Construction of a circRNA-miRNA-mRNA Regulated Pathway Involved in EGFR-TKI Lung Adenocarcinoma Resistance

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Abstract

Objectives: Epidermal growth factor receptor-tyrosine kinase inhibitors are widely used for lung epidermal growth factor receptor-positive lung adenocarcinomas, but acquired resistance is inevitable. Although non-coding RNAs, such as circular RNA and microRNA, are known to play vital roles in epidermal growth factor receptor-tyrosine kinase inhibitor resistance, comprehensive analysis is lacking. Thus, this study aimed to explore the circular RNA-microRNA-messenger RNA regulatory network involved in epidermal growth factor receptor-tyrosine kinase inhibitor resistance. **Methods:** To identify differentially expressed genes between the epidermal growth factor receptor-tyrosine kinase inhibitor sensitive cell line PC9 and resistant cell line PC9/ epidermal growth factor receptor-tyrosine kinase inhibitor resistance(PC9/ER), circular RNA, microRNA and messenger RNA microarrays were performed. Candidates were then identified to construct a circular RNA-microRNA-messenger RNA network using bioinformatics. Additionally, Gene Oncology and Kyoto Encyclopedia of Genes and Genomes pathway analyses were conducted to evaluate the network messenger RNA, setting up a protein-protein interaction network for hub-gene identification. Afterwards, RNA immunoprecipitation was performed to enrich microRNA, and quantitative real-time PCR was used to estimated gene expression levels. **Results:** In total, 603, 377, and 1863 differentially expressed circular RNA, microRNA, messenger RNAs, respectively, were identified using microarray analysis, constructing a circular RNA-microRNA-messenger RNA network containing 18 circular RNAs, 17 microRNAs and 175 messenger RNAs. Moreover, Gene Oncology and Kyoto Encyclopedia of Genes and Genomes pathway analyses showed that the most enriched biological process terms and pathways were related to epidermal growth factor receptor-tyrosine kinase inhibitor resistance, including Wnt and Hippo signaling pathways. Based on the competing endogenous RNA and protein-protein interaction network, circ-0007312 was showed to interact with miR-764 and both circ-0003748 and circ-0001398 were shown to interact with miR-628; both these microRNAs targeted MAPK1. Furthermore, circ-0007312, circ-0003748, circ-0001398, and MAPK1 were up-regulated, whereas miR-764 and miR-628 were downregulated in PC9/ER cells as compared to parental PC9 cells. We also found that circ-0007312 and miR-764 were positively expressed in plasma. **Conclusions:** Our original study associated with mechanism of target therapy in lung cancer provided a systematic and comprehensive regulation of circular RNA, microRNA and messenger RNA in epidermal growth factor receptor-tyrosine kinase inhibitor resistance. It was found that circ-0007312- miR-764-MAPK1, circ-0003748-miR-628-MAPK1, and circ-0001398-miR-628-MAPK1 axis may play key roles in epidermal growth factor receptor-tyrosine kinase inhibitor resistance.

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1   Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, with adenocarcinoma as the most common histological subtype.[1] In recent years, epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib (first generation) and osimertinib (third generation), have been widely used for lung adenocarcinoma patients harboring activated EGFR mutations (exon 18, 19, 20, and 21).[2,3] Although these treatments have effectively prolonged the survival of advanced lung adenocarcinoma patients, acquired resistance is still inevitable.[4,5] Since further strategies are lacking for EGFR-TKI-resistant lung adenocarcinoma patients, studies on resistance mechanisms are required.

Recently, it was discovered that non-coding RNA could affect lung adenocarcinoma proliferation, metastasis and drug resistance.[6–9] In particularly, circular RNA(circRNA) is a newly identified non-coding RNA mainly formed by the splicing of the 5′ end of the upstream exon and the 3′ end of the downstream exon.[10] Moreover, many circRNAs have been identified as competitive endogenous RNA (ceRNA) network regulators interacting with microRNAs(miRNAs) and other non-coding RNAs to compete with messenger RNA (mRNA) in miRNA adsorption.[11–14] Therefore, we predicted that ceRNA networks connecting these non-coding RNAs may play vital roles in EGFR-TKI resistance.

In our previous study, we constructed a PC9/ER cell line with gefitinib and osimertinib resistance, discovering that SHISA3 can restrain EGFR-TKI resistance in PC9/ER.[15] Given these data, we aimed to construct a ceRNA network of circRNA-miRNA-mRNA that was related to EGFR-TKI resistance by screening differentially expressed circRNAs, miRNAs and mRNAs. Furthermore, we conducted Gene Oncology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, setting up a protein-protein interaction (PPI) network to evaluate all mRNAs in the network to identify the biological functions of these molecules.

