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Long non-coding RNA CASC15 promotes proliferation and induces apoptosis of cervical cancer cells through targeting miR-101-3p

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Abstract. – OBJECTIVE: Cervical cancer (CC) is a common type of fatal gynecological cancer worldwide. The aim of this study was to identify the exact role of lncRNA CASC15 in the progression of CC and to explore the possible underlying mechanism.

PATIENTS AND METHODS: Real Time tative Polymerase Chain Reaction (RT-qPC as used to detect CASC15 expression in 4 C l lines, and 54 paired CC tissue samples. The r of CASC15 in CC were explored through apop sis assay, colony formation as l prolife ation assay *in vitro*, respec ermore, the underlying mechanism ASC15 CC was explored by luciferase r er gen and RNA immunoprecipitation as

RESULTS: CASC15 pre CC tissues was remarkably hi than tha diacent normal tissues. The kdown of 5 significantly inhibite proliferati whereas induced cell ptos *vitro*. Meanwhile, CC cell prolife tion was re bly promoted, and cell apop s was inhibit overexpression of CAS *in vitro*. In additi miR-101-3p was up-re ted a down-regulated after knockdo nd ov pression of CASC15 *in vitro*, resp ly rtherm bioinformatics analysis a hanism ays revealed that miR-3p w irec get of CASC15 in CC tu- nesis

CLUSIO CASC15 could promote proife n and inhibit apoptosis of CC cells by t 01-3p. Our findings suggested CASC15 might offer a new therapeutic inter- ntion for CC patients.

Key rds:
Long noncoding RNA, CASC15, Cervical cancer (CC), MiR-101-3p.

Introduction

vical can CC) ranks the fourth among fem les globally. Meanwhile, it is one of st prevalent cancers among Chinese women. Approximately 530,000 patients nosed with CC annually, with 275,000 eat Around 13% of CC patients have already been in advanced stages when first diagnosed. The 5-year survival rate for metastatic CC is 16.5%, accounting for 91.5% for localized CC[2]. Currently, basic therapeutic strategies for early-stage and locally advanced CC patients include surgery, chemotherapy, and radiotherapy. However, due to the heterogeneous manifestations of CC, the treatment for CC patients is limited. Moreover, the prognosis of patients with metastatic CC is very poor, with the median survival time of only 8 to 13 months[3]. Therefore, it is urgent to find out the underlying mechanism and to explore the novel treatment strategies for CC patients.

Long non-coding RNAs (lncRNAs) are a diverse group of transcripts with longer than 200 nucleotides in length and no protein-coding function. It has been reported that lncRNAs are differentially regulated in a variety of biological behaviors, including the progression of malignant tumors. For instance, lncRNA p23154 accelerates metastasis of oral squamous cell carcinoma cells through participating in glycolysis[4]. Through the regulation of miR-34c expression and targeting MUC2, lncRNA AF147447 represses proliferation and invasion of gastric cancer cells infect-

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ed with Helicobacter pylori[5]. Kim et al[6] have confirmed that long noncoding RNA MALAT1 suppresses breast cancer metastasis. The up-regulation of lncRNA LINC01510 is negatively associated with the prognosis of patients with colorectal cancer. Meanwhile, this may offer a potential independent prognostic biomarker for patients[7]. However, the exact function of CASC15 in CC development remains unknown. Therefore, the aim of this report was to investigate the role of SASC15 in CC and the underlying biological mechanism.

In our study, CASC15 was highly expressed in both CC tissues and cell lines. Moreover, further researches revealed that CASC15 regulated CC cell apoptosis and proliferation *via* targeting miR-101-3p.

Patients and Methods

Patients and Cell Lines

CC tissues were obtained from 54 patients who underwent surgery at Hubei Public Security County People's Hospital. All tissues were s d at –80°C for use. The written informed was obtained from each patient before su y. This study was approved by the Ethics Con tee of Hubei Public Security County Peop Hospital.

Cell Lines

Human CC cell lines HeL nd C4-1) and normal ce ica be l ce (NC104) (Chinese T Cultu ection, Chinese Academy c iences, Sh i, China) were cultured cco's Mod Eagle's Medium (DM 1, Hy South Logan, UT, USA) cont ing 10% fe vine serum (FBS; Gibco, P ville, MD, USA penicillin. Besides cells w re maintained in a humidified inc r with CO_2 at 37°C.

