Serum miR-21, miR-29a, and miR-125b Are Promising Biomarkers for the Early Detection of Colorectal Neoplasia

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Abstract

Purpose: Circulating microRNAs (miRNA) are emerging as promising diagnostic biomarkers for colorectal cancer, but their usefulness for detecting early colorectal neoplasms remains unclear. This study aimed to identify serum miRNA biomarkers for the identification of patients with early colorectal neoplasms.

Experimental Design: A cohort of 237 serum samples from 160 patients with early colorectal neoplasms (148 precancerous lesions and 12 cancers) and 77 healthy subjects was analyzed in a three-step approach that included a comprehensive literature review for published biomarkers, a screening phase, and a validation phase. RNA was extracted from sera, and levels of miRNAs were examined by real-time RT-PCR.

Results: Nine miRNAs (miR-18a, miR-19a, miR-19b, miR-20a, miR-21, miR-24, miR-29a, miR-92, and miR-125b) were selected as candidate biomarkers for initial analysis. In the screening phase, serum levels of miR-21, miR-29a, and miR-125b were significantly higher in patients with early colorectal neoplasm than in healthy controls. Elevated levels of miR-21, miR-29a, and miR-125b were confirmed in the validation phase using an independent set of subjects. Area under the curve (AUC) values for serum miR-21, miR-29a, miR-125b, and their combined score in discriminating patients with early colorectal neoplasm from healthy controls were 0.706, 0.741, 0.806, and 0.827, respectively. Serum levels of miR-29a and miR-125b were significantly higher in patients who had only small colorectal neoplasms (<5 mm) than in healthy subjects.

Conclusions: Because serum levels of miR-21, miR-29a, and miR-125b discriminated patients with early colorectal neoplasm from healthy controls, our data highlight the potential clinical use of these molecular signatures for noninvasive screening of patients with colorectal neoplasia. Clin Cancer Res; 21(18): 4234–42. ©2015 AACR.
Translational Relevance

Circulating microRNAs are emerging as promising biomarkers for various human diseases, including colorectal cancer. To reduce colorectal cancer–related mortality, diagnosis of the disease at curable stages, such as precancerous lesions and early cancers, is highly important. Although many studies have suggested the potential use of circulating microRNAs in colorectal cancer detection, their potential for detecting precancerous lesions remains poorly understood. In this study, we analyzed a large cohort of serum samples from patients with early colorectal neoplasms, in which the majority had only precancerous lesions, and demonstrated that expression levels of miR-21, miR-29a, and miR-125b could discriminate patients from healthy controls. Our findings of serum microRNAs as diagnostic biomarkers of precancerous colorectal lesions provide rationale for the further development of these molecular signatures as screening biomarkers for this fatal malignancy.

Serum miRNAs in the Early Detection of Colorectal Neoplasia

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Materials and Methods

Study subjects

Study subjects were prospectively enrolled at participating hospitals in Japan between January 2012 and May 2014. Case subjects consisted of patients who underwent endoscopic resection of early colorectal neoplasms. Early colorectal neoplasms included tubular adenomas, tubulovillous adenomas (TVA), serrated polyps, high-grade intraepithelial neoplasms (HGIN), and invasive cancers. Serrated polyps included sessile serrated polyps/adenomas, traditional serrated adenomas, and mixed serrated polyps. The majority of enrolled patients had only precancerous lesions, but some individuals with invasive cancers were enrolled because they underwent endoscopic resection based on an indefinite endoscopic diagnosis of intraepithelial neoplasia or invasive cancer, and pathologic examination of the resected specimen yielded the diagnosis of invasive cancer. Most of these invasive cancers showed only slight invasion into the submucosa. Clinicopathologic characteristics of the study subjects are summarized in Table 1. An advanced neoplasm was defined as either tubular adenoma or serrated polyp 10 mm or larger, TVA, HGIN, or an invasive cancer (8). Enrolled patients were categorized according to the histology of the index lesion. In patients with multiple colorectal neoplasms, the most advanced or largest lesion among equivalently advanced lesions was designated as the index lesion. Serum samples were drawn within 3 months before the endoscopic resection. Serum samples were also collected from asymptomatic healthy volunteers as controls. Volunteers were enrolled from employees, subjects for annual health checks without major abnormalities, and patients undergoing screening colonoscopy in the participating hospitals. Individuals with a personal history of colorectal cancer, malignant tumors in other organs, inflammatory bowel disease, familial adenomatous polyposis, or Lynch syndrome were excluded from both groups. Clinicopathologic information was obtained from medical charts and questionnaires filled out by the participants. Written informed consent was obtained from all subjects, and the study protocol was approved by the Institutional Review Board of participating institutions.

