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*Basic Study*

Overexpression of ELL-associated factor 2 suppresses invasion, migration and angiogenesis *via* signal transducer and activator of transcription 3/transforming growth factor beta 1 crosstalk in colorectal cancer

Feng ML *et al.* EAF2 suppresses STAT3/TGF- $\beta$ 1 crosstalk in CRC

## **Abstract**

### **BACKGROUND**

The androgen responsive gene, ELL-associated factor 2 (EAF2), expressed in benign prostate tissues, has been shown to play an important role in tumor suppression in a variety of malignant tumors. In addition, some scholars found that EAF2 frameshift mutations are associated with intratumor heterogeneity in colorectal cancer (CRC) and inactivation of EAF2 in microsatellite instability-H CRC. However, the molecular mechanism by which EAF2 is involved in CRC invasion and metastasis remains unclear.

### **AIM**

To determine the expression and clinical value of EAF2 protein in CRC. Furthermore, we studied the effects of EAF2 on the invasion, migration and angiogenesis of CRC cells *in vitro*.

### **METHODS**

In this study, we collected colorectal adenocarcinoma and corresponding adjacent tissues to investigate the clinical expression of EAF2 protein in patients with advanced CRC. Following, we investigated the effect of EAF2 on the invasion, migration and angiogenesis of CRC cells *in vitro* using plasmid transfection.

### **RESULTS**

Our findings suggest that EAF2 protein is low expressed in cancer tissues of patients with advanced CRC. And Kaplan-Meier survival analysis showed that the survival rate of the high EAF2 level group was higher than that of the low EAF2 level group.

### **CONCLUSION**

More results demonstrated that EAF2, as a tumor suppressor, may inhibit the invasion, metastasis and angiogenesis of CRC cells by regulating signal transducer and activator

of transcription 3/transforming growth factor- $\beta$ 1 crosstalk pathway, and play a cancer suppressive and protective role in the occurrence and development of CRC. It is of great significance to provide new ideas and theoretical basis for the targeted diagnosis and treatment of CRC.

**Key Words:** ELL-associated factor 2; Transforming growth factor- $\beta$ 1; Signal transducer and activator of transcription 3; Colorectal cancer; Invasion; Migration; Angiogenesis

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**Core Tip:** Androgen-responsive gene ELL-associated factor 2 (EAF2) plays an important role in tumor suppression in a variety of malignant tumors. And researches have shown that intratumor heterogeneity of EAF2 frameshift mutation in colorectal cancer (CRC). In this study, we collected colorectal adenocarcinoma and corresponding paracancerous tissue to explore the clinical expression of EAF2 protein in patients with advanced CRC. Following, we applied plasmid transfection technology to study the effects of EAF2 on the invasion, migration and angiogenesis of CRC cells *in vitro*. We found that EAF2 protein is low expressed in cancer tissues of patients with advanced CRC. And Kaplan-Meier survival analysis showed that the survival rate of the group with high EAF2 level was higher than that of the group with low EAF2 level. Moreover, as a tumor suppressor, EAF2 may play a tumor suppressive and protective role in the development of CRC by inhibiting the invasion, metastasis and angiogenesis of CRC cells *via* regulating the signal transducer and activator of transcription 3/transforming growth factor beta 1 crosstalk pathway. It is of great significance to provide new ideas and theoretical basis for targeting diagnosis and therapy of CRC.

## INTRODUCTION

Colorectal cancer (CRC) is one of the tumors with high morbidity and mortality in the world. The Global Cancer Statistics 2020 revealed that CRC was estimated to be the third most diagnosed malignant tumor (10%) and the second leading cause of cancer death (9.4%) worldwide<sup>[1]</sup>. As an important process in the development of CRC, tumor metastasis is a key factor affecting the survival rate of CRC. Approximately 35% of newly diagnosed CRC patients present metastatic disease, and about 50% of patients with non-metastatic disease eventually develop metastatic disease<sup>[2]</sup>. Most regrettable, the 5-year survival rate of patients with metastatic CRC is less than 10%<sup>[3]</sup>. There is a great need to elucidate the molecular mechanism of CRC invasion and metastasis, which is beneficial to explore new potential molecular markers and the direction of targeted therapy of CRC.

ELL-associated factor 2 (EAF2) was first identified as an androgen-responsive gene expressed by luminal epithelial cells in benign prostatic tissue<sup>[4,5]</sup>. Interestingly, EAF2 has been found to inhibit cell proliferation and tumor size in prostate cancer *in vivo*<sup>[6]</sup>, corresponding to that, knockdown of EAF2 in prostate cancer cells resulted in increased proliferation and migration<sup>[7]</sup>. In recent years, more and more researches have shown that EAF2 plays an important role in tumor suppression in a variety of malignant tumors<sup>[8]</sup>. Furthermore, there are scholars identified intratumor heterogeneity (ITH) of EAF2 frameshift mutation in CRC and the inactivation of EAF2 in microsatellite instability (MSI)-H CRC<sup>[9]</sup>. It suggests that EAF2 inactivation may occur during tumor progression rather than tumorigenesis. Besides, EAF2 gene silencing could modulate the cytotoxic response of colon cancer cell line HCT116 to statins<sup>[10]</sup>. However, studies on the expression and role of EAF2 protein in CRC are still lacking. The molecular mechanism of EAF2's involvement in CRC invasion and metastasis remains unclear.

