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Research Paper
Head and Neck Oncology

The role of SPP1 as a prognostic biomarker and therapeutic target in head and neck squamous cell carcinoma

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignancies and has a low 5-year survival rate. Mounting evidence suggests that oral potentially malignant disorders, such as oral leukoplakia (OLK), may progress to HNSCC. Given that OLK and HNSCC are often insidious and asymptomatic, the identification of markers of OLK malignant transformation and therapeutic targets in HNSCC is critical. Using various online tools and publicly available gene expression datasets, the secreted phosphoprotein 1 gene (*SPP1*) was identified as a significant differentially expressed gene among OLK, HNSCC, and non-cancerous tissues. *SPP1* mRNA levels were elevated in HNSCC tissues and were associated with cancer stage, tumor grade, and human papillomavirus infection status. High *SPP1* mRNA levels were correlated with poor overall survival of HNSCC patients. In contrast, *SPP1* mutations were not significantly associated with overall survival, although their frequency in HNSCC was very low (0.6%). Furthermore, *SPP1* expression levels in HNSCC were positively correlated with the infiltration of $CD4^+$ cells, macrophages, neutrophils, and dendritic cells. The study results suggest that *SPP1* may represent a diagnostic and prognostic biomarker, as well as a potential therapeutic target in HNSCC.

Key words: head and neck squamous cell carcinoma; *SPP1*; prognosis; target; bioinformatics.

Accepted for publication

Oral leukoplakia (OLK) is a term used to describe a predominantly white plaque of questionable risk that cannot be diagnosed as another known disease or disorder that carries no increased risk for cancer[1]. In humans, OLK is believed to be a precursor lesion of head and neck squamous cell carcinoma (HNSCC)[2]. As an oral potentially malignant disorder (OPMD), the malignant transformation rate for OLK ranges from 8% to nearly 18%[3]. Head and neck cancer is the sixth most common type of cancer, accounting for approximately 6% of all cancer cases worldwide[4]. In 2012, the global incidence of head and

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neck cancer was 529 500, and this is predicted to rise to 856 000 by 2035[5,6]. In Europe, the reported 5-year survival rate of head and neck cancer patients is less than 50%[7]. HNSCC represents the most common type of head and neck cancer, accounting for approximately 90% of head and neck malignancies[3].

The inactivation of tumor suppressor genes and activation of proto-oncogenes are critical genetic events that ultimately lead to the development of HNSCC. Various molecular techniques are currently used to identify genetic and epigenetic alterations in premalignant and invasive lesions, facilitating the delineation of a hypothetical model for HNSCC carcinogenesis[4]. The development and progression of HNSCC are thought to result from multiple stepwise alterations of cellular and molecular pathways in the squamous epithelium. Although a model of molecular progression from OPMD to invasive disease has been described previously[8], the precise mechanisms underlying the evolution of OPMD to invasive HNSCC remain poorly understood.

A key step to improving oral cancer outcomes is identifying the molecular factors driving disease initiation and progression, as these factors may represent good candidates for targeted therapies[9]. However, the accurate prediction of which OLK and other OPMDs may progress to HNSCC remains a significant clinical challenge[10]. Furthermore, despite recent progress in improving the prognosis of numerous human cancers, the prognosis of HNSCC has remained stagnant over the years. Since the 5-year survival rate is directly related to the disease stage at diagnosis, prevention and early detection efforts would not only decrease HNSCC incidence but also improve the long-term survival of HNSCC patients[11]. Nevertheless, early-stage OLK and HNSCC are often insidious and asymptomatic[11]. Therefore, the identification of biomarkers for malignant transformation of OLK, as well as prognostic biomarkers and therapeutic targets for HNSCC, is of high clinical importance.

Using microarray technology and bioinformatics, the aim of this study was to identify biomarkers of malignant transformation in OLK, as well as therapeutic targets and prognostic biomarkers in HNSCC. The secreted phosphoprotein 1 gene (*SPP1*) was identified as a gene differentially expressed among OLK, HNSCC, and non-malignant tissues. By conducting comprehensive bioinformatics analyses of *SPP1* expression in HNSCC using different large public databases, it was confirmed that *SPP1* may represent a useful therapeutic target and prognostic biomarker in HNSCC.

Materials and methods

Data acquisition from Gene Expression Omnibus (GEO)

GEO (https://www.ncbi.nlm.nih.gov/geo/) is a public functional genomics data repository containing high throughput gene expression and microarray data[12]. Four gene expression datasets (GSE85195[13], GSE26549[14], GSE30784[15], and GSE37991[16]) were downloaded from GEO, and the data of all samples classified according to the type of disease were analyzed. The probes were converted into gene symbols according to the annotation information of the platform. The GSE85195 dataset contained expression data from 15 OLK samples and 34 oral squamous cell carcinoma (OSCC) samples; GSE26549 contained 51 OLK samples and 35 OSCC samples; GSE30784 contained 167 OSCC samples and 45 non-cancerous samples; GSE37991 contained 40 OSCC samples and 40 non-cancerous samples.

