MicroRNA MIR21 (miR-21) and PTGS2 Expression in Colorectal Cancer and Patient Survival

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Abstract

Purpose: Prostaglandin-endoperoxide synthase 2 (PTGS2, cyclooxygenase-2; a target of aspirin) produces inflammatory mediator prostaglandin E2 (PGE2), and contributes to colorectal neoplasia development. PTGS2-driven inflammatory responses can induce tumor expression of microRNA MIR21 (miR-21) that can increase local PGE2 level by downregulating PGE2-metabolizing enzymes. We hypothesized that the prognostic association of tumor MIR21 expression level in colorectal carcinoma might depend on inflammatory tumor microenvironment and be stronger in tumors expressing high-level PTGS2.

Experimental Design: Utilizing 765 rectal and colon cancer specimens in the Nurses’ Health Study and the Health Professionals Follow-up Study, we measured MIR21 expression by quantitative reverse transcription PCR, and PTGS2 expression by immunohistochemistry. Cox proportional hazards regression model was used to assess statistical interaction between MIR21 and PTGS2 in colorectal cancer-specific survival analysis, controlling for potential confounders including microsatellite instability, CpG island methylator phenotype, LINE-1 methylation level, and KRAS, BRAF, and PIK3CA mutations.

Results: Tumor MIR21 expression level was associated with higher colorectal cancer-specific mortality (P_trend = 0.029), and there was a statistically significant interaction between MIR21 and PTGS2 (P_interaction = 0.0004). The association between MIR21 expression and colorectal cancer-specific mortality was statistically significant in PTGS2-high cancers (multivariable hazard ratio of the highest vs. lowest quartile of MIR21, 2.28; 95% confidence interval, 1.42–3.67; P_trend = 0.0004) but not in PTGS2-absent/low cancers (P_trend = 0.22).

Conclusions: MIR21 expression level in colorectal carcinoma is associated with worse clinical outcome, and this association is stronger in carcinomas expressing high-level PTGS2, suggesting complex roles of immunity and inflammation in tumor progression.

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Introduction

Colorectal cancers develop through the accumulation of genetic and epigenetic alterations and through tumor–host interactions including inflammatory responses and host immunity (1, 2). Prostaglandin-endoperoxide synthase 2 (PTGS2, cyclooxygenase-2) produces inflammatory mediator prostaglandin E2 (PGE2) and contributes to colorectal tumor development and progression (3–5). Randomized controlled trials and observational studies have demonstrated that regular use of aspirin (PTGS2 inhibitor) reduces the risk of colorectal neoplasia incidence and mortality (6, 7). Levels of PGE2 in the tumor microenvironment are likely influenced by PTGS2 that produces PGE2 (3). Previous studies suggest that cellular PTGS2 expression may influence effects of aspirin and selective inhibitors of PTGS2 on colorectal tumors (8, 9).

MicroRNAs (miRNAs) are small noncoding RNAs that post-transcriptionally regulate gene expression and have been shown to influence diverse physiologic and pathologic processes, including immunity, inflammation, and carcinogenesis (10). Accumulating evidence indicates that inflammatory responses can alter expression of miRNAs, some of which may contribute to tumor progression (11, 12). Among those miRNAs, MIR21 (miR-21) has been shown to promote inflammation-associated colorectal...
**Translational Relevance**

Accumulating evidence indicates that microRNAs are promising biomarkers and therapeutic targets in cancer. We examined an association of tumor MIR21 expression level with patient survival utilizing 765 colorectal cancer cases in two U.S. nationwide prospective cohort studies (the Nurses’ Health Study and the Health Professionals Follow-up Study). We found that tumor MIR21 expression level was associated with higher colorectal cancer–specific mortality independent of clinical, pathologic, and major tumor molecular features, including microsatellite instability, CpG island methylator phenotype, Kras, Braf, and Pik3ca mutations, and LINE-1 methylation level. In addition, this adverse prognostic association was stronger in colorectal cancers expressing high-level prostaglandin-endoperoxide synthase 2 (PTGS2, cyclooxygenase-2) that produces inflammatory mediator prostaglandin E2. Our population-based data suggest that MIR21 may serve as a potential therapeutic target, especially for colorectal cancers that express PTGS2 and may depend on inflammatory tumor microenvironment.

