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Original Article

MiR-92a expression profiling of exfoliated colonocytes isolated from feces for colorectal cancer screening

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Abstract: Objective: This study seeks to assess the efficacy of exfoliated colonocytes isolated from feces (ECIF) miR-92a as a clinical colorectal cancer diagnostic marker in a larger cohort. Methods: Clinicopathologic data from colorectal cancer patients and health controls that underwent colonoscopy, as well as patients of other cancers diagnosed, were included. A total of 963 Chinese participants were enrolled, with 292 (27.4%) having colorectal cancer, 140 (14.5%) having other types of cancer, e.g., pancreatic, liver, oral, bile duct, esophagus, and stomach cancer, 171 (17.8%) having infection in the intestine, rectal, stomach, appendix, and gastrointestinal ulcer, and 360 (37.4%) of healthy controls. ECIF samples were gathered and miR-92a levels were detected using TaqMan probe-based miR-92a real-time quantitative PCR (RT-qPCR) kit developed by Shenzhen GeneBioHealth Co., Ltd. Results: Through a series of experiments, we demonstrated that the Ep-LMB/Vi-LMB magnetic separation system is feasible, highly specific, and highly sensitive at a cutoff value of 1053 copies per 6 ng of ECIF RNA. ECIF miR-92a levels were significantly higher in colorectal cancer patients than in controls. Colorectal cancer detection sensitivity and specificity were 87.3% and 86.9% respectively. Furthermore, the performance of this miR-92a detection kit demonstrated that it is an effective tool for colorectal cancer, with a high sensitivity of 84.1%, even in early cancer stages (0, I, and II). Furthermore, tumor removal resulted in lower stool miR-92a levels (3.21±0.58 vs. 2.14±1.14, $P < 0.0001$, $n = 65$). Conclusion: Finally, the miR-92a RT-qPCR kit detects ECIF-increased miR-92a and could be used for colorectal cancer screening.

Keywords: Biomarker, colorectal cancer, miR-92a, screening

Introduction

Colorectal cancer (CRC), with an increasing global incidence, was ranked fourth in terms of malignant mortality rate [1]. Colorectal cancer frequently occurs with multiple genetic and epigenetic alterations in the early stages, allowing it to be screened at an early stage [2]. However, the lack of effective noninvasive screening techniques hampered the early detection of CRC, limiting early detection and possible improvement of the overall 5-year survival rate of CRC patients. With an early CRC diagnosis, e.g., stages I or IIA CRC, the 5-year survival rate could be 80%-92%, compared to only about 10% in the late stages [3-5].

To date, colonoscopy is the most commonly used screening tool for early lesions, but it is limited by poor patient compliance due to the risk of intestinal damage, and the results are limited by the rigorous operational requirements for complicated preparations and analysis [6]. The fecal occult blood test appears as a

noninvasive test to address the issue of poor patient compliance in the screening, but it is less effective in detecting early-stage CRC due to the lack of hemoglobin in stool in early CRC lesions [7]. Neoplasm-derived fecal proteins or mRNAs are also used for CRC diagnosis, but the inherent instability of the protein and mRNA can easily cause false negative results [8, 9].

Molecular tests have recently emerged as potential screening options. CRC-specific DNA tests, such as the fecal DNA test represented by Cologuard, or the cell-free blood DNA test represented by EpiProcolon [10, 11], are exhibiting a remarkable attitude in CRC screening due to their relatively stable performance. These DNA tests primarily focus on detecting DNA mutations such as KRAS and APC mutation [12, 13], gene promoter hypermethylation, such as BMP3, NDRG4, SDC2, or genomic sequence analysis [14-16]. Among molecular tests, microRNA has drawn significant attention as a potential noninvasive biomarker for the early detection of CRC [17]. MicroRNA, a type of small noncoding RNA, has been shown to act as either a tumor suppressor or an oncogene in the development and progression of CRC [18]. Though the function of microRNA in cancer is still unknown, studies have suggested that microRNA regulates roughly 30% of protein-encoding genes [19]. Recent studies have examined aberrant microRNA expression alterations in CRC, claiming the potential diagnostic and prognostic function of microRNA, which demonstrates very stable, reproducible, and consistent biomarker performance in serum, plasma, and feces [20-22].

Among emerging microRNAs studied for their association with CRC, miRNA-92a has emerged as a screening tool with relatively high sensitivity and specificity [23]. MiRNA-92a belongs to the miR-17-92 cluster (oncomir-1) and exhibits oncogenic function by targeting the BCL-2 family to regulate apoptosis [24, 25]. According to Ng et al., plasma levels of miR-92a are capable of distinguishing CRC from gastric cancer, inflammatory bowel disease, and normal subjects, with a sensitivity of 89% and specificity of 70% in differentiating CRC from normal controls with a receiver operating characteristic curve area of 88.5% [26]. Huang et al. also discovered that plasma miR-92a levels have a significant diagnostic value in CRC, with 84% sensitivity and 71.4% specificity. Because these changes can also be found in the stool, Huang et al. and Wu et al. investigated and confirmed the stability and reproducibility of miR-92a levels in the stool and discovered that the sensitivity was 71.6% and the specificity was 73.3% in distinguishing CRC from normal controls [27, 28]. Many studies have found that the progression of colorectal cancer is associated with microRNA abnormalities. MicroRNA in stool is highly stable, less expensive and non-invasive compared to colonoscopy, so we chose stool from CRC patients to study. We developed a CRC screening tool based on miR-92a levels in ECIF and tested its performance in detecting CRC.

