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Multiple serological biomarkers for colorectal cancer detection

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The aim of this study was to initiate a survey of human autoantibody responses to a panel of select colorectal tumor-associated antigens identified by previous serological analysis of a cDNA expression library and to subsequently identify multiple serological biomarkers for the detection of colorectal cancer. For screening of autoantibodies against colorectal tumor-associated antigens, sera from 94 colorectal cancer patients and 54 normal controls were analyzed by enzyme-linked immunosorbent assay using recombinant rCCCAP, rHDAC5, rP53, rNMDAR and rNY-CO-16 proteins as coating antigens. Seropositivity among colorectal cancer patients to the 5 individual coating antigens varied from 18.1% to 35.1%. Seropositivity to any of the 5 coating antigens was 58.5% and combining this analysis with evaluation of serum carcinoembryonic antigen ($\geq$5 ng/ml) significantly increased the seropositivity to 77.6%. Seropositivity of early-stage (Dukes' Stages A and B) colorectal cancer patients to CEA was 21.9%, and seropositivity to any of the 5 colorectal cancer-associated antigens was 53.7%, and the combination of these 2 measurements resulted in a higher diagnostic capacity (65.9%) than either marker alone. In conclusion, these results collectively indicated that combined detection of serum autoantibody profiles against our panel of colorectal tumor-associated antigens and the analysis of carcinoembryonic antigen provides a promising diagnostic biomarker for colorectal cancer, particularly among early-stage patients.

According to the survey of International Agency for Research on Cancer, colorectal cancer is the third most common cancer worldwide, with an estimated 1 million new cases and half a million deaths annually.[1] Detection of colorectal cancer at an early stage is critical for therapeutic success; however, it is necessary to point out that the symptoms of colorectal cancer are nonspecific, and many patients with early-stage colorectal cancer are asymptomatic. Clinically, the disease stage at initial diagnosis is the most important prognostic factor for patients with colorectal cancer. The prognosis for colorectal cancers diagnosed at an early stage (Dukes' Stages A and B) is superior, with a 5-year survival estimate of 97%. Unfortunately, about 60% of colorectal cancer patients are first diagnosed at an advanced stage (Dukes' Stages C and D) with an expected 5-year survival rate of 5%.[2–4] Therefore, improved detection methods are crucial to identify curable early-stage colorectal cancer.

Tumor markers are widely used for detection and monitoring of cancers in clinical laboratory tests. Although many biomarkers have been evaluated for colorectal cancer, only a small number have been recommended for clinical use. According to National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines, the approved clinical tumor markers for colorectal cancer include the carcinoembryonic antigen (CEA) for postoperative surveillance, the adenomatous polyposis coli gene for screening of familial adenomatous polyposis (FAP), mutation in the mismatch repair gene for screening hereditary nonpolyposis colorectal cancer (HNPCC) and the fecal occult blood test (FOBT) for screening asymptomatic subjects (recommended for patients $\geq$50 years of age).[3,5,6] Although FOBT is the least invasive screening method available, the major limitations of FOBT include relatively poor sensitivity for detecting early-stage lesions and a high false-positive rate.[5,7,8]

Other potential colorectal tumor markers such as CEA, CA 242, CA 19-9, CA 50, tissue plasminogen activator, tissue-polypeptide-specific antigen and tissue inhibitor of metalloproteinase 1 have also been studied extensively. However, none of these serological markers has demonstrated both

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high sensitivity and high specificity in clinical tests to detect early-stage colorectal cancer.[9–12] Despite the high false-positive rate and the low sensitivity for colorectal cancer detection, the serological marker CEA is still widely used in the diagnosis and follow-up of colorectal cancer patients.[13–15] Therefore, there is a need for the development of more sensitive and specific markers that can supplement CEA for the detection of this disease.

