European Review for Medical and Pharmacological Sciences 2019; 23: 8788-8794

Effects of LncRNA KCNQ1OT1 on proliferation and migration of ovarian cancer cells by Wnt/β-catenin

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Abstract. – OBJECTIVE: To explore the role of long noncoding ribonucleic acid (lncRNA) KCNQ1OT1 in the proliferation, apoptosis, and migration of ovarian cancer cells *via* Wnt/β-catenin.

MATERIALS AND METHODS: Ovarian cancer A2780 cells were divided into three groups, namely control group, KCNQ1OT1 overexpression group, and KCNQ1OT1 knockdown group. Next, the effect of KCNQ1OT1 on the proliferation of ovarian cancer A2780 cells was detected by cell counting kit-8 (CCK-8) assay. Wound healing assay and transwell assay were carried out to determine the influence of KCNQ1OT1 on the migration ability of ovarian cancer A2780 cells. The role of KCNQ1OT1 in the cell cycle of ovarian cancer A2780 cells was detected *via* flow cytometry. The impact of KCNQ1OT1 on the expression level of β-catenin protein in ovarian cancer A2780 cells was determined through Western blotting and fluorescence immunoassay.

RESULTS: The proliferation rate of cells was overtly decreased in KCNQ1OT1 knockdown group but significantly increased in KCNQ1OT1 overexpression group. The results of both wound healing and transwell assays showed that the migration ability of cells was reduced in KCNQ1OT1 knockdown group but raised in KCNQ1OT1 overexpression group. According to flow cytometry, the cell cycle was clearly arrested in the G0/G1 phase in KCNQ1OT1 knockdown group. The results of Western blotting and fluorescence immunoassay revealed that compared with that in control group, the expression level of β-catenin protein evidently declined in KCNQ1OT1 knockdown group, but it was notably elevated in KCNQ1OT1 overexpression group.

CONCLUSIONS: Increased lncRNA KCNQ1OT1 in ovarian cancer cells promotes the expression of β-catenin, thereby facilitating the proliferation and migration of ovarian cancer cells.

Key Words:
LncRNA KCNQ1OT1, Wnt/β-catenin, Ovarian cancer.

Introduction

Ovarian cancer remains a leading cause of the death from gynecological tumors so far[1]. It has no significant pain or other pathological features, and is already in the middle and advanced stages when diagnosed, bringing a lot of difficulties for its treatment. Currently, ovarian cancer can only be treated by some conventional treatment methods (surgical treatment and postoperative chemotherapy)[2]. However, the proliferation and differentiation of tumor cells cannot be controlled even if above conventional treatment methods are adopted. Therefore, finding out that the drugs are capable of controlling the growth of cancer cells becomes the main method to reduce the mortality rate of patients with ovarian cancer[3].

Long non-coding ribonucleic acids (lncRNAs) are non-coding RNAs with more than 200 nucleotides in length[4]. Kono et al[5] have shown that lncRNAs play important roles in such biological processes as cell proliferation, differentiation, apoptosis, and migration. KCNQ1OT1, also known as KCNQ1 overlapping transcript 1 or KCNQ1 opposite strand/antisense transcript 1, is located on human chromosome 11p15.5 and is a long non-coding RNA gene detected in the KCNQ1 *locus* that consists of 8-10 protein-coding genes[6]. Increasing evidence has proved that the lncRNA KCNQ1OT1 affects malignant tumors[7]. The lncRNA KCNQ1OT1 is expressed in many malignancies. Wnt is an acronym of "Wingless/Integrated" in the field of genetics. The Wnt signaling pathway is highly conserved in genetics among animals and similar but not identical in different species[8]. The Wnt gene is located on human chromosome 12q13. The Wnt pathway is one of the signal transduction systems regulated by the Wnt gene, and Wnt/β-catenin is a typical signal in the Wnt pathway. β-catenin binds to T

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cytokine/lymphocyte-enhancing factor in the nucleus to activate and express the target genes[9,10]. Therefore, in this study, the proliferation ability, wound healing path and migration ability and cell cycle of ovarian cancer cells and Wnt/β-catenin protein expression were compared among control group, KCNQ1OT1 overexpression group, and KCNQ1OT1 knockdown group to investigate whether Wnt/β-catenin and the lncRNA KCNQ1OT1 are related to the proliferation and metastasis of ovarian cancer cells.

