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Increased Expression of Calcineurin in Human Colorectal Adenocarcinomas

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Abstract Colorectal cancer (CRC) is the third most common cause of cancer death in the Western world. Calcineurin (CaN), a Ca^{2+}/calmodulin (CaM)-dependent protein phosphatase, is important for Ca^{2+}-mediated signal transduction. The main objective of this study is to examine the potential role of Ca^{2+}/CaM-dependent protein phosphatase in both normal and in invasive tumor components of human samples. In this study, we carried out 45 cases of CaN activity, 13 cases of CaN protein expression by Western blot analysis, and 6 cases for immunohistochemical analysis in both normal and invasive tumor components of human samples. Immunohistochemical analysis revealed that strong cytoplasmic staining of varying intensity was observed in colon tumors of all patients compared to normal mucosa. In addition, Western blot analysis revealed a prominent overexpressed immunoreactive band with an apparent molecular mass of 60 kDa catalytic alpha subunit (CaN A) as well as CaN Aα and β in colon tumor samples. Elevated CaN protein expression appears to be a possible link between Ca^{2+} signaling and oncogenic processes. J. Cell. Biochem. 95: 731–739, 2005.

Key words: signal transduction; calcineurin; Ca^{2+}/calmodulin; colorectal cancer; biomarker

Ca^{2+} acts as a second messenger in a number of different signaling pathways and plays a critical role in a myriad of different physiological and pathological processes, including oncogenesis [Richter, 1993; Missiaen et al., 2000]. Increase in intracellular [Ca^{2+}] by stimulating the entry of extracellular Ca^{2+} or by release of Ca^{2+} ions from intracellular stores leads to the binding of Ca^{2+} to calmodulin (CaM), which then converts CaM to an active conformation. This active CaM then associates with target protein(s) in a Ca^{2+}-dependent reversible manner causing a change in target protein activity [Cheung, 1980; Klee, 1988]. CaM-dependent enzymes participate in various physiological processes either directly or indirectly through the regulation of the activity of other cellular proteins by phosphorylation or dephosphorylation [Kakkar et al., 1999]. These enzymes are involved in cell proliferation and differentiation by participating in Ca^{2+}-dependent induction of gene transcription [Engels et al., 2000].

Calcineurin (CaN) is a serine/threonine protein phosphatase under the control of Ca^{2+}/CaM [Klee and Krinks, 1978]. CaN is a heterodimer composed of a catalytic alpha subunit (CaN A) and a Ca^{2+} binding regulatory beta subunit (CaN B). CaN A displays a multidomain structure with a catalytic domain at its N-terminus and a regulatory region at the carboxy-terminus. The regulatory region can be further subdivided into three distinct domains: a CaN B-binding domain, a CaM-binding domain, and an autoinhibitory region [Sharma et al., 1979; Klee et al., 1998]. Deletion of the autoinhibitory and CaM-binding domains results in a constitutively active form of the enzyme [Tokoyoda et al., 2000]. The immunosuppressive drugs FK506 and cyclosporine

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(CsA), when complexed to specific immunophilins (FKBP12 and cyclophilin A, specifically), bind CaN at multiple sites. Binding to these sites, which include the N-terminus of the CaN B-binding helix, the CaN B subunit, and the catalytic domain of CaN A [Kissinger et al., 1995], results in inhibition of CaN activity. CsA and FK506 are widely used for the therapy of a variety of diseases [Lee and Wurtman, 2000]. Enhancement of taxol-induced apoptosis in human urinary bladder cancer cells by FK506 and CsA is partly due to the inhibition of CaN [Nomura et al., 2002]. Data on the involvement of CaN in different types of cancer progression and metastases are limited. Higher CaN activity (two to five fold) and increased levels of anti-apoptotic proteins Bcl2 and Bcl-XL were reported in mitochondrial DNA-depleted human lung carcinoma A549 cells [Amuthan et al., 2002]. The activity of CaN was decreased by 75% and 85% in sera of patients diagnosed either for acute lymphoid leukemia or acute myeloid leukemia, respectively [Padma and Subramanyam, 2002]. The role that CaN plays in various tumor invasions is yet to be investigated in detail.

