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Clinical Cancer Research

Imaging, Diagnosis, Prognosis

The Prognostic Value of MicroRNAs Varies with Patient Race/Ethnicity and Stage of Colorectal Cancer

Liselle C. Bovell[1], Chandrakumar Shanmugam[1], Balananda-Dhurjati K. Putcha[1], Venkat R. Katkoori[1], Bin Zhang[2], Sejong Bae[3,4], Karan P. Singh[3,4], William E. Grizzle[1,4], and Upender Manne[1,4]

Abstract

Purpose: MicroRNAs (miRNA) have potential prognostic value for colorectal cancers; however, their value based on patient race/ethnicity and pathologic stage has not been determined. The goal was to ascertain the prognostic value of 5 miRNAs with increased expression in colorectal cancers of African American (black) and non-Hispanic Caucasian (white) patients.

Experimental Design: TaqMan quantitative real-time PCR was used to quantify expression of *miR-20a*, *miR-21*, *miR-106a*, *miR-181b*, and *miR-203* in paired normal and tumor colorectal cancer archival tissues collected from 106 black and 239 white patients. The results were correlated with overall survival based on patient race/ethnicity and pathologic stage. Because decisions about adjuvant therapy are important for stage III colorectal cancers, and because *miR-181b* seemed to have prognostic value only for stage III black patients, we assessed its prognostic value in a separate cohort of 36 stage III colorectal cancers of blacks.

Results: All 5 miRNAs had higher expression in colorectal cancers (>1.0-fold) than in corresponding normal tissues. High expression of *miR-203* was associated with poor survival of whites with stage IV colorectal cancers (HR = 3.00; 95% CI, 1.29–7.53), but in blacks it was an indicator of poor survival of patients with stages I and II colorectal cancers (HR = 5.63; 95% CI, 1.03–30.64). Increased *miR-21* expression correlated with poor prognosis for white stage IV patients (HR = 2.50; 95% CI, 1.07–5.83). In both test and validation cohorts, high *miR-181b* expression correlated with poor survival of only black patients with stage III colorectal cancers (HR = 1.94; 95% CI, 1.03–3.67).

Conclusion: These preliminary findings suggest that the prognostic value of miRNAs in colorectal cancers varies with patient race/ethnicity and stage of disease. *Clin Cancer Res; 19(14); 3955–65. ©2013 AACR.*

Introduction

In the United States, colorectal cancer is the third most common malignancy and the second leading cause of cancer-related deaths among men and women (1). Colorectal cancer is a heterogeneous disease, especially with respect to patient race/ethnicity, tumor stage, and genetic alterations that contribute to its progression. Identification of molecular determinants involved in the progression of colorectal cancers will aid in evaluating patient prognosis and/or provide targets for cancer prevention and/or therapy. MicroRNAs (miRNA) are implicated in the progression of and prognosis for colorectal cancers (2–5).

miRNAs are a family of small (20–24 nucleotides) single-stranded, noncoding RNAs that regulate gene expression at the posttranscriptional level (6). They are thought regulate protein production by binding to a complementary site in mRNA, preventing it from being translated or targeting it for destruction, or by transcriptional gene silencing at the chromatin level (7–9). The number of known miRNAs in humans now exceeds 1,500 (10). Each miRNA may regulate hundreds of mRNAs (11).

About half of the human miRNAs are located at cancer-associated regions of the genome, suggesting that they are involved in tumorigenesis (12). Based on cancer-associated alterations in their expression, miRNAs may act as tumor suppressors or oncogenes (13) and are implicated in tumor progression and metastasis (14). Many are dysregulated in human tumors (9). Microarray analyses have revealed specific downregulation of *let-7* expression in lung tumors, but not in breast or colon cancers (15), suggesting that the effects of miRNAs are organ specific.

Because of deletion of the p53 tumor suppressor gene in colon cancer cell lines, several miRNAs are characterized as abnormal. Among these, *miR-15b*, *miR-181b*, *miR-191*, and *miR-200c* are overexpressed in colorectal cancers

Authors' Affiliations: Departments of [1]Pathology, [2]Epidemiology, and [3]Medicine; and [4]Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama

Current address for B. Zhang: Department of Pediatrics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; current address for C.K. Shanmugam: Department of Pathology, Bhaskar Medical College, Moinabad, Andhra Pradesh, India; current address for V.R. Katkoori: Department of Surgery, Michigan State University, College of Human Medicine, East Lansing, Michigan.

