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TIMP4 serves as a novel potential prognostic biomarker for oral squamous cell carcinoma

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The abnormal expression of tissue inhibitor of metalloproteinase 4 (TIMP4) is associated with multiple human cancers. However, the role of TIMP4 in oral squamous cell carcinoma (OSCC) remains elusive. Therefore, this study explored the expression profile and prognosis of TIMP4 in OSCC. Tumor and adjacent non-tumor tissues were collected from OSCC patients, and protein expression of TIMP4 was detected through immunohistochemistry (IHC) and positive cell counting. Additionally, The Cancer Genome Atlas-Head and Neck Squamous Cell Carcinoma (TCGA-HNSCC) dataset was used to analyze TIMP4 expression further to determine its relationship with HNSCC clinical characteristics. Kaplan-Meier analysis was used to evaluate survival and prognosis. Meanwhile, the Tumor Immune Estimation Resource (TIMER) database was employed to assess the correlation between TIMP4 expression and tumor immune infiltration. Computational tools were also applied to investigate the involvement of TIMP4 in cancer pathways. Results showed that TIMP4 was decreased in OSCC and HNSCC compared with normal tissues. The decrease in TIMP4 was associated with cancer metastasis, immune suppression and HPV positive, clinical staging. Overall, these findings demonstrate that TIMP4 is significantly reduced in OSCC and HNSCC, and associated with a poor prognosis. Additional investigations are warranted to fully understand the therapeutic potential of TIMP4 in OSCC.

Keywords Oral squamous cell carcinoma, Tissue inhibitor of metalloproteinase 4 (TIMP4), Prognosis, Biomarker, Immune infiltration

According to the recent World Health Organization data, head and neck squamous cell carcinoma (HNSCC) is the sixth most common cause of cancer-related deaths worldwide and has become an important global health issue[1,2]. Oral squamous cell carcinoma (OSCC) is the most common type of HNSCC, accounting for about 70% of all HNSCCs[3,4]. Approximately 550,000 new cases and 260,000 deaths from OSCC are reported annually worldwide[5,6], showing a high-incidence trend. With changes in social lifestyle and habits, unhealthy biological factors such as smoking, alcohol consumption, betel nut chewing, long-term night shifts, and mental anxiety and tension, the incidence of OSCC is significantly increasing in recent years, especially in the younger population[7]. Therefore, improving the clinical treatment effect of OSCC and enhancing the patient's survival status with OSCC is paramount.

According to the recent clinical guidelines, the current treatment for OSCC involves a multidisciplinary treatment model that includes surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy[8,9], etc. However, even with this comprehensive approach, the improvement in clinical outcomes remains insignificant, with the 5-year survival rate for OSCC patients being about 60%[10]. Cervical lymph node and distant metastases are the main factors threatening patient survival[11]. Current techniques and research cannot meet the clinical needs of OSCC. Thus, filling the gaps in mechanism analysis, genetic research, and treatment plans is urgent.

Tissue inhibitors of metalloproteinases (TIMPs) form a specialized protein family that specifically curbs the enzymatic activity of matrix metalloproteinases (MMPs). This family consists of four distinct members: TIMP1, TIMP2, TIMP3, and TIMP4[12]. Through their binding to MMPs, TIMPs effectively prevent the breakdown of the extracellular matrix (ECM), thus playing an indispensable role in a wide array of physiological processes such as tissue remodeling, cell proliferation, migration, and apoptosis[13]. Currently, TIMP1, TIMP2, and TIMP3 have

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been extensively studied in oncology, with numerous investigations thoroughly documenting their functions and elucidating the complex mechanisms by which they operate[14,15]. In contrast, research on TIMP4 in the context of tumors remains relatively limited, and its precise role in specific cancers is not yet fully understood. For instance, Paul Nayim and colleagues demonstrated that the root metabolites of Imperata cylindrica exhibited cytotoxic effects on human cervical cancer cells. These metabolites suppressed cancer invasion and metastasis by downregulating the CD24 gene and upregulating the TIMP4 gene, thereby inhibiting the phosphoinositide-3-kinase-protein kinase B (PI3K/AKT) pathway and epithelial-mesenchymal transition[16]. Similarly, *Qi* et al. found that aloperine (ALO) downregulated the expression of MMP2 and MMP-9 mRNA and protein while upregulating TIMP4 mRNA and protein levels, thus inhibiting the migration, invasion, and adhesion of bladder cancer cells[17].However, some studies have reported high expression levels of TIMP4 in tumors. Han and co-workers observed that all TIMP family members were significantly overexpressed in human glioblastoma (GBM), with TIMP4 being associated with longer overall survival (OS) in GBM patients[18]. Collectively, these findings indicated that TIMP4 played a role in the progression and metastasis of various cancers, although this association was not universally consistent. The involvement of TIMP4 in the progression of OSCC has not yet been established, warranting further investigation.

Bioinformatics combines techniques from mathematics, informatics, statistics, and computer science to study biological problems[19]. It involves searching, processing, and utilizing biological data for sequence alignment, gene identification, gene assembly, protein structure prediction, gene expression, protein interaction prediction, and the development of evolutionary models[20]. It plays a huge role in genomics research, proteomics research, drug design, drug target identification, personalized medicine, and precision medicine[21]. With the rapid development of high-throughput sequencing technologies (such as RNA sequencing (RNA-seq), chromatin immunoprecipitation sequencing, whole-genome sequencing, etc.) in recent years, bioinformatics has become increasingly important in biomedical research, drug discovery, and precision medicine. The Cancer Genome Atlas (TCGA) is a landmark public-funded cancer genomics project, describing the genomes of over 200 types of cancer, involving relevant genetic data for over 30 major groups of human tumors, including OSCC[22], providing valuable materials for the analysis and study of OSCC.

The present study comprehensively analyzed TIMP4 expression in OSCC and HNSCC samples. Clinical pathological correlations between OSCC/HNSCC and tumor infiltration, survival and prognosis, and functional pathways were also examined. By elucidating the role of TIMP4 in OSCC and HNSCC, this study seeks to provide valuable insights into the molecular basis of this challenging cancer and potentially identify new avenues for therapeutic intervention.

