SERPINE1 as an independent prognostic marker and therapeutic target for Nicotine-related oral carcinoma

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Running Head: SERPINE1 in Nicotine-related oral carcinoma

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Conflict of interest: The authors declare that they have no potential conflicts of interest.

Financial disclosure:
This study was partially supported by the Guizhou Science and Technology Project (ZK2022-044), and the Cultivation project of Affiliated Hospital of Guizhou Medical University (I-2020-10 and gyfybsky-2021-60).

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Methodology: JL, HZ.
Visualization: HZ, XZ.
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Writing review & editing: JL, XZ.

Abbreviations
HNC, head and neck cancer; GEO, Gene Expression Omnibus; DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; TCGA, The Cancer Genome Atlas; GDSC, Genomics of Drug Sensitivity in Cancer; GSCA, Gene Set Cancer Analysis; IHC, immunohistochemistry; LNM,
lymph node metastasis; EMT, epithelial-mesenchymal transition; FDR, False Discovery Rate;

Highlights
1. There are many studies on nicotine addiction, but the carcinogenic mechanism is still unclear.
2. Data mining found that SERPINE1 may be the key gene of nicotine-related oral cancer.
3. SERPINE1 may be related to the proliferation, invasion, and chemosensitivity of cancer cells.
SERPINE1 as an independent prognostic marker and therapeutic target for nicotine-related oral carcinoma
Abstract:

**Background:** Nicotine is an ingredient of tobacco, and its exposure increases various cancer risks, including oral cancer. Previous studies have focused on its addictive properties, but its carcinogenic mechanism has rarely been studied. We aimed to explore the key genes in the process of nicotine promoting the occurrence and development of oral cancer through data mining and experimental verification.

**Methods:** This study involved three parts. First, the key genes related to nicotine-related oral cancer were screened out through data mining; second, the expression and clinical significance of the key gene in oral cancer tissues were verified by bioinformatics. Finally, the expression and clinical significance of the key gene in oral cancer were histologically investigated, and the effects of its expression on cell proliferation, invasion, and drug resistance were cytologically assessed.

**Results:** SERPINE1 was identified as the key gene, which was upregulated in nicotine-treated oral cells and may be an independent prognostic factor for oral cancer. SERPINE1 was enriched in various pathways such as TNF and Apelin pathways; and was related to the infiltration of macrophages, CD4+T cells, and CD8+T cells. Overexpression of SERPINE1 was associated with N staging and may be involved in hypoxia, angiogenesis, and metastasis. Knockdown of SERPINE1 in oral cancer cells resulted in weakened cell proliferation and invasion ability and increased sensitivity to Bleomycin and Docetaxel.
Conclusion: This study revealed *SERPINE1* as the key gene for nicotine-related oral cancer, indicating that *SERPINE1* may be a novel prognostic indicator and therapeutic target for oral carcinoma.

Keywords: Nicotine; Oral neoplasm; Bioinformatics; Metastasis; Prognosis
1. Introduction

Oral cancer is a malignant tumor that occurs in the oral cavity and adjacent tissues, which is the most common type of head and neck cancer (HNC), with its incidence gradually increasing [1]. Although the combination has been applied in multiple treatment methods, the prognosis is still poor, with a 5-year survival rate of less than 50% [1]. Risk factors for oral cancer mainly include smoking, alcohol consumption, betel nut chewing, and HPV virus infection, among which smoking is an established factor [2]. Smoking affects human health in several ways, which leads to the development of chronic diseases such as cardiovascular disease and cancer [3].

Nicotine, the main active and addictive ingredient in tobacco (chemical formula C10H14N2), is an alkaloid found in the Solanaceae plant and is highly toxic [4]. Research has focused on its addictive properties previously, but rarely on its carcinogenic properties [5]. However, studies showed that the possible carcinogenic effect of nicotine cannot be ignored. For example, nicotine may promote the progression of various cancers by acting on nicotinic choline receptors [6]. Activation of nicotinic acetylcholine receptors may stimulate cell proliferation and inhibit apoptosis, thus leading to tumorigenesis [7]. Moreover, nicotine exposure promotes cell metastasis and confers drug resistance in HNC [8]. Nevertheless, the carcinogenic mechanism of nicotine is very complex and still unclear.

