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The Use of a Colon Cancer Associated Nuclear Antigen CCSA-2 for the Blood Based Detection of Colon Cancer

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Abstract The early diagnosis of colorectal cancer (CRC) is central for effective treatment, as prognosis is directly related to the stage of the disease. Development of tumor markers found in the blood from patients, which can detect CRC at an early stage, should have a major impact in morbidity and mortality of this disease. The nuclear matrix is the structural scaffolding of the nucleus and specific nuclear matrix proteins (NMPs) have been identified as an ''fingerprint'' for various cancer types. Previous studies from our laboratory have identified four colon cancer associated NMPs termed colon cancer-specific antigen (CCSA)-2 to (CCSA)-5. The objective of the present study was to analyze the expression of one of these proteins, CCSA-2 in serum from various patient populations and to determine whether CCSA-2 antibodies could be used in a clinically applicable serum-based immunoassay specifically to detect colon cancer. Using an indirect ELISA, which detects CCSA-2, the protein was measured in the serum from 174 individuals, including healthy individuals, patients with colon cancer, patients with diverticulosis, colon polyps, inflammatory bowel disease (IBD) as well as other cancer types. With a predetermined cutoff absorbance of 0.6 OD we have successfully utilized this approach to develop an immunoassay that detected colon cancer. The immunoassay showed a sensitivity of 88.8% (24/27) and an overall specificity of 84.2% (106/127). This initial study showed the potential of CCSA-2 to serve as a highly specific blood based marker for colon cancer. Although potentially promising, the results of this study must be confirmed in larger independent validation studies. J. Cell. Biochem. 104: 286–294, 2008.

Key words: nuclear matrix proteins; colorectal cancer; tumor markers

Colorectal cancer (CRC) is one of the best characterized tumor types in regards to the multistep genetic progression pathway that has been elucidated. Despite our molecular understanding it is the second leading cause of cancer related death in the United States and the third most common cancer after lung and breast cancer worldwide [Parkin, 2001].

In 2007, more than 153,760 new cases will be diagnosed and more than 52,180 people will die from CRC in the USA [Jemal et al., 2007]. More than 50% of these deaths may have been prevented through the use of screening tests as the resulting early detection of the disease [Walsh and Terdiman, 2003]. The long natural history of CRC as it evolves from adenomatous polyps in the majority of cases provides opportunities for detection of early stage in cancer and for prevention of cancer by removal of polyps. Despite the potential for screening of CRC, only a minority of the population currently undergo screening program (www.cancer.org).

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The low rate of participation in CRC screening is critical to understand and is due to a number of actors, including patient discomfort, costs, and poor acceptability of current screening methods. Compliance to a serum test likely be better than tests involving feces and stool handling. An effective blood test, which ideally has a high specificity and sensitivity would be an ideal method to detect CRC and could lead to a reduction of the mortality and morbidity of CRC.

In order to identify highly specific tumor markers, investigators have focused attention on the structural changes that are associated with neoplastic transformation. Alterations in the cellular and nuclear structure are hallmarks of the carcinogenic process. These alterations are so prevalent in cancer cells that they are commonly used as a pathological marker of transformation. Nuclear shape reflects the internal nuclear structure and processes and is determined by the nuclear matrix [Pienta et al., 1989].

Most of the nuclear matrix proteins (NMPs) identified to date are common to all cell types, but several identified NMPs are tissue and cell line specific [Getzenberg, 1994]. This structure has many important functions like DNA organization, stabilization, and organization of gene regulatory complexes and synthesis of RNA, a variety of functions of which many have implications in cancer progression [Konety and Getzenberg, 1999].

Cell type-specific "fingerprinting" of aberrant NMPs and their appearance in cancer development has led to the analysis of NMP composition of a variety of tumors in an effort to determine whether these proteins can be developed as diagnostic and/or prognostic markers for cancer. Previously, we have identified specific NMP in prostate, bladder, renal, colon cancer, and colon cancer metastasis to the liver [Konety et al., 1998; Brunagel et al., 2004, 2002a,b; Van Le et al., 2004; Myers-Irvin et al., 2005; Paul et al., 2005]. This oncological "fingerprint" can be used as a specific and reliable diagnostic test, even when a distinction may not be made accurately on a histological basis alone [Getzenberg et al., 1991; Dhir et al., 2004a].

