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Plasma circular RNA panel acts as a novel diagnostic biomarker for colorectal cancer

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ABSTRACT

Background: Colorectal cancer (CRC) is one of the most common cancers worldwide, and emerging lines of evidence have implicated circular RNAs (circRNAs), a novel class of endogenous noncoding RNAs, in CRC development. However, whether plasma circRNAs might be novel diagnostic biomarkers for CRC remains unclear.
Methods: We investigated the plasma levels of selected circRNAs by quantitative real-time PCR (qRT-PCR). The presence of the candidate circRNAs was confirmed through RNase R assays, qRT-PCR and DNA sequencing, and their diagnostic value was evaluated using a receiver operating characteristic (ROC) curve.
Results: The plasma levels of three circRNAs (circ-CCDC66, circ-ABCC1 and circ-STIL) were significantly decreased in CRC patients (n = 45) compared with healthy controls (n = 61). The ROC curve analysis showed that the area under the ROC curve (AUC) of the three-circRNA panel was 0.780, which is higher than that of traditional protein biomarkers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19–9 (CA19-9). Combining the circRNA panel with CEA and CA19-9 might improve the ability to diagnose CRC (AUC = 0.855). In addition, the plasma circ-ABCC1 level was related to tumor growth and progression, and the plasma circ-CCDC66 and circ-ABCC1 levels were decreased in precursor lesions of CRC, including colon adenomas and adenomatous polyps. More importantly, circ-CCDC66 and circ-STIL were found to be useful for diagnosing early-stage CRC, and the three-circRNA panel improved the ability to diagnose CEA-negative and CA19-9-negative CRC.
Conclusion: Our study provides the first identification of a panel of three plasma circRNAs that could serve as a novel and independent diagnostic biomarker for CRC.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth leading cause of cancer-related death worldwide and thus places an increasingly important burden on the healthcare system [1]. The 5-year survival of CRC patients differs depending on the stage at diagnosis.

At present, imaging tests and endoscopic screening are widely used for CRC diagnosis [2]. However, the high cost, time-consuming nature, invasiveness and high operator variability of these methods have resulted in a poor compliance rate [3]. Despite the availability of some noninvasive and relatively convenient methods or biomarkers for diagnosing CRC, such as the fecal occult blood test (FOBT), carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), their diagnostic efficiency needs to be improved [4,5]. Thus, the identification of novel noninvasive biomarkers for the early diagnosis and prediction of CRC is urgently needed.

Circular RNAs (circRNAs) are a novel class of noncoding RNAs (ncRNAs) characterized by covalently closed loop structures with neither 5′–3′ polarity nor a polyadenylated tail. CircRNAs have potential as diagnostic biomarkers and play regulatory roles in various diseases, such as neurological diseases, cardiovascular diseases, immune diseases, and, especially, cancers [6–10]. Numerous recent studies have shown that several circRNAs exhibit aberrant expression during CRC development. Heyda et al. found that the global circRNA abundance was reduced in CRC tissues and negatively correlated with cell

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proliferation [11]. Weng et al. discovered that ciRS-7 expression was significantly upregulated in CRC tissues compared with matched normal mucosae. These results indicate that CiRS-7 is a promising prognostic biomarker of CRC and might thus be a therapeutic target in this cancer [12]. Circ-BANP is upregulated in CRC tissues and acts as a pro-proliferative circRNA [13]. Overall, these findings highlight possible roles for circRNAs in CRC development and progression.

CircRNAs are remarkably stable molecules due to their unique structure. Memczak et al. verified the existence of thousands of circRNAs in human peripheral whole blood [14], and circRNAs have also been detected in plasma samples [15]. Huang et al. demonstrated that circRNAs are enriched in cancer cell-produced exosomes and human serum exosomes (*exo*-circRNAs). Moreover, *exo*-circRNAs might distinguish patients with cancer from healthy controls [16]. These findings further indicate the potential of circRNAs as circulating biomarkers for cancer diagnosis. Several circulating circRNAs have been used as biomarkers to discriminate cancer patients from healthy controls [17–20]. However, the diagnostic value of plasma circRNAs in CRC remains unknown. In this study, we characterized the levels of several circRNAs in plasma from patients with CRC and healthy controls to explore the potential application of circRNAs in the detection of CRC.

2. Materials and methods

2.1. Patients and specimens

All samples used in this study were collected from patients at the 904th Hospital of The People's Liberation Army between September 2015 and December 2017, and the diagnosis of CRC was confirmed by histopathological analysis. Healthy, age- and sex-matched controls were recruited from volunteers undergoing physical examination. The training cohort comprised 15 CRC patients and 15 healthy controls, and the validation cohort comprised 30 CRC patients, 46 healthy controls, and 23 disease controls (colon adenomas and adenomatous polyps). Additionally, 20 CRC patients and 20 healthy controls were randomly selected from the overall set of samples to serve as a separate model group. The clinical characteristics of the patients are shown in Table 1. None of the patients received chemotherapy or radiotherapy prior to blood samples collection. Plasma samples were isolated from 4 mL of peripheral blood using BD Vacutainer tubes (EDTA-K2 acted as anticoagulation) (BD, New Jersey, USA) and subjected to the following two-step centrifugation protocol: the tubes were centrifuged at 800 × g for 10 min, and 1 mL aliquots of the supernatant plasma were transferred to 1.5 mL RNase-free tubes (AXYGEN, JIANGSU, China) and centrifuged at 16,000 × *g* for 3 min. The supernatant plasma was then transferred to new 1.5 mL RNase-free tubes and stored at −80 °C until use. All the participants in this study provided written informed consent before being enrolled in the study. This study was approved by the Institutional Ethics Committee of the 904th Hospital of The People's Liberation Army.

2.2. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from 200 μL of plasma was extracted using the miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. A synthetic *Caenorhabditis elegans* miRNA (cel-miR-39) (RiboBio, Guangzhou, China) at a final concentration of 50 nM was used as the spike-in normalization control. For circRNA detection, reverse transcription was performed using PrimeScript RT Master Mix (TaKaRa, Beijing, China). RT-PCR was performed using SYBR reagents (TOYOBO, Shanghai, China), and the data were normalized to cel-miR-39. The expression of cel-miR-39 was measured using a TaqMan MicroRNA Reverse Transcription kit and TaqMan MicroRNA Assays (Invitrogen, Carlsbad, CA, USA). The primer sequences are listed in Supplement Table 1 (Appendix). All qRT-PCRs were performed in triplicate using a Bio-Rad CFX96 real-time PCR system, and the relative expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method [21].

