EJGO European Journal of Gynaecological Oncology

CLC-3 Cl- channel-mediated invasion and migration of human ovarian cancer cells

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Summary

Objectives: To investigate the potential role of CLC-3, a member of the voltage-gated chloride channel (CLC) superfamily, in invasion and migration of ovarian cancer cell line SKOV3. *Materials and Methods:* CLC-3 antisense oligonucleotides were transfected into ovarian cancer cell line SKOV3, and its effects on cell invasion and migration were analyzed by using Transwell chamber assay and wound healing assay *in vitro*. The efficiency of CLC-3 antisense was determined with RT-PCR and Western blotting. The protein concentrations of matrix metalloproteinase (MMP)-2, MMP-9, and vascular endothelial growth factor (VEGF) were determined using ELISA kits. Cell volume measurements were performed. *Results:* Studies *in vitro* revealed that the CLC-3 antisense inhibited invasion and migration of ovarian cancer cell line SKOV3. CLC-3 antisense treatment decreased protein levels of MMP-2, MMP-9, and VEGF in culture medium of SKOV3 cells. In addition, the authors found that the capability for regulatory volume decrease (RVD) was much attenuated in SKOV3 cells transfected with CLC-3 antisense. *Conclusions:* These results strongly suggest that CLC-3 may get involved in proliferation, invasion, and migration of ovarian cancer cells and thus may be a useful therapeutic target.

Key words: Chloride channels; Invasion; Migration; Ovarian cancer.

Introduction

Ovarian cancer characterized by its incongruity between symptomatology and early-stage disease is one of the most lethal gynecologic malignant tumor. 75% of women with ovarian cancer are diagnosed with already widespread intraperitoneal metastasis, invasion, ascites, and the lack of effective therapies for advanced-staged disease [1, 2]. Thus, there is an imperative need to explore new therapeutic targets and a better understanding of the mechanisms involved in the invasion and metastasis of ovarian carcinoma. The factors involved in the invasion of ovarian cancer, however, are poorly understood.

The voltage-gated chloride channel (CLC) family plays an important role in the ionic and osmotic homeostasis of many cell types. CLCs are generally gated by transmembrane voltage, but also display gating in response to cell swelling and PH. In the present authors' previous study, the results strongly suggest that chloride channels may regulate proliferation and invasion of human ovarian cancer cell and endometrial cancer cell [3-5]. It is, however, unclear which chloride channel plays a key role in ovarian cancer cell invasion and migration. CLC-3, a most notably CLC family member, has been found to be specifically upregulated in glioma membranes and, in turn, facilitate cells divide or invade through extracellular brain spaces [6]. In fact, the CLC-3 channel attracts more and more attention, for it may play as another role in which the CLC-3 was reported to be a fundamental molecular candidate of volume-regulated Cl^- channel (VRC) [7,8]. VRC activation results in Cl^- efflux that is accompanied by cation and water efflux out of cells which causes subsequent regulatory volume decrease (RVD), a cellular defensive mechanism against hypotonic stress. There are ample evidences indicating that volume-activated chloride channels and subsequent RVD participate in the process of the proliferation, invasion, and migration of tumor cells [9-11]. Based on the emerging effects of CLC-3 itself on proliferation and invasion of cancer cells, and the strong link between the CLC-3 and VRC, the authors propose that CLC-3 Cl^- channel is a requirement for successful invasion and migration of ovarian cancer cell.

The lack of specific and potent pharmacological blockers of CLC-3 has been a major obstacle to further studies on CLC-3 function in human cancer. Therefore, the authors, in the present study, used antisense technology to knock down the expression of CLC-3 in human ovarian cancer cell line SKOV3 in an effort to explore the role and mechanisms of CLC-3 in the invasion and migration of ovarian cancer. They considered this an important undertaking because CLC-3 protein could represent a potential new target for cancer therapy.

Materials and Methods

Cell culture

Human epithelial ovarian cancer cell line SKOV3 was obtained from Basic Medicine Research Institute, Qilu Hospital, Shandong

Revised manuscript accepted for publication July 15, 2015

Eur. J. Gynaecol. Oncol. - ISSN: 0392-2936
XXXVII, n. 5, 2016
doi: 10.12892/ejgo3187.2016

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University, China. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and maintained at 37°C in a humid atmosphere of 5% CO_2 in air.

