Original Article
miRNA-708 functions as a tumor suppressor in colorectal cancer by targeting ZEB1 through Akt/mTOR signaling pathway

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Abstract: Background: Colon cancer, or colorectal cancer (CRC), is a type of cancer that develops from large bowel. Previous data has demonstrated that microRNAs (miRNAs) may be involved in the formation and progression of CRC. The deregulation of miR-708 has been identified in multiple types of cancer. However, to the best of our knowledge, there are no data concerning the expression and role of miR-708 in CRC. Methods: In this study, RT-PCR and Flow Cytometry were used to examine the expression and role of miR-708 and ZEB1 in proliferation and apoptosis. Transwell was used to examine the role of miR-708 and ZEB1 in invasion and migration. Western blot and qRT-PCR were conducted to determine the alteration of protein and miR-708 levels, respectively. Results: MiR-708 was significantly downregulated in CRC tissues and cell lines. The restoration of the expression of miR-708 suppressed cell proliferation, induced apoptosis, and reduced metastasis in CRC *in vitro*. Additionally, bioinformatics analysis predicted ZEB1 as a novel target gene of miR-708. Furthermore, ZEB1 was upregulated in CRC, which was negatively correlated with miR-708 expression. Further studies showed that the overexpression of miR-708 and silence of ZEB1 inhibited stage of CRC *via* inhibiting AKT/mTOR signaling pathway in CRC cells. Conclusion: Taken together, these results indicate that miR-708 plays an important role in suppressing the development of CRC by directly targeting ZEB1 through AKT/mTOR signaling pathway, suggesting that miR-708 is a novel, effective therapeutic target for treating patients with CRC.

Keywords: MicroRNAs, colorectal cancer, ZEB1, molecular mechanisms, miR-708

Introduction

Colon cancer, or colorectal cancer (CRC), begins as a tumor or tissue growth on the inner lining of the rectum or colon [1, 2]. Incidence of colon cancer ranks the 2nd place among all cancers in females and the 3rd place among cancers in males [3]. With proper treatment, the overall 5-year survival of patients with colon cancer is generally high [4]. Currently, CRC is confounded by the difficulty of early diagnosis, due to the lack of reliable cancer-specific diagnostic biomarkers. Profound changes at the cellular and subcellular levels, involving DNA, RNA, protein structure and function, are the initial factors for cancer development and progression [5]. Therefore, clarification of the mechanisms underlying the progression of CRC, which may pro-vide novel therapeutic targets for patients with this disease, is urgent.

MiRNAs are single-stranded, short non-coding RNA molecules, containing 22-25 nucleotides [6]; they post-transcriptionally regulate gene expression through base pairing to the 3' untranslated regions (3'-UTRs) of their target genes, causing translational inhibition or mRNA degradation, thereby decreasing protein expression [7]. A number of studies have demonstrated that miRNAs serve notable roles in tumorigenesis and tumor development through the regulation of a variety of pathways in physiological and pathological processes, including tumor cell proliferation, cycle, differentiation, angiogenesis, invasion and metastasis [8-10]. Specific expression profiles of miRNAs have been

Table 1. Primers used in real-time qRT-PCR

Gene	Number gene primer (5'→3')
N-cadherin	F: 5'-AGCCAACCTTAACTGAGGAGT-3'
	R: 5'-GGCAAGTTGATTGGAGGGATG-3'
E-cadherin	F: 5'-AAAGGCCCATTTCCTAAAAACCT-3'
	R: 5'-TGCGTTCTCTATCCAGAGGCT-3
ZEB1	F: 5'-TTGTAGCGACTGGATTTT-3'
	R: 5'-AGACGATAGTTGGGTCCCGGC-3'
Bax	F: 5'-CTTCAGGGTTTCATCCAGGA-3'
	R: 5'-TTACTGTCCAGTTCGTCCC-3
Bcl-2	F: 5'-GGTGGGGTCATGTGTGTGG-3'
	R: 5'-CGGTTCAGGTACTCAGTCATCC-3'
GAPDH	F: 5'-GGAGCGAGATCCCTCCAAAAT-3'
	R: 5'-GGCTGTTGTCATACTTCTCATGG-3'
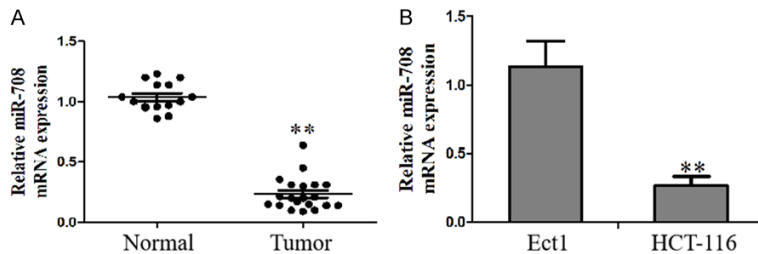
Figure 1. Expression status of miR-708 in CRC tissues and cell lines. A. RT-qPCR analysis was employed to determine miR-708 expression in 20 pairs of CRC and adjacent non-tumor tissues. B. The expression level of miR-708 in CRC cell lines (HCT-116) and a normal human cervix epithelial cell line (ect1/e6e7) was detected by using RT-qPCR. The assays were performed at least three times with similar results. Data are shown as the mean ± SD (n = 3) of one representative experiment. *P < 0.05 and **P < 0.01 *versus* normal groups.

shown in a variety of human cancers, including CRC [11-14]. Hence, miRNAs have the promising potential of being novel biomarkers for CRC diagnosis. MiR-708, one of the novel reported downregulated miRNAs, has been considered as a potential negative biomarker in the diagnosis of the progression of multiple types of cancer [15]. However, to the best of our knowledge, the expression pattern and roles of miR-708 in CRC remain unclear.

The epithelial-mesenchymal transition (EMT) is a vital morphogenic process during embryonic development, and is crucial for epithelial cancer cell acquisition of an invasive phenotype [16]. Zinc finger E-box-binding homeobox 1 (ZEB1) is an important regulator of EMT and plays vital roles in regulating metastasis, invasion and apoptosis to exert a negative impact on malignancy [17]. ZEB1 is a potent EMT acti-

vator [18] and can regulate cell stemness [19]. ZEB1 is correlated with lymphatic and distal metastasis [20] and influences metastasis *via* EMT in lung cancer [18]. More importantly, ZEB1 also regulates tumor metastasis through the ZEB1/EMT axis in hepatoma [21], breast cancer [21, 22], and colorectal cancer [23, 24]. Additionally, many researchers have demonstrated that AKT/mTOR plays an important role in tumorigenesis and tumor development, as well as other diseases [25]. One recent study reported that downregulation of miR-205 is associated with glioblastoma cell migration, invasion, and the epithelial-mesenchymal transition by targeting ZEB1 *via* the Akt/mTOR signaling pathway [26]. Although these studies imply that ZEB1 has an effect on tumors, the regulation effect of ZEB1 to CRC through Akt/mTOR signaling pathway in CRC remains unclear.

In this study, we detected miR-708 and ZEB1 expression in CRC, analyzed their roles in malignant phenotypes, and investigated their underlying mechanisms. Our results indicate that miR-708 can be developed as a molecular target to treat patients with CRC. These findings provide a novel strategy for the treatment of CRC in the future.