Our laboratory has recently demonstrated, that an antibody raised against the prostate cancer associated marker EPCA-2 is a sensitive and specific serum test for prostate cancer [Dhir et al., 2004b; Paul et al., 2005; Leman et al., 2007]. Additionally, an enzyme linked immunosorbent assay (ELISA) has been developed to detect a specific nuclear protein, BLCA-4 in the urine of individuals with bladder cancer. The test has shown to have a 96.4% sensitivity and 100% specificity [Konety et al., 2000].

Our previous studies describe the isolation of four NMPs (CCSA-2–CCSA-5) that are specifically expressed in colon cancer [Brunagel et al., 2002b]. One of these proteins, CCSA-2 was isolated by excising gel spots from negatively stained two-dimensional gels. The gels spots were then concentrated to obtain protein sequences and synthesized for antibody production.

Internal peptide sequencing of CCSA-2 resulted in four distinct peptides with sufficient amino acid sequence data. The four peptides along with the most significant matches obtained from BLAST analysis are described previously [Brunagel et al., 2002b]. Overall, while these data suggest that some regions of CCSA-2 may be common to other proteins, there is a high possibility of it being a novel uncharacterized protein.

The development of antibodies identifying aberrant NMPs in CRC could become clinically important assay with great specificity. The objective of this study was to investigate whether the NMP CCSA-2 can function as a highly specific and sensitive serum based biomarker for CRC.

Using an indirect ELISA approach, sera from patients with colon cancer were compared with serum samples from healthy donors, patients with diverticulosis/diverticulitis, patients with inflammatory bowel disease (IBD), patients with colon polyps, patients after curative treatment of colon cancer and patients with different cancers.

MATERIALS AND METHODS

Protein Sequencing

CCSA-2 was isolated according to an adaptation of a technique developed by Gevaert [1995]. Two-dimensional gels were negatively stained by 0.2 M imidazole and 0.3 M zinc chloride. The staining was stopped, and the protein gel spots were excised and frozen at -80°C. The spots were then stained with Coomassie blue and concentrated on an acrylamide/agarose gel and sequenced (Michigan State University).

Antibody Production

A standard protocol was followed in the production of monospecific antibodies raised against the CCSA-2 peptides in rabbits. Peptide sequences were chosen based upon the length of the sequence obtained as well as antigenicity. The peptide sequences were modified to contain a terminal cysteine for coupling purposes and conjugated to keyhole limpet hemocyanin or bovine serum albumin to increase immunoreactivity. Antibodies were produced at Biogenes Berlin (Germany) under an Institutional Animal Care and Use Committee approved protocol.

Patients

Serum samples were obtained from consenting patients under an Institutional Review Board—approved protocol. Serum samples from 174 patients were analyzed. After obtaining a blood sample, patients underwent a colonoscopy. Blood was collected with the blood collection system S-Monovette (Sarstedt, Nümbrecht, Germany). After collection, samples were centrifuge at 4,000 rpm. The supernatant was aliquoted in 2 ml tubes (Greiner Bio-one, Solingen, Germany,). The samples were stored at −80°C according to GLP (Good Laboratory Practice) conditions.

Of the patients studied, 27 were diagnosed with colon cancer. The control group consisted of 40 patients with a normal colon as evident by colonoscopy, 21 patients with a diverticulosis, 20 patients with colon polyps, 11 patients with an IBD, and 37 patients with different cancer types. Additionally nine patients 2–9 years after curative surgery for colon cancer were analyzed. The patient's characteristics are summarized in Table I.

Indirect ELISA

The detectability of CCSA-2 using the anti CCSA-2-antibody was assessed using serial dilutions of BSA-conjugated anti CCSA-2 antiserum against known concentrations of CCSA-2 peptide coated into a 96-well plate.

Using Nunc Immunoplate Maxisorb plates prepared with 50 μl coating solution (KPL, Baltimore, MD), 50 μl of serum per well, in triplicate, was allowed to incubated at room temperature with moderate shaking overnight. As a positive control, 50 μl of unlabeled rabbit immunoglobulin G (IgG), diluted with 50 μl coating solution (KPL), was plated overnight as well. The following day, all wells except the blank wells were blocked with 250 μl of Super Block Blocking Buffer (TBS; Pierce, Rockford, IL) for 45 min at 37°C. After blocking the wells, all wells were washed 3× with 250 μl reagent quality water before the addition of the primary antibody. The primary antibody for the sample wells consisted of 100 μl of diluted polyclonal antibody (previously described) in Super Block Blocking Buffer (Pierce). The negative control wells contained rabbit preimmune serum. Following a 2-h incubation period at 37°C with moderate shaking, the plate was emptied, washed with reagent quality water (250 μl, 3×), and then secondary antibody was added to all the wells for another 2 h. The secondary antibody applied was 1 mg/ml goat anti-rabbit IgG-horseradish peroxidase (human serum adsorbed) (KPL), diluted 1:5,000 in Super Block Blocking Buffer (Pierce). After washing the wells with reagent quality water (3 × 250 μl), 100 μl of 3,3′,5,5′-tetramethylbenzidine (KPL), was added to each well and allowed to react for 14 min and the absorbance was read at 650 nm on a Safire (Tecan, Germany) micro plate reader.

