# Chloride intracellular channels as novel biomarkers for digestive system tumors (Review)

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**Abstract.** Digestive system malignant tumors are common tumors, and the traditional treatment methods for these tumors include surgical resection, radiotherapy, chemotherapy, and molecularly targeted drugs. However, diagnosis remains challenging, and the early detection of postoperative recurrence is complicated. Therefore, it is necessary to explore novel biomarkers to facilitate clinical diagnosis and treatment. Accumulating evidence supports the crucial role of chloride channels in the development of multiple types of cancers. Given that chloride channels are widely expressed and involved in cell proliferation, apoptosis and cell cycle, among other processes, they may serve as a promising diagnostic and therapeutic target. Chloride intracellular channels (CLICs) are a class of chloride channels that are upregulated or downregulated in certain types of cancer. Furthermore, in certain cases, during cell cycle progression, the localization and function of the cytosolic form of the transmembrane proteins of CLICs are also altered, which may provide a key target for cancer therapy. The aim of the present review was to focus on CLICs as biomarkers for digestive system tumors.

**Contents**



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## Introduction

It is estimated that there will be >1.9 million new cases of cancer in the United States in 2021, accompanied by >608,000 deaths (1). Cancer of the digestive system has the second-highest number of new cases and cancer deaths (1). It is widely accepted that the normal function of the digestive system is essential for food digestion, residue excretion, nutrient absorption, and toxic substance discharge. These functions are dependent on the transport of large amounts of water, ions, and nutrients across the epithelium. These physiological processes are achieved via the uneven distribution of ions mediated by ion channels (2), such as absorption of glucose by sodium-glucose cotransporters in the small intestine (3). In addition to controlling the distribution of chloride inside and outside of the cell to maintain water-electrolyte balance, chloride channels also contribute to the regulation of intracellular volume and pH (4). Numerous studies have found that chloride intracellular channels (CLICs) have crucial roles in tumors of the digestive system and should be considered a potential diagnostic and therapeutic target for cancer (5-9). Therefore, the present review focused on CLICs as novel biomarkers for digestive system tumors.

## 2. Structural features of CLICs

In mammals, the CLIC family has seven members, CLIC1-6. Of these, CLIC5 has two alternative splice variants, CLIC5A and CLIC5B. CLICs exist as soluble globular proteins that can form ion channels in organelles and plasma membranes (10). However, to date, only crystal structures of soluble forms of CLICs have been obtained. CLICs comprise an N-terminal thioredoxin-like domain, which contains a mixture of α-helices and β-sheets, and an α-helical C-terminal domain. The N-terminal domain includes a putative transmembrane region (PTM) (4). In the three-dimensional folded structure, there is a similarity between soluble CLICs and ω class glutathione (GSH)-S-transferases (GSTs). CLICs, similar to ω class GST proteins, include a conserved glutaredoxin-like site and a reactive cysteine residue (Cys24 in CLIC1 and Cys35 in CLIC4) in mammals (11), suggesting that the function of CLICs can be regulated in a redox-dependent manner. Furthermore, the

CLIC structure includes an elongated cleft (or groove), similar to ω class GSTs, which can bind to glutathione (10). However, CLIC proteins have a very low affinity for glutathione (4). It can therefore be inferred that CLIC proteins use GSH-binding sites to target the CLICs to specific subcellular sites (4). The structure of CLIC1 is presented in Fig. 1. In addition to the N-terminus, C-terminus and PTM, which are common among CLICs, the secondary structure of CLIC1 is also shown. The primary tissues with physiological CLIC protein expression and digestive system tumors presenting abnormal expression of CLIC proteins are presented in Table I (12-36).

## 3. Functions of CLICs associated with cancer hallmarks

CLIC functions are summarized in Table II (12-44). This review focused on CLIC functions that may be associated with hallmarks of cancer. It is well known that tumor cells exhibit a variety of special biological behaviors, including apoptosis evasion, limitless replication potential, sustained angiogenesis, tissue invasion, and metastasis (45). Ion transport exerts crucial roles in tumor development and progression (46,47). The characteristic that distinguishes CLICs from other ion channels is their dimorphic existence. CLICs are located both in the cell membrane and the cytoplasm (10). Of note, CLICs are involved in cancer by regulating ion transport, but the characteristics of their non-ionic transport need to be further elucidated. All CLICs (CLIC1-6) function as ion channels to facilitate chloride flux across cell membranes and, as such, they provide a transcellular route for chloride transport across membranes (21,39,48-51). Both the overexpression of p64 in HeLa cells (52) and purified CLIC1 following its expression in *Escherichia coli* (53) resulted in the appearance of an outwardly rectifying anion channel.

$Cl^-$, the most abundant anion in nature, is involved in general biophysical processes, such as the transport of water and osmotic equilibrium (54). Thus far, it is known that for apoptosis to occur, cell volume, DNA fragmentation or division and apoptotic body formation should be tightly controlled (55). Cell volume changes are likely to be partly due to intra and extracellular ionic imbalance. Cell shrinkage is of great importance in the initial step during the apoptotic process and is mediated by the activation of ion channels that release $K^+$, $Cl^-$ and other organic molecules, followed by the extrusion of water out of the cells by osmotic pressure gradient (56).

