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miR-134-5p Promotes Stage I Lung Adenocarcinoma Metastasis and Chemoresistance by Targeting DAB2

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Despite surgery and adjuvant therapy, early-stage lung adenocarcinoma (LUAD) treatment often fails due to local or metastatic recurrence. However, the mechanism is largely unknown. Here, we report that increased expression levels of miR-134-5p and decreased levels of disabled-2 (DAB2) were significantly correlated with recurrence in stage I LUAD patients. Our data show that miR-134-5p overexpression or DAB2 silencing strongly stimulated LUAD cell metastasis and chemoresistance. In contrast, inhibition of miR-134-5p or overexpression of DAB2 strongly suppressed LUAD cell metastasis and overcame the insensitivity of chemoresistant LUAD cells to chemotherapy. In addition, we demonstrated that DAB2 is a target of miR-134-5p and that miR-134-5p stimulates chemoresistance and metastasis through DAB2 in LUAD. Taken together, these findings suggest that miR-134-5p and its target gene DAB2 have potential as a biomarker for predicting recurrence in stage I LUAD patients. Additionally, miR-134-5p inhibition or DAB2 restoration may be a novel strategy for inhibiting LUAD metastasis and overcoming LUAD cell resistance to chemotherapy.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide and causes one-quarter of all cancer deaths.[1] Non-small-cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases.[2] Recently, with the development of molecular targeted therapy and immunotherapy, the prognosis of advanced NSCLC has improved considerably.[3,4] In contrast, only a limited breakthrough has been achieved in the management of early-stage NSCLC. Patients with early-stage NSCLC mainly undergo surgery with or without adjuvant therapy; however, approximately 30%–40% of stage I NSCLC patients ultimately die of postoperative local or metastatic recurrence.[5] Lung adenocarcinoma (LUAD) is the major pathological subtype of NSCLC. Unfortunately, similar to other subtypes of NSCLC, there are no biomarkers that can predict recurrence and no specific treatment methods or drugs that can effectively prevent or inhibit the recurrence of stage I LUAD. Additionally, the mechanism of stage I LUAD recurrence remains largely unknown.

MicroRNAs (miRNAs) are small noncoding RNAs that play important roles in many normal biological processes, including cell proliferation, differentiation, and apoptosis.[6] miRNAs function through direct binding to the 3′ UTR of a target gene mRNA, which induces target gene mRNA cleavage or translational repression.[7,8] Accumulated evidence shows that deregulation of miRNAs has been implicated in many cancers, including LUAD, and dysregulated miRNA is an important factor leading to tumorigenesis and progression.[9–11] Importantly, studies have shown that even one aberrantly expressed miRNA is sufficient to cause tumor initiation and progression and that restoration or inhibition of that aberrantly regulated miRNA can dramatically suppress cancer progression, indicating that miRNAs have the potential to be an effective therapeutic target.[12,13] Thus, investigating the role of each miRNA that is dysregulated in cancer is very important for understanding cancer development, progression, and therapy.[12] Additionally, certain miRNAs have been indicated to be diagnostic and prognostic biomarkers in cancer clinical studies.[13,14]

In this study, we used our sample cohort and The Cancer Genome Atlas (TCGA) dataset to show that increased expression levels of miR-134-5p and decreased expression levels of disabled-2 (DAB2) are closely correlated with recurrence in patients with stage I LUAD. Additionally, we demonstrated that overexpression of miR-134-5p or silencing of DAB2 stimulates LUAD cell metastasis and chemoresistance, whereas inhibition of miR-134-5p or overexpression of DAB2 suppresses LUAD cell metastasis and chemoresistance. Furthermore, we demonstrated that miR-134-5p exerts its oncogenic role by directly targeting DAB2 in LUAD. Our findings suggest that the level of miR-134-5p and DAB2 has potential as a biomarker for predicting recurrence in patients with stage I LUAD and that inhibition of miR-134-5p or restoration of DAB2 expression may be a novel strategy for inhibiting early-stage LUAD progression.

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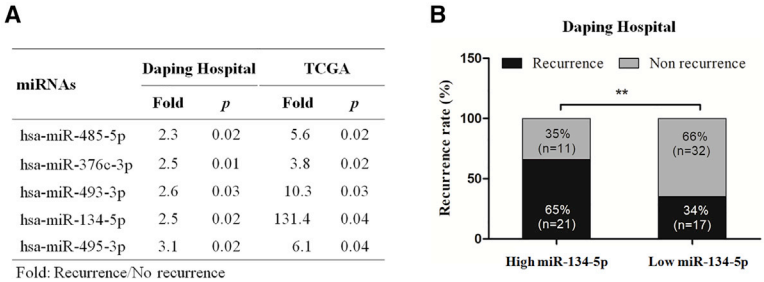
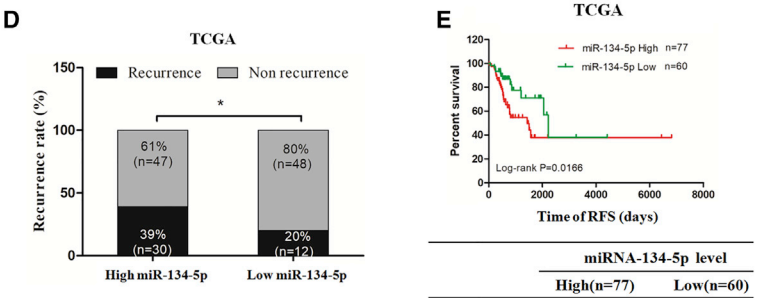
miRNAs	Daping Hospital		TCGA	
	Fold	*p*	Fold	*p*
hsa-miR-485-5p	2.3	0.02	5.6	0.02
hsa-miR-376c-3p	2.5	0.01	3.8	0.02
hsa-miR-493-3p	2.6	0.03	10.3	0.03
hsa-miR-134-5p	2.5	0.02	131.4	0.04
hsa-miR-495-3p	3.1	0.02	6.1	0.04

Fold: Recurrence/No recurrence



	miRNA-134-5p level	
	High(n=32)	Low(n=49)
Median time of RFS(months)	37.5	undefined

RFS: Recurrence-free survival



	miRNA-134-5p level	
	High(n=77)	Low(n=60)
Median time of RFS (days)	1433	2218

RFS: Recurrence-free survival

Figure 1. miR-134-5p Was Associated with Early Recurrence in Stage I LUAD Patients

(A) miRNAs that significantly increased in primary tumors from stage I LUAD patients who had relapsed within 40 months relative to that in those who had not relapsed within 40 months. (B) The miR-134-5p expression level was significantly associated with recurrence in stage I LUAD patients from the Daping Hospital cohort. (C) The miR-134-5p expression level was negatively correlated with the recurrence-free survival (RFS) rate and median time of RFS in stage I LUAD patients from the Daping Hospital cohort. (D) The miR-134-5p expression level was significantly associated with recurrence in stage I LUAD patients from the TCGA cohort. (E) The miR-134-5p expression level was negatively correlated with the RFS rate and median time of RFS in stage I LUAD patients from the TCGA cohort. *$p < 0.05$; **$p < 0.01$.

