MicroRNA and inflammatory gene expression as prognostic marker for overall survival in esophageal squamous cell carcinoma

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MicroRNAs (miRNAs) and inflammatory genes have a role in the initiation and development of esophageal squamous cell carcinoma (ESCC). In our study, we examined the potential of using miRNA and inflammatory gene expression patterns as prognostic classifiers for ESCC. Five miRNAs and 25 inflammatory-related genes were measured by quantitative reverse transcriptase PCR in tumor tissues and adjacent noncancerous tissues from 178 Chinese patients with ESCC. The expression levels of miR-21 (p = 0.027), miR-181b (p = 0.002) and miR-146b (p = 0.021) in tumor tissue and miR-21 (p = 0.003) in noncancerous tissue were associated with overall survival of patients. These data were combined to generate a miRNA risk score that was significantly associated with worse prognosis (p = 0.0001), suggesting that these miRNAs may be useful prognostic classifiers for ESCC. To construct an inflammatory gene prognostic classifier, we divided the population into training (n = 124) and test cohorts (n = 54). The expression levels of CRY61, CTGF and IL-18 in tumor tissue and VEGF in adjacent noncancerous tissue were modestly associated with prognosis in the training cohort (Z-score > 1.5 and were subsequently used to construct a Cox regression-based inflammatory risk score (IRS). IRS was significantly associated with survival in both the training cohort (p = 0.002) and the test cohort (p = 0.005). Furthermore, Cox regression models combining both miRNA risk score and IRS performed significantly better than models with either alone (p < 0.001 likelihood ratio test). Therefore, miRNA and inflammatory gene expression patterns, alone or in combination, have potential as prognostic classifiers for ESCC and may help to guide therapeutic decisions.

Key words: esophageal squamous cell carcinoma, microRNA, prognostic classifier, inflammations

Additional Supporting Information may be found in the online version of this article.

*Published 2012. This article is a US Government work and, as such, is in the public domain of the United States of America.

Grant sponsor: National Cancer Institute, Center for Cancer Research at the National Institutes of Health; Grant sponsor: National Science Foundation of China; Grant numbers: 30872937, 30930102; Grant sponsor: National Ministry of Science and Technology (973 Project); Grant number: 2011CB504301; Grant sponsor: National Science Foundation of Beijing; Grant number: 7100001

DOI: 10.1002/ijc.27954

History: Received 7 Jun 2012; Accepted 5 Oct 2012; Online 23 Nov 2012

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Esophageal cancer is the fifth leading cause of cancer-related deaths in men and the eighth leading cause in women worldwide.1 It is composed of two major histological subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EADC). ESCC usually arises from the squamous cells in the middle or upper one-third of the esophagus. EADC arises from glandular cells in the lower one-third of the esophagus or at the junction between the esophagus and stomach.2 Incidence of esophageal cancer varies geographically, with the highest incidence in Southern and Eastern Africa and Eastern Asia and the lowest incidence in Western and Middle Africa and Central America.3 A relatively large region of Asia has been referred to as the “esophageal cancer belt,” and this stretches from northern Iran through the central Asian republics to North-Central China. Approximately 90% of cases from this region present as ESCC.3,4 The high rate of esophageal cancer in these populations demonstrate an urgent need to identify causes, molecular classifiers and therapeutic targets for ESCC that can be used to reduce the burden of ESCC in these populations.
The prognosis of ESCC is quite poor. Treatment options for ESCC can include surgery, radiation therapy and various chemotherapeutic regimes. Identifying genes whose expression is associated with poor prognosis of patients with ESCC may lead to clinically useful tools for the management of ESCC in at least two ways. First, these genes may be used to develop molecular classifiers that can identify subgroups of patients that would benefit from more aggressive therapeutic interventions. These classifiers may also be able to stratify patients into appropriate arms of clinical trials to aid in the development and testing of new therapeutics. Second, if genes associated with poor prognosis have a mechanistic role in the initiation or progression of ESCC, they may also serve as novel therapeutic targets.

