J Appl Biotechnol Rep. 2024 September;11(3):1406-1420

doi 10.30491/jabr.2024.419473.1679

Journal of
Applied Biotechnology Reports

Original Article

Exploring the Genetic Signature of Oral Tongue Squamous Cell Carcinoma using Computational Systems Biology Approaches

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Received October 5, 2023; **Accepted** March 4, 2024; **Online Published** September 20, 2024

Abstract

Introduction: The survival rate for oral tongue squamous cell carcinoma (OTSCC) is the lowest compared to other types of carcinomas in the oral cavity. This study aims to employ novel and robust methodologies based on bioinformatics techniques to identify differentially expressed genes in OTSCC tissues compared to normal tongue samples to shed light on the underlying causes of OTSCC.

Materials and Methods: The dataset GSE13601 was obtained from the GEO, and the OPLS-DA method was applied to identify genes differentially expressed in OTSCC tissues compared to the normal healthy oral mucosa. A protein interaction map (PIM) was constructed, and hubs were identified. Survival analysis was performed to identify prognostic hub genes. The expression levels of the identified prognostic markers were evaluated at both mRNA and protein levels. Furthermore, bioinformatics web tools were used to perform pathway and GO annotation analyses.

Results: The study revealed 154 genes differentially expressed in OTSCC, with a statistically significant p <0.001 and a |Log2 fold change|>0.585. EIF2S1, EGF, NME1, NEDD8, EIF4A1, TOP1, and PSMA4 were found to have significant prognostic roles in OTSCC. The expression levels of all the identified prognostic markers were confirmed in HNSCC at the mRNA level and further validated the up-regulation of NME1, TOP1, and PSMA4 in HNSCC using immunohistochemical analysis. The FOXM1 was identified as the most important regulator of the hubs, while CDK2 was found to be the protein kinase acting on the transcription factors. The proteasome pathway and the regulation of ubiquitin-protein ligase activity were significantly enriched in OTSCC.

Conclusions: This study provides valuable insights into the pathogenesis of OTSCC and may assist in improving the prognosis of OTSCC patients.

Keywords: Biomarkers, Cancer, Oral, Prognosis, Tongue

Citation: Bayat Z, Samie L, Ansari M, Taherkhani A. Exploring the Genetic Signature of Oral Tongue Squamous Cell Carcinoma using Computational Systems Biology Approaches. J Appl Biotechnol Rep. 2024;11(3):1406-1420. doi:10.30491/jabr.2024.419473.1679

Introduction

Oral cancer (OC) is a prevalent malignancy, ranking as the sixth most common cancer worldwide. Among oral cancers, oral tongue squamous cell carcinoma (OTSCC) is the most frequently occurring type, accounting for 25-40% of all oral malignancies.[1-3] Unfortunately, OTSCC is associated with significant morbidity, mortality, and poor prognosis for affected patients.[4] The disease is characterized by its destructive behavior, high risk of metastasis to cervical lymph nodes, and an increased incidence of recurrence, particularly in younger individuals.[5-8] While surgery is the recommended treatment for early-stage OTSCC, adjuvant radiation therapy or chemo-radiotherapy is necessary in advanced cases.[9] However, even with treatment, 25-37% of early-stage OTSCC patients experience locoregional recurrences, and cancer-related deaths account for 19% of early-stage OTSCC patients.[10,11] However, the 5-year overall survival of the disease remains at approximately 65%.[12,13] Therefore, it is crucial to identify practical prognostic markers that can accurately predict OTSCC tumor behavior and guide the selection of appropriate treatment protocols.[2]

This study aims to employ novel and robust methodologies to identify differentially expressed genes in OTSCC tissues compared to normal tongue samples. The study seeks to establish a set of prognostic markers that can accurately predict OTSCC tumor behavior and guide the selection of appropriate treatment protocols. Additionally, the investigation delves into master regulators, protein kinases, signaling pathways, and biological processes implicated in the malignant transformation from normal tongue tissue to OTSCC. The study utilizes advanced techniques in re-analyzing the GSE13601 microarray dataset, originally developed by Estilo et al.,[14] to provide novel insights beyond the approaches employed in the original study. This dataset, encompassing gene expression profiles from OTSCC tissues and healthy mucosae collected between 1998 and 2002 at the Cancer Center of Memorial Sloan-Kettering, contributes

novel insights that extend beyond the approaches employed by Estilo et al.[14] The overall goal is to contribute to significant advancements in understanding and treating OTSCC by uncovering crucial molecular mechanisms underlying the disease.

Materials and Methods

Microarray Dataset

The gene expression dataset GSE13601 as a TXT file from the GEO (https://www.ncbi.nlm.nih.gov/geo/) was used for comprehensive analyses. The dataset included 31 OTSCC tissue and 26 normal mucosa samples based on the GPL8300 platform (Affymetrix Human Genome U95 Version 2 Array [HG_U95Av2]). Initially, Estilo et al.[14] created this dataset to examine the gene expression patterns in both OTSCC tissues and healthy mucosae of the upper aerodigestive tract. The goal of scrutinizing this dataset was to discover potential prognostic markers, TFs, protein kinases, signaling pathways, and biological processes associated with the development of OTSCC.

Multivariate Statistical Analysis

To identify discriminating genes between OTSCC and healthy mucosa samples, the robust Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) was used with the "ropls" package in the R environment (version 4.1.3; www.r-project.org). The cut-off score for DEGs was set as a p <0.001 and a 2/3>fold change>3/2 (|Log2 FC|> 0.585). This approach helped us identify genes significantly differentially expressed between OTSCC and healthy mucosa samples. This method aimed to identify prognostic markers that could assist in diagnosing and treating OTSCC.

PPI Network Analysis

To examine the potential relationships between DEGs, with a cut-off threshold of 0.4, the resource known as the STRING knowledge source was employed in version 11.5 (accessible at https://string-db.org/). The proteins that were found to be linked were subsequently imported into the Cytoscape 3.9.1 software (available at www.cytoscape.org) to conduct additional graph analysis and generate visual representations. The MCODE plugin was used to identify modules in the PPI graph, where condensed regions with specific features were assigned as significant modules.[15] These features included MCODE scores of more than three, a degree cut-off of 2, a minimum number of nodes of 10, a maximum depth of 100, a k-score of 2, and a node score cut-off of 0.2. The proteins in a common module are likely involved in common biological processes (BPs) or pathways,[16] and modules may contain a seed node with critical functions within the cluster.[17] Each protein's degree and betweenness centrality were calculated with the NetworkAnalyzer plugin. Nodes with betweenness and degree values above the mean of the vertices in the protein interaction map (PIM) were considered hub genes.

Measurement of Au NP Uptake

K562 cells were suspended in RPMI to a final concentration of 1×10^5 cells/well and incubated separately for 24 h with serial concentrations of Au NPs (20, 40, and 80 g/ml) in 5% CO_2 incubator. Then, the treated cells were washed three times with PBS to wash non-absorbed nanoparticles. Cells were then digested overnight at room temperature with 0.3 ml of 3% HCl in distilled HNO_3. After adding 5 µl of 5-ppm indium (as an internal reference) and 5 ml of matrix solution (2 percent HCl and 2 percent HNO_3), the Au NP content of each sample was determined and compared to the standard curve using an atomic absorption spectrometer (wavelength 280 nm, Model 603, Perkins Elmer, Waltham, MA).

Functional Enrichment Analysis

To better understand the molecular mechanisms involved in the etiology of OTSCC, functional enrichment analyses were conducted to unravel gene ontology (GO) annotations and signaling pathways affected by the disease. The online KEGG[18] and Reactome databases (available at https://reactome.org/)[19] were used for pathway enrichment analysis. The BP annotation enrichment analysis was conducted using the DAVID (https://david.ncifcrf.gov/)[20] and g:Plofiler (https://biit.cs.ut.ee/gprofiler/gost)[21] web servers. The enriched cellular components (CCs) and molecular functions (MFs) in OTSCC were also studied using the g:Plofiler. The genes within the identified modules were used for pathway and BP enrichment analyses, while all DEGs were considered for CC and MF annotation analyses.[15,22] The false discovery rate (FDR) was set to be less than 0.05.

Regulators of the Hubs

The atypical expression of TFs has been demonstrated to be critical in various biological conditions, including cancer. Therefore, identifying the primary TFs involved in the cancerous state can help researchers understand the underlying mechanisms of the disorder.[23] Our study used the iRegulon tool in Cytoscape[15] to identify several TFs regulating the hub genes. TFs with a normalized enrichment score (NES) of more than three were considered significant.[24] The Logos of binding profiles for TFs acting on the hub genes' expression were obtained with the JASPAR open-access tool (https://jaspar.genereg.net/).[25] The consensus sequence matching scores were calculated using R (version 4.1.3) according to the algorithm explained by Xiong.[26] The matching scores indicate the probability that consensus sequences fit the binding profiles as "2 scores" times more than by randomness.

