Published in final edited form as:

Immunogastroenterology. 2012 ; 1(1): 27–33. doi:10.7178/ig.1.1.7.

Reduced Expression of RNA Binding Protein CELF2, a Putative Tumor Suppressor Gene in Colon Cancer

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Abstract

Background & Aims—Colon cancer is the third leading cause of cancer death in both men and women in the United States. Every year, 160000 cases of colorectal cancer are diagnosed, and 57000 patients die. CUGBP, Elav-like family member 2 (CELF2) is an RNA binding protein that modulates various posttranscriptional events including RNA splicing, shuttling, editing, stability and translation. Previous studies have demonstrated that CELF2 expression is low in colon cancer cells. Furthermore, ectopic overexpression of CELF2 induces cells to undergo death by mitotic catastrophe. Based on the above observations, we hypothesized that CELF2 expression might be reduced during neoplastic transformation of colon cells.

Methods—Forty human colon cancer tissues along with 10 uninvolved normal colon tissues from cancer patients were utilized for immunohistochemical analysis of CELF2 expression.

Results—We have observed that CELF2 levels are reduced in colon tumor tissues when compared to the normal intestinal tissues. The data set suggests that RNA binding protein CELF2 could be a potential tumor suppressor protein. CELF2 was predominantly nuclear in normal cells, while the cancer tissues had diffused cytoplasmic staining.

Conclusion—CELF2 expression is consistently reduced during neoplastic transformation suggesting that it might play a crucial role in tumor initiation and progression.

Keywords

intestine; tumors; cell death

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Contributors

Satish Ramalingam: design and conducting the experiments; analysis and interpretation of the data; drafting of the article; final approval of the article. Prabhu Ramamoorthy: Conducting the experiments; analysis and interpretation of the data. Dharmalingam Subramaniam: analysis and interpretation of data. Shrikant Anant: conception and design; analysis and interpretation of the data; critical revision of the article for important intellectual content; final approval of the article.

Conflicts of interest

The authors declared no conflicts of interest.

Introduction

Cancer is the second most common cause of death in the United States. Even more disappointing is the fact that in 2012, about 1638910 new cancer cases are expected to be diagnosed of which approximately 577190 Americans would die.¹ This equates to a greater than 1500 deaths per day. Cancers are believed to occur through a combination of proto-oncogene activation and inhibition of tumor suppressor genes. With respect to tumor suppressor genes, they can function in many ways. They might inhibit the expression of proteins that drive the cells through cell cycle or they could promote apoptosis. When the expression of tumor suppressor genes is lost, the cells could divide in an uncontrollable manner thereby leading to cancer. Loss of tumor suppressor genes can occur through either mutation in the gene or epigenetic silencing thereby affecting activity or complete loss of expression due to deletion of the gene.

RNA binding protein CELF2 (CUGBP, Elav-like family member 2) was initially identified in neuroblastoma cells that were induced to undergo apoptosis by colchicine.² The protein encodes various functions at the posttranscriptional level including alternative splicing (e.g. Tau, troponin T), RNA editing (apolipoprotein B) and mRNA translation (cyclooxygenase-2, Mcl1).^{3–7} CELF2 contains 2 RNA recognition motifs (RRMs) near the N-terminus, a divergent domain loaded with alanine and glutamine residues, and a third RRM near the C-terminus.⁸ Based on deletion analyses, the divergent domain is believed to encode activation modules necessary for splicing activity.⁹

CELF2 is located in chromosome 10p13–p14 (Fig. 1) between Généthon markers D10S547 and D10S223.² Importantly, in gliomas chromosome 10 show monosomy in up to 60% and partial loss in up to 80% of high-grade gliomas.¹⁰ Also, cytogenetic studies have shown loss of an entire copy of chromosome 10 in majority of high-grade gliomas.^{11,12} However, very little work has been done on colon cancer and chromosome 10. Tomlinson and colleagues performed a genome-wide association study and identified colorectal susceptibility loci on chromosomes 10p14 and 8q23.3.¹³ Together, these data suggest the region encoding CELF2 has the potential for tumor suppression.