Materials and Methods

Instruments and Reagents

Human ovarian cancer A2780 cell line (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan), Dulbecco's Modified Eagle's Medium (DMEM, Shanghai Jinuo Biotechnology Co., Ltd., Shanghai, China), trypsin solution (Sigma-Aldrich, St. Louis, MO, USA), inverted microscope (Olympus, Tokyo, Japan) and fetal bovine serum (FBS; Gibco, Grand Island, NY, USA).

Cell Culture and Grouping

Human ovarian cancer A2780 cell lines were cultured in DMEM supplemented with 10% FBS in a cell incubator with 5% CO_2 at 37°C and then divided into control group, KCNQ1OT1 overexpression group, and KCNQ1OT1 knockdown group.

Cell Transfection

To investigate the effects of the long noncoding ribonucleic acids (lncRNAs) KCNQ1OT1 on the proliferation, apoptosis, and migration of ovarian cancer A2780 cells, the cells were transfected as follows. The full-length sequence of KCNQ1OT1 was synthesized and cloned into plasmid complementary deoxyribonucleic acid 3.1 (pcDNA3.1) plasmids (Invitrogen, Carlsbad, CA, USA). Ovarian cancer A2780 cells were evenly seeded in a 6-well plate at 1×10^7 cells/well. Next, the cells were transfected with KCNQ1OT1 overexpressing plasmids for 48 h using a Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the instructions of the kit. The KCNQ1OT1 expression level in the A2780 cells was knocked down *via* 24 h of transfection with sh-KCNQ1OT1 plasmids using a Lipofectamine 2000 kit in Opti-MEM I.

Detection of Proliferation Ability of Ovarian Cancer Cells Via CCK-8

The cells transfected were digested with 0.05% trypsin and seeded in a 96-well plate at 1×10^4 cells/well. Then, the original medium was discarded and the cells were synchronized for 12 h. The total reaction volume was 200 μL per well, with 6 replicates for each sample. After 72 h, 20 μL CCK-8 reaction solution (Dojindo, Kumamoto, Japan) was added, and the cells were incubated in a dark place at 37°C for 2 h. Thereafter, the cells were shaken on a micro-vibrator for 10 min. Lastly, the optical density (OD) at a wavelength of 450 nm was read using a microplate reader.

Determination of Migration Ability of Ovarian Cancer A2780 Cells Through Wound Healing Assay

After ovarian cancer A2780 cells were transfected successfully, they were digested with 0.05% trypsin solution and centrifuged. Next, the cells were collected and evenly inoculated into a 6-well plate at 1×10^7 cells/well. After the cells were attached to the wall, a 1 mL tip was used to evenly scratch them, with a consistent width of each wound as far as possible. Thereafter, the cells were rinsed gently with phosphate-buffered saline (PBS) three times to wash away the cell debris from scratching. Next, serum-free DMEM was added, and 6 h later, the distance of cell movement was recorded using the inverted microscope (100×).

Detection of Migration Ability of Ovarian Cancer Cells by Transwell Assay

Three groups of 200 μL ovarian cancer cell suspensions (5×10^5/mL) were prepared and added into the upper chamber of a transwell chamber (8 μm pore), and 300 μL DMEM complemented with 10% FBS were added to the lower chamber, with 5 replicates for each type of cells. Next, they were incubated at 37°C and 5% CO_2 for 12 h. Thereafter, the supernatant was discarded, and the cells that had not passed through the membrane were wiped off using wet cotton swabs, followed by staining with hematoxylin and observation using a microscope (400×).

