

Research and progress on ClC-2 (Review)

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Abstract. Chloride channel 2 (ClC-2) is one of the nine mammalian members of the ClC family. The present review discusses the molecular properties of ClC-2, including CLCN2, ClC-2 promoter and the structural properties of ClC-2 protein; physiological properties; functional properties, including the regulation of cell volume. The effects of ClC-2 on the digestive, respiratory, circulatory, nervous and optical systems are also discussed, in addition to the mechanisms involved in the regulation of ClC-2. The review then discusses the diseases associated with ClC-2, including degeneration of the retina, Sjögren's syndrome, age-related cataracts, degeneration of the testes, azoospermia, lung cancer, constipation, repair of impaired intestinal mucosa barrier, leukemia, cystic fibrosis, leukoencephalopathy, epilepsy and diabetes mellitus. It was concluded that future investigations of ClC-2 are likely to be focused on developing specific drugs, activators and inhibitors regulating the expression of ClC-2 to treat diseases associated

with ClC-2. The determination of CLCN2 is required to prevent and treat several diseases associated with ClC-2.

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1. Introduction

Chloride channels (ClCs) are a type of permeable channel protein for chloride ions or other anions on the cell membrane, and ClC proteins are encoded by genes of the ClC family. ClCs have nine family members, which are classified into three distinct subfamilies: ClC-1, ClC-2, ClC-Ka/K1 and ClC-Kb/K2; ClC-3, ClC-4 and ClC-5; and ClC-6 and ClC-7 (1). ClC-2 is one of the nine mammalian members of the ClC family, and was initially isolated from the rat heart and brain (2,3) and then from the rabbit heart (4,5). ClC-2 is a two-pore homodimeric, voltage-gated Cl⁻ channel (5-8). ClC-2 can be activated by hyperpolarization (3,9), cell swelling (2,9), extracellular hypotonicity (2) and extracellular acidification (9,10). ClC-2 is almost ubiquitously expressed (3,11), including in ureteric bud cells (12), intestine (13-17), gastric parietal cells (18,19), the liver (20), lung (11,21-26), rat retina (27), parotid acinar cells (28), guinea pig cardiac muscle (29), neuronal cells (30), rat and human airways (17), bovine trabecular meshwork (31), human trabecular meshwork (32,33) and rat trabecular meshwork (34). In addition, ClC-2 can regulate cell volume (2,4,35), control response to swelling (11,36-39), and regulates post-synaptic responses to GABA and glycine (11,40,41).

Although ClC-2 has a wide variety of properties and functions, reports of ClC-3 prior to 1994 are limited, with only six published between 1953 and 1994. Between 1994 and 2015,

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Abbreviations: ClCs, chloride channels; IGE, idiopathic generalized epilepsy; MLC, megalencephalic leukoencephalopathy with subcortical cysts; TLE, temporal lobe epilepsy; IBS, irritable bowel syndrome; CF, cystic fibrosis; LG, lacrimal glands; DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid; CFTR, cystic fibrosis transmembrane conductance regulator; HSN, hereditary sensory neuropathy; JAK2, janus kinase 2; JAK3, janus kinase 3; NEDD4-2, neural precursor cell expressed developmentally downregulated gene 4-like; SGKs, serine/threonine protein kinases; PIKfyve, FYVE finger-containing phosphoinositide kinase; GABA_AR, GABA_A receptor; EP (4), prostaglandin E2 receptor 4

Key words: chloride channel 2, molecular properties, biophysical properties, functional properties, regulation mechanism, chloride channel 2 diseases

CIC-2 attracted increasing attention as CIC-2 was understood to possess several molecular, functional and physiological properties, and be associated with several diseases, including degeneration of the retina, Sjögren's syndrome, age-related cataracts, degeneration of the testes, azoospermia, lung cancer, constipation, repair of impaired intestinal mucosa barrier, leukemia, cystic fibrosis, leukoencephalopathy, epilepsy and diabetes mellitus. However, reviews on CIC-2 are limited. The present review aimed to discuss the molecular, functional and physiological properties of CIC-2, in addition to mechanisms involved in the regulation of CIC-2 and diseases associated with CIC-2.

2. Molecular properties of CIC-2

CLCN2 and CIC-2 promoter. The CIC-2 protein is encoded by the CLCN2 gene, which is composed of 898 amino acids and is located in chromosome 3q27.1. The major transcription start site of the CIC-2 gene has been identified, and is localized 100 bp upstream of the putative translation initiation codon (21). A previous study (42) on the possible evolution of CIC-2 gene suggested that CIC-2 may have evolved by gene duplication, mutation and DNA rearrangement (27,43).

The CIC-2 promoter belongs to a GC-rich and TATA-less class (44). Within the 1,930-bp region, one of three CAAT boxes is close to the CIC-2 coding sequence, and the other two are at the middle (44). Within a 391-bp region upstream, the first three of four GC boxes are conserved in human CIC-2, as reported by Cid *et al* (21,44).

