Original Article
Long non-coding RNA ZEB2-AS1 promotes proliferation and inhibits apoptosis of colon cancer cells via miR-143/bcl-2 axis

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Abstract: Colon cancer (CC) is the third most common cancer and the fourth leading cause of cancer-associated death in the world. Long non-coding RNA (lncRNA) ZEB2-AS1 was reported to be dysregulated and play important roles in multiple human cancers. However, the expression level and functions of ZEB2-AS1 in colon cancer is unknown. Here, we firstly observed that ZEB2-AS1 was significantly upregulated in colon cancer and predicted a poor prognosis. Functional assays showed that silencing ZEB2-AS1 expression remarkably inhibited proliferation, suppressed cell cycle transition while induced apoptosis in CC cells. In addition, miR-143 was demonstrated to act as a tumor suppressor and predicted as a downstream target of ZEB2-AS1 in CC. Furthermore, bcl-2 was identified as a direct target of miR-143 and ZEB2-AS1 could regulate the expression of bcl-2 via miR-143 in CC. A rescue assay indicated that downregulation of miR-143 partly abolished the suppressive effect of ZEB2-AS1 silencing on CC cells proliferation. Collectively, our results revealed that ZEB2-AS1 was upregualted and functioned as an oncogene via regulating miR-143/bcl-2 axis in colon cancer. These findings suggest that ZEB2-AS1 may serve a novel biomarker in the diagnosis and a potential therapeutic target in the treatment of colon cancer.

Keywords: Colon cancer, lncRNA, ZEB2-AS1, miR-143, bcl-2

Introduction

Colon cancer (CC), one of the frequently malignant tumors deriving from digestive system, is the third most common cancer and the fourth leading cause of cancer-associated death in the world [1]. It is estimated that approximately 135,430 new cases of colon cancer are diagnosed and 50,260 deaths occurred each year in America [2]. Aging is considered as the most important risk factor for CC, obesity, smoking, drinking, lack of physical activity, inflammatory bowel diseases, and inherited genetic disorders are also the risk factors for the initiation of CC [3]. In China, with the development of economic and the improvement of people's living standards, the incidence and mortality rate of CC are both increasing in recent years [4]. Although a lot of improvements in the diagnosis and treatment of colon cancer has achieved during the past decades, the prognosis of CC patients is still unsatisfactory [5]. It is needed to identify novel biomarkers and therapeutic targets for colon cancer, so as to improve the outcome of CC patients.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs with more than 200 nucleotides in length and have no protein coding potential [6]. LncRNAs are aberrantly expressed and play pivotal roles by acting as tumor suppressors or promoter in various human cancers [7]. The lncRNA Zinc finger E-box-binding homeobox 2 antisense RNA 1 (ZEB2-AS1) is a newly identified lncRNA in hepatocellular carcinoma [8]. Growing evidence indicated that lncRNA ZEB2-AS1 may promote tumor progression in multiple human cancers. For example, Wu demonstrated that ZEB2-AS1 promoted tumorigenesis and development of bladder cancer through down-regulating tumor-suppressive miR-27b [9]. Guo also showed that ZEB2-AS1 promoted proliferation and inhibited apoptosis in lung cancer [10]. In addition, Gao reported

that ZEB2-AS1 promoted pancreatic cancer cells growth and invasion through regulating the miR-204/HMGB1 axis [11]. However, the expression level and functions of ZEB2-AS1 in colon cancer is unknown.

MicroRNAs (miRNAs) are a class of small non-coding RNAs with 18-25 nucleotides in length and exert their functions by directly binding to the 3'-untranslated region (UTR) of target mRNAs, which results in mRNA degradation or inhibition of protein translation [12, 13]. As we known, lncRNAs always play their roles via binding to some miRNAs so as to release the target mRNAs [14]. miR-143 has been demonstrated to be involved in the pathogenesis of human cancers and act as a tumor suppressor [15]. In colon cancer, recent studies indicated that miR-143 is decreased and may suppress tumor progression in CC [16, 17]. In the current study, we firstly explored the expression pattern of ZEB2-AS1 in CC tissue samples and cell lines. Then, we determined the functional effects of ZEB2-AS1 on CC cells proliferation and apoptosis. Furthermore, we found that ZEB2-AS1 exerted its roles via miR-143/bcl-2 axis in colon cancer.

Materials and methods

Tissue specimens

This study was approved by the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University. A total of 45 colon cancer tissues and adjacent normal tissues were obtained from patients who underwent surgical treatment for CC at our hospital. Both cancer tissues and adjacent normal tissues were confirmed by two pathologists independently. None of them had received any radiotherapy or chemotherapy before surgery. All the fresh tissue specimens were frozen in liquid nitrogen immediately after surgical resection and stored at -80°C until use. Every patient has signed an informed consent.

