의학박사 학위논문

CCSP-2 표적 나노바이오센서를 이용한 새로운 대장 종양 진단 혈액 검사

Novel blood-based detection of colorectal cancer and adenoma using a nanobiosensor targeting CCSP-2 (colon cancer secreted protein-2)

울산대학교대학원

의 학 과

이 효 정

CCSP-2 표적 나노바이오센서를 이용한 새로운 대장 종양 진단 혈액 검사

지도교수 명 승 재

이 논문을 의학박사 학위 논문으로 제출함

2018 년 2 월

울산대학교 대학원

의 학 과

이 효 정

이효정의 의학박사학위 논문을 인준함

심사위원 변 정 식 인

심사위원 명 승 재 인

심사위원 이 관 희 인

심사위원 김 상 엽 인

심사위원 주 진 명 인

울 산 대 학 교 대 학 원

2018 년 2 월

Abstract

Background A blood-based test for the early detection of colorectal cancer (CRC) would be an attractive alternative to current CRC screening tests. Colon cancer secreted protein-2 (CCSP-2) is a novel candidate blood marker for CRC, which was identified by gene expression microarray. The aims of this study were to assess the CCSP-2 expression patterns in a large number of human CRC tissues and to evaluate the possible clinical use of the CCSP-2 as a blood biomarker for the early diagnosis of CRC and adenomas.

Methods Immunohistochemical staining of CCSP-2 was performed in the colorectal tumor and paired normal tissue (10 advanced adenomas and 69 CRCs). The electric-field effect colorectal sensor (E-FECS), which is an ion-sensitive field-effect transistor under dual gate operation with nanostructure, was used to quantify CCSP-2 in human blood. Biosensor verification was performed on 10 controls and 10 CRC cases, and it was clinically validated on 30 controls, 30 advanced adenomas and 81 CRC cases.

Results CCSP-2 protein was homogenously expressed in all CRC and advanced adenoma tissues, whereas CCSP-2 was not detected in normal colorectal tissues. CCSP-2 levels in blood were significantly higher in the advanced adenoma and CRC patients compared with the controls (P = 0.005 and P = 0.001). There was no difference in the detection rate of CCSP-2 in blood between cancer stages (P = 0.067). The overall sensitivity of blood CCSP-2 in the diagnosis of colorectal tumors (including advanced adenomas and CRC) was 44.1%, and the specificity was 86.7% (AUC was 0.67, 95% confidence interval [CI] 0.57–0.76). The sensitivity and specificity for CRC were 44.4% and 86.7%, respectively, and the sensitivity and specificity for advanced adenomas were 43.3% and 86.7%, respectively.

Conclusions Our study showed that novel blood-based detection using a nanobiosensor targeting CCSP-2 could reveal about half of CRC and adenoma cases, irrespective of tumor stage. The utility of this method for CRC screening will require improved sensitivity, and further studies are needed to determine the role of this novel candidate blood marker in clinical practice.

Key words: CCSP-2, colorectal neoplasms, biomarkers, serum marker, field-effect biosensor

Table of contents

List of tables

List of figures

INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers, and it is the second-leading cause of cancer-related deaths worldwide.[1, 2] CRC cases and deaths can be prevented through screening, which can detect and treat precancerous lesions and early cancer.[3, 4] Current CRC screening guidelines recommend a colonoscopy or the fecal immunochemical test (FIT) as a first-tier option for screening.[5, 6] A colonoscopy is highly sensitive in the detection of CRC and adenomas, and it can remove adenomas during a single session. However, a colonoscopy involves high costs; significant resources; discomfort from bowel preparation; and a higher risk of complications, such as perforation and bleeding, compared to other screening tests.[5, 7] The FIT is a non-invasive test, and it is easy to perform and inexpensive, but it does not have sufficient sensitivity for adenoma detection.[8] A highly sensitive FIT-fecal DNA test has also been developed, but its high cost limits its use.[5, 9] Thus, a non-invasive and accurate test for the early detection of CRC and adenomas is needed.

Blood biomarkers are considerable potential tools for CRC screening. Several tests have been developed, but few biomarkers are currently available.[10-15] Colon cancer secreted protein-2 (CCSP-2) is a novel candidate blood marker, whose mRNA expression in human CRC tissues is marked upregulated with a mean 78-fold increase versus matched normal mucosa, and CCSP-2 protein can be detected in the plasma of a xenografted mouse.[16] Based on this CCSP-2 identification study, our group recently reported the utility of CCSP-2-targeting molecular imaging in the early diagnosis of CRC during a colonoscopy.[17] However, the expression patterns of CCSP-2 in CRC and adenoma tissues have not been fully evaluated, except in one study with a small sample size.[17] Moreover, the detection of CCSP-2 in the blood of human specimens has never been performed.

Biodetection tool is also important in targeting biomarkers for blood-based diagnoses. Ion-sensitive field-effect transistor under dual gate operation (DG ISFET), which tailors precisely the nanostructure of the transistor, is a promising biosensor due to its high sensitivity, reliability, and detection speed.[18, 19] It can detect trace amounts of the prostate cancer antigen annexin A3 in patient urine with a high reliability.[20] In this research, we used the DG ISFET chip, named the electric-field effect colorectal sensor (E-FECS), with a

disposable sensing gate to quantify CCSP-2 in human blood (Figure 1).

The aims of this study were to assess the CCSP-2 expression patterns in a large number of human CRC tissues and, for the first time, to verify the possible clinical use of CCSP-2 as a blood biomarker for the early diagnosis of CRC and adenomas.

MATERIALS AND METHODS

Study population

Fresh-frozen colorectal tumor tissues, paired normal colorectal tissues, and plasma specimens from patients with colorectal tumors (10 advanced adenomas greater than 1cm in size and 80 cancers) were provided by Asan Bio-Resource Center (BRC), Korea Biobank Network (2016-9[121]). These tumor samples were excised from patients during surgery, and the plasma specimens were obtained before surgery. We randomly divided these plasma and tumor samples into two groups for biosensor verification assaying (10 cases of stage II or III cancer) and clinical validation (80 cases: 10 advanced adenomas and 70 cancers).

To test the performance of the E-FECS according to the specimen preparation, we additionally collected 31 sera and 31 plasma specimens from 31 patients with colorectal tumors (20 advanced adenomas greater than 1cm in size and 11 cancers) who were prospectively enrolled from the outpatient clinic of the Department of Gastroenterology at the Asan Medical Center. These 31 blood samples were also included in the clinical validation set. The clinicopathologic characteristics of the patients in each of the colorectal tumor groups are indicated in Table 1.

Controls with no colorectal adenomas or cancer detected by complete colonoscopies were also prospectively enrolled. These subjects were excluded if they had any malignant disease; a family history of familial polyposis or Lynch syndrome; or any colorectal disease, such as inflammatory bowel disease. In total, 40 sera and 10 plasma specimens were obtained from 40 controls, and the controls were randomly divided into two groups for biosensor verification assaying (n = 10) and clinical validation (n = 30) (Table 1). Our study protocol was approved by the Institutional Review Board of the Asan Medical Center (protocol no. 2015-0969), and informed consent was obtained in all cases.