Kinases Enrichment Analysis

Protein kinases are recognized for their substantial

involvement in post-translational modification, whereby they attach a phosphate group to specific serine, threonine, and tyrosine residues of proteins. This process can result in activating or deactivating the targeted proteins.[27] Earlier research has established a connection between the upregulation of protein kinases and heightened cell proliferation rates, carcinogenesis, and the recurrence of cancer metastasis.[28-30] Therefore, inhibiting protein kinases has been considered a helpful approach to overcoming cancer.[31] In this study, the Kinase Enrichment Analysis 3 (KEA3) web tool (https://maayanlab.cloud/kea3/)[32,33] was used to determine protein kinases affecting the activity of TFs regulating the hub genes. Finally, a gene regulatory network (GRN) consisting of the hub genes, TFs, and protein kinases regulating the hubs was built.

Prognostic Hub Genes

To investigate the prognostic role of the hub genes in head and neck squamous cell carcinoma (HNSCC), the Gene Expression Profiling Interactive Analysis 2 (GEPIA2) database was used.[34] GEPIA2 applies expression analysis on the carcinoma-associated RNA sequencing databases TCGA[35] and GTEx[36] to provide reliable survival, boxplot, and correlation analyses in cancer patients compared to normal samples. The Kaplan-Meier curves and the Log-rank test were used to study the potential prognostic role of the hub genes. Prognostic markers in OTSCC were defined as genes with Log-rank test and hazard ratio (HR) p-values lower than 5%.

Validation of Prognostic Genes

The GEPIA2 and Human Protein Atlas (HPA) databases were utilized to compare the expression profiles of the prognostic markers identified in HNSCC tissues with those observed in healthy samples. The GEPIA2 database provided valuable information on the expression of genes in cancerous tissues compared to normal samples using RNA sequencing data from TCGA and GTEx databases.[34] The HPA database is an online resource that provides information on human proteins in cells, tissues, and organs using RNA sequencing, antibody-based immunohistochemical detection, and mass spectrometry-based proteomics.[37]

Results

DEGs in OTSCC

Our study used the Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) model to identify discriminating genes between OTSCC tissues and healthy mucosa samples. At a *p*-value of 0.001 and |Log2 FC> 0.585|, the OPLS model identified 154 determining genes, including 131 upregulated and 23 down-regulated genes (Supplementary Table 1). Figure 1a displays a volcano plot

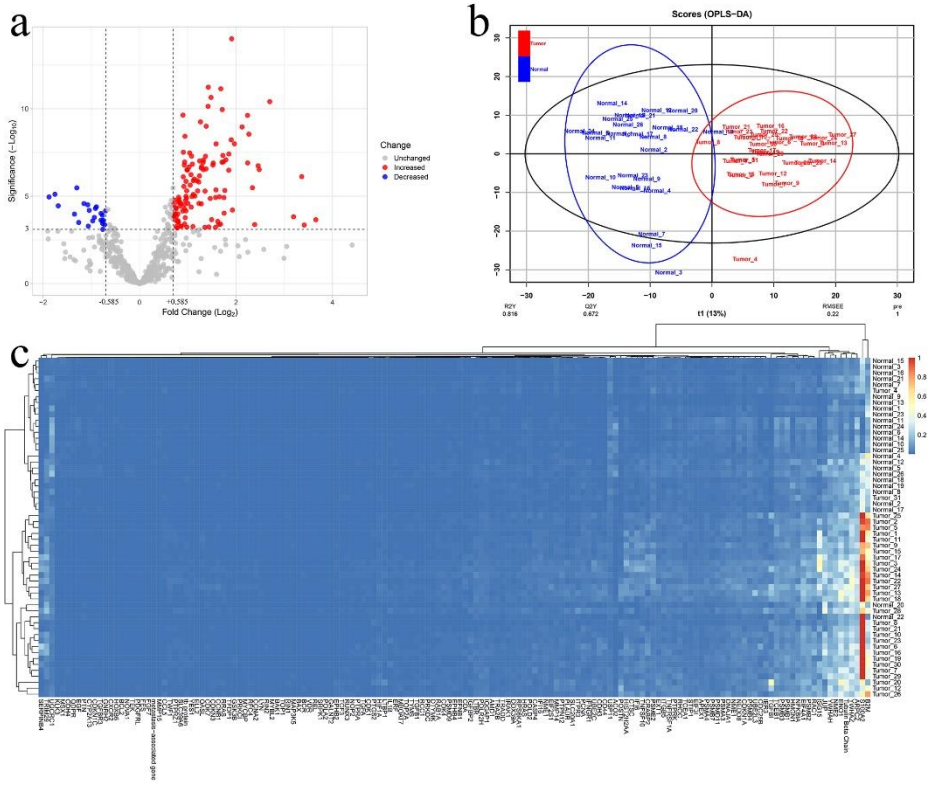

Figure 1. a) The volcano plot of genes in OTSCC tissues compared with the normal oral mucosa; b) Score plot in the orthogonal (y-axis) and predictive (x-axis) components of dataset GSE13601 achieved from the OPLS-DA analysis; c) Heat-map of DEGs in OTSCC tissues compared to the healthy control samples.

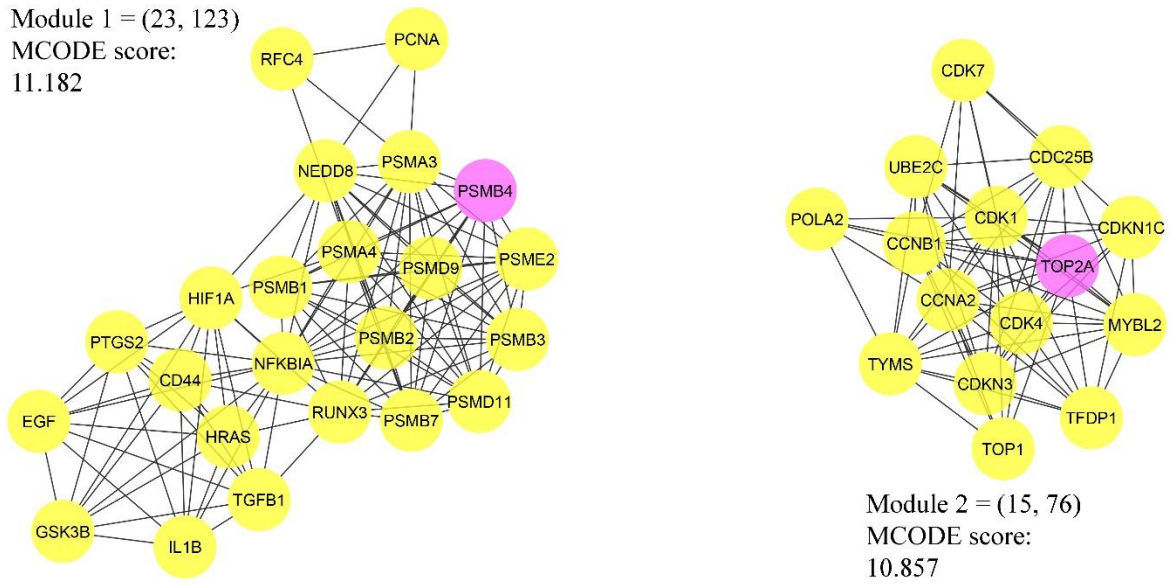

Figure 2. Module Analysis Using the MCODE Tool in the Cytoscape Illustrated Two Condensed Regions in the PPI Network Associated with OTSCC. Purple circles are seed nodes. PPI, protein-protein interaction; OTSCC, oral tongue squamous cell carcinoma.

