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Differential Expression of Circulating MiRNAs According To Severity of Colorectal Neoplasia

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Abstract

There is a need to develop a colorectal cancer (CRC) screening test that is noninvasive, cost effective, and sensitive enough to detect preneoplastic lesions. This case-control study examined the feasibility of using circulating extracellular miRNAs to differentiate a spectrum of colorectal neoplasia of various severity and hence for early detection of colorectal neoplasia.

Archived serum samples of 10 normal controls and 31 cases, including 10 with non-advanced adenoma, 10 with advanced adenoma, and 11 with CRC, were profiled for circulating miRNAs using next-generation sequencing. Multiple linear regression, adjusting for age, gender, and smoking status, compared controls and the three case groups for levels of 175 miRNAs that met stringent criteria for miRNA sequencing analysis. Of the 175 miRNAs, 106 miRNAs were down-regulated according to severity of neoplasia and showed a relative decrease in expression from controls to non-advanced adenoma to advanced adenoma to CRC ($P_{trend} < 0.05$). Pair-wise group comparisons showed that 39 and 80 miRNAs were differentially expressed in the advanced adenoma and CRC groups, as compared to the controls, respectively. Differences in miRNA levels between the non-advanced adenoma group and controls were modest.

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Our study found that expression of many miRNAs in serum was inversely correlated with the severity of colorectal neoplasia, and differential miRNA profiles were apparent in preneoplastic cases with advanced lesions, suggesting circulating miRNAs could serve as potential biomarkers for CRC screening.

INTRODUCTION

Screening for colorectal cancer (CRC) by fecal occult blood test (FOBT), fecal immunochemical test (FIT), flexible sigmoidoscopy (FSG), and colonoscopy can lower the incidence and mortality of colorectal cancer.[1-5] However, these recommended screening methods have shortcomings; FOBT and FIT have a low sensitivity (~40%) for detecting preneoplastic lesions,[4,5] while FSG and colonoscopy are limited by their high costs, the requirement of bowel preparation, and their invasive nature.[5,6] Therefore, compliance remains an issue for CRC screening. In 2012, only 65% of adults aged 50-75 years were up-to-date with the U.S. Preventive Services Task Force recommendations for CRC screening.[7] There is a pressing need to develop a screening test that is sensitive, noninvasive, and cost effective to detect biomarkers for both CRC and advanced preneoplastic lesions.

MiRNAs, small non-coding RNAs of ~22 nucleotides in length, mediate gene silencing by binding to specific mRNA targets and repressing translation of mRNA through degradation and/or translational inhibition.[8,9] As such, they have profound effects on regulating gene expression, including genes that are involved in tumorigenesis.[10] Extracellular miRNAs have the potential to serve as cancer biomarkers because they are derived from cells, show tissue specific expression,[11,12] and present in peripheral blood in a remarkably stable form.[13-15] In addition, tumors can be a source of circulating miRNAs.[13,16-18]

Because of these properties, a noninvasive blood test for assessment of extracellular miRNA markers in circulation could potentially be useful as a screening test for CRC. This is supported by observations that differential miRNA expression profiles were found between colorectal tumor and normal tissues,[19-22] that miRNAs dysregulated in tumor tissues also had aberrant levels in plasma of the same colorectal cancer patients,[22,23] and that circulating levels of specific miRNAs decreased after tumor resection.[22,23] Epidemiological studies have also shown differential circulating miRNA profiles between normal controls and individuals with either advanced adenoma or CRC.[22-33]

However, none of the previous epidemiological studies have examined whether circulating miRNA concentrations vary across the spectrum of neoplasia (i.e., from normal to non-advanced adenoma to advanced adenoma to CRC). If so, a screening test for miRNA markers may be feasible for prevention and early detection of CRC. This is the first case-control study of colorectal neoplasia to comprehensively profile circulating miRNAs in serum using next-generation sequencing and to correlate miRNA levels with severity of colorectal neoplasia.

