Long non-coding RNA ZEB1-AS1 promotes colon adenocarcinoma malignant progression via miR-455-3p/PAK2 axis

Xin Ni | Yuting Ding | Haitao Yuan | Jinmin Shao | Yan Yan | Rouyu Guo | Wenkang Luan | Min Xu

1Department of Gastroenterology, Affiliated Hospital of Jiangsu University, Zhenjiang, China
2Department of Rehabilitation, Changshu No. 2 People’s Hospital (The 5th Clinical Medical College of Yangzhou University), Changshu, China
3Department of General Surgery, Affiliated People’s Hospital of Jiangsu University, Zhenjiang, China
4Department of Liver Disease, Zhenjiang Third People’s Hospital, Zhenjiang, China
5Department of Plastic Surgery, Affiliated People’s Hospital of Jiangsu University, Zhenjiang, China

Correspondence
Min Xu, Department of Gastroenterology, Affiliated Hospital of Jiangsu University, 438 Jiefang Road, Zhenjiang, Jiangsu 212000, China.
Email: peterxu1974@163.com and Wenkang Luan, Department of Plastic Surgery, Affiliated People’s Hospital of Jiangsu University, 8 Dianli Road, Zhenjiang, Jiangsu 212000, China.
Email: luanwenkang@126.com

Funding information
The Health and Family Planning Science and Technology Key project Foundation of Zhenjiang city, Grant/Award Number: SHW2017004; National Natural Science Foundation of China, Grant/Award Number: 81672402 and 81802726; Provincial Natural Science Foundation of Jiangsu, Grant/Award Number: BK20171305

Abstract
Objective: The long non-coding RNA zinc finger E-box-binding homeobox 1 antisense 1 (ZEB1-AS1) acts as an oncogenic regulator in many human tumours. In the present study, we identify the role and potential molecular biological mechanisms of ZEB1-AS1 in colon adenocarcinoma (COAD).

Methods: QRT-PCR was used to detect the expression of ZEB1-AS1, miR-455-3p and p21-activated kinases 2 (PAK2) in COAD tissues. CCK8 assay, EdU assay, transwell assay and scratch wound assay were used to explore the biological function of ZEB1-AS1 in COAD cells. Bioinformatics, luciferase reporter assays and an RNA pull-down assay were used to demonstrate the mechanism of ZEB1-AS1. We further explore the role of ZEB1-AS1 in vivo though xenograft tumour assay.

Results: We found that ZEB1-AS1 expression was significantly up-regulated in COAD tissues, and high ZEB1-AS1 level was correlated with the poor prognosis of COAD patients. MiR-455-3p plays an anti-cancer role in COAD by targeting PAK2. We confirmed that ZEB1-AS1 promotes PAK2 expression by sponging miR-455-3p, thus facilitating COAD cell growth and metastasis.

Conclusions: To sum up, this result illustrates the novel molecular mechanism of ZEB1-AS1 in COAD and provides a new target for the diagnosis and treatment of COAD patients.
1 | INTRODUCTION

Colon adenocarcinoma (COAD), a type of colorectal cancer, is one of the most common causes of cancer-related mortality in worldwide. The incidence of COAD in Asian countries has risen sharply in the past decade. Although many treatments have been used for the management of such cancer, including surgical treatment, radiotherapy and chemotherapy, the 5-year overall survival rate is still poor. The main reason for poor prognosis in COAD patients is the lack of reliable biomarkers and therapeutic targets. Therefore, it is important to find the biomarkers of COAD and explore the potential molecular mechanism of COAD malignant progression.

Long non-coding RNAs (lncRNAs) play a crucial role in tumorigenesis in different ways, such as chromatin modification, transcription and post-transcriptional regulation. The aberrant expression of lncRNAs can be used as the biomarker in the diagnosis and prognosis of many human tumours. LncRNAs are also involved in the malignant progression of COAD, and some lncRNAs’ function in COAD have been confirmed.

Long non-coding RNA zinc finger E-box-binding homeobox 1 antisense 1 (ZEB1-AS1), located in physical contiguity with ZEB1, has been proved to be a cancer-related IncRNA. ZEB1-AS1 plays an oncogenic role in many cancers, such as hepatocellular cancer, glioma, gastric cancer and prostate cancer. However, the function and molecular biological mechanisms of ZEB1-AS1 in the malignant progression of COAD remain unclear.

