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Circulating plasma microRNAs in colorectal neoplasia: A pilot study in assessing response to therapy

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ABSTRACT

Introduction: Current serological surveillance markers to monitor colorectal cancer (CRC) or colorectal advanced adenomas (CAA) are hampered by poor sensitivity and specificity. The aim of this study is to identify and validate a panel of plasma microRNAs which change in expression after resection of such lesions.
Methods: A prospectively maintained colorectal surgery database was queried for patients in whom both pre- and post-procedural serum samples had been obtained. An initial screening analysis of CRC and CAA patients (5 each) was conducted using screening cards for 380 miRNAs. Four identified miRNAs were combined with a previously described panel of 7 miRNAs that were diagnostically predictive of CRC and CAA. Differential miRNA expression was assessed using quantitative real-time polymerase chain reaction(qRT-PCR).
Results: Fifty patients were included ($n = 27$ CRC, $n = 23$ CAA). There was no difference in age, gender, or race profile of CRC patients compared to CAA patients. Six miRNA were significantly increased after CRC resection (miR-324, let7b, miR-454, miR-374a, miR-122, miR-19b, all $p<0.05$), while three miRNAs were significantly increased following CAA resection (miR-454, miR-374a, miR-122, all $p<0.05$). Three miRNA were increased in common for both (**miR-454, miR-374a, miR-122**).
Discussion: The expression of miRNAs associated with neoplasia (either CRC or CAA) was significantly increased following surgical resection or endoscopic removal of CRC or CAA. Future studies should focus on the evaluation of these miRNAs in CRC and CAA prognosis.

Introduction

The majority of patients diagnosed with colorectal cancer (CRC) present with regional or distant stage disease [1]. There have been improvements in the survival of all patients with CRC, but the outcomes for patients with stage III or IV disease remain poor [1]. Although screening strategies have improved the early detection of CRC, there is a significant need to improve post-operative surveillance strategies [2].

The carcinoembryonic antigen (CEA) assay is the only plasma-based assay that is routinely available for post-operative surveillance and for monitoring response to therapy. It has also been evaluated for screening, but it lacks sufficient sensitivity (36–74%) and specificity (87%) to accurately detect CRC recurrence or recurrence of its precursor lesion, colorectal advanced adenoma (CAA) [3,4]. CAA is a well recognized precursor lesion to sporadic CRC [5,6] and is defined as an adenoma >0.6 cm diameter with high-grade dysplasia or with a villous component [7,8]. Endoscopic removal of such CAA prevents the formation of CRC, which, in turn, decreases CRC incidence [9,10].

MicroRNA (miRNA) are small non-protein coding RNA molecules that have been associated with the regulation of different disease processes and play an important role in oncogenesis by regulating gene expression [11,12]. MiRNAs are known to be actively released from cells and are found in body fluids such as plasma, saliva, feces and urine [13,14]. Plasma miRNAs have been identified as diagnostic and prognostic biomarkers in CRC [15,16].

We have previously investigated the use of miRNAs as a biomarker for the detection of CAA and CRC and have demonstrated that miRNAs were able to distinguish between individuals with CAA or CRC from healthy controls with higher sensitivity and specificity than currently used non-invasive screening methods [14].

Previous studies have demonstrated a change in the serum miRNA expression pre- and post-operation for both ovarian and breast can-

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cer. There are few studies that examine both CAA and CRC [17,18]. The identification of miRNAs that change in expression following endoscopic removal of CAA or tumor resection may have a potential novel role in monitoring these patients. We hypothesize that the expression of tumor-related miRNAs will significantly change following CRC resection or endoscopic CAA removal. The aim of this study was to identify and validate differentially expressed miRNAs between pre- and post-intervention serum samples in patients with CAA and CRC.

Methods

This study was approved by the University of Louisville Institutional Review Board (#12–025), and written informed consent was obtained from all patients. The study population consisted of consecutive patients recruited from the University of Louisville Colon and Rectal Surgery practice between 2016 and 2019, who had signed informed consent and in whom both pre-treatment and post-treatment serum samples were available.