Materials and methods

Analysis of the preparation and detection process of EpCAM and vimentin lipid magnetic beads

Figure 1 depicts the preparation and detection of double antibody-modified nanoparticle PLGA magnetic spheres prepared by EpCAM+ antibody or Vimentin+ antibody. Fe_3O_4 magnetic nanoparticles are encapsulated in PLGA polymer materials to create PLGA magnetic beads. The use of aminoacylated PLGA can provide NH_2 on the surface of PLGA beads, which is useful for modifying EpCAM+ or Vimentin+ antibodies. DSPE-PEG-NH_2 can not only act as an emulsion dispersion and surfactant, but it also provides more NH_2 groups on the surface of PLGA magnetic beads, making antibody coupling easier. The biodegradable PLGA matrix material can improve magnetic beads' biocompatibility and reduce cytotoxicity, allowing for the subsequent analysis and identification of ECIF. After mixing the collected fecal samples with PBS solution, EpCAM lipid magnetic beads (Ep-LMB) and vimentin lipid magnetic beads (Vi-LMB) were chosen for separation, and total nucleic acids were extracted within the ECIF. The expression level of mirNA-92a-related colorectal cancer indicators was detected using PCR reaction, and the association with early diagnosis and screening of colorectal cancer was established by evaluating the expression level of this indicator (**Figure 1**).

Human CRC cell lines SW480 and LoVo were purchased from ATCC (Manassas, VA, USA). SW480 cells were cultured in DMEM medium.

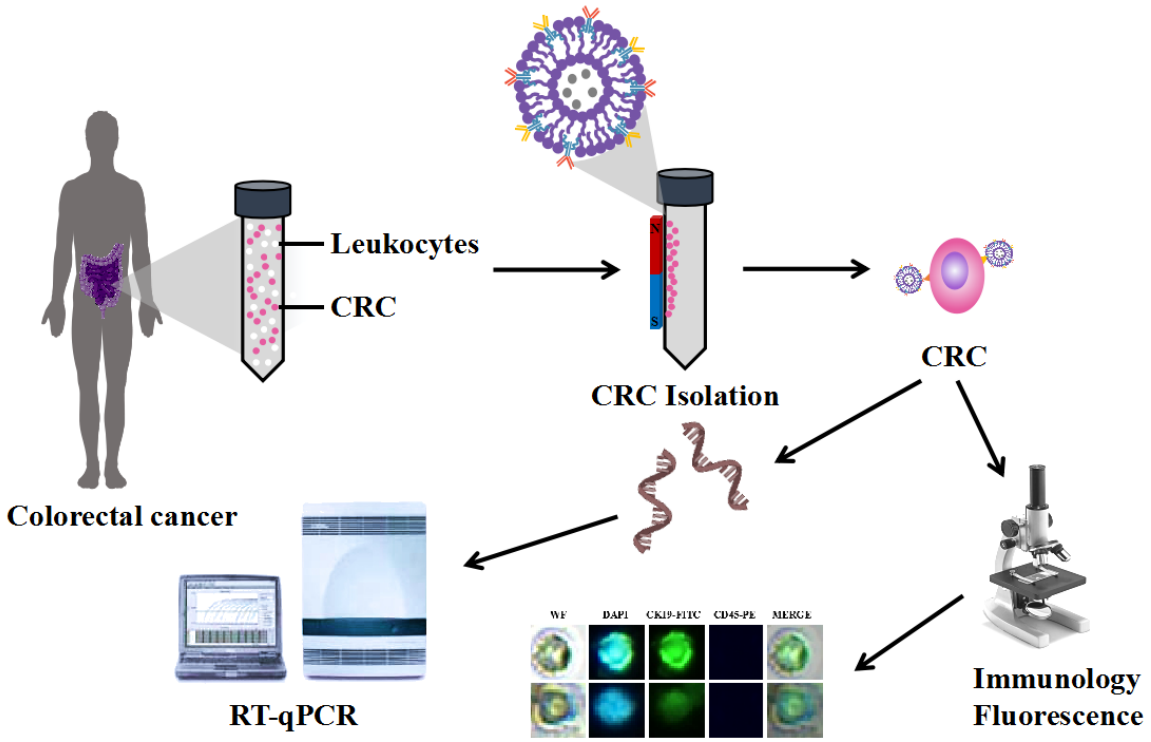

Figure 1. Preparation and experimental flow chart of Ep-LMB and Vi-LMB.

LoVo cells were maintained in F12-K. Normal colon epithelial cell line NCM460 was obtained from Rongbai (Shanghai, China) and maintained in DMEM-H medium containing 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. All cells were kept in a medium supplemented with 10% FBS (Invitrogen) at 37°C in a 5% CO_2 atmosphere.

Patients

The study adhered to the principles outlined in the Declaration of Helsinki. Between July 2015 and August 2017, 963 people were enrolled at Sun Yat-sen University Hospital, Shenzhen People's Hospital, and Tianjin People's Hospital. All participants signed written informed consent with the full understanding of this study before enrolling and the entire study was approved by the Institutional Review Board and Ethics Committee at each hospital. The inclusion criteria were: 1) Age between 18 and 80, regardless of gender; 2) Having a colonoscopy test and signing written informed consent with a full understanding of this study. Exclusive criteria were: 1) Watery or contaminated stool; 2) Participants who used drugs recently; 3) Participants who withdraw the consent; 4) Specimens without corresponding documents.

Characterization of EpCAM and vimentin lipid magnetic beads

Ten-μl Ep-LMB and Vi-LMB samples were diluted in 1-mL distilled water, and the particle size and potential of the magnetic spheres were measured using a BI-90Plus laser particle size meter/Zeta potentiometer. Another 10-μl Ep-LMB and Vi-LMB samples were diluted in 1 mL distilled water, and 50 μl were coated on mica sheets. After natural drying, the morphology of lipid magnetic spheres was examined by atomic force microscopy (AFM). 10-μl samples of Ep-LMB and Vi-LMB were diluted in 1-mL distilled water, and the immune lipid magnetic spheres were scanned by UV absorption spectroscopy using a UV spectrophotometer.

