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Upregulated lncRNA CACNA1G-AS1 aggravates the progression of colorectal cancer by downregulating p53

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Abstract. – OBJECTIVE: To investigate the role of long non-coding RNA (lncRNA) CACNA1G-AS1 in regulating proliferative and invasive abilities of colorectal cancer (CRC) cells by mediating p53, thus influencing the progression of CRC.

PATIENTS AND METHODS: CACNA1G-AS1 level in CRC tissues and adjacent normal tissues was first determined. Its level in CRC patients with different tumor stages was detected as well. Changes in proliferative and invasive abilities of HCT116 and SW480 cells influenced by CACNA1G-AS1 were evaluated. Subcellular distribution of CACNA1G-AS1 was analyzed. Through Western blot, RNA immunoprecipitation (RIP), and chromatin immunoprecipitation (ChIP) assay, the interaction between CACNA1G-AS1 and EZH2 was assessed. The biological function of the target gene of CACNA1G-AS1 was finally explored.

RESULTS: CACNA1G-AS1 was upregulated in CRC tissues compared to adjacent normal ones. Its level remained higher in CRC patients with stage III-IV compared to those with stage I-II. Knockdown of CACNA1G-AS1 reduced proliferative and invasive abilities of HTC116 and SW480 cells. CACNA1G-AS1 was mainly distributed in the nucleus. Moreover, CACNA1G-AS1 was verified to interact with EZH2. Knockdown of CACNA1G-AS1 or EZH2 upregulated p53 level and decreased the recruitment ability of EZH2 on p53. Finally, p53 knockdown could partially reverse the regulatory effect of CACNA1G-AS1 on the proliferative ability of HCT116 cells.

CONCLUSIONS: CACNA1G-AS1 downregulates p53 level by forming a carcinogenic complex with EZH2, thereby enhancing the proliferative and invasive abilities of CRC cells.

Key Words:
Colorectal cancer, CACNA1G-AS1, EZH2, P53.

Introduction

Colorectal cancer (CRC) is a common malignant tumor of the digestive tract, which is a serious threat to human health. It is reported[1-3] that the global incidence and mortality of CRC rank third, which are still on the rise each year. Although great strides on diagnosis, chemotherapy, targeted drugs, and biological therapy of CRC have been made, the 5-year survival of advanced CRC patients is very low[4]. Diagnosis and treatment of CRC as early as possible could remarkably improve the clinical outcome[5-7]. Hence, searching for diagnostic strategies and molecular hallmarks of CRC is well concerned.

Non-coding RNA (ncRNA) is widely expressed in organisms as a research hotspot. Based on the size of ncRNA, it is divided into long non-coding RNA (lncRNA) and short ncRNA (i.e. miRNA, siRNA, and piRNA)[8-10]. LncRNAs are 200-1000 kb nucleotides long and could not encode proteins due to the lack of an open reading frame. They were initially thought to be the "noise" of genomic transcription without any biological functions[11,12]. Later, diverse functions of lncRNAs have been discovered in the regulation of multiple biological processes[13-16]. Recent studies[10] have identified the crucial role of lncRNA in tumorigenesis and tumor progression. For example, overexpression of lncRNA MALAT1 stimulates the metastasis of NSCLC. HOTAIR aggravates several types of tumors by interacting with PCR2 and LSD1[8,17,18]. These findings provide new directions that lncRNA may be utilized as promising targets in tumor treatment.

CACNA1G-AS1 was first reported in 2015. By analyzing lncRNA expression profiles, CACNA1G-AS1 is validated to be important in keloid

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formation[19]. Li et al[20] proved that CACNA1G-AS1 is an oncogene in NSCLC. It accelerates the invasiveness and metastasis of NSCLC cells by up-regulating HNRNPA2B1, which provides a novel target for NSCLC prevention and treatment. In 2019, Yang et al[21] reported that CACNA1G-AS1 promotes the progression of nodular liver cancer by regulating the miR-2392/C1orf61 pathway. In this paper, we mainly explored the biological function of CACNA1G-AS1 in the malignant progression of CRC and the potential mechanism.

