

Serum miR-21 as a Diagnostic and Prognostic Biomarker in Colorectal Cancer

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Background The oncogenic microRNAs (miRNAs) *miR-21* and *miR-31* negatively regulate tumor-suppressor genes. Their potential as serum biomarkers has not been determined in human colorectal cancer (CRC).

Methods To determine whether *miR-21* and *miR-31* are secretory miRNAs, we screened expression in medium from 2 CRC cell lines, which was followed by serum analysis from 12 CRC patients and 12 control subjects. We validated expression of candidate miRNAs in serum samples from an independent cohort of 186 CRC patients, 60 postoperative patients, 43 advanced adenoma patients, and 53 control subjects. We analyzed *miR-21* expression in 166 matched primary CRC tissues to determine whether serum miRNAs reflect expression in CRC. Patient survival analyses were performed by Kaplan–Meier analyses and Cox regression models. All statistical tests were two-sided.

Results Although *miR-21* was secreted from CRC cell lines and upregulated in serum of CRC patients, no statistically significant differences were observed in serum *miR-31* expression between CRC patients and control subjects. In the validation cohort, *miR-21* levels were statistically significantly elevated in preoperative serum from patients with adenomas ($P < .001$) and CRCs ($P < .001$). Importantly, *miR-21* expression dropped in postoperative serum from patients who underwent curative surgery ($P < .001$). Serum *miR-21* levels robustly distinguished adenoma (area under the curve [AUC] = 0.813; 95% confidence interval [CI] = 0.691 to 0.910) and CRC (AUC = 0.919; 95% CI = 0.867 to 0.958) patients from control subjects. High *miR-21* expression in serum and tissue was statistically significantly associated with tumor size, distant metastasis, and poor survival. Moreover, serum *miR-21* was an independent prognostic marker for CRC (hazard ratio = 4.12; 95% CI = 1.10 to 15.4; $P = .03$).

Conclusions Serum *miR-21* is a promising biomarker for the early detection and prognosis of CRC.

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Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide. In the United States, CRC is the third most common cancer, with more than 143 000 new cases and more than 52 000 deaths each year (1). Several CRC screening tests, including fecal occult-blood testing and colonoscopy, have been available for years (2) and have aided in reducing the mortality associated with this disease (3–5). However, compliance with these screening tests has been far from adequate. Patients with metastatic disease frequently receive expensive cytotoxic chemotherapeutic regimens coupled with targeted monoclonal antibodies but with relatively modest benefits (6). Without a priori knowledge of which patients will experience tumor recurrence, there is inevitable overtreatment with agents associated with toxic side effects (7). These limitations underscore the need for novel biomarkers, particularly noninvasive biomarkers in serum or plasma, for diagnosis, prognosis, and prediction of response to chemotherapy.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that play a central role in the regulation of mRNA expression (8). The discovery that miRNA expression is frequently dysregulated in a cancer-specific manner provides an opportunity to develop these RNAs as biomarkers for cancer detection (9). Although most previous studies on miRNA expression have been performed on tissue specimens, some studies have shown diagnostic and prognostic potential for circulating miRNAs (10–14) because tumor-derived miRNAs can be present in blood and appear to be stably protected from endogenous ribonuclease activity in the circulation (15). Nonetheless, it is unclear whether expression profiles of circulating miRNAs reflect miRNA profiles of tumor tissues and to the best of our knowledge, no systematic investigation of the relationship between miRNA profiles in body fluids vs matched primary CRCs has thus far been undertaken. This is critical because increased expression of circulating miRNAs could be indicative of miRNAs secreted from a tumor, raising the overall diagnostic specificity of the biomarker.

MiR-21 is an oncogenic miRNA that modulates the expression of multiple cancer-related target genes such as *PTEN*, *TPM1*, and *PDCD* and has been shown to be overexpressed in various human tumors (16–18). In addition, *miR-21* expression is upregulated in CRC tissues, is elevated during tumor progression, and is also associated with poor survival and response to chemotherapy (19–22). The clinical significance of circulating *miR-21* levels in CRC remains unclear at this time. Although an earlier study was unable to use plasma *miR-21* as a biomarker because of low levels of detection using a direct amplification method (10), a more recent study demonstrated statistically significantly elevated plasma *miR-21* expression in CRC patients using TaqMan-based approaches (23). On the other hand, *miR-31* is another miRNA frequently overexpressed in CRC tissues and has been shown to be associated with tumor prognosis (19,24). Additionally, both *miR-21* and *miR-31* are frequently upregulated, even in premalignant lesions such as colonic adenomas, which are the target lesions of CRC screening (25–27). In light of these observations, we hypothesized that these two miRNAs might be good candidates for exploration as circulating biomarkers for the early detection and prognosis of CRC, assuming that the expression pattern for these miRNAs in serum mirrors that in the neoplastic tissues.

We have systematically investigated the expression of *miR-21* and *miR-31* in a two-phase study. In the first phase, we determined whether cultured CRC cells secrete these miRNAs into the culture medium, establishing their secretory potential. We then performed quantitative analyses of these miRNAs in a subset of serum samples from CRC patients and healthy control subjects to determine the feasibility of their detection in the circulation. In the second phase, using a large validation cohort comprised of matched serum and tissue samples from patients with colorectal neoplasia and serum from healthy control subjects, we evaluated the clinical significance of these miRNAs as potential biomarkers for diagnosis and prognosis of CRC patients.

Methods

Study Design

This study included analysis of 568 serum and tissue specimens that were obtained from healthy volunteers and consecutively enrolled patients with colorectal adenomas and cancers at the Mie University Medical Hospital, Japan, between January 1, 2005 and December 31, 2010. This study was designed as an initial screening phase and a subsequent validation phase. In the screening phase, oncogenic *miR-21* and *miR-31* were selected (16–18,24,28), and their expression was measured using TaqMan-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) using cell culture medium and matched serum and tissue samples. To determine the secretory potential of these miRNAs, two CRC cell lines, HCT116 and SW620, were cultured and a fraction of the culture medium was collected at 0, 24, and 48 hours after the initial seeding of cells in 10-cm dishes. In addition, a small set of preoperative serum samples were collected from 12 CRC patients and from 12 sex- and age-matched healthy subjects as control subjects. To further assess the specificity of miRNA expression in serum, CRC and adjacent normal tissues were analyzed from eight of the 12 CRC patients, from whom both matched normal and neoplastic tissues were available.