Detection of the Cell Cycle of Ovarian Cancer Cells Via Flow Cytometry

Three groups of A2780 ovarian cells were uniformly plated in a 6-well plate at 1×10^7 cells/well, cultured for 24 h, digested with 0.05% trypsin, fixed with 70% ethanol at 4°C, and placed in a refrigerator at 4°C for 24 h. Thereafter, the

cells were centrifuged for 5 min, and the supernatant was aspirated and washed with PBS for 5 min three times. Next, the cells were stained in the dark at 37°C for 30 min using a cell cycle Propidium Iodide (PI) staining kit (Beyotime Institute of Biotechnology, Shanghai, China) in strict accordance with the instructions of the kit. Lastly, a FACSCalibur flow cytometer was used for analysis.

Measurement of β-Catenin Protein Expression Level Through Western Blotting

The cells were digested with 0.05% trypsin and then collected. The proteins were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), subjected to ice-bath for 30 min and centrifuged, and the supernatant was extracted. The protein concentration was detected by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). After separation *via* 10% dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to a polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) *via* semi-dry experiment. Next, the membrane was blocked with 5% skim milk powder at room temperature for 1 h, incubated with primary β-catenin antibody (1:5000) for 24 h, and washed with TBST for 3 times (10 min/time), followed by incubation with secondary antibody at room temperature for 2 h. After that, the membrane was rinsed twice with Tris-Buffered Saline Tween-20 (TBST) and then once with TBS, with 10 min/time. Next, enhanced chemiluminescence (ECL) reagents were used for protein detection, with exposure in a dark room. Lastly, the relative expression level of proteins was analyzed using Image-Pro Plus v6 software (Media Cybernetics, Silver Spring, MD, USA).

Determination of β-Catenin Protein Expression by Fluorescence Immunoassay

Ovarian cancer cells in each group were evenly seeded in a 12-well plate at 5×10^5 cells/well, fixed with 4% paraformaldehyde for 10 min, washed with phosphate-buffered saline (PBS), treated with 0.5% Triton for 10 min, washed with PBS, blocked with 3% bovine serum albumin (BSA) for 1 h, washed with PBS, incubated for 24 h and washed with PBS 3 times. Next, the cells were added with goat anti-mouse IgG Alexa Flour 488 (1:200) for incubation in the dark at room temperature for 1 h, washed 3 times with PBS, added with 4,6-diacetyl -2-phenyl decanoate, stained in the dark at room temperature for 10 min and washed twice with PBS. Thereafter, the glass slides were covered with coverslips, followed by mounting with anti-fluorescent attenuating mounting medium. Lastly, a fluorescence microscope was employed for observation.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Data were expressed as mean ± standard deviation. Student's *t*-test was employed for statistical analysis, and $p<0.05$ suggested that the difference was statistically significant.

Results

Effect of KCNQ1OT1 on the Proliferation of Ovarian Cancer A2780 Cells Detected Via CCK-8 Assay

Results showed that compared with that in control group, the proliferation rate of cells was significantly decreased in KCNQ1OT1 knockdown group but remarkably elevated in KCNQ1OT1 overexpression group, showing statistically significant differences ($p<0.05$) (Figure 1).

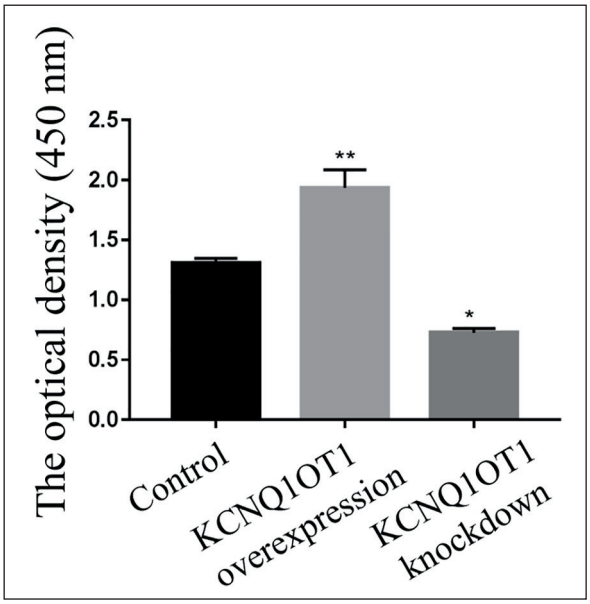

Figure 1. Effect of KCNQ1OT1 on cell proliferation detected *via* CCK-8 assay. **: $p<0.05$ *vs.* control group, *: $p<0.05$ *vs.* control group.

