Identification of Stage-associated MicroRNAs in Lung Adenocarcinoma Based on Microarray Data

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Abstract

Background: Lung adenocarcinoma (LUAD) is the most aggressive and most frequently seen histological variant of lung cancers, comprising nearly 45% of the overall cases of lung carcinoma and its incidence has been increased significantly worldwide in the last several decades. However, there was limited available evidence with respect to the signalling pathways and miRNAs related to the LUAD.

Methods: To identify the differentially-expressed miRNAs (DE-miRNAs) in different stages of LUAD, we performed a microarray on 514 LUAD and 54 normal tissues. At the mean time, the potentially targeted genes as well as the highly-enriched signaling pathways and the relevant protein–protein interaction (PPI) network were analyzed and constructed by using a series of bioinformatic methods. Moreover, the identified DE-miRNAs in different stages of the LUAD were verified by using the TCGA dataset.

Results : Overall 41 down-regulated -and 82 up-regulated DE-miRNAs were identified, of which 1,716 potential targeted genes were selected. Moreover, the enriched pathways of the above genes were screened by using the GO term and KEGG pathway analyses, including the AMPK signalling pathway, FoxO signalling pathway, MAPK signalling pathway, PI3K-Akt signalling pathway and hippo signalling pathway.

Conclusions: Our study could provide additional understanding of the underlying molecular events and increase the precision of prognostic prediction.

Background

Lung cancer (LC) is a life-threatening disease, with the highest mortality rate among all the malignant tumors worldwide [1]. In China, the disease burden of lung cancer is particularly significant in the elderly, with the highest incidence rate and leading mortality. It was estimated that lung cancer resulted in approximately 610,200 cases of death in 2015, that is, 1,670 deaths per day on average[2] . There are mainly two histological types of lung cancer, of which the non-small cell lung cancer accounts for 85% of all the LC cases. NSCLCs can be further subclassified into LUAD, large cell carcinoma and squamous cell carcinoma. Among them, LUAD is the most aggressive and frequently seen subtype, comprising about 45% of all the LC cases. During recent decades, the incidence of LUAD has been increasing significantly worldwide [3-6]. Although extensive efforts have been made in recent years to promote the development of target therapy, only a limited number of LUAD patients can be treated with potential targeted therapies[7-9]. Though advancements have been made regarding LC early detection, most patients won't be diagnosed until they reached advanced stage. Therefore, it is of great importance to explore novel diagnostic biomarkers and to develop efficient therapeutic methods for LUAD.

MicroRNAs (miRNAs) are non-coding small endogenous RNA molecules that are mostly composed of 21 nucleotides, possessing the function of silencing genes by regulating the post-transcriptional process of protein expression[10].The miRNA molecules can inhibit the translation of mRNAs or degrade the mRNA transcripts through bounding to the 3'-untranslated sections of target mRNAs, thus producing the effects

of gene expresision regulation. The miRNAs may impose oncogenic or tumor suppressing impacts on the development and progression of cancers[10]. There have been researches suggested that the miRNAs could be related to the tumor angiogenesis, invasion process, and changed metabolism in LUAD. More specifically, Li *et al.* demonstrated the promotion effects of the miR-93 on the development and metastasis of the NSCLCs through the inhibition of LKB1/PTEN/CDKN1A in the PI3K/Akt pathway[11]. Besides, other studies have also discovered the involvement of miRNA molecules in LC development and progression by targeting different genes, such as miR-199a-5p, miR-495[12], miR-9 [13]and so on. Due to the non-specific binding between the miRNAs and the target mRNAs, multiple pathways and protein-protein interaction networks related to carcinogenesis could be affected at the same time, as a result of which miRNAs are always considered as promising therapeutic targets for cancer treatment.

Given the significance of the miRNAs in the growth and metastasis of LUAD, previous studies have been focusing on the regulatory effects on targeted genes. However, limited evidence was available with respect to the exploration of miRNAs in different stages of the LUAD. Our study aimed to present the miRNAs with abnormal expression levels (DE-miRNAs) in T1/T2-stage and T3/T4-stage LUAD tissues respectively by adopting the microarray technique, and to explore the the related target genes as well as the potentially involved pathways through integrated bioinformatics analyses, and to construct the PPI networks of the predicted target genes, thus providing additional insights into the underlying molecular mechanisms of LUAD as well as the possible prognostic biomarkers for the treatment development of LUAD.

Methods

Evaluation of the expression of miRNAs via TCGA data

The sequences of the miRNAs in the LUAD tissues and adjacent normal tissues were downloaded from the public cancer-related data platform TCGA (https://cancergenome.nih.gov/). The levels of expression of the study miRNAs in the obtained specimens (514 LUAD tissues and 46 normal adjacent tissues) were determined by the Illumina HiSeq Systems, and the student's t-tests were performed to evaluate the differences between the LUAD specimens and normal tissues. Finally, the miRNAs with varied expression levels in two kinds of samples were identified as DE-miRNAs (p-values <0.05 and [logFC] >1).

Prediction of potential targeted genes

After the identification the the DE-miRNAs, the targeted genes and interactions were predicted by using the miRWalk2.0 database, which provides the information from 12 prediction softwares (e.g., Targetscan, miRanda and RNAhybrid)[14], and the genes achieved by at least 9 prediction softwares were considered as potential targeted genes for further analyses.

GO term and KEGG pathway analyses

In our study, the GO term [15]and KEGG pathway annotations[16] that were performed with the DAVID database (https://david.ncifcrf.gov/) and KEGG PATHWAY (http://www.genome.jp/kegg) were adopted for the analysis of functional enrichment of the potential targeted genes. The criteria of p-values less than 0.05 were considered as statistically significant for both analyses.

Construction of the PPI network

The interaction among the identified potentially targeted genes were mapped out based on the STRING database (http://string-db.org)[17]. The Cytoscape software v3.6 was adopted to identify the significant nodes of connection as well as the hub proteins with the criteria of a combined score more than 0.4. Thus, the PPI network was generated[18].

