



Identification of *CKS2* as a novel prognostic biomarker and potential therapeutic target for oral squamous cell carcinoma

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Background: The cyclin-dependent kinase subunit 2 (*CKS2*) is recognized to have a substantial impact on the pathogenesis and advancement of several malignant neoplasms. Nevertheless, its biological function and prognostic significance in oral squamous cell carcinoma (OSCC) have yet to be thoroughly investigated. Our primary objective was to clarify the contribution of *CKS2* in the progression and prognosis of OSCC.

Methods: We first conducted a thorough examination of online databases to investigate the expression of *CKS2*, and subsequently corroborated our discoveries by analyzing clinical specimens that we collected. According to the clinicopathological data, we then explored the prognostic significance of *CKS2*. Furthermore, we predicted the role of *CKS2* in OSCC progression by employing weighted gene co-expression network analysis (WGCNA) in conjunction with functional enrichment analysis. We conducted functional experiments *in vitro* to confirm our speculations. Additionally, we explored other potential functions of *CKS2* in immune infiltration, tumor mutation burden (TMB), and drug sensitivity. Finally, we established and validated a nomogram that effectively integrated *CKS2*-related genes and other relevant clinical factors.

Results: Our findings indicated a significant upregulation of *CKS2* expression in OSCC tissues compared to normal groups, which was positively associated with poor clinical outcomes. We also predicted and validated the role of *CKS2* in promoting proliferation by regulating the cell cycle. Additionally, its upregulation was significantly correlated to enhanced immune cell infiltration, high TMB, and increased sensitivity of anti-tumor agents. Following verification, the nomogram was conducted to quantify an individual's survival probability.

Conclusions: In general, our study indicates that *CKS2* is a novel prognostic biomarker and potential therapeutic target in OSCC.

Keywords: Cyclin-dependent kinase subunit 2 (*CKS2*); oral squamous cell carcinoma (OSCC); prognosis; immunity; cell proliferation

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Introduction

Oral squamous cell carcinoma (OSCC) is the most prevalent type of oral malignant neoplasms, representing over 90% of all cases. Despite the progress made in diagnostic and therapeutic approaches, the prognosis of OSCC has exhibited minimal advancement in recent decades (1,2). The 5-year survival rate of OSCC is still around 50% because of the high rate of metastasis and recurrence (3). Therefore, it is imperative to unravel the underlying molecular mechanisms and find innovative biomarkers to enhance the prognostic potential of OSCC.

Cyclin-dependent kinase subunit 2 (*CKS2*), a member of the human CKS family, is located on chromosome 9q22 (4). *CKS1*, a critical paralog of *CKS2*, shares 81% identical amino acids with *CKS2* but performs distinct functions. *CKS2* is a protein that interacts with cyclin-dependent kinases (CDK) and plays vital, conserved roles in the cell cycle (5). Previous research found that *CKS2* acts as a crucial nexus between the proteins necessary for meiotic chromosome recombination and those required for homologous chromosome segregation at meiotic anaphase (6). Further studies revealed that *CKS2* opposes *CKS1* in controlling cyclin A and CDK2 activity, thereby preserving replication reliability (7). Recent evidence showed that *CKS2* overlaps the DNA damage response barrier activated by oncoproteins and inhibits programmed cell death (8,9). Over the years, multiple studies have reported high expression levels of *CKS2* in various

malignant tumors, such as gastric cancer (10), hepatocellular carcinoma (11), pediatric retinoblastoma (12), colorectal cancer (13), prostatic cancer (9), lymphatic cancer (14), bladder cancer (15) and cervical cancer (16). These findings indicate a pivotal role of *CKS2* in malignant tumor progression. However, the precise role of *CKS2* in OSCC remains to be fully elucidated.

The primary aim of this study was to thoroughly investigate the role of *CKS2* in OSCC progression and prognosis. Specifically, we analyzed *CKS2* expression in OSCC samples as well as in paired adjacent non-cancerous tissues (ANCT). Meanwhile, we found that *CKS2* expression was correlated with the prognosis and some clinical characteristics of OSCC patients. Weighted gene co-expression network analysis (WGCNA) was performed to identify key modules associated with *CKS2* expression in OSCC. After that, we conducted Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA) to deepen our comprehension of the biological functions of *CKS2*. To confirm the effect of *CKS2* on cell proliferation, we carried out functional experiments in OSCC cell lines. Apart from the role in proliferation, we continued to explore other potential functions of *CKS2* in immune infiltration, tumor mutation burden (TMB), and drug sensitivity. Basing on the *CKS2*-related risk score and other clinical factors, we finally constructed and validated a risk model to predict the prognosis of OSCC patients. We present this article in accordance with the REMARK (17) and the TRIPOD reporting checklists (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-23-511/rc>).

Highlight box

Key findings

- Cyclin-dependent kinase subunit 2 (*CKS2*) is a novel prognostic biomarker and potential therapeutic target in oral squamous cell carcinoma (OSCC).

What is known and what is new?

- *CKS2* is significantly upregulated and is known to exert a substantial impact on the pathogenesis and progression of several malignant neoplasms.
- *CKS2* is recognized as a novel prognostic biomarker and potential therapeutic target in OSCC, as it promotes cell proliferation through the regulation of the cell cycle, correlates with immune infiltration and immunotherapy, enhances drug sensitivity, and offers a prognostic prediction value.

What is the implication, and what should change now?

- It is imperative to give clinical consideration to the *CKS2* expression in patients with OSCC, as it can aid in the prognostication of their condition and optimize the treatment strategy.

Methods

Clinical samples and OSCC cell lines

Totally 39 OSCC specimens and 25 ANCT specimens were obtained from the Hospital of Stomatology, Sun Yat-sen University. Additionally, we included 46 OSCC samples and five ANCT samples from the tissue chip (HOraC060PG01, Shanghai Outdo Biotech Co., China) in our analysis. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of the Hospital of Stomatology, Sun Yat-sen University (IRB AF/SC-07/v3.0; No. KQEC-2022-15-01) and informed consent was granted by each patient involved in this study.

The human OSCC cell lines CAL33, SCC1, SCC15, and SCC25 cells were utilized in the present study. Specifically,

SCC15 and SCC25 cells were procured from America Type Culture Collection. SCC1 cells were obtained from the University of Michigan, and CAL33 cells were provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). The Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), which included 10% fetal bovine serum (FBS, WISENT, Montreal, QC, Canada), was used to cultivate CAL33 and SCC1 cells. The SCC25 and SCC15 cell lines were cultured in a medium composed of DMEM/Hams F12 (DMEM/F12, Gibco, USA), supplemented with 400 ng/mL hydrocortisone (MACKLIN, Shanghai, China) and 10% FBS. All the cells were cultured at a temperature of 37 °C in a humidified incubator with 5% CO₂.

Data acquisition and pre-processing

Patients who met the following criteria were included in the study: (I) histologically verified primary OSCC; (II) sample size in the dataset was more than 70; (III) patients with complete RNA-seq data and survival data. We downloaded RNA-seq, clinical, and TMB data of OSCC patients (306 tumor samples and 30 normal controls) from the The Cancer Genome Atlas (TCGA) database (<https://www.cancer.gov/tcga>) (18). The RNA-seq data and associated clinical data were obtained from the GEO database, which included data on 45 normal samples and 167 OSCC samples (GSE30784), 29 normal samples and 74 OSCC samples (GSE42743), and 97 OSCC samples (GSE46163). The RNA-seq data was filtered, missing and duplicated data were removed before it was converted to log₂(TPM +1). The OSCC samples with complete clinical data in TCGA-OSCC and GSE4743 datasets were included in the development of the Nomogram.

CKS2 differential expression and prognosis correlation

The Gene Expression Profiling Interactive Analysis (GEPIA2) web server (<http://gepia2.cancer-pku.cn/>) was used to analyse gene expression in tumor and normal samples obtained from the TCGA and Genotype-Tissue Expression (GTEx) databases (19). We performed differential expression analysis and overall survival (OS) analysis of *CKS2* by the GEPIA2 on the 33 cancer subtypes in the TCGA pan-cancer datasets. After that, we utilized the R package “limma” to calculate *CKS2* mRNA expression between normal specimens and OSCC specimens in GSE30784, GSE42743, and TCGA

datasets. The relationships between the mRNA level of *CKS2* and several clinical features of the TCGA-OSCC and GSE41613 cohorts were also examined. The OSCC patients were divided into *CKS2*-high expression group and *CKS2*-low expression group based on the median value of *CKS2* expression. Kaplan-Meier survival analysis was then conducted to compare the OS and PFS in these two groups using the “Survival” package.