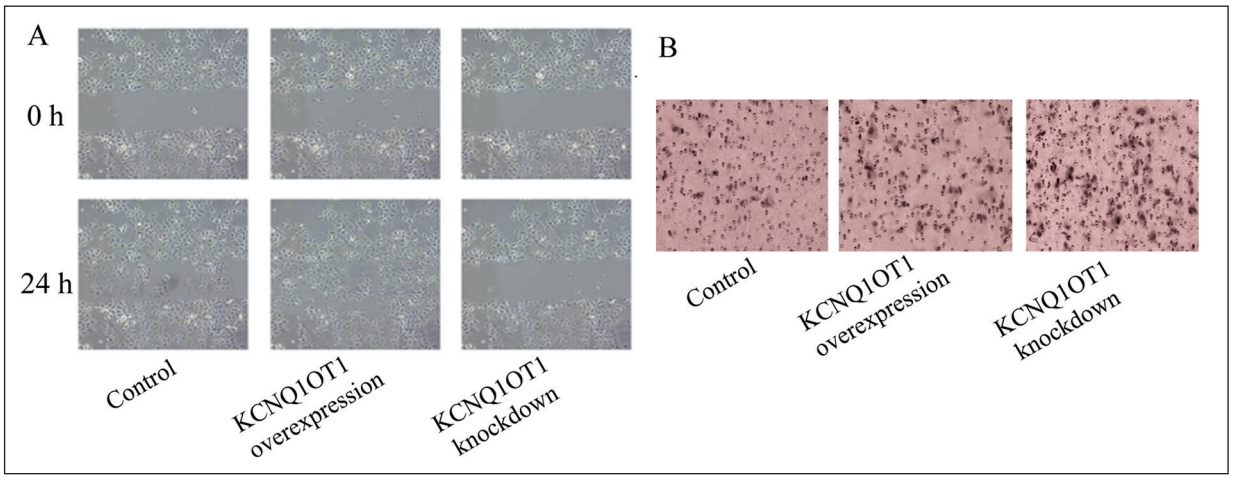

Figure 2. Influence of KCNQ1OT1 on migration ability of cells. **A,** Influence of KCNQ1OT1 on migration ability of cells detected *via* wound healing assay (magnification: 40×), **B,** Influence of KCNQ1OT1 on migration ability of cells determined through transwell assay (magnification: 40×).

Influence of KCNQ1OT1 on Migration Ability of Ovarian Cancer A2780 Cells

Wound healing assay and cell migration assays were performed to examine the effects of the lncRNA KCNQ1OT1 on vertical and horizontal migration abilities of ovarian cancer cells, respectively. In wound healing assay, pairwise comparison was conducted at 24 h after scratching, and it was discovered that KCNQ1OT1 knockdown group had the widest cell-free region in ovarian cancer cells, followed by KCNQ1OT1 overexpression group and then control group ($p<0.05$). The results of cell migration assay revealed that the number of migrating ovarian cancer cells was smaller in KCNQ1OT1 knockdown group than that in control group, and it was the largest in KCNQ1OT1 overexpression group, which was significantly largest than that in other two groups ($p<0.05$) (Figure 2).

Effect of KCNQ1OT1 on Cell Cycle of Ovarian Cancer A2780 Cells

The effect of the lncRNA KCNQ1OT1 on the cell cycle of ovarian cancer A2780 cells was detected, and the results (Figure 3 and Table I) indicated that the cell cycle was obviously arrested in the G0/G1 phase in KCNQ1OT1 knockdown group.

