Reciprocal Modifications of CLIC4 in Tumor Epithelium and Stroma Mark Malignant Progression of Multiple Human Cancers

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Abstract  

Purpose: CLIC4, a member of a family of intracellular chloride channels, is regulated by p53, c-Myc, and tumor necrosis factor-α. Regulation by factors involved in cancer pathogenesis, together with the previously shown pro-apoptotic activity of CLIC4, suggests that the protein may have a tumor suppressor function. To address this possibility, we characterized the expression profile, subcellular localization, and gene integrity of CLIC4 in human cancers and determined the functional consequences of CLIC4 expression in tumor epithelium and stromal cells.

Experimental Design: CLIC4 expression profiles were analyzed by genomics, proteomics, bioinformatics, and tissue microarrays. CLIC4 expression, as a consequence of crosstalk between stroma and epithelium, was tested in vitro by coculture of breast epithelial tumor cells and normal fibroblasts, and the functional consequences of CLIC4 expression was tested in vivo in xenografts of human breast tumor cell lines reconstituted with CLIC4 or mixed with fibroblasts that overexpress CLIC4 transgenically.

Results: In cDNA arrays of matched human normal and tumor tissues, CLIC4 expression was reduced in renal, ovarian, and breast cancers. However, CLIC4 protein levels were variable in tumor lysate arrays. Transcript sequences of CLIC4 from the human expressed sequence tag database and manual sequencing of cDNA from 60 human cancer cell lines (NCI60) failed to reveal deletion or mutations in the CLIC4 gene. On matched tissue arrays, CLIC4 was predominantly nuclear in normal human epithelial tissues but not cancers. With advancing malignant progression, CLIC4 staining became undetectable in tumor cells, but expression increased in stromal cells coincident with up-regulation of α-smooth muscle actin, suggesting that CLIC4 is up-regulated in myofibroblasts. Coculture of cancer cells and fibroblasts induced the expression of both CLIC4 and α-smooth muscle actin in fibroblasts adjacent to tumor nests. Introduction of CLIC4 or nuclear targeted CLIC4 via adenovirus into human breast cancer xenografts inhibited tumor growth, whereas overexpression of CLIC4 in stromal cells of xenografts enhanced tumor growth.

Conclusion: Loss of CLIC4 in tumor cells and gain in tumor stroma is common to many human cancers and marks malignant progression. Up-regulation of CLIC4 in tumor stroma is coincident with myofibroblast conversion, generally a poor prognostic indicator. Reactivation and restoration of CLIC4 in tumor cells or the converse in tumor stromal cells could provide a novel approach to inhibit tumor growth.

CLIC4 belongs to the chloride intracellular channel (CLIC) family of proteins with seven members (p64, CLIC1-CLIC5, and parchorin) that may be involved with multiple cellular functions, including regulating organellar volume, ionic homeostasis, and electroneutrality (1). CLIC members are localized in various cellular compartments, expressed in multiple tissue types, and have a major hydrophobic stretch that may be used to span the membrane. Soluble CLIC proteins reside in the cytoplasm but undergo a molecular rearrangement when localized to the cellular/organelle membrane and behave as an anion channel or channel regulator (2). CLIC4 has a putative Cl− selective channel activity exhibiting a single channel conductance, and other family members (parchorin, p64, CLIC1, and CLIC3) exhibit similar channel activities (3–6).

CLIC4 is ubiquitously expressed in various tissue types with high level of expression in skin, and up-regulation of CLIC4 is strongly associated with apoptosis in keratinocytes and other cell types (7, 8). In specific cell types, CLIC4 is found in multiple subcellular compartments, including inner mitochondrial membrane, trans-Golgi network, endoplasmic reticulum, large dense core vesicles, and actin cytoskeleton (3, 9–11). Cytoplasmic CLIC4 translocates to the nucleus in cells...
undergoing growth arrest or apoptosis in response to p53, DNA damage, and metabolic stress, and this nuclear trafficking is mediated by the nuclear localization signal in the CLIC4 sequence and the cellular nuclear import machinery (12). Furthermore, targeting CLIC4 to the nucleus of several cell types with a nuclear targeting vector accelerates apoptosis. The discovery that CLIC4 expression level and nuclear translocation may be important in cellular stress/apoptotic signaling suggested that CLIC4 expression and localization could be altered in human cancers. This possibility is reinforced by data showing regulation of CLIC4 expression by both p53 and c-Myc, two mediators of cancer pathogenesis in multiple tumor sites (8, 13). This study was undertaken to evaluate changes in CLIC4 gene integrity, transcript and protein expression, and subcellular localization in a series of human tumors and tumor cell lines. The results indicate that modification of CLIC4 expression, subcellular localization, and tissue compartment is frequent and common to many human tumor types, suggesting that CLIC4 is an important participant in human cancer development.