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MATERIAL AND METHODS

Study population

Thirty-one cases with various degree of colorectal neoplasia and 10 controls were identified from the Digestive Diseases Tissue Resource at the University of Pittsburgh. This program routinely collects and archives tissue and blood samples from individuals who participated in colonoscopy screening as well as from those who were diagnosed with CRC in doctors' offices. With informed consent, blood samples were collected before colonoscopy from screening participants or before treatment for colorectal cancer cases, processed within 1-2 hours of collection, and serum samples were stored at -80°C. The 31 cases in this study included 10 with non-advanced adenoma, 10 with advanced adenoma, and 11 colorectal cancer patients. Of the cancer patients, all had adenocarcinoma, 5 had stage I/II cancer, and 6 had stage III/IV cancer. The 10 controls were individuals who were screen negative by colonoscopy.

In addition to the 41 samples from cases and controls, duplicate aliquots from the same blood sample from six subjects were included to assess reproducibility of miRNA sequencing. All 47 samples were processed blindly without knowledge of the case-control status or identity of the duplicate samples. The study protocol was approved by the institutional review board of the two institutes involved in this study; it was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki), and informed consent was obtained.

Lab procedures

Total RNA was purified from 300 μL of serum using the miRNeasy serum/plasma kit (Qiagen). The median total RNA yield was 32 ng/10 L (range:15 - 61). From this, 10 ng of total RNA was used for small RNA cDNA library preparation. Quality of the library constructs was examined using High Sensitivity DNA Chip Kit (Agilent Technologies) for number of peaks, size distribution, and concentration of fragments. All samples showed a library peak around 145 bp, which corresponds to the 5'-3' adapter ligated miRNA constructs derived from the 20-30 nucleotides small RNAs.

MiRNA sequencing was done by Illumina Hi-Seq2000. To analyze sequencing data, we developed an automated analytical pipeline. Briefly, data provided from the HiSeq2000 sequencer in a standard fastq format were trimmed of adapter sequences and low quality reads (more than 3 low quality base-calls) through a C++ program.[34] These sequences were then collapsed to remove redundancy using the Galaxy Genome Browser tool fastx,[35] followed by alignment to the known human miRNA/small RNA database, miRBase (http://www.mirbase.org/), release 19 (August 2012).[36] Before statistical analysis, miRNA read counts were normalized using the reads per million (rpm) method, which divided read count of a given miRNA by the total number of miRNA reads in that sample and multiplied by a million.[37] Following normalization, we applied stringent criteria for a miRNA to be considered for statistical analysis, namely to be present in at least 50% of study samples in greater than 10 copy numbers. MiRNAs with less than 10 reads were not included due to the error rate of Illumina sequencing and stochastic variation in gene expression.[38] Of the 822

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unique miRNAs that were detected, 175 were retained for data analysis. The top two miRNAs that showed differential expression between cases and controls were verified by qRT-PCR.

Statistical analysis

We first assessed reproducibility of miRNA sequencing. Six individuals were sequenced twice. For each person, we calculated Spearman rank correlation coefficient between the duplicate values of 175 miRNAs. For these six subjects, one of the duplicate samples was randomly selected for further statistical analysis described below.

The effects of serum storage on miRNA sequencing were evaluated by Spearman rank correlations between serum storage duration (months between blood draw and RNA extraction) and total miRNA reads in 41 samples. In addition, for each of the175 miRNAs, we examined the Spearman rank correlation between its read count and serum storage time.

The four study groups (colorectal cancer, advanced adenoma, non-advanced adenoma, and controls) were first examined for differences in the established risk factors for colorectal neoplasia, namely age, gender, race/ethnicity, smoking status, and body mass index (BMI), by Mantel-Haenszel chi-square test for trend for categorical variables and by Kruskal-Wallis test for continuous variables. We then compared values of each miRNA among the four study groups using multiple linear regression adjusting for covariates significantly associated with colorectal neoplasia in this study population, including age, gender, and ever smoking. The outcome variable was the log2 transformed normalized miRNA read counts. The regression model generated three β coefficients; each represented the difference in mean miRNA values between a case group and the controls. As read counts were log2 transformed, the β coefficient was equivalent to the log ratio of geometric mean miRNA values of a case group versus that of the control group (i.e., log2 of fold-change). P values for global tests (evaluating significance of all 3 βs simultaneously) and 2-group comparisons (evaluating significance of each β) were obtained.

