

Imaging, Diagnosis, Prognosis

Inflammatory and MicroRNA Gene Expression as Prognostic Classifier of Barrett's-Associated Esophageal Adenocarcinoma

Giang Huong Nguyen^{1,2}, Aaron J. Schetter^{1,3}, David B. Chou^{2,4}, Elise D. Bowman¹, Ronghua Zhao⁵, Jason E. Hawkes^{1,2}, Ewy A. Mathé¹, Kensuke Kumamoto¹, Yiqiang Zhao^{1,6}, Anuradha Budhu¹, Nobutoshi Hagiwara⁷, Xin Wei Wang¹, Masao Miyashita⁷, Alan G. Casson⁵, and Curtis C. Harris¹

Abstract

Purpose: Esophageal cancer is one of the most aggressive and deadly forms of cancer; highlighting the need to identify biomarkers for early detection and prognostic classification. Our recent studies have identified inflammatory gene and microRNA signatures derived from tumor and nontumor tissues as prognostic biomarkers of hepatocellular, lung, and colorectal adenocarcinoma. Here, we examine the relationship between expression of these inflammatory genes and micro RNA (miRNA) expression in esophageal adenocarcinoma and patient survival.

Experimental Design: We measured the expression of 23 inflammation-associated genes in tumors and adjacent normal tissues from 93 patients (58 Barrett's and 35 Sporadic adenocarcinomas) by quantitative reverse transcription-polymerase chain reaction. These data were used to build an inflammatory risk model, based on multivariate Cox regression, to predict survival in a training cohort ($n = 47$). We then determined whether this model could predict survival in a cohort of 46 patients. Expression data for miRNA-375 were available for these patients and was combined with inflammatory gene expression.

Results: IFN- γ , IL-1 α , IL-8, IL-21, IL-23, and proteoglycan expression in tumor and nontumor samples were each associated with poor prognosis based on Cox regression [(Z-score) >1.5] and therefore were used to generate an inflammatory risk score (IRS). Patients with a high IRS had poor prognosis compared with those with a low IRS in the training ($P = 0.002$) and test ($P = 0.012$) cohorts. This association was stronger in the group with Barrett's history. When combining with miRNA-375, the combined IRS/miR signature was an improved prognostic classifier than either one alone.

Conclusion: Transcriptional profiling of inflammation-associated genes and miRNA expression in resected esophageal Barrett's-associated adenocarcinoma tissues may have clinical utility as predictors of prognosis. *Clin Cancer Res*; 16(23); 5824–34. ©2010 AACR.

Esophageal cancer (EC) is one of the most lethal malignancies of the digestive tract, and at the time of diagnosis, most patients are at an advanced stage (1). Despite advanced surgical techniques combined with

various treatment modalities, such as radiotherapy and chemotherapy, the overall 5-year survival rate of EC remains at 15% to 24%, highlighting the need for identification of prognostic biomarkers and new therapeutic strategies (2).

There are 2 main histologic subtypes of EC, squamous cell carcinoma (SCC) and adenocarcinoma (ADC). The incidence of ADC has increased remarkably over the past 2 decades and has supplanted SCC as the dominant phenotype in Western countries (3). ADC frequently arises from Barrett's esophagus (BE), a chronic inflammatory condition characterized by a change in the normal esophagus epithelium into intestinal metaplasia as a result of gastroesophageal reflux (4, 5) and genomic instability (6).

Both epidemiologic and functional studies have implicated chronic inflammation in the development of many human cancers including liver, esophagus, stomach, colon, and bladder cancer (7). During this process, deregulated cytokine production and signaling between the immune and neoplastic cells can alter cell growth, differentiation, and apoptosis (8, 9). We and others have reported that

Authors' Affiliations: ¹Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, ²Howard Hughes Medical Institute-National Institutes of Health Research Scholar, Howard Hughes Medical Institute, Chevy Chase, ³Cancer Prevention Fellowship Program, Office of the Director, National Cancer Institute, National Institutes of Health, Bethesda, and ⁴Mucosal Immunology Unit, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; ⁵Department of Surgery, University of Saskatchewan, Saskatoon SK, Canada; ⁶Peking University, Beijing, People's Republic of China; and ⁷Department of Surgery, Nippon Medical School, Tokyo, Japan

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Corresponding Author: Curtis C. Harris, Laboratory of Human Carcinogenesis, National Cancer Institute, National Institutes of Health, 37 Convent Drive, Building 37, Room 3068, Bethesda, MD 20892. Phone: (301) 496-2048; Fax: (301) 496-0497; E-mail: curtis_harris@nih.gov.

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Translational Relevance

In this report, we provide evidence that elevated expression of inflammatory genes in tumor and paired nontumor tissues was an independent prognostic marker for Barrett's-associated esophageal adenocarcinoma patients. We also report that combining independent markers can be an improved prognostic classifier than either one alone. By combining the inflammatory gene signature and miRNA-375 expression, we could better predict clinical outcome of Barrett's-associated adenocarcinoma patients. Our results suggest that this criteria defined here may have prognostic and therapeutic implications for the management of esophageal cancer patients.

inflammation-associated gene signatures can be used as indicators of prognosis and tumor burden in liver (10), lung (11), colorectal cancer patients (12–14).

Several studies demonstrated that expression of inflammatory genes is associated with EC progression and prognosis (15–20). In addition, experimental evidence indicates that tumor cells can produce cytokines to enhance their growth and to counteract the host immune response (21, 22). Most of these studies, however, examined relatively few genes and focused on patients with SCC, with far less published information on ADC. Furthermore, there are also limited data regarding the association between inflammation and prognosis in patients who receive preoperative adjuvant therapy.

Although inflammation-related cytokines have shown as potential diagnostic and prognostic markers, emerging data suggest that micro RNAs (miRNA) also provide independent prognostic information in cancer patients. miRNAs are small, noncoding RNA molecules that its expression signatures have been associated with the etiology and prognosis of many cancer types (23). Detection of miRNAs in patients with cancer has been shown to be associated with cancer progression and shortened survival (24). For example, we recently reported that Barrett's-associated adenocarcinoma (BAC) patients with reduced expression of miR-375 in cancerous tissue had worse prognosis (25) and tumor expression of miR-16-2, miR-30e, and miR-200a were individually associated with shorter overall and disease-free survival in esophageal ADC patients (26).