Study subjects

One hundred twenty samples from 60 patients were included in this study: 28 patients with CAA (defined as > 0.6 cm diameter with a villous component or high-grade dysplasia) [7,8], 30 patients with stage I-III CRC, and two patients with stage IV CRC (single and resectable liver metastasis). In the CAA group, the initial blood sample was taken at the time of endoscopy when the CAA was identified. In the CRC group, the initial sample was taken prior to any initial therapy. This follow-up specimen was obtained at the patient's next office visit or colonoscopy, whichever occurred first. Patients with CRC were staged according to the American Joint Committee on Cancer TNM staging system [19].

Peripheral whole blood was extracted from patients and plasma immediately isolated and stored at −80 °C for later use, as previously described [14,20].

miRNA screening

Screening studies were initially performed to determine differential expression of 384 miRNA between pre- and post-treatment samples in five patients with CRC and five patients with CAA (pre- and post-resection plasma samples of five CRC patients and pre- and post- endoscopic removal plasma samples of five CAA patients, $n = 20$ samples total). We have, however, previously reported on assay reproducibility with respect to plasma miRNA expression and demonstrated our experimental technique to be reproducible and reliable [21,22]. Total RNA was extracted from plasma (miRNeasy® Serum/Plasma Isolation Kit, Qiagen, Valencia, CA), after which the quantity and purity of each sample was confirmed using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific®, Middlesex, MA). Samples were deemed of good quality with an $A_{260/280}$ ratio of 1.8–2.2. For each sample ($n = 20$), the expression levels of 384 miRNAs were examined using the TaqMan® Low Density Array (TLDA) human miRNA card A following the manufacturer's protocol (Life Technologies, Carlsbad, CA). Complementary DNA (cDNA) was generated from total RNA samples using the TaqMan® miRNA Reverse Transcription kit (Life Technologies, Carlsbad, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific®, Middlesex, MA). The cycle threshold was set at 0.03. All experiments were performed by a single operator. Normfinder software was used to find the optimal endogenous reference gene on the TLDA cards [21]. As such, RNU6B and miR-16 were selected as the endogenous reference genes, with the mean used for statistical analysis.

Single assay miRNA validation

For miRNA single assay quantification, plasma was isolated from 50 patients, 25 with CAA and 25 with CRC, each with pre and post samples ($n = 100$ samples, 50 CAA and 50 CRC). Total RNA was converted to cDNA using TaqMan® miRNA Reverse Transcription kit and specific TaqMan® miRNA primers for each miRNA of interest and the endogenous reference miRNAs RNU6B and miR-16 (Life Technologies, Carlsbad, CA). Specific TaqMan® probes for each miRNA were then used to bind to complementary sequences on target cDNA during qRT-PCR, which was performed using the Step-One Plus qRT-PCR system (Life Technologies, Carlsbad, CA). All reactions were completed in duplicate and the cycle threshold set at 0.1 in order to perform statistical analysis.

Table 1
Patient demographics of the screening group.

Variables	Screening group ($N = 10$) N(%)
Age (Year)	57 (46 –72)
Gender	
Female	7 (70)
Male	3 (30)
Race	
Caucasian	10 (100)
African American	0 (0)
Type of neoplasia	
Colorectal advanced adenoma	5(50)
Colorectal adenocarcinoma	5(50)
Location of neoplasia	
Cecum	2(20)
Ascending colon	4(40)
Descending colon	1(10)
Rectum	3(30)

Continuous data presented as median (interquartile range). Categorical data presented as N(%).

Statistical analysis

Patient age and time from surgery to procedure were compared using a two-sample *t*-test or ANOVA, where appropriate. The frequencies for race and gender were compared using the chi-squared test, or Fischer's exact test, where appropriate. Expression levels of each individual miRNA were normalized to the mean of RNU6B and miR-16 (endogenous internal reference genes), in order to calculate ΔCt values. Differential expression between pre- and post-treatment samples were analyzed using the ΔΔCt method [22].

Results

Exploratory miRNA screening and selection of a miRNA panel

A flow diagram of the experimental procedure to identify a panel or miRNA for validation is shown in **Fig. 1**. Five patients with CAA and five patients with CRC were used in this exploratory analysis (**Table 1**). In these patients, the miRNA expression was compared between the post-treatment and pre-treatment blood samples **(Supplementary Table 1**). We identified four miRNAs (miR-324–5p, miR-454, Let 7b and miR-19b) from our screening cohort that were significantly increased in expression in the post-treatment sample compared to the pre-treatment samples in both CAA and CRC groups. These four miRNA were also selected on the basis of their biological relevance [23–26].