In the validation phase, changes in miRNA expression patterns in serum and tissues from CRC patients were validated in a large,

independent cohort of patients where preoperative sera (n = 186) and matched surgical tissues (n = 166) were collected from a pool of 200 CRC patients. Additionally, postoperative sera (day 7 postoperation) were collected from an independent set of 60 patients from whom matching preoperative sera were available to determine whether miRNA expression was altered subsequent to tumor resection. To better appreciate the diagnostic utility of these miRNAs, sera from 43 patients with advanced adenomas and 53 healthy control subjects were also collected. The serum specimens from healthy subjects were sex- and age-matched and each volunteer had a negative colonoscopic examination and no prior diagnosis of any other malignancy. The Tumor Node Metastasis (TNM) staging system from the American Joint Committee on Cancer was used for classification of pathological tumor staging of CRC (29). All CRC patients who underwent surgery were followed up for tumor recurrence at regular intervals for up to 5 years. During each annual hospital visit, all patients underwent a chest x-ray, colonoscopy, and abdominal computerized tomography. Survival time was calculated from the date of diagnosis to the date of death or last date of follow-up. Patients treated with radiotherapy or chemotherapy before surgery were excluded from the study. Patients with stage III/IV disease received 5-fluorouracil-based chemotherapy, whereas no adjuvant therapy was given to stage I/II CRC patients.

Ethics Statement

Both serum- and tissue-based specimen collection and studies were approved by the institutional review boards of Mie University Hospital, Japan, and Baylor University Medical Center, Dallas, Texas. All participants provided written consent and indicated willingness to donate their blood and tissue samples for research.

RNA isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction

MiRNA extraction from serum and culture media samples was performed with miRNeasy RNA isolation Kits (Qiagen, Valencia, CA), whereas miRNA extraction from Formalin-fixed and paraffin-embedded (FFPE) samples was performed using RecoverAll Total Nucleic Acid Isolation Kits (Ambion Inc, Austin, TX). TaqMan miRNA qRT-PCR (Applied Biosystems, Foster City, CA) were used to detect and quantify miRNA expression using the $2^{-\Delta\Delta Ct}$ method (for details, refer to [Supplementary Methods](#), available online).

Statistical Analyses

Results were expressed as mean \pm standard deviation. Mann–Whitney *U* and Kruskal–Wallis analyses of variance were used to evaluate statistical differences in serum or tissue miRNA expression between unpaired groups and multiple comparison groups, respectively. The Wilcoxon test was used to compare *miR-21* expression in paired serum samples obtained before surgical tumor resection and 7 days after surgical tumor resection. The Spearman correlation test was used to examine correlation between miRNA expression in serum and matched CRC tissues. Receiver operating characteristic (ROC) analysis was performed to determine the diagnostic performance of *miR-21* expression levels in distinguishing patients with colorectal adenomas or cancers from the healthy control subjects. Sensitivity against 100% minus specificity was plotted at each cutoff threshold, and the area under the curve (AUC) values that reflect the

probability of correctly identifying adenoma or CRC patients from control subjects were computed. The optimal cutoff thresholds for diagnosis were obtained by Youden index (30). In brief, the optimal cutoff threshold values were determined at the point on the ROC curve at which Youden's index (sensitivity + [100% – specificity]) was maximal. By using these optimal cutoff values, sensitivity, specificity, and positive and negative predictive values were calculated.

To validate the accuracy estimates of ROC curves and optimal cutoff threshold values and to adjust for optimism bias in discriminating CRC or adenoma patients from control subjects, the bootstrap bias correction and accelerated (BCa) bootstrap methods were performed (31). In general, there are no standard recommended methods for adjusting bias. However, we selected the BCa bootstrap method because it adjusts for both bias and skewness in the bootstrap distribution of data. For this analysis, we randomly included data from the original serum samples, followed by sensitivity and specificity determination for various cutoff thresholds. This process was repeated 1000 times, and resultant mean values (95% confidence interval [CI]) for sensitivity and specificity were computed. In addition, ROC convex hull (ROCCH) curves were generated using approaches that allowed the hull segment to be viewed as being generated by a tie between the plots of sensitivity and 100% minus specificity for various cutoffs from the original data and adjusted data by BCa bootstrap methods. Thereafter, the AUCs of ROCCH curves were calculated by Trapezoidal rule. Finally, a two-sided z test was used to compare the AUCs of two ROC curves (32). A multivariable logistic regression model was used to calculate odds ratios (ORs) for age- and sex-adjusted cases associated with CRC or adenoma according to serum miRNA levels.

We estimated that 154 patients were needed to achieve 80% power to substantiate more than 20% differences in prognostic outcome, a number that was much smaller than our cohort of 200 CRC patients. Survival analyses were performed using the Kaplan–Meier method, and the differences in survival were examined using log-rank tests. ROC curves were established to discriminate the patients with or without death, and the Youden index (30) was used to determine the optimal cutoff threshold of serum or tissue miRNAs levels to predict the overall survival. Cox's proportional hazard regression analyses were used to estimate hazard ratios (HRs) of death according to serum and tumor miRNA levels, unadjusted and adjusted for potential confounding factors for death, including age, sex, pathological differentiation, T stage, N stage, M stage, and serum carcinoembryonic antigen (CEA) levels. Assumptions of proportionality were confirmed for the Cox proportional hazards analyses by generating Kaplan–Meier survival curves (eg, high- vs low-expression groups) and by ensuring that the two curves did not intersect each other. All P values are two-sided; P less than or equal to .05 was considered statistically significant. All statistical analyses were carried out using Medcalc version 12.3.0 (Broekstraat 52, 9030; Mariakerke, Belgium).

Results

MiR-21 Expression in CRC Cell Culture Medium

In this initial experiment, we determined whether *miR-21* and *miR-31* act as secretory miRNAs and are excreted into the culture media by HCT116 and SW620 CRC cell lines. We observed that *miR-21* expression in the culture media from both cell lines increased with

time (24 and 48 hours; $P < .05$) and with increasing numbers of tumor cells ($P < .05$) (Figure 1A, HCT116; Figure 1B, SW620). However, *miR-31* expression levels did not reveal statistically significant changes in either cell line (Figure 1C, HCT116; Figure 1D, SW620), suggesting that *miR-21* but not *miR-31* is a secretory miRNA.

Tissue and Serum miR-21 Expression During Screening Phase

In the screening phase of the study, *miR-21* and *miR-31* expression were determined in a small set of eight CRCs and the adjacent normal mucosa. Both *miR-21* and *miR-31* levels were statistically significantly elevated in CRC tissues compared with adjacent normal control tissues (*miR-21*: $P < .01$; *miR-31*: $P < .05$) (Figures 1E and 1F). We next examined the feasibility of detecting the expression of circulating *miR-21* and *miR-31* in 24 serum samples from CRC patients ($n = 12$) and healthy control subjects ($n = 12$). We noted that *miR-21* levels were statistically significantly elevated in the sera of CRC patients ($P < .001$) (Figure 1G), whereas no statistically significant differences were noted in serum *miR-31* expression between CRC patients and control subjects ($P > .05$) (Figure 1H). Based on our result that only *miR-21* acts a secretory miRNA, we focused the rest of our study on *miR-21* for further assessment of its efficacy as a diagnostic and prognostic biomarker in patients with colorectal neoplasia.