Structural properties of CIC-2 protein. Despite wide functional diversity, CIC family members share a conserved protein structure, including a transmembrane region, which is involved in chloride anion transport, and two intracellular copies of the cystathionine- β -synthase (CBS) domain (CBS1 and CBS2) (5-8,45,46) and an N-terminus. Therefore, the protein structure of CIC-2 is the same in the other CIC family members (45,46), as shown in Fig. 1.

Several other studies on the structural properties of CIC-2 have reported that the CIC-2 dimer is the minimum functional structure (47), protons act independently from the possible effects of the N-terminus on gating (48) and the hetero-dimerization of CIC-2 can modify the unitary conductance of protopores (49).

3. Biophysical properties of CIC-2

In 1992, it was reported that CIC-2 expressed in *Xenopus* oocytes generates Cl^- currents, which activate slowly upon hyperpolarization and show a linear instantaneous current-voltage association (3). Further studies on the biophysical properties on CIC-2 have focused predominantly on Cl^- currents, including CIC-2-like inwardly rectifying Cl^- currents (3,50-54) and CIC-2 outwardly rectifying Cl^- currents (36,37,52,55-59).

CIC-2-like inwardly rectifying Cl^- currents have been detected in the human T84 cell line (50), drosophila CIC-2 variants in HEK-293 cells (51) and rat type IV spiral ligament fibrocytes (52). The conductivity sequence of the inwardly rectifying currents is $\text{Cl}^- \geq \text{Br}^- > \text{I}^-$ (3). CIC-2 inwardly rectifying Cl^- currents are inhibited by 4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid (DIDS) (3) and Cd^{2+} (50), and regulated by cell

swelling (53), extracellular pH, Cl^- and Ca^{2+} (52). However, the results of another study on CIC-2 inwardly rectifying Cl^- currents differed, reporting that CIC-2 did not significantly contribute to inward-rectifying anion conductance in the mouse choroid plexus (54).

CIC-2 outwardly rectifying Cl^- currents have been found in the T84 human adenocarcinoma cell line (36,37,55), human neurons (56), human parotid acinar cells (57) and rat type IV spiral ligament fibrocytes (52). The outwardly rectifying currents are characterized by a time-dependent decay at depolarizing voltages, and the anion permeability sequence $\text{I}^- > \text{Br}^- > \text{Cl}^- > \text{F}^-$, and the currents show sensitivity to 1,9-dideoxyforskolin, DIDS (58) and tamoxifen (55). CIC-2 outwardly rectifying Cl^- currents are regulated by extracellular pH, Cl^- and Ca^{2+} (52). Another study showed that CIC-2 may contribute to cell volume regulation following hyposmotic stress produced by outwardly rectifying Cl^- currents, however, cell volume regulation in T84 cells is independent of CIC-2 activity (59).

CIC-2 inwardly/outwardly rectifying Cl^- currents are simultaneously found in rat type IV spiral ligament fibrocytes (52), and they are modulated by extracellular pH, Cl^- and Ca^{2+} . According to previous studies, at least two chloride channels are involved in modulating membrane anion conductance (52). Another study on Cl^- currents in rabbit heart cells suggested that rabCIC-2 α /2 β may provide two homologous protein kinase A-activated chloride anion channels, with or without extracellular hypotonicity (60).

4. Functional properties of CIC-2

Previous studies have shown that CIC-2 has several functional properties, including effects on cell volume (2,4,20,35,61), the digestive system (11,16,18,19,62-64), the eye (65-67), respiratory system (11,24,26,68-70), circulatory system (35,71-75) and nervous system (76). In addition, several studies have reported the functions of the NH₂ terminus (77) and carboxy terminus (78) in CIC-2, and the similar functional properties between chloride-selective ion channels, including CIC-2, in protozoa (79-82).

Regulation of cell volume. The activation of CIC-2 in response to cell swelling and low extracellular pH is involved in the dissociation of an NH₂-terminal region of CIC-2 (10). The dephosphorylation of serine/threonine residues in the channel or an associated protein appears to be necessary for current activation following cell swelling in T84 cells (53) and for a CIC-2-like current in ascidian embryo cells during the cell cycle (83). Hypotonic cell swelling activates an outwardly rectifying anionic conductance, with different characteristics from those of CIC-2 previously reported (84). The endogenous currents suggest that protein tyrosine phosphorylation is involved in the signal transduction pathway in certain cell types (85-87), and that epidermal growth factor enhances the hypotonicity-induced anionic efflux (85). However, tyrosine kinase p56^{lck} activates these swelling-sensitive chloride channels without cell swelling, even during cell shrinkage, in lymphocytes (88).