Table 1. Clinicopathologic characteristics of controls and colorectal tumor patients

(A) Biosensor verification set

	Blood	
	Control (n = 10)	Cancer (n = 10)
Age, years, median (range)	60 (32–79)	63 (49–72)
Sex, male, no. (%)	6 (60.0%)	5 (50.0%)
Location, no. (%)*		
Proximal	-	2 (20.0%)
Distal	-	8 (80.0%)
Stage, no. (%)		
II	-	5 (50.0%)
III	-	5 (50.0%)
Differentiation, no. (%)		
Well	-	1 (10.0%)
Moderate	-	8 (80.0%)
Poor	-	1 (10.0%)
CEA, median (range), ng/mL	-	2.1 (1.3–9.2)

*Proximal colon includes cecum, ascending colon and transverse colon; Distal colon includes descending colon, sigmoid colon and rectum.

(B) Clinical validation set

	BRC stored samples (Tissue and blood)		**Prospectively collected samples (Blood)**		
	Adenoma (n = 10)	Cancer (n = 70)†	Control (n = 30)	Adenoma (n = 20)	Cancer (n = 11)
Age, years, median (range)	59 (42–70)	59 (30–80)	56 (38–70)	62 (33–78)	62 (51–75)
Sex, male, no. (%)	5 (50.0%)	38 (54.3%)	17 (56.7%)	12 (60.0%)	6 (54.5%)
Location, no. (%)*					
Proximal	4 (40.0%)	33 (47.1%)	-	11 (55.0%)	3 (27.3%)
Distal	6 (60.0%)	37 (52.9%)	-	19 (45.0%)	8 (72.7%)
Stage, no. (%)					
0	-	5 (7.1%)	-	-	5 (45.5%)
I	-	15 (21.4%)	-	-	1 (9.1%)
II	-	15 (21.4%)	-	-	2 (18.2%)
III	-	15 (21.4%)	-	-	1 (9.1%)
IV	-	20 (28.6%)	-	-	2 (18.2%)
Differentiation, no. (%)					
Well	-	10 (14.3%)	-	-	4 (36.4%)
Moderate	-	56 (80.0%)	-	-	63 (77.8%)

Table 1 (B) 계속

Poor	-	4 (5.7%)	-	-	7 (63.6%)
CEA, median (range), ng/mL	1.3 (0.8–2.4)	1.9 (0.3–1380.0)	-	-	1.4 (0.9–390.0)

BRC, Bio-Resource Center

*Proximal colon includes cecum, ascending colon and transverse colon; Distal colon includes descending colon, sigmoid colon and rectum.

† One colorectal cancer specimen without adequate cancer tissue was excluded from tissue immunostaining analysis.

Blood collection and processing

BRC stored plasma samples were collected in sodium citrate-containing tubes before surgery, and sent to and processed at the Asan BRC 1 h after collection. About 5 mL of blood was centrifuged at 1,900 × g at 4 °C for 10 min, and the supernatant was centrifuged again at 1,600 × g at 4 °C for 10 min to separate plasma from whole blood. The supernatants of the plasma samples were stored at -196°C until use. Prospectively collected plasma samples were collected in sodium citrate-containing tubes, and processed 1 h after collection. About 5 mL of blood was centrifuged at 1,900 × g at 4 °C for 20 min to separate plasma from whole blood. The supernatants of the plasma samples were stored at -80°C until use. Prospectively collected serum samples were collected using a serum-separating tube (SST), and processed 1 h after collection. About 3.5 mL of blood was centrifuged at 1,900 × g at 4 °C for 20 min to separate serum from whole blood. The supernatants of the serum samples were stored at -80°C until use.

Tissue microarray construction

For the CCSP-2 expression analysis, we established three tissue microarray slides using formalin-fixed paraffin-embedded tissues from 79 colorectal neoplasms and paired normal colorectal tissues. Representative areas of the tumors were marked on hematoxylin and eosin-stained slides. Duplicates of three cores from the tumor tissues and one core from the paired normal colorectal tissue measuring 1.5 mm in diameter were arrayed from the corresponding paraffin blocks into a recipient block using an arraying machine (Tissue microarrayer; Pathology Devices, Westminster, MD, USA). The first 4-μm sections from these arrays were examined for validation purposes, and additional sections were used for immunohistochemistry (IHC).

Immunohistochemistry

Immunohistochemical staining of CCSP-2 was performed using tissue microarray slides with the BenchMark XT automatic immunostaining device (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. The immunohistochemical

staining procedure was described in our previous study.[17] In brief, 4-μm tissue sections were transferred onto silanized charged slides, allowed to dry for 10 min at room temperature, and incubated for 20 min at 65°C. Antigens were retrieved by heating the sections for 64 min in Cell Conditioning 1 (CC1) buffer. After anti-CCSP-2 antibody (1:100) incubation for 32 min in the autoimmunostainer, detection was carried out with the Ventana OptiView DAB IHC Detection Kit (Ventana Medical Systems).

The expression profiles of CCSP-2 were scored according to staining intensity (0, negative; 1+, weakly positive; 2+, strongly positive), because the staining for CCSP-2 was homogenous in the stained core.

Enzyme-linked immunosorbent assay (ELISA)

The wells of microtiter plates were coated (O/N, 4°C) with 5 μg/ml of the CCSP-2 monoclonal antibody in 100 μl of a coating buffer (0.05 M Na2CO3, 0.05 M NaHCO3), and they were then blocked with 2% bovine serum albumin (BSA) in tris-buffered saline with Tween 20 (TBST) for 1 h at 37°C. In addition, 100 μl of the samples was loaded in triplicates and incubated for 1 h at room temperature, followed by the addition of 100 μl of antibody 1 (1:1,000, Cloud-Clone Corp.) for an additional 1 h at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:5,000) in a blocking buffer was added (1 h, room temperature), and the reaction was visualized through the addition of 50 μl of a chromogenic substrate (TMB) for 30 min. The reaction was stopped with 100 μl of 2M H2SO4, and absorbance at 450 nm was measured using an ELISA plate reader. Plates were washed five times with the washing buffer (TBS, pH 7.4, containing 0.1% [v/v] Tween 20) after each step. As a reference for quantification, a standard curve was established by a serial dilution of recombinant CCSP-2 protein (15 pg/mL–0.3 μg/mL, Figure 2).

Detection of CCSP-2 in blood with the highly sensitive E-FECS platform

1. Electrical characterization

Current versus voltage (*I-V*) measurements for CCSP-2 detection were taken using a

commercial Al/AgCl reference electrode and the Hewlett-Packard 4156B Precision Semiconductor Parameter Analyzer in a dark box to avoid light and noise interference. The sensing characteristics of the E-FECS were investigated by scanning the voltage on the bottom gate with a grounded top reference electrode.

2. Blood CCSP-2 measurement protocol

   A blood specimen was injected in duplicate onto the anti-CCSP-2-immobilized sensing membrane and allowed to react for 20 min. After washing out the previous media, a highly controlled phosphate-buffered saline (PBS) was injected, and the transfer curve was measured. As a reference for quantification, a standard curve was established through the serial dilution of recombinant CCSP-2 protein using undiluted PBS, untreated serum, and untreated plasma (100 ag/mL–1 μg/mL, Figure 3).

Statistical analysis

Differences in continuous variables between the two groups were evaluated using the Student's t-test or the Mann–Whitney U test, and differences in categorical variables were evaluated with the X^2 test or Fisher's exact test. Associations between two continuous variables were evaluated using bivariate correlation analysis. The areas under receiver operating characteristic (ROC) curves (AUCs) provided a measure of the overall performance of the diagnostic test. Statistical significance was defined as a P value of < 0.05, and the SPSS statistical software (version 20.0; SPSS, Chicago, IL) was used for all analyses.

