REVIEW

MicroRNA analysis of colorectal cancer using fecal and tissue samples

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Recently, several microRNAs (miRNAs) have been reported as promising biomarkers for cancer detection and tumor recurrence risk. Due to its stability, miRNA can be accessed from samples stored in severe conditions, such as feces or formalin-fixed paraffin-embedded (FFPE) tissue. Fecal miRNA extracted from the residuum of fecal occult blood tests (FOBTs) was assessed to determine whether a combination of this fecal miRNA test (FmiRT) with FOBT could improve the false-negative rate of colorectal cancer (CRC) screening compared with FOBT alone. Expression of miR-106a in patients with both positive and negative FOBTs was significantly higher than in healthy volunteers. To identify a high-risk group for recurrence, miRNAs were extracted from FFPE samples of patients with stage II CRC. Tumor recurrence occurred at a significantly higher rate in patients with increased miR-181c expression than in those with lower expression. The recurrence rate in patients with stage II CRC with higher expression of miR-181c was similar to that of patients with stage III CRC who had been treated by surgical resection alone. As miRNAs are stable in several severe storage conditions, such as in fecal and FFPE samples, they could be valuable, accessible biomarkers for CRC, for use both in cancer screening and as predictors of recurrence.

Keywords: colorectal cancer; microRNA; cancer screening; predictor of recurrence

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Introduction

Colorectal cancer (CRC) is the second highest cause of cancer-related mortality worldwide [1]. Patients with CRC have a much better chance of recovery with early detection and resection at an early stage. Thus, screening is important to reduce mortality. Indeed, the widespread adoption of the fecal occult blood test (FOBT) as a CRC screening test has enabled CRC incidence and mortality rates to decline for 2 decades [2-4]. During the past decade, disease-free survival among patients with advanced stage CRC has improved significantly, due to the introduction of adjuvant chemotherapy [5]. However, this therapeutic success has not been observed in patients with early stage CRC. The lack of simple and reliable relapse risk markers for patients with early stage CRC makes it difficult to identify patients in whom the hazards of adjuvant chemotherapy may be offset by benefits with respect to disease-specific survival [5].

MicroRNAs (miRNAs), which are small (18–25 nt) noncoding RNA molecules, regulate the function of specific mRNAs and play various roles in cancer progression. The function of miRNAs is to downregulate the expression of multiple target genes by degrading their corresponding mRNA, blocking gene expression via RNA interference [6, 7]. As of June 2014, a total of 2661 human mature miRNAs had been bioinformatically reported in miRBase 21 [8]. The high stability of miRNAs means that they can be preserved in poor conditions, such as fecal samples [9] and formalin-fixed paraffin-embedded (FFPE) sections stored for decades [10].

Several studies have suggested that miRNAs could be useful for the diagnosis of CRC using RNA extracted from feces [11-13], and could represent potential biomarkers for CRC recurrence in tissue samples [14, 15]. In addition, recent studies have demonstrated that several miRNAs play important roles in tumor invasion and metastasis [16, 17]. Thus, miRNAs, which can be readily analyzed from samples in severe storage conditions, are worthy of investigation as tumor biomarkers for CRC screening and recurrence.

Fecal miRNA test to detect false negative FOBT results

Several large-scale studies have demonstrated the low sensitivity of the FOBT, which can cause false-negative results in some patients with CRC, and false-positives in some healthy individuals [18-21]. Several attempts to use DNA- [22, 23] or RNA-based molecular biological methods [24-26] for the early detection of CRC have been reported. However, these methods have not proved superior to the FOBT in terms of sensitivity and specificity. However, several studies have suggested that miRNA directly extracted from feces could be useful for the diagnosis of CRC [11-13].

In our previous study, miRNA extracted from the residuum of FOBTs was assessed in a fecal miRNA test (FmiRT) to determine whether combining the FmiRT and the FOBT could improve the rate of false-negatives in CRC screening compared with the FOBT alone [27]. Fecal miRNA of sufficient quality for real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was extracted from the residuum of FOBTs stored at 4°C for up to 5 days [28], and significant positive correlations were observed between miRNAs extracted from feces and those extracted from the residuum of FOBT [27]. Chang *et al*. reported that several miRNAs showed high expressional correlations between tissue, plasma, and stool [29]. This indicates that such miRNAs might be clinically applicable for CRC detection.