Validation of the DE-miRNAs in TCGA Dataset

The same experimental methods were performed with the downloaded TCGA LUAD dataset for results validation given the reliability of the database in the filed of human cancer study.

Results

Identification of the DE-miRNAs as well as the potentially targeted genes

LUAD and normal tissue miRNAs expression profile was obtained from TCGA-LUAD. The microarray data had 283 Stage I LUAD tissues, 123 Stage II LUAD tissues, 84 Stage III LUAD tissues, 24 Stage IV LUAD tissues and 54 normal tissues. There are 147 DE-miRNAs were obtained between Stage I/II LUAD and normal tissues and 151 DE-miRNAs were obtained between Stage III/IV LUAD and normal tissues. Overall 123 miRNAs with differed expression levels were identified, including 41 down-regulated DE-miRNAs and 82 up-regulated DE-miRNAs (Fig. 1a, Additional file 1). In order to show the significantly differential distribution of the 123 DE-miRNAs, a heat map of the above identified miRNAs was drown using data profile TCGA as a reference (Fig. 1b). Additionally, 1716 target genes for identified miRNAs were achieved based on the miRWalk2.0 database, among which 1202 genes were for the up-regulated miRNAs while 514 ones were for the down-regulated miRNAs.

Enrichment analyses of DEGs

The GO functional enrichment analysis of the targeted genes was performed with the DAVID database, in which three functional groups were included (i.e. molecular function/MF, cell composition/CC, biological processes/BP) (Fig. 2). Among the target genes of the up-regulated miRNAs, the top 3 enriched BPs were positive regulation of RNA polymerase II promoter transcription, RNA polymerase II promoter transcription and negative regulation of RNA polymerase II promoter transcription, and the most enriched CCs were nucleoplasm, nucleus and cytoplasm, while the most enriched MFs included protein binding, transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding and RNA polymerase II core promoter proximal region sequence-specific DNA binding (Fig. 2a and b). Similarly, among the target genes of the down-regulated miRNAs, the top 3 enriched BPs were

positive regulation of RNA polymerase II promoter transcription, positive regulation of transcription, DNA-templated and osteoblast differentiation, and the mostly enriched CCs were nucleus, cytoplasm and cell-cell adheres junction, while the most enriched MFs included protein binding, RNA polymerase II core promoter proximal region sequence-specific DNA binding and transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding (Fig. 2c and d). All of the above results showed that majority of the DEGs were significantly enriched in RNA polymerase II promoter, cell part and binding.

Signaling pathway enrichment analysis

According to the KEGG pathway enrichment analysis, the potentially targeted genes of the up-regulated DE-miRNAs included FoxO signaling pathway, AMPK signaling pathway, transcriptional misregulation in cancer, pathways in cancer, adherens junction, axon guidance, PI3K-Akt signaling pathway, MAPK signaling pathway and regulation of actin cytoskeleton (Fig. 3a), whereas with respect to the down-regulated DE-miRNAs, the enriched KEGG pathways included adherens junction, ubiquitin mediated proteolysis, signaling pathways that regulate stem cell pluripotency, proteoglycans in cancer, pathways in cancer, hippo signaling pathway, SCLC and microRNAs in cancer (Fig. 3b).

Analyzing target genes in LUAD using PPI network and modular

According to the STRING database, great deal of interaction was observed among the identified target genes. A total of 1008 target genes (693 up-regulated and 315 down-regulated genes) of the 1716 key genes were selected to construct the PPI network. For the purpose of better display, the screeening was conducted respectively for the 15 hub nodes of which the DE-miRNAs were up-regulated or down-regulated to the most extent. Finally, the up-regulated nodes with the filtering of degree >= 37 were selected, including LRRK2, EP300, MAPK14, CDC42, HDAC2, RAC1, PIKFYVE, SIRT1, NFKB1, KAT2B, NRAS, RB1, ASH1L, UBE2D1 and HUWE1 (Fig. 4a), among which the LRRK2 demonstrated the most significant degree (degree = 93). Similarly, the down-regulated nodes with the filtering of degree >= 18 were selected, including NOTCH1, BCL2, FOS, MYB, ASH1L, UBE2D1, FBXW7, IGF1R, CUL3, WNT3A, SOCS1, PIKFYVE, HUWE1, INSR and UBE4A (Fig. 4b), among which the NOTCH1 was the most significant (degree = 40).

Validation of the DE-miRNAs by using the TCGA LUAD dataset

In order to verify our results, among the 123 DE-miRNAs identified in our study, 22 miRNAs were also found to be significant according to the downloaded LUAD TCGA dataset (e.g. mir-31, mir-196b, mir-133b, mir-215, mir-548v and mir-328) ($p < 0.05$) (Fig. 5 and Fig. 6).

Exploration of significant pathways associated with OS correlated microRNAs

In order to explore the pathogenesis of microRNA in LUAD, we selected two OS associated microRNAs, mir-9 and mir-486, which with the greatest significance in expression, and then explored their carcinogenic mechanism. By retrieving the mirwalk2.0 database, we found that two miRNAs were

associated with multiple pathways. Moreover, some significant target genes were also obtained and showed in Fig. 7.

Discussion

LUAD is commonly believed to be a burdensome malignant tumour with the highest prevalence. Currently surgeries and the radiotherapy plus adjuvant chemotherapy were considered as the most frequently used treatment strategies in clinical practice. Although there have been several studies exploring the underlying mechanisms of the develoment and progression of LUAD during the last decades, few advancement has been made regarding the prognosis.

In the present study, using bioinformatics methods to deeply analyze the gene expression profiles of TCGA-LUAD, a total of 123 DEGs, consisting of 82 upregulated and 41 downregulated DEGs were screen out between LUAD and normal samples. What's more, the STRING database indicated that a total of 1,008 targeted genes (693 up-regulated and 315 down-regulated genes) of the candidate key DE-miRNAs were selected to construct the PPI network. Besides, the most significant hub genes were obtained from the PPI network, among them, LRRK2 and NOTCH1 were identified as the most important node. Moreover, KEGG pathways enrichment for the targeted genes included AMPK signaling pathway, FoxO signaling pathway, PI3K-Akt signaling pathway, MAPK signaling pathway and hippo signaling pathway.

