

May 2007  
Volume 6

CENTER FOR CANCER RESEARCH  
**frontiers**  
IN SCIENCE

■ CARCINOGENESIS

## Tumor Cells and Stroma CLIC to Promote Cancer Progression

Suh KS, Crutchley JM, Koochek A, Ryscavage A, Bhat K, Tanaka T, Oshima A, Fitzgerald P, and Yuspa SH. Reciprocal modifications of CLIC4 in tumor epithelium and stroma mark malignant progression of multiple human cancers. *Clin Cancer Res* 13: 121–31, 2007.

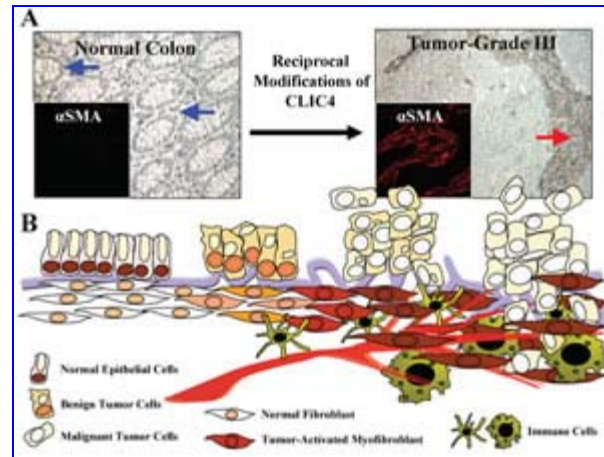
Intracellular chloride controls organelle volume, pH, and electrogenic balance and is crucial to regulate the integrity of intracellular organelles. Previous reports suggest that the regulation of chloride transport can influence tumor development and progression, but the changes may be specific to particular tumors and chloride channel families. We now report chloride intracellular channel-4 (CLIC4) has a more generalized pattern of cancer-associated changes in multiple human cancers.

CLIC4, one of seven members of the CLIC family, was discovered in a search for p53-regulated genes in differentiating keratinocytes (Fernandez-Salas E et al. *J Biol Chem* 274: 36488–97, 1999). Uniquely, soluble forms of CLIC proteins in the cytoplasm function in cell signaling pathways but undergo molecular and structural modifications in response to specific stimuli to autoinsert into the cellular/organelle membrane, where they behave as an anion channel or channel regulator. Therefore, CLIC proteins appear to be multifunctional, having both soluble and membrane activities. Cytoplasmic CLIC4 translocates to the nucleus in cells undergoing growth arrest or apoptosis in response to multiple stimuli, including metabolic or cytotoxic stress or physiological growth inhibitors. A C-terminus functional nuclear localization signal regulates nuclear trafficking. Overexpression of CLIC4 in the nucleus causes cell cycle arrest and accelerates apoptosis. CLIC4 is a direct downstream target of both p53 and c-Myc, two mediators of cancer pathogenesis in multiple tumors (Shiio Y et al. *J Biol Chem* 281: 2750–56, 2006) and is required for blood vessel tubular morphogenesis (Bohman S et al. *J Biol Chem* 280: 42397–404, 2005). These discoveries prompted us to evaluate the changes in *CLIC4* integrity, transcript and protein expression, and the subcellular localization of its product in a series of human tumors and test the impact of the *in vivo* results on tumor growth in experimental models.

In analyses of cDNA and tumor lysate arrays of matched human normal and tumor tissues representing all major human solid tumors, CLIC4 expression was often reduced in the tumor

extracts, particularly in ovary, renal, and breast cancers, but a specific expression pattern did not emerge. A more consistent pattern of change was detected in immunostained tissue arrays from multiple human solid epithelial cancers. Although CLIC4 was abundant and largely located within the nucleus in normal epithelium, CLIC4 was excluded from the nucleus and markedly reduced in the tumor epithelium. Conversely, CLIC4 was not highly expressed in the stroma of normal tissues but markedly upregulated in the tumor stroma, having been associated with myofibroblast conversion as indicated by co-expression of alpha smooth muscle actin ( $\alpha$ SMA). Thus, reciprocal modifications of CLIC4 in distinct tumor compartments would be difficult to detect in materials derived from whole tumor lysates. Transcript sequences of *CLIC4* from the human EST database and manual sequencing of cDNAs from NCI60 human cancer cell lines failed to reveal deletions or mutations in the gene, suggesting other genetic (e.g., methylation), post-transcriptional (e.g., mRNA stability), or post-translational (e.g., phosphorylation) changes may be responsible for the altered molecular expression of CLIC4 in cancer epithelium.

