Investigation of MicroRNA-Target Gene Regulatory Networks in Oral Squamous Cell carcinoma

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ABSTRACT — Oral squamous cell carcinoma (OSCC) is the seventh most common cancer worldwide. Despite improvement in its control, morbidity and mortality, rates have improved little in the past decades. To find differential miRNA and gene expression, GSE98463 and GSE3524 expression profiles were acquired from the Gene Expression Omnibus (GEO) database. Screened DE-miRNAs' transcription factors and target genes were predicted. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted to predict biological functions. Using Cytoscape software, protein–protein interaction (PPI) networks and hub genes were found in the miRNA-mRNA regulation network. Further hub gene expression and prognostic functions were examined. We examined 34 DE-miRNAs, 23 upregulated and 11 downregulated. Most screened DE-miRNAs may be regulated by transcription factor SP1. 2190 and 1061 predicted target genes were obtained for upregulated and downregulated DE-miRNAs, respectively. Subsequently, 452 upregulated DEGs and 221 downregulated DEGs were identified. Then, 18 and 58 potential downregulated and upregulated genes commonly appeared in target genes of DE-miRNAs and DEGs were selected for GO annotation and KEGG pathway enrichment analysis. The candidate target genes were significantly enriched for the Protein digestion and absorption, Relaxin signalling pathway, and AGE-RAGE signalling pathway. Construction and analysis of PPI network showed that MMP9 and COL1A1were recognized as hub genes with the highest connectivity degrees. Expression analytic result of the top 10 hub genes in OSCC using GEPIA database was generally identical with previous differential expression analysis for TCGA data and its expression was significantly associated with patients' overall survival. We constructed a putative oral cancer-related miRNA-mRNA regulation network in

this study to better understand molecular mechanisms and find novel treatment targets for OSCC. Further trials are needed to confirm our findings.

Keywords — Oral cancer, hub genes, transcription factor

I. INTRODUCTION (HEADING 1)

Oral cancer is one of the most prevalent cancers worldwide, accounting for about 354,864 new diagnoses and approximately 177,384 new deaths annually [1]. Generally, the term oral cancer identifies a subset of head and neck cancers arising in the lips, hard palate, upper and lower alveolar ridges, anterior two-thirds of the tongue, sublingual region, buccal mucosa, retro-molar trigone and floor of the mouth [2]. Among these cancers, the most frequent histotype is oral squamous cell carcinoma (OSCC) representing about 95% of all oral cancers [3]. Advances in surgical and medical treatments for OSCC over the past two decades have not improved the overall disease outcomes and OSCC continues to portend a poor prognosis.[4] Accordingly, OSCC remains a lethal disease for more than 50% of cases diagnosed annually mainly due to the high rate of local and regional recurrence. Tobacco chewing, smoking, alcohol drinking, prolonged UV exposure, and HPV are known oral cancer risk factors (HPV). Additionally, tumour suppressor genes, oncogenes, and regulatory genes may contribute to oral carcinogenesis. [5, 6] Genetic changes to TP53, NOTCH1, and PIK3CA influence epithelial cells and cause microenvironment changes such ROS buildup, cytokine overproduction, and epithelial to mesenchymal transition, causing uncontrolled cell proliferation, growth, and cancer. [7, 8] The molecular mechanisms of cancer and development must be understood to solve this problem. Many research organisations are studying OSCC pathogenesis, although the mechanisms of carcinogenesis and development are still unknown. Thus, OSCC mechanism investigations are needed to improve early diagnosis, focused therapy, and prognosis. Recent research has connected oral malignancies to epigenetic alterations such promoter/intragenic methylation and miRNA deregulation, which impact cellular homeostasis and physiological functions. [9,10,11] microRNAs (miRNAs) expression profile is becoming a leading cancer marker for diagnosis, staging, and treatment.[12] MicroRNAs, 22-nucleotide double-strand short RNAs, regulate gene expression post-transcriptionally and are involved in numerous regulatory circuits that affect developmental timing, apoptosis, cell proliferation, and differentiation. While messenger

RNA profiles are less helpful, miRNA expression profiles can be utilised to classify poorly differentiated cancers by reflecting cancer's developmental lineage and differentiation status.[13] OSCC pathogenesis is linked to miRNA activity. [14] In example, multiple investigations linked miRNA expression to OSCC clinical characteristics and outcomes. [15-17] Although, only miR-196a and miR-126 were found to connect with local recurrence insurgence and disease-free survival in OSCC. [18, 19] The GEO database was used to retrieve mRNA expression profiles (GSE3524) and datasets (GSE98463) to find DEGs and DEMs in OSCC versus normal samples. We then analysed miRNA-target gene networks. For subsequent analysis, transcription factors (TFs) related to important DEMs from the interaction network were found. This study identified OSCC's important genes, miRNAs, and TFs and built miRNA-target gene-TF regulatory networks to investigate the molecular mechanism using bioinformatics.

