MicroRNA *MIR21* and T Cells in Colorectal Cancer

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Abstract

The complex interactions between colorectal neoplasia and immune cells in the tumor microenvironment remain to be elucidated. Experimental evidence suggests that microRNA *MIR21* (miR-21) suppresses antitumor T-cell–mediated immunity. Thus, we hypothesized that tumor *MIR21* expression might be inversely associated with T-cell density in colorectal carcinoma tissue. Using 538 rectal and colon cancer cases from the Nurses' Health Study and the Health Professionals Follow-up Study, we measured tumor *MIR21* expression by a quantitative reverse-transcription PCR assay. Densities of CD3$^+$, CD8$^+$, CD45RO (PTPRC)$^+$, and FOXP3$^+$ cells in tumor tissue were determined by tissue microarray immunohistochemistry and computer-assisted image analysis. Ordinal logistic regression analysis was conducted to assess the association of *MIR21* expression (ordinal quartiles as a predictor variable) with T-cell density (ordinal quartiles as an outcome variable), adjusting for tumor molecular features, including microsatellite instability; CpG island methylator phenotype; *KRAS*, *BRAF*, and *PIK3CA* mutations; and LINE-1 methylation. We adjusted the two-sided α level to 0.012 for multiple hypothesis testing. Tumor *MIR21* expression was inversely associated with densities of CD3$^+$ and CD45RO$^+$ cells ($P_{trend} < 0.0005$). The multivariate odds ratio of the highest versus lowest quartile of *MIR21* for a unit increase in quartile categories of CD3$^+$ or CD45RO$^+$ cells was 0.44 [95% confidence interval (CI), 0.28 to 0.68] or 0.41 (95% CI, 0.26–0.64), respectively. Our data support a possible role of tumor epigenetic deregulation by noncoding RNA in suppressing the antitumor T-cell–mediated adaptive immune response and suggest *MIR21* as a potential target for immunotherapy and prevention in colorectal cancer. *Cancer Immunol Res; 4(1); 33–40. ©2015 AACR.*

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Introduction

Accumulating evidence indicates that innate and adaptive immunity influences tumor evolution (1). Attesting to an important role of T-cell–mediated adaptive immunity in inhibiting tumor progression, therapeutic antibodies specific for immune checkpoint molecules, including CTLA4, PDCD1 (programmed cell death 1; PD-1), and CD274 (programmed cell death 1 ligand 1; PD-L1), can effectively enhance antitumor T-cell activity in various cancers (2, 3). Emerging evidence suggests complex roles of tumor genetic alterations and tumor–host interactions in response to T-cell–based immunotherapies (4, 5). Although these immunotherapies appeared to be less effective for colorectal cancer, intense infiltrates of T cells in colorectal cancer tissue have been associated with better patient survival (6–8), and studies have suggested a potential role of immune checkpoint pathways in suppressing antitumor immune responses in a subset of colorectal cancers (9, 10). A high degree of microsatellite instability (MSI-high) in colorectal cancer is associated with intense infiltrates of T cells, as mismatch repair defects in MSI-high tumors cause numerous frameshift mutations and truncated proteins (neopeptides), which elicit antitumor T-cell–mediated adaptive immunity (11–13). However, MSI status is not the sole determinant of immune response to colorectal cancer, because the numbers of tumor-infiltrating T cells considerably overlap between MSI-high and microsatellite-stable (MSS) colorectal tumors (7, 9, 13). Hence, other factors may influence the antitumor immune response to colorectal cancer.

MicroRNAs (miRNA) are short noncoding RNAs (18–24 nucleotides in length) that play substantial roles in epigenetic gene regulation in diverse biologic and pathologic processes, including immunity and carcinogenesis (14, 15). Among various miRNAs, *MIR21* (miR-21) has been shown to play roles in immunity and colorectal carcinogenesis (16–18). In fact, high *MIR21* expression in colorectal cancer tissue has been associated with worse clinical outcome, suggesting *MIR21* as a prognostic tumor biomarker (19, 20). *MIR21* is expressed in colorectal cancer cells (20, 21), and *MIR21* increases amounts of IL10 and prostaglandin E_2 (PGE_2) in the tumor microenvironment *in vivo* (22–24). IL10 and PGE_2 can suppress antitumor T-cell–mediated adaptive immunity through inhibition of the antigen-presenting capacities of dendritic cells and recruitment of myeloid-derived suppressor cells into the tumor microenvironment (25, 26). Therefore, we hypothesized that higher *MIR21* expression might be associated with fewer T cells in colorectal cancer tissue. A better understanding of the relationship between miRNAs and immune cells in the tumor microenvironment may open opportunities to use miRNAs for immunotherapy and prevention of colorectal cancer.