In the present study, we investigated the CaN expression in human colon carcinoma progression using immunohistochemical approaches using polyclonal CaN antibody. We demonstrated that deeply invading human colon carcinoma cells showed higher levels of CaN activity and protein expression than normal mucosa.

MATERIALS AND METHODS

Materials

Nitrocellulose membranes were purchased from Bio-Rad Laboratories, Canada. The production and purification of the polyclonal CaN antibody has been described previously [Seitz et al., 2002]. Anti-CaN A (α and β) isoforms was purchased from Santa Cruz Biotechnology, Inc., USA. Anti-CaN and anti-β-actin were purchased from Sigma, Canada. Protein markers were purchased from Invitrogen, Canada. All other reagents were of analytical grade and were from BDH or Sigma, Canada.

Human Colorectal Specimens

With institutional approval and patient consent, the surgical pathology specimens from 45 patients who had undergone resection for colorectal adenocarcinoma were collected directly from the surgical operating room in a fresh state (before being immersed in tissue fixative). Following gross inspection, samples of tumors were dissected and immediately frozen at −80°C. Normal-appearing colonic mucosa far removed from the cancer was similarly dissected and frozen. Most of the remaining tissue was fixed in neutral buffered formaldehyde and processed for histological and immunohistochemical evaluation.

Preparation of Tissue Extracts

All procedures were carried out at 4°C, unless otherwise stated. Tissues were homogenized in 100 mM Tris-HCl, pH 7.4, containing 1 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mg/ml leupeptin. The crude homogenate was centrifuged for 30 min at 10,000 *g* and the supernatant was filtered through glass wool.

CaN Assay

CaN activity was assayed using *p*-nitrophenylphosphate (pNPP) as a substrate [Pallen and Wang, 1983; Lakshmikuttyamma et al., 2003, 2004a,b]. The reaction mixture contained 50 mM Tris-HCl, pH 7.0, 1 mM Ni^{2+}, 5 μg CaM, 3.4 mM pNPP, and CaN in a total volume of 1.0 ml. Ca^{2+}-independent activity of CaN was determined under identical conditions except 1 mM Ni^{2+} was substituted for 5 mM EGTA. The reaction was incubated at 30°C for 30 min. The reaction was initiated by the addition of pNPP and terminated by the addition of 75 mM K_2HPO_4. The pNPP hydrolysis was quantified by the increase of absorbance at 405 nm. One unit of phosphatase activity was defined as the amount of dephosphorylation resulting in an optical density of 0.1 at 30°C after 30 min incubation.

Immunohistochemical Method

Two tissue blocks were selected from each case, one from the tumor and the other from normal-appearing mucosa far removed from the cancer. Immunohistology was performed as described previously [Hsu et al., 1981; Seitz et al., 2002; Lakshmikuttyamma et al., 2003]. Human tissues fixed in 10% formaldehyde and dehydrated in ascending solutions of ethanol and xylene were embedded in paraffin. Five sections of 5-μm thickness were prepared from tissue blocks and placed on slides coated with

silane. The slides were kept at 55°C for 45 min in an oven to improve adherence of sections.

The sections were deparaffinized and rehydrated in xylene and descending concentrations of ethanol. Endogenous peroxidase was neutralized by 30-min incubation in 0.5% hydrogen peroxide in methanol. The antigens were unmasked by treating the sections with 2 mg pepsin per milliliter 0.01 N HCl for 45 min. This incubation time was determined with a series of trials and was found to yield maximum staining.

