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Original Article

Expression of STING and MIF in tumor infiltration lymphocytes as prognostic factors in patients with ESCC

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Abstract: STING and MIF are Tumor-immune related proteins act as immune regulating roles that effect the progression of cancer. In these studies, we aimed to detect the expression levels of STING and MIF in tumor cells and in lymphocytes in tumor microenvironments and their association with survivals of patients diagnosed with esophageal squamous cell carcinoma (ESCC). The expression levels of STING and MIF were accessed by immunochemistry staining in tumor tissues from 112 resected ESCC. Correlation analyses and independent prognostic outcomes were determined using Pearson's chi-square test. Independent prognostic outcomes were measured by Cox regression analysis. We found that STING high expression in TILs or MIF high expression in tumor cells or in tumor infiltrating lymphocytes (TILs) was significantly related to reduced disease-free survival (DFS) and overall survival (OS) of ESCC patients ($P<0.05$). Multivariate analysis indicated that the expression of STING and MIF in TILs were adverse independent prognostic factors in the whole cohort of patients ($P<0.05$). The expression of MIF in tumor cells or in TILs was significantly positively correlated with STING in TILs ($P<0.05$). The combined STING and MIF expression in TILs was strongly related to reduced DFS and OS of ESCC patients ($P<0.05$). Our studies indicated the expression levels of STING and MIF in TILs were independent predictive factors of survivals in patients with ESCC.

Keywords: STING, MIF, esophageal squamous cell carcinoma, tumor microenvironment

Introduction

Esophageal squamous cell carcinoma (ESCC) is a prevalent malignancy leading cause of cancer-related death worldwide [1]. ESCC is one of the malignant tumors with a five-year survival rate less than 30% [2]. Expect for the traditional prognostic factors such as TNM stage and cell differentiation, molecular markers such as IL-17, Foxp3 in tumor microenvironments have been studied for the prognoses of ESCC patients in recent studies [3-8]. However, novel prognostic markers in ESCC remain to be explored.

Stimulator of interferon genes (STING) a protein that is expressed in various cell types such as epithelial cells, T cells, macrophages and dendritic cells [9, 10]. Early studies found STING as a DNA sensor that is critical for activating type I interferon in response to invading DNA viruses or bacteria [11-14]. Recently, the role of STING in cancer generation and progression was investigated. In these studies, STING acts as a double-edged sword in favor or against tumor progression [15-18]. So far, the role of STING in ESCC has not been studied. Macrophage migration inhibitory factor (MIF) is a cytokine commonly expressed in diverse cell types including lymphocytes and endothelial cells [19]. Overexpression of MIF can be detected in many pathological conditions, such as autoimmunity and cancer [20, 21]. In many kinds of tumors, MIF was found to be associated with tumorigenesis, tumor metastasis and tumor anginogenesis [22-24]. MIF is considered as a link between inflammatory activation and cancer progression [25].

In our study, we investigated the expression of STING and MIF protein in tumor cells and TILs in 112 ESCC tissues by immunohistochemical staining. The correlations between the expression levels of STING and MIF in different cell types include tumor cells and TILs in tumor microenvironment and their prognostic values were assessed.

Materials and methods

Patients and tumor tissue samples

A total of 112 ESCC patients who underwent surgery at Sun Yat-Sen University Cancer Center in China from November of 2000 to December of 2002 were involved in this study. No patient had received any antitumor treatment prior to surgery. All patients had histologically confirmed primary ESCC. The follow-up data from the 112 patients with ESCC in this study were available and complete. OS was defined as the time interval from the date of surgery to the date of cancer-related death or the end of follow-up (December 2011). DFS was defined as the time interval from the date of surgery to the date of tumor progression. The study was approved by the Research Ethics Committee of the Sun Yat-Sen University Cancer Center.

Reagents and antibodies

Primary antibodies: mouse anti-human MIF (ab55445; Abcam, USA), rabbit anti-human STING (ab198951; Abcam, USA), and horseradish peroxidase-labeled antibody against a mouse/rabbit IgG (Envision; Dako, Glostrup, Denmark).

