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Long non-coding RNA OR3A4 facilitates cell proliferation and migration in colorectal cancer through the Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: Colorectal cancer (CRC) remains one of the most ordinary cancers worldwide. Recently, researches have suggested the important role of long noncoding RNAs (lncRNAs) in the progression of tumorigenesis. This study aims to identify how lncRNA OR3A4 functions in the development of CRC.

PATIENTS AND METHODS: OR3A4 expressions in 54 paired CRC tissues and CRC cell lines were detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). Moreover, the *in vitro* functions of OR3A4 in CRC were identified by performing proliferation assay, wound healing assay, and transwell assay. Besides, the underlying mechanism of OR3A4 in CRC development was explored through Western blot and RT-qPCR.

RESULTS: OR3A4 expression was significantly higher in CRC tissues than in adjacent normal ones. Cell proliferation, migration, and CRC were inhibited after OR3A4 was knocked down *in vitro*, which were promoted after upregulation of OR3A4. Moreover, OR3A4 could activate the Wnt/β-catenin pathway, thus influencing phenotypes of CRC cells.

CONCLUSIONS: OR3A4 enhances CRC cell proliferation and migration by activating the Wnt/β-catenin signaling pathway.

Key words:
long non-coding RNA OR3A4, Colorectal cancer, Wnt/β-catenin signaling pathway.

Introduction

Colorectal cancer (CRC) is the 3rd most prevalent malignancy in the world, and the 4th common reason of tumor-related death[1]. Moreover, the incidence and fatality rate of CRC have been rising every year. Various factors contribute to the development of CRC, including hereditary factors, smoking, drinking, high-fat diet, etc. It is estimated that 140,000 new cases were diagnosed of CRC in America in 2018 and over 50,000 CRC patients died in the same year, accounting for 8% of all cancer-related deaths[2]. About half of CRC patients eventually die of distant metastasis, especially metastasis. With the improved immunotherapy and gene therapy, the mortality of CRC has been reduced to a certain extent. However, 20%-40% of CRC patients experience distant metastasis at the initial diagnosis. The 5-year survival rate of postoperative CRC cases about 50%[3,4]. Therefore, it is crucial to further clarify the pathogenesis of CRC, thus improving efficacies of diagnosis and treatment of CRC.

In past several decades, numerous epigenetic changes and/or genetic mutations have been found out to be required to enhance the progression of carcinogenesis through functional disruption of oncogenes and tumor-suppressor genes. Long non-coding RNAs (lncRNAs) are longer than 200 nucleotides in length, which are one subtype of non-coding RNAs. LncRNA mainly regulates gene expressions and protein synthesis at epigenetic, transcriptional, and post-transcriptional levels. Abnormally expressed lncRNA is closely related to tumorigenesis and development of cancers. LncRNA HOTAIR enhances cell proliferation and invasion in cervical cancer by activating the Notch pathway, which may be a potential therapeutic target[5]. By sponging miR-27b-3p, lncRNA KCNQ1OT1 facilitates the proliferation and migration in the progression of non-small cell lung cancer by upregulating the expression of HSP90AA1[6].

Our study figured out that OR3A4 was remarkably overexpressed in both CRC tissues

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and cell lines. Moreover, knockdown of OR3A4 depressed the proliferation and migration of CRC cells *in vitro*. In addition, we found that the function of OR3A4 in CRC was also associated with the Wnt/β-catenin signaling pathway.

Patients and Methods

Tissue Specimens

A total of 52 paired CRC tissue samples and adjacent non-tumor tissue samples were sequentially gathered from CRC cases undergoing surgeries at the Linyi Cancer Hospital. All CRC cases were independently diagnosed by two pathologists. This research was approved by the Ethics Committee of Linyi Cancer Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

A total of 4 CRC cancer cell lines (HCT116, HT29, SW620, and SW480), and normal human colonic epithelial cells (NCM460) were purchased from the Chinese Academy of Science (Shan China). Cells were cultured in Roswel Memorial Institute-1640 (RPMI-1640; HyC e, South Logan, UT, USA) containing 10% feta vine serum (FBS; Gibco, Rockville, MD, US and 1% penicillin/streptomyci Aldric St. Louis, MO, USA). Cells e pla at 37°C in an incubator containin CO_2.

