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Suppression of ClC-3 channel expression reduces migration of nasopharyngeal carcinoma cells

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ABSTRACT

Recent studies suggest that chloride (Cl^-) channels regulate tumor cell migration. In this report, we have used antisense oligonucleotides specific for ClC-3, the most likely molecular candidate for the volume-activated Cl^- channel, to investigate the role of ClC-3 in the migration of nasopharyngeal carcinoma cells (CNE-2Z) *in vitro*. We found that suppression of ClC-3 expression inhibited the migration of CNE-2Z cells in a concentration-dependent manner. Whole-cell patch-clamp recordings and image analysis further demonstrated that ClC-3 suppression inhibited the volume-activated Cl^- current ($I_{Cl,vol}$) and regulatory volume decrease (RVD) of CNE-2Z cells. The expression of ClC-3 positively correlated with cell migration, $I_{Cl,vol}$ and RVD. These results strongly suggest that ClC-3 is a component or regulator of the volume-activated Cl^- channel. ClC-3 may regulate CNE-2Z cell migration by modulating cell volume. ClC-3 may be a new target for cancer therapies.

1. Introduction

Ion channels are expressed in a wide range of tissues where they have a variety of cellular functions, including proliferation, solute transport, volume control, enzyme activity, secretion, invasion, gene expression, excitation-contraction coupling and intercellular communication [1]. Recent studies have demonstrated that membrane ion channels play significant roles in tumor development and metastasis, and are essential for some cancer cell behaviors, such as proliferation, migration and apoptosis [2]. Voltage-gated sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) channels play a role in tumor metastasis and cell migration in breast cancer [3], melanoma [4] and fibrosarcoma [5]. Chloride (Cl^-) channels are also involved in tumor metastasis [6–8].

Depending on their gating mechanisms, Cl^- channels have been classified into five subtypes, one of which is the volume-activated chloride channel [9]. An apparently ubiquitous response to swelling in vertebrate cells is the activation of a volume-sensitive Cl^- current ($I_{Cl,vol}$) and a K^+ current. The outflow of Cl^- and K^+ through separate channels leads to a decrease in cell volume termed regulatory volume decrease (RVD) [10,11]. Volume-activated chloride channels also play a role in the migration of glioma cells [12]. Consistent with this, our previous work with nasopharyngeal carcinoma (CNE-2Z) cells have demonstrated that the pharmacological blockade of $I_{Cl,vol}$ inhibits CNE-2Z cell migration[13]; $I_{Cl,vol}$ regulates CNE-2Z cell migration by modulating RVD[14,15].

The channel protein that provides the $I_{Cl,vol}$ current is still unidentified, although several candidates have been proposed

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[9,10]. ClC-3, a member of the ClC family of voltage-gated Cl^- channels, is one such candidate. Recent studies in cardiac and vascular smooth muscle support the idea that ClC-3 is the molecular component involved in the activation or regulation of $I_{Cl,vol}$ [16,17]. ClC-3 protein expression has been found in different cancer cell types, including prostate cancer epithelial cells [18], PC12 cells [19] and glioma cells [20]. Previous studies have shown that ClC-3 is also expressed in CNE-2Z cells [21]. The role of endogenous ClC-3 in cell migration has not been investigated, however. In this study, we have used an antisense oligonucleotide specific to ClC-3 (ClC-3 antisense) to determine the role of this channel in the migration of nasopharyngeal carcinoma cells *in vitro*.

2. Materials and methods

2.1. Chemicals, reagents, and solutions

All chemicals were the highest grade available and purchased from Sigma (St. Louis, MO, USA). The solution in patch-clamp pipettes contained (in mM): 10 HEPES, 70 N-methyl-D-glucamine chloride (NMDG-Cl), 1.2 $MgCl_2$, 140 D-mannitol, 1 EGTA, and 2 ATP. The isotonic bath solution (300 mosmol/l) contained (in mM): 70 NaCl, 2 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, and 140 D-mannitol. The osmolarity in the pipette and isotonic bath solutions was adjusted to 300 mosmol/l with D-mannitol and monitored by an osmometer (Osmomat 30, Gonotec, Germany). The osmolarity of the 47% hypotonic bath solution was 160 mosmol/l (47% hypotonic, compared with the isotonic solution) and was obtained by omitting the D-mannitol from the isotonic bath solution. The pH of the pipette and bath solutions was adjusted with Tris base to 7.25 and 7.4, respectively.

2.2. Cell cultures

The poorly differentiated nasopharyngeal carcinoma cell line (CNE-2Z) was kindly provided by Professor Weiping Tang (Department of Pathology, Guangdong Medical College, China). Cells were grown in 25-cm^2 plastic tissue culture flasks in the RPMI 1640 medium with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. For volume measurements and current recordings, the cell suspension was plated onto round coverslips of 22-mm diameter (150 μl/coverslip) and incubated at 37 °C for 2–3 h before volume regulation experiments or current recordings.

2.3. Transfection of antisense and sense oligonucleotides

Antisense and sense oligonucleotides corresponding specifically to the initiation codon region of human ClC-3 mRNA were synthesized (Sangon, Shanghai, China) as reported by us previously [22]. The antisense sequence was 5′-TCCATTTGTCATTGT-3′; the sense sequence was 5′-ACAATGACAAATGGA-3′. The sequence of the missense oligonucleotide was 5′-TCTATTCCTGTATTG-3′ as reported by Wang et al. [23], which was consisted of the same bases as used in the antisense probe but in a random order, and did not recognize any known sequence available in GenBank. For the three oligonucleotides, the first three bases at either end were phosphorothioated. To examine their uptake by the CNE-2Z cells, the oligonucleotides were labeled with fluorescein at the fifth and the eleventh bases in the antisense and at the seventh and the fifteenth bases in the sense and at the fifth and the twelfth bases in the missense. Cells were transfected with antisense, sense or missense oligonucleotides in the presence of lipofectamine^{2000} (5 μl/ml, Life Technologies, Inc., Rockville, MD) in serum-free medium for 4.5 h and then in the medium containing 10% fetal bovine serum for additional time in different experiments.

