FRAS1-related extracellular matrix protein 2 drives the progression and poor prognosis of IL-1β-associated esophageal squamous cell carcinoma

CURRENT STATUS: UNDER REVIEW

Cell Communication & Signaling  ▪ BMC

Lin Wang
Zhongshan Hospital Fudan University

Jie Gu
Zhongshan Hospital Fudan University

Ronghua Liu
Fudan University School of Basic Medical Sciences

Wei Mao
Zhongshan Hospital Fudan University

Fengkai Xu
Zhongshan Hospital Fudan University

Zhonghe Liu
Zhongshan Hospital Fudan University

Huankai Shou
Zhongshan Hospital Fudan University

Qiaoliang Zhu
Zhongshan Hospital Fudan University

Chunlai Lu
Zhongshan Hospital Fudan University

Yiwei Chu
Fudan University School of Basic Medical Sciences

Qun Wang
Zhongshan Hospital Fudan University
Abstract

Background Chronic inflammation generates a tumor-supporting microenvironment, but it remains unclear whether and how the persistent inflammation drives genetic abnormalities leading to the occurrence and progression of esophageal squamous cell carcinoma (ESCC).

Methods By global RNA-sequncing, we screened genes related to ESCC with stimulation of the pro-inflammatory factor, interleukin (IL)-1β, and identified FRAS1-related extracellular matrix protein (FREM)2 as a oncogene. Flow cytometry was used to detect the expression of IL-1β and its receptor IL-1R1 in fresh ESCC specimens. The interaction between FREM2 and IL-1β signal was examined in vitro and in vivo. Levels of FREM2 and IL-1R1 were determined in ESCC tissue arrays derived from 299 patients, and their correlation with clinical outcomes was analyzed.

Results Multiple genes-related to ESCC occurrence and recurrence were elevated when exposed to the persistent stimulation of IL-1β. Among them, FRAS1-related extracellular matrix protein 2 (FREM2) was identified as a new oncogene in ESCC. IL-1β and its receptor IL-1R1 highly expressed in ESCC, especially in tumor cells. FREM2 was induced by IL-1β, and in turn bound to and stabilized IL-1R1, facilitating IL-1β signal transduction. The activation of downstream NK-κB and JNK signals mediated the tumor-promoting effect, while the reduction of FoxP1 was responsible for IL-1β-induced FREM2 transcription. High levels of FREM2 and IL-1R1 synergistically indicated the shorter survival time in patients.

Conclusion These results suggest FREM2 is an IL-1β-stimulated oncogene, which as a cofactor of IL1R1 promotes IL-1β-induced ESCC progression. Therapeutic
strategies targeting FREM2 is likely to prolong the survival of ESCC patients.

Background

Esophageal squamous cell carcinoma (ESCC) accounts for over 90% of esophageal cancer cases[1, 2]. Although previous studies have described complicated genetic and epigenetic alternations associated with ESCC progression[3], the underlying causes and mechanisms for these abnormalities have not been fully elucidated. There is increasing evidence reveal that factors leading to chronic irritation and inflammation of the esophagus, such as smoking, alcohol, and hot drinks and food, have been considered to initiate esophageal squamous cell dysplasia.[4–9]

Inflammation is acknowledged as one of important risks to induce ESCC.[10, 11] Regardless of its origin, inflammation in the tumor microenvironment has many tumor-promoting effects. The cellular effectors and mediators of inflammation are important constituents of the local environment of tumors. A growing number of reports indicate that chronic inflammation in some types of tumor is positively correlated with genetic alterations, such as DNA damage and gene mutations and amplification.[12]; [13–15] In other types of cancer, carcinogenic changes, such as activation of oncogenes or silencing of tumor suppressive genes, can in turn induce an inflammatory microenvironment or modulate inflammation-associated transcriptional programs, thus promoting tumors development. However, it is unclear which inflammatory factors and how to cause genetic abnormalities to initiate ESCC.

Interleukin (IL)-1β, a well-known pro-inflammatory cytokine, is abundant in many types of tumor. Upon IL-1β binding to its receptors (e.g., interleukin 1 receptor, type I [IL-1R1]), a cascade of pro-inflammatory gene expression is activated. Elevated
levels of IL-1β are correlated poor prognosis and shorter survival in patients with breast, lung, prostate, or pancreatic cancers as well as an increased risk of carcinogenesis.[16] This has been attributed to the induction of inflammatory responses, inhibition of the maturation and activation of tumor-infiltrating cells, or establishment of an immunosuppressive microenvironment.[17] However, there is little evidence that IL-1β directly causes genetic alterations in tumor.[18]

In the present study, we identified IL-1β and its receptor IL-1R1 was abundant in ESCC, especially in advanced patients. By screening for genetic alterations of ESCC, we identified multiple genes associated with IL-1β. Among them, FRAS1-related extracellular matrix protein (FREM)2 as a novel oncogene modulated and was modulated by IL-1β signal. IL-1β induced FREM2 overexpression in ESCC, and FREM2 enhanced IL-1β signal transduction by binding to and stabilizing IL-1R1. Both increased FREM2 and IL-1R1 were related to reduced survival time in ESCC patients, serving as prognostic biomarkers. Separating FREM2-IL1R1 complex may block IL-1β triggered ESCC initiation and progress, and be of promising in ESCC therapy.

Methods

Clinical specimens

Paraffin-embedded specimens (n = 299) were collected at Zhongshan Hospital, Fudan University (Shanghai, China) in 2007 with patient consent and with the approval of the local ethics committee. None of the patients received chemotherapy or radiotherapy before surgery. The specimens were selected solely based on the availability of complete clinicopathological and follow-up data for the patients. Overall survival was defined as the interval between surgery and death or between surgery and the last observation for surviving patients. Data were censored at the
last follow-up for living patients. An additional eight cases of paired frozen specimens and five cases of paired fresh ESCC samples were obtained from Zhongshan Hospital in 2017. Ethical approval was obtained from the Zhongshan Hospital Research Ethics Committee, and written informed consent was obtained from each patient. Tumor stage was determined according to seventh edition of the Union for International Cancer Control American Joint Committee on Cancer tumor-node-metastasis staging system.