Materials and Methods

Cell culture
Human breast cancer cell line MB231 was maintained in DMEM (BioWhittaker, Walkersville, MD) containing 8% fetal bovine serum (Gemini) and 20 units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA). Mouse dermal fibroblasts were isolated from newborn mice and maintained in DMEM as described previously (14). MCF10CA1a cells (human breast cancer cell line) were grown in DMEM/F12 supplemented with 5% horse serum (DMEM/F12, Invitrogen). MCF10CA1a breast epithelial cells and fibroblasts were cocultured by seeding 0.5 × 10⁶ of each type together in 100-mm tissue culture dishes with sterile coverslips in DMEM/F12 medium. NC160 tumor cell lines were cultured at the Developmental Therapeutics Program, National Cancer Institute (Frederick, MD).

Microarray blots
Human “matched” tumor cDNA microarray (Clontech, Mountain View, CA) was processed as described by the manufacturer using 33P (ICN Biomedicals, Irvine, CA)–labeled human CLIC4 and human ubiquitin DNA probes.

Immunoblots and tumor lysate array-blot analyses
Monospecific polyclonal anti–NH2-terminal CLIC4 antibodies were generated and used for immunoblots as described previously (7). A primary antibody against actin (Calbiochem, San Diego, CA) was used at dilutions suggested by the manufacturer. Anti-mouse, anti-rabbit, and anti-goat secondary antibodies conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) were used for visualization of the stained tissue.

Immunohistochemistry, immunofluorescence, and tissue microarray staining
Human tissue microarrays were obtained from multiple sources: TARP tissue arrays (National Cancer Institute), “matched” human tumor tissue arrays (Imgenex, San Diego, CA), Food and Drug Administration–standard normal and tumor tissue arrays (Biochain, Hayward, CA) and human cancer screen tissue arrays (Clonimics), prostate tumor arrays (Baylor Specialized Programs of Research Excellence), and human tissue arrays (Accumax) were used for CLIC4 expression and localization studies. The slides were subjected to immunohistochemical staining as described by the array manufacturers, and the stained slides were analyzed with bright-field microcopy using Leitz-DMRB (Leica, Heidelberg, Germany) and OpenLab (Improvision, Coventry, United Kingdom) software.

Fluorescent immunostaining of tissue arrays was done similarly to the immunohistochemical staining except that fluorescent-labeled secondary antibodies (FITC and Texas Red, Vector Lab, Burlingame, CA) were used and mounted with 4',6-diamidino-2-phenylindole–containing VectaMount (Vector Lab), α-Smooth muscle actin (αSMA) antibodies were purchased from Abcam (Cambridge, MA).

For nonradioactive in situ hybridization/staining, a specific region of mouse and human CLIC4 cDNA was chosen by combination analyses of sequence alignment to avoid detecting other CLIC members and genomic structure (exon/intron junction) inspection to avoid detecting the genomic sequence. PCR primers were designed to cross exons 5 and exons 6 (3′ untranslated region) to generate ~200-bp fragments from both mouse and human cDNAs. cDNAs encoding 3′ untranslated region sequences were generated by reverse transcription-PCR (RT-PCR) from a human brain cDNA library and used as a template for PCR. The PCR fragment was cloned into pGEM-Teasy (mouse) or pCRII (human), and Sp6 or T7 primers were used to generate digoxigenin-labeled sense and antisense strands with 200-bp length. The labeled strands and the protocols described by Basset-Sequin et al. (15) and DIG-High Primer Detection kit from Roche (Indianapolis, IN) were used for visualization of the stained tissue.

