



Circulating miR-17 as a promising diagnostic biomarker for lung adenocarcinoma: evidence from the Gene Expression Omnibus

Erna Jia^{1#}, Na Ren^{2#}, Rongkui Zhang³, Changyu Zhou¹, Jinru Xue²

¹Department of Gastroenterology, ²Department of Thoracic Surgery, China-Japan Union Hospital of Jilin University, Changchun, China;

³Department of Radiology, The First Hospital of Jilin University, Changchun, China

Contributions: (I) Conception and design: J Xue; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: E Jia, N Ren; (V) Data analysis and interpretation: E Jia, N Ren; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Jinru Xue. Department of Thoracic Surgery, China-Japan Union Hospital of Jilin University, No. 126, Xiantai Street, Changchun 130033, China. Email: jinruxue@jlu.edu.cn.

Background: A definitive preoperative diagnosis of lung adenocarcinoma (LUAD) is a clinical challenge. Informative and blood-based microRNAs (miRNAs) may provide new insights on LUAD screening and detection but are limited by suboptimal accuracy.

Methods: The raw expression levels of circulating miR-21, miR-155, miR-210, miR-126, miR-182, and miR-17 in LUAD cases and healthy controls from the Gene Expression Omnibus (GEO) database were obtained and analyzed to identify accurate diagnostic miRNAs biomarkers for LUAD detection. We applied a meta-analysis to determine the magnitude of statistically significant miRNAs in LUAD samples, based on estimation outcomes acquired by analyzing raw data. Furthermore, bioinformatics analysis was conducted to reveal the function of significant miRNAs in the comprehensive underlying mechanisms of LUAD biology.

Results: A total of 5 raw microarray datasets, including 87 LUAD samples and 83 healthy controls, were eligible. Through our analysis, the primary outcome measure was that circulating miR-17 showed a favorable accuracy in diagnosing LUAD, and the overall pooled area under the curve (AUC) value was 0.79. Furthermore, a total of 85 predicted target genes were chosen, and 29 gene ontology (GO) items and 3 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways showed a significant statistical difference, which demonstrated that the target genes are involved in the biological processes of LUAD. A protein-protein interaction (PPI) network analysis revealed 10 hub genes, namely *CCND2*, *E2F3*, *TNRC6B*, *AGO1*, *AAK1*, *RAB5B*, *LDLR*, *FBXO21*, *UBE3C*, and *MYLIP*, located in the center of the PPI network.

Conclusions: In conclusion, circulating miR-17 may be a quality diagnostic biomarker for LUAD screening, but confirmation by a large, rigorous clinical validation in a longitudinal setting is warranted.

Keywords: Lung adenocarcinoma (LUAD); miR-17; biomarker; bioinformatics

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Introduction

Lung adenocarcinoma (LUAD) has surpassed lung squamous cell cancer and large-cell carcinoma as the most common histological type of non-small cell lung cancer (NSCLC) and accounts for more than 85% of lung cancer

(LC) (1-3). The global incidence of LUAD has risen in the last few decades, especially in young patients, with the prevalence in young patients even reaching between 57.5% and 77.9% (4-10). Due to a lack of apparent symptoms, the 5-year survival rate of LUAD patients is less than 15% (11). However, the 5-year survival rate of LUAD patients with

stage I can reach 100% (12-14). Hence, the early detection and treatment of subjects with LUAD is associated with improved survival. Low-dose computed tomography (LDCT) is the method recommended for LUAD screening and surveillance by several guidelines (15-17). However, periodic LDCT tests for screening may expose individuals to a significant level of radiation, the impact of which is unknown but possibly harmful (18-21). Previous studies have reported that the high false-positive rates and poor cost-effectiveness of LDCT are still problematic for its use in the screening of LUAD (22,23). Therefore, an accurate, harmless, comparatively cost-effective screening tool is needed to facilitate the widespread early screening of LUAD.