To expand the scope of the miRNA to be studied on single assay analysis, we added other miRNAs found to be relevant to the study of colorectal neoplasia. Our group has previously reported a panel of seven miRNA (miR-21, miR-29c, miR-346, miR-374a, miR-372, miR-122 and miR-192), which were found to be highly diagnostically predictive of CRC and CAA and compared to other cancers (breast cancer, lung cancer, and pancreatic cancer) and to healthy controls [14]. These microRNA were found to be both biologically relevant to the study of colorectal neoplasia, but the changes in their expression following CAA or CRC resection has not been studied. Although the differences in these miRNA were not statistically significant on screening analysis,

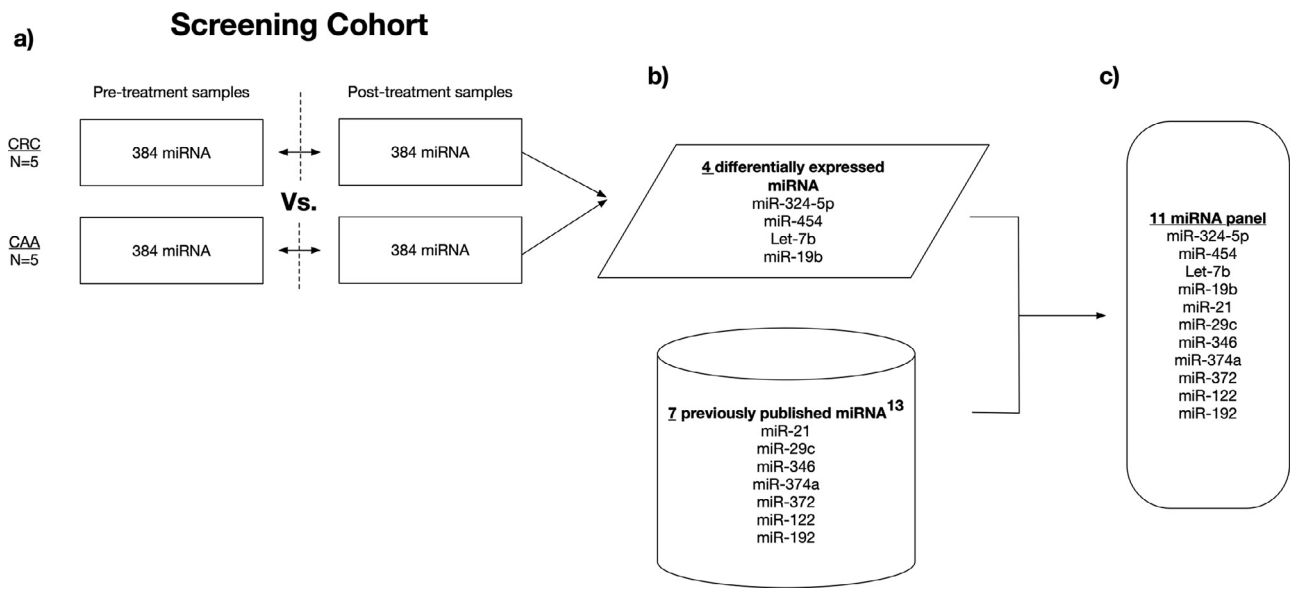

Fig. 1. Flow diagram describing the experimental procedure to identify the panel of 11 miRNA for validation. a) A pre-treatment and post-treatment serum sample was obtained for 5 patients with CRC and for 5 patients with CAA. The expression of 384 miRNA for each serum sample was performed to compare the miRNA expression in the post-treatment sample to the pre-treatment sample. **b)** Four miRNA were differentially expressed and combined with a previously reported panel of 7 miRNA.13 **c)** This produced a panel of 11 miRNA for further investigation.

Table 2
Patient demographics of the validation groups.

