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Silencing of long non-coding RNA CCHE1 inhibits the ovarian cancer SKOV3 cell invasion and migration and inactivates the p38-MAPK signaling pathway

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Abstract: Ovarian cancer (OC) is a major cause of cancer-related deaths among gynaecological malignancies. Emerging studies suggest that the long non-coding RNA (lncRNA) may be the potential biomarker for the diagnosis and prognosis of the cancer. The current study was carried out to investigate the role of lncRNA CCHE1 silencing in OC cell invasion and migration. Expression of lncRNA CCHE1 in normal ovarian cell Hose and OC cell lines HO 8910, A2780 and SKOV3 was detected. LncRNA were transfected with siRNA, and then the proliferation of cells was detected by using MTT assay. Cell invasion and migration was measured by using Transwell assay and scratch test, respectively. The protein levels of E-cadherin, N-cadherin, ERK, p38-MAPK and the phosphorylation of ERK and p38-MAPK in cells after siRNA transfection were detected by using Western blot analysis. Consequently, lncRNA CCHE1 expression was highly expressed in OC cell lines, especially in SKOV3 cells. siRNA1, siRNA2 and siRNA3 all decreased. lncRNA CCHE1 expression in SKOV3 cells and siRNA2 showed the best silencing efficacy. Silencing of lncRNA CCHE1 decreased proliferation, invasion and migration, and reduced the protein levels of N-cadherin, ERK, p38-MAPK and the phosphorylation of ERK and p38-MAPK, while reducing the protein level of E-cadherin in SKOV3 cells. Collectively, our study proved that the silencing of lncRNA CCHE1 could inhibit SKOV3 cell invasion and migration via inactivating the p38-MAPK signaling pathway.

Introduction

Ovarian cancer (OC) is deemed as one of the leading life-threatening gynecological malignancies, accounting for about 3% of all cancer incidences in female with 240,000 cases newly diagnosed and 150,000 death cases annually worldwide (Hua *et al.*, 2018). In China, the incidence and mortality of ovarian cancer have increased over the past decade (Chen *et al.*, 2016a; Chen, 2015). The majority OC patients show peritoneal metastases and suffer from perennial malignant ascites that lead to severe symptoms (Wolterink *et al.*, 2010). In the early stages of OC, the tumors are limited in the ovary, which are more likely to be cured with an over 90% 5-year survival rate (Wang *et al.*, 2019). However, owing to insufficient specific symptoms at early stage, OC is always diagnosed at advanced stage that is hard to be cured simply by surgical resection, and relapse commonly occurs during the clinical course of OC (Bareiss *et al.*, 2013). Despite efficient surgical interventions and the application of different anticancer drug combinations, the 5-year survival rate of patients with advanced stage OC was only 30% (Varughese *et al.*, 2011). Because of distinct biology of OC, the effective treatment for this disease remains limited (Hua *et al.*, 2018). Thus, it is urgent to develop novel therapeutic options for OC.

Long non-coding RNAs (lncRNAs) are defined as the molecules of transcribed RNA no less than 200 nucleotides as an important category of genes participating in diverse biological functions (Wang and Chang, 2011). LncRNAs can regulate proliferation, replication, invasion, metastasis and drug resistance of cancer cells, thus their abnormal expression may be an important promoter in cancer progression (Ning *et al.*, 2016; Wu *et al.*, 2018; Hauptman and Glavac, 2013), as well as in OC (Chen *et al.*, 2016b; Chen *et al.*, 2018b). Among the lncRNAs, the high-expressed long non-coding RNA (lncRNA) 1 (lncRNA CCHE1) of cervical carcinoma has been recognized as an oncogene in cervical cancer (Yang *et al.*, 2015), but its role in OC is rarely concerned. Activation of the extracellular-signal-regulated kinase/p-38 mitogen activated protein

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kinase (ERK/p38-MAPK) signaling pathway has been found to play a critical role in cancer development (Liu *et al.*, 2019; Yuan *et al.*, 2019; Fang *et al.*, 2018), including in OC (Zhang *et al.*, 2019a). Here, we hypothesized that CCHE1 may promote OC development with the involvement of the p38-MAPK pathway, and cell experiments were performed to identify this hypothesis.

