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Original Article

A fluorescent microsphere-based immunochromatographic strip is effective for quantitative fecal blood testing in colorectal cancer screening

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Abstract: *Background:* Colorectal cancer (CRC) represents a major health concern that can be screened for by the fecal immunochemical test (FIT), which detects blood in the stool. CRC detection sensitivity for hemoglobin (Hb) combined with transferrin (Tf) is higher than for hemoglobin alone. *Methods:* We developed a europium fluorescent microsphere-based quantitative lateral flow immunochromatography strip to detect fecal Hb and Tf. Performance was tested using fecal samples from 51 patients with CRC and 122 normal subjects. Test strips were generated using paired mouse anti-human Hb and mouse anti-human Tf monoclonal antibodies and tested using standard Hb and Tf samples. Fluorescence was observed at 365 nm and quantitatively measured using a portable fluorescent strip reader. *Results:* At cutoff values of 100 ng/mL (10 μg/g feces) and 25 ng/mL (2.5 μg/g feces) for Hb and Tf, respectively, the positive rates for Hb, Tf, and Hb+Tf in normal subjects were 6.56%, 5.74%, and 10.66%, respectively, compared to 88.24%, 64.71%, and 94.12% in patients with CRC. The sensitivity and specificity of the FIT combined detection technique were 87.5% and 89.2%, respectively, and the area under the curve (AUC) was 0.92. The sensitivity, specificity, and AUC for the Tf assay were 63.8%, 68.4%, and 0.759, respectively, and those for Hb testing were 69.7%, 70.2%, and 0.774, respectively. The AUC for Hb+Tf was significantly higher than those for Tf or Hb alone ($P < 0.001$). *Conclusions:* Fluorescent microsphere-based immunochromatographic strips sensitively detect fecal Hb and Tf, and sensitivity and specificity are improved for Hb+Tf. This system represents a rapid and portable alternative for on-site early CRC screening.

Keywords: Fluorescent microsphere, immunochromatographic strip, fecal blood test

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related death [1]. Early CRC screening substantially contributes to a downward trend in CRC incidence and mortality. Randomized controlled trials have shown that conducting annual fecal occult blood tests (FOBTs) and follow-up examinations, such as colonoscopy, can decrease CRC incidence by 30% and reduce CRC-related mortality by 50% [2, 3].

The 5-year survival rate among patients diagnosed with early-stage, localized CRC (Stages I and II) approaches 90%, whereas the survival rate for those diagnosed with late-stage CRC, which often involves metastasis to distal organs, is only 13.1%. Early CRC screening aims to prevent cancer morbidity and mortality by detecting significant lesions at an early stage before they become metastatic [4, 5].

Most CRC cases and premalignant adenomatous polyps are associated with the presence of blood in the feces, often in small quantities not easily observed directly. Various methods have been developed to detect blood in fecal specimens, including the guaiac FOBT (g-FOBT) and

the fecal immunochemical test (FIT). Researchers have suggested that the FIT can detect CRC and colorectal polyps with higher sensitivity than the g-FOBT and may be more acceptable to patients [6].

FITs can be either qualitative or quantitative. Qualitative FITs typically use a colloidal gold immunochromatographic strip that does not require specific instruments and is interpreted based on the appearance of test bands on the strip. For these reasons, qualitative FITs can be used for at-home testing. Quantitative FITs typically utilize immunoturbidimetry technology and are more sensitive and accurate than qualitative FITs; however, quantitative FITs require specialized devices and are often performed in a laboratory [7].

Currently, most FIT techniques use fluorescent labeling-based immunochromatographic assays. Fluorescence can contribute to decreasing the detection limit, but components of the fecal environment can also interfere with fluorescent signals. Several studies have demonstrated the advantages of using europium fluorescent microspheres as reporters in lateral flow immunochromatography tests, including the rapidity of analysis, practicality for on-site use, high accuracy, good reproducibility, and stability [8-10].

FITs can be used to detect the presence of fecal hemoglobin (Hb), and recent studies have suggested that combining Hb detection with the detection of transferrin (Tf) might increase FIT sensitivity for CRC screening. Tf is an iron-binding blood plasma protein that has been reported to be more stable in feces than Hb is [11].