In several tumor types studied, the extent to which CLIC4 was lost in tumor epithelium and upregulated in tumor stroma directly correlated with the stage of tumor progression (Figure 1). To test the functional relevance of CLIC4 changes in tumors, we injected human breast cancer cells into nude mice as subcutaneous xenografts. Inducing CLIC4 overexpression in tumor cells and, particularly, the nuclei of tumor cells by adenovirus transduction inhibited tumor growth. In contrast, grafting breast cancer cells together with fibroblasts engineered to overexpress CLIC4 enhanced tumor growth. These engineered fibroblasts upregulated  $\alpha$ SMA in response to CLIC4 overexpression *in vitro*, and the xenografts were rich in myofibroblasts *in vivo*. When human breast cancer cells were co-cultured with fibroblasts, CLIC4 was not detected in cancer cell foci but was upregulated in fibroblasts surrounding cancer foci along with  $\alpha$ SMA. Temporally, it appeared that CLIC4 was upregulated prior to the appearance of  $\alpha$ SMA in fibroblasts surrounding tumor foci. Thus, CLIC4 participates in the crosstalk between tumor cells and their surrounding stroma to induce a microenvironment conducive to enhanced growth, and a compartment-directed CLIC4 expression profile, in conjunction with the  $\alpha$ SMA profile, may be a useful addition to the diagnostic criteria in marking/grading tumors. Further, CLIC4 may be a novel molecular target with significant therapeutic potential for the following reasons: (1) CLIC4 reduction in the tumor mass is a consequence of epigenetic factors and therefore may be reversible, which could impede tumor growth. (2) CLIC4 is specifically excluded from the nucleus of cancer cells and not normal cells, so restoring expression and nuclear localization may be selectively toxic to tumor cells. (3) Modification of stromal CLIC4 expression may alter myofibroblast activity and/or angiogenesis in the tumor microenvironment and serve to diminish host factors that are recruited by tumor cells to enhance their growth.



**Figure 1.** Reciprocal modifications of chloride intracellular channel-4 (CLIC4) in tumor epithelium and stroma directly correlate with tumor progression. A) Immunostaining of CLIC4 in tissue sections from normal colon showing the predominant localization of CLIC4 in nuclei of crypt cells and lamina propria (blue arrows). In contrast, CLIC4 is excluded from tumor epithelium in advanced colon cancer and upregulated in tumor stroma (red arrow) where it is co-expressed with alpha smooth muscle actin ( $\alpha$ SMA). B) Diagram depicting the step-wise reciprocal modifications of CLIC4 (represented by brown color) in epithelial cells and stromal fibroblasts over the course of the multistage development of cancer at multiple organ sites.

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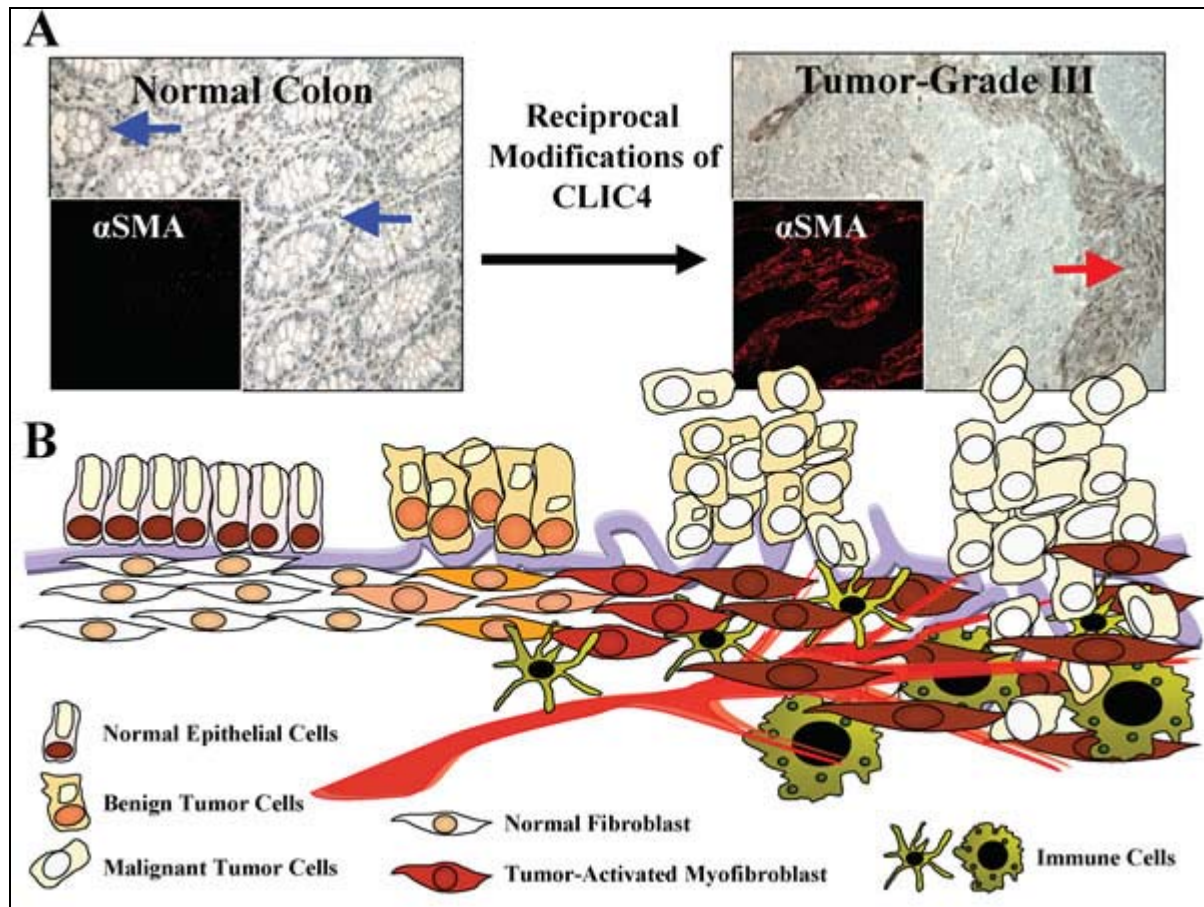
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