Original Article
C1orf63 silencing affects breast cancer cell proliferation, apoptosis, and cycle distribution by NF-κB signaling pathway

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Abstract: Objective: To investigate the effect of C1orf63 on breast cancer cell (BCC) proliferation, apoptosis, and cycle distribution and related mechanisms. Methods: The expression of C1orf63 was interfered with in BCC line MCF and cells were divided into a C1orf63 overexpression group, C1orf63 silence group, blank group, and empty group. The mRNA expression of C1orf63 and the proliferation, apoptosis, and cycle distribution of BCCs were detected. The mRNA expression levels of NF-κB signaling pathway factors (p-IκBα, CyclinD1, CDK4, Bcl-2, and Bax) in each group were also detected. Results: There was no significant difference between the blank group and empty group in the expression level of C1orf63 mRNA, cell proliferation rate, apoptosis rate, cell distribution rate, or mRNA expression levels of the NF-κB signaling pathway factors (all P>0.05). The expression levels of C1orf63 mRNA in the C1orf63 silenced group were lower than those in the other two groups (P<0.05). The cell proliferation rate, cell distribution in S phase and G2/M phase, and the mRNA expression levels of NF-κB signaling pathway factors (p-IκBα, CyclinD1, CDK4, and Bcl-2) in the C1orf63 silenced group at each time point were lower than those in the other two groups (all P<0.05). The apoptosis rate, cells in G1 phase, and the Bax mRNA expression level in C1orf63 silenced group at each time point were higher than those in the other two groups (all P<0.05). Conclusion: Down-regulation of C1orf63 acts on the NF-κB signaling pathway to regulate the expression of p-IκBα, CyclinD1, and CDK4, so as to inhibit BCC proliferation, promote cell apoptosis, and block the cell cycle.

Keywords: C1orf63, breast cancer cell, proliferation, apoptosis, cell cycle

Introduction

Breast cancer is one of the most common threats to women's health. The incidence of breast cancer is gradually increasing year by year and the patient's ages are becoming younger. Mortality rate is also increasing [1]. At present, the clinical treatment of breast cancer is still dominated by surgery in early stages, chemoradiotherapy, and targeted therapy. Although the above-mentioned treatment methods can extend the survival time of patients to a certain extent, the 5-year survival rate is low [2]. There is still a long way to go fin research on the occurrence, development, and treatment of breast cancer.

Currently, breast cancer pathogenesis is not fully clear, and there is no specific biomarker for the diagnosis of the disease; thus most patients have been in the mid to advanced stage when they were diagnosed, and they lose the optimal timing of treatment. Meanwhile, even though some the patients receive timely treatment, the recurrence rate is still high [3]. Therefore, it is extremely important to develop an efficacious and quick treatment method for breast cancer.

In this study, we investigated the role of d C1orf63 in the proliferation, apoptosis, and cell cycle distribution of breast cancer cells (BCCs), to provide a reference for clinical research on the pathogenesis of breast cancer.

Table 1. Primer sequences

Gene	Primer	Sequence
C1orf63	Forward primer	5'-GCAGCGAAAGCTCTAGGAAC-3'
	Reverse primer	5'-TTCAGGCTTTGCACCATTAC-3'
IκBα	Forward primer	5'-GAAGGAGCGGCTACTGGA-3'
	Reverse primer	5'-TTCTGGCTGGTTGGTGAT-3'
CyclinD1	Forward primer	5'-ACCTGAGGAGCCCCAACAAC-3'
	Reverse primer	5'-GCTTCGATCTGCTCCTGGC-3'
CDK4	Forward primer	5'-GAGGCGACTGGAGGCTTTT-3'
	Reverse primer	5'-GGATGTGGCACAGACGTCC-3'
Bax	Forward primer	5'-AAGCTGAGCAGTGCTCAAG-3'
	Reverse primer	5'-CAAAGTAGAAAAGGGCGACAAC-3'
Bcl-2	Forward primer	5'-ATGTGTGTGGAGAGCGTCAAC-3'
	Reverse primer	5'-AGAGACAGCCAGGAGAAATCAAAC-3'
GAPDH	Forward primer	5'-AACAGCCTCAAGATCATCAGCAA-3'
	Reverse primer	5'-GACTGTGGTCATGAGTCCTTCCA-3'

Table 2. Comparison of C1orf63 mRNA expression among the groups ($\overline{x} \pm sd$)