2.3. RNAse R treatment

Total RNA (5 μg) isolated from HEK-293 cells was incubated for 15 min at 37 °C in the presence or absence of 3 U/μg RNase R (Epicenter Technologies, Madison, WI, USA). The resulting RNA was purified using a RNeasy MinElute Cleanup Kit (QIAGEN, Hilden, Germany), and the circRNA expression levels were measured by qRT-PCR.

2.4. Electrophoresis and sequencing of qRT-PCR products

To verify the qRT-PCR products of the plasma circRNAs, 5 μL of the product mixture was subjected to 2% agarose gel electrophoresis. The qRT-PCR product of each circRNA was sent for DNA sequencing at Sangon Biotech Co., Ltd. (Sangon, Shanghai, China).

2.5. Serological CRC-associated protein biomarker analysis

The CEA and CA19-9 concentrations in the serum of patients with CRC, colon adenomas and adenomatous polyps and healthy controls were measured using a Cobas e602 system with the Elecsys CEA and CA19-9 Assay kit (Roche Diagnostics, Basel, Switzerland). The cutoff values for CEA and CA19-9 were 5 ng/mL and 37 U/mL, respectively. The detection limits for CEA and CA19-9 were 0.2 ng/mL and 0.6 U/mL, respectively, with a coefficient of variation (CV) < 5%.

2.6. Statistical analysis

The Mann–Whitney test was used to compare the differences in plasma circRNA expression between the CRC and control groups. ROC curves were used to assess the sensitivity and specificity of the circRNA biomarkers. Logistic regression was used to develop a combined circRNA panel. The optimal cutoff values for each circRNA and the circRNA panel were defined independently at the maximal (sensitivity + specificity)/2 value. Spearman correlation analyses were performed to examine the relationship between circRNA levels and clinical parameters. All statistical analyses were performed using SPSS 18.0 statistical software (IBM, Armonk, New York, USA). ROC curves were generated using MedCalc 11.0 (MedCalc Software, Ostend, Belgium), and graphs were generated using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Selection of candidate circRNAs as biomarkers for CRC and validation of the presence of circRNAs in plasma

CircRNAs are reportedly involved in CRC development and progression. We selected candidate circRNAs as potential plasma biomarkers based on the following criteria: 1) the circRNA is differentially expressed in CRC tissues and nonmalignant tissues; 2) the function of the circRNA has been confirmed *in vitro* or *in vivo*; and 3) the circRNA has not been analyzed in plasma. We selected circ-CCDC66 (chr3:56626997-56628056) [22], circ-ABCC1 (chr16:16101672-16162159) [23], circ-STIL (chr1:47745912-47748131) [24] and circ-USP3 (chr15:63824845-63855207) [11] for further investigation and these circRNAs were then validated by RNase R digestion and RT-PCR. First, total RNA isolated from HEK-293 cells was treated with RNase R, and the circRNAs were then amplified using divergent primers. The results showed that circ-CCDC66, circ-ABCC1, circ-STIL and circ-USP3 could be amplified by divergent primers and were resistant to RNase R digestion (Fig. 1A–B). A well-known circRNA circ-HIPK3 [25] and GAPDH linear RNA were used as the positive and negative controls,

Table 1
Clinicopathological characteristics of the patients in the training and validation cohorts.

	Training cohort			Validation cohort			
	CRC (n = 15)	Control (n = 15)	P value†	CRC (n = 30)	Precancerous lesions (n = 23)	Control (n = 46)	P value†
Gender, no. %							
Male	7(46.7)	7(46.7)	1	17(56.7)	13(56.5)	24(52.2)	0.907
Female	8(53.3)	8(53.3)		13(43.3)	10(43.5)	22(47.8)	
Age							
Median (IQR)	63 (56.5–70)	62 (54.5–69)	0.631	63 (57.25–69.75)	59 (52.5–63.5)	58 (52.25–65.75)	0.916
Tumor location							
Rectum	8(53.3)			14(46.7)			
Colon	7(46.7)			16(53.3)			
Pathological T category							
T1	1(6.7)			5(16.7)			
T2	1(6.7)			5(16.7)			
T3	6(40.0)			11(36.7)			
T4	7(46.7)			9(30)			
Lymph node metastasis							
Negative	7(46.7)			16(53.3)			
Positive	8(53.3)			14(46.7)			
Distant metastasis							
Negative	11(73.3)			27(90.0)			
Positive	4(26.7)			3(10.0)			
TNM stage							
I + II	6(40.0)			13(43.3)			
III + IV	9(60.0)			17(56.7)			
CEA							
< 5.0 ng/mL	11(73.3)			20(66.7)			
≥5.0 ng/mL	4(26.7)			10(33.3)			
CA19-9							
< 37.0 U/mL	10(66.7)			25(83.3)			
≥37.0 U/mL	5(33.3)			5(16.7)			

Abbreviations: CRC, colorectal cancer; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19–9.
† P values for gender and age based on comparisons of the distribution across the CRC and control subgroups in the training cohorts and across the CRC, precancerous lesions, and control subgroups in the validation cohorts. The P values were calculated using the Pearson chi-squared test.

respectively. The PCR products of these circRNAs in RNA isolated from patient plasma samples were verified by Sanger sequencing (Fig. 1C). CircRNAs lack a polyA tail for backsplicing, and to identify these circRNAs, we reverse-transcribed human plasma RNA with random 6-mers or oligo (dT) primers and then performed qRT-PCR assays. The results showed that circ-CCDC66, circ-ABCC1, circ-STIL and circ-USP3 could effectively be transcribed by random 6-mer but not oligo (dT) primers (Fig. 1D), which indicated that these circRNAs have no polyA tail. Taken together, these results suggest that circ-CCDC66, circ-ABCC1, circ-STIL and circ-USP3 are stable in the plasma of CRC patients.