2.4. Isolation of total RNA and RT-PCR

Total RNA was extracted from control cells and the cells transfected for 48 h. using Trizol (Invitrogen, Carlsbad, CA) and treated with DNase (Promega, Madison, WI) according to manufacturer's protocol. RNA was then extracted with phenol/chloroform/isoamyl alcohol, precipitated and resuspended in 1 mM sodium citrate of pH 6.4. Oligonucleotide primers for ClC-3 (GenBank accession no. NM001829) were designed with the designer programs "Primer" (Version 5.0, Whitehead Institute for Biomedical Research, Cambridge, MA) and "Oligo" (Version 6.0, National Bioscience, Plymouth, MN, USA). Forward and reverse PCR primer sequences were as follows: forward, 5′-TTGCCTACTATCACCACGAC-3′; reverse, 5′-GCATCTCCAACCCATTTACT-3′. RT-PCR was performed using the Qiagen One-Step RT-PCR kit (Qiagen, Valencia, CA). Reverse transcription and amplification was performed using a thermocycler (Biometre, Göttingen, Germany) that was programmed with the following parameters: (1) reverse transcription at 50 °C for 30 min; (2) PCR activation at 95 °C for 15 min; (3) 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min; and (4) final extension at 72 °C for 10 min. Aliquots of each PCR product were separated on a 2% agarose gel, stained with ethidium bromide and analyzed by illumination with ultraviolet and visible light (Multi Genius, Syngene, USA). The PCR products are in the linear range of amplification in the RT-PCR experiments.

2.5. Western blot analysis

To examine ClC-3 protein expression, CNE-2Z cells were washed with PBS once and lysed with the buffer containing Tris–Cl (50 mmol/l), NaCl (150 mmol/l), NaN_3 (0.02%), Nonidet P-40 (1%), SDS (0.1%), sodium deoxycholate (0.5%), leupeptin (5 μg/ml) and aprotinin (1 μg/ml). The protein content of cell lysates was quantified with Coomassie Brilliant Blue. The proteins samples were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH), which were then blocked at room temperature (24–26 °C) for 1 h in a solution containing (in mmol/l): 130 NaCl, 2.5 KCl, 10 Na_2HPO4, and 1.5 KH_2PO4, 0.1% Tween 20 and 5% BSA (pH 7.4). The membranes were incubated overnight at 4 °C with a polyclonal antibody directed against ClC-3, a gift from Dr. William J. Hatton [17] and then with a peroxidase-conjugated antibody (HRP-linked anti-rabbit secondary antibody) for 1 h at room temperature. Final detection was

accomplished with Western blot luminol reagent (SC-2048; Santa Cruz Biotechnology Inc., California, USA) as described by the manufacturer. The density of target bands was quantified by the computer-aided 1D gel analysis system.

2.6. Transwell migration assay

We used a modified transwell migration assay, as described [13]. Transwell chambers (Costar Corporation, Cambridge, MA) with polycarbonate membrane filters of 12-μm pore size were used to form dual compartments in a 24-well tissue culture plate. Fibronectin was used as a chemoattractant. Transfected or control cells (100 μl of suspension containing 3×10^5 cells) were added to each of the upper compartments. All assays were performed at 37 °C in humidified 5% CO_2/95% air for a period of 6 h. Then the cells on upper surface of the filter were removed by wiping with a cotton swab and the cells that invaded the lower surface were imaged under microscopy with a CCD camera.

The quantitation of migrated and non-migrated cells was made by a modified MTT method. Mitochondrial dehydrogenase enzymes in cells can convert a yellow water-soluble substrate 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue water insoluble formazan product, which can be solubilized by DMSO or other solvents and measured spectrophotometrically. The amount of formazan is directly proportional to the cell number. After the migration assay, the transwell chambers were placed into other wells of the 24-well culture plate containing 100 μl of 0.25% trypsin-EDTA for 5 min. The cells that had migrated onto the lower surface of the filter were gently pipetted and resuspended in medium. The suspension was added to the upper compartment of a new transwell insert. Non-migrated or migrated cells in inserts were incubated for an additional 4 h in medium contained 100 μl of MTT (5 mg/ml) per well. Then the inserts were placed onto a piece of filter paper to drain off the medium. After 100 μl of DMSO was added to each well, the colored solution (dissolved formazan) was transferred to a separate well in a 96-well culture plate and the absorbance (OD) was then measured at 570 nm. The rate of migration (%) = $OD_{mig}/(OD_{mig} + OD_{non}) \times 100\%$, where OD_{mig} is the OD value of migrated cells and OD_{non} is the OD value of non-migrated cells.

2.7. Whole cell current recording

The coverslip with CNE-2Z cells was placed in a microchamber mounted on an inverted microscope. Experiments were carried out at room temperature (20–24 °C). Standard whole-cell patch-clamp recordings were performed using a List EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). The resistance of the recording pipettes filled with the isotonic pipette solution was 4–6 MΩ. Cells were held at 0 mV (close to the chloride equilibrium potential), and then cycled through the 200 ms pulses of 0, ±40 and ±80 mV, with a 4-s interval between pulses. Whole cell currents were recorded by a computer through a laboratory interface (CED 1401, Cambridge, UK) with a sampling rate of 3 kHz and were analyzed by the EPC software package (CED, Cambridge, UK). The amplitude of currents was measured at 10 ms after the onset of each voltage step. Cell size was monitored simultaneously by a CCD camera. Cell capacitance was measured using the amplifier function.

2.8. Cell volume measurements

Cell volume was measured with the method described previously [15]. Coverslips with the prepared cells were fastened to the base of the recording chamber with non-melting hydrocarbon multipurpose grease and mounted on an inverted microscope (Olympus, Tokyo, Japan). The bath, with a volume of 0.5 ml, was perfused continually with a flow rate of 4 ml/min. Cell images were captured every 30 s by a CCD camera (Cohu, ALRAD Instruments Ltd., UK) and stored directly onto the computer. Image acquisition was controlled by the Scion analysis software (Scion Corporation, USA). Cell volume was calculated using the equations of $V = 4/3 \times S \times (S/\pi)^{1/2}$, where S is the area (μm^2). The level of regulatory volume decrease (RVD) was calculated using the equation:

$$\mathrm{RVD}\,(\%) = \frac{V_{max} - V_{min}}{V_{max} - V_0} \times 100,$$

where V_0 is the cell volume in isotonic solution before hypotonic shock, V_{max} the peak volume in hypotonic solutions and V_{min} is the volume before returning to isotonic solution.

2.9. Statistical analysis

The experimental data were expressed as mean ± standard error (number of observations). ANOVA and Student's *t*-tests were used to analyze the data, with statistical significance defined as $P < 0.05$. All experiments were repeated at least 3 times.

3. Results

3.1. Effects of antisense oligonucleotides on ClC-3 expression

Fluorescein-labeled oligonucleotides were added to the culture medium and incubated for 48 h in the presence or absence of transfection reagent. Oligonucleotide uptake was monitored by the fluorescence microscopy. Fluorescence was negligible in control cells, while the fluorescence in cells treated with transfection agent and either antisense, sense or missense oligonucleotide was greatly increased, confirming the proper uptake of oligonucleotides by these cells (Fig. 1).