In previous studies, we have demonstrated that overexpression of CELF2 in colon cancer cells results in reduced colony formation.⁵ Moreover, cell cycle analyses revealed that ectopic overexpression of CELF2 in cancer cells results in cells undergoing mitotic catastrophe wherein cells undergo apoptosis when they are transiting through the G2M phase of the cell cycle.⁵ These data suggest that CELF2 expression may be deleterious to the cancer cells. This prompted us to look at the expression profile of the protein in colon cancers.

Materials and Methods

Cell lines

HCT116 and SW480 cells (obtained from ATCC, Manassas, VA) were grown in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% antibiotic solution (Mediatech Inc, Manassas, VA). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Normal colon epithelial cells (FHC, CRL-1831) were grown in Ham's F12 medium 45%, Dulbecco's Modified Eagle Medium 45%, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferin, 100 ng/mL hydrocortisone, 10% fetal bovine serum and 1% antibiotic solution at 37°C in a humidified atmosphere of 5% CO₂.

Western blot Analysis

Lysates prepared from the cells were subjected to polyacrylamide gel electrophoresis and blotted onto Immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA). Antibodies were purchased from Cell Signaling Technology (Beverly, MA), Abcam Inc. (Cambridge, MA), GenScript USA Inc. (Piscataway, NJ), and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and specific proteins were detected by the enhanced chemiluminescence system (GE Health Care, Piscataway, NJ).

Immunohistochemistry

Tissue microarray slide having formalin fixed and paraffin-embedded normal colon and colon cancer tissue dissected from the patients was obtained from IMGENEX (San Diego CA). The slides were baked at 60°C for 60 min; deparaffinized; and rehydrated through graded alcohol rinses. Heat-induced antigen retrieval was performed by immersing slides in 10 mM citrate buffer (pH 6.0) and microwaving in a 750-watt microwave oven for 10 min. The slides were then cooled at room temperature for 20 min and rinsed abundantly in tap water. Nonspecific staining of slides was blocked with blocking reagent in the Histostain kit (Invitrogen, Grand Island, NY) for 30 min, and endogenous peroxidase activity was quenched using Peroxabolish from BioCare Medical (Concord, CA) for 30 min. Antigen was detected by overnight incubation at 4°C with a primary antibody at 1:100 dilution followed by a secondary antibody conjugated to a peroxidase complex (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody, Dako Cytomation, Glostrup, Denmark). Finally color development was done with 3,3'-diaminobenzidine (Pierce) as a chromogen to detect bound antibody complex. When relevant, slides were counterstained with hematoxylin. Standardization of the incubation and development times allowed an accurate comparison of expression levels in all cases. CELF2 primary antibody is a rabbit polyclonal antibody obtained from Sigma Genosys.

Immunofluorescence

FHC cells were plated on coverslips and transfected with siPORT™ NeoFX™ Transfection Agent (Applied Biosystems, Carlsbad, CA) and a siRNA to CELF2 (5': GCAAACCUUACUGAUCCUA-3') or a scrambled siRNA not matching any of the human genes for 72 h. The cells were fixed with 10% buffered formalin and then permeabilized with PBS containing 0.5% Triton X-100. For immunofluorescence, the cells were incubated with rabbit anti- CELF2 antibody (Sigma-Aldrich, St Louis, MO) followed by FITC-conjugated anti-rabbit IgG. The slides were mounted and examined in a Nikon Eclipse Ti microscope (Nikon Inc., Melville, NY).

Results

CELF2 expression in colonic normal and cancer epithelial cells

In order to determine the expression of CELF2 in tumor tissues, we needed an antibody that was good and produced reproducible results. Therefore, we tested antibodies available from various commercial sources. We identified that the antibody from Sigma Genosys to be reproducible. We also determined the expression of CELF2 in normal and cancer cell lines. For normal cell line, we used FHC, an immortalized cell line generated from fetal human colon.¹⁴ For cancer cells, we used two different cell lines, HCT116 and SW480. Western blot analysis reveals that CELF2 expression is decreased in cancer cells when compared to the normal colon cells (Fig. 2A). Furthermore, immunocytochemistry studies in HCT116 cells revealed that CELF2 is predominantly a nuclear protein (Fig. 2B). To confirm that the antibody has specificity, we also performed an immunofluorescence assay in FHC cells following transfecting the cells with siRNA for CELF2. In previous studies, we have

demonstrated specific inhibition of the protein with this siRNA.¹⁵ The data demonstrate that when CELF2 is downregulated the antibody does not have significant reactivity to the cells, suggesting specificity for the antibody (Fig. 3).