$Cl^-$ could have a critical role in the regulation of cell cycle progression and proliferation (57). The treatment of Jurkat leukemic T-lymphocyte cells with lectins, concanavalin A or phytohemagglutinin resulted in $Cl^-$ oscillations and increased intracellular chloride concentration [$(Cl^-)i$] (58). The lectin-induced $[Cl^-]i$ increase was blocked by anthracene-9 carboxylate (an inhibitor of $Cl^-$ channels) or through the removal of extracellular $Cl^-$, which inhibited Jurkat cell proliferation (58). Furthermore, $[Cl^-]i$ may affect the cell cycle through regulating the G1/S cell-cycle checkpoint and p21, which is a cyclin-dependent kinase inhibitor (57). Cell proliferation was reduced in the low $[Cl^-]i$ conditions following G0/G1 phase arrest in an MKN28 human gastric cancer (GC) cell line. The process involved p21 upregulation caused by low $[Cl^-]i$ but was independent of p53 (57). In addition, $[Cl^-]i$ was found to have an essential role in neurite growth in PC12 cells (59). A positive correlation was found between $[Cl^-]i$ and neurite length (60). For instance, inhibited $Na^+/K^+/2Cl^-$ cotransport 1 with bumetanide resulted in a decrease in $[Cl^-]i$ and neurite outgrowth (60).

In conclusion, $[Cl^-]i$ has been shown to inhibit proliferation and induce apoptosis. However, Heimlich and Cidlowski (61) reported that both the increased and decreased $[Cl^-]i$ had the same effect when Jurkat T-cells were exposed to UV-C, which resulted in a significant decrease in $[Cl^-]i$ and induced apoptosis through the activation of c-Jun N-terminal kinase (JNK). Those processes could be suppressed by the modulation of chloride flux through the reduction of extracellular chloride concentration or in the presence of chloride channel inhibitor disodium 4-acetamido-4'-isothiocyanato-stilben-2,2'-disulfonate, which resulted in an ~20-mM increase in $[Cl^-]I$ (61). The mechanisms underlying the biphasic effect of $[Cl^-]i$ need to be further elucidated.

CLICs not only have significant roles in cancer directly through chloride but also through interacting with other proteins and affecting cell signaling. Each CLIC member has unique functions that have been associated with hallmarks of cancer, including cell cycle, apoptosis, angiogenesis, migration and metastasis.

*CLICs and the cell cycle*. CLIC1 and CLIC4 are involved in cell cycle regulation (16,62). CLIC1 chloride conductance has been found to be altered during the cell cycle, and CLIC1 has been shown to be expressed only on the plasma membrane of Chinese hamster ovary (CHO)-K1 cells in the G2/M phase (16). In addition, the cell cycle has been shown to be arrested in the G2/M stage following treatment with chloride channel blockers indanyloxyacetic acid-94 (IAA-94) or anthracene-9-carboxylic acid (16). At the same stage, CLIC1 is selectively expressed on the plasma membrane (16). Mechanistically, the chloride flow causes the osmotic movement of water to alter the cell volume, which may in turn prevent cell division and/or the dissolution of the nuclear envelope (16). A recent study on the function of CLIC1 in medulloblastoma shed new light on its roles in cell cycle regulation (62). CLIC1-mediated chloride efflux may act synergistically with voltage-gated potassium channel-mediated potassium to decrease cell volume and control cell cycle progression. The abnormal increase in cell size beyond a certain threshold has been shown to lead to a high cytoplasm/nucleus (C/N) ratio, reduce macromolecule biosynthesis and hinder the cell cycle (63). CLIC1, whose activity is increased during mitosis, coordinates with potassium voltage-gated channel subfamily H member 5, a mitosis-specific protein localization on the plasma membrane, to regulate the C/N ratio and prevent cytoplasm dilution (62). Indeed, CLIC1 knockout inhibits the proliferation of tumor cells but does not affect mouse development, thereby increasing the survival of medulloblastoma-bearing mice (62). CLIC4 contributes to cell cycle arrest, possibly due to altering the $Cl^-$ levels and pH of the nucleus. Furthermore, CLIC4 protein knockdown induces cell cycle arrest in differentiating keratinocytes (34). When keratinocytes undergo growth arrest by differentiation *in vitro*, the cytoplasmic CLIC4 protein in actively proliferating keratinocytes translocates into the nucleus (34). Furthermore, nuclear $Cl^-$ is increased by targeting CLIC4 directly to the nucleus through adenoviral transduction in keratinocytes (34).

Table I. Expression for CLICs and their abnormal expression in digestive system tumors.