P values for trend tests were generated by coding and analyzing the four study groups in one linear variable. P values were adjusted for multiple comparisons. An arbitrary cutoff of 0.05 for false discovery rate (FDR) according to the Benjamini and Hochberg procedure was applied to determine statistical significance.[39]

RESULTS

Reproducibility of miRNA sequencing

Duplicate aliquots of the same serum sample from six subjects were processed and sequenced twice to assess reproducibility of miRNA sequencing. The correlations between duplicate values of 175 miRNAs in these six individuals ranged from 0.93 to 0.99 (median = 0.97), indicating excellent reproducibility of miRNA sequencing. Figure 1 shows the duplicate miRNA read counts of one subject.

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Effects of serum storage on miRNA levels

Median storage duration of the 41 serum samples was 29.4 months (range 11.1 – 90.2). There was little correlation between serum storage duration and total miRNA reads from sequencing (r = -0.06). Of the175 miRNAs, 166 (95%) of them had a weak to modest correlation (≤ 0.3) between their read counts and storage duration.

Differential miRNA expression according to severity of neoplasia

Median age of the 41 subjects was 61 years old (range 28 - 91), 49% were males, and 95% were Caucasians; 22 (54%) subjects ever smoked cigarettes, but only 4 (10%) were current smokers. Cases, particularly those with advanced adenoma or CRC, tended to be older and were more likely to be a male and an ever-smoker than controls (Table 1).

The geometric mean counts of 175 miRNAs in the cases and controls, their fold changes adjusted for age, gender, and smoking status, and FDR adjusted p values are shown in Supplemental Table 1. Heatmap in Figure 2 shows differential expression of miRNAs in the four study groups. Levels of 109 miRNAs were significantly correlated with the severity of colorectal neoplasia, as indicated by their p values for trend test (Supplemental Table 1). Of these, levels of 106 miRNAs were inversely associated with the extent of neoplasia and showed a relative decrease in miRNA levels from the controls to non-advanced adenoma to advanced adenoma to the CRC groups. As an example, Figure 3A shows boxplot of miRNA-30b ($P_{trend} = 1.9\times10^{-6}$), which was the most significant among the 106 miRNAs that were down-regulated in the cases. Three miRNAs were up-regulated in the cases with levels increasing with the severity of neoplasia (miRNA-486, miRNA-25, and miRNA-1180). Boxplot of miRNA-486 ($P_{trend} = 4.1\times10^{-4}$), the most significant of the three, is shown in Figure 3B.

Differential miRNA expression in pair-wise group comparisons

Two-group comparisons showed that the smallest differences in miRNA levels were between the non-advanced adenoma group and controls, and none of the miRNAs were significantly different between groups. On the other hand, 39 miRNAs were differentially expressed between the advanced adenoma and control groups, and 80 miRNAs were differentially expressed between the CRC and control groups (Supplemental Table 1). As examples, Table 2 shows the top 2 miRNAs (miRNA-30b and miRNA-30c) that were the most significantly different between the CRC and control groups and the top 2 miRNAs (miRNA-146a and miRNA-30d) that were differentially expressed in the advanced adenoma group. Expression levels of miRNA-30b and miRNA-146a were verified by qRT-PCR. The Spearman rank correlations between qRT-PCR and Illumina sequencing expression levels were 0.61 for miRNA-30b and 0.79 for miRNA-146a; qRT-PCR expression patterns in the study groups were consistent with the Illumina sequencing data (Supplemental Figure 1).