The sections were then blocked with 1% bovine serum albumin in PBS for 30 min, followed by incubation with anti-CaN polyclonal antibody at a concentration of 0.58 mg/ml for 90 min and with HRP-conjugated secondary antibodies (1:100–1:400; Dako Corp., Carpinteria, CA) for 45 min. The reactions were color developed with a peroxidase substrate kit (SK4600; Vector Laboratories, Burlingame, CA). Control experiments included omission of either the primary antibody or both primary and secondary antibodies to determine, respectively, non-specific binding of the secondary antibody and inhibition of endogenous tissue peroxidase. Some of the sections were stained with von Willebrand factor antibody (vWF; 1:200; Dako Corp.) to delineate blood vessels and to obtain another control. Some slides were counterstained for 2–4 min with methyl green. The slides were examined and images captured on an image analysis system (Northern Eclipse, Empix Imaging, Mississauga, Ontario). The extent of staining was graded on a 5-point scale from 0 to 4 as previously described [Moyana and Xiang, 1995; Raju et al., 1997], and the intensity of staining was noted as mild, moderate, or marked.

SDS–Polyacrylamide Gel Electrophoresis (PAGE) and Western Blot Analysis

Proteins isolated from human colon normal and cancer tissues were separated on 10% SDS-polyacrylamide gels according to the procedure described by Laemmli [1970]. For the protein expression of CaN in colon normal and cancer tissues, an equal amount (35 μg) of total cellular protein was resolved on a 10% SDS–PAGE. Gel transfer to nitrocellulose and membrane blocking were performed using standard procedures [Towbin et al., 1979]. The blot was incubated first with either CaN specific polyclonal (0.058 mg/ml) or CaN A (α and β) antibodies at a concentration of 0.001 mg/ml, washed, and probed with an anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) diluted 1:2,000. Membranes were then incubated in chemiluminescence reagent (NEN Life science products, USA) and exposed to Kodak X-OMAT Blue XB-1 film for detection. For quantitation, films were scanned and intensities determined using NIH Image software.

Other Method

Protein concentration was measured by the method of Bradford [1976] using bovine serum albumin as a standard.

Statistical Analysis

All the data are reported as the mean ± SEM. The differences between the mean values were tested for statistical significance by the two-tailed Student's *t*-test.

RESULTS

CaN Phosphatase Activity in CRC

To examine the potential role of CaN in the human CRC, we present 45 cases for CaN phosphatase activity and 13 cases for the evaluation of CaN protein expression in tumorigenesis. We analyzed CaN phosphatase activity in crude homogenate for both human normal colorectal mucosa and adenocarcinomas (Table I). Among 45 samples analyzed, we observed a higher CaN phosphatase activity (two to four fold) in 37 samples (82.2%) derived from colonic tumor as compared to the normal mucosa.

CaN Protein Expression by Western Blot Analysis

The specificity of the bovine CaN polyclonal antibody was carried out as described previously [Seitz et al., 2002; Lakshmikuttyamma et al., 2003, 2004a,b]. Bovine CaN polyclonal antibody is shown to cross-react with the human CaN (Fig. 1A). Furthermore, the detection of the CaN on the ELISA was prevented by pre-incubating the purified CaN with bovine brain CaN antibody (Fig. 1B).

In addition, we examined the levels of CaN protein expression in human normal colorectal mucosa and adenocarcinomas of 13 patients and 2 polyps. Equivalent amounts of protein from human normal colorectal mucosal and adenocarcinomas tissue were subjected to immunoblotting using anti-CaN (Fig. 2A). Western blot

