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Review

Chloride channels in cancer: Focus on chloride intracellular channel 1 and 4 (CLIC1 AND CLIC4) proteins in tumor development and as novel therapeutic targets ☆

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ABSTRACT

In recent decades, growing scientific evidence supports the role of ion channels in the development of different cancers. Both potassium selective pores and chloride permeabilities are considered the most active channels during tumorigenesis. High rate of proliferation, active migration, and invasiveness into non-neoplastic tissues are specific properties of neoplastic transformation. All these actions require partial or total involvement of chloride channel activity. In this context, this class of membrane proteins could represent valuable therapeutic targets for the treatment of resistant tumors. However, this encouraging premise has not so far produced any valid new channel-targeted antitumoral molecule for cancer treatment. Problematic for drug design targeting ion channels is their vital role in normal cells for essential physiological functions. By targeting these membrane proteins involved in pathological conditions, it is inevitable to cause relevant side effects in healthy organs. In light of this, a new protein family, the chloride intracellular channels (CLICs), could be a promising class of therapeutic targets for its intrinsic individualities: CLIC1 and CLIC4, in particular, not only are overexpressed in specific tumor types or their corresponding stroma but also change localization and function from hydrophilic cytosolic to integral transmembrane proteins as active ionic channels or signal transducers during cell cycle progression in certain cases. These changes in intracellular localization, tissue compartments, and channel function, uniquely associated with malignant transformation, may offer a unique target for cancer therapy, likely able to spare normal cells. This article is part of a special issue itled "Membrane Channels and Transporters in Cancers."

Contents

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1. Introduction

Over the last two decades, an important role for ion channels in tumor development and growth has been defined. It is now clear that an abnormal triggering of ion channel activity is important to support the high rate of proliferation of tumor cells [1]. Most of the studies have been focused on the role of potassium channels, but with the flow of time, chloride channels have gained a more prominent position in carcinogenesis [2]. Chloride channels are ubiquitously expressed, being localized both in plasma membrane and in intracellular organelles [3]. They have many different functions as the regulation of electrical excitability, trans-epithelial fluid transport, ion homeostasis, pH levels, and cell volume regulation, the latter being particularly important for cancer cells migration and infiltration [4–6]. These channels also participate in the regulation of the cell cycle, probably as key factors in the progression from G1 to S phase [7]. The pattern of chloride channel properties and modulation has been extensively reviewed by several authors describing voltage dependency, the possibility to be triggered by calcium or activated by several ligands and second messengers, and their primary role in cell volume regulation [8–12]. The different families of chloride channels retain a heterogeneous regulation of their activity, suggesting a multitask possibility of their participation in the tumorigenesis process. There are multiple examples of chloride channels participating in tumor development and progression, some of which will be surveyed in the next paragraphs.

An emerging class of chloride ionic channels involved in cancer development are the intracellular chloride channels (CLICs). So far, members of this protein family have been identified in several cancer types [13–34], but it is reasonable that, given their ubiquitous presence, CLIC proteins will be found in most of the tumors. Several CLIC proteins are overexpressed in cancer cells, and others are reduced when compared to normal corresponding tissues. The peculiarity of CLICs, in particular of CLIC1, is to be mainly present as a cytosolic protein in physiological conditions and to be transiently expressed in the cell membrane during stress conditions, like the increase of the oxidative levels of the cells [35] undergoing rearrangement of the protein structure [36,37]. Thus, as the three-dimensional configuration of CLIC proteins change, they are able to be functionally expressed as ion channels in the membrane. As an example, cytoplasmic CLIC1 protein translocates to the plasma membrane as an ion channel during the tumorigenic processes [18,23] as well as in other pathological conditions such as neurodegenerative disease progression [35,38]. In prolonged stress conditions, the functional expression and the membrane activity of CLIC1 is no longer transient but becomes chronic and thus could represent a unique pharmacological target [19].

Although most of our attention will be centered on the activity of CLICs channel proteins during tumorigenesis, and in particular on the functional role of CLIC1 and CLIC4 during cancer development, in the next paragraphs, we will briefly review the role of resident chloride ionic channels in the tumorigenic process.

1.1. Voltage-gated chloride channels

ClC-3 is a member of the ClC chloride channels and transporters family [10,12,39]. It has been suggested to be a molecular component involved in activation of volume-sensitive Cl^- currents [40] and to be closely related to cell proliferation, migration, apoptosis, and acidification of synaptic vesicles [7]. Volume-activated chloride channels play a crucial role in the process of regulatory volume decrease (RVD) induced by hypotonic stresses. RVD is a phenomenon that contributes to cell shape and volume changes required for cell migration, and so it also has an important role in cancer progression [41]. Furthermore, volume-regulated ion channels probably have a role in the angiogenic process [42]. An increase in swelling activated chloride current and ClC-3 expression has been observed in androgen-dependent prostate cancer epithelial cells as a consequence of overexpression of Bcl-2 [43].