RESULTS

High Expression Levels of miR-134-5p Are Closely Correlated with Early Recurrence in Stage I LUAD Patients

To screen miRNAs involved in early recurrence of stage I LUAD, we performed miRNA array analysis on specimens from stage I LUAD patients who had relapsed within 40 months after resection (n = 8) and who had not relapsed within 40 months after resection (n = 11)[5] because 80% of stage I LUAD recurrences occur within 40 months after surgery.[15] Through miRNA array analysis, we identified five miRNAs whose expression increased more than 2-fold in the early recurrence groups relative to that in the nonrecurrence groups (Figure 1A). Furthermore, this result was confirmed using a stage I LUAD dataset from TCGA. TCGA data analysis showed that the expression levels of the five miRNAs identified by array analysis were significantly increased in the group of stage I LUAD patients who relapsed within 40 months relative to those in the nonrelapse groups and that miR-134-5p was the miRNA with the greatest increase in the recurrence group (Figure 1A), suggesting that miR-134-5p may be associated with early recurrence of stage I LUAD. Thus, we further investigated the correlation between the miR-134-5p levels and recurrence in patients with stage I LUAD. Our data show that the stage I LUAD patient group with high miR-134-5p expression had a higher recurrence rate (Figure 1B) and a shorter median recurrence-free survival time than did the miR-134-5p-low group (Figure 1C). Such clinical results were confirmed using the TCGA dataset, and similar results were observed with our clinical data (Figures 1D and 1E). Taken together, these findings indicate that increased expression levels of miR-134-5p are closely associated with early recurrence of stage I LUAD.

miR-134-5p Significantly Stimulates LUAD Cell Metastasis

Metastasis and chemoresistance are the most common causes of cancer recurrence.[16] Thus, we investigated the effects of miR-134-5p on LUAD cell metastasis. To investigate whether miR-134-5p is directly involved in the regulation of LUAD cell metastasis, the LUAD cell lines A549 and H1299 were transfected with miR-134-5p mimics or a miR-134-5p inhibitor (Figure 2A) and were then subjected to western blot and Transwell assays. Our results showed that overexpression of miR-134-5p significantly inhibited epithelial-to-mesenchymal tranzition (EMT) marker protein E-cadherin (EMT negative regulator) (Figure 2B) expression and stimulated both the migration and invasion of H1299 and A549 cells (Figure 2C). In contrast, inhibition of miR-134-5p strongly stimulated E-cadherin expression (Figure 2B) and inhibited migration and invasion in both the H1299 and A549 cell lines (Figure 2C). Furthermore, to confirm the *in vitro*

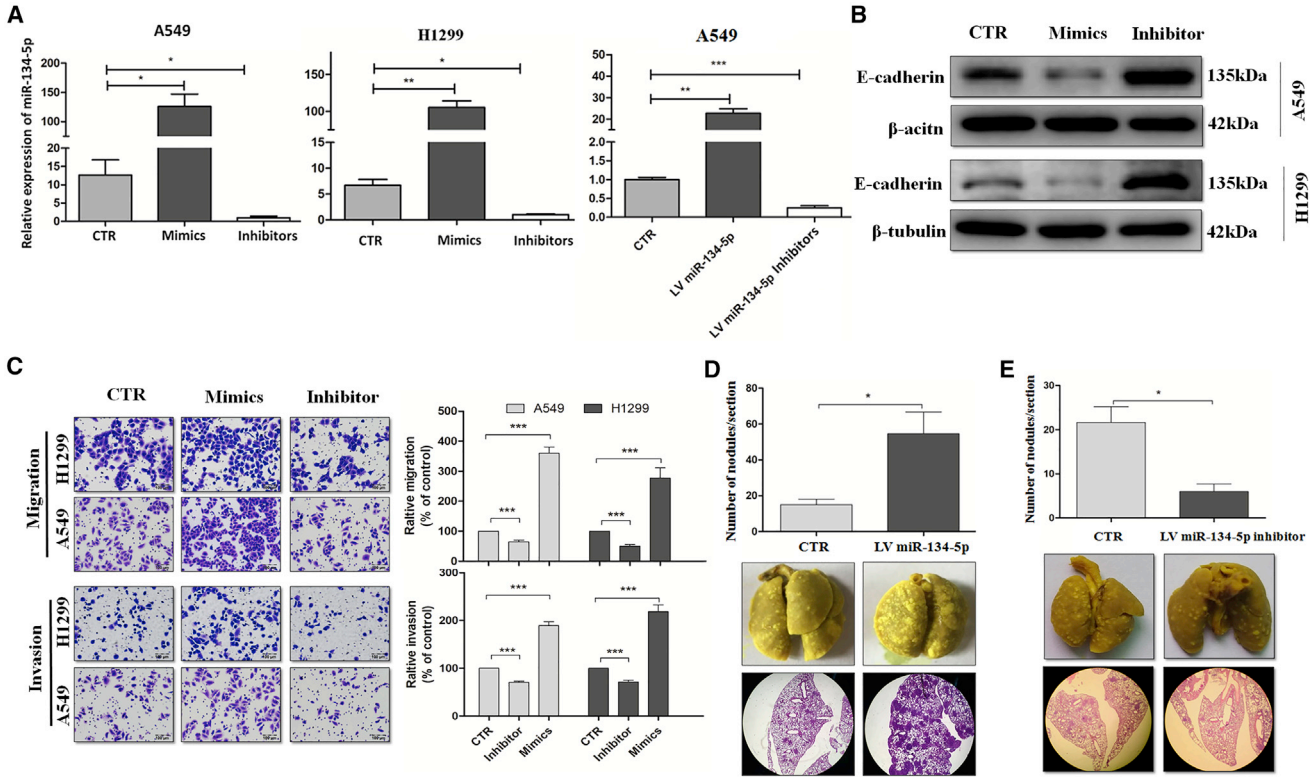


Figure 2. miR-134-5p Positively Regulated LUAD Cell Metastasis

(A) The miR-134-5p expression level was measured using qRT-PCR in the indicated cells. The indicated cells were transfected with the indicated oligonucleotides or infected with the indicated lentivirus. After 72 h, cells were subjected to qRT-PCR analysis. (B) miR-134-5p negatively regulated E-cadherin expression in LUAD cells. The indicated cells were transfected with miR-134-5p mimics or inhibitors. After 72 h of transfection, cells were subjected to western blot analysis. (C) miR-134-5p positively regulated LUAD cell invasion and migration. The indicated LUAD cells were transfected with negative control oligonucleotides, miR-134-5p mimics, or the miR-134-5p inhibitor. After 24 h of transfection, cells were subjected to invasion and migration assays. (D) Overexpression of miR-134-5p stimulated LUAD cell metastasis *in vivo*. A549 cells were infected with lentivirus expressing miR-134-5p or empty vector and injected into the tail vein of 6-week-old nude mice (n = 5 per group). The mice were sacrificed 3 weeks after cell injection, and nodules on the lung surface and in tissue sections were counted under a microscope. (E) Inhibition of miR-134-5p strongly suppressed LUAD cell metastasis *in vivo*. A549 cells were infected with lentivirus expressing the miR-134-5p inhibitor or control vector and injected into the tail vein of 6-week-old nude mice (n = 5 per group). Mice were sacrificed 3 weeks after cell injection, and nodules on the lung surface and in tissue sections were counted under a microscope. CTR, control; mimics, miR-134-5p; inhibitor, miR-134-5p antisense; LV miR-134-5p, lentivirus expressing miR-134-5p; LV miR-134-5p inhibitor, lentivirus expressing miR-134-5p antisense. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

experimental results *in vivo*, we generated A549 cell lines stably expressing miR-134-5p or the miR-134-5p inhibitor (Figure 2A). We then administered these cells to nude mice by tail vein injection. As shown in Figure 2D, overexpression of miR-134-5p significantly increased the formation of A549 cell-induced metastatic lesions in the lungs of mice compared to that induced by vector control cells. In contrast, inhibition of miR-134-5p strongly suppressed the formation of A549 cell-induced metastatic lesions in the lungs of mice compared to that induced by vector control cells (Figure 2E). These findings clearly indicate that miR-134-5p positively regulates LUAD cell metastasis.