Materials and methods

Clinical tissues

Paired CRC and adjacent non-tumor tissues were collected from 10 patients with CRC who had undergone surgical resection at Anhui Provincial Hospital. No patients had been treated with preoperative radiotherapy or chemotherapy. Tissue samples were collected after surgery, and immediately frozen in liquid nitrogen and stored at -80°C until for RNA and histopathological analyses. The study was appro-

A

B

C

D

| miR-708 NC | miR-708 inhibitor | miR-708 NC | miR-708 mimics |

Migration

E
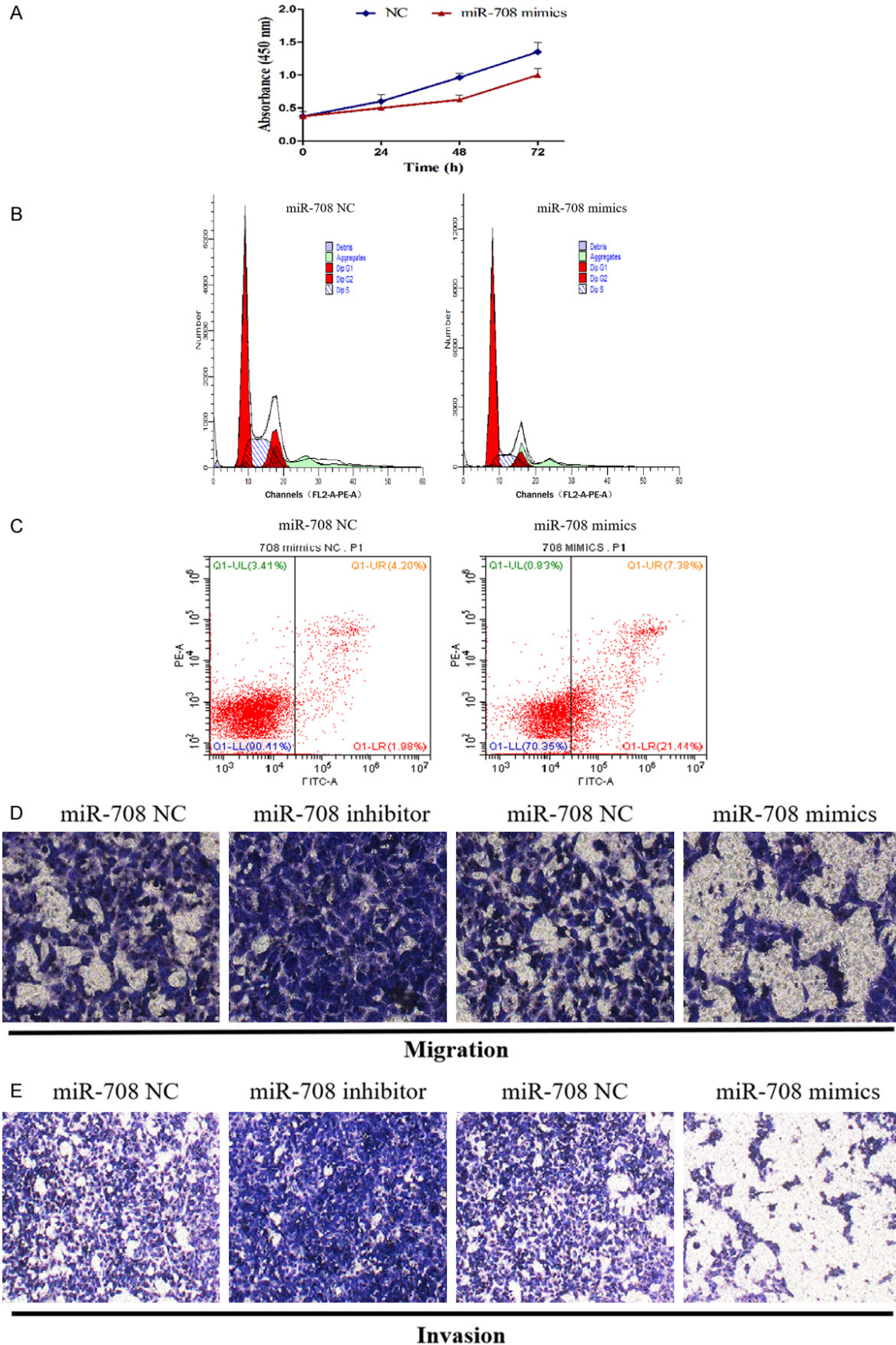
| miR-708 NC | miR-708 inhibitor | miR-708 NC | miR-708 mimics |

Invasion

Figure 2. miR-708 regulates the proliferation, apoptosis, migration, and invasion of HCT-116 cells. miR-708 mimics, miR-708 inhibitor or miR-NC was introduced into HCT-116 cells, and the transfected cells were used in the functional analyses. A-C. CCK-8 and flow cytometry assays were used to analyze the proliferation and apoptosis of HCT-116 cells following transfection with miR-708 mimics or miR-NC. D, E. The effects of miR-708 on the migration and invasion of HCT-116 cells were evaluated using Transwell assays. The assays were performed at least three times with similar results. Data are shown as the mean ± SD (n = 3) of one representative experiment. *P < 0.05 and **P < 0.01 *versus* normal groups.

ved by the Ethics and Scientific Committees of Anhui Institute of Cancer Research. The written informed consents were obtained from all patients.

Cell lines and cell culture

For routine cell culture, HCT-116 CRC cell lines and the Ect1/E6E7 normal liver epithelium cell line, purchased from the Shanghai Cell Collection (Shanghai, China), were maintained in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified air at 37°C with 5% of CO_2.

Cell transfection

To downregulate or upregulate the expression of miR-708 and ZEB1, miR-708 mimics, inhibitor, ZEB1-siRNA, or corresponding control RNA were used. The transfection was performed by using Lipofectamine™ 2000 (Invitrogen) according to the manufacture's protocols. Transfecction after 6 hrs, the culture medium was changed. Cells were reincubated in complete medium at 37°C for an additional 24 hrs. After 3 days, cells were collected for real-time polymerase chain reaction (RT-PCR) or Western blot analysis. MiR-708 mimics, miR-708 inhibitor and ZEB1-siRNA were purchased from GenePharma (Shanghai, China). The sequences are as following: miR-708 mimic was 5'-AAG GAG CUU ACA AUC UAG CUG GG-3'. The sequence of the miR-NC mimic was 5'-UUC UCC GAA CGU GUC ACG UTT-3'. The ZEB1 siRNA sequence was 5'-GCUGCCAAUAAGCAAACGA-3' and the NC siRNA sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'.

Cell counting kit-8 (CCK-8) assay

The Cell Counting Kit-8 (CCK-8; BestBio, Beijing) assay was used to verify the effect of miR-708 and ZEB1 expression in CRC on cell proliferation. A total of 3000 transfected cells

(100 μl) were seeded into each well of a 96-well plate. The cells were subsequently incubated at 37°C for 24, 48, 72 and 96 hrs and at each time point the CCK-8 assay was performed. A total of 10 μl CCK-8 reagent was added and incubated at 37°C for an additional 2 hrs. The absorbance was determined at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientifc, Inc.).

Migration and invasion assays

For the cell migration assay, 1×10^5 cells in serum-free DMEM were seeded in the upper chamber of the transwell insert (Millipore), and the lower chamber contained complete DMEM with 10% FBS as previously described [32, 33]. At 24 hrs after inoculation, the transwell chamber was collected and stained with crystal violet. For the cell invasion assay, the upper chamber of the transwell insert was pre-coated with Matrigel (BD), and incubated at 37°C for 3 h to form the Matrigel layer in the chamber. A total of 2×10^5 cells in serum-free DMEM were seeded in the upper chamber of the transwell insert, and the lower chamber contained complete IMDM with 20% FBS [34, 35]. At 24 hrs after inoculation, the transwell chamber was collected and stained with crystal violet. The experiment was performed in triplicate and repeated at least three times with the similar results.