Study design

Schematic representation of this study is illustrated in Fig. 1. Our study consisted of 3 parts: a systematic literature review for candidate miRNA biomarker selection, a screening phase, and a validation phase. The screening phase included patients with 24 early colorectal neoplasms and 25 healthy subjects and a validation phase included 136 patients with early colorectal neoplasms.
and 52 healthy subjects. In the screening phase, serum levels of 9 miRNAs (miR-18a, miR-19a, miR-19b, miR-20a, miR-21, miR-24, miR-29a, miR-92a, and miR-125b) were investigated by quantitative real-time RT-PCR in sera from 24 patients with early colorectal neoplasia and 25 healthy controls. In the validation phase, an independent cohort of 136 patients with early colorectal neoplasia and 52 healthy controls were used to examine serum levels of miR-21, miR-29a, and miR-125b.

### RNA extraction

RNA was extracted from serum samples using miRNeasy Serum/Plasma Kits (QIAGEN; catalog number 217184) according to the manufacturer’s instructions. Briefly, 250 μL of serum was thawed on ice and centrifuged at 16,000 × g at 4°C for 10 minutes to remove cellular debris. Thereafter, 200 μL of supernatant was lysed in 1,000 μL of QiAozol Lysis Reagent. After incubation for 5 minutes, 25 fmol of synthetic cel-miR-39 (Syn-cel-miR-39-3p miScript miRNA Mimic, QIAGEN; catalog number MSY0000010) was added to each sample as an external spiked-in control. Total RNA, including small RNA, was extracted and eluted in 30 μL of RNase-free water using a QIAcube devise (QIAGEN).

### Real-time RT-PCR

Serum levels of candidate miRNA biomarkers and cel-miR-39 were examined by real-time RT-PCR using TaqMan MicroRNA Assays (Applied Biosystems; catalog number 4440040) and performed in duplicate on the StepOne Plus system (Applied Biosystems) with the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Cycle threshold (Ct) values were calculated using StepOne Software v2.3 (Applied Biosystems). Expression levels of miRNAs were normalized to those of cel-miR-39 and determined by the 2^(-ΔΔCt) method. ΔCt was calculated as follows: ΔCt = Ct (miRNA of interest) – Ct (cel-miR-39). Then, ΔΔCt was calculated by using a sample from a healthy volunteer as a calibrator: ΔΔCt = ΔCt (tested sample) – ΔCt (calibrator).

### Power calculations and statistical analysis

Power calculations to determine the required sample sizes in the validation phase were performed by G’Power 3 (25) based on the data from the screening phase. To compare the serum miRNA levels between 2 groups, the Mann–Whitney U test was conducted. The Steel–Dwass test was used to perform all-paired multiple comparisons among 3 or more groups. Correlation between the size of the index lesion and the serum levels of miR-21, miR-29a, and miR-125b was analyzed by Spearman rank correlation coefficient (ρ). All P values were 2-sided and P < 0.05 was considered significant.
**Results**

