Autoantibodies to Ca^{2+} binding protein Calnuc is a potential marker in colon cancer detection

YAO CHEN[1], PING LIN[2,5], SUIMIN QIU[3], XUAN-XIAN PENG[4], KOKSUN LOOI[1], MARILYN GIST FARQUHAR[2] and JIAN-YING ZHANG[1]

[1]Department of Biological Sciences, The University of Texas at El Paso, 500 West University Avenue, El Paso, TX; [2]Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA; [3]Division of Surgical Pathology, Department of Pathology, The University of Texas Medical Branch, Galveston, TX, USA; [4]School of Life Sciences, Xiamen University, Xiamen, Fujian, P.R. China

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Abstract. Calnuc is a calcium (Ca^{2+}) binding protein found in both Golgi and cytoplasm, and it may play a role in G protein- and Ca^{2+}-regulated signal transduction events. This study was designed to investigate the possibility of whether Calnuc protein might be a tumor-associated antigen (TAA) that induces autoantibody response in human cancers, and to evaluate the feasibility of the Calnuc antigen-antibody system as a marker in cancer detection. Purified full-length recombinant Calnuc protein was used as an antigen in enzyme-linked immunoassay and Western blotting for the detection of autoantibodies in cancers. Sera from 447 patients with 9 different types of cancer were analyzed. Although the frequency of autoantibody to Calnuc was found to be 4.7% in total groups of cancer, it was not significantly different to that of normal individuals (1.2%). However, the frequency of autoantibody to Calnuc in colon cancer (11.5%) was significantly higher than that in normal individuals (1.2%). The expression analysis of Calnuc in multiple colon cancer tissues by immunohistochemistry on tissue array further confirmed the high specificity of Calnuc in colon cancer. Of 69 colon cancer tissue specimens examined, 41 tissues (59.4%) overexpressed Calnuc, while normal colon tissues did not show any expression of Calnuc. The subcellular distribution analysis of Calnuc examined by subcellular fractionation and immunofluorescence indicates that Calnuc is a membrane associated protein and mostly distributed in Golgi, which is consistent with previous reports. With adding Calnuc into a TAA array (including p53, c-myc, cyclin B1, cyclin D1), the cumulative frequency of antibody to multiple TAAs in colon cancer was raised to 65.4% which is significantly higher than the cumulative frequency in normal individuals (6.1%). This indicates that a mini-array of multiple TAAs which includes Calnuc might provide a novel non-invasive approach to enhance antibody detection for colon cancer diagnosis.

Correspondence to: Dr Jian-Ying Zhang, Department of Biological Sciences, The University of Texas at El Paso, 500 West University Avenue, El Paso, TX 79968, USA
E-mail: jzhang@utep.edu

Present address: [5]AVIVA Biosciences Corporation, San Diego, CA, USA

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Introduction

Studies in autoimmune diseases have provided abundant evidence suggesting that the autoantibodies are an antigen-driven response and can be viewed as reporters from the immune system revealing the identity of antigens which might play roles in the pathophysiology of the disease process (1,2). In recent years, there have been a steadily increasing number of studies describing and characterizing autoantibodies in cancer (3-5). Research on antibody immunity to cancer-associated proteins has received great attention. As the detection of antibody immunity to tumor antigens becomes more routine, investigators have begun to address specific clinical issues such as the role of antibody immunity as a marker for patients exposed to cancer, as a tool to monitor therapy, or as an indicator to predict disease prognosis (6). Although the mechanisms whereby these intracellular proteins provoke immune responses are not clear, there appears to be some relationship to tumorigenesis, since these antibodies are rarely found in normal people or nonmalignant disease conditions (2). Cancer autoimmunity may involve genetic mutations resulting in the production of tumor protein and defective tumor suppressor protein. The structural changes and abnormal expression of certain self-proteins, occurring during tumorigenesis, suggest mechanisms by which the immune system could be primed to perceive tumor-associated epitopes as foreign antigens (2). One of the most important criteria for cancer markers is the ability to distinguish cancer from inflammatory and benign diseases. Currently, although many cancer antigens such as α fetoprotein (AFP), carcino-embryonic-antigen (CEA), and prostate-specific-antigen (PSA) have been widely used clinically to identify cancers such as HCC, colorectal cancer, and prostate cancer, their increased level are also observed in benign conditions such as inflam-

mation, where the low sensitivity and specificity make them less reliable. The distinction between inflammation and cancer is hard to establish. Autoantibodies that are induced by tumor antigens, however, open a new gateway for cancer diagnosis. Since an increase in cancer antigens that occur temporarily with inflammation will not cause an increase in autoantibodies, the autoantibody detection can readily distinguish between cancer and inflammatory diseases (7). Another benefit of autoantibody in cancer detection is that a subtle biochemical change in a tumor cell might be able to produce a detectable level of autoantibodies before the emergence of the tumor phenotype and the released tumor antigens reaches a detectable level (8). A study on the comparison of the autoantibody-based enzyme immunoassay (EIA) to conventional antigen EIA kits stated that autoantibody EIA can significantly enhance the sensitivity and specificity of tumor markers (7). The question of why the immune response to tumor antigens is unable to eradicate tumor growth has expanded the complexity of molecular interactions between tumor cells and the immune system (9).