3.2. The plasma levels of circ-CCDC66, circ-ABCC1 and circ-STIL are decreased in CRC patients

To determine the levels of the four candidate circRNAs in the plasma of CRC patients, we performed qRT-PCR analyses using a training cohort comprising 15 CRC patients and 15 healthy controls. The levels of circ-CCDC66, circ-ABCC1 and circ-STIL in CRC patients were significantly lower than those in healthy controls, with fold changes of 0.58, 0.69 and 0.55, respectively (Fig. 2A). We excluded circ-USP3 from further consideration because no significant difference was found for plasma levels of this marker in the CRC patients *vs*. the healthy controls. We then analyzed the expression of the three remaining circRNAs using an independent validation cohort comprising 30 CRC patients and 46 healthy controls. The plasma levels of circ-CCDC66, circ-ABCC1 and circ-STIL were consistently decreased in CRC patients compared with those in healthy controls, with average fold decreases of 0.58, 0.43 and 0.65, respectively (Fig. 2B).

Subsequently, we performed a receiver operating characteristic (ROC) curve analysis and evaluated the sensitivity and specificity of each of these circRNAs for the diagnosis of CRC (including all stages). For the training and validation cohorts, the areas under the ROC curves (AUCs) for the above-mentioned three circRNAs ranged from 0.634 to 0.773 ($P < 0.05$). As shown in Fig. 2C, the AUCs of circ-CCDC66, circ-ABCC1 and circ-STIL obtained in the combined analysis of all the samples were 0.756 (95% confidence interval [21], 0.663–0.834; $P < 0.0001$), 0.663 (95% CI, 0.56–0.767; $P = 0.0041$) and 0.677 (95% CI, 0.579–0.764; $P = 0.0018$), respectively. Furthermore, a logistic regression analysis was performed to determine whether the combination of the three circRNAs exhibited higher predictive value than each individual circRNA. The AUC of the three-circRNA panel was 0.780 (95% CI, 0.689–0.854; $P < 0.0001$), with 64.4% sensitivity and 85.2% specificity (Fig. 2C, Supplement Table 2 (Appendix)). Compared with the widely accepted CRC protein biomarkers (CEA and CA19-9), the three-circRNA panel had an increased AUC value (panel: 0.780, CEA: 0.695, CA19-9: 0.678). Thus, combining the three-circRNA panel with CEA and CA19-9 may improve the capability to diagnose CRC patients (AUC = 0.855; Fig. 2D).

Then, we assessed the predictive value of the three-circRNA panel in 20 CRC patients and 20 healthy controls by random assignment. We were unaware of the patients' clinical characteristics at the time of the evaluation. Based on the logistic regression model, we correctly discriminated 14 of the 20 CRC samples (positive predictive value of 70%) and 16 of the 20 healthy control samples (negative predictive value of 80%). Taken together, our data suggest that the three-circRNA panel is a potential noninvasive biomarker for CRC.

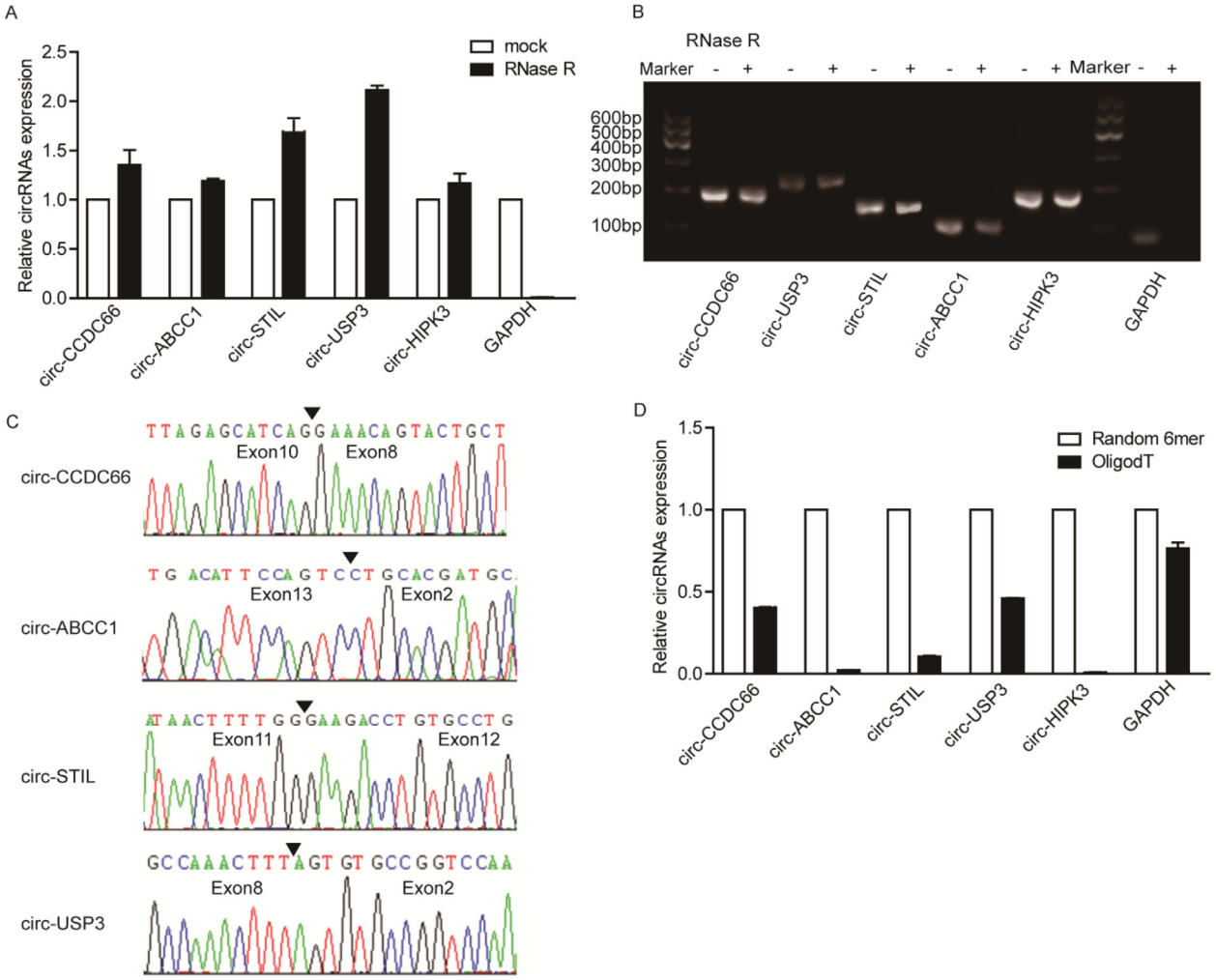

Fig. 1. The presence of selected circRNAs was validated. (A) Total RNA isolated from HEK-293 cells was treated or not treated (mock) with RNase R, and the expression levels of GAPDH and the selected circRNAs were then measured by qRT-PCR. (B) Gel electrophoresis of the qRT-PCR products pretreated with RNase R and the mock products was performed. (C) The qRT-PCR products of the plasma circRNAs were confirmed by Sanger sequencing. (D) cDNA of the selected plasma circRNAs was transcribed with random 6-mer or oligo (dT) primers and then detected by qRT-PCR.