RESULTS

CCSP-2 was homogenously expressed in all human colorectal adenoma and cancer tissues

The expression of CCSP-2 in CRC and adenoma cells was found to be cytoplasmic (Figure 4). CCSP-2 was homogenously expressed in all CRC and advanced adenoma tissues, whereas CCSP-2 was not detected in normal colorectal tissues. Of 70 CRC specimens, one was excluded because the tissues were inadequate for IHC, and of the remaining specimens,

30 (43.5%) were strongly positive (2+) and 39 (56.5%) were weakly positive (1+). Of the 10 adenoma specimens, four (40.0%) were strongly positive (2+) and six (60.0%) were weakly positive. The CCSP-2 expression in colorectal tissue did not differ between adenoma and CRC specimens (P = 1.000). Among CRC patients, the clinicopathologic characteristics did not differ between the weak-positive and strong-positive groups of CCSP-2 in the tissues except all poorly differentiated carcinoma were weakly positive (Table 2).

ELISA showed limited efficiency in the detection of CCSP-2 in blood

The standard curve according to recombinant CCSP-2 using ELISA is indicated in Figure 2. The detectable range of the ELISA was 4 ng/mL–30ng/mL. We measured CCSP-2 in blood using conventional ELISA, but CCSP-2 was not detected in all tested specimens (10 controls and 10 CRCs).

The highly sensitive E-FECS platform detected CCSP-2 in blood

The standard curve according to recombinant CCSP-2 using the E-FECS is indicated in Figure 3. The detectable range of the E-FECS was 100 ag/mL–10 ng/mL, and the E-FECS showed a good linearity, a high sensitivity, and a low limit of detection of less than 100 ag/mL.

We tested the E-FECS with a verification set of human blood specimens (10 serum specimens from controls and 10 plasma specimens from CRCs). The results of the detection voltage in human blood samples, and corresponding CCSP-2 concentrations calculated from the standard curve are presented in Figure 5. CCSP-2 was detected in five plasma samples of the CRC patients (50.0%) and in only one serum sample of the controls (10.0%) in the detectable range using the E-FECS.

Effects of blood preparation on the CCSP-2 measurement with the E-FECS

We compared the CCSP-2 measurements of serum and plasma specimens from 41 cases (10 controls, 20 advanced adenomas, and 11 cancers). Of the 10 controls, CCSP-2 was detected in only one, and the CCSP-2 concentrations were consistent in the serum and

Table 2. Comparison of the clinicopathologic characteristics between the weak-positive and strong-positive groups of CCSP-2 in the tissue of colorectal cancer patients

	Weak (n = 39)	**Strong (n = 30)**	***P***
Age, years, median (range)	58 (37–80)	64 (30–78)	0.877
Sex, male, no. (%)	21 (53.8%)	16 (53.3%)	0.966
Location, no. (%)*			0.751
Proximal	18 (46.2%)	15 (50.0%)	
Distal	21 (53.8%)	15 (15.0%)	
Stage, no. (%)			0.111
0	3 (7.7%)	2 (6.7%)	
I	5 (12.8%)	10 (33.3%)	
II	9 (23.1%)	6 (20.0%)	
III	8 (20.5%)	6 (20.0%)	
IV	14 (35.9%)	6 (20.0%)	
Differentiation, no. (%)			0.003
Well	2 (5.1%)	8 (26.7%)	
Moderate	33 (84.6%)	22 (73.3%)	
Poor	4 (10.3%)	0 (0.0%)	
CEA, median (range), ng/mL	1.8 (0.3–1380)	2.1 (0.5–132.0)	0.443

*Proximal colon includes cecum, ascending colon and transverse colon; Distal colon includes descending colon, sigmoid colon and rectum.

plasma. Overall, CCSP-2 measurements were higher in serum than in plasma (43.9% vs. 19.5%, $P = 0.018$; Figure 6).

Clinical validation of CCSP-2 as a blood biomarker for CRC diagnosis

CCSP-2 was detected more frequently in plasma from the advanced adenoma and CRC samples than in serum from the controls (43.3% and 44.4% vs. 13.3%; $P = 0.008$, Figure 7A). CCSP-2 levels were also significantly higher in the adenoma and CRC patients compared with the controls ($P = 0.005$ and $P = 0.001$, Figure 7A). The tissue IHC staining intensity and blood CCSP-2 levels showed no significant association ($P = 0.769$).

The results of the CRC samples are graphed by cancer stage in Figure 7B. CCSP2 was detected in all stages of colon cancer except stage 0 (Tis cancer), and the blood CCSP-2 positivity showed no differences between cancer stages ($P = 0.067$). A correlation between the level of CCSP-2 in blood and cancer stage was not found, the highest level of CCSP-2 in blood was seen in the patients with stage II cancer (5 ng/mL). Other clinicopathologic characteristics did not differ between the negative and positive groups of CCSP-2 in the blood of CRC patients (Table 3).

The overall sensitivity of blood CCSP-2 in the diagnosis of colorectal tumors (including adenomas and CRC) was 44.1%, and the specificity was 86.7% (AUC was 0.67, 95% confidence interval [CI] 0.57–0.76). The sensitivity and specificity for CRC were 44.4% and 86.7%, respectively (AUC was 0.67, 95% CI 0.57–0.77), and the sensitivity and specificity for advanced adenomas were 43.3% and 86.7%, respectively (AUC was 0.67, 95% CI 0.53–0.80).

Comparison and combination of CCSP-2 with Carcinoembryonic antigen (CEA)

Of the 81 CRC patients, the CEA values were available in 79 patients except two patients with stage 0 cancer (Tis cancer). Of these 79 patients, only 19 (24.1%) had an elevated serum CEA level, and the proportion of patients with an elevated CEA increased with more advanced tumor stage ($P < 0.001$, Figure 8). CCSP-2 gave a higher CRC detection rate compared to CEA (24.1% vs. 44.4%). The results of the CCSP-2 positivity and CEA

Table 3. Comparison of the clinicopathologic characteristics between the negative and positive groups of CCSP-2 in the blood of colorectal cancer patients

	Negative (n = 45)	**Positive (n = 36)**	***P***
Age, years, median (range)	61 (30–78)	59 (36–80)	0.949
Sex, male, no. (%)	22 (48.9%)	22 (61.1%)	0.273
Location, no. (%)*			0.177
Proximal	23 (51.1%)	13 (36.1%)	
Distal	22 (48.9%)	23 (63.9%)	
Stage, no. (%)			0.067
0	10 (22.2%)	0 (0.0%)	
I	8 (17.8%)	8 (22.2%)	
II	9 (20.0%)	8 (22.2%)	
III	6 (13.3%)	10 (27.8%)	
IV	12 (26.7%)	10 (27.8%)	
Differentiation, no. (%)			0.232
Well	11 (24.4%)	3 (8.3%)	
Moderate	31 (68.9%)	32 (88.9%)	
Poor	3 (6.7%)	1 (2.8%)	
CEA, median (range), ng/mL	1.5 (0.3–1380.0)	2.9 (0.8–132.0)	0.397

*Proximal colon includes cecum, ascending colon and transverse colon; Distal colon includes descending colon, sigmoid colon and rectum.

elevation were consistent in 50 patients (kappa value 0.230, $P = 0.022$). The combination of CCSP-2 and CEA improved CRC detection rate (24.1% vs. 53.2%), especially for early stage cancer (Figure 8).