EAF2 has been shown to attenuate transforming growth factor beta 1 (TGF- $\beta$ 1)-induced G1 cell cycle arrest and cell migration in a variety of tumor cells, such as renal carcinoma cells, human hepatocellular carcinoma cells, and breast cancer cells<sup>[11]</sup>. It is worth noting that blocking the TGF- $\beta$ 1 signaling pathway may be a therapeutic strategy

for attenuating tumor metastasis in CRC metastatic models<sup>[12]</sup>. In addition, <sup>12</sup> signal transducer and activator of transcription 3 (STAT3) pathway is involved in the process of TGF- $\beta$ 1-induced epithelial-mesenchymal transition (EMT), cell migration and invasion in malignant tumor<sup>[13,14]</sup>. As well, EAF2 knockout induced STAT3 phosphorylation (Tyr705) in prostate cancer cells, which drove tumor progression in prostate cancer<sup>[15]</sup>. Therefore, in this study, we investigated the effect of EAF2 on the STAT3/TGF- $\beta$ 1 pathway in CRC cells to explore the possible mechanism of EAF2 acting in CRC.

All tissues in the body need blood vessels to provide nutrients and oxygen to maintain growth and function<sup>[16]</sup>, so angiogenesis provides necessary functions in normal growth and biological development processes, such as <sup>11</sup> embryonic development, bone formation, and the function of ovaries and other endocrine glands<sup>[17]</sup>. Not only that, tumor growth and metastasis also require angiogenesis<sup>[18]</sup>. Abnormalities in vasculogenesis and spermatogenesis have been found in EAF2 knockout mice<sup>[19]</sup>. Besides, EAF2 negatively regulated the activity of angiogenic factor hypoxia inducible factor (HIF)-1 $\alpha$ <sup>[20]</sup>. Therefore, it may provide theoretical basis for anti-angiogenic treatment to explore the regulatory role of EAF2 in CRC angiogenesis. Activation of JAK2/STAT3 (Tyr705) signaling pathway in CRC cells promoted cell proliferation and angiogenesis by positively regulating HIF-1 $\alpha$ /vascular endothelial growth factor A (VEGFA)<sup>[21,22]</sup>. Meanwhile, HIF-1 $\alpha$  sumoylation may elevate the microvascular density and lumen size and promote tumor growth by amplifying TGF- $\beta$ /Smad signaling in tumor cells<sup>[23,24]</sup>. We further investigated the role of EAF2 in CRC angiogenesis *via* the STAT3/TGF- $\beta$ 1 pathway.

In this study, we aimed to determine the expression and clinical value of EAF2 protein in CRC. Furthermore, we studied the effects of EAF2 on the invasion, migration and angiogenesis of CRC cells *in vitro*. In addition, we preliminarily explored the mechanism by which EAF2 affects the biological function and angiogenesis of CRC cells *via* regulating STAT3/TGF- $\beta$ 1 pathway. It is of great significance to provide new ideas and theoretical basis for the targeted diagnosis and therapy of CRC.

## **MATERIALS AND METHODS**

### ***CRC patients and tissue samples***

We selected 70 cases of advanced CRC from the First Affiliated Hospital of China Medical University (Liaoning, China) between 2012 and 2015. This study was supported by the Institutional Review Board of the First Affiliated Hospital of China Medical University (Registration No. 2021-68-2, informed consent was waived). The 70 pairs of histological sections of colorectal adenocarcinomas and corresponding paracancerous tissue that had been surgically removed. In addition, another 8 pairs of fresh cancer and adjacent tissue were selected for western blot assay. None of patients received radiotherapy or chemotherapy prior to surgical resection. We defined tumor stages according to the American Joint Committee on Cancer/Union for International Cancer Control TNM staging system (8<sup>th</sup> Edition).

### ***Reagents***

Recombinant human TGF- $\beta$ 1 (mammalian derived) was bought from PeproTech (100-21) (United States). The antibodies used in this study include: Rabbit monoclonal anti-EAF2 (ab237753) (for IHC-P), rabbit monoclonal anti-EAF2 [EPR7117(2)] (ab151692) (for western blot) (United Kingdom, Abcam); rabbit monoclonal anti-TGF- $\beta$ 1 (56E4) (#3709), rabbit monoclonal anti-phospho-STAT3 (Tyr705) (#9145), rabbit monoclonal anti-STAT3 (#30835) (United States, Cell Signaling, Danvers, MA); rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (FL-335) (United States, Santa Cruz, CA).

