A transcriptional miRNA-gene network associated with lung adenocarcinoma metastasis based on the TCGA database

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Abstract. Lung adenocarcinoma is the most common subtype of non-small cell lung cancer (NSCLC), leading to the largest number of cancer-related deaths worldwide. The high mortality rate may be attributed to the delay of detection. Therefore, it is of great importance to explore the mechanism of lung adenocarcinoma metastasis and the strategy to block metastasis of the disease. We searched and downloaded mRNA and miRNA expression data and clinical data from The Cancer Genome Atlas (TCGA) database to identify differences in mRNA and miRNA expression of primary tumor tissues from lung adenocarcinoma that did and did not metastasize. In addition, combined with bioinformatic prediction, we constructed an miRNA-target gene regulatory network. Finally, we employed RT-qPCR to validate the bioinformatic approach by determining the expression of 10 significantly differentially expressed genes which were also putative targets of several dysregulated miRNAs. RT-qPCR results indicated that the bioinformatic approach in our study was acceptable. Our data suggested that some of the genes including PKM2, STRAP and FLT3, may participate in the pathology of lung adenocarcinoma metastasis and could be applied as potential markers or therapeutic targets for lung adenocarcinoma.

Introduction

Lung adenocarcinoma, the most common subtype of non-small cell lung cancer (NSCLC), leads to one million deaths each year, affecting an increasing percentage of the population over the past few years (1). Despite advances in suitable therapies, the 5-year survival of lung adenocarcinoma patients remains low. The high mortality rate may be attributed to the delay of detection, since patients are asymptomatic in early stages. Local and distant metastases occur in most cases by the time symptoms are obvious, resulting in treatment failure in advanced NSCLC (2). Therefore, it is of great importance to explore the mechanism of NSCLC metastasis and strategies to block metastasis of the disease.

As post-transcriptional modulators, microRNAs (miRNAs) are a class of endogenous, non-coding, single-stranded RNAs with 21-24 nucleotides (3). Dysregulation of miRNAs contributes to many pathological conditions, such as the initiation and progression of lung cancer. A number of studies have assessed the potential role of miRNA signatures to classify histological subtypes (4) or to predict diagnosis (5), metastasis, recurrence or survival of NSCLC patients (6-9). Wang *et al* revealed a set of 24 differentially expressed miRNAs between NSCLC metastatic primary loci and non-metastatic primary loci in a mouse model (9). Larzabal *et al* showed that miR-205 overexpression inhibited metastasis of NSCLC by targeting integrin α5 (a pro-invasive protein) upon TMPRSS4 blockade, which was confirmed by *in vivo* and *in vitro* experiments (10).

High throughput data, such as gene expression data from RNA sequencing or microarrays, could be widely used in the exploration of molecular mechanisms that drive tumor behavior. The Cancer Genome Atlas (TCGA) database, a publically available database, offers a multilayered view of genomic and epigenomic data of approximately 10,000 patient samples together with clinicopathological information across more than 30 human cancer types, which is a rich resource for data mining and biological discovery.

Based on the fact that the collection of lung adenocarcinoma specimens that have metastasized to other organs is difficult, investigation of the early molecular events underlying lung adenocarcinoma metastasis is difficult. Toward this end, we analyzed mRNA and miRNA expression data and clinical data derived from TCGA to identify differences in mRNA and miRNA expression in primary tumor tissues from lung adenocarcinoma that did and did not metastasize. In addition, combined with bioinformatic prediction, we constructed an miRNA-target gene regulatory network, suggesting the regulation of the beginning of lung adenocarcinoma metastasis.

Materials and methods

TCGA gene expression profiles. Lung adenocarcinoma level 3 mRNA and miRNA expression data, and the

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corresponding clinical information, were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). The tumor staging information for all patient samples was derived from TCGA clinical information. Gene expression data were available for 291 lung adenocarcinoma samples without metastases, and 248 lung adenocarcinoma samples with lymph node metastasis or distant metastases. The miRNA expression data were available for 186 lung adenocarcinoma samples without metastases, and 144 lung adenocarcinoma with metastases.

Ranking of differentially expressed genes and miRNAs. The raw expression data of all lung adenocarcinoma patients was downloaded, and transformed into log2 scale. Z-score normalization was also employed. The Limma (Linear Models for Microarray Data) package in R was used to identify the differentially expressed probe sets between the metastatic primary loci and non-metastatic primary loci by two-tailed Student's t-test, and p-values from the same platform were obtained. MetaMA package in R was used to combine p-values from different platforms, and the false discovery rate (FDR) was calculated for multiple comparisons using the Benjamini and Hochberg method. We selected differentially expressed mRNAs and miRNAs with criterion of FDR <0.05. Hierarchical clustering of differentially expressed genes was performed using the 'heatmap.2' function of the R/Bioconductor package 'gplots' (11).

Target gene prediction of differentially expressed miRNAs. To understand the potential association between differentially expressed mRNAs and miRNAs obtained in the study, we predicted the transcriptional targets of the identified miRNAs using the online tools of miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) (12) based on six bioinformatic algorithms (DIANAmT, miRanda, miRDB, miRWalk, PicTar and TargetScan). Target genes that were commonly predicted by ≥4 algorithms or experimentally validated based on miRWalk database, were considered as putative targets.

Construction of the regulatory network of miRNA-target genes. Given that miRNAs tend to decrease the expression of their target genes, we matched putative target genes with the list of differentially expressed genes between the metastatic primary loci and non-metastatic primary loci to increase the accuracy of target prediction. We selected miRNA-target pairs whose expression was inversely correlated, to subject to further investigation (13-15). We conducted miRNA-target gene interaction networks with miRNA-target gene interacting pairs, whose expression levels are inversely correlated, and the miRNA regulation networks were visualized by Cytoscape (16).

Functional annotation. Functional enrichment analysis is essential to uncover biological functions of miRNA target genes. To gain insight into the biological function of the miRNA target genes, we performed Gene Ontology (GO) classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis based on the online software GENECODIS (17). GO which includes three categories such as biological process, molecular function and cellular component, provides a common descriptive framework of gene annotation and classification for analyzing gene set data. The KEGG pathway enrichment analysis was also performed to detect the potential pathway of miRNA target genes based on the KEGG pathway database, which is a recognized and comprehensive database including all types of biochemistry pathways (18). FDR <0.05 was set as the cut-off for selecting significantly enriched functional GO terms and KEGG pathways.

Validating the expression of lung adenocarcinoma metastasis-associated miRNA target genes. Based on the published studies, hsa-mir-133a-1 and hsa-let-7d were closely linked to the metastatic ability of lung adenocarcinoma (19,20). In our study, SurvMicro (http://bioinformatica.mty.itesm.mx:8080/Biomatec/Survmicro.jsp), an online validation tool, was used to evaluate the association of miRNA expression with survival (21) in human cancer datasets.

Table I. List of primers designed for the RT-qPCR experiments.