The identification of altered expression patterns of tumor-associated autoantibodies for use as diagnostic biomarkers has been of interest to the field of cancer research. Previous studies have shown that humoral immune responses are triggered during certain stages of tumorigenesis.[16,17] In hepatocellular carcinomas, for example, several autoantibody responses have been detected during the transition from chronic liver disease to malignancy.[17,18] It is worth noting that antibody responses to tumor antigens such as p53 in early-stage colorectal cancer patients have also been reported,[19–21] suggesting that of serological autoantibodies may provide immunodiagnostic markers for improved early-stage cancer detection. Although the mechanism underlying the production of such autoantibodies is not completely understood,[22,23] it has been proposed that screening of tumor antigen-associated autoantibodies may enhance early detection of cancer.[19,21,24–29]

Serological responses to colorectal tumors are heterogenic. In colorectal cancer patients, elevated levels of serum autoantibodies against p53, p62, CEA, HER-2/*neu*, Ras, topoisomerase II-alpha, histone deacetylases 3 and 5, ubiquitin C-terminal hydrolase L3, tyrosinase, tropomyosin and cyclin B1 have been detected.[30] Previous observations have shown that these autoantibodies are only present in a limited proportion of patients (usually $< 40\%$) and that the sensitivity of detection was insufficient for use in routine diagnosis. However, this drawback could be overcome by using a panel of carefully selected tumor associated antigens to achieve the sensitivity and specificity required for clinical application.[30,31]

The serological analysis of recombinant cDNA expression libraries (SEREX) method was first used by Sahin *et al.*[26] in 1995 to analyze the humoral immune response to human cancer antigens. Since then, it has been applied to a variety of human cancer types resulting in the detection of more than 1,500 potential immunogenic antigens.[32,33] With respect to colon cancer, Scanlan *et al.* and Line *et al.*[28,33,34] successfully applied the SEREX approach to identify several colorectal tumor-associated antigens. However, the utility of the tumor-associated autoantibodies identified in their studies for clinical diagnosis of colorectal cancer has yet to be established.

The aim of this study was to develop an enzyme-linked immunosorbent assay (ELISA) using a panel of 5 select SEREX-defined recombinant fragment as coating antigens, to measure the prevalence of antibodies to those antigens in serum from patients with colorectal cancer and to evaluate the diagnostic potential of these antibodies for colorectal cancer detection. We determined that combined detection of antibodies to those 5 antigens and CEA largely improved the sensitivity of the assay to identify patients with early-stage colorectal cancer.

Material and Methods

Serum samples

Serum samples were obtained from 94 patients with colorectal cancer. As control, serum samples were obtained from 54 individuals undergoing annual health examinations and that had no evidence of malignancy. All serum samples were obtained from Chang Gang Memorial hospital (Lin-Kou, Taiwan, Republic of China) and informed consent was obtained from each subjects. Among the colorectal cancer patients, 52 (55.3%) were men and 42 (44.7%) were women, and the mean age was 64 years (range, 26-86 years). All colorectal cancer patients had histologically verified adenocarcinoma of the colon or rectum and were classified according to the Dukes' stage criteria: 7 (7.4%) patients were in Dukes' A stage, 34 (36.2%) were in Dukes' B stage, 38 (40.4%) were in Dukes' C stage and 15 (16%) were in Dukes' D stage. Retrospective blood samples for antibody screening and CEA measurements were taken preoperatively from all cancer patients and control sera were obtained from healthy subjects during routine annual health examinations. Sera were stored at -80°C until analysis. Serum CEA levels were measured at Chang Gang Memorial hospital using a luminescence immunoassay. Follow-up of all patients was performed retrospectively for at least 5 years, and the period from surgery to death was recorded. This study was approved by the Medical Ethics and Human Clinical Trial Committee at Chang Gung Memorial Hospital, Keelung, Taiwan.