Tissue and Serum miR-21 Expression During the Validation Phase

Patient Characteristics. The clinicopathological and other patient characteristics are summarized in Supplementary Table 1 (available online). There were no statistically significant differences observed in age between healthy control subjects (mean = 64 years; standard deviation [SD] = 12.9 years) and patients with adenomas (mean = 66 years; SD = 9.8 years) or CRCs (mean = 67 years; SD = 7.5 years; $P > .05$). Likewise, there were no sex differences between different groups, with 27 men and 26 women in the control group, 30 men and 13 women in the adenoma group, and 106 men and 80 women in the CRC group ($P > .05$). The median follow-up time period for CRC patients was 44 months (range = 2–84 months).

Serum miR-21 Expression in Patients With Colorectal Adenomas and Cancers.

To evaluate the diagnostic potential of *miR-21*, a total of 282 serum samples, including those from patients with CRC ($n = 186$), patients with adenomatous polyps ($n = 43$), and normal control subjects ($n = 53$) were examined. In comparison with healthy control subjects, the expression levels of serum *miR-21* demonstrated a stepwise increase in patients with adenomatous polyps ($P < .001$) (Figure 2A) and CRC ($P < .001$) (Figure 2A). Furthermore, when all CRC patients were segregated based upon TNM stage, the gradual increase in serum *miR-21* levels was clearly discernible, with statistically significantly higher expression levels in stage IV patients compared with stage I or II patients (both $P < .05$) (Figure 2A).

Next, we generated ROC curves to assess the potential usefulness of serum *miR-21* as a noninvasive biomarker for the early diagnosis of colorectal neoplasia. Our ROC analyses revealed that serum *miR-21* levels were robust in discriminating patients with CRC from control subjects, with an AUC value of 0.927 (95% CI = 0.886 to 0.956) (Figure 2B). Using a cutoff value of 0.0019,

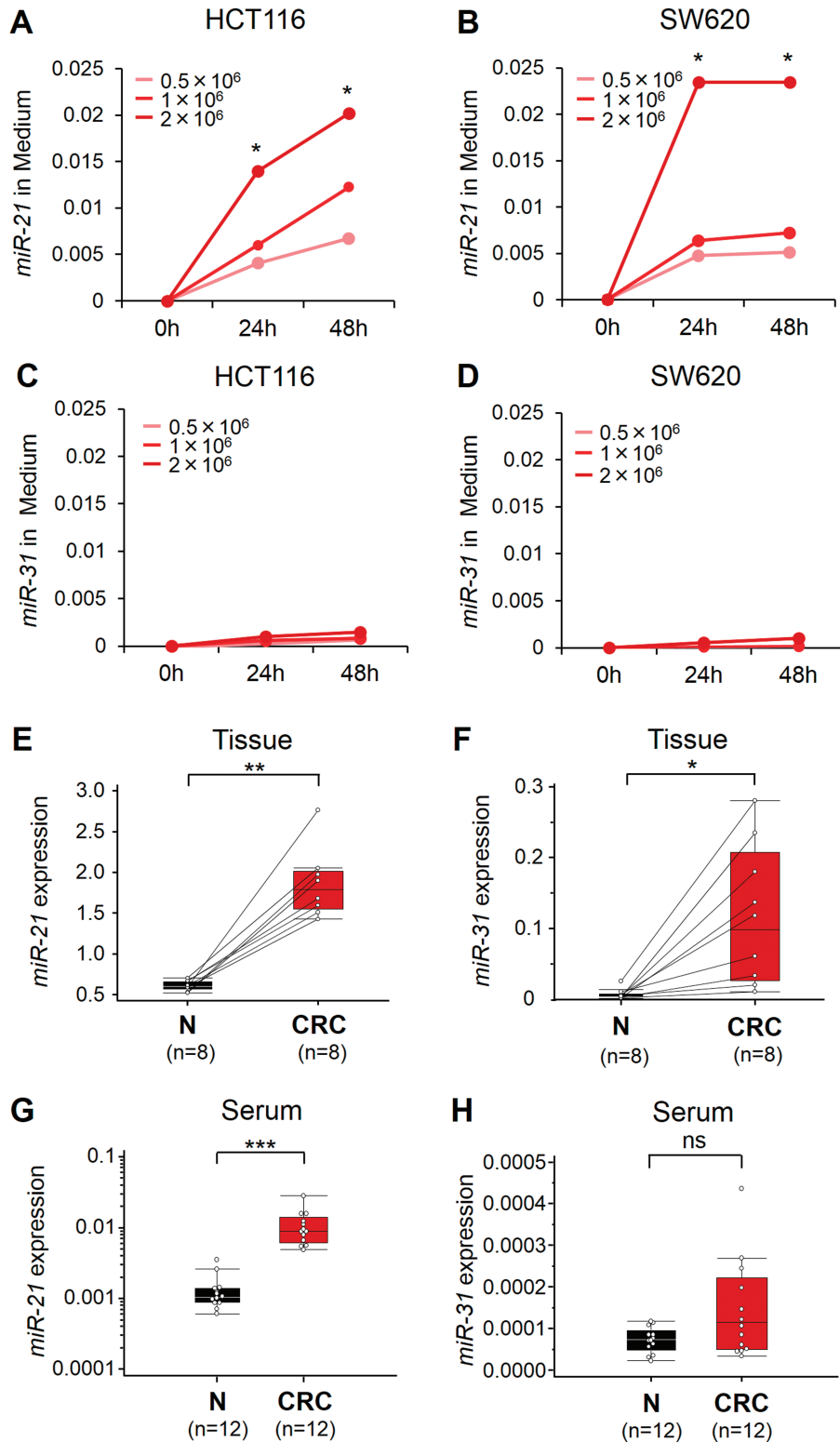


Figure 1. Expression of *miR-21* and *miR-31* in culture media of colorectal cancer (CRC) cell lines (HCT116 and SW620). *miR-21* levels in media of both HCT116 **A**) and SW620 **B**) increased with increased cell counts (0.5 – 2×10^6 cells/well) and longer incubation intervals, whereas *miR-31* levels did not change in either cell line **C**, **D**). The y-axis represents relative expression of *miR-21* and *miR-31* normalized to *cel-miR-39*. Initial screening for *miR-21* and *miR-31* expression in the screening phase, using a small subset of tissue and serum specimens from CRC patients, was done. Box plots are shown for *miR-21* expression **E**) and *miR-31* expression **F**) levels in primary

tumor tissues (CRC) and adjacent normal mucosa (N) from eight CRC patients. Box plots are shown for serum levels of *miR-21* **G**) and *miR-31* **H**) in mucosa from normal control subjects (N; $n = 12$) and CRC patients ($n = 12$). Boxes represent interquartile range, and the horizontal line across each box indicates median value. The y-axis represents relative expression of *miR-21* and *miR-31*, and data were normalized to *cel-miR-39* and *miR-16* expression in sera and tissue, respectively. Statistical analysis was performed using two-sided Wilcoxon and Mann–Whitney *U* tests. * $P < .05$; ** $P < .01$; *** $P < .001$; ns, not statistically significant.