Multiple studies focused on the role of ClC-3 in nasopharyngeal carcinoma. In 2004, Mao and colleagues [44] showed that volume-activated chloride current density in migrating human nasopharyngeal carcinoma CNE-2Z cells was higher than in non-migrating cells. In 2008, a positive correlation between the expression of ClC-3 and the rate of cell migration was highlighted, being associated with an inhibition of both volume-sensitive chloride currents and RVD when cells were transfected with ClC-3 antisense oligonucleotides [45]. Later on, another study revealed that ClC-3 down-regulation, by antisense treatment, arrested CNE-2Z cells in the S phase. The authors speculated that the expression of ClC-3 on intracellular vesicle membranes is necessary for vesicular acidification and may be related to DNA synthesis allowing the passage through the S restriction point [46]. Previously, Habela and colleagues [47] found that the activity of the volume-activated chloride channel is one of the important factors that regulate the passage of cells through the G1 restriction point and that the Cl^- current associated with RVD plays an important role in cell proliferation. Regarding the relationship between ClC-3 and the regulation of cell cycle in nasopharyngeal carcinoma CNE-2Z cells, basal and volume-activated chloride currents are increased by up-regulation of cyclin D1. Moreover, a direct interaction between cyclin D1 and ClC-3 was detected by the fluorescence resonance energy transfer (FRET) technique [7].

In HeLa cells, a human cell line derived from cervical cancer, the inhibition of ClC-3 protein expression arrests cell cycle in the S phase; from these experiments, it was concluded that volume-activated chloride channels play important roles in cell cycle-dependent migration of HeLa cells [48].

ClC-3 and other ClC channels, especially ClC-2 and ClC-5, are particularly important in glioma cells [2,49]. These three channels are expressed in glioma cell membranes, often associated with lamellipodia [49]. Glioma cell invasion [50] and migration [51] involve a coordinated reduction of cell volume. To achieve this state requires the activity of both potassium and chloride channels and transporters [51,52]. ClCs, in particular ClC-3, are the chloride channels involved in this operation. However, the blockade of a single ClC is not sufficient to reach a complete inhibition of glioma cell invasion, suggesting a cooperative role for these channels in the regulation of the volume of glioma cells [51, 53]. Exploiting the well-known activity of chlorotoxin (CTX), a blocker of small conductance chloride channels, successful phase I/II clinical trials for gliomas were reported using CTX-based bioconjugates [54].

1.2. Calcium-activated chloride channels

Although calcium-activated chloride channels (CaCCs) have been studied in the last 30 years, they still have controversial molecular identity. They play several important roles including epithelial secretion, olfactory transduction, membrane excitability, regulation of vascular tone, and photoreception [55].

Calcium-activated chloride channel regulator 2 (CLCA2) is predominantly expressed in trachea and lungs [56]. It has been shown that loss of CLCA2 expression in human breast cancer appears to be closely associated with tumorigenicity [57] and that the expression of CLCA2 was down-regulated in colon cancer [58].

More recently, CLCA2 was shown to be strongly induced by p53 in response to DNA damage, and the proposed mechanism to explain its inhibitory effects on cell proliferation and survival was correlated with its ability to acidify the cytosol [59]. These authors showed that human CLCA2 enhances chloride current in breast cancer cells and reduces pH to 6.7. This observation is in agreement with the proposed idea that some chloride channels are able to promote apoptosis by reducing intracellular pH. Further studies on breast cancer demonstrated that the inactivation of CLCA2 also enhances the expression and activation of focal adhesion kinase (FAK), a mechanism involved in the inhibition in cancer cell migration and invasion [60].

Another member of calcium-activated chloride channels family is anoctamin-1 (Ano1), a channel highly expressed in human interstitial

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cells of Cajal (ICC) in the gastrointestinal tract [61]. In 2004, it was demonstrated that Ano1 is ubiquitously expressed in gastrointestinal stromal tumors [62]. Since Ano1 is fundamental for proliferation of ICC [63], it was proposed that it could play the same role in gastrointestinal stromal tumor cells. The mechanism by which Ano1 regulates cells proliferation was reported to be strictly correlated with chloride entry at G1/S transition of the cell cycle. Recently, an increase in current density of Ano1 channels in human prostate cancer cells has been described, while the knockdown of ANO1 inhibits proliferation, invasion and, metastasis of these cells, decreasing tumor growth. The authors claimed that perturbed intracellular Ca^{2+} and Cl^{-} ion concentrations and cell volume are critical factors associated with androgen-independent prostate cancer pathogenesis [64].

1.3. CFTR

CFTR is a chloride channel activated by cAMP-dependent phosphorylation. Mutations of the *CFTR* gene affect the functioning of the channel, leading to cystic fibrosis and congenital absence of the *vas deferens*. CFTR is involved in multiple molecular pathways that modulate cell inflammation and apoptotic signaling, so it is possible that mutations in this gene could also modify the risk of development of cancer [65]. In 2010, McWilliams and colleagues [66] showed that patients carrying a *CFTR* disease-associated mutation display a modest increase risk of pancreatic cancer. In 2012, Peng and co-workers demonstrates that higher CFTR expression is associated with cervical cancer progression, proposing this channel as a novel tumor marker, prognostic indicator, and potential therapeutic target [67]. No evidence regarding the molecular mechanism by which the channel can be involved in cervical cancer cells has been to date proposed. On the other hand, mutations in the *CFTR* gene could also have a protective role in some tumors such as lung cancer [68], melanoma, colon, and breast cancer [69]. Furthermore, low expression of *CFTR* polymorphisms may contribute to a reduced risk of prostate cancer [70].