Combining a marker of miRNA with cytokines in a multimarker strategy appears to be a useful approach for improving risk assessment in cancer patients. Colorectal cancer patients with increased miR-21 expression and a high inflammatory risk score (IRS) have been shown to have an increased mortality risk compared with those with both reduced miR-21 expression and lower IRS (12). Integrating this multimarker approach into the routine assessment of colorectal cancer patients may allow clinicians to more accurately identify high-risk patients who may derive increased benefit from intensive management strategies.

These findings support a model that the tumor inflammatory microenvironment combined with miRNAs can be a prognostic biomarker for EC patients. In this study, we examined the expression of 23 inflammatory genes and use these data to develop an IRS that could be used as a prognostic classifier of ADC patients. We then used our previously reported data of miR-375 to determine whether the combination between this miRNA and IRS could be a better prognostic classifier than either alone. The possible role of multimarker gene signatures as a prognostic classifier of response to neoadjuvant chemoradiation therapy has also been examined.

Patients and Methods

Clinical samples

Our cohort comprised 93 patients who underwent surgical resection of primary esophageal ADC between 1993 and 2001 at the University of Maryland Medical Center, Baltimore Veterans Administration Medical Center (Baltimore, Maryland, USA) and the Queen Elizabeth II Health Science Centre (Halifax NS). The use of specimens from these centers was approved by Institutional Review Boards. All patients provided written consent, and samples were collected after surgical resection. The nontumor samples were taken from tumor-free margins of resected tumors and were histologically normal. Histopathologic diagnoses of BAC were verified on the basis of (hematoxylin and eosin) H&E-stained sections according to the Vienna Classification of gastrointestinal epithelial neoplasia (27). Diagnoses of BE were made if intestinal metaplasia was found histologically. All BACs originated in the lower esophagus or gastroesophageal junction, corresponding to ADC of the esophageal-gastric junction type. Tumor stage at the time of diagnosis and surgery was based on 2004 World Health Organization classification guidelines. Forty patients in the U.S. cohort received induction chemoradiation therapy, comprising Cisplatin and 5-fluorouracil concurrent with 45 to 50.4 Gy external beam radiation. Response to therapy was assessed according to Response Evaluation Criteria in Solid Tumors criteria (28). Cancer-specific mortality was used as the endpoint for all analyses.

RNA isolation and quantitative reverse transcription polymerase chain reaction analysis (qRT-PCR)

Surgical resected specimens used for RNA extraction were transported immediately to the laboratory and a senior pathologist provided representative sections of tumor and adjacent nontumor tissues, which were immediately snap-frozen in liquid nitrogen. *Paraffin-embedded slides* were H&E stained to confirm histologic diagnosis and determine tumor cellularity. Most samples have minimal 80% cancer cells with little or no necrosis. Total RNA was isolated using standard TRIZOL (Invitrogen) and then reverse transcribed using AB High-Capacity cDNA Archive Kit (Applied Biosystems or AB). Expression levels for individual inflammatory genes were evaluated in duplicate using Taqman Gene Expression Assays (AB). Primers for 23 genes were

purchased from AB, including interleukin 1 α (IL-1 α , assay ID Hs00174092_m1), IL-1 β (ID Hs00174097_m1), IL-2 (ID Hs00174114_m1), IL-5 (ID Hs00174200_m1), IL-6 (ID Hs00174131_m1), IL-8 (ID Hs00174103_m1), IL-10 (ID Hs00174086_m1), IL-12p35 (ID Hs00168405_m1), IL-12p40 (ID Hs00233688_m1), IL-15 (ID Hs00542571_m1), IL-17 (ID Hs00174383_m1), IL-18 (ID Hs00155517_m1), IL-21 (ID Hs00222327_m1), IL-22 (ID Hs00220924_m1), IL-23 (ID Hs00372324_m1), IL-24 (ID Hs01114274_m1), interferon gamma (IFN- γ , assay ID Hs00174143_m1), and tumor necrosis factor- α (TNF- α , assay ID Hs00174128_m1), the major histocompatibility complex class II antigens (HLA-DRA1, assay ID Hs00219575_m1; HLA-DPA1, assay ID Hs00410276_m1), annexin A1 (ANXA1, assay ID Hs00167549_m1), platelet proteoglycan (PRG1, assay ID Hs00160444_m1), colony stimulating factor 1 (CSF1, assay ID Hs00174164_m1), and 18S ribosomal RNA (18S rRNA, assay ID Hs99999901_s1). Double-stranded cDNA for each patient sample was amplified for 40 cycles using the TaqMan Universal PCR Master Mix using the manufacturer's protocol on the 7500HT Sequence Detection System (AB). For quality control, any samples with either an 18S rRNA cycle value greater than 20 or a gene cycle value greater than 36 were considered of poor quality and removed. In addition, a patient was removed from the analysis if either its tumor or its matched nontumor sample failed quality control. The tumor:nontumor ratios were then calculated using the comparative method ($2^{-\Delta\Delta Ct}$), where Ct = threshold cycle and $\Delta\Delta Ct$ = (Ct gene tumor - Ct 18S rRNA tumor) - (Ct gene nontumor - Ct 18S rRNA nontumor), as previously described (12).

MiRNA measurement

MicroRNA-375 expression level was previously measured in all patient samples and was described in detail there (25). Briefly, miRNA-375 expression level was measured by quantitative reverse transcriptase PCR (qRT-PCR) in triplicate using Taqman microRNA assays (Applied Biosystems) according to the manufacturer's instructions. High or low tumor miR-375 expression was defined on the basis of the median expression separately done for each cohort.

Statistical analysis

BRB ArrayTools version 3.6.0 (NCI, NIH) was used to explore the differential expression between tumor samples and their matched nontumor samples. A differentially expressed gene was considered to be up- or downregulated if $P \leq 0.05$ and false discovery rate (FDR) $\leq 5\%$, using the Wilcoxon matched pairs signed rank test.

The association between IRS, miRNA, and survival was analyzed by Kaplan-Meier and assessed for significance by the log-rank test using WINSTAT 2007 (R Fitch Software, Bad Krozingen).