Effect of CIC-2 on the digestive system. Nehrke *et al* (62) detected the regulatory volume decrease following cell

swelling, which showed that CIC-2 can control cell volume in mouse parotid acinar cells. Others functions of CIC-2 have been reported, including HCl secretion across the parietal cell secretory membrane in the rabbit gastric mucosa (19), mediating the basolateral membrane exit of Cl⁻ in the distal colon of guinea pigs (16), regulating gastric acid secretion (11,18,63), and affecting gastric glands and its cell layers (63). However, one study suggested that CIC-2 may not be a Cl⁻-transporting protein for gastric acid secretion in parietal cells (64).

Effect of CIC-2 on the eye. Previous studies have shown that CIC-2 has a protective effect on trabecular cells under pressure stress (65), can regulate the inward rectification in *Drosophila* retinal photoreceptors (66), and can modulate cellular volume, intracellular Cl⁻ and other cellular functions in trabecular meshwork cells (67).

Effect of CIC-2 on the respiratory, circulatory and nervous systems. CIC-2 is gestationally regulated and is predominantly expressed in the fetal lung (26,68). Cystic fibrosis transmembrane conductance regulator (CFTR)-independent fluid accumulation is induced by keratinocyte growth factor in fetal lung explants (69), and keratinocyte growth factor is necessary for CIC-2-like pH-sensitive Cl⁻ secretion in the fetal airway epithelia (24). It has been shown that CIC-2 affects fetal lung fluid production and lung cyst morphology (70).

It has been reported that a novel Cl⁻ inward rectifier channel is found in the cardiac atrial and ventricular myocytes of several species, including the guinea pig (71-73), mouse (71,73) and rat (72-75). The Cl⁻ inward rectifier channels have properties of activation by hypotonic cell swelling and extracellular acidification, inward rectification, time-dependent slow activation and Cd²⁺-sensitive inhibition (71-75). Experiments have shown that CIC-2 affects the positive chronotropic effect of acute exercise stress (73) and modulates cardiac pacemaker activity (73), but cannot alter intrinsic heart rate (73).

Another study (76) investigating the effect of CIC-2 on inhibitory interneurons showed that membrane voltage-/intracellular chloride-dependent CIC-2 can selectively regulate GABA_A receptor (GABA_A R)-mediated synaptic inputs from basket cells.

5. Mechanisms of CIC-2 regulation by regulators

Cell swelling can increase CIC-2 activity (2,9), and CIC-2 can be activated by hyperpolarization (3,9), extracellular hypotonicity (2) and extracellular acidification (9,10). Previous studies have shown that CIC-2 is also regulated by hormones (89-91), drugs (92-100), proteins (101-107), kinases (108-114), transcription factors (115,116), inhibitors (117-120), scorpion venom (121), α 1-adrenoceptor (122), *Plasmodium berghei* (123), adenosine triphosphate (124,125), ClH₃ (40,126-137), permeant anions (138,139), membrane cholesterol (140) and the tyrosine endocytosis motif (141).

Regulation of CIC-2 by hormones. Thyroid hormones (T3 and T4) can regulate renal function (142,143), Na⁺/K⁺ ATPase (144), Na⁺/H⁺ exchanger (145), and affect kidney growth and development (146). Previous studies have demonstrated that hyperthyroidism and hypothyroidism are associated with