Cell Transfection

ntivi x ing short-hairpin RNA (sh A) tar CASC15 was compounded and oned to pGPH1/Neo vector (GenePhar- m China). Subsequently, it was then nsfected into SiHa cells. CASC15 lentivirus s cloned into pGPH1/Neo vector (GenePhar- hanghai, China), and transfected into HeLa cel Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, the CASC15 expression level in transfected cells was performed using Real Time-quantitative Polymerase Chain Reaction (RT-qPCR).

RNA Extraction and RT-qPCR

TRIzol reagent (Invitrogen, sbad, CA, USA) was utilized to isolate tota in tissues and cells. Subsequently, the extracte l RNA was reverse-transcribed int mpleme de oxyribose nucleic acids As) in strict dance with Reverse T scriptio it (TaK Biotechnology Co., alia ina). rimers used for RT-qPC w llows: SC15 primers forwa -CAC ATC AAAC-CCAG-3′, re 5′-GAGGA GCTGTA-AGCC-3′; hyde 3-pho te dehydrogenase (G PDH) ers forward 5′-AGAAG-GCTGGGGCTCATT and reverse 5′-AGG-GG CACAGTCT . The thermal cycle w as follows: 30 sec at 95°C, 5 sec at 95°C for ycles, and 3 c at 60°C.

Cell Proliferation Kit-8 (CCK-8) Assay

Cell p ation of transfected cells in 96-well lates was monitored every 24 h by CCK-8 assay Molecular Technologies, Inc., Kuma- pan). The absorbance at 450 nm was monitored by a spectrophotometer (Thermo Scientific, Rockford, IL, USA).

Colony Formation Assay

1×10^3 CC cells were first seeded into 6-well plates.10 days later, the formed colonies were fixed with 10% formaldehyde for 30 min and stained with 0.5% crystal violet for 5 min. Finally, the number of formed colonies was counted by Nikon camera (Tokyo, Japan).

Flow Cytometric Analysis

Cell apoptosis was detected according to the instructions of Annexin-V-FITC (fluorescein isothiocyanate) apoptosis detection kit (BD, Franklin Lakes, NJ, USA). Briefly, harvested cells were washed twice with ice-cold. 100 µL flow cytometry binding buffer was then added in the cells. After 5 µL Annexin V/FITC and 5 µL Propidium Iodide (PI) were mixed at room temperature, the cells were stained with the mixture for 15 min in the dark. Each tube was added with 400 µL binding buffer. Finally, the apoptosis of cells was analyzed by FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Dual-Luciferase Reporter Assay

For luciferase assay, the 3′-UTR of CASC15 was cloned into the pGL3 vector (Promega, Madison, WI, USA), which was identified as wild-type (WT) 3′-UTR. Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used for the site-directed mutagenesis of the binding site of miR-101-3p in CASC15 3′-UTR, namely mutant (MUT) 3′-UTR. After that, the cells were transfected with WT-3′-UTR or MUT-3′-UTR and miR-ctrl or miR-101-3p for 48 h. Finally, the luciferase assay was conducted by dual luciferase reporter assay system (Promega, Madison, WI, USA).

RNA Immunoprecipitation (RIP) Assay

To confirm the endogenous relationship between CASC15 and miR-101-3p, RIP assay was performed using EZMagna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA). Transfected CC cells were collected and lysed with RIP lysis buffer containing protease inhibitor and RNase inhibitor. Then the cells were incubated with RIP buffer containing magnetic beads coated with 2 antibodies (Millipore, Billerica, MA, US was used as a negative control (input grou ter incubation for 2 h at 4°C, the co-precipi RNAs were isolated and measured by RT-qP analysis.

Statistical Analysis

The Statistical Produ Servi (SPSS) 18.0 (PASW S tisti cago, IL, USA) was for a stical analysis. The Student's *t*-test was used to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

Results

Expression Level of CASC15 in CC Tissues and Cells

Firstly, RT-qPCR w onducted to CASC15 expression i CC nts' tis and 4 CC cell lines res indicat d that CASC15 was sign am gulated C tissues when com d with a nt no tissues (Figure 1A). CASC15 exp CC cells was signifi her than tha NC104 cells as well (F re 1B).