In the present study, we found ZEB1-AS1 was significantly up-regulated in COAD tissues. We showed that miR-455-3p plays an anti-cancer role in COAD by targeting p21-activated kinases 2 (PAK2). PAK2 is a member of the P21-activated kinases (PAKs) family of serine/threonine kinases. PAKs, the effectors of the Rho family of small GTPases, participate in a variety of cellular signalling pathways. Accumulating evidence suggests that overexpression of PAK2 is involved in signalling pathways related to malignant progression of many malignant tumours. Furthermore, we demonstrated that ZEB1-AS1 function, as a sponge of miR-455-3p, lead to the loss of the inhibitory effect of miR-455-3p on PAK2, thus promoting the expression of PAK2. ZEB1-AS1 promotes the growth and metastasis of COAD via up-regulating PAK2 level. Thus, ZEB1-AS1 can act as a new special biomarker or target for the diagnosis and treatment of COAD patients.

2 | MATERIALS AND METHODS

2.1 | Human tissues

For this study, we collected 28 paired COAD tissues and adjacent normal tissues (ANT) from COAD patients at The Affiliated Hospital of Jiangsu University. Two pathologists independently diagnosed the pathological features of the tumour tissues. No patients received any neoadjuvant radiotherapy or chemotherapy before operation. This research was endorsed by the Research Ethics Committee of the Affiliated Hospital of Jiangsu University. The Cancer Genome Atlas (TCGA) COAD data are downloaded from the Genome Data Public Data Portal (https://portal.gdc.cancer.gov/).

2.2 | Cell lines and cell culture

The primary COAD cell lines (SW480, HT29, LS174T, HCT116 and DLD-1) and normal human colon histiocytes (CCD-18Co) were bought from American Type Culture Collection (ATCC). All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% foetal bovine serum (Thermo Fisher Scientific). COAD cells and normal human colon histiocytes were cultured in a humidified atmosphere of 37°C containing 5% CO2.

2.3 | Oligonucleotides and transfection

miR-455-3p mimic, miR-455-3p inhibitor and negative control (NC) were bought from GenePharma. The small interference RNAs (siRNAs) for ZEB1-AS1 and PAK2 was also obtained from GenePharma. The full length of PAK2 was amplified and inserted into a pcDNA3.1 vector (Invitrogen) to construct the PAK2 plasmid. Related oligonucleotides were transfected into COAD cells by using Lipofectamine 3000 (Invitrogen).

2.4 | Cell proliferation assay

For CCK-8 (Beyotime) assay, the COAD cells (5000 cells) were seeded in a 96-well plate, and 100 μL culture media supplemented with 10% CCK8 was added to each well and incubated at different times (12, 24, 36 and 48 hours). The absorbance (450 nm) was measured using microplate reader (Multiscan FC; Thermo Scientific). For EdU assay, EdU imaging kit (Life Technologies) was used to measure the DNA synthesis of COAD cells grown. EdU assay was carried out according to the reagent manufacturer’s instructions, and immunostaining and EdU results were visualized using Leica DMI3000B microscope. We counted the positive cells.

2.5 | Cell invasion and migration assays

For transwell assay, the COAD cells were digested and suspended in serum-free culture medium. These cells were placed on the top of the Matrigel-coated chambers (BD Biosciences), and culture medium containing 10% foetal bovine serum was added to the lower chamber as a chemical attractant. After 24 hours, the invading cells were stained and counted. For scratch wound assay, transfected COAD cells were added into a 6-well plates, and a 200 μL pipette tip was used to form wound gaps. Cells were photographed, and wound widths were recorded at 0 and 48 hours.
2.6 | Quantitative RT-PCR

RNA was extracted by using TRizol (Invitrogen), and reverse transcription was performed using different reverse transcription kits (Applied Biosystems). The PCR amplification reaction was carried out by using StepOnePlus system (Applied Biosystems) according to the set reaction conditions. Special primer of miR-455-3p was obtained from RiboBio, and U6 was used for normalization. The primers for ZEB1-AS1 are as follows: 5’-CCAGACACCTACACAACCTCC-3’ and 5’-GTGATCTCTACCT CGCTTGCTT-3’. The relative expression level was calculated using the 2^−△△Ct.

