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CircABCA13 acts as a miR-4429 sponge to facilitate esophageal squamous cell carcinoma development by stabilizing SRXN1

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Abstract

Circular RNAs (circRNAs) play a pivotal role in the tumorigenesis and progression of various cancers. However, the role and mechanisms of circABCA13 in esophageal squamous cell carcinoma (ESCC) are largely unknown. Here, we reported that circABCA13, a novel circular RNA generated by back-splicing of the intron of the ABCA13 gene, is highly expressed in ESCC tumor tissues and cell lines. Upregulation of circABCA13 correlated with TNM stage and a poor prognosis in ESCC patients. While knockdown of circABCA13 in ESCC cells significantly reduced cell proliferation, migration, invasion, and anchorage-independent growth, overexpression of circABCA13 facilitated tumor growth both in vitro and in vivo. In addition, circABCA13 directly binds to miR-4429 and sequesters miR-4429 from its endogenous target, SRXN1 mRNA, which subsequently upregulates SRXN1 and promotes ESCC progression. Consistently, overexpression of miR-4429 or knockdown of SRXN1 abolished malignant behavior promotion of ESCC results from circABCA13 overexpression in vitro and in vivo. Collectively, our study uncovered the oncogenic role of circABCA13 and its mechanism in ESCC, suggesting that circABCA13 could be a potential therapeutic target and a predictive biomarker for ESCC patients.

KEYWORDS
circABCA13, esophageal squamous cell carcinoma, miR-4429, SRXN1, Wnt/β-catenin

Abbreviations: BCA, bicinchoninic acid; circRNA, circular RNA; ESCC, esophageal squamous cell carcinoma; GEO, Gene Expression Omnibus; GSEA, gene set enrichment analysis; IHC, immunohistochemistry; LNM, lymph node metastasis; ROC, receiver operating characteristic; SRXN1, sulfiredoxin 1; TMA, tissue microarray.

1 | INTRODUCTION

Esophageal cancer is the sixth leading cause of cancer-related death in the world and is subdivided into two histological types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma.[1] ESCC patients in China constitute approximately half of all ESCC patients in the world.[2] Despite recent advances in the diagnosis and treatment of ESCC patients, the 5-year survival rate for ESCC patients is still low.[3] Hence, there is an urgent need to identify applicable therapeutic targets and biomarkers for ESCC diagnosis to improve clinical outcomes in ESCC patients.

Circular RNAs (circRNAs), one kind of long noncoding RNA, are generated by back-splicing, a process in which a spliceosome utilizes a 3′ splice site that is upstream of the selected 5′ splice site.[4] Due to their specific structure, circRNAs have the characteristic of long half-lives and are resistant to regular mechanisms of linear RNA decay.[5,6] Hence, circRNAs, such as circRNA-002178[7] and circRHOT1,[8] could be potential biomarkers for the diagnosis and prognosis evaluation of many cancers.[9,10] Moreover, increasing evidence has revealed that circRNAs can also participate in cancer development and progression.[11,12] For example, circRNA hsa_circRNA_104348 promotes hepatocellular carcinoma progression through activation of the Wnt/β-catenin pathway via the miR-187-3p/RTKN2 axis.[13] CircABCA13 (Circbase ID: has_circ_0001707) is generated by back-splicing from the intron of the ABCA13 gene (chr7:48541721–48542148) and was reported highly expressed in human cancer through high-throughput sequencing,[14–16] but there is no evidence to certify the role of circABCA13 in cancer development either in vitro or in vivo. Here, we explored a new mechanism by which circABCA13 regulates ESCC progression.

It's well known that miRNA could be adsorbed by circRNA to lose its inhibition on target genes.[17,18] MircoRNA-4429 (miR-4429) is reported to be sponged by Circ_0067680.[19] The 2–8 nucleotides of MiR-4429 have complementary base pairing with circABCA13 according to circBank database (http://www.circbank.cn), which suggests that miR-4429 may be involved in circABCA13-mediated ESCC progression. Furthermore, recent studies reported that sulfiredoxin-1 (SRXN1) could facilitate the metastasis of cervical cancer via the Wnt/β-Catenin signaling pathway,[20] and the miRWALK database also suggests a potential link between miR-4429 and SRXN1. Therefore, to explore the signaling pathway by which circABCA13 promotes SRXN1 expression via sponging miR-4429 will enrich the molecular mechanism of circRNA in ESCC progression.

The objective of this study was to identify candidate circRNAs involved in ESCC progression and investigate the underlying mechanism. The results from our study revealed that circABCA13 is upregulated in ESCC tissues and is positively associated with reduced survival among ESCC patients. Moreover, our results demonstrated that circABCA13 may play an oncogenic role in the tumorigenesis and progression of ESCC via the miR-4429/SRXN1 axis, which provides a new insight for clinical diagnosis and therapy in ESCC.

2 | MATERIALS AND SPECIMENS

2.1 | Fluorescence in situ hybridization

The location and expression level of circABCA13 in ESCC cells were detected with Cy3-labeled circABCA13 probes from Ribo™ FISH kits, as Chen et al. described[21] (RiboBio). The expression quantity of circABCA13 in ESCC tissues was determined by RNA FISH kits (Paraffin slice; GenePharma). Probes specific for circABCA13 were mixed and incubated overnight, and nuclei were then stained with DAPI. The sequences of the Cy3-labeled circABCA13 are listed in Table S1. The FISH images of the cells were captured using an Olympus BX43 fluorescence microscope. Images of tissue FISH in paraffin slices were collected by a Nanozoomer Digital Pathology scanner, and the results were evaluated based on mean fluorescence intensity via ImageJ software.

2.2 | Immunohistochemistry

Tissues were cut into 4-μM thick paraffin sections. We incubated the sections with primary antibodies against SRXN1 (1:50) and Ki67 (1:200) at 4°C overnight. Then, all sections were incubated in goat-anti-rabbit IgG-HRP secondary antibodies (ZSGB-BIO) for 1 h at room temperature and stained with diaminobenzidine reagent (ZSGB-BIO), and the nuclei were counterstained with hematoxylin. The immunohistochemistry (IHC) images were collected by a Nanozoomer Digital Pathology scanner, and the results were evaluated by mean fluorescence intensity via ImageJ Software.

2.3 | Western blot assay

Cells were lysed in RIPA (Beyotime) lysis buffer containing proteinase and phosphatase inhibitors. Protein concentrations were measured using bicinchoninic acid (BCA) assay (Beyotime) at 562 nm by a multifunctional enzyme-linked analyzer (BioTek). Protein samples were run on an SDS–PAGE gel at a concentration of 50 μg and transferred to PVDF membranes (Millipore). The membrane was incubated with the corresponding primary antibody at 4°C overnight and HRP-labeled secondary antibodies for 2 h for subsequent visualization analysis by a Tanon 5200 system. The antibodies used in this study are outlined in Table S2.