Simulation studies

The optimal conditions for capturing LOVO CRC cells from feces were determined through a series of simulation experiments. LOVO and SW480 single-cell suspensions were prepared separately, and 100 counted cells were added to a 7.5-ml PBS solution. The suspension was divided into the Ep-LMB group, Vi-LMB group, Ep-LMB/Vi-LMB group, and Vi-LMB/Ep-LMB groups, each of which was captured with the

prepared Ep-LMB and Vi-LMB magnetic beads. In Ep-LMB and Vi-LMB groups, 6-, 9-, 12-, 15-, and 18-μL magnetic beads were added to 7.5 ml mixtures, respectively. Ep-LMB/Vi-LMB group: 2-, 3-, 4-, 5-, 6-μL Ep-LMB was added to capture, followed by 2-, 3-, 4-, 5-, 6-μL Vi-LMB. Vi-LMB/Ep-LMB group: 2-, 3-, 4-, 5-, 6-μL Vi-LMB was added to capture, followed by 2-, 3-, 4-, 5-, 6-μL Ep-LMB. Each group was tested three times. In addition, 1 g feces from healthy volunteers was collected and mixed with 7.5 ml of the cell suspension to simulate the tumor cell capture experiment in feces. The Ep-LMB/Vi-LMB capture protocol was used to adjust LOVO and SW480 cells to different cell gradients of 10, 50, 100, 500, 1000, 5000, and 10000 cells, and the protocol's sensitivity was studied in a PBS system. The protocol's specificity was investigated in a simulated fecal system. Finally, magnetic beads with various antibody concentrations (0, 20, 40, 60, 80, and 100 μg) were used to capture LOVO cells in gradients, and the capture efficiency of the magnetic beads with various antibody concentrations was investigated in PBS and simulated fecal systems.

Exploration of cell capture time

In culture dishes, 1×10^4 LOVO cells and SW480 cells were seeded, 1 mL of cell culture medium was added, and the cells were cultured for 24 h at 37°C in a 5% CO_2 constant temperature incubator. After changing the culture medium, 20-μl Ep-LMB-FITC or Vi-LMB-FITC, 100-μl DAPI, and 100-μl Dil were added, and the dishes were photographed using a fluorescence microscope at 0, 5, 10, 15, and 20 min, respectively.

Fecal samples and isolation of exfoliated cells

One gram of feces was added to 10-ml PBS buffer and stirred to homogenate. The procedure was conducted under optimal conditions determined by the simulation study. Briefly, the mixture was incubated for 30 min at room temperature with 60 μl of EP-LMB and Vi-LMB after using a Ninon filter (pore size: 500 nm), then placed on a magnetic stand and continued to incubate for 15 min at room temperature, and the supernatant was discarded to obtain ECIF.

RNA extraction and real-time polymerase chain reaction

Each hospital collected ECIF samples and tested for miR-92a without knowing the clinical information. About 0.3-0.5 g ECIF was used for the miR-92a test. ECIF total RNA was extracted using the RNAColon® Nucleic Acid Extraction kit (GeneBioHealth, China) according to the manufacturer's instructions. Using this extraction kit, more pure RNA with higher OD260/280 can be obtained. The RNA concentration was then determined using the NanoDrop 1000 (ThermoFisher Scientific, US) and diluted to 6 ng/μl. Five microliters of diluted RNA were mixed with 5 μl of the reverse transcription components of the RNAColon® miR-92a Assay kit (GeneBioHealth, China), which was approved by National Medical Products Administration (NMPA) for clinical practice since 2018 (Registration number: 20183400108), and reverse transcribed to cDNA on a Bio-Rad T100 thermocycler (Bio-Rad, US) according to the manufacturer's instructions. Then, 2 μl of cDNA was mixed with 18 μl of the RNAColon® miR-92a assay kit's qPCR components, and the qPCR was performed on an ABI 7500 or a Roche LightCycler 480. Similar to Wu et al. and Yau et al., the miR-92a assay kit used TaqMan probe-based qPCR methods, while optimizing key reagents such as reverse transcriptase, primers, probe, and blocker to achieve better miR-92a detection performance [28-31]. Meanwhile, a standard curve (Ct vs. Copy Number) was created by calculating the copy number of each sample using a serially diluted miR-92a DNA solution.

Statistical analysis

The standard curve was developed by ABI 7500 and Roche 480 software (Version 2.0.6, California, US), and copy number was calculated using standard formulas with amplification efficiency and constant K generated by ABI 7500 and Roche 480. The sensitivity, specificity, and accuracy of copy numbers were calculated using standard formulas. A comparison of miR-92a levels among the CRC group, polyp group, and normal group was performed by the t-test. The SCIPY 1.7.3 package was used to process data and establish the receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) in the case of discriminating patients with CRC. The cutoff value of the miR-92a level was determined by the Youden index from ROC curves, which was determined at the top left point on the ROC curve where the difference between sensitivity and 1-specificity is maximal. Matplotlib 3.1.3

