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Transcriptional factor CCAAT enhancer binding protein beta inhibits epithelial-mesenchymal transition in cervical cancer via regulating attractin-like 1

Minjie Fang, Yayan Zhou, Zihuang Li, Xianming Li*

Department of Radiation Oncology, The Second Clinical Medical College of Jinan University, Shenzhen People's Hospital, Shenzhen, Guangdong 518020, China

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ABSTRACT

Cervical cancer (CC) is a malignancy seriously endangering women's life and health worldwide. GEPIA demonstrated that attractin-like 1 (ATRNL1) presents downregulation in CC tissue. Transcription factor CCAAT enhancer binding protein beta (CEBPB) was previously revealed to present depletion in CC tissue. We attempted to clarify molecular mechanism between ATRNL1 and CEBPB underlying CC progression. Bioinformatics, RT-qPCR and western blotting revealed expression characteristics of ATRNL1 in CC. RT-qPCR measured ATRNL1 and CEBPB levels in CC cell lines. Gain-of-function assays clarified role of ATRNL1 in CC cell behaviors. Bioinformatics, Pearson correlation, ChIP and luciferase reporter experiments assessed association of ATRNL1 and CEBPB in CC cells. Rescue assays assessed regulatory function of CEBPB-ATRNL1 in CC cellular processes. ATRNL1 showed depletion in CC tissue and cells at mRNA and protein levels. ATRNL1 upregulation repressed CC cell viability, migration and EMT. CEBPB bound to ATRNL1 promoter to transcriptionally upregulate ATRNL1 in CC cells. The impact of CEBPB elevation on CC cell viability, migration and EMT were countervailed by ATRNL1 depletion. ATRNL1 and CEBPB present depletion and serve as tumor suppressors in CC cells. ATRNL1 suppresses CC cell malignancy through CEBPB activation, which may provide a potential new direction for seeking therapeutic plans for CC.

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Introduction

Cervical cancer (CC), a malignancy seriously endangering women's life and health, ranks as the second in common tumor type among women worldwide (1). CC, the only gynecological malignancy with a clear etiology currently, has a close relation to persistent infection with high-risk human papilloma virus (HPV) (2). CC ranks second in cancer incidence and third in mortality among Chinese women aged 15-44 years (3). Cervical anatomy is easily exposed and has a long period of precancerous lesions (4), which facilitates clinical diagnosis and therapy of CC, thus CC is currently a malignancy with quite excellent clinical diagnosis and prevention. Nevertheless, there is still no breakthrough in the research on the pathogenesis of CC. At present, operation, chemotherapy and radiotherapy are the most commonly used treatments for CC (5). The 5-year survival rate of patients with advanced CC is not high, only approximately 50% (6). In recent years, the incidence of CC has a rising and younger trend (7, 8). Thus, in-depth research on pathogenesis of CC and clarification of new intervention biomarkers and therapeutic methods still have crucial clinical significance and application value.

Epithelial-mesenchymal transition (EMT) is a fundamental cellular phenomenon that plays an intrinsic role in tumor development (9). Recent literatures have indicated that EMT plays a key role in the progression of CC (10). EMT is considered a complex molecular and cellular program in which epithelial cells lose their differentiation features, including intercellular adhesion, apico-basal polarity, lack of cell viability and gain of mesenchymal features, such as viability and invasiveness (11). Therefore, understanding the potential mechanism underlying EMT in CC may provide novel sight for CC treatment.

The GEPIA database demonstrated that attractin-like 1 (ATRNL1) presents downregulation in cervical squamous cell carcinoma and cervical adenocarcinoma. Thus we suspected that ATRNL1 may exert a tumor-suppressor role in CC. Nevertheless, the upstream mechanism of ATRNL1 in CC remains elusive. Transcription factors, also known as trans-acting factors, are a group of proteins binding to cis-acting elements, enhancers or silencers located 50-5000 bp upstream of transcription initiation site and participating in modulating transcription efficacy of target genes (12). A transcription factor often regulates several genes simultaneously, and its binding sites on different genes are conserved to a certain extent, but not identical (13). It may be that multiple regions with similar sequences can become target-binding regions (14). CCAAT enhancer binding protein beta (CEBPB) is a crucial member of CCAAT enhancer-binding proteins (CEBPs) (15). Its C-terminus has a highly conserved DNA binding domain and a dimerization domain, which mainly gets involved in important life activities such as cell proliferation and differentiation, tumorigenesis and apoptosis, and inflammation via regulating target cell gene transcription (16). In uterine leiomyomas, CEBPB modulates aromatase ex-