| CLIC family member | Expression in normal tissues | Type of cancer | Expression | Association with prognosis | (Refs.) |
|---|---|---|---|---|---|
| CLIC1 | Glandular, stomach, small intestine, colon, bile duct, pancreatic duct, airway, the tail of the epididymis, renal, liver, brain | ESCC | Upregulated | Prediction of prognosis of patients with ESCC | (12-18) |
| | | GC | Upregulated | Prediction of lymph node metastasis, lymphatic invasion, perineural invasion and poor prognosis | |
| | | LC | Upregulated | Prediction of tumor size, metastasis and pTNM stage and decreased overall survival | |
| | | Gallbladder cancer | Upregulated | Prediction of histological grade, TNM stage, perineural invasion and decreased overall survival | |
| | | PC | Upregulated | Prediction of prognosis of patients with PC | |
| | | CRC | Upregulated | Prediction of prognosis of patients with CRC | |
| CLIC2 | Liver, heart and skeletal muscle, brain, lung and spleen, stomach and testis | ESCC | Unchanged | ND | (19-23) |
| | | GC | Downregulated | ND | |
| | | LC | Downregulated | ND | |
| | | PC | Upregulated | ND | |
| CLIC3 | Placental, lung, heart, renal, pancreatic, skeletal muscle (low expression) | ESCC | Downregulated | ND | (15,23-26) |
| | | GC | Upregulated | CLIC3 expression is inversely correlated with the depth of tumor invasion, and low CLIC3 expression is associated with prognosis in patients with GC | |
| | | LC | Upregulated | ND | |
| | | PC | Upregulated | Prediction of prognosis of patients with PC | |
| CLIC4 | Skin, brain, liver, testis, renal, lung, skeletal muscle | ESCC | Upregulated | ND | (15,23,27-32) |
| | | GC | Upregulated | ND | |
| | | LC | Upregulated | ND | |
| | | PC | Upregulated | Prediction of prognosis of patients with PC | |
| | | CRC | Upregulated | CLIC4 overexpression is a marker of colon cancer stem cells and is associated with poor prognosis | |
| CLIC5 | CLIC5A: Heart, renal, lung, placental, skeletal, inner ear. CLIC5B: Osteoclast ruffled membrane, heart, skeletal, muscle, renal, inner ear | GC | Downregulated | ND | (15,31,33-36) |
| | | LC | Downregulated | Prediction of prognosis of patients with LC | |
| | | PC | Upregulated | ND | |

Table I. Continued.

| CLIC family member | Expression in normal tissues | Type of cancer | Expression | Association with prognosis | (Refs.) |
|---|---|---|---|---|---|
| CLIC6 | Stomach, parietal cell, choroid plexus, salivary duct, lacrimalgland, renal, airway, and chorioretinal epithelial | GC | Downregulated | ND | (15,34) |
| | | LC | Upregulated | ND | |
| | | PC | Downregulated | ND | |

CLIC, chloride intracellular channel; ESCC, esophageal squamous cell carcinoma; GC, gastric cancer; LC, liver cancer; PC, pancreatic cancer; CRC, colorectal cancer; ND, not determined.

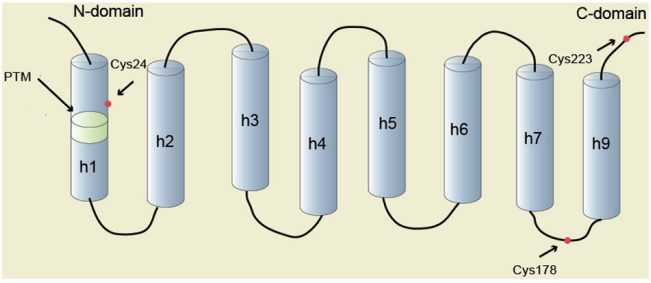


Figure 1. Putative structural model of CLIC1 protein in the soluble, reduced monomeric form. CLIC1 is 241 amino acid residues in length. Both N- and C-termini of CLIC1 are cytoplasmic. The structure of CLIC1 belongs to the glutathione S-transferase fold superfamily. CLIC1 has an active site cysteine residue (Cys24), which is rendered reactive by the protein itself. That differs from the classical enzymatic GST which activates the thiol group of the GSH. A total of three cysteines (Cys24, Cys178 and Cys223; shown in red) are conserved in all vertebrate CLIC proteins. The secondary structure of CLIC1 includes helices in h2-h7, h9 and loops in h1. The PTM is shown in yellow (residues, 25-46). The N-terminus domain is on the left (β-sheet plus helices h1, h2 and h3) and the all-helical C-terminus domain is on the right (helices h4-9). CLIC1, chloride intracellular channel 1; PTM, putative transmembrane region.

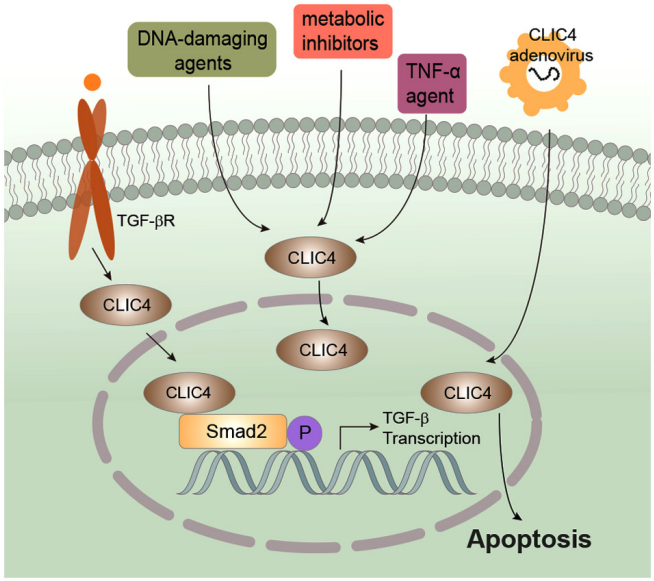


Figure 2. Factors promoting CLIC4 nuclear translocation. Cytoplasmic endogenous CLIC4 has been reported to translocate into the nucleus following treatment with DNA-damaging agents (etoposide, adriamycin and mitomycin), metabolic inhibitors (cycloheximide and actinomycin D), camptothecin, and TNF-α. In addition, targeting CLIC4 to the nucleus by adenoviral transduction accelerates cell apoptosis. CLIC4 also enhances TGF-β responsiveness by interfering with dephosphorylation of phosphorylated Smad signaling proteins. CLIC4, chloride intracellular channel 4.