MicroRNAs (miRNAs) are short, 20- to 24-nucleotide, noncoding RNAs that regulate the translation of specific genes. miRNAs have a causal role in many cancer types and can have either oncogenic or tumor suppressor functions. For example, abnormal expression of oncogenic miRNAs may decrease the translation of target tumor suppressor genes and contribute to the development or progression of cancer. There is interest in using miRNAs as both biomarkers and therapeutic targets for various cancer types. Expression levels of specific miRNAs have already shown potential as diagnostic and prognostic biomarkers for cancer. Our previous studies have found that altered expression of specific miRNAs is associated with prognosis of ESCC and adenocarcinoma of colon, lung and esophagus.

Chronic inflammation also has a role in cancer initiation and progression. In the tumor microenvironment, cytokines and chemokines are produced by infiltrating immune cells, tumor supporting cells and tumor cells, and they regulate several important inflammatory pathways. These pathways may be either protumorigenic or antitumorigenic. In the esophageal mucosa, chronic irritation and inflammation, such as that from cigarette smoking and alcohol abuse, can lead to the initiation and development of ESCC. Other risk factors related to chronic irritation and ESCC are esophageal achalasia and HPV infection. We have recently demonstrated that the mRNA expression patterns of inflammation-associated genes can be used as prognostic classifiers for liver cancer, lung cancer, colon cancer and esophageal adenocarcinoma. We have also demonstrated that miRNA and inflammatory gene classifiers were independently associated with prognosis for colon adenocarcinoma and EAC, suggesting that the combination of these classifiers could perform superior to either alone. Therefore, we hypothesized that a similar strategy could identify prognostic classifiers of ESCC.

In our study, we collected tumor and adjacent noncancerous tissues from 178 patients with ESCC from An Yang, Henan Province, China. The incidence of esophageal cancer in this region is 10-fold greater than the nationwide rate for China and 100-fold greater than the rate among Caucasian Americans. We focused exclusively on the squamous cell histology as it represents the majority of esophageal cancer in this region of the world. We measured the expression levels of five miRNAs and 25 inflammatory genes in these tissues to determine their potential as prognostic classifiers for ESCC. We hypothesized that the combination of these miRNAs and inflammatory genes could be prognostic classifiers of survival for patients with ESCC.

Patients and Methods
Clinical samples
Esophageal tumors and adjacent noncancerous specimens were collected from 185 patients who underwent surgical resection. Eligibility criteria for enrollment were as follows: (i) patients must be residents of An Yang, Henan Province, China, which is a high-risk area for ESCC; (ii) patients must be aged between 18 and 90 years and admitted to An Yang Cancer Hospital between April 30 and August 16, 2008; (iii) all tumors had to have a microscopically confirmed diagnosis of ESCC by two licensed pathologists and (iv) all patients must have signed informed consent forms. Our study was approved by the Medical Ethics Committee of Peking University (China) and the Institutional Review Boards of the National Institutes of Health (USA).

For each patient, primary tumor and its adjacent noncancerous specimens were collected during surgery, separated and cut by experienced pathologist. Tissues were immediately frozen and stored at $-80^\circ$C until extraction of total RNA. The adjacent noncancerous tissues were obtained from tumor-free margins and were at least 5 cm away from the tumor site. For each patient, detailed follow-up and clinicopathological characteristics (including gender, age, histopathology, TNM stage, adjuvant therapy, alcohol and cigarette consumptions and survival information) were collected.

Total RNA isolation
Total RNA of the paired cancerous and adjacent noncancerous tissues were extracted using the standard TRIzol protocol.
The inflammatory gene expression was calculated by normalizing the expression of each individual inflammatory gene using the threshold cycle method, where $Ct$ = threshold cycle and $\Delta Ct = (Ct$ endogenous control $- Ct$ gene).

qRT-PCR of miRNAs
Reverse transcription of miR-21, miR-181b, miR-146b, miR-223, miR-155 and RNU66 was performed according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA) with a reaction volume of 20 μl containing 1 μg of total RNA. The real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Each miRNA and endogenous control (RUN66) was measured in triplicate, and the average of the triplicate data was used. An overall quality control, CT values above 35 or measurements with standard deviations for the triplicates above 1 were excluded from further analysis.