**Materials and Methods**

**Study population**

We utilized the database of colorectal carcinoma cases within two U.S. nationwide prospective cohort studies [the Nurses’ Health Study (NHS) and the Health Professionals Follow-up Study (HPFS)], and examined a statistical interaction between tumor MIR21 and PTGS2 expression in survival analysis, controlling for potential confounders including major molecular features of colorectal cancer.

**RNA isolation and quantitative reverse transcription PCR for MIR21**

RNA was extracted from colorectal cancer tissue in whole-tissue sections of FFPE specimens with the use of RecoverAll Total Nucleic Acid Isolation Kit (Ambion Inc). Quantitative reverse transcription PCR assays for MIR21 and RNU6-2 were performed according to miScript PCR System protocol (Qiagen) after assay validation as described previously (20). Briefly, cDNA was synthesized with the use of miScript II RT Kit (Qiagen). Each reaction was performed in 25 μL solution containing 1 × final concentration QuantiTect SYBR Green PCR Master Mix (Qiagen) and each miScript Primer Assay (Qiagen) specific for MIR21 (catalog number, MS00009079) and RNU6-2 (catalog number, 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.

Our validation study has previously shown that the cycle threshold (Ct) values in the quantitative reverse transcription PCR assays for MIR21 and RNU6-2 decreased linearly with the amount of input cDNA using 10-fold dilution series from the same specimen (r2 > 0.99), and that the inter-assay coefficient of variation of Ct values from the same specimen in five different batches was ≤1% for MIR21 and RNU6-2 (20). Each specimen was analyzed in duplicate for each target in a single batch, and we used the mean of the two Ct values for each target. Spearman’s rank-correlation coefficient between the two Ct values (in duplicate runs) was 0.99 in quantitative PCR assays for MIR21 and RNU6-2 (20). MIR21 expression level in each specimen was calculated as a relative unitless value normalized with RNU6-2 using the 2^-ΔΔCt method (where ΔΔCt = “the mean Ct value of MIR21” – “the mean Ct value of RNU6-2”) as described previously (20).

**Immunohistochemistry for PTGS2 expression**

Immunohistochemistry (IHC) for PTGS2 (cyclooxygenase-2) was performed using anti-PTGS2 antibody (Cayman Chemical; dilution 1:300) as described previously (5, 8). A single pathologist (S. Ogino), unaware of other data, interpreted tumor PTGS2 expression level (absent, low, or high), compared with adjacent normal colonic epithelium. A random sample of 124 cancers was examined by a second pathologist (T. Morikawa), and concordance between the two observers was 0.85 (k = 0.69; ref. 18).
Representative sections from PTGS2-absent, PTGS2-low, and PTGS2-high tumors have been shown in our previous study (5).

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

DNA was extracted from archival colorectal cancer tissue blocks. Microsatellite instability (MSI) status was analyzed with use of 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487) as described previously (21). We defined MSI-high as the presence of instability in ≥30% of the markers, and MSI-low/microsatellite stable (MSS) as instability in <30% of the markers. Methylation analyses of long interspersed nucleotide element-1 (LINE-1; refs. 22, 23) and eight promoter CpG islands specific for CpG island methylator phenotype (CIMP; CACNA1G, CDKN2A, CRABP1, IGF2, MEH1, NEUROG1, RUNX3, and SOCS1; refs. 24, 25) were performed. PCR reaction and pyrosequencing were performed for KRAS (codons 12, 13, 61, and 146; refs. 26, 27), BRAF (codon 600; ref. 21), and PIK3CA (exons 9 and 20; ref. 28).

Statistical analysis

All statistical analyses were conducted using SAS (version 9.3, SAS Institute) and all P values were two-sided. Our primary hypothesis testing was a statistical interaction between tumor MIR21 and PTGS2 expression in relation to colorectal cancer-specific mortality. Neither MIR21 expression nor log-transformed values of MIR21 fit a normal distribution with the use of the Kolmogorov–Smirnov test for normality (P < 0.01). We conducted sequential test for a linear trend across ordinal quartile categories (1–4) of the tumor MIR21 expression level as a continuous variable in the Cox regression model. All other analyses including evaluation of individual hazard ratio (HR) estimates were secondary analyses. The statistical interaction was assessed by the Wald test on the cross-product term of tumor MIR21 expression [ordinal quartile categories (1–4)] and PTGS2 expression [ordinal categories; absent (1), low (2), and high (3)] variables in a Cox proportional hazards regression model. A two-sided α level was set at 0.05 for our primary hypothesis testing. For all of the primary and secondary analyses, we interpreted our results cautiously, given the exploratory hypothesis-generating nature of this study.