2   Materials and Methods

2.1   Patient and Cell-Free DNA (cfDNA)

Peripheral blood samples were obtained from 23 lung adenocarcinoma patients at the Peking University Cancer Hospital (Beijing, China). cfDNA was then extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (QIAGEN).

This original study for the EGFR-TKI resistance in lung cancer was approved by the Peking University Cancer Hospital Ethics Committee, and written informed consent was obtained from all participants prior to sample collection.

2.2   Cell Lines and Cell Culture

PC/ER cells were cultivated from parental PC9 cells after 6 months of gefitinib exposure, from 10nM to 10μM, and control cells were disposed in parallel with vehicle dimethyl sulfoxide. After PC9/ER cells were certified to be gefitinib- and osimertinib-resistant, bothPC9 and PC/ER were maintained in our laboratory and cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA), which was supplemented with 10% fetal bovine serum (Gibco BRL), 100U/mL penicillin, and 100μg/ml streptomycin. The cells were then incubated in an incubator (37°C) with 5% CO_2.

2.3   RNA Microarray Analysis

Following sample culture, circRNAs, miRNA, and mRNA microarrays were performed for the PC9 and PC9/ER cells, and total extracted RNA was quantified using NanoDrop ND-100. For circRNA microarray analysis, total RNA was digested using RNase R (Epiceter, Inc., Madison, WI, USA) to eliminate linear RNAs and enrich circRNAs. Afterwards, the enriched circRNAs were amplified and transcribed into fluorescent cDNA using a random priming method (Arraystar Super RNA Labeling Kit, Arraystar). The labeled cDNAs were then hybridized onto the Arraystar Human circRNA Array V2 (8 × 15K, Arraystar), and after washing the slides, the arrays were scanned using an Agilent Scanner G2505C.

The Agilent Human miRNA Microarray (release 21.0 8*60K, design ID: 070156) was used for the experiment, in which sample labeling, microarray hybridization, and slide washing were performed following the manufacturer's protocols. Briefly, total RNA was dephosphorylated, denatured, and labeled with cyanine-3-CTP. After purification, the labeled RNAs were hybridized to the microarrays, and the resulting arrays were scanned using an Agilent Scanner G2505C (Agilent Technologies) after washing.

mRNAs were scanned using an Agilent Microarray Scanner (Cat#G2565CA, Agilent technologies, Santa Clara, CA, USA),

and data were extracted using the Feature Extraction software version 10.7 (Agilent technologies, Santa Clara, CA, USA). Further, the raw data were normalized using the quantile algorithm, limma packages in R.

Significant circRNA, miRNA and mRNA alterations between PC9 and PC9/ER cells were detected using limma packages and when fold change (FC)≥1.5, as the selected FC cutoff.

2.4 CeRNA Network Construction

The circRNA-miRNA interactions were predicted using the Arraystar's homemade miRNA target prediction software based on TargetScan (http://www.targetscan.org) and miRanda, and the R package miRNA tap was used to predict the target mRNAs. Five databases were included in the analysis, including PicTar, DIANA, TargetScan, miRanda and miRDB, wherein miRNA-mRNA interactions were reported consistently in at least two of the five databases.

The circRNA-miRNA-mRNA network was constructed using the differentially expressed circRNAs, their potential targeted miRNAs and the target mRNAs of those miRNAs. Cytoscape (v3.7.2) was used for network visualization, only including differentially expressed circRNA/miRNA/mRNA with log2 FC ≥1.5 in the analysis. More specifically, a table of RNA interactions, including the source and target RNAs, as well as log2 FC in different groups, was created as the input. Once imported into the software, the source and target RNAs were used to construct the interaction network, with varying color and node shapes indicating the different attributes of the RNA entities.

2.5 PPI Network Construction and hub-Genes Identification

The PPI network was built with the ceRNA network using the STRING program, version 11.0. Afterwards, the edge file was downloaded and imported into Cytoscape, and the hub genes were clustered using the Cytoscape MCODE App, setting 3 as the "degree cutoff".

2.6 Pathway Enrichment

DAVAD (https://david.ncifcrf.gov/) was used for pathway enrichment analysis of the differentially expressed genes. The enriched GO and KEGG terms (adjusted p-value<.05) were displayed by GOplot using GOCircle function.