### ***Cell culture***

CRC cell lines (SW480, RKO, HCT116, HT29 and HIEC) and human normal colon cell line NCM460 were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). And these cells were cultured in RPMI-1640 medium (United States, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) (United States,

Gibco, Grand Island, NY) at 37 °C with 5% CO<sub>2</sub> in a humidified incubator. Human Umbilical Vein Endothelial Cells (HUVECs) were also purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China) and cultured in DMEM medium (United States, Gibco, Grand Island, NY) containing 10% FBS at 37 °C with 5% CO<sub>2</sub> in a humidified incubator.

### *Cell transfection*

The plasmid for overexpressing human EAF2 (GenBank no. NM\_018456) was designed and synthesized by GeneChem (Shanghai, China). And three plasmid targets for silencing human STAT3 (GenBank no. NM\_139276) were also provided by GeneChem (Shanghai, China). The sequences are listed in Table 1. The cell transfection was performed using the Lipofectamine 2000 Kit (United States, Invitrogen, Carlsbad, CA). Cells were plated into six-well plates and transfected with 4.0 µg plasmid DNA. A total of 6 h after transfection, cells were treated with medium containing 10% FBS.

### *Immunohistochemistry*

Typical CRC tissues and corresponding paracancer tissue sites were selected. The sections were dewaxed, rehydrated and heated using the pressure-cooker for antigen retrieval. And then we performed antigenic repair with citric acid. Subsequently, the sections were incubated overnight at 4 °C with rabbit monoclonal anti-EAF2 antibody (1:200 dilution, ab237753). Following, we incubated them with the secondary antibody for 40 min at 37 °C. Finally, all of them were stained with diaminobenzidine, counter-stained with hematoxylin, air-dried, dehydrated, and mounted.

Double blind reading was performed by two experienced pathologists. We calculated the final staining score by multiplying the scale score by the intensity score. Five high-power fields (magnification at 200 ×) were observed on each section. We divided the immunostaining intensity into four categories: 0 (negative immunostaining), 1 (weak immunostaining), 2 (moderate immunostaining), and 3 (strong immunostaining). And the proportion of positive cells scored from 0 to 4 as follows: (1) 0 (positive cells < 5%);



(2) 1 (positive cells 5% to 25%); (3) 2 (positive cells 26% to 50%); (4) 3 (positive cells 51% to 75%); and (5) 4 (positive cells > 75%).

#### *Western blot assay*

The tissues and cells were lysed in RIPA lysis buffer containing PMSF protease inhibitors. Protein concentrations were measured by BCA kit (China, Beyotime Institute of Biotechnology). Equal amounts of protein (50 µg per sample) were separated by 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes with 200 mA constant current. Primary antibodies for EAF2 (ab151692), TGF-β1, phospho-STAT3 (Tyr705), and STAT3 were used at a dilution ratio of 1:1000 and normalized to GAPDH which was used at a dilution ratio of 1:1000. Then anti-rabbit immunoglobulin G secondary antibody (1:5000) was used to incubate the membranes at 37 °C for 2 h. We detected the immunoreactive bands with ECL-Plus chemiluminescent detection HRP reagents (China, Beyotime Institute of Biotechnology) and using Microchemi 4.2 Bio-imaging system. Experiments were repeated at least three times under the same experimental conditions.

#### *Transwell invasion and migration assay*

Before invasion assay, Matrigel (BD Biosciences, San Diego, CA, United States) diluted at a ratio of 1:8 with serum-free medium was used to block the membranes at 37 °C for 6 h. A total of  $4 \times 10^4$  cells/well in 200 µL serum-free medium were seeded in the upper chambers of 8-µM pore transwell plates (3422, United States, Corning Incorporated, Corning, NY), and 600 µL medium containing 20% FBS was added to the lower chambers. For migration assay,  $2 \times 10^5$  cells/well were seeded into the upper chambers of an 8-µM pore transwell plates (3422) in 200 µL serum-free medium. The lower chambers were filled with 600 µL medium with 10% FBS.

After incubation for 72h, the non-invaded or non-migrated cells on the inner surface of the filter membrane were removed by scrubbing with a cotton swab, and the invaded

or migrated cells were fixed by methanol. Next, the cells were stained by 0.1% Crystal Violet. The membranes were moved from the chambers and fixed on cover slides. Invading or metastasizing cells were counted from five random fields under a light microscope (magnification at 200 ×). The experiments were repeated at least three times under the same experimental conditions.

#### ***Wound healing assay***

A total of  $2 \times 10^5$  cells/well were seeded into 6-well plates. Scratch wounds were generated using 200  $\mu$ L pipette tip when the cells reached 90% confluence. Afterwards, the cells were washed with phosphate buffered saline (PBS) three times, and then incubated in serum-2% medium. The scratch wounds were photographed at 0, 6 h, 12 h, 24 h, 48 h, 72 h in five selected regions. ImageJ software (Version 1.46r) was used to measure the wound area (magnification at 40 ×). The experiments were repeated at least three times under the same experimental conditions.