2.4 | Xenograft assay

To detect the role of circABCA13 in ESCC proliferation in vivo, 4-week-old female BALB/c nude mice (Vital River) were injected with approximately 4×10^6 KYSE30 cells stably expressing vector or circABCA13OE. To detect the role of the miR-4429/SRXN1 axis in proliferation acceleration in ESCC caused by circABCA13 overexpression in vivo, 4-week-old female BALB/c nude mice

were injected with approximately 2×10^6 KYSE410 vector cells or KYSE410 cells with stable circABCA13 overexpression, circABCA13 overexpression plus miR-4429 overexpression, or circABCA13 overexpression plus SRXN1 knockdown in rescue experiments. The mice were randomly divided into groups before injection. Tumor volume was measured twice a week and calculated according to the equation volume = (length × width^2)/2.

2.5 | Statistical analysis

GraphPad Prism 8.0 (GraphPad Software) was used for statistical analysis of all data. Differences between two groups were analyzed by Student's *t* test, and more than two groups were analyzed by one-way analysis of variance (ANOVA). The correlation of measurements was determined using Pearson's correlation analysis. All values are presented as the mean ± standard deviation (SD) of three independent experiments. Differences with a p value <0.05 were considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

3 | RESULTS

3.1 | Increased circABCA13 expression correlates with TNM stage and a poor prognosis in ESCC patients

To identify new circRNAs involved in ESCC progression, we analyzed the high-throughput sequencing data of 23 paired ESCC tumors and normal tissues from the Gene Expression Omnibus (GEO) dataset (GEO number: GSE130078) and a total of 211 upregulated and 124 downregulated circRNAs were identified using the cutoff of $\log_2|\text{fold change}|>1.0$ and $p<0.05$. CircABCA13, as one of the most significantly upregulated circRNAs in ESCC tissues, was selected for further analysis (Figure 1A). Sanger sequencing confirmed the junction sequence in the divergent primers crossing the predicted products (Figure 1B). Due to the circular structure of circRNAs,[22] circABCA13 was more stable than ABCA13 linear mRNA, which was further verified by actinomycin treatment (Figures 1C and S1A) and RNase R digestion (Figures 1D and S1B).

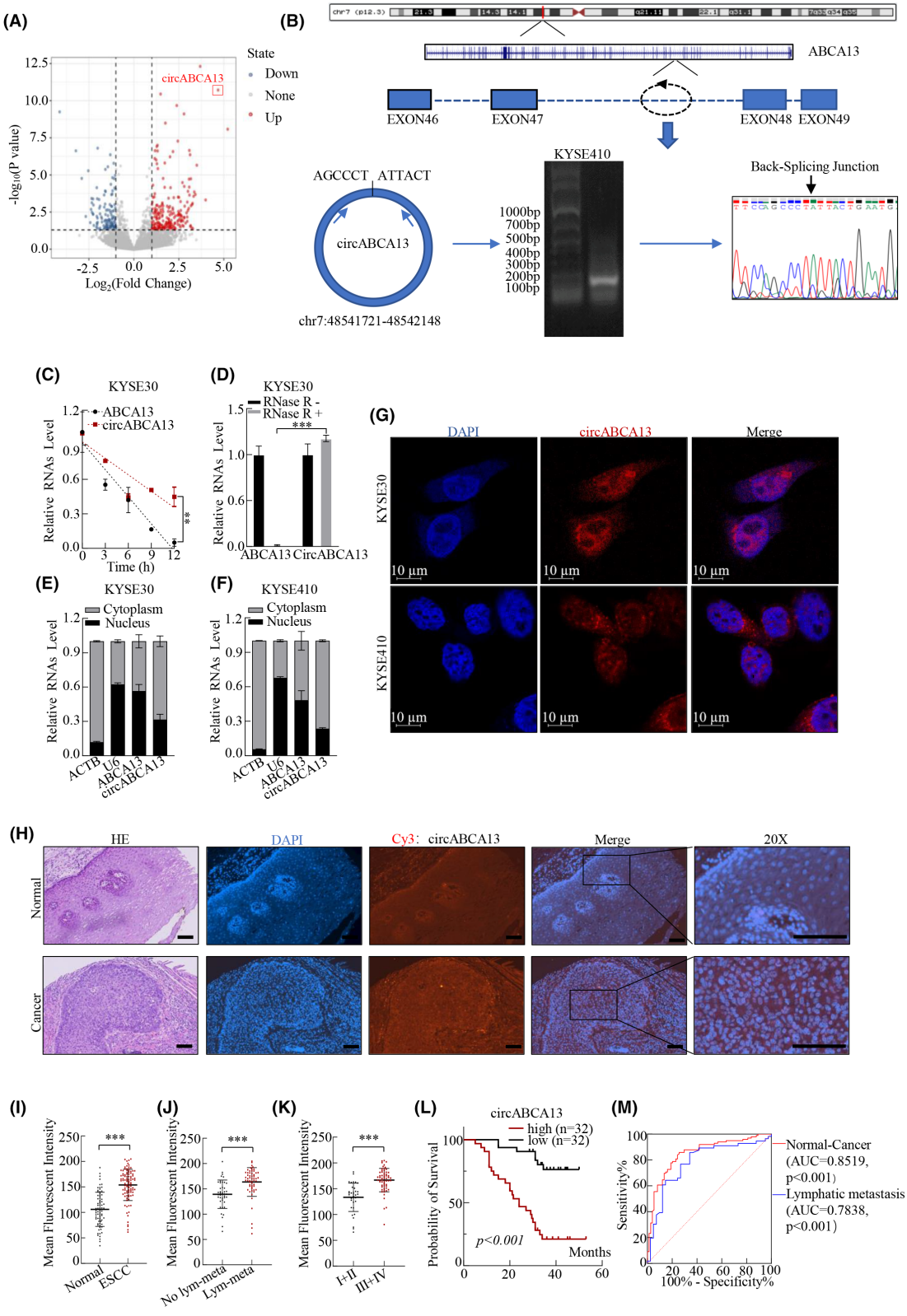
Increased expression of circABCA13 in ESCC was further confirmed by RT-qPCR in 16 paired ESCC tissues and normal tissues (Figure S1C). Additionally, analyzing samples from three ESCC patients with lymph node metastases revealed significantly higher expression in lymph nodes with ESCC invasion than in primary ESCC tumors (Figure S1D). Moreover, nuclear mass separation assays and FISH analyses showed that more than 50% of circABCA13 localized in the cytoplasm (Figure 1E–G). Collectively, these results indicate that circABCA13 is upregulated in ESCC and primarily localized in the cytoplasm.