3.3. Association of the circRNA biomarkers with CRC clinical characteristics, surgical treatment and precancerous lesions

We evaluated whether the plasma levels of the three circRNAs were associated with CRC clinical characteristics. Lower plasma levels of circ-ABCC1 correlated with significantly higher tumor stages, greater tumor burden, more lymph node metastasis and distal metastasis (Fig. 3A). However, the levels of circ-CCDC66 and circ-STIL in plasma had no correlation with tumor stage, lymph node metastasis or distal metastasis (data not shown).

We then determined the expression levels of the three circRNAs in 10 paired plasma samples from pre- and postoperative CRC patients. As shown in Fig. 3B, the plasma level of circ-CCDC66 was shown to have increased significantly in eight patients after surgery ($P < 0.05$), while levels of circ-ABCC1 and circ-STIL did not. These results indicate that circ-CDCC66 may be useful in predicting postoperative improvement in CRC patients.

Considering that in most patients, CRC develops from precancerous precursor lesions over a prolonged period, we further determined whether the expression of the three circRNAs changes as early as the precancerous lesion stage. The levels of both circ-CCDC66 and circ-ABCC1 in the plasma of patients with colon adenomas and adenomatous polyps (23 cases), which are well-defined precursor lesions of CRC, were significantly decreased when compared to those in healthy controls. However, the level of circ-STIL was unchanged in precancerous conditions (Fig. 3C). These data suggest that circ-CCDC66 and circ-ABCC1 can differentiate both precancerous lesions and malignant CRC from healthy control tissue. This ability is especially beneficial for the early diagnosis of CRC. Taken together, these results indicate that both circ-CCDC66 and circ-ABCC1 are valuable for identifying precancerous lesions. Moreover, circ-CCDC66 and circ-ABCC1 have significant value for identifying surgical treatment and CRC stage.

3.4. A two-circRNA panel is a biomarker of early-stage CRC

Early diagnosis and treatment are important for improving the overall survival (OS) of CRC patients. We measured the levels of the three circRNAs in the plasma of 19 patients with early-stage CRC (TNM stage I or II) and found that the plasma levels of circ-CCDC66 and circ-STIL were significantly decreased, with an average fold decrease of 0.575 and 0.311, respectively, in patients with early-stage CRC compared to that in healthy controls (Fig. 4A). We excluded circ-ABCC1 from further analysis because it had an AUC of 0.546 ($P > 0.05$). We subsequently attempted to combine a new two-circRNA panel comprising circ-CCDC66 and circ-STIL to evaluate the diagnostic value for early-stage CRC. We first measured the concentrations of recommended protein tumor biomarkers such as CEA and CA19-9 [26] in the serum of