Aberrantly expressed microRNAs (miRNAs) have been studied as candidate biomarkers for early detection of numerous cancers, including NSCLC (24,25). The possibility of cancer screening based on miRNAs markers detected in body fluids, such as in the blood, has recently become one of the prime focuses of research efforts. The expression profiles of miRNAs exist with remarkable stability in various types of body fluids, including blood (26), and reflect the tumor development lineage and differentiation stages, are related to the clinicopathological features of tumor (27,28), and can be used to identify tumor histological subtypes based on origin, histology, and chemical sensitivity (29-31). The less invasive sampling method of using circulating miRNAs biomarkers as surrogates is an attractive option for the molecular detection of LUAD which may revolutionize the screening and surveillance for LUAD.

Circulating miR-21, miR-155, miR-210, miR-126, miR-182, and miR-17 are the most frequently reported miRNAs diagnostic biomarkers for NSCLC. But the expression levels and deregulation directions are inconsistent in different studies (32-36), and whether these circulating miRNAs biomarkers can accurately identify LUAD or be applied for predictions in clinical practice is still inconclusive. In the present study, we conducted a systematic summary of the published microarray datasets that investigated the above-mentioned circulating miRNAs biomarkers for LUAD diagnosis based on the Gene Expression Omnibus (GEO) database. The primary purpose was to analyze the diagnostic performance of circulating miR-21, miR-155, miR-210, miR-126, miR-182, and miR-17 for patients with LUAD. The second aim was to clarify the biological processes and molecular regulatory mechanisms of these miRNAs in LUAD development via bioinformatics analysis.

We present the following article in accordance with the STREGA guideline checklist (available at <http://dx.doi.org/10.21037/tcr-19-3025>).

Methods

Selection of GEO dataset and data extraction

A systematic literature search was applied to identify microarray datasets assessing circulating miRNAs as diagnostic biomarkers for LUAD, NSCLC, and LC. We mined the GEO database for eligible datasets until June 26, 2019. The search strategy was as follows: ((lung OR pulmonary) AND (cancer OR carcinoma OR tumor OR tumor OR malignanc* OR neoplas* OR nodule OR adenoma* OR adenocarcinoma)) AND (microRNA* OR miRNA* OR miR*). We used the following filters to filter the search results again: “Homo sapiens”[porgn] AND “gse”[Filter]. The initial screening involved browsing the title and applying the following exclusion criteria: (I) unrelated to LUAD/NSCLC/LC, (II) non-human studies, (III) unrelated to the topic. The second screening used the following inclusion criteria: (I) datasets assaying the expression levels of miRNAs in serum, plasma, exosomes, and blood; (II) datasets comparing LUAD patients with healthy controls; (III) datasets with a sufficient amount of raw data to calculate the test performance parameters. Finally, the datasets including specific expression data in serum, blood, plasma, or exosomes of miR-21, miR-155, miR-210, miR-126, miR-182, and miR-17 for LUAD detection were enrolled as eligible datasets.

Two reviewers (Erna Jia and Na Ren) independently screened the involved datasets that remained after the filtering process described above, and disagreements between the reviewers were resolved by discussion and consensus. For each dataset, we extracted detailed information on dataset ID, first author, upload year, county, sample type, sample size, and expression value.

Statistical analysis

Means and standard deviations (SD) were calculated. A two-sample t-test was conducted for independent samples in random design studies, while standardized mean difference (SMD) was used to assess the relationship between circulating miRNA expression levels and LUAD. Heterogeneity was assessed by Cochran's Q Statistic and I^2 statistic with the Chi-squares test. Cochran's Q Statistic

with a P value less than 0.1 or a value of I^2 greater than 50% was considered to indicate significant heterogeneity. The sensitivity, specificity, and area under the curve (AUC) values of miRNAs with statistically significant differences between LUAD samples and healthy controls were calculated in each dataset, using true-positive (TP), false-positive (FP), true-negative (TN), and false-negative (FN) values estimated from the raw expression data. Then, the diagnostic accuracy of these miRNAs was estimated by pooled statistics. The summary receiver operator characteristic (SROC) curve was depicted. The pooled AUC was calculated to evaluate the diagnostic performance of statistically significant miRNAs discriminating LUAD cases from healthy controls. A P value of less than 0.05 was considered statistically significant.

All statistical analyses and plots were conducted via R x64 (version 3.5.3) and STATA 15.1 (Stata Corporation, College Station, Texas).