Mutation analyses
Biosinformaties. The open reading frame (ORF) sequences of the seven CLIC genes (CLIC1, CLIC2, CLIC3, CLIC4, CLIC5, CLIC5B, and CLIC6) were compared with the human expressed sequence tag database (updated December 10, 2004), using the tblastn program (version 2.2.10) with an e value cutoff of 0.00001. The expressed sequence tag sequences that showed similarity were sorted into readily identifiable cancer families based on the tissue type classification of the database entries. Additionally, whereas each positive database hit typically matched more than one CLIC family member, the bit scores and e values were used to assign each hit to a specific CLIC protein that represented the “best match.”

RT-PCR of NC160 human tumor cell lines. Total RNA samples of 60 different human tumor cell lines were prepared by National Cancer Institute Developmental Therapeutics Program. First-strand cDNA was generated from 1 μg total RNA per cell type by Superscript-III (Invitrogen) at 50°C according to the manufacturer’s instruction. A portion of the cDNA generated was then used for RT-PCR using CLIC4 gene-specific primer sets (set-A: forward, 5′-CACCTCCCGACACA-GACCACAGCCG-3′; reverse, 5′-GGAGTAGCTGGATGAAGCG-3′; set-B: forward, 5′-CAGCGACCCCTCGCGGCT-3′; reverse, 5′-GACATCTCTTTTTCAAAGCCG-3′). Set-A primers were used in RT-PCR for 20 cycles to generate a larger-size PCR fragment (899 bp) of CLIC4, and then a portion of the first RT-PCR product and primer set-B was used for PCR to generate only the ORF of CLIC4 (812 bp). High-fidelity PCR enzyme (Roche) was used for all PCR reactions. The final PCR product was purified by using the PCR purification kit (Qiagen, Valencia, CA) and sequenced at the National Cancer Institute DNA sequencing facility. DNA sequences were analyzed by Sequencer (Gene Codes Corp., Ann Arbor, MI) and Pairwise Blast (National Center for Biotechnology Information) followed by aligning the sequences with ClustalW Multiple Sequence Alignment program (National Center for Biotechnology Information).

Xenograft and adenovirus injection
MB231 cells (2 × 10⁶) or a mixture of MCF10CA1a (4 × 10⁵) and mouse fibroblasts (wild type or transgenic; 3 × 10⁶) were s.c. injected into the back of athymic mice. Xenograft studies were repeated at least twice with 10 mice per study group. For i.t. adenovirus injection studies, the tumor-bearing mice were injected twice a week with 200 μL PBS containing recombinant adenovirus (1 × 10⁹ particles per injection)
encoding green fluorescent protein (GFP), wild-type CLIC4 (Cyt-CLIC4), or nuclear-targeted CLIC4 (Nuc-CLIC4). Typically, three sites were injected per treatment. Production of CLIC4 recombinant adenovirus was described previously (12). Efficiency of recombinant adenovirus delivery was qualitatively assessed by fluorescent microscopy of the frozen sections of MB231 tumors that were injected with GFP adenovirus.

Results

CLIC4 expression is altered in specific human cancers. To examine the expression pattern of CLIC4 transcripts in a variety of human cancers, a membrane containing matched cDNAs derived from normal and tumor tissues of the same patient (Human Tumor Array, Clontech) was probed with 32P-labeled CLIC4 cDNA. The array data show that CLIC4 transcript level is frequently down-regulated in several human tumor types, especially in breast, ovary, and kidney (Fig. 1A). However, CLIC4 transcripts are up-regulated in some tumors, suggesting that suppression may be specific to a particular cell type within the tumor tissue. In some tissues (e.g., stomach), CLIC4 expression is low in both normal and the matched tumor, suggesting that basal CLIC4 expression varies among tissues as we have reported previously (7). Examination of the medical information associated with the tumor samples indicated that reduction of CLIC4 expression measured by array methodology is not correlated to patient age and tumor stage. It can be noted that spot intensity varies even among samples from normal tissues of different patients (Fig. 1A), suggesting that CLIC4 expression is not uniform in different tissue compartments or homeostatic states. In contrast to CLIC4 hybridization, blotting the membrane with an ubiquitin probe shows uniform hybridization in all of the cDNA spots (Fig. 1A), showing that equal amounts of cDNA are spotted on the array membrane. When the Oncomine database (Cancer Bioinformatics, Comprehensive Cancer Center, University of Michigan) was used as an independent bioinformatics approach, mean CLIC4 transcript level was reduced in selected studies, including lung (Fig. 1B), prostate (Fig. 1B), breast, sarcoma, and brain (data not shown).

