Association of Inflammation-Related and microRNA Gene Expression with Cancer-Specific Mortality of Colon Adenocarcinoma

Jason E. Hawkes,1,2 Carlo M. Croce,4 Suet Yi Leung,3 and Curtis C. Harris1

Abstract  
Purpose: Inflammatory genes and microRNAs have roles in colon carcinogenesis; therefore, they may provide useful biomarkers for colon cancer. This study examines the potential clinical utility of an inflammatory gene expression signature as a prognostic biomarker for colon cancer in addition to previously examined miR-21 expression.

Experimental Design: Quantitative reverse transcriptase-PCR was used to measure the expression of 23 inflammatory genes in colon adenocarcinomas and adjacent noncancerous tissues from 196 patients. These data were used to develop models for cancer-specific mortality on a training cohort (n = 57), and this model was tested in both a test (n = 56) and a validation (n = 83) cohort. Expression data for miR-21 were available for these patients and were compared and combined with inflammatory gene expression.

Results: PRG1, IL-10, CD68, IL-23a, and IL-12a expression in noncancerous tissue, and PRG1, ANXA1, IL-23a, IL-17a, FOXP3, and HLA-DRA expression in tumor tissues were associated with poor prognosis based on Cox regression (Z-score > 1.5) and were used to generate the inflammatory risk score (IRS). IRS was associated with cancer-specific mortality in the training, test (P = 0.01), and validation (P = 0.02) cohorts. This association was strong for stage II cases (P = 0.002). Expression of miR-21 was associated with IL-6, IL-8, IL-10, IL-12a, and NOS2a, providing evidence that the function of this microRNA and these inflammatory genes are linked. Both IRS and miR-21 expression were independently associated with cancer-specific mortality, including stage II patients alone.

Conclusion: IRS and miR-21 expression are independent predictors of colon cancer prognosis and may provide a clinically useful tool to identify high-risk patients.


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Colon adenocarcinoma is a leading cause of cancer mortality worldwide (1) and accounts for ~50,000 deaths annually in the United States (2). Although current adjuvant treatment modalities improve survival for tumor-node-metastasis (TNM) stage III colon cancer patients, whether stage II patients should be given these therapies remains controversial (3, 4). Some stage II patients will benefit from therapy, but therapy for others will harm quality of life with little therapeutic benefit. Therefore, it is important to develop biomarkers to identify high-risk, early-stage patients who may be suitable for therapeutic intervention.

Inflammation plays a key role in tumor initiation, progression, and metastasis (5, 6). Chronic inflammation is associated with increased rates of colon cancer for both ulcerative colitis and Crohn’s disease (7–9). Nonsteroidal anti-inflammatory drugs can reduce colon cancer risk (10). Inflammation-modulating cytokines affect tumor development through roles in cell proliferation, angiogenesis, and apoptosis (11). Cytokines can signal changes directly within the tumor or the tumor microenvironment to influence cancer progression (12). Because inflammation contributes to colon carcinogenesis, expression of inflammatory genes may serve as biomarkers for colon cancer.

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Infiltration of inflammatory cells in colorectal cancer has been associated with prognosis (13–15). Polymorphisms in inflammatory genes have been associated with colon cancer incidence and prognosis (16–18). Expression of inflammatory genes has also been associated with TNM staging and prognosis in colon cancer (19, 20). Previous studies identified unique expression signatures composed of a panel of inflammatory/immune-response genes that predict metastatic progression and survival of hepatocellular carcinoma (21) and lung adenocarcinoma (22) patients. Building on these findings in hepatocellular carcinoma and lung adenocarcinoma, we determined if expression of these inflammatory genes in tumors and the surrounding noncancerous tissue can be used as a prognostic biomarker for colon adenocarcinoma.

Combining multiple, independent prognostic biomarkers may improve the ability to identify cancer patients at high risk of disease progression and mortality. Therefore, adding an additional factor to an inflammatory gene biomarker may provide a more clinically useful biomarker than either alone. MicroRNA expression may serve this purpose. MicroRNAs are small, non-coding RNA molecules that have shown potential as biomarkers for colon cancer (23–26). Expression levels of microRNAs are altered in all cancers that have been studied. Alteration of specific microRNAs can alter tumor progression in mouse models (27), showing their potential to be causal factors in carcinogenesis. We recently reported that patients with tumors expressing high levels of an oncogenic microRNA, miR-21, have worse survival prognosis for stage II or stage III colon adenocarcinoma, showing its potential as a prognostic biomarker for colon cancer (28). The expression of miR-21 has previously been linked to inflammatory responses. Expression of miR-21 is increased following lipopolysaccharide-induced inflammation (29), and increased miR-21 expression occurs during T-cell differentiation (30). Interleukin 6 (IL-6), a proinflammatory cytokine, can drive miR-21 expression through a signal transducers and activators of transcription 3 (STAT3)-dependent mechanism (31). Because miR-21 expression is linked to inflammation and both miR-21 and inflammatory gene expression are linked to colon cancer, combining inflammatory gene biomarkers with miR-21 may improve their clinical utility.