Transient transfection of CNE-2Z cells with ClC-3 antisense oligonucleotides (20 μM) decreased the expression of ClC-3 mRNA by 77.5 ± 3.6% ($n = 4$) (Fig. 2A) in 48 h and, as expected, suppressed the expression of ClC-3 protein in a time-dependent manner (Fig. 2B). After transfection for 24, 48, and 72 h, the expression of native ClC-3 protein was inhibited by 25.3 ± 5.6%, 69.5 ± 6.3%, and 70.6 ± 5.7% ($n = 3$), respectively.

The suppression of ClC-3 protein expression by the ClC-3 antisense oligonucleotide was concentration dependent. When cells were transfected with ClC-3 antisense oligonucleotides at concentrations of 5, 10, 20, and 40 μM, the ClC-3 protein was decreased by 30.3 ± 3.2%, 53.3 ± 4.8%, 71.7 ± 5.2%

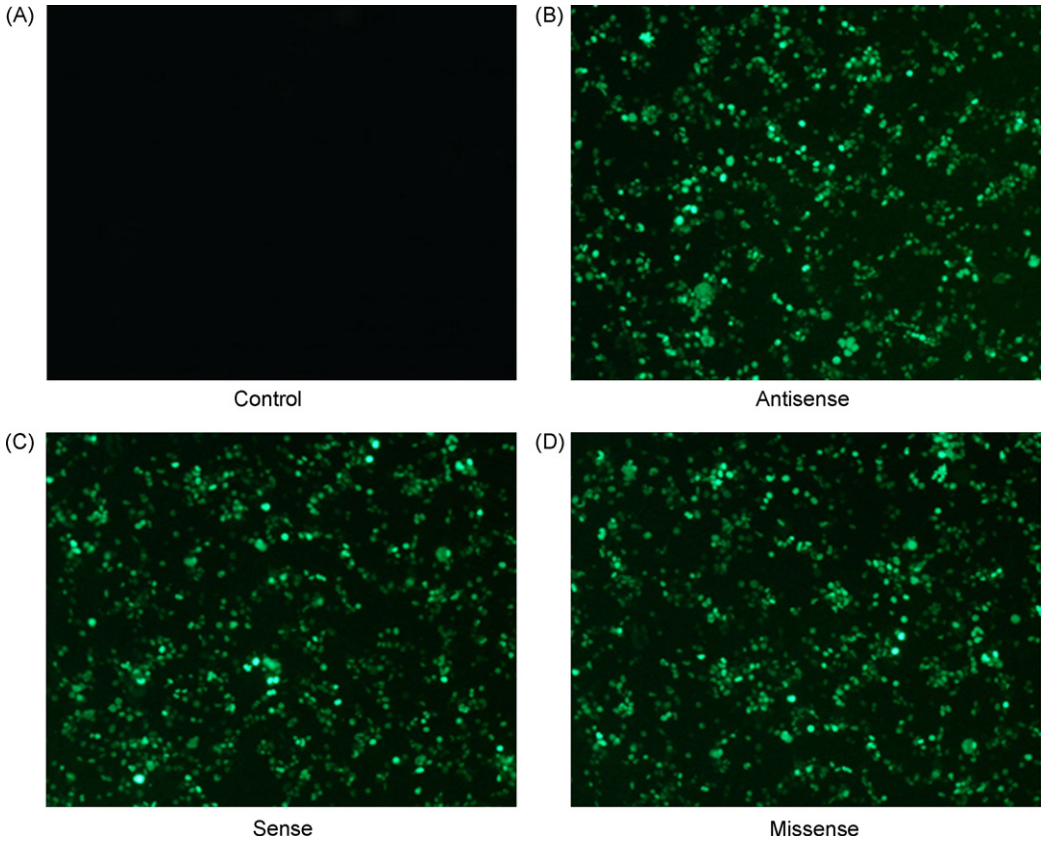

Fig. 1 – Uptake of oligonucleotides labeled with fluorescein by CNE-2Z cells (100×). CNE-2Z cells were transfected with 20 μM fluorescein-labeled ClC-3 antisense (B), sense (C) or missense (D) oligonucleotides in the presence of 5 μl/ml lipofectamine^{2000} for 48 h. No fluorescence was detectable in control cells (A, no additives), but strong fluorescence was detected in cells transfected with antisense, sense or missense oligonucleotides.

and 74.1 ± 5.4%, respectively (n = 3) (Fig. 2C). ClC-3 antisense at 20 μM produced the maximum effect (n = 5, $P > 0.05$ *vs.* 40 μM ClC-3 antisense). The IC_{50} for the inhibition of ClC-3 protein expression was 9.2 μM. To confirm the specificity of antisense oligonucleotides, the effects of transfection agent, sense and missense oligonucleotides were tested. The results showed that ClC-3 protein expression was not significantly altered by the transfection agent alone or by 48 h transfection of CNE-2Z cells with ClC-3 sense or missense oligonucleotides in the presence of the transfection agent (n = 3 for each group; $P > 0.05$, *vs.* control; Fig. 2C).

3.2. *Inhibition of CNE-2Z cell migration by ClC-3 antisense oligonucleotides*

Our previous studies have shown that the volume-activated Cl^- channel plays a role in CNE-2Z cell migration [13–15]. In this study, we demonstrated that the ClC-3 chloride channel was involved in the regulation of cell migration. The ability of CNE-2Z cells to migrate through polycarbonate filters was decreased significantly by ClC-3 antisense oligonucleotides (Fig. 3). Fig. 3A shows representative images of CNE-2Z cells that migrated across the filters in the control group (no additives) and in the groups transfected with 20 μM ClC-3 antisense or sense oligonucleotides in the presence of transfection agent lipofectamine^{2000} (5 μl/ml) for 48 h. Compared with the control group, the number of migrated cells was decreased by the antisense treatments in a concentration-dependent manner (Fig. 3A–C). Untransfected control cultures had a migration rate of 17.7 ± 2.0% (n = 8). 1 μM ClC-3 antisense did not hamper cell migration ($P > 0.05$, *vs.* control, n = 5), while the migration rate of cells transfected with 5, 10, 20 and 40 μM of ClC-3 antisense was significantly reduced to 14.5 ± 2.4%, 9.1 ± 1.6%, 7.3 ± 1.4% or 6.9 ± 1.5%, respectively (n = 5, $P < 0.01$; Fig. 3B and C). The IC_{50} for the inhibition of migration was 10.5 μM. It was also shown that the effect of 20 μM ClC-3 antisense oligonucleotides on cell migration rate could not be facilitated by the chloride channel blocker tamoxifen (15 μM).