Statistical Analysis

The data were compiled as mean ± standard error of the mean.

The normal distribution of the samples of each group was controlled by the Kolmogorov Smirnov test. To analyze differences between the groups, one-way analysis of variance (ANOVA) with the Dunnett's post hoc test was performed. The colon cancer group was taken as reference, statistical significance was assumed at $P < 0.05$.

All statistical analysis and receiver-operator characteristic (ROC) curve were performed using GrapPad Prism version 4.03 for Windows XP, GraphPad Software (San Diego, CA, www.graphpad.com).

RESULTS

Using anti-CC2 antibodies, an indirect ELISA was developed to measure the level of CCSA-2 in the serum from various patient populations. The average value for CCSA-2 in the serum of the 27 colon cancer patients was 0.73 ± 0.15 OD, whereas the average value for healthy individuals (control) was 0.53 ± 0.06 OD. Statistical analysis demonstrated a highly significant difference in serum CCSA-2 levels between the

TABLE I. Characteristics of Patients Studied

	Colon cancer, n = 27		Control, n = 40								
A: Tumor stage (UICC) and grade of the 27 colon cancer patients and control (normal colonoscopy) n = 40											
Female	13		23								
Male	14		17								
Age (mean)	66.3 years		59 years								
Age (range)	39–78 years		27–70 years								
Tumor stage UICC											
Tis (carcinoma in situ)	1										
I: $T_{\mathbf{1\text{-}2}}N_0M_0$	3										
II: $T_{\mathbf{3\text{-}4}}$ N_0M_0	10										
III: $T_{1\text{-}4}N_{\mathbf{1\text{-}2}}M_0$	9										
IV: $T_{1\text{-}4}N_{1\text{-}2}M_{\mathbf{1}}$	4										
Tumor grade											
G1	1										
G2	12										
G3	4										
	Diverticulosis/ diverticulitis n = 14	n = 7	Inflammatory bowel disease, n = 11 M. Crohn, n = 7; C. ulcerosa, n = 4	Colon polyps, n = 20	Other inflammatory disease, n = 9 Gastritis, cholezysto-lithiasis pancreatitis	Cholangioca., n = 6	Pancreatic cancer, n = 11	HCC, n = 3	Lung cancer, n = 4	After colon cancer, n = 9 2–9 years after curative surgery	gastric cancer, n = 13
B: All other patients profile											
Female	10	4	5	4	4	1	5	2	1	7	4
Male	4	3	6	16	5	5	6	1	3	2	9
Age (mean) years	64	59.6	47	69.6	62.7	57.7	47	64	65.9	63	66.9
Age (range) years	53–74	41–76	23–67	35–83	31–69	54–67	23–67	40–71	57–70	46–78	45–78

CC, cholangio carcinoma; HCC, hepatocellular carcinoma.

TABLE IIa. Dunnett multiple Comparison test

Population pairs	Significance (P)
Colon cancer group taken as reference	
Colon cancer vs. control	$P < 0.01$
Colon cancer vs. diverticulosis/itis	$P < 0.01$
Colon cancer vs. IBD	$P < 0.01$
Colon cancer vs. colon polyps	$P < 0.01$
Colon cancer vs. various cancer types	$P < 0.01$
Colon cancer vs. healthy patients after colon cancer	$P < 0.01$
Colon cancer vs. inflammatory disease	$P < 0.01$

colon cancer patients and each of the other patients groups (Tables IIa and IIb).

The receiver operating characteristic curves for CCSA-2 are shown in Figure 1A,B. The CCSA-2 assay was highly accurate in separating colon cancer from healthy control (area under the curve 0.94, 95% confidence interval, CI, 0.89–0.99; Table III). Additionally the ROC curve was highly accurate in separating colon cancer from healthy control and all other patients population (area under the curve 0.8938 95% CI, 0.83–0.94). Using the ROC curve the cut off level of 0.6 OD was selected (Fig. 2).