2.7 RNA Immunoprecipitation (RIP) Assay

Following the instructions of the RIP kit (BersinBio), the RIP assay was performed for the PC9 and PC9/ER cells. Cell lysates were obtained in a lysis buffer and were incubated with argonaute 2 (AGO2) or IgG overnight at 4 °C after DNA removal. The immunoprecipitated RNA was then extracted using phenol-chloroform-isopentanol, which was followed by quantitative real-time PCR (qRT-PCR) testing to determine relative RNA quantitation.

2.8 qRT-PCR

Total RNA was extracted from PC9 or PC9/ER cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and cDNA was synthesized from total RNA (2μg) using a commercial kit (EasyScript First-Strand cDNA Synthesis SuperMix, Transgen Biotech, Beijing, China). Furthermore, qRT-PCR was performed using the Go Taq qPCR Master Mix (Promega Corp., Madison, Wisconsin, USA). The relative circRNA, miRNA, and mRNA expression levels were normalized to those of GAPDH or U6, and FCs were calculated using the 2-$\triangle\triangle$Ct method. The PCR primers used in this study were as follows: hsa_circ_0007312, 5′-TAACGGTGACTAATGGTGT

TAAAGG-3′ (forward) and 5′-GATGCATTTACAAG CAAATCTGTGC-3′(reverse); hsa_circ_0003748, 5′-GAGAA GGCAGCCAACCAGAT-3′ (forward) and 5′-TCTTCTTC TAACTTATGGCTGCATG-3′ (reverse); hsa_circ_0001398, 5′-TGAGCTGATCAAGTTGACAGCATC-3′ (forward) and 5′-CAACTCAATAAAC

TTCAAGTACCGA-3′ (reverse); hsa-miR-764, 5′-GCA GGTGCTCACTTGTCCTCC

T-3′ (forward) and 5′-GCGAGCACAGAATTAA TACGAC-3′ (reverse); hsa-miR-628 to 5p, 5′-ATGC TGACATATTTACTAGAGG-3′ (forward) and 5′-GCGA GCACA

GAATTAATACGAC-3′ (reverse); MAPK1, 5′- TGTT CCCAAATGCTGACT-3′ (forward) and 5′-AACTTGAAT GGTGCTTCG −3′ (reverse).

2.9 Statistical Analysis

All experiments were performed in triplicates, wherein data are presented as mean ± standard deviation. Two-group differences were determined using t-test, and statistical significance was set at P <.05. All figures were analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

3 Results

3.1 circRNAs, miRNAs and mRNAs in the PC9/ER Cells with EGFR-TKI Resistance

To identify the essential genes that can regulate EGFR-TKI resistance, circRNA, miRNA, and mRNA microarrays were performed in EGFR-TKI resistant PC9/ER cells in comparison to parental PC9 cells. In total, 603 circRNAs were differentially expressed between PC9/ER and PC9 (FC≥1.5), including 384 upregulated and 219 downregulated circRNAs (Figure 1A), 98 upregulated and 135 downregulated miRNAs (Figure 1B), and 831 upregulated and 1032 downregulated mRNAs (Figure 1C).
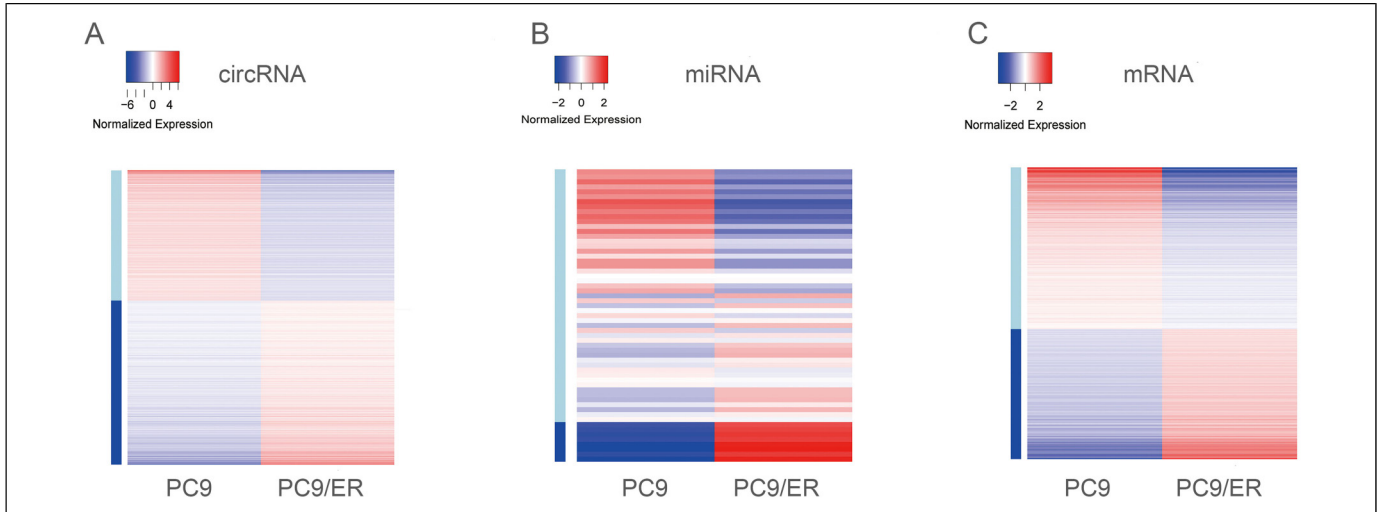
Figure 1. Heatmaps for differentially expressed circRNAs, miRNAs, and mRNAs between PC9 and PC9/ER cells. (A-C) Heatmaps for 603 differentially expressed circRNAs (A), with 384 upregulated and 219 downregulated (fold change [FC]≥1.5); differentially expressed miRNAs(B), with 178 upregulated and 199 downregulated (FC≥1.5); and 1863 differentially expressed mRNAs (C), with 831 upregulated and 1032 downregulated(FC≥1.5). All RNA microarrays were performed in duplicates.