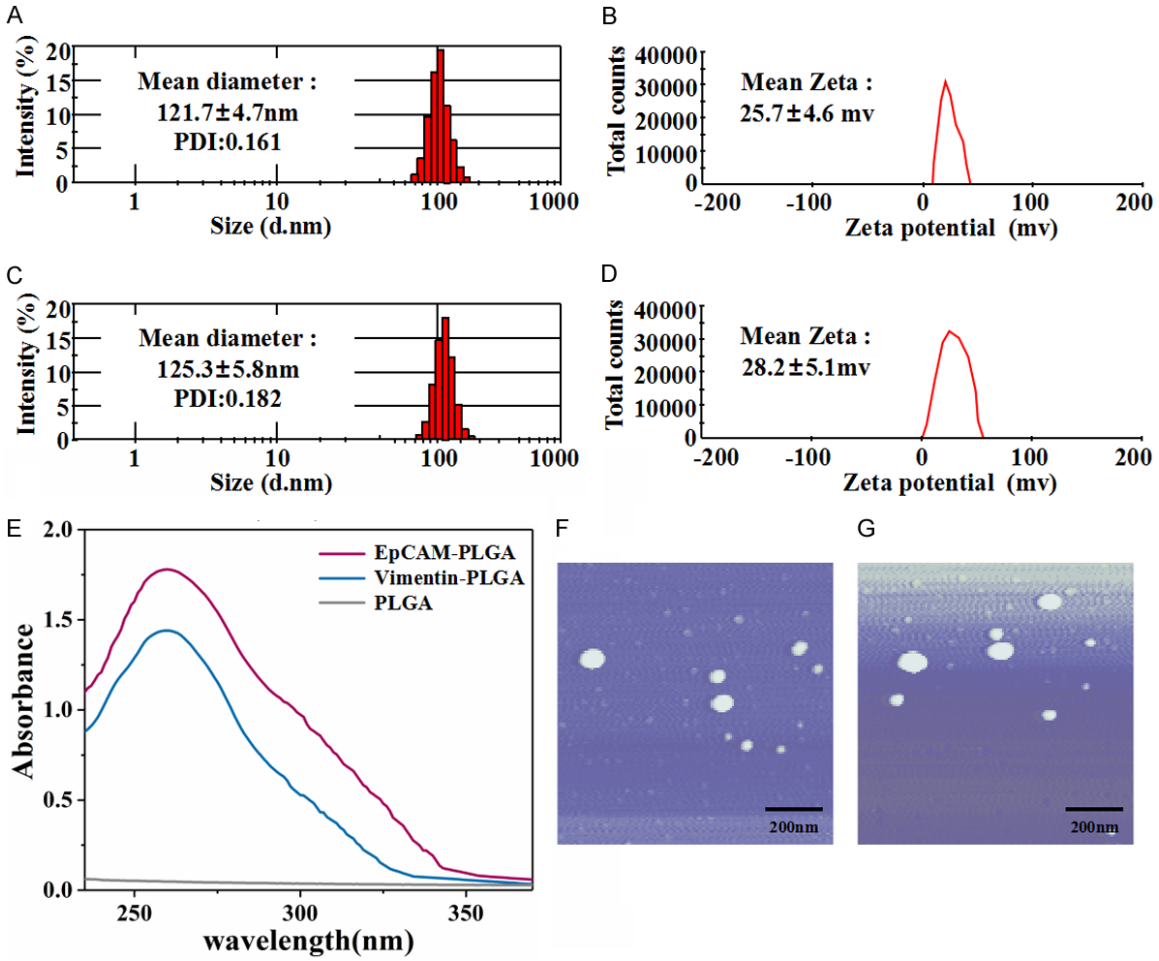

Figure 2. Characterization of Ep-LMB and Vi-LMB.

was used to demonstrate the relationship between miR-92a levels and lesion stages/locations. *P*-values < 0.05 were considered statistically significant.

Results

Performance verification and functional evaluation of EpCAM and vimentin lipid magnetic beads

The physicochemical properties of positively sorted nanoparticle magnetic spheres in the separation and detection system of colorectal exudated cells will greatly impact the ECIF sorting efficiency. **Figure 2A-D** show particle size analysis and Zeta potential analysis. The absorption peak of Ep-LMB appears at 277 nm in UV/visible spectrum (**Figure 2E**), while the broad absorption peak of Vi-LMB appears at 279 nm. **Figure 2F** and **2G** show the AFM image of Ep-LMB and Vi-LMB.

Binding time of the magnetic sphere to the cell

To investigate the optimal time for cells-immune lipid magnetic sphere interaction, Ep-LMB (**Figure 3A**) and Vi-LMB (**Figure 3B**) were fluorescently labeled with FITC and added to the culture dish, along with cell membrane probe Dil and nuclear fluorescent dye DAPI, which were stained and observed using a fluorescence microscope. The results showed that the FITC fluorescence signal in the cells gradually increased with time, indicating that the lipid magnetic spheres on the cell surface gradually increased, with the best effect achieved after 15 minutes of incubation.

Recovery rates of colonocytes using magnetic beads in the simulation study

Figure 3C and **3D** depict cell capture efficiency. Under the same cell concentration and amount of magnetic beads, adding two types of mag-