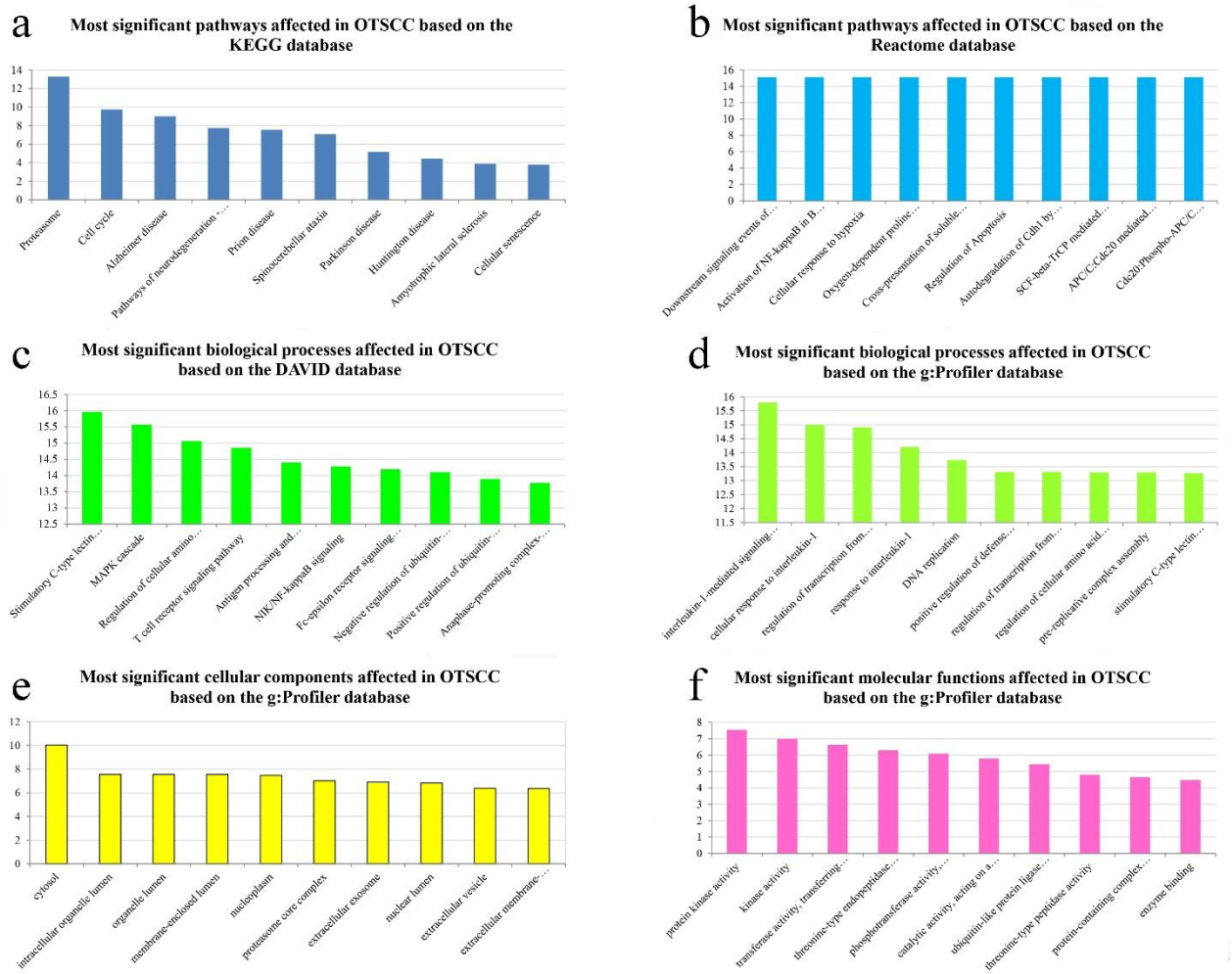

Figure 3. Most significant pathways (a and b), biological processes (c and d), cellular components (e), and molecular functions (f) enriched in OTSCC. X-axis shows the term's name. Y-axis demonstrates the minus value of the Log10 FDR. FDR, false discovery rate; OTSCC, oral tongue squamous cell carcinoma.

Table 1. Twenty-one Transcription Factors were Significantly Involved in the Phosphorylation of Hub Genes in OTSCC

TF	NES	No. targets	Targets
FOXM1	5.905	21	CCNA2, CDK1, CCNB1, RAC1, GSK3B, PSMA4, CDK4, TYMS, UBE2C, CDC25B, TOP2A, NFKBIA, EIF4A1, ENO1, TGFA, PCNA, LYN, NME1, YWHAZ, TGFB1, CDKN1A
CEBPG	5.774	8	LYN, IL1B, EIF4A1, CDKN1A, NFKBIA, PTGS2, TOP1, GSK3B
E2F1	5.765	21	PCNA, PSMA4, CDKN1A, EIF4A1, PSMA3, EIF2S1, EGF, CD44, TOP1, CDK1, TYMS, RUNX3, CDC25B, YWHAZ, ENO1, NME1, TGFB1, GSK3B, RAC1, HIF1A, TGFA
RELB	5.277	16	NFKBIA, CDC25B, CDKN1A, TGFA, RAC1, ISG15, TOP1, RUNX3, CD44, IL1B, PCNA, IQGAP1, EGF, GSK3B, LYN, YWHAZ
NFYA	4.99	16	GSK3B, TOP1, HIF1A, LYN, CDC25B, EGF, UBE2C, TOP2A, PCNA, YWHAZ, EIF4A1, CCNA2, CDK1, RUNX3, CDKN1A, PSMA3
SIN3A	4.476	25	EIF4A1, CDK7, NEDD8, PSMA4, PTGS2, GSK3B, NFKBIA, TOP1, ENO1, PCNA, TGFB1, CCNA2, YWHAZ, CDK1, UBE2C, CCNB1, CDK4, CDC25B, EIF2S1, NME1, TOP2A, LYN, TYMS, HIF1A, PSMA3
FOXN4	4.386	9	RUNX3, CDKN1A, LYN, NEDD8, CDK1, CDK4, EIF2S1, HIF1A, CDK7
ZNF513	4.252	15	CDKN1A, GSK3B, TOP2A, EIF4A1, ENO1, IQGAP1, TGFA, PCNA, YWHAZ, TGFB1, HIF1A, RAC1, RUNX3, TOP1, NME1
GRHL1	4.13	5	TGFA, HIF1A, PCNA, GSK3B, CDKN1A
MYBL2	4.022	15	TOP2A, NFKBIA, EIF4A1, TYMS, CDK4, NEDD8, NME1, CCNB1, UBE2C, PCNA, CCNA2, CDK1, ENO1, CDKN1A, GSK3B
NFIX	3.872	13	TGFB1, CDKN1A, EIF4A1, EGF, YWHAZ, LYN, IQGAP1, ENO1, GSK3B, IL1B, RUNX3, CD44, TGFA
SRF	3.773	7	CDKN1A, GSK3B, PCNA, NFKBIA, YWHAZ, RUNX3, TGFA
TP53	3.76	9	CDKN1A, NME1, PCNA, PSMA4, GSK3B, UBE2C, TYMS, PSMA3, ENO1
PAX3	3.711	11	ISG15, EIF4A1, PSMA3, GSK3B, CDKN1A, PSMA4, EGF, HIF1A, PCNA, RUNX3, YWHAZ
POLE4	3.689	15	TOP1, GSK3B, CDKN1A, YWHAZ, LYN, PTGS2, EIF4A1, ENO1, UBE2C, IQGAP1, PCNA, TOP2A, CDK1, RUNX3, EGF
TFDP1	3.678	7	CDK1, PCNA, TYMS, TOP2A, CCNB1, CCNA2, UBE2C
MEF2C	3.341	9	GSK3B, CDKN1A, RAC1, CDK1, ENO1, CDC25B, NME1, YWHAZ, LYN
STAT1	3.199	15	IL1B, RUNX3, IQGAP1, HIF1A, CDK1, EGF, LYN, CD44, TGFA, TOP1, CDKN1A, GSK3B, PCNA, PTGS2, NME1,
BHLHE40	3.188	6	HIF1A, CDKN1A, CDK4, ENO1, GSK3B, YWHAZ
ETS1	3.029	4	CDK1, CD44, CDKN1A, RUNX3
MAX	3.005	3	CDKN1A, TGFB1, RUNX3

OTSCC: oral tongue squamous cell carcinoma

of dataset GSE13601, generated using the Shiny apps web server accessible at https://huygens.science.uva.nl/.[38] The OPLS-DA model could clearly distinguish OTSCC tissues from healthy mucosa samples (Figure 1b) with R2X = 0.255, R2Y = 0.816, Q2 = 0.672. Also, an unsupervised hierarchical clustering analysis was performed using the R environment (version 4.1.3), which could distinguish the samples in the studied dataset based on the DEGs (Figure 1c).

Clustering, Functional Analysis, and Hub Genes

Our study transferred 140 connected DEGs, including 765 edges, to Cytoscape for further bioinformatics analyses. The MCODE algorithm identified two significant condensed regions in the PIM, including module 1 and module 2, with 23 and 15 genes, respectively (Figure 2). Using KEGG and Reactome data sources, 311 significantly enriched pathways in OTSCC were identified, in which 'proteasome' (KEGG: 03050) and 'downstream signaling events of B Cell Receptor' (R-HSA-1168372) had the lowest FDR based on the KEGG and Reactome, respectively. Further, 358 deregulated BPs in OTSCC based on the DAVID and g:Profiler databases were uncovered, in which the 'stimulatory C-type lectin receptor signaling pathway' (GO: 0002223) and 'interleukin-1-mediated signaling pathway' (GO:0070498) had the lowest FDR based on the DAVID and g:Profiler, respectively. The g:Profiler database also identified 41 CCs and 35 MFs that were significantly deregulated in OTSCC, in which 'cytosol' (GO: 0005829) and 'protein kinase activity' (GO: 0004672) had the most significant CC and MF, respectively. Figure 3 shows the most significantly affected pathways and GO annotations in OTSCC, based on their –Log10 FDR.