Although colonoscopy can detect early intestinal lesions, such as CRC and adenomas, the acceptance of the examination in clinical practice is not high; therefore, it is not useful as an early cancer screening test. The currently recommended methods for early screening of CRC are FOBTs, FIT, and FIT-DNA combination detection, among others. Because FIT is unaffected by peroxidase in food and has higher sensitivity in detecting CRC and progressive adenomas, studies show that the sensitivity of FIT for screening for CRC is 73.8% and 23.8%, and its specificity is 94.9%, achieving minimal cost and maximum benefit in early screening for CRC [12]. However, few studies have jointly examined Hb and Tf in feces. The purpose of this study was to develop an immunochromatographic strip using fluorescent nanoparticle labeling for the detection of fecal Hb combined with fecal Tf to evaluate the effectiveness of combined detection in the early screening of CRC. Levels of analytes were assessed using a portable fluorescence detector containing an ultraviolet (UV) light source, which is amenable to use in both clinical and laboratory settings.

Materials and methods

Fluorescent microspheres (Europium Chelate Microspheres, excitation: 365 nm; emission: 610 nm) with carboxyl groups (FCEU001) were purchased from Bangs Laboratories Inc (Fishers, IN). Goat anti-mouse IgG polyclonal antibody, paired mouse anti-human Tf monoclonal antibodies (mAbs), and paired mouse anti-human Hb mAbs were purchased from Zhuhai Bomei Biotechnology Co., Ltd. (Zhuhai, China). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, EDC (22980), MES Monohydrate (145224-94-8), and N-Hydroxysulfosuccinimide sodium salt, sulfo-NHS (A39269) were purchased from Thermo Fisher Scientific (Waltham, MA). Tween-20, PEG20000, and bovine serum albumin (BSA) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Ningbo Road, Shanghai). Sample pads, polyvinyl chloride (PVC) sheets, absorbent paper, and nitrocellulose film were purchased from Shanghai Jinbiao Biotechnology Co., Ltd. (Shanghai, China). All chemical reagents used were of analytical grade. The preparation process of colloidal gold test paper is shown in **Figure 1**.

Instruments

A three-dimensional film scoring colloidal gold sprayer (HM3030) was purchased from Shanghai Jinbiao Biotechnology Co., Ltd. A fluorescence spectrometer (emission: 365 nm, detection: 610 nm, FIC-S1) was purchased from Suzhou Hemai Precision Instrument Co., Ltd. (Suzhou, China). An ultrasonic dispenser (FS-100T) was obtained from Shanghai Bioanalysis Ultrasonic Instrument Co., Ltd. (Shanghai, China). The thermostatic drying oven and benchtop centrifuges are standard laboratory instruments.

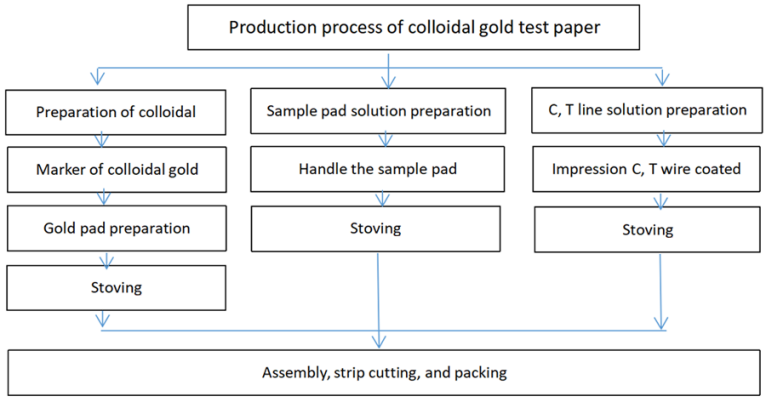

Figure 1. The preparation process of colloidal gold test paper.

Preparation of fluorescent microsphere-Ab conjugate

Fluorescent microspheres were prepared as follows. A 50 μl volume of fluorescent microsphere suspension was centrifuged at 12,000 rpm for 10 minutes, the supernatant was removed, the microspheres were resuspended in 200 μl MES buffer (50 mM, pH 6.0), and 20 μl each of the EDC coupling agent (10 mM) and sulfo-NHS (10 mM) were added, followed by incubation at room temperature for 0.5 hours. Following centrifugation at 12,000 rpm for 10 minutes, the supernatant was removed, the precipitate was resuspended in 200 μl borate buffer (20 mM, pH 8.0), and 50 μl of antibody was added (1 mg/mL). After incubation for 1 hour at room temperature, 40 μl BSA (10%) was added for blocking, incubated for 2 hours, and washed three times with MES buffer to remove excess free antibody, followed by centrifugation. The supernatant was removed, and the precipitate was resuspended in 200 μl storage buffer (20 mM Tris, 5% sucrose, 2% BSA, 1% PVP-K30, 0.01% NaN_3, pH 8.0) to obtain the conjugate complex.