Statistical analysis
A paired $t$-test was used to compare the differently expressed miRNAs and inflammatory genes in tumor and adjacent noncancerous tissues of all 178 patients with ESCC. To assess the association of miRNA and overall survival of patients with ESCC, miRNA expression data were binned into high and low based on median cutoff. Kaplan–Meier survival analysis was used to determine the association of miRNAs and overall survival ($p < 0.05$ assessed for significance by the log-rank test).

To develop a prognostic inflammatory gene classifier, all patients were randomly split into training ($n = 124$) and test cohorts ($n = 54$). More patients were included in the training cohort to make the risk model more robust. These two cohorts have similar clinicopathological characteristics (Table 1). Univariate Cox regression was used to select genes associated with overall survival in the training cohort using our previously reported criteria.

### Table 1. Characteristics of the patients

| Cohort (n) | All (n = 178) | Training (n = 124) | Test (n = 54) | $p$-value $^1$ |
|------------|--------------|-------------------|--------------|--------------|
| Gender, n (%) |              |                   |              |              |
| Male       | 108 (61)     | 77 (62)           | 31 (57)      | 0.62         |
| Female     | 70 (39)      | 47 (38)           | 23 (43)      |              |
| Age at enrollment (years) | |                   |              |              |
| Mean (SD)  | 62.2 (8.0)   | 62.7 (7.8)        | 61.2 (8.5)   | 0.25         |
| Range      | 34–84        | 40–84             | 34–78        |              |
| Alcohol consumption, $^2$ n (%) | |                   |              |              |
| Yes        | 23 (13)      | 16 (13)           | 7 (13)       | 1            |
| No         | 155 (87)     | 108 (87)          | 47 (87)      |              |
| Cigarette consumption, $^3$ n (%) | |                   |              |              |
| Yes        | 74 (42)      | 59 (48)           | 15 (28)      | 0.02         |
| No         | 104 (58)     | 65 (52)           | 39 (72)      |              |
| Survival, $^4$ n (%) | |                   |              |              |
| Yes        | 110 (62)     | 77 (62)           | 33 (61)      | 1            |
| No         | 68 (38)      | 47 (38)           | 21 (39)      |              |
| TNM staging, $^5$ n (%) | |                   |              |              |
| I          | 6 (3)        | 4 (3)             | 2 (4)        | 0.85         |
| IIa        | 94 (53)      | 68 (55)           | 26 (48)      |              |
| IIb        | 25 (14)      | 16 (13)           | 9 (17)       |              |
| III        | 53 (30)      | 36 (29)           | 17 (31)      |              |

$^1$p-values are from Fisher’s exact test or $t$-test where appropriate, which compares training and test cohorts. $^2$Alcohol consumption: drinking liquor at least twice per week for ≥12 months. $^3$Cigarette consumption: at least one cigarette per day for ≥12 months or ≥180 packs for 1 year. $^4$Data of 30 months follow-up. $^5$TNM staging at the time of surgery was based on the 2009 World Health Organization Classification.
Results
Expression of miRNAs altered in tumors and associated with overall survival in patients with ESCC

We have previously reported that the expression levels of miR-21, miR-181b, miR-146b, miR-155 and miR-223 were elevated in tumor tissue of ESCC and associated with survival of patients. Therefore, the expression levels of these miRNAs were measured in tumor and adjacent noncancerous tissues from the 178 Chinese patients with ESCC. We found that miR-21 (fold change = 3.57; \( p < 0.0001 \)), miR-181b (fold change = 1.64; \( p < 0.0001 \)), miR-146b (fold change = 1.52; \( p < 0.0001 \)), miR-155 (fold change = 1.52; \( p < 0.0001 \)) and miR-223 (fold change = 1.41; \( p = 0.01 \)) were all expressed at significantly higher levels in tumor tissues (Supporting Information Table S2). These results are consistent with a putative oncogenic role for these miRNAs.