Patients and Methods

Baseline Characteristics

A total of 30 paired CRC tissues and adjacent normal tissues were surgically harvested from 30 CRC patients admitted in The Second Hospital of Jilin University from December 2016 to October 2018. Tumor node metastasis (TNM) staging of enrolled CRC patients was recorded. None of these patients were preoperatively treated. This research was approved by the Ethics Committee of The Second Hospital of Jilin University. Signed written informed consents were obtained from all participants before the study.

Cell Culture and Transfection

CRC cell lines and colonic epithelial cell line provided by American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C. Cells in the logarithmic growth phase were selected for transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The fresh medium was replaced at 24 h. Sequences of transfection vectors were as follows: sh-CACNA1G-AS1 1#: 5'-AUGACGACCCUCCCCTGACC-3'; sh-CACNA1G-AS1 2#: 5'-UUGUUGGCCGGAGCACUAAU-3'; sh-CACNA1G-AS1 3#: 5'-GATAG AUUUGGGGAAGGACUU-3'; sh-EZH2: 5'-GAGGUUCAGACGAGCUGAUU-3'; sh-p53: 5'-GCUGCUCAGAUAGCGA UGGU-3'.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNA extraction was performed using the TRIzol method (Invitrogen, Carlsbad, CA, USA). The extracted RNA was quantified and reversely transcribed into complementary deoxyribose nucleic acid (cDNA), followed by PCR using the SYBR Green method (TaKaRa, Otsu, Shiga, Japan). Primer sequences were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5'-AGAAGGCTGGGGCTCATTTG-3', R: 5'-AGGGGCCATCCACAGTCTTC-3'; CACNA1G-AS1: F: 5'-CGTCCAGCTGCGAGCCAGC-3', R: 5'-AGCCTTCCTGTGACCTCATC-3'.

5-Ethynyl-2′- Deoxyuridine (EdU) Assay

Cells were seeded in the 96-well plate with 300 cells per well. Cells were labeled with 50 μmol/L EdU at 37°C for 2 h. After 30-min fixation in 4% paraformaldehyde, cells were incubated with phosphate-buffered saline (PBS) containing 0.5% Triton-100 for 20 min. After washing with PBS containing 3% bovine serum albumin (BSA), 100 μL of the dying solution was applied per well for 1-h incubation in dark and cells were counter-stained with 1×Hoechst 33342 for 30 min. EdU-positive cells were captured under a microscope (magnification 100×).

Transwell

The cell density was adjusted to 1×10^5/ml. 100 μL of suspension was applied in the upper side of transwell chamber (Millipore, Billerica, MA, USA) pre-coated with 100 μl of diluted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). In the bottom side, 600 μL of medium containing 10% FBS was applied. After 48 h of incubation, invasive cells were fixed in methanol for 30 min and dyed with 0.1% crystal violet for 10 min. Invasive cells in 6 randomly selected fields per sample were captured.

Cell Counting Kit-8 (CCK-8)

Cells were seeded in the 96-well plate with 1×10^4 cells per well. Absorbance (A) at 450 nm was recorded at the established time points using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

Determination of Subcellular Distribution

Cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR. U6 was the internal reference of the nucleus and GAPDH was the one of cytoplasm.

Western Blot

The total protein was extracted from cells or tissues using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 h, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image-Pro Plus (Media Cybernetics, Silver Springs, MD, USA).

RNA Immunoprecipitation (RIP)

Cells were treated according to the procedures of Millipore Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Promega, Madison, WI, USA). The cell lysate was incubated with anti-EZH2, or anti-IgG antibody at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/ml proteinase K containing 0.1% sodium dodecyl sulphate (SDS) to extract RNA. The magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. Finally, the extracted RNA was subjected to mRNA level determination using qRT-PCR.

Chromatin Immunoprecipitation (ChIP)

Cells were subjected to 10-min cross-link with 1% formaldehyde at room temperature. Subsequently, cells were lysed using lysis buffer and sonicated for 30 min. Finally, the sonicated lysate was immunoprecipitated with anti-EZH2, anti-H3K27me3 or anti-IgG.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

Upregulation of CACNA1G-AS1 in CRC

Relative to adjacent normal tissues, CACNA1G-AS1 was upregulated in CRC tissues (Figure 1A). According to tumor staging, CRC patients in stage III-IV presented a higher level of CACNA1G-AS1 than those in stage I-II (Figure 1B). Identically, CACNA1G-AS1 was highly expressed in CRC cell lines relative to that of the colonic epithelial cell line (Figure 1C). It is speculated that CACNA1G-AS1 was involved in the progression of CRC.