Results

TIMP4 protein expression is significantly downregulated in OSCC and HNSCC

The differential expression of TIMP4 was examined through various methods, including IHC analysis of multiple tumor and normal samples and analysis of multiple databases, to ensure the robustness and reliability of the results ($P<0.05$). IHC and positive cell count revealed a significant reduction in TIMP4 expression in OSCC compared with normal tissues (Fig. 1A-B). Meanwhile, staining analysis was performed on TIMP4-related proteins MMP-7 and Fibroblast activation protein (FAP), and the results confirmed high expression of MMP-7 and FAP in OSCC (Fig. 1C-F).

The significant difference in TIMP4 expression between normal and OSCC tissues was further confirmed using the TCGA database, results showed that the expression of TIMP4 was significantly lower in HNSCC (Fig. 2A-D). Concurrently, the TMER 2.0 database was utilized to evaluate TIMP4 expression in 32 malignant tumors. Compared with normal tissues, TIMP4 was significantly upregulated only in GBM tumors[23,24] and downregulated in 13 types of tumors, including HNSCC, lung adenocarcinoma, lung squamous cell carcinoma, bladder urothelial carcinoma, breast cancer (BRCA), cervical squamous cell carcinoma, kidney chromophobe, kidney renal papillary cell carcinoma, prostate adenocarcinoma, skin cutaneous melanoma, stomach adenocarcinoma, thyroid carcinoma, and uterine corpus endometrial carcinoma[25–27]. However, TIMP4 had no significant impact in the remaining 18 malignant tumors (Fig. 2E). This reaffirms the significant downregulation of TIMP4 in HNSCC and OSCC, and confirms that TIMP4 is not a pan-cancer gene, with its expression being specific to OSCC.

TIMP4 expression is associated with clinicopathological features and prognosis

Kaplan-Meier analysis showed that TIMP4 expression was significantly associated with the survival time and status. Intriguingly, the survival rate of the high TIMP4 expression group decreased during the early follow-up stages. However, patients in the high TIMP4 expression group had longer survival times and higher survival rates than those in the low expression group after the extension of the follow-up period ($P=0.007$) (Fig. 3A). Additionally, receiver operating characteristic (ROC) curve was plotted to further confirm the accuracy of the predictive results (Fig. 3B). Further correlation analysis between clinicopathological features of HNSCC and TIMP4 expression revealed that TIMP4 downregulation was associated with certain key tumor parameters, such as the human papillomavirus (HPV) status, clinical staging, and distant metastasis. However, no significant association was found among TIMP4 downregulation and age, gender, smoking, alcohol, cigarettes, or tumor size, etc. (Fig. 3C-K). Furthermore, we conducted an in-depth analysis of the relationship between TIMP4 expression and TP53 and NOTCH1 mutations. The results suggest that lower TIMP4 expression may be associated with a higher frequency of TP53 mutations, whereas changes in TIMP4 expression do not appear to influence the frequency of NOTCH1 mutations (Supp. Figure 4).

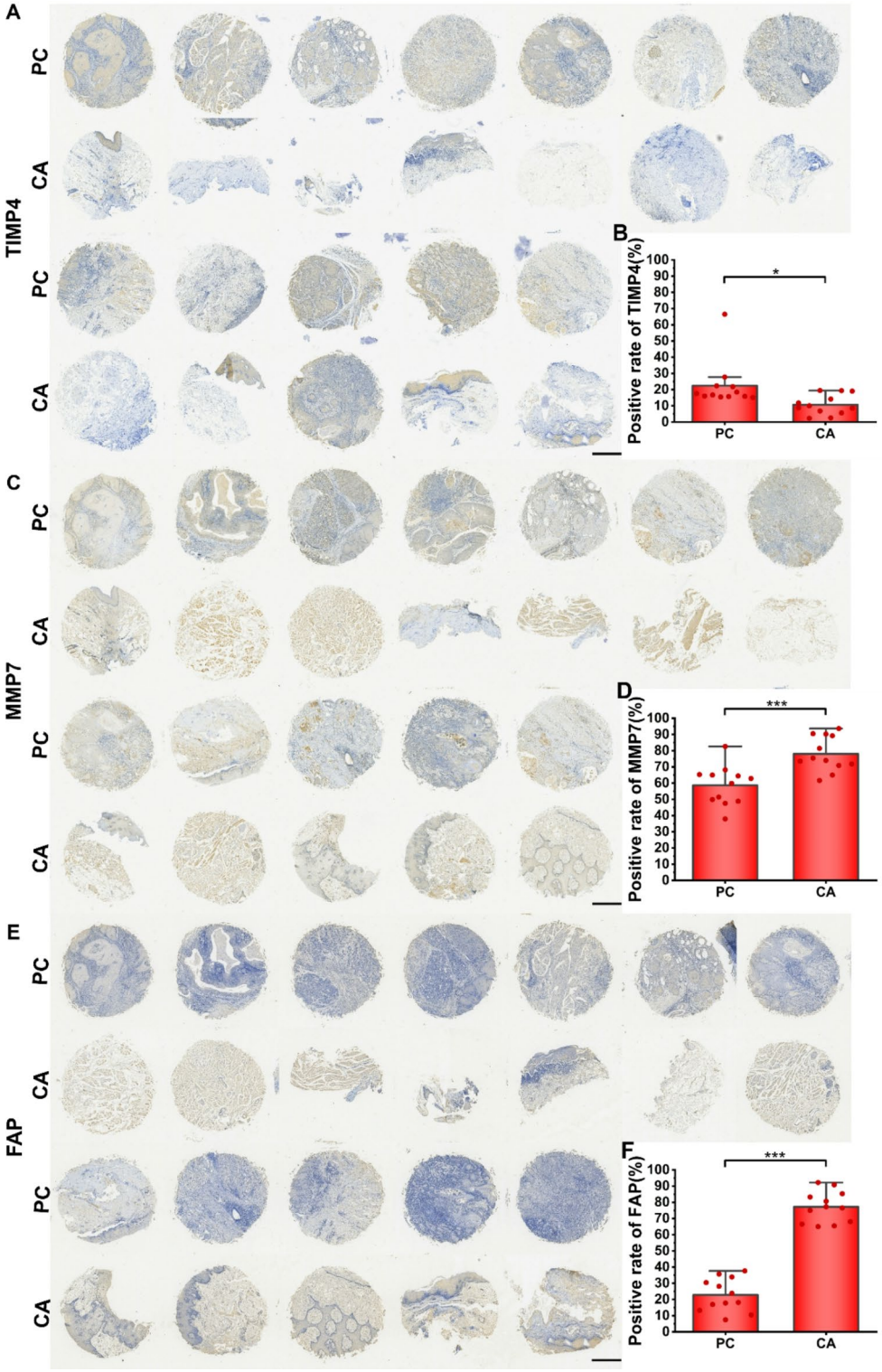

Fig. 1. Immunohistochemistry(IHC) and positive cell count expression of normal and OSCC tissues: (**A**,**B**) TIMP4, (**C**,**D**) MMP-7, and (**E**,**F**) FAP. Scale bar = 50 μm.