CELF2 protein expression in non-malignant and malignant colon epithelia

We have obtained a tissue microarray that contains 40 colon cancer samples and includes uninvolved normal colon tissue from 10 of these patients. There were a total of 28 male and 12 female patients in the array. The age of the patients ranged from 30–72 years, while the average age of the study group is 55.53 years. Furthermore, the study group had a wide range of different colon cancer stages. There were 2 stage I colon cancer patients, 10 stage IIa patients, 1 stage IIb, 8 stage IIb, 7 stage IIc, 8 stage IVa and 4 stage IVb patients (Table 1).

We evaluated changes in CELF2 expression during the transformation of normal colon epithelium to malignant epithelium using the immunohistochemical analysis. The samples were considered positive if there are more than 10% of the specimen was stained and were classified as exhibiting high (+++), moderate (++), low (+) immunoreactivity (staining intensity) for CELF2 (Table 2). Overall, there was high expression in the normal tissues compared to the cancer tissues (Fig. 4). Furthermore, there was high level of nuclear expression in normal tissues. However, in contrast in the cancer tissues the expression was found to be more diffuse and cytoplasmic (Fig. 4). These data suggest that not only is the expression reduced in the cancer tissues, but there is also a difference in localization. While the adjacent normal tissues demonstrated moderate to high levels of CELF2 expression, the cancer tissues demonstrated were predominantly low levels of expression (Table 3). Only three moderately differentiated and one poorly differentiated adenocarcinoma had expression levels that were in the moderate to high range (Table 2). Thus, CELF2 expression was significantly reduced in 36 (90%) of 40 colon cancer cases.

Discussion

The role of CELF2 in normal cells has been studied in various cell types including epithelial cells, neuronal cells and cardiac and skeletal muscle cells. CELF2 encodes various functions including regulating alternative splicing, RNA editing, RNA stability and RNA translation. In the heart, CELF2 variants have been identified to cosegregate with arrhythmogenic right ventricular disease.² Although not directly demonstrated, partial monosomy of the region of 10p where CELF2 is also located has been implicated in DiGeorge syndrome, a disease that is characterized with heart defects.¹⁶ In the skeletal muscle, elevated levels of CELF2 correlates with Duchene and Becker muscular dystrophies.¹⁷ Polymorphisms in CELF2 have also been associated with late onset Alzheimer's disease.¹⁸ However, association of CELF2 with cancers has not yet been identified. This is the first study demonstrating the reduction in the expression of the protein in colon tumor samples.

Cytogenetic studies have clearly shown losses of an entire copy of chromosome 10 in the majority of high-grade gliomas and prostate cancers.^{12,19} Loss of 10p has also been shown in other cancers such as medulloblastomas and primitive neuroectodermal tumors.²⁰ Recently a 3-year old girl with juvenile polyposis of infancy showed a paracentric inversion in 10q and a deletion in 10p.²¹ Similarly, loss of 10p has been found in pancreatic ductal adenocarcinoma.²² Undifferentiated endometrial sarcomas have also been associated with loss of 10p.²³ Moreover, there are various tumor suppressors such as KLF6, Hssh3bp1 are located in the short arm of chromosome 10, some of which are a very close to the region encoding CELF2.^{19,24} This led us to hypothesize that additional tumor suppressor genes may exist at chromosome 10p region and CELF2 could potentially be one such tumor suppressor protein. Earlier studies demonstrating that CELF2 inhibited cyclooxygenase-2

