

Received: 2021.01.23
Accepted: 2021.02.21
Available online: 2021.03.10
Published: 2021.07.02

Exploration of the Important Role of Microfibril-Associated Protein 4 Gene in Oral Squamous Cell Carcinoma

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Source of support: Departmental sources

Background: Oral squamous cell carcinoma (OSCC) is a common tumor of the head and neck. Its treatment usually requires multiple modalities. Currently, there are no molecular biomarkers to guide these treatment strategies. Studies have shown that microfibril-associated protein 4 (MFAP4) is potentially useful for non-invasive assessment of various diseases; however, its biological function in tumors is still unknown. In this study, we propose that MFAP4 is a new prognostic target for OSCC.

Material/Methods: First, we collected OSCC data (GSE25099 and GSE30784 datasets) from the Gene Expression Omnibus (GEO) database and compared the differential expression of *MFAP4* gene between the patients (tumor) and normal (control) groups. The comparison was done with University of California Santa Cruz Xena (<https://xenabrowser.net/Datapages/>), and we calculated the difference in *MFAP4* gene expression between normal and tumor tissues in a pan-cancer analysis. Then, we compared the 2 groups with high and low expression of *MFAP4* gene in terms of tumor mutation burden (TMB), miRNA regulation, and immune cell infiltration.





Results: We found that the expression of *MFAP4* gene was significantly decreased in tumors. Our research also showed that high expression of *MFAP4* was related to better prognosis of patients and may be related to tumor gene mutation, miRNA regulation, and infiltration of different immune cells.

Conclusions: Our work provides evidence that expression of *MFAP4* can be used as a prognostic biomarker for risk stratification of OSCC patients and elaborates on its relation with the regulation of TMB, miRNAs, and immune cell infiltration.

Keywords: **MFAP4 Protein, Human • Mouth Neoplasms • Prognosis**

Abbreviations: **TCGA** – The Cancer Atlas Genome; **GEO** – Gene Expression Omnibus; **MFAP4** – microfibril-associated protein 4; **Tem** – effector memory T cell; **NK** – natural killer; **GSEA** – gene set enrichment analysis; **GO** – gene ontology; **KEGG** – Kyoto Encyclopedia of Genes and Genomes; **TMB** – tumor mutation burden; **OSCC** – oral squamous cell carcinoma

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/931238>

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Background

Oral squamous cell carcinoma (OSCC) is one of the most common tumors of the head and neck. There are more than 300 000 new confirmed cases worldwide each year [1,2]. It is estimated that OSCC accounts for about 3% of all malignant tumors, and more than 13 000 patients with OSCC die in the United States each year [3]. The histological grade of OSCC can range from highly differentiated keratinizing carcinoma to undifferentiated non-keratinizing carcinoma with high metastatic tendency. Risk factors for OSCC include smoking, drinking, viral infections (eg, Epstein-Barr virus, human papillomavirus, and herpes simplex virus), chewing betel nuts, occupational exposure to carcinogens, immune deficiency, radiation, diet, and genetic susceptibility [4,5]. The treatment of OSCC is generally multimodal, including surgery followed by chemoradiotherapy (CRT). The epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab in combination with radiation can be used in HPV-negative OSCC where comorbidities prevent the use of cytotoxic chemotherapy. Immune checkpoint inhibitors such as pembrolizumab and nivolumab also play an important role in the treatment of OSCC and can be used for treatment of recurrent or metastatic OSCC and pembrolizumab is used as a primary treatment for unresectable tumors [6]. Currently, there are no molecular biomarkers to guide these treatment strategies.

Microfibril-associated protein 4 (MFAP4) is an extracellular matrix protein belonging to the fibrinogen-related domain family. This family includes several proteins involved in tissue homeostasis and innate immunity, such as fibrinogen C domain-containing protein 1 (FIBCD1), fibrin, and angiogenin [7-9]. *MFAP4* was initially identified as a gene that is commonly missing in patients with Smith-Magenis syndrome [10]. Studies have shown that *MFAP4* can be useful for non-invasive assessment of various diseases, such as chronic obstructive pulmonary disease [11,12], liver cirrhosis [13,14], diabetic neuropathy [15], and cardiovascular complications [16]. However, the biological function of *MFAP4* in tumors is still unknown. It is worth noting that a recent study reported that high expression of *MFAP4* mRNA was significantly associated with decreased overall survival of patients with neuroblastoma. However, the prognostic value of *MFAP4* expression in OSCC patients remains unclear.

In this study, we propose *MFAP4* as a new prognostic target for OSCC. We also studied its relationship with the regulation of tumor mutation burden (TMB), miRNAs, and immune cell infiltration.

Material and Methods

Differences in Expression of MFAP4

We used the GEOquery package to download 2 oral cancer datasets – GSE25099 and GSE30784 – from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo>). The GSE25099 dataset included 57 tumor samples and 22 normal samples, and the GSE30784 dataset included 17 samples of dysplasia, 45 normal samples, and 167 tumor samples. We compared the differences in *MFAP4* expression between normal and tumor samples. In addition, we downloaded the transcripts per kilobase million values from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression databases, which were uniformly processed with the Toil process of the University of California Santa Cruz (UCSC) Xena tool (<https://xenabrowser.net/datapages/>), and we compared the differences between groups (Figure 1). A Wilcoxon test was used to compare the difference between *MFAP4* expression in the tumor and normal groups, and the ggplot2 package was used for drawing graphs.

Survival analysis

We directly downloaded the head and neck squamous cell carcinoma (HNSC) head and neck cancer expression matrix (HTSeq-Counts and HTSeq-FPKM files) and clinical information from UCSC Xena (<https://xenabrowser.net/datapages/>). After we eliminated non-oral cancer and incomplete OS samples, 330 samples were included in the study as the experimental group. At the same time, we downloaded 76 samples of oral cancer patients from the GSE41613 dataset as a verification group. GEOquery package was used for data download. We performed survival analysis on the 2 groups of samples (Figure 2) to assess the relationship between difference in survival and expression of *MFAP4* between normal and tumor samples. Finally, we used tableone package to compare the differences between clinical phenotypes of TCGA after grouping (Supplementary Table 1). Survival and survminer packages were used for best separation grouping and survival analysis.