TIMP4 expression is involved in tumor immune infiltration

Furthermore, the TIMER2.0 database was utilized to further investigate the impact of TIMP4 on the immune microenvironment of HNSCC. Heatmap analysis revealed a negative correlation between TIMP4 expression and various immune cells (including CD8 + T cells, CD4 + memory T cells, and T follicular helper cells) (Fig. 4A). Further analysis of the proportion of all immune cells in patients showed a higher proportion of mast cells and a lower proportion of B cells (Fig. 4B). Additionally, the median TIMP4 (0.2386086) was used as the limit to divide

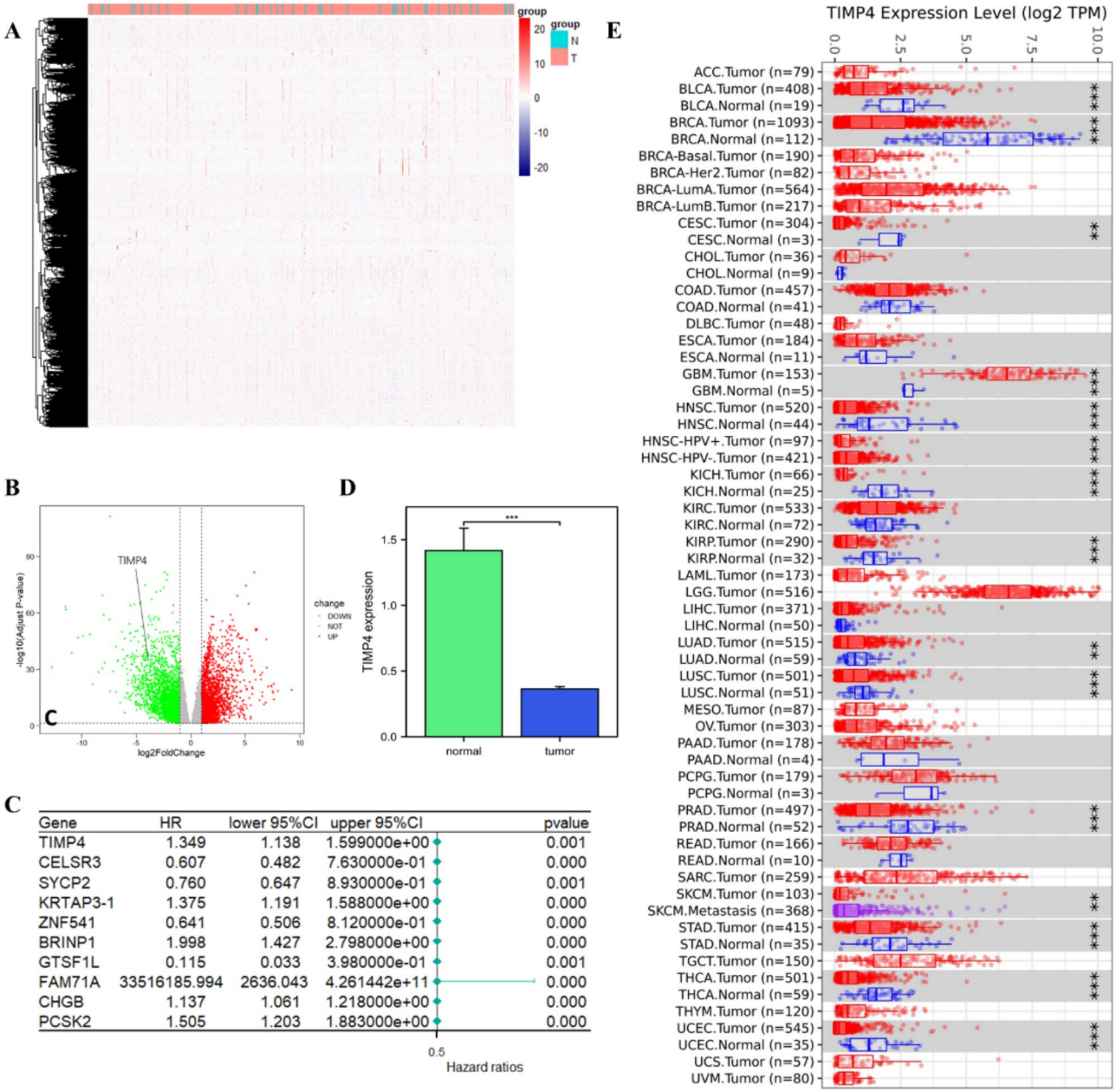

Fig. 2. TIMP4 expression in cancer. (**A**) Heatmap of differentially expressed RNA in The Cancer Genome Atlas (TCGA) dataset (blue: downregulated expression; red: upregulated expression). (**B**) Volcano plot of differentially expressed RNA levels of TIMP4 in the TCGA dataset. (**C**) Univariate Cox regression analysis of the top 10 genes affecting the prognosis of HNSCC patients ($P < 0.005$). (**D**) Expression levels of TIMP4 in tumor and normal tissues of HNSCC patients in the TCGA database. (**E**) Expression status of the TIMP4 gene in 32 malignant tumors.

the high and low expression groups, ssGSEA indicated that high-expression group had higher expression of 17 types of immune cells and natural killer T cells (Fig. 4C). Meanwhile, CIBERSORT analysis showed that low-expression group had a higher proportion of CD8 + T cells (Fig. 4D). Subsequent correlation analysis revealed a negative correlation between TIMP4 and the expression of CD8 + T cells (Fig. 4E) and memory B cells (Fig. 4F). Subsequently, IHC of tissue samples revealed that the surface marker CD39 of CD8 + T cells was highly expressed in tissues with low TIMP4 expression (Fig. 4G-H), confirming the aforementioned findings.