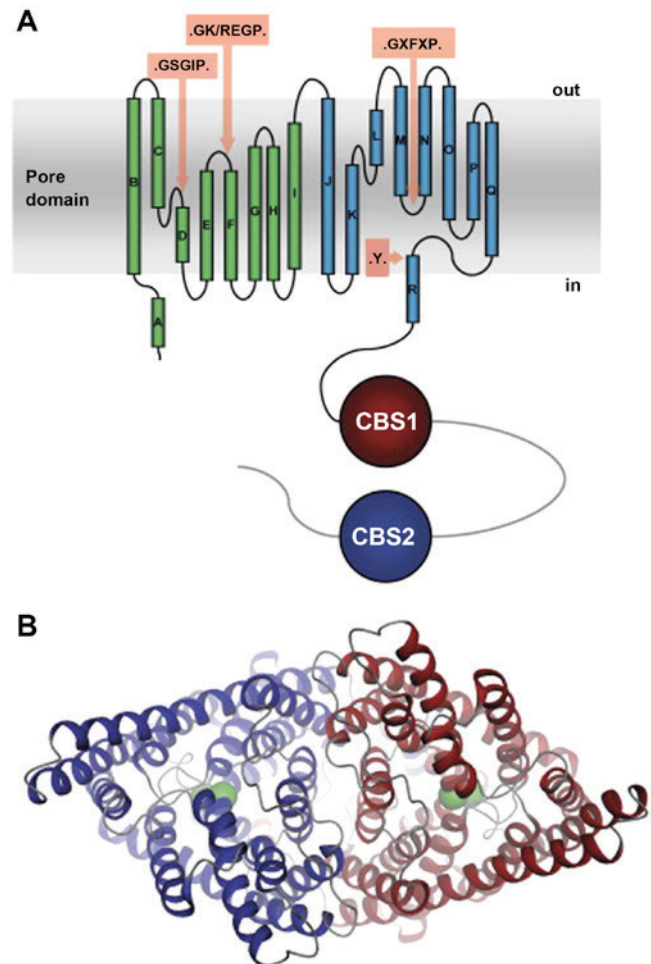


Figure 1. Basic structure of CIC-2 as a two-pore homodimeric channel. CIC-2 is a double-barreled channel with two identical, predominantly independent pores. (A) 18 α -helices are labeled A-R, and the two similar halves within the transmembrane domain (α -helices B-I and J-Q), which are oriented in opposite directions to the membrane, and are shown in green and cyan. The sequence regions, which contribute to the Cl⁻ selectivity filter, are indicated by orange arrows, and the respective conserved sequences are shown; CBS1 is colored red and CBS2 is colored blue. (B) Structure viewed from the extracellular side. The two subunits of the homodimeric protein are shown in red and blue, and bound anions are shown in green. CIC-2, chloride channel 2; CBS, cystathionine- β -synthase.

chloride (147,148), and the reversal of glomerular filtration by T4 is observed in patients with hypothyroidism (149). In addition, a previous study (89) on T4 suggested that CIC-2 is involved in chloride transport regulated by thyroid hormones in rat renal proximal tubules.

Estrogen is associated with a decrease in the urinary excretion of sodium and chloride (150-153). A study by Nascimento *et al* (90) showed that CIC-2 may be involved in estrogen-induced Cl⁻ transport in the rat kidney. Another study (91) showed that CIC-2 is also involved in the rat renal tubule transcellular chloride transport regulated by arginine vasopressin, a neurohypophysial hormone.

Regulation of CIC-2 by drugs. Lubiprostone can induce intestinal fluid secretion (154), treat constipation (155-157) and activate CIC-2 Cl⁻ currents in a concentration-dependent manner (94), and CIC-2 can recover mucosal barrier function in the ischemia-injured intestine (92). Studies have shown that the

activation of CIC-2 by lubiprostone can stimulate the recovery of intestinal barrier function in the ischemia-injured porcine ileum and colon (93), and may have a protective and therapeutic effect on murine models of colitis (99). The prostaglandin E2 receptor 4 [EP (4)] receptor (94-97) can stimulate CIC-2 and CFTR chloride channels, and it has been shown (98) that lubiprostone can activate CFTR by the EP (4) receptor in oocytes. The Chinese medicinal compounds Guanyin and diltiazem hydrochloride can decrease the mRNA and protein expression of CIC-2 in rats with myocardial ischemia reperfusion injury (100).

Regulation of CIC-2 by proteins. The functional expression and activation of CIC-2 is reduced by dynein (a protein complex) (101) and p34^{cdc2}/cyclin B (102), however, the interferon- γ glycoprotein can activate CIC-2 in lung epithelial cells through mRNA stabilization, and increase CIC-2 transcripts in Calu-3 cells (107). CIC-2 is regulated by protein phosphatase 1 (102), interactions between the actin cytoskeleton (a filamentous protein structure) and the N-terminus of CIC-2 (104). M phase-specific p34^{cdc2}/cyclin B can phosphorylate the ubiquitination of CIC-2 (103). Heat shock protein 90 can increase CIC-2 current amplitude and the intracellular Cl⁻ concentration, and enhance channel sensitivity to intracellular Cl⁻ (105). CIC-2 is also associated with the transmembrane glycoprotein, GlialCAM, in the brain, and GlialCAM can involve CIC-2 in the homeostasis of myelin, which is defective in leukodystrophy (106).

Regulation of CIC-2 by kinases. CIC-2 membrane abundance is increased by serum and glucocorticoid inducible kinases (SGKs) and decreased by NEDD4-2, an enzyme of the NEDD4 family (108). In rats, protein kinase A can directly phosphorylate CIC-2, whereas protein kinase C and Ca²⁺/calmodulin-dependent protein kinase II cannot (113). PIKfyve is a FYVE finger-containing phosphoinositide kinase (158) and is a potent stimulator of CIC-2-activity (112), which can contribute to the SGK1-dependent regulation of CIC-2 (112).