Cell culture

The colon cancer cell lines (HT-29, HCT116, SW620 and LoVo) and the normal colon epithelial cell line NCM460 were all purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; from Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). These cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

RNA extraction and qRT-PCR

Total RNA were extracted from tissue samples or cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and then reversed transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Dalian, China). Real-time PCR was performed using SYBR_Premix Ex Taq kit (TaKaRa) on an Applied Biosystems Prism 7300 System (Applied Biosystems, Foster City, USA) according to the manufacturer's guidelines. GAPDH was used as the internal control for ZEB2-AS1 and bcl-2. U6 were used as the internal controls for miR-143. The relative expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method. The primers were shown as follows: ZEB2-AS1, 5'-ATG-AAGAAGCCGCGAAGTGT-3' (forward) and 5'-CA-CACCCTA ATACACATGCCCT-3' (reverse); miR-143, 5'-AGTGCGTGTCGTGGTGT-3' (forward) and 5'-GCCTGAGATGAAGCACGTG-3' (reverse); bcl-2, 5'-GATGCAGTGCCGGCCTAAG-3' (forward) and 5'-TTCTCTTGTACGCACGAGCT-3' (reverse); GAPDH, 5'-AATCCCATCACCATCTTC-3' (forward) and 5'-AGGCTGTTGTCATACTTC-3' (reverse); U6, 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse); The experiments were performed in triplicate.

Cell transfection

Cell transfections were carried out by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. The siRNA ZEB2-AS1 and the negative controls were synthesized by GenePharma Co. (Shanghai, China). The sequence of si-ZEB2-AS1 was 5'-CAAAGG-ACACCTTTGGTTACCTGAA-3'. miR-143 mimics, inhibitors and their negative controls were purchased from RiboBio Co. (Guangzhou, China). The transfected cells were cultured at 37°C for 48 h and then harvested for further experiments.

Cell proliferation assay

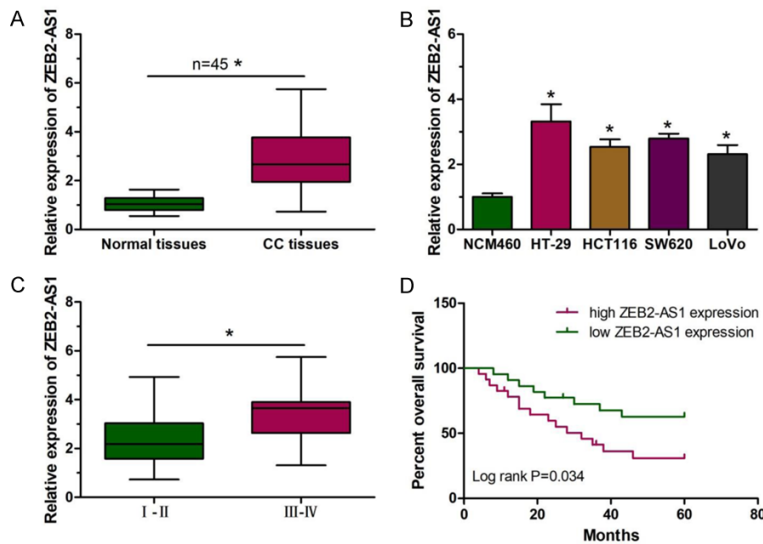Cell proliferation was analyzed by using a Cell Counting Kit-8 (CCK-8; meilunbio, Dalian, Chi-

Figure 1. ZEB2-AS1 was upregulated in colon cancer and indicated a poor prognosis. A. Expression of lncRNA ZEB2-AS1 in colon cancer tissues and adjacent normal tissues determined by qRT-PCR. B. ZEB2-AS1 expression in 4 CC cell lines and the normal colon epithelial cell line NCM460. C. The relative expression level of ZEB2-AS1 in CC tissues from patients with advanced clinical stage (III-IV) or early clinical stage (I-II). D. Kaplan-Meier survival curves of patients with high or low expression of ZEB2-AS1. Data were presented as mean ± SD. *$P < 0.05$.

na) according to the manufacturer's instruction. Briefly, the transfected cells were plated into 96-well plates at a density of 2×10^3 cells per well and maintained at 37°C for further culture. At each time point (0 h, 24 h, 48 h, 72 h), 20 µl of CCK-8 solution was added into each well and the plates were maintained at 37°C for 1 hours. Finally, the absorbance at 450 nm was measured by using a microplate reader (Bio-Rad, Hercules, CA, USA).

Apoptosis and cell cycle analysis

Cell apoptosis and cell cycle analysis were performed by using flow cytometry. Cell apoptosis rate was determined by using an Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, CA, USA) following the manufacturer's protocol. In brief, the cultured cells were harvested and resuspended in 200 µL of binding buffer. Then, cells were stained with Annexin V-FITC and propidium iodide (PI) for 15 min in darkness at room temperature. The apoptosis rate was finally detected using a BD FACS Calibur Flow Cytometer (BD Biosciences, CA, USA). For cell cycle analysis, the cultured cells were collected and fixed with 70% ethanol at 4°C overnight. Then, cells were washed twice with cold

PBS and stained with propidium iodide (PI, sigma) for 30 min at room temperature. The proportion of cells in each phase was determined by Calibur Flow Cytometer. Three independent assays were conducted.