Identification of differentially expressed genes (DEGs) using GEO2R

Differences in DEGs between OLK and OSCC and between OSCC and non-cancerous tissues were identified using GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/), which is an interactive web tool that allows users to compare two or more GEO datasets to identify DEGs across experimental conditions. The adjusted *P*-values and Benjamini and Hochberg false discovery rates were used to identify statistically significant genes and eliminate false-positives, respectively. Probe sets without corresponding gene symbols were removed, and genes with more than one probe set were averaged. Genes with a logFC (fold change) >1 and an adjusted *P*-value <0.05 were considered statistically significant DEGs.

ONCOMINE

ONCOMINE (www.oncomine.org) is an integrated online cancer DNA and RNA microarray database that facilitates genome-wide expression analyses in various cancer types[17]. The mRNA levels of *SPP1* were assessed in different cancer tissues and their corresponding adjacent non-malignant tissues. Statistical significance was determined using the Student *t*-test. The following criteria were used in the analyses: *P*-value <0.01, fold change >2, gene rank <10%.

Human Protein Atlas

The Human Protein Atlas (https://www.proteinatlas.org) is a database containing immunohistochemistry-based expression data for nearly 20 common cancer types, with at least 12 tumors for each tumor type[18]. The database allows users to identify tumor type-specific protein expression patterns. In this study, the protein levels of SPP1 were assessed in normal oral mucosa and head and neck cancer tissues.

UALCAN

UALCAN (http://ualcan.path.uab.edu) is an interactive web resource containing RNA-seq and clinical data of 31 cancer types from The Cancer Genome Atlas (TCGA) database. UALCAN can be used to analyze the transcription levels of genes of interest in tumor and non-malignant samples, and associate mRNA levels with clinicopathological characteristics[19]. Using UALCAN, the mRNA levels of *SPP1* in primary HNSCC tissues and the association between *SPP1* expression levels and clinicopathological features were assessed. Statistical significance was determined using the Student *t*-test; $P < 0.05$ was considered statically significant.

Kaplan–Meier Plotter

The prognostic value of *SPP1* mRNA expression levels in HNSCC was determined using Kaplan–Meier Plotter (http://kmplot.com/analysis/)[20]. Cancer patients were stratified into high and low *SPP1* expression groups based on the median values of mRNA expression. Kaplan–Meier curves, hazard ratios (HR), 95% confidence intervals (CI), and *P*-values were obtained from the Kaplan–Meier Plotter web tool. *P*-values <0.05 were considered statically significant.

cBioPortal

cBioPortal (www.cbioportal.org) is an online open-access resource for exploring, visualizing, and analyzing multidimensional cancer genomics data[21]. Using cBioPortal, the genomic profiles of *SPP1* were analyzed, including mutations and putative copy number alterations (CNA) from GISTIC (Genomic Identification of Significant Targets in Cancer). The relationship of *SPP1* mutations with overall survival (OS) of HNSCC patients

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was assessed by Kaplan–Meier analysis; the log-rank test was used to determine the significance of the survival in different groups, and *P*-values <0.05 were considered statically significant.

Tumor Immune Estimation Resource (TIMER)

TIMER (https://cistrome.shinyapps.io/timer/) is a reliable, intuitive tool that provides systematic evaluations of the infiltration of different immune cells and their clinical impacts[22]. 'Gene module' was used to evaluate the correlation between *SPP1* mRNA levels and immune cell infiltration. 'Survival module' was used to evaluate the correlation between clinical outcomes, immune cell infiltration, and *SPP1* expression levels.

Gene Expression Profiling Interactive Analysis (GEPIA2)

GEPIA2 (http://gepia2.cancer-pku.cn/) contains RNA sequencing data of 9736 tumors and 8587 normal samples from TCGA and GTEx, obtained using a standard processing pipeline[23]. GEPIA2 provides key interactive and customizable functions, including differential expression analysis, profiling plotting, correlation analysis, patient survival analysis, similar gene detection, and dimensionality reduction analysis. Using GEPIA2, similar gene detection analysis was performed to identify the top 100 similar genes of *SPP1* in HNSCC.

Metascape

Metascape (http://metascape.org) is a reliable, intuitive tool for gene annotation and pathway enrichment analysis[24]. Using Metascape, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the top 100 similar genes of *SPP1* were conducted. Additionally, enrichment analysis was performed for biological process, cellular component, and molecular function gene ontology (GO) terms. GO terms with a minimum overlap value of 3, *P*-value <0.05, and enrichment value <3 were considered as significantly enriched terms. Protein–protein interaction analysis was performed using BioGrid, InWeb_IM, and OmniPath. Furthermore, the Molecular Complex Detection (MCODE) algorithm was used to identify network hubs.

TRRUST analysis

TRRUST (https://www.grnpedia.org/trrust/) can be used to identify transcriptional regulatory networks based on 8444 transcription factor (TF)–target interactions for 800 human TFs[25]. Using TRRUST, TF potentially regulating *SPP1* were assessed.

LinkedOmics

LinkedOmics (http://www.linkedomics.org/) is a publicly available portal tool that provides comprehensive multi-omics data across 32 TCGA cancer types[26]. Using the 'LinkInterpreter' module, we performed kinase target and miRNA target enrichment analysis for SPP1 in HNSCC. Gene set enrichment analysis was also performed using gene sets containing at least three genes and a simulation factor of 500. Statistical significance was determined using the Spearman correlation test. *P*-values <0.01 were considered statistically significant.