We also repeated the analyses excluding 6 CRC cases with advanced stage III/IV disease to explore if serum miRNAs had the potential to detect early cancers. The results were similar (data not shown). For example, miRNA-30b and miRNA-30c were still among the top miRNAs with significant differential levels between stage I/II CRC cases and controls (FDR adjusted p values were .004 and .007, respectively).

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DISCUSSION

In this case-control study of colorectal neoplasia, we examined the feasibility of sequencing circulating extracellular miRNAs in archived serum samples and demonstrated that next-generation sequencing of miRNA had excellent reproducibility, and that archived serum samples were suitable for this technology. Serum storage duration did not affect quantity of total miRNA or levels of individual miRNAs in serum. Previous studies have also found circulating miRNAs to be present in a remarkably stable form that is resilient to freeze-thawing (as many as 10 cycles), RNase degradation, extended storage, and extreme temperature and pH.[13-15] These observations suggest that archived serum samples from existing cohort and case-control studies could provide valuable resources for efficient evaluation of circulating miRNAs as cancer biomarkers.

We also assessed the feasibility of using miRNAs, profiled by sequencing, to distinguish normal controls from cases with various degree of colorectal neoplasia. We found levels of many miRNAs to be inversely correlated with the extent of neoplasia. About 12 case-control studies in the literature compared circulating miRNAs between normal controls and cases with either CRC or advanced adenoma.[22-33] Most studies found up-regulation of miRNAs in cases, although specific miRNAs were rarely replicated except for miRNA-21 [22,31,33] and miRNA-29a [25,29,31] that were found to be over-expressed in CRC cases in three studies. Our results suggesting that serum concentrations of many miRNAs, including miRNA-21 and miRNA-29a, were down-regulated in the cases were inconsistent with those from previous studies. Nevertheless, down-regulation of miRNA expression globally in tumors as compared to normal tissues had been reported previously.[12]

This discrepancy could be due to the differences in the miRNA detection platform and type of blood sample. While other studies of colorectal neoplasia used qRT-PCR for a few specific miRNAs or microarrays, our study was the first to use miRNA next-generation sequencing. Moreover, almost all previous studies used plasma samples, but we used serum. It has been shown that for some miRNAs, their levels in plasma are different from those in serum.[40,41] MiRNA levels in plasma can be affected by centrifugation conditions, hence the amount of residual platelets and cellular debris that contribute miRNAs, and also by certain anticoagulants (e.g., citrate or heparin) that inhibit qRT-PCR.[9,40,42,43] Nevertheless, our results were unlikely to be attributed to systematic biases. This is because, in our study, miRNAs were not only down-regulated in cases but also decreased with the severity of colorectal neoplasia in a linear trend; blood samples of the adenoma cases and controls were collected before colonoscopy when neoplastic outcome was not known, and all serum samples were processed and sequenced without knowledge of the case-control status.

Our finding that many miRNAs had significantly lower circulating levels in the cases than controls raises a practical concern of whether down-regulated miRNAs could be utilized as cancer biomarkers in a clinical setting, if their low levels are difficult to be detected by qRT-PCR, a technology that is commonly available in clinical diagnostic laboratories, but less sensitive than next-generation sequencing. In our study, the top 2 miRNAs, namely miRNA-30b and miRNA-146a, that were down-regulated in CRC and advanced adenoma

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cases, respectively, were verified by qRT-PCR, demonstrating in principle that qRT-PCR could amplify and detect cancer biomarkers that circulate in a below normal level.