Difference Analysis and Enrichment Analysis

We divided the 330 samples from the TCGA dataset into 2 groups based on high or low expression of *MFAP4* (high: 253, low: 77). Then, we performed gene set enrichment analysis (GSEA), gene ontology (GO) enrichment analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. EdgeR package was used for differential analysis, and clusterProfiler package was used for enrichment analysis.

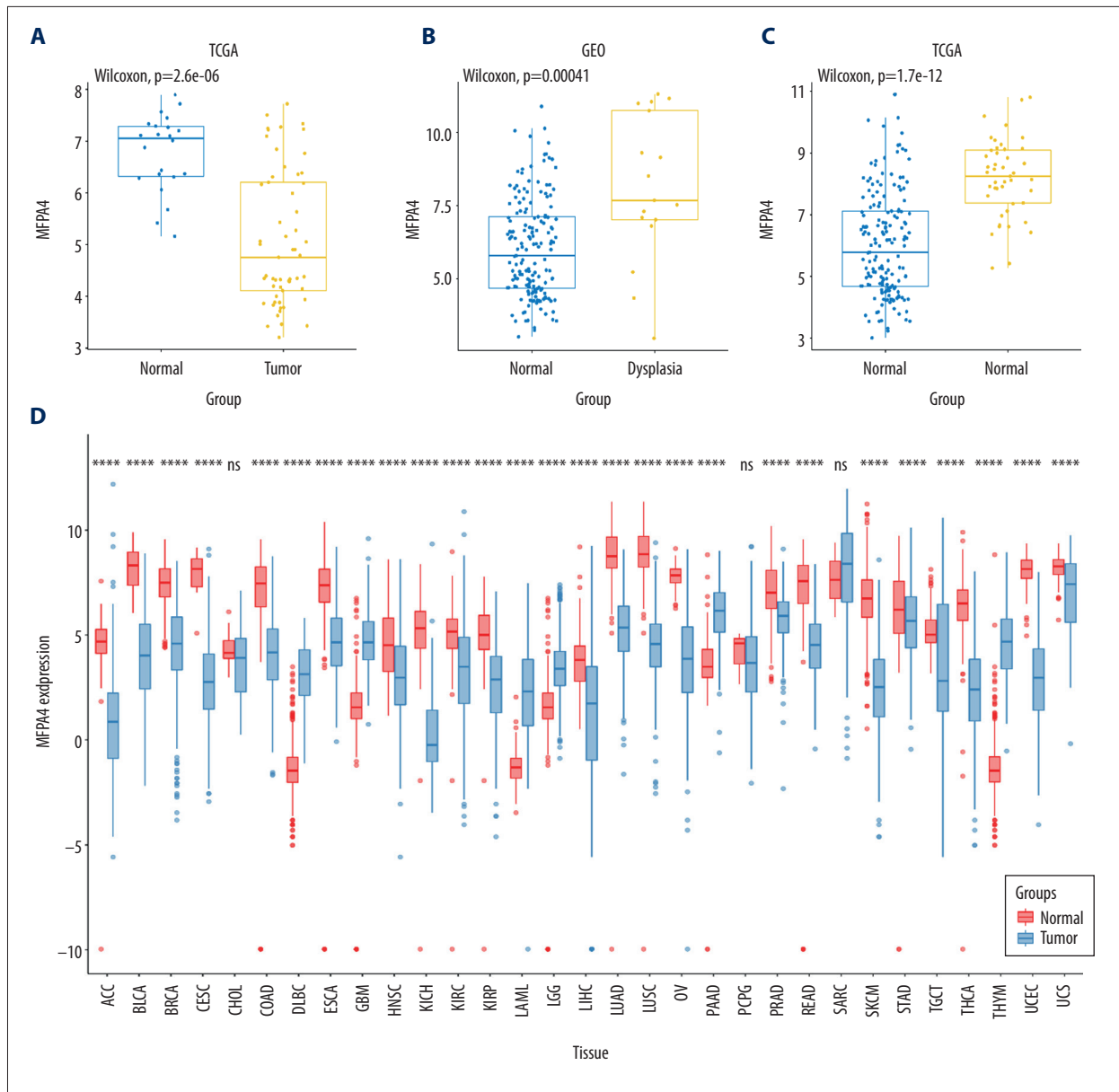


Figure 1. Expression of *MFAP4* in tumors. The GEO and TCGA datasets showed that the expression of *MFAP4* gene in tumors was lower than that in normal samples (A-C). Pan-cancer analysis showed that *MFAP4* gene had significantly lower expression in multiple tumors (D).

Relationship Between *MFAP4* Expression and TMB

TMB is becoming a new predictive biomarker for selecting patients who will benefit from immune checkpoint inhibitor therapy [17-19]. It is usually defined as the total number of somatically encoded mutations, and it is related to the emergence of new antigens that trigger anti-tumor immunity [20-22]. We downloaded the Mutation Annotation Format file for storing somatic variant information of HNSC patients from the TCGA database, and we screened tumor mutation data from 330 OSCC samples. We compared the mutation data of the high

and low *MFAP4* expression groups and mapped the mutation data of the top 6 genes. The highly mutated genes in the high *MFAP4* expression group were screened, and the first 5 mutated genes were compared and mapped. Maftools package was used to analyze mutation data [23].