3.2 Construction of ceRNA and PPI Networks Related to EGFR-TKI Resistance

In view of the interlaced regulation of coding and non-coding genes related to EGFR-TKI resistance, altered circRNA, miRNA, and mRNA in PC9/ER cells (log2FC≥1.5) were obtained to construct the ceRNA network. First, we predicted the interaction between circRNA and miRNA using a prediction software, including TargetScan and miRanda databases. Meanwhile, the interaction between miRNA and mRNA was predicted using the R package miRNAtap, which was based on five databases, including PicTar, DIANA, TargetScan, miRanda, and miRDB. Afterwards, a ceRNA circRNA-miRNA-mRNA network was generated using Cytoscape (version 3.7.2), including 18 circRNAs, 17 miRNAs, and 175 mRNAs (Figure 2A). The descriptions of these non-coding RNAs are illustrated in Supplemental Table 1.

The PPI network was then constructed using the mRNAs in ceRNA via STRING (version 11.0). After removing unconnected genes, the remaining 52 genes were used to establish the PPI network with a k-score of 1.5 as reference, wherein the results were visualized using Cytoscape (version 3.7.2) (Figure 2B). Exactly 10 groups of connections were observed, with MAPK1 and GNB4 located in the center of the main
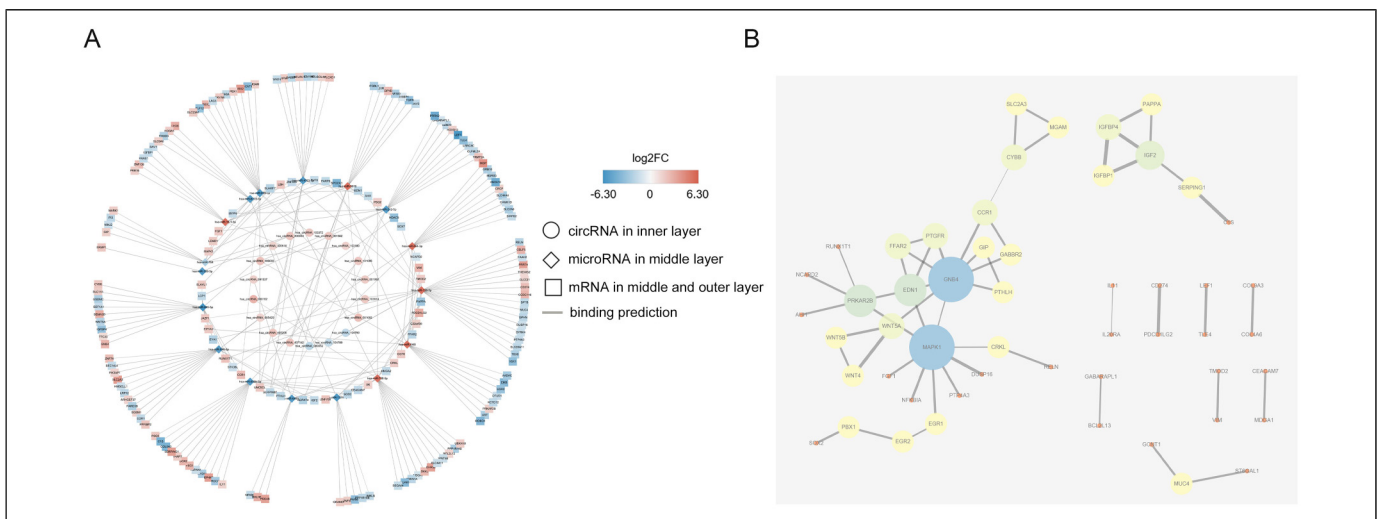

