



ORIGINAL ARTICLE

MicroRNA-331-3p inhibits epithelial-mesenchymal transition by targeting ErbB2 and VAV2 through the Rac1/PAK1/ β -catenin axis in non-small-cell lung cancer

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Abstract

MicroRNAs have been reported to play critical roles in the regulation of non-small-cell cancer (NSCLC) development, but the role of microRNA (miR)-331-3p in NSCLC is still unclear. In this study, the expression levels of miR-331-3p in NSCLC tumor tissues and adjacent normal tissues were examined by quantitative RT-PCR, and the relationship between miR-331-3p expression and patient clinicopathological characteristics was analyzed. The effects of miR-331-3p on epithelial-mesenchymal transition (EMT), migration, and metastasis of NSCLC cells were determined in vitro and vivo. Direct functional targets of miR-331-3p were identified by luciferase reporter assay, western blot assay, immunohistochemical staining, and rescue assay. The downstream pathway regulated by miR-331-3p was identified by immunofluorescence, immunoprecipitation, and Rac1 activity examination. Our results showed that miR-331-3p was significantly downregulated in NSCLC tumor tissues and was correlated with clinicopathological characteristics, and miR-331-3p could be an independent prognostic marker for NSCLC patients. Furthermore, miR-331-3p significantly suppressed EMT, migration and metastasis of NSCLC cells in vitro and in vivo. Both ErbB2 and VAV2 were direct functional targets of miR-331-3p. The activities of Rac1, PAK1, and β -catenin were regulated by miR-331-3p through ErbB2 and VAV2 targeting. These results indicated that miR-331-3p suppresses EMT, migratory capacity, and metastatic ability by targeting ErbB2 and VAV2 through the Rac1/PAK1/ β -catenin axis in NSCLC.

KEYWORDS

ErbB2, microRNA, non-small-cell lung cancer, Rac1, VAV2

1 | INTRODUCTION

Recently published epidemiology studies showed that lung cancer occupies the leading position for both estimated deaths and new cases

of human cancer in the United States and China.^{1,2} Approximately 80% of lung cancers are non-small-cell lung cancer (NSCLC), including adenocarcinoma and squamous cell carcinoma. Due to the failure of early interventions, relapse, and the lack of effective treatment

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for advanced cases, the prognosis of NSCLC is still poor, and the 5-year survival rate remains below 15%.³ Therefore, it is critical to identify the mechanisms of NSCLC development and explore innovative therapies for NSCLC patients.

MicroRNAs (miRNAs) are a group of highly conserved small non-coding RNAs with lengths of 20–24 nucleotides.⁴ As a rule, miRNAs can repress mRNA translation or promote RNA degradation by binding to the 3'-UTRs of their targets.⁵ Numerous pieces of evidence show that abnormal expression of miRNAs and their regulation play important roles in the initiation and progression of human cancers, such as esophageal carcinoma,^{6,7} hepatocellular carcinoma⁸ and breast carcinoma.⁹ Some miRNAs, such as miR-30a, are able to affect many types of malignancies and can be used as biomarkers for these tumors.¹⁰ To date, multiple miRNAs have been shown to have vital functions in the development of NSCLC,^{11,12} but few of these miRNAs could be a candidate for targeted therapy,¹³ and the specific underlying mechanisms for their effects in the tumorigenesis and progression of NSCLC are still not understood.

MicroRNA-331-3p is a miRNA that was reported to be abnormally expressed in several cancer types. However, the effect of miR-331-3p in different cancer types completely varies. *MiR-331-3p* functions as an oncogene in pancreatic cancer¹⁴ and hepatocellular cancer,¹⁵ but as a tumor suppressor gene in gastric cancer¹⁶ and cervical cancer.¹⁷ Although these studies described the effects of miR-331-3p in different types of cancer, the biological functions of miR-331-3p in NSCLC are still unknown. Herein, we found that the expression level of miR-331-3p was significantly lower in NSCLC tumor tissues than in adjacent normal tissues and negatively correlated with advanced stage and lymph node metastasis of NSCLC. Overexpression of miR-331-3p repressed the epithelial-mesenchymal transition (EMT) phenotype, migratory capacity, and metastatic ability of NSCLC cells *in vitro* and *in vivo*. Moreover, ErbB2 and VAV2 were identified as the target mRNAs of miR-331-3p, and the ErbB2/VAV2 complex and downstream Rac1/PAK1/ β -catenin activity were shown to be downregulated by miR-331-3p. Our findings confirmed the tumor suppressor activity of miR-331-3p in NSCLC and showed that VAV2 is a novel target of miR-331-3p.

2 | MATERIALS AND METHODS

2.1 | Ethics approval and consent to participate

This study was approved by the ethics committee of Xiangya Hospital, Central South University (CSU; Changsha, China), and written informed consent was obtained from all patients. All animal studies were approved by the Animal Ethics Committee and undertaken in accordance with the official recommendations of the Care and Use of Laboratory Animals of Xiangya Hospital, CSU.

2.2 | Patients and tissue specimens

From January 2011 to December 2012, a total of 80 pairs of NSCLC tumor tissues and adjacent normal tissues were gathered from patients

who underwent surgical lung resection at the Department of Thoracic Surgery, Xiangya Hospital, CSU. Patients did not receive any radiotherapy or chemotherapy before the lung resection operation. The tumor tissues were collected from the edge of NSCLC lesions and confirmed by at least 2 experienced pathologists. Specimens were rapidly frozen in liquid nitrogen and immediately transferred to a -80°C freezer immediately for subsequent experiments. The clinicopathological characteristics of all 80 patients were collected and analyzed.

All patients were followed up every 3 months by telephone or a visit by our research team for survival and recurrence inquiry until death or until the end of the investigation. In this study, the 5-year overall survival period (OS) was defined as the interval between the surgical lung resection date and the end date of follow-up or the date of death owing to recurrence of NSCLC and its associated complications. The 5-year disease-free survival period (DFS) was the interval between the surgical resection date and the follow-up deadline or the diagnosis of recurrence and/or metastasis of NSCLC by imaging examination.

2.3 | Cell lines and cell culture

Eight NSCLC cell lines (PC9, A549, H1299, CALU-1, H520, H1437, H460, and H1703), 293T and normal human bronchial epithelial cell lines (Beas-2b) were obtained from the Chinese Academy of Science Cell Bank (Shanghai, China). All cell lines were cultured in RPMI-1640 (Gibco, Billings, MT, USA) medium supplemented with 10% FBS (Gibco), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco) in a humidified incubator with 5% CO_2 at 37°C .

2.4 | Statistical analysis

All statistical analyses were carried out using SPSS version 25.0 software (IBM, New York, NY, USA). The results are expressed as the mean \pm SD. Student's *t* test was used to compare the differences between 2 groups. One-way ANOVA was used to assess the differences between 3 or more groups. The χ^2 -test was used to analyze the correlation between miR-331-3p expression and clinicopathological parameters. Spearman's rank correlation was used to determine the association between miR-331-3p and ErbB2/VAV2. The log-rank test was used to compare the patient Kaplan-Meier survival curves. The Cox proportional hazard regression model was used to determine factors that were independently associated with OS and DFS. $P < .05$ was considered statistically significant.

More details are described in Appendix S1.