To test our hypothesis, we analyzed two U.S. nationwide prospective cohort studies [the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS)] and examined tumor *MIR21* expression in relation to densities of CD3^{+}, CD8^{+}, CD45RO (PTPRC)[+], and FOXP3^{+} T cells in colorectal cancer tissue.

Materials and Methods

Study population

We used the databases of two U.S. nationwide prospective cohort studies, the NHS (121,701 women who enrolled in 1976) and the HPFS (51,529 men who enrolled in 1986; refs. 27, 28). Every 2 years, participants were sent follow-up questionnaires to gather information on health and lifestyle factors, and to identify newly diagnosed cancers and other diseases. Medical records were reviewed, and the cause of death was assigned by study physicians. The National Death Index was used to ascertain deaths of study participants and identify unreported lethal colorectal cancer cases. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from hospitals where participants with colorectal cancer had undergone tumor resection. Hematoxylin and eosin–stained tissue sections from all colorectal cancer cases were reviewed by a pathologist (S. Ogino), who was unaware of other data. Tumor differentiation was categorized as well to moderate or poor (>50% vs. ≤50% glandular area). On the basis of the availability of data on tumor *MIR21* expression and T-cell densities, a total of 538 colorectal cancer cases were included. Written informed consent was obtained from all study participants. Tissue collection and analyses were approved by the human subjects committee at the Harvard T.H. Chan School of Public Health and the Brigham and Women's Hospital (Boston, MA).

RNA isolation and quantitative RT-PCR for *MIR21*

RNA was extracted from colorectal cancer tissue and adjacent nontumor colonic mucosa in whole-tissue sections of FFPE specimens with the use of RecoverAll Total Nucleic Acid Isolation Kit (Ambion Inc.). The quantitative RT-PCR assays for *MIR21* and *RNU6-2* were performed according to miScript PCR System protocol (Qiagen). Briefly, complementary DNA (cDNA) was
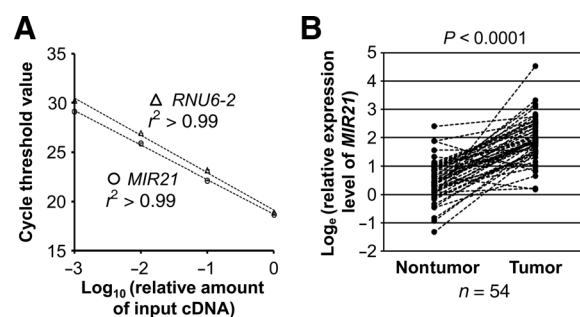

Figure 1.
MIR21 expression in colorectal cancer. A, quantitative RT-PCR assays for *MIR21* and *RNU6-2* using 10-fold dilution series (1:1,000, 1:100, 1:10, and 1:1) from the same specimen. Mean cycle threshold values (± SD) of triplicate runs and the coefficient of determination (r^2) in the assays for *MIR21* and *RNU6-2* are shown. cDNA, complementary DNA. B, *MIR21* expression in 54 pairs of colorectal cancer and adjacent nontumor colonic mucosa. A statistical analysis was performed using the two-sided Wilcoxon signed rank test.

synthesized with the use of miScript II RT Kit (Qiagen). Each reaction was performed in a 25-μL solution containing 1× final concentration QuantiTect SYBR Green PCR Master Mix (Qiagen) and each miScript Primer Assay (Qiagen) specific for *MIR21* (cat. MS00009079) and *RNU6-2* (cat. MS00033740) in a 96-well optical PCR plate. Amplification and detection of *MIR21* and *RNU6-2* were performed with the StepOnePlus Real-Time PCR Systems (Applied Biosystems) with the use of the following reaction conditions: 15 minutes at 95°C and 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 70°C. The cycle threshold (C_t) values in the quantitative RT-PCR for *MIR21* and *RNU6-2* decreased linearly with the amount of input cDNA using 10-fold dilution series from the same specimen ($r^2 > 0.99$; Fig. 1A). The interassay coefficient of variation of C_t values from the same specimen in five different batches was ≤1% for *MIR21* and *RNU6-2* in our validation study using five colorectal cancers (Table 1).