Figure 2. Constructed circRNA-miRNA-mRNA and protein-protein interaction networks. (A) The network consists of 18 circRNAs, 17 miRNAs, and 175 mRNAs. These circRNAs, miRNAs, and mRNAs are represented by circles, diamonds and squares, respectively, and their respective color is determined by fold changes. (B) Based on the differently expressed 175 mRNAs, 10 groups of interaction were found.
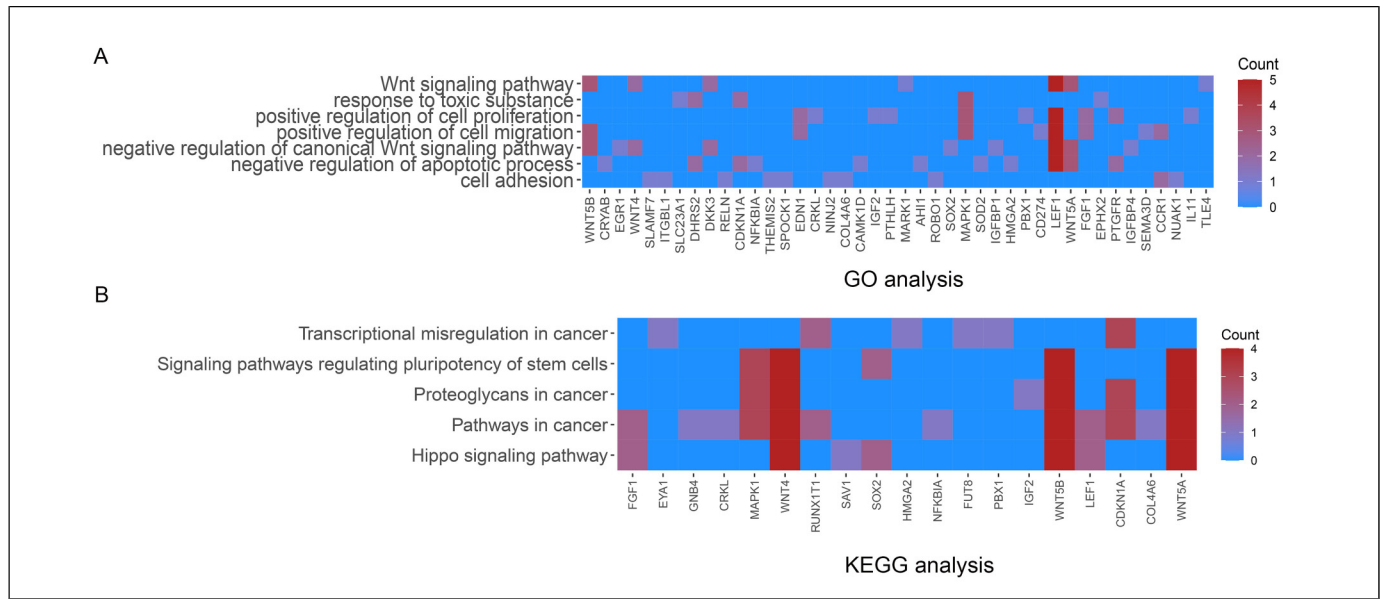
Figure 3. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomics (KEGG) analyses. (A) Heatmap for GO analysis of 175 mRNAs in the ceRNA network. (B) Heatmap for KEGG pathway analysis of 175 mRNAs in the circRNA-miRNA-mRNA regulatory network.

protein interaction, which was closely related to EGFR-TKI (Figure 2B). The complete list of proteins included in the PPI network can be found in Supplemental Table 2.

3.3 GO and KEGG Pathway Analyses of the ceRNA Network mRNAs

GO and KEGG pathway analyses were performed using the 175 mRNAs in the ceRNA network using DAVID functional analysis, of which only the enriched GO terms and KEGG pathways with P values <.05 were selected and ranked according to enrichment score. The most enriched GO terms were found to be associated with extracellular behavior, cell proliferation and apoptosis, cell adhesion and migration, Wnt signaling pathway, DNA binding regulation, and RNA polymerase II core promoter regulation, among others (Figure 3A, Supplemental Table 3). In contrast, the most enriched KEGG pathways were predominantly related to carcinogenetic pathways and proteoglycans, Hippo signaling pathway, transcriptional misregulation, and stem cell signaling pathways (Figure 3B, Supplemental Table 3). These results indicate that the enriched GO terms and KEGG pathways were associated with EGFR-TKI resistance in lung adenocarcinoma.

