Exploring stool-based microRNA-92a as a potential biomarker for colorectal cancer diagnosis

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Abstract
Background: Stool-based molecular markers have shown potential as a strategy for colorectal cancer (CRC) screening. This study aimed to evaluate the feasibility of using microRNA-92a expression as a biomarker for CRC in stool samples. Methods: The level of microRNA-92a was measured in stool samples from 210 CRC patients, 29 patients with advanced adenomas, 15 patients with other cancers, and 101 healthy controls, using real-time quantitative polymerase chain reaction. Receiver operating characteristic curves were used to evaluate sensitivity and specificity. Results: MicroRNA-92a expression was positive in 70.1% of CRC patients, 44.8% of advanced adenomas patients, and 36.6% of healthy controls, using a cut-off value of 31.5. The corresponding sensitivity and specificity for discriminating CRC from advanced adenomas were 66.9% and 63.4%, respectively. Moreover, stool-based microRNA-92a expression was better at detecting CRC cancers in the distal colon (sensitivity 82.1%) than the proximal colon (sensitivity 67.9%). There were no significant differences in clinical stage of CRC when comparing AUCs of each parameter (P > 0.05). Conclusion: These findings suggest that microRNA-92a expression in stool samples could serve as a promising non-invasive biomarker for CRC detection.

Keywords: colorectal cancer; advanced adenomas; microRNA-92a; stool-based; biomarkers
Background

China is one of the countries with the highest incidence of colorectal cancer (CRC) globally. The number of diagnosed cases increased from 400,700 in 2016 to 455,700 in 2020, with a compound annual growth rate of 3.3% \([1, 2]\). This trend indicates that as awareness of CRC screening among patients improves, new screening technologies are expected to help detect more cases before cancerous changes occur or in the early stages of cancer, thereby slowing down the rate of disease progression \([3, 4]\).

In the past decades, colonoscopy has been considered the standard for diagnosing colorectal cancer (CRC). However, its invasive nature, high equipment costs, and the need for skilled personnel have hindered its widespread application \([5]\). The most commonly used screening test is the fecal occult blood test (FOBT), but it has limitations in terms of low sensitivity, leading to a significant number of missed colorectal cancer cases \([6]\). Therefore, an ideal screening test should be minimally invasive, cost-effective, and have high compliance, sensitivity, and specificity \([7]\).

MicroRNAs (miRNAs) are small non-coding RNA molecules consisting of 18–25 nucleotides. They play a crucial role in regulating gene expression at the post-transcriptional level \([8, 9]\). Recent studies have highlighted the potential of miRNAs as reliable and non-invasive biomarkers in various tumor types, including CRC \([10, 11]\). Among these miRNAs, miR-92a, a component of the miR-17–92a gene cluster, has shown promising results. Studies have demonstrated that miR-92a is upregulated in tumor tissues, and its overexpression in CRC is associated with tumor growth, migration, metastasis, and an unfavorable prognosis \([12]\). This suggests that miR-92a plays a significant role in promoting the development and progression of CRC \([13, 14]\).

The detection of miR-92a in stool and plasma samples has emerged as a potential biomarker for CRC diagnosis.

In this study, we aimed to investigate the diagnostic potential of miR-92a in CRC. We collected clinical stool from a total of 210 CRC patients, 29 patients with advanced adenomas (AA), 15 patients with other types of cancer, and 101 healthy controls (HC). Firstly, we observed the difference of the miR-92a expression in patients with CRC, AA and other cancers and HC. Secondly, we investigate the diagnostic value of miR-92a between pre-surgery and post-therapy, different tumor size, different clinical stages. In the end, we evaluate the diagnostic value of miR-92a expression combined with FOBT in screening for CRC.

Materials and methods

Stool samples

To reduce the bias, we designed this experiment as a blinded assay. From January 2015 to June 2015, all stool samples were collected using the 30 mL disposable stool container before colonoscopy from subjects at the Sun Yat-sen University Cancer Center (Guangzhou, China). All colonoscopy was performed by an experienced endoscopic expert (Guo-Liang Xu) using the same diagnostic criteria. After receiving informed consent, fresh stool samples were collected in the morning and immediately frozen in liquid nitrogen and stored at \(-80^\circ C\) until RNA extraction. 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 of Sun Yat-sen University Cancer Center (approval number: SYL-202123-05).