All samples were randomly divided into a training or test cohort to identify a gene expression model associated with cancer-specific mortality. There were similar numbers for each subgroups presented in both the training and test cohorts. 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 (12). All genes were included for these purposes, and expression values for all analyses are continuous variables. For multivariate Cox regression models, missing values for genes were replaced with the average values. In the training cohort, selected genes were used to build multivariate models using tumor and nontumor ratios. Coefficients from these models were multiplied with gene expression values and summed to build risk scores. Individuals were defined as high risk if they had highest tertile scores for combined tumor and noncancerous ratios, separately for each cohort to account for possible confounding factors in clinical management from different institutions. Cox regression was carried out in Stata 9.2 (StataCorp LP). For survival analysis, only the year of diagnosis was used as a continuous variable, and all of the other variables were used as categorical variables. The proportional hazard assumption tested using Schoenfeld residual was met for all models reported. Multivariate models were based on stepwise addition and removal of clinical covariates found to be associated with poor survival in univariate models ($P < 0.10$). A Wald statistic of $P < 0.05$ was used as the criterion for inclusion in final multivariate models. All P values reported are 2-sided. Correlation analysis between miR-375 expression and cytokine genes was done using Stata 9.2 (StataCorp LP).

Results

Differential gene expression in esophageal cancer

Ninety-three patients (66 Americans and 27 Canadians) were recruited into this study and were randomly assigned to a training cohort ($n = 47$) or a test cohort ($n = 46$; Table 1). The median follow-up times for both cohorts were 72 months. Both cohorts were similar in age, gender, race, TNM stage, Barrett's, neoadjuvant treatment status, smoking, drinking status, and cancer-specific mortality (Table 1).

We measured the expression of 23 inflammation-related genes in all 93 tumor and paired nontumor tissues using qRT-PCR (Supplemental Table S1). Nineteen of these genes were selected from the previous studies of hepatocellular (10) lung (11) and colon carcinoma (12). We added 4 more genes (IL-18, IL-21, IL-22, and IL-24) on the basis of literature supporting their roles in esophageal carcinogenesis (29-33).

Inflammatory genes were differentially expressed between tumor and the adjacent nontumor tissues. Three genes, IL-8, IL-21, and IL-24, were significantly increased in tumors, whereas expression of 6 genes, ANXA1, IL-1 α , IL-2, IL-12p35, IL-18, and TNF α , were significantly decreased within the ADC tumor ($P \leq 0.05$; paired t test; FDR $\leq 5\%$; Supplemental Table S2). Of note, the top differentially expressed genes between tumor and nontumor were, IL-24 (9-fold increase) and ANXA1 (12-fold decrease). These findings reflect the possible diverse effects that these inflammatory genes can have in the tumor microenvironment of EC cells.

Inflammatory risk score and cancer-specific mortality in the training cohort

Similar to our previous analysis in colon cancer (12), we constructed a multigene signature using several genes with moderate association with prognosis in an attempt to develop an IRS in the training cohort that was then validated using the test cohort. A flow chart of this algorithm is shown in Figure 1. First, the 93 specimens (66 Americans and 27 Canadians) were randomly assigned to a training set ($n = 47$) or a test set ($n = 46$; Table 1). We then applied the Cox proportional hazard regression to each of the 23 inflammatory genes in the training cohort to identify essential genes that were correlated with cancer-related death. We identified 6 genes, IFN- γ , IL-1 α , IL-21, IL-23 (strong association), IL-8, and PRG (moderate association), that were associated with patient survival [(Z-score) > 1.5; Supplemental Fig. S1]. We then used these 6 genes to construct a signature by the risk score method. A risk score formula (IRS) was calculated as [(0.079 \times IFN- γ) + (0.085 \times IL-1 α) + (0.299 \times IL-21) + (0.072 \times IL-23) + (0.049 \times IL-8) - (0.01 \times PRG)]. This risk score method was used to calculate the IRS for all patients in the training cohort. We then ranked patients in the training cohort according to their IRS and divided them into a high-risk group (high IRS) and a low-risk group (low IRS) using the upper tertile risk-score as the cut off point. Patients with higher than the upper tertile IRS were considered as having a high IRS, whereas all others were considered low IRS. In the context of survival, we found patients with elevated IRS had a significantly shorter survival than patients with a low IRS ($P = 0.00026$, Kaplan–Meier log rank; Training cohort; Fig. 2A).

Validation of IRS as a prognostic classifier in the test cohort

To validate these findings in our test cohort, we used the same risk score formula obtained from the training cohort to calculate IRS for each of the 46 patients in the test cohort. We then dichotomized them into the high-risk group (high IRS) and the low-risk group (low IRS) using the same cut off point as in the training set. The test cohort was similar in all clinical characteristics to the training cohort (Table 1). Similar to what was observed in the training cohort, patients with elevated IRS had poor prognosis than patients with low IRS ($P = 0.01$, Kaplan–Meier log rank; Fig. 2B). Given the similarity seen in both the training and test cohorts, we decided to combine these 2 cohorts for any subgroup analysis. Likewise, patients with high IRS associated with poor survival than the ones with low IRS ($P = 1.9 \times 10^{-5}$; Kaplan–Meier log rank; Fig. 2C).

IRS is associated with patient survival within histologic subgroups

Because ADC can arise in very different biological settings, such as in the presence or absence of BE, we next examined the relationship between BAC (58 of 93 patients = 62%) and these inflammatory genes. Of all the genes tested, none was differentially expressed at a statistically significant level between BAC and SAC (Supplemental

Table 1. Characteristics of the population

	Training cohort ($n = 47$)	Test cohort ($n = 46$)	P^d
Age at surgery, y			
Mean (SD)	61.7 (10.6)	62.7 (10.4)	
Range	41-79	32-80	
Nationality, no.(%)			1.0
American	34 (72)	32 (70)	
Canadian	13 (28)	14 (30)	
Gender, no.(%)			0.8
Male	43 (91)	40 (87)	
Female	4 (9)	6 (13)	
Race, n (%)			1.0
White	46 (98)	45 (98)	
Non-white	1 (2)	1 (2)	
TNM staging ^a , no.(%)			0.48
0	10 (21)	10 (21)	
I	10 (21)	9 (20)	
II	11 (23)	12 (26)	
III	13 (28)	12 (26)	
IV	3 (7)	3 (7)	
History of Barrett's esophagus ^b , n (%)			0.68
Yes	27 (47)	31 (67)	
No	20 (53)	15 (33)	
Neoadjuvant treatment (CRT) ^c , n (%)			0.94
Yes	22 (47)	18 (39)	
No	25 (53)	28 (61)	
Drinking history, n (%)			0.64
Yes	32 (68)	33 (72)	
No	8 (17)	9 (19)	
Unknown	7 (15)	4 (9)	
Smoking history, n (%)			0.94
Yes	30 (64)	31 (67)	
No	9 (19)	8 (17)	
Unknown	8 (17)	7 (16)	

^aTNM staging at the time of surgery was based on the 2004 World Health Organization classification.