CACNA1G-AS1 Accelerated CRC Cells to Proliferate and Invade

To further analyze the potential function of CACNA1G-AS1 in CRC, we selected HCT116 and SW480 cells to perform *in vitro* experiments. Three shRNAs targeting CACNA1G-AS1 were constructed and their transfection efficacy was tested. The latter two (sh-CACNA1G-AS1 2# and sh-CACNA1G-AS1 3#) presented great transfection efficacy in HCT116 and SW480 cells (Figure 2A). The viability in HCT116 and SW480 cells at 48, 72 and 96 h was marked-

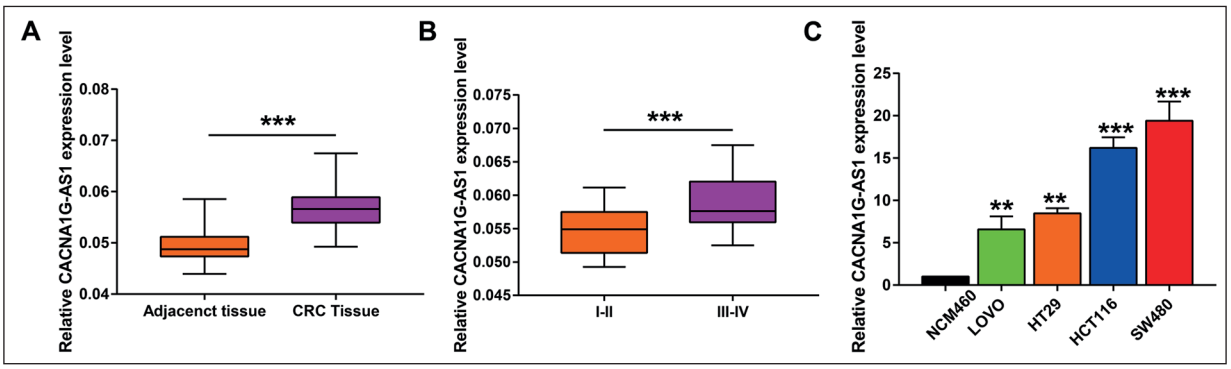

Figure 1. Upregulation of CACNA1G-AS1 in CRC. **A,** Relative level of CACNA1G-AS1 in adjacent normal tissues and CRC tissues. **B,** Relative level of CACNA1G-AS1 in CRC patients with stage III-IV and stage I-II. **C,** Relative level of CACNA1G-AS1 in CRC cell lines and colonic epithelial cell line.