**Cell lines**

The ECa109, TE1, ECa9706, and KYSE-150 cell lines were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Science (Shanghai, China), and the KYSE-510 cell line was purchased from National Institute of Biomedical Innovation (Osaka, Japan). The HEEpiC normal human esophageal epithelial cell (HEEC) line was obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). All cells were authenticated by short-tandem-repeat DNA profiling and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/l glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin sulfate.

**Flow cytometry**

Cells were labeled with fluorophore-conjugated antibodies according to manufacturer’s protocols. For intracellular labeling of IL-1β, cells were stimulated by incubation in RPMI 1640 medium containing 10% of FBS, 55 nM 2-mercaptoethanol (Life Technologies, Carlsbad, CA, USA), 0.5 mg/ml ionomycin (Peprotech, Rocky Hill, NJ, USA), 0.5 mg/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO, USA), and 2 mM monensin (eBioscience, San Diego, CA, USA).
at a concentration of $2 \times 10^6$ cells/2 ml/well for 5 h at 37 °C and 5% CO$_2$. The cells were then labeled for surface markers and cytokines using the Intracellular Fixation and Permeabilization Buffer Set (eBioscience) according to the manufacturer’s protocol.

**Tissue microarray and immunohistochemistry**

The procedures for tissue microarray analysis and immunohistochemistry are described in our previous study. Staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The percentage of stained cells was scored as 0 (0%), 1 (1–33%), 2 (34–66%), or 3 (67–100%). The sum of the intensity and percentage scores was used as the final staining score, which was defined as low (0–2) or high (3–6). The primary antibodies used are listed in Additional file 1.

**Supplementary Table 1.**

**CRISPR/Cas9-mediated depletion of FREM2 in ESCC cell lines**

All guide RNA were designed using the online optimized CRISPR design tool (http://crispr.mit.edu/) targeted the first exon of FREM2. Oligos synthesized were annealed and sub-cloned into Lentivirus vector PHY-701 (Hanyin Co. Shanghai, China) which was modified accoding to letiCRISPRv2(Addgene plasmid#52961).

Correct insertion was verified by sequencing sgRNA sequences #1 GTCCGGCGCGAGGATAACCC and #2 GGTACTAGACAACGACGCAC. The recombinant sgRNA expression lentivirus were prepared and titered to $5 \times 10^8$ TU/ml (transfection unit) by Hanyin Co. (Shanghai, China).ECa109 and ECa9706 cells grown to 50% confluence in 6-well plate were added sgRNA lentivirus and selected with puromycin (1 µg/ml) for 48 hours. Individual colonies were selected and genotyped by genomic DNA sequencing.
CRISPR/Cas9-mediated overexpression of FREM2 in ESCC cell lines

SgRNA oligos targeting FREM2 were synthesised and cloned into the vector U6-sgRNA-EF1A-dCAS9-SV40-p65-P2A-Puro by Hanyin Co. (Shanghai, China). U6-sgRNA-EF1A-dCAS9-SV40-p65-P2A-Puro or EF1A-MS2-N55K-SV40 NLS-P65-HSF1-T2A-Blasticidin-WPRE and lentiviral packages assisting plasmids psPAX2 and pMD2G were co-transfected into 293T cells, the viral supernatants were collected 72 h after transfection and titered to 10^8 TU/ml (transfection unit). Eca9706 and Eca109 cells were infected with the U6-sgRNA-EF1A-dCAS9-SV40-p65-P2A-Puro and EF1A-MS2-N55K-SV40 NLS-P65-HSF1-T2A-Blasticidin-WPRE lentivirus according to the manufacture’s instructions, and selected with puromycin (1 µg/ml) and blastcidin (10 mg/ml) for 48 hrs.

Cell proliferation assay

Cell proliferation was measured at different time points with Cell Counting Kit (CCK)-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s protocol. Briefly, cells were seeded in a 96-well plate at a density of 2 × 10^3 cells/well. CCK-8 solution was added to the wells at a final concentration of 10%, followed by incubation for 2 h before measurement of absorbance at 450 nm.

Cell migration assay

Cells were harvested and washed twice with phosphate-buffered saline (PBS), and 200 µl of cell suspension in serum-free medium (5 × 10^4 cells) were added to the upper compartment of transwell plates (8-µm pore size; Corning Inc., Corning, NY, USA). A 600-µl volume of medium containing 10% FBS was added to the lower chamber. After incubation for 24 h, cells that did not migrate through the pores were removed using cotton swabs, while those on the filter surface were fixed with
4% formaldehyde, stained with 0.4% crystal violet, and counted in five high-power microscope fields.

**Colony formation assay**

Cells (3 × 10^3) were seeded in a dish and cultured for 2 weeks, then washed three times with PBS and fixed for 20 min with 4% formaldehyde. After washing, the cells were stained with crystal violet in the dark for 20 min at room temperature. After two washes with tap water, the dishes were air-dried and photographed for colony counting.

**Mouse tumor xenograft model**

Nude mice (6–8 weeks old) were used for xenograft studies. Cells (5 × 10^6) were subcutaneously injected into the posterior bilateral flanks. The tumor surface area was measured at weekly intervals.

**Enzyme-linked immunosorbent assay (ELISA)**

Eight paired ESCC samples were homogenized in PBS, and IL-1β levels were measured using a commercial ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer’s instructions. Cytokine concentrations in the samples were calculated using standard curves based on known quantities of recombinant cytokines.