In this study, we set out to measure the expression of inflammatory genes in tumors and paired noncancerous tissue from 196 colon adenocarcinoma patients, and use these data to develop an inflammatory risk model that could be used as a prognostic biomarker for colon cancer. In addition, we used previously acquired data on miR-21 to address two specific questions. First, does miR-21 expression correlate with specific inflammatory genes as is predicted from mechanistic studies in cell culture? Second, does the combination of the inflammatory risk model with miR-21 expression have improved associations with cancer-specific mortality over either alone?

Materials and Methods

Tissue collection and RNA isolation. Pairs of primary colon tumor and adjacent noncancerous tissues came from 83 patients recruited from the University of Maryland Medical Center or Baltimore Veterans Administration Medical Center from 1993 to 2002, and from 113 patients recruited from Queen Mary Hospital in Hong Kong from 1991 to 2000. These patients have been described in a previous study (28). Cases with familial adenomatous polyposis were excluded. Tissues were grossly dissected and flash-frozen after surgery, prior to any adjuvant therapy. Detailed backgrounds for each tissue donor, including age, gender, clinical staging, tumor location, and survival time from diagnosis were collected. Final dates of follow-up were December 31, 2005 and August 16, 2004 for the NCI-Maryland and the Hong Kong cohorts, respectively. Tumor histopathology was classified according to the WHO Classification of Tumor system (1). Informed consent was given by all participants. This study was approved by the Institutional Review Board of the NIH, the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster, and the Institutional Review Board for Human Subject Research at the University of Maryland.

RNA isolation and quantitative reverse transcriptase-PCR of inflammatory genes. RNA from frozen tissue samples was extracted using standard TRIZOL (Invitrogen) methods. RNA was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems) with a 50 ng/μL final concentration. Expression levels of inflammatory genes were measured with custom-designed, Taqman low-density-array real-time PCR plates (Applied Biosystems) containing probes to 23 inflammatory genes: Annexin A1 (ANXA1; assay ID Hs00167549_m1), colon stimulating factor 1 (CSF1; assay ID Hs00174164_m1), MHC class II antigen DRα (HLA-DRα; assay ID Hs00195755_m1), MHC class II antigen Dpα1 (HLA-DPα1; assay ID Hs00410276_m1), IFN-γ (1D Hs00174143_m1), interleukin 1α (IL-1α; assay ID Hs00174092_m1), IL-1β (1D Hs00174097_m1), IL-2 (1D Hs00174114_m1), IL-4 (1D Hs00174122_m1), IL-5 (1D Hs00174200_m1), IL-6 (1D Hs00174131_m1), IL-8 (1D Hs00174103_m1), IL-10 (1D Hs00174086_m1), IL-12A (1D Hs00168405_m1), IL-12B (1D Hs00233688_m1), IL-15 (1D Hs00542571_m1), IL-17A (1D Hs00174383_m1), IL-23A (1D Hs00372324_m1), proteoglycan 1 (PRG1; assay ID Hs00160444_m1), nitric oxide synthase 2A (NOS2A; assay ID Hs00167257_m1), forhead box p3 (FOXP3; assay ID Hs00203958_m1), cluster of differentiation 68 (CD68; assay ID Hs00154355_m1), and tumor necrosis factor α (TNF-α; assay ID Hs00174128_m1), with 18 s rRNA (assay ID Hs99999901_s1) as a normalization control. Expression of inflammatory genes was measured while blinded to all clinical outcomes. For quality control, any tissue that had 18 s threshold cycle values >15 was considered poor quality and were removed. A patient was removed from this study if either noncancerous or paired tumor tissues failed quality control.