Target prediction analysis and bioinformatic analysis

Online prediction software, miRWalk 3.0 (Targetsca, miRTarBase, and miRDB), was used to predict the target genes of statistically significant miRNAs, and the genes that overlapped in all 3 databases were selected as the target genes. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of overlapped genes were analyzed by the online DAVID database, with a P value less than 0.05 and a false discovery rate (FDR) value more than 1.5 being considered as statistically significant. Furthermore, we used the online STRING database to construct protein-protein interaction (PPI) network analysis. A combined score of more than 0.9 was considered statistically significant, and we further used the MCODE plugin of Cytoscape to identify the hub genes.

Results

Results from the GEO database

Five microarray datasets, including GSE27486, GSE40738, GSE94536, GSE111803, and GSE93300 from the GEO database, were deemed eligible. Raw expression data were acquired from these 5 datasets, and means and SDs were calculated to estimate the expression state of miR-21, miR-155, miR-210, miR-126, miR-182, and miR-17 in LUAD cases and healthy controls via R x64. A meta-analysis merging all the qualified data was performed to calculate

the overall pooled SMD of those miRNAs in the LUAD cases and healthy controls. The results revealed that the expression level of miR-17 was higher in LUAD samples than in healthy controls ($P=0.03$), and the overall pooled SMD was -0.93 (95% CI, -1.78 , -0.08), presented visually in *Figure 1*. The Cochran-Q statistic ($P=0.02$) and the value of I^2 (71%) showed significant heterogeneity, necessitating the selection of a random-effects model for analysis (*Figure 1*). A two-sample *t*-test for independent samples was conducted for each dataset containing raw expression levels of miR-17, the results of which are displayed in *Figure 2* as intuitive boxplots, with the available data being summarized in *Table 1*. In order to draw a credible conclusion for the value of the miR-17 biomarker in LUAD detection, an SROC plot was depicted (*Figure 3*). Ultimately, the overall pooled sensitivity, specificity, and AUC values were 0.78, 0.71, and 0.79, respectively.

GO and KEGG analyses

A total of 85 overlapped genes were obtained. For the prediction of functional annotation information of overlapping genes by GO enrichment analysis (*Figure 4*), GO-BP included 11 statistical items, with the top 3 enriched items being endocytosis, cellular response to starvation, and regulation of calcium ion import; GO-MF included 10 statistical items, with the top 3 enriched items being protein binding, identical protein binding, and GTPase activity; GO-CC included 8 statistical items, with the top 3 enriched items being cytosol, cytoplasm, and intracellular membrane-bounded organelles. There were 3 statistically significant KEGG enrichment pathways: bladder cancer, cell cycle, and miRNAs in cancer, shown in *Figure 4*.

PPI network analysis

The PPI network contained 85 nodes and 12 edges ($P=0.00362$). The protein network diagram is presented in *Figure 5*. The MCODE plugin of Cytoscape identified 2 significant modules and 10 hub genes: *CCND2*, *E2F3*, *TNRC6B*, *AGO1*, *AAK1*, *RAB5B*, *LDLR*, *FBXO21*, *UBE3C*, and *MYLIP* (*Figure 6*). The standard settings were as follows: degree cutoff =2, node score cutoff =0.2, k-core =2, max depth =100.