Calnuc is a ubiquitous, EF-hand Ca^{2+} binding protein found in both Golgi and cytoplasm (11). Cytosolic Calnuc binds to Gαi3, whereas Golgi luminal Calnuc plays a key role in constitution of a Ca^{2+} storage pool (11). Golgi luminal Calnuc was found to be secreted outside the cell by which it may influence cell behavior via its Ca^{2+}-binding properties (10). These previous findings demonstrated that Calnuc might play an important role in G protein- and Ca^{2+}-regulated signal transduction events (11). In many ways cancer is a disease of mis-regulated signal transduction. For this reason, it is assumed that Calnuc protein might be involved in tumorigenesis. This study was designed to investigate the possibility of whether Calnuc protein might be a new TAA that induces an autoantibody response in human cancers, and further evaluate the usefulness of the Calnuc antigen-antibody system as a marker in cancer detection.

Materials and methods

Sera and antibodies. In this study, sera from 447 patients with different types of cancer and from 82 normal individuals who had no obvious evidence of malignancy were obtained from the serum bank of Tumor Cell Engineering Laboratory of Xiamen University (Fujian Province, P.R. China). Based on clinical information, all cancer sera were collected at the time of diagnosis, and patients did not receive any treatment with chemotherapy or radiotherapy. Normal control sera were collected during annual health examinations. This study was approved by the Institutional Review Board of Xiamen University and collaborating institutions. Polyclonal rabbit IgG antibody (F-5059) against recombinant full-length Calnuc was generated and affinity purified as previously described (11).

Cell culture. Three human colon cancer cells (LS174T, DLD-1, and HCT-116) were kindly provided by Dr John Carethers (UCSD, La Jolla, CA), and cultured in DME medium (high glucose) supplemented with 10% (v/v) FCS (Life Technologies, Gaithersburg, MD), 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Cells were used as 80% confluent monolayer for immunofluorescence assay.

Immunofluorescence assay. Colon cancer cells were fixed in 2% paraformaldehyde in phosphate buffer and permeabilized as previously described (11), and subsequently incubated with 0.1 g affinity purified anti-Calnuc IgG antibody at room temperature for 1 h, followed by incubation with Alexa Fluor 488 conjugated anti-rabbit F(ab')2. Specimens were examined with a Zeiss Axiophot microscope equipped for epifluorescence.

Subcellular fractionation. Cell lysates, postnuclear supernatants, membrane (100,000 x g pellets) and cytosolic fractions (100,000 x g supernatants) were prepared by centrifugation of postnuclear supernatants from colon cancer cells and analyzed by immunoblotting (11).

Purification of Calnuc full-length recombinant protein. Full-length Calnuc cDNA was subcloned into the pET28a vector (Novagen, Madison, WI) producing a fusion protein with NH2-terminal 6x histidine and T7 epitope tags (11). The recombinant protein was expressed in E. coli BL21 (DE3) and purified using nickel column chromatography. The protocol used for the high level expression and purification of 6x-histidine-tagged proteins was performed as described (Qiagen Inc., Valencia, CA). Eluting buffer (8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris, pH 4.5) was used to elute the recombinant protein. The purified recombinant protein was examined by SDS-PAGE. Full-length recombinants p53, c-myc, cyclin B1 and cyclin D1 were derived from our previous study (12).

Enzyme-linked immunosorbent assay (ELISA). Purified recombinant proteins were diluted in phosphate-buffered saline (PBS) to a final concentration of 0.5 μg/ml and coated onto a 96-well microtiter plate (Dynatech Laboratories, Alexandria, VA) at 200 μl per well, and 200 μl human sera which was 1:200 diluted were incubated in the antigen-coated wells. Horseradish peroxidase-conjugated goat anti-human IgG (Caltag Laboratories, San Francisco, CA) and the substrate 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (Boehringer Mannheim GmbH, Mannheim, FRG) were used as detecting reagents. Each serum sample was tested at least 2 times, and the average OD value at 405 nm was used for data analysis. The cutoff value designating positive reaction was the mean OD of 82 normal human sera (NHS) + 3SD. Each run of ELISA included 8 NHS selected to represent a range of absorbance above and below the mean of 82 normal human sera, and whose OD average is close to the mean OD of 82 normal human sera. The inclusion of these normal controls is to normalize all absorbance values in other runs of ELISA. All positive sera were further confirmed by Western blotting. The detailed protocol of ELISA was used as described by Rubin (13).