Primer	Primer sequence (5' to 3')	Product size (bp)
PKM2		
Forward	AAGTCTGGCAGGTCTGCTCAC	241
Reverse	TCAGCACAATGACCACATCTCC	
PTBP1		
Forward	TCCTTCTCCAAGTCCACCATCT	128
Reverse	AAAATCTCTGGTCTGCTAAGGTCAC	
FSCN1		
Forward	CGTCCAATGGCAAGTTTGTG	241
Reverse	GTGGAGTCTTTGATGTTGTAGGC	
STRAP		
Forward	GCAAACTGTGGTAGGAAAAACG	203
Reverse	ACTAACTGCAACATATGATTGACGC	
CISH		
Forward	TATTGGGGTTCCATTACGGC	235
Reverse	GCACAAGGCTGACCACATCC	
ESR2		
Forward	ATCACATCTGTATGCGGAACCTC	158
Reverse	AGTGAGCATCCCTCTTTGAACCT	
FLT3		
Forward	TATGTGACTTTGGATTGGCTCG	175
Reverse	CCAAGTGAGAAGATTTCCCACAGTA	
KIT		
Forward	TGAAGTGGATGGCACCTGAAAG	82
Reverse	CAAAGAAAAATCCCATAGGACCAGAC	
CNTN3		
Forward	TGAGCAATGGACATTTACTGGG	219
Reverse	GAGGCGTTTTCTTGGTGGTT	
BCL2		
Forward	GCCTTCTTTGAGTTCGGTGGG	107
Reverse	TGCCGGTTCAGGTACTCAGTCATC	
ACTIN		
Forward	ACTTAGTTGCGTTACACCCTT	156
Reverse	GTCACCTTCACCGTTCCA	

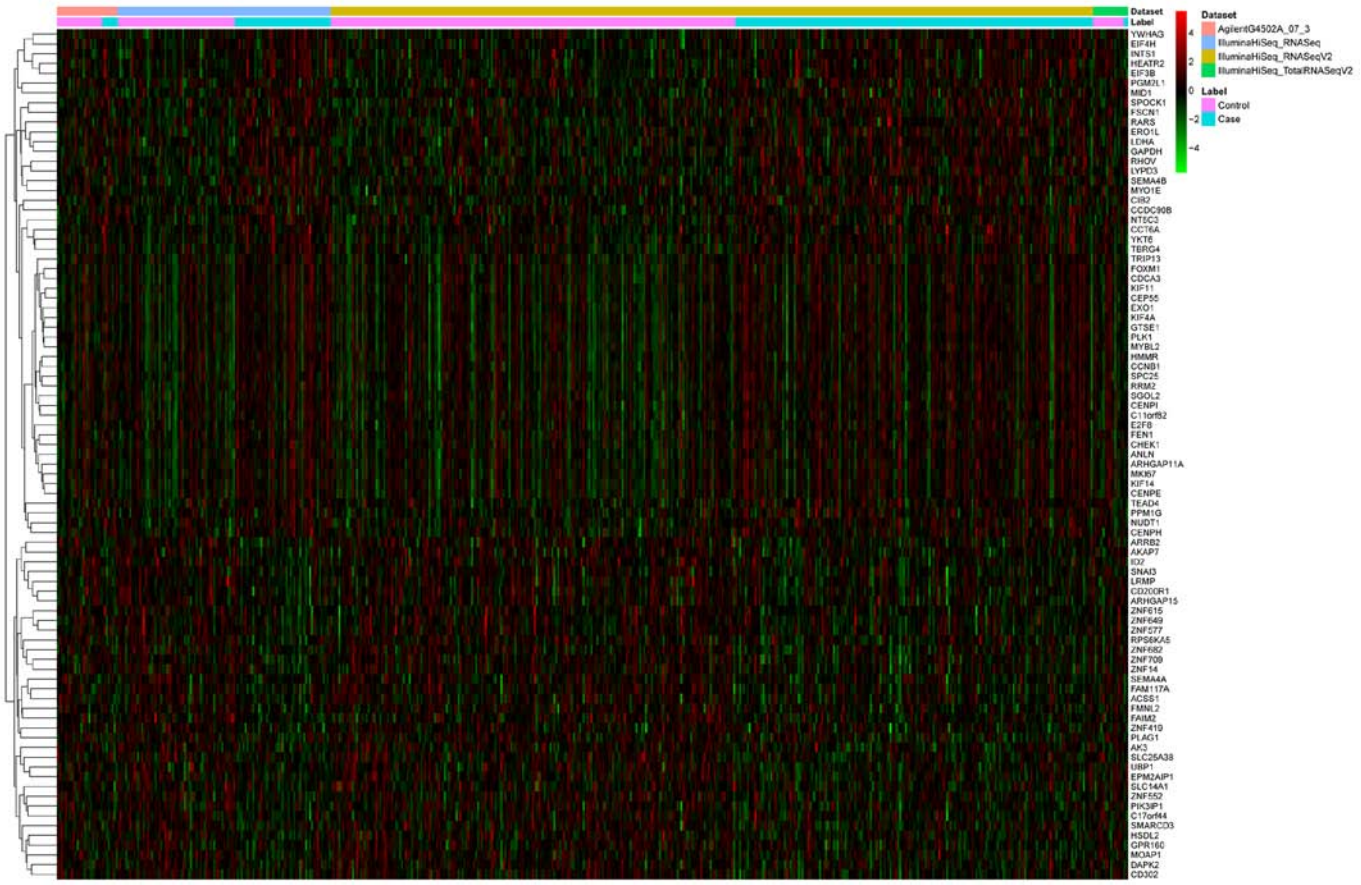

Figure 1. The hierarchical clustering of the top 100 differentially expressed genes between the metastatic primary loci and non-metastatic primary loci.

Furthermore, the expression of putative targets of hsa-mir-133a-1 (PKM2, PTBP1, FSCN1 and STRAP) and hsa-let-7d (KIT, BCL2, CNTN3, CISH, FLT3 and ESR2) was detected in primary tumor tissues from 10 lung adenocarcinoma patients by RT-qPCR, to validate the differential expression observed in the bioinformatic analysis. The $2^{-\Delta\Delta Ct}$ method was used to analyze the data of RT-qPCR. SPSS version 13.0 was used to perform all statistical analyses. The sequences of the primers used are provided in Table I.

Among the 10 patients included, 5 presented with metastasis and 5 were without metastasis. The clinical specimens were provided by Daping Hospital. Written informed consent forms were received from the patients or legal guardians of the patients. All protocols and documents were approved by the Medical Ethics Committee of Daping Hospital. Frozen sections were prepared from the tumor tissues for the cytological or histological diagnosis. The resected tumor tissues were stored in liquid nitrogen until RNA extraction.

Results

Differences in miRNA and mRNA expression in lung adenocarcinoma with or without metastasis. Based on clinical information 'AJCC pathologic tumor stage' in the TCGA lung adenocarcinoma, we classified these data into lung adenocarcinoma with or without metastasis. After the gene expression data were downloaded, we performed differential expression analysis of the genes and miRNAs between the metastatic primary loci and non-metastatic primary loci. Genes (1,257) were identified to be differentially expressed under a threshold of FDR <0.05, with 585 upregulated and 672 downregulated genes in the lung adenocarcinoma with metastasis. The hierarchical clustering of differentially expressed genes is shown in Fig. 1. Fifty-eight miRNAs were identified as differentially expressed under the threshold of FDR <0.05, with 44 upregulated and 14 downregulated miRNAs in the lung adenocarcinoma samples with metastasis (Table II).

The regulatory network of differentially expressed mRNAs and miRNAs associated with lung adenocarcinoma metastasis. To find the potential link between the differentially expressed mRNAs and miRNAs observed in the bioinformatic analysis, we predicted the putative targets of the identified miRNAs in the miRWalk database. Matching predicted putative targets with those found to be disregulated genes in metastatic primary tumors, miRNA-target gene pairs whose expression levels were inversely correlated were selected. As a result, we identified 1,154 miRNA-target gene pairs for the upregulated miRNAs with 61 pairs validated by previous experiments, and 474 miRNA-target gene pairs for the downregulated miRNAs with 57 pairs validated by previous experiments (Table III).