**RNA extraction and quantitative real-time (qRT-)PCR**

Total RNA was extracted from cells using TRizol reagent (Invitrogen, Carlsbad, CA, USA), and qRT-PCR was performed on an ABI 7500 thermocycler (Applied Biosystems, Foster City, CA, USA). Relative expression was normalized to that of β-actin. The primers used are listed in Additional file 2. Supplementary Table 2.

**Western blotting**
Esophageal tissue and whole cell extracts were analyzed by western blotting according to a standard protocol. The primary antibodies used are listed in Additional file 1. Supplementary Table 1.

Co-immunoprecipitation (co-IP)

The potential interaction between FREM2 and IL-1R1 was evaluated by co-IP. ECa109 and ECa9706 cells were washed twice with ice-cold PBS and then lysed with radioimmunoprecipitation assay lysis buffer. After removing insoluble material by centrifugation at 12,000 × g, the pre-cleared lysates were incubated overnight at 4 °C with primary antibody against FREM2 or IL-1R1 and pre-absorbed protein A-sepharose and G-sepharose beads (Pierce Biotechnology, Rockford, IL, USA). The precipitates were washed five times with lysis buffer, boiled in 5 × sodium dodecyl sulfate sample buffer for 5 min, and FREM2 and IL-1R1 were detected by western blotting.

Immunofluorescence analysis

EC109 and EC9706 cells were used to examine the subcellular localization of FREM2 and IL-1R1. Antibodies against FREM2 and IL-1R1 (both at 1:100 dilution) were used for immunodetection; nuclei were counterstained with 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). FREM2 and IL-1R1 localization was visualized by fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

Forkhead box (FOX)P1 overexpression

To construct the PCDNA3.1-FoxP1 plasmid, human FoxP1 (NM_001244814.1) was amplified by PCR and cloned between the BamHI and HindIII sites of the PCDNA3.1-MCS2 vector. Correct insertion of the fragment was confirmed by DNA sequencing. Primers used to detect PCDNA3.1-FoxP1 clones are used as follows: Forward (5’-3’):
CCGGGATCCGCCACCATGATGCAAGAATCTGGGACTGAGA; Reverse (5’-3’):
GCCCAAGCTTTCACTCCATGTCCTCGTTTACTGG. The plasmid was transfected into
ECa109 and ECa9706 cells using Lipofectamine 2000 (Invitrogen) according to the
manufacturer’s protocol.

Dual luciferase reporter assay

To construct the PGL3-basic-FREM2 promoter plasmid, fragments of the human
FREM2 (NM_207361.5) promoter including the putative FOXP1 binding sequence
were amplified by PCR and cloned between the XhoI and HindIII sites of the pGL3-
basic vector upstream of the luciferase reporter gene. Correct insertion of the
fragment was confirmed by DNA sequencing. Primers used to detect PGL3-basic-
FREM2 clones are used as follows: Forward (5’-3’):
CCGCTCGAGCTCAGTGCTGCTCTGAAATCGATG; Reverse (5’-3’):
GCCCAAGCTTTGTTCCGGCTGGAGGT. The dual luciferase reporter assay was
performed using a commercial kit (Promega, Madison, WI, USA) according to the
manufacturer’s protocol.

Chromatin (Ch)IP-quantitative PCR

ChIP was performed using an anti-FOXP1 antibody according to a standard protocol.
Independent chromatin immunoprecipitates were used for qRT-PCR; the primers are
used as follows: Forward (5’-3’): GTTAGGTAAGCTACTAGGTACTAGG; Reverse (5’-3’):
CACAGACTAAGATTAAGCAGTACAGAG.

Public database

We obtained RNA sequencing (RNA-seq) data from The Cancer Genome Atlas (TCGA;
released before May 15, 2015); this included data for 13 normal and 87 ESCC
samples. Reads per kilobase of exon model per million mapped reads values were
used as a measure of mRNA expression levels.

Statistical analysis

Data are presented as mean ± SEM of at least three experiments. Data were analyzed using SPSS v.22.0 (SPSS Inc., Chicago, IL, USA) and PRISM v.5.0 (GraphPad Inc., La Jolla, CA, USA) software. The Student’s unpaired t test or unpaired t test with Welch’s correction was used to analyze differences between two groups, and analysis of variance was used for comparisons between more than two groups. Pearson’s correlation coefficient was used to evaluate correlations between groups. Cumulative survival time was calculated by the Kaplan-Meier method and was analyzed with the log-rank test. Uni- and multivariate analyses were based on the Cox proportional hazards regression model. P < 0.05 was considered statistically significant.

Results

FREM2 is an IL-1β-stimulated gene and associated with ESCC occurrence and recurrence

We compared differentially expressed genes between ESCC tumor and corresponding non-tumor tissues by RNA-seq, and further screened ESCC-recurrence associated genes that were response to IL-1β stimulation. We identified 3111 differently expressed candidate genes in ESCC. Compared with our previous RNA-seq data for recurrent vs. non-recurrent cases[19], 34 genes were found to be upregulated and 20 were downregulated in the ESCC tumor and recurrence groups relative to the non-tissue and non-recurrent groups respectively (Fig. 1A, Additional file 3. Supplementary Fig. 1A and Additional file 4. Supplementary Table 3). We searched the 34 upregulated genes in TCGA database and confirmed that 16 protein
coding genes were significantly upregulated (Fig. 1A and Additional file 3. Supplementary Fig. 1B).