Preparation of recombinant colorectal tumor associated antigens

Five antigens previously identified by SEREX analysis-NY-CO-8 (centrosomal colon cancer autoantigen protein; CCCAP),[35] NY-CO-9 (histone deacetylase 5; HDAC5), NY-CO-13 (P53), NY-CO-16 and NY-CO-20 (glutamate binding subunit of the NMDA receptor; NMDAR) were selected for recombinant protein expression.[28,33,34] The cDNA sequences of these 5 colorectal tumor-associated antigens (TAA)were analyzed using GenoMax Version 3.4 software (Infor-Max, Bethesda, MD), and the encoded polypeptide fragment of each cDNA exhibiting the highest antigenic and hydrophilic potential was selected for the following cloning procedures. Total RNA was prepared from colorectal adenocarcinoma Lovo cells (ATCC, CCL-229, Manassas, USA) using the TRIzol™ reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized in a total volume of 20 μL using 2 μg of total RNA, 500 ng of oligo-dT primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The sequences of each primer were as follows: rCCCAP, 5′-AGAAGGATCCATCCGA-GAAGCTAAAACT-3′(sense) and 5′-ATACTCGAGGTG-CAGTTGGTGCTCAGATT-3′(antisense); rHDAC5, 5′-TCCAGAATTCCGACACGTTCATGCTAAA-3′(sense) and

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5′-ATAACTCGAGACAAGCTTCAGAGGCATCACA-3′(antisense); rP53, 5′-AGTCATATGGAGGAGCCGCAGTCAGATCCTAGC-3′(sense) and 5′-CTTGGATCCTCAGTCTGAGTCAGGCCCTTCTGTCTT-3′(antisense); rNY-CO-16, 5′-TCTGAATTCTCTGGCTTTGAGCCACCAGGA-3′(sense) and 5′-GTGTCTCGAGCCGTTCAATCTCTTCTTTGT-3′(antisense) and rNMDAR, 5′-ATCTGAATTCTCATTGTTGCATGAGCCCT-3′(sense) and 5′-CTGGCTCGAGTATTGGAATTTCACTGACA-3′(antisense). The PCR cycling conditions consisted of a single incubation step at 95°C for 10 min, followed by 30 cycles of 20 sec at 95°C, 30 sec at 56°C and 60 sec at 72°C. The reaction products were subcloned into pET29b (Novagen, Darmstadt, Germany) to produce a fusion protein containing N-terminal His_6 tag and T7 epitope tags. The recombinant proteins expressed in *Escherichia coli* BL21 (DE3) were purified using a HiTrap Chelating column (Pharmacia, Sunnyvale, CA). Expression of the 5 recombinant antigen fragments were examined using SDS-PAGE analysis followed by Coomassie blue staining to verify that the expression products had the expected molecular mass. In addition, the identity of the recombinant antigen fragments was confirmed by immunoblotting using a mouse monoclonal antibody against the His_6 tag (Santa Cruz Biotechnology, Santa Cruz, CA).

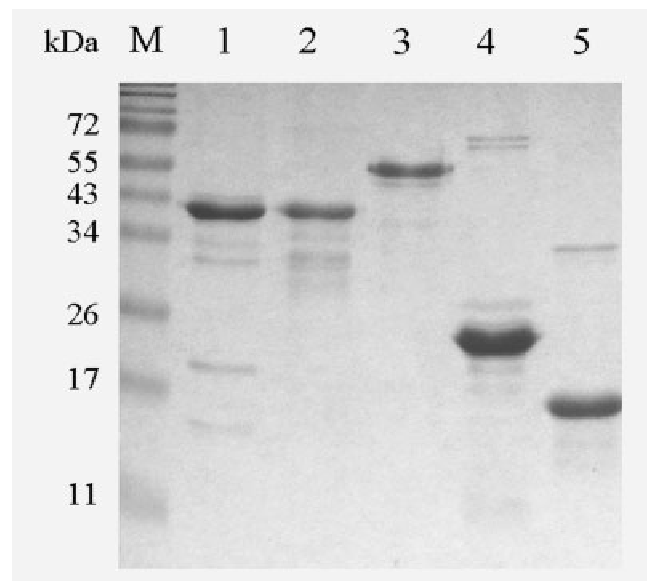

Figure 1. SDS-PAGE analysis following nickel chelate chromatography of the 5 His_6-tagged recombinant colorectal tumor-associated antigens. Recombinant protein bands were stained by Coomassie blue. M, molecular mass markers; Lane 1, rCCCAP antigen; Lane 2, rHDAC5 antigen; Lane 3, rP53 antigen; Lane 4, rNY-CO-16 antigen and Lane 5, rNMDAR antigen.