Measurement of miR-21. In a previous study, microRNA expression levels were measured in all of these patient samples and is described in detail there (28). Briefly, microRNA expression levels in the NCI-Maryland cohort were measured using microRNA microarrays (Ohio State microRNA microarray version 2.0). For the Hong Kong cohort,
expression of miR-21 was measured using quantitative reverse transcriptase-PCR (qRT-PCR) using Taqman microRNA assays (Applied Biosystems) according to manufacturer's instructions. High-expression cases for miR-21 were defined based on highest tertile separately for the microarrays and qRT-PCR results.

**Statistical analyses.** Expression data were imported into Biometric Research Branch Array Tools v3.6.0, and the median was normalized for the Hong Kong cohort and Maryland cohort separately. Paired t tests identified differentially expressed genes between tumor and noncancerous tissue for the Hong Kong and the Maryland cohorts separately. To account for multiple comparisons, only differences that were found and validated in each cohort separately (P < 0.05) were considered significant. GraphPad Prism v5.0 (Graphpad Software Inc.) was used for correlation analysis.

The Hong Kong cohort was divided randomly into a training cohort and a test cohort to identify a gene expression model associated with cancer-specific mortality. The Maryland cohort was used as the validation cohort for this model. Prior to beginning the analysis, the Hong Kong cohort was selected to divide into a training cohort and a test cohort because it was the larger of the two cohorts and would likely result in a model with improved accuracy compared with the smaller Maryland cohort. Univariate Cox regression analysis on the training cohort was used to select genes associated with cancer-specific mortality (|Z-score| >1.5; P < 0.13) to include in multivariate risk models using previously reported methods (32). All genes were included for these purposes because the microarray data from the Maryland cohort was considered less reliable. IL-4, IL-5, and IL-12b were excluded because they were missing data for >25% of the samples. The Bonferroni-Holm method (33) was used to adjust for multiple comparisons in the combined tumor and noncancerous regression models.

### Results

**Expression of inflammatory genes are systematically altered in colon adenocarcinoma.** This study used two independent cohorts, one consisting of 113 cases recruited from Hong Kong and a second cohort of 83 cases recruited from Maryland (Table 1). The median follow-up times were 84.6 and 80 months for patients in the Hong Kong and the NCI-Maryland cohorts, respectively. The cohorts were similar in TNM staging (P = 0.65, Fisher’s exact) and cancer-specific mortality

| Table 1. Characteristics of study populations and tumors |
|-----------------|-----------------|-----------------|
| **Age at enrollment (y)** | **Hong Kong cohort** | **Maryland cohort** |
| **Training (n = 57)** | **Test (n = 56)** | **Validation (n = 83)** |
| Mean (SD) | 57.8 (15.5) | 53.8 (14.1) | 64.4 (10.6) |
| Range | 32-84 | 30-82 | 32-87 |
| Gender, no. (%) | | | |
| Male | 30 (53) | 26 (46) | 65 (78) |
| Female | 27 (47) | 30 (54) | 18 (22) |
| Tumor location,* no. (%) | | | |
| Distal | 41 (72) | 49 (87.5) | 48 (59) |
| Proximal | 16 (28) | 7 (12.5) | 33 (41) |
| Adenocarcinoma histology, no. (%) | | | |
| Adenocarcinoma | 53 (93) | 52 (93) | 74 (89) |
| Mucinous adenocarcinoma | 4 (7) | 3 (5) | 8 (10) |
| Adenosquamous | 0 | 0 | 1 (1) |
| Signet ring cell and mucinous | 0 | 1 (2) | 0 |
| Adjuvant chemotherapy,† no. (%) | | | |
| Received | 22 (39) | 18 (32) | 22 (38) |
| Did not receive | 35 (61) | 38 (57) | 36 (62) |
| TNM staging,‡ No. (%) | | | |
| I | 2 (4) | 7 (13) | 8 (10) |
| II | 19 (33) | 18 (32) | 29 (35) |
| III | 27 (47) | 21 (38) | 36 (43) |
| IV | 9 (16) | 10 (18) | 9 (11) |
| Removed during quality control§ | 0 | 0 | 10 (12) |

*Distal includes tumors located in or distal to the descending colon. Proximal tumors include tumors in or proximal to the splenic flexure. Tumor location was available for all cases in the Hong Kong cohort and 81 cases in the Maryland cohort.

†Detailed information pertaining to receipt of chemotherapy was available for all patients in the Hong Kong cohort and 58 in the Maryland cohort. Chemotherapy was primarily fluorouracil-based (in forms of either i.v. fluorouracil or oral drugs including tegafur with uracil) with or without levamisole or leucovorin.