DISCUSSION

The present study assessed the CCSP-2 protein expression patterns in colorectal tumors from a large population and, for the first time, the ability of a novel blood marker, CCSP-2, to detect CRC and adenomas in human blood samples. The notable findings were that CCSP-2 was homogenously expressed in all human colorectal adenomas and cancer tissues, and a trace amount of CCSP-2 was present in the human blood of about half of the CRC and advanced adenoma patients, regardless of tumor stage.

A blood-based test for CRC detection would be convenient and highly compliant as an alternative to a colonoscopy or a stool-based test.[3, 4, 21)] Despite strong evidence that CRC screening reduces incidence rates and mortality, many people still do not undergo testing because of the inconvenience.[22)] Improved accuracy of blood-based CRC detection methods will save more patients. CEA, which is the only CRC tumor marker currently used in clinical practice, has proven useful in preoperative prognosis prediction and post-treatment monitoring for recurrence, but not in CRC screening.[23)] The pooled sensitivity of CEA for diagnosis of CRC was only 46%, and the pooled specificity of CEA was 89%.[24)] A variety of blood markers has been associated with CRC, but no markers have been shown to overcome the cost-effectiveness of the FIT.[10-15)]

CCSP-2 was identified by comparing candidate markers in colon cancers and normal colon epithelia through GeneChip gene expression microarrays, followed by northern blot analysis and real-time PCR.[16)] Our study confirms that CCSP-2 expressed in all stages of CRC and adenoma tissues at the protein level is consistent with previous observations that CCSP-2 was elevated in all colorectal tumors at the mRNA level.[16)] In particular, the upregulation of CCSP-2 in adenomas, precancerous lesions, suggests its potential role as a CRC screening marker. CCSP-2 was shown to encode the secreted protein that circulates stably and was detectable in the plasma of an animal model.[16)] These previous study results

motivated the present study, which was performed with the human blood of CRC and adenoma subjects.

We introduced the E-FECS, which is a highly sensitive DG ISFET biosensor with a nanostructure, to quantify CCSP-2 in blood to overcome the limited efficiency of conventional ELISA. The ISFET-based biosensor is an increasingly popular tool to quantify biomarkers.[25] DG ISFET has been found to improve the detection limit of single-gated devices as well.[18] E-FECS further precisely tailoring the nanostructure of the transistor, so the sensitivity is maximized and signal stability is improved.[20] In this study, we found that the E-FECS could stably detect CCSP-2 up to 100 ag/mL.

Our study showed that the E-FECS targeting CCSP-2 in blood could detect 44.4% of CRC cases with 86.7% specificity and 43.3% of advanced adenomas with 86.7% specificity. This result is not correlated with colorectal tumors that contain homogenous CCSP-2. It is possible that the amount of blood-circulated CCSP-2 was too low to be detected by the E-FECS, or protein degradation that can not be captured by the current antibody may have accounted for this result. The blood sample preparation method would also have affected the low detection rate of CCSP-2. We used plasma specimens from patients with colorectal tumors because the biobank storage specimens, which were quickly accessible for sample testing, were in plasma form. However, our data showed that CCSP-2 measurements were higher in serum than in plasma. Sodium citrate prevents coagulation by combining ionized calcium in a non-dissociated form, which might influence the measurement of circulating CCSP-2 in plasma sample.[26, 27]

In comparison with the performance of the FIT, which is one of the first recommended options for CRC screening, the E-FECS targeting CCSP-2 assay shows a CRC sensitivity of 44.4%, which is lower than the pooled sensitivity of the FIT (79%). The specificity of the E-FECS targeting CCSP-2 assay was 86.7%, compared with 94% for the FIT. Therefore, this novel blood-based detection method using a nanobiosensor targeting CCSP-2 has at present no utility in CRC and adenoma screening.

However, the interesting expression feature of CCSP-2, which upregulate from precancerous lesions, still suggests that CCSP-2 could be used as a biomarker for the early

detection of CRC. Further technical advances, such as applying new CCSP-2 peptides that could be detected more precisely than an antibody, or using the fragment antibody binding (Fab) region of an antibody to increase the voltage change in the E-FECS, would help to improve the rate of CCSP-2 detection in blood. Standardization of sample preparation will further increase the sensitivity of CCSP-2 detection. A multimarker method combining this novel single marker with other markers could also be used to develop a blood-based detection method for CRC.[12-14] In addition, the detection of CCSP-2 in stool for an early CRC diagnosis may be an interesting study as the next step, because CCSP-2 is expressed diffusely in the epithelium of a tumor.

Our study only assessed the performance of CCSP-2 in a screening setting, and it did not assess other possibilities, such as the notion that prognostic and predictive markers may influence the management of CRC. CCSP-2-targeting molecular imaging identify liver metastasis from CRC in mouse models, suggests that CCSP-2 expressed in the metastatic lesion of CRC.[17] In comparison with CEA, CCSP-2 could detect more CRC. A combination of CCSP-2 and CEA may improve the CRC detection capability in the post-treatment monitoring setting. Further studies are needed to determine the role of CCSP-2 in prognosis prediction, post-treatment monitoring or targeted therapy.

The function of CCSP-2 in colon carcinogenesis is still poorly understood. CCSP-2 encodes a secreted protein, and CCSP-2 protein has been reported to act as an extracellular matrix protein in animal studies.[28, 29] However, the function of CCSP-2 was not evaluated in human. Therefore, a mechanism study to identify the function of CCSP-2 in colon carcinogenesis should be conducted.

CONCLUSION

In conclusion, our study showed that novel blood-based detection using a nanobiosensor targeting CCSP-2 could reveal about half of CRC and advanced adenoma cases, irrespective of tumor stage. The utility of this method for CRC screening will require improved sensitivity, and further studies are needed to determine the role of this novel candidate blood marker in clinical practice.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA Cancer J Clin 2017;67 (1):7-30.
2. Torre LA, Siegel RL, Ward EM, Jemal A. Global Cancer Incidence and Mortality Ra tes and Trends--An Update. Cancer Epidemiol Biomarkers Prev 2016;25(1):16-27.
3. Zauber AG, Winawer SJ, O'Brien MJ, Lansdorp-Vogelaar I, van Ballegooijen M, Ha nkey BF, et al. Colonoscopic polypectomy and long-term prevention of colorectal-ca ncer deaths. N Engl J Med 2012;366(8):687-96.
4. Winawer SJ, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, et al. Prev ention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup. N Engl J Med 1993;329(27):1977-81.
5. Rex DK, Boland CR, Dominitz JA, Giardiello FM, Johnson DA, Kaltenbach T, et al. Colorectal Cancer Screening: Recommendations for Physicians and Patients From t he U.S. Multi-Society Task Force on Colorectal Cancer. Gastroenterology 2017;153 (1):307-23.
6. Lee BI, Hong SP, Kim SE, Kim SH, Kim HS, Hong SN, et al. Korean Guidelines for Colorectal Cancer Screening and Polyp Detection. Intest Res 2012;10(1):67-88.
7. Rabeneck L, Paszat LF, Hilsden RJ, Saskin R, Leddin D, Grunfeld E, et al. Bleeding and perforation after outpatient colonoscopy and their risk factors in usual clinical pr actice. Gastroenterology 2008;135(6):1899-906, 906.e1.
8. Lee JK, Liles EG, Bent S, Levin TR, Corley DA. Accuracy of fecal immunochemical tests for colorectal cancer: systematic review and meta-analysis. Ann Intern Med 20 14;160(3):171.
9. Imperiale TF, Ransohoff DF, Itzkowitz SH, Levin TR, Lavin P, Lidgard GP, et al. Mu ltitarget stool DNA testing for colorectal-cancer screening. N Engl J Med 2014;370 (14):1287-97.
10. Berretta M, Alessandrini L, De Divitiis C, Nasti G, Lleshi A, Di Francia R, et al. Ser um and tissue markers in colorectal cancer: State of art. Crit Rev Oncol Hematol 201 7;111:103-16.