Enzyme-linked immunosorbent assay

An indirect ELISA was performed according to a previous method.[36] Briefly, purified recombinant proteins were individually diluted in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic and a pH of 7.6) to a final concentration of 0.2 μg/ml and 200 μl was pipetted into each well of a microtiter plate (Costar, Corning, NY) for coating overnight at 4°C. Free binding sites were blocked with PBS containing 2.5% (w/v) bovine serum albumin. Serum samples were diluted (1:200) and incubated in the antigen-coated wells at room temperature for 2 hr, then incubated with 1:5,000 diluted horseradish peroxidase (HRP)-conjugated mouse monoclonal antibody against human IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hr followed by washing with PBS containing 0.05% Tween 20. The peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (KPL, Gaithersburg, MD) was used for detection. The optical density at 405 nm was determined for each sample using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA), and the cutoff value for determining a positive signal was designated as the mean absorbance of the 54 control serum samples plus 2 standard deviations (mean + 2 SD). Each sample was tested in triplicate, and 10 control sera were used in each experiment as an internal control.

Statistical analysis

Statistical analysis was performed using the SPSS program for Windows version 12.0 (SPSS Inc., Chicago, IL). Antibody reactions to SEREX-defined antigens in the serum of colon cancer patients and normal controls were analyzed using Student's *t* test to validate correlation with the presence of colorectal cancer. The χ^2 test was used to determine the significance of clinical parameter differences with respect to the presence or absence of antibody to colorectal cancer antigens. Assay sensitivity was defined as the percentage of diagnosed colorectal cancer patients that were correctly predicted to have colorectal cancer. Specificity was defined as the percentage of normal subjects that were correctly predicted to not have colorectal cancer. $p < 0.05$ was accepted as statistically significant.

Results

Seropositivity to the panel of 5 colorectal tumor-associated antigens among colorectal cancer patients

Using an indirect ELISA, 5 recombinant SEREX-defined colorectal tumor-associated antigens (Fig. 1) were applied to microtiter plates individually, and serum samples from 94 colorectal cancer patients and 54 normal controls were examined for the presence of autoantibodies against these individual antigens (Fig. 2). A positive signal was assigned to those samples with an optical density (OD) value greater than 2 SD above the mean optical density value of the 54 normal human serum samples.

As shown in Table 1, the frequency of colorectal cancer patient serum immune responses to any of the 5 individual colorectal tumor-associated antigens varied. The rNY-CO-16 antigen elicited the lowest response (18.1% sensitivity with 100% specificity) and rCCCAP elicited the highest response