2. The CLIC family

There are several ongoing clinical trials for different cancer types having as target membrane resident chloride channels [71]. However, all resident membrane chloride channels share the same archetype. The increase of current density or protein levels in the membrane does not represent *per se* a valid pharmacological target for antitumoral approaches. As we mentioned before, the inhibition of these channel activities could certainly interfere with tumor development but could also cause malfunctioning of important physiological processes.

Conversely, we believe that a valid alternative could be represented by some members of the CLIC family.

Among all the well-characterized chloride permeabilities, the chloride intracellular channel (CLIC) protein family has been the last discovered and still largely underexplored. These proteins are highly conserved in all vertebrates suggesting their involvement in basic biological functions.

The first identified intracellular chloride channel, p64, was isolated from microsomes of bovine kidney and trachea and showed chloride-selective channel function in lipid bilayers [72]. Distinct from membrane resident ion channels, these proteins can exist both as cytoplasmic soluble proteins and as integral membrane elements with ion channel activity. Membrane insertion occurs in response to different stimuli from increases of cytoplasmic oxidation to pH changes [73–75].

CLIC1 and CLIC4 were the first CLIC proteins to be cloned and functionally studied [76,77], and so far they remain the most characterized within the whole family. While CLIC1 was primarily identified in the nuclear membrane and then in the plasma membrane [76,78], CLIC4 subcellular localization varies with cell type being also localized to the inner mitochondrial membrane [34,79], Golgi [80], and endoplasmic reticulum [81]. Both channels are ubiquitously expressed with particularly high levels in specific tissues and are overexpressed in several tumors [13–34].

Although, as described in the previous paragraphs, chloride currents often play an important role in cancer development and tumor cell proliferation, the scenario highlighted for CLICs is peculiar. The cells can indeed modulate the rate of translocation of the channels and its functional insertion in the membranes as a consequence of perturbations in their homeostasis. For example, it has been shown that CLIC1 and CLIC4 membrane insertion is redox regulated [35–37,82,83].

Our leading idea envisages CLIC proteins like second messengers that can translocate to the cell membranes in response to modification of the basic cytoplasmic oxidative level. This mechanism can be transient or chronic according to the source and/or the time span of the perturbation. In our view, transient CLIC membrane translocation could be part of several physiological cell responses. On the contrary, chronic CLIC functional expression in the cell membranes can lead to severe pathological conditions and in particular to tumorigenic states of the cell [84]. What is particularly interesting is that translocation in response to external stimuli or internal changes in cell physiology may be unique for each CLIC family member and cell type. For example, redox changes in neuronal cells causes CLIC1 to migrate to the plasma membrane [35,38], whereas a similar change in macrophages leads to nuclear translocation of CLIC4 [85,86].

3. CLIC1 and CLIC4 in cancer

As the two channels have different patterns of expression in different tissues, they also have different levels of expression in different tumors. Moreover, it has been shown that the expression of CLIC4 transcript is regulated by p53 and tumor necrosis factor α (TNFα) and that it is a direct response gene for both c-myc and p53, two mediators of cancer pathogenesis in multiple tumor sites [34,87]. This regulation is typical of CLIC4, and it has never been demonstrated for CLIC1. Thus, it is likely that the functional roles of the two channels in tumor cells are not superimposable and that their expression is regulated by different pathways.

3.1. CLIC4 functional role in cancer

Multiple studies investigated the expression and the regulation of CLIC4 in cancer cells. Suh and colleagues [33,88] suggested that CLIC4 reduction could be a novel target for tumor therapy since the expression of CLIC4 antisense mRNA and the consequent reduction of CLIC4 protein inhibits the growth of human osteosarcoma cells *in vitro* and *in vivo*, increases apoptotic cells, and decreases cell proliferation. The antitumor effect induced by CLIC4 down-regulation in sarcoma cells was enhanced in combination with TNFα administration [89]. The specificity of CLIC4 in this response, however, was unclear as the antisense construct used in the study also reduced CLIC1 and CLIC5 so that the loss of multiple CLIC proteins may have contributed to the antitumor response.

A second study by this group clarified the natural pattern of CLIC4 expression in multiple human epithelial cancers. Using *in situ* methods on sections of human epithelial solid tumors, the study revealed that the loss of CLIC4 is prevalent in tumor cells, and the gain of its expression in tumor stroma is a common trait of many human cancers and marks malignant progression. The implication was that the reactivation and restoration of CLIC4 in tumor cells or the reduction in tumor stromal cells could provide a novel approach to inhibit tumor growth [89]. Progressing with these studies [26], the authors found that CLIC4 was lost early in tumor evolution and reconstituting tumor cells with exogenous CLIC4 repressed tumor growth. Both *in vitro* studies on tumor cell lines and *in vivo* experiments on tumor orthografts suggested that reconstitution of tumor cells with CLIC4 re-established their responsiveness to TGF-β-mediated growth inhibition. Previously, it was reported that CLIC4 is an integral part of TGF-β signaling and enhances TGF-β responsiveness through its interference with dephosphorylation of