2.7 | Western blot

Total proteins were extracted by using RIPA (KenGEN). A BCA Protein Assay Kit (Beyotime) was used to quantify the concentration of protein. Western blotting was carried out according to the previous described. PAK2 antibody was purchased from Abcam (1:5000) and was used to analyse PAK2 levels, and GAPDH (1:2500; Abcam) was used for normalization.

2.8 | Luciferase reporter assay

The fragment of PAK2 3’-UTR and ZEB1-AS1 containing the binding site of miR-455-3p was inserted into the pMIR-REPORT plasmid. COAD cells were cotransfected with miR-455-3p mimic and related reporter plasmids. Mutated plasmid was used as a control. Dual-Luciferase Reporter Assay System (Promega) was used to measure the luciferase activity.

2.9 | Isolation of RISC-associated RNA

Colon adenocarcinoma cells that overexpressed miR-455-3p were fixed with 1% formaldehyde. The cells were lysed using NETN buffer and cultured with Dynabeads protein A (Invitrogen) supplemented with IgG or anti-Pan-Ago and clone 2A8 antibody (Millipore). Proteinase K digestion was used to release immunoprecipitated RNA. RNA was extracted, purified by ethanol precipitation with glycogen and treated with DNase I.

2.10 | MS2-RIP assay

We used maltose-binding protein (MBP)-affinity purification to identify miRNAs that associated with ZEB1-AS1. The MS2-MBP was expressed and purified from E.coli according to Steitz laboratory method. Three bacteriophage MS2 coat protein-binding sites were inserted downstream of ZEB1-AS1 by using Stratagene QuikChange Site Directed Mutagenesis Kit. COAD cells were transfected with MS2-tagged ZEB1-AS1 to obtain miRNAs associated with the ZEB1-AS1. The cells were subjected to RIP analysis after 48 hours. The level of miR-455-3p was detected by qRT-PCR.

2.11 | RNA pull-down assay

Biotinylated miR-455-3p was bought from GenePharma, and biotinylated mutant and NC were used as controls. Biotinylated miR-455-3p, biotinylated mutant and NC were transfected into COAD cells. The cell lysates were incubated with M-280 streptavidin magnetic beads (Invitrogen). The bound RNA was extracted, and the ZEB1-AS1 level was detected by using qRT-PCR.

2.12 | Xenograft tumour assay

Fourteen mice (Beijing Laboratory Animal Center) were injected subcutaneously with HT29 cells, and the mice were divided into two groups after the tumours reached 50 mm3. ZEB1-AS1 siRNA or NC was injected into the inner region of the tumour every 4 days for 28 days. The tumour volume (length × width^2 × 0.5) was measured according to formulas every 4 days. The subcutaneous tumours were removed after 28 days. This research was endorsed by the Research Ethics Committee of the Affiliated Hospital of Jiangsu University. Immunohistochemistry staining of subcutaneous tumours was carried out according to the previously described Ref. and PAK2 antibody (1:150; Abcam) was used.

2.13 | Statistical analysis

The data are expressed as mean ± standard deviation (SD), and spss13.0 was used for data analysis. t test or one-way ANOVA was used to evaluate the statistical significance. Correlation analysis (spearman) was performed by using MATLAB. Kaplan-Meier analysis was used to plot survival curves. P < .05 had statistical significance considered to have statistical significance.

3 | RESULTS

3.1 | ZEB1-AS1 was increased in COAD, and high ZEB1-AS1 level was a risk factor for the prognosis of COAD patients

We initially analysed the expression of ZEB1-AS1 in 28 COAD tissues and ANT. ZEB1-AS1 was significantly increased in COAD tissues compared with ANT (Figure 1A). We discovered the same result by analysing the TCGA-COAD data set (Figure 1B). Meanwhile, primary COAD cell lines (SW480, HT29, LS174T, HCT116 and DLD-1) expressed higher ZEB1-AS1 levels compared with normal human colon histiocytes (CCD-18Co; Figure 1C). COAD patients with high ZEB1-AS1 expression (ZEB1-AS1 expression ratio > median ratio) had a poorer
survival (Figure 1D). TCGA-COAD data set also showed that ZEB1-AS1 overexpression was correlated with poor survival of COAD patients (Figure 1E). High ZEB1-AS1 level was closely related to the stage, lymph node metastasis and distant metastasis of COAD, but not to age, family history and sex (Table 1). These results indicated that ZEB1-AS1 maybe involved in the malignant progression of COAD.