Western blotting. Western blotting was performed essentially as described by Chan and Pollard (14). Purified recombinant Calnuc proteins were electrophoresed on SDS-PAGE and transferred to a nitrocellulose paper. After preblocking with PBS containing 5% nonfat dry milk and 0.05% Tween-20 (PBST) for 30 min at room temperature, the nitrocellulose strips were incubated for 90 min at room temperature with a 1:200 dilution of serum. Horseradish peroxidase-conjugated goat anti-human IgG (Caltag Laboratories, San Francisco, CA) was applied as secondary antibody at a 1:3,000 dilution.

Table I. Characteristics of 69 colon cancer tissue specimens.[a]

Variables	Results
Age	
Mean ± SD (years)	54.7±16.0
Range (years)	16-86
Gender	
Male (%)	56 (81.2)
Female (%)	13 (18.8)
Pathological grades	
Grade I (%)	20 (29.0)
Grade II (%)	25 (36.2)
Grade III (%)	14 (20.3)
Grade not available (%)	10 (14.5)

[a]Information in this table was provided by Cybridi Inc. (Bethesda, MD).

Table II. Frequency of autoantibody to Calnuc in human sera with diverse malignancies.[a]

Type of cancer	No. tested	Frequency (patients with Calnuc antibody/ total patients with disease)
Breast	39	0
Cervical	16	0
Colon	52	11.5% (6/52)[b]
Esophageal	70	5.7% (4/70)
Gastric	73	1.4% (1/73)
Hepatic	62	4.8% (3/62)
Lung	104	6.7% (7/104)
Nasopharyngeal	14	0
Ovarian	17	0
Total	447	4.7% (21/447)
NHS	82	1.2% (1/82)

[a]Cutoff value, mean + 3 SD of NHS; [b]p value relative to NHS, $p<0.05$. NHS, normal human sera.

Immunoreactive bands were detected using the ECL kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Immunohistochemistry (IHC) with tissue array. Superfrost plus tissue slides which contain 69 paraffin-embedded colon cancer tissue specimens and 3 normal colon tissue specimens were purchased commercially (Cybridi, Bethesda, MD) and used for Calnuc antigen detection. The general information regarding 69 colon cancer tissue specimens is shown in Table I. Antigen retrieval was performed by microwave-heating in a citrate-based antigen retrieval solution (BioGenex, San Ramon, CA) according to the manufacturer's recommendation. Non-specific protein binding sites were blocked by 1.5% normal horse sera for 30 min in a humidifier. Tissue sections were incubated with 1:1,000 diluted polyclonal rabbit anti-Calnuc (original concentration 6 μg/ml). Biotinylated secondary antibody and an ABC (Avidin-Biotinylated enzyme Complex) kit, and DAB (3,3'-diaminobenzidine) substrate kit were used as detecting reagents according to the manufacturer's recommendation (Vector Laboratories, Burlingame, CA). The slides were counterstained with hematoxylin, fixed by Scott's solution and dehydrolyzed with different concentrations of EtoH and Citrisolvent. Finally, the slides were mounted with permount mounting medium and observed under a microscope.

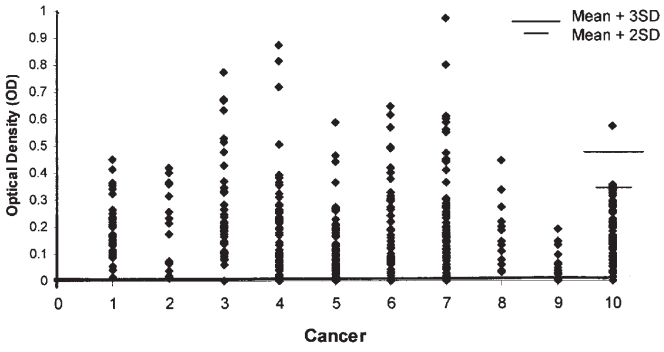

Figure 1. Titers of antoantibody to Calnuc in different cancers. The range of antibody titers to Calnuc (y-axis) is expressed as optical density (OD) obtained from enzyme-linked immunosorbent assay. The mean + 3SD of 82 normal human sera are shown in relationship to OD value of the cancer sera. X-axis represents the type of cancer sera: 1, breast; 2, cervical; 3, colon; 4, esophageal; 5, gastric; 6, hepatic; 7, lung; 8, nasopharyngeal; 9, ovarian; and 10, normal human sera.

