Identification of secretory factors associated with suppressive tumor microenvironment in esophageal cancer

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Abstract

Esophageal squamous cell carcinoma (ESCC) is one of the deadliest solid malignancies and has a poor survival rate worldwide. Suppressive tumor microenvironment is the main cause to promote tumor development, metastasis and poor survival time. Tumor-derived secretory factors could connect tumor tissues and components in the tumor microenvironment to promote tumor progression. Here, in our study, we analyzed multiple individual transcriptome databases and found a group of secretory factors derived from tumor tissues that have correlation with infiltrated immune cells in tumor microenvironment. These secretory factors are differently expressed in esophageal tumor tissues compared with adjacent normal tissues. Among them, MFAP2 as the most significant gene was positively correlated with a serial of suppressive infiltrated immune cells, higher pathological stage and poorer overall survival time. Furthermore, we got the consistent results when we collected tissue samples from patients with ESCC and detected the expression of MFAP2 by immunohistochemistry, immunofluorescence and qRT-PCR methods. Our comprehensive analyses deciphered the prognostic, immunological, and therapeutic value of MFAP2 in esophageal cancer management, thus providing a target for individual and precise therapy for combating esophageal cancer.

Introduction

Esophageal cancer (EC) is one of the most common cancers of the digestive system[1]. Esophageal squamous cell carcinoma (ESCC), the prevalent histologic subtype of EC in China is associated with a high degree of malignancy and poor prognosis[2]. In China, the morbidity and mortality rates of EC are ~30 times that of the United States, accounting for the third most common type of malignancy[1, 2]. Despite recent advances in diagnostics and therapeutics, the prognosis for esophageal cancer remains poor: the 5-year survival rate is approximately 15–25%[3]. The poor prognosis of EC is largely due to its high local recurrence and metastatic rates. The delayed diagnosis of metastasis shortens the optimal treatment period. Therefore, it is important to identify the molecular factors involving metastasis of EC to aid in the development of new treatment strategies for EC patients and lead to markedly improved clinical outcomes.

It has been widely acknowledged that pathological stage is significantly related with tumor microenvironment (TME)[4–6]. TME is not an inert component, it includes extracellular matrix (ECM), signaling molecules, immune and stromal cells, and adjacent non-tumorous tissue, contributes to cancer pathogenesis[7, 8]. Tumor tissues can actively interact with TME through secreting proteins, like growth factors, glycoproteins, inflammatory cytokines, enzymes and exosomes, which can act on the immune cells in the tumor microenvironment to facilitate tumor development[9–11]. Tumor-derived secretory factors exhibit a prompt role in the mechanism of tumor metastasis through cross-talk between tumor tissues and the extracellular matrix components, infiltrated immune cells in TME. This cross-talk could induce angiogenesis and inflammatory cell recruitment, ultimately favors the tumor development and metastasis[12, 13]. Hence, it is essential to explore the secretory proteins derived from tumor tissues that could mediate tumor development. To date, there are increasing studies on the regulation of TME.
mediated by secretory proteins from tumor tissues[14–18]. However, related studies in esophageal cancer are rarely reported.

Public RNA-seq datasets are a rich resource for elucidating the mechanisms of human diseases. Combining multiple individual datasets in one analysis will increase the statistical power and makes it possible to gain additional insight on underlying biological mechanisms[19–21]. Here, in order to identify the suppressive tumor microenvironment associated-secretory proteins from esophageal tumor tissues, we downloaded transcriptome data from different research team in public GEO datasets and followed by various bioinformatic analyses.

In our study, we found a group of secretory proteins that are differently expressed in esophageal tumor tissues compared with adjacent normal tissues. Among them, MFAP2 as the most significant gene was positively correlated with a serial of suppressive infiltrated immune cells, higher pathological stage and poorer overall survival time. Importantly, MFAP2 expression was validated in six pairs of tumor tissue samples by immunofluorescence, immunohistochemistry, and qRT-PCR. Our comprehensive analyses deciphered the prognostic, immunological, and therapeutic value of MFAP2 in esophageal management, thus providing a target for individual and precise therapy for combating esophageal cancer.

Materials And Methods

Public data collection

Publicly available gene-expression profiles were used to in our studies were downloaded from GEO (https://www.ncbi.nlm.nih.gov/gds) and TCGA datasets. GSE164158, GSE149612, GSE75241 were downloaded from GEO datasets. MFAP2 gene expression data and associated immune cells infiltrates in pan-cancer were downloaded from TIMER2 website (http://timer.cistrome.org/). MFAP2 gene expression and related pathological stage, overall survival time data are collected from TCGA (https://xenabrowser.net/datapages/).

