Circulating miR-34a Levels are Reduced in Colorectal Cancer

M. NUGENT, MD, N. MILLER, PhD,* AND M.J. KERIN, MCh
Department of Surgery, National University of Ireland, Galway, Ireland

**Introduction:** MicroRNAs (miRNAs) are small, non-coding RNA segments that regulate gene expression via post-transcriptional inhibition and have roles in cell differentiation, proliferation, and apoptosis. Expression differs between tumor and normal tissue in several malignancies. Most work has focused on tissue and cell expression with few reports of circulating miRNAs in colorectal cancer. Available biomarkers for colorectal cancer have limited sensitivity and specificity, thus there is a need for new markers.

**Aims:** This study aimed to identify miRNAs that are differentially expressed in the blood of colorectal cancer patients compared to controls and to establish if this is specific to colorectal cancer and thus could be utilized as potential tumor markers.

**Methods:** Blood samples were collected from 63 colorectal cancer patients and 45 controls. Expression of 7 target miRNAs (miR-143, miR-145, miR-21, miR-30a-3p, miR-31, miR-34a, and miR-92) was measured using RQ-PCR. Results were correlated with clinicopathological data and analyzed. Analysis of differentially expressed circulating miRNAs was expanded to include 62 patients with prostate, renal, breast, and melanoma cancers.

**Results:** Analysis of the relative quantification of the target miRNAs showed significantly reduced expression (P = 0.004) of miR-34a in colorectal cancer. MiR-34a was also significantly reduced in breast cancer (P = 0.019).

**Conclusion:** This study demonstrates significantly reduced expression of circulating miR-34a in colorectal and breast cancer. This may have future application as part of a biomarker profile.


**KEY WORDS:** colorectal cancer; microRNA; miR-34a; circulation; tumor markers

**INTRODUCTION**

Cancers of all types form the second most common cause of death in the developed world (after heart disease), with over 12 million new cases per annum worldwide and a predicted 27 million annual diagnoses expected by the year 2050 [1]. Colorectal cancer is the third most common cancer worldwide and second only to lung cancer as the leading cause of death from cancer in Western countries [2,5].

Prognosis in colorectal cancer is linked to stage at diagnosis, with 5-year survival rates ranging from over 93% for Stage I disease to less than 8% for Stage IV disease [6]. Many patients are symptom-free in the early stages therefore new diagnostic markers are needed to identify disease earlier [2,7].

The most commonly used tumor marker in colorectal cancer is carcinoembryonic antigen (CEA) which is widely used for post-operative surveillance and monitoring response to therapy. However CEA lacks sufficient sensitivity for use as a population screening tool or for detecting colorectal cancer recurrence in isolation [8–12]. Carbohydrate antigen 19.9 (CA19.9) has also been used as a tumor marker but is less sensitive than CEA for colorectal cancer [10]. Faecal markers such as occult blood also lack sensitivity and, although faecal DNA testing shows promise, it is not yet freely available [13]. Therefore there is a real need for sensitive and specific non-invasive biomarkers that can be exploited to detect early neoplastic changes in colorectal cancer.

MicroRNAs (miRNAs) are small (19–25 ribonucleotides), single-stranded non-coding RNAs encoded in plant, invertebrate and vertebrate genomes [4,14–19]. They regulate gene expression at the post-transcriptional level by acting on specific messenger RNA (mRNA) targets, inducing mRNA degradation or translational inhibition [14,15,20–24]. MiRNAs are involved in regulation of pathways in cell differentiation, cell cycle progression and apoptosis [16,25–27]. Each miRNA is assigned a numerical identifier and more than 900 have been identified in humans to date [15,28–30].

The recent discovery that miRNA expression is frequently dysregulated in malignancy has opened the possibility of utilizing these small molecules as tumor biomarkers [31].