Statistical analysis. To determine whether the frequency of autoantibodies to TAAs in each cohort of patients' sera was significantly higher than that in sera from normal individuals, the data were analyzed using the χ^2 test with Yates' correction. Two significant levels (0.05 and 0.01) were used. The method for calculating the sensitivity and specificity was based on a previously described methodology (15).

Results

Prevalence and titers of autoantibody to Calnuc in human cancer sera. Sera from patients with a variety of malignancies including breast, cervical, colon, gastric, esophageal, lung, hepatic and ovarian cancer were available for this study. The full-length Calnuc recombinant protein was used as an antigen. Among 447 Chinese cancer sera analyzed, the average frequency of positive reaction in ELISA was 4.7% (21/447) which showed no significant difference from normal human sera (1.2%). As shown in Table II, the frequency of antibody to Calnuc was 6.7% (7/104) in sera with lung cancer, 5.7% (4/70) in sera with esophageal cancer, and 4.8% (3/62) in sera with liver cancer. No positive reactivity was found in sera with breast cancer (0/39), cervical cancer (0/16), nasopharyngeal cancer (0/14) or ovarian cancer (0/17); there was no significant difference compared to the frequency in normal sera (1.2%). An interesting finding is that the frequency of antibody to Calnuc in colon cancer (11.5%) was significantly

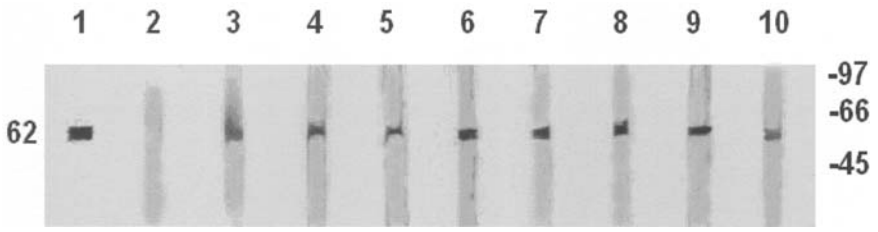

Figure 2. Representative Western blotting showing 8 positive reactions of ELISA positive cancer sera with recombinant Calnuc proteins. Lane 1, a positive control which shows the positive reactivity with rabbit polyclonal anti-Calnuc. Lane 2, the negative reactivity with a normal human serum. Lanes 3-10, eight representative cancer sera which were positive in ELISA, and also have strong reactivity with 62-kDa recombinant Calnuc protein in Western blotting.

