Suitability of Circulating miRNAs as Potential Prognostic Markers in Colorectal Cancer

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Abstract

miRNAs are crucial in cellular processes and have been shown to be abnormally expressed in cancer tissue and the circulation. Circulating miRNAs may serve as a novel class of minimally invasive biomarkers for prognosis. Within a first methodologic study, we evaluated the miRNA profile kinetics in the plasma of patients with colorectal cancer after surgical tumor removal to identify potential suitability as prognostic biomarkers. This pilot study is based on the ColoCare Study, a cohort study of newly diagnosed patients with stage I–IV colorectal cancer. Colorectal cancer pre- and postsurgical blood (2–7 days after surgery) and 6 months follow-up blood from 35 patients were examined and candidate miRNAs were investigated in the plasma. miRNA levels were measured by two-step qRT-PCR. Statistical analysis was performed using log-transformed normalized CT values using SAS 9.3. Comparing pre- and postsurgical miRNA levels revealed a statistically significant decrease of nine circulating miRNAs after surgery (miR92a, miR18a, miR320a, miR106a, miR16-2, miR20a, miR223, miR17, and miR143). Analyses of plasma levels over all three time points demonstrated a statistically significant decrease from presurgery to postsurgery and re-increase from postsurgery to the six months follow-up time point of four circulating miRNAs (miR92a, miR320a, miR106a, and miR18a). We were able to show for the first time that in plasma miRNA profiles change within days after colorectal cancer surgery. Our results underscore the role of the investigated miRNAs in colorectal cancer and their potential utility as prognostic biomarkers.

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Introduction

miRNAs are small, noncoding RNAs that function as regulators of many critical cellular processes, are known to be deregulated in cancer, and act either by oncogenic or tumor-suppressor function (1). Perfect or imperfect complementarity to 3′ untranslated regions on their target mRNAs results in repression of target gene expression posttranscriptionally. miRNA expression differs between normal and tumor tissue and varies among tissue types (2, 3). Tumor tissue–specific miRNAs can also be detected in the blood where they are surprisingly stable and are emerging as promising biomarkers (4–6). Tumor-derived miRNAs that have been released into the circulation have also shown a strong correlation with disease and prognosis in several types of cancer (7), including colorectal cancer (5). Noninvasive biomarker assays are desirable as they could be easily transferred into clinical screening routines for early cancer detection. Plasma miRNA levels could be monitored repeatedly and thereby serve as predictors of clinically relevant outcomes of recurrence, progression, and survival. The fecal occult-blood test is the most available noninvasive screening tool but has its limitation in low sensitivity. The current use of carcinoembryonic antigen (CEA) as blood-based tumor marker for colorectal cancer is suboptimal, too, due to its modest sensitivity, specificity, and high false-positive rate (8, 9).

However, the origin of circulating plasma miRNAs is still unclear. It is thought that they are released by cells in protein complexes or vesicles (10). To our knowledge, there are only three studies that have attempted to evaluate the use of miR18a, miR92a, miR29a, and miR17-3p as
molecular markers by comparing plasma levels of these miRNAs before and after surgical removal of the tumor (11–13). Huang and colleagues reported that levels of miR92a and miR29a were both significantly reduced in postoperative samples in 20 patients. Furthermore, both miRNAs could discriminate advanced adenomas and colorectal cancers from healthy controls. Following tumor resection, decreased levels of miR18a were observed in 21 patients with colorectal cancer (13). Ng and colleagues showed that miR92 and miR17-3p were significantly elevated in plasma from patients with colorectal cancer compared with controls in two independent populations. Plasma levels of both miRNAs were significantly reduced after surgery in 10 patients with colorectal cancer. These (12, 13) and other studies have analyzed the expression of specific miRNAs in the plasma of patients with colorectal cancer compared with healthy controls. Similarly, there have been a number of reports about the significance of miRNA as biomarker in colorectal cancer detections: miR92a, belonging to the miR17-92 cluster, was demonstrated to have reasonable sensitivity (76%) and specificity (64%) and thus might be useful as a screening tool. Circulating miRNAs that can predict recurrence and other outcomes for lung, prostate, and ovarian cancer have been identified (2, 14–16), and miR141 is promising in colorectal cancer reported to be significantly associated with stage IV colon cancer predicting poor survival (17). Pu and colleagues showed that plasma miR221 can predict poor survival and correlates with expression of p53 (15). There is only little known about how rapid changes, i.e., surgical removal of a colorectal tumor, are reflected in changing miRNA levels in the circulation. Therefore, our study fills an important gap by measuring the time course of candidate plasma miRNA patterns in a follow-up period of 6 months with three plasma-level measurements. The aim of this pilot study was to characterize the level of circulating miRNA in a time series before surgical removal of a colorectal tumor, within a week after surgery and 6 months after surgery.