11. Carter JV, Galbraith NJ, Yang D, Burton JF, Walker SP, Galandiuk S. Blood-based m icroRNAs as biomarkers for the diagnosis of colorectal cancer: a systematic review a nd meta-analysis. Br J Cancer 2017;116(6):762-74.

12. Rho JH, Ladd JJ, Li CI, Potter JD, Zhang Y, Shelley D, et al. Protein and glycomic p lasma markers for early detection of adenoma and colon cancer. Gut 2016.

13. Ciarloni L, Ehrensberger SH, Imaizumi N, Monnier-Benoit S, Nichita C, Myung SJ, et al. Development and Clinical Validation of a Blood Test Based on 29-Gene Expres sion for Early Detection of Colorectal Cancer. Clin Cancer Res 2016;22(18):4604-11.

14. Ciarloni L, Hosseinian S, Monnier-Benoit S, Imaizumi N, Dorta G, Ruegg C. Discov ery of a 29-gene panel in peripheral blood mononuclear cells for the detection of col orectal cancer and adenomas using high throughput real-time PCR. PLoS One 2015; 10(4):e0123904.

15. Church TR, Wandell M, Lofton-Day C, Mongin SJ, Burger M, Payne SR, et al. Pros pective evaluation of methylated SEPT9 in plasma for detection of asymptomatic col orectal cancer. Gut 2014;63(2):317-25.

16. Xin B, Platzer P, Fink SP, Reese L, Nosrati A, Willson JK, et al. Colon cancer secrete d protein-2 (CCSP-2), a novel candidate serological marker of colon neoplasia. Onco gene 2005;24(4):724-31.

17. Kim J, Do EJ, Moinova H, Bae SM, Kang JY, Hong SM, et al. Molecular Imaging of Colorectal Tumors by Targeting Colon Cancer Secreted Protein-2 (CCSP-2). Neopla sia 2017;19(10):805-16.

18. Lee IK, Jeun M, Jang HJ, Cho WJ, Lee KH. A self-amplified transistor immunosens or under dual gate operation: highly sensitive detection of hepatitis B surface antigen. Nanoscale 2015;7(40):16789-97.

19. Jang HJ, Ahn J, Kim MG, Shin YB, Jeun M, Cho WJ, et al. Electrical signaling of en zyme-linked immunosorbent assays with an ion-sensitive field-effect transistor. Bios ens Bioelectron 2015;64:318-23.

20. Jeun M, Park S, Kim Y, Choi J, Song SH, Jeong IG, et al. Self-Normalized Detection of ANXA3 from Untreated Urine of Prostate Cancer Patients without Digital Rectal

Examination. Adv Healthc Mater 2017;6(17).

21. Benning TM, Dellaert BG, Dirksen CD, Severens JL. Preferences for potential innov ations in non-invasive colorectal cancer screening: A labeled discrete choice experim ent for a Dutch screening campaign. Acta Oncol 2014;53(7):898-908.

22. Adler A, Geiger S, Keil A, Bias H, Schatz P, deVos T, et al. Improving compliance to colorectal cancer screening using blood and stool based tests in patients refusing scr eening colonoscopy in Germany. BMC Gastroenterol 2014;14:183.

23. Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, et al. ASC O 2006 update of recommendations for the use of tumor markers in gastrointestinal c ancer. J Clin Oncol 2006;24(33):5313-27.

24. Liu Z, Zhang Y, Niu Y, Li K, Liu X, Chen H, et al. A systematic review and meta-ana lysis of diagnostic and prognostic serum biomarkers of colorectal cancer. PLoS One 2014;9(8):e103910.

25. Choi J, Seong TW, Jeun M, Lee KH. Field-Effect Biosensors for On-Site Detection: Recent Advances and Promising Targets. Adv Healthc Mater 2017.

26. Biancotto A, Feng X, Langweiler M, Young NS, McCoy JP. Effect of anticoagulants on multiplexed measurement of cytokine/chemokines in healthy subjects. Cytokine 2 012;60(2):438-46.

27. Hsieh SY, Chen RK, Pan YH, Lee HL. Systematical evaluation of the effects of sam ple collection procedures on low-molecular-weight serum/plasma proteome profiling. Proteomics 2006;6(10):3189-98.

28. Richardson RJ, Gebauer JM, Zhang JL, Kobbe B, Keene DR, Karlsen KR, et al. AM ACO is a component of the basement membrane-associated Fraser complex. J Invest Dermatol 2014;134(5):1313-22.

29. Gebauer JM, Keene DR, Olsen BR, Sorokin LM, Paulsson M, Wagener R. Mouse A MACO, a kidney and skin basement membrane associated molecule that mediates R GD-dependent cell attachment. Matrix Biol 2009;28(8):456-62.

국문요약

배경: 대장 종양 조기 진단을 위한 혈액 검사는, 현재 대장암 선별검사의 단점을 극복하고 이에 대한 매력적인 대안이 될 것으로 기대되고 있다. Colon cancer secreted protein-2 (CCSP-2)는 유전자 발현 마이크로어레이 방법을 통해 확인된 대장암에 대한 새로운 후보 혈액 마커로서, 이 연구는 많은 수의 사람 대장암 조직에서 CCSP-2 발현 패턴을 알아보고, 대장 종양의 조기 진단을 위한 혈액 바이오마커로서의 CCSP-2 의 임상 적용 가능성을 평가하기 위해 시행되었다.

대상 및 방법: CCSP-2 의 면역 조직 화학 염색은 10 개의 진행성 선종과 69 개의 대장암 조직 및 짝을 이루는 정상조직에서 시행하였다. 사람 혈액 내 CCSP-2 는 나노바이오센서인 전장효과 대장암 센서 (E-FECS)를 사용하여 정량화 했다. 10 개의 대조군과 10 개의 대장암 검체에서 CCSP-2 표적 나노바이오센서 검증을 시행하고, 30 명의 대조군, 30 명의 진행성 선종, 81 명의 대장암 증례에서 이 새로운 검사의 효용성을 임상적으로 검증하였다.

결과: CCSP-2 단백질은 모든 대장암 및 선종 조직에서 균질하게 발현됨이 확인되었으나, 정상 대장 조직에서는 검출되지 않았다. 혈액 내 CCSP-2 농도는 대장암 및 진행성 선종 환자에서 대조군에 비해 유의하게 높았다 (P = 0.005 와 P = 0.001). 암 병기에 따른 혈액 내 CCSP-2 검출율 차이는 없었다 (P = 0.067). 대장 종양 (선종과 대장암 모두 포함)의 진단에서 혈액 CCSP-2 의 전체 민감도는 44.1% 였고, 특이도는 86.7% 였다 (AUC 는 0.67, 95% 신뢰구간 0.57- 0.76). 대장암에 대한 민감도와 특이도는 각각 44.4%와 86.7% 였으며, 진행성 선종에 대한 민감도와 특이도는 각각 43.3%와 86.7%였다.