Discussion

Our study aimed to evaluate the diagnostic accuracy of

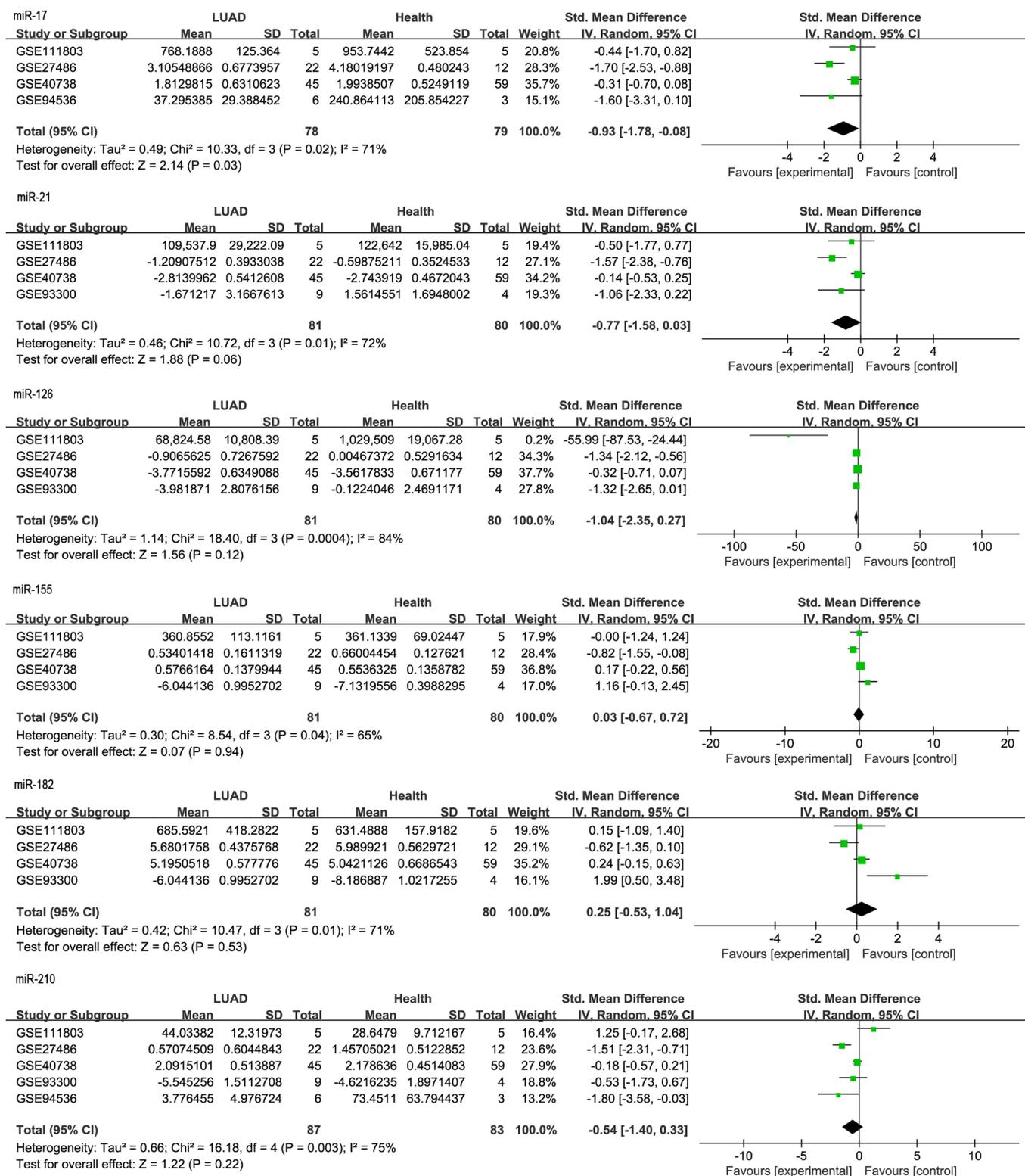


Figure 1 Forest plots for the meta-analysis of circulating miR-17, miR-21, miR-126, miR-155, miR-182, and miR-210 expression in LUAD cases compared with healthy controls. LUAD, lung adenocarcinoma.