In this study, several members in the miRNA-30 family (miRNA-30b, -30c, and -30d) were among the top miRNAs down-regulated in cases with either advanced adenoma or CRC. It is intriguing that low expression of the miRNA-30 family members have been reported in breast, lung, and colorectal cancers.[44-48] MiRNA-30b, the top miRNA down-regulated in CRC cases in our study, may function as a tumor suppressor – it had lower expression in CRC tissue than normal tissues, suppressed growth of CRC cell lines, and repressed expression of *KRAS*, *PIK3CD*, and *BCL2* genes.[47] MiRNA-30d was one of the top miRNAs down-regulated in cases with advanced adenoma in our study. MiRNA-30d targets GRP78, a protein that is commonly over-expressed in cancers as a major endoplasmic reticulum chaperone and signaling regulator, and has also been found to be down-regulated in colon tumors and a colon cancer cell line.[48]

MiRNA-146a was another top miRNA that was significantly down-regulated in cases with advanced adenoma. It is involved in innate immunity by inhibiting Toll-like receptor signaling and regulating cytokine responses. MiRNA-146a may function as a key negative regulator of inflammation [49-51] and could be involved in the pathogenesis of colorectal neoplasia, an inflammation-associated disease.[52-54] Of the miRNAs that were up-regulated among cases with advanced adenoma or CRC, MiRNA-486 was the most significant. Its role in carcinogenesis, however, is not quite clear. It was reported to suppress NF-κB-negative regulators, resulting in sustained NF-κB activity, in one study,[55] but shown to inhibit tumor growth in others.[56,57] Nevertheless, one study found up-regulation of miRNA-486 in colorectal cancer tissue to be associated with *KRAS* mutation.[58]

With a small sample size, this study was not designed to identify and validate diagnostic or early detection miRNA markers for colorectal neoplasia, but rather a proof of concept to demonstrate differential expression of circulating miRNAs according to severity of disease using next-generation sequencing, which, to the best of our knowledge, has not been studied previously. To further pursue the potential of measuring circulating miRNAs for CRC screening, future studies need to focus on several areas that are neglected by existing studies so far: (1) to discover differentially expressed circulating miRNA markers in cases targeted by screening, specifically a large sample size of individuals with early stage I-II CRC or advanced adenoma; (2) to identify and validate promising miRNA markers while adjusting for possible confounding factors, such as presence of inflammatory bowel disease or other inflammatory conditions, that could affect miRNA expression in study groups; (3) to evaluate whether serum levels of promising markers correlate with those in tissue and are specific for colorectal neoplasia; (4) to examine variation and latency of these promising miRNAs over time in serial blood samples; and (5) to assess the intended clinical utility of qRT-PCR in detecting promising miRNA markers in a target population for CRC screening. Given that most significant markers were down-regulated in individuals with disease in our study, it would be crucial for future studies to pinpoint the markers that, despite their levels in diseased individuals are below normal, can be detected reliably with high sensitivity by a clinical diagnostic technology.

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In summary, in this study of circulating miRNAs in colorectal neoplasia, we found that levels of circulating miRNAs correlated with severity of colorectal neoplasia. Moreover, cases with neoplastic lesions, as early as advanced adenoma and stage I/II CRC, had differential miRNA profiles compared to normal controls. These results, if confirmed and validated, suggest that miRNAs have the potential to be screening biomarkers for prevention and early detection of CRC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMI	body mass index
CRC	colorectal cancer
FDR	false discovery rate
FIT	fecal immunochemical test
FOBT	fecal occult blood test
FSG	flexible sigmoidoscopy
miRNA	micro-RNA
RT	reverse transcription

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Translational Significance

Our study found that levels of circulating miRNAs, determined by sequencing, correlated with severity of colorectal neoplasia. Cases with neoplastic lesions, as early as advanced adenoma and stage I/II cancer, had differential miRNA profiles compared to controls, suggesting that miRNAs have the potential to be screening biomarkers for prevention and early detection of colorectal cancer.

