Original Article

Low Expression of Chloride Channel Accessory 1 Predicts a Poor Prognosis in Colorectal Cancer

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BACKGROUND: Chloride channel accessory 1 (CLCA1) is a CLCA protein that plays a functional role in regulating the differentiation and proliferation of colorectal cancer (CRC) cells. Here we investigated the relationship between the level of CLCA1 and the prognosis of CRC. **METHODS:** First, the level of CLCA1 was detected quantitatively in normal and cancerous colonic epithelial tissues with immunohistochemistry. Next, the correlations between CLCA1 expression, pathological tumor features, and the overall survival rate of patients was analyzed. Finally, 3 publicly available data sets from the Gene Expression Omnibus were examined: normal CRC versus early CRC (GSE4107), primary CRC versus metastatic lesions (GSE28702), and low chromosomal instability versus high chromosomal instability (GSE30540). **RESULTS:** The expression of CLCA1 was decreased markedly in tumor specimens. CLCA1 expression was correlated significantly with the histological grade ($P<.01$) and lymph node metastasis ($P<.01$). A significantly poorer overall survival rate was found in patients with low levels of CLCA1 expression versus those with high expression levels ($P<.05$). The results confirmed that the low expression of CLCA1 in CRC was highly associated with tumorigenesis, metastasis, and high chromosomal instability. In addition, the loss of CLCA1 disrupted the differentiation of human colon adenocarcinoma cells (Caco-2) in vitro. **CONCLUSIONS:** These findings suggest that CLCA1 levels may be a potential predictor of prognosis in primary human CRC. Low expression of CLCA1 predicts disease recurrence and lower survival, and this has implications for the selection of patients most likely to need and benefit from adjuvant chemotherapy. ***Cancer* 2015;121:1570-80.** © 2015 The Authors. *Cancer* published by Wiley Periodicals, Inc. on behalf of *American Cancer Society*. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

KEYWORDS: chloride channel accessory 1 (CLCA1) expression, colorectal carcinoma, prognosis, cell differentiation.

INTRODUCTION

The chloride channel accessory (CLCA) family (also called the calcium-sensitive chloride conductance protein family) comprises 4 genes in humans and at least 6 genes in mice.[1-5] All members of the CLCA family map to the same region on chromosome 1p31-p22 and share a high degree of homology in size, sequence, and predicted structure, but they differ significantly in their tissue distribution. The human genome encodes 3 functional CLCA proteins, including CLCA1, CLCA2, and CLCA4. CLCA2 has been identified to be a p53-inducible inhibitor of cell proliferation and to be a marker of differentiated epithelium that is downregulated with tumor progression.[1,6] CLCA4 is downregulated in breast cancer cells, and low expression of CLCA4 indicates a poor prognosis for patients with breast cancer.[7] CLCA4 and CLCA1 are expressed in intestinal epithelium.[3,8-10] CLCA1 and CLCA4 may function as tumor suppressors and are associated negatively with tumorigenicity.[11] We have shown that CLCA1 contributes to differentiation and proliferation inhibition in colon cell lines,[10] but the role of CLCA1 in the prognosis of patients with colorectal cancer (CRC) remains unclear.

In cancer progression, individual cells undergo complex and important interactions with the extracellular environment through transmembrane signal transduction. These interactions regulate cancer cell proliferation, invasion, and metastasis. Ion channels are a crucial first step in this process, and they play emerging roles in carcinogenesis and tumor

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Jin Pu and Lin Cao conceived and designed the experiments. Jin Pu, Lin Cao, Colin D. McCaig, and Steven D. Heys wrote the article. Bo Yang and Jiaen Liu prepared the patient samples. Bo Yang, Lin Cao, Jiaen Liu, Gillian Milne, Wanhei Chan, and Yanjie Xu performed the experiments. Lin Cao, Jin Pu, Bo Yang, and Jiaen Liu analyzed the data.

The first 2 authors contributed equally to this article.

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progression.[11] For example, activation of the chloride current through specialized volume-regulated anion channels in response to cell swelling is one of the major mechanisms by which cells restore their volume after hypoosmotic stress (regulatory volume decrease, RVD).[12] This is important because there is a direct link between the apoptotic resistance conferred by the antiapoptotic Bcl-2 protein and the strengthening of RVD capability due to upregulation of the chloride current and swelling.[13] Therefore, further investigations will elucidate important roles for ion channels in cancer development and progression and potentially establish ion channels as effective discriminative markers and therapeutic targets.[14]

We have reported that CLCA1 is expressed in differentiated, growth-arrested mammalian epithelial cells but is downregulated during tumor progression.[10] We have identified CLCA1 as a regulator of the transition from proliferation to differentiation in Caco-2 cells. In this study, we have determined further that the expression of CLCA1 in human CRC intestinal tissue is associated with the primary tumor status (the degree of invasion of the intestinal wall), lymph node metastases, and the overall survival rate. CLCA1 also is correlated closely with tumor suppressor p53 and E-cadherin, which has been determined to influence the prognosis of CRC.[15-18] Our findings suggest that the expression level of CLCA1 may predict disease relapse and outcomes for patients with CRC.

MATERIAL AND METHODS

Patients

Thirty-six patients were diagnosed with CRC (26 with colon cancer and 10 with rectal cancer; Table 1), which was classified with the International Union Against Cancer TNM staging system and the Dukes staging system. All patients underwent surgical resection of their tumors at the 309th Hospital in Beijing, China. Ethical approval for the study was granted by the 309th Hospital's ethics committee. Informed written consent was obtained from all participants involved in the study.