To determine the relationship between IL-1β and these genes, we stimulated cultured ECa109 ESCC cells with IL-1β; as a result, seven genes (fibroblast growth factor [FGF]19, melanoma antigen family member [MAGE]A12, FREM2, N-terminal EF-hand calcium-binding protein 2, Keratin 77, IQ motif containing H and membrane palmitoylated protein 4) were upregulated (Fig. 1B). FREM2 was selected for further study because it was dramatically upregulated and there is little known about this gene in the context of ESCC. Results showed that IL-1β stimulation increased FREM2 protein level and the percentage of FREM2 + ESCC cells (Fig. 1C). Consistent with these observations, FREM2 expression was found to be increased in ESCC as compared to matched normal tissues no matter in mRNA level (Additional file 3. Supplementary Fig. 1C) or in protein level (Fig. 1D, Additional file 3. Supplementary Fig. 1D). As well as, FREM2 was overexpressed in five ESCC cell lines relative to normal HEECs (Fig. 1E). The flow cytometry analysis demonstrated that the percentage of FREM2+ cells was higher in tumor as compared to adjacent normal tissues (Fig. 1F). These results suggest that FREM2 has an oncogenic role in ESCC.

IL-1β and its receptor IL-1R1 are abounded in ESCC and mainly expressed by tumor cells.

An analysis of TCGA data revealed that IL-1β level was elevated in ESCC as compared to normal tissues (Additional file 5. Supplementary Fig. 2A), which was further confirmed by our results of ELISA (Additional file 5. Supplementary Fig. 2B) and western blotting (Additional file 5. Supplementary Fig. 2C). A flow cytometry analysis revealed that IL-1β expression was higher in cluster of differentiation
(CD)45$^+$ and CD45$^-$ cells of tumors (Fig. 2A), and was higher in epithelial cell
adhesion molecule (EPCAM)$^+$ epithelial cells than in CD45$^+$ lymph cells (Fig. 2B).
These results indicated that IL-1β expression was elevated in ESCC tumor tissues,
and that tumor cells, rather than lymphocytes, is the major source of IL-1β.
Since IL-1β exerts its effect through binding to IL-1R1, we evaluated IL-1R1
expression by western blotting and flow cytometry. Consistent with our observations
for IL-1β, IL-1R1 was overexpressed in ESCC tissue and was more highly expressed
in EPCAM$^+$ epithelial cells than in CD45$^+$ lymph cells (Fig. 2C-D). To determine the
role of IL-1β in tumorigenesis, we stimulated ECa109 ESCC cells with IL-1β and
evaluated cell proliferation and cell migration. Results showed that IL-1β stimulation
enhanced tumor cell proliferation as well as cell migration (Fig. 2E). These results
suggest that IL-1β enriched in ESCC tissues plays a tumor-promoting role, which
may be in both autocrine- and paracrine- manners.
FREM2 as a new oncogene is required for IL-1β-induced ESCC cell growth and
migration
To determine the biological function of FREM2 and its relationship with IL-1β-related
ESCC development, we constructed FREM2 knockout as well as overexpressed
ECa109 and ECa9706 cell lines using the clustered regularly interspaced short
palindromic repeats (CRISPR)/CRISPR-associated (Cas)9 system (Additional file 6.
Supplementary Fig. 3A-E). Functional analysis showed that loss of FREM2
suppressed ESCC cell proliferation and colony formation as well as migration
(Fig. 3A-C). Additionally, FREM2 knockout induced the apoptosis of ESCC cells
(Additional file 7. Supplementary Fig. 4A), but it had no effect on the cell cycle
(Additional file 7. Supplementary Fig. 4B). The effect of FREM2 knockout on ESCC
tumor growth was further examined in a xenograft model. Compared to the control group, tumor growth was markedly inhibited in the FREM2 knockout group (Fig. 3D). We also stably overexpressed FREM2 in ECa109 and ECa9706 cell lines (Fig. 3E), and confirmed that FREM2 overexpression increased cell proliferation and xenograft tumor growth (Fig. 3F-H). These results indicate that FREM2 functions as an oncogene in ESCC.

To further clarify the relationship between FREM2 and IL-1β, ESCC cells lacking FREM2 were stimulated with IL-1β. We found that ESCC cell proliferation and migration were both reduced under these conditions relative to control cells (Fig. 4A-B). The nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK)-c-Jun N-terminal kinase (JNK) pathways were activated by overexpressing FREM2, but inactivated after deleting FREM2 (Additional file 8. Supplementary Fig. 5 and Fig. 4C). Furthermore, proliferation and migration were decreased in ESCC cells treated with NF-κB/p65 and MAPK/JNK inhibitors (Fig. 4D-E). These results indicate that FREM2 promotes the activation of signaling pathways downstream of IL-1β (e.g., NF-κB and MAPK), thereby potentiating IL-1β-induced ESCC cell proliferation and migration.

**FREM2 enhances IL-1 signal transduction by binding to and stabilizing IL-1R1**

Furthermore, we investigated the mechanism by which FREM2 promotes IL-1β signal and subsequent ESCC progression. Results showed that FREM2 + epithelial cells had consistently higher expression of IL-1R1 in ESCC tissues (Fig. 5A), and cell lines (Fig. 5B). Moreover, the increase in FREM2 expression induced by IL-1β stimulation (Fig. 1C) was accompanied by upregulation of IL-1R1 (Additional file 9.
Supplementary Fig. 6A-C). A scatterplot of FREM2 and IL-1R1 protein levels in ESCC tissues revealed a significant positive correlation \( (r = 0.7154, P = 0.046; \text{Fig. } 5C) \).

To examine the correlation between FREM2 and IL-1R1 in ESCC cells in greater detail, we performed co-IP using an anti-FREM2 or -IL-1R1 antibody. In reciprocal co-IP assays, the FREM2 antibody pulled down IL-1R1 and vice versa (Fig. 5D). These results suggest that endogenous FREM2 forms a complex with IL-1R1. An immunofluorescence analysis revealed that FREM2 and IL-1R1 were colocalized in ESCC cells. Meanwhile, the fluorescence intensity of the two proteins increased synchronously in ECa109 and ECa9706 cell lines following stimulation with 1.5 nM IL-1\( \beta \) (Fig. 5E, Additional file 9. Supplementary Fig. 6D). Upon IL-1\( \beta \) stimulation, the protein level of IL-1R1 in FREM2-overexpressed cells increased markedly and stayed for a longer time (Fig. 5F, Additional file 9. Supplementary Fig. 6E). These results indicate that FREM2 and IL-1R1 directly interact to promote ESCC progression upon activation of IL-1\( \beta \) signaling.