Immunohistochemistry and evaluation of immunohistochemical staining

The formalin-fixed paraffin-embedded tumor tissues were cut continuously into 4-μm sections. The antigens were retrieved by heating the tissue slices in a pressure cooker for 8 min in EDTA (1 mmol/L, pH 8.0) solution. The sections were then immersed in a 0.3% hydrogen peroxide solution for 30 min. Slices were incubated with anti-MIF, anti-STING antibodies at 4°C overnight. A negative control was incubated with a normal murine IgG antibody. The sections were then incubated with a secondary antibody at room temperature for 30 min. Then the tissue sections were stained with DAB. Two independent observers blinded to the clinicopathological information scored the STING and MIF expression levels in tumor cells by assessing (a) the percentage of positive cells: (0, <5%; 1, 6 to 25%; 2, 26 to 50%; 3, 51 to 75%; 4, >75%) and (b) the staining intensity: (0, negative; 1, light yellow; 2, yellow; 3, brown). The score was the product of a × b. The levels of STING and MIF expression in lymphocytes were measured by counting the positively and negatively stained lymphocytes by a 400 × high-power microscopic for 5 fields and then calculating the mean positive percentage among the total lymphocytes per field.

Statistical analysis

Statistical analyses were performed with SPSS 16.0 software (SPSS Inc, Chicago, IL, USA). We divided the patients into two groups (a high-level group and a low-level group) based on the median values of different immunohistochemical variables. Pearson's chi-square test was applied to analyze the correlation between the patients' clinicopathological characteristics and immunohistochemical variants in different cell types. Clinical prognosis including disease-free survival and overall survival was analyzed by Kaplan-Meier analysis using the log-rank test according to the expression levels of STING and MIF examined in tumor cells and in TILs. Independent prognostic factors were identified by univariate and multivariate analyses using the Cox regression model. The correlations among the expression levels of STING and MIF in both tumor cells and TILs were tested by Pearson's correlation coefficient and linear regression analyses. In this study, a two-tailed *P*-value <0.05 was considered statistically significant.

Results

Expression patterns of STING and MIF in ESCC and their correlations with clinicopathological parameters

In this study, the median age of the 112 patients was 62 years, range from 35 to 90 years; 94 (83.9%) of the patients were males and 18 (16.1%) were females. There were 58 (51.8%) cases of Stage I and II tumors and 54 (48.2%) cases of Stage III and IV tumors based on the International Union against Cancer 2002 TNM staging system [27]. Of the 112 patients,