In addition to the topological analysis, the mean betweenness and degree values were calculated to be 0.01155 and 10.93, respectively. Based on this analysis, 36 genes with above-average degree and betweenness values were identified, which were assigned as hub genes and may be responsible for tumorigenesis in OTSCC. The STRING database was also used to identify internal connections among the hub genes (Figure 4).

GRN Construction

A total of 21 TFs that play a significant role in regulating the expression of hub genes were identified (Table 1). FOXM1, with the highest NES of 5.905, was found to regulate the expression of 21 hub genes, including CCNA2, CDK1, CCNB1, RAC1, GSK3B, PSMA4, CDK4, TYMS, UBE2C, CDC25B, TOP2A, NFKBIA, EIF4A1, ENO1, TGFA, PCNA, LYN, NME1, YWHAZ, TGFB1, and CDKN1A. The binding site logos of 17 regulators, including BHLHE40, CEBPG, E2F1, ETS1, GRHL1, MAX, MEF2C, MYBL2, NFIX, NFYA, PAX3, RELB, SRF, STAT1, TFDP1, TP53, and ZNF513, were identified in the JASPAR database (Figure 5).

Using the STRING database, ten protein kinases, including CDK2, CDK9, CDK8, TGFBR2, MAP2K7, SGK1,

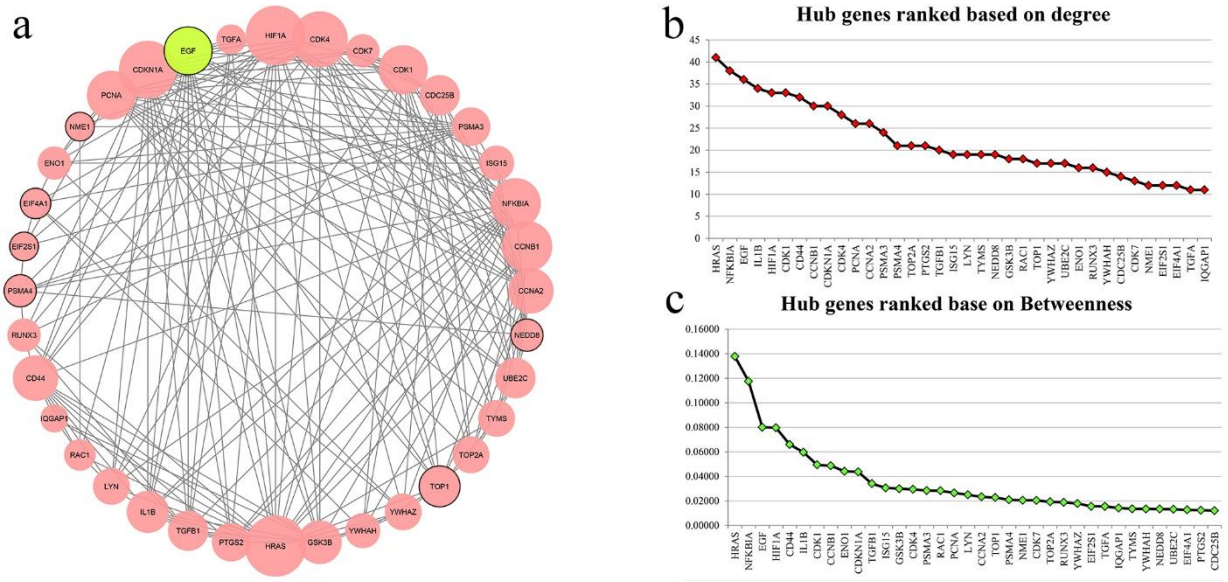

Figure 4. (a) Internal connections among hub genes. This network consisted of 36 nodes and 202 edges. Red and green vertexes show over-expressed and downregulated genes in OTSCC, respectively. The size of the nodes is positively correlated with their degrees. Nodes with dark borders demonstrated prognostic impact in HNSCC. (b) and (c) present hub genes based on their degree value and betweenness centrality. OTSCC, oral tongue squamous cell carcinoma; PPI, protein-protein interaction.

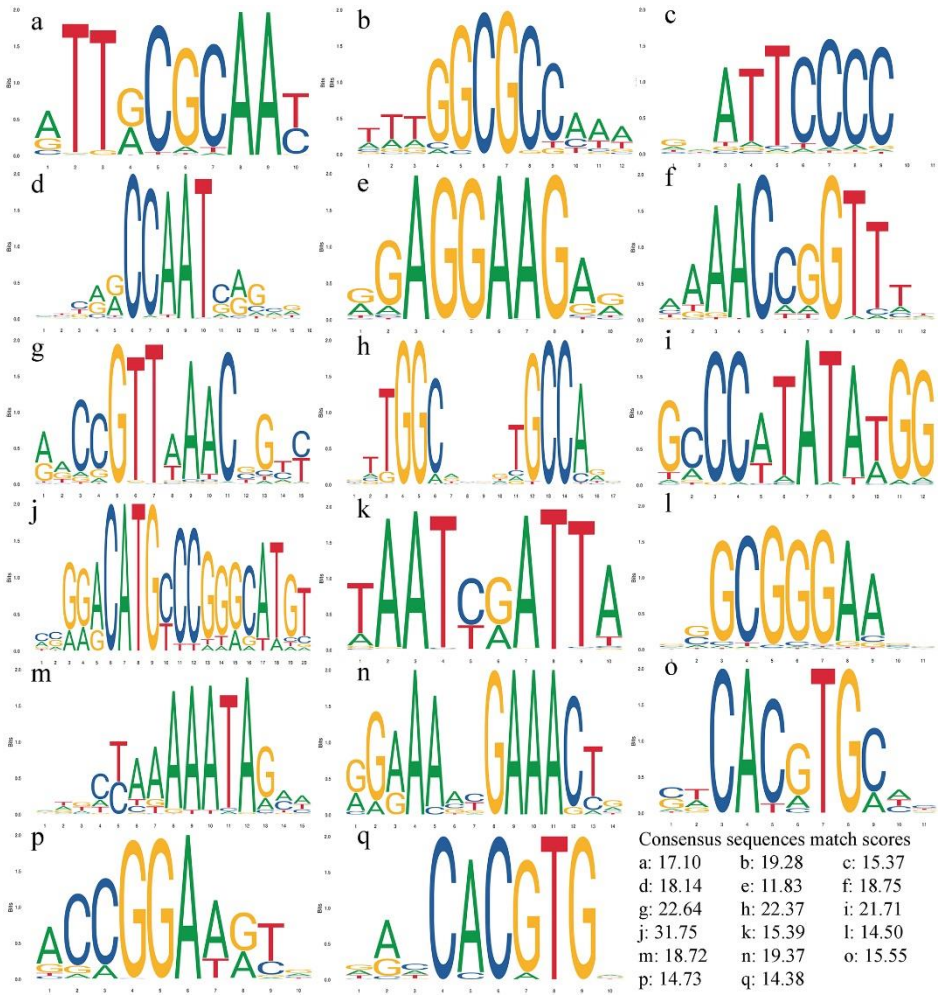

Figure 5. Logos for the binding profiles of (a) CEBPG (b) E2F1 (c) RELB (d) NFYA (e) ZNF513 (f) GRHL1 (g) MYBL2 (h) NFIX (i) SRF (j) TP53 (k) PAX3 (l) TFDP1 (m) MEF2C (n) STAT1 (o) BHLHE40 (p) ETS1 and (q) MAX.

Table 2. Top-10 Protein Kinases Affect Hub Genes Activity Identified by the KEA2 Web Server

Rank	Protein	FDR	Downstream targets
1	CDK2	0.000573	BHLHE40, CEBPG, E2F1, ETS1, FOXM1, FOXN4, MAX, MEF2C, MYBL2, NFYA, PAX3, POLE4, RELB, SIN3A, SRF, STAT1, TFDP1, TP53
2	CDK9	0.00102	E2F1, ETS1, FOXM1, MAX, MYBL2, RELB, SIN3A, SRF, STAT1, TP53
3	CDK8	0.00102	E2F1, ETS1, FOXM1, FOXN4, SIN3A, SRF, STAT1, TFDP1, TP53
4	TGFBR2	0.00102	BHLHE40, E2F1, ETS1, FOXM1, MAX, MEF2C, PAX3, RELB, SIN3A, SRF, STAT1, TP53, ZNF513
5	MAP2K7	0.00102	BHLHE40, E2F1, ETS1, FOXM1, MAX, MEF2C, MYBL2, PAX3, RELB, SRF, STAT1, TP53
6	SGK1	0.00102	BHLHE40, E2F1, ETS1, FOXM1, FOXN4, MEF2C, RELB, SIN3A, SRF, STAT1, TP53
7	ATM	0.00102	BHLHE40, E2F1, ETS1, FOXM1, GRHL1, MYBL2, NFYA, PAX3, POLE4, RELB, SIN3A, STAT1, TFDP1, TP53
8	FLT3	0.00102	BHLHE40, E2F1, ETS1, FOXM1, MEF2C, NFYA, RELB, SIN3A, STAT1, TP53
9	CDK1	0.00102	BHLHE40, E2F1, ETS1, FOXM1, FOXN4, MAX, MEF2C, MYBL2, NFYA, PAX3, RELB, SIN3A, SRF, STAT1, TFDP1, TP53
10	CLK3	0.00102	BHLHE40, E2F1, MEF2C, RELB, SIN3A, SRF, TP53