^bDiagnosis of Barrett's esophagus was verified on the basis of H&E-stained section according to the Vienna Classification of gastrointestinal epithelial neoplasia.

^cCRT composed of cisplatin and 5-fluorouracil concurrent with 45- to 50.4-Gy external beam radiation.

^dFisher's exact test.

Table S3). In the context of survival, we found BAC patients with elevated IRS had significantly poor prognosis than those with low IRS ($P = 0.0009$, Kaplan–Meier log rank; Fig. 3A). We observed a similar trend with SAC patients ($P = 0.0083$, Kaplan–Meier log rank; Fig. 3E).

Next, we examined the expression of these genes in a subset of 53 ADC patients in the combined cohort ($n = 93$) that did not receive neoadjuvant treatment (NAT). This

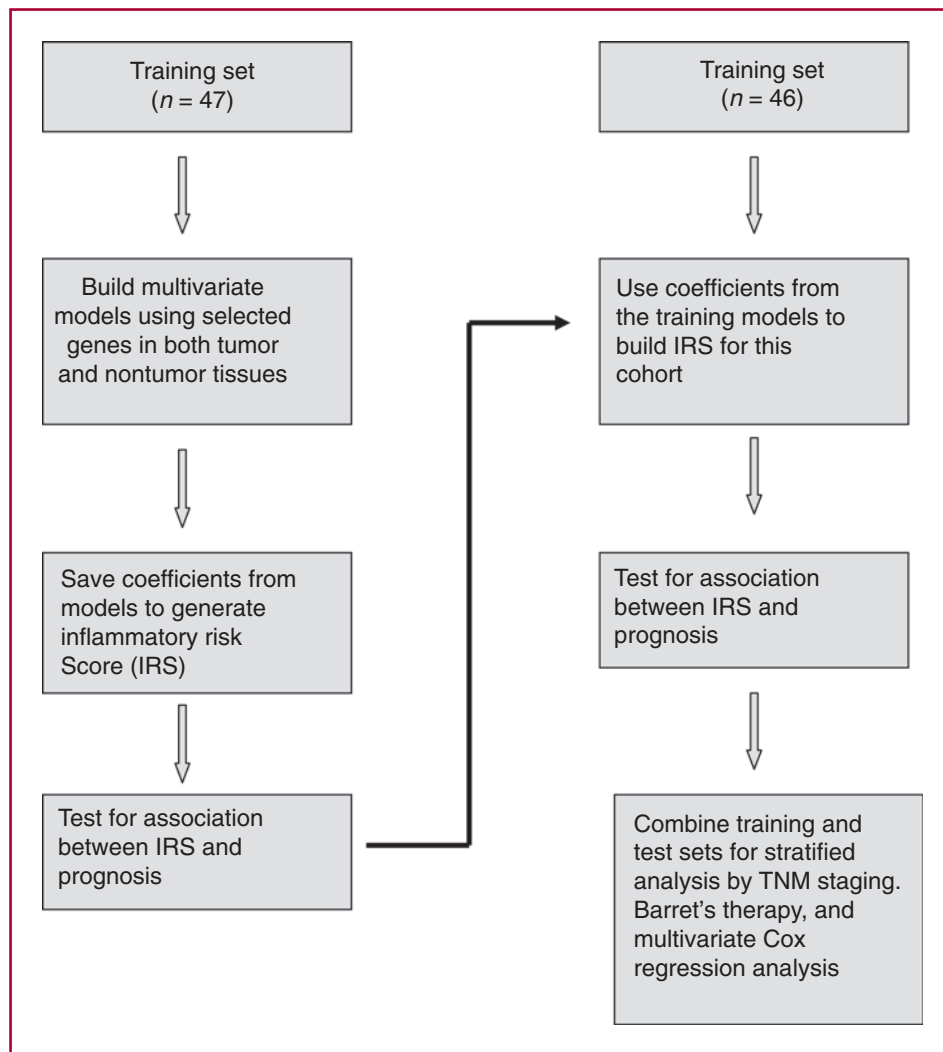


Fig. 1. Model for building inflammatory risk scores. Genes chosen for IRS in the training set were selected on the basis of univariate Cox regression. Multivariate Cox regression was used to build the IRS model. This model was then tested on an independent cohort (test cohort).

allows an assessment of the prognostic impact of these inflammatory genes, independent of any effects of therapy. In comparison with patients who had low IRS, that is, below the upper tertile, those with high IRS experienced a statistically significant reduction in cancer-specific survival ($P = 3.3 \times 10^{-6}$, Kaplan–Meier log rank; Supplemental Fig. 2A).

In our combined cohort, 40 of 93 patients (43%) received preoperative chemoradiation therapy, of which 15 (32.5%), 10 (25%), and 15 (32.5%) patients showed pathologic complete, partial, and nonrespondent to treatment, respectively. In the case of complete responders, there were still microscopic tumors seen on the slides. Kaplan–Meier showed that elevated IRS was associated with shorter survival, although it was not statistically significant (Supplemental Fig. 2B). In addition, minimal TNR difference among IFN- γ , IL-1 α , IL-21, IL-23, IL-8, and PRG was observed between NAT and NAT-naïve patients (Supplemental Table S4). Furthermore, NAT patients did not receive any survival benefit compared to NAT-naïve

patients [(hazard ratio, HR, 1.83; 95% confidence interval (95% CI), 0.80–4.16; $P = 0.15$]. In summary, high IRS was associated with poor prognosis and a similar trend was observed for both NAT-naïve and BAC patients.