*CLICs and apoptosis*. CLIC4 and CLIC5 participate in apoptosis. CLIC4 acts as an apoptotic effector and has a substantial role in p53 and c-Myc-mediated apoptosis (27). p53 overexpression or DNA damage mediates CLIC4 upregulation and induces apoptosis (27). Mechanistically, mitochondrial membrane potential is reduced by CLIC4 overexpression, followed by cytochrome c release into the cytoplasm and the activation of caspases to induce apoptosis (27). CLIC4 downregulation reduces p53-induced, but not Bax-induced apoptosis, indicating that the two pro-apoptotic proteins function independently (27). CLIC4 downregulation enhances autophagy and contributes to mitochondrial and endoplasmic reticulum stress-induced apoptosis under starvation (64). It has been shown that endogenous CLIC4 from the cytoplasm translocates into the nucleus under starvation, as well as after treatment with DNA-damaging agents (etoposide, adriamycin and mitomycin), metabolic inhibitors (cycloheximide and actinomycin D), camptothecin, tumor necrosis factor-α and transforming growth factor-β (Fig. 2) (64,65). CLIC4 nuclear translocation is a response to stress and may contribute to the initiation of apoptosis-related nuclear alterations (65).

CLIC5 is located at the inner mitochondrial membrane and CLIC4 in the outer mitochondrial membrane. CLIC5 has a direct role in the regulation of mitochondrial reactive oxygen species (ROS) generation (66). Mitochondria serve a significant role in lysosomal-mediated cell death (67). The ETS variant transcription factor 6 (ETV6)/RUNX family transcription factor 1 fusion gene results in childhood precursor B-cell acute lymphoblastic leukemia (68). The loss of ETV6 transcriptional repressor induces CLIC5 upregulation, ultimately leading to a decrease in lysosome-mediated apoptosis, indicating that CLIC5 activity facilitates an environment of oxidative stress induced by DNA damage accumulation, thereby contributing to the development of leukemogenesis (40).

*CLICs and angiogenesis*. The function of angiogenesis involves CLIC1, CLIC4 and CLIC5. The downregulation of CLIC1 reduces endothelial migration, capillary-like network formation, branching morphogenesis and capillary-like sprouting (23). Endothelial cell migration and adhesion depend on an appropriate amount of integrin expression (69). CLIC1 has an essential role in the regulation of the cell surface

Table II. Common and unique functions of CLIC family proteins.

| CLIC family member | Unique functions | Common functions | (Refs.) |
|---|---|---|---|
| CLIC1 | 1.Phagosomal acidification<br>2.Platelet aggregation<br>3. Positive regulation of osteoblast differentiation<br>4. Inflammation | 1. Chloride channel activity (CLIC1-CLIC6)<br>2. Endosomal trafficking (CLIC3 and CLIC4)<br>3. Glutaredoxin-like activity *in vitro* (CLIC1, CLIC2 and CLIC4)<br>4. Regulation of cell cycle (CLIC1 and CLIC4)<br>5. Regulation of mitochondrial membrane potential (CLIC1 and CLIC4)<br>6. Protein binding (CLIC1, CLIC2, CLIC3 and CLIC5B)<br>7. Angiogenesis (CLIC1, CLIC4 and CLIC5)<br>8. Mediating endothelial cell proliferation (CLIC1 and CLIC4)<br>9. Migration/metastasis (CLIC1, CLIC2, CLIC4 and CLIC5)<br>10. Apoptosis (CLIC4 and CLIC5) | (14,16,17,19,23,28-31) |
| CLIC2 | 1. Negative regulation of ryanodine-sensitive calcium-release channel activity<br>2. Regulation of cardiac muscle contraction by guiding the release of sequestered calcium ion<br>3. Regulation of release of sequestered calcium ions into cytosol by sarcoplasmic reticulum | | (15,17,20,31,32) |
| CLIC3 | Unknown | | (12,21) |
| CLIC4 | 1. Regulation of TGF-β signaling.<br>2. Keratinocyte differentiation<br>3. Establishment or maintenance of apical/basal cell polarity during mitosis and cytokinesis<br>4. Fertilization<br>5. Vacuolar acidification | | (13,18,24-27,33,34,35,37,38) |
| CLIC5 | 1. CLIC5 is vital for the formation of stereocilia in the inner ear and normal development of the organ of Corti<br>2. CLIC5 has a role in female pregnancy<br>3. CLIC5A has a role in membrane-ERM interaction in cilia, in the maintenance of podocyte and glomerular architecture and in actin-dependent membrane remodeling<br>4. CLIC5B has a role in actin-dependent membrane remodeling and in osteoclast differentiation | | (22,36,39-43) |
| CLIC6 | 1. D2 dopamine receptor binding<br>2. D3 dopamine receptor binding | | (24,44) |

CLIC, chloride intracellular channel.

expression of various integrins in angiogenesis, such as αVβ3, αVβ5 and subunits β1 and α3. In CLIC1$^{-/-}$ mice, CLIC1 knockdown resulted in a mild platelet dysfunction characterized by prolonged bleeding, and P2Y12 receptor signaling-related ADP stimulation led to a reduction in platelet activation (30). The activation of G(12/13) pathways by ADP regulates fibrinogen receptor activation in platelets and dense granule release (70).

