miR-423-3p activates FAK signaling pathway to drive EMT process and tumor growth in lung adenocarcinoma through targeting CYBRD1

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1 | INTRODUCTION

Lung cancer is a malignant tumor originated from the bronchial mucosa or glands of the lungs,1,2 which has characteristics of family clustering and genetic susceptibility.3,4 According to preventive pathology, lung cancer is classified into two categories: small cell carcinoma and non-small-cell carcinoma.5 Among them, non-small-cell carcinoma includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma.6 Lung adenocarcinoma (LUAD) is a type of lung cancer, accounting for 40%–55% in all lung cancer cases.
Symptomatically, LUAD is generally accompanied by coughing, wheezing, chest pain, hemoptysis, and brain metastasis. In addition, the occurrence of LUAD is highly related to smoking. Currently, drug therapy, molecular-targeted therapy, and surgical treatment are extensively utilized to alleviate LUAD. However, the high recurrence rate of LUAD is primarily responsible for the poor prognosis. Therefore, biomarkers with diagnostic and therapeutic significance are urgently required to predict and alleviate LUAD.

MicroRNA-423-3p (miR-423-3p) belongs to microRNAs, which are defined as small noncoding RNA molecules with single strand. Increasingly, studies have uncovered the functional role of microRNAs in various diseases, such as ischemia-reperfusion injury, human osteoarthritis, and psoriasis. In addition, the research reports about microRNAs have mainly focused on its regulation of multiple cancers, including osteosarcoma, melanoma, and colorectal cancer. As a member of microRNAs, miR-423-3p has been identified as a carcinogenic driver in various cancers. For example, Liu Z et al. revealed the promoted effect of miR-423-3p on cell proliferation and invasion in hepatic cancer, and Guo T et al. verified miR-423-3p as a promising biomarkers in the occurrence and progression of castration-resistant prostate cancer. Recently, miR-423-3p has been proved to participate in lung cancer progression, while the regulatory mechanisms through which miR-423-3p involved in LUAD remain uncovered.

Cytochrome B reductase 1 (CYBRD1) belongs to cytochrome b (561) family and is characterized with ferric reductase activity. Based on extensive references, CYBRD1 mainly contributed to the transportation of glucose and other sugars, metal ions, bile salts, and organic acids, as well as the absorption of mineral. CYBRD1 has been proved to be involved in the development of HFE hemochromatosis, chronic hepatitis C, and alcoholic liver disease. Additionally, Lemler DJ et al. demonstrated that DCYTB was a potential biomarker for breast cancer and exerted its functional effect through an iron-independent manner. However, the contribution of CYBRD1 to affecting LUAD has not been revealed.

Thus, we verified the expression levels of miR-423-3p and CYBRD1 and uncovered their regulatory mechanism. Moreover, we investigated the functional influence of miR-423-3p/CYBRD1 axis upon LUAD progression, providing a novel therapeutic candidate for the treatment of LUAD.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

Human lung adenocarcinoma cell lines NCI-H1299 and NCI-H157, as well as human bronchial epithelial cell line 16HBE, were obtained from ATCC (American Type Culture Collection) and were cultured in indicated medium in accordance with ATCC's instruction. For transfection, the miR-423-3p mimics, miR-423-3p inhibitor, miR-423-3p antagonir, pcDNA-CYBRD1, si-CYBRD1-1, and si-CYBRD1-2 were designed by GeneChem Corporation (Table S1).

2.2 | Cell counting kit-8 (CCK-8) and adhesion assay

Cell counting kit-8 was employed in the proliferation assay, NCI-H1299 and NCI-H157 cells were plated into 96-well incubation plates and maintained for 0, 24, 48, or 72 h, and then, the incubation plates were added with CCK-8 solution before 2 h of incubation. A microplate reader was implemented for the measurement of absorption of OD value at 450 nm.

For the estimation of cell adhesive capacity, type I collagen (BD Biosciences) was coated in 6-well plates, and then, the treated plates were utilized to culture NCI-H1299 and NCI-H157 cells for 1h. Then, MTT (Sigma-Aldrich) was supplemented after removing non-adherent cells. At 570 nm, the OD value was measured.

2.3 | Transwell

Transwell chambers were utilized for the estimation of NCI-H1299 and NCI-H157 cell invasive capacity. Briefly, the upper chamber was performed for the incubation of these two cell lines, while the lower chamber was supplemented with 10% FBS. Then, cells locating in the upper chamber were performed scrap, and meanwhile, the stain was achieved in the invaded cells derived from the lower chamber utilizing 0.1% crystal violet. An optical microscope (Olympus) was employed to finish the visualization.