Calculation of Differentially Expressed Genes

The limma R package was used to generate \( p \)-value/\( FDR \) and FoldChange (FC) for each gene between tumor tissues and adjacent normal tissues. This package takes the normalized expression matrix and the corresponding metadata as inputs. The package fits the negative binomial model to the dataset and perform Wald’s Test to calculate the \( p \)-value/\( FDR \) or significance that a gene is differentially expressed. Genes with an \( FDR < 0.05 \) and FoldChange > 2 were identified as differentially expressed genes (Table.S1).

Gene Oncology (GO) analysis

Using the R package, clusterProfiler (version 3.0.4)[22], gene ontology (GO) analysis was performed on the dataset. GO analysis varies from GSEA as it utilizes a different annotation set and accounts for gene length bias in detection of over/ under representation of genes. With the hg37 annotation set, we
performed enrichment analysis on our set of differentially expressed genes. We utilized log2(FC) and the FDR to determine significant genes for this analysis. Then we determined which GO terms were over or under-represented and visualized the data. We selected the significant, over-represented terms based on FDR < 0.05.

Gene Set Analysis

Gene set enrichment analysis (GSEA) was performed in the R environment using the fgsea package based on the raw gene expression matrices. This software utilizes Log2(FC) and gene identities to determine pathways and their level of expression. The software then used the resulting pathways and the Log2(FC) to perform its analysis. The difference between this type of analysis is that it looks at genes in an entire set instead of individual genes. The GSEA works by first calculating an enrichment score (ES) that represents the amount a gene is overrepresented. Next, a \( p \)-value is determined by permutating the genes in the set. The pathways were organized based on \( p \)-value and NES score, and we selected significant pathways based on these criteria. \( p \)-value under 0.05 represents statistical significance.

Tumor Tissue Collection

Six pairs of esophageal tumor tissues and adjacent non-tumorous tissue samples were collected from the First Affiliated Hospital of Nanjing Medical University. The clinical specimens were stored immediately in liquid nitrogen and kept at ~ 80 °C for further study. The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University and conducted following the Declaration of Helsinki.

Immunofluorescence and immunohistochemistry

For immunofluorescence and immunohistochemical staining, tissue samples were first made into 10 µm paraffin sections and then dehydrated. The slices were repaired with EDTA antigen retrieval solution, and then blocked with 3% BSA blocking solution. Sections were then incubated overnight at 4°C with anti-MFAP2 antibody (sc-166075, Santa Cruz) working solution. For immunofluorescent staining, an appropriate amount of fluorescent secondary antibody (Invitrogen) was added on the tissue, and incubated at room temperature for 1 hour. After counterstaining the sections with DAPI working solution, antifade mountant (Invitrogen) was added and covered the slide. For immunohistochemical staining, dropped the HRP secondary antibody on the slide, and incubated at room temperature for 30 minutes. Added DAB chromogenic solution to the slide, then counterstain the cell nuclei with hematoxylin, finally dehydrated and sealed the slice. Confocal microscopy study was performed on the Nikon C2 Plus confocal microscope (Nikon Corp, Japan).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).

Total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen, USA) and reverse-transcribed into cDNA using HiScript III RT SuperMix for qPCR (Vazyme, China). qRT-PCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme, China). The relative MFAP2 expression was normalized to GAPDH by the \( 2^{\Delta\Delta Ct} \) method. The sequences of the primer used in this study were listed in Table S3.
Statement

All expression data were downloaded from public datasets and all methods were performed in this manuscript in accordance with the relevant guidelines and regulations.

Results

Collection of gene expression data for esophageal cancer in GEO datasets

We collected gene expression data from Gene Expression Omnibus (GEO) using the key words “esophageal cancer” plus “expression profile” or “transcriptome”. Datasets were selected when includes gene expression matrix. GSE164158, GSE149612, and GSE75241 were selected for downstream analysis. Figure 1 showed the summarized workflow of our study. Firstly, we used these three datasets to calculate differentially expressed genes (DEGs) in esophageal tumor tissues. Then, we conducted gene oncology and gene set enrichment analysis (GSEA) based on DEGs to see any tumor microenvironment associated-pathways changed significantly. Last, we explored whether there are any secretory proteins in DEGs and investigated their correlation with pathological stage and overall survival time in online public datasets.