**FIGURE 1** ZEB1-AS1 was significantly increased in COAD and was found to be a risk factor for the survival of COAD patients. A, The expression of ZEB1-AS1 was measured in 28 COAD tissues and ANT tissues. B, The expression of ZEB1-AS1 was analysed in 471 COAD tissues and 41 normal samples by using TCGA database. C, The ZEB1-AS1 expression profile in primary COAD cell lines (SW480, HT29, LS174T, HCT116 and DLD-1) and normal human colon histiocytes (CCD-18Co). D, The overall survival curves of 28 COAD patients. E, The overall survival curves of COAD patients, data from TCGA database. *P < .05, **P < .01, ***P < .001. COAD, colon adenocarcinoma; ZEB1-AS1, zinc finger E-box-binding homeobox 1 antisense 1
3.2 ZEB1-AS1 promotes the COAD cell proliferation, invasion and migration

To further explore the biological function of ZEB1-AS1 on COAD cells, ZEB1-AS1 siRNA was transfected into SW480 and HT29 cells (Figure 2A). Reduction in ZEB1-AS1 significantly inhibited the proliferation ability of SW480 and HT29 cells (Figure 2B). Moreover, EdU assay revealed that ZEB1-AS1 knockdown SW480 and HT29 cells exhibited a marked decrease in the number of EdU-positive cells (Figure 2C). The invasive and migratory capacities of SW480 and HT29 cells were also repressed in ZEB1-AS1 siRNA transfected COAD cells (Figure 2D,E). These results indicated that ZEB1-AS1 can promote the growth and metastasis of COAD.

3.3 ZEB1-AS1 sponged miR-455-3p in COAD cells

Some lncRNAs play the role of competing endogenous RNAs (ceRNAs), which can bind to miRNAs to liberate mRNAs targeted by miRNAs. ZEB1-AS1 is expressed in both the nucleus and the cytoplasm and also has this effect. RT-qPCR assays revealed that ZEB1-AS1 was localized in cytoplasm and nuclear (Figure 3A). Starbase 3.0 (http://starbase.sysu.edu.cn) was used to found potential miRNAs, and miR-455-3p may bind to ZEB1-AS1. We constructed ZEB1-AS1 luciferase vectors containing the miR-455-3p binding sites (wild type and mutated; Figure 3B). MiR-455-3p mimics reduced the luciferase activity of wild-type ZEB1-AS1 vector in COAD cells (Figure 3C). MS2-RIP assay was used to further verify the direct interaction between miR-455-3p and ZEB1-AS1 in COAD. The MS2-tagged wild-type ZEB1-AS1 plasmid was enriched for miR-455-3p compared with the empty and mutant vectors (Figure 3D). Additionally, we performed an RNA pull-down assay with biotinylated miR-455-3p and found that ZEB1-AS1 was pulled down by biotinylated miR-455-3p in COAD cells (Figure 3E). The miR-455-3p level was decreased after transfection of ZEB1-AS1 siRNA in SW480 and HT29 cells (Figure 3F). Meanwhile, we analysed the level of miR-455-3p in 28 pairs of COAD tissues and ANT, and found that miR-455-3p was down-regulated in COAD tissues (Figure 3G). We also found a negative correlation between the expression of miR-455-3p and ZEB1-AS1 in COAD tissues (Figure 3H). TCGA-COAD database also reveals the same results (Figure 3I,J). All results showed that ZEB1-AS1 directly binds to miR-455-3p in COAD.