(COX-2) translation and overexpression of CELF2 induces cell death further point to the fact that the protein can act as a tumor suppressor protein.⁵ COX-2 is upregulated in colorectal adenomas and cancer, thereby suggesting that CELF2, by inhibiting COX-2, might prevent cancer development. The results presented in this manuscript demonstrating a reduction in CELF2 expression in colon cancer cell lines and colon tumor tissues further suggest that CELF2 could be a potential tumor suppressor protein, at least in the colon. The 36 colon cancer tissues showed a decrease in CELF2, while in the normal colon epithelium 7 out of 9 showed strong CELF2 protein expression. Further studies will provide additional information on whether CELF2 expression was reduced because of loss of the gene or due to decreased transcription of the gene or protein stability. Moreover, examination of CELF2 along with other crucial proteins regulated by CELF2 would provide interesting insights into colon cancer development and progression. In addition, CELF2 could be used as a target for therapeutic targeting of cancers. In this regard, curcumin, a dietary prevention agent was shown to induce CELF2 expression in pancreatic cancer cells thereby resulting in cells undergoing mitotic catastrophe.²⁵ Overall, these studies suggest that RNA binding protein CELF2 could be a potential tumor suppressor protein that is directly involved in the pathogenesis of colon tumors.

Acknowledgments

This study was supported by a grant from National Institute of Health.

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Summary Box

What is already known

- Colon cancer is the second leading cause of cancer related death.
- Chromosome 10 is deleted in various cancers.
- CUGBP, Elav-like family member 2 (CELF2) overexpression inhibits cell proliferation, and induces cell death.

What the new findings are

- CELF2 expression is decreased in colon cancer epithelial cells when compared to normal colon epithelial cells.
- In a collection of 40 colon tumor tissues, the authors confirm that CELF2 expression is decreased in 36 patients.
- CELF2 could be a potential tumor suppressor gene and elucidation of its molecular genetics during colorectal carcinogenesis is potentially of great clinical importance.

Figure 1.
CELF2 is located in chromosome 10. CELF2, CUGBP, Elav-like family member 2.

Figure 2.

CELF2 expression is decreased in colon cancer cells. A: Colon cancer cells have low CELF2 protein. Western blot analysis of lysates from normal colon and colon cancer cells demonstrated that CELF2 a 54 kDa protein expression is decreased in HCT116 and SW480 colon cancer cells when compared to FHC a normal colon epithelial cell; B: CELF2 is a predominantly nuclear protein. HCT116 colon cancer cells were allowed to grow on cover slips and immunostaining of CELF2 indicates a clear nuclear staining of CELF2 and a slight cytoplasmic signals. Nucleus is stained using DAPI. CELF2, CUGBP, Elav-like family member 2.

Figure 3.

Antibody to CELF2 is specific. FHC cells were transfected with CELF2-specific (siCELf2) or a scrambled siRNA (siScr) and the subjected to immunofluorescence for CELF2 protein expression. Data shows that CELF2 levels are significantly reduced in the cells following transfection with siCELf2. CELF2, CUGBP, Elav-like family member 2.

Figure 4. CELF2 expression is decreased in human colon tumor tissues. Immunohistochemical analysis of colon tumor samples and normal colon tissue indicates a decrease in CELF2 levels in cancer. CELF2 expression is significantly higher in the normal colon. Two each of normal and colon cancer tissues are presented here as representative images for hematoxylin-eosin and CELF2 staining. Image magnification for each column is shown above. CELF2, CUGBP, Elav-like family member 2.

Table 1

Clinical characteristics of colon cancer patients examined

Clinical characteristics	n
No. of patients	40
Sex (male/female)	(28/12)
Median age (range) (year)	(30–72) (55.53 [*])
Histologic malignancy grade	
Stage I	2
Stage IIa	10
Stage IIb	1
Stage IIIb	8
Stage IIIc	7
Stage IVa	8
Stage IVb	4

^{*} median age of 39 patients (as age of one patient is unknown); Tumor, node, metastasis (TNM) and stage: American Joint Committee on Cancer (AJCC) Cancer Staging Manual (6th Edition).