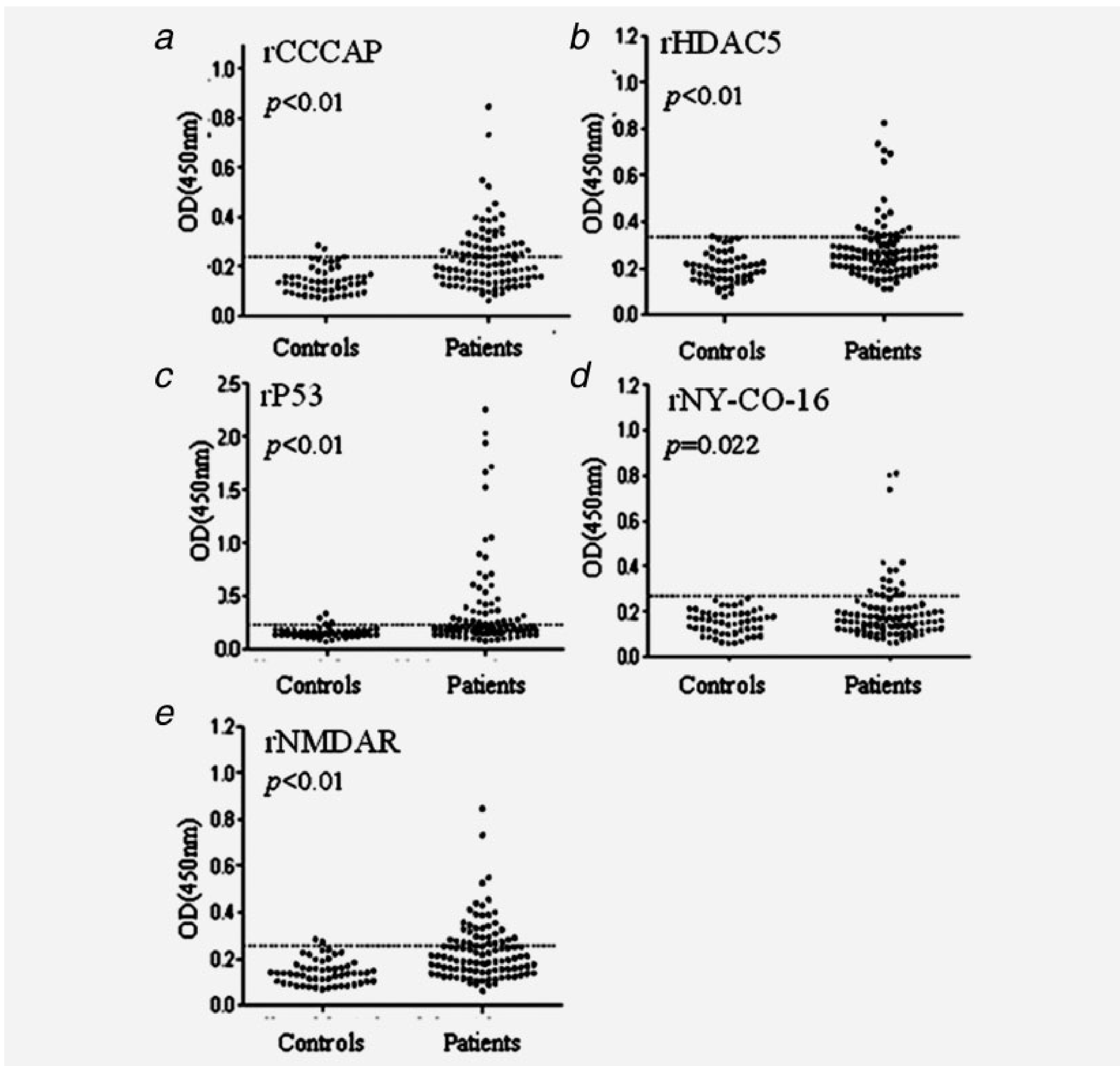

Figure 2. Comparison of autoantibodies against colorectal tumor-associated antigens in serum from colorectal cancer patients and healthy control donors using indirect ELISA analysis. The coating recombinant antigens used in the ELISA tests were rCCCAP (*a*), rHDAC5 (*b*), rP53 (*c*), rNY-CO-16 (*d*) and rNMDAR (*e*). Antibody titers to each of the 5 antigens, expressed as optical density (OD), are significantly higher in patients with colorectal cancer than in healthy controls. The horizontal line indicates the cutoff value for seropositivity. Seropositivity was defined as a signal $\geq$ 2 SD over the mean signal of 54 normal controls.

(35.1% sensitivity with 96.3% specificity). Among the colorectal cancer patients analyzed, 58.5% (55/94) of serum samples had antibodies against at least 1 of these 5 TAA. In this subset of seropositive patients, serum from 22 of 55 (40%) patients had antibodies that reacted with a single TAA, whereas 33 of 55 patients (60%) had antibodies that reacted with 2 or more of the 5 TAA. The seropositivity of control sera to each TAA was low, ranging from 0 to 3.7%, with a seropositivity of 7.4% when controls were examined using a cocktail of all 5 TAA.