Total RNA isolations by the automated method

Total RNA was extracted from stool samples using the nucleic acid isolation reagents (GeneBioHealth Co., Ltd., Shenzhen, China) and the Auto-Pure20B instrument (All sheng Co., Ltd., Hangzhou, China) according to the manufacturer’s instructions. Briefly, about 0.3–0.5 g of stool samples were immediately transferred to a sterilized 2 mL microtube (also frozen). After the addition of 0.9 mL Buffer A, the samples were homogenized in the vortex. Stool samples were subjected to centrifuge with high-speed at 12,000 g, 4 °C for 15 min to remove debris. Supernatants were subsequently added with 0.5 mL Buffer B and 0.2 mL trichloroethane in a sterilized 2 mL microtube. The samples were mixed again in the vortex and incubated at room temperature for 10 min. During the incubation, the samples were mixed in the vortex every 5 min. Then, a centrifugation step of 13,000 rpm at 4 °C for 10 min was made. The 0.6 mL aqueous phase was removed for a new sterilized microtube of 2 mL, and an equivalent volume of 100% ethanol and 20μL of magnetic beads (10 mg/mL) were added to the tube and mixed well then, the mixture was incubated for 5 min at room temperature. Finally, nucleic acids were obtained after adsorption, washing and elution.

Quantitative real-time reverse transcriptase-PCR

Single-stranded complementary DNA was synthesized from 600 to 1,000 ng of total RNA samples using the Real Time reverse transcription-PCR detection kit for miR-92a expression (GeneBioHealth Co., Ltd., Shenzhen, China). The complementary DNA then serves as the template for miR-92a-specific TaqMan real-time quantitative PCR using real-time quantitative PCR Master Mix (GeneBioHealth Co., Ltd., Shenzhen, China). All reactions were run in Applied Biosystems 7500 Fast Real-Time PCR System using miR-92a-specific primers. The amplification profile was denatured at 95 °C for 2 min, followed by 50 cycles of denaturation at 95 °C for 8 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. The comparative cycle threshold (CT) method was applied to quantify the miR-92a expression. A CT value of positive control is acceptable if less than 29. The positive control was measured in triplicate, and we then calculated the CT on average as CT positive control. The samples were interpreted as positive when the CT miR-92a of sample > CT positive control, while as negative when the CT miR-92a of sample ≤ CT positive control.

Data processing and statistical analysis

The main results were described as following: a positive result was a true positive if a neoplasm was found and a false positive if no neoplasm was found; a negative result was a true negative if no neoplasm was found and a false negative if a neoplasm was found depending on whether a colorectal neoplasm was found using endoscopy or surgery. The area under curves (AUCs) with 95% confidence intervals (CI) calculated by the receiver operating characteristic curve (ROCs) was performed to assess the sensitivity and specificity. We carried out statistical analyses using SPSS 16.0 software and considered a P value of 0.05 or less (two-sided) as being statistically significant.

Results

Clinicopathological characteristics

210 CRC patients, 29 AA patients, 15 other cancers patients and 101 HC were enrolled in this study. The main characteristics of the enrolled subjects are presented in Table 1. The comparison of miR-92a levels in stool samples from CRC patients, AA patients, other cancers, and HC is summarized in Figure 1. The sensitivity of miR-92a expression in CRC was significant higher than those patients with AA (P = 0.023), other cancers (P = 0.017) and HC (P < 0.001).

The sensitivity of stool miR-92a level in CRC

To explore the potential role of the stool-based miR-92a level as a reliable biomarker of CRC, we did a ROC analysis to evaluate the sensitivity and specificity in CRC compared to others. We found that the AUCs detected by the stool-based miR-92a expression were 0.71 in CRC (95% CI, 0.65–0.77) and 0.57 in AA (95% CI, 0.45–0.68), respectively. The sensitivities of 70.1% in CRC and 44.8% in AA were observed (P < 0.001, Figure 2). This suggests the stool miR-92a expression in distinguishing CRC from AA might be promising. Additionally, we compared the stool miR-92a expression in 153 and 57 CRC patients before and after surgery treatment, respectively. The stool miR-92a expression in post-surgery patients was significantly higher than those in pre-surgery samples (P < 0.001, unpaired t test, Figure 3A). The AUCs in pre-operation subgroup was greater than that.
in post-therapy subgroup suggesting the diagnosis efficacy of the stool miR-92a expression is superior in CRC patients with pre-operation (Figure 3B).

<table>
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<td>60.0 ± 11.6</td>
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**Figure 1** The association between stool miR-92a level and clinicopathological features. Comparison of levels of miR-92a level in stool samples from CRC patients (n = 210), AA patients (n = 29), other cancers (n = 15), and health controls (n = 101). Dot lines at the y-axis denote cut-off values = 31.5. Significant difference between groups was tested by the non-parametric t test. CRC, colorectal cancer; AA, advanced adenomas; HC, healthy controls.