The key clinical characteristics of the patients are summarized in Table 1. Normal specimens were obtained from 2 sources: 2 samples came from normal colon tissue of noncancer patients, and 4 samples were obtained from adjacent, grossly normal-appearing tissue taken at least 10 cm away from the cancer. This was in accordance with other research.[19] All tumor samples were obtained from surgical resection. After surgical resection of their tumors, patients were followed up with clinical examinations, abdominal ultrasonography or abdominal computer tomography scans, and carcinoembryonic antigen measurements

TABLE 1. Clinical Characteristics of Patients With Colorectal Cancer

Variable	n (%)
Patients	36 (100)
Men	25 (69.4)
Women	11 (30.6)
Primary tumor status	
T1	7 (19.4)
T2	5 (13.9)
T3	16 (44.4)
T4	8 (22.2)
Primary lymph node status	
N0	25 (69.4)
N1/N2	11 (30.6)
Dukes staging	
A	7 (19.4)
B	18 (50)
C	5 (13.9)
D	6 (16.7)
Histological grade	
I	10 (27.8)
II	13 (36.1)
III	13 (36.1)
Metastasis	
Local	4 (11.1)
Liver	2 (5.6)

every 6 months for the first 5 years. Thereafter, these investigations were performed annually until 5 years after the initial treatment. The patients who died as a result of any postoperative complications or non–cancer-related diseases were excluded from the survival analysis. A patient with a family history suggestive of hereditary nonpolyposis colon cancer syndrome was excluded. None of the patients included in this study had chemotherapy or radiotherapy before surgery. In all, 36 CRC patients were included in the survival analysis. Adjuvant chemotherapy was given to all CRC patients except those with stage I disease (cancer had not invaded the outermost layers of the colon or rectum and had not spread to lymph nodes or distant sites). The mFOLFOX6 chemotherapy regimen consisted of a 2-hour intravenous infusion of oxaliplatin (85 mg/m^2) and folinic acid (400 mg/m^2), which was followed by an intravenous bolus injection of 5-fluorouracil (400 mg/m^2) plus a 46-hour intravenous infusion of 5-fluorouracil (2400 mg/m^2); this was repeated every 2 weeks. After 4 cycles of therapy, all lesions were assessed with computer tomography.

Clinical Follow-Up

All patients were prospectively followed up according to the schedule described previously. The mean follow-up time in this study was 46 months (range, 7-52 months). The status of each patient was determined at the date of the last follow-up or at the end of a 5-year follow-up period, and if they were deceased, the cause of death was

ascertained from the medical records and/or death certificate information. At the initial diagnosis, 6 patients with CRC had metastases.

Histology and Colon Cancer Staging

Resected tumors were obtained immediately after surgical resection and were fixed in 10% pH-neutral formalin and embedded in paraffin. Paraffin-embedded tissue sections (5 μm thick) were cut serially and used for hematoxylin-eosin staining and immunohistochemical analysis. The patients were staged according to the International Union Against Cancer TNM staging system and the Dukes staging system (Table 1).[20] The Dukes staging system (used for colon cancer staging originally) was defined as follows: (A) tumor in the mucosa, (B) tumor in the muscle layer, (C) involvement of lymph nodes, and (D) distant metastases. Tumors were classified also by their degree of histological differentiation: (well differentiated [I]), moderately differentiated [II], or poorly differentiated [III]), the presence or absence of perineural invasion, the presence or absence of venous emboli, and the number of lymph nodes involved by the tumor.

Immunohistochemistry

After paraffin embedding, the tissue was cut into 5-μm-thick sections and mounted onto Superfrost Plus slides. The immunohistochemistry was performed on Leica Bond Max and included dewaxing and heat-induced epitope retrieval with Epitope Retrieval 1 (Leica Biosystems) at 100°C. Peroxidase block from the Bond Refine Detection System was used. The primary antibody CLCA1 (polyclone; Santa Cruz) was applied at a 1:100 dilution for 30 minutes at room temperature. The staining was completed with the Bond Polymer Refine Detection system. The Bond Polymer Refine Detection system contained a peroxide block, post primary, a polymer reagent, 3,3′-diaminobenzidine chromogen, and a hematoxylin counterstain (Leica Biosystems). p53, E-cadherin, phosphatase and tensin homolog (PTEN), and Ki-67 expression was evaluated according to the proportion of positively stained tumor cells.

Knockdown of CLCA1 and Western Blotting

Caco-2 cells (5×10^5; American Type Culture Collection) were cultured on collagen I–precoated 6-well plates for the indicated time. Cells were lysed with a cell lysis buffer (Sigma-Aldrich) and a protease inhibitor cocktail (Thermo Scientific) for western blot analysis. Knockdown of CLCA1 in Caco-2 cells has been described previously.[10] Briefly, a stealth RNAi small interfering RNA (siRNA) duplex with sense-strand sequences (5′-CAAUGCUACCCUGCCUCC AAUUACA-3′; Invitrogen, United Kingdom) was used to specifically target the CLCA1 gene. Caco-2 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with a final siRNA concentration of 150 nM. Nontargeting negative control siRNA was used for non–sequence-specific effects of these molecules. After 72 hours, cells were lysed for western blot analysis. The primary antibodies used for western blotting included anti-CLCA1 (1:1000; Santa Cruz), anti–E-cadherin (1:5000; BD), anti–intestinal alkaline phosphatase (anti-ALPI; 1:2000, Novus), and anti–glyceraldehyde 3-phosphate dehydrogenase (1:50,000; Santa Cruz). Membranes were incubated with the relevant primary antibodies overnight at 4°C. A secondary antibody with horseradish peroxidase (1:5000; Sigma-Aldrich, United Kingdom) was used, and the immunoblots were detected with WesternBright ECL (AGTC Bioproducts).