2.4 | Wound healing

To estimate the migratory capacity, NCI-H1299 and NCI-H157 transfected with indicated plasmids were maintained in 6-well plates for 1 day, followed by finishing the wounds utilizing a pipette tip. After another incubation for 1 day, a microscope (Olympus) was implemented to achieve the collection of images.

2.5 | Luciferase reporter assay

For the estimation of the luciferase activity, the wild-type CYBRD1 (CYBRD1-WT) and mutant-type CYBRD1 (CYBRD1-MUT) were, respectively, cloned into a pGL3 luciferase reporter vector (Promega Corporation) containing the Renilla luciferase gene. miR-423-3p mimics or NC mimics were co-transfected with CYBRD1-MUT or CYBRD1-WT luciferase reporter plasmid into NCI-H1299 cells, and the luciferase activity of CYBRD1 was measured utilizing Dual-Luciferase Reporter Assay (Promega).

2.6 | RNA immunoprecipitation (RIP)

To determine the relationship between miR-423-3p and CYBRD1, Magna RIP™ RNA Binding Protein Immunoprecipitation Kit
(Millipore) was introduced in RIP assay. In brief, RIP buffer was utilized for the lysis of NCI-H1299 cells, and the cell extract was partly used as an input and partly incubated with anti-Ago2 or anti-IgG-coated beads (Catalog No. 03–110; Millipore) 4°C for 6 h. After the cultivation of specimens with proteinase K buffer, immunoprecipitated RNA was isolated and then was purified. Finally, the CYBRD1 enrichment was measured by qRT-PCR.

### 2.7 | qRT-PCR

To determine the expression levels of miR-423-3p and CYBRD1 in LUAD, the total RNA was isolated adopting TRIzol reagent (Invitrogen), and reverse transcriptase kit (Invitrogen) was introduced for the cDNA synthesis of extracted samples. SYBR Green qPCR Super Mix-UDG (Invitrogen) was employed for qRT-PCR analysis. GAPDH and U6 were considered as internal control. The corresponding primer sequences are elaborated in Table S2.

### 2.8 | Western blotting (WB)

To detect the proteins levels, isolation of extracted proteins was performed utilizing SDS-PAGE, and then, the separated samples were transferred to PVDF membranes incubated in 5% skim milk. Next, these membranes were mixed with primary antibodies at 4°C overnight, mainly containing E-cadherin (ab76055, 1:200), N-cadherin (ab76011, 1:5000), vimentin (ab92547, 1:2000), CYBRD1 (ab66048, 1 ug/mL), p-FAK (ab81298, 1:1000), t-FAK (ab40794, 1:2000), p-Paxillin (ab109547, 1:5000), and t-Paxillin (ab32084, 1:3000) obtained from Abcam (UK) and then were cultured with corresponding secondary antibody conjugated by horseradish peroxidase. Finally, the measurement was conducted employing chemiluminescence system (Bio-Rad).

### 2.9 | RNA pull-down

To verify the correlation between miR-423-3p and CYBRD1, NCI-H1299 cell lysate was mixed with biotin-labeled miR-423-3p, Dynabeads™ M-280 Streptavidin (Invitrogen; Thermo Fisher Scientific) and streptavidin-coated magnetic beads (88817, Invitrogen) at 4°C overnight in RNA pull-down experiments. Then TRIzol® reagent was performed to finish the extraction of RNA fragments bound with beads. The CYBRD1 expression levels were quantified adopting qPCR.

### 2.10 | Xenograft model and IHC staining

To investigate the influence of miR-423-3p on LUAD tumor growth, BALB/c nude mice (5 weeks, 17–21 g) derived from Shanxi Provincial People’s Hospital were achieved injection with miR-423-3p-depleted NCI-H157 cells into the subcutaneous, thereby establishing xenografts tumor models. Then, nude mice were sacrificed mercilessly to collect the xenograft tumor tissues after 4 weeks of injection, and the weight and volume (volume (mm3) = (L×W2)/2 (L: length, W: width)) were measured. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was approved by Shanxi Provincial People’s Hospital.

Xenograft tumor tissues collected from mice were utilized to evaluate the expression of PCNA and CYBRD1 adopting IHC staining. In brief, citrate buffer was performed to achieve the antigen retrieval of tumor tissues, and permeabilization was finished using 0.1% Triton X-100, followed by mixing with anti-PCNA (ab29, 1:20000) and anti-CYBRD1 (ab28758, 2.5 ug/mL) at 4°C overnight. Then, hematoxylin counterstain was performed after the culture with secondary antibody. The images were captured adopting Eclipse 80i (Nikon).