To assess if the expression levels of any of these five miRNAs were associated with prognosis, we dichotomized the expression values of each miRNA into high and low groups based on median cutoff of their expression data. High expression of miR-21 (\( p = 0.03 \); Kaplan–Meier log rank), miR-181b (\( p = 0.002 \); Kaplan–Meier log rank) and miR-146b (\( p = 0.02 \); Kaplan–Meier log rank) was significantly associated with worse overall survival. In addition, high expression of miR-21 (\( p = 0.003 \); Kaplan–Meier log rank) was associated with worse overall survival in noncancerous tissues of patients.

Figure 1. miRNAs associated with overall survival of patients with ESCC in tumor and adjacent noncancerous tissues. To assess the prognostic role of miR-21, miR-181b, miR-146b, miR-155 and miR-223 in Chinese patients with ESCC, we dichotomized the expression values of each miRNA into high and low groups based on median cutoff of their expression data. In tumor tissue, high expression of miR-21 (\( p = 0.03 \); Kaplan–Meier log rank), miR-181b (\( p = 0.002 \); Kaplan–Meier log rank) and miR-146b (\( p = 0.02 \); Kaplan–Meier log rank) was associated with worse overall survival. In addition, high expression of miR-21 (\( p = 0.003 \); Kaplan–Meier log rank) was associated with worse overall survival in noncancerous tissues of patients.
IRS is associated with survival in patients with ESCC

We evaluated the expression of the inflammation-related genes for associations with patient survival. The construction of a multigene classifier using several genes with moderate associations can provide stronger associations with prognosis compared to a model using a single gene. Therefore, we generated a prognostic classifier based on the expression of multiple inflammation-related genes. Our strategy is outlined in Supporting Information Figure S1. The 178 patients with ESCC were split into a training cohort \((n = 124; 70\% \text{ of all patients})\) and a test cohort \((n = 54; 30\% \text{ of all patients})\) as discussed above, and these cohorts were similar in clinicopathological characteristics (Table 1). The expression levels of the inflammatory genes were similar compared to each cohort (Supporting Information Table S4). In the training cohort, we performed univariate Cox regression and ranked the 25 inflammatory genes according to their \(Z\)-scores. We selected genes with \([Z\text{-score}] > 1.5\) as a cutoff to include in a multivariate Cox regression model that was used to generate a prognostic classifier using similar criteria as a previous study.21 The formulas to calculate the IRS for each patients of training cohort are as follows: in cancerous tissue, risk \(=(0.071 \times CYR61) + (0.281 \times CTGF) + (-0.170 \times IL-18)\), and in adjacent noncancerous tissue, risk \(=(0.368 \times VEGF)\). Patients whose risk was higher than the median value for both tumor and noncancerous tissues were classified as high IRS group, and all the others were defined as low IRS group. Kaplan–Meier log-rank analysis showed that patients with high IRS had worse survival than patients with low IRS in the training cohort \((p = 0.002; \text{ Fig. 3})\). We then evaluated IRS in the test cohort. Similar results were found in the test cohort in that patients classified as high IRS group had worse survival outcomes than that of low IRS group \((p = 0.005, \text{ test cohort; Fig. 3})\), suggesting that IRS may be a useful prognostic classifier.