CNE-2Z cell migration was not significantly affected by 48 h treatment with the transfection agent lipofectamine^{2000} (5 μl/ml) alone or by transfection with ClC-3 sense (1, 5, 10, 20 and 40 μM) or missense (20 μM) oligonucleotides in the presence of transfection reagent lipofectamine^{2000} (5 μl/ml) for 48 h (Fig. 3A–C).

3.3. *The correlation between ClC-3 expression and cell migration*

We have demonstrated, as shown above, that ClC-3 antisense oligonucleotides inhibit ClC-3 expression and cell migration.

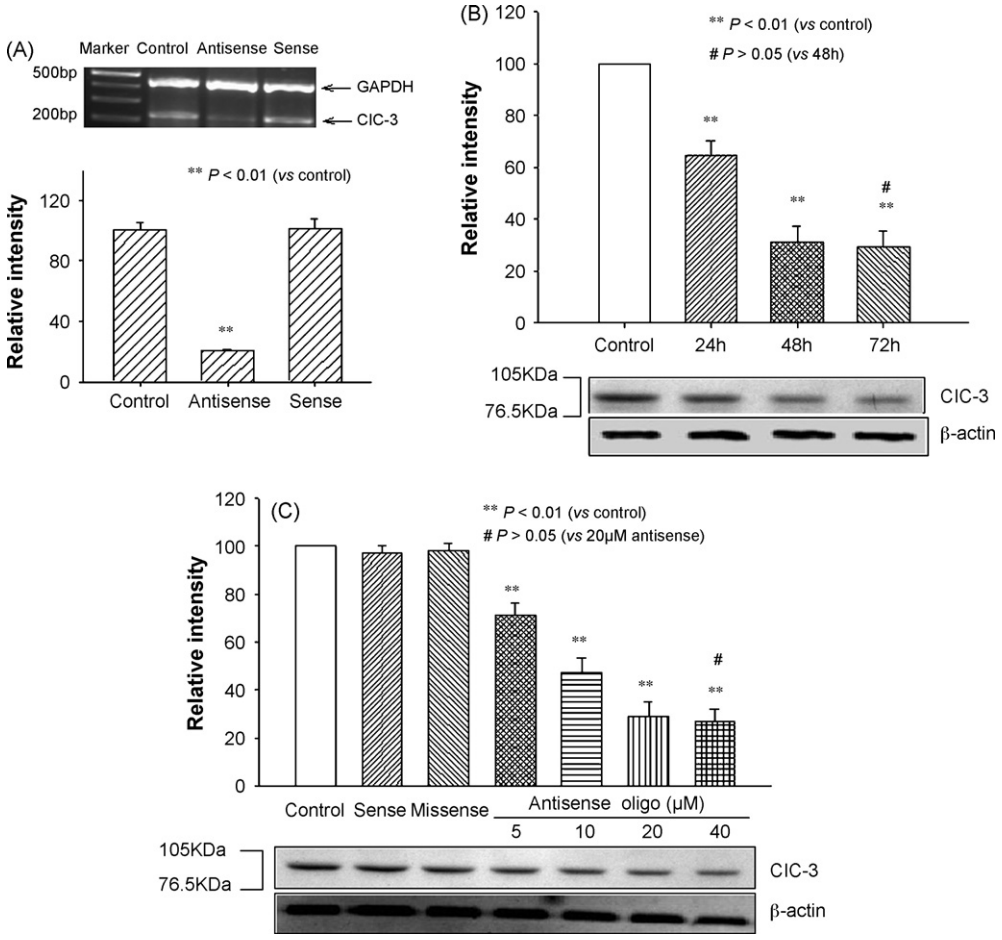

Fig. 2 – Effects of ClC-3 antisense oligonucleotide transfection on ClC-3 mRNA and protein expression in CNE-2Z cells. (A) Shows the results of RT-PCR performed on total RNA extracted from control cells or cells transfected with 20 μM ClC-3 antisense or sense oligonucleotides for 48 h and the intensity ratio of ClC-3 band to the standard band GAPDH. (B) Presents ClC-3 protein expression levels (intensity ratio of ClC-3 band to the standard band β-actin) analyzed by Western blot in control cells or cells transfected with ClC-3 antisense oligonucleotides (20 μM) for 24, 48 and 72 h. (C) Gives ClC-3 protein expression levels analyzed by Western blot in control cells or cells transfected with ClC-3 antisense (5, 10, 20 and 40 μM), sense (20 μM) or missense (20 μM) oligonucleotides for 48 h. ClC-3 antisense oligonucleotides inhibited the expression of ClC-3 mRNA and protein in the time-dependent and concentration-dependent manner. Sense and missense oligonucleotides failed to alter ClC-3 expression. Each point in the figure represents means ± S.E. from three different experiments.

We investigated further the relationship between ClC-3 expression and cell migration by comparing the ClC-3 protein expression level with the cell migration rate under the same treatments (Fig. 4). Both ClC-3 protein expression and cell migration rate were inhibited by the ClC-3 antisense oligonucleotide in a similar manner and a similar 50% inhibiting concentration (IC_{50}). The inhibitory effects of the antisense oligonucleotide on both ClC-3 expression and cell migration reached the peak level when the oligonucleotide concentration was 20 μM. Increasing the antisense concentration beyond 20 μM did not significantly increase the effects further. Furthermore, the ClC-3 protein expression level and the rate of cell migration were positively correlated (Fig. 4B). Linear regression analysis with the equation $f = y_0 + ax$ yielded a correlation coefficient of $r = 0.98$ ($y_0 = 0.6$, $a = 0.2$).

3.4. *Inhibition of volume-activated chloride currents by ClC-3 antisense oligonucleotides*

To further investigate the role of ClC-3 in cell migration, the patch-clamp technique was used to assess the effects of ClC-3 antisense oligonucleotides on $I_{Cl,vol}$. For the consistency and to activate most of the volume-activated chloride channels available in CNE-2Z cells, in this study, 160 mOsm solution (47% hypotonic) was used to induce $I_{Cl,vol}$ and RVD. The responses of CNE-2Z cells to 47% hypotonic and other hypotonic solutions have been tested in our previous studies in synchronized and non-synchronized CNE-2Z cells [24,25]. It was found that the 47% hypotonic solution activated a RVD that was near the maximal RVD level induced by hypotonic challenges (data not shown).