Materials and Methods

Cell culture

Normal ovarian cell line Hose and OC cell lines HO8910, A2780 and SKOV3 were purchased from Shanghai Institute of Biochemistry and Cell Biology, and Chinese Academic of Science (Shanghai, China). The cells were placed in a 37°C incubator containing 5% CO_2 (thromo3111, Jinan Beisheng Medical Instrument Co., Ltd., Shandong, China) and incubated in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS, Gibco Company, Grand Island, NY, USA), 50 U/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gioco) with the medium refreshed every 2 days. When the cell confluence reached 80%, the cells were detached with trypsin and passaged, and the cells in the logarithmic growth period were collected for the follow-up experiments.

Cell grouping and transfection

The well-grown SKOV3 cells in the logarithmic growth period were seeded into 6-well plates at the density of 1×10^5 cells/well. The cells were incubated in Dulbecco's Modified Eagle's Medium one day before transfection. The cells were allocated into 5 groups: the blank group, the small interfering-negative control group (si-NC, cells transfected with 50 nM si-NC), the siRNA 1/siRNA 2/siRNA 3 lncRNA CCHE1 groups (cells transfected with 50 nM siRNA 1/siRNA 2/siRNA 3 lncRNA CCHE1 respectively). All transfections were performed as per the instructions of the lipofectamin 2000 kit (11668-019, Invitrogen, New York, California, USA). Each group of plasmids (100 pmol for each) were diluted in 250 μL serum-free medium (ensure the final concentration at 50 nM), and 5 μL lipofectamin 2000 was diluted in another 250 μL serum-free medium, after which the two dilutions were fully mixed up, standing for 20 min, and then seeded into 6-well plates. Cells were incubated at 37°C with 5% CO_2 after transfection, and the medium was discarded 48 h later, and replaced as complete medium containing RPMI-1640 for another 48 h incubation. Next, the cells were collected for the following experiments. All transfection sequences and plasmids were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Each experiment was repeated for 3 times.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Forty-eight hours after transfection, the cells were collected, and then the total RNA of cells was extracted by using Trizol (No. 16096020, Thermo Fisher Scientific, New York, USA). Then the RNA was reversely transcribed into cDNA by using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara Holdings Inc., Tokyo, Japan). Next, qPCR was performed by using a SYBR® Premix Ex Taq™ II kit (Huai'an Xingzhi Biotechnology Co., Ltd., Jiangsu, China). The qPCR system included 25 μL SYBR® Premix Ex Taq™ II (2×), upstream (2 μL) and downstream (2 μL) PCR primers, 1 μL ROX Reference Dye (50×), 4 μL DNA template and 16 μL ddH_2O. Then the qPCR was run on ABI Prism® 7300 (Shanghai Kunke Instrument Co., Ltd., Shanghai, China). The PCR condition was as follows: predenaturation at 95°C for 10 min, followed by 32 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and a final extension at 72°C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was set as an internal reference, and then the relative expression of each target gene was expressed as $2^{-\Delta\Delta Ct}$, in which $\Delta Ct = Ct_{(target\ gene)} - Ct_{(GAPDH)}$, and $\Delta\Delta Ct = \Delta Ct_{(experimental\ group)} - \Delta Ct_{(control\ group)}$. The experiment was performed in triplicate. The primer sequences were shown in Tab. 1.