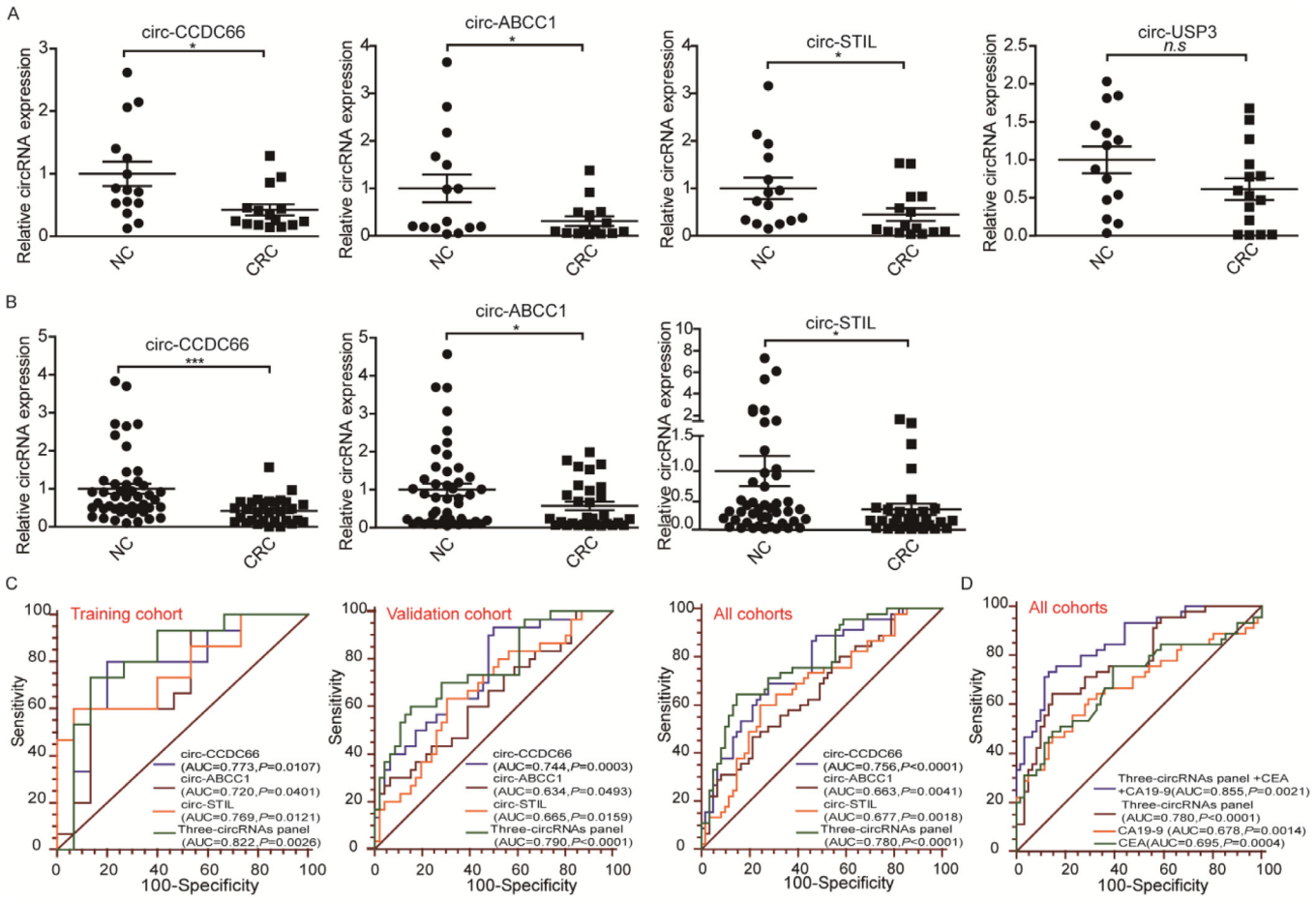

Fig. 2. The expression of selected circRNAs in the plasma of CRC patients was measured by qRT-PCR. (A) Expression of circ-CCDC66, circ-ABCC1, circ-STIL and circ-USP3 in the training cohort (n = 15 *vs.* 15). (B) Expression of circ-CCDC66, circ-ABCC1, circ-STIL and circ-USP3 in the validation cohort (n = 30 *vs.* 46). (C) AUC values were calculated obtained as a combined measure of the sensitivity and specificity of individual circRNAs and the three-circRNA panel in the various cohorts. cel-miR-39 was used as the external reference for all qRT-PCR assays. (D) Comparation of the AUC values between the three-circRNA panel and CEA or CA19-9 for CRC diagnosis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; *n.s.*, not significant.

the 19 patients with early-stage CRC. We found no significant difference in the CEA and CA19-9 levels between patients with early-stage CRC and healthy controls (Fig. 4B). Then, we evaluated the diagnostic value of circ-CCDC66, circ-STIL, CEA and CA19-9 in patients with early-stage CRC. The AUCs of circ-CCDC66 and circ-STIL were 0.746 (95% CI, 0.633–0.858; $P = 0.0013$) and 0.769 (95% CI, 0.657–0.881; $P = 0.0004$), respectively, higher than the AUCs of CEA (AUC = 0.604; 95% CI, 0.529–0.748; $P = 0.1731$) and CA19-9 (AUC = 0.644; 95% CI, 0.533–0.752; $P = 0.0605$). The combination of circ-CCDC66 and circ-STIL showed an AUC of 0.777 (95% CI, 0.671–0.863; $P = 0.0006$), which was higher than that of the combination of CEA and CA19-9 (AUC = 0.648; 95% CI, 0.533–0.752; $P = 0.0526$). In addition, incorporation of CEA and CA19-9 into the two-circRNA panel yielded the highest AUC value for predicting early-stage CRC (AUC = 0.786; 95% CI, 0.680–0.870; $P = 0.0002$) (Fig. 4C, Supplement Table 2 (Appendix). These results indicated that circ-CCDC66 and circ-STIL can be used for the early diagnosis of CRC and are superior to either CEA or CA19-9.

3.5. Circ-CCDC66, circ-ABCC1 and circ-STIL are useful biomarkers for CEA-negative or CA19-9-negative CRC

The serum levels of CEA and CA19-9 are not increased in some CRC patients.. Therefore, we explored the ability of the three circRNAs to diagnose CEA-negative or CA19-9-negative CRC. We measured the levels of the three circRNAs in 31 CEA-negative and 35 CA19-9-negative CRC patients (TNM stages I-III). The plasma levels of circ-CCDC66, circ-ABCC1 and circ-STIL in both CEA-negative and CA19-9-negative CRC patients were significantly lower than compared to thatthose in healthy controls (Fig. 5A-B).

We then assessed the diagnostic value of circ-CCDC66, circ-ABCC1 and circ-STIL for CEA- or CA19-9-negative CRC patients. As shown in Fig. 5C, the AUCs obtained for circ-CCDC66, circ-ABCC1 and circ-STIL in CEA-negative CRC patients were 0.76 (95% CI, 0.660–0.843; $P < 0.0001$), 0.632 (95% CI, 0.526–0.731; $P = 0.0385$) and 0.664 (95% CI, 0.558–0.759; $P = 0.0106$), respectively, and that for the three-circRNA panel was 0.775 (95% CI, 0.676–0.856; $P < 0.0001$). In CA19-9-negative CRC patients, the AUCs found for circ-CCDC66, circ-ABCC1 and circ-STIL were 0.764 (95% CI, 0.666–0.845; $P < 0.0001$), 0.651 (95% CI, 0.547–0.746; $P = 0.0141$) and 0.676 (95% CI, 0.573–0.768; $P = 0.0041$), respectively, and the AUC obtained with the three-circRNA panel was 0.778 (95% CI, 0.682–0.856; $P < 0.0001$) (Fig. 5C, Supplement Table 2, (Appendix)). These results suggest that the three-circRNA panel is useful for the diagnosis of CRC patients with normal CEA or CA19-9 levels.

Subsequently, the performance of the three circRNAs and CEA or CA19-9 in detecting CRC patients was compared. In the 45 CRC patients recruited in this study, revealed that CEA and CA19-9 discriminated 14 and 10 cases, with sensitivity values of 31.1% and 22.2%, respectively. While plasma circ-CCDC66, circ-ABCC1 and circ-STIL discriminated 40 (88.9%), 23 (51.1%) and 27 (60.0%) cases, respectively, the three-