Luciferase reporter assay

The 3'-UTR of ZEB2-AS1 and bcl-2 containing miR-143 binding sites were amplified by PCR and cloned into the pmir-GLO luciferase vector (Promega, USA) to generate the wild type 3'-UTR luciferase reporters (ZEB2-AS1 WT or bcl-2 WT). The mutant 3'-UTR luciferase reporters (ZEB2-AS1 Mut or bcl-2 Mut) with mutations in several key bases were prepared by the same approach. For luciferase reporter assay, CC cells were co-transfected with wild type or mutant 3'-UTR luciferase reporters and miR-143 mimics or negative controls with Lipofectamine 2000 reagent according to the manufacturer's protocols. Cells were harvested after transfection for 48 hours, and then the luciferase activity was measured and normalized to Renilla luciferase activity by using the Luciferase Reporter System (Promega, Madison, WI, USA). Each experiment was performed in three times.

Western blot

Total protein was collected from culture cells and stored at -20°C for western blot analysis. Equal amounts of 30 ug protein samples were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes (Millipore Sigma, USA). After blocked with 5% non-fat milk, membranes were washed three times and incubated with primary antibody (all from Abcam, Cambridge, UK) at 4°C overnight. Then, membranes were washed three times with PBST and further incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) for 1 h at room temperature. Finally, the relative band density was detected using an ECL Western blotting kit (Pierce, Rockford, IL, USA).
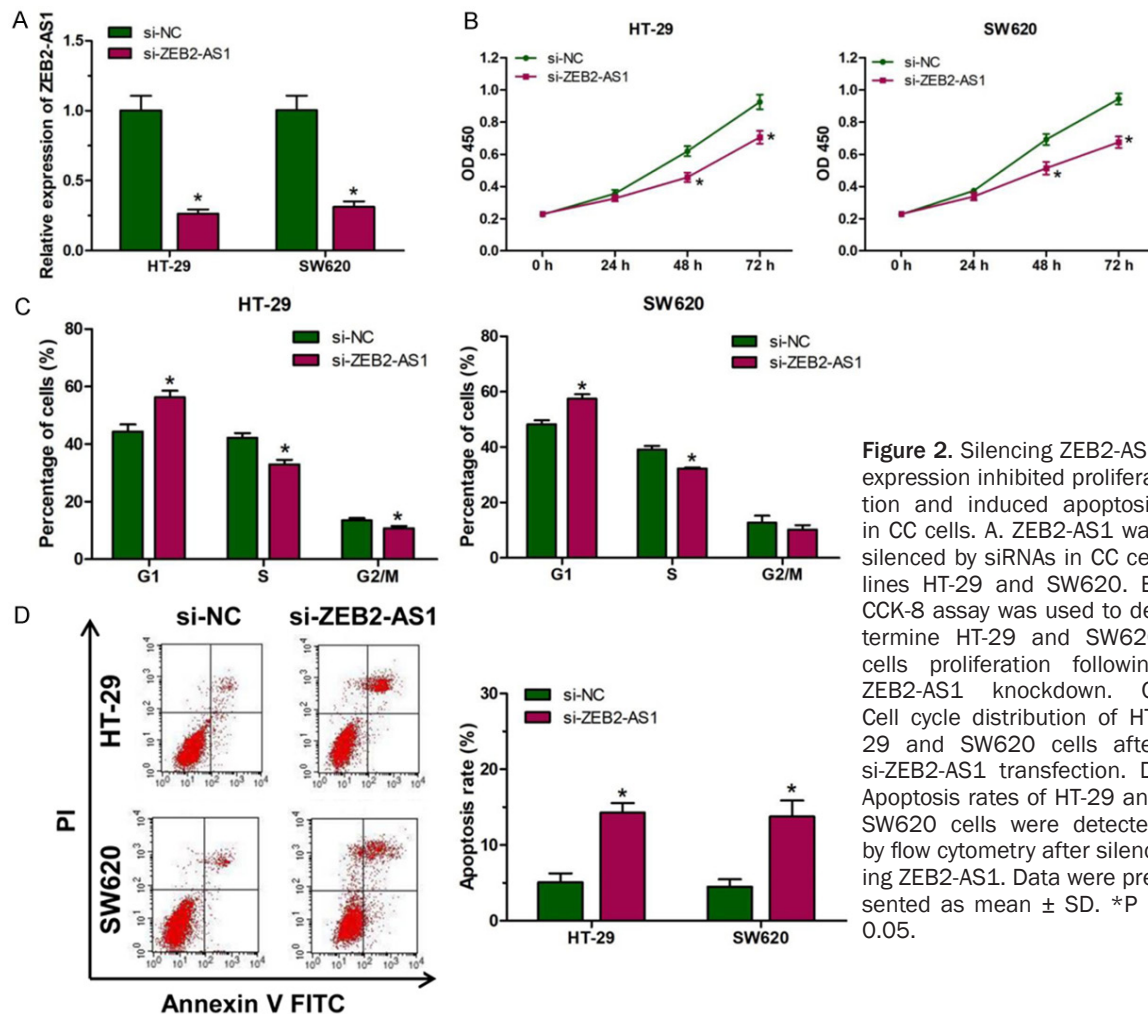
Figure 2. Silencing ZEB2-AS1 expression inhibited proliferation and induced apoptosis in CC cells. A. ZEB2-AS1 was silenced by siRNAs in CC cell lines HT-29 and SW620. B. CCK-8 assay was used to determine HT-29 and SW620 cells proliferation following ZEB2-AS1 knockdown. C. Cell cycle distribution of HT-29 and SW620 cells after si-ZEB2-AS1 transfection. D. Apoptosis rates of HT-29 and SW620 cells were detected by flow cytometry after silencing ZEB2-AS1. Data were presented as mean ± SD. *P < 0.05.