Western blot analysis

Cells were then detached with trypsin and harvested 48 h after transfection, and then the total proteins from cells were extracted by using radio-immunoprecipitation assay cell lysis buffer containing phenylmethylsulfonyl fluoride (R0010, Solarbio Science & Technology Co., Ltd., Beijing, China). The protein concentration was detected by using a bicinchoninic acid kit (Thermo Fisher Scientific) and adjusted with deionized water. The samples were mixed with loading buffer, warm bathed, and then 30 μg protein samples were added to each lane and run on the gel electrophoresis at 80 V for 2 h. The proteins were wet transferred onto polyvinylidene fluoride membranes (ISEQ0010, Millipore, Billerica, MA, USA) at 100 V for 2 h. The membranes were sealed with 5% skimmed milk at 4°C for 2 h, and then washed once with tris-buffered saline-tween (TBST) after discarding the sealing liquid. Next, the membranes were incubated with the following primary antibodies at 4°C overnight: E-cadherin (ab76055, 1:500), N-cadherin (ab18203, 1:1000), extracellular-signal-regulated kinases (ERK, ab3237, 1:1000), p-ERK (ab65142, 1:1000), p38 mitogen-activated protein kinase (p38-MAPK, ab197348, 1:1000), p-p38-MAPK (ab221011, 1:1000) and GAPDH (ab8226, 1:2000) (all purchased from Abcam, Cambridge, UK). After that, the membranes were washed 3 times with TBST (10 min for each), and then incubated with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (ab6721, 1:1000, Abcam). Then the membranes were rinsed with TBST and placed on clean glass plates. Equal volume of reagent A and reagent B in enhanced chemiluminescence kit (BB-3501, Shanghai BestBio, China) was fully mixed up in a dark room and then added on membranes. Then the membranes were exposed and imaged in a gel imager, photographed in a Bio-Rad image system (Bio-Rad, Inc., Hercules, CA, USA), and the grey value of each bad was analyzed by using the Image J software. The relative protein concentration was calculated via the ratio of the grey values of target protein band/GAPDH protein band, while the phosphorylation of protein was evaluated via the ratio of phosphorylated protein/total protein. The experiment was repeated for 3 times.

TABLE 1

Primer Sequences for RT-qPCR

Gene	Primer sequence
lncRNA CCHE1	F: 5'-CTTTCCGTTTCTTGTGATTGAG-3'
	R: 5'-AGGTGTTGGAGTAGGCTTAGCT-3'
GAPDH	F: 5'-GGGCTGCTTTTAACTCTGGT-3'
	R: 5'-GCAGGTTTTTCTAGACGG-3'

Note: RT-qPCR, reverse transcription quantitative polymerase chain reaction; lncRNA CCHE1, cervical carcinoma high-expressed long non-coding RNA (lncRNA) 1; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; F = forward; R = reverse.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The cells were detached, collected and counted 48h after transfection. Then the cells were seeded into 96-well plates at the density of 5×10^3 cells per well. The volume of each well was 100 μL, and 6 duplicated wells were set. Each well was added with 100 μL dimethyl sulphoxide, and then the optical density (OD) value at 570 nm of each well was read by using an enzyme-linked immunosorbent assay kit (Epoch microporous plate spectrophotometer, BioTek Instruments Inc., Winooski, VT, USA). A curve of cell viability was then produced with the time points set as the abscissa and the OD value as the ordinate.

Scratch test

48 hours after transfection, the cells were seeded into 6-well plates at the density of 5×10^5 cells per well. When the cell confluence reached nearly 90%, a scratch was produced in the midline of each well by using a sterile pipette, and each scratch was maintained at the same width. Next, the plates were further incubated in a serum-free medium, and the cells were photographed at 0h and 24 h after scratching respectively. The migrating distance of cells were detected by using an Image-Pro Plus Analysis software (Media Cybernetics, USA). Five fields of view were randomly selected and photographed. Three duplicated wells were set for each group. The experiment was repeated for 3 times.

Transwell assay

Transwells (JRDUN Biotechnogy (Shanghai) Co., Ltd., Shanghai, China) were placed into 96-well plates, and the apical chamber was coated with Martrigel (Shanghai Qcbio Science and Technologies Co., Ltd., Shanghai, China) diluent (1:8) and dried. Each group of cells were normally detached and washed twice with phosphate buffer saline (PBS), resuspended in RPMI 1640 medium, and adjusted to 1×10^5 cells/mL. Next, 200 μL cell suspension was filled into the aptical chamber and the basolateral chamber was filled with 600 μL RPMI1640 medium containing 20% FBS. Followed by 24 h normal incubation, the transwells were taken out and the cells on the inner side of the apical chamber were wiped away. Then the invaded cells were fixed with 4% polyformaldehyde (Beijing Leagene Biotechnology Co., Ltd., Beijing, China) for 15 min, stained with 0.5% crystal violet (Solarbio) for 15 min, washed with PBS for 3 times and observed under an inverted microscope (CKX53, Olympus Optical Co., Ltd., Tokyo, Japan) with 5 fields (200×) randomly selected, and then the number of invaded cells was calculated. The experiment was performed for three times.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS 22.0, IBM Corp. Armonk, NY, USA) was applied for data analysis. Kolmogorov-Smirnov test checked the data in normal distribution. Measurement data were expressed as mean ± standard deviation. Differences among multiple groups were compared by using one-way analysis of variance (ANOVA) and Tukey's post-hoc test was applied for pairwise comparisons after ANOVA. p was obtained from two-tailed test, and $p < 0.05$ was considered a statistically significant difference.