Janus kinase (JAK) 2 and JAK3 (tyrosine kinase) can downregulate the activation of CIC-2 and offset Cl⁻ exit (109,111), however, their functions and regulatory mechanism are different. JAK2 is involved in the signaling of leptin (159), growth hormones (160), erythropoietin, thrombopoietin, granulocyte colony-stimulating factor (161) and a variety of cytokines (161,162). JAK2 inhibitors can treat myeloproliferative disorders (163-170), and JAK2 can be activated by hyperosmotic shock (171,172). By contrast, JAK3 can promote proliferation and act against the apoptosis of lymphocytes and tumor cells (173-177). It is also present in acute megakaryoblastic leukemia (178,179). Therefore, cell proliferation and apoptosis is associated with Cl⁻ channel activity (110). In addition, the substitution of lysine by alanine can inactivate JAK3 (180). In addition to JAK2 and JAK3, SPS1-related proline/alanine-rich kinase and oxidative stress-responsive kinase 1 can also downregulate CIC-2 (114).

Regulation of CIC-2 by transcription factors. The Sp1 and Sp3 transcription factors can control the rate of transcription of genetic information from DNA to messenger RNA (181,182). Reducing interactions between Sp1 or Sp3 and the CIC-2

promoter can lead to a postnatal decrease in the expression of CIC-2 in lung epithelia (116). In addition, the glycosylation of SP1 produces the 105-kD isoform of SP1 and is involved in regulating the expression of CIC-2 (115).

Regulation of CIC-2 by inhibitors. Previous studies have shown that CIC-2 is inhibited by methadone (117), gating modifier of anion channels 2 (GATx2) (118), DIDS (119) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) (120). However, DASU-02, as an inhibitor of common chloride channels, cannot inhibit CIC-2 (117). The inhibitory effects on CIC-2 by these inhibitors are entirely different. Methadone can inhibit the activities of CIC-2, which shows that CIC-2 may be involved in chloride anion secretion (117). GATx2, as a peptide toxin inhibitor of CIC-2, can inhibit channel activation gating, but not other chloride channels or voltage-gated potassium channels (118). DIDS can significantly reduce the increased mRNA and protein levels of CIC-2 following ischemia-hypoxia damage (119). NPPB, as a CIC inhibitor, can affect the phagocytosis of human trabecular meshwork cells, which shows that CIC-2 may be involved in the regulation of its phagocytic process (120).

Other factors in the functional regulation of CIC-2. According to previous studies, in addition to the above-mentioned regulatory mechanisms, scorpion venom (121), α 1-adrenoceptor (122), *Plasmodium berghei* (123), adenosine triphosphate (124,125), ClH₃ (137), GABA_AR (40,126-128), permeant anions (138,139), membrane cholesterol (140) and β -cyclodextrin sensitive clusters (141) can also regulate or affect CIC-2. A peptide of scorpion venom can induce slower activation kinetics of CIC-2 (121). α 1-adrenoceptor activation can significantly reduce protein levels of CIC-2 in the villus and crypt epithelial cells from the acutely denervated jejunum, but not the innervated jejunum (122). The activation of CIC-2 is involved in the altered permeability caused by *Plasmodium berghei* infection in *Plasmodium berghei*-infected mouse CLCN2 (123). ATP not only decelerates CIC-2 common gating for sufficient electrical stability of neurons (124), it also alters its surface expression (125). Intra-/extra-cellular permeant anions can affect the V_m-dependence of CIC-2 (138). Specially, Cl⁻ as a permeant anion can increase pore occupancy, thus obstructing the closure of the protopore gate of CIC-2 (139). In addition, alterations in the (Cl⁻)_i between 10 and 200 mM can decelerate CIC-2 channel closing at a positive V_m (139). Membrane cholesterol can regulate the activation of CIC-2, and the increased activation of CIC-2 is involved in the relocalization of CIC-2 to detergent-soluble microdomains (140). β -cyclodextrin sensitive clusters with other molecules can maintain CIC-2 activity (141).

Previous studies have reported that ClH₃ encodes the *Caenorhabditis elegans* homologs of CIC-2 (129-131). ClH₃-dependent regulation not only alters the voltage dependence of CIC-2 channels and inhibits hereditary sensory neuropathy (HSN) excitability (137), but may also affect chloride influx (137) in *Caenorhabditis elegans*. However, the chloride efflux pathway of CIC-2 is associated with synaptic inhibition regulated by the GABA_AR (40,126-128). In addition, the HSNs can release acetylcholine, serotonin and multiple neuropeptides (133-136), which excite the VC motor

neurons (132). These functions of HSNs may be associated with *clh-3*-dependent regulation and the voltage dependence of *CIC-2* channels.

6. Diseases associated with *CIC-2*

Previous studies have shown that disruption and abnormality of *CIC-2* can cause a number of diseases, including ophthalmological disease (11,183-185), otorhinolaryngological disease (186), disease of the reproductive system (11,187), disease of the respiratory system (188), disease of the digestive system (189-194), disease of the hematological system (195), genetic diseases (23,25,26,42,44,74,76,196-198), diseases of the nervous system (30,40,41,168,199-212) and metabolic diseases (213). The majority of studies have focused on diseases of the nervous system.