3.4 ZEB1-AS1 acts as a ceRNA to promote PAK2 expression in COAD cells

We next applied bioinformatics software (TargetScan, miRDIP, Starbase, miRDB and miRPathDB) to predict the potential target of miR-455-3p (Figure 4A). PAK2 was found to have possible targets with miR-455-3p, and the 3’-UTR of PAK2 has the same binding sites that ZEB1-AS1 combined with miR-455-3p (Figure 4B). The luciferase activity of the wild-type PAK2 vectors was significantly decreased by miR-455-3p mimic in COAD cells (Figure 4C). We detected the PAK2 mRNA abundance in the Ago2/RNA-induced silencing complex (RISC) after overexpression of miR-455-3p.
Enrichment in the level of miR-455-3p and PAK2 that incorporated into RISC was observed in miR-455-3p mimic transfected cells by using RNA-ChIP analysis (Figure 4D). Overexpression of miR-455-3p repressed the expression of endogenous PAK2 protein (Figure 4E). These results suggest that PAK2 is the target of miR-455-3p in COAD. ZEB1-AS1 siRNA also inhibited the luciferase activity of the wild-type PAK2 vectors, and this inhibition can be reversed by miR-455-3p inhibitor (Figure 4C). Moreover, ZEB1-AS1
siRNA repressed the protein levels of PAK2 in COAD cells, and the effect of ZEB1-AS1 siRNA on PAK2 expression was also attenuated by cotransfection with the miR-455-3p inhibitor (Figure 4E). Twenty-eight COAD tissues collected by us, and TCGA-COAD data sets showed a positive correlation between ZEB1-AS1 and PAK2 mRNA level in COAD (Figure 4F,G). To sum up, these results indicated that ZEB1-AS1 up-regulated PAK2 expression by competitive binding miR-455-3p in COAD.

3.5 | ZEB1-AS1 promotes the growth and metastasis of COAD though sponging miR-455-3p to up-regulate PAK2 level

p21-activated kinases 2 can promote the proliferation and invasion of many cancer cells. We also confirmed the oncogenic effect of PAK2 in COAD (Figure S1A-E). We next studied the role
of miR-455-3p in COAD cells. The miR-455-3p mimics inhibited the proliferation, invasive and migratory ability of COAD cells (Figure 5A-D). The effects of miR-455-3p on the COAD cells proliferation, invasion and migration changes were rescued by the PAK2 plasmid (Figure S1A-E). These indicated that miR-455-3p plays an anti-cancer role by targeting PAK2 in COAD. To study whether ZEB1-AS1 plays its oncogenic role through sponging miR-455-3p, we cotransfected ZEB1-AS1 siRNA and miR-455-3p inhibitors into COAD cells. We found that the inhibitory effect of ZEB1-AS1 siRNA on growth and metastasis of COAD cells was reversed by miR-455-3p inhibitor (Figure 5A-D). As mentioned earlier, the miR-455-3p inhibitor also rescued the effect of miR-455-3p in COAD cells.
of ZEB1-AS1 siRNA on PAK2 level (Figure 4D). We used COAD xenograft model to explore the role of ZEB1-AS1 in vivo. The excision tumour of nude mice was shown in Figure 5E. As showed in Figure 5F, knockdown group of ZEB1-AS1 showed significant inhibition of tumour growth between 20 and 28 days. Compared with the control group, the weight of tumours in ZEB1-AS1 knockdown group was lighter (Figure 5G). The nude mice were sacrificed after 28 days, and the miR-455-3p level was increased and that of PAK2 was decreased in the ZEB1-AS1 knockdown group (Figure 5H,I). Overall, we demonstrated that ZEB1-AS1 modulates the malignant progress of COAD via sponging miR-455-3p to promote PAK2 expression.

**FIGURE 5** ZEB1-AS1 promotes the growth and metastasis of COAD by sponging miR-455-3p to up-regulate PAK2 expression. A, The proliferative ability of COAD cells was determined by CCK8 assay. B, The DNA synthesis of COAD cells grown was measured by EdU assay. Scale bar, 100 μm. C, The invasive capacity of COAD cells was assessed by the transwell assay. Scale bar, 50 μm. D, The migratory ability of COAD cells was assessed by the scratch wound assay. Scale bar, 200 μm. E, The excision tumour in nude mice of HT29 xenografts. F, Differences in tumour volume among groups. G, The tumour weight of excised tumour tissues. H, PCR identified miR-455-3p and ZEB1-AS1 expression changes. I, The expression of PAK2 was examined by immunohistochemical staining of sections from the xenograft model. Scale bar, 200 μm. *P < .05, **P < .01, ***P < .001. COAD, colon adenocarcinoma; PAK2, p21-activated kinases 2; ZEB1-AS1, zinc finger E-box-binding homeobox 1 antisense 1