TIMP4 and its network is strongly associated with HNSCC

Weighted gene co-expression network analysis (WGCNA) was performed on the target gene using the WGCNA package to gain a better understanding of the interactions involving TIMP4 (Fig. 5A-C). Correlation analysis between the modules and traits was conducted to identify modules related to both tumor and immune characteristics (Fig. 5D). The pink module was selected because TIMP4 was associated with patient survival as

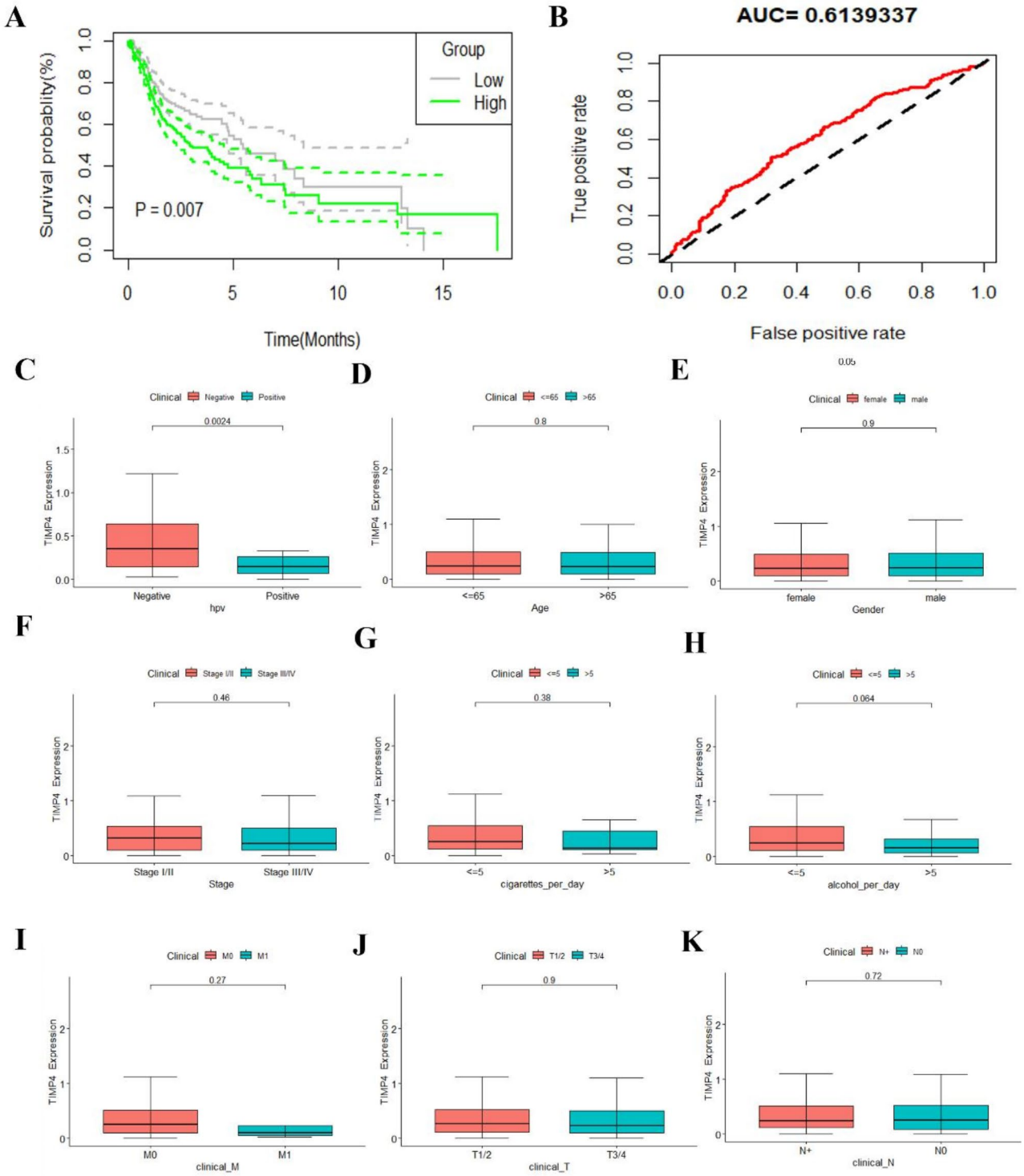

Fig. 3. Correlation between TIMP4 expression and clinical pathological features of HNSCC. (**A**,**B**) Kaplan-Meier plot indicates that low TIMP4 expression affects the long-term survival rate. Box-plot represents the relationship between TIMP4 expression and clinicopathological features, including (**C**)human papillomavirus (HPV) status, (**D**)age, (**E**)gender, (**F**)tumor stage, (**G**)cigarettes, (**H**)alcohol, (**I**)clincal_M, (**J**), clincical_T, and (**K**) clinical_N. The X-axis represents sample type and the Y-axis indicates levels of TIMP4 expression (**C–K**).

indicated by the Stroma Score ($R = 0.39$, $P = 2e\text{-}18$), ESTIMATE Score ($R = 0.28$, $P = 6e\text{-}10$), Tumor Purity ($R = -0.29$, $P = 1e\text{-}10$), and Immune Score ($R = 0.12$, $P = 0.01$). Subsequently, multiple genes co-expressed with TIMP4 were obtained from the pink module (Fig. 5E). Finally, the "limma" package was used for co-expression analysis of the TIMP4 gene, yielding four consistently co-expressed genes: RETREG1, AGT, SHISA4, and DTNA (Fig. 5F), providing references for studying the molecular mechanisms by which TIMP4 influences the progression