Corresponding Author: Upender Manne, Department of Pathology, University of Alabama at Birmingham; WTI-420A, Comprehensive Cancer Center, 1802 6th Avenue South, Birmingham, AL 35294. Phone: 205-934-4276; Fax: 205-934-4418; E-mail: manne@uab.edu

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Translational Relevance

The clinical utility of microRNAs (miRNA), especially for assessing patient prognoses and predicting the efficacy of therapy, is promising. Because most prior studies were conducted in white patient populations, the value of miRNAs in colorectal cancers based on race/ethnicity has not been assessed. We have showed that miRNAs have distinct prognostic value. Increased expression of *miR-21* and *miR-203* were associated with poor survival of whites with stage IV colorectal cancers, whereas *miR-203* was an independent indicator for blacks with early stage colorectal cancers. In contrast, *miR-181b* was an independent prognostic marker only for stage III colorectal cancers of blacks. These findings suggest that, for evaluation of the clinical utility of miRNAs in relation to colorectal cancers, patient race/ethnicity and tumor stage should be considered. Furthermore, these findings have clinical implications in identifying aggressive phenotypes of colorectal cancers and in identifying high-risk patients, thus maximizing the benefits of adjuvant therapy.

compared to normal colorectal tissues, and survival analyses indicate that patients with higher *miR-200c* expression have shorter survival times compared to patients with lower expression (2). Accumulation of *miR-143* and *miR-145* is downregulated in cells derived from colorectal cancers (16). *MiR-21* is expressed at high levels in colonic carcinoma cells, and high *miR-21* expression is associated with poor survival and decreased therapeutic response (3). In colorectal cancers, *miR-133b* and *miR-145* are downregulated and *miR-31*, *miR-96*, *miR-135b*, and *miR-183* are upregulated (17).

Although previous studies have highlighted the potential value of miRNA expression profiles in the prognosis for colorectal cancer patients (2–5), their value with respect to race/ethnicity and tumor stage is not known. In the present investigation, we determined the expression levels of 5 miRNAs (*miR-20a*, *miR-21*, *miR-106a*, *miR-181b*, and *miR-203*), which were the most highly overexpressed miRNAs among a panel of 389 miRNAs assayed in colorectal cancers (3). Furthermore, their prognostic value was analyzed in a consecutive, retrospective colorectal cancer patient cohort of 142 African Americans (blacks) and 239 non-Hispanic Caucasians (whites) with respect to race/ethnicity and tumor stage.

Materials and Methods

Patients and tissue sample collection

Included were 142 black and 239 white colorectal cancer patients who had undergone surgical resection for "first primary" sporadic colorectal cancer between 1985 and 2004 at the University of Alabama at Birmingham (UAB). Thus, this was an "unselected" patient population. Patients with multiple primaries within the colorectum, with multiple malignancies, or with a family or personal history of cancer (due to distinctive molecular pathways) were excluded. We excluded from the study population those patients who died within a week of their surgery; those with surgical margin involvement, unspecified tumor location, multiple primaries within the colorectum, or multiple malignancies; and those patients with a family history of hereditary nonpolyposis colorectal cancer, familial adenomatous polyposis (FAP), or personal histories of colorectal cancer. Because the information from the patient charts may not be reliable in identifying the familial versus sporadic nature of colorectal cancers, our cohort can be described as a "consecutive" patient population. The intent of using patients from this time period was to maximize postsurgery follow-up. Formalin-fixed, paraffin-embedded (FFPE) tissue blocks of colorectal cancers and their corresponding normal (benign colonic epithelial) surgical specimens were collected from UAB. Of 142 blacks, 106 were included as an initial test cohort and 36 as a separate validation cohort. The sample size and power were estimated based on a previous study (3). Demographic, clinical, and pathologic information was collected from medical records, physician charts, and pathology and radiology reports. These data included age, race, gender, tumor location, tumor-node-metastasis (TNM) stage, tumor size, survival times, status, and relapse. Hematoxylin and eosin–stained slides were reviewed by 2 pathologists (C.K. Shanmugam and W.E. Grizzle) together. Pathologic staging was done according to the criteria of the American Joint Commission on Cancer (18). Histologic grade was assessed according to World Health Organization criteria. Well and moderately differentiated tumors were pooled into a low-grade group and poor and undifferentiated tumors into a high-grade group (19). The Institutional Review Board of UAB approved this study.

Patient race/ethnicity and follow-up information

Information on patient race/ethnicity was obtained from their charts, and assignment was self-described or self-identified. We recognize, however, that there is some diversity in identification within any race/ethnic group.

Follow-up information was retrieved from the UAB Tumor Registry. Patients were followed either by the patient's physician or by the UAB Tumor Registry until their death or the date of the last documented contact within the study time frame. The Tumor Registry ascertained outcome (mortality) information directly from patients (or living relatives) and from the physicians of the patients through telephone and mail contacts. This information was further validated against the state Death Registry. Demographic data, including patient age at diagnosis, gender, race/ethnicity, date of surgery, date of the last follow-up (if alive), date of recurrence (if any), and date of death, were collected. The Tumor Registry updated follow-up information every 6 months, and follow-up of our retrospective cohorts ended in September 2012. The median follow-up periods for blacks and whites were 19 years (range 7–29 years) and 15 years (range 7–30 years), respectively.