Cell Transfection

Lentivirus ex ing sho pin RNA (shRNA) dire t OR3A4 provided by GenePha (Shan China). Negative control shR as also sy ed. Transfection of OR3A RNA or negative rol shRNA (NC) was ducted g Lipofectamine 2000 (Invitroge rls , CA, USA).

Len direct gainst OR3A4 was pro- by Ph (Shanghai, China). Scramble ctor synthesized. Then, OR3A4 le rus or sc mble vector (SV) was transfected cells using Lipofectamine 2000 as ell.

Extraction and Real ime-Quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) reagent was utilized to extract RNA from cultured CRC cells or tissues. After extraction, the RNA was reversely transcribed to complementary deoxyribonucleic acids (cDNAs) through reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan). Following were the primer sequences used for RT-qPCR: OR3A4, forwards 5′-CCTATCCCTTTCTCTAAGAA-3′ and 5′-ACTTCTGCAAAAACGTGCTG-3′ dehyde 3-phosphate dehydrogenas GAPDH), forward 5'-CCAAAATCAGAT GCAAT-GCTGG-3′ and reverse 5′TGA ATG-GACTGTGGTCATTCA-3′ qPCR per-formed three times in t llowing seq pre-denaturation at 95 r 1 m llowed 40 cycles at 95°C fo 15 3C 30 s, a 72°C for 30 s. The rel exp was ulated using the $2^{-\Delta\Delta Ct}$ hod.

Cell Proli ation say

Cell nting kit- CK-8) assay (Dojindo La s, Kumamot an) was performed t nitor cell viability. Briefly, 3 ×10^3 cells were s ed in 96-w lates and cultured overnight. O e other da lls were cultured with 10 μL of 8 for 2 An enzyme-linked immunosorbe ELISA) reader system (Multiskan Ascent, LabSystems, Helsinki, Finland) was used sure the absorbance at 450 nm.

Scratch Wound Assay

The cells, scratched with a plastic tip, were cultured in serum-free RPMI-1640. Each assay was repeated for three times independently. Wound closure was viewed at specific time points.

Transwell Assay

Transwell chambers with 8 μm pores were provided by Corning (Corning, NY, USA). 50 μL of Matrigel was utilized to precoat the membrane. Cells were applied on the upper chambers of a 24-well plate. The bottom chamber of the culture inserts was added with RPMI-1640 containing 20% FBS. These inserts were fixed with methanol for 30 min after cell culture for 24 h. Then, the inserts were stained by hematoxylin for 20 min. A light microscope (Olympus, Tokyo, Japan) was used to count the number of migratory cells.

Western Blot Analysis

After washed with precooled phosphate-buffered saline (PBS), cell samples were lysed with cell lysis solution (RIPA; Beyotime, Shanghai, China). Bicinchoninic acid (BCA; Abcam, Cambridge, MA, USA) was used to detect protein concentration. After being transferred on to a poly-

vinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), they were blocked in Tris-Buffered Saline and Tween-20 (TBST) in 5% skimmed milk for 2 h. Membranes were then incubated with primary antibodies at 4°C overnight, including antibodies against Wnt3a, β-catenin, Survivin, C-myc, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). On the other day, they were incubated with secondary antibody for 1 h at room temperature after being washed (3 × 10 min) with TBST. Image J (Media Cybernetics, Silver Springs, MD, USA) software was utilized to analyze the results.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 21.0 (IBM, Armonk, NY, USA) was conducted to perform all statistical analyses. The differences between two groups were compared by performing independent-sample *t*-test. $p<0.05$, considered as statistically significant.

Results

Expression Levels of OR3A4 in CRC Tissues and Cells

Matched CRC tissue samples and adjac normal samples were collecte was r markably upregulated in tiss samples compared to that in ad t sam (Figure 1A). Moreover, OR3A cancer cell lines an rmal colonic epithelial cells (NC) was de by RT-qPCR. Compared with the expression in NCM460, OR3A4 level in CRC cells was significantly higher (Figure 1B).