miR-134-5p Contributes to the Development of Chemoresistance in LUAD Cells

Next, we investigated the effects of miR-134-5p on the chemosensitivity of LUAD cells. First, we investigated the correlation between the miR-134-5p expression level and postoperative progression in stage I LUAD patients who received chemotherapy. Our clinical data showed that the stage I LUAD patient group with high miR-134-5p expression had a higher recurrence rate than did the group with low miR-134-5p expression (Figure 3A). In addition, our *in vitro* experiments showed that overexpression of miR-134-5p significantly suppressed CDDP-induced cell growth inhibition (Figure 3B) and apoptosis (Figure 3C; Figure S1) in both the A549 and H1299 LUAD cell lines. Consistent with the results of the *in vitro* experiment, the results of the A549 xenograft model experiment showed that overexpression of miR-134-5p significantly suppresses CDDP-induced LUAD growth inhibition (Figures 3D and 3E) and apoptosis (Figure 3F). Taken together, our findings suggest that aberrantly overexpressed miR-134-5p significantly contributes to chemoresistance development in LUAD.

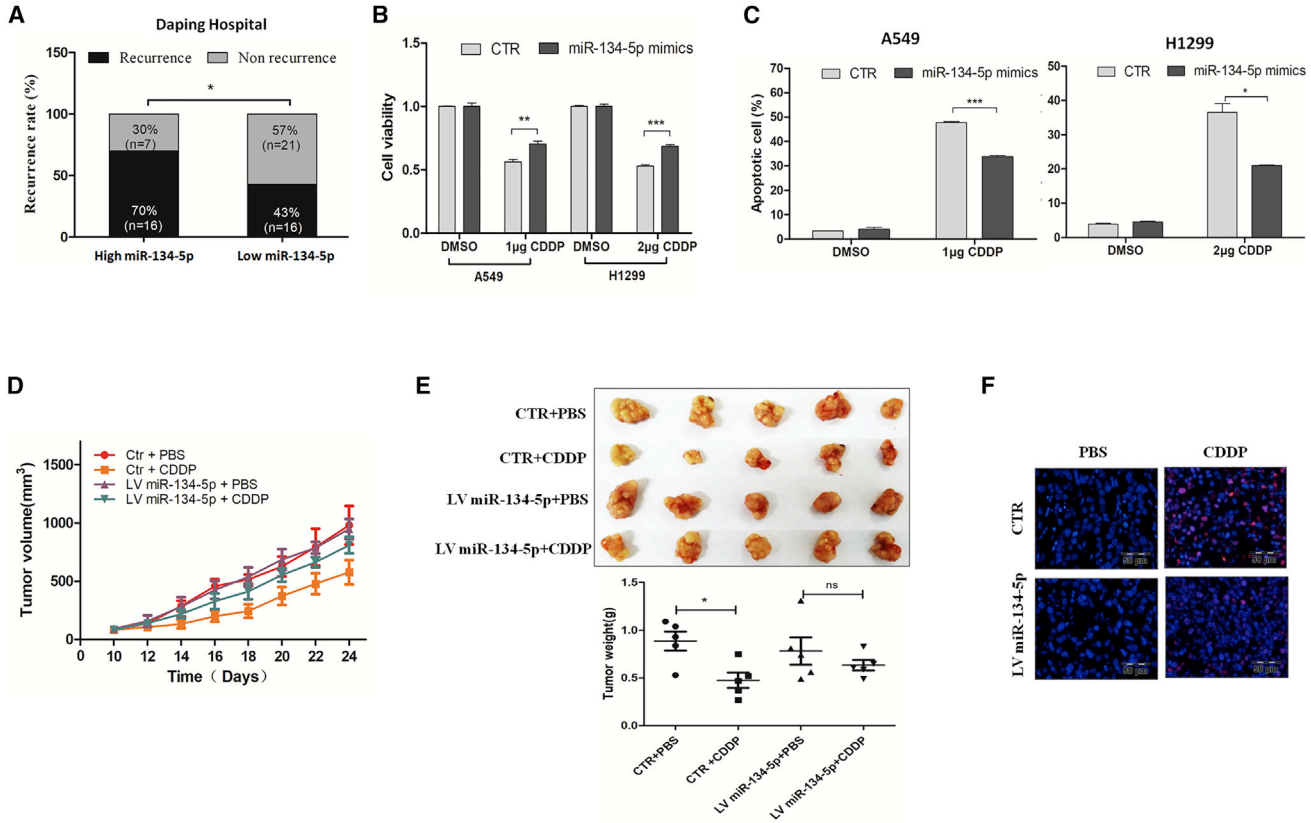


Figure 3. Overexpression of miR-134-5p Contributes to Stage I LUAD Chemoresistance

(A) Analysis of the Daping Hospital cohort showed that the miR-134-5p expression level was significantly correlated with recurrence in stage I LUAD patients who received chemotherapy (n = 60). (B) Overexpression of miR-134-5p suppressed CDDP-induced cell growth inhibition in both A549 and H1299 cells. After 48 h of transfection with the indicated oligonucleotides, cells were plated in a 96-well plate. Twelve hours after seeding, cells were incubated with or without the indicated concentration of CDDP for 48 h and were then subjected to CCK8 assay. (C) Overexpression of miR-134-5p suppressed CDDP-induced cell apoptosis in both A549 and H1299 cells. After 48 h of transfection with the indicated oligonucleotides, cells were plated in a six-well plate. Twelve hours after seeding, cells were incubated with or without the indicated concentration of CDDP for 24 h and were then subjected to apoptosis analysis. (D) miR-134-5p overexpression suppressed CDDP-induced tumor growth inhibition *in vivo*. miR-134-5p-overexpressing A549 cells and the corresponding control cells were used to generate xenograft tumors. After the mean tumor volume reached 100 mm^3, mice were started on treatment with either PBS or CDDP (2 mg/kg body weight), and the tumor volumes were measured every 2 days. (E) Mice were sacrificed after 2 weeks of CDDP treatment, and the tumors were weighed. (F) Apoptotic cells were detected via a TUNEL assay in the indicated xenograft tumor samples. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

The above data show that overexpression of miR-134-5p stimulates chemoresistance, prompting us to investigate whether miR-134-5p inhibition could overcome chemoresistance in LUAD. Here, we used the CDDP-resistant LUAD cell line A549/CDDP (Figure 4A) to examine the effects of miR-134-5p inhibition on LUAD chemoresistance, because A549/CDDP cells show higher expression levels of miR-134-5p than do the parental A549 cells (Figure 4B). As shown in Figure 4C, our cell viability assay showed that inhibition of miR-134-5p restores the sensitivity of A549/CDDP cells to CDDP treatment. Consistent with this finding, the flow cytometry analysis results showed that compared to CDDP treatment alone, inhibition of miR-134-5p significantly stimulated CDDP-induced A549/CDDP cell apoptosis (Figure 4D). Furthermore, these *in vitro* experiment results were confirmed *in vivo* using xenograft models that were generated by CDDP-resistant A549 cells. Our *in vivo* experiment show that combination of miR-134-5p inhibition and CDDP treatment significantly inhibited CDDP-resistant tumor growth compared to single treatment groups (Figure 4E). Taken together, our findings demonstrate that inhibition of miR-134-5p can overcome the insensitivity of chemoresistant LUAD cells to chemotherapy.