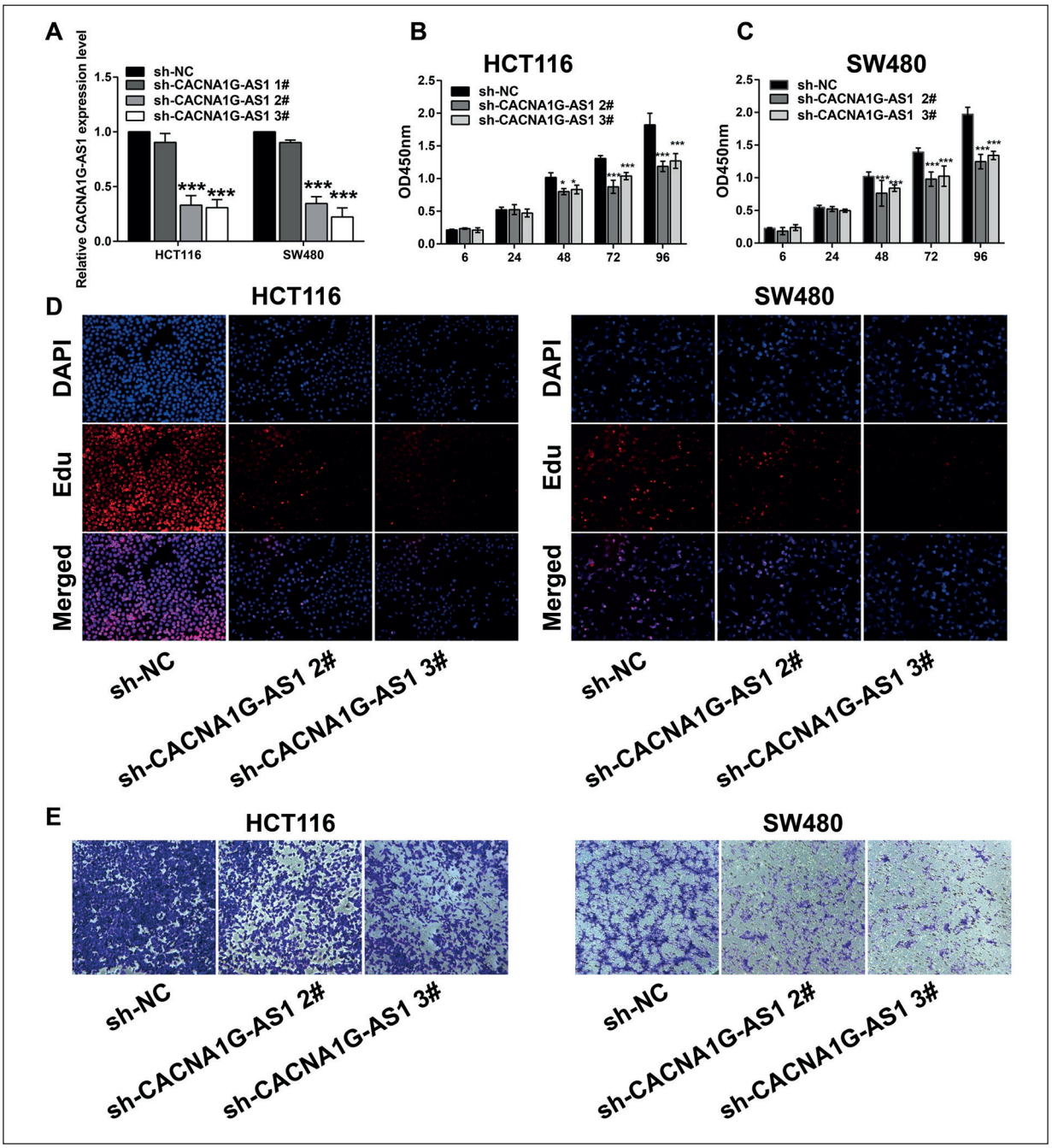

Figure 2. CACNA1G-AS1 accelerated CRC cells to proliferate and invade. **A,** Transfection efficacy of sh-CACNA1G-AS1 1#, sh-CACNA1G-AS1 2# and sh-CACNA1G-AS1 3# in HCT116 and SW480 cells. **B,** CCK-8 assay showed the viability in HCT116 cells transfected with sh-NC, sh-CACNA1G-AS1 2# or sh-CACNA1G-AS1 3#. **C,** CCK-8 assay showed the viability in SW480 cells transfected with sh-NC, sh-CACNA1G-AS1 2# or sh-CACNA1G-AS1 3#. **D,** EdU assay showed DAPI-labeled (blue), EdU-labeled (red) and merged cells in HCT116 and SW480 cells transfected with sh-NC, sh-CACNA1G-AS1 2# or sh-CACNA1G-AS1 3# (magnification: 100×). **E,** Transwell assay showed invasion in HCT116 and SW480 cells transfected with sh-NC, sh-CACNA1G-AS1 2# or sh-CACNA1G-AS1 3# (magnification: 40×).

ly reduced after the transfection of sh-CACNA1G-AS1 2# or sh-CACNA1G-AS1 3# (Figure 2B and C). Similarly, EdU assay revealed fewer EdU-positive cells after transfection of sh-CACNA1G-AS1 2# or sh-CACNA1G-AS1 3# in CRC cells (Figure 2D). The transwell assay revealed attenuated invasive abilities in HCT116 and SW480 cells transfected with sh-

CACNA1G-AS1 2# or sh-CACNA1G-AS1 3# (Figure 2E). We concluded that the knockdown of CACNA1G-AS1 in CRC cells attenuated their proliferative and invasive abilities.