Variables	Total ($N=50$) N(%)	CAA ($N=23$) N(%)	CRC ($N=27$) N(%)
Age (Year)	61 (49–70)	66 (51–73)	57 (47–67)
Gender			
Female	21 (42)	12 (52)	9 (32)
Male	29 (58)	11 (48)	18 (68)
Race			
Caucasian	44 (88)	19 (83)	25 (93)
African American	6 (12)	4 (17)	2 (8)
CAA/CRC location			
Cecum	9(18)	5(22)	4(15)
Ascending colon	9(18)	7(30)	2(7)
Hepatic flexure	1(2)	0(0)	1(4)
Transverse colon	2(4)	2(9)	0(0)
Splenic flexure	0(0)	0(0)	0(0)
Descending colon	3(6)	3(13)	0(0)
Sigmoid colon	10(20)	3(13)	7(26)
Rectum	16(32)	3(13)	13(48)
AJCC stage			
I	–	–	4 (15)
II	–	–	9 (33)
III	–	–	12 (44)
IV	–	–	2 (7)

CRC: Colorectal cancer, CAA: Colorectal advanced adenoma. Continuous data presented as median (interquartile range). Categorical data presented as N(%).

these seven miRNA were studied in addition to four miRNAs identified during screening. This produced an 11-miRNA panel to be assessed in the final study cohort.

Testing of the 11-miRNA panel

The demographics of the testing cohort are shown in Table 2. The cohort included 44/50 patients (88%) who were Caucasian; 29/50 (58%) were male. The median patient age at the initial blood draw was 61 years. The median number of months until retrieval of the follow-up blood sample in the CRC patients was 3 months, and 85% of patients had the follow-up blood sample taken within 12 months. The median length of follow up was 41 months (interquartile range: 25–49 months), and five patients had CRC recurrence. The median number of months to follow-up blood sample in the CAA patients was 15 months, and 78% of patients had the follow-up blood sample taken within 24 months. Fig. 2 is a flow diagram describing the experimental procedure to validate the panel of 11 miRNA.

CRC patients

Four of the miRNA identified in the screening cohort were significantly increased in expression post- CRC resection (**miR-324**: FC=2.65, $p=0.007$, **Let-7b**: FC=1.97, $p=0.009$, **miR-454**: FC=3.74, $p=<0.001$, **miR-19b**: FC=1.75, $p=0.04$) (Fig. 3). In addition, 3 miRNA from our original diagnostic panel were also increased in expression following resection (**miR-29c**: FC=1.68, $p=0.06$, **miR-374a**: FC=2.07, $p=0.04$, **miR-122**: FC=2.30, $p=0.04$) (**Supplementary Table 2,** Fig. 3). In order to account for the variability in time for obtaining follow-up blood samples, we performed several sensitivity analyses. First, we included only

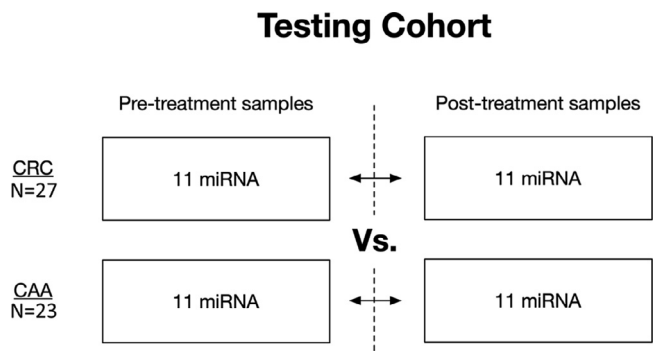

Fig. 2. Flow diagram describing the experimental procedure to validate the previously defined panel of 11 miRNAs. The expression of each of the 11 miRNA were compared between the post-treatment sample and pre-treatment sample of 27 patients with CRC and 23 patients with CAA.

patients who had follow-up blood samples collected within 6 months of surgery. miR-324, let7b, miR-454, miR-374a, and miR-19b were still significantly increased in expression (**Supplementary Table 3**). One other source of potential variation could be the fact that 4 CRC patients were receiving adjuvant chemotherapy at the time of the follow-up blood draw. This was, however, adjuvant chemotherapy to prevent recurrent disease, not to treat recurrent disease. The numbers of patients were too small to permit meaningful analysis.