Silencing of OR3A4 Repressed Cell Proliferation and Metastasis in CRC

We chose HT29 cell line for constr knockdown model of OR3A4. RT CR was utilized for detecting the OR3A pression (Figure 2A). Moreover, the re lt of 8 assay revealed that the cell gr ability T29 cells was remarkably r ssed after C was knocked down (F e 2B). atch wo assay showed that m gr er of HT2 cells was significantly crea ter OR was knocked dow gure 2C) th ore, the transwell a ed that the ber of metastatic HT2 cells emarkably reduced after OR3A knocked n (Figure 2D).

Overexpression of OR3A4 Promoted Cell Proliferation and Metastasis in CRC Cells

W480 cell l was selected for constructing overex sion model of OR3A4. Then, OR3A on was detected by RT-qPCR (Figure 3A). CCK-8 results revealed that the lity of SW480 cells significantly increased R3A4 was overexpressed (Figure 3B). Scratch wound assay showed that the migrated ability of SW480 cells significantly increased after OR3A4 was overexpressed (Figure 3C). Furthermore, transwell assay showed that the number of metastatic SW480 cells remarkably increased after OR3A4 was overexpressed (Figure 3D).

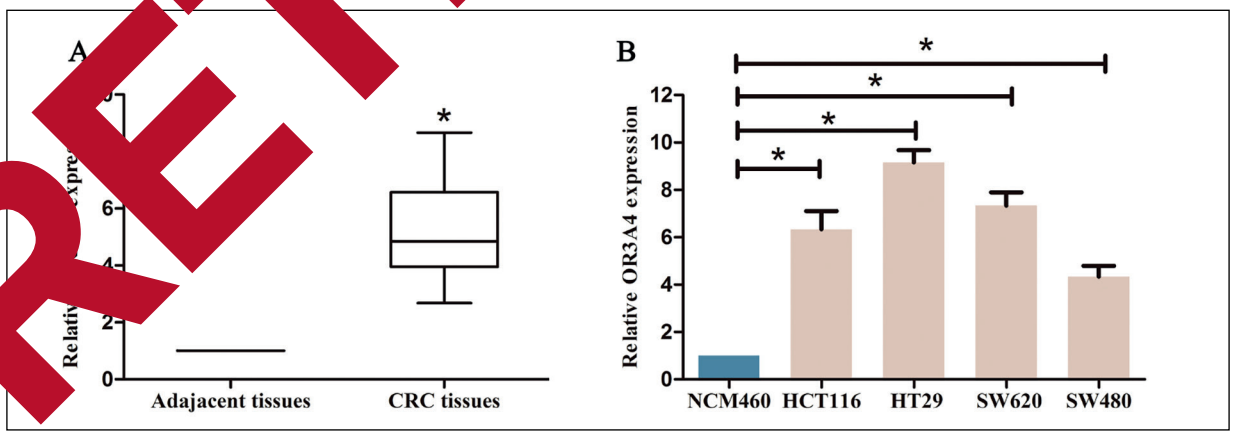

Figure 1. Expression levels of OR3A4 were upregulated in CRC tissues and cell lines. **A**, OR3A4 expression was significantly upregulated in the CRC tissues compared with adjacent tissues. **B,** Expression levels of OR3A4 relative to GAPDH were determined in the human CRC cell lines and normal human colonic epithelial cells (NCM460) by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