For analyses of colorectal cancer-specific mortality, deaths as a result of other causes were censored. To control for confounding, we used multivariable Cox proportional hazards regression models. In addition to tumor MIR21 expression level, the multivariable model initially included sex, age at diagnosis (continuous), 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), disease stage (I/II vs. III/IV), tumor differentiation (well/moderate vs. poor), MSI (high vs. MSI-low/MSS), CIMP (high vs. low/negative), KRAS (mutant vs. wild-type), BRAF (mutant vs. wild-type), PIK3CA (mutant vs. wild-type), and tumor LINE-1 methylation level (continuous). A backward elimination was carried out with P = 0.05 as a threshold, to select variables for the final model. For cases (3.0%) with missing information on LINE-1 methylation level, we assigned a separate indicator variable. For cases with missing information in any of the categorical covariates [family history of colorectal cancer in a first-degree relative (0.4%), tumor location (0.4%), disease stage (5.9%), tumor differentiation (0.1%), MSI (3.4%), CIMP (7.2%), KRAS (3.0%), BRAF (2.4%), and PIK3CA (9.4%)], we included these cases in the majority category of a given covariate to minimize the number of variables in multivariable Cox models. We confirmed that excluding the cases with missing information in any of the covariates did not substantially alter results (data not shown). The proportionality of hazards assumption was assessed by a time-varying covariate, using an interaction term of survival time and tumor MIR21 expression level variable (P = 0.63 for colorectal cancer-specific mortality and P = 0.11 for overall mortality). The Kaplan–Meier method was used to describe the distribution of colorectal cancer-specific survival and overall survival, and the log-rank test for trend was performed to assess a linear trend in survival probability across the ordinal quartile categories of tumor MIR21 expression level.

All cross-sectional univariable analyses for clinical, pathologic, and tumor molecular associations were secondary analyses, and we adjusted two-sided α level to 0.003 (= 0.05/15) by simple Bonferroni correction for multiple hypothesis testing. To assess associations between categorical data, the χ² test was performed. To compare mean age and mean LINE-1 methylation levels, an ANOVA assuming equal variances was performed.

Results

Clinical, pathologic, and tumor molecular associations

To test our primary hypothesis on the statistical interaction between tumor MIR21 and PTGS2 expression in colorectal cancer-specific survival analysis, we utilized the database of 765 colorectal cancer cases within the two prospective cohort studies. We measured tumor MIR21 expression level, using the quantitative reverse transcription PCR assay as described previously (20). Table 1 summarizes clinical, pathologic, and tumor molecular features according to tumor MIR21 expression level. High-level tumor MIR21 expression was associated with higher disease stage and BRAF mutation (P ≤ 0.0008 with adjusted α level of 0.003 for multiple hypothesis testing).

Association of tumor MIR21 expression level with colorectal cancer mortality

We examined the relationship between tumor MIR21 expression level and colorectal cancer mortality. In the 765 colorectal cancer cases, there were 429 deaths, including 231 colorectal cancer–specific deaths, during a median follow-up of 12.6 years (interquartile range: 9.8–17.3 years) for censored cases. In Kaplan–Meier analysis, tumor MIR21 expression level was associated with higher colorectal cancer–specific mortality (P = 0.0008 by the log-rank test for trend) and overall mortality (P = 0.001 by the log-rank test for trend; Fig. 1). Tumor MIR21 expression level was associated with higher colorectal cancer–specific mortality in univariable [P_{trend} = 0.0008] and multivariable Cox regression analyses [P_{trend} = 0.029; Table 2].

Interactive association of tumor MIR21 and PTGS2 expression level in survival analysis

In our primary hypothesis testing, we found a statistically significant interaction between tumor MIR21 and PTGS2 expression level in colorectal cancer–specific survival analysis (P_{interaction} = 0.0004; Table 3). Tumor MIR21 expression level was significantly associated with higher colorectal cancer–specific mortality in PTGS2-high cancers (P_{trend} = 0.0004) but not in PTGS2-absent/low cancers (P_{trend} = 0.22). Multivariable HRs for
Table 1. Clinical, pathologic, and tumor molecular features according to tumor MIR21 expression level in 765 colorectal cancer cases