* Corresponding author. Email: lixianming669369@163.com

pression through a complex cis-acting element, which is a vital factor in aromatase expression transcript (17), while aromatase inhibitors attenuate uterine leiomyoma growth, and are related to irregular uterine bleeding (18). CEBPB, activated by IL-1 and TNF-α, represses early HPV16 gene transcription, thereby affecting CC progression (19). Xing et al. detected cervical tissue cells of Uyghur women and found that CEBPB protein abundance presented depletion in cervical squamous cell carcinoma (20). We wondered whether transcriptional regulation of CEBPB had a relation to ATRNL1 expression status in CC.

Herein, we attempted to evaluate the association of ATRNL1 and transcription factor CEBPB in CC progression and clarify molecular mechanism between the underlying CC cells, which may provide a novel insight for targeted therapy of CC.

Materials and Methods

Specimen collection

A total of 25 CC patients treated in our hospital from April 2018 to March 2019 were enrolled in our research and 25 pair of CC tissue and adjacent non-tumor tissue specimens were obtained through surgery. All tissue specimens were stored at -80°C for further experiments. The ethics committee of our hospital approved our research and all the patients enrolled voluntarily signed informed consent.

Cell lines and cell culture

Human CC cell lines (SiHa, CaSki, HT-3 and C33A) and human normal cervical epithelial cell lines (HCerEpic) were provided from Cell Bank, Chinese Academy of Sciences (Shanghai, China). SiHa and C33A cells were cultured in MEM, CaSki cells were cultured in RPMI-1640 medium, HT-3 cells were cultured in McCoy's 5A medium, and HCerEpic cells were cultured in EMEM. All cell mediums were added with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C with 5% CO_2, and cells at logarithmic growth phase were taken for following assays.

RNA extraction and RT-qPCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA). ATRNL1 and CEBPB were reverse-transcribed through PrimeScript RT master mix (Takara, Japan) and designed primers normalizing to GAPDH. The PCR reaction was run in triplicate through 7500 Real-Time PCR System (Applied Biosystems, USA) using SYBR Premix Ex Taq II (Takara, Japan). The $2^{-\Delta\Delta Ct}$ method was performed to analyze relative expression.

Western blot

The logarithmic phase SiHa and C33A cells were taken, medium in the culture dish was aspirated and cells were stored in a sterile centrifuge tube. After centrifugation at 1200 r/min for 10 min, the lysate was added to resuspend cells. The protein concentration was determined by the BCA method. The 5 × SDS gel electrophoresis buffer was added and denatured at 100°C for 10 min. After being completely separated by electrophoresis, the protein was transferred to the PVDF membrane by semi-dry method. After blocked by 5% skimmed milk powder at room temperature for 2 h, the specific primary antibodies ATRNL1 (1/500, GTX66302, GeneTex) CEBPB (1/1000, ab32358, Abcam), E-cadherin (1/10000, ab40772, Abcam), N-cadherin (1/500, ab98952, Abcam), vimentin (1/1000, ab92547, Abcam) and GAPDH (1/2500, ab9485, Abcam) were added, and incubated overnight at 4°C. Then the secondary antibodies (1/2000, ab6728, Abcam) were added, incubated for another 2 h, and washed with TBS. Absorbance analysis was performed after color development to calculate the relative expression of each protein.

Cell transfection

Short hairpin RNA (shRNA) targeting ATRNL1 (sh-ATRNL1), ATRNL1 or CEBPB overexpression vector (OE-ATRNL1 or OE-CEBPB) or empty vector were provided by GenePharma (Shanghai, China). SiHa and C33A cells were cultured to approximately 80% confluence in plates and then transfected with designated plasmids using Lipofectamine 2000 according to the manufacturer's instructions. After 48 h of transfection, cells were harvested for next assays.

CCK-8

Cell viability was assessed with CCK-8 kit. Briefly, SiHa and C33A cells were seeded into each well of 96-well plates for culture plus CCK-8 (10 μL) at 48 h. Two hours post-incubation, optical density was measured with a microplate reader at 450 nm.