Most published studies to date have focused on tissue- and cell-based miRNA expression, however circulating miRNAs also appear to have altered expression levels in several malignancies. This was first reported in patients with diffuse large B-cell lymphoma who had elevated serum levels of miR-21, but alterations in the circulating miRNA profile have subsequently been reported in other cancers including prostate, breast, lung, and gastric [32–38]. At the outset of this study, there were only two small-published studies regarding miRNAs in the circulation of colorectal cancer patients, and we aimed to assess if the circulating miRNA profile is altered in individuals with colorectal cancer compared to healthy controls [39,40]. Since commencing this study, there have been a small number of studies published concerning miRNAs in the plasma of colorectal cancer patients, however the vast majority of studies to date regarding miRNAs and colorectal cancer relate to expression levels in tissues and cell lines [41].

This study aimed to identify specific miRNAs that are differentially expressed in the circulation of patients with colorectal cancer compared to healthy controls and establish if any such alteration in expression is specific to colorectal cancer or part of a generalized cancer phenomenon.


Conflicts of interest: The authors declare no competing interest.

*Correspondence to: N. Miller, PhD, Department of Surgery, Clinical Science Institute, University Hospital Galway, Ireland. Fax: +353-91494509. E-mail: nicola.miller@nuigalway.ie

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METHODS

Patient Cohort and Specimen Collection

Whole blood samples were collected prospectively from 170 participants including 63 colorectal cancer patients (46 colon and 17 rectal cancers), 19 breast cancer patients, 18 prostate cancer patients, 15 renal cell carcinoma patients, 10 malignant melanoma patients, and 45 healthy controls. All of the 125 cancer patients attended University Hospital Galway for management of their malignancy. Each case had a histologically confirmed diagnosis and the histological tumor profiles reflect those of typical cohorts for the respective malignancies. None had received neoadjuvant chemotherapy or radiotherapy prior to collection of the specimens. Samples were collected prior to any tumor resection. The controls were healthy individuals with no known malignancy or active inflammatory condition who provided equivalent whole blood samples. Details of the study cohort are summarized in Table I.

Prior written informed consent was obtained from each participant and ethical approval obtained from the ethics review board of Galway University Hospital. Ten milliliters of whole blood was collected from each participant in Vacuette EDTA K3E blood bottles (Grenier Bio-one, St. Gallen, Switzerland). These samples were stored at 4°C (unprocessed and in the original sample bottles) until RNA extraction was performed. Samples from both cancer patients and controls were collected over a period of 3 years.

RNA Extraction From Blood

Total RNA was extracted from 1 ml aliquots of whole blood using a modified trizol co-purification technique. For each 1 ml of whole blood, phase separation was performed by the addition of 3 ml of a modified trizol co-purification technique. For each 1 ml of whole blood, phase separation was performed by the addition of 3 ml of 1-bromo-4-methoxybenzene was then added to augment the RNA phase separation process. Total RNA was precipitated using isopropanol and washed with 75% ethanol prior to solubilization with 60 µl of nuclease free water. Thus each 1 ml of whole blood yielded 60 µl of total RNA solution, which was stored at −80°C.

RNA concentration was determined using a Nanodrop® Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The wavelength-dependent extinction coefficient ”33” was taken to represent the microcomponent of all RNA in solution. In general concentrations ranging between 20 and 300 ng/µl of miRNA were obtained for each sample.

Selection of miRNA Targets

A panel of 7 candidate miRNAs (miR-143, miR-145, miR-21, miR-30a-3p, miR-31, miR-34a, and miR-92) was chosen for investigation in blood samples from patients with primary colorectal cancer. These miRNAs were selected based on a review of the literature and the results of a miRNA microarray experiment on colorectal tumor tissues (previously conducted in our department) that identified several candidate miRNAs that were differentially expressed in tumor compared to adjacent (tumor associated normal) tissue [42,43]. These are summarized in Table II. As miR-34a was present in significantly lower levels in colorectal cancer patients, it was quantified in the blood samples from the other cancer patients in order to establish if this change was specific to colorectal cancer or part of a more generalized cancer-related phenomenon.