CA romoted t Pr feration of CC Cells

ccording to t expression level of CASC15 in ell lines, Si and HeLa cells were selected f knockdo nd overexpression of CASC15, resp ansfection efficiency was verified by CR (Figure 2A and 2B). CCK-8 ssay showed that the growth ability of CC cells arkably suppressed after CASC15 was do down (Figure 2C). However, the growth ability of CC cells was significantly promoted after CASC15 overexpression (Figure 2D). Similarly, the colony formation assay demonstrated that the number of colonies was significantly reduced *via* knockdown of CASC15 (Figure 2E). However, the number of colonies increased remarkably *via* overexpression of CASC15 (Figure 2F).

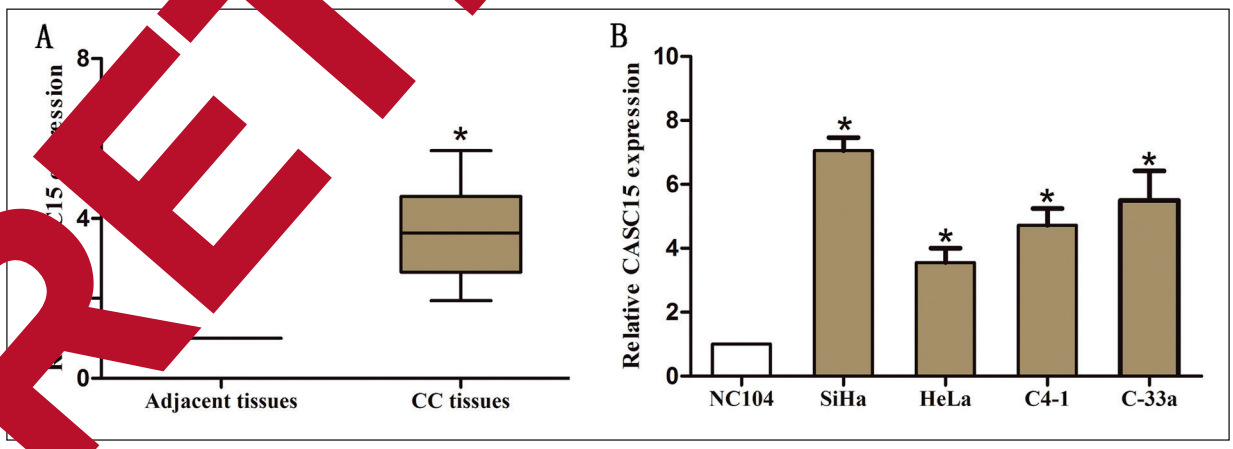

Figure 1. RT-qPCR was used to detect CASC15 expression in CC tissues and cell lines. **A,** CASC15 expression was remarkably higher in CC tissues than in corresponding normal tissues. **B,** CASC15 expression in SiHa, HeLa, C-33a, and C4-1 cells (human CC cell lines) was significantly higher than that of NC104 cells (normal cervical epithelium cell line). *$p<0.05$.