Our study results demonstrated the greatest alterations in the expression of hsa-miR-31 and hsa-miR-133b among all the identified DE-miRNAs, which confirmed the conclusions from previous studies regarding the clinical significance of the two miRNAs. Former studies have suggested the application of hsa-miR-31 in predicting LUAD prognosis [19] due to its significant correlation with the recurrence-free survival [20] and the 5-year overall survival (OS) in LUAD patients[21]. Additionally, miR-133b expression was proved to be correlated with tumour size and differentiation status of LUAD, disease-free survival and OS[22]. Moreover, it was proposed that miR-133b could reverse the cisplatin resistance to a great extent by targeting GSTP1 in cisplatin-resistant lung cancer cells[23]. Other studies also demonstrated the inhibitory effects of the miR-133b on the development and metastasis of NSCLCs by targeting MMP9 and FSCN1[24, 25]. More importantly, Chen et al. also demonstrated decreased expression level of miR-133b in advanced tumors and lymph node metastasis [26],which was consistent with our finding.

According to the GO term enrichment analysis, the most enriched BP for the target genes of the up-regulated DE-miRNAs was the positive regulation of RNA polymerase II promoter transcription, and the CC term was nucleoplasm, while the MF was associated with protein binding and transcriptional activator activity. Furthermore, the target genes of the down-regulated DE-miRNAs were enriched in the positive regulation of RNA polymerase II promoter transcription and positive regulation of transcription with respect to the BPs, in nucleus regarding the CCs, while the most enriched MF term was protein binding. Based on the KEGG analysis, a series of highly-enriched pathways that could be potentially associated with the LUAD were identified, including the FoxO signaling pathway, AMPK signaling pathway, transcriptional misregulation in cancer, pathways in cancer, adherens junction, axon guidance, PI3K-Akt

signaling pathway, MAPK signaling pathway and regulation of actin cytoskeleton for the up-regulated miRNAs. At the mean time, adherens junction, ubiquitin mediated proteolysis, signaling pathways that regulate stem cell pluripotency, proteoglycans in cancer, pathways in cancer, hippo signaling pathway, SCLC and microRNAs in cancer were identified for the down-regulated DE-miRNAs.

AMPK is proposed to be able to promote storage of energy and glucose uptake when activated by metabolic stress[27]. Previous study has demonstrated the involvements of SIRT1 and AMPK regarding the hypoxia-induced resistance of NSCLCs to cisplatin and doxorubicin[28]. AMPK/mTOR has been considered as a promising therapeutic target for NSCLCs[29] due to its great significance in the control of the growth, proliferation and autophagy processed of the tumor cells by regulating the activity of mTOR. FOXO has been reported to be involved in the modulation of apoptosis, cell cycle arrest and other biological processes in LUAD by regulating the expression levels of related genes[30]. In addition, previous study also suggested that FOXO3a could be activated in tumor cells and then improve the caspase-dependent apoptosis level to certain extent, which indicated the tumor suppression effect of FOXO3 in LUAD carcinogenesis[31]. The MAPK signalling pathway is widely believed to play an important role in tumourigenesis regulation. The reactivation of RAS-MAPK could also facilitate the acquired resistance in FGFR1-amplified lung cancer and lay a foundation for the blocking of upstream FGFR-MEK[32]. Recently, evidence suggested that Ghrelin could increase the proliferation of NSCLC tumor cells by regulating the ERK and PI3K/Akt/mTOR/P70S6K signalling pathways[33]. Moreover, the tetrahydrocurcumin was also discovered to be able to induce the autophagy of NSCLC cells through the PI3K/Akt/mTOR modulation[34]. Moreover, recent evidence also demonstrated the inhibitory effect of WWC3 on the epithelial-mesenchymal transition process in LC mediated by the activation of Hippo-YAP signaling pathway[35]. Extensive studies have shown that the hippo-YAP pathwaycould decrease the EGFR-TKI sensitivity in lung adenocarcinoma with primary or acquired EGFR-TKI resistance[36]. The enrichment pathway analyses performed in our study also confirmed the significant involvement of the above signaling pathways.

According to the constructed PPI network, the LRRK2 and NOTCH1 were identified as the genes with the highest connectivity among all the potentially targeted genes of the up-regulated or down-regulated miRNAs, which have been reported to be related to tumor malignancy in LUAD. LRRK2 is a member of the leucine-rich repeat kinase family that is encoded by the PARK8 gene[37], which is commonly composed by a WD40 domain, a GTPase domain, a RAS domain, a kinase domain, a leucine-rich repeat (LRR) domain, an ankyrin repeat (ANK) region, and an armadillo repeats (ARM) region. The enzyme mainly exist in the cytoplasm with a minority in the outer membrane of the mitochondria.The apoptosis of neuroblastoma cells and mouse cortical neurons could be induced by the expression of mutant LRRK2[38].The Notch signaling pathway can impose either tumour suppression or tumorigenic effects in SCLC [39].For instance, loss-of-function mutations of the NOTCH genes could inhibit the ectopic Notch activation, while, on the other hand, the potentials of EMT, cell motility and cell metastasis could be inhibited by the increased expression of Notch1 in SCLC.

We found that mir-486 had tumor suppressing effect in LUAD, and many previous studies support this conclusion. Yu et al. found that miR-486 inhibits cell proliferation and invasion through repressing GAB2 in LUAD[40], and miR-486played an anti-tumor function by targeting CDK4 in LUAD[41]. Moreover, previous studies also proved that miR-9 could promotes LUAD though different mechanism. Li and their team found that miR-9 promotes cell growth and metastasis in LUAD through the repression of TGFBR2[42], and may serve as biomarker and predicted the prognosis of LUAD. Increase in plasma miR-9 and decrease in miR-486 levels after NSCLC resection may indicate a good prognosis[43]. These data supported that miR-9 and miR-486 may be key therapeutic targets that were related to LUAD malignancy and outcome, while in-depth studies of their operating mechanism are still await for further exploration.