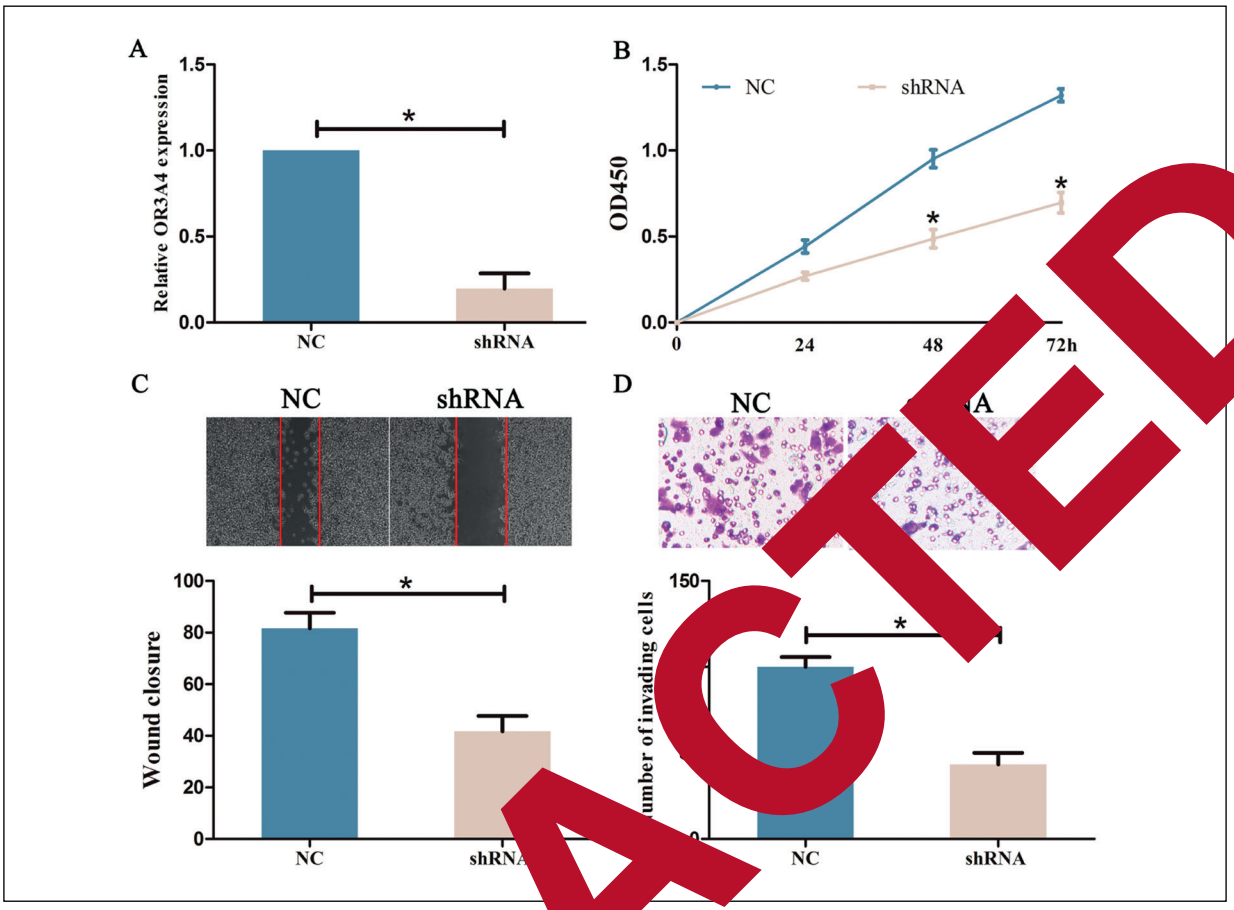

Figure 2. Knockdown of OR3A4 i liferatio gration and invasion in HT29 cells. **A**, OR3A4 expression in HT29 cells transfected with negati itro NA (NC) R3A4 shRNA (shRNA) was detected by RT-qPCR. GAPDH was used as an internal control. K-8 ass owed that ckdown of OR3A4 significantly repressed cell proliferation in HT29 cells. **C,** Scratch healing howed igrated length of HT29 cells in shRNA group was significantly reduced compared with NC group (ni l assay showed that knockdown of OR3A4 significantly repressed invasion in HT29 cells ignificati). The results represent the average of three independent experiments (mean ± standard error of the . *p<0.05, a ared with the control cells.

The Int tion Betw the Wnt/ tenin Signalin athway an R3A4 CRC

T ur the underlying mechanism of OR3A progr n of CRC, Western blot an -qP were conducted to detect ta prote e Wnt/β-catenin pathway such as t3a, C-myc, β-catenin, and Survivin. As re 4A, mRNA expressions of Wn-3a, C-myc, β-catenin, and Survivin were down-gulated after knockdown of OR3A4 in HT29 line. As shown in Figure 4B, the protein levels of Wnt3a, C-myc, β-catenin, and Survivin were identically downregulated after knockdown of OR3A4 in HT29 cell line. The reversed effects were also viewed in SW480 cells overexpressing OR3A4 (Figures 4C and 4D). These results suggested that OR3A4 promoted the development of CRC by regulating the Wnt/β-catenin signaling pathway.