CEA and colorectal tumor-associated antigens seropositivity in relation to clinicopathologic characteristics of colorectal cancer patients

The association between colorectal cancer patients with colorectal tumor-associated antigens seropositivity, and the

Table 1. Comparison of control and colorectal cancer patient seropositivity to each of 5 colorectal tumor–associated antigens[1]

	Fraction of seropositive samples			
Type of antigen	Colorectal cancer	Normal control	Sensitivity	Specificity
rCCCAP	33/94	2/54	35.1%	96.3%
rHDAC5	19/94	2/54	20.2%	96.3%
rP53	23/94	1/54	24.5%	98.1%
rNY-CO-16	17/94	0/54	18.1%	100%
rNMDA	19/94	2/54	20.2%	96.3%
Any of 5 antigens[2]	55/94	4/54	58.5%	92.6%

[1]Analysis determined using ELISA. Seropositivity was defined as a signal $\geq$ 2 SD over the mean signal of 54 normal controls. [2]Five colorectal tumor-associated antigens were recombinant centrosomal colon cancer autoantigen protein (rCCCAP), recombinant histone deacetylase 5 (rHDAC5), recombinant P53 (rP53), recombinant NY-CO-16 and recombinant glutamate binding subunit of the NMDA receptor (rNMDAR), respectively.

Table 2. The association between clinical stage of colorectal cancer and serum CEA levels and the presence of autoantibodies to any of 5 colorectal tumor-associated antigens

		Number of seropositive samples (%)		
Dukes' stage	Number tested	CEA level[1] (I)	Any of 5 antigens[2] (II)	Combination (I and II)
A	7	0 (0%)	5 (71.4%)	5 (71.4%)
B	34	9 (26.5%)	17 (50.0%)	22 (64.7%)
C	38	18 (47.4%)	22 (57.9%)	31 (81.5%)
D	15	13 (86.7%)	11 (73.3%)	15 (100%)
Early stage (A and B)	41	9 (21.9%)	22 (53.7%)	27 (65.9%)
Advanced stage (C and D)	53	31 (58.4%)	33 (62.2%)	46 (86.7%)
All stage	94	40 (42.5%)	55 (58.5%)	73 (77.6%)

[1]CEA level cutoff was set at >5 ng/ml. [2]Seropositivity to any of the 5 antigens — rCCCAP, rHDAC5, rP53, rNY-CO-16 and rNMDAR was defined as a signal $\geq$ 2 SD over the mean signal of 54 normal controls.

Dukes' stage was analyzed (Table 2). Our data revealed that 5/7 (71.4%), 17/34 (50%), 22/38 (57.9%) and 11/15 (73.3%) of serum samples from patients in Dukes' stages A, B, C and D, respectively, were positive for 1 or more of the 5 TAA autoantibodies. Furthermore, 22/41 (53.7%) and 33/53 (62.3%) serum samples from patients with early stage and advanced stage colorectal cancer, respectively, were positive for 1 or more of the 5 TAA autoantibodies. The average overall survival of patients with serum antibodies against any of the 5 TAA was 60 months, whereas survival of patients lacking these serum antibodies was 66 months. However, this difference was not statistically significant ($p = 0.328$, by Kaplan-Meier test). Similarly, seropositivity to 1 or more of the 5 TAA was also not statistically associated with gender, age, tumor location or Dukes' classification in colorectal cancer patients (data not shown). However, our data show that the anti-TAA assay was significantly powerful than serum CEA to detect tumor from several groups such as age $\geq$ 70 years, left-sided colon (splenic flexure of transverse colon, descending colon, rectosigmoid and rectum) and early-stage colorectal cancer patients (Table 3).

For colorectal cancer patients, the median serum CEA level was 3.68 ng/ml (range, 0.5–1,120 ng/ml). When a cutoff value of 5.0 ng/ml was used, the percent of positive serum samples became higher as the disease stage advanced. As seen in Table 2, 0%, 26.5%, 47.4% and 86.7% of serum samples from patients in Dukes' stages A, B, C and D, respectively, were CEA positive. CEA seropositivity was 21.9% (9/41) for patients in the early-stage group and 58.4% (31/53) for patients in the advanced-stage group. The difference between those 2 groups was significant ($p = 0.001$).

For colorectal cancer detection (Table 2), the CEA assay and analysis of serum autoantibodies against any of the 5 TAA had sensitivities of 42.5% and 58.5%, respectively. When results from the CEA and Anti-TAA analyses were combined, the sensitivity increased to 77.6%. This increase was statistically significant ($p < 0.001$). In addition, for early-stage colorectal cancer patients, combination of the assays resulted in a seropositivity of 65.9% (27/41) compared with 21.9% (9/41) seropositivity for the CEA assay alone and 53.7% (22/41) for the anti-TAA assay alone.