Relationship Between *MFAP4* Expression and MicroRNA

We used the TCGAbiolinks package to download the miRNA expression profiles of OSCC samples and divided them into 2 groups (high *MFAP4* expression: 251, low *MFAP4*

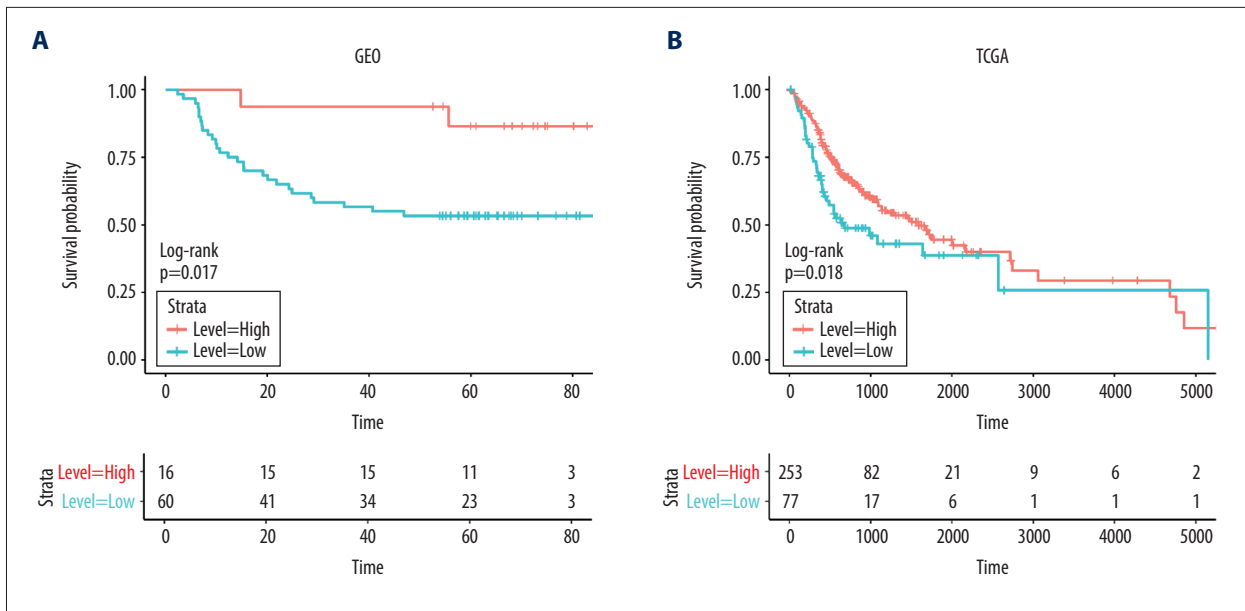


Figure 2. (A, B) The prognostic relationship between *MFAP4* and OSCC. In the TCGA and GEO datasets, high expression of *MFAP4* was related to better prognosis of patients.

expression: 75). Then, edgeR package was used for differential analysis to identify miRNAs that were related to *MFAP4*, and to analyze the correlation between them.

Relationship Between *MFAP4* and Immune Cells

Single-sample GSEA (ssGSEA) is a method for quantifying infiltrating immune cells [24]. We analyzed data on immune cell infiltration of OSCC samples in TCGA and GEO databases, based on the most commonly used immune cell marker genes [25]. Differences in immune cell infiltration between the low- and high-expression groups were identified. Gene Set Variation Analysis package was used to quantify 24 types of immune cells. We compared the differences in quantified immune cells between the 2 groups and selected different immune cells in TCGA and GEO databases, including eosinophils, macrophages, mast cells, natural killer (NK) cells, T cells, T helper cells, and effector memory T (Tem) cells.

Correlation of *MFAP4* and Immune Checkpoints

Immune checkpoints were reported in previous research, and we evaluated the relationship between *MFAP4* and immune checkpoints to assess the importance of *MFAP4*. The relationship was evaluated with Pearson correlation.

Ethics Statement

The data of this study are from GEO and TCGA datasets, and did not involve animal experiments or human specimens. There were no ethics-related issues.

Results

MFAP4 Expression Was Low in Tumor Samples

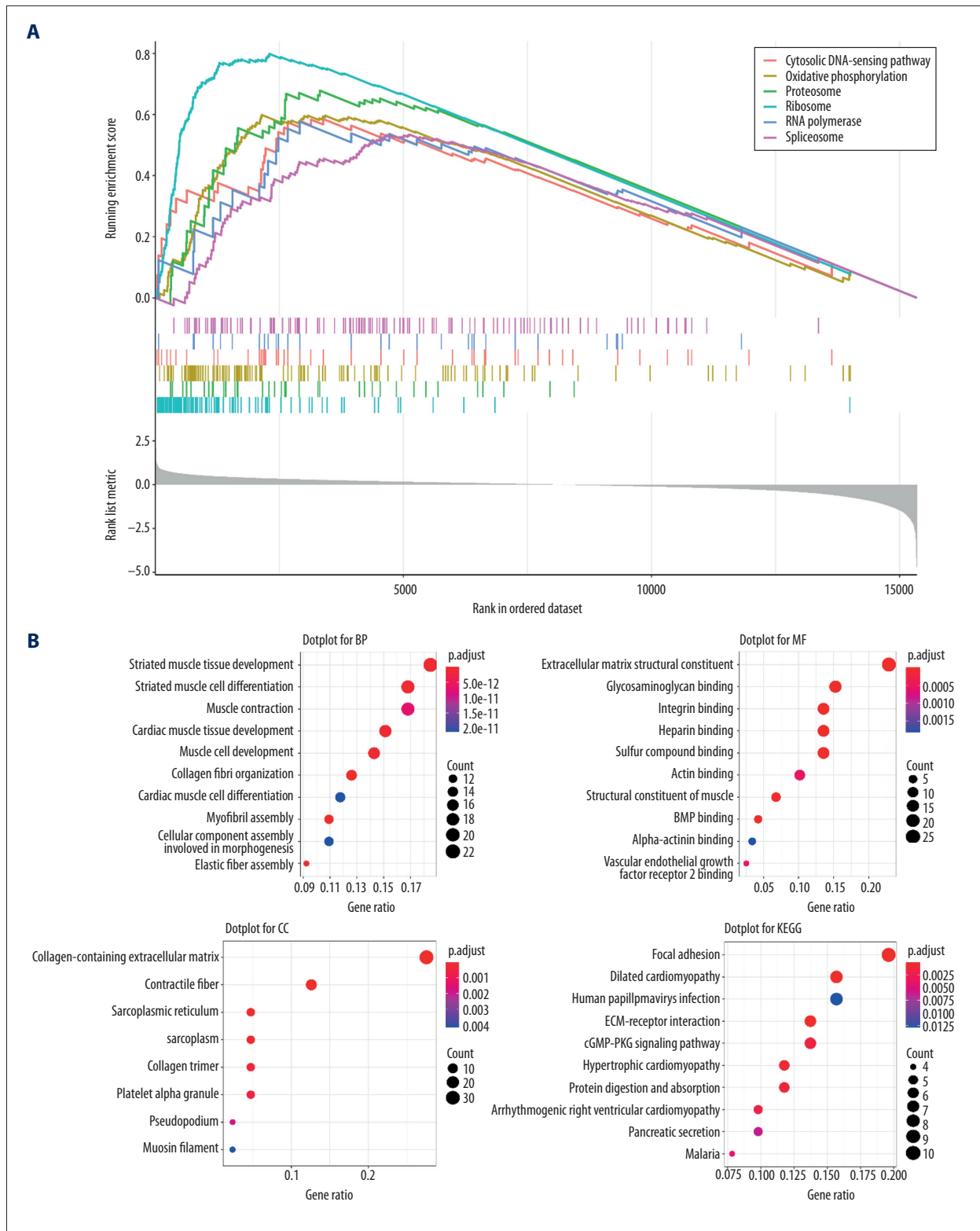
We performed Wilcoxon testing to assess the differential expression of *MFAP4* between tumor samples and normal samples in the GSE25099 and GSE30784 datasets. We found that the expression of *MFAP4* was lower in tumor samples, and box plots were drawn to show the difference. TCGA network researchers described the molecular characteristics of tumors in 11 160 patients with 33 cancer types and identified many molecular subtypes. In these cancers, we calculated the difference in expression of *MFAP4* between tumor and normal samples, and the results are shown in **Figure 1**.