Quantitative Analysis

Slides were assessed with light microscopy. All slides were analyzed by 2 independent observers (L.C. and J.P.) who were blinded to the clinical data. For each colon carcinoma, staining was evaluated on separate slides, which included the core and the invasive edge of the tumor, respectively. Slides were examined at ×400 (40× objective and 10× ocular) and were analyzed via the counting of all cells present in them (at least 5000 cells). All of the slides were reviewed independently by each researcher twice. Discrepancies between investigators (<10% of the cases) required a third joint observation with a conclusive agreement.[21] The expression of CLCA1 was scored as the ratio of the number of positive cells to the total number of cells (stained cells/total number evaluated). The samples were considered CLCA1-positive if any positive staining was detected unless there were only rare, isolated single cells. To translate a continuous variable of CLCA1 expression into a clinical decision, it is necessary to stratify patients into 2 groups that may have different prognoses. Currently, there is no standard method or standard software for biomarker cutoff determination.[22] However, our approach was to use X-tile 3.6 plot software (developed by Camp et al[23]), which provides a single, global assessment of every possible way of dividing a population into low-, medium-, and high-level marker expression. Using an X-tile plot, we determined the significant, optimal cut point to be 30% ($P = .0224$). Therefore, we used 30% CLCA1 positive staining as a cutoff value to unequivocally categorize cases into 2 groups: high expression of CLCA1 (>30% of cells stained) and low expression of CLCA1

(none or ≤30% of cells stained). This was in accordance with other research.[21,24]

Microarray Data Analysis

The microarray data sources were obtained from the Gene Expression Omnibus (GEO).[25] Three data sets (series accession numbers GSE4107, GSE28702, and GSE30540) were not subjected to any additional normalization because all had been normalized when we obtained them.[19] In GSE4107, early CRC specimens (n = 12) and adjacent, grossly normal-appearing tissue (n = 10) at least 8 cm away were collected routinely and archived from patients undergoing colorectal resection at the Singapore General Hospital.[19] In the GSE28702 study, 83 patients with unresectable CRC, including 56 patients with primary CRC and 27 patients with metastatic lesions in the liver (23 tumors), lungs (1 tumor), and peritoneum (3 tumors), were recruited from April 2007 to December 2010 at Teikyo University Hospital and Gifu University Hospital.[26] All CRC samples were obtained before mFOLFOX6 therapy. GSE30540 included 25 chromosomal instability–high (CIN-high) CRC patients and 10 chromosomal instability–low (CIN-low) CRC patients.[27] We analyzed the expression of CLCA1 in these published microarray data sets with the GEO software. The identity of genes across microarray data sets was established with public annotations primarily based on Unigene.[28]

Statistical Analysis

Statistical analysis was performed with Excel and Prism. The equality of group means and comparisons between proportions were analyzed with an unpaired Student *t* test and chi-square test, respectively. Univariate statistical analysis was performed with a log-rank test (Mantel-Cox). X-tile 3.6.1 plot software (http://medicine.yale.edu/lab/rimm/research/software.aspx) was used to determine the cutoff point of the CLCA1 expression level for separating all patients into 2 groups to examine the impact of CLCA1 expression on prognosis. The curves were plotted with the product-limit method (Kaplan-Meier) and were analyzed with Spearman correlation coefficients and Wilcoxon tests for all survival analyses. For covariates retained in the model, relative hazards with 95% confidence intervals were estimated. Differences with a *P* value of .05 or less were considered to be statistically significant.

RESULTS

Patients and Tumors

The characteristics of the 36 CRC patients in the study cohort are shown in Table 1. There were 11 women (30.6%) and 25 men (69.4%). The median age was 55.5 years with a range of 25 to 80 years. In terms of the anatomical location of the tumors, 15 (41.7%) were in the ascending colon, 2 (5.6%) were in the transverse colon, 3 (8.3%) were in the left colon, 6 (16.7%) were in the sigmoid colon, and 10 (27.8%) were in the rectum. Twenty-six patients (72.2%) had moderately or poorly differentiated tumors, with the remaining 10 (27.8%) having well-differentiated cancers. The Dukes staging was as follows: (A) 7 or 19.4%, (B) 18 or 50%, (C) 5 or 13.9%, and (D) 6 or 16.7%.

Expression of CLCA1 and Clinical Grades of CRCs

The level of expression of CLCA1 with respect to tumor staging is shown in Table 2. There was a high level of CLCA1 expression in normal colonic epithelium in stark contrast to the tumor tissue (Fig. 1A). The mean percentage of CLCA1-positive cells was 88% in the normal samples (n = 6) and 54% in the tumor samples (n = 36). In addition, the expression pattern of CLCA1 was predominantly membranous and cytoplasmic in normal colonic epithelium, but this pattern was altered in the tumor area, which showed an absence of cytoplasmic and/or membranous staining (Fig. 1A). Furthermore, we analyzed the relationship between the level of expression of CLCA1 and the primary tumor status (T1-T4), lymph nodes status (N0 or N1/N2), Dukes stage (A-D), and histological grade (well, moderately, or poorly differentiated). This analysis showed that the CLCA1 expression in noncancerous control mucosa samples was significantly higher than that in samples with tumors. There also was a significant difference between normal tissue and early CRC tissue (T1, $P < .05$). In addition, in the more advanced tumor stages (T3 and T4), the expression of CLCA1 was reduced in comparison with earlier stage tumors (T1/T2 vs T3/T4, $P < .01$; Fig. 1B and Table 2). Furthermore, CLCA1 expression levels in primary tumors were reduced significantly when the patients had positive lymph nodes (N1/N2, $P < .01$; Fig. 1C). In Dukes stage A and B tumors, there was much higher expression of CLCA1 in comparison with Dukes stage C and D tumors ($P < .01$; Fig. 1D and Table 2). An analysis by histological grades also showed that the expression of CLCA1 was reduced significantly in poorly differentiated tumors versus well-differentiated tumor ($P < .01$; Fig. 1E and Table 2). Our data indicate that low CLCA1 expression levels are associated with an advanced tumor stage, a less differentiated tumor histological grade, and metastases in regional lymph nodes.