Results

Identification of DEGs in OLK, HNSCC, and non-cancerous tissues

After standardization of the microarray data, 5645 DEGs were identified in GSE85195, seven in GSE26549, 2532 in GSE30784, and 2106 in GSE37991. Only four genes (*DCT*, *TYRP1*, *SPP1*, *FMO2*) were shared DEGs among the four datasets (Supplementary Material Fig. S1).

Aberrant expression of DEGs in HNSCC patients

The *DCT*, *TYRP1*, *SPP1*, and *FMO2* mRNA levels in HNSCC and non-malignant tissues were assessed using ONCOMINE. *SPP1* mRNA levels were significantly higher in HNSCC tissues compared with non-malignant tissues, whereas the mRNA levels of *DCT*, *TYRP1*, and *FMO2* were significantly lower in HNSCC (Supplementary Material Fig. S2). Hence, a further evaluation was performed to determine the potential prognostic and therapeutic value of *SPP1* in HNSCC. A detailed comparison of *SPP1* mRNA levels in HNSCC and normal tissues in different ONCOMINE datasets is shown in Table 1. In the Ginos dataset, *SPP1* was overexpressed in HNSCC tissues compared with non-malignant tissues with a fold change of 43.614 ($P = 1.31 \times 10^{-20}$)[27]; in the Peng[28] and Cromer[29] datasets, it was found that *SPP1* mRNA expression was 11.215 ($P = 8.04 \times 10^{-24}$) and 25.599 ($P = 3.81 \times 10^{-6}$) times higher in HNSCC than in non-malignant tissues, consistent with previous findings[30–33].

Analyses in the Human Protein Atlas revealed that SPP1 protein levels were low in normal oral mucosa tissues (3/3), whereas head and neck cancer tissues (3/4) exhibited moderate SPP1 protein levels (Supplementary Material Fig. S3), confirming SPP1 overexpression in HNSCC.

Association between *SPP1* mRNA levels and clinicopathological characteristics of HNSCC patients

SPP1 expression was further analyzed by UALCAN based on the TCGA database, which is a comprehensive archive of tumor data. It was confirmed that *SPP1* mRNA levels were significantly higher in HNSCC than non-malignant tissues ($P = 2.825 \times 10^{-12}$; Fig. 1A). Next, the relationship between *SPP1* mRNA levels and the clinicopathological characteristics of HNSCC patients were analyzed, including cancer stage, tumor grade, and human papillomavirus (HPV) status. *SPP1* mRNA levels were significantly associated with cancer stage, with advanced-disease patients expressing higher levels of *SPP1* (Fig. 1B). Patients with stage 4 HNSCC exhibited the highest *SPP1* mRNA levels. Similarly, *SPP1* mRNA levels were significantly associated with the tumor grade (Fig. 1C). HNSCC patients with tumor grade 2/3 showed the highest *SPP1* mRNA levels. Interestingly, *SPP1* mRNA levels were significantly associated with HPV status: the 41 HPV-positive HNSCC patient cases had lower *SPP1* mRNA levels than the 80 HPV-negative patient cases (Fig. 1D).

Prognostic value of mRNA expression of *SPP1* in HNSCC patients

Next, Kaplan–Meier analyses were performed to assess the prognostic value of *SPP1* mRNA levels in HNSCC patients. Interestingly, *SPP1* mRNA levels were significantly associated with the HNSCC patient prognosis (Fig. 2). Notably, high *SPP1* mRNA levels were associated with poor OS in HNSCC (HR 1.33, 95% CI 1.02–1.74, $P = 0.035$).

Relationship between *SPP1* mutations and HNSCC patient survival

The frequency of *SPP1* mutations in HNSCC patients was analyzed using cBioPortal. Interestingly, a low *SPP1*

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Table 1. mRNA levels of *SPP1* in different types of HNSCC tissues and normal tissues at the transcriptome level (ONCOMINE).

Type	Fold change	*P*-value	*t*-test	References
Head and neck squamous cell carcinoma (oral cavity, oropharynx, hypopharynx, larynx, sinus) vs normal	43.614	1.31×10^{-20}	17.052	Ginos et al.[27]
Oral cavity squamous cell carcinoma vs normal	11.215	8.04×10^{-24}	14.489	Peng et al.[28]
Hypopharyngeal squamous cell carcinoma vs normal	25.599	3.81×10^{-6}	10.741	Cromer et al.[29]
Tongue squamous cell carcinoma vs normal	2.576	2.16×10^{-6}	5.671	Ye et al.[30]
Tongue squamous cell carcinoma vs normal	15.528	2.52×10^{-9}	7.018	Estilo et al.[31]
Oral cavity squamous cell carcinoma vs normal	4.598	1.79×10^{-4}	4.535	Toruner et al.[32]
Tongue squamous cell carcinoma vs normal	5.325	7.68×10^{-8}	5.997	Talbot et al.[33]

HNSCC, head and neck squamous cell carcinoma.

mutation rate (0.6%; 3/496) was found in HNSCC patients (Supplementary Material Fig. S4A, B). Kaplan–Meier analysis revealed that *SPP1* mutations had no significant impact on OS ($P = 0.207$; Supplementary Material Fig. S4C). However, given that all three patients with *SPP1* alterations were censored alive (12, 34, and 66 months), the relationship between *SPP1* mutations and HNSCC patient survival merits further investigation.