WGCNA

The R package “WGCNA” (20) was utilized for the analysis of the top 25% of the most variant genes in the TCGA dataset and GSE30784 dataset. To ensure data integrity, the “goodSamplesGenes” function was implemented. Following the confirmation of the optimal soft threshold value (β), the expression matrix was transformed into multiple modules via the “blockwiseModules” function. Subsequently, we determined the correlation coefficients for *CKS2* expression level and eigengene in the module. In conclusion, the pivotal module was identified for further analysis.

Functional enrichment analysis

The GO and KEGG analysis of the hub module gene were performed using the R package “clusterProfiler” (21,22).

We applied the GSEA to analyze the enriched biological functions and pathways between the groups with low and high *CKS2* expression (23). Gene sets with FDR and P value less than 0.05 were considered highly enriched.

Immunohistochemistry (IHC) staining

Expression of the *CKS2* protein was investigated by immunostaining in OSCC and paired ANCT. After dewaxing, dehydrating, and antigen repairing, we blocked the tissue slices with goat serum (AR0009, BosterBio, China). Next, we incubated the tissue slices overnight at 4 °C with a primary antibody against *CKS2* (1:100; ab155078, Abcam). The sections were subsequently treated with secondary antibodies for 30 minutes at 25 °C and stained with diaminobenzidine (DAB, GK600510, Gene Tech, China). The percentage of positively stained cells (0–100%) and the intensity of tissue staining (0: no staining, 1: weak staining, 2: moderate staining, and 3: strong staining) were estimated, and the staining index (ranging from 0 to 300) was used to calculate the immunoscore (percentage of positive cells multiplied by staining intensity).

Small interfering RNA (siRNA) and lentiviral transfection

CAL33 and SCC25 cells were seeded and incubated for approximately 24 hours until they reached the logarithmic growth phase. Following the product instructions, we applied the Pepmute Transfection Reagent (Signagen, Rockville, MD, USA) to transfect 30 nM *CKS2*-specific si-RNAs or negative controls (si-NC) in each well. The two independent siRNA sequences are: si-*CKS2*#1: 5'-GAGTCTAGGCTGGGTTTCATTA-3', si-*CKS2*#2: 5'-CTTGGTGTCCAACAGAGTCTA-3'.

The lentiviral vector pCDH-CMV-MCS carrying the *CKS2* coding sequence was employed to achieve stable overexpression of *CKS2*. According to the product instructions, cotransfection of the 293T cells were achieved using the lentivirus packaging plasmids psPAX2, pMD2G (GenePharma, China), and *CKS2* plasmids. An empty vector was used as the control group. After 48 hours of transfection, the supernatant containing virus was collected, filtered, concentrated, and then transfected into SCC15 and SCC1 cells with 3 µg/mL Polybrene (40804ES76, Yeasen, China). After infection, puromycin (P8230, Solarbio, China) was used to screen the cells for 14 days (SCC1: 2 µg/mL, SCC15: 2.5 µg/mL).

Western blot

The cells were lysed using the RIPA buffer (CW2333S, CWBIO, China). The proteins were measured and distinguished through the process of electrophoresis on a 15% SDS-PAGE gel. Following this, the proteins were transferred onto PVDF membranes (ISEQ00010, Millipore, USA). After blocking them with 5% bovine serum albumin (0332, Amresco) at room temperature for one hour, the membranes were incubated with primary antibodies against *CKS2* (1:2,000; ab155078, Abcam) and β-actin (1:1,500; 30102ES60, Yeasen, China) overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (GK600510, Gene Tech, China) for one hour at room temperature. Finally, the bands were quantified using Image-J (<https://imagej.nih.gov/ij/download.html>) after exposure to a chemiluminescent HRP substrate.

RNA extraction and quantitative real-time PCR (qRT-PCR)

According to the guidelines provided by the manufacturer, we extracted the RNA using the RNazol reagent (RN190, Molecular Research Center, USA) and then reverse transcribed it to cDNA through utilization of the the

Hifair™ III 1st Strand cDNA Synthesis SuperMix (11141ES60, Yeasen, China). In order to conduct the qPCR reactions, we utilized the SYBR Green Master Mix (11201ES08; Yeasen, China) in the LightCycler 96 System (Roche, Basel, Switzerland). The $2^{-\Delta\Delta C_t}$ method was used to determine the relative RNA expression of *CKS2* compared to the control. The following are the primer sequences we utilized: β-*ACTIN* forward: CTACCTCATGAAGATCCTCACCGA, reverse: TTCTCCTTAATGTCACGCACGATT; *CKS2* forward: TTCGACGAACACTACGAGTACC; reverse: GGACACCAAGTCTCCTCCAC.

Cell counting kit-8 (CCK-8) assay

CAL33, SCC25, SCC1, and SCC15 cells were seeded into 96-well plates at a density of 2,000 cells per well. The CCK-8 (40203ES80, Yeasen, China) was utilized to quantify the proliferation of cells at 0, 24, 48, 72, and 96 hours. The growth curve was eventually produced using the absorbance values obtained from a microplate reader (Bio-Rad, USA) operating at 450 nm.

Colony formation assay

Following the corresponding treatment, 800 cells of CAL33, SCC25, SCC1, and SCC15 were seeded in each well of 6-well plates. Following a 10–14-day culture period to facilitate colony formation, the cells were immobilized with methanol and subsequently stained with crystal violet. Upon examination under a microscope, the colonies were quantified.

Immune infiltration and TMB analysis

The CIBERSORT algorithm was employed to calculate the abundances of 22 immune cells in TCGA-OSCC cohort. Subsequently, the association between the abundances of these 22 immune cells and *CKS2* expression was examined. To investigate the correlation between *CKS2* expression and TMB in OSCC, the Spearman's correlation was computed in the TMB analysis.

Drug sensitivity analysis

To estimate the IC₅₀ value of frequently used anti-tumor agents in the TCGA-OSCC cohort, tumor drug response data were collected from the Genomics of Drug Sensitivity in

Cancer (GDSC) (<https://www.cancerRxgene.org>) (24). The R package “oncopredict” was applied to explore the relationship between *CKS2* expression and drug sensitivity (25). P value <0.05 was considered statistically significant.

Establishment of a risk model based on prognostic genes associated with CKS2

By conducting differential analysis on *CKS2* expression and univariate Cox regression analysis, the prognostic genes related to *CKS2* were identified in the TCGA-OSCC cohort. Moreover, we seek out those genes with optimal performance and calculate their coefficients using least absolute shrinkage and selection operator (LASSO) Cox regression algorithm. Following this, the risk score for each sample was calculated by multiplying the expression of each gene by its respective regression coefficient (β), and adding them together. Patients with OSCC were divided into high- and low-risk groups according to the median value of the risk score.

Development and verification of the nomogram

To estimate the predictive accuracy of the risk score in the TCGA-OSCC cohort, we calculated the area under the ROC curve (AUC) at 1, 3, and 5 years. Additionally, the prognostic value of the risk score and other clinical features (including age, gender, stage, T, and N) was evaluated using univariate and multivariate COX regression analyses. Based on the risk score and these clinical factors, we formulated a nomogram utilizing the R package “rms” nomogram to predict the 1-, 3-, and 5-year survival probabilities of OSCC patients. The calibration curves and ROC were utilized to verify it. Furthermore, we validated the nomogram in the external validation dataset GSE42743 using the same methodology.

Statistical analysis

R software (version 4.2.2, <https://www.Rproject.org>) and GraphPad Prism 9.0 software were used for statistical analysis in this study. Wilcoxon test was employed to compare the expression of *CKS2* among the two groups. Correlation assessment was conducted by calculating Pearson’s *r* value. Mann-Whitney *U*, Kruskal-Wallis, and Chi-square tests were used to assess the association between *CKS2* expression and clinicopathological parameter. One way ANOVA and student’s *t*-test were utilized to compare the control group and the experimental group. A P value of

0.05 or less was considered statistically significant.