3.4 Validation of circRNA-miRNA-mRNA Between PC9 and PC9/ER Cells

Based on ceRNA and PPI network analyses, we focused on the upregulated circRNAs, followed by the targeted miRNAs and mRNAs in EGFR-TKI- resistant cells. Based on bioinformatic analysis, circ-0007312 was demonstrated to interact with miR-764, and both circ-0003748 and circ-0001398 were found

to interact with miR-628. Moreover, these two miRNAs were related to MAPK1 in the ceRNA network(Figure 4A). We then detected the circRNAs, miRNAs, and mRNAs in parental PC9 and PC9/ER cells using qRT-PCR, showing that circ-0007312, circ-0003748, circ-0001398, and MAPK1 were increased, whereas miR-764 and miR-628 were decreased in PC9/ER cells as compared to those in PC9 cells (Figure 4B, C, and D). To validate circRNA-miRNA-mRNA signaling, we performed an RIP assay to analyze whether miR-628 and miR-764 could be enriched using AGO2 immunoprecipitation. As shown in Figure 4E, miR-628 and miR-764 were enriched in AGO2 immunoprecipitation when compared to the IgG control, indicating that AGO2 protein can directly bind to miR-628 and miR-764 in PC9 cells. Consistent with higher miR-764 and miR-628 expressions in PC9 cells, both were immunoprecipitated using AGO2 RIP in PC9 cells as compared to those in PC9/ER cells (Figure 4F). Based on all these findings, it has been suggested that these circRNAs, miRNAs, and mRNAs are essential in the ceRNA network associated with EGFR-TKI resistance.