Antisense knockdown assay

The antisense and sense oligonucleotides corresponding specifically to the initiation codon region of the human CLC-3 mRNA were synthesized as reported previously [12]. The antisense sequence was 5′-TCC ATT TGT CAT TGT-3′. The sense had the sequence 5′-ACA ATG ACA AAT GGA-3′. The first three bases at either end in both oligonucleotides were phosphorothioated. To examine the uptake of oligonucleotide by the cells, the oligonucleotides were labeled with fluorescence. SKOV3 cells were incubated for 24 hours to allow them to reach 70% confluency. Cells were then washed in triplicate with serum-free RPMI-1640 and incubated with sense (100 μg/ml) or antisense oligonucleotide (25, 50, and 100 μg/ml) in serum-free RPMI-1640 containing five-μg/ml lipofectamine 2000 at 37°C. The medium was then replaced with 10%FBS RPMI-1640 medium six hours later before culturing at 37°C for 48 hours.

Semiquantitative reverse transcription-PCR of CLC-3

Total RNA was isolated from the cultured SKOV3 cells using the Trizol reagent and reverse-transcribed into first strand cDNA using M-MLV reverse transcriptase. The cDNA was used for subsequent PCR using primers specific for human CLC-3 as reported previously [13]. The sequences of the primer used were as follows; CLC-3 (235 bp product), sense, 5′-GGCAGCATTAACAGTTC-TACAC-3′; antisense, 5′-TTCCAGAGCCACAGGCATATGG-3′.β-actin (247 bp product) which was used as a loading control, sense, 5′-AACTCCATCATGAAGTGTGA-3′; antisense, 5′-ACTCCTGCTTGCTGATCCAC-3′. PCR cycling conditions of were as follows: an initial denaturation step of 94°C for five minutes, 94°C for one minute, annealing at 58°C for one minute, and elongation at 72°C for one minute. A final elongation step of ten minutes at 72°C occurred on the last cycle. PCR reactions were cycled 40 times.

Western blotting of CLC-3

For Western blotting analysis, the protein content of SKOV3 cell lysates was separated by SDS-PAGE and transferred to nitrocellulose membranes. The membrane was then immunoblotted with rabbit anti-CLC-3 antibody (1:300) overnight at 4°C followed by reaction with goat anti-rabbit antibody linked to horseradish peroxidase. Final detection was carried out with chemiluminescent reagent.

Electrophysiology and cell volume measurement

Electrophysiological experiments and cell volume measurements were performed as described previously [9, 12]. The whole-cell mode of the patch-clamp technique was used to measure membrane potentials and membrane currents. Currents were monitored with an EPC-9 patch clamp amplifier. Patch electrodes had a resistance of between three to five MΩ. Once the whole cell configuration was established, the cell was held at 0 mV, and test potentials were applied from -100 to +80 mV for 400 ms, with an increment of +20 mV at five-second interval between steps. The pipette solution was composed of (in mM): 105 N-methyl-D-glucamine chloride (NMDG-Cl), 1.2 $MgCl_2$, 10 Hepes, 1 EGTA, 70 D-mannitol, and 2 ATP. The isotonic bath solution contained (in mM): 105 NaCl, 0.5 $MgCl_2$, 2 CaCl2, 10 Hepes, and 70 D-mannitol. The hypotonic bath solution was achieved by omitting the D-mannitol from the solution, reaching an osmolarity of 230 mosmol/l. The pH of the pipette and bath solutions was adjusted to 7.25 and 7.4, respectively, with Tris base. SKOV3 cell images were captured every 60 seconds by an inverted microscope with a CCD camera and stored directly onto the computer. Cell volume (V) was calculated from the imaging area (S) with the following equation: $V = 4/3 \times S \times (S/\pi)^{1/2}$. The RVD was calculated with the following relation: $RVD\ (\%) = (V_{max}-V_{min})/(V_{max}-V_0) \times 100\%$, in which V_0 is the cell volume in isotonic solution before hypotonic stress, V_{max} is the peak volume in hypotonic solution, and V_{min} is the volume before return to isotonic solution. All experiments were performed at room temperature (22-26°C).