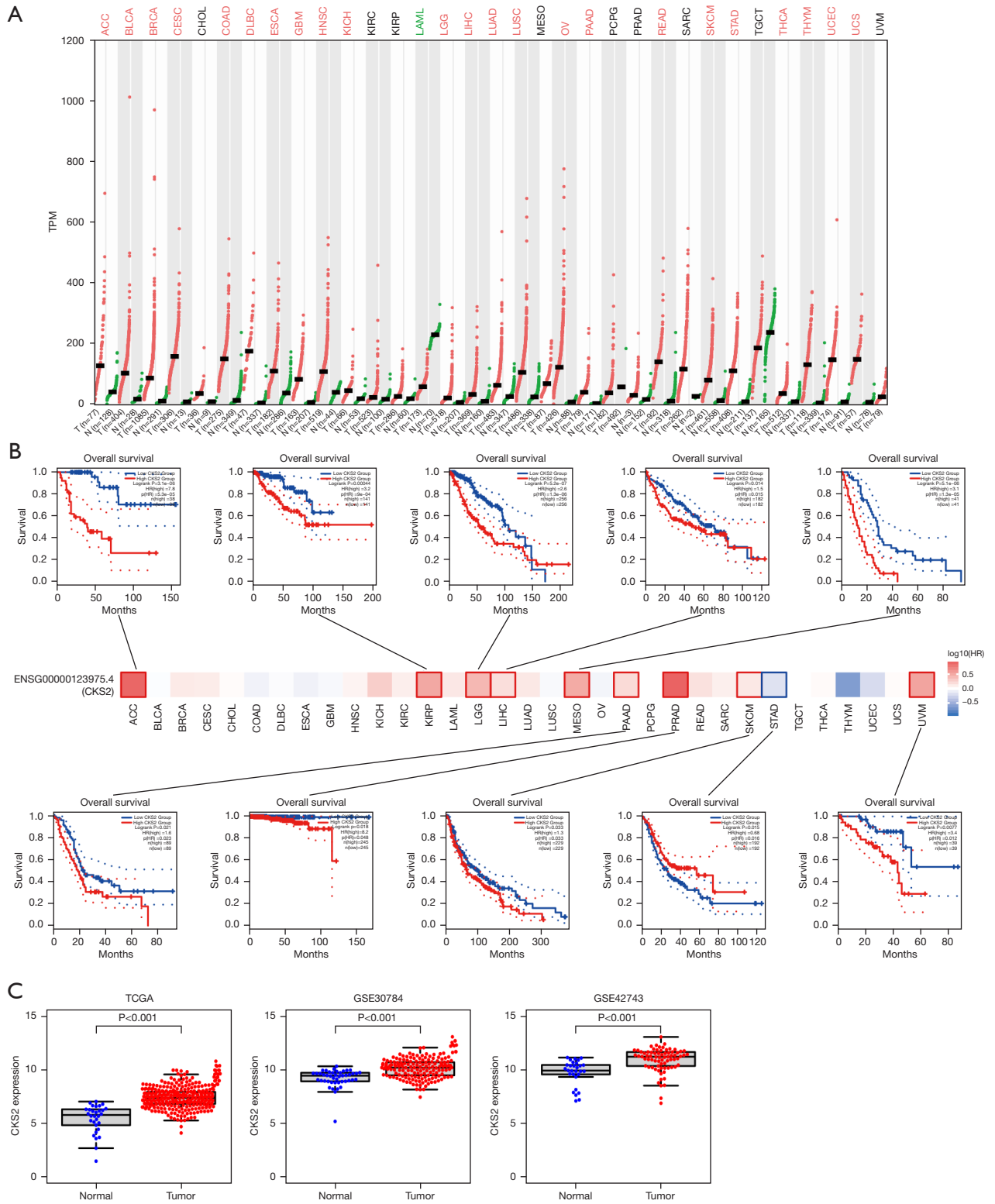
Results

CKS2 is overexpressed in OSCC and correlated with poor prognosis

Firstly, we used the GEPIA web server to explore the expression level and the prognostic significance of *CKS2* in a comprehensive pan-cancer assessment. Our analysis encompassed RNA-seq and clinical data of 33 cancer subtypes gathered from the GTEx and TCGA databases. Notably, in comparison to normal tissue, we observed a substantially elevated expression level of *CKS2* in tumor tissue across the most subtypes (*Figure 1A*). Additionally, through our OS analysis, we concluded that high *CKS2* expression was a predictor of a poor prognosis in adrenocortical cancer (ACC), kidney papillary cell carcinoma (KIRP), brain lower grade glioma (LGG), liver hepatocellular carcinoma (LIHC), mesothelioma (MESO), pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), skin cutaneous melanoma (SKCM), and uveal melanoma (UVM) (*Figure 1B*). Based on our pan-cancer analysis, we proceeded to study *CKS2* expression specifically in OSCC. We compared data obtained from TCGA, GSE30784, and GSE42743 datasets, which demonstrated a significant upregulation of *CKS2* in OSCC samples when compared with normal tissue samples (*Figure 1C*, $P < 0.001$). In addition, the IHC staining analysis of the clinical samples corroborated our findings (*Figure 1D, 1E*, $P < 0.001$).

We separated the 306 OSCC samples chosen from the TCGA dataset into groups with high and low *CKS2* mRNA expression levels, using the median expression level of *CKS2* as the dividing point. Our findings indicate that elevated *CKS2* expression is notably correlated with higher grade (*Figure 1F*, $P < 0.01$), male gender (*Figure 1G*, $P < 0.05$), older age (*Figure 1H*, $P < 0.05$), higher N stage (*Figure 1I*, $P < 0.05$), and greater positivity of lymph nodes (*Figure 1J*, $P < 0.05$).

Considering that *CKS2* expression was found to be high in OSCC and this high expression was associated with unfavorable clinical characteristics, we proceeded to investigate the prognostic significance of *CKS2* in both TCGA-OSCC and GSE41613 datasets. Through Kaplan-Meier survival analysis of OS and PFS, we observed that elevated *CKS2* expression levels were significantly linked to a poorer prognosis (*Figure 1K*, $P = 0.0018$; *Figure 1L*, $P = 0.001$; *Figure 1M*, $P = 0.013$).



