Initial Analyses of Colon Cancer–Specific Antigen (CCSA)-3 and CCSA-4 as Colorectal Cancer–Associated Serum Markers

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Abstract

Colon cancer–specific antigen (CCSA)-3 and CCSA-4 are novel colon cancer markers identified by focused proteomic analysis of nuclear structural proteins. The goal of these studies was to evaluate serum-based CCSA-3 and CCSA-4 in the detection of individuals with preneoplastic and neoplastic lesions using ELISAs. Serum samples from 107 subjects undergoing colonoscopy, 28 subjects with colorectal cancer, and 125 subjects with benign disease or other types of cancer were evaluated. Individuals who underwent colonoscopy were classified into mutually exclusive categories, including normal colon, hyperplastic polyp, nonadvanced adenoma, and advanced adenoma. Sensitivity and specificity for both CCSA-3 and CCSA-4 were evaluated using receiver operating characteristic (ROC) curves. At a cutoff of 2 μg/mL for CCSA-3 and 0.3 μg/mL for CCSA-4, each marker detected all 28 colorectal cancers, for a sensitivity of 100% (lower 95% confidence bound, 89.8%). The sensitivity for detection of the combined end point of colorectal cancer and advanced adenoma for CCSA-3 was 89.1% [95% confidence interval (95% CI), 76.4–96.4%] and for CCSA-4 was 84.8% (95% CI, 71.1–93.7%) and 91.3% (95% CI, 79.2–97.6%) for either marker positive. The specificity in individuals with normal, hyperplastic polyps, or nonadvanced adenomas was 82.0% (95% CI, 72.4–89.4%) and 91.0% (95% CI, 83.0–96.0%) for CCSA-3 and CCSA-4, respectively. ROC curves for CCSA-3 and CCSA-4 reveal an area under the curve of 0.94 (95% CI, 0.90–0.98%). In these initial analyses, CCSA-3 and CCSA-4 show promise as potential serum markers for detection of colorectal cancer and advanced adenomas. [Cancer Res 2007;67(12):5600–5]

Introduction

Colorectal cancer is one of the world's most common neoplasms. Colorectal cancer is thought to evolve from adenomatous polyps, a precursor lesion that when accompanied by acquired genetic mutations in tumor suppressor genes, oncogenes, and others can advance to invasive cancer (1, 2). Because the evolution of the adenoma-carcinoma sequence takes 5 to 10 years or longer (1), there is ample opportunity for early intervention. Data from randomized trials of fecal occult blood testing show that screening for colorectal cancer can reduce mortality by detecting cancer at an earlier stage (3, 4). Colonoscopy is the most accurate screening test, but it is as yet unclear whether its improved efficacy is outweighed by the additional complications, inconvenience, and cost (5). Despite the proven benefits of screening, surveys show that <50% of eligible subjects are up to date with current screening recommendations (6).

A signature of cancer cells is change in nuclear structure and architecture. Alterations in nuclear matrix proteins have been identified in various cancers, including breast, prostate, bladder, lung, ovarian, and squamous carcinoma of the head and neck (7). We have previously identified colon cancer–specific nuclear matrix proteins that were present in cancer tissue, but not found in normal adjacent tissue nor in the tissue of subjects without colon cancer (8). Antibodies against these proteins have been analyzed along the continuum of normal mucosa, adenomatous polyp, and cancer tissue (9). Using ELISAs for the detection of two colorectal cancer–specific proteins, colon cancer–specific antigen (CCSA)-3 and CCSA-4, expression in the serum of subjects undergoing colonoscopy, in subjects with colorectal cancer, and in control subjects with other cancers and benign diseases was examined.

The main objective of this study is to determine the performance characteristics of the CCSA-3 and CCSA-4 immunoassays. This study examines the performance of both CCSA-3 and CCSA-4 in relevant clinical populations but the sensitivity and specificity values obtained are not necessarily reflective of a screening population.