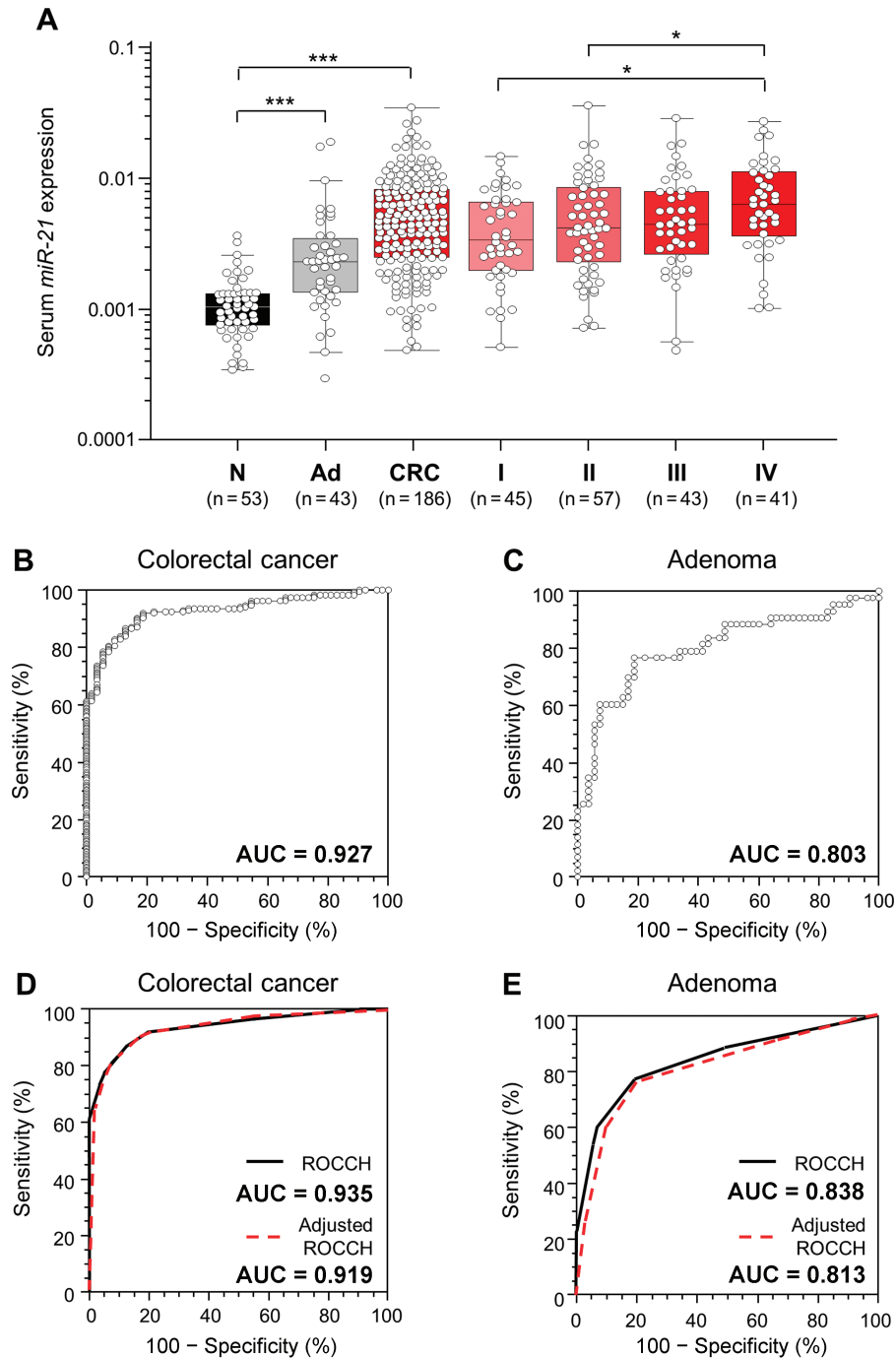


Figure 2. *MiR-21* expression levels in serum samples (n = 282). **A** Box plots represent serum *miR-21* levels in healthy control subjects (N; n = 53) and patients with adenomatous polyps (Ad; n = 43) and different Tumor Node Metastasis (TNM) stages (I, II, III, and IV) of colorectal cancer (CRC) (n = 186). The y-axis (log₁₀ scale) represents relative expression of *miR-21* normalized to *cel-miR-39*. **Boxes** represent the interquartile range, and the **horizontal line** across each box indicates median values. Statistically significant differences were determined using the Mann-Whitney *U* test and Kruskal-Wallis tests. **P* < .05; ****P* < .001. Receiver operating characteristics (ROC) curve analysis using serum *miR-21* for distinguishing patients with colorectal neoplasms from normal control subjects was performed. **B** Serum *miR-21* yielded an area under the curve (AUC) value of 0.927 (95% confidence interval [CI] = 0.89 to 0.96), with 82.8% sensitivity and 90.6% specificity in distinguishing CRC from

normal control subjects. **C** Serum *miR-21* yielded AUC values of 0.803 (95% CI = 0.71 to 0.88) with 76.8% sensitivity and 81.1% specificity in discriminating adenomas from normal control subjects. ROC convex hull (ROCCH) curve analysis using raw data and bootstrap bias correction and accelerated (BCa) bootstrap bias-corrected data for distinguishing patients with colorectal neoplasms from normal control subjects was performed. **D** AUC values derived from ROCCH curves of original and BCa bootstrap-corrected samples were 0.935 (95% CI = 0.812 to 0.982) and 0.919 (95% CI = 0.867 to 0.958), respectively, in distinguishing CRC patients from normal control subjects. **E** AUC values derived from ROCCH curves of original and BCa bootstrap-corrected samples were 0.838 (95% CI = 0.619 to 0.964) and 0.813 (95% CI = 0.691 to 0.910), respectively, in distinguishing adenoma patients from normal control subjects.

the sensitivity, specificity, and positive and negative predictive values were 82.8%, 90.6%, 96.3%, and 60.8%, respectively, to identify a patient with CRC (Supplementary Table 2, available online). Even more important from a screening perspective, serum *miR-21* levels could reliably differentiate patients with advanced adenomatous polyps from healthy control subjects, as evidenced by an AUC value of 0.803 (95% CI = 0.669 to 0.869) (Figure 2C). With a cutoff value of 0.0013, the sensitivity, specificity, and positive and negative predictive values were 76.8% and 81.1%, 76.7%, and 81.1%, respectively (Supplementary Table 2, available online).

To validate the accuracy estimates of ROC curves and optimal cutoff values for discriminating patients with colorectal adenoma or cancer from healthy control subjects, we performed an internal validation by BCa bootstrap methods. The results obtained with the original and the BCa bootstrap samples were in good agreement (Supplementary Tables 2 and 3, available online). With serum *miR-21* at 0.0013 (95% CI = 0.0009 to 0.00134), the sensitivity and specificity were 91.9% and 81.1%, respectively, to identify a patient with CRC, and the sensitivity and specificity were 81.13% and 76.74%, respectively, for a patient with colorectal adenoma using a cutoff value of 0.0013 (95% CI = 0.0010 to 0.00134) (Supplementary Table 3, available online).