결론: 이번 연구는 CCSP-2 표적 나노바이오센서를 사용한 새로운 혈액 검사가 종양의 진행 단계에 관계 없이 대장 종양의 약 절반 정도를 진단할 수 있음을 증명하였다. 대장암 선별검사를 위해 이 검사법을 사용하기 위해서는 민감도의 향상이 필요하며, 임상에서 이 새로운 혈액 마커의 역할을 검증하기 위한 추가 연구가 필요하다.

중심단어: CCSP-2, 대장 종양, 바이오마커, 혈액 마커, 전장효과 바이오센서

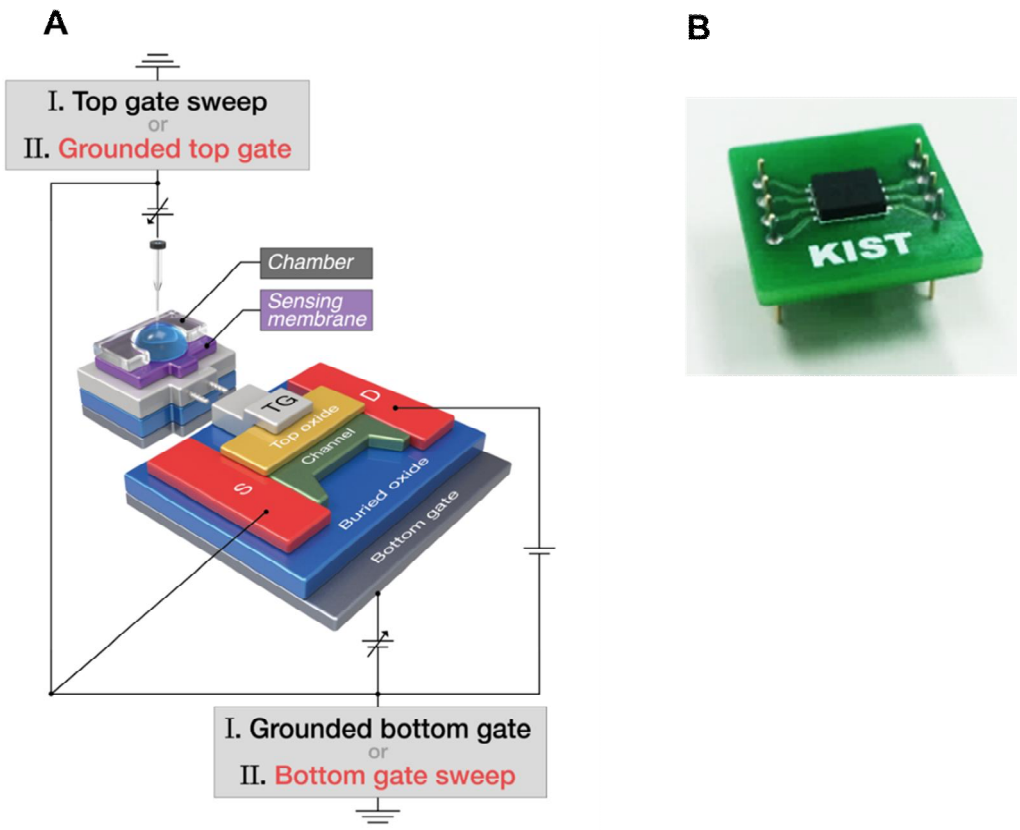

Fig. 1. (A) Schematic image of the electric-field effect colorectal sensor (E-FECS) with a disposable sensing membrane, (B) real image of the E-FECS.

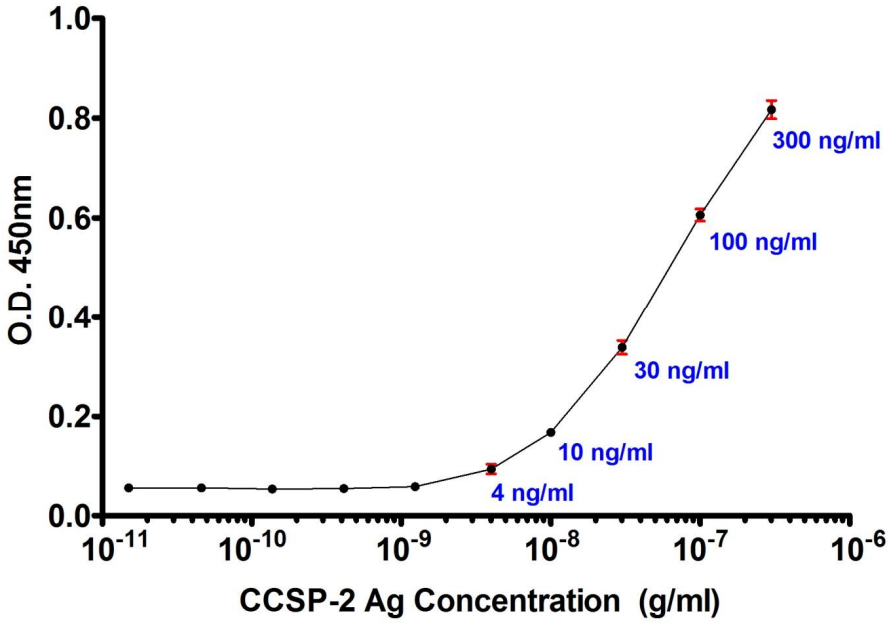

Fig. 2. Standard curve for recombinant CCSP-2 using ELISA (15 pg/mL–0.3 μg/mL).