### 2.11 | Informatic analysis

GSE135918 and GSE27486 datasets from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The expression of target miRNAs in LUAD tumor tissues and normal tissue, and clinicopathological parameters (smoker, age, pathologic stage) were downloaded from The Cancer Genome Atlas (TCGA-LUAD) datasets (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). The survival and receiver operating characteristic (ROC) curves were evaluated utilizing miRNA expression levels. The mRNA and protein levels of CYBRD1 and overall survival in LUAD were analyzed through GEPIA2 (http://gepia.cancer-pku.cn/). UALCAN database (http://ualcan.path.uab.edu/index.html) was used to evaluate the tumor grade. Gene Set Enrichment Analysis (GSEA) was utilized to investigate the CYBRD1-related signaling pathway.

### 2.12 | Statistical analysis

In this work, all data were showed as mean ±SD and were calculated utilizing GraphPad Prism and SPSS. For the difference analysis of groups, Student’s t-test and one-way analysis of variance (ANOVA) were introduced. The overall survival was analyzed utilizing Kaplan–Meier strategy. p < 0.05 presents statistical significance.

### 3 | RESULTS

#### 3.1 | Association between miR-423-3p expression and clinicopathological parameters in LUAD patients

To screen the upregulated miRNAs in LUAD patients, we first utilized two cancer datasets GSE135918 and GSE27486 and obtained 7 intersected genes, including hsa-miR-513a-5p, hsa-miR-513b,
hsa-miR-423-3p, hsa-miR-519d, hsa-miR-483-5p, hsa-miR-1298, and hsa-miR-512-5p (Figure 1A). Among them, we noticed that miR-423-3p was obviously upregulated in LUAD tissues according to TCGA-LUAD database analyses (Figure 1B). In addition, we found that the expression level of miR-423-3p was higher in smoked LUAD patients aged over 40 than that in aged <40 patients (Figure 1C). Moreover, survival curve exhibited that the high expression of miR-423-3p was closely associated with poor survival in non-smoked, aged over 65, and pathological stage 1 LUAD patients (Figure 1D). Interestingly, the ROC curves indicated that miR-423-3p had high clinical diagnostic value for LUAD patients with AUC =0.826. More specifically, the diagnostic efficiency of miR-423-3p reached a significantly high levels in pathological stage 1, aged over 65, and non-smoked LUAD patients, respectively, with AUC =0.825, AUC =0.820, and AUC =0.819 (Figure 1E). Collectively, we suggested that miR-423-3p was highly expressed in LUAD and closely correlated with age, smoking, and pathological stage of LUAD patients.

3.2 miR-423-3p drives LUAD cell proliferation, invasion, adhesion, and EMT

Considering the upregulation of miR-423-3p in LUAD based on informatic analysis, we further examined the expression levels of miR-423-3p in LUAD cell lines (NCI-H1299 and NCI-H157) and human bronchial epithelial cell line 16HBE, and confirmed that miR-423-3p expression was higher in LUAD cell lines than in 16HBE cell (Figure 2A). To further uncover the functional influence of miR-423-3p on LUAD progression, we constructed miR-423-3p-upregulated NCI-H1299 cells and miR-423-3p-depleted NCI-H157 cells (Figure 2B). CCK-8 assay exhibited an accelerated proliferation rate after miR-423-3p overexpression, while miR-423-3p depletion caused markedly inhibited effect upon it (Figure 2C). Similarly, the migratory and invasive capacities of LUAD cells were considerably enhanced by miR-423-3p overexpression and depleted miR-423-3p restrained it (Figure 2D,E). Moreover, upregulated miR-423-3p resulted in the upregulation of N-cadherin and vimentin and the
downregulation of E-cadherin, and meanwhile inhibited miR-423-3p exerted reverse impact, concluding that miR-423-3p facilitated the EMT process in LUAD (Figure 2F). In addition, NCI-H1299 cell adhesive ability was promoted by overexpressing miR-423-3p and was obviously hindered by miR-423-3p depletion (Figure 2G). Overall, it was inferred that miR-423-3p played a carcinogenic role in LUAD.

3.3 | miR-423-3p targets CYBRD1 in LUAD

To reveal the targeting genes downstream miR-423-3p, we utilized TarBase and GEPIA2 databases to predict the differently downregulated intersection genes and finally screened 42 intersected genes (Figure 3A). We found that CYBRD1 was negatively correlated with
miR-423-3p (Figure 3B). TCGA database further displayed that the mRNA and protein levels of CYBRD1 were markedly upregulated in LUAD tissues (Figure 3C), which could be validated by qPCR assay in LUAD cell lines (Figure 3D).