Invasion and migration assays

The invasiveness of ovarian cancer cells was assayed using Transwell chambers. Briefly, polycarbonate filter (pore size, eight μm) was coated with 100 μl of Matrigel. SKOV3 cells were treated with oligonucleotide at indicated concentrations for 48 hours, and 1.0×10^5 cells with or without oligonucleotide were then introduced into the upper compartment. After incubation for 24 hours at 37°C, cells that had not penetrated the filter were removed with a cotton swab, and invaded cells on the lower surface of the filter were fixed with ice-cold methanol and stained. Results are presented as the mean number of invaded cells of five field ± SD of three independent experiments. To assess cellular migration potential, the protocol described above was used, except that Matrigel was omitted and complete medium was added in the lower compartment as a chemoattractant.

Wound healing assay

To study directional cell migration, the wound healing assay was employed. SKOV3 cells were treated with oligonucleotide at indicated concentrations for 48 hours, and were then plated in six-well plates. When the cells grew into confluency, artificial wounds were created by scraping with a micropipette tip. The wound closure was monitored at 0 and 36 hours between oligonucleotide treated and control group. Images of the wounds were taken digitally with an inverted microscope.

Determination of matrix metalloproteinase (MMP) and vascular endothelial growth factor (VEGF) concentrations

SKOV3 cells were incubated in the presence or absence of oligonucleotide for 48 hours. Conditioned media were then centrifuged to remove debris and stored at −80 °C. The protein concentrations of total MMP-2 (pro- and active MMP-2), total MMP-9 (pro- and active MMP-9), and VEGF were determined using ELISA kits. The concentrations of active MMP-2 and MMP-9 were determined using MMP collagenase activity assay kits according to the manufacturer's instructions. All assays were performed in triplicate.

Statistics

Data were presented as the mean ± standard error. Student's *t*-test was used for statistical analyses and differences were considered significant at $p < 0.05$.

Results

CLC-3 antisense downregulated CLC-3 RNA and protein expression in SKOV3 cells

Because of not existing specific CLC-3 chloride channel blockers, the authors used antisense oligonucleotide specifically against CLC-3 to inhibit CLC-3 expression. To determine the uptake of oligonucleotide by SKOV3

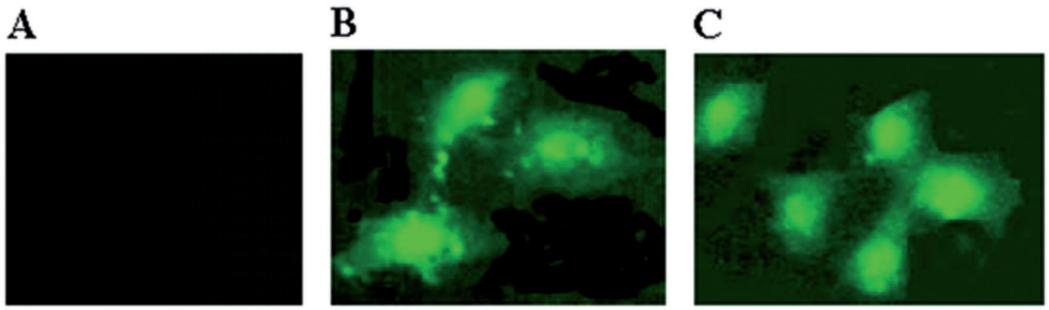

Figure 1. — Representative images of fluorence in SKOV3 cells (x400). After six hours transfection with oligonuceotides (labeled with fluorescein), the fluorescence was detected, indicating oligonucleotides have been uptaken by SKOV3. A: untransfected SKOV3 cells. B: SKOV3 cells transfected with 100 μg/ml CLC-3 sense. C: SKOV3 cells transfected with 100 μg/ml CLC-3 antisense.