3 | RESULTS

3.1 | MicroRNA-331-3p was downregulated in NSCLC tissues and inversely associated with prognosis

To explore the expression level of miR-331-3p in NSCLC tissues, we evaluated the expression of miR-331-3p in 80 pairs of frozen NSCLC tumor tissues and adjacent normal lung tissues, which were

obtained at least 5 cm away from the tumor edge, by quantitative RT-PCR (qRT-PCR). Our results showed that the expression level of miR-331-3p was significantly lower in NSCLC tumor tissues than in adjacent normal tissues. Among the 80 patients, miR-331-3p expression in 45 of the patients (56.3%) was at least 50% lower in tumor tissues than in the matching normal lung tissues (Figure 1A). The overall expression data showed that normal lung tissues had a nearly 2-fold higher miR-331-3p level than that of adjacent tumor tissues (-0.97 ± 1.52 vs -2.02 ± 1.52 , $P < .0001$; Figure 1B).

Patients were then divided into 2 groups according to the relative miR-331-3p expression level in tumor tissues (\log_2 fold change of tumor tissues/adjacent normal tissues ≤ -1 or \log_2 fold change > -1), and clinicopathological data of these patients were collected. The expression of miR-331-3p in NSCLC tumor tissues was significantly inversely correlated with lymphatic metastasis and clinical TNM stage (Table 1). Patients who had a lower miR-331-3p expression in tumor tissues had a higher probability of lymphatic metastasis and a more advanced TNM classification.

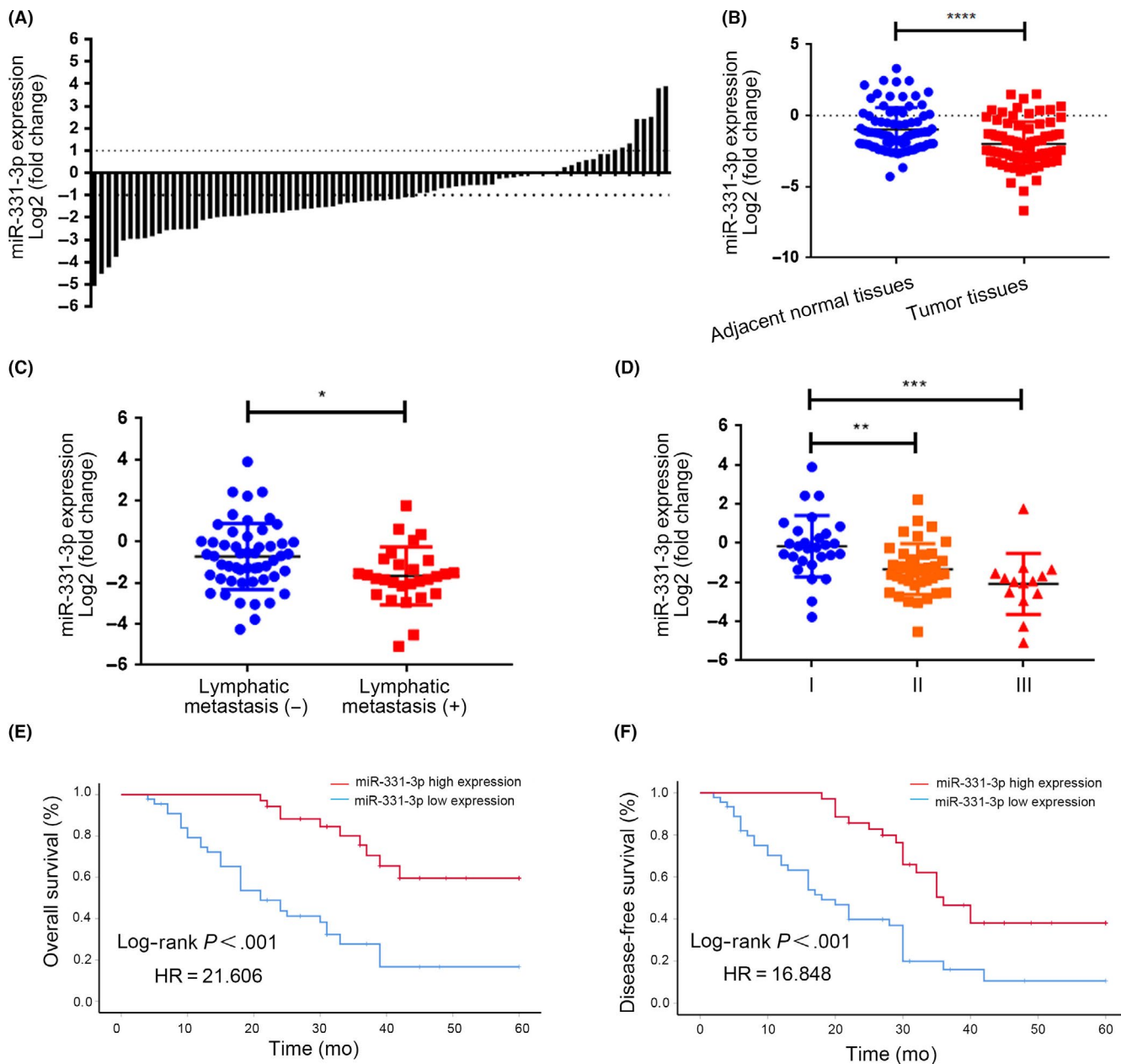


FIGURE 1 Expression level of microRNA (miR)-331-3p in non-small-cell lung cancer (NSCLC) tumor and adjacent normal tissues and its clinical significance. A, Relative expression level of miR-331-3p in NSCLC tumor tissues from 80 patients. B, RT-PCR results showed that the overall expression level of miR-331-3p in NSCLC tumor tissues was significantly higher than in adjacent normal tissues. C, D, Metastatic or advanced TNM stage NSCLC tumors had lower miR-331-3p expression levels compared with those of nonmetastatic or early TNM stage NSCLC tumors. E, F, Kaplan-Meier analysis showed that the 5-y overall survival and disease-free survival for NSCLC patients with low miR-331-3p levels were significantly shorter than those of patients with high miR-331-3p levels. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. HR, hazard ratio

Variables	n	miR-331-3p expression		P value
		High expression	Low expression	
Gender				
Male	65	28	37	>.9999
Female	15	7	8	
Age (y)				
<60	50	22	28	>.9999
≥60	30	13	17	
Smoking history				
Yes	51	24	27	.4874
No	29	11	18	
Pathological type				
Adenocarcinoma	36	16	20	>.9999
Squamous carcinoma	44	19	25	
Tumor differentiation				
High	8	3	5	.6468
High-middle	11	4	7	
Middle	29	16	13	
Middle-low	15	6	9	
Low	17	6	11	
Tumor size (cm)				
>3	65	28	37	>.9999
≤3	15	7	8	
Lymphatic metastasis				
Yes	28	7	21	.018
No	52	28	24	
TNM classification				
I	28	22	6	<.0001
II	38	12	26	
III+IV	14	1	13	

^aThe bold values indicates the P values which are less than 0.05.

Additionally, patients who had lymphatic metastasis or advanced TNM classification had a lower miR-331-3p expression level (metastasis (+): -1.66 ± 1.38 , metastasis (-): -0.72 ± 1.59 , $P = 0.01$; I: -0.15 ± 1.54 , II: -1.33 ± 1.28 , III: -2.08 ± 1.50 ; I vs II: $P < 0.01$; I vs III: $P < 0.001$; Figure 1C,D). Moreover, whether miR-331-3p expression correlated with patient survival was investigated. Our results showed that NSCLC patients with lower miR-331-3p expression in NSCLC tissues had much shorter OS and DFS compared with those of NSCLC patients with higher miR-331-3p expression (Figure 1E,F). Furthermore, the Cox proportional hazards regression model was used to determine whether miR-331-3p could be an independent prognostic marker for NSCLC patients. The multivariate survival analysis data showed that tumor differentiation, clinical TNM stage, and miR-331-3p expression reached significance for the OS of NSCLC patients, and clinical TNM stage and miR-331-3p expression reached significance for disease-free survival (Table 2). Taken together, our results showed that

TABLE 1 Correlations of microRNA (miR)-331-3p with clinicopathological features of patients with non-small-cell lung cancer

miR-331-3p was remarkably downregulated in NSCLC tumor tissues and had an inverse correlation with lymphatic metastasis and clinical TNM stage. MicroRNA-331-3p expression was correlated with OS and DFS of NSCLC patients and could act as an independent prognostic marker for NSCLC patients.