Conclusions

Our study presented a primary profile of the DE-miRNAs in different LUAD stages by using a series of integrated bioinformatic analyses. Overall 41 down-regulated -and 82 up-regulated DE-miRNAs were identified, of which 1,716 potential targeted genes were selected. Moreover, the enriched pathways of the above genes were screened by using the GO term and KEGG pathway analyses, including the AMPK signalling pathway, FoxO signalling pathway, MAPK signalling pathway, PI3K-Akt signalling pathway and hippo signalling pathway. Our study confirmed the conclusion proposed by former studies, that hsa-miR-31, hsa-miR-133b, LRRK2 gene and NOTCH1 gene could be used as promising therapeutic targets for the LUAD. Our study provided additional insights into the progression and malignancy of the LUAD. However, further explorations were needed with respect to the specific mechanism of the carcinogenesis and tumor suppression effects related to the significant miRNAs in different stages.

Abbreviations

LUAD: Lung adenocarcinoma;miRNA: microRNA;hsa:Homo sapiens;GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes;miRFA: miRNA functional analysis;PPI: Proteinprotein interaction; TCGA:The cancer genome atlas; TNM: tumor-node-metastasis;seq:Sequencing

Declarations

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Authors' contributions

GG conceived the study. JR,WW,PG, SY, MY,JL and XC analyzed data. JR, WW and GG wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

All used data was obtained from the databases referenced.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Additional File

Additional file 1. 123 DE-miRNAs were identified from dataset, including 41 downregulated DE-miRNAs and 82 upregulated DE-miRNAs.

Figures

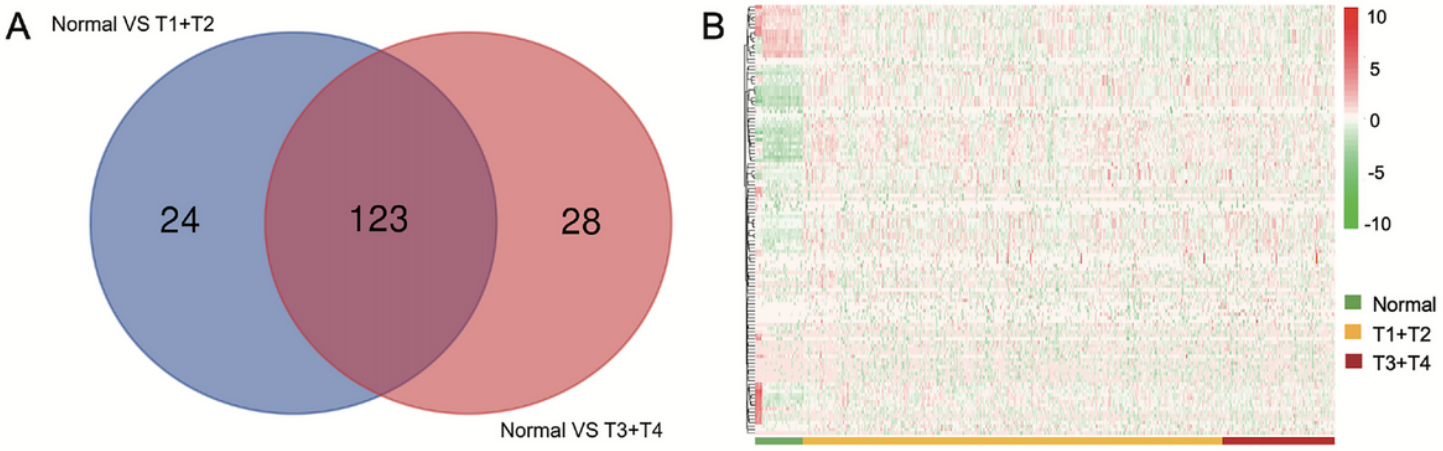

Figure 1

DE-miRNAs in lung adenocarcinoma. (A) Venn diagram presents overlapping relationships, and 123 differentially expressed miRNAs were identified. Red circle stands for the miRNAs with varied express levels in T1/T2 stage tumours and normal tissues, and circle in blue represented for the miRNAs with varied express levels in T3/T4 stage tumors and normal tissues. (B) Cluster analysis of differentially expressed miRNAs using data profile from TCGA, a total of 46 normal tissue and 514 lung adenocarcinoma samples are presented as a heat map.