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RNA isolation and qRT-PCR

Total RNA was extracted from macrodissected tumor and corresponding normal FFPE samples using TRIzol Reagent (Invitrogen). The quality of RNA was determined with a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific). Quantitative real-time PCR (qRT-PCR) of miRNAs was conducted using TaqMan miRNA assays (Applied Biosystems) and was conducted by two-step RT-PCR according to the manufacturer's protocol. Briefly, cDNA synthesis was first accomplished using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The template consisted of 10 ng of total RNA, and miRNA-specific primers were used (provided in TaqMan MicroRNA kits). This PCR reaction was in a 15-μL-reaction mixture and was done on the iQ 5 Real-Time PCR Detection System (Bio-Rad) for 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 85°C. The synthesized target cDNA was amplified using sequence-specific primers from the TaqMan kits. This PCR reaction was accomplished in a 20-μL mixture (1.33 μL of template cDNA) and was conducted for 10 minutes at 95°C, 15 seconds at 95°C, and 1 minute at 60°C for 40 cycles. Signals were collected at the end of each cycle.

Relative expression values were calculated using the comparative C_T method and by normalizing with miRNA *RNU6B* as an endogenous control. Fold change = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T(C_{T\text{miRNA}} - C_{T\text{RNU6B}})_{\text{tumor}} - \Delta C_T(C_{T\text{miRNA}} - C_{T\ \text{RNU6B}})_{\text{normal}}$. The experiments were done in triplicate, and the investigators were blinded to clinical data and survival outcome information until completion of all assays.

Statistical analyses and validation

Sample size and power calculations. The sample size and power analysis were estimated based on a prior study of miRNAs in colorectal cancers (3). In that analysis, increased expression of *miR-21* was a poor prognostic indicator of survival, and 26 of 72 patients (36%) were positive for its overexpression. The HR was 2.5 ($P = 0.01$). Thus, we proposed to evaluate 96 samples. A minimum of 96 samples in each racial group will provide enough power to detect an HR $\geq$ 2.2. Therefore, the sample size ($n = 96$) was sufficient to identify a statistically significant prognostic value for miRNA expression.

Statistical analysis. Chi-square tests were used to assess the univariate associations of baseline characteristics with miRNA expression for black and white patients, separately. The baseline characteristics included demographic variables (age and gender) and pathologic variables (tumor location, size, grade, nodal status, distant metastasis, and stage). Cluster analyses, conducted to define miRNA expression cutoff points, were based on Euclidean distances, which are geometric distances in multidimensional space. This method was chosen to establish cut-off points based on numerical technique. The cutoff of each miRNA was calculated for blacks, whites, and combined patients separately, but remained constant within each race throughout the study. Descriptive statistics were used to describe the basic features of the fold change of each miRNA (Table 2). The type I error rate of each test was controlled at <0.05. All analyses were conducted (by B. Zhang and S. Bae) with SAS application version 9.2 (SAS Institute Inc.).

Deaths due to colorectal cancer were the outcomes (events) of interest. Survival analysis was used to model the relationship between the time to death due to colorectal cancer and miRNA expression. Those patients who died of any cause other than colorectal cancer and those who were alive at the end of the study were considered to be censored. A log-rank test and Kaplan–Meier survival curves were used to compare patient survival in the high and low expression groups for each miRNA. Multivariate Cox regression survival models were built by including all 5 miRNAs and the known confounding covariates (age, sex, tumor location, tumor stage, tumor size, and tumor grade). To detect the relationship for patients with different tumor stages, survival analyses were also conducted for patient groups with each tumor stage (I + II, III, and IV) separately. The final Cox model was obtained based on stepwise selection criteria. All the above analyses were conducted for black, white, and combined patient populations separately. The final Cox model was obtained based on stepwise selection criteria. All the above analyses were conducted for black, white, and combined patient populations separately, and Benjamini–Hochberg corrections were used to adjust for multiple comparisons testing errors (20). This method is an accepted approach for adjusting for multiple comparisons, and similar corrections have been used in other studies of cancer biomarkers (21, 22).

Results

miRNA expression profiles of colorectal cancers

The characteristics of black (test and validation) and white patient cohorts are given in Table 1. Expression levels of 5 miRNAs in the combined population, blacks, and whites are given separately in Table 2. We recently reported that all 5 miRNAs are stable in FFPE tissues stored over long periods of time (23). For the combined population, all miRNAs had higher expression in colorectal cancers compared to normal tissues (>1.0-fold; range 1.33–2.10). All 5 had higher expression in colorectal cancers from blacks (range 3.97–5.98) as compared to colorectal cancers from whites (1.06–2.03; Table 2).

Survival analyses

Data on both univariate Kaplan–Meier and multivariate Cox regression survival analyses of the combined patient population as well as patient groups categorized according to race/ethnicity, tumor stage, and miRNA status are provided in Tables 3 and 4. Univariate survival curve figures are provided only for test and validation data on *miR-181b* to show its independent prognostic value in blacks with stage III colorectal cancers.