High levels of FREM2 and IL-1R1 correlate with shorter survival in patients

To assess the clinical relevance of our findings, we carried out immunohistochemical analysis using tissue arrays of 299 ESCC patients (Table 1). Elevated expression of FREM2 and IL-1R1 was observed in 154 (51.5%) and 160 (53.5%) patients. Upregulation of FREM2 was significantly correlated with advanced T stage \( (P = 0.002) \), lymph node metastasis \( (P = 0.041) \), and advanced clinical stage \( (P = 0.045) \). High IL-1R1 expression level was related to T stage \( (P = 0.022) \). It is worth noting that high expression of FREM2 was related to upregulation of IL-1R1 \( (P = 0.026) \). FREM2 and IL-1R1 immunopositivity was almost exclusively confined to the tumor
cells and was observed in the cell membrane and cytoplasm (Fig. 5G); the signal intensity was higher in ESCC than in non-tumor tissues. We divided the 299 patients into three groups: those with high expression of FREM2 and IL-1R1 (FREM2\textsuperscript{high}/IL-1R1\textsuperscript{high}); high expression of either FREM2 or IL-1R1; and low expression of FREM2 and IL-1R1 (FREM2\textsuperscript{low}/IL-1R1\textsuperscript{low}). Results showed that elevated expression of both FREM2 and IL-1R1 was correlated with T stage (P < 0.001), lymph node metastasis (P = 0.023), and clinical stage (P = 0.018).

Table 1
The correlation between FREM2/IL-1R1 and clinicopathological characteristics in 299 esophageal squamous cell carcinomas.

| Characteristics | FREM2 expression | IL-1R1 expression | Combined expression | P     |
|-----------------|------------------|-------------------|---------------------|-------|
|                 | Low | High | Low | High | Low/Low | Low/High | High/High |       |
| Gender          | 231 | 68   | 112 | 33   | 119/35  | 127/33   | 0.995     | 0.018 |
| Age ≤60        | 156 | 143  | 79  | 66   | 77/67   | 84/76    | 0.904     | 0.888 |
| Smoking status  | 136 | 163  | 64  | 81   | 72/82   | 66/94    | 0.115     | 0.621 |
| Complications   | 242 | 57   | 119 | 26   | 123/31  | 132/28   | 0.460     | 0.984 |
| Differentiation | 182 | 117  | 94  | 51   | 88/66   | 91/69    | 0.129     | 0.158 |
| T stage         | 117 | 182  | 70  | 75   | 47/107  | 53/107   | 0.022     | < 0.001|
| LN metastasis   | 178 | 121  | 95  | 50   | 83/71   | 89/71    | 0.140     | 0.023 |
| Tumor stage     | 189 | 110  | 100 | 45   | 89/65   | 95/65    | 0.140     | 0.018 |
| FREM2/IL-1R1 expression | 145 | 154  | 77  | 68   | 62/92   | 62/92    | 0.026     | < 0.001|
A univariate analysis revealed that T stage (P < 0.001), lymph node metastasis (P < 0.001), tumor stage (P < 0.001), and combined expression of FREM2 and IL-1R1 (P = 0.013) were significantly associated with OS. The 5-year OS rate of FREM2\textsuperscript{low} patients was higher than that of FREM2\textsuperscript{high} patients (49.8% vs. 40.7%, P = 0.037). In addition, the IL-1R1\textsuperscript{low} group had a higher 5-year OS rate than the IL-1R1\textsuperscript{high} group (51.7% vs. 37.9%, P = 0.014). The 5-year OS rate in the FREM2\textsuperscript{high}/IL-1R1\textsuperscript{high} group was only 35.0%, which was lower than the rates in the FREM2\textsuperscript{low}/IL-1R1\textsuperscript{low} group (56.8%) and the group with either high FREM2 or high IL-1R1 expression (51.6%) (P = 0.011). A multivariate Cox proportional hazards model indicated that high expression levels of both FREM2 and IL-1R1 are independent predictors of OS (P = 0.042) (Table 2).

| Characteristic | Univariate analysis | Multivariate analysis |
|---------------|---------------------|----------------------|
|               | HR                  | 95% CI               | P        | HR                | 95% CI               | P        |
| Gender (Male vs. Female) | 1.378               | 0.937–2.054          | 0.102   |                   |                      |          |
| Age (year) (≤ 60 vs. >60) | 0.816               | 0.598–1.114          | 0.200   |                   |                      |          |
| Smoking status (Non-smokers vs. Smokers) | 0.822               | 0.600–1.126          | 0.221   |                   |                      |          |
| Complications (No vs. Yes) | 0.977               | 0.656–1.453          | 0.907   |                   |                      |          |
| Differentiation (Well/ Moderate vs. Poor) | 0.780               | 0.569–1.068          | 0.121   |                   |                      |          |
| T stage (III-IV vs. I-II) | 2.066               | 1.466–2.907          | <0.001  | 1.994              | 1.324–3.003          | 0.001   |
| LN metastasis (Yes vs. No) | 2.924               | 2.128–4.000          | <0.001  | 6.497              | 3.395–12.434         | <0.001  |
| Tumor stage (III-IV vs. 0-II) | 2.326               | 1.698–3.185          | <0.001  | 3.003              | 1.499–6.024          | 0.002   |
| Combined expression of FREM2 and IL-1R1 High/Low vs. Low/Low High/High vs. Low/Low | 1.503 | 0.993–2.275 | 0.013 | 1.186 | 0.775–1.814 | 0.096 | 0.432 | 0.042 |

Abbreviations and Notes: OS, overall survival; 95%CI, 95% confidence interval; Multivariate analysis, COX proportional hazards regression model. Characteristics were adopted for their prognostic significance by univariate analysis with forward stepwise selection (Forward; likelihood rat
The decrease of FOXP1 is responsible for the overexpression of FREM2 induced by IL-1β.