Discussion

The selection of discriminative antigens for serum antibody screening is critical for diagnostic success. In this study, we were interested in increasing the sensitivity and specificity of autoantibody-based diagnostic markers for detection of colorectal cancer by selecting TAA with improved immunoreactivity. Antigens that were defined during previous SEREX technique[28,33,34] and that exclusively reacted with serum

Table 3. Comparison of CEA and autoantibody seropositivity to clinicopathologic characteristics of colorectal cancer patients

Clinicopathologic characteristics	Number tested	Seropositivity		p Value[3]
		Any of 5 antigens[1]	CEA[2]	
Gender				
Male	52	27 (51.9%)	21 (40.4%)	0.325
Female	42	28 (66.7%)	19 (45.2%)	0.078
Age (years)				
<70	57	29 (50.9%)	24 (42.1%)	0.453
≥70	37	26 (70.3%)	16 (43.2%)	0.034
Tumor location[4]				
Right-sided colon	7	2 (28.6%)	2 (28.6%)	1.00
Left-sided colon	87	53 (60.9%)	38 (43.7%)	0.033
Dukes stage				
Early stage (A and B)	41	22 (53.7%)	9 (22.0%)	0.006
Advanced stage (C and D)	53	33 (62.3%)	31 (58.5%)	0.843
All stages (A–D)	94	55 (58.5%)	40 (42.6%)	0.041

[1]Seropositivity to any of the 5 antigens — rCCCAP, rHDAC5, rP53, rNY-CO-16 and rNMDAR was defined as a signal ≥ 2 SD over the mean signal of 54 normal controls. [2]CEA level cutoff was set at >5 ng/ml. [3]Using Chi-square test. [4]Right-sided colon: cecum, ascending colon and hepatic flexure of ascending colon; left-sided colon: splenic flexure of transverse colon, descending colon, rectosigmoid and rectum.

antibodies derived from colorectal cancer patients but not with serum from normal controls were selected as candidate antigens for evaluation in this study. In our preliminary study, we have tested those antigens for their diagnostic potential. We found that some antigens such as NAP1L1 (nucleosome assembly protein 1-like 1), EPRS (Glu-Pro tRNA synthetase), C21ORF2 (chromosome 21 open reading frame 2) or survivin did not elicit sufficient immune responses for clinical use and were therefore excluded from the present study. Finally, we selected and constructed 5 specific recombinant colorectal tumor associated antigens-rCCCAP, rP53, rHDAC5, rNMDAR and 1 unknown colorectal cancer-associated rNY-CO-16 protein as screening antigens in this study.

Of the 5 SEREX-based antigens examined in this study, serum samples from colorectal cancer patients reacted to rCCCAP with the highest frequency (35.1%) and to rNY-CO-16 with the lowest frequency (18.1%). Similarly, Scanlan *et al.*[34] demonstrated a frequency of serological response to 13 colorectal cancer-related antigens of 3–8% and Shebzukhov *et al.*[37] identified HDAC3 as a novel serological antigen for colon cancer detection with a 5% serological response frequency. A compilation of anti-p53 studies indicates the reaction frequency among colorectal cancer patient serum samples was 15–24.6%.[20,34] Collectively, these data indicate that the seroprevalence of autoantibodies to an individual antigen is low in colorectal cancer patients; this differs from the high frequency of humoral immune response to the annexin I/II antigen observed among lung cancer patients (60%)[38] and to the PHF-3 antigen among glioblastoma multiforme patients (60%).[39] Together, these data suggest that analysis of serum antibodies to a multiple-antigen panel, rather than a single antigen, improves the diagnostic capability for detection of colorectal cancer.

