The miR-23a/27a/24-2 cluster promotes postoperative progression of early-stage non-small cell lung cancer

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Even with optimal surgery, many early-stage non-small cell lung cancer (NSCLC) patients die of recurrence. Unfortunately, there are no precise predictors for postoperative recurrence in early-stage NSCLC, and the recurrence mechanism is still unclear. In this study, we found that simultaneous overexpression of all miRNAs in the miR-23a/27a/24-2 cluster was closely associated with postoperative recurrence, β-catenin upregulation and promoter methylation of p16 and CDH13 in early-stage NSCLC patients. In addition, *in vitro* and *in vivo* experiments show that overexpression or inhibition of all miRNAs in the miR-23a/27a/24-2 cluster significantly stimulated or inhibited NSCLC cell stemness, tumorigenicity and metastasis. Furthermore, we demonstrated that the miR-23a/27a/24-2 cluster miRNAs activated Wnt/β-catenin signaling by targeting their suppressors and stimulated promoter methylation-induced silencing of p16 and CDH13 by affecting DNA methylation-related genes expression. Our findings suggest that simultaneous high expression of all miRNAs in the miR-23a/27a/24-2 cluster represents a new biomarker for predicting postoperative recurrence in early-stage NSCLC. The miR-23a/27a/24-2 cluster miRNAs stimulate early-stage NSCLC progression through simultaneously stimulating Wnt/β-catenin signaling, and promoter methylation-induced tumor suppressor genes silencing. In addition, simultaneous inhibition of all miRNAs in the miR-23a/27a/24-2 cluster may be a useful strategy for treatment of early-stage NSCLC recurrence.

INTRODUCTION

Lung cancer remains the leading cause of cancer incidence and mortality worldwide.[1] Non-small cell lung cancer (NSCLC) is the main subtype of lung cancer and accounts for 75%–80% of all cases, and approximately 30% of NSCLC patients are diagnosed with early-stage NSCLC (stage I and II).[2] Surgery is the standard treatment for early-stage NSCLC; however, 15% of stage I and 40% of stage II NSCLC patients develop postoperative recurrence, which is the major cause of treatment failure.[3] Thus, identifying biomarkers for predicting recurrence after curative resection will be beneficial for patient care. Unfortunately, biomarkers that can accurately predict postoperative recurrence of early-stage NSCLC have not been identified and the underlying mechanisms of recurrence are still unclear.

Recent studies have found that several factors are associated with the postoperative progression of early-stage NSCLC, including high expression of β-catenin.[4] β-catenin is a central signal transducer of Wnt/β-catenin signaling, and hyperactivated Wnt/β-catenin signaling stimulates cancer stem cell (CSC) development.[5,6] Importantly, CSCs play a crucial role in promoting tumor recurrence because these cells present self-renewal, high invasiveness, high tumorigenicity, and can differentiate into all tumor cell types.[7] In addition, studies have shown that aberrant methylation in the promoters of certain tumor suppressor genes (TSGs) and the abnormal promoter methylation-induced downregulation of TSGs are associated with postoperative progression of early-stage NSCLC, including p16 and cadherin 13 (CDH13).[8–10] Thus, investigating the deregulation mechanism of Wnt/β-catenin signaling and promoter methylation of TSGs are important for understanding the postoperative progression of early-stage NSCLC and promoting the future development of novel therapeutic strategies.

miRNAs are small noncoding RNAs that inhibit gene expression through translational repression and mRNA degradation by binding to the 3′ UTR of target genes.[11] Studies show that most types of cancer present dysregulated miRNA expression and that aberrantly expressed miRNAs are correlated with postoperative recurrence,[12] Wnt/β-catenin signaling hyperactivation,[6] and abnormal methylation-induced TSG downregulation in lung cancer.[13] These findings suggest that miRNAs may represent potential biomarkers for predicting postoperative recurrence of NSCLC and could contribute to recurrence by activating Wnt/β-catenin signaling and abnormal methylation-induced TSG silencing.

In this study, we demonstrated that simultaneously upregulated expression level of all miRNAs in the miR-23a/27a/24-2 cluster is independent predictor for postoperative recurrence and poor outcome
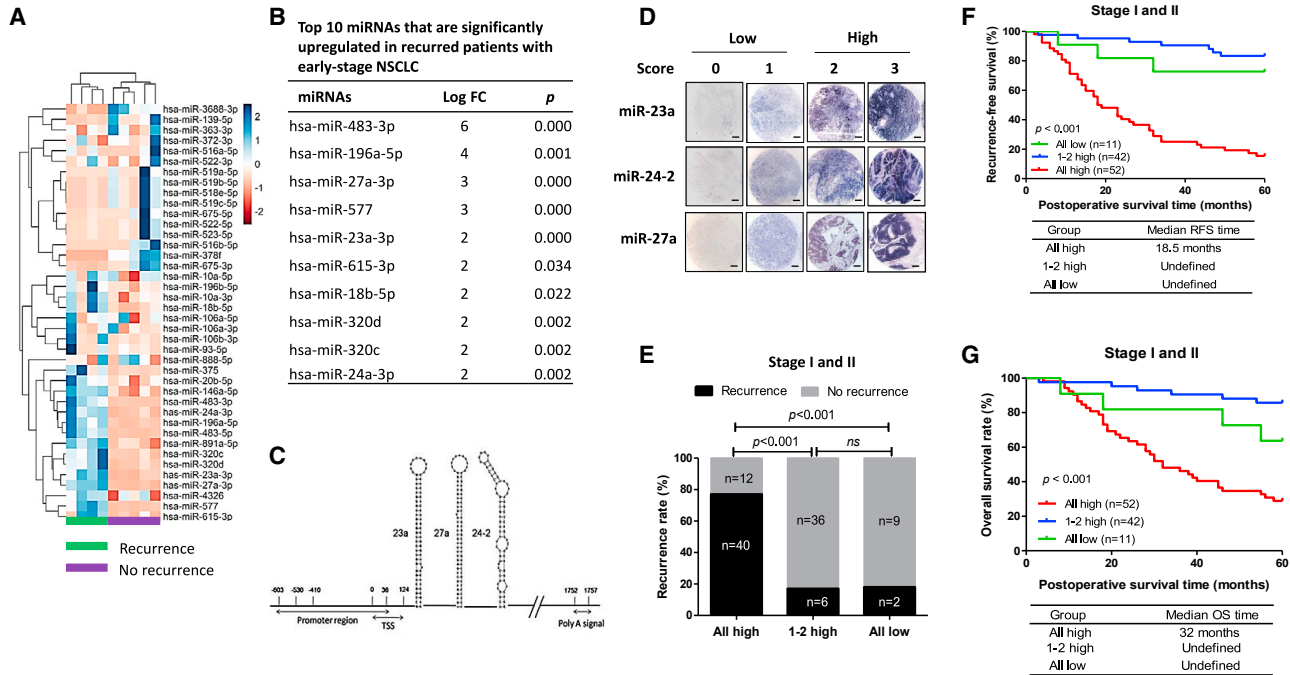
Figure 1. Simultaneously upregulated expression levels of miR-23a, miR-27a, and miR-24-2 in primary tumors closely associated with recurrence and poor prognosis in postoperative early-stage NSCLC

(A) Heatmap showing that miRNAs were differentially expressed in primary tumors from patients with early-stage NSCLC who relapsed within 40 months after surgery (n = 4) and those who did not relapse within 5 years after surgery (n = 5). (B) List of the top 10 miRNAs that were significantly upregulated in primary tumors from patients with early-stage NSCLC who relapsed within 40 months after surgery compared with those who did not relapse within 5 years after surgery. (C) The structure of pre-miR-transcript of hsa-miR-23a/27a/24-2 cluster showed that the hsa-miR-23a/27a/24-2 cluster contained three miRNAs: hsa-miR-23a, hsa-miR-27a, and hsa-miR 24-2. (D) miRNA expression level of the miR-23a/27a/24-2 cluster in primary tumor tissues of early-stage NSCLC are detected using *in situ* hybridization. (E) Simultaneous high expression of miR-23a, miR-27a, and miR-24-2 closely correlated with postoperative recurrence in patients with early-stage NSCLC. (F) Simultaneous high expression of miR-23a, miR-27a, and miR-24-2 in primary tumors significantly correlated with poor recurrence-free survival in patients with early-stage NSCLC. (G) Simultaneous high expression of miR-23a, miR-27a, and miR-24-2 in primary tumors significantly correlated with poor overall survival in patients with early-stage NSCLC. RFS, recurrence-free survival; OS, overall survival. Scale bars, 200 μm.

in early-stage NSCLC patients. Overexpression of all miRNAs in the miR-23a/27a/24-2 cluster-stimulated NSCLC progression by simultaneously stimulating Wnt/β-catenin signaling and promoter methylation-induced TSG downregulation.