Each specimen was analyzed in duplicate for each target in a single batch, and we used the average of the two C_t values for each target. The Spearman rank-correlation coefficient between the two C_t values (in duplicated runs) was 0.99 in quantitative PCR assays for *MIR21* and *RNU6-2*. The *MIR21* expression level in each specimen was calculated as a relative unitless value normalized with *RNU6-2* using the $2^{-\Delta C_t}$ method (where $\Delta C_t = $ "the average C_t value of *MIR21*" − "the average C_t value of *RNU6-2*") as previously described (29).

Analyses of MSI, DNA methylation, and *KRAS*, *BRAF*, and *PIK3CA* mutations

DNA was extracted from archival colorectal cancer tissue blocks. MSI status was analyzed with the use of 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487), as previously described (30). We defined MSI-high as the presence of instability in ≥30% of the markers, and MSI-low/MSS as instability in <30% of the markers. Methylation analyses of long interspersed nucleotide element-1 (LINE-1; ref. 31, 32) and eight promoter CpG islands specific for CpG island methylator phenotype (CIMP; *CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3,* and *SOCS1*; refs. 33, 34) were performed. PCR reaction and pyrosequencing were performed for *KRAS* (codons 12, 13, 61,

Table 1. Interassay coefficients of variation in quantitative RT-PCR assays for *MIR21* and *RNU6-2*

| | Targets in quantitative RT-PCR assays | | | |
| | *MIR21* | | *RNU6-2* | |
	Mean cycle threshold ± SD	Interassay coefficient of variation (%)	Mean cycle threshold ± SD	Interassay coefficient of variation (%)
Specimen 1	19.5 ± 0.06	0.28	19.9 ± 0.13	0.65
Specimen 2	19.5 ± 0.07	0.36	21.2 ± 0.15	0.73
Specimen 3	19.3 ± 0.09	0.44	21.0 ± 0.18	0.86
Specimen 4	20.0 ± 0.09	0.46	22.1 ± 0.09	0.39
Specimen 5	18.0 ± 0.10	0.55	21.0 ± 0.12	0.59
Mean coefficient of variation (%)		0.42		0.64

NOTE: Interassay coefficient of variation of cycle threshold values from the same specimen were assessed by repeating assays in five different batches with the use of five colorectal cancers.

and 146; refs. 35, 36), *BRAF* (codon 600; ref. 30), and *PIK3CA* (exons 9 and 20; refs. 37, 38).

Immunohistochemistry and quantification of the density of T cells

We constructed a tissue microarray (TMA) and conducted immunohistochemistry for CD3, CD8, CD45RO (one of the PTPRC protein isoforms), and FOXP3 (7). We used an automated scanning microscope and the Ariol image analysis system (Genetix) to measure densities (cells/mm^2) of CD3$^+$, CD8$^+$, CD45RO$^+$, and FOXP3$^+$ T cells in colorectal cancer tissue as previously described (7).

Statistical analysis

All statistical analyses were conducted using SAS (version 9.3, SAS Institute), and all *P* values were two-sided. Neither *MIR21* expression, T-cell density, nor log-transformed values of *MIR21* or T-cell density fit a normal distribution with the use of the Kolmogorov–Smirnov test for normality ($P \leq 0.048$). Thus, we tested our primary hypothesis using a linear trend test in an ordinal logistic regression model to assess associations of tumor *MIR21* expression (an ordinal quartile predictor variable as a continuous variable) with the density of CD3$^+$, CD8$^+$, CD45RO$^+$, or FOXP3$^+$ T cells in colorectal cancer tissue (an ordinal quartile outcome variable). Because we tested four primary hypotheses (for CD3$^+$, CD8$^+$, CD45RO$^+$, and FOXP3$^+$ T cells as outcome variables), we adjusted two-sided α level to 0.012 (= 0.05/4) by simple Bonferroni correction. All other analyses, including evaluation of individual odds ratio (OR) estimates, represented secondary analyses. In those secondary analyses, in view of multiple comparisons, we interpreted our data cautiously, in addition to the use of the adjusted α level of 0.012.