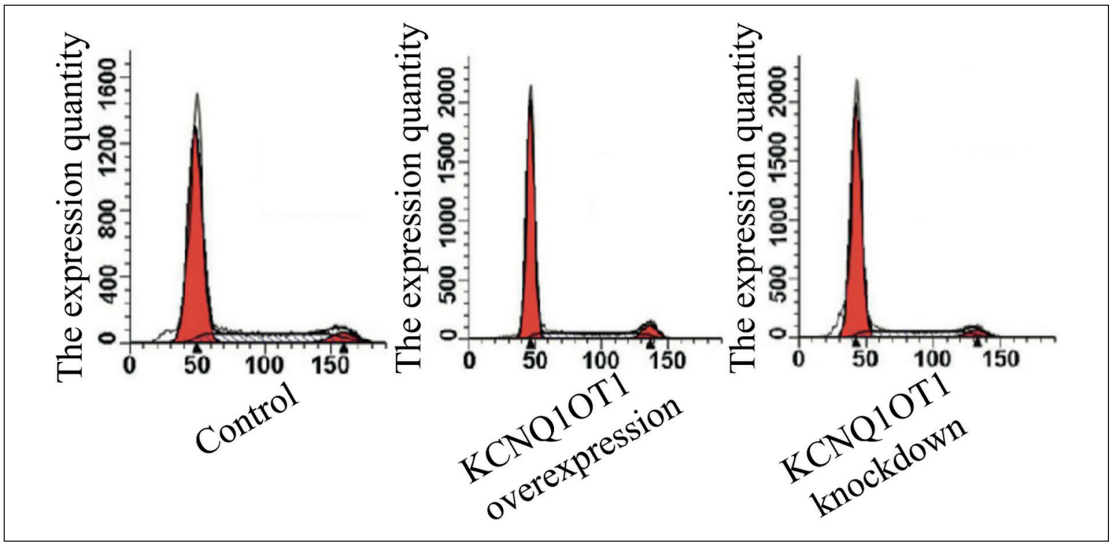

Figure 3. Effect of KCNQ1OT1 on cell cycle of ovarian cancer A2780 cells.

Table I. Effect of KCNQ1OT1 on cell cycle of ovarian cancer A2780 cells.

	G0/G1	S	G2
Control group	46.84%	19.71%	33.45%
KCNQ1OT1 overexpression group	45.32%	18.73%	35.95%
KCNQ1OT1 knockdown group	76.31%**	17.92%	5.77%

Impact of KCNQ1OT1 on β-Catenin Protein Expression Level Detected by Western Blotting

The results (Figure 4) of Western blotting assay showed that compared with that in control group, the expression level of β-catenin protein was notably lowered in KCNQ1OT1 knockdown group, but markedly raised in KCNQ1OT1 overexpression group, and the differences were statistically significant ($p<0.05$).

Effect of KCNQ1OT1 on the Expression Level of β-Catenin Protein Detected by Cellular Fluorescence Immunoassay

The TOPFlash fluorescence reporter system is capable of displaying the changes of the β-catenin signaling pathway through fluorescent signals. It was found that in comparison with control group, KCNQ1OT1 knockdown group had an overtly reduced expression level of β-catenin protein, while KCNQ1OT1 overexpression group exhibited a clearly increased expression level of β-catenin protein, showing statistically significant differences ($p<0.05$) (Figure 5).

Discussion

Ovarian cancer, one of the major malignant tumors in women, ranks the top in both morbidity and mortality rates[11,12]. The main hallmarks of malignant tumors are invasion and metastasis, and the invasion and metastasis of cancer cells are leading causes of the death of most patients with tumors[13]. Until today, no great achievement is made in the research on the pathogenesis of ovarian cancer, and the cause of ovarian cancer is undetermined, so that the prevention and treatment of ovarian cancer cannot be further studied[14,15]. Hence, further studying the pathogenesis of ovarian cancer helps faster find methods inhibiting the proliferation of ovarian cancer and reduces the mortality rate of patients with ovarian cancer at the same time[16,17].