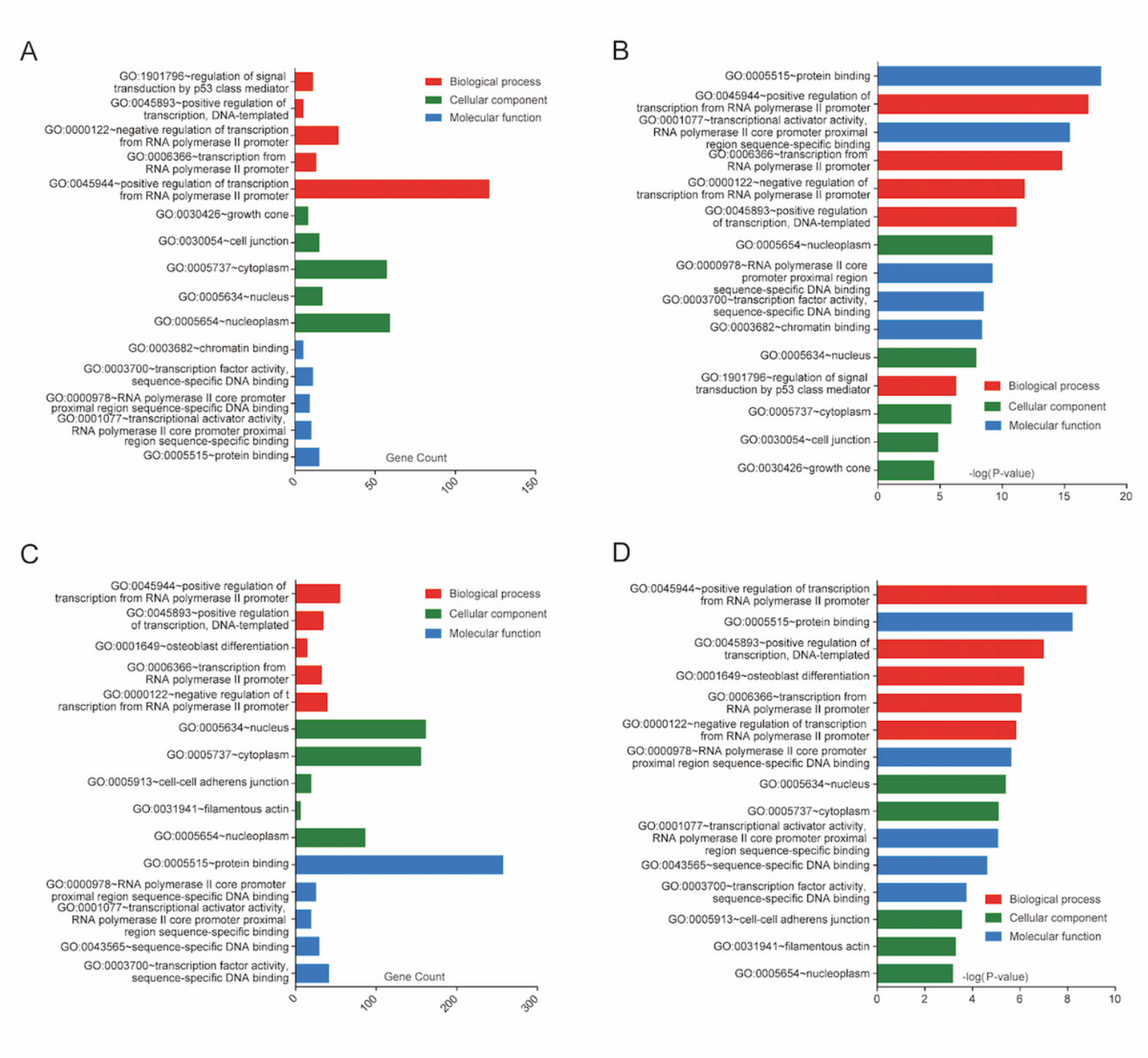

Figure 2

Gene Ontology analysis and significant enriched GO terms of the potentially targeted genes of the identified miRNAs in lung adenocarcinoma. The 5 mostly enriched terms of each group were showed ($p<0.05$). (A) GO analysis classified the upregulated differentially expressed miRNAs targets into 3 categories (MF, BP and CC). (B) Highly enriched GO terms of up-regulated differentially expressed miRNAs targets in lung adenocarcinoma based on their functions. (C) GO analysis classified the targets of down-regulated differentially expressed miRNAs into 3 groups. (D) Significant enriched GO terms of downregulated differentially expressed miRNAs targets in lung adenocarcinoma based on their functions.