DAB2 Is a Target of miR-134-5p in Stage I LUAD

To investigate the mechanism underlying the effect of miR-134-5p on LUAD cells, we performed mRNA sequencing analysis using miR-134-5p-overexpressing A549 cells and the corresponding parental cells. Through mRNA sequencing analysis, we identified 144 genes that were downregulated in miR-134-5p-overexpressing A549 cells by more than 2-fold relative to their expression in control cells (Figure 5A). Among them, through a search of the miRNA target prediction databases microRNA.org and TargetScan, we demonstrated six

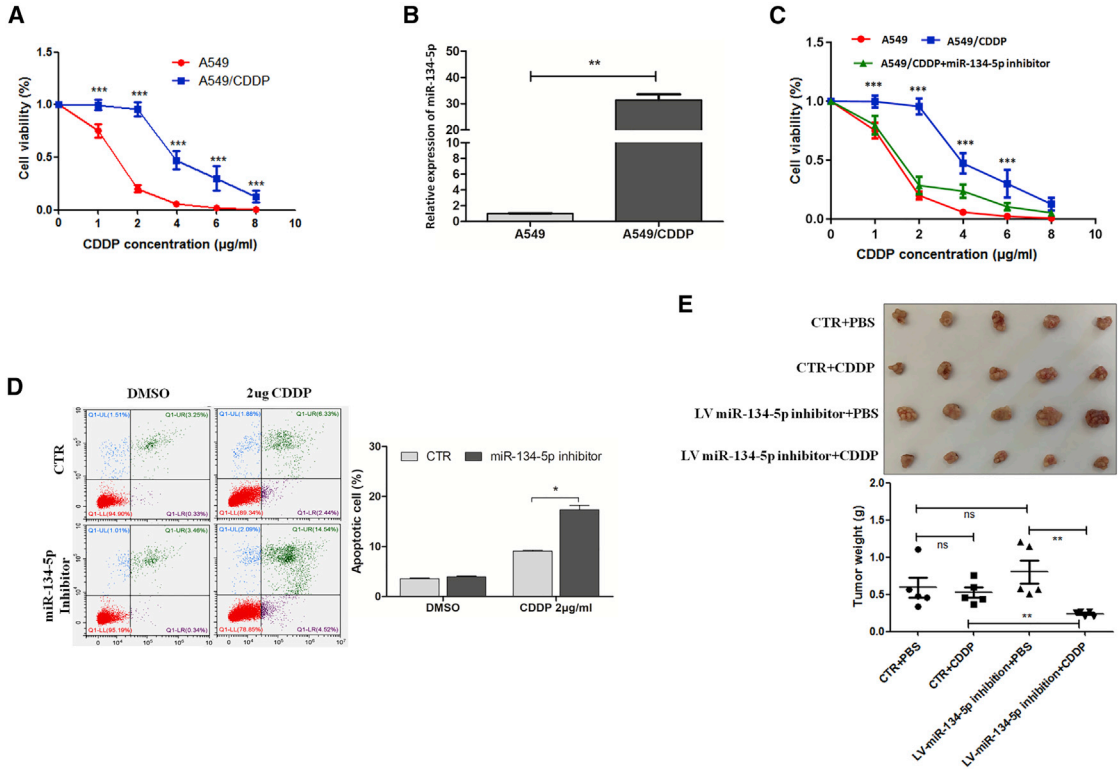


Figure 4. Inhibition of miR-134-5p Overcomes the Insensitivity of CDDP-Resistant A549 Cells to CDDP Treatment

(A) A549/CDDP cells exhibit resistance to CDDP treatment compared to their parental cells. The indicated cells were treated with the indicated concentration of CDDP for 48 h and were then subjected to a cell viability assay. (B) miR-134-5p expression was measured by qRT-PCR in A549/CDDP cells and their parental cells. (C) Inhibition of miR-134-5p significantly enhanced the sensitivity of A549/CDDP cells to CDDP treatment. A549/CDDP cells were transfected with the miR-134-5p inhibitor, treated with the indicated concentration of CDDP for 48 h, and subjected to a cell viability assay. (D) Inhibition of miR-134-5p significantly stimulated CDDP-induced apoptosis in A549/CDDP cells. A549/CDDP cells were transfected with the miR-134-5p inhibitor, treated with the indicated concentration of CDDP for 48 h, and subjected to apoptosis analysis. (E) Inhibition of miR-134-5p enhanced the sensitivity of CDDP-resistant tumors to CDDP treatment inhibition *in vivo*. Expressing miR-134-5p-inhibitor A549/CDDP cells and the corresponding control cells were used to generate xenograft tumors. After the mean tumor volume reached 100 mm^3, mice were started on treatment with either PBS or CDDP (2 mg/kg body weight). Mice were sacrificed after 2 weeks of CDDP treatment, and the tumors were weighed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant.

genes for which the 3′ UTR contained miR-134-5p binding sites (Figure 5A). Furthermore, by clinical samples analysis, we investigated the association between these six genes and miR-134-5p expression levels in stage I LUAD. Our clinical data show that the DAB2 expression level was negatively correlated with the miR-134-5p level in stage I LUAD (Figure 5B). In addition, previous studies have shown that cancer suppressor gene DAB2 downregulation induces EMT progression, thereby stimulating cancer metastasis.[17,18] These findings suggesting that DAB2 may be a target gene of miR-134-5p involved in the regulation of miR-134-5p on stage I LUAD metastasis. In fact, our *in vitro* experiments showed that overexpression of miR-134-5p significantly inhibited DAB2 expression in both A549 and H1299 LUAD cells (Figure 5C). Consistent results were also observed in xenograft tumors generated by miR-134-5p-overexpressing A549 cells and vector control cells (Figure 5D). In addition, our luciferase activity assay showed that overexpression of miR-134-5p significantly inhibited luciferase expression driven by the wild-type 3′ UTR of DAB2; however, it did not affect luciferase expression driven by the mutant 3′ UTR of DAB2 (Figures 5E and 5F), indicating that DAB2 is a target of miR-134-5p and that miR-134-5p inhibits DAB2 expression through directly binding to the 3′ UTR of DAB2 in LUAD cells.

DAB2 Is Negatively Correlated with Early Recurrence in Stage I LUAD and Stimulates LUAD Cell Metastasis and Chemoresistance

The above results indicate that miR-134-5p plays an important role in the early recurrence of stage I LUAD metastasis and chemoresistance and that DAB2 is a target of miR-134-5p in stage I LUAD. These data further suggest that DAB2 may negatively regulate metastasis and chemosensitivity in stage I LUAD. In fact, the TCGA dataset analysis results show that stage I LUAD patients in the high-DAB2 group had longer median recurrence-free survival times and lower recurrence rate than did those in the low-DAB2 group (Figures 6A and 6B).