Cell cycle and apoptosis analysis

For the cell cycle analysis, cells were incubated with 10 or 20 μmol/L naringin for 24 hrs and fixed in 75% ethanol at 4°C overnight. After resuspension in 10 μg/ml PI, cell cycle stages were determined using a Coulter Epics XL Flow Cytometry System (Beckman-Coulter, Miami, FL). For the apoptosis analysis, the samples were incubated with Annexin V-FITC/PI according to the manufacturer's recommended protocol (SigmaAldrich). Cell-bound fluorescence was analyzed using a Coulter Epics XL Flow Cytometry System (Beckman-Coulter, Miami, FL).
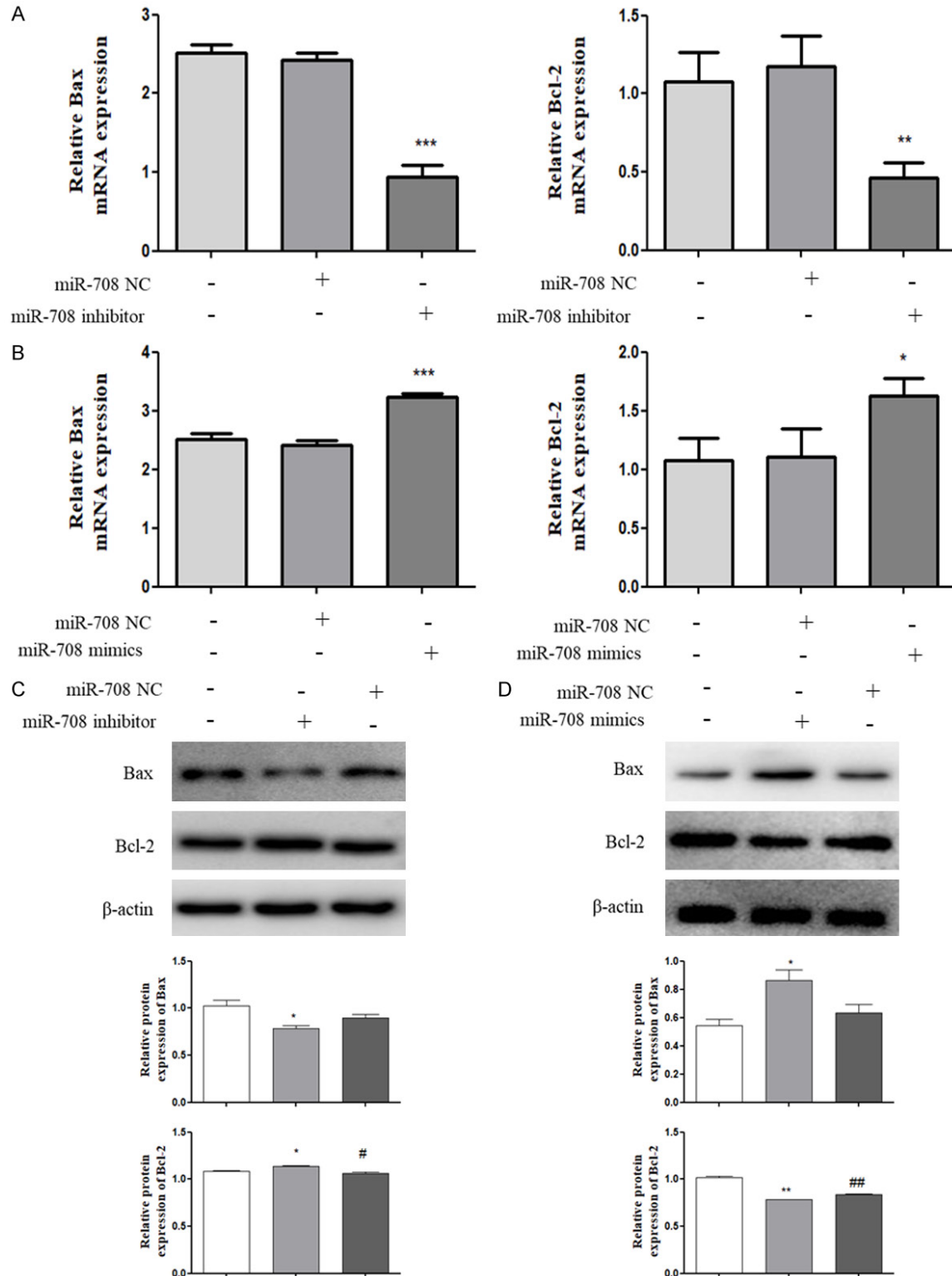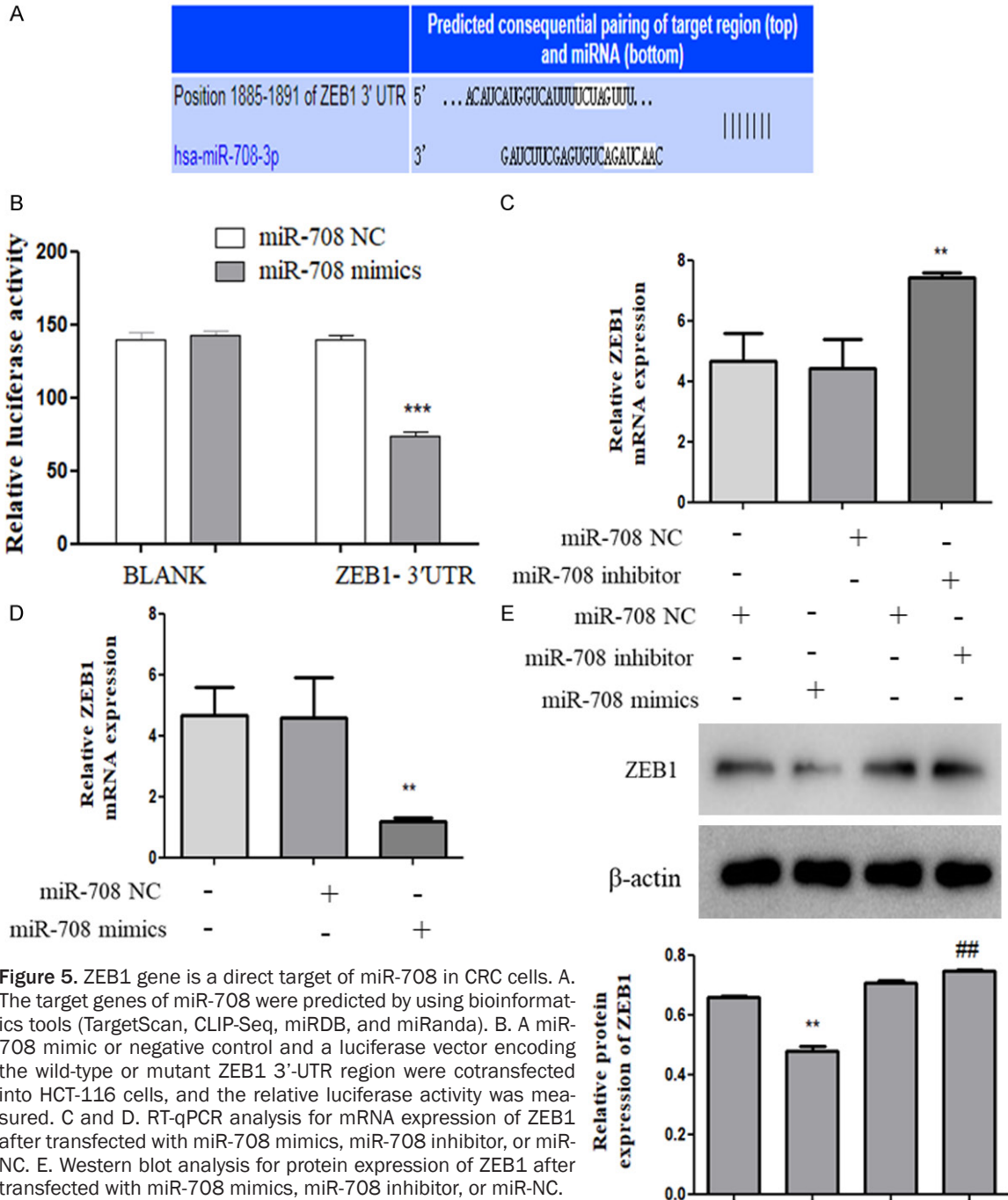
Figure 3. The effect of miR-708 expression on apoptosis protein expression in CRC cell lines. A and B. RT-qPCR was performed to evaluate the Bax and Bcl-2 mRNA expression after transfection of HCT-116 cells with miR-708 mimics, miR-708 inhibitor and miR-NC. C and D. Western blot was performed to evaluate the Bax and Bcl-2 protein expression after transfection of HCT-116 cells with miR-708 mimics, miR-708 inhibitor and miR-NC. The assays were performed at least three times with similar results. Data are shown as the mean ± SD (n = 3) of one representative experiment. *P < 0.05 and **P < 0.01 *versus* normal groups.
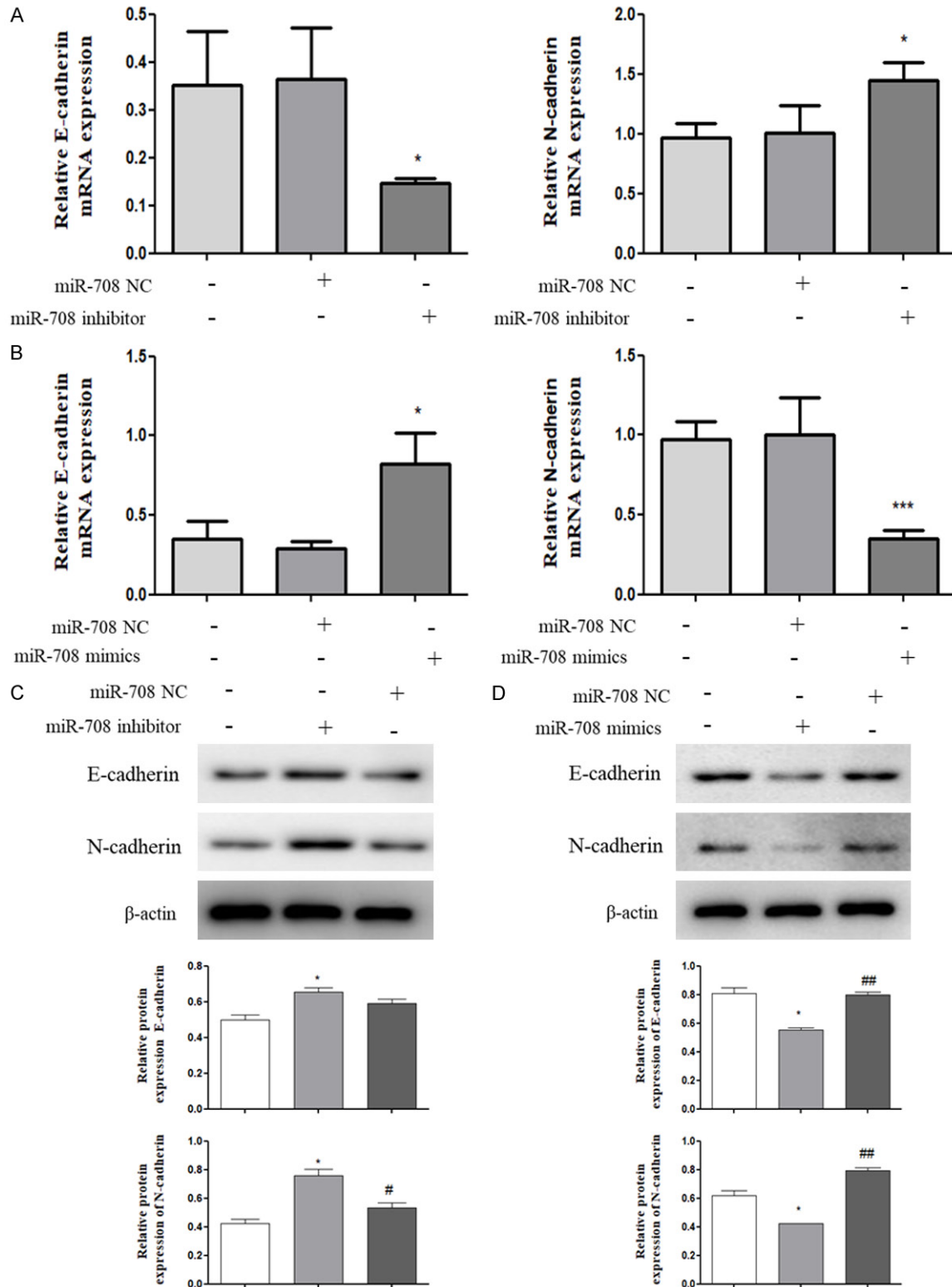
Figure 4. The effect of miR-708 expression on E-cadherin and N-cadherin expression in CRC cell lines. A and B. RT-qPCR was performed to evaluate the E-cadherin and N-cadherin mRNA expression after transfection of HCT-116 cells with miR-708 mimics, miR-708 inhibitor and miR-NC. C and D. Western blot was performed to evaluate the E-cadherin and N-cadherin protein expression after transfection of HCT-116 cells with miR-708 mimics, miR-708 inhibitor and miR-NC. The assays were performed at least three times with similar results. Data are shown as the mean ± SD (n = 3) of one representative experiment. *P < 0.05 and **P < 0.01 *versus* normal groups.