To further evaluate the relationship between circABCA13 and the clinicopathologic characteristics of ESCC patients, we performed tissue microarray (TMA)-based FISH in 99 ESCC tissues and 74 normal tissues. Consistent with the results obtained from RT-qPCR, increased circABCA13 levels were observed in ESCC tissues compared with normal tissues (Figure 1H,I), and circABCA13 was upregulated to a greater degree in patients with lymph node metastases or high T grades (Figures 1J and S1E,F, and Table 1). Moreover, higher levels of circABCA13 in ESCC were associated with higher TNM stage (III + IV) and decreased overall patient survival rates ($p<0.01$) (Figure 1K,L). Finally, the receiver operating characteristic (ROC) curve analysis revealed that circABCA13 had high accuracy in detecting ESCC (Area Under Curve = 0.8519, $p<0.001$) and ESCC with lymph node metastases (Area Under Curve = 0.7838, $p<0.001$), with sensitivity values of 0.859 and 0.768, respectively, and specificity values of 0.743 and 0.732, respectively (Figure 1M). Together, these results suggest that circABCA13 is overexpressed in ESCC and may be a potential biomarker in ESCC for predicting tumor progression and patient prognosis.

3.2 | CircABCA13 acts as an oncogene and promotes the malignant characteristics of ESCC

We next investigated whether circABCA13 promotes the malignant characteristics of ESCC cells in vitro. The circABCA13 levels were assessed in four ESCC cell lines (KYSE30, KYSE150, KYSE410, and KYSE510) and two normal esophageal epithelial cell lines (HEEC, HET-1A). While circABCA13 was barely detectable in the two normal esophageal epithelial cell lines, significantly higher expression of circABCA13 was observed in all four ESCC cell lines, with the highest level in KYSE150 cells and the lowest level in KYSE30 cells (Figure 2A). Overexpression or knockdown of circABCA13 in ESCC cells was achieved by transfecting KYSE30 and KYSE410 cells with circABCA13 overexpression lentiviruses, and KYSE150 and KYSE410 with circABCA13 knockdown lentiviruses. Successful overexpression or knockdown of circABCA13 was verified by qRTPCR (Figures 2B,C and S2A,B). The effects of altered circABCA13 expression on cell viability and proliferation were assessed by CCK-8 assays and colony formation assays. While overexpression of circABCA13 significantly increased the viability of KYSE30 and KYSE410 cells (Figure 2D–F), knockdown of circABCA13 efficiently decreased the viability of KYSE150 and KYSE410 cells (Figure S2C–E). Additionally, a soft agar assay revealed that overexpression of circABCA13 promoted anchorage-independent growth (Figure 2G), and knockdown of circABCA13 suppressed anchorage-independent growth (Figure S2F). Furthermore, ESCC migration and invasion were significantly enhanced in circABCA13-overexpressing cells but reduced in circABCA13-knockdown cells (Figures 2H,I, S2G,H, and S3A–D).

The abnormal activation of cell cycle-related proteins accelerates tumor progression,[23] and the occurrence of epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM) remodeling caused by MMPs are characteristics positively correlated with tumor



invasion and metastasis.[24–26] CircABCA13 overexpressing obviously upregulated cyclin D1, cyclin D3, N-cadherin, MMP-2, and MMP-9 and downregulated E-cadherin (Figures 2J and S3E), while the opposite effects were observed in circABCA13 knockdown cells. A mouse xenograft assay verified that circABCA13 could promote tumor growth in vivo, including tumor volume and tumor weight (Figures 2K,L and S3F). Moreover, the proliferation index stained by Ki67 was also increased in the circABCA13 overexpression group compared with the vector group, further supporting the oncogenic role of circABCA13 in ESCC cells (Figure 2M). Together, these results demonstrated that circABCA13 promotes ESCC progression in vitro and in vivo.

FIGURE 1 Identification and characterization of circABCA13 in esophageal squamous cell carcinoma (ESCC) cells and tissues. (A) Volcano plot of circular RNA (circRNA) profiles from GSE130078. (B) Schematic of ABCA13 and circABCA13 genomic loci. (B) Sanger sequencing of PCR products amplified by divergent primers (185 bp) of circABCA13. (C) Relative RNA stability of circABCA13 and linear ABCA13 in KYSE30 cells after treatment with actinomycin D. (D) Relative RNA levels of circABCA13 and linear ABCA13 in KYSE30 cells treated with RNase R. (E, F) Quantification of the relative RNA levels of circABCA13 and linear ABCA13 in the cytoplasm and nucleus of KYSE30 and KYSE410 cells. (G) FISH images of the subcellular localization of circABCA13 in KYSE30 or KYSE410 cells. (H) FISH and H&E staining of ESCC tissues ($n = 99$) and normal tissues ($n = 74$) (scale bar = 100 μm). (I) Mean fluorescence intensity of circABCA13 in ESCC tissues and normal tissues. (J) Mean fluorescence intensity of circABCA13 in ESCC tissues with or without lymphatic metastasis. (K) Mean fluorescence intensity of circABCA13 in I + II stage and III + IV stage ESCC tissues. (L) Kaplan–Meier curve representing the overall survival rate of 64 ESCC patients (Hazard Ratio = 5.886, $p < 0.001$). (M) Receiver operating characteristic analysis showing the ability of circABCA13 expression to distinguish between ESCC, normal, and lymphatic metastasis samples. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

TABLE 1 Correlations between circABCA13 expression in ESCC tissues and clinicopathologic characteristics of 99 ESCC patients

Clinicopathologic characteristics	Cases ($n = 99$)	CircABCA13 level	
		Mean ± SEM (mean fluorescent intensity)	p value
Gender			
Male	80	151.10 ± 30.49	0.482
Female	19	146.32 ± 30.59	
Age (years)			
≤50	4	163.10 ± 11.58	0.544
>50	95	153.53 ± 31.04	
LNM status			
No	43	138.96 ± 29.25	<0.001***
>50	56	165.40 ± 24.65	
T status			
T1 + T2	18	133.69 ± 34.84	0.002**
T3 + T4	81	158.41 ± 27.57	
TNM stage			
I + II	38	133.30 ± 30.94	<0.001***
III + IV	61	166.76 ± 22.11	

Abbreviations: ESCC, esophageal squamous cell carcinoma; LNM, lymph node metastasis; T, tumor; TNM, tumor-node-metastasis.