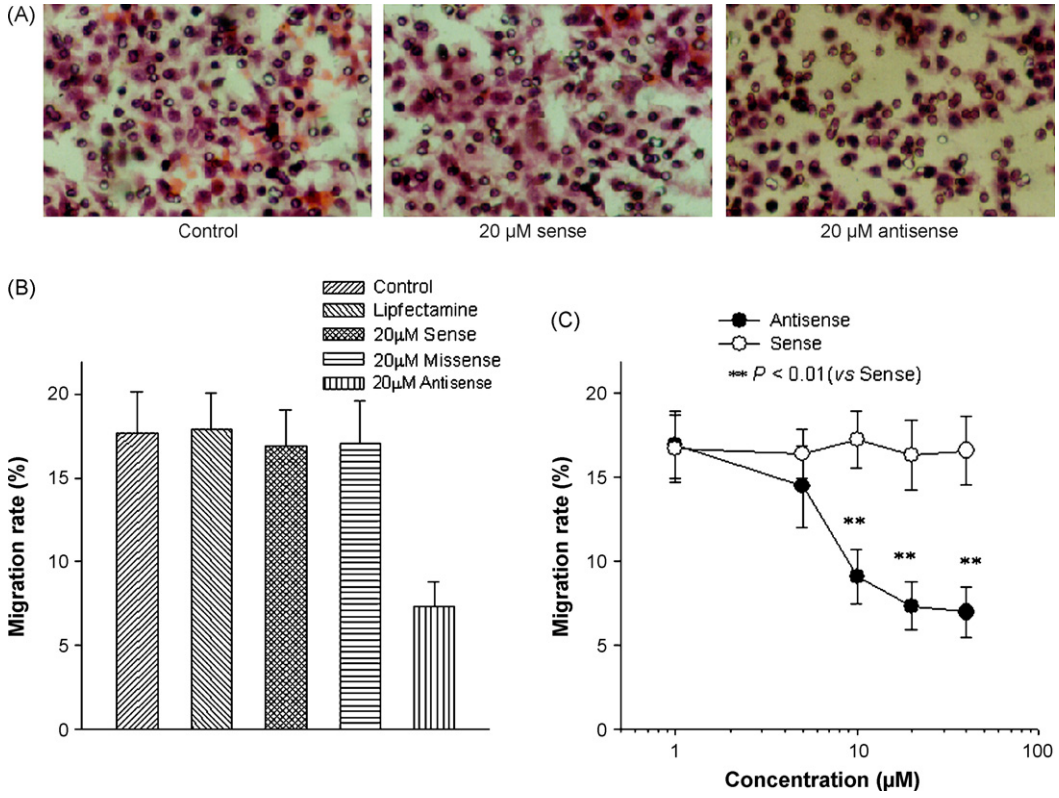

Fig. 3 – Effects of ClC-3 antisense oligonucleotides on CNE-2Z cell migration. After transfected with ClC-3 antisense (5, 10, 20 and 40 μM), sense (5, 10, 20 and 40 μM) or missense (20 μM) oligonucleotides plus 5 μl/ml lipofectamine^{2000} or 5 μl/ml lipofectamine^{2000} alone for 48 h, the migration rate of control (no additives) and transfected cells was measured. The number of migrated cells was counted by CCD camera imaging (100× magnification) and assessed by the MTT assay. (A) Representative micrographs of the migrated cells on the bottom side of filters. (B) Comparison of cell migration rate of different treatments. (C) Concentration-dependent inhibition of cell migration by ClC-3 antisense oligonucleotides. Data were the mean ± S.E. of 5 experiments.

Whole cell currents under both isotonic (300 mosmol/l) and hypotonic (160 mosmol/l) conditions were recorded from CNE-2Z cells 48 h after transfection with 1, 5, 10, 20 or 40 μM ClC-3 antisense oligonucleotides, (Fig. 5). The currents recorded from both control and transfected cells were small under isotonic conditions. The hypotonic-activated Cl^- currents recorded from cells transfected with ClC-3 antisense oligonucleotides were quite different from

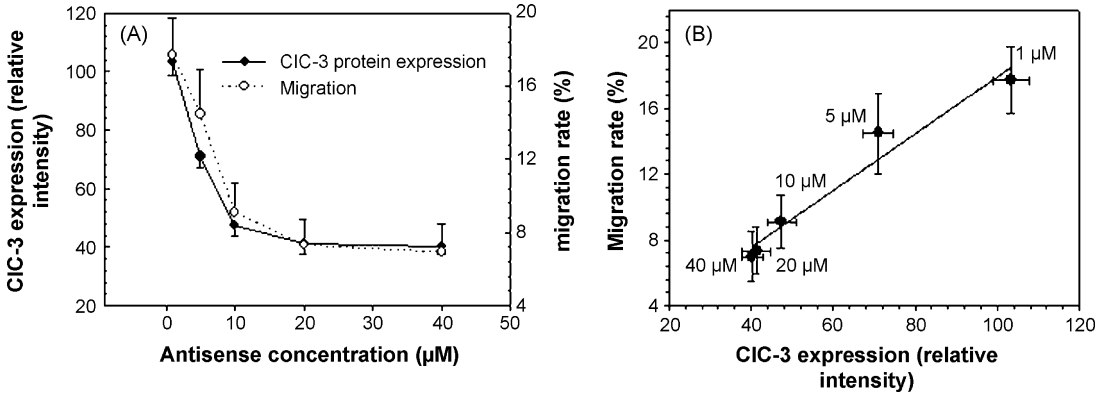

Fig. 4 – The correlation between ClC-3 expression and cell migration. Incubation of CNE-2Z cells in various concentrations of ClC-3 antisense oligonucleotides for 48 h caused a concentration-dependent reduction of ClC-3 protein expression and cell migration (A). A positive correlation between ClC-3 expression and cell migration was obtained by plotting the migration rate against the ClC-3 expression level under the same treatments and by fitting the data with the equation $f = y_0 + ax$ (B). The fitting yielded a linear correlation coefficient of $r = 0.98$ ($P < 0.01$; $y_0 = 0.6$, $a = 0.2$).