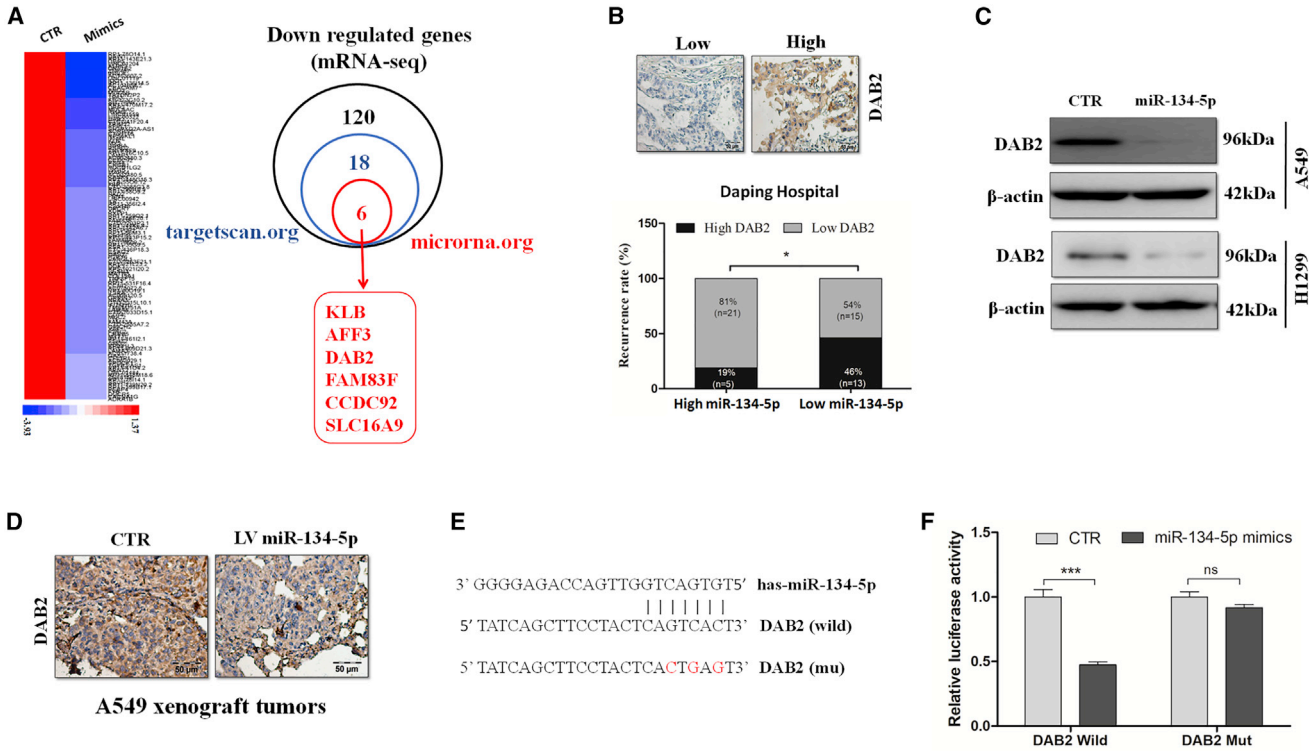


Figure 5. DAB2 Is a Target of miR-134-5p in Stage I LUAD

(A) Candidate targets of miR-134-5p in LUAD cells. mRNA sequencing identified 144 genes (heatmap) that were downregulated more than 2-fold in miR-134-5p-overexpressed A549 cells relative to that in control cells. Then, through a search of the miRNA databases TargetScan and microRNA.org, six candidate target genes of miR-134-5p were identified. (B) The level of miR-134-5p was negatively correlated with DAB2 expression level in primary tumors of stage I LUAD. The miR-134-5p and DAB2 expression levels were measured by qRT-PCR and immunohistochemistry, respectively, in stage I LUAD tissues (n = 54). (C) Overexpression of miR-134-5p significantly suppressed DAB2 expression in both A549 and H1299 cells. The indicated cells were transfected with miR-134-5p (mimic) or control oligonucleotides (CTR). After 72 h of transfection, cells were subjected to western blot analysis. (D) Immunohistochemical analysis showed that DAB2 expression was strongly inhibited in xenograft tumors generated by miR-134-5p-overexpressing A549 cells. (E) Predicted binding sites of miR-134-5p in the wild-type 3′ UTR of DAB2. Mutations in this 3′ UTR are highlighted in red. (F) Luciferase activity of the reporter driven by the wild-type or mutant 3′ UTRs of DAB2 in A549 cells cotransfected with control oligonucleotides (CTR) or miR-134-5p mimics. Luciferase reporter constructs containing the wild-type (DAB2-WT) or mutant 3′ UTR of DAB2 (DAB2-MT) were transfected into A549 cells with or without miR-134-5p mimics. After 48 h of transfection, luciferase intensity was assessed. *$p < 0.05$; ***$p < 0.001$; ns, not significant.

Additionally, we found significantly decreased expression of DAB2 in CDDP-resistant A549/CDDP cells compared to that in the parental cells (Figure 6C). In addition, *in vitro* experiments showed that silencing DAB2 significantly stimulated LUAD cell migration, whereas overexpression of DAB2 strongly reduced LUAD cell migration (Figures 6D and 6E). Silencing DAB2 also suppressed the CDDP-induced inhibition of LUAD cell growth, whereas overexpression of DAB2 enhanced the CDDP-induced inhibition of LUAD cell growth (Figures 6F and 6G). More importantly, overexpression of DAB2 overcame the insensitivity of CDDP-resistant A549/CDDP cells to CDDP treatment (Figure 6H). These data suggest that DAB2 is an important regulator of metastasis and chemosensitivity in early-stage LUAD.

miR-134-5p Plays an Oncogenic Role through DAB2 in LUAD

Next, we investigated whether DAB2 directly contributes to miR-134-5p-induced metastasis and chemoresistance in LUAD. Our data showed that overexpression of DAB2 blocked the miR-134-5p-induced inhibition of E-cadherin expression (Figure 7A) and stimulation of migration (Figure 7B) and chemoresistance in H1299 cells (Figure 7C). In contrast, DAB2 knockdown suppressed the inhibition of miR-134-5p-induced stimulation of E-cadherin expression (Figure 7D) and inhibition of migration (Figure 7E) and chemoresistance (Figure 7F). Taken together, these findings suggest that DAB2 is an important player controlling the effects of miR-134-5p on LUAD metastasis and chemoresistance.