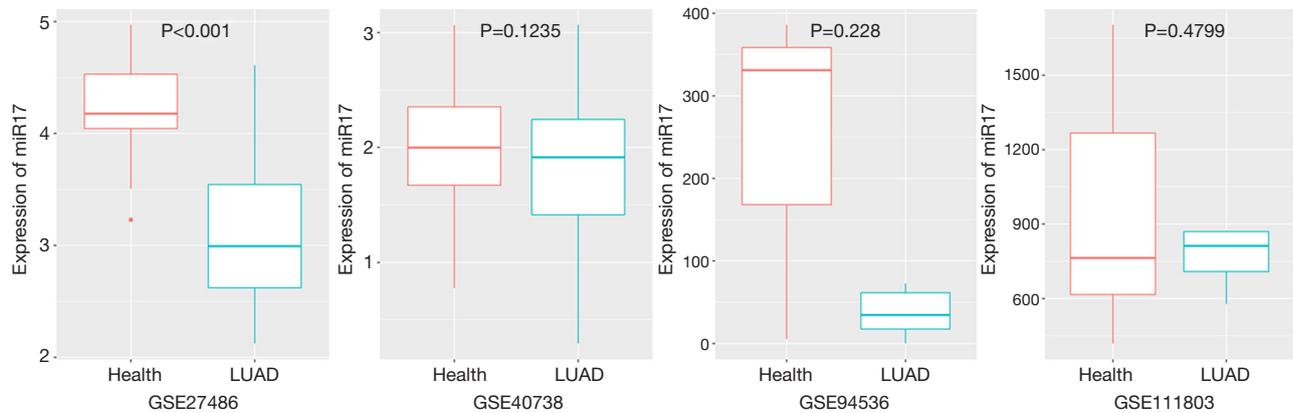


Figure 2 Box plots for the expression levels of circulating miR-17 in LUAD samples and healthy controls based on the GEO database. LUAD, lung adenocarcinoma; GEO, Gene Expression Omnibus.

Table 1 The relevant information of 5 eligible microarray datasets from the GEO database

PMID	First author	Year	Country	Sample type	LUAD N	Healthy N	T value	P value	TP	FP	TN	FN
GSE27486	Patnaik SK	2011	USA	Blood	22	12	5.3684	8.59E-06*	20	2	10	2
GSE40738	Patnaik SK	2017	USA	Blood	45	59	1.5555	0.1235	18	27	46	13
GSE94536	Li LL	2017	China	Plasma	6	3	1.7042	0.228	6	0	2	1
GSE111803	Fang H	2019	China	Exosome	5	5	0.77029	0.4799	5	0	2	3
GSE93300	Liu X	2019	China	Plasma	9	4	-	-	-	-	-	-
GSE93300	Liu X	2019	China	Plasma	9	4	-	-	-	-	-	-

*, indicates the P value was less than 0.05. LUAD, lung adenocarcinoma; GEO, Gene Expression Omnibus; TP, positive value; FP, false positive; TN, true negative; FN, false negative.

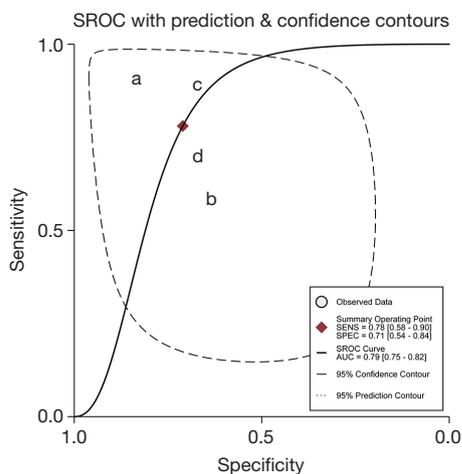


Figure 3 SROC curve for circulating miR-17 in the diagnosis of LUAD. a,b,c,d indicate GSE27486, GSE40738, GSE94536 and GSE111803. SROC, summary receiver operator characteristic; LUAD, lung adenocarcinoma.

circulating miR-21, miR-155, miR-210, miR-126, miR-182, and miR-17 for patients with LUAD, based on raw expression levels of these miRNAs from the GEO database. With raw qualified data estimation outcomes, circulating miR-17 was more highly expressed in LUAD samples compared with healthy controls. The results revealed that circulating miR-17 had high diagnostic accuracy in discriminating LUAD cases from healthy controls. However, the eligible microarray datasets were limited, and there was moderate heterogeneity. Subsequently, bioinformatics analysis was performed to investigate the molecular role of miR-17 in the pathogenesis of LUAD, and 10 hub-genes were selected from the predicted target genes.