Combined IRS and miR-375 is a better prognostic classifier of cancer-specific mortality in BAC patients

Quantitative real-time PCR data for miR-375 expression were available for all 93 ADC tumor and nontumor samples from our previous study (25). We previously reported that reduced tumor expression of miR-375 was associated with poor prognosis in BAC, but not SAC patients (25). In this study, we showed that elevated IRS was associated with poor survival in ADC patients. Because both of these studies shared the same patient population, we asked whether or not combining these 2 signatures (IRS and miRNA) could improve prognostic utility of either method alone.

IRS and miRNA-375 were not associated with one another ($P = 0.402$ for all cases or $P = 0.661$ for all Barret's

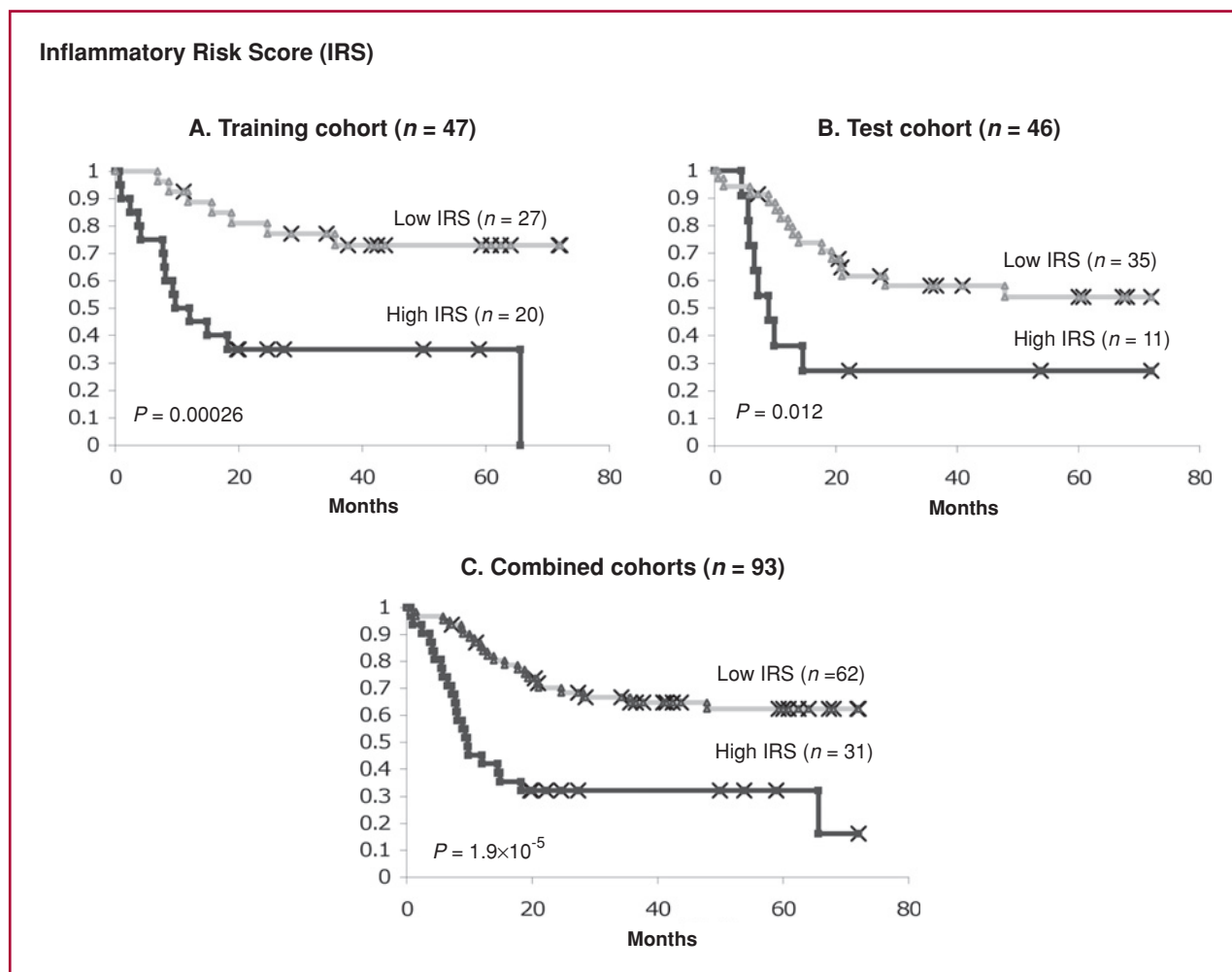


Fig. 2. High IRS is associated with poor prognosis in the training, test, and a combined cohort. High IRS expression was classified on the basis of the highest tertile using tumor and nontumor ratio. A, the association of high IRS with poor prognosis in the training cohort. B, high IRS associated with poor survival was validated in a test cohort. C, the association of high IRS and poor survival in a combined cohort. Log-rank P values are from Kaplan–Meier analysis.

cases, Fisher's exact test). Therefore, combination of these 2 markers might provide a good survival prediction power over either method alone. Furthermore, we used linear regression to examine the association of miR-375 expression with inflammatory genes in tumor and nontumor tissues. None showed to be either positively or negatively correlated with miR-375 expression in a statistical manner. Next, we analyzed miR-375 in tumor samples of all patients using the median expression cut off where a high value was defined as having higher than the median value for all samples in the same cohort, consistent with our previous method (25). As we previously reported, low miR-375 expression was associated with poor survival in BAC patients ($P = 0.0088$, Kaplan–Meier log rank; Fig. 3B). Given that no significant association between miR-375 and survival observed in SAC patients ($P = 0.677$, Kaplan–Meier log rank; Fig. 3F), we decided to focus on BAC for further analysis from here on.

We then performed an analysis of IRS and miR-375 in the BAC group. Patients with high IRS/low miR-375 had the worst prognosis. Patients with either high IRS/high miR-375 or low IRS/low miR-375 had an intermediate prognosis. Patients with low IRS/high miR-375 had the best prognosis (Fig. 3C). From the survival curve shown in Figure 3C, we found that the survival curve for the low IRS/low miR-375 superimposed the curve for the high IRS/high miR-375. Therefore, we combined these 2 groups into a new group called "Intermediate risk". The high IRS/low miR-375 was defined as "High risk" and the low IRS/high miR-375 was defined as "Low risk". Patients classified as either high risk or low risk had significantly worst survival than the intermediate risk group ($P = 0.042$ and $P = 0.025$, Kaplan–Meier log rank; respectively Fig. 3D). The "High risk" group was expected to have a shorter survival than the "Low risk" group ($P = 1.6 \times 10^{-5}$, Kaplan–Meier log rank; Fig. 3D).