This experiment was repeated at least three times.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). GraphPad Prism 6.0 (San Diego, CA) and SPSS 18.0 software (Chicago, IL) were used to perform all statistical analysis. The difference between groups was analyzed by using Student t test or ANOVA. Kaplan-Meier curves were calculated and the log-rank test was performed for the survival data analysis. $P < 0.05$ was considered statistically significant.

Results

ZEB2-AS1 was upregulated in colon cancer and indicated a poor prognosis

The expression level of lncRNA ZEB2-AS1 was firstly detected in colon cancer tissues and cell lines by using qRT-PCR. Our data showed that ZEB2-AS1 was significantly upregulated in CC tissues compared with adjacent normal tissues (**Figure 1A**). ZEB2-AS1 expression in 4 CC cell lines was also markedly increased compared with that in normal colon epithelial cell line NCM460 (**Figure 1B**). Besides, we observed that ZEB2-AS1 expression level was significantly higher in patients with advanced clinical stage (III-IV phase) than that with early clinical stage (I-II phase) (**Figure 1C**). Patients were divided into high ZEB2-AS1 expression group (n = 21) and low ZEB2-AS1 expression group (n = 20) according to the relative ZEB2-AS1 expression level in CC tissue samples. Kaplan-Meier curves and log-rank test of overall survivals showed that patients with high ZEB2-AS1 expression had a relative poor overall survival than that with low ZEB2-AS1 expression (**Figure 1D**). These results showed that ZEB2-AS1 was upregulated in colon cancer and indicated a poor prognosis.
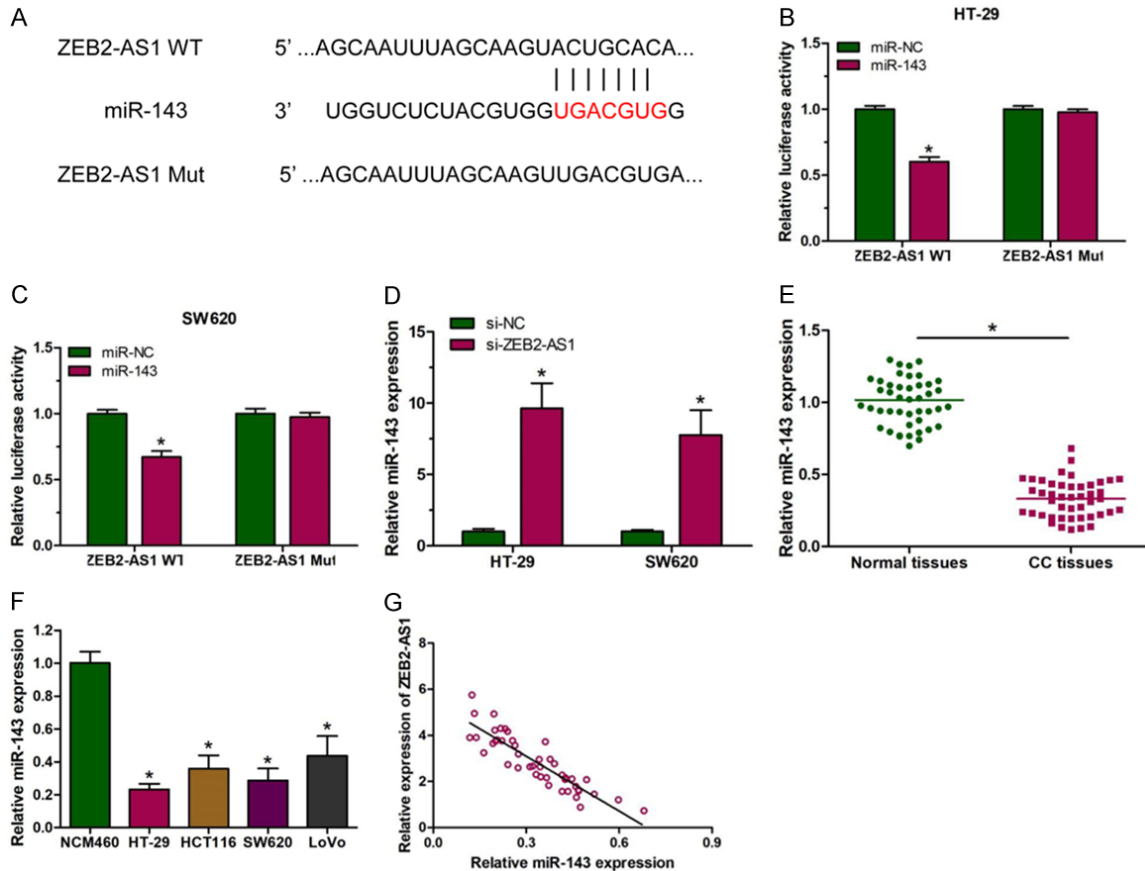
Figure 3. ZEB2-AS1 acted as a sponge for miR-143 in CC cells. A. The putative binding sites of ZEB2-AS1 and miR-143 were predicted using Starbase prediction website. B and C. Luciferase reporter assay was conducted in HT-29 and SW620 cells to verify the direct binding between miR-143 and ZEB2-AS1. D. The expression of miR-143 was detected in HT-29 and SW620 cells after ZEB2-AS1 knockdown. E. miR-143 expression in colon cancer tissues and adjacent normal tissues. F. miR-143 expression in 4 CC cell lines and the normal colon epithelial cell line NCM460. G. The correlation between miR-143 and ZEB2-AS1 expression in CC tissues. Data were presented as mean ± SD. *P < 0.05.