Prognostic significance of miR-20a and miR-106a. Univariate survival analyses showed that high expression of *miR-20a* was associated with shorter overall survival (OS) in

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Table 1. Clinicopathological and molecular features of colorectal cancer patients in study

Variable	Blacks		Whites
	Test cohort	Stage III validation cohort	
	N = 106 (%)	*N* = 36 (%)	*N* = 239 (%)
Age group (years)			
<65	52 (49)	13 (36)	117 (49)
≥65	54 (51)	23 (64)	122 (51)
Gender			
Female	71 (67)	18 (50)	104 (44)
Male	35 (33)	18 (50)	135 (56)
Tumor location			
Proximal colon	28 (26)	19 (53)	114 (48)
Distal colon	69 (65)	12 (33)	69 (29)
Rectum	9 (9)	5 (14)	56 (23)
Depth of tumor invasion			
pT0	0 (0)	0 (0)	0 (0)
pT1	4 (4)	0 (0)	8 (3)
pT2	11(10)	2 (6)	29 (12)
pT3	73 (69)	27 (75)	165 (69)
pT4	18 (17)	7 (19)	37 (15)
Nodal status[a]			
N_0	57 (54)	0 (0)	132 (55)
N_{1-3}	49 (46)	36 (100)	97 (41)
Distant metastasis[b]			
M0	89 (85)	36 (100)	199 (83)
M1	16 (15)	0 (0)	40 (17)
Tumor stage			
I	9 (9)		29 (12)
II	46 (43)		100 (42)
III	35 (33)	36 (100)	71 (30)
IV	16 (15)		39 (16)
Tumor grade			
Low	86 (81)	31 (86)	185 (78)
High	20 (19)	5 (14)	53 (22)
Tumor size (cm)[c]			
≤5	66 (62)	18 (50)	152 (64)
>5	40 (38)	16 (44)	87 (36)

NOTE: *N*, total number of cases; %, percentage of *N*.
[a]Information missing for 10 white cases.
[b]Information missing for 1 black case (test).
[c]Information missing for 2 black cases (validation).

the combined patient population when all stages were considered (log-rank, $P = 0.02$) and especially for stage III black colorectal cancer patients (log-rank, $P = 0.017$; Table 3). Similarly, high expression of *miR-106a* was associated with shorter OS for the combined population; for blacks when all stages were considered; and for blacks with stage III colorectal cancers (log-rank, $P = 0.005$, $P = 0.049$, and $P = 0.039$, respectively; Table 3). In our multivariate analyses, however, these 2 miRNAs were not established as independent markers (Table 4).

Prognostic significance of miR-21. Increased expression of *miR-21* correlated with shorter OS of only stage IV patients (HR = 3.25; 95% CI, 1.37–7.72; $P = 0.008$; Table 4), especially for white patients with stage IV colorectal cancers (log-rank, $P = 0.023$; Table 3 and HR = 2.50; 95% CI, 1.07–5.83; $P = 0.034$; Table 4).

Prognostic significance of miR-181b. Although high expression of *miR-181b* was associated with shorter OS of the combined patient population (log-rank, $P = 0.004$; Table 3), it was an independent prognostic marker only for black patients (log-rank, $P = 0.003$; Table 3 and HR = 3.55; 95% CI, 1.50–8.41; Table 4), especially for those with stage III colorectal cancers (test-set, log-rank, $P = 0.008$; Table 3 and Fig. 1A; HR = 7.94; 95% CI, 1.60–39.30; Table 4).

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Table 2. Expression of miRNAs in colorectal cancers

miRNA	Combined ($N = 345$) FC[a,b] (Q1–Q3)[c]	Blacks ($N = 106$) FC[a,b] (Q1–Q3)[c]	Whites ($N = 239$) FC[a,b] (Q1–Q3)[c]
miR-20a	2.05 (0.72–7.31)	4.14 (1.51–27.3)	1.60 (0.61–4.78)
miR-21	2.10 (0.92–5.38)	3.79 (0.85–11.2)	2.03 (1.00–4.10)
miR-106a	2.00 (0.61–8.14)	4.33 (1.06–47.5)	1.58 (0.58–4.58)
miR-181b	1.33 (0.59–3.97)	3.97 (0.68–22.8)	1.06 (0.58–2.34)
miR-203	2.10 (0.40–9.06)	5.98 (0.69–71.5)	1.49 (0.38–5.70)

Abbreviations: *N*, total number of cases; FC, fold change.

[a]Threshold cycle (C_T) is the unit of measurement in qRT-PCR to measure relative gene expression. Difference in C_T ($\Delta\Delta C_T$) = tumor change in Ct minus paired nontumor change in C_T.

[b]Fold change calculated from $\Delta\Delta$Ct, where fold change = $2^{-(\text{median } \Delta\Delta CT)}$. Median fold change values are represented.

[c]Q1 and Q3 for interquartile range of fold change values.