MiR-17, as an oncogene in numerous tumors, is the most prominent member in the miR-17-92 cluster (37). MiR-17-92 clusters are essential regulators of fundamental cellular processes like differentiation, metastases, apoptosis,

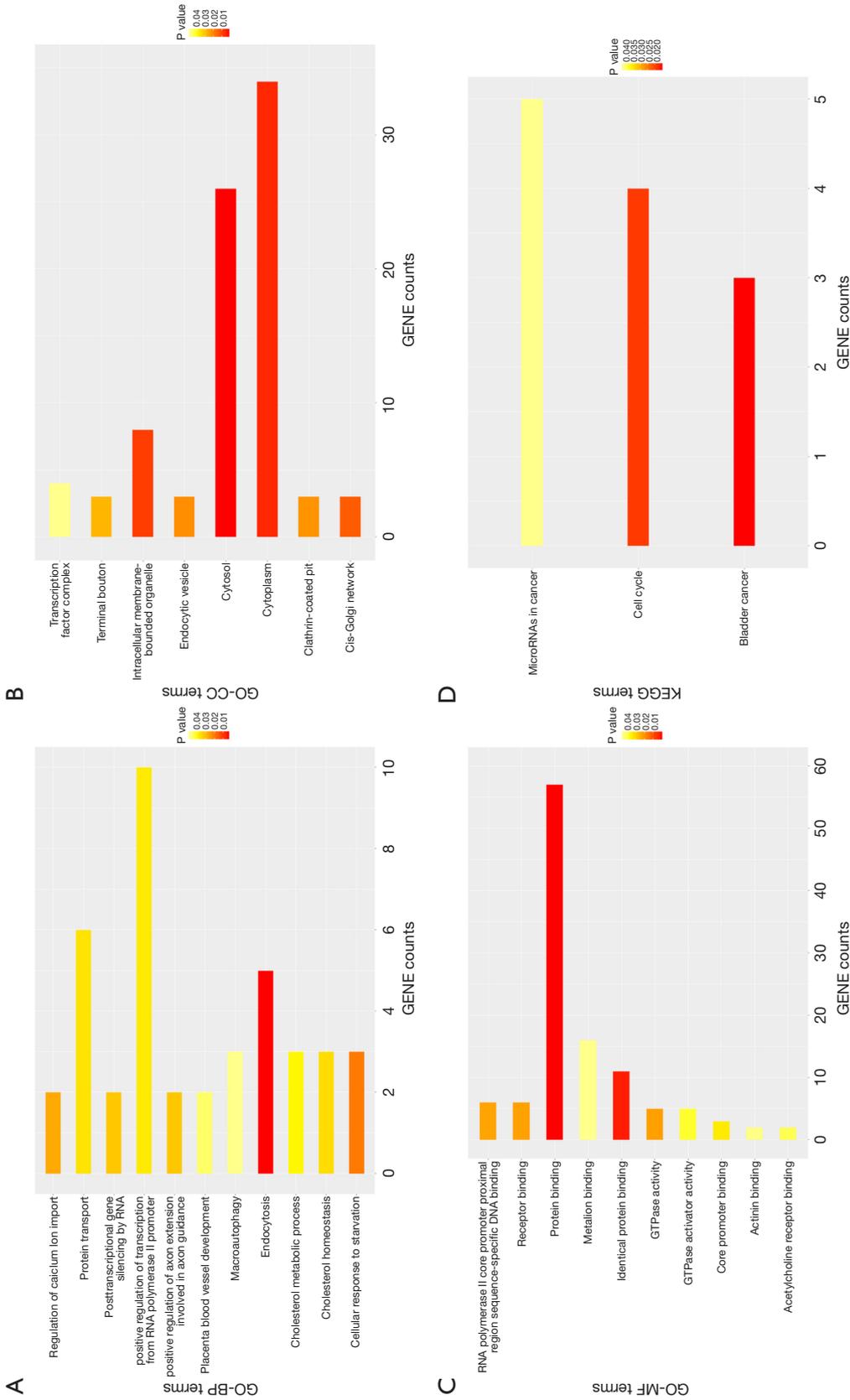


Figure 4 Statistically significant GO items and KEGG pathways. (A) Biological processes (BP); (B) cellular components (CC); (C) molecular functions (MF); (D) KEGG pathways. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