ROCCH curves with BCa bootstrap bias correction data in both CRC patients vs control subjects and adenoma patients vs control subjects were very similar to those without bias correction (Figure 2, D and E). In addition, AUC values obtained from ROCCH analysis from the original and bootstrap bias-corrected samples for identifying a patient with CRC were non-statistically significant (original AUC = 0.935, 95% CI = 0.812 to 0.982; BCa corrected AUC = 0.919, 95% CI = 0.867 to 0.958; $P = .80$) (Supplementary Table 4, available online). In a similar manner, no statistically significant differences were observed in ROCCH-derived AUC values for discriminating between two samples with colorectal adenomas (original AUC = 0.838, 95% CI = 0.619 to 0.964; BCa bootstrap AUC = 0.813, 95% CI = 0.691 to 0.910; $P = .84$) (Supplementary Table 4, available online).

In addition, multivariable logistic regression analyses revealed that serum *miR-21* could be used as a potential diagnostic

biomarker for the identification of patients with CRC or adenomas after adjustment for patients' age and sex ($P < .001$ for both) (Table 1). The odds ratio for patients with serum *miR-21* was 43.3 (95% CI = 17.53 to 107.13), and the odds ratio for patients with adenomas was 6.62 (95% CI = 2.63 to 16.88) (Table 1).

Correlation Between Serum and Tissue *miR-21* Expression in CRC Patients.

Next, we asked whether *miR-21* expression in matching tumor tissues and serum samples correlated with any of the other clinicopathological data. Table 2 illustrates that *miR-21* levels in both tumor tissues and matched serum were statistically significantly higher in CRC patients with larger tumor size ($P = .01$ and $P = .004$, respectively) and those with distant metastases ($P = .02$ and $P = .01$, respectively). Tissue levels of *miR-21* expression associated with CRC clinical stage (stage I vs IV: $P < .05$; stage II vs IV: $P < .05$) (Figure 3A). However, *miR-21* levels in both tumor tissues and matched serum samples did not correlate with specific tumor location within the colorectum.

To further enhance the specificity of our assay and validate that circulating *miR-21* expression accurately reflects concentrations found in CRC tissues, we determined the relationship between *miR-21* levels in primary CRC tissues and matched serum from individual CRC patients. Interestingly, we observed a statistically significantly positive correlation between *miR-21* expression in primary CRC lesions and matched serum samples from these patients ($\rho = 0.315$; 95% CI = 0.17 to 0.45; $P < .001$; Spearman's correlation analysis) (Figure 3B).

Thereafter, we analyzed paired pre- and postoperative serum samples in a subset of 60 CRC patients who underwent surgical resection of their tumors. In the 60 CRC patients, 45 underwent potentially curative resection, whereas 15 had multiple hepatic metastases and underwent primary resection to prevent bleeding and bowel obstruction (noncurative resection). It was interesting to note that serum levels of *miR-21* statistically significantly plummeted after surgery in the same subset of patients ($P < .001$) (Figure 4A). Furthermore, when data were analyzed based on potentially curative vs noncurative surgeries, postoperative reductions in serum *miR-21* levels occurred exclusively

Table 1. Multivariable logistic analyses for serum *miR-21* levels and various diagnostic factors in patients with colorectal cancer (CRC) and adenomas*

Variables	OR (95% CI)	P
CRC patients vs control subjects		
Age, >67 y vs ≤67 y†	1.69 (0.73 to 3.92)	.22
Sex, female vs male	1.81 (0.78 to 4.22)	.17
<i>miR-21</i> in serum, >0.0019 vs ≤0.0019‡	43.3 (17.53 to 107.13)	<.001
Adenoma patients vs control subjects		
Age, >67 y vs ≤67 y†	0.54 (0.21 to 1.39)	.20
Sex, female vs male	1.32 (0.53 to 3.34)	.55
<i>miR-21</i> in serum, >0.0013 vs ≤0.0013‡	6.62 (2.63 to 16.88)	<.001

* CI = confidence interval; OR = odds ratio.

† Median age was 67 years.

‡ The cutoff values of serum *miR-21* in CRC patients vs control subjects and adenoma patients vs control subjects were derived by receive operating characteristic curves with Youden's index.

Table 2. Clinical significance of *miR-21* expression in serum and tissue specimens from colorectal cancer patients*

Variables	Serum <i>miR-21</i> expression, mean ± SD (n = 186)	P	Tissue <i>miR-21</i> expression, mean ± SD (n = 166)	P
Age, y†				
≤67	0.0062 ± 0.0047 (n = 88)	.22	3.16 ± 1.46 (n = 80)	.07
>67	0.0059 ± 0.0052 (n = 98)		3.64 ± 1.73 (n = 86)	
Sex				
Male	0.0059 ± 0.0053 (n = 106)	.30	3.23 ± 1.46 (n = 100)	.36
Female	0.0062 ± 0.0044 (n = 80)		3.52 ± 1.72 (n = 66)	
Tumor location				
Right side	0.0073 ± 0.0065 (n = 59)	.16	3.38 ± 1.45 (n = 56)	.70
Left side	0.0058 ± 0.0048 (n = 127)		3.33 ± 1.57 (n = 110)	
Tumor type				
Colon cancer	0.0063 ± 0.0055 (n = 110)	.89	3.50 ± 1.51 (n = 101)	.07
Rectal cancer	0.0062 ± 0.0054 (n = 76)		3.11 ± 1.52 (n = 65)	
Tumor size, cm‡				
≤5	0.0048 ± 0.0035 (n = 91)	.004	3.13 ± 1.47 (n = 78)	.01
>5	0.0070 ± 0.0056 (n = 95)		3.67 ± 1.71 (n = 88)	
Serosal invasion				
Negative	0.0051 ± 0.0041 (n = 58)	.08	2.82 ± 1.37 (n = 47)	.004
Positive	0.0064 ± 0.0052 (n = 128)		3.64 ± 1.66 (n = 119)	
Lymphatic invasion				
Negative	0.0058 ± 0.0041 (n = 46)	.83	3.04 ± 1.52 (n = 39)	.02
Positive	0.0061 ± 0.0051 (n = 140)		3.53 ± 1.64 (n = 127)	
Venous invasion				
Negative	0.0058 ± 0.0045 (n = 108)	.53	3.36 ± 1.53 (n = 91)	.60
Positive	0.0064 ± 0.0054 (n = 78)		3.47 ± 1.73 (n = 75)	
Lymph node metastasis				
Negative	0.0055 ± 0.0042 (n = 106)	.12	3.24 ± 1.48 (n = 92)	.11
Positive	0.0068 ± 0.0057 (n = 80)		3.62 ± 1.76 (n = 74)	
Distant metastasis				
Negative	0.0055 ± 0.0045 (n = 145)	.01	3.21 ± 1.37 (n = 131)	.02
Positive	0.0078 ± 0.0060 (n = 41)		4.18 ± 2.19 (n = 35)	

* SD = standard deviation.

† The median age was 67 years.

‡ The median tumor size was 5 cm.