Relationship between *SPP1* and immune cell infiltration in HNSCC patients

Comprehensive analyses of the relationship between *SPP1* expression levels and immune cell infiltration were performed using the TIMER database. *SPP1* expression was positively correlated with the infiltration of $CD4^+$ cells (correlation = 0.198, $P = 1.21 \times 10^{-5}$), macrophages (correlation = 0.36, $P = 3.42 \times 10^{-16}$), neutrophils (correlation = 0.125, $P = 6.36 \times 10^{-3}$), and dendritic cells (correlation = 0.293, $P = 4.99 \times 10^{-11}$; Fig. 3A). Importantly, after correcting for $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells, it was found that B cells ($P = 0.045$) and *SPP1* levels ($P = 0.031$) were significantly associated with the clinical outcome of HNSCC patients (Fig. 3B).

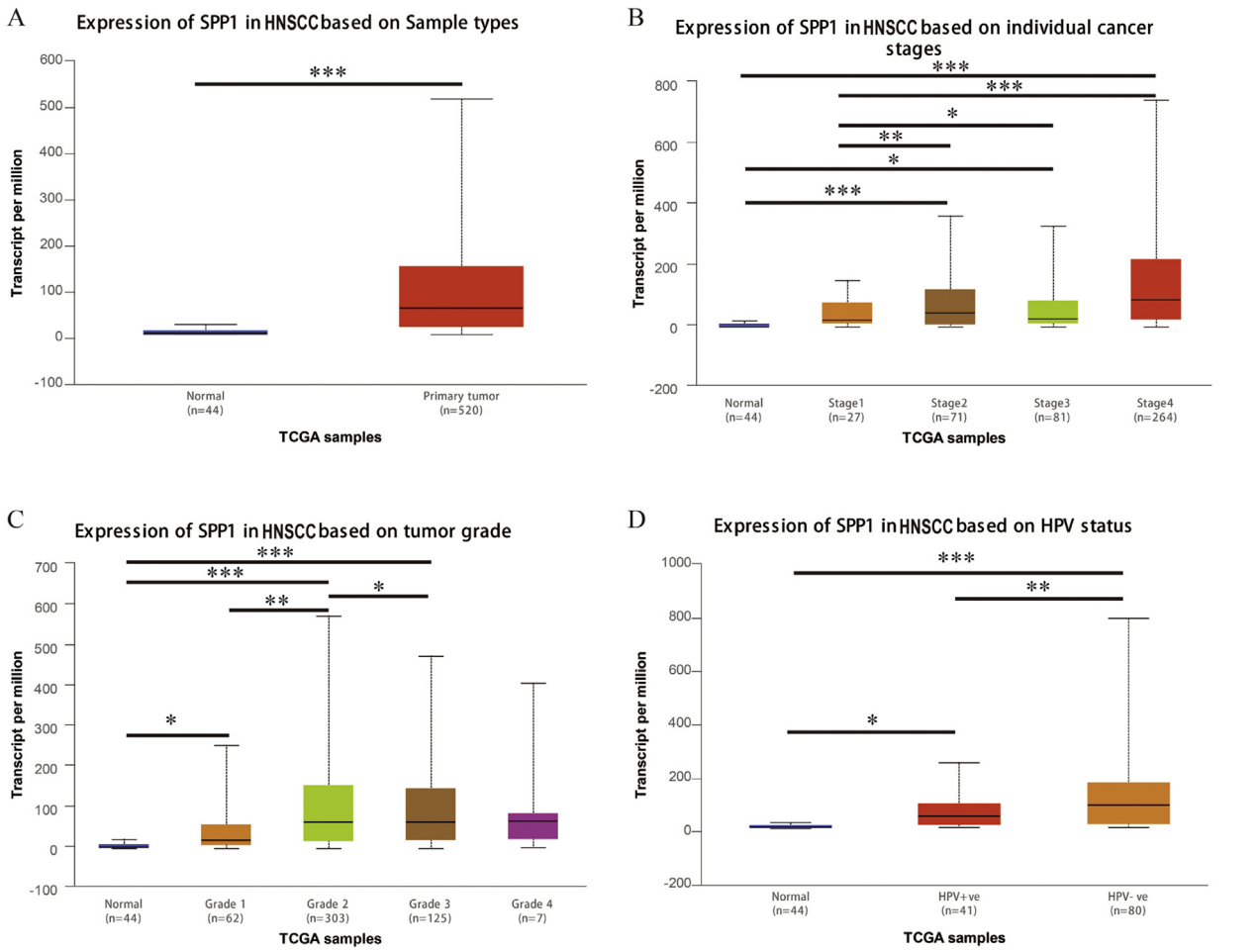

Fig. 1. Transcription of *SPP1* and relationship between mRNA expression of *SPP1* and clinicopathological parameters in HNSCC (UALCAN). The transcriptional level of *SPP1* in HNSCC tissues was significantly elevated (A). The mRNA expression of *SPP1* was significantly correlated with patients' individual cancer stages (B), tumor grades (C), and HPV status (D). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