‡For one patient in the Maryland cohort, it was unclear if that patient had stage III or stage IV colon cancer, therefore this patient was removed from analyses stratifying by TNM stage.

§Cases with poor quality data from quantitative RT-PCR for either the tumor or nontumorous tissue were removed.
(P = 0.46, Kaplan-Meier log rank) with 5-year survival rates of 49.5% (Hong Kong cohort) and 59.7% (NCI-Maryland cohort). Along with the racial, cultural, and geographic differences of the two cohorts, the Maryland cohort was considerably older, with a higher percentage of men.

We measured the expression of 23 inflammatory genes in primary colon tumor and paired noncancerous tissues using low-density-array real-time PCR. Eighteen of these genes were selected because they were included in previous studies of hepatocellular carcinoma (21) and lung adenocarcinoma (22). The additional five genes (IL-17A, IL-23A, CD68, NOS2A, and FOXP3) were selected based on literature supporting their roles in colonic inflammation or cancer (34–36). IL-4 and IL-5 were not detectable in the majority of tissues and were removed from all further analyses.

Inflammatory gene expression was systematically altered in tumors. Expression of the 21 inflammatory genes could distinguish tumor from noncancerous tissue pairs with 99% or 100% accuracy based on nearest centroid or 3-nearest neighbors class prediction algorithms, respectively (10-fold cross-validation repeated 100 times) using the Hong Kong cohort. Unsupervised hierarchical clustering of the 21 genes separated tissues into two distinct groups: one composed of 97% tumor tissue and the other composed of 99% noncancerous tissue (Fig. 1A). Of the 21 inflammatory genes examined, the expression of 18 was altered in tumors in the Hong Kong cohort (P < 0.05; paired t-test, Supplementary Table S1). Of these, IL-8 showed the largest fold-increase in tumors at ∼13-fold higher levels in tumors whereas IL-2 showed the largest reduction in tumor with ∼80% less in tumors. These results indicate a systematic change in the expression of inflammatory genes during tumorigenesis.

We next analyzed the NCI-Maryland cohort. Fold changes in tumors for these inflammatory genes were consistent with the Hong Kong cohort (Pearson R = 0.96; Fig. 1B), indicating that these changes in gene expression are likely representative of the majority of colon adenocarcinomas. Expression of IL-8, IL-23a, IL-1a, IL-1b, FOXP3, IL-17a, IFN-γ, and IL-6 was significantly increased in tumors from both cohorts, whereas expression of IL-2, IL-15, IL-10, IL-12a, CSF1, HLA-DPA1, HLA-DRA, TNF-a, and CD68 was significantly decreased in tumors from both cohorts.

Colon adenomas represent an early, precancerous lesion of the colon. Changes in inflammatory gene expression in adenoma tissues may indicate early changes in the inflammatory state that can lead to cancer. We evaluated the expression levels of the 23 inflammatory-related genes in 18 pairs of colon adenomas and nonadenoma tissues. Although there was limited
power to detect differences in expression of these genes due to using a limited number of tissues, we found similar changes in gene expression in adenoma as compared with the colon cancer tissues (Supplementary Table S1). When using the Hong Kong cohort as a reference, expression changes in colon adenomas were consistent with colon cancer tissues for these inflammatory genes (Pearson $R = 0.91; P < 0.0001$). Of the 10 genes significantly decreased in tumors, all showed decreased expression in adenomas and seven of these (IL-2, IL-10, IL-12a, CSF1, HLA-DPA1, HLA-DRA, and PRG1) were significantly reduced. Of the eight genes significantly increased in tumors, all eight were increased in adenomas and six of these (IL-8, IL-23a, IL-1a, IL-1b, FOXP3, and IL-17a) were significantly increased. Similar to colon cancer tissues, IL-8 showed the greatest increase and IL-2 showed the greatest decrease in adenoma tissues.