CACNA1G-AS1 Upregulated p53 by Recruiting EZH2

Subcellular distribution analysis illustrated that CACNA1G-AS1 was mainly distributed in the nucleus of HCT116 cells (Figure 3A). Transfection of sh-CACNA1G-AS1 2# or sh-CACNA1G-AS1 3# remarkably upregulated the protein level of p53 in HCT116 and SW480 cells (Figure 3B and C). Moreover, the RIP assay revealed higher enrichment of CACNA1G-AS1 in anti-EZH2 relative to anti-IgG, suggesting the interaction between CACNA1G-AS1 and EZH2 (Figure 3D). Notably, the protein level of p53 was markedly upregulated by the transfection of sh-EZH2 (Figure 3E). Furthermore, ChIP assay showed that the immunoprecipitants of anti-EZH2 and anti-H3K27me3 were reduced after transfection of sh-CACNA1G-AS1 2#, indicating that EZH2 was recruited by CACNA1G-AS1 (Figure 3F). Collectively, the knockdown of CACNA1G-AS1 attenuated the recruitment ability of EZH2 on p53 and thus upregulated p53 alleviated the progression of CRC.

P53 Knockdown Partially Reversed the Regulatory Effect of CACNA1G-AS1 on CRC Cells

To further identify the role of p53 in the progression of CRC, we constructed sh-p53 and tested its transfection efficacy in HCT116 cells (Figure 4A). Of note, the decreased number of EdU-positive cells in HCT116 cells transfected with sh-CACNA1G-AS1 2# was partially re-