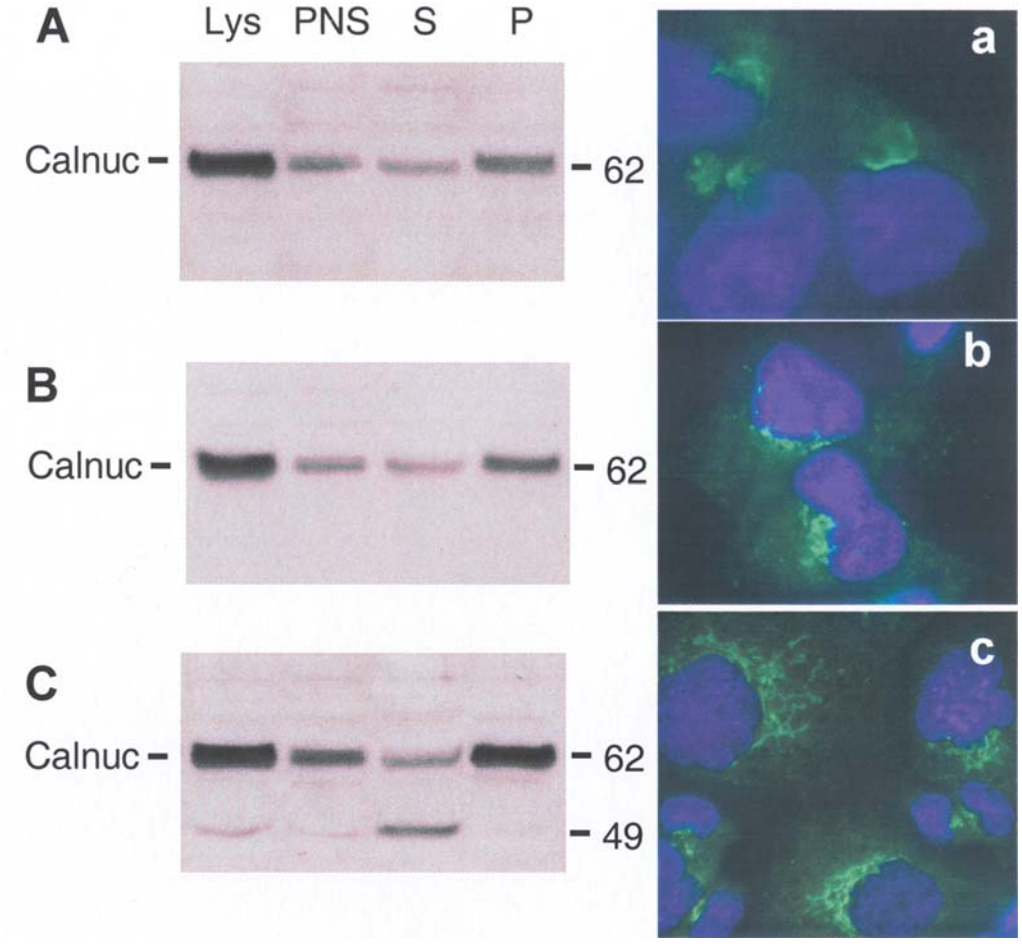

Figure 3. Distribution of Calnuc is examined on whole cell lysates (Lys), postnuclear supernatants (PNS), cytosolic (S) and membrane associated (P) fractions. LS174T (A) and DLD-1 (B) cells show 62-kDa calnuc in both cytosolic (40%) and membrane-associated (60%) fractions. In HCT-116 cells (C), though 62-kDa calnuc is found in cytoplasm (10%) and membrane (90%) fractions, an additional 49-kDa band was also visualized in cytoplasm. Localization of Calnuc in the Golgi is shown in all three cell lines including LS174T (a), DLD-1 (b) and HCT-116 (c).

higher than that in normal human sera (1.2%). The ranges of antibody titers to Calnuc in different cancers are shown in Fig. 1. All the ELISA positive sera were confirmed by Western blotting. As shown in Fig. 2, the eight representative positive sera with antibody to Calnuc in ELISA also showed strong reactivities with a 62-kDa Calnuc recombinant protein, whereas the normal serum which is negative in ELISA had no reactivity in Western blot analysis.

Distribution of endogenous Calnuc in colon cancer cells. To examine the distribution of Calnuc, both studies of subcellular fractionation and immunofluorescence assay were performed on three human colon cancer cell lines including LS174T, DLD-1, and HCT-116. Subcellular fractionation showed that both LS174T (A) and DLD-1 (B) cells have a similar distribution of 62-kDa calnuc with 40% in cytosolic fraction (S) and 60% on the membrane (P), which is consistent to what we reported previously (11). However, in HCT-116 cells (C), besides 62-kDa protein found in both cytoplasm (10%) and membrane fraction (90%), an additional band of 49-kDa protein was also found in cytoplasm. Immunofluorescence assay indicated that membrane associated endogenous Calnuc localizes in the Golgi of LS174T (a), DLD-1 (b), and HCT-116 (c), respectively (Fig. 3).