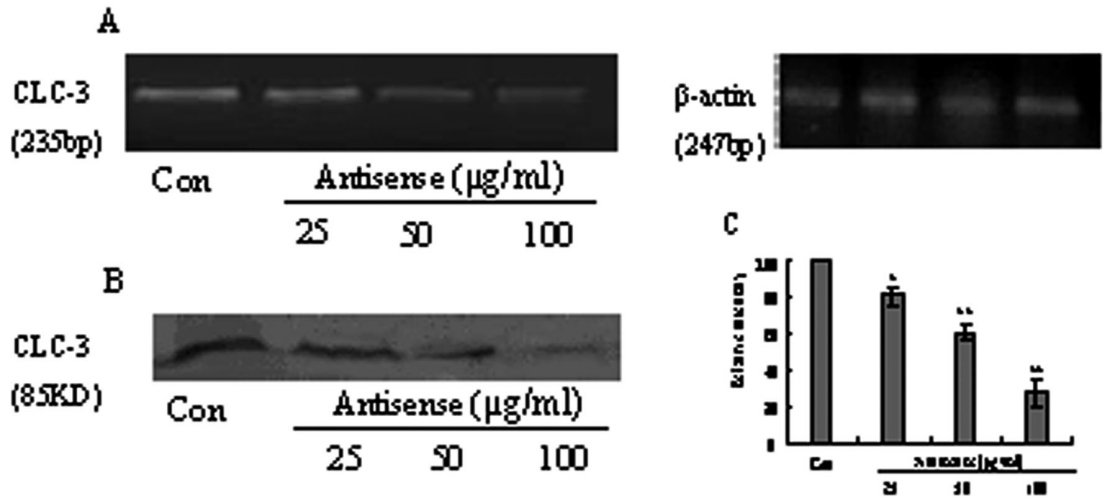

Figure 2. — CLC-3 antisense reduced CLC-3 RNA and protein expression in SKOV3 cells. Expression of CLC-3 in SKOV3 cells was detected by semiquantitative PCR using primers specific for CLC-3 and β-actin. Analysis of gray scale demonstrates it. CLC-3 antisense inhibits CLC-3 RNA expression in a dose-dependent manner. A: Western blotting analysis showing that CLC-3 antisense decreased CLC-3 protein espression in a dose-dependent manner also in B and C. *$p < 0.05$; **$p < 0.01$ *vs.* control.

cells, the oligonucleotides were labeled with fluorescence. As shown in Figure 1, under control conditions (no additives) the fluorescence in the cells was negligible, but the fluorescence in cells treated with antisense and sense were greatly increased, which confirmed the uptake of oligonucleotide by SKOV3 cells. Semiquantitative RT-PCR assay showed the PCR product of CLC-3 was decreased by 24.5 ± 4.2% ($p < 0.01$), 48.3 ± 5.6% ($p < 0.01$), and 76.6 ± 8.3% ($p < 0.01$) compared with control in SKOV3 cells treated with 25, 50, and 100 μg/ml CLC-3 antisense, respectively (Figure 2a). As shown in Figure 2b and 2c, CLC-3 protein levels were significantly downregulated in cells transfected with CLC-3 antisense in a dose-dependent manner also. However in cells treated with sense and lipofectamine, there exhibited no interference effects on CLC-3 RNA and protein expression in SKOV3 cells ($p > 0.05$) (data not shown).

Role of CLC-3 in volume-regulated Cl^- channel and cell volume regulation

The authors used antisense oligonucleotide to determine the role of CLC-3 in the activation of the VRC, in which the whole-cell currents of the SKOV3 cells at isotonic and hypotonic conditions were recorded by employing patch-clamp technique. The whole-cell currents were small under isotonic solution. Exposure to hypotonic solution (230 mosmol/l) activated a volume-regulated Cl^- current in SKOV3 cells. As shown in Figure 3, the current amplitude of VRC was significantly decreased in hypotonic solution after antisense treatment. Results displayed that cell volume was stable under isotonic solutions. Exposure of SKOV3 cells to a hypotonic solution caused an increase in relative cell volume after which gave rise to a RVD, while cells were exposed in hypotonic stress. Treating the cells with CLC-3 antisense

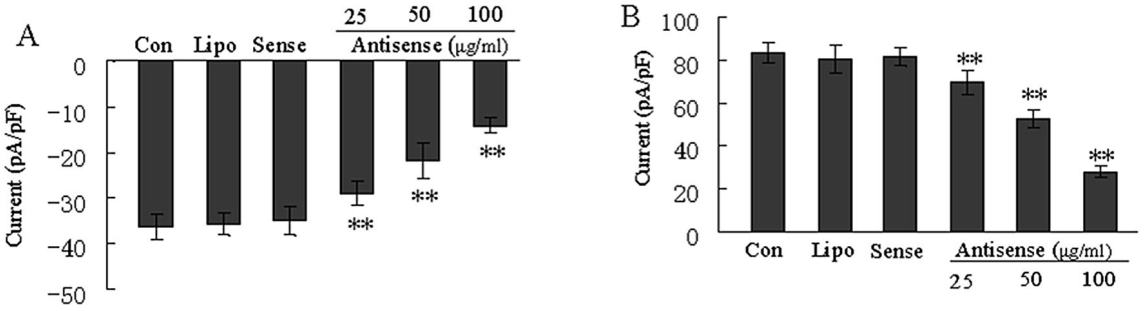

Figure 3. — Effects of CLC-3 antisense on volume-regulated Cl^- current in SKOV3 cells. Exposure of the cells to hypotonic solution activated a volume-regulated Cl^- current, and the mean outward and inward currents were measured using the patch-clamp technique as described in Materials and Methods, which showed that CLC-3 antisense inhibited the mean currents of SKOV3 cells in a dose-dependent manner. A: mean currents densities measured at -80 mV. B: mean current densities measured at +80 mV. (n=26~35), $^{**}p < 0.01$ compared with control cells.