Significant differences in the relative expression levels of 9 selected miRNAs were observed between patients with CRC and healthy volunteers ($P < 0.05$). In particular, miR-106a expression was significantly higher in both positive and negative FOBT patients than in healthy volunteers ($P = 0.001$). The sensitivity and specificity of the FmiRT using miR-106a expression were 34.2% and 97.2%, and those of the FOBT were 60.7% and 98.1%, respectively. The overall sensitivity and specificity of the new combined screening method were 70.9% and 96.3%, respectively. One quarter of patients with CRC with false-negative FOBTs were identified as true positives by the addition of the FmiRT using fecal miR-106a [27].

Those findings suggest that miR-106a is a promising molecular marker to identify patients with CRC with false-negative FOBT results. Combined FmiRT/FOBT screening might improve the sensitivity of CRC detection.

Using miRNA as a predictive marker of recurrence in stage II CRC

The standard treatment for stages I–III CRC is surgical resection. The effectiveness of adjuvant chemotherapy for patients with stage III CRC has been demonstrated [30, 31]. However, the effectiveness of adjuvant chemotherapy for patients with stage II CRC has not been established to date. However, the recurrence rates of patients with stage II CRC who undergo surgery alone are 12%–37% [32-36]. According to the guidelines of the American Society of Clinical Oncology [37] and the European Society for Medical Oncology [38], high-risk patients with stage II CRC are defined by clinicopathological factors. However, there are no robust biomarkers suitable for predicting recurrence in patients with stage II CRC. To identify patients with stage II CRC who would benefit from adjuvant chemotherapy, the identification of adequate biomarkers would be an important advancement in clinical settings.

To identify biomarkers to predict the recurrence of stage II CRC, miRNAs extracted from the tissue samples of patients with stage II CRC who had undergone surgical resection were assessed [39]. Initially, miRNAs that were expressed at higher levels in cancer tissues compared to normal tissues were identified using a highly sensitive miRNA microarray. Of 1719 miRNAs, 105 showed significantly greater expression in cancer tissues than in normal tissues ($P < 0.01$). Among the 105 miRNAs, 15 showed significantly higher expression in patients with recurrence than in those without recurrence ($P < 0.05$). The expression of the 15 candidate miRNAs was analyzed. In a univariate analysis, tumor recurrence occurred at significantly higher rates in patients