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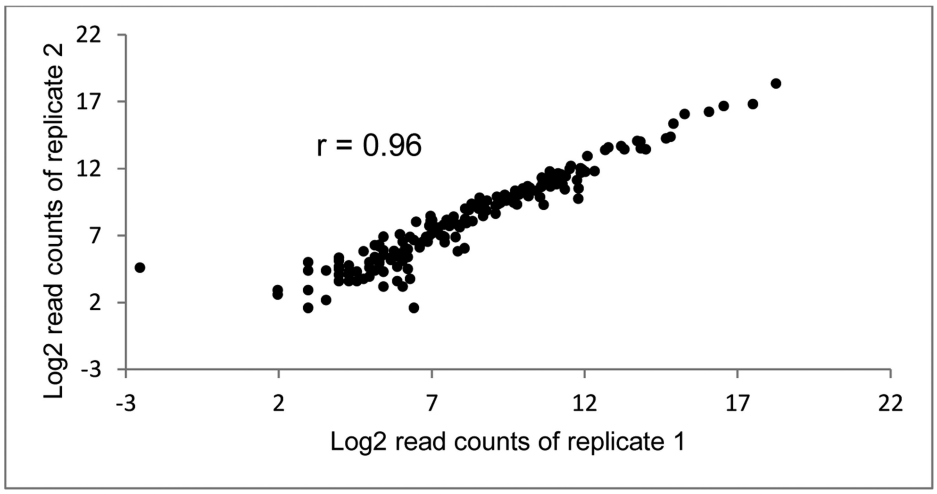

Figure 1.
Replicate read counts of 175 miRNAs in one sample

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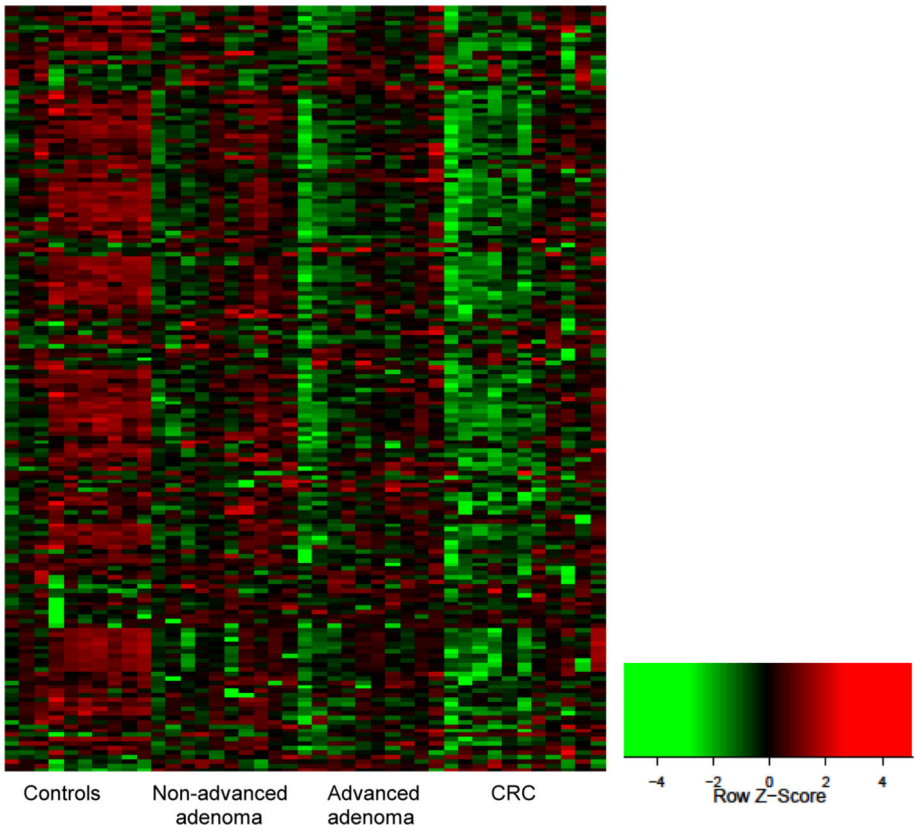

Figure 2.