TABLE 2. Expression Level of CLCA1 and Status of Primary Tumors

	Cases			Primary Tumor Status			Primary Lymph Nodes			Dukes Staging			Histological Grade			Relapse/ Death
CLCA1 %	All	Male	Female	T1/T2	T3/T4	P^a	N0	N1/N2	P^a	A/B	C/D	P^a	I/II	III/IV	P^a	
Low (≤30%)	22	16 (44.4)	6 (16.7)	4 (11.1)	18 (50)		12 (33.3)	10 (27.8)		12 (33.3)	10 (27.8)		10 (27.8)	12 (33.3)		7 (19.4)
High (>30%)	14	9 (25)	5 (13.9)	8 (22.2)	6 (16.7)	<.01	13 (36.1)	1 (2.8)	<.01	13 (36.1)	1 (2.8)	<.01	13 (36.1)	1 (2.8)	<.01	0
Total	36	25	11	12	24		25	11		25	11		23	13		7

Abbreviation: CLCA1, chloride channel accessory 1.
[a] Expression levels were compared with the Pearson chi-square test.

Reduced Expression of CLCA1 Is an Indicator of the Likelihood of Disease Relapse and Poorer Survival

The median disease-free survival (DFS) for all patients was 34.5 months. The postoperative median DFS for patients with high expression of CLCA1 was 40 months, whereas that of patients with low CLCA1 expression was 23 months. CRC patients with reduced CLCA1 expression had a higher risk of disease relapse and death than patients with high CLCA1 expression ($P < .01$; Table 2). Kaplan-Meier analysis was used to evaluate the correlation between the survival of patients with CRC and the level of expression of CLCA1. Patients were divided into 2 groups: CRC patients with high CLCA1 expression (>30%) and those with low CLCA1 expression (≤30%). Our data showed that the DFS for CRC patients whose tumors had preserved CLCA1 expression was significantly higher than the DFS for patients with low CLCA1 expression ($P < .05$; Fig. 2A). Kaplan-Meier analysis was also performed with stratification by the characteristics of the primary tumor and lymph node metastases. We classified patients into groups with T1/T2 (n = 12) and T3/T4 tumors (n = 24) because of the case number and better prognosis with T1/T2 tumors versus T3/T4 tumors[29] and with lymph node–negative tumors versus lymph node–positive tumors. Our results also showed that the primary tumor stage was correlated with the DFS of CRC patients. The DFS of patients with T3/T4 tumors was reduced significantly in comparison with the DFS of patients with T1/T2 tumors ($P < .05$; Fig. 2B).

However, the lymph node status (N0 vs N1/N2) with respect to the DFS of this group of CRC patients showed a difference with a P value of .06 (Fig. 2C). Ki-67 has been studied as a prognosticator for CRC,[30] but we showed that there was no significant association between the expression levels of Ki-67 and the survival of CRC patients (Fig. 2D). Overall, our results demonstrated that the CLCA1 expression level was a prognostic factor for the survival of patients with CRC in the univariate analysis. Patients with CRC characterized by high levels of CLCA1 expression had a favorable prognosis, whereas patients with CRC with low CLCA1 expression had a poorer survival rate.

Correlation Between CLCA1 and PTEN, p53, E-Cadherin, and Ki-67 in CRC Patients

Abnormal expression or mutations of PTEN and p53, E-cadherin, and Ki-67 are associated with a poor prognosis for patients with CRC.[15,18,30-32] We also checked the