Sample pad treatment

We soaked the sample pad with the sample pad treatment solution (20 mM PB, 0.2% casein, 0.5% PVP-K30, 1% sucrose, 0.1% TWEEN-20, 0.05% Triton X-100, 0.01% NaN_3, pH 8.0) for 10 minutes, allowed it to air dry it at 37°C overnight, and kept it sealed. We diluted the coupling complex with storage buffer (20 mM Tris, 5% sucrose, 2% BSA, 1% PVP-K30, 0.01% NaN_3, pH 8.0) using an appropriate ratio and sprayed the mixture onto the treated sample pad at the speed of 8 μl/cm using a sprayer. The sample pad was allowed to air dry at 37°C for 1 hour and was sealed for storage.

Nitrocellulose film coating

We diluted each antibody to an appropriate concentration with antibody coating buffer (20 mM PB, 1% sucrose, 0.01% NaN_3, pH 8.0) and sprayed the diluted antibody onto the nitrocellulose film at a speed of 1 μl/cm to generate two test lines and one control line, in that order. The film was air-dried at 37°C for 2 hours and sealed for storage.

Antibody spraying and immobilization on nitrocellulose film

This immunochromatographic assay is based on the double antibody sandwich method. Paired mouse anti-human Tf mAbs and paired mouse anti-human Hb mAbs were selected for the sample test, and a goat anti-mouse IgG polyclonal antibody was selected as the control. We labeled one of the paired antibodies with fluorescent microspheres, and the other paired antibody was immobilized on the nitrocellulose film as a test line. The goat anti-mouse IgG polyclonal antibody was also sprayed onto the nitrocellulose film as the control line.

Strip assembly

We cut the PVC sheet into 4 mm × 60 mm pieces to serve as strip support and cut the sample pad into 4 mm × 10 mm pieces, the absorbent paper into 4 mm × 20 mm pieces, and the antibody-coated nitrocellulose membrane into 4 mm × 40 mm pieces. We applied a sample pad, an antibody-coated nitrocellulose membrane, and an absorbent paper onto the strip support to form a test strip. We loaded the test strips into test strip cases to form a test card and sealed them for storage.

Data collection

For each stool sample, 20 mg was added to 2 mL sample dilution buffer, premixed at room

Table 1. Participants' information

Normal controls (n=122)		
Sex (n)	Male	68
	Female	54
Age (Years)	Range	32-76
	Median	54
Colorectal cancer (n=51)		
Sex (Cases)	Male	31
	Female	20
Age (Years)	Range	28-84
	Median	62
Stage (n)	0	10
	I	14
	II	13
	III	8
	IV	6
Location (n)	Colon	20
	Rectum	31

temperature, and then added to the test card. Antigens in the sample that passed through the nitrocellulose membrane were captured by the mAbs on either the test (T) or control (C) line, resulting in a strong fluorescence signal that was visible under a 365 nm light source within 15 minutes. A fluorescent detector was used to measure the strength of the fluorescence signal and calculate the analyte quantity using built-in analysis software.

Sensitivity and linear range

Serially diluted standard samples were spiked into blank feces and used to test the sensitivity and linear range of the test. The fluorescence intensity ratio between the T and C lines was used as the test result.

Stool sample collection

Stool sample collection was performed at the Anyang Tumor Hospital, Anyang, China. Verbal informed consent was obtained from each participant. This study was approved by the Medical Ethics Committee of Anyang Tumor Hospital. All patients were first diagnosed with colorectal disease, which was confirmed by pathology or colonoscopy. The tumor staging process followed the 7th edition of the American Joint Committee on Cancer staging manual, revised in 2010. The control group comprised healthy individuals who were tested in the physical examination center of Anyang Cancer Hospital. The identities of all fecal samples were blinded to the investigators of this study. Each participant was asked to collect two stool samples from different bowel movements without any specific recommendations for dietary or medicinal restrictions. Fecal samples were collected before bowel preparation for either colonoscopy or surgical operation. Participants were asked to store stool-filled containers under refrigerated conditions until they were submitted to the laboratory. Upon receipt, the containers were immediately frozen at -20°C.

We received 244 qualified stool samples from 122 normal subjects (with no obvious clinical abnormalities) and 102 qualified stool samples from 51 patients with histopathologically diagnosed CRC. All participants were Asian, specifically Han Chinese (**Table 1**).