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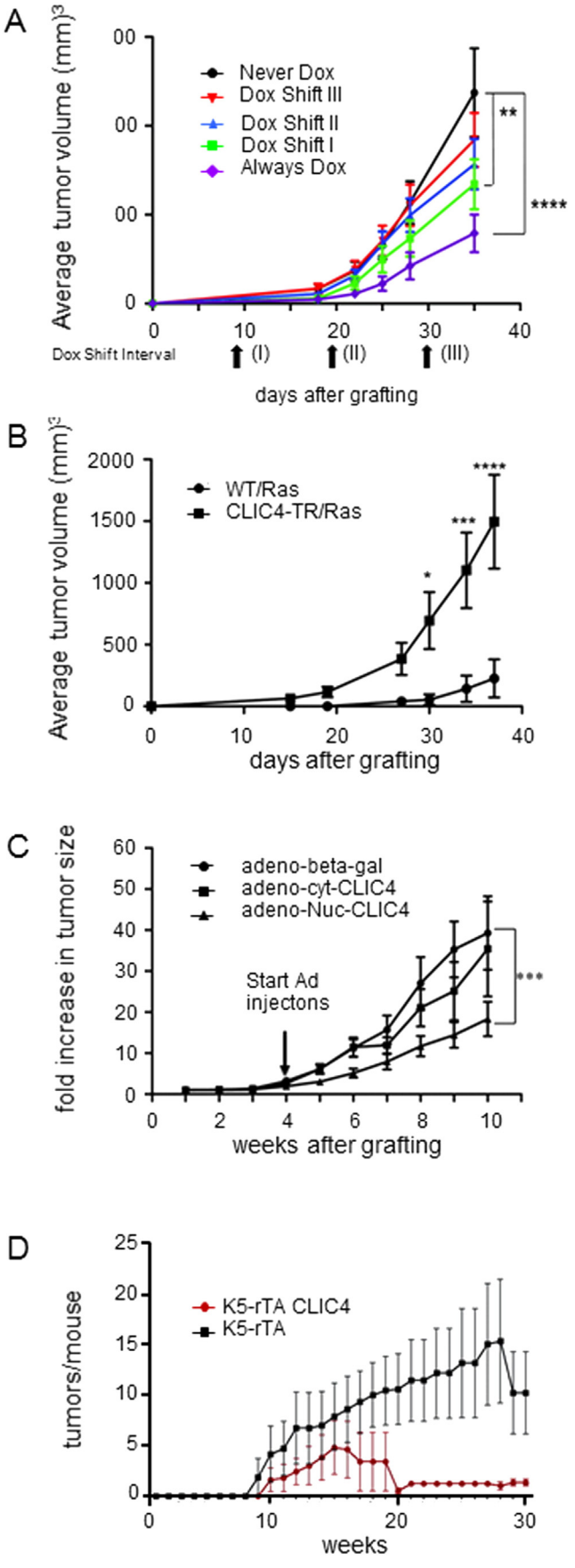

phospho-Smad signaling [90]. The results from these studies strongly implicated CLIC4 in the suppression of tumor cell growth and suggested that its loss in tumor epithelium is a requirement for tumor progression.

Ronnov-Jessen and co-workers had first suggested an important function for CLIC4 in the myofibroblast stroma of breast cancer patients where it was the most up-regulated transcript after TGF-β treatment [91]. Yao and colleagues [28] also explored TGF-ß1-induced fibroblast-to-myofibroblast transdifferentiaton in ovarian cancer and concluded that a ROS-CLIC4 up-regulated pathway is required in this process. In a cutaneous cancer model, Shukla et al. [17] concluded that TGF-β induced myofibroblast conversion was mediated through activation of p-38 by interfering with dephosphorylation of phospho-p38 in similarity to its function on p-Smad activation. Further, these authors showed that tumor orthografts would not grow on recipient mice lacking CLIC4. A potential stromal function for CLIC4 is also suggested by its important participation in angiogenesis where it is required for endothelial tube formation through acidification of vacuoles during angioneogenesis [92]. Together, these studies indicate that drugs designed to inhibit CLIC4 might have therapeutic potential by targeting tumor stroma.

What we can conclude is that for now there are few hypotheses about the biological mechanisms that can be the consequence of the protein's regulation in diverse cancer cells. Moreover, all these studies analyze the total level of CLIC4 protein without distinguishing the membrane and the cytosolic fractions and thus its activity as a channel. In this way, it is not known whether the modified level of expression of the protein in these cells is also correlated with an alteration in the chloride current. CLIC4 is a protein only recently discovered and studied, and so there is still a long road to go to understand its role and the mechanisms that involve this protein in tumor generation and progression. Moreover, its intracellular distribution in the cellular organelles makes the analysis of its membrane distribution challenging.

While the exact mechanisms by which CLIC4 functions as a tumor suppressor or stromal activator are still open questions, increasing evidence is accumulating showing that its expression and activity has clinical relevance. Tumor profiling in bladder cancer [93], uterine leiomyoma [94], glioma [95], and melanoma [96] among others has documented significant changes in CLIC4 expression during tumor formation. Proteome analysis revealed that CLIC4 is reduced in human primary lung cancers and lung cancer cell lines, and the restoration of CLIC4 in the cell lines inhibited growth [16]. CLIC4 expression was reported to be a marker of colon cancer stem cells and associated with a poor prognosis [15]. CLIC4 also enhanced MMP9 expression and invasion in cancer cell lines escaping photodynamic therapy [97]. Of particular interest, high circulating CLIC4 was identified as a biomarker in patients with ovarian cancer [19], and CLIC4 was present in exosomes