*Ophthalmological and otorhinolaryngological disease associated with *CIC-2*.* Severe degeneration of the retina occurs in *CIC-2*-knockout mice, although no other notable eye abnormalities are present (11). Possible reasons for degeneration of the retina caused by *CIC-2*-knockout include the following (11): i) *CIC-2* disruption causes the death of photoreceptor cells; ii) depletion of *CIC-2* may impair the transport and alter the ionic environment of photoreceptors. Other studies on ophthalmological diseases associated with *CIC-2* include Sjögren's syndrome (183,184,214-218) and age-related cataracts (185).

Sjögren's syndrome is a chronic autoimmune disease, and previous studies have shown that eye diseases caused by Sjögren's syndrome are primarily through the destruction of lacrimal glands (LGs) (214), keratoconjunctivitis sicca (215). In addition, LG pathologic properties in Sjögren's syndrome appear in rabbits induced by autoimmune dacryoadenitis (216-218). Acini and interlobar ducts have the lowest mRNA abundance of *CIC2γ*, and the intralobar duct has the highest (183,184), and alterations in *CIC2γ* may alter lacrimal secretion, particularly Cl^- transport (184). The causes of age-related cataracts include aging, inheritance, local nutritional disorders, immune and metabolic abnormalities, trauma, poisoning and radiation. According to a study by Ouyang (185), the expression level of *CIC-2* is associated with the development of age-related cataracts.

Reports on otorhinolaryngological diseases associated with *CIC-2* are limited, however, Li *et al* reported on the pathogenesis of nasal polyps. The study (186) showed that *CIC-2* was not expressed in normal nasal mucosa, however, *CIC-2* proteins were expressed in epithelial cells and sub-epithelial mucous glands in patients affected with nasal polyps. This suggested that *CIC-2* is involved in the pathogenesis of nasal polyps.

*Diseases of the reproductive system associated with *CIC-2*.* In previous studies, a number of diseases of the reproductive system have been associated with *CIC-2*, including degeneration of the testes (11) and azoospermia (187,219,220).

It was shown that severe degeneration of the testes appears in *CIC-2*-knockout mice, however, no other reproductive abnormalities are observed (11). A study by Bösl *et al* suggested that *CIC-2* disruption causes the death of germ cells, impaired

transport and alterations to the ionic environment of germ cells, and that germ cells rely on the transepithelial transport mediated by Sertoli cells (11).

Azoospermia is a medical condition in which males do not have a measurable level of sperm in their semen, and is associated with low levels of fertility or infertility. Its pathogenesis involves CFTR disruption in CF, affecting male fertility (219). In addition, the male sterility resulting from reduced fluid volume is associated with disruption of Cl^- channels from other *CICN* genes (220). Another study (187) by Edwards *et al* reported that *CICN2nmf240* homozygotes have azoospermic symptoms severe degradation of spermatogenesis, and short-ages of spermatocytes, spermatoblasts and sperm.

*Diseases of the respiratory system associated with *CIC-2*.* Reports on diseases of the respiratory system associated with *CIC-2* are limited. A previous study (188) reported that *CIC-2* may be important in the invasion, development and occurrence of lung cancer, and that *CIC-2* may be a novel molecular target for clinical therapy in non-small cell lung cancer.

*Diseases of the digestive system associated with *CIC-2*.* There have been several reports on diseases of digestive system associated with *CIC-2*, including constipation (18,26,189-193,221) and repair of impaired intestinal mucosa barrier (194).

CFTR is major chloride channel regulating chloride secretion in the small intestine (221), however, *CIC-2* can also regulate chloride secretion (18,26). Hypotonicity can also activate chloride currents in the rat ileum (189). Several studies have reported that *CIC-2* is expressed in the intestinal epithelium, in T84 human intestinal cells, rat intestinal tissue (3) and the murine duodenum (190). In addition, previous studies have shown that *CIC-2* can regulate chloride secretion in rodent neonatal airways (26), and regulate gastric chloride secretion (18). However, another study (191) described contradictory findings, reporting that *CIC-2* was not involved in Cl^- secretion, but was involved in Cl^- absorption in the distal colon. According to these studies, *CIC-2* inhibitors may be used to treat constipation by decreasing NaCl and water absorption in the colon (191).

Irritable bowel syndrome (IBS) is characterized by chronic abdominal pain, discomfort and bloating associated with altered bowel habits, including diarrhea and/or constipation. Current therapy for constipation caused by IBS has side effects, including deterioration of the condition or electrolyte disturbances (192). Previous studies have shown that, in addition to the above-mentioned side effects, lubiprostone can also increase gut motility and frequency of stool passage, relieve abdominal pain and discomfort (192), stimulate chloride secretion and improve bowel function (193).