Results

LncRNA CCHE1 is highly expressed in OC cells

To identify the expression difference of lncRNA CCHE1 between OC cells and normal ovarian cells, we detected the lncRNA CCHE1 expression in normal ovarian cell line Hose and OC cell lines HO 8910, A2780 and SKOV3. The results showed that compared to the Hose cell line, the lncRNA CCHE1 expression was notably elevated in OC cell lines, especially in SKOV3 cells (all $p < 0.05$) (Fig. 1). Thus, SKOV3 cell line was selected for the following experiments.

Silencing of lncRNA CCHE1 reduces SKOV3 cell proliferation

As lncRNA CCHE1 was highly expressed in OC cells, we treated CCHE1 with siRNA 1, siRNA2 and siRNA3 respectively to investigate the roles of CCHE1 knockdown in OC cell growth. Firstly, we measured the transfection efficiencies of 3 siRNA, the results (Fig. 2A) suggested that the lncRNA CCHE1 expression both declined after siRNA1, siRNA2 and siRNA3 transfections. Among them, siRNA2 showed the best silencing efficiency on lncRNA CCHE1 expression, thus siRNA2 was applied for lncRNA CCHE1 intervention in the followed-up experiments. Next, the cell proliferation was measured by using MTT assay, and the results (Fig. 2B) identified that the cell proliferation showed little difference between the si-NC group and the blank group ($p > 0.05$). Compared to the si-NC group, the cell proliferation significantly declined in the siRNA2 lncRNA CCHE1 group at 48 h and 72 h (all $p < 0.05$) respectively, indicating that the silencing of lncRNA CCHE1 could inhibit the proliferation of SKOV3 cells.

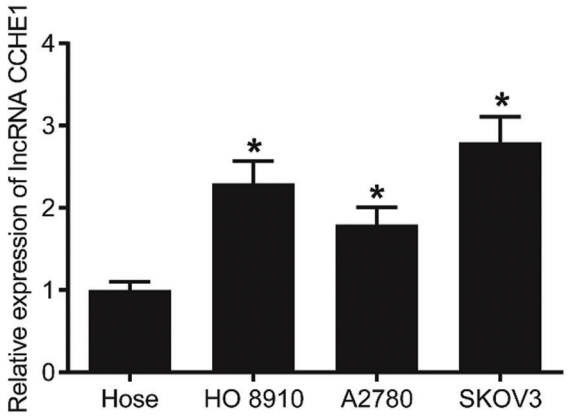

FIGURE 1. LncRNA CCHE1 is highly expressed in OC cells. lncRNA CCHE1 expression in normal ovarian cell line Hose and in OC cell lines HO 8901, A2780 and SKOV3 were detected by using RT-qPCR. *, compared to the Hose cells, $p < 0.05$. Repetition = 3.

Silencing of lncRNA CCHE1 suppresses SKOV3 cell migration

The cell migration was detected by using scratch test. The results suggested that (Fig. 3) the cell migration showed no major difference between the si-NC group and the blank group ($p > 0.05$). Compared to the si-NC group, the migration of SKOV3 cells in the siRNA2 lncRNA CCHE1 group was significantly inhibited at 24 h ($p < 0.05$), indicating that the silencing of lncRNA CCHE1 suppressed the migration of SKOV3 cells.

Silencing of lncRNA CCHE1 inhibits SKOV3 cell invasion

The Transwell assay was performed to evaluate the invasion of SKOV3 cells in each group. The results (Fig. 4) suggested that the number of invaded cells presented little difference between the blank group and the si-NC group ($p > 0.05$). Compared to the si-NC group, the number of invaded cells notably declined in the siRNA 2 lncRNA CCHE1 group ($p < 0.05$), indicating the silencing of lncRNA CCHE1 could inhibit SKOV3 cell invasion.