Fecal sample test

The tests were conducted at room temperature. For each fecal sample, 20 mg was added to 2 mL fecal dilution buffer (50 mM PB, 0.5% TWEEN-20, 0.1% PVP-K30, 0.01% NaN_3, pH 7.2), and 75 μl of the resulting mixture was added to the sample loading hole of the test strip case. After 15 minutes, the result was read by a fluorescence spectrometer.

Statistical analysis

The Chi-square test was conducted to determine the significance of differences between groups. $P < 0.05$ in a two-tailed test was considered significant. The sensitivity, specificity, and usefulness (via the area under the curve [AUC]) of the CRC detection were calculated using the receiver operator characteristic (ROC) curve. SPSS version 23 for Windows was used to perform all statistical analyses.

Results

Optimization of the immunochromatographic strip test

The optimization process included (1) antibody selection and purification, (2) selection of the model film, (3) labeling the ratio of antibody to gold, (4) optimization of the T line's raw materials to improve the binding ratio, and (5) optimization of the sample pad. The immunochro-

Figure 2. Representative example of the developed test strip. A strip installed in the test card showed a result 15 min after the addition to the card of 75 μL sample containing hemoglobin and transferrin. The fluorescent bands were observed under 365 nm ultraviolet light.

matographic strips were structured using an immune sandwich method. One of the paired mouse anti-human Hb mAbs was immobilized onto the nitrocellulose film and served as test line 1, and one of the paired mouse anti-human Tf mAbs was immobilized onto the nitrocellulose film and served as test line 2. Goat anti-mouse IgG polyclonal antibody was immobilized onto the nitrocellulose film as the control line. The other paired mouse anti-human Hb and mouse anti-human Tf mAbs were conjugated with fluorescent microspheres to detect Hb and Tf, respectively.

All combinations of the paired antibodies were tested to optimize the intensity and duration of the fluorescent signals emitted by each test line and the control line. We assembled the strips, installed test cards, and tested the strips using standard Hb and Tf samples. The fluorescent signal was measured under 365 nm UV light (**Figure 2**), indicating that the test strips were assembled correctly.

Sensitivity and calibration curves

For quantitative tests, a standard curve was established using 12 serially diluted concentrations of Hb (1.59-3250 ng/mL) and Tf (0.61-1340 ng/mL) that were spiked into pre-measured stool quantities, and each dilution was tested using 20 strips. The fluorescence intensity was measured using a portable fluorescent strip reader. The T/C value was calculated, and the average value of each dilution was taken. The sensitivity for fecal Hb was 3.17 ng/mL, and the sensitivity of fecal Tf was 1.21 ng/mL. The calibration curves were linear in the range of 25-1625 ng/mL for Hb (**Figure 3A**) and in the range of 9.6-155 ng/mL for Tf (**Figure 3B**).

We tested 244 fecal samples collected from 122 normal subjects and 102 fecal samples collected from 51 patients with CRC. The concentrations of Hb and Tf were calculated for each sample. For normal subjects, the median fecal Hb concentration was 32 ng/mL (range: 0-976 ng/mL), and the median fecal Tf concentration was 2 ng/mL (range: 0-95 ng/mL). For patients with CRC, the median fecal Hb concentration was 856 ng/mL (range: 0-2888 ng/mL), and the median fecal Tf concentration was 18 ng/mL (range: 0-154 ng/mL). **Figure 4** shows the distribution of fecal Hb and Tf concentrations.

Diagnostic performance

Positive rates in normal subjects and patients with CRC: We tested each of the two fecal samples obtained from each individual and used the highest values from the two tests. We set cutoffs of 100 ng/mL (10 μg/g feces) for Hb and 25 ng/mL (2.5 μg/g feces) for Tf. The results are shown in **Table 2**. The positive rate for Hb+Tf was higher than that for Hb and Tf among patients with CRC, and the differences were significant (94.12% vs. 88.24% and 64.71%, respectively, n=51, X=17.000, P=0.000). In contrast, the positive rates for the healthy subjects remained low for all (10.66% vs. 6.56% and 5.74%, respectively, n=122), and the differences were not significant (X=2.398, P=0.302).