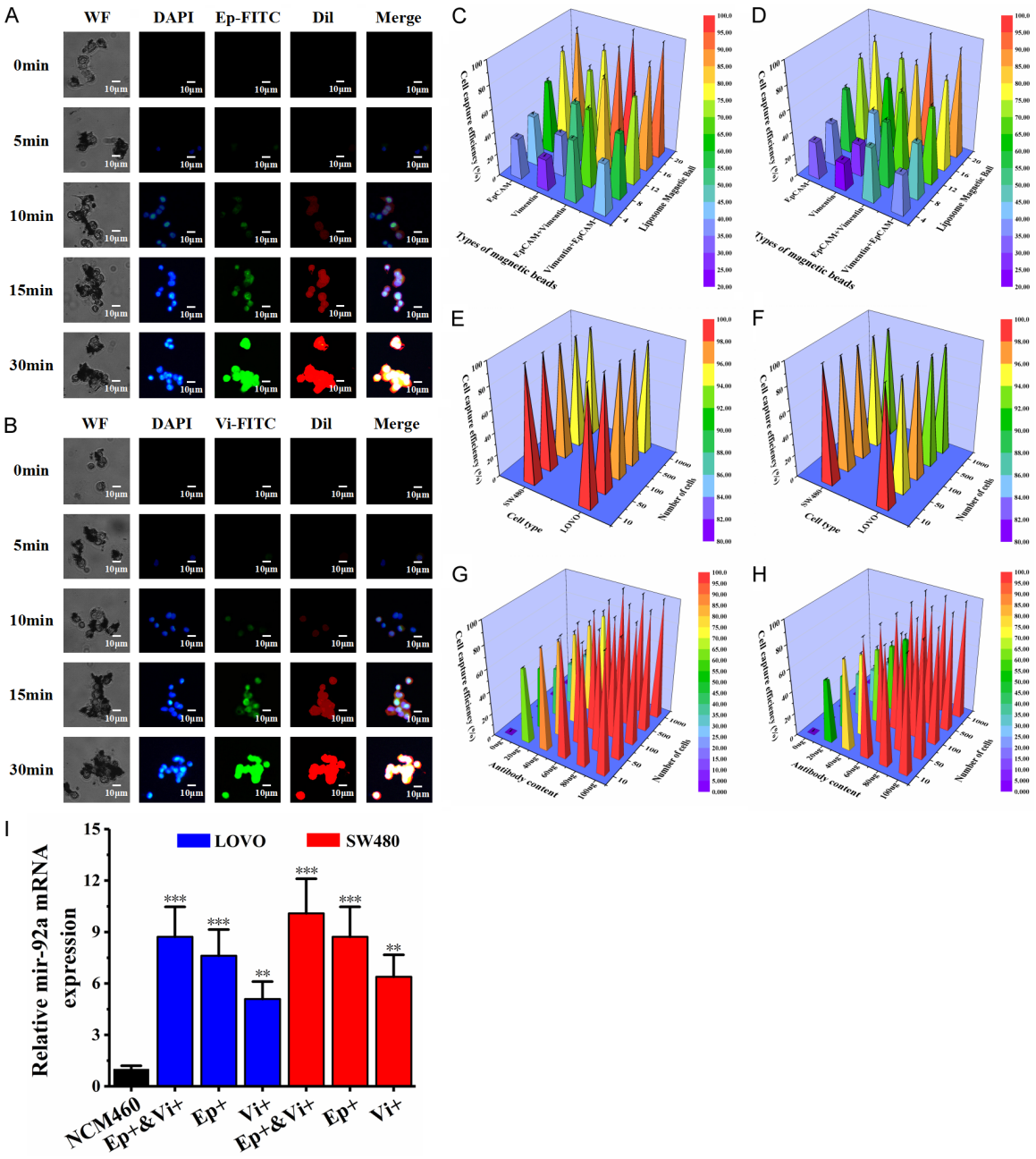

Figure 3. Simulation studies of the capture efficiency of Ep-LMB and Vi-LMB on colorectal cancer tumor cells. A. Changes in Ep-LMB-FITC binding to LOVO cells over time; B. Changes in VI-LMB-FITC binding to LOVO cells over time; C. Different capture schemes and amounts of magnetic spheres were used to test the capture efficiency of LOVO cells in PBS; D. Test the capture efficiency of LOVO cells in simulated feces using different capture schemes and different amounts of magnetic spheres; E. Test the sensitivity of the sorting system in PBS with two types of colorectal cancer cells; F. Test specificity of the sorting system using two colorectal cancer cell lines in a simulated fecal system; G. Capture efficiency of LOVO cells by magnetic spheres containing different antibody contents in PBS system; H. Capture efficiency of LOVO cells by magnetic spheres containing different antibody contents in a simulated fecal system; I. Analysis of the detection results of miR-92a relative expression in tumor cells captured by Ep-LMB and/or Vi-LMB.

netic beads sequentially improved LOVO cell capture efficiency over magnetic bead sorting alone. The order of adding magnetic beads during sequential sorting did not affect sorting efficiency. The best capture strategy was to add 5-μL Ep-LMB/Vi-LMB sequentially, which

Table 1. Clinicopathological characteristics of subjects

	Normal	CRC	Others
No. of cases	360	292	311
Age at enrollment (years)			
Mean ± SD	50.0±12.6	58.8±12.0	49.3±14.7
Gender (No. of cases)			
Male (%)	151 (41.9%)	170 (58.2%)	182 (58.5%)
Female (%)	209 (58.1%)	122 (41.8%)	129 (41.5%)
Tumor histology (No. of cases)			
Adenocarcinoma		145 (49.7%)	
Mucinous adenocarcinoma		5 (1.7%)	
Unknown		142 (48.6%)	
Location of tumor (No. of cases)			
Proximal		45 (15.4%)	
Distal		216 (74.0%)	
Unknown		31 (10.6%)	
TNM stage[a] (No. of cases)			
0, I, II, and III		203 (69.5%)	
IV		48 (16.4%)	
Unknown		41 (14.1%)	

Notes: [a]The 8th Edition of the AJCC Cancer Staging Manual. Abbreviations: CRC, Colorectal Cancer; SD, Standard Deviation.

resulted in a capture rate of > 90% (**Figure 3C**). The results were confirmed in the simulated stool, although the capture efficiency was reduced due to the high level of impurities in the simulated stool system, however the capture rate remained > 90% (**Figure 3D**). We chose Ep-LMB/Vi-LMB sequential capture scheme to examine the capture rate of LOVO cells in PBS, and the results showed that there was no significant difference in the capture rate of Ep-LMB/Vi-LMB sequential capture for the two types of tumor cells (P > 0.05), and the sensitivity was 96.12% (**Figure 3E**). The detection in simulated feces results revealed that there was no significant difference in the capture rate of Ep-LMB/Vi-LMB sequentially captured tumor cells (P > 0.05), and the specificity was 95.32% (**Figure 3F**). The results of a cell gradient experiment with varying antibody content revealed that the capture efficiency in the PBS system and simulated fecal systems was greatest when the antibody content was 60 μg (**Figure 3G** and **3H**).

Analysis of the relative expression of miR-92a in tumor cells captured by EpCAM and vimentin lipid magnetic beads

After a series of verifications of the preparation process and functional properties of the constructed Ep-LMB and Vi-LMB, NCM460, LOVO, and SW480 cell lines were chosen. It was used to detect the capture and sorting efficiency of Ep-LMB and Vi-LMB on the above cells, as shown in **Figure 3I**. The results showed that when Ep-LMB alone or Vi-LMB alone, Ep-LMB, and Vi-LMB are captured in sequence, they can capture more colorectal cancer cells. The relative miR-92a mRNA expression in LOVO and SW480 is 8.72 and 9.79, respectively, which can be used for the isolation of exfoliated cells from feces.