Silencing ZEB2-AS1 expression inhibited proliferation and induced apoptosis in CC cells

In order to investigate the biological functions of lncRNA ZEB2-AS1 in colon cancer, we used siRNAs to silence the expression of ZEB2-AS1 in CC cell lines HT-29 and SW620. The knockdown efficiency was confirmed by qRT-PCR analysis (**Figure 2A**). Proliferation of the treated cells was determined by CCK-8 assay. Data showed that ZEB2-AS1 silencing significantly inhibited HT-29 and SW620 cells proliferation (**Figure 2B**). The apoptosis rate and cell cycle distribution of transfected CC cells were measured by flow cytometry. Our results showed that knockdown of ZEB2-AS1 in CC cells significantly suppressed cell cycle transition (**Figure 2C**). Data from apoptosis analysis indicated

that the apoptosis rate in CC cells was obviously increased following ZEB2-AS1 knockdown (**Figure 2D**). These results revealed that lncRNA ZEB2-AS1 may serve as an oncogene in the development of colon cancer.

ZEB2-AS1 acted as a sponge for miR-143 in CC cells

Previous evidences showed that lncRNAs always act as competing endogenous RNAs (ceRNAs) for miRNAs to regulate the downstream target genes. Thus, we investigated whether lncRNA ZEB2-AS1 function as a sponge for a certain miRNA in CC cells. Firstly, the bioinformatics prediction tool StarBase (http://starbase.sysu.edu.cn/) predicted that miR-143 contains the potential binding site for ZEB2-
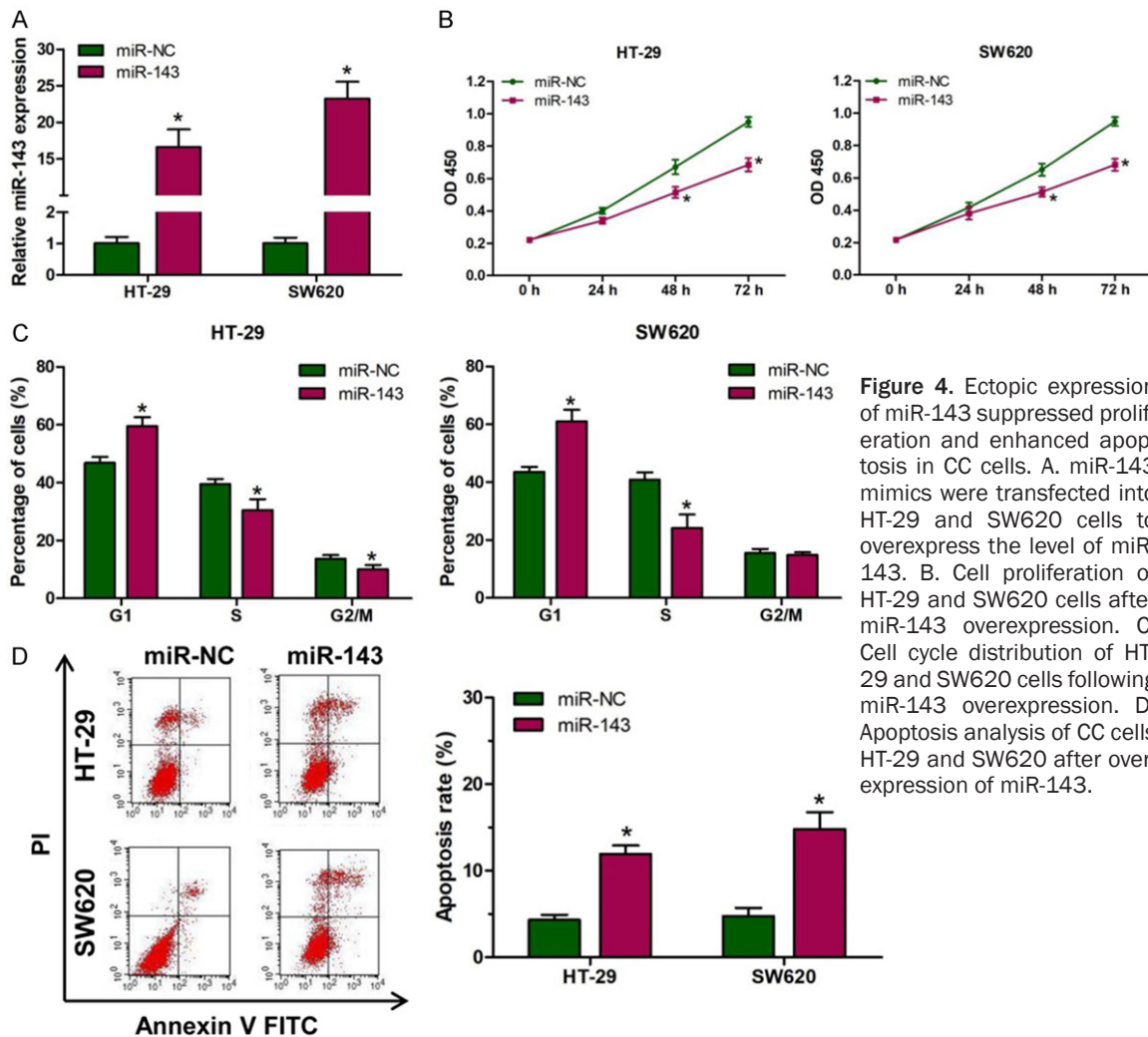