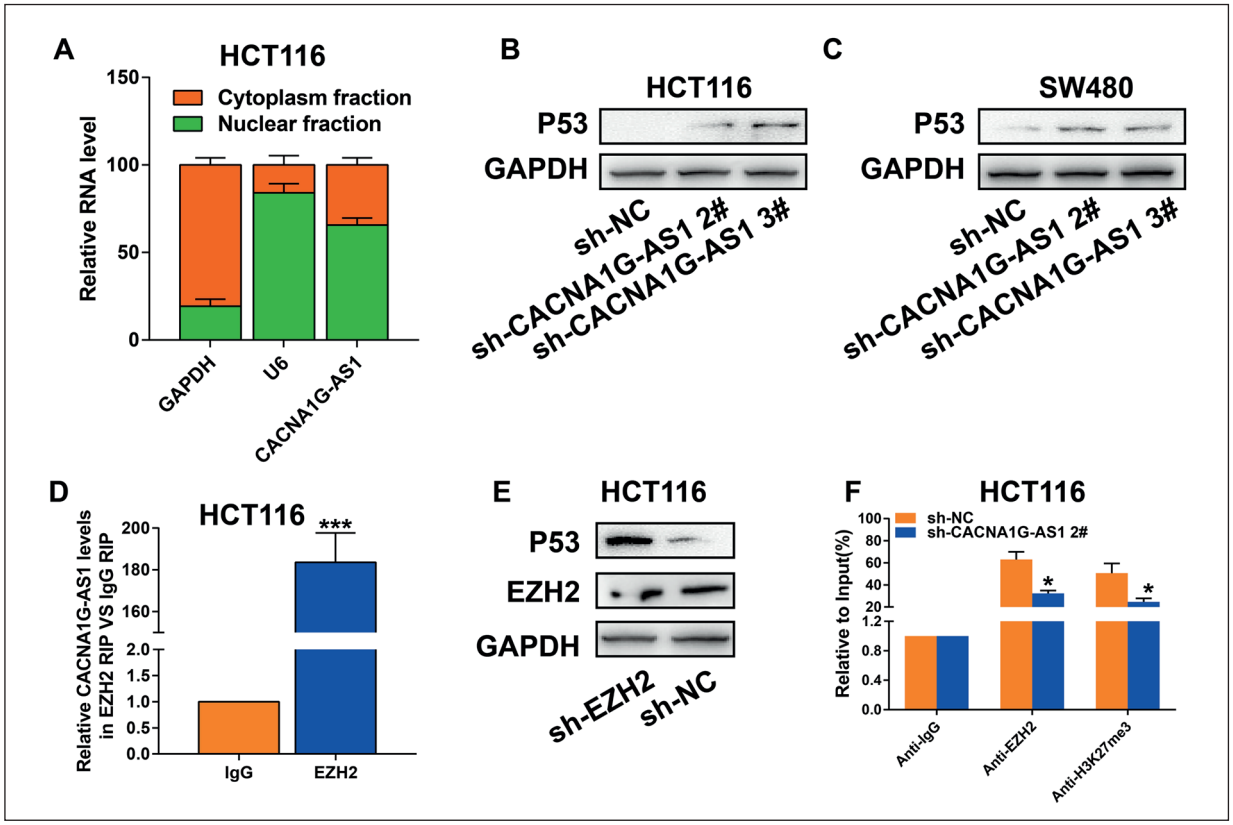

Figure 3. CACNA1G-AS1 upregulated p53 by recruiting EZH2. **A,** Subcellular distribution of CACNA1G-AS1 in cytoplasm fraction and nuclear fraction. GAPDH and U6 were served as cytoplasmic and nuclear internal reference, respectively. **B,** Protein level of p53 in HCT116 cells transfected with sh-NC, sh-CACNA1G-AS1 2# or sh-CACNA1G-AS1 3#. **C,** Protein level of p53 in SW480 cells transfected with sh-NC, sh-CACNA1G-AS1 2# or sh-CACNA1G-AS1 3#. **D,** RIP assay showed enrichment of CACNA1G-AS1 in anti-IgG and anti-EZH2. **E,** Protein levels of p53 and EZH2 in HCT116 cells transfected with sh-NC or sh-EZH2. **F,** ChIP assay showed relative immunoprecipitant of HCT116 cells transfected with sh-NC or sh-CACNA1G-AS1 2# in anti-IgG, anti-EZH2, and anti-H3K27me3.