KEA: kinase enrichment analysis; FDR: false discovery rate

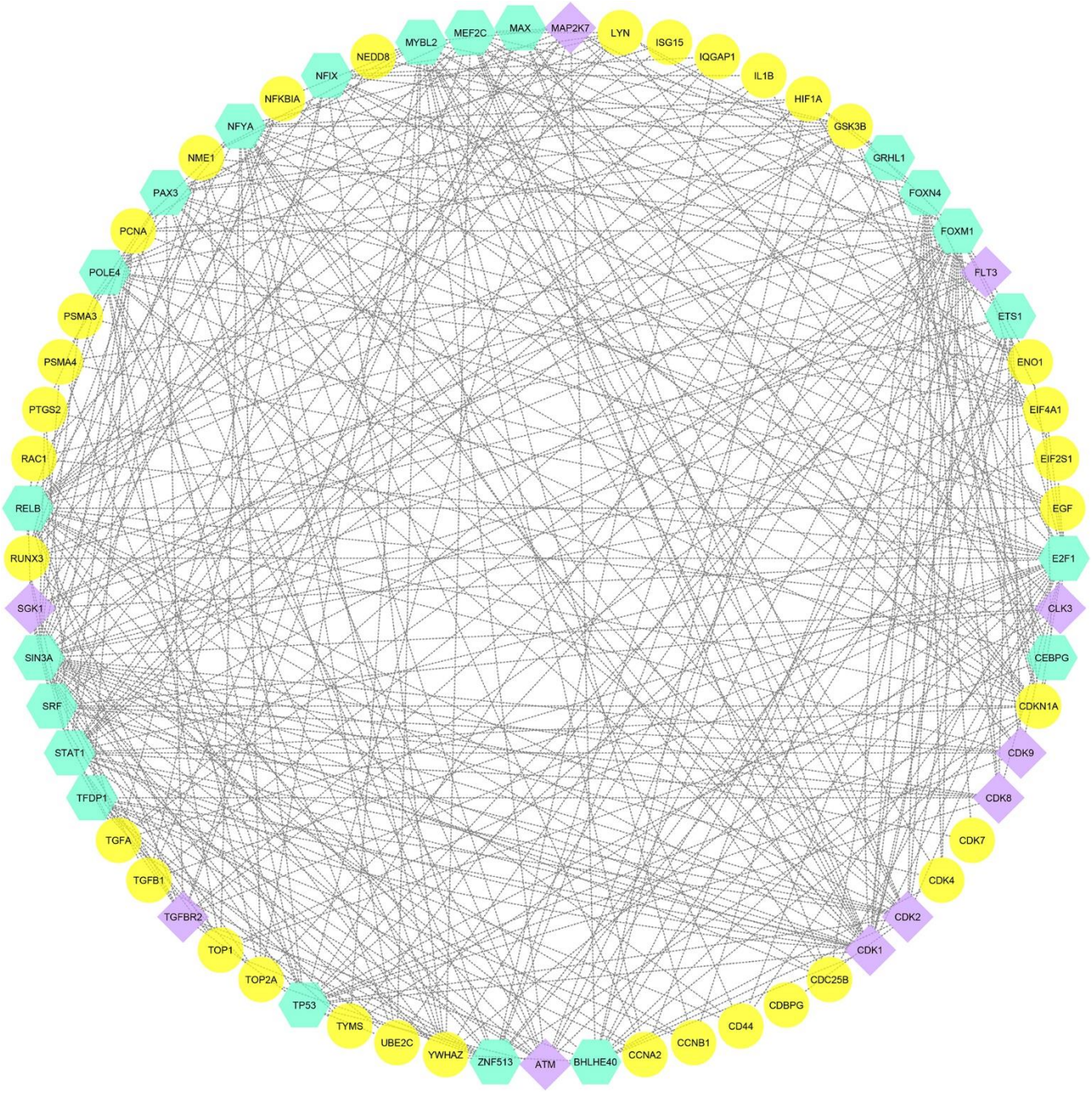

Figure 6. Gene Regulatory Network in OTSCC. Yellow (circle), cyan (hexagon), and purple (diamond) nodes present hub genes, transcription factors, and protein kinases taking part in the etiology of the disease, respectively. The nodes are sorted by their names.

ATM, FLT3, CDK1, and CLK3, that significantly regulate the TFs were identified (FDR <0.05) (Table 2) according to the KEA2 server. Finally, a GRN was constructed, including 34 hub genes, 21 TFs, 10 protein kinases, and 370 edges (Figure 6).

Prognostic Markers

It was found that overexpression of seven hub genes, including EIF2S1, EGF, NME1, NEDD8, EIF4A1, TOP1, and PSMA4, was significantly associated with a poor prognosis in patients with OTSCC (Log-rank test and HR *p* < 0.05). Furthermore, the combination of EIF2S1, EGF, NME1, NEDD8, EIF4A1, and TOP1 was found to be the most significant prognostic panel with an HR of 1.8 and an HR *p*-value of 0.000028 (Table 3 and Figure 7).

Validation Study

Our findings were consistent with the expression analysis from GEPIA2, which showed that EGF had lower expression in HNSCC than in normal tissues. In contrast, EIF2S1, NME1, NEDD8, EIF4A1, TOP1, and PSMA4 had higher expression

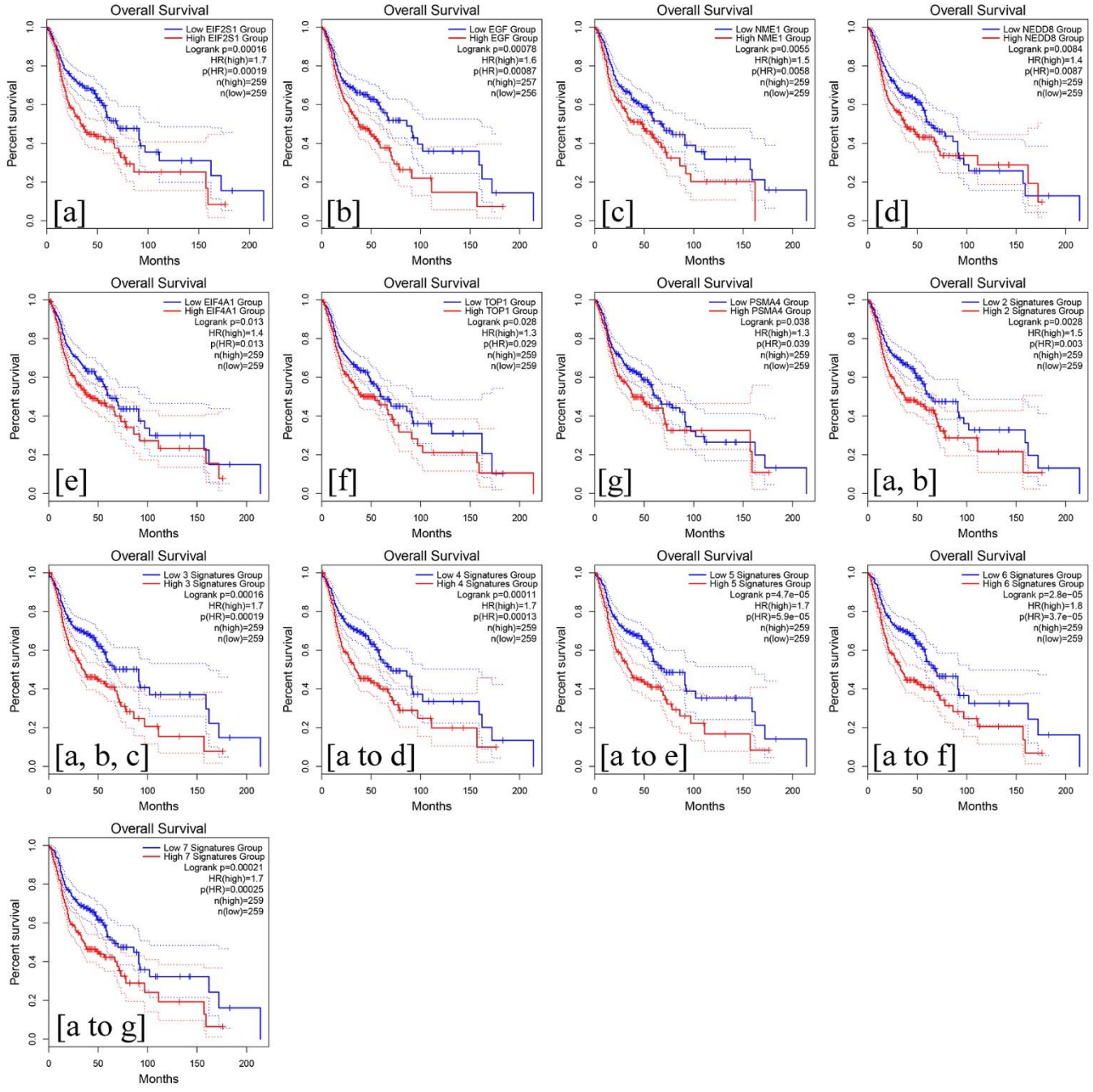

Figure 7. Prognostic Role of EIF2S1 (a) EGF (b) NME1 (c) NEDD8 (d) EIF4A1 (e) TOP1 (f) and PSMA4 (g) was Found to be Significant in HNSCC. The prognostic impact of the Combination of genes is also exhibited. The X-axis and Y-axis present the survival time of HNSCC patients and the survival probability, respectively. The dotted lines are 0.95 confidence intervals. HNSCC, head and neck squamous cell carcinoma.