Materials and Methods

Sample population. Serum samples (n = 125) were collected from patients undergoing colonoscopy or colon cancer surgery at the University of Pittsburgh Medical Center (UPMC) as part of the studies for the National Cancer Institute Early Detection Research Network. The study population included those presenting for screening colonoscopies as well as symptomatic subjects including those for evaluation of rectal bleeding, fecal occult blood, and change in bowel habits. All subjects provided informed consent under protocols approved by the Institutional Review Board of the University of Pittsburgh (Table 1). For subjects undergoing colonoscopy, samples were generally drawn from a fasting participant, immediately before the colonoscopy procedure, and thus before knowledge of the colonoscopy result. An adenoma was defined as advanced if it contained villous features (villous or tubulovillous adenomas), was large (≥1 cm as measured by the pathologist or by the endoscopist when removed in a piecemeal fashion), or had high-grade dysplasia. Based on the histologic results of the colonoscopy, subjects were classified into one of four mutually exclusive, hierarchical categories: normal colonoscopy (n = 30), hyperplastic polyp (n = 23), nonadvanced adenoma (n = 36), and advanced adenoma (n = 18). To increase recruitment of subjects with advanced adenoma, some subjects with polyps ≥1.0 cm were recruited and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Table 1. Sample population characteristics

	UPMC						JHH			
	Normal (n = 30)	Hyperplastic (n = 23)	Nonadvanced adenoma (n = 36)	Advanced adenoma (n = 18)	Colorectal cancer (n = 18)	All subjects (n = 125)	Benign disease (n = 58)	Other cancer (n = 67)	Colon cancer (n = 10)	All subjects (n = 135)
Gender (% female)	56.7	47.8	27.8	38.9	50.0	43.2	53.4	26.9	20.0	37.8
Race (% Caucasian)	93.3	91.3	100.0	100.0	83.3	94.4	N/A	N/A	N/A	N/A
Age (y)										
Median	61	54	63	57	66	61	52	65	63	60
Range	34–73	37–79	38–86	48–80	49–93	34–93	17–85	37–82	47–87	17–87
Family history										
CRC* (%)	26.7	30.4	13.9	11.1	16.7	20.0	N/A	N/A	N/A	N/A
Personal cancer history (%)										
Colorectal cancer	0.0	4.3	2.8	0.0	100.0	4.0	N/A	N/A	N/A	N/A
Other cancer	13.3	17.4	19.4	11.1	5.6	14.4	N/A	N/A	N/A	N/A
Any cancer	13.3	21.7	22.2	11.1	100.0	18.4	N/A	N/A	N/A	N/A
CCSA-3 (μg/mL)										
Mean ± SD	0.86 ± 0.38	1.15 ± 0.58	1.79 ± 0.51	2.49 ± 0.66	3.54 ± 1.35	1.80 ± 1.13	1.14 ± 0.46	1.42 ± 0.56	2.71 ± 0.65	1.39 ± 0.66
Range	0.51–1.79	0.48–2.77	0.92–3.01	1.28–3.60	2.24–7.47	0.48–7.47	0.26–1.79	0.23–2.92	2.07–4.02	0.23–4.02
CCSA-4 (μg/mL)										
Mean ± SD	0.13 ± 0.05	0.14 ± 0.10	0.23 ± 0.13	0.39 ± 0.17	0.74 ± 0.33	0.29 ± 0.26	0.13 ± 0.06	0.15 ± 0.07	0.70 ± 0.21	0.18 ± 0.17
Range	0.04–0.24	0.04–0.47	0.07–0.60	0.15–0.68	0.31–1.31	0.04–1.31	0.01–0.31	0.04–0.42	0.52–1.16	0.01–1.16

NOTE: Serum samples were collected from 260 individuals.
Abbreviations: CRC, colorectal cancer; N/A, not available.
*Defined as a first-degree relative with colorectal cancer.

blood was drawn after the colonoscopy procedure was completed and the effect of the conscious sedation had abated. Over 92% of subjects with normal, hyperplastic, or nonadvanced adenoma were drawn preprocedure. Eight of 18 subjects with an advanced adenoma were drawn after procedure, usually within 45 min of the completion of the colonoscopy. Of the 18 cancer subjects recruited at the University of Pittsburgh, 11 had colon and 7 had rectal cancer; 2 had blood drawn precolonoscopy, and 16 were drawn preoperatively, in the holding area immediately before the cancer surgery. Samples were stored at −80°C before analysis.