TABLE I. CaN Phosphatase Activity in Human Colorectal Adenocarcinomas

	CaN Phosphatase activity, Unit/mg of protein			
	In presence of Ni^{2+}/CaM		In presence of EGTA	
Case no.	Normal	Tumor	Normal	Tumor
1	8.13 ± 0.12	36.00 ± 0.07	3.85 ± 0.24	8.56 ± 0.17
2	6.52 ± 0.20	16.52 ± 0.24	1.95 ± 0.09	3.78 ± 0.21
3	7.82 ± 0.18	14.55 ± 0.55	2.60 ± 0.04	3.95 ± 0.15
4	7.28 ± 0.22	9.56 ± 0.31	2.25 ± 0.31	1.98 ± 0.08
5	5.89 ± 0.15	6.87 ± 0.38	1.78 ± 0.13	1.90 ± 0.19
6	7.56 ± 0.23	14.82 ± 0.18	2.65 ± 0.25	3.63 ± 0.20
7	10.87 ± 0.17	19.87 ± 0.46	3.58 ± 0.38	4.59 ± 0.22
8	9.22 ± 0.09	21.54 ± 0.33	2.87 ± 0.49	5.19 ± 0.48
9	8.88 ± 0.19	23.44 ± 0.51	2.75 ± 0.15	6.15 ± 0.10
10	10.86 ± 0.12	26.34 ± 0.23	3.59 ± 0.19	6.36 ± 0.31
11	9.51 ± 0.13	19.56 ± 0.52	3.02 ± 0.59	4.25 ± 0.35
12	7.90 ± 0.09	29.32 ± 0.57	2.75 ± 0.12	7.33 ± 0.42
13	9.11 ± 0.18	27.55 ± 0.39	3.35 ± 0.22	6.12 ± 0.37
14	7.32 ± 0.21	18.53 ± 0.12	2.49 ± 0.46	4.75 ± 0.22
15	10.56 ± 0.25	22.35 ± 0.48	3.28 ± 0.31	5.93 ± 0.29
16	8.49 ± 0.13	19.19 ± 0.46	2.93 ± 0.51	4.82 ± 0.15
17	5.98 ± 0.18	20.35 ± 0.59	2.15 ± 0.49	5.25 ± 0.33
18	9.65 ± 0.10	18.50 ± 0.25	3.21 ± 0.24	4.21 ± 0.23
19	9.23 ± 0.26	22.58 ± 0.34	3.65 ± 0.12	5.67 ± 0.27
20	6.32 ± 0.15	19.88 ± 0.70	2.11 ± 0.22	5.55 ± 0.32
21	8.33 ± 0.22	25.41 ± 0.24	2.95 ± 0.06	6.29 ± 0.33
22	10.18 ± 0.18	21.90 ± 0.65	3.59 ± 0.15	5.54 ± 0.25
23	7.35 ± 0.19	17.10 ± 0.57	2.87 ± 0.15	4.88 ± 0.22
24	8.10 ± 0.16	19.35 ± 0.71	2.95 ± 0.14	3.80 ± 0.10
25	7.25 ± 0.23	21.39 ± 0.53	2.22 ± 0.13	4.82 ± 0.17
26	9.54 ± 0.13	20.15 ± 0.77	2.95 ± 0.2	6.72 ± 0.66
27	8.82 ± 0.24	21.35 ± 0.45	2.54 ± 0.07	5.01 ± 0.59
28	6.67 ± 0.21	17.65 ± 0.97	2.35 ± 0.45	3.83 ± 0.08
29	10.35 ± 0.15	23.45 ± 0.40	3.15 ± 0.25	9.35 ± 0.34
30	10.91 ± 0.52	19.33 ± 0.61	3.25 ± 0.20	6.15 ± 0.37
31	9.35 ± 0.41	17.29 ± 0.85	3.05 ± 0.14	4.99 ± 0.35
32	7.23 ± 0.24	19.37 ± 0.51	2.26 ± 0.15	5.93 ± 0.07
33	9.19 ± 0.58	21.12 ± 0.94	3.27 ± 0.13	5.85 ± 0.21
34	9.34 ± 0.69	23.18 ± 0.27	3.77 ± 0.06	6.43 ± 0.20
35	8.65 ± 0.76	18.18 ± 0.35	3.08 ± 0.21	5.34 ± 0.39
36	9.16 ± 0.40	20.56 ± 0.26	3.32 ± 0.17	5.43 ± 0.27
37	10.28 ± 0.73	18.05 ± 0.80	3.65 ± 0.10	4.43 ± 0.23
38	9.32 ± 0.14	17.39 ± 0.34	2.83 ± 0.10	4.32 ± 0.32
39	9.29 ± 0.79	22.36 ± 0.66	3.71 ± 0.20	7.98 ± 0.29
40	6.15 ± 0.58	19.39 ± 0.42	2.28 ± 0.34	5.25 ± 0.25
41	7.39 ± 0.91	19.13 ± 0.24	2.84 ± 0.2	5.69 ± 0.23
42	7.88 ± 0.50	18.95 ± 0.68	3.01 ± 0.12	5.90 ± 0.07
43	8.15 ± 0.47	19.83 ± 0.08	2.87 ± 0.09	6.98 ± 0.21
44	10.07 ± 0.67	21.34 ± 0.90	2.95 ± 0.31	6.08 ± 0.21
45	9.32 ± 0.57	19.13 ± 0.28	3.47 ± 0.32	5.25 ± 0.43