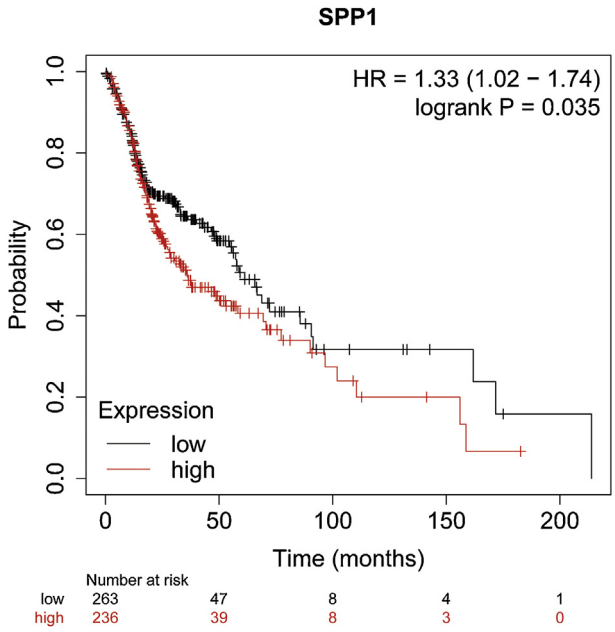

Fig. 2. Prognostic value of mRNA expression of *SPP1* in HNSCC patients (Kaplan–Meier Plotter). Higher mRNA expression of *SPP1* was associated with poorer overall survival in HNSCC patients.

Functional enrichment analysis of *SPP1* co-expressed genes in HNSCC patients

Using GEPIA2, the top 100 genes that are co-expressed with *SPP1* in HNSCC were identified. GO and KEGG pathway enrichment analyses revealed that the top 20 GO terms and KEGG pathways could be classified into four functional groups: GO biological process (12 items), GO molecular function (four items), GO cellular component (two items), and KEGG pathways (two items; Supplementary Material Fig. S5A, B and Table S1)[34–38]. *SPP1* co-expressed genes were enriched in cell activation and metabolism-related biological processes, including myeloid leukocyte activation, pentose biosynthesis, macrophage activation, positive regulation of cytokine secretion, and icosanoid metabolism. Enriched molecular functions included xenobiotic transmembrane transporter activation, indanol dehydrogenase activation, and complement binding, while ficolin-1-rich granule and secretory granule lumen were the enriched cellular components. Lysosome and glutathione metabolic pathways were the two KEGG pathways enriched among the *SPP1* co-expressed genes.

To understand more fully the role of *SPP1* co-expressed genes in HNSCC, a protein–protein interaction enrichment analysis was performed using Metascape. The two most significant MCODE components were extracted from the protein–protein interaction network; pathway and biological process enrichment analyses were performed for each MCODE component. The pentose phosphate pathway (pentose phosphate cycle), glucose 6-phosphate metabolic pathway, leukocyte degranulation, and regulated exocytosis were significantly enriched (Supplementary Material Fig. S5C, D).

Transcriptional regulators, kinase targets, and miRNA targets of *SPP1* in HNSCC

Next, the potential transcriptional regulators, kinase targets, and miRNA targets of *SPP1* were explored using the TRRUST and LinkedOmics databases. Twelve transcription factors that potentially regulate *SPP1* expression were identified (Table 2)[39–49]. CEBPA, ERG, FOXD3, POU5F1, and TFCP2 were predicted to activate *SPP1* expression, whereas HDAC1, HTATIP2, and ING4 were predicted to repress its expression. LYN and FYN were identified in the SPP1-kinase network, and 11 miRNAs (*MIR-328*, *MIR-498*, *MIR-452*, *MIR-218*, *MIR-96*, *MIR-137*, *MIR-324-5P*, *MIR-511*, *MIR-126*, *MIR-18A/MIR-18B*, *MIR-212/MIR-132*) were found in the SPP1-miRNA network (Table 3).