In this study, we also evaluated whether a panel of 5 TAA could enhance the diagnostic capacity of an ELISA-based colorectal cancer assay. We found that 58.5% of serum samples from colorectal cancer patients elicited an immune response to 1 or more of the 5 TAAs examined. This was higher than an earlier study which showed that, for colorectal cancer patients, the percent of serum samples with antibodies against any 1 of 7 selected antigens was between 4.4% and 15.6%, and the percentage reached 51.1% when all 7 antigens were analyzed in combination.[31] Another similar result was reported in which signature autoantibodies against a combination of prostate-specific antigens were analyzed for prostate cancer detection.[40] These results point out that the serological reactivity to an individual TAA is relative low and that combining multiple biomarkers significantly improves the diagnostic power of colorectal cancer detection assays. An important question concerning the antigenicity should be taken into consideration when interpretation of our results. In comparison with full-length antigen proteins, the seropositivity may decrease because of losing some conformational epitopes when using partial recombinant protein as screening antigens. The main purpose of this study is to set up an ELISA format for rapid screening autoantibodies in sera of colorectal cancer patients. The sensitivity and specificity of our antigens panel could be improved by using more specific or full-length of candidate antigens in the future.

For colorectal cancers, recent studies have reported that some serum antibodies were correlated with clinicopathologic characteristics such as tumor location, histological differentiation grade and patient survival rate.[41,42] Overall, we did not

find a significant association of clinicopathologic characteristics with serum antibodies against the 5 TAA examined. However, for some unknown reasons, the cancer diagnostic power was significantly better than serum CEA from particular colorectal cancer patients (Table 3). One possible explanation of this situation was that the CEA and anti-TAA antibodies were caused by different pathological mechanisms.

We also examined the prevalence of autoantibodies to our panel TAA in sera from patients with non-colorectal cancers and explored the possibility that patients with colorectal and non-colorectal cancers exhibit distinct autoantibody profiles. Intriguingly, using our panel of 5 TAA, seropositivity varied for different types of cancers (Table 1 and Supplement Table 1). Specifically, a higher percentage of seropositivity was observed among colorectal cancer patients compared with that of non-colorectal cancer patients (>17.1% *vs.* <10%, respectively). This phenomenon suggests that our assay may provide organ-specific diagnostic capability. However, this inference needs to be substantiated using a larger number of patient samples.

Several studies have shown that detection of serum autoantibodies against tumor-associated antigens might be useful in early-stage cancer diagnosis. For example, serum anti-P53 antibodies were detected before clinical detection of lung cancer[43] and oral cancer,[44] and anti-HER-2/*neu* antibodies were detected in serum from early-stage breast cancer patients.[45] Although several colorectal tumor-associated serum autoantibodies have been identified, none of them has sufficient sensitivity for detection of colorectal cancer at an early stage.[10,30]

CEA, a glycoprotein consisting of ~60% carbohydrate with a molecular mass of 180–200 kDa, is one of the most widely used tumor markers worldwide and certainly the most frequently used marker for detection of colorectal cancer.[46,47] Using 5 μg/l CEA concentration as the cutoff for detection, Wanebo *et al.*[48] found that CEA seropositivity was 3%, 25%, 45% and 65% for patients with Dukes' stages A, B, C and D colorectal cancer, respectively. From a screening perspective, we deemed it necessary to demonstrate expression of serum antibodies against our panel of 5 TAA in early-stage colorectal cancer patients and to determine how these responses overlap with the serum CEA biomarker. We found that combining results from the CEA assay with the anti-TAA assay increased the detection of early-stage colorectal cancer from 21.9% to 65.9% (Table 2). Similarly, a study by Holten-Anderson *et al.*[42] has shown that combining serum CEA and metallopeptidase inhibitor 1 measurements resulted in a 66% detection frequency among early-stage colon cancer patients. Our findings demonstrated that autoantibody signatures may possess potential to supplement other serological markers for improved diagnosis of early-stage colorectal cancer, especially for CEA-negative individuals.

In conclusion, our studies further confirm and extend previous observations that autoantibody responses to tumor antigens do occur in early-stage colorectal cancer patients and that our panel of multiple colorectal tumor-associated antigens enhances tumor associated autoantibodies detection. Detection of these antibodies against colorectal tumor-associated antigens provides a noninvasive approach for colorectal cancer diagnosis, especially when used together with CEA analysis. Future analysis using a large cohort prospective study is needed to validate the utility of our panel of colorectal tumor-associated antigens for colorectal cancer detection.

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