Table 2

Immunohistochemical analysis of CELF2 in primary colon carcinomas

Patient	Histological type	pTNM	Immunohistochemistry ^a
1	Mucinus adenocarcinoma	T3N2M0	+
2	Adenocarcinoma, moderately differentiated	T3N1M0	+
3	Adenocarcinoma, well differentiated	T3N1M0	+
4	Adenocarcinoma, moderately differentiated	T3N0M0	++
5	Adenocarcinoma, moderately differentiated	T3N0M0	+
6	Mucinus adenocarcinoma	T3N1M0	+
7	Adenocarcinoma, well differentiated	T3N1M0	+
8	Adenocarcinoma, well differentiated	T3N1M0	+
9	Adenocarcinoma, well differentiated	T3N1M0	+
10	Adenocarcinoma, moderately differentiated	T3N0M0	+
11	Adenocarcinoma	T3N0M1	+
12	Adenocarcinoma, moderately differentiated	T2N2M0	+
13	Adenocarcinoma, well differentiated	T3N0M0	+
14	Adenocarcinoma, well differentiated	T3N1M0	+
15	Mucinus adenocarcinoma	T3N1M0	+
16	Adenocarcinoma, well differentiated	T3N0M0	+
17	Adenocarcinoma, moderately differentiated	T3N1M0	+++
18	Adenocarcinoma, moderately differentiated	T4N0M0	+
19	Adenocarcinoma, moderately differentiated	T3N0M1	+
20	Adenocarcinoma, moderately differentiated	T3N0M0	+
21	Adenocarcinoma, moderately differentiated	T3N0M0	+
22	Adenocarcinoma, moderately differentiated	T3N2M0	+
23	Adenocarcinoma, well differentiated	T3N1M1	+
24	Mucinus carcinoma	T3N0M0	+
25	Adenocarcinoma, poorly differentiated	T4N0M0	+++
26	Adenocarcinoma, moderately differentiated	T3N0M0	+
27	Adenocarcinoma, moderately differentiated	T3N0M0	+
28	Adenocarcinoma, moderately differentiated	T3N2M1	++
29	Adenocarcinoma, well differentiated	T3N1M0	+
30	Adenocarcinoma, well differentiated	T3N0M0	+
31	Adenocarcinoma, moderately differentiated	T4N1M0	+
32	Adenocarcinoma, poorly differentiated	T3N2M0	+
33	Adenocarcinoma, moderately differentiated	T3N2M0	+
34	Adenocarcinoma, moderately differentiated	T4N1M1	+
35	Adenocarcinoma, moderately differentiated	T3N2M0	+
36	Adenocarcinoma, poorly differentiated	T3N2M0	+
37	Adenocarcinoma, moderately differentiated	T3N2M1	+
38	Adenocarcinoma, moderately differentiated	T4N2M0	+
39	Adenocarcinoma, moderately differentiated	T4N2M0	+

Patient	Histological type	pTNM	Immunohistochemistry ^a
40	Adenocarcinoma, moderately differentiated	T4N1M1	+
41	Normal colon (adjacent to cancer, match of #33)		+++
42	Normal colon (adjacent to cancer, match of #34)		+++
43	Normal colon (adjacent to cancer, match of #9)		+++
44	Normal colon (adjacent to cancer, match of #1)		++
45	Normal colon (adjacent to cancer, match of #2)		+++
46	Normal colon (adjacent to cancer, match of #3)		+++
47	Normal colon (adjacent to cancer, match of #4)		+++
48	Normal colon (adjacent to cancer, match of #5)		++
49	Normal colon (adjacent to cancer, match of #24)		+++

^a Samples were considered positive if more than 10% of the specimen was stained and were classified as exhibiting high (+++), moderate (++), low (+), immunoreactivity (intensity) for CELF2 tumor, node, metastasis (TNM) and stage: American Joint Committee on Cancer (AJCC) Cancer Staging Manual (6th Edition). CELF2, CUGBP, Elav-like family member 2.

Table 3

CELF2 stain scoring

Histology type	Immunohistochemistry immunoreactivity for CELF2		
	Low expression (+)	Moderate expression (++)	High expression (+++)
Normal	-	2	7
Cancer	36	2	2

CELF2, CUGBP, Elav-like family member 2.