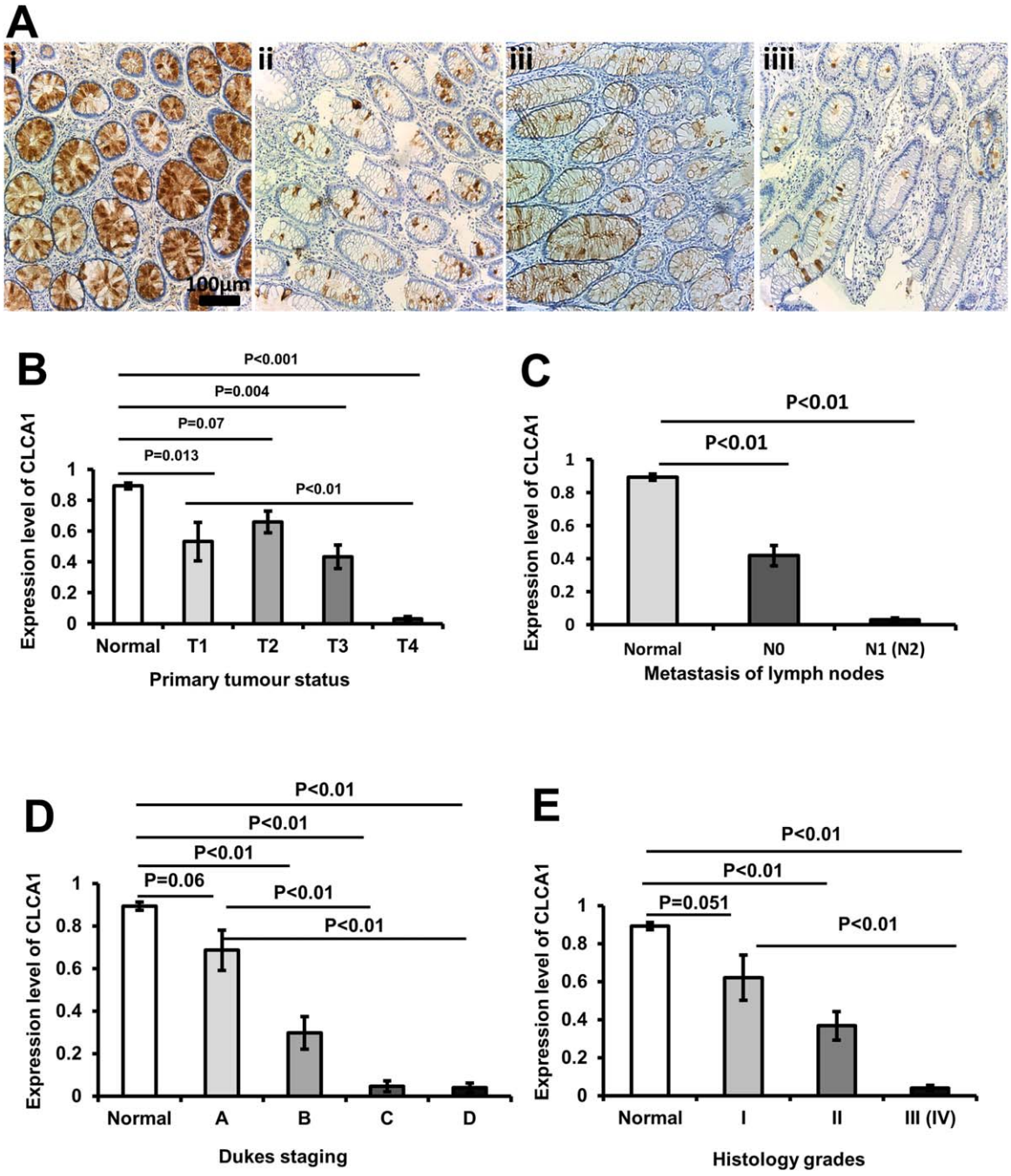

Figure 1. The expression of CLCA1 correlates with tumor stages, histological grades, and lymph node metastasis in colorectal cancer. (A) CLCA1 in normal colonic tissue showed (i) preservation of high levels of expression (brown) on cell membranes and in the cytoplasm, (ii) reduced expression on membranes and in the cytoplasm in colon cancer tissue, (iii) reduced expression in cytoplasm only in colon cancer tissue, and (iiii) reduced expression in rectal cancer. CLCA1 expression was strongly associated with (B) a different tumor status, (C) lymph node metastasis, (D) Dukes staging, and (E) histological grades. Expression levels of CLCA1 were quantified via the ratio of positive cells to total cells. Data are presented as means and standard errors of the mean. CLCA1 indicates chloride channel accessory 1.

expression of PTEN, p53, Ki-67, and E-cadherin in patients and compared the relationship between expression levels of CLCA1 and these 4 tumor-associated genes. We found that 16 of 28 CRC patients (57%) had high expression (>50% score) of Ki-67 cancer cells. However, no correlation was found between the levels of CLCA1

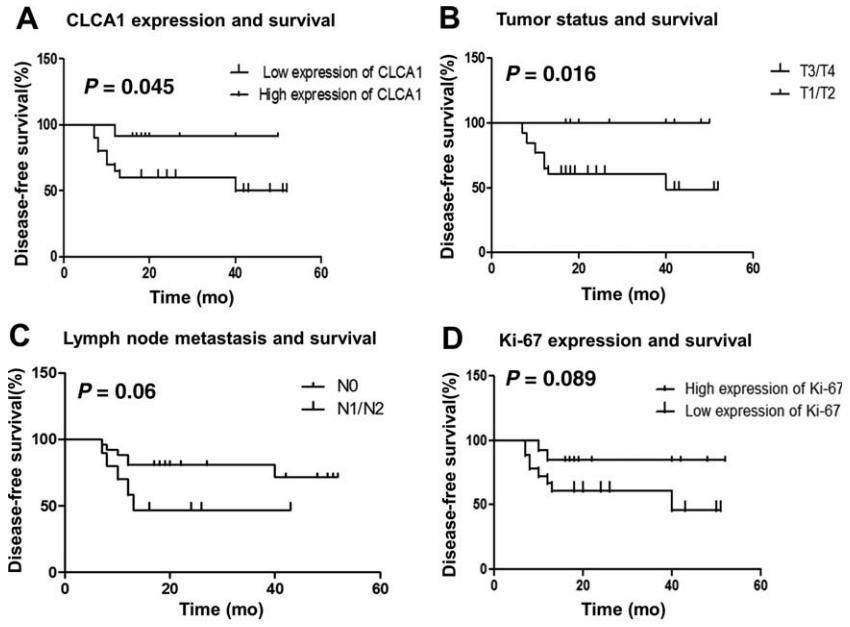

Figure 2. CLCA1 expression levels and disease-free survival for CRC patients. Kaplan-Meier curves of disease-free survival are shown for CRC patients: (A) different CLCA1 expression ($P < .05$), (B) tumor status ($P < .05$), (C) lymph node metastasis ($P > .05$), and (D) Ki-67 expression ($P > .05$). The differences between curves were analyzed with the log-rank test. CLCA1 indicates chloride channel accessory 1; CRC, colorectal cancer.