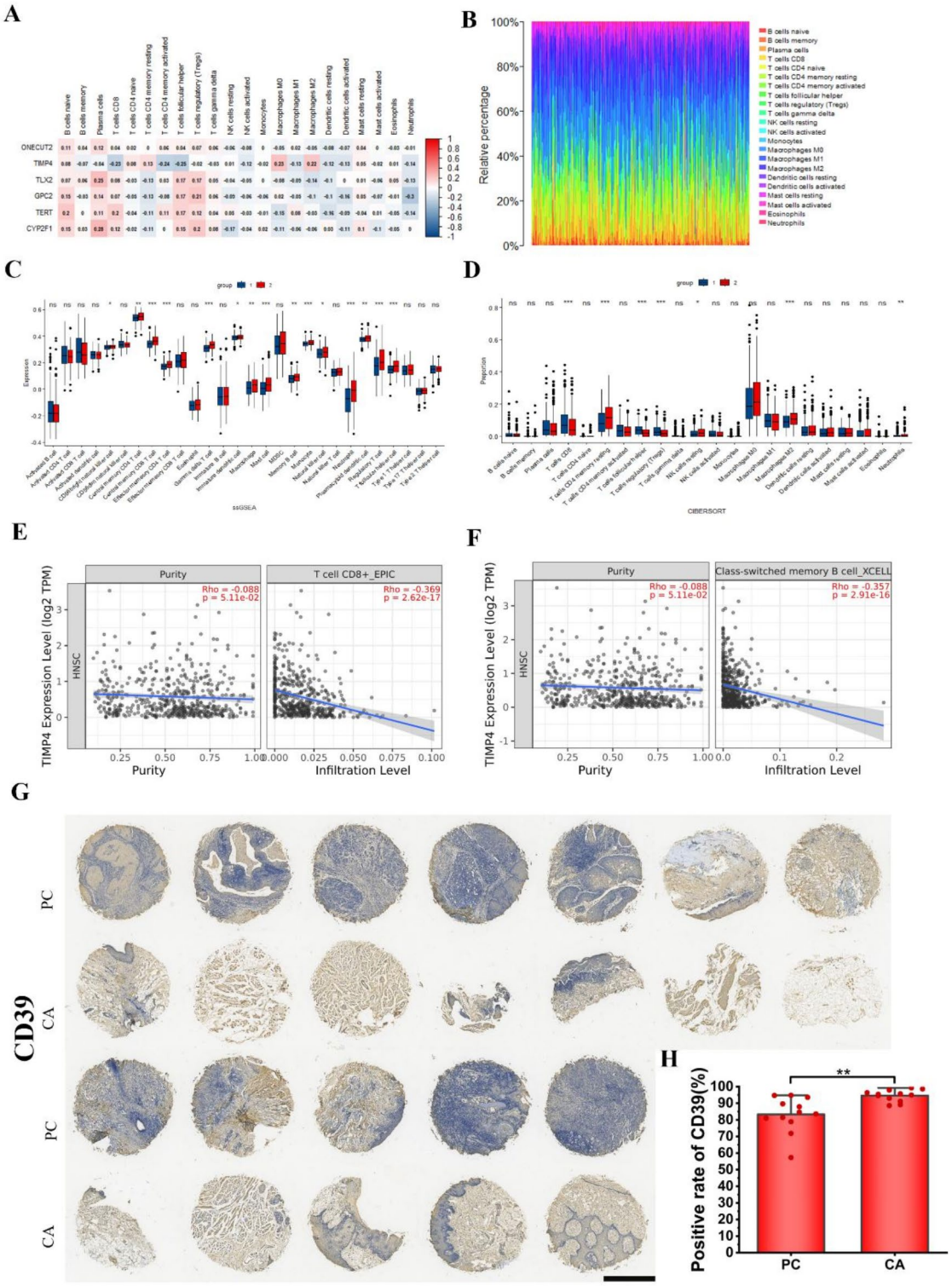

Fig. 4. Immune-related analysis of the TIMP4 gene. (**A**) Heatmap of the correlation between six genes (ONECUT2, TIMP4, TLX2, GPC2, TERT, and CYP2F1) and 22 immune cell types. (**B**) Heatmap showing the proportion of each of the 22 immune cells in high-and low TIMP4 expression groups. (**C**) Differential expression of the TIMP4 gene in 28 immune cell types between high- and low-expression groups. (**D**) Proportions of 22 immune cells in high- and low-expression groups. (**E**) Correlation between the TIMP4 gene and CD8 + cells using partial Spearman correlation analysis with purity adjustment based on TIMER. (**F**) Correlation between the TIMP4 gene and memory B cells using partial Spearman correlation analysis with purity adjustment based on TIMER. (**G,H**) IHC and positive count of CD39. Scale bar = 50 μm.