Using the 770 miRNA-target gene pairs, an miRNA-target gene regulatory network was constructed (Fig. 2). In the miRNA-target gene regulatory network, we identified the

Table II. Summary of the differentially expressed miRNAs.

miRNAs	P-value	miRNAs	P-value	miRNAs	P-value
Upregulated miRNAs					
hsa-mir-1293	6.62E-04	hsa-mir-381	1.17E-02	hsa-mir-147b	3.00E-02
hsa-mir-105-2	1.87E-03	hsa-mir-365-1	1.50E-02	hsa-mir-1285-1	3.13E-02
hsa-mir-365-2	2.82E-03	hsa-mir-550a-1	1.52E-02	hsa-mir-105-1	3.25E-02
hsa-mir-193a	3.22E-03	hsa-mir-539	1.70E-02	hsa-mir-432	3.43E-02
hsa-mir-196b	3.64E-03	hsa-mir-433	1.76E-02	hsa-mir-9-3	3.68E-02
hsa-mir-1185-1	3.86E-03	hsa-mir-509-2	1.88E-02	hsa-mir-376c	3.92E-02
hsa-mir-944	8.07E-03	hsa-mir-3191	2.09E-02	hsa-mir-2116	4.35E-02
hsa-mir-767	8.36E-03	hsa-mir-382	2.15E-02	hsa-mir-379	4.40E-02
hsa-mir-193b	8.48E-03	hsa-mir-665	2.27E-02	hsa-mir-513a-1	4.53E-02
hsa-mir-3942	8.49E-03	hsa-mir-582	2.31E-02	hsa-mir-1276	4.76E-02
hsa-mir-9-1	8.65E-03	hsa-mir-889	2.40E-02	hsa-mir-212	4.89E-02
hsa-mir-9-2	9.43E-03	hsa-mir-421	2.48E-02	hsa-mir-516a-2	4.92E-02
hsa-mir-655	1.14E-02	hsa-mir-744	2.72E-02	hsa-mir-98	4.96E-02
hsa-mir-409	1.15E-02	hsa-mir-653	2.86E-02	hsa-let-7d	4.98E-02
hsa-mir-496	1.16E-02	hsa-mir-134	2.96E-02		
Downregulated miRNAs					
hsa-mir-133a-1	1.07E-02	hsa-mir-518a-1	2.77E-02	hsa-mir-338	3.21E-02
hsa-mir-3065	1.11E-02	hsa-mir-552	2.94E-02	hsa-mir-577	3.24E-02
hsa-mir-3186	1.42E-02	hsa-mir-30b	3.02E-02	hsa-mir-622	3.95E-02
hsa-mir-135a-2	2.08E-02	hsa-mir-660	3.03E-02	hsa-mir-3202-2	4.16E-02
hsa-mir-1250	2.35E-02	hsa-mir-302a	3.17E-02		

top 10 miRNAs which regulated the most target genes, such as hsa-miR-7, hsa-miR-182, hsa-miR-324-3p, hsa-miR-139-5p, hsa-miR-130b, hsa-let-7f, hsa-miR-18a, hsa-miR-188-5p, hsa-let-7d, and hsa-miR-590-5p, and the target genes such as RPS6KA3, TSC1, AIM1, GAS7, GFOD1, GGA2, IGF1, IL28RA, and INSR were regulated by the most miRNAs.

GO classification and KEGG pathways of the miRNA target genes. We performed the GO classification and KEGG pathway enrichment analysis for miRNA target genes whose expression was differentially expressed. We found that lymphoid progenitor cell differentiation (GO:0002320, FDR=4.75E-04) and hematopoietic progenitor cell differentiation (GO:0002244, FDR=2.08E-03) were significantly enriched for biological processes. While for molecular functions, signaling receptor activity (GO:0038023, FDR=9.24E-04) and signal transducer activity (GO:0004871, FDR=8.77E-04) were significantly enriched (Table IV). The most significant pathway in our KEGG analysis was DNA replication (FDR=1.78E-04). Furthermore, T cell receptor signaling pathway (FDR=1.12E-03) and endocytosis (FDR=1.17E-03) were also highly enriched (Table V).

Validating the expression of lung adenocarcinoma metastasis-associated miRNA target genes. By the online tool of SurvMicro, survival analysis was performed to evaluate the correlation between miRNA expression level (hsa-miR-133a-1 and hsa-let-7d) and the overall survival time of the lung adenocarcinoma patients. With the data from TCGA database, Kaplan-Meier curves indicated that the expression of hsa-mir-133a-1 was significantly correlated with the overall survival time of lung adenocarcinoma patients (P=0.03654), while the expression of hsa-let-7d was not significantly correlated with the overall survival time of the lung adenocarcinoma patients (P=0.114) (Fig. 3).

We performed RT-qPCR for the putative target genes of hsa-mir-133a-1 and hsa-let-7d, which exhibited significantly differential expression between the primary tumor tissues from lung adenocarcinoma with metastasis and that from the lung adenocarcinoma patiemts without metastasis in the bioinformatic analysis. In the RT-qPCR assay, most of the target genes showed similar expression patterns to what was observed in the bioinformatic analysis. The expression levels of the selected upregulated and downregulated genes are shown in Fig. 4. Among the upregulated genes in the lung adenocarcinoma with metastasis, PKM2 and STRAP mRNA expression was significantly increased (P=0.04, P=0.05), and PTBP1 mRNA expression was also increased, but not obviously, while FSCN1 mRNA expression was decreased. Among the downregulated genes in the lung adenocarcinoma with metastasis, FLT3 mRNA expression was significantly decreased (P=0.023), and BCL2, ESR2, and KIT mRNA expression was also mildly decreased. In contrast, CISH mRNA expression was significantly increased (P=0.027). CNTN3 mRNA expression appeared unchanged between the metastatic primary loci and the non-metastatic primary loci.

Table III. miRNA-target gene pairs whose expression levels are inversely correlated.

miRNA	Status (miRNAs)	No. of miRNA target genes	Target mRNAs
hsa-mir-133a-1	Downregulated	7	PTBP1, PKM2, NKX2-3, STRAP, FSCN1, TGFBI, PA2G4
hsa-mir-135a-2	Downregulated	2	SLC27A4, FOXM1
hsa-mir-302a	Downregulated	67	MCM7, SLC27A4, ELOVL6, RPE, CKAP4, KPNA2, SEMA3C, POLQ, KCND2, SLC45A2, MCM10, ASF1B, PBK, ST8SIA2, USP42, E2F7, CCT7, ERLIN1, BLCAP, PDAP1, CD109, KDELC2, ESCO2, DDX10, DHCR7, DSG3, E2F2, TMEM184A, C11orf82, GGA2, PSD4, NUP62, PANX1, PGM2L1, RACGAP1, HOXA11, CYP27C1, ACADVL, KRT14, MCM4, MCM6, NEDD4, MRPS16, NIP7, CYCS, PON2, RNF216, CDCA8, CEP55, OGFOD1, SYT13, KIAA1609, GNPNAT1, BRCA1, UPK1B, C1orf135, SHCBP1, FBXL18, MFAP5, CDT1, C10orf58, BARX2, STC2, EXO1, NCAPD2, CDC25A, ECT2
hsa-mir-30b	Downregulated	100	MRPS24, DPP7, NKX2-3, F2, TRIP13, AP2A1, MYO1E, GARS, PLK1, UTP15, ZNF384, KDELC2, DSG2, DSP, AURKB, ABCF2, POLD2, TXNRD1, NCL, MYBL2, CARHSP1, SFXN1, CHORDC1, NEDD4, PTBP1, RARS, GAPDH, PTGFRN, IQGAP3, GNPNAT1, LMNB2, POLA2, NT5C3, DLGAP4, GALNT2, CYP24A1, LASS3, MESDC2, DPY19L1, GRM5, INCENP, KIF11, PELO, ERRFI1, PON2, AVEN, HECW2, RRM2, YWHAG, SLC7A5, ARHGAP11A, LPGAT1, CHST1, PSME3, CIB2, CCT4, ERLIN1, C1QL1, PDAP1, CBX3, SLC5A11, CPD, TICAM1, ESCO2, DLX1, TTLL12, RNASEH1, C16orf73, ABCA12, GPR110, PGM2L1, HDLBP, HNRNPA2B1, HOXA11, KCND2, MID1, MTHFD1, NEFM, ORC2L, CYCS, ANLN, CDCA8, PGM2, DEPDC1B, KIF15, SH3GL1, CENPH, STC1, TFAP2A, UPK1B, ZIC2, C1orf135, CALB2, MLF1IP, CALU, FAM136A, PRC1, HN1L, KIAA0101, E2F7
hsa-mir-338	Downregulated	1	CCT4
hsa-mir-552	Downregulated	52	CYP24A1, FOXC1, CYCS, UBFD1, C10orf58, EIF2S2, ABCF2, CARM1, CENPF, YKT6, CDCA5, WBSCR22, CD109, CPD, AP2A2, ENO1, TMEM184A, EXTL3, TTLL12, NCAPH, FOSL2, GNG4, PGM2L1, HOXA10, BIRC5, CYP27C1, CHST6, MCM4, MKI67, MRPS16, PKM2, ERRFI1, PNPLA2, PTBP1, SYT13, NKX3-2, RFC2, BCAN, GNPNAT1, FSCN1, BRCA1, TGM4, TULP3, GLT25D1, MAFK, USP5, ST8SIA2, CDT1, LMNB2, CDK5R2, HN1L, DDX21
hsa-mir-1276	Upregulated	15	CNOT6L, CHIC2, PIK3CG, C1orf190, BDH2, BCL2, SLC14A1, HSDL2, PSTPIP2, CD5, PKHD1L1, ITM2A, LPIN2, MTSS1, EPM2AIP1
hsa-mir-1293	Upregulated	5	NMUR1, ITIH4, ROBO2, DNALI1, FCRLA
hsa-mir-134	Upregulated	74	KIT, KLHDC1, SEC14L3, IL16, PCMTD2, SLC14A1, FCRL5, RCL1, ATP8A1, RRH, CHKA, WIF1, ERMAP, FMNL2, CYP2R1, CNR1, CSF3R, PRICKLE2, ZNF25, LNX2, DENND3, MMRN1, P2RX2, PDZD2, GPD1L, ISCU, CBX7, CNOT6L, SLC41A1, GCET2, PDE7B, ZNF776, ZNF615, SLC25A42, S100A7A, FAM19A2, ID2, RSPO2, C3orf62, KCNJ8, CLEC12B, SMAD6, MOCS1, UNC13C, MTHFR, GIMAP6, CDON, CECR1, PLCB2, GNG2, RSBN1, TNFRSF19, PRKCQ, MOSPD1, SLC24A3, BCL2, SLAMF1, UBP1, C18orf1, ZFP161, C21orf2, ZNF20, DNALI1, BTG2, ZNF552, C1orf21, PIGY, MPDZ, LIMD1, ZNF468, IL33, RPS6KA5, TP53INP1, LPIN2
hsa-mir-147b	Upregulated	3	MACROD2, LY9, NFIX