To determine if changes in CLIC4 gene expression detected in the cDNA microarray correspond with the protein expression, a membrane containing human tissue lysates derived from matched normal and tumor tissue was probed with CLIC4 antibody (Fig. 1C). Similar to the cDNA array data, CLIC4 protein expression varied among the normal samples of the same tissue type, although all spots represent an equal amount of protein. Furthermore, CLIC4 protein levels also varied in tumors when compared with the matching normal tissues (Fig. 1C), implying that altered CLIC4 expression in tumors may be dependent on the abundance of cell types that comprise the major portion of the tumors analyzed. From the limited samples analyzed, CLIC4 protein may be reduced in kidney
Cancer, but a particular expression pattern was not detected in other tumors (Fig. 1C). Consistent with the cDNA data, the pathology reports associated with the tissue samples on the array indicate that altered CLIC4 expression was not dependent on patient age and tumor stage.

**CLIC4 expression is diminished in tumor cells and up-regulated in tumor stroma.** The variable expression patterns for CLIC4 in lysed whole tumors prompted us to examine CLIC4 in human tumor material *in situ* using matched tissue arrays that are immunostained with a specific CLIC4 antibody. *In vivo*, CLIC4 is nuclear in multiple normal epithelial tissues (e.g., esophagus, kidney, and colon), consistent with the slow cycling of most epithelial cells (12). In general, stromal CLIC4 expression is very low. In tumors from the great majority of multiple human tissues, three distinct and common changes can be observed. Nuclear CLIC4 is lost; CLIC4 levels are reduced in the tumor epithelium; and CLIC4 expression is markedly up-regulated in tumor stroma (Fig. 2A-C). Specifically in kidney, CLIC4 is also located in the apical portion of distal tubular cells and their nuclei (Fig. 2B). Most notably, total CLIC4 in tumor cells decreases with increasing tumor grade as shown in Fig. 3 for colon cancer but seen in most other cancers studied. Conversely, total stromal CLIC4 increases with tumor grade (Fig. 3A-D). These results indicate that highly proliferative cancer cells first exclude or reduce CLIC4 in the nucleus and then suppress CLIC4 in the entire cell mass. To test if restoring CLIC4 levels in tumor cells would influence tumor growth, xenografts of the highly malignant breast cancer cell line MB231 were established in nude mice. Wild-type CLIC4 and nuclear-targeted CLIC4 were introduced into established tumors by i.t. injection of recombinant adenovirus or a control viral vector expressing GFP (Fig. 4A and B). Immunofluorescent microscopy of frozen tissue sections indicate that many cells from xenograft tumors are efficiently infected by the GFP vector.
recombinant virus (Fig. 4A). In the same setting, the expression of either wild-type or nuclear-targeted CLIC4 retards the growth of xenograft tumors significantly compared with the GFP control vector. In addition, targeting CLIC4 to the nucleus is more effective for tumor inhibition than raising the cytoplasmic level of CLIC4 in the tumor.

CLIC4 is not deleted or mutated in human tumors. Transmembrane and nuclear localization signal motifs regulate the subcellular localization of CLIC4 (12). Blast analysis of the sequences of the seven CLIC proteins against the entire human expressed sequence tag database revealed 2,049 sequences that showed significant similarity to CLIC genes. Of these 2,049 sequences, 602 were further grouped into 23 distinct cancer types based on tumor and tissue annotations associated with each entry (Table 1). Within each cancer group, the matched sequences were further subdivided into each CLIC family member, and DNA sequences were examined for mutations within the ORF region. Multiple sequence alignment data show that CLIC1 contained a variant at position 168, where the leucine in the sequence KFLDG changes to serine, and this mutation (T→C) was occasionally (12 of 552 cases) found in cancer types examined. However, no mutations were found in CLIC4 (29 ORF) and other CLIC members when sequences were examined for frequent or consistent differences between the original CLIC genes and the cancer-derived expressed sequence tag sequences. To verify the bioinformatics data by an independent method, human CLIC4 ORF was amplified by RT-PCR using the total RNA extracted from NCI-60 tumor cell lines, and the amplified DNA fragment was then sequenced for analysis. When DNA sequences were examined by multiple sequence alignment, mutations were not detected in CLIC4 ORF region in any of 60 tumor cell lines. These two independent mutation analyses showed that the altered subcellular localization or diminished expression of CLIC4.