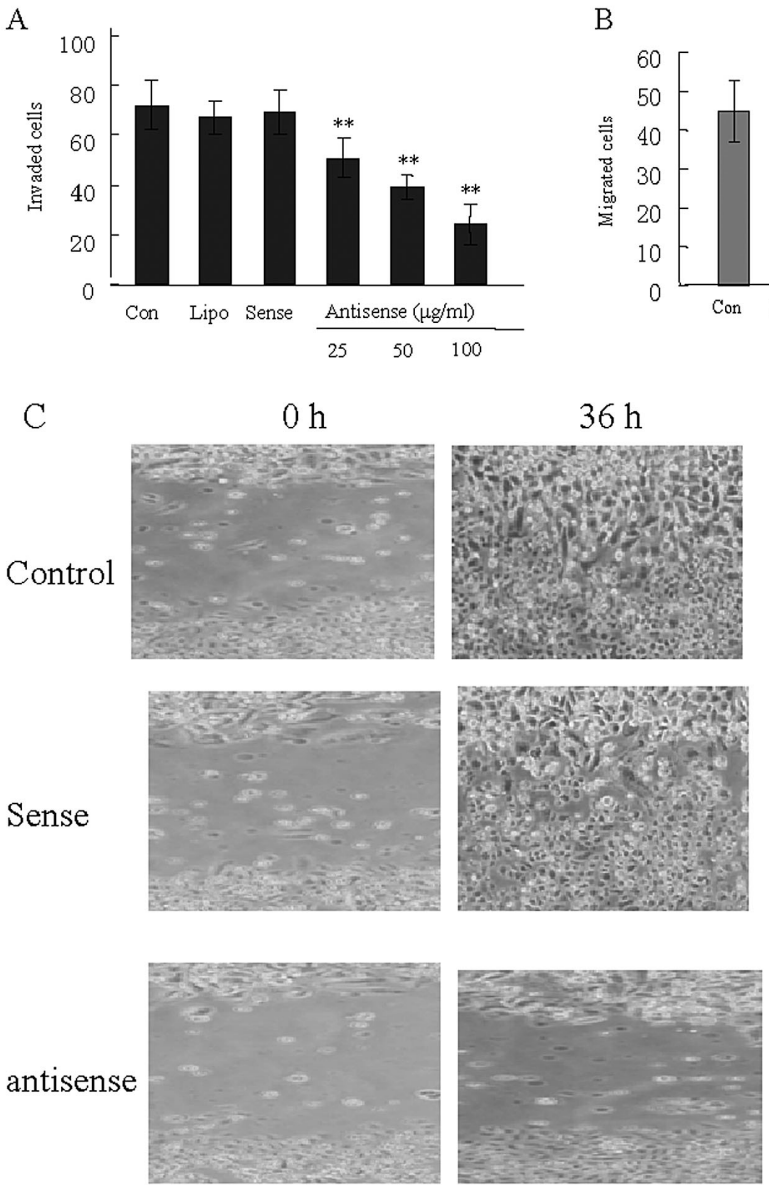

Figure 4. — Effects of CLC-3 antisense on invasion and migration of SKOV3 cells. Transwell invasion and migration assays were performed on the cells trasnfected with oligonucleotide and control cells. The cell numbers of CLC-3 antisense treated group were significantly less than control group, which suggested that CLC-3-targeting antisense can inhibit SKOV3 cell invasion and migration in a dose-dependent manner. A, B: $^{**}p < 0.01$ compared control. Wound healing assay also illustrated that SKOV3 cells transfected with CLC-3 antisense exhibited significantly decreased capacity of migration relative to control cells (C).