RESULTS
High expression of all miRNAs in the miR-23a/27a/24-2 cluster is associated with postoperative recurrence and poor prognosis in early-stage NSCLC patients

To identify the miRNAs associated with postoperative recurrence of early-stage NSCLC, we performed small RNA sequencing using primary tumors from early-stage NSCLC patients who relapsed within 40 months after surgery and patients who did not relapse within 5 years after surgery, because 80% of early-stage NSCLC recurrences occur within 40 months after surgery.[14] The miR-23a/27a/24-2 cluster includes miR-23a, miR-27a, and miR-24-2,[15] and previous studies have shown these miRNAs play an oncogenic role in gastric cancer[16] and glioma through synergistic effects.[17] But there is no research reported about miR-23a/27a/24-2 cluster and NSCLC recurrence. Our mRNA sequencing results showed that miR-23a, miR-27a, and miR-

24-2 are highly expressed in the primary tumors of the early-stage NSCLC patients who had postoperative relapse and were in the top 10 molecules with the greatest changes (Figures 1A–1C), suggesting that these miRNAs may be involved in the postoperative progression of early-stage NSCLC. To prove this hypothesis, based on the hybridization scores of the miR-23a/27a/24-2 cluster miRNAs in primary tumors (Figure 1D), 105 postoperative early-stage NSCLC patients (stage I–II) were divided into the following three groups: all miRNAs with high expression, all miRNAs with low expression, and high expression of one to two miRNAs in the miR-23a/27a/24-2 cluster. Then, the correlation between miRNA expression levels and postoperative progression of early-stage NSCLC patients was analyzed. As shown in Figure 1E, 77% of the patients in the all miRNA high-expression group experienced postoperative recurrence, and they accounted for 83% of the total relapsed cases. However, only 14% of the patients in the one to two miRNA high-expression group and 18% of the patients in the all miRNAs low expression group experienced postoperative recurrence (Figure 1E). Notably, patients in the all miRNAs high expression group had a shorter median recurrence-free survival (RFS) time and a lower RFS rate than the patients

Table 1. Multivariable analyses of prognostic factors for overall and recurrence-free survival in stage I or II NSCLC patients

Variable	Recurrence-free survival		Overall survival	
	HR (95% CI)	p	HR (95% CI)	p
Gender	1.03(((0.52–2.06)	0.93	0.62((0.33–1.17)	0.14
Age	0.83 ((0.45–1.53)	0.54	0.66((0.39–1.11)	0.12
Histologic type	1.14(((0.60–2.17)	0.69	0.83((0.49–1.40)	0.48
T status	0.80((0.45–0.92)	0.04	0.88 (0.56–1.39)	0.59
N status	1.75 (1.58–2.29)	0.02	1.51 (1.29–1.90)	0.02
miR level	2.04 (1.38–5.95)	0.01	1.96 (1.35–2.65)	0.01
Methylation	1.45 (1.23–1.89)	0.02	1.47 (1.27–1.81)	0.01
β-catenin level	1.28 (1.01–1.84)	0.03	1.45 (1.12–1.73)	0.04

HR, hazard ratio; CI, confidence interval; methylation, simultaneous methylation of p16 and CDH13; miR level, simultaneous high expression of miR-23a, miR-27a, and miR-24-2.

in the other two groups (Figure 1F). Similar results were observed in the 5-year overall survival rate analysis (Figure 1G). In addition, our multivariable analyses showed that simultaneous high expression of all miRNAs in the miR-23a/27a/24-2 cluster is an independent predictor for postoperative recurrence and poor prognosis in early-stage NSCLC patients (Table 1). Altogether, our findings indicate that high expression level of all miRNAs in the miR-23a/27a/24-2 cluster may represent a biomarker for predicting postoperative recurrence and poor prognosis in early-stage NSCLC. In addition, simultaneous overexpression of all miRNAs in the miR-23a/27a/24-2 cluster may have synergistic stimulatory effects on early-stage NSCLC progression.

Overexpression of all miRNAs in the miR-23a/27a/24-2 cluster significantly promoted NSCLC progression

To investigate whether miRNAs in the miR-23a/27a/24-2 cluster play a synergistic role in NSCLC progression, we selected NSCLC cell lines with high or low expression of all miRNAs in the miR-23a/27a/24-2 cluster (Figure S1A) and transfected them with inhibitor or mimics of the miR-23a/27a/24-2 cluster miRNAs (Figure S1B), respectively. Then, these cells were subjected to malignancy analysis. Our results showed that overexpression or inhibition of all miRNAs in the miR-23a/27a/24-2 cluster more significantly promoted or suppressed NSCLC cell sphere formation (Figure 2A), soft agar colony formation (Figure 2B), and cell invasion and migration than single miRNA (Figures 2C and 2D). These findings indicate that simultaneous overexpression of all miRNAs in the miR-23a/27a/24-2 cluster has a synergistic effect in promoting NSCLC cell stemness, tumorigenicity, and metastasis. In addition, inhibition of all miRNAs in the miR-23a/27a/24-2 cluster may be an effective strategy for inhibiting NSCLC progression.

Furthermore, we confirm the effects of the miR-23a/27a/24-2 cluster miRNAs on NSCLC progression *in vivo*. First, we generated NSCLC cell lines that stably express high or low levels of the miR-23a/27a/24-2 cluster miRNAs (Figure S1C). Then, using these cell lines we generated subcutaneous xenograft models. Our experiments showed that NSCLC cells that overexpressed the miR-23a/27a/24-2 cluster displayed higher tumorigenicity than the control (Figure 3A). Notably, only NSCLC cells overexpressing the miR-23a/27a/24-2 cluster formed visible tumors when 5×10^3 cells were implanted (Figure 3A), and obviously upregulated expression levels of cancer stemness markers, such as CD133 and ALDH1, compared with the control (Figure 3B). In contrast, inhibition of the miR-23a/27a/24-2 cluster (Figure S1C) dramatically inhibited NSCLC cell tumorigenicity (Figure 3C). Then, we investigated the effects of the miR-23a/27a/24-2 cluster on NSCLC metastasis *in vivo*. As shown in Figure 3D, tumor nodule formation on the lung surface significantly increased or decreased with overexpression or inhibition of the miR-23a/27a/24-2 cluster compared with their control in tail vein injection lung metastasis models. Similar results were further observed in orthotopic models (Figure 3E). These findings suggest that overexpression of the miR-23a/27a/24-2 cluster strongly stimulates NSCLC progression *in vivo* and that inhibition of the miR-23a/27a/24-2 cluster is an effective strategy for the treatment of NSCLC.

Overexpression of all miRNAs in the miR-23a/27a/24-2 cluster significantly downregulated suppressors of Wnt/β-catenin signaling and upregulated β-catenin in NSCLC

To investigate the oncogenic mechanism of the miR-23a/27a/24-2 cluster miRNAs in NSCLC, we performed mRNA sequencing using NSCLC cells overexpressing all miRNAs in the miR-23a/27a/24-2 cluster and their control cells. The results showed that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster increased the mRNA expression levels of β-catenin (CTNNB1) and its downstream genes CD44 and c-Myc (Figure 4A). In addition, gene set enrichment analysis results show that overexpression of the miR-23a/27a/24-2 cluster miRNAs associated with activation of Wnt/β-catenin signaling pathway in NSCLC cells (Figure 4B). Similar results were observed in immunohistochemistry (IHC) analysis of tumor tissues in a xenograft model (Figure 4C). Importantly, western blot analysis showed that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster more significantly upregulated β-catenin, CD44, and c-Myc than single miRNA overexpression (Figure 4D). In contrast, inhibition of all miRNAs of the miR-23a/27a/24-2 cluster more significantly downregulated the expression of β-catenin, CD44, and c-Myc than inhibition of the single miRNA (Figure 4D). This synergistic effect on β-catenin expression was further confirmed by immunofluorescence (IF) in NSCLC cells (Figure 4E), suggesting that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster significantly activates Wnt/β-catenin signaling through a synergistic effect in NSCLC.