Pairwise comparisons among groups showed that there were statistically significant differences in OD value between control group and KCNQ1OT1 overexpression group as well as between control group and KCNQ1OT1 knockdown group. The proliferation rate was significantly higher in KCNQ1OT1 overexpression group than

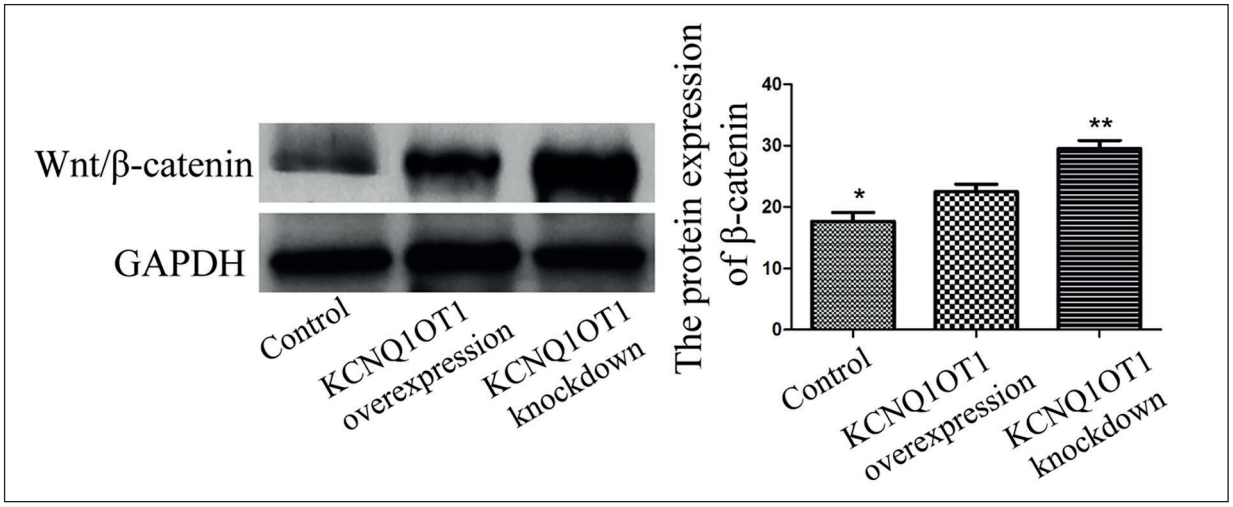

Figure 4. Impact of KCNQ1OT1 on β-catenin protein expression level detected by Western blotting assay. *: $p<0.05$ *vs.* control group, **: $p<0.05$ *vs.* control group.