The expression of CLIC4 is required at multiple stages of angiogenesis. CLIC4 is necessary for endothelial cell hollowing, a process required for vessel formation during ischemia and embryogenesis (71). CLIC4 promotes endothelial cell proliferation and regulates endothelial morphogenesis (38,72). CLIC4 downregulation was demonstrated to decrease cell proliferation, capillary-like sprouting, lumen formation and capillary network formation, all of which was promoted by CLIC4 upregulation (25). CLIC4$^{-/-}$ mice exhibited defective angiogenesis *in vivo* (38,71,73). Compared with wild-type mice, CLIC4$^{-/-}$ mice demonstrated abnormal collateral circulation in response to ischemic injury (71,73), and the native cerebral collateral density was reduced, leading to severe infarctions (71), smaller kidneys with fewer glomeruli, less dense peritubular capillary networks (74), and retinal angiogenesis defects (38). Furthermore, CLIC4/CLIC5A-mediated ezrin, radixin, moesin activation is necessary for the maintenance of the glomerular capillary architecture (75).

*CLICs and cell cortex-associated migration and metastasis*. CLIC1, CLIC2, CLIC4 and CLIC5 interact with the cell cortex (76-79). Cell adhesion has a vital role in cancer progression and metastasis. Metastasis is a multi-step process, where cells lose their original tissue contacts across the extracellular matrix (ECM), invade into the surrounding tissue, enter into the blood and/or lymphatic system, extravasate in a distant organ and form new tumors (80). Therefore, tumor cells are significantly influenced by cell-cell and cell-ECM adhesion (80). In order to migrate, cells must interact with their environment through adhesion receptors, such as integrins, and form adhesion complexes that respond to different extracellular cues (81).

CLIC1 and CLIC4 bridge the cortical actin cytoskeleton and the plasma membrane for cytokinesis (76). The downregulation of CLIC4 and CLIC1 result in abnormal blebbing at the polar cortex and regression of the cleavage furrow during late cytokinesis, ultimately forming multinucleated cells (76). CLIC2 is decreased in most endothelial cells in blood vessels of cancer tissue (77). In human umbilical vein endothelial cells (HUVECs), CLIC2 downregulation helps human cancer cells to transmigrate through a HUVEC monolayer (77). CLIC4 is implicated in various actin-based processes, such as integrin trafficking and cell adhesion (78). Mechanistically, CLIC4 regulates the Ras homolog family member A/mouse homolog of diaphanous 2-regulated signaling network to integrate cortical actin assembly and membrane protrusion by binding to profilin-1 (78). In addition, in HeLa and MDA-MB-231 cells, CLIC4 downregulation suppresses cell spreading, cell-matrix adhesion and integrin signaling (82). CLIC4 is recruited to β1 integrin at the plasma membrane and RAB35-positive endosomes by lysophosphatidic acid stimulation. Furthermore, CLIC4 impedes the RAB35-dependent regulation of β1 integrin trafficking by decreasing RAB35 activity (82). In renal glomerular podocyte foot processes, CLIC5A, one of two alternative splicing variants of CLIC5, is a component of the ezrin (EZR)/sodium-hydrogen exchanger regulatory factor 2 (NHERF-2)/podocalyxin cytoskeletal complex (79). Furthermore, at the cell cortex, similar to EZR, CLIC5A may have an essential role in the assembly and/or maintenance of F-actin-based structures (83). CLIC5A is a component of the EZR-NHERF2-podocalyxin complex in glomeruli. In CLIC5-deficient mice, the cytoskeletal association of EZR and NHERF2 was diminished (79). Mechanistically, the interaction of CLIC5A with PI(4,5)P2-generating kinases led to clustered plasma membrane PI(4,5)P2 accumulation, followed by the promotion of EZR activation and actin-dependent cell surface remodeling (79).

## 4. CLICs and digestive system tumors

*CLICs and esophageal cancer*. In esophageal squamous cell carcinoma (ESCC), CLIC1 expression is upregulated in both cell lines (TE2, TE5, TE8, TE9, TE15, KYSE70, KYSE150, KYSE170, and KYSE790), and tissue samples (5). Furthermore, CLIC1 is present in the cytoplasm of cancer cells (5). CLIC1 knockdown inhibited the proliferation of tumor cells, and CLIC1 regulated apoptosis through the Toll-like receptor 2/JNK pathway (5). Furthermore, cell cycle arrest was found to occur in the sub-G1phase following CLIC1 knockdown (5). However, as previously mentioned, CLIC1 is only observed at the plasma membrane of G2/M phase cells, and blockade of CLICK1 by chloride channel blockers IAA-94 and anthracene-9-carboxylic acid resulted in the arrest of CHO-K1 cells in the G2/M phase of the cell cycle (16). Whether this contradictory result was due to the different cell lines or different mechanisms requires further investigation. In addition, patients with strong expression of CLIC1 exhibited a significantly lower 5-year overall survival than those with weak expression of CLIC1 (sample size, 61) (5). In a cohort of 45 patients with ESCC, CLIC3 was found downregulated, CLIC4 was upregulated and CLIC2 unchanged, as detected by quantitative PCR and western blotting (5). The specific functions of CLICs in ESCC, as well as differences in expression, require further study. Squamous cell carcinoma is a common type of esophageal cancer, and CLIC4 has been demonstrated to inhibit the growth of squamous cell carcinoma, and the degree of reduction in CLIC4 coincided with the progression of squamous cell tumors from benign to malignant (5). However, it remains unclear whether CLIC4 is involved in the development and progression of ESCC.