Furthermore, mRNA sequencing results also showed that overexpression of all miNRAs in the miR-23a/27a/24-2 cluster simultaneously inhibited multiple suppressors of the Wnt/β-catenin signaling, namely, nemo-like kinase (NLK), glycogen synthase kinase 3β (GSK3β), and CTNNBIP1 (Figure 4A). Notably, overexpression of all miRNAs in the miR-23a/27a/24-2 cluster more significantly inhibited NLK, GSK3β, and CTNNBIP1 expression than single
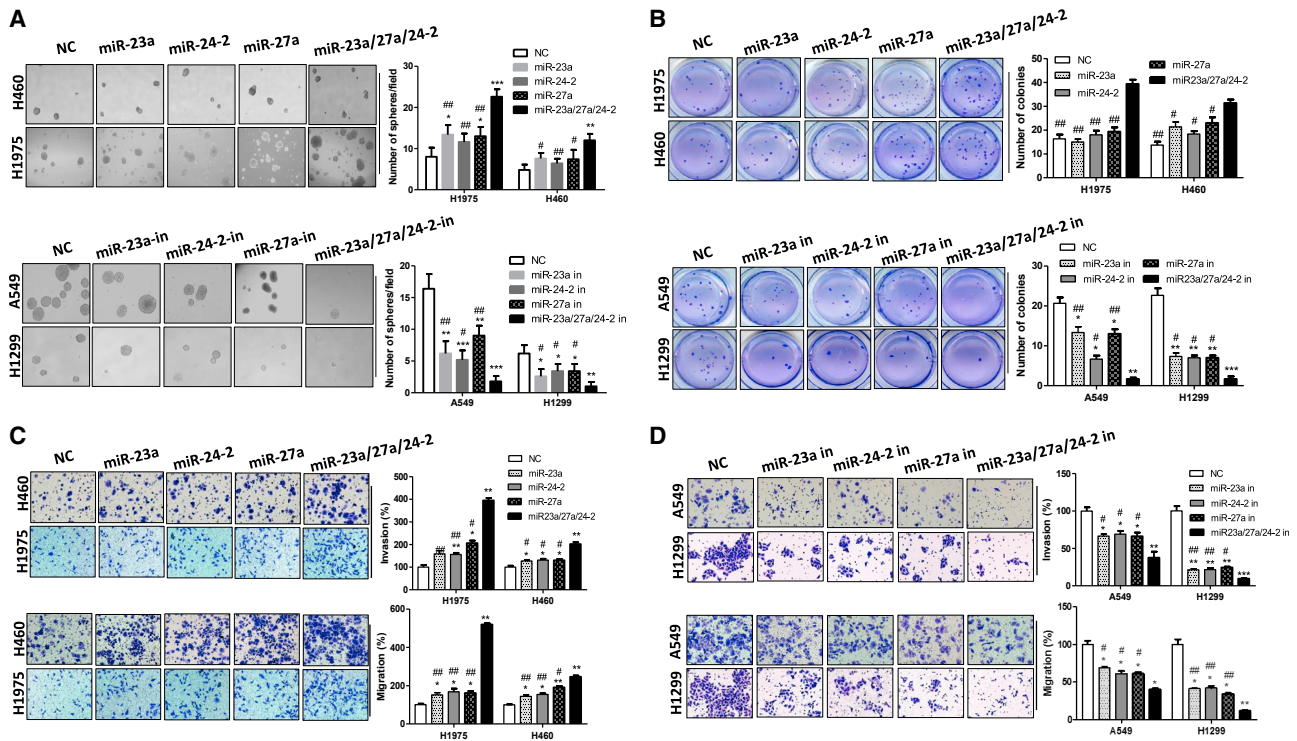
Figure 2. miRNAs in the miR-23a/27a/24-2 cluster play a synergistic role in promoting cancer stemness, soft agar growth, invasion, and migration of NSCLC cells

(A) Compared with individual miRNA, the overexpression or inhibition of all miRNAs in the miR-23a/27a/24-2 cluster more significantly stimulated or inhibited the sphere formation of NSCLC cells. (B) Compared with individual miRNA, the overexpression or inhibition of all miRNAs in the miR-23a/27a/24-2 cluster more significantly stimulated or inhibited the soft agar colony formation of NSCLC cells. (C) Compared with individual miRNA, the overexpression of all miRNAs in the miR-23a/27a/24-2 cluster more significantly stimulated invasion and migration of NSCLC cells. (D) Compared with individual miRNA, the inhibition of all miRNAs in the miR-23a/27a/24-2 cluster more significantly suppressed invasion and migration of NSCLC cells. Indicated cells were transfected with the indicated miRNA mimics and were then subjected to functional analysis. Indicated cells were transfected with the indicated miRNA mimics. After 48 h of transfection, cells were subjected to analysis. NC, negative control oligonucleotides; in, inhibitor; *p < 0.05 compared with NC; **p < 0.01 compared with NC; ***p < 0.001 compared with NC; #p < 0.05 compared with the miR-23a/27a/24-2 group; ##p < 0.01 compared with miR-23a/27a/24-2 group; ###p < 0.001 compared with the miR-23a/27a/24-2 group. Scale bars, 100 µm.

miRNAs, and only overexpression of all miRNAs in the miR-23a/27a/24-2 cluster simultaneously inhibited the expression of NLK, GSK3β, and CTNNBIP1 (Figure 4F). Then, using computational algorithm analysis, we determined that each 3′ UTR of NLK, GSK3β, and CTNNBIP1 contains sequences that can bind with two to three miRNAs of the miR-23a/27a/24-2 cluster (Figure 4G). In addition, a luciferase assay showed that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster can simultaneously more effectively inhibit the luciferase regulated by 3′ UTR of NLK, GSK3β, and CTNNBIP1 (Figure 4H). However, we did not observe a difference between the control and overexpression of all miRNAs in the miR-23a/27a/24-2 cluster groups when luciferase was regulated by mutated 3′ UTRs of the target genes (Figure 4H). Furthermore, using an Ago2 RNA immunoprecipitation (RIP) assay we demonstrated that miR-23a/27a/24-2 cluster miRNAs increase interaction between the Ago2 complex and mRNA target genes (Figure S2). These findings indicate that miRNAs in the miR-23a/27a/24-2 cluster inhibit the expression of NLK, GSK3β, and CTNNBIP1 by directly binding to their 3′ UTRs

in NSCLC. Together, these data suggest that each miRNA of the miR-23a/27a/24-2 cluster simultaneously targets multiple suppressors of Wnt/β-catenin signaling, and that multiple miRNAs of the miR-23a/27a/24-2 cluster simultaneously target the same suppressor of Wnt/β-catenin signaling. Therefore, multiple suppressors of Wnt/β-catenin signaling are simultaneously suppressed more effectively by overexpression of all miRNAs of the miR-23a/27a/24-2 cluster.