II. MATERIAL AND METHODS

A. Microarray Data

The gene expression profile was downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The GSE3524 dataset was from the study of Toruner et al. [20], and the dataset was based on the platform of GPL96 (Affymetrix Human Genome U133A Array). This dataset comprised kidney samples from 16 OSCC patients and four normal, healthy participants. The miRNA expression profile under accession number GSE98463 was contributed by Chamorro et al. [21]. The dataset was downloaded from the GEO database according to the platform of the GPL21572 [miRNA-4] Affymetrix Multispecies miRNA-4 Array. MiRNA expression was analyzed in the urine sediments of 8 OSCC patients and eight healthy individuals. Raw data from the GSE3524 and GSE98463 datasets were read using the “affy” package in R (version 3.5.3, R Core Team, Vienna, Austria). The RMA algorithm was used for background correction and data normalization.

B. Data processing and differential expression analysis

Microarray data were processed and analysed in R. By comparing with the OSCC patients and normal individuals, we calculated the differentially expressed mRNA (DE-mRNA) from GSE3524 dataset and different expressed miRNA (DE-miRNAs) from GSE98463 dataset by “limma” package in R. In order to decreasing the false discovery rate, the p values were adjusted using the Benjamin and Hochberg method, the thresholds were adj.p < 0.05 and logFC > 1.5 (upregulated) or logFC < -1.5 (downregulated).

C. Prediction of Potential Transcription Factors, and Target Genes of DE-miRNAs

The upstream transcription factors of screened DE-miRNAs were predicted using FunRich software, which is a tool used mainly for functional enrichment and interaction network analysis of genes and proteins. [22] The screened upregulated and downregulated DE-miRNAs were typed into this software. The top 10 predicted transcription factors were shown. Subsequently, miRNet database(https://www.mirnet.ca), an easy-to use tool for comprehensive statistical analysis and functional interpretation of data from miRNAs studies, was employed to predict the downstream target genes of screened DE-miRNAs.[23] The miRNA–gene interaction data of miRNet were collected from miRTarBase v7.0, TarBase v7.0 and miRecords, catalogs that predict and validate miRNA–gene interactions. [24-26] The validated miRNA–gene interactions are manually curated and experimentally validated, and the predicted miRNA–gene interactions are produced by established miRNA target prediction programs. The screened upregulated and downregulated DE-miRNAs were entered into the web platform, and the data of the potential target genes of the upregulated and downregulated DE-miRNAs were downloaded. Then, these data were input into Cytoscape 3.10.1 software to access the DE-miRNA-target gene network. [27]

D. Functional enrichment analysis of candidate genes

Gene Ontology (GO) is widely used to produce gene annotation terms [28]. The Kyoto Encyclopaedia of Genes and Genomes (KEGG) is extensively applied in pathway enrichment analyses [29]. The candidate DEGs were further evaluated for their corresponding biological meaning using GO annotations retrieved from the Gene Ontology Consortium, and the biological pathway information was mapped based on the KEGG database. The GO terms and KEGG pathways were analysed with DAVID (http://david.ncifcrf.gov/) an online tool that can be utilized to perform a functional analysis and pathway enrichment analysis for discovering the relationships among the selected gene sets. [30] Gene Ontology (GO) analysis and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed by the DAVID online program.

E. PPI Network Construction of candidate genes and Analysis of Modules

The Search Tool for the Retrieval of Interacting Genes (STRING) (https://string-db.org/) was used to establish the PPI networks of candidate [31]. The STRING database was used to evaluate the correlations among DE-mRNAs. The overlapping DE-mRNAs were mapped in STRING to generate a network with functional interactions. Cytoscape software (version 3.10.1) was used to visualize the PPI network [32]. Then, the top 10 hub genes in the PPI network were identified according to degree using Cytoscape Software.