Fig. 3. CLIC4 down-regulation in the epithelium and up-regulation in the stroma increases with higher tumor grade. Matched normal and tumor tissues were immunostained with CLIC4 antibody. Tissues from rectum representing normal (A), tumor grade 1 (B, T-I), grade 2 (C, T-II), and grade 3 (D, T-III). CLIC4 localized to the nucleus in normal epithelium and grade 1 tumors (blue arrow) and the interstitial or stromal compartment of normal and tumor tissues (red arrow). Left, lower magnification (×10) of the tissues. Dotted boxes, origin of right.
frequently observed in tumor tissue samples was not due to an alteration in the CLIC4 gene, but most likely due to transcriptional or pretranslational or posttranslational modification.

CLIC4 colocalizes with α-SMA in stroma of human tumors. Strong up-regulation of CLIC4 in tumor stroma was a consistent finding in many human cancer types and suggested an important function for CLIC4 in the tumor microenvironment. A previous report suggested that CLIC4 is up-regulated in myofibroblastic differentiation (16). The up-regulation of α-SMA during myofibroblast differentiation is a characteristic of stromal reaction in cancer (17). To determine if CLIC4 is coexpressed with α-SMA in normal tissues or cancers, a number of normal and cancerous tissues were analyzed by double immunostaining on tissue arrays (Fig. 5A-C). As seen in the immunohistochemical studies, most normal tissues express CLIC4 in epithelial cells, whereas CLIC4 expression is down-regulated in tumor cells. In the normal tissues, α-SMA is primarily detected in the vascular walls where CLIC4 is also expressed (see, e.g., Fig. 5B and C). Previous studies have indicated that endothelial CLIC4 also participates in vascular tubulogenesis (18). Both α-SMA and CLIC4 are markedly up-regulated and colocalized in the stroma of selected tumor types (Fig. 5D), suggesting that CLIC4 is a common marker for myofibroblast differentiation and likely participates in the formation of myofibroblasts. Immunohistochemical staining of CLIC4 in infiltrating ductal carcinoma of human breast showed similar staining patterns as seen with the immunofluorescence methods (Fig. 5E). To investigate whether α-SMA expression is a consequential outcome of CLIC4 expression, primary human fibroblasts were infected with the recombinant CLIC4 adenovirus; α-SMA protein level was significantly up-regulated upon CLIC4 overexpression (Fig. 5F). In situ hybridization of kidney tumor sections indicates that up-regulation of CLIC4 in the tumor stroma is transcriptionally mediated, whereas transcripts for CLIC4 are rare in the tumor cells (Fig. 6A). To determine if the induction of stromal CLIC4 and α-SMA involve an interaction with tumor cells, normal mouse fibroblasts were cocultured with human breast tumor cell line MCF10CA1a or normal breast epithelial cells and monitored for CLIC4 and α-SMA expression. Figure 6B reveals that adjacent to tumor islands, fibroblasts coexpress α-SMA and CLIC4, whereas the proteins are undetectable in tumor cells. This response was not elicited in coculture with normal breast epithelial cells. With increasing distance from tumor islands, only cytoplasmic CLIC4 is detected in fibroblasts, and with even greater distance, neither marker is detected. This interaction was confirmed in an in vivo xenograft model where human dermal fibroblasts were genetically modified by infection with retrovirus-encoding GFP.