Overexpression of all miRNAs in the miR-23a/27a/24-2 cluster inhibited DNA methylation-regulated TSG expression

As shown in Figure 4A, the mRNA sequencing data also showed that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster inhibited the mRNA expression of TSGs that are negatively correlated with lung cancer recurrence and frequently downregulated in lung cancer due to promoter hypermethylation, including p16, CDH13, and WIF1.[10,18,19] In addition, overexpression of all miRNAs in the miR-23a/27a/24-2 cluster upregulated DNA methyltransferase 3B, an enzyme that promotes DNA methylation, but inhibited mRNA
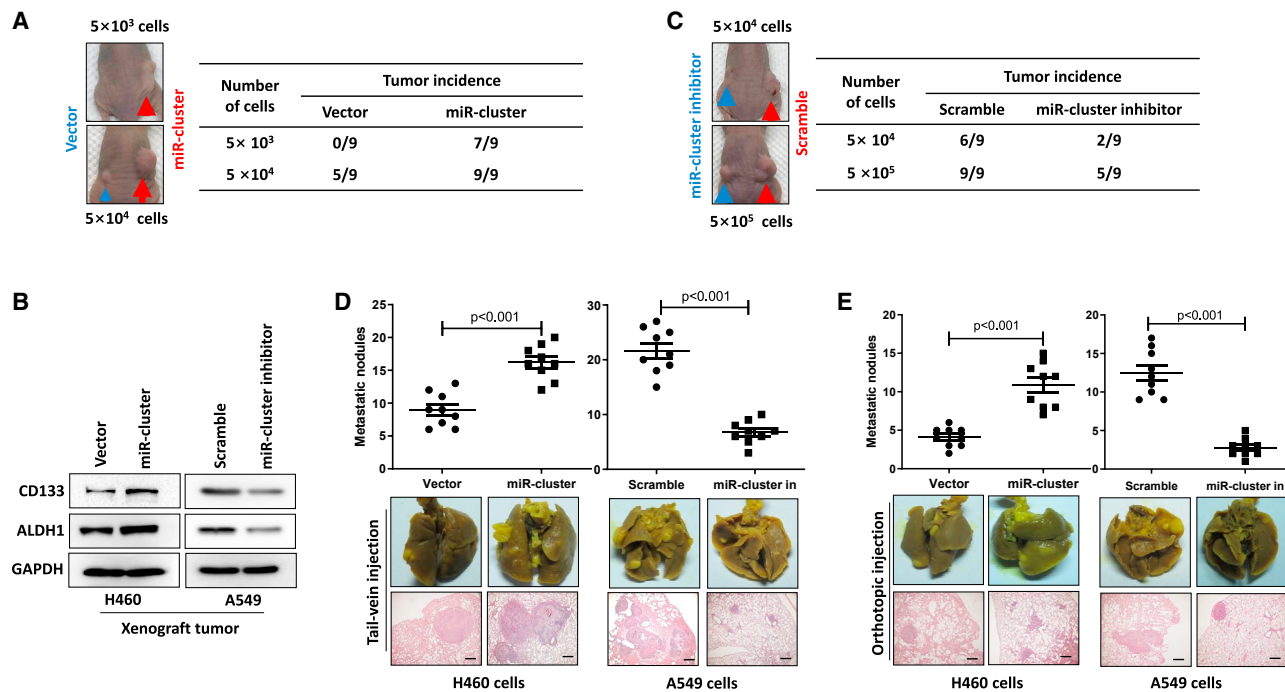
Figure 3. Simultaneous overexpression of miR-23a, miR-27a, and miR-24-2 enhanced the tumorigenicity, stemness, and metastasis of NSCLC cells *in vivo*
(A) Simultaneous overexpression of miR-23a, miR-27a, and miR-24-2 significantly enhanced NSCLC cell tumorigenicity in nude mice (n = 9 per group). Empty vector (Vector) or miR-23a/27a/24-2 cluster expression vector (miR-23a/27a/24-2)-transfected H460 cells were implanted in nude mice. (B) The stem cell markers CD133 and ALDH1 were significantly increased or decreased in tumors with simultaneous overexpression or inhibition of miR-23a, miR-27a, and miR-24-2 compared with vector control, respectively. (C) Simultaneous inhibition of miR-23a, miR-27a, and miR-24-2 significantly inhibited NSCLC cell tumorigenicity in nude mice (n = 9 per group). Scramble or expressing antisense nucleotides of miR-23a, miR-27a, and miR-24-2 plasmid (miR-23a/27a/24-2 in)-transfected A549 cells were implanted in nude mice. (D) Tail vein or (E) orthotopic injection lung metastatic model experiments show that miRNAs in the miR-23a/27a/24-2 cluster positively regulated NSCLC cell lung metastasis. n = 9 per group. Scale bars, 100 μm.

expression of the DNA demethylation enzyme tet methylcytosine dioxygenase 1 (TET1) in NSCLC cells (Figure 4A). These results were further confirmed by IHC analysis in xenograft tumor tissues (Figure 5A). Notably, western blot analysis showed that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster more significantly suppressed p16, CDH13, WIF1, and TET1 protein expression and more significantly increased DNMT3B expression in NSCLC cells than single miRNA overexpression (Figure 5B). In addition, only overexpression of all miRNAs in the miR-23a/27a/24-2 cluster simultaneously inhibited the expression of CDH13, p16, WIF1, and TET1 and upregulated DNMT3B expression in NSCLC cells (Figure 5B). Importantly, overexpression of the miR-23a/27a/24-2 cluster significantly increased promoter methylation of WIF, CDH13, and WIF1 in human pulmonary alveolar epithelial cell line (HPA-Epic) cells (Figure 5C). In addition, treatment with the DNA methylation inhibitor 5-azad (Figure 5D) or the combination of TET1 overexpression and DNMT3B silencing (Figure 5E) dramatically restored WIF1, CDH13, and p16 expression, which was inhibited by the miR-23a/27a/24-2 cluster miRNAs. These findings indicate that the overexpression of all miRNAs in the miR-23a/27a/24-2 cluster promotes DNA methylation regulated by DNMT3B and TET1 through a synergistic effect, thereby significantly inhibiting TSG expression in NSCLC.

Next, we investigated the mechanism by which miRNAs in the miR-23a/27a/24-2 cluster regulate the expression of DNMT3B and TET1. Our data showed that the miR-23a/27a/24-2 cluster inhibited the expression of the DNMT3B-negative regulator forkhead box O3 (FOXO3) (Figures 4A, 5A, and 5B) and that overexpression of FOXO3 inhibited the miR-23a/27a/24-2 cluster-induced upregulation of DNMT3B in NSCLC cells (Figure 5F). We then found that the 3′ UTRs of FOXO3 and TET1 contain binding sequences for one to three miRNAs of the miR-23a/27a/24-2 cluster (Figure 5G). In addition, a luciferase assay showed that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster significantly inhibited luciferase activity regulated by the 3′ UTR of TET1 or FOXO3 compared with the control (Figure 5H). However, this did not affect the mutated 3′ UTR of TET1- or FOXO3-regulated luciferase expression (Figure 5H). Furthermore, miR-23a/27a/24-2 cluster miRNAs increased the interaction between the Ago2 complex, and the mRNAs of FOXO3 and TET1 were detected by Ago2 RIP assay (Figure S2). Together, these findings indicate that the miR-23a/27a/24-2 cluster downregulates TET1 and FOXO3 expression by directly targeting their 3′ UTRs and upregulates DNMT3B by inhibiting FOXO3.
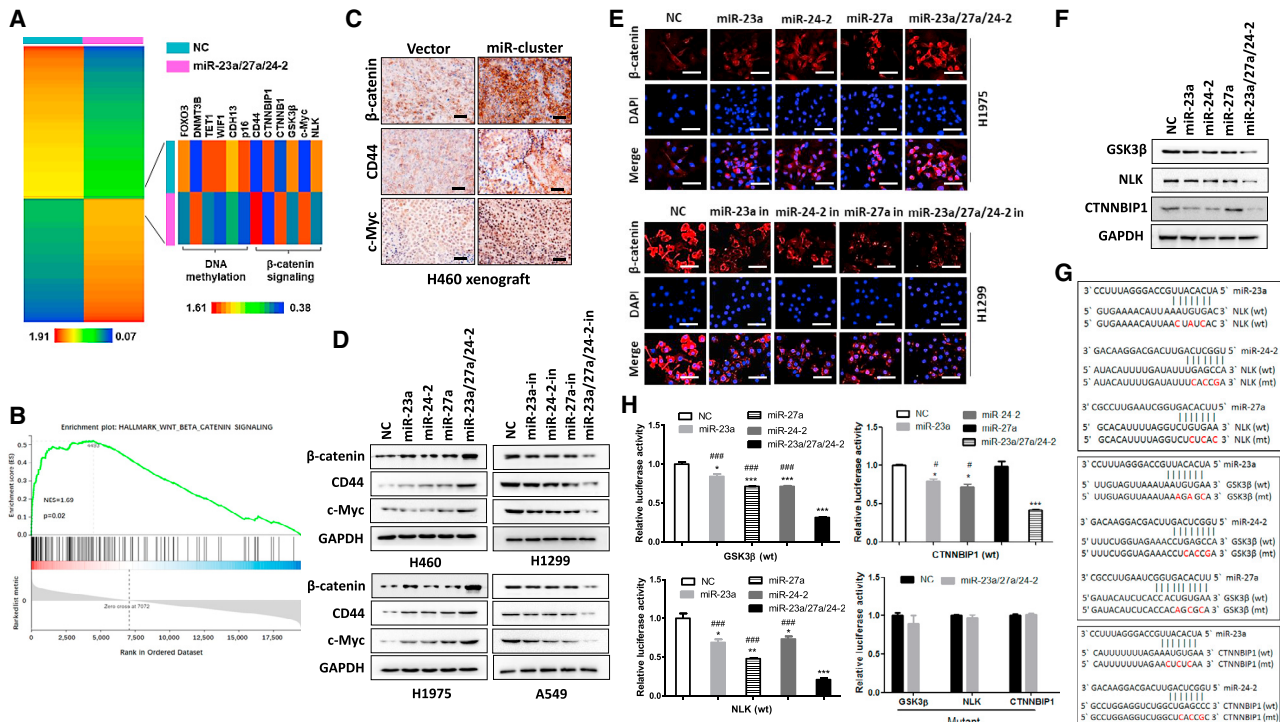
Figure 4. The synergistic effect of miRNAs in the miR-23a/27a/24-2 cluster on the activation of Wnt/β-catenin signaling in NSCLC