In addition to the serum samples from the University of Pittsburgh, 135 serum samples from the Departments of Pathology and Urology at Johns Hopkins Hospital (JHH) were also studied. These samples consisted of individuals with colon cancer (n = 10), as well as individuals with other benign diseases and cancers [n = 67; breast cancer (n = 10), liver cancer (n = 10), pancreatic cancer (n = 10), renal cancer (n = 9), bladder cancer (n = 9), lung cancer (n = 9), and prostate cancer (n = 10)], and individuals with benign diseases [n = 58; breast disease (n = 9), liver disease (n = 10), pancreatic disease (n = 10), renal disease (n = 10), lung disease (n = 9), and prostate disease (n = 10)]. In subjects who underwent surgery, these samples were collected before surgery and processed promptly after collection and stored at 4°C for a maximum of 48 h before freezing at −70°C. Both genders are well represented and, as expected, subjects are in an appropriate age range for colon testing (Table 1). There was a relatively high rate of family history of colorectal cancer in first-degree relatives in subjects with the normal and hyperplastic polyps on colonoscopy (Table 1). Stages of colorectal cancer were well represented, stage I (n = 6), stage II (n = 9), stage III (n = 9), stage IV (n = 3), and unstaged (n = 1).

CCSA-3 and CCSA-4 indirect ELISA. Serum samples were plated in 96-well Nunc Immunoplate MaxiSorb plates. Each plate consisted of samples from different groups; each sample was plated in duplicate at room temperature overnight with shaking. Serum samples from all groups were mixed and run in batches at the same time. Each plate consisted of samples from various control, polyp, and colon cancer groups. Samples were aspirated and blocked in Super Block (Pierce). The blocking buffer was removed and then washed with 1× TBS with 0.05% Tween 20 (TBS-T). Antibodies for CCSA-3 and CCSA-4 were added and incubated for 2 h at 37°C. The plates were rinsed and secondary antibody (KPL, Inc.) was added and incubated at 37°C for 2 h. The plates were washed with TBS-T followed by incubation with room temperature TMB substrate (KPL) for 30 s. The plates were then read at 630 nm using Pherastar plate reader (BMG Lab Tech).

Statistical analysis. We used exact methods based on the binomial distribution to obtain upper and lower bounds for 95% confidence intervals (95% CI) surrounding estimates of test sensitivity and specificity. When study results indicated perfect (100%) sensitivity or perfect (100%) specificity in a subgroup of interest, we report the lower 95% confidence bound. Otherwise, we report a symmetrical 95% CI. We used the area under the empirical receiver operating characteristic (ROC) curve to summarize the ability of a CCSA-3 or CCSA-4 test result to discriminate a colorectal cancer or advanced adenoma patient from a colonoscopy patient with findings no worse than nonadvanced adenoma. We used a bootstrap method with 500 iterations to estimate the 95% CI, for the area under the ROC curve (10).

Results

Detection of colorectal cancer by CCSA-3 and CCSA-4. We first examined the specificity of both CCSA-3 and CCSA-4 antibodies used in these studies by evaluating their cross-reactivity with several proteins, such as early prostate cancer antigen (EPCA) and EPCA-2, both of which are prostate cancer–specific nuclear

matrix proteins. None of these proteins showed any cross-reactivity with either CCSA-3 or CCSA-4 (Supplementary Fig. S1).