When grafted together with MCF10CA1a cells, the GFP-marked fibroblasts were detected in the stroma surrounding tumor nests where they expressed high levels of CLIC4 (Fig. 6C). To further verify the immunostaining and the in vivo data, mouse fibroblasts derived from a CLIC4-overexpressing transgenic mouse line 4 or wild-type mouse fibroblasts were mixed with MCF10CA1a tumor cells and used in xenograft studies (Fig. 6D-F). Approximately 3-fold more CLIC4 is expressed by transgenic fibroblasts relative to wild-type fibroblasts in vitro (Fig. 6D). In a xenograft model, tumors retaining the fibroblasts overexpressing CLIC4 had a growth advantage over the wild-type fibroblast control, and both mixed xenograft models produced larger tumors than the MCF10CA1a tumor cells alone (Fig. 6E). Injection of wild-type control or transgenic fibroblast alone did not produce tumors (data not shown). CLIC4 is also up-regulated in the stroma surrounding the tumor nests in the xenograft tumors most prominently in tumors grafted with transgenic fibroblasts (Fig. 6F, inset). Double immunostaining of the xenograft tumors with CLIC4 and α-SMA antibodies further suggested that α-SMA and CLIC4 are coexpressed in the tumor stroma formed by the grafted fibroblasts (Fig. 6F). Together, these data indicate that the induction of CLIC4 and α-SMA in fibroblasts results from a tumor-stromal interaction; each is induced independently, and Figs. 5F and 6B together suggest that CLIC4 induction likely precedes the induction of α-SMA in myofibroblast differentiation.

Discussion

Maintenance of intracellular chloride is an essential cellular function to control volume, pH, and electrogenic balance, and this is critical to cell viability. Five families of chloride channels regulate chloride flux in mammals (1). However, only a few reports have examined changes in chloride channels in cancer cells (19). Voltage-gated chloride channels CLC-2, CLC-3, and

4 Suh et al., unpublished data.
CLC-5 are localized to various intracellular organelles and expressed at high levels in patient biopsies from low- and high-grade malignant gliomas (20). Glycine-gated chloride channels in endothelial cells inhibit the stimulatory effect of vascular endothelial growth factor on blood vessel growth (21). Calcium-activated chloride channels CLCA1 and CLCA2 are significantly down-regulated in 80% of colorectal carcinomas and >90% of highly proliferating tumor cells (22, 23). Reconstitution of CLCA2 expression in highly malignant cell lines reduced Matrigel invasion in vitro and prevented the growth or metastasis of tumor cells transplanted s.c. in nude mice (24, 25). Expression and function of the KCl cotransporter is essential for growth and invasion of cervical cancer cells, and inhibition of KCl transporter activity prevents the growth of cervical cancer cells in vivo (26). Together, these results suggest that the regulation of chloride transport can influence tumor development and progression, but the changes may be specific to particular tumors and particular chloride channel families.

We now report a more generalized pattern of cancer-associated changes for CLIC4. In a study of multiple human solid cancers, we find that CLIC4 expression is commonly reduced in the tumor epithelium, is excluded from the nucleus of cancer cells (in contrast to its location in the nucleus of non-cancerous differentiated epithelial cells), and is highly up-regulated in the tumor stroma, associated with myofibroblast differentiation. The extent of these changes in general correlates directly with the stage of tumor progression in selected tumor types studied. Furthermore, our studies suggest that restoration of CLIC4 levels, and particularly CLIC4 in the nucleus of tumor cells, inhibits tumor growth. Coculture studies suggest that the up-regulation of CLIC4 in myofibroblast transformation of tumor stroma precedes the expression of αSMA and is induced by factors released from tumor cells. Enhancement in growth of xenograft tumors with fibroblasts overexpressing CLIC4 versus control suggests that up-regulation of CLIC4 in tumor stroma participates in tumor progression. These reciprocal changes of CLIC4 expression in tumor epithelium and stroma explain why tumor lysate studies did not show a consistent expression pattern and suggest that whole tumor lysates should be evaluated cautiously when used to identify a particular marker.