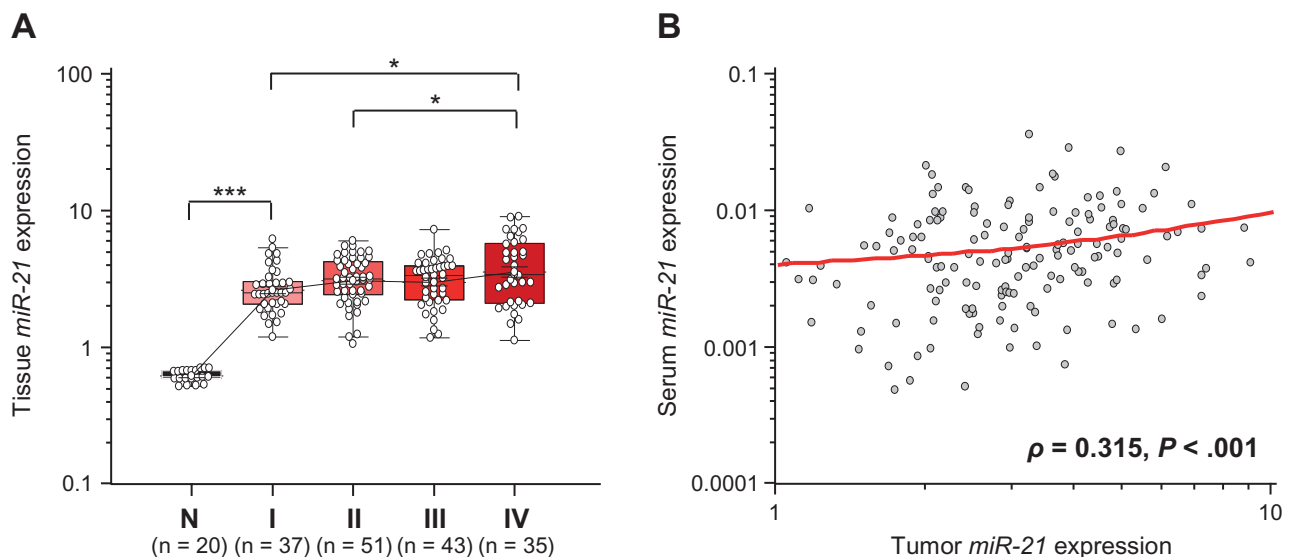


Figure 3. Validation of *miR-21* expression in matched tissue samples (n = 186). **A**) Box plots illustrating tissue *miR-21* levels in different Tumor Node Metastasis (TNM) stages (I,II,III, and IV) of colorectal cancers (CRCs) (n = 166) and adjacent normal mucosa (N; n = 20). The y-axis (log₁₀ scale) represents relative expression of *miR-21* normalized to *miR-16* in tissue samples. **Boxes** represent the interquartile range, and the **horizontal line** across each box indicates the median

value. Statistically significant differences were determined using the Mann–Whitney *U* test and Kruskal–Wallis tests. **P* < .05; ****P* < .001. **B**) Scatter plots showing the correlation between relative expression of *miR-21* levels in serum (y-axis: log₁₀ scale) and matched tumor tissues (x-axis: log₁₀ scale) obtained from 154 CRC patients. A positive correlation was found by Spearman correlation ($\rho = 0.315$; 95% CI = 0.17 to 0.45; *P* < .001).

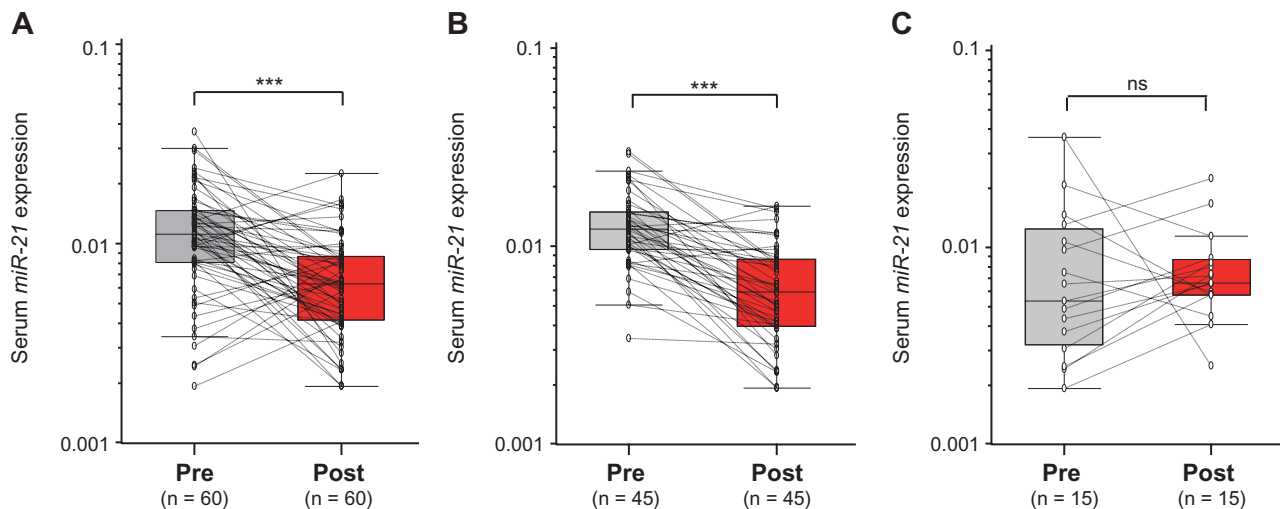


Figure 4. Alterations in serum *miR-21* expression levels in patients with colorectal cancer (CRC) before surgery (Pre) and 7 days after post-surgical removal of primary tumors (Post). **A)** Comparison of serum *miR-21* levels from all CRC patients (n = 60). **B)** Comparison of serum *miR-21* levels in 45 CRC patients who underwent potentially curative surgeries. **C)** Comparison of serum *miR-21* levels in 15 CRC patients

who underwent noncurative surgeries. The y-axis (log₁₀ scale) represents relative expression of *miR-21* normalized to *cel-miR-39*. Boxes represent the interquartile range, and the horizontal line across each box indicates the median value. Statistically significant differences were determined using the Wilcoxon test. ****P* < .001; ns, not statistically significant.