Table III. Continued.

miRNA	Status (miRNAs)	No. of miRNA target genes	Target mRNAs
hsa-mir-193b	Upregulated	42	BCL2, KIT, UPRT, CAMTA1, CBX7, MMP19, UBP1, RAMP3, UNC13B, MGAT4A, UNC45B, PDIK1L, PRICKLE2, TAPT1, FAIM2, CNOT6L, PPP1R16B, GATA6, CRB2, C3orf62, RHOH, MAP3K3, CD244, PLAG1, C21orf29, POU2AF1, SNRK, TRIM68, NXF3, WDR48, SLC4A5, C18orf1, EVI5, C1orf21, CAMKK1, KBTBD8, ATOH8, VAMP8, NMT2, TP53INP1, AATK, SPNS3
hsa-mir-196b	Upregulated	53	FLT3, BCL2, GATA6, AQP4, ZMYND11, EPS15, WDR37, CAPN7, ATRNL1, PDE7B, SMAD6, MGAT4A, CYP2U1, UNC45B, PRICKLE2, KLHDC8B, GPD1L, SLC41A1, PPP1R16B, SERP1, HLF, SNAI3, RSPO2, C3orf62, MAOA, MTHFR, CDON, PLAG1, PLCB2, BCL11A, BEST2, TNFRSF19, MRPS25, COL14A1, WNT2B, TMEM50B, SLC25A20, EEA1, ZNF577, ATOH8, PRSS12, FAM125B, LIMD1, CREB3L1, ACVR2A, VAPA, MS4A1, TP53INP1, FHL5, AATK, LPIN2, WSCD2, LPPR4
hsa-mir-212	Upregulated	92	RPS6KA5, REM1, ANKRD29, PRICKLE2, SOSTDC1, PDE7B, SERP1, MAOA, SLC24A3, TMEM50B, BTG2, SLC25A20, USP38, KLRG1, CXCR6, WASF3, FGL2, MGAT4A, CYP2U1, PIK3IP1, FMNL2, CNR1, C1orf88, MAP3K8, CPT1B, UPRT, GAB3, GPR155, BTLA, ZNF483, TAPT1, ENPP4, KLRK1, CLASP2, GLT25D2, TBC1D9, GPD1L, CAMTA1, FOSB, CNOT6L, PPP1R16B, NAP1L5, GPR160, SESN1, ZNF615, FAM19A2, IKBKB, IL12B, IL16, IMPG1, B3GNT8, MAP3K3, MLLT3, UNC13C, AK3, CDON, EMCN, PLAG1, C21orf29, GFOD1, LAX1, PCMTD2, FBXW7, PNRC2, MOSPD1, LHX9, GALNTL1, WDR48, STIM2, PTPN4, SLC4A5, RSU1, SDF2, C18orf1, ZFP161, ZNF708, ZNF80, C1orf21, TCF7L1, SYT15, HSDL2, KBTBD8, PDE8B, LIMD1, LARGE, VAPA, NMT2, TP53INP1, CHST10, TOX, ELMO1, SEMA4A
hsa-mir-365-1	Upregulated	1	BCL2
hsa-mir-365-2	Upregulated	1	BCL2
hsa-mir-376c	Upregulated	51	PDIK1L, SMYD1, ALCAM, GNG2, KLHL9, ARID4A, VLDLR, ACYP2, TESK2, FBLN5, RCBTB2, SLAMF6, KLHDC1, UNC45B, ZNF483, NLRC3, DAPK2, PLA2G4F, C1orf101, GCET2, D4S234E, SERP1, SNAI3, FAM19A2, FAM19A1, MAP3K3, GIMAP6, AK3, C3orf18, PLAG1, P2RY13, RCOR3, MOSPD1, ST6GAL1, ZNF649, C18orf1, ZFP161, ZNF10, ZNF708, ZNF614, NDFIP1, C1orf21, IL33, CABLES1, GPR55, MS4A1, WSCD2, TOX, MTSS1, FLT3, RORB
hsa-mir-379	Upregulated	52	ATP8A2, CYP2U1, KLHL9, C7, KLRG1, NMUR1, UNC13B, ZNF211, INMT, SCGB3A2, C1orf88, GAB3, ANKRD29, PRICKLE2, ALCAM, DENND3, FAIM2, SLC41A1, C1orf101, HSPB7, ZNF776, C13orf15, GZMK, HLF, S100A7A, ID4, SLCO4C1, IL16, KIT, ARRB1, UNC13C, GIMAP6, CDON, C9orf68, SLC25A36, SPTLC3, BEX4, WNT2B, C18orf1, ZNF10, DNALI1, C1orf21, ACSS1, IL18RAP, CD5, LARGE, VAPA, GPR55, MS4A1, CD40LG, WSCD2, LPPR4
hsa-mir-381	Upregulated	102	KIT, DLC1, WDR37, MMRN1, ID2, CNTN3, SLC25A36, SLC24A3, PCDH20, ACVR2A, MERTK, FBLN5, CXCR6, WASF3, MGAT4A, CYP2U1, FMNL2, KLHDC1, CNR1, CNTFR, C1orf88, CD200R1, MAP3K8, UPRT, PDIK1L, SMYD1, BTLA, ZNF483, PRICKLE2, SUSD3, F11, ENPP4, SACM1L, SORCS3, SWAP70, CLASP2, TBC1D9, GPD1L, ANKRD12, CAMTA1, CAPN7, CNOT6L, SLC41A1, GCET2, NAP1L5, PDE7B, SERP1, ZNF776, ZNF615, GPR34, GRIA1, C13orf15, ZC3H7A, GZMK, HTR2A, ID4, RSPO2, KCNT2, RBPJ, SLCO4C1, IKBKB, IL12B, KCNA4, RHOH, MLLT3, NINJ1, PLAG1, BCL11A, PMM1, P2RY13, SETD4, FAM105A, SNRK, LAX1, TRIM68, PCMTD2, TNFRSF19, KLHL9, BEX4, WDR48, SLC4A5, ARID4A, PRDM16, SLAMF1, BTG1, VLDLR, C18orf1, ZNF10, ZNF136, DNALI1, LRRC27, FAM117A, EEA1, HSDL2, KBTBD8, CD1D, VAPA, RCSD1, TP53INP1, KIAA0408, MTSS1, NFIB