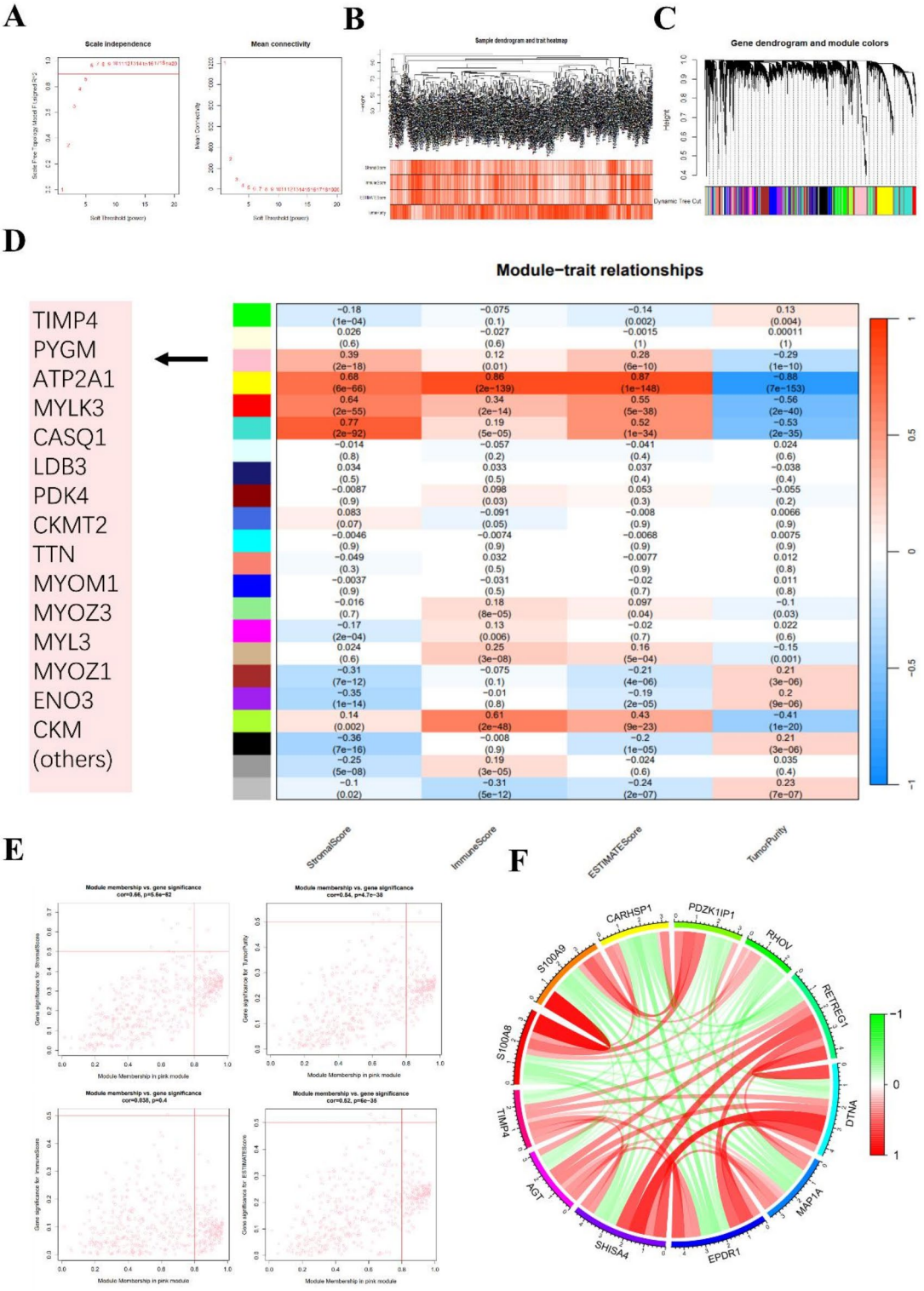

Fig. 5. Co-expression analysis of TIMP4. (**A**) Analysis of the soft power network topology. (**B**) Hierarchical clustering dendrogram of 546 HNSCC patients. (**C**) Gene map and module colors. (**D**) Heatmap of the correlation between module eigengenes and high/low TIMP4 expression groups and ESTIMATE scores. (**E**) Scatter plot of red module signature genes. (**F**) Co-expression analysis of TIMP4 using the "limma" package and genes shared in the pink module.

of OSCC and HNSCC. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was employed to further investigate the signaling pathways associated with the TIMP4 protein. The histogram illustrated the top 40 significant signaling pathways, which encompassed calcium signaling pathway (including the PI3K-Akt signaling pathway), myocardial contraction, vascular smooth muscle contraction, and extracellular matrix (ECM)-receptor interaction (Supp. Figure 5A-B).

Discussion

Oral cancer is the most common type of head and neck malignant tumor, with over 90% of cases being OSCC[28],early OSCC is often mistaken for oral inflammation or ulcers, and most patients are diagnosed at an advanced stage, the curative effects are often unsatisfactory. Given its high treatment costs, chewing and swallowing dysfunctions, and poor prognosis[29,30], exploring the pathogenesis of OSCC is imperative to improve clinical treatment outcomes.

In recent years, the role of TIMP4 in tumor progression has received extensive attention[31].TIMP4 plays a crucial role in numerous biological processes and has significant regulatory effects on angiogenesis and tumor progression[32],which primarily acts as a protease inhibitor, suppressing the activity of MMPs and promoting the synthesis and degradation of ECM regulated by cells[33]. Previous evidence has shown that TIMP4 expression is closely associated with the occurrence and development of various types of cancer, including BRCA, cervical cancer, colon cancer, and other solid tumors, and may serve as a new therapeutic target. However, studies exploring the role of TIMP4 in the progression of OSCC are relatively scarce. The current comprehensive study utilized the TCGA-HNSCC dataset to thoroughly investigate the expression profile of TIMP4 and its clinical significance in HNSCC, and findings were validated using OSCC samples.

The present study found that TIMP4 expression was significantly decreased in OSCC and HNSCC. It is noteworthy that TIMP4 dysregulation does not occur in all cancer types, underscoring the significance of exploring its role in OSCC. Kaplan-Meier analysis further emphasized its clinical relevance, suggesting a significant impact on OS rates. Compared with patients with high TIMP4 expression, those with low TIMP4 expression had higher OS rates in the early follow-up period but a poorer long-term prognosis, which may be related to the exhaustion of tumor-associated immune cells with prolonged follow-up time[34]. Notably, TIMP4 downregulation exhibited a complex association with key clinical and pathological features of HNSCC, including cancer metastasis, immune suppression and HPV positive, clinical staging, and TP53 mutant status, etc. This complexity underscores the multifaceted role of TIMP4 in HNSCC progression and immune response. For instance, TIMP4 downregulation may enhance the invasive potential of cancer cells and contribute to an immunosuppressive tumor microenvironment[35]. TP53 mutation status interrelate with TIMP4 downregulation, influencing the overall prognosis and therapeutic response of HNSCC patients. Understanding these associations is crucial for developing targeted therapies and improving patient outcomes[36]. Regrettably, the limited sample size restricts further correlation analysis.

In tumor tissues, cancer cells must adhere to adhesion proteins of the ECM, and by enzymatically degrading the ECM, these cells migrate to distant sites and invade surrounding normal tissues, achieving eventual metastasis. Therefore, the dynamic process of ECM degradation and repair often occurs during tumor progression. MMP-7, also known as matrilysin, is a member of the MMP family with certain substrate specificity, primarily acting on the ECM, basement membrane, and cell membrane surface molecules. Studies have shown that MMP-7 is distributed at the invasive front of OSCC and is associated with tumor aggressiveness[37]. Another gene, FAP, is also involved in the development of tumors. FAP is a serine protease that is highly expressed in tumor-associated fibroblasts and can degrade type I collagen and exert dipeptidyl peptidase activity. Several studies have pointed out that FAP is highly expressed in BRCA, gastric cancer, pancreatic ductal adenocarcinoma, colorectal cancer, and cervical cancer cells. Wang et al. found that FAP was upregulated in OSCC compared with benign tissue samples[38]. Moreover, its expression level is closely associated with the OS of OSCC patients[39]. Therefore, MMP-7 and FAP may show certain interactive effects with TIMP4 during the dynamic changes of the ECM. Our preliminary IHC findings also confirmed the high expression of MMP-7 and FAP in OSCC.