Fig. 1. CLIC4 expression inhibits growth of mouse and human squamous tumor orthografts. (A) SP-1/F-Tet-On CLIC4-HA cells (doxycycline inducible CLIC4-HA) were placed as orthografts on a prepared skin bed on nude mice, and mice were started on a doxycycline diet at various times after grafting. Tumor measurements were made weekly with calipers in three dimensions and recorded as volume. (B) Keratinocytes were isolated from wild type FVB/N or CMV-CLIC4-HA (FVB/N) transgenic mouse skin and transduced with retrovirus encoding v-ras^{Ha} in culture. After 8 days in culture, cells were placed as orthografts on nude mice and tumor size was measured weekly. (C) SCC-13 human squamous cancer cells were placed subcutaneously on the flank of nude mice. When tumors were palpable at 4 weeks, adenoviruses encoding Beta-gal, native CLIC4 or nuclear targeted CLIC4 were injected into the tumors at three sites each week. Tumors were measured weekly and reported as the fold increase in size relative to the starting size at week 4. Each group in grafting experiments consisted of 10 mice. Statistical evaluation used 2-way ANOVA with Bonferroni post-test comparison. $^{*}p = 0.05$, $^{**}p = 0.01$, $^{***}p = 0.001$, $^{****}p = 0.0001$. (D) Six- to seven-week-old FVB/N single transgenic K5-rTA or K5-rTA-Tet-O-CLIC4 double transgenic mice with doxycycline inducible CLIC4 targeted to the epidermis by the keratin 5 (K5) promoter were initiated once with DMBA, placed on a doxycycline diet 24 h later, and promoted with TPA for 20 weeks. Tumor multiplicity was monitored each week for 30 weeks. Each group consisted of 6 mice and error bars are SEM. Because of the small number of mice in the study, statistical significance could not be achieved. One mouse with tumors in the CLIC4 group expired at week 20. Reprinted with permission of Oxford University Press from Suh et al. CLIC4 is a tumor suppressor for cutaneous squamous cell cancer. Carcinogenesis 33:986-995, 2012.

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released from human ovarian cancer cell lines [98,99]. The level of circulating exosomes in ovarian cancer patients is directly related to disease progression [100]. Thus, in addition to an important function in cancer pathogenesis, CLIC4 is immerging as a potential biomarker to monitor tumor progression and recurrence in multiple human cancers.

The loss of CLIC4 in tumor epithelium is particularly worthy of note in squamous tumors of the esophagus [101] and cervix (Yuspa et al., unpublished data). To model this, CLIC4 was examined in squamous cancer development on mouse skin [26]. During progression from benign to malignant tumors induced on mouse skin by dimethylbenz(a)anthracene (DMBA) followed by multiple applications of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the expression of CLIC4 is diminished in tumor epithelium. This is paralleled by loss of CLIC4 protein in mouse and human tumor cell lines as they progress from benign to malignant *in vitro*.

Soluble CLIC4 translocates from the cytoplasm to the nucleus under cell stress conditions such as DNA damage, senescence, or metabolic stress from drugs [102]. Transduction of keratinocytes with an oncogenic mutant of HRAS is an early event required for skin tumor induction. Remarkably, associated with the earliest signs of HRAS-induced transformation, the translocation of CLIC4 to the nucleus in response to DNA damage is prevented. Since nuclear CLIC4 is required for its ability to enhance TGF-β signaling, the absence of CLIC4 in the nucleus could interfere with the growth inhibition in response to TGF-β common to many tumor cells. In fact, reconstituting nuclear CLIC4 via adenovirus transduction restored TGF-β-mediated growth inhibition to tumor cells. Mechanistically, it had been shown that nuclear translocation of CLIC4 occurred in response to S-nitrosylation of a critical cysteine to expose a nuclear localization signal [85]. By using auranofin to block thioredoxin reductase and restoring a nitrosylating environment in tumor cells, nuclear translocation of CLIC4 is restored. The importance of CLIC4 in tumor cell growth was emphasized in a number of *in vivo* studies of tumor orthografts (Fig. 1). Under multiple conditions in which CLIC4 expression can be elevated in tumor grafts, tumor growth is inhibited. Furthermore, in transgenic mice in which CLIC4 is expressed to high levels in the epidermis, tumor induction by DMBA/TPA is inhibited. These data suggest that CLIC4 in tumor cells is a tumor suppressor and understanding the mechanism of repression of CLIC4 in squamous tumors is a priority for potential therapy.

3.2. CLIC1 functional role in cancer

Concerning CLIC1 and its expression in cancers, CLIC1 protein levels are reportedly increased in human breast ductal carcinoma [103], gastric cancer [31], gallbladder metastasis [104], colorectal cancer [29], nasopharyngeal carcinoma [105], ovarian cancer [106], hepatocellular carcinoma[107], and high-grade gliomas [22]. All these analyses propose CLIC1 as a tumor marker, sometimes detectable even in the plasma of patients and so very useful in the clinic. In 2004, Huang proposed that the overexpression of CLIC1 in liver cancer might alter cell division rate and/or antiapoptosis signaling, resulting in cellular transformation.