A pilot study evaluating the CCSA-3 and CCSA-4 assays in 25 sera samples showed 100% sensitivity and specificity in distinguishing colorectal cancer from subjects with normal or hyperplastic polyps (data not shown). Absorbance levels were translated into peptide concentrations using equations generated from fitting known peptide concentrations to a sigmoidal curve resulting in cutoff points for CCSA-3 (2 μg/mL) and CCSA-4 (0.3 μg/mL). Samples were run on multiple plates over time and the assays resulted in values with tight concordance (coefficient of variation within 10%). On each plate, samples for each of the patient groups were run and these were distributed across the plates. Once the assay characteristics and cutoffs for this subset of samples were established and reproducible, we expanded our studies into larger sample population.

Our results show that the established cutoffs and the assays for both CCSA-3 and CCSA-4 work reproducibly and consistently. The mean levels of CCSA-3 and CCSA-4 are presented for each sample population (Table 1), and the individual values on a log scale are represented in dot-plot graphs (Fig. 1*A* and *B*).

Analysis of the sensitivity and specificity at the cutoff values of 2.0 μg/mL for CCSA-3 and 0.3 μg/mL for CCSA-4 assays is reported in Table 2. Both CCSA-3 and CCSA-4 individually detected all 28 colorectal cancers for a sensitivity of 100% (lower 95% bound, 89.8%). The sensitivity for detection of colorectal cancer and