Figure 5. ZEB1 gene is a direct target of miR-708 in CRC cells. A. The target genes of miR-708 were predicted by using bioinformatics tools (TargetScan, CLIP-Seq, miRDB, and miRanda). B. A miR-708 mimic or negative control and a luciferase vector encoding the wild-type or mutant ZEB1 3'-UTR region were cotransfected into HCT-116 cells, and the relative luciferase activity was measured. C and D. RT-qPCR analysis for mRNA expression of ZEB1 after transfected with miR-708 mimics, miR-708 inhibitor, or miR-NC. E. Western blot analysis for protein expression of ZEB1 after transfected with miR-708 mimics, miR-708 inhibitor, or miR-NC.

Dual luciferase reporter assay

The 3'-UTR fragments of ZEB1 containing putative wild-type (wt) and mutant (mut) miR-708-binding sites were constructed by Changsha Yingrun Co., Ltd., inserted into the pMIRRE-PORT miRNA Expression Reporter vector (Ambion; Thermo Fisher Scientifc), and named pMIR-ZEB1-3'-UTR wt and pMIR-ZEB1-3'-UTR mut, respectively. Cells were plated into 24-well plates 1 night before transfection. miR-708 mimics or miR-NC were co-transfected with pMIR-ZEB1-3'-UTR wt or pMIR-ZEB1-3'-UTR mut into cells using Lipofectamine 2000 reagent according to the manufacturer's recommendations. Transfected cells were collected after 48 hours of incubation at 37°C and then luciferase activity was measured using a dual-luciferase reporter assay system (Promega Corporation, Fitchburg, WI, USA) in accor-
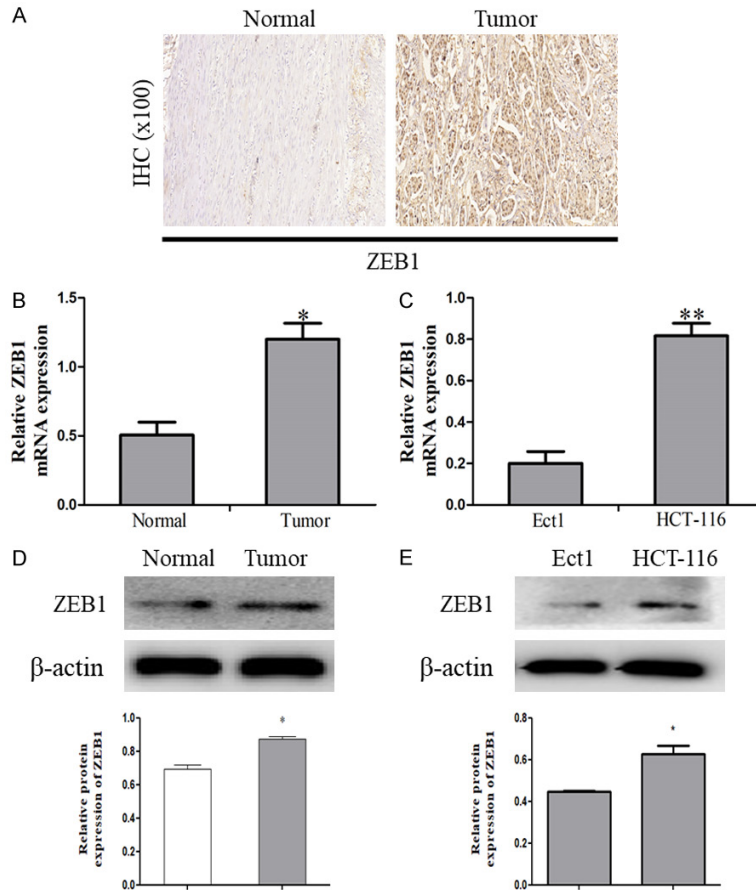
Figure 6. Expression status of ZEB1 in CRC tissues and cell lines. A. Immunohistochemistry staining with ZEB1 was performed to detect the expression of ZEB1 in CRC tissues. Representative images of Immunohistochemistry staining are presented (× 100). B and C. The mRNA expression of ZEB1 was analyzed by using a RT-qPCR analysis in CRC tissues and cell lines. D and E. The protein expression of ZEB1 was analyzed by using the Western blot analysis in CRC tissues and cell lines.