Analysis of Relationship Between survival and *MFAP4* Expression

In addition to the 330 samples included in the study as the experimental group, we downloaded 76 samples of oral cancer patients from the GSE41613 dataset as a verification group. We drew the survival curve of *MFAP4* expression between the 2 groups and found that high expression of *MFAP4* gene was related to better prognosis of patients (**Figure 2**).

GSEA, GO terms, and KEGG Pathway Analysis

After performing GSEA on both the high and low *MFAP4* expression groups, we selected the first 6 enriched pathways for mapping (**Figure 3A**). The cytosolic DNA-sensing, oxidative phosphorylation, proteasome, ribosome, RNA polymerase,



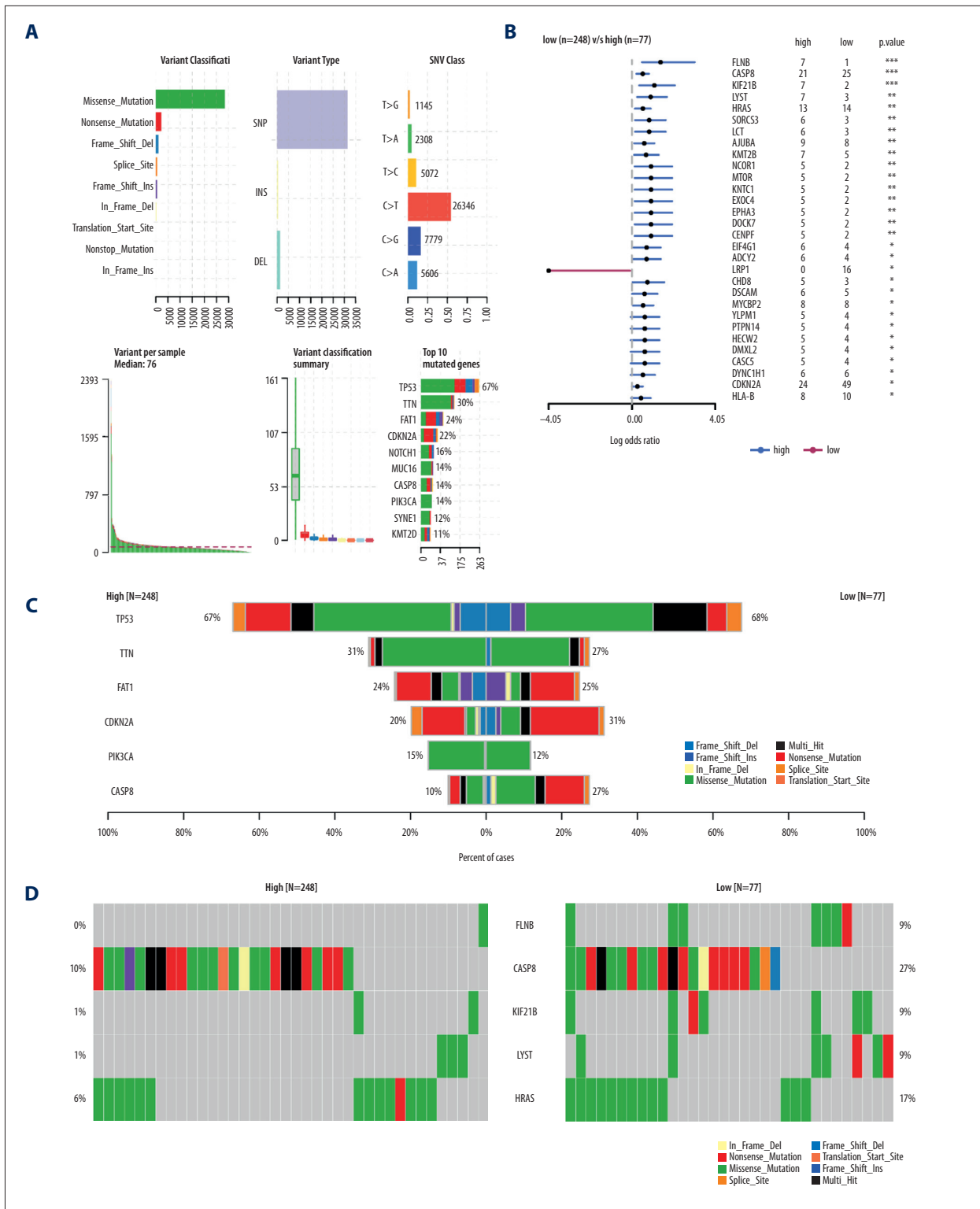


Figure 4. The relationship between *MFAP4* and TMB. **(A)** Overview of mutation data of OSCC tumors in the TCGA database. **(B)** Group comparison of the first 6 mutant genes. **(C)** Forest plot of genes that were highly mutated in the high *MFAP4* expression group compared to the low expression group. **(D)** Comparison of mutation data of the top 5 mutant genes between the high and low *MFAP4* expression groups.