#### ***Tube formation assay***

Before tube formation assay, Matrigel was placed in a 96-well plate at 37 °C for 1 h. And the conditioned medium from treated RKO cells was collected and centrifuged at 2000 rpm for 10 min to remove debris. HUVECs with  $1.0 \times 10^4$  cells/well were seeded in Matrigel coated 96-well plate and cultured in conditioned medium. After 6 h, capillary morphogenesis was evaluated by inverted microscopy (magnification at 100 ×). Then, we analyzed the images for evaluating the branch points by means of the angiogenesis analyzer that is developed for the Image J software (Version 1.46r).

#### ***HUVEC transwell migration assay***

For HUVEC migration assay,  $1 \times 10^5$  HUVECs were plated in the upper chambers of an 8- $\mu$ M pore transwell plates (3422) with 200  $\mu$ L serum-free medium; 600  $\mu$ L conditioned medium were placed in the lower chambers. After 6 h, the cells were fixed by methanol and non-migrated cells were removed from the inner surface of the filter membrane.

The cells on the lower surface of the membrane were stained with 0.1% Crystal Violet, and the numbers of migrated cells were counted from five fields (magnification at 200 ×) under an optical microscope. The experiments were repeated at least three times under the same experimental conditions.

#### *HUVEC scratch assay*

For scratch assay,  $2 \times 10^5$  HUVECs/well were seeded in 6-well plate. When cells reached about 90% confluence, a 200 µL tip was used to scratch the cells. The cells were then washed with PBS three times and incubated with 2 mL conditioned medium per well. The scratch was photographed at 0 h, 12 h and 24 h time points under microscope in five selected regions (magnification at 40 ×). ImageJ software (Version 1.46r) was used to measure the wound area. Experiments were repeated at least three times under the same experimental conditions.

#### *Statistical analysis*

The results in this study were analyzed by SPSS software (Version 26). Pearson's chi-squared test was adopted to evaluate the correlation between EAF2 protein and clinicopathological parameters. The expression differences of EAF2 between paired adenocarcinomas and para-cancerous tissue were analyzed by Wilcoxon sign rank test. receiver operating characteristic (ROC) curve was used to determine the Youden index to delimit the high and low expression of EAF2 in tissues. The data were shown as mean  $\pm$  SD. Student's *t* test was used for analyzing the differences between two groups, while for differences among three or more groups, one-way ANOVA was performed. The Kaplan-Meier method was used to estimate the cumulative survival of patients, and the Log-rank test was used to test the significance of the differences in survival. Cox proportional hazards regression model was used for multivariate analysis to evaluated the independent prognostic effect of EAF2 protein on survival. All the statistics were subjected to ANOVA followed by Bonferroni test. All *P* values were two-sided and considered statistically significant when  $P < 0.05$ .

## **RESULTS**

### ***EAF2 protein expression was decreased in advanced CRC tissue***

To assess the expression and localization of EAF2 protein in CRC specimens, we examined the expression of EAF2 at protein level in paired adenocarcinomas and paracancerous tissue by immunohistochemical analysis and Western blot assay. Immunohistochemical analysis of 70 pairs of colorectal adenocarcinomas and corresponding paracancerous tissue showed that EAF2 protein was mainly localized in the cytoplasm of colorectal epithelial cells (Figure 1A). The median EAF2 score was 6.0 in tumor tissue and 6.8 in adjacent non-tumor tissue, with a median difference of -2.0. Moreover, Wilcoxon signed-rank test of paired samples showed that EAF2 expression was lower in tumor tissue than that in non-tumor tissue ( $Z = -3.727$ ,  $P < 0.001$ ).<sup>6</sup> Additionally, we used western blot assay to measure the level of EAF2 protein expression in 8-matched pairs of fresh colorectal adenocarcinomas and corresponding paracancerous tissue. The results also revealed a lower level of EAF2 protein expression ( $P = 0.012$ ) in tumor tissue compared with adjacent non-tumor tissue (Figure 1B). According to the Youden index determined by ROC curve, the cut-off value delimiting high and low expression of EAF2 (dependent variable is non-cancer) was determined to be 6.2 ( $P < 0.001$ ). In this study, we found EAF2 Low-expression in 58 of 70 CRC tissues.

We then assessed the relationship between EAF2 protein expression and major clinicopathological characteristics. Statistical results showed that the expression of EAF2 protein in CRC tissue<sup>8</sup> was negatively correlated with distant metastasis ( $r = -0.268$ ,  $P = 0.025$ ) and carcinoembryonic antigen (CEA) ( $r = -0.249$ ,  $P = 0.038$ ), but not with other clinical characteristics, such as age, sex, primary tumor site, tumor size, tumor histological differentiation, degree of differentiation, vasculolymphatic<sup>1</sup> and/or perineural invasion, tumor stage, tumor invasion depth, lymph node status, and carbohydrate antigen 19-9 (CA19-9), P53 and CDX2 (Table 2). High expression of CEA, CA19-9, P53 and CDX2 were defined as a score of  $\geq 3$ . Low expression of them were defined as a score of  $< 3$ .