cDNA Synthesis and Real-Time Relative Quantification PCR (RQ-PCR)

For each sample, 100 ng of miRNA was reverse transcribed into cDNA using MultiScribe™ reverse transcriptase and gene-specific stem-loop primers which target the mature miRNA sequence. RQ-PCR was performed using TaqMan™ probes which bind to a complementary sequence in the target gene between the forward and reverse primers. The RQ-PCR was performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using default thermal cycling conditions. MiR-26b cDNA synthesized from pooled normal breast tissue was included on each 96-well plate as an inter-assay control and calibrator. A negative control (nuclease-free water) was also included on each plate. All reactions were performed in triplicate. The threshold standard deviation accepted for intra- and inter-assay replicates was 0.3.

Raw fluorescence data (Ct values) generated by the real-time PCR instrument were exported to qBasePlus software (Biogazelle, Ghent, Belgium), the purpose of which was to scale raw data to an internally defined calibrator and an endogenous control gene to generate relative quantities. MiR-425 was used as the endogenous control gene as this was the most stably expressed of five candidates endogenous controls (let-7a, miR-10b, miR-16, miR-425, miR-31).

<table>
<thead>
<tr>
<th>Target miRNA</th>
<th>Previous association with CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-143</td>
<td>Decreased in colorectal &amp; other tumours</td>
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<tr>
<td>miR-145</td>
<td>Decreased in colorectal &amp; breast tumours</td>
</tr>
<tr>
<td>miR-21</td>
<td>Increased in colorectal &amp; other tumours</td>
</tr>
<tr>
<td>miR-30a-3p</td>
<td>Decreased in colorectal &amp; bladder tumours</td>
</tr>
<tr>
<td>miR-31</td>
<td>Increased in colorectal tumours</td>
</tr>
<tr>
<td>miR-34a</td>
<td>Decreased in colorectal tumours</td>
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<tr>
<td>miR-92</td>
<td>Increased in colorectal tumours</td>
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TABLE I. Candidate miRNA Targets Selected for Investigation

<table>
<thead>
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<th>Target miRNA</th>
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<tr>
<td>miR-143</td>
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<tr>
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<td>Increased in colorectal &amp; other tumours</td>
</tr>
<tr>
<td>miR-30a-3p</td>
<td>Decreased in colorectal &amp; bladder tumours</td>
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<tr>
<td>miR-34a</td>
<td>Increased in colorectal tumours</td>
</tr>
<tr>
<td>miR-92</td>
<td>Increased in colorectal tumours</td>
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TABLE II. Demographic and Clinicopathological Details of the Study Participants (n = 170)

<table>
<thead>
<tr>
<th></th>
<th>Colorectal cancer (n = 63)</th>
<th>Breast cancer (n = 19)</th>
<th>Prostate cancer (n = 18)</th>
<th>Renal cancer (n = 15)</th>
<th>Melanoma (n = 10)</th>
<th>Controls (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>71.2</td>
<td>58</td>
<td>60.6</td>
<td>65.7</td>
<td>52.9</td>
<td>53.4</td>
</tr>
<tr>
<td>Age range</td>
<td>35–90</td>
<td>48–79</td>
<td>50–68</td>
<td>31–78</td>
<td>17–78</td>
<td>27–89</td>
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<td>Gender</td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>44 (70%)</td>
<td>0</td>
<td>18 (100%)</td>
<td>8 (53%)</td>
<td>5 (50%)</td>
<td>11 (24%)</td>
</tr>
<tr>
<td>Female</td>
<td>19 (30%)</td>
<td>19 (100%)</td>
<td>0</td>
<td>7 (47%)</td>
<td>5 (50%)</td>
<td>34 (76%)</td>
</tr>
<tr>
<td>Stage of disease</td>
<td></td>
<td></td>
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<tr>
<td>In-situ</td>
<td>12%</td>
<td>5%</td>
<td>5%</td>
<td>1%</td>
<td>0%</td>
<td>Not applicable</td>
</tr>
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<td>I</td>
<td>5%</td>
<td>5%</td>
<td>1%</td>
<td>4%</td>
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<td>II</td>
<td>22%</td>
<td>64%</td>
<td>9%</td>
<td>53%</td>
<td>5%</td>
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<td>21%</td>
<td>8%</td>
<td>53%</td>
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<td>16%</td>
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and miR-454) analyzed in a subset of samples from 36 colorectal cancer patients and 20 controls. A number of different endogenous controls have been reported as suitable for use in relative quantification of miRNAs, however some of these have not been fully validated in blood, therefore we performed this analysis prior to further experimentation in order to ensure accurate quantification. These candidates endogenous controls were selected based on data from previous microarray experiments showing good stability in tissues [42].