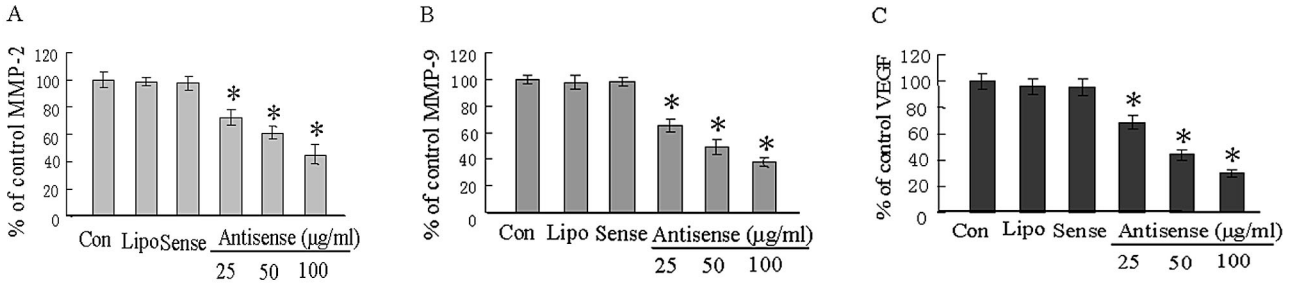

Figure 5. — CLC-3 antisense decreased protein levels of MMP-2, MMP-9, and VEGF. SKOV3 cells transfected with CLC-3 antisense were cultured for 48 hours, and then the levels of MMP-2, MMP-9, and VEGF proteins in culture supernatants were measured by ELISA. Results showed that the CLC-3 antisense obviously reduced production of MMP-2 (A), MMP-9 (B), and VEGF (C) proteins in a dose-dependent manner. *$p < 0.05$, compared with untreated controls.

did not significantly change the cell swelling process under the same hypotonic conditions, whereas, the process of RVD was obviously blocked by the antisense treatment. The CLC-3 antisense at 25, 50, and 100 μg inhibited RVD by 29.8 ± 3.6% (n=26, $p < 0.01$), 56.4 ± 4.1% (n=28, $p < 0.01$), and 71.3 ± 4.9% (n=33, $p < 0.01$), respectively, which were similar to the decreased magnitude of CLC-3 protein induced by CLC-3 antisense. There were no effects of CLC-3 sense and lipofectamine on the RVD (data not shown).

Effects of CLC-3 antisense on invasion and migration of SKOV3 cells in vitro

To investigate the roles that CLC-3 plays in the invasive and migrated behavior of SKOV3 cells, the authors used Transwell migration assay and wound healing assay frequently used to assess cell chemotaxis and invasiveness. The number of cells that had migrated through the filter and into the lower chamber was counted and compared. The results showed that CLC-3 antisense induced a dose-dependent regulation on invasion and migration: cells treated with antisense showed significantly lower numbers of invaded and migrated cells (Figure 4a, 4b). However, the CLC-3 sense and lipofectamine had no significant effect on invasion and migration of SKOV3 cells. The authors also assessed SKOV3 cell migration in response to antisense using a wound healing assay. As shown in Figure 4c, similar sized wounds were created in monolayer SKOV3 cells. Thirty-six hours after wound creation, the scars of control group and sense group had already closed. In contrast, after transfection with indicated concentrations of CLC-3 antisense, the speed of wound closure was much slower and the scars were still unchanged in SKOV3 cells at 36-hour post-transfection. All these data indicated that CLC-3 antisense treatment can significantly inhibit the cell invasion and migration of SKOV3 cells.

CLC-3 antisense decreased production of MMP-2, MMP-9, and VEGF in SKOV3 cells

MMPs degrade most components of the extracellular matrix (ECM), and MMP-2 and MMP-9 are believed to play a critical role in ovarian cancer invasion [14]. Moreover, data have shown that MMP-2 and MMP-9 can induce the release of VEGF which may also contribute to ovarian cancer metastasis [15]. Thus the present authors next determined whether CLC-3-inhibited cell invasion resulted from reduction of MMPs and VEGF. Their results showed that the CLC-3 antisense significantly reduced the release of the MMP-2, MMP-9, and VEGF proteins in a dose-dependent manner (Figure 5). CLC-3 antisense also dose-dependently induced a 31.5% to 70.3% decrease in active MMP-9 and a 37.6% to 69.1% decrease in active MMP-2 levels in SKOV3 supernatants 48 hours after transfection. However, CLC-3 sense and lipofectamine had no significant effect on production of MMP-2, MMP-9, and VEGF or active MMP-2 and MMP-9.