Materials and Methods

Study population/sample collection

Thirty-five patients of the ColoCare study recruited at the Heidelberg University Clinic between December 2011 and May 2012 were included in this study. Newly diagnosed patients with colon or rectal cancer (any stage), age ≥18 years, with surgical resection at the University Heidelberg Surgical Clinic were recruited. Blood samples (ethylenediaminetetraacetic acid) were taken from patients the day before surgery, within one week after surgery (2–7 days, mean = 4.1 days) and six months after surgery. Preparation of plasma was performed within 4 hours after blood draw by retaining supernatant after centrifugation (2,500 g; 15 minutes) and was stored in aliquots at −80°C. Clinical data were abstracted from the clinical information system available at the National Center for Tumor Diseases (NCT). Study procedures were approved by the University of Heidelberg’s ethics committee and all patients provided informed consent.

miRNA isolation and quantification

RNA was isolated from cryopreserved 100 μL plasma samples following a modified protocol of the Qiagen miRNeasy Mini Kit (18). As the miRNA yield cannot be measured accurately by spectrophotometry (18), a spike-in control, synthetic nonhuman miRNA (cel-miR39 from C. elegans), was used. A literature search and own preliminary data revealed candidate miRNAs that were potentially related to colorectal cancer, especially those also found in the circulation: miR92a (6, 11, 12, 17, 19–24), miR18a (6, 19–21, 23, 25, 26), miR320a (24), miR106a (6, 27, 28), miR16-2, miR20a (6, 21–24, 28, 29), miR223 (22), miR143 (6, 20, 22, 23, 30–33), miR17 (12, 21–23, 30), miR22a (11, 25, 26), miR221 (15), miR145 (6, 20, 22–24, 30, 33), miR21 (6, 19, 20, 28, 30–32, 34), miR125b (20), miR141 (17), miR133a (6, 22, 32), miR31 (20, 22, 23, 30, 32, 33). One of the major problems in plasma miRNA analysis is the lack of consensus housekeeping sequences. In this study, miR16-2 and miR223 were evaluated as potential endogenous references but as the expression levels of both of these miRNAs were significantly decreased after surgical removal of the tumor, both were regarded as candidates.

Reverse transcription was carried out by the Taqman miRNA Reverse Transcription Kit with miRNA-specific stem-loop primers for the target miRNAs. Real-time PCR was performed using TaqMan MicroRNA Assays. RT product was directly transferred from the RT 96-well plate to a new PCR 96-well plate. All reactions were performed in duplicates (7900HT Fast Real-Time PCR System).

Data analysis

SDS 2.4, RQ manager 1.2 (both Applied Biosystems), and Microsoft Excel were used for calculation of 2^−ΔCT analyses. Automatic baseline and threshold settings were used for all targets. miRNA expression levels were analyzed using calculation with the ΔCT method. All mean CT values were normalized to cel-miR39, by subtracting the mean CT value of cel-miR39 from each target’s mean CT value. Then, calculation of 2^−ΔCT values followed. Statistical analysis was performed using log-transformed normalized CT values using a paired t test for the comparison of pre- and postsurgery levels and repeated-measures ANOVA for comparison of all 3 consecutive time points. We used analysis of covariance (ANCOVA) models including stage, tumor site, age at surgery, and gender to investigate associations between characteristics or clinicopathologic parameters and miRNA levels at each time point. All statistical analyses were performed using the SAS software 9.3 (SAS Institute). P values of 0.05 or less were considered to be statistically significant.
Results

The study population is described in Table 1. miRNA levels were measured in all patients at all three time points, with the exception of 1 patient who died before the 6 months blood draw. In the first step of analysis, pre- and postsurgical plasma miRNA levels were compared. T test analyses revealed a statistically significant decrease of levels of nine circulating miRNAs [miR92a (P < 0.0001), miR18a (P < 0.0001), miR320a (P = 0.0002), miR106a (P = 0.0003), miR16-2 (P = 0.0007), miR20a (P = 0.0002), miR223 (P = 0.02), miR17 (P = 0.02), miR143 (P = 0.02), data not shown] from presurgery to postsurgery. The levels of the other candidate miRNAs did not change significantly between these time points. In the next step, all three time points were compared using ANOVA. Significant differences of levels in both measurement intervals (pre- vs. postsurgery and postsurgery vs. six months follow-up) were observed for four circulating miRNAs [miR92a (P = 0.001), miR320a (P = 0.002), miR106a (P = 0.002), miR18a (P = 0.003); see Fig. 1]. The changes of levels of the other candidate miRNAs were only significant for one time interval or not at all.

Multivariate analyses revealed statistically significant associations between patient characteristics or clinicopathologic parameters and miRNA levels at specific time points. At the postsurgery time point, expression levels of miR18a were significantly reduced in tumors of the rectum compared with tumors of the colon (P = 0.02, data not shown). Older age was significantly associated with higher levels of miR106a (P = 0.01) and high levels of miR20a (P = 0.01) at the 6 months time point (see Fig. 2). We did neither observe an association of miRNA expression level at any time point with stage of the disease nor with chemotherapy treatment.