F. Hub Gene Expression Analysis

The expression levels of top 20 hub genes were further validated using the Gene Expression Profiling Interactive Analysis (GEPIA), which is a newly developed interactive web server for analysing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects. [33] Hub genes with $|\log2FC| > 1.5$ and $P < 0.05$ were considered as statistically significant.

G. Survival analysis of hub gene

To further investigate the influence of the hub gene on tumor prognosis, we employed a method of sample stratification based on the median value. This resulted in the formation of two distinct groups: one with high-expression levels and the other with low-expression levels. The survival curves of hub genes (DEGs) were constructed using GEPIA database

III. RESULT

A. Identification of DEGs and DEMs

With the objective to ascertain differentially expressed miRNAs (DEMs) and differentially expressed genes (DEGs) between normal and cancer samples, we conducted an analysis on miRNA datasets (GSE98463) as well as one mRNA datasets (GSE3524). Differentially expressed genes (DEGs) and microRNAs (miRNAs) exhibited statistically significant increases (P value < 0.05 and logFC > 1.5) or decreases (P value < 0.05 and logFC < -1.5). Ultimately, our analysis yielded a total of 673 differentially expressed genes (DEGs), with 452 DEGs exhibiting downregulation and 221 DEGs displaying upregulation. Additionally, we identified 34 differentially expressed miRNAs (DEMs), 11 downregulated and 23 upregulated genes were found.

B. Prediction of Upstream Transcription Factors of DE-miRNAs

In this study, upstream transcription factors of candidate upregulated and downregulated DE-miRNAs were predicted by using FunRich software. The top 10 transcription factors for upregulated and downregulated DE-miRNAs were presented in Fig 1A, B, respectively. For upregulated DE-miRNAs, the top 10 transcription factors were SP1, EGFR1, SP4, POU2F1, NKX6-1, SOX1, FOXA1, MEF2A, YYA1, and RORA. For downregulated DE-miRNAs, the

top 10 transcription factors were EGR1, SP1, SP4, POU2F1, SOX1 NR1H3, GFI1, FEV, SPI1 and HMX1

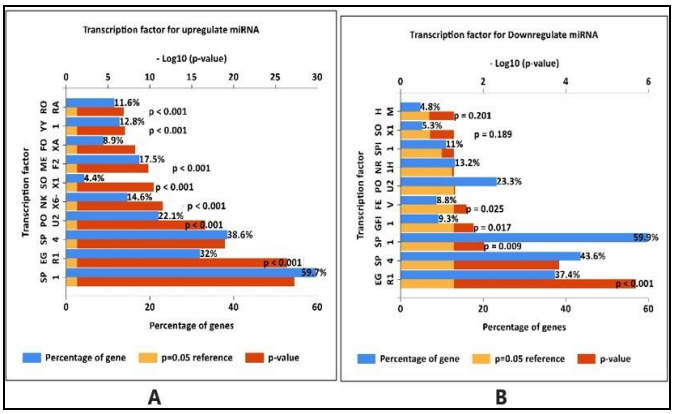

Fig.1: Predicted transcription factors of DE-miRNAs. (A) Transcription factors of upregulated DE-miRNAs; (B) transcription factors of downregulated DE-miRNAs.

C. Prediction of Downstream Target Genes of DE-miRNAs

We used the miRNet database to predict the potential target genes of the DE-miRNAs, as miRNAs exert their biological effects mainly by directly targeting the 3' untranslated regions of mRNAs. A total of 3252 target genes were identified from the miRNet database. The potential target genes for the upregulated DE-miRNAs included 2190 genes associated with 23 miRNAs, and the potential target genes for the downregulated DE-miRNAs included 1062 genes associated with 11 miRNAs. The upregulated DEmiRNA- target gene network was established and is presented in Fig. 2A, and the downregulated DE-miRNAtarget gene network was established and is presented in Fig. 2B. Additionally, the degrees of target genes for the DE-miRNAs are listed in Table1.