We performed multivariable ordinal logistic regression analysis to control for potential confounders. The multivariable model initially included age (continuous), sex, year of diagnosis (continuous), family history of colorectal cancer in a first-degree relative (present vs. absent), tumor location (proximal colon vs. distal colon vs. rectum), tumor differentiation (well to moderate vs. poor), MSI (high vs. MSI-low/MSS), CIMP (high vs. low/negative), *KRAS* (mutant vs. wild-type), *BRAF* (mutant vs. wild-type), and *PIK3CA* (mutant vs. wild-type), and LINE-1 methylation level (continuous). For cases with missing information in any of the covariates, we assigned a separate ("missing") indicator variable. A backward stepwise elimination with a threshold of $P = 0.05$ was used to select variables in the final models. We assessed the proportional odds assumption in the ordinal logistic regression model, which was generally satisfied ($P > 0.05$).

All cross-sectional univariable analyses for clinical, pathologic, and molecular associations (with variables listed in Table 2) were secondary exploratory analyses, and we adjusted the two-sided α level to 0.003 (= 0.05/14) by simple Bonferroni correction for multiple hypothesis testing. To assess associations between the ordinal categories (first to fourth quartile) of tumor *MIR21* expression and categorical data, the χ^2 test was performed. To compare mean age and mean LINE-1 methylation levels, an ANOVA assuming equal variances was performed.

Results

MIR21 expression in colorectal cancer

To test the hypothesis of an inverse relationship between *MIR21* expression and T-cell infiltration in colorectal cancer tissue, we measured *MIR21* expression with RT-PCR assays on 538 colorectal cancer cases within the NHS and the HPFS databases. In 54 pairs of colorectal cancer and adjacent nontumor colonic mucosa, *MIR21* expression was generally higher in colorectal cancer than in paired adjacent nontumor colonic mucosa (Wilcoxon signed rank test, $P < 0.0001$; Fig. 1B).

Table 2 shows the clinical, pathologic, and molecular features of the 538 cases according to tumor *MIR21* expression. Higher tumor *MIR21* expression was associated with *BRAF* mutation ($P = 0.003$; with adjusted α level of 0.003 for multiple hypothesis testing).

Association of tumor *MIR21* expression with T-cell density in colorectal cancer tissue

We measured the densities of CD3$^+$, CD8$^+$, CD45RO$^+$, and FOXP3$^+$ T cells in colorectal cancer tissue by immunohistochemistry and image analysis. Supplementary Table S1 shows pairwise correlations between the densities of CD3$^+$, CD8$^+$, CD45RO$^+$, and FOXP3$^+$ T cells. Except for between CD8$^+$ and FOXP3$^+$ T cells ($P = 0.16$), all of the other pairwise correlations were statistically significant (with Spearman rank correlation coefficients ranging from 0.18 to 0.48; all $P < 0.0001$).

Table 3 shows a distribution of colorectal cancer cases according to tumor *MIR21* expression (quartiles) and the density of T cells in colorectal cancer tissue (quartiles). In our primary hypothesis testing, we conducted univariable and multivariable ordinal logistic regression analyses to assess the associations of tumor *MIR21* expression (as an ordinal quartile predictor variable) with the density of CD3$^+$, CD8$^+$, CD45RO$^+$, or FOXP3$^+$ T cells in colorectal cancer tissue (an ordinal quartile outcome variable; Table 4 and Supplementary Table S2 with all covariates). Tumor *MIR21* expression was inversely associated with the densities of CD3$^+$ T cells and CD45RO$^+$ T cells in univariable and

Table 2. Clinical, pathologic, and molecular features according to tumor *MIR21* expression in 538 colorectal cancer cases