Discussion

OLK is a common OPMD that can progress to HNSCC. Mounting evidence sug-

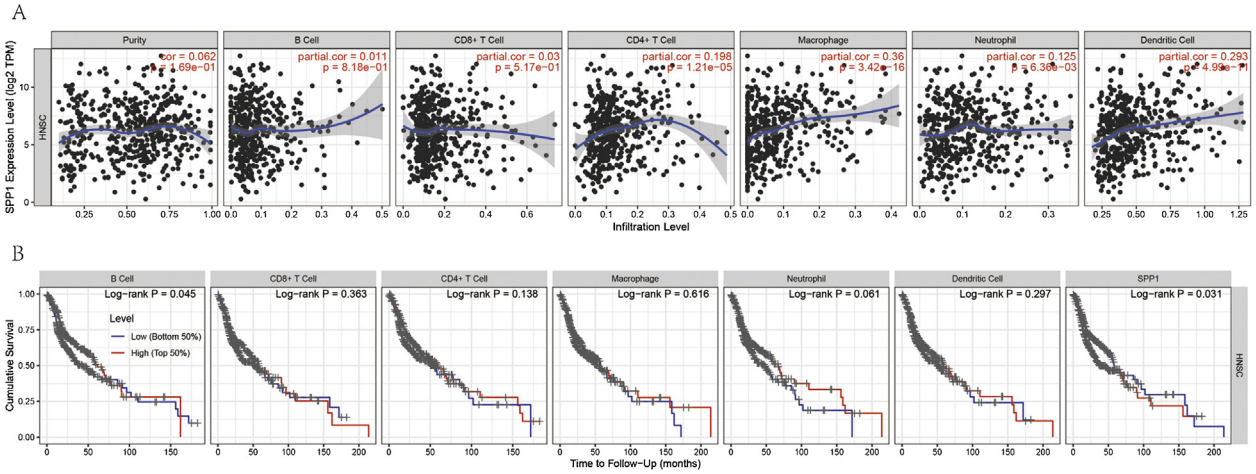

Fig. 3. Correlation between different expression levels of *SPP1* and immune cell infiltration (TIMER). *SPP1* expression was positively associated with the infiltration of $CD4^+$ cells, macrophages, neutrophils, and dendritic cells (A). The Cox proportional hazards model was used, correcting for the following confounding factors: $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, dendritic cells. B cells and *SPP1* expression were significantly associated with the clinical outcome of HNSCC patients (B).

Table 2. Transcription factors that regulate *SPP1* (TRRUST).

Transcription factors	Description	Mode of regulation	References
CEBPA	CCAAT Enhancer Binding Protein Alpha	Activation	Liu et al.[39]
ERG	ETS Transcription Factor ERG	Activation	Flajollet et al.[40]
FOXD3	Forkhead Box D3	Activation	Guo et al.[41]
POU5F1	POU Class 5 Homeobox 1	Activation	Guo et al.[41]
TFCP2	Transcription Factor CP2	Activation	Yoo et al.[42], Yoo et al.[43]
HDAC1	Histone Deacetylase 1	Repression	Sharma et al.[44], Pazolli et al.[45]
HTATIP2	HIV-1 Tat Interactive Protein 2	Repression	Zhao et al.[46], Tong et al.[47]
ING4	Inhibitor of Growth Family Member 4	Repression	Colla et al.[48]
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1	Unknown	Wang et al.[49]
POU2F1	POU Class 2 Homeobox 1	Unknown	Wang et al.[49]
POU2F2	POU Class 2 Homeobox 2	Unknown	Wang et al.[49]
SP1	Sp1 Transcription Factor	Unknown	Wang et al.[49]

Table 3. The enriched target networks of *SPP1* in HNSCC (LinkedOmics).

Enriched target	Description	Leading edge number	*P*-value
Kinase_LYN	LYN proto-oncogene, Src family tyrosine kinase	19	0
Kinase_FYN	FYN proto-oncogene, Src family tyrosine kinase	18	0
MIR-328	AGGGCCA	24	0
MIR-498	GCTTGAA	34	0
MIR-452	TGCAAAC	35	0
MIR-218	AAGCACA	104	0
MIR-96	GTGCCAA	81	0
MIR-137	AAGCAAT	68	2.004×10^{-3}
MIR-324-5P	GGGATGC	14	4.3011×10^{-3}
MIR-511	AAAGACA	54	6×10^{-3}
MIR-126	TAATAAT	75	6×10^{-3}
MIR-18A, MIR-18B	GCACCTT	32	6.0852×10^{-3}
MIR-212, MIR-132	GACTGTT	45	8.0808×10^{-3}

HNSCC, head and neck squamous cell carcinoma.

gests that HNSCC initiation and progression result from multiple stepwise alterations of cellular and molecular pathways in the squamous epithelium[8]. Chu et al.[50] performed a salivary proteomic analysis and identified complement factor H (CFH), fibrinogen alpha chain (FGA), and alpha-1-antitrypsin (SERPINA1) as potential salivary markers of HNSCC. NANOG has also been reported as a clinically relevant biomarker of early-stage HNSCC[51]. Furthermore, epidermal growth factor receptor gene (*EGFR*) copy number alterations have been proposed as a potential marker of OPMD malignant transformation[52]. However, the mechanisms underlying the progression from OPMD to invasive cancer remain elusive[10]. Understanding the molecular processes involved in the development and progression of HNSCC is of high clinical importance. The combination of clinical, histopathological, and molecular examinations will significantly advance the prevention, early detection, and treatment of HNSCC[53]. Therefore, the discovery of biomarkers of OLK malignant transformation and the identification of prognostic biomarkers and therapeutic targets for HNSCC are crucial.

In this study, four mRNA microarray datasets were analyzed to identify DEGs among OLK, OSCC, and non-cancerous tissues. Four DEGs were common among the four datasets. Since *SPP1* was the only shared DEG upregulated in HNSCC, its potential value in HNSCC prognosis and treatment was further analyzed. *SPP1* encodes secreted phosphoprotein 1, a glycosylated phosphoprotein found in all body fluids and the proteinaceous matrix of mineralized tissues, delivering signals either as a cell attachment protein or as a secreted molecule. *SPP1* expression is enhanced by numerous toxicants[54] and *SPP1* is involved in various biological processes, including the regulation of gene expression, monocyte/macrophage migration, and inhibition of apoptosis, potentially promoting cell survival in response to toxicant injury[54]. Although *SPP1* upregulation has been reported in various malignancies[54], its role in HNSCC is poorly understood. To obtain further insight into the role of *SPP1* in HNSCC, *SPP1* expression patterns in HNSCC were analyzed, as well as its relationship with clinicopathological characteristics, immune cell infiltration, and the patient prognosis.