Transwell migration

SiHa and C33A cells in the upper chamber were seeded in transwell chamber containing 200 μL of a serum-free DMEM medium. The medium in the lower chamber possessed 10% FBS for attracting cells. The cells were stained through 0.5% crystal violet for 20 min after 48 h of incubation. The cells were photographed and counted in five random distinct fields.

Immunofluorescence (IF)

SiHa and C33A cells were seeded into 6-well plates. After 24 h of incubation, cells received fixation with 4% paraformaldehyde for 20 min and then washed using PBS three times. The 0.3% TritonX-100 broke cell membrane and 1% BSA buffer with 5% anti-goat serum blocked cells under room temperature for 1 h. FITC-labeled RNA probes applied were anti-E-cadherin (1/500, ab40772, Abcam), anti-N-cadherin (1/200, ab98952, Abcam) and anti-vimentin (2 μg/ml, ab92547, Abcam). Olympus Fluo View FV1000 confocal microscope was applied for images.

Xenograft tumor mouse models

The animal care and use ethics committee of our hospital approved the animal experiments. A total of 8 nude mice (4 weeks, 18 g) were divided into two groups randomly (n = 3). In brief, 0.1 mL of C33A cell suspension with lentivirus expressing ATRNL1 or empty vector was subcutaneously injected into the left flank of nude mice. Tumor volume = length × width^2 × 0.5. Mice were sacrificed 7-8 weeks post-injection. Tumors were photographed and weighed and then stored at -80°C for subsequent use.

Immunochemistry (IHC)

Dewaxed and rehydrated tumor slides received treatment of 3% hydrogen peroxide and 5% BSA. The slides received incubation with primary antibody anti-E-cadhe-

rin (1/500, ab40772, Abcam), anti-N-cadherin (1/200, ab98952, Abcam) and anti-vimentin (1/200, ab92547, Abcam) overnight, and received subsequent incubation with a secondary antibody for 30 min at room temperature. Sections were counterstained with hematoxylin and observed through microscopy.

ChIP

Formaldehyde was added into 10-cm dishes with SiHa and C33A cells for incubation at 37°C for 10 min. The cells were scraped into tubes after being washed by PBS and pelleted at 2000 rpm at 4°C for 5 min. SDS lysis buffer dissolved cells. An ultrasound equipment was utilized for shearing DNA. NaCl (5 M) was added into tubes to reverse crosslink at 65°C overnight. The supernatant was harvested after centrifugation at 13,000 rpm at 4°C for 10 min. The ChIP Dilution Buffer diluted cell supernatant and Salmon Sperm DNA attenuated non-specific background. The anti-CEBPB and anti-IgG were added into specimens with rotation at 4°C overnight. Protein A Agarose collected antibody complexes at 4°C for 4 h followed by gentle centrifugation. Low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer and TE buffer washed agarose in order. Then agarose was washed by elution buffer and the supernatant received treatment with NaCl to reverse histone-DNA crosslinking by heating at 65°C for 4 h and Proteinase K to remove proteins in the mixture at 45°C for 1 h. DNA was extracted via phenol extraction combined with ethanol precipitation.

Luciferase reporter gene

pGL4-CMV-Luc2 plasmid was utilized to construct the pGL4-ATRNL1 promoter-WT and pGL4-ATRNL1 promoter-MUT plasmids. The plasmids were then subcloned into SiHa and C33A cells seeded into 24-well plates with OE-CEBPB or OE-NC, respectively. After 48 h of transfection, cells were harvested and measured with the Luciferase Assay Kit using a Veritas microplate luminometer.

Statistical analysis

SPSS 20.0 software processed data. The data were expressed as mean ± standard deviation (m ± s). The mean of samples between groups was compared using t-test, and that of multiple groups through one-way analysis of variance followed by Tukey's post hoc test. Pearson curve assessed the association of ATRNL1 level with CEBPB level in CC tissue. All assays were conducted three times. The difference was statistically significant upon $P < 0.05$.