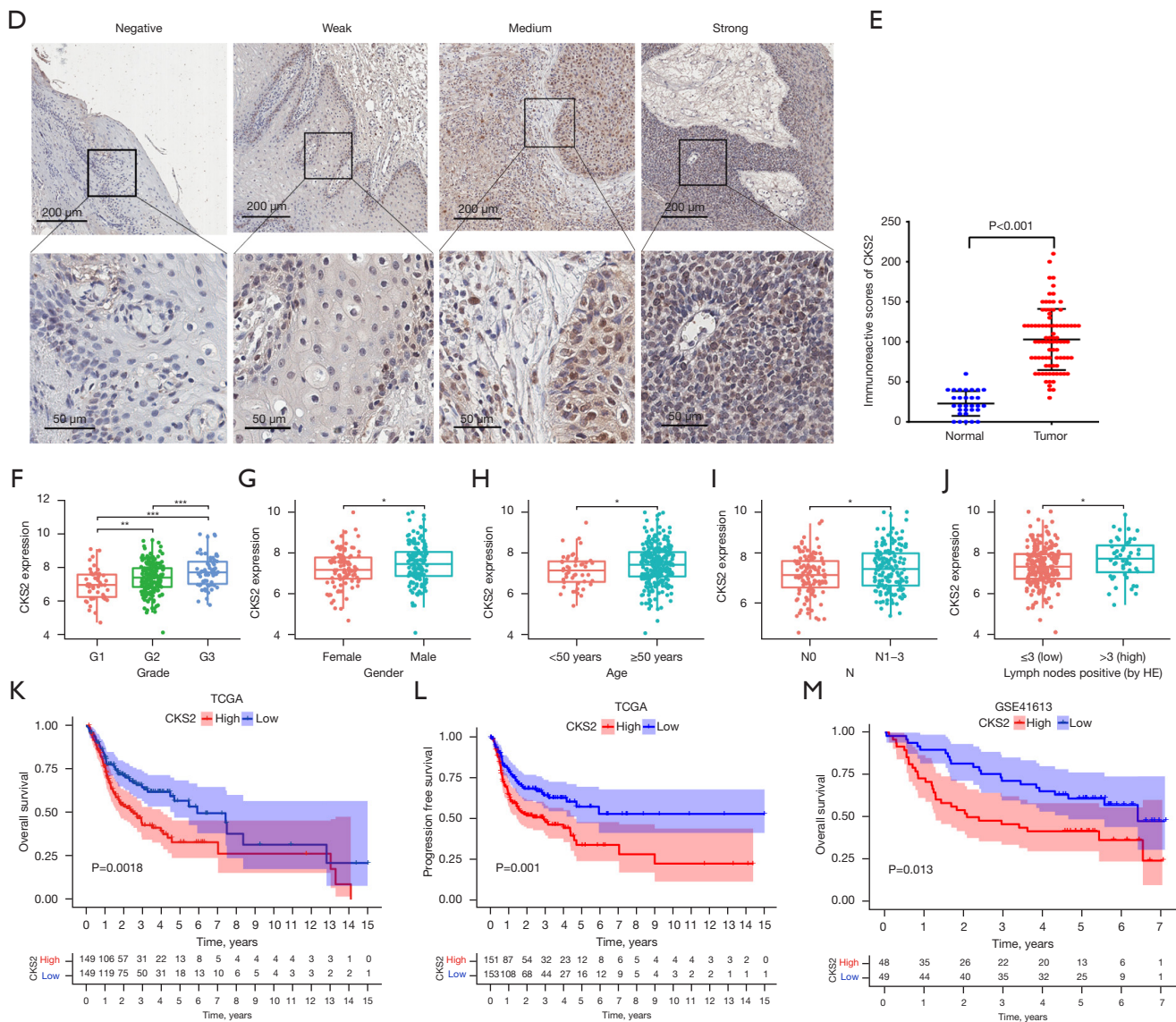


Figure 1 *CKS2* was overexpressed in OSCC tissues and associated with poor prognosis. (A) Differential expression of *CKS2* in 33 different tumor tissues and paired normal tissues from TCGA and GTEx databases. Each dot represents the expression of samples. (B) The prognostic impact of *CKS2* expression level based on the survival heatmap, showing significance in ACC, KIRP, LGG, LIHC, MESO, PAAD, PRAD, SKCM, STAD and UVM. (C) Differential analysis of *CKS2* expression between OSCC and normal samples in OSCC-TCGA, GSE30784, and GSE42743 datasets. (D) Representative images of immunohistochemistry staining for *CKS2* in ANCT and OSCC tissues. Magnification at 50 \times (up) and 200 \times (down). (E) Immunohistochemical staining score of *CKS2* in ANCT (n=30) and OSCC (n=85) tissues. (F-J) The correlation between *CKS2* expression and clinical features in TAGA-OSCC cohort, including the grade, gender, age, N, and lymph nodes positive (by hematoxylin-eosin staining). (K-L) Kaplan-Meier survival curves of overall survival (K) and progression free survival (L) based on TCGA-OSCC patients with high- and low-expression *CKS2*. (M) Kaplan-Meier survival curves of overall survival based on GSE41613 dataset. Differences between the two groups were compared using a log-rank test. *, P<0.05; **, P<0.01; ***, P<0.001. *CKS2*, cyclin-dependent kinase subunit 2; TCGA, The Cancer Genome Atlas; N, node; HE, hematoxylin and eosin; OSCC, oral squamous cell carcinoma; GTEx, Genotype-Tissue Expression; ACC, adrenocortical cancer; KIRP, kidney papillary cell carcinoma; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; MESO, mesothelioma; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; SKCM, skin cutaneous melanoma; STAD, stomach cancer; UVM, uveal melanoma; ANCT, adjacent non-cancerous tissues.

Construction of a weighted co-expression network and the detection of the key module associated with CKS2

Using the WGCNA method, we aimed to uncover gene modules that cooperatively express and investigate their relationship with *CKS2* expression. Initially, we produced clustering dendrograms and eliminated outliers before selecting a power of $\beta=5$ to attain the desired scale-free topology (Figure S1A,S1B). As the clustering dendrogram presented, we merged modules with an eigenvalue similarity >0.75 for further examination (Figure S1C,S1D). The WGCNA algorithm recognized 18 modules and 12 modules in GSE30784 and TCGA-OSCC cohorts, respectively. In both datasets, the red module with the largest correlation coefficient with *CKS2* ($r=0.58$, $P=1\times 10^{-28}$; $r=0.65$, $P=6\times 10^{-21}$) was recognized as the key module for further analysis (Figure 2A,2B and website: <https://cdn.amegroups.cn/static/public/tcr-23-511-1.xlsx>, website: <https://cdn.amegroups.cn/static/public/tcr-23-511-2.xlsx>).

Functional enrichment analysis

We conducted KEGG enrichment and GO annotation analyses on the red module to explore the pathways associated with *CKS2* in OSCC. The GO analysis results demonstrated that genes in the red module were significantly correlated with cell division (Figure 2C,2D and website: <https://cdn.amegroups.cn/static/public/tcr-23-511-3.xlsx>, website: <https://cdn.amegroups.cn/static/public/tcr-23-511-4.xlsx>). The KEGG analysis results indicated that genes in the red module were predominantly enriched in the cell cycle (Figure 2E,2F and website: <https://cdn.amegroups.cn/static/public/tcr-23-511-5.xlsx>, website: <https://cdn.amegroups.cn/static/public/tcr-23-511-6.xlsx>).

Furthermore, we conducted GSEA between groups with high and low *CKS2* expression using the R package “clusterProfiler”. A similar outcome was obtained from the analysis, which revealed that group with high *CKS2* expression was primarily enriched with “Cell Cycle” and “DNA Replication” (Figure 2G,2H and website: <https://cdn.amegroups.cn/static/public/tcr-23-511-7.xlsx>, website: <https://cdn.amegroups.cn/static/public/tcr-23-511-8.xlsx>).

CKS2 promotes the proliferation of OSCC cells

Based on the aforementioned analysis, our hypothesis

suggests that the regulation of cell cycle by *CKS2* promotes the proliferation of OSCC cells. To validate our conjecture, we developed cellular models for *CKS2* knockdown and overexpression. We transfected siRNA, which was specifically designed to target *CKS2*, into CAL33 and SCC25 cell lines to suppress *CKS2* expression. Additionally, we constructed a lentiviral vector that carries the *CKS2* coding sequence to stably overexpress *CKS2* in SCC1 and SCC15. The knockdown and overexpression effects of *CKS2* were determined using PCR and Western blotting (Figure 3A,3B). Following this, we conducted colony formation and CCK-8 assays to assess the cell growth. The results indicate that downregulation of *CKS2* considerably reduced cell proliferation and clonogenicity in CAL33 and SCC25 cells. Conversely, upregulation of *CKS2* significantly amplified cell proliferation and clonogenicity in SCC1 and SCC15 (Figure 3C-3E).

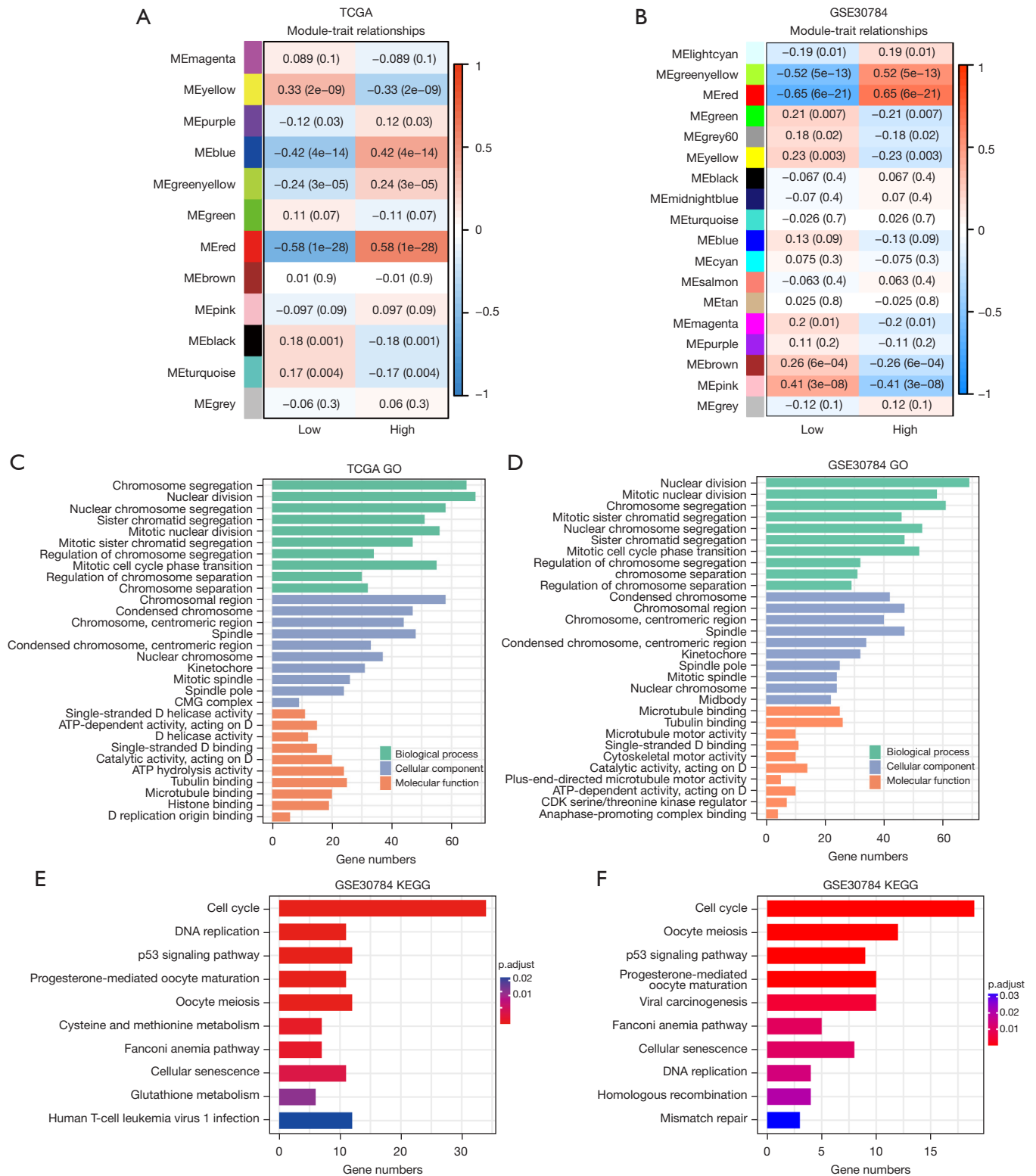
Immunological significance of CKS2 in OSCC