Devoll et al.[55] reported that *SPP1* was not expressed in the normal oral epithelium. Intracellular and intercellular immunoreactivity was observed in 75% of hyperplasias, 57% of dysplasias, 54% of carcinomas in situ, and 67% of OSCCs[55]. Coppola et al.[56] observed high cytoplasmic SPP1 levels in 100% of gastric carcinomas, 85% of colorectal carcinomas, 82% of transitional cell carcinomas of the renal pelvis, 81% of pancreatic carcinomas, 72% of renal cell carcinomas, 71% of lung and endometrial carcinomas, 70% of esophageal carcinomas, 58% of HNSCC, and 59% of ovarian carcinomas[56]. Additionally, Le et al.[57] showed that SPP1 plasma levels were elevated in HNSCC patients with hypoxic tumors. In the present study, *SPP1* was identified as a significant DEG among OLK, HNSCC, and non-cancerous tissues. Importantly, *SPP1* mRNA levels were significantly higher in HNSCC tissues than in non-malignant tissues, suggesting that *SPP1* might represent a potential OLK malignant transformation and HNSCC biomarker.

Celetti et al.[58] showed that *SPP1* expression was strongly correlated with advanced stage, high grade, metastatic

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disease, and poor survival in patients with laryngeal squamous cell carcinoma. Similarly, Coppola et al.[56] found that the SPP1 score was significantly correlated with tumor stage in bladder ($P = 0.01$), colon ($P = 0.004$), kidney ($P = 0.0001$), larynx ($P = 0.035$), mouth ($P = 0.046$), and salivary gland ($P = 0.011$) tumors. In the present study, it was found that *SPP1* mRNA levels in HNSCC were significantly associated with cancer stage, tumor grade, and HPV status. Polat et al.[59] reported that head and neck cancer patients exhibited elevated SPP1 plasma levels 24 hours after surgery. Four weeks after tumor resection, SPP1 plasma levels decreased to baseline levels mirroring the pre-treatment situation. This prolonged increase in SPP1 plasma levels was attributed to wound healing[59]. Interestingly, Overgaard et al.[60] found that high SPP1 plasma levels were associated with poor outcomes after radiotherapy in patients with head and neck cancer. They also found that high plasma levels of SPP1 could predict clinically relevant hypoxia and identify patients who could benefit from hypoxia modulation during radiotherapy[60]. Qin et al.[61] found that nasopharyngeal carcinoma patients with high *SPP1* expression had a lower OS rate compared with those patients who had low *SPP1* expression. Consistently, we found that high *SPP1* mRNA levels were associated with poor OS in HNSCC patients. These findings suggest that SPP1 may represent a therapeutic target and prognostic biomarker in HNSCC.

Figure 2 shows the relationship between the expression of *SPP1* and the prognosis of HNSCC. The survival curve shows that between about 2 years and 8 years of HNSCC, the survival rate of patients with high expression of *SPP1* is lower than that of patients with low expression; however, at about 8 years, the curves overlap. Then after 8 years, the survival rate of patients with high expression of SPP1 is much lower than that of patients with low expression of *SPP1*. We speculate that there were some confounding factors in the analysis of the prognosis of HNSCC with different *SPP1* expression. There might be other risk factors that have not been considered, and new findings may be identified in a stratified analysis. It can be seen in Fig. 1 that there were differences in the expression of *SPP1* in the various stages, grades, and HPV infection states of those with HNSCC, which suggests that SPP1 may play a complex role in the tumorigenesis and prognosis of HNSCC. Therefore, the simple classification of high and low expression of *SPP1* can only be used as a potential prognostic indicator of HNSCC. However, data in the database were not sufficiently detailed to allow for a stratified analysis of the prognosis. The definite role of SPP1 in the prognosis of HNSCC may need to be verified by multicenter clinical data with large samples.

Briones-Orta et al.[62] assessed the relevance of *SPP1* gene variants in cancer progression and metastasis. Overexpression of individual *SPP1* splice variants was often associated with an unfavorable prognosis. For particular cancer types, the detection of specific *SPP1* splice variants predicted the patient prognosis, suggesting that *SPP1* splice variant analysis could guide treatment decision-making and predict patient survival[62]. In the present study, a low *SPP1* mutation rate (0.6%) was observed in HNSCC patients; genetic alterations in *SPP1* were not associated with HNSCC patient survival. However, given that the number of patients with *SPP1* mutations was extremely low, the relationship between *SPP1* mutations and HNSCC patient prognosis merits further investigation. Additionally, future studies are required to address the association between *SPP1* expression and the HNSCC patient prognosis when other variables, such as genetic variations and comorbidities, are taken into account.