3.2 | MicroRNA-331-3p inhibited migratory capacity, metastatic ability, and EMT activation of NSCLC cells

The clinicopathological statistics showed a remarkable relationship between miR-331-3p expression and metastasis. Therefore, we evaluated the function of miR-331-3p on the metastatic capacity in NSCLC cells. First, we examined miR-331-3p expression in NSCLC cell lines (PC9, A549, H1299, CALU-1, H520, H1703, H1437, and H460) and a normal bronchus epithelial cell line (Beas-2b). Our results showed that NSCLC cells had significantly lower expression

TABLE 2 Cox regression analysis of overall survival (OS) and disease-free survival (DFS) in 80 patients with non-small-cell lung cancer

	Univariate analysis			Multivariate analysis		
	OS		DFS	OS		DFS
	HR (95% CI)	P	HR (95% CI)	HR (95% CI)	P	HR (95% CI)
Age	1.278 (0.661-2.470)	.465	1.355 (0.753-2.437)	1.028 (0.484-2.186)	.942	1.06 (0.552-2.033)
Gender	1.193 (0.528-2.694)	.671	0.937 (0.482-1.823)	2.029 (0.702-5.867)	.191	1.12 (0.494-2.539)
Smoke history	0.926 (0.489-1.753)	.814	0.897 (0.510-1.576)	0.672 (0.325-1.39)	.284	0.7 (0.378-1.297)
Tumor differentiation		.007		.047	.034	
Low	1.000		1.000	1.000		1.000
Middle-low	1.145 (0.454-2.886)	.774	0.977 (0.403-2.365)	0.828 (0.275-2.492)	.737	0.882 (0.314-2.482)
Middle	0.476 (0.169-1.338)	.159	0.444 (0.169-1.165)	0.533 (0.146-1.943)	.34	0.613 (0.193-1.951)
Middle-high	0.266 (0.097-0.725)	.010	0.367 (0.155-0.868)	0.201 (0.057-0.707)	.012	0.359 (0.127-1.012)
High	0.345 (0.099-1.200)	.094	0.486 (0.174-1.353)	0.59 (0.153-2.273)	.443	0.756 (0.249-2.289)
Tumor size	0.438 (0.171-1.119)	.084	0.610 (0.297-1.255)	0.458 (0.159-1.315)	.147	0.586 (0.264-1.3)
Lymphatic metastasis	0.439 (0.236-0.816)	.009	0.502 (0.289-0.871)	1.108 (0.516-2.38)	.792	0.946 (0.479-1.869)
TNM classification		<.001		<.001	.007	
I	1.000		1.000	1.000		1.000
II	0.117 (0.046-0.299)	<.001	0.187 (0.085-0.411)	0.144 (0.043-0.484)	.002	0.268 (0.098-0.734)
III	0.465 (0.224-0.965)	.04	0.534 (0.270-1.058)	0.404 (0.159-1.026)	.057	0.562 (0.238-1.325)
miR-331-3p expression	4.698 (2.276-9.696)	<.001	3.063 (1.716-5.466)	3.426 (1.524-7.702)	.003	2.355 (1.23-4.509)

CI, confidence interval; HR, hazard ratio.

Bold values are $P < 0.05$.

of miR-331-3p compared with normal bronchus cells. Among them, A549 (with the lowest miR-331-3p expression) and H1299 (with the highest miR-331-3p expression) were selected for further assays (Figure 2A). These 2 cell lines were infected with lentivirus to either cause overexpression or knockdown of miR-331-3p expression. The efficiency of the infected lentivirus was confirmed by qRT-PCR (Figures 2B,C and S1).

Transwell assays and wound healing assays were carried out to assess the role of miR-331-3p in NSCLC migration and invasion. Our results showed that A549 cells with elevated expression levels of miR-331-3p had fewer migratory (invasive) cells in the Transwell assay (migration: WT, 61.82 ± 9.67 ; negative control [NC], 71.23 ± 14.26 ; miR-331-3p, 16.44 ± 2.71 ; $P < .01$; invasion: WT, 89.45 ± 35.85 ; NC, 107.38 ± 41.72 ; miR-331-3p, 32.64 ± 9.89 ; $P < .001$) (Figure 2D) and a significantly slower rate of closure in the wound healing assay compared with those of WT and NC cells (24 hours: WT, 37.43 ± 7.30 ; NC, 42.25 ± 7.69 ; miR-331-3p, 16.23 ± 8.91 ; 48 hours: WT, 58.36 ± 25.96 ; NC, 57.25 ± 13.37 ; miR-331-3p, 28.38 ± 14.80 ; $P < .05$) (Figure 2E). Reduced miR-331-3p expression in H1299 cells generated opposite results. Anti-miR-331-3p-transfected H1299 cells showed more migratory (invasive) cells in the Transwell assay (migration: WT, 88.43 ± 13.95 ; NC, 57.88 ± 10.86 ; anti-miR-331-3p, 210.08 ± 39.29 ; $P < 0.001$; invasion: WT, 79.5 ± 11.09 ; NC, 77.43 ± 18.02 ; anti-miR-331-3p, 275.77 ± 35.73 ; $P < 0.01$) (Figure 2F) and a faster rate of closure in the wound healing assay compared with those of the NC group (24 hours: WT, 29.10 ± 6.67 ; NC, 39.57 ± 9.42 ; anti-miR-331-3p, 60.02 ± 10.75 ; 48 hours: WT, 54.35 ± 10.32 ; NC, 62.16 ± 7.20 ; anti-miR-331-3p, 91.08 ± 11.23 ; $P < 0.001$) (Figure 2G). Furthermore, EMT-specific markers were examined in the virus-infected A549 and H1299 cells. Interestingly, miR-331-3p upregulated A549, showing observably enhanced expression of the epithelial marker E-cadherin and remarkably reduced expression of the mesenchymal markers N-cadherin and vimentin. As expected, the reduction of miR-331-3p levels in H1299 cells resulted in E-cadherin downregulation and N-cadherin/vimentin upregulation (Figure 2H). Together, these results indicated that miR-331-3p acts as an EMT suppressor and impairs the migratory and metastatic capacity of tumor cells in NSCLC.