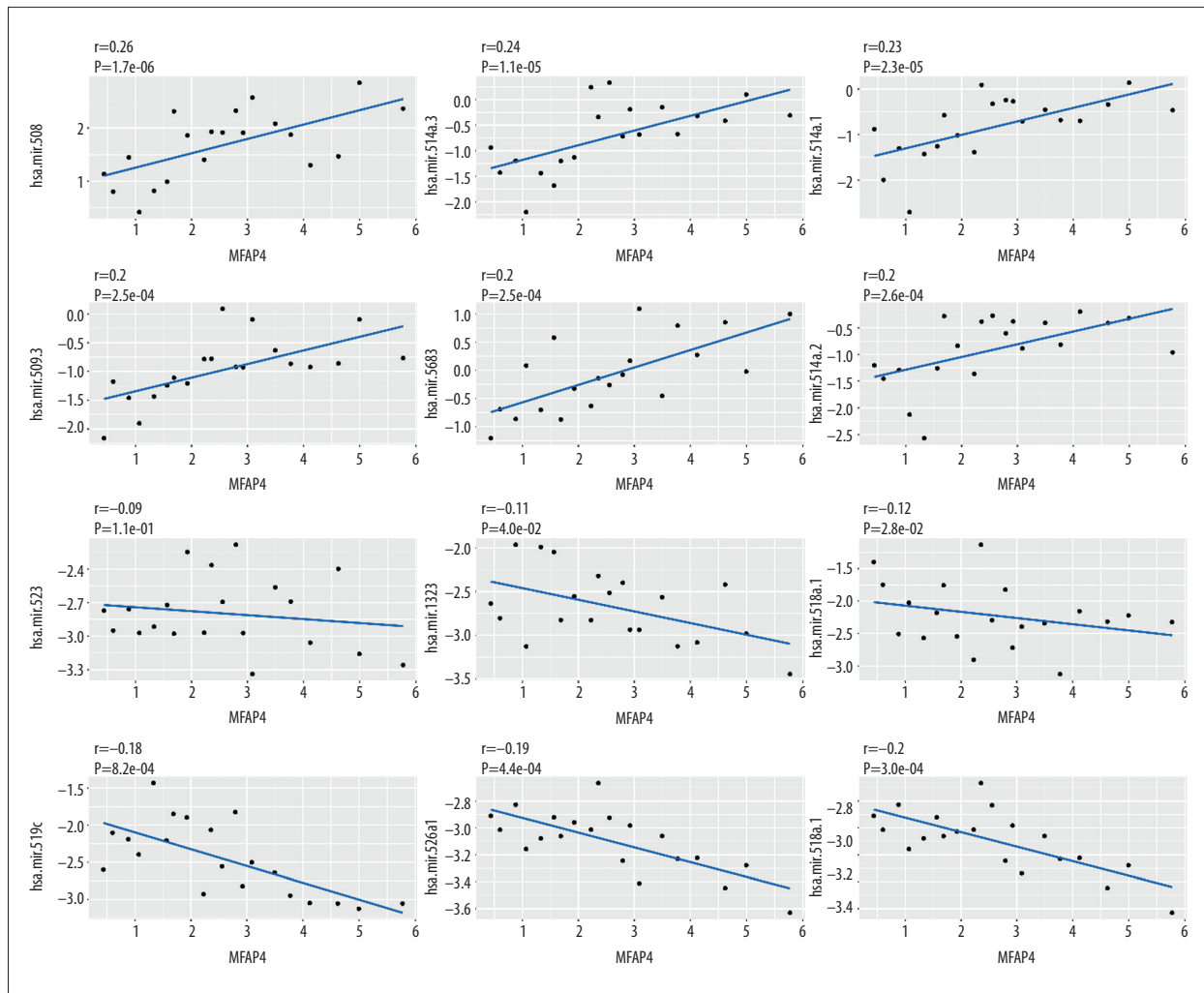


Figure 5. Correlation between miRNA and *MFAP4*. The top 6 positively correlated miRNA and top 6 negatively correlated miRNA showed good linear relationships with *MFAP4*.

and spliceosome pathways were enriched in GSEA. Finally, we performed functional enrichment GO and KEGG analyses on the genes that differed (criteria were $|\log_{2}FC| > 2$ and $P < 0.05$; a total of 121 differentially expressed genes were included) (Figure 3B). In GO analysis, genes that were involved in cell functions such as striated muscle tissue development, extracellular matrix structural constituents, and collagen-containing extracellular matrix were enriched. In KEGG pathway analysis, genes mainly involved in focal adhesion, dilated cardiomyopathy, and human papillomavirus infection were enriched.

Relationship Between *MFAP4* and TMB

We downloaded 330 OSCC samples and data on OSCC tumor mutations from the TCGA database (Figure 4A). Maftools package was used to calculate the TMB between the high and low *MFAP4* expression groups, and to compare the differences between the groups. We also compared and plotted the graph

of the top 6 mutation groups (Figure 4B). Forest plots were used to show the differential mutation genes between the high and low *MFAP4* expression groups (Figure 4C). Compared with the low expression group, the top 10 highly mutated genes in the high *MFAP4* expression group were *FLNB*, *CASP8*, *KIF21B*, *LYST*, *HRAS*, *LCT*, *SORCS3*, *AJUBA*, *KMT2B*, and *CENPF*. Finally, we compared the data of the first 5 mutant genes between the 2 groups. The results are shown in Figure 4D.

Identification of miRNAs Related to *MFAP4*

With the criteria set as $|\log_{2}FC| > 2$ and P value < 0.05 , 23 differential miRNAs were included in the Pearson correlation analysis of the relationship between miRNA and *MFAP4* in both the high- and low-*MFAP4* expression groups. We determined the positive correlation and negative correlation of the top 6 miRNAs, and the results are shown in Figure 5.