Combination of miRNA score and IRS improves survival models

There was a modest, increased association between being classified as high miRNA score and high IRS score \((\text{odds ratio} = 2.05; p = 0.048)\). We next determined if the combination of these two classifiers improved associations with prognosis. Multivariate analysis showed that IRS and miRNA risk score in all 178 patients with ESCC were independently associated with patient survival (Table 2). Likelihood ratio tests demonstrated that the Cox regression models using both IRS and miRNAs performed significantly better than models with only IRS or miRNA risk score alone \((p < 0.001)\), suggesting that the combination of these classifiers is superior to either alone. Kaplan–Meier analysis gave similar results. Patients were classified based on both IRS and miRNA risk score. Patients who scored high for both were classified as high risk. Patients who scored high for either IRS or miRNA risk score were classified as intermediate. Those who were low for both IRS and miRNA risk score were classified as low. The intermediate group has significantly worse survival
than the low-risk group ($p = 0.04$), whereas the high-risk group was significantly worse than the intermediate group ($p < 0.0001$) (Figure 4). These data suggest that miRNA risk score and IRS could be used in combination as a prognostic classifier for ESCC.

**Discussion**

In our study, we analyzed the expression levels of five miRNAs and 25 inflammatory-related genes for their association with survival in 178 Chinese patients with ESCC. The expression levels of miR-21, miR-181b, miR-146b, miR-155 and miR-223 were each elevated in cancerous tissues similar to our previous study on ESCC.\(^8\) These results are consistent with the suggested oncogenic roles for these miRNAs. For example, miR-21 is an oncogenic miRNA that is increased in most solid tumors, including ESCC.\(^8\) Increased expression of miR-21 in the absence of other genetic defects is sufficient at causing malignancies in mice,\(^27\) whereas deletion of miR-21 reduces KRAS-driven oncogenic transformation.\(^28\)
can target many important tumor suppressor genes in a variety of cancer cell lines, including PTEN, TP53, PDCD4, and Sprouty2. The targeting of PTEN by miR-21 is especially relevant to ESCC in that miR-21 expression can repress PTEN protein levels in an ESCC cell line and that PTEN protein levels were inversely correlated in ESCC tumors. miR-181b has been reported to be elevated in a variety of cancers and can target important oncogenes such as BCL2 and TIMP3. miR-155 is a highly studied miRNA that is implicated in both inflammation and cancer, and high levels of miR-155 can lead to lymphomas in mice. miR-146b was reported to be elevated in thyroid cancer and nonsmall cell lung cancer and can repress SMAD4. With the known mechanistic role of miRNAs in cancer, our results support that miR-21, miR-181b, miR-146b, miR-155 and miR-223 have a role in ESCC and therefore may be useful therapeutic targets for the treatment of ESCC.

Our previous study of populations from the United States and Japan suggested that the expression of miR-21, miR-181b, miR-146b, miR-155 and miR-223 had potential as prognostic classifiers for ESCC. In our study, the increased expression of miR-21, miR-181b and miR-146b in tumors and the increased expression of miR-21 in noncancerous tissues were associated with prognosis in Chinese patients with ESCC. Increased expression of each of these miRNAs has also been reported to be associated with poor prognosis in other cancer types, which further demonstrates their potential as prognostic classifiers. Elevated miR-146b in lung squamous cell carcinomas was associated with worse survival prognosis, whereas increased miR-181b expression is associated with worse prognosis in gastric and colon adenocarcinomas. Elevated expression of miR-21 has been found to be associated with worse prognosis in at least 10 other cancer types, including colon cancer, lung cancer, breast cancer, pancreatic cancer, tongue cancer, gastric cancer, head and neck cancer, chronic lymphocytic leukemia, melanoma and astrocytomas. Interestingly, we validated the finding that the expression of miR-21 in nontumor tissue is associated with prognosis of ESCC. This suggests that miR-21 may have role in the tumor stroma. All of these results highlight the potential of using miRNA expression patterns as prognostic classifiers. The expression of miR-21 is also associated with therapeutic outcome in colon and gastric cancers, whereas miR-181b is associated with therapeutic outcome in gastric cancer. This highlights the potential of these miRNAs to guide therapeutic decisions. Importantly, we demonstrate here that the combination of miRNA expression pattern is a stronger prognostic classifier than each individual miRNA.