(A) Heatmap showing that simultaneous overexpression of miR-23a, miR-27a, and miR-24-2 significantly affected expression of many genes in NSCLC cells, including β-catenin signaling and DNA methylation-related genes. (B) Gene set enrichment analysis showing the correlation between the miR-23a/27a/24-2 cluster miRNA level and the Wnt/β-catenin signaling pathway in NSCLC cells. (C) Immunohistochemistry analysis showing that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster (miR-cluster) upregulates the expression of β-catenin, CD44, and c-Myc in NSCLC. NSCLC tissues were from H460 xenograft model. Scale bars, 100 μm. (D) Western blot analysis showed that miRNAs in the miR-23a/27a/24-2 cluster play a synergistic role in the regulation of β-catenin and its downstream target gene expression. The indicated cells were transfected with the indicated miRNA mimics. After 72 h of transfection, cells were subjected to western blot analysis. (E) Immunofluorescence analysis showing that miRNAs in the miR-23a/27a/24-2 cluster positively regulate the expression of β-catenin in NSCLC cells. Scale bars, 50 μm. (F) Western blot analysis showing that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster effectively suppressed the expression of NLK, GSK3β, and CTNNBIP1 in H1975 cells. H1975 cells were transfected with the indicated miRNA mimics. After 72 h of transfection, cells were subjected to western blot analysis. (G) Sequence alignment of miRNAs in the miR-23a/27a/24-2 cluster with the 3′ UTRs of the NLK, GSK3β, and CTNNBIP1 in NSCLC cells. (H) miRNA luciferase reporter assay showing that miRNA overexpression of the miR-23a/27a/24-2 cluster synergistically suppressed the 3′ UTRs of NLK or GSK3β- or CTNNBIP1-regulated luciferase expression in NSCLC cells.

The miR-23a/27a/24-2 cluster plays oncogenic roles by simultaneously stimulating Wnt/β-catenin signaling and methylation of TSG promoters

Next, we investigated whether Wnt/β-catenin signaling and DNA hypermethylation were directly involved in the oncogenic role of the miR-23a/27a/24-2 cluster. As shown in Figure 6, β-catenin silencing or 5-azad treatment partly inhibited overexpression of the miR-23a/27a/24-2 cluster-induced stimulation of sphere formation (Figure 6A), soft agar colony formation (Figure 6B), and cell invasion and migration (Figure 6C) in NSCLC cells. However, the combination of β-catenin silencing and 5-azad treatment more completely blocked the overexpression of miR-23a/27a/24-2 cluster-induced stimulation of sphere formation (Figure 6A), soft agar colony formation (Figure 6B), and cell invasion and migration (Figure 6C) than single treatments. In addition, IHC analysis of xenograft tumors showed that inhibition of the miR-23a/27a/24-2 cluster inhibited β-catenin expression but increased the expression of p16 and CDH13 (Fig-

ure 6D). Together, these findings indicate that miR-23a/27a/24-2 cluster miRNAs depend on Wnt/β-catenin signal activation and DNA hypermethylation-induced TSG downregulation to promote NSCLC.

Clinical relevance of the miR-23a/27a/24-2 cluster with β-catenin expression and promoter methylation of p16 and CDH13 in early-stage NSCLC

Finally, we examined the correlation between the expression of the miR-23a/27a/24-2 cluster miRNAs and β-catenin expression and/or promoter methylation of p16 and CDH13 in primary tumors of early-stage NSCLC. First, we showed that high expression levels of β-catenin and simultaneous methylation of p16 and CDH13 promoters are two independent predictors for poor prognosis in early-stage NSCLC patients (Table 1) and closely correlated with postoperative recurrence, low RFS, and low overall survival (Figure S3). In addition, we indicated that high expression of β-catenin (Figure 7A) or
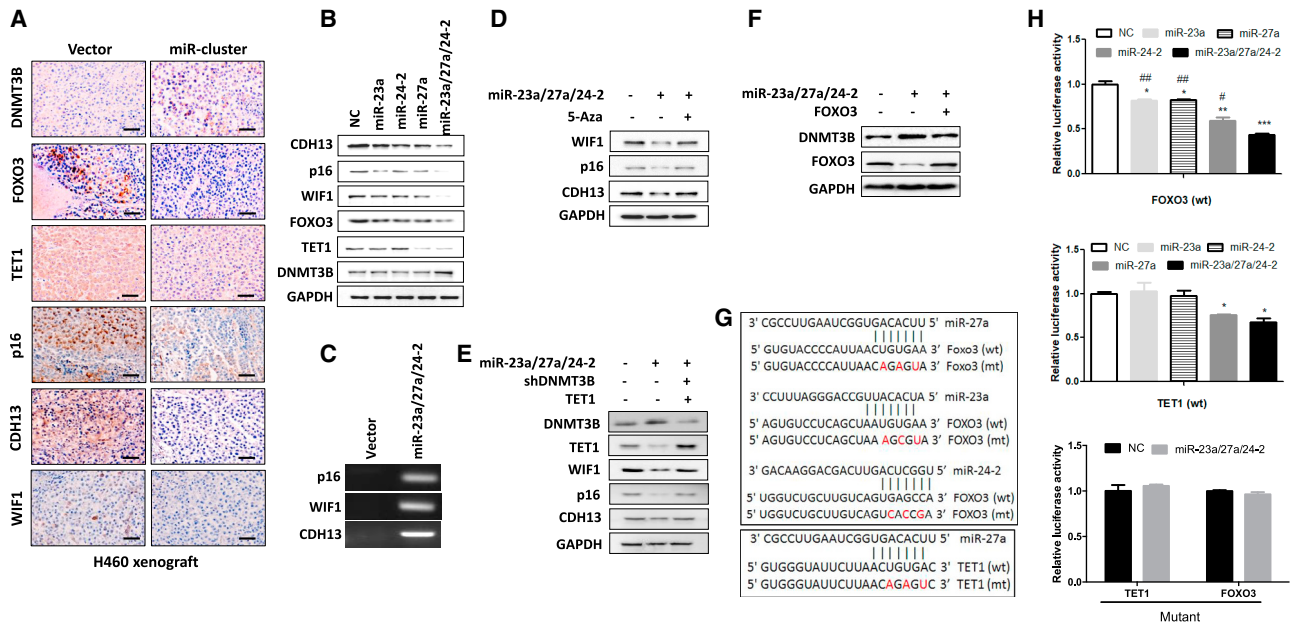
Figure 5. miRNAs in the miR-23a/27a/24-2 cluster inhibit TSGs expression through synergistically increasing their promoter methylation in NSCLC cells