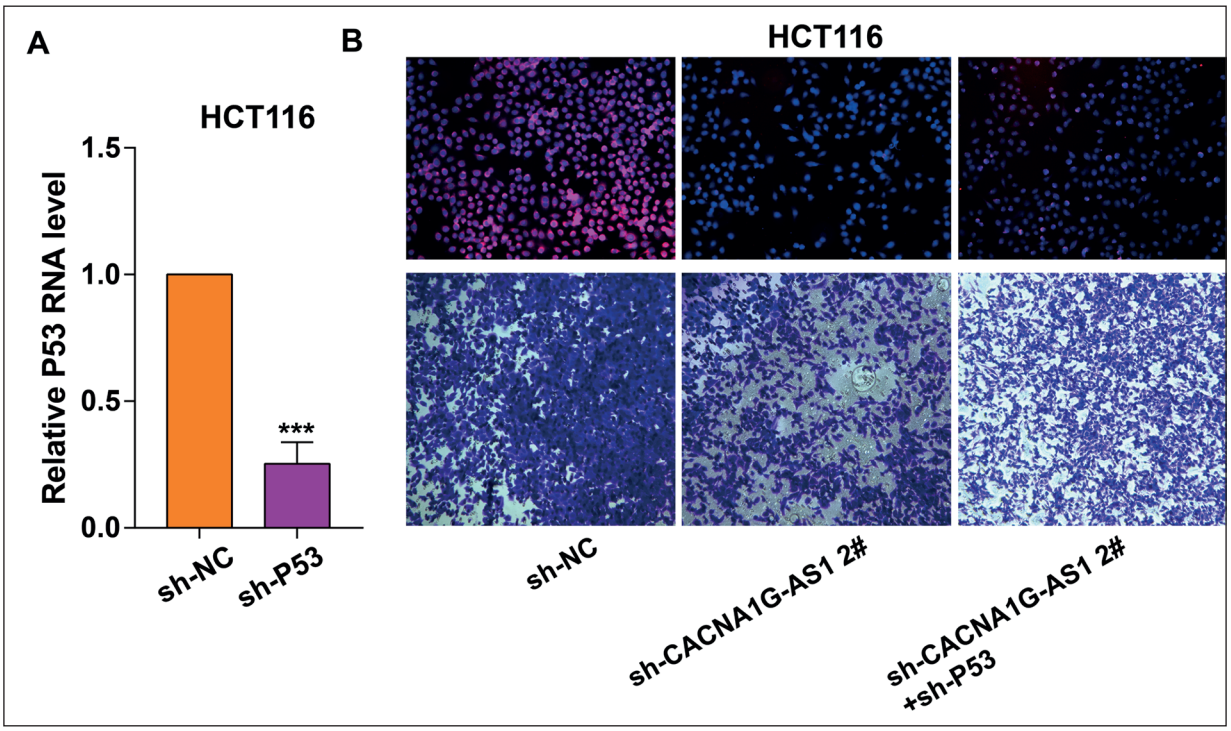

Figure 4. P53 knockdown partially reversed the regulatory effect of CACNA1G-AS1 on CRC cells. **A,** Transfection efficacy of sh-p53 in HCT116 cells. **B,** EdU assay showed DAPI-labeled (blue), EdU-labeled (red) and merged cells in HCT116 cells transfected with sh-NC, sh-CACNA1G-AS1 2# or sh-CACNA1G-AS1 2#+sh-p53 (magnification: 40×).

versed after co-transfection of sh-p53 (Figure 4B). It is suggested that CACNA1G-AS1 mediated the cellular behaviors of CRC cells by regulating p53 level.

Discussion

With the further explorations on lncRNAs, they are identified to participate in epigenetic regulation, chromatin modification, transcriptional activation, etc.[13-16]. LncRNAs also exert vital functions in the occurrence and progression of tumor diseases[8,17]. Differentially expressed lncRNAs in tumor cells are discovered through sequencing technology. These lncRNAs may be potential tumor hallmarks for improving the therapeutic efficacy[22]. In recent years, CACNA1G-AS1 is found to be abnormally expressed in several types of tumors[19-21]. This work first examined the expression pattern of CACNA1G-AS1 in CRC. Subsequently, its regulatory effect on cellular behaviors of CRC was specifically assessed. As the data showed, CACNA1G-AS1 was upregulated in CRC tissues and cell lines. The knockdown of CACNA1G-AS1 attenuated the proliferative and invasive abilities of CRC cells.

As an important tumor suppressor, p53 contributes to induce tumor cell apoptosis, maintain genomic stability, and inhibit tumor angiogenesis[23,24]. Over 50% of malignant tumors present p53 mutations[23,25]. We speculated whether CACNA1G-AS1 was capable of influencing p53 level in CRC cells. As expected, CACNA1G-AS1 or EZH2 knockdown upregulated p53 level in CRC cells. Notably, the knockdown of p53 partially reversed the inhibited proliferative ability in HCT116 cells with CACNA1G-AS1 knockdown. Collectively, CACNA1G-AS1 co-regulated p53 level alongside with EZH2, thus aggravating the malignant progression of CRC. Our conclusions provide a promising target for clinical treatment of CRC.