The CaN phosphatase activity was analyzed in presence of Ni^{2+}/CaM and EGTA as described under Materials and Methods. Each value is the mean of three experiments and each experiment was performed three times.

analysis showed a prominent immunoreactive band with an apparent molecular mass of approximately 60 kDa reacting with CaN antibody in 11 patients out of 13 patients (84.6%). The intensities of CaN A expression was determined using NIH Image software (NIH at http://rsb.info.nih.gov/nih-image/download.html). This demonstrates the overexpression of CaN A in colon tumor. In contrast, CaN A was only weakly expressed in samples from normal mucosa (Fig. 2A). Quantitative analysis of the 60 kDa band revealed a two to three fold higher expression of CaN in colorectal tumors compared to respective normal mucosa. Furthermore, Western blot analysis revealed an immunoreactive band of approximately 18 kDa, indicating that CaN B was detectable in both normal mucosa and colorectal adenocarcinomas. However, no significant change in protein expression was observed (Fig. 2A). It has been shown that CaM-regulated enzymes exist in multiple isoforms with unique features and characteristics [Rusnak and Mertz, 2000] and CaN A also has been demonstrated to exist in isoforms [Rusnak and Mertz, 2000]. Therefore, the expression of the two major isoforms of CaN

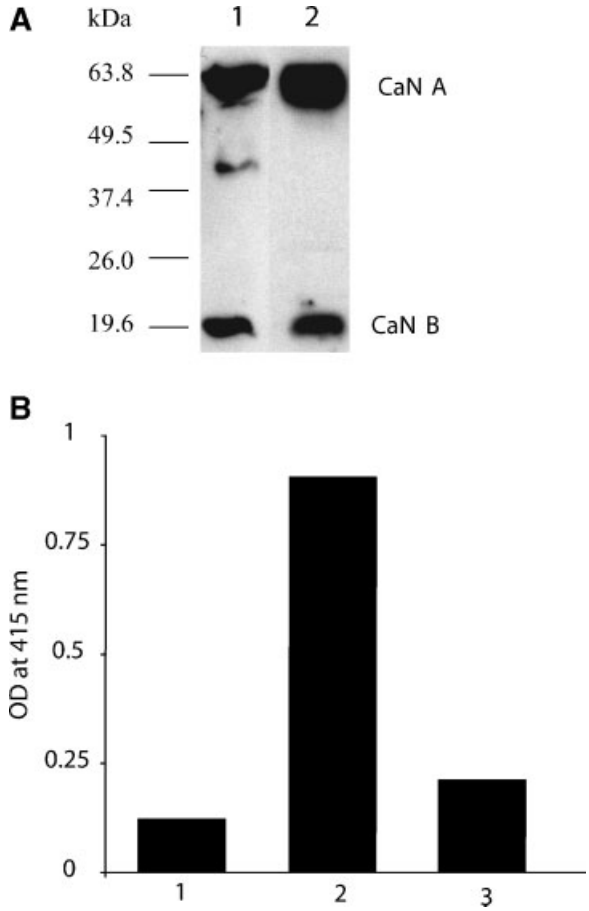

Fig. 1. **A**: Specificity of CaN. **1**: Purified bovine brain CaN and (**2**) human colorectal cancer (CRC). **B**: Enzyme immunoassay of purified bovine brain CaN. **1**: Anti-CaN alone, (**2**) anti-CaN and bovine brain CaN (800 ng), and (**3**) anti-CaN (23 ng) and bovine brain CaN (800 ng) pre-incubated overnight at 4°C.