Figure 4. Ectopic expression of miR-143 suppressed proliferation and enhanced apoptosis in CC cells. A. miR-143 mimics were transfected into HT-29 and SW620 cells to overexpress the level of miR-143. B. Cell proliferation of HT-29 and SW620 cells after miR-143 overexpression. C. Cell cycle distribution of HT-29 and SW620 cells following miR-143 overexpression. D. Apoptosis analysis of CC cells HT-29 and SW620 after overexpression of miR-143.

AS1 (**Figure 3A**). To confirm the direct binding between miR-143 and ZEB2-AS1, a luciferase reporter assay was performed with wild type or mutant ZEB2-AS1 3'-UTR luciferase vector (ZEB2-AS1 WT or ZEB2-AS1 Mut) in HT-29 and SW620 cells. Data showed that miR-143 overexpression significantly decreased the luciferase activity of wild type ZEB2-AS1 (ZEB2-AS1-WT), rather than that of mutant ZEB2-AS1 (ZEB2-AS1-Mut) in both CC cell lines (**Figure 3B**, **3C**). Furthermore, miR-143 expression level was markedly increased following ZEB2-AS1 knockdown in CC cells (**Figure 3D**). In addition, we found that miR-143 expression was obviously decreased in CC tissues and cell lines compared with control (**Figure 3E**, **3F**). A correlation analysis between miR-143 and ZEB2-AS1 expression in CC tissues revealed that miR-143 was inversely correlated with ZEB2-AS1 level in colon cancer tissues (**Figure 3G**).

These results suggested that ZEB2-AS1 functioned as a sponge for miR-143 in CC cells.

Ectopic expression of miR-143 suppressed proliferation and enhanced apoptosis in CC cells