The tumor microenvironment includes a multifaceted dynamic system composed of cancer cells, a complex cytokine environment, immune cells, and physical and chemical characteristics[40]. In terms of immune cells, tumor-infiltrating CD8 + T cells are considered one of the main T cell subsets responsible for mediating effective anti-tumor responses[41]. The function of tumor-infiltrating CD8 + T cells is associated with improved survival outcomes in patients with lung cancer, BRCA, and malignant pleural mesothelioma. However, T-cell exhaustion often occurs in the tumor microenvironment due to the long-term activation of the immune response, leading to cancer immune evasion[42]. CD39, an enzyme expressed on the surface of tumor-infiltrating CD8 + T cells, can weaken the function of effector T cells by hydrolyzing extracellular adenosine triphosphate and blocking effector responses in lymphocytes. Kang et al. pointed out that CD39 upregulation may be associated with T-cell exhaustion, thereby reducing the OS of liver cancer patients[43]. Intriguingly, our immune-related analysis demonstrated that the low TIMP4 expression group tends to have stronger immune infiltration than the high TIMP4 expression group, particularly CD8 + T cells. Interestingly, IHC analysis revealed that CD39 was highly expressed in OSCC, potentially involved in T-cell exhaustion, which suppresses the immune response.

Functional analysis has uncovered the intricate network of TIMP4, emphasizing its close association with oncogenes and key cellular processes that are vital to cancer pathogenesis. Our study identified four co-expressed genes based on WGCNA analysis: RETREG1, DTNA, SHISA4, and AGT, which have been previously implicated in various types of cancer. Reticulophagy regulator 1 (RETREG1) is generally considered a tumor suppressor gene that is involved in the regulation of endoplasmic reticulum stress and apoptosis. Decreased expression of RETREG1 is correlated with tumor aggressiveness and poor prognosis in certain cancer types. Acting as a tumor suppressor, RETREG1 can modulate the turnover of the endoplasmic reticulum by selective autophagy to regulate cancer cell growth and proliferation[44]. Dystrobrevin alpha (DTNA)-encoded protein is part of the

dystrophin-associated protein complex, which plays a crucial role in the maintenance of the cytoskeleton and cell signaling. The abnormal expression of DTNA may be associated with tumor development and metastasis in some cancers. Changes in DTNA expression are related to BRCA and glioma aggressiveness and patient clinical outcomes[45]. Shisa family member 4 (SHISA4) is a member of the Shisa family proteins, which play essential roles in various biological processes, including cell proliferation, differentiation, and apoptosis. SHISA4 expression may be correlated with tumor grading and patient survival rates in glioblastoma and other types of cancer[46]. Angiotensinogen (AGT) regulates the renin-angiotensin-aldosterone system (RAAS). Besides, AGT expression may be associated with tumor angiogenesis, invasion, and metastasis. Elevated expression of AGT is correlated with tumor progression and poor prognosis in gastric, lung, and colorectal cancers[47]. These findings deepen our understanding of the potential impact of TIMP4 on cancer development and emphasize its multifaceted role in cellular mechanisms. Particularly, our findings provide more insights into the multifaceted role of TIMP4 in OSCC, and a full understanding of its potential impact on disease progression and patient outcomes may provide new therapeutic avenues for OSCC patients. The KEGG analysis offers an in-depth overview of the biological processes involving TIMP4 in head and neck squamous cell carcinoma (HNSCC). For instance, the extracellular matrix (ECM)-receptor interaction pathway plays a pivotal role in cellular adhesion and migration, functions that are frequently impaired in cancer. Elucidating the interactions within these pathways in HNSCC can provide valuable insights for developing targeted therapies and enhancing patient outcomes[48,49].

Nevertheless, this study has some limitations. First, this study included a small sample size; thus, more large-scale studies are warranted to validate our findings. In addition, the specific molecular mechanisms by which TIMP4 impacts the progression of OSCC have not been verified through cellular and animal experiments, which merits further investigation considering the identified association between TIMP4 and tumor progression. These experiments may further elucidate the molecular pathways and mechanisms of TIMP4 in OSCC.

Conclusion

In summary, this study highlights the significant downregulation of TIMP4 mRNA and protein expression in HNSCC, particularly in OSCC. Further analysis of TCGA data and samples from OSCC patients established an association between TIMP4 dysregulation and key clinical and pathological features, including cancer metastasis, immune suppression and HPV positive, clinical staging, etc. Moreover, low TIMP4 expression emerged as a prognostic indicator and was associated with poorer long-term OS in OSCC and HNSCC patients. The functional analysis unraveled various associations of TIMP4 with oncogenes and proteins that are crucial to cancer pathogenesis, suggesting its potential as a prognostic biomarker and therapeutic target in OSCC. However, further exploration and clinical research are needed to validate these findings.

Materials and methods

Patient recruitment and sample collection

All study protocols were approved by the Ethics Committee of the Affiliated Tumor Hospital of Chongqing University (Ethics Number: CZLS2024198-A) and were performed in accordance with Declaration of Helsinki. The OSCC and normal samples used for the experiment were collected from the operating room (normal tissues were obtained from parts close to the primary tumor site) at the Affiliated Tumor Hospital of Chongqing University. Signed informed consent was obtained from all patients before OSCC and normal samples were collected and analyzed. Patients with tumor recurrence, secondary oral metastasis from systemic tumors, a history of previous radiotherapy and chemotherapy, multiple primary tumors, and other severe systemic diseases were excluded from the study. These phenomena may alter the biological characteristics of tumor cells and other cells, thereby complicating the investigation of their intrinsic properties. Additionally, the expression levels of TIMP4 were affected, further complicating the interpretation of the results. Such changes may compromise the consistency of the study design and reduce the reliability of the findings. The sample inclusion process and clinical data are shown in supplementary picture 1 (Supp. Figure 1).