3.5 Validation of circRNAs and miRNAs in the Plasma of Lung Adenocarcinoma Patients

To investigate the potential prediction for disease progression of circRNA and miRNA, we collected plasma derived from advanced lung adenocarcinoma patients. The cfDNAs were then extracted from the plasma samples, and qRT-PCR was performed to detect circ-0007312, circ-0003748, circ-0001398, miR-628, and miR-764 expressions. We found that circ-0007312 and miR-764 were positively expressed in 14 and 20 patients, respectively; however, circ-0003748, circ-0001398, and miR-628 could not be detected in plasma (Figure 5A and B), indicating that only circ-0007312 and
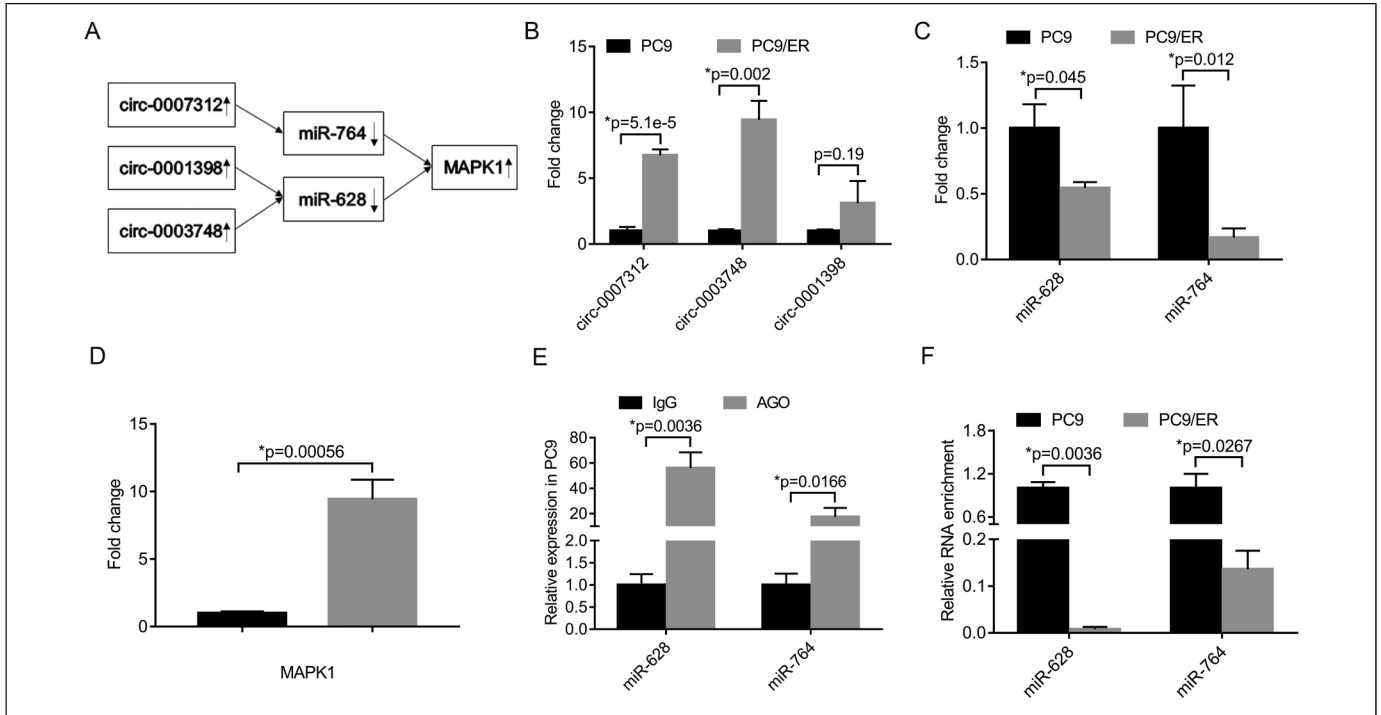
Figure 4. Validation of essential ceRNA network genes. (A) Interactions of several key circRNAs, miRNAs, and mRNAs. (B-D) circ-0007312, circ-0001396, circ-0003746 (B, n = 3), miR628, miR764 (C, n = 3), and MAPK1 (D, n = 3) are detected in PC9 and PC9/ER cells using qRT-PCR. (E) Immunoprecipitation of miR628 and miR764 in PC9 cells using RIP assay (n = 2). (F) Immunoprecipitation of miR628 and miR764 in PC9 cells and PC9/ER cells using RIP assay (n = 2). * mean: p <.05.
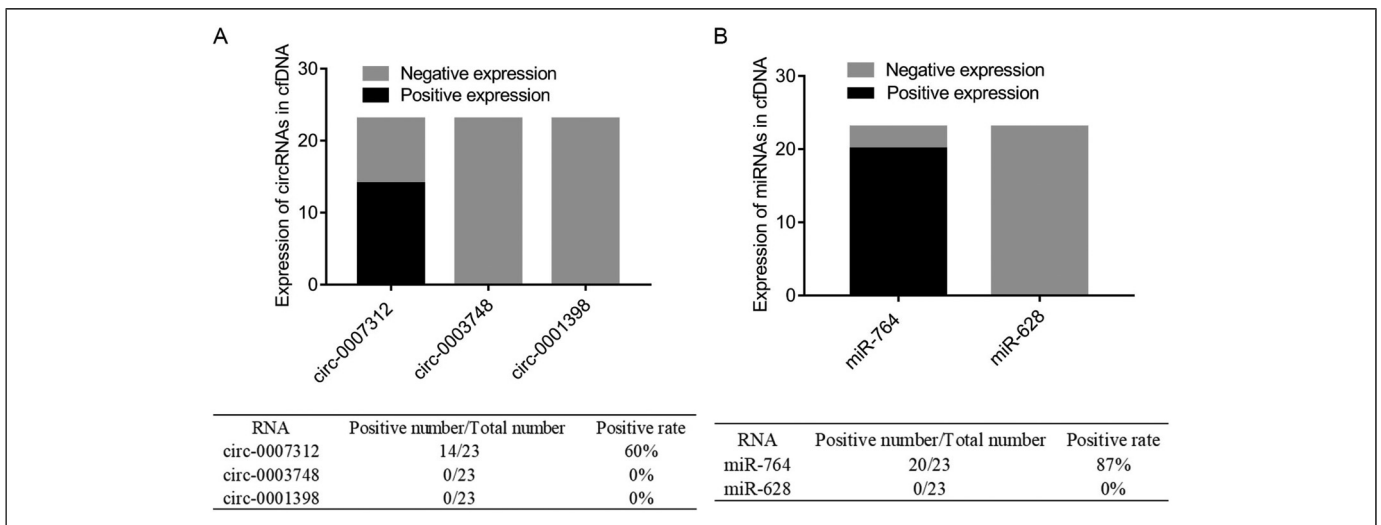

Figure 5. An illustration of the miRNA and circRNA expressions in cfDNAs. (A) The expressions of circ-0007312, circ-0003748, and circ-0001398 in cfDNAs using qRT-PCR. (B) The expressions of miR764 and miR628 in cfDNAs using qRT-PCR.

miR-764 have the potential to be novel disease progression biomarkers in lung adenocarcinoma patients.