Results

Downregulation of ATRNL1 in CC tissue and cells

The GEPIA database (http://gepia.cancer-pku.cn) demonstrated that ATRNL1 presents downregulation in cervical squamous cell carcinoma and cervical adenocarcinoma tissue relative to controls (Figure 1A). To clarify ATRNL1 role underlying CC, we validated ATRNL1 expression status in CC tissue from 25 patients using RT-qPCR. As a result, ATRNL1 presented a marked depletion in CC tissue relative to controls (Figure 1B). Bioinformatics by proteinatlas demonstrated that gene ATRNL1 presented major nuclear distribution in CC cells (Figure 1C), suggesting that ATRNL1 may present downregulation due to upstream transcriptional regulation. Moreover, mRNA status in normal human tissues from GTEx (https://www.genome.gov/Funded-Programs-Projects/Genotype-Tissue-Expression-Project), Illumina (https://www.illumina.com), and BioGPS (http://biogps.org/dataset) depicted that ATRNL1 predominately distributed in genital organ tissue, including in uterus (Figure 1D). RT-qPCR depicted that ATRNL1 presented a remarkable downregulation in CC cell lines (SiHa, CaSki, HT-3 and C33A) relative to control cell line (HCerEpic) (Figure 1E). Western blotting showed a similar trend of ATRNL protein abundance in CC cells (Figure 1F). Collectively, ATRNL1 presents depletion in CC tissue and cells and may serve as a tumor-suppressor molecule in CC.

ATRNL1 suppresses CC malignant phenotypes in vitro and in vivo

Due to ATRNL1 showing downregulation in CC tissue and cells, we hypothesized that ATRNL1 may function as a tumor suppressor in CC cell malignancy. Thus, we carried out gain-of-function assays in SiHa and C33A cells. First, ATRNL1 received successful overexpression in SiHa and C33A cells via OE-ATRNL1 plasmid (Figure 2A). A similar trend of ATRNL1 protein abundance in CC cells validated the successful overexpression (Figure 2B). Then, CCK-8 assessed CC cell viability. As a result, ATRNL1 upregulation resulted in a remarkable viability reduction in SiHa and C33A cells (Figure 2C). Moreover, transwell migration assay demonstrated the decreased migratory CC cells under ATRNL1 upregulation (Figure 2D). Malignant

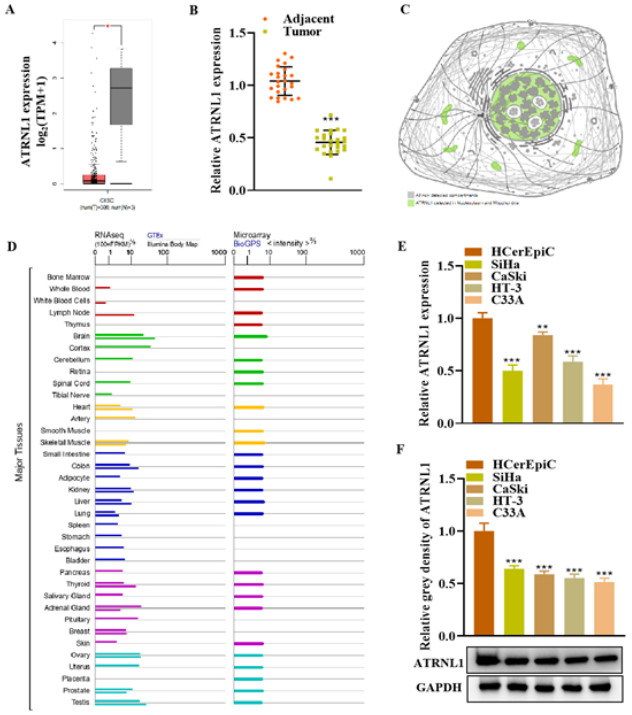

Figure 1. ATRNL1 showed downregulation in CC tissue and cells. A, GEPIA revealed ATRNL1 level in CC tissue and non-tumor tissue. B, RT-qPCR measured ATRNL1 level in CC tissue (n = 25) and adjacent non-tumor tissue. C, HUMAN PROTEIN ATLAS indicated subcellular localization of ATRNL1. D, mRNA expression in normal human tissues from GTEx, Illumina, and BioGPS for ATRNL1. E-F, RT-qPCR and western blotting measured ATRNL1 mRNA level and protein abundance in CC cell lines (SiHa, CaSki, HT-3 and C33A) and control cell line HCerEpic. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