Characteristic[a]	Total (N = 538)	Tumor *MIR21* expression (quartile)				P[b]
		Q1 (lowest; n = 135)	Q2 (second; n = 134)	Q3 (third; n = 134)	Q4 (highest; n = 135)	
Mean age ± SD, y	67.6 ± 8.3	66.7 ± 8.2	67.2 ± 8.5	68.8 ± 8.1	67.5 ± 8.3	0.19
Sex						0.044
Men	185 (34%)	50 (37%)	44 (33%)	56 (42%)	35 (26%)	
Women	353 (66%)	85 (63%)	90 (67%)	78 (58%)	100 (74%)	
Year of diagnosis						0.006
Prior to 1995	201 (38%)	63 (47%)	55 (41%)	42 (32%)	41 (30%)	
1996–2000	202 (38%)	43 (32%)	54 (40%)	44 (34%)	61 (45%)	
2001–2008	131 (24%)	28 (21%)	25 (19%)	45 (34%)	33 (25%)	
Family history of colorectal cancer in a first-degree relative						0.21
Absent	422 (79%)	105 (78%)	111 (83%)	96 (74%)	110 (82%)	
Present	109 (21%)	29 (22%)	22 (17%)	34 (26%)	24 (18%)	
Tumor location						0.30
Cecum	96 (18%)	20 (15%)	22 (17%)	27 (20%)	27 (20%)	
Ascending to transverse colon	173 (32%)	39 (29%)	40 (30%)	43 (32%)	51 (38%)	
Splenic flexure to sigmoid	149 (28%)	39 (29%)	44 (33%)	31 (23%)	35 (26%)	
Rectosigmoid and rectum	117 (22%)	36 (27%)	27 (20%)	33 (25%)	21 (16%)	
Disease stage						0.009
I	110 (21%)	37 (28%)	29 (22%)	23 (18%)	21 (16%)	
II	173 (34%)	44 (34%)	45 (35%)	44 (35%)	40 (31%)	
III	164 (32%)	31 (24%)	44 (34%)	48 (39%)	41 (32%)	
IV	67 (13%)	18 (14%)	11 (8.5%)	10 (8.0%)	28 (21%)	
Tumor differentiation						0.24
Well to moderate	486 (91%)	121 (90%)	127 (95%)	119 (89%)	119 (88%)	
Poor	51 (9.5%)	13 (9.7%)	7 (5.2%)	15 (11%)	16 (12%)	
MSI status						0.26
MSI-low/MSS	440 (84%)	114 (87%)	115 (86%)	107 (82%)	104 (79%)	
MSI-high	86 (16%)	17 (13%)	18 (14%)	24 (18%)	27 (21%)	
MLH1 hypermethylation						0.15
Absent	461 (87%)	118 (89%)	121 (91%)	109 (83%)	113 (85%)	
Present	69 (13%)	14 (11%)	12 (9.0%)	23 (17%)	20 (15%)	
CIMP status						0.015
Low/negative	440 (83%)	113 (86%)	119 (89%)	108 (82%)	100 (75%)	
High	90 (17%)	19 (14%)	14 (11%)	24 (18%)	33 (25%)	
BRAF mutation						0.003
Wild-type	444 (84%)	120 (90%)	116 (88%)	110 (82%)	98 (75%)	
Mutant	86 (16%)	13 (9.8%)	16 (12%)	24 (18%)	33 (25%)	
KRAS mutation						0.11
Wild-type	311 (59%)	76 (58%)	67 (51%)	88 (66%)	80 (61%)	
Mutant	216 (41%)	55 (42%)	64 (49%)	46 (34%)	51 (39%)	
PIK3CA mutation						0.86
Wild-type	408 (83%)	99 (84%)	104 (82%)	105 (82%)	100 (85%)	
Mutant	82 (17%)	19 (16%)	23 (18%)	23 (18%)	17 (15%)	
Mean LINE-1 methylation level (%) ± SD	61.6 ± 9.6	61.6 ± 8.4	59.6 ± 10.4	62.1 ± 10.2	63.0 ± 9.1	0.032

Abbreviations: Q1 to Q4, quartile 1 to quartile 4.

[a]Percentage indicates the proportion of cases with a specific clinical, pathologic, or molecular feature in colorectal cancer cases with each tumor *MIR21* expression. There were cases that had missing values for any of the characteristics except for age and sex.

[b]To assess associations between the ordinal categories (first to fourth quartile) of tumor *MIR21* expression and categorical data, the χ^2 test was performed. To compare mean age and mean LINE-1 methylation levels, an ANOVA was performed. We adjusted the two-sided α level to 0.003 (= 0.05/14) by simple Bonferroni correction for multiple hypothesis testing.

multivariable ordinal logistic regression analyses (all $P_{trend} <$ 0.0005; with adjusted α level of 0.012 for multiple hypothesis testing). Compared with cases in the lowest quartile of tumor *MIR21* expression, those in the highest quartile were inversely associated with the densities of CD3$^+$ T cells [multivariable OR, 0.44; 95% confidence interval (CI), 0.28–0.68; for a unit increase in quartile categories] and CD45RO$^+$ T cells (multivariable OR, 0.41; 95% CI, 0.26–0.64; for a unit increase in quartile categories). Tumor *MIR21* expression was not significantly associated with the density of CD8$^+$ or FOXP3$^+$ T cells ($P_{trend} >$ 0.03 in univariable analysis with adjusted α level of 0.012). We also used tumor *MIR21* expression after adjusting for cellularity in colorectal cancer tissue, and observed similar associations of tumor *MIR21*

expression with the density of T cells (Supplementary Methods and Supplementary Table S3).