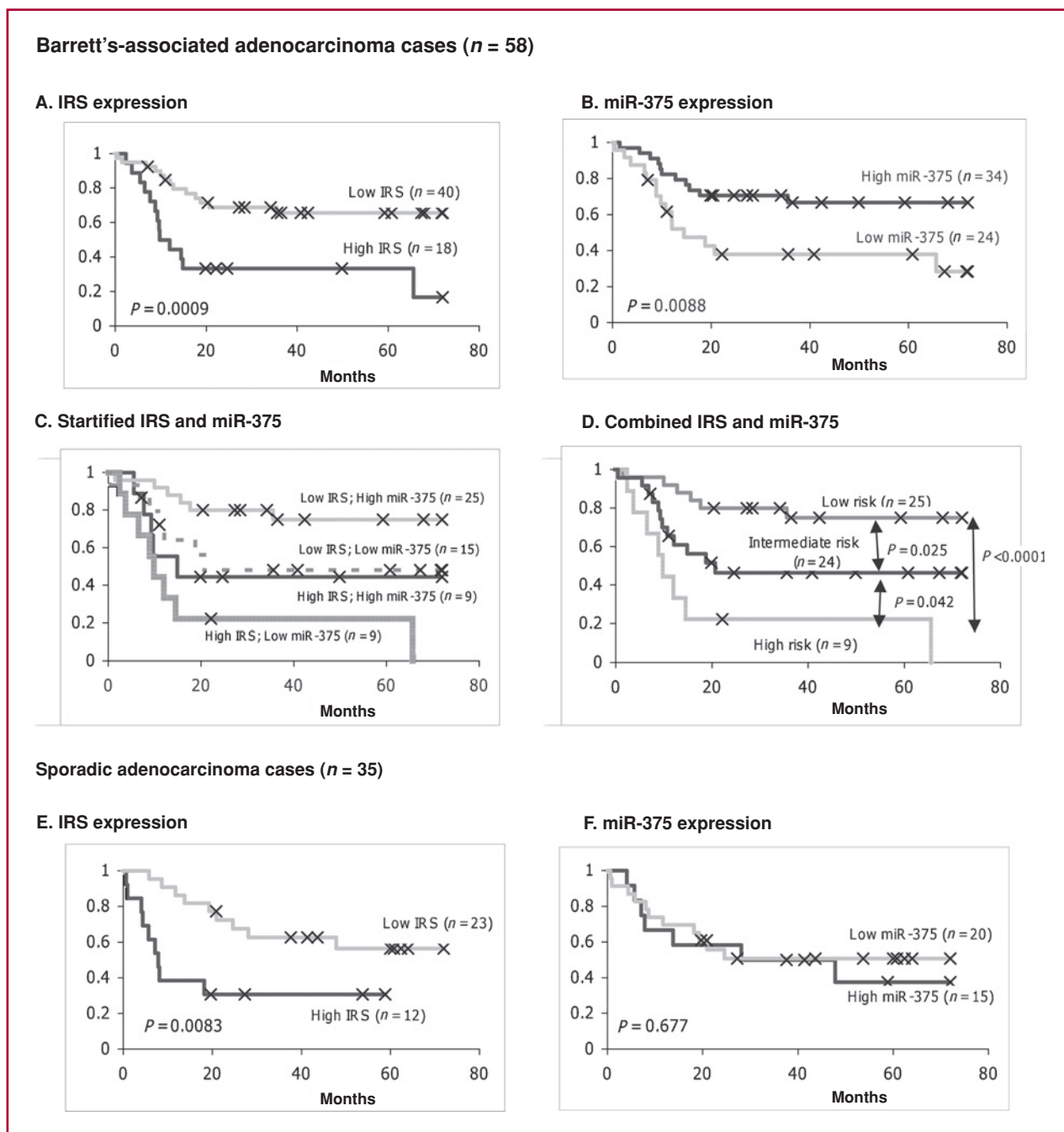


Fig. 3. Combined IRS and miR-375 expression predicts esophageal cancer-specific mortality better than either alone. Graphs are Kaplan–Meier analysis between expression and survival. For Barrett's-associated adenocarcinoma: A, Kaplan–Meier (KM) analysis for IRS. B, KM for miR-375. C, stratified IRS and miR-375 together. D, combined IRS and miR-375. For sporadic adenocarcinoma: E, KM for IRS. F, KM for miR-375. High IRS expression was classified on the basis of the highest tertile using tumor and nontumor ratio. High miR-375 expression was defined according to the higher than median expression in tumor samples.

To further determine the additive predictive power of the combined method after accounting for all clinical variables in BAC patients, Cox proportional hazards analysis was performed using tumor stage, age, and therapy status in the 58 BAC patients cohort, which demonstrated that high IRS [HR, 4.04; 95% confidence interval (95% CI), 1.80–9.09],

low miR-375 (HR, 2.78; 95% CI, 1.27–6.10), and TNM stage (HR, 4.27; 95% CI, 1.78–10.21) were each associated with poor prognosis (Table 2; Univariate). Multivariate regression analysis indicated that both IRS (HR, 3.74; 95% CI, 1.62–8.63) and miR-375 (HR, 2.73; 95% CI, 1.17–6.39) were independent of one another and TNM

Table 2. Cox regression analysis of IRS and miR-375 expression with cancer-specific mortality of Barrett's-associated ADC sample ($n = 58$)

Variables	Univariate		Multivariate ^a	
	HR (95% CI)	P	HR (95% CI)	P
IRS (high/low)	4.04 (1.80–9.09)	0.001	3.74 (1.62–8.63)	0.002
miR-375 (low/high)	2.78 (1.27–6.10)	0.011	2.73 (1.17–6.39)	0.021
Tumor stage (III-IV/I-II)	4.27 (1.78–10.21)	<0.001	6.84 (2.40–19.51)	<0.001
Age (<65/>65)	0.86 (0.47–1.57)	0.62		
Therapy (yes/no)	1.83 (0.80–4.16)	0.15		

NOTE: All analyses are adjusted for nationality (American vs. Canadian).

