanwei lung cancer. In some degree, our result might reflect the miRNAs in Xuanwei lung cancer. Thirdly, the seventh Exiqon miRCURY LNA microRNA Array (v.18.0) were applied in our analysis. Three thousand one hundred capture probes were included in the assay, which had high specificity and sensitivity, even for miRNAs riched for AT. Moreover, they provided good reproducibility, 99% correlation between arrays and >5 orders of magnitude of dynamic range. Finally, false positive results were detected in high throughput microarray detection. Top 4 upregulated and downregulated miRNAs were validated by qPCR in 30 parried tissues and the results were consisted with microarray. Suggesting the miRNAs profile identified by microarray is reliable.

Also, some limitation should be acknowledged in our analysis. Firstly, we did not conducted miRNA microarray analysis in same tissue at the same time, which will make it easier to predict the function of dysregulated miRNA and identify their associated coding RNA. Secondly, false positive results may exist in our analysis. Although we conducted qPCR validation, only top 4 most upregulated and downregulated miRNAs were validated. In reaming differentially expressed miRNAs, the consistence with microarray was still unclear. Further validation should be done in future.

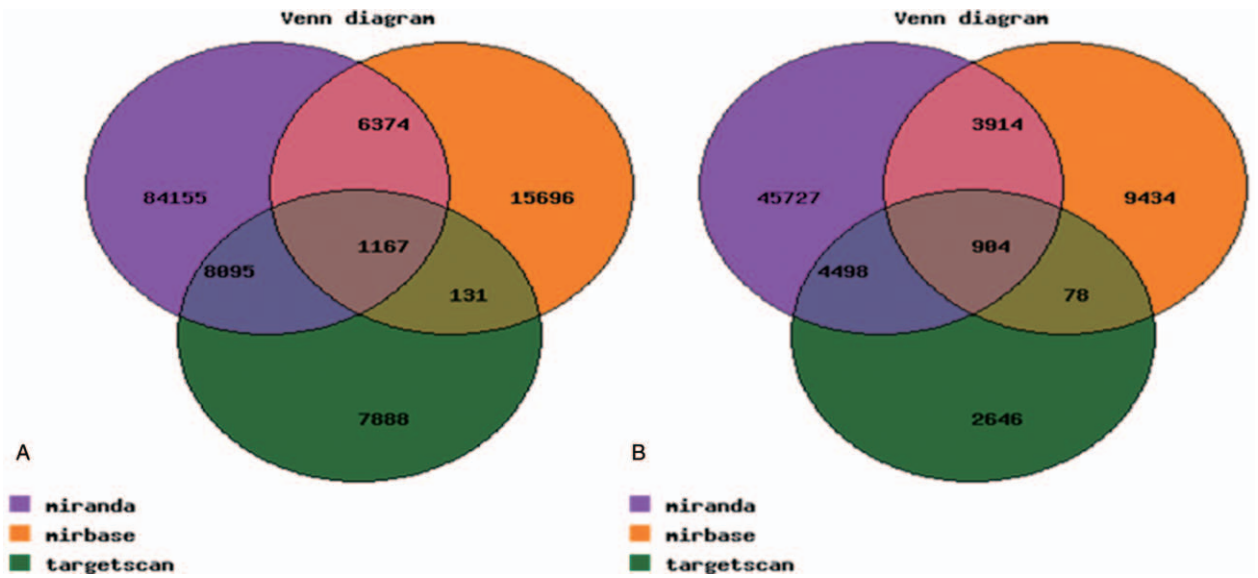

Figure 3. The predicted putative target genes by upregulated and downregulated miRNAs in from 3 databases (miRanda, miRBase, and TargetScan).