Because *miR-181b* seemed to have a prognostic value for stage III, especially for black patients, both in univariate and multivariate analyses, and studies showing that markers are useful in determining prognosis of minority populations are rare, we validated the prognostic value of *miR-181b* in a separate cohort of 36 stage III colorectal cancers of blacks. Moreover, traditional pathologic features coupled with novel molecular markers could identify aggressive phenotypes within stage III tumors and aid in identifying high-risk patients to maximize the benefits of adjuvant therapy. The demographic and tumor characteristics for this validation cohort are given in Table 1. Because of nonavailability of follow-up information, one case in the validation cohort was excluded from survival analyses.

Similar to the findings of the test cohort of stage III black patients, univariate analysis showed that high expression of *miR-181b* was associated with short patient survival in the validation cohort (log-rank, $P = 0.005$; Fig. 1B). Because the findings of multivariate Cox regression analysis of the validation cohort were similar to findings for the test cohort (HR = 2.75; 95% CI, 1.17–6.48; data not shown), and to increase our sample size to increase statistical power, the test and validation cohorts were pooled for multivariate analysis. Multivariate analyses showed that blacks with stage III colorectal cancers with high expression of *miR-181b* were 1.94 times more likely to die of colorectal cancer than patients in the combined population who had low *miR-181b* expression (HR = 1.94; 95% CI, 1.03–3.67; Table 5).

Prognostic significance of miR-203. High expression of *miR-203* in colorectal cancers correlated with short survival in different groups of patients (Tables 3 and 4). Increased *miR-203* expression had an independent prognostic value for all colorectal cancer patients (combined population, log-rank, $P = 0.002$; HR = 1.49; 95% CI, 1.03–2.17), but especially for blacks with early stage (I + II) colorectal cancers (log-rank, $P = 0.021$; HR = 5.63; 95% CI, 1.03–30.64) and for whites with stage IV disease (log-rank, $P = 0.029$; HR = 3.01; 95% CI, 1.30–6.98).

Discussion

A panel of miRNAs that are overexpressed in colorectal cancers (3) was analyzed to determine their prognostic value for black and white colorectal cancer patients. The results show an increase in the expression of all the miRNAs in colorectal cancers, which is consistent with previous reports (2, 3, 24, 25). Although the prognostic value of these miRNAs has been investigated in various cancers, including colorectal cancers (2, 3, 24, 25), none of the earlier studies dealt with race/ethnicity and tumor stage. The current investigation shows that the prognostic value of different miRNAs varies with tumor stage and patient race/ethnicity. The results show a greater fold increase for all 5 miRNAs in colorectal cancers of blacks than for their white counterparts.

In normal prostate tissues, *miR-301*, *miR-219*, *miR-26a*, *miR-1b-1*, and *miR-30c-1* are expressed three times higher in blacks than in whites (26). Similarly, prostate cancer cell lines generated from blacks have higher expression of *miR-26a* compared to lines derived from whites of similar stage and pathologic grade (27). These results suggest that racial differences exist in the expression levels of some miRNAs. Plausible mechanisms for aberrant expression of miRNAs include the alteration of miRNA copy numbers, epigenetic modification of miRNAs and/or miRNA processing proteins, and single-nucleotide polymorphisms (SNP) in miRNA genes (28–31). Because SNPs are specific to race/ethnicity (32), associations between SNPs in miRNAs or in their target genes may contribute to distinct phenotypic features in different race/ethnic groups. Also, dysregulations of specific miRNAs are associated with stage of the disease and survival in several malignancies (3, 33–36). Furthermore, miRNA expression profiles can reflect specific stages in tumor progression (37); and several miRNAs, referred to as "metastamirs," are involved in tumor metastasis, even though some of these may not have obvious roles in tumorigenesis (38). These reports support our findings related to race- and stage-specific miRNAs in the prognosis of colorectal cancer patients.

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Table 3. Univariate survival analyses based on miRNA status

Race	miRNA	Log rank *P*-value[a,b]	Total (*N*)	Low (*N*)	High (*N*)
Combined					
All stages	*miR-20a*	0.020	344	233	111
	miR-21	0.223	344	232	112
	miR-106a	0.005	344	231	113
	miR-181b	0.004	344	234	110
	miR-203	0.002	344	233	111
Stage I + II	*miR-20a*	0.236	184	136	48
	miR-21	0.396	184	125	59
	miR-106a	0.236	184	136	48
	miR-181b	0.303	184	138	46
	miR-203	0.360	184	136	48
Stage III	*miR-20a*	0.877	105	68	37
	miR-21	0.567	105	69	36
	miR-106a	0.158	105	63	42
	miR-181b	0.125	105	64	41
	miR-203	0.045	105	63	42
Stage IV	*miR-20a*	0.965	55	29	26
	miR-21	0.155	55	38	17
	miR-106a	0.585	55	32	23
	miR-181b	0.722	55	32	23
	miR-203	0.920	55	34	21
Blacks					
All stages	*miR-20a*	0.065	106	72	34
	miR-21	0.149	106	72	34
	miR-106a	0.049	106	72	34
	miR-181b	0.003	106	72	34
	miR-203	0.005	106	71	35
Stage I + II	*miR-20a*	0.368	55	36	19
	miR-21	0.287	55	38	17
	miR-106a	0.471	55	38	17
	miR-181b	0.189	55	38	17
	miR-203	0.021	55	39	16
Stage III	*miR-20a*	0.017	35	26	9
	miR-21	0.331	35	24	11
	miR-106a	0.039	35	25	10
	miR-181b	0.008	35	24	11
	miR-203	0.071	35	22	13
Stage IV	*miR-20a*	0.879	16	10	6
	miR-21	0.891	16	10	6
	miR-106a	0.766	16	9	7
	miR-181b	0.891	16	10	6
	miR-203	0.879	16	10	6
Whites					
	miR-20a	0.978	238	161	77
	miR-21	0.680	238	161	77
	miR-106a	0.415	238	160	78
	miR-181b	0.123	238	162	76
	miR-203	0.696	238	163	75
Stage I + II	*miR-20a*	0.873	129	94	35
	miR-21	0.399	129	93	36
	miR-106a	0.486	129	96	33
	miR-181b	0.371	129	99	30
	miR-203	0.408	129	97	32