(A) Immunohistochemistry analysis shows that the miR-23a/27a/24-2 cluster inhibited TET1, FOXO3, p16, CDH13, and WIF1 expression, while it increased DNMT3B expression in NSCLC tissues from xenograft models. (B) Western blot analysis showing that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster synergistically inhibited the expression of WIF1, p16, and CDH13 in NSCLC cells. H1975 cells were transfected with the indicated miRNA mimics. After 72 h of transfection, cells were subjected to western blot. (C) Methylation-specific PCR showing that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster significantly stimulated promoter methylation of p16, WIF1, and CDH13. Methylation analysis was performed using the HPA-Epic cell line stably expressing all miRNAs in the miR-23a/27a/24-2 cluster. (D) Western blot analysis showing that 5-azad treatment restored the expression of WIF1, p16, and CDH13, which were inhibited by overexpression of all miRNAs in the miR-23a/27a/24-2 cluster in NSCLC cells. H1975 cells were transfected with the indicated miRNA mimics. After 12 h of transfection, cells were treated with 2 μM 5-azad for 72 h and then subjected to western blot. (E) Western blot analysis showing that combination of DNMT3B silencing and TET1 overexpression restored the expression of WIF1, p16, and CDH13, which were inhibited by overexpression of all miRNAs in the miR-23a/27a/24-2 cluster in NSCLC cells. H1975 cells were transfected with the indicated miRNA mimics and/or plasmid. After 72 h of transfection, cells were subjected to western blot. (F) Western blot analysis showing that overexpression of FOXO3 suppressed miR-23a/27a/24-2 cluster-induced high expression of DNMT3B in NSCLC cells. H1975 cells were transfected with the indicated plasmid or/and miRNA mimics. After 72 h of transfection, cells were subjected to western blot. (G) Sequence alignment of miRNAs in the miR-23a/27a/24-2 cluster with the 3′ UTRs of FOXO3 and TET1 in NSCLC cells. (H) miRNA luciferase reporter assay showing that miRNA overexpression of the miR-23a/27a/24-2 cluster suppressed the 3′ UTRs of TET1- and FOXO3-regulated luciferase expression in NSCLC cells. Scale bars, 100 μm.

methylation of both p16 and CDH13 promoters (Figure 7B) in early-stage NSCLC are closely correlated with the high expression of all miR-NAs in the miR-23a/27a/24-2 cluster. Notably, compared with the other two groups, more patients in the all miRNA high expression group showed high expression of β-catenin and methylation of the p16 and CDH13 promoters (Figure 7C). In addition, the patient group with high expression of β-catenin and methylation of the p16 and CDH13 promoters showed the highest postoperative recurrence rate (Figure 7D) and the lowest survival rate (Figure 7E) compared with the patient group with high β-catenin expression or methylation of both the p16 and CDH13 promoters. Importantly, 81% of postoperative relapsed patients with β-catenin overexpression and methylation of the p16 and CDH13 promoters showed simultaneous overexpression of all miRNAs in the miR-23a/27a/24-2 cluster (Figure 7F). Altogether, these clinical data indicate that activation of β-catenin signaling and methylation of p16 and CDH13 promoters play an important role in early-stage NSCLC postoperative recurrence. Overexpression of the miR-23a/27a/24-2 cluster is likely an important

contributor to stimulating Wnt/β-catenin signaling and p16 and CDH13 promoter methylation in early-stage NSCLC.

DISCUSSION

We report for the first time that simultaneous high expression of all miRNAs in the miR-23a/27a/24-2 cluster is a powerful biomarker for predicting postoperative recurrence and prognosis in early-stage NSCLC. Previous studies have shown that upregulated expression levels of individual miRNAs in the miR-23a/27a/24-2 cluster are associated with the progression of various cancers.[20–23] However, our clinical data showed that all miRNAs in the miR-23a/27a/24-2 cluster are simultaneously upregulated in the primary tumors of early-stage NSCLC patients with postoperative recurrence, and that simultaneous high expression of all miRNAs in the miR-23a/27a/24-2 cluster is more closely correlated with postoperative recurrence and poor prognosis in early-stage NSCLC compared with high expression of individual miRNAs (Figure 1). In addition to the miR-23a/27a/24-2 cluster, high expression of β-catenin and simultaneous methylation
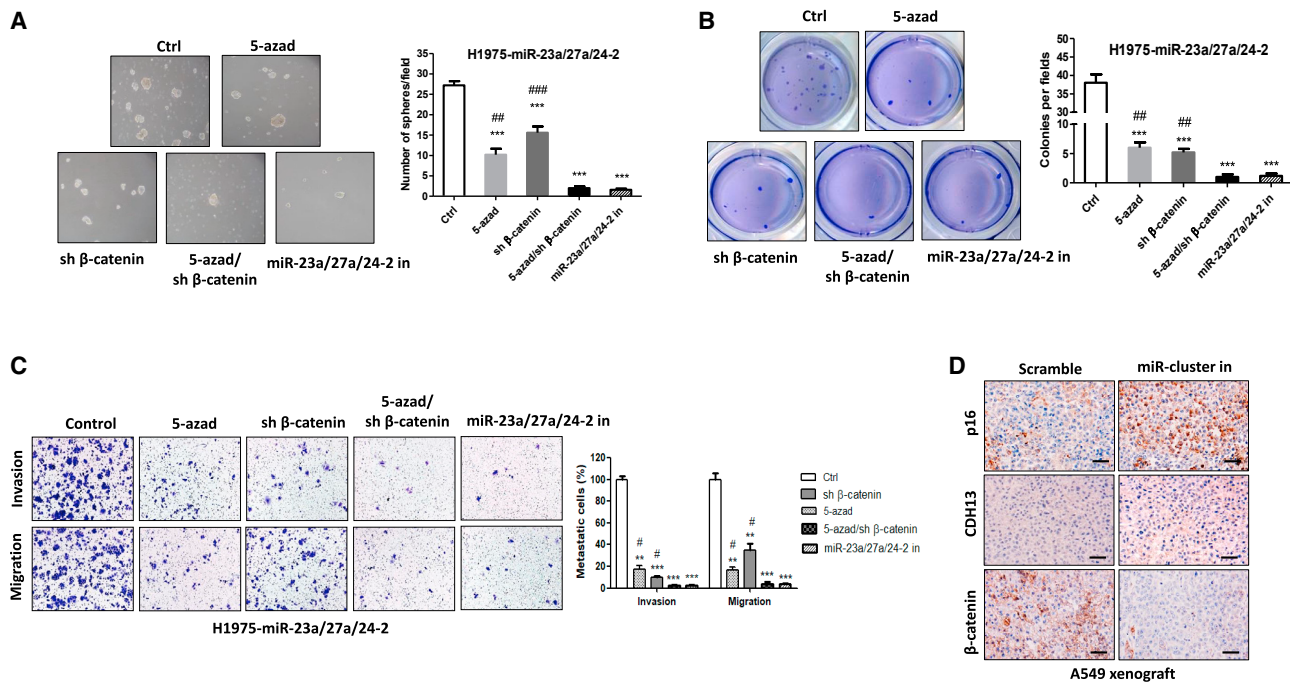
Figure 6. miRNAs in the miR-23a/27a/24-2 cluster play oncogenic roles through stimulating Wnt/β-catenin signaling and DNA hypermethylation in NSCLC

(A) Combination of β-catenin silencing and 5-azad treatment more significantly inhibited miR-23a/27a/24-2 cluster-stimulated sphere formation, (B) soft agar colony formation, and (C) invasion and migration of NSCLC cells. (D) Immunohistochemistry analysis shows that inhibition of all miRNAs in the miR-23a/27a/24-2 cluster increased p16 and CDH13 expression, while it suppressed β-catenin expression in NSCLC. NSCLC tissues were from A549 xenograft models. Scale bars, 100 μm.

of the p16 and CDH13 promoters were independent biomarkers for recurrence and prognosis in postoperative early-stage NSCLC patients (Figure S3; Table 1). These findings are consistent with the results reported previously by other research groups. Xu et al. reported that high expression of β-catenin is an independent marker of poor prognosis of NSCLC after surgery,[24] and Brock et al. reported that simultaneous methylation of both the p16 and CDH13 promoters is a biomarker for recurrence and poor prognosis of early-stage NSCLC.[8] However, the highlight of this study are our clinical data showing that simultaneous high expression of all miRNAs in the miR-23a/27a/24-2 cluster is a more powerful biomarker for predicting postoperative recurrence in early-stage NSCLC than high β-catenin expression or p16 and CDH13 promoter methylation. Our clinical data showed that postoperative relapsed patients in the β-catenin high expression group and the p16 and CDH13 promoter methylation groups accounted for 73% and 50% of the total relapsed cases (Figures S3A and S3D), respectively, whereas the number of postoperative relapsed patients in the all miRNA high expression group accounted for 83% of the total relapsed cases (Figure 1E). However, the number of patients included in this study was not large. Thus, before our findings can be used in clinical practice, they must be further verified in large-sample prospective research cohorts.