Patient characteristics

A total of 963 Chinese participants were enrolled at three hospitals after providing written informed consent. Sun Yat-sen University Hospital enrolled 257 (26.7%), Shenzhen Peoples' Hospital enrolled 519 (53.9%), and Tianjin People's Hospital enrolled 187 (19.4%). Among 519 participants from Shenzhen People's Hospital, 32 postsurgery CRC cases were re-enrolled. Hence, all 963 patients had results and the corresponding colonoscopy tests. **Table 1** shows the clinicopathological characteristics of the eligible population, which included CRC patients (n = 292) and healthy controls (n = 360). A total of 292 participants who had colonoscopy tests were found to have

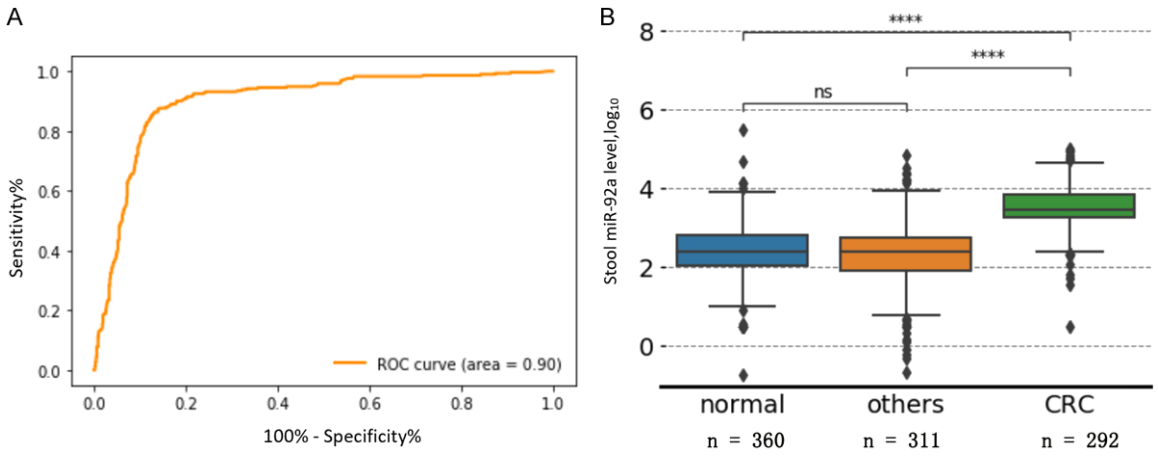

Figure 4. ECIF miR-92a distinguishes between CRC and other control groups. A. Receiver operating characteristics curve analysis using ECIF miR-92 for CRC discrimination. B. ECIF miR-92a levels in colorectal cancer (CRC) patients, normal controls, and others were compared (nonprecancerous gastrointestinal disease patients).

Table 2. Characteristics of miR-92a test sensitivity

	Case Number	Stool miR-92a Test	
		Positive (No.)	Sensitivity (%)
Cancer lesion location			
Proximal CRC	45	39	86.7
Distal CRC	216	186	86.1
Location unknown CRC	31	30	96.8
Cancer lesion TNM stage[a]			
Stages 0, I, and II	132	111	84.1
Stages III and IV	119	108	90.8
Stage unknown	41	36	87.8
		Positive (No.)	Specificity (%)
Health controls	360	57	84.2
Non-CRC diseases	311	31	89.5
Postsurgery	33	6	81.8

Notes: [a]The 8th Edition of the AJCC Cancer Staging Manual. Abbreviation: CRC, Colorectal Cancer.

colorectal cancer (prevalence, 27.4%). Among these participants, 203 (69.5%) had cancers that were not yet in stage IV (n = 48, 16.4%). We labeled cases as "unknown" if there was no tumor location, tumor stage, or polyp type information on the clinical case record, or if the clinical case record was not clear enough for us to determine this information (**Table 1**).

Sensitivity and specificity of exfoliated colonocytes isolated from feces miR-92a test in colorectal cancer patients

The discrimination between colorectal cancer (n = 292) and a combination of non-CRC cancer patients, polyp patients, nonprecancerous gastrointestinal disease patients, and healthy normal controls (total, n = 671) enrolled at three hospitals were assessed using ROC curves. The AUC under the ROC curve was 0.90 (**Figure 4A**). A cutoff value was chosen that maximized the sum of sensitivity and specificity. At a cutoff value of 1053 copies per 6 nanograms of extracted stool total RNA, miR-92a was detected positive in 255 out of 292 (87.3%) patients with CRC, 57 out of 360 (15.8%) healthy controls, 45 out of 311 (14.5%) patients with non-CRC or nonprecancerous diseases, and 14 out of 65 (21.5%) postsurgery patients. Except for postsurgery patients, the overall sensitivity was 87.3% (255/292) and the overall specificity was 86.9% (746/901) (**Table 2**). To see if there were batch effects or biases in these three clinical centers, we examined the data separately. As shown in the Supplementary Tables 1, 2, 3, the sensitivity and specificity of miR-92a were 86.0% and 88.9% in Tianjin People's Hospital, 87.8% and 88.8% in Shenzhen People's Hospital, and 87.4% and 76.4% in Sun Yat-sen University Hospital, respectively. This means that no obvious bias existed between clinical centers.