Aberrant expression of some genes may mediate the carcinogenesis of nicotine. For example, nicotine exposure can elevate the expression of α5 nicotinic acetylcholine receptor and Survivin in lung cells, which plays an important role in the
occurrence and development of lung adenocarcinoma [9]. Besides, nicotine exposure can regulate the abnormal expression of Prx1 and its interacting proteins CFL1 and PPP2R1A, thereby promoting the transformation of oral normal cells into cancer cells [10]. However, most of the literature focuses on a single gene or pathway, which may lead to biases in our understanding of nicotine's oncogenic mechanisms. Therefore, it is necessary to comprehensively study its molecular mechanism, which may help improve our understanding of oral cancer prevention and treatment.

In this study, we aimed to explore the dysregulated genes associated with nicotine-related oral cancer and screen out the key genes by data mining. Then, the expression and function of the key genes were further verified through big data analysis and experiments.

2. Materials and methods

2.1 Screening of the key gene in Nicotine-related oral cancer

2.1.1 Screening of the differentially expressed genes (DEGs)

Datasets regarding nicotine-treated oral cells were retrieved from the Gene Expression Omnibus (GEO) database (nebi.nlm.nih.gov/geo/). The DEGs between the nicotine-treated experimental and control groups were obtained by using the GEO2R [11]. The cut-off criteria of adjusted P-value < 0.05 and log2 |fold-change| >1.5 were used for identification of the DEGs.

2.1.2 Functional enrichment analysis of the DEGs
Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted to annotate the functions of the DEGs. The DAVID database (david.ncifcrf.gov) was used. P<0.05 was considered statistically significant.

2.1.3 Screening of the hub genes

To screen for the hub genes/proteins, the DEGs were submitted to STRING (cn.string-db.org) for calculation. The genes with a composite score of more than 0.4 were chosen. The Cytoscape was used for further calculation and visualization. Genes were sorted by degree and betweenness, respectively, and the top 15 genes were considered the hub genes. A Venn diagram was used to obtain the intersection of the two gene sets. The GEPIA tool (gepia.cancer-pku.cn) based on the TCGA (The Cancer Genome Atlas) database was used for evaluation.

2.2 Validation of the key genes through bioinformatics analysis

2.2.1 Expression levels of the key gene in HNC

The HNC cohort from the TCGA database was used. The associations of the key gene (SERPINE1) expression with the confounding factors were assessed. Univariate and multivariate Cox regression analysis was applied to verify the prognostic values of SERPINE1 and clinicopathological factors in HNC.

The prognostic value and possible functions of SERPINE1 in HNC were also assessed. The GEO database, ProggeneV2 (www.progtools.net/gene/), and TNMplot (tnmplot.com/analysis/) were used.
2.2.2 Prediction of intergenic interactions of *SERPINE1*

The NetworkAnalyst tool ([www.networkanalyst.ca](http://www.networkanalyst.ca)) was used to predict the target genes of *SERPINE1*.

2.2.3 Immune correlation analysis of *SERPINE1*

The TIMER (timer.cistrome.org/) and CIBERSORT (cibersortx.stanford.edu) algorithms were used to investigate whether *SERPINE1* expression was related to the tumor immune microenvironment.

2.2.4 Single-cell functional analysis

The CancerSEA database [12] was used to learn the roles of *SERPINE1* in individual HNC cells.

2.2.5 Drug sensitivity prediction

The possible effect of *SERPINE1* expression on the drug sensitivity was predicted by using The Genomics of Drug Sensitivity in Cancer (GDSC) database and the Gene Set Cancer Analysis (GSCA) [13] was used to help visualize the results.

2.3 Validations of the key genes by experimental assays

The experiment was approved by the ethics committee of our university. The tissue microarray was commercially purchased, and the consent of the patients had been obtained when collecting samples.