Apart from its role in proliferation, we further investigated other potential functions of *CKS2* in the regulation of OSCC. Initially, we employed the CIBERSORT algorithm to illustrate the presence of 22 immune cells infiltrating the TCGA-OSCC cohort, wherein significant differences were observed between the high and low *CKS2* expression groups (Figure 4A,4B). Correlation analyses were also conducted between *CKS2* expression and the 22 immune cells (Figure 4C-4G). Our findings suggest that *CKS2* has a positive correlation with the infiltration of macrophage M1 ($R=0.23$, $P=5.2\times 10^{-5}$) and macrophage M2 ($R=0.13$, $P=0.026$), while exhibiting a negative correlation with immune infiltration of dendritic cells resting ($R=-0.15$, $P=0.0091$), mast cells resting ($R=-0.25$, $P=1.2\times 10^{-5}$), and neutrophils ($R=-0.22$, $P=1\times 10^{-4}$).

Emerging evidence shows that TMB is a novel prognostic biomarker for immunotherapy in various cancers. We collected TMB data from the TCGA-OSCC cohort and included it in the analysis along with *CKS2* expression. As a result, high expression of *CKS2* was positively related to TMB (Figure 4H, $R=0.23$, $P=5.8\times 10^{-5}$).

Drug sensitivity analysis

Antitumor drugs play a vital role in OSCC therapy. Hence, we explored the potential association between *CKS2* expression level and anti-tumor drug sensitivity. Following the NCCN guidelines, we focused on the recommended



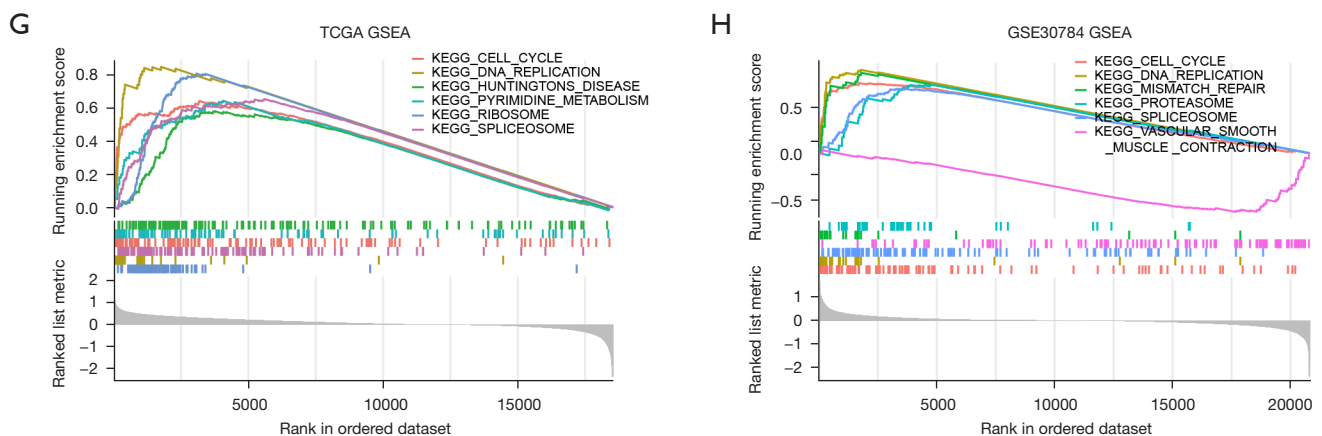


Figure 2 Identification of the key module related to *CKS2* and functional enrichment analysis in TCGA-OSCC and GSE30784 datasets. (A,B) The correlation coefficients and P values of module-trait relationships. Each row corresponds to a module eigengene. (C,D) GO analysis for *CKS2*-related key module. (E,F) KEGG enrichment analysis for *CKS2*-related key module. (G,H) GSEA results show the significant enriched pathway based on *CKS2* expression. *CKS2*, cyclin-dependent kinase subunit 2; TCGA, The Cancer Genome Atlas; OSCC, oral squamous cell carcinoma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, The Gene Set Enrichment Analysis.

therapeutic drugs, which included cisplatin, carboplatin, 5-FU, paclitaxel, docetaxel, hydroxyurea, cetuximab, vincristine, cyclophosphamide, and methotrexate (26). Our findings indicated that a high level of *CKS2* expression was significantly correlated with a low IC50 of cisplatin, 5-FU, paclitaxel, vincristine, cyclophosphamide, and docetaxel (Figure 5A-5F). Furthermore, a high level of *CKS2* expression was also significantly correlated with a low IC50 of pevonedistat, rapamycin, and MK-1775, suggesting that they could be potentially effective treatments (Figure 5G-5I).

A nomogram based on CKS2-related genes

The study above showed that *CKS2* has significant functions in OSCC, leading us to investigate its clinical predictive value. By utilizing a cutoff of $\log_{2}FC > 1$ and an adjusted P value of < 0.05 , we detected 110 genes related to *CKS2* expression level in the TCGA-OSCC cohort (website: <https://cdn.amegroups.com/static/public/tcr-23-511-9.xlsx>). Univariate Cox regression models indicated that six genes, namely *COL6A5* (HR =0.454, 95% CI: 0.229–0.902, $P=0.024$), *SERPINA9* (HR =0.737, 95% CI: 0.576–0.944, $P=0.016$), *HOXB9* (HR =1.136, 95% CI: 1.000–1.289, $P=0.050$), *GABRA* (HR =2.294, 95% CI: 1.259–4.179, $P=0.007$), *AKAP14* (HR =1.656, 95% CI: 1.097–2.500, $P=0.016$), and *CKS2* (HR =1.358, 95% CI: 1.134–1.625, $P<0.001$), were linked to OSCC patients' prognosis (Figure

6A). LASSO regression analysis determined five *CKS2*-related genes to create a prognostic gene model (Figure 6B,6C). The *CKS2*-derived risk score = $(-0.5676 * COL6A5 \text{ expression}) + (-0.2141 * SERPINA9 \text{ expression}) + (0.5742 * GABRA \text{ expression}) + (0.4183 * AKAP14 \text{ expression}) + (0.1560 * CKS2 \text{ expression})$. 253 samples in TCGA-OSCC and 74 samples in GSE42743 were selected to construct the nomogram. Our research in univariate Cox regression analysis further revealed that the risk score (HR =3.209, 95% CI: 2.059–5.002, $P<0.001$), T (HR =1.470, 95% CI: 1.209–1.787, $P<0.001$), N (HR =1.552, 95% CI: 1.266–1.902, $P<0.001$), and age (HR =1.019, 95% CI: 1.003–1.036, $P=0.021$) were significantly correlated with OSCC prognosis (Figure 6D). A multivariate Cox regression analysis was conducted, which indicated that the risk score (HR =2.265, 95% CI: 1.426–3.598, $P<0.001$), T (HR =1.543, 95% CI: 1.141–2.086, $P=0.005$), N (HR =1.511, 95% CI: 1.213–1.880, $P<0.001$), and age (HR =1.021, 95% CI: 1.003–1.040, $P=0.026$) were independent prognostic indicators of OSCC (Figure 6E). Notably, the risk score based on *CKS2* exhibited greater predictive potential for OSCC compared to conventional clinical indicators such as age, gender, T, N, and stage (Figure 6F). Finally, we developed a nomogram that effectively integrates the risk score and related clinical factors, within the training set, to predict the OS of OSCC patients (Figure 6G). The points of each factor add up to the total points, which indicate