To explore the biological effects of miR-143 on CC cells, we transfected miR-143 mimics into HT-29 and SW620 cells to overexpress the level of miR-143 (**Figure 4A**). Results from CCK-8 assay showed that overexpression of miR-143 significantly suppressed CC cells proliferation (**Figure 4B**). Cell cycle analysis demonstrated that more cells were arrested at G0/G1 phase following miR-143 overexpression (**Figure 4C**). Besides, the apoptosis rates in CC cells were markedly increased after miR-143 overexpression (**Figure 4D**). These results showed that miR-143 may act as a tumor suppressor in colon cancer development.
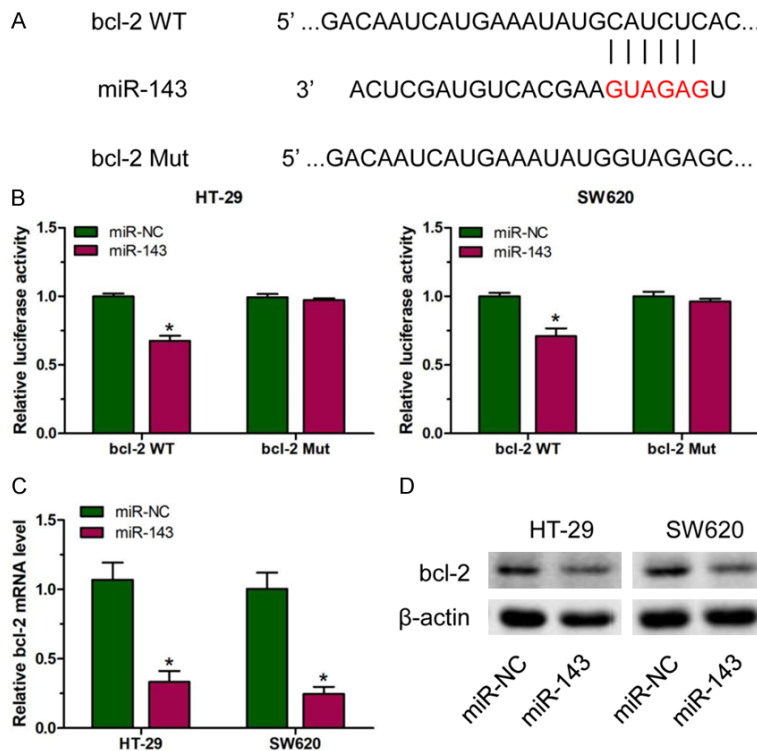
Figure 5. Bcl-2 was a direct target of miR-143 in colon cancer. A. Bcl-2 was a predicted as a direct target of miR-143 by TargetScan. B. Luciferase reporter assay was conducted in HT-29 and SW620 cells to verify the direct binding between miR-143 and bcl-2. C. The mRNA levels of bcl-2 in CC cells detected by qRT-PCR following miR-143 overexpression. D. Western blot was performed to measure the protein expression of bcl-2 after miR-143 overexpression. Data were presented as mean ± SD. *$P < 0.05$.

Bcl-2 was a direct target of miR-143 in colon cancer

Our results indicated that miR-143 was downregulated in colon cancer and acted as a tumor suppressor. Here, we further explored the possible target gene of miR-143 in CC. Using TargetScan tool, we found that bcl-2 was a direct target of miR-143 (**Figure 5A**). Then, a luciferase reporter assay was conducted to verify that. Our results showed that the luciferase activities were markedly decreased in CC cells when cotransfected with miR-143 mimics and wild-type bcl-2 vectors (bcl-2 WT), but no change when cotransfected with miR-143 mimics and mutant bcl-2 vectors (bcl-2 Mut) (**Figure 5B**). In addition, we detected the mRNA level and protein expression of bcl-2 in HT-29 and SW620 cells after miR-143 overexpression. Data showed that miR-143 overexpression significantly decreased the mRNA and protein levels of bcl-2 in CC cells (**Figure 5C**, **5D**). These

results indicated that bcl-2 was a direct target of miR-143 in CC cells.

ZEB2-AS1 promoted CC cells proliferation via miR-143/ bcl-2 axis

Based on the results above, we hypothesized that lncRNA ZEB2-AS1 may promote CC cells proliferation via miR-143/bcl-2 axis. Thus, we transfected miR-143 inhibitors into CC cells to downregulate miR-143 expression after ZEB2-AS1 knockdown (**Figure 6A**). Then, CCK-8 assay proved that miR-143 downregulation partly reversed the suppressive effect of ZEB2-AS1 knockdwon on CC cells proliferation (**Figure 6B**). In addition, we examined the mRNA and protein levels of bcl-2 in CC cells when transfected with ZEB2-AS1 siRNA or/and cotransfected with miR-143 inhibitors. Data showed that the mRNA and protein levels of bcl-2 were dramatically decreased after ZEB2-AS1 siRNA transfection, while these changes were partly abolished by co-transfecting with miR-143 inhibitors (**Figure 6C**, **6D**). These results suggested that ZEB2-AS1 promoted cell proliferation via miR-143/bcl-2 axis in colon cancer.