Table 3. A total of seven hub genes significantly revealed prognostic impact in OTSCC

A, Single-gene				
Gene symbol (gene label)	**HR (high)**	**P (Log-rank test)**	**P (HR)**	**Log2 FC, OTSCC/Normal**
EIF2S1 (A)	1.7	0.00016	0.00019	1.15
EGF (B)	1.6	0.00078	0.00087	-0.8
NME1 (C)	1.5	0.0055	0.0058	1.43
NEDD8 (D)	1.4	0.0084	0.0087	0.8
EIF4A1 (E)	1.4	0.013	0.013	1.4
TOP1 (F)	1.3	0.028	0.029	1.2
PSMA4 (G)	1.3	0.038	0.039	0.94
B, Combination of genes				
Prognostic panel	**HR (high)**	**P (Log-rank test)**	**P (HR)**	
A	1.7	0.00016	0.00019	
A+B	1.5	0.0028	0.003	
A+B+C	1.7	0.00016	0.00019	
A+B+C+D	1.7	0.00011	0.00013	
A+B+C+D+E	1.7	0.000047	0.000059	
A+B+C+D+E+F	1.8	0.000028	0.000037	
A+B+C+D+E+F+G	1.7	0.00021	0.00025	

HR: hazard ratio; OTSCC: oral tongue squamous cell carcinoma

in cancer tissues than in healthy controls (Figure 8). Additionally, our immunohistochemical analyses showed that NME1, TOP1, and PSMA4 had higher expression in HNSCC compared to the normal oral mucosa. Specifically, NME1 expression was moderate and increased in five and two of the seven HNSCC samples, respectively, while NME1 expression was not detected in two of the five normal oral mucosa samples. Furthermore, TOP1 expression was high and low in three and two of the five HNSCC samples. In comparison, three of the six normal oral mucosa samples displayed elevated expression of TOP1, and the other three showed low expression levels of TOP1. Lastly, PSMA4 expression was not detected in one of the eight HNSCC samples, while the expression levels of PSMA4 were low, medium, and high in two, three, and two of the cancerous samples, respectively. Additionally, PSMA4

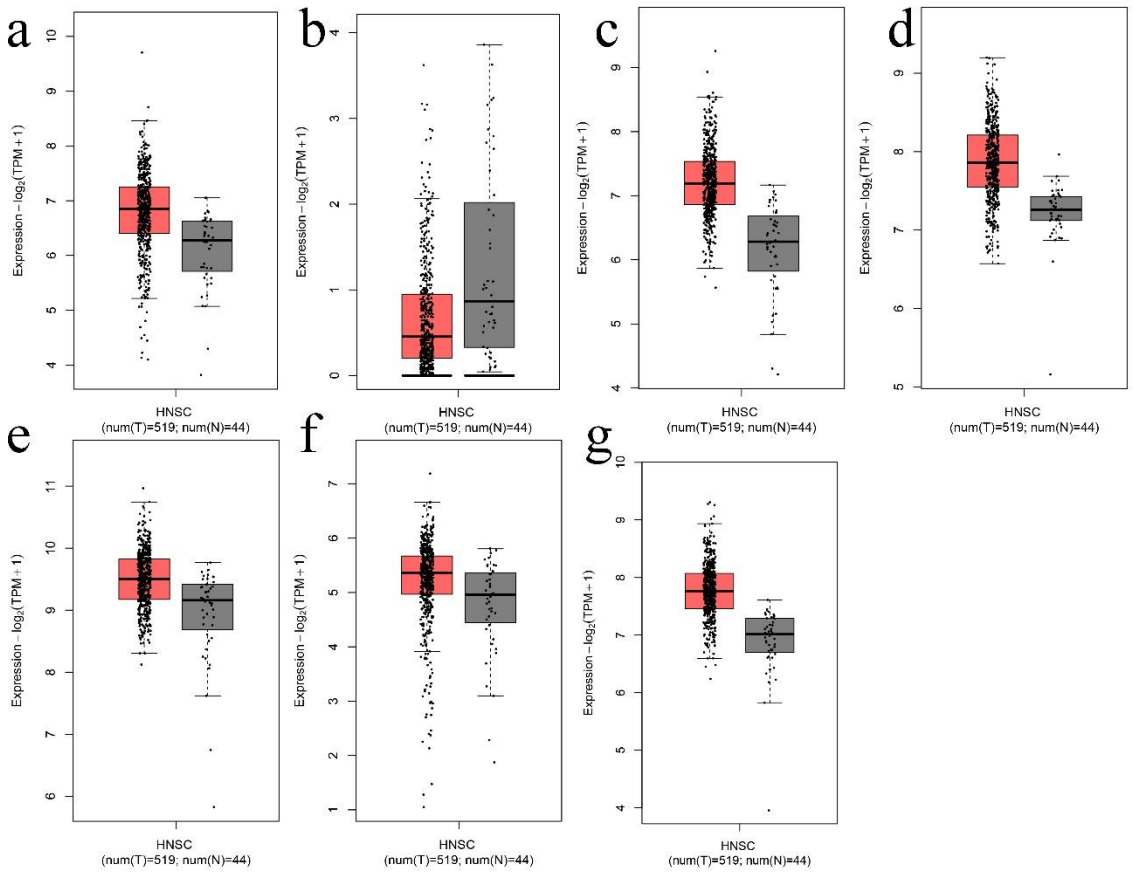

Figure 8. Validation of Prognostic Markers in OTSCC from the Expression Analysis in the GEPIA2 Database. Box plots are based on 519 HNSCC samples (red color) and 44 normal tissues (gray color) that demonstrate down-regulation of EGF (b) and up-regulation of EIF2S1 (a), NME1 (c), NEDD8 (d), EIF4A1 (e), TOP1 (f), and PSMA4 (g) in HNSCC. OTSCC, oral tongue squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma.