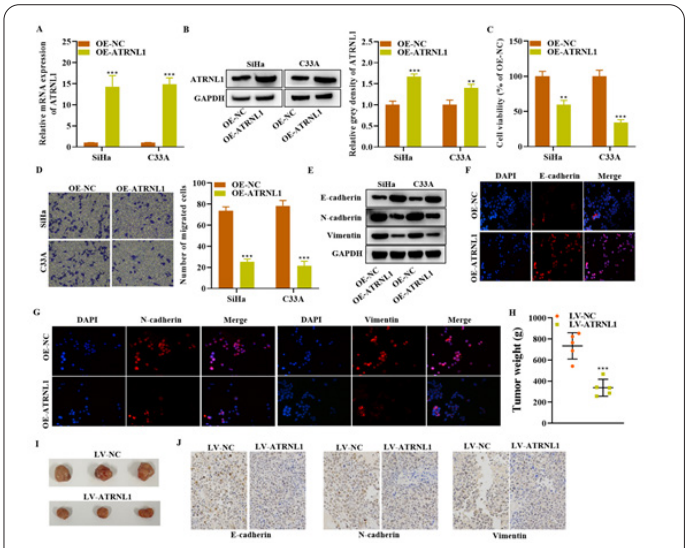

Figure 2. ATRNL1 suppressed CC malignant phenotypes in vitro and in vivo. A-B, RT-qPCR and western blotting detected overexpression efficacy of OE-ATRNL1 or empty vector in CC cells. C, CCK-8 assessed CC cell viability under transfection with OE-ATRNL1 or empty vector. D, Transwell assessed CC cell migration under indicated transfection. E, Western blotting evaluated EMT-related protein abundance in CC cells under indicated transfection. F-G, IF staining evaluated EMT-related protein distribution in CC cells under indicated transfection. H. Mouse tumor weight under indicated transfection. I. Representative pictures of mouse tumors (n = 3). J. IHC of EMT-related protein distribution in CC mouse tissue under indicated transfection. ***P<0.001.

tumor invasion and migration is one of the main manifestations of tumor malignant transformation, and EMT is a crucial mechanism of tumor invasion and migration (21). Thus we detected EMT-related protein abundance and distribution in CC cells via western blotting and IF staining. As a result, ATRNL1 elevation led to N-cadherin and vimentin downregulation in SiHa and C33A cells which resulted in E-cadherin upregulation in SiHa and C33A cells (Figure 2E). The results presented consistency with IF staining results (Figure 2F-G). To further clarify ATRNL1 role in CC progression, we conducted gain-of-function assays in vivo. The stably transected C33A cells with lentiviral vectors overexpressing ATRNL1 (LV-ATRNL1) and controls were subcutaneously injected into mice. As a result, ATRNL1 overexpression led to a decrease in mouse tumor weight and size (Figure 2H-I). IHC demonstrated that N-cadherin and vimentin presented downregulation while E-cadherin presented elevation after LV-ATRNL1 injection (Figure 2J). Collectively. ATRNL1 may exert an inhibitory impact on CC progression.

CEBPB transcriptionally upregulates ATRNL1 in CC cells

It has been revealed that CEBPB protein abundance presents depletion in cervical squamous cell carcinoma (20), and CEBPB represses early HPV16 gene transcription, thereby affecting CC progression (19). hTFtarget database (http://bioinfo.life.hust.edu.cn/hTFtarget) predicted that CEBPB is a putative transcriptional factor of ATRNL1. Thus, we hypothesized that CEBPB may be involved in CC progression via transcriptional regulation of ATRNL1. RT-qPCR depicted that CEBPB presented a marked depletion in CC tissue relative to controls (Figure 3A). Pearson correlation curve illustrated that CEBPB level presented a positive association with ATRNL1 level in CC tissue (Figure 3B). GTEx database on the GEPIA website demonstrated that CEBPB level indeed presents a positive association with ATRNL1 level in CC tissue (Figure 3C). Western blotting depicted that CEBPB protein abundance presented a remarkable downregulation in CC cells relative to controls (Figure 3D). Moreover, OE-CEBPB transfection elevated CEBPB and ATRNL1 protein abundance in SiHa and C33A cells (Figure 3E), suggesting that CEBPB exerts a positive regulation of ATRNL level in CC cells. Thus we clarified the molecular mechanism between ATRNL1 and CEBPB underlying CC cells. ChIP illustrated that the ATRNL1 promoter showed a marked enrichment in anti-CEBPB rather than anti-IgG (Figure 3F), further supporting a binding of ATRNL1 promoter to CEBPB in CC cells. Furthermore, JASPAR predicted the binding site of CEBPB in ATRNL1 promoter region and displayed the putative DNA motif of CEBPB. CEBPB may bind to ATRNL1 promoter at gatttcacagt (Figure 3G). After mutation of binding sequence gatttcacagt, luciferase reporter experiments were conducted. As a result, CEBPB upregulation led to elevation in luciferase activity of ATRNL1 promoter-WT while showing no impact on the mutant group (Figure 3H). Collectively, CEBPB transcriptionally induces ATRNL1 via targeting ATRNL1 promoter.