Group	C1orf63 mRNA
Blank group	0.62±0.15
Empty group	0.64±0.16
C1orf63 overexpression group	1.06±0.27[a,b]
C1orf63 silenced group	0.12±0.03[a,b,c]
F	13.644
P	0.001

Note: Compared with the blank group, [a]P<0.05; compared with the empty group, [b]P<0.05; compared with the C1orf63 overexpression group, [c]P<0.05.
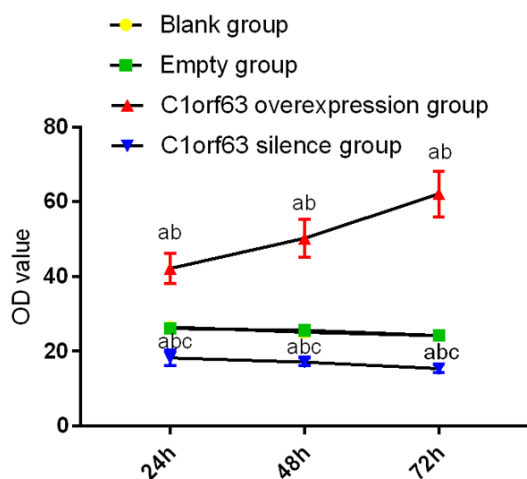
Figure 1. Proliferation of cells in each group at 24 h, 48 h, and 72 h. Compared with the blank group, [a]P<0.05; compared with the empty group, [b]P<0.05; compared with the C1orf63 overexpression group, [c]P<0.05.

Materials and methods

Materials

Cell lines: The Human BCC line MCF-7 was purchased from Shanghai Xinyu Biotechnology Co., Ltd.

Main reagents and instruments: Fetal bovine serum was purchased from Wuhan Punoxai Life Technology Co., Ltd. RPMI 1640 complete medium was purchased from Shanghai Caiyou Industrial Co., Ltd. Trypsin was purchased from Beijing Keriky Biotechnology Co., Ltd. RT buffer was purchased from Xiamen Huijia Biotechnology Co., Ltd. Rabbit anti-rat C1orf63 antibody was purchased from Wuhan Boote Biotechnology Co., Ltd. Rabbit anti-rat p-IκBα antibody was purchased from Shanghai heng Fiji Biological Technology Co., Ltd. Mouse anti-rat CyclinD1 antibody was purchased from Wuhan Boote Biotechnology Co., Ltd. Mouse anti-rat CDK4 antibody was purchased from Shanghai Hengfei Biotechnology Co., Ltd. Rabbit anti-human Bcl-2 antibody was purchased from Shenzhen Haodi Huatuo Biotechnology Co., Ltd. Rabbit anti-rat Bax antibody was purchased from Shanghai Zhenyu Biotechnology Co., Ltd. TRIzol reagent was purchased from Wuhan Purity Biotechnology Co., Ltd. Thermo Reverse Transcription Kit was purchased from Beijing Jiehuibo Biotechnology Co., Ltd. PCR Kit was purchased from Xiamen Huijia Biotechnology Co., Ltd. DxFLEX flow cytometer was purchased from Beckman Coulter Trading (China) Co., Ltd. PI dye solution was purchased from Wuhan Seville Biotechnology Co., Ltd. MTT kit was purchased from Shanghai Qunji Biotechnology Co., Ltd. AnnexIV-FITC/PI Double Staining Cell Apoptosis Detection Kit was purchased from Anhui Jingke Biotechnology Co., Ltd.

Methods

Cell culture and grouping: The human BCCs were cultured in RPMI 1640 medium containing penicillin/streptomycin combination and 10% fetal bovine serum at a temperature of 37°C, 5% CO_2. The medium should be changed every 3 days. When the cells reached about 70%-80% confluence, they were sub-cultured into three groups: blank group (without any

Table 3. Comparison of apoptosis rates in the groups at different time points ($\bar{x} \pm sd$)

Group	Apoptosis rates		
	24 h	48 h	72 h
Blank group	16.77±2.11	19.82±2.15	20.55±2.59
Empty group	16.79±2.13	19.57±2.23	20.68±2.64
C1orf63 overexpression group	14.68±2.09[a,b]	12.51±2.16[a,b]	10.15±2.07[a,b]
C1orf63 silence group	37.68±3.25[a,b,c]	56.59±3.95[a,b,c]	75.29±4.37[a,b,c]
F	3.339	11.377	14.879
P	0.001	0.001	0.001

Note: Compared with the blank group, [a]$P<0.05$; compared with the empty group, [b]$P<0.05$; compared with the C1orf63 overexpression group, [c]$P<0.05$.

treatment), empty group (treated with C1orf63 empty vector), C1orf63 overexpression group (treated with C1orf63 overexpression vectors), and C1orf63 silence group (treated with C1orf63 silencing vectors). The cells growing in the logarithmic growth phase were used for subsequent experiments.