2.3.1 Tissue sample analysis

A tissue microarray of oral cancer patients (HOraC060PG01) was purchased from Shanghai Outdo Biotech Company. The characteristic of the patients was described
previously [14]. **SERPINE1** protein expression was measured by immunohistochemistry (IHC) assay. IHC staining was performed according to the manufacturer's instructions. Protein staining intensity was scored from 0 to 3, with 0 indicating negative, 1 weak, 2 moderate, and 3 strong. The percentage of positively stained cells was scored from 0 to 4, where 1 represents (1-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). Samples were scored as the product of percent staining and staining intensity, ranging from 0-12. A score of not less than 6 was considered "high" and the rest was considered "low".

### 2.3.2 Cell culture

CAL27, SAS, HSC-3 (oral cancer cell lines), and DOK (human dysplastic oral keratinocyte) from American Type Culture Collection were stored in the laboratory. Cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂.

DOK was intermittently treated with a low dose of Nicotine (1µM) for 6 months according to the reference [15]. The treated cells were named DOK/NIC.

### 2.3.3 Cell line analysis

**SERPINE1** was overexpressed in oral cancer tissues. Thus, the loss-of-function strategy was used to explore its roles. The RNA interference technique was utilized. Stable **SERPINE1**-knockdown cell lines were established according to the methods described recently [16]. Two cancer cell lines, CAL27 and SAS, were chosen for further exploration. The cells with stable **SERPINE1**-silence were named sh-**SERPINE1**-CAL27 and sh-**SERPINE1**-SAS, whereas the relevant controls were
(named sh-NC-CAL27 and sh-NC-SAS, respectively.

The mRNA and protein expressions of \textit{SERPINE1} in these cells were detected by qRT-PCR and western blot assays (Anti-PAI1 Rabbit monoclonal antibody, Abcam). The cell proliferation was assayed by CCK-8 and colony formation test. The invasive abilities of the cells were evaluated by transwell invasion assay.

2.4. Statistical analysis

For continuous variables, differences between groups were assessed using t-tests, ANOVA, or Wilcoxon rank sum tests, depending on the specific type of data. If ratio comparisons were involved, the chi-square test was chosen. Overall survival curves were calculated using the Kaplan-Meier method, and differences in survival rates were determined using the log-rank test. Multivariate Cox regression analysis was performed when multiple possible clinical factors were considered. A P-value or False Discovery Rate (FDR) of less than 0.05 was considered statistically significant. The statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc; USA)

3. Results

3.1 Screening for the key gene

3.1.1 Screening of the DEGs

GSE89923 [17], one gene expression profile that met the inclusion criteria, was identified. The dataset was retrieved and downloaded from the GEO database. The dataset contained six samples of normal human oral cells exposed to nicotine and
six samples of control cells. They were performed on the GPL570 platform of Affymetrix Human Genome U133 Plus 2.0 Array. The DEGs were screened from the comparison of the two groups. Finally, 123 up-regulated genes and 109 down-regulated ones were screened out (Figure 1A).

3.1.2 Functional annotation of the DEGs

We performed a GO analysis to investigate the possible biological processes of the DEGs. The results showed that the DEGs were enriched in hundreds of terms. Figure 1B listed the top 20 terms, such as cell adhesion, epidermal development, neutrophil chemotaxis, epithelial cell differentiation, and neutrophil chemotaxis. KEGG analysis showed the pathway terms. Figure 1C listed the top 20 terms, such as Pathways in cancer, IL-17 signaling pathway, TNF signaling pathway, Transcriptional misregulation in cancer, TGF-beta signaling pathway, and NF- kappa B signaling pathway.

3.1.3 SERPINE1 was selected as a key gene

The interactions of the DEGs were further explored using the STRING and Cytoscape software. In the network, genes were sorted by their degree and betweenness values, respectively (Figure 1D-E and Table 1). The top 15 hub genes were selected separately for Venn analysis (Figure 1F). The intersection contained 8 genes, including EGFR, CDH1, CXCL8, S100A7, SERPINE1, LCN2, EP300, and P13. The prognostic values of these genes in HNC were evaluated by using the GEPIA tool. SERPINE1 was eventually identified as a key gene for its possible effect on the prognosis of HNC patients and its high expression in HNC tissues (Table 1).
3.2 Validation of the roles of the key gene in HNC by bioinformatics methods