CAA patients

In contrast, two of the miRNA in the screening cohort were increased in expression post-CAA excision (**miR-324**: FC=1.68, $p = 0.09$ and **miR-454**: FC=2.12, $p = 0.02$) (Fig. 4). Similar to CRC resection, **miR-374a** (FC=2.18, $p = 0.007$) and **miR-122** (FC=3.96, $p = 0.004$) from our original diagnostic panel were also significantly increased in expression (**Supplementary Table 4**, Fig. 4). An additional sensitivity analysis was performed, including only patients who had follow-up blood samples obtained within 18 months of surgery. miR-374a and miR-122 were still significantly increased in expression (**Supplementary Table 5**).

Association with clinical outcomes

As the purpose of this study was to examine whether plasma miRNA expression changed following resection of either CAA or CRC, the examination for associations with clinical outcomes is limited and only descriptive due to sample size. In this patient cohort, 5 of the 27 CRC patients developed recurrent disease a median of 12 months after surgery. For these 5 patients with disease recurrence, the time from surgery to date of post-operative blood draw was less than 2.6 months. At the very early time point sampled in this study, obvious differences in miRNA expression between those patients who developed recurrent disease and those that did not were not apparent **(Supplementary Table 6**). Clearly, further studies with serial patient sampling over time are required.

Discussion

The aim of this study was to identify neoplasia-associated miRNAs that change in expression following CAA removal or CRC resection and that could potentially be used for therapeutic surveillance or monitoring. Four miRNA were selected from a screening analysis and combined with our previous panel of seven miRNA that had been found to be diagnostically predictive of colorectal neoplasia. On validation analysis, six