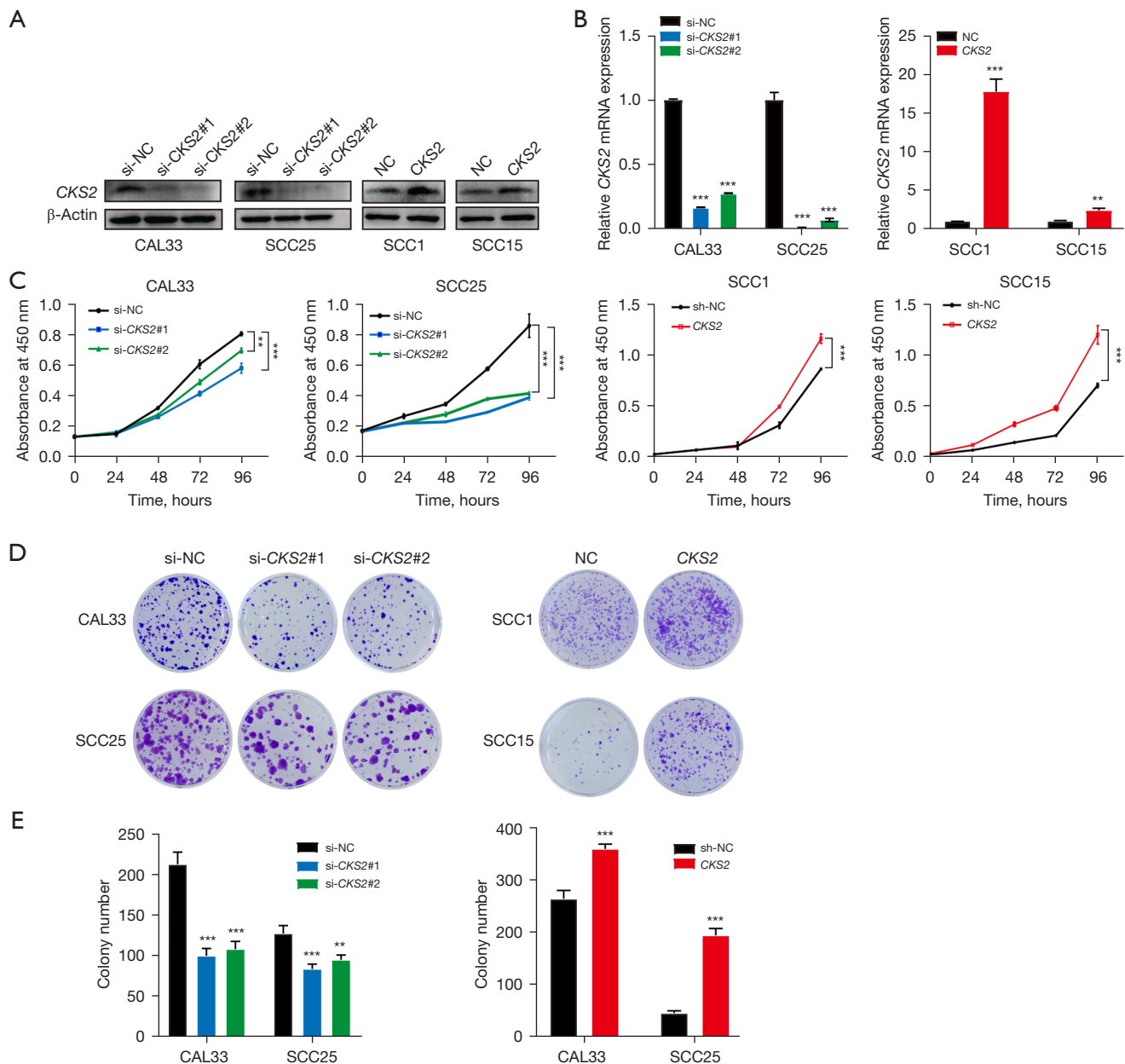


Figure 3 *CKS2* promotes proliferation of OSCC by regulating cell cycle. (A,B) Western blot and RT-qPCR were used to detect the *CKS2* expression after transfection in OSCC cell lines. (C) CCK-8 assay showed that down regulation of *CKS2* expression inhibited the proliferation of CAL33 and SCC25 cells, up-regulation of *CKS2* expression promoted the proliferation of SCC1 and SCC15 cells. (D,E) Clone formation assay showed that down regulation of *CKS2* expression inhibited the proliferation of CAL33 and SCC25 cells, up-regulation of *CKS2* expression promoted the proliferation of SCC1 and SCC15 cells. Cells were stained with crystal violet. Magnification at 1×. **, $P < 0.01$; ***, $P < 0.001$. OSCC, oral squamous cell carcinoma; *CKS2*, cyclin-dependent kinase subunit 2; TCGA, The Cancer Genome Atlas; RT-qPCR, quantitative reverse transcription PCR; CCK-8, cell counting kit-8; NC, negative control.

1-, 3-, and 5-year survival probabilities. The ROC curves confirmed the prediction accuracy of the nomogram in predicting OSCC patients' survival outcomes (Figure 6H).

And the calibration curves revealed that the predicted OS results were highly consistent with actual results for the 1-, 3-, and 5-year periods (Figure 6I). In addition, data from

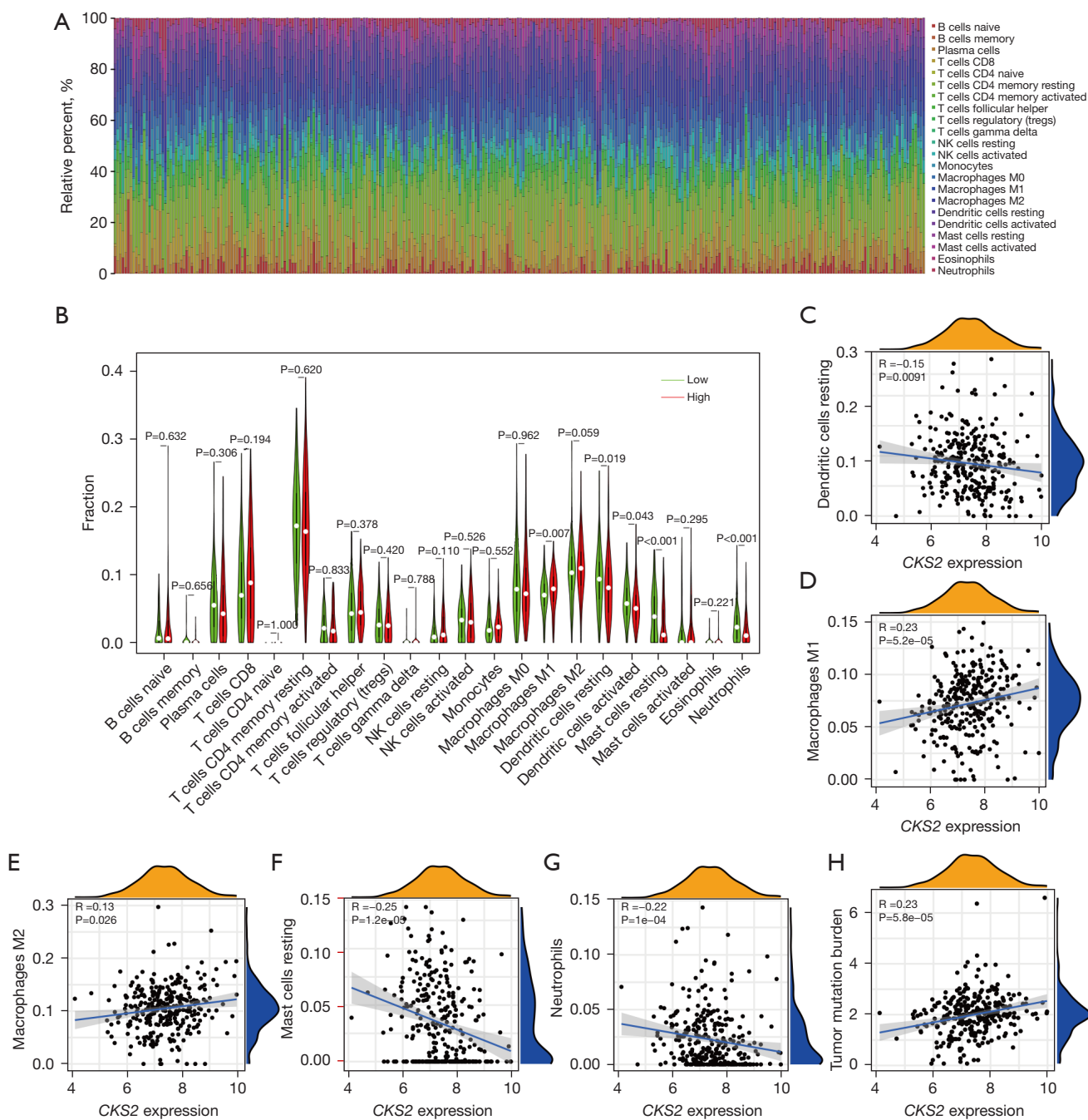


Figure 4 Immunological significance of *CKS2* in OSCC. (A) The distribution of infiltrating immune cells in 306 TCGA-OSCC samples based on *CKS2* expression. (B) The differences in the infiltrating levels of 22 immune cells between the high- and low-*CKS2* expression groups. (C-G) The correlations between the *CKS2* expression and the enrichment of five core immune cells. (H) The correlations between the *CKS2* expression and the tumor mutation burden. *CKS2*, cyclin-dependent kinase subunit 2; OSCC, oral squamous cell carcinoma; TCGA, The Cancer Genome Atlas.