3.2.1 Expression of SERPINE1 in pan-cancer and its prognostic value in HNC cancer

The TIMER database was used to analyze the expression of SERPINE1 in pan-cancer. As displayed in Figure 2A, the expression of SERPINE1 was significantly higher in HNC, BRCA, COAD, ESCA, KIRC, READ, and other tumor tissues than that in normal tissues, respectively. Then the survival curves based on the TCGA and GSE68585, respectively, showed that the overall survival time of the HNC patients bearing high SERPINE1 expression was shorter than that of patients harboring low SERPINE1 expression (Figure 2B-C).

We evaluated the relationship between SERPINE1 expression and the clinicopathological features in HNC. As shown in Table 2, the expression of SERPINE1 was significantly associated with the N-stage and the number of lymph nodes (P < 0.05), indicating that SERPINE1 expression might have a correlation with lymph node metastasis (LNM).

Through multivariate-cox analysis of different variables in HNC patients, the data revealed that SERPINE1 expression level was an independent prognostic factor for HNC (Figure 2D).

The plot from the TNMplot tool showed that high SERPINE1 expression might have a relationship with enhanced metastatic abilities of oral cancer cells (Figure 2E).

3.2.2 Intergenic interaction prediction for SERPINE1
SERPINE1 may closely interact with 57 genes (Figure 2F). GO analysis and KEGG pathway enrichment analysis were also conducted. The top GO terms included Metabolic processes, Growth, Biological regulation, developmental processes, cellular processes, immune system processes, and cellular component tissue biogenesis (Figure 2G). The KEGG terms mainly involved Transcriptional misregulation in cancer, TGF-β signaling pathway, Apelin signaling pathway, Cell cycle, and Thyroid hormone signaling pathway (Figure 2H).

3.3.3 Analysis of SERPINE1 gene expression and tumor microenvironment

Evidence indicates that immune cells play an important role in tumor development, metastasis, and drug resistance [18]. We further explored the relationship between SERPINE1 and tumor immune cells. Different algorithms were used to calculate the relationship between the SERPINE1 expression levels and the infiltration of different immune cells.

As shown in Figure 3A, there were significant associations between SERPINE1 expression and the infiltration levels of dendritic cells, macrophages, CD4+ T cells, and CD8+ T cells (P<0.05). The CIBERSORT algorithms showed that SERPINE1 expression might correlate with the infiltration levels of CD4+ T cells, NKT cells, and macrophages in HNC samples (Figure 3B).

The above results confirmed that SERPINE1 might affect the infiltrates of immune cells in the cancer microenvironment.

3.2.4 Correlation analysis of SERPINE1 with the functional status of cancer cells

To further learn the possible roles of SERPINE1 in HNC monocytes, the single-
cell database CancerSEA was selected for assessment [12]. The results showed that \textit{SERPINE1} was positively correlated with metastasis (r=0.50, p<0.05), angiogenesis (r=0.35, p<0.05), hypoxia (r=0.42, p<0.05), and EMT (epithelial-mesenchymal transition) (r=0.36, p<0.05, \textbf{Figure 3C}).

\textbf{3.2.5 Drug sensitivity prediction}

To predict the possible effect of \textit{SERPINE1} expression on the chemosensitivity of cancer cells, the data from the GDSC database were evaluated. As shown in \textbf{Figure 3D}, \textit{SERPINE1} expression was negatively correlated with the sensitivity of cancer cells to several agents, such as 17-AAG, Bleomycin, and CHIR-99021. Among these drugs, Bleomycin (r=-0.347, FDR<0.01) and Docetaxel (r=-0.346, FDR<0.01) were clinically used for the treatment of oral cancer. Thus, they were chosen for further validation.