**Inflammatory risk score is associated with cancer-specific mortality.** We evaluated the expression of these inflammatory genes for associations with cancer-specific mortality. Constructing a

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Fig. 2. Strategy for building inflammatory risk scores. Genes were selected for inclusion in the risk score based on univariate Cox regression on the Hong Kong training cohort. Multivariate Cox regression on the training cohort was used to build the risk models. This model was then tested on the Hong Kong test and Maryland validation cohorts.
multi-gene signature using several genes with moderate associations can provide more accurate predictions than a model using a single gene. Therefore, we used univariate Cox regression to identify genes with moderate associations with prognosis following previously established methodologies (32). We randomly split the Hong Kong cohort into a training cohort (n = 57) and a test cohort (n = 56; Fig. 2). These cohorts were similar in clinical characteristics, including age at enrollment, gender, and TNM staging. Based on univariate Cox regression on the training cohort, expression of PRG1, IL-10, CD68, IL-23a, and IL-12a in noncancerous tissue, and PRG1, ANXA1, IL-23a, IL-17a, FOXP3, and HLA-DRA in tumors was moderately associated with cancer-specific mortality ([Z-score] >1.5; using criteria from ref. 32; Supplementary Fig. S1). These genes were selected to construct a multigene risk signature. Using the training cohort, multivariate Cox regression was done on selected genes to develop risk models. The noncancerous risk model was \[ (0.855 \times \text{PRG1}) + (0.720 \times \text{IL-10}) + (0.458 \times \text{CD68}) + (-0.494 \times \text{IL-23a}) + (-0.635 \times \text{IL-12a}) \] = risk score. The tumor risk model was \[ (1.321 \times \text{PRG1}) + (0.840 \times \text{ANXA1}) + (0.123 \times \text{IL-23a}) + (0.484 \times \text{IL-17a}) + (0.367 \times \text{FOXP3}) + (-0.373 \times \text{HLA-DRA}) \] = risk score. Individuals having higher than median values for both models were classified as having high inflammatory risk score (IRS). All others were considered low. When evaluated separately, patients classified as high IRS had significantly worse cancer-specific mortality for the Hong Kong training cohort, the Hong Kong test cohort (P = 0.01;

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**A Inflammatory Risk Score (IRS) by each cohort separately**

<table>
<thead>
<tr>
<th></th>
<th>Hong Kong Training Cohort</th>
<th>Hong Kong Test Cohort</th>
<th>Maryland Validation Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS</td>
<td>Low High</td>
<td>Low High</td>
<td>Low High</td>
</tr>
<tr>
<td>No. at risk</td>
<td>40  37  26  26  14</td>
<td>Low 32  28  21  19  14</td>
<td>Low 47  38  25  19  9</td>
</tr>
<tr>
<td>Months</td>
<td>20  40  60  80</td>
<td>20  40  60  80</td>
<td>20  40  60  80</td>
</tr>
</tbody>
</table>

**B Combined Hong Kong Test and Maryland Validation Cohorts**

<table>
<thead>
<tr>
<th></th>
<th>All Cases</th>
<th>Stage I cases</th>
<th>Stage II cases</th>
<th>Stage III cases</th>
<th>Stage IV cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS</td>
<td>Low High</td>
<td>Low High</td>
<td>Low High</td>
<td>Low High</td>
<td>Low High</td>
</tr>
<tr>
<td>No. at risk</td>
<td>79  66  54  41  28</td>
<td>Low 10  10  10  8  2</td>
<td>Low 33  31  27  22  18</td>
<td>Low 30  25  17  11  8</td>
<td>Low 5  0  0  0  0</td>
</tr>
<tr>
<td>Months</td>
<td>20  40  60  80</td>
<td>20  40  60  80</td>
<td>20  40  60  80</td>
<td>20  40  60  80</td>
<td>20  40  60  80</td>
</tr>
</tbody>
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**Fig. 3.** IRS is associated with cancer-specific mortality in TNM stage II patients. A, IRS in Hong Kong training, Hong Kong test, and NCI-Maryland validation cohorts separately. B, combined analysis of Hong Kong test and NCI-Maryland validation cohorts, stratified by TNM stage. For one patient in the Maryland Cohort, it was unclear if that patient had stage III or stage IV colon cancer and therefore was removed from analyses stratifying by TNM stage.
Kaplan-Meier log rank), and NCI-Maryland validation cohort (P = 0.02, Kaplan-Meier log rank; Fig. 3A).

To evaluate the potential use of IRS as a biomarker, we did a stratified analysis by TNM staging. For these analyses, the Hong Kong training cohort was excluded to prevent overfitting. The Hong Kong test and NCI-Maryland validation cohorts were combined. IRS was associated with TNM stage (P = 0.03, Fisher's exact test). Patients with more advanced TNM stage were more likely to be classified as high IRS. Four of 14 (29%) stage I, 9 of 42 (21%) stage II, 16 of 46 (35%) stage III, and 9 of 14 (64%) stage IV cases were classified as high IRS. High IRS was associated with poor cancer-specific mortality for all patients (P = 0.0003, Kaplan-Meier log rank; Fig. 3B). When stratified by TNM stage, IRS was associated with cancer-specific mortality in stage II cases (P = 0.002, Kaplan Meier log rank; Fig. 3B). IRS was not associated with prognosis in stage I, stage III, or stage IV patients.