Hyperactivation of Wnt/β-catenin signaling[6] or promoter methylation of TSGs, including p16 and CDH13,[8] are important factors

that promote NSCLC recurrence. In this study, clinical data showed that high β-catenin expression and/or methylation of p16 and CDH13 promoters in early-stage NSCLC were closely correlated with high expression of all miRNAs in the miR-23a/27a/24-2 cluster (Figure 7). In addition, a series of *in vitro* and *in vivo* experiments showed that NSCLC progression promoted by miR-23a/27a/24-2 cluster miRNAs dependent on both Wnt/β-catenin signaling activation and DNA methylation-induced TSG downregulation (Figures 2, 3, 4, 5, and 6), indicating that the miR-23a/27a/24-2 cluster miRNAs play an oncogenic role by simultaneously stimulating Wnt/β-catenin signaling and methylation-induced TSG downregulation.

Aberrantly expressed miRNAs usually activate Wnt/β-catenin signaling by inhibiting suppressors of Wnt/β-catenin signaling in lung cancer.[6] Here, we demonstrated that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster can simultaneously inhibit the expression of CTNNBIP1, NLK, and GSK3β by directly targeting their 3′ UTR (Figure 4) and the expression of WIF1 through a DNA methylation mechanism (Figures 5C-E). Low expression of CTNNBIP1,[25,26] NLK,[27] and WIF1[28,29] has been identified in NSCLC, and their downregulation is closely related to Wnt/β-catenin signaling hyperactivation and poor progression of NSCLC patients. Studies have shown that CTNNBIP1 binds to β-catenin, thus blocking the interaction of β-catenin and TCF/LEF;[25] NLK inhibits the
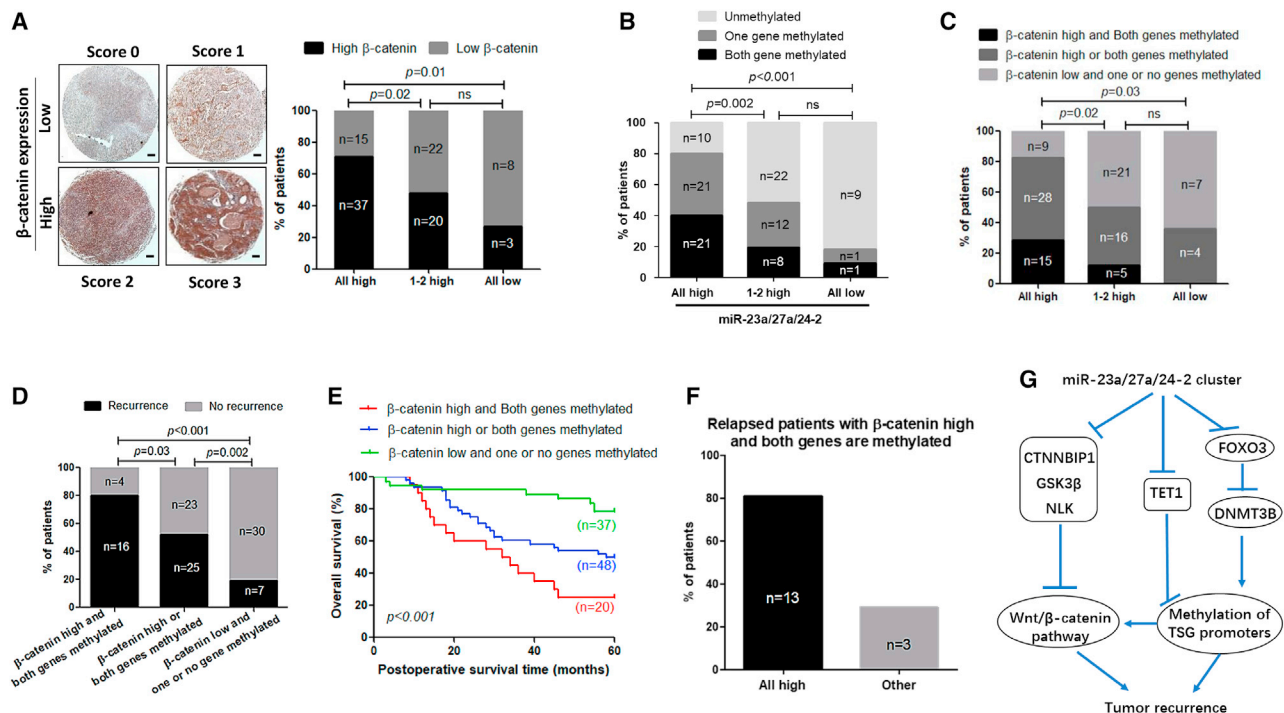
Figure 7. Clinical relevance of miRNA expression of the miR-23a/27a/24-2 cluster with β-catenin and methylation of the p16 and CDH13 promoters in early-stage NSCLC tissue

(A) High expression level of all miRNAs in the miR-23a/27a/24-2 cluster correlated with high expression level of β-catenin in early-stage NSCLC. β-catenin expression levels in early-stage NSCLC were examined by immunohistochemistry analysis. (B) High expression level of all miRNAs in the miR-23a/27a/24-2 cluster correlated with methylation of the promoters of both p16 and CDH13 in early-stage NSCLC. (C) Simultaneous high expression of β-catenin and methylation of p16 and CDH13 more frequently presented in the early-stage NSCLC patients group with high expression of all miRNAs in the miR-23a/27a/24-2 cluster. (D) Postoperative recurrence was more frequently presented in early-stage NSCLC patients group with simultaneously high expression of β-catenin and methylation of p16 and CDH13. (E) Simultaneous high expression of β-catenin and methylation of p16 and CDH13 more closely correlated with lower overall survival in early-stage NSCLC patients. (F) In relapsed patients group with β-catenin high expression and methylation of p16 and CDH13, 81% of patients presented high expression of all miRNAs in the miR-23a/27a/24-2 cluster. (G) A schematic model of the regulation of early-stage NSCLC recurrence by miRNAs in the miR-23a/27a/24-2 cluster. Scale bars, 100 μm.

binding of the β-catenin/TCF/LEF complex to its transcriptional response elements;[30–34] and WIF1 suppresses Wnt/β-catenin signaling by binding to Wnt and negatively correlates with lung tumorigenesis.[35] In addition, GSK3β also suppresses Wnt/β-catenin signaling by promoting β-catenin degradation and inhibiting lung cancer metastasis.[36,37] These findings indicate that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster activates Wnt/β-catenin signaling by simultaneously suppressing the expression of WIF1, GSK3β, NLK, and CTNNBIP1.

We also elucidated the mechanism by which overexpression of all miRNAs in the miR-23a/27a/24-2 cluster affects the methylation of TSGs in NSCLC. DNMT3B is a DNA methyltransferase that is upregulated in lung cancer and is correlated with poor prognosis.[38] In contrast, TET1 has a demethylation role and is frequently downregulated in various malignancies.[39] Notably, studies have shown that DNMT3B upregulation and/or TET1 downregulation are associated with promoter hypermethylation-induced downregulation of TSGs, including WIF1,[40] p16,[41,42]

and CDH13.[43] Here, we indicated that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster inhibited TET1 expression by directly binding to the 3′ UTR and upregulated DNMT3B expression by inhibiting FOXO3 expression by directly binding to its 3′ UTR (Figures 5A, 5B, 5F–5H). In addition, overexpression of all miRNAs in the miR-23a/27a/24-2 cluster downregulated the expression of WIF1, p16, and CDH13, although the expression was restored by inhibition of DNA methylation or the combination of DNMT3B silencing and TET1 overexpression (Figures 5D and 5E). These findings suggest that overexpression of all miRNAs in the miR-23a/27a/24-2 cluster inhibits the expression of p16, CDH13, and WIF1 through DNMT3B- and TET1-regulated methylation in NSCLC.