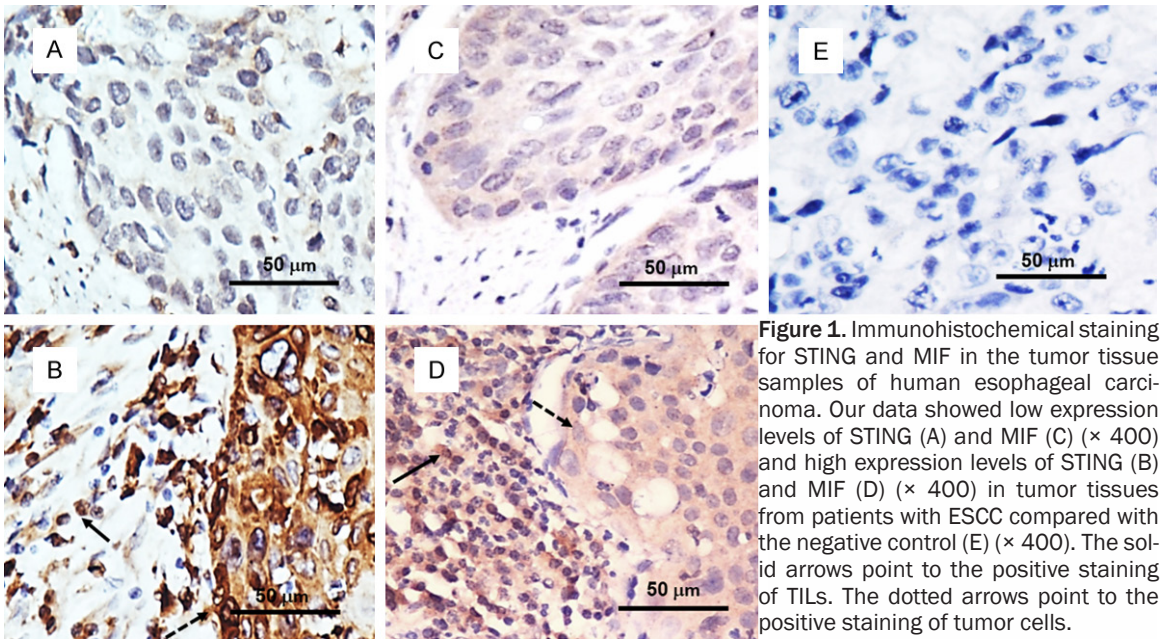

Figure 1. Immunohistochemical staining for STING and MIF in the tumor tissue samples of human esophageal carcinoma. Our data showed low expression levels of STING (A) and MIF (C) (× 400) and high expression levels of STING (B) and MIF (D) (× 400) in tumor tissues from patients with ESCC compared with the negative control (E) (× 400). The solid arrows point to the positive staining of TILs. The dotted arrows point to the positive staining of tumor cells.

Table 1. Association of the expression of STING, MIF and clinicopathologic parameters in 112 patients with ESCC

Clinicopathologic parameter	Total case	Expression in tumor cells				Expression in lymphocytes			
		High level expression of STING (%)	P	High level expression of MIF (%)	P	High level expression of STING (%)	P	High level expression of MIF (%)	P
Age									
≤62 (y)	56	31 (55.4%)	0.345	26 (46.4%)	0.450	31 (55.4%)	0.257	26 (46.4%)	0.450
>62 (y)	56	26 (46.4%)		30 (53.6%)		25 (44.6%)		30 (53.6%)	
Gender									
Female	18	9 (50.0%)	0.934	6 (33.3%)	0.123	7 (38.9%)	0.303	8 (51.1%)	0.607
Male	94	48 (50.9%)		50 (53.2%)		49 (52.1%)		48 (44.4%)	
T status									
T1-2	32	14 (43.8%)	0.339	14 (43.8%)	0.403	15 (46.8%)	0.676	11 (34.4%)	0.036*
T3-4	80	43 (53.8%)		42 (52.5%)		41 (51.3%)		45 (56.3%)	
N status									
N0	52	28 (53.8%)	0.561	23 (44.2%)	0.256	25 (48.1%)	0.705	22 (42.3%)	0.130
N1	60	29 (48.3%)		33 (55.0%)		31 (51.7%)		34 (56.7%)	
Clinical stage									
I-II	58	31 (53.4%)	0.575	28 (48.3%)	0.705	30 (51.7%)	0.705	27 (46.6%)	0.449
III-IV	54	26 (48.1%)		28 (51.9%)		26 (48.1%)		29 (53.7%)	

Note: *P<0.05, Pearson's X^2 test.

83 (74.1%) had died, 86 (76.8%) had suffered the relapse or progression of the disease. The patients' clinical characteristics are listed in Additional file 1: Table S1.

The expression levels of STING and MIF were detected in tumor tissues from 112 patients with ESCC. STING and MIF was mainly expressed in the cytoplasm of tumor cells and TILs (**Figure 1**). The mean percentage and the range of the percentage for MIF or STING expression in TILs per field were 33% (range, 0 to 92%) and 31% (range, 0 to 85%), respectively (Additional file 1: Table S2).