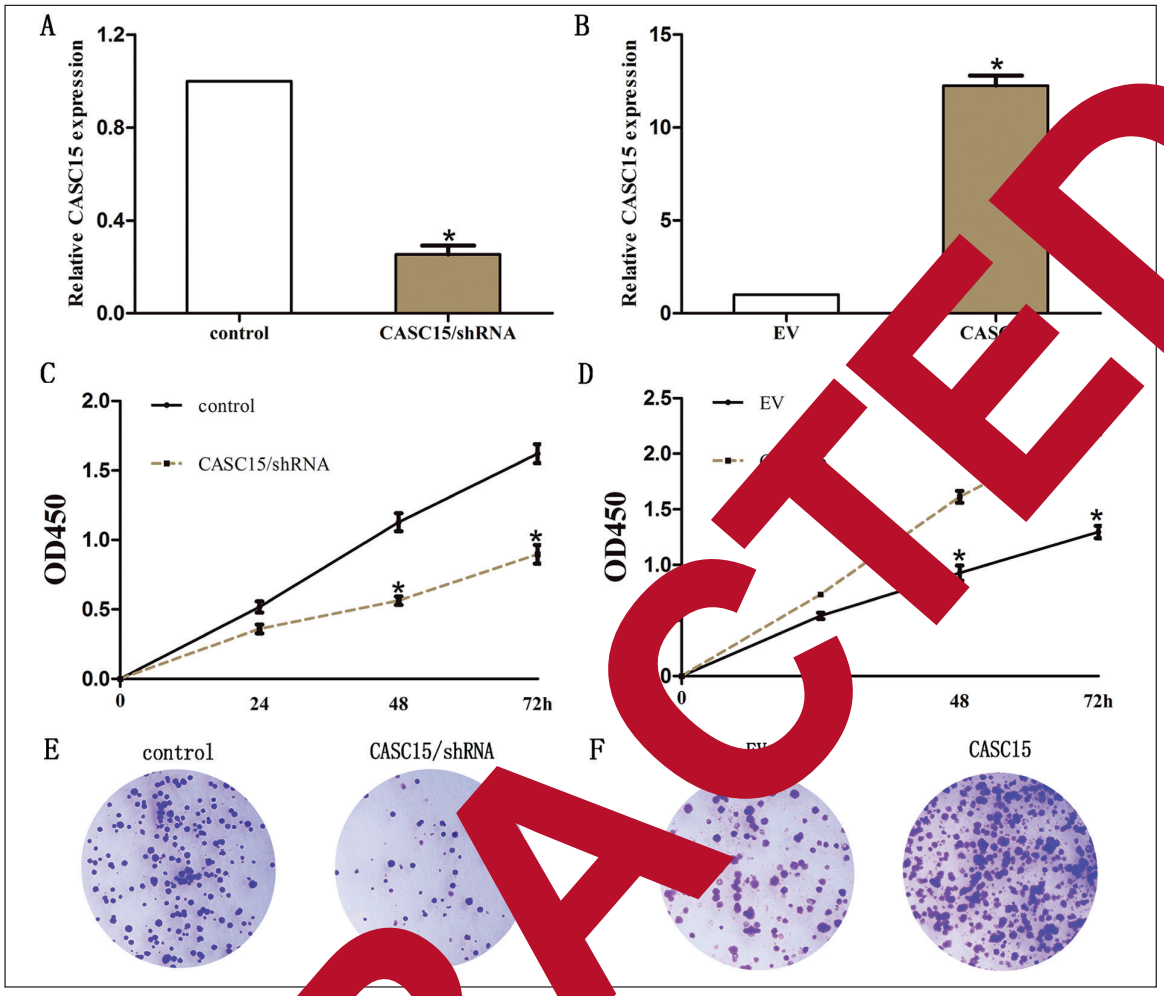

Figure 2. CASC15 promoted cell proliferation. **A,** RT-qPCR detected CASC15 expression in CC cells transfected with CASC15 shRNA (CASC15/shRNA) or negative control). GAPDH was used as an internal control. **B,** RT-qPCR detected CASC15 expression in CC cells transfected with CASC15 lentivirus (CASC15) or empty vector (EV). GAPDH was used as an internal control. **C,** CCK8 assay showed that the silence of CASC15 significantly inhibited the proliferation of CC cells. **D,** CCK8 assay showed that overexpression of CASC15 significantly promoted proliferation of CC cells. **E,** Colony formation assay showed that the number of colonies in CC cells was significantly reduced *via* silence of CASC15 (magnification: 10×). **F,** Colony formation assay showed that the number of colonies in CC cells significantly increased *via* overexpression of CASC15 (magnification: 10×). The results represented the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$.

CASC15 Inhibited the Apoptosis of CC Cells

Flow cytometric analysis was performed to detect the apoptosis rate of transfected CC cells. The results revealed that the apoptosis rate of SiHa cells significantly increased after CASC15 was knocked down (Figure 3A). However, the apoptosis of HeLa cells was significantly reduced after CASC15 overexpression (Figure 3B).