### *The survival analysis of EAF2 expression in patients with advanced CRC*

Kaplan-Meier survival analysis and Log-rank testing for overall survival (OS) showed that the survival rate of the group with high EAF2 level was higher than that of the group with low EAF2 level ( $P = 0.026$ ). Figure 2 shows the Kaplan-Meier survival curve of advanced CRC patients with high and low EAF2 expression. We used Cox proportional-hazards model to evaluate the effect of EAF2 protein on OS in patients with advanced CRC. From univariate analysis, we found that angiolymphatic and/or perineural invasion ( $P = 0.003$ ), tumor stage ( $P = 0.012$ ), tumor invasion depth ( $P = 0.011$ ), lymph node status ( $P = 0.017$ ), distant metastasis ( $P < 0.001$ ) and EAF2 protein expression level ( $P = 0.038$ ) were significantly correlated with OS (Table 3). The clinicopathological characteristics with  $P < 0.3$  in univariate analysis were included in multivariate analysis, and the independent prognostic effect of EAF2 protein on OS was assessed by adjusting for confounding factors. However, multivariate analysis proved that tumor invasion depth ( $P = 0.035$ ) and distal metastasis ( $P < 0.001$ ) were independent prognostic factors for OS (Table 3).

### *Overexpression of EAF2 inhibited the invasion and migration of CRC cells*

We cultured CRC cells *in vitro* to determine the expression of EAF2 protein and further investigate its effects on the biological function of CRC cells. The expression level of EAF2 protein in human CRC cell lines (SW480, RKO, HCT116, HT29 and HIEC) were observably lower than that in normal colorectal epithelial cells (NCM460) (Figure 3). There has been a considerable decrease in colon cancer cell line RKO cells, with a reduction of 64.11% in EAF2 protein expression ( $P < 0.001$ ) (Figure 3). Then, we used RKO cells in subsequent experiments.

Following, we overexpressed EAF2 protein by plasmid transfection technique to investigate the effects on invasion and migration of human colon cancer cell line RKO cells. EAF2 overexpression vector significantly increased the protein expression level in RKO cells, which was 2.25-fold higher than the control group (Figure 4A,  $P < 0.001$ ).

Results of transwell invasion assay with Matrigel-coated membranes showed that EAF2 overexpression significantly weakened the invasion ability of RKO cells (Figure 4B,  $P < 0.001$ ). Analogously, cells transfected with EAF2-overexpressing plasmids also showed a poorer migration capacity than the control group in the transwell migration assay (Figure 4B,  $P < 0.001$ ). In addition, as shown in Figure 4C, EAF2 overexpression reduced the wound healing ability of RKO cells ( $P < 0.001$ ).

#### ***Overexpression of EAF2 suppressed the activity of STAT3/TGF- $\beta$ 1 crosstalk pathway***

In this study, we set out to investigate the molecular mechanism of EAF2 on RKO cells *in vitro*. Phosphorylated STAT3 (Tyr705) and TGF- $\beta$ 1 protein expression levels were significantly increased in human CRC cell lines (SW480, RKO, HCT116, HT29 and HIEC) compared with normal colorectal epithelial cells (NCM460) (Figure 3). The upregulation of STAT3 phosphorylated protein (Tyr705) (with 1.585-fold increased,  $P < 0.001$ ) and TGF- $\beta$ 1 protein (with 2.485-fold increased,  $P < 0.001$ ) in RKO cells was statistically significant.

Compared with control cells, TGF- $\beta$ 1 protein expression level was significantly decreased in RKO cells transfected with EAF2-overexpressing plasmid (Figure 5A). Meanwhile, we also found that overexpression of EAF2 remarkably decreased phosphorylated STAT3 (Tyr705) levels in RKO cells, but not total STAT3 Levels (Figure 5A).

And then we knocked down STAT3 gene by transfecting with STAT3 siRNA (Figure 6A). We found that inhibition of STAT3 phosphorylation (with reduction of 60.25%,  $P < 0.001$ ) resulted in a significant decrease in TGF- $\beta$ 1 protein expression ( $P < 0.001$ ) in RKO cells but not in EAF2 ( $P = 0.228$ ) (Figure 6B). Furthermore, we treated RKO cells with TGF- $\beta$ 1 recombinant protein to clarify the regulation of TGF- $\beta$ 1 on EAF2 protein expression and STAT3 phosphorylation. The results revealed that TGF- $\beta$ 1 recombinant protein reversed the decrease of phosphorylated STAT3 (Tyr705) induced by EAF2 overexpression ( $P < 0.001$ ) (Figure 5A). However, our results showed that TGF- $\beta$ 1

recombinant protein did not affect the expression of EAF2 protein ( $P = 0.099$ ) in RKO cells (Figure 5A).