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Table 3. Univariate survival analyses based on miRNA status (Cont'd)

Race	miRNA	Log rank *P*-value[a,b]	Total (*N*)	Low (*N*)	High (*N*)
Stage III	*miR-20a*	0.513	70	42	28
	miR-21	0.089	70	42	28
	miR-106a	0.919	70	38	32
	miR-181b	0.509	70	42	28
	miR-203	0.811	70	39	31
Stage IV	*miR-20a*	0.482	39	25	14
	miR-21	0.023	39	26	13
	miR-106a	0.184	39	26	13
	miR-181b	0.752	39	21	18
	miR-203	0.029	39	27	12

NOTE: Combined population includes all stages and all races/ethnic groups.

[a]Log-rank, *P*-value estimated from Kaplan–Meier univariate analysis.

[b]Adjustment for multiple comparisons has been made for the *P* values using the Benjamini–Hochberg method.

In this investigation, there was no substantial correlation between increased *miR-21* levels and poor patient prognosis when all stages were considered, as previously reported (3, 39, 40). However, once the population was stratified by patient race/ethnicity and tumor stage, high expression of *miR-21* was associated with poor prognosis of white patients with stage IV colorectal cancers. In this study, miRNAs were isolated from macrodissected tumor tissues, whereas prior studies evaluated miRNAs from whole colorectal cancer tissues (3, 27, 39). Highly expressed in the stroma of colorectal cancers, *miR-21* is associated with shorter disease-free survival (41). Moreover, there is a differential pattern of expression of miRNAs during colorectal cancer progression (42). These factors may have contributed to the inconsistency between our findings and those of previous studies. Similar to our findings, the high expression of *miR-21* in advanced stages of colorectal cancers correlates with distant metastases and shorter patient survival (39, 40, 43). Furthermore, highly expressed *miR-21* is more common in stage IV colorectal cancers than in stages II and III colorectal cancers (44). These reports support our finding of *miR-21* as an independent prognostic indicator of patients with stage IV colorectal cancers.

Increased *miR-181b* was identified and validated as an independent marker of poor prognosis for black patients with stage III colorectal cancers. There are high levels of miR-181b in colorectal cancers (2) and in sessile serrated adenomas, know aggressive lesions, relative to hyperplastic polyps (45). Even though there is high *miR-181b* expression in colorectal cancers, it was considered to have no prognostic value (46). Our findings, however, suggest that, for stage III black patients, *miR-181b* is a prognostic marker that could aid in identifying high-risk patients who would benefit from adjuvant therapy. *MiR-181b* acts as an epigenetic switch to inhibit the tumor suppressor *CYLD*, thereby increasing NF-κB activity and maintaining the transformed state of tumor cells (47). *In silico* analyses have predicted several target genes of *miR-181b* that are involved in cell-cycle regulation, cell signaling, and chemosensitivity (2). SNPs in the *miR-181b* binding sites of the targets may be a reason for increased expression of *miR-181b* in colorectal cancers. For example, in one of the proposed targets of *miR-181b*, GATA6, which has an oncogenic function in gastrointestinal cancers (48), 3 SNPs have been identified (49). Because SNPs are race/ethnic specific and have prognostic value in colorectal cancers (50), there may be an association between *miR-181b* and SNPs in its targets that contributes to colorectal cancer carcinogenesis in black patients. However, the underlying mechanisms for the prognostic value of *miR-181b* in black patients with stage III colorectal cancers need to be explored.

Our study has found, for the combined population, that high expression of *miR-203* is an independent marker of poor prognosis of colorectal cancers. Furthermore, *miR-203* is an independent marker of poor prognosis for blacks with stage I or II disease, but, in whites, it is a marker for stage IV disease. In colorectal cancer patients <40 years of age, there is increased expression of *miR-203* that correlates with aggressive tumor behavior (25). Elevated expression of *miR-203* is a predictor of poor prognosis for pancreatic adenocarcinoma (51, 52) and for breast cancer (53). In contrast, there is decreased expression of *miR-203* in lung cancer cells, and it inhibits proliferation and invasion (54). These findings suggest that the role of *miR-203* varies with the type and stage of cancer and patient race.