Using a cutoff value from 0.6 OD the sensitivity was 88.8%; 24 from 27 colon cancer patients are detectable in serum with CCSA-2 and the specificity was 92.5%, 37 healthy individuals from 40 were identified with the assay as correctly negative. The overall specificity was 84.2%, 106 of the 127 individuals were diagnoses as normal were below the cut off (Table IV; Fig. 3).

DISCUSSION

The early diagnosis of CRC and the early detection of recurrence are central to the effective treatment of this disease. There is a consensus that CRC screening is effective and it can be prevented in many cases. Due to CRC screening the incidence of CRC has dropped in recent years, possibly due to the screening program [Mandel, 2005]. There is less consensus regarding optimal screening strategies, as sensitivity and specificity, and patient acceptance, limit current options. To overcome these barriers a range of approaches, including proteomics based testing, stool genetic testing, radiological imaging, and enhanced endoscopies has been the focus of intense research.

Presently, colonoscopy with a sensitivity of 97% and a specificity of 98% and a sensitivity of adenomas of at least 1 cm diameter of around 90% [Pickhardt et al., 2003; Winawer et al., 2003], is considered the gold standard for colon cancer diagnosis and offers the potential to both, find and remove premalignant lesions, but it is associated with high cost, patient discomfort, complication, and variable sensitivity given through the experience of the endoscopies.

A useful diagnostic assay must be sensitive and must detect the cancer in an early tumor

TABLE IIb. Summary of Data

Group	N	Mean	Standard deviation	Standard error of mean	Median
Colon cancer	27	>0.7359	>0.1576	>0.03034	>0.6900
Control	40	>0.5324	>0.06086	>0.009624	>0.5300
IBD	11	>0.5390	>0.06725	>0.02028	>0.5660
Colon polyps	20	>0.5686	>0.1329	>0.02971	>0.5400
Diverticulosis/itis	21	>0.5529	>0.09961	>0.02174	>0.5500
Other cancer type	37	>0.5687	>0.1445	>0.02375	>0.5450
After colon cancer	9	>0.4882	>0.05895	>0.01965	>0.4860
Inflammatory disease	9	>0.5573	>0.09594	>0.03198	>0.5340

Group	Minimum	Maximum	95% Confidence interval	
			From	To
Colon cancer	>0.5480	>1.250	>0.6735	0.7983
Control	>0.3990	>0.7200	>0.5129	0.5518
IBD	>0.4390	>0.6470	>0.4938	0.5841
Colon polyps	>0.3400	>0.8480	>0.5064	0.6308
Diverticulosis/itis	>0.4000	>0.7700	>0.5075	0.5982
Other cancer type	>0.3688	>1.031	>0.5204	0.6169
After colon cancer	>0.3920	>0.5690	>0.4429	0.5335
Inflammatory disease	>0.4390	>0.7300	>0.4836	0.6311