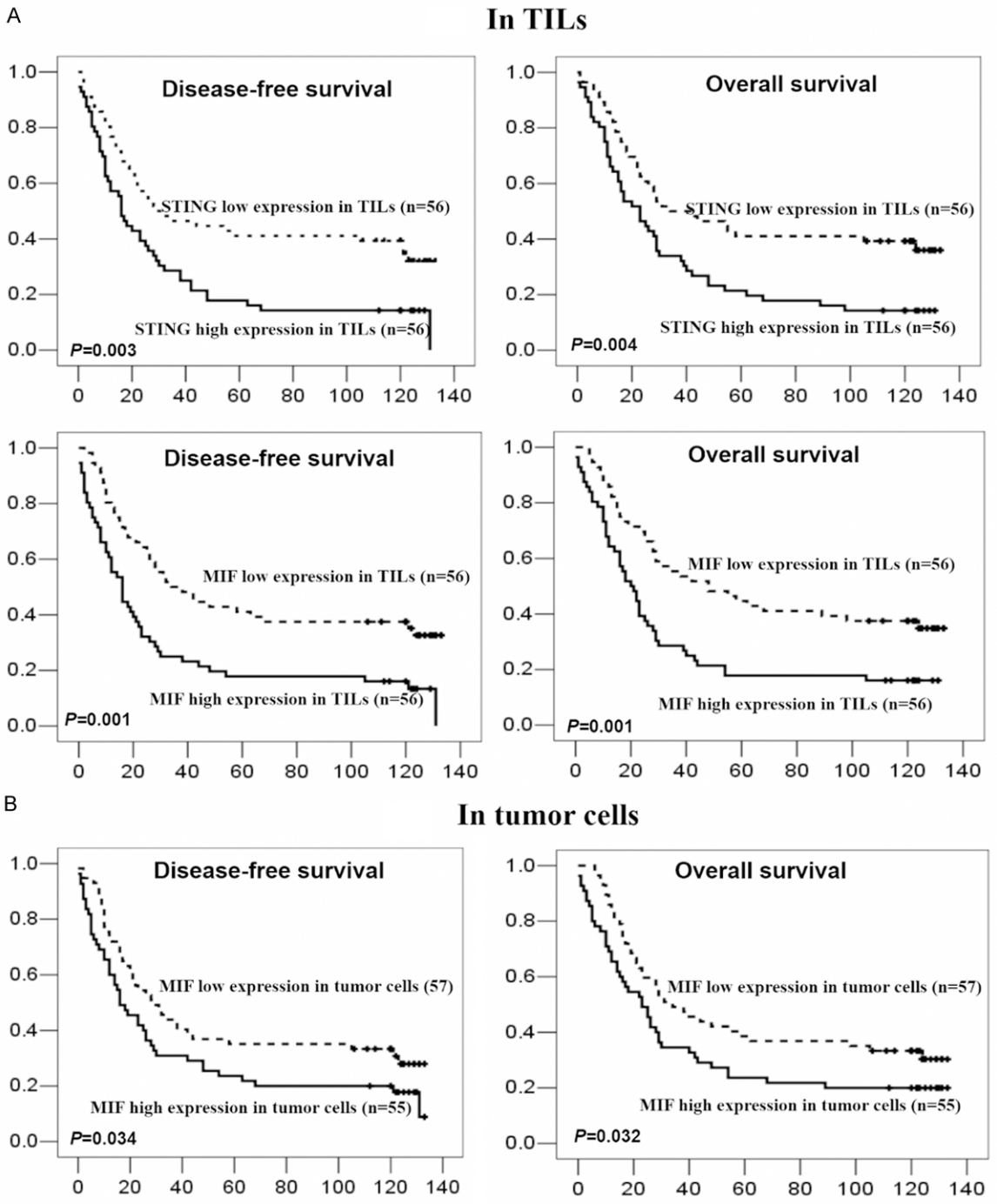

Figure 2. Kaplan-Meier survival analysis in patients with ESCC. A: Disease-free survival and overall survival curves for patients according to the low and high expression levels of STING and MIF in TILs. B: Disease-free survival and overall survival curves for patients according to the low and high expression levels of STING and MIF in tumor cells.