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total no. (n = 765)</th>
<th>Quartile 1 (lowest) (n = 192)</th>
<th>Quartile 2 (n = 190)</th>
<th>Quartile 3 (n = 192)</th>
<th>Quartile 4 (highest) (n = 191)</th>
<th>P&lt;sub&gt;B&lt;/sub&gt;</th>
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<td>Mean age ± SD (year)</td>
<td>62.5 ± 8.9</td>
<td>67.2 ± 8.6</td>
<td>68.2 ± 9.1</td>
<td>69.6 ± 7.8</td>
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<td>Men</td>
<td>328 (43%)</td>
<td>84 (44%)</td>
<td>79 (42%)</td>
<td>96 (50%)</td>
<td>69 (36%)</td>
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<td>Women</td>
<td>437 (57%)</td>
<td>108 (56%)</td>
<td>111 (58%)</td>
<td>96 (50%)</td>
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<td>Prior to 1995</td>
<td>276 (36%)</td>
<td>88 (46%)</td>
<td>70 (37%)</td>
<td>58 (30%)</td>
<td>60 (31%)</td>
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<tr>
<td>1996-2000</td>
<td>250 (33%)</td>
<td>54 (28%)</td>
<td>66 (33%)</td>
<td>60 (31%)</td>
<td>70 (37%)</td>
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<td>2001-2008</td>
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<td>50 (26%)</td>
<td>54 (28%)</td>
<td>74 (39%)</td>
<td>61 (32%)</td>
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<td>Absent</td>
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<td>158 (84%)</td>
<td>151 (79%)</td>
<td>148 (78%)</td>
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<tr>
<td>Present</td>
<td>157 (21%)</td>
<td>44 (23%)</td>
<td>31 (16%)</td>
<td>40 (21%)</td>
<td>42 (22%)</td>
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<tr>
<td>Tumor location</td>
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<td>Cecum</td>
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<td>25 (13%)</td>
<td>30 (16%)</td>
<td>41 (21%)</td>
<td>38 (20%)</td>
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<td>Ascending to transverse colon</td>
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<td>48 (25%)</td>
<td>59 (31%)</td>
<td>62 (32%)</td>
<td>73 (39%)</td>
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<td>Splenic flexure to sigmoid</td>
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<td>66 (35%)</td>
<td>60 (32%)</td>
<td>44 (23%)</td>
<td>46 (24%)</td>
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<tr>
<td>Rectosigmoid and rectum</td>
<td>170 (22%)</td>
<td>52 (27%)</td>
<td>40 (21%)</td>
<td>45 (24%)</td>
<td>33 (17%)</td>
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<tr>
<td>I</td>
<td>171 (24%)</td>
<td>56 (32%)</td>
<td>45 (25%)</td>
<td>39 (21%)</td>
<td>31 (17%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>236 (33%)</td>
<td>56 (32%)</td>
<td>65 (37%)</td>
<td>60 (33%)</td>
<td>55 (30%)</td>
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<tr>
<td>III</td>
<td>213 (29%)</td>
<td>39 (22%)</td>
<td>50 (28%)</td>
<td>66 (36%)</td>
<td>58 (32%)</td>
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</tr>
<tr>
<td>IV</td>
<td>100 (14%)</td>
<td>26 (14%)</td>
<td>18 (10%)</td>
<td>17 (9.3%)</td>
<td>39 (21%)</td>
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<td>173 (91%)</td>
<td>179 (94%)</td>
<td>175 (91%)</td>
<td>167 (87%)</td>
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<td>Poor</td>
<td>70 (9.2%)</td>
<td>18 (9.4%)</td>
<td>11 (5.8%)</td>
<td>44 (23%)</td>
<td>33 (17%)</td>
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<td>PTGS2 expression</td>
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<td>34 (18%)</td>
<td>32 (17%)</td>
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<tr>
<td>Present</td>
<td>647 (84%)</td>
<td>190 (91%)</td>
<td>153 (85%)</td>
<td>153 (81%)</td>
<td>118 (62%)</td>
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<td>MSI-low/MSS</td>
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<td>169 (91%)</td>
<td>156 (85%)</td>
<td>154 (84%)</td>
<td>148 (80%)</td>
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<td>17 (9.0%)</td>
<td>28 (15%)</td>
<td>30 (16%)</td>
<td>37 (20%)</td>
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<td>617 (80%)</td>
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<td>154 (86%)</td>
<td>141 (78%)</td>
<td>133 (73%)</td>
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<tr>
<td>Present</td>
<td>98 (12%)</td>
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<td>24 (13%)</td>
<td>34 (19%)</td>
<td>42 (22%)</td>
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<td>Low/negative</td>
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<td>154 (87%)</td>
<td>154 (86%)</td>
<td>141 (81%)</td>
<td>133 (73%)</td>
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<tr>
<td>High</td>
<td>128 (18%)</td>
<td>24 (13%)</td>
<td>24 (13%)</td>
<td>34 (19%)</td>
<td>42 (22%)</td>
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<td>Wild-type</td>
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<td>171 (91%)</td>
<td>163 (87%)</td>
<td>154 (82%)</td>
<td>141 (76%)</td>
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<tr>
<td>Mutant</td>
<td>110 (16%)</td>
<td>17 (9.0%)</td>
<td>24 (13%)</td>
<td>33 (18%)</td>
<td>44 (24%)</td>
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<td>112 (60%)</td>
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<td>128 (68%)</td>
<td>111 (60%)</td>
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<tr>
<td>Mutant</td>
<td>294 (40%)</td>
<td>74 (40%)</td>
<td>88 (48%)</td>
<td>59 (32%)</td>
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<tr>
<td>PIK3CA mutation</td>
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<td>Wild-type</td>
<td>577 (78%)</td>
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<td>149 (85%)</td>
<td>144 (81%)</td>
<td>142 (85%)</td>
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<tr>
<td>Mutant</td>
<td>198 (22%)</td>
<td>26 (15%)</td>
<td>30 (17%)</td>
<td>34 (19%)</td>
<td>26 (15%)</td>
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</tr>
<tr>
<td>Mean LINE-1 methylation level (%) ± SD</td>
<td>62.1 ± 9.3</td>
<td>61.0 ± 9.0</td>
<td>60.7 ± 9.8</td>
<td>62.7 ± 9.3</td>
<td>63.8 ± 8.9</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Abbreviations: CIMP, CpG island methylator phenotype; LINE-1, long interspersed nucleotide element-1.