**$p < 0.01$; ***$p < 0.001$.

3.3 | CircABCA13 increases SRXN1 levels and subsequently activates the Wnt/β-catenin pathway

To elucidate the mechanism by which circABCA13 promotes the malignant characteristics of ESCC, we performed transcriptome profiling of KYSE30 cells expressing either circABCA13 overexpression or vector control by RNA-seq. As shown in Figure 3A and Table S6, 131 upregulated genes and 47 downregulated genes were identified in circABCA13-overexpressing cells compared to vector control cells (the screening threshold was set as $\log_2|\text{fold change}| > 0.8$, $p < 0.05$). We further screened the upregulated genes in a high-expressed CircABCA13 group compared with a low-expressed CircABCA13 group of the ESCC dataset which comes from GSE130078, and subsequently intersects with the upregulated genes in overexpressed circABCA13 KYSE30 cells by RNA-seq data, as showed in Figure S4A, five genes were performed as potential upregulated genes induced by circABCA13 overexpression. Only SRXN1 RNA expression was consistent with RNA-seq results KYSE30 or KYSE410 (Figures 3B,C and S4B–E), and the protein level further confirmed the upregulation effect of circABCA13 on SRXN1 (Figures 3D and S4F). As we know, SRXN1 is widely reported to boost the progression in kinds type of cancers,[20,27] To determine whether increased SRXN1 by circABCA13 contributes to the tumor progression acceleration, SRXN1 knockdown was performed in circABCA13-overexpressing KYSE30 and KYSE410 cells by utilizing specific siRNAs (Figure S5A–C). In terms of functional phenotype, knockdown of SRXN1 dramatically abolished the promotion of circABCA13 overexpressing in ESCC cells including cell proliferation, anchorage-independent growth, cell migration, and invasion (Figures 3E–G and S5D–F), suggesting that SRXN1 act as a downstream gene of circABCA13 and

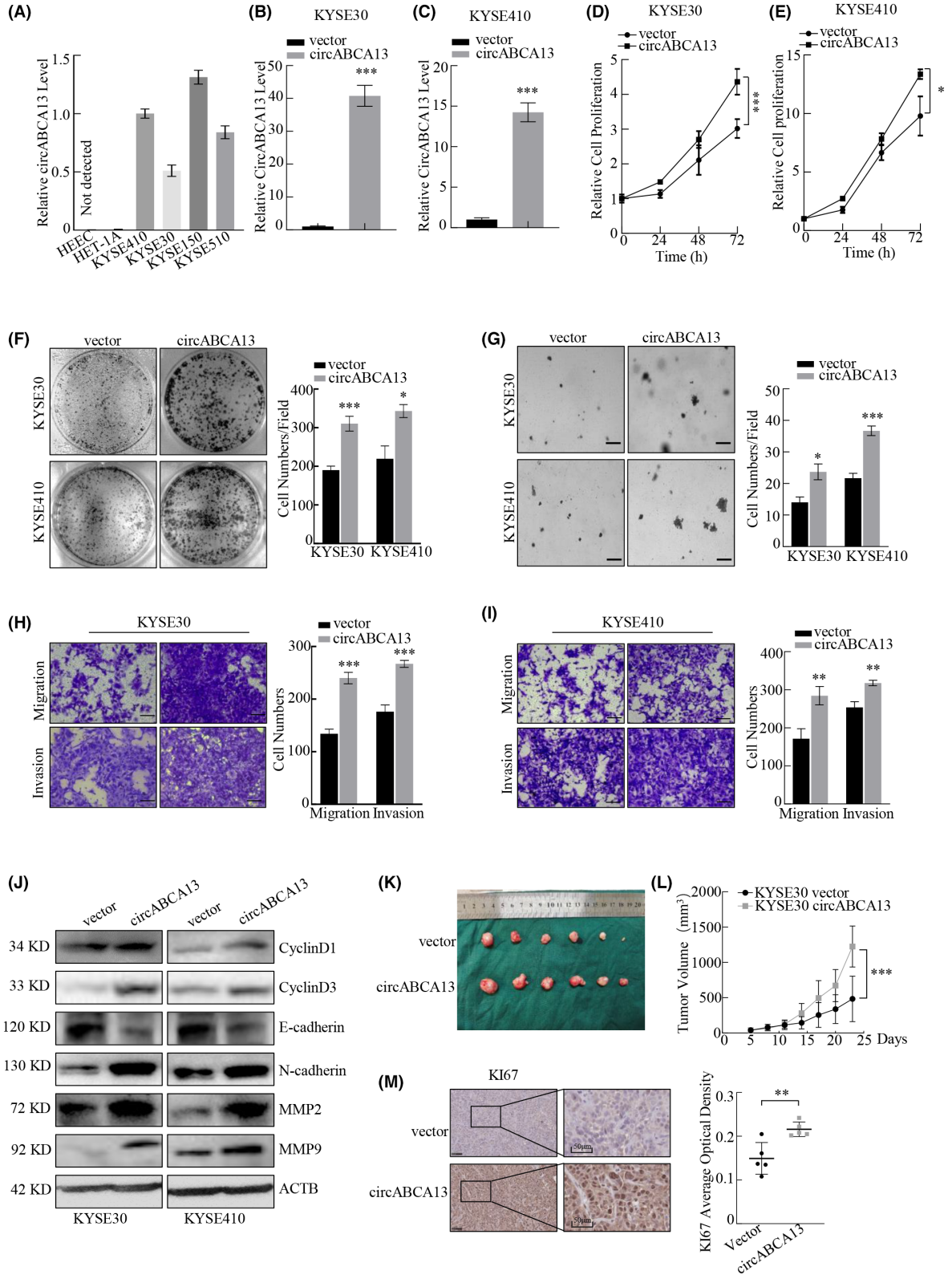


FIGURE 2 CircABCA13 acts as an oncogene and regulates proliferation, migration, and invasion in vitro and in vivo. (A) CircABCA13 expression levels in esophageal cell lines. (B, C) Relative circABCA13 expression levels in vector- or circABCA13-overexpressing KYSE30 cells (B) and KYSE410 cells (C). (D, E) Relative cell proliferation in vector- and circABCA13-overexpressing (D) and KYSE410 cells (E) for 72 h. (F) Colony formation assay and quantification for vector- and circABCA13-overexpressing KYSE30 and KYSE410 cells. (G) Soft agar assay in vector- and circABCA13-overexpressing KYSE30 and KYSE410 cells (scale bar = 50 μm). (H, I) Transwell assays showing the migration and invasion of KYSE30 cells and KYSE410 cells (scale bars = 100 μm). (J) Western blot analysis of MMP-2, MMP-9, E-cadherin, N-cadherin, Cyclin D1, and Cyclin D3 protein levels in vector- and circABCA13-overexpressing KYSE30 and KYSE410 cells. (K) Images of tumor xenografts in nude mice ($n = 6$). (L) Quantification of tumor volume in the vector and circABCA13-OE groups. (M) Immunohistochemistry staining of Ki67 in the vector and circABCA13-OE groups of tumor xenografts. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

mediates circABCA13-induced tumor progression. Next, gene set enrichment analysis (GSEA) analysis of RNA-seq data was used to explore the regulated pathway results from circABCA13, and the results are shown in Figure 3H. The Wnt signaling pathway was highly enriched in circABCA13-overexpressing KYSE30 cells. Since SRXN1 was reported to promote cervical cancer progression via the Wnt/β-catenin pathway,[20] we next demonstrated that overexpression of circABCA13 increased the levels of β-catenin protein and phosphorylated GSK3β^{Ser9}, an inactive form of GSK3β that is unable to phosphorylate β-catenin,[28,29] and the upregulation of β-catenin and p-GSK3β^{Ser9} caused by circABCA13 overexpression was reversed by knockdown of SRXN1 (Figure 3I). These data suggest that circABCA13 upregulates SRXN1 to facilitate the oncogenic characteristics of ESCC by activating the Wnt/β-catenin pathway. In addition, SRXN1 levels were elevated in ESCC tissues compared with normal tissues according to TMA-based IHC (Figure 3J,K). Similar to the results for circABCA13, SRXN1 levels were higher in patients with lymph node metastasis (Figure S6A) and advanced tumor stage (Figure S6B) than in those without lymph node metastasis and those with low tumor stage. Pearson's correlation analysis showed that SRXN1 levels exhibited a positive correlation with circABCA13 levels (Figure 3M). Taken together, SRXN1 plays a crucial role in the oncogenesis of circABCA13 and activated Wnt/β-catenin pathway conjunction with circABCA13 in ESCC.