Discussion

In recent years, accumulating evidence suggest that a lot of long non coding RNAs are involved in the initiation and development of multiple human cancers including colon cancer. For example, the lncRNA prostate cancer-associated ncRNA transcript 6 (PCAT6) was demonstrated to be upregulated and promote cell proliferation by regulating anti-apoptotic protein in colon cancer [18]. Bo et al. reported that lncRNA AFAP1-AS1 was significantly elevated in colon cancer, and its high expression promoted the progression of colon cancer and predicted poor prognosis [19]. Wu et al. showed that lncRNA FAL1 expression was remarkably
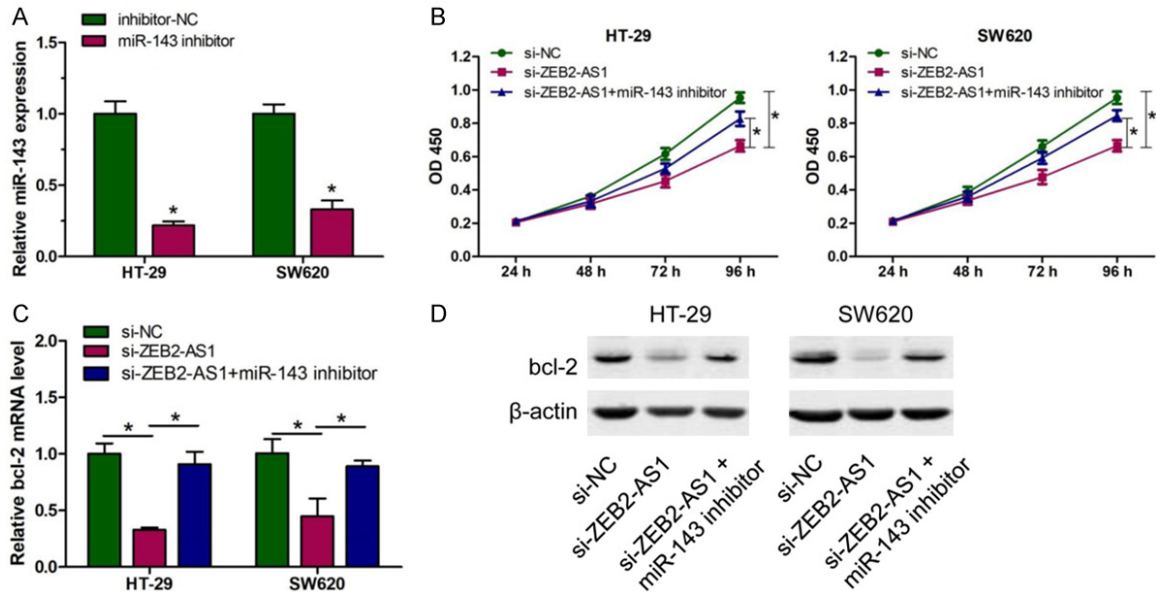
Figure 6. ZEB2-AS1 promoted CC cells proliferation via miR-143/bcl-2 axis. A. miR-143 inhibitors were transfected into CC cells HT-29 and SW620 to downregulate miR-143 expression after ZEB2-AS1 knockdown. B. CCK-8 assay was conducted to analyze cell proliferation of the treated CC cells. C. The mRNA levels of bcl-2 in HT-29 and SW620 cells after miR-143 inhibitors and si-ZEB2-AS1 co-transfection. D. The protein expression of bcl-2 in HT-29 and SW620 cells after miR-143 inhibitors and si-ZEB2-AS1 co-transfection. Data were presented as mean ± SD. *P < 0.05.

up-regulated in colon tumor tissues. Knockdown of lncRNA FAL1 attenuated cell proliferation and stimulated cell apoptosis [20]. These studies reveal that lncRNAs may participant in various biological processes of colon cancer and are correlated with prognosis of CC patients. Therefore, identifying some novel lncRNAs which are involved in the progression of CC is helpful to develop new therapeutic strategies and improve the clinical outcome for patients with colon cancer.

The aim of this study was to explore the expression level and biological roles of a novel lncRNA ZEB2-AS1 in colon cancer. By using qRT-PCR, we found that ZEB2-AS1 was significantly upregulated in colon cancer tissues and cells compared with normal tissues and normal colon epithelial cells. Survival analysis showed that patients with high ZEB2-AS1 expression had a relative poor overall survival than that with low ZEB2-AS1 expression. Then, small interfering RNA targeting ZEB2-AS1 was used to silence its expression in CC cells and a series of functional assays were conducted. We found that knockdown of ZEB2-AS1 remarkably inhibited proliferation, suppressed cell cycle transition while promoted apoptosis in CC cells HT-29

and SW620. These results indicated that high expression of lncRNA ZEB2-AS1 promoted progression of colon cancer and predicted a poor prognosis.

It is well established that lncRNAs can serve as a competing endogenous RNA (ceRNA) of miRNAs to regulate the downstream targets gene of miRNAs [21]. Here, we predicted that miR-143 has the binding site of ZEB2-AS1 by using bioinformatics analysis. The luciferase reporter showed that ZEB2-AS1 can directly bind to miR-143. In addition, miR-143 expression was downregulated and inversely correlated with ZEB2-AS1 level in CC tissues. Functional assays indicated that miR-143 acted as a tumor suppressor in the progression of colon cancer. Moreover, bcl-2 was identified as a direct target of miR-143 in CC. By using rescue assays, we observed that ZEB2-AS1 can promote cell proliferation and regulate the expression level of bcl-2 by acting as a ceRNA of miR-143 in CC cells. These results suggest that lncRNA ZEB2-AS1 promoted tumor progression via regulating the miR-143/bcl-2 axis in colon cancer.

In summary, our current study showed that lncRNA ZEB2-AS1 was upregulated in colon

cancer and predicted a poor prognosis. ZEB2-AS1 could function as an oncogene via regulating miR-143/bcl-2 axis in the initiation and progression of colon cancer. These findings suggest that ZEB2-AS1 may serve a novel biomarker in the diagnosis and a potential therapeutic target in the treatment of colon cancer.

Disclosure of conflict of interest

None.

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