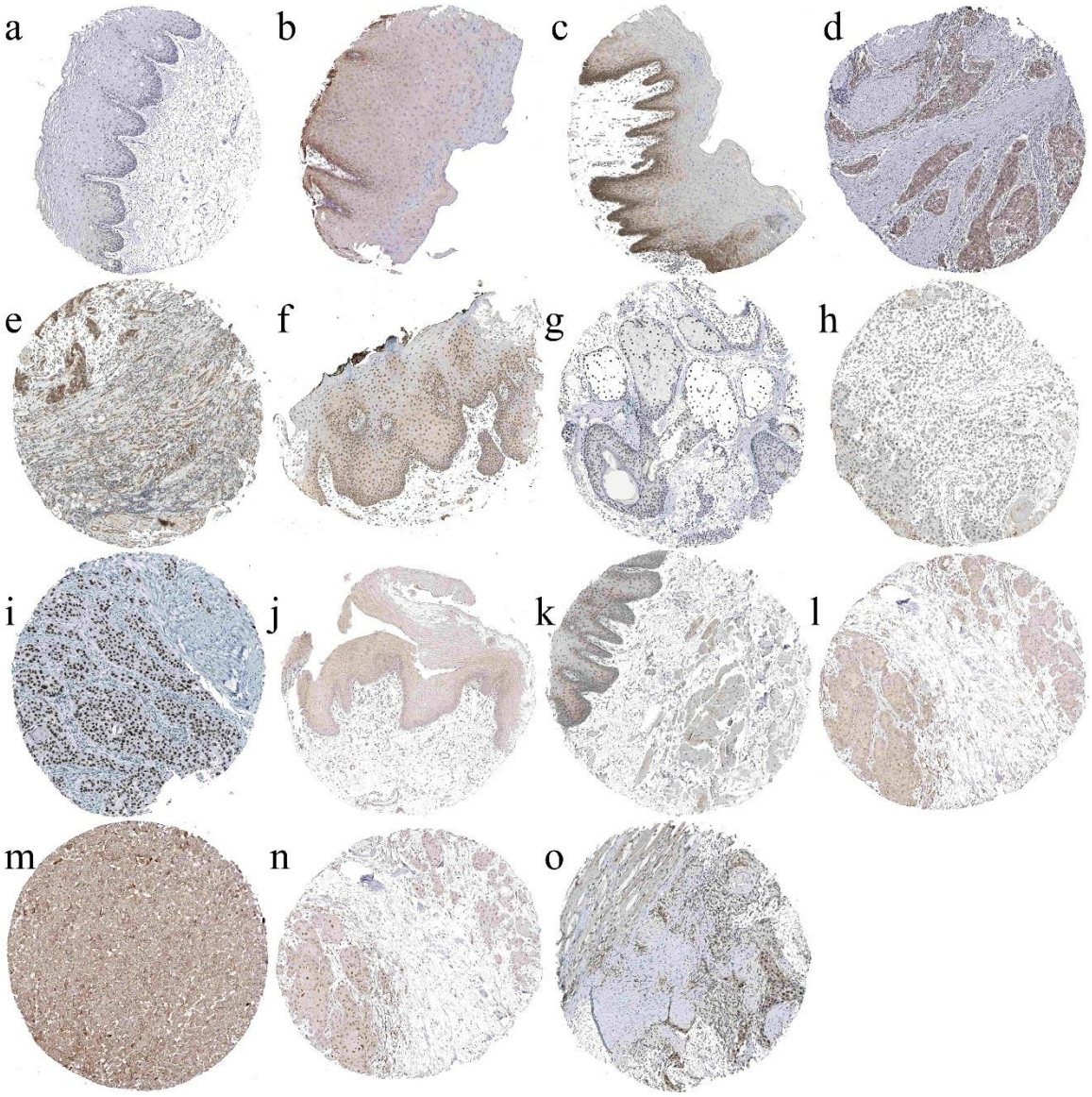

Figure 9. Validation of Prognostic Markers in OTSCC from Protein Expression Analysis in the HPA Database. NME1 expression was not detected in a normal oral mucosa sample (a). Intermediate (b) and high (c) expression levels of NME1 in normal samples. Moderate (d) and high (e) NME1 expression levels in HNSCC samples. Low (f) and high (g) expression levels of TOP1 in normal oral mucosa samples. TOP1 expression was low (h) and high (i) in HNSCC samples. The expression of PSMA4 was not detected (j) and low (k) in normal oral mucosa samples. The expression levels of PSMA4 were not detected (l), low (m), medium (n), and high (o) in cancerous samples. HPA, human protein atlas.

expression was not detected in one of the five normal oral mucosa samples, while the expression levels of PSMA4 were low in four of the normal oral tissues (Figure 9).

Discussion

Oral squamous cell carcinoma (OSCC) is a prevalent subtype of malignancy in the oral cavity, and OTSCC is the most common subtype and has the worst prognosis among patients.[15] The re-analysis of raw data obtained through genomics and transcriptomics techniques holds the promise of uncovering novel insights that might not have been initially addressed in the primary publications. Therefore, apart from statistical analysis, we conducted various bioinformatics analyses, including PPI network, GRN, gene set enrichment, and kinase enrichment analyses, to enhance the depth of our data mining results. This collective analysis contributes to a more comprehensive understanding of the OTSCC pathogenesis.

The present study uncovered DEGs, hub genes, upstream regulators, protein kinases, modules, and signaling pathways involved in the malignant transformation from normal tongue mucosa to OTSCC. The number of DEGs varies based on factors such as p-value, Fold Change, and the statistical methodology employed for analysis. Estilo et al.[14] identified 77 DEGs with criteria: $p < 0.05$ and $0.5 > FC > 2$, utilizing JMP statistical software version 4.0.0 (SAS Institute, Inc.). In contrast, our study revealed 154 DEGs, employing criteria: $p < 0.001$ and $2/3 > FC > 3/2$. Additionally, we

utilized OPLS-DA, a robust method for "Omics" data analysis.[17,39-42] We acknowledge the validity of Estilo et al.'s[14] results. Different methodologies offer unique advantages, contributing collectively to more robust findings.

The common set of DEGs between our study and Estilo et al.'s[14] research was identified through interactive Venn diagrams accessible at https://bioinformatics.psb.ugent.be/webtools/Venn/ (Figure 10). As a result, seven genes—CD44, EXT1, UBE2L3, PSMB2, TGFBI, HIF1A, and EIF4A1—were consistently dysregulated in both studies, as outlined in Table 4.

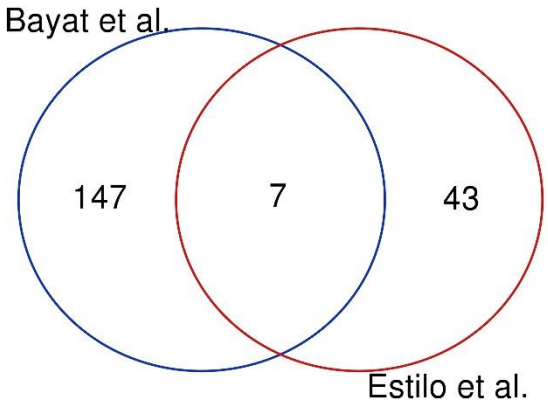

Figure 10. Common Differentially Expressed Genes between Different Studies.

Estilo et al.[14] decided to assess the potential prognostic significance of three specific genes (GLUT3, HSAL2, and PACE4) in patients with OTSCC. Their rationale for this selection was rooted in the observation that these three genes exhibited a two-fold or greater overexpression in OTSCC compared to normal tongue tissues. Furthermore, their study noted that 30.6%, 24.5%, and 26.5% of patients demonstrated elevated levels of GLUT3, HSAL2, and PACE4, respectively. In contrast, our analysis delved into the potential impact of 36 genes on the prognosis of OTSCC patients. The criteria for selecting this set of genes were twofold: these 36 genes were initially identified as DEGs in OTSCC compared to normal tongue mucosa. Secondly, all 36 genes were recognized as hub genes in the PPI network associated with the pathogenesis of OTSCC, substantiated by their significant centrality within the network. In addition, Estilo et al.[14] derived their survival data from 49 voluntary patients participating in their study. Conversely, our analysis drew upon the GEPIA2 database to assess the prognostic role of the 36 genes in OTSCC patients. Notably, the GEPIA2 dataset encompassed information from 259 patients, offering a comprehensive perspective on the prognostic implications of the selected genes.

Table 4. Comparison of Differentially Expressed Genes between the Study Conducted by Estilo et al. [14] and Our Results

Study	Total	Genes
Bayat et al. Estilo et al.	7	CD44 EXT1 UBE2L3 PSMB2 TGFBI HIF1A EIF4A1
Bayat et al.	147	VDR ELL2 RIN2 CDH3 PSMB1 PRKCI CDK4 CDK1 NFKBIA PSMA4 IFI16 ENO1 IQGAP1 PTN TYMS POLD2 PTPRK TNFSF10 TCF3 EIF3I ARPC2 CYP2A13 TubulinBetaChain IRAK1 BCL2 TCEB1 FES DTYMK PSMD9 RAC1 HRAS CCL5 IER3 YWHAH TWF1 REL TRIM29 IFNGR1 PDGFRL RCC1B JUP MSX1 DDX39A CCNB1 MMP15 PSMD11 NOP2 PSME2 TGFB1 TOP2A SLC20A1 CASP4 UQCRC1 APEX1 TGFBR3 EGF metastasis-associatedgene CXCR2 BAK1 CDKN1A CRABP2 RPS6KA1 SRPK1 RFC4 RUNX3B LTBP1 CCNA2 CDA tbprotein HMGN1 EPHB2 STIP1 PSMB4 PLAUR CDH4 CDKN3 MYCBP PCNA NME1 LYN YES1 CDC25B PTPN12 PSMA3 S100A2 PTPRZ1 EIF2S1 EFNB1 NME2 MAP3K5 HOXB6 IFI6 GSK3B CDK7 BAX OASL TNFRSF1A KLK3 CTSCB POLA2 TEK AMD1 PSMB7 ITGB5 NOVA1 POSTN EPHB4 DNAJA1 MYBL2 TCEA1 TGFA PTGS2 LTBR RAB1A B2M RHOC MMP14 PTPRA FGF7 GALNT2 TRA2B TRADD UBE2C TFDP1 POLR2H IL1B ISG15 ERF BCR ACO2 IGFBP2 USP11 HIST2H2AA NEDD8 PRKDC SERPINB4 MBOAT7 ITPR3 GNRH2 RRAS TOP1 YWHAZ CDKN1C SLC39A6 PSMB3 QDPR RIN1
Estilo et al.	43	DAPK2 ANXA2 UGCG TGM6 FABP5 GMPS MYO1B TSPAN8 TMSB10 PPIA CNGB1 MMP7 UMPS MMP122 PGAM1 AP2S1 MTSS1 AMOT UBE2S MSN EIF3B ACAA2 PDXK SHC1 PI3 N4BP1 RCAN2 GPD1L GIGYF1 H2BC13 SELENBP1 MMP1 TYMP MYO10 GSTP1 NNT GARS1 TAGLN2 RAN H2AC18 PLA2G2A PCMT1 PIK3IP1