Heatmap of expression of 175 miRNAs (rows) in 41 serum samples (columns) Red=High relative expression. Green= Low relative expression

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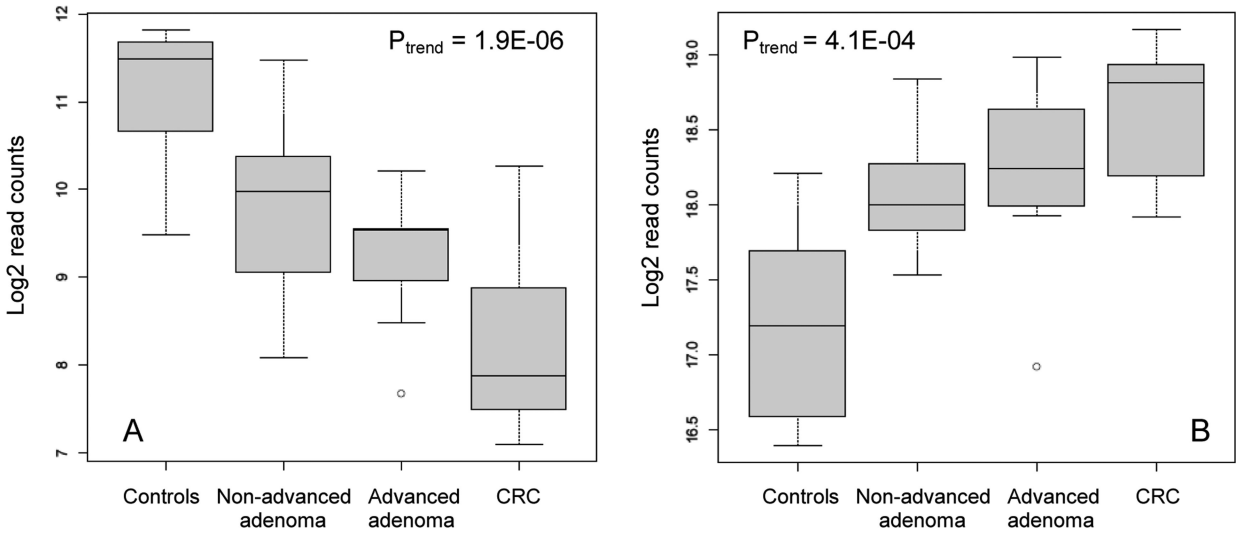

Figure 3.

Boxplots of miRNA-30b (A) and miRNA-486 (B) read counts in the study groups

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Table 1

Characteristics of cases with colorectal neoplasia and controls

	Controls (n=10)	Non-advanced adenoma (n=10)	Advanced adenoma (n=10)	Colorectal cancer (n=11)	P value
Median age (IQR)*	54.5 (52-65)	59.5 (57-72)	57.5 (54 – 79)	68.0 (61 – 87)	0.093
Median BMI (IQR)*	30.7 (25.8-31.2)	29.3 (26.6 – 34.3)	31.0 (23.8 – 36.5)	31.5 (27.0 – 37.1)	0.851
Male, n (%)	4 (40)	2 (20)	6 (60)	8 (73)	0.047
Ever smoked, n (%)	3 (30)	3 (30)	8 (80)	8 (73)	0.012

*IQR = Inter-quartile range.

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Table 2

The top miRNAs differentially expressed in cases with advanced adenoma or CRC compared to controls

	Geometric mean count			Fold change		FDR adjusted P value	
miRNA	**Controls**	**Advanced adenoma**	**CRC**	**Advanced adenoma vs controls**	**CRC vs controls**	**Advanced adenoma vs controls**	**CRC vs controls**
30b	2309	616	288	0.34	0.17	0.028	0.4×10^{-3}*
30c	4241	1074	540	0.30	0.14	0.034	1.9×10^{-3}*
146a	6442	519	973	0.09	0.13	0.7×10^{-3}†	4.9×10^{-3}
30d	30130	12646	13104	0.41	0.39	1.7×10^{-2}†	3.1×10^{-3}

*MiRNA-30b and miRNA-30c had the lowest FDR adjusted p values when the CRC group was compared to the controls.

†MiRNA-146a and miRNA-30d had the lowest FDR adjusted p values when the advanced adenoma group was compared to the controls.