Identification of differentially expressed genes in esophageal cancer

In order to identify the suppressive tumor microenvironment associated-secretory proteins in esophageal cancer, we first explored the gene expression profiles in esophageal cancer and adjacent normal tissues. We used limma package in Rstudio to calculate the statistically significant DEGs in all three datasets. For each dataset, we got many DEGs (Fig. 2A, B and C, Table.S1). In order to enhance the statistical power, we tried to find any overlapped DEGs in these three datasets. 384 overlapped DEGs were identified, including 134 upregulated genes and 250 downregulated genes (Fig. 2D and Table.S1). Normalized expression data from these 384 DEGs were used to perform unsupervised hierarchical clustering which showed a clear separation between tumor tissues and adjacent normal tissues in respective datasets (Fig. 2E, F and G).

Differentially expressed genes mainly involved in extracellular matrix (ECM) organization in tumor tissue

To further explored these DEGs’ function, we did gene oncology (GO) analysis which could perform enrichment analysis on different gene sets. As Fig. 3A showed, the most significant biological process was extracellular matrix organization. Moreover, gene set enrichment analysis also showed extracellular matrix related function was over-presented in tumor tissues compared with adjacent normal tissues (Fig. 3B and C). While, pathway activity associated with metabolism of xenobiotics by cytochrome P450
was decreased in tumor tissue (Fig. 3D). These results indicate these DEGs mainly involved in extracellular matrix organization in tumor tissues. Given to the role of extracellular matrix (ECM) for immune cells in the tumor microenvironment[23], these results remind us that some of these DEGs may also affect infiltrated immune cells in the tumor microenvironment.

**MFAP2 gene positively regulated suppressive tumor microenvironment**

To find the direct factors that affect infiltrated immune cells, we explored all DEGs to see whether there are secretory proteins that could secrete into tumor microenvironment from tumor tissues. We screened 384 DEGs with known secreted proteins from The Human Protein Atlas website (https://www.proteinatlas.org/). We found there are nearly 17% (67/384) DEGs are secretory proteins (Table.S2). Given to the key role of myeloid-derived suppressor cells (MDSCs) in infiltrated suppressive immune cells, we used MDSCs as a representative to evaluate the effect of these 67 secretory DEGs on infiltrated immune cells. In Table.S2 & Fig. 4A & B, we found the up-regulated gene MFAP2 in esophageal cancer showed the most significant positive correlation with MDSCs. Moreover, we also found MFAP2 gene showed positive with other suppressive infiltrated immune cells, like cancer associated fibroblast (CAF) (Fig. 4C), neutrophils (Fig. 4D), macrophage M0 (Fig. 4E), and stromal cells (Fig. 4F). Meanwhile, its expression showed a negative correlation with CD8⁺ T cells (Fig. 4G), which is a type of tumor-suppressing immune cell. All of these results suggested MFAP2 acts as an oncogene to facilitate the development of tumor through recruiting suppressive infiltrated immune cells and deactivating cytotoxic T cells in esophageal cancers.

**MFAP2 gene induced higher pathological stage and poorer overall survival in esophageal cancer**

In order to investigate whether the up-regulation of MFAP2 will involve in the metastatic process in esophageal cancer, we evaluated the correlation of MFAP2 expression and tumor pathological stages in esophageal cancer. We found MFAP2 gene positively correlated with higher pathological stage (Fig. 5A). Moreover, we found its increased expression will promote tumor proliferation (Fig. 5B). While, it has no correlation with metastatic lymph nodes (Fig. 5C). Furthermore, we collected metadata from esophageal cancer in TCGA and compared the MFAP2 gene expression and overall survival time. As Fig. 5D showed, higher MFAP2 expression associated with a shorter overall survival time. All these data strongly suggest the MFAP2 gene could induce a higher pathological stage and poorer overall survival in esophageal cancer.

**MFAP2 gene as a general oncogene in pan-cancer**

To further validate MFAP2 expression and its role in the TME of other tumors, we analyzed MFAP2 expression and its correlation with assorted immune cell infiltrates in pan-cancer using several algorithms
including CIBERSORT, XCELL, EPIC, QUANTISEQ and TIDE. Firstly, we found MFAP2 was significantly increased in most of tumor tissues when compared with adjacent normal tissues (Fig. 6A), not only in esophageal cancer. Then we saw a strongly positive correlation between MFAP2 expression and cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs), macrophages in most of tumor tissues (Fig. 6B). Meanwhile, its expression was negatively correlated with CD8 + T cells (include naïve, central memory, and effector memory) in various cancer types (Fig. 6C). These data indicated secretory protein MFAP2 acts as a general oncogene in pan-cancer that could induce a suppressive tumor microenvironment and lead to tumor metastasis and development.