4   Discussion

Although EGFR-positive lung adenocarcinomas can benefit from EGFR-TKI treatment, drug resistance inevitably develops.[16,17] At present, several mechanisms involved in EGFR-TKI resistance have been identified, including aberrant gene expression, EGFR-depend and -independent signaling pathway activation, and small cell lung cancer transformation.[4,16] Currently, we analyzed the role of the circRNA-miRNA-mRNA network in EGFR-TKI-resistant lung adenocarcinoma cells.

Recent studies have demonstrated that non-coding RNAs, including circRNAs and miRNAs, play vital roles in drug resistance.[9,18] In particular, a study indicated that hsa_circ_0004015 could promote gefitinib resistance in non-small cell lung cancer via the miR-1183/PDPK1 signaling pathways.[7] Another study proved that epigenetic miR-483 to 3p silencing promoted acquired gefitinib resistance and epithelial-mesenchymal transition in non-small cell lung cancer by targeting integrin β3.[19] In our study, we first obtained differential expression profiles of circRNAs, miRNAs and mRNAs between the EGFR-TKIs sensitive cell line PC9 and resistant cell line PC9/ER. As a result, a total of 603 differentially expressed circRNAs, 377 differentially expressed miRNAs, and 1863 differentially expressed mRNAs were found, indicating that many circRNAs and miRNAs were involved in EGFR-TKI resistance.

Given the complex interactions among circRNAs, miRNAs, and mRNAs,[9,20,21] it is meaningful to construct a comprehensive circRNA-miRNA-mRNA network to explore their impact on EGFR-TKI resistance. In order to clarify their mutual relationships and identify potential key molecules related to EGFR-TKI resistance, we constructed a ceRNA network based on the interactions between these differentially expressed non-coding RNAs, consisting of 18 circRNA, 17 miRNAs, and 175 mRNAs. Notably, the circRNAs involved in this network have not yet been reported in previous studies. Among these genes, circ-0007312, circ-0003748, and circ-0001398 were upregulated, interacting with downregulated miR-764 and miR-628 in the EGFR-TKI-resistant cells. In relation to the downregulated miRNAs, long non-coding RNA Mirt2 has been reported to inhibit apoptosis and alleviate myocardial infarction by regulating miR-764-PDK1.[22] Another study showed that miR-764 can downregulate the pro-survival factor Ninjurin 2 to protect neuronal cells death and hydrogen peroxide-induced apoptosis.[23] Furthermore, a study showed that glioma cells proliferation, migration and invasion were promoted, and apoptosis was suppressed by the long non-coding RNA AGAP2-AS1 through miR-628-PTN axis regulation.[24] All these studies have indicated that miR-764 and miR-628 are associated with apoptosis and are closely linked to drug resistance. Moreover, our study found that circ-0007312 and miR-764 can be detected in plasma, indicating their potential to be prognostic biomarkers.

Based on the GO analysis using the network mRNAs, many GO terms were associated with EGFR-TKI resistance, including the Wnt signaling pathway, positive regulation of cell migration, and negative regulation of apoptotic process. Meanwhile, most enriched KEGG pathways were also closely related to EGFR-TKI resistance, including the Hippo signaling pathway and signaling pathways regulating stem cells pluripotency. Lee et al. in particular, reported that Hippo pathway effector YAP inhibition could restore EGFR-TKI sensitivity in lung adenocarcinomas.[25] Another recent study also proved that autophagy could decrease osimertinib cytotoxicity by inducing stem cell-like properties in lung cancer.[26] Furthermore, we constructed a PPI network using the 175 network mRNAs, forming 10 groups of connections that were involved, with MAPK1 and GNB4 located in the center of the main connection. As previously mentioned, MAPK1 was induced in EGFR-TKI-resistant PC9/ER cells, and miR-764 and miR-628 have been proposed to inhibit MAPK1. This finding is consistent with that of previous studies reporting the involvement of MAPK-ERK pathway activation in EGFR-TKI resistance.[27,28]

In summary, this study provided a ceRNA network of circRNA-miRNA-mRNA based on microarray and bioinformatics analyses. Of these network non-coding RNAs, circ-0007312-miR-764-MAPK1, circ-0003748-miR-628-MAPK1, and circ-0001398- miR-628-MAPK1 in the network may play key roles in EGFR-TKI resistance.

Ethics Statement

This study was approved by the Peking University Cancer Hospital Ethics Committee (Beijing, China). The approval number is 2020KT51. And the date of approval to our manuscript is in 2020 on May 07. This original study for the EGFR-TKI resistance in lung cancer was approved by the Peking University Cancer Hospital Ethics Committee, and written informed consent was obtained from all participants prior to sample collection.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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