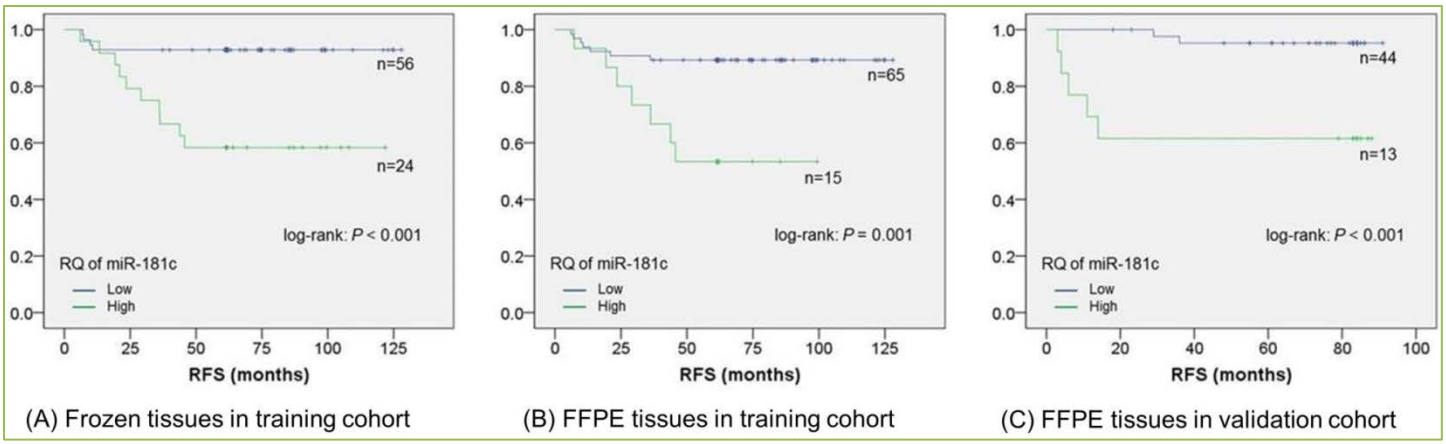

Figure 1. Relapse-free survival of patients with stage II CRC. A) Kaplan–Meier method for the two groups in the training cohort using frozen tissue that showed both higher and lower miR-181c expressions. RFS was significantly worse for patients with higher miR-181c expressions than for those with lower miR-181c expressions. B) Kaplan–Meier method for the two groups in the training cohort using FFPE tissue that showed both higher and lower miR-181c expressions. RFS was significantly worse for patients with higher miR-181c expressions than for those with lower miR-181c expressions. C) Kaplan–Meier method for the two groups in the validation cohort that showed both high and low miR-181c expressions. RFS was significantly worse for patients with higher miR-181c expressions than for those with lower miR-181c expressions. The differences were analyzed by log-rank test. $P < 0.05$ denotes a statistically significant difference. Reprinted with permission [39].

with increased expression of let-7a, -7d, -7e, miR-23c, -26b, -128a, -151-5p, and -181c; a tumor depth of T4; and mucinous carcinoma histological type (P < 0.05). In a multivariate analysis including pathological factors, increased expression of miR-181c was an independent predictive factor of recurrence [odds ratio (OR): 9.43, 95% confidence interval (CI): 2.57–34.48, P = 0.001], and also an independent predictive factor of worse relapse-free survival (RFS) [hazard ratio (HR): 6.62, 95% CI: 2.08–21.28, P = 0.001].

However, it is difficult to obtain frozen tissues and to store them long term. Fortunately, miRNAs are preserved in poor conditions, even detectable in FFPE sections stored for 20 years [10]. Thus, FFPE tissues were analyzed in comparison with their corresponding frozen tissues in the same cohort. In a univariate analysis, tumor recurrence occurred at significantly higher rates in patients with increased expression of let-7d, -7e, miR-26b, -128a, -148b, -151-5p, and -181c; a tumor depth of T4; and mucinous carcinoma histological type (P < 0.05). Interestingly, the increased expression of miR-181c was also an independent predictive factor of recurrence [OR: 7.46, 95% CI: 1.97–28.57, P = 0.003], and an independent predictive factor of worse RFS [HR: 4.74, 95% CI: 1.66–13.51, P = 0.001] in a multivariate analysis.

Since the miRNA expression analysis showed similar results between frozen and FFPE tissues in the cohort, another cohort with patients with stage II CRC who had undergone surgical resections were enrolled to validate the analysis using frozen tissues and FFPE tissues. The expression of miR-181c extracted from FFPE cancer tissues was analyzed. In the validation cohort, tumor recurrence occurred at a significantly higher rate in patients with higher miR-181c expression than in those with lower expression (P = 0.001). Moreover, the RFS was significantly worse for patients with higher miR-181c expression than for those with lower miR-181c expression (P < 0.001).

In a combined analysis using FFPE tissues from the two cohorts, the recurrence rates were 42.9% and 8.3% with higher and lower expression of miR-181c, respectively **(Figure 1)**. The recurrence rate in patients with stage II CRC with higher expression of miR-181c was similar to that of patients with stage III CRC who had been treated by surgical resection alone [40, 41]. In our study, the expression of miR-181c in patients with stage II CRC without recurrence was not significantly different from that in stage III patients with CRC without recurrence (P = 0.297). Moreover, miR-181c expression in patients with stage II CRC with recurrence was similar to that in stage III patients with recurrence ($P = 0.825$) [39]. Furthermore, in patients with lower miR-181c expression, the recurrence rate of patients with stage II CRC was similar to or lower than that of patients with stage I CRC [42].

These findings suggest that patients with stage II CRC with higher miR-181c expression could be candidates for adjuvant chemotherapy, and that higher expression of miR-181c may be a useful recurrence predictor for CRC.

Conclusions

Taken together, the evidence suggests that miRNAs, which are stable in several severe storage conditions, could be useful biomarkers for CRC, both in initial cancer screening and in recurrence prediction.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Author contributions

Study concepts and design: Nobuyoshi Yamazaki, Yoshikatsu Koga; Analysis and interpretation of data: Nobuyoshi Yamazaki, Yoshikatsu Koga; Drafting of the manuscript: Nobuyoshi Yamazaki, Yoshikatsu Koga, Yasuhiro Matsumura

Abbreviations

CRC: colorectal cancer; FFPE: formalin-fixed, paraffin-embedded; FmiRT: fecal microRNA test; FOBT: fecal occult blood test; HR: hazard ratio; miRNAs: microRNAs; OS: odds ratio; RFS: relapse-free survival; RT-PCR: real-time reverse transcription-polymerase chain reaction.

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