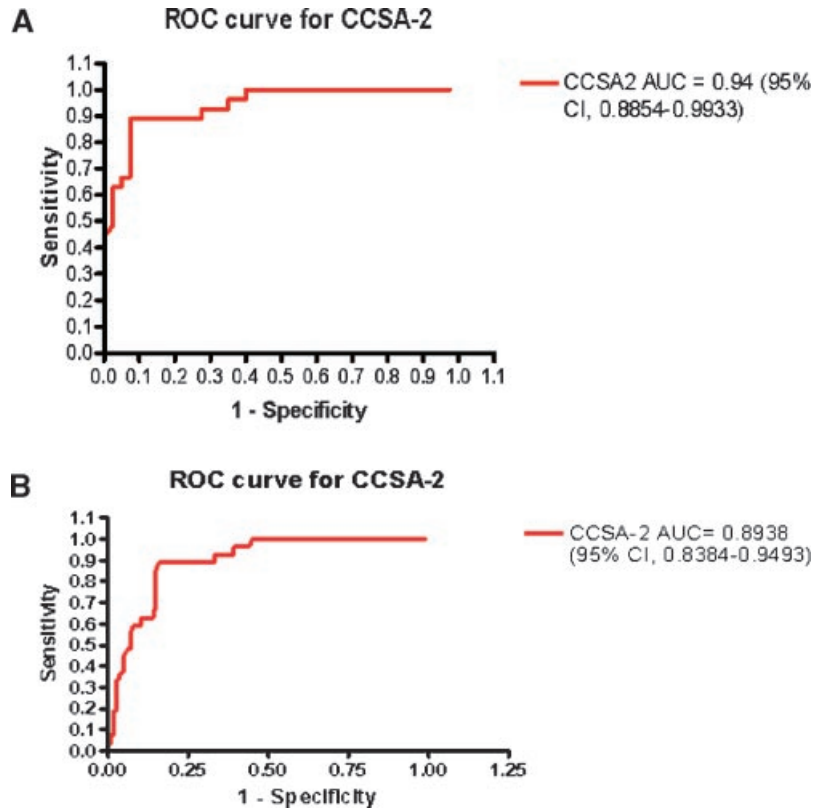

Fig. 1. **A**: Receiver-operator characteristic (ROC) curve for CCSA-2 in separating normal healthy patients and colon cancer patients. AUC: area under the ROC curve. **B**: Receiver-operator characteristic (ROC) curve for CCSA-2 in separating colon cancer patients from all other patients including healthy controls.

stage. Also it must have a high specificity to minimize false positives that necessitate cost or invasive examination and additional scares the patient and the families needlessly [Ahlquist, 1997]. That one biomarker will accomplish all these criteria will be almost impossible, but the combination of specific markers could have the possibility to meet the condition for a useful screening test in CRC.

TABLE III. Area Under the ROC Curve

1. ROC analyses for CCSA-2 in separating control individuals (normal colon) from colon cancer patients	
Area	0.9394
Std. error	0.02751
95% Confidence interval	0.8854–0.9933
P-value	<0.0001
Data	
Control	40
Colon cancer patient	27
2. ROC analyses for CCSA-2 in separating control individuals (normal colon) and all other patients from colon cancer patients	
Area	0.8938
Std. error	0.02831
95% Confidence interval	0.8384–0.9493
P-value	<0.0001
Data	
Control and all other patients	127
Colon cancer patient	27

This study shows that the ELISA, that detects serum based CCSA-2, is both sensitive and specific for colon cancer. In addition, this is the first time, that CCSA-2 has been detected in the serum from patients with advanced adenomas, confirming tissue data we could found in previous studies in colon polyps [Brunagel et al., 2004]. The serum based ELISA with CCSA-2 antibody demonstrated a sensitivity of 88.8% and considering the entire study population, a specificity of 84.2%.

Three of the colon cancer patients, two had a tumor stage UICC II and one patient UICC III were under the cut off point and therefore considered to be negative for CCSA-2. So far, we have no explanation, why these patients do not appear to express CCSA-2 in the serum. Previously studies have shown that CCSA-2 is expressed in 80% of colon cancer tissues (13). With the presumption that not all colon cancers may be express the NMP CCSA-2, we understood the limitations and the development of

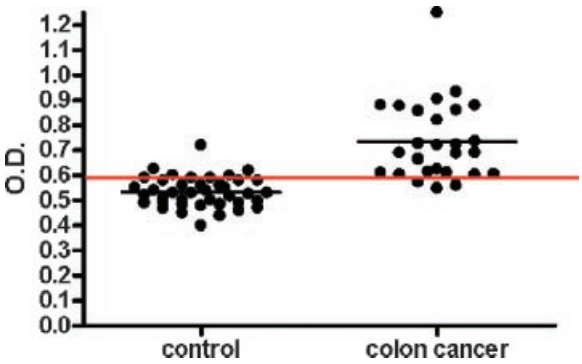

Fig. 2. Serum analysis of CCSA-2 in colon cancer and control. Using the ROC curve a cut off represented by red line of 0.6 OD, results in the optimal balance between sensitivity and specificity.

additional serum marker based on the other identified NMPs CCSA-3,4,5 could close this gap [Leman et al., 2007].

Based on evidence from epidemiological and pathological studies, most sporadic colon cancers are thought to develop from benign adenomas. Presently, there is no clear way of identifying which adenomas will become malignant. There is consent, that progression is associated with severe dysplasia, patient age, size of adenoma, and histological types [O'Brien et al., 1990]. Adenomas that are >1 cm, show severe dysplasia and/or villous architecture are described as advanced.

In previous studies, we demonstrated the expression of CCSA-2 in advanced polyps [Brunagel et al., 2004]. Four serum levels from patients with colon polyps are above the cut off point, three of which have advanced adenomas.