4 | DISCUSSION

Long non-coding RNA is a non-coding RNA with a length greater than 200 nucleotides. The abnormal expression of lncRNAs plays a key role in the malignant progression of many malignant tumours. ZEB1 is a transcription factor that promotes invasion and metastasis of cancer cells. LncRNA ZEB1-AS1, located physically adjacent to ZEB1, has been reportedly contributed to various cancers progression. For instance, ZEB1-AS1 facilitated hepatocellular carcinoma cell growth and motility, and promoted tumour metastasis in vivo. ZEB1-AS1 also promotes the proliferation and migration capacities of prostate cancer cells. It has demonstrated that the high
ZEB1-AS1 level indicates poor prognoses of colorectal cancers.\(^{31}\) However, the role and potential mechanism of ZEB1-AS1 in COAD have not been determined. Here, we found that ZEB1-AS1 was increased in COAD and conferred a poor prognosis to COAD patients. ZEB1-AS1 increased the proliferation, invasion and migration ability of COAD cell. Next, we further explored the potential molecular mechanism of ZEB1-AS1 in COAD.

Numerous studies have shown that certain specific IncRNAs function as ceRNAs, which can sponge miRNAs to regulate the target genes expression of miRNAs.\(^{32,33}\) Accumulated evidence indicates that ZEB1-AS1 also functions as a ceRNA in many human tumours. For instance, ZEB1-AS1 promotes the tumorigenesis of glioma by regulating the miR-200c/141-ZEB1 axis.\(^{34}\) ZEB1-AS1 also facilitates melanoma progression by regulating miR-1224-5p.\(^ {28}\) Here, we showed that ZEB1-AS1 directly binds to miR-455-3p in COAD cells.

miR-455-3p plays a role of tumour suppressor in many tumours.\(^ {25,36}\) In this study, we proved that miR-455-3p inhibits the proliferation, invasion and migration of COAD cells via targeting PAK2. PAKs, a member of the STE20 serine/threonine kinases family, play an important role in regulating the changes in actin cytoskeleton structure and cell morphology.\(^ {27}\) PAK2, a member of PAKs family, is associated with malignant progression of human cancer.\(^ {22}\) Recent study revealed that PAK2 could mediate tumour cell proliferation, invasion, apoptosis and so on.\(^ {38,39}\) We confirmed the oncogenic effect of PAK2 in COAD. We discovered that the PAK2 3’-UTR shares the binding sites of miR-455-3p with ZEB1-AS1. Moreover, we confirmed that ZEB1-AS1 promotes PAK2 expression by competitive binding miR-455-3p in COAD. The inhibitory effect of ZEB1-AS1 siRNA on COAD cells can be rescued by cotransfecting miR-455-3p inhibitor. ZEB1-AS1 also promotes COAD progression in vivo via miR-455-3p/PAK2 axis.

In summary, ZEB1-AS1 plays a critical role in malignant progression of COAD. ZEB1-AS1 facilitated the growth and metastasis of COAD cells by competitive binding miR-455-3p and promoted PAK2 expression by releasing the PAK2 mRNA transcripts. Understanding the potential molecular mechanism of ZEB1-AS1 in COAD is important to improve the knowledge of molecular biological basis of COAD development and identify novel special biomarkers or therapeutic target for COAD patient.

ACKNOWLEDGEMENTS

This study was funded by National Natural Science Foundation of China (81802726, 81672402), Provincial Natural Science Foundation of Jiangsu, China (BK202171305), The Health and Family Planning Science and Technology Key project Foundation of Zhenjiang city (SHW2017004).

CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

AUTHOR CONTRIBUTIONS

WKL and MX conceived and designed the experiments; XN, YTD, HTY, JMS, RYG and YY performed the experiments. WKL, HTY and MX provided the technical support. XN, YTD, MX and HTY analysed and interpreted the data. XN, WKL and YTD wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Wenkang Luan https://orcid.org/0000-0001-9922-3340

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.