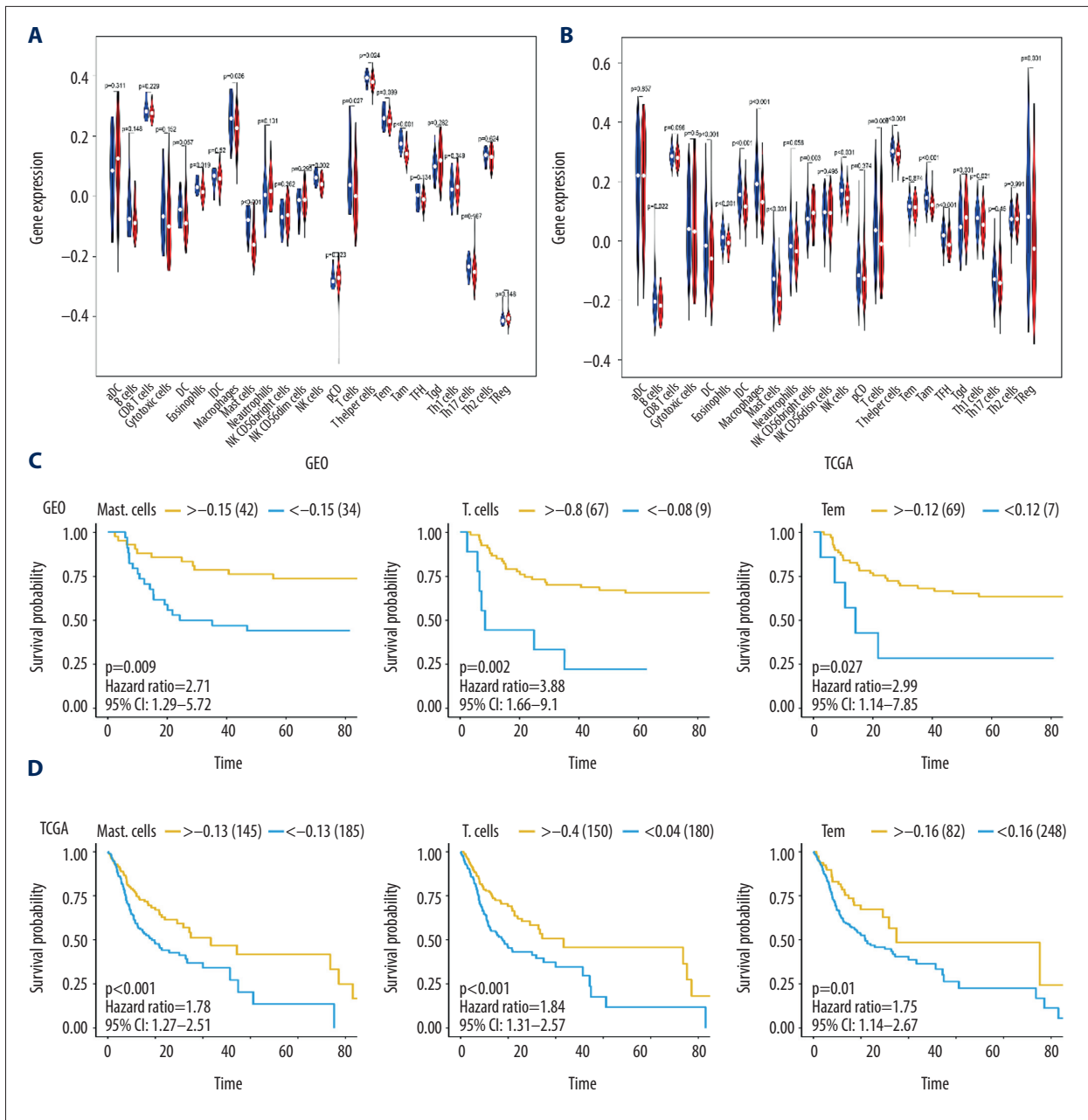


Figure 6. The relationship between *MFAP4* gene expression and tumor immune cell infiltration. **(A, B)** Differences in immune cell infiltration between samples from TCGA and GEO databases. Seven immune cells showed statistical difference in the *MFAP4* expression group. **(C, D)** The infiltration of 3 immune cells was related to the prognosis of the patients.

Relationship Between *MFAP4* and Immune Infiltration

We used ssGSEA to quantify immune cell infiltration in OSCC samples from TCGA and GEO databases, and compared the high- and low-*MFAP4* expression groups (Figure 6A, 6B). After grouping the samples according to best separation and analyzing the survival information of each patient with respect to the type of infiltrating immune cell, we found that 3 immune cell types (mast cells, T cells, and Tem cells) were related to survival (Figure 6C, 6D).

Correlation of *MFAP4* and Immune Checkpoints

With Spearman’s rank correlation analysis, we used the most commonly recognized and used immune cell markers to calculate the correlation of immune checkpoints with the *MFAP4* gene [25]. These immune checkpoints included CD28 (GEO, r=0.332; TCGA, r=0.532), CD80 (GEO, r=-0.111; TCGA, r=0.221), CD86 (GEO, r=0.030; TCGA, r=0.291), CTLA4 (GEO, r=0.235; TCGA, r=0.221), INPP5D (GEO, r=0.296; TCGA, r=0.376), INPL1