Positive rates between colon and rectum cancer cases: The positive rates for Hb were similar for colon (n=20, 83.33% positive) and rectal cancer cases (n=31, 92.59% positive). In contrast, much lower positive rates were observed for Tf among individuals with colon and rectal cancer (54.17% vs. 74.07%), although these differences were not significant. The positive rate for Hb+Tf was higher than that for Hb and Tf among patients with rectal cancer, and the differences were statistically significant (96.30% vs. 92.59% and 74.07%, respectively, n=27, X=7.073, P=0.029) (**Table 3**).

Positive rates in different CRC stages: Compared to patients diagnosed with early-stage CRC (Stages 0-I), the positive rates were higher among patients diagnosed with late-stage CRC (Stages II-IV) for both Hb (79.17% vs. 96.30%) and Tf (58.33% vs. 70.37%). The posi-

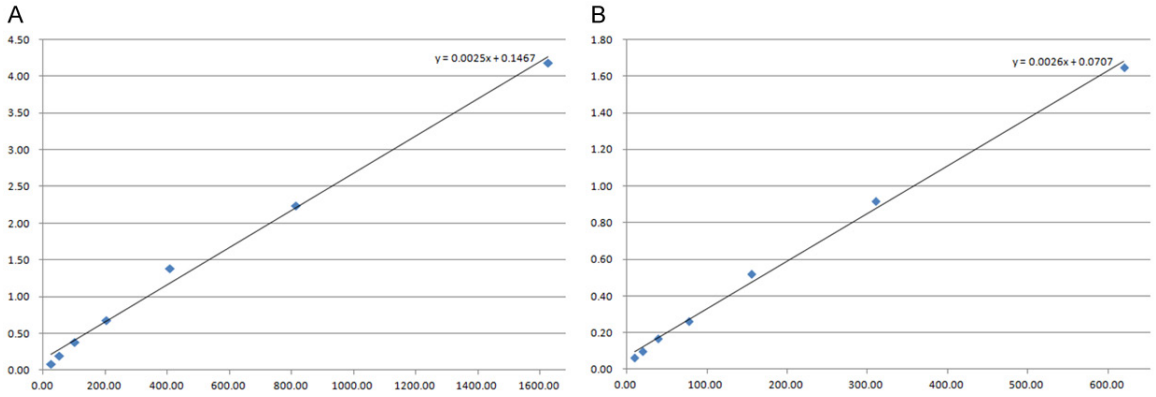

Figure 3. Linearity evaluation of the developed test using hemoglobin and transferrin standards. A total of 12 dilutions were used for each standard (0-3250 ng/mL for hemoglobin and 0-1340 ng/mL for transferrin). The calibration curves are linear in the ranges of 25-1625 ng/mL for hemoglobin (A) and 9.6-155 ng/mL for transferrin (B).