**Significant upregulation of MFAP2 in the esophageal cancer tissues**

In order to validate the results from bioinformatic assays, we collected esophageal tumor and adjacent normal tissues to detect the expression of MFAP2. The results from immunofluorescent staining showed that the expression of MFAP2 protein in esophageal tumor tissues was significantly up-regulated compared with adjacent tissues, and much of them were observed in the extracellular matrix (Fig. 7A). Immunohistochemical staining also showed the same result (Fig. 7B). Furthermore, we extracted mRNA from tumor tissue for qRT-PCR purpose, and the results showed that the MFAP2 gene mRNA expression in esophageal tumor tissues was about 23-times higher than that in the adjacent normal tissues (Fig. 7C). These data indicated the expression of MFAP2 was significantly upregulated in the esophageal tumor tissues.

**Discussion**

Esophageal squamous cell carcinoma (ESCC) is the main malignant pathological subtype of EC and contributes to a large proportion of morbidity and mortality in China. Although the incidence of ESCC has decreased because of improvements in dietary habits in high-risk areas in recent years, patients still exhibit poor prognosis with limited therapeutic targets[24]. Analysis of public esophageal cancer transcriptome profile revealed that MFAP2 as a secretory protein is overexpressed in esophageal tumor tissues and is associated with suppressive tumor microenvironment, higher pathological stage, and poorer overall survival time. Furthermore, we found MFAP2 actually acts as a general oncogene in various cancer type which could as an indicator for prognostic purpose.

Recent evidence already found that some of secretory proteins from tumor tissues could induce tumor development and metastasis[25]. Targeting these secretory proteins may give some insight for development more effective therapy. Here, we identified a serial of secretory proteins associated with suppressive tumor microenvironment in esophageal cancer. Among them, MFAP2 gene as the most significant gene correlated with various infiltrated immunosuppressive cells. Our strategy verified the possibility that identify the candidate prognostic marker or therapeutic target in tumor microenvironment in different cancer types. Similar workflow could be used in other cancer types to find the general factor that drive the suppressive tumor microenvironment.
In the tumor microenvironment (TME), immunosuppressive players (T regulatory cells, myeloid-derived suppressor cells, and cancer associated fibroblasts) are recruited by the tumor-derived factors to support tumor growth[26, 27]. Members of the transforming growth factor beta (TGF-β) superfamily are known to be the main inducers of cancer-associated fibroblasts (CAFs) activation[28]. Likewise, activin A has showed the ability to induce a secretory phenotype in CAFs via the SMAD-2-mediated transcriptional regulation of genes encoding extracellular matrix (ECM) components and ECM regulators[29]. Here, we found MFAP2 gene, also as a secretory protein played a significant role in regulating suppressive infiltrated immune cells. Further studies on underlying molecular mechanism that MFAP2 gene on infiltrated immune cells should be conducted in various cancer types.

MFAP2 (microfibril associated protein 2) is an ECM matrix protein that plays a vital role in elastin production, microfibril assembly, matrix remodeling and energy metabolism[30]. Recently, aberrant expression of MFAP2 has been reported in several cancer types: stomach adenocarcinoma, gastric cancer, colon adenocarcinoma and hepatocellular carcinoma. Most of these researches focused on its upregulation in tumor tissues and their association with poor prognosis[31–34]. Here, we established the connection between MFAP2 gene upregulation and poor prognosis via suppressive tumor microenvironment. We found MFAP2 was positively correlated with various infiltrated immune cells: myeloid-derived suppressor cells (MDSCs), cancer associated fibroblasts (CAFs), neutrophils, and negatively regulated CD8+ T cells. All of these will induce a suppressive tumor microenvironment around the tumor tissues to support tumor development and metastasis. We speculate that MFAP2 protein plays a tumor-promoting role in the tumor microenvironment by interacting with other secretory proteins, such as TGF-β[35], to promote the proliferation of immunosuppressive cells. The detailed regulatory mechanism needs to be further explored.

In summary, we found a group of secretory proteins that are differently expressed in esophageal tumor tissues compared with adjacent normal tissues. Among them, MFAP2 as the most significant gene was positively correlated with a serial of suppressive infiltrated immune cells, higher pathological stage and poorer overall survival time. Our comprehensive analyses deciphered the prognostic, immunological, and therapeutic value of MFAP2 in esophageal management, thus providing a target for individual and precise therapy for combating esophageal cancer.