*Percentage (%) indicates the proportion of cases with a specific clinical, pathologic, or tumor molecular feature in colorectal cancer cases with each quartile category of tumor MIR21 expression level. There were cases that had missing values for any of the characteristics except for age, sex, and year of diagnosis.

**To assess associations between the ordinal quartile categories of tumor MIR21 expression level and categorical data, the χ² test was performed. To compare mean age and mean LINE-1 methylation levels, an ANOVA was performed. We adjusted two-sided α level to 0.003 (= 0.05/15) by simple Bonferroni correction for multiple hypothesis testing.

of the highest versus lowest quartile of MIR21 expression for colorectal cancer–specific mortality were 2.28 (95% confidence interval CI, 1.42–3.67) in PTGS2-high cancers and 0.61 (95% CI, 0.34–1.10) in PTGS2-absent/low cancers (Table 3).

Interaction of tumor MIR21 expression level and regular aspirin use after diagnosis in survival analysis of stage I to III patients

As a secondary analysis, we examined the relationship between regular aspirin use after diagnosis and colorectal cancer mortality according to tumor MIR21 expression level among 579 patients with stage I to III colorectal cancer (Supplementary Methods and Table S1). No statistically significant interaction between tumor MIR21 expression level and postdiagnosis aspirin use was observed in colorectal cancer–specific or overall survival analysis (P<interaction > 0.20; Supplementary Table S1); however, statistical power was limited.

**Discussion**

We conducted this study to test the hypothesis that the association of tumor MIR21 expression level in colorectal cancer tissue...
with worse clinical outcome might be stronger in cancers expressing high-level PTGS2. Utilizing the database of the 765 colorectal cancer cases in the two U.S. nationwide prospective cohort studies, we found that tumor MIR21 expression level was associated with higher colorectal cancer–specific mortality, consistent with previous studies by other investigators (15). Our population-based data have provided evidence for the prognostic significance of tumor MIR21 expression level in colorectal cancer, independent of clinical, pathologic, and major tumor molecular features. In addition, there was a statistically significant interaction between tumor MIR21 and PTGS2 expression level in the survival analysis. As we hypothesized, the adverse prognostic association of tumor MIR21 expression level in colorectal cancer was stronger in PTGS2-high cancers than in PTGS2-absent/low cancers. In our secondary analysis, there was no statistically significant difference in the prognostic association of postdiagnosis aspirin use by MIR21 expression level. However, statistical power was limited in our analysis of stage I to III patients, to minimize ascertainment bias in aspirin use data collection after cancer diagnosis.