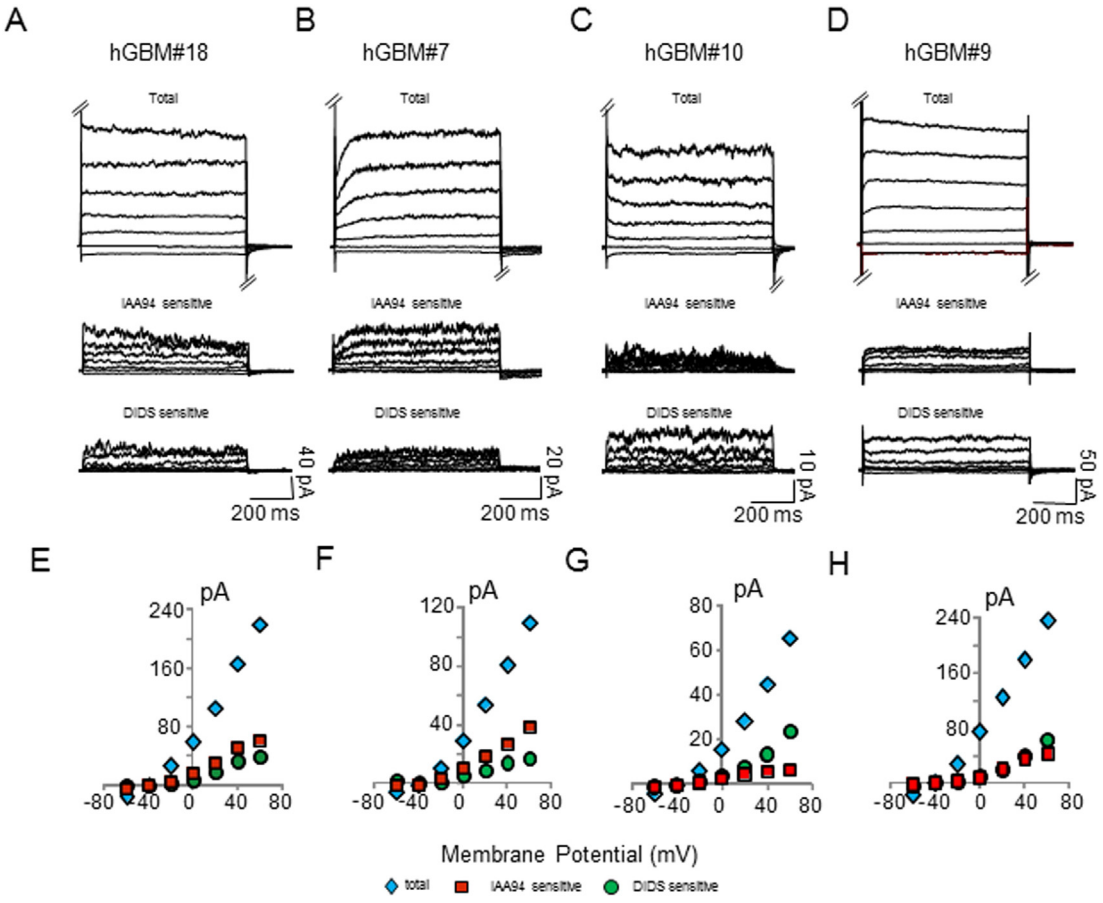

Fig. 2. Native chloride currents in stem/progenitor cells from four different human glioblastomas (GBMs). (A–D) Representative ionic currents (total, IAA94 and DIDS current) from perforated patches of CSCs isolated from four different patient postsurgical specimens of grade IV glioblastoma: hGBM#9 (A), hGBM#10 (B), hGBM#18 (C), hGBM#7 (D). Cells were initially perfused with bath solution to record the total current of the cell (top traces). Successively, ionic currents were recorded during perfusion of 100 μM IAA94 (CLIC1 inhibitors) and 100 μM IAA94 + 200 μM DIDS (Cl^- channels inhibitor that does not affect CLIC1 current). By analytical subtraction of the ionic current after 100 μM IAA94 addition from the total current of the cell, we obtained the IAA94-sensitive current (middle traces); the DIDS-sensitive current was calculated by subtracting the current after 200 μM DIDS addition from the current after IAA94 effect (bottom traces). (E–H) Current/voltage relationships from the data in panels A–D, respectively. Total current (blue diamond) is plotted together with the current amplitude of IAA94-sensitive current (red squares) and DIDS-sensitive current (green circle) (Angelini, M., Savalli, N. and Mazzanti, M. unpublished results).