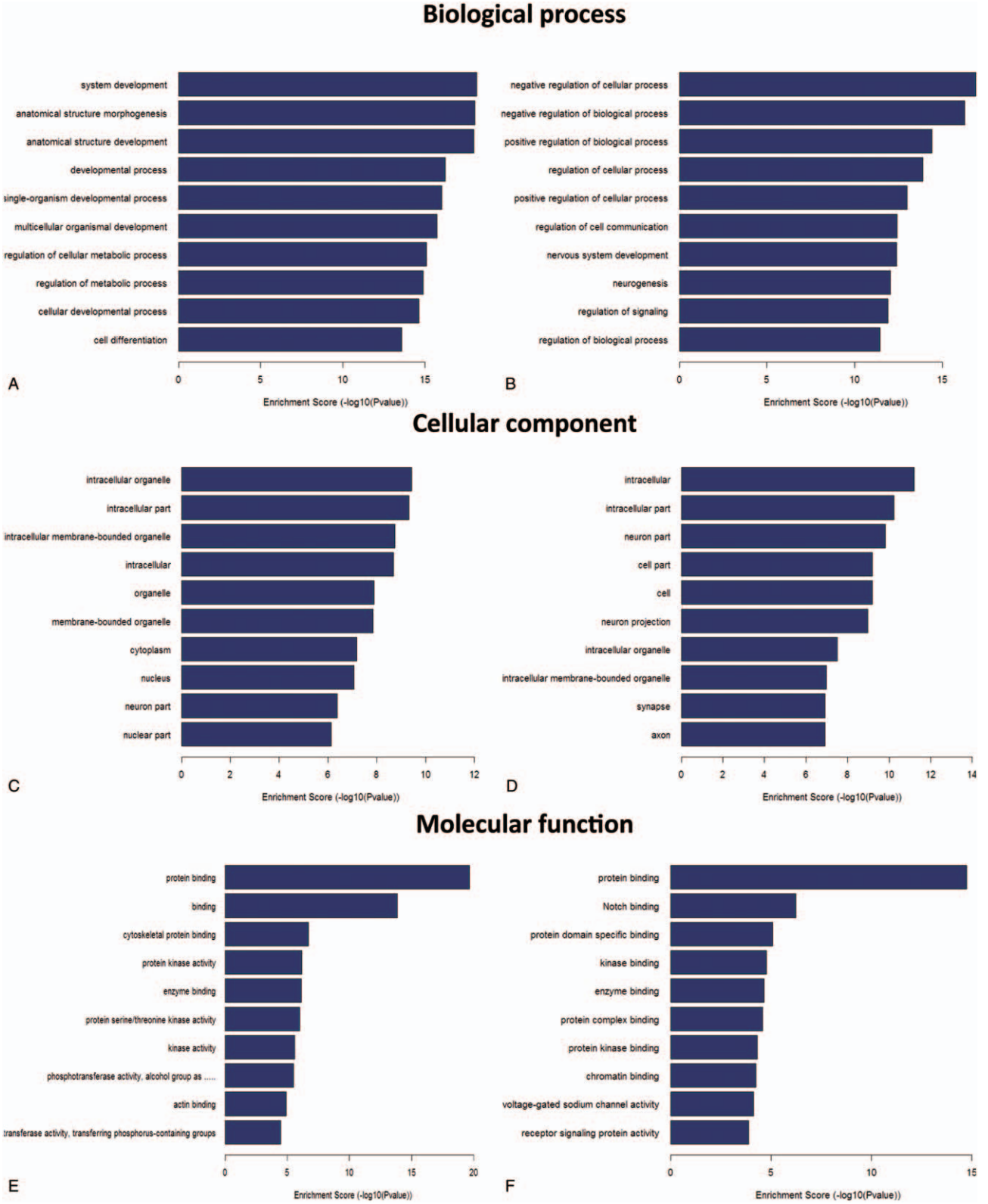

Figure 4. Top 10 significant functional GO terms of the predicted miRNA target genes. The bar plot shows the enrichment scores (–log 10 [*P*-value]) of the significant enrichment GO terms. The ontology covers 3 domains: biological process (BP), cellular component (CC), and molecular function (MF). (*P*-value $\leq$.05 is recommended). GO = gene ontology.

Table 3

The most enriched cancer related pathway by miRNA targets ($P < .05$, FDR < 0.1).

Pathway ID	Definition	Fisher-*P* value	FDR	Enrichment score
Upregulated miRNAs				
hsa04022	cGMP-PKG signaling pathway—*Homo sapiens* (human)	.000154318	0.0151231	3.8116
hsa04010	MAPK signaling pathway—*H sapiens* (human)	.000546228	0.0326345	3.2626
hsa04510	Focal adhesion—*H sapiens* (human)	.000555008	0.0326345	3.2557
hsa05202	Transcriptional misregulation in cancer—*H sapiens* (human)	.00104443	0.0511771	2.9811
hsa04915	Estrogen signaling pathway—*H sapiens* (human)	.001329757	0.054697	2.8762
hsa04015	Rap1 signaling pathway—*H sapiens* (human)	.001674397	0.054697	2.7761
hsa04310	Wnt signaling pathway—*H sapiens* (human)	.002120699	0.0566805	2.6735
hsa04390	Hippo signaling pathway—*H sapiens* (human)	.002438897	0.059753	2.6128
hsa04151	PI3K-Akt signaling pathway—*H sapiens* (human)	.003699495	0.0725101	2.4319
hsa05205	Proteoglycans in cancer—*H sapiens* (human)	.005178756	0.089562	2.2858
Downregulated miRNAs				
hsa04024	cAMP signaling pathway—*H sapiens* (human)	.000210082	0.0617641	3.6776

5. Conclusion

The current study described the miRNAs expression profiles of lung adenocarcinoma in Xuanwei. Whether they could serve as biomarkers in XWLC? What their potential role and mechanism? Further studies should be done.

Author contributions

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Investigation: Zhenghai Shen.
Methodology: Zhenghai Shen.
Project administration: Yunchao Huang.
Writing – original draft: Zaoxiu Hu.
Writing – review & editing: Xiaoxiong Wang.

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