Previous studies have indicated that CLIC4 transcripts are up-regulated substantially in breast cancer myofibroblasts in response to transforming growth factor-β (16). Furthermore, growth of breast cancer cells in vitro is enhanced when cocultured with serum-stimulated fibroblasts that have a gene expression profile of myofibroblasts, including marked up-regulation of CLIC4 (27). The exact role played by CLIC4 in stromal fibroblasts remains to be determined. Our studies indicate that up-regulation of stromal CLIC4 is common to many tumors and correlates with increasing malignant behavior of the cancer cells. Myofibroblasts contribute essential functions in tumor growth under experimental testing, including secretion of matrix metalloproteinases (28), promotion of angiogenesis (29), promotion of tumor cell growth and invasion (27, 29), and chemokine release (30). A recent report suggests that secretion of stromal cell–derived factor 1 by tumor myofibroblasts recruits endothelial cells and stimulates tumor growth (29). Stimulation of CLIC4 by this factor would also be of consequence because CLIC4 and αSMA also colocalize in the muscularis of vascular structures in both normal and tumor tissue, and CLIC4 has a shown role in

Table 1. Mutations of CLIC family members in human cancers

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NOTE: The DNA sequences of the seven CLIC family members were compared with the human expressed sequence tag database, and the expressed sequence tag sequences (total 602) that showed similarity were sorted into readily identifiable cancer types and then further subdivided into CLIC isoforms. From 25 total mutations, only the mutations found in the ORF of CLIC isoforms are shown at the bottom.
tubular morphogenesis of blood vessels (18). The relationship of CLIC4 and αSMA then seems to be of functional consequence in several physiologic and pathologic situations. Previously, CLIC4 was reported to interact with components of the cytoskeleton in a complex with dynamin, tubulin, β-actin, and 14-3-3 proteins (9), suggesting that CLIC4 is involved in cytoskeleton function in addition to its channel activity. This characteristic of CLIC4 may explain its importance in tumor stroma because overexpression of recombinant CLIC4 in fibroblasts reduces cell motility (16), a property that may enhance the interaction of myofibroblasts with adjacent tumor cells.

In tumor lysates, a reduction in CLIC4 expression was common to many tumor types but with substantial variability among and within tumor specimens. Analysis of tumor sections in situ revealed that variability in lysates was due to the opposing changes of loss of expression in tumor epithelium and gain of expression in tumor stroma. The loss of CLIC4 in tumor epithelium was pervasive across almost all major cancer types. For example, systematic immunohistochemical analyses of tumors with various grades in the format of tissue microarrays (270 kidney cores from 190 cases, 189 esophagus cores from 66 cases, 189 colon cores from 67 cases, and 189 rectum cores from 63 cases) showed a loss of CLIC4 in ~80% of the...
cases ($P < 0.05$; 0.94-1.0 of 95% confidence interval with a probability for true negative 0.7-0.87; by VassarStat software) as cancers progress. Similar data were also obtained from breast, lung, prostate, and other tumor types (368 tumor tissue cores from 225 cases) by immunofluorescence analysis using anti-CLIC4 and anti-αSMA antibodies. Together, these data suggest that the loss of CLIC4 in tumor epithelium and the reciprocal increase in the tumor stroma are common events during tumor progression. Based on the cDNA array results, particularly in breast, ovary, and kidney, and in situ hybridization, reduced expression in tumor cells is transcriptionally regulated. The underlying mechanisms that suppress CLIC4 expression are not known at this time.

We considered that intrinsic alterations in CLIC4 through mutations or genomic rearrangements could alter expression. CLIC4 is localized to the tip region of the p-arm of chromosome 1 at 1p36.11 (4D2.3 region in mouse) where DNA aberrations are frequent in multiple human cancer types. An analysis of the human genomic database has revealed that several cancer-related genetic aberrations are within close proximity of CLIC4. However, sequencing of CLIC4 cDNA from the NCI60 tumor cell lines and human expressed sequence tag sequence analysis indicate that the CLIC4 gene is not deleted, and the CLIC4 sequence is highly preserved in human cancers. Moreover, neither the nuclear localization signal nor the transmembrane domains of CLIC4 was mutated. The lack of deletions or mutations in the CLIC4 gene suggests that CLIC4 at some level is essential for cell viability. Nevertheless, we cannot rule out that aberrations near the CLIC4 genetic locus may affect regulation of CLIC4 expression and alter the protein level; however, another mechanism of gene silencing seems more likely. If the chloride channel function of CLIC4 is its essential contribution to normal physiology, then other CLIC family members or other chloride channel families may compensate and maintain the intracellular ionic homeostasis in tumors. However, little is published on