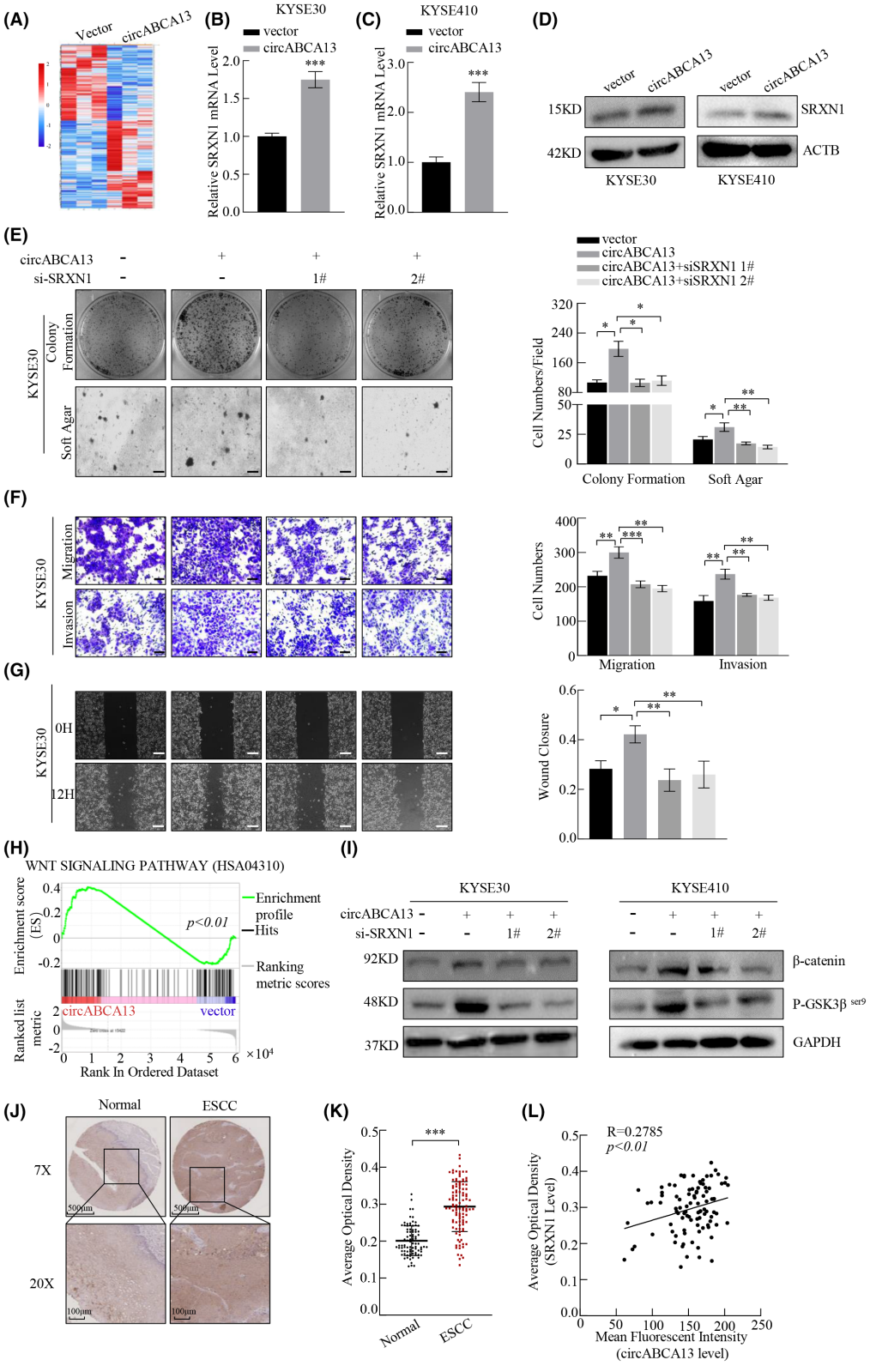
3.4 | CircABCA13 upregulates SRXN1 by acting as a sponge for miR-4429 in ESCC

Since circABCA13 upregulates SRXN1 to facilitate malignant characteristics in ESCC, and is located in cytoplasm, circRNA was also known served as a miRNA sponge in cytoplasm, we focused on the miRNA that binds to both circABCA13 and SRXN1 mRNA. There were 1546 miRNAs that may bind with SRXN1 mRNA from the miRWalk database, as well as 30 miRNAs that may bind with CircABCA13 predicted by the circBank database. Furthermore, the intersection of these two gene sets displayed 19 common miRNAs shared by circABCA13 and SRXN1 (Figure 4A), among which miR-4429 was the only miRNA that exhibited an inverse relationship with circABCA13 levels in ESCC cells (Figure 4B–D). To determine whether circABCA13 directly binds to miR-4429, an RNA pull-down assay indicated that circABCA13 sense probes were able to bind more miR-4429 rather than circABCA13 antisense probes (Figure 4E). Additionally, the luciferase activity in cells co-transfected with miR-4429 mimics and the wild-type circABCA13 luciferase reporter was significantly lower than that in cells co-transfected with miR-4429 mimics and the mutant circABCA13 luciferase reporter (Figure 4F,G), further supporting a direct interaction between miR-4429 and circABCA13. To assess whether miR-4429 is involved in SRXN1 upregulation by circABCA13, we constructed a luciferase reporter containing the full-length SRXN1 and three truncations of the SRXN1 3′UTR, each of which had a potential binding site for miR-4429 (Figure 4H). CircABCA13 overexpression increased the luciferase activity of the SRXN1 3′UTR reporter, while co-transfection with miR-4429 mimics significantly decreased circABCA13-induced luciferase activity. Consistent results were observed in the UTR-1 region, suggesting that the (421–921 bp) region was most efficient for miR-4429 targeting of the whole SRXN1 3′UTR (Figure 4I). Next, we constructed a SRXN1 UTR-1 region luciferase reporter containing a mutant miR-4429 binding site (Figure S6C) and the results showed that the mutated SRXN1 UTR-1 luciferase reporter lost the promotion luciferase activity in circABCA13 overexpression and also abolished the inhibition by miR-4429 mimics transfection (Figure 4J). In addition, the miR-4429 mimics reversed the SRXN1 mRNA expression upregulation (Figure 4K) and protein expression (Figures 4L and S6D) induced by circABCA13 in both KYSE30 and KYSE410 cells. In summary, circABCA13 may serve as a miR-4429 sponge to upregulate SRXN1, facilitating ESCC cell proliferation, migration, and invasion.