A (α and β) were examined by probing CaN A isoforms polyclonal antibodies with normal and tumor colorectal cancer (CRC) samples (Fig. 2B,C). We observed that there was a higher expression of CaN Aα (Fig. 2B) and CaN Aβ (Fig. 2C) in colorectal tumors compared to respective normal mucosa.

Immunohistochemical Analysis

Furthermore, to support the analysis of Ca^{2+}/CaM dependent activity and protein expression in human colorectal adenocarcinoma, we carried out the immunohistochemical analysis of six cases using the polyclonal CaN antibody to detect Ca^{2+}/CaM-dependent phosphatase in colorectal adenocarcinoma patients (Table II). Mucosa dissected far from cancerous tissue taken from respective patients was considered as normal. Immunohistochemical studies of normal mucosa showed weak staining with CaN antibody (Fig. 3Ai). Strong immunoreactivity was observed for all specimens dissected from cancerous mucosa (Fig. 3Aiii). While the intensity of staining varied from patient to patient, a marked difference was observed when compared to normal mucosa (Fig. 3Ai). The staining appeared to be cytoplasmic rather than nuclear (Fig. 3B). In polyps, a moderate staining was observed and the degree of immunoreactivity was less than that found in tumor tissue (Fig. 3Aii).

DISCUSSION

Our immunohistochemistry results strongly suggest that CaN expression is related to colon carcinoma development. Very weak staining was detected in all of the tested normal colonic mucosas. Thus, the overexpression of CaN in tumor cells can be regarded as a characteristic feature of colon cancer. This is supported by the increased level of phosphatase activity of CaN and higher levels of protein expression in colorectal adenocarcinomas.

The major signaling pathway of CaN in the development of malignancy may be through NFAT. In T-cells, CaN-NFAT signaling represents a critical event for mediating cellular activation and the immune response [Sugiura et al., 2001]. Cell-mediated immune responses are an essential aspect of tumor-host interactions in CRC. The progression from pre-cancerous (adenomatous) colon polyps to malignant CRC depends on a complex pathway involving the activities of activated T lymphocytes [Song et al., 2000]. The important role of CaN in T-cell activation is underscored by the effects of immunosuppressive drugs, such as CSA and FK506, which inhibit CaN activity [Cho et al., 2003]. The activation of T-cell receptors recruit a large array of necessary intracellular signaling mediators such as mitogen-activated protein kinase family members, tyrosine kinases (Fyn-T, Lck, Syk, and Zap70) NF-kB, Jak/Stat, PKCθ, and various cytokines [Chu et al., 1998; Kuo and Leiden, 1999; Pages et al., 1999; Dong et al., 2000; Sun et al., 2000]. The elevated levels of IL-8 gene expression in human colonic epithelial cells occur through the activation of nuclear factor-kappa B (NF-kB) [Gewirtz et al., 2000] and are regulated by CaM-dependent CaN [Yu et al., 2001]. Elevated levels of circulating plasma cytokines IL-6 and IL-8 have been reported in cancer patients [Takahashi