We were unable to analyze associations with therapeutic outcome. Of the 34 stage II patients for whom we had information about receipt of adjuvant therapy, only 7 received it. Only one of these seven patients was classified as high IRS. Therefore, we did not have sufficient power to analyze associations between IRS and therapeutic outcome.

miR-21 expression is associated with the expression of IL-6, IL-8, IL-10, IL-12a, and NOS2a. Expression of miR-21 was available for these samples from a previous study (28). miR-21 has previously been shown to be associated with inflammation. Therefore, we used linear regression to examine the associations of miR-21 expression with inflammatory genes in these tissues. This was evaluated in noncancerous tissues, tumor tissues, and then a combination of all tissues adjusting for tumor status (Supplementary Table S2). In the combined model, expression of IL-6 (P < 0.0005), IL-8 (P < 0.0005), and IL-10 (P = 0.002) was positively associated, and IL-12a (P < 0.0005) and NOS2a (P = 0.0025) expression was negatively associated with miR-21 expression. Only IL-6 and IL-12a expression was statistically significant (P < 0.05) in both the tumor and noncancerous tissues, separately.

IRS and miR-21 expression are independently associated with cancer-specific mortality, including stage II patients. We previously reported that high miR-21 expression in tumors was associated with poor prognosis in colon adenocarcinoma (28). That study utilized the same patients as the current study and provides an opportunity to combine miR-21 and IRS to determine if together they have improved prognostic utility. High miR-21 expression was defined in our previous publication where the highest tertile (≥3.3-fold higher than average noncancerous tissue) is defined as high (28). Survival information for the NCI-Maryland cohort was updated from that study to include an additional 12 months of available survival information. Consistent with our previous report, high miR-21 expression is associated with cancer-specific mortality using all cases (P < 0.0001, Kaplan-Meier log rank) or stage II cases (P = 0.006, Kaplan Meier log rank; Fig. 4). Due to the association between IL-6 and miR-21 expression, we investigated if combining IL-6 and miR-21 expression data into survival models would alter the association between miR-21 and cancer-specific mortality, and found that it did not (data not shown).

Although IRS and miR-21 expression were each associated with prognosis, they were not associated with one another (P = 0.83, Fisher's exact). Therefore, combination of these biomarkers may identify high-risk patients that would be misclassified by a single end point. We did a stratified analysis of miR-21 and IRS (Fig. 3). Patients with low miR-21 expression and low IRS had the best prognosis. Patients with high miR-21/low IRS or low miR-21/high IRS had an intermediate prognosis. Patients with high miR-21/high IRS had the worst prognosis. This was true when observing all cases or stage II cases alone. Upon combining intermediate groups, patients classified as high for either miR-21 or IRS score had significantly worse cancer-specific mortality than those classified as low miR-21/low IRS for all cases (P = 0.0002, Kaplan-Meier
log rank) or stage II cases ($P = 0.002$, Kaplan-Meier log rank). Patients classified as high for both miR-21 and IRS had worse survival than patients classified as high for either using all cases ($P = 0.002$, Kaplan-Meier log rank) or stage II alone ($P = 0.02$, Kaplan-Meier log rank). Only two stage II patients were classified as high IRS and high miR-21. Therefore, one should be cautious interpreting the poor outcome of these stage II patients and future studies will explore this association.

Univariate Cox regression analysis for all cases found that high IRS [hazard ratio (HR), 2.4; 95% confidence interval (95% CI), 1.4-4.2], high miR-21 (HR, 3.0; 95% CI, 1.7-5.1), and TNM staging (HR, 4.7; 95% CI, 2.5-8.8) were each associated with poor prognosis (Table 2). Multivariate analyses showed that both high IRS (HR, 2.2; 95% CI, 1.3-3.8) and high miR-21 (HR, 3; 95% CI, 1.7-5.2) were independent of one another and TNM staging. Additionally, the multivariate model including IRS, TNM staging, and miR-21 did significantly better than the model without miR-21 ($P < 0.001$, likelihood ratio test). When restricting the analysis to stage II cases, univariate analyses showed that high IRS (HR, 5.4; 95% CI, 1.7-17.2) and high miR-21 (HR, 4.8; 95% CI, 1.4-16.1) were each associated with poor prognosis. Multivariate analysis showed that high IRS (HR, 7.5; 95% CI, 2.2-25.6) and high miR-21 (HR, 6.5; 95% CI, 1.9-21.9) were each associated with prognosis independent of one another. A multivariate model including both IRS and miR-21 in stage II patients did significantly better than a model including only IRS ($P = 0.004$, likelihood ratio test). Therefore, IRS and miR-21 expression may be used together as a prognostic biomarker for stage II colon adenocarcinoma.