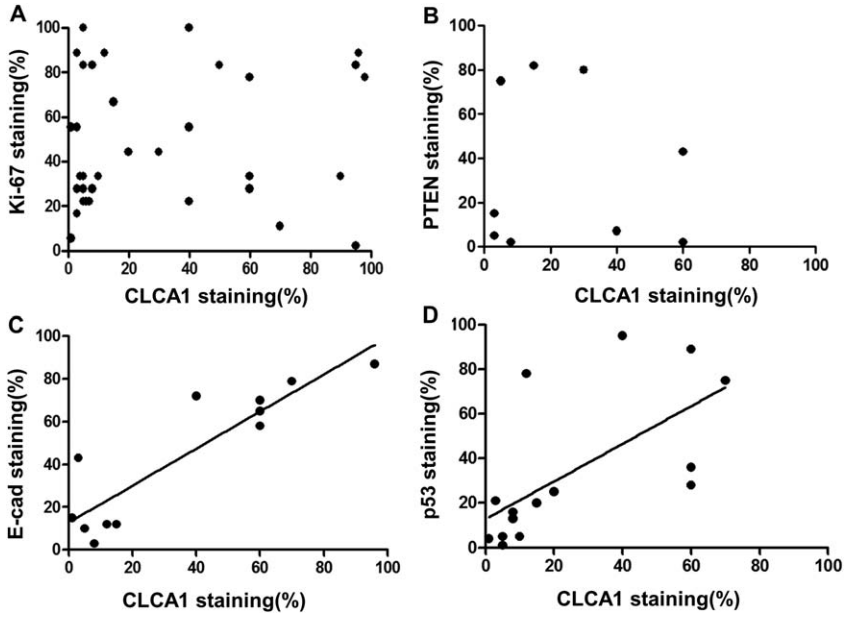

Figure 3. Correlation between the expression of CLCA1 and known prognostic markers for colorectal cancer. Expression levels of CLCA1, Ki-67, E-cad, PTEN, and p53 were analyzed through the percentages of positively staining cells. The correlations of CLCA1 expression with (A) Ki-67 (n = 28, $R^2 = 0.18$, $P = .29$), (B) PTEN (n = 9, $R^2 = -0.02$, $P = .95$), (C) E-cad (n = 12, $R^2 = 0.8$, $P = .0016$), and (D) p53 (n = 15, $R^2 = 0.78$, $P = .0007$) are shown, and strong positive correlations between high CLCA1 expression and high E-cad and p53 expression levels are indicated. CLCA1 indicates chloride channel accessory 1; E-cad, E-cadherin; PTEN, phosphatase and tensin homolog.

and Ki-67 expression in the primary tumors ($P = .29$; Fig. 3A). Furthermore, we investigated the correlation between the expression of PTEN and CLCA1 and found no association between the expression levels of these 2 molecules ($P = .95$; Fig. 3B). However, CLCA1 expression levels correlated strongly and positively with the level

of p53 and E-cadherin expression ($P < .01$; Fig. 3C,D). The downregulation of E-cadherin at the membrane indicated a poor outcome.[15] p53 is a tumor suppressor in CRC. Mutations of p53 are associated with worse survival, and normal levels of p53 are required for CRCs to respond to chemotherapy.[16] These data further confirm that low expression of CLCA1 appears to correlate strongly with a poor prognosis of CRC.

Confirmation of CLCA1 Expression in CRC With Microarray Data Sets

To further validate our data, we analyzed the 3 public, independent microarray data sets from GEO. Our analysis showed that the expression level of CLCA1 was inhibited significantly in early CRC specimens (normal vs early CRC, $P < .05$; Fig. 4A). In comparison with nonmetastatic specimens, the expression of CLCA1 was downregulated significantly in metastatic CRC ($P < .01$; Fig. 4B). CIN-high showed the worst survival for CRC patients.[27] Our results showed that CRC with the CIN-high phenotype had lower expression of CLCA1 than CRC with the CIN-low phenotype ($P = .015$; Fig. 4C). Therefore, the results from these 3 independent microarray sets were identical to the results from our clinical analysis data.

CLCA1 Regulates the Differentiation of Human CRC Cells

Culturing to confluence induces the spontaneous differentiation of human colon adenocarcinoma cells (Caco-2).[11,33-35] Using this model, we investigated further the functional role of CLCA1 in the differentiation of Caco-2 cells. First, we detected the expression of CLCA1 and the differentiation marker E-cadherin in a confluent culture. We found that the expression of both CLCA1 and E-cadherin was increased in a time-dependent manner (Fig. 5A). These data suggest that the expression of CLCA1 may contribute to the spontaneous differentiation of Caco-2 cells. Next, we used stealth siRNA (^{siRNA}CLCA1) to knock down the expression of CLCA1 in Caco-2 cells. After 72 hours of transfection, cells were tested for the expression of CLCA1, ALPI, and E-cadherin by western blotting. We found that knockdown of CLCA1 inhibited expression levels of ALPI and E-cadherin significantly (Fig. 5B).[10] These results indicate that CLCA1 expression plays a key role in the regulation of the spontaneous differentiation of Caco-2 cells.