Designing and synthesis of C1orf63 sequence: The mRNA sequence of human C1orf63 was obtained from the GenBank database. The interference sequence was designed using the Ambion's online design software. Then, the BLAST software was used to analyze the specificity of the design sequence, on the basis of the general design principles of siRNA. One pair of the sequence was selected and sent to the Tianjin Saier Biotechnology Co., Ltd for synthesis and lentivirus packaging. si-C1orf63 siRNA sequences: upstream: 5'-GCTAGGCTTCAAATG-GCAAT-3', downstream: 5'-CATCGAGCTGCTCG-TAGCCA-3'; oe-C1orf63 sequence: upstream: 5'-GCTAGTCCCTGGAGTAATTC-3', downstream: 5'-ACTGCCCAGTGGGACCTGAA-3'.

Cell transfection: The MFC-7 cells were cultured in a 6-well plate with 2 * 10^5 cells/well. The cells were cultured at 37°C and 5% CO_2 for 24 h. The lentivirus transfection was carried out when the cells reached about 50% confluence and 2 ml of culture medium consisted of lentivirus suspension and DMEM (1:1) was added into each well. The stable oe-C1orf63 cells (C1orf63 overexpression group) and si-C1orf63 cells (C1orf63 silenced group) were obtained after screening. The specification of recombinant lentivirus suspension (oe-C1orf63, si-C1orf63) was 1.0 * 10e^11 pfu/mL. Design, synthesis, and lentivirus packaging of C1orf63 sequence was performed by Tianjin Saier Biotechnology Co., Ltd.

qRT-PCR: The fluorescence quantitative PCR method was used to detect the mRNA expression of C1orf63 and p-IκBα, CyclinD1, CDK4, Bcl-2 and Bax. Total RNA was extracted by TRIzol and used to synthesize cDNA template according to the instructions of Thermo Reverse Transcription Kit. The PCR reaction system (20 μL) contained 1 μL cDNA template, 10 μL SYBR Primer Ex TaqTMII (2 ×), 0.8 μL PCR Forward Primer (10 μM), 0.8 μL PCR Reverse Primer (10 μM), 0.4 μL Rox Reference Dye II (50 ×), 7 μL RNase H_2O. The PCR reaction condition was set as 95°C for 1 min, 95°C for 15 s, 60°C for 1 min, 30 cycles, and preserved at 4°C. GAPDH was the internal reference. The mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method. All the primer sequences were designed and synthesized by Shanghai Sangon Biological Engineering Co., Ltd. The primer sequences are shown in **Table 1**.

MTT assay: Cell proliferation was detected by MTT assay. Cells were cultured in a 96 well plate at a density of 7 * 10^3 cells/well, and the culture medium was 200 μL per well. The cells were cultured for 2-4 days, then incubated with MTT for 4 h. After that, the cell supernatant in the wells was discarded directly and 150 μL DMSO was added into each well. Finally, absorption value was measured by the ELISA reader at the wavelength of 570 nm. Cell proliferation rate (%) = (cell group OD value - reference group OD)/reference group OD * 100.

Flow cytometry analysis: Cells was washed twice with PBS and fixed by 5 mL of 70% precooled ethanol and incubated at 4°C for at least 18 h. Then, the fixed cells were centrifuged and washed twice with PBS, and the cell concentration was adjusted to 1 × 10^6 cells/mL. For the detection of apoptosis, AnnexIV-FITC/PI Double Staining Cell Apoptosis Detection Kit was used and the experiment was performed according to the kit's instruction.