Discussion

In this study, we investigated variations in levels of 16 circulating miRNAs within a specified time period as a potential result of surgical removal of a tumor. Comparing pre- and postsurgical miRNA levels in plasma revealed a statistically significant decrease of nine circulating candidate miRNAs. Assuming that miRNAs are released into the circulation by tumor cells, this substantial change in the plasma profile underscores the role of these miRNAs in colorectal carcinogenesis. Thus far, only few studies have investigated circulating miRNAs in patients with colorectal cancer, even less have evaluated plasma miRNAs at pre- and postsurgery time points (12, 13). Decreased levels of miR92a and miR17-3p were reported in the plasma of patients with colorectal cancer (n = 10) postsurgery compared with presurgery (12). Similar, also miR200c and miR18a were lower in postoperative compared with preoperative samples of 21 patients (13). Furthermore, miR92a could distinguish colorectal cancer and advanced adenoma from normal controls, with a sensitivity of >62% and specificity of >84% (12). In our pilot study, miR92a and miR17 were also significantly decreased after surgery (miR92a: P = 0.0001; miR17: P = 0.02). Huang and colleagues reported that circulating miR92a and miR29a can discriminate patients with colorectal cancer and advanced adenoma from healthy controls (11). Also, its expression was associated with more advanced tumor–lymph node–metastasis (TNM) stage. miR29a was evaluated in our study, and the change between pre- and postsurgery time points was suggestive (P = 0.07). miR18a previously was reported as elevated in the plasma of patients with colorectal cancer (13, 25, 26), consistent to the findings of our study (P < 0.0001). Other studies investigating circulating miRNAs in colorectal cancer have shown elevated miR21 (34), miR141 (17) and miR221 (15). Elevated miR221 levels were associated with a worse prognosis in terms of overall survival (15). Cheng and colleagues (17) demonstrated that high plasma levels of miR141 are associated with stage IV colorectal cancer, predict poor survival, and complement CEA.

Results of the three-time-point ANOVA showed that plasma levels of miR92a, miR320a, miR106a, and miR18a, which had decreased after surgical removal of the tumor, increased in the period between postsurgery and 6 months postsurgery, reaching presurgery levels. This may be an intriguing finding and requires further follow-up, including investigations of miRNA levels as potential biomarkers of recurrence.

In this study, 27 of expected 34 blood samples were collected for analyses of the 6 months follow-up, as the 5 remaining patients did not keep their follow-up appointments. Postsurgery blood draws were implemented.
within a week after removal of the tumor (range: 2–7 days). Also, the 6 months follow-up has to be interpreted as a range of several weeks (mean, 218 days; SD, 35 days; range, 154–304 days); delays were in large parts attributable to the protocol, which required at least 2 weeks after the last chemotherapy cycle before blood draw.

Our study had several strengths and limitations: To our knowledge, it is the first investigation characterizing miRNA levels in patients with colorectal cancer at more than two time points before and after surgery. We performed a normalization step to the value of the spike-in cel-miR39 to remove technical variance. However, we were not able to normalize for biologic variance because the miRNAs intended as endogenous controls were also varying by time point. Pritchard and colleagues reported that miRNAs expressed by red blood cells (e.g., miR92a and miR16) are significantly elevated in hemolyzed blood samples (35). They also noted a positive correlation between miRNAs expressed by myeloid cells (e.g., miR223) and corresponding white blood cell counts. The plasma used to perform our study was visually free of hemolysis. However, because we did not quantify hemoglobin, we cannot exclude the occurrence and influence of small levels of hemolysis.

The sample size of this methodologic study was limited which may also be a possible explanation for the lack of statistically significant associations with clinicopathologic parameters. The long-term goal of the ColoCare Study will be to establish noninvasive blood-based biomarkers, which can predict clinically relevant outcomes such as recurrence, progression, and survival. If one hypothesizes that miRNAs circulating in the plasma actually derive from tumor cells and considering the increase of plasma miRNA levels 6 months after surgery in this study,}

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**Figure 1.** miRNA expression levels at presurgery, postsurgery, and 6 months time point (ANOVA). Horizontal lines, significance between intervals.
clinically undetectable growth of metastases may be a possible cause. However, it is still unknown which mechanism leads to a release of miRNAs into the plasma and where these miRNAs originate. Besides including a larger population, future studies may consider including validation samples or study tumor tissue of the same patients simultaneously. Thus, as long as it is not clear which factors influence plasma miRNA profiles, studies about circulating miRNAs should be regarded with caution. Nevertheless, our results on the time course of candidate miRNAs’ levels before and after surgery substantiate to some extent their role in colorectal cancer and their potential as future biomarkers of prognosis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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