In addition, we evaluated the correlation between the expression levels of miR-134-5p and several other genes in stage I LUAD, because contrary to our findings, previous studies have shown that miR-134-5p plays an anti-metastasis and anti-chemoresistance role by targeting ABCC1, TAB1, PAK2, DPYD, ITGB1, EGFR, CCND1, and FOXM1.[16,19–25] However, we did not observe a correlation between the expression levels of these genes and miR-134-5p in

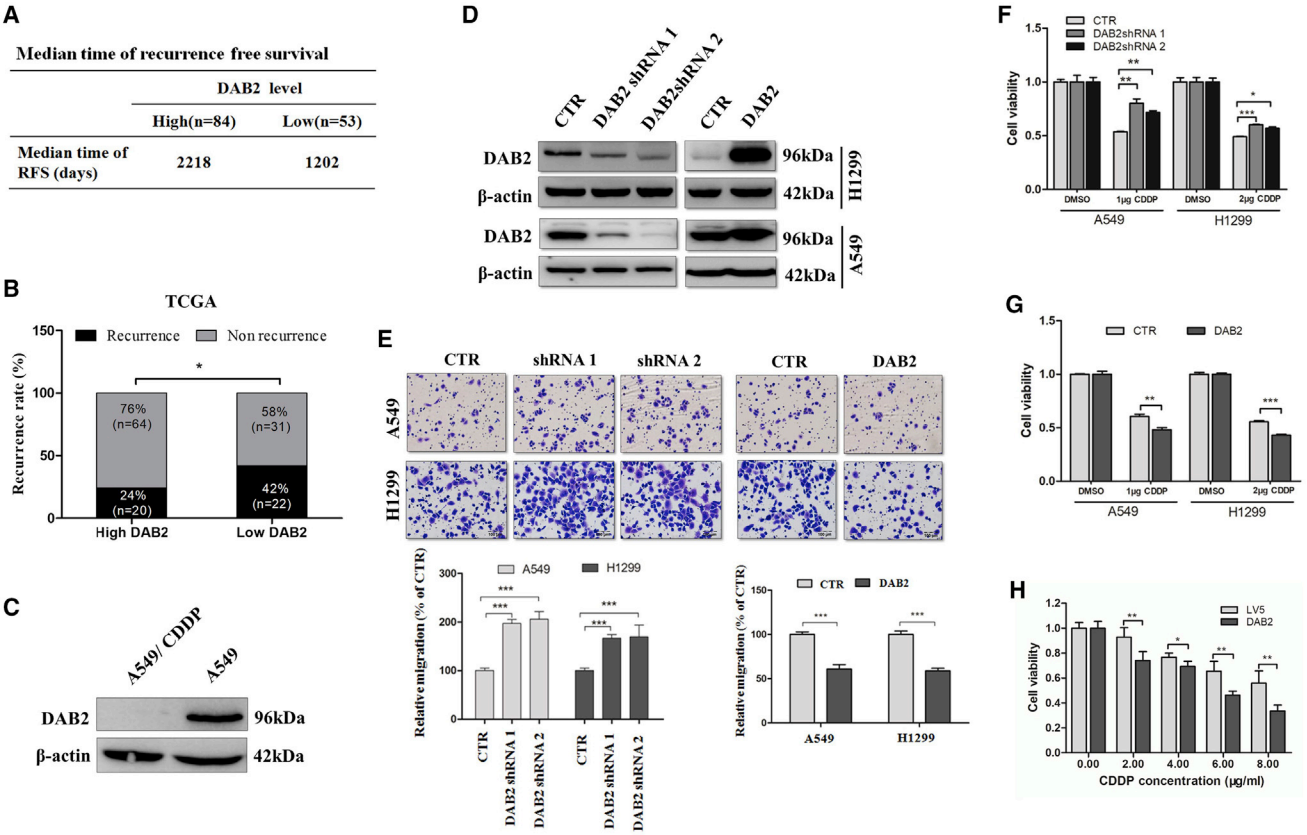


Figure 6. DAB2 Expression Level Was Negatively Correlated with Stage I LUAD Progression

(A) Analysis of the stage I LUAD TCGA dataset showed that the DAB2 expression level was significantly associated with the median recurrence-free survival time in patients with stage I LUAD (n = 137). (B) Analysis of the stage I LUAD TCGA dataset showed that the DAB2 expression level was negatively correlated with the recurrence rate in stage I LUAD patients (n = 137). (C) DAB2 expression was significantly decreased in A549/CDDP cells relative to that in the parental A549 cells. (D) The indicated cells were infected with the indicated lentivirus. After 72 h of infection, cells were subjected to western blotting. (E) DAB2 negatively regulated the migration of both A549 and H1299 cells. Cells were infected with the indicated lentivirus. After 48 h of infection, cells were subjected to a migration assay. (F) Knockdown of DAB2 suppressed CDDP-induced cell growth inhibition in both A549 and H1299 cells. After 48 h of infection with the indicated lentivirus, cells were plated in a 96-well plate. Twelve hours after seeding, cells were incubated with or without the indicated concentration of CDDP for 48 h and were then subjected to a CCK8 assay. (G) Overexpression of DAB2 enhanced CDDP-induced cell growth inhibition in both A549 and H1299 cells. After 48 h of infection with the indicated lentivirus, cells were plated in a 96-well plate. Twelve h after seeding, cells were incubated with or without the indicated concentration of CDDP for 48 h and were then subjected to a CCK8 assay. (H) Restoration of DAB2 enhanced the sensitivity of the CDDP-resistant cell line A549/CDDP to CDDP treatment. A549/CDDP cells were infected with the indicated lentivirus and plated in a 96-well plate. Twelve hours after seeding, cells were incubated with or without the indicated concentration of CDDP for 48 h and were then subjected to a CCK8 assay. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

LUAD (Figure S2), suggesting that these genes are not major targets in stage I LUAD.

DISCUSSION

Identification of prognostic factors that can stratify patients according to clinical and biological markers may help in selecting adequate treatment strategies.[26] In this study, by using our clinical sample cohort and TCGA dataset, we found that increased expression levels of miR-134-5p in primary tumors were significantly correlated with early recurrence in stage I LUAD patients. In addition, we used the TCGA dataset to demonstrate that the expression level of the miR-134-5p target gene DAB2 was negatively correlated with recurrence in I LUAD patients. These findings suggest that the expression levels of miR-134-5p and DAB2 have potential as a biomarker for predicting early recurrence in stage I LUAD patients. However, this result needs to be further confirmed in larger samples.

In general, stage I LUAD is treated with surgery combined with radiation or chemotherapy. However, treatment for stage I LUAD often fails due to local or metastatic recurrence,[27] suggesting that metastasis and chemoresistance significantly contribute to recurrence. Recent studies have shown that aberrantly expressed miRNAs plays a key role in the metastasis and therapeutic resistance of a variety of cancers. For example, Gullà et al.[28] reported that increased expression of miR-221 was associated with drug resistance in multiple myeloma and that inhibition of miR-221 can overcome drug resistance of