CEBPB suppresses CC cell malignancy via ATRNL1 elevation

To clarify the CEBPB-ATRNL1 regulatory pattern in CC cellular processes, we conducted rescue experiments. We performed co-transfection using sh-ATRNL1 in OE-CEBPB-transfected SiHa and C33A cells. We discovered that the decreased CC cell viability (Figure 4A), migratory

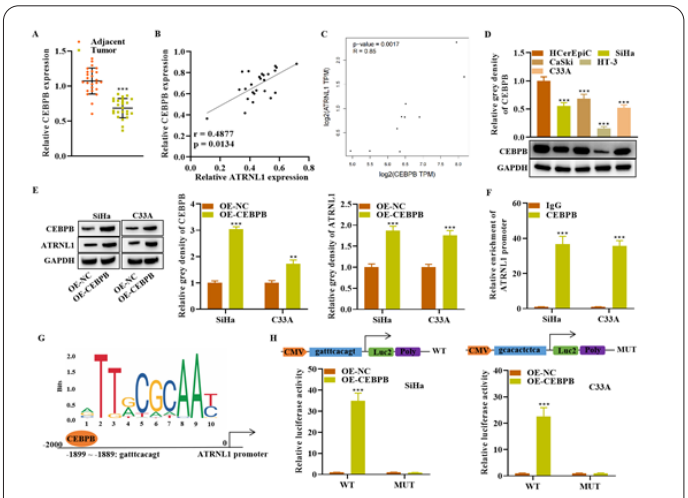

Figure 3. CEBPB transcriptionally upregulated ATRNL1 in CC cells. A, RT-qPCR measured CEBPB level in CC tissue (n = 25) and adjacent non-tumor tissue. B. CEBPB and ATRNL level association in CC tissue through Pearson analysis. C. GEPIA showed CEBPB and ATRNL level association in CC tissue. D, RT-qPCR and western blotting measured CEBPB mRNA level and protein abundance in CC cell lines and controls. E, Western blotting detected overexpression efficacy of OE-ATRNL1 or empty vector as well as its impact on ATRNL1 in CC cells. F, ChIP detected ATRNL1 promoter enrichment in RISCs bound to anti-CEBPB or anti-IgG in CC cells. G, Bioinformatics predicted binding sequence of CEBPB on ATRNL1 promoter and DNA motif of CEBPB. H, Luciferase reporter assessed binding effect of ATRNL1 promoter and CEBPB in CC cells. ***P<0.001.

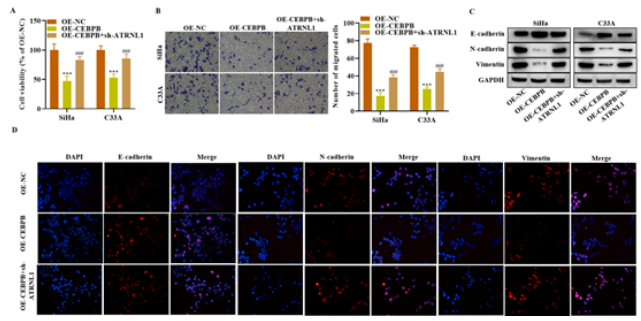

Figure 4. CEBPB suppressed CC cell malignancy via ATRNL1 elevation. A, CCK-8 assessed CC cell viability under transfection with empty vector, OE-CEBPB or OE-CEBPB+sh-ATRNL1. B, Transwell evaluated CC cell migration under indicated transfection. C, Western blotting evaluated EMT-related protein abundance in CC cells under indicated transfection. D, IF staining evaluated EMT-related protein distribution in CC cells under indicated transfection. ***P<0.001, ###P<0.001.

capability (Figure 4B) and EMT progress (Figure 4C-D) due to CEBPB overexpression were all rescued through ATRNL1 downregulation. Collectively, CEBPB represses CC cell malignant phenotypes via ATRNL1 upregulation.