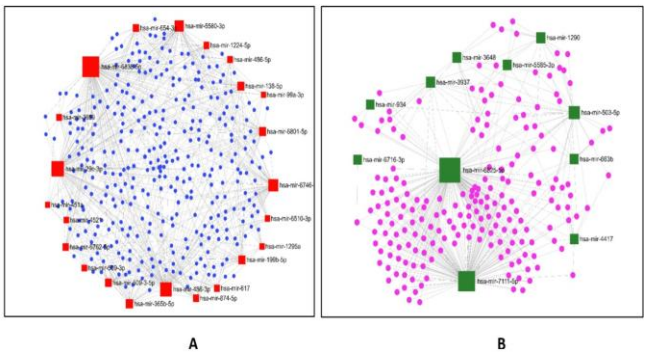

Fig 2.: Predicted target genes of the DE-miRNAs. (**A**) Upregulated DE-miRNA-target gene network constructed using miRNet. The red rectangles represent the upregulated DE-

miRNAs, and the blue ellipses represent the target genes. (**B**) Downregulated DE-miRNA-target gene network constructed using miRNet. The green rectangles represent the downregulated DE-miRNAs, and the pink ellipses represent the target genes.

Table 1. Degrees of the target genes of the DE-miRNAs.

	miRNA ID	Degree
Upregulated miRNA	hsa-mir-199b-5p	31
	hsa-mir-138-5p	33
	hsa-mir-29c-3p	76
	hsa-mir-451a	9
	hsa-mir-486-5p	20
	hsa-mir-509-3p	14
	hsa-mir-617	17
	hsa-mir-99a-3p	10
	hsa-mir-486-3p	68
	hsa-mir-654-3p	28
	hsa-mir-509-3-5p	28
	hsa-mir-1224-5p	25
	hsa-mir-1295a	13
	hsa-mir-3659	21
	hsa-mir-4521	13
	hsa-mir-5580-3p	52
	hsa-mir-365b-5p	32
	hsa-mir-6510-3p	14
	hsa-mir-874-5p	23
	hsa-mir-6746-5p	53
	hsa-mir-6762-5p	23
	hsa-mir-6801-5p	35
	hsa-mir-6838-5p	108
Downregulated miRNA	hsa-mir-503-5p	32
	hsa-mir-934	3
	hsa-mir-663b	11
	hsa-mir-1290	14

	hsa-mir-3648	11
	hsa-mir-3937	16
	hsa-mir-4417	11
	hsa-mir-5585-3p	9
	hsa-mir-6716-3p	1
	hsa-mir-6825-5p	163
	hsa-mir-7111-5p	146

D. Identification of candidate target genes.

Abundant studies have proved the inverse relationship between miRNAs and target genes.[34] Our study screened out 2190 target genes for upregulated miRNAs and 1061 target genes for downregulated miRNAs. We also identified 221 upregulated and 452 downregulated DE-mRNAs. After analysing DE-mRNAs and target genes of DE-miRNAs, 58 and 18 candidate DEGs for upregulated and downregulated DE-miRNAs were identified, respectively (Fig 3 A and B).

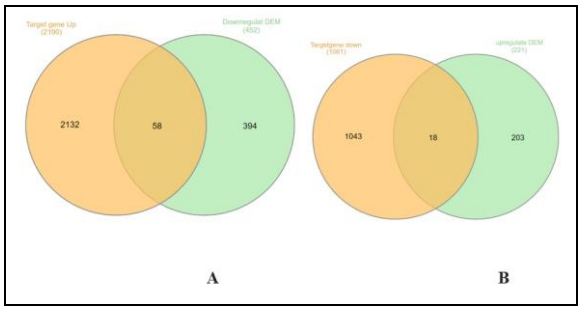

Fig 3. Identification of candidate target genes. (**A**) The intersection of the predicted target genes of the upregulated DE-miRNAs and downregulated DE-mRNAs. (**B**) The intersection of the predicted target genes of the downregulated DE-miRNAs and upregulated DE-mRNAs.

E. Functional enrichment analysis of candidate genes

To explore the functions of those DEGs in depth, the biological process, pathway annotation and molecular function were revealed using DAVID Gene Functional Classification Tool.