Subsequently, we found that the mRNA and protein levels of CYBRD1 were significantly attenuated by miR-423-3p overexpression, but were enhanced by miR-423-3p depletion (Figure 3E). Meanwhile, in CYBRD1-WT plasmid transfected NCI-H1299 cells, the luciferase activity of CYBRD1 was efficiently suppressed, while the cells transfected with CYBRD1-MUT had not be impacted (Figure 3F). Furthermore, RIP assay demonstrated that upregulated miR-423-3p could remarkably enrich CYBRD1 in anti-AGO2 complexes (Figure 3G), and the RNA fragments of CYBRD1 could be obviously bound by biotin-labeled miR-423-3p according to RNA pull-down results (Figure 3H). Overall, these data indicated that miR-423-3p could competitively bind to the 3′UTR of CYBRD1 to inhibit its expression in LUAD.

3.4 | Confirms the pathological relevance of CYBRD1 in LUAD- and CYBRD1-related signaling pathways

To further investigate the correlation between CYBRD1 expression and clinicopathological parameters in LUAD, we searched TCGA database and observed that CYBRD1 was significantly downregulated in LUAD smokers aged over 40, and the expression level of CYBRD1 was higher in non-smoked LUAD than smoked ones (Figure 4A). Meantime, with the rise of LUAD tumor stage and grade, the protein levels of CYBRD1 reduced accordingly, indicating that the CYBRD1 expression was negatively correlated with the stage of LUAD tumor (Figure 4A). Additionally, we noticed that the low expression of CYBRD1 indicated poor overall survival and disease-specific survival, and CYBRD1 had a high clinical diagnostic value for LUAD with AUC = 0.886 (Figure 4B), implying that CYBRD1 may act as an indispensible role in LUAD occurrence and progression.

To find out the potential CYBRD1-associated signaling pathway, we first introduced GSEA database and identified 5 primary terms, which were mainly enriched in the negative regulation of epithelial cell apoptotic process, epithelial tube branching in lung morphogenesis, the negative regulation of epithelial cell proliferation, cell cycle, and focal adhesion (Figure 4C). To reveal the CYBRD1-mediated specific signaling pathways, we constructed CYBRD1-upregulated NCI-H157 cells and CYBRD1-silenced NCI-H1299 cells. CYBRD1 silence obviously activated the expression of FAK signaling pathway in NCI-H1299 cells, while CYBRD1 overexpression restrained it (Figure 4D). In summary, CYBRD1 expression was negatively associated with clinicopathological parameters of LUAD and may impact LUAD development through FAK signaling pathway.

FIGURE 3 | MicroRNA-423-3p inhibits CYBRD1 in LUAD. (A) TarBase and GEPIA2 databases to predict the targeting genes downstream miR-423-3p in LUAD. (B) The correlation analysis between miR-423-3p and CYBRD1 expression. TCGA database (C) and qPCR (D) were adopted to determine the expression of CYBRD1 in LUAD. (E) Western blotting and qPCR were used to measure CYBRD1 expression. (F) Dual-luciferase reporter assay was performed to detect the luciferase activity in NCI-H1299 cells. RIP (G) and RNA pull-down (H) assays were introduced for the validation of correlation between CYBRD1 and miR-423-3p. *p < 0.5, **p < 0.01, ***p < 0.001. Data represent at least three independent sets of experiment.
3.5 | miR-423-3p accelerates LUAD cell proliferation, invasion, adhesion, and EMT through targeting CYBRD1

To verify whether miR-423-3p plays a carcinogenic role in LUAD through mediating CYBRD1, we co-transfected miR-423-3p and CYBRD1 overexpression plasmids into NCI-H157 cells. CCK-8 results clarified that CYBRD1 overexpression suppressed NCI-H157 cell proliferation, which could be rescued by upregulated miR-423-3p (Figure 5A). Consistently, wound-healing and Transwell assays also revealed the crucial role of miR-423-3p in recovering the impairment of migration and invasion induced by CYBRD1 (Figure 5B,C). In addition, overexpressing CYBRD1 efficiently hindered the EMT process, FAK/Paxillin pathways, and adhesive viability, while upregulated...
miR-423-3p rescued it (Figure 5D,E). Our findings validated that miR-423-3p could facilitate LUAD cell progression and activate the FAK signaling pathway via inhibiting CYBRD1.