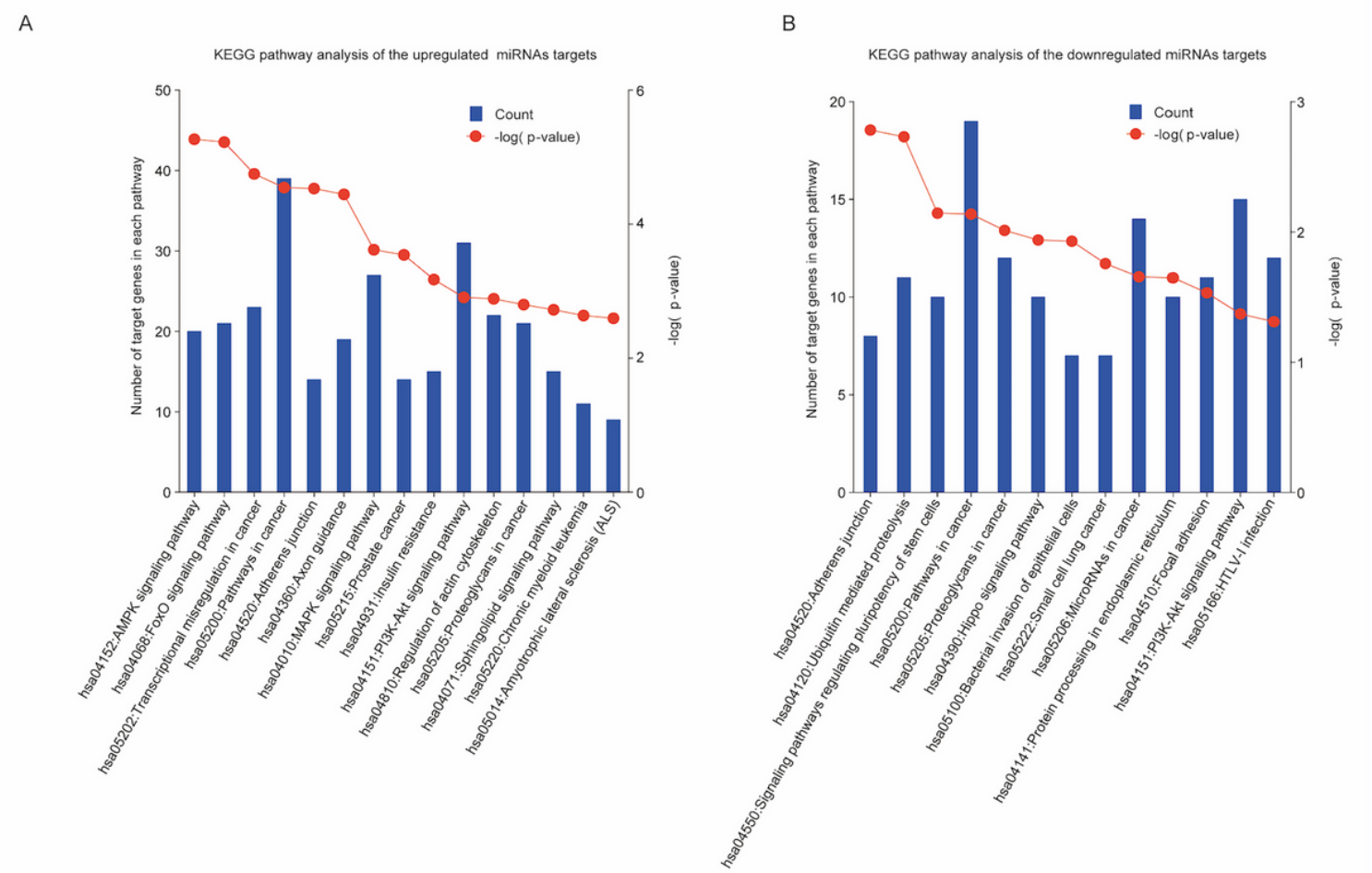

Figure 3

Significantly enriched pathway terms of differentially expressed miRNAs targets in lung adenocarcinoma. (a) KEGG pathway enrichment analysis of upregulated miRNAs target genes in lung adenocarcinoma. ($p<0.05$). (b) KEGG pathway enrichment analysis of downregulated miRNAs targets in lung adenocarcinoma. ($p<0.05$).

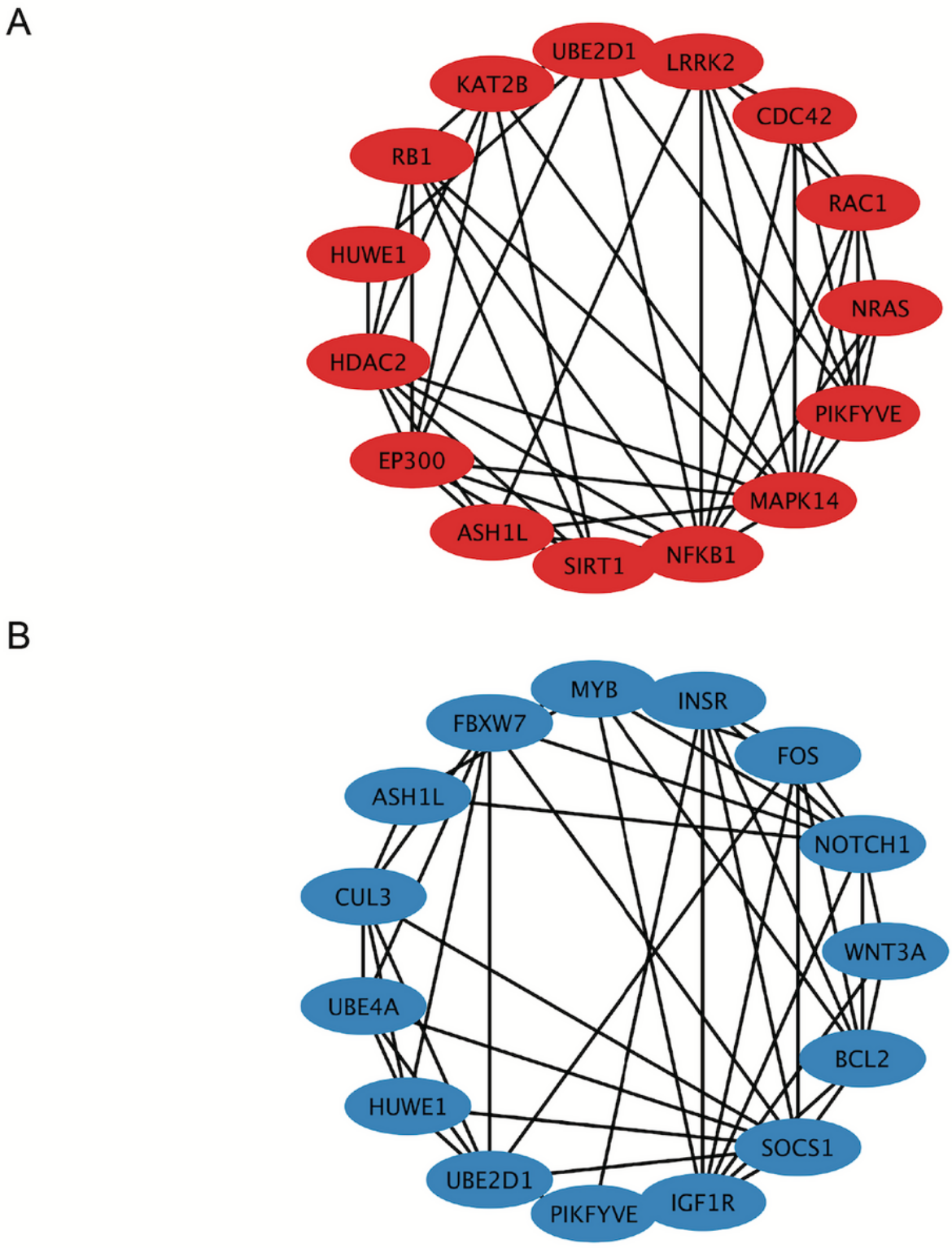

Figure 4

Gene regulatory network of the 15 hub targeted genes with the highest significance in lung adenocarcinoma (orange: targets of up-regulated miRNAs; blue: targets of down-regulated miRNAs). (A) Up-regulated miRNAs. (B) Down-regulated miRNAs.