Although this study did not find an independent prognostic value for *miR-20a* or *miR-106a*, in univariate analyses, their increased expression correlated with poor survival in the combined population. A meta-analysis of 20 studies on miRNA expression levels in colorectal cancers found upregulation of *miR-20a* in more than one study (55). High levels of *miR-20a* in gastric and gastrointestinal cancers correlate with reduced survival (56, 57). *miR-106a* highly is expressed in metastatic colorectal cancer cell lines (58),

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Table 4. Cox multivariate regression analysis to evaluate the independent prognostic value of miRNAs in colorectal cancers

Combined				Blacks				Whites			
Prognostic variables	**Indicator of poor prognosis**	**HR[a] (95% CI)**	***P* value[c]**	**Prognostic variables**	**Indicator of poor prognosis**	**HR[a] (95% CI)**	***P* value[c]**	**Prognostic variables**	**Indicator of poor prognosis**	**HR[a] (95% CI)**	***P* value[c]**
All stages ($N = 344$)				All stages ($N = 106$)				All stages ($N = 238$)			
miRNA				miRNA				Tumor stage			
miR-203	High	1.49 (1.03–2.17)	0.036	*miR-181b*	High	3.55 (1.50–8.41)	0.004	II vs. I	II	3.55 (1.08–11.63)	0.038
Tumor stage				Tumor stage				III vs. I	III	6.22 (1.90–20.37)	0.002
II vs. I	II	1.60 (0.75–3.40)	0.224	II vs. I	II	0.80 (0.27–2.40)	0.701	IV vs. I	IV	26.43 (7.91–88.32)	<0.0001
III vs. I	III	2.97 (1.41–6.28)	0.004	III vs. I	III	2.38 (0.83–6.79)	0.107				
IV vs. I	IV	10.11 (4.66–21.93)	<0.0001	IV vs. I	IV	4.19 (1.31–13.41)	0.017	Tumor grade			
Tumor grade				Tumor location				High vs. low	High	1.54 (0.99–2.38)	0.057
High vs. low	High	1.60 (1.13–2.28)	0.009	Proximal colon vs. Rectum	Proximal colon	1.60 (1.13–2.28)	0.061				
				Distal colon vs. Rectum	Distal colon	1.43 (0.33–6.21)	0.644				
				Tumor grade							
				High vs. low	High	2.21 (1.14–4.29)	0.019				
Stage I + II ($N = 184$)				Stage I + II ($N = 55$)				Stage I + II ($N = 129$)			
Tumor grade				miRNA				Tumor grade			
High vs. low	High	1.68 (0.86–3.28)	0.132	*miR-203*	High	5.63 (1.03–30.64)	0.046	High vs. low	High	1.52 (0.60–3.83)	0.382
				Tumor grade							
				High vs. low	High	3.67 (1.30–10.37)	0.015				
Stage III ($N = 105$)				Stage III ($N = 35$)				Stage III ($N = 70$)			
miRNA				miRNA				Tumor grade			
miR-203	High	1.90 (1.05–3.42)	0.034	*miR-181b*	High	7.94 (1.60–39.30)	0.012	High vs. low	High	1.40 (0.63–3.12)	0.416
Tumor grade				Tumor grade							
High vs. low	High	1.27 (0.66–2.42)	0.480	High vs. low	High	0.28 (0.03–2.42)	0.249				
Stage IV ($N = 55$)				Stage IV[b] ($N = 16$)				Stage IV ($N = 39$)			
miRNA								miRNA			
miR-21	High	3.25 (1.37–7.72)	0.008					*miR-21*	High	2.50 (1.07–5.83)	0.034
miR-203	High	2.19 (1.10–4.33)	0.026					*miR-203*	High	3.01 (1.30–6.98)	0.011
Tumor grade								Tumor grade			
High vs. low	High	3.76 (1.85–7.63)	0.0003					High vs. low	High	3.12 (1.29–7.53)	0.012

[a]Adjusted for miRNA expression levels, age, gender, TNM tumor stage, tumor location within the colorectum, tumor grade, tumor size, and race (for analyses of the Combined patient population); CI, confidence interval.

[b]Because the small number of available cases, multivariate analyses was not completed for stage IV black colorectal cancer patients.

[c]Adjustment for multiple comparisons has been made for the *P* values using the Benjamini–Hochberg method.