3.5 | CircABCA13 promoted ESCC progression via the miR-4429/SRXN1 axis in vitro and in vivo

To investigate whether miR-4429 plays a role in circABCA13-promoted ESCC progression, miR-4429 mimics were transfected into circABCA13-overexpressing cells (Figure S7A,B). CCK-8 and colony formation assays showed that transfection of miR-4429 mimics reduced circABCA13-induced cell proliferation in both KYSE30 and KYSE410 cells (Figures 5A and S7C,D). Moreover, the enhancement of cell migration and invasion as well as the capacity to undergo anchorage-independent growth were completely abolished in cells transfected with miR-4429 (Figures 5B–D and S7E). These results indicate that circABCA13 promotes the development of ESCC cells by functioning as a miR-4429 sponge. Moreover, the increase in the protein level of SRXN1 caused by overexpression of circABCA13 could be rescued by miR-4429 mimic transfection (Figures 4K,L and S6D). Western blot analysis confirmed that the upregulation of β-catenin and GSK3β^{Ser9} caused by circABCA13 overexpression was rescued after miR-4429 transfection (Figure 5E). Taken together, these results indicate that circABCA13 promotes the activation of the Wnt signaling pathway by sponging miR-4429.

To determine the functional role of circABCA13/miR-4429/SRXN1 in vivo, KYSE410 cells stably transfected with vector, circABCA13, circABCA13 plus shSRXN1, circABCA13 plus miR-4429 mimics were subcutaneously injected into nude mice and analyzed for tumor growth. In comparison to the control group, the group with overexpression of circABCA13 showed increased ESCC cell proliferation, as evidenced by increased tumor volume and weight as well as increased expression of the proliferation index marker Ki67. Overexpression of miR-4429 or downregulation of SRXN1 attenuated the increases in tumor volume (Figure 5F,G), tumor weight (Figure S7F), and Ki67 levels (Figure 5H). Moreover, SRXN1 protein expression was rescued by miR-4429 mimics in xenograft tissues (Figure 5I). Taken together, these results demonstrate that the circABCA13/miR-4429/SRXN1 axis promotes ESCC progression both in vitro and in vivo (Figure 6).



4 | DISCUSSION

Recent studies have suggested that circRNAs can be either oncogenic or tumor-suppressive and have great potential to serve as biomarkers for tumor diagnosis, progression, and prognosis prediction.[30,31] In this study, we demonstrated the oncogenic role of circABCA13 and its underlying mechanism in ESCC progression. circABCA13 expression was upregulated in ESCC tissues and cell lines compared with normal controls. Upregulation of circABCA13 correlated with advanced TNM stage and a poor prognosis in ESCC

FIGURE 3 CircABCA13 accelerated esophageal squamous cell carcinoma cell proliferation, migration, and invasion by upregulating SRXN1. (A) Clustered heatmap of significant differentially expressed genes in KYSE30 cells with stable circABCA13 overexpression ($n=3$) or vector group ($n=3$). (B, C) Relative SRXN1 mRNA expression levels in KYSE30 (B) and KYSE410 (C) cells with stable circABCA13 overexpression. (D) SRXN1 protein levels incircABCA13 overexpressed KYSE30 and KYSE410 cells. (E–G) Rescue experiments showed that knockdown of SRXN1 could partially reverse the oncogenic function of circABCA13 in colony formation assays and soft agar assays. (E, scale bars = 50 μm), Transwell assays (F, scale bars = 100 μm), and wound healing assays (G, scale bars = 200 μm) in KYSE30 cells. (H) Gene set enrichment analysis from RNA-seq showed the activation of the Wnt signaling pathway. (I) Western blotting analyses show that knockdown of SRXN1 rescued the activation of the Wnt signaling pathway. (J) Representative immunohistochemistry images of ESCC tissues and normal tissues. (K) Relative protein levels of SRXN1 in ESCC tissues and normal tissues, ***$p<0.001$. (L) Pearson's correlation analysis of the relative expression of circABCA13 and SRXN1 ($r=0.2785$, $p<0.001$). *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

patients. Overexpression of circABCA13 in ESCC cells promoted proliferation, migration, and invasion both in vitro and in vivo, whereas knockdown of circABCA13 inhibited the malignant characteristics of ESCC cells. Moreover, RNA sequencing analysis revealed a potential mechanism for circABCA13 and revealed that the Wnt signaling pathway was closely correlated with circABCA13. Additionally, we found that circABCA13 could upregulate SRXN1 by sponging miR-4429. Rescue experiments demonstrated that the circABCA13/miR-4429/SRXN1 axis promoted the progression of ESCC by activating the Wnt/β-catenin pathway.

Clinically, esophageal cancer is diagnosed in an advanced stage due to insidious early symptoms, and late diagnosis is associated with a poor prognosis.[32] Therefore, it is necessary to find novel biomarkers that provide more insight for ESCC diagnosis and prognosis evaluation. Recently, emerging evidence has indicated that circRNAs play critical roles in various tumors.[33] We demonstrated that circABCA13 expression was elevated in the tumor tissues of ESCC patients. In our study, we found that circRNAs were more stable than linear RNAs. Due to its stable circular structure, circRNA has greater potential to serve as a biomarker for tumor diagnosis and prognosis than other molecular markers.[10,34] Our results showed that elevated circABCA13 expression in ESCC tissues is significantly correlated with advanced ESCC clinical stages. Additionally, ROC and Kaplan–Meier analyses further supported that circABCA13 may be an important clinical marker for evaluating lymph node metastasis and prognosis in ESCC patients. Further studies including a larger number of clinical samples should be performed to verify the efficacy of circABCA13 as a biomarker.

Recent studies have suggested that circRNAs play oncogenic roles in the occurrence and development of ESCC tumors.[35] Through functional experiments, we confirmed that circABCA13 accelerated ESCC proliferation, promoted anchorage-independent growth, and enhanced cell migration and invasion in vitro. Consistently, mouse xenograft models further verified the oncogenic role of circABCA13 in vivo. To elucidate the mechanism behind circABCA13-mediated ESCC progression, we explored the potential downstream genes regulated by circABCA13 via RNA-seq. SRXN1 was identified as the downstream gene of circABCA13 and further verified through qRT–PCR. SRXN1 is a newly discovered antioxidant enzyme that has protective functions against oxidative stress,[36] and it has been shown to be closely correlated with the progression of several types of cancer.[37] Interestingly, the findings from this study support the idea that upregulation of SRXN1 is positively associated with circABCA13 in ESCC tissues and, vice versa, knockdown of SRXN1 has inhibitory effects on ESCC progression in vitro and in vivo. The activation of β-catenin to initiate the transcription of its specific downstream target genes has been reported to be associated with ESCC.[38] In our study, we found that the Wnt/β-catenin pathway may play a critical role in the process by which circABCA13 promotes ESCC progression after GSEA of RNA-seq data. SRXN1 was previously reported to promote cell invasion and migration in cervical cancer by activating the Wnt/β-catenin signaling pathway.[39,40] We revealed that the upregulation of β-catenin and p-GSK3β^{Ser9} caused by circABCA13 overexpression was significantly reversed by SRXN1 knockdown. Together, our results suggest that circABCA13 activates the Wnt/β-catenin pathway by upregulating SRXN1 to facilitate the malignant characteristics of ESCC cells.