3.3 | MicroRNA-331-3p suppressed tumor metastasis of NSCLC cells in vivo

After identifying the inhibitory effect of miR-331-3p on NSCLC cell migration and invasion in vitro, we examined whether miR-331-3p had an impact on NSCLC metastasis in vivo. MicroRNA-331-3p-overexpressing A549 and NC cells were injected into the tail vein of nude

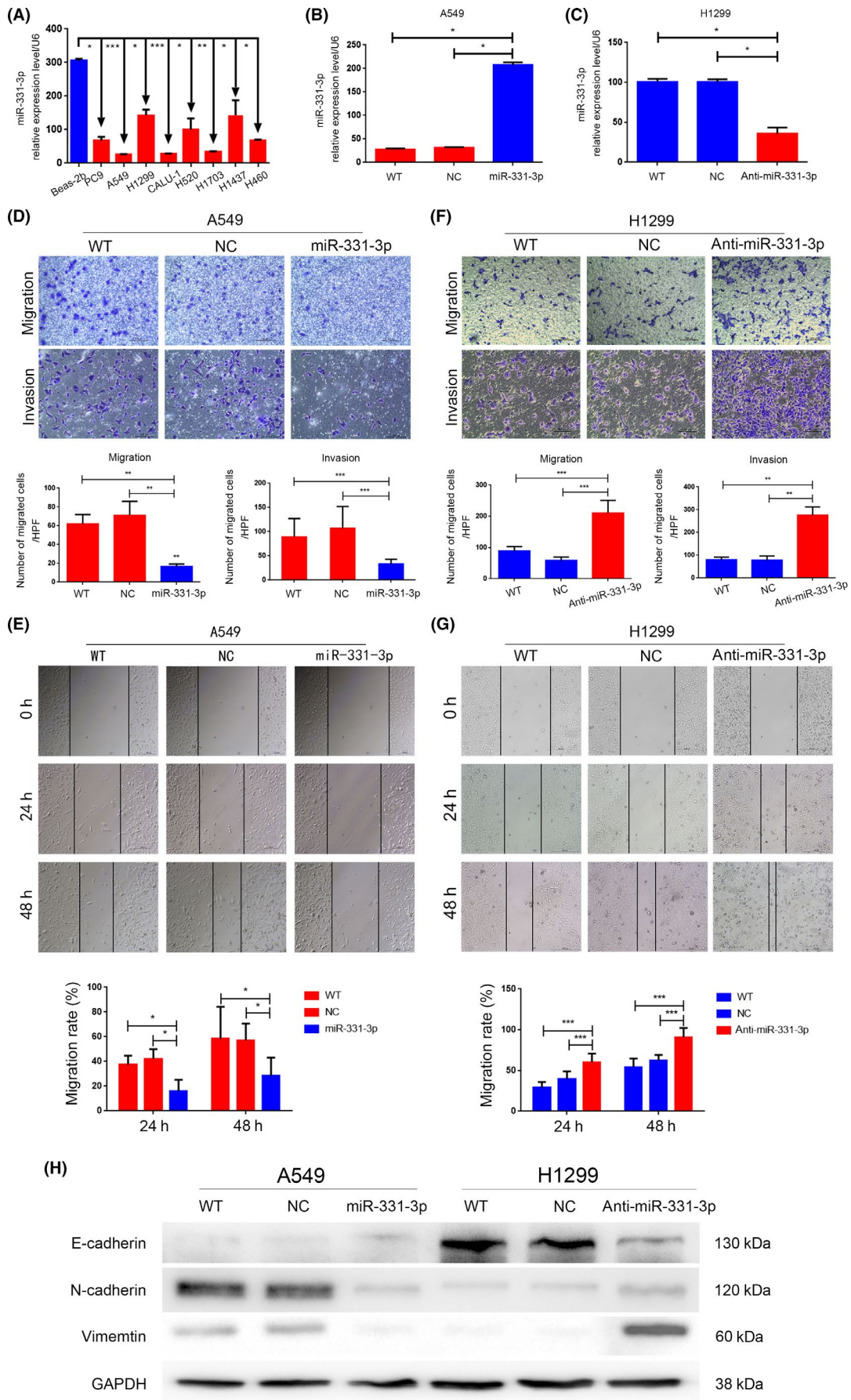
mice. After 28 days, all mice underwent bioluminescence imaging and were killed for the next assays (Figure 3). Our results showed that miR-331-3p-overexpressing A549-injected mice had weaker luminescence intensity compared with those injected with NC cells (0.023 ± 0.004 vs 0.053 ± 0.006 ; $P < 0.05$) (Figures 3D,E, and S2). The lungs of killed mice were collected, and the metastatic nodes were counted. The number of intrapulmonary metastatic nodes in NC mice was significantly higher than that of the miR-331-3p overexpression group (15.00 ± 1.87 vs 3.25 ± 1.09 ; $P < 0.05$) (Figure 3A-C). The immunohistochemistry results showed that the metastatic nodes from the miR-331-3p group had noticeably lower expression of ErbB2 and VAV2 compared with those of the NC (Figure 3A).

3.4 | Both ErbB2 and VAV2 are direct targets of miR-331-3p

To gain further insight into the molecular mechanisms by which miR-331-3p regulates NSCLC cell migration, we used 4 miRNA target databases (miRDB,¹⁸ miRWalk,¹⁹ TargetScan,²⁰ and miRTarBase²¹) to predict the putative targets of miR-331-3p. When we combined the results of the 4 databases, 23 genes were identified as miR-331-3p target candidates (Figure 4A, Table S1). Among them, carcinogenesis-related genes such as *ErbB2* and *VAV2* were of special interest to us. *ErbB2* and *VAV2* are canonical oncogenes, and bioinformatic tools predicted highly complementary sites in the 3'-UTR of both genes for the seed region of miR-331-3p. To validate these two targets, we established WT luciferase reporter constructs that included the 3'-UTR of the *ErbB2* and *VAV2* genes and mutant type (Mut) reporter constructs, which contained mutant binding sequences of miR-331-3p (Figure 4B). Then miR-331-3p and these constructs were cotransfected into the 293T cell line. Compared with the miR-NC transfected cells, 293T cells transfected with miR-331-3p showed significantly decreased luciferase activity compared with the WT constructs. In contrast, this repression was not observed with the transfection of the Mut constructs. These phenomena were observed in both the *ErbB2* and *VAV2* targeted assays (Figure 4C). Thus, the luciferase assays confirmed the direct binding of the *ErbB2* and *VAV2* 3'-UTR to the seed region of miR-331-3p.

Furthermore, western blot analysis was carried out, and the results showed that both *ErbB2* and *VAV2* were dramatically suppressed in the miR-331-3p-overexpressing A549 cells compared with those of the WT or NC cell lines. Otherwise, miR-331-3p-knockdown H1299 cells showed enhancement of *ErbB2* and *VAV2* compared with those of untreated cells or the blank virus group (Figure 4D). Interestingly, although western blot analysis showed a regulatory relationship between *ErbB2*/*VAV2* and miR-331-3p,

FIGURE 2 MicroRNA (miR)-331-3p suppressed the migration, invasion, and epithelial-mesenchymal transition (EMT) phenotype of non-small-cell lung cancer (NSCLC) cells. A, Quantitative RT-PCR results showed miR-331-3p levels in 8 NSCLC cell lines and normal bronchus cell lines. B,C, MiR-331-3p expression levels in miR-331-3p/anti-miR-331-3p-transfected A549 and H1299 cells. D,E, Representative images and quantification of the Transwell assay and wound healing assay using miR-331-3p-transfected A549 cells. F,G, Representative images and quantification of the Transwell and wound healing assays using anti-miR-331-3p-transfected H1299 cells. H, Western blot assay showing E-cadherin, N-cadherin, and vimentin protein levels in miR-331-3p/anti-miR-331-3p-transfected NSCLC cells. * $P < .05$ vs negative control (NC) group, ** $P < .01$ vs NC group, *** $P < .001$ vs NC group



qRT-PCR did not detect observable ErbB2/VAV2 mRNA changes at the transcriptional level after miR-331-3p-related treatment in either A549 or H1299 cells (Figure 4E). These results suggest that miR-331-3p silences ErbB2 and VAV2 expression by inhibiting the translation of their mRNAs. Afterward, we examined ErbB2/VAV2 expression in NSCLC specimen tissues by immunohistochemistry. We found an inverse correlation between miR-331-3p and ErbB2/VAV2 expression in NSCLC tumor tissues (ErbB2, $r = -0.435$, $P < .0001$; VAV2, $r = -0.2774$, $P = 0.0127$; Figure 4F,G). Taken together, our data indicated that both ErbB2 and VAV2 were direct targets of miR-331-3p.