Discussion

CRC is a popular malignancy throughout the world. In China, the incidence of CRC ranks third among all malignant tumors[7]. The main management of CRC includes surgery, chemotherapy, and radiotherapy. However, the therapeutic effect is unsatisfactory. Deep understanding of the pathogenesis of CRC will help to find out effective therapeutic targets[8]. It has been found that the evolution of CRC is closely related to many molecules, including lncRNAs. Although lncRNAs

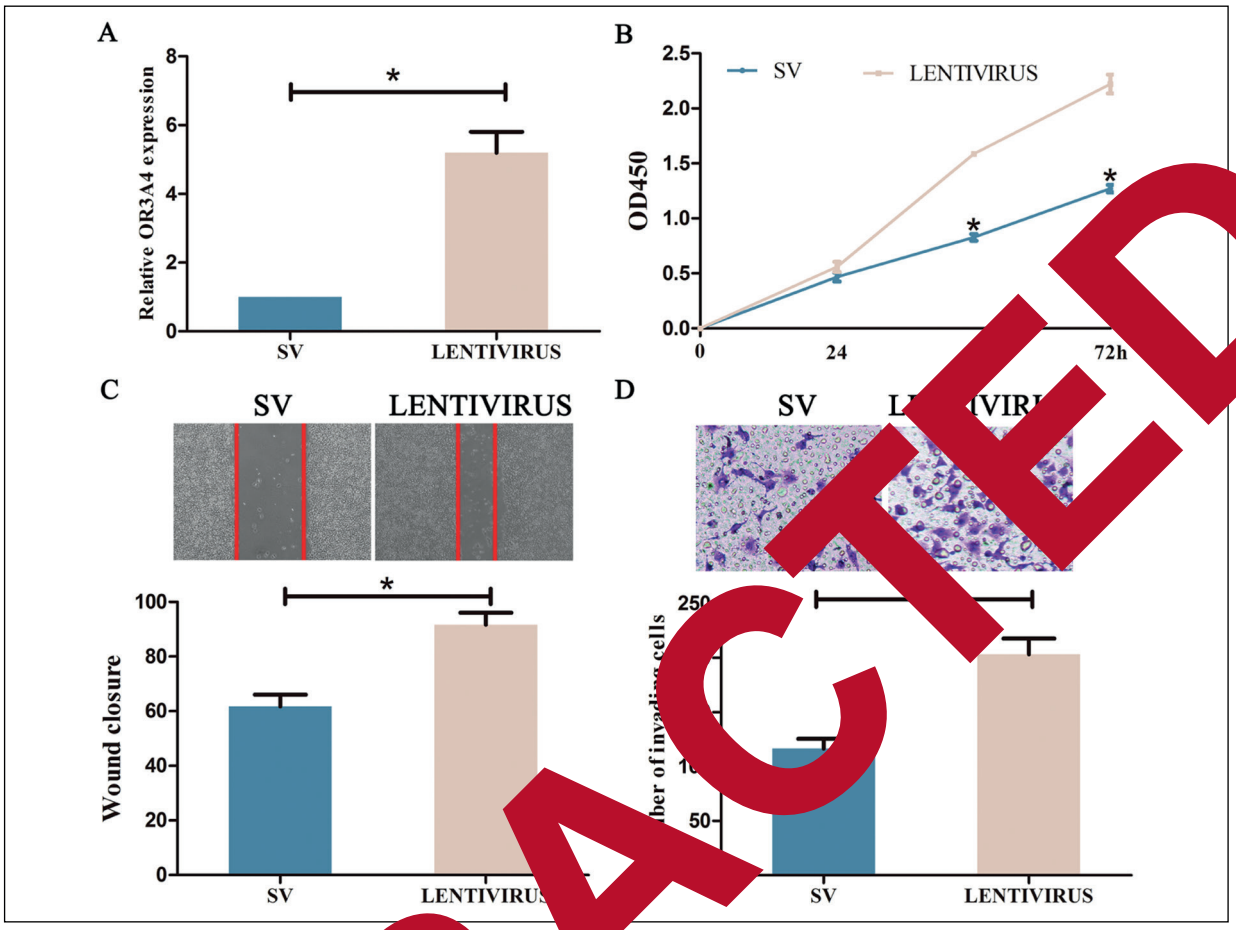

Figure 3. Overexpression of OR3 proliferat migration and invasion in SW480 cells. **A**, OR3A4 expression in SW480 cells transfected with able ve SV) or O 4 lentivirus (lentivirus) was detected by RT-qPCR. GAPDH was used as an internal control K8 ass wed that o erexpression of OR3A4 significantly promoted cell proliferation in SW480 cells. **C,** Scratch ea say migrated length of SW480 cells in lentivirus group significantly increased compared with roup (ation: 40×). **D,** Transwell assay showed that overexpression of OR3A4 significantly promoted invasion in 0 cells (m ation: 40×). The results represent the average of three independent experiments (mean ± standard e mean). *p s compared with the control cells.