**Figure 2** Receiver operating characteristics (ROCs) curves were used to discriminate patients with CRC, AA and CRC&AA. A cut-off value of 31.5 was best distinguished in patients with CRC, and the AUC value was 0.71, AA and CRC&AA yield an AUC value of 0.57 and 0.69, respectively. CRC, colorectal cancer; AA, advanced adenomas; AUC, receiver operating characteristic curve.
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2023;6:e23024 demonstrated and was alone become AUCs <
of the stool was found TNM expression a with in induce tool stool-based together, = fecal data a and the series < expression we Figure 3 Comparison of levels of miR-92a in samples from postoperative patients (n = 153) and the pre-therapy samples (n = 57). (A) Mean ± SEM of postoperative patients were 29.78 ± 0.17, Mean ± SEM of pre-therapy patients were 31.19 ± 0.34. The lines denote the medians. Dot lines at the y-axis denote cut-off values. A significant difference between the two groups was examined by Unpaired t test (P < 0.001). (B) Receiver operating characteristics (ROCs) curves were used to discriminate postoperative patients and the pre-therapy samples. Stool miR-92 yielded a ROC curve value of 0.75 (95% CI, 0.693–0.819), sensitivity of 76.5%, and specificity of 72% in discriminating CRC of postoperative patients. Stool miR-92 yielded a ROC curve value of 0.57 (95% CI, 0.479–0.662), sensitivity of 40.4%, and specificity of 50% in discriminating pre-therapy samples. miR-92a, microRNA-92a; AUC, receiver operating characteristic curve.

Correlation between the stool miR-92a expression and clinical characteristics
According to the tumor location and diameter, TNM stage, we also did the ROC analysis for evaluation of diagnosis efficacy of the stool-based miR-92a expression. The stool miR-92a level was demonstrated a higher sensitivity for distal CRC than proximal CRC (82.1% versus 67.9%, P < 0.001; Figure 4A). The sensitivity of the stool miR-92a level detecting tumor diameter between 1 cm to 3 cm was 82.1%, while 78.1% in the maximum tumor diameter ≥ 3 cm (P < 0.001, Figure 4B). Interestingly, only one case harboring miR-92a expression was identified in the maximum tumor diameter ≤ 1 cm. No difference of the AUCs was found when detecting TNM stage using the stool-based miR-92a expression (Figure 4C–4F).

Comparison of the stool-based miR-92a expression and fecal occult blood test for detection of CRC
The most common and noninvasive method for screening CRC is the FOBT. We then investigated the value of miR-92a level by combined the stool miR-92a level with FOBT for CRC. According to the FOBT results and miR-92a expression, we obtained the data including 153 CRC patients and 93 HC. The sensitivity, specificity and AUC values for CRC diagnosis were 79.1%, 61.3% and 0.72 (95% CI, 0.63–0.77) using miR-92a expression, and 43.8%, 87.1% and 0.65 (95% CI, 0.59–0.72) using FOBT method, respectively (P < 0.0001, Figure 5). When it was combined the stool miR-92a expression with FOBT in parallel, the sensitivity, specificity and AUC values were 88.9%, 54.9% and 0.72 (95% CI, 0.65–0.79) (P < 0.0001, Figure 5); whereas combined in series, the sensitivity, specificity and AUC values were 34.0%, 93.6% and 0.64 (95% CI, 0.57–0.71) (P = 0.003, Figure 5). A comparison of the ROC curves showed that the AUC of the two-marker combination (miR-92a and FOBT) in parallel was equal to miR-92a alone (P > 0.05). Importantly, the AUC of miR-92a expression alone was greater than the two-marker combination in series and FOBT alone (P < 0.05). These findings indicate that the stool miR-92a expression could be superior to FOBT alone or the combination in the accuracy of CRC diagnosis.

Discussion
In this study, the relative expression of the stool-based miR-92a is increased in patients with CRC, compared with the patients with AA and other malignancies, and the lowest in HC [15]. The stool-based miR-92a expression was decreased in patients after surgery and significantly affected by tumor diameter, location and TNM staging, which were associated with the normal-adenoma-carcinoma order [16]. Taken together, the stool-based miR-92a level may have the potential to become a screening tool in high-risk populations with precancerous colorectal lesions [17, 18].

miRNAs has been shown to remain a high proportion of its original level in a 72 h period [19]. Stool-based miRNAs have high stability over mRNA and their detection is reproducible. Therefore, it ensures that subsequent quantitative analysis is less likely to be affected by miRNAs degradation. As a known onco-miRNA, the miR-92a could promote cell proliferation, suppress apoptosis, induce tumor angiogenesis, and accelerate tumor progression [20]. Several previous studies have been shown that the relative expression of miR-92a in
tissues, plasma and stool of CRC patients were significantly higher than those of HC [21, 22]. In our study, miR-92a has the best diagnostic efficiency in patients with CRC and HC when using a cut-off value of 31.5 Ct in real-time PCR. The corresponding sensitivity and specificity for patients with CRC were 70.1% and 63.4%, respectively, and the AUC value was 0.71, which are consistent with a previous study.