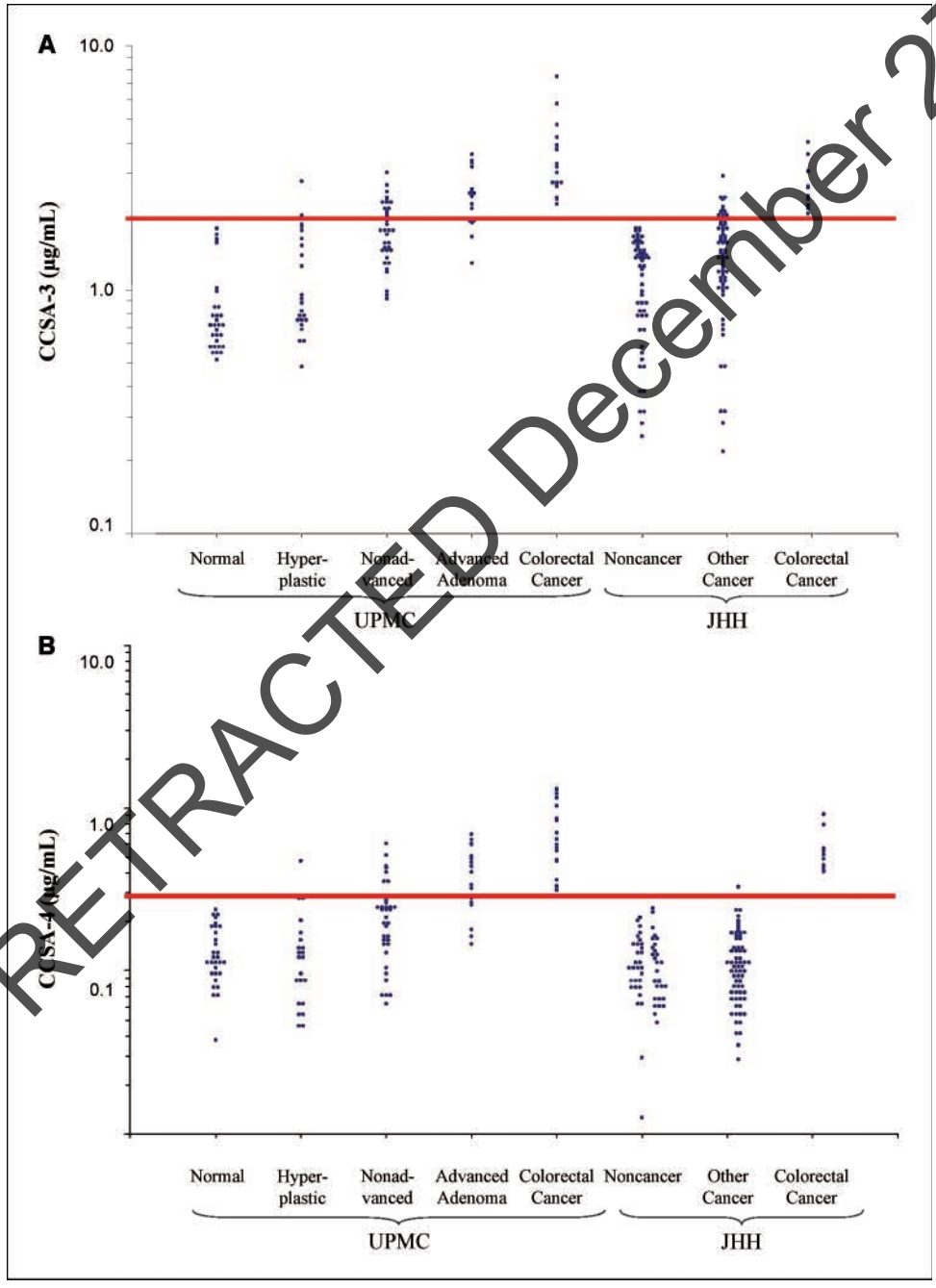


Figure 1. CCSA-3 and CCSA-4 levels in serum samples collected from 260 individuals. *A,* natural logarithm values of CCSA-3 in serum samples. *B,* natural logarithm values of CCSA-4 in the sera. Serum concentration for each subject is indicated by patient subgroup. Cutoff values (*red line across each graph*). Cutoff for CCSA-3 is 2 μg/mL and for CCSA-4 is 0.3 μg/mL.

Table 2. Sensitivity and specificity for CCSA-3 and CCSA-4 at specified cutoffs

Group	CCSA-3 ≥2 μg/mL		CCSA-4 ≥0.3 μg/mL		CCSA-3 ≥2 μg/mL or CCSA-4 ≥0.3 μg/mL	
	Sensitivity, *n* (%)	95% CI	Sensitivity, *n* (%)	95% CI	Sensitivity, *n* (%)	95% CI
Colorectal cancer (UPMC and JHH)	28/28 (100)	89.8	28/28 (100)	89.8	28/28 (100)	89.8
Advanced adenoma (UPMC) and colorectal cancer (UPMC/JHH)	41/46 (89.1)	76.4–96.4	39/46 (84.8)	71.1–93.7	42/46 (91.3)	79.2–97.6
Advanced adenoma (UPMC)	13/18 (72.2)	46.5–90.3	11/18 (61.1)	35.8–82.7	14/18 (77.8)	52.4–93.6
Nonadvanced and advanced adenoma (UPMC)	27/54 (50)	36.1–63.9	18/54 (33.3)	21.1–47.5	31/54 (57.4)	43.2–70.8
Group	CCSA-3 <2 μg/mL		CCSA-4 <0.3 μg/mL		CCSA-3 <2 μg/mL and CCSA-4 <0.3 μg/mL	
	Specificity, *n* (%)	95% CI	Specificity, *n* (%)	95% CI	Specificity, *n* (%)	95% CI
Normal, hyperplastic (UPMC)	51/53 (96.2)	87.0–99.5	52/53 (98.1)	89.9–99.9	51/53 (96.2)	87.0–99.5
Normal, hyperplastic, nonadvanced adenoma (UPMC)	73/89 (82.0)	72.4–89.4	81/89 (91.0)	83.0–96.0	70/89 (78.7)	68.7–86.6
Benign (JHH)	58/58 (100)	95.0	57/58 (98.3)	90.8–99.9	57/58 (98.3)	90.8–99.9
Other cancer (JHH)	56/67 (83.6)	72.5–91.5	64/67 (95.5)	87.5–99.1	53/67 (79.1)	67.4–88.1

advanced adenoma, or the combination of lesions that are most advanced and the most important to detect from a screening standpoint, for CCSA-3 is 89.1% (95% CI, 76.4–96.4%) and for CCSA-4 is 84.8% (95% CI, 71.1–93.7%). If either marker above threshold is considered positive, the sensitivity is 91.3% (95% CI, 79.2–97.6%). The point estimate for sensitivity for detection of advanced adenoma alone was 77.2% and 77.8% for CCSA-3 and CCSA-4, respectively, and the sensitivity for nonadvanced and advanced adenoma combined was 50% and 57.4% (Table 2).