For the detection of cell cycle, 1 mL of cell suspension was taken out, and the cells were centrifuged and resuspended in 1 mL of PI dye
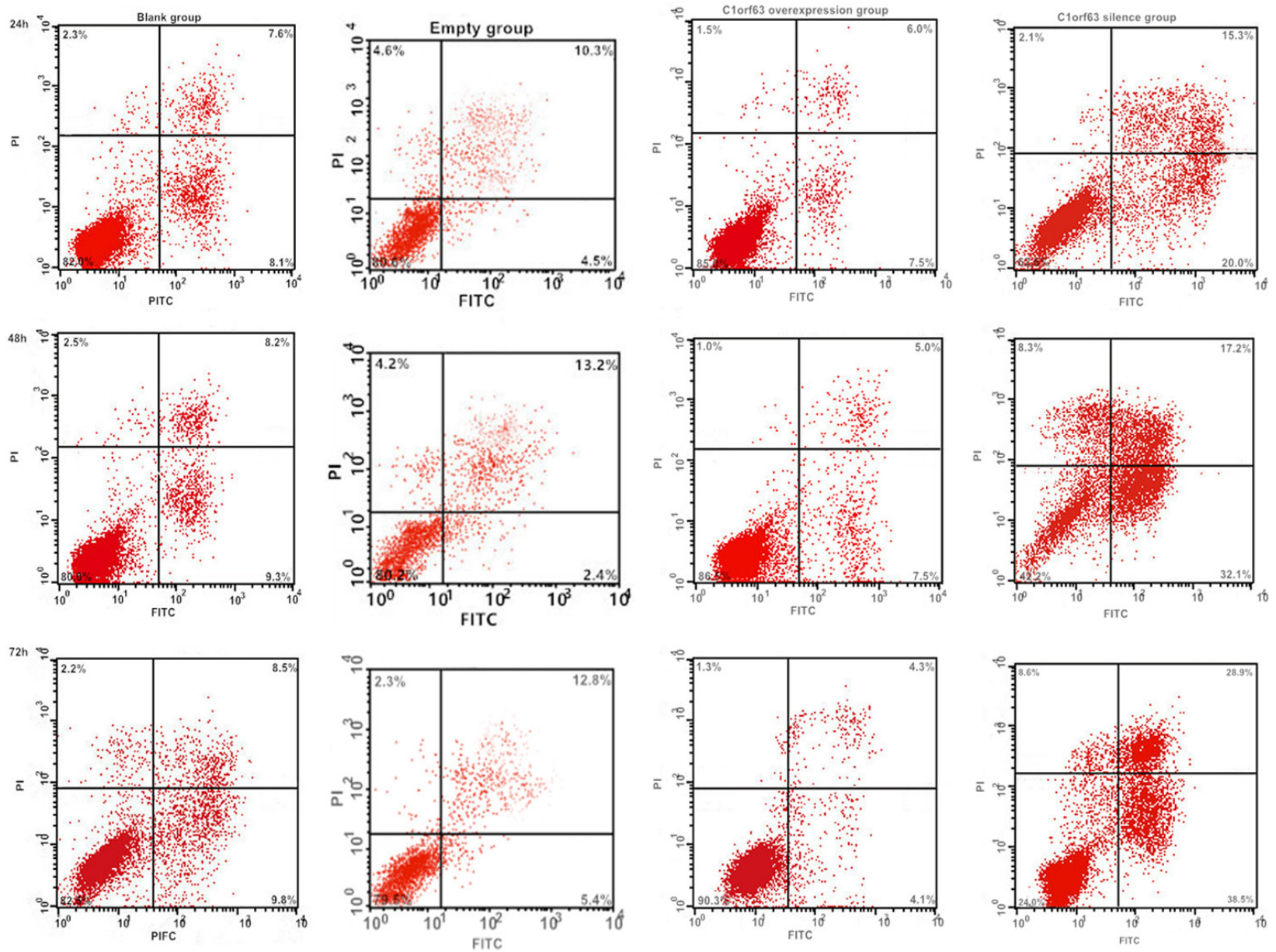
Figure 2. Flow cytometry analysis of apoptosis in the groups at 24 h, 48 h, and 72 h.

Table 4. Comparison of cell cycle distribution in the groups ($\bar{x}$ ± sd)

Group	G1	S	G2/M
Blank group	57.12±1.69	15.26±1.25	9.98±0.15
Empty group	57.16±1.82	15.52±1.22	10.12±0.46
C1orf63 overexpression group	52.26±2.25[a,b]	30.28±3.25[a,b]	13.26±1.02[a,b]
C1orf63 silence group	80.23±3.16[a,b,c]	10.25±1.02[a,b,c]	7.63±0.69[a,b,c]
F	8.192	20.461	15.091
P	0.001	0.001	0.001

Note: Compared with the blank group, [a]P<0.05; compared with the empty group, [b]P<0.05; compared with the C1orf63 overexpression group, [c]P<0.05.

solution. The cells were incubated at 4°C in the dark for 30 min. After that, the cell sample was tested on DxFLEX flow cytometer at a wavelength of 488 nm.