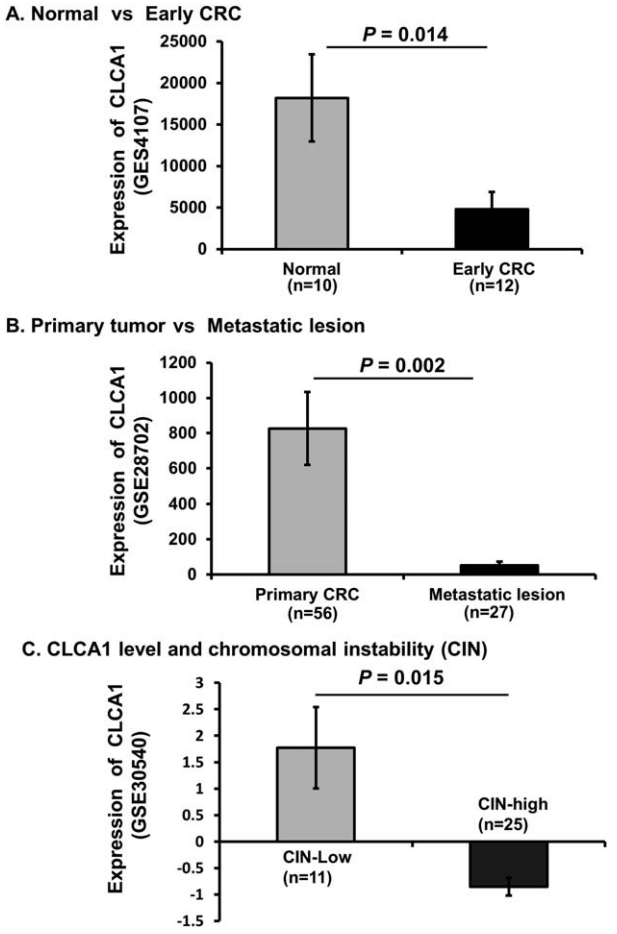

Figure 4. Publicly available microarray data sets for validation. (A) In public microarray data set GSE4107, we analyzed the expression of CLCA1 in normal colon mucosa and CRC tissues. The results showed that the expression of CLCA1 was inhibited significantly in early CRC patients. (B) In the GSE28702 microarray gene set, low expression of CLCA1 was associated with CRC metastasis. (C) The analysis of the GSE30540 microarray gene set showed that low CLCA1 expression was associated with CRC with a CIN-high signature, and this indicated significantly poorer survival in comparison with a CIN-low signature. CIN indicates chromosomal instability; CLCA1, chloride channel accessory 1; CRC, colorectal cancer.

DISCUSSION

In 2010, 40,695 people in the United Kingdom were diagnosed with bowel cancer, and 15,708 died from the disease. In CRC, one of the important challenges is the accurate prediction of which patients will experience disease relapse after surgery and which patients will require and most benefit from adjuvant therapies. At present, the TNM staging system for tumors is the gold standard for determining the prognosis of patients with CRC. The staging system is dependent on the extent of local invasion, the degree of lymph node involvement, and the presence or absence of distant metastases. However, this system can cause problems, especially with respect to treatment decisions.[36] For example, the number of paraffin blocks containing tumor tissue (tumor blocks) will affect directly the likelihood of

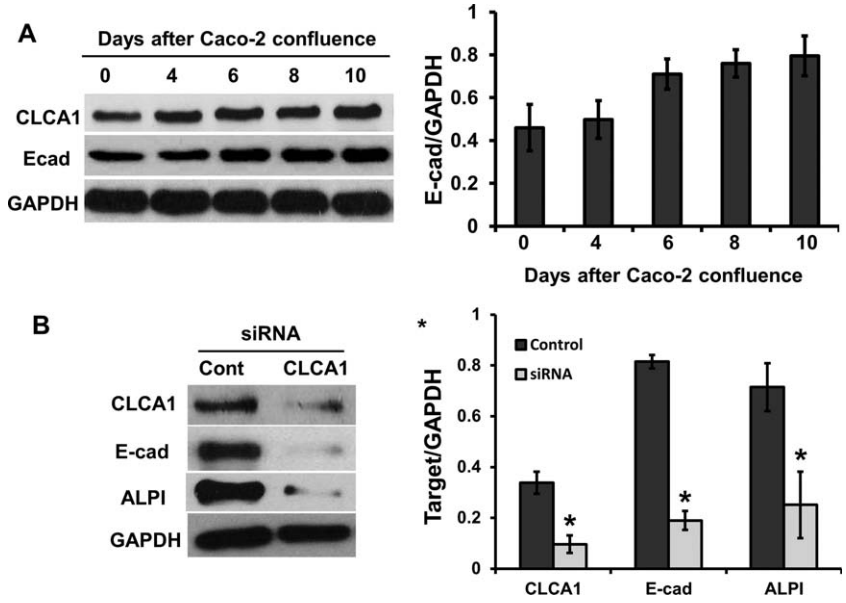

Figure 5. CLCA1 contributes to the differentiation of colorectal cancer cells. Human colon adenocarcinoma cells (Caco-2) were cultured to become confluent for 10 days. (A) The expression of CLCA1 was upregulated for 4 days in a confluent culture and lasted for up to 10 days of culturing. The mature epithelial marker E-cad increased in a time-dependent manner. The histogram shows the relative intensity of E-cad expressed as a ratio with respect to the GAPDH control. (B) Caco-2 cells were transfected transiently with 150 nM ^{siRNA}CLCA1 and blotted for CLCA1, E-cad, and ALPI. ^{siRNA}CLCA1 effectively inhibited CLCA1 and downregulated the expression of E-cad and ALPI. The histogram shows the relative intensity of CLCA1, E-cad, and ALPI expressed as a ratio with respect to the GAPDH control. Data are presented as means and standard errors of the mean. All results were analyzed on the basis of 3 independent experiments. ALPI indicates intestinal alkaline phosphatase; CLCA1, chloride channel accessory 1; Cont, control; E-cad, E-cadherin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; siRNA, small interfering RNA.