*CLICs and GC*. In GC, CLIC1 is upregulated and correlates with lymph node metastasis, lymphatic invasion, perineural invasion, and poor patient prognosis (6,84,85). Following CLIC1 knockdown in GC cells SGC-7901, the expression levels of integrin α3, αv and β1 *in vivo*, as well as the phosphorylation of PI3K/AKT, ERK, and p38, were found to be decreased, while integrin α1 was found to be increased; it was therefore hypothesized that the mechanism of CLIC1 in the progression of GC may be associated with the regulation of integrin family proteins, leading to the sequential regulation of PI3K/AKT, mitogen-activated protein kinase (MAPK)/ERK, and MAPK/p38 pathways (86). CLIC1 upregulation could

mediate, at least partly, the ability to enhance invasion and metastasis of GC following the knockdown of proteasome activator subunit 2 (87). As aforementioned, one of the hallmarks of malignancy is angiogenesis, which provides blood supply to the tumor tissue (36). One of the functions of CLICs is their participation in angiogenesis (41,47). Unregulated angiogenesis leads to a constant hypoxia-reoxidation (H-R) state, thus increasing ROS production, providing a substrate for further undifferentiation (88). Similarly, CLIC1 regulates GC cell migration and invasion through the ROS-mediated p38 MAPK signaling pathway (84). This is further confirmed by the fact that blocking CLIC proteins with chloride channel blocker IAA-94 reduces ROS production (89). CLIC1 is closely associated with GC resistance to vincristine, and exosome-mediated transfer of CLIC1 can induce the development of vincristine resistance *in vitro*, possibly through the upregulation of P-glycoprotein and Bcl-2 (90). Tissue microarray analysis using 107 GC specimens revealed that CLIC3 was inversely correlated with pathological tumor depth, that a lower CLIC3 expression was linked to a worse prognosis, and that CLIC3 functioned as a chloride channel on the plasma membrane of GC cells (91).

*CLICs and liver cancer.* In hepatitis B virus X protein-positive HepG2 cells, CLIC1 protein accumulation was found to promote hepatocellular carcinoma (HCC) development (92). CLIC1 has been reported to be upregulated in HCC (93), and CLIC1 overexpression in liver tumor tissues was significantly correlated with tumor size, metastasis, and pTNM stage. In addition, CLIC1 overexpression was found to be associated with poor prognosis (7,93). In mouse hepatoma ascites, CLIC1 is expressed in the cytoplasm and plasma membrane in both the Hca-F lymphatic metastasis cell line with a high metastatic potential and the Hca-P lymphatic metastasis cell line with a low metastatic potential. Furthermore, two-dimensional difference-gel electrophoresis revealed that CLIC1 expression was higher in both Hca-F cells and plasma membranes, compared with Hca-P (94). Consistently, another study found that the expression of CLIC1 mRNA and protein in Hca-F cells was higher compared with that in Hca-P cells (2 and 1.6-fold, respectively), and the migration and invasion ability were significantly decreased following CLIC1 downregulation, thus demonstrating that CLIC1 may be a key factor in the development of lymphatic metastasis (95). Furthermore, CLIC1 is upregulated in HCC tissues with portal vein tumor thrombus (96). CLIC1 overexpression was confirmed to decrease maspin expression and increase vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP) 2, MMP12, and MMP13 expression (96). These results revealed that, in HCC, CLIC1 upregulation was significantly associated with vascular invasion, and that CLIC1 may control the mechanism of HCC invasiveness by targeting maspin (96). CLIC1 downregulation by RNA interference significantly enhanced the expression of tumor metastasis genes annexin A7 and gelsolin *in vitro*; conversely, annexin A7 and gelsolin downregulation enhanced the expression of CLIC1 *in vitro* and *in vivo* (97). These data illustrated that CLIC1 may function by regulating the expression of annexin A7 and gelsolin in the migration and invasion of liver cancer (97). Furthermore, at the transcriptome level, microRNA (miR)-124 directly reduced CLIC1 expression, further inhibiting cell migration, and invasion in HCC cells, but without affecting cell proliferation (98). Alternatively, the levels of CLIC1 can be regulated by miR-122-5P (7).

It is not clear whether CLICs act as ion channels to affect liver cancer. Of note, the chloride channel blocker 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) can effectively inhibit the proliferation of liver cancer cells (99). DIDS induces G1 arrest by downregulating the protein expression levels of cyclin D1 and cyclin E (99). DIDS reduces the protein expression levels of α-fetoprotein, suggesting that it may be able to improve the prognosis of HCC in patients (99). CLIC1 has a vital role, similar to that of proto-oncogene, that may be targeted in the development of novel tumor treatments with chloride channel blocker IAA-94 (100). However, the high concentration of IAA-94 required for its function, as well as its poor specificity for CLIC1, have affected its use as a CLIC1-specific drug for cancer therapy. Further therapeutic development requires a specific and potent CLIC1 inhibitor. If the structure of soluble CLIC1 were characterized, the rational design of small molecules or peptides for CLIC1 inhibition would provide new avenues for blocking the function of CLIC1 (62). Function inhibition may also be achieved by hyperpolarization using native CLIC1 chloride channels, suggesting a therapeutic modality that does not require gene therapy (101).

CLICs are involved in cytoskeleton formation, while cell deformation is required during tumor invasion and metastasis (102). CLIC2 knockdown in HUVECs allows human cancer cells to migrate through HUVEC monolayers (77). CLIC2 was found to be downregulated in fibrotic and advanced HCC tissues (77). CLIC5 can be used as a biological indicator to predict the prognosis of HCC together with EZR and podocalyxin-like (PODXL) (proteins associated with invasion, migration, and poor prognosis of various types of cancer). It has been found that CLIC5 forms a complex with EZR and PODXL, and that it is required for podocyte structure and function (103). In HCC, EZR, PODXL, and CLIC5 are overexpressed (103). Furthermore, migration and invasion were found to be decreased when the expression of CLIC5 and PODXL was inhibited in Huh7 cells (103).