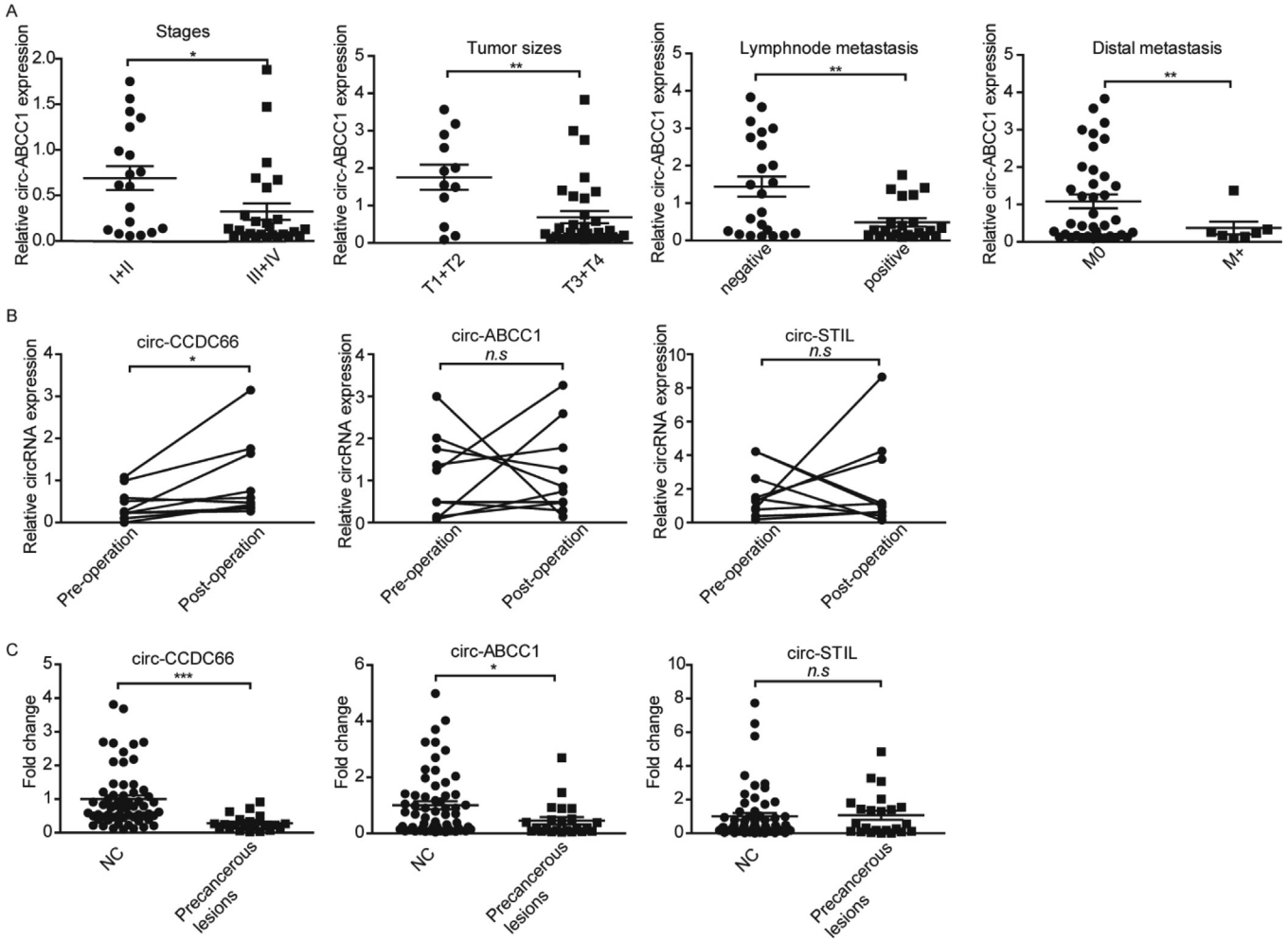

Fig. 3. Association of circRNA biomarkers with CRC clinical characteristics, surgical treatment and precancerous lesions. (A) Analysis of the correlations of the circ-ABCC1 levels with clinical characteristics (n = 45). (B) The levels of circ-CCDC66, circ-ABCC1 and circ-STIL in 10 paired plasma samples from pre- and postoperative CRC patients were analyzed by qRT-PCR. (C) The plasma levels of circ-CCDC66, circ-ABCC1 and circ-STIL in precancerous lesions were analyzed by qRT-PCR. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; *n.s.*, not significant.

circRNA panel discriminated 29 (64.4%) cases. The patients detected by each circRNA or three-circRNA panel were not completely overlapped. Among the 45 CRC patients, 13 patients could be diagnosed by either individual or combined circRNAs. The sensitivity values of the three-circRNA panel and individual circRNA were higher than those of CEA and CA19-9 separately (Fig. 5D). For a new biomarker, sensitivity and specificity should be comprehensively analyzed to evaluate the effect of the diagnostic biomarker. According to the ROC curve (Supplement Table 2, (Appendix)), the AUC of three individual circRNAs are better for CRC detection than CEA and CA19-9, and the AUC of three-circRNA panel is higher than individual circRNA. Taken together, our data suggest that the three-circRNA panel is potentially a noninvasive and efficient diagnostic biomarker for CRC.

3.6. Correlation analysis between the three circRNAs, CEA and CA19-9 in the plasma of patients with CRC

We performed a correlation analysis to determine whether the three circRNAs were correlated with each other. As shown in Supplement Table 3, (Appendix), significant correlations were found between circ-STIL and circ-CCDC66 ($P < 0.001$) and between circ-CCDC66 and circ-ABCC1 ($P < 0.001$). We also calculated the correlation between the three-circRNA panel and the protein biomarkers CEA and CA19-9. As shown in Supplement Table 4 (Appendix), the plasma CEA and CA19-9 levels were correlated, with a correlation coefficient of 0.338 ($P < 0.001$). Notably, no correlation was detected between the three-circRNA panel and the traditional biomarkers. These results suggest that the three-circRNA panel could be an independent biomarker for CRC.

4. Discussion

Currently, the FOBT is the most widely used noninvasive screening test for CRC [3]. Although the FOBT does reduce deaths from CRC due to earlier detection, the major disadvantage of the FOBT is its low sensitivity for both CRC (25%–38%) and advanced adenomas (16%–31%) [4]. Blood biomarkers, such as CEA, CA19-9, CA242, CA72-4, tissue polypeptide antigen (TPA) and tissue polypeptide-specific antigen (TPS), have also been developed, but a lack of sensitivity and specificity precludes their use for the early detection of CRC [5]. Thus, the identification of more reliable diagnostic biomarkers that would allow the early and confident identification of CRC patients is very desirable.