Western blot: The expression of proteins related to NF-kappa B cell signaling pathways, including p-I kappa alpha, cyclinD1, and CDK4 B as well as Bcl-2, and Bax were detected by western blot. The total protein was extracted and quantified by BCA method. Then, 50 μg protein of each sample was mixed with 2X SDS buffer. The electrophoresis was performed to separate the protein mixture according to molecular weight, and the proteins were transferred on to the PVDF film. After blocking with skimmed milk, the membranes were incuabated with primary antibodies including p-I kappa alpha, cyclinD1, and CDK4 B, Bcl-2, and Bax at 4°C overnight. GAPDH was used as the internal reference. The membranes were washed 3 times and incubated with secondary antibody (1:10,000). The membranes were developed by DBA and the protein expression was quantified by Image J software.

Statistical analysis

SPSS21.0 software was used for statistical analysis. The measurement data were expressed as ($\bar{x}$ ± sd). One-way ANOVA was used for the comparison of multiple groups and the LSD-t test was used for comparison between two groups. P<0.05 indicated a significant difference.

Results

Comparison of C1orf63 mRNA expression among the groups

As shown in **Table 2**, there was no difference between the blank group and the empty group in the C1orf63 mRNA expression level

(P>0.05). Compared with the blank group and the empty group, the mRNA expression of C1orf63 was significantly increased in the C1orf63 overexpression group, but significantly decreased in the C1orf63 silenced group (P<0.05), indicating successful transfections.

Comparison of cell proliferation among the groups at different time points

As shown in **Figure 1**, there was no difference between the blank group and the empty group in the cell proliferation rate at 24 h, 48 h and 72 h (all P>0.05). Compared with the blank group and the empty group, the cell proliferation rate in the C1orf63 overexpression group was significantly higher, but significantly lower in the C1orf63 silenced group at at 24 h, 48 h and 72 h (all P<0.05). The cell proliferation rate in the C1orf63 silenced group was significantly lower than that in the C1orf63 overexpression group at 24 h, 48 h, and 72 h (all P<0.05).

Comparison of apoptosis rate in the groups at different time points

As shown in **Table 3** and **Figure 2**, there was no difference between the blank group and the empty group in the apoptosis rate at 24 h, 48 h and 72 h (all P>0.05). Compared with the blank group and the empty group, the apoptosis rate was significantly lower in the C1orf63 overexpression group, but significantly higher in the C1orf63 silenced group at at 24 h, 48 h, and 72 h (all P<0.05). The apoptosis rate in the C1orf63 silenced group was higher than that of the C1orf63 overexpressed group at 24 h, 48 h, and 72 h (all P<0.05).