\textbf{3.3 Validation of \textit{SERPINE1} expression in oral cancer by experimental assays}

\textbf{3.3.1 Expression of \textit{SERPINE1} mRNA in nicotine-exposed oral cells and oral cancer cells}

The mRNA expression of \textit{SERPINE1} was detected in nicotine-exposed oral cells and oral cancer cells. The results showed that \textit{SERPINE1} mRNA expression was significantly higher in DOK/NIC and oral cancer cell lines than that in the control cells (\textbf{Figure 4A}), suggesting that treatment of nicotine might upregulate \textit{SERPINE1} expression in oral cells and \textit{SERPINE1} might be an oncogene for oral carcinoma.

\textbf{3.3.2 Expression of \textit{SERPINE1} protein in oral cancer tissues}
A tissue microarray comprising oral cancer samples was used for detection. **Figure 4B** showed that *SERPINE1* protein expression was significantly higher than that in the controls (P<0.05).

*SERPINE1* expression was markedly higher in samples with LNM than that in samples without LNM (P<0.05, **Figure 4C**). No associations were presented in comparisons regarding age, sex, and T-stages (**Figure 4D-G**).

### 3.3.3 Cell proliferation, invasive abilities, and drug sensitivity assessment

Both the mRNA and protein expression levels of *SERPINE1* in the *SERPINE1*-silenced cells were markedly lower than those in the control groups (P<0.05, **Figure 5A-B**).

The cell proliferation assays showed that the cell viabilities (**Figure 5C**) and colony formation capabilities (**Figure 5D**) were significantly weakened in the *SERPINE1*-knockdown cells (sh-*SERPINE1*-CAL27 and sh-*SERPINE1*-SAS) relative to the controls (P<0.05). Moreover, the invasive abilities of the *SERPINE1*-knockdown cells were significantly lower than that of the control cells (P<0.05, **Figure 5E**). The results implied that overexpression of *SERPINE1* might correlate with enhanced cell proliferation and invasive abilities of oral cancer cells.

To preliminarily verify the effect of *SERPINE1* expression on the chemosensitivity of oral cancer cells, cells were treated with 10 μg/ml Bleomycin and 10 nM Docetaxel for 48 hours, respectively. The results showed that the cell viability of *SERPINE1*-silenced cells was significantly lower than that of the controls (P<0.05, **Figure 5F**), indicating that overexpression of *SERPINE1* might confer the
chemoresistance of oral cancer cells to chemotherapy drugs.

4. Discussion

In the present study, SERPINE1 has been identified as the key gene that might be involved in nicotine-induced oral carcinogenesis and cancer progression. Overexpression of SERPINE1 in oral cancer tissues might be correlated with the LNM, which predicted poor prognosis. Thus, the downregulation of SERPINE1 in oral cancer cells resulted in weakened cell proliferation and cell invasive abilities. Moreover, SERPINE1 silencing resulted in increased cell sensitivity to Bleomycin and Docetaxel.

SERPINE1, a plasminogen activator inhibitor (PAI-1) protein-coding gene, locates in the long arm of chromosome 7 (7q21.3-q22) and encodes the SERPINE1 protein, a member of the serine protease inhibitor (serpin) superfamily, which rapidly inhibits fibrinogenesis and mediates a variety of pathological processes such as inflammation and cancer [19]. SERPINE1 is highly expressed in a variety of tumor tissues [20]. The evidence was substantially in line with the results of the present study that SERPINE1 was overexpressed in oral cancer cells and was linked with poor clinical outcomes in patients.

In silico analyses showed that SERPINE1 expression might be associated with the LNM of oral cancer. The single-cell functional analysis indicated that SERPINE1 expression was positively correlated with hypoxia, EMT, angiogenesis, and
metastasis. The occurrence of LNM in oral cancer patients usually predicts a poor prognosis [21]. Evidence showed that hypoxia might result in the infiltration of relevant cells including immune cells in cancer tissues, which facilitates angiogenesis and thus expedites cancer metastasis [22]. In addition, hypoxia can induce the expression of relevant genes, and then stimulate cells to undergo EMT through some factors such as FGF1, thereby promoting angiogenesis [23]. Thus, there seemed to be an inseparable relationship among hypoxia, EMT, and angiogenesis, which together promote the invasion and metastasis of cancer cells. Studies showed that a hypoxic environment can induce high expression of SERPINE1, which in turn promotes angiogenesis by activation of the EMT process, enhancing the proliferation, invasion, and invasion abilities of cancer cells [24, 25]. In the present study, after silencing SERPINE1 expression in oral cancer cells, not only was it observed that the cell proliferation ability was weakened, but the cell invasive ability was also significantly reduced. This result suggests that SERPINE1 expression enhances the malignant properties of oral cancer cells, while targeting SERPINE1 may significantly reverse the malignant phenotype of the cancer cells.