proliferation, invasion, DNA repair, and autophagy (38), which are implicated in the regulation of many oncogenes involved in distinct and opposing pathways (39-41). The carcinogenic role of miR-17-92 clusters in different cancers has been confirmed, and they could interact with the transcription factors E2Fs and c-Myc, which play critical roles in cell-cycle regulation during tumorigenesis (42,43). As reported, circulating miR-17 is significantly

expressed in many cancers, including breast cancer, gastric cancer, prostate cancer, and NSCLC, and thus can be an efficient non-invasive biomarker for the screening of cancer patients (25,44-47). For the first time, the present study demonstrated that circulating miR-17 has a strong ability to distinguish between LUAD cases and healthy controls, supporting its potential use as a new clinical biomarker for the screening and detection of LUAD patients. To confirm the true clinical utility for screening and diagnosis of LUAD, further validation should preferably be done in the context of large-scale prospective research among multi-site collaborative studies.

Bioinformatics analysis was applied to gain a more comprehensive understanding of miR-17 in LUAD biology. Several core GO terms and signal pathways were illuminated in the underlying molecular interaction mechanism of miR-17 in LUAD based on GO enrichment and KEGG pathway analysis. Ten hub genes, namely *CCND2*, *E2F3*, *TNRC6B*, *AGO1*, *AAK1*, *RAB5B*, *LDLR*, *FBXO21*, *UBE3C*, and *MYLIP*, were identified as candidate target genes involved in the regulation of crucial biological processes in LUAD. Apart from *TNRC6B* and *MYLIP*, other genes have been reported to have a close association with tumorigenesis, progression, invasion, metastasis, recurrence, and the therapy strategy of LC (48-55). Researchers should thus endeavor to identify the specific relationship between these hub genes and LUAD.

Exosomes, plasma and blood are currently accepted sample modalities for non-invasive detection of miRNAs, but there are different miRNA profiles from different

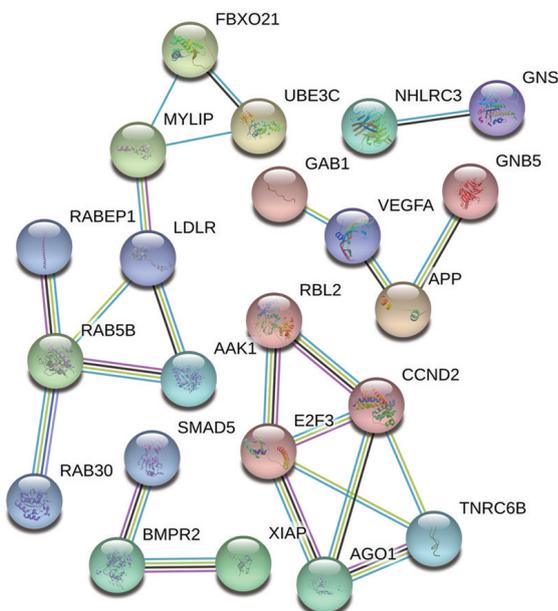


Figure 5 PPI networks of the overlapped predicted target genes of miR-17. PPI, protein-protein interaction.