**Candidate miRNA biomarker selection**

Because one of the primary goals of our study was identification of diagnostic biomarkers for early colorectal neoplasms, we first selected miRNAs that had been reported to be dysregulated in early colorectal neoplasia tissues (11, 13, 26–30). We found 175 miRNAs that have been reported to be dysregulated in early steps during colorectal carcinogenesis. To narrow our candidate list exclusively to secretory miRNAs that may be detectable in blood, we next selected miRNAs dysregulated in serum or plasma from patients with colorectal cancer and/or colorectal adenoma (16–24). We chose only miRNAs showing consistent dysregulation in tissues and serum or plasma among multiple studies and identified 20 such miRNAs. We thereafter excluded 6 miRNAs that were discovered solely by microarrays in small sample numbers and not validated by subsequent quantitative methods such as real-time RT-PCR. Through this approach, 14 miRNAs remained as candidate biomarkers (Supplementary Table S1). Five miRNAs (miR-409-3p, miR-424, miR-575, miR-601, and miR-760) were further excluded because serum expression levels of these miRNAs were too low to be accurate quantified by real-time RT-PCR in our preliminary experiment (data not shown). As a result, 9 miRNAs (miR-18a, miR-19a, miR-19b, miR-20a, miR-21, miR-24, miR-29a, miR-92, and miR-125b) were selected as candidate biomarkers for the early detection of colorectal neoplasms.

**The screening phase**

In the screening phase, expression levels of the above-mentioned 9 miRNAs in serum samples from 24 patients with early colorectal neoplasia and 25 healthy controls were examined. As shown in Fig. 2, serum levels of miR-21 ($P = 0.0007$), miR-29a ($P < 0.0001$), and miR-125b ($P = 0.020$) were significantly higher in patients with early colorectal neoplasia than in healthy controls.

**The validation phase**

First, we performed power calculations by G*Power 3 (25) to determine the sample size required in the validation phase. On the basis of the miR-125b data in the screening phase, which showed the smallest effect size among the three candidate miRNAs, we estimated a minimal sample size of 122 patients with early colorectal neoplasia and 30 healthy controls would be required to achieve 0.95 power.

Finally, we used serum samples from 136 patients with early colorectal neoplasia and 52 healthy controls (Table 1) to validate the discriminatory capability of miR-21, miR-29a, and miR-125b. As shown in Fig. 3A, serum levels of miR-21 ($P < 0.0001$), miR-29a ($P < 0.0001$), and miR-125b ($P < 0.0001$) were significantly higher in patients with early colorectal neoplasia than in healthy controls. As shown in Supplementary Fig. S1, increased levels of 3 miRNAs were significant even when we excluded 8 patients with colorectal cancers and compared only patients with noninvasive colorectal neoplasms to healthy subjects. When we divided patients with colorectal neoplasia into either having advanced neoplasias or not (non–advanced neoplasias), expression levels of miR-21 and miR-29a were significantly elevated in patients with advanced neoplasia compared with non–advanced neoplasia patients and healthy controls, whereas miR-125b levels increased in both non–advanced neoplasia and advanced colorectal neoplasia.
neoplasia patients compared with healthy controls (Fig. 3B). Serum levels of miR-21 and miR-29a significantly correlated with the size of the index lesions whereas miR-125b showed no correlation (Fig. 3C).

Next, we generated ROC curves to evaluate the performance of the 3 miRNAs as serum biomarkers for the detection of early colorectal neoplasms. AUC values for serum miR-21, miR-29a, and miR-125b in discriminating patients with early colorectal neoplasia from healthy controls were 0.706 (95% CI, 0.635–0.770), 0.741 (95% CI, 0.673–0.802), and 0.806 (95% CI, 0.742–0.860), respectively. To discriminate patients with advanced neoplasia from individuals without advanced neoplasia (i.e., patients with non–advanced neoplasia colorectal neoplasms plus healthy controls), AUC values for serum miR-21, miR-29a, and miR-125b were 0.708 (95% CI, 0.638–0.772), 0.731 (95% CI, 0.662–0.793), and 0.690 (95% CI, 0.618–0.755), respectively. Combination of 3 miRNAs showed further improvement in AUC = 0.826 (95% CI, 0.765–0.878) for all early colorectal neoplasms and AUC = 0.759 (95% CI, 0.691–0.818) for advanced neoplasia (Fig. 4). The results were almost identical when we excluded 8 patients with colorectal cancer from the analyses (Supplementary Fig. S2).