A previous study (194) by Chen *et al* reported that the activation of *CIC-2* can activate tight junction proteins and repair impaired intestinal mucosa barrier. In addition, *CIC-2* and tight junction proteins are involved in maintenance of the intestinal mucosal barrier, and acute biliary obstruction-induced destruction of the intestinal mucosa barrier is associated with *CIC-2* in enterocytes (194).

*Diseases of the hematological system associated with *CIC-2*.* There have been few studies on diseases of the hematological

system associated with CIC-2. A previous study reported the expression of swelling-and/or pH-regulated CIC-2 chloride channels in human leukemia (195). In addition, it was suggested that the molecular identification of chloride channels may provide a novel approach for the treatment of leukemia (195).

Genetic diseases associated with CIC-2. According to previous reports, there have been several studies on the association between CF and CIC-2 (3,11,23,25,26,68,70,196-198,222,223). Several studies have reported that CIC-2 may be an alternative pathway for chloride anion secretion in CF (3,11,23), and that CIC-2 is a potential target for therapy in CF (25,197). CF is a genetic disease, which primarily affects the lungs, in addition to the pancreas, liver, kidneys and intestine (222). CF is caused by mutations in the gene encoding CFTR, the only member of the ABC transporter family known to be a cAMP-activated chloride channel (223). CIC-2 is a candidate alternative chloride channel in respiratory epithelia (196). CIC-2 is also involved in lung morphogenesis (26,68), and can conduct chloride in mature respiratory epithelia (23,25,197). CIC-2 mRNA and protein are expressed in unaffected tissues in CF, which may make up for defects in the expression of CFTR (68).

However, another previous study (198) reported that CIC-2 is unlikely to be a candidate rescue channel in CF, as disruption of CIC-2 and CFTR channel genes did not cause morphological alterations in the intestine, lung or pancreas affected by CF; neither disrupted CIC-2 or CFTR reduced Cl⁻ secretion (198). In addition, two studies (70,196) refer to the importance of detecting modifications of CF by CIC-2, and underlined the importance of examining potential polymorphisms in subjects affected with CF and potential mutations in the coding region of CIC-2.

Diseases of the nervous system associated with CIC-2. There have been more studies on diseases of the nervous system associated with CIC-2, compared with other diseases associated with CIC-2. Studies on diseases of the nervous system associated with CIC-2 have predominantly focused on leukoencephalopathy (199-202,224) and epilepsy (30,40,203-212,225).

Leukoencephalopathy can refer specifically to any of the following diseases: Progressive multifocal leukoencephalopathy, toxic leukoencephalopathy, leukoencephalopathy with vanishing white matter, leukoencephalopathy with neuroaxonal spheroids, reversible posterior leukoencephalopathy syndrome, megalencephalic leukoencephalopathy with subcortical cysts (MLC) and hypertensive leukoencephalopathy. Among these, MLC is a rare type of leukodystrophy, which is characterized by macrocephaly emerging in the first years of life (224).

The disruption of CIC-2 can cause fluid accumulation resulting in myelin vacuolation in mice, similar to that observed in humans affected with MLC from mutations in MLC1 or GlialCAM. GlialCAM is a CIC-2 binding partner and the first auxiliary subunit of CIC-2 (199). According to a study by Jeworutzki *et al* (199), neither the stimulation of GlialCAM on CIC-2 currents, nor mislocalization of this Cl⁻ channel were found to damage glial Cl⁻ transport. Mutated GlialCAM in MLC can target CIC-2 to cell contacts in glia and activate its currents (200). A study by Jeworutzki *et al* (200) showed that GlialCAM can target the common gate deficient CIC-2 mutant, E211V/H816A, to cell contacts without

altering its function. Another study suggested that CIC-2 is not important for MLC1 or GlialCAM localization in the brain (201), but that it is involved in the pathogenesis of MLC (201).

However, a separate study (202) reported different conclusions, as there was no evidence that the CLCN2 gene is associated with MLC. Despite not referring to the association between the CLCN2 gene and MLC, this study demonstrated that mice lacking the CIC-2 protein had white matter abnormalities with vacuole formation in myelin sheaths, similar to the intramyelinic vacuoles in MLC.