***Overexpression of EAF2 inhibited the invasion and migration of CRC cells by down-regulating the activity of STAT3/TGF- $\beta$ 1 crosstalk pathway***

We attempted to further determine whether EAF2 regulates the biological function of CRC cells by regulating the activity of STAT3/TGF- $\beta$ 1 crosstalk pathway. We used TGF- $\beta$ 1 recombinant protein to treat the RKO cells transfected with EAF2-overexpressing plasmid for 24 h. As a result, the invasiveness and metastasis capability of RKO cells weakened by EAF2 overexpression was partially reversed by TGF- $\beta$ 1 recombinant protein (Figures 5B and 5C).

***Overexpression of EAF2 inhibited CRC angiogenesis***

In this study, we preliminarily investigated the effects of RKO cells overexpressing EAF2 on migration and tube formation abilities of HUVECs. Firstly, HUVECs were cultured with DMEM medium containing 10% serum<sup>[25]</sup>. After 2-6 generations of cells, HUVECs were subcultured into corresponding petri dishes. We then treated HUVECs with conditioned medium from RKO cells transfected with EAF2-overexpressed plasmids (EAF2-OV group) or empty control plasmids (EAF2-NC group) for 24 h. Compared with the control group (RKO-C group), whose conditioned medium was collected from RKO cells cultured with 10% serum for 24 h, the tube formation (Figure 7A) and migration (Figures 7B and 7C) of HUVECs in the EAF2-OV group were significantly reduced, while the differences in the EAF2-NC group were not statistically significant. Our results indicated that overexpression of EAF2 protein may inhibit angiogenesis induced by CRC cells *in vitro*.

***Overexpression of EAF2 regulated CRC angiogenesis via STAT3/TGF- $\beta$ 1 signaling pathway***



STAT3-related pathway and non-specific angiogenic factor TGF- $\beta$ 1 play a proangiogenic role in tumor angiogenesis. Our study showed that overexpression of EAF2 inhibits the activity of the STAT3/TGF- $\beta$ 1 crosstalk pathway in RKO cells. Hence, we investigated whether EAF2 inhibits CRC angiogenesis through the STAT3/TGF- $\beta$ 1 signaling pathway. We used TGF- $\beta$ 1 recombinant protein to treat RKO cells transfected with or without EAF2-overexpressed plasmids for 24 h, and collected conditioned medium to culture HUVECs. We aimed to investigate whether TGF- $\beta$ 1-related pathways are involved in the molecular mechanism of EAF2 overexpression regulating angiogenesis in RKO cells *in vitro*. Compared with RKO-C group, the tube formation (Figure 8A) and migration (Figures 8B and 8C) of HUVECs in the EAF2-OV group were significantly decreased. Interestingly, when HUVECs were cultured with conditioned medium from EAF2-overexpressing RKO cells treated with TGF- $\beta$ 1 recombinant protein, the inhibition of EAF2 overexpression on tube formation (Figure 8A) and migration (Figures 8B and 8C) of HUVECs was reversed. Together, these data confirmed our hypothesis that EAF2 overexpression inhibits CRC angiogenesis by suppressing the activity of the STAT3/TGF- $\beta$ 1 crosstalk pathway *in vitro*.

## **DISCUSSION**

Previous researches have supported the role of EAF2 as a tumor suppressor in multiple human tissues<sup>[6]</sup>. EAF2 knockout mice developed a higher frequency of B-cell lymphoma, lung adenocarcinoma, hepatocellular carcinoma, and prostatic intraepithelial neoplasia<sup>[6]</sup>. EAF2 and its homologue EAF1 were originally identified as binding partners of the fusion protein ELL (11-19 Lysine-rich leukemia) associated with myeloid leukemia<sup>[26,27]</sup>, stimulating the extension activity of ELL. However, EAF2 mutations produce truncated EAF2 mutants, which may be associated with the pathogenesis of MSI-H cancers<sup>[9]</sup>. Recently, further studies have found ITH in EAF2 frameshift mutations in CRC and the inactivation of EAF2 in MSI-H CRC<sup>[9]</sup>, suggesting that EAF2 mutations may occur during tumor progression rather than during



tumorigenesis. However, the expression, role and molecular mechanism of EAF2 protein in CRC remain unclear.

<sup>1</sup> This study collected colorectal adenocarcinoma and paired adjacent tissues to investigate the clinical expression of EAF2 protein in patients with advanced CRC. Compared with non-tumor tissue, the results of immunohistochemistry and western blot assay showed that the level of EAF2 protein in CRC tissue was significantly lower. And immunohistochemical results indicated that EAF2 protein was mainly expressed in cytoplasm. Meanwhile, *in vitro* experiments also confirmed that EAF2 expression level in CRC cells was lower than that in normal colorectal epithelial cells. Further analysis of the correlation between EAF2 expression level and clinicopathological characteristics revealed that EAF2 protein level was negatively correlated with distant metastasis and CEA expression level in CRC tissues. These results suggest that EAF2 protein may be related to the invasion and metastasis of CRC. However, further studies are needed to determine the mechanism of decreased EAF2 protein expression in CRC.