It is worth noting that SERPINE1 expression was predicted to have an association with the sensitivity of cancer cells to a series of drugs. Among the significantly related drugs, Bleomycin and Docetaxel have been applied in the clinical treatment of HNC, and therefore they were selected for further verification. Bleomycin as a drug in electrochemical therapy has been used in the treatment of HNC, which can exert cytotoxic effects in cancer cells by inducing reactive oxygen
species production; however, aberrant expressions of some genes, such as CoQ(10), can increase the resistance of oral cancer cells to Bleomycin [26]. Docetaxel has been used in first-line chemotherapy for HNC, as well as in the treatment of recurrent and metastatic HNC. Likewise, the emergence of resistance to Docetaxel presents a challenge for the treatment of oral cancer [27]. The results of the present study showed that after silencing SERPINE1 in oral cancer cells, the sensitivity of the cells to both Bleomycin and Docetaxel was significantly increased, suggesting that high expression of SERPINE1 may reduce the sensitivity of cancer cells to chemotherapy drugs.

To explore the carcinogenic mechanism of nicotine, a functional enrichment analysis of the DEGs related to nicotine-associated carcinogenesis was performed. These genes are enriched in many cancer-related signaling pathways, such as the IL-17 signaling pathway, TNF signaling pathway, and TGF-beta signaling pathway. These pathways may play important roles in tumorigenesis and development. For instance, IL-17 promotes tumorigenesis by regulating Beclin-1 ubiquitination [28]. The TNF signaling pathway mediates the mesenchymal transition of glioblastoma and plays a key role in its progression [29]. The data showed that several signaling pathways with different functions might be involved in the occurrence and development of nicotine-related oral cancer. The genes closely related to SERPINE1 were also functionally annotated, which were enriched in multiple signaling pathways. These pathways were scattered across various aspects of cellular functions, and they were not observed to be concentrated in any area. The data suggest that
*SERPINE1* may exert biological activities in tumorigenesis and development through multiple different signaling pathways. However, its specific mechanism needs further experimental verification.

We noted that *SERPINE1* expression in oral cancer may be associated with the infiltration of immune cells, including dendritic cells, macrophages, CD4+ T cells, and CD8+ T cells. The immune microenvironment was involved in the occurrence and progression of cancer. In general, CD8+ T-cell infiltration indicates a better prognosis in several tumors [30], while overexpression of *SERPINE1* predicts a poor prognosis. Nevertheless, a positive correlation between CD8+ T and *SERPINE1* expression was observed. The discrepancy might be due to the reason that *SERPINE1* promotes the infiltration of CD8+ T cells as only a part of its effect, but the influence of this part on the overall prognosis was not sufficient to counteract its other effects (such as the infiltration of tumor-associated macrophages) that may be detrimental to the prognosis. Therefore, the influencing factors of tumor prognosis should be comprehensively analyzed. These results suggest that there may be a correlation between *SERPINE1* expression and various immune cell infiltrations. However, the mechanism by which *SERPINE1* affects immune cell infiltration is unclear and needs to be further clarified in future experimental studies.

Several limitations might exist in the present study. First, the experimental validation only concerned in vitro assays. The roles of the genes were not verified in vivo. Second, the study focused on the effect of nicotine on the oral cavity. Future studies considering the larynx and hypopharynx may enhance our understanding of
the carcinogenic effects and mechanisms of nicotine. Third, whether other carcinogens of tobacco such as benzopyrene and NO can also cause the alteration of SERPINE1 expression is uncertain and has not been addressed in this study. Future studies need to explore this issue because it would help to deepen our understanding of the mechanism of nicotine carcinogenesis. Fourth, although this study explored the correlation between nicotine exposure and SERPINE1 expression through bioinformatics analysis and cytological verification, the causal relationship between them is not sufficient, and the mechanism of nicotine regulating SERPINE1 expression is still unclear. These problems should be taken into account in future experiments.