Silencing of lncRNA CCHE1 inactivates the ERK signaling pathway and reduces N-cadherin expression while increasing E-cadherin expression in SKOV3 cells

The above findings triggered us to further figure out the underlying mechanism involved in the lncRNA CCHE1 silencing-related events. We detected the protein levels of p38-MAPK signaling pathway-related factors ERK and p38-MAPK, and the levels of E-cadherin and N-cadherin. The results (Fig. 5) suggested that the protein levels of the above factors presented no major difference between the si-NC group and the blank group cells (all $p > 0.05$). Compared to the si-NC group, the phosphorylation of ERK and p38-MAPK was significantly reduced and the N-cadherin protein decreased while the E-cadherin protein level increased in the siRNA 2 lncRNA CCHE1 group (all $p < 0.05$). These results identified that the silencing of lncRNA CCHE1 could inactivate the p38-MAPK signaling pathway and inhibit SKOV3 cell invasion by regulating the invasion-related factors.

Discussion

The 5-year overall survival rate of OC patients has not been improved significantly in the past two decades due to limited therapy (Gao *et al.*, 2019). LncRNAs have been found to play an essential biological role in cancer development including OC (Qiu *et al.*, 2019; Zhang *et al.*, 2019b). In the current study, we investigated the roles of lncRNA CCHE1 in OC with the conclusion that the silencing of lncRNA CCHE1 could inhibit SKOV3 cell invasion and migration. The functions of lncRNA CCHE1 in OC were possibly mediated with the involvement of the ERK/p38-MAPK signaling pathway.

Initially, our study found that lncRNA CCHE1 was highly expressed in OC SKOV3 cells when compared with that in normal ovarian cells. The silencing of lncRNA CCHE1 was found to reduce SKOV3 cell proliferation, invasion and migration with the protein level of E-cadherin increased and the level of N-cadherin decreased. LncRNA CCHE1 was firstly found to be highly expressed in cervical cancer cells and promote the cell proliferation (Peng *et al.*, 2016; Yang *et al.*, 2015; Chen *et al.*, 2017; Aalijahan and Ghorbian, 2019). Likewise, it has been documented that lncRNA CCHE1 promoted proliferation, invasion and metastasis of non-small lung cancer cells (Liao *et al.*, 2018), gastric cells (Xu *et al.*, 2018) and colorectal carcinoma cells (Gaballah *et al.*, 2019). E-cadherin and N-cadherin are well-known epithelial mesenchymal transformation (EMT)-related biomarkers, (Zhou *et al.*, 2019). E-cadherin is a

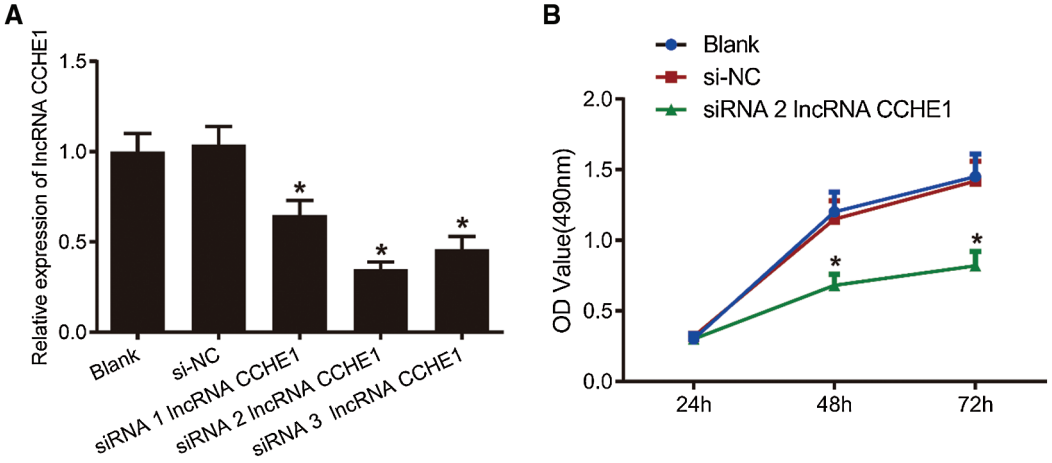

FIGURE 2. The silencing of lncRNA CCHE1 could reduce the proliferation of SKOV3 cells. A, lncRNA CCHE1 expression of SKOV3 cells after siRNA transfections was detected by using RT-qPCR; B, SKOV3 cell proliferation was detected by using MTT assay. *, compared to the si-NC group, $p < 0.05$. Repetition = 3.