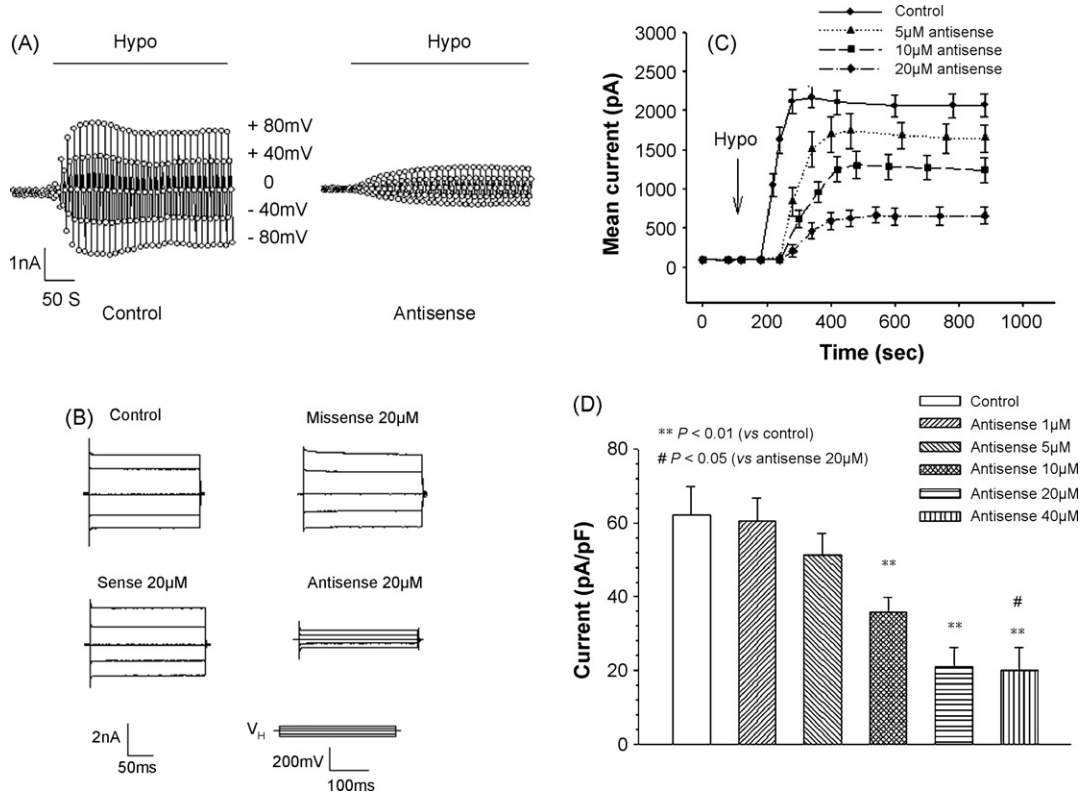

Fig. 5 – Inhibition of volume-activated chloride currents by ClC-3 antisense oligonucleotides. Whole-cell path clamp recordings were performed on CNE-2Z cells after transfection with 0, 1, 5, 10, 20 or 40 μM ClC-3 antisense oligonucleotides in the presence of lipofectamine^{2000} (5 μl/ml) for 48 h. Cells were cycled through the 200 ms pulses of 0, ±40 and ±80 mV (V_H). (A) Shows the typical time course of the Cl^- current activated by the hypotonic solution (Hypo, 160 mosmol/l) in the control cell (Control, no additives) and in the cell treated with 20 μM ClC-3 antisense oligonucleotide (Antisense). (B) Presents the typical traces of the hypotonic-activated Cl^- current in control cells and in cells treated with 20 μM missense, sense or antisense oligonucleotides. (C) and (D) show respectively the time course of whole-cell currents and the density of peak currents induced by the hypotonic solution (at +80 mV step) in control cells and in cells transfected with different concentrations of antisense oligonucleotides. The arrow in (C) shows the time point of exposure to the hypotonic solution. Data in (C) and (D) were the mean ± S.E. of 21 cells in control and 7, 6, 6, 11 and 15 cells in antisense groups (1, 5, 10, 20 and 40 μM, respectively).

those of control cells, however (Fig. 5A). In the antisense group, the hypotonic-induced currents were activated more slowly and the peak currents were diminished (Fig. 5A, right panel, and Fig. 5B). The whole-cell current activated by the hypotonic solution (at the +80 mV step) was reduced from a peak of 2200 ± 100 pA ($n = 24$) in the control cells to 580 ± 100 pA ($n = 22$; $P < 0.01$) in the cells transfected with ClC-3 antisense oligonucleotides at 20 μM (Fig. 5C). The remained current could not be inhibited by tamoxifen (15 μM). Treatments with sense or missense oligonucleotides (20 μM sense or missense oligonucleotides plus lipofectamine^{2000} of 5 μl/ml) for 48 h did not affect the hypotonic-activated current (Fig. 5B; $n = 12$, $P > 0.05$).

As expected, the effect of ClC-3 antisense oligonucleotides on the hypotonic-activated Cl^- current was concentration-dependent in both the whole-cell level (Fig. 5C) and the current density (Fig. 5D). The IC_{50} for antisense inhibition of the hypotonic-activated current was 9.6 μM. There was no significant difference in current density between 20 and 40 μM antisense treatments.

3.5. *Inhibition of RVD by ClC-3 antisense oligonucleotides*

We monitored the regulatory volume decrease (RVD) of CNE-2Z cells following hypotonic shocks by microscopy and imaging with a CCD digital camera. While cell size was stable in the isotonic solution, exposing cells to a 47% hypotonic solution resulted in cell swelling (Fig. 6A). Swelling was evident in 30–60 s and reached a peak in 1–2 min. After peak swelling, cell volume decreased gradually (RVD), even though cells were still bathed in the hypotonic solution (Fig. 6A). Treating cells with 20 μM ClC-3 antisense oligonucleotide plus lipofectamine^{2000} (5 μl/ml) for 48 h did not significantly change the cell swelling process (Fig. 6A), but inhibited significantly the RVD process under the same hypotonic condition. RVD capacity was decreased from 56.4 ± 3.5% in the control group to 22.4 ± 3.4% in the treatment group, with an inhibitory rate of 60.4 ± 2.7%, as indicated by the failure of the cell volume to return to the control level (Fig. 6A). The inhibition of RVD mediated by ClC-3 antisense oligonucleotides was concentration-dependent with an IC_{50} of 10.8 μM (Fig. 6B and C). There