then reverse transcribed, and RT-PCR was performed using the ABI PRISM 7900 sequence detection system (Biosystems). The PCR amplification was performed in a reaction system containing cDNA, forward and reverse primers, 2 × SYBR Green qPCR Super Mix, and distilled water. PCR was carried out for 40 cycles of 95°C for 10 s and 60°C for 30 s. Primer sequences for detection of mRNA expression were synthesized (**Table 1**). The relative expression of the amplified RNA samples was calculated using the 2^{-DDCT} method. All the reactions were repeated at least three times.
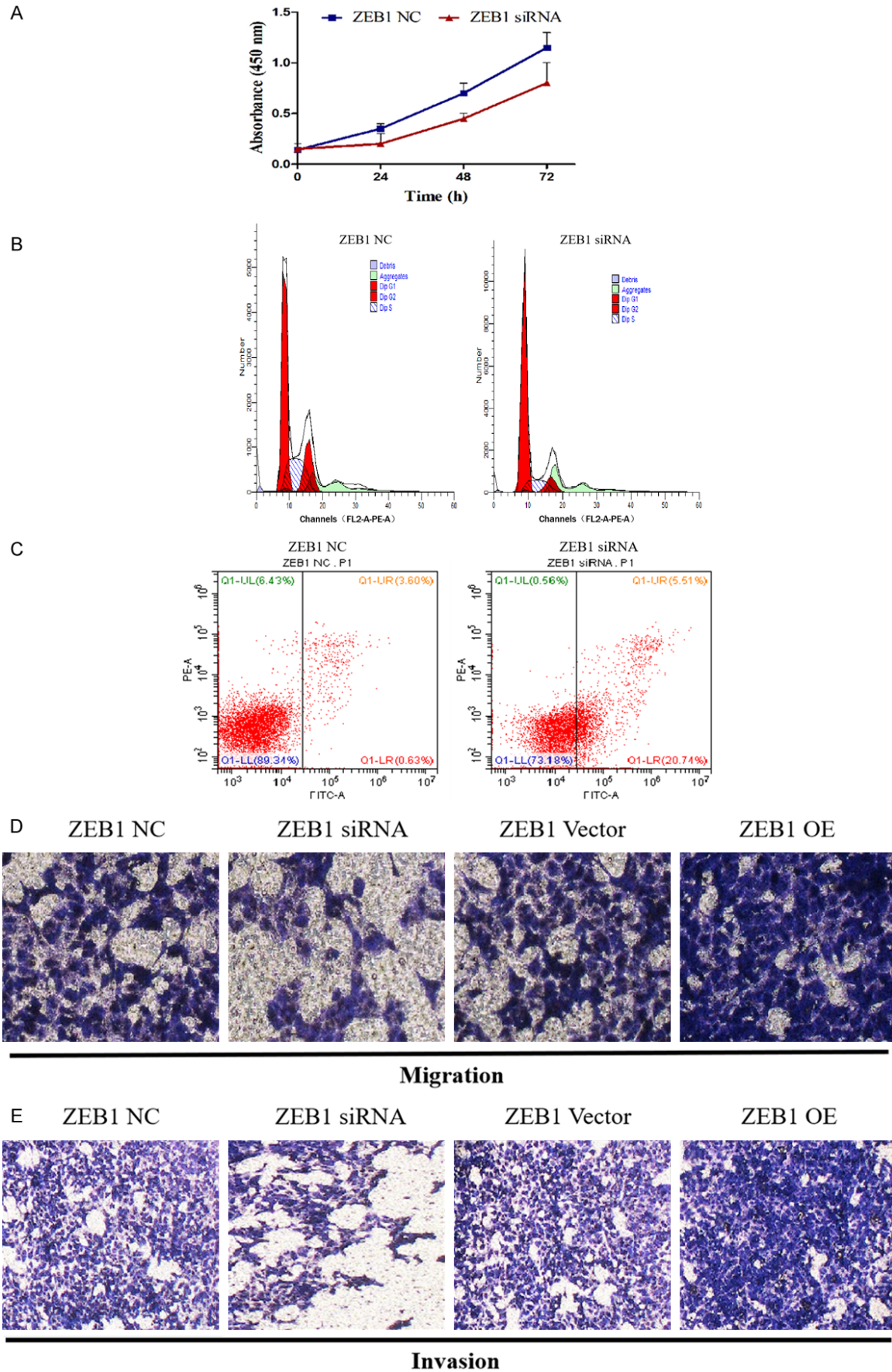
Western blot analysis

Total proteins were collected with RIPA lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and protein concentrations were measured with the BCA method (Beyotime, Jiangsu, China). A total of 40 µg protein were subjected to 4-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Sigma St. Louis, MO, USA), following the proteins were electrophoretically transferred to polyvinylidene fluoride membranes (Roche, Indianapolis, IN, USA). Then, the membranes were blocked in 5% milk, followed by incubation with primary antibodies for ZEB1 (Abcam, Cambridge, MA, USA), E-cadherin (Abcam), N-cadherin (Abcam), p-AKT (Abcam), or p-mTOR (Abcam) and subsequent incubation with the appropriate secondary antibodies (Cell Signaling Technology, Beverly, MA, USA). GAPDH (US Biological, Swampscott, MA, USA) was used as a loading.

Statistical analysis

All data were analyzed using SPSS version 21.0 software (SPSS Inc, Chicago, Illinois). Measurement data was presented as mean ± standard deviation (SD). Comparisons between the 2

dance with the manufacturer's instructions. Firefly luciferase activity was normalized to that of Renilla luciferase.

Histopathology

For routine histology, the CRC tissues were preserved in 10% formalin (Solarbio, Beijing, China) and embedded in paraffin and cut into 50-mm thick sections, which were prepared and stained with hematoxylin and eosin (H&E) and IHC staining by using standard methods. All images of pathological changes were captured with the Olympus BX-145 microscope (Olympus, Japan).

Real-time PCR (RT-PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen). 1 µg of the total RNA was

Figure 7. ZEB1 regulates the proliferation, apoptosis, migration, and invasion of HCT-116 cells. ZEB1-siRNA, ZEB1 OE and NC were introduced into HCT-116 cells, and the transfected cells were used in the functional analyses. A-C. CCK-8 and flCC cytometry assays were used to analyze the proliferation and apoptosis of HCT-116 cells following transfection with ZEB1 siRNA and ZEB1 NC. D, E. The effects of miR-708 on the migration and invasion of HCT-116 cells were evaluated using Transwell assays. The assays were performed at least three times with similar results. Data are shown as the mean ± SD (n = 3) of one representative experiment. *P < 0.05 and **P < 0.01 versus normal groups.

groups were measured by *t* test. Comparisons among multiple groups were conducted using 1-factor analysis of variance (ANOVA). *P <* 0.05 was considered to be of statistical significance.

Results

MiR-708 expression is decreased in CRC tissues and cell lines

To determine the expression status of miR-708 in CRC, RT-qPCR was conducted to detect miR-708 expression in CRC and adjacent non-tumor tissues. Compared to that in adjacent non-tumor tissues, miR-708 showed low expression in CRC tissues (**Figure 1A**). Additionally, the expression level of miR-708 in CRC cell line (HCT-116) and a normal human liver epithelium cell line (Ect1) was determined by RT-qPCR. MiR-708 was downregulated in CRC cell lines relative to that in Ect1 (**Figure 1B**). These results suggest that downregulation of miR-708 may be closely related with the malignant biological feature of CRC.

MiR-708 inhibits CRC cell proliferation, migration, and invasion, and promotes cell apoptosis in vitro

As miR-708 was expressed at low levels in CRC, miR-708 may serve important roles in CRC initiation and progression. To confirm this hypothesis, HCT-116 cells were transfected with miR-708 mimics, miR-708 inhibitor or NC control. Subsequently, the regulatory influence of miR-708 on the proliferation of CRC cells was examined by using flow cytometry and CCK8 kits. HCT-116 cells transfected with miR-708 mimics exhibited obvious growth suppression compared to cells transfected with NC. In contrast, knockdown of miR-708 showed a much higher viability compared with the cells transfected with NC (**Figure 2A**). Interestingly, overexpression of miR-708 inhibited cell proliferation *via* cell cycle arrest at the G1 phase (**Figure 2B**). The role of miR-708 in regulating CRC cell ap-

optosis was then investigated. Flow cytometry analysis revealed that miR-708 upregulation significantly promoted the apoptosis of HCT-116 cells. Conversely, overexpression of miR-708 promoted the apoptosis in HCT-116 cells (**Figure 2C**). Furthermore, migration assays performed to determine the effect of miR-708 on the metastasis and invasion of CRC cells. Ectopic miR-708 expression attenuated the migratory and invasive abilities of HCT-116 cells, and the migratory and invasive abilities were promoted in HCT-116 transfected with miR-708 inhibitor (**Figure 2D** and **2E**).