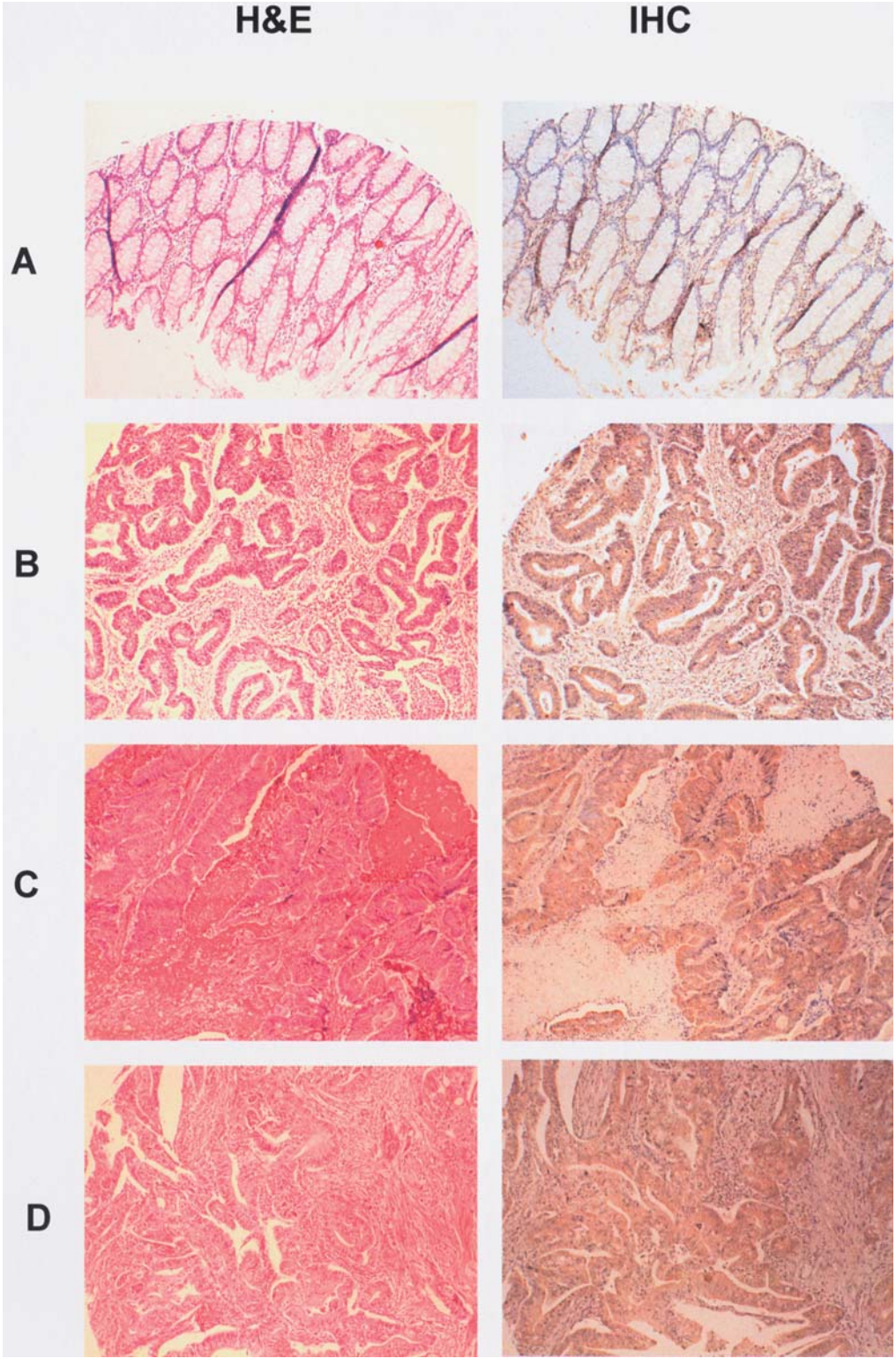

Figure 4. Expression of Calnuc in colon cancer tissues examined by immunohistochemistry (IHC). Tissue-array slide was stained with rabbit anti-Calnuc antibody at a 1:1,000 dilution. H&E (hematoxylin and eosin) staining was performed to show the histopathology of the cancer specimen. A, Calnuc negatively stained colon tissue (normal colon tissue, #72). B, Calnuc positively stained colon tissue (colon adenocarcinoma, well differentiated, #53). C, Calnuc positively stained colon tissue (colon adenocarcinoma, moderately differentiated, #60). D, Calnuc positively stained colon tissue (colon adenocarcinoma, well differentiated, #63). Of the 69 colon cancer tissue specimens examined, increased expression of Calnuc was observed in 41 (59.4%) specimens (magnification x100).

Expression of Calnuc protein in colon cancer tissues by immunohistochemistry on tissue array. The above data show that the frequency of autoantibody to Calnuc in sera from patients with colon cancer was significantly higher than that in sera from patients with other cancers and normal individuals. In order to investigate whether Calnuc can appear at a high frequency of expression in colon cancer tissues, the expression of Calnuc in solid colon cancer tissues and normal colon tissue was examined by immunohistochemistry with tissue array slide. Fig. 4 shows an example of one normal colon tissue

Table III. Frequency of antibodies to five tumor-associated antigens p53, c-myc, cyclin Bl, cyclin Dl and Calnuc in colon cancer.[a]

Samples	No.	Number (%) of autoantibodies to:					
		p53	c-myc	cyclin Bl	cyclin Dl	Calnuc	Any of five antigens
Colon cancer	52	5 (9.6)[b]	8 (15.4)[c]	17 (32.7)[c]	11 (21.2)[c]	6 (11.5)[b]	34 (65.4)[c]
NHS	82	2 (1.2)	0 (0.0)	2 (2.4)	1 (1.2)	1 (1/82)	5 (6.1)

[a]Cutoff value, mean + 3SD of 82 NHS. P values between cancer and NHS were calculated to be [b]<0.05 or [c]<0.01. NHS, normal human sera.

which was negatively stained with Calnuc (A) and three different colon cancer tissues which were positively stained with Calnuc (B, C and D). Of the 69 colon cancer specimens, 41 (59.4%) tumor samples showed positive staining. Three normal tissues did not show any positive staining of Calnuc. The frequency of Calnuc positive staining was 65% (13/20) in grade I colon cancer, 64% (16/25) in grade II colon cancer, and 64.3% (9/14) in grade III colon cancer. Due to the small sample size of tissues with different grades in this commercial tissue array slide, it is still difficult to establish a statistically significant correlation between Calnuc expression and tumor grade.