**Statistical Analysis**

Data were analyzed using the software package SPSS (version 18). Due to the magnitude and range of relative miRNA expression levels observed, results data were log transformed for analysis (natural log). All datasets were normally distributed, as verified by the Kolmogorov–Smirnov Z-test. For all two-sample comparisons the two-sample t-test was used to assess differences in mean expression levels. One-way analysis of variance (ANOVA), followed by Tukey HSD post hoc test, was used to compare the mean expression levels of miR-34a across different stages of disease and between different cancer types. Pearson’s correlation was used to assess the relationship between miR-34a and the pre-operative CEA level. All tests were two tailed and results with a $P < 0.05$ were considered statistically significant.

**RESULTS**

**Detection of miRNAs in the Circulation of Colorectal Cancer Patients and Controls**

All seven of the chosen miRNA targets amplified satisfactorily during RQ-PCR and thus were readily detectable at the concentrations used in these assays. The $C\text{\textsubscript{i}}$ value is the amplification cycle number at which the fluorescence generated within a reaction rises above a defined threshold fluorescence [44]. The seven miRNAs attained raw $C\text{\textsubscript{i}}$ values ranging between 17 and 34.

**Relative Quantities of miRNAs**

Expression of each miRNA in the colorectal cancer (n = 63) and control (n = 45) groups is represented in Figure 1.

Expression of miR-143, miR-145, miR-21, miR-30a-3p, miR-31, and miR-92 did not differ significantly between the two groups ($P = 0.327, 0.958, 0.136, 0.417, 0.415,$ and 0.113, respectively).

MiR-34a expression was significantly reduced in the colorectal cancer group ($P = 0.004$). As this study tested seven separate targets of interest on the same cohort, a Bonferroni correction was applied to adjust the accepted cut-off level of significance to 0.007. The $P$-value of 0.004 remained statistically significant after application of this correction.

As miR-34a was significantly reduced in the cancer group, further analysis (using ANOVA) was performed to assess the relationship of miR-34a with stage of disease. There was no significant difference found in this analysis ($P = 0.21$), illustrated in Figure 2.

In order to explore the potential of using miR-34a as a specific biomarker for colorectal cancer, miR-34a expression levels in the circulation of 63 colorectal cancer patients were compared with those of 62 patients with other types of cancer patients and 45 healthy control subjects. Circulating levels miR-34a were found to be significantly reduced in both colorectal and breast cancer groups ($P = 0.038$ and 0.019, respectively), with no significant changes in the remaining cancer groups. This is illustrated in Figure 3. There was no relationship between circulating miR-34a levels and stage or grade of tumors in the colorectal cancer and breast cancer cohorts ($P = 0.297$ and 0.321, respectively).

**DISCUSSION**

This study confirms that several miRNAs are present and readily detectable in whole blood specimens. This appears to be the case even when the samples have been stored for variable periods of time (as some of the samples included in this study were, due to the time taken to accrue sufficient samples for the study) whether at 4°C in the unprocessed whole blood form (in the original EDTA vacuette containers) or in the form of extracted RNA stored at −80°C. This miRNA stability has important practical implications for future clinical applications of miRNA-based assays.