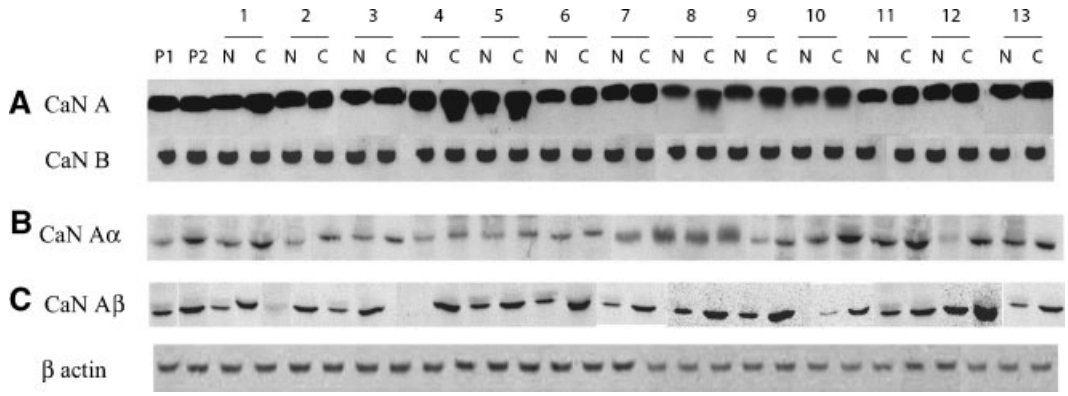

Fig. 2. Western blot analysis of human colorectal normal, polyps, and tumor tissue extracts probed with (**A**) anti-CaN, (**B**) anti-CaN Aα, and (**C**) anti-CaN Aβ. Equal amount (35 μg) of proteins was loaded onto each lane of an SDS–PAGE and immunoblotted with anti-CaN as described under Materials and Methods. β-Actin was used as an internal control for Western blot analysis. The data presented are representative of at least three separate experiments.

et al., 2003]. RelA/NF-kB, an important inhibitor of apoptosis increased significantly in the transition from adenoma with low dysplasia to adenocarcinoma in colorectal tumorigenesis [Yu et al., 2003]. It has been reported that CaN may regulate integrin-dependent cell adhesion and spread of Colo201 cells [Mohri et al., 1998]. Furthermore, CaN may also be involved in the regulation and establishment of new adhesive interactions in HT29 cells [Haier and Nicolson, 2000]. The available reports indicate that CaN is likely to play a significant role in the development of colorectal adenocarcinomas by signaling various molecules causing cell proliferation.

The involvement of CaN with various apoptotic factors and whether this Ca^{2+}/CaM-dependent phosphatase has any role in inhibiting apoptosis is unclear. Simizu et al. [2000] reported that Bcl-2 inhibits CaN-mediated Fas ligand expression in anti-tumor drug-treated baby hamster kidney cells [Simizu et al., 2000]. Recently Ca^{2+}-dependent interaction of CaN with Bcl-2 was reported in neuronal tissues [Erin et al., 2003]. CaN bound to Bcl-2 is an active phosphatase but is unable to promote the nuclear translocation of NFAT, a transcription-factor required for induction of interleukin-2 expression, suggesting a mechanism by which Bcl-2 suppresses NFAT activity. Direct interaction between Bcl-2 and CaN has been reported in BHK-21 cells transfected with both proteins [Shibasaki et al., 1997]. Dephosphorylation of Ser70 of Bcl-2 by CaN is required for Bcl-2 to have anti-apoptotic activity. The overexpressed CaN in human colorectal adenocarcinoma may provide protection against apoptosis by dephosphorylating Bcl-2 [Shitashige et al., 2001].

TABLE II. Clinico-Pathological Information of Human Colorectal Adenocarcinomas

						Staining grade	
Case no.	Age	Sex	Tumor	Normal	Metastasis	Normal mucosa	Tumor mucosa
1	88	Male	pT3	pN1	pMx	0–1	3
2	32	Female	pT4	pN2	pMx	1	4
3	60	Male	pT2	No	pMx	1	3
4	45	Male	pT3	pN1	pMx	1	4
5	71	Male	pT2	pN0	pMx	1	3
6	49	Female	pT3	pN1	pM1	1	4

Pathologic staging (pTNM): Primary tumor (pT)—pT2, tumor invades muscularis propria; pT3, tumor invades through the muscularis propria into the subserosa or the non-peritonealized pericolic or perirectal soft tissues; pT4, tumor directly invades other organs or structures and/or tumor penetrates the visceral peritoneum.
Regional lymph nodes (pN)—pN0, no regional lymph node metastasis; pN1, metastasis in 1–3 regional lymph nodes; pN2, metastasis in four or more regional lymph nodes; No, no grade.
Distant metastasis (pM)—pMX, cannot be assessed; pM1, distant metastasis.
Staining grades (semi-quantitative scale)—percentage of cells stained and degree of staining, weak, moderate, and strong: 0%–24% of cells stain positive, 25%–49% of cells stain positive, 50%–74% of cells stain positive, 75%–100% of cells stain positive.