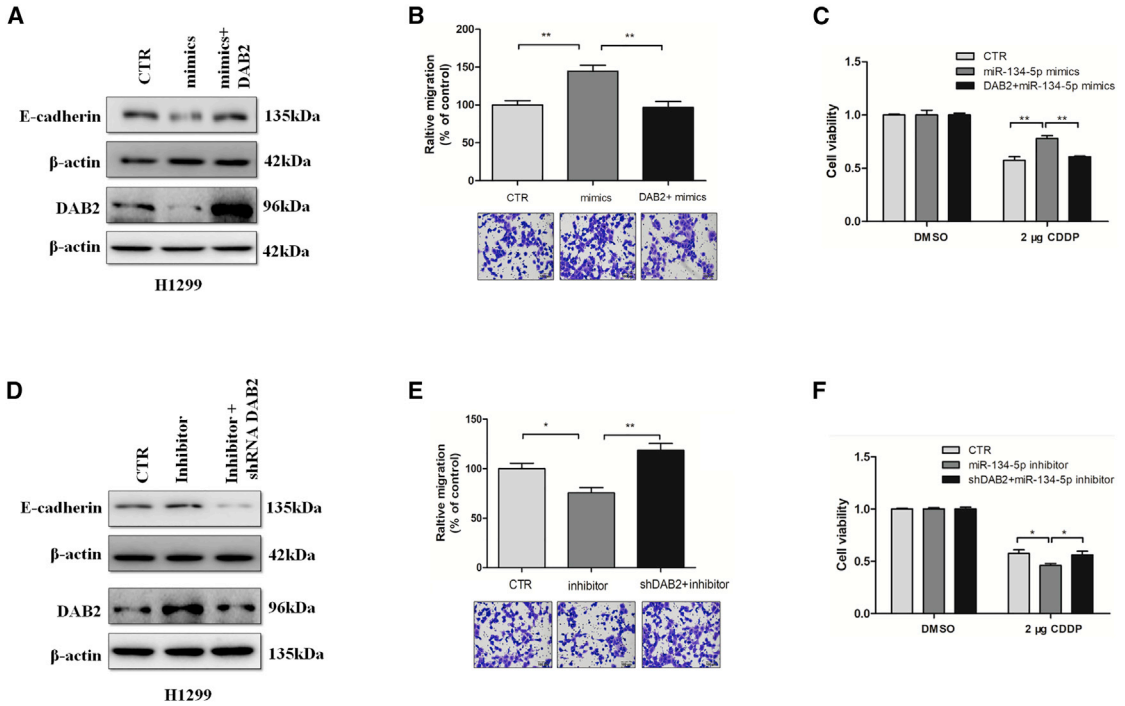


Figure 7. miR-134-5p Stimulates LUAD Cell Metastasis and Chemoresistance through DAB2

(A) Restoration of DAB2 expression suppressed the miR-134-5p-induced inhibition of E-cadherin expression in H1299 cells. miR-134-5p mimics were transfected into H1299 cells with or without the DAB2 expression vector. After 72 h of transfection, cells were subjected to western blot analysis. (B) Restoration of DAB2 expression inhibited the miR-134-5p-induced stimulation of H1299 cell invasion. miR-134-5p mimics were transfected into H1299 cells with or without the DAB2 expression vector. After 48 h of transfection, cells were subjected to an invasion assay. (C) Restoration of DAB2 expression overcame miR-134-5p-induced chemoresistance in H1299 cells. miR-134-5p mimics were transfected into H1299 cells with or without the DAB2 expression vector. After 48 h of transfection, cells were incubated with the indicated concentration of CDDP for 48 h and were then subjected to a cell viability assay. (D) Silencing DAB2 inhibited the miR-134-5p inhibition-induced upregulation of E-cadherin expression in H1299 cells. miR-134-5p inhibitors were transfected into H1299 cells with or without the DAB2 shRNA expression vector. After 72 h of transfection, cells were subjected to western blot analysis. (E) Silencing DAB2 inhibited miR-134-5p inhibition-suppressed H1299 cell invasion. H1299 cells were transfected with the miR-134-5p inhibitor and/or DAB2 shRNA-expressing lentivirus. After 48 h of transfection, cells were subjected to a cell invasion assay. (F) Silencing DAB2 suppressed the miR-134-5p inhibition-enhanced sensitivity of H1299 cells to CDDP treatment. H1299 cells were transfected with the miR-134-5p inhibitor and/or DAB2 shRNA-expressing lentivirus. After 48 h of transfection, cells were subjected to a cell viability assay.

multiple myeloma; Wang et al.[29] reported that overexpression of miR-96 promotes cellular sensitivity to CDDP and poly ADP-ribose polymerase (PARP) inhibition in several type cancers; Gasparini et al.[30] reported that overexpression of miR-155 enhances the sensitivity of breast cancer cells to irradiation; and Xu et al.[12] reported that decreased expression of miR-382 was significantly associated with osteosarcoma lung metastasis and that overexpression of miR-382 can inhibit osteosarcoma lung metastasis. In this study, we used a series of *in vitro* and *in vivo* experiments to provide evidence that increased expression of miR-134-5p significantly contributes to LUAD metastasis and chemoresistance development. The oncogenic role of miR-134-5p was also identified by other research groups previously in lung cancer. Zhang et al.[31] reported that miR-134-5p stimulates the proliferation of lung cancer cells. Chen et al.[32] also reported that increased expression of miR-134-5p promotes cell proliferation and inhibits apoptosis in lung cancer. More importantly, our data clearly show that inhibition of miR-134-5p significantly inhibits LUAD cell metastasis and overcomes chemoresistance *in vitro* and *in vivo*. Taken together, these findings suggest that miR-134-5p functions as a tumor stimulator in LUAD and contributes to early recurrence of stage I LUAD through stimulating metastasis and chemoresistance. Additionally, they suggest that inhibition of miR-134-5p may be a useful strategy for the treatment or prevention of stage I LUAD metastasis and chemoresistance.

In contrast to our results, several reports show that the expression of miR-134-5p is significantly downregulated in NSCLC and negatively associated with NSCLC progression.[22] This is possible because some miRNAs exhibit different expression patterns in different types of cancers and play different roles.[9,33] In contrast to a previous NSCLC study, we investigated the effects of miR-134-5p on LUAD, a subtype of NSCLC, and focused on stage I LUAD. miRNAs play their roles through target genes, and a single miRNA can target many different genes while functioning as both a tumor suppressor and tumor stimulator.[34] Even the same miRNA in the same tumor plays opposite roles at different stages by targeting different genes.[35] These findings

suggest that the role of a miRNA depends on its target gene. Here, we used a stage I LUAD dataset from TCGA to investigated the correlation between the expression levels of miR-134-5p and some oncogenes that were previously reported as target genes of miR-134-5p.[16,19–25] However, we observed no correlation between the expression levels of miR-134-5p and these oncogenes in stage I LUAD, suggesting that these oncogenes may not be primary targets for miR-134-5p in stage I LUAD.

Here, we also clarified the cancer stimulation mechanism of miR-134-5p in stage I LUAD. Previous studies have shown that DAB2 is a tumor suppressor gene, which is significantly downregulated in lung cancer, and that reduced DAB2 expression is closely associated with lung cancer development and progression.[36,37] Studies also show that downregulated DAB2 is associated with recurrence in esophageal squamous cell carcinoma[38] and promotes EMT in breast cancer through upregulating transforming growth factor β (TGF-β).[17] Previous studies show that TGF-β is a stimulator of EMT and causes E-cadherin downregulation and N-cadherin upregulation.[39] Our data also show that downregulated DAB2 is closely associated with recurrence of stage I LUAD, and that DAB2 positively regulates E-cadherin expression and negatively regulates metastasis of LUAD cells. In addition, we identified DAB2 as a novel target gene of miR-134-5p in stage I LUAD. Our clinical data showed that DAB2 expression is inversely correlated with miR-134-5p expression in stage I LUAD specimens and that overexpression of miR-134-5p expression in LUAD cells leads to suppression of DAB2 expression. In addition, the luciferase reporter assay showed that miR-134-5p directly targets the 3′ UTR of DAB2. Furthermore, our data indicate that restoration of DAB2 expression blocked miR-134-5p overexpression-induced LUAD cell metastasis and chemosensitivity, whereas DAB2 silencing suppressed the inhibition of miR-134-5p-induced LUAD cell metastasis and chemoresistance. These findings clearly suggest that DAB2 is a key downstream effector of miR-134-5p that mediates miR-134-5p-induced LUAD cell metastasis and chemoresistance.