*CLICs and gallbladder cancer (GBC).* In human GBC, CLIC1 expression is upregulated compared with normal tissues, and high CLC1 expression is associated with histological grade, TNM stage, perineural invasion ($P<0.05$), and decreased overall survival ($P<0.001$) (104). In fact, CLIC1 expression and histological grade are independent risk factors for overall survival (104). CLIC1 knockdown promoted apoptosis and inhibited proliferation, migration and invasion of GBC cells (105). CLIC1 overexpression promoted GBC-SD18L cell motility and invasion and, conversely, CLIC1 knockdown significantly reduced the *in vitro* GBC-SD18H cell motility and invasion, indicating that CLIC1 may play an essential role in the metastasis of gallbladder cancer (8). At the transcript level, CLIC1 is a direct target gene of hsa-miR-372 and miR-122 (7,106). In GBC, hsa-miR-372 is downregulated and correlates with the aggressive and progressive behavior of tumors by affecting CLIC1 expression (106). Urothelial cancer associated 1 (UCA1) was found to promote bile duct carcinoma

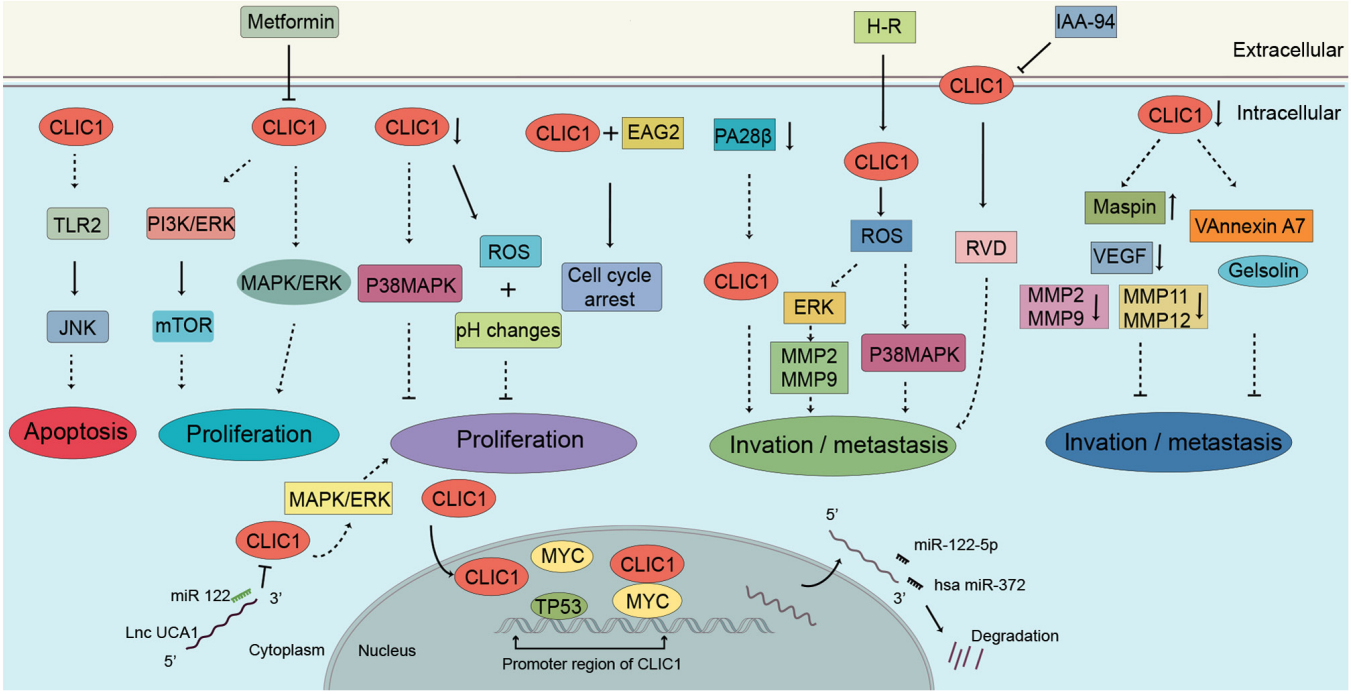


Figure 3. Roles of CLIC1 in cancer. CLIC1 is involved in multiple biological behaviors associated with cancer, including cell proliferation, apoptosis, invasion and metastasis. Regulation of CLIC1 expression by targeting CLIC1 mRNA is also shown. TP53 is the upstream transcription factor of CLIC1. CLIC1, chloride intracellular channel 1; TLR2, toll-like receptor 2; JNK, c-Jun N-terminal kinase; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; EAG2, potassium voltage-gated channel subfamily H member; PA28β, proteasome activator subunit 2; H-R, hypoxia-reoxidation; MMP, matrix metalloproteinase; RVD, regulatory volume decrease; VEGF, vascular endothelial growth factor; miR, microRNA; Lnc, long non-coding RNA; UCA1, urothelial cancer associated 1.