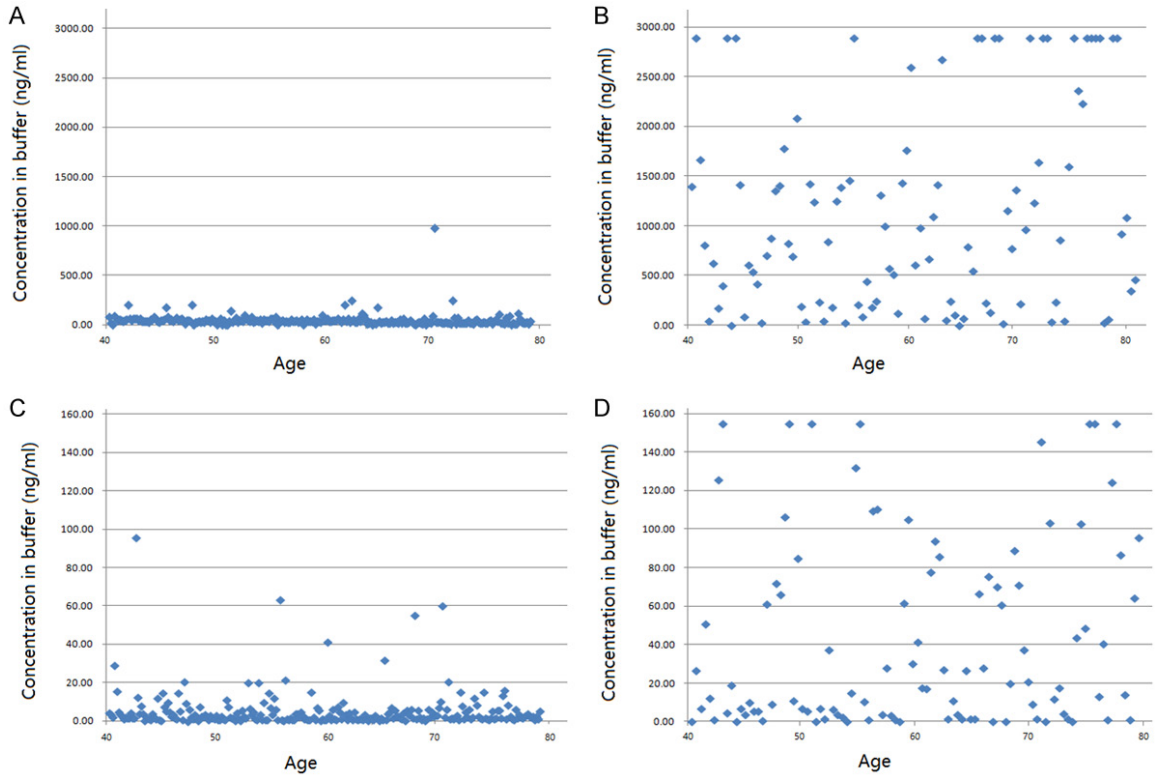

Figure 4. Clinical fecal sample test results. All fecal samples were tested for hemoglobin and transferrin. The concentration measured for each sample was plotted according to age. A. Fecal hemoglobin concentrations in normal subjects. B. Fecal hemoglobin concentrations in patients with colorectal cancer. C. Fecal transferrin concentrations in normal subjects. D. Fecal transferrin concentrations in patients with colorectal cancer.

tive rate for Hb+Tf was higher than that for Hb or Tf among patients with early-stage and late-stage CRC, but the differences among patients with early-stage CRC were not statistically significant (X=4.333, P=0.115), whereas the differences among patients with late-stage CRC were significant (100.00% vs. 96.30% and 70.37%, respectively, n=27, X=14.250, P=0.001) (**Table 4**).

Positive rates in one fecal sample test: We randomly selected one of the two samples for each participant to calculate the Hb and Tf positive rates for one fecal sample test. The

Table 2. Hemoglobin and transferrin positive rates among the 122 normal subjects and 51 colorectal cancer patients

	Cases, n	Hb, n (%)	Tf, n (%)	Hb+Tf, n (%)
Normal	122	8 (6.56)	7 (5.74)	13 (10.66)
CRC	51	45 (88.24)	33 (64.71)	48 (94.12)*

Patients were subjected to two fecal sample tests, and the larger value was collected. Cutoffs were 100 ng/mL (10 μg/g feces) for hemoglobin (Hb), 25 ng/mL (2.5 μg/g feces) for transferrin (Tf). *P < 0.05. CRC: colorectal cancer.

Table 3. Hemoglobin and transferrin positive rates for colorectal cancer

	Cases, n	Hb, n (%)	Tf, n (%)	Hb+Tf, n (%)
Colon	24	20 (83.33)	13 (54.17)	22 (91.67)
Rectum	27	25 (92.59)	20 (74.07)	26 (96.30)*

*P < 0.05. Hb, hemoglobin; Tf, transferrin.

Table 4. Hemoglobin and transferrin positive rates according to early and late stages of colorectal cancer

	Cases, n	Hb, n (%)	Tf, n (%)	Hb+Tf, n (%)
Stage 0-I	24	19 (79.17)	14 (58.33)	21 (87.50)
Stage II-IV	27	26 (96.30)	19 (70.37)	27 (100.00)*

*P < 0.05. Hb, hemoglobin; Tf, transferrin.

Table 5. Hemoglobin and transferrin positive rates in one fecal sample test

	Cases, n	Hb, n (%)	Tf, n (%)	Hb+Tf, n (%)
Normal	122	6 (4.92)	3 (2.46)	8 (6.56)
CRC	51	40 (78.43)	20 (39.22)	42 (82.35)*

*P < 0.05. Hb, hemoglobin; Tf, transferrin; CRC, colorectal cancer.

Table 6. Hemoglobin and transferrin positive rates using adjusted cutoffs

	Cases, n	Hb, n (%)	Tf, n (%)	Hb+Tf, n (%)
Normal	122	4 (3.28)	4 (3.28)	7 (5.74)
CRC	51	35 (68.63)	24 (47.06)	39 (76.47)*

*P < 0.05. Hb, hemoglobin; Tf, transferrin; CRC, colorectal cancer.

positive rate for Hb+Tf was higher than that for Hb and Tf among patients with CRC, and the differences were statistically significant (82.35% vs. 78.43% and 39.22%, respectively, n=51, X=26.118, P=0.000). The differences among healthy subjects were not significant (6.56% vs. 4.92% and 2.46%, respectively, n=122, X=2.344, P=0.310) (**Table 5**).