**Serum miR-21, miR-29a, and miR-125b levels and characteristics of early colorectal neoplasms.** Given that serum miR-125b levels were significantly elevated in non–advanced neoplasia, which consisted of tubular adenomas and serrated polyps less than 10 mm in size, we determined whether the levels of miRNAs were elevated in patients with even smaller colorectal neoplasms. We calculated the total diameters of all colorectal neoplasms detected in an individual and found that 11 of 136 patients had colorectal neoplasms with total diameter of 5 mm or less. Serum expression levels of miR-29a and miR-125b were significantly higher in patients who only had small colorectal neoplasms (total diameter of colorectal neoplasms ≤ 5 mm) than in healthy controls (P = 0.022 and P = 0.001, respectively; Fig. 5A).

We analyzed serum miRNA expression levels according to histologic subtypes of colorectal neoplasms. Compared with healthy controls, patients with tubular adenomas and HGINs showed significantly higher serum levels of all 3 miRNAs, whereas patients with TVAs had significantly elevated levels of serum miR-29a and miR-125b (Fig. 5B).

Finally, we examined the association between serum levels of miR-21, miR-29a, and miR-125b and clinicopathologic factors. As shown in Supplementary Table S2, there were no significant associations between serum levels of the 3 miRNAs and age, gender, the presence of concurrent disorders, location, or morphology of the colorectal neoplasms.

**Discussion**

In this study, we analyzed a cohort of 237 serum samples in independent screening and validation sets and found that the levels of miR-21, miR-29a, and miR125b in serum could discriminate patients with early colorectal neoplasms from healthy controls. To our knowledge, this is the first study to systematically investigate circulating miRNA biomarkers focused on the identification of precancerous colorectal neoplasms—the optimal target lesion for a colorectal cancer screening strategy.
Although genome-wide miRNA profiling approaches such as microarrays have been successfully used for the discovery of novel miRNA biomarkers for the early detection of colorectal neoplasms, the outcomes have been largely inadequate for clinical decision making due to the limited number of studies and small numbers of samples analyzed. In fact, a few recent studies have found multiple miRNAs that could discriminate patients with precancerous adenomas from healthy controls by using microarray; however, none of these studies identified common overlapping miRNA biomarkers, raising concerns about the discovery
and validation methodologies used in these reports (18–20). In the current study, we started with a systematic literature review rather than genome-wide approaches for the selection of candidate miRNAs. This enabled us to choose miRNAs that have been found by independent researchers using different patient cohorts. Because reproducibility is one of the most critical components in the discovery of clinically relevant biomarkers, we believe our strategy has an advantage in finding more generally applicable markers.

The most remarkable findings of our study were that serum miR-21, miR-29a, and miR-125b could discriminate patients with early colorectal neoplasia from healthy controls. By performing a power calculation, we were able to analyze a sufficient number of subjects to reach this conclusion with appropriate statistical power. miR-125b showed the best performance among the 3 miRNAs to discriminate patients with early colorectal neoplasia from healthy subjects. Serum miR-125b levels were elevated in non–advanced adenoma patients (i.e., tubular adenomas and/or serrated polyps smaller than 10 mm) compared with healthy controls, and no significant increase was observed in patients with advanced neoplasia. Surprisingly, elevated levels of miR-125b were seen even in patients whose total diameters of colorectal neoplasms were 5 mm or less. Thus, the increase in serum miR-125b levels most likely reflects an earlier step during colorectal carcinogenesis. In contrast, serum levels of miR-21 and miR-29a were elevated significantly in patients with advanced neoplasia compared with healthy controls and non–advanced neoplasia patients. Considering that the most relevant lesions in colorectal cancer screening would be precancerous lesions that possess higher risks of progressing to cancers, miR-21 and miR-29a might be better biomarkers to detect more risky lesions such as advanced neoplasia.