Three normal individuals showed an increased level of CCSA-2 in there serum. Regarding the colonoscopy report, the examination was not difficult and the colon clean. Reviewing the literature 4% of polyps or carcinoma are overseen in a colonoscopy especially in the right colon [Bressler et al., 2004]. In these cases and additionally in the cases with diverticulosis and IBD we can just speculate, if there was something overseen. However, especially in cases where colonoscopy is difficult, a serum marker, which could detect early colon cancer and furthermore advanced adenomas, would be very helpful.

Regarding the other cancers types, 9 patients out of 37 have an expression of CCSA-2 in the serum above the cut off point. Three patients with cholangiocarcinoma (3/6), one patient with lung cancer (1/4), four patients with gastric cancer (4/13), and one patient with hepatocellular carcinoma (1/3). None of the 11 patients with pancreatic cancer had an expression of CCSA-2 above the cut off point. There is no correlation of the tumor stage and the CCSA-2 expression (correlation coefficient (r) = −0.1687, r squared = 0.02847).

So far we have no explanation for the expression of CC2 in other cancer types.

To evaluate the effect of the removal of the colon cancer by surgery on the serum CCSA-2 value, samples were obtained from nine patients after colon cancer surgery 2–9 years after curative surgery. All nine individuals considered to be normal after curative colon cancer surgery.

Additionally, patients with benign inflammatory disease like pancreatitis and gastritis and diverticulitis and patients with IBD were studied. One patient with IBD (1/11), four patients with diverticulosis (4/21), and two patients with benign inflammatory disease (2/9) had CCSA-2 values above the cut off point. We could not observe a correlation between the elevated CCSA-2 levels and the grade of the inflammation.

Further studies are needed to examine the expression on CCSA-2 in other disease. Nevertheless the overall specificity of CCSA-2 is 84.2%, shown it is a specific marker for colon cancer. This is the first study demonstrating the ability of CCSA-2 antibodies to specifically identify colon cancer patients in a clinically applicable test. However, clinical trials need to be performed, for evaluation of the sensitivity

TABLE IV. Specificity/Sensitivity of Blood CCSA-2 Assay

	No. of samples <0.6 OD/total no. samples	Specificity %
Donors	>37/40	>92.5
All populations	>106/127	>84.2
	No. of samples >0.6 OD/total no. samples	Sensitivity %
Colon cancer	>24/27	>88.8

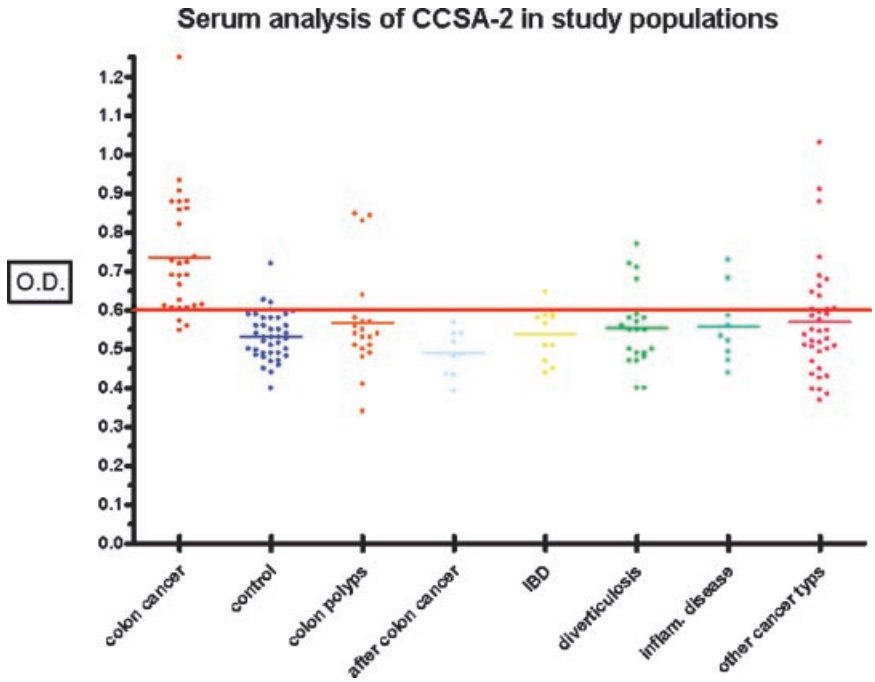

Fig. 3. Serum analysis of CCSA-2 in study populations. Total of 174 serum samples screened for CCSA-2 in indirect ELISA. Cut off value of 0.6 OD. Represented by red line across graph. Line in between the patients groups represents the median value of the group.

and specificity in independent validation studies in a larger population of patients.

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