In our exploratory analyses, higher tumor *MIR21* expression was significantly associated with higher colorectal cancer–specific mortality ($P_{trend} = 0.003$), whereas higher CD8$^+$ T-cell density was significantly associated with lower colorectal cancer–specific mortality ($P_{trend} = 0.012$; Supplementary Methods and Supplementary Table S4).

Discussion

We conducted this study to test the hypothesis that tumor *MIR21* expression might be inversely associated with the density

Table 3. Distribution of colorectal cancer cases according to tumor *MIR21* expression and the density of T cells

		Tumor *MIR21* expression (quartile)				
	Total	Q1 (lowest)	Q2 (second)	Q3 (third)	Q4 (highest)	P_{trend}[a]
CD3$^+$ cell density (quartile)						0.0004
Q1 (0–115 cells/mm^2)	130 (25%)	26 (20%)	29 (22%)	31 (25%)	44 (33%)	
Q2 (116–252 cells/mm^2)	129 (25%)	26 (20%)	30 (23%)	35 (28%)	38 (29%)	
Q3 (253–533 cells/mm^2)	130 (25%)	42 (32%)	33 (25%)	30 (24%)	25 (19%)	
Q4 (≥534 cells/mm^2)	129 (25%)	38 (28%)	38 (30%)	28 (23%)	25 (19%)	
CD8$^+$ cell density (quartile)						0.27
Q1 (0–66 cells/mm^2)	128 (25%)	23 (18%)	31 (24%)	40 (32%)	34 (26%)	
Q2 (67–185 cells/mm^2)	127 (25%)	42 (33%)	26 (20%)	28 (23%)	31 (24%)	
Q3 (186–410 cells/mm^2)	128 (25%)	30 (24%)	34 (26%)	28 (23%)	36 (27%)	
Q4 (≥411 cells/mm^2)	127 (25%)	31 (25%)	39 (30%)	27 (22%)	30 (23%)	
CD45RO$^+$ cell density (quartile)						0.0002
Q1 (0–183 cells/mm^2)	131 (25%)	24 (18%)	30 (23%)	31 (24%)	46 (35%)	
Q2 (184–430 cells/mm^2)	130 (25%)	32 (25%)	33 (25%)	39 (31%)	26 (20%)	
Q3 (431–805 cells/mm^2)	131 (25%)	26 (20%)	37 (27%)	30 (24%)	38 (29%)	
Q4 (≥806 cells/mm^2)	130 (25%)	48 (37%)	33 (25%)	27 (21%)	22 (16%)	
FOXP3$^+$ cell density (quartile)						0.032
Q1 (0–14 cells/mm^2)	124 (25%)	25 (20%)	31 (26%)	32 (27%)	36 (28%)	
Q2 (15–25 cells/mm^2)	124 (25%)	29 (23%)	24 (20%)	34 (28%)	37 (28%)	
Q3 (26–48 cells/mm^2)	124 (25%)	38 (31%)	27 (22%)	27 (22%)	32 (25%)	
Q4 (≥49 cells/mm^2)	123 (25%)	32 (26%)	38 (32%)	28 (23%)	25 (19%)	

Abbreviations: Q1 to Q4, quartile 1 to quartile 4.
[a]P_{trend} value was calculated by the linear trend test across the ordinal (first to fourth quartile) categories of tumor *MIR21* expression as a continuous variable in a univariable ordinal logistic regression model for the density of CD3$^+$, CD8$^+$, CD45RO$^+$, or FOXP3$^+$ T cells (an ordinal quartile outcome variable). Because we assessed four primary outcome variables, we adjusted the two-sided α level to 0.012 (= 0.05/4) by simple Bonferroni correction.

of T cells in colorectal cancer tissue in a human population. We demonstrated that miRNA expression analysis, by RT-PCR assay, on FFPE tissue specimens was feasible and robust, in agreement with results from previous studies (19, 20). Using the database

of the 538 colorectal cancer cases in the two U.S. nationwide prospective cohort studies, we found that tumor *MIR21* expression was inversely associated with the densities of CD3$^+$ and CD45RO$^+$ T cells in human colorectal cancer tissue. Our first-line