Conclusions

LncRNA CACNA1G-AS1 downregulates p53 level by forming a carcinogenic complex with EZH2, thereby enhancing the proliferative and invasive abilities of CRC cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Moore JS, Aulet TH. Colorectal cancer screening. Surg Clin North Am 2017; 97: 487-502.
2) Sopik V, Phelan C, Cybulski C, Narod SA. BRCA1 and BRCA2 mutations and the risk for colorectal cancer. Clin Genet 2015; 87: 411-418.
3) Thomas J, Ohtsuka M, Pichler M, Ling H. MicroRNAs: clinical relevance in colorectal cancer. Int J Mol Sci 2015; 16: 28063-28076.
4) Roncucci L, Mariani F. Prevention of colorectal cancer: how many tools do we have in our basket? Eur J Intern Med 2015; 26: 752-756.
5) Chang L, Chang M, Chang HM, Chang F. Expending role of microsatellite instability in diagnosis and treatment of colorectal cancers. J Gastrointest Cancer 2017; 48: 305-313.
6) Faury S, Koleck M, Foucaud J, M'Bailara K, Quintard B. Patient education interventions for colorectal cancer patients with stoma: a systematic review. Patient Educ Couns 2017; 100: 1807-1819.
7) Yue B, Qiu S, Zhao S, Liu C, Zhang D, Yu F, Peng Z, Yan D. LncRNA-ATB mediated E-cadherin repression promotes the progression of colon cancer and predicts poor prognosis. J Gastroenterol Hepatol 2016; 31: 595-603.
8) Hu X, Sood AK, Dang CV, Zhang L. The role of long noncoding RNAs in cancer: the dark matter matters. Curr Opin Genet Dev 2018; 48: 8-15.
9) Kung JT, Colognori D, Lee JT. Long noncoding RNAs: past, present, and future. Genetics 2013; 193: 651-669.
10) Sun T. Long noncoding RNAs act as regulators of autophagy in cancer. Pharmacol Res 2018; 129: 151-155.
11) Kim TK, Hemberg M, Gray JM. Enhancer RNAs: a class of long noncoding RNAs synthesized at enhancers. Cold Spring Harb Perspect Biol 2015; 7: a018622.
12) Schmitz SU, Grote P, Herrmann BG. Mechanisms of long noncoding RNA function in development and disease. Cell Mol Life Sci 2016; 73: 2491-2509.
13) Sun W, Yang Y, Xu C, Guo J. Regulatory mechanisms of long noncoding RNAs on gene expression in cancers. Cancer Genet 2017; 216-217: 105-110.
14) Tang Y, Cheung BB, Atmadibrata B, Marshall GM, Dinger ME, Liu PY, Liu T. The regulatory role of long noncoding RNAs in cancer. Cancer Lett 2017; 391: 12-19.
15) Yu B, Shan G. Functions of long noncoding RNAs in the nucleus. Nucleus 2016; 7: 155-166.
16) Zhou JF, Wang HG, Ma TH, Yan W, Wu SN, Shi YT, Yang XZ. Long noncoding RNA LINC01510 is highly expressed in colorectal cancer and predicts favorable prognosis. Eur Rev Med Pharmacol Sci 2018; 22: 7710-7715.
17) Bhan A, Soleimani M, Mandal SS. Long noncoding RNA and cancer: a new paradigm. Cancer Res 2017; 77: 3965-3981.
18) Bolha L, Ravnik-Glavac M, Glavac D. Long noncoding RNAs as biomarkers in cancer. Dis Markers 2017; 2017: 7243968.
19) Liang X, Ma L, Long X, Wang X. LncRNA expression profiles and validation in keloid and normal skin tissue. Int J Oncol 2015; 47: 1829-1838.
20) Li Y, Liang X, Wang P, Long X, Wang X, Meng Z. Long non-coding RNA CACNA1G-AS1 promotes calcium channel protein expression and positively affects human keloid fibroblast migration. Oncol Lett 2018; 16: 891-897.
21) Yang J, Li C, Li H, E C. LncRNA CACNA1G-AS1 facilitates hepatocellular carcinoma progression through the miR-2392/C1orf61 pathway. J Cell Physiol 2019: Mar 25. doi: 10.1002/jcp.28477. [Epub ahead of print]
22) Feng Y, Zhang Y, Ying C, Wang D, Du C. Nanopore-based fourth-generation DNA sequencing technology. Genomics Proteomics Bioinformatics 2015; 13: 4-16.
23) Bykov VJ, Zhang Q, Zhang M, Ceder S, Abrahmsen L, Wiman KG. Targeting of mutant p53 and the cellular redox balance by APR-246 as a strategy for efficient cancer therapy. Front Oncol 2016; 6: 21.
24) Sullivan KD, Galbraith MD, Andrysik Z, Espinosa JM. Mechanisms of transcriptional regulation by p53. Cell Death Differ 2018; 25: 133-143.
25) Prokocimer M, Molchadsky A, Rotter V. Dysfunctional diversity of p53 proteins in adult acute myeloid leukemia: projections on diagnostic workup and therapy. Blood 2017; 130: 699-712.