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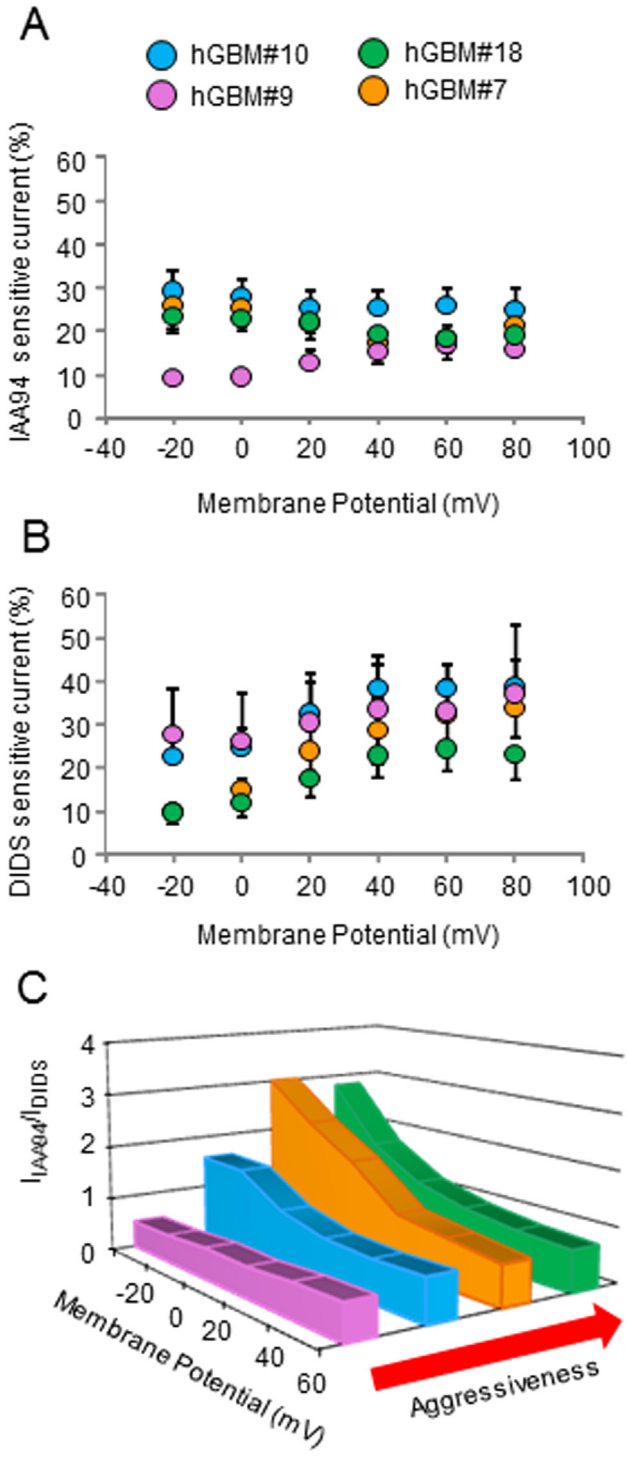

Fig. 3. Positive correlation between the relative abundance of CLIC1-mediated current and glioblastoma aggressiveness. Averages of the ratio of IAA94-sensitive currents (A) or DIDS-sensitive currents (B) over the total current of the same cell, in stem/progenitor cells isolated from hGBM#9 (pink, $n = 6$), hGBM#10 (blue, $n = 15$), hGBM#18 (green, $n = 12$), and hGBM#7 (orange, $n = 6$). (C) The 3D plot correlating the relative abundance of CLIC1-mediated current (I_{IAA94}/I_{DIDS}), the membrane potential, and the tumor aggressiveness (defined as the latency of tumor formation occurring after inoculation of CSCs in nude mice). For example, at 0 mV, the I_{IAA94}/I_{DIDS} ratio was 0.5 ± 0.12 ($n = 6$) in hGBM#9 cells, 1.54 ± 0.35 ($n = 15$) in hGBM#10 cells, 1.74 ± 0.09 ($n = 6$) in hGBM#18 cells, and 2.32 ± 0.42 ($n = 12$) in hGBM#7 cells. In the tumors analyzed in this study, the average survival of nude mice inoculated with CSCs was 152 ± 2 days for hGBM#9, 128 ± 2 days for hGBM#10, 88 ± 4 days for hGBM#18, and 55 ± 4 days for hGBM#7. Note that the more aggressive is the glioblastoma, the more CLIC1-mediated current is represented compared to the other Cl^- currents in the cells. (Angelini, M., Savalli, N. and Mazzanti, M. unpublished results).

In mouse hepatocarcinoma cells, CLIC1 is overexpressed [108,109] and contributes to promoting migration and invasion. Surprisingly, the down-regulation of CLIC1 enhanced proliferative activity, increased the ratio of cells entering G2/M phase, and decreased percentage of apoptosis [108]. This is in accord with previous studies [110] in which treatment with CLIC1 blocker IAA94 led to arrest of CHO-K1 cells in the G2/M stage of the cell cycle. These results indicate that CLIC1 is a critical factor in the development of lymphatic metastasis but that further investigations are still needed to explore the molecular mechanism in tumor occurrence and development.

Two recent studies [14,23] on the function of CLIC1 in LOVO cells, a human colon adenocarcinoma cell line characterized by a high metastatic potential, suggested that CLIC1 expression is related to the metastatic potential of colon cancer cells. Moreover, they analyzed the role of CLIC1 as an ion channel by suppressing its current with IAA94 or knocking-down CLIC1 expression. In both cases, migration and invasion of colon cancer cells were inhibited, an effect attributed to a decrease in RVD (regulatory volume decrease) capacity. The relationship between CLIC1 and ROS levels in LOVO cells has also been studied [14]. The inhibition of CLIC1 channel activity by IAA94 reduces the intracellular ROS production during the hypoxia-reoxygenation treatment, leading to decreased cell migration. CLIC1 and ROS cross-talk can possibly be involved also in other tumors since an alteration in the oxidative state of the cell is typical of cancer cells, and it has been shown by our group that not only CLIC1 translocation is regulated by ROS but also that CLIC1 chloride current is necessary to support ROS production by NADPH oxidase [35]. It is known that changes in ROS levels are fundamental for the progression of the cell cycle [111,112]. One hypothesis is that ROS increase could regulate CLIC1 membrane insertion or, conversely, the boost of CLIC1 chloride current could sustain ROS production necessary for the progression through the cell cycle. The possibility to regulate the insertion of a channel in the plasma membrane in response to specific signals and to have an increasing pool of cytoplasmatic proteins ready to undergo structural modifications and membrane insertion could be very useful for tumor development that is characterized by a high rate of proliferation and migration.