CASC15 Promoted CC Tumorigenesis via miR-101-3p

MiRNAs containing complementary bases with CASC15 were predicted by conducting DIANA LncBASE Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted). As a tumor suppressor gene, miR-101-3p was predicted as a target miRNA of CASC15 in CC (Figure 4A). A significantly higher expression of miR-101-3p was observed in CC cells of CASC15 shRNA group than that of the control group (Figure 4B). However, a lowly expressed miR-101-3p was indicated in CC cells of CASC15 lentivirus group when compared with the empty vector group (Figure 4C). Subsequent luciferase assay showed that co-transfection of miR-101-3p and CASC15-

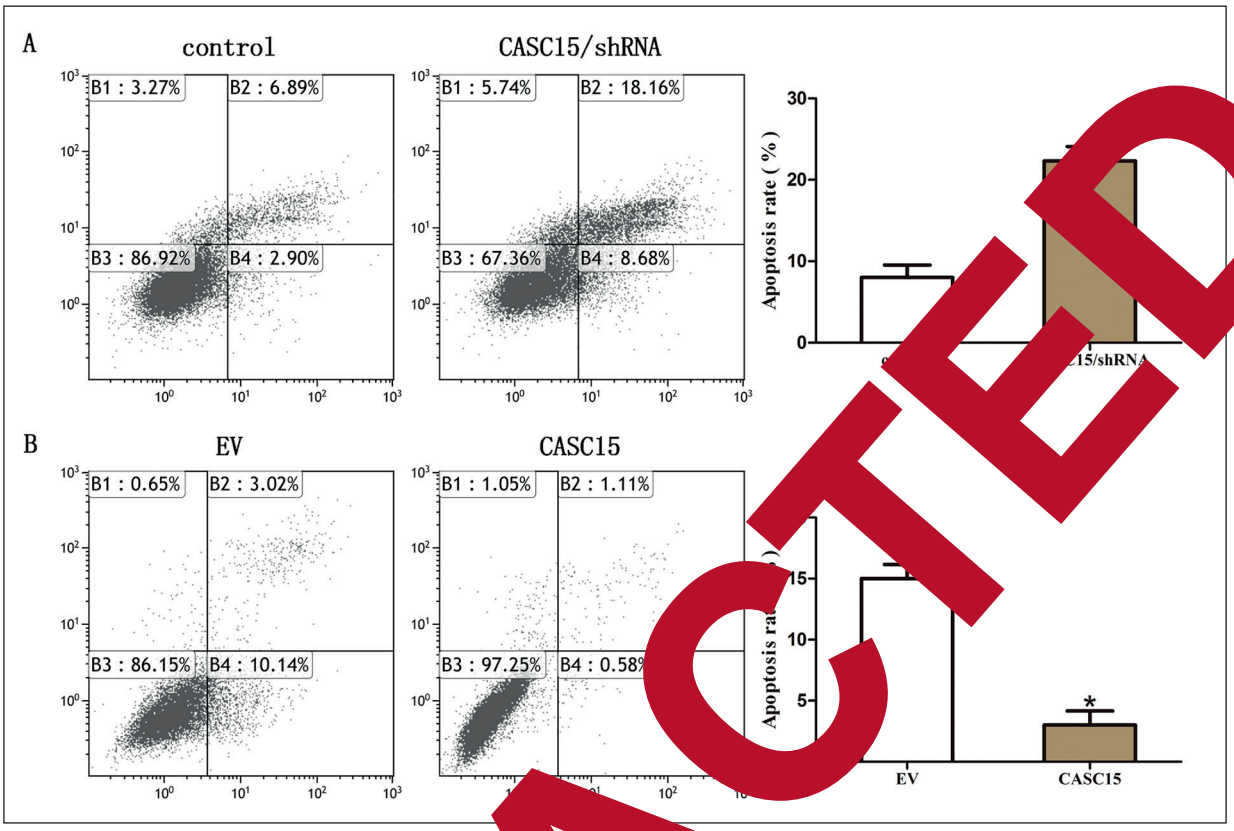

Figure 3. CASC15 inhibited CC cell apoptosis. **A,** F…ytom…ssay revealed that the apoptosis rate of CC cells significantly increased *via* silence of CASC15. **B,** Flow …analysis assay revealed that the apoptosis rate of CC cells remarkably decreased *via* overexpression of CASC15. …ts represented the average of three independent experiments (mean ± standard error of the mean). *…

WT remarkably depress… lucife… However, no significa… ch… in luciferase activity… co-t… tion of miR-101-3p and CASC… MUT (Figu…). The results of the RIP… monstrated …ASC15 and miR-101… were… ificantly enriched in Ago2-cont…ing beads …pared with input group (F… e 4E).