Table III. Continued.

miRNA	Status (miRNAs)	No. of miRNA target genes	Target mRNAs
hsa-mir-382	Upregulated	59	DLC1, ATP8A1, MAP3K8, CRHBP, CAPN7, GPR160, ZNF615, ID4, SLCO4C1, CABC1, ROBO2, CD96, MGAT4A, CISH, CNR1, PPM1M, MACROD2, GPR155, BTLA, PRICKLE2, TAPT1, EPS15, ALCAM, SORCS3, CAMTA1, CNOT6L, PPP1R16B, RSPO2, IL10RA, IL12B, ITGAD, B3GNT8, MAOA, LRP2BP, KLHL9, MOSPD1, SLC24A3, SLC4A5, PRDM16, PCDH20, C18orf1, ZNF708, TMEM50B, CA3, ZNF614, C1orf21, SYT15, CLDN2, IL33, CD1D, RCSD1, HMGN3, GRAP2, ITM2A, AKAP7, CHST10, TOX, NFIB, PPP3CC
hsa-mir-421	Upregulated	79	CBX7, TESK2, CLASP2, SOSTDC1, RHOH, SLC24A3, NDFIP1, KBTBD8, B3GALT2, VAPA, TSPAN32, CXCR6, WASF3, FRS3, FGL2, MGAT4A, ERMAP, CNR1, GAB3, PDIK1L, SMYD1, DBH, ZNF540, PRICKLE2, NLRC3, ZNF25, ENPP4, ANKRD12, CAMTA1, CNOT6L, SLC41A1, PDE7B, SERP1, SLC46A3, NKIRAS1, ZC3H7A, FAM19A2, RSPO2, SLCO4C1, IL10RA, IL12B, AQP4, C3orf62, NR3C2, MTHFR, GIMAP6, CDON, BCL11A, FBXO42, RSBN1, LAX1, SLC25A36, PCMTD2, LRP2BP, GALNTL1, WDR48, STIM2, PTPRB, RSU1, GZF1, MRPS25, C18orf1, BTG2, ZNF614, LRRC27, FCRL5, SYT15, ZNF577, LIMD1, CLDN2, ACVR2A, GPR55, NMT2, AKAP7, USP6NL, TOX, LPPR4, RORB, ACTN2
hsa-mir-432	Upregulated	79	CXCR6, SLCO4C1, GNG2, CA3, C1orf21, HSDL2, PPAP2A, RCL1, CD96, DLC1, WASF3, CHKA, MGAT4A, ADH1B, CNR1, MAP3K8, CPT1B, MACROD2, UNC45B, ANKRD29, GPR155, NLRC3, TAPT1, F11, TMEM130, KLRK1, SORCS3, GPD1L, CAMTA1, PPP1R13B, FOS, SLC41A1, MYRIP, SETBP1, NAP1L5, GPR160, HSPB7, GNG7, SNAI3, ID4, RSPO2, IL16, LY9, MAP3K3, MLLT3, GIMAP6, P2RX1, C3orf18, REV1, HMGCLL1, RSBN1, PPP1R1A, SLC25A36, TMEM57, FBXW7, SPTLC3, GIMAP5, LRP2BP, C1orf183, BCL2, PRDM16, GZF1, C5, UBP1, C18orf1, ZNF80, EVI5, DENND1C, ATOH8, FAM125B, BZRAP1, GPR55, MS4A1, ENTPD3, CD40LG, WSCD2, USP6NL, TOX, PDZD2
hsa-mir-433	Upregulated	55	NR0B2, ZNF211, RAB40B, TAGAP, NLRC3, ZNF615, NINJ1, RSBN1, PTPN4, PRDM16, MRPS25, ZNF649, ZNF91, BTG2, ACVR2A, RAMP3, ATP8A1, UNC13B, FGL2, FCRL3, CNR1, CD200R1, GAB3, PDIK1L, BTLA, DMP1, GPC5, SACM1L, SWAP70, ANKRD12, CAMTA1, CNOT6L, NAP1L5, ZNF776, IL16, UNC13C, C9orf68, TRIM68, FBXW7, TNFRSF19, LRP2BP, SLC4A5, PTPRB, BCL2, ZNF708, EVI5, LRRC27, C1orf21, KBTBD8, ZNF439, VAPA, NMT2, AKAP7, KIAA0408, LPPR4
hsa-mir-496	Upregulated	49	KIT, ANKRD12, FAM55D, ZNF20, ZNF136, MGAT4A, CNR1, ADHFE1, CR2, CRHBP, GPR155, ZNF781, P2RX2, TBC1D9, C20orf194, SETBP1, GATA6, CLUL1, HSPB7, ANGPT1, ZNF776, GPR34, S100A7A, SLCO4C1, C3orf62, CDON, EMCN, P2RY13, HMGCLL1, LAX1, SUSD2, BEX4, BDH2, BCL2, MRPS25, SPATA20, SLC14A1, ZNF708, ZNF614, C1orf21, EEA1, ZNF160, IL33, VAPA, BZRAP1, ITM2A, LPPR4, NFIB, PAQR8
hsa-mir-513a-1	Upregulated	1	DNAH8
hsa-mir-539	Upregulated	101	KIT, KLRG1, WASF3, PIK3IP1, MACROD2, BTLA, CLASP2, PDE7B, ZNF776, ID4, RSPO2, PNRC2, C7, NDFIP1, FCRL5, SYT15, PRSS12, ZNF266, RCBTB2, ZPBP2, CNR1, CNTFR, MAP3K8, UNC45B, SMYD1, GPR155, ZNF483, DLG4, DMP1, FAIM2, PDZD2, GLT25D2, ANKRD12, PPP1R13B, FOS, PIK3R5, FOSB, CCNDBP1, SLC41A1, GCET2, ATRNL1, SEC14L3, ANGPT1, S100A7A, SLCO4C1, C3orf62, CLEC12B, MAOA, NR3C2, MTHFR, GIMAP6, P2RX1, CNTN3, CDON, PHF7, CECR1, PIK3CG, BCL11A, PNOC, C21orf29, C1orf190, GNG2, PALMD, SLC25A36, SPTLC3, GIMAP5, TNFRSF19, MOSPD1, CPA6, TNFRSF17, RSU1, NAPB, USP4, C18orf1, DNALI1, EVI5, BTG2, DENND1C, ZNF614, LRRC27, C1orf21, CDADC1, EEA1, C15orf48, KBTBD8, EMR3, ST3GAL5, FAM125B, LIMD1, ZNF439, CLDN2, CD1D, C22orf32, CD5, VAPA, MS4A1, NMT2, TP53INP1, AKAP7, CD69, USP6NL

Table III. Continued.