Inflammation has an etiological role in the development and progression of esophageal cancer. We developed a prognostic classifier of inflammatory-related genes (IRS) based on the expression of CTGF, CYR61, IL-18 and VEGF. These genes are also functionally linked to cancer. CTGF and CYR61 are two members of a group of matricellular proteins of extracellular matrix referred to as the CCN (CyR61/CTGF/Nov) family. The CCN family has important roles in inflammation, wound healing and in cancer progression via several important signaling pathways, including insulin-like factor, transforming growth factor-β, and Wnt signaling. Elevated expression of CTGF has been reported in high tumor grade and metastatic ESCC tumor samples, whereas high expression of CYR61 can enhance ESCC tumor cell progression. IL-18 has duel effect on cancer. As known as an immune activator, IL-18 contributes to antitumor effect through activation of NK and T cells. VEGF can contribute to the tumor proliferation, angiogenesis and metastasis by promoting stromal degeneration, inducing endothelial cell proliferation and migration and enhancing vascular permeability. In ESCC, the expression of VEGF is associated with lymph node metastases, depth of tumor invasion, distance metastases, pathological grade of malignancy and prognosis.

We developed two independent prognostic classifiers for ESCC based on the expression of inflammatory-related genes or miRNAs and demonstrated that these classifiers may be used in combination to better predict a patient's survival risk. These associations were found in a relatively large cohort from rural China, none of which have received

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Table 2. Univariate and multivariate Cox regression analyses on miR score and inflammatory risk score with overall survival in 178 patients with ESCC

| Variable (comparison/referent) | Univariate analysis | Multivariate analysis1 |
|--------------------------------|--------------------|------------------------|
|                                | HR (95% CI)        | p-value HR (95% CI)    |
| IRS1 (high/low)                | 2.88 (1.78–4.67)   | <0.0005 2.48 (1.48–4.17) |
| miR score2 (high/low)          | 2.89 (1.65–5.07)   | <0.0005 2.72 (1.55–4.78) |
| TNM stage (III and IV/II and IIa) | 1.34 (0.83–2.16)   | 0.230 1.02 (0.62–1.70)  |
| Age4 (≥62 years/ <62 years)    | 0.89 (0.56–1.44)   | 0.646 |
| Gender (male/female)           | 1.51 (0.91–2.50)   | 0.115 |
| Current alcohol consumption (yes/no) | 1.77 (0.95–3.32)   | 0.072 |
| Current smoker (yes/no)        | 1.12 (0.69–1.81)   | 0.640 |

1Multivariate analysis including IRS, miR score, and TNM stage. 2Inflammatory risk score classifier. 3miR score is defined as high if two or more of the miRs were higher than median expression. 4The median age was 62 years for the cohort, and therefore, we dichotomized age as higher or lower than 62 years.

Int. J. Cancer: 132, 2901–2909 (2013) 2012 UICC
preoperative or postoperative chemotherapy. Therefore, it is likely that differences in therapy could confound these results. A limitation of our study is that a single portion of the tumor is sampled for each patient, one could improve the performance of the classifiers. Another limitation of our study is that follow-up time for these patients was only 30 months due to the recent accrual times of the tissues. It will be important for future studies to investigate additional populations for longer periods of time to determine if these associations are broadly applicable to all patients with ESCC. If so, the next step will be to determine the best ways these classifiers can be used to select proper postoperative therapies for ESCC or to assign individuals to appropriate arms of prospective clinical trials. Future work should also include sensitivity and specificity analyses of each classifier as well as determine the optimal way to combine these biomarkers to further improve sensitivity and specificity.

There is a need to develop prognostic biomarkers and identify therapeutic targets for ESCC. Our study suggests that expression patterns of miR-21, miR-181b and miR-146b, alone or in combination with IRS, can be used as prognostic classifiers for patients with ESCC. If these miRNAs or inflammatory genes are mechanistically involved in ESCC cancer progression, development of therapies based on these genes may be appropriate. Our study supports future work investigating these areas.

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