An overwhelming amount of evidence has demonstrated that circRNAs in tissue samples are associated with tumor development and progression [27,28]. In CRC, circRNAs are associated with clinicopathological characteristics of CRC patients, such as differentiation, TNM classification and distant metastasis, and might even play crucial roles in multiple processes associated with malignant phenotypes [29]. Due to their presence and stability in human blood, circRNAs could be

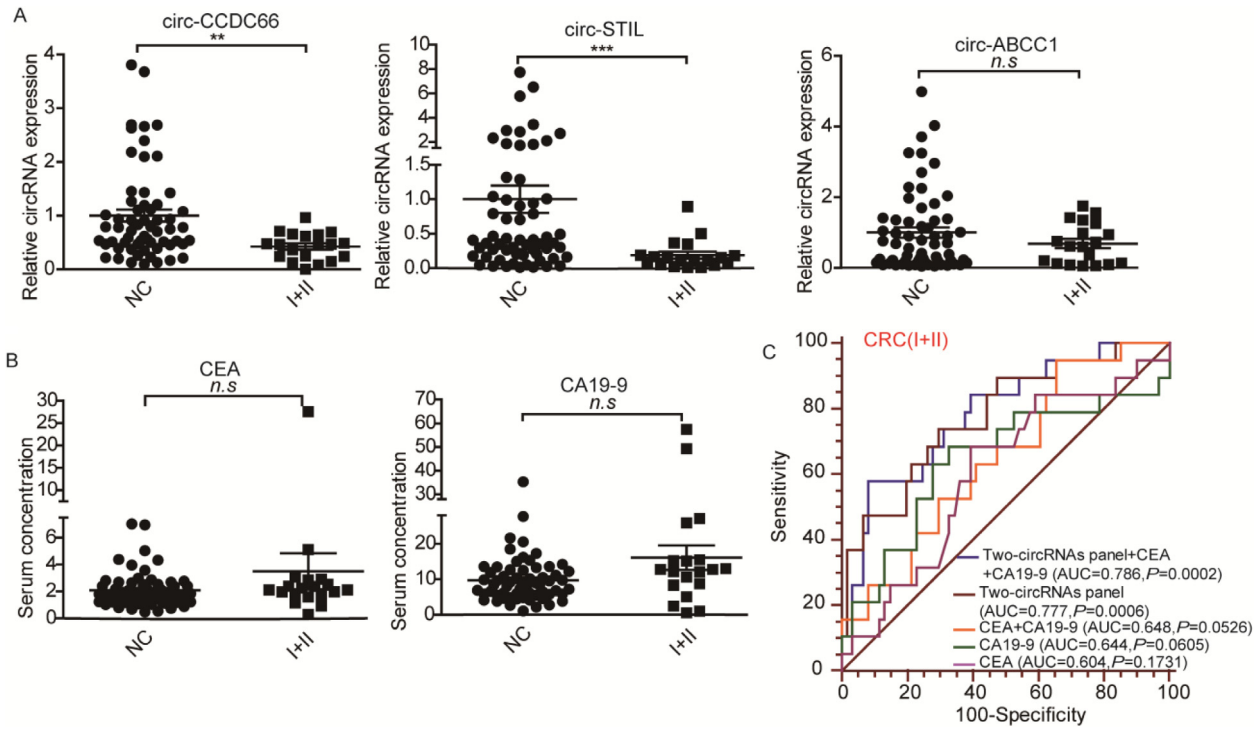

Fig. 4. The value of the three circRNAs for diagnosing early-stage CRC was evaluated. (A) Plasma expression of circ-CCDC66, circ-ABCC1, circ-STIL and circ-USP3 in healthy controls and patients with early-stage CRC (n = 61 *vs.* 19). (B) Serum concentrations of CEA and CA19-9 in healthy controls and patients with early-stage CRC (n = 61 *vs.* 19). (C) AUC values were calculated as a combined measure of the sensitivity and specificity of the circRNAs, CEA and CA19-9 in early-stage CRC. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; *n.s*, not significant.

used as noninvasive biomarkers. Recently, plasma circRNAs have been demonstrated to serve as biomarkers in several diseases such as type 2 diabetes mellitus [30], rheumatoid arthritis (RA) [31] and cancers [18–20,32].

Here, we first found that a panel of three circRNAs (circ-CCDC66, circ-ABCC1 and circ-STIL) exhibited a significant decrease in the plasma of CRC patients compared with that of healthy controls and was a potential independent predictive biomarker for CRC. Moreover, the level of circ-ABCC1 was negatively related to the tumor burden, tumor progression stage, and lymph node and distant metastasis statuses. CRC formation is a complex and dynamic process. Notably, our results indicate that the expression of plasma circ-CCDC66 and circ-ABCC1 was decreased in precursor lesions of CRC, such as colon adenomas and adenomatous polyps. Combining the three-circRNA panel with the traditional serum tumor protein biomarkers CEA and CA19-9 might improve the capability for diagnosing CRC patients.

For the diagnosis of CRC using blood biomarkers, improving the detection rate of early CRC and reducing the rate of missed diagnoses are two urgent problems to be solved. We successfully combined a two-circRNA panel comprising circ-CCDC66 and circ-STIL for the diagnosis of early-stage CRC (AUC = 0.777). Combining the two-circRNA panel with CEA and CA19-9 further improved the prediction rate of early-stage CRC. Moreover, our results showed that circ-CCDC66, circ-ABCC1 and circ-STIL could be useful biomarkers for CEA-negative or CA19-9-negative CRC.

To our knowledge, this study constitutes the first analysis of the application of plasma circRNAs as potential predictive and diagnostic biomarkers for CRC. The findings might improve our understanding of the roles of circRNAs in CRC. However, we recognize that some limitations of the current study need to be considered. First, we focused on three circRNAs based on a screen of the reported literature; thus, we cannot exclude the possibility that other circRNAs might also have vital diagnostic value in CRC. Considering this possibility, high-throughput methods such as microarrays and next-generation sequencing should be used to characterize the global landscape of plasma circRNAs in a cohort of human subjects and thus identify the most valuable diagnostic and prognostic factors for CRC. Second, the sample size in this single-center study was relatively small, and the geographical distribution of the participants was relatively narrow. Thus, these data need to be confirmed with a larger number of samples and multiple centers. Third, our study included only pre- and postoperative follow-up. A longer prospective study should be conducted to provide additional information on clinical outcomes. Despite these limitations, the sensitivity and specificity of the three-circRNA panel have been shown to be higher than those of other known CRC biomarkers.