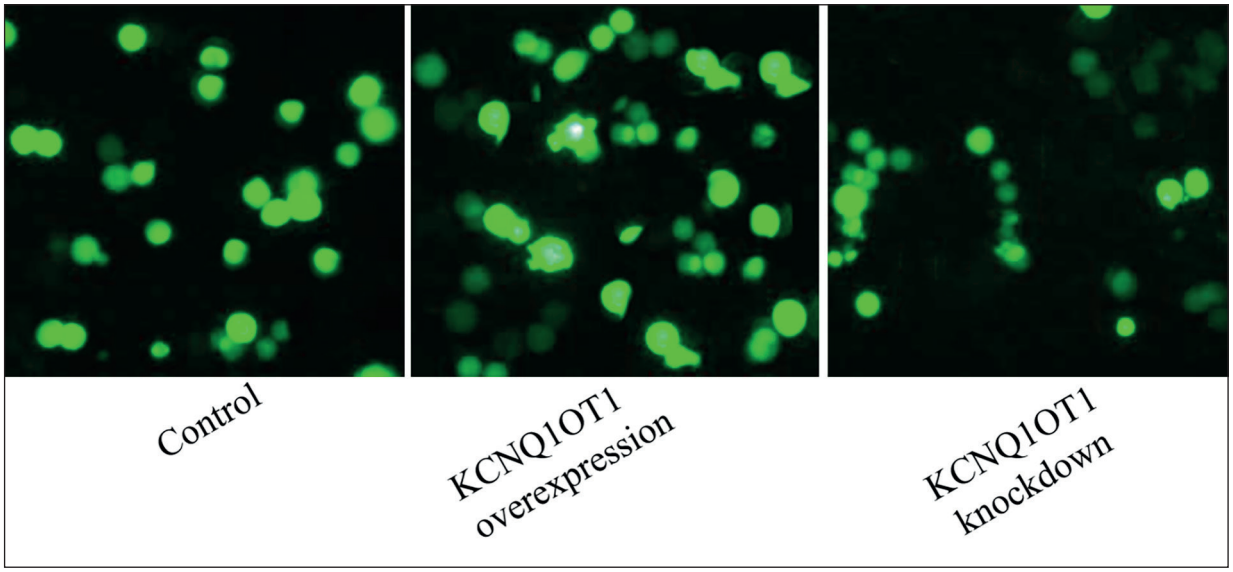

Figure 5. Protein expression of β-catenin detected by cellular fluorescence immunoassay (magnification: 100×).

that in control group (p<0.05), while it was evidently lower in KCNQ1OT1 knockdown group than that in control group (p<0.05), indicating that the inhibition of the lncRNA KCNQ1OT1 suppresses the proliferation of ovarian cancer cells. This result is consistent with the findings (down-regulating lncRNA KCNQ1OT1 expression can overtly repress cell proliferation and invasion) of the study (lncRNA KCNQ1OT1 mediates the growth of hepatocellular carcinoma by functioning as a competitive endogenous RNA of miR-504) by Li et al[18]. The effect of lncRNA KCNQ1OT1 on the cell cycle of human ovarian cancer cells was determined, and the results showed that KCNQ1OT1 knockdown group had the shortest G1, S and G2 phases in ovarian cancer cells, and the proportions of cells in G1, S and G2 phases were significantly lower in control group than those in KCNQ1OT1 overexpression group (p<0.05). It was found in wound healing assay that KCNQ1OT1 knockdown group had the widest cell-free region in ovarian cancer cells at 24 h after scratching, followed by KCNQ1OT1 overexpression group and then control group (p<0.05). The results of cell migration assay revealed that the number of migrating ovarian cancer cells was smaller in KCNQ1OT1 knockdown group than that in control group, and it was the largest in KCNQ1OT1 overexpression group (p<0.05). The above results suggest that the lncRNA KCNQ1OT1 inhibits the horizontal and vertical migration of ovarian cancer cells, which is in line with the findings (the inhibition of lncRNA KCNQ1OT1 expression weakens cell migration ability) of the research (Cancer-testis antigens in ovarian cancer: implication for biomarkers and therapeutic targets) by Xie et al[19].

The Western blotting assay was performed for the Wnt/β-catenin protein in ovarian cancer cells in control group, KCNQ1OT1 overexpression group, and KCNQ1OT1 knockdown group. According to the gray analysis, the Wnt/β-catenin protein expression was the highest in KCNQ1OT1 knockdown group but the lowest in KCNQ1OT1 overexpression group, and the expression of Wnt/β-catenin protein was significantly higher in KCNQ1OT1 overexpression group than that in control group (p<0.05). The fluorescence reporter system displayed that the number of fluorescent dots was the smallest in KCNQ1OT1 knockdown group, i.e., the Wnt/β-catenin protein expression was the lowest in KCNQ1OT1 knockdown group, while the number of fluorescent dots was increased in KCNQ1OT1 overexpression group compared with that in control group, namely, the Wnt/β-catenin protein expression was increased significantly (p<0.05). The lncRNA KCNQ1OT1 promotes the activation of the Wnt/β-catenin signaling pathway and participates in the development and progression of various malignant tumors. This result is consistent with the findings (down-regulating Wnt/β-catenin and downstream factors inhibit the invasion and metastasis of ovarian cancer cells) of the study (Proliferation and differentiation of cancer cells *via* Wnt/β-catenin pathway) of Zhan et al[20].

Conclusions

We found that raised lncRNA KCNQ1OT1 in ovarian cancer cells facilitates the expression of Wnt/β-catenin, thus accelerating the proliferation cells and the progression of ovarian cancer cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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