Discussion

The incidence and mortality of CC ranks fourth among female malignancies (22). Since there is currently no nationwide cancer screening in China, the incidence of CC in China has not presented depletion in the past few decades (23). In recent years, targeted therapy has gradually become the focus of therapy for CC (24). Herein, ATRNL1 was for the first time demonstrated to present a marked downregulation in CC tissue specimens and cell lines via combining bioinformatics and RT-qPCR. Nevertheless, ATRNL1 role has not been researched in cancer, including CC. We hypothesized that ATRNL1 exerted an inhibitory role underlying CC progression due to its downregulation in CC. Furthermore, ATRNL1 overexpression suppressed CC cell proliferative and migratory capabilities and EMT. ATRNL1 overexpression also suppressed CC tumor growth and EMT in vivo. It was suggested that ATRNL1 repressed CC cell malignant phenotypes and tumorigenesis, and thus hindered CC progression. The findings were consistent with our hypothesis that ATRNL1 functioned as a tumor-suppressor molecule in CC cells.

Moreover, bioinformatics demonstrated the major nuclear distribution in CC cells, suggesting the potential transcriptional function of upstream molecules on ATRNL1 aberrant expression status. Bioinformatics revealed that CEBPB was a putative transcription factor of ATRNL1. The CEBP family is a subfamily of the basic leucine zipper protein family (25). So far, different laboratories have discovered and named multiple CEBP members. The common feature of CEBP family members is that their protein molecules include three similar structural components: a leucine zipper at the C-terminus, a transcriptional activation domain at the N-terminus and a DNA-binding domain in the middle (26-28). The CEBP family exerts a variety of biological functions, involving energy metabolism, liver regeneration, cell cycle, inflammatory response and pathology of various diseases, including cancers. CEBPB was first discovered by Akria et al and named NF-IL6 (29). CEBPB and other CEBP proteins have a highly homologous carboxyl-terminal (C-terminal) basic leucine zipper region, which can form dimers with other proteins to perform DNA binding role, and its amino-terminal (N-terminal) has an activation domain and inhibition domain (30, 31). Thus we hypothesized that CEBPB may be involved in CC progression via transcriptional regulation of ATRNL1. As reported previously, CEBPB is implicated in the progression of various cancers, such as gastric cancer, breast cancer and colorectal cancer (32-34). It has been revealed that CEBPB protein abundance presents depletion in cervical squamous cell carcinoma (20), and CEBPB represses early HPV16 gene transcription, thereby affecting CC progression (19). Consistent with previous studies, CEBPB presented a marked depletion in CC tissue and cells and possessed a positive association with ATRNL1 level in CC tissue. Moreover, CEBPB exerted a positive modulation of ATRNL1 protein translation in CC cells. Mechanistically, CEBPB bound to ATRNL1 promoter at gatttcacagt region in CC cells. Furthermore, ATRNL1 depletion rescued the decreased CC cell proliferative and migratory capabilities and EMT under CEBPB upregulation. These findings suggested that CEBPB bound to ATRNL1 promoter to transcriptionally activate ATRNL1, thereby hindering CC cell malignancy and tumorigenesis. Consistent with our finding, Y-L Hu et al have pointed that CEBPB is downregulated in CC and inhibits CC cell proliferation and invasion (35).

There are several limitations in our study. First, the samples of our study were relatively small. Second, the specific mechanism of CEBPB for regulating the transcription of ATRNL1 was unclear. Therefore, our study needs to perform more experiments to perfect our study in the future.

In conclusion, ATRNL1 and CEBPB present depletion and serve as tumor suppressors in CC cells. ATRNL1 suppresses CC cell malignancy through CEBPB activation, providing a potential new direction for seeking therapeutic plans for CC.

Informed consent

The authors report no conflict of interest.

Availability of data and material

We declared that we embedded all data in the manuscript.

Authors' contributions

Minjie Fang, Yayan Zhou, Zihuang Li, Xianming Li

FM conducted the experiments and wrote the paper; ZY and LZ analyzed and organized the data; LX conceived, designed the study and revised the manuscript.

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