Associations between clinicopathological parameters and immunohistochemical variables

The associations between clinicopathological parameters and immunohistochemical variables in different cell types of 112 ESCC patients are detailed in **Table 1**. Patients were divided into high-expression level group and low-expression level group based on the medians of immunohistochemical variable values in tumors and TILs. High expression level of MIF in TILs was correlated with T status (P=0.036),

Table 2. Univariate analysis of DFS and OS in 112 patients with ESCC

Variables	DFS (n=136)		OS (n=136)	
	HR (95% CI)	P	HR (95% CI)	P
Age, years (≤62/>62)	0.754 (0.492-1.154)	0.193	0.822 (0.534-1.266)	0.373
Gender (male/female)	0.502 (0.265-0.951)	0.034*	0.406 (0.203-0.812)	0.011*
Tumor (T) status (1-2/3-4)	1.710 (1.040-2.812)	0.035*	1.739 (1.039-2.909)	0.035*
Nodal (N) status (0/1)	2.270 (1.455-3.540)	<0.001*	2.141 (1.365-3.357)	0.001*
TNM stage (I-II/III-IV)	2.081 (1.352-3.202)	0.001*	1.297 (1.044-1.613)	0.019*
MIF in tumor cells (low/high)	1.570 (1.026-2.402)	0.038*	1.594 (1.034-2.457)	0.035*
STING in tumor cells (low/high)	1.079 (0.706-1.651)	0.724	1.098 (0.713-1.690)	0.672
MIF in lymphocytes (low/high)	2.081 (1.352-3.202)	0.001*	2.000 (1.290-3.102)	0.002*
STING in lymphocytes (low/high)	1.883 (1.223-2.900)	0.004*	1.868 (1.205-2.896)	0.005*

Note: *p* value is determined by log-rank test. *$P<0.05$.

Table 3. Multivariate Cox analyses for DFS and OS of 112 patients with ESCC

Variables	DFS (n=112)		OS (n=112)	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Gender (male/female)	-	-	0.422 (0.206-0.864)	0.018*
Nodal (N) status (0/1)	2.863 (1.206-6.794)	0.017*	2.950 (1.202-7.239)	0.018*
MIF in Lymphocytes (low/high)	1.733 (1.086-2.766)	0.021*	1.684 (1.049-2.705)	0.031*
STING in Lymphocytes (low/high)	1.587 (1.088-2.748)	0.021*	1.759 (1.103-2.805)	0.018*

Note: The Cox proportional hazards regression model contained the significantly different factors in univariate analysis, including gender, WHO grade, T status, N status and TNM stage. HR, hazard ratio; CI, confidence interval; *P<0.05.

whereas the expression levels of MIF in tumor cells or the expression levels of STING in tumor cells and in TILs were not related to any of the clinicopathological parameters.

Association between STING and MIF expression and clinical outcome

The median survival time of the 112 patients was 26 months (range: 0 to 133 months). The cumulative five-year OS rate and DFS rate of the patients in this study were 30% and 29%, respectively (data not shown). The statistical analysis showed a significant negative correlation between DFS, OS and the expression levels of MIF in tumor cells and TILs. Negative correlation between DFS, OS and the expression levels of STING in TILs was also detected ($P<0.05$, **Figure 2**).

Univariate and multivariate analyses of STING and MIF expression level as prognostic factors

The univariate analysis indicated that high expression level of MIF ($P=0.034$ and $P=0.032$) in tumor cells was significantly correlated with reduced DFS and OS. High expression level of MIF ($P=0.001$ and $P=0.001$) in TILs was also significantly associated with decreased DFS and OS. High expression level of STING ($P=0.003$ and $P=0.004$) in TILs was also notably correlated with decreased DFS and OS, whereas the expression levels of STING in TILs were not significantly correlated with reduced DFS and OS (**Table 2**). Clinicopathological parameters such as gender, Tumor status, nodal status and TNM stage were also of prognostic value in univariate analysis. Furthermore, according to the multivariate Cox model analysis, we observed that the expression levels of MIF or STING in TILs were independent predictors of DFS and OS (**Table 3**).

Correlation between STING and MIF expression

Pearson's correlation coefficient and a linear regression analysis were used to evaluate the correlations between the expression levels of STING and MIF in tumor cells and TILs. STING expression levels in TILs were significantly positively correlated with MIF expression levels in tumor cells and in TILs ($P=0.022$, $R=0.217$ and $P=0.016$, $R=0.226$, respectively). Besides, the expression levels of MIF in tumor cells were positively correlated with MIF expression levels