To our knowledge, this is the first report of altered circulating miR-34a expression in colorectal cancer. In this study, miR-34a levels were significantly lower in colorectal cancer patients compared to controls. Reduced miR-34a expression has been previously demonstrated in colorectal cancer tissue samples and miR-34a is thought to be regulated by the P53 tumor suppressor gene and have a role in apoptosis in several malignancies [45–48]. Thus a finding of reduced expression in the circulation of the cancer cohort, while novel, is consistent with previous findings in other tissue types.

While recent blood-based miRNA reports, including the present study, clearly show that malignancy alters miRNA levels in the circulation, it is still unclear how tumor associated miRNAs make their way into the bloodstream. It has been suggested that tumor miRNAs may be present in circulation as a result of tumor cell death and lyses, or alternatively, that tumor cells release miRNAs into the tumor microenvironment, where they enter newly formed blood vessels, and thereby make their way into the circulation [16,38,49].

Many cancer-associated miRNAs that are detectable and quantifiable in circulating fluids appear to be relatively non-specific, particularly those such as miR-21 which has been associated with many different types of malignancy and is sometimes referred to as a “general oncomir” for this reason [50].

This study included multiple different cancer types and the results indicate that, while miR-34a did not appear to behave as a “general oncomir” (i.e., was not dysregulated in all the cancer types tested), the reduction seen in circulating levels is not specific to colorectal cancer as this was also seen in the breast cancer cohort. This provides some partial support to the hypothesis that certain miRNAs are site specific, but certainly does not suggest that lower miR-34a levels are colorectal-cancer specific. It should be noted however, that one of the main limitations of this study is the small numbers in the non-colorectal cancer groups and a different picture may emerge in a study with greater power. Previous authors have suggested that a tumor-specific profile based on a panel of miRNAs may have greater sensitivity and specificity than use of any single miRNA [31,51,52]. Therefore, it is possible that miR-34a combined with another miRNA or miRNAs as part of a “biomarker panel” may together provide sufficient sensitivity and specificity to distinguish colorectal cancer cases from controls and from other types of malignancies, although further work needs to be done in order to clarify this.

Levels of specific miRNAs in serum or whole blood have been shown to be resistant to degradation by endogenous ribonucleases and stable through a number of freeze-thaw cycles [33,35]. Although there is limited data to date regarding temporal stability of miRNAs in unprocessed blood samples, they have been demonstrated to be present in similar levels in formalin-fixed paraffin-embedded (FFPE) colorectal tissues stored for periods from 6 to 28 years and to remain at consistent levels in RNA extracted from lymphocytes and stored at −80°C after periods of 14 days and 10 months [53,54].

Further evaluation of blood-based miRNAs in larger cancer cohorts is necessary to validate these findings, and to further elucidate the feasibility of developing circulating miRNA assays specific for individual cancers.

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Fig. 1. Expression levels of each miRNA in colorectal cancer patients (n = 63) and controls (n = 45). There was a significant difference in miR-34a expression (P = 0.004) but not for any other miRNA. The boxes show the interquartile range and median, whiskers indicate the range and outliers are depicted with the symbol (*)..

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CONCLUSION

Many features of miRNAs, such as their low complexity, stability, and ease with which they are amplified and quantified, make these molecules promising candidates as biomarkers to reflect various physiological and pathological states. The results presented here showing significantly altered circulating miRNA levels in colorectal and breast cancer patients compared to healthy individuals highlights the potential of these molecules as novel non-invasive biomarkers for cancer. However, a more useful biomarker in the clinical setting would be one which is truly specific for a particular cancer type, which was only partially demonstrated in the case of miR-34a, given that levels were altered in both colorectal and breast cancer cases (though not in melanoma, prostate or renal cancers). Further prospective evaluation of blood-based miRNAs, in colorectal and other cancers is needed to explore the potential of circulating miRNAs to emerge as clinically useful novel biomarkers for cancer.

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REFERENCES

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