Despite the limitations, the present study has revealed that SERPINE1 might be a key gene that plays a crucial role in the genesis and development of Nicotine-related oral cancer, raising the possibility of SERPINE1 as a prognostic factor and a therapeutic target for oral carcinoma. Future experiments are needed to in-depth explore the molecular mechanisms.
Reference


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Legends

**Figure 1** (A) Heatmap of the DEGs screened from dataset GSE89923. The horizontal axis represents the names of the genes, and the right vertical axis represents the samples. Red represents the upregulated genes and blue represents the downregulated ones. (B-C) Enrichment analysis of the DEGs by GO (B) and KEGG (C). (D-E) The top 15 genes in a PPI network of the DEGs are sorted by Degree (D) and Betweenness (E) values. (F) Venn analysis of the top 15 hub genes ranked by the Degree and Betweenness values, respectively. The intersection contained 8 genes.

**Figure 2** (A) The expression of SERPINE1 in pan-cancer based on the TCGA database. *P<0.05; **P<0.01; ***P<0.001. SERPINE1 expression was significantly higher in HNC tissues than that in normal tissues (P < 0.001). (B-C) The survival curves showed that HNC patients with high SERPINE1 expression had a shorter overall survival time than those with low SERPINE1 expression (P < 0.05). B: TCGA; C: GSE65858. (D) Multivariate COX regression analysis with different variables indicated SERPINE1 as an independent prognostic factor for HNC (P < 0.05). (E) TNMplot presented that high expression of SERPINE1 was associated with metastasis (P<0.05). (F) The predicted targets of SERPINE1. The interaction plot showed 57 target genes that may be closely related to SERPINE1. (G-H) GO (G) and KEGG (H) enrichment analyses of the 57 genes.

**Figure 3** (A) The association between SERPINE1 expression and tumor purity as well
as the infiltration levels of several immune cells (TIMER algorithm). (B) Analysis of the relationship between the expression of SERPINE1 and the infiltration levels of 22 types of immune cells by the Cibersort algorithm. The darker color indicates a higher correlation (*P<0.05). (C) The correlation of SERPINE1 expression with malignant phenotypes in HNC tissues. Scatter plots showed positive correlations between SERPINE1 expression and malignant phenotypes, such as (a) Metastasis, (b) Hypoxia, (c) EMT, and (d) Angiogenesis. (D) Relationship between SERPINE1 expression and the drug sensitivity of cancer cells. Red represents a positive correlation while blue stands for a negative correlation.

**Figure 4** (A) The mRNA expression of SERPINE1 was higher in nicotine-treated oral cells (DOK/NIC) and oral cancer cell lines (Cal27, SAS, HSC-3) than that in DOK cells, respectively (*P<0.05). (B) The IHC scores of SERPINE1 protein expression in oral cancer tissues were markedly higher than that in the normal controls (*P<0.05). (C) The expression scores of SERPINE1 protein were higher in cancer samples with LNM than that without LNM (*P<0.05). (D) The scores of SERPINE1 expression were higher in the samples with high pathological stages than that with low stages (*P<0.05). (E-F) No associations were presented concerning Age (E), Sex (F), and T-stages (G) (ns, P>0.05).

**Figure 5** (A) The mRNA expression of SERPINE1 was significantly downregulated in the SERPINE1-silenced oral cancer cells (sh-SERPINE1-Cal27 or sh-SERPINE1-
SAS) compared with that of the control cells (sh-NC-Cal27 and sh-NC-SAS) (*P<0.05). (B) The changing trend of SERPINE1 protein expression was in line with the mRNA expression. (C) The cell proliferation abilities of the SERPINE1-silenced cancer cells were significantly lower than that of the control cells (*P<0.05). (D) The number of colony formations in the SERPINE1-silenced cells was significantly less than that in the control cells (*P<0.05). (E) The invasive abilities in the SERPINE1-silenced cells were significantly inhibited compared with those in the control cells (*P<0.05). (F) The administration of Bleomycin or Docetaxel significantly resulted in decreased cell viabilities in SERPINE1-silenced cells compared with the control cells (*P<0.05).