Colorectal cancers are a heterogeneous group of diseases that result from the accumulation of differing sets of genomic and epigenomic alterations, and tumor–host interactions (29–35). Therefore, research on tumor biomarkers is important for clinical medicine and public health (36–39). In the current study, high-level tumor MIR21 expression was associated with BRAF mutation, which has been associated with clinical outcome in colorectal cancer (40–43). An integrative analysis of multiple gene expression datasets of colorectal cancer by Guinney and colleagues (44) has suggested four major tumor subtypes. The majority of BRAF-mutated colorectal cancers have been included in one tumor subtype that is also associated with MSI-high and high-level antitumor immunity. Our current study has shown the association of BRAF mutation in colorectal cancer with high-level tumor MIR21 expression, which may potentiate the PTGS2/PGE2 pathway and suppress antitumor immunity (20). However, lack of gene expression profiling data precluded our use of colorectal cancer subtyping scheme described by Guinney and colleagues (44).

Table 2. Tumor MIR21 expression level and colorectal cancer mortality

<table>
<thead>
<tr>
<th>MIR21 expression level</th>
<th>Colorectal cancer–specific mortality</th>
<th>Overall mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cases</td>
<td>No. of events</td>
</tr>
<tr>
<td>Quartile 1 (lowest)</td>
<td>192</td>
<td>51</td>
</tr>
<tr>
<td>Quartile 2</td>
<td>190</td>
<td>44</td>
</tr>
<tr>
<td>Quartile 3</td>
<td>192</td>
<td>61</td>
</tr>
<tr>
<td>Quartile 4 (highest)</td>
<td>191</td>
<td>75</td>
</tr>
<tr>
<td>( P_{\text{trend}} )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The multivariable Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer in parent or sibling, tumor location, disease stage, tumor differentiation, microsatellite instability, CpG island methylator phenotype, KRAS, BRAF, and PIK3CA mutations, and long interspersed nucleotide element-1 (LINE-1) methylation level. A backward elimination with a threshold of \( P = 0.05 \) was used to select variables in the final models.

*Test for a linear trend was conducted across ordinal quartile categories (1 to 4) of tumor MIR21 expression level as a continuous variable in the Cox regression model.
Although the mechanisms underlying the association of tumor MIR21 expression with BRAF mutation in colorectal cancer remain uncertain, experimental evidence suggests that activation of the RAF–MAPK signaling pathway may increase MIR21 expression level (45), and that BRAF mutation may potentiate the STAT3 signaling pathway that has been shown to increase MIR21 expression level (11, 46). Taken together, BRAF mutation might increase MIR21 expression level through the activation of the MAPK and/or the STAT3 signaling pathways, although additional experimental studies are needed to test this hypothesis. Emerging evidence suggests that PTGS2-derived PGE2 may suppress antitumor T-cell response, and PTGS2 inhibitors may enhance the efficacy of therapeutic antibodies specific for immune checkpoint molecules in BRAF-mutated melanoma (47). Hence, it would be intriguing for future investigations to explore potential influences of tumor MIR21 and PTGS2 expression on the efficacy of the immune checkpoint inhibitors in colorectal cancers.

PTGS2 produces inflammatory mediator PGE2, which has been shown to promote colorectal tumor progression (3–5). Recent experimental data suggest that inflammatory responses induce MIR21, which in turn increases local level of PGE2 by suppressing degradation of PGE2 (11–14, 16, 17). These lines of experimental evidence may be consistent with the current-population-based data suggesting that the adverse prognostic association of tumor MIR21 expression level in colorectal cancer is stronger in cancers expressing high-level PTGS2. Experimental evidence also suggests that PTGES (prostaglandin E synthase or microsomal prostaglandin E synthase-1 [mPGES-1]) catalyzes the conversion of prostaglandin H2 (PGH2) to PGE2, and that HPGD [hydroxyprostaglandin dehydrogenase 15-(NAD); or 15-PDGH], SLCO2A1 (solute carrier organic anion transporter family member 2A1 or prostaglandin transporter), and ABCC4 (ATP binding cassette subfamily C member 4 or multidrug resistance-associated protein 4) regulate PGE2 degradation (3). Hence, additional future studies of tumor expression of HPGD and the other molecules involved in the PGE2 biosynthetic pathways in relation to MIR21 expression in colorectal cancer are needed. miRNA-targeting therapies for human disease including cancer are currently being investigated (48). In light of our findings, future investigations may be warranted to explore a potential strategy of inhibiting MIR21 in treatment for colorectal cancers expressing high-level PTGS2.