3.5 | Biological functions of miR-331-3p driven by ErbB2/VAV2 inhibition

To investigate whether miR-331-3p inhibited NSCLC cell metastasis by mediating ErbB2 and VAV2, we promoted or repressed ErbB2 and VAV2 expression in miR-331-3p/anti-miR-331-3p-transfected NSCLC

cells. ErbB2 and VAV2 mimic/shRNA plasmids were constructed and cotransfected into A549/H1299 cells with miR-331-3p/anti-miR-331-3p (Figures 5A and S3a). Then the Transwell and wound healing assays were carried out. Our results showed that the restoration of neither ErbB2 nor VAV2 alone could fully recover the inhibitory effect on metastasis of miR-331-3p in A549 cells. Elevation of ErbB2 or VAV2 alone partly attenuated miR-331-3p-mediated inhibition of migration and invasion. However, when both ErbB2 and VAV2 were elevated, the migratory and invasive capacities of miR-331-3p-transfected A549 cells were completely restored. It is noteworthy that 4 μ M of 1A-116, a specific Rac1 inhibitor, significantly suppressed the migratory and invasive capacities after elevation of both ErbB2 and VAV2 in the miR-331-3p-transfected A549 cells (Figure 5B-E). In contrast, the inhibition of ErbB2 or VAV2 alone only partially blocked the effect of anti-miR-331-3p on the migration of NSCLC cells. After blocking both ErbB2 and VAV2, the migratory capacity of anti-miR-331-3p-transfected H1299 cells was fully suppressed to levels comparable to that of the NC level (Figure S3b-e). Overall, our results

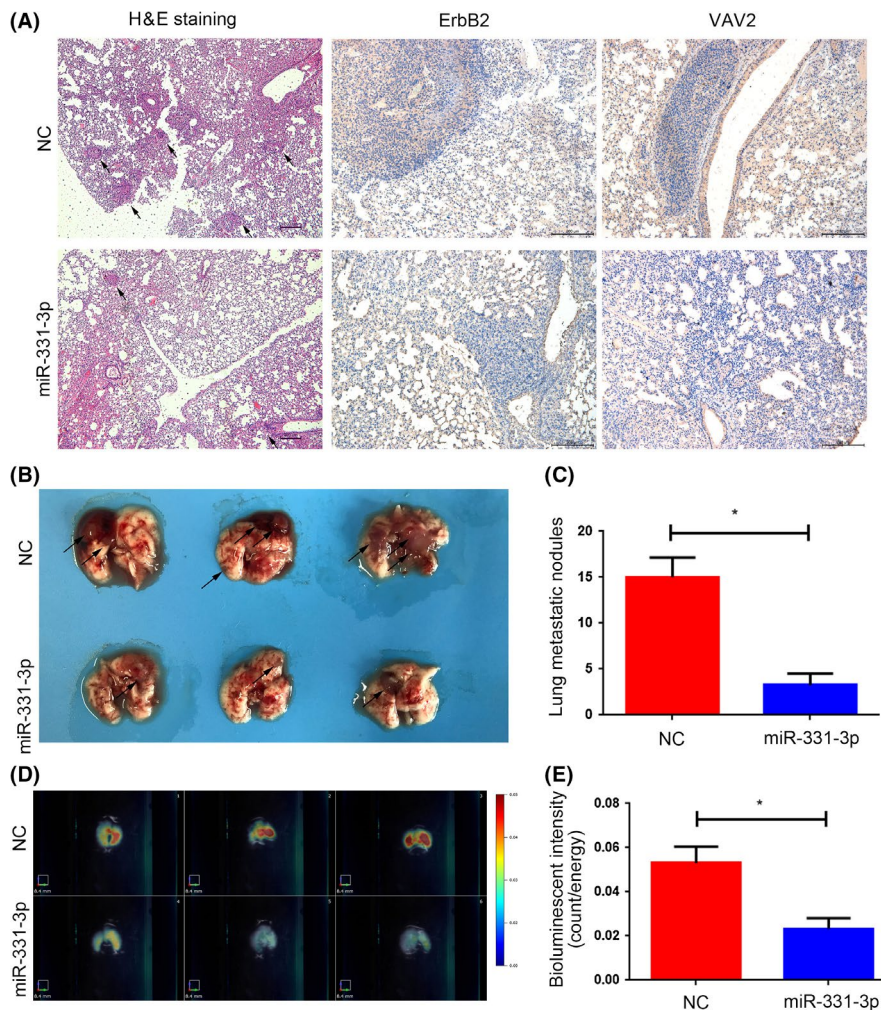


FIGURE 3 MicroRNA (miR)-331-3p inhibited the in vivo metastasis of non-small-cell lung cancer cells. A, Representative micrographs of metastatic tumor nodes in mouse lungs from i.v. metastasis assay. Arrows indicate the metastatic nodes with H&E staining, and the expression levels of ErbB2 and VAV2 were determined by immunohistochemistry. B,C, Representative photographs and quantification of metastatic tumor nodes in mouse lungs from the i.v. metastasis assay. D,E, Representative luciferase images and quantification of average luciferase intensity of lungs in the i.v. metastasis assay. * $P < .05$ vs negative control (NC) group