do not di y encode p s, they are crucial in the ulation of gene ex ions at different leve Meany , they are closely related to deve en metabolic, evolutionary, and biological ses of t rs. Compelling evidence how t ln As play an important role in cinoge CRC. Through regulation of th iR-1254- NT11 signal pathway, lncRNA facilitates cells proliferation and invasion in CRC, which may offer a new therapeutic target for CRC[9]. LncRNA RUNX1-IT1 s as a tumor suppressor in CRC by inhibition of cell proliferation and migration[10]. Upregulation of lncRNA LINC01510 is negatively associated with the prognosis in CRC patients, which may offer a potential independent prognostic biomarker[11]. By modulation of cell proliferation activity and the ERK/COX-2 pathway, lncRNA CCHE1 functions as an oncogene in the progression of CRC[12]. OR3A4 (Olfactory receptor family 3 subfamily A member 4) has recently been revealed to be abnormally expressed in several cancers, which may offer a potential therapeutic target and diagnostic biomarker. For instance, OR3A4 is significantly overexpressed in ovarian cancer, which promotes the metastasis of ovarian cancer cells through inhibiting KLF6[13]. Overexpression of OR3A4 facilitates cell proliferation and metastasis in breast cancer through epithelial-mesenchymal transition[14]. By regulating the AGGF1/akt/mTOR signaling, OR3A4 plays an important role in angiogenesis of hepatocellular carcinoma[15]. OR3A4 promotes angiogenesis and tumorigenesis in gastric cancer, which may offer