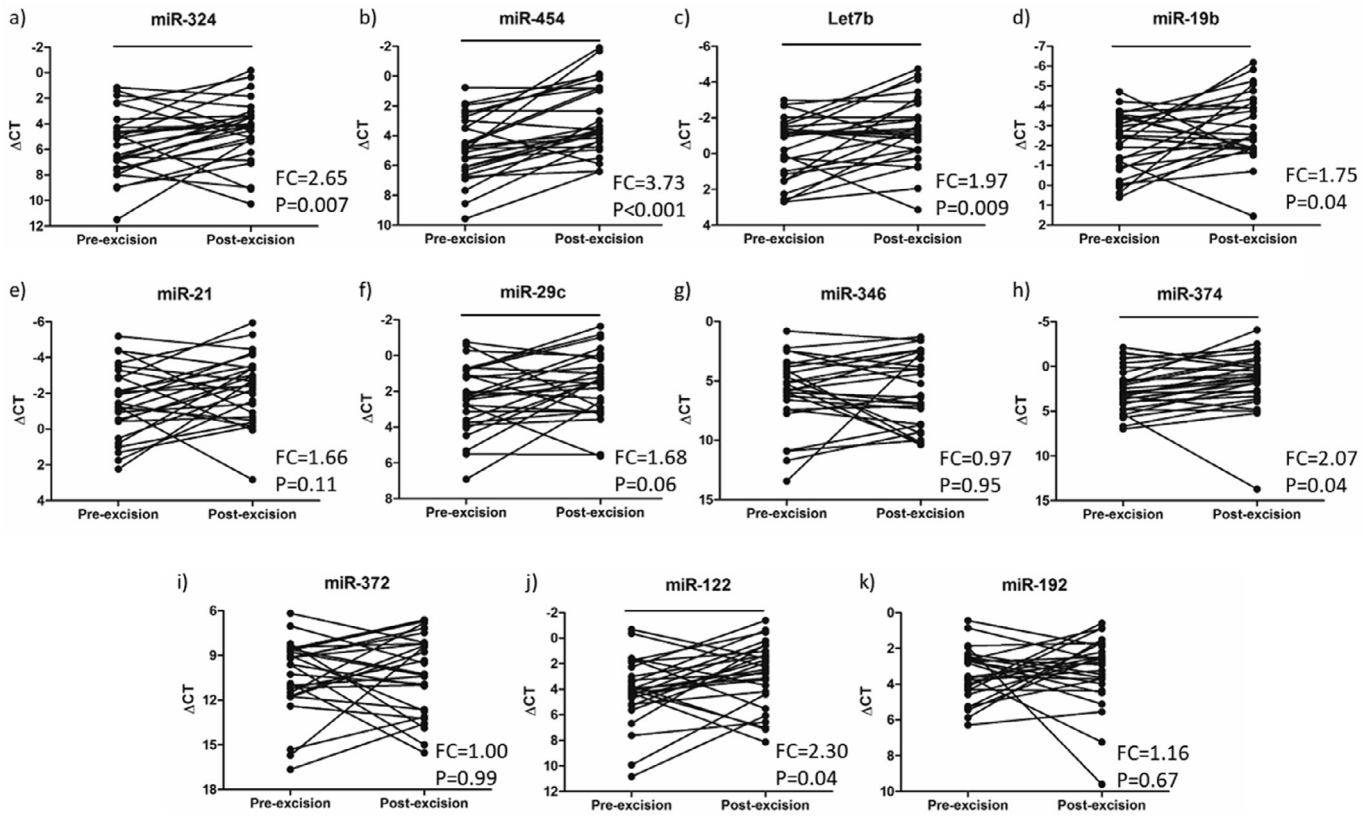

Fig. 3. Scatter plots comparing the pre-treatment and post-treatment expression of each individual miRNA in the CRC cohort. Seven of the miRNA were increased in expression following CRC resection. a-d) Four of the miRNA identified from the screening were significantly increased in expression; miR-324: FC=2.65, $p = 0.007$, Let-7b: FC=1.97, $p = 0.009$, miR-454: FC=3.73, p=<0.001, miR-19b: FC=1.75, $p = 0.04$.) e-k) Three of the miRNA from our previously described diagnostic panel were increased in expression (miR-29c: FC=1.68, $p = 0.06$, miR-374a: FC=2.41, $p = 0.04$, miR-122: FC=2.42, $p = 0.045$). FC- Fold change.