Discussion

…ently, …sing number of lncRNAs ha… een di…ed for their critical roles in reg…ing the d…velopment of cancers, includ-in… been already found that lncRNA …G3 serves as a tumor suppressor gene and in-…its the growth in CC by regulating miR-21-5p[8]. …NA WT1-AS inhibits the aggressiveness of cer…l cancer cells *via* regulating p53 expression by sponging miR-330-5p[9]. LncRNA DANCR promotes cervical cancer progression by up-regulating ROCK1 *via* sponging miR-335-5p[10]. In addition, overexpression of lncRNA NNT-AS1 facilitates the proliferation and invasion of CC cells *via* Wnt/β-catenin signaling pathway[11].

Cancer susceptibility candidate 15 (CASC15), also known as LINC00340, was initially identified in silico as an active lncRNA. CASC15 is located on chromosome 6p22.3. Lessard et al[12] have indicated that the overexpression of CASC15 promotes metastatic progression and phenotype switching of melanoma. By regulating the expression of SOX4, CASC15 has been found to participate in RUNX1-rearranged acute leukemia[13]. CASC15 enhances the metastasis and hepato-carcinogenesis of hepatocellular carcinoma (HCC). Meanwhile, CASC15 is correlated with poor prognosis of HCC patients[14]. In addition, CASC15 regulates gastric cancer cell proliferation, migration, and epithelial-mesenchymal transition (EMT) by targeting CDKN1A and ZEB1[15]. However, the biological role of CASC15 in the tumorigenesis of CC remains unclear.