To elucidate the mechanism through which the miR-708 regulated migration, invasion and apoptosis, we examined relevant proteins by using RT-qPCR and Western blot. As shown in **Figure 3B** and **3D**, the overexpression of miR-708 led to increased Bax mRNA and protein level and decreased Bcl-2 mRNA and protein level in HCT-116 cells, whereas miR-708 knockdown reduced Bax and increased Bcl-2 expression in HCT-116 cells (**Figure 3A** and **3C**). These data are in line with the observed apoptosis caused by the miR-708 mimics. Additionally, the mRNA and protein levels of N-cadherin and E-cadherin were reduced by the miR-708 mimics in HCT-116 cells (**Figure 4B** and **4D**). In contrast, silence of miR-708 increased the mRNA and protein levels of N-cadherin and E-cadherin in HCT-116 cells (**Figure 4A** and **4C**). These results suggest that miR-708 plays a tumor suppressive role in the growth and metastasis of CRC.

ZEB1 is a direct target of miR-708 in CRC

To clarify the molecular mechanism responsible for the tumor suppressor activity of miR-708 in CRC, bioinformatics analysis was performed to identify the potential targets of miR-708. Among these putative targets, ZEB1 was selected for additional analysis (**Figure 5A**), as it had previously been demonstrated to contribute to multiple cellular processes, including cell growth, apoptosis, migration, invasion, metas-
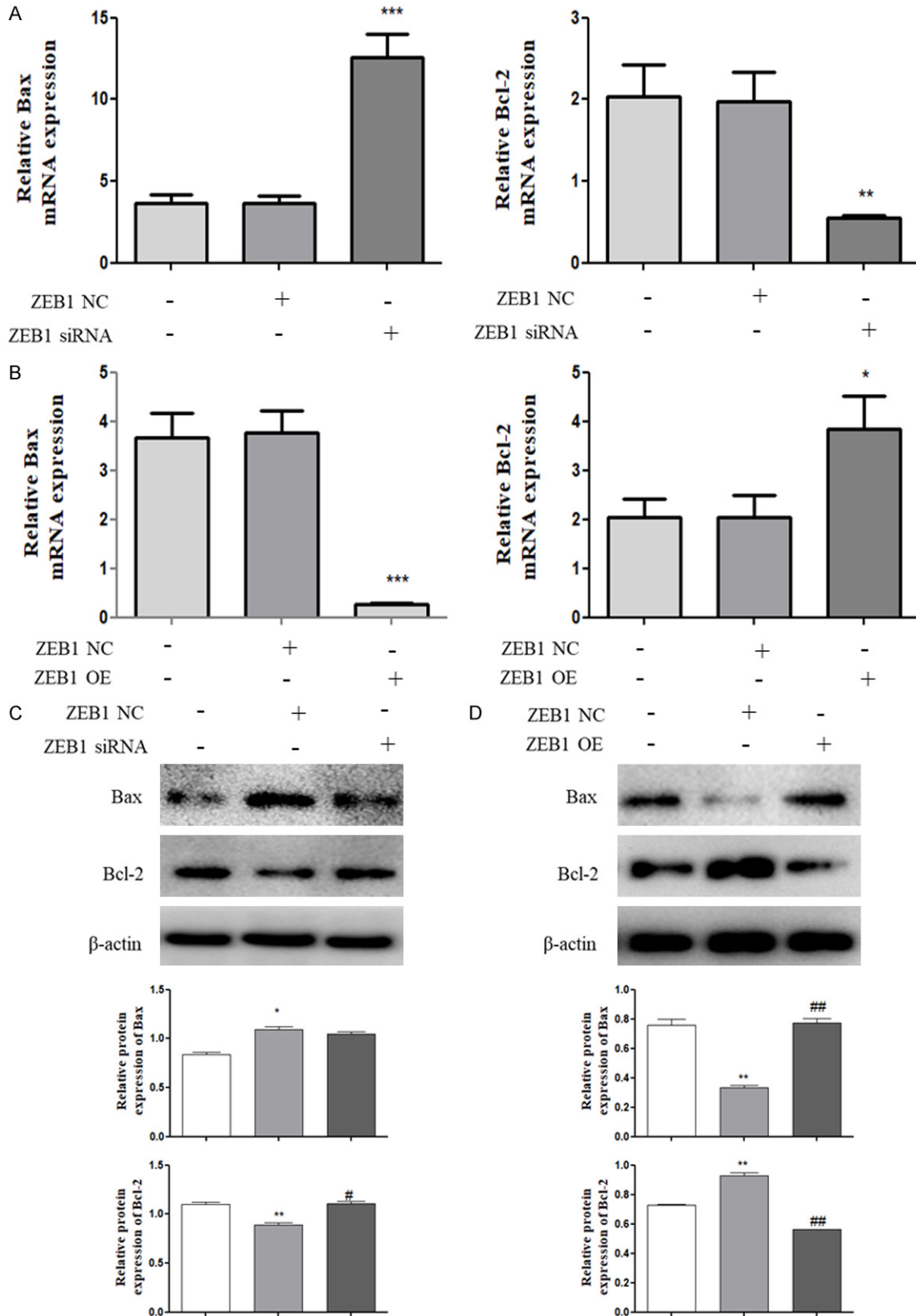
Figure 8. The effect of ZEB1 expression on apoptosis protein expression in CRC cell lines. A and B. RT-qPCR was performed to evaluate the Bax and Bcl-2 mRNA expression after transfection of HCT-116 cells with ZEB1-siRNA, ZEB1 OE and NC. C and D. Western blot was performed to evaluate the Bax and Bcl-2 protein expression after trans-

fection of HCT-116 cells with ZEB1-siRNA, ZEB1 OE and NC. The assays were performed at least three times with similar results. Data are shown as the mean ± SD (n = 3) of one representative experiment. *P < 0.05 and **P < 0.01 versus normal groups.

tasis, tumor development and tumor progression [27-30]. A luciferase reporter assay was then conducted to determine whether miR-708 directly binds to the 3'-UTR of ZEB1 in HCT-116 cells. As shown in **Figure 4B**, miR-708 overexpression noticeably reduced the luciferase activity in HCT-116 cells (**Figure 5B**). To confirm the regulatory effect of miR-708 on ZEB1 expression, RT-qPCR and Western blot were performed to determine ZEB1 expression in HCT-116 cells in response to the changes in miR-708 overexpression and miR-708 knockdown. RT-qPCR and Western blot analysis revealed that restoration of miR-708 expression suppressed the mRNA (**Figure 5C** and **5D**) and protein (**Figure 5E**) expression of ZEB1 in HCT-116 cells. Collectively, these results demonstrated that ZEB1 is a direct target of miR-708 in CRC.

Upregulation of ZEB1 is negatively correlated with miR-708 expression in vivo and vitro

To further evaluate the relationship between miR-708 and ZEB1 in CRC, the expression of ZEB1 was determined by using Immunohistochemistry, RT-qPCR in CRC, adjacent non-tumor tissues and healthy controls. We performed IHC staining to examine the expression of ZEB1. CRC patients that displayed higher ZEB1 expression (**Figure 6A**). Additionally, the expression level of ZEB1 mRNA was significantly higher in CRC tissues and cell lines (**Figure 6B** and **6C**). Western blot analysis revealed that ZEB1 protein level was upregulated in CRC tissues and cell lines (**Figure 6D** and **6E**). These results document that upregulation of ZEB1 expression in CRC tissues is, at least partly, caused by low miR-708 expression.

ZEB1 promotes CRC cell proliferation, migration, and invasion, and promotes cell apoptosis in vitro

Because ZEB1 was identified as a direct target gene of miR-708 in CRC, we then investigated the functional roles of ZEB1 in CRC. ZEB1 siRNA and ZEB1 plasmid were introduced into HCT-116 cells to reduce its expression and increase its expression. The CCK-8 assay revealed that downregulation of ZEB1 led to a decreased proliferative ability of HCT-116 cells (**Figure 7A**).

Interestingly, suppression of ZEB1 inhibited cell proliferation *via* cell cycle arrest at the G1 phase (**Figure 7B**). Flow cytometry analysis indicated that the apoptosis rate of HCT-116 cells was promoted by ZEB1 silence compared to the NC siRNA (**Figure 7C**). Furthermore, the number of migrated and invaded cells was significantly reduced in ZEB1 siRNA transfectants compared to that in NC siRNA-transfected HCT-116 cells. Whereas, opposite results were obtained by using ZEB1 over-expression (**Figure 7D** and **7E**). These results demonstrate that the effects of ZEB1 knockdown on CRC cells were similar to that of miR-708 overexpression, further suggesting that ZEB1 is implicated in tumor progression of CRC cells.