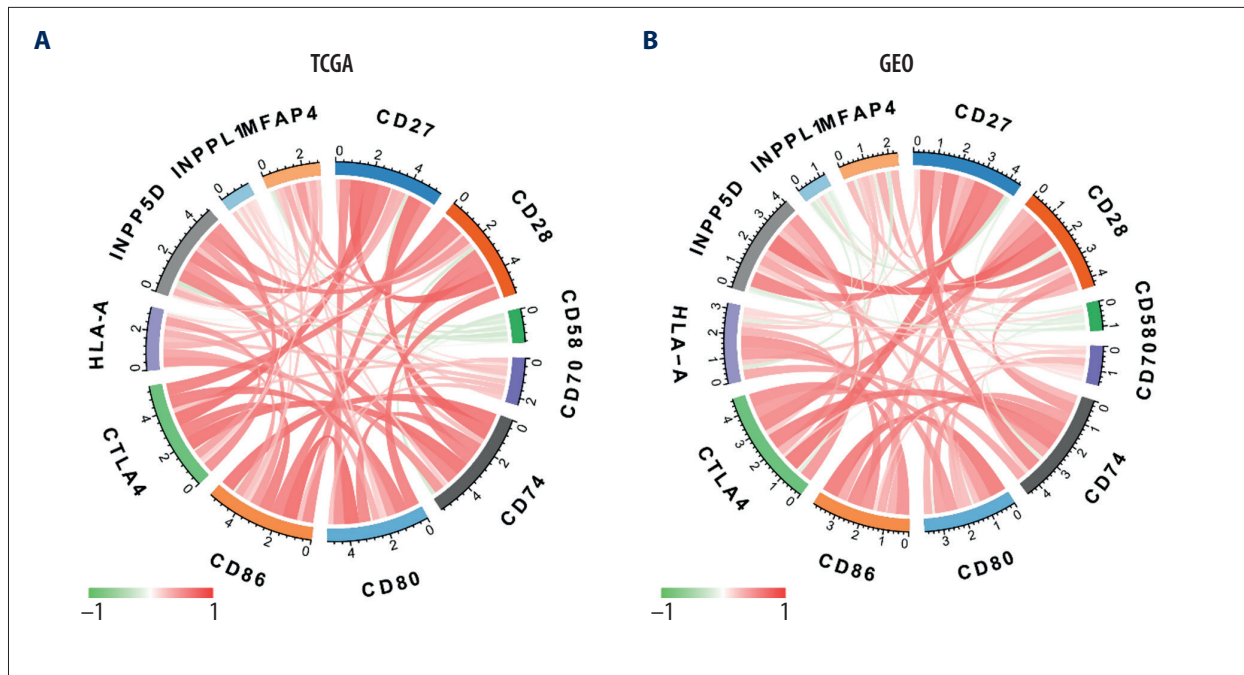


Figure 7. (A, B) Diagram of the relationship between *MFAP4* and immune targets.

(GEO, $r=0.213$; TCGA, $r=0.266$), CD58 (GEO, $r=-0.140$; TCGA, $r=-0.198$), CD27 (GEO, $r=0.399$; TCGA, $r=0.403$), CD70 (GEO, $r=0.045$; TCGA, $r=0.057$), HLA-A (GEO, $r=-0.198$; TCGA, $r=-0.079$), and CD74 (GEO, $r=0.352$; TCGA, $r=0.235$) (Figure 7). We drew a graph to show the correlation of the relationship of each 2 genes; the color and width of each line represent the correlation coefficient.

Discussion

OSCC is one of the most common tumors of the head and neck and seriously affects health. Its treatment usually requires multiple modalities, including surgery followed by chemoradiotherapy (CRT), epidermal growth factor receptor (EGFR) monoclonal antibody combined with radiation and immune checkpoint inhibitors, but patients often have a poor prognosis nonetheless. Therefore, it is very important to determine new therapeutic targets for the treatment of OSCC patients. This study proposed that *MFAP4* is a new prognostic target for OSCC and also studied its relation with the regulation of TMB, miRNAs, and immune cell infiltration.

MFAP4 is a stromal cell protein belonging to the fibrinogen-associated protein superfamily. This family also includes ficolins, fibroleukin, FIBCD1, angiopoietins, and tenascins, which play multifaceted roles in innate immunity, development of the cardiovascular system, and normal functioning of the endothelium [26,27]. However, there are no relevant reports on the role of *MFAP4* in OSCC. In this study, we found that the

MFAP4 gene showed low expression in oral cancer tumor samples and other tumors, and high expression of *MFAP4* was related to better prognosis.

After establishing this connection between *MFAP4* gene and OSCC, we then further explored the mechanism of influence of *MFAP4* on OSCC and found that the cytosolic DNA-sensing, oxidative phosphorylation, proteasome, ribosome, RNA polymerase, and spliceosome pathways were enriched on GSEA. At the same time, we assessed differences in enrichment with GO and KEGG functions. GO was enriched in cell functions such as striated muscle tissue development, extracellular matrix structural constituents, and collagen-containing extracellular matrix. KEGG analysis showed enrichment in focal adhesion, dilated cardiomyopathy, and human papillomavirus infection. Therefore, studying these pathways will help to clarify the underlying mechanisms of the development, proliferation, and invasion of oral cancer cells and also help to predict the progression of cancer.

TMB is becoming a new predictive biomarker for selecting patients who will benefit from immune checkpoint inhibitor therapy [17-19]. It is usually defined as the total number of somatically encoded mutations and is related to the emergence of new antigens that trigger anti-tumor immunity [20-22]. When we compared the mutation data of the high- and low-*MFAP4* expression groups, we found that the Nonsense_Mutation and Missense_Mutation of *FLNB* mutant genes in the low *MFAP4* expression group samples increased significantly. The Frame_Shift_Ins and Multi_Hit mutations of *CASP8* mutant genes in

the low-*MFAP4* expression group were significantly different from those in the high-expression group. Our results therefore indicate that the *MFAP4* gene may affect the occurrence of oral cancer by affecting the mutations of these genes.

MicroRNAs are small non-coding RNAs that have been recently identified as regulators of gene expression through binding to the 3' untranslated regions (UTRs) of target mRNA [28]. This results in mRNA degradation or translational inhibition. Emerging evidence has shown that microRNAs are abnormally expressed in various cancers [29], and deregulated microRNA expression is strongly associated with tumor initiation, promotion, and progression [30,31]. Each microRNA can have multiple target genes, and several microRNAs can also regulate the same gene. Therefore, this complex regulatory network not only regulates the expression of multiple genes through one microRNA but also finely regulates the expression of a certain gene through the combination of several microRNAs. In view of the important relationship between *MFAP4* gene and oral cancer, we further explored the relationship between it and microRNA. We obtained 23 differentially expressed microRNAs, calculated the correlation between these differentially expressed microRNAs and *MFAP4* expression, and finally selected the first 6 positively correlated and the last 6 negatively correlated microRNAs, and then drew the correlation diagram. Previous studies have shown that *NFKB1* may be negatively regulated by miR-508-3p and plays an important role in the occurrence of gastric cancer [32]. The positive regulatory relationship between *MFAP4* and miR-508-3p was discovered for the first time in the present study, and it may be helpful to understand the mechanism of oral cancer. Only a few studies have found relationships of other positive regulations, such as with hsa.mir.514a.3, hsa.mir.514a.1, hsa.mir.514a.2, hsa.mir.509.3, and hsa.mir.5683, with cancer. Therefore, the relationship between microRNAs and oral cancer needs further exploration. Hsa-miR-519c decreases endogenous ABCG2 mRNA and protein levels by acting through a putative miR-519c binding site located within the longer 3UTR region found only in the parental cells [33]. There are relatively few studies on the relationship between other negative regulatory microRNAs and cancer, and the regulatory relationship of microRNA with *MFAP4* gene and the mechanism of oral cancer development still needs further research and verification.