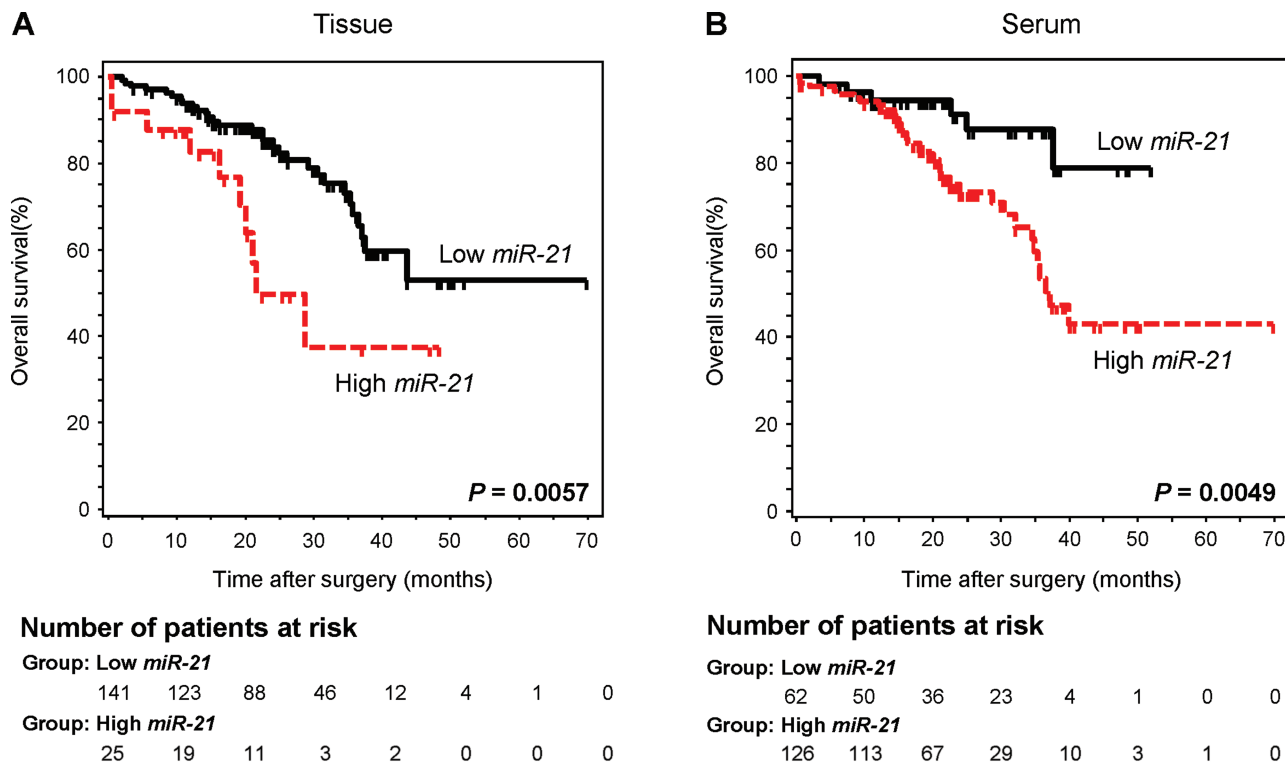


Figure 5. Kaplan–Meier survival analysis in colorectal cancer (CRC) patients based upon *miR-21* expression in primary tumors and matched serum samples. **A)** The overall survival rate in CRC patients with high *miR-21* expression in tumor tissue (n = 25) was statistically significantly lower than that for those with low *miR-21* expression (n = 141) (>3.7 vs ≤3.7; *P* = .006; log-rank test). **B)** The overall survival rate in CRC patients with

high serum *miR-21* expression (n = 126) was statistically significantly lower than that for those with low serum *miR-21* expression (n = 62) (>0.003 vs ≤.003; *P* = .005; log-rank test). Cutoff values for *miR-21* expression in serum and primary tumor tissues were determined from the receiver operating characteristic curves by using Youden’s index. The number of patients at risk are shown below each curve at various timepoints.

Number of patients at risk

Group: Low <i>miR-21</i>							
141	123	88	46	12	4	1	0
Group: High <i>miR-21</i>							
25	19	11	3	2	0	0	0

Number of patients at risk

Group: Low <i>miR-21</i>							
62	50	36	23	4	1	0	0
Group: High <i>miR-21</i>							
126	113	67	29	10	3	1	0

Table 3. Univariate and multivariable analyses of factors predictive of poor overall survival in colorectal cancer patients*

Variables	Univariate†		Multivariable‡	
	HR (95% CI)	P	HR (95% CI)	P
Age#, >67 y vs ≤67 y	0.72 (0.43 to 1.37)	.37	1.23 (0.57 to 2.67)	.59
Sex, female vs male	1.02 (0.56 to 1.86)	.92	2.07 (0.86 to 4.96)	.10
Pathological T, T3/4 vs T1/2	8.97 (2.19 to 36.70)	.002	2.38 (0.30 to 29.77)	.31
Pathology, poor diff. vs diff.	2.26 (1.05 to 4.84)	.04	2.39 (0.57 to 9.92)	.23
Lymph node metastasis, yes vs no	17.1 (6.18 to 47.80)	<.001	6.83 (1.69 to 28.36)	.008
Distant metastasis, yes vs no	35.6 (14.40 to 86.60)	<.001	21.7 (5.92 to 79.99)	<.001
CEA§, >5 ng/mL vs ≤5 ng/mL	4.84 (2.15 to 10.89)	.001	1.03 (0.33 to 3.22)	.94
<i>miR-21</i> in tissue¶, >3.7 vs ≤3.7	2.66 (1.29 to 5.45)	.01	0.59 (0.21 to 1.63)	.31
<i>miR-21</i> in serum¶, >0.0031 vs ≤0.0031	3.25 (1.36 to 7.73)	.003	4.12 (1.10 to 15.40)	.03

* CEA = carcinoembryonic antigen; CI = confidence interval; diff. = differentiation; HR = hazard ratio.

The median age was 67 years.

§ Cutoff value for CEA is 5 ng/mL (as per American Association of Clinical Oncology recommendations).

¶ Cutoff values of *miR-21* in tissue and serum are derived from receiver operating characteristic curve with Youden's index.

† Univariate analysis was performed using clinical data available from 200 patients, serum *miR-21* data from 186 patients, and tissue *miR-21* data from 166 colorectal cancer patients, respectively.

‡ Multivariable analysis was performed using data from 153 colorectal cancer patients from whom matched matching data were available for all clinico-pathological factors as well as serum and tissue *miR-21* expression results.

among patients with potentially curative surgeries ($P < .001$) (Figure 4B). Contrariwise, no statistically significant differences were observed in *miR-21* levels before or after surgery in patients with noncurative resections ($P = .72$) (Figure 4C). Collectively, these data underscore the importance of serum *miR-21* expression as a highly specific biomarker for the diagnosis of colorectal neoplasia.

Association of Serum *miR-21* Expression With Survival in Patients With CRC.

To further evaluate whether serum *miR-21* levels can serve as a predictor of patient outcome, we performed Kaplan–Meier survival analysis. As anticipated, patients with higher levels of *miR-21* in the tumor tissues had statistically significantly worse overall survival ($P = .006$; log-rank test) (Figure 5A). Moreover, a similar pattern of increased *miR-21* concentrations associated with statistically significantly decreased overall survival was observed when the analysis used serum *miR-21* expression levels ($P = .005$; log-rank test) (Figure 5B).