To elucidate the mechanism through which the ZEB1 regulated migration, invasion and apoptosis, we examined relevant proteins by using Western blot. As shown in **Figure 8A** and **8C**. The suppression of ZEB1 led to increased Bax mRNA and protein levels and decreased Bcl-2 levels in HCT-116 cells, whereas ZEB1 upregulation reduced Bax and increased Bcl-2 mRNA and protein in HCT-116 cells (**Figure 8B** and **8D**). Additionally, the mRNA and protein of N-cadherin and E-cadherin were reduced by the ZEB1 siRNA in HCT-116 cells (**Figure 9A** and **9C**). In contrast, overexpression of ZEB1 increased the expression of N-cadherin and E-cadherin in HCT-116 cells (**Figure 9B** and **9D**). These data are in agreement with the observed apoptosis caused by the ZEB1 siRNA. These results further highlight that ZEB1 plays a tumor suppressive role in the growth and metastasis of CRC cells.

MiR-708 can regulate AKT/mTOR pathway by targeting ZEB1

AKT/mTOR signaling pathway was reported to play a crucial role in multiple type tumors, suppression of AKT/mTOR signaling may limit cancer cells invasion and proliferation [25]. Therefore, we sought to explore how miR-708 would influence AKT/mTOR pathway by regulating ZEB1. We noted that overexpression of the miR-708 in HCT-116 cells led to a decrease in protein expression of p-AKT and p-mTOR (**Figure 10A**). Correspondingly, we observed an increase in protein level of these genes in the
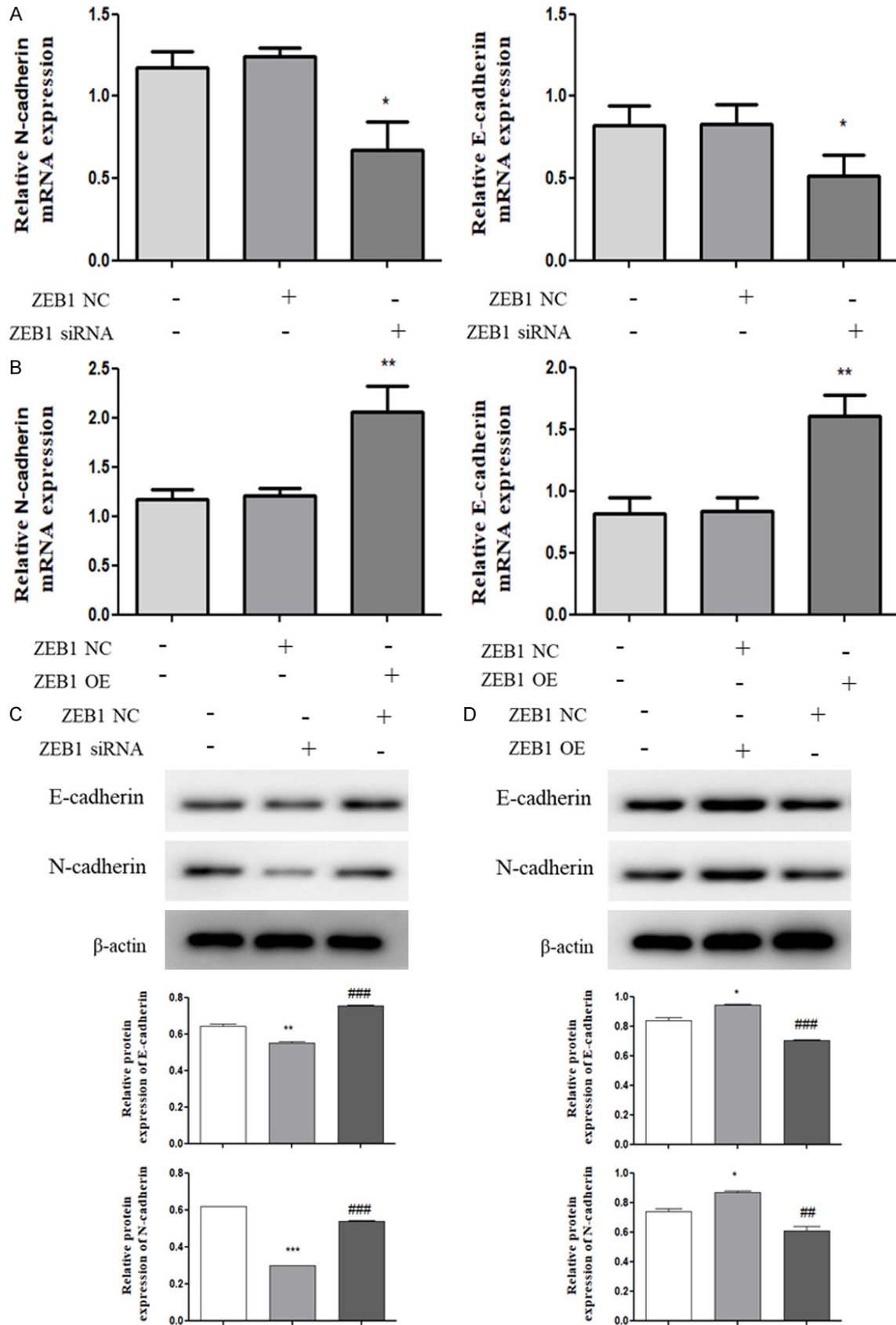
Figure 9. The effect of ZEB1 expression on E-cadherin and N-cadherin expression in CRC cell lines. A and B. RT-qPCR was performed to evaluate the E-cadherin and N-cadherin mRNA expression after transfection of HCT-116 cells with ZEB1-siRNA, ZEB1 OE and NC. C and D. Western blot was performed to evaluate the E-cadherin and N-cadherin protein expression after transfection of HCT-116 cells with ZEB1-siRNA, ZEB1 OE and NC. The assays were performed at least three times with similar results. Data are shown as the mean ± SD (n = 3) of one representative experiment. *P < 0.05 and **P < 0.01 versus normal groups.

presence of miR-708 inhibitor (**Figure 10B**). To further explore whether the effect of miR-708 on AKT/mTOR pathway is related to ZEB1, we transfected cells with ZEB1 siRNA and ZEB1 OE, and detected the protein expression of p-AKT and p-mTOR. As expected, all were diminished in the HCT-116 cells transfected with ZEB1 siRNA (**Figure 10C**). In contrast, overexpression of ZEB1 increased the expression of p-AKT and p-mTOR (**Figure 10D**). Taken together, these data indicate that the negative regulation of miR-708 on AKT/mTOR signaling pathway through ZEB1.

Discussion

Therapies using miRNA are a promising means of regulating the pathways involved in CRC. The major finding of the current study is that miR-708 was significantly reduced in CRC tissues and HCT-116. Our results further showed that overexpression of miR-708 and silence of ZEB1 can inhibit CRC cell proliferation, migration, and invasion, and promote cell apoptosis. In addition, we clearly demonstrate that miR-708 regulated ZEB1 gene expression by specifically interacting with the 3'-UTR of ZEB1 mRNA to inhibit the expression of ZEB1. Interestingly, our data suggested that miR-708 regulated the stage of CRC cells by targeting ZEB1 through AKT/mTOR signaling pathway. Therefore, we deduce that the upregulation of miR-708 promotes apoptosis and inhibits proliferation, migration, and invasion of CRC cells.

MicroRNAs (miRNAs) represent a group of highly conserved, short non-coding RNA molecules of ~22 nucleotides in length [31]. Previous studies have indicated that 1/2 of miRNAs are located in the fragile or oncogene-associated regions of chromosomes, suggesting that abnormally expressed miRNAs are associated with carcinogenesis and cancer progression [32]. miRNAs regulate gene expression at the post-transcriptional level *via* sequence-specific binding to the 3'-UTR of target mRNAs [33, 34]. A single miRNA is able to regulate various mRNAs. MiRNAs may therefore present a novel

therapeutic target in the treatment of human cancer, including CRC [35]. To investigate the function of miRNAs in carcinogenesis and cancer progression, aberrant miRNA expression must be validated, and the biological role of miRNA in cancer initiation and progression investigated [36, 37]. miR-708 is dysregulated in multiple types of cancer, and previous studies reported that miR-708 was downregulated in renal cell carcinoma and prostate cancer [38, 39]. Guo et al [40] observed that miR-708 expression is low in human glioblastoma. By contrast, miR-708 was observed to be upregulated in non-small cell lung cancer, childhood common precursor B-cell acute lymphoblastic leukemia and bladder carcinoma [41, 42]. However, the expression status and specific roles of miR-708 in CRC remain largely unknown. Here, we found that miR-708 was downregulated in CRC, and the decreased miR-708 expression was significantly correlated with metastasis. Functional analyses demonstrated that miR-708 overexpression restricted CRC cell proliferation, promoted apoptosis, and attenuated migration and invasion as well as hindered tumor growth *in vivo*. Hence, this miRNA may be developed as a diagnostic biomarker and valuable therapeutic agent for patients with CRC.