Positive rates with cutoff adjustments: Adjusting the cutoff values used for Hb (200 ng/mL) and Tf (50 ng/mL) lowered the positive rates for both normal subjects and for patients with CRC. The results are shown in **Table 6**. The positive rate for Hb+Tf was higher than that for Hb or Tf among patients with CRC, and the differences were statistically significant (76.47% vs. 68.63% and 47.06%, respectively, n=51, X=10.276, P=0.006), whereas the positive rates for healthy subjects remained low for all (5.74% vs. 3.28% and 3.28%, respectively, n=122, X=3.390, P=0.184), and the differences were not significant.

ROC curves

The sensitivity and specificity of the FIT combined detection technique were assessed using ROC curves. For the combined FIT detection, the sensitivity and specificity were 87.5% and 89.2%, respectively, and the AUC was 0.92 (**Figure 5A**). The sensitivity, specificity, and AUC of the Tf assay were 63.8%, 68.4%, and 0.759, respectively (**Figure 5B**); for Hb testing, they were 69.7%, 70.2%, and 0.774, respectively (**Figure 5C**). The AUC for Hb+Tf was higher than that for Tf and Hb (P < 0.001), indicating that combined FIT testing had high efficacy for CRC testing.

Discussion

Colorectal cancer (CRC) is a serious problem and can be fatal. Early screening has been shown to effectively reduce disease-related mortality by facilitating treatment before lesions become cancerous and refractory. CRC screening methods are mainly divided into 2 categories: natural population screening and opportunistic screening. The former usually includes large-scale screening for all people of a certain age and intends to detect early lesions and improve the treatment effect. Appropriate intervention by screening precancerous lesions in the natural population aims to reduce tumor incidence and improve the population's health. The disadvantages of natural population screening are its considerable require-

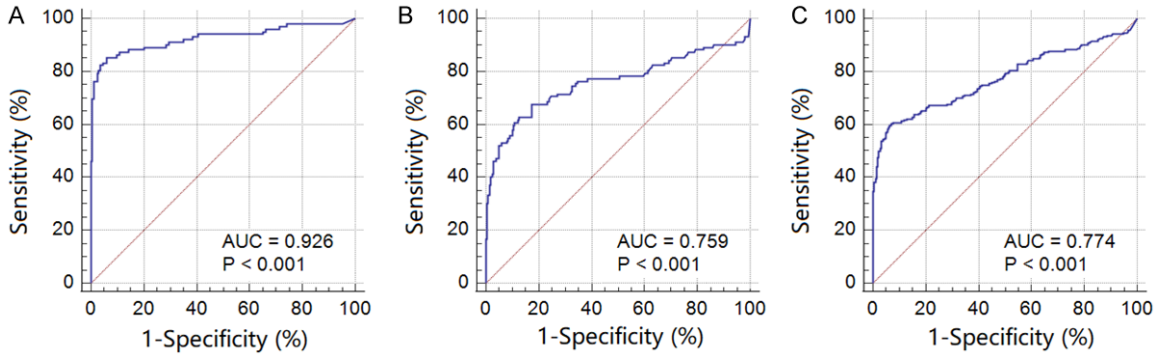

Figure 5. Receiver operator characteristic curves for the detection of patients with colorectal cancer. A. Hemoglobin combined with transferrin. B. Hemoglobin only. C. Transferrin only. AUC, area under the curve.

ments for labor and material resources and its low target population compliance. Opportunistic screening (also called individual screening in clinical practice) is a clinical-based method in which patients are screened for target diseases during unrelated medical treatment or routine physical examination. This is a relatively convenient and practical screening method that requires no additional personnel or financial support and has satisfactory subject compliance. China's abundant population and medical resources pose a challenge to disease prevention, so opportunistic screening is the most suitable screening model for China's national conditions and medical system. Currently, several screening products detect fecal Hb as a marker of early colorectal cancer, including the FIT. However, the sensitivity and specificity of these products require further improvement, and recent studies indicate that the simultaneous detection of both fecal Hb and Tf can increase the sensitivity of colorectal cancer screening.