Evaluation of the addition of Calnuc into multiple TAA array in enhancing antibody detection for diagnosis of colon cancer. As shown in Table III, antibodies to four other TAAs such as p53, c-myc, cyclin B1, and cyclin D1, which have been extensively tested in different cancers, were also used to test this group of colon cancer samples in the current study, and the frequency of antibody to individual TAA ranged from 9.6% (5/52) to 32.7% (17/52) in colon cancer. When sequentially adding the TAA to the panel of five TAAs including Calnuc, the cumulative frequency was raised to 65.4% (34/52), which was significantly higher than the cumulative frequency of antibody in normal individuals (6.1%). The sensitivity and specificity in the detection of colon cancer was 65.4% and 93.9%, respectively. The contribution of Calnuc to this mini-array in increasing the sensitivity of colon cancer detection also suggests that Calnuc plays a role in the tumorigenesis of colon cancer.

Discussion

Autoimmune responses have been frequently observed in patients with malignancies, and been thought to be driven by some tumor-associated antigens which might be involved in cellular functions related to tumorigenesis (2). The identification of panels of tumor antigens that elicit a humoral response may have utility in cancer detection, diagnosis, or in establishing prognosis (16). Interpreting the specificity of an observed humoral or cellular immune response to tumor antigens has become the critical issue in human tumor immunology (17). The molecular cloning of tumor antigens recognized by autoantibodies has opened a new era in tumor immunology and the list of defined immunogenic human tumor antigens is growing rapidly (3,18,19). Such antigens may also have utility in cancer immunotherapy, and provide simple and reliable end points for monitoring the immmunogenecity of cancer vaccines (2,19).

Our previous study demonstrated that frequencies of antibodies against seven TAAs such as p53, p62, Koc, c-myc, cyclin B1, Survivin, and IMP1 range from 7.1-26.8% in lung cancer, 10.8-24.6% in hepatocellular carcinoma (HCC), 8.8-18.7% in gastric cancer, and 4.4-17.8% in colon cancer. With the concern that no single tumor associated antigen can account for a large majority of immunoreactivity in various cancers, the value of individual TAA as a tumor marker was tempered. However, this drawback can be overcome by using a mini-array of multiple TAAs. It was noted, in the same study, with the successive addition of antigen to a final total of seven TAAs, that there was a stepwise increase of positive antibody reaction to 67.9% in lung cancer, 56.9% in HCC, 52.7% in gastric cancer, and 51.1% in colon cancer (12,20). It was hypothesized that various combinations of selected antibody-antigen systems would be useful for diagnosis of certain types of cancer. With the rapid increase in the number of the identified TAAs, the judicious selection of antigens to be included in panels or arrays of TAAs is extremely important because not all cellular protein recognized as antigens by one type of cancer sera are cancer specific. Some can also be recognized by other types of cancer or non-cancer disorder (20). Our primary interest is to identify and characterize more TAAs, test the frequency of antibodies to individual TAA in various types of cancer and further investigate the possibility of a 'customized TAA array' as a diagnostic tool for certain types of cancer (12,20,21).

Calnuc, also called Nucleobindin, was originally identified as a 55-kDa, DNA- and calcium-binding leucine zipper protein. It participates in constitution of a Ca^{2+} storage in the Golgi, as well as in other biological processes that involve DNA-binding and protein-protein interactions. It was thought to be a transcription factor based on its ability to bind DNA fragments *in vitro* (22,23). In previous study, Calnuc was isolated from the sera of mice prone to the autoimmune disorder systemic lupus erythematosis (SLE), as well as the growth media of a lymphocyte cell line established from these mice (24,25). Kubota and coworkers found that Calnuc might enhance anti-DNA antibody production when added to cultures of autoimmune MRL/lpr mouse spleen cells, and normal mice injected with Calnuc developed thymic apoptosis and many of the symptoms of SLE (26-28). It was assumed that over-expression and secretion of the Calnuc protein might be

involved in the activation of autoreactive T cells and play a role in the pathogenesis of these autoimmune diseases (29). However, Calnuc was not only well-associated with autoimmune disease, the higher expression of Calnuc was also observed in cancer. For example, a study on gastric adenocarcinoma has demonstrated that in 50 gastric adenocarcinomas with lymph node metastasis, 56% of cases showed a positive reaction to Calnuc, which was much higher compared to that in 50 gastric adenocarcinomas without lymph node metastasis (10%) (30). In another study, Kubota and his colleagues tested the expression of Calnuc in non-Hodgkin's lymphoma by immunohistochemical staining with anti-human Nuc monoclonal antibody; >90% of tumor cells were stained with anti-Nuc, and 65% were in a high grade of histological malignancy, 54% in the intermediate grade and 22% in the low grade (31). All of the above information indicates that overexpression of Calnuc might be associated with the activation or proliferation of the tumor cells. In the present study, the possible relationship of Calnuc to cancer is related to the observation of a significantly higher prevalence of antibody to Calnuc in colon cancer sera (11.5%) compared to the relatively low frequency in normal human sera (1.2%). In examination of archival paraffin-embedded colon cancer tissue-array, 59.4% of cancer specimens show high expression of Calnuc, whereas three normal colon tissues did not show any positive expression. This indicates that aberrant Calnuc expression might cause the antibody reaction and contribute to the malignant transformation of colon cancer. Analysis on the frequency of positive Calnuc staining in colon cancer tissues with different grades has not revealed any significant correlation between Calnuc expression and cancer grades due to the limited number of tissue specimens in this study, especially the small sample size of tissues with grade III. For a better understanding of the correlation more extensive study is needed.