Validation of the direct targets of miR-708 is important for understanding its roles in the progression of CRC and may be helpful for identifying promising therapeutic approaches. Therefore, we investigated the molecular mechanisms responsible for the tumor suppressive actions of miR-708 in CRC. First, bioinformatics analysis was conducted to search for potential targets of miR-708. One highly conserved putative binding site was observed at the 3'-UTR of ZEB1. ZEB1 is a member of the deltaEF1 family of two handed zinc-finger factors [43]. ZEB1 was reported to be overexpressed in a variety of human malignant tumors, such as thyroid cancer, lung cancer, colorectal cancer, and endometrial cancer [44, 45]. Emerging studies have shown that ZEB1 is closely related to the carcinogenesis and progression of CRC by
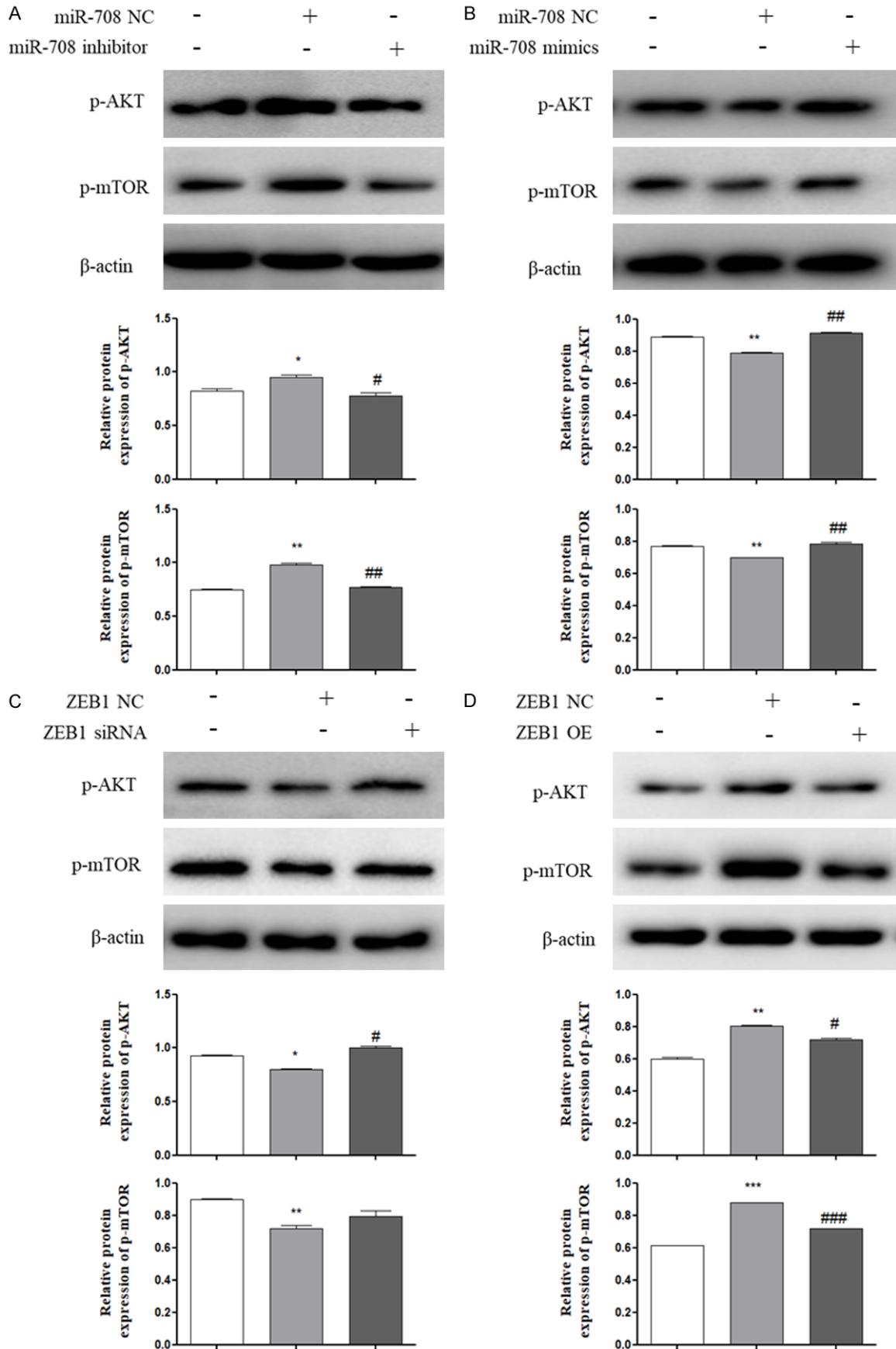
Figure 10. The effect of ZEB1 expression on Akt/mTOR signaling pathway in CRC cell lines. A and B. Western blot analyzed the Akt/mTOR signaling related proteins in HCT-116 cells transfected with miR-708 mimics, miR-708 inhibitor and miR-NC. C and D. Western blot analyzed the Akt/mTOR signaling related proteins in HCT-116 cells transfected with ZEB1-siRNA, ZEB1 OE and NC.

affecting cell proliferation, migration, invasion, epithelial-mesenchymal transition, and motility. Moreover, a luciferase reporter assay, RT-qPCR, and Western blot analysis revealed that miR-708 directly binds to the 3'-UTR of ZEB1 and thus inhibits ZEB1 expression in CRC cell lines. ZEB1 was upregulated in CRC tissues and cell lines, which is negatively correlated with miR-708 expression. Inhibition of ZEB1 mimicked the tumor suppressing roles of miR-708 overexpression in CRC cell lines. Finally, rescue experiments confirmed that the downregulation of ZEB1 was essential for miR-708 to be effective in the malignant phenotypes of CRC cells. These results clearly demonstrate that ZEB1 is a direct and functional downstream target of miR-708 in CRC. ZEB1 knockdown using miR-708-based targeted therapy may be a suitable therapeutic strategy for the prevention and treatment of patients with CRC.

Increasing evidence shows that the activation of AKT/mTOR pathway is crucial for EMT process [46, 47]. Mechanistically, phosphorylation of AKT could activate mTOR to control motility, invasion and phenotype transformation of cells and contribute to EMT [25]. Previous study showed that miR-200c suppresses the expression of ZEB1 through AKT/mTOR signaling pathway in non-small cell lung cancer [48]. Furthermore, Src promotes the process of EMT by upregulation of ZEB1 and ZEB2 through AKT signaling pathway in gastric cancer cells [49]. It has been reported that miRNAs may function as a tumor suppressive miRNA and play an important role in inhibiting the HCC tumorigenesis by modulating the AKT/mTOR pathway [50]. In our present study, we found that miR-708 was negatively regulated expression of ZEB1 during the stage of CRC. MiR-708-mimics and ZEB1-siRNA treatment significantly decreased the phospho-AKT and phospho-mTOR expression. Therefore, miR-708 might down-regulated ZEB1 through AKT/mTOR signaling pathway, which promoted apoptosis and inhibited proliferation, migration, and invasion of CRC and subsequent protection against CRC. In general, the specific regulatory mechanism by which miR-708 inhibited stages of CRC is still to be determined future.

In conclusion, the present study demonstrates that miR-708 is significantly downregulated in CRC and associated with increased tumor stage. miR-708 decreases cell proliferation, migration and invasion by directly targeting ZEB1 mRNA through AKT/mTOR signaling pathway in CRC. Identification of miR-708 targets may provide an in-depth understanding of the potential underlying mechanisms of carcinogenesis and progression in CRC. miR-708 may be a novel target for future CRC therapy.

Conclusions

The present study demonstrates that knockdown of miR-708 could suppress oncogenic properties and inhibit migration and invasion of CRC cells, the underlying mechanism of which was closely associated with the inhibition of migration and invasion of CRC at least partially by targeting ZEB1 through Akt/mTOR signaling pathway. The findings will provide the theoretical basis for recognizing miR-708 as a potential therapeutic target in CRC.

Acknowledgements

Disclosure of conflict of interest

None.

Abbreviations

MiRNAs, microRNA; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum.

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