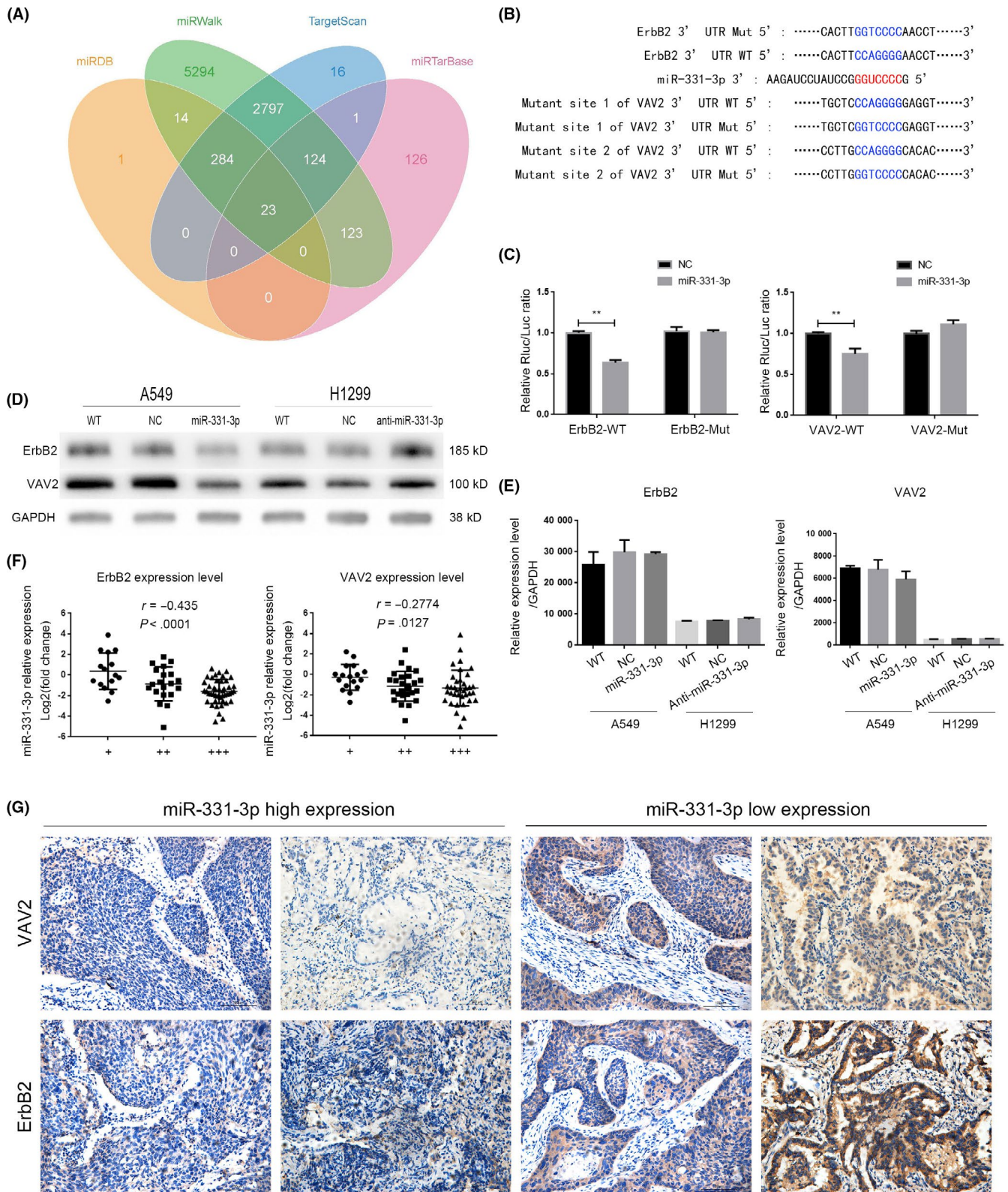


FIGURE 4 Both ErbB2 and VAV2 are direct targets of microRNA (miR)-331-3p. A, Four databases were used to predict targets of miR-331-3p. B, Schematic depicting the construction of the WT or mutant (Mut) ErbB2/VAV2 3'-UTR vectors. C, Luciferase reporter assay showed a direct binding association between ErbB2/VAV2 and miR-331-3p. D, Western blot assay showing the ErbB2/VAV2 protein levels in miR-331-3p-transfected A549 and anti-miR-331-3p-transfected H1299 cells. E, Quantitative RT-PCR showed the relative mRNA quantitation of ErbB2/VAV2 in miR-331-3p-transfected A549 and anti-miR-331-3p-transfected H1299 cells. F, Inverse correlation between the expression of miR-331-3p and ErbB2/VAV2 in non-small-cell lung cancer (NSCLC) tumor tissues. G, Representative immunohistochemistry photographs showing ErbB2/VAV2 expression in NSCLC tumor tissues with high/low miR-331-3p expression. ** $P < 0.01$

indicated that miR-331-3p impaired the migratory ability of NSCLC cells by repressing the expression of ErbB2 and VAV2.

3.6 | MicroRNA-331-3p inhibited EMT of NSCLC cells through the ErbB2/VAV2/Rac1/PAK1/ β -catenin signaling pathway

In previous experiments, we confirmed that miR-331-3p exerted its antimetastatic function by targeting ErbB2 and VAV2. *ErbB2* is a well-studied oncogene and has been proved to be a Rac1 activator in breast cancer²² and gastric cancer,²³ but its Rac1 activator function in NSCLC is not fully understood. VAV2 is also known as a direct activator of Rac1,^{24,25} and has been proved to form a complex with ErbB2.^{26,27} Taken together, we hypothesized that miR-331-3p suppressed the migratory ability of NSCLC by targeting ErbB2 and VAV2 through Rac1 signaling. To confirm this hypothesis, we evaluated the colocalization of ErbB2 and VAV2 by immunofluorescence. Our results showed that ErbB2 and VAV2 were colocalized in the cytoplasm, and ErbB2 was also observed in the cell nucleus (Figure 6A). Then, coimmunoprecipitation was carried out to confirm the direct interaction of ErbB2/VAV2/Rac1 in NSCLC cells. Our data showed that ErbB2 directly bound to VAV2 and Rac1 (Figure 6B,D). Furthermore, Rac1 activity was examined to confirm the regulation of Rac1 by miR-331-3p. We found that Rac1 activity was significantly repressed in the miR-331-3p-overexpressing A549 cells, and this repression was attenuated by ErbB2/VAV2 mimic transfection. The simultaneous elevation of ErbB2 and VAV2 fully recovered the Rac1 activity in miR-331-3p-transfected A549. Anti-miR-331-3p transfection remarkably promoted Rac1 activation. This intensification was partly blocked by ErbB2/VAV2 shRNA transfection, and silencing both ErbB2 and VAV2 repressed Rac1 activity to a level lower than that of knockdown of the individual proteins (Figure 6C). Moreover, the activity of PAK1 and β -catenin, a target of Rac1 and an EMT-related downstream molecule, was examined by western blotting. Our data confirmed the inverse correlation of phospho-PAK1 levels and phospho- β -catenin levels with miR-331-3p in both A549 and H1299 cells, and the regulation of ErbB2 and VAV2 attenuated the effects of miR-331-3p/anti-miR-331-3p. In addition, E-cadherin, N-cadherin, and vimentin were examined as EMT markers, and western blot assays showed that the restoration of ErbB2 and VAV2 blocked the anti-EMT effect of miR-331-3p in NSCLC cells (Figure 6E,F). Taken together, our results support the hypothesis that miR-331-3p suppresses EMT and metastasis in NSCLC by regulating ErbB2/VAV2 expression through the Rac1/PAK1/ β -catenin signaling pathway (Figure 6G).

4 | DISCUSSION

Non-small-cell lung cancer has become one of the deadliest malignancies in the world, but the molecular mechanisms involved in NSCLC development are not fully understood. Recent studies revealed that miRNAs play vital roles in the tumorigenesis and

progression of NSCLC.²⁸ In the present study, we focused on the antimetastatic function of miR-331-3p in NSCLC. The abnormal expression of miR-331-3p has been found in many types of carcinomas, but the evidence on its biological functions in the tumorigenesis of these cancers is conflicting. Our previous study showed that miR-331-3p promotes proliferation and metastasis of hepatocellular carcinoma,¹⁵ and other researchers found similar effects of miR-331-3p in pancreatic cancer.¹⁴ However, other researchers discovered antitumor functions of miR-331-3p in urothelial cancer,²⁹ gastric cancer,¹⁶ and cervical cancer.¹⁷ The functions of miR-331-3p in NSCLC were also ambiguous,^{30,31} and little was known about the targets and signaling pathways regulated by miR-331-3p in NSCLC. In the present study, we confirmed the downregulation of miR-331-3p in NSCLC tumor tissues and found that the relative expression level of miR-331-3p in NSCLC tumor tissues had a correlation with metastasis stage, clinical TNM stage, overall survival, and disease-free survival. The Cox regression multivariate analysis results showed that the expression of miR-331-3p in tumor tissues could be an independent prognostic marker for NSCLC patients. Next, we showed that overexpression of miR-331-3p suppressed the migration and invasion of NSCLC cells and that the EMT process was also inhibited by miR-331-3p elevation. In addition, the downregulation of miR-331-3p promoted the EMT-induced migratory and invasive capacity of NSCLC cells. Although *miR-331-3p* plays a role as a cancer suppressor gene and could be a potential prognostic marker in NSCLC, its therapeutic application would be limited because of its various functions across multiple cancer types. Lung-targeted pharmaceutical approaches should be used to enhance the targeting ability of miR-331-3p.