Furthermore, Cox proportional hazard regression analyses revealed that in the univariate analysis, poor prognosis in CRC patients was associated with high levels of *miR-21* in both tumor and serum ($P = .01$ and $P = .003$, respectively), high levels of carcinoembryonic antigen (CEA > 5 ng/mL; $P = .001$), high T stage (T3/T4; $P = .002$), lymph node metastasis ($P < .001$), poorly differentiated tumors ($P = .04$), and distant metastasis ($P < .001$) (Table 3). More important, multivariable analysis demonstrated that high levels of serum *miR-21*, but not high concentrations of *miR-21* in tumor tissues or high CEA levels, served as an independent prognostic marker for indicating overall survival in CRC patients (HR = 4.12; 95% CI = 1.10 to 15.4; $P = .03$).

Discussion

Most approaches aimed at the identification of diagnostic or prognostic miRNAs in the circulation have focused on miRNAs that are frequently overexpressed (12,15,33). However, relatively few miRNAs that are abundantly expressed in cancer cells have been

detectable in the circulation (34), and nearly 30% of the released miRNAs do not mirror the cellular expression profiles originating in specific cancer cells (35). These reports underscore the need for more careful evaluation of criteria depending upon whether these miRNAs are retained in the cell or are released into the systemic circulation to provide a robust substrate for a noninvasive diagnostic strategy.

Data from our initial screening phase revealed two important observations. First, *miR-21* levels in cell culture medium increased statistically significantly in a time- and cell number-dependent manner, establishing the secretory nature of this miRNA for development as a noninvasive biomarker of the early detection of colorectal neoplasia. Second, a statistically significant correlation was observed in *miR-21* expression between matching serum and tissues from a small subset of CRC patients, validating the specificity of *miR-21* expression in the circulation. Increased *miR-21* expression was thereafter successfully validated in a large, independent set of matching serum and tissue samples. Although our observation of increased *miR-21* expression in CRC tissues is consistent with previous reports (19–22), our results are the first to demonstrate that high levels of *miR-21* in both primary CRC tissues and matched serum samples are associated with large tumor size, distant metastasis, and advanced TNM stage. Another interesting feature of our study was the existence of a statistically significant correlation between *miR-21* expression in primary lesions and those in sera. The fact that the circulating *miR-21* in serum of CRC patients is likely produced by the CRCs was further strengthened by our observation of a statistically significant drop in serum *miR-21* expression in postoperative serum vis-à-vis preoperative samples after potentially curative surgery in patients with CRC.

This study is the first to demonstrate the potential role of serum *miR-21* in the early detection of colorectal neoplasia. This is supported by the markedly high AUC values of 0.919 derived from comparisons between CRC patients and healthy control subjects (sensitivity = 91.9%; specificity = 81.1%). Even more important from a CRC screening perspective, serum *miR-21* expression levels

demonstrated AUC values of 0.813, associated with 81.1% sensitivity and 76.7% specificity, for discerning patients with adenomatous polyps from healthy control subjects, an observation that is novel and perhaps represents the highest levels of sensitivity and specificity for any serum biomarker aimed at the identification of colorectal adenomas in a noninvasive manner. In addition, the odds ratio for case subjects with high levels of *miR-21* expression being associated with CRC was 43.3 (95% CI = 17.53 to 107.13), which is an extraordinary performance for a noninvasive biomarker compared with recently reported data for other circulating miRNAs (OR = 18.7) (11) or a positive first guaiac FOBT (OR = 7.6) (36). Another important feature of our study was that we observed detectable levels of serum *miR-21* in premalignant adenomas, even though these lesions are not fully vascularized. Thus, our study highlights that *miR-21* expression can be exploited not only as a promising noninvasive biomarker for early detection of CRC but also for the identification of clinically meaningful adenomas—a critical target lesion for any CRC screening strategy.

Another interesting finding of our study is that *miR-21* expression in serum also serves as a prognostic biomarker for CRC. Our results are consistent with previous studies that have demonstrated the potential of tissue *miR-21* as a prognostic and predictive marker for response to chemotherapy in CRC (20,22,37). However, our findings that high levels of serum (rather than tissue) *miR-21* indicate a poor prognosis in patients with CRC is an important step forward in the identification of a noninvasive biomarker for this disease. Furthermore, the multivariable Cox proportional hazards model illustrated that high expression of serum *miR-21* was an independent prognostic variable, whereas the prognostic values of *miR-21* expression in tumors and CEA levels were statistically significantly compromised by other clinical factors. Therefore, serum levels of *miR-21* might not only diagnose neoplasia but also help predict metastases or tumor recurrence with higher accuracy than *miR-21* expression in the tumor tissue.

This study clearly highlights that the relative expression of serum *miR-21* was statistically significantly different between case patients (for both adenomas and cancers) and control subjects. Unlike tissue or cellular miRNAs, at present there is no consensus housekeeping miRNA for normalizing the expression of circulating miRNAs (38,39); therefore, measurement of relative expression levels of circulating miRNAs has been a common approach in published studies. In spite of this limitation, we circumvented this concern using two approaches. First, we used the same amount of starting serum (200 μ L) from each patient for every quantitation. Second, to further ensure the technical aspects of our assay, including variability in serum RNA extraction and polymerase chain reaction amplification efficiencies, we used the recently described approach for normalization of experimental miRNA data using spiked-in synthetic, nonhuman mature miRNA from *Caenorhabditis elegans* (40). Although our method of quantifying relative expression [$2^{-\Delta\text{Ct}}$; $\Delta\text{Ct} = \text{Ct}(\text{miRNA of interest}) - \text{Ct}(\text{cel-miR-39})$ [this miRNA used as a normalizer for sample to sample variation]] of serum miRNAs was quite robust, absolute quantitation of serum *miR-21* expression levels may further improve the translation of these data into a clinically viable diagnostic test for the early detection of colorectal neoplasia in the immediate future.

Although our current assay may become a promising screening tool for colorectal neoplasia, we acknowledge two potential limitations of using *miR-21* as a single biomarker for the early detection of colorectal neoplasia. First, circulating expression of *miR-21* has been described in many solid cancers besides CRC, including glioblastoma, pancreatic cancer, and breast cancer, underscoring the need for being vigilant about organ and disease specificity while investigating *miR-21* as solitary biomarker for CRC. As a consequence, it might be challenging to differentiate whether circulating *miR-21* expression is specifically associated with CRC itself or if this is a common phenomenon that manifests during progression of any cancer as a result of perturbations in the host immune response (41). In this study, we have addressed this issue by demonstrating that a statistically significant correlation existed between tissue and serum levels of *miR-21* across all stages of colorectal neoplasia and that *miR-21* levels fell after surgical removal of the colorectal tumors in a subset of patients, highlighting the specificity of *miR-21* as a specific biomarker for colorectal neoplasia. Second, although it is highly unlikely to have a substantial impact, use of serum *miR-21* expression levels as a diagnostic and prognostic biomarker must be validated in diverse ethnic populations because the clinical materials analyzed in this study were solely from patients of Japanese origin.

In conclusion, our results provide compelling evidence for the potential usefulness of serum *miR-21* as a noninvasive screening and prognostic tool in patients with colorectal neoplasia, a concept that can be incorporated into routine clinical practice in the not-so-distant future pending validation in large-scale prospective trials.

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Notes

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