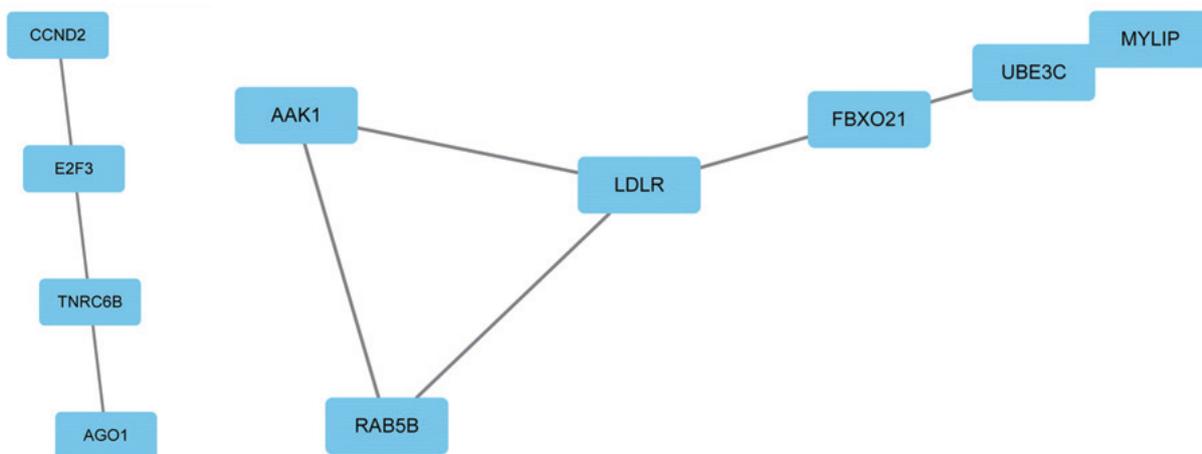


Figure 6 The top 2 modules and top 10 hub genes from the PPI network. PPI, protein-protein interaction.

sample types. Hemolysis can lead to the release of intracellular miRNAs into extracellular that contaminate extracellular miRNAs concentrations, as intracellular miRNA concentrations are higher than extracellular ones (56,57). In addition, the miRNA concentrations can be affected by cell debris which is not completely removed, if the centrifugal speed and time of preparation procedure is insufficient (58). However, strong studies have recently verified that exosomes derived from tumor cells can be specially identified by surface markers, which effectively avoid the contamination of abovementioned factors (59,60). Therefore, more and more researches have been focusing on exosome miRNAs using for diagnosing cancers including LUAD. In the present study, subgroup analysis of sample types was not conducted due to few datasets included, we only assessed the diagnostic performance of the abovementioned miRNAs in all circulation sample types. MiRNAs extraction and standardize quantitative methods are also the source of heterogeneity (58,61). Now there are several kinds of miRNA extraction kits, the miRNeasy kit is the recommended detection kit (62), but not all studies used this kit. U6 snRNA, cel-miR-39, and miR-16 are the generally standardize references, but it is not unified. In the future, the uniform preparation procedure, miRNA extraction method and standardize quantitative reference should be applied in large sample studies to identify the diagnostic accuracy of circulation miRNAs in LUAD.

There are several strengths and a few deficiencies in this study. Our study integrated raw expression data from the GEO database to identify the diagnostic ability of circulating miR-21, miR-155, miR-210, miR-126, miR-182, and miR-17 for LUAD detection. We estimated the mean and SD based on raw expression data, and a meta-analysis based on the estimation outcomes was conducted to give an overall conclusion. It is plausible that circulating miR-17 can be a valid biomarker for LUAD detection and screening. However, several datasets showing negative outcomes and poor diagnostic performance were included in this meta-analysis. As circulating miRNA biomarkers entail lower costs, less radiation exposure, better tolerance, and more convenient specimen extraction. Our study focused on identifying those circulating miRNA biomarkers that may be useful for the detection of LUAD. Moreover, our study identified several signal pathways and hub genes of miR-17 in the underlying molecular mechanisms of LUAD development, which helped further clarify this exploration. In the evaluation of circulating miRNAs, raw

expression data in each dataset, and the inconsistencies of the specimens, detection platforms, operating protocols, and demographics might have been unavoidable confounding factors, causing moderate heterogeneity, although we did apply a random-effects model. The number of enrolled samples in the 5 datasets ranged greatly from 3 to 59, and this might also have influenced our results. Finally, 10 hub genes of miR-17 were identified from predicted target genes in present study, but, due to budgetary constraints, we were unable to validate these hub genes.

Conclusions

Our estimations focused on studies distinguishing LUAD cases from healthy controls based on the raw expression levels of circulating miR-21, miR-155, miR-210, miR-126, miR-182, and miR-17; all of these miRNAs were mostly reported as potential diagnostic biomarkers in previous studies. By combining the original estimations, a meta-analysis showed a significant diagnostic capacity of circulating miR-17 for LUAD detection. Also, we discovered several underlying pathways and 10 hub genes of miR-17 in LUAD biological processes through bioinformatics analysis. Although circulating miR-17 revealed high diagnostic accuracy for LUAD detection in our study, it still cannot be used in the clinical settings for LUAD screening and surveillance. Thus, a large set of samples, used in prospective and experimental research with a longitudinal study design, is needed to validate the findings, and will be a challenging but promising task.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr-19-3025>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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