We acknowledge limitations of our study. First, data on cancer recurrence were limited in the two cohorts. However, colorectal-cancer-specific mortality can be considered as a reasonable cancer-specific outcome in a population-based study with long-term follow-up, because median survival for recurrent (metastatic) colorectal cancer was approximately 10 to 20 months during the time period of this study (49). Second, data on cancer treatment were also limited. However, distributions of chemotherapy use and its regimen would unlikely substantially differ according to tumor MIR21 and PTGS2 expression in resected specimens, because these data were not available for treatment decisions. We recognize that another limitation of our current study is the lack of a widely accepted, standardized classification scheme for tumor PTGS2 expression levels. We assessed tumor PTGS2 expression by IHC through the central, blinded review of tumor specimens with rigorous comparison with internal controls. The interobserver agreement for tumor PTGS2 expression levels (0.85; $\kappa = 0.69$) was reasonably good. Any random misclassification of tumor PTGS2 expression status would have driven our results towards the null hypothesis. Despite this limitation, we were able to demonstrate the significant interaction between MIR21 and PTGS2 expression in colorectal cancer mortality analysis.

The strengths of our study include the use of our molecular pathologic epidemiology (50–52) database of rectal and colon carcinoma cases in the two U.S. nationwide, prospective cohort studies, which integrates clinicopathologic features, long-term survival data, and tumor molecular features including miRNA

### Table 3. Tumor MIR21 expression level and colorectal cancer mortality according to PTGS2 expression

<table>
<thead>
<tr>
<th>MIR21 expression level</th>
<th>No. of cases</th>
<th>No. of events</th>
<th>Univariable HR (95% CI)</th>
<th>Multivariable HR (95% CI)</th>
<th>No. of events</th>
<th>Univariable HR (95% CI)</th>
<th>Multivariable HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTGS2-absent/low cancer</td>
<td>73</td>
<td>25</td>
<td>1.00 (0.69–1.46)</td>
<td>1.00 (0.69–1.46)</td>
<td>33</td>
<td>1.00 (0.69–1.46)</td>
<td>1.00 (0.69–1.46)</td>
</tr>
<tr>
<td>PTGS2-high cancer</td>
<td>119</td>
<td>26</td>
<td>1.00 (0.69–1.46)</td>
<td>1.00 (0.69–1.46)</td>
<td>60</td>
<td>1.00 (0.69–1.46)</td>
<td>1.00 (0.69–1.46)</td>
</tr>
</tbody>
</table>

 Abbreviations: CI, confidence interval; HR, hazard ratio.

a The multivariable Cox regression model included sex, age, year of diagnosis, family history of colorectal cancer in parent or sibling, tumor location, disease stage, tumor differentiation, microsatellite instability, CpG island methylator phenotype, KRAS, BRAF, and PIK3CA mutations, and long interspersed nucleotide element-1 (LINE-1) methylation level. A backward elimination with a threshold of $P = 0.05$ was used to select variables in the final models.

b Test for a linear trend was conducted across ordinal quartile categories (1 to 4) of tumor MIR21 expression level as a continuous variable in the Cox regression model.

The interaction values (two-sided) were calculated by the Wald test on the cross-product term of tumor MIR21 expression (ordinal quartile categories (1 to 4)) and PTGS2 expression (ordinal categories; absent (1), low (2), and high (3)) variables in the Cox regression model.
MIR21 expression in colorectal cancer tissue. This population-based colorectal cancer database enabled us to rigorously examine the interactive prognostic association of tumor MIR21 and PTGS2, 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, which increase generalizability of our findings.

In conclusion, tumor MIR21 expression level is associated with higher colorectal cancer mortality independent of clinical, pathologic, and tumor molecular features, and this association is stronger in cancers expressing high-level PTGS2. Additional prospective studies are needed to validate these findings from the current exploratory, hypothesis-generating study. Upon validation, our population-based data may inform future research to develop strategies for colorectal cancer prevention and treatment through targeting MIR21 and the PTGS2/PGE2 pathway.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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