In this study, we developed novel immunochromatographic test strips using europium chelate-coated fluorescent nanospheres and tested their ability to quantitatively measure fecal Hb and Tf. The sensitivity and specificity of the combined (Hb+Tf) FIT detection technique were 87.5% and 89.2%, respectively, better than those of Hb and Tf separately. The method showed a good signal-to-noise ratio, low detection limits, and a wide linear range. The results were consistent with studies reported by other researchers using europium fluorescent nanospheres [9, 10]. The sensitivity for fecal Hb detection using this method was 1.164 ng/mL, and the sensitivity for fecal Tf detection was 1.164 ng/mL.

We evaluated the diagnostic performance of the developed strips using fecal samples from patients with CRC and normal subjects. Fecal Hb had a superior diagnostic performance among patients with CRC compared to fecal Tf (88.24% vs. 64.71%). By contrast, Hb combined with Tf increased the positive rate among patients with CRC (94.12%), which aligns with the results reported by previous studies indicating the enhanced power of assays combining Hb with Tf for the early screening of CRC. These findings also demonstrate that the strips we designed and manufactured performed excellently. However, a larger-scale experiment remains necessary to further validate these results.

One critical consideration when performing CRC screening in clinical practice is balancing the consequences of missing CRC or advanced neoplasia with the medical costs associated with repeated testing among patients at different risks of developing CRC. Lowering the cutoff value used to characterize a positive FIT can increase diagnostic sensitivity but also increase the false-positive rate [13, 14].

The present study offers a quantitative FIT that is sufficiently sensitive to quantify exact fecal Hb and Tf levels and could be used to optimize cutoff values based on the test results from different target populations. Cutoff value optimization is expected to significantly balance detection sensitivity and the false-positive rate, reducing medical costs. However, this task is challenging and will require additional studies using large populations of healthy volunteers and patients at different CRC stages.

Some studies have evaluated the performance of one-sample FIT compared against protocols using two or more samples and found significant differences in the detection rates. Data from these studies indicate that performing FITs using two or more samples increases the positive rate across all CRC screening practices [15-17].

The present study suggests that the use of two samples in FITs that include both Hb and Tf resulted in a significant increase in the positive rate among patients with CRC; however, the positive rate also increased among normal subjects when using this protocol. Previous studies showed that the combined use of fecal Hb with fecal Tf increased sensitivity but reduced specificity [18-20]. The results in the present study were consistent with those of prior studies. Our results showed that the combination of both biomarkers led to an increase in the sensitivity of CRC detection from 88.24% to 94.12% but reduced specificity from 90.44% to 89.34%, although these differences were not significant. This issue requires additional study to determine how best to apply these biomarkers in CRC screening.

The present study has several limitations. First, the numbers of both healthy controls and patients with CRC participating in this study were relatively low, possibly impairing the accuracy of the results. Further validation of the effectiveness of the test strips for early CRC screening using larger numbers of both healthy subjects and patients with CRC would likely improve the accuracy and reliability of the test strip results. Second, all of our participants were of Han Chinese descent, and testing outcomes may differ across populations with distinct genetic backgrounds. The test strips must be validated in different ethnic groups to determine the generalizability of these findings. Third, the healthy subjects and patients with CRC were characterized with different median ages (54 versus 62, as shown in **Table 1**). Whether age affects the evaluation results of the test strips remains unknown. Furthermore, the 51 CRC patients were categorized into different disease stages, and age differences were observed across disease stages. Similar differences in the sex distribution may also exist, which requires further investigation.

Future improvements could be made to the test system, including the identification and combination of a third CRC protein marker with Hb and Tf, which is expected to further improve the sensitivity and specificity of the test strip for early diagnosis of CRC. In addition, the use of higher-quality antibodies in the test strip could increase sensitivity and specificity for all marker proteins. The development of new technologies is likely to reveal other potential methods to improve the performance of the proposed system, and our test strip system could be further adapted for the detection of other marker proteins in feces and other specimen types, including urine, sweat, and saliva, to screen for several diseases. FIT combined detection has the advantages of being non-invasive and achieving good patient compliance as well as high sensitivity and specificity and is expected to become an important auxiliary means for the early diagnosis and screening of CRC. The small sample size of this study and the narrow range of the considered regions somewhat affect the credibility and representativeness of the study data, which we will address in follow-up studies to obtain more accurate and generalizable results.

Conclusions

A fluorescent microsphere-based immunochromatographic strip was successfully developed for the rapid and highly sensitive detection of fecal Tf and Hb and can be used for early CRC screening.

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Disclosure of conflict of interest

None.

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