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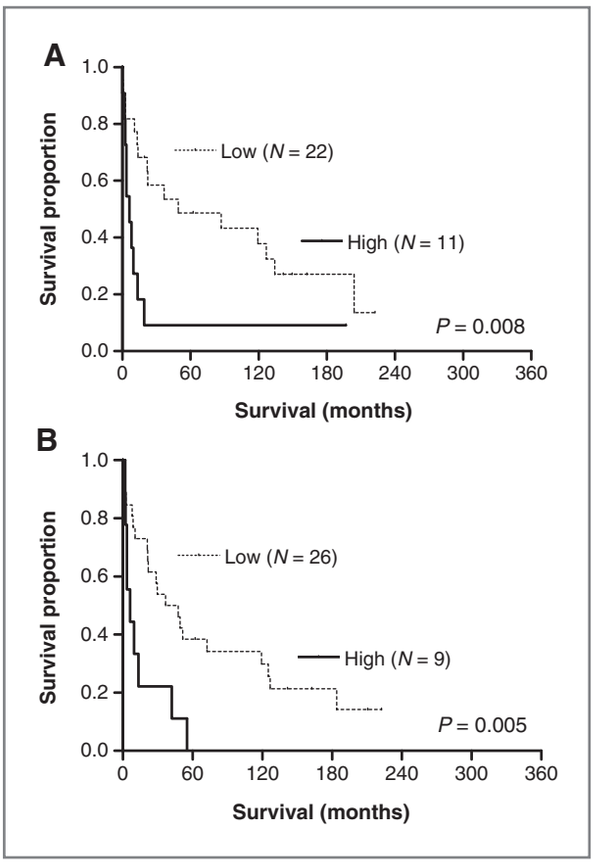

Figure 1. Kaplan–Meier univariate survival analysis based on *miR-181b* expression levels in the test (A) and validation (B) cohorts of black stage III colorectal cancer patients.

colorectal cancers tissues (3, 59), and stool samples of colorectal cancer patients (60). With respect to colorectal cancer prognosis, conversely to what we found, low expression of *miR-106a* was associated with decreased survival in a Spanish colorectal cancer population (61).

We acknowledge that there are limitations of our study. To begin, this study was conducted with a sample set collected at a single medical center, thus, it is not a population-based study. The samples used were remnants of diagnostic tissues that were processed and archived for more than two decades. Thus, there may be biases related to tissue collection and processing (62). For the discovery and validation of biomarkers, reference sample sets that are collected following standardized protocols are ideal. Furthermore, the validation studies should ideally be conducted on a sample cohort collected from a different institution. However, because limited resources and the exploratory nature of this study, both our test and validation cohorts were collected from the same institution. Nevertheless, our future studies will focus on validating these findings in reference colorectal cancer samples collected from blacks and whites of different geographical regions of the United States. Finally, although we used self-identified race to categorize patients into blacks and whites, we recognize that there is some diversity in identification within any race or ethnic group.

Table 5. Cox multivariate regression analysis to evaluate the independent prognostic value of *miR-181b* in the combined test and validation cohorts of black stage III colorectal cancers ($N = 70$)

Prognostic variables	Indicator of poor prognosis	HR (95% CI)[a]	*P* value[b]
miRNA			
miR-181b	High expression	1.94 (1.03–3.67)	0.043
Tumor grade			
High vs. low	High	1.89 (0.91–3.91)	0.092

[a]Adjusted for miRNA expression levels, age, gender, TNM stage, tumor location within the colorectum, tumor grade, and tumor size; CI, confidence interval.
[b]Adjustment for multiple comparisons has been made for the *P* values using the Benjamini–Hochberg method.

The present investigation is the first to evaluate the prognostic value of *miR-20a*, *miR-21*, *miR-106a*, *miR-181b*, and *miR-203* in colorectal cancers based on patient race/ethnicity and tumor stage. These miRNAs were expressed greater than twofold higher in primary colorectal cancers of black patients than in their white counterparts. Furthermore, *miR-21* is an independent prognostic marker for stage IV colorectal cancers of whites; *miR-181b* is an independent prognostic marker for stage III colorectal cancers of blacks; and *miR-203* is an independent prognostic marker of blacks with early-stage colorectal cancers and for whites with late-stage colorectal cancers. Although these findings need to be validated in prospective studies, the results warrant that race/ethnicity of patients and stage of the disease should be considered in assessing the clinical utility of miRNAs in colorectal cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L.C. Bovell, C. Shanmugam, S. Bae, W.E. Grizzle, U. Manne
Development of methodology: L.C. Bovell, B.D.K. Putcha, B. Zhang, S. Bae, U. Manne
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.C. Bovell, C. Shanmugam, U. Manne
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.C. Bovell, C. Shanmugam, V.R. Katkoori, B. Zhang, S. Bae, K.P. Singh, W.E. Grizzle, U. Manne
Writing, review, and/or revision of the manuscript: L.C. Bovell, C. Shanmugam, B. Zhang, S. Bae, K.P. Singh, W.E. Grizzle, U. Manne
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.C. Bovell, W.E. Grizzle, U. Manne
Study supervision: W.E. Grizzle, U. Manne

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The Prognostic Value of MicroRNAs Varies with Patient Race/Ethnicity and Stage of Colorectal Cancer

Liselle C. Bovell, Chandrakumar Shanmugam, Balananda-Dhurjati K. Putcha, et al.

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