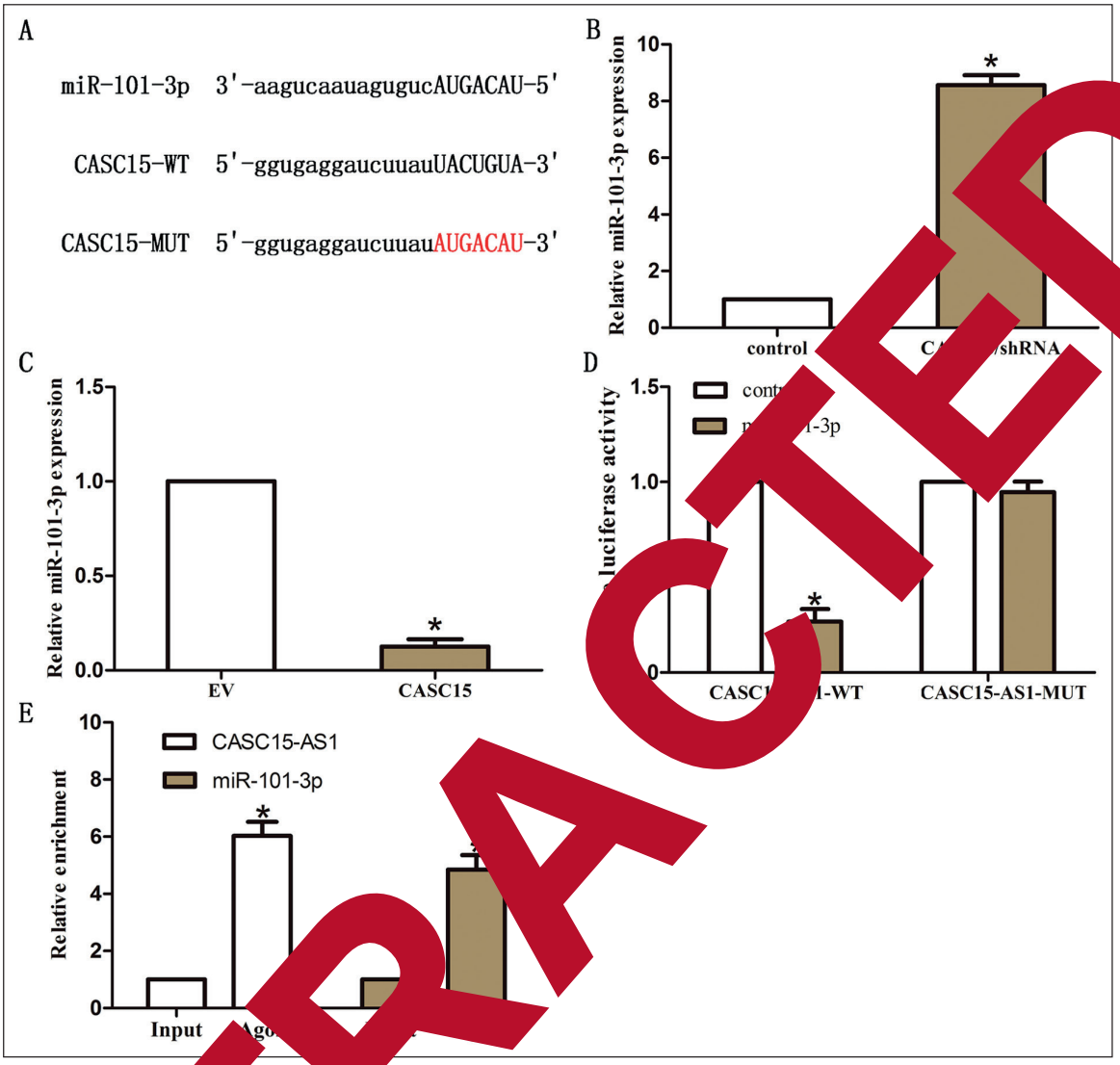

Figure 4. MiR-101-3p expression was [illegible]ted by the silence of CASC15 in CC. **A,** The binding sites of miR-101-3p on CASC15. **B,** MiR-101-3p expression signifi[illegible]increased in CASC15/shRNA group compared with control group. **C,** MiR-101-3p expressio[illegible]decreased in CASC15 group compared with EV group. **D,** Co-transfection of miR-101-3p and CASC15-WT strongly decre[illegible]iferase activity, while co-transfection of miR-control and CASC15-WT did not change the luciferase a[illegible]. **E,** RIP assay [illegible]d the enrichment of CASC15 and miR-101-3p in Ago2-containing beads compared with input gro[illegible]e results represente[illegible]erage of three independent experiments. The data were presented as mean ± standard error o[illegible]ean. *$p < 0.05$, as compar[illegible]d with control cells.

In th[illegible]y, the [illegible]ts showed that CASC15 signifi[illegible]gulated in both CC tissues an[illegible]ls. Aft[illegible]SC15 was knocked down, CC cell[illegible]oliferation was suppressed, whereas cell a[illegible]omoted. Similarly, the overexpression of CASC15 showed an opposite effect on CC cell proliferation and apoptosis. All these results indicated that CASC15 acted as an oncogene in CC by regulating cell proliferation and apoptosis.

An increasing number of studies have proved evidence that lncRNAs function as molecular sponges or ceRNAs in regulating miRNA expression and biological function. Scholars have identified the aberrant expression of miR-101-3p and its targeted genes in cancers. By serving as a ceRNA of miR-101-3p, lncRNA SPRY4-IT1 enhances the proliferation and invasion of colorectal cancer cells[16]. Moreover, by sponging miR-101-3p, lncRNA SPRY4-IT1 functions as an oncogene in bladder cancer by up-regulating EZH2[17]. By silencing p27 and sponging miR-101-3p, lncRNA SNHG6 facilitates the invasion and proliferation

of gastric cancer cells through EMT[18]. By targeting MALAT-1, miR-101-3p depresses tumorigenesis of non-small cell lung cancer by blocking PI3K/AKT signal pathway[19].

Our work showed that miR-101-3p was up-regulated after the knockdown of CASC15, while miR-101-3p was down-regulated after overexpression of CASC15 *in vitro*. Bio-informative analysis and functional experiments indicated that miR-101-3p could directly bind to CASC15. Our findings suggested that CASC15 realized its function in CC development by targeting miR-101-3p.

Conclusions

The results of this study indicated that CASC15 promotes proliferation and inhibits apoptosis of CC cells by targeting miR-101-3p. Our findings suggested that CASC15 might offer a new therapeutic intervention for CC patients.

Conflict of Interest

The Authors declare that they have no conflict of in[illegible]ts.

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