Precancerous colorectal neoplasms contain various histologic subtypes, including serrated polyps, tubular adenomas, TVAs, and HGINs. Because different types of colorectal neoplasms possess different molecular alterations and possibly different miRNA signatures, it is important to consider the relevance of biomarker miRNAs in each subtype of precancerous lesion. Our data showed that serum miR-21, miR-29a, and miR-125b had similar elevations in patients with tubular adenomas, TVAs, and HGINs, suggesting their potential in detecting these precancerous colorectal neoplasms. Although levels of miR-21, miR-29a, and miR-125b were elevated in a subset of patients with serrated polyps, another subset of precancerous lesion with different molecular characteristics (31), it was difficult to draw conclusions due to the small numbers of subjects with serrated polyps in this study.

In addition to the performance of individual miRNA biomarkers, a biomarker panel created by combining the expression of 3 miRNAs demonstrated enhanced performance in detecting patients with advanced neoplasia with an AUC value of 0.759 and was comparable to the multistage stool testing (AUC = 0.73) reported by Imperiale and colleagues (8). Compared with stool DNA and hemoglobin testing, serum miRNA testing has several potential advantages as a screening method for early colorectal neoplasms. First, our approach is simple and requires only the quantification of the serum levels of 3 miRNAs by real-time RT-PCR, whereas the stool DNA testing requires transporting stool to a centralized laboratory, multiple assays of DNA mutation and methylation, and a hemoglobin immunoassay. Second, serum miRNAs in this study showed no significant difference according to the location of colorectal neoplasms, suggesting their superiority in detecting colorectal neoplasms regardless of their location. By contrast, stool DNA and hemoglobin testing were less sensitive for proximal lesions. Finally, as stool samples are collected by the subjects themselves, quality control may be easier using blood rather than stool. In addition, the use of blood samples may lead to better compliance than expecting patients to properly collect and ship stool.

There are some potential limitations of our study. First, the healthy volunteers in our study were younger than patients with colorectal neoplasia. To minimize the bias caused by the age differences between the 2 groups, we assigned healthy volunteers...
of a similar age range with subjects undertaking colorectal cancer screening in the general population to the validation set. Second, hemolysis is known to affect levels of circulating biomarker miRNAs (32). As we saw some hemolyzed samples in our study subjects, hemolysis might confound our results. Impacts of confounding factors, including hemolysis, should be evaluated in future studies. Third, our study lacked an independent, large validation cohort, which must be considered in future investigations to further appreciate the clinical significance of the findings reported in this study.

In conclusion, we conducted a systematic investigation to identify circulating miRNA biomarkers for colorectal cancer screening and found that serum miR-21, miR-29a, and miR-125b levels could discriminate patients with early colorectal neoplasia from healthy controls. Our data highlight the capability of serum miRNAs to detect precancerous colorectal neoplasms, suggesting the potential clinical use of these molecular signatures for noninvasive screening of patients for colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

The authors thank Dr. Yoshitaka Konda, Dr. Chihiaru Kawanami, Dr. Shuizuru Yazumi, Dr. Shinya Ohashi, Dr. Shigehiko Fujii, Hiromi Yoshida, Yukie Nakai, Mihoko Tsurumaki, Noriko Fujita, Mayumi Mizuguchi, Hoki Matsumoto, and Eriko Ban for their support in patient enrollment and sample collection.

Grant Support

The present work was supported by grant R01 CA72851 and CA 181572 from the National Cancer Institute, NIH, and funds from the Baylor Research Institute to A. Goel and C.R. Boland. This study was also supported by a pilot project grant from the Charles A Sammons Cancer Center, Baylor University Medical Center to A. Goel.

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Received October 28, 2014; revised April 23, 2015; accepted May 9, 2015; published OnlineFirst June 2, 2015.

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