Generally, miRNAs exert their functions by direct cleavage and/or translational inhibition of target mRNAs. Recently, it has been reported that miR-331-3p has several targets in different cancers, such as PHLPP in hepatocellular cancer,¹⁴ ST7L in pancreatic cancer¹⁴ and NRP2 in cervical cancer.¹⁷ ErbB2 was also reported to be a target of miR-331-3p,¹⁶ but the signaling pathway that was regulated by miR-331-3p that targeted ErbB2 was unknown. Our results confirmed the regulation of miR-331-3p on ErbB2 and discovered that VAV2 was a novel direct target of miR-331-3p.

ErbB2 (*HER2*) was identified as a classical oncogene that promotes tumorigenesis in many malignancies, especially breast cancer, and the involvement of ErbB2 in NSCLC has also been known for many years.^{32,33} However, clinical studies targeting *ErbB2* mutations have yielded disappointing results. Elevated ErbB2 expression was found in many NSCLC patients, but the role of ErbB2 overexpression in lung cancer remains unclear.³⁴ There are various pathways that are regulated by ErbB2 that are involved in EMT, migration, and invasion of cancers. Among them, Rac1 activation-induced migratory enhancement by ErbB2 was reported in breast cancer and ovarian tumor cells.^{26,27} The VAV family of proteins is a group of well-studied guanine nucleotide exchange factors for Rho GTPases, and VAV2 is a widely expressed member of this group.^{24,35} Rac1 was proven to be the most important Rho GTPase target of VAV2, and ErbB2 was confirmed to form

A549

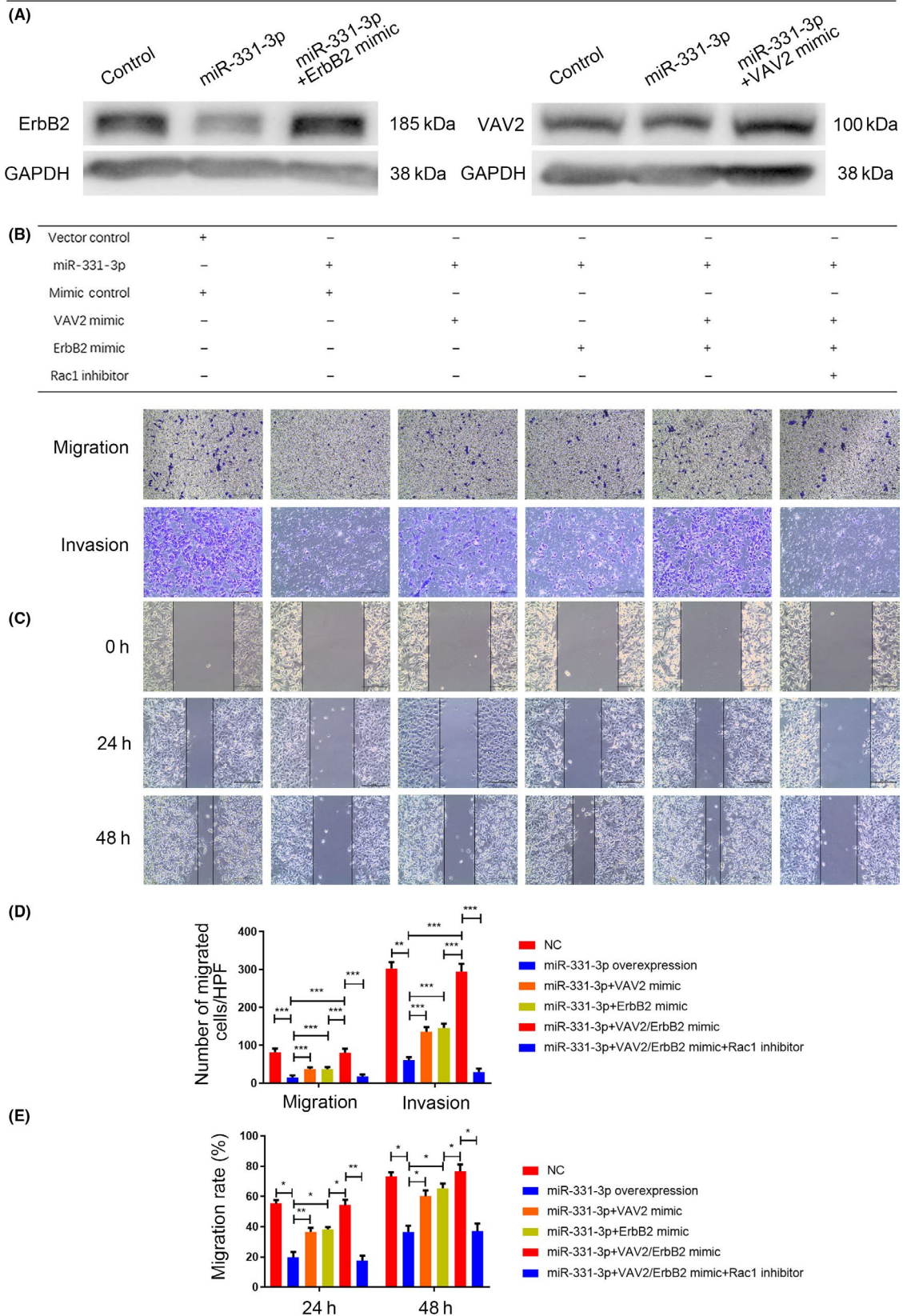
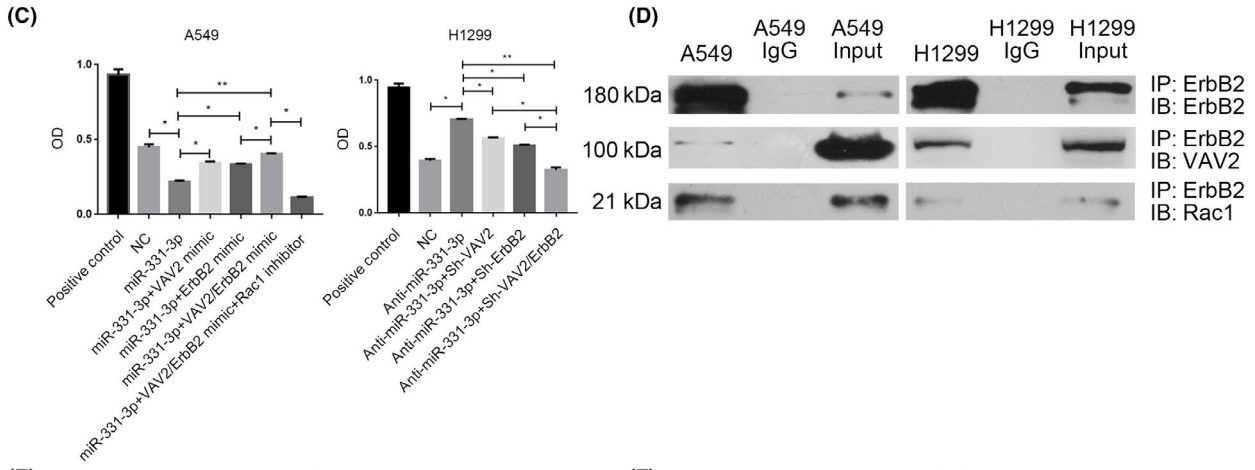
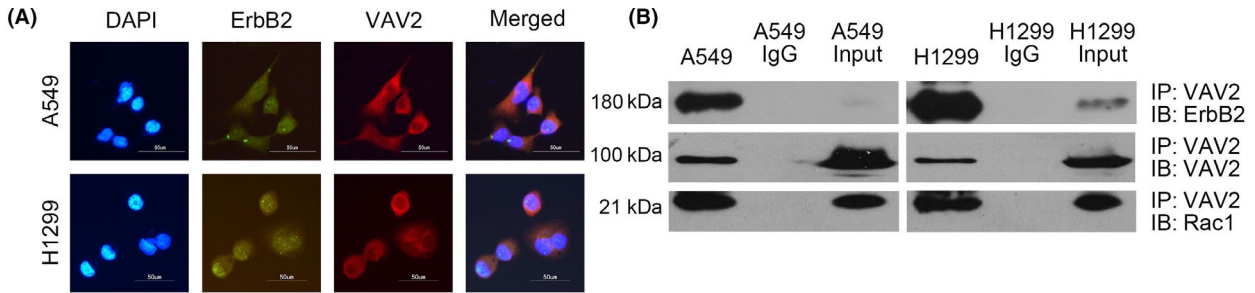