### 3.6 miR-423-3p depletion hinders LUAD tumor growth in vivo

To further confirm whether miR-423-3p impacts LUAD tumor growth in vivo, we established xenograft nude model through injecting miR-423-3p-silenced NCI-H157 cells. We noticed that miR-423-3p silence resulted in a distinctive reduction in LUAD tumor size (Figure 6A,B) and weight (Figure 6C). In addition, depleted miR-423-3p caused the downregulation of miR-423-3p and the upregulation of CYBRD1 in xenograft tumor tissues (Figure 6D). IHC assay displayed that tumor growth-related factor PCNA was evidently restrained in xenograft tumor formed by miR-423-3p-silenced cells, while CYBRD1 was upregulated (Figure 6E). Taken together, we validated that miR-423-3p depletion could exert repressive effect on LUAD tumor growth in vivo.

### 4 DISCUSSION

Lung adenocarcinoma is a malignant tumor originating from bronchial mucosal epithelium and mucous glands, with poor prognosis and high recurrence rate. Up to date, enormous biomarkers have been revealed to be capable of impacting the occurrence and progression of LUAD. Among them, microRNAs have been regarded as indispensable mediators in LUAD. For example, Yu F and colleagues demonstrated that exosomal miR-31-5p derived from hypoxic tumor targeted SATB2 and activated MEK/ERK signaling to facilitate tumor metastasis in LUAD, and Zhan J et al. demonstrated that miR-3130-5p could drive the growth, metastasis, and EMT progress in LUAD through directly mediating NDUFS1.

In addition, Liu H et al. revealed the carcinogenic role of miR-301b-3p in inducing LUAD cell proliferation, migration, and invasion via inhibiting Dlk1. Consistent with previous research, we found that miR-423-3p was highly expressed in LUAD and the high expression miR-423-3p predicted poor survival probability in non-smoked, aged over 65, and pathological stage 1 LUAD patients. More importantly, we also determined miR-423-3p as a carcinogenic driver due to its promoted effect on LUAD cell proliferation, migration, invasion, and adhesion, and in vivo, we demonstrated that miR-423-3p silence could efficiently restrain xenograft tumor growth. EMT has been identified as the transformation of epithelial-to-mesenchymal cells, which impart the capacity of cells to migrate and invade. Herein, we found that miR-423-3p caused the upregulation of EMT-related proteins (N-cadherin and vimentin), thereby accelerating the EMT progress, which further explained the reason why miR-423-3p promotes LUAD cell proliferation and migration.

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**Figure 5**  MicroRNA-423-3p facilitates LUAD cell proliferation, invasion, adhesion, and EMT via inhibiting CYBRD1. NCI-H157 cells were co-transfected with miR-423-3p and CYBRD1 overexpression plasmids. (A) CCK-8 was performed to detect the proliferation rate of NCI-H157 cells. Wound-healing (B) and Transwell (C) assays were, respectively, introduced to estimate NCI-H157 cell migration and invasion. Western blotting (D) and adhesion (E) assays were respectively employed to examine EMT-related protein expression and adhesive rate. *p < 0.5, **p < 0.01, ***p < 0.001. Data represent at least three independent sets of experiment.
According to previous research, CYBRD1 has been proved mainly involved in the regulation of the transportation of metal ions; in addition, Velázquez-Fernández D et al. revealed that CYBRD1 was downregulated in adrenocortical neoplasms. In our work, we found that CYBRD1 was obviously downregulated in LUAD tissues based on TCGA-LUAD database. Combining with bioinformatic analysis and cell experiments, CYBRD1 expression was negatively correlated with miR-423-3p, and miR-423-3p could competitively bind to the 3'-UTR of CYBRD1 to repress its expression. Moreover, the low expression of CYBRD1 predicted poor overall survival and disease-specific survival in LUAD, and CYBRD1 depletion caused the activation of FAK signaling pathway. FAK/Paxillin pathway has been demonstrated to be responsible for cell adhesion, and as a confirmation, we found that silencing CYBRD1 could restrain LUAD cell adhesive capacity. Furthermore, we verified that CYBRD1 overexpression could impair the LUAD cell proliferation, invasion, adhesion, and EMT process, which could be rescued by miR-423-3p overexpression, determining that miR-423-3p could drive LUAD progression via CYBRD1.

To summarize, we highlighted the upregulation of miR-423-3p and the downregulation of CYBRD1 in LUAD and determined CYBRD1 as the target gene of miR-423-3p. Functionally, miR-423-3p facilitated LUAD cell proliferation, invasion, adhesion, and EMT progress via binding to CYBRD1. These findings verified miR-423-3p/CYBRD1 as the promising and actionably biomarkers for diagnosing and alleviating LUAD.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT
My manuscript has no associated data.

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SUPPORTING INFORMATION

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