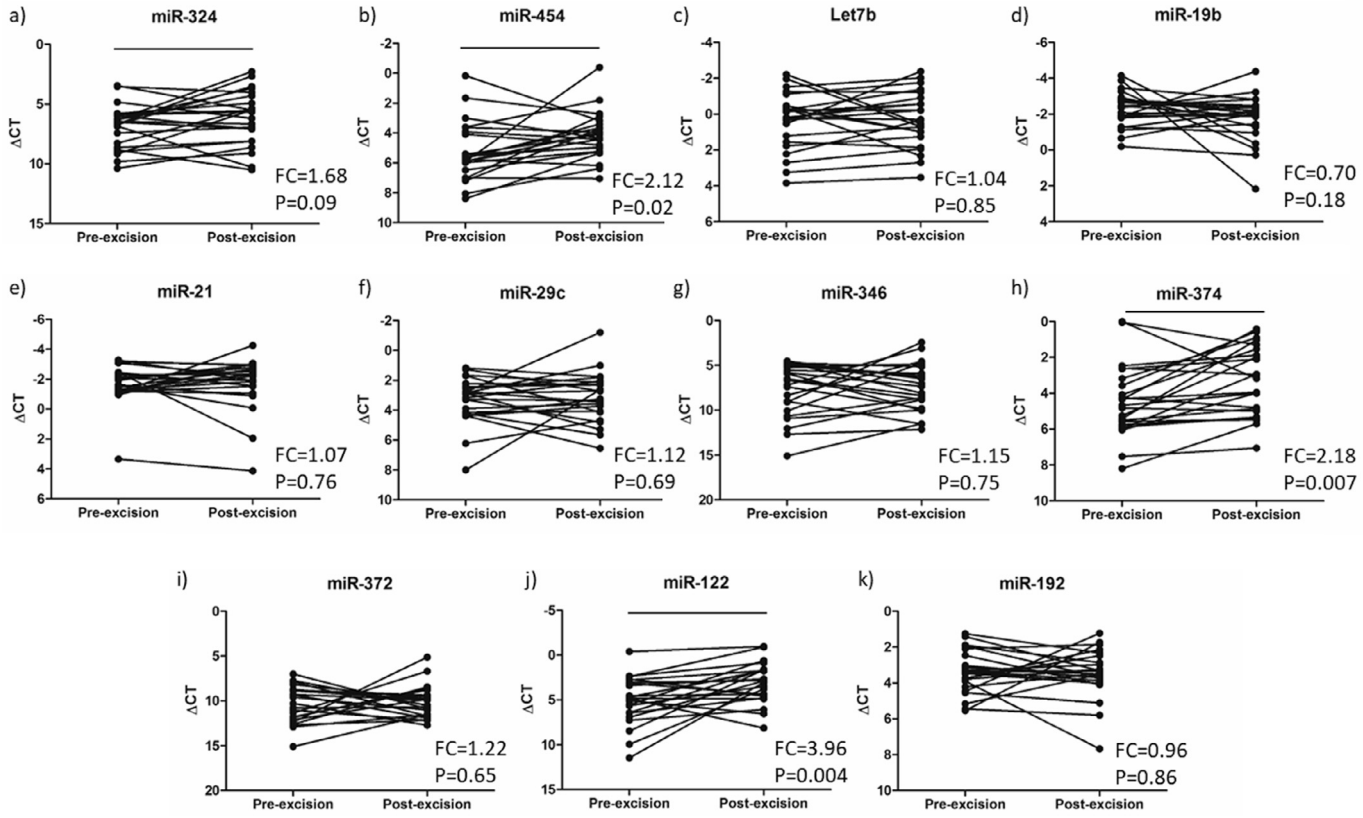

Fig. 4. Scatter plots comparing the pre-treatment and post-treatment expression of each individual miRNA in the CAA cohort. Four of the miRNA were increased in expression following CAA removal. a-d) Two of the miRNA identified from the screening were increased in expression (miR-324: FC=1.68, $p = 0.09$ and miR-454: FC=2.12, $p = 0.02$.) e-k) Two of the miRNAs from our previously described diagnostic panel were significantly increased in expression (miR-374a [FC=2.18, $p = 0.007$] and miR-122 [FC=3.96, $p = 0.004$]) FC-Fold change.

of the miRNA were significantly increased in expression following CRC resection and three miRNA were increased in expression following CAA removal. As expected, the magnitude of fold change for the CRC patients was greater than that for the CAA patients. This study demonstrates the potential use of measuring serial miRNAs (e.g., following CRC resection or CAA removal as a surveillance biomarker).

Due to normal variation in patient care, there was some variability in the time from initial sample retrieval to that of the follow-up blood sample in both the CRC and CAA patients. Despite this, several miRNA were significantly increased in expression and remained significantly increased in expression on sensitivity analysis. This demonstrates the clinical utility and practicality of miRNAs as potential biomarkers, as this variability in time is a normal component of patient care. Our previous study demonstrated the utility of measuring serum miRNA in the diagnosis of colorectal neoplasia [14], but it did not examine the use of this panel for measuring response to therapy. The current study demonstrates the use of measuring serum miRNA as potential biomarkers of treatment response.

The fold changes of the identified miRNA were increased in expression following resection in both the CRC and CAA groups. This is contradictory to some studies. Serum miR-21 was previously shown to decrease in expression at day 7 post-surgical resection in CRC patients [29]; however, the median time from surgery to the follow-up blood sample in our study was 2.6 months. A recent study on circulating tumor DNA demonstrated an initial drop in DNA expression following resection and an increase in expression over time in patients who subsequently have disease recurrence, and to a lesser extent, in those who do not have disease recurrence [30]. The longer time to follow-up blood sample may account for the overall differences between the pre- and post-blood samples. The increase in miRNA expression may be a result of the removal of the CAA or CRC lesion, which was inhibiting miRNA expression from other tissues/organs.

As expected, the magnitude of the miRNA fold change for patients with CRC was greater than that of the CAA patients. In addition, the physiological challenge of surgery (e.g., sympathetic nervous system activation, endocrine, immunologic, and haematologic changes) is significantly greater than that of an endoscopic CAA removal. In trauma surgery, there are short- and long-term changes in gene expression following an initial insult [27,28]. This may, in part, explain the larger magnitude of gene expression in miRNAs associated with cellular survival [29], growth [30,31], and healing[31] in patients undergoing abdominal surgery when compared to patients undergoing endoscopic CAA removal. Serial samples of CRC patients in the immediate postoperative period, and over a longer time period, is warranted to identify longitudinal changes in miRNA expression and to verify these results.