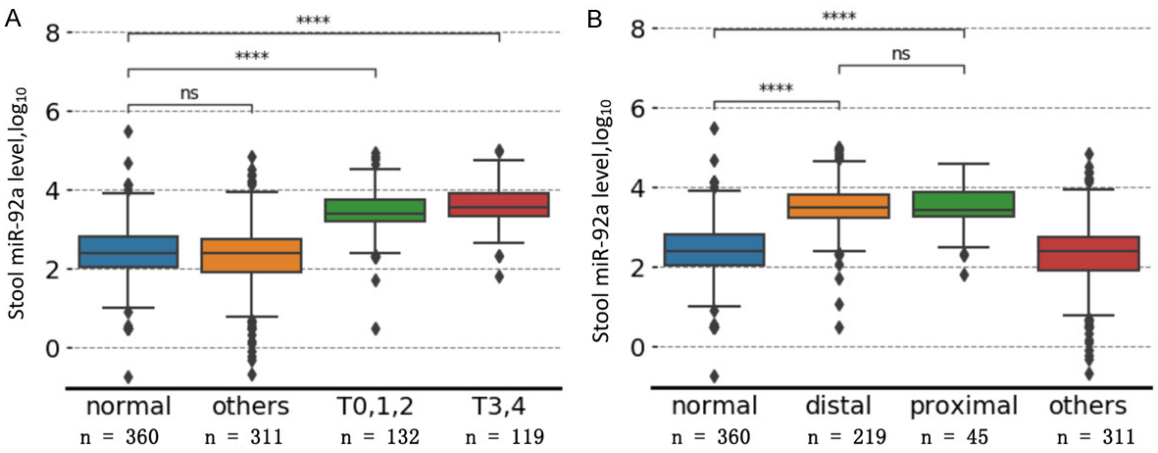

Figure 5. Association between ECIF miR-92a level and CRC lesion stage (A) and location (B). T0, 1, 2 means early-stage CRC; T3, 4 means late-stage CRC.

The sensitivity and specificity of stool miR-92a were also assessed concerning tumor location and stage. The location grouping criteria were as follows: 1) Lesions in the ileocecal region/ascending colon/ascending transverse colon/transverse colon or distance to anal ≥ 60 cm or right side colon are classified as proximal; 2) Lesions in the transverse-descending colon/descending colon/sigmoid rectum or distance to anal < 60 cm or left side colon are classified as distal; 3) Multiple occurred lesions are grouped based on the location of index lesions; 4) Lesions with no further information are categorized as unknown. As shown in **Table 2**, the sensitivity for patients with proximal CRC was 86.7%, while it was 86.1% for patients with distal CRC. In terms of lesion stages, miR-92a sensitivity for CRC 0, I & II, CRC III & IV was 84.1% and 90.8%, respectively (**Table 2**).

Elevated levels of exfoliated colonocytes isolated from feces miR-92a in colorectal cancer patients

To determine the expression level of ECIF miRNA using RT-qPCR, a relative quantitation with standard curve calibration was used for miRNA quantitation in the current study. As shown in **Figure 4B**, the ECIF miR-92a level was significantly higher in CRC ($P < 0.0001$) when compared to that in healthy controls and other patients (e.g., non-CRC patients including gastric cancer patients, liver cancer patients, etc., and nonprecancerous gastrointestinal disease patients including enteritis patients, colonitis patients, gastritis patients, etc.).

Then we want to know how early CRC can be detected by miR-92a. To accomplish this, we compared miR-92a levels in stages I-IV CRC and a normal control group. **Figure 5A** demonstrated that there were statistically significant differences between all stages I-IV CRC and normal controls, but no statistical difference between others and normal controls.

Furthermore, ECIF-based miR-92a levels of proximal (3.47±0.58, n = 45) and distal (3.52±0.59, n = 216) CRC were significantly higher than those in healthy controls (2.41±0.71, n = 360) (**Figure 5B**). The distal patients' ECIF miR-92a level was slightly higher than that in proximal patients, but no statistical difference was found between these two groups. These findings show that ECIF miR-92a is associated with CRC and can detect both distal and proximal CRC.

Among participants enrolled in Shenzhen People's Hospital, 65 participants with CRC were sampled and stool miR-92a levels were detected both before (n = 33) and after (n = 33) surgery. The expression of miRNA-92a decreased dramatically after surgery (from 3.21±0.58 to 2.14±1.14, P < 0.00005) (**Figure 6A**). At a cutoff value of 1053 copies per 6 nanograms of stool total RNA, 22 of 33 cases (66.7%) were detected as positive using presurgery samples, while only 6 of these 33 cases (18.2%) remained positive (**Figure 6B**). These findings indicated the decrease of miR-92a levels after surgery for CRC patients.

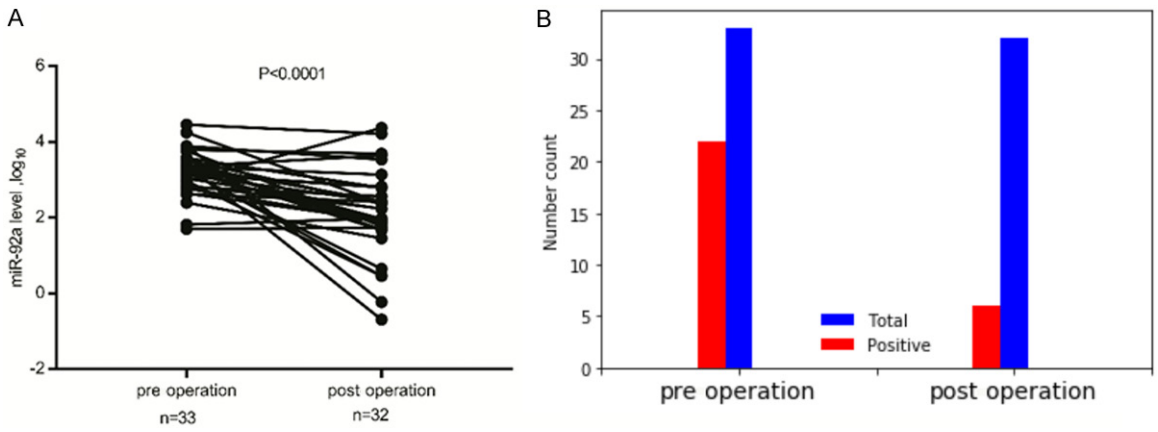

Figure 6. Stool miR-92a levels decreased after surgery. A. postoperative CRC patients had lower miR-92a levels; B. Comparison of stool miR-92a level of colorectal cancer (CRC) patients before and after surgery.