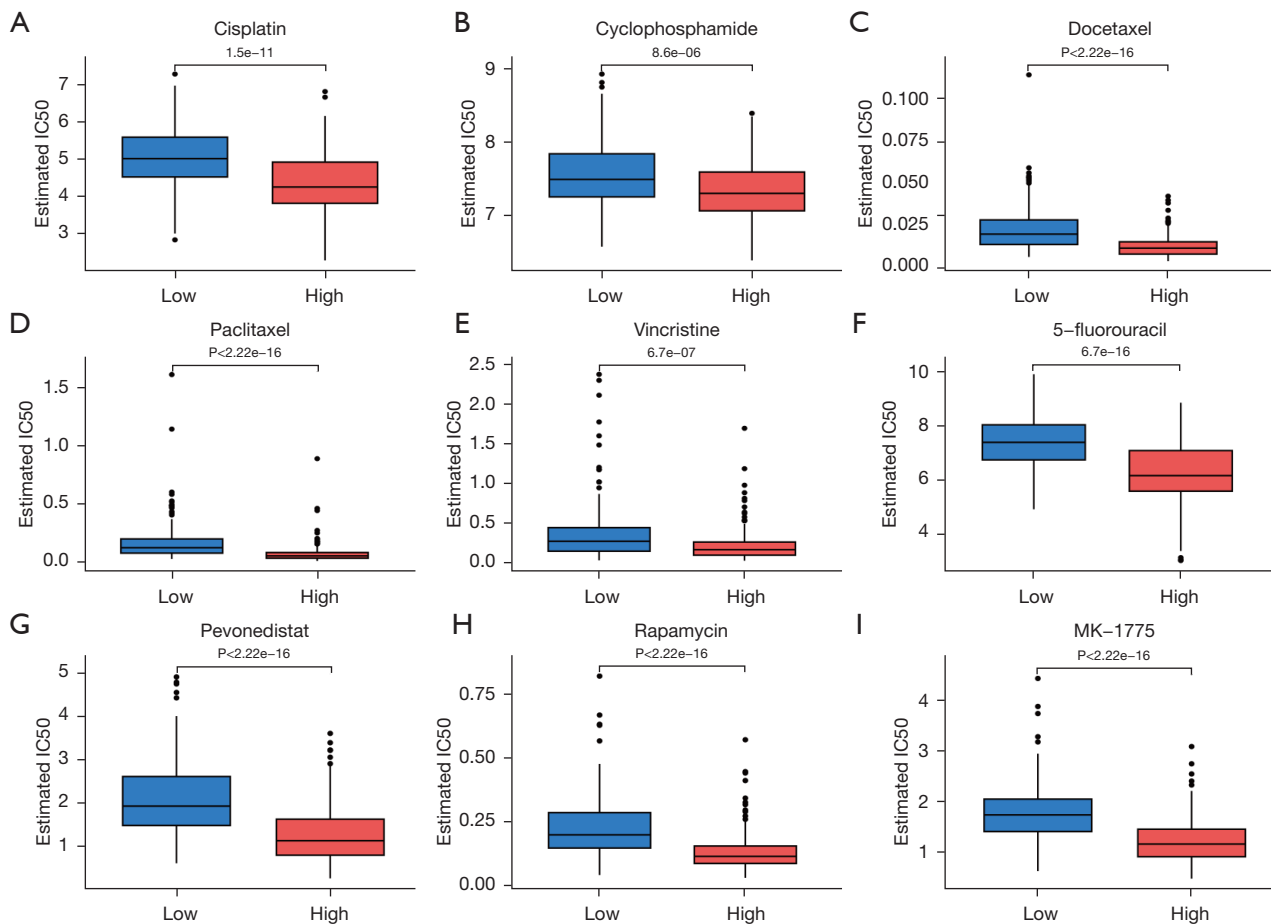


Figure 5 Drug prediction of IC50 differences in *CKS2* high and low group based on the GDSC database. (A-F) High level of *CKS2* expression was significantly correlated with low IC50 of recommended therapeutic drugs in OSCC. (G-I) Other commonly used anti-tumor drugs most associated with the expression level of *CKS2*. IC50, half maximal inhibitory concentration; *CKS2*, cyclin-dependent kinase subunit 2; GDSC, Genomics of Drug Sensitivity in Cancer; OSCC, oral squamous cell carcinoma.

GSE42743 was utilized to validate the nomogram, which indicated the nomogram possessed the potential to function as a quantitative tool for predicting OS in patients with OSCC (Figure 6J, 6K).

Discussion

As one of the most common malignant tumors globally, OSCC is typically treated with surgery, chemotherapy, radiotherapy, or a combination of these modalities (26). Unfortunately, despite these efforts, around 50% of OSCC patients eventually succumb to recurrence or metastasis (3). Given the high heterogeneity of OSCC, identifying new biomarkers could lead to significant clinical benefits for OSCC patients, as evidenced by recent studies (27). *CKS2*,

a protein that has been shown to be highly expressed and correlated with unfavourable prognosis in various cancers such as colorectal cancer, epithelial ovarian cancer, and osteosarcoma (28-30), is among the promising candidates. However, the precise roles of *CKS2* in OSCC remain unclear, which prompted us to investigate it. Our study unveiled that *CKS2* was overexpressed in OSCC samples and associated with unfavorable prognosis. Through WGCNA and functional enrichment analysis, we discovered that *CKS2* may promote OSCC cell proliferation by modulating the cell cycle, a finding that was validated through functional assays conducted in OSCC cell lines. Additionally, we examined the roles of *CKS2* in immune infiltration, TMB, and drug sensitivity. Finally, an established and validated risk model based on prognostic