<sup>2</sup> Besides, Kaplan-Meier survival analysis showed that the survival rate of the group with high EAF2 levels was higher than that of the group with low EAF2 levels. Although multivariate analysis did not suggest that EAF2 expression level was an independent survival prognostic marker in patients with advanced CRC, univariate analysis suggested that it was an influential factor of survival and prognosis in patients with advanced CRC. However, more cases and more comprehensive clinical studies are needed to further explore this question.

In addition, phospho-STAT3 (Tyr705) and TGF- $\beta$ 1 protein were highly expressed in CRC cells in this study. Studies have shown that TGF- $\beta$ 1 is highly expressed in CRC tissue, and overexpression of TGF- $\beta$ 1 facilitates invasion and migration in CRC cells *in vitro*<sup>[28]</sup>. Furthermore, blocking the TGF- $\beta$ 1 signaling pathway in CRC metastatic models may serve as a therapeutic strategy to weaken tumor metastasis *in vivo*<sup>[20]</sup>. Importantly, STAT3 acts as a positive regulator to activate TGF- $\beta$ 1 to induce tumor EMT and metastasis<sup>[14]</sup>. Meanwhile, STAT3 is one of the major oncogenic pathways activated in

CRC, and its activation can be detected in CRC tissue, primary CRC cells or CRC cell lines<sup>[29-31]</sup>.

Further studies have revealed that EAF2 attenuates TGF- $\beta$ 1-induced G1 cell cycle arrest and cell migration, which has been demonstrated in renal carcinoma cells, human hepatocellular carcinoma cells, and breast cancer cells<sup>[11]</sup>. Besides, phospho-STAT3 (Tyr705) immunostaining is correlated with down-regulation of EAF2 in human prostate cancer specimens<sup>[15]</sup>. EAF2 knockout induces STAT3 phosphorylation (Tyr705) *in vivo* and *in vitro*, suggesting that EAF2 is a repressor of STAT3 signaling pathway<sup>[15]</sup>. In our study, we found that overexpression of EAF2 decreased the levels of phosphorylated STAT3 (Tyr705) and TGF- $\beta$ 1 protein in CRC cells. In addition to this, we also found that silencing STAT3 reduced TGF- $\beta$ 1 protein expression. More than that, TGF- $\beta$ 1 recombinant protein eliminated the reduction of phosphorylated STAT3 (Tyr705) induced by EAF2 overexpression. These results indicate that EAF2 overexpression may inhibit the activity of STAT3/TGF- $\beta$ 1 crosstalk pathway in CRC cells.

We found that EAF2 overexpression restrains the invasion and metastasis of CRC cells. It makes sense to further explore the role of EAF2 in regulating the activity of STAT3/TGF- $\beta$ 1 crosstalk pathway in CRC cell invasion and metastasis. The results revealed that the invasiveness and metastasis capability of CRC cells weakened by EAF2 overexpression could be partially reversed by TGF- $\beta$ 1 protein. Therefore, it was concluded that EAF2 overexpression inhibited the invasion and migration of CRC cells by down-regulating the activity of the STAT3/TGF- $\beta$ 1 crosstalk pathway. It is possible that therapeutic drugs targeting the STAT3/TGF- $\beta$ 1 signaling pathway may be effective for CRC subsets with down-regulated EAF2, elevated STAT3 phosphorylation, and up-regulated TGF- $\beta$ 1.

Tumor angiogenesis is critical not only for tumor growth, but also for tumor progression and metastasis<sup>[32]</sup>. Tumor angiogenesis is regulated by multiple angiogenic factors and angiogenic inhibitors<sup>[21]</sup>. The activation of the STAT3 pathway contributes to angiogenesis by increasing the expression of angiogenic factors, such as VEGFA,

interleukin (IL)-8, HIF-1 $\alpha$ , or IL-6<sup>[33]</sup>. Non-specific angiogenic factor TGF- $\beta$ 1 plays a proangiogenic role in CRC angiogenesis<sup>[34]</sup>. TGF- $\beta$ 1 signaling inactivation caused by dnTGFBR1 in tumor cells can reduce microvessel density and lumen sizes, and decrease tumor growth<sup>[35]</sup>. In keloid tissues sumoylation of HIF-1 $\alpha$  may increase the stability and an amplified effect on TGF- $\beta$ /SMAD signaling<sup>[23]</sup>. Matrigel plug angiogenesis assay showed that EAF2 knockout mice responded to VEGF with significantly enhanced neovascularization<sup>[19]</sup>. And EAF2 is a negative regulator of HIF-1 $\alpha$  activity<sup>[20]</sup>. EAF2 binds to and stabilizes von Hippel-Lindau protein and then disrupts HIF-1 $\alpha$ -mediated hypoxia signaling pathway<sup>[19,36]</sup>. Abnormal vasculogenesis occurred in EAF2 knockout mice<sup>[19]</sup>. We hypothesized that EAF2 may regulate CRC angiogenesis through the STAT3/TGF- $\beta$ 1 pathway. HUVECs was used in this study for evaluating the impact of RKO cells on angiogenesis. Besides, endothelial progenitor cell is also a good endothelial model for studying tumor-induced angiogenesis<sup>[37]</sup>.