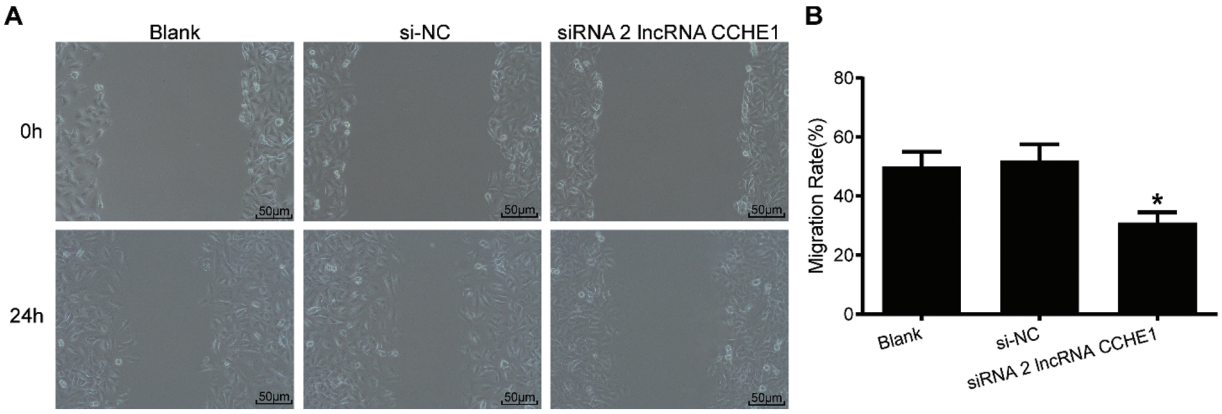

FIGURE 3. The silencing of lncRNA CCHE1 suppresses SKOV3 cell migration.
A, the image of cell migration was measured via scratch test; B, the histogram of the migration rate of cells. *, compared to the si-NC group, $p < 0.05$. Repetition = 3.

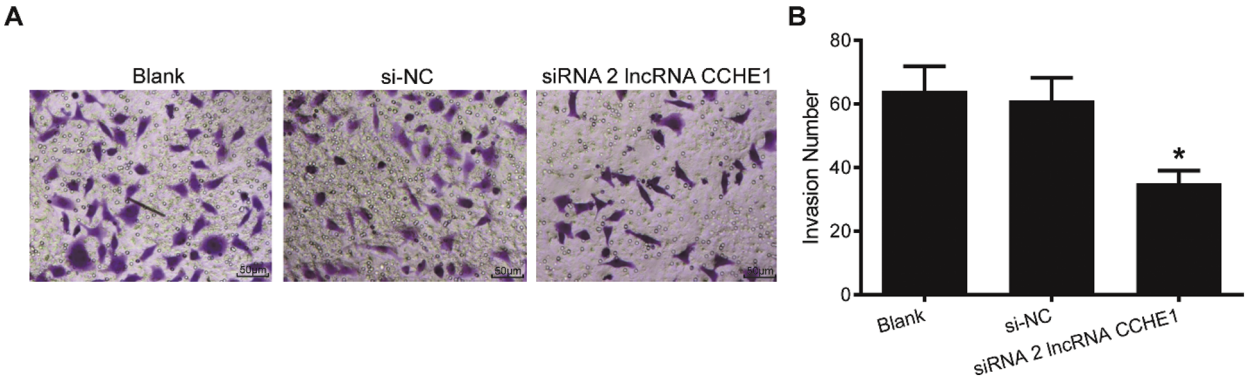

FIGURE 4. Silencing of lncRNA CCHE1 inhibits SKOV3 cell invasion.
A, the image of cell invasion was measured by using Transwell assay and observed under a microscope (200×); B, the histogram of the number of invaded cells. *, compared to the si-NC group, $p < 0.05$. Repetition = 3