Hsiao et al. found that circ-CCDC66 is upregulated in colon polyps and cancer tissues and functions as an oncogene through the regulation of a subset of oncogenes [22]. In addition, circ-STIL is upregulated in CRC tissues compared with adjacent precancerous tissues and promotes cell proliferation, migration and invasion [24]. However, we demonstrated that the expression of these two circRNAs was decreased in the plasma of CRC patients. This contradiction indicates that the molecular mechanism underlying the secretion of plasma circRNAs remains uncertain. CircRNAs can originate not only from cancer cells but also cells in the tumor microenvironment, immune cells or cells from other organs [29]. A large body of evidence shows that non-coding RNAs (ncRNAs) (including miRNAs, lncRNAs and circRNAs) could be selectively secreted into the blood and that the expression of ncRNAs in plasma differs from that in tissues, which is a phenomenon related to their active secretion mechanism and function [33]. Therefore, more variable sample sources of these three circRNAs should be examined, and the roles of the above-mentioned circRNAs need further investigation.

In conclusion, we identified a plasma circRNA panel that might serve as a novel diagnostic biomarker for CRC. Combination of the three-circRNA panel with traditional protein biomarkers will improve the sensitivity and specificity of CRC diagnosis. The three-circRNA panel is helpful for diagnosing early-stage CRC and CEA-/CA19-9-

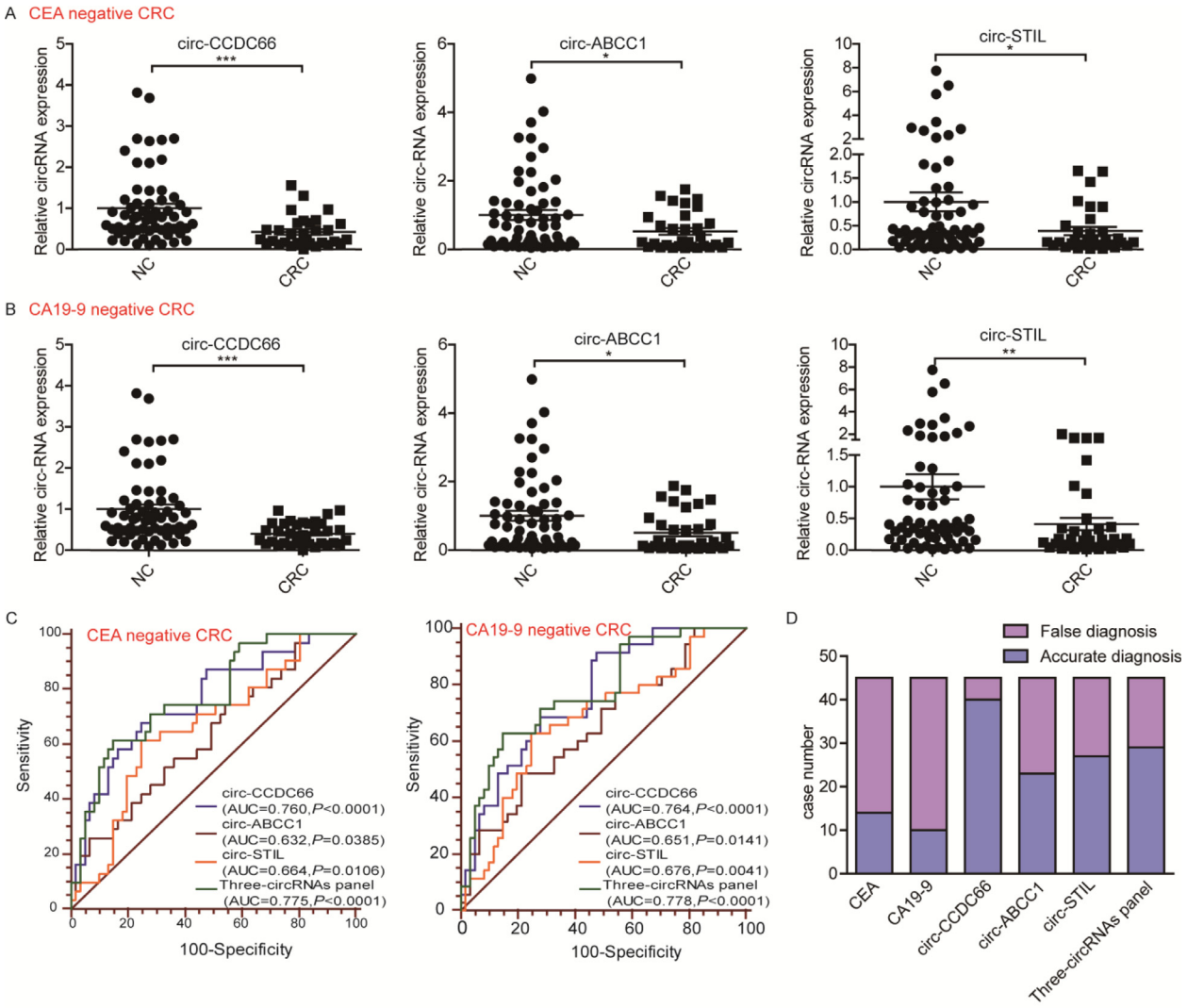

Fig. 5. The ability of the circRNA panel to identify CEA-negative or CA19-9-negative CRC was evaluated. (A) Plasma expression of circ-CCDC66, circ-ABCC1, circ-STIL and circ-USP3 in healthy controls and patients with CEA-negative CRC (n = 61 *vs.* 31) (B) Plasma expression of circ-CCDC66, circ-ABCC1, circ-STIL and circ-USP3 in healthy controls and patients with CA19-9-negative CRC (n = 61 *vs.* 35) (C) AUC values were calculated as a combined measure of the sensitivity and specificity of the circRNAs in the CEA- and CA19-9-negative groups. (D) The detection rates of CEA, CA19-9, the three circRNAs and the three-circRNA panel in 45 patients with CRC were compared. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

negative CRC. A prospective, multicenter study is currently underway to confirm the potential utility of the circRNA panel. Understanding the detailed molecular mechanisms and biological functions of these three circRNAs in CRC is necessary. We anticipate that in the next few decades, a substantial number of circRNA biomarkers will be available for the overall clinical management of CRC.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2019.10.012.

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