As demonstrated in many other studies, cancer has long been recognized as a multi-step process which involves not only genetic changes conferring growth advantage but also factors which disrupt regulation of growth and differentiation. Different types of cancer might confer different autoantibody profiles (12,20). Characterization of these TAAs might help us to define factors involved in the multiple stages of tumorigenesis (12,20). In the present study, although Calnuc showed a higher immunogenicity in colon cancer, the frequency of antibody to Calnuc in colon cancer (11.5%) still did not reach the level of sensitivity that could be routinely useful in cancer diagnosis, which is probably due to the multi-factor nature of colon cancer. However, a novel strategy, as demonstrated in our study, a mini-array of multiple TAAs including Calnuc may greatly increase the sensitivity of antibody detection in colon cancer. By linking the response to several antigens together, the sensitivity and specificity of the test increase considerably, presumably because the chance that a patient will respond to at least one of the antigens is increased (3). This indicates that a critical selection of a panel of cancer specific tumor associated antigens provides a promising way to enhance cancer diagnosis. The contribution of Calnuc to the mini-array in enhancing colon cancer detection also suggests that Calnuc might play a role in malignant transformation and might be considered as a new diagnostic marker and therapeutic target in colon cancer.

The possible function of Calnuc in carcinogenesis might be of interest. There are two pools of Calnuc protein in the cells. The intra-Golgi pool of Calnuc was shown to be important in maintaining the Golgi Ca^{2+} stores through Ca^{2+} binding to its EF-hands (32). The cytoplasmic pool is more likely to regulate other proteins through direct interaction, as with other Ca^{2+}-binding proteins such as calmodulin, troponin C, and recoverin located in the cytoplasm (33). It was observed that cell shape, adhesion, and motility were affected by Ca-regulated pathways, which depended on Ca^{2+}-binding proteins (34). Previous study has reported that one ER Ca^{2+} binding protein calreticulin, which shows significant sequence homology to Calnuc, affects cell adhesion via the regulation of expression of proteins which is important in adhesion, as well as via its effects on intracellular signaling pathways (35-38). It was hypothesized that Calnuc might play a similar role to calreticulin in the cells, by affecting the expression of other cell adhesion molecules, which could contribute to the cancer metastasis. Meanwhile, Calnuc binds to Gαi3 in a Ca^{2+}- and Mg^{2+}-dependent manner (39), and undergoes a conformational change after binding Ca^{2+} (40). This suggests, in analogy to calmodulin and other calcium sensors, a putative role for Calnuc in regulation of G subunits (41). Calnuc binds to the C-terminal α5-helix of Gαi3 (39), and therefore might interfere with binding of putative receptors to G subunits (41), then affecting the activation of different functions such as cell growth, proliferation, and migration. Adam and his colleagues have provided such evidence in their study (42). They have identified a breast cancer cell membrane-associated protein BCMP84, which is a member of the S100 family of proteins. BCMP84 shows plasma membrane localization in both breast cancer-derived cell lines and breast cancer tissues but cytosolic expression in non-tumor cells. The nucleobindin (CALNUC) protein identified by yeast two-hybrid cloning is considered as one of the BCMP84-interacting proteins. This finding suggests that BCMP84 can interact with Calnuc which in turn can bind G proteins in a calcium-dependent manner, and it may also provide a possible mechanism by which BCMP84 can associate with the plasma membrane in cancer cells and suggests a role for BCMP84 in G protein-coupled signal transduction events (42). Furthermore, it will be important to determine the functional consequences of the interaction.

In conclusion, the results of the higher frequency of Calnuc antibody in colon cancer, and of overexpression of Calnuc in colon cancer tissue, together with its specific cellular function such as G protein binding and Ca^{2+} signaling regulation, provide evidence that Calnuc may play a role in tumorigenesis of colon cancer. A mini-array of five TAAs including Calnuc greatly increases the sensitivity of antibody detection in colon cancer. Whether Calnuc can be used as a marker for diagnosis of certain types of cancer such as colon cancer still needs to be investigated. Future studies including analysis of gene mutation, and production of transgenic mice, may provide insights into how the Calnuc protein is involved in the malignancy.

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