*Comparison of cell cycle distribution among the groups*

As shown in **Table 4** and **Figure 3**, there was no difference between the blank group and empty group in the cell distribution rate in each phase (all P>0.05). Compared with the blank group and empty group, the cell distribution rate at G1 phase was significantly lower in the C1orf63 overexpression group, but significantly higher in the C1orf63 silenced group (all P<0.05); the
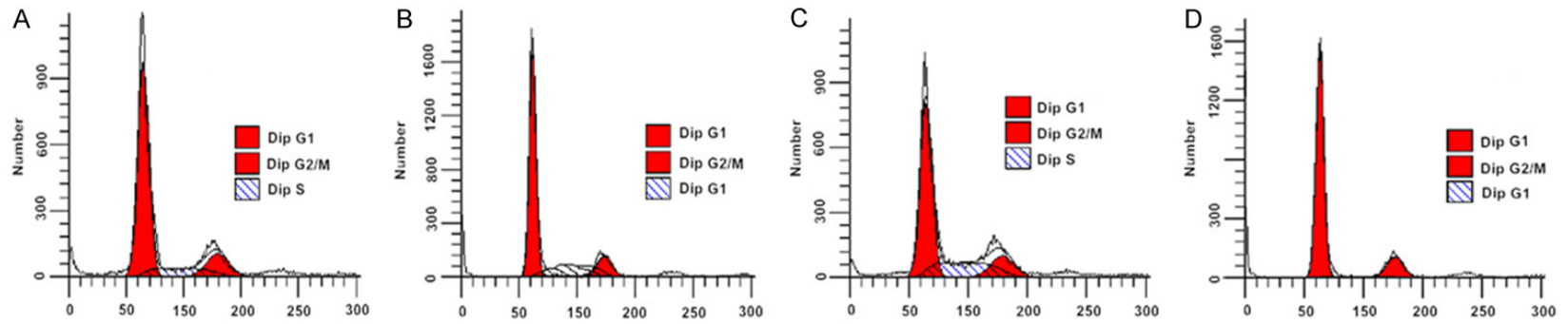
Figure 3. Flow cytometry analysis of cell cycle distribution in the groups. A: Blank group; B: Empty group; C: C1orf63 overexpression group; D: C1orf63 silenced group.
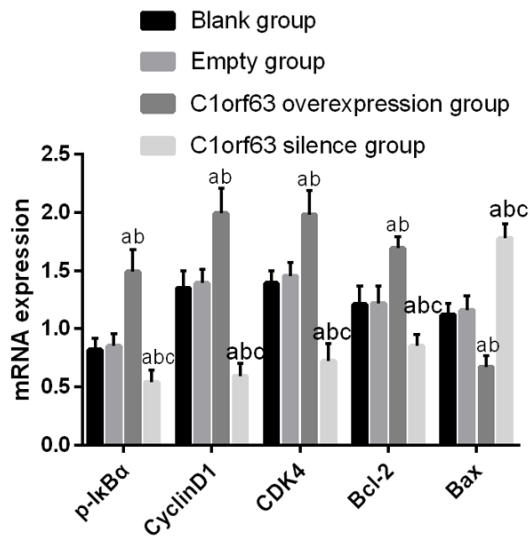
Figure 4. Comparison of mRNA expression levels of NF-κB signaling pathway factor in each group. Compared with the blank group, [a]P<0.05; compared with the empty group, [b]P<0.05; compared with the C1orf63 overexpression group, [c]P<0.05.

cell distribution rate at S and G2/M phase was significantly higher in the C1orf63 overexpression group, but significantly lower in the C1orf63 silenced group (all P<0.05). The cell distribution rate at G1 phase in the C1orf63 silenced group was higher than that in the C1orf63 overexpression group, while the cell distribution rates at S and G2/M phase in C1orf63 silenced group were lower than those in the C1orf63 overexpression group (all P<0.05).

Comparison of mRNA and protein expression of NF-κB signaling pathway factors in each group

As shown in **Figures 4**, **5**, there was no significantdifference between the blank group and empty group in the mRNA and protein expression levels of NF-κB signaling pathway factors, (p-IκBα, cyclinD1, CDK4, and Bcl-2 all P>0.05). Compared with the blank group and empty group, the mRNA and protein expression levels of p-IκBα, CyclinD1, CDK4, and Bcl-2 were significantly higher in the C1orf63 overexpression group, but significantly lower in the C1orf63 silenced group (all P<0.05); the Bax mRNA and protein expression levels were significantly lower in the C1orf63 overexpression group but significantly higher in the C1orf63 silenced group (all P<0.05). The mRNA and protein expression levels of p-IκBα, CyclinD1, CDK4,

and Bcl-2 in the C1orf63 silenced group were lower than those in the C1orf63 overexpression group. But, the Bax mRNA and protein expression levels in the C1orf63 silenced group were higher than those in the C1orf63 overexpression group (all P<0.05).

Discussion

Breast cancer is a common malignant tumor. The incidence of female breast cancer is rising gradually, and breast cancer has become the leading cause of female mortality [4, 5]. C1orf63 is a newly discovered gene that may be involved in regulating the cell cycle. High expression of C1orf63 indicates a poor prognosis of breast cancer, suggesting that C1orf63 may promote the occurrence and development of breast cancer [6]. Studies have shown that increased proliferation and decreased apoptosis of cancer cells are key factors leading to the occurrence and development of malignant tumor. Therefore, down-regulation of C1orf63 may be an effective way to inhibit the proliferation and promote the apoptosis of BCCs [7].