To investigate the mechanism by which IL-1β induces FREM2 upregulation, we searched for transcription factors that regulate FREM2 expression (Additional file 10. Supplementary Table 4). Since FOXP1 is the major transcriptional regulator of FREM2, we performed a dual luciferase reporter assay using cells co-transfected with FREM2 promoter reporter and FOXP1 plasmids. As expected, FOXP1 repressed luciferase activity, implying a direct interaction between FOXP1 and FREM2 promoter in ESCC cells (Fig. 6A). FOXP1 level was decreased relative to that in normal ESCC tissue (Fig. 6B, C). This inverse association between FREM2 and FOXP1 strongly suggests that FOXP1 negatively regulates FREM2 transcription.

The results of the ChIP assay confirmed that the direct interaction between FOXP1 and the FREM2 promoter was abrogated in the presence of IL-1β (Fig. 6D).

Furthermore, luciferase activity was decreased in cells transfected with FOXP1 overexpression or control plasmid under IL-1β stimulation (Fig. 6E). FOXP1 overexpression caused a reduction in FREM2 protein level, which was partly reversed in the presence of IL-1β (Fig. 6F-G). These data indicate that IL-1β regulates FREM2 expression via FOXP1 in ESCC cells (Fig. 6H).

Discussion

Chronic inflammation contributes to tumor initiation and development. However, little is known about the interaction between inflammatory factors and oncogenes in ESCC. In the present study we identified FREM2 as a novel oncogene in ESCC that is upregulated by IL-1β and whose protein product forms a complex with IL-1R1. Increased expression of FREM2 enhanced the activation of IL-1β downstream
signaling by stabilizing IL-1R1. We also showed that the regulation of FREM2 expression by IL-1β was FOXP1-dependent. Both FREM2 and IL-1R1 were prognostic factors for poor OS.

IL-1β is known to exert tumorigenic effects in various malignancies. [20]; [21] A previous study reports that IL-1β is overexpressed in ESCC specimens and correlated with advanced clinical stage of ESCC.[18] Our results confirm these observations but also demonstrate that IL-1β exerts its tumorigenic effects through regulation of an oncogene. Consistently, IL-1β stimulation leads to upregulation of genes involved in breast cancer cell survival [22] This study identified seven genes that were upregulated in ESCC tissues and are closely related to IL-1β. Among them, FGF19 belongs to the FGF protein family whose members have mitogenic activity and are involved in tumor growth and invasion.[23-25] MAGEA12 is a member of the melanoma antigen protein family and associated with advanced tumor stage and reduced survival time[26, 27] Significantly, Ours is the first report to identify FREM2 as an novel oncogene in ESCC.

FREM2 is a member of Fras1/Frem family of ECM proteins that are involved in epithelial-mesenchymal cohesion during embryonic development.[28] Mutations in FREM2 have been linked to Fraser syndrome. Although FREM2 has been implicated in gliosarcoma and ovarian carcinoma,[29, 30] its biological function is largely unknown. We found here that FREM2 was overexpressed in malignant ESCC, which was significantly associated with lymph node metastasis, advanced clinical stage, and worse overall survival. FREM2 can serve as a prognostic biomarker in ESCC. Moreover, our results reveal the new relationship between FREM2 and IL-1 signal. Toll-like and IL-1 receptor regulator, a splice variant of FREM1, was shown to associate with IL-1R1 to amplify IL-1-induced activation of NF-κB and inflammation-
related genes,[31] while FREM2 mediated the formation of a ternary complex that included FREM1 and FRAS1.[32] In our study, we found that FREM2 and IL-1R1 were upregulated and formed a complex to stabilize IL-1 signal transduction and ESCC progression.

FOXP1 is a member of the P subfamily of FOX transcription factors.[33] Although FOXP1 has been linked to different types of cancer,[34] there have been no reports to date about its role in ESCC. The results of our study also support a tumor suppressor role for FOXP1 since it was downregulated in ESCC tissue. Moreover, FOXP1 overexpression partly abrogated IL-1β-induced transcriptional activation of FREM2. FOXP1 transcriptional activity has been reported to be suppressed in various epithelial malignancies either through downregulation of the mRNA or protein level or by aberrant localization in the cytoplasm.[35] We observed that nuclear expression of FOXP1 was significantly reduced under stimulation with IL-1β, consistently with these earlier findings.

In this study, IL-1β expression was higher in epithelium-derived tumor cells than in CD45⁺ lymphocytes in ESCC tissues. We speculate that this is not only due to increased lymphocyte infiltration but also because IL-1β production was enhanced, resulting in genetic alterations in tumor cells.

Conclusion

Clarifying the interaction between IL-1β and oncogenes can lead to improved strategies for ESCC prevention and treatment. In particular, our results suggest that as a novel oncogene that amplifies IL-1β signaling, FREM2 is a promising new target for blocking inflammation-associated ESCC progression.
abbreviations
ChIP, Chromatin Immunoprecipitation;
Co-IP, Co-Immunoprecipitation;
ESCC, esophageal squamous cell carcinoma;
FOXP1, forkhead box P1;
FREM2, FRAS1 related extracellular matrix protein 2;
IL-1β, interleukin 1 beta;
IL-1R1, interleukin-1 receptor type 1;
MAPK, mitogen-activated protein kinase;
NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells;
RPKM, Reads Per Kilobase per Million mapped reads;
TCGA, The Cancer Genome Atlas

declarations

**Ethics approval and consent to participate** This study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University, Shanghai, China. Written informed consent was obtained from every patient participating in this research. All the animal experiments were performed according to the protocol of the Fudan Committee on Animal Care and in strict accordance with the Institutional Animal Care guidelines.