The findings of our investigation indicated that elevated expression levels of seven markers, namely EIF2S1, EGF, NME1, NEDD8, EIF4A1, TOP1, and PSMA4, were notably linked to an unfavorable prognosis in individuals diagnosed with OTSCC. EIF2A, or EIF2S1, is a single polypeptide chain with a molecular weight of 65 kDa found in eukaryotes.[43] Recent studies have shown that EIF2A plays a significant role in tumor initiation and development. Sendoel et al.[44] reported that through studying The Cancer Genome Atlas (TCGA), the EIF2A locus is amplified in several human carcinomas, including 29% of lung SCC, 15% of HNSCC, and 15% of esophageal carcinoma. These findings suggest that the overexpression of EIF2A may contribute to the pathogenesis and progression of these cancers.

Previous studies have demonstrated that OSCC commonly exhibits up-regulation of the EGF receptor (EGFR), resulting in hyperactivation of the EGF-EGFR signaling pathway, enhanced drug resistance, tumor development, and poor prognosis in patients with OSCC. As a result, targeting EGFR using small molecules and monoclonal antibodies has been considered a therapeutic strategy for treating patients with advanced OSCC in Japan.[45] Of interest, our results indicated that individuals with OTSCC had down-regulated levels of EGF relative to healthy controls (FC = 0.57; p = 0.000097), which could result from the tumor mass response. These results add to the complexity of EGFR

signaling in OTSCC and suggest that further investigation is necessary to understand the role of EGF in the disease entirely.

In a study by Whang et al.,[46] Nm23-H1 (NME1) expression levels were investigated in primary OSCC and metastatic lymph nodes using immunohistochemistry and Western blot analysis. The authors also utilized invasion assays and transwell migration to analyze cell motility and migration. The results showed that the expression levels of Nm23-H1 were significantly lower in metastatic lymph nodes compared to primary OSCC. Furthermore, an inverse correlation was observed between Nm23-H1 expression and the aggressive behavior of OSCC cells. The authors' conclusions established that Nm23-H1 is critical in inhibiting metastasis. Therefore, the diminished expression of Nm23-H1 is linked to an unfavorable prognosis in individuals with OSCC who have metastatic lymph nodes.

Neural precursor cell-expressed developmentally down-regulated 8 (NEDD8) belongs to the superfamily of ubiquitin-like proteins and is involved in the cell cycle through its covalent ligation to cullin proteins.[47,48] A study by Chairatvit et al.[46] showed that NEDD8 conjugation is upregulated in human oral carcinoma cells, leading to increased cell growth. Furthermore, a direct correlation was observed between the expression of NEDD8 conjugation and the proliferation rate in oral carcinoma cells. These findings suggest that NEDD8 is critical in regulating cell growth and proliferation in oral carcinoma. Further investigation into the mechanisms underlying the effects of NEDD8 may provide insights into potential therapeutic targets for treating oral carcinoma.

EIF4A1, also known as DEAD-box helicase, is essential for unwinding the 5′ untranslated region (5′UTR) of mRNA, facilitating ribosome binding and translation initiation. Previous studies have shown that the expression levels of EIF4A1 are linked to several oncogenes, including SMAD2, TGFβ1, CBC25B, c-myb, and c-myc.[49] A study by Zhao et al.[47] reported that OSCC patients with high EIF4A1 expression levels had a high recurrence rate. Furthermore, in patients with early-stage OSCC, heightened expression levels of EIF4A1 were positively correlated with an unfavorable prognosis.

In a study conducted by Liu et al.,[50] it was discovered that the expression of TOP1 and TOP2 was increased at the protein and mRNA levels in liver cancer tissues relative to normal tissues.

PSMA4, a type-4 alpha proteasome subunit, has been implicated in promoting cancer cell proliferation. Earlier research has demonstrated that PSMA4 is upregulated at the mRNA level in lung cancer tissues compared to healthy controls.[51] Additionally, Wang et al.[52] conducted a case-control study in China to investigate the relationship between PSMA4 polymorphism and lung cancer. According to the investigation, the SNP rs12901682 in PSMA4 is a significant risk factor for the development of lung cancer. These findings offer valuable information regarding the potential involvement of PSMA4 in the development of lung carcinoma, emphasizing the significance of genetic factors in the pathogenesis of the disease. Further research is needed to fully understand the mechanisms underlying the effects of PSMA4 on OTSCC development and progression.

Further analysis revealed that 21 genes regulate the transcription of hubs, with forkhead box protein M1 (FOXM1) showing the most significant result with an NES of 5.905. FOXM1 is an oncogene that plays a crucial role in several essential processes, including cell cycle progression, proliferation, and aging.[53] Previous studies have shown that up-regulation of FOXM1 is associated with a poor prognosis in various cancers, including glioblastoma,[54] breast cancer, and gastric cancer.[55] Recently, Bayat et al.[15] reported that FOXM1 significantly regulates critical genes involved in the etiology of salivary gland carcinoma. Moreover, Harada et al.[56] demonstrated that increased expression of FOXM1 in tumor tissues compared to healthy control samples was significantly associated with a poor prognosis in patients with OSCC. These findings suggest that FOXM1 may play a crucial role in the pathogenesis and progression of various cancers, including OSCC, and could serve as a potential therapeutic target for treating the disease.

Several protein kinases were found to be significantly associated with TF phosphorylation, with four of the top 10 protein kinases based on their FDR being cyclin-dependent kinase (CDK) genes, including CDK2 (FDR = 0.00057), CDK9 (FDR = 0.00102), CDK8 (FDR = 0.00102), and CDK1 (FDR = 0.00102). Negata et al.[57] have reported that the expression ratios of CDK/CDK inhibitors could serve as biomarkers for the risk of locoregional and hematogenous dissemination in OSCC. The study showed a direct correlation between high CDK2/CDKN1A expression ratios and distant metastasis (p <0.0001). Notably, protein kinase activity was found to be the most significant molecular function enriched in OTSCC (Figure 3f).

Several biological processes are affected in OTSCC, with the proteasome pathway (KEGG:03050) and regulation of ubiquitin-protein ligase activity (GO:0051436 and GO:0051437) being among the top-10 significant pathways and biological processes, respectively, based on FDR. The ubiquitin-proteasome system (UPS) regulates cell growth, apoptosis, and survival. Therefore, UPS inhibition has been considered a promising strategy for cancer therapy, particularly in combating chemoresistance in human cancers.[58]

The current study has a primary limitation in that the stage of each sample in the dataset GSE13601[14] was not separately available for the research team. Although most of the observations used in the dataset were in the early stages of OTSCC, the lack of information about the stage of each

sample may have influenced the results. Therefore, the present findings would be more valuable if they were fully achieved from the early stages of OTSCC. In future studies, it is recommended to analyze datasets focusing specifically on the early stages of OTSCC and compare the findings with the present results. This could provide a deeper understanding of the molecular mechanisms underlying early-stage OTSCC and potentially lead to the identification of novel biomarkers and therapeutic targets for the disease.

Conclusion

The present study identified 154 genes, including 132 upregulated and 23 down-regulated genes, to be differentially expressed in OTSCC tissues compared to the normal oral mucosa. OTSCC patients with EIF2S1, EGF, NME1, NEDD8, EIF4A1, TOP1, and PSMA4, had a negative prognosis. The GEPIA2 database confirmed the mRNA expression levels of all prognostic markers, while immunohistochemical analysis revealed overexpression of NME1, TOP1, and PSMA4 at the protein level in HNSCC tissue samples compared to healthy controls. The study also revealed FOXM1 and CDK2 as the most significant TF and protein kinase involved in the transcription of hub genes and phosphorylation of TFs, respectively. Additionally, the most effective pathways and BPs affected in OTSCC were associated with the proteasome pathway and the regulation of ubiquitin-protein ligase activity. These discoveries may aid in predicting the prognosis of individuals with OTSCC and contribute to a better understanding of the molecular mechanisms involved in the disease.

Authors' Contributions

All authors equally contributed to this study.

Ethical Approval

The Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (ethics no. IR.UMSHA.REC.1400.650) approved the current study.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

The authors would like to appreciate the Dental Research Center, Deputy of Research and Technology and Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan - Iran, for their support. This paper was extracted from the thesis of Mohammadmahdi Ansari.

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