miRNA	Status (miRNAs)	No. of miRNA target genes	Target mRNAs
hsa-mir-582	Upregulated	1	SMAD6
hsa-mir-9-1	Upregulated	9	CD19, CEBPA, BCL2, RASSF1, CBX7, ATP8A2, CISH, GRIA1, IGFALS
hsa-mir-9-2	Upregulated	8	ATP8A2, CD19, RASSF1, BCL2, GRIA1, CEBPA, IGFALS, CISH
hsa-mir-9-3	Upregulated	7	ATP8A2, CD19, BCL2, IGFALS, GRIA1, CEBPA, CISH
hsa-mir-98	Upregulated	3	ESR2, CISH, CNTN3
hsa-let-7d	Upregulated	61	ACVR2A, BCL2, FOSB, FLT3, PGC, KIT, CNTN3, ESR2, ATP8A2, CXCR4, CEBPA, FBXW7, CACNA2D2, CISH, MTSS1, RASSF1, MGAT4A, USP38, ADAMTS8, WDR37, RUFY3, RSPO2, COL14A1, WASF3, CYP2U1, SLAMF6, CNR1, CD200R1, GAB3, GPR155, DAPK1, ZNF540, NLRC3, KLHDC8B, EPS15, ENPP4, CLASP2, DAPK2, PPP1R16B, HSPB7, CRB2, HLF, SNAI3, TMEM110, P2RX1, PLCB2, GFOD1, BEST2, SFTPB, MRPS25, C18orf1, ZNF10, ZNF614, C1orf21, TCF7L1, SYT15, EEA1, ZNF577, MPDZ, FAM125B, TP53INP1

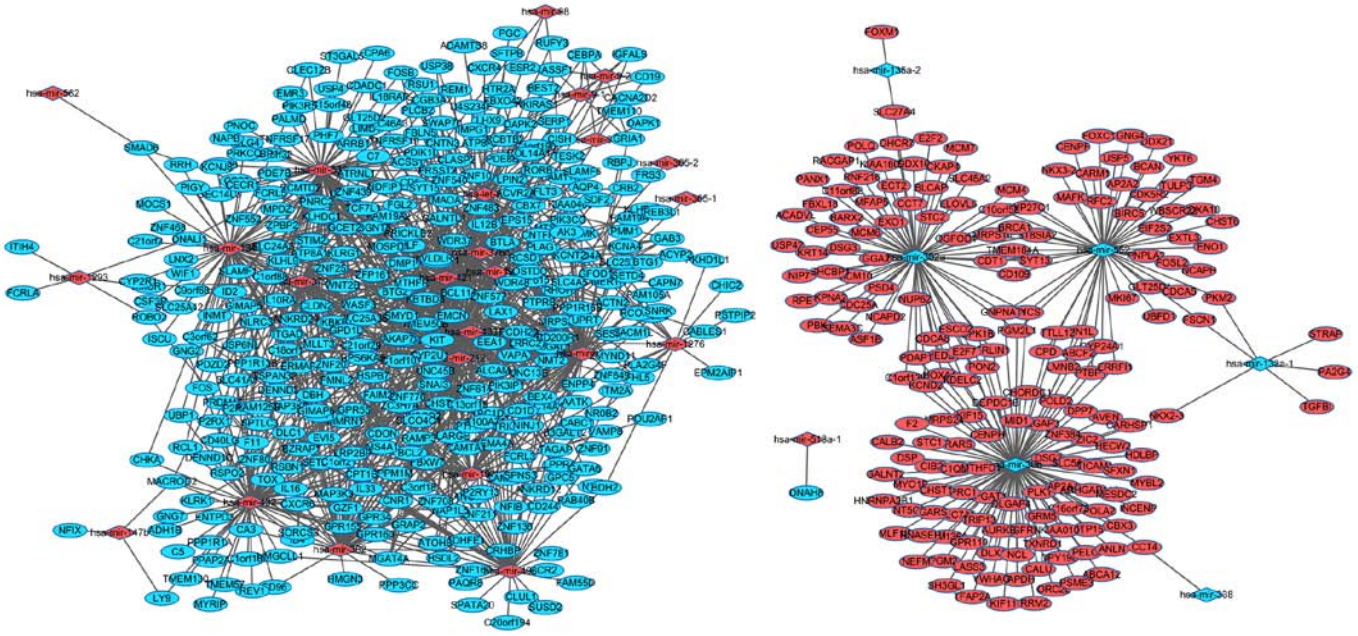

Figure 2. The regulatory network between miRNAs and target genes associated with lung adenocarcinoma metastasis. The diamonds and ellipses represent the miRNAs and genes, respectively. The red and green colors represent relatively high and low expression, respectively.

Discussion

Due to a substantially lower cost, high-throughput gene expression data are becoming widely available. A key question in the field is how to use the data to identify both biological drivers and strong metastatic markers. In this study, we compared miRNA and mRNA expression data differences between lung adenocarcinoma with metastasis and lung adenocarcinoma without metastasis from the TCGA data portal with the aim to screen the miRNAs and genes associated with lung adenocarcinoma metastasis.

In line with previous findings, some of the identified differentially expressed miRNAs were found to be implicated in lung adenocarcinoma metastasis, such as hsa-mir-98, hsa-let-7d, hsa-mir-134, hsa-mir-196b, hsa-mir-381, hsa-mir-133a, hsa-mir-552, hsa-mir-944, hsa-mir-550, and hsa-mir-655. miR-98 was also found to be overexpressed in lung cancer cell lines, and it binds the 3'UTR of Fus1, a tumor suppressor, to inhibit protein expression (22). Despite no reports related to lung adenocarcinoma metastasis, a recent study uncovered the regulatory roles of miR-98 in melanoma metastasis (23). miR-134 and miR-655, belonging to the same cluster on chromosome 14q32, were demonstrated to be involved in TGF-β1-induced EMT which is recognized as a key element of cell invasion, migration, and metastasis, by directly targeting MAGI2, a scaffold protein required for PTEN (24). Directly

Table IV. GO functional annotation of the predicted miRNA target genes.

GO ID	GO term	FDR
Biological process		
GO:0002320	Lymphoid progenitor cell differentiation	4.75E-04
GO:0002244	Hematopoietic progenitor cell differentiation	2.08E-03
GO:0070661	Leukocyte proliferation	1.44E-02
GO:0002521	Leukocyte differentiation	2.32E-02
GO:0046425	Regulation of JAK-STAT cascade	1.94E-02
GO:0048639	Positive regulation of developmental growth	1.65E-02
GO:0007166	Cell surface receptor signaling pathway	1.88E-02
GO:0043552	Positive regulation of phosphatidylinositol 3-kinase activity	2.20E-02
GO:0002318	Myeloid progenitor cell differentiation	1.96E-02
GO:0048070	Regulation of developmental pigmentation	1.76E-02
GO:0030318	Melanocyte differentiation	1.60E-02
GO:0050931	Pigment cell differentiation	1.47E-02
GO:0090218	Positive regulation of lipid kinase activity	1.36E-02
GO:0060563	Neuroepithelial cell differentiation	1.26E-02
GO:0048534	Hematopoietic or lymphoid organ development	1.23E-02
GO:0001932	Regulation of protein phosphorylation	1.20E-02
GO:0042113	B cell activation	1.53E-02
GO:0031399	Regulation of protein modification process	1.67E-02
GO:0002065	Columnar/cuboidal epithelial cell differentiation	1.73E-02
GO:0042509	Regulation of tyrosine phosphorylation of STAT protein	1.64E-02
GO:0042531	Positive regulation of tyrosine phosphorylation of STAT protein	1.57E-02
GO:1903727	Positive regulation of phospholipid metabolic process	1.50E-02
GO:0046427	Positive regulation of JAK-STAT cascade	1.43E-02
GO:0071310	Cellular response to organic substance	1.38E-02
GO:0050853	B cell receptor signaling pathway	1.41E-02
GO:0050769	Positive regulation of neurogenesis	1.64E-02
GO:0051094	Positive regulation of developmental process	1.67E-02
Molecular function		
GO:0038023	Signaling receptor activity	9.24E-04
GO:0004871	Signal transducer activity	8.77E-04
GO:0004888	Transmembrane signaling receptor activity	8.24E-04
GO:0004872	Receptor activity	1.40E-03
GO:0060089	Molecular transducer activity	1.57E-03
GO:0005057	Receptor signaling protein activity	8.40E-03
GO:0002020	Protease binding	1.02E-02
GO:0004714	Transmembrane receptor protein tyrosine kinase activity	8.93E-03
GO:0042803	Protein homodimerization activity	1.36E-02
GO:0019199	Transmembrane receptor protein kinase activity	3.21E-02
GO:0042802	Identical protein binding	3.54E-02
GO:0046983	Protein dimerization activity	3.64E-02
GO:0044389	Ubiquitin-like protein ligase binding	4.93E-02
GO:0031625	Ubiquitin protein ligase binding	4.58E-02
GO:0004713	Protein tyrosine kinase activity	4.60E-02