detecting submucosal, mesocolic, mesorectal, or peritoneal invasion, and the greater the number of tumor blocks dissected, the higher the expected T stage.[36] Hence, additional prognostic biomarkers are needed urgently for the improved management of CRC patients.[37]

The high tissue specificity of transcription of some CLCAs[38] suggested initially that the detection of their expression in specific tissues might be useful for early diagnosis, as a means of molecular staging, and for postoperative surveillance.[11] In this study, primary CRC tumors showed significantly lower expression of CLCA1 in both membranes and cytoplasm in comparison with the normal colonic tissues. This may suggest that reduced CLCA1 expression is a biomarker of malignant cells. Patients in different stages of disease also show a big discrepancy in survival. Molecules involved in cancer relapse and prognosis might serve as markers for the early detection of metastasis and as a measure for therapeutic intervention (eg, N-Myc downstream-regulated gene 2, E-cadherin, and p53).[15,16,18,39] In CRC, CLCA1 and CLCA4 are downregulated significantly in approximately 80% of patients.[11] The loss of expression of both CLCA1 and CLCA4 during tumorigenesis suggests that strong activation of either might inhibit the survival of tumor cells.[4] Our findings showed that CLCA1 expression was downregulated significantly in CRC and highly associated with known high-risk factors such as the tumor status, lymph node metastasis, Dukes grade, and histologic staging. Our data suggested that CLCA1 expression also decreased progressively from good to poor differentiation and with the progression of tumor stages. Specifically, the expression of CLCA1 in advanced CRC was decreased further in comparison with early stages of CRC. This indicated that the level of CLCA1 expression in primary CRC might be associated with its prognosis.

Several molecular prognostic factors, such as p53, Ki-67, K-ras, and E-cadherin, are being evaluated in CRC patients,[15,16,30,31] and it is still not possible to predict accurately the probability of recurrence of CRC in patients after surgery.[32] Therefore, it is necessary to find reliable and sensitive markers that can help us to make well-informed decisions regarding which patients should receive chemotherapy and which should not. Here we have shown that the level of CLCA1 expression is correlated with the expression of known predictors: E-cadherin and p53. CLCA2 has been reported to be a p53-inducible inhibitor of cell proliferation and to be downregulated with tumor progression.[1,6] Downregulation of E-

cadherin at the membrane indicates a poorer prognosis.[15] These results further support the notion that CLCA1 may be a tumor suppressor and may be associated with progressive potential.

Kaplan-Meier survival analysis is defined as the probability of surviving for a given length of time, and it is used to compare the fraction of patients living for a certain amount of time after treatment.[40] To further validate our hypothesis, we used Kaplan-Meier analysis to test the prognostic sensitivity of CLCA1 in CRC. Although we analyzed a small group of 36 patients, we found that CRC patients with high CLCA1 expression levels had better DFS than CRC patients with low CLCA1 expression levels. The tumor stage as a univariate prognostic factor in CRC also correlated with the survival time of this group of patients. Higher CLCA1 expression proved to be correlated with longer survival for patients with CRC. Extending DFS means the prevention or delay of recurrence or metastasis, and this is a clinical benefit. To validate the prognostic potential of CLCA1, we analyzed the expression of CLCA1 in normal and early CRC, primary CRC and metastases, and CIN-high and CIN-low phenotypes from 3 independent microarray data sets. Our results further confirmed that low expression of CLCA1 correlated with the tumorigenesis of CRC, metastasis, and a CIN-high gene signature. In this respect, our findings indicate that measurements of CLCA1 expression may help to identify patients at high risk of a poorer prognosis. Therefore, an assessment of CLCA1 levels could contribute to an accurate prediction of the prognosis and recurrence probability of patients after potentially curative surgery and, consequently, to individualized treatment for each patient.

Recent studies have shown that CLCA1 could increase cell differentiation through the regulation of the proliferation-to-differentiation transition.[10] CLCA2 also has been reported to suppress the epithelial-mesenchymal transition in breast cancer.[1] The CLCA1 precursor is approximately 900 amino acids long with 1 proteolytic cleavage site after the amino-terminal signal sequence. Eventually, 2 products of 90 and 30 to 40 kDa play functional roles.[3,4,8,9] Using cultured Caco-2 monolayers as a model, we found that CLCA1 promotes intestinal epithelial differentiation through enhancement of E-cadherin and ALPI expression (Fig. 5).[10] In some tumor types, including CRC, E-cadherin expression is often downregulated in highly invasive, poorly differentiated carcinomas.[41,42] Decreased E-cadherin expression through the inhibition of CLCA1 might block cell differentiation and increase the likelihood of distant metastasis. Thus, the loss of expression of CLCA1 appears to be an important step in tumorigenic progression. Although the clinical role and therapeutic effect of CLCA1 are still to be investigated, the current study will continue to improve our understanding of the biological profile and behavior of CRC.

In summary, we have shown that aberrant CLCA1 expression is a sign of a poor outcome in CRC. Our data indicate that low levels of CLCA1 in primary CRC might be a powerful predictor of disease relapse and prognosis. Although further prospective studies will be needed to determine the actual clinical utility of this observation, our findings indicate that CLCA1 might be a sensitive prognostic marker for evaluating the recurrence, early metastasis, and prognosis of CRC. Moreover, it might also be a potential therapeutic target for molecular therapy.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosure.

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