^aMultivariate analysis include IRS, miR-375, and TNM staging. Multivariate analysis used stepwise addition and removal of clinical covariates were found to be associated with survival in univariate models ($P < 0.10$) and final models include only those variables significantly associated with survival (Wald statistics, $P < 0.05$).

staging. We found that the multivariate model, including IRS, miR-375, and TNM staging was a significantly better prognostic classifier than a model of without IRS or a model without miR-375 ($P = 0.003$ and $P = 0.026$, likelihood ratio test; respectively). These results indicate that IRS and miR-375 have potential to be used in combination as a prognostic classifier of BAC, although this result requires validation in another cohort.

In this study, both IRS and miR-375 were significantly associated with cancer-specific mortality only in patients who did not receive any neoadjuvant therapy (NAT-naïve; Fig. 4). Therefore, we restricted our analysis to this group of NAT-naïve patients to investigate the association of IRS, miR-375, and survival independent of any therapeutic effects in BAC. In our combined cohort, 39 of 58 BAC patients did not receive any preoperative chemoradiation therapy. Similar to what was seen before, either high IRS or low miR-375 was associated with poor survival ($P = 0.0021$ and $P = 0.032$, Kaplan–Meier log rank; Figs. 4A, B; respectively). We then performed an analysis of IRS and miR-375 in the BAC NAT-naïve group. Risk groups were defined as they were for the BAC analysis. Patients defined as low risk had the best prognosis (Fig. 4C). Patients classified as high risk had the worst prognosis than low risk group ($P = 0.001$; Kaplan–Meier log rank; Fig. 4D).

Significant association in SAC NAT-naïve patients was observed between IRS and survival ($P = 0.0004$, Kaplan–Meier log rank; Fig. 4E), but not miR-375 and survival ($P = 0.881$, Kaplan–Meier log rank; Fig. 4F). In summary, we found significant association between IRS and miR-375 with survival in the BAC NAT-naïve patients. Therefore, combining IRS and miRNA expression might be used together as a prognostic classifier of Barrett's-associated esophageal adenocarcinoma patients.

Discussion

In this study, we analyzed 23 inflammation-associated genes in ADC patients with respect to survival. We identified gene expression signatures containing PRG, IL-1 α , IL-

8, IL-21, IL-23, IFN- γ , and miR-375 that were associated with survival in BAC patients. We confirmed these findings in a test cohort of ADC patients. Patients with a high IRS in their tumor and nontumor samples and a low miR-375 in its tumors had shortened survival independent of other clinical and pathologic parameters. These findings suggest that inflammatory genes and microRNA may play an important role in the molecular pathogenesis, clinical cancer progression, and prognosis of BAC patients.

Our findings here are consistent with other reports evaluating the connection between inflammation and cancer. Our previous studies support a role for inflammatory genes, as both tumor and nontumor tissues play important roles in tumor progression and metastasis. For example, a 17-cytokine gene signature (primarily composed of T_h1 and T_h2 cytokines) obtained from noncancerous hepatocellular carcinoma tissues predicted metastasis and recurrence (10). Similar gene signatures obtained from both tumor and nontumor tissues were used to predict prognosis in lung and colon ADC (11, 12). In the present study, 6 of these inflammatory genes are associated with survival outcome in EC. This suggests that the patterns of inflammatory gene expression among lung, liver, colon, and esophagus are similar and support the mechanistic relationships between inflammation and carcinogenesis.

Our findings are also consistent with published studies on inflammation and EC. For example, elevated IFN- γ expression was correlated with advanced SCC stage (20). Cytokines, such as IL-8 and IL-1 β , were markedly increased in biopsies from ADC patients (15, 18). Metaplastic Barrett's epithelial cells were shown to produce IL-8, IL-10, and IL-1 β (34). Other groups also found an association between T_h1 inflammation and the pathogenesis of Barrett's dysplasia (35, 36). For example, IL-1 β enhances Barrett's metaplasia by increasing CDX1 (a regulator of intestinal epithelial development; 37).

ANXA1 and IL-24 identified in this study showed the highest fold difference in tumors compared with nontumor tissues, suggesting their clinical utility as potential diagnostic markers. Although this finding does not