The process of tumor immune cell infiltration has been well explained in a previous article [24]. In this study, we screened 7 common differentially infiltrating immune cells (eosinophils, macrophages, mast cells, NK cells, T cells, T helper cells, Tem) from the data of GEO and TCGA. After grouping by best separation, we found that high infiltration of tumors by mast cells, T cells, and Tem cells was related to better prognosis. Mast cells are immune cells that are present in all classes of vertebrates [34]. They have a widespread distribution in close

proximity to epithelia, fibroblasts, blood, lymphatic vessels, and nerves [35]. Mast cell progenitors enter the circulation and complete their maturation in tissues. These cells are involved in several physiological and inflammatory processes, including organ development [36], wound healing [45], heart function [37], and tumorigenesis [38-42]. Encouragingly, in some models, T cell-mediated immune response can efficiently control tumors. In the last 2 decades, a large number of antigens have been identified in tumors that can be recognized by T cells [43-46], suggesting a potential role of T lymphocytes in anti-tumor immune response. Functional cytolytic T cells have been shown to develop action against tumor cells that express self-tumor antigens in some patients with melanoma [47], pancreatic cancer [48], and Epstein-Barr virus-associated malignancies [49]. We therefore analyzed the correlation between the immune targets of these immune cells and *MFAP4*, and found that *MFAP4* was related to the immune targets of CD27, CD28, CD58, CD70, CD74, CD80, CTLA4, HLA-A, INPP5D, and INPPL1. This result provides a new prospect for further studying the immune infiltration mechanism in relation to the *MFAP4* gene and OSCC.

Some studies have shown miR-24-3-p, CD163, and CD204 have predictive value in OSCC [50,55]. He found miR-24-3p can maintain the proliferation of OSCC cells through targeting PER1 [50]. Kubota suggested that CD 163⁺ CD204⁺ tumor-associated macrophages (TAMs) could play an important role in the invasion and metastasis of OSCC by T cell regulation via IL10 and PD-L1 production [55]. In the present study, the prognosis of patients was significantly different with different expression levels of *MFAP4*, which indicated that *MFAP4*, like miR-24-3-p, CD163, and CD204, has potential as a biomarker of oral squamous cell carcinoma. Similarly, we also know that it is difficult to accurately predict the prognosis of patients based on expression of a single gene, so a single gene could be combined with other prognostic genes, TMB, immune infiltration, and so on to study which combination most improves the prediction effect. In this study, we found that the top 6 positively correlated miRNA and the top 6 negatively correlated miRNA showed good linear relationships with *MFAP4*, 7 immune cells showed a significant difference in the *MFAP4* expression group, and the infiltration of 3 immune cells was related to the prognosis of the patients. Thus, the prognostic value of *MFAP4* lies not only in its use as a separate predictive marker, but in its combination with other factors.

Conclusions

Our research clarified the relationship between *MFAP4* and oral cancer (particularly OSCC). We found that the *MFAP4* gene is related to the prognosis of OSCC and is also closely related to TMB. We also identified some miRNAs that are related

to MFAP4, which is of great significance for exploring the impact of OSCC mutations. Finally, we found a connection between mast cells, T cells, and Tem cells and *MFAP4* in terms of immune infiltration and their relationship to the prognosis of tumors. These findings suggest that MFAP4 may be a promising therapeutic target for OSCC.

Acknowledgements

We thank the researchers who gave their data for this analysis. We gratefully acknowledge their contributions.

Ethical Statement

The data of this study are from the GEO and TCGA databases, and do not involve animal experiments or human specimens, and there are no ethics-related issues.

Availability of Data and Material

The data of this study are from the GEO and TCGA databases.

Conflict of Interest

None.

Supplementary Data

Supplementary Table 1. Comparison of clinical phenotypes in different groups.

n	High 144	Low 186	p
Anatomic neoplasm subdivision (%)			NA
Alveolar ridge	6 (4.2)	12 (6.5)	
Base of tongue	11 (7.6)	12 (6.5)	
Buccal mucosa	9 (6.2)	13 (7.0)	
Floor of mouth	33 (22.9)	27 (14.5)	
Hard Palate	4 (2.8)	3 (1.6)	
Lip	1 (0.7)	2 (1.1)	
Oral cavity	38 (26.4)	34 (18.3)	
Oral tongue	42 (29.2)	83 (44.6)	
Clinical M (%)			0.709
M0	138 (95.8)	173 (94.5)	
M1	0 (0.0)	2 (1.1)	
MX	6 (4.2)	8 (4.4)	
Clinical N (%)			0.800
N0	73 (52.1)	94 (53.4)	
N1	24 (17.1)	34 (19.3)	
N2	41 (29.3)	47 (26.7)	
N3	2 (1.4)	1 (0.6)	
Clinical T (%)			0.028
T1	4 (2.8)	16 (8.9)	
T2	41 (29.1)	63 (35.2)	
T3	36 (25.5)	46 (25.7)	
T4	60 (42.6)	54 (30.2)	

Supplementary Table 1 continued. Comparison of clinical phenotypes in different groups.

n	High	Low	p
	144	186	
Clinical stage (%)			0.066
Stage I	1 (0.7)	10 (5.6)	
Stage II	31 (22.0)	47 (26.3)	
Stage III	32 (22.7)	36 (20.1)	
Stage IV	77 (54.6)	86 (48.0)	
OS=1 (%)	81 (56.2)	69 (37.1)	0.001
DSS=1 (%)	45 (33.6)	47 (26.3)	0.170
DFI=1 (%)	10 (29.4)	13 (21.3)	0.456
PFI=1 (%)	64 (44.4)	70 (37.6)	0.216

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