(BDC) cell migration and invasiveness, while miR-122 inhibited their progression (84). CLIC1, as a downstream target gene of miR-122, has the opposite effect. The ERK/MAPK signaling pathway is activated following the upregulation of long non-coding RNA UCA1 (84). UCA1 promoted the metastasis of BDC cells and the activation of the ERK/MAPK pathway by regulating the expression of miR-122 and its downstream gene CLIC1, therefore expanding the options for targeted treatment of cholangiocarcinoma (107). In addition, in GBC, serum carbohydrate antigen 19-9 concentration is positively correlated with the expression levels of troponin T1, slow skeletal type, MMP-9 and CLIC3 (108). Of note, metformin markedly inhibits the proliferation and viability of GBC cells, promotes apoptosis and increases the number of early apoptotic cells (109). Metformin has been further shown to exert growth inhibitory effects by inhibiting p-AKT activity and the Bcl-2 family (85). Of note, either the dysfunction or downregulation of CLIC1 could partially reduce the antitumor effect of metformin, whereas upregulation of CLIC1 could increase drug sensitivity (110).

*CLICs and pancreatic cancer.* In the pancreas, inhibition of CLIC4 increases β-cell survival, likely due to increased levels of Bcl-2, Bcl-xl, and Bad phosphorylation, and the overexpression of Bcl-2 or Bcl-xl in β-cells increases their resistance to cytokine-induced apoptosis (111). Using *in silico* modeling to construct an interactome of the CLIC gene family, CLIC1, CLIC3, and CLIC4 have been identified as prognostic markers of overall survival in pancreatic ductal adenocarcinoma (PDAC) compared with healthy controls. Among them, the expressions of CLIC1-CLIC3, CLIC4-CLIC5, and CLIC5-CLIC6 have been found to be positively correlated (112). Similar to these findings, CLIC1 protein expression was significantly increased in tumor samples from patients with resected PDAC compared with normal tissues (67.1% vs. 25.7%, respectively; P<0.001) (113). In addition, CLIC1 overexpression was found to be associated with a higher histological grade, larger tumor size, and worse overall survival [hazard ratio, 5.822; 95% confidence interval (CI), 1.329-15.628; P=0.016) (113). Furthermore, the treatment of pancreatic cancer cell lines with CLIC1-targeting small interfering RNA oligonucleotides significantly reduced cell proliferation, anchorage-independent growth, and cell migration on soft agar (9). CLIC3 drives pancreatic cancer invasiveness by cooperating with RAB25 to regulate α5β1 integrin recycling from late endosomes to the plasma membrane (114). Similarly, CLIC3 predicts lymph node metastasis and poor prognosis in inoperable cases of PDAC (12). CLIC4 and Indian hedgehog have been found to be significantly correlated with tumor grade, lymph node metastasis, tumor invasion, and poor overall survival (115). Of note, HOXA distal transcript (HOTTIP), a long non-coding RNA, is upregulated in PDAC (92), and, by identifying canonical HOTTIP/HOXA13 targets, CLIC5 was found to be crucial for PDAC cell growth and cell invasion (116). However, the oncogenic pathway mediated by HOTTIP is not fully understood.

*CLICs and colorectal cancer (CRC).* CLIC1 is overexpressed in CRC tumors (117,118) and is associated with poor

prognosis (118). CLIC1 is involved in the metastasis of LOVO colon cancer cells by regulating the ROS/ERK pathway during H-R and regulatory volume decrease (RVD)-mediated chloride channel function (117). Previous studies showed that functionally suppressing CLIC1 using the CLIC1 blocker IAA-94 or CLIC1 knockdown (117,119) inhibited the migration and invasion of colon cancer cells, and the effect was attributed to a decrease in RVD capacity (117). On the other hand, the inhibition of CLIC1 channel activity by IAA94 reduced intracellular ROS production during H-R treatment, resulting in decreased cell migration (96). ROS can regulate CLIC1 translocation and, conversely, CLIC1 chloride current is required for ROS production by NADPH oxidase (120). An increase in CLIC1 current could sustain ROS production, which is necessary for cell cycle progression. This contributes to the development of tumors. It has been reported that CLIC4 expression is a marker of colon cancer stem cells and is associated with poor outcome (121). CLIC4 has also been shown to enhance MMP-9 expression and invasiveness in cancer cell lines evading photodynamic therapy (122). A previous study (121) demonstrated that three proteins, CLIC4, endoplasmic reticulum protein 29 (ERp29), and diablo IAP-binding mitochondrial protein (DIABLO, also known as SMAC), have been identified in metastatic cancer stem-like cells of CRC. The protein expression levels of this three-protein panel (CLIC4, ERp29, and DIABLO) enabled the classification of the validation cohort into risk stratification of colorectal cancer (121).

## 5. Conclusion

CLICs have an integral role in the process of tumorigenesis by participating in various physiological processes. In particular, the role of CLIC1 in cancer has been summarized in Fig. 3. The expression levels of CLICs can be used as a diagnostic and prognostic marker for digestive system tumors. However, there is currently a lack of studies based on CLIC knockout mice in digestive system tumors. The understanding of the role of CLICs in health and tumors is incomplete. It is also necessary to clarify whether CLICs function as chloride channels or as proteins under specific disease conditions, and to understand their interactions and exact molecular basis of the complex signaling network activated by CLICs. Subsequently, the potential functional continuity of CLICs between the cytoplasm and membrane needs to be explored. The development of conformation-specific drug inhibitors and CLIC protein activity modulators may herald new and more effective avenues for the treatment of cancer.

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## Availability of data and materials

Not applicable.

## Authors' contributions

HW wrote the manuscript. JA and CL participated in information collection, analysis, and organization. SH and JW primarily revised and finalized the manuscript. BT revised the manuscript for clarity and style and critically revised the article for important intellectual content. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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