**Declarations**

**Ethical Approval**

The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University and conducted following the Declaration of Helsinki.

**Competing interests**

Authors declare no conflict of interest.
Author contributions

LS, JL and HC designed the article structure. JL, DW were responsible for collecting data, writing the manuscript. YY, ZL contributed to data analysis and statistical methods. HC, ZZ and LS contributed to data interpretation and manuscript revision. JL, DW, LZ contributed to manuscript revision. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

Public datasets can be downloaded from GEO website according to each accession number available in the paper.

References


Figures
Figure 1

Schematic workflow of study

Flowchart depicting the approach to identify the secretory factors associated with suppressive tumor microenvironment in esophageal cancer
Figure 2

Identification of differentially expressed genes in esophageal cancer

(A, B, C) Volcano plot of differentially expressed genes between esophageal tumor tissues and adjacent normal tissues. Fold Change $> 2$ & $FDR < 0.05$ were set as screening criteria. Genes that have both a significant $FDR$ (lower than 0.05) and a fold change (higher than 2) are represented as red dot. Genes that
either have a significant $FDR$ (lower than 0.05) or a fold change (higher than 2) are represented as blue and green dot. Gray dot means genes neither have a significant $FDR$ nor fold change.

(D) Venn diagram depicting the overlapped differentially expressed genes between three different GEO datasets.

(E, F, G) Unsupervised hierarchical clustering heatmap showed all differentially expressed genes in three different GEO datasets in esophageal cancer.
Figure 3

Differentially expressed genes mainly involved in extracellular matrix (ECM) organization in tumor tissue

(A) Gene oncology analysis for all differentially expressed genes. y-axis means different biological process; x-axis represents gene count involved in each biological process.
(B) Different active pathways in tumor tissues and adjacent normal tissues based on GSEA analysis.

(C) Extracellular matrix (ECM)-receptor interaction showed a higher activity in tumor tissues (NES=-2.25, FDR=8.25e-09).

(D) Metabolism of xenobiotics by cytochrome P450 showed a higher activity in adjacent normal tissues (NES=2.04, FDR=3.7e-04).

Figure 4

Association between MFAP2 and suppressive tumor microenvironment in esophageal cancer.

(A) Relative mRNA expression of MFAP2 in esophageal tumor tissues and adjacent normal tissues. Red rectangle represents tumor tissue; Grey rectangle means normal tissue. *, p<0.05.

Correlation between MFAP2 gene expression and infiltrated immune cells in tumor microenvironment: MDSCs (B), CAFs (C), Neutrophils (D), M0 Macrophage (E), Stromal cells (F), CD8+ T cells (G). Correlation coefficient Rho and p-value were labeled at the upright corner.
**Figure 5**

MFAP2 gene induced higher pathological stage and poorer overall survival.

(A) Upregulation of MFAP2 gene in higher pathological stage in esophageal tumor tissues. **, p<0.01.

(B) Increased expression of MFAP2 gene in higher primary tumor stages in esophageal tumor tissues. **, p<0.01.

(C) Expression profile of MFAP2 at different stage of metastatic lymph nodes.
(D) Correlation between MFAP2 expression and overall survival time in esophageal tumor tissues. x-axis means overall survival time. y-axis represents survival probability. Different color means the expression level of MFAP2.

Figure 6
Association between MFAP2 and suppressive tumor environment in pan-cancer. (A) Overview of MFAP2 gene expression in various cancer type. Red rectangle means tumor tissue, blue represent adjacent normal tissue.

(B) Correlations of MFAP2 gene expression with cancer associated fibroblast, myeloid-derived suppressor cells, CD8+ T cell, macrophage.

(C) Correlations of MFAP2 gene expression with CD8+ T cells (naïve, central memory, and effector memory).
Figure 7

Upregulation of MFAP2 in the esophageal cancer tissues.

(A) Immunofluorescent staining of MFAP2 protein expression in esophageal carcinoma (ESCC) and adjacent normal tissues (ANT). DAPI was dyed with blue and MFAP2 were labeled with red. Scale bars, 50μm.
(B) Immunohistochemical staining of MFAP2 expression in ESCC and ANT. Scale bars, 50μm.

(C) MFAP2 mRNA expression in ESCC and ANT by qRT-PCR. ***, p<0.001.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Table.S1DifferentiallyexpressedgenesfromthreeGEOdatasets.xlsx
- Table.S2SecretoryproteinsinDEGs.xlsx
- Table.S3ThesequencesofqRTPCRprimers.docx