targeting oncogenic receptors, such as IGF-1R, TGFBR1, and EGFR, miR-133a inhibits cell invasiveness and cell growth in lung cancer cells. Furthermore, an *in vivo* animal model showed that miR-133a markedly suppressed the metastatic ability of lung cancer cells (19). An miRNA microarray revealed that the expression level of miR-552 in colorectal cancer metastases in the lung was 39 times higher than that of primary lung adenocarcinomas, suggesting its possible roles in cancer metastases (25). miR-944 affected NSCLC cell growth, proliferation, and invasion by targeting a tumor suppressor,

Table V. KEGG pathway enrichment analysis of the predicted miRNA target genes (top 15).

ID	Items	Count	FDR	Genes
hsa03030	DNA replication	7	1.78E-04	MCM4, RNASEH1, MCM7, POLD2, RFC2, MCM6, POLA2
hsa04660	T cell receptor signaling pathway	9	1.12E-03	IKBKB, FOS, PIK3R5, PPP3CC, PRKCQ, CD40LG, GRAP2, PIK3CG, MAP3K8
hsa04144	Endocytosis	12	1.17E-03	CXCR4, KIT, AP2A2, NEDD4, SH3GL1, PSD4, SMAD6, ARRB1, EEA1, EPS15, FAM125B, AP2A1
hsa04380	Osteoclast differentiation	5	1.19E-03	IKBKB, FOS, PIK3R5, PPP3CC, PIK3CG
hsa05145	Toxoplasmosis	5	1.23E-03	IKBKB, CYCS, PIK3R5, BCL2, PIK3CG
hsa04210	Apoptosis	5	1.23E-03	IKBKB, CYCS, PIK3R5, BCL2, PIK3CG
hsa04110	Cell cycle	3	1.24E-03	MCM4, MCM7, MCM6
hsa05222	Small cell lung cancer	5	1.26E-03	IKBKB, PIK3R5, E2F2, BCL2, PIK3CG
hsa05210	Colorectal cancer	7	1.27E-03	CYCS, TCF7L1, FOS, PIK3R5, BIRC5, BCL2, PIK3CG
hsa04620	Toll-like receptor signaling pathway	5	1.27E-03	IKBKB, FOS, PIK3R5, PIK3CG, MAP3K8
hsa05142	Chagas disease (American trypanosomiasis)	6	1.28E-03	IKBKB, FOS, TICAM1, PIK3R5, IL12B, PIK3CG
hsa04722	Neurotrophin signaling pathway	4	1.29E-03	IKBKB, PIK3R5, BCL2, PIK3CG
hsa05200	Pathways in cancer	17	1.31E-03	IKBKB, CYCS, TCF7L1, FOS, PIK3R5, KIT, BIRC5, WNT2B, E2F2,CSF3R, FLT3, RASSF1, BCL2, DAPK1, CEBPA, PIK3CG, DAPK2
hsa05221	Acute myeloid leukemia	7	1.39E-03	IKBKB, TCF7L1, PIK3R5, KIT, FLT3, CEBPA, PIK3CG
hsa04640	Hematopoietic cell lineage	8	1.43E-03	KIT, CSF3R, CD1D, FLT3, CR2, CD19, CD5, MS4A1

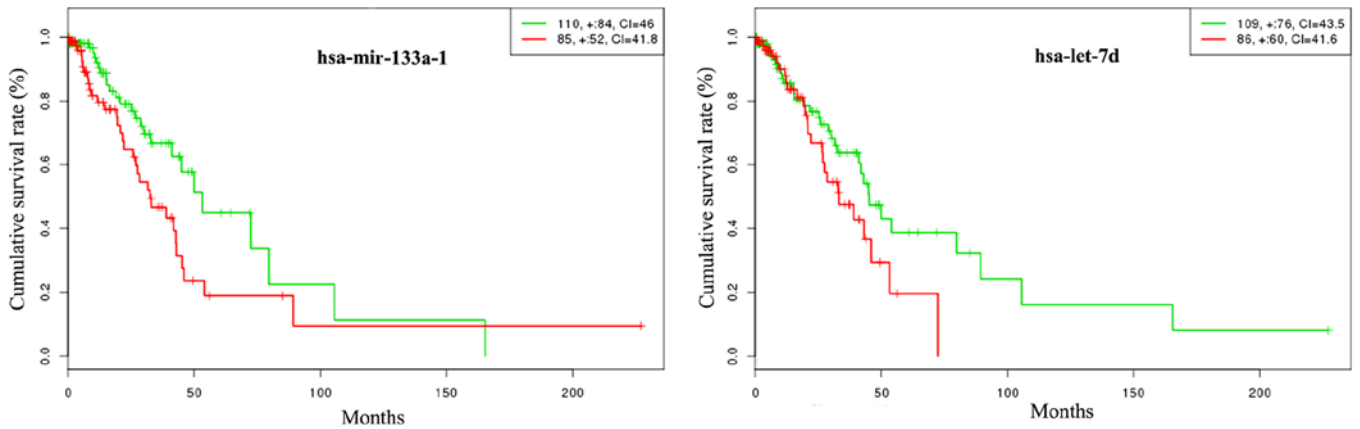

Figure 3. Kaplan-Meier survival curves show the correlation between the miRNA expression levels (hsa-mir-133a-1 and hsa-let-7d) and the overall survival time of lung adenocarcinoma patients.

SOCS4. miR-944 was found to be associated with lymph node metastasis by determining its expression in 52 formalin-fixed paraffin-embedded SCC tissues (26).

More importantly, two miRNAs, hsa-mir-133a and hsa-let-7d, were found to be involved in lung adenocarcinoma metastasis by previous studies. Consequently, we performed survival analysis to evaluate the correlation between miRNA expression level and the overall survival time of lung adenocarcinoma patients, and we found that the expression of hsa-mir-133a was significantly correlated with the overall survival of lung adenocarcinoma, while the expression of hsa-let-7d was not significantly correlated with the survival of lung adenocarcinoma.

miR-133a was identified to function as a tumor suppressor in NSCLCs directly targeting several membrane receptors including IGF-1R, TGFBR1 and EGFR. In addition, by using the *in vivo* animal model, ectopically expressing miR-133a markedly suppressed the metastatic ability of the lung cancer cells, which may be applied in the clinical therapy of lung cancer in the future. Accordingly, NSCLC patients with higher expression levels of miR-133a had longer survival rates compared with those with lower miR-133a expression levels (19).