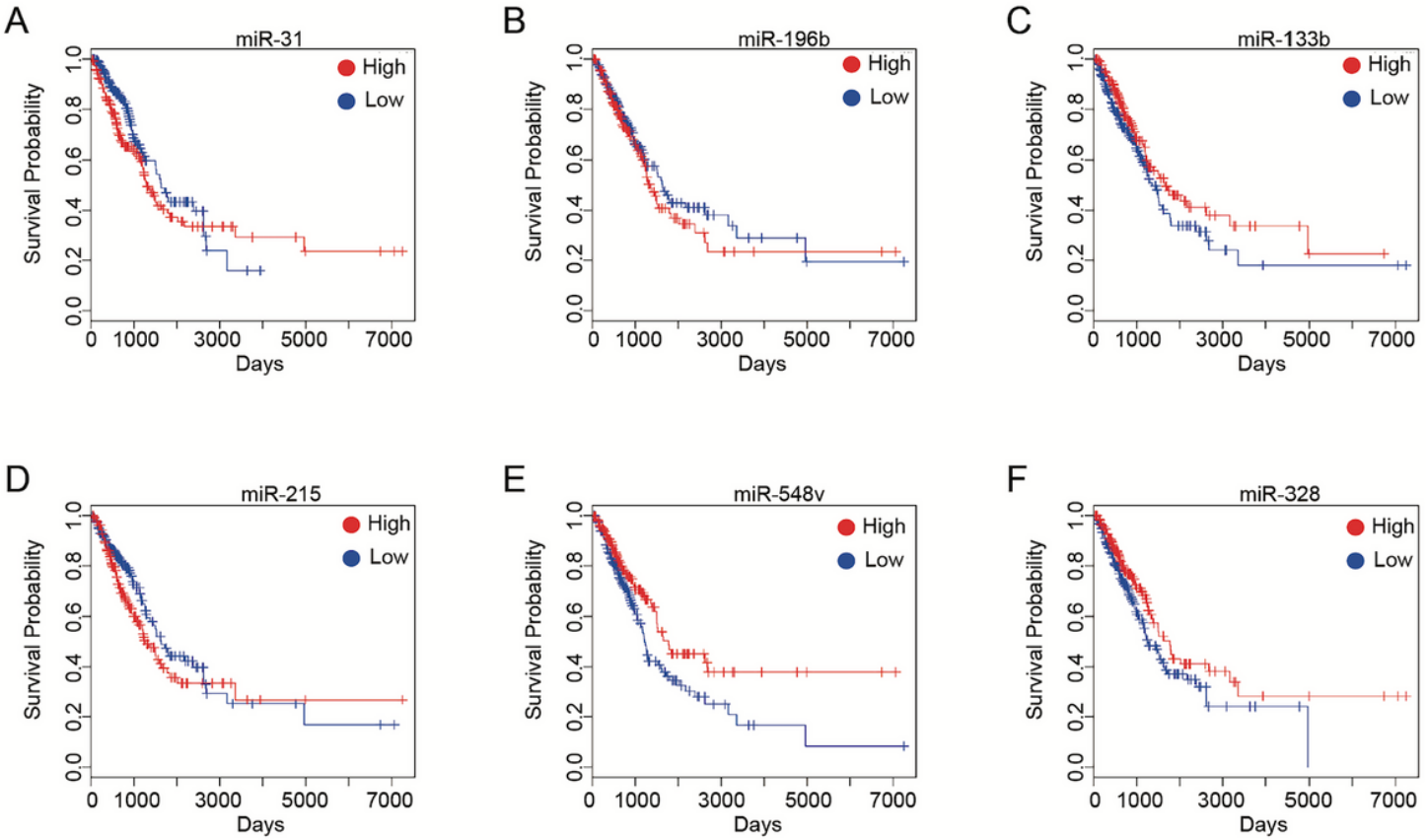

Figure 5

Expression of top six differentially expressed miRNAs (from TCGA) correlates with survival in lung adenocarcinoma. The medial expression was set as the cut-off point ($p<0.05$). (A) KM survival curves of mir-31. (B) KM survival curves of mir-196b. (C) KM survival curves of mir-133b. (D) KM survival curves of mir-215. (E) KM survival curves of mir-548v. (F) KM survival curves of mir-328.