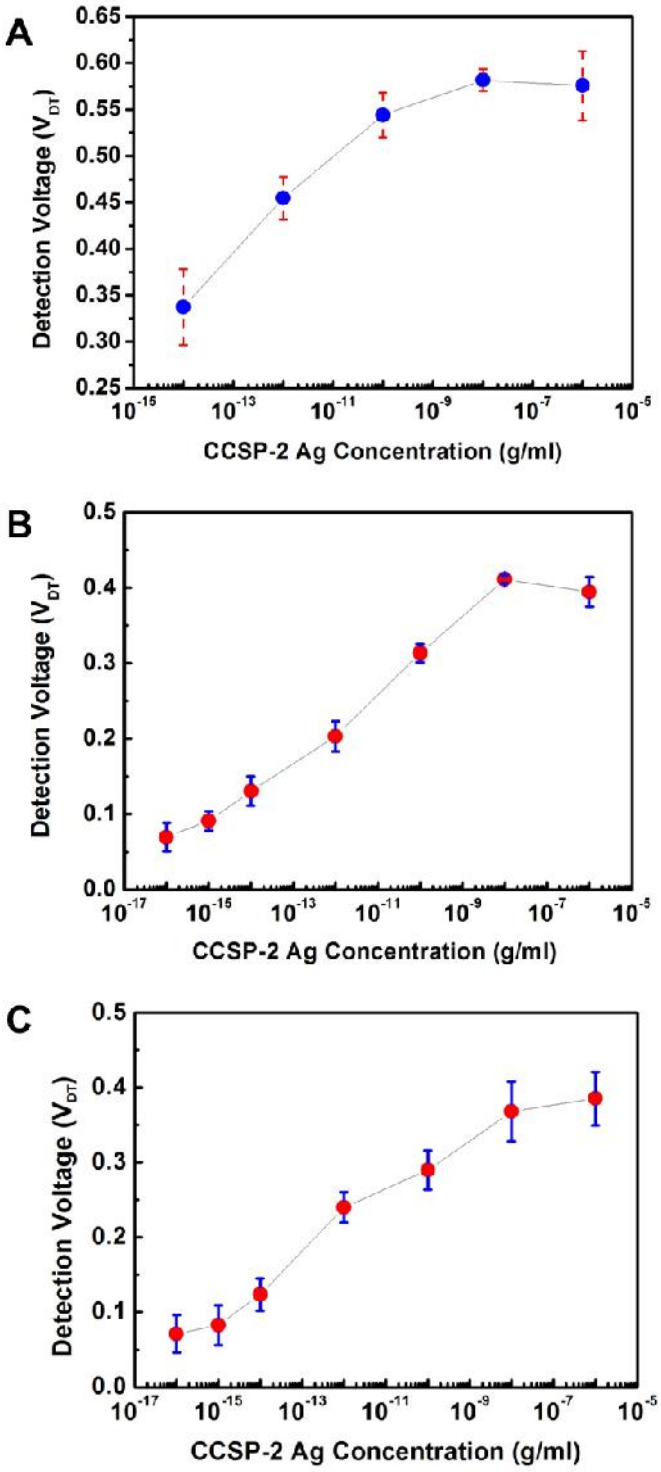

Fig. 3. Standard curve for recombinant CCSP-2 using the E-FECS (100 ag/mL–1 μg/mL) (A) with 1×PBS, (B) with serum, and (C) with plasma as a buffer.

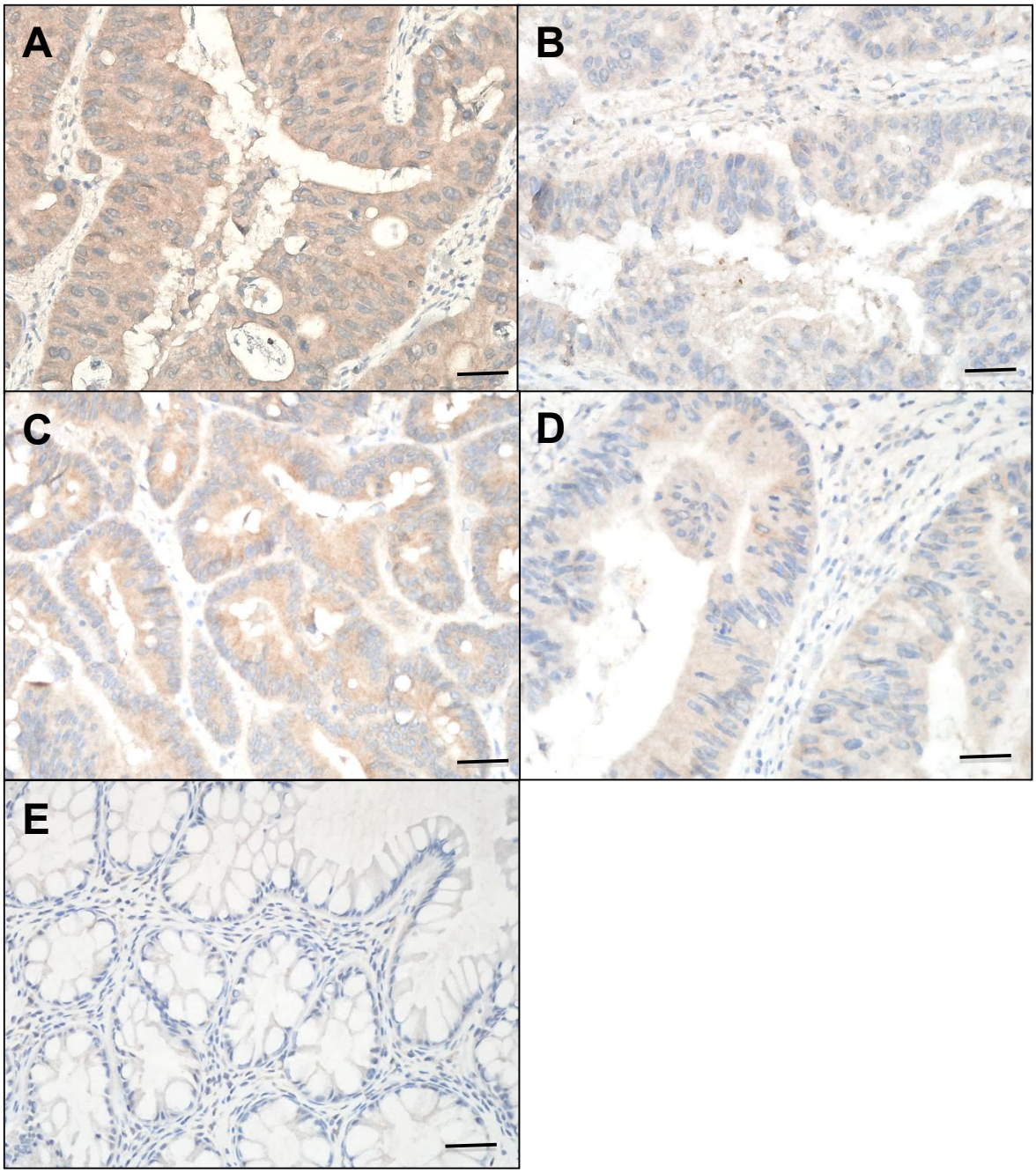

Fig. 4. Representative immunostaining results of CCSP-2 in human colorectal tissues (A) Strong positive in cancer (B) Weak positive in cancer (C) Strong positive in adenoma (D) Weak positive in adenoma (E) No signal in normal tissue. Original magnification, x200; Scale bar, 100 μm

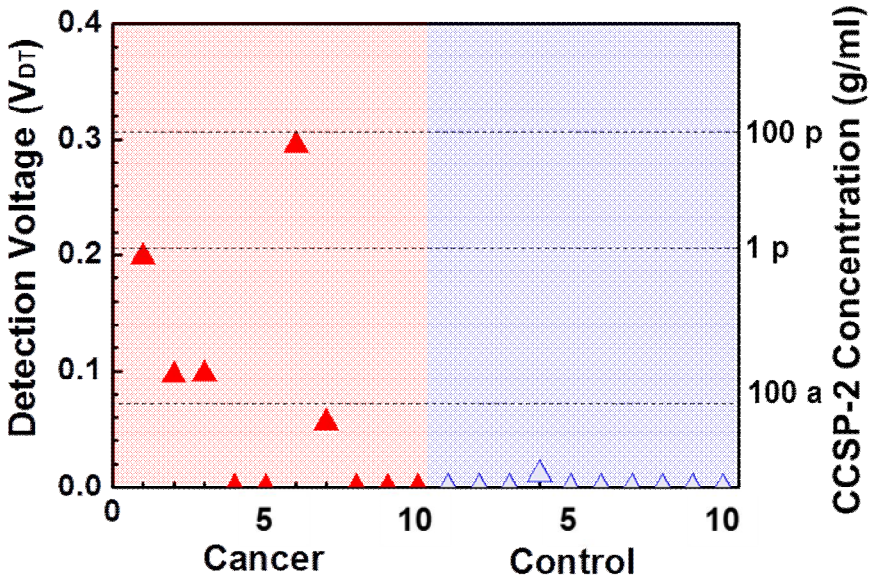

Fig. 5. The results of detection voltage for human blood samples and corresponding CCSP-2 concentrations calculated from the standard curve (the E-FECS test assay for blood CCSP-2).