**Table 2.** Cox regression of inflammatory risk score and miR-21 expression with cancer-specific mortality on combined Hong Kong test cohort and Maryland validation cohort

<table>
<thead>
<tr>
<th>Variable (comparison/referent)</th>
<th>All cases, regardless of TNM stage</th>
<th>Stage II cases, adjusted for cohort membership</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate analysis*</td>
<td>Multivariate analysis†</td>
</tr>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>$P$</td>
</tr>
<tr>
<td>IRS (high/low)</td>
<td>2.4 (1.4-4.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-21 expression (high/low)</td>
<td>3.0 (1.7-5.1)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Tumor stage (III-IV/I-II)</td>
<td>4.7 (2.5-8.8)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Age in y (50/&lt;50)</td>
<td>1.1 (0.6-2.1)</td>
<td>0.82</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>1.9 (1.0-3.5)</td>
<td>0.06</td>
</tr>
<tr>
<td>Tumor location (proximal/distal)</td>
<td>0.8 (0.4-1.7)</td>
<td>0.58</td>
</tr>
</tbody>
</table>

*Univariate analysis is adjusted for cohort membership only.
†Multivariate analysis is adjusted for cohort membership, IRS, miR-21 expression, and (where appropriate) TNM stage. Multivariate analysis used stepwise addition and removal of clinical covariates found to be associated with survival in univariate models ($P < 0.10$) and final models include only those covariates that were significantly associated with survival (Wald statistic, $P < 0.05$). miR-21 measurements were available for 115 of 117 patients, including all 42 stage II patients, and only those patients are included in multivariate analyses.

**Discussion**

We found systematic changes in inflammatory gene expression in colon tumors. Of the eight inflammatory genes consistently increased in tumors, seven (IL-8, IL-23a, IL-1a, IL-1b, IL-17a, INFγ, and IL-6) are proinflammatory cytokines and the other is FOXP3, a marker for regulatory T cells. These results are consistent with other reports evaluating their expression in colon cancer (37). Therefore, there are predictable changes in inflammatory gene expression in colon tumors, consistent for a role for these genes in carcinogenesis. We found similar changes in gene expression in colon adenomas. This indicates that changes in the inflammatory state may be an early event in colon carcinogenesis.

Expression of inflammatory genes was associated with miR-21 expression. The association of IL-6 and IL-12a expression was statistically significant in both the tumor and noncancerous tissues, separately. IL-6 is thought to drive the expression of miR-21 in a STAT3-dependent mechanism (31). Our results are consistent with that model and provide evidence that this mechanism may be relevant to colon cancer. There is also a predicted binding site for miR-21 in the 3‘ untranslated region of IL-12a as indicated by Targetscan 5.0 (38) and miRanda (39). IL-12a has a negative correlation with miR-21, which is consistent with a pattern for a miR-21 target. Based on this finding, mechanistic studies should be done to determine if IL-12a is a target of miR-21. The interaction between miR-21 and inflammatory genes may play an important role in colon carcinogenesis. Although the associations between miR-21 and inflammatory genes may play an important role in colon carcinogenesis. Although the associations between miR-21 and inflammatory genes may play an important role in colon carcinogenesis.
explained by these models. This indicates that other mechanisms for gene regulation contribute to the expression of these genes. For example, the mir-21 promoter contains putative binding sites for the transcription factors AP-1, Ets/PU.1, SRF, TP53, C/EBPa, and STAT3 (40), and miR-21 expression can also be influenced by epidermal growth factor receptor activity (41). Therefore, it is likely that the expression of miR-21 is influenced by many of these and other factors in the context of cancer. The expression of inflammatory genes is altered in colon adenocarcinoma. Although this study does not address the causal relationship between changes in inflammatory gene expression and carcinogenesis, these changes are consistent with published work showing a potential causal relationship between altered expression of inflammatory genes and carcinogenesis. For example, IL-8 showed the highest fold-increase in both colon tumors and adenomas in our study. Previous studies have shown IL-8 to be a proinflammatory chemokine that is expressed at elevated levels in tumors (42). IL-8 expression has been shown to enhance cell proliferation, cell survival, and angiogenesis through induction of the multiple signaling pathways. Conversely, IL-2 showed the largest reduction in tumors and adenomas. IL-2 expression had been found to inhibit tumor growth in vivo, and high dose IL-2 therapy has shown some promise to reduce tumor burden in patients (43) exhibiting a causal role between aberrant expression of IL-2 and cancer.