In summary, we combined clinical and experimental studies to determine the clinical significance and role of miR-134-5p and DAB2 in the early recurrence of stage I LUAD. Our work provides new insight into the mechanisms of miR-134-5p in early-stage LUAD metastasis and chemoresistance. Our findings may also aid in the development of potential therapeutics for the treatment of early-stage LUAD recurrence.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS) and cell culture medium were purchased from Biological Industries (Cromwell, CT, USA). Glutaraldehyde, puromycin, and cisplatin (CDDP) were purchased from Sigma (St. Louis, MO, USA). A miRNeasy FFPE (formaldehyde-fixed, paraffin-embedded) kit was obtained from QIAGEN (Hilden, Germany). The miR-134-5p mimics, miR-134-5p inhibitor, negative control oligonucleotides, miR-134-5p primer set, and U6 small nuclear RNA (RNU6) primer set were obtained from RiboBio (Guangzhou, China). A High-Capacity cDNA Reverse Transcription Kit and a QuantiTect SYBR Green PCR kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Lentiviral vectors expressing miR-134-5p or inhibitor of miR-134-5p were obtained from GeneChem (Shanghai, China). Lentiviral vectors expressing DAB2 or short hairpin RNA (shRNA) targeting DAB2 were obtained from GenePharma (Shanghai, China). A cell counting kit-8 (CCK8) kit and an apoptosis assay kit were purchased from Biosharp (Wuhan, China) and GeneCopoeia (Guangzhou, China), respectively. Migration and invasion assay kits were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Anti-β-actin and anti-β-tubulin antibodies were obtained from Abcam (Cambridge, MA, USA). An anti-E-cadherin antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). TRIzol and an anti-DAB2 antibody were obtained from Invitrogen (Carlsbad, CA, USA). A dual-luciferase assay system and terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay kit were purchased from Beyotime (Shanghai, China). A miRNAs array was obtained from Affymetrix (Santa Clara, CA, USA).

Cell Culture and Human Specimens

The CDDP-resistant LUAD cell line A549/CDDP was obtained from the Cancer Research Institute of XinQiao Hospital, Third Military Medical University. Other LUAD cell lines used in this study were purchased from the American Type Culture Collection. All LUAD cells were grown in RPMI 1640 medium supplemented with 10% FBS at 37°C in an atmosphere of 95% air and 5% CO_2.

LUAD specimens were obtained from 81 stage I LUAD patients during surgery at Daping Hospital and Research Institute of Surgery. This study was conducted in accordance with the guidelines of the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Daping Hospital and Research Institute of Surgery, Third Military Medical University. Clinical data of the patients enrolled in this study were acquired from medical records and telephone follow-up interviews. The patients' characteristics from the Daping Hospital cohort are summarized in Table S1. The TCGA dataset for stage I LUAD patients was obtained from http://gdac.broadinstitute.org and https://xena.ucsc.edu, and the patients' characteristics are summarized in Table S2.

miRNAs Array

For the miRNA array analysis, total RNA was extracted from FFPE tissue samples using the RNA Prep Pure FFPE Kit according to the manufacturer's instructions. Only tissues containing more than 70% tumor cells were used for RNA isolation. The patients' characteristics are summarized in Table S3. Gene expression profiling was performed using Affymetrix GeneChip miRNA 4.0 arrays according to the manufacturer's instructions.

Real-Time qRT-PCR Analysis

Total RNA was isolated from cells using TRIzol reagent according to the manufacturer's protocol, and reverse transcription (RT) was performed using the High-Capacity cDNA Reverse Transcription Kit.

PCR amplification of miR-134-5p and RNU6 was performed with a specific primer set. The relative expression of miR-134-5p was normalized to RNU6 expression. PCR amplification of DAB2 and GAPDH was performed with the QuantiTect SYBR Green PCR kit. The primers used for DAB2 amplification were 5′-GTAGAAA CAAGTGCAACCAATGG-3′ and 5′-GCCTTTGAACCTTGCTAA GAGA-3′; the primers used for GAPDH amplification were 5′-GG AGCGAGATCCCTCCAAAAT-3′ and 5′-GGCTGTTGTCATACTT CTCATGG-3′.

Transwell and Cell Viability Assays

Indicated cells were transfected with the indicated oligonucleotides and plasmid for 48 h. Then, 1×10^4 cells in serum-free growth medium were seeded in the upper wells of Transwell chambers. The lower wells contained the same medium supplemented with 10% serum. After 24 h, cells that had invaded to the lower side of the chamber were fixed with 2.5% glutaraldehyde, stained with 0.1% crystal violet, dried, and counted.

For the cell viability assay, indicated cells were plated in 96-well plates at density 1×10^4 per well. After 12 h of seeding, cells were treated with indicated drugs for 48 h. Then, cell viability was determined using CCK8 reagent according to the manufacturer's instructions.

Western Blot and Immunohistochemical Assays

Western blotting and immunohistochemical assays were performed as described previously.[40]

Apoptosis Analysis

The indicated cells were seeded in six-well cell culture plates. Twelve hours after seeding, cells were treated with the indicated concentration of CDDP. Twenty-four hours later, cells were harvested and stained with annexin V and 7-aminoactinomycin D (7-AAD) according to the manufacturer's protocol, followed by flow cytometric analysis. Apoptotic cells in tumor tissues were detected using an *In Situ* Cell Death Detection Kit according to the manufacturer's instructions.

Luciferase Reporter Assay

In brief, fragments of the DAB2 3′ UTR that were predicted to interact with miR-134-5p were amplified by PCR from human genomic DNA and inserted into the *Mlu*I and *Hind*III sites of the miRNA expression reporter vector. For the luciferase reporter experiments, the indicated cells were seeded into 24-well cell culture plates at a concentration of 1×10^4 cells per well. The next day, the cells were transfected with the indicated reporter plasmids expressing firefly luciferase. The Renilla luciferase plasmid was cotransfected as the transfection control. Cells were lysed 48 h after transfection, and luciferase activity was measured by the Dual-Luciferase assay system according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity.

Animal Experiments

Xenograft models were generated using A549 cells infected with expressing miR-134-5p or miR-134-5p inhibitor lentivirus. For the tumor growth assay, 2×10^6 of the indicated cells in 0.1 mL of PBS were subcutaneously injected into 6-week-old female nude mice (five mice per group). When the tumors reached a size of approximately 100 mm^3, the mice were started on treatment with either PBS or CDDP (2 mg/kg body weight). The treatment was administered every other day. After 2 weeks, the mice were sacrificed and the tumors were weighed. For the lung metastasis experiments, a total of 1.5×10^6 of the indicated cells were suspended in 0.1 mL of PBS and injected into the lateral tail vein of 6-week-old female nude mice (six mice per group). Three weeks after injection, all mice were sacrificed, the lungs were embedded with paraffin and sliced for H&E staining, and tumor foci in the sections were counted. The mice were obtained from Shanghai Laboratory Animal Center, and all animal care and experimentation procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee of the Army Military Medical University.

Statistical Analysis

All data are presented as the means ± SD, and significant differences between treatment groups were analyzed by a Student's t test or one-way ANOVA using GraphPad Prism. The recurrence-free survival rate of patients with stage I LUAD was calculated using a Kaplan-Meier survival analysis. Differences were considered statistically significant at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.09.025.

AUTHOR CONTRIBUTIONS

C.-X.X. and D.W. designed the experiments; L.Z., P.H., and Q.L. performed experiments; all authors analyzed data; and C.-X.X wrote the paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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