Functionally, the role of noncoding circRNAs as miRNA sponges has been previously demonstrated.[17] Our bioinformatic analyses verified that miR-4429 was a circABCA13 target, which was confirmed by dual-luciferase reporter assays and qRT–PCR. MiR-4429 has been demonstrated to inhibit the progression of human tumors and interact with the Wnt/β-catenin pathway. For example, miR-4429 suppresses the proliferation of prostate cancer cells by targeting distal-less homeobox 1 and inactivating the Wnt/β-catenin pathway.[41] However, the role of miR-4429 in ESCC remains largely unknown. Our study suggested that miR-4429 could target the SRXN1 3′UTR and further confirmed that the miR-4429/SRXN1 axis plays a critical role in the oncogenic function of circABCA13. The exact mechanism by which SRXN1 influences the Wnt/β-catenin pathway in ESCC deserves further investigation.

In conclusion, the results from our study demonstrate that upregulation of a novel circRNA, circABCA13, in ESCC was associated with the progression and prognosis of ESCC. CircABCA13 could facilitate the malignant characteristics of ESCC by sponging miR-4429, a novel microRNA that binds to the SRXN1 3′UTR, and by regulating SRXN1 expression by binding to the SRXN1 UTR to activate the Wnt/β-catenin pathway. Our findings provide insight into the underlying mechanisms of ESCC progression and suggest that circABCA13 may serve as a prognostic biomarker and potential therapeutic target for ESCC patients.

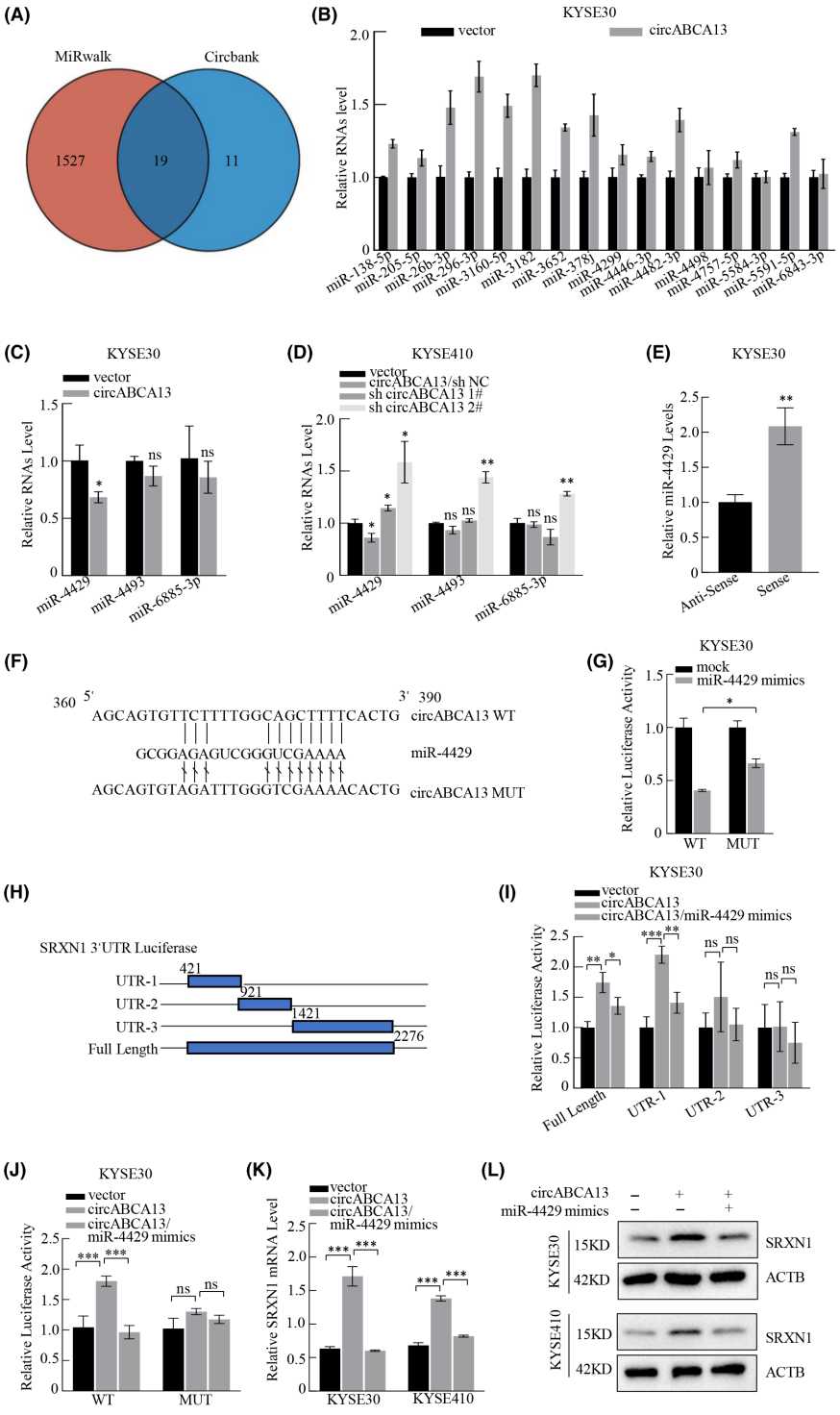


FIGURE 4 CircABCA13 upregulated SRXN1 and activated the Wnt/β-catenin pathway by functioning as a sponge for miR-4429 in vitro. (A) The Venn diagram shows that 19 miRNAs were selected to combine with circABCA13 and SRXN1. (B–D) Relative 19 miRNA expression levels in KYSE30 cells (B), KYSE410 cells (C) with stable circABCA13 overexpression or vector group, and KYSE410 cells (D) with stable circABCA13 knockdown. (E) The results of RNA pull-down assays in KYSE30 cells. (F) A schematic of the design of the luciferase assay. The predicted seed-recognition site in the corresponding circABCA13 sequence is marked. (G) Relative luciferase activity of the circABCA13 reporter plasmid in KYSE30 cells transfected with miR-4429 mimics. (H, I) Relative luciferase activity of the whole SRXN1 UTR and the three truncations in vector, circABCA13-OE, and circABCA13-OE with miR-4429 mimic-transfected KYSE30 cells. (J) Relative luciferase activity of the SRXN1 UTR1 wild-type reporter plasmid and mutant plasmid in vector, circABCA13-OE, and circABCA13-OE with miR-4429 mimic-transfected KYSE30 cells. (K) Relative SRXN1 mRNA expression in vector, circABCA13-OE, and circABCA13-OE with miR-4429 mimic-transfected KYSE30 and KYSE410 cells. (L) SRXN1 protein expression in vector, circABCA13-OE, and circABCA13-OE with miR-4429 mimic-transfected KYSE30 and KYSE410 cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