**Fig. 6.** Induction of CLIC4 and αSMA in fibroblasts results from a tumor stromal interaction. **A.** CLIC4 transcripts (black arrow) in the tumor stroma of a kidney tumor (clear cell carcinoma) in tissue arrays were detected by nonradiolabeled in situ hybridization. Inset, lower magnification (×10). **B.** Malignant breast cancer cell line MCF10CA1a or normal breast cell line MCF10A (inset, control) were cocultured with primary fibroblasts for 2 d and immunostained with CLIC4 (red) and αSMA (green) antibodies. White dotted line, colonies of cancer cells and normal breast epithelial cells. **C.** Human dermal fibroblasts were transduced with a retrovirus encoding GFP, mixed with MCF10CA1a cells and xenografted to nude mice. Tumor sections were immunostained for GFP and CLIC4. D, lysates of cultured primary dermal fibroblasts from CLIC4 transgenic (TR) and wild-type (WT) mice were immunoblotted with anti-CLIC4 and anti-actin antibodies. E, fold increase in CLIC4 was determined by the ImageMaster densitometry program and normalized against the actin signal. MCF10CA1a cancer cells alone or mixed with primary fibroblasts (transgenic or wild type) were s.c. injected into nude mice, and the xenograft tumors were measured weekly. Each group represents 10 mice. Points, mean tumor measurements; bars, SD. **F.** Tumors from (E) were excised, sectioned, and immunostained for CLIC4 and αSMA.
chloride regulation in cancer where changes in cell volume, pH, and oxygenation suggest that the physiologic environment may require specific modifications in ion transport to maintain growth and viability (31).

Our immunostaining studies revealed predominant nuclear localization of CLIC4 in the epithelium of multiple normal tissues. In vitro, targeting CLIC4 to the nucleus by genetic or pharmacologic methods is associated with an apoptotic response in many cell types, particularly when CLIC4 levels are also increased (12). However, most normal epithelia in vivo are in a G0 resting state, and we have found a number of situations in vitro (differentiation, senescence, and growth inhibition) where endogenous nuclear CLIC4 is associated with cell cycle arrest. CLIC4 therefore may contribute to the control of cell cycle, either through an influence on the ionic milieu of the nucleus or an independent function. An ion channel independent function of CLIC4 in the nucleus is supported by the recent finding that CLIC4 localizes at the centrosome and midbody of mitotic cells (32). Furthermore, our prior work revealed that nuclear CLIC4 is predominantly in the nucleoplasm (12), where it would not be expected to participate in ion transport. Whatever specific functions are carried out by nuclear CLIC4 are obviously detrimental to cancer cells as CLIC4 is uniformly excluded from the nucleus of tumor cells even at early stages of malignant progression. Precisely how CLIC4 is excluded from the nucleus of cancer cells in the absence of alterations in the nuclear localization signal or cotransporters must await an understanding of factors that control nuclear translocation under physiologic situations. Both CLIC1 and CLIC4 are subject to redox regulation with major structural rearrangements and functional activities dependent on redox state (33, 34). Because cancer cells frequently exhibit altered redox regulation (35, 36), this control of CLIC4 subcellular localization should be considered.

Subcellular translocation of important signaling molecules is a new paradigm in sorting out changes that are relevant to malignant transformation. For instance, immunohistochemical studies showed that cell cycle regulatory or proapoptotic proteins p21<sup>CIP1</sup> (37) and p27<sup>KIP1</sup> (38, 39), KLF4/GKLF (40), Apaf-1 (41), cRel (42), p33ING1 (43), supressin (44), and nucleophosmin (45) were localized in the nucleus of normal tissues. Localization of CLIC4 in the epithelium of multiple normal tissues suggests that such proteins are uniformly excluded from the nucleus of tumor cells even at early stages of malignant progression. As cancer cells frequently exhibit altered redox regulation (35, 36), this control of CLIC4 subcellular localization should be considered.

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Reciprocal Modifications of CLIC4 in Tumor Epithelium and Stroma Mark Malignant Progression of Multiple Human Cancers

Kwang S. Suh, John M. Crutchley, Arash Koochek, et al.


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