FIGURE 5 Rescue assays confirmed that ErbB2 and VAV2 were functional targets of microRNA (miR)-331-3p in A549 cells. A, ErbB2 and VAV2 expression in the miR-331-3p and ErbB2/VAV2 mimic-cotransfected A549 cells. B-E, Representative photographs and quantification of Transwell and wound healing assays in A549 cells cotransfected with miR-331-3p and ErbB2/VAV2 vectors. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. HPF, high-power field; NC, negative control



(E) A549

Vector control	+	-	-	-	-	-
miR-331-3p	-	+	+	+	+	+
Mimic control	+	+	-	-	-	-
VAV2 mimic	-	-	+	+	+	+
ErbB2 mimic	-	-	-	+	+	+
Rac1 inhibitor	-	-	-	-	-	+

(F) H1299

Vector control	+	-	-	-	-
Anti-miR-331-3p	-	+	+	+	+
shRNA-control	+	+	-	-	-
shRNA-VAV2	-	-	+	-	+
shRNA-ErbB2	-	-	-	+	+

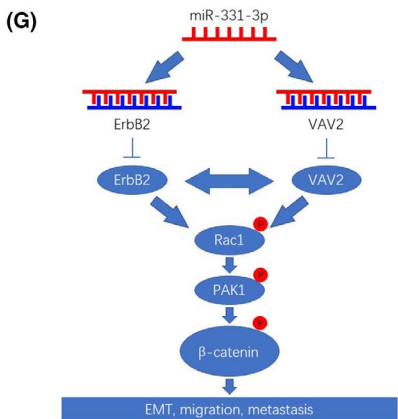
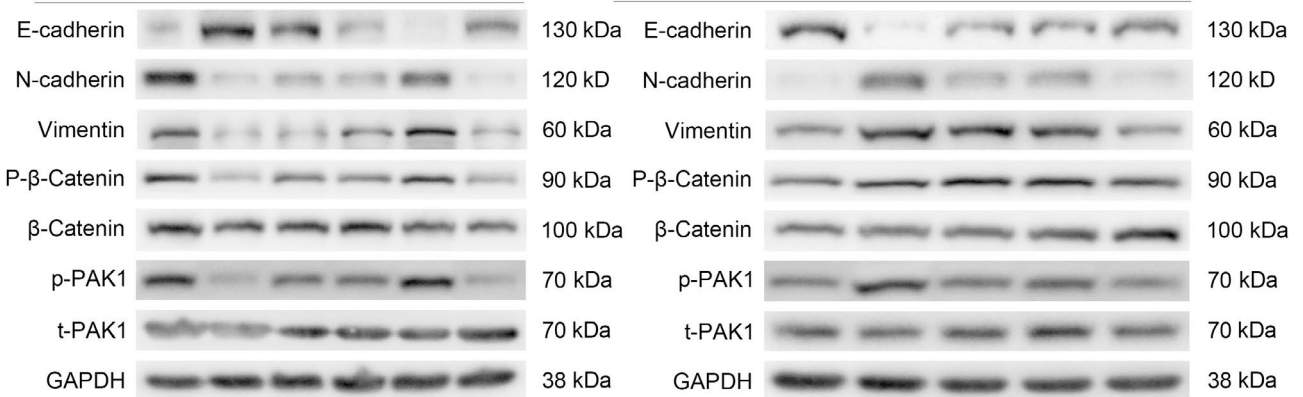


FIGURE 6 MicroRNA (miR)-331-3p inhibited the Rac1/PAK1/ β -catenin pathway and epithelial-mesenchymal transition (EMT) by targeting ErbB2 and VAV2 in non-small-cell lung cancer (NSCLC) cells. A, Immunofluorescence assay showed the colocalization of ErbB2 and VAV2. B, D, Coimmunoprecipitation assay showing the direct binding of ErbB2 with VAV2 and Rac1 in NSCLC cells. C, Rac1 activity assay results showing that miR-331-3p inhibited Rac1 activation by targeting ErbB2 and VAV2 in both A549 and H1299 cells. E, F, Western blot assay showing the activity of PAK1, and the phosphorylation of β -catenin and EMT suppressed by miR-331-3p. Restoration of ErbB2 and VAV2 attenuated this suppression in NSCLC cells. G, MiR-331-3p suppresses EMT, migration, and metastasis of NSCLC cells by targeting ErbB2/VAV2 through the Rac1/PAK1/ β -catenin pathway. * $P < 0.05$; ** $P < 0.01$. IB, immunoblot; IP, immunoprecipitant; OD, optical density

a complex by binding with VAV2 and Rac1; activated Rac1 promoted the phosphorylation of PAK1, which regulated the migration and invasion of cancer cells.²⁶ To date, this pathway has not been proven in NSCLC. Our research first found that both ErbB2 and VAV2 were direct targets of miR-331-3p and that miR-331-3p functions not by degrading target mRNAs but by suppressing the translation of ErbB2/VAV2 mRNAs. Moreover, we first indicated that miR-331-3p exerted its antimigration function in NSCLC by direct regulation of the ErbB2/VAV2/Rac1 pathway.

The EMT process comprises a spectrum of intermediate states between epithelial and mesenchymal phenotypes and plays a critical role in the invasion and metastasis of lung cancer.^{28,36,37} In this study, we examined key biomarkers of EMT, such as E-cadherin, N-cadherin, and vimentin, and found that miR-331-3p suppressed EMT activation in NSCLC cells. The regulation of ErbB2 and VAV2 expression was proved to attenuate the EMT repression by miR-331-3p in NSCLC cells. Numerous pieces of evidence have shown that Rho proteins, especially Rac1 activation, have a vital role in both junctional assembly and disassembly.³⁸ Our data showed that Rac1 activity was significantly repressed, and the phosphorylation of PAK1, a downstream effector of Rac1, was remarkably impaired after miR-331-3p transfection. Furthermore, the activity of β -catenin, another key effector of EMT, was also impeded in miR-331-3p-overexpressing NSCLC cells. These pathway disruptions could be attenuated by ErbB2 and/or VAV2 elevation. Taken together, our results indicated that miR-331-3p suppressed the EMT process in NSCLC cells by targeting ErbB2/VAV2 through the Rac1/PAK1/ β -catenin pathway.

Our findings reveal that miR-331-3p is downregulated in NSCLC tumor tissues, and its expression level has a correlation with tumor metastasis and patient survival. MicroRNA-331-3p suppresses EMT activation, migration, and invasion of NSCLC cells by targeting ErbB2/VAV2 through the Rac1/PAK1/ β -catenin pathway.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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