There is significant interest in the identification of biomarkers to detect tumor recurrence. Serial CEA measurement for surveillance following resection is recommended for colorectal cancer, but it has variable sensitivity and specificity [3,32]. A previous murine study demonstrated decreases in blood miRNA expression following surgery, radiation, and chemotherapy for implanted tumors [33]. Ng et al. sought to confirm changes in 2 miRs (miR-17–3p and miR-92) whose expression had been identified to be increased in CRC as compared to controls. Relative expression of these 2 miRs was found to decrease in the 7-day postoperative sample compared to the preoperative sample [34]. Similar studies have demonstrated the differential expression of miRNA in blood following resection in other malignancies such as cervical cancer and hepatocellular carcinoma [17,35]. Leidinger et al. conducted a longitudinal analysis of 1205 plasma miRNAs in 26 lung cancer patients after lung resection at 8 time points over an 18-month period compar-

ing them to 12 controls. They identified 16 miRNAs, including 10 positively and 6 negatively correlated miRNA [36]. Some authors have used a miRNA-sequencing approach to identify differentially expressed miRNAs, beyond that of miRNAs classically associated with tumorigenesis [37]. *Latchana* et al. compared the plasma miRNA expression profile in patients before and after surgical resection of metastatic melanoma ($n = 6$) and identified 2 significant miRNAs for further analysis [37]. The ability to track miRNA expression as a surrogate marker for disease progression has also been explored in breast cancer, where miR-155 was found to be significantly correlated with chemotherapy response [18]. In the current study, we identified and validated six miRNAs in CRC, and three miRNA in CAA, which increased in expression following CRC resection or CAA removal. These studies highlight the relevant clinical utility of miRNA in the care of patients with colorectal neoplasms. The finding that some of the miRNAs are significantly increased following CAA removal is important as studies have shown that a history of adenomas is associated with an increased risk of colorectal cancer [38]. Therefore, a blood-based assay to follow patients with CAA may be of signficiant public health importance.

The measurement of stool DNA expression as a method for screening has come to the forefront in recent years [39]. The expression of miRNA in urine and fecal matter has also been explored in cancer patients. Fecal miR-20a-5p, miR-21–3p, and miR-141 were increased in the stool of patients with CRC, and the expression returned to that of control patients following CRC resection [40]. Similarly, urine miR-146a-5p expression decreased following transurethral resection of bladder cancer [41]. Measurement of miRNAs from other bodily fluids could be an alternative way to improve the sensitivity and specificity of current cancer screening programs.

There are limitations to this study. While all of the samples were paired, our sample size was modest. Due to normal patient variation in the scheduling of post-operative follow-up visits, there was variability in the time to obtaining follow-up blood samples. This represents a study limitation as previous studies have demonstrated that dynamic changes occur in circulating tumor DNA levels post-operatively. This requires further study. In addition, the miRNA screening cards used in this study are restricted to well-characterized miRNA with a biological reason for inclusion. They, therefore, do not quantify the expression of miRNA that would be captured using a next-generation sequencing approach. We do not include data on miRNA expression over time in a cohort of healthy individuals; however, MacLellan et al. have shown miRNA expression in healthy controls have little variability when examining levels in matched samples over up to a 17-month interval [42]. Our results are contrary to some studies examining perioperative miRNA. Serial longitudinal measurement of blood miRNA expression is warranted to investigate dynamic plasma miRNA expression and whether changes in miRNA expression correlate with tumor recurrence or originate from other organ and cell sources that may increase their relative contribution in the absence of the insult. This hypothesis requires further study. Future studies will focus on the measurement of miRNAs in patients who have tumor recurrence as a basis for identifing markers for recurrent disease.

Conclusion

This exploratory study identified miRNAs that increase in expression following CRC resection or CAA removal. It highlights the potential role that plasma miRNA measurements have in the non-invasive monitoring of patients with colorectal neoplasms.

Declaration of Competing Interest

All authors declare no conflict of interest or relevant financial disclosure.

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Susan Galandiuk: *Funding acquisition, Study Concept, Design, and Methodology, Supervision, Drafting of manuscript and final approval*

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2020.100962.

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