Our findings also indicate that the increase in positive ratio of the stool miR-92a was more obvious in the groups with tumor located at distal colon than in the groups with tumor located at proximal stomach. One possible explanation is that the miRNA was degraded as the amount of time taken in the stool, the less time remain in the gut, the less time contact with degrade-related enzymes. Another explanation might be that the miRNA released by the distal colon cancer concentrations in the surface of stool, easy to collect and detect.

Furthermore, we conducted an analysis to examine the relationship between stool-based miR-92a levels and tumor burden. Interestingly, we observed that the expression of miR-92a was higher in pre-surgery patients compared to post-therapy patients. However, there were no statistically significant differences observed among different TNM stages groups, which is consistent with previous studies. It is worth noting that colorectal cancer patients, particularly during the initial rounds of chemotherapy, tend to experience a significant shedding of tumor cells, which can contribute to the increased presence of miR-92a in their feces. It is important to acknowledge that the results obtained in this study may be influenced by the relatively small sample size. Therefore, further investigations with a larger number of cases are warranted to validate and expand upon these findings.

In this study, we found the AUC similarity in parallel combination and considered the practical implications of parallel vs. series combinations. We primarily considered the correlation with tumor size or staging, as early-stage colorectal cancer is difficult to detect, whether it is through the miR-92a content in feces or the presence of blood in feces. In contrast, late-stage colorectal cancer has a larger tumor volume, making it easier to detect using any method. In clinical practice, for patients suspected of having colorectal cancer, we often use multiple methods in combination for testing, primarily to establish a definitive diagnosis and prevent misdiagnosis.

Figure 4: Receiver operating characteristic curve analysis was employed to determine the cut-off value for the overexpression of stool miR-92a based on patient characteristics. The sensitivity and specificity for each clinical outcome were plotted. (A) Association between stool miR-92a level and tumor location. (B) Maximum tumor diameter. (C) TNM stage. (D) T stage. (E) N stage. (F) M stage. AUC, receiver operating characteristic curve.
While differences in sensitivity were observed, other factors influencing miR-92a expression need to be considered. First, dietary factors need to be considered. Since all participants did not have controlled diets, the presence of peroxides in dietary components could also lead to false positives. Second, gut age, which refers to the balance of various bacteria in the gut, is important in predicting the aging status of the gut and the probability of developing modern lifestyle diseases, thus assessing one’s health condition. Third, factors related to intestinal motility should be taken into account. Weakened intestinal motility can lead to prolonged stool retention in the colon, resulting in difficulties in defecation and constipation. Fourth, intestinal bleeding should be considered. In patients with colorectal cancer, rectal bleeding occurs due to tumor rupture and bleeding, or it may be caused by the rupture of the colorectal tumor, leading to the mixing of blood and stool.

One limitation of this study is the relatively small number of samples, which may influence the power of statistical tests. The relative expression of stool miR-92a showed an up-regulating tendency in advanced adenoma patients compared with health cohorts, but this tendency did not dramatically increase because the number of patients in the group is insufficient. Secondly, the diagnostic efficiency of stool miR-92a for CRC diagnosis is not very specific, in part from its lack of specificity. Therefore, it is necessary to combine the detection of miR-92a with other tumor markers which can efficiently reduce false negative results on the next research step. Thirdly, tissue and serum groups that verify CRC patients with the positive expression of stool miR-92a are scanty.

In conclusion, we found that there were obvious differences in the stool-based miR-92a expression between patients with CRC and healthy cohorts, stool miR-92a might be a novel potential biomarker in the diagnosis of colorectal cancer.

References


Figure 5 ROC curves comparing stool miR-92a level (AUC = 0.70, P < 0.0001), FOBT (AUC = 0.65, P < 0.0001), the two markers combined (in parallel) (AUC = 0.72, P < 0.0001) and the combination of the two markers series (AUC = 0.64, P = 0.003) in patients with CRC. FOBT, fecal occult blood test; miR-92a, microRNA-92a; AUC, receiver operating characteristic curve.