**Consent for publication** Not applicable

**Availability of data and materials** The datasets generated and/or analysed during the current study are available in the Gene Expression Omnibus (GEO) under accession code GSE119436 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=
Competing interests The authors declare that they have no competing interests.

Funding: This paper was supported by National Nature Science Foundation of China 81572295 to Z.L., 81872291 to D.G., and 81601362 to R.L, as well as Shanghai Municipal Commission of Health and Family Planning 20184Y0089 and 2018YQ16 to R.L. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions: L.W. and J.G performed the research, discussed and analyzed the data, and wrote the paper. Z.L., R.L and D.G. designed the research, discussed and analyzed the data, and wrote the paper. F.X., W.M., Z.L., Q.Z. and H.S. performed the research and developed the methods. C.L. Q.W. and Y.C. revised the paper and provided materials.

All authors read and approved the final manuscript.

Acknowledgements: Thanks to The Cancer Genome Atlas Database, to all of the patients who contributed to this study, and to the support of the National Science Foundation of China and Shanghai Municipal Commission of Health and Family Planning.

References

1. W Chen, R Zheng, PD Baade, S Zhang, H Zeng, F Bray, et al: Cancer statistics in China, 2015. CA Cancer J Clin 2016, 66:115-32.

2. LA Torre, F Bray, RL Siegel, J Ferlay, J Lortet-Tieulent, A Jemal: Global cancer statistics, 2012. CA Cancer J Clin 2015, 65:87-108.

3. Y Song, L Li, Y Ou, Z Gao, E Li, X Li, et al: Identification of genomic alterations
in oesophageal squamous cell cancer. *Nature* 2014, 509:91-5.

4. X Castellsague, N Munoz, E De Stefani, CG Victora, R Castelletto, PA Rolon, et al: Independent and joint effects of tobacco smoking and alcohol drinking on the risk of esophageal cancer in men and women. *Int J Cancer* 1999, 82:657-64.

5. Y Lin, Y Totsuka, Y He, S Kikuchi, Y Qiao, J Ueda, et al: Epidemiology of esophageal cancer in Japan and China. *J Epidemiol* 2013, 23:233-42.

6. WR Tang, ZJ Chen, K Lin, M Su, WW Au: Development of esophageal cancer in Chaoshan region, China: association with environmental, genetic and cultural factors. *Int J Hyg Environ Health* 2015, 218:12-8.

7. Y Chen, Y Tong, C Yang, Y Gan, H Sun, H Bi, et al: Consumption of hot beverages and foods and the risk of esophageal cancer: a meta-analysis of observational studies. *Bmc Cancer* 2015, 15:449.

8. R Lin, C Zhang, J Zheng, D Tian, Z Lei, D Chen, et al: Chronic inflammation-associated genomic instability paves the way for human esophageal carcinogenesis. *Oncotarget* 2016, 7:24564-71.

9. XL Wei, FH Wang, DS Zhang, MZ Qiu, C Ren, Y Jin, et al: A novel inflammation-based prognostic score in esophageal squamous cell carcinoma: the C-reactive protein/albumin ratio. *Bmc Cancer* 2015, 15:350.

10. F Balkwill, A Mantovani: Inflammation and cancer: back to Virchow? *Lancet* 2001, 357:539-45.

11. D Hanahan, RA Weinberg: The hallmarks of cancer. *Cell* 2000, 100:57-70.

12. X Liu, M Zhang, S Ying, C Zhang, R Lin, J Zheng, et al: Genetic Alterations in Esophageal Tissues From Squamous Dysplasia to Carcinoma. *Gastroenterology* 2017, 153:166-177.

13. G Landskron, M De la Fuente, P Thuwajit, C Thuwajit, MA Hermoso: Chronic
inflammation and cytokines in the tumor microenvironment. *J Immunol Res* 2014, 2014:149185.

14. D Hanahan, RA Weinberg: Hallmarks of cancer: the next generation. *Cell* 2011, 144:646-74.

15. J Massague, AC Obenauf: Metastatic colonization by circulating tumour cells. *Nature* 2016, 529:298-306.

16. TG Shrihari: Dual role of inflammatory mediators in cancer. *Ecancermedicalscience* 2017, 11:721.

17. JV Fernandes, RN Cobucci, CA Jatoba, TA Fernandes, JW de Azevedo, JM de Araujo: The role of the mediators of inflammation in cancer development. *Pathol Oncol Res* 2015, 21:527-34.

18. MF Chen, MS Lu, PT Chen, WC Chen, PY Lin, KD Lee: Role of interleukin 1 beta in esophageal squamous cell carcinoma. *J Mol Med (Berl)* 2012, 90:89-100.

19. ZW Lin, J Gu, RH Liu, XM Liu, FK Xu, GY Zhao, et al: Genome-wide screening and co-expression network analysis identify recurrence-specific biomarkers of esophageal squamous cell carcinoma. *Tumour Biol* 2014, 35:10959-68.

20. AM Jimenez-Garduno, MG Mendoza-Rodriguez, D Urrutia-Cabrera, MC Dominguez-Robles, EA Perez-Yepez, JT Ayala-Sumuano, et al: IL-1beta induced methylation of the estrogen receptor ERalpha gene correlates with EMT and chemoresistance in breast cancer cells. *Biochem Biophys Res Commun* 2017, 490:780-785.

21. J Ma, J Liu, Z Wang, X Gu, Y Fan, W Zhang, et al: NF-kappaB-dependent microRNA-425 upregulation promotes gastric cancer cell growth by targeting PTEN upon IL-1beta induction. *Mol Cancer* 2014, 13:40.

22. M Mendoza-Rodriguez, RH Arevalo, EM Fuentes-Panana, JT Ayala-Sumuano, I
Meza: IL-1beta induces up-regulation of BIRC3, a gene involved in chemoresistance to doxorubicin in breast cancer cells. *Cancer Lett* 2017, 390:39-44.