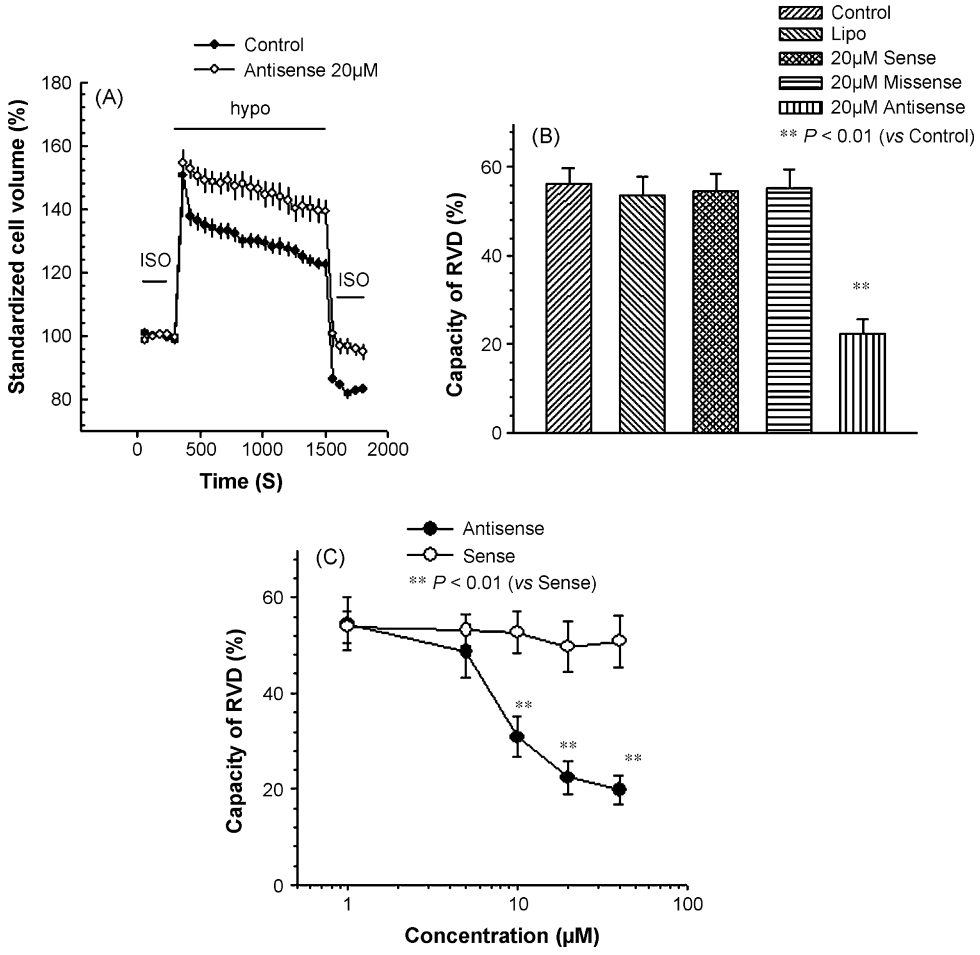

Fig. 6 – Inhibition of RVD by ClC-3 antisense oligonucleotides. The volume change of CNE-2Z cells was monitored by a CCD camera and was analyzed by an image analysis software. CNE-2Z cells were transfected with ClC-3 antisense, sense or missense oligonucleotides plus 5 μl/ml lipofectamine^{2000} or 5 μl/ml lipofectamine^{2000} alone for 48 h. (A) The time course of RVD activated by the 47% hypotonic solution (Hypo) in control and antisense-transfected cells. Iso, Cells in isotonic condition. (B) The effects of antisense, sense and missense oligonucleotides and lipofectamine^{2000} (Lipo) alone on the RVD capacity of CNE-2Z cells. (C) Concentration-dependent inhibition of RVD by antisense, but not sense oligonucleotides. Each point in the figures represents the mean ± S.E. of 20–27 experiments.

was no significant changes of RVD when cells were transfected with 20 μM ClC-3 sense or missense oligonucleotides plus lipofectamine^{2000} (5 μl/ml), or with lipofectamine^{2000} alone for 48 h (Fig. 6B).

3.6. *The correlation between ClC-3 expression and $I_{Cl,vol}$ and RVD*

As shown above, ClC-3 antisense oligonucleotides inhibited both $I_{Cl,vol}$ and RVD of CNE-2Z cells. We examined further the relationships between ClC-3 protein expression and either $I_{Cl,vol}$ or RVD. The concentration dependence of antisense oligonucleotide-mediated inhibition of ClC-3 expression was in excellent agreement with both $I_{Cl,vol}$ and RVD (Fig. 7). Plotting the magnitude of either $I_{Cl,vol}$ or RVD against the level of ClC-3 protein expression and fitting the data with the equation $f = y_0 + ax$ indicated that the ClC-3 expression level was positively correlated to the magnitude of $I_{Cl,vol}$ or RVD capacity, with a linear correlation coefficient (r) of 0.98 ($y_0 = -3.8$, $a = 0.7$) or 0.97 ($y_0 = 2.2$, $a = 0.5$), respectively ($P < 0.01$, Fig. 7B and D).

4. Discussion

ClC-3, a member of the voltage-gated ClC Cl^- channel family, is proposed to be responsible for the $I_{Cl,vol}$ [22,26–28], although the molecular identity of volume-activated chloride channel are still under debate [16]. Our previous work has demonstrated that ClC-3 is functionally expressed in CNE-2Z cells [21]. We have also found that two nonspecific chloride channel blockers, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and tamoxifen, inhibit the migration of CNE-2Z cells [13,15], suggesting that ClC-3 may play a role in cell migration.

Antisense oligonucleotides provide an effective tool for studying the function of genes and proteins in living cells [29,30]. Nevertheless, RNA interference, an alternative technique, has been increasingly applied in this field [31]. In this