Klement et al.[63] reported that *SPP1* was highly expressed in colon carcinoma cells and $CD11b^{+}Ly6C^{lo}Ly6G^{+}$ myeloid cells found in the tumor microenvironment; they concluded that SPP1 may act as an immune checkpoint, contributing to CTLA-4/PD-1/PD-L1-independent immune suppression and resistance to immune checkpoint inhibitors[63]. In this study, we found that *SPP1* expression levels were positively correlated with the infiltration of $CD4^{+}$ cells, macrophages, neutrophils, and dendritic cells.

Increasing evidence supports the potential usefulness of SPP1 as a therapeutic target in HNSCC. Celetti et al.[58] reported the involvement of SPP1 in mitogenic signaling and the migration of carcinoma cells. In the present study, it was found that myeloid leukocyte activation (GO:0002274), xenobiotic transmembrane transporter activation (GO:0042910), and lysosomal function (hsa04142) were enriched among the top 100 *SPP1* co-expressed genes. We also identified CEBPA among other transcription factors potentially regulating *SPP1* expression. Further, kinases and miRNAs that might regulate *SPP1* expression and function were identified, affecting the ability of SPP1 to promote HNSCC progression.

There are three isoforms of SPP1: OPN-a, OPN-b, and OPN-c, which play different roles in various cancers[64]. OPN-c has been identified as an indicator of invasive cancer and a prognostic marker of breast precancerous lesions[65]. Sun et al.[66] found that all *SPP1* transcripts promoted tumorigenesis in vivo. *SPP1* transcripts might regulate monocyte activation by increasing the expression of transforming growth factor β1 (TGF-β1) and monocyte chemoattractant protein 1 (MCP-1), and different transcripts have been shown to have similar effects on monocyte differentiation[66]. The interaction between SPP1 and CD44v6 can promote the metastasis of colorectal cancer. The expression of OPN-b and OPN-c has been found to be upregulated in gastric cancer, OPN-b may protect gastric cancer cells from apoptosis, and OPN-c may be involved in cancer metastasis[67]. Similarly, OPN-b has been observed to inhibit the apoptosis of glioma cells, while OPN-c has been shown to be involved in the invasion of glioma cells[68]. Soft tissue sarcoma patients with high levels of OPN-a and OPN-b were found to have an increased risk of death, while patients who underwent radiotherapy were found to have a higher risk of death with high levels of OPN-b and OPN-c[69]. However, there are no reports on the three isoforms of SPP1 (OPN-a, OPN-b, and OPN-c) in regard to HNSCC, so this represents a further direction of investigation, to explore the role of SPP1 in tumorigenesis and the progression of HNSCC.

There are some limitations to this study. First, as this study was based on a bioinformatics analysis with an in silico methodology, the role of SPP1could not be determined completely; hence, this study provides only a preliminary extensive analysis of SPP1. In order to elucidate its role, further exploration through studies with a more rigorous and detailed experimental design is required. Moreover, squamous cell carcinoma still lacks signicantly effective prognostic biomarker or therapeutic target, which need more studies. The investigation of SPP1 in this study could serve as a reference for finding more useful markers of squamous cell carcinoma in the future. Second, DEGs were derived from the GEO database, with the cancer datasets screened for OSCC. In TCGA, ONCOMINE, and the other databases, the disease type was only shown as HNSCC and not further subdivided into OSCC and oropharyngeal squamous cell carcinoma, which is considered a distinct subtype because of the high rate of HPV infection. Therefore, in silico analysis based on these databases could only pre-

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sume the potential effect of SPP1 in HNSCC. SPP1 might lead to prognostic differences in oropharyngeal squamous cell carcinoma. However, this will require further database development and supporting data from large multicenter clinical studies to elucidate. Third, it was found that the mutation rate of *SPP1* in HNSCC was very low, and no relationship between *SPP1* mutation and the prognosis was found. This is only a preliminary demonstration of the association between *SPP1* mutation and the prognosis from the cBioPortal. In order to further study the association between *SPP1* mutation and the prognosis, a multicenter clinical study with a large sample is necessary, and the mutation in normal controls should also be considered.

In summary, *SPP1* was identified as a significant DEG among OLK, HNSCC, and non-cancerous tissues. *SPP1* mRNA levels were elevated in HNSCC tissues. Importantly, *SPP1* expression levels were correlated with cancer stage, tumor grade, and HPV status in HNSCC patients. High *SPP1* mRNA levels predicted poor HNSCC patient survival. In contrast, *SPP1* mutations could not predict HNSCC patient survival, although the frequency of *SPP1* mutation was extremely low. Moreover, *SPP1* expression levels were correlated with the infiltration of different immune cells, including $CD4^+$ cells, macrophages, neutrophils, and dendritic cells. The results presented here provide further insight into the role of SPP1 in the initiation and progression of HNSCC and suggest that SPP1 is a promising biomarker for HNSCC and a potential therapeutic target. However, large cohort studies are required to confirm the study findings and validate the clinical usefulness of SPP1 as a prognostic factor or therapeutic target in HNSCC.

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Competing interests

All authors declare that there were no conflicts of interest with regard to the contents of this article.

Ethical approval

Not applicable.

Patient consent

Not applicable.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijom.2021.07.022.

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