23. KH Tiong, BS Tan, HL Choo, FF Chung, LW Hii, SH Tan, et al: Fibroblast growth factor receptor 4 (FGFR4) and fibroblast growth factor 19 (FGF19) autocrine enhance breast cancer cells survival. *Oncotarget* 2016, 7:57633-57650.

24. S Wang, D Zhao, R Tian, H Shi, X Chen, W Liu, et al: FGF19 Contributes to Tumor Progression in Gastric Cancer by Promoting Migration and Invasion. *Oncol Res* 2016, 23:197-203.

25. Y Teng, H Zhao, L Gao, W Zhang, AY Shull, C Shay: FGF19 Protects Hepatocellular Carcinoma Cells against Endoplasmic Reticulum Stress via Activation of FGFR4-GSK3beta-Nrf2 Signaling. *Cancer Res* 2017, 77:6215-6225.

26. T Yanagi, K Nagai, H Shimizu, SI Matsuzawa: Melanoma antigen A12 regulates cell cycle via tumor suppressor p21 expression. *Oncotarget* 2017, 8:68448-68459.

27. J Wu, J Wang, W Shen: Identification of MAGEA12 as a prognostic outlier gene in gastric cancers. *Neoplasma* 2017, 64:238-243.

28. E Pavlakis, R Chiotaki, G Chalepakis: The role of Fras1/Frem proteins in the structure and function of basement membrane. *Int J Biochem Cell Biol* 2011, 43:487-95.

29. M Nagaishi, YH Kim, M Mittelbronn, F Giangaspero, W Paulus, B Brokinkel, et al: Amplification of the STOML3, FREM2, and LHFP genes is associated with mesenchymal differentiation in gliosarcoma. *Am J Pathol* 2012, 180:1816-23.

30. H Haslene-Hox, E Oveland, K Woie, HB Salvesen, H Wiig, O Tenstad: Increased WD-repeat containing protein 1 in interstitial fluid from ovarian carcinomas
shown by comparative proteomic analysis of malignant and healthy
gynecological tissue. Biochim Biophys Acta 2013, 1834:2347-59.

31. X Zhang, F Shephard, HB Kim, IR Palmer, S McHarg, GJ Fowler, et al: TILRR, a
novel IL-1RI co-receptor, potentiates MyD88 recruitment to control Ras-
dependent amplification of NF-kappaB. J Biol Chem 2010, 285:7222-32.

32. D Kiyozumi, N Sugimoto, K Sekiguchi: Breakdown of the reciprocal stabilization
of QBRICK/Frem1, Fras1, and Frem2 at the basement membrane provokes
Fraser syndrome-like defects. Proc Natl Acad Sci U S A 2006, 103:11981-6.

33. HB Koon, GC Ippolito, AH Banham, PW Tucker: FOXP1: a potential therapeutic
target in cancer. Expert Opin Ther Targets 2007, 11:955-65.

34. M Katoh, M Igarashi, H Fukuda, H Nakagama, M Katoh: Cancer genetics and
genomics of human FOX family genes. Cancer Lett 2013, 328:198-206.

35. AH Banham, N Beasley, E Campo, PL Fernandez, C Fidler, K Gatter, et al: The
FOXP1 winged helix transcription factor is a novel candidate tumor suppressor
gene on chromosome 3p. Cancer Res 2001, 61:8820-9.

Figures

Figure 1

FREM2 is an IL-1β-stimulated gene and associated with ESCC occurrence and recu
IL-1β and its receptor IL-1R1 abundant in ESCC tissues are mainly expressed by tumor cells and promote the cell growth and migration. (A) Percentages of IL-1β-expressing cells among CD45+ and CD45− cells from normal or tumor tissues, as detected by flow cytometry. (B) IL-1β expression in epithelial cells (EPCAM+ CD45−), immune cells (EPCAM− CD45+), and other cells (EPCAM− CD45−), as detected by flow cytometry. (E) Proliferation and migration of ECa109 cells treated with 1.5 nM IL-1β or left untreated, as determined with the CCK-8 and transwell assays, respectively. Data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

FREM2 functions as an oncogene in ESCC. (A) Western blot analysis of FREM2 protein level in siCtrl- and ovFR2, FREM2 overexpression. Data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

FREM2 is required for IL-1β-induced ESCC cell proliferation and migration. Cell proliferation (A) and migration (B) of siCtrl and siFR2 cells treated with 1.5 nM IL-1β; cells without IL-1β stimulation served as the control. (C) Protein levels of P65, phosphorylated (p-)P65, JNK, and p-JNK, in siCtrl and siFR2 cells with or without 1.5 nM IL-1β stimulation, as determined by western blotting. Cell proliferation (D) and migration (E) of cells stimulated with 1.5 nM IL-1β and treated with JNK inhibitor (SP600125, 50 μM) or NF-κB inhibitor (BAY11-7082, 10 μM); unstimulated cells served as the control. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 5

FREM2 interacts with IL-1R1 to enhance IL-1 signal transduction, and predicts poor prognosis in ESCC patients. The co-localization of FREM2 (red) and IL-1R1 (green) was increased after 1.5 nM IL-1β stimulation in ECa109 cell lines. (F) IL-1R1 expression in ovCtrl and ovFR2 cells of ECa109 cell lines treated with 1.5 nM IL-1β, as detected by flow cytometry. (G) Photomicrographs of normal and tumor tissues from patients with ESCC. Overall survival rates were assessed with Kaplan-Meier survival estimates and the log-rank test. *P < 0.05, **P < 0.01, ***P < 0.001.
**Figure 6**

FOXP1 is a transcriptional inhibitor mediating the overexpression of FREM2 induced by IL-1β.

**Supplementary Files**

This is a list of supplementary files associated with the primary manuscript. Click to download.