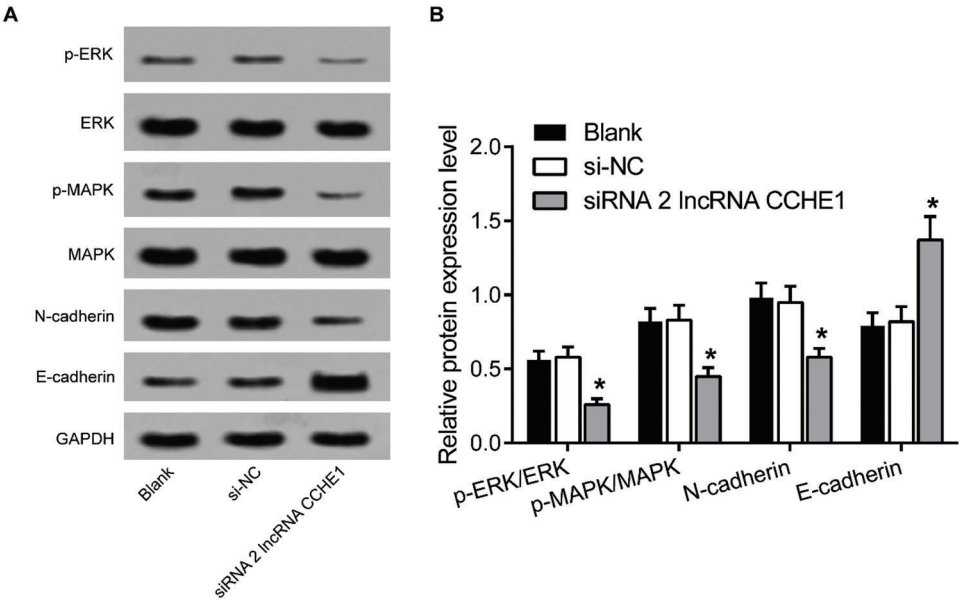

FIGURE 5. Silencing of lncRNA CCHE1 inactivates the ERK signaling pathway, reduces N-cadherin expression while increases E-cadherin expression in SKOV3 cells.
A-B, the protein bands (A) and the protein levels (B) of p38-MAPK pathway-related factors and invasion-related factors were detected by using Western blot analysis. *, compared to the si-NC group, $p < 0.05$. Repetition = 3.

junction protein between cells and always down-regulated or disappeared during EMT progress (Zhao *et al.*, 2014). Moreover, it has been documented that E-cadherin may switch into N-cadherin during EMT (Rogers *et al.*, 2013). These findings have further identified that the silencing of lncRNA CCHE1 could reduce SKOV3 invasion and migration from the molecule perspective.

Then the focus of the study shifted to find out the underlying mechanisms involved in the events above. Importantly, our study found that the silencing of lncRNA CCHE1 could decrease the

protein levels of ERK and p38-MAPK in SKOV3 cells. The ERK/MAPK signaling pathway could regulate many cellular processes including differentiation, proliferation, cell death and survival in all eukaryotes (Whelan *et al*., 2012). Up-regulation of MAPK/ERK has been documented to promote the aggressiveness of OC cells (Chen *et al*., 2018a). Likewise, the activation of ERK/P38-MAPK has been suggested to be involved in the promotion of OC metastasis (Lu *et al*., 2016). It has been suggested that lncRNA CCHE1 could elevate the ERK expression and promote tumor metastasis and vascular invasion in colorectal carcinoma (Gaballah *et al*., 2019). Similarly, lncRNA CCHE1 has been identified to promote the carcinogenesis of hepatocellular carcinoma via activating the ERK/MAPK signaling pathway (Peng and Fan, 2016). Here, our study identified that the silencing of lncRNA CCHE1 might reduce OC cell invasion and migration via inactivating the ERK/p38MAPK signaling pathway.

To conclude, our study has provided evidences that lncRNA CCHE1 could inhibit invasion and migration of OC SKOV3 cells partly via down-regulation of the ERK/p38-MAPK signaling pathway. These findings may offer new insights in the prevention and treatment of OC. However, the potential molecular mechanisms by which CCHE1 affected the ERK/MAPK still remain unclear, so our studies in the near future will focus on studying the detailed underlying molecular mechanisms involved in the events.

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Conflicts of Interest: We declare no conflicts of interest.

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