In this study, the expression of C1orf63 was regulated in the BCCs to observe the effects of C1orf63 on the biologic behavior of BCCs. We found that down-regulation of C1orf63 could inhibit the proliferation and promote apoptosis in a time-dependent manner. Previous studies have shown that blocking cancer cells at G0 phase can increase the apoptosis of cancer cells [8-11]. Some scholars have noted that blocking the cycle distribution of cancer cells is of great significance to promote apoptosis [12, 13]. In our study, we found that the proportion of BCCs in G0 phase was relatively high when the expression of C1orf63 was down-regulated. This indicated that down-regulation of C1orf63 could block the cell cycle of breast cancer, thus promoting the apoptosis of BCCs to some extent.

The expression of NF-κB signaling pathway factors (p-IκBα, CyclinD1, CDK4, Bcl-2 and Bax) is closely related to apoptosis. p-IκBα is a nuclear transcription factor that is commonly expressed in the cytoplasm of a variety of cells. p-IκBα participates in various physiologic and pathologic processes, such as cell proliferation and apoptosis. p-IκBα is a protein family composed of a complex polypeptide subunit [14, 15].
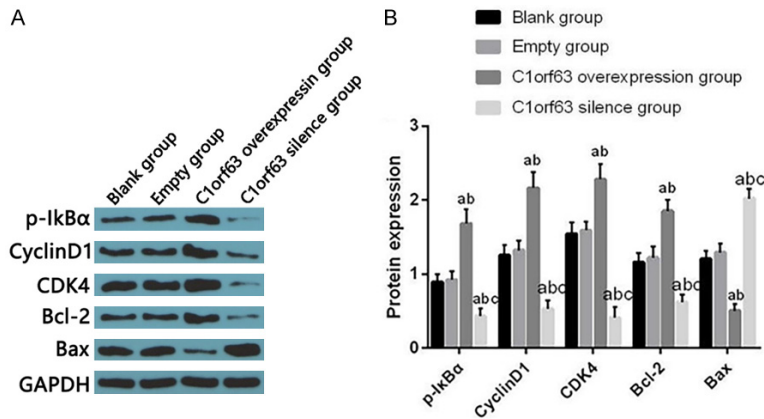
Figure 5. Comparison of protein expression of NF-κB signaling pathway factor in each group. A: Respresentive western blot images of NF-κB signaling pathway factors; B: The quantification results of protein expression of NF-κB signaling pathway factors. Compared with the blank group, [a]P<0.05; compared with the empty group, [b]P<0.05; compared with the C1orf63 overexpression group, [c]P<0.05.

CyclinD1 plays an important role in cell cycle regulation, which can push cells into the late G1 phase and regulate cell cycle proteins [16]. The estrogen-independent proliferation of BCCs is caused by CyclinD1 and other G0/G1 cyclins. The changes in cyclinD1 and CDK4 play an important role in the occurrence of ovarian cancer, and the balance between these two oncogenes is very important for gene therapy of ovarian cancer [17]. CDK4 is the regulatory center of the cell cycle in the G0-S phase. The amplification of CDK4 has been found to be related to a variety of human tumors and cell lines, suggesting that the occurrence of tumor is closely related to abnormal expression of CDK4 [18]. Bcl-2 plays a critical role in the negative regulation of apoptosis, and it can prolong the survival time of cells by inhibiting apoptosis [19]. Bax does not induce apoptosis directly, but it triggers apoptosis by significantly accelerating the transmission of cell death signals [20]. Bcl-2 and Bax form heterodimers, and their balance is a vital factor for cell apoptosis as the interaction between them determines the threshold of cell death [21, 22]. In the study of Yue et al., they found that NF-κB/cyclinD1 signaling pathway is involved in inhibition of the proliferation of BCCs [23]. The results of our study showed that down-regulation of C1orf63 acts on the NF-κB signaling pathway to regulate the expressions of downstream factors (p-IκBα, CyclinD1, CDK4, Bcl-2, and Bax), to further inhibit cancer cell proliferation, promote cancer cell apoptosis, and block the cell cycle. These results were inconsistent with the above studies.

However, the mechanism has not been studied deeply, and there are no clinical data. Therefore, the results of this study need to be further confirmed, and some experiments must be done to further clarify the regulation effects of C1orf63 on breast cancer, so as to provide a new direction for clinical diagnosis and treatment.

In conclusion, C1orf63 inhibits the NF-κB signaling pathway to regulate the expression of downstream factors (p-IκBα, CyclinD1, CDK4, Bcl-2, and Bax), so as to inhibit BCC proliferation, promote cancer cell apoptosis, and block the cell cycle.

Disclosure of conflict of interest

None.

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