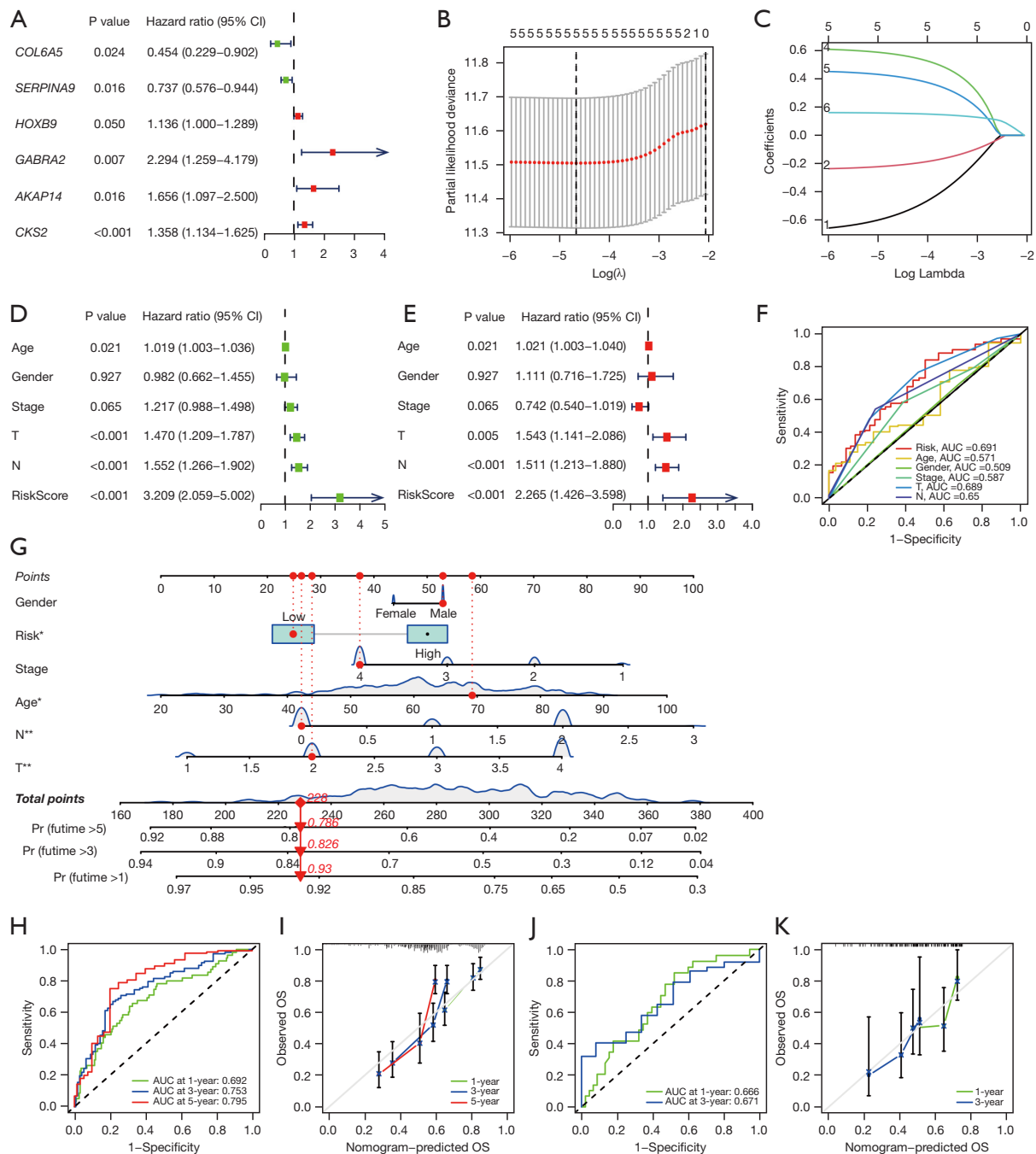


Figure 6 Construction and validation of a nomogram based on *CKS2*-related genes. (A) Forest plot of univariate Cox regression analysis of 6 hub genes associated with *CKS2* expression and OS in TCGA-OSCC cohort. (B,C) LASSO regression analysis screened 5 *CKS2*-related genes. (D,E) Uni- and multivariate Cox regression models are conducted to uncover the association of clinical features and *CKS2*-derived risk score with OSCC survival outcome. (F) The ROC curves show the predictive power of *CKS2*-derived risk score and clinical features on the prognosis of OSCC patients. (G) Construction of prognostic nomogram for predicting OS of OSCC patients based on TCGA dataset. (H,I) The ROC curves and calibration curves for 1-, 3-, and 5-year in the TCGA-OSCC training set. (J,K) The ROC curves and calibration curves for 1- and 3-year in the GSE42743 testing set. *, $P < 0.05$; **, $P < 0.01$. T, tumor; N, node; AUC, area under the curve; CI, confidence interval; OS, overall survival; *CKS2*, cyclin-dependent kinase subunit 2; TCGA, The Cancer Genome Atlas; OSCC, oral squamous cell carcinoma; LASSO, least absolute shrinkage and selection operator; ROC, receiver operating characteristic.

CKS2-related genes was developed in light of the abundant and significant functions played by *CKS2*.

The pan-cancer analysis findings indicated that *CKS2* was highly expressed in most subtypes, and negatively correlated with OS, consistent with studies on adrenocortical carcinoma (31), lower-grade glioma (32), hepatocellular carcinoma (11), pancreatic adenocarcinoma (33), and skin cutaneous melanoma (34). Both several independent datasets and the IHC assay data confirmed upregulation of *CKS2* in OSCC samples. Notably, the IHC assay showed that *CKS2* was prominently stained in the region close to the stratum basale of normal epithelial tissue. It can be hypothesized that this result could be attributed to the actively proliferating cells in the stratum basale. The Kaplan-Meier survival analysis demonstrated that increased levels of *CKS2* expression were linked to unfavorable OS and PFS. Despite the lack of statistical significance between *CKS2* expression and the OS rate of head and neck squamous cell carcinoma (HNSC), it was postulated that this outcome might have resulted from the limited sample size and non-OSCC HNSC. Additional analysis revealed a significant association between high *CKS2* expression level and older age, male gender, higher grade, and greater lymph node positivity—all of which are commonly observed adverse clinical features in carcinoma.

To investigate the role of *CKS2* in OSCC, we conducted WGCNA and functional enrichment analyses in GSE30784 and TCGA-OSCC cohorts. The results of GSEA and KEGG analyses showed that *CKS2* mainly activates cell cycle and DNA replication pathways, which was consistent with the findings of GO analysis that showed enrichment in chromosome segregation and nuclear division of hub module genes. Recently, *CKS2* was found to induce cell cycle progression and cell proliferation through downregulating p21, p53, and PTEN in non-small cell lung cancer (35). A study has reported that the interaction between CDK2 and CKS1 or CKS2 provides cells with partial resistance to inhibitory tyrosine phosphorylation mediated by the intra-S-phase checkpoint, enabling them to continue DNA replication even in the presence of replicative stress (8). Several studies have shown that *CKS2* is essential for cell proliferation (12,33,36). These findings indicated that upregulation of *CKS2* might promote OSCC growth and proliferation by promoting the cell cycle. Our *in vitro* experiments confirmed that downregulation of *CKS2* reduced the proliferation and clonogenicity of OSCC cells, while overexpression of *CKS2* significantly enhanced them. To summarize, *CKS2* potentially expedites the proliferation

of OSCC cells through its promotion of the cell cycle.

Substantial evidence suggests that the tumor microenvironment (TME) is widely recognized as a critical contributor to cancer initiation and promotion of tumor growth (37). To gain further insight into the potential effects of *CKS2* on OSCC, we conducted an immune infiltration analysis on the TCGA-OSCC cohort. The results revealed that *CKS2* expression was positively correlated with tumor-associated macrophages (TAMs), including Macrophage M1 and M2, and negatively associated with the infiltration of resting dendritic cells, resting mast cells, and neutrophils. It is worth noting that TAMs are known to contribute to tumor initiation, progression, angiogenesis, and metastasis (38,39). Recent studies have shown that targeting TAMs enhances immunotherapy (40-42). In light of these findings, *CKS2* is probably involved in the formation of the immune-tolerant microenvironment. Besides, *CKS2* may activate dendritic cells and mast cells by regulating the cell cycle.

Moreover, our investigation revealed that elevated *CKS2* expression in the TCGA-OSCC cohort was positively correlated with TMB, which has been shown to be a valuable biomarker for immunotherapy in certain cancer types (43). While additional verification is required, it is plausible that *CKS2* has multifaceted roles within the OSCC TME and is linked to the effectiveness of the antitumor immune response.

Due to the use of genomics to predict medication responses and the development of precise gene editing technologies, new evidence points to intriguing potential in the search for improved possible treatments for OSCC patients (27). The analysis of drug sensitivity revealed that TCGA-OSCC patients with high *CKS2* expression were sensitive to commonly used OSCC therapeutic agents, including cisplatin, 5-FU, paclitaxel, vincristine, cyclophosphamide, and docetaxel. From this perspective, *CKS2* may serve as a biomarker to identify the most sensitive therapeutic medications, thus minimizing adverse effects of anti-tumor treatments and improving OS in OSCC patients.

Clinical prognostic models are already widely used for many human diseases (44-46). However, there were methodological differences in model development and validation (47). In accordance with the REMARK reporting checklist and TRIPOD checklist, we finally established a nomogram based on *CKS2*-related genes and other clinical factors from the TCGA-OSCC cohort. In 1-, 3-, and 5-year AUC and calibration analyses, the nomogram

presents customized and precise survival prediction results. Furthermore, we applied the GSE42743 dataset for further validation. Due to the limitation of sample size and follow-up time, we only performed 1- and 3-year analyses, which showed the same results.

However, there are still some limitations in the study. Considering the nomogram was constructed and verified based on retrospective data from public databases, it is necessary to collect additional prospective clinical data for validation. Additionally, the role of *CKS2* in cell cycle regulation, TMB, and drug sensitivity necessitates further validation through a comprehensive set of cellular experiments.

Conclusions

In summary, our research demonstrated that *CKS2* was upregulated and associated with poor prognosis in OSCC. Moreover, *CKS2* influences the malignant progression and treatment of OSCC through various mechanisms, including cell cycle, proliferation, immune infiltration, TMB, and drug sensitivity. All these findings indicated that *CKS2* was a novel prognostic biomarker and potential therapeutic target in OSCC.

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Footnote

Reporting Checklist: The authors have completed the REMARK and TRIPOD reporting checklists. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-23-511/rc>

Data Sharing Statement: Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-23-511/dss>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-23-511/coif>). The authors

have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The ethics committee at the Hospital of Stomatology, Sun Yat-sen University granted approval for this study (No. KQEC-2022-15-01). Informed consent was granted by each patient involved in this study.

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