Table 4. The association of tumor *MIR21* expression with the density of T cells

		Univariable OR (95% CI)	Multivariable OR (95% CI)[a]
Model for CD3$^+$ cell density ($n = 518$, as an outcome variable)			
MIR21 expression	Q1 (lowest)	1 (reference)	1 (reference)
	Q2 (second)	0.88 (0.57–1.36)	0.85 (0.55–1.31)
	Q3 (third)	0.67 (0.43–1.04)	0.59 (0.37–0.92)
	Q4 (highest)	0.47 (0.31–0.73)	0.44 (0.28–0.68)
	P_{trend}[b]	0.0004	<0.0001
Model for CD8$^+$ cell density ($n = 510$, as an outcome variable)			
MIR21 expression	Q1 (lowest)	1 (reference)	1 (reference)
	Q2 (second)	1.14 (0.74–1.77)	1.25 (0.80–1.96)
	Q3 (third)	0.72 (0.46–1.12)	0.76 (0.48–1.19)
	Q4 (highest)	0.89 (0.58–1.38)	0.99 (0.63–1.54)
	P_{trend}[b]	0.27	0.50
Model for CD45RO$^+$ cell density ($n = 522$, as an outcome variable)			
MIR21 expression	Q1 (lowest)	1 (reference)	1 (reference)
	Q2 (second)	0.70 (0.46–1.09)	0.72 (0.46–1.12)
	Q3 (third)	0.57 (0.37–0.89)	0.54 (0.34–0.84)
	Q4 (highest)	0.45 (0.29–0.70)	0.41 (0.26–0.64)
	P_{trend}[b]	0.0002	<0.0001
Model for FOXP3$^+$ cell density ($n = 495$, as an outcome variable)			
MIR21 expression	Q1 (lowest)	1 (reference)	1 (reference)
	Q2 (second)	0.98 (0.63–1.54)	0.93 (0.59–1.46)
	Q3 (third)	0.73 (0.47–1.14)	0.61 (0.39–0.96)
	Q4 (highest)	0.66 (0.42–1.02)	0.55 (0.35–0.86)
	P_{trend}[b]	0.032	0.003

Abbreviations: Q1 to Q4, quartile 1 to quartile 4.
[a]The multivariable ordinal logistic regression analysis model initially included age; sex; year of diagnosis; family history of colorectal cancer in parent or sibling; tumor location; tumor differentiation; MSI; CpG island methylator phenotype; *KRAS*, *BRAF*, and *PIK3CA* mutations; and LINE-1 methylation level. A backward stepwise elimination with a threshold of $P = 0.05$ was used to select variables in the final models. Variables remaining in the final multivariable ordinal logistic regression models are shown in Supplementary Table S2.
[b]P_{trend} value was calculated by the linear trend across the ordinal (first to fourth quartile) categories of *MIR21* expression as a continuous variable in the ordinal logistic regression model for the density of CD3$^+$, CD8$^+$, CD45RO$^+$, or FOXP3$^+$ T cells (an ordinal quartile outcome variable). Because we assessed four primary outcome variables, we adjusted the two-sided α level to 0.012 (= 0.05/4) by simple Bonferroni correction.

population-based data support an immunosuppressive role of *MIR21* in colorectal cancer.

High densities of CD3^{+} pan-T cells and T-cell subpopulations (CD8^{+}, CD45RO^{+}, and FOXP3^{+} T cells) in colorectal carcinoma have been associated with better patient survival, indicating a major role of T-cell–mediated adaptive immunity in inhibiting colorectal tumor progression (39–41). Therefore, both tumor molecular and immunity analyses are increasingly important in cancer research and clinical practice. miRNAs play substantial roles in carcinogenesis and immunity and are potential biomarkers or therapeutic targets (42). One possible mechanism of the immunosuppressive effect of *MIR21* is based on its ability to suppress the expression of PDCD4, which normally inhibits the translation of IL10 mRNA. Without this suppression, more IL10 is present in the tumor microenvironment (22, 23), which inhibits the antigen-presenting capacities of dendritic cells (25). Tumor *MIR21* expression has been shown to inversely correlate with tumor PDCD4 expression assessed by immunohistochemistry on human colorectal cancer tissue (43, 44). Taken together from these findings, it seems to be plausible that *MIR21* may suppress antitumor immune responses through increased IL10 in colorectal cancer. In addition, emerging evidence indicates that *MIR21* suppresses tumor expression of HPGD [hydroxyprostaglandin dehydrogenase 15-(NAD); or 15-PDGH], which converts PGE_2 to its biologically inactive metabolite (24). Hence, *MIR21* may increase PGE_2 in the tumor microenvironment, which can lead to suppression of antitumor T-cell–mediated adaptive immunity (26). Our human population-based data, along with these lines of experimental evidence, support the hypothesis that *MIR21* suppresses antitumor T-cell–mediated immune response to colorectal cancer, although additional studies are needed to clarify the exact mechanism. miRNA-targeting therapies for human disease, including cancer, are currently being investigated (42, 45, 46). In light of our findings, it would be intriguing for future research to explore a potential strategy of inhibiting *MIR21* and thus its immunosuppressive effect in immunotherapy and prevention for colorectal cancer.