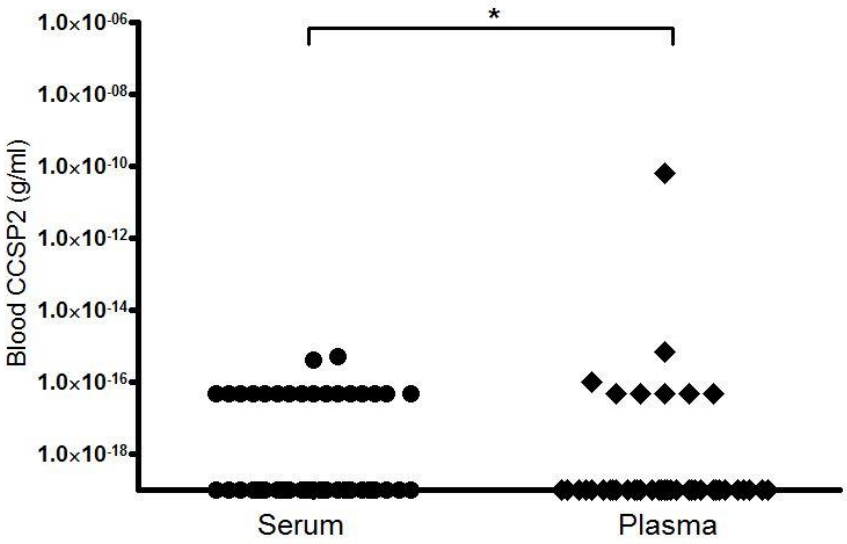

Fig. 6. Comparison of the CCSP-2 concentrations in serum to plasma preparations (*$P < 0.05$).

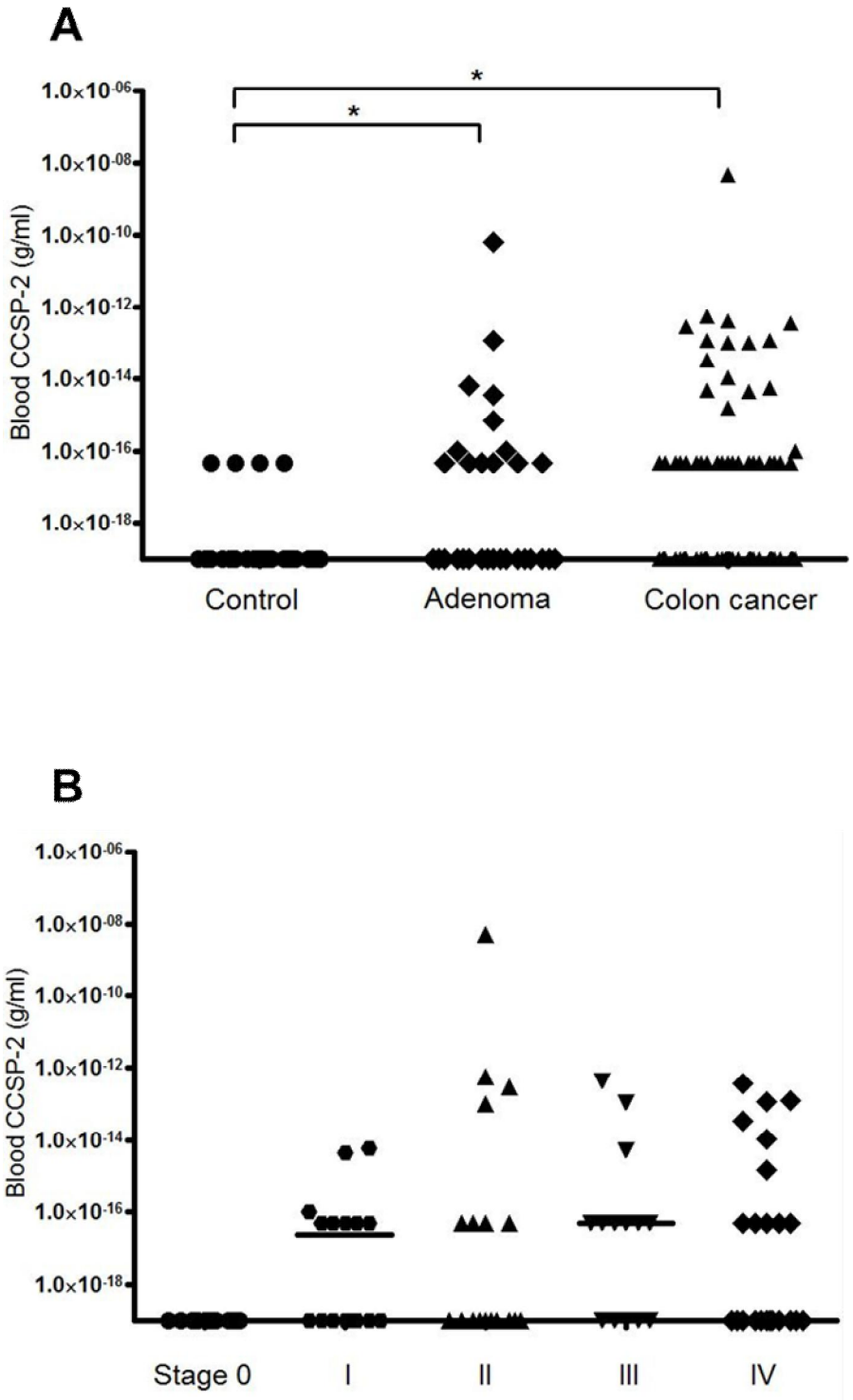

Fig. 7. Blood CCSP-2 measurement with the E-FECS. (A) In controls (n = 30) and in patients with colorectal adenoma (n = 30) and cancer (n = 81) (*$P < 0.01$), (B) the results of CCSP-2 from colorectal cancer graphed by cancer stage.

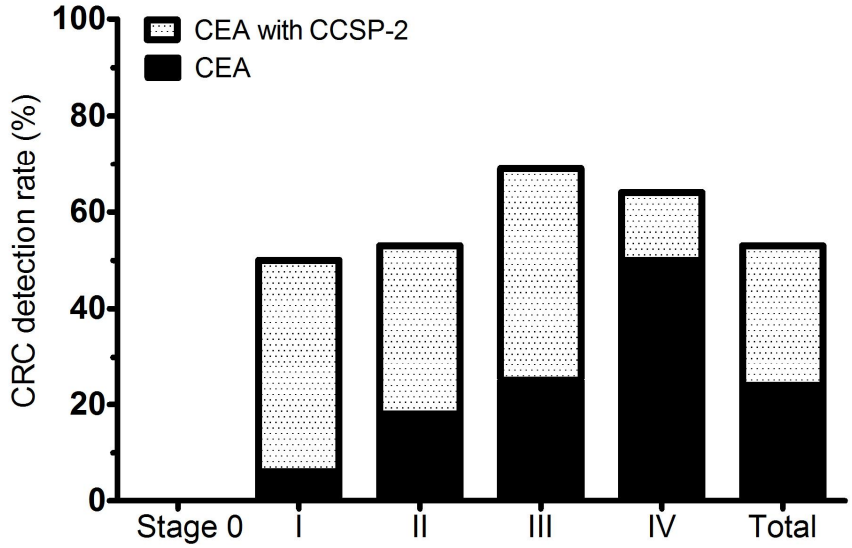

Fig. 8. The efficacy of CEA, used alone or in combination with CCSP-2, for the detection of colorectal cancer (CRC).