Table 1 The hub genes in the intersection based on a Venn analysis

Table 2 Association between SERPINE1 expression and clinicopathological factors in oral cancer (TCGA)
Table 1 The hub genes in the intersection based on a Venn analysis

<table>
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<th>Betweenness</th>
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<td>0.0025</td>
</tr>
<tr>
<td>S100A7</td>
<td>44</td>
<td>74.98333</td>
<td>2426.04387</td>
<td>UP</td>
<td>3.15E-03</td>
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<td>EP300</td>
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<td>72.65</td>
<td>1837.0896</td>
<td>UP</td>
<td>1.29E-03</td>
<td>0.41</td>
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<tr>
<td>LCN2</td>
<td>40</td>
<td>73.81667</td>
<td>801.31089</td>
<td>Down</td>
<td>1.01E-07</td>
<td>0.28</td>
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<tr>
<td>PI3</td>
<td>36</td>
<td>71.48333</td>
<td>1090.78971</td>
<td>UP</td>
<td>7.06E-07</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Note: Up/Down: Based on the TCGA cohort, compared with the normal group, the gene expression trends in HNC samples.
P (Exp): P value for the comparison of gene expression differences between HNC and normal groups.
P (HR): In the HNC cohort, the p-value of the Hazard Ratio for the effect of gene expression on prognosis.
Table 2 Association between SERPINE1 expression and clinicopathological factors in oral cancer (TCGA)

<table>
<thead>
<tr>
<th>Features</th>
<th>Classification</th>
<th>Number</th>
<th>SERPINE1 expression [Median (min, max)]</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&gt;65</td>
<td>129</td>
<td>12.6184 (6.5269,16.9370)</td>
<td>1.145</td>
<td>0.2521</td>
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<tr>
<td></td>
<td>≤65</td>
<td>213</td>
<td>12.7642 (8.3665,16.5453)</td>
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</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>106</td>
<td>12.9465 (6.5269,16.9730)</td>
<td>0.983</td>
<td>0.3254</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>237</td>
<td>12.6700 (8.3665,16.5453)</td>
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</tr>
<tr>
<td>T-stage</td>
<td>T1-T2</td>
<td>129</td>
<td>12.5206 (6.5269,16.9730)</td>
<td>1.475</td>
<td>0.1403</td>
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<tr>
<td></td>
<td>T3-T4</td>
<td>203</td>
<td>12.8323 (8.3665,16.5453)</td>
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<tr>
<td>N-Stage</td>
<td>N0</td>
<td>124</td>
<td>12.5670 (8.3665,16.9730)</td>
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<tr>
<td></td>
<td>N1-N3</td>
<td>163</td>
<td>12.9081 (8.5971,16.5453)</td>
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<tr>
<td>Number of lymph node</td>
<td>NO</td>
<td>173</td>
<td>12.4969 (6.5269,16.9730)</td>
<td>3.165</td>
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<td>170</td>
<td>12.9481 (8.5971,16.9481)</td>
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<td>M-stage</td>
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<td>12.7072 (6.5269,16.9730)</td>
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<td>0.6141</td>
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<tr>
<td></td>
<td>M1</td>
<td>2</td>
<td>13.1597 (12.0129,14.3065)</td>
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<tr>
<td>Histologic grade</td>
<td>G1-G2</td>
<td>259</td>
<td>12.7133 (8.3665,16.9730)</td>
<td>0.624</td>
<td>0.5326</td>
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<tr>
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<td>G3-G4</td>
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<td>12.7518 (6.5269,15.9377)</td>
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<tr>
<td>Clinical stage</td>
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<td>12.5206 (6.5269,16.9730)</td>
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<tr>
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<td>III- IV</td>
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<td>12.7867 (8.3665,16.5453)</td>
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</tbody>
</table>

Note: SERPINE1 expression values referred to the Transcript per million values