Epilepsy comprises a set of neurological diseases characterized by epileptic seizures, and a heterogeneous disorder characterized by recurrent unprovoked seizures, which affect ~1-3% of the population during their lifetimes (225). CIC-2 mRNA and protein are found in neurons and astrocytes (30,40,203). In addition, CIC-2 protein is present at the end feet of astrocytes contacting blood vessels and neurons close to inhibitory synapses (30). Inwardly rectifying hyperpolarization-activated CIC-2-like currents are found in hippocampal pyramidal cells (40,127) and in astrocytes (204-207). In hippocampal pyramidal cells and astrocytes (208), CIC-2 may have different effects. In neurons, CIC-2 can prevent the accumulation of chloride anions above equilibrium due to the activation of CIC-2 by intracellular Cl⁻ (41,127). Under these conditions, GABA_A R activity may become excitatory (208). A previous study (208) reported that loss of function mutations of CIC-2 lead to increased excitability in certain neurons, and that hyperpolarization-activated chloride currents are detected in cortical astrocytes, but absent in tissues from CIC-2-null mice (207). Another study (211) showed that several CIC-2 sequence abnormalities previously found in patients affected with epilepsy are likely to represent innocuous polymorphisms, detected by sequencing of a large collection of human DNA and electrophysiological analysis.

Several other studies have reported that a susceptibility locus for idiopathic generalized epilepsy (IGE) is on chromosome 3q26 (the location of the CLCN2 gene) (209), and three mutations on CIC-2 cosegregated with IGE with autosomal dominant inheritance (210) have been identified.

In addition to the above-mentioned studies on the association between epilepsy and CIC-2, an association between temporal lobe epilepsy (TLE) and CIC-2 has been reported. TLE with spontaneous recurrent attacks, and learning and memory disabilities, is associated with neurodegeneration, abnormal reorganization of the circuitry and loss of functional suppression in hippocampus (212). A study by Ge *et al* reported that CIC-2 contributes towards tonic inhibition, modulated by $\alpha 5$ subunit-containing GABA_A Rs in the CA1 area (212).

Metabolic diseases associated with CIC-2. Few studies on metabolic diseases associated with CIC-2 have been performed, however, a study on diabetes mellitus was found. Diabetes mellitus is a metabolic disease, which generally causes chronic delayed wound healing. In a study by Pan *et al* (213), a high glucose concentration inhibited keratinocyte migration by downregulating CIC-2, suggesting CIC-2 may be important during delayed wound healing processes. In addition, the study reported that CIC-2 is an important

modulator of cell migration in keratinocytes, although it did not discuss how CIC-2 is involved in keratinocyte migration.

7. Conclusion

With the continuous progress in experimental and clinical studies on the CIC family, it is clear that the activation of CIC-2 occurs via hyperpolarization (3,9), cell swelling (2,9), extracellular hypotonicity (2) and extracellular acidification (9,10), and that the expression of CIC-2 is ubiquitous (3,11) in ureteric bud cells (12), the intestine (13-17), gastric parietal cells (18,19), the liver (20), the lungs (21-25), rat retina (27), parotid acinar cells (28), guinea pig cardiac muscle (29), neuronal cells (30), rat and human airways (17), bovine trabecular meshwork (31), human trabecular meshwork (32,33) and rat trabecular meshwork (34). Although there has been progress in understanding the CLCN2 gene, the molecular structure of the CIC-2 protein, the structure of CIC-2 chloride channels, and the functional properties and mechanisms regulating CIC-2, they remain to be fully elucidated and there have been contradictions in previous studies (226). According to previous studies, it is known that the disruption of CIC-2 can lead to several diseases. Although CIC-2 is associated with the pathogenesis of several diseases, the association between pathogenesis and CIC-2 remains to be fully elucidated.

At present, >6,500 types of genetic disease have been identified, among which ~3,000 are caused by a single gene defect. In this review, the mechanisms underlying the association between the CLCN2 gene and diseases, including azoospermia and IGE, were discussed. In the future, investigations focused on the determination of the CLCN2 gene may identify novel methods to treat and prevent several diseases associated with CIC-2. In addition, as the overexpression and underexpression of CIC-2 can cause diseases, the development of specific CIC-2 activators and inhibitors, and understanding the mechanism of action between the functional properties of CIC-2 and these activators and inhibitors, is required in future investigations to regulate the expression of CIC-2.

As described above, CIC-2 can be regulated by hormones, drugs and scorpion venom. Certain hormones in humans are produced in small quantities, however, they have a substantial effect on health, with underproduction or overproduction leading to a variety of diseases. In addition, scorpion venom can be used to treat cancer and lower blood pressure, and is applied for hemostasis, anticoagulation, as an analgesic and a nerve growth factor. Therefore, future investigations focused on the regulatory mechanisms of hormones, drugs and scorpion venom may assist in developing specific drugs for treating diseases associated with CIC-2.

In conclusion, CIC-2 is important in several diseases and, in order to fully elucidate the structure and function of CIC-2, and the mechanisms regulating CIC-2 associated with disease treatment, examining the associations between CIC-2 and regulators, including hormones, proteins, kinases, transcription factors, scorpion venom, adenosine triphosphate, clh-3, permeant anions, membrane cholesterol, tyrosine endocytosis motif and α 1-adrenoceptor is required to develop novel treatment strategies. Therefore, further investigations are required in the future.

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