GO biological process (BP) functional enrichment analysis showed that the candidate target genes were significantly enriched collagen fibril organization, extracellular matrix organization, skeletal system development, cellular response to amino acid stimulus, cellular response to transforming growth factor beta stimulus. GO molecular function (MF) function enrichment analysis showed that the candidate target genes were significantly enriched extracellular matrix structural constituent, extracellular matrix structural constituent conferring tensile strength, platelet-derived growth factor binding, SMAD binding, identical protein binding. GO cellular component (CC) function enrichment analysis showed that the candidate target genes were significantly enriched for the endoplasmic reticulum lumen, collagen trimer, extracellular matrix, extracellular exosome and basement membrane. KEGG pathway enrichment analysis of the candidate target genes was then conducted. The candidate target genes were significantly enriched for the Protein digestion and absorption, Relaxin signaling pathway, AGE-RAGE signaling pathway in diabetic complications, Cellular senescence, Amoebiasis, Platelet activation and Focal adhesion. Fig.4

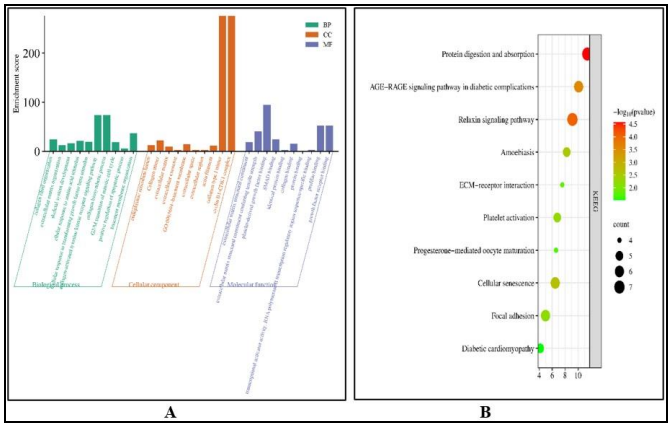

Fig4. Functional enrichment for candidate target genes. (A) The top 10 enriched BP cc AND mf items of candidate genes; (B) The top 10 enriched KEGG pathways for candidate genes.

F. PPI Network Construction of candidate genes and Analysis of Modules

Next, we mapped these candidate target genes into the STRING database. Based on the information from this public database, we constructed PPI network of these genes consists 76 node and 148 edges. (Fig 5) The top 10 hub genes were listed in Table 2.

TABLE 2: Hub genes in the PPI networks.

Name	Degree
MMP9	16
COL1A1	16
CTGF	14
SPARC	13
COL1A2	14
COL4A2	12
FBN1	12
COL3A1	14
COL4A1	13
COL5A2	11

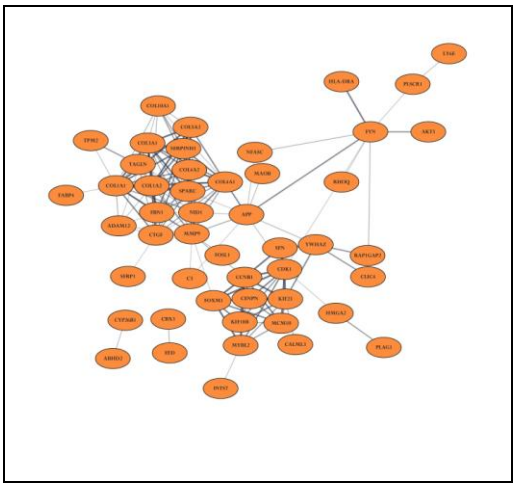

Fig. 5 PPI network of the candidate target genes.

G. Validation of Hub gene expression levels

Then, the GEPIA database was used to compare crucial hub gene expression in oral squamous cell carcinoma (OSCC) tissues to normal oral mucosa in the TCGA dataset. Fig 6 shows that all hub genes were upregulated in 519 oral squamous cell carcinoma (OSCC) samples compared to 44 normal tissue samples.

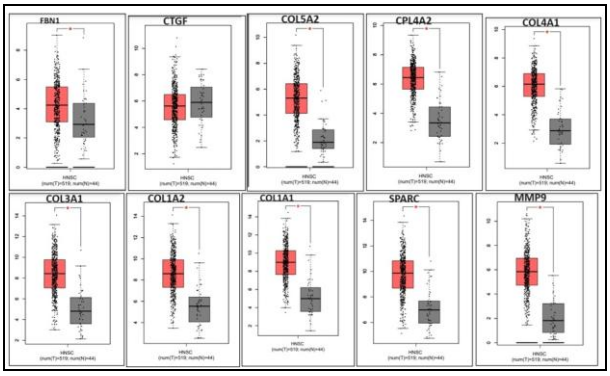

Fig 6. The GEPIA database shows the relative expression of DEGs in primary OSCC tumours compared to adjacent normal tissue.