An important role for CLIC1 as a chloride channel is specifically associated with the development of glioblastoma (GBM), the most aggressive and frequent brain tumor. In these tumors the bulk of malignant cells is generated by a rare fraction of self-renewing, multipotent cancer stem cells (CSCs) responsible for tumor origin, progression, and recurrence [113,114]. These subpopulations of cells have shown intrinsic resistance to therapy, being then capable to repopulate the tumor after surgical or chemotherapeutic treatment [115]. CLIC1 is highly expressed in glioblastoma and both mRNA and protein levels are increased in high grade in comparison to low grade brain tumors or control (non-tumor) brain tissue [18,22]. Upon CLIC1 silencing, both proliferative capacity and self-renewal properties in vitro were impaired. Moreover, immunodeficient mice injected into the *nucleus caudatus* with CLIC1-silenced CSCs, survived longer than non-treated CSCs-injected control mice [18]. However, RNA interference experiments *per se* are not able to distinguish whether CLIC1 is active as a cytoplasmic component or if the ability to modulate CSCs proliferation and migration is due to its plasma membrane ion channel property. To address this specific task, Setti and co-workers [18] showed not only that the IAA94-sensitive membrane current was drastically reduced in CLIC1 silenced human glioma CSCs but also that CSC neurospheres, treated for 48 h with NH_2-CLIC1 antibody, active as a CLIC1 channel blocker only from outside the cell [78], compromised cancer development in injected mice. In recent unpublished experiments, Angelini and colleagues calculated the ratio between CLIC1-mediated current (I_{IAA94}) and other Cl^- currents (I_{DIDS}) obtained from perforated-patch experiments in CSCs isolated from four different human glioblastoma postsurgical specimens (Fig. 2). The relative large amount of CLIC1-mediated current positively correlates with tumor aggressiveness as shown in Fig. 3.

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These results support the idea that the abundance of CLIC1 protein in the plasma membrane is a precise sign of a cell in unbalanced physiological condition. This condition could be a transient event in a regular function of the cell life. However, if the protein overexpression becomes chronic as in glioblastoma CSCs, CLIC1 activity could be instrumental to the progress of the pathological state. More important, if the modulatory action is represented by the chloride ion channel, CLIC1 could be considered a privileged therapeutic target for CSCs in glioblastoma as well as in other untreatable tumors.

A recent report strongly supported this hypothesis showing that CLIC1 activity can be pharmacologically regulated, discriminating among CSCs and normal stem cells. It was indeed shown that metformin, an antidiabetic drug able to affect CSC viability [116], is a powerful inhibitor of CLIC1 conductance [117]. Importantly, this effect was evident only in glioblastoma CSC-enriched cultures, while umbilical cord-derived mesenchymal stem cells (MSC) were unaffected. The differential inhibitory effects of metformin on CLIC1 activity in CSCs and MSCs closely correlated with the drug-induced reduction of cell viability that was observed in the tumor cells but not in normal stem cells [117]. Moreover, in light of the proposed role of over-activation of CLIC1 in glioblastoma cells due to its sustained membrane localization, in MSCs that are not sensitive to the antiproliferative effects of metformin, CLIC1 was mainly confined to a cytosolic localization in an inactive form not reachable by metformin [117]. These results strongly support the potential role of CLIC1 as a pharmacological target that allows the discrimination between normal and tumor cells.

4. Conclusion and remarks

Ionic channels have always been considered extremely valid pharmacological targets. Since they are involved in crucial physiological mechanisms, malfunctioning membrane ionic permeabilities are known to be responsible for several pathological states. The CLIC protein family, even in its peculiar expression, should be incorporated into this protein class. The dimorphic nature of CLIC proteins offers the possibility to be involved in cellular functions as cytoplasmic reagents or as membrane ion conducting elements as well-documented by the two examples reported above.

Understanding the involvement of CLIC family proteins in health and disease is still in a developing state. There are evident technical and conceptual difficulties slowing down our ability to understand the functional role of CLIC proteins in the biology of the cell. The close sequence and structural homology among the family members confounds the interpretation of the results. Furthermore, the different three-dimensional structures acquired by the individual proteins could be seen as an obstacle to separate CLIC cytoplasmic and membrane functions. However, the discovery of specific inhibitors, in particular conformation antibodies, not only for individual family members but also for the different protein conformations would have a strong impact in the field. The next step of the research should concentrate our efforts to uncover if there is a functional continuity between the cytoplasmic and the membrane form of the protein and determine if the two protein conformations are active and localized in the different cell environments depending on the cell state. The identification of conformation-specific pharmacological inhibitors and/or regulators of CLIC protein activities could open novel and more efficient ways for cancer treatment.

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