We also evaluated the application potential of the miR-23a/27a/24-2 cluster as a therapeutic target for NSCLC. Previous studies have shown that inhibition of Wnt/β-catenin signaling or abnormal DNA methylation can suppress NSCLC progression.[44,45] However, our clinical data show that 33% of early-stage NSCLC patients with

Table 2. Characteristics of stage I or II NSCLC patients

Variable	No. of patients (n = 105)			
	All high (n = 52)	1 or 2 high (n = 42)	All low (n = 11)	p
Gender				0.33
Male	37	29	10	
Female	15	13	1	
Age				0.06
≤60	29	13	6	
>60	23	29	5	
Smoking				
Yes				
No				
Histology				0.76
Adenocarcinoma	27	24	5	
Non-adenocarcinoma	25	18	6	
Stage				0.13
I	24	25	3	
II	28	17	8	
T status				0.93
T1	8	9	2	
T2	37	27	8	
T3	7	6	1	
N status				0.87
N0	36	36	7	
N1	16	6	4	

postoperative recurrence showed high expression of β-catenin and methylation of p16 and CDH13 promoters (Figure 7D), suggesting that inhibition of Wnt/β-catenin signaling or DNA methylation alone may not be effective in treating such cases. Importantly, our series of experiments showed that inhibition of the miR-23a/27a/24-2 cluster dramatically suppressed NSCLC progression (Figures 2 and 3), decreased the expression of β-catenin, and increased p16 and CDH13 expression (Figure 6D). Together, these findings suggest that targeting the miR-23a/27a/24-2 cluster may be a useful strategy for the treatment or prevention of postoperative early-stage NSCLC recurrence.

In summary, our study clearly indicates that simultaneous high expression of all miRNAs in the miR-23a/27a/24-2 cluster represents a new biomarker for predicting recurrence and poor prognosis in postoperative early-stage NSCLC patients. Simultaneous Wnt/β-catenin signaling activation and DNA methylation-induced TSG downregulation are important factors leading to postoperative recurrence of early-stage NSCLC, which is closely related to overexpression of all miRNAs in the miR-23a/27a/24-2 cluster. In addition, simultaneous inhibition of all miRNAs in the miR-23a/27a/24-2 cluster may be an effective novel strategy for the treatment of early-stage NSCLC recurrence (Figure 7G).

MATERIALS AND METHODS

Materials

5-Aza-2′-deoxycytidine, 4′,6-diamidino-2-phenylindole, crystal violet, epidermal growth factor, human basic fibroblast growth factor, insulin, and Texas red-conjugated anti-mouse antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Transwell chambers for invasion and migration assay was obtained from Corning (Corning, NY, USA). All cell culture medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). TRIzol reagent and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against GSK3β, β-catenin, FOXO3, p16, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against NLK, TET1, and CDH13 were obtained from Abcam (Cambridge, MA, USA). Catein beta interacting protein 1 (CTNNBIP1) antibody was from CUSABIO Technology (Wuhan, Hubei, China). miRNA mimics, inhibitors, primers, and the quantitative real-time PCR kit were purchased from RiboBio (Guangzhou, Guangdong, China). DIG-labeled miRNA probes and miRNA *in situ* hybridization (ISH)-related reagents were obtained from Zoonbio Biotechnology (Nanjing, Jiangsu, China) and Roche (Mannheim, Germany), respectively. The dual luciferase reporter assay kit was purchased from Promega (Madison, WI, USA). The peroxidase substrate kit and DNA extraction mini kit were obtained from Vector Laboratories (Peterborough, UK) and QIAGEN (Valencia, CA, USA), respectively.

Cell culture and human specimens

HPA-EpiC was obtained from ScienCell (Carlsbad, CA, USA) and NSCLC cell PC-9 was kindly provided by Dr. Shen (Jilin University, China). Other NSCLC cell lines used in this study were purchased from the American Type Culture Collection (Rockville, MD, USA). All cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS.

NSCLC specimens were collected from 105 patients with early-stage NSCLC during surgery at Daping Hospital under a protocol approved by the ethical review committees (Table 2).

RNA analysis

Total RNAs were isolated from cells or tissues using TRIzol reagent, and then subjected to miRNA and mRNA profiling. Small RNA sequencing and mRNA sequencing were performed on the Illumina HiSeq 2500 platform and Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA), respectively.

Transwell, soft agar, and sphere assay

Cells were transfected with the indicated nucleotides. After 48 h of transfection, transwell, sphere,[46] and soft agar assays[47] were performed as described previously.

IHC, miRNA ISH, and IF

Tissues were fixed in 10% neutral buffered formalin, paraffin processed, sectioned at 4 μm, and then subjected to IHC and ISH

analysis. IHC was performed as described previously,[48] and miRNA ISH was performed as described by Nuovo.[49] ISH score was performed as described by Guo et al. previously.[50] In brief, cytoplasmic staining was interpreted based on the intensity as negative (0), weak (1), moderate (2), and strong (3). Cases showing 0 and 1 intensity of staining were considered as low expression, and cases showing 2 and 3 intensity of staining were considered as high expression. IHC and ISH results were scored independently by three pathologists without knowledge of the clinicopathological findings. For IF analysis, cells were grown in a coverslip and transfected with the indicated nucleotides. After 72 h of transfection, IF was performed as described previously.[51]

Western Blot analysis
Western blot was performed as described previously.[48]

miRNA luciferase report assay
The full-length 3′ UTR of GSK3β, NLK, CTNNBIP1, TET1, and FOXO3 were amplified by PCR from human genomic DNA and inserted into the pMIR-REPORT miRNA expression reporter vector (expressing firefly luciferase). For reporter assay, cells were transfected with the indicated reporter plasmids and the indicated oligonucleotides. The Renilla luciferase expression vector was co-transfected as a transfection control. After 72 h of transfection, luciferase activity was measured by the Dual Luciferase Assay system and the firefly luciferase activity was normalized to the activity of Renilla luciferase.

RIP assay and methylation-specific PCR
For RIP assay, Ago2-flag plasmid and the indicated miRNAs were co-transfected into the indicated cells. Following 48 h of incubation, cells were subjected to the RIP assay. The RIP assay was performed using Flag antibody as described by Keene et al.[52] RIP was followed by quantitative RT-PCR.

For methylation-specific PCR, genomic DNA was extracted from human samples and cell lines using a DNA extraction kit, then subjected to methylation-specific PCR. PCR was performed as described by Wang et al. previously.[53] All primer pairs are provided in Table S1.

Animal experiments
All animal experiments were conducted in the Animal Experiment Center of Daping Hospital, and approved by the Institutional Animal Care and Use committee at Army Medical University. In this study, we used 6-week-old male nude mice and the number of animals in each group was nine. For the tumorigenesis experiment, the indicated amount cells in 0.1 mL of phosphate-buffered saline (PBS) were injected subcutaneously into the back of mice. At 6 weeks after the cells injection, mice were sacrificed. For the orthotopic tumor implantation, 1×10^5 cells in 20 μL of PBS containing 10 ng Matrigel were injected into the pleural cavity of mice. The mice were sacrificed 1 month after implantation and lung surface tumor foci were counted. For the tail vein injection metastatic model, 5×10^5 cells in 0.1 mL PBS were injected into the lateral tail vein of mice. One month after cell injection, the mice were sacrificed, the lungs were collected, and the tumor nodules on the lung surface were immediately counted under the microscope. Then, the lung was divided into two parts, the left lung (two lobes) was stored at −80°C for further extraction of proteins and mRNA, and the right lung (three loves) was fixed in 10% neutral buffered formalin for H&E and IHC analysis.

Statistical analysis
Data are presented as mean ± standard deviation and differences were considered statistically significant at a p value of less than 0.05. Statistical differences between treatment groups were analyzed by one-way analysis of variance and Duncan's multiple range test using SAS statistical software. The survival rate of NSCLC patients was calculated using Kaplan-Meier survival analysis.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.12.014.

AUTHOR CONTRIBUTIONS
H.J. conceived the experiments and supervised the work. H.J. wrote the manuscript. X.F. and S.T. performed *in vitro* and *in vivo* experiments. B.D. and S.T. collected human samples and performed immunohistochemistry experiments and analysis. Q.L. performed statistical analysis. Q.-Y.T. provided some reagents, and participated in human sample collection and clinical data analysis.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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