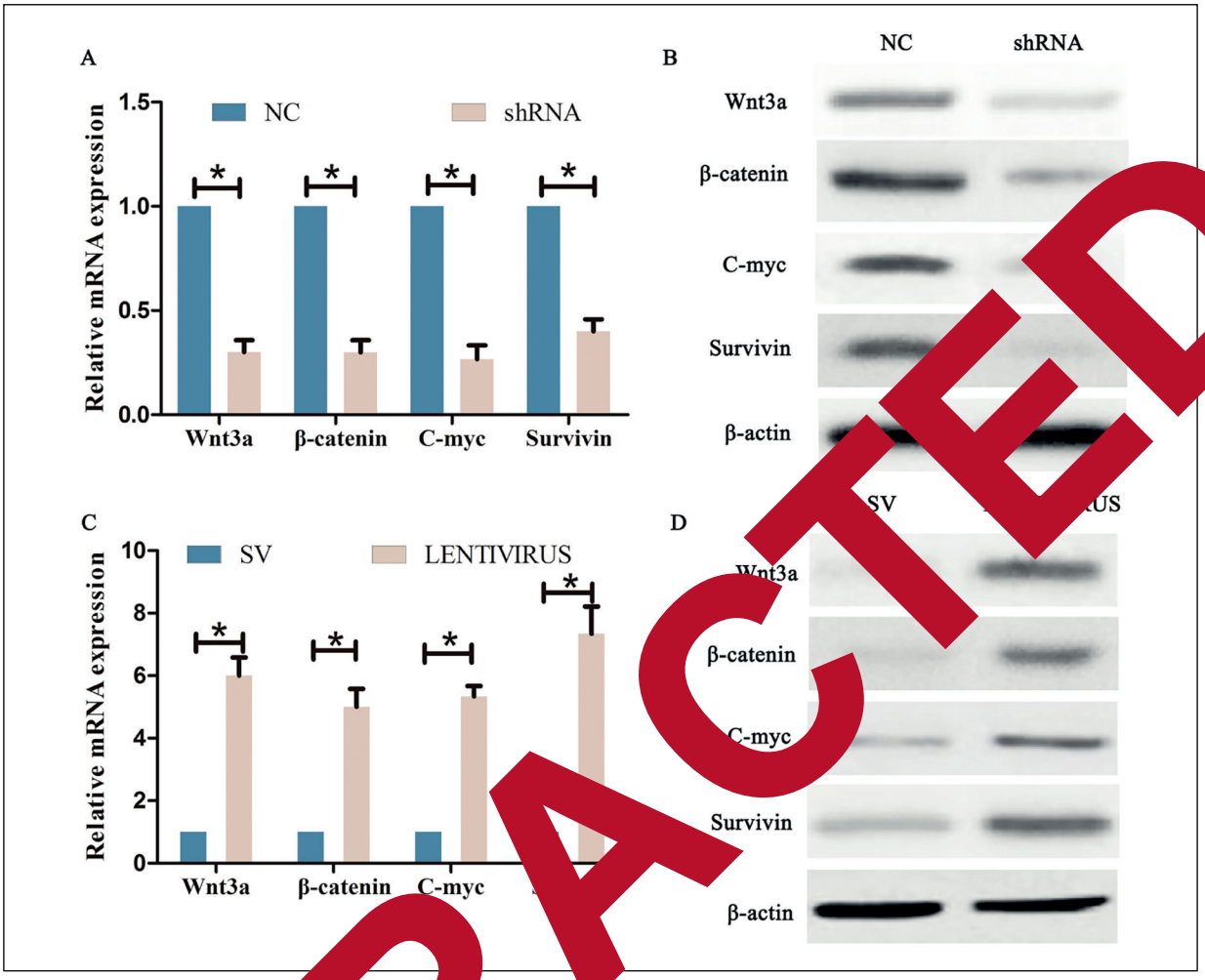

Figure 4. The association betw[illegible]OR3A[illegible]/β-catenin signaling pathway in CRC. **A**, RT-qPCR results revealed that the expressions of tar[illegible]ote[illegible]nt/β-c[illegible]signaling pathway were downregulated in shRNA group compared with NC group. **B,** We[illegible]blot assa[illegible]ts revealed that the expressions of target proteins in the Wnt/β-catenin signaling pathway were down[illegible]ed in shRNA[illegible] compared with NC group. **C,** RT-qPCR results revealed that the expressions of target proteins[illegible]/β-catenin sig[illegible]g pathway were upregulated in lentivirus group compared with SV group. **D,** Western blot [illegible]ay res[illegible]ealed that t[illegible]e expressions of target proteins in the Wnt/β-catenin signaling pathway were upregulated [illegible]ntivirus grou[illegible]pared with SV group. The results represent the average of three independent experiments. Data are [illegible]nted as the mean [illegible]dard error of the mean. *p<0.05.

a novel potential therapeutic strategy[16]. In this study, it was found that OR3A4 was upregulated in CRC tissues and cells. Moreover, OR3A4 knockdown suppressed cell growth, migration, and invasion in CRC. Conversely, overexpression of OR3A4 in CRC yielded the opposite trends. These data indicated that OR3A4 functioned as an oncogene and promoted the tumorigenesis of CRC. The Wnt/β-catenin signaling pathway is conserved in evolution, which participates in many pathological and physiological processes, including cell growth, development, tissue regeneration in tumors, and diseases[17,18]. Previous studies have shown that the Wnt/β-catenin signaling pathway is abnormally activated in cancer tissues which may participate in the progression of tumorigenesis. By activating Wnt/β-catenin signaling inhibitors, DKK1 and SFRP2, TET1 serves as a tumor suppressor in ovarian cancer by inhibition of epithelial-mesenchymal transition[19]. By modulating the Wnt/β-Catenin/Axin2 signaling, c-Myb facilitates cell invasion and migration in breast cancer[20]. Herein, the interaction between the Wnt/β-catenin pathway and OR3A4 in CRC development was explored. After OR3A4 was knocked down *in vitro*, target proteins in

the Wnt/β-catenin pathway were downregulated, and opposite results were obtained after overexpression of OR3A4. Collectively, OR3A4 might promote tumorigenesis of CRC by activating the Wnt/β-catenin pathway.

Conclusions

To sum up, we first clarified that OR3A4 could enhance CRC cell proliferation and metastasis by activating the Wnt/β-catenin pathway. The findings implied that OR3A4 could function as a promising marker for CRC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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