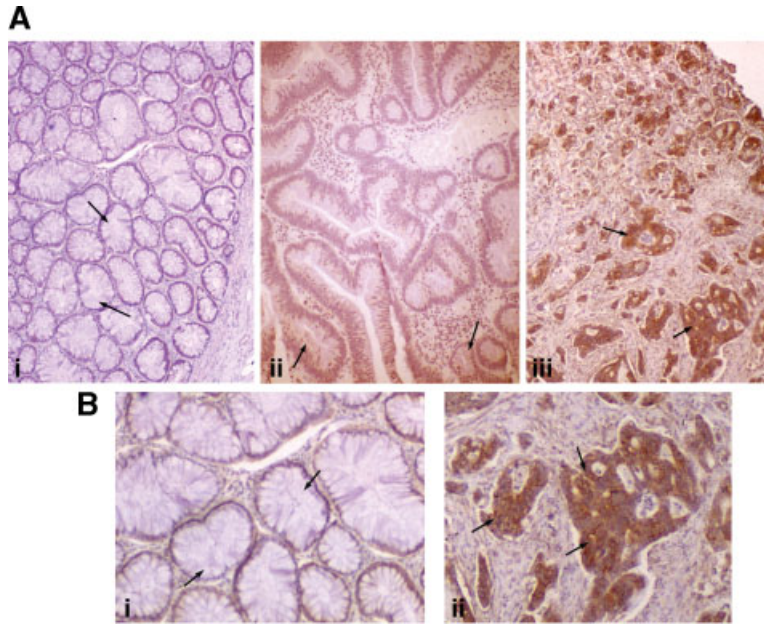

Fig. 3. **A**: Immunoperoxidase staining of CaN in human colorectal normal mucosa and adenocarcinoma. **i**: Section from normal mucosa far removed from tumor showing a weak degree of focal staining with anti-CaN (see arrows) (immunoperoxidase; original magnification, ×120). **ii**: Section from polyps showing a moderate degree of antibody reactivity (immunoperoxidase; original magnification, ×120). **iii**: Section from colorectal adenocarcinoma showing a strong immunoreactivity with CaN antibody in most of the tumor cells (immunoperoxidase; original magnification, ×120). **B**: High power view depicting the cytoplasmic staining of (**i**) normal and (**ii**) tumor tissues (immunoperoxidase; original magnification, ×600).

Further work will be needed to explain the interaction of CaN and Bcl-2 that results in cell proliferation in colon tumor tissues.

In apoptosis, proteolysis is very important in altering or disabling the function of many enzymes involved in signal transduction. Recently, we observed higher expression of *m*-calpain, a Ca^{2+}-activated cysteine protease in human colorectal adenocarcinomas [Lakshmikuttyamma et al., 2004c]. Furthermore, we demonstrated the in vitro proteolysis of brain CaN by *m*-calpain and the enhancement of its phosphatase activity after proteolysis [Lakshmikuttyamma et al., 2004a]. It may be possible that *m*-calpain plays a role in enhancing the expression of CaN in colon tumor.

In conclusion, we have demonstrated the increased activity and protein expression of CaN in colorectal adenocarcinomas. Ca^{2+} signaling has diverse cellular functions ranging from transmembrane and cytoplasmic signal transduction to gene regulation. The increased activity and overexpression of this Ca^{2+}/CaM-dependent phosphatase suggests a possible link between Ca^{2+} signaling and oncogenic processes. Future work directed to the interaction of CaN and various apoptotic factors in colon cancer cells will provide a greater understanding of the molecular pathways affecting overexpression of this enzyme. In addition, continuing research will increase our knowledge about Ca^{2+} signaling and metastatic processes.

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