Expression of inflammatory genes in tumors and the surrounding noncancerous tissues is associated with prognosis in colon adenocarcinoma. This cooperation of tumor and noncancerous expression of inflammatory genes was observed in our previous investigation of lung adenocarcinoma (22). Higher expression of IL-10 in noncancerous tissues was associated with worse survival in that study and the current study. IL-10 is an anti-inflammatory cytokine that can suppress cell-mediated immunity (44). Therefore, elevated IL-10 in noncancerous tissue may create an inflammatory environment primed for metastasis and disease progression.

High levels of IL-23a and IL-12a in the noncancerous tissue were associated with improved survival. Both are members of the IL-12 family of proinflammatory cytokines (45). IL-12 activity is important for host resistance to tumors (46), therefore high levels of IL-12 in the tumor microenvironment may lead to resistance of tumor progression and metastasis through induction of IFN-γ and activation of natural killer cells and cytotoxic T cells. In contrast, elevated levels of IL-23a and IL-17a in cancerous tissues were associated with worse survival and may promote a microenvironment that suppresses any host antitumor response. IL-23a can stimulate Th17 cells to increase the production of IL-17a, and overexpression of IL-17a in cervical cancer (47), non–small cell lung cancer (48), or fibrosarcoma (49) cell lines increases tumor formation and/or tumor growth in xenograft mouse models. These cytokines are associated with a Th17 response. Therefore, a Th17 response in tumors may create a favorable condition for tumor progression.

There are limitations to the current study. First, patients with mucinous or adenosquamous histologies were excluded from this study; therefore IRS may not be applicable to these patients. Additionally, the IRS was built using relatively few cases (n = 53). Developing molecular signatures of these genes on larger cohorts may strengthen the accuracy and precision of this biomarker, although the validation of this biomarker in two independent cohorts shows its potential clinical utility. It will be important to begin exploring the relationship between IRS and other clinical covariates, such as microsatellite instability status, p53 mutational status, or K-ras mutational status, to determine if a combination of these markers can provide more clinically useful information. It will also be useful to do immunohistochemistry on patient samples to determine localization patterns of these inflammatory genes to gain insights into the cell types responsible for this gene signature. This will provide mechanistic insights as to how the combination of these genes may contribute to the worse prognosis in high-IRS patients.

We found the association between IRS and survival to be strongest in TNM stage II patients. The reason for this association in stage II patients and not stage III/IV patients is unclear. One possibility is that high IRS is associated with a favorable inflammatory environment for metastasis. This is consistent in that we see more advanced-stage patients are more likely to be classified as high IRS. In this context, it may be a useful marker only in stage II patients, for which metastasis is not yet detectable by current clinical methods. In stage III or IV patients, metastasis is evident and therefore an IRS score to predict metastasis is not meaningful.

Cancer immunotherapy is a promising field of research for colon cancer (50). As in any therapy, successful stratification of patients into groups that are more or less likely to respond will increase the chances of developing successful immunotherapies. IRS is based on the expression of inflammatory genes, and the expression of these genes is likely to be correlated with the current state of the immune system. It is possible that IRS may be associated with a patient's response to immunotherapy. Although future investigation of this is needed, there is a potential that IRS, or a similar inflammatory gene biomarker, may be able to identify patients more or less likely to respond to immunotherapy.

There is a need for better ways of diagnosing early-stage colon cancer patients with undetectable micrometastases. Therefore, we propose that a subset of stage II patients would benefit from therapeutic intervention as their disease will likely progress; but for others, therapeutic intervention unnecessarily harms quality of life and continued screening would be sufficient. We found IRS was significantly associated with prognosis in stage II patients. Previously, we identified miR-21 as a prognostic biomarker for stage II patients (28). The combination of IRS and miR-21 expression was a better predictor of prognosis than either alone. Therefore, IRS and miR-21, alone or in combination, have potential to help diagnose stage II patients and assist in choosing treatment options. Prospective studies to evaluate this potential are warranted.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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