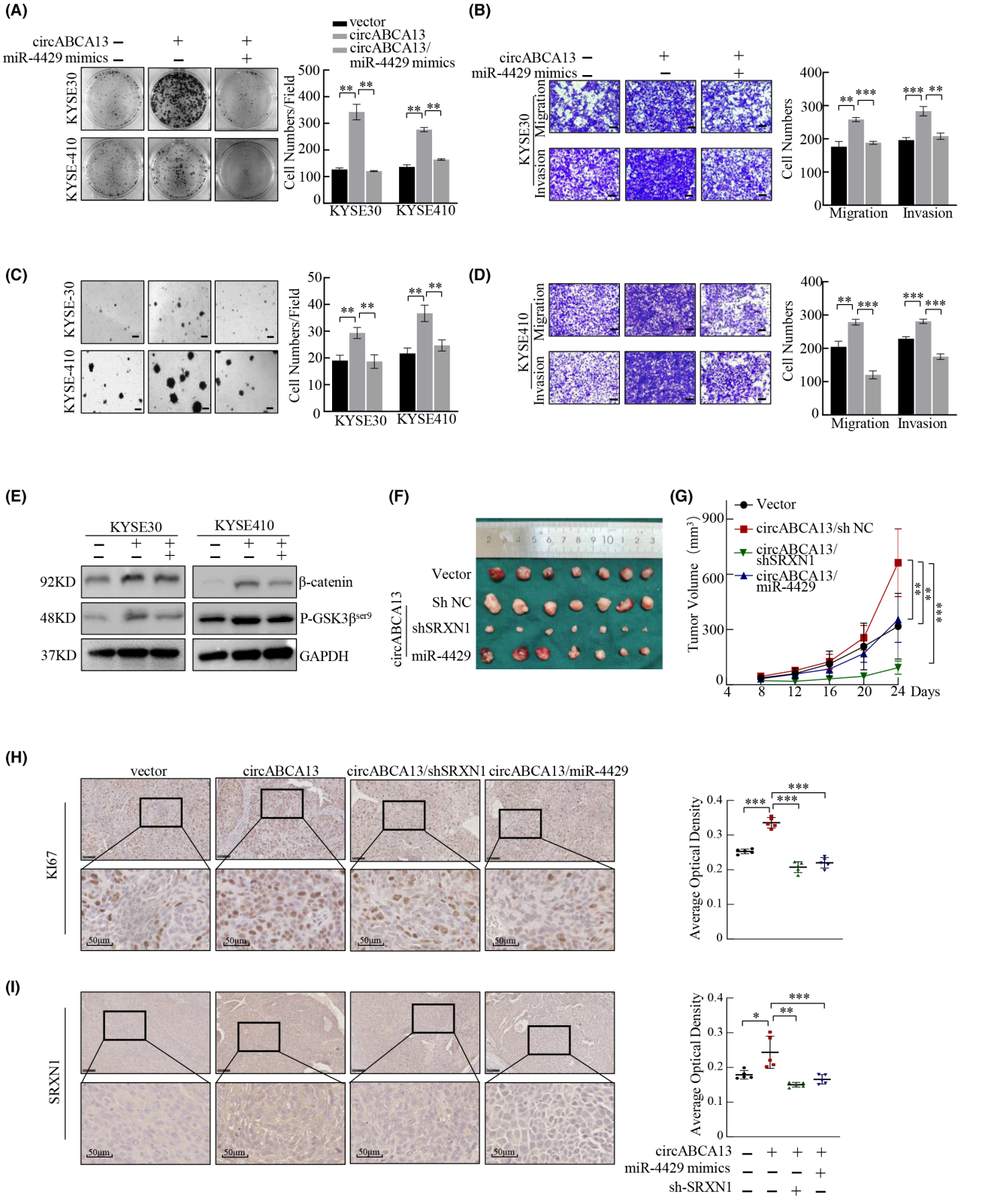


FIGURE 5 SRXN1 is regulated by the circABCA13/miR-4429 interaction to promote esophageal squamous cell carcinoma tumor progression in vitro and in vivo. (A–D) Rescue experiments showed that the transfection of miR-4429 mimics reduced the oncogenic function of circABCA13 in colony formation assays (A), soft agar assays (C, scale bars = 50 μm), and Transwell assays (B, D, scale bars = 100 μm) in both KYSE30 and KYSE410 cells. (E) Western blot analysis shows that transfection with miR-4429 mimics could rescue the activation of the Wnt signaling pathway in both KYSE30 and KYSE410 cells. (F) Images of subcutaneous xenograft tumors formed by KYSE410 cells stably transfected as indicated in nude mice. (G) Tumor volumes were measured for each group. (H, I) Immunohistochemistry staining of Ki67 (H) and SRXN1 (I) in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

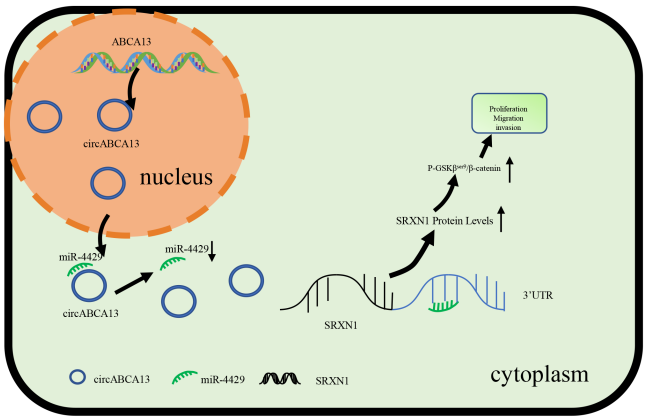


FIGURE 6 Mechanistic diagram of the CircABCA13/miR-4429/SRXN1 axis in esophageal squamous cell carcinoma (ESCC). The mechanism diagram shows that during ESCC proliferation and progression, overexpressed circABCA13 sponges miR-4429, upregulates SRXN1, and activates the Wnt signaling pathway.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: This study was approved by the Ethics Committee of The Second Hospital of Shandong University.

Informed Consent: Patients were informed consent for obtaining samples and the study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Ethics Committee of the Second Hospital of Shandong University.

Registry and the Registration No. of the study/trial: KYLL-2022A038 and KYLL-2022P038.

Animal Studies: All animal experiments were approved by the Ethics Committee of The Second Hospital of Shandong University.

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SUPPORTING INFORMATION

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

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