Let-7d, one of the members of the let-7 family, is deregulated in many types of cancers (27-30). Let-7d is involved in cellular differentiation, epithelial-to-mesenchymal transition

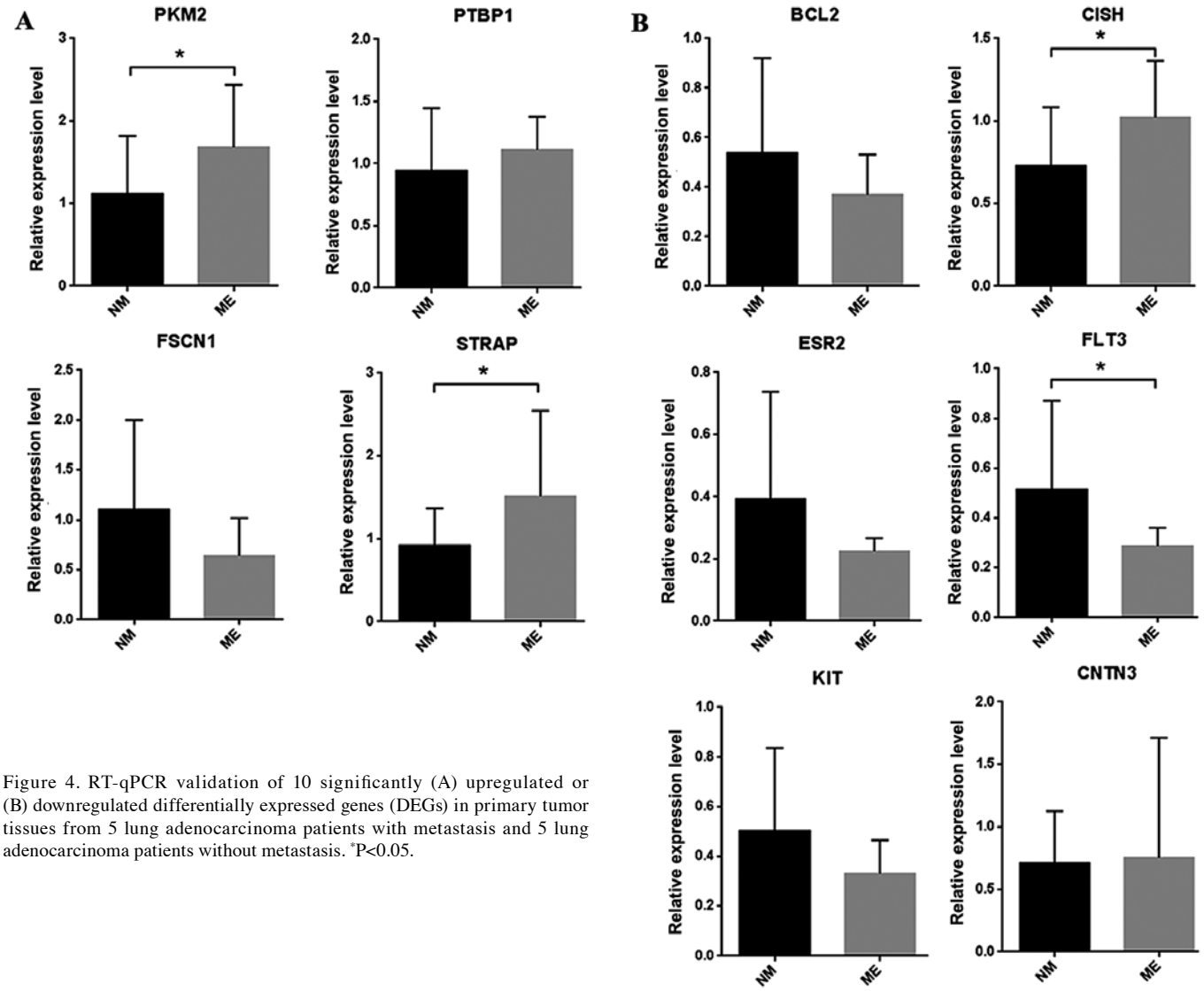

Figure 4. RT-qPCR validation of 10 significantly (A) upregulated or (B) downregulated differentially expressed genes (DEGs) in primary tumor tissues from 5 lung adenocarcinoma patients with metastasis and 5 lung adenocarcinoma patients without metastasis. *P<0.05.

(EMT) as a switch between EMT and MET (mesenchymal-epithelial transition), and regulates tumor-initiating cell (TIC) formation (31). Mairinger *et al* showed that let-7d expression was significantly associated with overall survival in pulmonary neuroendocrine tumors, suggesting its important role in metastasis and tumor progression (20).

The putative targets of hsa-mir-133a and hsa-let-7d were subjected to RT-qPCR validation in primary tumor tissues from 5 lung adenocarcinoma patients with metastasis and 5 lung adenocarcinoma patients without metastasis. The result of the RT-qPCR assay revealed that most of the genes showed similar expression patterns to what were observed in the bioinformatic analysis, suggesting that the findings in the bioinformatic analysis of the differential gene expression data were credible. PKM2, as a common putative target of both hsa-mir-133a and hsa-mir-552, was significantly upregulated at the mRNA level in the lung adenocarcinoma with metastasis. Interesting, the protein level of PKM2 was upregulated in the lung adenocarcinoma tissues compared with the paired surrounding normal tissue, which was also correlated with chemotherapy resistance, the severity of epithelial dysplasia, as well as a relatively poor prognosis (32,33), indicating that PKM2 could be used as a tumor marker for diagnosis and, in particular, as a potential target for cancer therapy.

STRAP, a putative target of hsa-mir-133a, was significantly increased at the mRNA level in lung adenocarcinoma with metastasis. As a WD domain-containing protein, STRAP inhibits TGF-β signaling through interaction with receptors and Smad7, and promotes growth and enhances tumorigenicity. Similarly, strong upregulation of STRAP was also observed in lung tumors by immunoblot analyses (34).

FLT3, a common putative target of hsa-let-7d, hsa-mir-196b and hsa-mir-376c, was significantly upregulated at the mRNA level among the downregulated genes in the lung adenocarcinoma with metastasis. It was found that in a mouse model of lung metastases treatment with the Flt3 ligand significantly reduced the number of lung metastases after laparotomy or radiation therapy, and thus prolonged survival (35,36). Unlike the findings in the bioinformatic analysis, CISH mRNA expression was significantly increased. As negative feedback regulators of JAK-STAT and several other signalling pathways, CISH, a putative common target of hsa-let-7d, hsa-mir-382, hsa-mir-9-3, hsa-mir-9-2 and hsa-mir-9-1, was recently found to be involved in the development of many solid

organ and haematological malignancies (37). Prostate cancer LNCaP-S17 cells were resistant to exogenous IL-6-induced neuroendocrine differentiation due to increased levels of CISH and SOCS7 that blocked activation of the JAK2-STAT3 pathways (38).

Functional enrichment analysis of miRNA target genes showed that those genes were highly correlated with carcinogenesis. The most significantly enriched pathway based on the KEGG database was DNA replication. Based on the fact that cancer cells are characterized by uncontrolled cell proliferation properties, deregulation of DNA replication may promote the process of carcinogenesis. In addition, in cancer cells several DNA replication-initiation proteins, such as CDC6 and minichromosome maintenance (MCM) proteins, were overexpressed (39,40).

There are two limitations in this study. Firstly, the expression levels of the miRNAs were not determined in the metastatic primary loci compared with non-metastatic primary loci of lung adenocarcinomas. Secondly, the small size of the clinical samples for RT-qPCR validation was also a limitation of our study, which may be the reason that the RT-qPCR results were not significant. Given that surgery is not recommended for the traditional stage IIIB-IV patients with lung adenocarcinoma classified according to the taxonomy of cancer staging (TNM) system, it is difficult to obtain specimens of metastatic lung adenocarcinoma. Thus, detailed further studies are needed to investigate the selected miRNAs and the corresponding target genes in lung adenocarcinomas metastasis.

In summary, by comparing the difference in the miRNA and mRNA expression profiling in lung adenocarcinomas with and without metastases, an miRNA-regulated network involved in lung adenocarcinoma metastasis was identified combined with the bioinformatic analysis. RT-qPCR results indicated that the bioinformatic approach in our study was acceptable. Our results may contribute to the identification of markers which could be used to detect lung adenocarcinoma metastasis and assess prognosis.

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