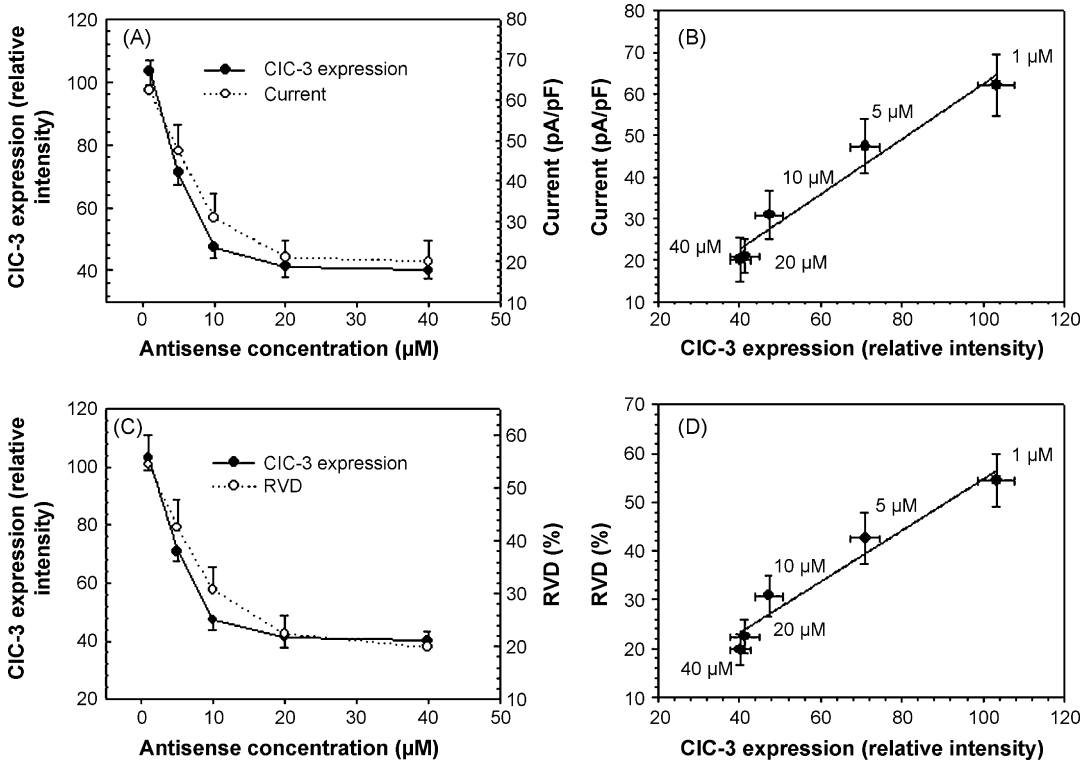

Fig. 7 – The correlation between ClC-3 protein expression and $I_{Cl,vol}$ or RVD. Transfection of CNE-2Z cells with ClC-3 antisense oligonucleotides (1, 5, 10, 20 and 40 μM) for 48 h caused a concentration-dependent reduction of ClC-3 protein expression, $I_{Cl,vol}$ and RVD (A, C). (B) and (D) shows the positive correlation between ClC-3 expression and $I_{Cl,vol}$ or RVD, respectively. $I_{Cl,vol}$ or RVD was plotted against the level of ClC-3 protein expression. Fitting the data in (B) or (D) with the equation, $f = y_0 + ax$, resulted in a linear correlation coefficient (r) of 0.98 ($y_0 = -3.8$, $a = 0.7$) or 0.97 ($y_0 = 2.2$, $a = 0.5$), respectively ($P < 0.01$).

study, we investigated the role of ClC-3 in cell migration by inhibiting the functional expression of ClC-3 through the transfection of antisense oligonucleotides in CNE-2Z cells. We first verified the successful uptake of oligonucleotides. We then showed that ClC-3 antisense but not sense or missense oligonucleotides, inhibited the expression of ClC-3 protein and decreased CNE-2Z cell migration in a concentration-dependent manner. Finally, by analyzing the inhibitory effects of various concentrations of ClC-3 antisense oligonucleotides, we showed a positive correlation between the expression of ClC-3 and the rate of cell migration. These results provide compelling evidence that ClC-3 is involved in CNE-2Z cell migration.

Cell migration is one of the key events in tumor metastasis and malignant transformation and is frequently accompanied by enhanced mobility of tumor cells. Tumor cells must undergo several morphological changes in cell shape and volume as they navigate through narrow, tortuous extracellular spaces [32]. The translocation of cell processes has been suggested to require local volume increases in those parts of the cell that are in the active process of locomotion [33,34]. Moreover, it is believed that Cl^- and K^+ enter at the leading edge of lamellipodia, leading to local swelling that is obligatory for translocation [35]. An increase in cell volume usually initiates the regulatory volume decrease (RVD) process through activation of ion (K^+ and Cl^-) channels and transporters, which returns the cell to its normal volume. The activation of $I_{Cl,vol}$ is crucial for RVD [10,25]. Recently, Cl^- efflux through the volume-activated Cl^- channel was shown to be required for migration of glioma cells [12]. In our previous studies, chloride channel blockers, which inhibit $I_{Cl,vol}$, also inhibited the migration of CNE-2Z cells with a similar IC_{50} [13]. Both the rate of RVD and the functional expression of the volume-activated Cl^- channel were up-regulated in nasopharyngeal carcinoma CNE-2Z cells that had migrated [14,15]. These data suggest that this chloride current facilitates the changes in cell volume and morphology that are required for cell migration. The ClC-3 channel may underlie the $I_{Cl,vol}$ that modulates RVD and therefore cell migration. This hypothesis is supported by the direct evidence reported herein. Transfection of CNE-2Z cells with ClC-3 antisense oligonucleotides inhibited both $I_{Cl,vol}$ and RVD in the concentration-dependent manner with similar IC_{50}. These results support the notion that ClC-3 is a fundamental molecular component of the volume-activated chloride channel in CNE-2Z cells and is crucial for RVD. Disruption of ClC-3 function might result in impaired control of $I_{Cl,vol}$ and RVD, abnormality in the control of cell size and shape and subsequent decrease of cell migration ability.

The results in this study show that the ClC-3 antisense oligonucleotide suppressed about 70% of $I_{Cl,vol}$ and the remained current could not be inhibited by tamoxifen.

Furthermore, the migration rate was inhibited only by about 59% in the CNE-2Z cells transfected with the ClC-3 antisense oligonucleotide. These data suggest that the ClC-3 channel is the main, but not the only component accounting for the $I_{Cl,vol}$ associated with cell migration in CNE-2Z cells. There may be other types of volume-activated channels that are not sensitive to tamoxifen present in CNE-2Z cells. It has been reported that there are at least three types of volume-activated chloride channels or channel regulators in bovine ciliary epithelial cells [36–38].

ClC-3 channels may also affect cell migration by other pathways, due to its differential expression in different cell types [39]. ClC-3 channels are expressed in intracellular vesicles, as well as on the cell membrane, and are involved in intracellular compartment or endosomal acidification and chloride accumulation [40,41]. Intracellular ClC-3 channels exists as monomers, but the ClC-3 channels on cell membranes are present as dimers [42]. The expression of monomeric ClC-3 on intracellular vesicular membrane may affect cell migration by regulating intracellular vesicle acidification or other intracellular signaling pathways.

In conclusion, we have shown that transient transfection of CNE-2Z cells with ClC-3 antisense oligonucleotides inhibited the expression of ClC-3, cell migration, the $I_{Cl,vol}$, and RVD. The expression levels of ClC-3 were closely related to cell migration, $I_{Cl,vol}$, and RVD. ClC-3 may thus function as a volume-activated chloride channel that regulates cell migration by modulating cell volume. ClC-3 may also provide a new therapeutic target for interventions of tumor metastasis.

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