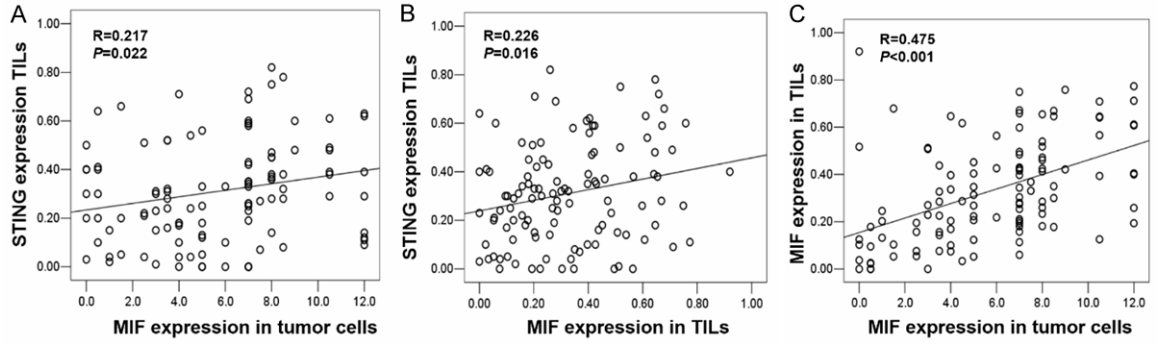

Figure 3. Scatter dot plots and correlation analysis between the STING and MIF expressions in different cell populations. A: The expression of MIF in tumor cells were significantly positively correlated with STING expression in TILs (P=0.022, R=0.217). B: The expression levels of MIF in TILs were significantly positively correlated with STING expression in TILs (P=0.016, R=0.226). C: The expression of MIF in tumor cells were significantly positively correlated with MIF expression in TILs (P<0.001, R=0.475).

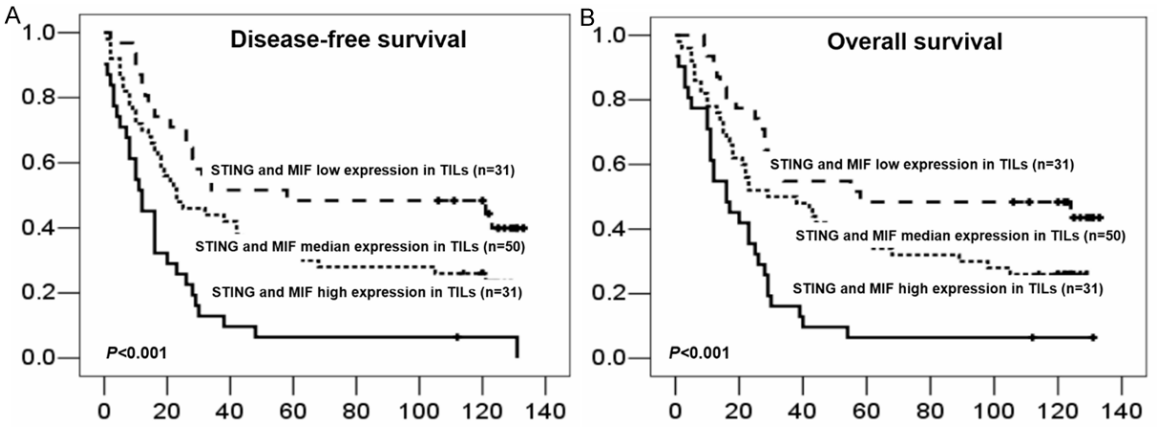

Figure 4. Survival curves for ESCC patients according to their expression levels of STING and MIF in tumor cells. A and B: DFS and OS curves for patients according to the combined low expression level, single high expression level and combined high expression level of STING and MIF in TILs.

in TILs (P<0.001, R=0.475), as shown in **Figures 3A** and **4B**. Furthermore, our study showed that the combined high expression of STING and MIF in TILs was strongly associated with reduced DFS and OS (**Figure 4**).