Discussion

Expression of microRNAs is regulated by matching sequences of non-coding regions of target genes [28]. Zhang et al. found that upregulation of miR-92a expression was closely associated with lymph node metastasis in CRC patients [29]. Ke et al. found that miR-92a promotes colon cancer metastasis through activation of the PI3K/AKT pathway [30]. MicroRNA-92a (miR-92a) is induced by oxidative stress in endothelial cells (ECs) and is involved in angiogenesis [31]. These studies demonstrate that mir 92a plays an important role in tumorigenesis. It is noteworthy to demonstrate the use of stool-based miR-92a as a clinical application for CRC screening. A previous study used a TaqMan probe-based RT-qPCR assay for stool-based miR-92a levels and found that it had moderate sensitivity (~71.6%) and specificity (~73.3%) in 88 CRC patients [32]. REColon® miR-92a Assay kit (GeneBioHealth, China) is a commercial in vitro diagnostic kit targeting miR-92a in feces as a biomarker for colorectal cancer. The kit has been approved for clinical practice in China (S1). According to the technical review report issued by National Medical Products Administration (China), the sensitivity of the reagent was 71.76% and the specificity was 90.23% after a total of 1306 clinical samples were evaluated in three hospitals (S2). This kit has been approved by the authorities for clinical application. In this study, we tested the feasibility of ECIF-based miR-92a as a biomarker for CRC screening in a large cohort (n = 963) using an improved method. Sensitivity is the most important characteristic of screening tests because the primary purpose of such testing is to rule out diseases such as cancer. In our study, the ECIF-based miR-92a test had a sensitivity of about 87.3% for detecting colorectal cancer. Although high sensitivity is the most important feature of cancer screening tests, specificity is also important because it affects the number of people who have positive test results, the majority of whom will have false positive results due to cancer's low prevalence. The specificity of ECIF-based miR-92a is high, reaching 86.9%. Plasma-based miRNA and stool-based miRNA have high stability and reproducible detection. In this study, we discovered that the expression level of miR-92a in stool from CRC tissues was significantly higher than that of non-CRC tissues. This data agreed with previous reports [33]. We chose a cutoff value of 1060 copies per 6 ng to distinguish CRC patients from healthy individuals based on the ROC curve. miR-92a in our study demonstrated a higher discriminating ability of test sensitivity (100%) and specificity (89.7%) than Koga et al studies [34]. The improved detection performance of miR-92a in our current studies is most likely due to the optimization of ECIF RNA extraction and the optimized miR-92a primers, probe, and blocker used in the miR-92a detection kit. Of course, the difference in key reagents used in the kit, such as reverse transcriptase, may also play a role in better performance. To demonstrate the principle of the enhanced stool-based miR-92a derived from the primary malignancy, miRNA in ECIF was compared between pre and postsurgery.

After tumor removal, a significantly dropped level of miR-92a was reported by us. These findings imply that the high levels of miR-92a in CRC patients' ECIF are derived from neoplastic cells. This is, to the best of our knowledge, the first multicenter clinical study evaluating microRNA biomarkers for CRC diagnosis. Our findings pave the way for future research into the use of microRNA biomarkers to diagnose CRC or other cancers. Although these findings suggest that ECIF miR-92a is a useful tool for CRC screening, our current study has some limitations. First, we did not compare miR-92a to commonly used biomarkers (such as FIT and other microRNAs [35-37], as well as other emerging biomarkers, including DNA methylation biomarkers [10, 14, 15], gut microbes [38-40], etc.). Second, our current study is a follow-up. We do not know whether an ECIF-based miR-92a test can effectively screen high CRC-risk populations from average-risk populations in a larger prospective cohort. Therefore, these limitations prompted us to conduct future comparative studies to see if combining several biomarkers can improve sensitivity and specificity and if miR-92a performs well in prospective cohorts.

Conclusions

Finally, this study demonstrated the viability of using ECIF miR92 as a noninvasive tool for CRC detection. The ECIF-based miRNA-92a demonstrated excellent stability and reproducibility. ECIF miR-92a has an acceptable sensitivity and specificity for CRC detection. Because the current trend in ECIF-based tests is to incorporate multiple heterogeneous markers to increase test sensitivity, this study justifies incorporating ECIF-based miRNA markers like miR-92a into an already existing molecular marker panel.

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Informed consent was obtained from all individual participants included in the study.

Disclosure of conflict of interest

None.

Abbreviations

AUC, Areas Under the Curve; CRC, Colorectal Cancer; FOBT, Fecal Occult Blood Test; ROC, Receiver Operating Characteristic; RT-qPCR, Real-Time Quantitative PCR; ECIF, Exfoliated Colonocytes Isolated from Feces.

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Supplementary Table 1. Sensitivity and specificity of stool miR-92a detection in Tianjin People's Hospital

miR-92a Results	Colonoscopy & Pathological Results		Total
	Positive	Negative	
Positive	45	49	94
Negative	11	233	244
Total	56	282	338
Sensitivity	45/56 = 80.36%		
Specificity	233/282 = 82.62%		

Supplementary Table 2. Sensitivity and specificity of stool miR-92a detection in Shenzhen People's Hospital

miR-92a Results	Colonoscopy & Pathological Results		Total
	Positive	Negative	
Positive	95	65	160
Negative	18	370	388
Total	113	435	548
Sensitivity	95/113 = 84.07%		
Specificity	370/435 = 85.06%		

Supplementary Table 3. Sensitivity and specificity of stool miR-92a detection in Sun Yat-sen University Hospital

miR-92a Results	Colonoscopy & Pathological Results		Total
	Positive	Negative	
Positive	138	42	180
Negative	33	142	175
Total	171	184	355
Sensitivity	138/171 = 80.70%		
Specificity	142/184 = 77.17%		