Immunohistochemistry (IHC) and positive cell count

OSCC and normal specimens were fixed with 4% formaldehyde, dehydrated, paraffin-embedded, and then sectioned into 4-μm slices. Tissue sections were deparaffinized and rehydrated in xylene and graded ethanol. Tissue sections were then treated with 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase. Subsequently, antigen retrieval was performed using a pressure cooker with 0.01 M citrate buffer (pH 6.0) for 15 min. Sections were incubated at 4 °C overnight with primary antibodies, including rabbit anti-human TIMP4 (1:200; Aifang biological, Hunan, China), rabbit anti-human MMP-7 (1:100; Aifang biological, Hunan, China), and rabbit anti-human fibroblast activation protein (FAP) (1:200; Aifang biological, Hunan, China), followed by incubation with secondary antibodies at room temperature for 1 h. Finally, sections were visualized using 3,3'-diaminobenzidine (DAB). Using Visiopharm software, regions of interest (ROIs) were defined as required, all cells within the ROI were intelligently identified, signal expression information on each cell was read, and positive cell determination was performed. The output of positive cell and total cell counts were used to calculate the cell positivity rate.

Gene screening using the TCGA database

Analysis of the TCGA dataset highlighted the primary impact of OSCC in HNSCC, which accounts for approximately 70% of the samples and specifically affects certain oral anatomical regions, including the tongue, lips, palate, gingiva, floor of the mouth, oropharynx, and other unspecified areas[50]. The expression of TIMP4 was strategically explored utilizing the TCGA database s(https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga), providing a comprehensive analysis with strong correlations and insights

targeted at OSCC in a broader context of HNSCC. RNA expression data of 612 HNSCC samples were retrieved from the TCGA database, and 546 relatively complete RNA-seq datasets were selected. Next, differential analysis and Cox regression analysis were performed to screen for significant genes. Subsequently, survival analysis was conducted based on the survival information of each sample. After screening multiple genes, the significant tumor suppressor gene TIMP4 was identified. Weighted co-expression and correlation analyses of all genes were then performed, along with clinical and immune-related analyses.HNSCC samples date in TCGA and TIMP4 screening process See supplementary picture 2 (Supp. Figure 2). Prior to the formal TIMP4 analysis, we compared the expression of TIMP1, TIMP2, and TIMP3 using IHC and TCGA data based on the same approach above.Results showed significant and distinct TIMP4 expression levels in OSCC and normal tissues (Supp. Figure 3).

Expression analysis of TIMP4

Firstly, the Tumor Immune Estimation Resource 2.0 (TIMER 2.0) database was utilized to assess the expression of TIMP4 across 32 different types of cancer, presenting the results in a panoramic red-blue heatmap. Additionally, publicly available HNSCC-related RNA-seq data and associated clinical information were obtained from the TCGA database. R packages "limma" and "DESeq2" were employed to analyze the differential expression of TIMP4 between tumor and adjacent non-tumor tissues using $|\text{Log2 fold change}| > 1$ and adjusted p-value < 0.05 as the screening criteria. HNSCC data were then divided into two groups: tumor and normal groups. Finally, data were visualized using "ggplot2" and "ggpubr" R packages.

Analysis of survival outcomes and clinicopathological characteristics

Kaplan-Meier survival analysis was conducted to examine the prognostic significance of TIMP4 mRNA levels in OSCC patients, and the accuracy of the results was confirmed using the area under the curve (AUC) values. The correlation between TIMP4 expression and clinicopathological features in HNSCC was also investigated.

Tumor infiltration analysis and IHC

Correlation analysis of 22 types of immune cells for six genes (ONECUT2, TIMP4, TLX2, GPC2, TERT, and CYP2F1) selected in the Cox regression was performed using the "corrplot" package, and a heatmap was generated. Subsequently, "parallel" and "preprocessCore" packages were utilized to analyze the respective proportions of all samples in the 22 immune cells. Patient samples were then divided into a high-expression group (group 1) and a low-expression group (group 2) based on the expression levels of TIMP4. Next, "tidyr" and the "GSVA" packages were used to organize and analyze the data, and "ggsci" and "ggpubr" packages were utilized for visualization. Single-sample gene set enrichment analysis (ssGSEA) and CIBERSORT analyses were performed based on the expression levels in 28 immune cells and the proportion differences in the 22 immune cells. Finally, the immune cell correlation analysis was verified on the TIMER2.0 website. The relevant protein marker rabbit anti-human CD39 (1:100; Aifang biological, Hunan, China) was selected and previous tissue samples for IHC and positive cell count were used for validation (see subsection 2.2 for specific steps).

Weighted gene co-expression network analysis (WGCNA) and Kyoto Encyclopedia of genes and genomes (KEGG) analysis

WGCNA is a systems biology method for describing gene association patterns across different samples. It is used to identify gene sets that change in a highly coordinated manner and candidate biomarker genes or therapeutic targets based on the intramodular connectivity of the gene sets and their correlation with phenotypes[51]. Differentially expressed genes were processed with padj < 0.05 obtained through the "WGCNA" R package. To ensure that the constructed co-expression network is close to a scale-free distribution, the "hclust" function in the R environment was used for hierarchical clustering to assess the presence of any obvious outliers. Furthermore, the "pickSoftThreshold" function was used to select an appropriate soft threshold/beta to determine the presence of any obvious outliers. The soft power was set at 5. Thereafter, 22 modules were obtained and their correlations with cluster, stromal scores, immune scores, ESTIMATE scores, and tumor purity were calculated. Module membership (MM) indicates the correlation between genes and the module, and gene significance (GS) indicates the correlation between genes and the trait. Genes with $|\text{MM}| > 0.8$ and $|\text{GS}| > 0.4$ were considered key module genes. Multiple genes were identified for further research by calculating MM and GS. For the identified differentially expressed gene sets, KEGG enrichment analysis was performed using the Cluster Profiler package in the R environment[52]. Additionally, utilizing the ggplot2 package for visualization and integration[53], it was determined that only pathways with p-values less than 0.05 were considered significantly enriched.

Statistical analysis

The log-rank test was employed to compare the Kaplan-Meier survival curves. The T-test was used to compare the high- and low-expression groups of differential genes, and $P < 0.05$ was considered statistically significant.

Data availability

1. The TCGA database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) was used to strategically investigate TIMP4 expression;2. The Tumor Immune Estimation Resource 2.0 (TIMER 2.0) database was utilized to assess the expression of TIMP4 across 32 different types of cancer.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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