Discussion

CLC-3, an important member of the CLC superfamily, plays a crucial role in a variety of cellular processes, including cell volume regulation, endosomal acidification, and organellar PH modulation [16]. Besides physiological functions, it was implicated that upregulation of CLC-3 mRNA and protein is an adaptive response of inflamed pulmonary artery, which enhances the viability of PASMCs against reactive oxygen species [17]. Moreover, CLC-3, recently, has been found to be overexpressed in the human prostatic carcinoma cell NE-LNCaP, which protects cells against apoptotic stimuli and increases cell viability [18]. Most attractively, CLC-3 has been considered the most likely molecular candidate of the VAC [7, 8, 19] whose activation leads to subsequent RVD, a cellular defensive mechanism against hypotonic shock, although the role of CLC-3 as a volume-regulated Cl^- channel remains contro-

versial [20]. The present data showed that ovarian cancer cell line SKOV3 notably expressed CLC-3 by RT-PCR and Western blotting assays. CLC-3 antisense significantly suppressed the Cl^- currents induced by the hypotonic challenge at all the voltage steps applied, and attenuated the RVD response of ovarian cancer cell SKOV3 in a dose-dependent manner. These results provided evidence to support that CLC-3 participates in the generation of volume-regulated Cl^- current in the ovarian cancer epithelial cells.

The novel findings from this study are that the CLC-3 knockdown through antisense strategy significantly reduced invasion and migration of ovarian cancer cell line SKOV3 *in vitro*. The authors consider two possible interpretations for the potent antitumor effect of CLC-3 antisense. Firstly, and most simply, the CLC-3 antisense changes the regulatory mechanisms of cell volume control. Cell invasion and migration need substantial changes of cell volume, and thus volume regulatory mechanisms are certainly activated [21]. Moreover some studies have testified that volume-activated Cl^- channels mediating cell shape-volume changes contribute to the proliferation and invasion of cancer cells [9-11]. Recently, more and more studies support the notion that ClC-3 is the most likely molecular candidate involved in the activity or regulation of volume-regulated Cl^- channel [7, 8, 19]. The present study demonstrated that CLC-3 antisense abolished hypotonicity-induced Cl^- currents and downregulated VRC, thereby retarding RVD. The invasion of SKOV3 cells are thus inhibited by CLC-3 knockdown because of capacity decrease of cell volume regulation. Secondly, and perhaps more importantly, CLC-3 appears to be associated with MMPs and VEGF. In carcinogenesis, the invasion and metastatic capacity of ovarian cancer cells requires activation of proteolytic enzymes to destroy biologic barriers such as the basement membrane (BM). MMPs, a family of structurally related zinc-dependent endopeptidases, can collectively digest almost all ECM and BM components [22]. Thus, MMPs are largely involved in promoting angiogenesis and tumor metastasis. VEGF plays an important role in increasing vascular permeability and promoting angiogenesis, and is thus believed to play an important role in tumor metastasis [23]. Interestingly, investigations have shown that MMP-2 and MMP-9 can induce the release of VEGF in ovarian tumor cell lines, hence suggesting that an interplay between VEGF and the gelatinases may be a factor in progression of ovarian tumors [15, 24]. Meanwhile, VEGF stimulation causes the complicated interaction and activation of extracellular MMPs to facilitate malignant tumor cell invasion and migration through the BM [25]. Therefore, the coexpression and possible interaction between MMPs and VEGF may be a key factor in progression of ovarian tumors. Here the authors demonstrated that CLC-3 antisense significantly decrease protein levels of MMP-2, MMP-9, and VEGF in a dose-dependent manner, and reduce the activity of MMP-2 and MMP-9, therefore preventing invasion of SKOV3 cells. These findings indicate a novel function of CLC-3 in ovarian cancer invasion, the regulation of MMP-2, MMP-9, and VEGF. To the authors' knowledge, there has not been any previous study to indicate a relationship between CLC and MMPs and VEGF. They will focus in future studies on elucidating how the CLC-3 affects MMPs and VEGF products in ovarian cancer cells.

In summary, the present findings indicate that CLC-3 may be essential for invasion and migration of ovarian cancer cell SKOV3. It is likely that CLC-3 modulates invasion and migration of SKOV3 cells through RVD, MMP, and VEGF changes. Therefore, the promising role for CLC-3 chloride channel in invasion and migration merits further investigation which may provide additional insights into its potential as a new molecular target for ovarian cancer therapy.

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