H. Survival analysis of Hub Genes

We divided differentially expressed genes associated with oral squamous cell carcinoma (OSCC) into two groups based on median expression levels to determine median expression. Next, we examined survival rates between these two groups. Survival differences were found between hub genes high and low expression groups. Compared to the low-expression group, the high-expression group had a worse prognosis and survival rate. Fig 7

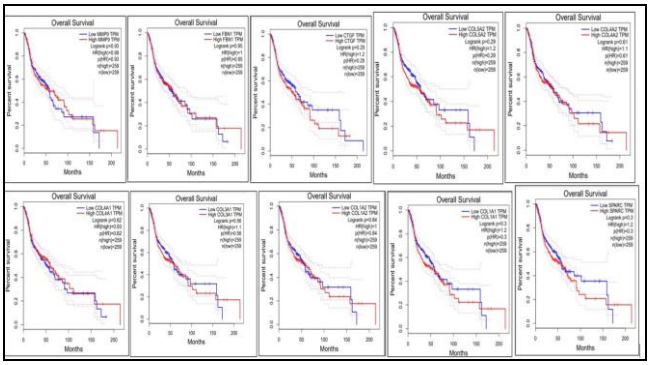

Fig.7. Overall survival analysis of hub genes in oral squamous cell carcinoma. Gene Expression Profiling Interactive Analysis was used to create the curves based on data from The Cancer Genome Atlas. ($p < 0.01$).

IV. DISCUSSION

New research is shedding light on the functions of microRNAs in various malignancies, including OSCC. MicroRNAs (miRNAs) play an important role in tumour development and have been demonstrated in many studies to act as oncogenes or tumour suppressors in cancer

cells. [35, 36] There is a lot of interest in miRNA dysregulation as a possible prognostic marker because of the important role it plays in cancer and metastasis. Nevertheless, picking important targets for cancer detection and therapy remains difficult due to the large number of miRNAs involved in tumour cell control. In this study, differential miRNAs and mRNAs expression analysis of OSCC from the same clinical samples in GEO database were performed. 58 up-regulated and 18 down-regulated candidate genes were identified from the integration data of DE-mRNAs and target genes of DE-miRNAs. KEGG pathway enrichment analysis revealed that candidate genes were mainly enriched in the Protein digestion and absorption, Relaxin signalling pathway, AGE-RAGE signalling pathway in diabetic complications, Cellular senescence, Amoebiasis, Platelet activation and Focal adhesion. Hub genes including MMP9,COL1A1,CTGF,SPARC,COL1A2, COL4A2, FBN1,COL3A1,COL4A1 and COL5A2were identified through PPI network analysis. Hub genes were all increased at both mRNA expression levels and upregulation of Hub genes indicated the poor prognosis.

Although an integrated analysis has been performed in silico and a potential miRNA-mRNA regulatory network has been constructed in this present study, some limitations exist. The sample size of GEO dataset included in this study is not big enough. First, this study used just one miRNA and one mRNA dataset with limited sample sizes. Second, we screened DE-miRNAs and DE-mRNAs from a web database with data from numerous sources to eliminate the limitations of a single-centre study, but a single study is still needed to confirm and screen the regulatory network. Verifying miRNAs and mRNAs in the same samples is optimal. In conclusion, we present a possible comprehensive mechanism of miRNA-mRNA regulatory axis in OSCC pathogenesis and created a candidate OSCC-related regulatory network, which may support fundamental and clinical research on OSCC and its treatment.

CONCLUSION

By shedding light on the functions performed by miRNA- target gene interactions, our results may help to explain the molecular mechanisms of OSCC. They may suggest which genes and miRNAs could be beneficial in the diagnosis, treatment, and prognosis of OSCC. A total of ten genes including MMP9, COL1A1, CTGF, SPARC, COL1A2, COL4A2, FBN1, COL3A1, COL4A1 and COL5A2 were identified as OSCC biomarkers, and Protein digestion and absorption, Relaxin signalling pathway, AGE-RAGE signalling pathway in diabetic

complications, Cellular senescence, and Focal adhesion were revealed to be important mechanisms of OSCC. One drawback of our study is that we only used the Kaplan-Meier plotter database to forecast the hub genes' prognostic value. Another restriction is that we only used a small number of samples. Additional research is required to understand the pathways and mechanisms behind these genes, and their predictive value in patients with OSCC must be investigated.

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