The specificity in subjects with either a normal colon on colonoscopy or only hyperplastic polyps was 96.2% for CCSA-3 and 98.1% for CCSA-4 (Table 2). The specificity for individuals with normal, hyperplastic polyps, or nonadvanced adenomas was 82.0% (95% CI, 72.4–89.4%) and 91.0% (95% CI, 83.0–96.0%) for CCSA-3 and CCSA-4, respectively (Table 2), and 78.7% (95% CI, 63.4–81.0%) for both tests being below threshold. False-positive tests in subjects from JHH with benign disease were 0% for CCSA-3 and 1.7% for CCSA-4 and in subjects with cancer other than colorectal cancer were 16.4% for CCSA-3 and 6% for CCSA-4 (Table 2).

ROC analyses. We also evaluated the markers using ROC curves, which permit an analysis of the tradeoff between sensitivity and specificity at variable cut points. The ROC curves used colon cancers (n = 28) and advanced adenomas (n = 18) as the end point for detection compared with colonoscopy-assessed normals, hyperplastic polyps, and nonadvanced adenomas (total n = 89). For the detection of colorectal cancer and advanced adenoma, the area under the curve (AUC) for the CCSA-3 assay is 0.94 (95% CI, 0.90–0.98; Fig. 2*A*). Cutpoint C (2.06 μg/mL) corresponds closely to the value we selected for analysis, 2.0 μg/mL. Lowering the cutpoint to point D, or 1.87 μg/mL, would increase the sensitivity for detection of colorectal cancer and advanced adenoma to 96% but lower the specificity to 81%.

A ROC curve for CCSA-4 showed a similar AUC of 0.94 (95% CI, 0.90–0.98; Fig. 2*B*). Using cut point C, or 0.25 μg/mL, would generate a sensitivity of 94% for detection of colorectal cancer and advanced adenoma but lower the specificity to 84%.

Although combining CCSA-3 and CCSA-4 to improve sensitivity by allowing either test to be positive did improve sensitivity, the added benefit was rather small and was insignificant. As shown in Table 2, the utilization of both assays resulted in a higher sensitivity for individuals with advanced adenomas and colorectal cancers (91.3%) but slightly lowered the specificity for normal, hyperplastic, and nonadvanced adenoma populations (78.7%). Although the ROC curve for the combined CCSA-3 and CCSA-4 (Supplementary Fig. S2) has an AUC of 0.957 (95% CI, 0.91–0.98), it is not statistically significant when compared with the AUC of CCSA-3 (P = 0.122) and CCSA-4 (P = 0.239). This is not surprising because the Pearson correlation coefficient for CCSA-3 and CCSA-4 was 0.65 (95% CI, 0.58–0.72), indicating that the assays are highly correlated.

We found no significant association between cancer stage or between adenoma size and CCSA-3 or CCSA-4 concentration (data not shown). In control subjects without evidence of colorectal neoplasia (UPMC normal and hyperplastic polyp, JHU benign disease, and JHU other cancer; total n = 178), CCSA-3 and CCSA-4 distributions were not statistically different in persons less than 60 versus 60 or more years of age. Men had higher CCSA-3 values than women (median, 1.39 versus 0.95 μg/mL; P = 0.003). In neoplasia-free subjects from UPMC (UPMC normal and hyperplastic; total n = 53), CCSA-3 and CCSA-4 distributions did not differ according to timing of blood draw, history of polyps, or personal history of cancer. However, median CCSA-3 and CCSA-4 values were higher in persons with than in persons without a family cancer history [CCSA-3, 1.27 versus 0.74 μg/mL (P = 0.03); CCSA-4, 0.16 versus 0.11 (P = 0.04)]. Sensitivity and specificity levels did not significantly change when the 25 subjects in the pilot phase were excluded from the analysis.