In this study, our results show that EAF2-overexpressed CRC cells can inhibit tube formation and migration of HUVECs. Besides, the inhibition of EAF2 overexpression on tube formation and migration of HUVECs may be reversed by TGF- $\beta$ 1 recombinant protein. This suggests that EAF2 overexpression inhibited CRC angiogenesis by suppressing the activity of the STAT3/TGF- $\beta$ 1 crosstalk pathway. However, this study still has some limitations, and lacks further studies on vascular regulatory factors released by vascular endothelial cells and changes in tumor microenvironment mediated by EAF2 and its downstream STAT3/TGF- $\beta$ 1 crosstalk pathway.

## **CONCLUSION**

In conclusion, our findings suggest that EAF2 protein is under-expressed in cancer tissue of patients with advanced CRC. And Kaplan-Meier survival analysis showed that the survival rate of the group with high EAF2 levels was higher than that of the group with low EAF2 levels. Moreover, as a tumor suppressor, EAF2 may play a tumor suppressive and protective role in the development of CRC by inhibiting the invasion, metastasis and angiogenesis of CRC cells. The regulation of EAF2 on the biological

functions of CRC cells may be realized by regulating the STAT3/TGF- $\beta$ 1 crosstalk pathway (Figure 9). Further research on methods and technologies to increase EAF2 in CRC will be one of the future research directions, and exploration of upstream factors such as related non-coding RNAs. It may provide new diagnostic markers and novel therapeutic targets for CRC.

## **ARTICLE HIGHLIGHTS**

### ***Research background***

There are few studies on the expression and role of ELL-associated factor 2 (EAF2) protein in colorectal cancer (CRC). The molecular mechanism of EAF2's involvement in CRC invasion and metastasis remains unclear.

### ***Research motivation***

EAF2 may play a tumor suppressive and protective role in the development of CRC by inhibiting the invasion, metastasis and angiogenesis of CRC cells.

### ***Research objectives***

We assessed the expression and localization of EAF2 protein in CRC specimens. Meanwhile, we cultured CRC cells *in vitro* to determine the expression of EAF2 protein and further investigate its effects on the biological function of CRC cells.

### ***Research methods***

We used immunohistochemistry and western blot assay to detect the expression of EAF2 protein in CRC tissues from 70 patients. In addition, we applied plasmid transfection technology to study the effects of EAF2 on invasion, migration and angiogenesis of CRC cells *in vitro*.

### ***Research results***

Our findings suggest that EAF2 protein is low expressed in cancer tissues of patients with advanced CRC. And Kaplan-Meier survival analysis showed that the survival rate of the group with high EAF2 level was higher than that of the group with low EAF2 level. In addition, EAF2 affects the biological functions of CRC cells by regulating the STAT3/TGF- $\beta$ 1 crosstalk pathway.

### *Research conclusions*

As a tumor suppressor, EAF2 may play a tumor suppressive and protective role in the development of CRC by inhibiting the invasion, metastasis and angiogenesis of CRC cells via regulating the signal transducer and activator of transcription 3/transforming growth factor beta 1 crosstalk pathway. It may provide new diagnostic markers and novel therapeutic targets for CRC.

### *Research perspectives*

Our data is beneficial to elucidate the molecular mechanism of CRC invasion and metastasis as well as explore new potential molecular markers and the direction of targeted therapy.

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Xing Liu, Zhu Chen, Gang Ouyang, Tieshan Song, Huageng Liang, Wei Liu, Wuhan Xiao. "ELL Protein-associated Factor 2 (EAF2) Inhibits Transforming Growth Factor  $\beta$  Signaling through a Direct Interaction with Smad3", Journal of Biological Chemistry, 2015  
Crossref

21 words — < 1%
- 5

Niannian Liu, Jian Shi, Xin Ge, Li Zhang, Sai Chen, Dongmei Zhang, Xu Miao. "Pyruvate Kinase M2 is Highly Expressed in Cutaneous Squamous Cell Carcinoma and Promotes its Progression", Research Square Platform LLC, 2021  
Crossref Posted Content

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- 6

Ting Yang, Wei-Cong Chen, Pei-Cong Shi, Man-Ru Liu, Tao Jiang, Hu Song, Jia-Qi Wang, Rui-Zhi Fan, Dong-Sheng Pei, Jun Song. "Long noncoding RNA MAPKAPK5-AS1 promotes colorectal cancer progression by cis-regulating the nearby gene MK5 and acting as a let-7f-1-3p sponge", Research Square Platform LLC, 2020

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["Sunday, 2 September 2007", European Heart Journal, 09/02/2007](#)  
Crossref

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[Minghui Ou, Xuezhi Sun, Jiancheng Liang, Fei Liu, Liqin Wang, Xiaomin Wu, Jie Tu. "A polysaccharide from Sargassum thunbergii inhibits angiogenesis via downregulating MMP-2 activity and VEGF/HIF-1 \$\alpha\$  signaling", International Journal of Biological Macromolecules, 2017](#)  
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Crossref

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