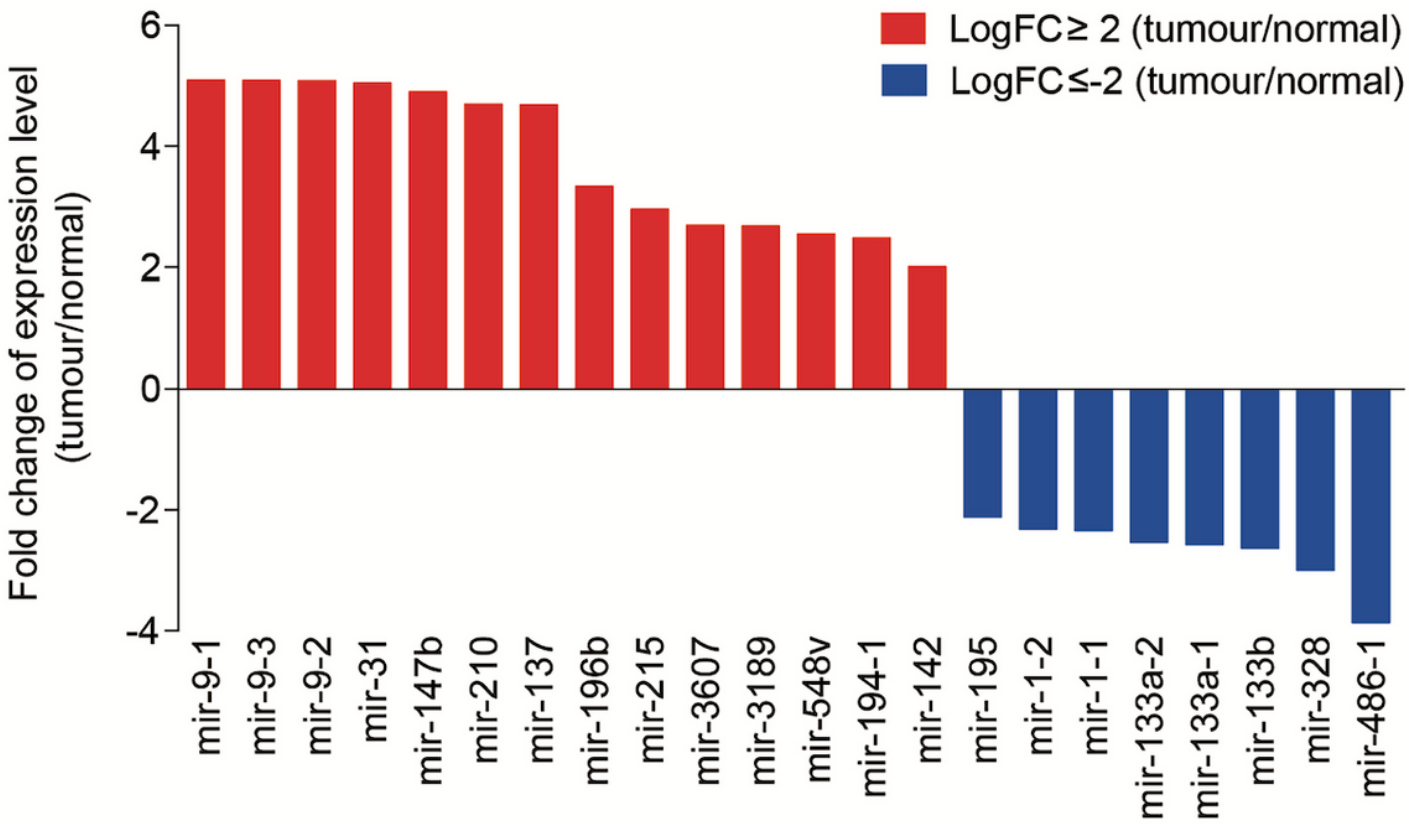

Figure 6

Differentially expressed microRNAs with significantly survival between lung adenocarcinoma and adjacent normal tissues (from TCGA). Differentially expressed microRNAs with more than fourfold change or 0.25-fold change.

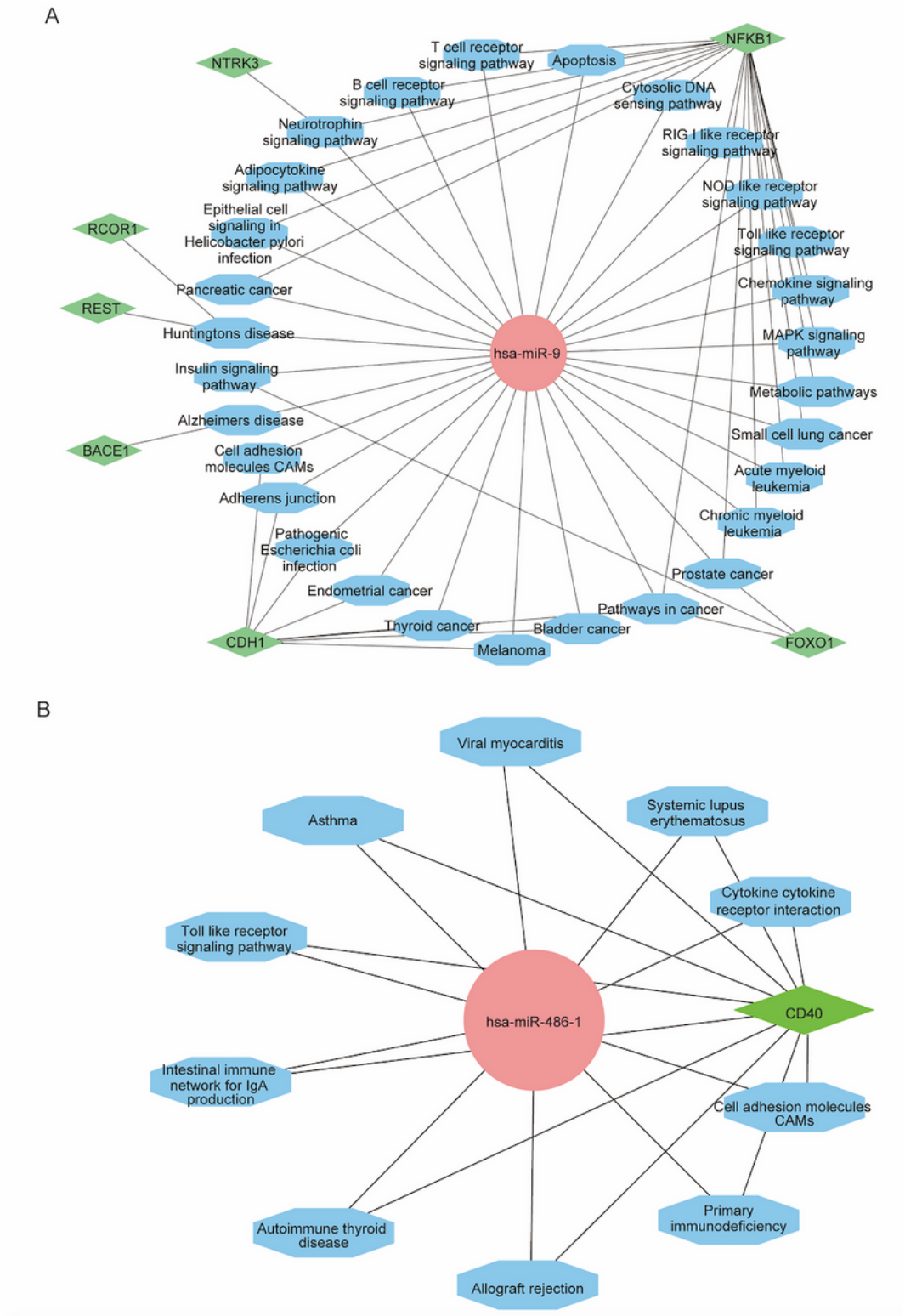

Figure 7

Significant pathways associated with OS correlated microRNAs. (A) Potentially targeted genes and enriched pathways of the most up-regulated microRNA. (B) Potentially targeted genes and enriched pathways of the most down-regulated microRNA.

Supplementary Files

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- Additionalfile1.docx