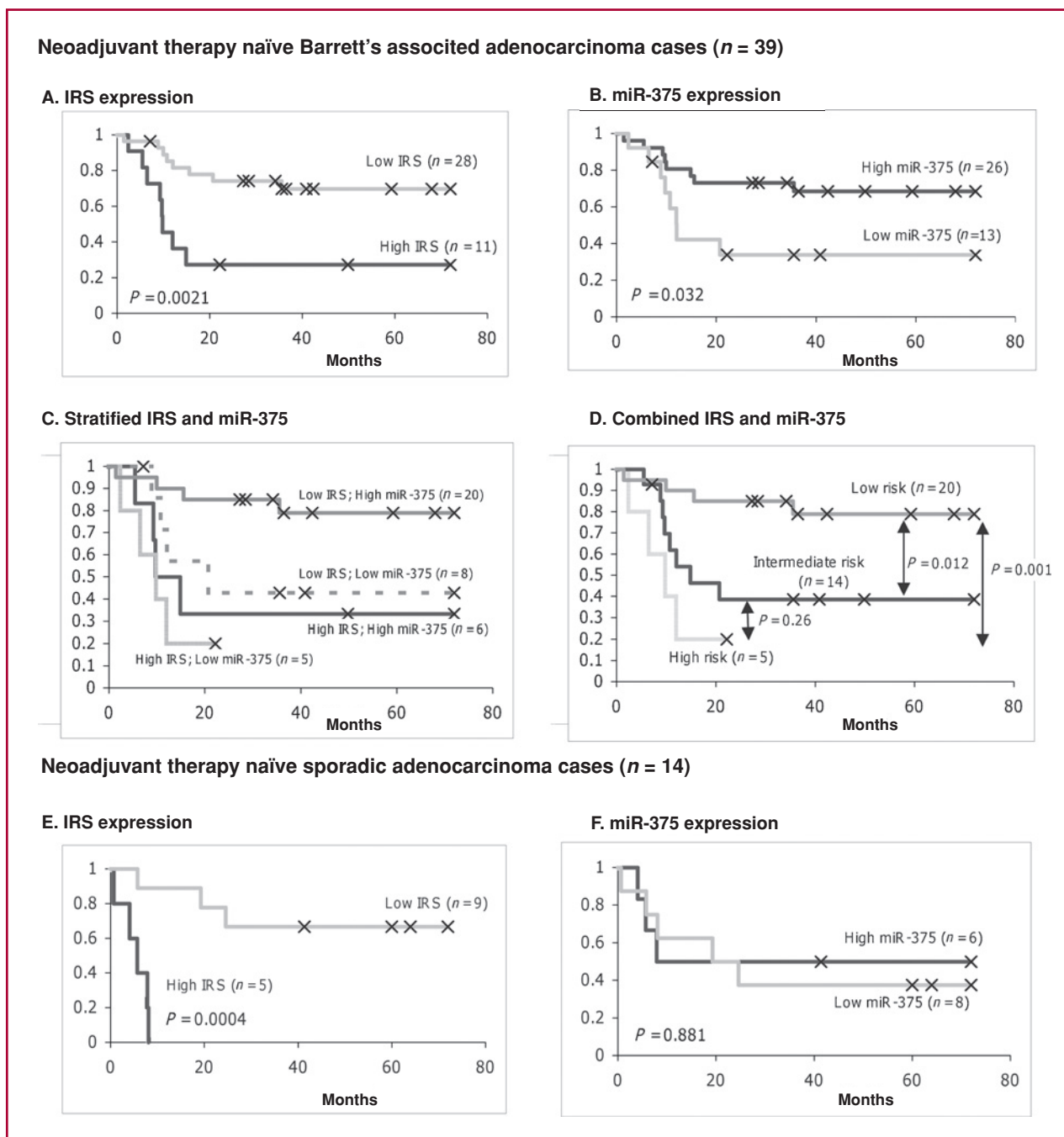


Fig. 4. Combined IRS and miR-375 expression predicts esophageal cancer-specific mortality in patients without any neoadjuvant treatment. Graphs are Kaplan–Meier analysis between expression and survival. For Barrett's-associated adenocarcinoma: A, Kaplan–Meier (KM) analysis for IRS. B, KM for miR-375. C, stratified IRS and miR-375 together. D, combined IRS and miR-375. For sporadic adenocarcinoma: E, KM for IRS. F, KM for miR-375. High IRS expression was classified on the basis of the highest tertile using tumor and nontumor ratio. High miR-375 expression was defined according to the higher than median expression in tumor samples.

address the causal relationship between inflammation and cancer, these changes are consistent with data in the current literature. For example, we showed in this study that ANXA1 had the largest reduction in esophageal tumors. Its expression has been shown to be reduced in a number of cancers including those from the esophagus

origin (38–40). ANXA1 is a mediator of apoptosis and inhibitor of cell proliferation in variety of cancer settings (41). Conversely, IL-24 had the largest fold increase of all genes tested in our ADC samples. A newly discovered member of the IL-10 family, IL-24 was originally described in the setting of melanoma, where it functions

as a tumor suppressor (42). Its antitumor activities have since been expanded to include many different cancer models (43, 44). To our knowledge, there are no published studies of IL-24 and EC. Although our finding might appear counterintuitive, the antitumor effect of IL-24 is almost always tested in the setting of forced overexpression by the cancer cells themselves and the physiologic roles of IL-24 is not well understood. Whether IL-24 has any influence to the biology of ADC is beyond the scope of this study and would, therefore, warrant further investigation to access the etiologic role of IL-24 in esophageal carcinogenesis.

The current clinical-pathologic staging method has limited success in predicting patient survival. For patients with identical clinical-pathologic characteristics or different histologic subtypes, great uncertainties remain regarding how some patients will be cured whereas other patients will have cancer recurrence, metastasis, or death after multimodalities treatment. Applying our IRS and miR-375 signature to the combined samples of the training and the test cohorts, we found a clear separation between the low- and the high-risk curves. We showed that the combined signature can distinguish high-risk versus low-risk Barrett's patients only. Given the fact that the difference in survival between sporadic and BAC has rarely been examined before and thus is of great interest. This finding may potentially enable doctors to identify and select high-risk patients for effective therapy in addition to standard surgery to improve the treatment outcome of BAC patients.

Neoadjuvant therapy is now common practice in the treatment of EC given that more than 50% of patients present with bulky tumors and radiographic metastasis at the time of diagnosis (45). Although EC patients may respond to NAT, overall survival remains generally poor with the exception of a subset of patients who achieve a complete pathologic response. However, there is currently no clinically useful indicator to select patients who should receive additional interventions. Unfortunately, because of the lack of pretreatment tissues in this study, we were unable to investigate therapeutic responses in NAT patients. In patients with resected tumors, particularly in the absence of any NAT, the major determinant of long-term cancer-specific survival is expected to be micro-metastatic disease. Therefore, our observation that IRS and miR-375 were good prognostic classifiers in the BAC NAT-naïve patients was an important finding because it might help identify individuals that are likely to have micrometastasis and would therefore, require more aggressive adjuvant therapy. Although this specific observation requires an

independent validation, the biomarkers defined here might aid in the identification of patients who did not receive any neoadjuvant therapy and therefore, should be treated more aggressively post surgical resection.

A strength of this study lies in the fact that a small number of genes and miRNA was measured using real-time qRT-PCR, a technique that is considered the most sensitive, specific, fast, and economical RNA quantification method. This gene/miRNA set might be clinically applicable procedure. This methodology could be easily translated to the clinic by quantifying RNA from surgical specimens (46). A constraint of this study, on the other hand, is that there are still possibilities of false positives in the selection of these genes for the correlation of clinical outcome in ADC patients. Therefore, the potentially clinical application of this study should be examined in a larger independent cohort, although the validation of this signature in our test cohort provides confidence in the validity of this gene signature as a predictor of clinical outcome.

In conclusion, our finding provides evidence that elevated expression of inflammatory genes in association with miRNA could predict clinical outcome in an independent cohort. Our results suggest that this criteria defined here may have prognostic and therapeutic implications for the management of EC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Giang Huong Nguyen, Aaron J. Schetter, David B. Chou, et al.

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