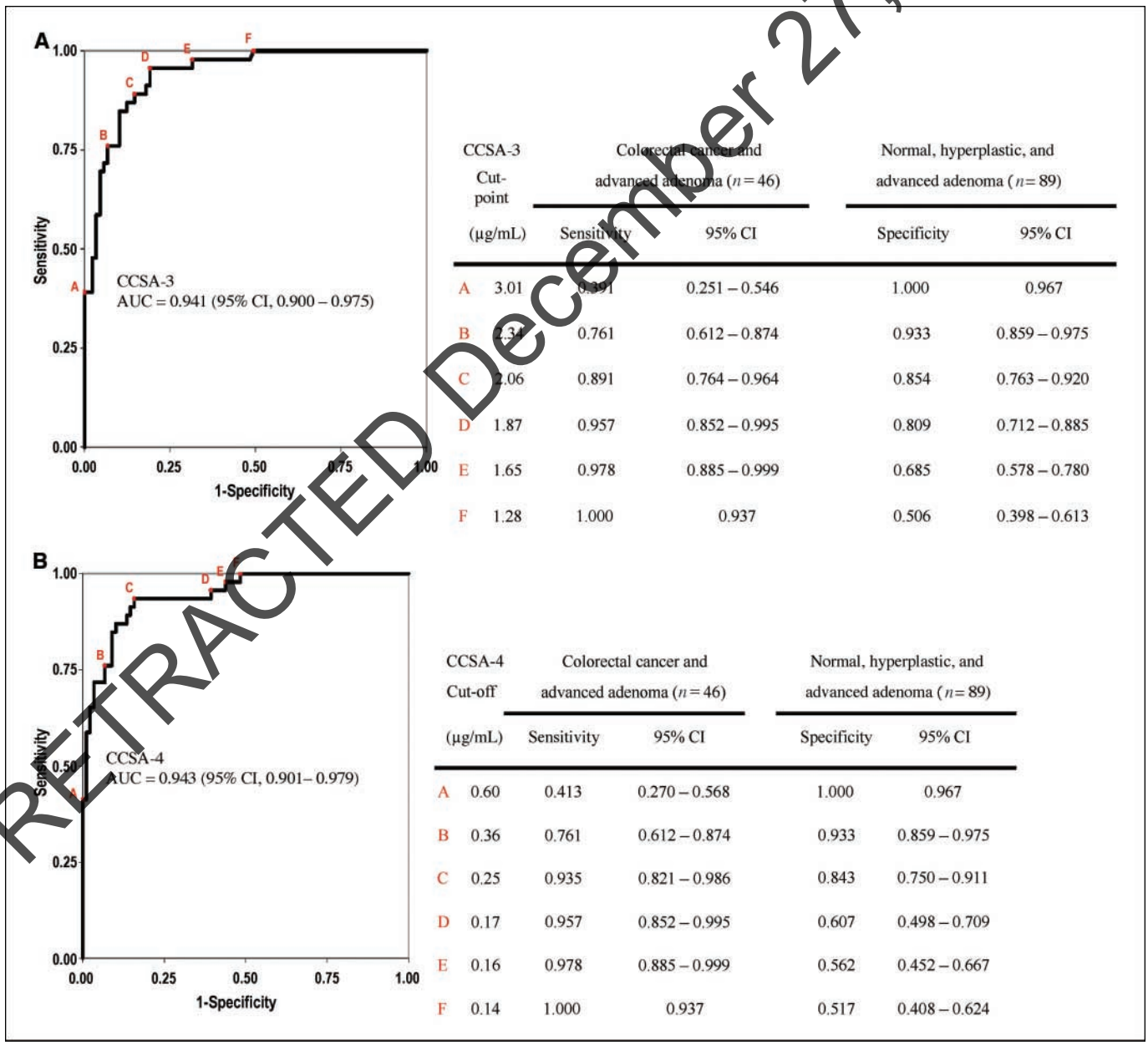
Discussion

This report describes initial analyses on two novel serum-based assays, CCSA-3 and CCSA-4, for detection of colorectal cancer. Developed via a proteomic approach focused on nuclear matrix proteins specific to colorectal cancer (8), our previous analyses on colon polyps and cancer tissues revealed that CCSA-3 and CCSA-4 were present in 83% to 100% of advanced adenomas and in all