Higher tumor *MIR21* expression was associated with *BRAF* mutation in the present study, which has not been examined in colorectal cancer before. Oncogenic mutation of *BRAF* activates the MAPK signaling pathway (47). Experimental evidence suggests that activation of the RAF–MAPK signaling pathway may increase *MIR21* expression in cancer (48). Taken together from these findings, *BRAF* mutation might increase *MIR21* expression through activation of the MAPK signaling pathway, although additional experimental studies are needed to test this hypothesis.

One limitation of this study is its cross-sectional nature. Hence, we cannot exclude a possibility of reverse causation. It is possible that the interaction of T cells with tumor cells might cause low expression of *MIR21* in tumors. However, our specific hypothesis was based on several lines of experimental evidence, indicating that *MIR21* suppresses T-cell–mediated immune response to tumor (22–26). Because experimental systems cannot perfectly recapitulate the complexities of human tumors or the immune system, analyses of human population are essential in translational medicine. Another limitation is measurement of *MIR21* expression in colorectal cancer tissue, which contains a mixture of neoplastic and nonneoplastic cells, including immune cells. Nonetheless, a number of studies have shown that *MIR21* is expressed in neoplastic cells, but not substantially in immune

cells (20, 21). We also recognize the limitations in evaluating T cells in human colorectal cancer tissue. We evaluated the well-characterized T-cell markers, such as CD3, CD8, CD45RO, and FOXP3, with the use of TMA immunohistochemistry and computer-assisted image analysis to objectively quantify the T-cell densities in a large number of cases. The favorable prognostic associations of the densities of these T-cell populations in our cohort studies were consistent with the results of previous studies in other populations (6, 8), suggesting that the density of T cells, as assessed by immunohistochemistry, might be considered a reliable measure of the adaptive immune response to colorectal tumors.

Strengths of this study include the use of our molecular pathological epidemiology database of more than 500 colorectal cancer cases in the two U.S. nationwide, prospective cohort studies, which integrates epidemiologic exposures, clinicopathologic features, key tumor molecular features, and immune reaction status in colorectal cancer tissue (49, 50). This population-based colorectal cancer database enabled us to rigorously examine the association of tumor *MIR21* expression with the density of T cells, controlling for potential confounders. In addition, our colorectal cancer specimens were derived from a large number of hospitals in diverse settings across the United States (but not based on a limited number of hospitals), which increases the generalizability of our findings. As another strength, we used robust laboratory assays, including miRNA analysis and tissue image analysis that could objectively quantify specific T cells in tumor tissue.

In conclusion, tumor *MIR21* expression is inversely associated with the densities of CD3^{+} and CD45RO^{+} T cells in colorectal cancer tissue. Our data support a possible role of *MIR21* in downregulating antitumor T-cell–mediated adaptive immunity, and suggest *MIR21* as a potential target for immunotherapy and immunoprevention in colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

The former Editor-in-Chief of this journal (Glenn Dranoff) is an author on this article. In keeping with the AACR's editorial policy, the peer review of this submission was managed by a senior member of *Cancer Immunology Research*'s editorial team; a member of the AACR Publications Committee rendered the final decision concerning acceptability.

Disclaimer

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Use of Standardized Official Symbols

We use HUGO (Human Genome Organisation)–approved official symbols for genes and gene products, including BRAF, CACNA1G, CD3, CD8, CD274, CDKN2A, CRABP1, CTLA4, FOXP3, HPGD, IGF2, IL10, KRAS, MIR21, MLH1, NEUROG1, PDCD1, PDCD4, PIK3CA, PTPRC, RNU6-2, RUNX3, and SOCS1; all of which are described at www.genenames.org. Gene names are italicized, and gene product names are not italicized.

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