Discussion

Immune cells in tumor microenvironment play an important role in the generation and development of cancer [26]. The roles of STING in tumor microenvironment are Contradictory. In some studies, STING was found to be a protective factor that prevented the generation and progression of cancer. The activation of STING in phagocytes was reported to leading to T cell responses and function as an antitumor role [27]. STING mediates protection against colon cancer by recognizing intestinal DNA damage and promoting wound repair in the colon [28, 29]. Radiation-induced cancer cell death stimulated STING-dependent cytosolic DNA sensing resulted in type I interferon-dependent antitumor responses [30]. STING agonists were capable to cause tumor regression and showed potent antitumor therapeutic effects [31]. In other reports, the negative role of STING has been uncovered in the prevention of inflammation-induced cancer. DNA leakage in the cytoplasm caused by DNA damage activates STING-mediated inflammation and finally leads to skin carcinogenesis [32]. Besides, studies found STING is capable to influence the function of immunosuppressive cells include T regulatory cells and Myeloid-derived Suppressor Cells (MDSCs) in tumor microenvironment. A study on tongue squamous cell carcinoma indi-

cated that activated STING promoted the generation of several immunosuppressive cytokines including IL-10, IDO and CCL22, and enhanced the recruitment of Foxp3$^+$ regulatory T cells (Tregs) via the c-jun/CCL22 signaling [33, 34]. Moreover, a study on STING ligand c-di-GMP revealed that, Low doses of c-di-GMP increased the production of IL12 by MDSCs, whereas a high dose of c-di-GMP killed the tumor cells directly [35].

Here, we discussed about the immunosuppressive potential of STING in tumor microenvironment via Tregs and MDSCs. Interestingly, MIF is also considered as an immunosuppressive factor in cancer in variety of studies. In some studies, MIF promotes tumor progression by increasing the prevalence of MDSCs in tumor microenvironment [36]. MIF can also inhibit the differentiation of MDSCs to normal monocytes and promote the immunosuppressive function of MDSCs [37, 38]. Moreover, a study on tumor-bearing mice indicates that MIF promotes tumor growth by promoting the generation of Tregs generation through the regulation of IL-2 production [39]. Our data confirmed the roles of STING and MIF in TILs in facilitating the carcinogenesis and cancer progression. We also investigated the expression of STING and MIF in tumor cells. But no significant difference was found in the survival of patients in high and low expression of STING in tumor cells. It may be explained by the contradictory role of STING in tumorigenesis. Further investigations are needed to uncover the function of STING in tumor cells and the molecular mechanisms of STING in tumor induced immunity.

Our data provides novel prognostic indicators of STING and MIF in TILs in predicting the survival of patients with ESCC. The expression pattern of STING in TILs in ESCC was firstly described. Our results indicate that STING has different biological functions in tumor cells and in TILs. Importantly, the expression levels of STING and MIF in tumor cells and in TILs were positively associated (**Figure 3**). Our results showed for the first time that combined high expression of STING and MIF in TILs strongly indicate a reduced DFS and OS (**Figure 4**). The relationship between STING and MIF is still unclear and waiting to be explored. Besides, the immune regulatory function of STING and MIF in tumor microenvironment is an interesting project that deserves further investigation.

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Disclosure of conflict of interest

None.

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Table S1. Clinical characteristics of the 112 patients With ESCC

Characteristic	No. (%)
Total cases	112 (100%)
Age	
Median	62
Range	35-90
Gender	
Male	94 (83.9%)
Female	18 (16.1%)
Tumor (T) status	
T1	4 (3.6%)
T2	28 (25.0%)
T3	77 (68.8%)
T4	3 (2.7%)
Lymphoid Nodal (N) status	
N0	52 (46.4%)
N1	60 (53.6%)
Distant metastasis (M) status	
M0	107 (95.5%)
M01	5 (4.5%)
TNM stage	
I	3 (2.7%)
IIa-IIb	55 (49.1%)
III	49 (43.8%)
IV	5 (4.5%)
Progression or Relapse	
No	26 (23.2%)
Yes	86 (76.8%)
Death	
No	29 (25.9%)
Yes	83 (74.1%)

Table S2. Descriptive statistics of immunohistochemical variables in 112 patients

Variable	In tumor cells		In TILs	
	Low expression level (%)	High expression level (%)	Mean percentage (%)	Range of percentages (%)
MIF	56 (50%)	56 (50%)	33	0-92
STING	56 (50%)	56 (50%)	31	0-85