colon cancer specimens (9). These results suggest that both CCSA-3 and CCSA-4 are expressed before the onset of cancer and thus may useful as markers of early detection. Despite the fact that both CCSA-3 and CCSA-4 are nuclear proteins, we speculate that they are released into the blood by cellular breakdown or apoptosis and are quite stable once they get there (11). The mechanisms by which CCSA-3 and CCSA-4 are found in the serum are under study.

The increase in mean values from nonadenomatous pathology (hyperplastic and normal) to nonadvanced adenomatous pathology and further increase with advanced adenoma and cancer mirrors the pathologic development of disease along the adenoma-carcinoma sequence. Advanced adenomas were collected prospectively from subjects undergoing colonoscopy, in a similar fashion to the normals and those with hyperplastic polyps, further substantiating that the higher levels observed with advanced adenomas represent a real finding. Cancers collected at two different participating clinical centers gave similar results as did controls. Finally, other cancers and benign conditions generally did not exceed the threshold values for a positive test, supporting the specificity of these assays for colorectal pathology. In our analyses, we did not observe any significant variations in the serum levels of the proteins despite some differences in when the samples were collected. Finally, these assays are based on ELISA methods, which are easily standardized and reproducible.

Although both proteins may be closely related or isoforms due to their similar molecular weights but slightly different isoelectric focusing points, the fact that the cutoff values are different from



CCSA-3 Cut-point		Colorectal cancer and advanced adenoma ($n = 46$)		Normal, hyperplastic, and advanced adenoma ($n = 89$)	
	(μg/mL)	Sensitivity	95% CI	Specificity	95% CI
A	3.01	0.391	0.251 – 0.546	1.000	0.967
B	2.34	0.761	0.612 – 0.874	0.933	0.859 – 0.975
C	2.06	0.891	0.764 – 0.964	0.854	0.763 – 0.920
D	1.87	0.957	0.852 – 0.995	0.809	0.712 – 0.885
E	1.65	0.978	0.885 – 0.999	0.685	0.578 – 0.780
F	1.28	1.000	0.937	0.506	0.398 – 0.613

CCSA-4 Cut-off		Colorectal cancer and advanced adenoma ($n = 46$)		Normal, hyperplastic, and advanced adenoma ($n = 89$)	
	(μg/mL)	Sensitivity	95% CI	Specificity	95% CI
A	0.60	0.413	0.270 – 0.568	1.000	0.967
B	0.36	0.761	0.612 – 0.874	0.933	0.859 – 0.975
C	0.25	0.935	0.821 – 0.986	0.843	0.750 – 0.911
D	0.17	0.957	0.852 – 0.995	0.607	0.498 – 0.709
E	0.16	0.978	0.885 – 0.999	0.562	0.452 – 0.667
F	0.14	1.000	0.937	0.517	0.408 – 0.624

Figure 2. Empirical ROC curves for CCSA-3 and CCSA-4. ROC analyses for CCSA-3 (*A*) and CCSA-4 (*B*) in separating normal, hyperplastic, and nonadvanced adenomas from cancer and advanced adenoma.

one another suggests that CCSA-3 may be more abundant in serum compared with CCSA-4. Studies determining the complete protein sequence and identification of the genes that encode CCSA-3 and CCSA-4 are in progress. Determining how these proteins work could enhance our understanding of the biological events that foster the evolution from adenoma to carcinoma.

These results represent characterization of an assay and are not designed to determine the performance of this assay in screening patients for colorectal cancer in that the populations studied in this article are not representative of distributions found in a screening population. Future studies should, as best possible, evaluate an unselected screening population. In conclusion, our findings show that CCSA-3 and CCSA-4 show promise as potential serum markers for detection of colorectal cancer and advanced adenoma. Although further validation and study is needed, the promise of a blood test for colorectal cancer may be upon us.

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